β 1 integrins regulate fibroblast chemotaxis through control of N-WASP stability

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Chemotactic migration of fibroblasts towards growth factors, such as during development and wound healing, requires precise spatial coordination of receptor signalling. However, the mechanisms regulating this remain poorly understood. Here, we demonstrate that β 1 integrins are required both for fibroblast chemotaxis towards platelet-derived growth factor (PDGF) and growth factor-induced dorsal ruffling. Mechanistically, we show that β 1 integrin stabilises and spatially regulates the actin nucleating endocytic protein neuronal Wiskott–Aldrich syndrome protein (N-WASP) to facilitate PDGF receptor traffic and directed motility. Furthermore, we show that in intact cells, PDGF binding leads to rapid activation of β 1 integrin within newly assembled actin-rich membrane ruffles. Active β 1 in turn controls assembly of N-WASP complexes with both Cdc42 and WASP-interacting protein (WIP), the latter of which acts to stabilise the N-WASP. Both of these protein complexes are required for PDGF internalisation and fibroblast chemotaxis downstream of β 1 integrins. This represents a novel mechanism by which integrins cooperate with growth factor receptors to promote localised signalling and directed cell motility.

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Introduction

Chemotaxis is the process whereby cells undergo directed and persistent migration towards an external gradient of diffusible chemoattractant such as growth factors (Insall, 2010). Integrins are the main family of transmembrane receptors that mediate binding to extracellular matrix (ECM) proteins and are known to cooperate with a number of growth factor receptors to promote optimal downstream signalling in a wide range of contexts (Streuli and Akhtar, 2009; Schwartz, 2010). In adherent cells, both β 1 and β 3

integrins have been shown to act in synergy with, among others, the tyrosine kinase epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptors (PDGFRs), respectively, to promote efficient cell adhesion, spreading, or migration in response to ligand (Woodard *et al*, 1998; Roberts *et al*, 2001; Sturge *et al*, 2002). This has been proposed to occur through both direct and indirect associations between receptor types downstream of either ECM or growth factor binding. Early studies suggested that endocytic recycling of integrins from the rear to the front of cells was required for efficient directed migration towards extracellular stimuli (Bretscher, 1989). Subsequent experiments have revealed that integrins can undergo rapid recycling within new protrusions at the front of migrating cells under the control of Rab GTPases leading to persistent migration (Caswell *et al*, 2007; White *et al*, 2007).

Growth factor receptor internalisation is known to occur upon ligand binding (Lemmon and Schlessinger, 2010). In the case of EGFR and PDGFR, binding to respective ligands induces rapid autophosphorylation of each receptor resulting in signal transduction, receptor internalisation, and either degradation or recycling. The balance between the latter two appears to be dictated in part by the concentration and exposure time to the growth factor, cell type, and adhesion conditions. Furthermore, locally regulated integrin traffic has recently been shown to mediate increased levels of EGFR at the leading edge of tumour cells (Caswell *et al*, 2008). However, the spatial localisation of these cooperating receptor families in cells undergoing chemotaxis and the signalling mechanisms mediating this crosstalk remain poorly understood.

This suggests that integrins may directly influence growth factor signalling at the receptor level as well as through synergistic signalling pathways.

Here, we show that fibroblasts require β 1 integrin to maintain the stability of the endocytic actin nucleating protein N-WASP and that this in turn is required for efficient PDGF uptake and cell chemotaxis. We demonstrate that N-WASP is locally bound to WASP-interacting protein (WIP) at the leading edge of polarised chemotaxing cells in a β 1-dependent manner. Mechanistically, we show that PDGF directly mobilises and activates β 1 integrin, which in turn is required for local activation of Cdc42 and N-WASP to permit efficient chemotaxis. This data provide a novel spatially regulated relatively direct signalling link between integrin and growth factor receptors that promotes directed fibroblast migration.

Results and discussion

β 1 integrin is required for fibroblast chemotaxis towards PDGF

β 1 integrin has previously been shown to be required for normal migration in a wide range of cell types both

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in vitro and *in vivo*. Furthermore, mice lacking $\beta 1$ integrin specifically in keratinocytes or endothelial cells show defects in polarised, directed migration *in vivo* (Grose *et al*, 2002; Raghavan *et al*, 2003; Zovein *et al*, 2010). However, the mechanism by which this integrin contributes to cellular responses to chemotactic growth factors remains unclear. To address this question, we used fibroblasts (we have previously generated) isolated from $\beta 1$ floxed mice to generate $\beta 1^{+/+}$ (not cre treated), $\beta 1$ null fibroblasts ($\beta 1^{-/-}$ cre recombinase treated) or $\beta 1^{-/-}$ rescued with human $\beta 1$ tagged with GFP ($\beta 1$ -GFP) (Parsons *et al*, 2008). $\beta 1^{-/-}$ fibroblasts were able to adhere to fibronectin and form focal adhesions in a manner similar to $\beta 1^{+/+}$ cells, although spread cell area was smaller in the cells lacking $\beta 1$ integrin (Figure 1A; Supplementary Figure S1A). To determine whether $\beta 1$ was required for normal motility, $\beta 1^{+/+}$, $\beta 1^{-/-}$, and $\beta 1$ -GFP cells were imaged by phase contrast time-lapse microscopy over 16 h. Surprisingly, analysis of cell tracks over time demonstrated that $\beta 1^{-/-}$ cells exhibited significantly faster random migration speeds compared with $\beta 1^{+/+}$ cells but showed no change in directional persistence (Figure 1B; Supplementary Figure S1B). Moreover, time-lapse analysis of the same cells within Dunn chemotaxis chambers revealed that $\beta 1^{-/-}$ cells failed to migrate towards the PDGF gradient (Figure 1C) in agreement with findings from a previous study in $\beta 1$ -deficient embryonic stem cells (Sakai *et al*, 1998). Importantly, these phenotypes were restored in $\beta 1^{-/-}$ cells rescued with $\beta 1$ -GFP (Figure 1B and C; Supplementary Figure S1A). In contrast to $\beta 1^{+/+}$ cells (mean speed $1.2 \mu\text{m}/\text{min}$, Figure 1B), comparison of unstimulated $\beta 1^{-/-}$ cells with those exposed to either a gradient or a global stimulation with PDGF demonstrated no difference in migration speeds between conditions (data not shown). This suggests that $\beta 1$ integrin is required for normal fibroblast migratory responses in part by suppressing the rate of motility, coupled with cooperative signalling downstream of PDGF stimulation.

