

Activation of MK5/PRAK by the atypical MAP kinase ERK3 defines a novel signal transduction pathway

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Extracellular signal-regulated kinase 3 (ERK3) is an atypical mitogen-activated protein kinase (MAPK), which is regulated by protein stability. However, its function is unknown and no physiological substrates for ERK3 have yet been identified. Here we demonstrate a specific interaction between ERK3 and MAPK-activated protein kinase-5 (MK5). Binding results in nuclear exclusion of both ERK3 and MK5 and is accompanied by ERK3-dependent phosphorylation and activation of MK5 *in vitro* and *in vivo*. Endogenous MK5 activity is significantly reduced by siRNA-mediated knockdown of ERK3 and also in fibroblasts derived from ERK3^{-/-} mice. Furthermore, increased levels of ERK3 protein detected during nerve growth factor-induced differentiation of PC12 cells are accompanied by an increase in MK5 activity. Conversely, MK5 depletion causes a dramatic reduction in endogenous ERK3 levels. Our data identify the first physiological protein substrate for ERK3 and suggest a functional link between these kinases in which MK5 is a downstream target of ERK3, while MK5 acts as a chaperone for ERK3. Our findings provide valuable tools to further dissect the regulation and biological roles of both ERK3 and MK5.

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Introduction

Mitogen-activated protein kinase (MAPK) cascades are highly conserved signal transduction pathways, which relay a wide variety of extracellular signals to elicit an appropriate biological response (Garrington and Johnson, 1999). In mammalian cells and tissues, these include proliferation, differentiation, inflammation and apoptosis. MAPK activation requires the phosphorylation of both the threonine and tyrosine residues of a conserved T-X-Y motif within the activation loop of the kinase by a dual-specificity MAPK kinase (MKK or MEK) (Marshall, 1994). Mammalian MAPKs are subdivided into three major classes based on sequence homology and differential activation by agonists (Davis, 2000; Kyriakis and Avruch, 2001; Johnson and Lapadat, 2002). These include the growth factor-activated extracellular signal-regulated kinase (ERK) 1 and 2 and two families of stress-activated MAPKs, the c-Jun amino-terminal kinases (JNKs) and the p38 MAPKs.

ERK3 was first identified by sequence homology to ERK1, showing identities as high as 83 and 72% within kinase subdomains V and IX, respectively, and with a subdomain structure placing it firmly within the MAPK family (Boulton *et al*, 1991; Zhu *et al*, 1994; Cheng *et al*, 1996; Meloche *et al*, 1996). Despite this, ERK3 exhibits striking differences compared with other MAPK isoforms. Most notably, the highly conserved T-E-Y motif is substituted by S-E-G and ERK3 also contains a unique C-terminal extension. Until recently, almost nothing was known about the regulation and function of ERK3. ERK3 mRNA is widely distributed in adult tissues, but is markedly upregulated during mouse development (Turgeon *et al*, 2000). In addition, ERK3 mRNA is also upregulated during differentiation of P19 embryonal carcinoma cells towards the neuronal or muscle lineages (Boulton *et al*, 1991). Taken together, these studies suggest a possible role for ERK3 during embryogenesis and cellular differentiation.

Many known MAPK substrates are not phosphorylated by ERK3 *in vitro*, indicating that it targets distinct and perhaps highly specific proteins *in vivo* (Cheng *et al*, 1996). Furthermore, serine 189 within the S-E-G motif of ERK3 is constitutively phosphorylated *in vivo*, indicating that ERK3 is not regulated like other MAPK family members (Cheng *et al*, 1996; Julien *et al*, 2003). The latter idea was reinforced by the finding that ERK3 is highly unstable, with rapid turnover mediated by ubiquitination and proteosomal degradation (Coulombe *et al*, 2003, 2004). Interestingly, ERK3 is stabilised on differentiation of both PC12 and C2C12 cells into the neuronal and muscle lineage, respectively, and expression of stable mutants of ERK3 caused cell cycle arrest in NIH3T3 cells. This suggests that rather than being regulated by inducible phosphorylation, the biological activity of ERK3 may be determined by its cellular abundance (Coulombe *et al*, 2003). Despite these advances, further progress in elucidating

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the regulation, biochemical activity and physiological function of ERK3 is hampered by ignorance of its cellular targets.

We and others reported that both the activity and sub-cellular localisation of MAPK-activated protein kinase-5 (MK5 or PRAK) is subject to regulation by p38 (Seternes *et al*, 2002; New *et al*, 2003). However, these experiments relied on overexpression of MK5 and p38 in mammalian cells, and recent studies of endogenous MK5 do not support the idea that p38 is a physiological regulator of MK5 (Shi *et al*, 2003). In particular, endogenous MK5 was not activated in response to stimuli that activate p38, no interaction was detected between endogenous MK5 and p38 and, unlike MK2, MK5 did not display chaperoning properties towards p38 (Shi *et al*, 2003).

To address the interaction of MK5 with the MAPK family of enzymes, we performed a yeast two-hybrid screen in which MK5 was assayed against a comprehensive panel of MAPKs. Surprisingly, ERK3, but not p38, emerged as a specific binding partner for MK5. Binding was direct and the interaction of endogenous MK5 and ERK3 was confirmed. Interestingly, this also promoted the nuclear export of both ERK3 and MK5 and increased the activity of MK5 towards peptide substrates both *in vitro* and *in vivo*. To establish that ERK3 regulates MK5 *in vivo*, we employed siRNA to knock down endogenous ERK3 expression in HeLa cells and also

derived mouse embryo fibroblasts (MEFs) from ERK3^{-/-} mice. In both cases, we observed a significant reduction in the activity of endogenous MK5. Furthermore, we show that the elevated levels of ERK3 protein seen during PC12 cell differentiation are accompanied by increased MK5 activity *in vivo*. Conversely, experiments in which siRNA was used to knock down endogenous MK5 revealed that loss of MK5 protein was accompanied by a dramatic fall in the levels of endogenous ERK3, which could be rescued by ectopic expression of MK5. Finally, we demonstrate that activated MK5 is able to phosphorylate ERK3 both *in vitro* and *in vivo*, indicating that in addition to being an activator of MK5, ERK3 is also a physiological target for MK5. Our results identify the first physiological target for ERK3 in mammalian cells and indicate a strong functional link between these two protein kinases.

Results

MK5 interacts specifically and directly with ERK3

Yeast two-hybrid assays were used to determine the specificity of interactions between MK5 and a panel of nine distinct MAPK isoforms *in vivo*. Binding was determined by the activation of GAL4-dependent ADE2/HIS3/*lacZ* reporters. Activation of ADE2/HIS3 was assessed by selection on syn-

