Spatiotemporal regulation of ERK2 by dual-specificity phosphatases

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SUMMARY

Although many stimuli activate extracellular signal-regulated kinases 1 and 2 (ERK1/2), the kinetics and compartmentalization of ERK1/2 signals are stimulus-dependent and dictate physiological consequences. ERKs can be inactivated by dual-specificity phosphatases (DUSPs), notably the MAPK-phosphatases (MKPs) and atypical DUSPs, that can both dephosphorylate and scaffold ERK1/2. Using a cell imaging model (based on knock-down of endogenous ERKs and add-back of wild-type or mutated ERK2-GFP reporters), we explored possible effects of DUSPs on responses to transient or sustained ERK2 activators (EGF and PDBu, respectively). For both stimuli, a D319N mutation (which impairs DUSP binding) increased ERK2 activity and reduced nuclear accumulation. These stimuli also increased mRNA levels for eight DUSPs. In an siRNA screen, twelve out of sixteen DUSPs influenced ERK2 responses. These effects were evident amongst nuclear-inducible MKP, cytoplasmic ERK MKP, JNK/p38 MKP and atypical DUSP subtypes and, with the exception of the nuclear-inducible MKPs, were paralleled by corresponding changes in Egr-1 luciferase activation. Simultaneous removal of all JNK/p38MKPs or nuclear-inducible MKPs revealed them as positive and negative regulators of ERK2 signaling, respectively. The effects of JNK/p38 MKP siRNAs were not dependent on protein neosynthesis but were reversed in the presence of JNK and p38 kinase inhibitors, indicating DUSP-mediated cross-talk between MAPK pathways. Overall, our data reveal that a large number of DUSPs influence ERK2 signaling. Together with the known tissue-specific expression of DUSPs and the importance of ERK1/2 in cell regulation, our data support the potential value of DUSPs as targets for drug therapy.

Keywords: DUSP/ERK/PKC/EGF/MKP/phosphatase/high-content

INTRODUCTION

The extracellular signal-regulated kinase 1/2 (ERK1/2) pathway forms a major part of the mitogen activated protein kinase (MAPK) network, and is activated by a diverse array of extracellular cues (1-3). Activated ERK1/2 can phosphorylate a growing list of substrate proteins in the nucleus and cytoplasm, and represents a focal point of integration in cellular responses (1-3). The specificity of biological outcome from ERK1/2 stimuli is commonly achieved through tight control of the duration, magnitude and localization of ERK1/2 signals (2;3).

Activation of ERK1/2 commonly causes its translocation from the cytoplasm to the nucleus, which is necessary for the transcription of many immediate early genes such as c-Fos, c-Jun and early growth response gene-1 (Egr-1) (4-8). In fibroblasts and epithelial cells, sustained ERK1/2 activity causes expression and stabilisation of immediate early gene products, culminating in G1/S transition (6-9). This does not occur in cells where nuclear localization of ERK1/2 is prevented (10). In contrast, transient ERK1/2 signals similarly cause the transcription of immediate early genes, but this is not sustained, and the protein products are rapidly degraded (6-8). Thus, both the signal duration and localization of ERK1/2 determine cell fate.
The dual specificity phosphatases (DUSPs) are an important family of proteins that influence spatiotemporal aspects of ERK1/2 signaling. Two major groups in this family are the MAPK phosphatases (MKPs) and the highly related atypical DUSPs (11;12). The MKP group consists of ten proteins which can remove activating Thr and Tyr phosphate groups from ERK1/2 and/or the other major MAPKs, c-Jun N-terminal kinase (JNK) and p38, and thus act in direct opposition to activating signals from upstream kinases (13;14). The MKPs are characterized by their variable N-terminal MAPK binding region which governs substrate specificity and stability of interaction (14-18). This region includes the D (docking)-domain motif and can determine whether DUSPs remain associated with ERK1/2 following dephosphorylation (16-19). The MKPs are further divided into subgroups according to subcellular localization and substrate specificity. DUSPs 1, 2, 4 and 5 constitute the nuclear inducible MKPs, all of which are able to dephosphorylate ERK1/2 and, with the exception of DUSP5, can also dephosphorylate JNK and/or p38 (18;20-22). DUSP6, 7 and 9 preferentially dephosphorylate ERK1/2, are not restricted to the nucleus and are termed the cytoplasmic ERK MKPs (23-25). DUSP8, 10 and 16 have greater activity towards JNK and/or p38 and are known as the JNK/p38 MKPs (26-28). In contrast, the atypical DUSPs are smaller, lack obvious MAPK targeting motifs and dephosphorylate a diverse group of substrates (11;12). However, members of this group are known to act directly on MAPK family members. Notably, DUSP3 dephosphorylates ERK1/2 (29;30), while DUSP18 and 22 dephosphorylate JNK or its upstream activators (31-33). A recent genome-wide phosphatase screen revealed that a number of DUSPs are essential for cell survival (34). These include DUSPs that can directly dephosphorylate ERKs, highlighting the potential value of DUSP targeting as a means to control ERK1/2 activity and cell fate (35-38).

We recently began to explore spatiotemporal aspects of ERK2 activation using a high-content imaging-based model in which endogenous ERKs are knocked-down with siRNAs and a GFP fusion protein reporter is added back with recombinant adeno virus (Ad) expressing wild-type (WT) ERK2-GFP or a similar construct mutated to prevent D-domain binding. We showed that ERK2 dephosphorylation and trafficking is coordinately regulated by DUSPs 1, 2 and 4 in a stimulus-specific manner and that whereas DUSP2 and 4 dephosphorylate and scaffold ERK2 in the nucleus, DUSP1 dephosphorylates ERK2 and releases it for return to the cytoplasm (19). Here, we have used this model to explore possible effects of other DUSP family members on ERK responses to transient or sustained ERK activators (EGF and PDBu, respectively). We show that a surprisingly large number of these enzymes (twelve out of sixteen in an siRNA screen) are able to shape ERK2 responses. These include members of each DUSP subgroup. The effects of the siRNAs were stimulus-specific and were mostly inhibitory. For most DUSPs, the reduction in ERK2 activity and/or nuclear localization was paralleled by decreases in ERK-dependent transcription, but this was not the case for the nuclear inducible MKPs. When all four members of the nuclear inducible MKP subgroup were knocked out simultaneously, levels of ERK2-GFP in the nucleus were decreased, while both active nuclear ERK2 levels and ERK-dependent transcription were greatly increased. In contrast, targeting of JNK/p38 MKPs reduced ERK2 activation, ERK2-GFP nuclear accumulation and ERK-dependent transcription. These siRNA effects were prevented by pharmacological JNK or p38 kinase inhibitors, indicating the JNK/p38MKP siRNA effects were mediated by JNK/p38 activation. These data indicate opposing collective functional roles of the nuclear-inducible MKPs and JNK/p38 MKPs during ERK2 regulation.