$\beta 1^{-/-}$ fibroblasts show defective PDGF uptake

To determine whether loss of response to PDGF was due to changes in levels of PDGFR in $\beta 1^{-/-}$ cells, we analysed the amount and localisation of this receptor in both cell types. Data showed no difference in total protein levels or localisation of PDGFR between $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cells (Figure 2A and C). Furthermore, there was no significant co-localisation observed between $\beta 1$ integrin (or other focal adhesion markers) and PDGFR, suggesting that these receptors do not reside in significantly overlapping compartments under normal growth conditions (Figure 2B). There was also no difference in cell surface levels of PDGFR, as measured by FACS, between $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cells (Figure 2C). PDGFR is known to undergo degradation upon acute treatment with PDGF and we therefore speculated that $\beta 1$ integrin could instead have a role in regulating PDGF signalling at this level. Analysis of total levels of PDGFR over a time course of treatment with PDGF revealed that, in contrast to $\beta 1^{+/+}$, $\beta 1^{-/-}$ cells failed to degrade the PDGFR following 6 h of exposure to ligand despite similar levels of receptor at time 0 (Figure 2D). Moreover, short-term treatment with PDGF led to enhanced tyrosine phosphorylation of PDGFR in $\beta 1^{-/-}$ compared with wild-type cells (Figure 2E and F) and basal levels of phosphorylated PDGFR were increased in $\beta 1^{-/-}$ compared with wild-type cells (Figure 2E) hinting at defective internalisation of PDGFR in $\beta 1^{-/-}$ cells. This suggests that $\beta 1$ integrin promotes efficient PDGFR signalling and internalisation, the latter of which has recently been shown to be essential for directed cell migration (Kawada *et al*, 2009). To determine whether the altered PDGFR signalling in $\beta 1^{-/-}$ cells also translated into defective ligand uptake, we analysed internalisation of biotin-labelled PDGF in $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cells over time. Data from ELISAs showed a significant reduction in the internalisation of PDGF, but not transferrin receptor, in $\beta 1^{-/-}$ versus $\beta 1^{+/+}$ cells (Figure 2G; Supplementary Figure S2A) suggesting that the increased PDGFR phosphorylation observed in cells lacking

Figure 1 $\beta 1$ is required for fibroblast chemotaxis towards PDGF. (A) Example confocal images of $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cells stained for vinculin. Scale bars are $10 \mu\text{m}$. (B) Random migration speed of $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cells plated in growth media. Error bars are $\pm$ s.e.m. throughout. $N =$ at least 100 cells for each condition. * $P < 0.05$ (C) Cells assayed for chemotaxis towards PDGF in Dunn chambers. Circular rose plots show the proportion of cells with migratory direction lying within each 20° interval (PDGF source at top of histogram). The arrow represents the mean direction of migration; the grey segment represents the 95% confidence interval determined by the Rayleigh's test. $P < 0.01$ between $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cells.

Figure 2 $\beta 1$ regulates uptake of PDGF. (A) Western blots of $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cell lysates probed for PDGFR or GAPDH (control). (B) FACS analysis of $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cells probed for cell surface levels of PDGFR. (C) Example confocal images of $\beta 1^{-/-}$ cells stained for PDGFR-Alexa 568 and phosphotyrosine (PY)-Alexa633. Merged images are shown. Scale bars are 10 μ m. (D) Western blots of lysates from $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cells treated with PDGF for indicated times and probed for PDGFR and GAPDH. Graph shows quantification of levels from four experiments $\pm$ s.e.m. (E) Western blots of lysates from $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cells treated with PDGF for indicated times and probed for PY or PDGFR. (F) Immunoprecipitation of PDGFR from lysates of $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cells with or without 5 min PDGF. Blots probed for PY or PDGFR. (G) Biotinylated PDGF uptake over time in lysates from $\beta 1^{+/+}$ and $\beta 1^{-/-}$ cells measured by ELISA. Error bars are $\pm$ s.e.m. throughout. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

$\beta 1$ integrin may have functional consequences specifically for efficient signalling from and uptake of the growth factor.

Loss of $\beta 1$ integrin results in N-WASP degradation

Internalisation and trafficking of PDGFR has previously been shown to be dependent upon a number of different endocytic and actin-binding proteins including the Rab and dynamin GTPases, Arp2/3, cortactin, WAVE complex proteins, and N-WASP (Boyle *et al*, 2007; Kawada *et al*, 2009; Abella *et al*, 2010b). To determine whether $\beta 1$ integrin may regulate PDGFR function through control of these molecules, we analysed levels of a number of these proteins in $\beta 1^{+/+}$

and $\beta 1^{-/-}$ cells (Supplementary Figure S2B–D). Of the proteins analysed, the only striking difference was in the levels of N-WASP, which was significantly reduced in the $\beta 1^{-/-}$ cells compared with controls (Figure 3A). We saw a similar decrease in N-WASP levels in NIH3T3 fibroblasts treated with $\beta 1$ integrin siRNA suggesting the effects seen in $\beta 1^{-/-}$ cells may be a general one for fibroblasts and not due to other compensatory mechanisms in these cells (Supplementary Figure S2E). Previous studies in tumour cells have demonstrated that $\beta 1$ and $\beta 3$ integrins can cooperate through control of endocytic proteins to promote recycling of growth factors receptors, and thus cell invasion

Figure 3 $\beta 1$ regulates N-WASP stability. (A) Western blots of $\beta 1/\beta 3 +/+$ and $\beta 1/\beta 3 -/-$ cell lysates probed for N-WASP or GAPDH. Numbers are densitometry values normalised to $+/+$ cells. (B) Immunoprecipitation of N-WASP (or control IgG) from lysates of $\beta 1 +/+$ and $\beta 1 -/-$ cells probed for ubiquitin or N-WASP. (C) Western blots of lysates from $\beta 1 +/+$ and $\beta 1 -/-$ cells $\pm$ MG132 (20 μ M). Blots probed for N-WASP or GAPDH. Numbers are densitometry values normalised to $+/+$ cells. (D) Immunoprecipitation of N-WASP (or control IgG) from lysates of $\beta 1 +/+$ and $\beta 1 -/-$ cells $\pm$ PP2 (10 μ M) or DMSO (vehicle control) probed for phosphotyrosine (PY) or N-WASP. (E, F) Immunoprecipitation and western blots for specified proteins from lysates of $\beta 1 +/+$ and $\beta 1 -/-$ cells. (G) Confocal images of $\beta 1$ -GFP cells starved or treated with PDGF for 5 min and stained for N-WASP (red). Insets shown are highlighted by white boxes. (H) Immunoprecipitation of PDGFR (or control IgG) from lysates of untreated or wiskostatin treated $\beta 1 +/+$ cells probed for PY.

(Caswell *et al*, 2008, 2009). Interestingly, however, analysis of lysates from $\beta 3$ null fibroblasts showed no changes in N-WASP levels, suggesting that $\beta 1$ integrins may have a specific role in regulating N-WASP stability (Figure 3A). Furthermore, as total levels of $\beta 3$ integrin subunit are not altered between $\beta 1 +/+$ and $\beta 1 -/-$ cells (Supplementary Figure S2B) it is unlikely that the defects in these cells are simply due to over compensation by $\beta 3$ integrins. However, given the known transdominant crosstalk between these integrins (Calderwood *et al*, 2004) we cannot rule out that $\beta 1 -/-$ cells may exhibit subtle changes in activation status of $\beta 3$ integrins that may in turn contribute to cellular responses to growth factors.

