

CORRECTION

A crucial role for DOK1 in PDGF-BB-stimulated glioma cell invasion through p130Cas and Rap1 signalling

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In the Abstract and Introduction, TERF2IP was incorrectly given as an alternative name for Rap1. This should have referred to the small GTPase Rap1 (which has two isoforms, Rap1a and Rap1b).

We apologise to the readers for any confusion that this error might have caused.

RESEARCH ARTICLE

A crucial role for DOK1 in PDGF-BB-stimulated glioma cell invasion through p130Cas and Rap1 signalling

Angela Barrett^{1,*}, Ian M. Evans^{1,*}, Antonina Frolov¹, Gary Britton¹, Caroline Pellet-Many¹, Maiko Yamaji¹, Vedanta Mehta¹, Rina Bandopadhyay², Ningning Li³, Sebastian Brandner³, Ian C. Zachary¹ and Paul Frankel^{1,‡}

ABSTRACT

DOK1 regulates platelet-derived growth factor (PDGF)-BB-stimulated glioma cell motility. Mechanisms regulating tumour cell motility are essential for invasion and metastasis. We report here that PDGF-BB-mediated glioma cell invasion and migration are dependent on the adaptor protein downstream of kinase 1 (DOK1). DOK1 is expressed in several glioma cell lines and in tumour biopsies from high-grade gliomas. DOK1 becomes tyrosine phosphorylated upon PDGF-BB stimulation of human glioma cells. Knockdown of DOK1 or expression of a DOK1 mutant (DOK1FF) containing Phe in place of Tyr at residues 362 and 398, resulted in inhibition of both the PDGF-BB-induced tyrosine phosphorylation of p130Cas (also known as BCAR1) and the activation of Rap1 (also known as TERF2IP). DOK1 colocalises with tyrosine phosphorylated p130Cas at the cell membrane of PDGF-BB-treated cells. Expression of a non-tyrosine-phosphorylatable substrate domain mutant of p130Cas (p130Cas15F) inhibited PDGF-BB-mediated Rap1 activation. Knockdown of DOK1 and Rap1 inhibited PDGF-BB-induced chemotactic cell migration, and knockdown of DOK1 and Rap1 and expression of DOK1FF inhibited PDGF-mediated three-dimensional (3D) spheroid invasion. These data show a crucial role for DOK1 in the regulation of PDGF-BB-mediated tumour cell motility through a p130Cas–Rap1 signalling pathway.

KEY WORDS: Cell motility, PDGF signalling, DOK1, p130Cas, GTPase

INTRODUCTION

The acquisition of increased cell motility gives tumour cells the capacity to invade their surrounding tissue, and is described as one of the ‘hallmarks of cancer’ (Hanahan and Weinberg, 2011). However, specific chemotactic signalling pathways involved in the regulation of tumour cell motility and three-dimensional (3D) invasion are not well understood. Platelet derived growth factor (PDGF)-BB is an important ligand regulating cell motility in both non-transformed and cancer cells (Jones and Cross, 2004). The expression of PDGF and PDGF receptor is deregulated in a variety of cancers, including glioblastoma, melanoma, breast

carcinoma, prostate carcinoma and various forms of leukaemia (Heldin and Westermark, 1999).

Glioblastoma is a highly invasive primary malignant brain tumour. Glioblastoma cells migrate and proliferate preferentially along fibre tracts and blood vessels, resulting in a diffuse infiltration of brain tissue. It has been proposed that glioma cells are attracted to blood vessels by growth factors produced by endothelial cells, such as PDGF-BB (Farin et al., 2006). PDGF-BB plays an important role in regulating a variety of glioma cell functions, including motility, survival and proliferation (Shih and Holland, 2006). PDGF-BB stimulates cell migration, through the recruitment of adaptor proteins, protein kinases, protein phosphatases and small GTPases into large multi-protein complexes required for the regulation of cell motility (Takahashi et al., 2008). Although these complexes have been described, it is not yet fully understood how these signalling molecules are coordinated to produce chemotactic responses to PDGF.

Downstream of kinase 1 (DOK1) is a 62-kDa protein that is phosphorylated by both receptor and non-receptor tyrosine kinases (Carpino et al., 1997; Yamanashi and Baltimore, 1997). DOK1 has a modular domain structure, with an N-terminal pleckstrin homology (PH) domain, followed by a phosphotyrosine-binding (PTB) domain and containing multiple tyrosine residues in the C-terminal region (Mashima et al., 2009). It has been proposed that DOK1 has a role as a scaffold or adaptor protein in the formation of multi-molecular complexes, spatially and/or temporally regulating other signalling molecules to produce coordinated cellular responses (Mashima et al., 2009).

Previously, we reported that tyrosine phosphorylation of p130Cas (also known as BCAR1) plays a key role in the PDGF-BB-induced migration of U87 glioma cells and vascular smooth muscle cells (Evans et al., 2011; Pellet-Many et al., 2011). Here, we investigated the role of DOK1 in PDGF-BB-mediated cell motility and chemotactic p130Cas signalling in malignant glioma cells. We found that PDGF-BB stimulates DOK1 phosphorylation on Tyr 362 and 398, and that phosphorylation at these residues is crucial for PDGF-BB-stimulated tyrosine phosphorylation of p130Cas. Furthermore, DOK1 mediates PDGF-BB-induced activation of the small GTPase Rap1 (also known as TERF2IP), through a pathway dependent on p130Cas tyrosine phosphorylation, and PDGF-BB stimulation of glioma cell migration and 3D radial invasion are dependent on DOK1 and Rap1. Taken together with the expression of DOK1 in tumour biopsies from high-grade gliomas, these results indicate that DOK1 plays a crucial role in regulating a p130Cas–Rap1 pathway in PDGF-BB-mediated glioma cell motility, with implications for the mechanisms underlying the pathogenesis of human glioblastoma.

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RESULTS

DOK1 has been reported to play both positive and negative roles in tumour progression (Berger et al., 2010; Hosooka et al., 2001; Mercier et al., 2011), yet very little is known about DOK1 protein expression in human tumours. We examined multiple human glioma cell lines for their responsiveness to PDGF-BB stimulation and DOK1 expression (Fig. 1A). DOK1 mRNA expression in cancer was examined by searching the continuously updated Oncomine database, based on analysis of cancer versus normal, cancer versus cancer and Cancer Outlier Profile Analysis (COPA) (MacDonald and Ghosh, 2006). Analysis of cancer versus normal showed that DOK1 mRNA was moderately overexpressed in both breast and kidney cancers, whereas it was moderately reduced in lung cancer (supplementary material Fig. S1A). In addition to COPA, we also investigated mRNA expression using The Cancer Genome Atlas (TCGA) data sets that are available through Oncomine. We found significant increases in DOK1 mRNA expression in lung, breast and brain cancers (supplementary material Fig. S1B). Additionally, we explored the Human Protein Atlas for DOK1 expression in glioma. We found that DOK1 is expressed in the majority of glioma samples and importantly is not detected in normal brain (<http://www.proteinatlas.org/>

ENSG00000115325/cancer). Considering these results, we decided to look at DOK1 protein levels in biopsies from human gliomas. We found that the expression of DOK1 protein was elevated in glioblastoma multiforme (GBM) biopsies (ranging from grades 2–4) compared with normal brain tissue, which showed very little expression (Fig. 1B).

Having established that DOK1 is expressed in several human glioma tumour cell lines and in human glioma biopsies, we decided to investigate the role of DOK1 in PDGF-BB-stimulated glioma cell invasion. PDGF-BB treatment of U87MG cells stimulated a marked increase in the phosphorylation of DOK1 at Tyr 362 and 398. This increase was transient, peaking between 5 and 15 min post-treatment, and it declined to basal levels after 60 min (Fig. 2A). The timecourse of DOK1 tyrosine phosphorylation was very similar to that of PDGF-BB-induced tyrosine phosphorylation of p130Cas, an adapter protein that plays a key role in the migration of U87MG cells in response to PDGF-BB (Evans et al., 2011). Because the PDGF receptor α (PDGFR α) has been shown to be upregulated in gliomas (Martinho et al., 2009), we investigated responses to PDGF-AA-mediated activation of PDGFR α signalling in U87MG cells. Although PDGF-BB stimulation caused a robust increase in

