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Visualising and quantitating the spatiotemporal regulation of Ras/ERK signalling by dual-specificity mitogen-activated protein phosphatases (MKPs).

Christopher J. Caunt[1], Andrew M. Kidger[2] and Stephen M. Keyse[2*]

[1]Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom and [2]Cancer Research UK Stress Response Laboratory, Division of Cancer Research, Jacqui Wood Cancer Centre, Ninewells Hospital & Medical School, University of Dundee, Dundee DD1 9SY, United Kingdom.

***Corresponding Author s.m.keyse@dundee.ac.uk**

Running Head: Spatiotemporal Regulation of ERK Signalling

1. Summary

The spatiotemporal regulation of the Ras/ERK pathway is critical in determining the physiological and pathophysiological outcome of signalling. Dual-specificity mitogen-activated protein kinase (MAPK) phosphatases (DUSPs or MKPs) are key regulators of pathway activity and may also localise ERK to distinct subcellular locations. Here we present methods largely based on the use of high content microscopy to both visualise and quantitate the subcellular distribution of activated (*p*-ERK) and total ERK in populations of mouse embryonic fibroblasts derived from mice lacking DUSP5, a nuclear ERK-specific MKP. Such methods in combination with rescue experiments using adenoviral vectors encoding wild type and mutant forms of DUSP5 have allowed us to visualise specific defects in ERK regulation in these cells thus confirming the role of this phosphatase as both a nuclear regulator of ERK activity and localisation.

i. Key words

DUSP5, Ras/ERK signalling, spatiotemporal regulation, high-content microscopy, adenoviral expression

1. Introduction

The core Ras/extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) signalling pathway is comprised of a three-component module in which a member of the Raf family of MAPK kinase kinases (MKKKs or MEKKs) phosphorylates and activates a MAPK kinase (MKK or MEK). The latter is a dual-specificity (Thr/Tyr) protein kinase, which phosphorylates both residues within the conserved T-X-Y activation loop motif to activate ERK1 and ERK2 (***1***). This pathway mediates a wide variety of physiological outcomes in response to extracellular stimuli. These include cell proliferation, differentiation, transformation, migration and survival (***2-4***). In addition, Ras/MAPK signalling is frequently deregulated in human cancers, often due to activating mutations in upstream pathway components such as receptor tyrosine kinases (RTKs), the Ras family of small GTPases, the Braf MAPK kinase kinase and MAPK kinases (***5***). Thus, Ras/ERK signalling components are a major focus of anticancer drug development with a range of small molecule inhibitors of RTKs, Braf and MEK either in development or clinical use (***6,7***).

The diversity of physiological outputs of Ras/ERK signalling is due to the coordinated phosphorylation of a wide range of cellular ERK substrates. These include transcription factors, protein kinases, metabolic enzymes and cytoskeletal proteins (***8,9***). Clearly, because these substrates either reside in or are associated with distinct subcellular compartments, the spatial as well as the temporal control of Ras/ERK signalling is a critical determinant of biological outcome. This may explain, at least in part, why ERK activation can result in quite different endpoints within the same cell type. A good example of the latter is the ability of nerve growth factor (NGF) to cause sustained activation and nuclear localisation of ERK in rat PC12 cells leading to differentiation and neurite outgrowth, while exposure to epidermal growth factor (EGF) instead results in transient ERK activation and drives cell proliferation (***10***).

Exactly how the spatial organisation of the Ras/ERK pathway is regulated is still unclear and likely to be complex. Various mechanisms have been proposed to underpin the growth factor-induced nuclear translocation of ERK itself, including activation–dependent dimerization, active/passive transport and the phosphorylation of additional regulatory sites on ERK that promote its association with importin7

(***11***). In addition, it is clear that a diverse array of proteins can act as ERK binding partners and retain ERKs in specific subcellular locations. The latter include MKK/MEK, scaffold proteins such as β-arrestin and kinase suppressor of Ras (KSR), phosphoprotein enriched in astrocytes-15 (PEA-15) and cytoskeletal elements such as microtubule and actin filaments (***12***). One class of ERK regulatory proteins that clearly plays a role in controlling both the activity and localisation of ERK1 and ERK2 are the dual-specificity MAPK phosphatases (DUSPs or MKPs) exemplified by DUSP5 and DUSP6/MKP-3 (***13***).

DUSP6/MKP-3 is a cytoplasmic ERK-specific MKP that binds tightly to the common docking (CD) domain of ERKs via a kinase interaction domain (KIM) located within the amino-terminal non-catalytic domain of the protein (***14-16***). The latter domain also carries a functional leucine-rich nuclear export signal (NES), which mediates the cytoplasmic localisation of the protein (***17***). DUSP6/MKP-3 is a transcriptional target of ERK itself and acts as a negative feedback regulator of pathway activity (***18***). Once induced in response to ERK activation, DUSP6/MKP-3 would be expected to bind to, dephosphorylate and retain ERK in the cytoplasm. Indeed, overexpression experiments have confirmed the ability of DUSP6/MKP-3 to act in this way to anchor ERKs in the cytosol: -this anchoring function requires both a functional KIM and NES within the DUSP6/MKP-3 protein (***17,19***). In contrast to DUSP6/MKP-3, DUSP5 is an inducible nuclear ERK-specific MKP, which is also transcriptionally regulated by ERK signalling and has been proposed to act as a nuclear anchor for ERK (***20,21***). Again, overexpression experiments confirm the ability of DUSP5 to anchor inactive ERK within the cell nucleus and this is also dependent on the integrity of both the KIM and a nuclear localisation signal (NLS) located within the amino terminal domain of DUSP5 (***20***).