**EXPERIMENTAL PROCEDURES**

**Engineering of Plasmids and Viruses**

Adenoviral (Ad) shuttle vectors of wild-type (WT), Y261A and D319N ERK2-GFP in pacAd5 CMV K-N pA were constructed as described (19). A 1.2kb SaII fragment of the Egr-1 promoter from an Egr-1-Luc vector (39) was subcloned into an XhoI digest of pAd5-Luc2 (made initially by subcloning an Spel-BamHI fragment of pGL4.17 into pacAd5 K-N pA). Viruses were generated from shuttle vectors as described (40). Briefly, 4.5µg of shuttle vectors were digested alongside 1.5µg of pacAd5 9.2-100 sub360 backbone vector.
(donated by Prof. Beverly Davidson, University of Iowa, Iowa City, USA) with PacI. Cut shuttle and backbone vectors were then mixed and transfected into low passage HEK293 cells using Superfect (Qiagen, Crawley, UK). Cells were left to allow recombination between shuttle and backbone vectors. Verification of recombination was performed by restriction digest and sequence analysis, and Ad vectors were grown to high titre and purified according to standard protocols (41). The Ad CMV β-galactosidase vector was a gift from Prof. James Uney (University of Bristol, UK).

Cell Culture and Transfection
HeLa cells were cultured in 10% FCS-supplemented Dulbecco’s modified Eagle’s medium (DMEM). For 96-well plate experiments, cells were transfected with 1nM non-targeting control siRNAs or siRNAs targeted to non-coding regions of ERK1/2 as described (19;42). For DUSP siRNA transfection, 10nM SMARTpool or non-targeting control siRNA mixtures (Dharmacon, Cramlington, UK) were included in transfections. Sixteen hours after siRNA transfection, cells were transduced with 2x10^6 pfu/ml Ad WT or D319N ERK2-GFP vector in DMEM with 10% FCS. For luciferase assays, Ad Egr-1-luciferase and Ad CMV β-galactosidase vectors were included at 1x10^6 pfu/ml. The Ad-containing medium was removed after 4–6 h and replaced with fresh DMEM supplemented with 0.1% FCS. The cells were then maintained for 16–24 h in culture prior to stimulation with EGF (Calbiochem, San Diego, CA, USA) or PDBu (phorbol 12, 13-dibutyrate, Sigma, Poole, UK). In inhibitor studies, cells were pre-treated for 30min with 10µM SP600125 (Ascent Scientific, Weston-super-Mare, UK), 20µM SB203580 (Calbiochem) or 30µM cyclohexamide (Sigma). Expression levels of GFP-tagged fusions were compared by western blotting (ref. 1, see also supplementary Fig. 1) as well as comparison of mean cell fluorescence in microscopy assays (as demonstrated in Fig. 2C).

Western Blotting
HeLa cells were simultaneously plated and transfected in 6-well plates (2.5x10^5 cells/well) with 1nM ERK1/2 siRNAs and 10nM control or SMARTpool siRNAs prior to Ad transduction as above. Following treatment noted in figure legends, cells were lysed as described (19;41), prior to western blotting. Total and ppERK1/2 were detected using polyclonal rabbit anti-total ERK1/2 and rabbit anti-ppERK1/2 (1:1000; Cell Signaling Technology, Hitchin, UK), respectively. Loading controls were assayed by staining parallel blots with mouse anti-α-tubulin (Sigma).

Quantitative PCR
HeLa cells were simultaneously plated and transfected in 6-well plates (2.5x10^5 cells/well) with 1nM ERK1/2 siRNAs and 10nM control or SMARTpool siRNAs prior to Ad transduction as described above. Cells were either kept in 10% FCS DMEM, or kept in reduced serum media prior to stimulation with 10nM EGF or 1µM PDBu. Extraction of total RNA was performed using an RNeasy kit according to manufacturer’s instructions (Qiagen). Contaminating genomic DNA was removed from columns using an additional DNase (Qiagen) digestion step. Complementary DNA was then prepared for 1µg of each total RNA sample using a cloned AMV first-strand synthesis kit according to manufacturer’s instructions (Invitrogen). cDNAs were then quantified relative to expression of human GTPase-activating protein (hGAP) using the following primers:

- hGAP: 5’-GGG AAG GTG AAG GTC GGA GT-3’ and 5’-GAG TTA AAA GCA GCC CTG GT-3’
- DUSP1: 5’-CAA CGA GGC CAT TGA CTT CAT AG-3’ and 5’-CAA ACA CCC TTC CAT CTC CA-3’
- DUSP2: 5’-CAU AAA ACC AGC CGC TCC GCC TCC GAC-3’ and 5’-CCA GGA ACA GCC CTG GTG A-3’
- DUSP3: 5’-GCG CTT ACT TTG AAA GGG CTG-3’ and 5’-TGC CGC ATC ATG AGG TAG G-3’
- DUSP4: 5’-CTG GTT CAT GGA AGC CAT TGA GT-3’ and 5’-CGG CCG CAG CAG TCC-3’
- DUSP5: 5’-GCG CCG GTC TAC TTC CTC A-3’ and 5’-GGG TTT TAC ATC CAC GCA ACA-3’
- DUSP6: 5’-GCA TCC TGC CTC ACC TCT ACC-3’ and 5’-CTG CAG CGA AG-3’
- DUSP7: 5’-CTG CAG AAG CTG CGA-3’ and 5’-CCT GGA ATC TGC TGA AGC CT-3’
- DUSP8: 5’-GCA TCC TGC CTC ACC TCT-3’ and 5’-CCA GCC AAG CAA TGT ACC AAG-3’
- DUSP9: 5’-GGT CTC GGC GTC TAC ACC TCT-3’ and 5’-CCA GAA TGA GAG CTG TGC ATC-3’
- DUSP10: 5’-GCC TGC TGA TGC-3’ and 5’-GCA TCC TCC TGC TCT ACC TCT-3’
- DUSP11: 5’-GCC TGC TGC TGA TGC-3’ and 5’-GCA TCC TCC TGC TCT ACC TCT-3’
PCR primers were mixed with 50ng RT-PCR template and SYBR green PCR master mix (Applied Biosystems, Warrington, UK), and the comparative C_{T} method was used to detect relative expression curves on an ABI PRISM 7500 detection system (Applied Biosystems).

Semi-automated Image Acquisition and Analysis
Cells were transfected with siRNA, transduced with Ad vectors and plated as described above on Costar plain black-wall 96-well plates (Corning, Arlington, UK). Following treatment with EGF or PDBu (and/or inhibitors), cells were washed in ice-cold phosphate buffered saline (PBS) before fixation and staining for ppERK1/2 and imaging as described (19). Image acquisition in each well was performed on an IN Cell Analyzer 1000 microscope, using a x10 objective (GE Healthcare, Amersham, UK). Analysis of ppERK1/2 staining and localization was performed using the Dual Area Object Analysis algorithm in the IN Cell Analyzer Workstation (IN Cell Investigator, GE Healthcare) using DAPI and ppERK1/2 images. ERK2-GFP localization and ppERK2 staining was simultaneously analysed using the Multi-target Analysis algorithm (IN Cell Investigator, GE Healthcare) using ERK2-GFP, ppERK2 and DAPI images (ERK2-GFP and DAPI images were used to define whole-cell and nuclear regions, respectively). Single cells expressing superphysiological levels of ERK2-GFP were excluded from analysis (approx. 20% of cells) using appropriate gating parameters to prevent misleading localization data (19). 300-500 cells per field were typically analysed, and up to four fields per well were captured in experiments performed in duplicate or quadruplicate, meaning that in each experiment data were normally derived from at least 1000 individual cells per time-point. Imaging data are reported as ppERK2 intensity (mean fluorescence intensity per cell) or as a ratio of nuclear to cytoplasmic intensity (N:C ratio) of either ERK2-GFP or ppERK2 signal.

