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**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**

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Short title: Feedback regulation of FGF signalling by DUSP6/MKP-3

Abbreviations used: AER, apical ectodermal ridge; CHIP, chromatin immunoprecipitation; DUSP, dual-specificity phosphatase; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility shift assay; ER, oestrogen receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2; Ets, E-twenty six specific sequence; FGF, fibroblast growth factor; FGFR, FGF-receptor; 4-HT, 4-hydroxytamoxifen; JNK, cJun amino-terminal kinase; KIM, kinase interaction motif; MAPK, mitogen-activated protein kinase; MKK or MEK, MAPK kinase; MKP, mitogen-activated protein kinase phosphatase; NF $\kappa$ B, nuclear factor kappa-B; NES, nuclear export signal; SRF, serum response factor; TCF, ternary complex factor TOR, target of rapamycin;

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## SYNOPSIS

DUSP6 (dual-specificity phosphatase 6) also known as MKP-3 (mitogen-activated protein kinase [MAPK] phosphatase -3) specifically inactivates ERK1/2 *in vitro* and *in vivo*. DUSP6/MKP-3 is inducible by fibroblast growth factor (FGF) 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 signaling levels is unknown. Here 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 E-twenty-six-specific sequence (Ets) 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 recapitulates the specific pattern of endogenous DUSP6/MKP-3 mRNA expression in the chicken neural plate, where its activity depends on FGFR 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.

## KEYWORDS:

DUSP6, MAP kinase, MKP-3, transcription, FGF, phosphatase

## INTRODUCTION

Dual-specificity phosphatase 6 (DUSP6) or mitogen-activated protein kinase [MAPK] phosphatase-3 (MKP-3) is the prototypic 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 extracellular-signal regulated kinases (ERKs) 1 and 2 *in vitro* and *in vivo* [3, 4]. Specific recognition and binding to ERK2 is mediated by a conserved kinase interaction motif (KIM) within the amino-terminal non-catalytic domain of DUSP6/MKP-3 and this region of the protein also contains a conserved nuclear export signal (NES), which is responsible for the cytoplasmic localisation of this phosphatase [5, 6]. The specificity of DUSP6/MKP-3 for dephosphorylation and inactivation of the ERK1 and 2 MAPKs is further enhanced 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 fibroblast growth factor (FGF) signalling in developing mouse and chicken embryos. These include the limb bud and branchial arch mesenchyme, midbrain/hindbrain isthmus and hair and mammary placodes [9] and early neural plate [10]. Further experiments involving tissue ablation and transplantation in chicken embryos, identified the apical ectodermal ridge (AER) 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 organiser 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 down stream 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 penetrant skeletal dwarfism, premature fusion of cranial sutures (craniosynostoses) and deafness, all of which are consistent with increased levels of FGF signalling [14].

While 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 FGF receptor is responsible for mediating DUSP6/MKP-3 transcription, with essential roles proposed for both the ERK and PI3-kinase pathways [10-13, 15]. The majority of this data was 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 MAP kinase 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 FGF-basic, human FGF-4 and mouse FGF-8b were purchased from R&D systems. SU5402 and LY294002 were from Calbiochem. PD184352 was kindly provided by Professor Sir Philip Cohen (University of Dundee). Antibodies against ERK, phospho-ERK, p38, phospho-p38, JNK, phospho-JNK and phospho-Akt were purchased from Cell Signaling. The antisera raised against Ets family proteins were from Santa Cruz. 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 recognise 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 WT and DUSP6 knockout mouse embryos (data not shown). Chemicals were purchased from Sigma, unless indicated otherwise. All tissue culture reagents were obtained from Gibco BRL.

### Cell lines and tissue culture

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, penicillin (100U/ml) and streptomycin (100U/ml).  $\Delta$ -Raf-1:ER\* NIH3T3 cells were maintained in phenol red-free Dulbecco's modified Eagle's high glucose medium containing penicillin (100U/ml), streptomycin (100U/ml), glutamine and 10% FBS supplemented with 2  $\mu$ g of puromycin ml<sup>-1</sup>.  $\Delta$ -Raf-1:ER\* was induced with either 10 nM or 100 nM 4-Hydroxytamoxifen (4-HT) as indicated. For inhibitor studies, cells were cultured in petri dishes for 48h and serum starved overnight. Inhibitors SU5402 (50  $\mu$ M), PD184352 (2  $\mu$ M) and LY294002 (10  $\mu$ M), were added 30 minutes prior to the

addition of FGFs (30ng/ml) and then incubated for an additional 5h. Cells were then 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 RNAeasy kit (Quiagen) according to the manufacturer's instructions and 200ng of RNA was reverse transcribed in a final volume of 50 $\mu$ l using Taqman reverse transcription reagents (Applied Biosystems). 4ng of cDNA was then subjected to 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 x Taqman Universal PCR mix (Applied Biosystems) under the following conditions 50°C (2 min), 95°C (10 min), 92°C (15 sec) and 60°C (1 min) using an ABI Prism 7700 sequence detector. Fluorescence output was directly proportional to the concentration of input cDNA and was normalised against  $\beta$ -actin (Mm00607939\_s1).

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

The upstream genomic sequence of DUSP6 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). 3 hours 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 24h before the Dual-Luciferase® Reporter (DLR) Assay (Promega) was performed according to the manufacturer's instructions. pGL3Basic was used as negative (no promoter) control and pRL-TK Renilla was used to normalise for transfection efficiency (Promega). A 6399 bp Bam *HI*-Bam *HI* 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 PCR reactions to generate deletion mutants, which were subsequently cloned into pGL3Basic as Kpn I - Bam *HI* fragments. Point mutations within the DUSP6/MKP-3 promoter were introduced into the 507 bp promoter-reporter construct. Ets binding mutants were made using complementary primers 5'-CTTATCCGGAGCGGAAATTCCTTTC-3' in which the central core Ets binding site GGA was mutated to TTC (triple mutant) according to 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 was altered to GAA using complementary primers 5' GGAGCGGAAATGAATTTCCGTTTTTG-3'. The forkhead mutant (FKHD) was made using complementary primers 5'-GTTGCAGCTTGTTTGCCTGGG-3' in which the central FKHD binding site (underlined) was altered to CCCC. Wild type and mutant (Ets2  $\Delta$ 410-425 and Ets2T72A) forms of Ets2 were expressed as HA-epitope tagged proteins in the mammalian expression vector pSG5. The NFkappaB reporter construct 3 x  $\kappa$ B ConA luciferase (3 x  $\kappa$ B), its control (lacking the NfkappaB binding sites) ConA luciferase (ConA) and the expression vectors encoding the dominant negative I $\kappa$ B $\alpha$  mutant (RSV-Mad3) and  $\beta$ -galactosidase (RSV- $\beta$ Gal) were kindly provided by Dr Neil Perkins (University of Dundee).

