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The armadillo protein p0071 is involved in Rab11-dependent recycling

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ABSTRACT

p0071 is an intercellular junction protein of the p120 catenin family. We have identified Rab11a as a novel interaction partner of p0071. p0071 interacted preferentially with active Rab11a. Knockdown experiments revealed an interdependent regulation of both proteins. On the one hand, p0071 depletion induced a perinuclear accumulation of Rab11, suggesting a role of p0071 in the anterograde transport of Rab11 from the pericentrosomal region to the plasma membrane but not in retrograde transport. p0071 as well as Rab11 depletion increased transferrin receptor recycling indicating that p0071-induced Rab11 mislocalization interfered with Rab11 function and shifted recycling from the slow Rab11-dependent pathway to the fast Rab4-dependent pathway. When p0071 or Rab11 depletion was combined with a Rab4 knockdown the effect was reversed. On the other hand, Rab11a depletion increased p0071 recycling to cell contacts thereby identifying p0071 as a Rab11 cargo protein. This correlated with increased intercellular adhesion. Thus, we propose that p0071 has a key role in regulating recycling through the Rab11-dependent perinuclear recycling compartment, and links the regulation of adherens junctions to recycling to allow dynamic modulation of intercellular adhesion.

KEY WORDS: p0071, PKP4, Rab11, Transferrin receptor, Recycling

INTRODUCTION

p0071 (also known as PKP4) is a member of the armadillo subfamily of p120 catenins, which included p120 catenin (p120ctn; CTNND1), NPRAP/δ-catenin (CTNND2), ARVCF (armadillo repeat gene deleted in velo-cardio-facial syndrome) and the more distantly related plakophilin 1–3 (Hatzfeld, 2005). Like all p120-catenins, p0071 localizes to adherens junctions (AJs), mediated by the interaction with the juxtamembrane domain of classical cadherins. The regulation of cadherin turnover seems to be a common function of the p120 catenins (Carnahan et al., 2010; Hatzfeld, 2005). However, despite some redundancy, each p120 catenin fulfills unique tasks in cell signaling. p0071 interacts with RhoA and the Rho-GEF Ect2 to regulate Rho signaling during cytokinesis. In conjunction with Ect2, p0071 stimulates RhoA activation to facilitate formation and contraction of the contractile ring (Keil et al., 2007; Wolf et al., 2006). Furthermore, p0071 interacts with the microtubule-associated motor protein KIF3B, a subunit of the heterotrimeric KIF3 complex. p0071 is transported through KIF3B to the midbody, and the proper localization of p0071 by KIF3B is required for its role in actin reorganization during cell division (Keil et al., 2009). Localization of p0071 at cell–cell contact also depends on KIF3B (Keil et al., 2013).

In order to determine additional functions of p0071 we identified Rab11a as a putative interaction partner of p0071 in a yeast-two hybrid screen. Rab11a is a small GTPase of the Ras superfamily (Sakurada et al., 1991). Rab GTPases define vesicle identity and ensure that cargo proteins are delivered to their correct destinations at the right time. Among the multiple vesicle transport pathways that have been extensively analyzed, recycling of endocytic vesicles is essentially regulated by Rab4, Rab5 and Rab11 (Agola et al., 2011; Li and DiFiglia, 2012; Stenmark, 2009).

These proteins coordinate the return of endocytosed proteins and lipids to the plasma membrane by two alternative routes (Maxfield and McGraw, 2004). Basically, endocytosed proteins are first delivered to sorting or early endosomes from where they either recycle back to the plasma membrane or they are delivered to late endosomes and lysosomes for subsequent degradation. Pulse–chase experiments revealed both a fast and a slow pathway for protein recycling: the fast route depends on Rab4, which mediates recycling directly from early endosomes to the plasma membrane. In the slow route that depends on Rab11, proteins first pass the early endosomes and then the perinuclear recycling endosome before they are transported back to the cell surface (Li and DiFiglia, 2012; Maxfield and McGraw, 2004; Ren et al., 1998; Sönnichsen et al., 2000; Stenmark, 2009). The mechanism of sorting and the proteins involved in determining which recycling route is used remain largely unknown (Daro et al., 1996; Li and DiFiglia, 2012). Rab11 generally localizes to the perinuclear recycling endosome and participates in the slow recycling of endocytosed proteins, such as the transferrin receptor (TfnR) (Ullrich et al., 1996). Interestingly, Rab11 also participates in the transport and sorting of newly synthesized E-cadherin by recycling endosomes as well as in the recycling of endocytosed E-cadherin (Bryant et al., 2007; Delva and Kowalczyk, 2009; Lock and Stow, 2005). Thus, Rab11 is involved in the regulation of cadherin-mediated intercellular adhesion.

Rab11 fulfills its functions by interacting with numerous proteins (Kelly et al., 2012), including the Rab11 family interacting proteins (FIPs). These effectors, namely Rip11/FIP5, FIP2, RCP/FIP1, FIP3 and FIP4 are recruited by Rab11 in a GTP-dependent manner (Hales et al., 2001; Horgan and McCaffrey, 2009; Wallace et al., 2002a). They regulate multiple membrane trafficking events and are, together with microtubule (KIF3B, cytoplasmic dynein 1 motor protein complex)- and actin-associated motor proteins (MyoVb), essential for the

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correct localization of Rab11-positive recycling endosomes (Hales et al., 2002; Horgan et al., 2010; Horgan et al., 2004; Lapierre et al., 2001; Schonteich et al., 2008; Wallace et al., 2002b). Although KIF3B and dynein 1 are required for the perinuclear localization of the recycling endosome, MyoVb appears to participate in plasma-membrane-directed transport, although a function in retrograde transport has also been reported (Lanzetti, 2007; Powelka et al., 2004; Provance et al., 2004).

The spatiotemporal regulation of Rab11 activity is of particular importance. However, so far, only one GTPase-activating protein (GAP), Evi5, has been identified (Dabbeekeh et al., 2007). Among the numerous GDP–GTP exchange factors (GEFs) known to regulate Rab GTPases (Barr and Lambright, 2010) no GEF that activates Rab11 has been identified despite a systematic characterization of the DENN (differentially expressed in neoplastic and normal cells) domain subfamily of Rab GEFs (Yoshimura et al., 2010).

Notwithstanding the progress made in revealing motor proteins and effectors, superordinated factors and processes that control Rab11 are largely unknown. Here, we describe p0071 as a novel interaction partner of Rab11a and identify a two-way regulatory mechanism involving p0071 and Rab11. On the one hand, p0071 controls the localization and the recycling function of Rab11a and is thus involved in the decision of whether cargo proteins are recycled through the slow or the fast route. On the other hand, Rab11a regulates intercellular adhesion by controlling p0071 and E-cadherin recycling.

RESULTS

p0071 and Rab11a interact

In a yeast-two-hybrid screen for p0071-interacting proteins we identified full-length Rab11a as a putative binding partner of the p0071 armadillo domain (p0071-arm). GST-pulldown experiments confirmed this finding and excluded the p0071 N- and C-terminal domains as Rab11a interaction domains. Dominant-active Rab11a (Rab11a-DA; Q70L) precipitated p0071-arm more efficiently than the wild-type protein (Rab11a-WT), whereas almost no interaction was observed between p0071-arm and dominant-negative Rab11a (Rab11a-DN; S25N; Fig. 1A). The co-purification of FLAG-tagged p0071-WT and GFP-tagged Rab11a-WT from HEK293 lysates suggested the presence of a complex containing both proteins (Fig. 1B). Again, p0071 preferentially interacted with GFP–Rab11a-DA (Fig. 1B). Because Rab11a activation is a transient process and the interaction of p0071 with Rab11a depended on its activation, we were not able to co-purify the endogenous proteins from lysates of untreated cells. The co-purification of endogenous p0071 and Rab11 was, however, possible when p0071 was internalized by EGTA treatment. Under these conditions, a proportion of Rab11 co-precipitated with p0071 (Fig. 1C), confirming our assumption that this interaction is transient under physiological conditions and may require additional stimuli. This is supported by the finding that p0071 and Rab11 co-localized only partially in the perinuclear region, although both proteins showed an obvious enrichment in this region in sub-confluent HeLa cells (Fig. 2A).
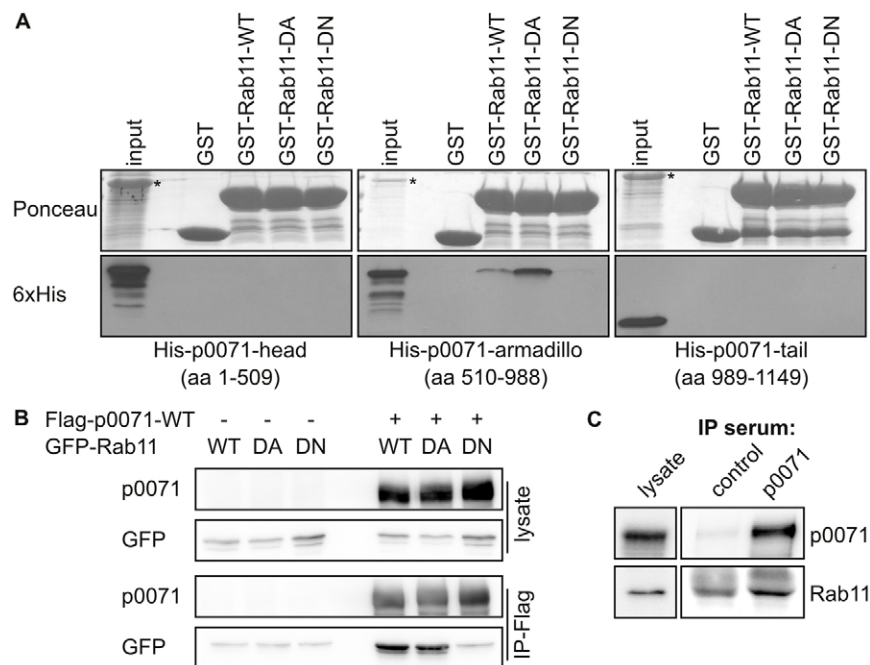