Luciferase Assays
Cells were transfected with siRNA, transduced with Ad vectors and plated as described above on Costar plain black-wall 96-well plates (Corning), but including Ad Egr-1-Luciferase and Ad CMV β-galactosidase reporter vectors. Following treatment with EGF or PDBu (and/or inhibitors), cells were washed in ice-cold PBS, lysed and assessed for luciferase activity by chemical luminescence following the addition of luciferin substrate (Promega, Southampton, UK). β-galactosidase activity was used to correct luciferase activity for transduction efficiency, as measured following the addition of chlorophenol red-β-D-galactopyranoside substrate (Roche, East Sussex, UK).

RESULTS AND DISCUSSION
Comparison of EGF and PKC-induced ERK2 responses
To examine the spatial and temporal aspects of ERK2 regulation, we have used siRNAs targeted to non-coding regions to knock-down endogenous ERK1/2 and recombinant Ad to restore ERK2 expression with fusion proteins of wild type (WT) or the phosphatase-resistant D319N ERK2-GFP. When combined with antibody staining for dually phosphorylated, active ERK1/2 (ppERK1/2) and a DAPI stain to identify nuclear regions, this system allows direct monitoring of both total and phosphorylated forms of ERK2-GFP in nuclear and cytoplasmic compartments (Fig. 1A). The key readouts from this assay are: 1. whole-cell...
ppERK2 intensity, which reflects ERK2 activation state irrespective of scaffolding or localization, 2. ppERK2 nuclear to cytoplasmic (N:C) ratio, which indicates changes in the compartmentalization and scaffolding of active ERK2, and 3. the N:C distribution of ERK2-GFP, a readout for changes in ERK2 distribution irrespective of activation state. Combining these with an Egr-1 luciferase reporter assay (as a downstream readout for ERK1/2-dependent transcriptional activation) we initially determined the effects of ERK1/2 siRNA transfection and transduction with Ad ERK2-GFP on responses to EGF and the PKC-activating phorbol ester, PDBu. In cells transfected with control siRNAs, 5min stimulation with EGF caused robust and dose-dependent increases of ppERK1/2 staining, and 6h stimulation caused induction of Egr-1 transcription. Potencies (log EC50 values) were identical for both endpoints (-10.3, Fig. 1B, left and right panels). Transfection with ERK1/2 siRNAs had no effect on cell number or the expression of α-tubulin in microscopy or western blotting assays (Supplementary Fig. 1 and (19)), but ERK1/2 expression and maximal effect of EGF on ppERK1/2 and Egr-1 luciferase were reduced by >85% (Fig. 1B, left and right panels and Supplementary Fig. 1). Subsequent transduction with Ad ERK2-GFP restored ERK2 expression levels (as judged by western blotting for ERK1/2, Supplementary Fig. 1). It also restored the whole-cell ppERK2 and Egr-1 luciferase responses, so that maximal responses to EGF and log EC50 values were indistinguishable between cells receiving control siRNAs and those receiving ERK1/2 siRNAs and Ad ERK2-GFP together. Monitoring of the ERK2-GFP nuclear-cytoplasmic (N:C) ratio revealed that increases in ERK2-GFP nuclear distribution paralleled ppERK2 responses in the same cells (Fig. 1B, left and middle panels). Similar profiles were seen in parallel experiments with PDBu, but with a log EC50 of -6.3 (not shown).

We next used the knock-down and add-back system to define time-courses of EGF and PDBu treatment. EGF caused a rapid and transient increase in whole cell ppERK2 levels, which was paralleled by a transient relocalization of ERK2-GFP to the nucleus in the same cells (Fig. 1C, left and middle panels). EGF also mediated strong activation of the Egr-1 luciferase reporter, peaking at approximately a 100-fold induction over basal levels after 4-6h stimulation (Fig. 1C, Right panel). PDBu caused a comparably rapid ppERK2 response to that of EGF (maximum at 5-15min) with a subsequent reduction to approximately 40% of peak values for the remainder of the experiment (Fig. 1C, left panel). PDBu also caused a similar rapid increase in N:C ERK2-GFP ratio to EGF, but rather than paralleling the activation profile, ERK2-GFP nuclear localization was sustained, peaking at 120min (Fig. 1C, middle panel). This echoes previous findings that transient ERK2 activation does not mediate sustained nuclear accumulation of ERK2 (19;43). In contrast, PDBu causes sustained ERK2 activation as well as sustained nuclear localization of dephosphorylated ERK2 ((19) and images in Fig. 3C, Fig. 5 and Fig. 7B). The more sustained ppERK2 response to PDBu is associated with more pronounced activation of Egr-1 luciferase. These responses reached approximately 240-fold induction after 6h stimulation, almost 2.5 times higher that that induced by EGF (Fig. 1C, right panel). Together, these data reveal that out knock-down, add-back and imaging based model recapitulates key features of ERK1/2 signaling seen with more conventional models (e.g. western blotting, (6-9)). These include relative potencies, duration of responses and effects on nucleocytoplasmic distribution in cells stimulated with EGF and PDBu. The parallel effects on Egr-1-dependent transcription also demonstrate that the transient and sustained ERK1/2 activation modes are interpreted by the cell at the level of immediate early gene transcription (6-9).

Effects of D-domain interference on ERK2 activation, trafficking and Egr-1 transcription

The D319N mutation within the common docking domain of ERK2 is analogous to the sevenmaker gain of function mutation in Drosophila (44), which perturbs ERK2’s ability to associate with D-domain binding partners without affecting intrinsic kinase activity (15;42). These partners include all MKPs (1;14;16), making the D319N variant a useful tool to study the influence of DUSP interaction on ERK2 responses. We first assessed the dose-dependence of EGF and PDBu effects under conditions of ERK1/2 knock down and reconstitution with WT or D319N ERK2-GFP. We found that D319N mutation did not
affect the potency (log EC\textsubscript{50} values) of EGF or PDBu effects on ppERK2, ERK2-GFP N:C ratio, and Egr-1 luciferase activity (Fig. 2A, B, D and not shown). However, maximal ERK2-GFP N:C and Egr-1 luciferase responses were significantly altered. As shown (Fig. 2B), EGF-mediated changes in ERK2-GFP N:C ratio at 5min stimulation were reduced by up to 50% by D319N mutation, while whole cell ERK2-GFP intensity (N+C) values remained unchanged (indicating comparable whole cell expression levels, Fig. 2C), but Egr-1 luciferase responses were increased approximately 2.5-fold (Fig. 2D). In contrast, inhibition of D-domain interactions had no effect on 5min ppERK2 responses to EGF (Fig. 2A). Similar trends were observed in cells stimulated with PDBu (not shown).

