Rab35 regulates cadherin-mediated adherens junction formation and myoblast fusion

Sophie Charrasse\(^a\), Franck Comunale\(^a\), Sylvain De Rossi\(^b\), Arnaud Echard\(^c\), and Cécile Gauthier-Rouvière\(^a\)

\(^a\)Centre de Recherche de Biochimie Macromoléculaire, Universités Montpellier 2 et 1, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 5237, 34293 Montpellier, France; \(^b\)Montpellier RIO Imaging, BioCampus Montpellier, 34094 Montpellier, France; \(^c\)Membrane Traffic and Cell Division Laboratory, Institut Pasteur, Centre National de la Recherche Scientifique, URA2582, 75015 Paris, France

ABSTRACT Cadherins are homophilic cell–cell adhesion molecules implicated in many fundamental processes, such as morphogenesis, cell growth, and differentiation. They accumulate at cell–cell contact sites and assemble into large macromolecular complexes named adherens junctions (AJs). Cadherin targeting and function are regulated by various cellular processes, many players of which remain to be uncovered. Here we identify the small GTPase Rab35 as a new regulator of cadherin trafficking and stabilization at cell–cell contacts in C2C12 myoblasts and HeLa cells. We find that Rab35 accumulates at cell–cell contacts in a cadherin-dependent manner. Knockdown of Rab35 or expression of a dominant-negative form of Rab35 impaired N- and M-cadherin recruitment to cell–cell contacts, their stabilization at the plasma membrane, and association with p120 catenin and led to their accumulation in transferrin-, clathrin-, and AP-2–positive intracellular vesicles. We also find that Rab35 function is required for PIP5KI\(_γ\) accumulation at cell–cell contacts and phosphatidylinositol 4,5-bisphosphate production, which is involved in cadherin stabilization at contact sites. Finally, we show that Rab35 regulates myoblast fusion, a major cellular process under the control of cadherin-dependent signaling. Taken together, these results reveal that Rab35 regulates cadherin-dependent AJ formation and myoblast fusion.

INTRODUCTION

Cadherins are highly conserved transmembrane receptors that mediate calcium-dependent cell–cell adhesion and form adherens junctions. They play essential roles during embryonic development by regulating cell differentiation, growth, and migration and in the maintenance of tissue architecture in adult life (Takeichi, 1995; Halbleib and Nelson, 2006; Harris and Tepass, 2011). Perturbation of cadherin function is associated with cancer invasion and metastasis (Christofori, 2003). Cadherins mediate homotypic cell–cell adhesion through their extracellular domain (Troyanovsky, 2005), whereas their cytoplasmic domains interact with a range of proteins that link cadherins to the cytoskeleton and to cell signaling pathways (Kemler, 1993; Perez-Moreno et al., 2003).

Formation of cell–cell contacts is a multistep process that includes cadherin association with catenins, delivery of cadherin–catenin complexes to the plasma membrane (PM), lateral diffusion in the PM toward cell–cell contact sites, cadherin oligomerization, homotypic interactions, and association of cadherin complexes with their intracellular partners and the actin cytoskeleton. Moreover, cadherin-based cell–cell contacts are dynamic adhesive structures, and the trafficking and turnover of cadherins to and from the PM play an important role in this dynamic behavior (Yap et al., 2007; Schill and Anderson, 2009; Baum and Georgiou, 2011). Much attention has been focused on the role of the Rho and Arf families of small GTPases in cadherin-dependent adhesion (Braga et al., 2000; Fukata and Kaibuchi, 2001; Palacios et al., 2001; Lozano et al., 2003), whereas the implication of the Rab family of small GTPases that includes 60 proteins is much less known. Rab GTPases define specific trafficking routes within the secretory and endocytic pathways by controlling several transport steps, such as vesicle formation, motility, docking, and fusion (Zerial and McBride, 2000; Fukata and Kaibuchi, 2001; Palacios et al., 2001; Lozano et al., 2003).
In 2001; Stenmark, 2009). In particular, Rab11 has been involved in E-cadherin recycling and apical membrane formation in mammals and Drosophila (Desclouzeaux et al., 2008; Roeth et al., 2009), Rab5 and Rab7 in lysosomal targeting of E-cadherin in Src-induced epithelial-to-mesenchymal transition (Palacios et al., 2005), and Rab5 and Rab11 in N-cadherin trafficking during neuronal migration (Kawauchi et al., 2010). To determine whether some Rab family members might play a role in cadherin-dependent adhesion, we analyzed their localization and found that Rab35 strongly accumulated at cell–cell contacts. Rab35 is ubiquitously expressed and localizes at the PM and in endocytic compartments and controls a fast endocytic recycling pathway (Kouranti et al., 2006; Patino-Lopez et al., 2008). Rab35 has also been involved in cytokinesis, phagocytosis, and neurite outgrowth (Kouranti et al., 2006; Chevallier et al., 2009; Dambournet et al., 2011; Egami et al., 2011; Kobayashi and Fukuda, 2012). Moreover, several types of cargo, such as T-cell and major histocompatibility class I (MHC)I receptors, KCa2.3 Ca2+-activated K+ channels, and the oocyte receptor in Caenorhabditis elegans, require Rab35 for their recycling (Patino-Lopez et al., 2008; Allaire et al., 2010; Gao et al., 2010). In addition to its role in the regulation of membrane trafficking, Rab35 modulates actin organization directly through its effectors, by controlling Rac1 and Cdc42 localization at the PM, or through Arf6 (Zhang et al., 2009; Shim et al., 2010; Dambournet et al., 2011; Egami et al., 2011; Kobayashi and Fukuda, 2012).

