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RESEARCH ARTICLE

PDK1 regulates focal adhesion disassembly by modulating endocytosis of αvβ3 integrin

Laura di Blasio[1,2,‡], Paolo Armando Gagliardi[1,2], Alberto Puliafito[1,2], Roberto Sessa[1,2,3], Giorgio Seano[1,2,4], Federico Bussolino[1,2,*] and Luca Primo[1,2,*,‡]

ABSTRACT

Non-amoeboid cell migration is characterised by dynamic competition among multiple protrusions to establish new adhesion sites at the cell's leading edge. However, the mechanisms that regulate the decision to disassemble or to grow nascent adhesions are not fully understood. Here we show that, in endothelial cells, 3-phosphoinositide-dependent protein kinase 1 (PDK1) promotes focal adhesion (FA) turnover by controlling endocytosis of integrin αvβ3 in a PI3K-dependent manner. We demonstrate that PDK1 binds and phosphorylates integrin αvβ3. Downregulation of PDK1 increases FA size and slows down their disassembly. This process requires both PDK1 kinase activity and PI3K activation but does not involve Akt. Moreover, PDK1 silencing stabilises FA in membrane protrusions decreasing migration of endothelial cells on vitronectin. These results indicate that modulation of integrin endocytosis by PDK1 hampers endothelial cell adhesion and migration on extracellular matrix, thus unveiling a novel role for this kinase.

KEY WORDS: PDK1, PI3K, Endothelial cells, Focal adhesion, Integrin endocytosis

INTRODUCTION

In the currently accepted model of non-amoeboid cell migration, initiation of cell locomotion involves a directional protrusion to form a lamellipodium, which is stabilised through integrin-mediated adhesions (Lee et al., 1993).

In this process, integrins mediate dynamic interactions between the actin cytoskeleton and the extracellular matrix (ECM) (Hynes, 1992), driving cell migration in many physiological and pathological contexts, including tissue regeneration and repair, embryonic development, inflammation, tumour angiogenesis and cancer metastasis (Lauffenburger and Horwitz, 1996); (Ridley et al., 2003); (Friedl and Wolf, 2010).

Integrins are organised into large protein clusters named focal adhesions (FAs), which are complex structures that form at sites of integrin attachment to the ECM (Burridge and Chrzanowska-Wodnicka, 1996). Nascent adhesions (also called focal contacts) at the leading edge of migrating cells either mature into stabilised FAs or turn over. Whereas FA formation mechanisms are largely known (Zamir and Geiger, 2001), the driving mechanisms for FA disassembly are not yet well understood. In the tail, FAs may be disassembled or left on the substratum as 'footprints' (Regen and Horwitz, 1992); (Smilenov et al., 1999). Microtubules contribute to FA disassembly either through the modulation of Rho GTPase signalling (Broussard et al., 2008) or through a FAK−dynamin pathway, independently of Rho and Rac activity (Ezratty et al., 2005). Clathrin and some of its adaptors (e.g. AP-2 and Dab2) are also involved in this process, by mediating integrin endocytosis from disassembling adhesion sites (Chao and Kunz, 2009); (Ezratty et al., 2009).

During cell migration, integrins are continuously endocytosed and recycled back to the plasma membrane (Pellinen and Ivaska, 2006); (Caswell and Norman, 2008); (Caswell et al., 2009). In recent years it has been demonstrated that trafficking is used to restrict integrins to cell protrusions (Caswell et al., 2007) and to guide the recycling of other receptors, such as epidermal growth factor receptor (EGFR) (Caswell et al., 2008) and vascular endothelial growth factor receptor (VEGFR) (Reynolds et al., 2009).

Among integrins, αvβ3 is particularly important in the vascular system (Avraamides et al., 2008; Hynes, 2007). This integrin is expressed at low levels in quiescent endothelial cells, and is upregulated during normal and tumour angiogenesis, which made it an attractive therapeutic target (Brooks et al., 1994). Indeed, αvβ3 and αvβ5 antagonists are being used in clinical trials as anti-angiogenic drugs, including the humanised monoclonal antibody Vitaxin and the RGD-mimetic Cilengitide (Gutheil et al., 2000); (Nabors et al., 2007). However, neither αv-deficient nor β3/β5 double-deficient mice displayed a vascular network with obvious developmental defects (Bader et al., 1998); (Hodivala-Dilke et al., 1999); (Huang et al., 2000). Thus, these and others studies suggest that regulation of αvβ3 integrin plays a crucial role in angiogenesis.

We have recently shown that, in endothelial cells, the Ser/Thr kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1) is required for directional motility (Primo et al., 2007). PDK1 phosphorylates kinases of the AGC family, including Akt (Alessi et al., 1997), and contains a C-terminal pleckstrin homology (PH) domain (Mora et al., 2004) that binds with high affinity to the phosphoinositide 3-kinase (PI3K) products, phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and phosphatidylinositol (3,4)-bisphosphate (PIP2) (Anderson et al., 1998). Although several effectors have been proposed to be activated by PDK1 during cell migration, the exact molecular mechanism is not known (Primo et al., 2007); (Pinner and Sahai, 2008); (Liu et al., 2009); (Gagliardi et al., 2014).

[1]Candiolo Cancer Institute FPO-IRCCS, Candiolo, 10060 Turin, Italy. [2]Department of Oncology, University of Torino, 10126 Turin, Italy. [3]Present address: UC Berkeley, 689 Minor Hall, Berkeley, CA 94720, USA. [4]Present address: Edwin Steele Laboratory for Tumor Biology, Harvard Medical School, Massachusetts General Hospital, Boston, MA 02114, USA.
*These authors contributed equally to this work

‡Authors for correspondence (laura.diblasio@ircc.it; luca.primo@ircc.it)

Received 7 January 2014; Accepted 24 December 2014

Journal of Cell Science

Journal of Cell Science (2015) 128, 863–877 doi:10.1242/jcs.149294

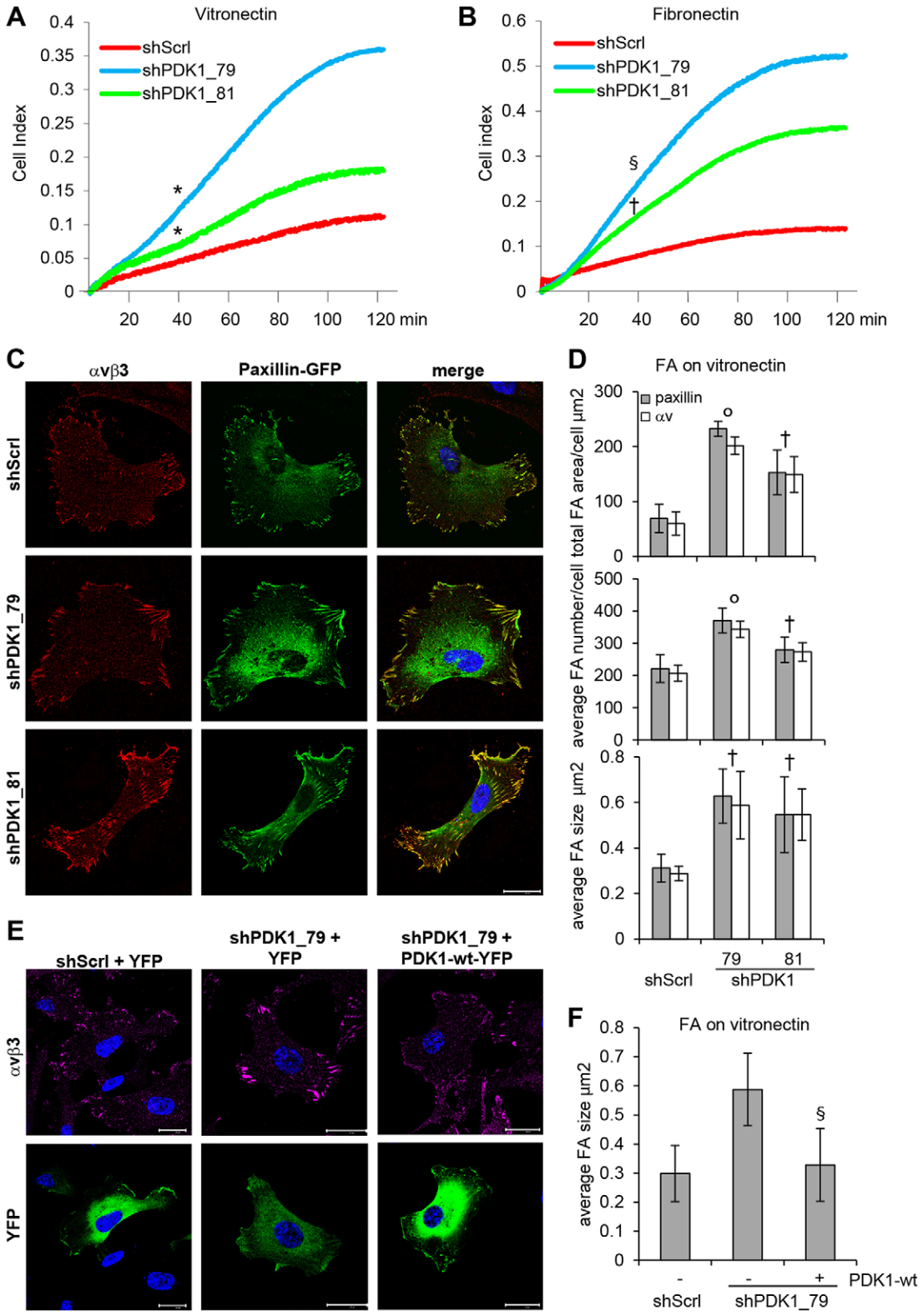

Fig. 1. See next page for legend.

Here we present evidence for a completely new function of PDK1. We found that PDK1 promotes FA turnover on vitronectin by controlling the endocytosis of integrin αvβ3 in a PI3K-dependent manner. Notably, PDK1 dynamically localises to FAs just before their disassembly, and interacts with αvβ3, modulating adhesion and migration of endothelial cells.

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Fig. 1. PDK1 regulates αvβ3-dependent adhesion of endothelial cell. (A,B) Control (shScrl) and PDK1-knockdown (shPDK1_79 and 81) endothelial cells adhering on vitronectin (A) or fibronectin (B) was monitored for 2 hours using the xCELLigence system. Data were plotted as the mean cell index from three wells at each time points; *P*-values were calculated at 40 minutes, *$P<0.0005$, §$P<0.005$, †$P<0.05$ for knockdown endothelial cells versus control endothelial cells. (C) Paxillin–GFP (green) transfected control (shScrl) and PDK1-knockdown (shPDK1_79 and 81) endothelial cells were seeded on vitronectin and stained with anti-αvβ3 (red, DAPI in blue). Scale bar: 20 μm. (D) Quantification of paxillin- or αv-positive FA area/cell, number/cell and average size. Data were plotted as the mean±s.d.; °$P<0.001$, †$P<0.05$ for knockdown endothelial cells versus control endothelial cells. (E) Control (shScrl) endothelial cells transfected with YFP alone (green) and shPDK1_79 endothelial cells transfected with YFP alone or PDK1-wt-YFP were seeded on vitronectin and stained with anti-αvβ3 (magenta, DAPI in blue). Scale bars: 50 μm. (F) Quantification of average FA size. Data were plotted as the mean±s.d.; §$P<0.005$ for shPDK1_79 endothelial cells transfected with PDK1-wt versus shPDK1_79 endothelial cells.

RESULTS

αvβ3-mediated cell adhesion is modulated by PDK1

To efficiently and persistently migrate, cells need to modulate integrin-mediated contacts with the surrounding ECM molecules. Therefore, we investigated whether PDK1 regulates adhesion on the ECM by reducing expression of PDK1 with short hairpin RNAs (shRNA). We identified two shRNAs, referred to as shPDK1_79 and shPDK1_81, that reduced PDK1 protein levels by ~90% and ~80%, respectively, compared with endothelial cells that expressed not-targeting shRNA (shScrl) (supplementary material Fig. S1A). Endothelial cell adhesion was analysed in real time by measuring cellular impedance with the xCELLigence system (see Materials and Methods) on provisional ECM proteins vitronectin and fibronectin. Endothelial cells with reduced PDK1 levels (shPDK1_79 and shPDK1_81) showed significantly increased adhesion ability, on both vitronectin and fibronectin, compared to control cells (Fig. 1A,B). However, reduced PDK1 levels did not significantly change the spreading area of endothelial cells, excluding the possibility that cellular impedance is affected by cell spreading (supplementary material Fig. S1B,C). In order to exclude off-target effects of shRNAs that were used to silence PDK1, we transduced shPDK1_79 cells with a PDK1-wt cDNA resistant to silencing (supplementary material Fig. S1D). PDK1-wt re-expression rescued the effect of PDK1 silencing, reducing the ability of shPDK1_79 to adhere on vitronectin (supplementary material Fig. S1E).

