

# Rab5 activation promotes focal adhesion disassembly, migration and invasiveness in tumor cells

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Accepted 7 June 2013

Journal of Cell Science 126, 3835–3847

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doi: 10.1242/jcs.119727

## Summary

Migration and invasion are essential steps associated with tumor cell metastasis and increasing evidence points towards endosome trafficking being essential in this process. Indeed, the small GTPase Rab5, a crucial regulator of early endosome dynamics, promotes cell migration *in vitro* and *in vivo*. Precisely how Rab5 participates in these events remains to be determined. Considering that focal adhesions represent structures crucial to cell migration, we specifically asked whether Rab5 activation promoted focal adhesion disassembly and thereby facilitated migration and invasion of metastatic cancer cells. Pulldown and biosensor assays revealed that Rab5-GTP loading increased at the leading edge of migrating tumor cells. Additionally, targeting of Rab5 by different shRNA sequences, but not control shRNA, decreased Rab5-GTP levels, leading to reduced cell spreading, migration and invasiveness. Re-expression in knockdown cells of wild-type Rab5, but not the S34N mutant (GDP-bound), restored these properties. Importantly, Rab5 association with the focal adhesion proteins vinculin and paxillin increased during migration, and expression of wild-type, but not GDP-bound Rab5, accelerated focal adhesion disassembly, as well as FAK dephosphorylation on tyrosine 397. Finally, Rab5-driven invasiveness required focal adhesion disassembly, as treatment with the FAK inhibitor number 14 prevented Matrigel invasion and matrix metalloproteinase release. Taken together, these observations show that Rab5 activation is required to enhance cancer cell migration and invasion by promoting focal adhesion disassembly.

**Key words:** Rab5, Focal adhesion, Cell migration, Invasion

## Introduction

Cancer cell migration and invasion are essential steps in the metastatic cascade, but their underlying mechanisms remain to be fully defined (Hanahan and Weinberg, 2011; van Zijl et al., 2011). In this context, the importance of endosome trafficking and membrane dynamics in metastasis has become more generally accepted (Mosesson et al., 2008). Endocytosis is involved in a wide variety of cellular processes, including uptake and internalization of solutes and components of the extracellular matrix (ECM) (Conner and Schmid, 2003), regulation of signaling induced by extracellular ligands (Ivaska and Heino, 2011), trafficking of cell surface receptors, such as integrins (Caswell and Norman, 2006; Caswell et al., 2009; Pellinen and Ivaska, 2006), and rearrangement of the actin cytoskeleton (Lanzetti et al., 2004; Palamidessi et al., 2008). Endocytosis and intracellular trafficking are coordinated by a family of small GTPases termed the Rabs. Rab proteins are classified according to their function and localization in different intracellular compartments and membrane domains (Stenmark, 2009).

Among the Rab proteins, Rab5 is one of the best characterized, as it participates in a multitude of cellular functions, including vesicle formation, early endosome fusion, early-to-late endosome maturation and motility along microtubules (Christoforidis et al., 1999; Hoepfner et al., 2005; Nielsen et al., 1999; Rink et al.,

2005; Rubino et al., 2000). Therefore, Rab5 is considered a master regulator of early endosome dynamics. As a small GTPase, Rab5 cycles between a GDP- (inactive) and GTP-bound form (active). In addition to its canonical role in endocytosis, Rab5 has been implicated in other cellular processes, such as cell adhesion and migration (Torres and Stupack, 2011). In so doing, Rab5 is necessary for local actin remodeling, as it recruits the Rac1 guanine-nucleotide-exchange factor (GEF) Tiam1 to early endosomes, promoting Rac1-GTP loading and formation of circular dorsal ruffles (Lanzetti et al., 2004; Palamidessi et al., 2008). Indeed, Rab5-mediated activation of Rac1 is required for tumor cell migration *in vitro* and *in vivo* (Palamidessi et al., 2008; Torres et al., 2010). Additionally, Rab5 associates with  $\beta 1$  integrins, and regulates the rates of integrin internalization and recycling (Pellinen et al., 2006; Torres et al., 2010). Interestingly, Rab5 accumulates at the leading edge of migrating cells and promotes formation of lamellipodia (Palamidessi et al., 2008; Spaargaren and Bos, 1999; Torres et al., 2010; Torres et al., 2008). Most studies investigating this migration-promoting role of Rab5 have evaluated total Rab5 protein expression, but not its activity. Therefore, further research is needed to understand the precise role of Rab5 activity in the migration process.

Cell migration is a multi-step process, which involves cell polarization, membrane protrusion at the leading edge, formation

of adhesion complexes, cell body contraction and release of adhesion complexes at the cell rear (Ridley et al., 2003). Thus, spatio-temporal regulation of focal adhesion (FA) dynamics is essential for cell migration (Broussard et al., 2008). In this regard, the focal adhesion kinase (FAK) has been associated with both FA assembly and disassembly, with phosphorylation on Y397 being essential in promoting FA turnover (Hamadi et al., 2005). Despite the well-known mechanisms underlying FA formation and maturation, events leading to FA disassembly remain to be fully characterized. Recent evidence indicates that microtubule-induced FA disassembly is mediated by a FAK- and clathrin-dependent mechanism involving the accessory and adaptor proteins dynamin, AP-2 and DAB2 (Chao and Kunz, 2009; Ezratty et al., 2009; Ezratty et al., 2005). Here, the FA component integrin  $\beta 1$  is known to be transported via Rab5-positive early endosomes, both in non-stimulated cells (Pellinen et al., 2006; Torres et al., 2010) and following microtubule-induced disassembly of FAs (Ezratty et al., 2009). Despite such evidence, the precise role of Rab5 in FA turnover remains unknown. This is important, in light of recent evidence suggesting a role for FAs as restricted sites for ECM degradation, in addition to invadopodia structures (Wang and McNiven, 2012). Accordingly, both FAs and FAK activity have been associated with tumor invasiveness (Mon et al., 2006; Segarra et al., 2005; Stokes et al., 2011; Zeng et al., 2006), but further insight into the underlying mechanisms is required.

Here, we show that Rab5 is activated during tumor cell spreading and migration and that Rab5 activity is required for these processes. Rab5 associated with FA components, including vinculin, paxillin and integrin  $\beta 1$ , during cell migration. Indeed, Rab5 activity regulated the rates of FAK phosphorylation-dephosphorylation on Y397, FA disassembly and cell invasion. As a consequence, Rab5-mediated FA disassembly is necessary for tumor cell invasiveness. In summary, Rab5 activation promotes tumor cell migration and invasion by regulating FAK activation and FA dynamics.

## Results

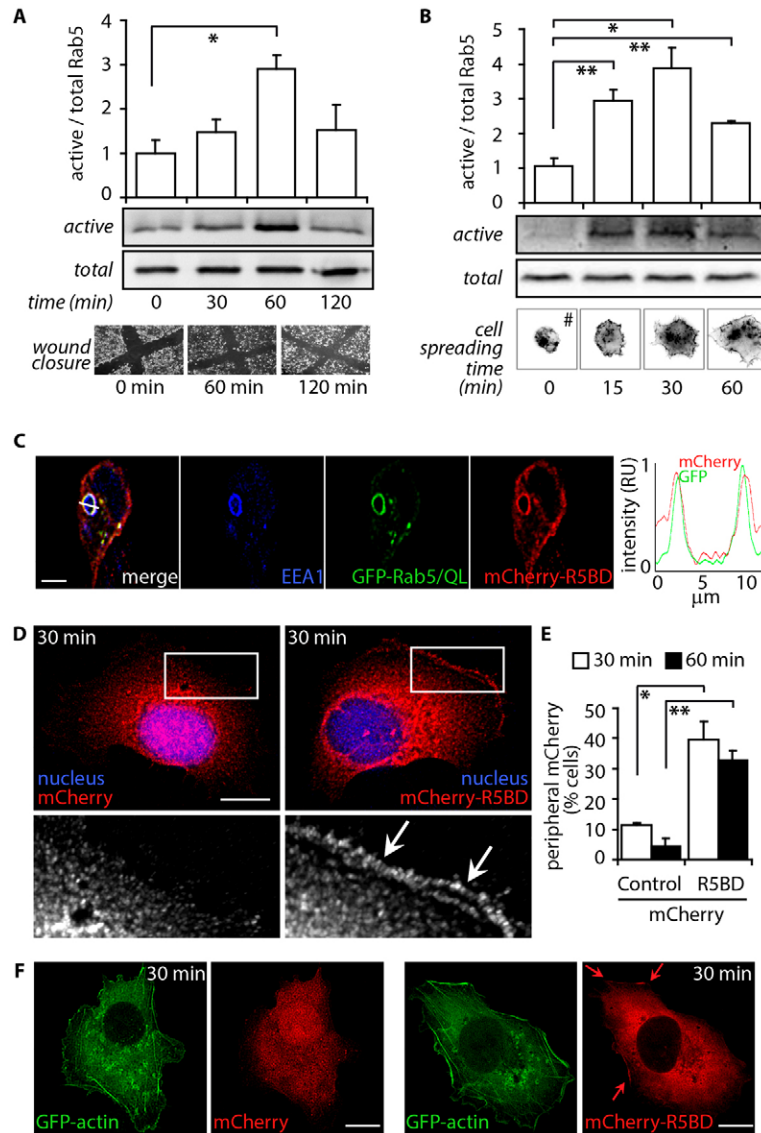
### Rab5 activity is required for tumor cell migration

