UNIVERSITY^{OF} BIRMINGHAM University of Birmingham Research at Birmingham

Quantitative Phosphoproteomics Reveals a Role for Collapsin Response Mediator Protein 2 in PDGF-Induced Cell Migration

Almuntafeky, Adil Rashid Sarhan; Szyroka, Justyna; Begum, Shabana; Tomlinson, Michael; Hotchin, Neil; Heath, John; Cunningham, Debbie

DOI: 10.1038/s41598-017-04015-x

License: Creative Commons: Attribution (CC BY)

Document Version Publisher's PDF, also known as Version of record

Citation for published version (Harvard):

Almuntafeky, ARS, Szyroka, J, Begum, S, Tomlinson, M, Hotchin, N, Heath, J & Cunningham, D 2017, 'Quantitative Phosphoproteomics Reveals a Role for Collapsin Response Mediator Protein 2 in PDGF-Induced Cell Migration', *Scientific Reports*, vol. 7, 3970. https://doi.org/10.1038/s41598-017-04015-x

Link to publication on Research at Birmingham portal

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

•Users may freely distribute the URL that is used to identify this publication.

•Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.

•User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?) •Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

SCIENTIFIC REPORTS

Received: 21 October 2016 Accepted: 16 May 2017 Published online: 21 June 2017

OPEN Quantitative Phosphoproteomics **Reveals a Role for Collapsin Response Mediator Protein 2 in PDGF-Induced Cell Migration**

Adil R. Sarhan 1,2, Justyna Szyroka¹, Shabana Begum¹, Michael G. Tomlinson¹, Neil A. Hotchin¹, John K. Heath¹ & Debbie L. Cunningham

The Platelet Derived Growth Factor (PDGF) family of ligands have well established functions in the induction of cell proliferation and migration during development, tissue homeostasis and interactions between tumours and stroma. However, the mechanisms by which these actions are executed are incompletely understood. Here we report a differential phosphoproteomics study, using a SILAC approach, of PDGF-stimulated mouse embryonic fibroblasts (MEFs). 116 phospho-sites were identified as up-regulated and 45 down-regulated in response to PDGF stimulation. These encompass proteins involved in cell adhesion, cytoskeleton regulation and vesicle-mediated transport, significantly expanding the range of proteins implicated in PDGF signalling pathways. Included in the downregulated class was the microtubule bundling protein Collapsin Response Mediator Protein 2 (CRMP2). In response to stimulation with PDGF, CRMP2 was dephosphorylated on Thr514, an event known to increase CRMP2 activity. This was reversed in the presence of micromolar concentrations of the protein phosphatase inhibitor okadaic acid, implicating PDGF-induced activation of protein phosphatase 1 (PP1) in CRMP2 regulation. Depletion of CRMP2 resulted in impairment of PDGF-mediated cell migration in an in vitro wound healing assay. These results show that CRMP2 is required for PDGFdirected cell migration in vitro.

Platelet Derived Growth Factor (PDGF) was first identified in platelet extracts as a potent mitogen for fibroblasts and endothelial cells¹. The PDGF family of ligands comprises four related gene products: PDGFA, PDGFB, PDGFC and PDGFD which can form both homodimers and heterodimers providing a repertoire of PDGF isoforms². The PDGF family of ligands elicit their biological effects by interacting with two structurally related high affinity receptors of the tyrosine kinase family: PDGFR alpha and PDGFR beta³. PDGF-AA, AB, BB and CC induce PDGFR alpha homo-dimerisation; PDGF-BB and DD induce formation of PDGFR beta homodimers, and PDGF-AB, BB, CC and DD induce formation of PDGFR alpha/PDGFR beta heterodimers². PDGF-BB homodimers are therefore universal PDGFR ligands capable of eliciting the full repertoire of PDGFR-mediated biological responses.

PDGF receptors are expressed by cells of mesenchymal origin and the cognate PDGF ligands are expressed by overlying epithelial cells indicative of paracrine signalling from epithelium to the underlying mesenchyme. For example, in the lung, PDGFR alpha is expressed in the lung mesenchyme and PDGFA is expressed in the epithelium⁴. This pattern is seen in multiple organs and tissues such as gut, kidney, gonads, somites and nervous system⁵. Extensive genetic studies of PDGF and PDGFR function in the mouse confirms that PDGF signalling from epithelial cells via PDGFRs is absolutely required for the expansion and migration of specific mesenchymal cell populations⁶. For example, in the kidney, PDGF signalling is required both for the multiplication of mesangial cell precursors and their migration into the glomerular space⁵. In the lung, PDGF is required for the expansion of myofibroblasts and their migration along the alveolar epithelium during lung morphogenesis⁷. In the central

¹School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom. ²Present address: MRC Protein Phosphorylation and Ubiquitylation Unit, College of Life Sciences, University of Dundee, Dow Street, Dundee, DD1 5EH, United Kingdom. Correspondence and requests for materials should be addressed to D.L.C. (email: d.cunningham@bham.ac.uk)

nervous system (CNS) oligodendrocyte precursor (O2A) cells require PDGF signalling both for population expansion and migration through the spinal cord⁸.

PDGF signalling also has significant functions in adult tissue homeostasis and regeneration, acting on cells of mesenchymal origin. PDGF is an established mitogen and chemoattractant for mesenchymal derived cells *in vitro*⁹ and has been studied for its ability to promote wound healing through promoting the migration, proliferation and extracellular matrix deposition by fibroblasts¹⁰. Ectopic expression of PDGFB in the kidney glomerulus induces expansion of bone marrow derived mesenchymal cells to form a severe mesanagial glomerulopathy and fibrosis¹¹. PDGF-BB is a mitogen for vascular smooth muscle cells and activation of PDGF signalling is required for pulmonary arterial hypertension (PAH) and pulmonary arterial remodelling¹².

The PDGF signalling axis is also implicated in regulation of tumour/stromal cell interactions^{13, 14}. PDGF-BB produced by tumours of epithelial origin acts upon adjacent connective tissue cells, pericytes and vascular smooth muscle cells to regulate tumour angiogenesis and tumour-associated fibrosis¹⁵. PDGF-activated perivascular cells also promote metastasis via tumour-associated macrophages and act on tumour cells that have undergone epithelial-mesenchymal transition (EMT) to promote migration through the extracellular matrix^{16,17}.