Here we show that Rab35 is recruited to cell–cell contacts in a cadherin-dependent manner. Rab35 knockdown dramatically affects N-, M-, and E-cadherin recruitment to cell–cell contacts and the PM and leads to accumulation of cadherins in intracellular vesicles in both myoblasts and HEla cells. Absence of Rab35 activity decreases the accumulation of phosphatidyl inositol 4,5-bisphosphate (PI(4,5)P2) and PIP5Kα at cell–cell contacts, a change that also participates in the loss of cadherins at these sites. We thus identify Rab35 as a new regulator of adherens junction (AJ) formation.

RESULTS

Rab35 localizes at cell–cell contacts and associates with cadherin complexes

To investigate the possible involvement of Rab family members in cadherin-dependent adhesion, we expressed wild-type Rab4, Rab5, Rab7, Rab8, Rab11, and Rab35 fused to green fluorescent protein (GFP) in C2C12 mouse myoblasts and HEla cells and then monitored their localization and that of N- and M-cadherin. In both cell lines, only Rab35 accumulated at cell–cell contact sites, where it colocalized with N- and M-cadherin (Figure 1, A and B, for myoblasts; Supplemental Figure S1, A and B, for HEla cells).

Moreover, cadherins triggered Rab35 recruitment to cell–cell contact sites. Indeed, in mouse L cells, which do not express endogenous cadherins, Rab35 did not accumulate at cell contacts. Conversely, upon expression of exogenous N-, M-, or E-cadherin, Rab35 was recruited to cell contacts, where it colocalized with the expressed cadherin (Figure 1C). This is specific for Rab35, because none of the other tested Rab family members (Rab4, Rab5, Rab7, and Rab11) was recruited to cell–cell contacts in a cadherin-dependent manner (Supplemental Figure S1C).

Finally, in immunoprecipitation experiments using anti-N- or M-cadherin antibodies and whole-cell extracts of C2C12 myoblasts and HEla cells that express wild-type Rab35 (Rab35WT) fused to GFP, Rab35 was immunoprecipitated together with endogenous N-cadherin (Figure 1D, a and c) or M-cadherin (Figure 1Db), as revealed by Western blot analysis. Similarly, endogenous Rab35 was immunoprecipitated together with M-cadherin in C2C12 myoblasts (Supplemental Figure S1D). Rab35 was not found in the M-cadherin complex, whereas the Rab35 effector fascin was.

These findings show that Rab35 is complexed with N- and M-cadherin and colocalizes with these cadherins at cell contact sites. They also indicate that Rab35 recruitment at cell–cell contacts is induced by cadherins.

Rab35 is involved in AJ formation

To get insights into Rab35 function at cell–cell contact sites, we generated by retroviral infection stable C2C12 cell lines in which Rab35 expression was inactivated by RNA interference. Two Rab35 short hairpin RNAs (shRNAs; Rab35 shRNA1 and Rab35 shRNA2) were used to generate a pool (Figure 2A) and various cell clones that were selected using a resistance marker (Supplemental Figure S2, A and B). As controls, parental and C2C12 myoblasts expressing a luciferase shRNA (Luc shRNA) were used (Fortier et al., 2008). We first investigated whether Rab35 inactivation influenced N- and M-cadherin localization. In parental and Luc shRNA C2C12 myoblasts, N- and M-cadherin accumulated at cell–cell contacts (Figure 2B, a and b and g and h, respectively; arrows). In contrast, their accumulation at cell–cell contacts was perturbed in Rab35 shRNA C2C12 myoblasts (Figure 2B, c–f and i–l, arrowheads), whereas their intracellular localization increased (Figure 3). We confirmed this result by expressing a dominant-negative mutant of Rab35 (Rab35S22N) in C2C12 myoblasts (Supplemental Figure S2C). Again, N- and M-cadherin localization at cell–cell contacts was disrupted in 60% of C2C12 and HeLa cells expressing Rab35S22N (Supplemental Figures S2C and S3 and Supplemental Videos S1–S4). Moreover, cadherins accumulated in intracellular vacuoles (asterisks in Supplemental Figure S2C and Supplemental Figures S4 and S5) that were induced by Rab35S22N expression, as previously shown for other cargoes (Kouranti et al., 2006; Patino-Lopez et al., 2008; Chesneau et al., 2012). Thus cadherin localization at cell–cell contacts was perturbed in Rab35-knockdown cells and upon expression of Rab35S22N.