### EMSA and ChIP assays



[<sup>32</sup>P]-labelled probes were generated by annealing complementary oligonucleotide pairs which generate 5'-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 according to the manufacturer's instructions. Oligos used were MKP-3 (wild-type) 5'-GGGCTTATCCGGAGCGG AAATTCCTTT-3' and 5'-CGGAAAGGAATTTCCGCTCCGGATAA G-3'; MKP-3m (Ets-site mutant) 5'-GGGCTTATCCGGAGCTTCAATTCCT-3' and 5'-CGGAAAG GAATTGAAGCTCCGGATAG-3'; E74 (canonical wild-type Ets site) 5'-GGAGCTGAATAACCGGAAGTAACTCAT-3' and 5'-GGGATGAGTTACTTCCGG TTATTCAGC-3'; E74m (mutant Ets site) 5'-GGAGCTGAATAACCGTAAGTAACT CAT-3' and 5'-GGGATGAGTTACTTACGGTTATTCAGC-3'. Nuclear extracts were prepared and electrophoretic mobility-shift assays (EMSA) were performed exactly as described by Feng et al [21].

ChIP assays were performed exactly as described by Boyd et al [22]. Briefly, approximately 10<sup>7</sup> NIH3T3 cells were used per immunoprecipitation. Following sonication, 100  $\mu$ l chromatin samples were diluted in 1ml of buffer containing 0.1%w/v SDS, 167mM NaCl, 1.1%v/v triton X100, 1mM EDTA, 16.7 mM Tris 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). Precleared samples were then immunoprecipitated overnight with 2  $\mu$ g of the following antibodies, Ets1 (sc350x), Ets2 (sc351x), Erm81 (sc22807x) and the unrelated anti-HA (sc805x) antiserum (all Santa Cruz) in the presence of 20  $\mu$ l pre-blocked protein G. Immune complexes were washed 5 times in modified RIPA buffer (20 mM Tris pH 8.0, 1mM EDTA, 1.0%v/v NP40, 0.4M LiCl, 0.7% w/v sodium deoxycholate and protease inhibitors). The DNA was then eluted in TE 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 (Quiagen). PCR reactions (40  $\mu$ l) were then performed under the following conditions 95°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec (30 cycles) using 3  $\mu$ l of eluted DNA as

template and the following primers either ChIPFO2 5'-TGTGAACTCTAAACAGAAGGAAACAC-3' and ChIPRE2 5'-ACAGGTTGTGTTGATGAATTGTTAAT-3' which amplify nucleotides -555 to -764 containing the putative Ets site or, as a control, ChIPFO3 5'-CAGCGACTGGAATGAGAACA-3' and ChIPRE3 5'-GGTGCCTGATTAACCCTTGA-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 x TBE and stained with ethidium bromide.

### Embryo culture and electroporation

The wild-type 507 bp DUSP6/MKP-3 promoter fragment, and the corresponding Ets site (GGA to TTC) and forkhead (TGTT to CCCC) mutants were subcloned into ptkd2EGFP [23] as Kpn I - Bgl II fragments. Fertile hen's eggs (High Sex x Rhode Island Red) were incubated at 38°C to yield embryos of stage HH10 (Hamburger and Hamilton, 1951). Electroporation, with appropriate constructs was carried *in ovo* using standard techniques [24] and fluorescence was monitored after 4h. For bead experiments, embryos were put in EC culture [25], electroporated, as described in [10] and incubated for 1h before beads (presoaked for 1h) presenting inhibitors (5mM SU5402-Calbiochem, 20mM PD184352, 20mM LY294002-Calbiochem 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 prior to fixation in 4% paraformaldehyde in PBS overnight at 4°C.

### In situ hybridisation and immunocytochemistry of chick embryos

A standard in situ hybridisation 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 antibody from Cell Signalling Technology [26].

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## RESULTS

### DUSP6/MKP-3 is an FGF inducible protein in NIH3T3 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 PI3-kinase in mediating this response.

To dissect the molecular mechanisms, which underlie 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 NIH3T3 fibroblasts. In contrast, DUSP6/MKP-3 was absent in Cos-1, HeLa and HEK293T cells (data not shown). Because NIH3T3 cells have been widely used in studies of inducible gene expression, we determined if levels of DUSP6/MKP-3 are increased following FGF treatment (Figure 1A). Elevated levels of DUSP6/MKP-3 protein were detected following exposure to basic FGF2 (FGFb), 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 has previously been shown to result in activation of both ERK1/2 and PI3-kinase [28]. Consistent with this, we observe increased phosphorylation of both ERK1/2 and the Akt protein kinase, a downstream target of the PI3-kinase pathway, in response to FGF2. Phosphorylation of both ERK and Akt is apparent after 30min following addition of FGF2, reaches peak levels after 1h and decline thereafter (Fig. 1B, upper panel). As expected, Akt phosphorylation is abolished by LY294002

(10 $\mu$ M), a specific inhibitor of PI3-kinase signalling (Fig. 1B, lower panel) Elevated levels of DUSP6/MKP-3 protein are detected approximately 60 min after exposure to FGF2 and persist for at least 8h (Figure 1B, upper panel) while DUSP6/MKP-3 mRNA levels are increased within 15 min of FGF exposure, reach maximum levels after approximately 90 min and decline thereafter (Figure 1C).

### **FGF-dependent activation of ERK2 but not PI3-kinase is essential for induction of DUSP6/MKP-3 mRNA and protein**

To dissect out the signalling events responsible for FGF-inducible DUSP6/MKP-3 expression we have employed chemical inhibitors at concentrations where specificity is optimal. Firstly, cells were treated with SU5402 (50  $\mu$ M), a specific FGFR inhibitor [29], and then exposed to FGFs for 5h (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 PI3-kinase 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 inhibitor PD184352 (2  $\mu$ M), which is a more specific and potent inhibitor of the MAPK pathway than PD098059 [30, 31]. In contrast to 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  $\mu$ M) 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 pathway using rapamycin (100nm) or phospholipase-C activity using U-73122 (4 $\mu$ M) (data not shown). Finally, we used Real-Time PCR to measure relative levels of DUSP6/MKP-3 mRNA in FGF-treated NIH3T3

cells in the absence and presence of either PD184352 or LY294002. Clearly inhibition of the ERK MAPK pathway, but not PI3-kinase activity, blocks FGF-inducible DUSP6/MKP-3 mRNA expression (Figure 2D).