In order to study the precise role of DUSP5 in regulating ERK activation and function, we recently generated mice in which the DUSP5 gene was deleted. These animals and cells derived from them were then used to ask two key questions about the role of DUSP5. Firstly, is ERK signalling deregulated in the absence of DUSP5, if so how does this affect the distribution of ERK/phospho-ERK and what biological endpoints are affected? Secondly, does DUSP5 play a role in modulating the oncogenic potential of mutant Ras oncogenes *in vivo*? To address the latter, we used the DMBA/TPA

model of skin carcinogenesis and found that loss of one or both copies of DUSP5 greatly sensitised mice to the development of skin papillomas. At the cellular level, we were able to show that loss of DUSP5 caused increased levels of nuclear phospho-ERK and gene expression studies identified a small cohort of ERK-dependent transcripts that were upregulated in cell lacking DUSP5. Prominent amongst the latter was SerpinB2, a protease inhibitor previously linked to susceptibility to DMBA/TPA-induced carcinogenesis. Surprisingly, deletion of serpinB2 completely reversed the sensitivity of mice lacking DUSP5 to DMBA/TPA indicating that DUSP5 acts as a tumour suppressor in this model by regulating nuclear ERK signalling and supressing the ERK-dependent expression of serpinB2 (***22***).

In order to study the precise spatiotemporal regulation of ERK activation in murine cells lacking DUSP5, we made extensive use of quantitative high-content microscopy to detect the subcellular distribution of endogenous total ERK and phosphorylated, activated ERK over time, either after stimulation with phorbol ester or expression of mutant Hras. This technique uses automated fluorescence microscopy to analyse thousands of individual cells per condition, thus allowing an accurate quantitation of the spatiotemporal regulation of ERK activation in cell populations (***23***). To link this regulation to the biochemical activities and ERK-binding properties of DUSP5 itself, we also employed adenoviral vectors expressing either wild type or mutant forms of DUSP5 under the control of the early growth response-1 (EGR-1) promoter. This allowed us to perform rescue experiments in which ERK-dependent DUSP5 expression was recapitulated in DUSP5 knockout cells at physiologically relevant levels. This differs from previous approaches, principally utilising DUSP5 overexpression under the control of constitutive promoters whose activity is divorced from ERK regulation, which do not reveal the same dynamic functions of DUSP5 as a feedback regulator. These techniques, together with more conventional biochemical fractionation methods to study the distribution of activated ERK are described in detail below and should be broadly applicable to the study of other MKPs which act to regulate ERK signalling such as DUSP1/MKP-2, DUSP2, DUSP4/MKP-2 and DUSP6/MKP-3.

2. Materials

2.1 MEF cell culture

1. Primary mouse embryo fibroblasts (MEFs) (*see* **Note 1**).
2. Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX supplement, pyruvate and 4.5g/l D+glucose (Invitrogen).
3. Clear bottomed, black-walled 96-well plates (Corning Costar plate 3904).
4. Foetal bovine serum (FBS)
5. Trypsin (0.25%) and ethylenediamine tetra-acetic acid (EDTA) mix dissolved in phosphate buffered saline (PBS)
6. PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.47 mM KH_2PO_4, pH 7.4.
7. Tetra-decanoyl phorbol acetate (TPA) is stored in dimethyl sulphoxide (DMSO) in single use aliquots at -20ºC. Working stock is prepared by dilution in PBS.

2.2 Adenoviral generation and purification

1. Human embryonic kidney (HEK)293 cells (*see* **Note 2**).
2. Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX supplement, pyruvate and 4.5g/l D+glucose (Invitrogen).
3. Foetal bovine serum (FBS)
4. PacI restriction enzyme and 'Cutsmart' enzyme buffer (New England Biolabs) for digestion of Adenoviral backbone and shuttle vectors.
5. 2x HBS solution for calcium phosphate transfection: rinse a clean bottle x5 with ddH_2O, to remove residual detergent, then make up 280mM NaCl, 10mM KCl, 1.5mM $Na_2HPO_4 \cdot 12H_2O$, 12mM D+glucose, 50mM HEPES buffer. pH to 7.05 and filter through 0.22µm syringe-driven filter into sterile 50ml falcon tubes. Store in 20-50ml aliquots at -20ºC.
6. 2M $CaCl_2$ for calcium phosphate transfection: make up and filter through 0.22µm syringe-driven filter. Store in 5ml aliquots at -20ºC.
7. 100mM Tris-Cl, pH 7.5.