N-WASP has previously been shown in neurons to undergo ubiquitin-dependent degradation following phosphorylation at tyr253 (Y253 in mice and Y256 in humans; Suetsugu *et al*, 2002). To investigate whether this was occurring in $\beta 1 -/-$ cells, we immunoprecipitated endogenous N-WASP from both $\beta 1 +/+$ and $\beta 1 -/-$ cells and probed for the presence of ubiquitin and phosphotyrosine. Data revealed that N-WASP in $\beta 1 -/-$ cells were highly ubiquitinated and phosphorylated compared with $\beta 1 +/+$ controls and this phosphorylation was inhibited in cells treated with the Src family kinase (SFK) inhibitor PP2 (Figure 3B and D). This is in agreement with other reports showing that both SFK and Abl/Arg kinases can phosphorylate N-WASP (Torres and Rosen,

2006; Miller *et al*, 2010). Ubiquitin-reactive N-WASP was detected at a molecular weight only slightly higher than that seen in straight western blots, as opposed to a smear, suggesting that addition of a small number of ubiquitin molecules to this protein is sufficient to target for degradation. The SFK-dependent increase in N-WASP phosphorylation was not due to enhanced Src activation in $\beta 1 -/-$ cells as western blot and immunofluorescence analysis of active Src species showed no clear difference between control and $\beta 1 -/-$ cells (Supplementary Figure S3A and B). Furthermore, treatment of $\beta 1 -/-$ cells with an inhibitor of proteasome function (MG132) rescued levels of N-WASP back to those seen in $\beta 1 +/+$ cells (Figure 3C) and increased ubiquitination of N-WASP in both cell lines (Supplementary Figure S2F), suggesting that $\beta 1$ integrin is required for protecting N-WASP from degradation. Treatment with PP2 resulted in increased levels of N-WASP in $\beta 1 -/-$ cells, but did not fully restore levels back to those seen in $\beta 1 +/+$ cells (Supplementary Figure S3C). As PP2 is known to also inhibit other kinases, including Abl/Arg and FAK, it is possible that these may also contribute towards integrin-dependent regulation of N-WASP through alternative feedback pathways thus making this data more difficult to interpret. Of note, basal N-WASP phosphorylation and ubiquitination were only seen at very low levels in $\beta 1 +/+$ cells, suggesting that a small proportion of N-WASP is degraded in normal adherent fibroblasts.

To determine whether $\beta 1$ regulates N-WASP and PDGFR through formation of a tripartite complex, we immunoprecipitated $\beta 1$, N-WASP, or PDGFR from $\beta 1 +/+$ or $\beta 1 -/-$ cells and probed for each respective binding partner. IP's of each protein from $\beta 1 +/+$ cells demonstrated that N-WASP was in a complex with $\beta 1$ and PDGFR, but that these receptors were not in a complex with one another (Figure 3E). This is in agreement with a previous report showing N-WASP and $\beta 1$ can also form a complex in human carcinoma cells (Sturge *et al*, 2002). However, fluorescence lifetime imaging microscopy (FLIM) analysis of fluorescence resonance energy transfer (FRET) between $\beta 1$ -GFP and N-WASP-mCherry demonstrated no direct association between these proteins in fibroblasts (as compared with $\beta 1$ -talin internal positive control as we have previously shown (Parsons *et al*, 2008; Worth *et al*, 2010)), suggesting additional adaptors act to link these molecules in a complex (Supplementary Figure S4, top panels). Basal levels of N-WASP in complex with PDGFR were lower in $\beta 1 -/-$ cells compared with controls, presumably as a result of decreased levels of N-WASP in these cells. Analysis of PDGF stimulated demonstrated higher levels of N-WASP in complex with $\beta 1$ coupled with increased co-localisation at the plasma membrane compared with serum-starved cells (Figure 3F and G). Finally, treatment of $\beta 1 +/+$ cells with the N-WASP inhibitor wiskostatin (Peterson *et al*, 2004) resulted in enhanced phosphorylated PDGFR similar to that seen in $\beta 1 -/-$ cells (Figure 2F) further

implying a role for functional N-WASP in regulating PDGFR signalling (Figure 3H). These data combined suggest that $\beta 1$ integrin is required for protecting N-WASP from Src-induced phosphorylation and subsequently protein degradation and that formation of a dynamic complex between $\beta 1$, N-WASP, and PDGFR may be required for efficient cellular responses to PDGF.

$\beta 1$ is required for dorsal ruffle formation

To determine whether $\beta 1$, N-WASP, and PDGFR were redistributed upon PDGF stimulus we analysed the localisation of all three proteins in dorsal ruffles. Dorsal ruffles are actin-rich structures formed in a number of different cell types in response to growth factor stimulus and have been proposed as important sites of growth factor receptor endocytosis and signalling (Orth and McNiven, 2006; Orth *et al*, 2006; Abella *et al*, 2010a). N-WASP has previously been shown to have a role in regulating PDGF-induced dorsal ruffles in fibroblasts (Legg *et al*, 2007), but the role of integrin signalling in this process remains unknown. Confocal analysis of endogenous $\beta 1$, PDGFR, and N-WASP localisation demonstrated that all three proteins were recruited to dorsal ruffles in $\beta 1 +/+$ cells (Figure 4A). Quantification of dorsal ruffles in $\beta 1 +/+$ cells or those treated with wiskostatin confirmed a clear requirement for N-WASP in the formation of PDGF-induced ruffles (Figure 4B). Remarkably, despite having normal levels of PDGFR and showing activation of this receptor upon PDGF

Figure 4 $\beta 1$ integrins are required for dorsal ruffling and PDGF uptake. (A) Example images of dorsal ruffles in $\beta 1$ -GFP cells stained for N-WASP or PDGFR (red) and actin (blue). (B) Quantification of dorsal ruffle formation following 5 min PDGF (30 ng/ml, % cells) in $\beta 1 +/+$ cells $\pm$ wiskostatin (1 μ M for 5 min) or $\beta 1 -/-$. (C) Images from movies of $\beta 1 +/+$ ($\pm$ N-WASP siRNA) or $-/-$ cells expressing GFP-dynamin plated in imaging chambers. Cells were incubated with PDGF-Alexa568 for 5 min, washed and left at 37°C for 30 min to permit PDGF uptake. Cells were then imaged by confocal microscopy with one frame acquired every 2 s. Merged images of GFP and Alexa568 channels are shown from specified time intervals with insets showing PDGF-568 channel only. See also Supplementary movies 1, 2, and 3. * $P < 0.001$.

treatment, β 1 $^{-/-}$ cells failed to assemble dorsal ruffles in response to PDGF (Figure 4B) further suggesting a role for β 1 in regulating localised PDGFR signalling, possibly through N-WASP.