Since fibronectin is the ligand of several integrins – whereas vitronectin binds specifically αvβ3 and αvβ5 (Hynes, 2002) – we sought to investigate whether PDK1 regulates αvβ3/αvβ5-mediated cell adhesion. To this end, we analysed the distribution of these integrins and the morphology of FAs on vitronectin (supplementary material Fig. S1F). In endothelial cells, αvβ3 integrin clearly localised to FAs (supplementary material Fig. S1F); in contrast, αvβ5 showed negligible accumulation in FAs, neither in basal culture condition nor when cells were stimulated with VEGF (supplementary material Fig. S1F) (Friedlander et al., 1995). Then, we concentrated our attention on the effect of PDK1 silencing on αvβ3 and αvβ5 localisation in endothelial cells plated on vitronectin (Fig. 1C). In shScrl-endothelial cells, αvβ3 localised mainly to small FAs along the cell periphery. Conversely, endothelial cells transduced with shPDK1_79 or shPDK1_81 showed longer and thicker αvβ3-positive FAs, sometimes located along the whole basal side of the cells (Fig. 1C). Moreover, PDK1 silencing increased average size, total area and number of αvβ3-positive FAs (Fig. 1D). In contrast, the distribution of αvβ5 integrin in control and PDK1-silenced cells did not change (supplementary material Fig. S1G). The re-expression of PDK1-wt in shPDK1_79 endothelial cells plated on vitronectin restored the physiological pattern of αvβ3-positive FAs (Fig. 1E,F).

When plated on fibronectin, shPDK1 endothelial cells displayed FAs that were similar to those observed in cells plated on vitronectin (supplementary material Fig. S2A right panels, and Fig. 2B). In contrast, when endothelial cells were plated on collagen type-I, PDK1 silencing had no effects on FA organisation (supplementary material Fig. S2A, left panels, and B). Interestingly, the αvβ3 integrin inhibitor cyclo [Arg-Gly-Asp-D-Phe-Val] (cRGDfV) completely neutralised the effect of PDK1 silencing on vitronectin (supplementary material Fig. S1H,J), whereas we could not measure any effect on collagen type-I (supplementary material Fig. S1I,K). These results indicate that PDK1 downregulation increases endothelial cells adhesion on vitronectin by regulating organisation and size of αvβ3-containing FAs.

PDK1 regulates FA disassembly

Since an enlargement of FAs may indicate a change in the turnover kinetics of their components (Webb et al., 2002), we further investigated the role of PDK1 in this process, by taking advantage of a method to monitor the synchronised disassembly of adhesions (Bershadsky et al., 1996); (Ezratty et al., 2005). This method is based on the finding that microtubule depolymerisation – promoted by nocodazole treatment – stimulates the formation of FAs. Upon nocodazole removal, microtubule regrow, and FAs rapidly and synchronously disassemble. By adapting this approach, we found that 1-hour treatment of endothelial cells with nocodazole stabilised paxillin and αvβ3-containing FAs and, concurrently, completely disassembled microtubules (supplementary material Fig. S2C and Fig. 2A,C). Then, ~30 minutes after nocodazole washout, while microtubules regrew, FAs disassembled (supplementary material Fig. S2C and Fig. 2B,C). Upon microtubule disassembly, control and PDK1-knockdown endothelial cells showed similar FA size (Fig. 2A,C). Moreover, consistently with the idea that depletion of PDK1 affects FA turnover, we found that FA disassembly was slower in PDK1-knockdown endothelial cells than in control endothelial cells, even though microtubules regrowth was normal (Fig. 2B,C). Conversely, αvβ5 integrin was neither accumulated in paxillin-positive FAs after nocodazole treatment nor clearly changed its localisation during nocodazole washout (supplementary material Fig. S2D). Thus, these data suggest that PDK1 promotes the disassembly of FAs.

PDK1 associates with αvβ3 integrin and dynamically localises to FAs

PDK1 usually localises throughout the cytoplasm and accumulates at the plasma membrane through the binding with PIP3 produced by PI3K (Mora et al., 2004). Thus, we wondered whether PDK1 moved to FAs during their disassembly. Paxillin–GFP-expressing endothelial cells were treated with nocodazole and PDK1 and αv integrin localisation was analysed by immunofluorescence staining and confocal microscopy (Fig. 3A,B). Cells treated with nocodazole showed an enrichment of PDK1 in FAs, identified by paxillin–GFP and anti-αv integrin staining (Fig. 3A, white arrows). When nocodazole was removed, PDK1 quickly disappeared from FAs (Fig. 3B). In untreated cells, cultured in basal medium and plated on vitronectin, the colocalisation of PDK1 with paxillin and

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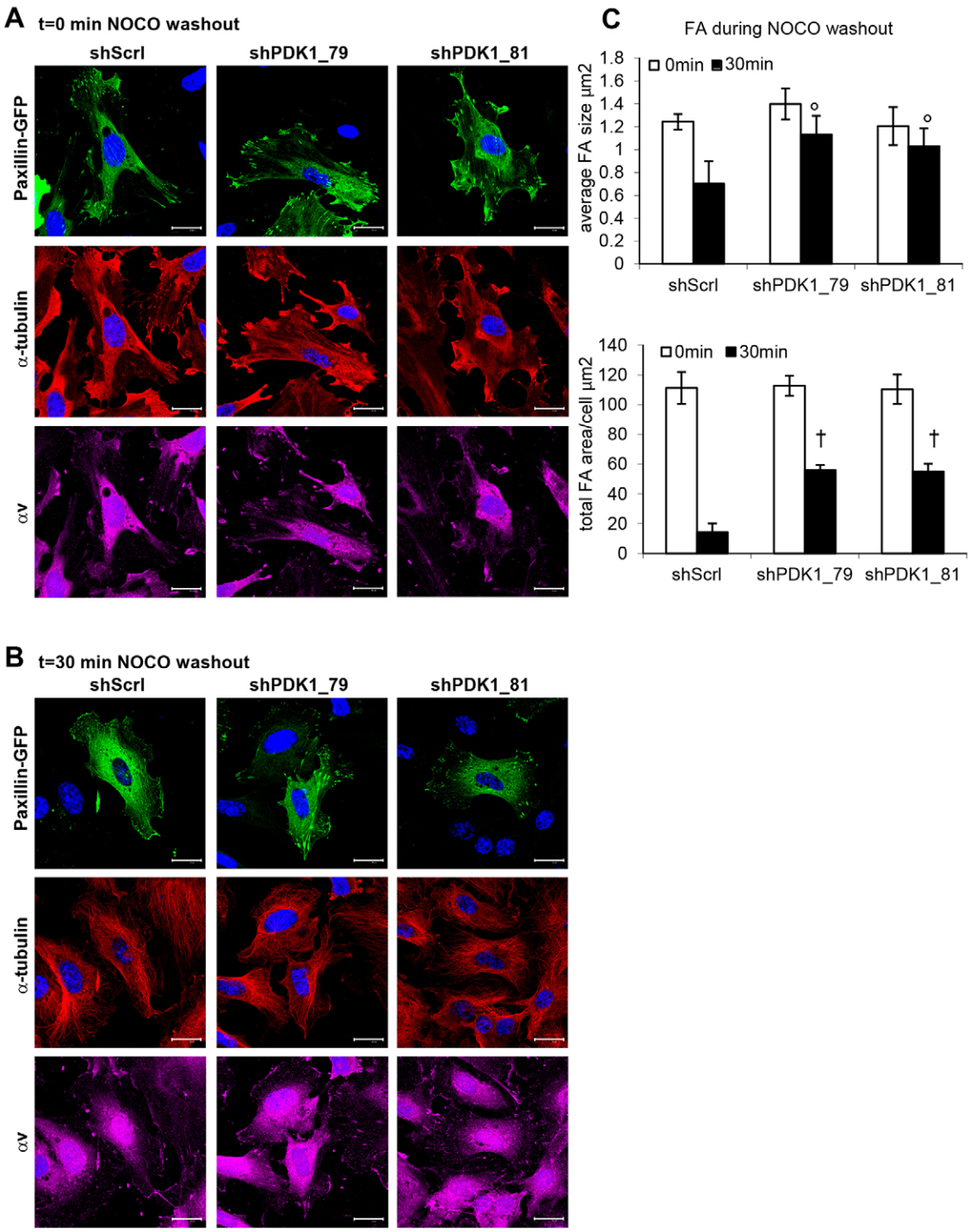

Fig. 2. PDK1 regulates FA disassembly on vitronectin. (A,B) Control (shScrl) and PDK1 knockdown (shPDK1_79 and 81) endothelial cells transfected with Paxillin–GFP (green) that had been seeded on vitronectin, were treated with nocodazole (t=0 min NOCO washout, A). Then the drug was washed out (t=30 min NOCO washout; B) and cells were stained with anti-α tubulin (red) and anti-αv (magenta, DAPI in blue). Scale bars: 20 µm. (C) Quantification of FA area/cell and average FA size. Data were plotted as the mean±s.d.; $^{\circ}P<0.001$, $^{\dagger}P<0.05$ for knockdown endothelial cells versus control endothelial cells.

αvβ3 was still visible albeit less pronounced (Fig. 3C). Such qualitative observations were corroborated by quantifying the amount of PDK1 or αv integrin found in single FAs with respect to the cytoplasmic region. The results of this quantitative analysis confirmed the qualitative observations (for further details see supplementary material Fig. S3A,B, and Materials and Methods).

PDK1 localisation to FAs led us to investigate whether PDK1 and αvβ3 integrin physically associated in nocodazole-treated cells. We co-immunoprecipitated endogenous PDK1 with αvβ3 integrin in nocodazole-treated endothelial cells and found the amount of co-immunoprecipitated PDK1 to be increased in endothelial cells overexpressing PDK1 (Fig. 3D and supplementary material Fig. S2E). After nocodazole removal,

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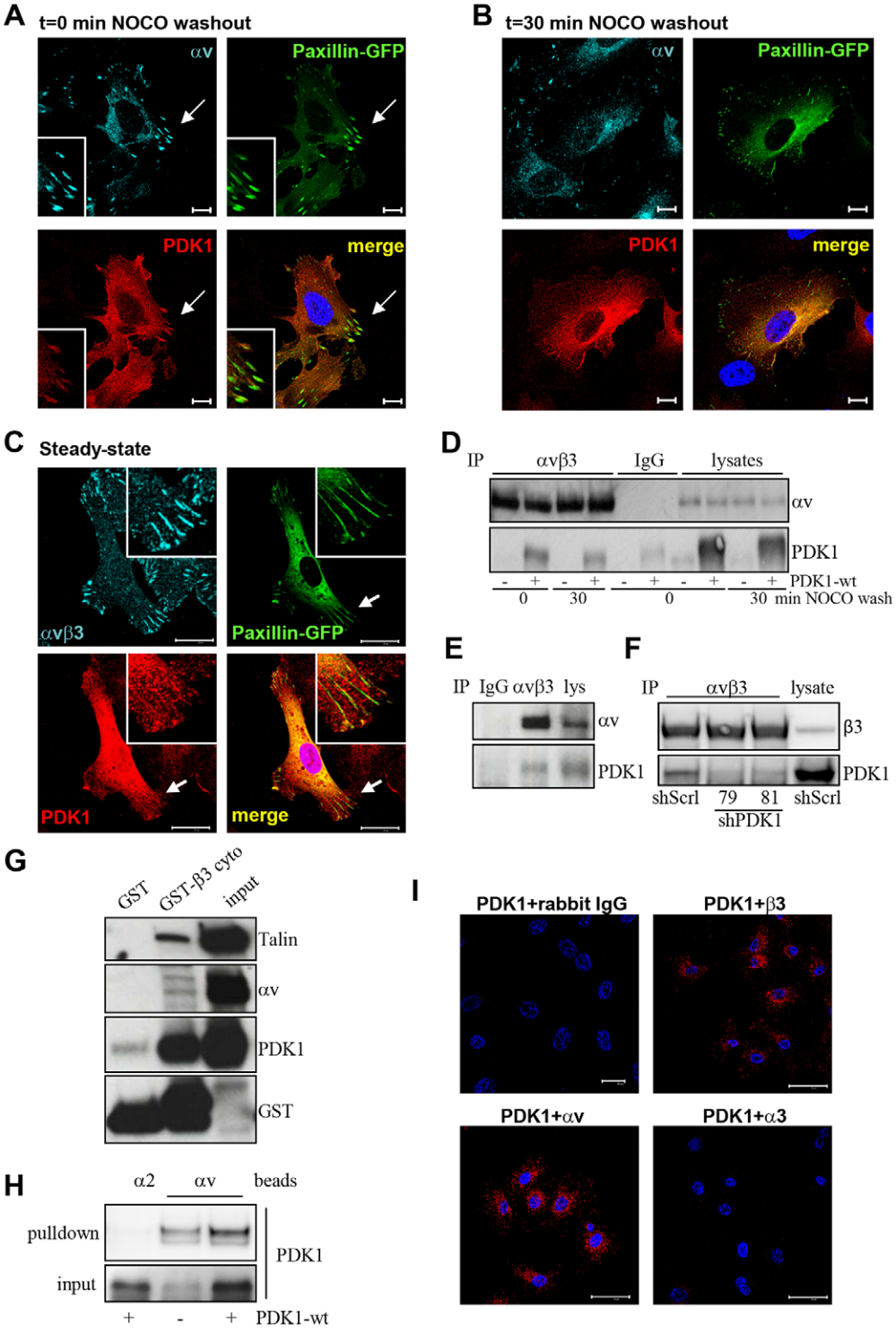

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αvβ3 integrin was no longer able to co-immunoprecipitate with endogenous PDK1 while it still maintained, although to a lesser degree, the ability to co-immunoprecipitate with overexpressed PDK1 (Fig. 3D and supplementary material Fig. S2E).