Fig. 1. Rab11 interacts with p0071. (A) The recombinant p0071 armadillo domain interacted directly with Rab11a-WT and -DA. Glutathione–agarose-immobilized GST or GST–Rab11a variants [wild-type (WT), dominant active (DA), dominant negative (DN)] were incubated with bacterial lysates containing p0071 domains (head, armadillo, tail) fused to 6× His tag. The precipitates were analyzed by western blotting using 6×His-tag-directed antibodies. *Chaperone Cpn60 from ArcticExpress cells. (B) FLAG-tagged p0071-WT co-precipitated with EGFP–Rab11a-WT and -DA. Lysates from HEK293 cells expressing GFP–Rab11a variants (WT, DA, DN) together with FLAG-tagged p0071-WT were incubated with anti-FLAG–agarose. The precipitates (IP-FLAG) were analyzed for GFP and p0071 by western blotting. Lysates from HEK293 cells co-transfected with constructs coding for the Rab11a variants and the FLAG-tag alone were used as a specificity control. (C) Endogenous Rab11 was co-precipitated with endogenous p0071. HeLa cells were incubated in medium containing 4 mM EGTA for 60 minutes and subsequently lysed. Immunoprecipitations (IPs) used either guinea pig anti-p0071 serum (p0071) or pre-immune serum (control) derived from the same animal. The precipitates were analyzed for the presence of p0071 and Rab11 by western blotting. The background in both control and p0071 IPs resulted from a cross reactivity of the secondary antibody with the light chain of the used antibodies.
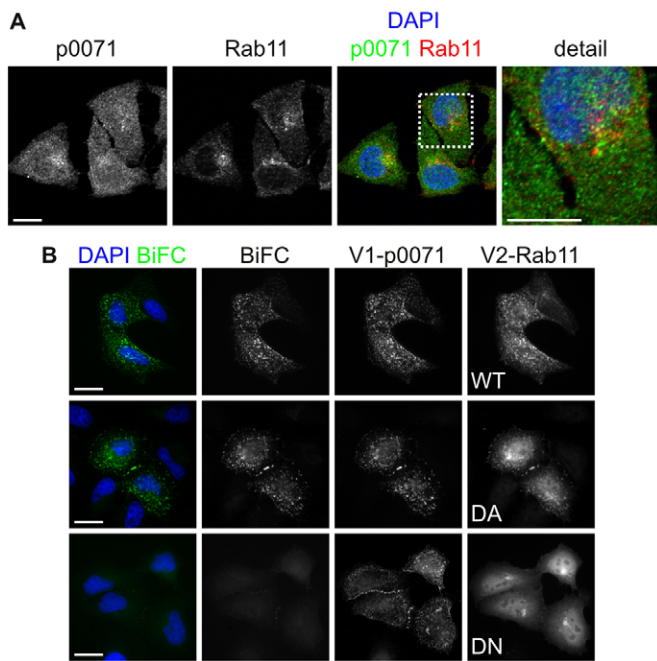
Fig. 2. p0071 and Rab11 partially colocalize. (A) Partial colocalization of p0071 and Rab11 at the perinuclear region. Non-confluent HeLa cells were immunostained for endogenous p0071 and Rab11. Scale bars: 20 µm; enlargement 10 µm. (B) Bimolecular fluorescence complementation (BiFC) of p0071-WT (V1-p0071) and Rab11a (V2-Rab11) in HeLa cells. The expression of the non-fluorescent fusion proteins was visualized by FLAG-tag (V1-p0071)- or HA-tag (V2-Rab11a-WT, -DA, -DN)-directed antibodies. The fluorescence of the reconstituted Venus protein was imaged with identical exposure times. Scale bars: 20 µm.

Bimolecular fluorescence complementation (BiFC) revealed strong signals in HeLa cells co-expressing Venus1 (V1)-p0071-WT and Venus2 (V2)-Rab11a-WT or V2-Rab11a-DA but not V2-Rab11a-DN. Although V1-p0071-WT and V2-Rab11a-WT formed punctate BiFC complexes in the cytoplasm (Fig. 2B, upper row) with perinuclear accumulation in some cells (not shown), BiFC complexes of V2-Rab11a-DA and V1-p0071-WT also occurred at cell borders. A perinuclear accumulation of BiFC complexes as seen in V2-Rab11a-WT cells appeared with a lower frequency. In good accordance with the GST-pulldown approaches, V1-p0071-WT and V2-Rab11-DN coexpressing cells showed only very faint BiFC signals (Fig. 2B, lower row) again confirming that p0071 preferentially interacts with active Rab11a. Similar results were obtained in MCF-7 cells, in which the membrane association of p0071–Rab11a complexes was more pronounced (supplementary material Fig. S1).

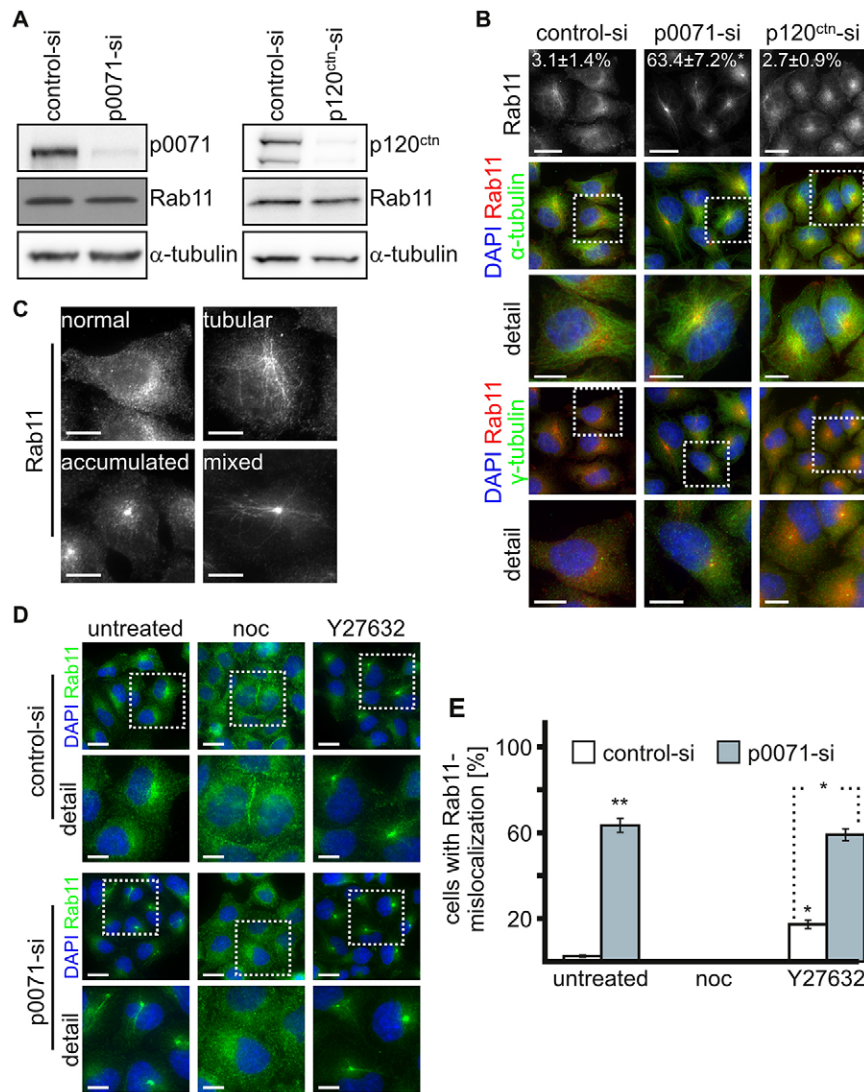**p0071 regulates the localization of Rab11**
On the basis of the preferred interaction of p0071 with active Rab11a, p0071 could be involved in the spatiotemporal regulation of Rab11a either by acting as a Rab11a effector, a Rab11a GAP, or as a scaffold to support Rab11a function. Thus, in order to ascertain how, or if p0071 regulates Rab11, we used a pool of three siRNAs to suppress the expression of p0071. In HeLa cells, western blot analyses confirmed a specific downregulation of p0071, while the expression of Rab11 remained unaffected (Fig. 3A; supplementary material Fig. S2A). Strikingly, the localization of Rab11 was affected. In the majority of cells, Rab11a was concentrated in the perinuclear region where it

colocalized with γ-tubulin-labeled centrosomes. In some cells, a tubular appearance of Rab11 that partially colocalized with α-tubulin was observed (Fig. 3B; p0071 siRNA). This localization of Rab11 was rarely observed in control siRNA-transfected cells (Fig. 3B). To quantify this phenotype the proportion of cells with mislocalized Rab11 was determined (Fig. 3C; accumulated, tubular and mixed phenotype). Whereas almost two-thirds of p0071-siRNA-transfected cells showed mislocalization of Rab11, only 3.1% of control cells did (control-si: 3.1%, s.d. = 1.4%; p0071-si: 63.4%, s.d. = 7.2%; $P<0.001$, $n=3$). To exclude the possibility of off-target effects we also tested the siRNAs individually for their ability to induce a Rab11 relocalization. All three siRNAs strongly reduced p0071 expression, with minor differences in efficiency, and induced the Rab11 mislocalization phenotype in a p0071-concentration-dependent manner (supplementary material Fig. S2A,B). Since the siRNA pool mediated the most efficient p0071 knockdown and strongest Rab11 phenotype, we used this in all subsequent experiments. The untypical localization of Rab11 upon p0071 knockdown was confirmed in a GFP-Rab11a-WT-expressing cell line, excluding the possibility of immunostaining artifacts (supplementary material Fig. S2C).

In order to exclude a cell-line-specific effect we extended our analyses to MCF-7 cells. As shown in supplementary material Fig. S2E, approximately one third of control-siRNA-transfected MCF-7 cells showed a perinuclear Rab11 accumulation. Upon p0071 depletion significantly more cells exhibited accumulated Rab11 suggesting that p0071 may regulate Rab11 localization in various cell lines (supplementary material Fig. S2D,E). We also analyzed whether p120[ctn], the only relative of p0071 that is expressed in HeLa cells, was able to regulate the localization of Rab11. In contrast to the p0071 knockdown, the knockdown of p120[ctn] did not induce a Rab11 mislocalization (Fig. 3A,B), suggesting that a role in the regulation of Rab11 is unique to p0071.

The accumulation of Rab11 at the centrosomes and its partial tubulin association upon p0071 knockdown suggests that the altered Rab11 localization depends on the microtubule cytoskeleton. To investigate the role of microtubules in Rab11 localization, control- and p0071-siRNA-transfected HeLa cells were treated with nocodazole. Nocodazole treatment completely dispersed the Rab11 accumulation observed upon p0071 depletion (Fig. 3D,E), suggesting that this localization depends on microtubule-based transport of Rab11 along intact microtubules. Furthermore, in nocodazole-treated control cells, Rab11 accumulated at cell–cell contacts as described previously (Casanova et al., 1999), whereas a similar localization was never observed in p0071-depleted cells. Thus, p0071 might mediate tethering of Rab11 vesicles at the plasma membrane.

p0071 regulates the actin cytoskeleton through the GTPase RhoA during cytokinesis (Keil et al., 2007; Wolf et al., 2006). Although not characterized in detail, depletion of p0071 appears to alter actin dynamics also in interphase. To investigate whether inhibition of RhoA signaling might affect Rab11 localization, we treated HeLa cells with the ROCK inhibitor Y27632. Approximately 17% of control siRNA-transfected cells treated with the drug showed the same Rab11 accumulation as observed in p0071-depleted cells not treated with the ROCK inhibitor (Fig. 3D). Although we observed fewer cells with Rab11 mislocalization compared with the p0071 knockdown, this experiment indicates a role of Rho signaling in Rab11 regulation. The fact that Y27632 treatment of p0071-depleted cells did not reinforce the accumulation of Rab11 (Fig. 3E), further supports the hypothesis that both factors target the same process.