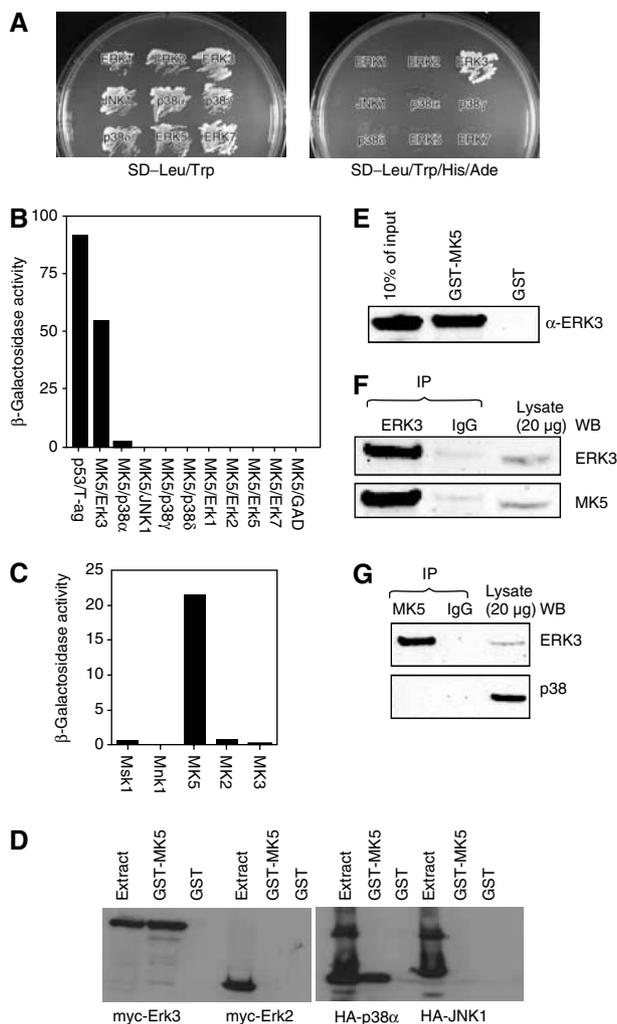


Figure 1 MK5 interacts specifically with ERK3. (A) Yeast two-hybrid assay. pGBKT7-MK5 was transformed into PJ69-2A and mated with Y187 expressing the indicated GAL4AD fusions. Yeast diploids expressing both DB and AD fusions were selected on synthetic dropout (SD) medium deficient for leucine and tryptophan (SD-leu/trp, left panel). Leu/trp positives were restreaked onto SD minus leucine, tryptophan, histidine and adenine (SD-leu/trp/his/ade, right panel), and protein-protein interactions were assessed by growth on this medium. (B) Semiquantitative analysis of induction of the β -galactosidase reporter for MK5 and the indicated MAPKs. Interactions between SV40 large T antigen and p53 and MK5 and GAL4-AD alone are used as positive and negative controls, respectively. Assays were performed in triplicate, and the results of a representative experiment are shown. (C) pGBKT7 expressing GAL4 DB fusions of the indicated MAPKAP-kinases were transformed into PJ69-2A and mated with Y187 expressing the GAL4 AD fusion pGADT7.ERK3. Semiquantitative analysis of induction of the β -galactosidase reporter was performed in triplicate and the results of a representative experiment are shown. (D) Lysates of COS-1 cells transfected with either Myc-tagged ERK3, Myc-tagged ERK2, HA-tagged p38 α or HA-tagged JNK-1 were mixed with either GST-MK5 or GST alone. Bound MAPKs were detected by Western blotting using an anti-Myc antibody (ERK3 and ERK2, left panel) and an anti-HA antibody (p38 α and JNK-1, right panel). (E) Direct interaction between ERK3 and MK5 *in vitro* assayed by GST pull-down. Recombinant ERK3 (1 μ g) was mixed with 2 μ g of either GST-MK5 or GST alone and glutathione agarose. Bound ERK3 was detected using an anti-ERK3 antibody. (F) Co-immunoprecipitation of ERK3 and MK5 in RAW 264.7 cells using an anti-ERK3 antibody. ERK3 was immunoprecipitated from cell lysates using a monoclonal anti-ERK3 antibody and protein G-Sepharose and immunoprecipitates analysed by Western blotting using either a polyclonal ERK3 antibody (upper panel) or a polyclonal MK5 antibody (lower panel). Control immunoprecipitations were performed using preimmune IgG antibodies. Total cell lysate (20 μ g) was also analysed in parallel. (G) Co-immunoprecipitation of ERK3 but not p38 in RAW 264.7 cells using an anti-MK5 antibody. MK5 was immunoprecipitated from cell lysates using a polyclonal anti-MK5 antibody and protein G-Sepharose. Immunoprecipitates were then analysed by Western blotting using either a monoclonal ERK3 antibody (upper panel) or a monoclonal p38 antibody (lower panel). Control immunoprecipitations were performed using preimmune IgG antibodies. Total cell lysate (20 μ g) was also analysed in parallel.

thetic dropout medium deficient for adenine and histidine and the strength of interaction was quantified in a β -galactosidase assay. MK5 interacts specifically with ERK3 with no significant interaction detected with p38 α , p38 δ , p38 γ , JNK1, ERK1, ERK2, ERK5 or ERK7 (Figure 1A and B). To further examine the specificity of interaction between ERK3 and MK5, we used five MAPK-activated protein kinases (MAPKAP-kinases) as baits in the two-hybrid assay. These include MK2 and MK3, which are most highly related to MK5 (45 and 46% identity, respectively). ERK3 interacted specifically with MK5 showing no significant binding to MK2, MK3, MSK1 or MNK1 (Figure 1C). To confirm the specificity of MAPK binding, we used GST pulldown assays employing both transfected cell lysates and purified recombinant proteins. In agreement with the yeast two-hybrid results, MK5 binds to ERK3 and not to either ERK2 or JNK1 (Figure 1D). Furthermore, this interaction is direct as it is also detected using purified recombinant ERK3 and GST-MK5 (Figure 1E). In agreement with our previous study (Seternes *et al*, 2002), MK5 also interacted with p38 α (Figure 1D). Finally, to determine the ability of endogenous MK5 to interact with either ERK3 or p38, we performed co-immunoprecipitation experiments using the RAW246.7 mouse macrophage cell line. Endogenous MK5 was readily detected in immunoprecipitates of endogenous ERK3 (Figure 1F) and endogenous ERK3, but not p38 was readily detected in immunoprecipitates of endogenous MK5 (Figure 1G). We conclude that MK5 binds specifically and directly to ERK3 both *in vitro* and *in vivo*. In contrast, although we could detect interactions between MK5 and p38 *in vitro*, we found no evidence of interaction between endogenous MK5 and p38.

Coexpression of ERK3 and MK5 causes the redistribution of both proteins from the nucleus to the cytoplasm

To examine the subcellular distributions of MK5 and ERK3, we expressed either wild-type ERK3 or an EGFP-MK5 fusion protein in HeLa cells. As described previously (Seternes *et al*, 2002), EGFP-MK5 is predominantly nuclear when expressed in these cells (Figure 2A). In contrast, ERK3 is distributed in both the nuclear and cytoplasmic compartments (Figure 2B). However, on coexpression, a profound change in the subcellular localisation of ERK3 and MK5 was seen, with both proteins now excluded from the nucleus (Figure 2C). Identical results were obtained in NIH3T3, HEK293 and COS-1 cells (data not shown). The kinase activities of either protein are not required for relocalisation, as 'kinase-dead' mutants of either MK5 or ERK3 are also predominantly cytoplasmic (Figure 2D). To examine the requirement for specific protein-protein interactions between MK5 and ERK3, we used two truncations of ERK3. The first of these (ERK3 1-330) no longer interacts with MK5 in GST pulldown assays, while the second (ERK3 1-340) readily binds to MK5 (Figure 3A). Although both proteins exhibit subcellular distributions that are indistinguishable from the full-length protein (Figure 3B), only ERK3 1-340 causes the redistribution of MK5 from the nucleus to the cytoplasm when the two proteins are coexpressed (Figure 3C).

We next determined if an active nuclear export pathway is essential for relocalisation of MK5 and ERK3. Both proteins were coexpressed in HeLa cells either in the absence or presence of leptomycin B (LMB), an inhibitor of CRM1-

dependent nuclear export (Wolff *et al*, 1997; Kudo *et al*, 1998). LMB blocks the redistribution of both MK5 and ERK3, indicating that a leucine-rich NES present on either or both of these proteins is required to mediate the relocalisation seen on coexpression (Figure 4A and B). This is consistent with a recent report demonstrating CRM1-dependent export of ERK3 (Julien *et al*, 2003).

The export of MK5 in response to ERK3 is reminiscent of the effect of coexpressing p38 (Seternes *et al*, 2002). To ask if both events are mediated by a similar mechanism, we used mutated and truncated forms of MK5, which show differential binding to ERK3 and p38. Mutation of the nuclear localisation sequence in MK5 (GSTMK5mutNLS) reduces its ability to interact with p38 in GST pulldown assays, but permits interaction with ERK3 (Figure 5A and B). Conversely, a truncated form of MK5 (EGFP-MK5 1-423) no longer binds to ERK3, but can bind to p38 (Figure 5A and B). The latter form of MK5 is efficiently exported from the nucleus in response to activation of the p38 pathway (MKK6E/E + p38) but is unresponsive to expression of ERK3 (Figure 5C). To examine any possible connection between p38 activity and nuclear export of ERK3, we also coexpressed ERK3 alone with MKK6E/E + p38. We observed no change in ERK3 localisation, nor was this affected by treatment of cells with sodium arsenite (data not shown). Finally, the nuclear export of MK5 in response to activation of p38 requires phosphorylation of Thr182 (Seternes *et al*, 2002; New *et al*, 2003). To determine if this is also required for the relocalisation of MK5 by ERK3, we coexpressed a mutant form of MK5 in which this site was mutated to alanine (EGFP-MK5T182A) and either ERK3 or p38 plus MKK6E/E. As observed previously, MKK6E/E + p38 failed to cause nuclear export of MK5T182A (Figure 5D). In contrast, this mutant was exclusively cytoplasmic when coexpressed with ERK3, thus demonstrating no requirement for T182 phosphorylation for MK5 to relocalise in response to ERK3 binding.

ERK3 phosphorylates and activates MK5 both *in vitro* and *in vivo*

The interactions between ERK3 and MK5 strongly suggest an enzyme-substrate relationship between these two proteins. To investigate this, we expressed and purified wild-type ERK3 and a 'kinase-dead' mutant (ERK3 D171A) in insect Sf9 cells. When analysed by SDS-PAGE, both proteins migrated as a single band of approximately 100 kDa, indicating that they are full length and undegraded (Figure 6A). Furthermore, both proteins were recognised by an anti-phospho antibody against serine 189 of the S-E-G motif, demonstrating that this residue is constitutively phosphorylated in Sf9 cells (Figure 6A). We also produced ERK3 in which S189 was mutated to glutamic acid (S189E) and this serves as a negative control for the anti-phospho S189 antibody (Figure 6A).