The association of PDK1 with αvβ3 was confirmed also in untreated cells (Fig. 3E). Concordantly to colocalisation experiments, the amount of PDK1 that co-immunoprecipitated with αvβ3 under basal culture conditions was very limited (Fig. 3E). The specificity of PDK1/αvβ3 co-immunoprecipitation was further demonstrated by experiments showing the absence of PDK1 in αvβ3 integrin that had been immunoprecipitated from PDK1-silenced cells (Fig. 3F). In addition, endogenous PDK1 was not immunoprecipitated by anti-α2β1 antibody, suggesting that PDK1 can bind specifically αvβ3 integrin (supplementary

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Fig. 3. PDK1 transiently localises to FAs and associates with αvβ3 integrin. (A,B) Endothelial cells that had been seeded on vitronectin and transfected with paxillin–GFP (green), were treated with nocodazole (t=0 min NOCO washout, A). Then the drug was washed out (t=30 min NOCO washout, B) and cells were stained with anti-αv integrin (turquoise) and anti-PDK1 (red, DAPI in blue). The merged images originate from the overlap of paxillin and PDK1 staining; arrows indicate zoomed-in regions. Scale bars: 10 μm. (C) Endothelial cells that had been seeded on vitronectin and transfected with paxillin–GFP (green), were fixed after 2 hours of adhesion and stained with anti-αvβ3 integrin (turquoise) and anti-PDK1 (red, DAPI in blue). The merged images originate from the overlap of paxillin and PDK1 fluorescence; arrows indicate the zoomed regions. Scale bars: 20 μm. (D) Empty vector-infected (−) or PDK1-overexpressing (+) endothelial cells were treated with nocodazole (t=0 min NOCO wash). After nocodazole removal (t=30 min NOCO wash), endothelial cells were lysed and αvβ3 was immunoprecipitated; immunocomplexes and corresponding lysates were immunoblotted with the indicated antibody. (E) Wild-type endothelial cells were lysed and αvβ3 was immunoprecipitated; immunocomplexes and corresponding lysate were immunoblotted with the indicated antibody. (F) Control (shScrl) and PDK1 knockdown (shPDK1_79 and 81) endothelial cells were lysed and αvβ3 was immunoprecipitated; immunocomplexes and corresponding lysates were immunoblotted with the indicated antibody. (G) Wild-type endothelial cells were lysed and pull-down assays were carried out using the cytoplasmic tail of β3 integrin fused to GST; immunocomplexes and corresponding lysate were immunoblotted with the indicated antibody. (H) Endothelial cells infected with empty vector (−) or overexpressing PDK1 (+) were lysed and peptide pull-down assays were carried out using a synthetic peptide corresponding to the cytoplasmic part of αv and α2 integrin. Western blots shown are representative of four experiments performed with similar results. (I) Endothelial cells were seeded on vitronectin and then analysed in PLAs with the following antibodies: anti-PDK1 and anti-αv (PDK1+αv), anti-PDK1 and anti-β3 (PDK1+β3), anti-PDK1 and anti-α3 (PDK1+ α3) and anti-PDK1 and rabbit IgG (PDK1+rabbit IgG) as a negative control. DAPI is in blue, red spots represent PLA signals. Scale bars: 20 μm for PDK1+rabbit IgG, 50 μm for other images.

material Fig. S2G). Moreover, in HEK 293T cells that express αv but not β3 integrin (Taherian et al., 2011), PDK1 co-immunoprecipitated with αvβ3 only in cells transfected with β3 integrin (supplementary material Fig. S2F). To further confirm the interaction, we performed pull-down experiments with the αv and β3 cytoplasmic tails (Fig. 3G,H). The GST-fused cytoplasmic tail of β3, which maintains the ability to interact with talin, efficiently associated with PDK1 (Fig. 3G). Moreover, a biotinylated peptide of the cytoplasmic tail of αv integrin, but not of α2 integrin, was able to co-precipitate with both overexpressed and endogenous PDK1 (Fig. 3H). For reasons that remain to be investigated, we could hardly detect αvβ3 integrin by PDK1 immunoprecipitation, even in nocodazole-treated cells (supplementary material Fig. S2H).

The *in vivo* association of PDK1 with αvβ3 was also analysed in proximity ligation assays (PLAs). PLA-positive spots were detected in presence of anti-PDK1 together with either anti-αv or anti-β3 antibodies, both at the periphery and in the cytoplasm of cells that had been plated on vitronectin (Fig. 3I). Only a small number of spots or no spots were detected with anti-PDK1 antibody plus anti-β3 antibody or plus rabbit IgG, respectively (Fig. 3I). Taken together, these results indicate that PDK1 associates with the cytoplasmic domain of αvβ3 integrin and dynamically localises to FAs.

PDK1 controls αvβ3 endocytosis during FA disassembly

To elucidate the mechanism by which PDK1 regulates FA disassembly, we first tested whether PDK1 knockdown affected the activity of FAK, a key regulator of FA turnover. However, the phosphorylation of FAK on Tyr397, which is crucial for FAK activation and FA disassembly (Webb et al., 2002) was not affected by PDK1 silencing (supplementary material Fig. S3C). Recently, it has been demonstrated that FA disassembly requires endocytosis of integrins (Chao and Kunz, 2009); (Ezratty et al., 2009). Since we observed that the plasma membrane expression of β3 integrin was slightly increased in PDK1-silenced endothelial cells (supplementary material Fig. S3D), we sought to determine whether PDK1 regulated endocytosis of αvβ3 integrin, thereby affecting FA turnover. We then used both the antibody-chase (Fig. 4A,B) and biochemical internalisation assay (Fig. 4C) to determine whether PDK1 regulated endocytosis of αvβ3 integrin.

In the antibody-chase assay, we observed by indirect immunofluorescence that the amount of anti-β3 antibody internalised after acid wash was reduced in PDK1-silenced cells (Fig. 4A, 37℃ plus acid wash, B). As expected, cells treated in the same manner but kept at 4℃ did not internalise αvβ3 integrin (Fig. 4A, 4℃). To better characterise this effect, we performed a quantitative biochemical internalisation assay based on surface protein biotinylation, followed by cell surface reduction and detection of internalised biotinylated integrin using a capture enzyme-linked immunoadsorbent assay (capture ELISA) procedure (Roberts et al., 2001). This experiment, performed in the presence of primaquine, which inhibits receptor recycling, showed that PDK1 silencing decreased the amount of αvβ3 integrin internalised by endothelial cells (Fig. 4C). In contrast, the internalisation of α5 integrin was not affected by PDK1 knockdown either in presence or not of primaquine (supplementary material Fig. S3E).

Different αvβ3 integrin internalisation routes have been described. Actually, αvβ3 integrin can be internalised in a cholesterol- and caveolin1-dependent manner by clathrin-coated structures or by macropinocytosis (Caswell et al., 2009). To define which αvβ3 internalisation routes are predominant in endothelial cells and potentially regulated by PDK1, we performed antibody-chase internalisation assay of β3 integrin in the presence of different endocytosis inhibitors (supplementary material Fig. S4A–C). Cyclodextrin, which blocks cholesterol-dependent endocytosis, strongly inhibited β3 internalisation. In contrast, the macropinocytosis inhibitor Amiloride had no influence on β3 endocytosis. Treatment of endothelial cells with Dynasore, which inhibits dynamin-mediated endocytosis, had only a minor effect (supplementary material Fig. S4A,B).

Hence, we tested whether PDK1 modulates αvβ3 integrin endocytosis during nocodazole washout. Simultaneous to nocodazole removal, anti-β3 antibody was added to the cells. (Fig. 4D–G). In shScrl endothelial cells, 30 minutes after nocodazole washout, β3 integrin was endocytosed and simultaneously FA disassembled (Fig. 4D,G and supplementary material Fig. S3F). In contrast, β3 integrin internalisation during nocodazole washout was impaired in PDK1-silenced endothelial cells, resulting in more stable FAs (Fig. 4E–G, and supplementary material Fig. S3F). It is worth noting that during FA disassembly, β3 integrin was internalised by previously described endocytosis routes (Caswell et al., 2009). Indeed, after nocodazole washout, β3 integrin localised to Rab4- and Rab5-positive early endosomes, and partially to Rab11-positive recycling endosomes, irrespectively of primaquine addition (supplementary material Fig. S3G–I).

In biochemical internalisation assay, PDK1-silencing significantly reduced the β3 integrin internalisation after nocodazole removal. As shown in Fig. 4H, the addition of primaquine increased the percentage of internalised β3 integrin during nocodazole washout;

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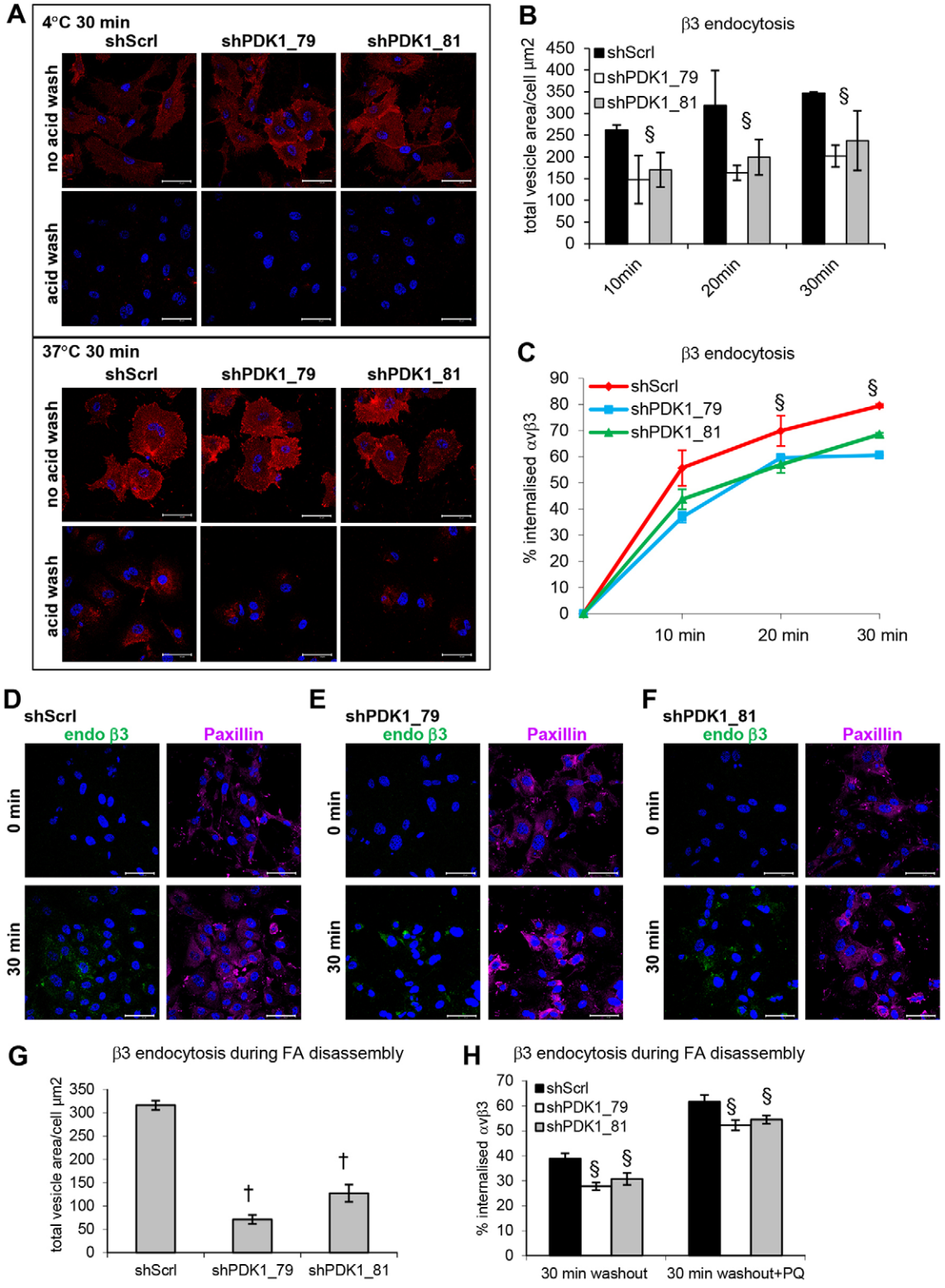

Fig. 4. See next page for legend.

notably, PDK1-knockdown endothelial cells still displayed reduced ability to internalise β3 integrin (Fig. 4H). Therefore, PDK1 controls αvβ3 integrin endocytosis, which takes place during FA disassembly.