Of importance, we did not detect any significant difference in the total expression levels of N- and M-cadherin in cells expressing Rab35 shRNA1 and 2 in comparison to parental and control Luci shRNA C2C12 myoblasts (Figure 2C). To further quantify N- and M-cadherin levels at the cell surface of Luci shRNA and Rab35 shRNA C2C12 myoblasts, we used a cell surface biotinylation assay (Figure 2D). Quantification of the results showed that Rab35 knockdown decreased the amount of both N- and M-cadherin at the PM. Because cadherin stability at cell–cell contacts is dependent on the binding of p120 catenin (Davis et al., 2003; Xiao et al., 2003), we then analyzed whether Rab35 silencing decreased p120 catenin association with N- and M-cadherin. Although the level of p120 catenin was not modified in Rab35 shRNA C2C12 myoblasts (Figure 2Ea), p120 catenin association with N-cadherin (Figure 2Eb) and M-cadherin (Figure 2Ec) was significantly decreased. In contrast, the association of β-catenin with M-cadherin was not modified in Rab35 shRNA C2C12 myoblasts.

Moreover, the reduced accumulation of cadherins at the PM of Rab35 shRNA C2C12 myoblasts was accompanied by increased N- and M-cadherin accumulation in transferrin-labeled recycling compartments (Figure 3A). Similar results were obtained upon Rab35S22N expression (Supplemental Figure S5). Accumulation of N- and M-cadherin in transferrin-labeled recycling compartments was even higher when cell–cell contact disruption induced by Rab35S22N was enhanced by ethylene glycol tetraacetic acid (EGTA) treatment (Supplemental Figure S5). As previously described (Rahajeng et al., 2012), functional inhibition of Rab35 led to...
FIGURE 1: Rab35 colocalizes and is complexed with N- and M-cadherin at cell–cell contacts. (A, B) C2C12 myoblasts were transfected with GFP-tagged Rab4, Rab5, Rab7, Rab8, Rab11, and Rab35, stained for N-cadherin (A) or M-cadherin (B) expression, and analyzed by confocal microscopy. Arrows show colocalization of cadherins and GFP-Rab35 at cell contact sites. Quantification of the two signals was performed along the white line shown in the “merge” panels by line scan (MetaMorph software). Bar, 10 μm. (C) Mouse L cells that express plasmids encoding either RFP-Rab35WT alone or with N-, M-, or E-cadherin/GFP. Arrows show cadherins and Rab35 accumulation at cell–cell contacts. Bar, 10 μm. (D) Cell lysates from control and GFP-Rab35WT-transfected C2C12 myoblasts (a, b) and HeLa cells (c) were immunoprecipitated using anti-N- or M-cadherin (+) or an irrelevant (−) antibodies and immunoblotted to assess the presence of cadherins and GFP-Rab35.
**FIGURE 2:** Rab35 is required for N- and M-cadherin accumulation at cell–cell contacts. (A) Cell lysates (30 μg) of control Luci shRNA and Rab35 shRNA C2C12 myoblasts were assessed by Western blot analysis for expression of Rab35 and α-tubulin. (B) N- and M-cadherin localization was analyzed by indirect immunofluorescence in parental and Luci shRNA (controls; a and b, and g and h), Rab35 shRNA1 C2C12 myoblasts (c and d, and i and j) and Rab35 shRNA2 C2C12 myoblasts (e and f, and k and l). Arrows show cadherin accumulation at cell contact sites, and arrowheads show absence of cadherin accumulation at cell contact sites. Bar, 10 μm. (C) Cell lysates (30 μg) of parental, Luci shRNA, and Rab35 shRNA C2C12 myoblasts cultured in growth medium (GM) were assessed by Western blot analysis for expression of N-cadherin, M-cadherin, tubulin, and Rab35. (D) Luci shRNA (control) and Rab35 shRNA C2C12 myoblasts were cell surface biotinylated at 4°C. Biotinylated N- and M-cadherin were recovered onto streptavidin beads. The N- and M-cadherin contents in total and biotinylated fractions were analyzed by immunoblotting. The histogram represents the quantification of biotinylated cadherins at the PM normalized to the total amount of cadherins calculated from at least three independent experiments. (E) a, Expression of N- and M-cadherin, p120 and β-catenin, α-tubulin, and Rab35 was analyzed by Western blotting in cell lysates from parental (control) and Rab35 shRNA C2C12 myoblasts. b, c, Cell lysates of control and Rab35 shRNA C2C12 myoblasts were immunoprecipitated using anti-N-cadherin (b) or anti-M-cadherin (c) antibodies and probed for the presence of p120 and β-catenin. (∼) Control immunoprecipitations were performed using irrelevant immunoglobulins G.
accumulation of transferrin in the perinuclear region (Figure 3A, d and j). These N- or M-cadherin–containing endocytic vesicles were positive for AP-2, clathrin, and to a lesser extent Rab5 (Figure 3B) but not for other endocytic markers, such as EEA1, Rab4, and Rab11 (unpublished data). Accumulation of N- or M-cadherin in AP-2–, clathrin–, and Rab5-positive vesicles was higher in C2C12 myoblasts that express Rab35S22N than in cells expressing Rab35WT (Figure 3B).

Taken together, these data show that Rab35 silencing does not affect the total expression level of N- and M-cadherin but perturbs their localization at the PM and their stabilization at cell–cell contacts.