To determine if ERK3 is capable of activating MK5 *in vitro*, we incubated either wild-type ERK3 or kinase-dead (ERK3D171A) protein with inactive recombinant MK5 in the presence of ATP and Mg²⁺ and monitored MK5 activity using PRAKTide (KKLRRTLSVA, derived from glycogen synthase). Wild-type ERK3, but not ERK3D171A, caused a significant increase in MK5 activity towards this substrate (Figure 6B). Furthermore, an antiserum specific for phosphorylation of

the regulatory site (T182) within the activation loop of MK5 demonstrates that activation of MK5 by ERK3 was accompanied by phosphorylation of Thr182 (Figure 6C). The ERK3

protein in which the putative regulatory serine (S189) of the S-E-G motif was mutated to glutamic acid (ERK3S189E) also activated MK5, albeit with lower efficiency, and activation was also accompanied by phosphorylation of Thr182 (Figure 6B and C). We conclude that ERK3 is capable of activating MK5 *in vitro*, and that this activation requires ERK3 kinase activity and is accompanied by phosphorylation of the key regulatory site (Thr182) of MK5. Furthermore, the result obtained using the S189E mutant of ERK3 suggests that phosphomimetic substitution of this residue is able to activate, at least in part, the kinase activity of ERK3 towards MK5.

To explore the ability of ERK3 to activate MK5 under more physiological conditions, HeLa cells were transfected with expression constructs encoding EGFP-MK5, either alone or in combination with either Myc-tagged wild-type or kinase-dead ERK3. EGFP-MK5 was then immunoprecipitated from these cells and assayed for activity towards PRAKTide as before. As positive controls for these experiments, EGFP-MK5 was also coexpressed with either p38 alone or p38 plus MKK6E/E, the latter combination having previously been demonstrated to activate MK5 in these cells (Seternes *et al*, 2002). Under these conditions, wild-type ERK3 but not the kinase-dead (D171A) mutant activates MK5. Furthermore, the level of MK5 activity is comparable with that seen following activation of p38 (Figure 7A) and is also dependent on phosphorylation of Thr182 in MK5, as mutation of this residue abolishes activation by ERK3 (Figure 7B). Finally, given the ability of p38 to activate MK5, it was critical to demonstrate that ERK3 was not activating MK5 indirectly by activating the p38 pathway. However, treatment of cells with SB203580, a specific p38 inhibitor (Cuenda *et al*, 1995), had no effect on the activation of MK5 achieved by cotransfection of ERK3. In contrast, the activation of MK5 by p38 plus MKK6E/E was completely abolished by this drug (Figure 7C). We conclude that ERK3 is capable of activating MK5 *in vivo*, that activation is mediated by phosphorylation of Thr182 in MK5 and that this is not dependent on the activity of p38.

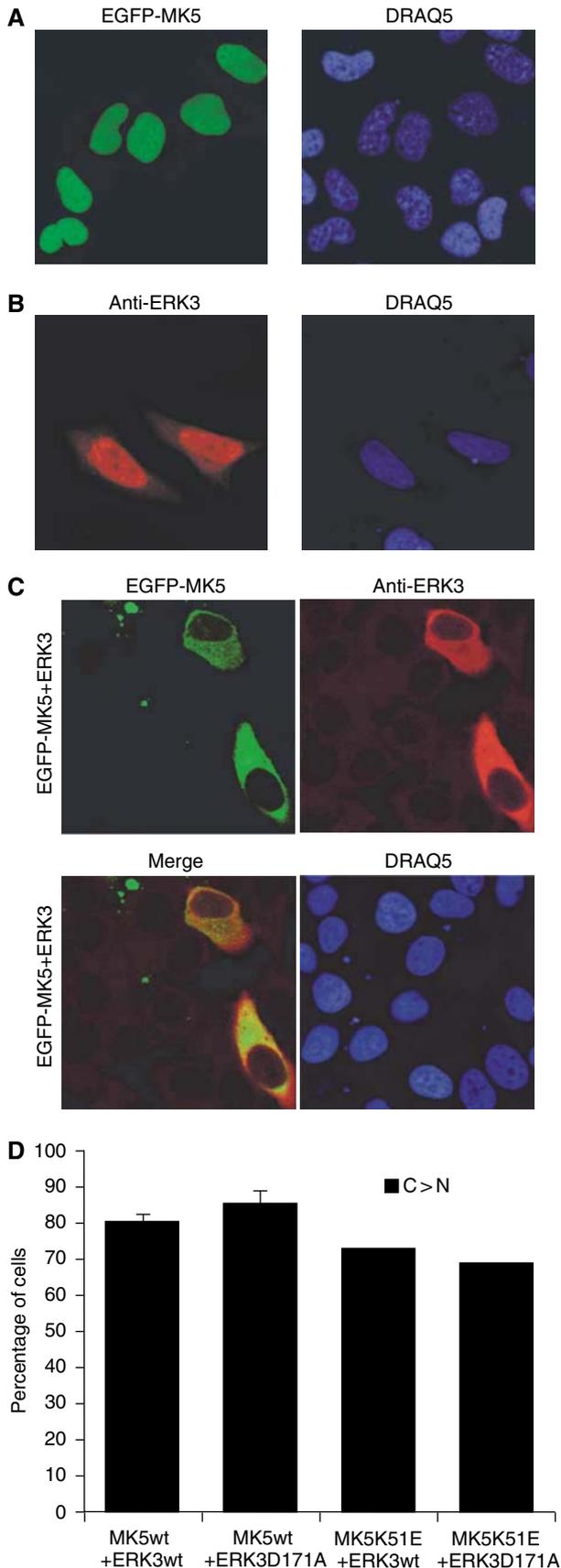
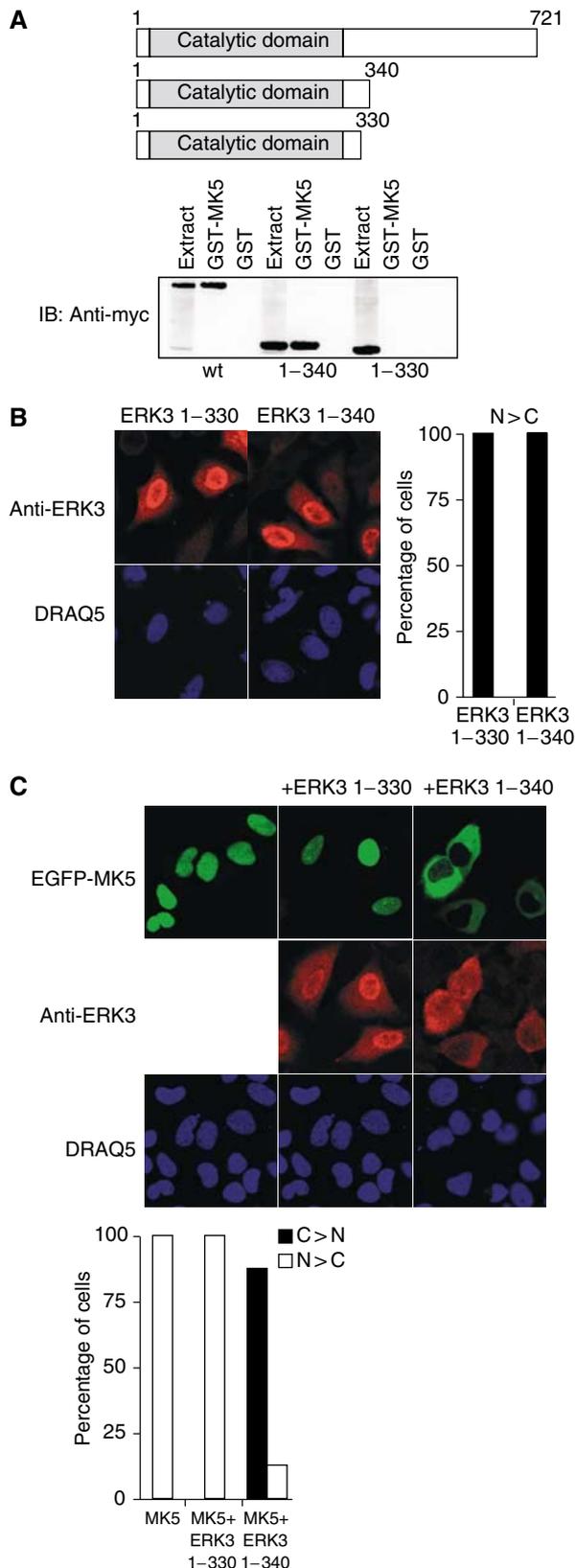