Both PH domain and kinase activity of PDK1 are required for controlling integrin αvβ3 endocytosis

PDK1 is characterised by a centrally located kinase domain, a C-terminal PH domain and a docking site, named PIF-binding

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Fig. 4. PDK1 controls αvβ3 endocytosis during FA disassembly. (A) Control (shScrl) and PDK1 knockdown (shPDK1_79 and 81) endothelial cells were plated on vitronectin and incubated with anti-β3 antibody either at 4°C as control or at 37°C in presence of primaquine to allow internalisation of antibody-bound integrin (red, DAPI in blue); as a control of β3 surface staining, acid wash was done or not. Scale bars: 50 μm. (B) Quantification of the total vesicle area/cell after 10, 20 and 30 minutes of endocytosis. Data were plotted as the mean±s.d.; $^{\S}P<0.005$ for knockdown endothelial cells versus control endothelial cells. (C) Endocytosis of αvβ3 in presence of primaquine was evaluated in control (shScrl) and PDK1 knockdown (shPDK1_79 and 81) endothelial cells after 10, 20 and 30 minutes. Internalised biotinylated integrin was quantified by ELISA. Data were plotted as mean±s.d. of the percentage of total biotinylated integrin αvβ3 that had been internalised; $^{\S}P<0.005$ for knockdown endothelial cells versus control endothelial cells. (D–F) Control (shScrl) and PDK1 knockdown (shPDK1_79 and 81) endothelial cells that had been seeded on vitronectin, were treated with nocodazole (t=0 min). Then, during the drug washout (t=30 min), cells were incubated with anti-β3 antibody in presence of primaquine and internalisation of antibody-integrin complexes were followed by indirect immunoflourescence. FA disassembly was confirmed with anti-paxillin antibody (magenta, internalised β3 integrin in green, DAPI in blue). Scale bars: 50 μm. (G) Quantification of vesicle area/cell. Data were plotted as mean±s.d.; $^{\dagger}P<0.05$ for knockdown endothelial cells versus control endothelial cells. (H) Control (shScrl) and PDK1 knockdown (shPDK1_79 and 81) endothelial cells were treated with nocodazole and αvβ3 endocytosis was followed during drug washout with or without primaquine. Internalised biotinylated integrin was quantified by ELISA. Data were plotted as the mean±s.d. of the percentage of total biotinylated αvβ3 integrin that had been internalised; $^{\S}P<0.005$ for knockdown endothelial cells versus control endothelial cells.

pocket, which is located on the small lobe of the kinase domain (Tian et al., 2002); (Biondi et al., 2001). To determine which region is required for αvβ3 endocytosis regulation by PDK1, we infected shPDK1_79 endothelial cells with lentivirus encoding different PDK1 mutants resistant to silencing (Gagliardi et al., 2012) (supplementary material Fig. S4D). Then, shScrl endothelial cells and shPDK1_79 endothelial cells expressing PDK1 wild-type (wt), PDK1 constitutively anchored to the plasma membrane (caax), PDK1 lacking the PH domain (ΔPH), PDK1 kinase-dead (KD) or PDK1 with a mutation on the PIF pocket (L155E) were assayed in the antibody-chase and biochemical internalisation assays (Fig. 5A,B and supplementary material Fig. S4E, respectively). Re-expression of PDK1-wt, PDK1-caax and PDK1-L155E was able to recover the normal level of αvβ3 endocytosis; by contrast, PDK1-ΔPH and PDK1-KD did not rescue the defective αvβ3 integrin internalisation caused by endogenous PDK1 silencing (Fig. 5A,B; supplementary material Fig. S4E and not shown). The same results were obtained when we tested αvβ3 internalisation during FA disassembly (supplementary material Fig. S4F). Moreover, the PDK1-ΔPH mutant lost the ability to localise to the plasma membrane in proximity of FAs (Fig. 5C) and co-immunoprecipitated with αvβ3 less efficiently than PDK1-wt or PDK1-KD (supplementary material Fig. S4G and not shown). Thus, membrane localisation through PIP3 binding and kinase activity are necessary for PDK1 to regulate αvβ3 endocytosis.

PI3K but not Akt inhibition reduces integrin αvβ3 endocytosis

Following PI3K activation and production of PIP3, both PDK1 and Akt localise to the plasma membrane by means of their PH domains. The proximity of the two proteins at plasma membrane allows the phosphorylation of the activation loop of Akt by PDK1 (Collins et al., 2003); (McManus et al., 2004). Thus, we verified whether the inhibition of either PI3K or Akt with chemical compounds was able to phenocopy the effect of PDK1 silencing on αvβ3 endocytosis (Fig. 5D and supplementary material Fig. S4H). The block of PI3K activity with LY294002 slightly but significantly inhibited endocytosis of antibody-bound β3 integrin (Fig. 5D and supplementary material Fig. S4H). Surprisingly, Akt inhibition increased the rate of αvβ3 internalisation (Fig. 5D and supplementary material Fig. S4H). Neither of the two inhibitors had effects on the endocytosis of transferrin (Fig. 5D). Taken together, these data demonstrate that PI3K activation is necessary for αvβ3 endocytosis and that PDK1 regulates this process in a PI3K-dependent way, but without the requirement of downstream activation of Akt.

β3 integrin is phosphorylated by PDK1

Given that kinase activity of PDK1 was required for the regulation of αvβ3 integrin endocytosis, we wondered whether PDK1 was able to phosphorylate β3 integrin cytoplasmic domain in cultured cells. It has been previously shown that the threonine residue at position 753 (Thr753) of β3 integrin is phosphorylated *in vitro* by PDK1 and Akt (Kirk et al., 2000). Therefore, we verified whether PDK1 was able to phosphorylate β3 integrin also *in vivo*. In absence of specific antibodies against phosphorylated (anti-phospho) Thr753, we used an anti-phospho Akt-substrate antibody recognising peptides and proteins that containing phosphorylated serine and threonine residues preceded by lysine or arginine at positions −5, such as Thr753 of β3. We validated this antibody by expressing the cytoplasmic tail of integrin β3 in HEK 293T cells and treating the cells with the protein phosphatase 2A (PP2A) inhibitor calyculin A or PDK1 inhibitor BX-795 (Fig. 6A). The cytoplasmic tail of β3 integrin showed basal phosphorylation, which was enhanced by treatment with calyculin A (Fig. 6A, Cal). By contrast, treatment with BX-795 reduced phosphorylation of the β3 integrin cytoplasmic tail, suggesting this antibody being specific for phosphorylated Thr753 (Fig. 6A, PDKi). Thus, we analysed the phosphorylation of endogenous β3 integrin immunoprecipitated from endothelial cells. When treated with PDK1 inhibitor, endothelial cells showed reduced phosphorylation of β3 (Fig. 6B). These data were further confirmed by PLA experiments performed with anti-β3 and the same anti-phospho Akt-substrate used in the immunoblot (Fig. 6C). Untreated cells showed more spots of β3 integrin phosphorylated at Thr753 than BX-795-treated cells (61±8 spots/cell vs 36±5, $n=10$); by contrast, the inhibition of Akt had only a partial effect (42±7 spots/cell) (Fig. 6C). In addition, we observed a reduced phosphorylation of β3 immunoprecipitated from PDK1-silenced endothelial cells compared to control endothelial cells (Fig. 6D).

To determine whether Thr753 and its phosphorylation by PDK1 is important for β3 endocytosis, we infected endothelial cells with either wild-type β3 integrin or mutant β3 integrin in which Thr753 had been mutated to Ala (T753A). Both wild-type and T753A β3 integrin were correctly targeted to the plasma membrane and localised to FAs (supplementary material Fig. S4I,J and Fig. 6E). As expected, the Ser/Thr phosphorylation of T753A β3 integrin was reduced compared to wild-type protein (Fig. 6F). Thus, we analysed internalisation of ectopic wild-type β3 integrin and its T753A mutant (Fig. 7A–D; supplementary material Fig. S3J). Whereas wild-type β3 integrin internalization was comparable to that of the endogenous protein, T753A β3 integrin showed a reduced rate of endocytosis (Fig. 6A,B) and less accumulation in endocytic vesicles (Fig. 6C,D and supplementary material Fig. S3J). Thus, PDK1 phosphorylates the β3 cytoplasmic tail and this phosphorylation is important for its endocytosis.

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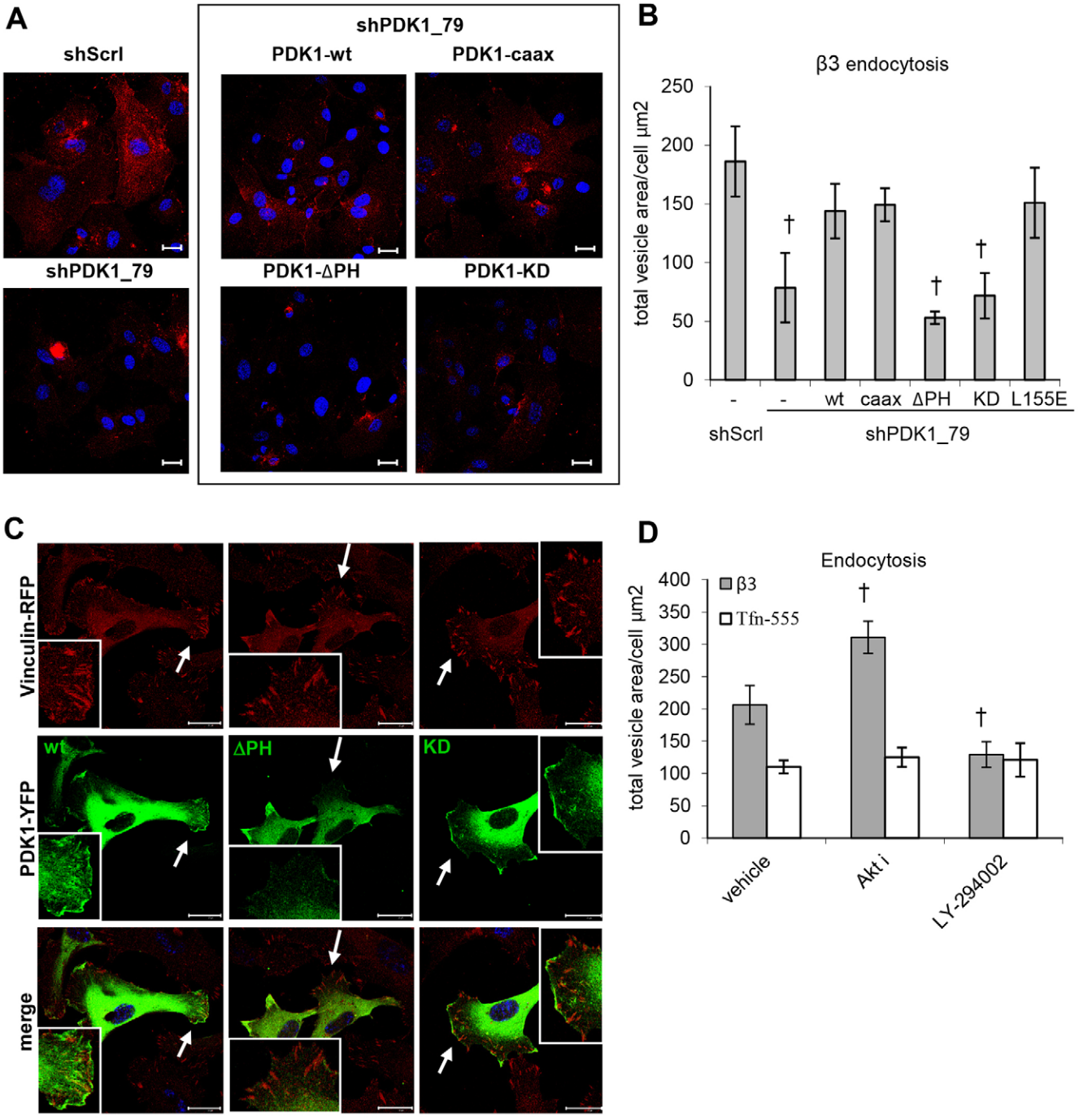