### **Erk1/2 activation is both necessary and sufficient for induction of DUSP6/MKP3**

While the above experiments indicate that ERK signalling is essential for FGF-mediated induction of DUSP6/MKP-3 we wished to know if activation of ERK alone is sufficient to trigger expression of this gene. To address this question we obtained mouse 3T3 fibroblasts that stably express a fusion between a constitutively active form of the Raf-1 MAPK kinase kinase (MKKK) and a mutant form of the oestrogen receptor ( $\Delta$ -Raf-1:ER\*). These  $\Delta$ -Raf-3T3 cells respond to 4-hydroxytamoxifen (4-HT) by activation of Raf, which selectively activates MAPK kinase thus causing activation of ERK [32]. Clearly treatment of  $\Delta$ -Raf-3T3 but not parental wild-type NIH3T3 cells with 4-HT (10 or 100 nM) leads to ERK activation and induction of DUSP6/MKP-3 protein while 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, A and B). 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 approximately 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 E-twenty-six (Ets) family of transcription factors, NFkappaB, PBX1-related homeobox factors and the SRY-box containing factor SOX5 (Figure 4A). Of these, the Ets factor binding site is of particular interest as a number of Ets family members including Elk1, Ets1 and Ets2, are known targets of ERK1/2 signalling [34, 35]. In addition, forkhead is a downstream target of PI3-kinase 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 *Bam* HI 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 approximately 100 bp of 5' UTR. This was cloned into a luciferase-reporter construct and, following transfection into NIH3T3 cells, transcriptional activity was assayed in either the absence or presence of FGF2, FGF4 or FGF8 (Figure 4, B and C). 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 (1009 bp), -866 to -359 (507 bp), -755 to -359 (396 bp), -700 to -364 (346 bp) and -655 to -359 (296 bp) demonstrated an increased transcriptional response following exposure of cells to FGFs. In contrast, reporters containing sequences between -605 and -359 (246 bp) and -550 to -359 (191 bp) were completely unresponsive to FGF treatment (Figure 4, B and C). Interestingly, the 50 bp interval between

constructs 296 and 246 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 PI3-kinase pathway**

We next assessed whether FGF-responsive DUSP6/MKP-3 transcription was dependent on signalling through either the ERK or the PI3-kinase pathway. The 507 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 NFkappaB binding sites. This plasmid was transfected into NIH3T3 cells, which were then stimulated with FGF2, FGF4 or FGF8 either in the absence or presence of PD184352 or LY294002. FGF-inducible promoter activity is strongly inhibited by the MAPK kinase inhibitor PD184352, while this activity is resistant to inhibition of PI3-kinase activity (Figure 5A). Identical results were obtained with the longer (6399 bp) reporter construct containing nucleotides -6758 to -359 (data not shown). In an alternative approach, we co-transfected the 507 bp reporter with either MEK-EE, a constitutively active mutant of MAPK kinase either alone or together with human DUSP6/MKP-3 itself. The activated form of MAPK kinase 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 behaves identically 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 the  $\Delta$ -Raf-3T3 cells with the 507 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 PI3-kinase 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 PI3-kinase in NIH3T3 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 factor-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 507 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 important to investigate a possible role for this signalling pathway in mediating the response to FGF. We first determined if expression of a dominant negative mutant of  $\kappa$ B $\alpha$  (Mad3 super-repressor) affected the FGF-mediated activation of the DUSP6/MKP-3 promoter. Clearly 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 3 x NF $\kappa$ B binding sites (3 x  $\kappa$ B) was completely unresponsive to FGF. In contrast, TNF $\alpha$  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 296 bp DUSP6/MKP-3 reporter. However, mutation of this site within our 507 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 NFkappaB nor forkhead plays a role in mediating this response.

### **The 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 electrophoretic mobility-shift assays (EMSAs) using a labelled, double stranded oligonucleotide probe spanning the conserved Ets site (MKP-3). These experiments revealed a specific protein complex, which is 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 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) oligo containing a canonical Ets binding site. A complex, which co-migrated with that seen using the wild-type MKP-3 oligo, was also seen using the wild-type canonical Ets site (Ets) but not a mutant oligo (Etsm) as a probe (Figure 7A). This complex was competed away by both wild-type Ets (E) oligo and, albeit less effectively, by the wild-type MKP-3 oligo (M). Further experiments showed that the complex detected using the MKP-3 oligo 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 (data 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 chromatin immuno-precipitation (ChIP) assays.

For these experiments NIH3T3 cells were cultured in 10% FCS 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 approximately  $2 \times 10^7$  cells was then 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 then amplified by PCR, separated by acrylamide 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 factor-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 PCR reactions. We could detect significant association of both Ets1 and Ets2 with the Ets binding site while no detectable signal was seen using either the control antibody or an antibody against ERM81, a member of the closely related *pea3* sub-family of Ets factors. Additional ChIP experiments using antibodies specific for Elk-1 also resulted in a failure to detect binding to the DUSP6/MKP-3 promoter (data not shown). Finally, we assessed the impact of expressing wild-type and mutant forms of Ets2 on the activity of the 507 bp DUS6/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 (Ets2DBDmut) or a mutant form of Ets2 (Ets2T72A) 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 507 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 containing unstable EGFP (507ptkd2EGFP) [23]. Day 1.5 chick embryos (Hamburger and Hamilton stage 10, ten somite embryos) (Figure 8A) were then co-electroporated with this plasmid and a plasmid encoding CMV promoter-driven RFP (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 neural tube flanked by somites (Figure 8A and B) [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 RFP) and not in the rostral neural tube, where only RFP was detected (after 4-6h incubation) (Figure 8C, 14/14 cases). This 507 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, 6/11 cases) or absent (5/11 cases). In contrast, mutation of the putative Forkhead binding site does not reduce EGFP expression (Figure 8E, 6/6 cases).

We next sought to test whether the 507 bp DUSP6/MKP-3 promoter fragment is regulated by FGF-mediated ERK1/2 activation in caudal neural tissue. Embryos were co-electroporated with the 507ptkd2EGFP and RFP 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 Methods). Blocking activation of either FGFRs or just ERK1/2 inhibited MKP3 promoter activity (6/6 and 8/8 cases respectively) while control beads did not attenuate EGFP expression. Finally, to test if the 507bp promoter is regulated by PI3-kinase signalling, we electroporated embryos with the 507ptkd2EGFP and RFP constructs, then cultured in the presence of beads soaked in the PI3-kinase inhibitor LY294002. In all cases, there was no

loss of EGFP suggesting that MKP3/DUSP6 expression in the caudal neural plate is not downstream of this signalling pathway (Fig. 8I, 5/5 cases)".

Stage 2(a) POST-PRINT

## DISCUSSION

In vertebrates the induction of DUSP6/MKP-3 in response to FGF during early development represents the best-characterised 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 crosstalk between two intracellular signalling pathways is controversial, as essential roles have been proposed for both ERK MAPK and PI3-kinase signalling in regulating DUSP6/MKP-3 expression [10-13, 15]. The majority of this data was obtained using two different pharmacological inhibitors of MAPK kinase (PD098059 or PD184352) and LY294002, a specific inhibitor of PI3-kinase. 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 now utilised cultured NIH3T3 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 data clearly show that FGF treatment of NIH3T3 fibroblasts induces DUSP6/MKP-3 expression at the level of both mRNA and protein. Furthermore, while inhibition of ERK1/2 activity blocks this expression completely, we can find no evidence for the involvement of PI3-kinase in this process. This data is reinforced by the observation that conditional activation of the ERK1/2 MAPK pathway in cells expressing  $\Delta$ -Raf-1ER\* 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, which contained 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 missing 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 factor-binding site. Many growth factor-activated genes such as c-Fos are regulated by Serum response factor (SRF) a MADS-box transcription factor. SRF forms a ternary complex with Ets family members such as Elk1 also known as ternary complex factors (TCFs), whose activity is controlled by MAPK signaling [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 EMSA assays 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 NIH3T3 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-factor 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 most likely a reflection of the conservation of the regulatory sequences revealed by our promoter analysis. Scanning genomic fragments for enhancer activity utilising 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 mouse 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 Ets 2 as binding to the DUSP6/MKP-3 promoter in NIH3T3 cells and the question arises as to their expression in the tissues we have analysed here. 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 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 PI3-kinase 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 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 genes 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, these include 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.

