Competitive binding of Rab21 and p120RasGAP to integrins regulates receptor traffic and migration

Anja Mai,[1,2,4] Stefan Veltel,[1,2] Teijo Pellinen,[2] Artur Padzik,[1] Eleanor Coffey,[1] Varpu Marjomäki,[3] and Johanna Ivaska[1,2,4]

[1]Turku Centre for Biotechnology, Turku 20521, Finland
[2]VTT Technical Research Centre of Finland, Medical Biotechnology, Turku 20520, Finland
[3]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä 40500, Finland
[4]Department of Biochemistry and Food Chemistry, University of Turku, Turku 20521, Finland

Integrin trafficking from and to the plasma membrane controls many aspects of cell behavior including cell motility, invasion, and cytokinesis. Recruitment of integrin cargo to the endocytic machinery is regulated by the small GTPase Rab21, but the detailed molecular mechanisms underlying integrin cargo recruitment are yet unknown. Here we identify an important role for p120RasGAP (RASA1) in the recycling of endocytosed α/β1-integrin heterodimers to the plasma membrane. Silencing of p120RasGAP attenuated integrin recycling and augmented cell motility. Mechanistically, p120RasGAP interacted with the cytoplasmic domain of integrin α-subunits via its GAP domain and competed with Rab21 for binding to endocytosed integrins. This in turn facilitated exit of the integrin from Rab21- and EEA1-positive endosomes to drive recycling. Our results assign an unexpected role for p120RasGAP in the regulation of integrin traffic in cancer cells and reveal a new concept of competitive binding of Rab GTPases and GAP proteins to receptors as a regulatory mechanism in trafficking.

Introduction

Membrane trafficking of receptors between the plasma membrane and intracellular membrane–enclosed organelles is fundamentally important for the maintenance of cell polarity, regulation of signal transduction, and cell migration (Scita and Di Fiore, 2010). Targeted delivery of the cargo receptors is controlled by Rab GTPases. These small GTPases have specific subcellular localizations and regulate endocytic processes like cell surface receptor trafficking via recruitment of specific effector molecules (Zerial and McBride, 2001; Schwartz et al., 2007). To date, the majority of the characterized Rab effectors are recruited to GTP-bound Rabs, but there are also rare examples of effectors preferring GDP-bound Rabs (Stenmark, 2009).

Integrins are a family of cell adhesion receptors that are used by cells to assemble and recognize their functional ECM. Integrins are heterodimers composed of α- and β-subunits. In addition to mediating attachment to the ECM, integrins also function as bi-directional signaling molecules that have the capability to transmit signals from the outside of the cell to the inside and vice versa (Hynes, 2002). Both integrin subunits are important for integrin function and have been shown to affect integrin signaling via specific interactions with cytosolic proteins (Liu et al., 2000; Legate et al., 2009).

In adherent cells, integrins are constantly endocytosed and recycled back to the plasma membrane (Pellinen and Ivaska, 2006; Caswell and Norman, 2008; Caswell et al., 2009). Integrin trafficking has been established as a critical process for cell migration, turnover of focal adhesions, cell division, cell invasion, and even for tumor dissemination downstream of mutant p53 (Muller et al., 2009). Integrin traffic is known to involve transit through specific Rab-positive compartments in the cell, and both integrin heterodimer composition as well as extracellular stimuli influence the traffic (Caswell and Norman, 2006; Muller et al., 2009). The Rab5 family members Rab5 and Rab21 have been shown to be important for receptor entry (Pellinen et al., 2006; Caswell et al., 2009). For Rab21, this involves association with integrin α-subunits (Pellinen et al., 2006). After entry to the Rab5/Rab21 endosomes, integrins are recycled to the

A. Mai and S. Veltel contributed equally to this paper.

Correspondence to Johanna Ivaska: Johanna.ivaska@vtt.fi

Abbreviations used in this paper: EGFR, epidermal growth factor receptor; FP, fluorescence polarization; GAP, GTPase-activating protein; MEF, mouse embryonic fibroblast.

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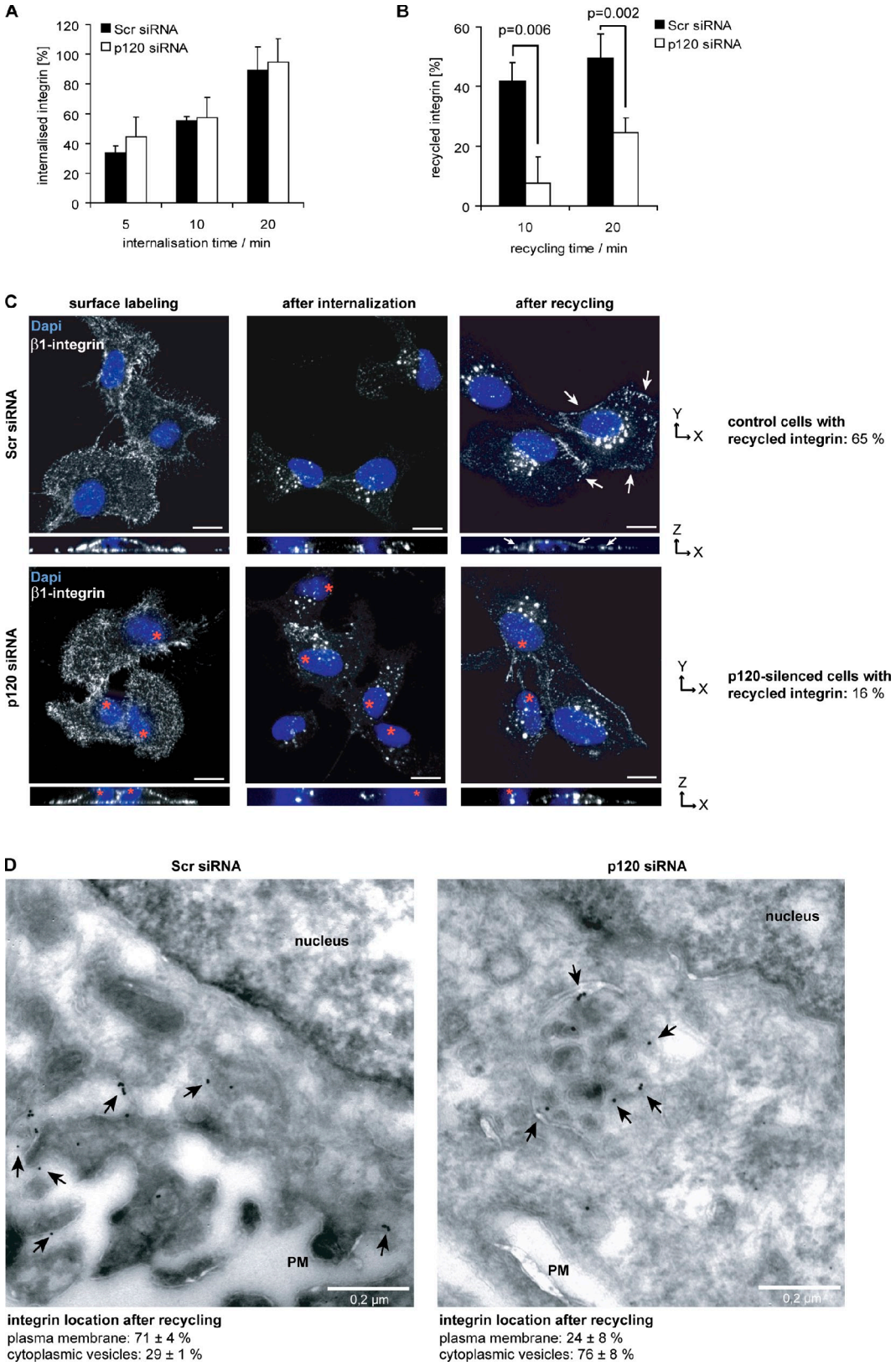


Figure 1. **p120RasGAP is required for β1-integrin recycling to the plasma membrane.** (A) Serum-starved cells were surface labeled with cleavable biotin. Internalization was allowed for the times indicated. After cleavage of surface remaining biotin, the cells were lysed and immunoprecipitated with anti–β1-integrin antibody, immunoblotted with anti-biotin antibody, followed by reprobing with anti-β1-integrin antibody. The graph shows biotinylated integrin relative to the total amount of surface-biotinylated integrin normalized against immunoprecipitated β1-integrin (mean ± SEM; three independent experiments). (B) Cells were treated as in A, but after internalization (30 min) and removal of cell-surface biotin, recycling (indicated times) was enabled by serum stimulation followed by a second cell-surface biotin cleavage. Recycling was assessed by the decrease of biotinylated integrin (mean ± SEM; six independent experiments). (C) Surface integrins of starved control (Scr) or p120RasGAP-silenced cells (asterisk, based on 3′ Alexa 647–conjugated

plasma membrane via a Rab4-dependent mechanism (αVβ3-integrin) or Rab11-positive recycling endosomes (β1-integrins; Roberts et al., 2001, 2004). Interestingly, increased integrin recycling correlates with invasion and metastasis in vitro and in vivo (Muller et al., 2009). Integrin trafficking is critically dependent on the ability of the Rabs to switch from GTP- to GDP-bound forms as not only inactive GDP-locked, but also GTP-locked mutants of Rab21, Rab5, Rab4, and Rab11 block integrin traffic (Roberts et al., 2001, 2004; Powelka et al., 2004; Pellinen et al., 2006). However, very little is known about the specific GTPase-activating proteins (GAPs) that would catalyze this GTP hydrolysis.

P120RasGAP (RASA1) is a well-known GAP that functions as a negative regulator of Ras signaling downstream of several growth factor receptors (Kazlauskas et al., 1990; Cooper and Kashishian, 1993; Jones et al., 2006). In addition, it has been suggested to function as a GAP for Rab5 (Liu and Li, 1998). From its predominantly cytoplasmic localization, p120RasGAP can be recruited to the plasma membrane in response to growth factors and integrin engagement (Huang et al., 1993; Sharma, 1998). This translocation is facilitated by the SH2- and SH3-protein interaction domains of p120RasGAP that mediate binding to platelet-derived growth factor receptor (PDGFR), focal adhesion kinase (FAK), and p190RhoGAP at the plasma membrane, and the internalized epidermal growth factor receptor (EGFR) on endosomes (Wang et al., 1996; Pamonsinlapatham et al., 2009; Tomar and Schlaepfer, 2009). P120RasGAP has also been shown to regulate cell motility. In fibroblasts, p120RasGAP has been shown to function as a positive regulator of cell migration (Kulkarni et al., 2000) and this has been largely attributed to the recruitment of p190RhoGAP to the plasma membrane and subsequent transient inactivation of RhoA, which facilitates cell spreading (Tomar and Schlaepfer, 2009; Tomar et al., 2009). In contrast, in epithelial cells silencing of p120RasGAP has been shown to increase migration (Pamonsinlapatham et al., 2008).