We have previously observed that ligation of  $\beta 1$  integrins leads to GTP-loading of Rab5 in neuroblastoma cells (Torres et al., 2010). Given that Rab5 is suggested to represent a key regulator of cell migration, we sought to evaluate the activation of this small GTPase during migration of metastatic cancer cells. To that end, confluent monolayers of MDA-MB-231 breast cancer cells were wounded repetitively with a steel comb and allowed to migrate, as previously reported (Urrea et al., 2012). The percentage of cells adjacent to the wounded area was estimated to represent 20% of the cells remaining in the monolayer. Rab5-GTP levels, detected by pulldown assays, increased substantially during cell migration, in a time-dependent manner, with a peak of activity 60 min after wounding and a subsequent decrease at 120 min (Fig. 1A, graph and middle panels). Importantly, fluctuations were not associated with premature wound closure at time points evaluated (Fig. 1A, lower panels), as MDA-MB-231 cells are known to be highly motile (Urrea et al., 2012). In order to confirm the observations shown in Fig. 1A, cells in suspension were seeded onto fibronectin-coated plates to induce cell spreading, which permits evaluating initial steps of migration (Fig. 1B, lower panels). Because maximal MDA-MB-231 cell spreading was observed at 60 min, experiments were performed within this

time-frame (Fig. 1B, see below). As expected, Rab5-GTP levels increased steadily during cell spreading, with a peak of activity at 30 min (Fig. 1B, upper and middle panels). However, these data do not provide any information about the precise location of active Rab5. These results indicate that cell migration and spreading are accompanied by activation of Rab5. In order to determine the location of activated Rab5, MDA-MB-231 cells were transfected with the modified pEGFP-C1-mCherry-R5BD plasmid (see Materials and Methods for details), which encodes the Rab5-binding domain (R5BD) that binds GTP-loaded Rab5 (Liu et al., 2007; Torres et al., 2008; Vitale et al., 1998). Importantly, mCherry-R5BD, but not mCherry alone was recruited to giant early endosomes when induced by the active mutant GFP-Rab5/Q79L (Fig. 1C; supplementary material Fig. S1A). Moreover, mCherry-R5BD partially co-localized with endogenous Rab5, but showed a little co-localization with the inactive mutant GFP-Rab5/S34N (supplementary material Fig. S1A,B). As shown in Fig. 1D, cell spreading induced peripheral accumulation of mCherry-R5BD, but not mCherry alone. Significant re-distribution of mCherry-R5BD was observed at different time points of cell spreading (Fig. 1E; supplementary material Fig. S1C). Recruitment of mCherry-R5BD to the cell edge and actin-based protrusions was confirmed by co-transfection with GFP-actin (Fig. 1F). Of note, no peripheral accumulation of the inactive mutant GFP-Rab5/S34N was observed during cell spreading (supplementary material Fig. S1D). Altogether, these results suggest that localized activation of Rab5 might be required for cancer cells to migrate.

The requirement of Rab5 activity in migration of metastatic cancer cells was then evaluated. Endogenous Rab5 was targeted by shRNA in MDA-MB-231 cells (shRNA sequence F10). Rab5 was downregulated by 70%, with no significant changes in late or early endocytic proteins (Fig. 2A, lower panels; supplementary material Fig. S2A). Rab5 downregulation was accompanied by a 50% decrease in cell migration (Fig. 2A, upper panel). Of note, shRNA-targeting of total Rab5 led to an 82% decrease in Rab5-GTP levels, as compared with control shRNA (supplementary material Fig. S2B), which is larger than the decrease observed for total Rab5. These observations are in agreement with previous results suggesting the existence of a positive-feedback loop in Rab5 activation (Del Conte-Zerial et al., 2008). Given that Rab5-GTP levels increased during cell spreading (Fig. 1B), we sought to evaluate the role of Rab5 in cell spreading. Accordingly, Rab5 downregulation led to a decrease in the spreading area at different time points (Fig. 2B,C).

To extend our findings in MDA-MB-231 cells, endogenous Rab5 was targeted by two shRNA sequences in A549 lung carcinoma cells (shRNA sequences B5 and F10). Rab5 was downregulated by 91% and 78% with sequence B5 and sequence F10, respectively, with no significant changes in the early endocytic protein EEA1 (Fig. 2D, lower panel). As expected, cell migration was substantially decreased with both shRNA sequences (Fig. 2D, upper panel). Importantly, in recovery experiments with Rab5 constructs that are resistant to shRNA sequence B5, expression of wild-type Rab5, but not the S34N mutant (GDP-bound Rab5) restored A549 cell migration (Fig. 2E). Similar results were observed by expressing GFP-tagged shRNA-resistant Rab5 constructs (supplementary material Fig. S2C). It is worth mentioning that the shRNA sequence F10 (expressed in both MDA-MB-231 and A549 cells) could not be used in recovery experiments, as it targets recombinant Rab5.



**Fig. 1. Rab5 activation during cell migration.** (A) MDA-MB-231 cells were grown to confluence and monolayers were wounded multiple times with a steel comb. Cells were allowed to migrate for different times and whole-cell lysates were prepared. Rab5-GTP levels were determined by using the R5BD pulldown assay. Upper graph, relative Rab5-GTP levels normalized to total Rab5 by scanning densitometry are shown as the fold increase with respect to time point '0'. Data represent the average of three independent experiments (mean  $\pm$  s.e.m.;  $*P < 0.05$ ). Middle panels, representative western blot images. Lower panels, representative images of cell monolayers 0, 60 and 120 min after wounding. (B) MDA-MB-231 cells in suspension were allowed to attach and spread onto fibronectin-coated plates (2  $\mu\text{g}/\text{ml}$ ) for different times. Whole-cell lysates were prepared and Rab5-GTP levels were determined by the R5BD pulldown assay. Upper graph denotes relative Rab5-GTP levels as obtained in A. Data represent the average of three independent experiments (mean  $\pm$  s.e.m.). Statistically significant differences are shown ( $**P < 0.01$ ;  $*P < 0.05$ ). Middle panels are representative images obtained by western blotting. Lower panels are representative images of every time point, except time point 0 (indicated as #), as a typical cell requires at least 5 min to adhere. (C) MDA-MB-231 cells were co-transfected with both pEGFP-C1-Rab5/Q79L (active Rab5) and mCherry-R5BD (see Materials and Methods for details). Post-transfection (24 h), samples were fixed, stained with anti-EEA1 polyclonal antibody (blue) and imaged for mCherry-R5BD (red) and GFP-Rab5/Q79L (green) by confocal microscopy. Images were captured at a distance from the ventral zone of the z-axis of the cell, in order to obtain clearly defined endosomes. A representative deconvoluted image is shown. The graph on the right represents a plot of the intensity profile, corresponding to the signals of GFP-Rab5/Q79L (green) and mCherry-R5BD (red), of a set of pixels distributed on a line drawn across vesicles (shown in the merged panel). Data are expressed as relative units versus length in microns. (D,E) MDA-MB-231 cells were transfected with mCherry alone or mCherry-R5BD. Post-transfection (24 h), cells in suspension were allowed to spread onto fibronectin-coated coverslips (2  $\mu\text{g}/\text{ml}$ ) and evaluated after either 30 or 60 min. Samples were fixed, stained with DAPI (blue) and mCherry localization (red) was determined by confocal microscopy. Images were captured at the ventral zone of the z-axis of the cell. (D) Representative deconvoluted images are shown for 30 min of spreading. Lower panels are magnifications of boxed areas. Arrows indicate peripheral mCherry-R5BD staining. (E) The percentage of cells exhibiting peripheral accumulation of mCherry was determined as described in the Materials and Methods section. Data represent the average of three independent experiments (mean  $\pm$  s.e.m.;  $*P < 0.05$ ,  $**P < 0.01$ ). (F) MDA-MB-231 cells were co-transfected with GFP-actin and the mCherry or mCherry-R5BD constructs. Post-transfection (24 h), cells in suspension were allowed to spread onto fibronectin-coated coverslips (2  $\mu\text{g}/\text{ml}$ ) for 30 min, then fixed and analyzed by confocal microscopy. Representative deconvoluted images are shown. Arrows indicate peripheral mCherry-R5BD staining. Scale bars: 10  $\mu\text{m}$ .

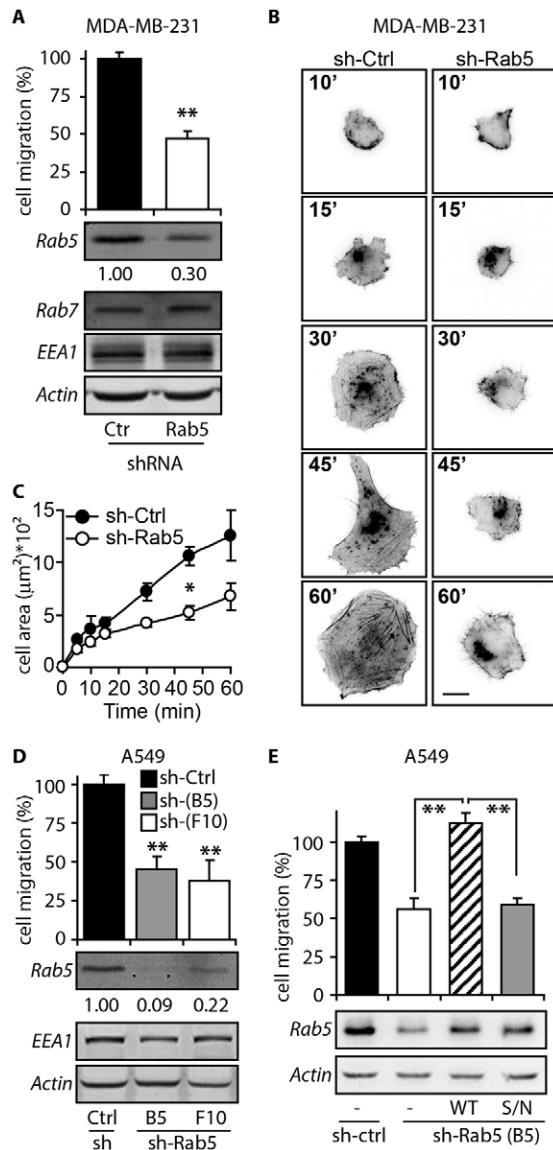
Thus, for subsequent experiments involving Rab5 re-expression, A549 cells transduced with the shRNA-B5 sequence were employed.