Despite the biological significance of these activities, little is known about the downstream intracellular pathways that mediate these responses beyond canonical RTK signalling pathways such as PLC, AKT and MAPK/ERK². To gain further insight into how PDGFs mediate their biological effects we analysed a differential phosphoproteomics dataset of PDGF-stimulated mouse embryo fibroblasts (MEFs)¹⁸. This shows that, in MEFs, PDGFR activation elicits a broad range of phosphorylation events mediated by downstream Ser/ Thr kinases which impact on mediators of cytoskeletal function and cell motility. In particular, we show that Collapsin response mediator protein-2 (CRMP2) is dephosphorylated in response to PDGF and is required for PDGF-induced cell migration. CRMP2 (also known as dihydropyrimidinase-like 2) is highly expressed in the nervous system and mediates cellular responses downstream of ephrins, neurotrophins and semaphorins, such as neuronal polarity, growth and axonal guidance^{19, 20}.

Collectively these results significantly expand the range of proteins and phosphorylation events implicated in PDGF signalling pathways and reveal potential new targets for therapeutic intervention in PDGF signalling. They also reveal a role for CRMP2 in PDGF-induced cell migration which has broad implications for tissue repair and tumour development.

Results

PDGF-induced changes in the phosphoproteome. We have recently published quantitative SILAC proteomics data identifying changes in the phosphoproteome and proteome of mouse embryonic fibroblasts (MEFs) due to the absence of the intracellular phosphatase domain of Leukocyte Common Antigen-related (LAR) protein tyrosine phosphatase (PRIDE Accession PXD002545^{18,21}). As part of our analysis of LAR function WT MEFs were stimulated with PDGF, and here we have reanalysed this part of the dataset to identify novel PDGF-dependent phosphorylation events. Full details of the experimental setup and procedures used to obtain the phosphoproteomics and proteomics datasets have been previously described¹⁸. Methodology relevant to the PDGF-dependent dataset are summarised in Fig. 1A. The Pearson's correlation coefficient for the phosphopeptide ratios measured across the three biological replicates ranged from 0.77 to 0.93 indicating good biological reproducibility (Fig. 1B). Within the proteomic dataset, a total of 703 proteins were identified in two or more biological replicates. No significant differences in protein abundance were observed between unstimulated and PDGF stimulated (7 min) cells (Fig. 1C). In the phosphoproteome dataset, 989 phosphosites from 611 proteins were detected with high localization scores (localization probability >0.75; score difference >5) in three or more experimental replicates (Supplementary Table S1). Within the phosphoproteome dataset, we identified 896 (90.6%) serine, 76 (7.7%) threonine and 17 (1.7%) tyrosine phosphorylation sites (Fig. 1D). These results are an accurate reflection of the relative abundance of serine: threonine: tyrosine phosphorylation events in the cell, previously reported to be 88:11:1, and are comparable to those detected downstream of other receptor tyrosine kinases^{22, 23}. Being a tyrosine kinase, activation of PDGFR will initiate cell signalling via tyrosine phosphorylation. PDGF-induced tyrosine phosphorylation events have been previously studied using enrichment strategies to increase the relative abundance of tyrosine phosphorylated peptides^{24, 25}. As our aim was to gain an understanding of the global signalling landscape regulated by PDGF our enrichment strategy was not selective for either serine, threonine or tyrosine, enabling novel insight into the breadth of biological processes regulated by PDGF. From the 989 phosphopeptides identified, 161 showed a significant change in phosphorylation status upon PDGF stimulation (p < 0.05; > 1.5-fold change) (Fig. 2; Supplementary Table S2). A total of 116 phosphosites were upregulated and 45 downregulated upon PDGF treatment. Cellular compartment analysis revealed that these PDGF-regulated phosphosites are present on proteins localised throughout the cell, indicating the diversity of PDGF signalling (Fig. 3).

Within this dataset we identified proteins that have previously been shown to be involved in PDGF signalling. For example, the ERK1/2 MAP kinases have been extensively studied and shown to be hyperphosphorylated in response to growth factors such as PDGF^{26–28}. In agreement with these studies, we found increased phosphorylation of ERK1 on Tyr205 (L_{+PDGF}/M_{-PDGF} ; 15.10) and Thr203 (L_{+PDGF}/M_{-PDGF} ; 14.13), and ERK2 on Tyr185 (L_{+PDGF}/M_{-PDGF} ; 16.77). The phosphorylation of these sites on ERK1/2 is essential for kinase activity. Phosphorylated ERK1/2 mediates diverse physiological functions such as cell growth, survival, cell adhesion, migration and differentiation^{26–28}. We have also identified an increase in the phosphorylation of Bcl2-associated agonist of cell death (BAD) at Ser136 upon PDGF stimulation (Supplementary Table S2). This site is a target for Akt downstream of PDGF and phosphorylation of Ser136 on BAD leads to enhanced cell survival²⁹.

Novel PDGF-regulated phosphosites. From 161 phosphosites, we identified 110 (67%) that have not previously been reported as PDGF-regulated sites (PhosphoSitePlus)³⁰ and are therefore considered novel. Within



Log₂ fold change (L_{+PDGF}/ M_{-PDGF})

Figure 1. Global quantitative proteomics and phosphoproteomics of PDGF signalling in mouse embryo fibroblasts (MEFs). (**A**) Schematic overview of experimental design. SILAC labelled MEFs were stimulated with PDGF for $7 \min (+)$ or left unstimulated (-). (**B**) Heat map of Pearson correlation coefficients and multi scatter plots demonstrating reproducibility between the phosphoproteome of biological and technical replicates. (**C**) Volcano plots showing the ratio (log2 fold change) and significance $(-\log_{10} \text{Benjamini-Hochberg}$ adjusted *p*-value) of the proteome dataset. (**D**) Numbers of serine, threonine and tyrosine phosphorylation sites identified within our PDGF-regulated phosphoproteome dataset.

the phosphoproteomics data set we observed that phosphorylation of Ser303 on insulin receptor substrate 2 (IRS2) increased upon PDGF stimulation (Fig. 2). IRS2 is an adaptor protein that binds to the insulin receptor and regulates insulin signalling pathways. PKC isoforms, β and δ , or angiotensin II can induce phosphorylation of Ser303 on IRS2 in endothelial cells leading to inhibition of insulin signalling³¹. This suggests a novel role for PDGF as a negative regulator of insulin signalling, possibly via PKC, known to be activated downstream of PDGF through PLC $\gamma^{32, 33}$.