Here we identify p120RasGAP as a novel regulator for integrin traffic. We found that p120RasGAP regulates cell motility by controlling the return of endocytosed integrins to the plasma membrane. We identified a direct interaction of the GAP-domain of p120RasGAP with the membrane-proximal conserved sequence found in most integrin α-subunits. This binding site overlaps with that of Rab21, and p120RasGAP competes with Rab21 for integrin binding. In the absence of p120RasGAP, integrins remain bound to Rab21 and are increasingly retained in EEA1-positive endosomes instead of actively returning to the plasma membrane. These results assign an unexpected role for p120RasGAP in the regulation of integrin traffic and cell motility.

Results

Recycling of β1-integrins requires p120RasGAP

We have previously shown that Rab21 regulates endo/exocytic traffic of α/β1-integrin heterodimers in several cell types by associating with integrin α-subunits. Perturbation of Rab21 function leads to impaired cell motility and cytokinesis (Pellinen et al., 2006, 2008). Very little is known about which GTPase regulators are relevant for integrin traffic. P120RasGAP has been implicated in the inactivation of Rab5, a small GTPase with high sequence homology to Rab21 (Liu and Li, 1998). In addition, p120RasGAP has been shown to regulate cell migration (Kulkarni et al., 2000; Pamonsinlapatham et al., 2008) and polarity (Tomar et al., 2009) and to localize to endosomes (Trahey et al., 1988). Therefore, we sought to determine whether p120RasGAP would regulate integrin traffic. We silenced p120RasGAP in MDA-MB-231 breast cancer cells (in these cells, traffic of β1-integrins is Rab21 dependent; Pellinen et al., 2006, 2008) using two independent siRNAs. Both siRNAs readily silenced p120RasGAP expression without influencing expression of β1-integrin, Rab21, or p190RhoGAP (Fig. S1 A). Using a well-established biochemical assay to investigate endocytosis and recycling of cell surface–labeled integrins (Roberts et al., 2001), we found that silencing of p120RasGAP had no significant effect on the endocytosis rate of β1-integrins (Fig. 1 A). However, the recycling rate of β1-integrins was significantly reduced in p120RasGAP-silenced cells compared with control siRNA-transfected cells (Fig. 1 B). Importantly, transfection with two different siRNAs against p120RasGAP reduced integrin recycling to a similar extent (Fig. S1 B).

We then analyzed β1-integrin traffic with an antibody-based assay (Powelka et al., 2004). Cell surface β1-integrin was labeled with an antibody, and its itinerary fate was followed in control or p120RasGAP-silenced cells. In both cases, integrins were efficiently internalized from the membrane (Fig. 1 C). In control cells, serum induced the recycling of internalized integrin back to the plasma membrane (65% of 43 analyzed cells recycled integrin to the plasma membrane). In contrast, p120RasGAP-siRNA–transfected cells displayed a reduced ability to recycle integrins back to the plasma membrane (16% of 38 analyzed cells recycled integrin to the plasma membrane; Fig. 1 C). We also observed by electron microscopy (EM) that after consecutive internalization and recycling steps of cell surface–bound, gold-labeled anti-β1 antibody, β1-integrin predominantly returned to the plasma membrane only in control cells (71 ± 4% of integrin recycled, $n = 50$ cells). In p120RasGAP-silenced cells, however, β1-integrins remained mainly intracellular in cytoplasmic vesicles (24 ± 8% recycled integrin, $n = 35$ cells; Fig. 1 D). Taken together, these results indicate that efficient recycling of internalized β1-integrins depends on p120RasGAP.

p120RasGAP-siRNA) were labeled with anti–β1-integrin antibody (gray). Internalization (30 min) was followed by serum-induced recycling for 30 min (arrows; recycled integrins at the plasma membrane). Numbers indicate the percentage of cells with recycled integrins ($n = 38–43$ cells). Images represent projections of 25–35 planes per stack viewed along the x-y or x-z axis. (D) Recycling assay done as in C, but with 10-nm gold–conjugated anti–β1-integrin antibody and detected with electron microscopy (PM, plasma membrane). Arrows point to gold-labeled integrins. Integrin location after recycling is indicated ($n = 35–50$ cells scored). Bars: (C)10 µm; (D) 0.2 µm.

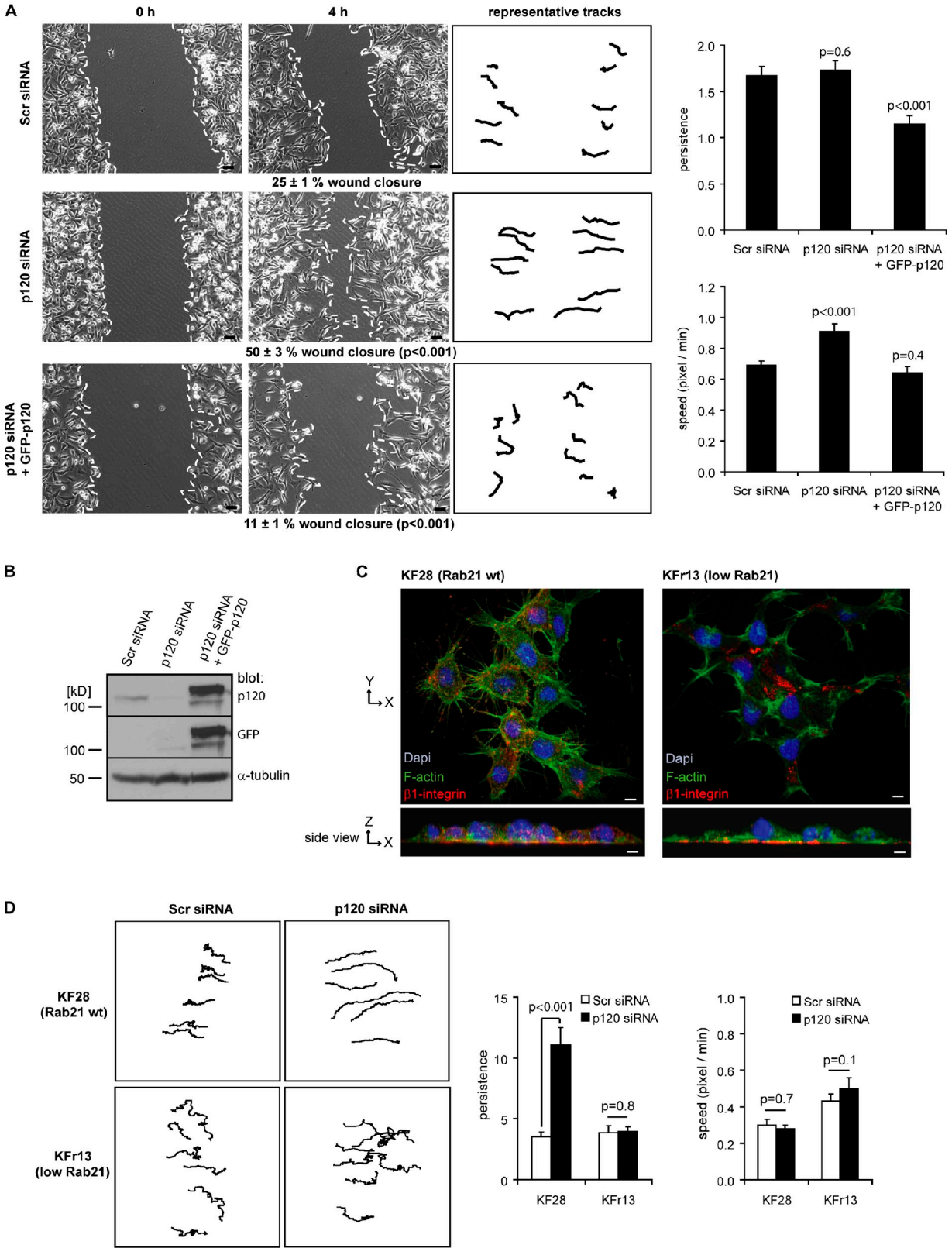


Figure 2. **p120RasGAP regulates cell migration downstream of Rab21-driven integrin endocytosis.** (A) Migration analysis of control (Scr), p120-silenced MDA-MB-231 cells, or cells that were first silenced for p120RasGAP and then rescued by GFP-p120 expression (p120 siRNA + GFP-p120) by scratch-wound assay. Shown are representative wounds at the indicated times and percentage of wound closure (wound area after [4 h] related to wound area before [0 h] imaging; mean ± SEM, *n* = 10 wounds per treatment). Cell motility has been analyzed in more detail by tracking the location of cells (*n* = 50 cells per treatment) over time and measuring both the persistence time (persistence) using the calculated mean squared displacement (MSD) of tracked cells as well as the migration speed (distance migrated per minute). Bar, 100 µm. (B) Western blot analysis with the indicated antibodies from cell lysates of the

P120RasGAP regulates cell migration

Integrin traffic and cell migration are tightly regulated cellular processes. Recent data has linked reduced integrin recycling with increased directional cell motility (White et al., 2007; Caswell et al., 2008; Muller et al., 2009). Because loss of p120RasGAP attenuated integrin recycling in MDA-MB-231 cells, we analyzed movement of p120RasGAP- and control-siRNA–transfected cells using time-lapse microscopy. Silencing of p120RasGAP increased speed of migration in MDA-MB-231 cells (Fig. S2 A). Furthermore, compared with control cells, silencing of p120RasGAP stimulated the migration of MDA-MB-231 cells into a wound (Fig. 2, A and B). Importantly, the stimulated migratory phenotype could be reversed by reexpression of siRNA-resistant, GFP-tagged p120RasGAP in p120-silenced cells (Fig. 2, A and B). Enhanced wound closure upon p120RasGAP silencing could also be observed in other cell lines, such as human (telomerase immortalized foreskin fibroblasts, TIFFs) and mouse fibroblasts (mouse embryonic fibroblasts, MEFs; Fig. S2, B and C). In these cell lines the more rapid wound closure was a function of increased migration speed and persistence. These migratory changes are likely to be due to alterations in integrin traffic because we did not detect any significant changes in cell surface expression or activation status of β1-integrins in p120RasGAP-silenced cells compared with control cells (Fig. S3).

Because we have shown earlier that migration of MDA-MB-231 cells is dependent on Rab21-mediated integrin endocytosis (Pellinen et al., 2006), we sought to assess the link between p120RasGAP siRNA-induced cell motility and Rab21-regulated integrin traffic in more detail. For this purpose, we turned to another cell model. KFr13 ovarian carcinoma cells harbor a chromosomal deletion of the *RAB21* gene locus resulting in reduced Rab21 expression compared with their parental KF28 cell line with an intact *RAB21* locus (Fig. S4 A; Pellinen et al., 2008). We found that in KFr13 cells, the absence of Rab21 results in poor endocytosis of β1-integrins (Fig. S4 B) leading to a basal plasma membrane localization of the majority of integrins (Fig. 2 C). Importantly, silencing of p120RasGAP induced persistent cell migration only in KF28 cells, but failed to stimulate directional motility in KFr13 cells (Fig. 2 D). This is indicative of a role for p120RasGAP in regulating migration downstream of Rab21-mediated integrin endocytosis.