### Association of Rab5 with focal adhesion components

Previous work has suggested that Rab5 associates with  $\beta 1$  integrins in different cellular settings (Pellinen et al., 2006; Torres et al., 2010). The precise role of this association in cancer cell migration remains elusive. Given that integrin ligation precedes formation of large supra-molecular complexes known as focal adhesions (FAs) (Parsons et al., 2010), we evaluated whether Rab5 associates with FA proteins by immunoprecipitation. In addition to the expected association of Rab5 with  $\beta 1$  integrin, we also detected the presence of Rab5 in a complex with paxillin, FAK and vinculin (Fig. 3A,B). Interestingly, this association between Rab5 and FA proteins was increased during cell migration, as shown in multi-wound assays (Fig. 3A). Moreover, Rab5 associated with paxillin, vinculin and  $\beta 1$  integrin in inverse co-immunoprecipitation experiments (Fig. 3B). Given that detection of FA proteins in Rab5

precipitates was limited, we sought to increase formation of stable FAs, in order to confirm these findings. To this end, FAs were stabilized by microtubule depolymerization with nocodazole, as previously reported (Ezratty et al., 2009; Ezratty et al., 2005). As expected, microtubule depolymerization led to an increased number of mature FAs (see next paragraphs, Fig. 5; supplementary material Fig. S4). Treatment with nocodazole increased co-immunoprecipitation of Rab5 and vinculin (Fig. 3C). Accordingly, co-localization of Rab5 and paxillin-positive FAs was increased by treatment with nocodazole (Fig. 3D). To test this association in a more relevant model, localization of Rab5 and FAs was evaluated in cells undergoing spreading. Limited but detectable co-localization between Rab5 and paxillin was found at both FAs and as yet undefined intracellular compartments (Fig. 3E; supplementary material Fig. S3A). Likewise, Rab5 was found to co-localize with both paxillin and vinculin in cells migrating towards a wounded area (supplementary material Fig. S3B,C). The relative amount of FAs containing Rab5 was estimated as  $9.8 \pm 3.5\%$  (supplementary material Fig. S3C).

To evaluate the selectivity of the association between Rab5 and FAs, additional Rab proteins were investigated. These included Rab11, a known component in the recycling pathway for integrin  $\beta 1$  (White et al., 2007), Rab21, which associates directly with  $\beta 1$  integrins (Pellinen et al., 2006), and EEA1, a Rab5 effector. Co-immunoprecipitation data indicated that



**Fig. 2. Rab5 activity is required for tumor cell migration and spreading.** (A) MDA-MB-231 cells were treated with either a control shRNA (number 1864) or an shRNA targeting Rab5 (sequence F10). Stably transduced cells were used for analysis of cell migration and protein expression by western blotting. Upper graph, cell migration was assessed after 2 h in Boyden chambers. Data were normalized to shRNA-control cells and are shown as the average of three independent experiments (mean  $\pm$  s.e.m.; \*\* $P < 0.01$ ). Lower panels are representative images obtained by western blotting. Rab5 levels were quantified by scanning densitometry analysis and normalized to actin (numerical data below panel). Residual Rab5 levels in shRNA-Rab5 cells ( $0.30 \pm 0.1$ ) were calculated from three independent measurements (mean  $\pm$  s.e.m.). Likewise, residual Rab7 ( $1.19 \pm 0.5$ ) levels in shRNA-Rab5 cells were quantified ( $n = 3$ ). (B,C) MDA-MB-231 cells in suspension were allowed to spread onto fibronectin-coated coverslips ( $2 \mu g/ml$ ) and evaluated at different time points. Samples were fixed, stained with phalloidin-Rhodamine and analyzed by spinning disk microscopy. In B, representative images are shown. Scale bar:  $10 \mu m$ . (C) The area of spreading cells was calculated with the Image J software, as indicated in the Materials and Methods. Data were averaged from three independent experiments (mean  $\pm$  s.e.m.; \* $P < 0.05$ ). (D) A549 cells were treated with either a control shRNA (number 1864) or two different shRNAs targeting Rab5 (sequences B5 and F10). Stably transduced cells were used for the analysis of cell migration and protein expression by western blotting, as indicated in A. Upper graph, cell migration was measured in Boyden chambers (mean  $\pm$  s.e.m.). Statistically significant differences are shown in comparison with shRNA-control (\*\* $P < 0.01$ ). Lower panels are representative images obtained by western blotting. Residual Rab5 levels (numerical data below panel) in shRNA-Rab5, sequence B5 ( $0.09 \pm 0.04$ ) and sequence F10 ( $0.22 \pm 0.08$ ) were calculated from three independent measurements (mean  $\pm$  s.e.m.). Likewise, residual EEA1 levels in shRNA-Rab5, sequence B5 ( $0.70 \pm 0.3$ ) and sequence F10 ( $1.34 \pm 0.3$ ) were quantified ( $n = 3$ ). (E) A549 cells treated with shRNA-control or shRNA-Rab5 (sequence B5) were transfected with the plasmids pcDNA3.1(+) or pcDNA3.1(+) encoding either wild-type (WT) or mutant Rab5 (inactive S34N, S/N). Cell migration was assessed after 2 h in Boyden chambers and total protein levels were evaluated by western blot. Upper graph, cell migration analysis (mean  $\pm$  s.e.m.,  $n = 3$ ). Statistically significant differences are shown (\*\* $P < 0.01$ ). Lower panels are representative images obtained by western blotting.

EEA1, but not Rab11 or Rab21 associated with vinculin (Fig. 3F; supplementary material Fig. S3D,E). Importantly, as a control, both Rab5 and Rab21 were found to associate with  $\beta 1$  integrin (Fig. 3F).

Because pulldown and biosensor assays indicated that active Rab5 undergoes re-localization to the leading edge of migrating cells (Fig. 1), we evaluated co-localization of the mCherry–R5BD construct with FAs. mCherry–R5BD was found to co-localize with paxillin-positive FAs in cells undergoing spreading (Fig. 3G). Moreover, both endogenous Rab5 and mCherry–R5BD co-localized with paxillin at the leading edge of migrating cells (supplementary material Fig. S3F). Recruitment of Rab5 to FAs was further confirmed by fractionation analysis. Rab5 was

readily detected in FA-enriched fractions from MDA-MB-231 and A549 cells undergoing spreading (Fig. 3H). Of note, ectopically expressed GFP–Rab5/Q79L and, to a lesser extent, GFP–Rab5 wild-type, but not GFP–Rab5/S34N, accumulated in FA-enriched fractions (supplementary material Fig. S3G). Taken together, these results indicate that a fraction of Rab5, mainly in the active form, is associated with FA complexes in migrating cells.

### Rab5 activity is required for focal adhesion disassembly

Given that Rab5 is activated during migration and this is essential for cancer cell migration and spreading, we sought to identify a possible mechanism to explain such behavior. We focused on

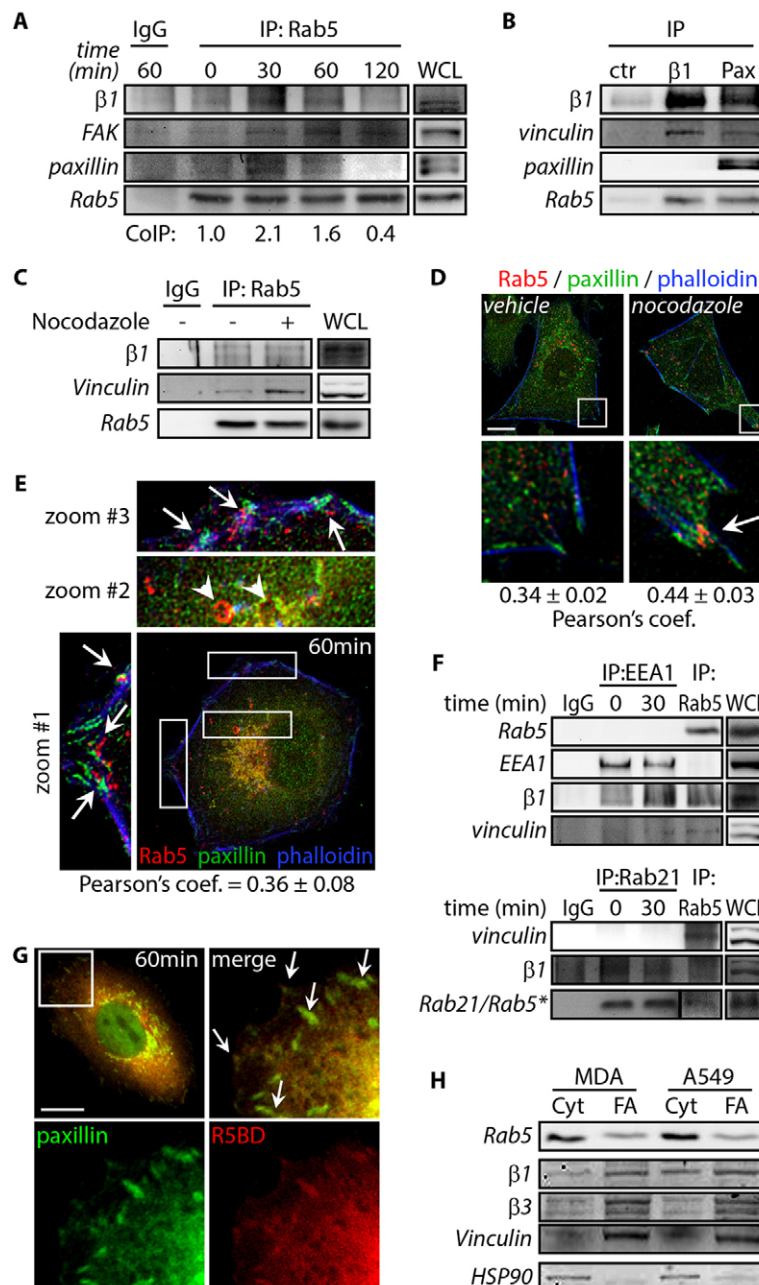


Fig. 3. See next page for legend.

evaluating FA disassembly for the following reasons: first, FA dynamics is a key event in coordinating cell migration (Broussard et al., 2008); second, downregulation of Rab5 does not affect cell adhesion or 'stickiness' towards ECM components (supplementary material Fig. S2D), but does affect cell spreading (Fig. 2B); third, Rab5 downregulation does not affect integrin  $\beta 1$  levels at steady state (supplementary material Fig. S2E); and fourth, Rab5 was reported to regulate integrin internalization and recycling (Torres et al., 2010). Thus, we hypothesized that Rab5 enhances FA disassembly and thereby promotes cell migration. To test this hypothesis, different approaches were developed. First, recombinant Rab5 and FA proteins were co-expressed in MDA-MB-231 cells undergoing spreading, in order to track their