Figure 2 Coexpression of MK5 and ERK3 leads to relocalisation of both proteins. (A) HeLa cells were transfected with expression vector encoding EGFP-MK5. EGFP fluorescence was visualised directly (green channel on left) and cell nuclei were visualised by DRAQ5 staining (blue channel on right). (B) HeLa cells transfected with expression vector encoding ERK3. After 24 h, ERK3 expression was visualised by staining with an anti-ERK3 antibody and Alexa 594 anti-sheep antibody (red channel on left), while cell nuclei were visualised by DRAQ5 staining (blue channel on right). (C) HeLa cells were cotransfected with expression vectors encoding EGFP-MK5 and ERK3 and after 24 h cells were fixed. EGFP-MK5 was visualised directly (green channel on left), and ERK3 was visualised by staining with an anti-ERK3 antibody and Alexa 594 anti-sheep antibody (red channel on right). A merged image of the green and red channels is shown (lower left) and nuclei were visualised by DRAQ5 staining (blue channel on lower right). (D) HeLa cells were cotransfected with the indicated expression vectors and EGFP-MK5 and ERK3 were visualised as above. In all, 100 cells coexpressing both EGFP-MK5 and ERK3 from three independent transfections were counted and the distribution of both proteins was scored. The results are presented as the % of cells in which both EGFP-MK5 and ERK3 were predominantly cytosolic (C > N) and mean values with associated errors are shown. In all experiments, several fields of cells were examined and representative images are shown.

The activation of MK5 by ERK3 requires specific protein-protein interaction

To explore the necessity for physical docking between these two proteins in mediating the activation of MK5, we used the truncated forms of ERK3 characterised in Figure 3. As before,



wild-type ERK3 led to significant activation of MK5. However, ERK3 1-330, which fails to bind to MK5, does not (Figure 7D). In contrast, ERK3 1-340, which retains the ability to bind to MK5, activates MK5 as efficiently as the wild-type protein (Figure 7D). Finally, the truncated form of MK5 (MK5 1-423), which is unable to bind to ERK3, but retains binding towards p38, is activated on coexpression of p38 and MKK6E/E but not by ERK3 (Figure 7E). We conclude that specific but distinct interactions between ERK3 and MK5 are necessary for both relocalisation and for the phosphorylation and activation of MK5 by ERK3.

Loss of endogenous ERK3 protein causes a significant decrease in the activity of MK5, while elevated levels of ERK3 in differentiating PC12 cells are accompanied by an increase in MK5 activity

Although our overexpression studies suggest a functional link between ERK3 and MK5, they do not prove that ERK3 is a physiological activator of MK5. To address this, we first performed siRNA-mediated knockdown of endogenous ERK3 in HeLa cells. Using three distinct siRNAs directed against ERK3, we achieved significant reduction in the levels of endogenous ERK3 protein (Figure 8A). In contrast, levels of the related ERK2 MAPK were unchanged, as were levels of endogenous MK5. Immune complex kinase assays revealed that ERK3 knockdown resulted in a significant reduction (30-50%) in endogenous MK5 activity. To confirm an absolute link between ERK3 and MK5, we obtained primary MEFs derived from wild-type (ERK3^{+/+}), heterozygous (ERK3^{+/-}) and null (ERK3^{-/-}) animals. ERK3 protein levels were either reduced (ERK3^{+/-}) or absent (ERK3^{-/-}) in these cells, while MK5 protein levels were unchanged. However, endogenous MK5 activity was reduced by approximately

Figure 3 Amino acids 330-340 of ERK3 are required for interaction with and relocalisation of MK5. (A) COS-1 cells were transfected with expression vectors encoding Myc-tagged full-length, or C-terminal truncation mutants of ERK3 encoding either amino acids 1-340 or 1-330 of the protein. Cell lysates were then mixed with either recombinant GST-MK5 or GST alone. Binding of ERK3 was detected by Western blotting using an anti-Myc monoclonal antibody. (B) HeLa cells were transfected with expression vectors encoding either amino acids 1-330 or 1-340 of ERK3. ERK3 was visualised by staining with an anti-ERK3 antibody and an Alexa 594-coupled secondary (anti-sheep) antibody (red channel in upper panels) and cell nuclei were visualised by DRAQ5 staining (blue channel in lower panels). In all, 100 cells expressing the indicated mutant of ERK3 from each of three independent transfections were counted and the distribution of the ERK3 protein was scored. The results are presented as the % of cells in which the ERK3 protein was predominantly nuclear (N>C) and mean values with associated errors are shown. (C) HeLa cells were cotransfected with vectors encoding EGFP-MK5 and either ERK3 1-330 or 1-340. EGFP-MK5 was visualised directly (green channel in upper panels), ERK3 was visualised by staining with an anti-ERK3 antibody and an Alexa 594-coupled secondary (anti-sheep) antibody (red channel in middle panels) and cell nuclei were visualised by DRAQ5 staining (blue channel in lower panels). A total of 100 cells expressing either EGFP-MK5 alone or coexpressing both EGFP-MK5 and the indicated mutant of ERK3 from three independent transfections were counted and the distribution of MK5 was scored. The results are presented as the % of cells in which EGFP-MK5 was either predominantly nuclear (N>C) or predominantly cytoplasmic (C>N) and mean values with associated errors are shown. In all experiments, several fields of cells were examined and representative images are shown.

25% in the ERK3 heterozygous and 50% in the ERK3 null cells (Figure 8B). Our results demonstrate that ERK3 is responsible for a significant fraction of endogenous MK5 activity in mammalian cells. Finally, ERK3 protein accumulates during the differentiation of PC12 cells in response to

nerve growth factor (NGF) (Coulombe *et al*, 2003). To determine if this influences MK5 activity, we exposed PC12 cells to NGF for 4 days and monitored differentiation as characterised by neurite outgrowth. These cells were also analysed for expression levels of ERK3, ERK2, p38 and MK5, and MK5 activity was assayed following immunoprecipitation. After 4 days of exposure to NGF, the levels of endogenous ERK3 protein increased significantly, while the levels of endogenous MK5 appeared to be stable (Figure 8C). However, the kinase activity of the endogenous MK5 protein increases approximately four-fold, indicating that increased levels of ERK3 protein result in activation of MK5 (Figure 8C). It is possible that this could be the result of p38 activation, as this MAPK has been implicated in PC12 cell differentiation in response to NGF (Morooka and Nishida, 1998). However, MK5 activity was unaffected by exposure of these cells to SB203580 and thus is not dependent on p38 (data not shown).

Chaperoning or nuclear-cytoplasmic cotransport activity of MK5 is required to maintain levels of endogenous ERK3

Deletion of MK2 causes a reduction in p38 protein, due to a lack of chaperoning and/or nucleo-cytoplasmic cotransport of p38 (Kotlyarov *et al*, 2002). In contrast, loss of MK5 has no effect on levels of p38, indicating that MK5 does not perform a similar function (Shi *et al*, 2003). To examine the effect of reducing MK5 on ERK3 protein levels, we have employed siRNA-mediated knockdown. Using this approach, we achieve a significant reduction in the levels of MK5 protein and this is reflected in a reduction of approximately 90% in endogenous MK5 kinase activity. In contrast, no change is seen in the levels of the closely related kinase MK2 (Figure 9A). Furthermore, in cells treated with MK5 siRNA, we consistently observe a dramatic reduction in the level of endogenous ERK3 protein (Figure 9B) and this can be rescued by expression of either an siRNA-resistant (murine) wild-type MK5 or T182A mutant of MK5.