Previous studies with fibroblasts have shown that p120RasGAP–p190RhoGAP complex formation regulates cell polarity and migration via transient inactivation of RhoA at the plasma membrane (Peacock et al., 2007). However, in MDA-MB-231 cells, silencing of p120RasGAP did not activate RhoA (Fig. S5 A). Furthermore, overexpression of a SH2-SH3-SH2 fragment of p120RasGAP (RFP-232), which abolishes the interaction of endogenous p120RasGAP with EGFR, PDGFR, and p190RhoGAP (Bradley et al., 2006), had no significant effect on migration in these cells (Fig. S5 B). Thus, it is unlikely that increased migration of p120RasGAP-silenced MDA-MB-231 cells would be due to altered RhoA activity or Ras signaling.

P120RasGAP is not a GAP for Rab21

The fact that p120RasGAP regulates cell migration downstream of Rab21 suggests that both proteins somehow jointly regulate integrin trafficking. Therefore, we tested whether p120RasGAP could function as a GAP for Rab21. We performed an in vitro GTP-hydrolysis assay with γ-[^{32}P]GTP. However, the GAP domain of p120RasGAP (p120 GAP) was not able to increase the intrinsic GTP-hydrolysis of Rab21 (Fig. 3 A) although it had strong GAP activity on H-Ras, an established substrate of p120RasGAP (Pawson, 1995; Scheffzek et al., 1997). Based on these data, p120RasGAP does not regulate integrin traffic by influencing Rab21 activity.

Rab21 binds to the cytoplasmic tail of α2-integrin (Pellinen et al., 2006). However, it has remained unknown whether this is a nucleotide-dependent direct interaction. To investigate this, we set up a fluorescence polarization (FP)–based protein–protein interaction assay (Veltel et al., 2008) using the membrane-proximal region of α2-integrin cytoplasmic tail (residues 1154–1166) coupled to the fluorophor EDANS (5-[(2′-aminoethyl)amino]naphthalene sulfonic acid) and purified, recombinant Rab21 loaded with either GDP or the nonhydrolyzable GTP-analogue GppNHp. The success of the nucleotide exchange and the induction of the GTP-bound conformational change in GST-Rab21*GppNHp were confirmed with a pull-down assay using APPL1, which specifically interacts with GTP-bound Rab21 (Fig. 3 B; Zhu et al., 2007). The purity of all recombinant proteins used for in vitro assays in this study has been verified by SDS-PAGE (Fig. 3 C). Interestingly, an FP-based binding assay showed that an α2-integrin peptide (α2-cons, containing the conserved sequence of the integrin α2-tail and five additional residues that had to be added to retain solubility and support α-helix formation [WKLGFFKRKYEKM, residues 1154–1166 of the α2-subunit]) binds identically to both Rab21*GppNHp and Rab21*GDP, but not to GST (Fig. 3 D). Addition of higher concentrations of GST (up to 50 µM) to the fluorescent peptide did not increase the anisotropy, indicating that the increase in anisotropy binding signal of the integrin peptide to Rab21 is not due to protein concentration artifacts. Furthermore, also in pull-down experiments β1-integrin heterodimers associated with both Rab21*GppNHp and Rab21*GDP (Fig. 4 B). These data suggest that Rab21 interacts with the integrin cargo in an unusual, nucleotide-independent manner. However, because the Rab21GDP mutant has been shown to block integrin endocytosis (resulting in plasma membrane–localized integrins) and the Rab21GTP mutant to inhibit integrin recycling (resulting in intracellular accumulation of integrins) (Pellinen et al., 2006), it is likely that yet unidentified nucleotide

experiment shown in A. (C) KF28 and KFr13 cells were fixed and immunostained as indicated. Images represent projections covering in total 10–13 µm viewed along the x-y or x-z axis. Bar, 10 µm. (D) Migration of p120RasGAP- or control- (Scr) silenced KF28 and KFr13 cells at the edge of a scratch wound was analyzed using time-lapse microscopy. Shown are representative tracks. Speed and directionality of motility was scored as described in A (mean ± SEM, *n* = 56–68 cells, combined data from two separate experiments).

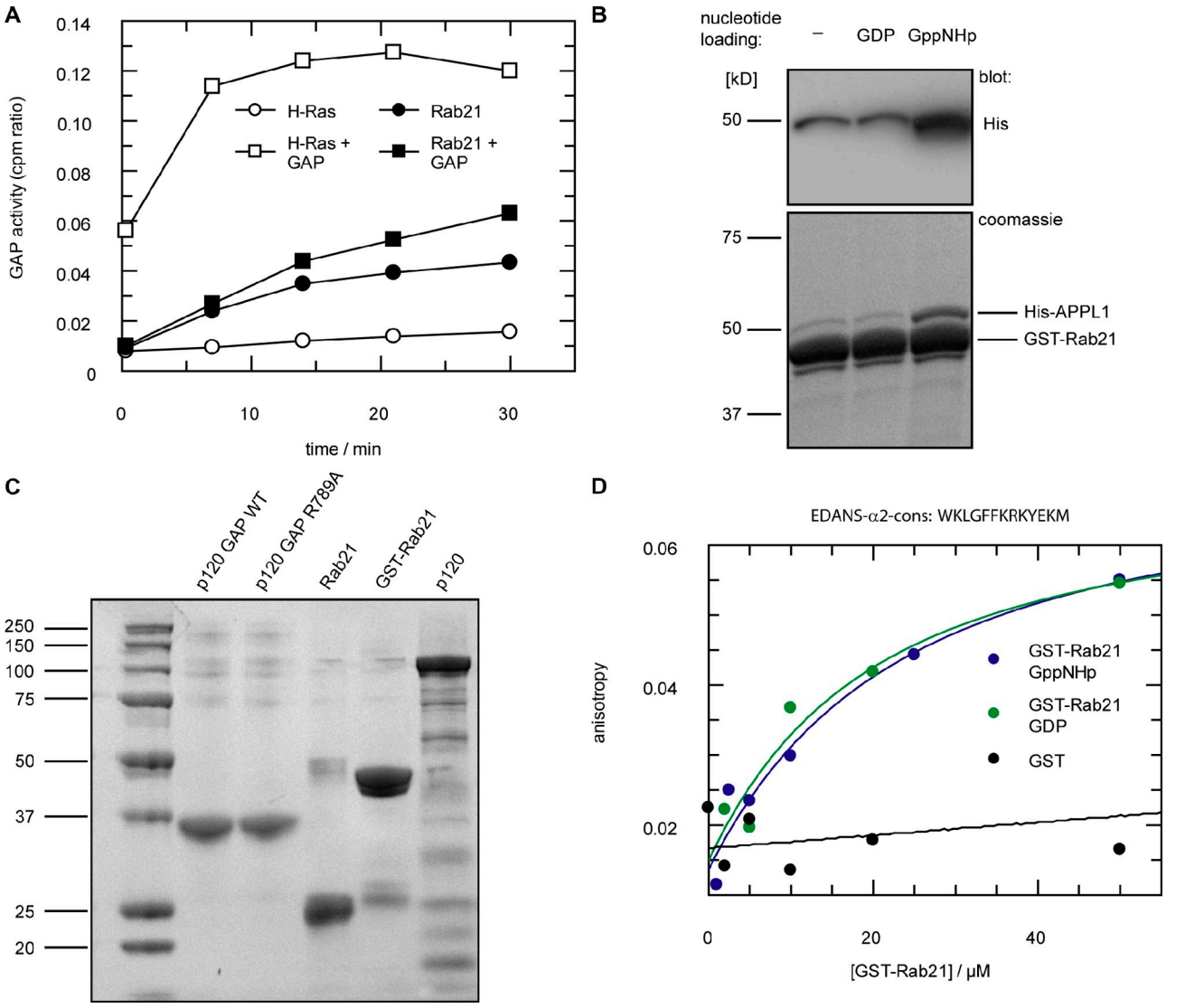


Figure 3. **p120RasGAP does not function as a GAP for Rab21.** (A) The GTP-hydrolysis–stimulating activity of the recombinantly purified GAP domain of p120RasGAP (p120 GAP) toward Rab21 and H-Ras was measured in a radioactive GAP-assay. 1 μM of Rab21 or H-Ras bound to γ-[^{32}P]GTP was incubated with 5 μM of p120 GAP. The release of the radioactive labeled reaction product $^{32}P_i$ was detected at the indicated time points by scintillation counting and compared with the total counts per minute used in the assay (cpm ratio). (B) His-APPL1 binding to GST-Rab21–bound beads was studied to confirm successful loading of Rab21 with the GTP analogue GppNHp for the experiment shown in D. (C) Coomassie-stained SDS-PAGE of all recombinant purified proteins used in this study. 7 μg of each protein was loaded to a 10% SDS gel. All proteins are highly pure and degradation could only be observed to some extent for p120. (D) A fluorescence polarization (FP)–based assay was applied to detect binding between 5 μM EDANS-conjugated α2-integrin cytoplasmic domain peptide and different concentrations of GST or GST-Rab21 loaded either with GDP or GppNHp. Complex formation was deduced from an increase in anisotropy signal.

status-dependent Rab21 effectors are also important for the adequate trafficking of integrin cargo downstream of Rab21. For example, in case of the Rab21 homologue Rab5, effector proteins like EEA1 or Rabenosyn are recruited to a Rab5*GTP-signaling platform to ensure induction of endosomal membrane fusion in concert with SNARE proteins (Ohya et al., 2009).

The GAP domain of p120RasGAP interacts directly with integrin α-cytoplasmic tails

To investigate how p120RasGAP regulates integrin traffic, we examined whether p120RasGAP associates with integrins in cells. Reciprocal immunoprecipitations with anti-β1 and anti-p120 RasGAP antibodies demonstrated that a fraction of β1-integrin is in a complex with p120RasGAP in cells at any given moment (Fig. 4 A). In pull-down experiments β1-integrin associated strongly with recombinant GST-p120RasGAP (p120) and GST-p120RasGAP GAP domain (p120 GAP WT), as well as with a catalytically inactive GAP mutant (p120 GAP R789A). This suggests that the GAP domain of p120RasGAP alone is sufficient for association with β1-integrins, and that p120 GAP activity is not needed for this association (Fig. 4 B).