To further define the role of β 1 and N-WASP in regulating PDGF uptake, we performed confocal time-lapse imaging of live β 1 $+/+$ cells, those treated with control or N-WASP siRNA and β 1 $^{-/-}$ cells transfected with dynamin-GFP. These cells were then incubated with PDGF directly labelled with Alexa568 for 30 min to permit visualisation of uptake of the growth factor. Time-lapse movies demonstrated that PDGF-568 was efficiently taken up into GFP-dynamin-positive vesicles with very little labelled PDGF remaining at the cell surface after this time, indicating that the majority of uptake occurs within this time frame in normal fibroblasts (Figure 4C, top panels; Supplementary movie 1). Conversely, β 1 $^{-/-}$ cells treated for the same time period retained high levels of the PDGF-568 at the plasma membrane, and relatively low or diffuse PDGF within trafficking dynamin-positive vesicles (Figure 4C, middle panels; Supplementary movie 2). Furthermore, β 1 $+/+$ cells treated with N-WASP siRNA show a very similar phenotype to that seen in β 1 $^{-/-}$ cells (Figure 4C, bottom panels; Supplementary movie 3). These data further demonstrate that both β 1 and N-WASP are required for efficient uptake of PDGF in fibroblasts. Interestingly, although integrins are classically considered to be active only at sites of ECM contact, a recent study has shown that active β 1 is mobilised rapidly in ruffling membrane structures coincident with F-actin polymerisation (Galbraith *et al*, 2007). While the function of active β 1 integrins at these sites is currently unclear, it is suggestive of non-ECM engaged active integrins having a role within highly dynamic sites of membrane remodelling such as is seen during endocytosis.

WIP overexpression rescues β 1 $^{-/-}$ cell chemotaxis defects

WIP is an actin-binding protein that has previously been shown to bind to and potentially stabilise N-WASP, thus influencing downstream actin assembly and membrane curvature (Vetterkind *et al*, 2002; Takano *et al*, 2008). To determine whether WIP could compensate for β 1 in regulating N-WASP stability in fibroblasts, we generated β 1 $^{-/-}$ cells stably overexpressing WIP-mCherry. Western blotting of whole cell lysates demonstrated that overexpression of WIP was sufficient to restore N-WASP levels and localisation to the membrane in β 1 $^{-/-}$ cells (Figure 5A and B). Furthermore, WIP overexpression in β 1 $^{-/-}$ cells partially rescued the defect in ligand-induced PDGF receptor degradation (Figure 5C) and PDGF uptake (Figure 5D), suggesting that WIP-dependent stabilisation of N-WASP is required for efficient PDGF receptor signalling. WIP can also bind to cortactin and the adaptor protein Nck1 and has been shown to have an additional role in fibroblast adhesion and spreading through regulation of actin assembly, as well as in the formation of dorsal ruffles (Ramesh and Geha, 2009; Cortesio *et al*, 2010). However, WIP-expressing β 1 $^{-/-}$ cells treated with wiskostatin were unable to efficiently internalise PDGF, indicating that the partial rescue of growth factor uptake in these cells is due to WIP binding to N-WASP rather than N-WASP-independent effects (Figure 5D). Surprisingly, WIP overexpression was also sufficient to restore the ability of β 1 $^{-/-}$

cells to undergo chemotaxis towards PDGF, although not to rescue the increased migration speed seen in these cells (Figure 5E). This suggests that β 1 integrin regulates chemotaxis through N-WASP rather than through specific and direct adhesion-dependent mechanisms, and that this is uncoupled from β 1 regulation of cell migration speed. WIP overexpression also partially rescued the ability of β 1 $^{-/-}$ cells to form dorsal ruffles in response to PDGF (12% versus 24% in β 1 $+/+$ cells; Figure 5F, refer to Figure 4B). As was seen in PDGF uptake experiments, this rescue was dependent on WIP binding to N-WASP, as expression of WIP-deltaWBD N-WASP binding mutant in β 1 $^{-/-}$ cells did not rescue dorsal ruffle formation (Figure 5F). Furthermore, overexpression of two other N-WASP binding proteins implicated in PDGF receptor traffic, Nck or cortactin, was not able to rescue dorsal ruffle formation in β 1 $^{-/-}$ cells. Interestingly, transient overexpression of N-WASP Y253E or Y253F constructs (phospho-mimic or phospho-dead, respectively) in β 1 $^{-/-}$ cells also failed to rescue dorsal ruffle formation, suggesting that a balance and possibly cycle of N-WASP phosphorylation is required to permit efficient PDGFR signalling. Of note, the recent study by Legg *et al* (2007) showed that N-WASP $^{-/-}$ cells have both reduced numbers and smaller 'collapsed' forms of dorsal ruffles, suggesting that loss of N-WASP alone does not abolish dorsal ruffle formation. As the authors point out in this paper, this may be due to contribution of other factors (such as Src, dynamic, or cortactin) in these knockout cells, possibly as a result of transformation or more directly due to compensatory mechanisms from these molecules. Importantly, the data presented in our study suggest that restoring levels of N-WASP in β 1 $^{-/-}$ cells (by overexpressing WIP) can only partially rescue dorsal ruffle formation, which suggests that β 1 integrin can contribute to the assembly of these structures in an N-WASP-independent manner. This is in agreement with the model suggested in Legg *et al*, that manipulation of N-WASP alone is not sufficient to regulate dorsal ruffle formation.

To directly define the role of WIP in stabilising N-WASP in fibroblasts, we treated β 1 $+/+$ cells with siRNA to knockdown endogenous WIP. Western blot analysis of lysates from WIP-depleted cells demonstrated a significant reduction in N-WASP levels confirming WIP-dependent stabilisation of this protein (Figure 6A). Further, functional analysis demonstrated reduced PDGF uptake and defective chemotaxis in WIP knockdown cells (Figure 6B and C). These data combined with that shown in Figure 5 strongly suggest that WIP is required for N-WASP stability and subsequent N-WASP-dependent regulation of fibroblast responses to PDGF.

Active β 1 integrin promotes polarised N-WASP-WIP complex formation during chemotaxis

Previous studies have shown that, in some contexts, growth factors can activate integrins either directly or through binding to canonical receptors (Streuli and Akhtar, 2009). To define whether PDGF might act to trigger β 1 activation at the membrane, we treated serum-starved β 1 $+/+$ cells with PDGF alone or in combination with the PDGF receptor inhibitor AG1296 and stained using a conformation-sensitive antibody to recognise active β 1 integrins. Quantification of antibody intensity from a number of images demonstrated a significant increase in active β 1