ERK3 is a substrate for activated MK5 *in vitro* and *in vivo*

MK5 was first characterised as a kinase, which phosphorylated the small heat shock protein Hsp27 *in vitro* and *in vivo* (New *et al*, 1998). However, mice lacking MK2 suffer almost complete loss of Hsp27 kinase activity, making it extremely unlikely that Hsp27 is a physiological substrate for MK5 (Kotlyarov *et al*, 1999). Given that ERK3 and MK5 form a complex, which results in MK5 activation, we wanted to determine if ERK3 was itself a substrate for activated MK5. To examine this, we incubated activated MK5 and MK2 (1 U/ml) with either recombinant wild-type ERK3 or Hsp27, together with [γ - 32 P]ATP and Mg $^{2+}$. We clearly observe that MK5, but not MK2, is able to phosphorylate ERK3 *in vitro*, while both kinases phosphorylate Hsp27 (Figure 10A). The stoichiometry of ERK3 phosphorylation by MK5 was also determined and demonstrated that ERK3 is not only a preferred substrate for MK5 over MK2, but is also efficiently phosphorylated to 1.5 mol/mol. These results suggest that ERK3 is a target for activated MK5 *in vivo*. To address this, we coexpressed either Myc-tagged wild-type or kinase-dead ERK3 with either EGFP alone, wild-type MK5 or MK5L337G, a constitutively active mutant of MK5 (Seternes *et al*, 2002). Cells were then labelled with

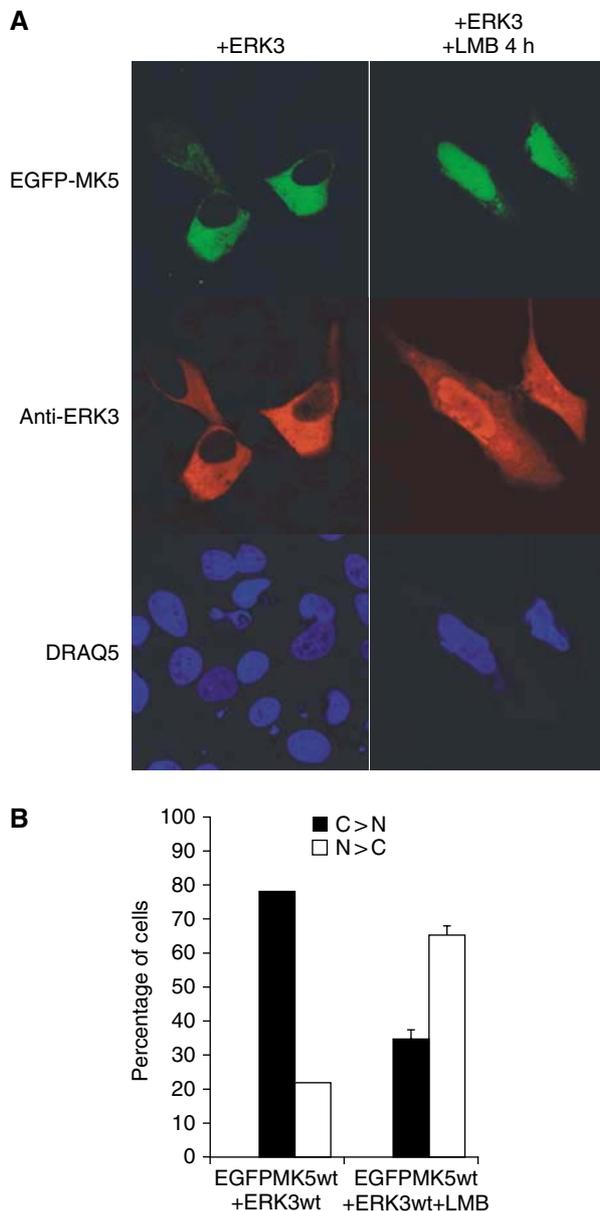
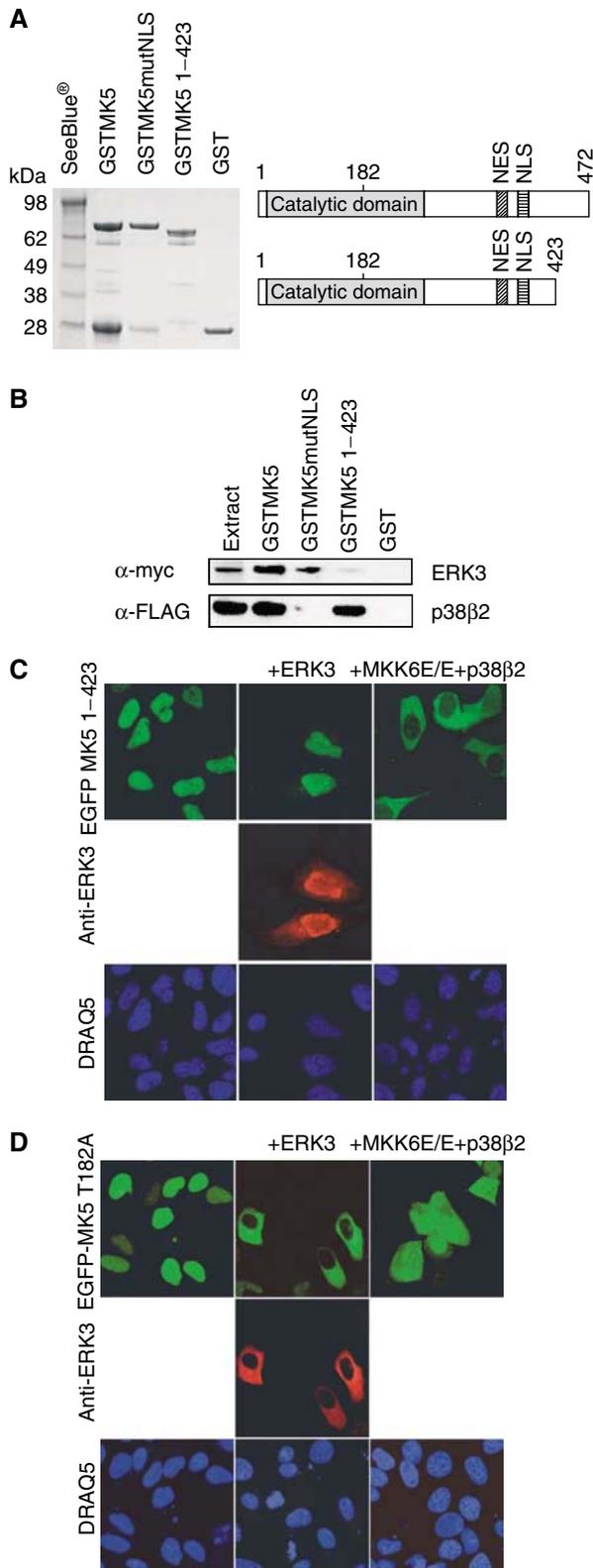


Figure 4 Relocalisation of both MK5 and ERK3 requires a CRM1-dependent nuclear export pathway. (A) HeLa cells were cotransfected with expression vectors encoding EGFP-MK5 and ERK3. After 24 h, cells were either left untreated (left panels) or exposed to LMB at a final concentration of 5 ng/ml for 4 h (right panels). Following fixation, EGFP-MK5 was visualised directly (green channel in upper panels), ERK3 was visualised by staining with an anti-ERK3 antibody and an Alexa 594-coupled secondary (anti-sheep) antibody (red channel in middle panels) and cell nuclei were visualised by DRAQ5 staining (blue channel in lower panels). Several fields of cells were examined and representative images are shown. (B) A total of 100 cells coexpressing both EGFP-MK5 and ERK3 from each of three independent transfections were counted and the distribution of both proteins was scored. The results are presented as the % of cells in which both EGFP-MK5 and ERK3 were either predominantly cytosolic (C > N) or predominantly nuclear (N > C) and mean values with associated errors are shown.

[³²P]orthophosphate and ERK3 was immunoprecipitated and analysed by SDS-PAGE. Both wild-type and kinase-dead ERK3 are phosphorylated by MK5, indicating that ERK3 is a physiological target for activated MK5 in mammalian cells (Figure 10).



Discussion

We have established a functional link between the atypical MAPK ERK3 and the activity and localisation of MK5. MK5 was first characterised as p38-regulated/activated protein kinase (PRAK) (New *et al*, 1998; Ni *et al*, 1998). Subsequent work indicated that both the activity and the subcellular localisation of MK5 could be regulated by interaction with p38 (Seternes *et al*, 2002; New *et al*, 2003). However, these interactions were either demonstrated *in vitro* or following overexpression of p38 and MK5 in cultured cells. Subsequent attempts to demonstrate functional interactions between endogenous p38 and MK5 have proven difficult as highlighted by recent studies of endogenous MK5 activities in both wild-type and MK5 null mice (Shi *et al*, 2003). Firstly, endogenous MK5 activity was not increased by treatments that activated both endogenous p38 and its *bona fide* substrate MK2. Secondly, attempts to show binding of endogenous p38 using tandem affinity purification (TAP) of MK5 failed, whereas p38 was readily detected following TAP of MK2. Finally, p38 protein levels were unaffected by deletion of MK5, whereas loss of MK2 caused a significant reduction in p38, indicating that MK2, but not MK5, exhibits chaperoning properties towards p38 (Shi *et al*, 2003).