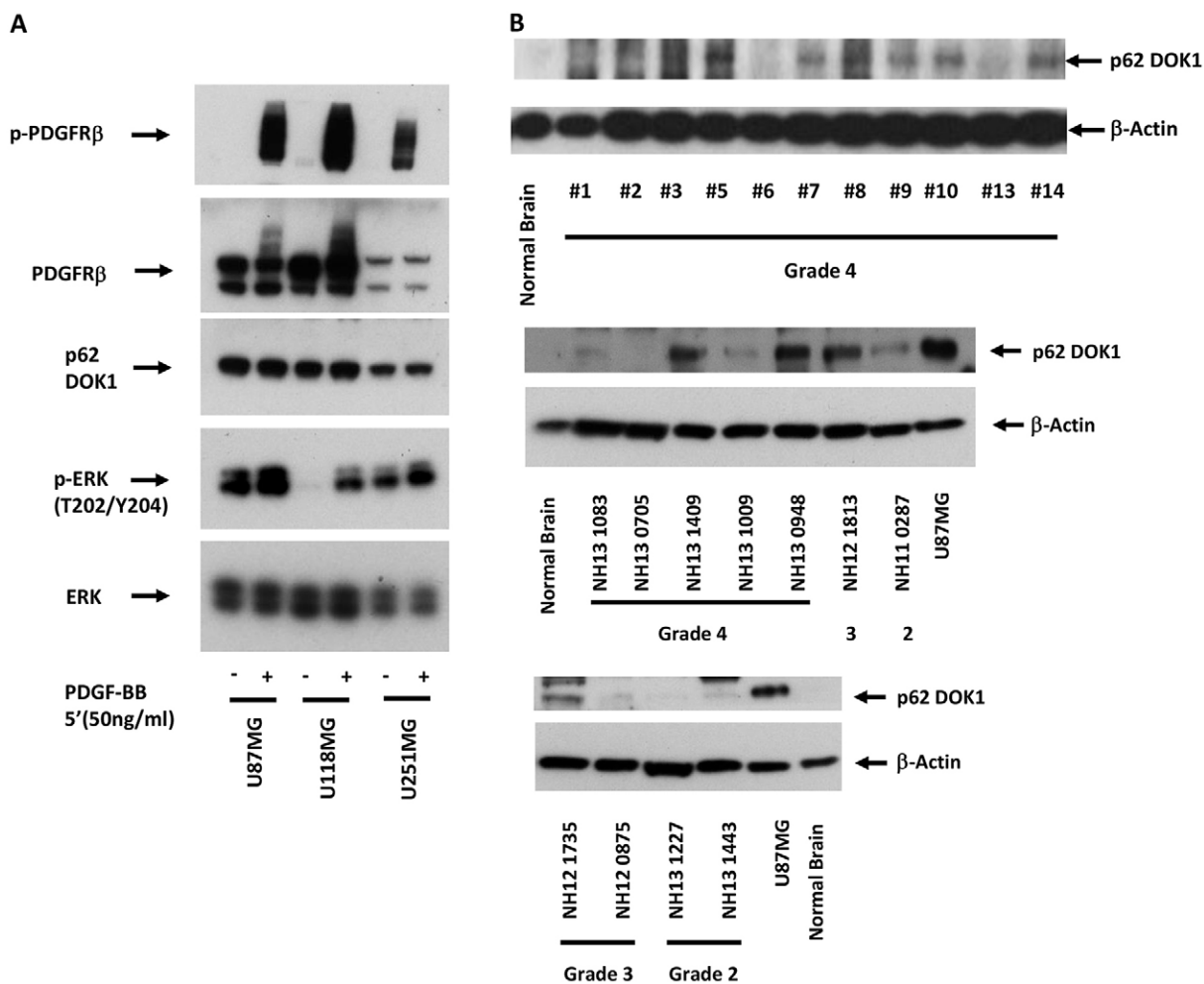


Fig. 1. DOK1 expression in human glioma cell lines and human malignant glioma biopsies. (A) DOK1 is expressed in multiple human glioma cell lines. Levels of total and phospho-PDGFR β and total and phospho-ERK with (+) or without (–) a 5-min treatment with 50 ng/ml PDGF-BB are also shown. (B) Protein lysates of several biopsies of human GBM (stages 2, 3 and 4, all provided with separate code numbers) separated by gel electrophoresis and probed using antibodies against DOK1 and β -actin. Blots shown here and in all subsequent figures are representative of at least three separate experiments.

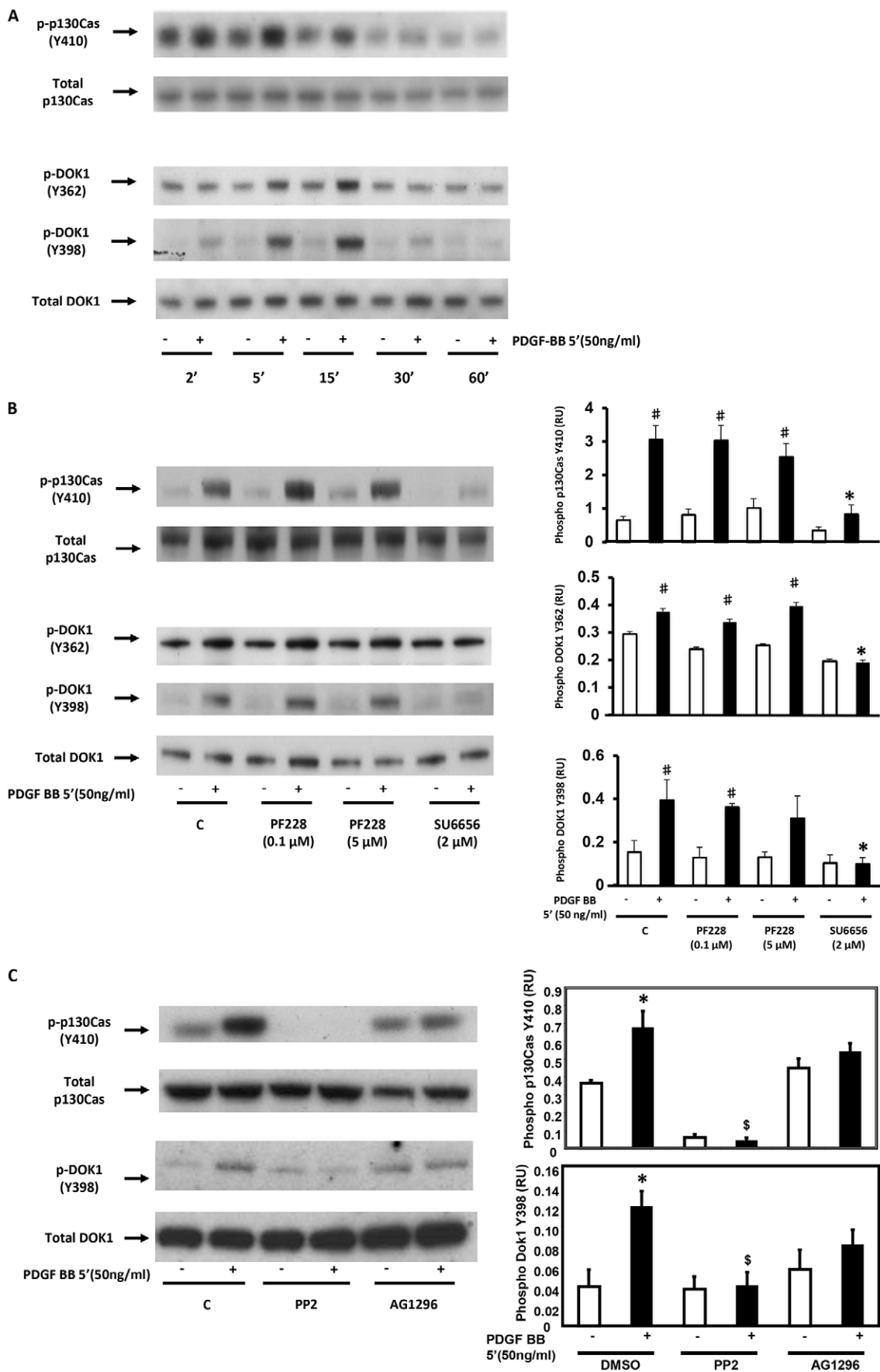


Fig. 2. See next page for legend.

Fig. 2. Timecourse of PDGF-BB-stimulated DOK1 and p130Cas tyrosine phosphorylation and the requirement for SRC family kinases.

