

# Negative-feedback regulation of FGF signalling by DUSP6/MKP-3 is driven by ERK1/2 and mediated by Ets factor binding to a conserved site within the *DUSP6/MKP-3* gene promoter

Maria EKEROT\*, Marios P. STAVRIDIS†, Laurent DELAVAIN\*, Michael P. MITCHELL‡, Christopher STAPLES\*, David M. OWENS\*, Iain D. KEENAN\*, Robin J. DICKINSON\*, Kate G. STOREY† and Stephen M. KEYSE\*<sup>1</sup>

\*Cancer Research UK Stress Response Laboratory, Biomedical Research Centre, Level 5, Ninewells Hospital and Medical School, Dundee DD1 9SY, U.K., †Division of Cell and Developmental Biology, College of Life Sciences, University of Dundee, Dundee DD1 5EH, U.K., and ‡Bioinformatics & Biostatistics Group, Cancer Research UK, 44 Lincoln's Inn Fields, London WC2A 3PX, U.K.

DUSP6 (dual-specificity phosphatase 6), also known as MKP-3 [MAPK (mitogen-activated protein kinase) phosphatase-3] specifically inactivates ERK1/2 (extracellular-signal-regulated kinase 1/2) *in vitro* and *in vivo*. DUSP6/MKP-3 is inducible by FGF (fibroblast growth factor) signalling and acts as a negative regulator of ERK activity in key and discrete signalling centres that direct outgrowth and patterning in early vertebrate embryos. However, the molecular mechanism by which FGFs induce *DUSP6/MKP-3* expression and hence help to set ERK1/2 signalling levels is unknown. In the present study, we demonstrate, using pharmacological inhibitors and analysis of the murine *DUSP6/MKP-3* gene promoter, that the ERK pathway is critical for FGF-induced *DUSP6/MKP-3* transcription. Furthermore, we show that this response is mediated by a conserved binding site for the Ets (E twenty-six) family of transcriptional regulators and that the Ets2 protein, a known target of ERK signalling,

binds to the endogenous *DUSP6/MKP-3* promoter. Finally, the murine *DUSP6/MKP-3* promoter coupled to EGFP (enhanced green fluorescent protein) recapitulates the specific pattern of endogenous *DUSP6/MKP-3* mRNA expression in the chicken neural plate, where its activity depends on FGFR (FGF receptor) and MAPK signalling and an intact Ets-binding site. These findings identify a conserved Ets-factor-dependent mechanism by which ERK signalling activates *DUSP6/MKP-3* transcription to deliver ERK1/2-specific negative-feedback control of FGF signalling.

**Key words:** dual-specificity phosphatase 6 (DUSP6), fibroblast growth factor (FGF), mitogen-activated protein kinase (MAPK), mitogen-activated protein kinase phosphatase-3 (MKP-3), phosphatase, transcription.

## INTRODUCTION

DUSP (dual-specificity phosphatase) 6 or MKP [MAPK (mitogen-activated protein kinase) phosphatase]-3 is the prototypical member of a subfamily of three cytoplasmic MKPs, which also includes *DUSP7/MKP-X* and *DUSP9/MKP-4* [1,2]. These enzymes all display a high degree of substrate selectivity for the ERKs (extracellular-signal-regulated kinases) 1 and 2 *in vitro* and *in vivo* [3,4]. Specific recognition and binding to ERK2 is mediated by a conserved KIM (kinase interaction motif) within the N-terminal non-catalytic domain of DUSP6/MKP-3 and this region of the protein also contains a conserved NES (nuclear export signal), which is responsible for the cytoplasmic localization of this phosphatase [5,6]. The specificity of DUSP6/MKP-3 for dephosphorylation and inactivation of the ERK1 and ERK2 MAPKs is enhanced further by ERK-induced conformational change within the catalytic domain of MKP-3, which leads to greatly enhanced phosphatase activity *in vitro* [7,8].

The first clues as to the physiological role of MKP-3 came from the observation that *DUSP6/MKP-3* mRNA is expressed at many sites of FGF (fibroblast growth factor) signalling in developing

mouse and chicken embryos. These include the limb bud and branchial arch mesenchyme, midbrain/hindbrain isthmus, hair and mammary placodes [9], and early neural plate [10]. Further experiments involving tissue ablation and transplantation in chicken embryos identified the AER (apical ectodermal ridge) and Hensen's node as tissue sources of FGF which are essential for the expression of *DUSP6/MKP-3* in the developing limb bud and neural plate respectively [10,11]. In addition, FGF signalling is also responsible for the expression of *DUSP6/MKP-3* in the murine isthmus organizer during neural tube development and in developing chick somites [12,13]. These studies suggest that DUSP6/MKP-3 is a negative regulator of FGF signalling during vertebrate development, which may work to set the levels of ERK signalling downstream of this signalling pathway. This conclusion is supported by the results of a recent mouse knockout experiment. *DUSP6/MKP-3*-null embryos display elevated ERK phosphorylation in limb bud mesenchyme and present with variably penetrating skeletal dwarfism, premature fusion of cranial sutures (craniosynostoses) and deafness, all of which are consistent with increased levels of FGF signalling [14].

Abbreviations used: AER, apical ectodermal ridge; ChIP, chromatin immunoprecipitation; ConA, concanavalin A; DMEM, Dulbecco's modified Eagle's medium; DUSP, dual-specificity phosphatase; EGFP, enhanced green fluorescent protein; Elk1, Ets-like kinase 1; EMSA, electrophoretic mobility-shift assay; ER, oestrogen receptor; ERK, extracellular-signal-regulated kinase; Ets, E twenty-six; FBS, fetal bovine serum; FGF, fibroblast growth factor; FGFR, FGF receptor; FKHD, Forkhead mutant;  $\beta$ Gal,  $\beta$ -galactosidase; HA, haemagglutinin; HH10, Hamburger and Hamilton stage 10; 4-HT, 4-hydroxytamoxifen; I $\kappa$ B $\alpha$ , inhibitor of nuclear factor  $\kappa$ B  $\alpha$ ; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKK, MAPK kinase; MKKK, MAPK kinase kinase; MKP, MAPK phosphatase; mRFP, monomeric red fluorescent protein; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PBX1, pre-B-cell leukaemia transcription factor 1; PI3K, phosphoinositide 3-kinase; RSV, rous sarcoma virus; SRF, serum-response factor; SRY, sex-determining region Y; TNF $\alpha$ , tumour necrosis factor  $\alpha$ .

<sup>1</sup> To whom correspondence should be addressed (email s.m.keyse@dundee.ac.uk).

Although the link between FGF signalling and *DUSP6/MKP-3* expression is now well established, the precise molecular mechanism by which this occurs is unknown. In particular, it is unclear which of the intracellular signalling pathways that lie downstream of the FGFR (FGF receptor) is responsible for mediating *DUSP6/MKP-3* transcription, with essential roles proposed for both the ERK and PI3K (phosphoinositide 3-kinase) pathways [10–13,15]. The majority of these data were obtained in a variety of embryonic tissues often using different pharmacological inhibitors of these pathways and this may account for some of the contradictory data obtained [16].

In the present study, we have used a cell culture model to overcome the limitations of drug delivery using bead implantation in chicken embryos to address the nature of the intracellular signalling pathways involved in FGF-mediated *DUSP6/MKP-3* expression. This has been combined with a bioinformatic and functional dissection of the *DUSP6/MKP-3* gene promoter and has enabled us to define a mechanism by which signalling through the ERK MAPK pathway interacts with a conserved regulatory region within the proximal promoter of the gene to effect negative-feedback control of FGF signalling *in vitro* and in the developing chick embryo.

## EXPERIMENTAL

### Reagents

Recombinant human FGF2 (basic FGF), human FGF4 and mouse FGF8b were purchased from R&D Systems. SU5402 and LY294002 were from Calbiochem. PD184352 was kindly provided by Professor Sir Philip Cohen (MRC Protein Phosphorylation Unit, University of Dundee). Antibodies against ERK, phospho-ERK, p38, phospho-p38, JNK (c-Jun N-terminal kinase), phospho-JNK and phospho-Akt were purchased from Cell Signaling Technology. The antisera raised against Ets (E twenty-six) family proteins were from Santa Cruz Biotechnology. The polyclonal antibody against *DUSP6/MKP-3* was raised in sheep using purified recombinant *DUSP6/MKP-3* protein as an antigen. The specificity and sensitivity of this antiserum was verified by immunoblotting of recombinant *DUSP6/MKP-3*, its ability to recognize *DUSP6/MKP-3*, but not the related phosphatases encoded by *DUSP7* and *DUSP9*, when expressed in Cos-1 cells and Western blotting of protein lysates from wild-type and *DUSP6*-knockout mouse embryos (results not shown). Reagents were purchased from Sigma, unless indicated otherwise. All tissue culture reagents were obtained from Invitrogen.

### Cell lines and tissue culture

NIH 3T3 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% newborn calf serum, penicillin (100 units/ml) and streptomycin (100 units/ml).  $\Delta$ -Raf-1:ER\* NIH 3T3 cells [NIH 3T3 fibroblasts that stably express a fusion between a constitutively active form of the Raf-1 MKKK (MAPK kinase kinase) and a mutant form of the ER (oestrogen receptor)] were maintained in Phenol Red-free DMEM containing glucose (4500 mg/l), penicillin (100 units/ml), streptomycin (100 units/ml), 2 mM glutamine and 10% FBS (fetal bovine serum) supplemented with 2  $\mu$ g/ml puromycin.  $\Delta$ -Raf-1:ER\* NIH 3T3 cells were induced with either 10 nM or 100 nM 4-HT (4-hydroxytamoxifen) as indicated. For inhibitor studies, cells were cultured in Petri dishes for 48 h and were serum-starved overnight. Inhibitors SU5402 (50  $\mu$ M), PD184352 (2  $\mu$ M) and LY294002 (10  $\mu$ M) were added 30 min before the addition of FGFs (30 ng/ml) and cells were then incubated for an addi-

tional 5 h. Cells were lysed, and proteins were analysed using the NuPAGE electrophoresis system (4–12%) (Invitrogen) and Western blotting. For analysis of *DUSP6/MKP-3* mRNA levels, RNA was isolated from cells using an RNeasy kit (Qiagen) according to the manufacturer's instructions, and 200 ng of RNA was reverse-transcribed in a final volume of 50  $\mu$ l using Taqman reverse transcription reagents (Applied Biosystems). A 4 ng sample of cDNA was analysed by quantitative PCR using pre-developed assay primers and probes for *DUSP6/MKP-3* (Mm00650255\_g1; Applied Biosystems). Real-time PCR was performed in the presence of 0.6 $\times$  Taqman Universal PCR mix (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, 92°C for 15 s and 60°C for 1 min using an ABI Prism 7700 sequence detector. Fluorescence output was directly proportional to the concentration of input cDNA and was normalized against  $\beta$ -actin (Mm00607939\_s1).