Rab35 controls PI(4,5)P2 accumulation at cell–cell contact sites

We previously demonstrated PI(4,5)P2 accumulation and colocalization with N- and M-cadherin at cell contacts in C2C12 myoblasts (Taulet et al., 2009; Bach et al., 2010). Given that Rab35 regulates the localization of PI(4,5)P2 at the PM (Kouranti et al., 2006), we then assessed whether Rab35 knockdown affected its accumulation and colocalization with N-cadherin in C2C12 myoblasts. To this aim, we transfected parental and Rab35 shRNA C2C12 myoblasts with a construct that expresses the PH domain of PLCδ fused to GFP (PH-PLCδ-GFP) that specifically binds to PI(4,5)P2 (Varnai and Balla, 1998). PI(4,5)P2 accumulated at the PM and at cell–cell contacts in control cells (Figure 4Aa, arrow) but not in Rab35 shRNA C2C12 myoblasts (Figure 4Ac, arrowhead). To confirm that Rab35 regulates PI(4,5)P2 accumulation at cell contacts, we analyzed the localization of PH-PLCδ-GFP during cell–cell contact reformation upon Ca2+ recovery after chelation with EGTA. Rab35 knockdown affected PI(4,5)P2 accumulation at cell–cell contacts also after Ca2+ recovery (Figure 4A, compare b and d). Specifically, whereas 70% of cell–cell contacts showed PI(4,5)P2 enrichment in control cells, this dropped to 30% in Rab35 shRNA C2C12 myoblasts. Expression of the Rab35 dominant-negative mutant Rab35S22N in C2C12 myoblasts also reduced PI(4,5)P2 accumulation at cell–cell contacts (Figure 4B). In contrast, no perturbation of PI(4,5)P2 localization at cell–cell contacts was observed after expression of Rab35WT or Rab35Q67L, a constitutively active form of Rab35. Moreover, both Rab35 isoforms colocalized with PI(4,5)P2 at cell–cell contacts (Figure 4B). We also analyzed PI(4,5)P2 levels at cell–cell contacts using an antibody that specifically detects this phosphoinositide. To perform a more
accurate quantification of PI(4,5)P$_2$ at cell–cell contacts, we expressed red fluorescent protein (RFP) fused to a CAAX sequence that addresses RFP to the PM to normalize the signal given by the anti-PI(4,5)P$_2$ antibody. Line-scan analysis confirmed the decrease of PI(4,5)P$_2$ expression at cell–cell contacts in Rab35-depleted cells (representative line scan shown in Figure 4C).

These data show that Rab35 is required for PI(4,5)P$_2$ accumulation at cell–cell contacts.

PI(4,5)P$_2$ is required for cadherin accumulation at cell–cell contacts

PI(4,5)P$_2$, N- and M-cadherin (Taulet et al., 2009; Bach et al., 2010), and Rab35 (this study) accumulate at cell contacts in C2C12 myoblasts. Moreover, recruitment and activation of PIP5K$_\gamma$ at sites of N-cadherin ligation, resulting in PI(4,5)P$_2$ production, have been reported (El Sayegh et al., 2007). We thus asked whether PI(4,5)P$_2$ was required for N- and M-cadherin accumulation at cell–cell contacts. To decrease PI(4,5)P$_2$ level in cultured myoblasts, cells were treated with agents that reduce (calcimycin, LiCl) or mask (neomycin) PI(4,5)P$_2$ (Griffin and Hawthorne, 1978; Hallcher and Sherman, 1980; Gabev et al., 1989; Laux et al., 2000). Whereas in control cells, N- and M-cadherin accumulated at cell–cell contacts (Figure 5A, a and e, arrows), treatments that perturbed PI(4,5)P$_2$ disrupted cadherin accumulation at cell–cell contacts (Figure 5A, b–d and f–h, arrowheads). We then analyzed the effect of PI(4,5)P$_2$ reduction on N- and M-cadherin localization by using stable C2C12 cell lines in which PIP5K$_\gamma$ expression was silenced by RNA interference (El Sayegh et al., 2007; Ling et al., 2007; Bach et al., 2010). Moreover, PI(4,5)P$_2$ expression at cell–cell contacts was significantly lower in PIP5K$_\gamma$ shRNA myoblasts than in control cells (Figure 5B). Both N- and M-cadherin accumulation at cell–cell contacts was perturbed in PIP5K$_\gamma$ shRNA myoblasts compared with control Luci shRNA C2C12 cells (Figure 5C, a–c). Similarly, Rab35 was not recruited to cell–cell contacts in PIP5K$_\gamma$ shRNA myoblasts (Supplemental Figure S6). This is in agreement with our data showing that cadherins recruit Rab35 to cell–cell contact sites (Figure 1C). Moreover, whereas the level of p120 catenin was not modified in PIP5K$_\gamma$ shRNA C2C12 myoblasts (Figure 5Da), p120 catenin association with N-cadherin (Figure 5Db) and M-cadherin (Figure 5Dc) was decreased. Conversely, β-catenin binding was not