8. CsCl -saturated 100mM Tris-Cl, pH 7.5.
9. 1:0.6 ratio 100mM Tris-Cl:CsCl-saturated 100mM Tris-Cl, pH 7.5.
10. 13x51mm 'Quick-Seal' polyallomer ultracentrifuge tubes (Beckman Coulter).
11. NVTi 90 ultracentrifuge rotor (Beckman Coulter).
12. L-80 preparative ultracentrifuge (Beckman Coulter)
13. 21G and 25G needles and 5ml syringes.
14. PBS containing 3% Sucrose.
15. 0.5-3.0ml 5kDa cut-off 'Slide-a-Lyser' dialysis cassettes (Pierce).

2.3 Immunofluorescence staining of ERK1/2, p-ERK1/2 and Myc

1. Paraformaldehyde (PFA) made up as a 4% stock solution in PBS (*see* **Note 3**).
2. 100% methanol.
3. Normal goat serum stored in single use aliquots at -20 ºC. Working stock is prepared by dilution into a 2.5% solution with PBS and 0.01% Sodium Azide.
4. Anti ERK1/2 rabbit monoclonal antibody (clone 137F5) (Cell Signaling Technology)
5. Anti *p*-ERK1/2 mouse monoclonal antibody (clone MAPK-YT) (Sigma).
6. Anti Myc epitope rabbit monoclonal antibody (clone 71D10) (Cell Signaling Technology).
7. Alexa 488-conjugated, highly cross-adsorbed goat anti-mouse secondary antibody (Invitrogen).
8. Alexa 546-conjugated, highly cross-adsorbed goat anti-rabbit secondary antibody (Invitrogen).
9. 3mM DAPI (4,6-diamidino-2-phenylindole) stock in water. Dilute 1:5000 in PBS for working solution (600nM).

2.4 Subcellular Fractionation and Immunoblot Analysis

1. NE-PER nuclear and cytoplasmic extraction reagents (ThermoScientific).
2. Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (100X) (ThermoScientific).
3. Bradford Assay Reagent (ThermoScientific).

4. NuPAGE® LDS Sample Buffer (4X) (ThermoScientific).
5. NuPAGE® Novex® 4-12% Bis-Tris Protein Gels, 1.0 mm (ThermoScientific).
6. Immobilon-FL PVDF 0.45um Blotting Membrane (Millipore).
7. Anti ERK1/2 rabbit monoclonal antibody (clone 137F5) (Cell Signaling Technology).
8. Anti ppERK1/2 rabbit monoclonal antibody (clone D13.14.4E) (Cell Signaling Technology).
9. Anti DUSP5 sheep polyclonal antibody (*see* **Note 4**).
10. Anti UBF sheep polyclonal antibody (*see* **Note 5**).
11. Anti MEK rabbit polyclonal antibody (Cell Signaling Technology).
12. Alexa Fluor® 680 conjugate, Donkey anti-Sheep IgG (H+L) Secondary Antibody (ThermoScientific).
13. DyLight™ 680 Conjugate, Goat anti-rabbit IgG (H+L) secondary antibody (Cell Signaling).

3. Methods

We have previously described methodology for sensitive detection of ERK and phosphorylated ERK in HeLa cells using high content microscopy (***23***). Here, we describe similar approaches for studying ERK regulation by DUSP5 in primary MEFs by DUSP5 knockout and reconstitution using adenoviral (Ad) expression of Myc-tagged DUSP5, or mutants of DUSP5. An additional layer of complexity is added by the heterogeneity in cell shape and size in the non-monoclonal population, which presents new challenges for automated analysis. High quality monoclonal antibodies are available, which specifically recognise dual phosphorylated ERK1/2 (but neither mono-phosphorylated form), ERK1/2 (irrespective of phosphorylation state) and the Myc epitope tag, which are validated for use in immunofluorescence imaging and flow cytometry. The use of high-content microscopy and analysis is well suited to studying spatiotemporal aspects of DUSP5-ERK regulation, which requires quantitative assessment of ERK phosphorylation and subcellular distribution in multiple knockout rescue and stimulus conditions in parallel. Antibody detection allows multiplexed single cell assessment of *p*-ERK1/2, ERK1/2 and Myc

intensity and distribution in the same samples, as suitable antibodies for detection are available as mouse or rabbit monoclonals. Our microscopy results were cross- validated using immunoblotting of biochemically fractionated cell lysates, thus we have also included the methodology for this complementary approach here.

3.1 Generation of Recombinant Adenovirus

This method is based on the Iowa Gene Transfer Vector Core 'RapAd™' system (***24)***. All plasmids may be obtained from them directly:

http://www.medicine.uiowa.edu/vectorcore/products/

The RapAd™ plasmids used to make serotype 5 recombinant adenoviral vectors have left-hand ITR, E1a, and partial E1b sequence deletions. This modification greatly reduces the chances of wild-type non-recombinant virus from contaminating the final preparation. The backbone vector, pacAd5 9.2-100, contains the entire adenoviral genome, but lacks the first 9.2 map units, and must recombine with the shuttle vector, pacAd5 K-N pA, via homology regions flanking the multiple cloning site in the presence of the E1a protein (expressed by the HEK293 helper cell line) in order to undergo viral replication and enter a lytic cycle. Therefore, the virus will not replicate in cells that do not harbour the E1a gene, and may be used safely as a vector delivery system for transgenes of interest (*see* **Note 2**).