### Analysis of the *DUSP6/MKP-3* promoter *in silico*

The upstream genomic sequence of *DUSP6/MKP-3* was inspected within the February 2006 (NCBI build 36) assembly of the murine genome using the UCSC genome browser [17], and a candidate region was selected such that the maximum number of vertebrate species genomes aligned to the mouse. The corresponding multiple species alignment was extracted using the Vertebrate Multiz Alignment & Conservation track [18] within the UCSC genome browser. The alignments were then screened for conserved transcription factor-binding sites using MatInspector [19] and a vertebrate factor subset of Genomatix's proprietary database.

### Luciferase reporter assays

Cells were transfected with luciferase reporters using Lipofectamine™ Plus (Invitrogen). At 3 h post-transfection, cells were starved overnight in 0.5% serum. The following day, cells were stimulated with FGFs (all at 30 ng/ml) either alone or together with the indicated inhibitors and then incubated for an additional 24 h before the DLR (Dual-Luciferase® Reporter) Assay (Promega) was performed according to the manufacturer's instructions. pGL3Basic was used as a negative (no promoter) control and pRL-TK *Renilla* was used to normalize for transfection efficiency (Promega). A 6400 bp BamHI/BamHI genomic fragment lying upstream of the *DUSP6/MKP-3* start codon and including the putative transcriptional start site was cloned into pGL3Basic. This full-length promoter was used as a template in PCRs to generate deletion mutants, which were subsequently cloned into pGL3Basic as KpnI/BamHI fragments. Point mutations within the *DUSP6/MKP-3* promoter were introduced into the 508 bp promoter-reporter construct. Ets-binding mutants were made using complementary primers 5'-CTTATCCGGAGCGGAAATTCCTTTC-3' in which the central core Ets-binding site GGA (underlined) was mutated to TTC (triple mutant) as described by Withers and Hakomori [20], or as single mutants TGA (first base), GTA (second base) or GGC (third base) respectively. The palindromic Ets-binding site TCC (underlined) was altered to GAA using complementary primers 5'-GGAGCGGAAATGAATTTCCGTTTTTG-3'. The Forkhead mutant (FKHD) was made using complementary primers 5'-GTTGCAGCTTGTTCCTGACTGGG-3' in which the central FKHD-binding site (underlined) was altered to CCCC. Wild-type and mutant (Ets2  $\Delta$ 410–425 and Ets2 T72A) forms of Ets2 were expressed as HA (haemagglutinin) epitope-tagged proteins in the mammalian expression vector pcDNA3. The NF- $\kappa$ B (nuclear factor  $\kappa$ B) reporter construct 3 $\times$   $\kappa$ B-ConA (concanavalin A)-luciferase (3 $\times$   $\kappa$ B), its control (lacking the

NF- $\kappa$ B-binding sites) ConA-luciferase (ConA) and the RSV (rouv sarcoma virus) expression vectors encoding the dominant-negative I $\kappa$ B $\alpha$  (inhibitor of NF- $\kappa$ B  $\alpha$ ) mutant (RSV-Mad3) and  $\beta$ -galactosidase (RSV- $\beta$ Gal) were kindly provided by Dr Neil Perkins (University of Dundee).

### EMSA (electrophoretic mobility-shift assay) and ChIP (chromatin immunoprecipitation) assays

<sup>32</sup>P-labelled probes were generated by annealing complementary oligonucleotide pairs which generate 5'  $\rightarrow$  3' overhangs and subsequent radiolabelling by incubation with Klenow polymerase and [ $\alpha$ -<sup>32</sup>P]dCTP. Probes were purified on Probequant G-50 columns (GE Healthcare) according to the manufacturer's instructions. Oligonucleotides used were MKP-3 (wild-type), 5'-GGGCT-TATCCGGAGCGGAAATTCCTT-3' and 5'-CGGAAAGGA-ATTTCCGCTCCGATAAG-3'; MKP-3m (Ets-site mutant), 5'-GGGCTTATCCGGAGCTTCAATTCCT-3' and 5'-CGGAAA-GGAATTGAAGCTCCGGATAG-3'; E74 (canonical wild-type Ets site), 5'-GGAGCTGAATAACCGGAAGTAACTCAT-3' and 5'-GGGATGAGTTACTCCGGTTATTCAGC-3'; E74m (mutant Ets site), 5'-GGAGCTGAATAACCGTAAGTAACTCAT-3' and 5'-GGGATGAGTTACTTACGGTTATTCAGC-3'. Nuclear extracts were prepared and EMSAs were performed exactly as described by Feng et al. [21].

ChIP assays were performed exactly as described by Boyd et al. [22]. Briefly, approx. 10<sup>7</sup> NIH 3T3 cells were used per immunoprecipitation. Following sonication, 100  $\mu$ l of chromatin samples were diluted in 1 ml of buffer containing 0.1% SDS, 167 mM NaCl, 1.1% (v/v) Triton X-100, 1 mM EDTA, 16.7 mM Tris/HCl (pH 8.0) with protease inhibitors and pre-cleared twice for 1 h at 4°C with 25  $\mu$ l of Protein G saturated with BSA and salmon sperm DNA (Upstate). Pre-cleared samples were then immunoprecipitated overnight with 2  $\mu$ g of the anti-Ets1 (sc350x), anti-Ets2 (sc351x), anti-Erm81 (sc22807x) and the unrelated anti-HA (sc805x) antibodies (all Santa Cruz Biotechnology) in the presence of 20  $\mu$ l of pre-blocked Protein G. Immune complexes were washed five times in modified RIPA buffer [20 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1% (v/v) Nonidet P40, 0.4 M LiCl, 0.7% sodium deoxycholate and protease inhibitors]. The DNA was then eluted in TE (10 mM Tris/HCl and 1 mM EDTA, pH 7.5) containing 1% (w/v) SDS, incubated overnight at 55°C in the presence of proteinase K (100  $\mu$ g/ml) and purified using a PCR purification column (Qiagen). PCRs (40  $\mu$ l volumes) consisting of 30 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 30 s were carried out using 3  $\mu$ l of eluted DNA as the template and either ChIPFO2, 5'-TGTGAACCTCTAAACAGAAGGAAACAC-3', and ChIPRE2, 5'-ACAGGTTGTGTTGATGAATTGTTAAT-3', which amplify nucleotides -555 to -764 containing the putative Ets site, or, as a control, ChIPFO3, 5'-CAGCGACTGGAATGA-GAACA-3', and ChIPRE3, 5'-GGTGCCTGATTAACCCT-TGA-3', which amplify nucleotides +330 to +550 lying downstream of the Ets-binding site. PCR products were analysed using a 2% agarose gel run in 0.5 $\times$  TBE (Tris/borate/EDTA) and stained with ethidium bromide.

### Embryo culture and electroporation

The wild-type 508 bp *DUSP6/MKP-3* promoter fragment, and the corresponding Ets site (GGA to TTC) and FKHD (TGTT to CCC) were subcloned into an EGFP (enhanced green fluorescent protein)-expressing plasmid ptkd2EGFP [23] as KpnI/BglII fragments. Fertile hen's eggs (High Sex  $\times$  Rhode Island Red) were incubated at 38°C to yield embryos of Hamburger and Hamilton stage 10 (HH10, ten somite) [23a]. Electroporation with appropriate constructs was carried out *in ovo* using

standard techniques [24] and fluorescence was monitored after 4 h. For bead experiments, embryos were put in culture [25], electroporated as described in [10] and incubated for 1 h before beads (pre-soaked for 1 h) presenting inhibitors (5 mM SU5402, 20 mM PD184352, 20 mM LY294002 or DMSO vehicle) were implanted next to the caudal neural plate. Embryos were left to develop for 4–5 h in a humidified 38°C incubator before being checked for fluorescence and photographed before fixation in 4% (w/v) paraformaldehyde in PBS overnight at 4°C.

### *In situ* hybridization and immunocytochemistry of chick embryos

A standard *in situ* hybridization protocol was used to detect *DUSP6/MKP-3* mRNA in the chick embryo [10]. Whole-embryo immunocytochemistry to detect dual-phosphorylated and activated ERK1/2 was carried out as described previously using an anti-dpERK1/2 (dual-phosphorylated ERK1/2) antibody from Cell Signaling Technology [26].

## RESULTS

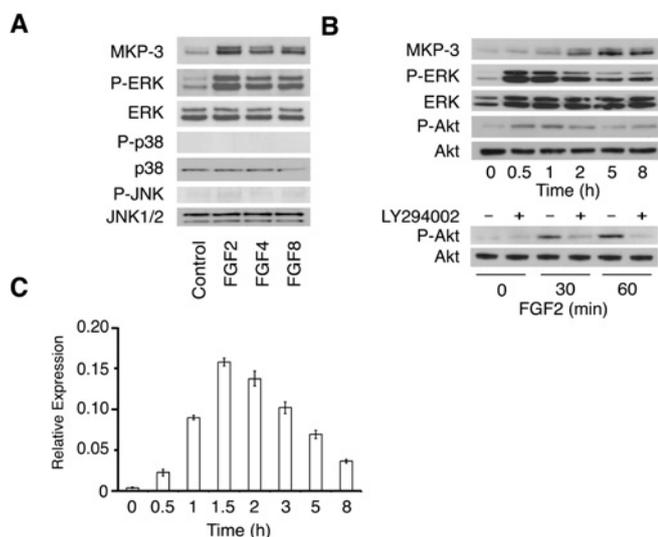
### DUSP6/MKP-3 is an FGF-inducible protein in NIH 3T3 cells

Studies in vertebrate embryos have provided compelling evidence for the involvement of FGFR-mediated signalling in the induction of *DUSP6/MKP-3* mRNA expression during early development. However, it is uncertain exactly how this occurs with studies invoking essential roles for both ERK and PI3K in mediating this response.