We characterized these differences further in time-course studies using maximally stimulating concentrations of EGF or PDBu. Early, peak levels of ppERK2 responses were unaltered by D319N mutation but responses to both stimuli were greatly increased at later time-points (Fig. 3A and 3B, left panels and Fig. 3C). Comparing the distribution of the ppERK2 signal intensity in the nucleus and cytoplasm (ppERK2 N:C) revealed that prolongation of the whole-cell ppERK2 signal by D319N mutation is associated with an increased proportion of ppERK2 in the nucleus (Fig. 3A and 3B, middle left panels and Fig. 3C). In contrast, the ERK2-GFP N:C ratios from the same cells show that D319N actually reduces the total amount of ERK2-GFP in the nucleus (Fig. 3A and 3B, middle right panels and Fig. 3C). The D319N mutation also increased effects of both stimuli on Egr-1 transcription, almost doubling responses at 4-6h (Fig. 3A and 3B, right panels). Taken together, these data show that D319N-mediated inhibition of phosphatase binding does not increase the sensitivity of ERK2-GFP to low concentrations of stimuli (see also ), but increases ERK2-dependent transcriptional responses by prolonging its activation and increasing the proportion of active ERK2 in the nucleus. The corresponding decrease in ERK2-GFP N:C ratio is consistent with the fact that, in these cells, high levels of nuclear accumulation are due to D-domain dependent scaffolding and signal termination by nuclear inducible MKPs (19).

**Regulation of DUSP transcription and ERK2 responses**

Since D319N mutations inhibit ERK2 association with MKP family DUSPs (14;16;19), the data above are indicative of a major role for them in shaping ERK2 responses. Many ERK1/2 stimuli induce the transcription of nuclear-inducible MKPs to act in negative feedback loops, but the involvement of other DUSPs has not been extensively explored in this context. We first assessed expression and knock-down of DUSPs 1-16, 18, 19, 21 and 22 using quantitative PCR (qPCR) and siRNAs. Of these twenty transcripts, we found that DUSP13, 15 and 21 mRNAs were not detectable in these cells, and that DUSP8 mRNA could not be reduced by more than 30% following siRNA transfection (not shown), so these enzymes were excluded from further analysis. The qPCR revealed that a large number of the remaining DUSP transcripts (nine out of sixteen) were increased by EGF and/or PDBu (Fig. 4A). PDBu had a greater effect than EGF on both the number of transcripts regulated (six compared to three) and, in some cases, the magnitude of transcription (Fig. 4A). Thus, although transcriptional regulation appeared characteristic of the nuclear-inducible MKPs, it also occurred amongst the cytoplasmic ERK MKPs (DUSP6), the JNK/p38 MKPs (DUSP10) and the Atypical DUSPs (DUSP14) (Fig. 4A). ERK2 activity and signal duration clearly plays a major role in these transcriptional responses, as evidenced by the sensitivity to ERK1/2 knock-down (inhibiting effects on DUSP1 and 2 transcription) and D319N ERK2-GFP expression (enhancing transcription of DUSPs 2, 5 and 6) (Fig. 4A).

To investigate possible roles of these DUSPs, we reduced the mRNA levels of each DUSP through siRNA targeting. Assessment of mRNA levels 48h after siRNA transfection revealed at least 70% reduction in mRNA levels for each of the sixteen genes tested (Supplemental Data, table 1). These DUSP knock-down conditions were used in conjunction with ERK1/2 siRNAs and Ad WT ERK2-GFP to assess DUSP regulation of ERK2. None of the siRNAs significantly altered whole cell ERK2-GFP expression levels, cell number or the proportion of apoptotic cells (Supplemental Data, table 1) arguing against non-specific cytotoxic effects. Forty-eight hours after
transfection, cells were either left untreated (basal), or stimulated with EGF (5 and 120min) or PDBu (15 and 120min) prior to assessment of ppERK2 staining and ERK2-GFP localization. As shown (Fig. 4B), we found twelve phosphatases that had a significant effect on PDBu and/or EGF-stimulated ERK2 phosphorylation and/or compartmentalization (Fig. 4B). None of the siRNAs had a measurable effect on whole cell ppERK2 levels in unstimulated cells (Fig. 4B, left panel), but siRNAs to DUSPs 3, 5, and 10 significantly reduced the ERK2-GFP N:C ratio, and DUSP2 and 7 siRNAs increased ERK2-GFP nuclear localization under basal conditions (Fig. 4B, right panel). siRNAs to DUSPs 3, 9, 10 and 16 all reduced ppERK2 and/or ERK2-GFP responses to both EGF and PDBu (Fig. 4B). No siRNAs increased ppERK2 responses to EGF or PDBu, but siRNAs to DUSPs 5, 7, 12, 14 and 18 specifically inhibited the PDBu-mediated ppERK2 response (particularly at later time-points) without altering the EGF response at either time-point (Fig. 4B). As expected (19), siRNAs to DUSPs 2 and 4 reduced the ERK2-GFP N:C ratio in cells stimulated with PDBu without measurably altering the ppERK2 response, nor any aspect of the EGF response (Fig. 4B). DUSP1 knock-down was the sole condition that increased the ERK2-GFP N:C ratio in stimulated cells. DUSP3 and 9 siRNAs were the only treatments to significantly reduce the effect of both EGF and PDBu on ppERK2 levels and ERK2-GFP N:C ratio (Fig. 4B). The striking findings from these experiments are firstly that many DUSPs from both the MKP and atypical groups play specific roles in shaping ERK2 responses, with little overlap or redundancy of function (illustrated in representative images in Fig. 5). Secondly, the unifying theme of action across each subgroup of DUSPs was that the knock-downs inhibited ligand effects on ppERK2 and/or ERK2-GFP N:C ratio. However, the nuclear-inducible MKPs stood out as the only group which had pronounced stimulus-specific effects on ERK2-GFP N:C ratio without an overall effect on phosphorylation state.

Effects of DUSP knock-down on stimulus-specific induction of Egr-1
As most DUSP knock-downs reduced ppERK2 responses and/or ERK2-GFP N:C ratio, we focused on a selection of DUSPs showing a range of effects on ERK2-GFP activation and trafficking to see if they had corresponding effects on Egr-1 transcription. We compared EGF and PDBu-mediated Egr-1 induction in the presence and absence of siRNAs to DUSPs 1, 2, 3, 9, 10 and 16 (Fig. 6). Knock-down of DUSPs 3, 9, 10 and 16 reduced PDBu effects on Egr-1 luciferase, paralleling their ability to reduce the PDBu effect on ppERK2 and/or ERK2-GFP N:C response. Similar effects were seen in EGF-stimulated cells, suggesting that the effects of these DUSPs were not stimulus-specific (Fig. 6). For each of these DUSPs, effects of knock-down on ERK2 activity were predictive of functional outcome: a reduction in ppERK2 response led to a reduction in transcriptional activation. In contrast, knock-down of the nuclear inducible MKPs, DUSP1 and 2, did not affect ppERK2 responses, but increased and decreased ERK2-GFP N:C localization, respectively (Fig. 6). They also had stimulus-specific effects on Egr-1 transcription. DUSP2 knock-down caused a 40% increase in PDBu-stimulated Egr-1 luciferase (without altering the EGF-mediated response) and DUSP1 knock-down had no effect on the levels of Egr-1 luciferase activity under any condition (Fig. 6).