Figure 5 WIP rescues chemotaxis and dorsal ruffling in $\beta 1^{-/-}$ cells. (A) Western blots of $\beta 1^{+/+}$, $\beta 1^{-/-}$, and $\beta 1^{-/-}$ + WIP cell lysates probed for N-WASP or GAPDH. Numbers are densitometry values normalised to loading and $\beta 1^{+/+}$ cells. (B) Example images of $\beta 1^{+/+}$, $\beta 1^{-/-}$, and $\beta 1^{-/-}$ + WIP cells treated with PDGF (30 ng/ml, 5 min) and stained for endogenous N-WASP. (C) Western blots of lysates from $\beta 1^{-/-}$ and $\beta 1^{-/-}$ + WIP cells treated with PDGF for indicated times and probed for PDGFR and GAPDH. Graph shows quantification of levels from four experiments. (D) Biotinylated PDGF uptake over time in lysates from $\beta 1^{-/-}$ and $\beta 1^{-/-}$ + WIP cells measured by ELISA. (E) Cells assayed for chemotaxis towards PDGF in Dunn chambers. Circular rose plots show the proportion of cells with migratory direction lying within each 20° interval (PDGF source at top of histogram). The arrow represents the mean direction of migration; the grey segment represents the 95% confidence interval determined by the Rayleigh's test. $P < 0.05$ between $\beta 1^{-/-}$ and $\beta 1^{-/-}$ + WIP cells. Graph shows migration speed of $\beta 1^{-/-}$ or $\beta 1^{-/-}$ + WIP cells treated with PDGF. (F) Quantification of dorsal ruffle formation following 5 min PDGF (30 ng/ml, % cells) in $\beta 1^{-/-}$ cells expressing specified proteins. Error bars are $\pm$ s.e.m. throughout. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

in large ruffles of cells treated with PDGF, but not with PDGF plus AG1296 (Figure 7A). Staining for total $\beta 1$ in parallel samples showed no difference between treatments

(not shown). This data demonstrate that PDGF can act to mobilise active $\beta 1$ indirectly via signalling through PDGFR.

Figure 6 WIP is required for PDGF uptake and chemotaxis. (A) Western blots of lysates from $\beta 1 + / +$ cells treated with control siRNA (conSi) or two individual siRNA to target WIP, probed with specified antibodies. Numbers are densitometry values normalised to $\beta 1 + / +$ cells. (B) Biotinylated PDGF uptake over time measured by ELISA in lysates from $\beta 1 + / +$ cells transfected with con or WIP siRNA. Error bars are $\pm$ s.e.m. (C) Cells assayed for chemotaxis towards PDGF in Dunn chambers. Circular rose plots show the proportion of cells with migratory direction lying within each 20° interval (PDGF source at top of histogram). The arrow represents the mean direction of migration; the grey segment represents the 95% confidence interval determined by the Rayleigh's test. $P < 0.05$ between control and WIP siRNA transfected cells.

We next sought to determine whether the interaction between N-WASP and WIP was dependent on PDGF and mobilisation of active $\beta 1$ integrin. To this end, we analysed FRET between GFP-N-WASP and WIP-mCherry in $\beta 1 + / +$ cells treated with PDGF in the presence or absence of a function-blocking antibody against $\beta 1$ integrin or an IgG control. Analysis demonstrated a basal, predominantly cytoplasmic interaction between N-WASP and WIP. PDGF induced a significant increase in N-WASP-WIP binding at the membrane, but not in cells where $\beta 1$ integrin function was blocked (Figure 7B), suggesting active $\beta 1$ is required for PDGF-induced association of these molecules at the plasma membrane. Moreover, analysis of FRET between N-WASP and WIP in live cells undergoing chemotaxis towards PDGF in a Dunn chamber demonstrated a highly localised, dynamic interaction between these proteins within new protrusions formed towards the PDGF gradient (Figure 7C). Furthermore, confocal analysis of parallel fixed samples stained with anti-active $\beta 1$ showed a higher correlation between active integrin and N-WASP-WIP at the leading edge of polarised cells compared with the back (Figure 7D). This data combined suggest that a gradient of PDGF can activate $\beta 1$ integrin and this in turn promotes local assembly of N-WASP-WIP complex. This complex is then required for N-WASP stability and subsequent regulation of PDGF receptor endocytosis and ligand uptake. This novel, tightly spatially regulated cooperative signalling link between PDGFR and $\beta 1$ integrin is likely to be important in other cell types undergoing directed migration.

$\beta 1$ integrin regulates Cdc42 activation and binding to N-WASP

N-WASP activation is known to be at least partly dependent upon binding the small GTPase Cdc42 and we and others have previously shown this complex to be important for endocytosis (Hussain *et al*, 2001; Parsons *et al*, 2005; Bu *et al*, 2010). To determine whether $\beta 1$ integrin may contribute to N-WASP function through control of Cdc42, we transfected $\beta 1 + / +$ and $\beta 1 - / -$ cells with the Cdc42 Raichu FRET biosensor to analyse activation of this GTPase *in situ* in fixed cells (Itoh *et al*, 2002). Acceptor photobleaching revealed a significant increase in FRET efficiency (indicative of high

Cdc42 activation) in $\beta 1 + / +$ cells in PDGF-treated cells, which was blocked in cells pre-treated with AG1296 demonstrating that PDGF activates this GTPase (Figure 8A). However, PDGF treatment did not activate Cdc42 in $\beta 1 - / -$ cells or in $\beta 1 + / +$ cells treated with anti- $\beta 1$ blocking antibodies, suggesting that $\beta 1$ is required in some part for PDGF-induced Cdc42 activity (Figure 8A). The dependence upon $\beta 1$ for Cdc42 activation following PDGF treatment was confirmed by biochemical analysis of levels of GST-PAK-CRIB domain bound to active Cdc42 in $\beta 1 + / +$ and $\beta 1 - / -$ cells (Figure 8B). Of note, Cdc42 activation was previously shown to be suppressed in $\beta 1 - / -$ T-cells (Makrogiannelli *et al*, 2009) implying a general role for this integrin in controlling local activation of this GTPase.

To determine whether $\beta 1$ integrins regulate N-WASP through formation of a direct complex with either Cdc42 or WIP, we performed FRET/FLIM analysis on $\beta 1$ -GFP cells expressing either Cdc42-mRFP or WIP-mCherry. However, neither protein showed direct binding to $\beta 1$ integrin, indicating that other proteins or complexes are required to mediate this effect (Supplementary Figure S4, bottom panels). There are a number of potential candidate proteins that may have a role in signalling to N-WASP downstream of integrin activation. Notably, focal adhesion kinase (FAK) is known to be activated upon integrin engagement and is proposed to associate with, and phosphorylate, N-WASP to regulate localisation (Wu *et al*, 2004). Active integrins can also recruit a number of adaptor proteins such as Cas, which can in turn associate with Nck and Cdc42, and potentially localised recruitment of N-WASP (Funasaka *et al*, 2010). Sites of active integrin clustering are also known to be rich in phospholipids and phosphate kinases, which may act to recruit N-WASP as well as contribute towards N-WASP activation through PIP2. It will be interesting in future to dissect the contribution of these candidates in controlling integrin-dependent growth factor responses in both fibroblasts and other cells to provide more mechanistic insight into this crosstalk.