To assess whether p120RasGAP binds integrins directly and whether the interaction is mediated via the α- or the β-subunit, we performed a set of biochemical and biophysical binding assays. First, using biotinylated integrin peptides we found that recombinant, purified p120 GAP domain interacts

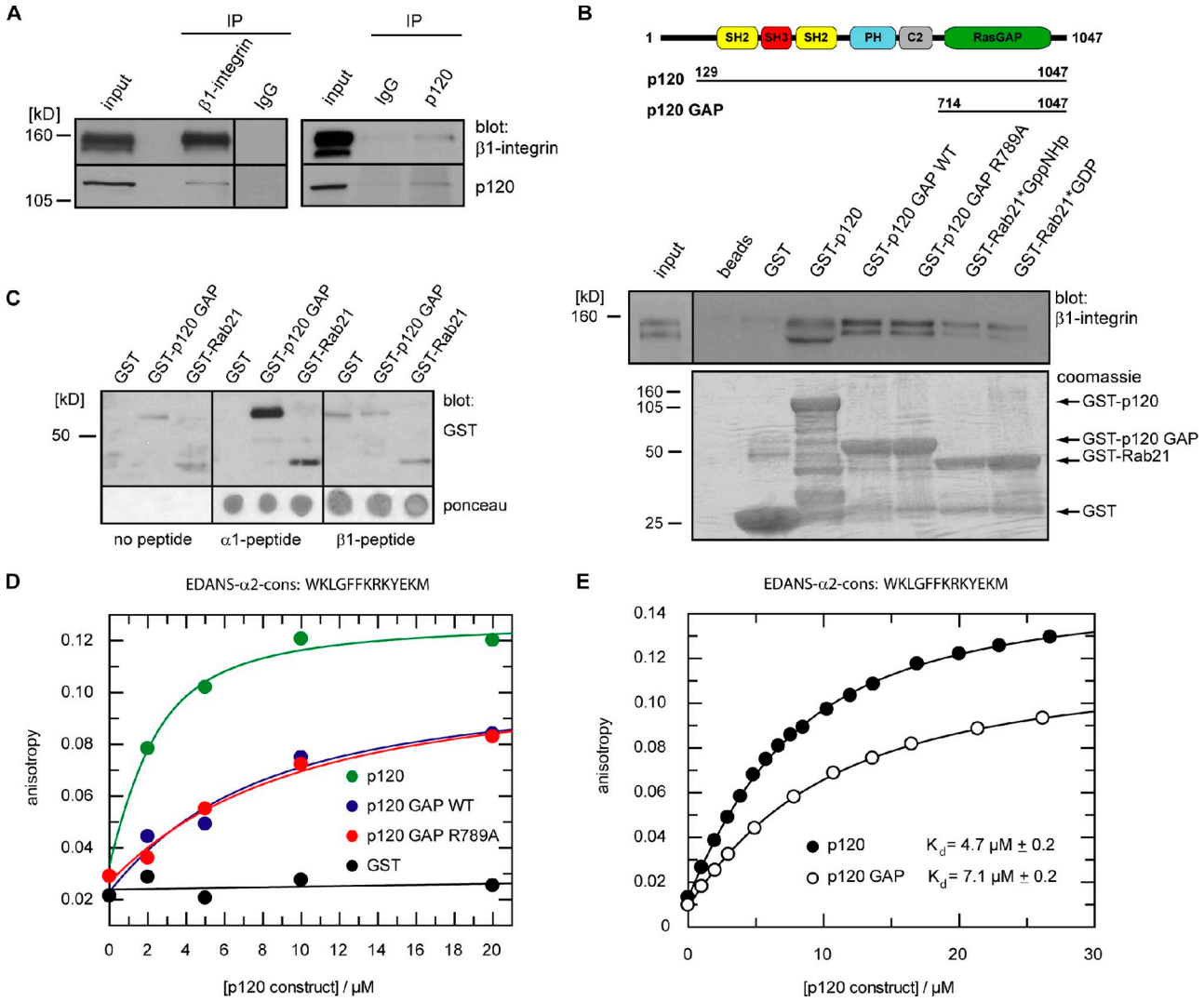
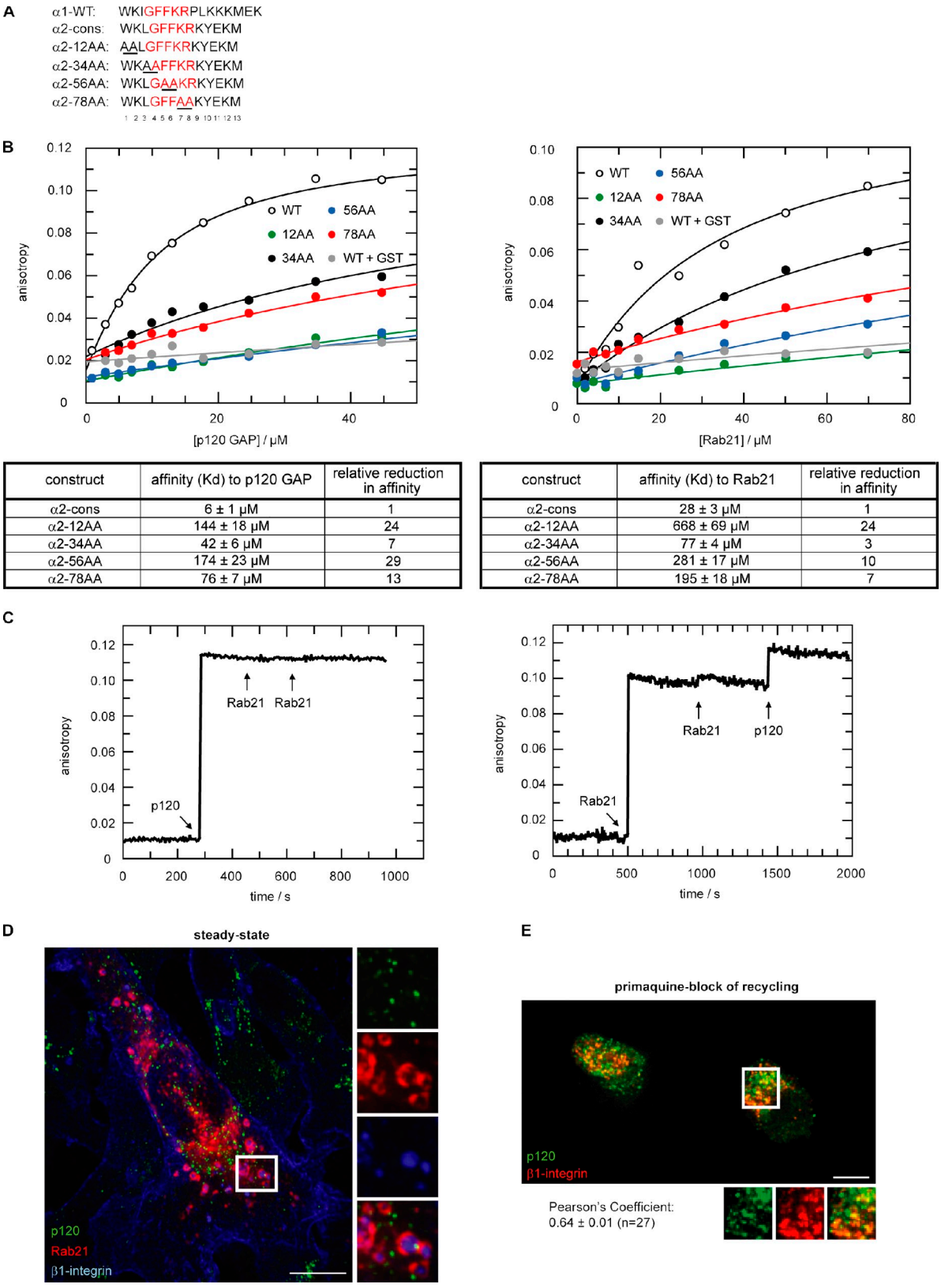


Figure 4. **p120RasGAP GAP domain interacts with integrin α-tails.** (A) Lysates from MDA-MB-231 cells were subjected to immunoprecipitation with the indicated antibodies. (B) Schematic illustration of the p120RasGAP constructs used. Cell lysate was incubated with immobilized GST or GST-tagged Rab21 and p120RasGAP constructs. Bound proteins were probed for β1-integrin heterodimers. Coomassie-stained SDS-PAGE was used to check for equal loading of the GST fusion proteins. (C) Biotinylated peptides corresponding to the cytoplasmic domain of either α1- or β1-integrin were immobilized and incubated with purified GST-tagged proteins as stated in the figure. Ponceau staining demonstrates usage of equal amounts of the biotinylated peptides. (D) Fluorescence polarization (FP)–based assay to measure the binding between 5 μM EDANS-α2 integrin peptide and different concentrations of the indicated recombinant proteins. Note that in FP-based assays the stronger increase of anisotropy with the full-length p120RasGAP protein is due to higher molecular weight of the protein compared with only the GAP domain of p120. (E) 5 μM of the EDANS-α2-integrin peptide was titrated with increasing concentrations of p120 or p120 GAP to determine equilibrium dissociation constants (K_d).

directly with integrin α1-subunit cytoplasmic tail, but not with integrin β1-cytoplasmic tail (Fig. 4 C). Second, using surface plasmon resonance–based technology (BIAcore) we detected an interaction between p120 GAP domain and α2-cytoplasmic tail peptide (Fig. S5 C), but no interaction was detected with the β1-cytoplasmic tail (unpublished data). The fact that p120 GAP interacts with both α1- and α2-cytoplasmic tails is indicative of an interaction with the conserved membrane-proximal sequence WKLGFFKR that is common to most α-subunits (Hynes, 2002). Residues in this sequence have earlier been shown to be critical for Rab21 association with integrins (Pellinen et al., 2006).

To confine the integrin-binding site on p120RasGAP, we performed a FP-based assay between the α2-cons peptide and purified recombinant proteins. In this experiment, we detected a direct interaction between the integrin α2-cons peptide and the full-length p120RasGAP as well as the GAP domain of p120RasGAP alone (Fig. 4 D). Supporting our results from the GST pull-down experiments, the catalytically inactive GAP mutant R789A was able to directly bind to the integrin peptide in the same way as the wild-type GAP protein. Furthermore, with FP-based titration experiments we also found that the GAP domain alone displays a similar binding affinity to the integrin α-tail as the full-length protein, indicating that the GAP domain is the primary binding site for the conserved segment of the α2-integrin tail (Fig. 4 E).