After ERK1/2, the next most significant increase in phosphorylation was observed for Ser72 of Ras-related protein Rab7a (Fig. 2). Rab7a is a small GTPase involved in the regulation of endo-lysosomal trafficking³⁴. Phosphorylation of Rab7a on Ser72 inactivates the protein, thereby impairing the recruitment to endosomal membranes and delaying the transport of EGFR from early to late endosomes³⁵. This suggests that PDGFR activates an as yet unidentified kinase which phosphorylates Ser72 on Rab7a indicating an intersection between two major RTKs, PDGFR and EGFR in the regulation of endocytosis. In addition, we observed increased phosphorylation of Slit-Robo GTPase-activating protein 3 (srGAP3) on Ser858 (Supplementary Table S2), a RhoGAP that functions downstream of the Robo receptor and is involved in the development of the nervous system^{36, 37}. This site is phosphorylated by protein kinase A (PKA), promoting GAP activity towards Rac1 and inhibition of actin reorganisation in neurons³⁸.



Figure 2. Volcano plots showing the magnitude and significance of differential phosphopeptide abundance in PDGF-stimulated versus unstimulated cells. Phosphopeptides are deemed significantly up- or down-regulated if they have an adjusted p value < 0.05 and exhibit a > 1.5 fold-change in abundance.

.....

Following stimulation, 45 novel PDGF-regulated phosphosites were observed to be significantly down-regulated including Rho-associated protein kinase 2 (ROCK2; Ser1362), Neurofibromin (NF1; Ser2517), CLIP-associated protein 1 (CLASP1; Ser1193), Parva (Ser14), collapsin response mediator protein 2 (CRMP2; Thr514) and collapsin response mediator protein 4 (CRMP4; Thr514) (Figs 2 and 3; Supplementary Table S2). These proteins are mainly involved in regulation of nervous system homeostasis including development, axon formation and growth cone guidance³⁹⁻⁴⁴, suggesting a common set of PDGF effectors exist in both fibroblasts and the nervous system and demonstrating that, in addition to the autophosphorylation and phosphorylation of proteins downstream of PDGFR, PDGF can also induce the dephosphorylation of key effectors, some of which regulate cell motility.

Functional analysis of the PDGF-regulated phosphoproteome. Gene Ontology (GO) analysis of the phosphoproteins regulated by PDGF revealed a number of enriched GO biological processes (Fig. 4A; Supplementary Table S3). The dominant enriched GO terms were associated with cytoskeletal organisation, MAP kinase signalling, regulation of microtubules and cell adhesion, all processes involved in cell migration. Actin cytoskeleton and microtubule proteins enriched in our phosphoproteome dataset include LIM domain and actin-binding protein 1 (Lima1), Rho guanine nucleotide exchange factor 17 (Arhgef17), Band 4.1-like protein 1 (Epb4111) and Stathmin (Stmn1) (Supplementary Table S3). We observed an increase in abundance of phosphopeptides belonging to these proteins in response to PDGF stimulation (Supplementary Table S2). We also note the enrichment of biological processes associated with cell adhesion. Reticulon-4 (Rtn4; also known as Nogo), a protein involved in the regulation of neuron development such as neurite growth and axon-axon adhesion^{45, 46} was phosphorylated on Ser105 in response to PDGF stimulation (Supplementary Table S2). Paxillin (PXN), an adaptor protein involved in modulation of focal adhesions and cell adhesion pathways, is one of the PDGF-regulated proteins^{47,48}. PDGF is known to regulate PXN activity by promoting the phosphorylation of Tyr118^{49–51}. We have not identified this site in our phosphoproteomics dataset, but did identify an increase in phosphorylation of Ser83 (Supplementary Table S2). It has been reported that p38MAPK and ERK1/2 can phosphorylate PXN at Ser83 to regulate neurite extension and chemotaxis in neurons^{52, 53}.

KEGG pathway analysis of PDGF-regulated proteins revealed enrichment of signalling pathways implicated in cell migration including focal adhesion, regulation of actin cytoskeleton, adherens junctions and transendothelial cell migration (Fig. 4B; Supplementary Table S4)^{54–56}. In addition, KEGG analysis of the dataset also identified neurotrophin signalling and axon guidance as PDGF-regulated processes (Fig. 4B). To identify which kinases are activated upon PDGF stimulation, we used the Kinase Enrichment Analysis (KEA) tool⁵⁷. KEA is a web-based software tool for the prediction of kinase-substrate interactions and links mammalian proteins with the upstream kinases that potentially phosphorylate them. KEA analysis revealed a significant enrichment of substrates for ribosomal protein S6 kinase (RSK2) (Fig. 4C; Supplementary Table S5). RSK2 promotes cell motility and invasion by mediating the activity of integrins and actin cytoskeleton rearrangement^{58–61}. RSK2 can also promote migration in epithelial cells through the FGFR2-p38 kinase axis pathway⁶². KEA analysis also identified other over-represented kinases including dual specificity mitogen-activated protein kinase kinase (MEK1) and c-Jun N-terminal kinase (JNK1) (Fig. 4C). The activities of both kinases are known to be regulated by growth factors such as PDGF and they are involved in regulation of many cellular functions including cell migration^{63, 64}.