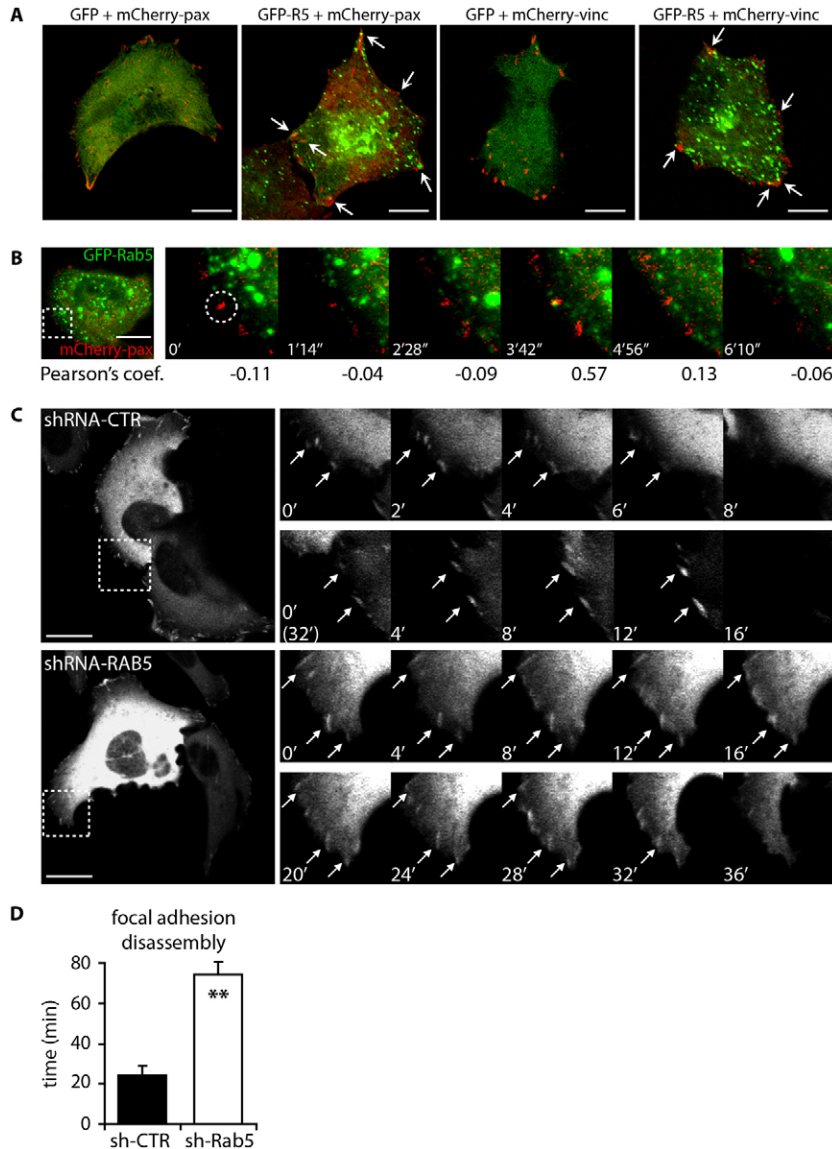
**Fig. 3. Association of Rab5 with focal adhesion components in migrating cells.**

(A) MDA-MB-231 cells were grown to confluence and monolayers were wounded multiple times with a steel comb. Cells were allowed to migrate and whole-cell lysates (WCL) were prepared at different times. Rab5 was immunoprecipitated with a polyclonal antibody and samples were analyzed by western blotting. For comparison, 50  $\mu$ g of whole-cell lysates were analyzed. Control immunoprecipitation (IP) experiments were performed with an irrelevant IgG at 60 min. Relative levels of paxillin were quantified in immunoprecipitates by scanning densitometry of western blots and were normalized to Rab5 immunoprecipitated and total paxillin in whole-cell lysates (IP paxillin/IP Rab5/total paxillin). Numerical data below each panel indicate the fold increase in paxillin levels (relative to 0 min) after 30 min ( $2.1 \pm 0.7$ ), 60 min ( $1.6 \pm 0.7$ ) and 120 min ( $0.4 \pm 1.0$ ), as calculated from three independent experiments (mean  $\pm$  s.e.m.). (B) MDA-MB-231 cells were wounded multiple times, as in A, and both integrin  $\beta 1$  and paxillin were immunoprecipitated after 30 min, with polyclonal antibodies. Samples were analyzed by western blotting. (C) MDA-MB-231 cells were incubated with either vehicle [DMSO (–)] or 10  $\mu$ M nocodazole (+) in serum-free medium for 4 h, in order to stabilize focal adhesions. Then, whole-cell lysates were prepared, Rab5 was immunoprecipitated and samples were analyzed by western blotting. For comparison, 50  $\mu$ g of whole-cell lysates were analyzed. Representative images from two independent experiments are shown. (D) MDA-MB-231 cells were grown on glass coverslips and incubated with 10  $\mu$ M nocodazole in serum-free medium for 4 h, as indicated in C. Samples were fixed and stained with polyclonal anti-paxillin (green) and monoclonal anti-Rab5 (red) antibodies. Actin fibers were stained with phalloidin (blue). Samples were analyzed by confocal microscopy. Representative deconvoluted images are shown from two independent experiments. Lower panels are magnifications of boxed areas. Arrows indicate co-localization at focal adhesions (Pearson's coefficient is indicated as numerical data below each panel,  $n=3$ ). (E) MDA-MB-231 cells in suspension were allowed to attach and spread onto fibronectin-coated coverslips (2  $\mu$ g/ml) for 60 min. Samples were fixed and incubated with monoclonal anti-Rab5 (red) and polyclonal anti-paxillin antibodies (green). Actin fibers were stained with phalloidin (blue). Samples were analyzed by confocal microscopy. Representative deconvoluted images are shown from two independent experiments. Upper and left panels are magnifications of boxed areas. Arrows indicate co-localization at focal adhesions; arrowheads indicate co-localization at intracellular compartments (Pearson's coefficient is indicated as numerical data,  $n=3$ ). (F) Confluent monolayers of MDA-MB-231 cells were wounded multiple times with a steel comb. Cells were allowed to migrate for 0 and 30 min and whole-cell lysates were prepared. EEA1, Rab5 and Rab21 were immunoprecipitated and samples were analyzed by western blotting. For comparison, 50  $\mu$ g of whole-cell lysates were analyzed. Images are representative from two independent experiments. Blots for Rab5 and Rab21 are shown in the same panel (Rab21/Rab5\*), separated by a black line. (G) MDA-MB-231 cells were transfected with mCherry–R5BD (red) and allowed to spread onto fibronectin-coated coverslips (2  $\mu$ g/ml) for 60 min. Samples were fixed and stained for paxillin (polyclonal antibody, green). Samples were analyzed by confocal microscopy. Representative deconvoluted images are shown from two independent experiments. Arrows indicate co-localization at focal adhesions (Pearson's coefficient =  $0.89 \pm 0.03$ ,  $n=3$ ). (H) Focal-adhesion-enriched (FA) and cytosolic (Cyt) fractions were analyzed by immunoblotting. Data are representative of two independent experiments. Scale bars: 10  $\mu$ m.

dynamics by live-cell imaging. Both mCherry–paxillin and mCherry–vinculin accumulated at FAs, whereas GFP–Rab5 showed a punctated distribution. In agreement with co-localization data of endogenous Rab5, paxillin and vinculin (Fig. 3), a sub-population of GFP–Rab5 endosomes co-localized with mCherry–paxillin and mCherry–vinculin-positive FAs (Fig. 4A). Thereafter, we tracked the dynamics of GFP–Rab5 and mCherry–paxillin in live cells by time-lapse microscopy. GFP–Rab5-positive endosomes showed partial co-localization with mCherry–paxillin. Intriguingly, co-localization was short-lived and followed by collapse of both structures (Fig. 4B, supplementary material Movie 1). These observations suggest that Rab5 is connected with FA disassembly. To test this possibility, MDA-MB-231 cells treated with shRNA-control or shRNA-Rab5 were transfected with mCherry–paxillin. Subsequently, cells were serum-starved and pulsed with 10% serum to induce cell spreading and FA disassembly. As shown by time-lapse microscopy, mCherry-labeled FAs readily disassembled in shRNA-control cells, whereas shRNA-Rab5 cells depicted delayed kinetics (Fig. 4C; supplementary material Movies 2, 3). Rab5 knockdown was followed by a threefold increase in the time necessary for FA disassembly as compared with shRNA-control cells (Fig. 4D).

As an additional method to evaluate the requirement of Rab5 for FA disassembly, FAs were stabilized by microtubule depolymerization with nocodazole, as mentioned. Treatment with nocodazole led to depolymerization of microtubules in MDA-MB-231 cells (supplementary material Fig. S4A, no treatment, 'NT', versus nocodazole treatment followed by no removal, '0 min'), whereas removal of this drug led to microtubule re-growth with similar kinetics in both shRNA-control- and shRNA-Rab5-treated cells (supplementary material Fig. S4B,C). Such treated cells were then analyzed for FA (vinculin staining) and stress fiber formation (phalloidin staining). Treatment with nocodazole (Fig. 5A, NT versus 0 min) induced FA formation in both shRNA-control and shRNA-Rab5 cells, whereas nocodazole removal decreased the number of FAs in a time-dependent manner in both cell lines (Fig. 5A). However, the kinetics of FA disassembly was substantially delayed in shRNA-Rab5 cells as compared with control cells (Fig. 5A,B). Rab5 downregulation led to a threefold decrease in  $V_0$  (initial velocity of FA disassembly), as compared with shRNA-control cells (Fig. 5B, inset data). Noteworthy, microtubule-induced FA disassembly was preceded by enhanced co-localization between GFP–Rab5 and mCherry–paxillin (supplementary material Movie 4), as well as mCherry–R5BD and GFP–vinculin (supplementary material Movie 5).