(A) U87MG cells (~80% confluent) were incubated in SFM for ~18 h prior to treatment with either SFM plus vehicle control (–) or SFM plus 50 ng/ml PDGF-BB (+) for the indicated times (min). Cell lysates were prepared and immunoblotted as indicated. (B) U87MG cells (~80% confluent) were incubated in SFM for ~18 h prior to pre-incubation for 30 min with PF573228 (PF228) at 0.1 μ M or 5 μ M, SU6656 at 2 μ M or the vehicle control (0.05% DMSO, 'C'), followed by treatment with SFM control (–) or 50 ng/ml PDGF-BB (+) for 5 min. Cell lysates were then prepared, blotted and probed with the indicated antibodies. Representative blots of at least three separate experiments are shown in the left-hand panel. Quantification of tyrosine phosphorylation was performed by densitometry using ImageJ, as shown in the right-hand panel. Data from at least three independent experiments are presented as phosphorylation relative units (RU), and show the mean \pm s.e.m. Data are normalised to either total p130Cas (top panel) or DOK1 (middle and lower panels). * P <0.01 (compared with PDGF-BB-stimulated control, 'C'); [#] P <0.05 (compared with SFM control, –). (C) U87MG cells (~80% confluent) were incubated in SFM for ~18 h prior to pre-incubation for 30 min with either the Src family kinase inhibitor PP2 (10 μ M), the PDGFR inhibitor AG 1296 (10 μ M) or the vehicle (0.05% DMSO, 'C'), followed by treatment with SFM control (–) or 50 ng/ml PDGF-BB (+) for 5 min. Cell lysates were then prepared, blotted and probed with the indicated antibodies. Representative blots of at least three separate experiments are shown (left-hand panel). Quantification of tyrosine phosphorylation was performed as in B (right-hand panel). ^{\$} P <0.01 (compared with PDGF-stimulated control, 'C'); * P <0.05 (compared with SFM control, –).

PDGFR β tyrosine phosphorylation (Fig. 1A), no increase in PDGFR α tyrosine phosphorylation was detectable in response to either PDGF-AA or PDGF-BB (supplementary material Fig. S2A). Furthermore, although PDGF-BB increased p130Cas tyrosine phosphorylation and ERK activation in U87MG cells, PDGF-AA had no effect on these signalling events in these cells. By contrast, PDGF-AA treatment of human coronary artery smooth muscle cells increased tyrosine phosphorylation of PDGFR α , as we reported previously (Pellet-Many et al., 2011) (supplementary material Fig. S2B).

We next looked at the role of SRC family tyrosine kinases (SFKs), which are known to become activated in response to PDGF-BB stimulation. Treatment with the SFK inhibitor SU6656 resulted in the inhibition of PDGF-BB-stimulated DOK1 phosphorylation on Tyr 362 and 398 (Fig. 2B), residues within the SRC substrate consensus sequence, YXXP. SU6656 also strongly inhibited p130Cas tyrosine phosphorylation (Fig. 2B). To confirm the specificity of the SRC inhibitor and signalling through PDGFR, we treated cells with an additional selective SFK inhibitor, PP2, and the selective PDGFR inhibitor AG 1296. Pre-treatment of U87MG cells with PP2 or AG 1296 significantly reduced PDGF-BB-stimulated tyrosine phosphorylation of both DOK1 and p130Cas (Fig. 2C). By contrast, treatment of the U87MG cells with the potent FAK family inhibitor, PF573228, at concentrations that specifically block the activity of FAK kinase and PYK2 (also known as PTK2B) (Slack-Davis et al., 2007), had no effect on PDGF-BB-stimulated tyrosine phosphorylation of DOK1 and p130Cas (Fig. 2B). Phosphatidylinositol 3-kinase (PI3K) activity has been reported to be required for DOK1 tyrosine phosphorylation in haematopoietic cells and for DOK1 membrane localisation in fibroblasts (van Dijk et al., 2000; Zhao et al., 2001). Treatment of U87MG cells with the PI3K inhibitor LY294002 strongly reduced PDGF-stimulated DOK1 phosphorylation at Tyr 362 and Tyr 398, and also reduced AKT activity compared with the vehicle control (Fig. 3A). Furthermore, immunofluorescence microscopy indicated that

LY294002 treatment reduced the amount of DOK1 that was localised at the cell membrane in U87MG cells following PDGF-BB treatment (Fig. 3B).

The results in Fig. 2 suggested that DOK1 could be an important endogenous mediator of p130Cas signalling in the regulation of cell motility. This was examined by testing the effect of DOK1 silencing on PDGF-BB-stimulated p130Cas tyrosine phosphorylation. Treatment of U87MG cells with PDGF-BB strongly increased the level of p130Cas tyrosine phosphorylation after 5 min, an effect that was markedly reduced when cells were treated with multiple siRNAs directed against DOK1 (Fig. 4A). These results were further supported by the finding that PDGF-BB-induced tyrosine phosphorylation of p130Cas was similarly inhibited by multiple DOK1 siRNAs in the glioma cell line U251MG (supplementary material Fig. S3A). Inhibition of p130Cas tyrosine phosphorylation by DOK1 knockdown was selective, because PDGF-BB-mediated phosphorylation of ERK and AKT, two signalling molecules with well-established roles in regulating cell migration, were unaffected by treatment with DOK1 siRNA (Fig. 4B). We also examined the effect of PDGF-BB treatment on the localisation of DOK1 and p130Cas in U87MG cells, by performing co-immunofluorescent staining. PDGF-BB increased the localisation of DOK1 and p130Cas to the cell membrane, accompanied by a significant increase in the colocalisation of these proteins (Fig. 5), which was blocked by pre-treatment with the selective PDGFR inhibitor AG 1296 (supplementary material Fig. S2C), indicating that PDGF-BB has a similar effect on cellular redistribution of DOK1 and p130Cas.

We next investigated a possible role for DOK1 and p130Cas in regulating Rap1 activity in glioma cells. PDGF-BB treatment of U87MG cells strongly increased Rap1-GTP levels, as determined by pulldown of active GTP-bound Rap1. This effect was significantly reduced in cells that were either transfected with DOK1 siRNAs (Fig. 6A) or that overexpressed the non-phosphorylatable p130Cas15F mutant (Fig. 6B) (Evans et al., 2011) compared with controls. Because the Rac1 GTPase has also been shown to become activated downstream of p130Cas, we investigated a possible role for DOK1 in Rac1 activation. PDGF-BB treatment of U87MG cells modestly increased Rac1-GTP levels, as determined by pulldown of active GTP-bound Rac1, but this effect was not reduced in cells that were transfected with DOK1 siRNA (data not shown).

These results led us to examine whether DOK1 tyrosine phosphorylation at Tyr 362 and 398 was important for mediating PDGF-BB signalling through p130Cas and Rap1. To do this, we infected U87MG cells with adenoviruses encoding either wild-type DOK1 (Ad.DOK1) or DOK1 with Tyr 362 and 398 mutated to phenylalanine (Ad.DOK1FF). As shown in Fig. 6C, expression of Ad.DOK1FF significantly decreased PDGF-BB-stimulated p130Cas tyrosine phosphorylation, whereas expression of Ad.DOK1 had no effect. Ad.DOK1FF expression also resulted in a significant decrease in Rap1 activation in response to PDGF-BB, whereas Ad.DOK1 had no effect on Rap1-GTP levels (Fig. 6D).

The role of DOK1 in PDGF-BB-mediated chemotactic migration was assessed using a transwell migration assay. In both U87MG and U251MG glioma cells, knockdown of either DOK1 or Rap1 significantly inhibited the ability of these cells to migrate towards PDGF-BB (Fig. 7A,B; supplementary material Fig. S3B). We further investigated the role of DOK1 in PDGF-BB-stimulated motility using a 3D spheroid assay. We generated