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## FIGURE LEGENDS

**Figure 1 The expression of DUSP6/MKP-3 mRNA protein and mRNA is inducible by fibroblast growth factor in NIH3T3 cells.** (A) NIH3T3 cells were serum starved overnight before exposure to FGF2, FGF4 or FGF8 (all at 30ng/ml) for 5h. Cells were then lysed and proteins were analysed by SDS-PAGE and Western blotting using antisera against MKP-3, phospho-ERK, ERK, phospho-p38, p38, phospho-JNK and JNK (B) NIH3T3 cells were serum starved overnight and then exposed to FGF2 (30ng/ml) for the indicated time before lysis and analysis of proteins by SDS-PAGE and Western blotting using antisera against MKP-3, phospho-ERK, ERK, phospho-Akt or Akt (upper panel). NIH3T3 cells were serum starved overnight and then exposed to FGF2 (30ng/ml) for the indicated time either in the absence or presence of the PI3-kinase inhibitor LY294002 (10 $\mu$ M) before lysis and analysis of proteins by SDS-PAGE and Western blotting using antisera against phospho-Akt or Akt (lower panel) (C) NIH3T3 cells were serum starved overnight before exposure to FGF2 (30ng/ml) for the indicated time. 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 mean values with associated errors.

**Figure 2 The induction of DUSP6/MKP-3 protein and mRNA by FGFs is blocked by chemical inhibitors of either the FGF-receptor tyrosine kinase or MAPK kinase, but not by a specific inhibitor of PI3-kinase activity in NIH3T3 cells.** NIH3T3 cells were serum starved overnight before exposure to FGF2, FGF4 or FGF8 (all at 30ng/ml) for 5h either in the absence (DMSO vehicle only) or presence of (A) the FGF-receptor inhibitor SU5402 (50 $\mu$ M), (B) The MAPK kinase inhibitor PD184352 (2 $\mu$ M) or (C) The PI3-kinase inhibitor LY294002 (10 $\mu$ M) before lysis and analysis of proteins by SDS-PAGE and Western blotting using antisera against MKP-3, phospho-ERK, ERK, and

phospho-AKT. (D) NIH3T3 cells were serum starved overnight before exposure to FGF2, FGF4 or FGF8 (all at 30ng/ml) for 90 min either in the absence (DMSO vehicle only) or 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 mean values with associated errors.

**Figure 3 ERK activation is both necessary and sufficient for the induction of DUSP6/MKP-3 protein in NIH3T3 cells.** (A) Either  $\Delta$ -Raf 3T3 or NIH3T3 cells were serum starved overnight and then exposed to the indicated concentration of 4-hydroxytamoxifen (4-HT) in either the absence or presence of PD184352 (2 $\mu$ M). As a control, cells were exposed to FGF4 (30ng/ml). After 5h incubation, cells were lysed and proteins were analysed by SDS-PAGE and Western blotting using antisera against MKP-3, phospho-ERK and ERK. (B)  $\Delta$ -Raf 3T3 cells were serum starved overnight and then exposed to either the indicated concentration of 4-hydroxytamoxifen (4-HT) or FGF4 (30ng/ml) in the absence or presence of the FGF-receptor inhibitor SU5402 (50 $\mu$ M). After 5h incubation, cells were lysed and proteins were analysed by SDS-PAGE and Western blotting using antisera against MKP-3, phospho-ERK, and ERK.

**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 (forward strand) or black hatched (reverse strand) and identified as follows: FKHD (Forkhead family), ELK1 (Ets factor binding site), NF $\kappa$ B (Nuclear Factor kappa-B), PBXC (PBX1 - MEIS1 complexes), HNF1 (Hepatic Nuclear Factor 1), SOX5 (Sox/SRY-sex/testis determining and

related HMG box factors), RFX1 (Regulatory Factor X 1), CAAT box (CAAT box: promoter element in some genes located about 75-80 base pairs 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 expressed sequence tags (ESTs) for DUSP6/MKP-3. (B) Schematic 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 *Bam* H1 restriction enzyme sites used to subclone the longest (6399 bp) genomic fragment into the firefly luciferase reporter plasmid. The position of the conserved region containing the putative transcription factor binding sites is also indicated. (C) The constructs indicated in (B) were co-transfected into NIH3T3 cells along with pRL-TK Renilla to normalise 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 24h after which cells were lysed and luciferase assays performed. DpGL3Basic acted as a negative (promoterless) control. Luciferase assays were performed in quadruplicate and mean values (Firefly to Renilla ratios) are presented graphically with associated errors.

**Figure 5 FGF-dependent DUSP6/MKP-3 promoter activity is mediated by activation of ERK and not by activation of the PI3-kinase pathway.** (A) The 507 bp DUSP6/MKP-3 reporter construct was co-transfected into NIH3T3 cells along with pRL-TK Renilla to normalise 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) either in the

absence or presence of either vehicle (DMSO), PD184352 (2 $\mu$ M) or LY294002 (10 $\mu$ M) for an additional 24h. Cells were then lysed and luciferase assays performed in quadruplicate. Results are presented graphically as mean values (Firefly to Renilla ratios) with associated errors. (B) Either the 507 bp DUSP6/MKP-3 reporter construct or a plasmid encoding a GAL4-Elk1 fusion protein together with a GAL4-dependent luciferase reporter was co-transfected into NIH3T3 cells along with pRL-TK Renilla to normalise for transfection efficiency and either empty pSG5 expression vector or pSG5 encoding either a constitutively active mutant of MAPK kinase (MEKEE) or DUSP6/MKP-3 itself. After 24h, cells were lysed and luciferase assays performed in quadruplicate. Results are presented graphically as mean values (Firefly to Renilla ratios) with associated errors. (C)  $\Delta$ -Raf 3T3 cells were co-transfected with the 507 bp DUSP6/MKP-3 reporter construct and pRL-TK Renilla to normalise for transfection efficiency. Cells were then starved overnight and either left untreated or treated with either FGF4 (30ng/ml) or 4-hydroxytamoxifen (4-HT, 100nM) in the presence or absence of either vehicle (DMSO), PD184352 (2 $\mu$ M) or LY294002 (10 $\mu$ M). After 24h, cells were lysed and luciferase assays performed in quadruplicate. Results are presented graphically as mean values (Firefly to Renilla ratios) with associated errors.