Taken together, these data suggest that the GAP domain of p120RasGAP interacts directly with integrin α-cytoplasmic tails, but the catalytic activity of p120RasGAP is not needed for the interaction.



construct	affinity (Kd) to p120 GAP	relative reduction in affinity
α2-cons	6 ± 1 μM	1
α2-12AA	144 ± 18 μM	24
α2-34AA	42 ± 6 μM	7
α2-56AA	174 ± 23 μM	29
α2-78AA	76 ± 7 μM	13

construct	affinity (Kd) to Rab21	relative reduction in affinity
α2-cons	28 ± 3 μM	1
α2-12AA	668 ± 69 μM	24
α2-34AA	77 ± 4 μM	3
α2-56AA	281 ± 17 μM	10
α2-78AA	195 ± 18 μM	7

Figure 5. **p120RasGAP and Rab21 bind integrin α-tails mutually exclusive.** (A) Illustration of the integrin mutant peptides used. (B) Quantitative determination of affinity-binding constants of p120RasGAP and Rab21 with either wild-type (WT) α2-integrin peptide or α2-peptide mutants as stated in the figure. Affinity values in the table are mean values (±SEM) from five independent measurements. The graphs show representative titration curves to determine affinities. (C) FP-based competition assay of Rab21 (25 kD) and p120 (100 kD) for binding to the integrin α2-tail. Left: 5 μM EDANS-α2 peptide, 50 μM p120, and two times 10 μM Rab21 were added consecutively. Right: first, 60 μM Rab21 was added to 5 μM EDANS-α2 peptide. Saturation of the peptide with Rab21 is shown by adding further 10 μM Rab21. Addition of only 10 μM p120 leads then to an increase of anisotropy. (D) Three-color immunostaining of mDsRed-Rab21 (red)–transfected MDA-MB-231 cells with p120RasGAP in green and β1-integrin in blue (note that this is steady-state integrin distribution

Rab21 and p120RasGAP compete for integrin binding

As p120RasGAP and Rab21 interact directly with the conserved α-subunit membrane-proximal sequence (Fig. 4 D and 3 D, respectively), it is possible that their binding sites on the integrin α-tail overlap. To analyze this in more detail, we determined and compared the binding affinities of either Rab21 or p120 GAP domain to the integrin α2-cons peptide and different mutant peptides comprising alanine substitutions within this sequence (Fig. 5 A) in FP-based assays (Fig. 5 B). Assuming that there is only one binding site on both binding partners, the affinity of the interactions with the wild-type integrin α-tail peptide was 6 µM for p120 GAP domain and 28 µM for Rab21 (Fig. 5 B). Interestingly, the introduction of specific alanine substitutions within the WKLGFFKR sequence reduced the binding affinity to Rab21 and p120RasGAP in a similar way. Alterations in residues WK and FF resulted in a strong reduction of binding affinity to both proteins. The KR motif seemed also to be important, but to a minor extent. These findings confirm that p120RasGAP and Rab21 interact with overlapping binding sites on integrin α-subunit cytoplasmic tails. The data are also consistent with our previous notion that the Arg1161 within the GFFKR sequence of α-subunits impairs integrin–Rab21 association in cells (Pellinen et al., 2006, 2008).

The overlapping binding sites of the two proteins suggested that their interaction with the integrin could be mutually exclusive and, based on differences in binding affinity, also competitive. Accordingly, we found that Rab21 and p120RasGAP do not form a ternary complex with the α-cytoplasmic domain of integrins (Fig. 5 C). Addition of Rab21 to the α2-cons peptide, which was already saturated with p120RasGAP (anisotropy = 0.115), did not change the anisotropy value (Fig. 5 C, left). This demonstrates that Rab21 is neither able to bind to a preformed α2–p120RasGAP complex nor displace p120RasGAP from this complex. In contrast, addition of full-length p120RasGAP to an α2-cons peptide that is saturated with Rab21 leads to an additional increase of anisotropy signal (Fig. 5 C, right). The final anisotropy value (0.115) is identical to the value obtained in the experiment where the EDANS-α2 peptide is saturated with p120RasGAP alone. Thus, the 100-kD p120RasGAP protein was able to replace the lower molecular weight Rab21 from the integrin α-cytoplasmic domain under these in vitro conditions. These data demonstrate that p120RasGAP can compete with Rab21 for binding to integrins most likely due to its higher affinity toward the integrin α-tail.

In line with the mutually exclusive integrin binding of Rab21 and p120RasGAP, we observed very little overlap of Rab21 and p120RasGAP immunostaining in MDA-MB-231 cells under steady-state conditions (Fig. 5 D). Integrins were detected in Rab21-positive endosomes alongside smaller Rab21-negative vesicles. P120RasGAP was frequently detected adjacent to integrin-Rab21 endosomes but rarely colocalized with Rab21. The limited overlap between integrins and p120RasGAP in cells could be due to the fact that the complex of the two represents a transient intermediate along the integrin endo/exocytic trafficking route. To test this, we labeled cell surface integrins with an antibody and allowed for internalization in the presence of the recycling inhibitor primaquine (Roberts et al., 2001). This significantly increased the overlap of integrins with p120RasGAP (Fig. 5 E). Therefore, these data indicate that p120RasGAP is an endosomal protein that transiently overlaps with endocytosed integrin en route to the plasma membrane.

P120RasGAP binding to integrins is required for integrin recycling

The data above indicated that the competitive binding of Rab21 and p120RasGAP to integrins is an important feature of the mechanism through which integrin recycling is enabled by p120RasGAP. To test this, we produced a p120RasGAP mutant that does not associate with integrins (Fig. S5 D) due to the deletion of the GAP domain (GFP-p120 ΔGAP). We investigated the ability of this mutant, alongside p120RasGAP WT and the GAP-deficient p120RasGAP R789A mutant, to rescue the reduced integrin recycling in p120RasGAP-silenced cells. Using the same antibody-based recycling experiment as in Fig. 1 C, we found that GFP-p120RasGAP WT and R789A mutant fully rescued integrin traffic in p120RasGAP-silenced cells (Fig. 6 A). In contrast, GFP alone or GFP-p120RasGAP ΔGAP had no significant effect on integrin recycling. Similar results were obtained when the effect of the same constructs were analyzed for their effect on migration of p120RasGAP-silenced cells (Fig. 6 B). GFP and GFP-p120RasGAP ΔGAP had no significant effect on migration whereas reexpression of full-length WT p120RasGAP or R789A mutant significantly attenuated migration (Fig. 6, C and D), suggesting that the catalytic activity of p120RasGAP is not needed for the regulation of cell migration. Thus, p120RasGAP binding to integrins, but not the GAP activity, is required for p120RasGAP-dependent stimulation of integrin recycling in cells.

P120RasGAP is required for integrin exit from Rab21- and EEA1-positive endosomes

Finally we asked what happens to integrins that cannot redistribute to the plasma membrane in the absence of p120RasGAP. To assess this issue, we used the antibody-based trafficking assay in conjunction with costaining of endogenous Rab21 (Fig. 7 A). We found strong colocalization of Rab21 and integrins in p120RasGAP-silenced cells, both after internalization and recycling (Fig. 7 A). In contrast, in control cells there was less overlap between integrins and Rab21, indicating that the endocytosed integrins had either trafficked to the recycling endosomes (as described earlier; Powelka et al., 2004) or they

in cells, not internalized pool of integrin from the cell surface like in Figs. 1 C and 7 A). Bar, 10 µm. (E) To increase colocalization of p120RasGAP and integrins in MDA-MB-231 cells, integrin trafficking was allowed for 45 min in the presence of primaquine (inhibits recycling) after labeling of surface integrins with anti–β1-integrin antibody (red). Cells were then fixed, permeabilized, and stained for endogenous p120RasGAP (green). The determination of the Pearson's correlation coefficient was done with ImageJ software (n = 27 cells).

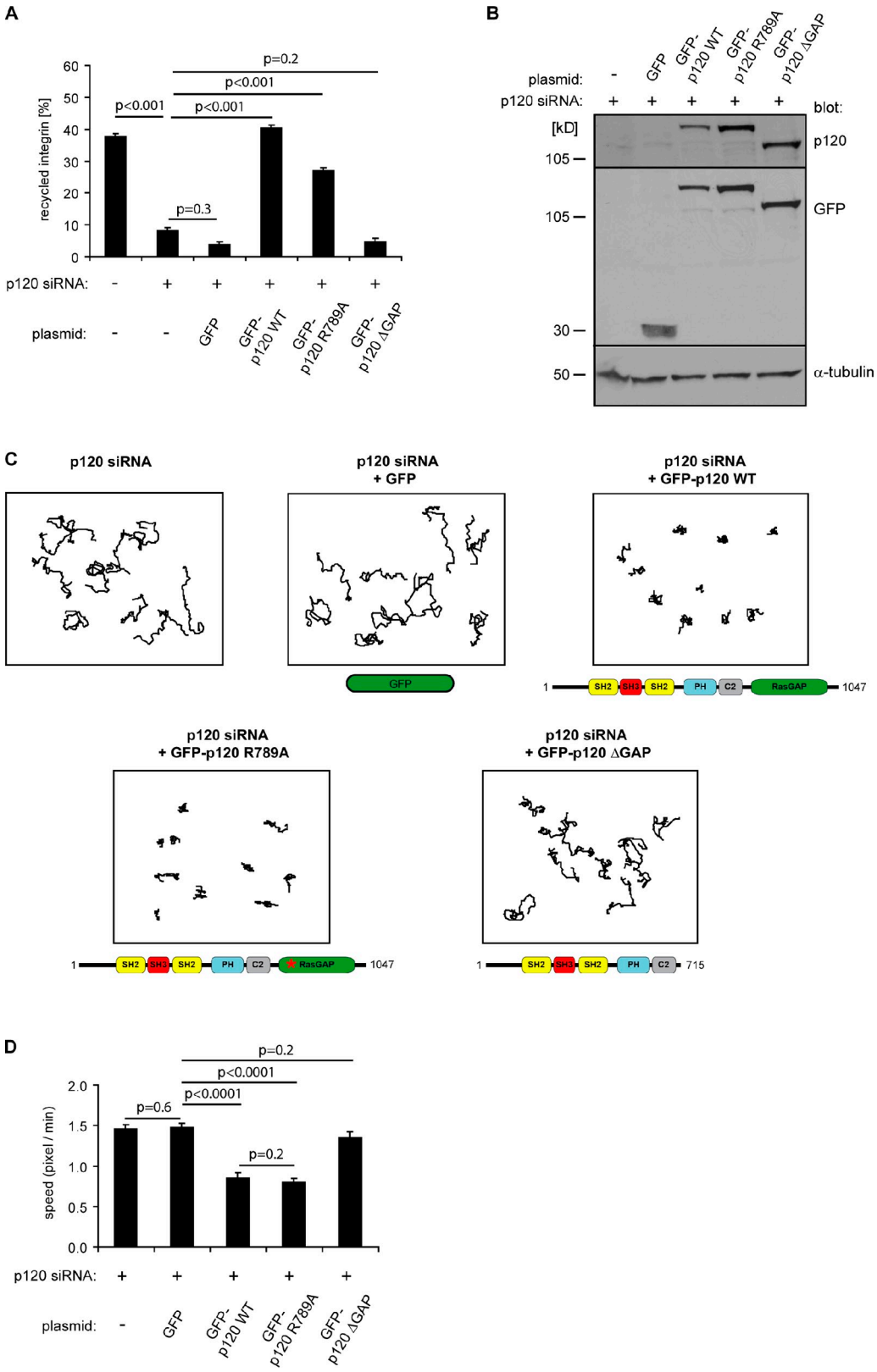


Figure 6. **p120 GAP domain, but not p120RasGAP catalytic activity, is crucial for the regulation of cell migration.** (A) MDA-MB-231 cells were first silenced with 3′ Alexa 647–conjugated p120RasGAP-siRNA for 2 d followed by transfection with the indicated GFP constructs for 24 h. The cells were then labeled with anti–β1-integrin antibody, and integrin trafficking was allowed for 60 min. In immunostainings of p120RasGAP-siRNA and GFP-positive cells, the amount of integrin recycling to the cell surface was determined by measuring both internal and total integrin staining (n = 15–20 cells each treatment from three independent experiments). (B) Western blot analysis from experiment in C demonstrating silencing and rescue efficiency. (C) P120RasGAP-silenced MDA-MB-231 cells were transfected with the indicated GFP constructs and then allowed to migrate on tissue culture plates for 10 h. Time-lapse images were taken every 10 min for 10 h. Shown are representative track plots. (D) Quantitation of the migration speed of tracked cells from three independent experiments shown in C (mean ± SEM, n = 30 cells per treatment).

were recycled more efficiently back to the plasma membrane. Next we stained the p120RasGAP-silenced cells with markers for the early and the recycling endosomes. We found that the endocytosed integrin was accumulating in EEA1-positive structures in the p120RasGAP-silenced cells, and only limited overlap was detected with Rab11 or Rab coupling protein RCP (Fig. 7 B). Immuno-EM also showed that in control-siRNA transfected MDA-MB-231 cells, anti-integrin antibody-labeled surface integrins were after consecutive internalization and recycling steps predominantly found at the plasma membrane and hardly colocalized with Rab21 (Fig. 7 C). However, p120RasGAP-silenced cells showed significantly more integrin remaining in close proximity with Rab21-labeled structures.