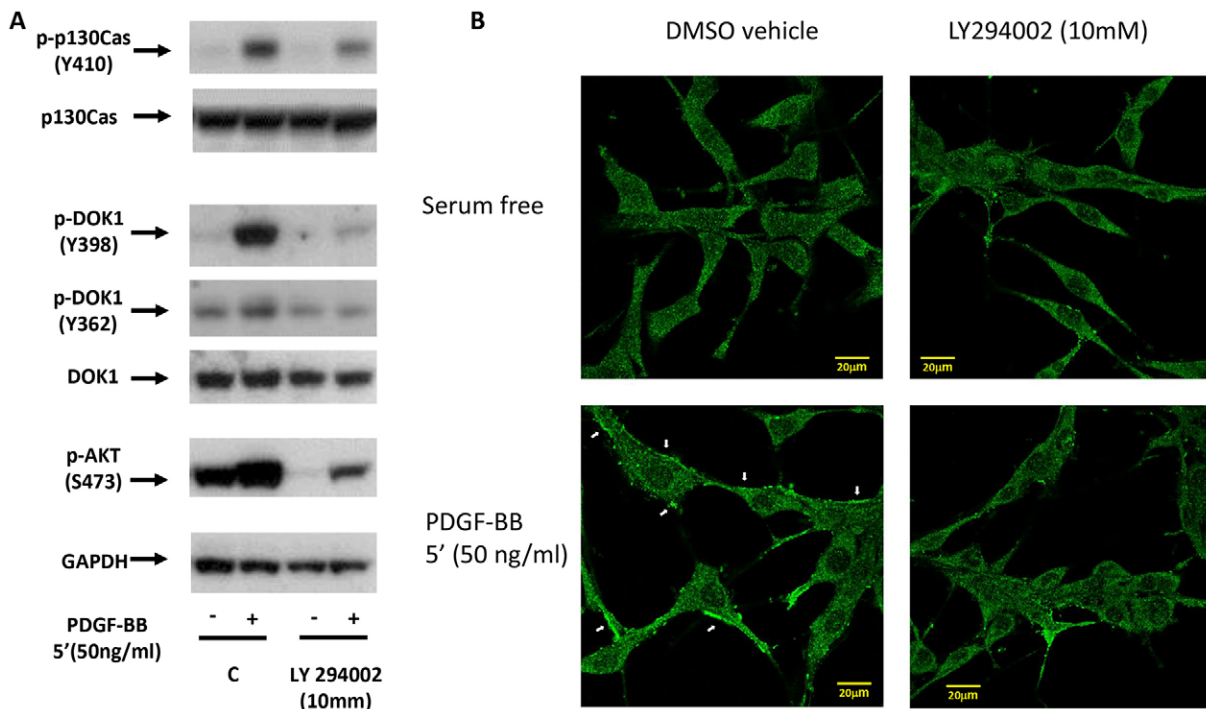


Fig. 3. PI3K is required for DOK1 phosphorylation and recruitment to the membrane. (A) U87MG cells (~80% confluent) were incubated in SFM for ~18 h prior to pre-incubation for 30 min with LY294002 (10 μM) or the vehicle (0.05% DMSO, 'C'), followed by treatment with SFM vehicle control (-) or 50 ng/ml PDGF-BB (+) for 5 min. Cell lysates were then prepared, blotted and probed with the indicated antibodies. (B) U87MG cells were seeded onto glass coverslips and incubated in SFM for ~18 h prior to pre-incubation for 30 min with LY294002 (10 μM) or the vehicle (0.05% DMSO), followed by treatment with SFM vehicle control (-) or 50 ng/ml PDGF-BB (+) for 5 min. Confocal imaging was performed as described in Materials and Methods, with DOK1 staining shown in green. Images are representative of at least three separate experiments. Arrows indicate areas of increased DOK1 localisation to the membrane upon PDGF-BB treatment.

U87MG spheroids and embedded them in collagen I plugs supplemented and overlaid with either serum-free medium (SFM) or SFM containing PDGF-BB. PDGF-BB treatment for 48 h resulted in enhanced radial invasion compared with the control spheroids incubated in SFM, and the effect of PDGF-BB was significantly reduced in spheroids generated from cells treated with DOK1 siRNAs (Fig. 7C). We next examined the role of DOK1 phosphorylation in PDGF-BB-mediated radial invasion. Ad.DOK1FF expression in U87MG cells significantly inhibited invasion induced by PDGF-BB, whereas Ad.DOK1 had no significant effect on radial invasion compared with the Ad.LACZ control (Fig. 7D). We also investigated the contribution of each of the tyrosine residues (Tyr 362 and Tyr 398) on PDGF-BB-stimulated DOK1 signalling by infecting cells with adenoviruses expressing the single DOK1 point mutations Y362F and Y398F. Expression of either DOK1 Y362F or DOK1 Y398F in U87MG cells caused a significant decrease in PDGF-BB-stimulated p130Cas tyrosine phosphorylation and 3D radial invasion (supplementary material Fig. S4; Fig. 7D). The role of Rap1 in radial invasion was addressed by silencing Rap1 expression using targeted siRNAs. Three different Rap1-specific siRNAs all significantly reduced the stimulation of radial U87MG spheroid cell invasion in collagen I (Fig. 7E).

DISCUSSION

The role of DOK1 in tumorigenesis is still emerging and remains unclear. Some studies suggest a role for DOK1 as a tumour suppressor or negative regulator of tumour progression (Berger et al., 2010; Mercier et al., 2011). By contrast, there are reports

that DOK1 plays a positive role in tumour progression and motility (Hosooka et al., 2001; Mercier et al., 2011). Indeed, in a gene expression profiling study of invasive carcinoma cells versus primary mammary tumours, DOK1 was found to be significantly upregulated in the invasive cell population (Wang et al., 2004). We used COPA, an algorithm developed to identify an oncogene expression profile where high gene expression is seen in a subset of samples in the total population within a cancer type (MacDonald and Ghosh, 2006), to analyse DOK1 expression in various human cancers. The value of this analysis is highlighted by the ERBB2 oncogene, which is overexpressed in 25% of breast cancers. Standard analysis does not indicate significant ERBB2 mRNA upregulation, whereas COPA reveals a strong and significant upregulation of ERBB2. Using COPA, we found reduced levels of DOK1 in several cancers, particularly leukaemia, but COPA also revealed that overexpression of DOK1 is strongly associated with brain, kidney and liver cancer and lymphoma. In addition, we used OncoPrint to search TCGA, which contains high-resolution genetic information on several types of cancers. Data sets are presented for individual cancer types as well as comparisons with corresponding normal (non-cancerous) tissue samples. We found significant increases in DOK1 mRNA expression in several cancers, including GBM versus normal brain. Although increased mRNA expression suggests a role for DOK1 in specific cancers, there are limitations in correlating this to protein expression and function. We therefore investigated DOK1 protein expression in cancer using the Human Protein Atlas. In agreement with DOK1 mRNA expression, we found that DOK1 protein is expressed in the

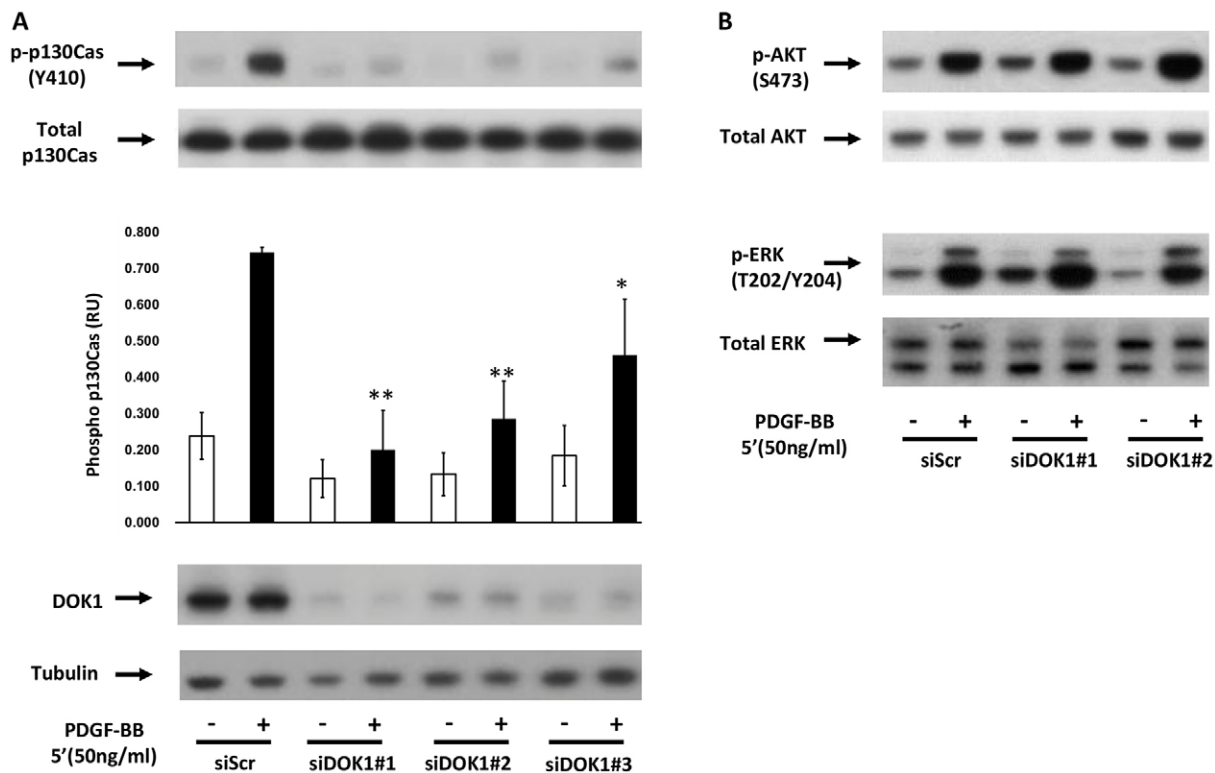


Fig. 4. DOK1 specifically regulates PDGF-BB-stimulated tyrosine phosphorylation of p130Cas. (A) U87MG cells were transfected with three independent siRNAs targeting DOK1 (siDOK1, 25 nM) or with control scrambled siRNA (siScr, 25 nM). At 48 h post-transfection, cells were incubated in SFM for ~18 h prior to treatment with SFM containing vehicle control (-) or 50 ng/ml PDGF-BB (+) for 5 minutes. Cell lysates were then prepared, blotted and probed with the indicated antibodies. Quantification of p130Cas phosphorylation was performed by densitometry using ImageJ. Data from three independent experiments are presented as p130Cas phosphorylation relative units (RU) and shown as the mean \pm s.e.m. Data are normalised to total p130Cas. * $P < 0.05$; ** $P < 0.01$ (compared with ligand-stimulated siScr). (B) U87MG cells were treated as above and probed with the indicated antibodies.

majority of the available glioma samples and is not detected in normal brain. Furthermore, our results showed higher DOK1 protein expression in several grade 2, 3 and 4 glioma biopsies compared with normal brain tissue, in which DOK1 protein expression was undetectable.