**Figure 6 A conserved Ets-factor binding site is required for FGF-inducible DUSP6/MKP-3 promoter activity.** (A) NIH3T3 cells were co-transfected with either the wild-type 507 bp DUSP6/MKP-3 reporter construct or the indicated mutants together with pRL-TK Renilla to normalise 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 24h, cells were lysed and luciferase assays performed in quadruplicate. Results are presented graphically as mean values (Firefly to Renilla ratios) with associated errors. (B) NIH3T3 cells were co-transfected with the indicated reporter constructs along with either RSV-Mad3 (20 or 100ng) or as a control

RSV- $\beta$ Gal and pRL-TK Renilla to normalise for transfection efficiency. Cells were then serum starved overnight (0.5% serum) and either left untreated or exposed to either FGF4 (30 ng/ml) for 24h or TNF $\alpha$  (10ng/ml) for 6h. Cells were then lysed and luciferase assays performed in quadruplicate. Results are presented graphically as mean values (Firefly to Renilla ratios) with associated errors. (C) NIH3T3 cells were co-transfected with either the wild-type 507 bp DUSP6/MKP-3 reporter construct or a 507 bp reporter in which the forkhead-binding site was mutated (FKHD) together with pRL-TK Renilla to normalise 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) either in the absence (DMSO) or presence of PD184352 (2 $\mu$ M). After 24h, cells were lysed and luciferase assays performed in quadruplicate. Results are presented graphically as mean values (Firefly to Renilla ratios) with associated errors.

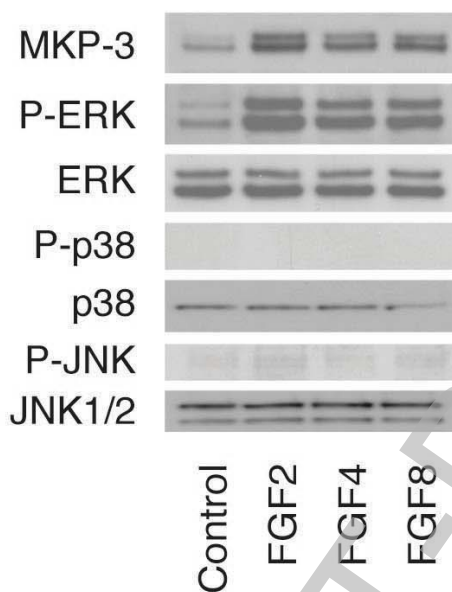
**Figure 7 Ets-family transcription factors bind to the endogenous DUSP6/MKP-3 gene promoter.** (A) EMSA assays were performed using the following labelled oligonucleotide probes; wild type MKP-3 or an Ets site mutant MKP-3 (MKP-3m); wild-type canonical Ets-factor binding site (E74) or its corresponding Ets site mutant (E74m). Labelled probes were then incubated with nuclear extract from NIH3T3 cells either in the absence or 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 visualised by electrophoresis and autoradiography. (B) Chromatin immunoprecipitation (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 factor binding site and the non-specific control reactions are shown, as are the controls lacking template and the results of PCR reactions performed using the input chromatin as template. (C) NIH3T3

cells were either transfected with the 507 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 MAP kinase phosphorylation site (Ets2 T72A) or wild-type Ets2. Following transfection, cells were cultured overnight in medium containing 10% serum. Cells were then lysed and luciferase assays performed in triplicate. Results are presented graphically as mean values (Firefly to Renilla ratios) with associated errors.

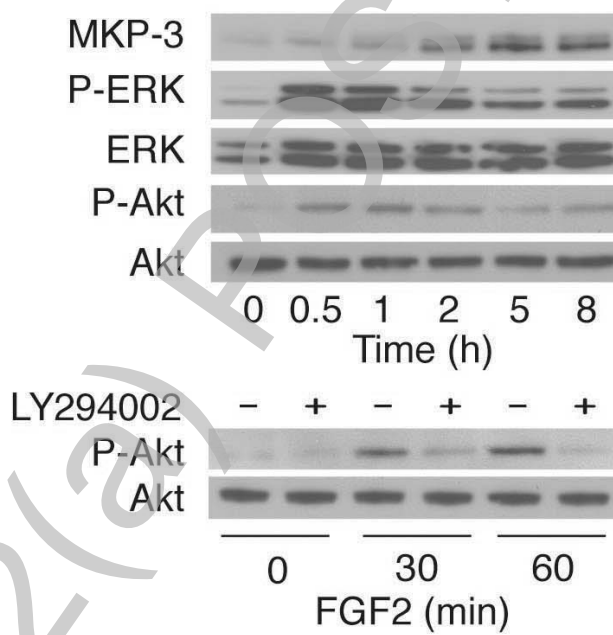
**Figure 8 The 507 bp DUSP6/MKP-3 promoter fragment directs transgene expression in an endogenous DUSP6/MKP-3 domain.** (A) Schematic diagram of the chick embryo at 1.5 days. Boxed area indicates approximate field in (B-I). (B) Comparison of ERK1/2 phosphorylation and DUSP6/MKP-3 mRNA expression in the caudal region of the day 1.5 chick embryo. (C) Day 1.5 chick embryo co-electroporated with a plasmid constitutively expressing RFP (CAGmRFP) and the 507 bp fragment of the DUSP6/MKP-3 promoter driving expression of EGFP (507ptkd2EGFP) only within the endogenous DUSP6/MKP-3 domain. (D) Embryo co-electroporated with plasmid 507ptkd2EGFP in which the Ets binding site is mutated (EtsMut) and CAGmRFP shows reduced EGFP expression. (E) Co-electroporation of plasmid 507ptkd2EGFP in which the forkhead binding site is mutated (FKHDMut) and CAGmRFP. (F-H). Embryos were co-electroporated with the wild-type DUSP6/MKP-3 promoter fragment (507ptkd2EGFP) and CAGmRFP before beads soaked in either DMSO (F), 5mM SU5402 (G), 20mM PD184352 (H) or 20mM LY294002 (I) were placed next to the electroporation site. Both SU5402 and PD18432, but not either LY294002 or DMSO alone, cause a local down-regulation of DUSP6/MKP-3 promoter activity. \*indicates grafted bead and the arrowhead indicates the position of the last formed somite, nt = neural tube, cnp = caudal neural plate.