Taken together, these data support a model whereby β1-integrins normally traffic in cells along a Rab21- and p120RasGAP-dependent pathway (Fig. 8 A). Inactivation of Rab21 (by expression of a Rab21GDP mutant) does not interfere with the ability of Rab21 to bind to the integrins but endocytosis is nevertheless blocked, possibly due to impaired recruitment of other required Rab21 effectors (Fig. 8 B). In contrast, in the absence of p120RasGAP integrins are endocytosed normally but remain in Rab21- and EEA1-positive early endosomes. Thus, due to the lack of p120RasGAP competition, integrins then fail to redistribute back to the cell surface via recycling endosomes (Fig. 8 C).

Discussion

In the present study we show that p120RasGAP is required for efficient recycling of endocytosed α/β1-integrin heterodimers to the plasma membrane. Our results demonstrate that in the absence of p120RasGAP endocytosed β1-integrin is increasingly retained in Rab21- and EEA1-positive endosomes resulting in increased cell motility. Mechanistically, p120RasGAP and Rab21 compete for binding to overlapping sites on integrin α-subunit cytoplasmic domains in vitro and we find it plausible that similar competition functions in cells, too. Therefore, our results have led us to propose a model in which p120RasGAP replaces Rab21 on the α-tail of endocytosed integrin, which in turn triggers receptor trafficking via recycling endosomes back to the plasma membrane (Fig. 8). The ability of p120RasGAP to compete with Rab21 for integrin binding could be based on its more than four times higher affinity for the integrin α-tail (Fig. 5 B) and the endosomal localization of p120RasGAP.

The molecular details described in this study contain several novel and unexpected features that broaden our view of the role of small Rab GTPases and GAP proteins in receptor trafficking. (1) The interaction between Rab21 and integrins is direct and not specific to a nucleotide-bound conformation. For several Rab proteins, recruitment of cargo is controlled via effector proteins that interact with Rab proteins in a nucleotide-dependent manner and recognize specific cargo (Stenmark, 2009). (2) Release of Rab21 from integrin does not require p120 GAP activity, but is regulated via competitive binding of p120RasGAP to the integrin α-tail. To the best of our knowledge this is a fundamentally new mechanism to regulate the release of Rab-endocytosed cargo from an endocytic compartment. (3) Integrin α-tail interacts with the GTPase-activating GAP domain (not the classical protein–protein interaction domains) of p120RasGAP. Our data propose a mechanism involving competitive binding of Rab21 and p120RasGAP to integrins.

Thus far, Rab25 and Rab21 are rare examples of Rab family members that directly interact with cell surface receptors, namely integrins (Pellinen et al., 2006, 2008; Caswell et al., 2007). Of these, Rab25 interacts with integrin β-subunits in a GTP-dependent manner. We demonstrate here that Rab21 interacts with integrins in a nucleotide-independent manner, suggesting that a mechanism other than the common switching between GDP- and GTP-bound forms would be important in regulating the interaction between Rab21 and integrin. However, because we know that both GDP- and GTP-locked Rab21 mutants severely impair integrin traffic (Pellinen et al., 2006, 2008), it is likely that the control of the endocytosis step is not solely exerted at the level of the Rab21–integrin interaction but via recruitment of other yet unidentified Rab21 effectors. This is plausible because Rab proteins function via recruiting many effector proteins in a nucleotide-dependent manner.

The other unexpected feature is the interaction between the integrin α-subunit and the GAP domain of p120RasGAP. P120RasGAP is a well-known multi-functioning protein with several well-described protein–protein interactions mainly mediated via the structural domains found in the N terminus (Pamonsinlapatham et al., 2009). Because binding of integrins to the GAP domain of p120RasGAP would be compatible with concomitant recruitment of growth factor receptors, FAK, p190RhoGAP, Src, p62Dok, filamin, annexin A6, and calpain small subunit-1 to the N-terminal domains of the protein, it is possible that p120RasGAP may emerge as an important signaling hub mechanistically linking integrins to several important cellular signaling pathways.

Recent studies have established regulation of β1-integrin traffic as a critical determinant between random and directional modes of migration (White et al., 2007; Caswell et al., 2008; Muller et al., 2009). We demonstrate here a new function for p120RasGAP in the joint regulation of integrin traffic and cell migration. Earlier work with MEFs isolated from p120RasGAP-null mice indicated that loss of p120RasGAP would impair directional cell motility (Kulkarni et al., 2000). It is possible that acute silencing (resulting in ∼90% reduction in p120RasGAP protein) changes cell behavior somehow fundamentally differentially than loss of the protein in a knock-out animal where compensatory mechanisms may evolve during development. However, the data in Fig. S2 demonstrate clearly that siRNA-mediated silencing on p120RasGAP in human and mouse fibroblasts, under identical conditions that were used earlier, results in increased cell motility. Interestingly, a recent study indicates that loss of p120RasGAP, due to a miRNA switch, induces angiogenesis in vivo (Anand et al., 2010). Because cell migration is involved in angiogenesis, these findings would be fully compatible with the notion of p120RasGAP as a negative regulator of cell migration.

In different cell types, inhibition of αvβ3-integrin regulates complex formation between α5β1-integrin, RCP (Rab11 coupling protein), and EGFR in recycling endosomes and

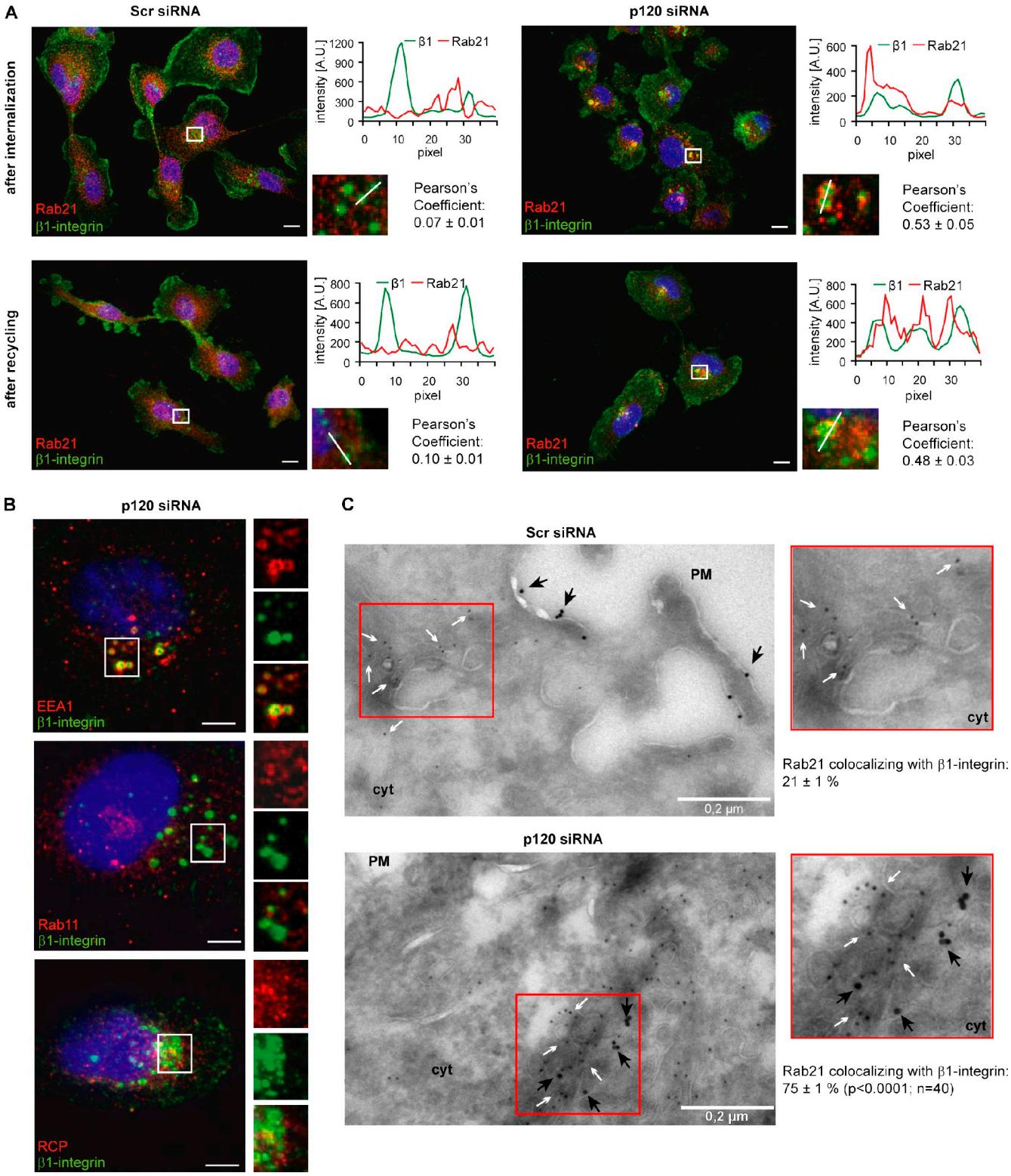


Figure 7. **Integrins accumulate in Rab21 endosomes upon p120RasGAP silencing.** (A) Surface integrins of starved control (Scr) or p120RasGAP-silenced MDA-MB-231 cells were labeled with anti–β1-integrin antibody (green). Cells were allowed to undergo internalization or recycling as described in Fig. 1 C. Cells were stained for endogenous Rab21 (red) and nuclei (DAPI). Adjacent graphs show the results of line scan analysis of the enlarged areas shown in the figure. Bar, 10 µm. Images were further analyzed for colocalization (Pearson's correlation coefficient) of Rab21 and β1-integrin in endosomes (mean ± SEM, *n* = 15 cells). (B) To characterize the compartment in which integrins accumulate upon loss of p120RasGAP, surface integrins in p120-silenced MDA-MB-231 cells were first labeled with anti–β1-integrin antibody (green) and then allowed to undergo recycling. After fixation and permeabilization, the cells were stained for either EEA1, Rab11, or RCP (red) as indicated in the figure. (C) Rab21-EGFP–expressing cells, either control or p120RasGAP silenced, were surface labeled with 10-nm gold–conjugated anti–β1-integrin antibody (black arrows). Subsequently, cells were allowed to undergo internalization followed by serum-induced recycling for 30 min, respectively. Rab21-EGFP was co-labeled with anti-GFP antibody and 5-nm protein A gold (white arrows). Shown are electron microscopic images of cells from frozen thin cryosections (PM, plasma membrane; cyt, cytosol). The quantitation of the ratio of Rab21 gold particles colocalizing with integrins on endosomes (mean ± SEM, *n* = 40 cells) is indicated. Bar, 200 nm.