Functional profiling of DUSP Family Effects on ERK Signaling
The data outlined above reveal the stimulus-specific shaping of ERK2 responses by members of each DUSP class. They also demonstrate the importance of assaying localization as well as phosphorylation state when screening for changes in ERK2 regulation. This appears to be especially pertinent when dealing with the nuclear inducible MKPs, presumably because they can both dephosphorylate and scaffold ERK2 (18;19) and because knock-down of one can lead to compensatory changes in another. For example, we have found that knock-down of DUSP1 increases PDBu-stimulated DUSP2 expression, just as knock-down of DUSP2 increases PDBu-stimulated DUSP1 expression (19). In light of such compensation we found that function of these proteins could best be revealed by simultaneous knock-down of multiple nuclear-inducible DUSPs (19). Extending this we have combined siRNAs to knock-down all nuclear-inducible MKPs (DUSPs 1, 2, 4 and 5), all cytoplasmic ERK MKPs (DUSPs 6, 7 and 9) or both JNK/p38 MKPs (DUSPs 10
and 16) before determining PDBu effects. As shown (Fig. 7A and C, left panels, and representative images in B), knock-down of the nuclear-inducible MKPs did not alter whole cell ppERK2 responses but caused a marked prolongation of PDBu effects on the ppERK2 N:C ratio whilst reducing its effects on the ERK2-GFP N:C ratio and greatly enhancing its effect on transcriptional activation of Egr-1. These data demonstrate the importance of the nuclear-inducible MKPs (collectively) as inhibitors of sustained ERK signaling by virtue of their ability to inactivate and scaffold ERK within the nucleus. In contrast, siRNAs targeting the cytoplasmic ERK MKPs did not alter PDBu effects on whole cell ppERK2 responses or on the ppERK2 N:C ratio and had only a modest inhibitory effect on ERK2 distribution (reducing the PDBu effect on ERK2-GFP N:C ratio at 240 min only). They also failed to alter PDBu-stimulated Egr-1 luc activity, arguing against a major role for cytoplasmic ERK MKPs in shaping of sustained ERK signaling in this model. Knock-down of the JNK/p38 MKPs also failed to alter PDBu effects on ppERK2 distribution but it did reduce the PDBu effect on the whole cell ppERK2 levels and ERK2-GFP N:C ratio (significant reductions of both at 60-120 min) and also inhibited the PDBu effect on Egr-1 luc activity. Thus, in this model the JNK/p38 MKPs act (together) as positive regulators of ERK2 signaling, supporting Egr-1 luciferase activity by enhancing ERK2 phosphorylation. This is in sharp contrast to the nuclear-inducible MKPs that act (collectively) as negative regulators of ERK2 signaling, primarily by reducing the proportion of active ERK2 within the nucleus (Fig. 7).

Relevance of protein neosynthesis and JNK or p38 MAPKs for JNK/p38 MKP effects on ERK2 Signaling.

We next explored mechanisms of JNK/p38 MKP action, focussing first on relevance of protein neosynthesis. We have previously shown that the sustained effects of PDBu on whole cell ppERK2 levels and ERK2-GFP N:C ratio and its more transient effect on ppERK2 N:C ratio are all increased and/or prolonged by the protein synthesis inhibitor, cyclohexamide (CHX). In contrast, when EGF is used to elicit transient ERK activation, CHX has little or no effect (ref. (19) and Supplemental data, Fig. 2). These effects are thought to reflect prevention of nuclear-inducible MKP neosynthesis as evidenced by the close parallels between effects of CHX and knock-down of nuclear-inducible MKPs as well as with effects of the D319N mutation that inhibits ERK2 binding to nuclear-inducible MKPs (Fig. 3). To determine the relevance of protein synthesis for JNK/p38 effects we repeated the JNK/p38 siRNA experiments (shown in Fig. 7) in the presence and absence of CHX. As shown (Fig. 8) CHX caused the expected prolongation of PDBu effects on ppERK2 levels and nuclear localization of ppERK2, whilst inhibiting its effects on ERK2-GFP N:C localization. JNK/p38 siRNAs reduced PDBu effects on whole cell ppERK2 levels and on ERK2-GFP distribution, but did not alter its effects on ppERK2 N:C ratio (Fig. 8). JNK/p38 siRNAs also caused a pronounced inhibition of PDBu effects on each of these imaging end-points in the presence of CHX (Fig. 8). The fact that effects of JNK/p38 knock-down are maintained or increased by CHX argues that shaping of ERK2 responses by JNK/p38 MKPs is not dependent upon protein neosynthesis in spite of the fact that PDBu increased DUSP10 transcription (Fig. 4A). This is in direct contrast to the nuclear-inducible MKPs, for which neosynthesis is clearly necessary (13;14;18;19;43;45).

Changes in JNK/p38 activity can have profound effects on the ERK MAPK pathway (46), and a recent study demonstrated that impairment of ERK1/2 activation in DUSP2-/- mice was due to increased JNK activity (45). The observation that JNK/p38 MKPs influence ERK2 signaling in this model implies that PDBu effects on ERK2 may be influenced by cross-talk from concomitantly activated p38 and JNK MAPKs. To address this possibility we determined effects of pharmacological inhibition of JNK (using SP600125) and p38 (using SB203580) on ERK2 signaling and also determined the effects of the JNK/p38 MKP siRNAs in the presence of these inhibitors. We found that when used alone, these inhibitors did not measurably influence responses to PDBu (Fig. 9). As expected, knock-down of the JNK/p38 MKPs inhibited PDBu effects on whole cell ppERK2 levels, ERK2-GFP N:C ratio and Egr-1 luciferase responses without altering its effects on ppERK2 N:C ratios (Fig. 7 and Fig. 9).
However, the knock-down effects on Egr-1 activation and ERK2-GFP nuclear localization were reversed by pharmacological inhibition of JNK or p38 activity: Figure 9 shows data for 120 min stimulation with PDBu, and similar effects were seen with 30 or 60 min stimulation (not shown). Thus, the observed effects of JNK/p38 MKP siRNAs are, at least partially, dependent upon activation of JNK and/or p38 MAPKs. These data therefore identify the JNK/p38 MAPKs as modulators of ERK2 signaling and as the pertinent targets for JNK/p38 MKPs in this respect.