In the present study, we used a yeast two-hybrid assay to study interactions between MK5 and MAPKs and we identified ERK3, but not p38, as a specific binding partner for MK5. Furthermore, although we could readily demonstrate the presence of endogenous MK5 in ERK3 immunoprecipitates and endogenous ERK3 in MK5 immunoprecipitates, we repeatedly failed to detect endogenous p38 in the latter. Therefore, despite our ability to detect robust binding between p38 and MK5 both *in vitro* and when overexpressed in mammalian cells, the results of our protein-protein interac-

Figure 5 ERK3 and p38 mediate the translocation of MK5 through different mechanisms. **(A)** Recombinant GSTMK5, GSTMK5mutNLS, GSTMK5 1-423 and GST alone were expressed and purified from *Escherichia coli*. Each protein (2 μg) was then analysed by SDS-PAGE and Coomassie blue staining. This material represents the input in the GST pull-down assay described below and also those presented in Figures 1 and 3. **(B)** Lysates from COS-1 cells transfected with expression vectors encoding either Myc-tagged ERK3 or FLAG-tagged p38β2 were mixed with the indicated GST fusion proteins. Bound MAPKs were detected using an anti-Myc antibody (ERK3, upper panel) and an anti-FLAG antibody (p38β2, lower panel). The experiment was performed three times and a representative result is shown. **(C)** HeLa cells were cotransfected with expression vectors encoding a C-terminal truncation of EGFP-MK5 (EGFPMK5 1-423) and either ERK3 or constitutively active MKK6 together with p38β2 (MKK6E/E + p38β2). After 24 h, cells were fixed and EGFP-MK5 was visualised directly (green channel in upper panels), and ERK3 was visualised by staining with an anti-ERK3 antibody and an Alexa 594-coupled secondary (anti-sheep) antibody (red channel in the middle panel). Cell nuclei were visualised by DRAQ5 staining (blue channel in lower panels). **(D)** HeLa cells were cotransfected with expression vectors encoding EGFP-MK5 T182A, in which the phospho-acceptor site Thr182 was substituted by alanine together with either ERK3 or MKK6 plus p38β2 (MKK6E/E + p38β2). After 24 h, cells were fixed and EGFP-MK5 was visualised directly (green channel in upper panels), and ERK3 was visualised by staining with an anti-ERK3 antibody and an Alexa 594-coupled secondary (anti-sheep) antibody (red channel in middle panels). Cell nuclei were visualised by DRAQ5 staining (blue channel in lower panels). In all experiments, several fields of cells were examined and representative images are shown.

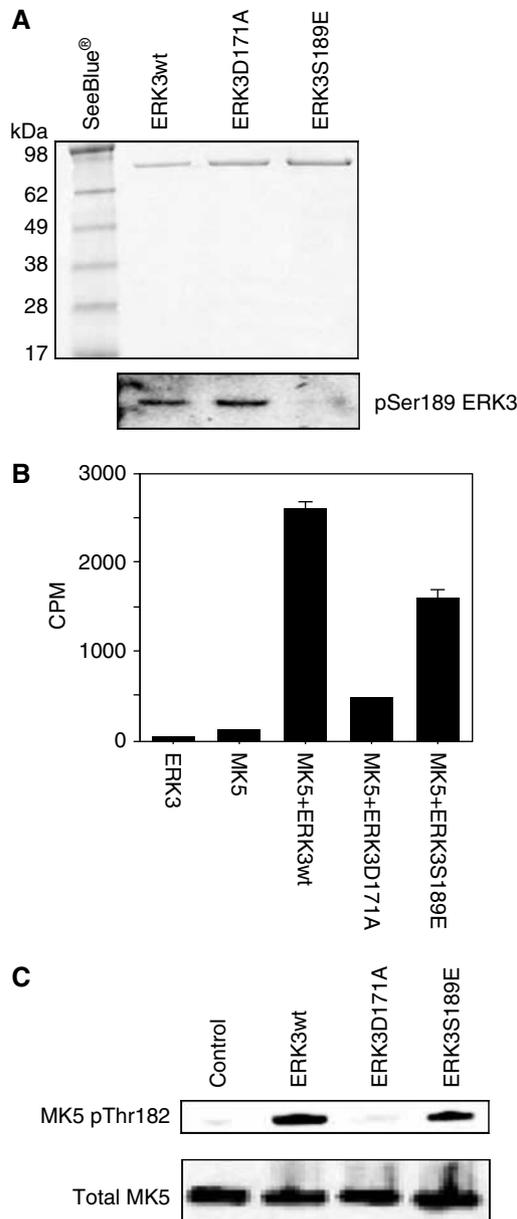


Figure 6 ERK3 activates MK5 *in vitro* by phosphorylating Thr182 within the activation loop of the kinase. **(A)** Recombinant wild-type ERK3 (ERK3wt), a kinase-dead mutant of ERK3 (ERK3D171A) or a mutant in which serine189 within ERK3 was mutated to glutamic acid (ERK3S189E) were expressed and purified from insect Sf-9 cells. Proteins (2 µg) were then analysed using SDS-PAGE and Coomassie blue staining (upper panel). Proteins were also analysed by SDS-PAGE and Western blotting using a phospho-specific antibody raised against S189 of ERK3 (lower panel). **(B)** Activation of recombinant MK5 was assayed *in vitro* by incubation with either wild-type ERK3, the kinase-dead mutant of ERK3 (ERK3D171A) or ERK3S189E using PRAKTide as substrate and activities compared with those of either ERK3 or MK5 alone. Assays were performed in quadruplicate and mean activities are presented here as counts per minute (CPM) incorporated with associated errors. **(C)** Recombinant MK5 (1 µg) was incubated either alone or with 6 µg of each of the indicated recombinant ERK3 proteins in the presence of ATP and Mg²⁺. After 60 min, a sample of MK5 (250 ng) was analysed by SDS-PAGE and Western blotting using a phospho-specific antibody raised against Thr182 (upper panel). As a loading control, the samples were also analysed by SDS-PAGE and Western blotting using an anti-MK5 antibody (lower panel).

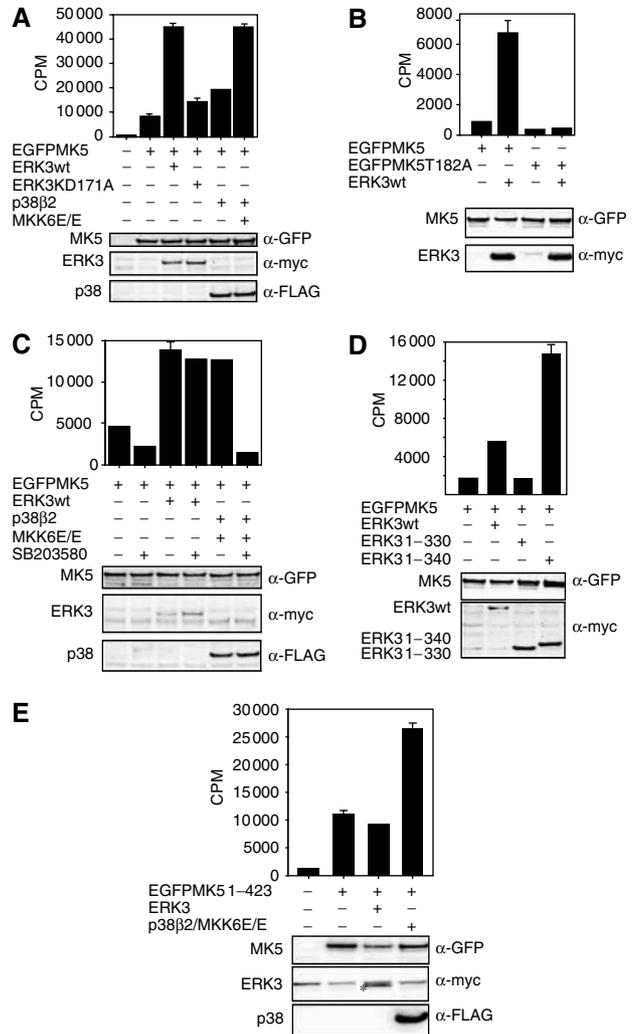


Figure 7 ERK3 activates MK5 *in vivo*. **(A)** HeLa cells were transfected with the indicated expression plasmids. After 24 h, cells were lysed and EGFPMK5 activity was assayed in an immune complex kinase assay using PRAKTide as substrate. Activity is expressed as counts per minute (CPM) incorporated. **(B)** HeLa cells were transfected with either wild-type MK5 (EGFPMK5) or the mutant EGFPMK5 T182A, either alone or in combination with wild-type ERK3 (ERK3wt). After 24 h, cells were lysed and the activity of the EGFPMK5 fusion protein was assayed as before. **(C)** HeLa cells were transfected with the indicated plasmids as in (A). After 24 h, cells were treated (where indicated) with 10 µM of SB 203580 for 2 h before cell lysis and assay of EGFPMK5 kinase as before. **(D)** HeLa cells were transfected with EGFPMK5 either alone or in combination with either wild-type ERK3 (ERK3wt), a C-terminal truncation encoding residues 1-330 of ERK3 (ERK3 1-330) or a C-terminal truncation encoding residues 1-340 of ERK3 (ERK3 1-340). After 24 h, cells were lysed and the activity of the EGFPMK5 fusion protein was assayed as before. **(E)** HeLa cells were transfected with a C-terminal truncation of MK5 (EGFPMK5 1-423) either alone or in combination with either wild-type ERK3 (*) or expression plasmids encoding p38β2 and MKK6E/E. After 24 h, cells were lysed and the activity of the EGFPMK5 1-423 fusion protein was assayed as before. All experiments were performed at least three times, and the results of a single representative experiment are shown. Kinase assays were performed in quadruplicate and mean values with associated errors are presented. Expression of wild-type and mutant forms of MK5 and ERK3 and of p38 was verified by Western blotting of cell lysates using appropriate antibodies (lower panels in A-E).