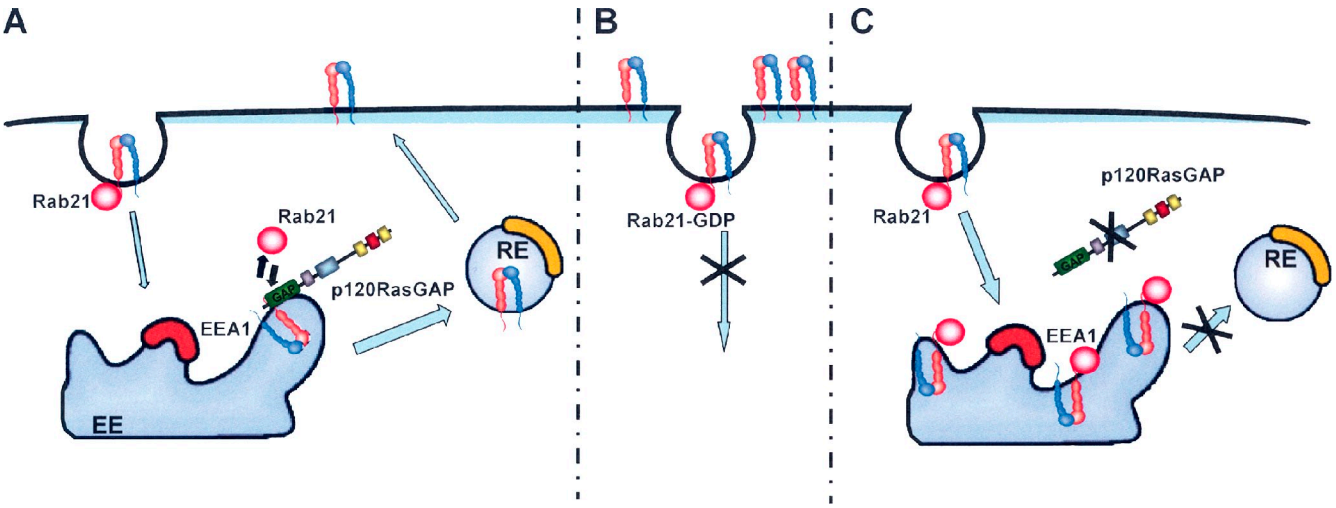


Figure 8. **Model of the mechanism of integrin trafficking jointly controlled by Rab21 and p120RasGAP.** (A) Rab21 mediates endocytosis of integrins by binding directly to the cytoplasmic tails of the α-integrin subunits. After internalization, p120RasGAP competes for Rab21 binding sites on the integrin α-tail. The replacement of Rab21 by p120RasGAP on early endosomes then triggers the recycling of integrins back to the plasma membrane. (B) Expression of a dominant-negative Rab21 (GDP-locked mutant) impedes integrin internalization and results in diminished cell migration (Pellinen et al., 2006). (C) Loss of p120RasGAP, on the other hand, results in the accumulation of integrins inside the cell in Rab21-positive early endosomes. Yellow bar indicates Rab11.

subsequent recycling of these receptors to the plasma membrane. This results in increased random motility and invasion in 3D (Caswell et al., 2007, 2008; White et al., 2007; Muller et al., 2009). P120RasGAP is known to bind to activated EGFR via its N-terminal SH2 domains (Pamonsinlapatham et al., 2009). Here we demonstrate an interaction between integrin α-subunit and the C-terminally located GAP domain of p120RasGAP. Thus, one could envisage the existence of an endosomal recycling complex where p120RasGAP would bridge integrins and EGFR, and Rab11-dependent recruitment of RCP to the complex would trigger recycling to the membrane. Previous studies have shown that recycled RCP and EGFR strongly colocalize in protrusions at the cell front (Caswell et al., 2008). Because p120RasGAP recruitment to membranes has been linked with transient RhoA attenuation and subsequent formation of protrusions via Rac activation (Tomar and Schlaepfer, 2009), the concept of p120RasGAP as a critical regulator of integrin traffic would be in line with its role in facilitating formation of protrusions. This could be relevant to several important biological processes like cancer cell invasion and metastasis. Furthermore, our novel concept of competitive binding between Rab GTPases and GAP proteins as a regulatory mechanism involved in receptor trafficking may be important in endocytic traffic of other receptors as well.

Materials and methods

Antibodies and DNA constructs

Commercial antibodies against the following antigens were used: Rab21 (Abnova), β1-integrin (P5D2, P4G11, and AIIB2 [Developmental Studies Hybridoma Bank]; 12G10 [Abcam]; 9EG7 and MAB13 [BD]; MAB2252 [Millipore]), p120RasGAP and p190RhoGAP (BD), α-tubulin (Santa Cruz Biotechnology, Inc.), RhoA (Cell Signaling Technology), RFP (MBL), and GST, GFP, and fluorescently conjugated secondary antibodies (Invitrogen). Anti-RCP antibody was provided by Jim Norman (The Beatson Institute for Cancer Research, Glasgow, UK).

Full-length murine Rab21 was cloned into a pEGFP-C2 vector as described previously (Pellinen et al., 2006). Human p120RasGAP was cloned into a pEGFP-C2 vector via EcoRI–ApaI. Human p120RasGAP 714–1047 GAP domain (p120 GAP), Rab21 15–225, and APPL1 5–419 were cloned into pGEX vectors. Mutations resulting in the R789A mutant respectively the p120 ΔGAP construct were inserted using site-directed mutagenesis primer. RFP and RFP-232 constructs (Peacock et al., 2007) were gifts from Anthony J. Koleske (Yale University, New Haven, CT), pGEX-4T1 p120RasGAP 129–1047 (p120) from A. Wittinghofer (Max Planck Institute for Molecular Physiology, Dortmund, Germany), and pET15b-APPL1 5–419 from X.C. Zhang (Oklahoma Medical Research Foundation, Oklahoma City, OK).

Biotin-conjugated integrin peptides corresponding to the cytoplasmic domain of integrins (α1, α2, β1) were custom synthesized by Genecust, EDANS-labeled peptides by GenScript. The α2-peptide contained the cytoplasmic sequence of α2-integrin (WKLGFFKRKYEKM) with the conserved α-specific sequence being underlined. The α1-peptide comprised the conserved α-sequence and the α1-integrin–specific sequence in double repeat (WKIGFFKRPLKKKMEKRPLKKKMEK). The β1-peptide enclosed the β1-integrin cytoplasmic sequence (WKLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK).

Cell lines and siRNA transfections

MDA-MB-231 cells (American Type Culture Collection) were grown in DME 4500 plus 1% nonessential amino acids, 1% glutamine, and 10% FBS. KF28 and KFr13 cells (Kikuchi et al., 1986) were a gift from Dr. N. Sasaki (National Defense Medical College, Saitama, Japan) and were grown in RPMI plus 1% glutamine, 1% Hepes buffer, 1 mM sodium pyruvate, 24% glucose, and 10% FBS. MEF (mouse embryonic fibroblast) cells were cultured in DME 4500 plus 1% glutamine and 10% FBS. Human TIFF cells (telomerase immortalized foreskin fibroblasts), a gift from Jim Norman, were cultured in DME 4500 plus 20% FBS, 1% glutamine, and 20 mM Hepes buffer.

Two different siRNAs targeting p120 (sense, 5′-GGAAGAAGAUCCACAUGAATT-3′ and 5′-GCUCCCAUAUACCAUUAAATT-3′; QIAGEN), Rab21 (sense, 5′-GGCAUCAUUCUUAACAAAGTT-3′; Invitrogen), or scramble control siRNA (Invitrogen) were transfected at 100-nM concentration to cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence

MDA-MB-231, KF28, and KFr13 cells were plated onto acid-washed glass coverslips. Cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100 and 2% BSA in PBS for 20 min, and then blocked with 2% BSA in PBS. Primary antibodies were used at 5–10 mg/ml and incubated overnight at 4°C. Alexa-conjugated secondary antibodies, at a concentration of 5 μg/ml, were incubated at RT for 1 h. Coverslips were mounted with Mowiol containing Dabco and DAPI to counterstain nuclei. Immunofluorescent samples were analyzed with an inverted widefield microscope (Carl Zeiss) with a confocal unit, Orca-ER camera

(Hamamatsu Photonics), Plan-Neofluar 63× oil/1.4 NA objective (Carl Zeiss), and SlideBook 5.0 imaging software (Intelligent Imaging Innovations, Inc.).

Immunoprecipitation, pull-down, protein interaction, and Western blot analysis

MDA-MB-231 cells were lysed in PBS, 1.3% n-Octyl-β-D-glucopyranoside, 1% NP-40, 0.5% BSA, 1 mM EDTA, and protease and phosphatase inhibitor cocktails (Complete and PhosStop; Roche). Equal amounts of cleared lysate were either subjected to immunoprecipitation or pull-down analysis.

For immunoprecipitation, lysates were incubated with the indicated antibodies bound to protein G–Sepharose beads. Immunoprecipitates were washed three times with cell lysis buffer diluted in PBS 1:3. Precipitated proteins were resolved with SDS-PAGE followed by Western blotting analysis.

For pull-down analysis, cell lysates were incubated at 4°C for 2 h with either GST or GST-tagged Rab21, p120 or p120 GAP WT, or R789A mutant bound to glutathione-Sepharose beads, or respectively purified recombinant GST fusion proteins were incubated with biotinylated integrin peptides bound to streptavidin-Sepharose. After washing the beads twice with diluted lysis buffer, pulled-down proteins were detected by SDS-PAGE and Western blot analysis.