**PI(4,5)P$_2$ accumulation at cell–cell contacts**

(A) Parental and Rab35 shRNA C2C12 myoblasts were transfected with PH-PLCδ-GFP and left either untreated (a and c) or treated with EGTA for 20 min and then incubated in Ca$^{2+}$-containing medium for 60 min (EGTA/Rec., b and d). Arrows show accumulation and arrowheads show absence of accumulation of PH-PLCδ-GFP at cell contact sites. Bar, 10 μm. The histogram represents the percentage of cells in which PH-PLCδ-GFP accumulated at cell–cell contacts. At least three independent experiments were analyzed and 50 cells counted for each experiment. (B) C2C12 myoblasts were cotransfected with PH-PLCδ-GFP and RFP-fused Rab35WT (a–c), Rab35Q67L (d–f), or Rab35S22N (g–i). Arrows show Rab35 and PH-PLCδ-GFP accumulation, and arrowheads show absence of accumulation at cell contact sites. Bar, 10 μm. The histogram represents the proportion of cells in which PH-PLCδ-GFP accumulated at cell contact sites. At least three independent experiments were analyzed. Fifty cells were counted for each experiment. (C) Parental and Rab35 shRNA C2C12 myoblasts were transfected with the RFP-fused CAAX construct (b and e) and stained using an anti-PI(4,5)P$_2$ antibody (a and d). Arrows show PI(4,5)P$_2$ and CAAX-RFP accumulation and arrowheads show absence of accumulation at cell contact sites. Bar, 10 μm. Quantification of the two signals was performed along the white line shown in the merge panels (c and f) by line scan (MetaMorph software).

**FIGURE 4:** PI(4,5)P$_2$ accumulation at cell–cell contacts is reduced in Rab35-knockdown myoblasts. (A) Parental and Rab35 shRNA C2C12 myoblasts were transfected with PH-PLCδ-GFP and left either untreated (a and c) or treated with EGTA for 20 min and then incubated in Ca$^{2+}$-containing medium for 60 min (EGTA/Rec., b and d). Arrows show accumulation and arrowheads show absence of accumulation of PH-PLCδ-GFP at cell contact sites. Bar, 10 μm. The histogram represents the percentage of cells in which PH-PLCδ-GFP accumulated at cell–cell contacts. At least three independent experiments were analyzed and 50 cells counted for each experiment. (B) C2C12 myoblasts were cotransfected with PH-PLCδ-GFP and RFP-fused Rab35WT (a–c), Rab35Q67L (d–f), or Rab35S22N (g–i). Arrows show Rab35 and PH-PLCδ-GFP accumulation, and arrowheads show absence of accumulation at cell contact sites. Bar, 10 μm. The histogram represents the proportion of cells in which PH-PLCδ-GFP accumulated at cell contact sites. At least three independent experiments were analyzed. Fifty cells were counted for each experiment. (C) Parental and Rab35 shRNA C2C12 myoblasts were transfected with the RFP-fused CAAX construct (b and e) and stained using an anti-PI(4,5)P$_2$ antibody (a and d). Arrows show PI(4,5)P$_2$ and CAAX-RFP accumulation and arrowheads show absence of accumulation at cell contact sites. Bar, 10 μm. Quantification of the two signals was performed along the white line shown in the merge panels (c and f) by line scan (MetaMorph software).
FIGURE 5: PI(4,5)P₂ accumulation at cell–cell contacts is required for N- and M-cadherin localization at cell contact sites. (A) C2C12 myoblasts were cultured in GM supplemented with agents that reduce (50 μM calcimycin or 1 mM LiCl) or mask (1 mM neomycin) PI(4,5)P₂, and after 16 h, N- and M-cadherin localization was analyzed by indirect immunofluorescence. Arrows show cadherin accumulation at cell contact sites, and arrowheads show absence of cadherin accumulation at cell contact sites. Bar, 10 μm. (B) Control Luci shRNA (a–c) and PIP5Kγ shRNA C2C12 myoblasts (d–f) were transfected with the RFP-fused CAAX construct and stained using an anti-PI(4,5)P₂ antibody. Bar, 10 μm. Quantification of the two signals was performed along the white line shown in the “merge” panels by line scan (MetaMorph software). The PI(4,5)P₂/CAAX ratio was quantified in 50 cells. (C) N- and M-cadherin localization was analyzed at cell contact sites, and arrowheads show perturbation of cadherin accumulation at cell contact sites. Bar, 10 μm. (D) a, Expression of N- and M-cadherin, p120 and β-catenins, and α-tubulin was analyzed by Western blotting in cell lysates of control Luci shRNA and PIP5Kγ shRNA C2C12 myoblasts. b, c, Cell lysates of Luci shRNA and PIP5Kγ shRNA C2C12 myoblasts were immunoprecipitated using anti–N-cadherin (b) or anti–M-cadherin (c) antibodies and probed for the presence of β- and p120 catenins. (–) Control immunoprecipitations were always performed using irrelevant immunoglobulins G.
Rab35 regulates adherens junctions

The weak association of N- and M-cadherin with p120 catenin when PI(4,5)P₂ is reduced reflects an unstable cadherin complex at cell junctions.