Fig. 3. p0071 regulates Rab11 localization.
(A) Western blot analysis of the p0071 and p120ctn knockdown. HeLa cells were transfected with control, p0071 or p120ctn siRNA pools and lysed after 48 hours. Equal amounts of total protein were loaded and analyzed by western blotting. α-tubulin was used as a loading control. (B) Depletion of p0071 alters the localization of Rab11. Control-, p0071- or p120ctn-siRNA-transfected HeLa cells were fixed with TCA and immunostained for Rab11, α- and γ-tubulin. The relative amount of cells with altered Rab11 localization is indicated. Scale bars: 20 μm; detail: 10 μm. *$P<0.001$, $n=3$. (C) Different localization patterns of Rab11 observed in HeLa cells. HeLa cells were immunostained for Rab11 and its localization was classified as indicated. Cells with tubular Rab11 localization (tubular), Rab11 accumulation in the perinuclear region (accumulated) and combinations of both localization patterns (mixed) were subsequently designated as cells with Rab11 mislocalization. Scale bars: 10 μm. (D) Localization of Rab11 depends on microtubule and actin cytoskeletons. Control- and p0071-siRNA-transfected cells were treated for 2 hours with 3 μg/ml nocodazole (noc) or 20 μM Y27632, and subsequently fixed and immunostained for Rab11. Scale bars: 20 μm; detail: 10 μm. (E) Quantification of the Rab11 mislocalization. The percentage of cells showing Rab11 mislocalization ± s.d. is shown for control- and p0071-siRNA-transfected cells. *$P<0.01$, **$P<0.001$; $n=3$.

Taken together, these experiments indicate that the transport of Rab11 vesicles from the pericentrosomal region to the plasma membrane depends on p0071 and on actin organization. Accordingly, p0071 depletion alters the balance between the microtubule-based transport of Rab11 towards the perinuclear region and the escape from this region.

To analyze this in more detail we treated HeLa cells for 2 hours with nocodazole and examined the redistribution of Rab11 upon nocodazole release (Fig. 4). In control siRNA-transfected cells, Rab11 was transiently enriched in the pericentrosomal region at 10 minutes after nocodazole release. Approximately 50% of these cells showed a punctate accumulation at the centrosome. By 20 minutes after nocodazole release, Rab11 was again dispersed over the cell with enrichment but negligible accumulation at the perinuclear region as seen in untreated cells. This indicates that Rab11 vesicles pass the pericentrosomal compartment rather fast and move on towards the plasma membrane. In comparison, p0071-depleted cells had a much more pronounced punctate accumulation of Rab11 at the centrosomes at 10 minutes and 20 minutes after nocodazole release (Fig. 4A,B), suggesting that the translocation of Rab11 towards the perinuclear region is microtubule dependent and p0071 independent. In contrast, the

translocation from this region back to the plasma membrane appears to require p0071.

One explanation for the altered Rab11 localization in response to the p0071 knockdown is a transport defect. Since Rabs are typically transported in their active form (Stenmark, 2009) we speculated that p0071 might function as a linker between activated Rab11 and a cytoskeleton-associated motor protein thus facilitating Rab11-dependent transport from the perinuclear region to the plasma membrane. p0071 interacts with the kinesin family member KIF3B, and its localization at the midbody (Keil et al., 2009) and at cell–cell contacts (Keil et al., 2013) requires KIF3B. KIF3B has also been implicated in Rab11 transport, but a direct interaction between KIF3B and Rab11a has not been demonstrated (Schonteich et al., 2008). Therefore, we tested whether p0071 could function as a linker to mediate KIF3B-dependent Rab11 transport. However, KIF3B knockdown did not induce a pericentrosomal accumulation of Rab11 in untreated cells or in cells released from nocodazole (Fig. 4A,B), although KIF3B expression was strongly reduced (supplementary material Fig. S3A). Unaffected Rab11 levels exclude the possibility that reduced levels of Rab11 mask the phenotype (supplementary material Fig. S3A). This indicates that Rab11 accumulation was not caused by a KIF3B-dependent
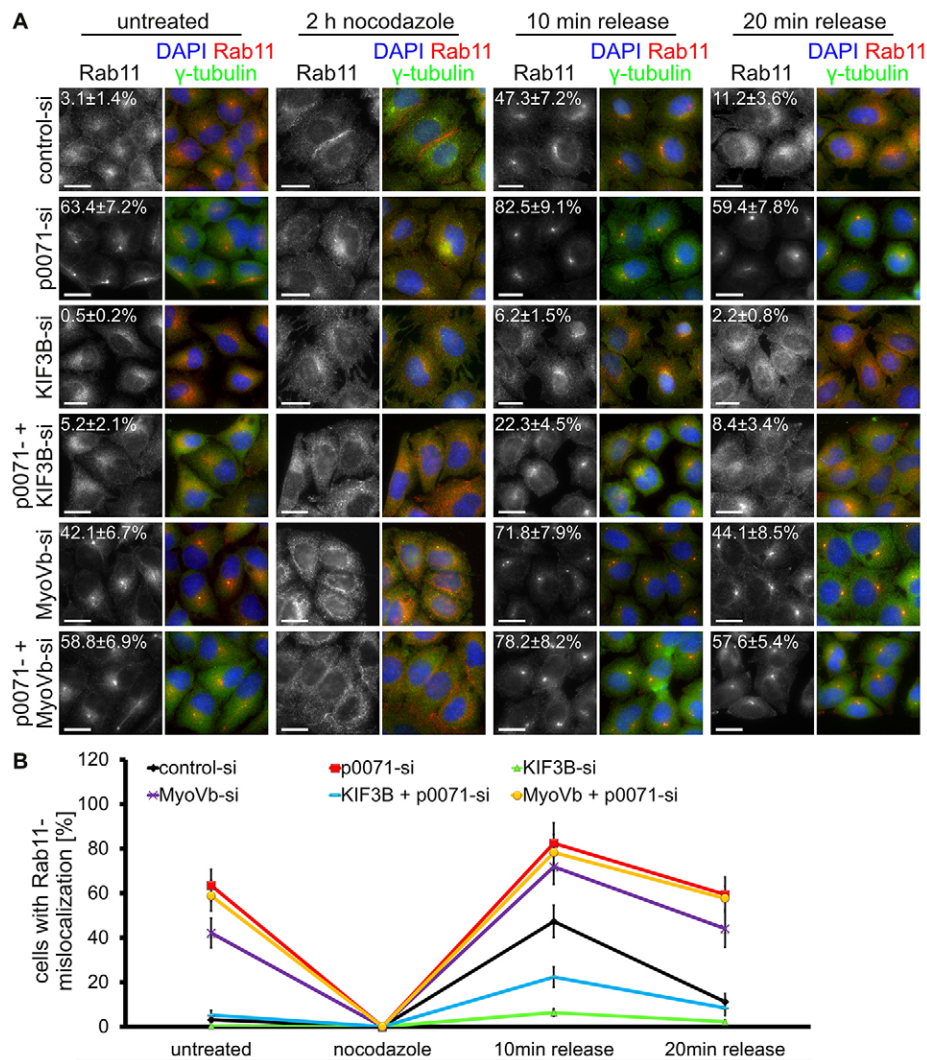
Fig. 4. Role of motor proteins in directing Rab11 localization. (A) p0071, KIF3B and MyoVb participate in the localization of Rab11. At 48 hours after transfection with the indicated siRNAs, HeLa cells were incubated for 2 hours with 3 µg/ml nocodazole. After washing, cells were incubated in normal medium for the indicated time and then immunostained for Rab11, α- and γ-tubulin. The relative amount of cells showing Rab11 mislocalization is indicated. Scale bars: 20 µm. (B) Diagram showing Rab11 mislocalization during nocodazole release. After release from nocodazole, a substantial number of control cells transiently accumulated Rab11, which were almost completely gone 10 minutes later. The p0071 or the MyoVb knockdown as well as the combined knockdown of both induced Rab11 mislocalization in untreated cells to a similar extent. Release from nocodazole significantly increased Rab11 accumulation in these cells compared to the control. In KIF3B-depleted cells almost no Rab11 mislocalization was observed even after nocodazole release. The combined knockdown of KIF3B plus p0071 prevented Rab11 mislocalization in untreated cells as well as in cells released from nocodazole.

transport defect. Moreover, a knockdown of both p0071 and KIF3B almost completely rescued the Rab11 accumulation as observed after p0071 depletion. The reduced Rab11 accumulation in double knockdown cells was also observed in cells that underwent nocodazole treatment and subsequent release (Fig. 4A,B).

p0071 may be involved in actin-dependent transport of Rab11 vesicles, as MyoVb has been identified as a motor and effector of Rab11 (Hales et al., 2002; Lapierre et al., 2001). Therefore, we next investigated whether MyoVb is involved in the p0071-dependent Rab11 localization. The effect of MyoVb depletion (supplementary material Fig. S3A) strongly resembled the Rab11 mislocalization phenotype caused by the p0071 knockdown in the nocodazole release experiment (Fig. 4A,B). The depletion of both p0071 and MyoVb did not increase the number of cells with Rab11 accumulation compared with p0071 knockdown alone, indicating that p0071 and MyoVb might converge in the regulation of Rab11 (Fig. 4A,B).

p0071 participates in the slow Rab11-dependent recycling pathway

We next investigated whether p0071 also participates in the recycling of the TfnR, a well-characterized Rab11 cargo protein (Grant and Donaldson, 2009). TfnR is essential for regulating iron uptake of cells. Briefly, the iron–transferrin complex bound to the TfnR is internalized by receptor-mediated endocytosis. Iron release occurs in endosomes at low pH. Subsequently TfnR complexed with apotransferrin is recycled back to the plasma membrane, where apotransferrin is released (Aisen, 2004).

HeLa cells were incubated with Alexa-Fluor-555-conjugated transferrin (Tfn-A555) on ice to prevent internalization of TfnR-bound Tfn-A555 and subsequently unbound Tfn-A555 was removed by washing. The knockdown of p0071 or Rab11a provoked an increase in the cell-surface-bound Tfn-A555 (Fig. 5A), suggesting an increase in cell surface receptor. Incubation at 37℃ induced the endocytosis of Tfn-A555. After

30 minutes at 37℃, most of the internalized Tfn-A555 had left the EEA1-positive compartment in control cells. In contrast, Tfn-A555 largely colocalized with EEA1 in p0071- and Rab11a-depleted cells, suggesting that in the absence of p0071 or Rab11a, release of the TfnR from the early endosomes to the recycling endosome was disturbed (Fig. 5A). Together with the increased surface localization of the receptor this suggests that recycling of the TfnR occurred directly from early endosomes to the plasma membrane.