Protein expression and purification

p120, p120 GAP WT, or R789A mutant and Rab21 were expressed as GST fusion proteins in the *Escherichia coli* strain Rosetta BL21DE3. Protein expression was induced at OD_{600} of 0.5 with 250 µM IPTG overnight at RT. Proteins were purified on glutathione-Sepharose beads (GSH) preequilibrated with purification buffer PB (50 mM Tris, pH 7.5, 150 mM NaCl, and 3 mM β-mercaptoethanol) at 4°C. After washing with PB, fusion proteins were eluted with 30 mM glutathione in PB or cleaved with thrombin or PreScission protease (50 U). After separation of cleaved GST by GSH beads, proteins were concentrated, flash-frozen in liquid nitrogen, and stored at −80°C. His-APPL1 5–419 was purified similar to earlier publications (Zhu et al., 2007). In brief, His-tagged APPL1 was expressed like the GST fusion proteins, but purified on Ni-NTA beads. After washing the beads with PB plus 20 mM imidazole, the His-tagged protein was eluted using PB plus 250 mM imidazole, rebuffered to PB, concentrated, flash-frozen in liquid nitrogen, and stored at −80°C

GAP assay

To monitor GTP hydrolysis in vitro, a charcoal-based assay was performed as described previously (Veltel et al., 2008). For the nucleotide exchange, Ras was incubated with radioactive γ-[^{32}P]GTP and nonlabeled GTP for 15 min on ice in 10 µl measurement buffer (MB; 20 mM Tris, pH 7.5, 50 mM NaCl, and 3 mM β-mercaptoethanol) plus 8 mM EDTA. To start the reaction 60 µl p120GAP in MB plus 5 mM $MgCl_2$ was added at 25°C. Final concentrations were 1 µM Ras, 5 µM p120GAP, 500 nM GTP, and 10 nM γ-[^{32}P]GTP (3.5 µCi). Because Rab21 has a high internal GTP hydrolysis rate and shows nucleotide exchange only at room temperature, Rab21 was incubated in MB plus EDTA without GTP for 10 min at RT before adding GTP and γ-[^{32}P]GTP. 30 s after adding the nucleotides, the reaction was started by adding the GAP protein as in the Ras sample.

Aliquots of 10 µl were taken at different time points and mixed with 300 µl of charcoal solution (50 g/liter charcoal in 20 mM phosphoric acid) to stop the reaction. The charcoal-bound proteins and nucleotides were pelleted and the amount of free $^{32}P_i$ in 100 µl of the supernatant was determined by scintillation counting.

Protein interaction analysis using fluorescence polarization

Time-resolved polarization measurements were performed in a Quanta Master spectrofluorimeter (Photon Technology International) with excitation at 340 nm and emission at 490 nm. All measurements were performed in MB. Various concentrations of the interaction partners were added gradually to 5 µM of EDANS–α2-peptide. Increase in anisotropy was integrated over 3 min. Data analysis, fitting, and plotting were done with Grafit 6.0 (Erithracus Software). K_d values were calculated as described previously (Kraemer et al., 2001).

For affinity determination of p120 GAP and Rab21 to the α2-alanine scanning mutants, 5 µM of EDANS-labeled mutant peptides were incubated with serial concentrations of the interacting proteins in MB containing 0.001% Tween 20. Samples were analyzed in a 384-well plate format in an ENVISION 2100 multi-label plate reader (PerkinElmer) using 355-nm excitation and 500-nm emission filter. Every experiment was repeated five times. Data analysis was performed as described above.

Protein interaction analysis using surface plasmon resonance

Real-time protein–protein binding was analyzed with BIAcore (GE Healthcare). 500 RU of the biotinylated α2-peptide were captured on a streptavidin surface (Sensor chip SA). Pre-conditioning of the chip surface was done by three consecutive injections of 1 M NaCl in 50 mM NaOH. Binding reactions were performed in HBS-P running buffer (0.01 M Hepes, pH 7.4, 0.15 M NaCl, and 0.0005% Surfectant P20).

The response data were analyzed for serial p120 GAP concentrations ranging from 0.5 to 3 µM at a flow rate of 30 µl/min. Signal changes on the control surface (without peptide) as well as nonspecific binding responses caused by the running buffer itself were subtracted from the α2-p120 GAP binding response curves.

Biotinylation-based internalization and recycling assays

To follow the internalization of β1-integrins, either control- or p120RasGAP-silenced cells were placed on ice, washed once with ice-cold PBS, and surface proteins were then labeled with 0.2 mg/ml NHS-SS-biotin (Thermo Fisher Scientific) in PBS at 4°C for 30 min. Labeled cells were subsequently transferred to prewarmed serum-free medium, and internalization was allowed at 37°C for the times indicated. Surface-remaining biotin was removed by cleavage with 60 mM MesNa in MesNa buffer (50 mM Tris-HCl, pH 8.8, and 100 mM NaCl in PBS) at 4°C for 30 min followed by quenching with 100 mM iodoacetamide (IAA) for 15 min on ice. Cells were lysed and subjected to immunoprecipitation using anti–β1-integrin antibody. Endocytosed integrins were detected with anti-biotin HRP-linked antibody (Cell Signaling Technology) in immunoblots. Re-probing with anti–β1-integrin antibody after stripping was used for normalization.

Recycling assays were done similarly but after allowing maximal internalization for 30 min and removal of surface-remaining biotin labels, recycling was enabled by adding complete medium to the cells and incubation at 37°C for the times indicated. Subsequent MesNa cleavage and IAA quenching removed biotin labels of proteins that have been recycled to the cell surface.

Antibody-based internalization and recycling assays

Endocytosis and recycling of integrins were analyzed by an assay adapted from Powelka et al. (2004). In brief, transfected cells were allowed to adhere onto collagen I–coated coverslips for 30 min at 37°C. Surface integrins were thereafter labeled with anti–β1-integrin antibody for 1 h at 4°C. Labeled cells were subsequently transferred to prewarmed serum-free medium, and integrin trafficking (endocytosis and recycling) was allowed at 37°C for 1 h. The cells were fixed, permeabilized, and stained with a secondary antibody to detect the anti–β1-integrin antibody followed by immunofluorescence analysis. Fluorescence intensity was scored from the entire cell and from the cell interior (without plasma membrane staining) with ImageJ and percentage of endocytosed integrin was determined based on the ratio of intercellular/total integrin.

Immunogold labeling–based recycling assay

Recycling assay was performed with control or p120RasGAP-silenced cells cotransfected with Rab21-EGFP as described already for the antibody-based assay but here, surface integrins were labeled with K20 anti–β1-integrin antibody (Beckman Coulter) conjugated with 10-nm gold, prepared and conjugated as described by Slot and Geuze (2007). In brief, 10-nm gold sol was prepared by mixing 1% gold chloride solution with 1% trisodium citrate solution containing tannic acid (Mallincrodt Inc.) at 60°C. Gold sol was stabilized with 6 µg/ml K20 mAb at pH 9.0 and pelleted by centrifugation (30 min, 20,400 rpm, SW-28 rotor [Beckman Coulter]). After p120RasGAP silencing and Rab21-EGFP cotransfection, K20 mAb gold conjugate was bound to cells on ice in serum-free medium. Integrin antibody gold conjugate was allowed to internalize for 30 min in serum-free conditions followed by 30 min with serum to allow recycling. Cells were then prepared for cryosectioning according to Slot and Geuze (2007). In brief, cells were fixed at room temperature with 4% paraformaldehyde, scraped off the dish, and pelleted by centrifugation. Drop of 12% gelatin in PBS was added on the pellet at 37°C, mixed gently and pelleted (5 min at 15,000 *g*) after 10 min incubation. After solidification of the gelatin mixture with cells on ice, suitable blocks were cut and placed in 2.1 M sucrose for cryoprotection, mounted on aluminum specimen carriers, and frozen in liquid nitrogen. Immunolabeling on thin frozen sections was performed according to Slot and Geuze (2007) using 10% fetal calf serum as a blocker of nonspecific binding. Rab21-EGFP was detected by using rabbit antibodies against GFP (Invitrogen) and 5-nm protein A gold (from Dr. George Posthuma, University Medical Center Utrecht, Utrecht, Netherlands). The nontransfected cells were used as a control to find out conditions that gave no background labeling.

Colocalization of Rab21 with K20 was calculated from ~40 randomly picked Rab21-positive cells after both p120RasGAP and mock silencing, and from 210 and 330 K20-positive structures, respectively. The ratio of Rab21-EGFP small gold particles colocalizing with K20 gold-positive structures was calculated in both conditions. Nonparametric Mann-Whitney *U* test was used to evaluate the statistical significance of labeling differences.

To calculate the location of K20 β1-integrin label after p120RasGAP and mock silencing, altogether 2000 and 1300 K20 gold conjugates were counted, respectively. Location was divided between peripheral (label within 200-nm distance from the plasma membrane) and cytoplasmic labeling.

Migration assay

MDA-MB-231 cells were seeded sparsely on tissue-culture plastic or allowed to migrate on collagen I–coated tissue culture plates for 12 h in the presence of 10% FBS. Phase–contrast images were taken with an inverted wide-field microscope (AxioCam MRm camera, EL Plan-Neofluar 20×/0.5 NA objective, 4 frames per hour [Carl Zeiss]) equipped with a heated chamber (37°C) and CO_2 controller (4.8%). Image processing was done with MetaMorph imaging software. Migration was analyzed in roughly 40 cells by measuring the X and Y coordinates of tracked cells over time. The hence determined mean squared displacement (MSD) was calculated (MSD = $2S^2P[t - P(1 - e^{-t/P})]$ with S, cell speed; P, persistence time; and t, time), and this was used to determine the persistence of motility (Palecek et al., 1997). Migration speed was scored as the distance migrated (pixel) per minute.

Scratch-wound assay

Cells were plated on tissue-culture plastic, allowed to grow to a density of ~90%, and serum-starved overnight. Wounds were applied with a pipette tip and washed thoroughly with PBS. Wound closure was thereafter imaged in serum-free medium for the indicated times using an inverted wide-field microscope (AxioCam MRm camera, EL Plan-Neofluar 10×/0.5 NA objective, pictures taken every 10 min [Carl Zeiss]) equipped with a heated chamber (37°C) and CO_2 controller (4.8%). Image processing was done with ImageJ software. Wound closure efficiency was calculated as percentage of wound area after the imaging (indicated times) compared with imaging starting time (0 h). Cell motility was analyzed in more detail by determining the displacement of cells over time and calculating migration speed and directionality as described for the migration assay.

Rho activation assay

Rho activation in transfected MDA-MB-231 cells was measured in Rhotekin Rho-binding domain pull-downs from cell lysates according to the manufacturer's instructions (Rho activation assay; Cytoskeleton).

Online supplemental material

Fig. S1 shows additional trafficking experiments with p120RasGAP siRNA-transfected cells. Fig. S2 shows additional migration experiments with p120RasGAP siRNA-transfected cells. Fig. S3 shows FACS analysis of cell surface integrin levels in p120RasGAP siRNA-transfected cells. Fig. S4 shows that Loss of Rab21 impairs integrin endocytosis in ovarian carcinoma cells. Fig. S5 shows effects of p120RasGAP silencing and dominant-negative p120RasGAP on RhoA activity and Ras signaling. Binding assays demonstrating that p120RasGAP interacts with the integrin α-tail via the GAP domain. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201012126/DC1.

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