CRMP2 is a novel PDGF-regulated protein. We elected to further analyse the role of collapsin response mediator protein 2 (CRMP2) as an example of a novel PDGF-induced dephosphorylation target found within



Figure 3. PDGF-regulated proteins and phosphosites. Proteins containing PDGF-regulated phosphosites were clustered according to their subcellular localisations as listed in the COMPARTMENTS database. PDGF-regulated phosphosites on each protein are shown in blue and those that are novel PDGF-regulated sites are bordered in red.

our dataset. CRMP2 regulates cytoskeletal remodelling, neurotransmission and axon guidance via its ability to promote microtubule assembly^{65–67}. Consistent with this, GSK3 β has been shown to phosphorylate CRMP2 at Thr509, Thr514 and Ser518 thereby inhibiting CRMP2 activity leading to microtubule depolymerization and destabilization^{68, 69}. Within our dataset a doubly phosphorylated peptide from CRMP2 (pThr514; pSer518) was significantly downregulated following PDGF stimulation (Fig. 5A; Supplementary Table S2). Western blotting with a phosphospecific antibody recognising the highly conserved Thr514 residue confirmed that phosphorylated cells (Fig. 5B–D). These data clearly show that PDGF regulates the dephosphorylation of CRMP2 at Thr514, an event known to activate the protein.

It has been previously shown that PP1 or PP2A can dephosphorylate CRMP2 at Ser518, Thr514 or Thr509^{70,71}. We therefore tested the effect of okadaic acid on CRMP2 phosphorylation at Thr514 following PDGF stimulation in MEFs. PP2A is inhibited at low concentrations ($<1\mu$ M) of okadaic acid, whereas higher concentrations ($>1\mu$ M) inhibit PP1^{72,73}. Western blot analysis showed no significant changes in PDGF-regulated CRMP2 dephosphorylation when okadaic acid was added at low (0.01 μ M or 1 μ M) concentrations (Fig. 6A). However, higher concentrations of okadaic acid (1.5 μ M) inhibited the dephosphorylation of CRMP2 at Thr514 in response to PDGF stimulation (Fig. 6A,B). Together, these observations suggest that PDGF signalling acts via PP1 to dephosphorylate CRMP2 at Thr514.

Α

cytoskeleton organization	single-organism organelle organization		cellular component organization		nent c	t cellular component morphogenesis		MAPK import into nucleus	protein localization		cellular localizatio	n regula cellul	positive regulation of cellular process		regulation of cellular process	
					n			extrinsic apoptotic signaling	cell migrat	acromolect	tosphorylat	positive regulat	positive regulation of biological process		n of Lile- ocess	
	cellular component biogenesis		protein depolymerization			regulation of organelle organization		pathway	5					gative biologi	gulatior crotubt sed pro	
					on			organism cellular localization	localizatic of cell	regulation localizatio signal transductio		regulation of biological process		ຍິ່ວ ຊິ negativ regulat cellular	negative regulation of cellular process	
actin cytoskeleton organization	macromolecular complex	cellular developmental		cellula compo disass	nent embly	regulation of anatomical structure morphogenesis		single-organism cellular process		regulation of cellular		mesenchyme development		cell adh	cell adhesion	
	subunit organization	nesis			collular			movement of cell		component biogenesis		ssenchyma II ferentiatior	achea ormation	substrate adhesion- dependent cell		
regulation of cellular component organization	protein	Inatomica	norphoge	anatomical structure developmer	compone	nent esis	ent junction sis assembly	or subcellular component				₫ 8 ĝ		spreading		
	complex assembly	cell differentiation		pment	cell juncl organiza	tion ation	basement membrane assembly			biol adh	ogical esion	cellular process	localization		ule- develop process	
organelle organization				system develo	regulatio cellular	on of	positive regulation of developmental	actin filam	ent-	łł	tse to ning	ε	loco	motion	microtub based process	
	cell	regulation of developmenta process		e ion of nent ation	compone	ent	process	based pro	cess of association of association of a statement o	se to nal grov	- respor -contai und	organisı s	biolo	odical	ycle	
	merpriegeneele			negativ regulat compol organiz	regulatio	on of sis	organ development			respon epiderr factor	cellular oxyger compo	single-	regulation		cell c	
cytoskeleton organ	nization	sin	gle-or	ganism cel	ular proc	cess										
MAPK import into nucleus		response to growth factor														

regulation of microtubule-based

process

в

mesenchyme development

cell adhesion



MYLK AXL

Figure 4. PDGF regulates distinct biological processes and signalling pathways. (A) Proteins containing the 161 PDGF regulated phosphosites were analysed in REVIGO to identify enriched GO Terms. Enriched biological processes are visualised as a Tree map. Clusters of related terms are displayed using the same colour and the size of the box corresponds to the $-\log 10$ p-value in that category. (B) Proteins containing the PDGFregulated phosphosites were analysed in DAVID to identify enriched KEGG pathways. (C) PDGF-regulated phosphopeptides were searched using the Kinase enrichment analysis (KEA) tool. The network shows the enriched predicted upstream kinases and the node size corresponds to the number of PDGF-regulated phosphopeptides predicted to be phosphorylated by that kinase.

CRMP2 depletion inhibits cell migration in MEFs. CRMP2 has been widely studied in the nervous system where it regulates neuronal migration and axon guidance^{74,75}. CRMP2 also regulates polarisation and migration of T lymphocytes within the immune system^{76, 77}. However, the regulation of cell migration by CRMP2 outside the nervous and immune systems has not been studied. PDGF induced cell migration has been widely



Figure 5. CRMP2 is a novel PDGF-regulated protein. (**A**) Tandem mass spectrum of the CRMP2 T514/S518 phosphopeptide. (**B**) Schematic representation of the domain structure of CRMP2 and the highly conserved amino acid motif containing Thr514. (**C**) MEFs were stimulated with 20 ng/mL PDGF for 7 min. CRMP2, CRMP2 Thr514, ERK1/2, ERK 1/2 Thr202/Tyr204 and Alix in whole cell lysates were analysed by Western blotting. For uncropped images of blots see Supplementary Fig. S2. (**D**) Western blots were quantified and ratios of pCRMP2 to CRMP2 were plotted (**P < 0.01).





studied in fibroblasts, revealing roles for Rac1, MAPK/ERK and JNK kinases^{64,78}. To establish if CRMP2 has a role in PDGF induced cell migration in MEFs we examined the consequences of RNAi-mediated depletion of CRMP2 in a standard *in vitro* scratch wound healing assay. Confluent MEFs were transfected with a CRMP2 directed SMARTpool of siRNAs and non-silencing control siRNA, serum starved and wounded in the presence of PDGF (Fig. 7). The results of this experiment clearly show that siRNA mediated depletion of CRMP2 (Fig. 7A) significantly inhibits the rate of wound closure in the presence of PDGF (Fig. 7B,C). This was confirmed using three individual siRNAs against CRMP2 (Supplementary Fig. S1). These observations confirm that CRMP2, identified in our dataset as a novel PDGF downstream effector, is required for PDGF-stimulated cell migration in MEFs.