To dissect the molecular mechanisms underlying FGF-inducible *DUSP6/MKP-3* expression in a highly tractable system, we screened a panel of mammalian cell lines for expression of the protein. Detectable levels of DUSP6/MKP-3 were seen in HepG2 (human hepatoma) cells and mouse NIH 3T3 fibroblasts. In contrast, DUSP6/MKP-3 was absent in Cos-1, HeLa and HEK-293T (human embryonic kidney) cells (results not shown). Because NIH 3T3 cells have been widely used in studies of inducible gene expression, we determined whether levels of DUSP6/MKP-3 are increased following FGF treatment (Figure 1A). Elevated levels of DUSP6/MKP-3 protein were detected following exposure to FGF2, FGF4 and FGF8, and this correlated with increased phosphorylation of ERK2. In contrast, none of the FGF treatments led to phosphorylation of either p38 or JNK. We note that DUSP6/MKP-3 is resolved as a doublet in these immunoblots. This reflects the use of alternative translational start sites (at codons 1 and 14) within the *DUSP6/MKP-3* mRNA [27]. Exposure of mouse fibroblasts to FGF was shown previously to result in activation of both ERK1/2 and PI3K [28]. Consistent with this, we observed increased phosphorylation of both ERK1/2 and the Akt protein kinase, a downstream target of the PI3K pathway, in response to FGF2. Phosphorylation of both ERK and Akt was apparent 30 min after addition of FGF2, reached peak levels after 1 h and declined thereafter (Figure 1B, upper panels). As expected, Akt phosphorylation was abolished by LY294002 (10  $\mu$ M), a specific inhibitor of PI3K signalling (Figure 1B, lower panel). Elevated levels of DUSP6/MKP-3 protein were detected approx. 1 h after exposure to FGF2 and persisted for at least 8 h (Figure 1B, upper panel) while *DUSP6/MKP-3* mRNA levels were increased within 30 min of FGF exposure, reached maximum levels after approx. 90 min and declined thereafter (Figure 1C).

### FGF-dependent activation of ERK2, but not PI3K, is essential for induction of *DUSP6/MKP-3* mRNA and protein

To dissect the signalling events responsible for FGF-inducible *DUSP6/MKP-3* expression, we have employed chemical

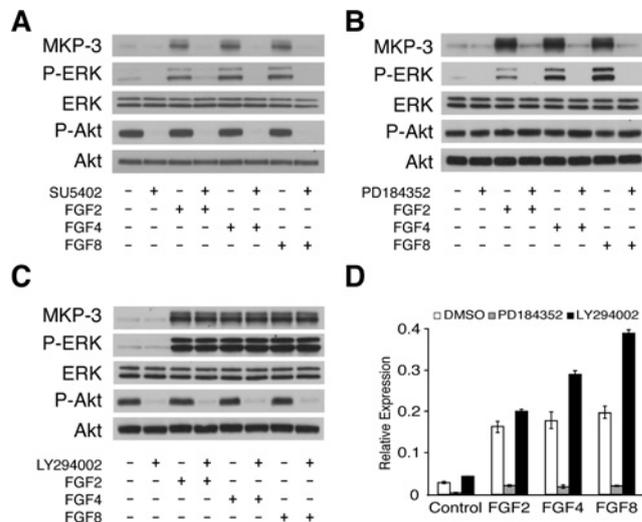


**Figure 1** The expression of *DUSP6/MKP-3* mRNA and protein is inducible by FGF in NIH 3T3 cells

(A) NIH 3T3 cells were serum-starved overnight before exposure to FGF2, FGF4 or FGF8 (all at 30 ng/ml) for 5 h. Cells were then lysed, and proteins were analysed by SDS/PAGE and Western blotting using antisera against MKP-3, phospho-ERK (P-ERK), ERK, phospho-p38 (P-p38), p38, phospho-JNK (P-JNK) and JNK. (B) NIH 3T3 cells were serum-starved overnight and then exposed to FGF2 (30 ng/ml) for the indicated time before lysis and analysis of proteins by SDS/PAGE and Western blotting using antisera against MKP-3, phospho-ERK (P-ERK), ERK, phospho-Akt (P-Akt) or Akt (upper panels). NIH 3T3 cells were serum-starved overnight and then exposed to FGF2 (30 ng/ml) for the times indicated in either the absence or the presence of the PI3K inhibitor LY294002 (10 μM) before lysis and analysis of proteins by SDS/PAGE and Western blotting using antisera against phospho-Akt (P-Akt) or Akt (lower panel). (C) NIH 3T3 cells were serum-starved overnight before exposure to FGF2 (30 ng/ml) for the times indicated. Cells were then lysed, and cellular RNA was prepared. *DUSP6/MKP-3* mRNA levels were then analysed using real-time PCR. Assays were performed in triplicate and relative *DUSP6/MKP-3* mRNA levels are presented as means  $\pm$  S.E.M.

inhibitors at concentrations where specificity is optimal. First, cells were treated with SU5402 (50 μM), a specific FGFR inhibitor [29], and then exposed to FGFs for 5 h (Figure 2A). As expected, this drug blocked both the FGF-dependent phosphorylation of ERK and phosphorylation of the Akt protein kinase, a target of the PI3K pathway. SU5402 also blocked the induction of *DUSP6/MKP-3* protein by FGF2, FGF4 and FGF8, clearly demonstrating a requirement for FGFR activation in this response. We next employed the specific MEK (MAPK/ERK kinase) inhibitor PD184352 (2 μM), which is a more specific and potent inhibitor of the MAPK pathway than PD98059 [30,31]. In contrast with SU5402, this drug did not affect phosphorylation of Akt, but completely blocked the FGF-mediated activation of ERK1/2 and the induction of *DUSP6/MKP-3* protein (Figure 2B).

LY294002 (10 μM) had no effect on either FGF-induced ERK activation or *DUSP6/MKP-3* expression, but, as expected, it caused a dramatic reduction in the levels of phospho-Akt (Figure 2C). In addition to these inhibitors, we also determined that FGF-mediated induction of *DUSP6/MKP-3* was unaffected by inhibition of either the TOR (target of rapamycin) pathway using rapamycin (100 nM) or phospholipase C activity using U-73122 (4 μM) (results not shown). Finally, we used real-time PCR to measure relative levels of *DUSP6/MKP-3* mRNA in FGF-treated NIH 3T3 cells in the absence and presence of either PD184352 or LY294002. Clearly, inhibition of the ERK MAPK pathway, but not PI3K activity, blocks FGF-inducible *DUSP6/MKP-3* mRNA expression (Figure 2D).



**Figure 2** The induction of *DUSP6/MKP-3* protein and mRNA by FGFs is blocked by chemical inhibitors of either the FGFR tyrosine kinase or MEK, but not by a specific inhibitor of PI3K activity in NIH 3T3 cells

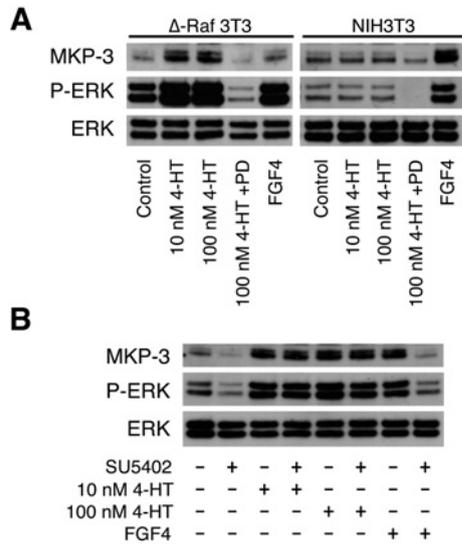
NIH 3T3 cells were serum-starved overnight before exposure to FGF2, FGF4 or FGF8 (all at 30 ng/ml) for 5 h either in the absence (DMSO vehicle only) or presence of (A) the FGFR inhibitor SU5402 (50 μM), (B) the MEK inhibitor PD184352 (2 μM) or (C) the PI3K inhibitor LY294002 (10 μM). Cell lysates were analysed by SDS/PAGE and Western blotting using antisera against MKP-3, phospho-ERK (P-ERK), ERK, phospho-Akt (P-Akt) and Akt. (D) NIH 3T3 cells were serum-starved overnight before exposure to FGF2, FGF4 or FGF8 (all at 30 ng/ml) for 90 min in either the absence (DMSO vehicle only) or the presence of the indicated inhibitor. Cells were then lysed, and cellular RNA was prepared. *DUSP6/MKP-3* mRNA levels were then analysed using real-time PCR. Assays were performed in triplicate and relative *DUSP6/MKP-3* mRNA levels are presented as means  $\pm$  S.E.M.

### ERK1/2 activation is both necessary and sufficient for induction of *DUSP6/MKP-3*

Although the experiments described above indicate that ERK signalling is essential for FGF-mediated induction of *DUSP6/MKP-3*, we wished to know whether activation of ERK alone is sufficient to trigger expression of this gene. To address this question, we obtained mouse NIH 3T3 fibroblasts that stably express a fusion between a constitutively active form of the Raf-1 MKKK and a mutant form of the ER ( $\Delta$ -Raf-1:ER\* NIH 3T3 cells). These  $\Delta$ -Raf-1:ER\* NIH 3T3 cells respond to 4-HT by activation of Raf, which selectively activates MKK (MAPK kinase), thus causing activation of ERK [32]. Treatment of  $\Delta$ -Raf-1:ER\* NIH 3T3 cells, but not parental wild-type NIH 3T3 cells with 4-HT (10 or 100 nM) leads to ERK activation and induction of *DUSP6/MKP-3*, whereas both cell lines respond normally to FGF4 (Figure 3A). As expected, both the induction of *DUSP6/MKP-3* and activation of ERK in response to 4-HT are blocked by PD184352, but are insensitive to inhibition of FGFR activity by SU5402 (Figure 3). We conclude that activation of ERK is both necessary and sufficient to trigger expression of *DUSP6/MKP-3*.

### The murine *DUSP6/MKP-3* promoter contains a conserved region that is required for FGF-responsive transcription

The use of comparative genome analysis to detect non-coding DNA sequence conservation within the 5'-flanking regions of orthologous genes across species is a useful tool in the identification of key regulatory elements [33]. An analysis of this region of the *DUSP6/MKP-3* genes from mouse, rat, human, *Xenopus*, zebrafish and *Fugu* identified a highly conserved region extending



**Figure 3** ERK activation is both necessary and sufficient for the induction of DUSP6/MKP-3 protein in NIH 3T3 cells

(A) Either  $\Delta$ -Raf-1:ER\* NIH 3T3 cells ( $\Delta$ -Raf 3T3) or NIH 3T3 cells were serum-starved overnight and then exposed to the indicated concentration of 4-HT in either the absence or the presence of PD184352 (PD; 2  $\mu$ M). As a control, cells were exposed to FGF4 (30 ng/ml). After 5 h of incubation, cells were lysed, and proteins were analysed by SDS/PAGE and Western blotting using antisera against MKP-3, phospho-ERK (P-ERK) and ERK. (B)  $\Delta$ -Raf-1:ER\* NIH 3T3 cells were serum-starved overnight and then exposed to either the indicated concentration of 4-HT or FGF4 (30 ng/ml) in the absence or presence of the FGFR inhibitor SU5402 (50  $\mu$ M). After 5 h of incubation, cells were lysed, and proteins were analysed by SDS/PAGE and Western blotting using antisera against MKP-3, phospho-ERK (P-ERK) and ERK.

approx. 250 bp upstream of the putative transcriptional start site for *MKP-3* (Figure 4A). This putative promoter does not contain a TATA box, but, in common with many TATA-less promoters, it does contain a CAAT box in the reverse (ATTG) orientation. Interestingly, this region also contains consensus-binding sites for a number of different transcription factor families that occur in all five species in the same order and orientation. These include sites for Forkhead, the Ets family of transcription factors, NF- $\kappa$ B, PBX1 (pre-B-cell leukaemia transcription factor 1)-related homeobox factors and the SRY (sex-determining region Y)-box containing factor SOX5 (Figure 4A). Of these, the Ets-binding site is of particular interest as a number of Ets family members, including Elk1 (Ets-like kinase 1), Ets1 and Ets2, are known targets of ERK1/2 signalling [34,35]. In addition, Forkhead is a downstream target of PI3K signalling, and Meis1, which can form complexes with PBX1, is a retinoic acid-inducible protein in the proximal domain of the developing limb [36,37].