These observations were further supported by analysis of FAK phosphorylation on Y397, as readout. Phospho-Y397-FAK levels were increased by treatment with nocodazole in both shRNA-control and shRNA-Rab5 cells. Removal of nocodazole resulted initially in dephosphorylation of FAK followed by re-phosphorylation on Y397 in a time-dependent manner (Fig. 5C). Rab5 downregulation delayed the kinetics of both FAK dephosphorylation and phosphorylation (Fig. 5C,D). These effects were recapitulated in A549 cells, where Rab5 targeting by a different shRNA sequence was followed by delayed kinetics of FA disassembly and FAK dephosphorylation (data not shown). Importantly, and in agreement with cell migration data, expression of wild-type Rab5, but not the GDP-bound mutant S34N in Rab5 knockdown cells, accelerated the rate of



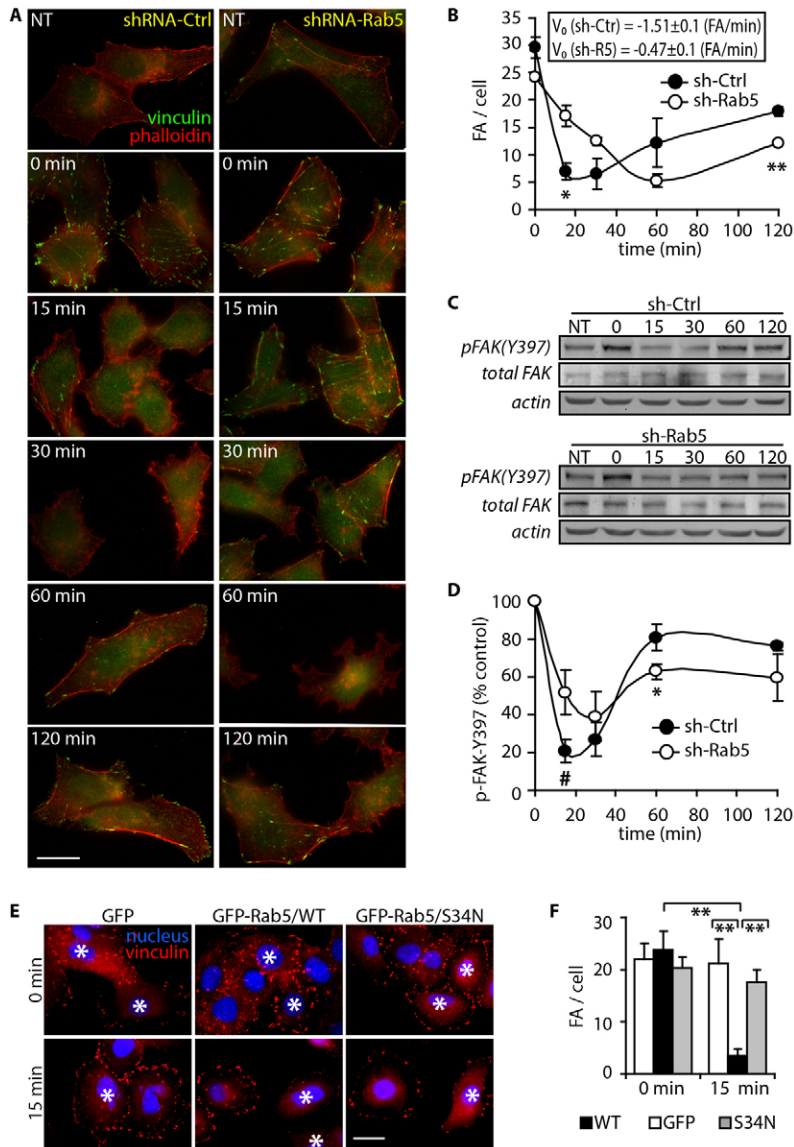
**Fig. 4. Rab5 is required for focal adhesion disassembly in tumor cells.** (A) MDA-MB-231 cells were grown on glass-coverslips and co-transfected with GFP, GFP-Rab5, mCherry-paxillin or mCherry-vinculin, as indicated. Post-transfection (24 h), samples were fixed and imaged for mCherry (red) and GFP (green) by confocal microscopy. Representative images are shown. Arrows indicate co-localization at focal adhesions. Pearson's coefficients were obtained as follows: GFP + mCherry-Paxillin ( $0.29 \pm 0.08$ ); GFP-Rab5 + mCherry-paxillin ( $0.50 \pm 0.11$ ); GFP + mCherry-vinculin ( $0.35 \pm 0.08$ ); GFP-Rab5 + mCherry-vinculin ( $0.58 \pm 0.12$ ). (B) MDA-MB-231 cells were grown on glass-bottomed tissue culture dishes and co-transfected with GFP-Rab5 and mCherry-paxillin. Post-transfection (24 h), cells were serum-starved for 2 h, pulsed with 10% serum and then recorded by time-lapse microscopy for 60 min (74 seconds between frames). A magnification of the boxed area is shown at selected time points. A region of interest (ROI) is encircled at 0 min. Pearson's coefficients were calculated for ROIs at indicated time points and quantifications are shown as numerical data below each panel. (C) MDA-MB-231 cells treated with shRNA-control or shRNA-Rab5 were transfected with mCherry-paxillin for 24 h. Subsequently, cells were serum-starved for 2 h, pulsed with 10% serum and recorded by time-lapse microscopy for 80 min (120 seconds between frames). Magnifications of boxed areas are shown at selected times, as indicated. Arrows indicate representative mCherry-paxillin-positive focal adhesions that undergo disassembly. (D) Kinetics of focal adhesions was measured from the experiments shown in C, scoring at least ten mCherry-positive focal adhesions per experiment. Data represents the mean of three independent experiments (mean  $\pm$  s.e.m.;  $**P < 0.01$ ). Scale bars: 10  $\mu$ m.

FA disassembly following nocodazole removal (Fig. 5E,F; supplementary material Fig. S5A). Taken together, these data suggest that Rab5 activity favors FA disassembly in cancer cells.

#### Tumor invasiveness depends on Rab5 activation and focal adhesion disassembly

Rab5 expression has been previously suggested to correlate with tumor cell invasion (Liu et al., 2011). Precise mechanisms underlying such connection remain to be defined. Accordingly, Rab5 downregulation in both MDA-MB-231 and A549 cells impaired cell invasion in Matrigel (Fig. 6A,B). Because tumor cell invasiveness depends on ECM remodeling by matrix metalloproteinases (MMPs), we evaluated secretion and activity of MMP9 and MMP2 as an additional way to determine the invasive potential. Rab5 knockdown was accompanied by decreased MMP9 and MMP2 activation (Fig. 6C). Importantly, as these data and those shown in Figs 4 and 5, indicate that Rab5 regulates FA disassembly and cancer cell invasion, it was tempting to speculate that both effects might be interconnected. In this regard, recent reports have suggested that tumor cell

invasion is regulated by the activity of both FAs and FAK (Mon et al., 2006; Segarra et al., 2005; Stokes et al., 2011; Zeng et al., 2006). We thus evaluated the hypothesis that Rab5, by regulating FA dynamics, promoted cancer cell invasion. To test this, A549 shRNA-Rab5 cells were reconstituted with GFP-Rab5, and cell invasion was assessed in the absence and presence of the FAK inhibitor number 14. Note that in these experiments, inhibition of FAK activity was used as a way to interfere with FA dynamics, in contrast to those measurements of phosphorylated FAK in FA disassembly experiments, where this was used as readout of FA maturation. First, it should be noted that FAK inhibitor number 14 reduced FAK phosphorylation on Y397 and prevented FA disassembly (Fig. 6D). As anticipated, cell invasion, and MMP2 and MMP9 release induced by overexpression of wild-type Rab5 were substantially decreased by inhibiting FA disassembly with FAK inhibitor number 14 (Fig. 6E). In addition, decreased invasion in Rab5 knockdown cells was rescued by expression of the constitutively active mutant of FAK, GFP-FAK/Y180A-M183A (supplementary material Fig. S5B). Taken together, these data suggest that Rab5 stimulates tumor cell invasiveness, at least in part by promoting the disassembly of FAs in a FAK-dependent manner.



**Fig. 5. Rab5 promotes microtubule-induced focal adhesion disassembly.** (A) MDA-MB-231 cells treated with either shRNA-control (number 1864) or shRNA-Rab5 (sequence F10) were grown on glass coverslips and incubated with 10  $\mu$ M nocodazole in serum-free medium for 4 h, in order to stabilize focal adhesions. Then, nocodazole was washed-out and at different time points, samples were fixed and stained with anti-vinculin antibody (green) and phalloidin (red), to identify focal adhesions and actin fibers, respectively. Samples were analyzed by spinning disk microscopy. Representative images are shown from three independent experiments. (B) The number of focal adhesions per cell was quantified from images obtained as in A. At least ten images per condition were analyzed (each containing roughly 5–10 cells). Data represent the average of three independent experiments (mean  $\pm$  s.e.m.). Inset data represent the values for initial velocity ( $V_0$ ) of focal adhesion disassembly, calculated from each curve. Statistically significant differences are shown (\* $P$ <0.05, \*\* $P$ <0.01). (C) FAK phosphorylation on Y397 was evaluated in MDA-MB-231 cells treated with nocodazole, as indicated in A. Whole cell lysates were prepared and analyzed by western blot. Representative images are shown. NT, no treatment. (D) Relative levels of FAK phosphorylated on Y397 were quantified by scanning densitometry and normalized to actin. Data represent the average of three independent experiments (mean  $\pm$  s.e.m.; \* $P$ <0.05). #Note that for time 15 min,  $P$ =0.06. (E) A549 cells treated with shRNA-Rab5 (sequence B5) were transfected with either empty pEGFP-C1 or pEGFP-C1 encoding wild-type (WT) or mutant Rab5 (inactive, S34N). Focal adhesions were synchronized with nocodazole (0 min), as described in A, and disassembly was recorded at 15 min after nocodazole removal. Focal adhesions were detected by vinculin staining (red). Nuclei were stained with DAPI (blue). Samples were analyzed by spinning disk microscopy. GFP-expressing cells are indicated with an asterisk. Representative images from two independent experiments are shown. (F) The number of focal adhesions per cell was quantified in the GFP-transfected population of cells. At least ten images per condition were analyzed. Data are means  $\pm$  s.e.m. from three independent experiments (\*\* $P$ <0.01). Scale bars: 10  $\mu$ m.