To determine whether Cdc42 binding is required for N-WASP-mediated chemotaxis, we analysed fibroblasts isolated from N-WASP $- / -$ mice (Snapper *et al*, 2001) expressing GFP alone, or WT, H211/214D or Y253E N-WASP-GFP in Dunn chemotaxis chambers containing PDGF. As we may

Figure 7 Active $\beta 1$ localises the N-WASP–WIP complex to polarised protrusions. **(A)** Example images of $\beta 1$ $+/+$ cells treated with PDGF (30 ng/ml, 5 min) $\pm$ AG1296 (PDGFR inhibitor, 2 μ M) co-stained for actin or active $\beta 1$ integrin. Graph is quantification of active $\beta 1$ integrin intensity staining in each. $N \geq 40$ cells over three experiments. **(B)** Example images of FLIM analysis of FRET between N-WASP–GFP and WIP–mCherry in $\beta 1$ $+/+$ cells. Pseudocolour lifetime images show normal lifetime as blue (~ 2.3 ns) and FRET as red. Graph shows quantification of FRET efficiency. $N \geq 12$ cells for each. **(C)** Example of images from analysis of FRET between GFP–N-WASP and WIP–mCherry in live $\beta 1$ $+/+$ cell migrating towards PDGF gradient (arrow) over time in a Dunn chamber. Arrowheads show FRET at protruding cell front. **(D)** Example image of localisation of N-WASP, WIP, and active $\beta 1$ integrin in a fixed $\beta 1$ $+/+$ cell migrating in a Dunn chamber. Graph shows quantification of Pearson's correlation co-efficient between specified proteins at front versus rear of cells undergoing chemotaxis towards PDGF. * $P < 0.001$.

Figure 8 $\beta 1$ integrin regulates Cdc42 activation and binding to N-WASP. (A) Analysis of Cdc42 activation using Raichu biosensor FRET probes. Example images of FRET of acceptor photobleaching analysis of Cdc42 CFP/YFP FRET biosensor in $\beta 1^{+/+}$ or $\beta 1^{-/-}$ cells starved or treated with PDGF (5 min) are shown. Graph shows cumulative FRET efficiencies from these experiments and additional samples where cells were pre-incubated with $\beta 1$ blocking antibodies or AG1296. (B) Analysis of Cdc42 activation in $\beta 1^{+/+}$ or $\beta 1^{-/-}$ cells using PAK CRIB domain GST pulldowns. Example blots for GST-bound (active) Cdc42 and total Cdc42, and are representative of three independent experiments. (C) Cells assayed for chemotaxis towards PDGF in Dunn chambers. Circular rose plots show the proportion of cells with migratory direction lying within each 20° interval (PDGF source at top of histogram). The arrow represents the mean direction of migration; the grey segment represents the 95% confidence interval determined by the Rayleigh's test. (D) Migration speed of N-WASP $^{-/-}$ cells from experiments shown in (C). Error bars are $\pm$ s.e.m. throughout. N = at least 65 cells for each condition. $*P < 0.05$. (E) Biotinylated PDGF uptake over time in lysates from wild-type (WT) and N-WASP $^{-/-}$ cells measured by ELISA. (F) Analysis of FRET by FLIM between WT or H211/214D N-WASP-GFP and WIP-mCherry expressed in N-WASP $^{-/-}$ fibroblasts. Example images of lifetime maps of individual cells (left) and cumulative histogram (right) are shown. Error bars are $\pm$ s.e.m. throughout. $N > 9$ cells for each condition.

have predicted from our previous findings in $\beta 1$ null cells, N-WASP $^{-/-}$ cells were unable to undergo chemotaxis towards PDGF despite having similar levels of PDGFR at the cells surface (Figure 8C; Supplementary Figure S5B). This chemotaxis defect was rescued by re-expression of N-WASP-GFP but not the mutant forms of N-WASP that are unable to bind Cdc42 or undergo de-phosphorylation at Y253 (Figure 8C). Similarly, phospho-mutant N-WASP was unable to rescue defects in dorsal ruffle formation in these cells

(Supplementary Figure S5B). Furthermore, siRNA knock-down of N-WASP in $\beta 1^{+/+}$ cells resulted in a decrease in formation of dorsal ruffles (Supplementary Figure S5C). This suggests that both binding to Cdc42 and/or a cycle of phosphorylation at Y253 is required for N-WASP-dependent responses to PDGF. In agreement with a previous report (Misra *et al*, 2007), the migration speed of N-WASP $^{-/-}$ cells was significantly higher than those rescued with GFP-N-WASP or wild-type cells, a phenotype we also see in

β1^{-/-} cells (Figure 1B and D). Interestingly, expression of either N-WASP mutant partially rescued the migration speed defect in N-WASP^{-/-} cells (Figure 8D), suggesting that N-WASP regulation of chemotaxis is not necessarily coupled to motility rate. N-WASP^{-/-} cells did not show differences in basal cell surface levels of PDGFR, but demonstrated defective uptake of PDGF, further confirming a role for N-WASP in controlling PDGFR function (Figure 8E; Supplementary Figure S5A). Of note, PDGF uptake in N-WASP^{-/-} cells was severely impaired only at later time points, suggesting that loss of N-WASP alone acts to delay or limit the extent of uptake of PDGF, rather than completely inhibit entry.

As our data suggested that binding of N-WASP to WIP was important for cellular responses to PDGF (Figure 5), we next aimed to determine whether Cdc42 has a role in regulating this complex. To this end, we co-expressed WT or H211/214D (non-Cdc42 binding) N-WASP-GFP and WIP-mCherry in N-WASP^{-/-} cells and analysed interactions between these proteins by FRET/FLIM. Data demonstrated that both N-WASPs associated equally well with WIP, suggesting that

N-WASP binding to WIP and Cdc42 is not mutually exclusive events (Figure 8F). Taken together, these data demonstrate that β1 is required for Cdc42 activation and that N-WASP is required for efficient fibroblast chemotaxis towards PDGF in a Cdc42-dependent manner.

Active β1 integrin is required for N-WASP-Cdc42 binding and PDGF uptake

Having determined that N-WASP-Cdc42 interaction was required for chemotaxis, we sought to further clarify the role of β1 integrin in regulating formation of this signalling axis. To measure N-WASP-Cdc42 binding in intact cells, we measured FRET between GFP-N-WASP and Cdc42-mRFP (as we have done previously (Parsons *et al*, 2005)) expressed in N-WASP^{-/-} cells pre-treated with either control or β1 function-blocking antibodies. FRET efficiency was significantly lower in cells where β1 activation was blocked, confirming our hypothesis that β1 acts upstream of Cdc42 and N-WASP (Figure 9A). Furthermore, mutation of Y253 in N-WASP abrogated binding to Cdc42 back to levels seen in

Figure 9 Active β1 integrin is required for N-WASP-Cdc42 binding and PDGF uptake. **(A)** Analysis of FRET by FLIM between WT, Y256E, or H211/214D N-WASP-GFP and Cdc42-mRFP expressed in N-WASP^{-/-} fibroblasts. Where specified, cells were pre-treated with control IgG or anti-β1 integrin blocking antibodies. **(B)** Analysis of FRET between WT N-WASP-GFP and Cdc42-mRFP expressed in N-WASP^{-/-} fibroblasts either starved or incubated with PDGF-Cy5 +/- β1 blocking antibody. Right hand panels show image of Cy5 PDGF. **(C)** Diagrammatic representation of proposed model for β1 integrin regulation of chemotaxis. **P* < 0.001.