Summary
Multiple DUSPs clearly contribute to the regulation of ERK1/2, and at least twelve DUSPs can dephosphorylate ERK1/2 in vitro (13;14). However, the prediction of DUSP regulation of ERK1/2 in cells is complicated by a number of factors. Firstly, they can often dephosphorylate multiple MAPK substrates (13;14). Secondly, their transcription can be rapidly regulated in a stimulus-specific manner and thirdly, their expression is highly cell-type specific (Fig. 4A and (13;14;19;45)). These properties make them exciting therapeutic targets, and a number of DUSP inhibitors have been discovered in recent years (35-38). It is important to recognise, however, that DUSPs may influence many interconnecting signaling and feedback pathways so their loss or inhibition can have positive or negative effects on ERK1/2 signaling (13;14;19;45). This underlines the need to explore DUSP function within intact systems but we know of no previous work in which mammalian DUSPs have been systematically knocked down in order to assess their influence on ERK1/2 signaling. Here, we have done so and have determined the effects of siRNA-mediated knock-down of individual DUSPs on multiple aspects of ERK2 regulation. Our data reveal that a surprisingly large number of DUSPs influence ERK2 signaling in this model. These include members of the nuclear inducible MKPs (DUSPs 1, 2, 4 and 5). They also include MKPs of the cytoplasmic ERK1/2 selective group (DUSPs 7 and 9) and the JNK/p38-selective group (DUSPs 10 and 16), as well as four of the atypical DUSPs.

Although a screen of this nature cannot reveal mechanisms, it did reveal a remarkable degree of specificity. The functional profiles of DUSP knock-downs were almost all distinct when the stimulation conditions (stimulus and time) and end-points (ppERK2 levels, ERK2-GFP N:C ratio and Egr-1 transcription) were taken into consideration (Fig. 4, Fig. 5 and Fig. 6). The other obvious finding is that the effects of DUSP knock-down (with the exception of the nuclear inducible MKPs) were all negative: DUSP knock-down caused a decrease in ppERK2 response and a corresponding decrease in Egr-1 transcription (Fig. 6). This is in stark contrast to the experiments we performed expressing a D319N variant of ERK2-GFP to inhibit interactions with phosphatases, which had a pronounced enhancing effect on ERK2 activity (Fig. 2 and Fig. 3). In this comparison it is important to note that this mutation will only affect the binding of phosphatases that contain D-domains, and will only affect association with ERK2, while the catalytic activity of the DUSPs and their effect on other MAPKs will be left intact. In contrast, the siRNA knock-downs represent the impact of selective phosphatase inhibition. These data may therefore prove useful in the design and use of isotype-specific DUSP inhibitors and inhibitors that reduce DUSP-ERK1/2 interaction, as the outcomes can clearly be very different. This study also sounds a cautionary note in screening assays for ERK1/2 regulation. By monitoring two endpoints (ppERK2 and ERK2-GFP N:C) with two stimuli (EGF and PDBu) and two time-points (for each stimulus) we have found that twelve of the sixteen DUSPs tested influence ERK2 signaling. Had we performed a single end-point assay screening for effects on ERK2-GFP N:C ratio after 15 min stimulation with PDBu, we would have obtained only one hit and, if we had just screened for effects on the acute ERK2-GFP N:C response to EGF, we would have obtained none.

Following-up the screen with more mechanistic studies we have found that the nuclear-inducible MKPs act (collectively) as negative regulators of ERK2 signaling whereas the JNK/p38 MKPs act (together) as positive regulators. Interestingly, they do so by distinct mechanisms as the nuclear-inducible MKPs inhibit ERK-dependent transcription by reducing the proportion of
ppERK2 in the nucleus (but without altering the whole cell level of active kinase) whereas the JNK/p38 MKPs support ERK-dependent transcription by increasing whole-cell ppERK2 levels. Moreover, the JNK/p38 MKPs appear to shape PDBu-mediated ERK2 signaling by acting upon JNK and p38 MAPKs. Together, our findings support the notion that DUSPs represent non-redundant regulators of ERK2 signal regulation in response to external stimuli. The degree of specificity revealed in this study support the use of multiple-endpoint microscopy-based assays to resolve effects of DUSP regulation of ERK1/2. Most importantly, the specificity revealed in this investigation may well prove important in the exploitation of DUSPs as therapeutic targets.
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FOOTNOTES

* This work was supported by Wellcome Trust Project and Equipment Grants (062918, 076557 and 078407).

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2The abbreviations used in this manuscript are: PKC, protein kinase C, ERK, extracellular signal-regulated kinase, MAPK, mitogen-activated protein kinase, MEK, MAPK/ERK kinase, EGF, epidermal growth factor, MKP, MAPK phosphatase, DUSP, dual-specificity phosphatase, JNK, c-Jun N-terminal kinase, D-, docking, siRNA, short inhibitory RNA, GFP, (enhanced) green fluorescent protein, PDBu, phorboleth dibutyrate, CHX, cyclohexamide, SP, SP600125, SB, SB203580, Ad, adenovirus, PBS, phosphate buffered saline, qPCR, quantitative polymerase chain reaction, hGAP, human GTPase-activating protein, DAPI, 4'-6-Diamidino-2-phenylindole, N:C, nuclear:cytoplasmic, N+C, nuclear+cytoplasmic.
FIGURE LEGENDS

Figure 1
High content imaging methods for studying ERK1/2 regulation. (A) Cells were transfected with ERK1/2 siRNAs and transduced with Ad ERK2-GFP. Following treatment, cells were stained and images acquired for DAPI, ERK2-GFP and ppERK2 stains, as described in Experimental Procedures. Top panels show whole fields of acquired images, and bottom panels show blown-up images of areas denoted by white squares. Outlines on lower panels denote the segmentation of individual cells according to DAPI and ERK2-GFP intensity using Multi-target Analysis Software. Cells without outlines indicate cells excluded from analysis either for expressing superphysiological levels of ERK2-GFP, or failing to meet other criteria needed for accurate segmentation. Bars: 100µm. (B) Cells were transfected with control (ctrl) siRNAs, ERK1/2 siRNAs or ERK1/2 siRNAs as well as Ad ERK2-GFP as indicated. Cells were stimulated for 5min with indicated concentrations of EGF before fixation, ppERK1/2 staining, image acquisition and analysis as described in (A) to assess whole cell levels of ERK1/2 phosphorylation (ppERK, left panel) and nucleo-cytoplasmic distribution of ERK2-GFP (ERK2-GFP N:C, middle panel). For Egr-1 luciferase assays, Ad Egr-1 luciferase and Ad CMV β-galactosidase vectors were also added to cells before stimulation with EGF for 6h and assay of luciferase activity (Egr-1 Luc) as described in Experimental Procedures, and are expressed as fold change compared to unstimulated conditions. (C) Cells were transfected with ERK1/2 siRNAs and transduced with Ad ERK2-GFP (with or without Ad Egr-1 luciferase and Ad CMV β-galactosidase), prior to stimulation with 10nM EGF or 1µM PDBu, as indicated, in time-course studies. Cells were fixed and assessed for ppERK2 levels (left panel) and ERK2-GFP N:C localization (middle panel) simultaneously, or lysed and assayed for luciferase activity (right panel) as described in (B). Data shown are from four separate experiments (mean ±SEM, n=4). *= p<0.05 and **= p<0.01, comparing PDBu and EGF-stimulated cells using two-way ANOVA and Bonferroni post-hoc tests.