Figure 1

**A**



**B**



**C**

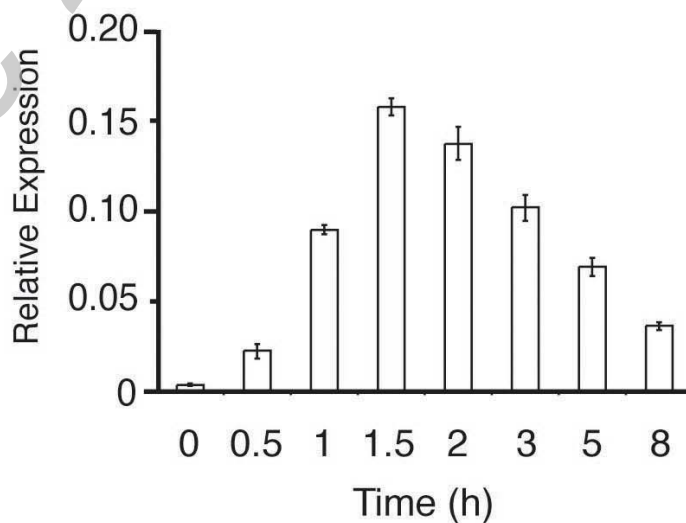




Figure 2

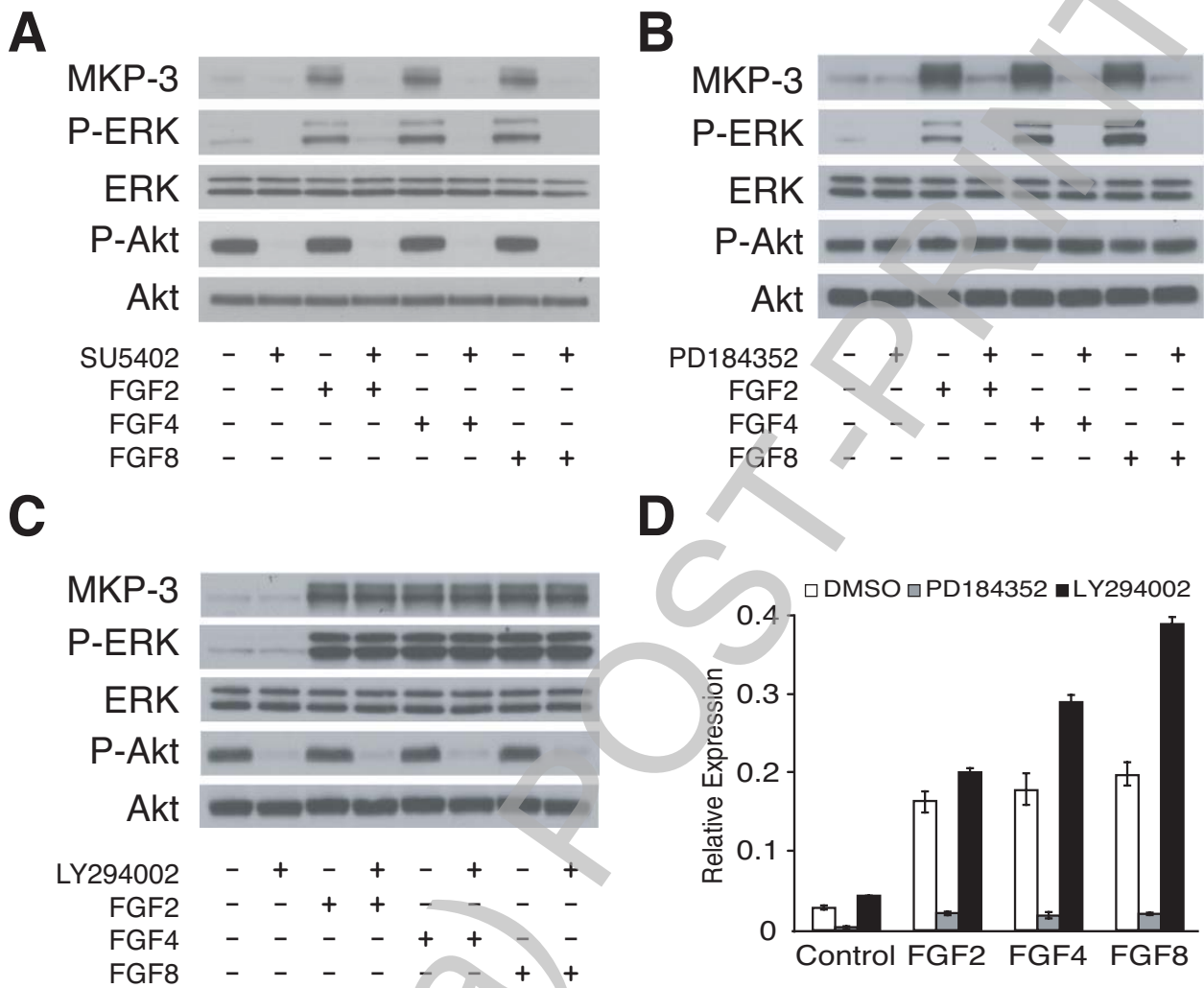


Figure 3

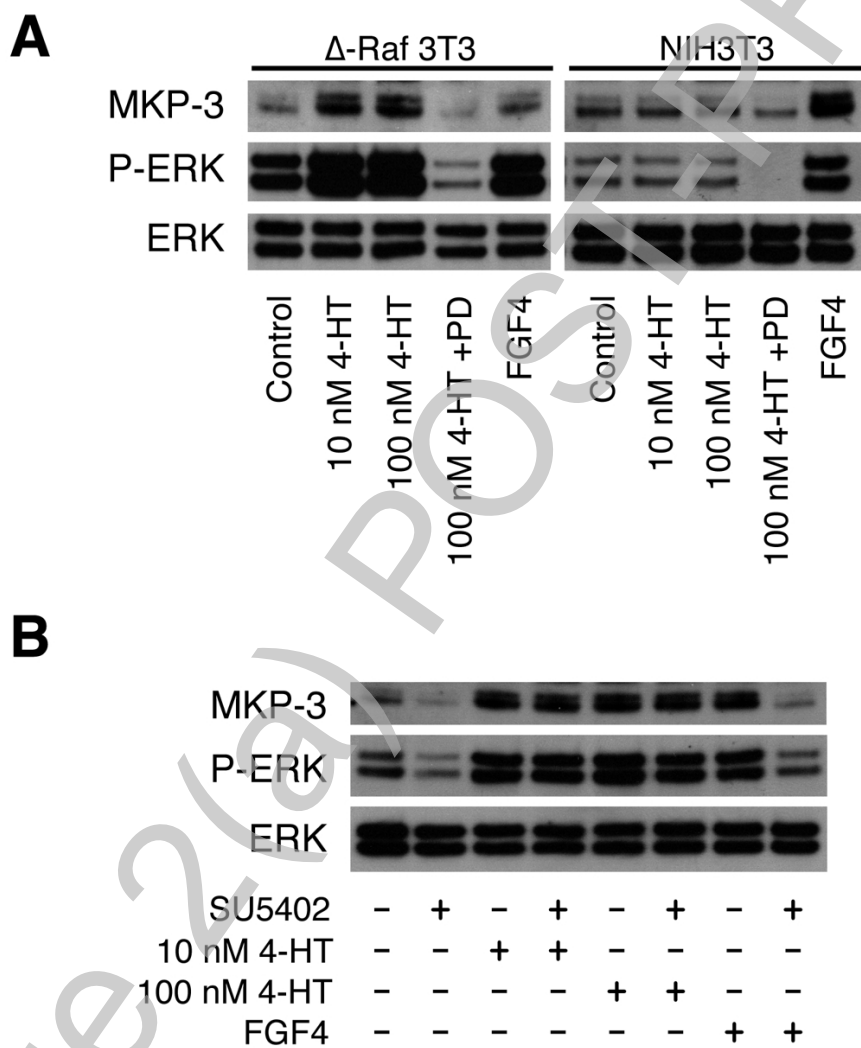
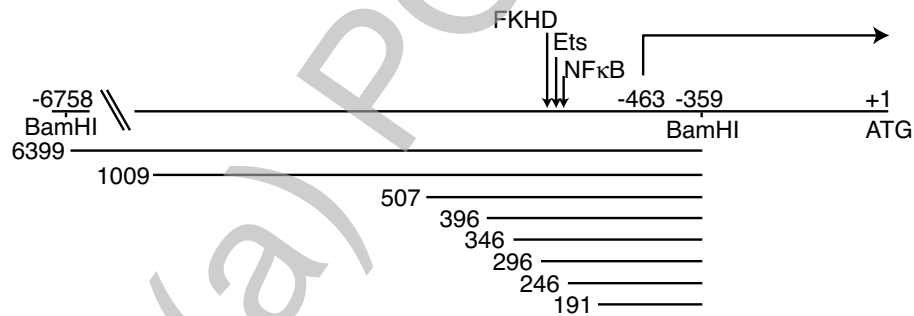