Figure 7. CRMP2 plays a role in PDGF-induced cell migration. (**A**) Representative western blot showing levels of CRMP2 in MEFs following transient transfection with non-silencing control siRNA (NSC), or with CRMP2 siRNA (siCRMP2). Alix was used as a loading control. For uncropped images of blots see Supplementary Fig. S4. (**B**) Transfected MEFs were wounded using an automated scratch maker and stimulated with 20 ng/ ml PDGF. Images of five separate wounds were taken at 0, 10, 20, 30, 40, 50, and 60 h post wounding using the IncuCyte ZOOM imaging system. (**C**) The percentage of the wound open at each time point was quantified using ImageJ. Data are presented as average % wound open and error bars represent the standard error of the mean (n = 3; **P < 0.001).

Discussion

PDGF ligands and their cognate receptors are firmly established as key mediators of mesenchymal cell migration in developmental, homeostatic and pathological processes^{2–5}. However, the downstream effectors of the PDGF pathway are incompletely characterised. In order to identify PDGF-regulated protein phosphorylation events and expand the range of PDGF mediators, we undertook a study of PDGF-stimulated MEFs using a differential phosphoproteomics approach¹⁸. The aim of this study was to significantly expand the repertoire of PDGF pathway effectors and to identify novel candidates that might mediate PDGF induced cell migration. Here we report the identification of 989 phosphosites from 611 proteins in our screen. Out of these, 116 phosphopeptides were significantly upregulated in PDGF-stimulated cells, many of which are novel PDGF-regulated phosphosites. Phosphoproteins regulated by PDGF play a role in cellular signalling pathways that mediate cell adhesion, endocytosis and vesicular trafficking, cytoskeletal organization and nuclear function.

We also report the identification of 45 phosphopeptides that are significantly down-regulated in response to PDGF stimulation. These include proteins such as CLASP1 and CRMP2. CRMP2 is of interest as an effector of PDGF-mediated cell migration as it has a well- established role as mediator of axon guidance, growth cone collapse and cell migration in neurons^{67, 75, 79}. Phosphoproteomic and western blotting analysis indicated that, in MEFs, PDGF mediates dephosphorylation of CRMP2 at Thr514. In neuronal cells this site is phosphorylated by GSK3 β leading to destabilization of microtubules and neurite degeneration, whereas the dephosphorylated form of CRMP2 Thr514 promotes microtubule stabilisation and sustains neurite outgrowth^{68, 69, 80}. This indicates that dephosphorylation of CRMP2 involves the activation of a protein phosphatase(s). We showed that micromolar concentrations of okadaic acid, a serine/threonine phosphatase inhibitor, restored the phosphorylation of CRMP2 at Thr514. This is most likely due to preferential inhibition of protein phosphatase 1 (PP1)^{72, 73}. CRMP2 is not however dephosphorylated in non-stimulated cells showing that PDGF pathway activation is essential for the CRMP2 directed activity of this phosphatase. Given the intersection of CRMP2 in neuronal cells and MEFs we were interested to learn if CRMP2 was required for PDGF induced cell migration. Depletion of CRMP2 resulted in impairment of cell migration in a scratch wound assay in the presence of PDGF showing that it is required for PDGF induced cell migration in MEFs.

Our quantitative phosphoproteomics study has significantly expanded the known repertoire of PDGF effectors and provided a resource for further experimental studies. The novelty of our findings is highlighted by the fact that we have identified a role for CRMP2 in PDGF-regulated cell migration, a fundamental biological process closely linked to developmental processes and progression of disease.

Methods

Phosphoproteomics and bioinformatics. Details of the experimental setup for the quantitative phosphoproteomics experiments have been previously described¹⁸. Within the MaxQuant output, phosphorylation sites were considered to be localised correctly if the localisation probability was at least 0.75 (75%) and the score difference at least 5. Significance testing was performed in the Perseus software environment, which is part of MaxQuant (Perseus version 1.5.0.15; www.perseus.framework.org), using a Student's t-test on log 2 transformed ratios and controlled with a Benjamini-Hochberg FDR threshold of 0.05. Peptides quantified in three or more experimental repeats were deemed significantly changed and regulated by PDGF if they had a p-value of <0.05 and a ratio of <0.667 or >1.5 (at least a 1.5-fold change in abundance). The COMPARTMENTS database⁸¹ was used to assign proteins to subcellular localisations. Protein network visualization was performed using Cytoscape (version 3.3.0)⁸². DAVID (Database for Annotation, Visualization and Integrated Discovery)⁸³ was used to identify over-represented KEGG pathways^{54–56} (threshold count, 2; EASE score, 0.05) and REVIGO⁸⁴ was used for identification and visualisation of over-represented GO terms. Kinases upstream of the identified PDGF-regulated phosphopeptides were predicted using Kinase Enrichment Analysis (KEA)⁵⁷.

Reagents and antibodies. Antibodies against CRMP2 (#9393), CRMP2 Thr514 (#9397), and phospho-threonine (#9381) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against ERK1/2 (sc-514302) and ERK Thr202/Tyr204 (sc-136521) were purchased from Santa Cruz Biotechnology (Dallas, TX), and against tubulin (T6199) from Sigma-Aldrich Company Ltd (U.K.). The anti-Alix antibody was a gift from Carl Hendrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden)⁸⁵. Goat anti-mouse-IgG IRDye-conjugated antibody (925-68070) and goat anti-rabbit-IgG (925-68071) HRP-conjugated antibodies were from LI-COR Biosciences (Lincoln, NE). Recombinant human PDGF-BB (#8912) was purchased from Cell Signaling Technology and okadaic acid was purchased from Calbiochem (Hertfordshire, UK).