Although DOK1 has been implicated in the regulation of epithelial and smooth muscle cell motility (Lee et al., 2004; Ling et al., 2005), the mechanisms involved in regulating DOK1 and its downstream signalling pathways in tumour cell motility are not well understood. In this study, PDGF-BB stimulation resulted in a transient increase in DOK1 tyrosine phosphorylation, indicating that DOK1 phosphorylation is regulated by the PDGF-BB signalling pathway. The exact mechanism of DOK1 tyrosine phosphorylation is unclear, with reports of both SFK-dependent and SFK-independent mechanisms (Liang et al., 2002; Senis et al., 2009). Our data demonstrate that, in glioma cells, PDGF-BB-induced DOK1 tyrosine phosphorylation is dependent on SFK. Furthermore, treatment with a PI3K inhibitor strongly decreased PDGF-BB-stimulated DOK1 tyrosine phosphorylation and also reduced DOK1 localisation at the cell membrane, consistent with previous findings in other cell types (van Dijk et al., 2000; Zhao et al., 2001). This suggests that PI3K is required for DOK1 recruitment to the cell membrane, and such recruitment might be needed for its phosphorylation by SFK.

We recently reported that tyrosine phosphorylation of the adaptor protein p130Cas plays a key role in PDGF-BB-dependent migration of glioma cells and vascular smooth muscle cells (Evans et al., 2011; Pellet-Many et al., 2011). DOK1 has been

reported to associate with p130Cas upon stimulation of type 1 Fc ϵ R1 receptors (Fc ϵ R1) in mast cells, but the nature of this association is unclear, and the effect on p130Cas tyrosine phosphorylation was not determined (Abramson et al., 2003). Indeed, we attempted to co-immunoprecipitate p130Cas with endogenous and tagged DOK1, but were unable to observe any association between DOK1 and p130Cas, in contrast to the results reported by Abramson et al. Nevertheless, we show herein that DOK1 is required for PDGF-BB-induced p130Cas tyrosine phosphorylation and that both DOK1 and p130Cas colocalise to the membrane following stimulation with PDGF-BB. Furthermore, DOK1 has a selective role in regulating PDGF-BB-stimulated tyrosine phosphorylation of p130Cas, because DOK1 knockdown did not significantly affect other major receptor tyrosine kinase (RTK) signal transduction pathways, including activation of the ERK and AKT signalling pathways.

Through its multiple domains, p130Cas is thought to function as a scaffold for the assembly of signalling complexes that are required for remodelling of the cytoskeleton during cell motility (Barrett et al., 2013; Defilippi et al., 2006). A key role of p130Cas is in the formation of complexes with exchange factors for Ras family small GTPases (Di Stefano et al., 2011). In particular, p130Cas, through its N-terminal SH3 domain, binds to the adaptor protein Crk, and both p130Cas and Crk associate with the guanine nucleotide exchange factor (GEF) C3G (also known as RAPGEF1). This results in the recruitment of the small GTPase Rap1, and its activation through exchange of GDP for GTP. In turn, Rap1 activation drives multiple signalling pathways that are

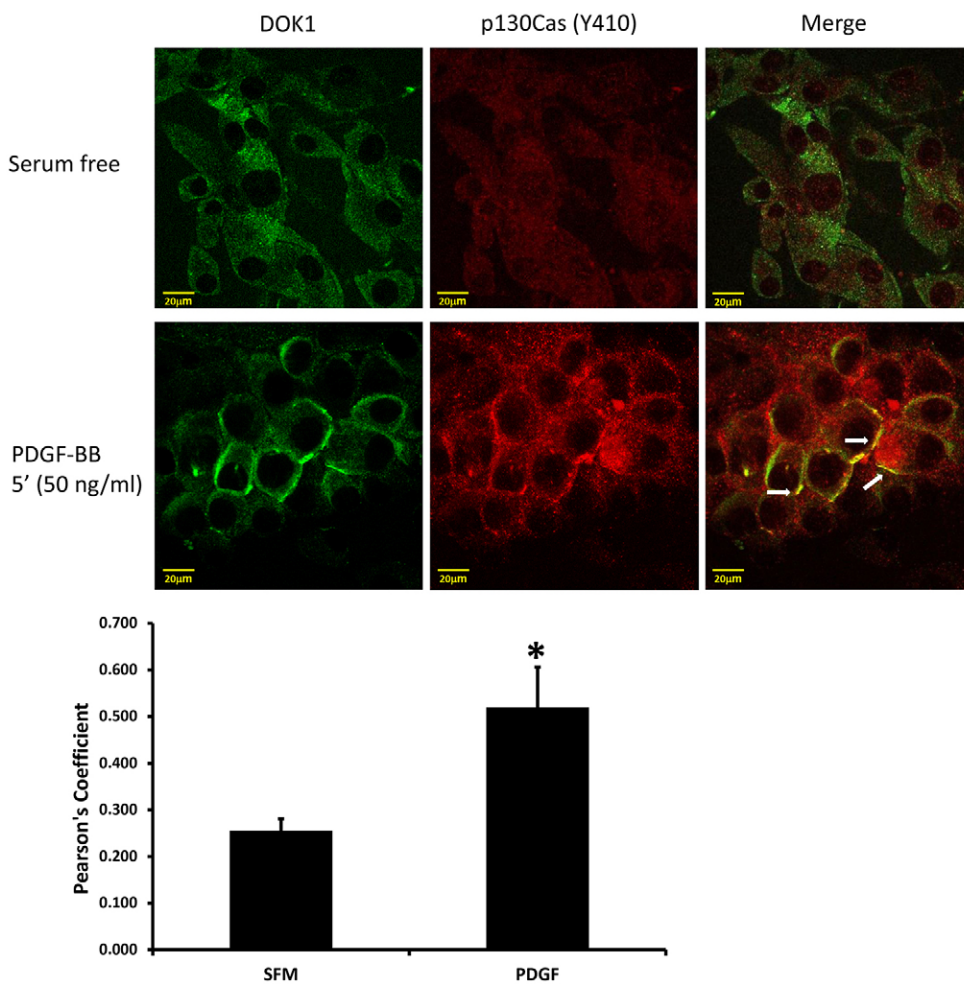


Fig. 5. PDGF-BB-mediated increase in colocalisation of DOK1 and phosphorylated p130Cas. U87MG cells were seeded onto glass coverslips and incubated in SFM for ~18 h prior to treatment with SFM control (serum free) or 50 ng/ml PDGF-BB for 5 min. Confocal imaging was performed as described in Materials and Methods, with DOK1 staining shown in green and phospho-p130Cas (Y410) shown in red (upper panel). Merged images show co-staining of DOK1 and phospho p130Cas (yellow) and are representative of at least three separate experiments. Arrows indicate membrane regions and areas of increased colocalisation upon PDGF-BB treatment. Quantification of DOK1 and phospho-p130Cas colocalisation (as described in Materials and Methods) is shown (lower panel) and represents data from three independent experiments expressed as Pearson's coefficient of colocalisation. Data show the mean \pm s.e.m. * $P < 0.05$, compared with SFM control (two-tailed Student's *t*-test).

required for RTK-mediated cell motility (Boettner and Van Aelst, 2009; Frische and Zwartkruis, 2010). Our finding that either DOK1 knockdown or overexpression of Ad.DOK1FF inhibits p130Cas tyrosine phosphorylation and Rap1 activation supports the conclusion that PDGF-BB-induced DOK1 tyrosine phosphorylation plays a key role in mediating a signalling pathway involving p130Cas and Rap1.

Consistent with its role in Rap1 activation, assays of chemotaxis and 3D invasion showed that DOK1 is required for PDGF-BB-stimulated cell motility. Examination of chemotactic tumour cell invasion in a 3D environment has proven to be difficult, owing to the difficulties in establishing a defined chemotactic gradient. We sought to overcome this problem by using the spheroid invasion model with exogenously supplied PDGF-BB. Although this is not a model of movement towards a single chemotactic source, it does produce directional radial movement outwards from the rim of the spheroid core. DOK1 knockdown or expression of Ad.DOK1FF inhibited spheroid outgrowth, as did Rap1 silencing. These results further establish an important role for DOK1 in regulating PDGF-BB-mediated signalling, which is essential for cell motility and invasion.