To validate this hypothesis and to test whether indeed TfnR would accumulate at the plasma membrane because of faster recycling we quantified the amount of cell surface receptor. Surface proteins were biotin-labeled for 45 minutes on ice and subsequently precipitated using avidin agarose. Although the overall amount of TfnR was not affected upon p0071 or Rab11a knockdown, the amount of surface-exposed TfnR was increased (Fig. 5B). In agreement, in a pulse–chase experiment, we found decreased intracellular TfnR ligand (Biotin-Tfn) in both p0071- and
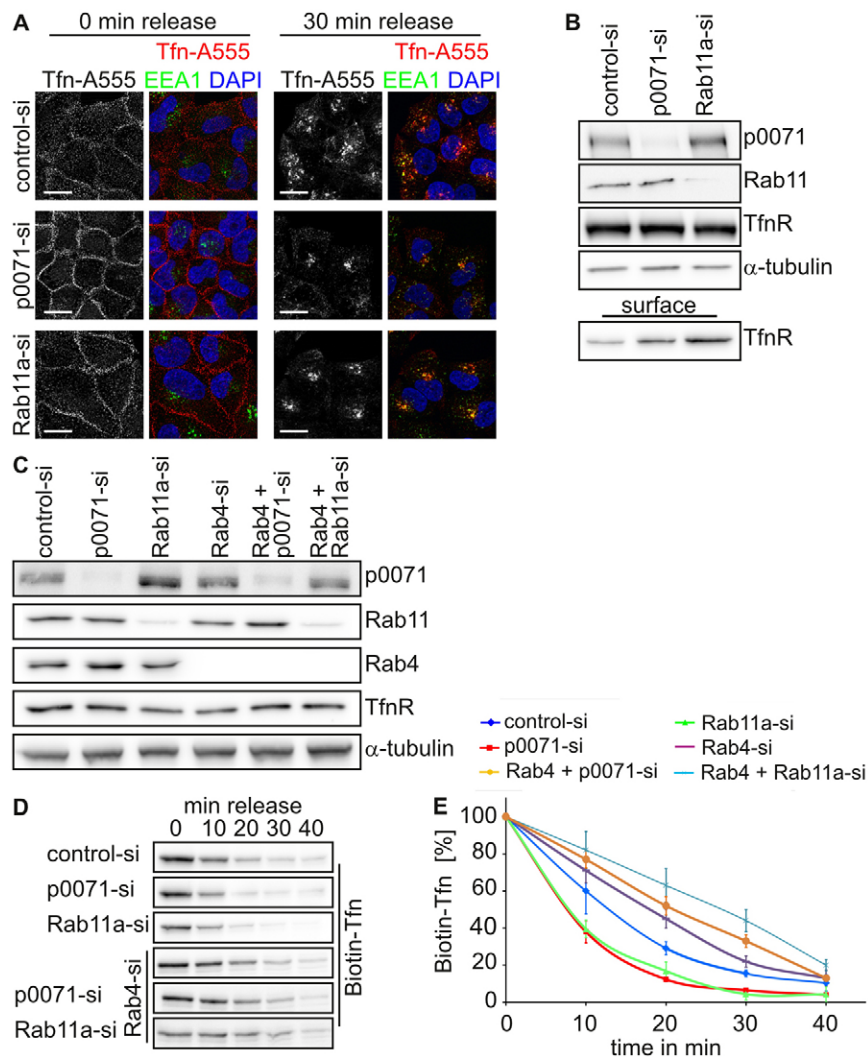

Fig. 5. p0071 interferes with Rab11-dependent slow recycling of the transferring receptor. (A) Depletion of either p0071 or Rab11 induced recycling of Tfn-A555 through early endosomes. Control, p0071 or Rab11a siRNA-transfected HeLa cells were incubated with Alexa-Fluor-555-conjugated transferrin (Tfn-A555, red) for 45 minutes on ice, washed and either fixed for immunofluorescence analyses or incubated at 37℃ for 30 minutes before fixation in formaldehyde. Cells were immunostained for the early endosomal marker EEA1 (green). Scale bars: 20 μm. (B) Depletion of either p0071 or Rab11 increased the amounts of surface TfnR. Control-, p0071- or Rab11a-siRNA-transfected HeLa cells were surface biotinylated for 45 minutes on ice. Subsequently, biotinylated proteins were precipitated using avidin agarose and the amount of surface TfnR was analyzed by western blotting. To monitor knockdown efficiencies of siRNAs and overall TfnR expression, equal protein amounts were loaded and analyzed by western blotting. α-tubulin was used as loading control. (C) Expression of p0071, Rab11, Rab4 and TfnR in siRNA-transfected HeLa cells. HeLa cells were transfected with the indicated siRNAs and lysed 48 hours later. Equal amounts of protein were loaded and the expression of p0071, Rab11, Rab4 and TfnR was analyzed by western blotting. α-Tubulin was used as loading control. (D) Depletion of either p0071 or Rab11a shifts the recycling of TfnR to the Rab4-dependent fast pathway. HeLa cells transfected with the indicated siRNAs were loaded with Biotin-Tfn, washed and incubated in medium containing unconjugated Tfn for the indicated time. Before lysis, surface-bound Biotin-Tfn was stripped off using an acidic wash buffer. The amount of residual intracellular Biotin-Tfn was analyzed by western blotting; representative blots are displayed from at least four independent experiments. (E) Quantification of intracellular Biotin-Tfn of siRNA-transfected HeLa cells compared with control cells. The relative amount of residual Tfn-Biotin (means ± s.d.) compared with time point zero was plotted against the time in minutes for HeLa cells transfected with the indicated siRNAs ($n \geq 4$). The recycling of Biotin-Tfn was significantly increased upon p0071 or Rab11a deletion during the first 30 minutes ($P < 0.05$). The additional knockdown of Rab4 prevented the observed acceleration significantly during the same period ($P < 0.05$).

Rab11a-depleted cells (Fig. 5C–E), supporting our assumption of accelerated TfnR recycling. To investigate whether the fast recycling pathway is preferred after either p0071 or Rab11a depletion we used knockdown of Rab4, which mediates fast endocytic recycling directly from early endosomes (Stenmark, 2009). The knockdown of Rab4 alone increased the amount of intracellular Biotin-Tfn (Fig. 5E), suggesting that constitutive Tfn recycling uses the Rab4- as well as the Rab11-dependent pathway. The combined knockdown of Rab4, plus p0071 or Rab11a significantly increased intracellular Biotin-Tfn compared with the single p0071 or Rab11a knockdown, thus reversing the effect observed after p0071 or Rab11 depletion. This indicates that the p0071 and the Rab11 knockdown indeed shift recycling towards the Rab4-dependent fast pathway (Fig. 8A).

p0071 is recycled through the slow Rab11-dependent pathway

Upon siRNA-mediated Rab11a depletion, we observed an ~70% increase in the level of p0071 in HeLa cells compared with control cells (Fig. 5B,C; Fig. 6A,B). The same effect was observed using two distinct siRNAs for Rab11a (supplementary material Fig. S3B). Interestingly, the combined knockdown of Rab11a and Rab4 restored p0071 levels (Fig. 5C) suggesting that p0071 itself might be recycled by the slow Rab11-dependent pathway. A loss of Rab11 function would shift p0071 recycling to

the faster route thereby increasing its association with cell–cell contact sites. At the plasma membrane, p0071 might become stabilized by its interaction with cadherins. This would finally lead to an increase of total p0071 levels in the cell. To analyze this in more detail, we first investigated whether p0071 localization was affected by Rab11a. The knockdown of Rab11a induced an increase of cell–cell-contact-associated p0071 in HeLa cells (Fig. 6C; supplementary material Fig. S4B). p0071 is a constituent of AJs that interacts with classical cadherins. Because MCF-7 cells have more pronounced AJs than HeLa cells we examined the recycling of AJ components in this cell line. Upon Rab11a depletion, p0071 was enriched at cell–cell contacts, to a greater extent than in control cells (Fig. 6D; supplementary material Fig. S3C,D; Fig. S4A). As demonstrated by western blotting the knockdown of Rab11a did not alter the expression of E-cadherin, p120[ctn] or β-catenin (Fig. 6E).

Using EGTA treatment, we induced internalization of components of AJs and analyzed the restoration of AJs upon adding Ca^{2+}-containing medium. Although all investigated AJ components were colocalized within the same compartments after internalization, E-cadherin and p120[ctn] relocalized much faster to cell–cell contacts than p0071. At 30 minutes after Ca^{2+} addition, p0071 was still largely trapped in vesicular structures in control cells (Fig. 6D; supplementary material Fig. S4A). In contrast,
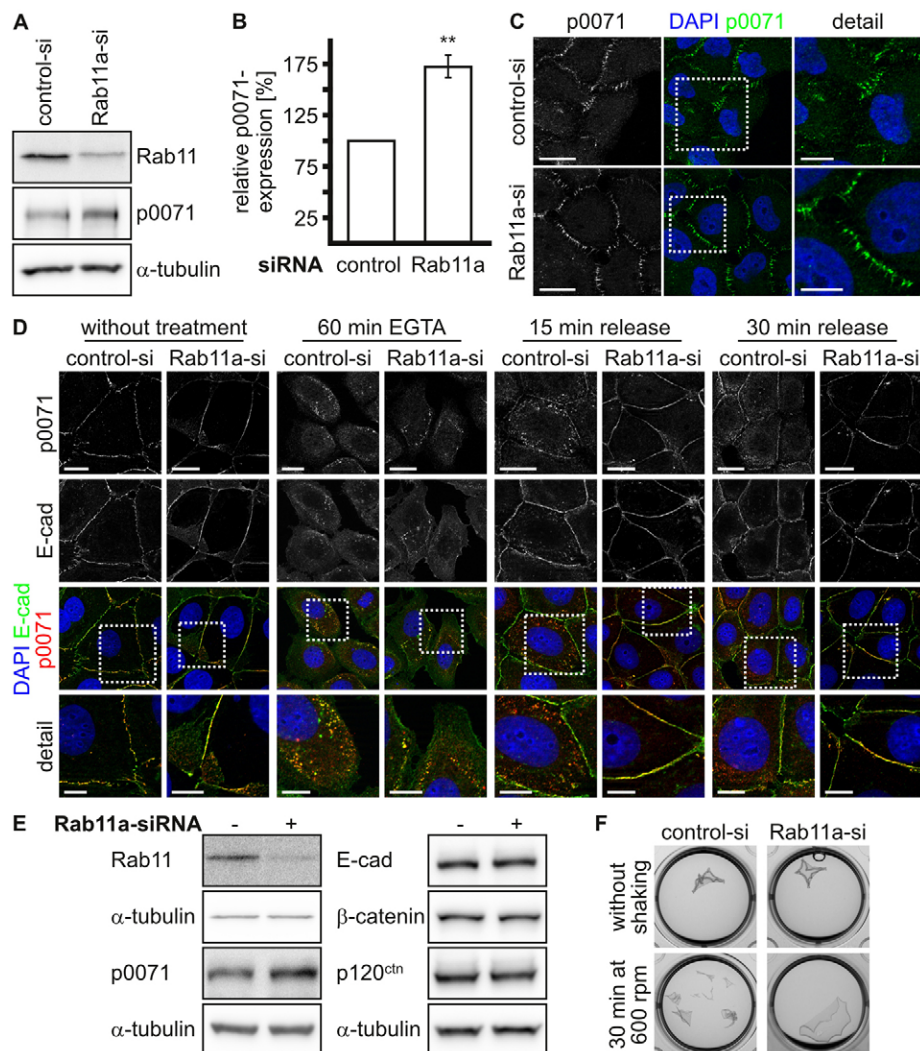