Cell culture. Mouse embryonic fibroblasts (MEFs) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 10 mg/mL streptomycin, and 250 μ g/mL amphotericin B. For growth factor stimulation experiments, cells were grown to 80% confluence and starved for 16 h in serum-free DMEM before being treated with 20 ng/mL recombinant PDGF-BB at 37 °C. In some experiments, cells were incubated with either 0.01 μ M, 1 μ M or 1.5 μ M okadaic acid.

Cell lysis and immunoblotting. Cell lysates were prepared, separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted using previously described protocols¹⁸. Immunoblots were visualised using fluorescence detection on the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln NE). Densitometric analysis was carried out using Imagel⁸⁶.

RNA interference. Cells were transiently transfected with 80 pmol of SMARTpool or individual ONTARGETplus siRNA for murine CRMP2 (L-041965-01-0005, J-041965-09-002, J-041965-10-002, J-041965-11-002; Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX (Life Technologies, Paisley, UK) as described elsewhere⁸⁷. Nonsilencing control (NSC) siRNA was used as a control for each experiment.

Scratch wound assay. Cells were plated in 96-well cell culture plates and cultured to confluency. Cells were serum starved overnight. Scratch wounds were made using an automated wound maker (Essen BioScience, Hertfordshire, UK), cells were stimulated with 20 ng/ml PDGF, and images collected at hourly intervals post-wounding using an IncuCyte ZOOM imaging system (Essen BioScience, Hertfordshire, UK).

Data Availability. The mass spectrometry proteomics data, including the output from MaxQuant⁸⁸, used in this analysis have been previously deposited on the Proteome Xchange Consortium via the PRIDE partner repository with the dataset identifier PXD002545^{18,21}.

References

- 1. Waterfield, M. D. *et al.* Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. *Nature* **304**, 35–39 (1983).
- 2. Heldin, C. H. & Lennartsson, J. Structural and functional properties of platelet-derived growth factor and stem cell factor receptors. *Cold Spring Harb Perspect Biol* **5**, a009100 (2013).
- Heldin, C. H., Ostman, A. & Ronnstrand, L. Signal transduction via platelet-derived growth factor receptors. *Biochimica et biophysica acta* 1378, F79–113 (1998).
- 4. Ataliotis, P. & Mercola, M. Distribution and functions of platelet-derived growth factors and their receptors during embryogenesis. *Int Rev Cytol* **172**, 95–127 (1997).
- 5. Lindahl, P. et al. Paracrine PDGF-B/PDGF-Rbeta signaling controls mesangial cell development in kidney glomeruli. *Development* **125**, 3313–3322 (1998).
- 6. Betsholtz, C. Insight into the physiological functions of PDGF through genetic studies in mice. *Cytokine & growth factor reviews* **15**, 215–228 (2004).
- 7. Bostrom, H. *et al.* PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* **85**, 863–873 (1996).
- 8. Calver, A. R. et al. Oligodendrocyte population dynamics and the role of PDGF in vivo. Neuron 20, 869–882 (1998).