1. Clone fragment of interest into pacAd5 K-N pA promoterless shuttle vector (in our case the EGR-1 promoter and DUSP5-Myc cDNA). This vector contains a Pac1 digestion site, Amp resistance, and large sections of the adenoviral genome necessary for viral replication.
2. Plate low passage (<50) University of Iowa HEK293 cells in DMEM+10% FBS, 24 h prior to transfection with backbone and shuttle vectors. Cells should be 50-60% confluent in a T25 for the transfection (on day of transfection).

3. Digest 1.5 µg of pacAd5 9.2-100 backbone vector with 5U of PacI in Cutsmart buffer in a total volume of 25 µl. Digest 4.5 µg pacAd5 EGR-1pDUSP5-Myc shuttle vector with 5U of PacI in Cutsmart buffer in a total volume of 25 µl. Incubate digests at 37ºC overnight.
4. Combine PacI-digested shuttle and pacAd5 9.2–100 backbone, and make up to 140 µl total volume with sterile ddH_2O.
5. Add 20 µl of 2 M $CaCl_2$. Mix well.
6. Add 160 µl of 2x HBS. Add drop-wise over a period of ~20 sec (the drop-wise addition is important to ensure precipitate formation). Incubate at room temperature for 20 min. Do not shake or mix the solution or the precipitate will not form properly.
7. Mix well by pipetting up and down and transfer to HEK293 cells by adding to media. Return to 37ºC, 5% CO_2 for 3 – 4 h (can leave the transfection medium on O/N).
8. Remove media from cells and wash once with PBS.
9. Add 5 ml of DMEM+10% FBS and return to 37ºC, 5% CO_2
10. Check daily for presence of plaques-these will appear initially as foci of clearance in the cell monolayer, where cells appear healthy apart from rounded cells with the appearance of 'bunches of grapes' at the periphery of the plaque. Gaps in the monolayer are easy to distinguish as cells will appear healthy and well spread at the periphery of the area devoid of cells. If plate is ready for harvest (> 60% of cells lifted, then tapping the flask gently removes virtually all cells). If not, return to incubator.
11. If media is low or appears exhausted before plaques start to form, add 2 ml media on day 7 and 10. Recombination and plaque formation is usually obvious by 14 days.
12. To harvest: gently tap the side of the culture flask to remove residual cells and transfer media and cells into a 5 ml sterile tube. Snap-freeze the cells in liquid N_2 and store at -80^{o}C.
13. Re-inoculate a T75 flask of HEK293 cells (in 15 ml medium) with 0.3-0.5 ml of viral lysate from the initial prep to check for functional virus alongside a non-infected control flask, and a control flask of cells that are not transformed with the E1a gene (e.g. HeLa cells). A genuine adenovirus

will cause lysis of the HEK293 flask within 24-72 h, and no effect should be seen on cell viability in either control flask. Harvest this lysate in a 50 ml tube as above. Snap-freeze the lysate in liquid N_2 and store at -80^{o}C for future large-scale amplification.

3.2 Large-scale amplification and purification of recombinant adenovirus

~~*Amplification of adenovirus*~~

1. Grow 10 x T175 flasks of low passage (<50) HEK293 cells to 60% confluency. To achieve 60% confluency, split 1 x T175 flask into 10 x T175 flasks. Cells will be 60% confluent 1-2 days post-split.
2. Infect each flask with 0.5 ml of viral lysate/supernatant (from previous steps above)
3. Inoculate for 24-72 h until the cells are about to detach from the flask. Maximal yields are achieved if cells are harvested just prior to cell lysis, so it is important the cells are not all detached already: this means they have already released virus to the supernatant.
4. Tap the side of the flask to dislodge the cells. Collect the cells in the media into 50 ml tubes.
5. Spin down the cells at 1000g for 10 min.
6. Discard the supernatant, save some 5-15 ml aliquots by snap-freezing in liquid N_2 and storing at -80^{o}C for future large scale preps if necessary.
7. Resuspend the cell pellets and pool them in a total volume of 3 ml 100 mM Tris-HCl, pH 7.5.
8. Snap-freeze in liquid N_2 and store at -80^{o}C until ready for purification.

~~*Purification of adenovirus*~~

9. Thaw the harvested virus and cells (3 ml) on ice. This process can be accelerated using a 37 ^{o}C waterbath, but it is important the mix does not warm to more than 4^{o}C. Snap-freeze in liquid N_2 and thaw (repeat process again a further 3 times to lyse cells).