Fig. 6. Localization of p0071 is regulated by Rab11. (A) Rab11 depletion increased p0071 expression in HeLa cells. HeLa cells were transfected with the indicated siRNAs and lysed 48 hours later. Equal amounts of protein were loaded and the expression of p0071 and Rab11 was analyzed by western blotting. α-Tubulin was used as loading control. (B) Quantification of p0071 expression upon Rab11a knockdown. **$P<0.005$; $n=5$. (C) Rab11a depletion caused increased amounts of p0071 at cell–cell contacts in HeLa cells. Control- or Rab11a-siRNA-transfected HeLa cells were immunostained for p0071 (green). Scale bars: 20 μm; detail 10 μm. (D) Depletion of Rab11a altered the appearance and recycling of E-cadherin and p0071 at cell–cell contacts. MCF-7 cells were transfected with control or Rab11a siRNA. Subsequently, they were either left untreated or incubated for 60 minutes with EGTA followed by a release for the indicated time, and immunostained for p0071 and E-cadherin. Scale bars: 20 μm, detail 10 μm. (E) Expression of AJ proteins in MCF-7 cells. Equal amounts of control- and Rab11a-siRNA-transfected cell lysates were loaded and the expression of the indicated proteins was analyzed by western blotting. α-tubulin was used as loading control. (F) The intercellular adhesive strength was increased upon Rab11a depletion in MCF-7 cells. Epithelial sheets of control or Rab11a-depleted MCF-7 cells were detached using dispase. The stability of the monolayers was determined by applying mechanical stress (30 minutes at 600 r.p.m.).
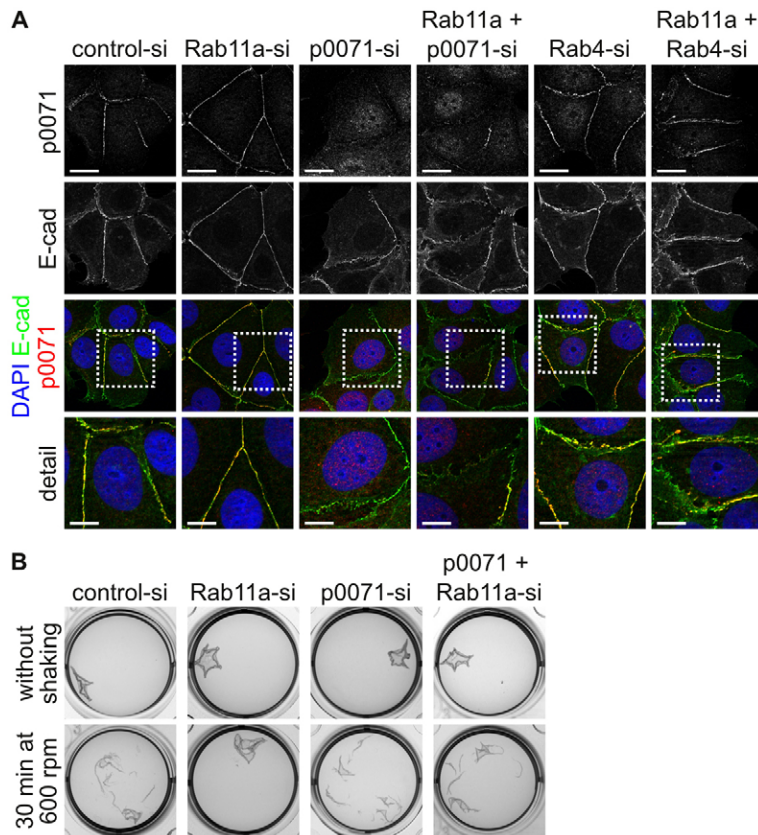
Fig. 7. The effect of Rab11a depletion on AJs depends on p0071 and Rab4. (A) Rab11a depletion-induced alterations in AJ organization depend on p0071 and Rab4. MCF-7 cells were transfected with the indicated siRNAs and 48 hours later immunostained for p0071 (red) and E-cadherin (green). Scale bars: 20 μm; detail 10 μm. (B) The strengthening of intercellular adhesion upon Rab11a depletion is mediated by p0071. Epithelial sheets of cells transfected with the indicated siRNAs were detached using dispase. The integrity of the monolayers was determined by applying mechanical stress (30 minutes at 600 r.p.m.).

Rab11-depleted cells restored cell–cell-contact-associated p0071 much faster. Already at 15 minutes after Ca^{2+} addition, p0071 was almost completely recycled back to the membrane. E-cadherin and $p120^{ctn}$ also appeared to recycle somewhat faster, but more striking was, again, the straight shape of the newly formed AJs (Fig. 6D; supplementary material Fig. S4A). The acceleration of p0071 recycling after the Rab11a knockdown was also observed in HeLa cells although reformation of AJs was in general slower than in MCF-7 cells (supplementary material Fig. S4B).

To elucidate whether the increase in p0071 and the modulation of AJs correlated with increased intercellular adhesive strength, we released epithelial sheets of control or Rab11a siRNA-transfected MCF-7 cells using dispase and subjected them to mechanical shear stress as previously described (Hudson et al., 2004). Whereas epithelial sheets of control cells lost their integrity after 30 minutes of shaking, epithelial sheets of Rab11a-depleted cells stayed intact (Fig. 6F). To confirm that the increased adhesive strength observed upon Rab11a depletion was due to elevated p0071 association with cell contact sites, we combined Rab11a and p0071 depletion. E-cadherin staining in those cells appeared fuzzy compared with Rab11a-depleted cells, suggesting that p0071 is responsible for the altered E-cadherin localization observed upon Rab11a knockdown (Fig. 7A). Moreover, the increase in intercellular adhesive strength induced by Rab11a depletion was reversed by simultaneous p0071 depletion (Fig. 7B). Our results suggest that Rab11a depletion shifts p0071 recycling to the fast Rab4-dependent pathway. To confirm this, we combined Rab11a and Rab4 depletion, which should prevent fast recycling. Indeed, this did restore p0071 expression levels, as well as its localization (Fig. 5C; Fig. 7A), indicating that p0071 is recycled preferentially through the Rab11a-dependent slow pathway.

Thus, we conclude that a Rab11a knockdown shifts recycling of p0071 from the slow Rab11-dependent pathway to the faster Rab4-dependent route, leading to p0071-dependent AJ reformation that is accompanied by increased intercellular adhesive strength (Fig. 8B).

DISCUSSION

Despite the identification of several effectors and motor proteins involved in Rab-dependent vesicle transport, it remains unknown how exactly the localization of the particular Rab GTPases and/or endosomes is regulated and which factors determine the recycling pathway of cargo proteins. Here we have identified the armadillo protein p0071 as a novel interaction partner for Rab11a and show the interdependent regulation of these proteins. On the one hand, p0071 depletion induces a mislocalization of Rab11 which results in a shift of cargo protein recycling from the slow Rab11-dependent to the fast Rab4-dependent pathway. On the other hand, p0071 is recycled through the Rab11-dependent route. In this context, Rab11 participates in the surveillance of intercellular adhesion mediated by AJs.

p0071 as a regulator of Rab11 localization and function

The knockdown of p0071 was accompanied by a strong accumulation of Rab11 in the perinuclear region, clearly indicating that p0071 plays a role in Rab11 localization (Figs 3, 4; supplementary material Fig. S2; Fig. S3A). Interestingly, a similar phenotype has been described by Hehnly and co-workers who showed that Rab11 accumulated at membrane-free centrosomes isolated from Rab11 GAP Evi5-depleted cells (Hehnly et al., 2012). This suggests that activated Rab11 becomes trapped at the centrosome, which is supported by the finding that the Rab11 cargo protein TfnR is similarly localized in
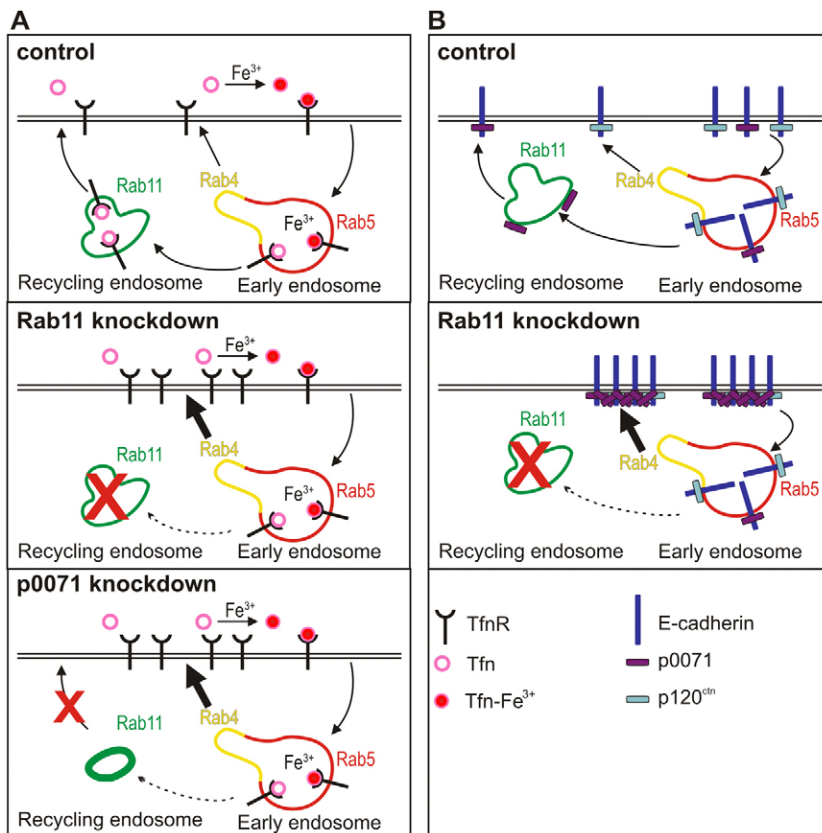
Fig. 8. Model summarizing the roles of p0071 in recycling. (A) Transferrin recycling in control, Rab11- and p0071-depleted cells. Rab11 knockdown interferes with the integrity of the recycling endosome and shifts Tfn recycling to the Rab4-dependent fast pathway. p0071 depletion causes Rab11 accumulation and interferes with recycling endosome dynamics and function again resulting in a shift of Tfn recycling to the Rab4-dependent short route. (B) p0071 recycling in control and Rab11 knockdown cells. In untreated cells p0071 recycling is retarded compared to E-cadherin and p120ctn because it passes the recycling endosome. Rab11 depletion interferes with recycling endosome integrity and function, and shifts p0071 recycling to the fast pathway. As a consequence, more p0071 accumulates at the membrane and triggers a reorganization of the AJs resulting finally in a stabilization of intercellular cohesion. The model has been adapted from Linford et al. (Linford et al., 2012).