Figure 4

**A**



**B**



**C**

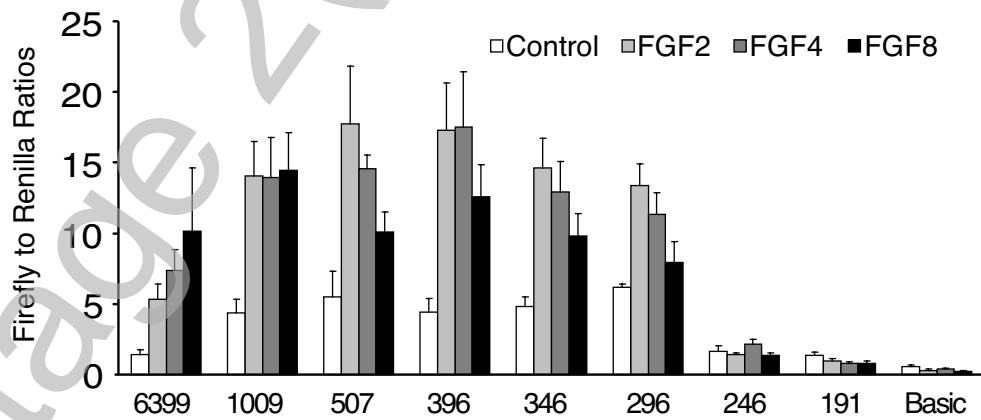
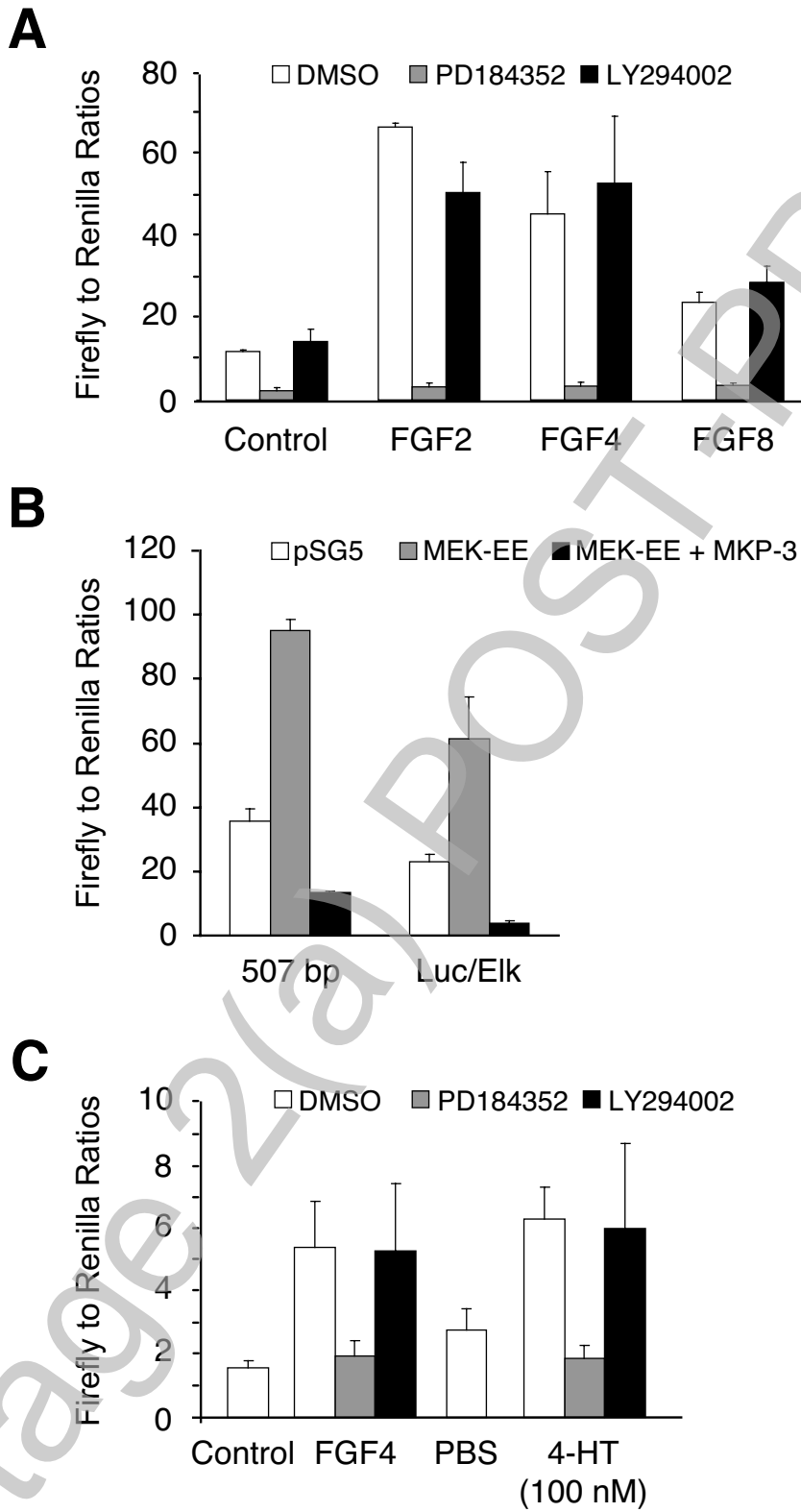
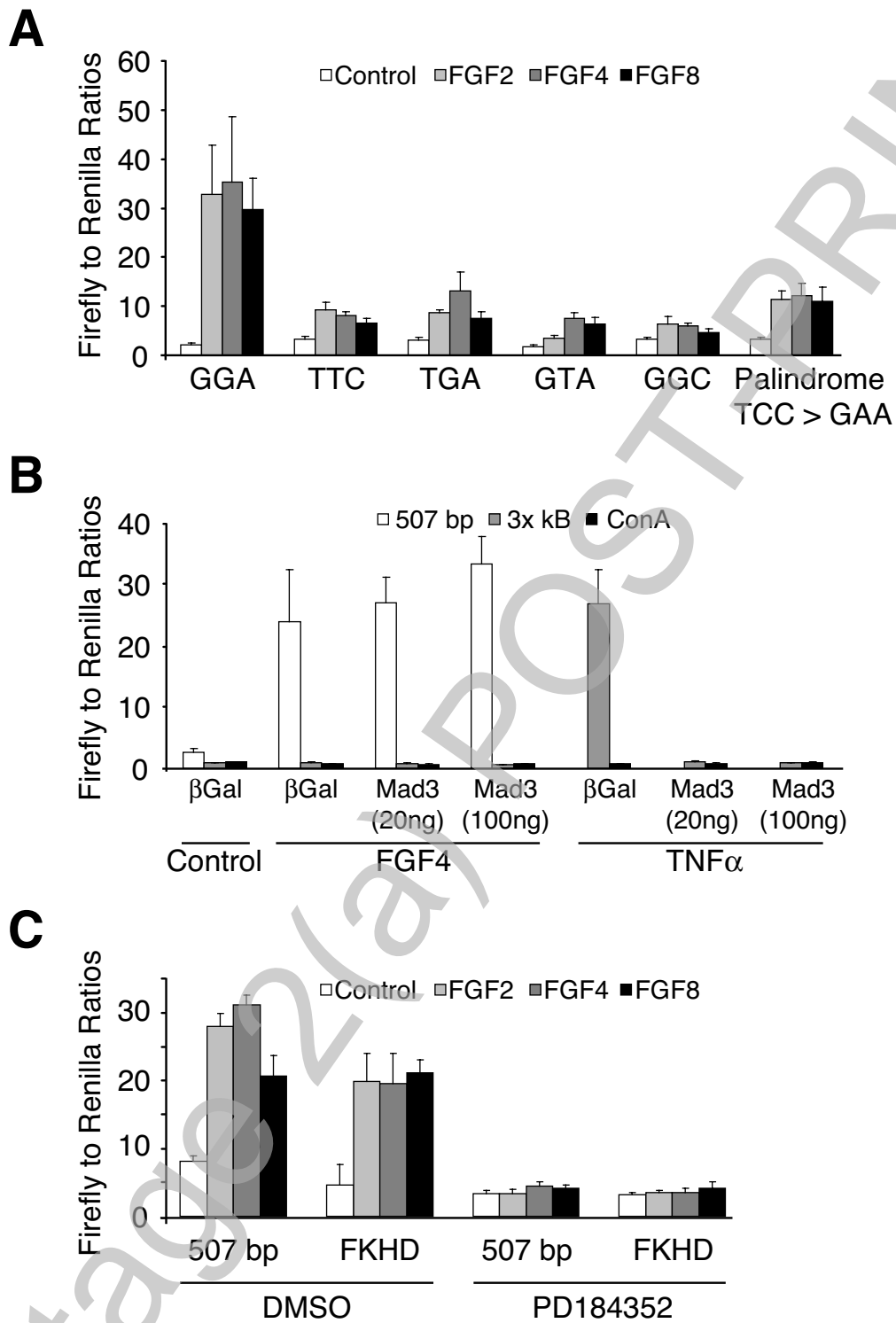