Fig. 5. PDK1 needs both the PH domain and kinase activity to control αvβ3 integrin endocytosis. (A) shPDK1_79 endothelial cells infected with lentivirus carrying different PDK1 mutants resistant to silencing (PDK1-wt, PDK1-caax, PDK1-ΔPH, PDK1-KD) and control endothelial cells (shScrl) were plated on vitronectin and incubated with anti-β3 antibody in presence of primaquine to allow internalisation of antibody-bound integrin (red, DAPI in blue). Scale bars: 20 µm. (B) Quantification of vesicle area/cell of experiment shown in A. Data were plotted as the mean±s.d.; $^{\dagger}P<0.05$ for knockdown endothelial cells versus control endothelial cells. (C) endothelial cells were transfected with Vinculin–RFP (red) in combination with different YFP-fused mutants of PDK1 (wt, ΔPH and KD, in green), seeded on vitronectin and fixed after 2 hours of adhesion; arrows indicate the zoomed regions. Scale bars: 20 µm. (D) Endocytosis of β3 and Tfn-555 was performed as described in A with wild-type endothelial cells in presence of Akt inhibitor (Akti), LY294002 or vehicle; then total vesicle area/cell was quantified. Data were plotted as mean±s.d.; $^{\dagger}P<0.05$ for treated cells versus control endothelial cells.

Endothelial cell migration and FA dynamics in membrane protrusions are controlled by PDK1

Next, we sought to determine whether PDK1 is necessary to regulate FA dynamics in endothelial cells, in particular for those forming in protruding regions. Therefore, we analysed paxillin-positive FAs using TIRF microscopy in live control and PDK1-silenced endothelial cells seeded on vitronectin (Fig. 8A,B and supplementary material Movies 1, 2). Control cells had adhesive structures heterogeneous in size, and migrated by assembling and disassembling focal structures within protrusions (Fig. 8A). The

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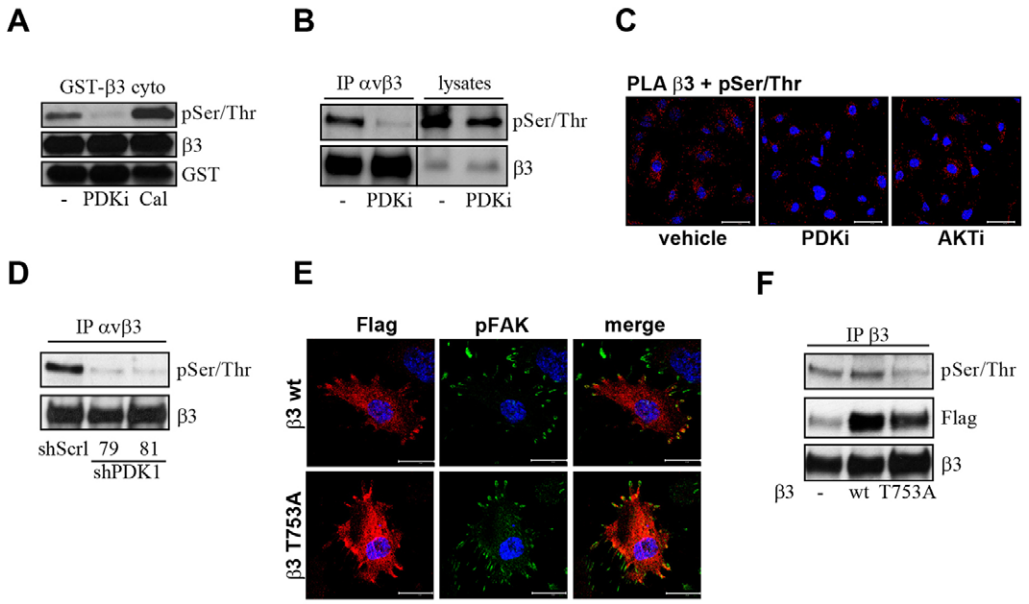

Fig. 6. The cytoplasmic tail of β3 integrin is phosphorylated by PDK1. (A) HEK 293T cells expressing GST-β3 cyto were treated for 1 hour with either the PDK1 inhibitor BX-795 (PDKi) or calyculin A (Cal); then lysed and purified with Glutathione Sepharose; GST-proteins were immunoblotted with the indicated antibody. (B) endothelial cells treated for 1 hour with the PDK1 inhibitor BX-795 (PDKi) were lysed and αvβ3 was immunoprecipitated; immunocomplexes and corresponding lysates were immunoblotted with the indicated antibody. (C) Endothelial cells that had been seeded on vitronectin, were treated with either the PDK1 inhibitor BX-795 (PDKi) or Akt inhibitor (AKTi) for 1 hour and a PLA assay was performed with the following antibodies: anti-β3/anti-pSer/Thr Akt-substrate. DAPI is in blue, red spots represent PLA signals. Scale bars: 50 μm. (D) Control (shScrl) and PDK1 knockdown (shPDK1_79 and 81) endothelial cells were lysed and αvβ3 was immunoprecipitated; immunocomplexes were immunoblotted with the indicated antibody. (E) β3 wild-type- and β3 T753A-expressing endothelial cells that had been seeded on vitronectin, were fixed after 2 hours of adhesion and stained with anti-phospho FAK (green) and anti-FLAG (red, DAPI in blue). Scale bars: 20 μm. (F) β3 wild-type- and β3 T753A-expressing endothelial cells were lysed and β3 was immunoprecipitated; immunocomplexes were immunoblotted with the indicated antibody.

majority of FAs in control endothelial cells was small and dot-like; by contrast, shPDK1_79 endothelial cells displayed a substantial number of FAs longer than 0.75 μm (Fig. 8B). To quantify the effect of PDK1 silencing, we compared silenced and control cells by isolating the images of several protrusions per filmed cell. Each protrusion was identified as a subregion of the cell expanding and retracting. Images were then segmented and the ratio of large and long to small and round adhesive structures was calculated for each protrusion. Typical protrusions are composed of small dot-like structures normally identified as focal complexes that can either disassemble or mature into elongated and thick structures usually referred to as FAs. shPDK1_79 endothelial cells had substantially larger and thicker adhesive structures in the protrusions (Fig. 8C). Notably, the re-expression of PDK1 wild-type in shPDK1_79 endothelial cells completely recovered the normal aspect of FAs (Fig. 8C; supplementary material Movie 3); by contrast, re-expression of PDK1-ΔPH had no effect on FA structure (Fig. 8C; supplementary material Movie 4).

It is known that alterations of FA dynamics affect the ability og the cell to directionally migrate. Thus, we evaluated the effect of PDK1 silencing in endothelial cells that were stimulated to migrate on vitronectin. PDK1 knockdown strongly reduced the ability of the cells to migrate compared to normal endothelial cells (Fig. 8D). We, therefore, conclude that PDK1, through PIP3-binding, modulates FA dynamics in cell protrusions and regulates endothelial cells motility.

DISCUSSION

In this study, we showed that endocytosis of β3 integrin is controlled by PDK1, which associates with and phosphorylates this integrin, promoting its internalisation. The ability of endothelial cells to adhere to and to directionally move on the ECM strictly relies on the integrin trafficking and FA dynamics. This prevailing idea is supported by several pieces of evidence demonstrating that integrin recycling contributes to cell migration (Caswell and Norman, 2006); (Pellinen and Ivaska, 2006); (Nishimura and Kaibuchi, 2007) and that integrins are endocytosed into Rab-labeled endocytic compartments during stimulation with growth factor (Roberts et al., 2001); (Pellinen et al., 2008). Integrin internalisation occurs through both clathrin-dependent and clathrin-independent mechanisms (Ezratty et al., 2009); (Nishimura and Kaibuchi, 2007); (Fabbri et al., 2005); (Shi and Sottile, 2008); (Gu et al., 2011). Nevertheless, how integrin endocytosis and recycling are dynamically regulated in a spatially and temporally restricted manner is not completely clear. Under our experimental conditions αvβ3 integrin internalisation mainly depends on membrane lipid-rafts but not on macropinocytosis.

Our experimental evidence suggests that PDK1 regulates αvβ3 integrin endocytosis by directly associating with the integrin. Moreover, kinase-inactive PDK1 is unable to stimulate integrin endocytosis, suggesting that a phosphorylated substrate is involved in this process. However, inactivation of Akt, one of the most relevant PDK1 substrates, does not reduce integrin endocytosis rate, indicating an Akt-independent role for PDK1 in this process. Accordingly we were able to detect PDK1-dependent phosphorylation on Thr753 of β3 integrin, a position that has been previously identified as an *in vitro* PDK1 phosphorylation target (Kirk et al., 2000). Although it has been reported that phosphorylation of this threonine/serine-rich region decreases the binding of integrins for filamin, the importance of

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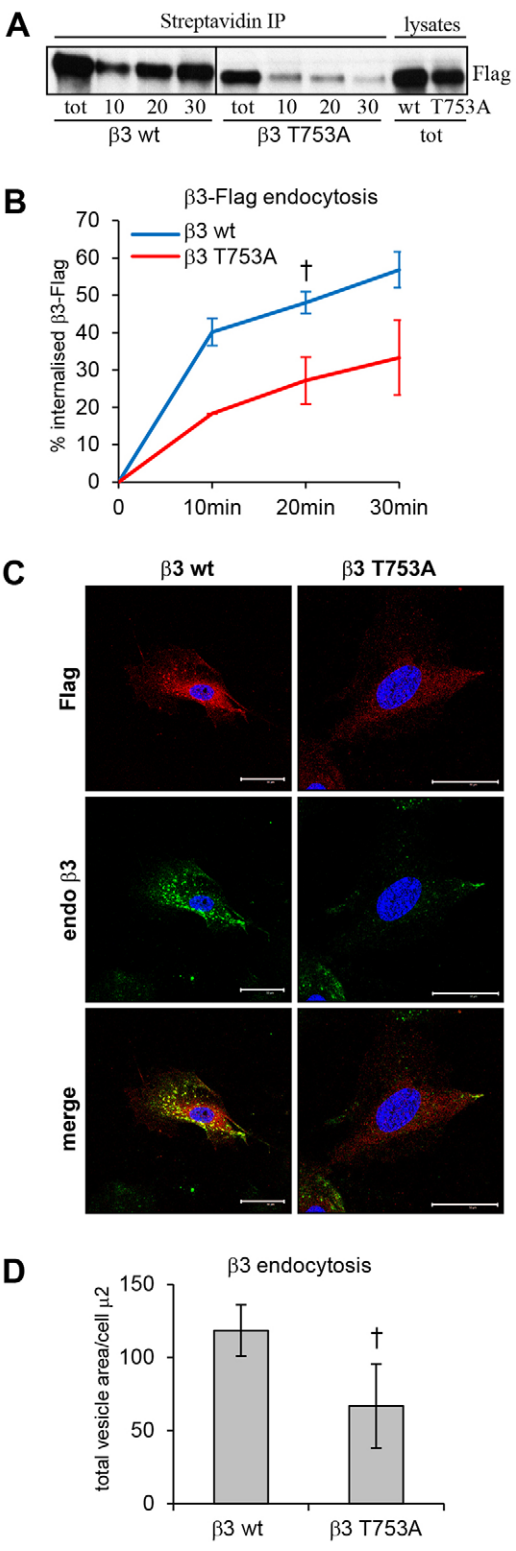

Fig. 7. Thr753 of β3 integrin regulates its endocytosis. (A,B) Endocytosis of β3-FLAG in presence of primaquine was evaluated in β3 wild-type- and β3 T753A-expressing endothelial cells after 10, 20 and 30 minutes. Total (tot) and internalised biotinylated integrin was quantified by streptavidin-agarose immunoprecipitation and anti-FLAG immunoblotting (A). Data were plotted as the mean±s.d. of the percentage of total biotinylated β3-FLAG integrin that had been internalised (B); $^{\dagger}P<0.05$ for β3 T753A- endothelial cells versus β3 wild-type endothelial cells. (C) β3 wild-type- and β3 T753A-expressing endothelial cells were plated on vitronectin and incubated with anti-β3 antibody at 37°C in presence of primaquine to allow internalisation of antibody-bound integrin (green, DAPI in blue); as a control ectopic β3 integrin was stained with anti-FLAG antibody (red). Scale bars: 50 µm. (D) Quantification of total vesicles area/cell after 30 minutes of endocytosis. Data were plotted as the mean±s.d.; $^{\dagger}P<0.05$ for β3 T753A-expressing endothelial cells versus β3 wild-type-expressing endothelial cells.

this phosphorylation site *in vivo* remains to be determined (Kiema et al., 2006).