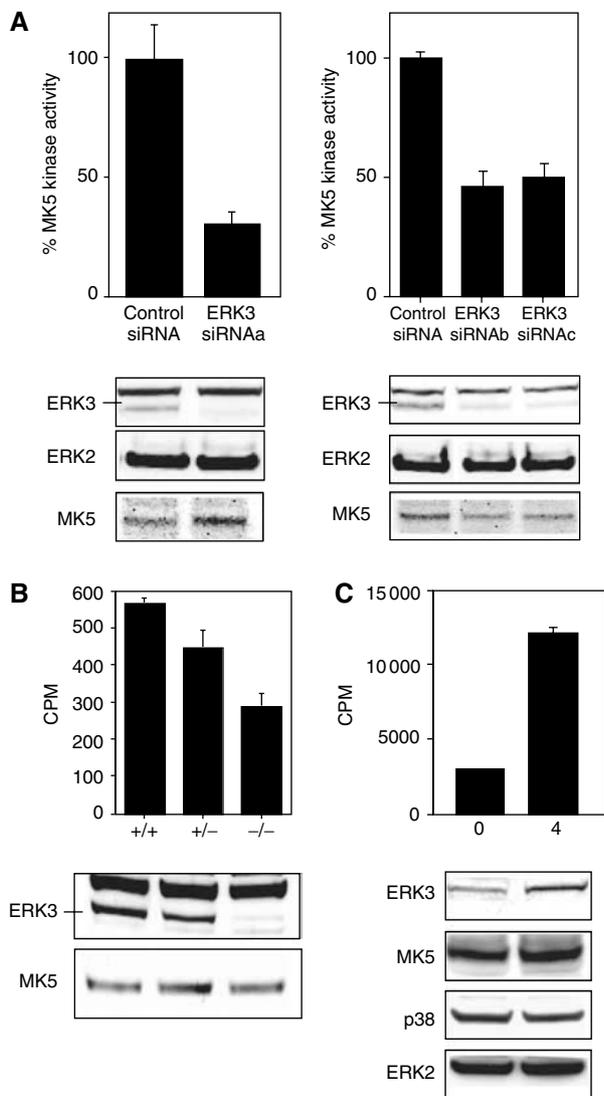


Figure 8 ERK3 is a physiological activator of MK5. (A) SiRNA-mediated knockdown of ERK3 decreases endogenous MK5 activity. HeLa cells were transfected with 10 nM of either a control (scrambled) siRNA or three distinct siRNAs against human ERK3. After 24 h, cells were lysed and endogenous MK5 was immunoprecipitated. MK5 activity was then assayed exactly as before and results are presented as relative kinase activity (%), where the activity in cells transfected with control siRNA is 100%. Total cell lysates (20 µg) were also analysed by SDS-PAGE and Western blotting to determine the levels of endogenous ERK3, ERK2 and MK5 using appropriate antibodies (lower panels). (B) MK5 activity is reduced in ERK3 knockdown fibroblasts. Primary MEFs derived from wild-type (+/+), ERK3 heterozygous (+/-) or ERK3 null (-/-) mice were lysed and endogenous MK5 was immunoprecipitated. MK5 activity was then assayed as before. Total cell lysates (20 µg) were also analysed by SDS-PAGE and Western blotting to determine the levels of endogenous ERK3 and MK5 using appropriate antibodies (lower panels). (C) Differentiation of PC12 leads to increased ERK3 expression and MK5 activity. PC12 cells were either untreated (0) or stimulated with NGF (100 ng/ml) for 4 days (4) before cells were lysed. MK5 was immunoprecipitated and MK5 activity was then assayed as described before. Total lysates (20 µg) were also analysed by SDS-PAGE and Western blotting to determine the levels of ERK3, MK5, p38 and ERK2 expression using appropriate antibodies (lower panels). All experiments were performed at least three times, and the results of a single representative experiment are shown. Kinase assays were performed in quadruplicate and mean values with associated errors are presented.

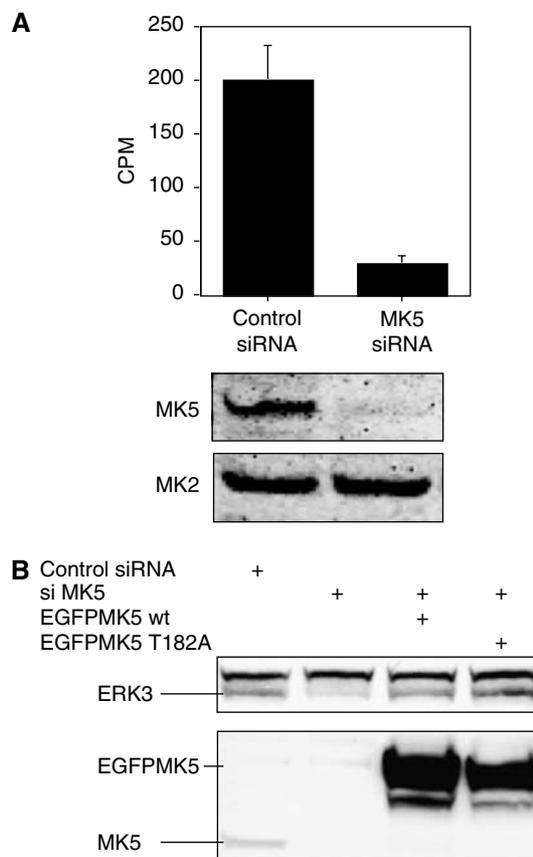


Figure 9 SiRNA-mediated knockdown of MK5 causes loss of endogenous ERK3 protein. (A) HeLa cells were transfected with 100 nM of the indicated siRNAs and lysed after 48 h. MK5 was immunoprecipitated using a polyclonal anti-MK5 antibody and the activity of the MK5 was assayed in an immune complex kinase assay as before. Experiments were performed at least three times, and the results of a single representative experiment are shown. Kinase assays were performed in quadruplicate and mean incorporations (CPM) with associated errors are presented. Cell lysates (20 µg) were also analysed by SDS-PAGE and Western blotting using antibodies against MK5 and MK2, respectively (lower panels). (B) HeLa cells were transfected with 100 nM of the indicated siRNAs. After 24 h, the cells were transfected with 1 µg of the indicated expression plasmids and 24 h later, the cells were harvested. Total lysates (20 µg) were analysed by SDS-PAGE and Western blotting using a rabbit polyclonal ERK3 antibody (E3-CT4, upper panel) and a polyclonal MK5 antibody, respectively (lower panel).

tion studies strongly favour a functional relationship between endogenous MK5 and the atypical MAPK ERK3 but not p38. This conclusion is further strengthened by our demonstration that this interaction causes both proteins to relocate from the nucleus to the cytoplasm. Furthermore, recombinant ERK3, in which serine 189 within the S-E-G motif is phosphorylated, is able to activate MK5 *in vitro* and expression of wild-type ERK3, but not a kinase-dead mutant, is also able to activate MK5 *in vivo*. The activation of MK5 by ERK3 is absolutely dependent on the phosphorylation of Thr182 within the activation loop of MK5, requires specific protein-protein interactions between ERK3 and MK5 and is completely unaffected by a potent and specific inhibitor of p38.

To demonstrate a definitive physiological link between ERK3 and MK5 activation, we have used several approaches.