Figure 2
Influence of D-domains on the potency of ERK2 signaling. Cells transfected with ERK1/2 siRNAs were transduced with Ad wild-type (WT) or D319N-mutated ERK2-GFP and analysed for activation, localization and transcriptional activation as follows: (A, B and C) Cells were stimulated in 96-well plates with the indicated concentrations of EGF for 5min and stained before image acquisition and analysis (as described in Fig. 1) for the calculation of whole-cell ppERK2 intensity (A), the ERK2-GFP N:C ratio (B) and whole-cell ERK2-GFP (N+C) levels (C). (D) Cells were additionally transduced with Ad Egr-1 luciferase and Ad CMV β-galactosidase vectors before stimulation with indicated concentrations of EGF for 6h and prior to lysis and luciferase assay (as described in Fig. 1) for the assessment of Egr-1 promoter activity. Data shown were obtained from three separate experiments, each with duplicate wells (mean ±SEM, n=3). *= p<0.05 and **= p<0.01, comparing WT to D319N conditions, according to two-way ANOVA and Bonferroni post-hoc tests.

Figure 3
Enhancement of ERK2 signaling by D319N mutation of ERK2-GFP. (A, B and C) Cells transfected with ERK1/2 siRNAs were transduced with Ad wild-type (WT) or D319N-mutated ERK2-GFP as indicated prior to stimulation with 10nM EGF (A) or 1µM PDBu (B and C) for the times indicated. Cells were stained before image acquisition and analysis (as described in Fig. 1) for the calculation of whole-cell ppERK2 intensity (A and B, left panels), ppERK2 N:C ratio (A and B, middle-left panels) and the ERK2-GFP N:C ratio (A and B, middle-right panels). Cells were additionally transduced with Ad Egr-1 luciferase and Ad CMV β-galactosidase vectors before stimulation to assess Egr-1 induction by luciferase assay as described in Fig. 1 (A and B, right panels). Data shown were obtained from three separate experiments, each with duplicate wells (mean ±SEM, n=3). *= p<0.05 and **= p<0.01, comparing WT to D319N conditions, according to two-way ANOVA and Bonferroni post-hoc tests. (C) Representative
cropped images, collected under conditions described in (A) and (B) showing differences in ERK2-GFP distribution (top panels) and ppERK2 levels (bottom panels) following stimulation with 1µM PDBu as indicated. Bar: 50µm.

**Figure 4**
Stimulus and ERK-dependence of DUSP transcription and effects of DUSP siRNAs on ERK2 signaling. (A) Cells were transfected in 6-well plates with control siRNAs (Ctrl) or ERK1/2 siRNAs and transduced with Ad wild-type (WT) or D319N-mutated ERK2-GFP as indicated. Cells were either left unstimulated (basal) or treated with 10nM EGF (left panel) or 1µM PDBu (right panel) for 120min. Total RNA isolates were analyzed for relative levels of DUSP2 mRNA using qPCR protocols described in Experimental Procedures. Data shown are average values from three independent experiments represented as fold change from basal levels, and presented as a heat map. DUSPs are grouped according to sequence similarity and substrate specificity and data included are values found to differ significantly from control (basal) conditions using one-way ANOVA and Dunnet’s post hoc test, accepting p<0.05 as significant. (B) Cells were transfected in 96-well plates with 1nM ERK1/2 siRNAs and 10nM control (Ctrl) or siRNA SMARTpools targeting individual DUSPs (as indicated) before addition of Ad ERK2-GFP. Cells were stimulated with 1µM PDBu or 10nM EGF as indicated, prior to staining and imaging (as described in Fig. 1). Data are expressed in the heat map as the extent of difference above or below control values for each condition and time-point for ppERK2 intensity (left panel) and ERK2-GFP N:C ratio (right panel) from four separate experiments performed in duplicate. Targets are again grouped according to sequence similarity and substrate specificity. Statistical analysis was performed using one-way ANOVA and Dunnet’s post-hoc test, accepting p<0.05 as significant. Non-significant changes are shown as white blocks for both experiments.

**Figure 5**
DUSP siRNAs affect spatiotemporal ERK2-GFP regulation. Cells were transfected in 96-well plates with 1nM ERK1/2 siRNAs and 10nM control (Ctrl) or siRNA SMARTpools targeting individual DUSPs (as indicated) before addition of Ad ERK2-GFP. Cells were stimulated with 1µM PDBu as indicated, prior to staining and imaging (as described under Figure 1). Representative cropped images are shown for each condition from ERK2-GFP (top panels) and ppERK2 images (bottom panels). Bar: 50µm.

**Figure 6**
Comparison of ERK2-GFP activation, localization and regulation of Egr-1 following DUSP knock-down. Cells were transfected with 1nM ERK1/2 siRNAs and 10nM control (Ctrl) or siRNA SMARTpools targeting individual DUSPs (as indicated) before addition of Ad ERK2-GFP. Top and middle panels: cells were either left unstimulated (basal), or treated with 1µM PDBu or 10nM EGF for 120min as indicated, prior to staining and imaging (as described in Fig. 1). Bottom panel: cells were additionally transduced with Ad Egr-1 luciferase and Ad CMV β-galactosidase vectors before stimulation with 1µM PDBu or 10nM EGF for 6h as indicated to assess Egr-1 induction by luciferase assay (as described in Fig. 1). Data are expressed as average ppERK2 values (top panel), ERK2-GFP N:C ratio (middle panel) and fold change in Egr-1 luciferase activity compared to unstimulated ctrl siRNA-transfected cells (bottom panel) and were obtained from three separate experiments, each with duplicate observations (mean ±SEM, n=3). *= p<0.05 and **= p<0.01, comparing DUSP and control siRNA conditions for each stimulus, according to one-way ANOVA and Dunnet’s post-hoc test.

**Figure 7**
Contribution of DUSP subfamilies to ERK2 regulation. (A) Cells were transfected with ERK1/2 siRNAs and either 40nM control siRNAs (Ctrl si), 40nM nuclear inducible MKP siRNAs (Nuc Ind MK Psi, 10nM each of DUSP1, 2, 4 and 5 siRNAs), 40nM cytoplasmic ERK MKP siRNAs (Cyt ERK MKP si, 10nM each of DUSP6, 7, 9 and ctrl siRNAs) or 40nM JNK/p38 siRNAs (JNK/p38 MKP si, 10nM each of
DUSP10 and 16 siRNAs with 20nM ctrl siRNAs) as indicated. Cells were transduced with Ad ERK2-GFP prior to stimulation with 1µM PDBu for the times indicated. Cells were stained before image acquisition and analysis (as described in Fig. 1) for the calculation of whole-cell ppERK2 intensity (top panels), ppERK2 N:C ratio (middle panels) and the ERK2-GFP N:C ratio (lower panels). (B) Representative cropped images for Ctrl siRNA and Nuc Ind MKP siRNA conditions (as indicated) collected as described in (A, left panels) showing differences in ERK2-GFP (top panels) and ppERK2 (bottom panels) distribution following stimulation with 1µM PDBu as indicated. Bar: 50µm. (C) Cells treated as described in (A) were additionally transduced with Ad Egr-1 luciferase and Ad CMV β-galactosidase vectors before stimulation with PDBu for times indicated to assess Egr-1 induction by luciferase assay (as described in Fig. 1). Data shown in A and C were obtained from four separate experiments, each with duplicate wells (mean ±SEM, n=4). *= p<0.05 and **= p<0.01, comparing Ctrl siRNA to test conditions, according to two-way ANOVA and Bonferroni post-hoc tests.