Taken together, these results show for the first time that DOK1 and p130Cas play an important role in the regulation of PDGF-BB-stimulated Rap1 signalling in U87MG glioma cells. The finding that Tyr 362 and 398 are crucial for PDGF-BB-stimulated p130Cas tyrosine phosphorylation and Rap1 activation suggests

an important role for the signalling protein(s) that bind either one or both of these residues. Furthermore, these data show that the Ad.DOK1FF mutant behaves in a dominant-negative manner, possibly by competing with endogenous DOK1 binding partners to form non-functional complexes. The adaptor proteins Crk and NCK have been reported to bind to DOK1 and to p130Cas (Defilippi et al., 2006; Noguchi et al., 1999). However, knockdown (~80%) of either Crk or NCK with targeted siRNAs had no significant effect on PDGF-BB-stimulated tyrosine phosphorylation of p130Cas or DOK1, suggesting that they might act further downstream (data not shown).

It is likely that DOK1 associates with other as-yet-unidentified binding partner(s). DOK1 was originally described as a binding protein and substrate for the Abl tyrosine kinase (Yamanashi and Baltimore, 1997), and an Abl pathway has been implicated in regulating cell motility (Woodring et al., 2004). We therefore investigated the role of Abl using siRNA-mediated knockdown. However, we were unable to demonstrate a significant effect of silencing Abl on PDGF-BB-mediated stimulation of DOK1 and p130Cas tyrosine phosphorylation (data not shown). This suggests that Abl might not be important for DOK1-mediated PDGF-BB signalling through the p130Cas pathway described here. However, these results do not preclude a role for Abl in DOK1-dependent signalling in glioma cells, and this warrants further investigation. We found that expression of the single mutants DOK1 Y362F and, to a lesser extent, DOK1 Y398F

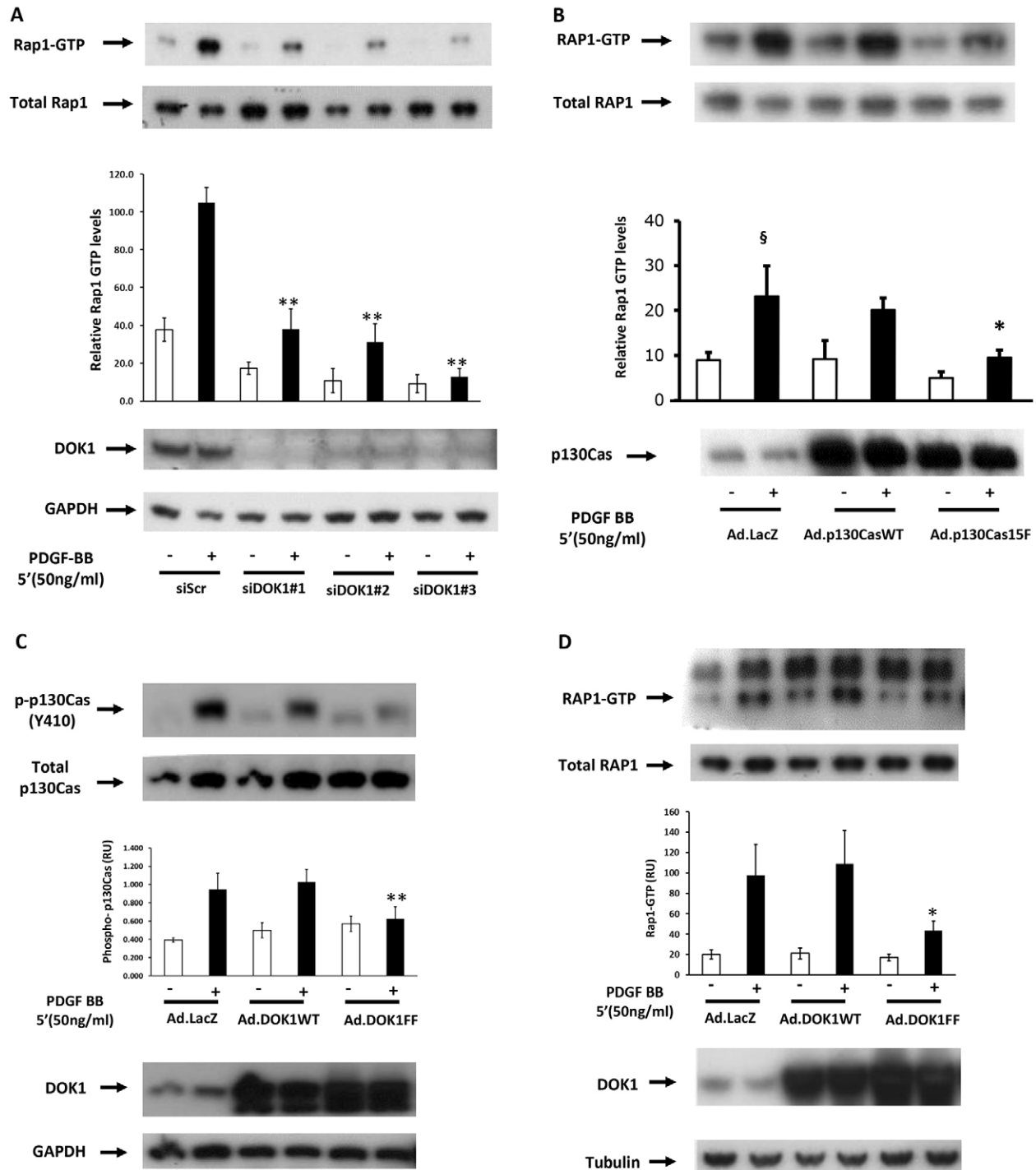


Fig. 6. DOK1 and p130Cas are required for Rap1 activation. (A) U87MG cells were transfected with three independent siRNAs targeting DOK1 (siDOK1, 25 nM) or with control scrambled siRNA (siScr, 25 nM). At 48 h post-transfection, cells were incubated in SFM for ~18 h prior to treatment with SFM containing vehicle control (–) or 50 ng/ml PDGF-BB (+) for 5 min. Cell lysates were prepared and assayed using a Rap1 activation assay, as described in Materials and Methods. Rap1-GTP and whole-cell extract samples were blotted and probed with the indicated antibodies. Quantification of Rap1-GTP levels was performed by densitometry using ImageJ. Data from three independent experiments are presented as Rap1-GTP relative units (RU) and show the mean ± s.e.m. Data are normalised to total Rap1. ** $P < 0.01$ (compared with ligand-stimulated siScr). (B) U87MG cells (~80% confluent) were infected with Ad.LacZ, Ad.p130Cas or Ad.p130Cas15F at MOIs of 250. At 48 h after infection, cells were treated as described above and quantification of Rap1-GTP levels was performed as for A. * $P < 0.05$ (compared with ligand-stimulated Ad.LacZ); § $P < 0.05$ (compared with Ad.LacZ SFM control). (C) U87MG cells (~80% confluent) were infected with Ad.LacZ, Ad.DOK1 or Ad.DOK1FF at MOIs of 200. At 48 h after infection, cells were incubated in SFM for ~18 h prior to treatment with SFM control (–) or 50 ng/ml PDGF-BB (+). Cell lysates were then prepared, blotted and probed with the indicated antibodies, and p130Cas phosphorylation was quantified. ** $P < 0.01$ (compared with Ad.LacZ plus PDGF-BB). (D) U87MG cells were treated as in C, lysed in Rap1 activation buffer and analysed using a Rap1 activation assay as described in Materials and Methods. Quantification of Rap1-GTP levels was performed as for A; * $P < 0.05$ compared with Ad.LacZ plus PDGF-BB.