To perform a functional analysis of the *DUSP6/MKP-3* promoter, we first isolated a 6.4 kb BamHI fragment containing nucleotides  $-6758$  to  $-359$  (where the A of the ATG start codon is +1) of the 5' flanking sequence from the murine gene. This included the putative transcriptional start site and approx. 100 bp of the 5'-UTR (untranslated region). This was cloned into a luciferase-reporter construct, and, following transfection into NIH 3T3 cells, transcriptional activity was assayed in either the absence or presence of FGF2, FGF4 or FGF8 (Figures 4B and 4C). A robust increase in promoter activity was seen in response to all three FGFs. We then performed a deletion analysis of this FGF-responsive promoter. Reporter constructs containing sequences from  $-1368$  to  $-359$  (1010 bp),  $-866$  to  $-359$  (508 bp),  $-755$  to  $-359$  (397 bp),  $-700$  to  $-359$  (342 bp) and  $-655$  to  $-359$  (297 bp) demonstrated an increased transcriptional response

following exposure of cells to FGFs. In contrast, reporters containing sequences from  $-605$  to  $-359$  (247 bp) and  $-550$  to  $-359$  (192 bp) were completely unresponsive to FGF treatment (Figures 4B and 4C). Interestingly, the 50 bp interval between constructs 297 and 247 falls within the conserved region identified in our *in silico* promoter analysis, indicating that it contains regulatory sequences essential for FGF-inducible *DUSP6/MKP-3* transcription.

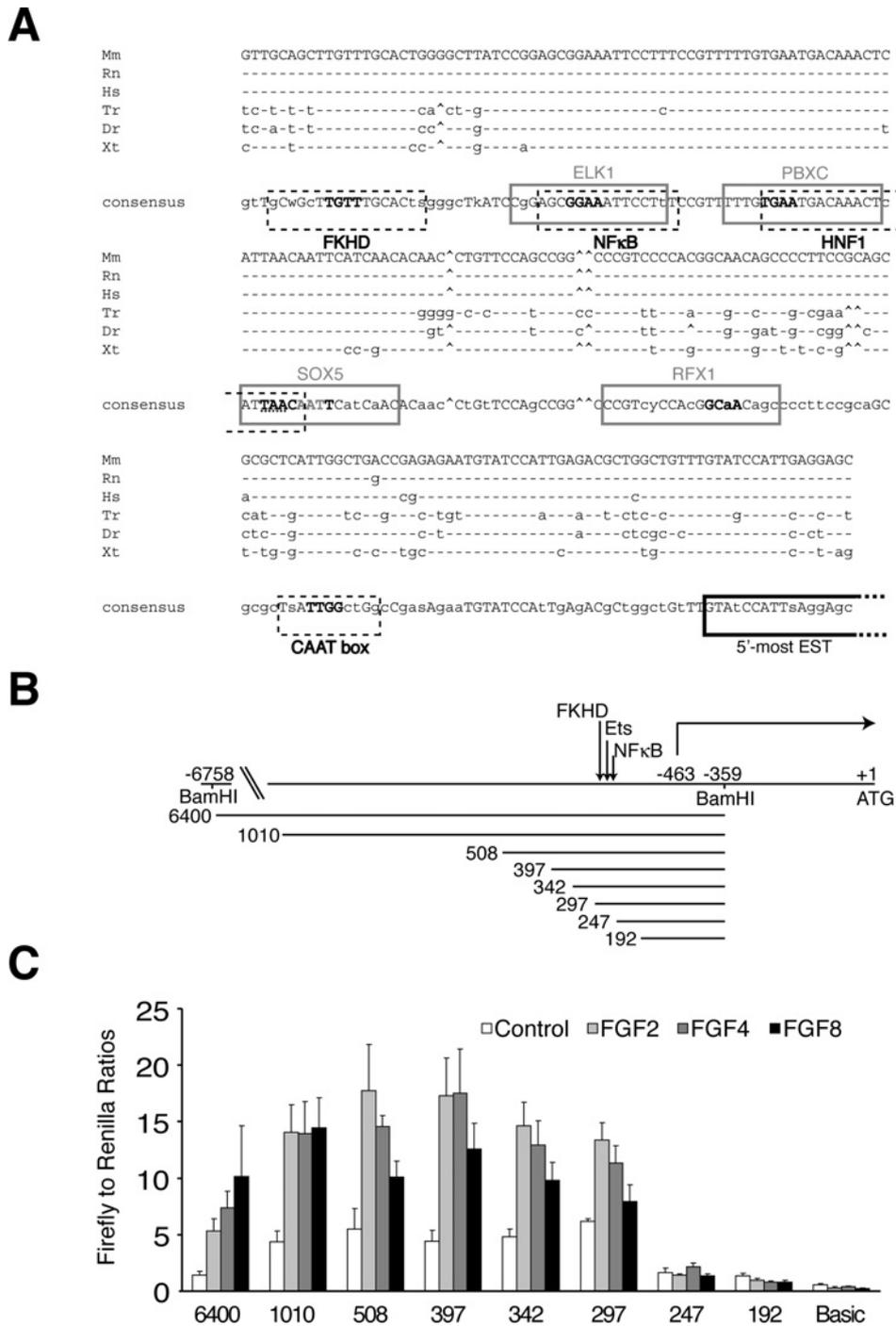
#### FGF-dependent *DUSP6/MKP-3* reporter activity is mediated by activation of ERK and not by activation of the PI3K pathway

We next assessed whether FGF-responsive *DUSP6/MKP-3* transcription was dependent on signalling through either the ERK or the PI3K pathway. The 508 bp *DUSP6/MKP-3* reporter (nucleotides  $-866$  to  $-359$ ) was selected for these experiments as this is both FGF-responsive and contains the whole of the conserved region identified in our *in silico* analysis which includes the putative Forkhead-, Ets- and NF- $\kappa$ B-binding sites. This plasmid was transfected into NIH 3T3 cells, which were then stimulated with FGF2, FGF4 or FGF8 either in the absence or presence of PD184352 or LY294002. FGF-inducible promoter activity was strongly inhibited by the MEK inhibitor PD184352, while being refractory to inhibition of PI3K signalling (Figure 5A). Similar results were obtained with the longer (6400 bp) reporter construct containing nucleotides  $-6758$  to  $-359$  (results not shown). In an alternative approach, we co-transfected the 508 bp reporter with either MEK-EE, a constitutively active mutant of MKK, alone or together with human *DUSP6/MKP-3* itself. The activated form of MKK caused a significant increase in reporter activity and this was abrogated by co-expression of *DUSP6/MKP-3*. In this regard, our *MKP-3* promoter behaved similarly to a model ERK-responsive transcriptional readout in the form of a GAL4-dependent luciferase reporter co-transfected with a GAL4-Elk1 fusion protein (Figure 5B). Finally, we transfected  $\Delta$ -Raf-1:ER\* NIH 3T3 cells with the 508 bp reporter and stimulated these cells with either FGF4 or 4-HT. Both agonists caused a significant increase in *DUSP6/MKP-3* reporter activity, and this was blocked by the MEK inhibitor PD184352, but not by the PI3K inhibitor LY294002 (Figure 5C). We conclude that the FGF-dependent activation of the *DUSP6/MKP-3* gene promoter is dependent on the activation of ERK1/2, but not PI3K, in NIH 3T3 cells and that this activity accurately reflects the ERK-dependence of FGF-mediated induction of both endogenous *DUSP6/MKP-3* protein and mRNA.

#### ERK-dependent *DUSP6/MKP-3* transcription requires a conserved Ets-binding site

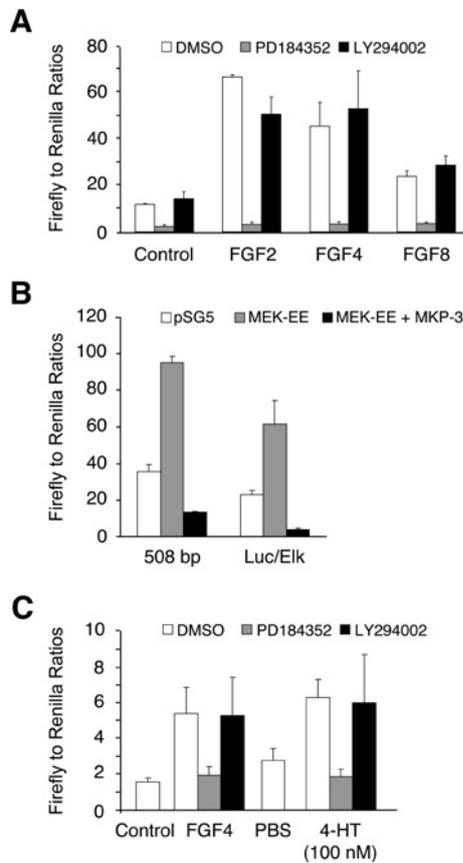
As noted previously, the region of the *DUSP6/MKP-3* promoter identified in our deletion analysis contains putative binding sites for a number of transcription factors. Of these, the most obvious target for signalling through the ERK pathway is an Ets site, which overlaps with a potential binding site for NF- $\kappa$ B (Figure 4A). To investigate the importance of this sequence, we mutated the three core bases of the Ets site either singly or in combination within the context of the 508 bp reporter. All of these changes led to a significant reduction in promoter activity in response to FGF2, FGF4 and FGF8 (Figure 6A). In addition, we noticed that the Ets site is actually palindromic (CGGAAATTCCT) and mutation of this second motif (TCC to GAA) also causes a significant reduction in *DUSP6/MKP-3* promoter activity (Figure 6A).