These results indicate that Rab5 promotes FA disassembly, cell migration and invasion, and that this is accompanied by increased MMP2 and MMP9 activity. To evaluate whether degradation of the ECM is required for these events, the effect of the MMP2 and MMP9 inhibitor II was evaluated in migration, invasion and FA disassembly assays. As anticipated, MMP inhibition was followed by decreased Rab5-driven invasion (Fig. 6F, right panel). Intriguingly, Rab5-dependent cell migration was partially decreased by MMP inhibition (Fig. 6F, left panel), whereas Rab5-induced FA disassembly was not affected by treatment with the inhibitor (supplementary material Fig. S5C). These data suggest that Rab5-dependent invasion depends on MMP-dependent degradation of the ECM. Moreover, invasiveness required cell migration and FA disassembly, but conversely, Rab5-mediated FA disassembly did not depend on ECM degradation.

## Discussion

Cancer cell migration and invasion are essential steps in metastasis that remain partially understood. In this regard, the role of endosome trafficking and membrane dynamics in

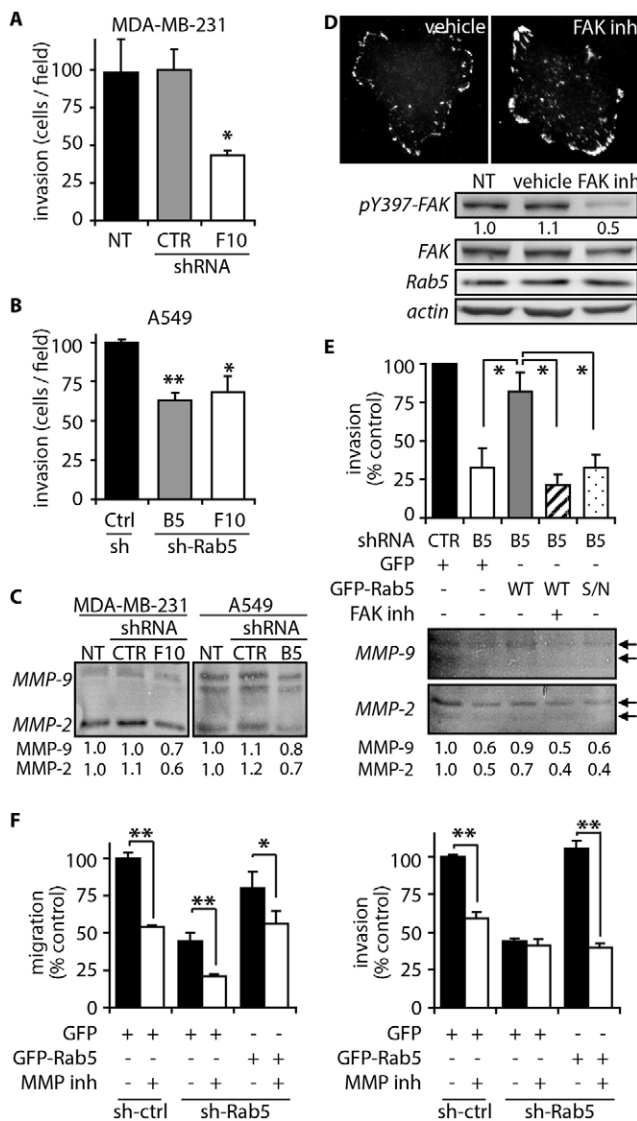
metastasis is emerging as a crucial issue in cancer research. Consequently, a major goal of ongoing and future studies centers on characterizing key players involved in regulating endocytic trafficking of metastatic cancer cells. Increasing evidence shows that Rab5 is involved in the migration of both normal and tumor cells. Expression of Rab5 leads to increased cell motility (reviewed by Torres and Stupack, 2011), integrin trafficking (Pellinen et al., 2006; Torres et al., 2010) and cytoskeleton rearrangement (Lanzetti et al., 2004; Palamidessi et al., 2008). Indeed, several reports have suggested a correlation between Rab5 expression, metastatic potential and poor patient prognosis (Fukui et al., 2007; Yang et al., 2011; Yu et al., 1999; Zhao et al., 2010). These reports favor the view that Rab5 is involved in tumor progression and metastasis.

How precisely Rab5 promotes cell migration has remained elusive. Here, we observed that upon migration, Rab5 was activated in metastatic cancer cells. By using two alternative approaches, based on wound-healing and spreading assays, a similar tendency for Rab5 activation was observed, although kinetics differed. This could be explained in two ways: first, even

though both assays measure initial steps in cell migration, certain differences cannot be avoided, such as ECM composition. The wound healing assay measures cell migration towards remnant ECM at the wounded area, whereas the spreading assay measures cell expansion on fibronectin-coated surfaces. Second, in the wound healing assay, ~20% of the whole cell monolayer is stimulated to migrate, whereas spreading assays involve the whole cell population. Such differences could explain why Rab5 activity increases more rapidly in the spreading assay. It remains unknown whether Rab5 activation is required to initiate or sustain cell migration. We favor the second possibility, because data obtained with the mCherry-R5BD construct showed that active Rab5 accumulates at the leading edge of migrating cells during all time points evaluated (Fig. 1D; supplementary material Fig. S1C). This is in line with previous reports showing re-localization of Rab5-positive endosomes to the periphery of cells that are undergoing directional migration (Torres et al., 2010). Importantly, the present study provides evidence indicating that it is the active pool of Rab5 that undergoes substantial re-localization during cell migration. In this regard, future studies

will be needed to understand the precise role of different sub-cellular pools of active Rab5 in cell migration and spreading. Alternative approaches, including live-cell imaging and (fluorescence resonance energy transfer) FRET will help tracking the precise localization of active Rab5 and effectors in real time. Our findings suggest that a spatially restricted pool, rather than total protein is more relevant to cell migration. The identity of upstream regulators that sense these 'mitogenic stimuli' and promote Rab5 activation remains unknown. Interesting candidates include Rab5 GTPase-activating proteins (GAPs) (such as phosphatidylinositol 3-kinase regulatory subunit  $\alpha$ ) and Rab5 GEFs (such as RIN2, RN-Tre or alsin).

Previous studies have shown accumulation of Rab5 at FAs by both fractionation and total internal reflection fluorescence (TIRF) analysis (Torres et al., 2010) (V.A.T. and D.S., unpublished data). However, to our knowledge, this study represents the first to show that Rab5 associates in a complex with FA components. Whether this association involves a direct interaction between Rab5 and FA proteins or rather involves an indirect association mediated by the formation of complexes with integrins, needs to be evaluated further. Both scenarios are



**Fig. 6. Rab5-driven tumor invasiveness depends on GTP loading and requires focal adhesion disassembly.** (A) Invasion of MDA-MB-231 cells treated with either shRNA-control (number 1864) or shRNA-Rab5 (sequence F10) was measured in Matrigel assays. Data were normalized to shRNA-control cells and the average of three independent experiments is shown (mean  $\pm$  s.e.m.; \* $P$ <0.05). (B) Invasion of A549 cells treated with either shRNA-control (number 1864) or shRNA-Rab5 (sequences B5 and F10) was measured as in A (mean  $\pm$  s.e.m.). Statistically significant differences are shown for sequence B5 (\*\* $P$ <0.01) and sequence F10 (\* $P$ <0.05), both in comparison with shRNA-control. (C) Gelatinase activity was measured in supernatants obtained from serum-starved MDA-MB-231 and A549 cells, in order to detect metalloproteinase MMP2 and MMP9 activities by zymography analysis. Data are representative of two independent experiments. Numbers below each lane indicate the relative activity for MMP2 and MMP9, as determined by scanning densitometry. Values are normalized to the protein concentration in supernatants. Data are shown as the mean of two independent experiments, with respect to their controls (NT, no treatment). (D) A549 cells were treated with either vehicle (DMSO, control) or 5  $\mu$ M of FAK inhibitor number 14. Samples were used for vinculin staining (upper panels) or western blot analysis (lower panels). Phospho-Y397-FAK levels were quantified by scanning densitometry analysis and normalized to total FAK (numerical data below panel). Quantifications for samples treated with vehicle ( $1.07 \pm 0.1$ ) and FAK inhibitor number 14 ( $0.54 \pm 0.1$ ) are shown ( $n=3$ , mean  $\pm$  s.e.m.). (E) A549 cells treated with shRNA-control (number 1864) or shRNA-Rab5 (sequence B5) were transfected with GFP, GFP-Rab5/wild-type (WT) or GFP-Rab5/S34N (S/N). Transfected cells were used for invasion (upper panel) and zymography assays (lower panels), as indicated. Upper panel, invasion was measured in Matrigel, in the presence of either vehicle (DMSO) or 5  $\mu$ M of FAK inhibitor number 14. Data are shown as the percentage of invasion with respect to shRNA-control cells transfected with GFP, and represent the average of three independent experiments (mean  $\pm$  s.e.m.; \* $P$ <0.05). Lower panel, MMP2 and MMP9 activities were measured by zymography analysis. Numbers below each lane indicate the relative activity for MMP2 and MMP9, determined by scanning densitometry, as shown in C. Data are shown as the mean of two independent experiments. (F) A549 cells treated with shRNA-control or shRNA-Rab5 (sequence B5) were transfected with either GFP or GFP-Rab5. Transfected cells were used for migration assays in Boyden chambers (left graph) and invasion assays in Matrigel (right graph), in the presence of either vehicle [DMSO (-)] or the MMP2 and MMP9 inhibitor II (+). Data are shown as the percentage of shRNA-control cells transfected with GFP, and represent the average of three independent experiments (mean  $\pm$  s.e.m.; \*\* $P$ <0.01; \* $P$ <0.05).

possible, as Rab5 was previously shown to interact with  $\beta 1$  integrins (Pellinen et al., 2006; Torres et al., 2010) and this interaction might account for the association with paxillin and vinculin. Further studies, such as pulldown of purified recombinant proteins, two-hybrid or proximity ligation assays (PLA), might be desirable to evaluate such possibilities. Regardless of whether the interaction was direct or indirect, basal association of Rab5 with paxillin, vinculin and integrin  $\beta 1$  in resting cells was further increased during migration, as shown by immunoprecipitation, immunofluorescence and fractionation assays. Our immunofluorescence studies showed a limited detection of Rab5 at FAs, leading to the hypothesis that Rab5 recruitment to FAs is transient and occurs within a narrow timeframe. We propose that Rab5 is partially recruited to FAs and that the nature of this association is transient and restricted to specific sites. This is supported by our live-cell imaging studies; a sub-population of GFP–Rab5-positive endosomes underwent co-localization with paxillin-positive FAs, and intriguingly co-localization events were followed by collapse of both structures (Fig. 4). The amount of mCherry–paxillin-positive structures that co-localized with GFP–Rab5 was estimated as roughly 69%. These data suggest that a significant amount of FAs underwent previous association with Rab5-containing endosomes followed by disassembly. However, we cannot exclude the participation of alternative mechanisms of FA disassembly.