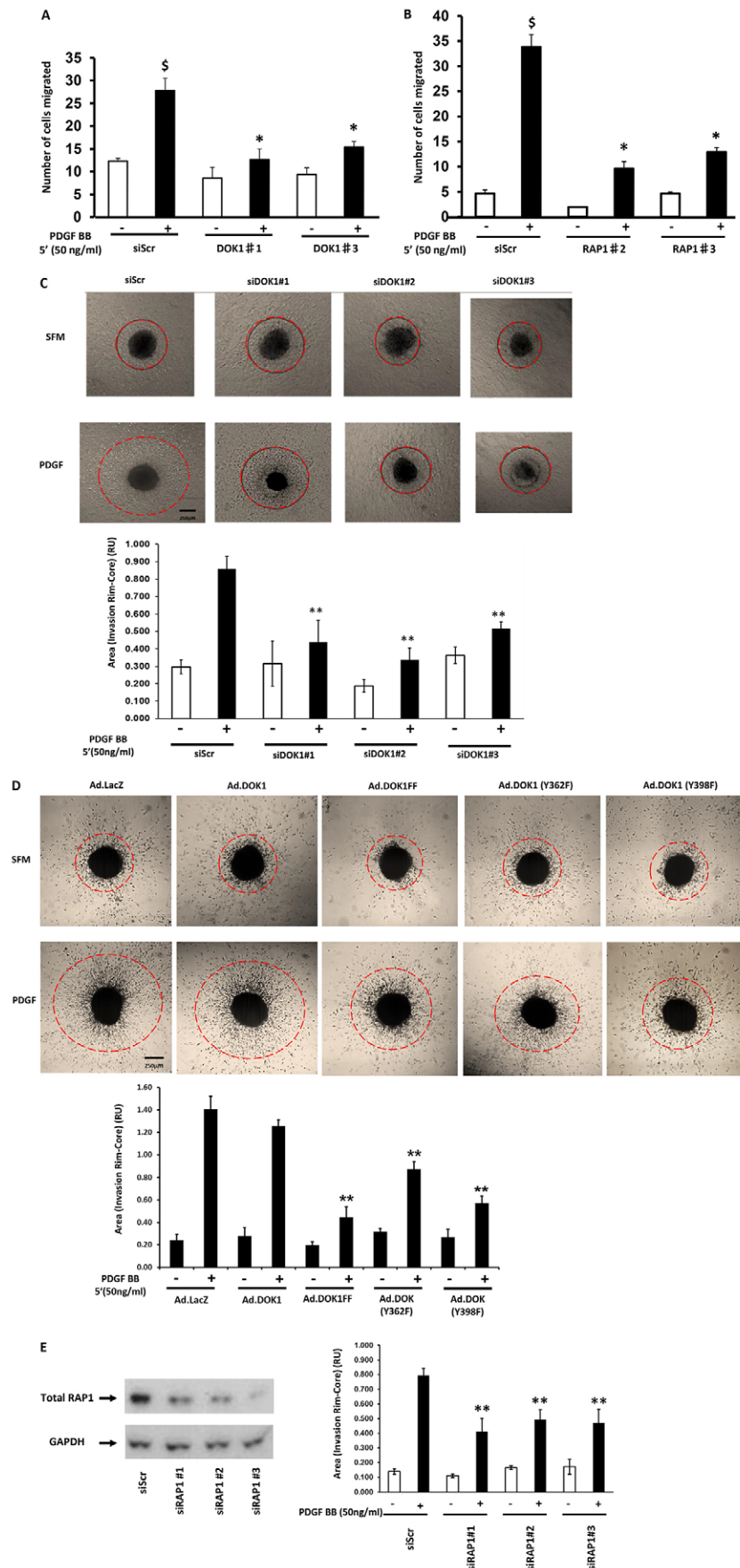


Fig. 7. DOK1 and Rap1 are required for PDGF-BB-stimulated chemotactic motility and 3D radial invasion of U87MG spheroids. (A) U87MG cells were transfected with three independent siRNAs targeting DOK1 (siDOK1, 25 nM) or with control scrambled siRNA (siScr, 25 nM). At 48 h post-transfection, cells were used in a transwell migration assay as detailed in Materials and Methods. Data are representative of three separate experiments and show the mean \pm s.e.m., expressed as the number of cells migrating per field. * $P < 0.01$ (compared with siScr); $^{\$}P < 0.05$ (compared with siScr SFM control). (B) U87MG cells were transfected with three independent siRNAs targeting Rap1 (siRap1, 25 nM) or with control scrambled siRNA (25 nM). At 48 h post-transfection, cells were used in a transwell migration assay as detailed in Materials and Methods. Data are presented as for A. * $P < 0.01$ (compared with siScr); $^{\$}P < 0.05$ (compared with siScr SFM control). (C) U87MG cells were transfected with three independent siRNAs targeting DOK1 (25 nM) or with control scrambled siRNA (25 nM). ** $P < 0.01$ (compared with siScr plus PDGF-BB). (D) U87MG cells (~80% confluent) were infected with Ad.LacZ, Ad.DOK1, Ad.DOK1FF, Ad.DOK1 Y362F or Ad.DOK1 Y398F at MOIs of 200. ** $P < 0.01$ compared with Ad.LacZ plus PDGF-BB. (E) U87MG cells were transfected with three independent siRNAs targeting Rap1 (25 nM) or with control scrambled siRNA (25 nM). Representative levels of Rap1 and GAPDH expression are shown (left-hand panel). ** $P < 0.01$ (compared with siScr plus PDGF-BB). (C–E) At 24 h post infection or transfection, cells were re-suspended and equal amounts of cells were used to generate spheroids as described in Materials and Methods. At 24 h after spheroid production, spheroids were imbedded in a collagen gel and incubated in SFM containing vehicle control (–) or 50 ng/ml PDGF-BB (+) for an additional 48 h. Spheroids were fixed in 4% paraformaldehyde and invasion was determined by measuring the area corresponding to the invasion rim (red dashed line) minus the area of the core for at least four different spheroids per condition. Data from at least three independent experiments are presented as relative area units (RU) and show the mean \pm s.e.m.

significantly decreased PDGF-BB-stimulated p130Cas tyrosine phosphorylation and 3D radial invasion, indicating that phosphorylation of either Tyr 362 or Tyr 398 is required for DOK1 function in PDGF-BB signalling. This is likely due to the requirement of specific DOK1-binding proteins to form a multi-protein signalling complex required for Rap1 activation and cell motility. Understanding the precise role of these important tyrosine residues will be the basis of future investigations.

This is the first study clearly establishing a role for DOK1 in the positive regulation of tumour cell motility. Our findings that DOK1 regulates PDGF-BB-mediated glioma cell motility through a novel signalling pathway involving p130Cas and Rap1 significantly advance our understanding of DOK1 signalling and function.

MATERIALS AND METHODS

Cell culture

U87 and U251 glioma cells were cultured in DMEM containing 10% fetal calf serum (FCS) supplemented with penicillin-streptomycin (1:100; P4333, Sigma).

Derivation of surgical biopsies

All patients gave informed consent before the surgical intervention. The storage of human tissue is governed by the Human Tissue Act (UK; HTA Licence numbers 12054 and 12198). The use of tissue and cells was approved by the National Hospital Ethics Committee (LREC 08/0077 and 02/N093). Neurosurgical biopsies were obtained at the operating theatre or in post-mortem and immediately transferred into the Department of Neuropathology, where they were dissected for tissue processing. Samples were homogenised in RIPA buffer with protease and phosphatase inhibitors (Roche) using sonication. The homogenates were then spun at 10,000 *g* for 10 min, and the protein concentration of the resulting supernatant was measured using the Bio-Rad protein assay kit.

Antibodies, reagents and siRNA

Antibodies against PYK2, phospho-PYK2 (Y402), phospho-p130Cas (Y410), ERK, phospho-ERK (T202/Y204), phospho-Src (Y416) and phospho-PDGFR- β (Y751) were from Cell Signaling Technology (Danvers, MA). Antibodies against phospho-DOK1 (Y398), DOK1 N-terminus (E-16), phospho-PDGFR- α (Y754), PDGFR- α (C-20), PDGFR- β , FAK (A-17), Rap1, GAPDH (V-18) and tubulin (TU-02) were from Santa Cruz Biotechnology (Heidelberg, Germany). Secondary antibodies against mouse, goat and rabbit IgGs were also from Santa Cruz Biotechnology. Antibody against the DOK1 C-terminus was from Abcam (Cambridge, UK), antibody against total DOK1 was from Abnova (Taipei City, Taiwan) and anti-p130Cas monoclonal antibody was from BD Transduction Laboratories (Oxford, UK). Antibody against phospho-DOK1 (Y362) was from ECM Biosciences (Versailles, KY) and antibody against phospho-FAK (Y397) was from Invitrogen (Paisley, UK). Alexa-Fluor-486-conjugated donkey anti-goat-IgG, Alexa-Fluor-546-conjugated donkey anti-rabbit-IgG and Alexa-Fluor-555-phalloidin were from Invitrogen (Paisley, UK). The Src inhibitor SU6656, the Src/Abl inhibitor 1-naphthyl PPI and the Fak/Pyk2 inhibitor PF573228 were all purchased from Tocris Bioscience (Bristol, UK). PDGF-BB was purchased from Peprotech (London, UK).