Because the conserved Ets-binding site overlaps with a putative site for the NF- $\kappa$ B transcription factor (Figure 4A), it was



**Figure 4 Bioinformatic and functional analysis of the murine *DUSP6/MKP-3* gene promoter**

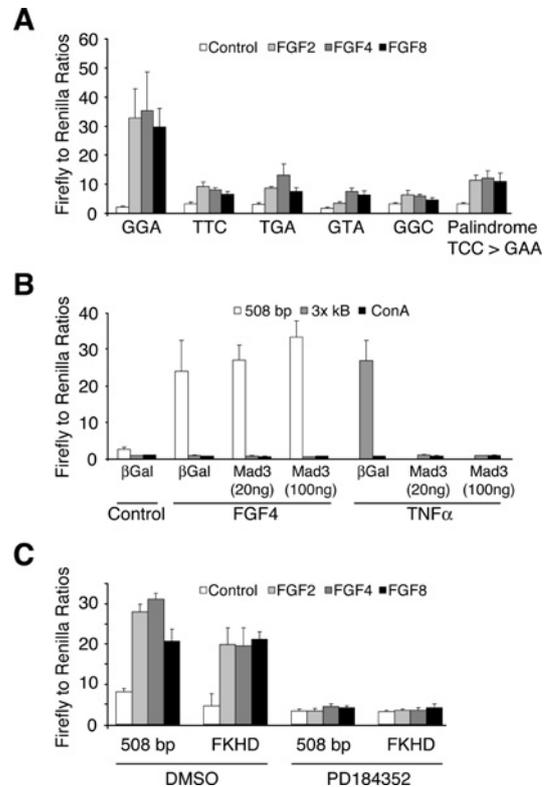
(A) DNA sequence alignment of a conserved region identified within the proximal promoter of the *DUSP6/MKP-3* gene in vertebrates: Mm (*Mus musculus*), Rn (*Rattus norvegicus*), Hs (*Homo sapiens*), Tr (*Takifugu rubripes*), Dr (*Danio rerio*) and Xt (*Xenopus tropicalis*). Conserved transcription factor-binding sites are boxed, either grey-lined (forward strand) or black broken-lined (reverse strand) and identified as follows: FKHD (Forkhead family), ELK1 (Ets-binding site), NF- $\kappa$ B, PBXC (PBX1–Meis1 complexes), HNF1 (hepatic nuclear factor 1), SOX5 [Sox/SRY–sex/testis determining and related HMG (high-mobility group) box factors], RFX1 (regulatory factor X1), CAAT box (CAAT box: promoter element in some genes located approx. 75–80 bp upstream of the start site for transcription). Core bases are shown in bold, and, where these overlap, the nucleotides belonging to each site are identified to indicate orientation (either bold grey or hatched underlined). The putative transcriptional start site is also indicated (boxed in black). The latter is based on mapping the 5'-most extent of annotated ESTs (expressed sequence tags) for *DUSP6/MKP-3*. (B) Schematic diagram showing the 5' boundaries of the *DUSP6/MKP-3* promoter–reporter constructs employed in the deletion analysis. Nucleotides are numbered with the A of the ATG start codon designated as +1. The putative transcriptional start site (–463) is indicated as are the BamHI restriction enzyme sites used to subclone the longest (6400 bp) genomic fragment into the firefly luciferase reporter plasmid. (C) The constructs indicated in (B) were co-transfected into NIH 3T3 cells along with pRL-TK *Renilla* to normalize for transfection efficiency. Cells were then starved overnight in 0.5% serum. The following day, cells were either left untreated or stimulated with the indicated FGF (all at 30 ng/ml) for an additional 24 h, before cells were lysed, and luciferase assays were performed. pGL3Basic acted as a negative (promoterless) control. Luciferase assays were performed in quadruplicate, and results are mean  $\pm$  S.E.M. firefly/*Renilla* ratios.



**Figure 5** FGF-dependent *DUSP6/MKP-3* promoter activity is mediated by activation of ERK and not by activation of the PI3K pathway

(A) The 508 bp *DUSP6/MKP-3* reporter construct was co-transfected into NIH 3T3 cells along with pRL-TK *Renilla* to normalize for transfection efficiency. Cells were then starved overnight in 0.5% serum. The following day, cells were either left untreated or stimulated with the indicated FGF (all at 30 ng/ml) in either the absence or the presence of vehicle (DMSO), PD184352 (2  $\mu$ M) or LY294002 (10  $\mu$ M) for an additional 24 h. Cells were then lysed, and luciferase assays were performed in quadruplicate. (B) Either the 508 bp *DUSP6/MKP-3* reporter construct or a plasmid encoding a GAL4-Elk1 fusion protein together with a GAL4-dependent luciferase reporter (Luc/Elk) was co-transfected into NIH 3T3 cells along with pRL-TK *Renilla* to normalize for transfection efficiency and either empty pSG5 expression vector or pSG5 encoding either a constitutively active mutant of MEK (MEK-EE) or *DUSP6/MKP-3* itself. After 24 h, cells were lysed, and luciferase assays were performed in quadruplicate. (C)  $\Delta$ -Raf-1:ER\* NIH 3T3 cells were co-transfected with the 508 bp *DUSP6/MKP-3* reporter construct and pRL-TK *Renilla* to normalize for transfection efficiency. Cells were then serum-starved overnight and either left untreated or treated with either FGF4 (30 ng/ml) or 4-HT (100 nM) in the presence or absence of vehicle (DMSO), PD184352 (2  $\mu$ M) or LY294002 (10  $\mu$ M). After 24 h, cells were lysed, and luciferase assays were performed in quadruplicate. Results are mean  $\pm$  S.E.M. firefly/*Renilla* ratios.

important to investigate a possible role for this signalling pathway in mediating the response to FGF. We first determined whether expression of a dominant-negative mutant of *I $\kappa$ B $\alpha$*  (Mad3 super-repressor) affected the FGF-mediated activation of the *DUSP6/MKP-3* promoter. Co-expression of this protein had no effect on FGF-inducible *DUSP6/MKP-3* transcription (Figure 6B). In addition, an NF- $\kappa$ B-responsive reporter containing three NF- $\kappa$ B-binding sites (3 $\times$   $\kappa$ B) was completely unresponsive to FGF. In contrast, TNF $\alpha$  (tumour necrosis factor  $\alpha$ ) stimulated this reporter, and its activity was completely inhibited by co-expression of the Mad3 super-repressor (Figure 6B). Finally, a putative site for the Forkhead (FOXO) family of transcription factors lies within the 5' boundary of our FGF-responsive 297 bp *DUSP6/MKP-3* reporter. However, mutation of this site within our



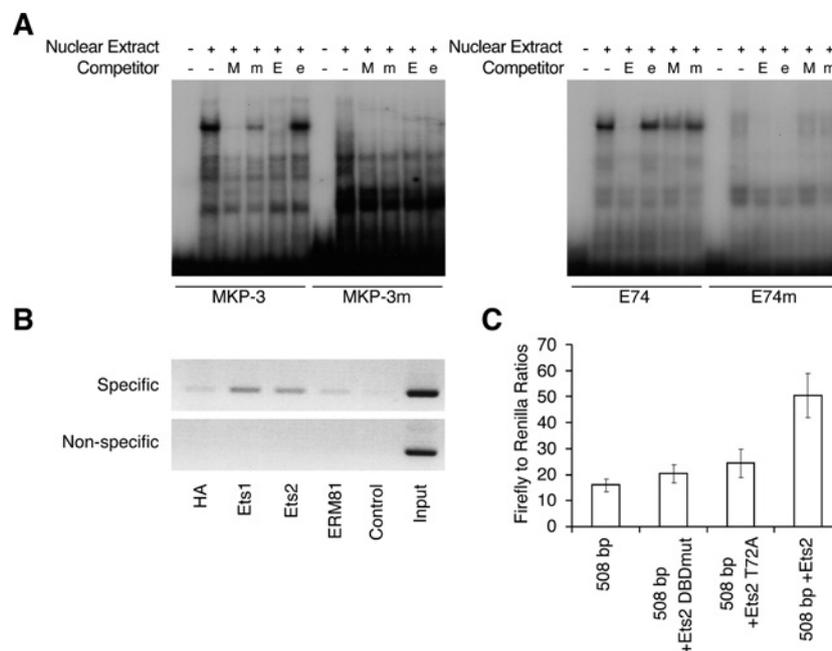
**Figure 6** A conserved Ets-binding site is required for FGF-inducible *DUSP6/MKP-3* promoter activity

(A) NIH 3T3 cells were co-transfected with either the wild-type 508 bp *DUSP6/MKP-3* reporter construct or the indicated mutants together with pRL-TK *Renilla* to normalize for transfection efficiency. Cells were then starved overnight in 0.5% serum. The following day, cells were either left untreated or stimulated with the indicated FGF (all at 30 ng/ml). After 24 h, cells were lysed, and luciferase assays were performed in quadruplicate. (B) NIH 3T3 cells were co-transfected with the indicated reporter constructs along with either RSV-Mad3 (20 or 100 ng) or as a control RSV- $\beta$ Gal and pRL-TK *Renilla* to normalize for transfection efficiency. Cells were then serum-starved overnight (0.5% FBS) and either left untreated or exposed to either FGF4 (30 ng/ml) for 24 h or TNF $\alpha$  (10 ng/ml) for 6 h. Cells were then lysed, and luciferase assays were performed in quadruplicate. (C) NIH 3T3 cells were co-transfected with either the wild-type 508 bp *DUSP6/MKP-3* reporter construct or a 508 bp reporter in which the Forkhead-binding site was mutated (FKHD) together with pRL-TK *Renilla* to normalize for transfection efficiency. Cells were then starved overnight in 0.5% FBS. The following day, cells were either left untreated or stimulated with the indicated FGF (all at 30 ng/ml) either in the absence (DMSO) or presence of PD184352 (2  $\mu$ M). After 24 h, cells were lysed, and luciferase assays were performed in quadruplicate. Results are mean  $\pm$  S.E.M. firefly/*Renilla* ratios.

508 bp reporter had no effect on FGF-mediated activation, and transcriptional activity remained sensitive to inhibition of the ERK1/2 MAPK pathway by PD184352 (Figure 6C). We conclude that a functional Ets site is required for FGF-inducible *DUSP6/MKP-3* transcription and that neither NF- $\kappa$ B nor Forkhead plays a role in mediating this response.