10. Centrifuge at 3000g for 10 min at 4^{o}C to remove cell debris.
11. Transfer the supernatant to an ultracentrifuge tube using a 5 ml syringe and 21G needle
12. Add 0.6 volumes of CsCl-saturated 100 mM Tris-HCl (prepare by adding CsCl to 100 mM Tris-HCl, pH 7.5 until salt precipitates and no longer enters solution).
13. Fill the tube(s) with 1 volume 100 mM Tris : 0.6 volumes CsCl-saturated 100 mM Tris.
14. Balance tubes to within 0.01 g of each other.
15. Close the tubes using a heat sealer.
16. Add balance caps to the tubes and place tubes in rotor. Coat the rotor screw-tops with 'Spinkote' lubricant and screw on.
17. Close the caps to 120 psi using a torque wrench.
18. Centrifuge at 65,000 rpm for 6-8 h.
19. Remove tubes from rotor using tweezers.
20. Place in a stand in such a position to ensure that the viral band will be visible. Insert 2 x 25G needles into the top of the tube to equalise the pressure.
21. Remove the white viral particle band with a 19-21G needle and a 5 ml syringe. Insert the needle just below the viral band, taking care only to puncture one side of the tube, slowly insert the needle and then angle the needle upwards into the viral particle band. Slowly remove the viral particles using the syringe plunger.
22. Transfer the viral particles into a fresh ultracentrifuge tube and fill with 1 volume 100 mM Tris : 0.6 volumes CsCl-saturated 100 mM Tris.
23. Balance, heat seal, place in rotor as above and centrifuge for a second time overnight.
24. Remove the virus particle band as above, and inject into a 'Slide-a-Lyser' dialysis cassette. Remove air from the cassette to allow maximal use of membrane surface area and efficient dialysis. Mark the entry point used for injection of the viral particles into the cassette, as the same one should not be used twice.

25. Insert the cassette into a floating holder and dialyse for 3 h with gentle stirring in PBS containing 3% w/v sucrose. Use 1 litre of PBS-sucrose and change every hour (3 litres total needed).
26. Remove virus from cassette using a needle inserted into a different corner from that used to introduce the virus. Aliquot into sterile 500 µl tubes at 20 µl/tube.
27. Snap freeze the aliquots in liquid N_2 and store at -80^{o}C.

3.3 Culture, adenoviral infection and staining of MEFs for ERK1/2, p-ERK1/2 and Myc

1. Wash subconfluent flasks of wild-type (WT) and DUSP5 knockout (KO) MEFs x2 with sterile PBS. Add 2 ml/flask of trypsin/EDTA mix, and incubate at 37°C for 5 min, or until cells have detached from the bottom of the flask. Tap the side of the flask to dislodge remaining cells. Add 10 ml of DMEM containing 10% FBS to stop the action of trypsin. Transfer to 15 ml centrifuge tubes and spin at 800g for 5 min at room temperature. Discard the supernatant and resuspend cells in DMEM containing 10% FBS.
2. Dilute cells to a suspension containing 4,000 cells per 100 µl of 10% FBS/DMEM suspension. Plate cells on to 96-well back-wall imaging plates at 100 µl. Return cells to the incubator overnight.
3. Infect cells with empty adenoviral vector containing no transgene, or adenovirus expressing DUSP5-Myc (or alternatively a mutant, such as R53/54A DUSP5 [***20***], which cannot associate with or dephosphorylate the ERK kinase target, despite being catalytically active) under the control of the EGR-1 promoter. Defrost a virus aliquot and dilute to 0.1-3x10^{6} plaque forming units (pfu) per ml for re-expression of DUSP5-Myc in DUSP5 KO MEFs. Titres of 1-3x10^{5} pfu/ml are usually sufficient to restore endogenous levels of DUSP5 expression in MEFs. Remove media from cells and replace with 10% FBS DMEM containing adenovirus at 100 µl/well. Return cells to the incubator for 4-6 h.

4. Remove media from all cells, wash once with PBS and replace with 90 µl/well 10% FBS DMEM overnight for stimulation with TPA the next day. For serum stimulus, replace with 90 µl/well DMEM without FBS to serum starve cells overnight. Add 10 µl of 10x TPA or FBS to cells for required periods.
5. Tip off media from cells and fix by adding 50 µl/well 4% PFA in PBS (*see* **Note 6**). Incubate on a rocking platform for 10 min at room temperature.
6. Tip off PFA and permeabilise the cells by adding 50 µl/well of –20^{o}C methanol. Incubate for 5 min in the freezer.
7. Remove methanol and wash cells with 100 µl/well PBS at room temperature.
8. Add 25 µl/well of 2.5% v/v normal goat serum and 0.01% w/v sodium azide in PBS (blocking buffer) and incubate plates at room temperature on a rocking platform for 2 h.
9. Tip away blocking buffer and add 25µl/well 1:200 dilution of primary mouse anti-*p*-ERK1/2 and either rabbit anti-ERK1/2 or rabbit anti-Myc antibody diluted in blocking buffer. Incubate overnight on a rocking platform at 4^{o}C.
10. Retrieve antibody for reuse if required. Wash cells 3 times with 100 µl/well PBS at room temperature.
11. Remove PBS and add 25µl/well 1:300 dilution of either or both Alexa 546 or 488 labelled goat anti-mouse or anti-rabbit secondary antibody diluted in blocking buffer. Incubate for 90 min on a rocking platform at room temperature.
12. Retrieve antibody for reuse if required. Wash cells 3 times with 100 µl/well PBS at room temperature.
13. Add 100 µl/well of 300 nM DAPI diluted in PBS. Store at 4^{o}C until ready to image.