Finally, we analyzed PIP5KIγ localization in Rab35-knockdown cells. PIP5KIγ accumulated at cell–cell contacts in control cells, where it colocalized with N- and M-cadherin (Figure 6A, a–c and g–i, arrows). In contrast, in Rab35 shRNA C2C12 myoblasts, PIP5KIγ was no longer accumulated at cell–cell contacts, whereas its punctuated intracellular localization increased (Figure 6A, d–f and j–l). To analyze in more detail PIP5KIγ distribution, we cotransfected C2C12 myoblasts with hemagglutinin (HA)-tagged PIP5KIγ and either RFP-Rab35WT or RFP-Rab35S22N. PIP5KIγ was mostly found at the PM and accumulated at cell–cell contacts when coexpressed with Rab35WT (Figure 6B, a–c). Conversely, it colocalized with Rab35S22N in clusters located outside cell–cell contacts (Figure 6B, d–f). Moreover, N-cadherin was also found in these clusters with PIP5KIγ and Rab35S22N (Figure 6C). Taken together, these data show that 1) Rab35 is required for PIP5KIγ accumulation at cell–cell contacts and 2) PI(4,5)P₂ generated at cell–cell contacts is required for cadherin-dependent intercellular junction formation.

**Rab35 knockdown inhibits myoblast differentiation**

To analyze the functional consequence of Rab35 knockdown, and because N- and M-cadherins are two major regulators of myogenesis induction and myoblast fusion (Charrasse et al., 2002, 2006), we asked whether Rab35 knockdown impaired myogenesis. First, C2C12 cells were grown to 80% confluency in growth medium (GM) and then shifted to differentiation medium (DM) for 4 d. Rab35 expression level increased during the differentiation process (Figure 7A). Then parental, Luci shRNA, and Rab35 shRNA C2C12 myoblasts were induced to differentiate, and the expression of myogenin, troponin T, and myosin heavy chain (three myogenic markers) and myotube formation were analyzed. We observed a little decrease in the expression of these myogenic markers in Rab35 shRNA myoblasts (Figure 7B). These results were confirmed using four different Rab35 shRNA clones (Rab35 shRNA A1 and A7, Rab35 shRNA2 A1 and A10, unpublished data). Analysis of myogenin and troponin T expression by immunofluorescence after 2 d in DM also confirmed these results (unpublished data). In addition, time-lapse imaging of parental and Rab35 shRNA myoblasts

---

**FIGURE 6:** Rab35 is required for PIP5KIγ accumulation at cell–cell contacts. (A) Control Luci shRNA (a–c and g–i) and Rab35 shRNA C2C12 myoblasts (d–f and j–l) were transfected with HA-tagged PIP5KIγ and stained for N-cadherin (a–f) or M-cadherin (g–l) and for HA tag detection. Images were acquired using a wide-field microscope and deconvolved using the Huygens system image restoration software. Insets show enlarged selected areas. Arrows show colocalization of cadherins and PIP5KIγ at cell–cell contacts. Bar, 10 μm. (B) C2C12 myoblasts were transfected with HA-tagged PIP5KIγ and RFP-Rab35WT (a–c) or RFP-Rab35S22N (d–f) and stained for HA tag detection. Images were acquired using a wide-field microscope and deconvolved using the Huygens system image restoration software. Insets show enlarged selected areas. Arrow shows colocalization of Rab35WT and PIP5KIγ at cell–cell contacts. Quantification of the two signals was performed along the white line shown in the “merge” panels by line scan (MetaMorph software; c and f). Bar, 10 μm. (C) C2C12 myoblasts were transfected with N-cadherin–RFP, HA-tagged PIP5KIγ, and GFP-Rab35WT (a–d) or RFP-Rab35S22N (e–h) and stained for HA tag detection. Images were acquired using a wide-field microscope and deconvolved using the Huygens system image restoration software. Insets show enlarged selected areas; merge panels show colocalization of the three proteins in the selected areas. Arrow shows colocalization of N-cadherin, Rab35WT, and PIP5KIγ at cell–cell contacts or N-cadherin, Rab35S22N, and PIP5KIγ in clusters outside cell–cell contacts (d and h). Bar, 10 μm.
revealed that Rab35 silencing perturbed myoblast fusion (Supplementary Video S5; left, parental C2C12 cells; right, Rab35 shRNA2 myoblasts). A significant reduction in the number of myotubes and in the fusion index was observed in Rab35 shRNA myoblasts after 4 d in DM (Figure 7C, d–g) in comparison to controls (Figure 7C, a–c). Comparable results were obtained with the different Rab35 shRNA clones and pooled cells. Moreover, these findings were confirmed using C2C12 myoblasts that express the dominant-negative mutant Rab35S22N-GFP after selection by fluorescence-activated cell sorting (FACS) or with a resistance marker (G418; (Figure 7C, h and i). These data show that Rab35 is required for myogenesis and more specifically for myotube formation and fusion.