Given that Rab5 is recruited to the leading edge of migrating cells, it might also be involved in FA disassembly at sites of cell protrusions, where new adhesion contacts are established. Indeed, our data indicate that Rab5 downregulation is associated with decreased protruding activity (supplementary material Movies 2, 3), which is in agreement with previous reports (Palamidessi et al., 2008; Torres et al., 2010). Accordingly, Rab5-positive endosomes showed high motility and accumulated at areas of cell protrusions (supplementary material Movie 6).

Our study evaluated the effect of Rab5 in FA dynamics. However, other Rab proteins and molecules involved in intracellular trafficking could also be involved in these phenomena. Interesting candidates include Rab11, which represents a known recycling pathway component for integrin  $\beta 1$  (White et al., 2007), Rab21, which associates directly with  $\beta 1$  integrins (Pellinen et al., 2006), and EEA1, a Rab5 effector. On the basis of immunoprecipitation and expression assays, we exclude the involvement of Rab11 and Rab21. Intriguingly, despite the fact that EEA1 was shown to co-immunoprecipitate with vinculin, this protein failed to co-localize with FAs. A possible explanation for this is that, although EEA1 is a Rab5 effector, EEA1 is mostly restricted to early endosomes, whereas Rab5 can be recruited to both early endosomes and the plasma membrane. Thus, it is likely that the effects of Rab5 on FAs documented here were independent of EEA1. Of note, although no significant changes were observed in levels of caveolin-1 and syndecan-4 proteins, their involvement in the events described here cannot be excluded, particularly owing to their reported participation in integrin and GTPase recycling (Morgan et al., 2013; Nethe et al., 2010), which are known to promote FA dynamics.

Taken together, data presented in this study provide substantial evidence indicating that Rab5 promotes FA disassembly, thereby affecting cancer cell migration. Accordingly, Rab5 accelerates the rates of both FAK dephosphorylation and phosphorylation, further suggesting a role in FA dynamics. Thus, several models

can be proposed for Rab5-dependent FA disassembly. One such scenario is that Rab5 promotes the internalization of entire FA complexes within vesicles and early endosomes. Evidence supporting this possibility includes the observation that both paxillin and vinculin (Figs 3, 4), as well as integrin  $\beta 1$  (Pellinen et al., 2006; Torres et al., 2010), have been shown to co-localize with Rab5-positive early endosomes. Here, FAK would undergo dephosphorylation on Y397 – and hence inactivation – either before or after FA internalization. The second scenario is that Rab5 directly accelerates the rates of FAK phosphorylation and/or dephosphorylation on Y397, thereby affecting the dynamic stability of FAs and rendering FAs more sensitive to external stimuli for disassembly. This possibility is supported by data presented in Fig. 4C,D, where Rab5 accelerated the rates of both FAK phosphorylation and dephosphorylation on Y397. In this scenario FAK activity would promote Rab5-induced FA disassembly, given that FAK phosphorylation on Y397 is known to be involved in FA disassembly (Hamadi et al., 2005).

Metastasis not only depends on enhanced cell migration, but also the ability of tumor cells to invade the surrounding matrix. Rab5 has been previously suggested to promote tumor invasiveness (Liu et al., 2011). These studies were descriptive in showing that Rab5 expression correlates with increased invasion. Currently, no data are available concerning how Rab5 might be involved in tumor cell invasion. Here, we found that Rab5 downregulation was accompanied by decreased invasiveness and MMP release. Importantly, we provide evidence for a link between Rab5-mediated invasiveness and FA disassembly, which is also dependent on Rab5. Because FAK is involved in MMP2 and MMP9 release (Mon et al., 2006; Sein et al., 2000; Shibata et al., 1998) and tumor cell invasiveness (Mon et al., 2006; Segarra et al., 2005; Stokes et al., 2011; Zeng et al., 2006), we evaluated the possibility that Rab5 and FAK collaborated in promoting invasiveness. Indeed, we observed that FAK activation is required for Rab5-driven cell invasion, given that inhibition of FAK autophosphorylation on Y397 decreased invasiveness of tumor cells expressing Rab5. Moreover, expression of constitutively active FAK sufficed to restore invasiveness of Rab5 knockdown cells. These findings coincide with evidence suggesting a role for FAs and FAK in tumor cell invasion. Intriguingly, recent evidence suggests that FAK-mediated FA assembly, but not FAK activity, which rather promotes FA disassembly, is required for invasiveness (Wang and McNiven, 2012). Whether FAK itself or FAK-mediated FA formation and/or dynamics are essential for tumor invasiveness remains a matter of debate. Hence, further studies are required in order to determine the precise role of both FAK activation and FA disassembly in Rab5-promoted invasiveness, as both events appear to be linked in our studies.

## Materials and Methods

### Materials

Monoclonal anti-Rab5 (sc46692), polyclonal anti-Rab5 (sc28570) and antibodies for Rab7 (sc10767), Rab11 (sc9020), EEA1 (sc33585) and integrin  $\beta 1$  (sc8978) were from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies included anti-phospho-Y397-FAK (number 3283, Cell Signaling Technology), anti-vinculin (number V4505, Sigma-Aldrich, St Louis, MO), anti- $\alpha$ -tubulin (number CP06, Calbiochem, La Jolla, CA) and anti-paxillin (number 610620, BD Biosciences, San Diego, CA). Goat anti-rabbit and goat anti-mouse antibodies coupled to horseradish peroxidase (HRP) and anti-actin antibody (number A5316) were from Bio-Rad Laboratories (Hercules, CA). Geneticin (number 11811), phalloidin–Rhodamine, Alexa-Fluor-488- and Alexa-Fluor-568-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA). Tissue culture medium, antibiotics and fetal bovine serum (FBS) were from GIBCO Life

Technologies (Grand Island, NY) and HyClone Laboratories (Logan, UT). Glutathione–Sepharose-4B was from GE Healthcare (Piscataway, NJ). The EZ-ECL chemiluminescent substrate and protein A/G beads were from Pierce Chemical (Rockford, IL). Rab5 lentiviral short hairpin RNAs (shRNA) were from Open Biosystems (Huntsville, AL). The FAK inhibitor compound number 14 was from Tocris Bioscience (R&D Systems). The MMP2 and MMP9 inhibitor II was from Calbiochem (La Jolla, CA).

### Plasmids

The pcDNA3.1(+) plasmids encoding wild-type Rab5 and the mutants S34N (high affinity for GDP) and Q79L (GTPase-deficient) were as described previously (Torres et al., 2008). The pEGFP-C1 plasmids encoding wild-type Rab5, Rab5/S34N and Rab5/Q79L were kindly provided by Dr Francisca Bronfman (Pontificia Universidad Católica de Chile, Chile). The mCherry–paxillin, mCherry–vinculin, pEGFP-C1-actin and pEGFP-C1-FAK/Y180A/M183A constructs were kindly provided by Dr David Schlaepfer (University of California, San Diego, USA). The modified pEGFP-C1-mCherry plasmid harboring a deletion of GFP gene sequence, but encoding mCherry instead was also provided by Dr David Schlaepfer. The Rab5-binding domain (R5BD) previously described (Liu et al., 2007; Torres et al., 2008; Vitale et al., 1998) was sub-cloned into the modified pEGFP-C1-mCherry plasmid by PCR amplification. To this end, the pCMV-SPORT6 plasmid encoding human Rabaptin5 (Invitrogen) was used as a template, with the primers 5'-CGCCCGGG-AGCTAAGGCTACCGTTGAACA-3' (forward, *Sall* restriction site) and 5'-CCCCCGGGTCATGTCTCAGGAAGCTGGT-3' (reverse, *Bam*HI restriction site). The PCR product was digested and subcloned by blunt-end ligation into pEGFP-C1-mCherry. Orientation was confirmed by sequencing.

### Cell culture

MDA-MB-231 human breast cancer cells and A549 lung carcinoma cells were cultured in DMEM-F12 and DMEM-high glucose, respectively, supplemented with 10% FBS and antibiotics. Rab5 targeting was performed as previously described, by using shRNA constructs targeting Rab5A (Torres et al., 2010). Of note, shRNA constructs used in this study (shRNA sequence number B5, shRNA sequence number F10; Open Biosystems) selectively target Rab5A, but not Rab5B or Rab5C. Control cells were infected with a lentivirus encoding a nonspecific shRNA sequence (plasmid 1864; Adgene, Cambridge, MA). Rab5 downregulation in MDA-MB-231 cells was performed by using the shRNA sequence number F10, whereas for A549 cells both shRNA sequences number B5 and number F10 were used. Sequence number B5 could not be used for MDA-MB-231 cells, as cells did not survive transduction with this shRNA sequence. Stable cell lines were selected and maintained in puromycin-containing culture medium.