Rab11a-DA-expressing cells (Ren et al., 1998). Although we do not know whether the Rab11 that accumulated upon p0071 depletion was active or inactive, we suggest that p0071 is involved in the regulation of Rab11 activity required for the escape of Rab11-positive vesicles from the pericentrosomal region. Because p120-catenins appear to be platforms for the regulation of Rho GTPases by interacting with their GEFs, GAPs and effectors (Anastasiadis, 2007; Keil et al., 2013; Menke and Giehl, 2012), an analogous principle might apply to p0071 and Rab11. However, we were unable to show a direct role of p0071 in regulating Rab11 activity through GST-pulldown experiments with the FIP4 effector as bait (supplementary material Fig. S5). This assay reports the global Rab11 activation status of whole cell lysates which also contain the relevant GEFs and GAPs. Thus, the activation status may change during incubation. Therefore, our results do not exclude local effects. Interestingly, the phenotype observed upon p0071 knockdown is more striking than that of overexpressed Rab11a-DA. We therefore conclude that the regulation of Rab11 activation by p0071 probably contributes to, but does not fully explain, the observed phenotype.

Another possibility of how p0071 could be involved in regulating Rab11 localization is that is has a role in Rab11 transport. Two microtubule-dependent motors have been implicated in the transport of Rab11-positive vesicles, namely KIF3B and the cytoplasmic dynein 1 motor protein complex (Horgan et al., 2010; Schonteich et al., 2008). Based on the known relationship between p0071 and KIF3B (Keil et al., 2009) we hypothesized that p0071 might serve as a linker to connect Rab11 to the KIF3B motor. However, knockdown of KIF3B prevented the pericentrosomal accumulation of Rab11 that transiently occurs upon nocodazole release (Fig. 4) thus

producing an opposing phenotype. Moreover, the p0071-knockdown-induced Rab11 mislocalization was significantly reduced when both Kif3b and p0071 were depleted, indicating that KIF3B is involved in the retrograde transport of Rab11 vesicles towards the perinuclear region, whereas p0071 is required for the anterograde transport to the cell periphery. In good accordance with our observations, Schonteich and colleagues described a peripheral accumulation of TfnR following a KIF3B knockdown (Schonteich et al., 2008), again suggesting that KIF3B primarily mediates the retrograde transport of Rab11 towards the perinuclear recycling endosome.

Actin cytoskeleton remodeling is required for a variety of processes during membrane trafficking (Lanzetti, 2007) and Rab11-dependent processes such as the recycling of β1-integrin were shown to depend on actin (Powelka et al., 2004). In support of this, the ROCK inhibitor Y27632 induced a mislocalization of Rab11 in the pericentrosomal region that strongly resembled the effect of the p0071 knockdown (Fig. 3D). ROCK mediates Rho-induced stress fiber formation (Narumiya et al., 2000) and actin filament contractility by regulating myosin light chain and its phosphatase (Street and Bryan, 2011). Rab11 localization also depends on a myosin motor protein, MyoVb. The overexpression of the motor-deficient MyoVb tail domain results in Rab11 compaction in the perinuclear area (Lapierre et al., 2001), resembling the phenotype that we observed upon p0071 or MyoVb knockdown (Fig. 4). This suggests that p0071 and MyoVb contribute to the regulation of Rab11 localization in a common pathway. Because MyoVb directly interacts with Rab11 (Lapierre et al., 2001) it is not likely that p0071 acts as a linker between MyoVb and Rab11 cargo vesicles.

Another possibility is that disturbed actin dynamics upon p0071 depletion prevent the transport of Rab11a through MyoVb.

This would link the regulation of Rho signaling through p0071 to its role in regulating the endocytic recycling pathway. Many studies suggest that Rho-GTPase-driven actin cytoskeleton remodeling participates in membrane trafficking and endocytic recycling and that the regulation of Rho and Rab family GTPases is interconnected (de Curtis and Meldolesi, 2012; Harris and Tepass, 2010). In this context, p0071 could function as a scaffold to coordinate signaling through Rho and Rab GTPases.

Functionally, mislocalization of Rab11 upon p0071 depletion correlated with accelerated recycling of TfnR (Fig. 5C–E) that resulted from a shift of the Rab11-dependent slow recycling through recycling endosomes to Rab4-dependent fast recycling through early endosomes (summarized in Fig. 8A). This suggests that accumulated Rab11 in the perinuclear region in p0071-depleted cells does not participate in the recycling of endocytosed proteins. Studies using Rab11 mutants suggest that Rab11 is required for sorting of cargo proteins from the early endosome to the recycling endosome (Ren et al., 1998). Accordingly, the knockdown of Rab11 would prevent early endosome to recycling endosome transport of TfnR-bearing vesicles (Fig. 8A). Several studies reported decreased TfnR recycling after Rab11-WT, -DA or -DN overexpression without compensation by the fast recycling pathway, as shown in our study (Ren et al., 1998; Ullrich et al., 1996). However, overexpressed Rab11 and its mutants could sequester endogenous regulators and effectors that might be rate limiting in the fast pathway as described for the regulation of Rho GTPases (Heasman and Ridley, 2008). One effector that could mediate the suggested crosstalk between Rab4 and Rab11 and is implicated in recycling of TfnR is RCP/FIP1 (Lindsay et al., 2002). One study examined TfnR recycling upon Rab11 knockdown (Takahashi et al., 2012). Contradictory to our results, it showed an accumulation of TfnR underneath the plasma membrane correlating with decreased TfnR exocytosis. Although we never observed TfnR accumulation underneath the membrane in either p0071- or Rab11-depleted cells, we cannot exclude that different cellular systems might contribute to these observations. However, studies (as discussed below) on proteins participating in Rab11-dependent slow recycling support our findings. The knockdown of centriolin, FIP3, DLIC or D-AKAP-2 shifted the recycling of TfnR to the faster route (Eggers et al., 2009; Hehnly et al., 2012; Horgan et al., 2010). Moreover, the knockdown of KIF3B or Rip11/FIP5 led to increased TfnR recycling through the early endosome because of a disturbed transport of vesicles to the pericentrosomal recycling endosome (Schonteich et al., 2008). This suggests that a complex network of proteins regulates the balance between the slow and fast recycling routes to maintain appropriate cell surface localization of adhesion and signaling receptors.

p0071 as a Rab11 cargo protein
Interestingly, depletion of Rab11 significantly increased the levels of p0071 in HeLa and MCF-7 cells. This correlated with an increase of junctional p0071. Despite the fact that the expression of all other examined AJ components was unaffected by the Rab11a knockdown, the overall appearance of AJs was altered and intercellular adhesion was increased (Fig. 6; supplementary material Fig. S3C,D; Fig. S4). As observed for the recycling of the TfnR, the combined knockdown of Rab11a and Rab4 restored the p0071 expression levels and the appearance of AJ (Fig. 5C; Fig. 7A) suggesting that p0071 recycling is Rab11-dependent and

that Rab11a depletion induced the faster Rab4-dependent pathway. In agreement with our findings (Fig. 6; supplementary material Fig. S4), several studies indicate that catenins are internalized along with E-cadherin (Bryant et al., 2007). It is also known that depletion of p120^{ctn} induces lysosomal E-cadherin degradation, whereas the association with p120^{ctn} may redirect E-cadherin to the plasma membrane (Delva and Kowalczyk, 2009; Kowalczyk and Reynolds, 2004). Based on our finding that Rab11a depletion predominantly affected p0071 (Figs 6, 7; supplementary material Fig. S4), we propose that the E-cadherin–p120^{ctn} complex is directly redistributed to cell–cell contacts after removing EGTA, whereas the recycling of p0071 occurs through Rab11 and the recycling endosome. In this context, p0071 may function as a Rab11 regulator. The combined knockdown of Rab11a and p0071 reversed the Rab11a-depletion-induced stabilization in cell–cell adhesion indicating that this gain of adhesive strength depended on p0071.

Langevin and co-workers have shown that DE-cadherin transport to the plasma membrane occurs through Rab11-positive recycling endosomes and depends on the exocyst complex. Moreover, armadillo and Rab11 interacted with the exocyst components Sec15 and Sec10, respectively (Langevin et al., 2005). E-cadherin is also transported through the recycling endosome in polarized and non-polarized cells. The expression of Rab11-DN prevented basolateral sorting of E-cadherin leading to disturbed monolayer maturation in MDCK cells (Desclozeaux et al., 2008; Lock and Stow, 2005). Consistent with our data the recycling of endocytosed E-cadherin occurred through EEA1-positive early endosomes and Rab11-positive recycling endosomes (Bryant et al., 2007; Paterson et al., 2003). Our data indicate that the majority of E-cadherin internalized upon EGTA treatment is recycled quickly back to the plasma membrane, probably in association with p120^{ctn} (Fig. 8B). However, most of the p0071 accumulated without detectable E-cadherin association, suggesting a different recycling route for p0071 through the Rab11-dependent slow pathway. In this context p0071 and Rab11 might function in common to regulate dynamic cadherin-mediated cell–cell adhesion.