Although no clear evidence of a direct PDK1 involvement in the integrin function is currently available, an interaction between PDK1 and the Ca^{2+}-activated tyrosine kinase Pyk2 in regulating the integrity of FAs has been observed (Taniyama et al., 2003). Furthermore, the homologues of mammalian PDK1 in *Saccharomyces cerevisiae* (Pkh1 and Pkh2) has been shown to be involved in the endocytic trafficking pathway (Friant et al., 2001); (deHart et al., 2002), but the molecular details are yet to be elucidated.

By contrast, the crucial role of localising PDK1 to the membrane through activation of PI3K in regulating both substrate specificity and spatially restricted activation has been clearly established (Currie et al., 1999). Notably, the PH domain of PDK1, which preferentially binds PIP3 at the membrane (McManus et al., 2004), is required for αvβ3 integrin endocytosis. Consistently, inhibition of PI3K activity reduces endocytosis of αvβ3 integrin, although less than PDK1 silencing. Therefore, these results support a model in which both membrane translocation mediated through the PH domain and interaction with the integrin are involved in endocytosis. A similar mechanism has been described for kindlin-2 (officially known as FERMT2), which interacts through its FERM domain with integrin αIIbβ3 but requires binding of PIP3 to its PH domain for the regulation of integrin activity (Liu et al., 2011).

Although the process of FA disassembly remains to be fully elucidated, integrin endocytosis and microtubule targeting are known to regulate FA turnover (Ezratty et al., 2005); (Nishimura and Kaibuchi, 2007). In the absence of PDK1, endothelial cells showed FAs with altered morphology and that were more stable than in normal endothelial cells. This phenotype can be attributed to altered FA dynamics (Chao and Kunz, 2009). The slow FA disassembly during microtubule re-growth and the reduced integrin endocytosis in endothelial cell knockdown for PDK1 suggest that FA disassembly – instead of assembly – is regulated by PDK1. Under normal culture condition PDK1 is only relatively weakly enriched in FAs. Nevertheless, when FA turnover was synchronised by treatment with nocodazole, PDK1 was clearly localised to FAs, and rapidly disappeared during nocodazole washout. This is consistent with the highly dynamic association between PDK1 and integrin αvβ3 in close proximity to the plasma membrane.

Whether and how integrin endocytosis contributes to FA dynamics is still debated, but recent evidence shows a crucial role for endocytosis in FA disassembly induced by nocodazole washout (Ezratty et al., 2009). Accordingly, PDK1 could regulate FA size and turnover by affecting integrin endocytosis. In this context, the role of the PH domain of PDK1 is particularly interesting. If PDK1 lacks the ability to bind PIP3, it is no longer able to promote integrin endocytosis or FA disassembly. From this point of view, the PIP3 binding ability of PDK1 could contribute to control FA dynamics to a specific intracellular compartment, for example, defined by the localised PI3K activity

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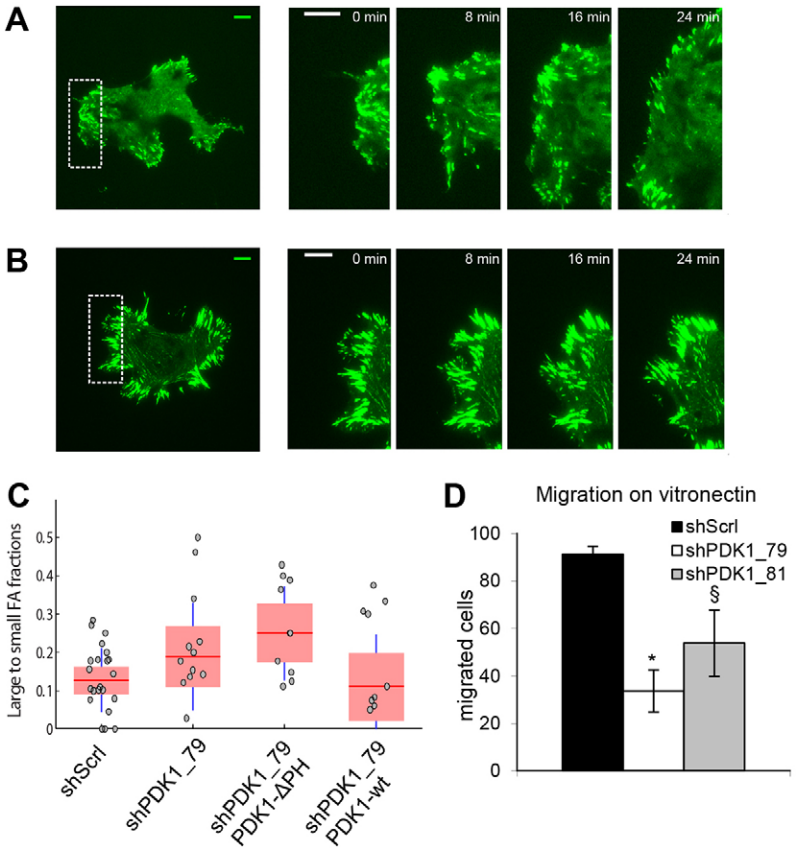

Fig. 8. Adhesive structures in membrane protrusions and migration towards vitronectin are regulated by PDK1. (A,B) Representative shScrl (A) and shPDK1_79 (B) endothelial cells transduced with paxillin–GFP and imaged using TIRF microscopy are shown. The area marked by the dashed rectangle is enlarged in the right panels and representative snapshots of the expanding protrusion were taken every 8 minutes for 25 minutes. Scale bars: 10 μm. (C) Images of several protrusions per filmed silenced and control endothelial cells were analysed. Each protrusion was identified as a subregion of the cell expanding and retracting. Images were then segmented and the ratio of large-and-long to small-and-round adhesive structures was calculated for each protrusion. Each point in the plot thus represents one protrusion. shScrl (n=22 protrusions with Na=779, where Na is the total number of identified adhesive structures) versus shPDK1_79 (n=12, Na=409, P=0.0124); shScrl versus shPDK1_79_ΔPH (n=10, Na=344, $P=7.5623\times10^{-4}$). Statistical tests were performed using one-tailed t-test with a significance level of 0.05. (D) Control (shScrl) and PDK1-knockdown endothelial cells (shPDK1_79 and shPDK1_81) were used in a Transwell migration assay, in presence of vitronectin coating. Data were plotted as the mean±s.d.;*P<0.0005, §P<0.005 for knockdown versus control endothelial cells.

in response to chemotactic stimulation. Although this concept has not been specifically tested here, the rostro-caudal PIP3 gradient in polarised cells, maintained by PTEN activity in the rear of the cell, could be transformed in a PDK1-dependent manner into differentially behaving cell–matrix adhesion sites.

The process of endothelial migration through provisional matrix, such as fibronectin and vitronectin, is a critical event in the formation of new vessels in pathological conditions (Silva et al., 2008). High expression levels of αvβ3 integrin characterise angiogenic endothelium, and blocking αv integrin function by disrupting ligand binding can produce an anti-angiogenic effect (Brooks et al., 1994). Recently, trafficking of αvβ3 integrin to and from the cell surface, has been demonstrated to influence persistent cell migration, endothelial cell motility and angiogenesis (Caswell et al., 2007); (di Blasio et al., 2010); (Reynolds et al., 2009). Accordingly, our results show that an alteration of αvβ3 endocytosis causes defects in endothelial cell migration on provisional ECM molecules such as vitronectin. Taken together, these pieces of evidence suggest that PDK1 regulation of αvβ3 endocytosis directly affects angiogenesis, integrating PI3K signalling activated by membrane receptors with ECM dynamic interaction.

MATERIALS AND METHODS

Cell culture and transfection

Human endothelial cells were isolated from umbilical cord veins and cultured as previously described (Primo et al., 2007). HEK 293T cells were grown in IMDM (Sigma) supplemented with 10% FBS, L-Glutamine (2 mM, Sigma) and antibiotics. Endothelial cells were transfected by using Lipofectamine Plus reagent (Life Technologies) according to the manufacturer's instructions. The following constructs were used: paxillin–GFP, vinculin–RFP, Rab4–GFP, Rab5–GFP, Rab11–GFP (kindly provided by Serini G.) and different mutants of PDK1 fused to YFP (Gagliardi et al., 2014).

Lentivirus-driven PDK1 shRNA and PDK1 cDNA infection

Short hairpin RNAs (shRNAs) against human PDK1 (two constructs, TRCN0000039779 and TRCN0000039781 named shPDK1_79 and shPDK1_81, respectively) were purchased from TRC-Hs1.0 library (Sigma). Self-inactivating lentiviral particles were produced as previously described (Gagliardi et al., 2012).

Silencing-resistant MYC-tagged PDK1 wild-type, PDK1-caax, PDK1-ΔPH, PDK1-KD (K110N) and PDK1-L155E, previously cloned into PINCO retroviral vector (Primo et al., 2007), were subcloned into a third-generation lentiviral vector pCCL.sin.cPPT.polyA.CTE.eGFP.minh CMV.hPGK (Amendola et al., 2005); (Follenzi et al., 2000) as described in (Gagliardi et al., 2012).

β3 integrin point mutation and cloning

β3 integrin, previously cloned into pcDNA3 vector (Woods et al., 2004), was subcloned into the third-generation lentiviral vector PWPXL (http://tronolab.epfl.ch/) with In-Fusion® HD EcoDry™ Cloning-Kit (Clontech Laboratories). To add the FLAG-tag and to mutate Thr753 to Ala (T753A), the following primers were designed: 'β3 FW' 5′-GCCTAAG-CTTACGCGTATGCGAGCGCGACCGAGGCCC-3′, 'β3 wt RE' 5′-AGGTTGATTATCATATGTTACTTGTCATCGTCATCCTTGTAATC-AGTGCCCCGGTACGTGATATTGGTGAAGGTAGA-3′, and 'β3 T753A RE' 5′-AGGTTGATTATCATATGTTACTTGTCATCGTCATCCTTG-TAATCAGTGCCCCGGTACGTGATATTGGTGAAGGCAGA-3′. The acceptor plasmid PWPXL was digested at *Mlu*I and *Nde*I sites.

Adhesion assay

Cell adhesion was evaluated with xCELLigence system (Roche). 96-well microtiter plates that are specifically designed to measure cellular impedance (E-Plate, Roche) were coated with vitronectin, fibronectin or

collagen type-I (0.25 µg/ml) for 1 hour at 37℃ and then saturated with 3% BSA. 1×10^4 endothelial cells were resuspended in 0.1 ml of serum-free medium and transferred on E-Plates after background measurement. When used, the αvβ3 integrin inhibitor cRGDfV (200 nM, Merck-Millipore) was added before transferring cells on E-Plates. The extent of cell adhesion and spreading, measured as changes in impedance, was monitored every 15 seconds for a period of 2 hours. The measured impedance was expressed as relative impedance (Cell Index). The Cell Index at each time point is defined as $(Rn-Rb)/(15\ \Omega)$, where Rn is the cell-electrode impedance of the well when it contains cells and Rb is the background impedance of the well with the medium alone. As a control, the same number of cells was plated on 96-wells plates coated with vitronectin, fibronectin or collagen type-I. After 2 hours of adhesion, cells were fixed, stained and photographed to measure to spreading area.

FA disassembly assay

This assay was performed as described in Ezratty et al., 2005 with modifications. Briefly, endothelial cells were seeded on vitronectin-coated glass coverslips (1 µg/ml) and treated with 4 µM nocodazole (Sigma) for 1 hour to completely depolymerise microtubules. The drug was washed out with serum-free medium, and microtubules were allowed to re-polymerise for 30 minutes. Cells were then fixed with 4% paraformaldehyde in PBS for 10 minutes followed by immunofluorescence staining.