The following siRNAs were purchased from Applied Biosystems (Warrington, UK): siDOK1-1, 5'-GGGCCCTTATGATCTGCCT-3'; siDOK1-2, 5'-GGATGCATGGTGGTCCAA-3'. The following siRNAs were purchased from Qiagen (Crawley, UK): AllStars negative control; siDOK1-3, 5'-CCGCTGGACTGCAAAGTGAT-3'; siRap1A-1, 5'-AAAGTCAAAGATCAATGTAA-3'; siRap1A-2, 5'-AAGTCGATTGC-CAACAGTGTA-3'; siRap1A-3, 5'-CCCAACGATAGAAGATTCTTA-3'.

siRNA transfection

U87 and U251 glioma cells at 60% confluence were transfected by using Lipofectamine 2000 (Invitrogen), with a final siRNA concentration of 25 nM.

Adenoviral construction and infection

Adenoviruses expressing wild-type DOK1, DOK1FF (Y362F, Y398F), DOK1 Y362F and DOK1 Y398F were generated using the GATEWAY™ vector (pAd/CMV/V5-DEST; Invitrogen) and were verified by DNA sequencing. Prior to cloning into the pENTR3C vector, the indicated mutations in DOK1FF were generated using the QuikChange Multi-Site-Directed Mutagenesis Kit (Agilent Technologies, Cheshire, UK). The following primers were used for multi-site-directed mutagenesis, designed and used according to the manufacturer's instructions: Y362F, 5'-CCCAAAGAGGATCCCATCTTTGATGAACCTGAGGGC-CTG-3'; Y398F, 5'-CGGGTGAAGGAGGAGGGCTTTGAGCTCCCC-TACAACCT-3'. DOK1 adenoviral expression vectors (pAd/CMV/V5-DEST; Invitrogen) were generated by recombination, and adenovirus was produced by transfection into host HEK293A cells (Invitrogen). Viral particles were purified by caesium chloride centrifugation, and the virus titre was determined by immunoassay (QuickTiter Adenovirus Titer Immunoassay kit; Cell Biolabs, San Diego, CA). Adenovirus was stored at -20°C. U87MG cells were infected with adenovirus expressing either LacZ (Ad.LacZ), DOK1 (Ad.DOK1) or DOK1FF (Ad.DOK1FF) at a multiplicity of infection (MOI) of 200.

Immunoblotting

For immunoblotting, cells were lysed in a solution containing 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, complete protease inhibitor (Roche) and phosphatase inhibitors I and II (Sigma), and the resulting cell lysates were analysed by SDS-PAGE on 4–12% Bis-Tris gels (Nupage, Invitrogen), followed by electrotransfer onto polyvinylidene fluoride (PVDF) membranes (Invitrogen). Membranes were blocked with 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween-20 in phosphate-buffered saline (PBS-T) for 1 h at room temperature, before being probed with the primary antibody by overnight incubation at 4°C, followed by incubation for 1 h at room temperature with a horseradish peroxidase (HRP)-linked secondary antibody (Santa-Cruz Biotechnology) and detection using ECL reagents (GE Healthcare, Little Chalfont, UK), following the manufacturer's protocol. Immunoblots were quantified by scanning of films with a calibration strip and analysis by densitometry using ImageJ (National Institutes of Health).

Immunofluorescent staining and confocal imaging

For immunofluorescent staining, cells were fixed in 4% paraformaldehyde in PBS for 60 min followed by permeabilisation in 0.2% Triton X-100 for 30 min. Antibody incubations were performed overnight at 4°C in 1% bovine serum albumin (BSA), 0.1% Tween 20 in PBS. Confocal imaging was performed using a LEICA SPE2 upright microscope running LEICA-LAS software using sequential imaging capture. Co-localisation of DOK1 and phosphorylated p130Cas (Y410) was quantified using the ImageJ plugin JACoP (Bolte and Cordelières, 2006). Both the DOK1 and Y410 p130Cas channels were analysed, and the resultant Pearson's coefficient of colocalisation was determined.

Rap1 activation assay

Rap1-GTP levels were determined using a specific activation assay. U87MG cells were treated as indicated and harvested in activation buffer [50 mM Tris-HCl, 10% glycerol, 1% NP-40, 5 mM MgCl₂, 100 mM NaCl, 1 mM tris-(2-carboxyethyl)phosphine (TCEP), EDTA-free complete protease inhibitor and phosphatase inhibitors]. The GST-Rap1 binding domain of RalGDS was coupled to glutathione-Sepharose beads (GE Healthcare Life Sciences, Buckinghamshire, UK). Lysates were incubated with the bead-bound probe to precipitate GTP-bound Rap1. Rap1-GTP was released from the beads upon the addition of 2× SDS buffer. Western blotting was carried out on pulldown samples and normalised to total Rap1 levels as determined by western blot analysis of the whole-cell lysate.

Transwell chemotactic migration assay

This assay was performed as described previously (Evans et al., 2011). Briefly, transwell cell culture inserts (Falcon; BD Biosciences, Oxford, UK) were inserted into a 24-well plate. Serum-free medium

supplemented with or without PDGF-BB or vehicle was added to the bottom chamber, and U87 or U251 glioma cells in suspension (1.5×10^5 cells/well in serum-free DMEM) were added to the top chamber and incubated at 37°C for 6 h. Cells that had not migrated or had only adhered to the upper side of the membrane were removed before membranes were fixed and stained with a Reastain Quik-Diff kit (IBG Immucor, West Sussex, UK). Cells that had migrated to the lower side of the membrane were counted in four random fields per well at 20× magnification using an eyepiece indexed graticule.

3D spheroid invasion assay

Spheroids were generated using the metho-cellulose technique as described previously (Augustin, 2004). Virus infection and siRNA transfection were carried out on U87 cells as described previously (Evans et al., 2011). Following the infection and transfection period, cells were trypsinised and 3×10^5 cells/ml were suspended in a 4:1 (v/v) mixture of 10% FCS in DMEM and methylcellulose. Spheroids were produced by pipetting 100 µl of the cell suspension into a well of a 96-well round-bottomed non-tissue-culture plate and incubating for 24 h (37°C, 5% CO₂). Spheroids were collected and embedded in collagen I plugs (2.1 mg/ml) prepared from fibrillar bovine collagen I (3.1 mg/ml; PureCol) by dilution in DMEM, in accordance with the manufacturer's protocol (Nutacon, The Netherlands). The collagen I solution was supplemented with either dH₂O (–) or 50 ng/ml PDGF-BB (+). Plugs were overlaid with SFM (–) or SFM plus 50 ng/ml PDGF-BB (+). Spheroids were allowed to invade for 48 h, followed by fixation in 4% formaldehyde. Spheroid invasion was determined by measuring the circular area of the spheroid core and the rim of invasion using ImageJ. The rim of invasion was determined as the circular distance from the edge of the core to the edge of contiguous invading cells (Stein et al., 2007).

ELISA assay for PDGFR activity

An immobilised capture antibody specific for human PDGFR recognises both tyrosine phosphorylated and nonphosphorylated PDGFR. After washing away unbound material, an HRP-conjugated detection antibody specific for phosphorylated tyrosine was used to detect only the tyrosine-phosphorylated receptor, using HRP. The capture antibody was diluted to a working concentration of 4 µg/ml in PBS without carrier protein, and 100 µl was immediately added to a 96-well microplate for overnight incubation at room temperature. The next day, each well was washed four times with washing buffer (0.05% Tween 20 in PBS, pH 7.2) and blocked for 2 h with 300 µl of PBS containing 1% BSA. Wells were washed again three times before adding 100 µl of lysate prepared in the following diluent: 1% Nonidet P40, 20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA and 1 mM activated Na₃VO₄. The same diluent without protein was used as a blank. The plate was left to incubate for 2 h at room temperature and washed again. Then, 100 µl of detection antibody diluted to the manufacturer's recommendations in 20 mM Tris-HCl pH 7.2, 137 mM NaCl, 0.05% Tween 20 and 0.1% BSA was added directly to the well, before incubation for a further 2 h. Finally, after three further washes, 100 µl of substrate solution was added to each well for 20 min, followed by incubation with 50 µl of stop solution (1M H₂SO₄). The absorbance of each well was determined immediately, using a microplate reader set to 450 nm with a wavelength correction of 595 nm. Total PDGFR levels and equal loading was assessed by western blotting.

Statistical analysis

The data displayed on the graphs are means, with error bars representing the standard error of the mean (s.e.m.). Statistical analysis was performed by two-way analysis of variance (ANOVA) or *t*-test, as appropriate. *P* < 0.05 was considered significant.

Competing interests

The authors declare no competing interests.

Author contributions

A.B., I.M.E. and P.F. designed the study, performed the experiments, analysed the data and wrote the paper. A.F., G.B., C.P.M. and M.Y. performed the experiments.

A.F. and G.B. contributed equally. V.M., R.B., N.L. and S.B. generated essential reagents. I.C.Z. designed the study, analysed the data and wrote the paper.

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.135988/-DC1>

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