**Figure 8**
Effects of protein synthesis inhibition on JNK/p38 MKP regulation of ERK2. (A, B and C) Cells were transfected with 1nM ERK1/2 siRNAs and either 40nM control siRNAs (Ctrl si) or 40nM JNK/p38 siRNAs (JNK/p38 MKP si, 10nM each of DUSP10 and 16 siRNAs with 20nM ctrl siRNAs) as indicated. Cells were transduced with Ad ERK2-GFP prior to treatment with 30µM cyclohexamide (CHX) as indicated for 30min prior to stimulation with 1µM PDBu for the times indicated. Cells were stained before image acquisition and analysis (as described in Fig. 1) for the calculation of whole-cell ppERK2 intensity (A), ppERK2 N:C ratio (B) and the ERK2-GFP N:C ratio (C). Data shown in all panels were obtained from four separate experiments, each with duplicate wells (mean ±SEM, n=4). *= p<0.05 and **= p<0.01, comparing Ctrl and JNK/p38 siRNA conditions to those with CHX, according to two-way ANOVA and Bonferroni post-hoc tests.

**Figure 9**
Effects of JNK and p38 kinase inhibition on JNK/p38 MKP regulation of ERK2. Cells were transfected with 1nM ERK1/2 siRNAs and either 40nM control siRNAs (Ctrl si) or 40nM JNK/p38 siRNAs (JNK/p38 MKP si, 10nM each of DUSP10 and 16 siRNAs with 20nM ctrl siRNAs) as indicated. Cells were transduced with Ad ERK2-GFP prior to treatment with 10µM SP600125 (SP) or 20µM SB203580 (SB) as indicated for 30min prior to stimulation with 1µM PDBu for 120min. Cells were stained before image acquisition and analysis (as described in Fig. 1) for the calculation of whole-cell ppERK2 intensity (middle panel) and the ERK2-GFP N:C ratio (lower panel). Cells in the upper panel were treated as described in above, but were additionally transduced with Ad Egr-1 luciferase and Ad CMV β-galactosidase vectors before stimulation with 1µM PDBu for 6h prior to assessment of Egr-1 induction by luciferase assay (as described in Fig. 1). Data shown were obtained from three separate experiments, each with triplicate wells (mean ±SEM, n=3). *= p<0.05 and **= p<0.01, comparing JNK/p38 siRNA conditions with and without SP or SB, according to one-way ANOVA and Dunnet’s post-hoc tests.

**Figure 10**
Model of ERK2 regulation by nuclear inducible and JNK/p38 MKPs. ERK2 activation and translocation to the nucleus causes neosynthesis of the nuclear inducible MKPs (DUSP1, 2, 4 and 5) which that collectively mediate both dephosphorylation and scaffolding of ERK2 in the nucleus. Previous studies have revealed that all nuclear inducible MKPs can contribute to ERK2 dephosphorylation, but only DUSP2, 4 and 5 stably associate with ERK2, while DUSP1 inactivates and releases ERK2 for reactivation in the cytosol (18;19). This presumably facilitates sustained ERK2 signals in the face of persistent upstream stimuli. In contrast, we find that the JNK/p38 MKPs (DUSP10 and 16) have a positive role in ERK2 regulation by inactivating JNK and p38 kinases. The proteins that mediate negative cross-talk between the JNK, p38 and ERK2 pathways have not been identified in this system, but have been defined in others (46).
Supplementary Figure 1
A knock-down and add-back model for studying ERK regulation. Cells were transfected with control siRNAs (ctrl), ERK1/2 siRNAs, or ERK1/2 siRNAs and transduced with Ad ERK2-GFP, prior to stimulation with 1µM PDBu as indicated. Samples were immunoblotted for α-tubulin (bottom panel) total ERK1/2 (middle panel) and ppERK1/2 (top panel) as described in Experimental Procedures and (19).

Supplementary Figure 2
Effects of protein synthesis inhibition on ERK2 activation and traffic. (A and B) Cells were transfected in 96-well plates with ERK1/2 siRNAs and transduced with Ad ERK2-GFP prior to stimulation with 10nM EGF (A) or 1µM PDBu (B). Cells were left untreated (Ctrl) or treated with 30µM cyclohexamide (CHX) as indicated for 30min before stimulation, staining and imaging (as described under Fig. 1) for the calculation of whole-cell ppERK2 intensity (left panels), ppERK2 N:C ratio (middle panels) and the ERK2-GFP N:C ratio (right panels). Data shown were obtained from four separate experiments, each with duplicate wells (mean ±SEM, n=4). *p<0.05 and **p<0.01, comparing Ctrl to CHX conditions, according to two-way ANOVA and Bonferroni post-hoc tests. (C) Representative cropped images for Ctrl and CHX conditions (as indicated) collected as described in (B) showing differences in ERK2-GFP (top panels) and ppERK2 (bottom panels) distribution following stimulation with 1µM PDBu as indicated. Bar: 50µm.
Figure 1

(A) DAPI, ERK2-GFP, ppERK2

(B) Egr-1 Luc

(C) ppERK2, ERK2-GFP N:C

Figure 1A shows representative images of DAPI, ERK2-GFP, and ppERK2 under different conditions. Figure 1B illustrates the expression levels of Egr-1 Luc under various treatments. Figure 1C depicts the time-dependent changes in ppERK2 and ERK2-GFP N:C levels in response to EGF and PDBu.
Figure 2

A

B

C

D

**[EGF] log M**

**ppERK2**

**ERK2-GFP N:C**

**ERK2-GFP N+C**

**Egr-1 Luc**

**WT**

**D319N**

* * *
Figure 3

A

B

C

WT ERK2-GFP

D319N ERK2-GFP

ERK2-GFP

pERK2

Basal 15min PDBu 120min PDBu

Basal 15min PDBu 120min PDBu

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Figure 5

- Ctrl siRNA
- DUSP1 siRNA
- DUSP2 siRNA
- DUSP9 siRNA

ERK2-GFP

ppERK2

Basal

PDBu 120min
Figure 9

![Bar graphs showing Egr-1 Luc, ppERK2, and ERK2-GFP N:C](image)

- **Egr-1 Luc**: Ctrl > SP > SB
- **ppERK2**: Ctrl > SP = SB
- **ERK2-GFP N:C**: Ctrl = SP > SB

Control (Ctrl), SP, SB

Ctrl si, JNK/p38 MKP si
Negative regulation of ERK by nuclear inducible MKPs, causing dephosphorylation and scaffolding

Positive regulation of ERK by JNK/p38 MKPs through dephosphorylation of JNK and p38
Spatiotemporal regulation of ERK2 by dual-specificity phosphatases
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J. Biol. Chem. published online July 23, 2008

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