Putative function of the reciprocal regulation
We hypothesize that the two-way regulatory mechanism described here contributes to maintenance of AJ-mediated adhesion under steady state conditions: at elevated (cytoplasmic) p0071 levels, its recycling occurs preferentially through the slow Rab11-dependent pathway. This slow transport would result in a slight decrease of AJ-associated p0071 and thus prevent the modulation of AJs upon minor changes in p0071 levels. Upon reduction of cytoplasmic p0071 levels, this mechanism would ensure that sufficient p0071 localizes at the membrane by accelerating its return transport. The same mechanism could also function to regulate AJ plasticity. Increased translocation of p0071 to the cytoplasm by, presently unknown, post-translational modifications would shift recycling to the slow pathway, thereby facilitating AJ reorganization without the loss of intercellular adhesion.

MATERIALS AND METHODS
Antibodies
p0071-directed antibodies have been described previously (Wolf et al., 2006). The following commercially available antibodies were used: rabbit anti-Rab4a, mouse anti-MyoVb, rabbit anti-KIF3B (SCBT), rabbit anti-GFP (Rockland), mouse anti-His (Qiagen), mouse anti-FLAG, mouse anti-α-tubulin, rabbit anti-γ-tubulin (Sigma-Aldrich), rabbit anti-HA (Rockland), mouse anti-Rab11 (detects Rab11a and Rab11b), mouse

anti-p120^{ctn}, mouse anti-EEA1, mouse anti-β-catenin, mouse anti-E-cadherin (BD), mouse anti-TfnR (Invitrogen), rat anti-α-tubulin (Millipore).

Secondary antibodies were donkey anti-mouse and anti-guinea-pig conjugated to horseradish peroxidase and donkey anti-mouse, anti-rabbit, anti-guinea pig and anti-rat conjugated to DL488, Cy3 and DL649 (Rockland).

cDNA constructs
p0071 constructs have been described previously (Hatzfeld et al., 2003; Keil et al., 2009). p0071 head (amino acids 1–509), p0071 arm (510–988) and p0071 tail (989–1149) were subcloned into pRSET (Invitrogen). p0071-WT (1–1149) was subcloned into pV1-FLAG. The Rab11a-WT was amplified from HeLa cDNA and subcloned into pEGFP-C2 (BD), pGex 5.1 (GE Healthcare), pFLAG-C2 and pV2-HA. Rab11a mutants were generated by site-directed mutagenesis to replace Q70 with L (DA) or S25 with N (DN), respectively. The BiFC vectors have been previously described (Wolf et al., 2010). pFLAG-C2 was derived from pEGFP-C2 by replacing EGFP with the FLAG tag.

Cell culture, transfection, RNA interference and cell treatment
MCF-7, HeLa and HEK293 cells were grown in DMEM containing 10% FCS. Plasmids and siRNAs were transfected using Lipofectamine 2000 (Invitrogen). siRNAs (supplementary material Table S1) were purchased from MWG or Sigma-Aldrich. Knockdown efficiencies were determined by western blotting.

Cells were incubated in medium containing 3 µg/ml nocodazole (Sigma-Aldrich) or 20 µM Y27632 (Millipore) for 2 hours at 37℃ and subsequently processed as indicated. For the analyses of AJ reassembly, cells were treated for 60 minutes with 4 mM EGTA.

Fluorescence microscopy
For fluorescence microscopy cells were seeded on coverslips, treated as indicated, fixed for 10 minutes in 3.7% formaldehyde at room temperature (RT) or in methanol at −20℃ and permeabilized in 0.5% Triton X-100 in PBS for 15 minutes at RT. Cells fixed in 10% trichloroacetic acid (TCA; 15 minutes on ice) were permeabilized for 5 minutes in 0.5% Triton X-100 in PBS at RT. After blocking in 1% dry-milk in PBS, specimens were stained with the appropriate antibodies.

All images were taken using a Zeiss Axio Observer.Z1 microscope equipped with a CCD camera (AxioCam MRm Rev. 3) and either a Plan-Apochromat 63× 1.40 NA oil DIC or the Plan-Apochromat 20× 0.8 NA objective, controlled with the Zeiss ApoTome and the AxioVision Rel. 4.7 software. ImageJ was used for image processing.

Quantification of Rab11 mislocalization
HeLa cells were transfected with the indicated siRNAs. 48 hours after transfection cells were either directly immunostained or treated as stated and then immunostained for Rab11. At least 600 cells in three independent experiments were counted using a Zeiss Axio Observer.Z1 microscope equipped with a Plan-Neofluar 40× 1.30 NA oil objective.

GST pulldown, FLAG immunoprecipitation and BiFC
GST, GST-Rab11a-WT, -DA, -DN and His-p0071-head, -arm, -tail were expressed in *Escherichia coli* ArcticExpress (DE3) RIL (Agilent Technologies) according to the manufacture's protocol. GST pulldown and FLAG immunoprecipitation were performed as previously described (Keil et al., 2009).

For BiFC analyses HeLa or MCF-7 cells were transfected with the indicated constructs. 12 hours after transfection cells were fixed using formaldehyde and immunostained with FLAG- and HA-tag-directed antibodies. Images of cells expressing both FLAG- and HA-tagged fusion proteins were taken with equal exposure times (500 mseconds) to enable a comparison of BiFC efficiencies.

Co-immunoprecipitation
HeLa cells were treated with 4 mM EGTA for 60 minutes and subsequently lysed in IP buffer containing 20 mM Tris-HCl (pH 8.0),

137 mM NaCl, 1% NP40 and 2 mM EDTA supplemented with 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM Pefabloc, and 5 mM NaF. p0071 was precipitated using a guinea-pig-derived anti-p0071 antiserum (PSL). The preimmune serum of the same animal served as a specificity control. The precipitates were analyzed by western blotting for the presence of Rab11. As secondary antibody for the detection of immunoprecipitated endogenous Rab11, we used mouse-IgG-TrueBlot® ULTRA (Rockland).

Analyses of Tfn recycling
Analyses of Tfn recycling were performed essentially as described (Altschuler et al., 1998; Irie et al., 2005). Briefly, siRNA-transfected HeLa cells were serum starved for 60 minutes and incubated with 30 µg/ml Alexa-Fluor-555–Tfn (Invitrogen) for 45 minutes on ice. After washing, cells were either fixed in formaldehyde and processed for microscopy or incubated in normal medium at 37℃ for 30 minutes before fixing and processing.

For quantification of TfnR recycling siRNA-transfected HeLa cells were serum starved for 60 minutes and subsequently incubated with 30 µg/ml Biotin-Tfn (Sigma-Aldrich) for 45 minutes at 37℃ in serum-free medium. After washing, cells were incubated in normal medium containing 200 µg/ml unconjugated transferrin for the indicated time. To strip unincorporated transferrin from the cell surface, cells were treated prior to lysis with ice-cold acid solution (0.2 M acetic acid, 0.5 M NaCl) for 10 minutes on ice and then washed with PBS. Residual intracellular transferrin was detected by western blotting with HRP-conjugated avidin (Sigma) and subjected to densitometric quantification.

Surface biotinylation
To analyze surface expression of TfnR, 48 hours after siRNA transfection HeLa cells were incubated with 5 mM Sulfo-NHS-Biotin (Thermo Scientific) for 45 minutes on ice. Subsequently cell were washed three times with ice-cold PBS containing 100 mM glycine and lysed in avidin binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate supplemented with 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM Pefabloc and 5 mM NaF). Biotinylated proteins were precipitated using avidin agarose (Thermo Scientific). The amount of biotinylated and precipitated TfnR was analyzed by western blotting.

Dispase assay
Dispase assays were performed as described previously (Hudson et al., 2004). Epithelial sheets of siRNA-transfected HeLa cells were detached using 4 U/ml dispase in normal medium for 30 minutes at 37℃. Released monolayers were subjected to mechanical stress using an orbital shaker at 600 r.p.m. Images were taken using a Sony Alpha57 camera equipped with a SAL-18135 DT 18–135 mm f/3.5–5.6 objective. Photoshop CS5 and ImageJ were used for image processing.

Statistical analyses
To determine the statistical significance of the quantification of Rab11 mislocalization, p0071 expression and Biotin-Tfn recycling unpaired Student's *t*-tests were performed.

Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.132266/-/DC1

References

Agola, J. O., Jim, P. A., Ward, H. H., Basuray, S. and Wandinger-Ness, A. (2011). Rab GTPases as regulators of endocytosis, targets of disease and therapeutic opportunities. *Clin. Genet.* **80**, 305-318.

Aisen, P. (2004). Transferrin receptor 1. *Int. J. Biochem. Cell Biol.* **36**, 2137-2143.

Altschuler, Y., Barbas, S. M., Terlecky, L. J., Tang, K., Hardy, S., Mostov, K. E. and Schmid, S. L. (1998). Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms. *J. Cell Biol.* **143**, 1871-1881.

Anastasiadis, P. Z. (2007). p120-ctn: A nexus for contextual signaling via Rho GTPases. *Biochim. Biophys. Acta* **1773**, 34-46.

Barr, F. and Lambright, D. G. (2010). Rab GEFs and GAPs. *Curr. Opin. Cell Biol.* **22**, 461-470.

Bryant, D. M., Kerr, M. C., Hammond, L. A., Joseph, S. R., Mostov, K. E., Teasdale, R. D. and Stow, J. L. (2007). EGF induces macropinocytosis and SNX1-modulated recycling of E-cadherin. *J. Cell Sci.* **120**, 1818-1828.

Carnahan, R. H., Rokas, A., Gaucher, E. A. and Reynolds, A. B. (2010). The molecular evolution of the p120-catenin subfamily and its functional associations. *PLoS ONE* **5**, e15747.

Casanova, J. E., Wang, X., Kumar, R., Bhartur, S. G., Navarre, J., Woodrum, J. E., Altschuler, Y., Ray, G. S. and Goldenring, J. R. (1999). Association of Rab25 and Rab11a with the apical recycling system of polarized Madin-Darby canine kidney cells. *Mol. Biol. Cell* **10**, 47-61.

Dabbeekeh, J. T., Faitar, S. L., Dufresne, C. P. and Cowell, J. K. (2007). The EVI5 TBC domain provides the GTPase-activating protein motif for RAB11. *Oncogene* **26**, 2804-2808.

Daro, E., van der Sluijs, P., Galli, T. and Mellman, I. (1996). Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling. *Proc. Natl. Acad. Sci. USA* **93**, 9559-9564.

de Curtis, I. and Meldolesi, J. (2012). Cell surface dynamics - how Rho GTPases orchestrate the interplay between the plasma membrane and the cortical cytoskeleton. *J. Cell Sci.* **125**, 4435-4444.

Delva, E. and Kowalczyk, A. P. (2009). Regulation of cadherin trafficking. *Traffic* **10**, 259-267.

Desclozeaux, M., Venturato, J., Wylie, F. G., Kay, J. G., Joseph, S. R., Le, H. T. and Stow, J. L. (2008). Active Rab11 and functional recycling endosome are required for E-cadherin trafficking and lumen formation during epithelial morphogenesis. *Am. J. Physiol.* **295**, C545-C556.