#### ERK-responsive transcription factors Ets1 and Ets2 bind to the endogenous *DUSP6/MKP-3* gene promoter

Our results thus far implicate a member or members of the Ets group of transcription factors in mediating ERK-dependent *DUSP6/MKP-3* transcription in response to FGFs. To explore this further, we performed EMSAs using a labelled double-stranded oligonucleotide probe spanning the conserved Ets site (*MKP-3*). These experiments revealed a specific protein complex, which was not seen when labelled mutant oligonucleotide (*MKP-3m*) is used as a probe (Figure 7A). This complex is effectively competed away by unlabelled wild-type *MKP-3* oligonucleotide (M), but not



**Figure 7** Ets-family transcription factors bind to the endogenous *DUSP6/MKP-3* gene promoter

(A) EMSAs were performed using the following labelled oligonucleotide probes: wild-type *MKP-3* or an Ets site mutant *MKP-3* (*MKP-3m*), and wild-type canonical Ets-binding site (*E74*) or its corresponding Ets-binding site mutant (*E74m*). Labelled probes were then incubated with nuclear extract from NIH 3T3 cells in either the absence or the presence of the following unlabelled competitor DNAs: wild-type *MKP-3* (M), mutant *MKP-3* (m), wild-type *E74* (E) or the corresponding Ets site mutant (e). Following incubation, mobility-shifts were visualized by electrophoresis and autoradiography. (B) ChIP assays were performed using an unrelated control antibody (HA) and antibodies specific for Ets1, Ets2 or ERM81. Specific PCR products corresponding to the region of the *DUSP6/MKP-3* promoter containing the putative Ets-binding site and the non-specific control reactions are shown, as are the controls lacking template and the results of PCRs performed using the input chromatin as template. (C) NIH 3T3 cells were either transfected with the 508 bp *DUSP6/MKP-3* reporter alone or together with either a deletion mutant of Ets2 lacking a functional DNA-binding domain (Ets2 DBDmut), a mutant Ets2 protein lacking a conserved MAPK phosphorylation site (Ets2 T72A) or wild-type Ets2. Following transfection, cells were cultured overnight in medium containing 10% FBS. Cells were then lysed, and luciferase assays were performed in triplicate. Results are mean  $\pm$  S.E.M. firefly/*Renilla* ratios.

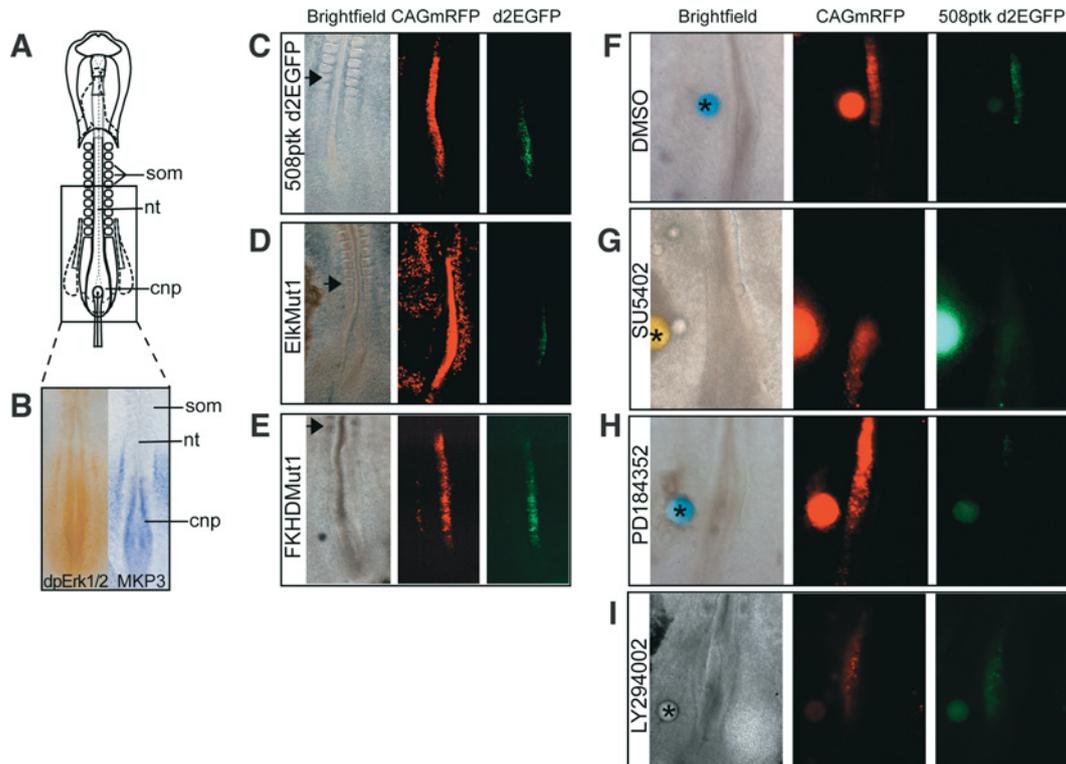
by a mutant oligonucleotide with a single substitution in the Ets site (m). This complex is also effectively competed by a wild-type (E) but not a mutant (e) oligonucleotide containing a canonical Ets-binding site. A complex that co-migrated with that seen using the wild-type *MKP-3* oligonucleotide, was also observed using the wild-type canonical Ets site (*E74*), but not a mutant oligonucleotide (*E74m*) as a probe (Figure 7A). This complex was competed away by both wild-type Ets (E) oligonucleotide and, albeit less effectively, by the wild-type *MKP-3* oligonucleotide (M). Further experiments showed that the complex detected using the *MKP-3* oligonucleotide was present when nuclear extracts from either control or FGF-treated cells was used, indicating that protein(s) may be constitutively bound to the *DUSP6/MKP-3* promoter (results not shown). With this information in hand, we decided to take a candidate approach to examine the binding of individual Ets factors to the endogenous *DUSP6/MKP-3* promoter using ChIP assays.

For these experiments, NIH 3T3 cells were cultured in 10% serum before treatment with 1% formaldehyde for 10 min to cross-link DNA and proteins *in vivo*, followed by cell lysis and the preparation of chromatin. Chromatin from approx.  $2 \times 10^7$  cells was sonicated to an average length of 600 bp before immunoprecipitation with either a non-specific (HA) antibody or specific antisera against Ets factors. Precipitated DNA was amplified by PCR, separated by agarose gel electrophoresis, and visualized by ethidium bromide staining (Figure 7B). Two sets of oligonucleotide PCR primers were used, one of which was specific for the Ets-binding site and, as a control, primers that annealed to sequences upstream of the Ets-binding site. Non-precipitated (input) chromatin was a positive control for these PCRs. We

could detect significant association of both Ets1 and Ets2 with the Ets-binding site, whereas no detectable signal was seen using either the control antibody or an antibody against ERM81, a member of the closely related Pea3 subfamily of Ets factors. Additional ChIP experiments using antibodies specific for Elk1 also resulted in a failure to detect binding to the *DUSP6/MKP-3* promoter (results not shown). Finally, we assessed the impact of expressing wild-type and mutant forms of Ets2 on the activity of the 508 bp *DUSP6/MKP-3* luciferase reporter. Co-transfection of wild-type Ets2 caused a significant increase in reporter activity. In contrast, co-expression of either a deletion mutant of Ets2 ( $\Delta 410-425$ ) lacking a functional DNA-binding domain (Ets2 DBDmut) or a mutant form of Ets2 (Ets2 T72A) lacking a critical MAPK phosphorylation site [38] did not increase *DUSP6/MKP-3* reporter activity (Figure 7C).

#### The murine *DUSP6/MKP-3* promoter is regulated appropriately by endogenous signals which depend on the FGF/MAPK pathway

To test whether the 508 bp fragment of the *DUSP6/MKP-3* promoter is appropriately activated in the context of a developing embryo, we placed this sequence into a reporter construct driving expression of destabilized *EGFP* (508ptkd2EGFP) [23]. HH10 (ten somite) embryos (Figure 8A) were then co-electroporated with this plasmid and a plasmid encoding CMV (cytomegalovirus) promoter-driven *mRFP* (monomeric red fluorescent protein) (CAGmRFP), which identifies all electroporated cells. At this stage *DUSP6/MKP-3* is expressed in the caudal neural plate, a region of high ERK1/2 activity, but not more rostrally in the



**Figure 8** The 508 bp *DUSP6/MKP-3* promoter fragment directs transgene expression in an endogenous *DUSP6/MKP-3* domain

(A) Schematic diagram of the HH10 chick embryo. The boxed area indicates approximate field in (B–I). (B) Comparison of ERK1/2 phosphorylation and *DUSP6/MKP-3* mRNA expression in the caudal region of the HH10 chick embryo. dpErk1/2, dual-phosphorylated ERK1/2. (C) HH10 chick embryo co-electroporated with a plasmid constitutively expressing mRFP (CAGmRFP) and the 508 bp fragment of the *DUSP6/MKP-3* promoter driving expression of EGFP (508ptkd2EGFP) only within the endogenous *DUSP6/MKP-3* domain. (D) Embryo co-electroporated with plasmid 508ptkd2EGFP in which the Ets-binding site is mutated (EtsMut1) and CAGmRFP shows reduced EGFP expression. (E) Co-electroporation of plasmid 508ptkd2EGFP in which the Forkhead-binding site is mutated (FKHDMut1) and CAGmRFP. (F–H) Embryos were co-electroporated with the wild-type *DUSP6/MKP-3* promoter fragment (508ptkd2EGFP) and CAGmRFP before beads were soaked in DMSO (F), 5 mM SU5402 (G), 20 mM PD184352 (H) or 20 mM LY294002 (I) were placed next to the electroporation site. Both SU5402 and PD184352 cause a local down-regulation of *DUSP6/MKP-3* promoter activity. In contrast, neither LY294002 nor DMSO alone have any significant effect on *DUSP6/MKP-3* promoter activity. Asterisks (\*) indicates grafted bead, and the arrowhead indicates the position of the last formed somite. nt, neural tube; cnp, caudal neural plate; som, somite.

neural tube flanked by somites (Figures 8A and 8B) [26]. Introduction of these constructs into the neuroepithelium revealed that this *DUSP6/MKP-3* promoter region drives EGFP expression within the endogenous *DUSP6/MKP-3* domain (where cells co-expressed EGFP and mRFP) and not in the rostral neural tube, where only mRFP was detected (after 4–6 h of incubation) (Figure 8C, 14 of 14 cases). This 508 bp sequence is thus sufficient to recapitulate the expression pattern of the endogenous *DUSP6/MKP-3* gene. When the Ets-binding site is mutated, expression of EGFP is either much reduced (Figure 8D, six of 11 cases) or absent (five of 11 cases). In contrast, mutation of the putative Forkhead-binding site does not reduce EGFP expression (Figure 8E, six of six cases).

We next tested whether the 508 bp *DUSP6/MKP-3* promoter fragment is regulated by FGF-mediated ERK1/2 activation in caudal neural tissue. Embryos were co-electroporated with the 508ptkd2EGFP and CAGmRFP constructs, then cultured in the presence of locally applied beads soaked in either DMSO vehicle control (Figure 8F), the FGFR inhibitor SU5402 (Figure 8G) or the MEK inhibitor PD184352 (Figure 8H) (see the Experimental section). Blocking activation of either FGFRs or just ERK1/2 inhibited *MKP-3* promoter activity (six of six and eight of eight cases respectively), whereas control beads did not attenuate EGFP expression. Finally, to test whether the 508 bp promoter is regulated by PI3K signalling, we electroporated embryos with the 508ptkd2EGFP and CAGmRFP constructs, then cultured them

in the presence of beads soaked in the PI3K inhibitor LY294002 (Figure 8I). In all cases, there was no loss of EGFP, suggesting that *DUSP6/MKP-3* expression in the caudal neural plate is not downstream of this signalling pathway (five of five cases).