Figure 5



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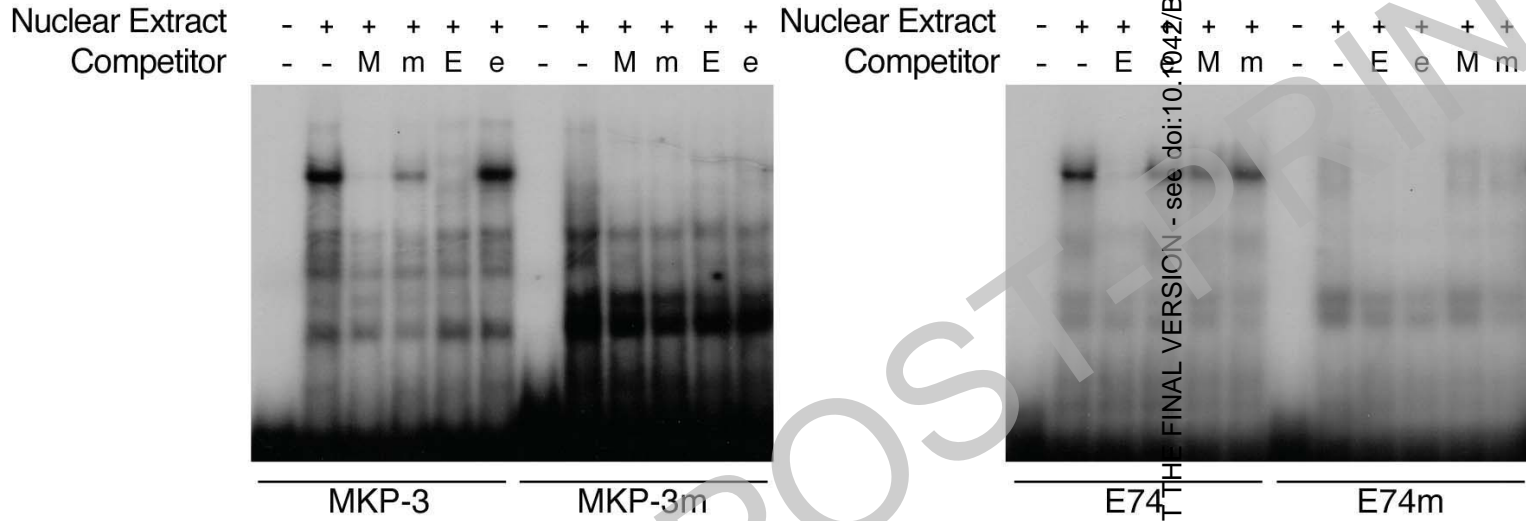
Figure 6



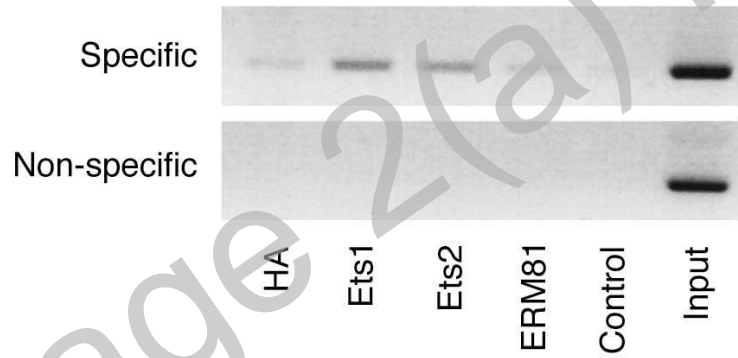
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Figure 7

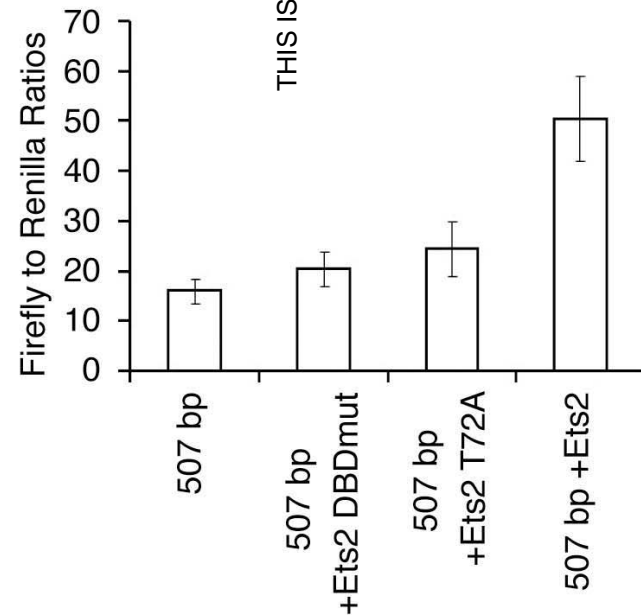
**A**



**B**



**C**



**Figure 8**