### Immunofluorescence

Immunofluorescence protocols were performed as previously described (Avalos et al., 2004; Urra et al., 2012). Samples were visualized by confocal microscopy, using either an Olympus IX81 spinning disk microscope or a Carl Zeiss LSM-Pascal 5 confocal microscope.

### Focal adhesion analysis

For focal adhesion synchronization, cells were grown on glass coverslips, starved overnight in medium containing 1% serum and treated with 10  $\mu$ M nocodazole in serum-free medium for 4 h to depolymerize microtubules, as previously described (Ezratty et al., 2005). Nocodazole was washed-out with serum-free medium and cells were incubated at 37°C for the indicated periods of time. Subsequently, cells were fixed and prepared for immunofluorescence staining. Focal adhesion analysis was performed as previously described (Avalos et al., 2004; Urra et al., 2012). The number of focal adhesions per cell was quantified at all time points, as follows. Images were processed after subtracting threshold levels owing to diffuse vinculin staining, by using the Image J software. Then, fluorescence pixels owing to vinculin accumulation in punctate and elongated structures were normalized with respect to the number of cells analyzed (judged by nuclear staining). Other parameters, such as focal adhesion size and shape were not measured. Data were normalized to the number of focal adhesions in control cells.

### Spreading assay

Cells in suspension were allowed to attach and spread onto fibronectin-coated coverslips (2  $\mu$ g/ml) for the indicated periods of time. Samples were prepared for immunofluorescence and stained with phalloidin–Rhodamine. At least 10 images per condition were analyzed (typically 5–10 cells per image). Cell spreading was evaluated on the basis of phalloidin staining, by using the Image J software. Upon threshold adjustment, the number of pixels per image was measured and normalized with respect to the total cell number. Cell spreading at 60 min was used as internal standard, in order to perform the pixel-to- $\mu$ m<sup>2</sup> conversion, based on the scale of the image (in  $\mu$ m).

### Microtubule depolymerization analysis

Microtubules were stained by immunofluorescence (green channel) and the signal was converted into grayscale format. Then, microtubule re-growth was calculated by using the Image J software. To this end, the plug-ins 'find edges', 'invert' and 'sharpen' were sequentially applied to all data and then, the threshold was adjusted (representative images made following this procedure are shown in supplementary material Fig. S4B). Finally, the 'integrated density' was measured and data were shown as the percentage of non-treated controls.

### Image analysis

#### Deconvolution of confocal images

Images obtained by confocal microscopy were subjected to iterative deconvolution using the Huygens Professional software (Version 3.7.0p3; SVI, Hilversum, The Netherlands) using theoretical point-spread functions. The signal-to-noise ratio was adjusted in each image to obtain optimum restoration. Deconvolution was limited to a maximum of 40 iterations.

#### Measurement of the incidence of cells with peripheral mCherry

To analyze the distribution of mCherry and mCherry–R5BD, cells were transiently transfected for 24 h, re-suspended and then allowed to spread on fibronectin-coated (2  $\mu$ g/ml) coverslips for different periods of time. mCherry was imaged by confocal microscopy (Carl Zeiss LSM-Pascal 5). Analysis was performed as previously described for total Rab5 (Torres et al., 2008). Images were randomly chosen, and entire cells were scored as 'positive for peripheral mCherry staining' or 'negative for peripheral mCherry staining' based on the detection of mCherry signal at the cell periphery. Colors were separately analyzed with the Image J software (split channels menu), with no threshold adjustments, and events were recorded by two observers that were blind to the transfection undertaken. The percentage of 'positive cells' was calculated with respect to the total cell number. At least eight images per sample were analyzed. Total cell number was counted by nuclear staining (DAPI).

#### Co-localization analysis

Co-localization data and plots for the intensity profile were evaluated in original and deconvoluted images, obtained by confocal microscopy (Carl Zeiss LSM-Pascal 5). Analysis was performed with the Image J software, by using the 'Dynamic Profile' and 'JACoP' plugins.

### Time-lapse microscopy

Time-lapse microscopy and live-cell imaging was performed as described previously (Urra et al., 2012). Cells were transiently transfected with mCherry–vinculin, mCherry–paxillin or GFP–Rab5 in glass bottomed tissue culture plates (MatTek, Mattek Corporation). Post-transfection (24 h), cells were serum-starved by 2 h, followed by a pulse with 10% serum to stimulate cell spreading. Cells were visualized in a confocal microscope (FluoView FV1000, Olympus). Images were captured for at least 1 h. For FA analysis, FA structures were defined by size with the Image J software. mCherry-positive structures (ranging from 20 to 800 pixels) were screened. FA disassembly was evaluated from the time-lapse series, by quantifying the intensity of pixels with the Image J software.

### Western blotting

Cells were washed twice with cold PBS and lysed in 0.2 mM HEPES (pH 7.4) buffer containing 0.1% SDS, phosphatase inhibitors (1 mM Na<sub>3</sub>VO<sub>4</sub>), as well as a protease inhibitor cocktail. Total protein extracts (50  $\mu$ g/lane, unless indicated) were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Blots were blocked with 5% milk in 0.1% Tween-PBS and then probed with antibodies. Bound primary antibodies were detected with HRP-conjugated secondary antibodies and the EZ-ECL system.

### Immunoprecipitation

Rab5 immunoprecipitation was as previously described (Torres et al., 2008). Basically, this method was developed to minimize GTP hydrolysis and optimize recovery of active Rab5, by using short incubation periods. Essentially, cell extracts were prepared in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40 and protease inhibitors. After 5 min incubation on ice, samples were centrifuged at 13,000 *g* for 1 min at 4°C and post-nuclear supernatants (500  $\mu$ g total protein) were immunoprecipitated with polyclonal antibodies immobilized on protein A/G beads for no longer than 30 min. Immunoprecipitated samples were solubilized in Laemmli buffer, boiled, separated by SDS-PAGE and analyzed by western blotting.

### Rab5-GTP pulldown assay

Cells were lysed in a buffer containing 25 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% NP-40, 10% glycerol, 1 mM dithiothreitol and protease inhibitors. Extracts were incubated for 5 min on ice and clarified by centrifugation (10,000 *g*, 1 min, 4°C). Post-nuclear supernatants were used for pulldown assays with 30  $\mu$ g of GST–R5BD pre-coated GSH beads per condition. Beads were

incubated with supernatant for 15 min at 4°C in a rotating shaker. Thereafter, beads were collected, washed with lysis buffer containing 0.01% NP-40 and samples were analyzed by western blotting.

#### Transwell migration assay

Assays were performed in Boyden chambers (Transwell Costar, 6.5-mm diameter, 8-µm pore size). The bottom sides of the inserts were coated with 2 µg/ml fibronectin. Cells ( $5 \times 10^4$ ) re-suspended in serum-free medium were plated onto the top of each chamber insert and medium supplemented with 10% serum was added to the bottom chamber. After 2 h, inserts were removed, washed and cells that migrated to the bottom side of the inserts were stained with 0.1% Crystal Violet in 2% ethanol and counted in an inverted microscope.

#### Matrigel assay

Assays were performed in Matrigel (number 354480, BD Biosciences, San Diego, CA), following the manufacturer's instructions. Briefly, the inserts were pre-hydrated for 2 h in serum-free medium. Cells ( $3 \times 10^4$ ) re-suspended in serum-free medium were plated onto the top of each chamber insert and medium supplemented with 10% serum was added to the bottom chamber. After 24 h, inserts were removed, washed and cells that migrated to the bottom side of the inserts were stained with 0.1% Toluidine Blue and counted in an inverted microscope.

#### Zymography

Cells were serum-starved for 16 h and supernatants were analyzed by zymography to determine the enzymatic activity of MMP2 and MMP9. Samples were resolved in 6% polyacrylamide gels co-polymerized with gelatin (1 mg/ml). Samples (25 mg protein) were incubated for 30 min in sample buffer (0.4 M Tris-HCl, pH 6.8, containing 5% SDS, 20% glycerol, 0.03% Bromophenol Blue) under non-reducing conditions, at room temperature. After electrophoresis, gels were incubated in 2.5% Triton X-100 for 1 h at room temperature, in order to eliminate residual SDS. Gels were then incubated by 24 h in metalloproteinase test buffer (150 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub>) at 37°C. Gels were fixed and stained with Coomassie Blue R-250.

#### Adhesion assay and fractionation of focal adhesions

Cell adhesion and fractionation assays were performed as described previously (Torres et al., 2010). For fractionation assays, cells that adhered onto fibronectin-coated plates (2 µg/ml, 45 min) were analyzed.

#### Statistical analysis

Data were compared in unpaired Student's *t*-tests by using the GraphPad Prism 5 software (San Diego, CA). At least three independent experiments were analyzed. *P* < 0.05 was considered significant.

#### Acknowledgements

We acknowledge Dr Francisca Bronfman for providing the pEGFP-C1 plasmids encoding the Rab5 constructs. Dr David Schlaepfer is gratefully acknowledged for the insightful discussion of our work and for having provided us with the mCherry-paxillin, mCherry-vinculin, pEGFP-C1-actin and pEGFP-C1-FAK/Y180A/M183A constructs.

#### Author contributions

P.M. and V.T. conceived and designed the experiments; P.M., R.O., J.D. and V.T. performed the experiments; P.M., R.O., J.D. and V.T. analyzed the data; A.Q., L.L., D.S. and V.T. contributed reagents/materials/analysis tools; P.M., A.Q., L.L., D.S. and V.T. wrote the paper.

#### Funding

This work was supported by the National Fund for Scientific and Technological Development (FONDECYT) Initiation [grant number 11100287 to V.T.]; the National Commission for Science and Technology (CONICYT) 'Insertion of Young Postdoctoral Researchers in the Academy' [grant number 79090021 to V.T.]; FONDECYT [grant number 1130250 to A.Q.]; Biomedical Neuroscience Institute from Iniciativas Científicas Milenio [grant number P09-015-F to L.L.]; FONDECYT [grant number 1110149 to L.L.] and CONICYT PhD fellowships (to J.D. and R.O.).

Supplementary material available online at

<http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.119727/-DC1>

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