Immunofluorescence microscopy

To analyse the FAs, endothelial cells were plated on glass coverslips coated with vitronectin, fibronectin or collagen type-I (1 µg/ml). After 3 hours of adhesion, cells were fixed and stained as previously described (di Blasio et al., 2010). The following antibodies were used: anti-αvβ3 (MAB1976, Millipore), anti-paxillin (BD Biosciences), anti-α tubulin (Santa Cruz Biotechnology), anti-PDK1 (Sigma and BD Biosciences), anti-β5 (Cell Signaling Technology and Millipore), anti-αv (AB1923, Millipore), anti-phospho FAK (Millipore) and anti-FLAG (Cell Signaling Technology). Analysis was performed using a confocal laser-scanning microscope (TCS SP2 with DM IRE2; Leica) equipped with a 63×/1.40 HCX Plan-Apochromat oil-immersion objective. Confocal images are the maximum projections of a *z*-section of ~1.50 µm. The images were arranged and labelled using Photoshop software (Adobe). Size, area/cell and number/cell of FA were quantified in 50 cells/sample, captured with the same zoom, from three independent experiments, using Image J.

To measure the degree of colocalization of PDK1 and αvβ3 integrin, we used paxillin staining to define ROIs corresponding to FAs by filtering with a high-pass filter to enhance regions that contain high amounts of paxillin–GFP followed by manual thresholding. Average PDK1 and αvβ3 intensities within each FA were divided by the average intensity within cells. Cells that underwent nocodazole washout were not added in the quantification as FAs were absent in these cells and high paxillin staining would not be a well-defined, biologically known ROI.

To generate simulated random images, first a random image was generated and multiplicative noise was added to it. Finally, median filtering was applied to recreate patches of the same size of those found in PDK1 images. A representative image is shown in supplementary material Fig. S3A.

The internalisation of β3 integrin was performed as previously described (di Blasio et al., 2010). When indicated, 24 hours before the assay, endothelial cells were transfected with GFP-tagged Rab proteins. To visualise β3 tagged with FLAG, anti-FLAG antibody was used (Cell Signaling Technology). The same protocol was followed for the analysis of Tfn-555 (20 µm/ml, Life Technologies) endocytosis. The inhibitors used are: Amiloride (50 µM, Sigma), Dynasore (80 µM, Sigma), Cyclodextrin (5 mM, Sigma), LY-294002 (10 µM, Sigma), Akti (InSolution™ Akt inhibitor VIII, 5 µM, Calbiochem).

The vesicle area/cell was quantified in 50 cells/sample, captured with the same zoom, from three independent experiments, using ImageJ.

Immunoprecipitation and western blots

After 1-hour treatment with nocodazole in 10% FBS medium at 37℃ (4 µm, Sigma), cells were washed with serum-free medium and either transferred to ice (0 minutes washout) or to 37℃ with fresh 10% FBS medium for 30 minutes (30 minutes washout). Then cells were washed twice with cold PBS, and lysed in EB buffer as previously described (di Blasio et al., 2010). 1 mg of proteins was incubated with anti-αvβ3 (MAB1976, Millipore), anti-PDK1 (BD Biosciences), anti-α2β1 (Millipore), anti-β3 (N20, Santa Cruz Biotechnologies) or negative control mouse IgG for 2 hours; immune complexes were recovered on anti-mouse IgG-Agarose (Sigma). To analyse the membrane localisation of wild-type and T753A mutant of β3 integrin, we surface-labelled endothelial cells at 4℃ with 0.2 mg/ml N-Hydroxysulfosuccinimide (NHS)-SS-biotin (Pierce) in PBS for 30 minutes and then immunoprecipitated all biotinylated proteins by using streptavidin-agarose conjugate (Millipore). Proteins were separated by SDS-PAGE, blotted and incubated with primary antibody (anti-PDK1 [Cell Signaling Technology], anti-αv [AB1930, Millipore], anti-β3 [N20, Santa Cruz Biotechnologies], anti-β1 [Millipore], anti-pSer/Thr Akt-substrate [Cell Signaling Technology], anti-FLAG [Cell Signaling Technology]).

Peptide and GST pull-down assays

Peptide pull-down assays were carried out using synthetic, N-terminally biotin-coupled peptide corresponding to the cytoplasmic portion of αv and α2 integrin (biotin-YRMGFFKRVRPPQEEQEREQLQPHENGEGNSET and biotin-WKLGFFKRKYEKMTKNPDEIDETTELSS, Genscript) as previously described (Schiller et al., 2013).

For GST pull-down assays, the cytoplasmic tail of β3 integrin was cloned into pDONR/zeo using Gateway BP Clonase (Life technologies). To perform the BP recombination we previously performed a PCR reaction using the following primers: 'attB1-β3 cyto' 5′-GGGGACAAG-TTTGTACAAAAAAGCAGGCTTAACCATGAAACTCCTCATCACC-ATCCACGAC-3′, and 'attB2-β3 cyto' 5′-GGGGACCACTTTGTAC-AAGAAAGCTGGGTCAGTGCCCCGGTACGTGATATTGGT-3′,. The construct was then transferred through Gateway LR Clonase in the pDEST27 (GST-N-Term Tag, Life Technologies). pDEST27 and pDEST27-β3 cyto constructs were transfected into HEK 293T cells with calcium phosphate (Promega) and GST and GST-β3 cyto were isolated and used to pull-down proteins from endothelial cell extracts as described in Gagliardi et al., 2014. To analyse the phosphorylation of the β3 cytoplasmic tail, HEK 293T cells expressing the pDEST27-β3 cyto construct were treated with either the PDK1 inhibitor BX-795 (1 µm, Axon Medchem) or calyculin A (10 nM, Merck-Millipore) for 1 hour; GST-β3 cyto was isolated with Glutathione Sepharose 4B beads and phosphorylation was analysed by western blotting.

Proximity ligation assay (PLA)

PLA was performed according to the Olink® Bioscience's protocol using Duolink® II reagents, with minor changes. After blocking and staining with primary antibodies (anti-PDK1 + anti-αv, anti-PDK1 + anti-β3 [C-20, Santa Cruz Biotechnologies], anti-PDK1 + anti-α3 [AB1920, Millipore] and PDK1 + a specific rabbit IgG as negative control), PLA anti-mouse MINUS and anti-rabbit PLUS or PLA anti-goat MINUS and anti-rabbit PLUS probes were diluted 1:5 in PBS 1% donkey serum and incubated for 1 hour at 37℃ in a pre-heated humidity chamber. Subsequent ligation and amplification steps were performed using Duolink® II Detection Kit according to the manufacturer's protocol.

To study the phosphorylation of β3 integrin, endothelial cells that had been seeded on vitronectin, were treated with either the PDK1 inhibitor BX-795 or Akt inhibitor for 1 hour and then analysed as described above using anti-β3 + anti-pSer/Thr Akt substrate antibodies and PLA anti-goat MINUS and anti-rabbit PLUS probes.

Endocytosis assay

Endocytosis assay were performed as previously described in (Roberts et al., 2001) and modified in di Blasio et al., 2010. When endocytosis during nocodazole washout was assayed, endothelial cells were treated with nocodazole as described above and then labelled with biotin. Levels of biotinylated integrin were determined by capture-ELISA with anti-αvβ3 (MAB1976, Millipore), anti-β3 (BD Biosciences) and anti-α5 (BD Biosciences). To analyse the endocytosis of wild-type and T753A mutant of β3 integrin, total biotinylated proteins were immunoprecipitated by

Streptavidin-agarose conjugate and then ectopic β3 was visualised by SDS-PAGE electrophoresis and immunoblotting with anti-FLAG antibody. Bands intensities from three independent experiments were quantified using Image J software.

Flow cytometry

To detect levels of β3 integrin cell surface expression, cells were detached with EDTA 10 mM in PBS and incubated with primary antibody (anti-β3, BD Biosciences) for 30 minutes at 4℃, and Alexa Fluor 488-conjugated secondary antibody at 4℃ for 30 minutes. Cells were analysed on a Beckman Coulter Cyan ADP.

Total internal reflection fluorescence (TIRF) microscopy

Endothelial cells transfected with paxillin–GFP were seeded at low density on glass-bottomed plates (MatTek Corporation) coated with vitronectin (0.75 µg/ml). After 1 hour of adhesion, the cells were placed on an inverted microscope equipped with a humidified chamber at 37℃ and 5% CO_2, and visualised using True MultiColor Laser TIRF Leica AM TIRF MC (Leica Microsystems).

To quantify FA size and shape, we employed image analysis algorithm developed in (Zamir et al., 1999); (Berginski et al., 2011). Briefly, images of paxillin–GFP endothelial cells, acquired by means of TIRF microscopy, were filtered with a high-pass filter to enhance high paxillin–GFP regions. The width of the filter used was 1.3 µm. Filtered images were thresholded by an empirically determined value chosen to identify adhesive structures. Identified structures were then filtered to eliminate segmentation errors. In particular, objects with a major-to-minor axis ratio >5 and that were bigger than 5 μm^2 were disregarded. Threshold values used to discriminate between large/long and small/round adhesions were 0.75 µm long and a major-to-minor axis ratio of 2.5. In practice, these two ratios correspond to the adhesions that are large and elongated, and adhesions that are small and round, respectively. Image analysis routines were written in MATLAB.

Cell migration assay

Endothelial cells were starved overnight and then seeded on the upper side of 8-µm pore filters of Transwell chambers (Falcon, 5×10^4 cells/well) coated on both sides with vitronectin (5 µg/ml). After 5 hours of incubation at 37℃ and in 5% CO_2, cells on the upper side of the filters were mechanically removed and those that had migrated to the lower side of the filters were fixed in 2.5% glutaraldehyde for 30 minutes, and stained with 0.1% Crystal Violet. Four random fields of each sample in the lower surface of the filters were counted at 10×magnification.

Competing interests

The authors declare no competing or financial interests.

Author contributions

L.d.B. and L.P. conceived the project and designed experimental details. L.d.B. performed experiments. P.A.G. set up PDK1 silencing and GST pull-down experiments. A.P. performed analysis of focal adhesions in live cells. G.S. set up the confocal microscopy analysis. R.S. set up the FACS analysis. L.d.B. and L.P. wrote the manuscript with contributions from all authors. F.B. and L.P. obtained funding and supervised the study.

Funding

This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) [grant numbers IG 10133 to F.B., 14635 and 9158 to L.P.]; Fondazione Piemontese per la Ricerca sul Cancro (Intramural grant) [grant number 5x1000 2008 to L.P.]; MIUR- Fondo Investimenti per la Ricerca di Base RBAP11BYNP (Newton) (to F.B. and L.P.); University of Torino- Compagnia di San Paolo, grant RETHE (to F.B.), grant GeneRNet (to L.P.), FP7-ICT-2011-8 Biloba [grant number 318035]; and CNR (grant ''Personalized Medicine''). P.A.G. is supported by a triennial FIRC fellowship [grant number 15026].

Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.149294/-/DC1

References

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr. Biol.* **7**, 261-269.

Amendola, M., Venneri, M. A., Biffi, A., Vigna, E. and Naldini, L. (2005). Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. *Nat. Biotechnol.* **23**, 108-116.

Anderson, K. E., Coadwell, J., Stephens, L. R. and Hawkins, P. T. (1998). Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. *Curr. Biol.* **8**, 684-691.

Avraamides, C. J., Garmy-Susini, B. and Varner, J. A. (2008). Integrins in angiogenesis and lymphangiogenesis. *Nat. Rev. Cancer* **8**, 604-617.

Bader, B. L., Rayburn, H., Crowley, D. and Hynes, R. O. (1998). Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. *Cell* **95**, 507-519.

Berginski, M. E., Vitriol, E. A., Hahn, K. M. and Gomez, S. M. (2011). High-resolution quantification of focal adhesion spatiotemporal dynamics in living cells. *PLoS ONE* **6**, e22025.

Bershadsky, A., Chausovsky, A., Becker, E., Lyubimova, A. and Geiger, B. (1996). Involvement of microtubules in the control of adhesion-dependent signal transduction. *Curr. Biol.* **6**, 1279-1289.

Biondi, R. M., Kieloch, A., Currie, R. A., Deak, M. and Alessi, D. R. (2001). The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *EMBO J.* **20**, 4380-4390.

Brooks, P. C., Clark, R. A. and Cheresh, D. A. (1994). Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science* **264**, 569-571.

Broussard, J. A., Webb, D. J. and Kaverina, I. (2008). Asymmetric focal adhesion disassembly in motile cells. *Curr. Opin. Cell Biol.* **20**, 85-90.