FIG 1 ABOUT HERE PLEASE

3.4 High content microscopy and analysis

These procedures are optimised for use with the GE Healthcare IN Cell Analyzer 2000 fluorescence microscope and proprietary “IN Cell” Investigator software. However, the same outcomes are achievable using most platforms. A schematic overview of the workflow involved in performing the analysis is given in Figure 1 and representative images and quantitative data are shown in Figure 2B

1. Plates must firstly be equilibrated to room temperature prior to imaging. This prevents condensation forming on the bottom of the plate and plate expansion during the run, which affect image consistency and quality.
2. In the microscope software 'acquisition mode' set-up, be sure to select the Costar plate type 3904 from the list of options (or alternative if a different culture plate is being used). If the plate is not in the database, then the plate dimensions will need to be added using the 'plate manager' button in the drop-down menu (*see* **Note 7**).
3. Dock the plate into the microscope, using the 'insert/eject plate' button in the toolbar ensuring that well A1 is in the top left-hand corner of the plate-holder tray.
4. Set up an acquisition protocol to capture 3 fluorescent images per well, selecting excitation filters: 'DAPI' (350±25nm), 'FITC' (490±10nm) and 'Cy3' (543±11nm) and emission filters: 'DAPI' (455±25nm), 'FITC' (525±10nm) and 'Cy3'(604±32nm). Select the 'QUAD1' polychroic mirror and '2-D deconvolution' options. The latter applies an image processing function, which sharpens the images but does not influence the linear dynamic range of the image detection, and does not incur a time penalty. We often use the '2x2 binning' function, which pools fluorescence detection from four detectors per pixel (*see* **Note 8**). Set exposure times of 0.1, 0.3 and 0.2 sec for DAPI, Alexa 488 and Alexa 546 images, respectively.
5. In the 'focus' window in the wizard, select the 'laser autofocus' (hardware autofocus) option and click the 'auto-offset' button above the list of filters and exposure times. The microscope should automatically find the optimal distance above the plastic-PBS interface of the sample plate at

which to capture images with greatest in-focus clarity in the field of view. Problems at this stage normally arise from incorrect plate settings.

6. Move to several different wells by clicking on the plate map image in well areas. Click the 'AF' (autofocus) button next to each filter setting to ensure the autofocus/offset settings give in-focus images in a range of wells in different areas of the plate. Also check the pixel histograms by clicking on the button that looks like an artist's palette to ensure all detectors are acquiring fluorescence values within the dynamic range of the camera. If some pixels are saturated, the exposure time must be reduced. Other options for brightfield acquisition and the order in which wells are acquired are optional. Similarly, if a minimum cell number of required per well, an 'on the fly' cell count may be included based on the DAPI stain to ensure the microscope carries on acquiring images until the number has been achieved.
7. In the field selection button to the right of the plate image, select the number of fields per well to be acquired (usually 2-4 fields of view, giving roughly 500-1000 cells acquired/well).
8. Once the acquisition protocol is set up, it is not usually changed between assays, apart from to redefine the 'auto-offset' at the start of each session.
9. Acquire images by pressing the 'run protocol' button in the toolbar. Specify the file-path for the images (usually a server mapped to the computer) and type in an appropriate name for the experiment. Images will be directly saved to the server.
10. Once images are saved, proceed to image analysis. Note that this does not require the microscope- just a computer with access to the images. Open IN Cell Investigator software (or equivalent, such as 'Cell Profiler'). We use a bespoke analysis protocol for our staining procedure, so we include our settings merely as a guide.
11. Click on the 'View/Analyze Image Stack' button and navigate to image stack using the dialogue box.
12. Open the 'Protocol Explorer' window and define the first 'Target set' in a new protocol as 'nuclei', choosing 'DAPI' as the input image (channel 1). Apply a 'Denoising-anisotropic diffusion'

Preprocess function to the image to make segmentation of individual nuclei easier, using a 'kernel size of 3 and a sensitivity of 6. Choose 'Object segmentation' parameters as a kernel size of 13 and a sensitivity of 24. Add a first Post-process function of 'Fill holes' and a second of 'Sieve' with settings of 'keep objects > 13μm^2'.