**DISCUSSION**

AJs are very dynamic structures, and their continuous remodeling is vital for the maintenance of tissue integrity, morphogenetic movements, delamination, and epithelial-to-mesenchymal transition (Harris and Tepass, 2011). Here we report that the Rab35 GTPase is a new regulator of cadherin-dependent AJ formation through regulation of cadherin trafficking and stabilization at cell–cell contacts. Endocytosed cadherins can undergo multiple fates, including degradation or recycling back to the PM. In Rab35-depleted cells, cadherin localization at the cell surface was reduced after 4 d in DM addition, Bar, 30 μm. The histogram represents the fusion index in control myoblasts, the indicated Rab35 shRNA pools and clones, and C2C12 myoblasts that express Rab35S22N. The results are representative of three independent experiments. At least 3000 nuclei were counted for each experiment.

**FIGURE 7:** Inhibition of Rab35 expression by RNA interference affects myogenesis and myotube formation. (A) Protein extracts (50 μg/well) from C2C12 myoblasts, collected at the indicated time points (GM, growth medium; D, days after switching to differentiation medium, DM) were immunblotted for assessing Rab35 and actin expression. (B) Cell lysates (30 μg) of Luci shRNA (control) and Rab35 shRNA C2C12 myoblasts cultured in GM or DM for the indicated time were assessed by Western blot analysis for expression of Rab35, myogenin, troponin T, MHC, and actin. Results are representative of three independent experiments using cells that express Rab35 ShRNA1 or 2. (C) Phase contrast images of parental C2C12 (a), Luci shRNA C2C12 (b), GFP-expressing (vector alone) C2C12 cells sorted by FACS (c), Rab35 shRNA C2C12 pools (d, shRNA1; e, shRNA2) and clones (f, shRNA1 clone A7; g, shRNA2 clone A10), and GFP-Rab35S22N–expressing C2C12 myoblasts (h, sorted by FACS; i, selected by G418 resistance) 4 d after DM addition. Bar, 30 μm. The histogram represents the fusion index in control myoblasts, the indicated Rab35 shRNA pools and clones, and C2C12 myoblasts that express Rab35S22N. The results are representative of three independent experiments. At least 3000 nuclei were counted for each experiment.
undergo endocytic processing through several internalization routes from the PM (Delva and Kowalczyk, 2009; Schill and Anderson, 2009; Harris and Tepass, 2011). Rab35 does not generally control the recycling of all transmembrane proteins but allows selective recycling of some cargoes from early endosomes. Indeed, it was previously demonstrated that Rab35 controls recycling of MHC class I (Allaire et al., 2010; Chesneau et al., 2012) but not of β1-integrin from early endosomes (Allaire et al., 2010). Until now, only few cargoes that require Rab35 for their recycling had been identified, and here we demonstrate that cadherins are among them.

To characterize the compartments in which cadherins are located after Rab35 depletion, various GFP-tagged Rab family members were expressed, such as Rab4a, 5, 7, 8, and 11. With the exception of Rab5, none of the tested Rab members localized in the compartment in which cadherins accumulated. We observed an enlargement of Rab5-positive early endosomes, as described (Allaire et al., 2010). A clear colocalization upon Rab35 depletion between cadherins and transferrin was detected (Figure 3D). This is consistent with studies linking Rab35 to the recycling of transferrin back to the PM (Kouranti et al., 2006; Patino-Lopez et al., 2008; Chesneau et al., 2012). Finally, we demonstrate that cadherins and clathrin heavy chain or AP-2 colocalize in cells that express dominant-negative Rab35. This is not surprising because Rab35 and connexin, a GEF for Rab35, are both expressed in clathrin-coated vesicles (Allaire et al., 2010).

Besides its role in cadherin localization at the PM, Rab35 could also affect cadherin stabilization at cell–cell contacts through a decrease of PI(4,5)P2 production at cell–cell contact sites. We confirm here, as previously shown in epithelial cells (Ling et al., 2007), that the production of PI(4,5)P2 at cell–cell contact sites is important for cadherin stability. Recruitment and activation of PIP5KI at sites of N- and E-cadherin ligation result in PI(4,5)P2 production (Akiyama et al., 2005; El Sayegh et al., 2007; Ling et al., 2007). PIP5KII interacts directly with the cytoplasmic tail of cadherins and is required for E-cadherin–mediated AJ assembly. Moreover, expression of a kinase-inactive form of PIP5KII showed that PI(4,5)P2 generation is required for AJ assembly (Ling et al., 2007) and local actin assembly at AJs (El Sayegh et al., 2007). When Rab35 is inhibited, PIP5KII is not accumulated at cell–cell contacts and is sequestered in membrane vesicles with cadherins and Rab35. The colocalization of PIP5KII with cadherins in these structures is not surprising, because cadherin and PIP5KII are associated (El Sayegh et al., 2007) and are relocated to similar intracellular areas when cell–cell contacts are disrupted upon reduction of calcium ion concentration (Ling et al., 2007). As a consequence, PI(4,5)P2 production at cell–cell contacts is impaired. These results argue for a role of Rab35 in the control of cadherins and PIP5KII localization at cell–cell contacts. In stable C2C12 cell lines in which PIP5KII expression was silenced by RNA interference we observed 1) a decrease in the amount of PI(4,5)P2 at cell–cell contacts (Figure 5B) and 2) perturbation of N- and M-cadherin accumulation at cell–cell contacts (Figure 5C). The perturbation of N- and M-cadherin accumulation at cell–cell contacts in PIP5KII shRNA myoblasts is always less pronounced than in Rab35 shRNA myoblasts. This indicates that inhibition of PIP5KII affects only PI(4,5)P2 production at cell–cell contacts, which then perturbs cadherin stabilization. Conversely, inhibition of Rab35 affects both cadherin and PIP5K delivery to the plasma membrane and thus has a more pronounced effect on cadherin accumulation at cell–cell contacts. Indeed, in addition to the inhibition of PIP5K and PI(4,5)P2 production at cell–cell contacts, which impairs cadherin stabilization, Rab35 inhibition also blocks cadherin delivery into vesicles/clusters outside cell–cell contacts.