Burridge, K. and Chrzanowska-Wodnicka, M. (1996). Focal adhesions, contractility, and signaling. *Annu. Rev. Cell Dev. Biol.* **12**, 463-519.

Caswell, P. T. and Norman, J. C. (2006). Integrin trafficking and the control of cell migration. *Traffic* **7**, 14-21.

Caswell, P. and Norman, J. (2008). Endocytic transport of integrins during cell migration and invasion. *Trends Cell Biol.* **18**, 257-263.

Caswell, P. T., Spence, H. J., Parsons, M., White, D. P., Clark, K., Cheng, K. W., Mills, G. B., Humphries, M. J., Messent, A. J., Anderson, K. I. et al. (2007). Rab25 associates with alpha5beta1 integrin to promote invasive migration in 3D microenvironments. *Dev. Cell* **13**, 496-510.

Caswell, P. T., Chan, M., Lindsay, A. J., McCaffrey, M. W., Boettiger, D. and Norman, J. C. (2008). Rab-coupling protein coordinates recycling of alpha5beta1 integrin and EGFR1 to promote cell migration in 3D microenvironments. *J. Cell Biol.* **183**, 143-155.

Caswell, P. T., Vadrevu, S. and Norman, J. C. (2009). Integrins: masters and slaves of endocytic transport. *Nat. Rev. Mol. Cell Biol.* **10**, 843-853.

Chao, W. T. and Kunz, J. (2009). Focal adhesion disassembly requires clathrin-dependent endocytosis of integrins. *FEBS Lett.* **583**, 1337-1343.

Collins, B. J., Deak, M., Arthur, J. S., Armit, L. J. and Alessi, D. R. (2003). In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *EMBO J.* **22**, 4202-4211.

Currie, R. A., Walker, K. S., Gray, A., Deak, M., Casamayor, A., Downes, C. P., Cohen, P., Alessi, D. R. and Lucocq, J. (1999). Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1. *Biochem. J.* **337**, 575-583.

deHart, A. K., Schnell, J. D., Allen, D. A. and Hicke, L. (2002). The conserved Pkh-Ypk kinase cascade is required for endocytosis in yeast. *J. Cell Biol.* **156**, 241-248.

di Blasio, L., Droetto, S., Norman, J., Bussolino, F. and Primo, L. (2010). Protein kinase D1 regulates VEGF-A-induced alphavbeta3 integrin trafficking and endothelial cell migration. *Traffic* **11**, 1107-1118.

Ezratty, E. J., Partridge, M. A. and Gundersen, G. G. (2005). Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat. Cell Biol.* **7**, 581-590.

Ezratty, E. J., Bertaux, C., Marcantonio, E. E. and Gundersen, G. G. (2009). Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. *J. Cell Biol.* **187**, 733-747.

Fabbri, M., Di Meglio, S., Gagliani, M. C., Consonni, E., Molteni, R., Bender, J. R., Tacchetti, C. and Pardi, R. (2005). Dynamic partitioning into lipid rafts controls the endo-exocytic cycle of the alphaL/beta2 integrin, LFA-1, during leukocyte chemotaxis. *Mol. Biol. Cell* **16**, 5793-5803.

Follenzi, A., Ailles, L. E., Bakovic, S., Geuna, M. and Naldini, L. (2000). Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat. Genet.* **25**, 217-222.

Friant, S., Lombardi, R., Schmelzle, T., Hall, M. N. and Riezman, H. (2001). Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. *EMBO J.* **20**, 6783-6792.

Friedl, P. and Wolf, K. (2010). Plasticity of cell migration: a multiscale tuning model. *J. Cell Biol.* **188**, 11-19.

Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A. and Cheresh, D. A. (1995). Definition of two angiogenic pathways by distinct alpha v integrins. *Science* **270**, 1500-1502.

Gagliardi, P. A., di Blasio, L., Orso, F., Seano, G., Sessa, R., Taverna, D., Bussolino, F. and Primo, L. (2012). 3-phosphoinositide-dependent kinase 1 controls breast tumor growth in a kinase-dependent but Akt-independent manner. *Neoplasia* **14**, 719-731.

Gagliardi, P. A., di Blasio, L., Puliafito, A., Seano, G., Sessa, R., Chianale, F., Leung, T., Bussolino, F. and Primo, L. (2014). PDK1-mediated activation of MRCKα regulates directional cell migration and lamellipodia retraction. *J. Cell Biol.* **206**, 415-434.

Gu, Z., Noss, E. H., Hsu, V. W. and Brenner, M. B. (2011). Integrins traffic rapidly via circular dorsal ruffles and macropinocytosis during stimulated cell migration. *J. Cell Biol.* **193**, 61-70.

Gutheil, J. C., Campbell, T. N., Pierce, P. R., Watkins, J. D., Huse, W. D., Bodkin, D. J. and Cheresh, D. A. (2000). Targeted antiangiogenic therapy for cancer using Vitaxin: a humanized monoclonal antibody to the integrin alphavbeta3. *Clin. Cancer Res.* **6**, 3056-3061.

Hodivala-Dilke, K. M., McHugh, K. P., Tsakiris, D. A., Rayburn, H., Crowley, D., Ullman-Culleré, M., Ross, F. P., Coller, B. S., Teitelbaum, S. and Hynes, R. O. (1999). Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J. Clin. Invest.* **103**, 229-238.

Huang, X., Griffiths, M., Wu, J., Farese, R. V., Jr and Sheppard, D. (2000). Normal development, wound healing, and adenovirus susceptibility in beta5-deficient mice. *Mol. Cell. Biol.* **20**, 755-759.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687.

Hynes, R. O. (2007). Cell-matrix adhesion in vascular development. *J. Thromb. Haemost.* **5 Suppl. 1,** 32-40.

Kiema, T., Lad, Y., Jiang, P., Oxley, C. L., Baldassarre, M., Wegener, K. L., Campbell, I. D., Ylänne, J. and Calderwood, D. A. (2006). The molecular basis of filamin binding to integrins and competition with talin. *Mol. Cell* **21**, 337-347.

Kirk, R. I., Sanderson, M. R. and Lerea, K. M. (2000). Threonine phosphorylation of the beta 3 integrin cytoplasmic tail, at a site recognized by PDK1 and Akt/PKB in vitro, regulates Shc binding. *J. Biol. Chem.* **275**, 30901-30906.

Lauffenburger, D. A. and Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.

Lee, J., Ishihara, A. and Jacobson, K. (1993). How do cells move along surfaces? *Trends Cell Biol.* **3**, 366-370.

Liu, Y., Wang, J., Wu, M., Wan, W., Sun, R., Yang, D., Sun, X., Ma, D., Ying, G. and Zhang, N. (2009). Down-regulation of 3-phosphoinositide-dependent protein kinase-1 levels inhibits migration and experimental metastasis of human breast cancer cells. *Mol. Cancer Res.* **7**, 944-954.

Liu, J., Fukuda, K., Xu, Z., Ma, Y. Q., Hirbawi, J., Mao, X., Wu, C., Plow, E. F. and Qin, J. (2011). Structural basis of phosphoinositide binding to kindlin-2 protein pleckstrin homology domain in regulating integrin activation. *J. Biol. Chem.* **286**, 43334-43342.

McManus, E. J., Collins, B. J., Ashby, P. R., Prescott, A. R., Murray-Tait, V., Armit, L. J., Arthur, J. S. and Alessi, D. R. (2004). The in vivo role of PtdIns(3,4,5)P3 binding to PDK1 PH domain defined by knockin mutation. *EMBO J.* **23**, 2071-2082.

Mora, A., Komander, D., van Aalten, D. M. and Alessi, D. R. (2004). PDK1, the master regulator of AGC kinase signal transduction. *Semin. Cell Dev. Biol.* **15**, 161-170.

Nabors, L. B., Mikkelsen, T., Rosenfeld, S. S., Hochberg, F., Akella, N. S., Fisher, J. D., Cloud, G. A., Zhang, Y., Carson, K., Wittemer, S. M. et al. (2007). Phase I and correlative biology study of cilengitide in patients with recurrent malignant glioma. *J. Clin. Oncol.* **25**, 1651-1657.

Nishimura, T. and Kaibuchi, K. (2007). Numb controls integrin endocytosis for directional cell migration with aPKC and PAR-3. *Dev. Cell* **13**, 15-28.

Pellinen, T. and Ivaska, J. (2006). Integrin traffic. *J. Cell Sci.* **119**, 3723-3731.

Pellinen, T., Tuomi, S., Arjonen, A., Wolf, M., Edgren, H., Meyer, H., Grosse, R., Kitzing, T., Rantala, J. K., Kallioniemi, O. et al. (2008). Integrin trafficking regulated by Rab21 is necessary for cytokinesis. *Dev. Cell* **15**, 371-385.

Pinner, S. and Sahai, E. (2008). PDK1 regulates cancer cell motility by antagonising inhibition of ROCK1 by RhoE. *Nat. Cell Biol.* **10**, 127-137.

Primo, L., di Blasio, L., Roca, C., Droetto, S., Piva, R., Schaffhausen, B. and Bussolino, F. (2007). Essential role of PDK1 in regulating endothelial cell migration. *J. Cell Biol.* **176**, 1035-1047.

Regen, C. M. and Horwitz, A. F. (1992). Dynamics of beta 1 integrin-mediated adhesive contacts in motile fibroblasts. *J. Cell Biol.* **119**, 1347-1359.

Reynolds, A. R., Hart, I. R., Watson, A. R., Welti, J. C., Silva, R. G., Robinson, S. D., Da Violante, G., Gourlaouen, M., Salih, M., Jones, M. C. et al. (2009). Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors. *Nat. Med.* **15**, 392-400.

Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T. and Horwitz, A. R. (2003). Cell migration: integrating signals from front to back. *Science* **302**, 1704-1709.

Roberts, M., Barry, S., Woods, A., van der Sluijs, P. and Norman, J. (2001). PDGF-regulated rab4-dependent recycling of alphavbeta3 integrin from early endosomes is necessary for cell adhesion and spreading. *Curr. Biol.* **11**, 1392-1402.

Schiller, H. B., Hermann, M. R., Polleux, J., Vignaud, T., Zanivan, S., Friedel, C. C., Sun, Z., Raducanu, A., Gottschalk, K. E., Théry, M. et al. (2013). β1- and αv-class integrins cooperate to regulate myosin II during rigidity sensing of fibronectin-based microenvironments. *Nat. Cell Biol.* **15**, 625-636.

Shi, F. and Sottile, J. (2008). Caveolin-1-dependent beta1 integrin endocytosis is a critical regulator of fibronectin turnover. *J. Cell Sci.* **121**, 2360-2371.

Silva, R., D'Amico, G., Hodivala-Dilke, K. M. and Reynolds, L. E. (2008). Integrins: the keys to unlocking angiogenesis. *Arterioscler. Thromb. Vasc. Biol.* **28**, 1703-1713.

Smilenov, L. B., Mikhailov, A., Pelham, R. J., Marcantonio, E. E. and Gundersen, G. G. (1999). Focal adhesion motility revealed in stationary fibroblasts. *Science* **286**, 1172-1174.

Taherian, A., Li, X., Liu, Y. and Haas, T. A. (2011). Differences in integrin expression and signaling within human breast cancer cells. *BMC Cancer* **11**, 293.

Taniyama, Y., Weber, D. S., Rocic, P., Hilenski, L., Akers, M. L., Park, J., Hemmings, B. A., Alexander, R. W. and Griendling, K. K. (2003). Pyk2- and Src-dependent tyrosine phosphorylation of PDK1 regulates focal adhesions. *Mol. Cell. Biol.* **23**, 8019-8029.

Tian, X., Rusanescu, G., Hou, W., Schaffhausen, B. and Feig, L. A. (2002). PDK1 mediates growth factor-induced Ral-GEF activation by a kinase-independent mechanism. *EMBO J.* **21**, 1327-1338.

Webb, D. J., Parsons, J. T. and Horwitz, A. F. (2002). Adhesion assembly, disassembly and turnover in migrating cells – over and over and over again. *Nat. Cell Biol.* **4**, E97-E100.

Woods, A. J., White, D. P., Caswell, P. T. and Norman, J. C. (2004). PKD1/PKCmu promotes alphavbeta3 integrin recycling and delivery to nascent focal adhesions. *EMBO J.* **23**, 2531-2543.

Zamir, E. and Geiger, B. (2001). Molecular complexity and dynamics of cell-matrix adhesions. *J. Cell Sci.* **114**, 3583-3590.

Zamir, E., Katz, B. Z., Aota, S., Yamada, K. M., Geiger, B. and Kam, Z. (1999). Molecular diversity of cell-matrix adhesions. *J. Cell Sci.* **112**, 1655-1669.