the H211/214D negative control, suggesting that phosphorylation of N-WASP is also an important regulatory factor in controlling assembly of this complex (Figure 9A). As our data also show that β1 integrins regulate phosphorylation and degradation of N-WASP (Figures 3 and 5) this strongly implies a role for β1 integrin in driving the assembly and stability of the N-WASP–Cdc42 complex. Finally, to define whether formation of this complex was functionally relevant in terms of PDGF ligand uptake, we analysed N-WASP–Cdc42 FRET in cells treated with PDGF-Cy5 to permit direct visualisation of growth factor localisation with respect to FRET. Images demonstrate that 30 min of PDGF stimulation resulted in a significant increase in N-WASP–Cdc42 binding, with the PDGF localised at the plasma membrane and in vesicles where the highest FRET levels were also seen (Figure 9B). Treatment with a function-blocking antibody to β1 resulted in loss of PDGF-stimulated N-WASP–Cdc42 binding, and retardation of the PDGF-Cy5 signal at the plasma membrane (Figure 9B, bottom panels). As we have also shown that PDGF stimulates β1 activation (Figure 7A), this data further support a role for β1 in regulating growth factor-induced assembly of both Cdc42–N-WASP and N-WASP–WIP complexes, leading to PDGF uptake and efficient cell chemotaxis.

In summary, our data reveal a novel mechanism of cross-talk between PDGF receptor and β1 integrins through a mechanism involving N-WASP. Our comprehensive analysis of signalling complexes in intact cells supports a model (Figure 9C) whereby PDGF binding to its canonical receptor leads to activation of both β1 integrin and Cdc42. This stimulates a transient dynamic increase in N-WASP binding to both WIP and Cdc42 at the leading edge of chemotaxing cells, and/or at circular dorsal ruffles resulting in efficient localised endocytosis of PDGF and downstream cellular signalling. β1 integrins are also required to locally modulate phosphorylation of N-WASP, through Src or Abl family kinases, which is required both for the stability of the protein and for N-WASP-dependent chemotaxis. This data provide novel insight into the assembly of these key signalling protein complexes in migrating cells and highlights the complex, dynamic interplay between integrins and growth factors that dictate activation of spatial signalling events and ultimately drive directed cell migration.

Materials and methods

Antibodies and reagents

Antibodies were from the following suppliers: Mouse anti-vinculin, anti-talin, anti-GAPDH (Sigma) mouse anti-total β1 and anti-human active β1 (12G10) (Chemicon) rabbit anti-β3 (Serotec); mouse anti-paxillin, mouse anti-Transferrin receptor and rabbit anti-Arp3 and dynamin 2 (BD Biosciences); Rabbit anti-PDGFR and anti-N-WASP (Cell Signaling); Rabbit anti-Rab4A and Goat anti-WIP (Santa Cruz Biotechnology); Rabbit anti-Rab5A (Abcam); Rabbit anti-Rab11 (Zymed); Mouse anti-phosphotyrosine (4G10) (Upstate Biotechnology); Src, Src416(P), Nck and WAVE1 (Biosource); control IgG (Dako); secondary HRP antibodies for western blotting all from Dako; secondary fluorescently labelled Alexa antibodies were all from Invitrogen. Phalloidin-Alexa was from Invitrogen (Molecular probes), wiskostatin and PP2 (Calbiochem); human plasma fibronectin (BD Biosciences). Recombinant murine PDGF-BB (PeproTech), and AG1296 (used at 2 μM, Merck).

Constructs and siRNA

N-WASP–GFP was as in Parsons *et al* (2005). This backbone was used to generate mCherry versions and Y253E/F mutants. The latter were generated using the following primers and carried out using

Stratagene Quickchange kit according to the manufacturers instructions:

Y253E (forward): AACTTAAAGACAGAGAAACATCAAAAGTTTATA GAAGACTTCATTGAAAAACAGGAGG; (reverse) CCTCCTGTTTTT TCAATGAAGTCTTCTATAACTTTTGATGTTTCTCTGCTTTAAAGT

Y253F (forward) ACTTAAAGACAGAGAAACATCAAAAGTTATAT TTGACTTCATTGAAAAACAGG; (reverse) CCTGTTTTTCAATGAA GTCAAATATAACTTTTGATGTTTCTCTGCTTTAAAGT. WIP and WIP-delWBD–mCherry were subcloned into pHR9SIN-SEW lentiviral expression plasmid and viruses were generated in 293T packaging cells as in Demaison *et al* (2002). Cortactin–GFP and Nck–GFP were gifts from L Machesky (Beatson Institute, Glasgow) and M. Way (Cancer Research UK LRI, London), respectively. Dynamin–GFP was a gift from J Rappoport (University of Birmingham, UK). Cdc42–mRFP was subcloned from Cdc42–GFP as in Parsons *et al* (2005). Cdc42 CFP/YFP Raichu FRET probe was a kind gift from M Matsuda (Kyoto University). ON-TARGET PLUS siRNA oligonucleotides to target N-WASP, β1 integrin, and WIP were all from Dharmacon.

Cell culture and transfections

β1+/+, β1–GFP, β1–/–, and β3–/– cells were derived and cultured in DMEM containing 10% FCS plus penicillin, streptomycin, glutamine, and 20 U/ml IFNγ (all from Sigma) and maintained at 33°C and 5% CO₂ as previously described (Parsons *et al*, 2008; Worth *et al*, 2010). NIH 3T3 cells were cultured in DMEM containing 10% FCS plus penicillin, streptomycin, and glutamine. N-WASP+/+ and N-WASP–/– cells were a kind gift from S Snapper and were derived as in Snapper *et al* (2001). Cells were cultured at 37°C and 5% CO₂ in DMEM containing 10% FCS plus penicillin, streptomycin, and glutamine. All transfections were carried out using FuGene 6 reagent (Roche) or Dharmafect1 (for siRNA Dharmacon) according to the manufacturer's instructions. Cells were plated at 40% confluency in 6-well plates and transfected using 0.5–2 μg/well of DNA (depending upon construct used) or 5 nmol siRNA suspended in OptiMem (Gibco).

Immunoprecipitation, GST pull-downs, and western blotting

Cells were lysed in RIPA buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 10 μM sodium fluoride, 1 μM okadaic acid with protease inhibitor complex (Calbiochem)). Cells were centrifuged at 13 000 r.p.m. for 5 min, lysate protein concentration was determined with the BCA assay (Pierce). For western analysis, protein was loaded onto polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked in TBS-T and 5% non-fat milk and incubated with specified primary antibodies in TBS-T overnight at 4°C. The immunoblots were washed in TBS-T and incubated for 2 h with HRP-conjugated secondary antibody. Membranes were then washed with TBS-T and visualised with ECL substrate reagent (Pierce). For immunoprecipitation, lysates were pre-cleared with protein A/G beads (Santa Cruz) and incubated with indicated antibodies followed by incubation with protein A/G beads for 2 h at 4°C. Beads were washed three times in lysis buffer before analysing by western blotting. For Cdc42 activation assays, PAK-CRIB–GST beads (Invitrogen) were incubated with lysates at 4°C for 30 min, washed in lysis buffer before analysing by western blotting. Whole cell lysates were analysed alongside for total Cdc42 levels.