13. Create a new 'Target set' called 'Cells'. Set up a 'Preprocess macro', which links the following image transformations and filters, using the *p*-ERK1/2 image (channel 2) as an input, and exporting the output image to channel 6: information equalisation > histogram equalisation > smooth > smooth. This has the effect of slightly blurring the image and enhancing boundary definition between stained cells and background. Choose 'Object segmentation' parameters as a kernel size of 27 and a sensitivity of 75. Add a first Post-process function of 'Sieve' with settings of 'Keep objects > 40μm^2' a second of 'Clump break' based on 'Nuclei' targets to ensure only single cells (one nucleus per cell) are counted, a third of 'Fill holes' and finally 'Border object removal-all borders'.
14. Create 'Target linking-one to one linking' between 'Nuclei' and 'Cells' target sets using 90% overlap of 'Nuclei' within 'Cells' as acceptable, defining the linked objects as 'Nuclei_Cells'.
15. If necessary, apply a flat field correction to acquired images in the Alexa 488 and 546 channels (*p*-ERK1/2 and either ERK1/2 or Myc images, respectively), using 'Shading removal-QSM' and an offset value of 30-50 in multiplicative mode.
16. Define measures (for example, average pixel intensity or cell area) by using the 'Add user defined measure' function by right-clicking in the 'Measures' panel (highlighted when the icon if the same name is selected underneath the 'Nuclei_Cells' icon). Double click the desired measure, then double click the linked 'Nuclei_Cells target path' to link the measure to the relevant objects. Apply statistical functions from the drop-down menu in the 'Measures' panel as necessary (e.g. 'Sum', 'Mean' etc.) to summarise population-level data. Ensure that the data is acquired from the relevant source image using the drop-down menu in the 'Measures' panel. For example, any measures

relevant to p-ERK images must be linked to either the original acquired images in channel 2 or their flat field corrected derivatives.

FIGURE 2 ABOUT HERE PLEASE

3.5 Culture, adenoviral infection and subcellular fractionation of MEFs for immunoblot

These procedures are optimised for use with NE-PER nuclear and cytoplasmic extraction reagents (ThermoScientific) and fluorescent immunoblots imaged on a Li-Cor Odyssey imaging system (LI-COR Biosciences) (*see* **Note 9**)

1. Perform steps 1-4 of subheading 3.3 scaled up for use in 10 cm tissue culture treated plates. Seed $5x10^5$ WT and KO MEFs per 10 cm plate in 10 ml DMEM containing 10% FBS. Infect with the required titre of adenovirus in 5 ml DMEM containing 10% FBS to minimise the amount of virus required.
2. Following the required stimulations remove media from all cells and wash twice with PBS. Harvest cells by adding 5 ml/plate of trypsin/EDTA mix, and incubate at 37°C for 5 min. Tap the flask to dislodge the remaining cells, add 10 ml of DMEM containing 10% FBS to quench the trypsin, then centrifuge at 500g for 5 min at room temperature. Discard the supernatant and resuspend cells in 1 ml PBS.
3. Transfer the cell suspension to 1.5 ml micro-centrifuge tubes and pellet by centrifugation at 500g for 3 min. Resuspend cells in 100 µl ice-cold CER I buffer (containing Halt protease and phosphatase inhibitors) from the NE-PER nuclear and cytoplasmic extraction reagent kit (ThermoScientific).
4. Continue to use the NE-PER nuclear and cytoplasmic extraction reagents (ThermoScientific) to isolate the cytoplasmic and nuclear fractions according to the manufacturer's instructions. Utilise the minimum reagent volumes recommended to obtain concentrated protein samples in the cellular fractions.

5. Perform a Bradford assay on the isolated cellular fractions to determine protein concentrations. The cytoplasmic fraction is usually 3-4 times more concentrated than the nuclear fraction. Normalise the protein concentrations within the samples and add NuPAGE LDS sample buffer (ThermoScientific) and boil at 95°C for 5 min.
6. Resolve equivalent quantities of protein by SDS-PAGE using NuPAGE® Novex® 4-12% gradient 4-12% Bis-Tris protein gels, transfer onto Immobilon-FL PVDF membranes, and block in 5% milk for 30 min at room temperature on a rocking platform.
7. Tip away blocking buffer and wash cells 3 times for 5 min with PBS-Tween at room temperature.
8. Incubate with the required primary antibodies, diluted in 5% BSA, overnight at 4°C including:
    a. anti-DUSP5 (1:2,000) (*see* **Note 4**)
    b. anti-pERK (1:1,000)
    c. anti-ERK (1:1,000)
    d. anti-MEK (1:1,000)
    e. anti-UBF (1:1,000) (*see* **Note5**).
9. Retrieve antibody for reuse if required. Wash cells 3 times for 5 min with PBS-Tween at room temperature.
10. Place in corresponding fluorescent-tagged secondary antibodies, diluted in 5% milk, for 1 h at room temperature on a rocking platform, avoiding exposure to light.
11. Retrieve antibody for reuse if required. Wash cells 3 times for 5 min with PBS-Tween at room temperature.
12. Scan the membrane utilising the 680nm channel on a Li-Cor Odyssey imaging system (LI-COR Biosciences) to visualised reactive bands. Representative results of a fractionation experiment are shown in Fig 2A

4. Notes

1. A number of standard methods are available for the isolation and culture of primary MEFs from 12.5 to 13.5 postcoitum (p.c.) mouse embryos. In addition, Pierce now produce a commercial mouse embryonic fibroblast isolation kit (Cat No 88279) which claims to have improved performance over standard trypsin-based tissue digestion in the establishment of MEFs in culture.