## DISCUSSION

In vertebrates, the induction of *DUSP6/MKP-3* in response to FGF during early development represents the best-characterized link between a specific growth factor signalling axis and expression of an MKP [9,10]. However, whether *DUSP6/MKP-3* induction represents a negative-feedback control or an example of regulated cross-talk between two intracellular signalling pathways is controversial, as essential roles have been proposed for both ERK MAPK and PI3K signalling in regulating *DUSP6/MKP-3* expression [10–13,15]. The majority of these data were obtained using two different pharmacological inhibitors of MEK (PD98059 or PD184352) and LY294002, a specific inhibitor of PI3K. Furthermore, chemicals were delivered by implantation of beads pre-soaked in 10–20 mM solutions of drug into chicken embryos [10,11]. This makes the effective concentrations of inhibitor delivered to tissues difficult to assess and raises questions about both the potency and specificity of pathway inhibition in these studies [16]. In the present study, we have utilized cultured NIH 3T3 cells to study FGF-inducible expression of *DUSP6/MKP-3*,

allowing a more precise delivery of drug and direct biochemical readout of its effects.

Our results clearly show that FGF treatment of NIH 3T3 fibroblasts induces *DUSP6/MKP-3* expression at the level of both mRNA and protein. Furthermore, although inhibition of ERK1/2 activity blocks this expression completely, we can find no evidence for the involvement of PI3K in this process. These results are reinforced by the observation that conditional activation of the ERK1/2 MAPK pathway in  $\Delta$ -Raf-1:ER\* NIH 3T3 cells also increases levels of DUSP6/MKP-3 protein, indicating that ERK activation is both necessary and sufficient to trigger expression of this phosphatase. To probe the molecular mechanism by which ERK activation might influence *DUSP6/MKP-3* expression levels, we performed both a bioinformatic and a functional analysis of the murine *DUSP6/MKP-3* gene promoter.

Comparative genome analysis revealed a conserved region within the proximal promoter of *MKP-3/DUSP6* containing a number of transcription factor-binding sites. This analysis included sequences from mammal, amphibian and fish genomes. Most of the work performed previously to analyse the signalling pathways involved in the regulation of *DUSP6/MKP-3* by FGF has been performed in chicken embryos [10,11]. However, we were unable to include the chicken *DUSP6/MKP-3* homologue in our analysis, as the 5'-flanking region of the gene is lacking from the current genome sequence release ([http://www.ensembl.org/Gallus\\_gallus/index.html](http://www.ensembl.org/Gallus_gallus/index.html)).

Of the putative regulatory elements within this conserved region, the most obvious candidate with respect to an interface with the ERK MAPK pathway is a conserved Ets-binding site. Many growth factor-activated genes such as c-Fos are regulated by SRF (serum-response factor), a MADS-box transcription factor. SRF forms a ternary complex with Ets family members such as Elk1, also known as TCFs (ternary complex factors), whose activity is controlled by MAPK signalling [34,39]. However, close examination of the *DUSP6/MKP-3* promoter reveals no cognate binding sites for SRF, indicating that the Ets site is functioning autonomously.

We can detect the constitutive binding of protein(s) to this Ets consensus sequence in EMSAs and, by using ChIP analysis, the Ets family proteins Ets1 and Ets2 were found to be associated with this binding site *in vivo*. The latter observation provides a mechanistic link between *DUSP6/MKP-3* transcription and ERK1/2 activity. The observation that two distinct Ets family members can associate with the *DUSP6/MKP-3* promoter in NIH 3T3 cells also raises questions of binding specificity. A recent genome-wide analysis of Ets protein binding revealed unexpected levels of redundant occupancy [40]. Furthermore, this degree of redundancy was increased if the Ets-binding site was proximal to the transcriptional start site, as is the case in *DUSP6/MKP-3*. The Ets site within *DUSP6/MKP-3* is also palindromic, and core bases within both half-sites are required for FGF-responsive *DUSP6/MKP-3* transcription. Interestingly, the stromelysin-1 gene promoter also contains a head-to-head Ets-binding site palindrome [41]. This site has recently been demonstrated to mediate stromelysin-1 expression in response to the tumour promoter PMA, and the Ets1 transcription factor is found to be constitutively associated with this site *in vivo* [42]. It will be interesting to explore further mechanistic similarities in the regulation of these two growth factor-regulated genes, both of which involve the Ets family of transcription factors acting independently of SRF as a conduit for ERK1/2 signalling.

The expression of the *DUSP6/MKP-3* gene in response to tissue sources of FGF is observed in mouse, chicken and zebrafish embryos, and this is probably a reflection of the conservation of the regulatory sequences revealed by our promoter analysis.

Scanning genomic fragments for enhancer activity utilizing embryo electroporation in the chicken can identify such regions. This method makes use of reporter constructs in which expression of *EGFP* is driven by DNA sequences from the locus of interest [23]. Using this approach, we have clearly shown that the region of the murine *DUSP6/MKP-3* promoter encompassing the conserved Ets-binding site recapitulates the endogenous domain of *DUSP6/MKP-3* expression in chicken embryo caudal neural tissue. Furthermore, this expression is dependent on the integrity of the Ets-binding site and is sensitive to inhibition of the ERK MAPK pathway. We have identified Ets1 and Ets2 as binding to the *DUSP6/MKP-3* promoter in NIH 3T3 cells, and the question arises as to their expression in the tissues we have analysed in the present study. We have not determined the pattern of *Ets1* and *Ets2* mRNA expression in the chicken embryo. However, between day 8.5 and day 10.5 of mouse embryonic development, *Ets2* mRNA is expressed in tail bud, pre-somitic mesenchyme and limb bud mesenchyme in a pattern which is almost indistinguishable from that which we observe for *DUSP6/MKP-3* mRNA, which in turn correlates with sites of active FGF signalling [9,43].

In conclusion, we have provided compelling evidence that activation of the ERK MAPK pathway and not the PI3K pathway drives expression of *DUSP6/MKP-3* mRNA and protein in response to FGFs in mammalian cells. This, together with our previous studies in developing mouse and chicken embryos [10,16], strongly supports the idea that DUSP6/MKP-3 performs a key role as a negative-feedback regulator of FGF-activated ERK1/2 signalling. In addition, our functional studies of the mouse gene promoter provide the first evidence of a molecular mechanism by which ERK1/2 signalling impinges on transcription factors that are responsible for mediating *DUSP6/MKP-3* expression. *DUSP6/MKP-3*, along with genes such as *Sprouty* and *Sef*, belong to the FGF synexpression group of FGF antagonists [44]. Although the FGF-inducible expression of *Sprouty* and *Sef* has also been linked with activation of ERK1/2 [45,46], both the mechanism by which this occurs and their mode of action as inhibitors of FGF signalling remain unclear [47–50]. Our present results thus make *DUSP6/MKP-3* the best understood of the FGF synexpression group in terms of a molecular mechanism for negative-feedback control of FGF signalling. Finally, *DUSP6/MKP-3* gene expression is also reported to be responsive to other agonists during early development, including maternal  $\beta$ -catenin signalling and retinoic acid [51,52]. The tools we have developed in the present study should facilitate a more extensive analysis of the signalling pathways that interact with the transcription machinery to regulate *DUSP6/MKP-3* expression.

We thank Professor Andy Sharrocks (School of Biological Sciences, University of Manchester, Manchester, U.K.) for reagents and advice on performing ChIP analysis, Dr Neil Perkins (School of Life Sciences, University of Dundee) for reagents and advice on the NF- $\kappa$ B signalling pathway, Dr Simon J. Cook (Babraham Institute, Cambridge, U.K.) for providing the NIH 3T3 cell line expressing  $\Delta$ -Raf-1:ER\* and Professor H. Kondoh (Graduate School of Frontier Biosciences, University of Osaka, Osaka, Japan) for the ptk2EGFP plasmid. We are also grateful to Professor Cheryl Tickle (School of Life Sciences, University of Dundee) for many helpful discussions throughout the course of this work. K. G. S. and research in the Storey Laboratory is supported by grants from the MRC (Medical Research Council) (G0200220 and G0600234). M. P. S. holds a Collaborative Career Development Fellowship in Stem Cell Research (G113/18) in the Storey Laboratory. S. M. K. and work in the Cancer Research UK Stress Response Laboratory are supported by Cancer Research UK and by an MRC Ph.D. studentship (to C. S.).

## REFERENCES

- 1 Camps, M., Nichols, A. and Arkinstall, S. (2000) Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J.* **14**, 6–16
- 2 Dickinson, R. J. and Keyse, S. M. (2006) Diverse physiological functions for dual-specificity MAP kinase phosphatases. *J. Cell Sci.* **119**, 4607–4615