Flow cytometry (FACs)

Cells were plated in 6 cm dishes and were serum-starved for 6 h before scrapping in PBS. Cells were centrifuged at 259 g for 3 min followed by fixation in 500 μl 4% PFA/PBS for 30 min on ice. Cells were then washed (three times in 500 μl of PBS) and blocked on ice for 30 min in 100 μl 5% BSA/PBS. Primary antibody incubation was overnight at 4°C in 100 μl 5% BSA/PBS then the cells were washed. Cells were incubated with the secondary antibody on ice for 1 h in 100 μl 5% BSA/PBS and then washed again and re-suspended in 500 μl FACS Flow buffer. Cells were analysed on BD FACSCalibur flow cytometer (BD Biosciences) with CellQuest Pro software. Gates were set to exclude outlying cells and then histograms were plotted to show cell count against intensity of staining. The mean value for cells only incubated with secondary antibody was deducted from the mean values for the other samples to compensate for non-specific intensity values. The experiment was repeated three times.

PDGF uptake assay

PDGF-BB was biotinylated according to manufacturers instructions (Roche). Cells were incubated on ice with PDGF-biotin following serum starvation followed by incubation at 37°C for indicated time periods. Cells were then washed three times in cold PBS, followed by an acid wash (twice with cold 0.2 M glycine buffer containing 0.15 M NaCl (pH 3.0)) followed by a further wash in cold PBS. Cells were then lysed and incubated on StreptaWell ELISA plates (Roche) and biotin levels measured according to manufacturers instructions.

Dorsal ruffle assays, confocal microscopy, and quantification of immunostaining

For dorsal ruffle assays, cells were serum-starved in OPTIMEM (Invitrogen) for 5 h followed by treatment with PDGF (30 ng/ml) for 5 min. Images were acquired using a Nikon A1R confocal microscope equipped with CFI Plan Fluor $\times$ 40 oil objective. Dorsal ruffles were counted in all cells in at least 10 fields of view per experiment. For quantification of total and active (12G10) integrin intensity, coverslips were imaged using identical laser settings between samples to permit comparison. Specificity of integrin antibodies was confirmed using β 1^{-/-} cells as control samples. In both experimental set-ups, look-up tables were used to ensure that pixel saturation was not reached and intensity per pixel was quantified using Image J software following common background subtraction.

Random migration and Dunn chamber assays

Phase contrast time-lapse imaging of cells as performed on a Zeiss Axio100 microscope equipped with Sensicam CCD camera (PCO Cooke), motorised stage (Ludl) and excitation/emission filters (Chroma) and filter wheels (Ludl). Images were acquired using a $\times$ 10 phase objective. Random migration was performed on cells plated on 12-well tissue culture plates. Chemotaxis was studied using Dunn chambers as in Zicha *et al* (1997). Briefly, both outer and inner wells were filled with OPTIMEM. Cells were grown on glass coverslips overnight followed by serum starvation for at least 5 h in OPTIMEM I. Coverslips were inverted onto the Dunn Chamber and sealed on three sides with hot wax mixture (Vaseline:paraffin:beeswax, 1:1:1). The media was removed from the outer well by capillary action and was rinsed with OPTIMEM before filling with OPTIMEM containing 50 ng/ml PDGF-BB. The chamber was then sealed with wax and mounted on a Zeiss Axio100 inverted microscope. Images were acquired as above, taking a frame every 10 min for 16 h using IQ acquisition software (Andor). Subsequently, all cells in the acquired time-lapse sequences were tracked using Andor Bioimaging Tracking. Tracking resulted in the generation of a sequence of position coordinates relating to each cell in each frame, motion analysis was then performed on these sequences using Mathematica 6 notebooks (Wolfram). Rose plots show the proportion of cells with migratory direction lying within each 20° interval. The arrow represents the mean direction of migration and the grey segment represents the 95% confidence interval determined by Rayleigh's test.

Live endocytosis assays

β 1^{+/+} or β 1^{-/-} cells were transfected with dynamin-GFP (Parsons *et al*, 2005) and plated into glass-bottomed imaging chambers. Cells were then incubated with 30 ng/ml PDGF labelled with Alexa-568 (Molecular Probes, as per manufacturers instructions) for 15 min on ice followed by gentle washing with cold PBS and replaced with Optimem. Cells were then left at 37°C to permit uptake for 30 min. Images were then acquired using a Nikon A1R confocal microscope equipped with CFI Plan Fluor $\times$ 40 oil objective. Frames were acquired every 2 s for 3 min using both

488 nm and 561 nm laser illumination in linescan mode. Resultant movies were compiled and analysed in Nikon NIS Elements software and exported as AVI for presentation purposes.

Raichu probe ratio FRET

Analysis of Cdc42 activation in intact cells was performed using the Cdc42 Raichu probe as in Itoh *et al* (2002) using acceptor photobleaching FRET as we have done previously (Carmona-Fontaine *et al*, 2008). Briefly, cells were transfected with the Cdc42 Raichu probe, fixed and imaged using a Zeiss LSM 510 META laser scanning confocal microscope and a $\times$ 63 Plan Apochromat NA 1.4 Ph3 oil objective. The CFP and YFP channels were excited using the 440-nm diode laser and the 514-nm argon line, respectively. The two emission channels were split using a 545-nm dichroic mirror, which was followed by a 475–525 nm bandpass filter for CFP and a 530 nm longpass filter for YFP (Chroma). Time-lapse mode was used to collect one pre-bleach image for each channel followed by bleaching with 50 iterations of the 514-nm argon laser line at maximum power (to bleach YFP). A second post-bleach image was then collected for each channel. Control non-bleached areas were acquired for all samples in the same field of view as bleached cells to confirm specificity of FRET detection. Pre- and post-bleach CFP and YFP images were then imported into Mathematica 6 for processing.

FRET/FLIM

Fluorescence lifetime imaging was performed and data analysed as described previously (Parsons *et al*, 2008). Time-domain FLIM was performed with a multi-photon microscope system as described previously (Parsons *et al*, 2008). Fluorescence lifetime imaging capability was provided by time-correlated single photon counting electronics (Becker and Hickl, SPC 700). Widefield acceptor (mRFP) images were acquired using a CCD camera (Hamamatsu) at <100 ms exposure times. Data were analysed as previously described (Parsons *et al*, 2008) using TRI2 software (developed by Dr Paul Barber). Histogram data are plotted as mean FRET efficiency from >10 cells per sample. ANOVA was used to test statistical significance between different populations of data. Lifetime images of example cells are presented using a pseudocolour scale whereby blue depicts normal GFP lifetime (no FRET) and red depicts lower GFP lifetime (areas of FRET).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: SJK, TMES, DW, and MP all performed the experiments; JM and GEJ contributed with reagents and had input into experimental design; SJK and MP designed the project and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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