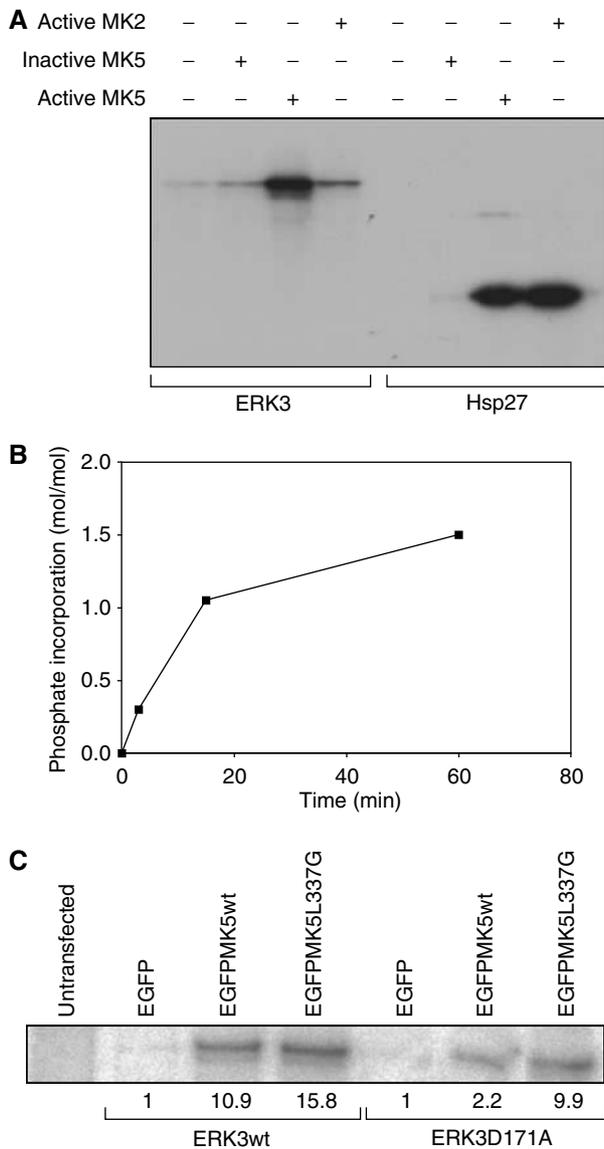


Figure 10 ERK3 is a substrate for MK5 *in vitro* and *in vivo*. (A) In all, 5 μ g of either recombinant ERK3 (left) or Hsp27 (right) was incubated with either 1 U/ml activated MK5 or MK2 or 1 μ g inactive MK5 for 20 min in the presence of Mg^{2+} and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Radiolabelled proteins were then analysed by SDS-PAGE and autoradiography. (B) ERK3 was phosphorylated at 2 μ M by 3 U/ml active MK5. Samples were removed at the indicated times and analysed by SDS-PAGE and autoradiography. Radiolabelled bands were excised and subjected to scintillation counting before calculation of the stoichiometry of ERK3 phosphorylation (phosphate incorporation in mol/mol). (C) Phosphorylation of ERK3 by MK5 *in vivo*. HeLa cells were either untransfected or cotransfected with expression plasmids encoding either Myc-tagged wild-type or kinase-dead ERK3 together with expression vectors encoding either EGFP alone, EGFP/MK5 or constitutively active MK5 (EGFP/MK5337G). After 24 h, cells were labelled for 4 h with $[\text{}^{32}\text{P}]\text{orthophosphate}$, lysed and ERK3 was immunoprecipitated using an anti-Myc monoclonal antibody. Immunoprecipitates were analysed by SDS-PAGE and radiolabelled ERK3 proteins were visualised and quantitated using a PhosphorImager. Incorporation of ^{32}P is expressed here relative to the amount detected in ERK3 when coexpressed with EGFP alone.

Firstly, we employed siRNA to knock down endogenous ERK3 protein in HeLa cells. Secondly, we obtained MEFs from animals in which the ERK3 gene has been deleted by homologous recombination. In both models, we see a sig-

nificant reduction in endogenous MK5 activity. Finally, in agreement with recent studies (Coulombe *et al*, 2003), we have shown that PC12 cells induced to differentiate by NGF contain elevated levels of ERK3 protein and that this correlates with a significant increase in endogenous MK5 kinase activity.

Deletion of ERK3 does not abolish MK5 activity, causing a maximum reduction of approximately 40–50%. This suggests the existence of additional MK5 regulators in these cells. One possibility is that bound ERK3 prevents endogenous p38 from activating MK5. This might explain the finding that MK5 can be activated by p38 only when overexpressed, and suggests that in the absence of ERK3, p38 might interact with and activate endogenous MK5. We find that the sequence determinants for interaction between either ERK3 and MK5 or p38 and MK5 are distinct, and that mutated or truncated forms of both partner proteins exist, which affect interaction with ERK3 but not p38 and *vice versa*. Furthermore, whereas p38-mediated export of MK5 requires phosphorylation of Thr182 within MK5, ERK3-mediated export of MK5 did not require this modification. This indicates that although the relocalisation of MK5 by both ERK3 and p38 occurs via a CRM1-dependent export pathway, it is not mediated by a common mechanism. However, in preliminary experiments, we have been unable to demonstrate any effect of the p38 inhibitor SB203580 on endogenous MK5 activity in HeLa cells expressing specific siRNA against ERK3, indicating that p38 is not responsible for this residual MK5 activity.

A second possible regulator of MK5 activity was uncovered by a recent genomic analysis of ERK3. This revealed that, in addition to ERK3 (*MAPK6*), mice and humans possess a functional gene designated *MAPK4*, which encodes a 63 kDa ERK3 homologue (Turgeon *et al*, 2002). p63^{MAPK} displays 73% homology to ERK3 within the kinase domain and also contains the S-E-G motif. At present, nothing is known about the regulation and function of p63^{MAPK} , but expression is detected in human heart, brain and lung (Gonzalez *et al*, 1992). Future studies will examine the role of p63^{MAPK} in regulating MK5 activity.

Having demonstrated that ERK3 can activate MK5 *in vivo*, we wished to explore the possibility that, in addition to being activated by ERK3, MK5 also plays some role in regulating the function of ERK3 itself. This might be analogous to the dual role of MK2, which acts both as a substrate for p38 and as a chaperone to stabilise p38 protein levels (Kotlyarov *et al*, 2002). To approach this question, we have used siRNA to target endogenous MK5 in HeLa cells and have clearly shown that we can achieve a significant reduction in both protein level and associated MK5 kinase activity. Furthermore, loss of MK5 causes a dramatic reduction in the levels of endogenous ERK3 protein, which can be rescued by expression of siRNA-resistant murine MK5. In agreement with our results, a significant reduction in ERK3 protein levels is also detected in MEFs derived from MK5 knockout mice (Kotlyarov and Gaestel, personal communication). More interestingly, in the C57/B6 genetic background, MK5-deficient mice show embryonic lethality with incomplete penetrance, which results from embryonic death at about E11, the developmental stage where ERK3 expression is maximum (Kotlyarov and Gaestel, personal communication).

Finally, we have gone on to show that ERK3, in addition to activating MK5 and forming a complex that is translocated

from the nucleus to the cytoplasm, is also a potential substrate for the kinase activity of MK5. ERK3 is very efficiently phosphorylated by activated MK5 *in vitro* and *in vivo*. As yet it is unclear what the function of this modification might be. However, our demonstration that the T182A mutant of MK5 efficiently rescues the loss of ERK3 seen when endogenous MK5 is depleted and also relocalises to the cytoplasm as efficiently as the wild-type protein indicates that the kinase activity of MK5 towards ERK3 is not required for either the chaperoning or cotransport activities of MK5 towards ERK3.

In conclusion, we have presented convincing evidence for a functional link between the atypical MAPK ERK3 and the serine/threonine kinase MK5 and have identified the latter protein as the first *bona fide* physiological substrate for ERK3. At present, the functional consequences of this interaction and the resulting activation of MK5 are not clear. In the case of ERK3, elevated levels of protein are associated with the process of cellular differentiation (Boulton *et al*, 1991; Coulombe *et al*, 2003), and we have demonstrated a possible link between ERK3 accumulation and MK5 activity. ERK3 expression is also induced when human carcinoma cell lines are plated on type-IV collagen and correlated with decreased cell proliferation. Furthermore, overexpression of ERK3 inhibited cancer cell growth, migration and invasion (Crowe, 2004). All of the above point towards a negative role for ERK3 with respect to cell proliferation and this would be compatible with a key role for ERK3 and possibly MK5 during the process of tissue and cell differentiation. Our study provides important tools and suggests further experiments to probe the relationship between ERK3 and MK5 and the consequences of MK5 activation.

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Materials and methods

siRNA

All siRNAs were purchased as validated or predesigned from Ambion and transfected into HeLa cells using Lipofectamine 2000 according to the manufacturer's instructions. Precise details of the siRNAs used can be found in Supplementary data.

Localisation studies

Cells were fixed using 4% paraformaldehyde (PFA) and 0.3% Triton X-100 for 5 min and 4% PFA for 20 min. Cells were then incubated for 2 h with 3% BSA in phosphate-buffered saline before incubation with anti-ERK3 antibody at a final concentration of 2 µg/ml for 1 h. Immunostaining was developed using Alexa 594-conjugated anti-sheep IgG (1:400; Molecular Probes). Cell nuclei were visualised by staining with DRAQ5 (Biosstatus Ltd, Leicestershire, UK). Images were collected using a Zeiss LSM510 confocal microscope. A detailed description of all other methods employed in this study can be found in Supplementary data of this paper.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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