- 3 Groom, L. A., Sneddon, A. A., Alessi, D. R., Dowd, S. and Keyse, S. M. (1996) Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. *EMBO J.* **15**, 3621–3632
- 4 Muda, M., Theodosiou, A., Rodrigues, N., Boschert, U., Camps, M., Gillieron, C., Davies, K., Ashworth, A. and Arkinstall, S. (1996) The dual specificity phosphatases M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases. *J. Biol. Chem.* **271**, 27205–27208
- 5 Nichols, A., Camps, M., Gillieron, C., Chabert, C., Brunet, A., Wilsbacher, J., Cobb, M., Pouyssegur, J., Shaw, J. P. and Arkinstall, S. (2000) Substrate recognition domains within extracellular signal-regulated kinase mediate binding and catalytic activation of mitogen-activated protein kinase phosphatase-3. *J. Biol. Chem.* **275**, 24613–24621
- 6 Karlsson, M., Mathers, J., Dickinson, R. J., Mandl, M. and Keyse, S. M. (2004) Both nuclear-cytoplasmic shuttling of the dual specificity phosphatase MKP-3 and its ability to anchor MAP kinase in the cytoplasm are mediated by a conserved nuclear export signal. *J. Biol. Chem.* **279**, 41882–41891
- 7 Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U. and Arkinstall, S. (1998) Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase. *Science* **280**, 1262–1265
- 8 Stewart, A. E., Dowd, S., Keyse, S. M. and McDonald, N. Q. (1999) Crystal structure of the MAPK phosphatase Pyst1 catalytic domain and implications for regulated activation. *Nat. Struct. Biol.* **6**, 174–181
- 9 Dickinson, R. J., Eblaghie, M. C., Keyse, S. M. and Morriss-Kay, G. M. (2002) Expression of the ERK-specific MAP kinase phosphatase *PYST1/MKP3* in mouse embryos during morphogenesis and early organogenesis. *Mech. Dev.* **113**, 193–196
- 10 Eblaghie, M. C., Lunn, J. S., Dickinson, R. J., Munsterberg, A. E., Sanz-Ezquerro, J. J., Farrell, E. R., Mathers, J., Keyse, S. M., Storey, K. and Tickle, C. (2003) Negative feedback regulation of FGF signalling levels by Pyst1/MKP3 in chick embryos. *Curr. Biol.* **13**, 1009–1018
- 11 Kawakami, Y., Rodriguez-Leon, J., Koth, C. M., Buscher, D., Itoh, T., Raya, A., Ng, J. K., Esteban, C. R., Takahashi, S., Henrique, D. et al. (2003) MKP3 mediates the cellular response to FGF8 signalling in the vertebrate limb. *Nat. Cell Biol.* **5**, 513–519
- 12 Smith, T. G., Sweetman, D., Patterson, M., Keyse, S. M. and Munsterberg, A. (2005) Feedback interactions between MKP3 and ERK MAP kinase control scleraxis expression and the specification of rib progenitors in the developing chick somite. *Development* **132**, 1305–1314
- 13 Echevarria, D., Martinez, S., Marques, S., Lucas-Teixeira, V. and Belo, J. A. (2005) Mkp3 is a negative feedback modulator of Fgf8 signalling in the mammalian isthmus organizer. *Dev. Biol.* **277**, 114–128
- 14 Li, C., Scott, D. A., Hatch, E., Tian, X. and Mansour, S. L. (2007) Dusp6 (Mkp3) is a negative feedback regulator of FGF-stimulated ERK signalling during mouse development. *Development* **134**, 167–176
- 15 Gomez, A. R., Lopez-Varea, A., Molnar, C., de la Calle-Mustienes, E., Ruiz-Gomez, M., Gomez-Skarmeta, J. L. and de Celis, J. F. (2005) Conserved cross-interactions in *Drosophila* and *Xenopus* between Ras/MAPK signalling and the dual-specificity phosphatase MKP3. *Dev. Dyn.* **232**, 695–708
- 16 Smith, T. G., Karlsson, M., Lunn, J. S., Eblaghie, M. C., Keenan, I. D., Farrell, E. R., Tickle, C., Storey, K. G. and Keyse, S. M. (2006) Negative feedback predominates over cross-regulation to control ERK MAPK activity in response to FGF signalling in embryos. *FEBS Lett.* **580**, 4242–4245
- 17 Karolchik, D., Baertsch, R., Diekhans, M., Furey, T. S., Hinrichs, A., Lu, Y. T., Roskin, K. M., Schwartz, M., Sugnet, C. W., Thomas, D. J. et al. (2003) The UCSC Genome Browser Database. *Nucleic Acids Res.* **31**, 51–54
- 18 Blanchette, M., Kent, W. J., Riemer, C., Elnitski, L., Smit, A. F., Roskin, K. M., Baertsch, R., Rosenbloom, K., Clawson, H., Green, E. D. et al. (2004) Aligning multiple genomic sequences with the threaded blockset aligner. *Genome Res.* **14**, 708–715
- 19 Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M. and Werner, T. (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* **21**, 2933–2942
- 20 Withers, D. A. and Hakomori, S. I. (2000) Human  $\alpha(1,3)$ -fucosyltransferase IV (*FUTIV*) gene expression is regulated by elk-1 in the U937 cell line. *J. Biol. Chem.* **275**, 40588–40593
- 21 Feng, Y., Goulet, A. C. and Nelson, M. A. (2004) Identification and characterization of the human *Cdc212* gene promoter. *Gene* **330**, 75–84
- 22 Boyd, K. E., Wells, J., Gutman, J., Bartley, S. M. and Farnham, P. J. (1998) c-Myc target gene specificity is determined by a post-DNA binding mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13887–13892
- 23 Uchikawa, M., Ishida, Y., Takemoto, T., Kamachi, Y. and Kondoh, H. (2003) Functional analysis of chicken *Sox2* enhancers highlights an array of diverse regulatory elements that are conserved in mammals. *Dev. Cell* **4**, 509–519
- 23a Hamburger, V. and Hamilton, H. (1951) A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92
- 24 Itasaki, N., Bel-Vialar, S. and Krumlauf, R. (1999) 'Shocking' developments in chick embryology: electroporation and *in ovo* gene expression. *Nat. Cell Biol.* **1**, E203–E207
- 25 Chapman, S. C., Collignon, J., Schoenwolf, G. C. and Lumsden, A. (2001) Improved method for chick whole-embryo culture using a filter paper carrier. *Dev. Dyn.* **220**, 284–289
- 26 Lunn, J. S., Fishwick, K. J., Halley, P. A. and Storey, K. G. (2007) A spatial and temporal map of FGF/Erk1/2 activity and response repertoires in the early chick embryo. *Dev. Biol.* **302**, 536–552
- 27 Dowd, S., Sneddon, A. A. and Keyse, S. M. (1998) Isolation of the human genes encoding the Pyst1 and Pyst2 phosphatases: characterisation of Pyst2 as a cytosolic dual-specificity MAP kinase phosphatase and its catalytic activation by both MAP and SAP kinases. *J. Cell Sci.* **111**, 3389–3399
- 28 Hadari, Y. R., Gotoh, N., Kouhara, H., Lax, I. and Schlessinger, J. (2001) Critical role for the docking-protein FRS2 $\alpha$  in FGF receptor-mediated signal transduction pathways. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 8578–8583
- 29 Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J. (1997) Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955–960
- 30 Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., Teclé, H., Barrett, S. D., Bridges, A., Przybranowski, S. et al. (1999) Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. *Nat. Med.* **5**, 810–816
- 31 Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105
- 32 Samuels, M. L., Weber, M. J., Bishop, J. M. and McMahon, M. (1993) Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. *Mol. Cell. Biol.* **13**, 6241–6252
- 33 Aparicio, S., Morrison, A., Gould, A., Gilthorpe, J., Chaudhuri, C., Rigby, P., Krumlauf, R. and Brenner, S. (1995) Detecting conserved regulatory elements with the model genome of the Japanese puffer fish, *Fugu rubripes*. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1684–1688
- 34 Yang, S. H., Sharrocks, A. D. and Whitmarsh, A. J. (2003) Transcriptional regulation by the MAP kinase signalling cascades. *Gene* **320**, 3–21
- 35 Sharrocks, A. D. (2001) The ETS-domain transcription factor family. *Nat. Rev. Mol. Cell Biol.* **2**, 827–837
- 36 Arden, K. C. and Biggs, 3rd, W. H. (2002) Regulation of the FoxO family of transcription factors by phosphatidylinositol-3 kinase-activated signalling. *Arch. Biochem. Biophys.* **403**, 292–298
- 37 Mercader, N., Leonardo, E., Piedra, M. E., Martinez, A. C., Ros, M. A. and Torres, M. (2000) Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. *Development* **127**, 3961–3970
- 38 McCarthy, S. A., Chen, D., Yang, B. S., Garcia Ramirez, J. J., Cherwinski, H., Chen, X. R., Klagsbrun, M., Hauser, C. A., Ostrowski, M. C. and McMahon, M. (1997) Rapid phosphorylation of Ets-2 accompanies mitogen-activated protein kinase activation and the induction of heparin-binding epidermal growth factor gene expression by oncogenic Raf-1. *Mol. Cell. Biol.* **17**, 2401–2412
- 39 Treisman, R. (1995) Journey to the surface of the cell: Fos regulation and the SRE. *EMBO J.* **14**, 4905–4913
- 40 Hollenhorst, P. C., Shah, A. A., Hopkins, C. and Graves, B. J. (2007) Genome-wide analyses reveal properties of redundant and specific promoter occupancy within the *ETS* gene family. *Genes Dev.* **21**, 1882–1894
- 41 Baillat, D., Begue, A., Stehelin, D. and Aumercier, M. (2002) ETS-1 transcription factor binds cooperatively to the palindromic head to head ETS-binding sites of the stromelysin-1 promoter by counteracting autoinhibition. *J. Biol. Chem.* **277**, 29386–29398
- 42 Baillat, D., Leprivier, G., Régner, D., Vintonenon, N., Bègue, A., Stéhelin, D. and Aumercier, M. (2006) Stromelysin-1 expression is activated *in vivo* by Ets-1 through palindromic head-to-head Ets binding sites present in the promoter. *Oncogene* **25**, 5764–5776
- 43 Risteovski, S., Tam, P. P., Hertzog, P. J. and Kola, I. (2002) Ets2 is expressed during morphogenesis of the somite and limb in the mouse embryo. *Mech. Dev.* **116**, 165–168
- 44 Niehrs, C. and Meinhardt, H. (2002) Modular feedback. *Nature* **417**, 35–36
- 45 Ozaki, K., Kadomoto, R., Asato, K., Tanimura, S., Itoh, N. and Kohno, M. (2001) ERK pathway positively regulates the expression of Sprouty genes. *Biochem. Biophys. Res. Commun.* **285**, 1084–1088
- 46 Furthauer, M., Lin, W., Ang, S. L., Thisse, B. and Thisse, C. (2002) Sef is a feedback-induced antagonist of Ras/MAPK-mediated FGF signalling. *Nat. Cell Biol.* **4**, 170–174

- 47 Kovalenko, D., Yang, X., Chen, P. Y., Nadeau, R. J., Zubanova, O., Pigeon, K. and Friesel, R. (2006) A role for extracellular and transmembrane domains of Sef in Sef-mediated inhibition of FGF signalling. *Cell. Signalling* **18**, 1958–1966
- 48 Ren, Y., Cheng, L., Rong, Z., Li, Z., Li, Y., Li, H., Wang, Z. and Chang, Z. (2006) hSef co-localizes and interacts with Ras in the inhibition of Ras/MAPK signalling pathway. *Biochem. Biophys. Res. Commun.* **347**, 988–993
- 49 Ren, Y., Li, Z., Rong, Z., Cheng, L., Li, Y., Wang, Z. and Chang, Z. (2007) Tyrosine 330 in hSef is critical for the localization and the inhibitory effect on FGF signalling. *Biochem. Biophys. Res. Commun.* **354**, 741–746
- 50 Martinez, N., Garcia-Dominguez, C. A., Domingo, B., Oliva, J. L., Zarich, N., Sanchez, A., Gutierrez-Eisman, S., Llopis, J. and Rojas, J. M. (2007) Sprouty2 binds Grb2 at two different proline-rich regions, and the mechanism of ERK inhibition is independent of this interaction. *Cell. Signalling* **19**, 2277–2285
- 51 Moreno, T. A. and Kintner, C. (2004) Regulation of segmental patterning by retinoic acid signalling during *Xenopus* somitogenesis. *Dev. Cell* **6**, 205–218
- 52 Tsang, M., Maegawa, S., Kiang, A., Habas, R., Weinberg, E. and Dawid, I. B. (2004) A role for MKP3 in axial patterning of the zebrafish embryo. *Development* **131**, 2769–2779

---

Received 2 November 2007/5 March 2008; accepted 6 March 2008

Published as BJ Immediate Publication 6 March 2008, doi:10.1042/BJ20071512