2. Low passage HEK293 cells (<20) were obtained from Iowa Gene Transfer Vector Core, to ensure the strong E1A expression necessary for adenoviral propagation. There are quite large differences in HEK293 cell phenotype between different sources, which are likely due to subtle differences in selection pressure between the thousands of labs culturing this immortalised line. Therefore, some HEK293 lines more efficiently generate recombinant virus following co-transfection, while others form better monolayers for cell biological studies. Here, the strongest indicator of their utility as a 'helper' cell line is the expression of the E1A gene.

3. To make up 4% PFA, weigh PFA in a fume cupboard and add to PBS on a stirrer. Add concentrated NaOH drop wise until PFA dissolves, then restore to pH 7.2. Store in aliquots at -20ºC and defrost thoroughly before use.

4. We used an “in house” sheep polyclonal antibody raised against recombinant protein produced in *E. coli* to detect DUSP5 by Western blotting (***20***). Commercial antibodies against DUSP5 are available, but are of variable quality. Specificity for DUSP5 should be verified by siRNA knockdown and Western blotting before use. The authors can provide samples of the antiserum used here on request.

5. We used a non-proprietary antibody against upstream binding factor (UBF) as our marker for the nuclear fraction. However, several commercial antibodies are available for this purpose. These include antibodies against lamin A, poly ADP ribose polymerase (PARP), histone deacetylase-2 (HDAC-2) and the transcription factor SP1.

6. All preparation of DNA and digestion, as well as the actual transfections, should be carried out under aseptic conditions in a class II sterile flow cabinet designed for cell culture. All materials and solutions that have been in contact with adenovirus or HEK293 helper cells must be sterilised by immersion in 1% hyperchlorite or other peroxygen-based commercial disinfectant, such as 2% Virkon (Fisher Scientific).

7. Plate dimensions are usually available from the plate manufacturer, but an actual measure of plate thickness and skirt height can also be obtained using the 'laser autofocus trace' tool.

8. The '2x2 binning' function increases sensitivity and decreases image file size from 8Mb to 2Mb but reduces image resolution. We have shown this does not influence the quality of data gathering from cells the size of MEFs acquired using a 10x lens and measuring fluorescence changes in nuclear and cytoplasmic compartments, and actually improves signal/noise ratios.

9. The same outcomes are achievable using a range of commercially available subcellular fractionation kits and reagents and standard (ECL) immunoblotting procedures.

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Figure legends

Figure 1. *Image acquisition and analysis workflow using high content microscopy.* Mouse embryo fibroblasts were stained with up to 3 fluorophores (in this case, using DAPI, *p*-ERK and Myc antibodies, as described in main text) and imaged using a 10 objective at 3-4 fields/well (capturing typically 100-500 cells/field) in 96-well plates using an IN Cell Analyzer 2000 microscope (GE Healthcare). Duplicate, triplicate or quadriplicate wells per condition were used in individual experiments. Custom analysis algorithms were defined using IN Cell Developer software (GE Healthcare) to capture data related to cell and nuclear area, shape and stain distribution and intensity. Typically, the DAPI stain was used to define a nuclear mask and *p*-ERK stain was used as a common readout between experiments to define the cell perimeter. Image processing functions were applied to enhance object definition as necessary. Mean, max and sum values per field, per well and per cell were captured and plotted in figures as appropriate.

Figure 2. *Comparison of spatiotemporal ERK regulation using cell fractionation and high content microscopy.* Primary MEFs derived from wild-type (WT) or DUSP5 knockout (KO) mice were maintained in 10% FBS prior to stimulation with 100ng/ml TPA phorbol ester as indicated. (*A*) Wild type and DUSP5 KO MEFs were lysed and fractionated before analysis by SDS-PAGE and Western blotting using the antibodies indicated. Upstream binding factor (UBF) and MAP kinase kinase (MEK) were used as markers to verify efficient separation into nuclear (N) and cytoplasmic (C) fractions respectively. Immunoblotting with antibodies against phosphorylated ERK (*p*-ERK) and total ERK clearly indicates that loss of DUSP5 leads to an increase in nuclear *p*-ERK. (*B*) Representative images of WT and DUSP5 KO MEFs treated for 1h with TPA prior to immunostaining for *p*-ERK, ERK and DAPI. Scale bar = 75 μm. Note that cropped images are only 20% of the area of a single field of view acquired per well. (*C*) Graphs represent population average normalised fluorescence values for nuclear (Nuc) and cytoplasmic (Cyt) *p*-ERK intensity, or nuclear (Nuc) ERK intensity derived from 3 separate experiments, each of which contained 2-4 replicate wells per condition. Data are shown as mean ±SEM, n = 3. The data clearly show the increase in nuclear *p*-ERK, which results from deletion of DUSP5, but also reveal the role of

DUSP5 as a nuclear anchor for total ERK, with the knockout cells failing to accumulate total ERK in the cell nucleus at later times after stimulation. For further details of these and rescue experiments using adenoviral vectors encoding wild type and mutant DUSP5 see (***22***)