- 9. Siegbahn, A., Hammacher, A., Westermark, B. & Heldin, C. H. Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes, and granulocytes. *The Journal of clinical investigation* **85**, 916–920 (1990).
- 10. Werner, S. & Grose, R. Regulation of wound healing by growth factors and cytokines. Physiological reviews 83, 835-870 (2003).
- 11. Ostendorf, T., Boor, P., van Roeyen, C. R. & Floege, J. Platelet-derived growth factors (PDGFs) in glomerular and tubulointerstitial fibrosis. *Kidney Int Suppl (2011)* **4**, 65–69 (2014).
- Zhang, L. et al. Platelet-derived growth factor (PDGF) induces pulmonary vascular remodeling through 15-LO/15-HETE pathway under hypoxic condition. Cellular signalling 24, 1931–1939 (2012).
- 13. Cao, Y. Multifarious functions of PDGFs and PDGFRs in tumor growth and metastasis. *Trends in molecular medicine* **19**, 460–473 (2013).
- 14. Heldin, C.-H. Targeting the PDGF signaling pathway in tumor treatment. Cell Communication and Signaling 11, 1-18 (2013).
- 15. Tsai, J. H. & Yang, J. Epithelial-mesenchymal plasticity in carcinoma metastasis. Genes & development 27, 2192-2206 (2013).
- Tan, E. J., Olsson, A. K. & Moustakas, A. Reprogramming during epithelial to mesenchymal transition under the control of TGFbeta. Cell Adh Migr 9, 233–246 (2015).
- 17. Yang, Y. et al. The PDGF-BB-SOX7 axis-modulated IL-33 in pericytes and stromal cells promotes metastasis through tumourassociated macrophages. Nat Commun 7, 11385 (2016).
- Sarhan, A. R. et al. Regulation of Platelet Derived Growth Factor Signaling by Leukocyte Common Antigen-related (LAR) Protein Tyrosine Phosphatase: A Quantitative Phosphoproteomics Study. Molecular & Cellular Proteomics 15, 1823–1836 (2016).
- Yamashita, N. et al. Regulation of spine development by semaphorin3A through cyclin-dependent kinase 5 phosphorylation of collapsin response mediator protein 1. The Journal of neuroscience: the official journal of the Society for Neuroscience 27, 12546–12554 (2007).
- Uchida, Y. et al. Semaphorin3A signalling is mediated via sequential Cdk5 and GSK3beta phosphorylation of CRMP2: implication
 of common phosphorylating mechanism underlying axon guidance and Alzheimer's disease. Genes Cells 10, 165–179 (2005).
- Vizcaino, J. A. et al. The PRoteomics IDEntifications (PRIDE) database and associated tools: status in 2013. Nucleic Acids Res 41, D1063–1069 (2013).
- Olsen, J. V. *et al.* Global, *in vivo*, and site-specific phosphorylation dynamics in signaling networks. *Cell* 127, 635–648 (2006).
 Worthington, J., Spain, G. & Timms, J. F. Effects of ErbB2 Overexpression on the Proteome and ErbB Ligand-specific
- Worthington, J., Spain, G. & Timms, J. F. Effects of EF0B2 Overexpression on the Proteome and Er0B Ligand-specific Phosphosignaling in Mammary Luminal Epithelial Cells. *Mol Cell Proteomics* 16, 608–621 (2017).
- 24. Amanchy, R. et al. Identification of c-Src tyrosine kinase substrates in platelet-derived growth factor receptor signaling. Mol Oncol 3, 439–450 (2009).
- Kratchmarova, I., Blagoev, B., Haack-Sorensen, M., Kassem, M. & Mann, M. Mechanism of divergent growth factor effects in mesenchymal stem cell differentiation. Science 308, 1472–1477 (2005).
- 26. Nishimoto, S. & Nishida, E. MAPK signalling: ERK5 versus ERK1/2. EMBO reports 7, 782-786 (2006).
- 27. Qi, M. & Elion, E. A. MAP kinase pathways. Journal of cell science 118, 3569-3572 (2005).
- 28. Yao, Z. & Seger, R. The ERK signaling cascade-views from different subcellular compartments. Biofactors 35, 407-416 (2009).
- 29. Datta, S. R. et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91, 231-241 (1997).
- Hornbeck, P. V. et al. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. Nucleic Acids Res 40, D261–270 (2012).
- Park, K. et al. Serine phosphorylation sites on IRS2 activated by angiotensin II and protein kinase C to induce selective insulin resistance in endothelial cells. Mol Cell Biol 33, 3227–3241 (2013).
- Li, W. et al. Stimulation of the platelet-derived growth factor beta receptor signaling pathway activates protein kinase C-delta. Molecular and Cellular Biology 14, 6727–6735 (1994).
- 33. Moriya, S. et al. Platelet-derived growth factor activates protein kinase C epsilon through redundant and independent signaling pathways involving phospholipase C gamma or phosphatidylinositol 3-kinase. Proceedings of the National Academy of Sciences 93, 151–155 (1996).
- 34. Wang, T., Ming, Z., Xiaochun, W. & Hong, W. Rab7: role of its protein interaction cascades in endo-lysosomal traffic. *Cell Signal* 23, 516–521 (2011).
- Shinde, S. R. & Maddika, S. PTEN modulates EGFR late endocytic trafficking and degradation by dephosphorylating Rab7. Nature communications 7, 10689 (2016).
- 36. Wong, K. et al. Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway. Cell 107, 209–221 (2001).
- 37. Ma, Y. *et al*. The inverse F-BAR domain protein srGAP2 acts through srGAP3 to modulate neuronal differentiation and neurite outgrowth of mouse neuroblastoma cells. *PloS one* **8**, e57865 (2013).
- Li, M. et al. Fasting and Systemic Insulin Signaling Regulate Phosphorylation of Brain Proteins That Modulate Cell Morphology and Link to Neurological Disorders. The Journal of biological chemistry 290, 30030–30041 (2015).
- Chen, M. et al. Fasudil and its analogs: a new powerful weapon in the long war against central nervous system disorders? Expert Opin Investig Drugs 22, 537–550 (2013).
- 40. Blaise, S. et al. In vivo evidence that TRAF4 is required for central nervous system myelin homeostasis. PloS one 7, e30917 (2012).
- Montani, L. et al. Neuronal Nogo-A modulates growth cone motility via Rho-GTP/LIMK1/cofilin in the unlesioned adult nervous system. The Journal of biological chemistry 284, 10793–10807 (2009).
- 42. Charrier, E. et al. Collapsin response mediator proteins (CRMPs): involvement in nervous system development and adult neurodegenerative disorders. Mol Neurobiol 28, 51-64 (2003).
- Aizawa, H. et al. Phosphorylation of cofilin by LIM-kinase is necessary for semaphorin 3A-induced growth cone collapse. Nat Neurosci 4, 367–373 (2001).
- 44. Trovo-Marqui, A. B. & Tajara, E. H. Neurofibromin: a general outlook. Clin Genet 70, 1-13 (2006).
- 45. Petrinovic, M. M. *et al.* Neuronal Nogo-A regulates neurite fasciculation, branching and extension in the developing nervous system. *Development* **137**, 2539–2550 (2010).
- Mathis, C., Schroter, A., Thallmair, M. & Schwab, M. E. Nogo-a regulates neural precursor migration in the embryonic mouse cortex. *Cereb Cortex* 20, 2380–2390 (2010).
- Huang, C., Rajfur, Z., Borchers, C., Schaller, M. D. & Jacobson, K. JNK phosphorylates paxillin and regulates cell migration. *Nature* 424, 219–223 (2003).
- 48. Schaller, M. D. Paxillin: a focal adhesion-associated adaptor protein. Oncogene 20, 6459-6472 (2001).
- Panetti, T. S. Tyrosine phosphorylation of paxillin, FAK, and p130CAS: effects on cell spreading and migration. Front Biosci 7, d143-150 (2002).
- 50. Abedi, H. & Zachary, I. Signalling mechanisms in the regulation of vascular cell migration. Cardiovasc Res 30, 544–556 (1995).
- 51. Sarhan, A. R. et al. LAR protein tyrosine phosphatase regulates focal adhesions through CDK1. J Cell Sci 129, 2962-2971 (2016).
- Lee, S. H., Hollingsworth, R., Kwon, H. Y., Lee, N. & Chung, C. Y. beta-arrestin 2-dependent activation of ERK1/2 is required for ADP-induced paxillin phosphorylation at Ser(83) and microglia chemotaxis. *Glia* 60, 1366–1377 (2012).
- Huang, C., Borchers, C. H., Schaller, M. D. & Jacobson, K. Phosphorylation of paxillin by p38MAPK is involved in the neurite extension of PC-12 cells. *The Journal of cell biology* 164, 593–602 (2004).
- 54. Kanehisa, M. & Goto, S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28, 27-30 (2000).

- Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M. & Tanabe, M. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res 44, D457–462 (2016).
- 56. Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y. & Morishima, K. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res* **45**, D353–D361 (2017).
- 57. Lachmann, A. & Ma'ayan, A. KEA: kinase enrichment analysis. Bioinformatics 25, 684-686 (2009).
- 58. Sulzmaier, F. J. & Ramos, J. W. RSK isoforms in cancer cell invasion and metastasis. *Cancer research* 73, 6099–6105 (2013).
- Gawecka, J. E. et al. RSK2 protein suppresses integrin activation and fibronectin matrix assembly and promotes cell migration. The Journal of biological chemistry 287, 43424–43437 (2012).
- 60. Kang, S. *et al.* p90 ribosomal S6 kinase 2 promotes invasion and metastasis of human head and neck squamous cell carcinoma cells. *The Journal of clinical investigation* **120**, 1165–1177 (2010).
- 61. Lee, C. J. et al. Magnolin inhibits cell migration and invasion by targeting the ERKs/RSK2 signaling pathway. BMC Cancer 15, 576 (2015).
- 62. Czaplinska, D. *et al.* Phosphorylation of RSK2 at Tyr529 by FGFR2-p38 enhances human mammary epithelial cells migration. *Biochimica et biophysica acta* 1843, 2461–2470 (2014).
- 63. Westerlund, N. *et al.* Phosphorylation of SCG10/stathmin-2 determines multipolar stage exit and neuronal migration rate. *Nature neuroscience* 14, 305–313 (2011).
- 64. Zhan, Y. et al. Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression. Arterioscler Thromb Vasc Biol 23, 795–801 (2003).
- 65. Arimura, N., Menager, C., Fukata, Y. & Kaibuchi, K. Role of CRMP-2 in neuronal polarity. J Neurobiol 58, 34-47 (2004).
- 66. Liu, B. P. & Strittmatter, S. M. Semaphorin-mediated axonal guidance via Rho-related G proteins. *Curr Opin Cell Biol* **13**, 619–626 (2001).
- 67. Inagaki, N. et al. CRMP-2 induces axons in cultured hippocampal neurons. Nat Neurosci 4, 781-782 (2001).
- Wakatsuki, S., Saitoh, F. & Araki, T. ZNRF1 promotes Wallerian degeneration by degrading AKT to induce GSK3B-dependent CRMP2 phosphorylation. *Nature cell biology* 13, 1415–1423 (2011).
- 69. Gu, Y., Hamajima, N. & Ihara, Y. Neurofibrillary tangle-associated collapsin response mediator protein-2 (CRMP-2) is highly phosphorylated on Thr-509, Ser-518, and Ser-522. *Biochemistry* **39**, 4267–4275 (2000).
- 70. Zhu, L. Q. et al. Protein phosphatase 2A facilitates axonogenesis by dephosphorylating CRMP2. The Journal of neuroscience: the official journal of the Society for Neuroscience **30**, 3839–3848 (2010).
- Cole, A. R. et al. Relative resistance of Cdk5-phosphorylated CRMP2 to dephosphorylation. The Journal of biological chemistry 283, 18227–18237 (2008).
- 72. Cohen, P., Holmes, C. F. & Tsukitani, Y. Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem Sci* 15, 98–102 (1990).
- Cohen, P., Klumpp, S. & Schelling, D. L. An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. *FEBS Lett* 250, 596–600 (1989).
- Ip, J. P., Fu, A. K. & Ip, N. Y. CRMP2: functional roles in neural development and therapeutic potential in neurological diseases. *Neuroscientist* 20, 589–598 (2014).
- 75. Yoshimura, T. et al. GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. Cell 120, 137-149 (2005).
- 76. Varrin-Doyer, M. *et al.* Human T lymphotropic virus type 1 increases T lymphocyte migration by recruiting the cytoskeleton organizer CRMP2. *J Immunol* **188**, 1222–1233 (2012).
- 77. Vincent, P. *et al.* A role for the neuronal protein collapsin response mediator protein 2 in T lymphocyte polarization and migration. *J Immunol* **175**, 7650–7660 (2005).
- Doanes, A. M., Irani, K., Goldschmidt-Clermont, P. J. & Finkel, T. A requirement for rac1 in the PDGF-stimulated migration of fibroblasts and vascular smooth cells. *Biochem Mol Biol Int* 45, 279–287 (1998).
- 79. Ip, J. P. *et al.* alpha2-chimaerin controls neuronal migration and functioning of the cerebral cortex through CRMP-2. *Nat Neurosci* 15, 39–47 (2012).
- Soutar, M. P., Thornhill, P., Cole, A. R. & Sutherland, C. Increased CRMP2 phosphorylation is observed in Alzheimer's disease; does this tell us anything about disease development? *Curr Alzheimer Res* 6, 269–278 (2009).
- Binder, J. X. *et al.* COMPARTMENTS: unification and visualization of protein subcellular localization evidence. *Database (Oxford)* 2014, bau012 (2014).
- Shannon, P. et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 13, 2498–2504 (2003).
- Huang da, W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4, 44–57 (2009).
- 84. Supek, F., Bosnjak, M., Skunca, N. & Smuc, T. REVIGO summarizes and visualizes long lists of gene ontology terms. *PloS one* **6**, e21800 (2011).
- Lennartsson, J., Wardega, P., Engstrom, U., Hellman, U. & Heldin, C. H. Alix facilitates the interaction between c-Cbl and plateletderived growth factor beta-receptor and thereby modulates receptor down-regulation. *J Biol Chem* 281, 39152–39158 (2006).
- Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9, 671–675 (2012).
 Lock, F. E. & Hotchin, N. A. Distinct roles for ROCK1 and ROCK2 in the regulation of keratinocyte differentiation. *PloS one* 4, e8190 (2009).
- Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteomewide protein quantification. Nat Biotechnol 26, 1367–1372 (2008).

Acknowledgements

The authors would like to acknowledge Cleidiane Zampronio and Jinglei Lu (Functional Genomics, Proteomics and Metabolomics Facility, University of Birmingham) for technical support. We thank Alexandra Mazharian (Institute of Cardiovascular Sciences, University of Birmingham, UK) for supplying the okadaic acid. ARS is supported by a scholarship from the Higher Committee for Education Development in Iraq (HCED). The IncuCyte ZOOM imaging system was purchased through a British Heart Foundation Infrastructure Grant.

Author Contributions

Conceived and designed the experiments: A.R.S., M.G.T., N.A.H., J.K.H., D.L.C. Performed the experiments: A.R.S., S.B., J.S., D.L.C. Analysed the data: A.R.S., J.S., D.L.C. Wrote the paper: A.R.S., N.A.H., J.K.H., D.L.C.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-04015-x

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017