Eggers, C. T., Schafer, J. C., Goldenring, J. R. and Taylor, S. S. (2009). D-AKAP2 interacts with Rab4 and Rab11 through its RGS domains and regulates transferrin receptor recycling. *J. Biol. Chem.* **284**, 32869-32880.

Grant, B. D. and Donaldson, J. G. (2009). Pathways and mechanisms of endocytic recycling. *Nat. Rev. Mol. Cell Biol.* **10**, 597-608.

Hales, C. M., Griner, R., Hobdy-Henderson, K. C., Dorn, M. C., Hardy, D., Kumar, R., Navarre, J., Chan, E. K., Lapierre, L. A. and Goldenring, J. R. (2001). Identification and characterization of a family of Rab11-interacting proteins. *J. Biol. Chem.* **276**, 39067-39075.

Hales, C. M., Vaerman, J. P. and Goldenring, J. R. (2002). Rab11 family interacting protein 2 associates with Myosin Vb and regulates plasma membrane recycling. *J. Biol. Chem.* **277**, 50415-50421.

Harris, K. P. and Tepass, U. (2010). Cdc42 and vesicle trafficking in polarized cells. *Traffic* **11**, 1272-1279.

Hatzfeld, M. (2005). The p120 family of cell adhesion molecules. *Eur. J. Cell Biol.* **84**, 205-214.

Hatzfeld, M., Green, K. J. and Sauter, H. (2003). Targeting of p0071 to desmosomes and adherens junctions is mediated by different protein domains. *J. Cell Sci.* **116**, 1219-1233.

Heasman, S. J. and Ridley, A. J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat. Rev. Mol. Cell Biol.* **9**, 690-701.

Hehnly, H., Chen, C. T., Powers, C. M., Liu, H. L. and Doxsey, S. (2012). The centrosome regulates the Rab11- dependent recycling endosome pathway at appendages of the mother centriole. *Curr. Biol.* **22**, 1944-1950.

Horgan, C. P. and McCaffrey, M. W. (2009). The dynamic Rab11-FIPs. *Biochem. Soc. Trans.* **37**, 1032-1036.

Horgan, C. P., Walsh, M., Zurawski, T. H. and McCaffrey, M. W. (2004). Rab11-FIP3 localises to a Rab11-positive pericentrosomal compartment during interphase and to the cleavage furrow during cytokinesis. *Biochem. Biophys. Res. Commun.* **319**, 83-94.

Horgan, C. P., Hanscom, S. R., Jolly, R. S., Futter, C. E. and McCaffrey, M. W. (2010). Rab11-FIP3 links the Rab11 GTPase and cytoplasmic dynein to mediate transport to the endosomal-recycling compartment. *J. Cell Sci.* **123**, 181-191.

Hudson, T. Y., Fontao, L., Godsel, L. M., Choi, H. J., Huen, A. C., Borradori, L., Weis, W. I. and Green, K. J. (2004). In vitro methods for investigating desmoplakin-intermediate filament interactions and their role in adhesive strength. *Methods Cell Biol.* **78**, 757-786.

Irie, F., Okuno, M., Pasquale, E. B. and Yamaguchi, Y. (2005). EphrinB-EphB signalling regulates clathrin-mediated endocytosis through tyrosine phosphorylation of synaptojanin 1. *Nat. Cell Biol.* **7**, 501-509.

Keil, R., Wolf, A., Hüttelmaier, S. and Hatzfeld, M. (2007). Beyond regulation of cell adhesion: local control of RhoA at the cleavage furrow by the p0071 catenin. *Cell Cycle* **6**, 122-127.

Keil, R., Kiessling, C. and Hatzfeld, M. (2009). Targeting of p0071 to the midbody depends on KIF3. *J. Cell Sci.* **122**, 1174-1183.

Keil, R., Schulz, J. and Hatzfeld, M. (2013). p0071/PKP4, a multifunctional protein coordinating cell adhesion with cytoskeletal organization. *Biol. Chem.* **394**, 1005-1017.

Kelly, E. E., Horgan, C. P. and McCaffrey, M. W. (2012). Rab11 proteins in health and disease. *Biochem. Soc. Trans.* **40**, 1360-1367.

Kowalczyk, A. P. and Reynolds, A. B. (2004). Protecting your tail: regulation of cadherin degradation by p120-catenin. *Curr. Opin. Cell Biol.* **16**, 522-527.

Langevin, J., Morgan, M. J., Sibarita, J. B., Aresta, S., Murthy, M., Schwarz, T., Camonis, J. and Bellaïche, Y. (2005). Drosophila exocyst components Sec5, Sec6, and Sec15 regulate DE-Cadherin trafficking from recycling endosomes to the plasma membrane. *Dev. Cell* **9**, 365-376.

Lanzetti, L. (2007). Actin in membrane trafficking. *Curr. Opin. Cell Biol.* **19**, 453-458.

Lapierre, L. A., Kumar, R., Hales, C. M., Navarre, J., Bhartur, S. G., Burnette, J. O., Provance, D. W., Jr, Mercer, J. A., Bähler, M. and Goldenring, J. R. (2001). Myosin vb is associated with plasma membrane recycling systems. *Mol. Biol. Cell* **12**, 1843-1857.

Li, X. and DiFiglia, M. (2012). The recycling endosome and its role in neurological disorders. *Prog. Neurobiol.* **97**, 127-141.

Lindsay, A. J., Hendrick, A. G., Cantalupo, G., Senic-Matuglia, F., Goud, B., Bucci, C. and McCaffrey, M. W. (2002). Rab coupling protein (RCP), a novel Rab4 and Rab11 effector protein. *J. Biol. Chem.* **277**, 12190-12199.

Linford, A., Yoshimura, S., Nunes Bastos, R., Langemeyer, L., Gerondopoulos, A., Rigden, D. J. and Barr, F. A. (2012). Rab14 and its exchange factor FAM116 link endocytic recycling and adherens junction stability in migrating cells. *Dev. Cell* **22**, 952-966.

Lock, J. G. and Stow, J. L. (2005). Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin. *Mol. Biol. Cell* **16**, 1744-1755.

Maxfield, F. R. and McGraw, T. E. (2004). Endocytic recycling. *Nat. Rev. Mol. Cell Biol.* **5**, 121-132.

Menke, A. and Giehl, K. (2012). Regulation of adherens junctions by Rho GTPases and p120-catenin. *Arch. Biochem. Biophys.* **524**, 48-55.

Narumiya, S., Ishizaki, T. and Uehata, M. (2000). Use and properties of ROCK-specific inhibitor Y-27632. *Methods Enzymol.* **325**, 273-284.

Paterson, A. D., Parton, R. G., Ferguson, C., Stow, J. L. and Yap, A. S. (2003). Characterization of E-cadherin endocytosis in isolated MCF-7 and chinese hamster ovary cells: the initial fate of unbound E-cadherin. *J. Biol. Chem.* **278**, 21050-21057.

Powelka, A. M., Sun, J., Li, J., Gao, M., Shaw, L. M., Sonnenberg, A. and Hsu, V. W. (2004). Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. *Traffic* **5**, 20-36.

Provance, D. W., Jr, Gourley, C. R., Silan, C. M., Cameron, L. C., Shokat, K. M., Goldenring, J. R., Shah, K., Gillespie, P. G. and Mercer, J. A. (2004). Chemical-genetic inhibition of a sensitized mutant myosin Vb demonstrates a role in peripheral-pericentriolar membrane traffic. *Proc. Natl. Acad. Sci. USA* **101**, 1868-1873.

Ren, M., Xu, G., Zeng, J., De Lemos-Chiarandini, C., Adesnik, M. and Sabatini, D. D. (1998). Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosomes. *Proc. Natl. Acad. Sci. USA* **95**, 6187-6192.

Sakurada, K., Uchida, K., Yamaguchi, K., Aisaka, K., Ito, S., Ohmori, T., Takeyama, Y., Ueda, T., Hori, Y., Ohyanagi, H. et al. (1991). Molecular cloning and characterization of a ras p21-like GTP-binding protein (24KG) from rat liver. *Biochem. Biophys. Res. Commun.* **177**, 1224-1232.

Schonteich, E., Wilson, G. M., Burden, J., Hopkins, C. R., Anderson, K., Goldenring, J. R. and Prekeris, R. (2008). The Rip11/Rab11-FIP5 and kinesin II complex regulates endocytic protein recycling. *J. Cell Sci.* **121**, 3824-3833.

Sönnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J. and Zerial, M. (2000). Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J. Cell Biol.* **149**, 901-914.

Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. *Nat. Rev. Mol. Cell Biol.* **10**, 513-525.

Street, C. A. and Bryan, B. A. (2011). Rho kinase proteins—pleiotropic modulators of cell survival and apoptosis. *Anticancer Res.* **31**, 3645-3657.

Takahashi, S., Kubo, K., Waguri, S., Yabashi, A., Shin, H. W., Katoh, Y. and Nakayama, K. (2012). Rab11 regulates exocytosis of recycling vesicles at the plasma membrane. *J. Cell Sci.* **125**, 4049-4057.

Ullrich, O., Reinsch, S., Urbé, S., Zerial, M. and Parton, R. G. (1996). Rab11 regulates recycling through the pericentriolar recycling endosome. *J. Cell Biol.* **135**, 913-924.

Wallace, D. M., Lindsay, A. J., Hendrick, A. G. and McCaffrey, M. W. (2002a). The novel Rab11-FIP/Rip/RCP family of proteins displays extensive homo- and hetero-interacting abilities. *Biochem. Biophys. Res. Commun.* **292**, 909-915.

Wallace, D. M., Lindsay, A. J., Hendrick, A. G. and McCaffrey, M. W. (2002b). Rab11-FIP4 interacts with Rab11 in a GTP-dependent manner and its overexpression condenses the Rab11 positive compartment in HeLa cells. *Biochem. Biophys. Res. Commun.* **299**, 770-779.

Wolf, A., Keil, R., Götzl, O., Mun, A., Schwarze, K., Lederer, M., Hüttelmaier, S. and Hatzfeld, M. (2006). The armadillo protein p0071 regulates Rho signalling during cytokinesis. *Nat. Cell Biol.* **8**, 1432-1440.

Wolf, A., Krause-Gruszczynska, M., Birkenmeier, O., Ostareck-Lederer, A., Hüttelmaier, S. and Hatzfeld, M. (2010). Plakophilin 1 stimulates translation by promoting eIF4A1 activity. *J. Cell Biol.* **188**, 463-471.

Yoshimura, S., Gerondopoulos, A., Linford, A., Rigden, D. J. and Barr, F. A. (2010). Family-wide characterization of the DENN domain Rab GDP-GTP exchange factors. *J. Cell Biol.* **191**, 367-381.