Functionally, we found that Rab35 is required for myoblast fusion to occur and observed that Rab35 expression level increased during myoblast fusion. Until now the only Rab involved in myoblast fusion was Rab11 in Drosophila (Bhui and Roy, 2009). The defect in myoblast fusion we observed in C2C12 myoblasts in which Rab35 was silenced could be explained by the decrease of cadherin accumulation and PI(4,5)P2 production at cell–cell contacts. Indeed, myoblast fusion is characterized by PI(4,5)P2 accumulation at cell–cell contact sites (Bach et al., 2010). PI(4,5)P2 decrease at contact sites might thus impair the recruitment at fusion sites of PH domain–containing proteins involved in the regulation of myoblast fusion, such as Trio, Rac1, and Arf6, through the reorganization of the actin cytoskeleton and of PM dynamics (Macia et al., 2008). Moreover, Rab35 could participate in the control of myoblast fusion through the regulation of EHD1, one of the four carboxyl-terminal Eps15 homology domain-containing (EHD) proteins. Indeed, EHD proteins have a role in endocytic regulation and have been linked to several Rab family members through their association with mutual effectors (Naslavsky and Caplan, 2011). Of interest, Rab35 controls early endosomal recruitment of EHD1 (Allaire et al., 2010), a protein that was recently shown to be involved in myoblast fusion (Posey et al., 2011).

In conclusion, we report here that Rab35 controls cadherin-mediated AJ formation and myoblast fusion. Given that Rab35 regulates cadherin function, it will now be interesting to investigate whether dysfunction of Rab35 might be associated with cancer development or with skeletal muscle pathologies such as muscular dystrophies.

MATERIALS AND METHODS

Cell culture

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum. Culture and differentiation of C2C12 mouse myoblasts were done as described (Charrasse et al., 2006). PIP5KII shRNA C2C12myoblasts were described previously (Bach et al., 2010).

EGTA was used at 2 mM for 1 h, and then cells were rinsed in GM for 1 h. Calcinymine (50 μM; Sigma-Aldrich, St. Louis, MO), LiCl (1 mM; Sigma-Aldrich), and neomycin (1 mM; Sigma-Aldrich) were added to GM for 16 h.

DNA constructs and RNA interference

shRNA constructs were made using the retroviral vector pSIREN-RetroQ according to the manufacturer's protocol (BD Biosciences, San Diego, CA). To suppress endogenous Rab35 expression, the annealed double-strand oligonucleotides GATCGGacaacttggcgaaacagcTTCAAGAGAGcttgttccgaagtgtgctTTTTTTACCGCTG (top) and AATTACCGCGTAAAAAGGacaacttggcgaacagcTCTCTG AAGctgtttccgaagtgtgctCG (bottom) were inserted into the RNAi-Ready pSIREN-RetroQ vector (Clontech, Mountain View, CA) to produce Rab35 shRNA1. Bold letters correspond to oligonucleotides 680–688 of the mouse Rab35 cDNA sequence (NM-198163). For Rab35 shRNA2, the sequence in bold letters of the top strand of the shRNA1 was replaced by GATCCGccttcagcagcagcagcTTCAAGAGAGctgttccgaagtgtgctTTTTTTACCGCTG (top) and AATTACCGCGTAAAAAGGacaacttggcgaacagcTCTCTG AAGctgtttccgaagtgtgctCG (bottom) were inserted into the RNAi-Ready pSIREN-RetroQ vector (Clontech, Mountain View, CA) to produce Rab35 shRNA1. Bold letters correspond to oligonucleotides 255–273 of the cDNA sequence (NM-198163). For Rab35 shRNA2, the sequence in bold letters of the top strand of the shRNA1 was replaced by GATCCGccttcagcagcagcTTCAAGAGAGctgttccgaagtgtgctTTTTTTACCGCTG (top) and AATTACCGCGTAAAAAGGacaacttggcgaacagcTCTCTG AAGctgtttccgaagtgtgctCG (bottom) were inserted into the RNAi-Ready pSIREN-RetroQ vector (Clontech, Mountain View, CA) to produce Rab35 shRNA1. Bold letters correspond to oligonucleotides 680–688 of the mouse Rab35 cDNA sequence (NM-198163). For Rab35 shRNA2, the sequence in bold letters of the top strand of the shRNA1 was replaced by GATCCGccttcagcagcagcTTCAAGAGAGctgttccgaagtgtgctTTTTTTACCGCTG (top) and AATTACCGCGTAAAAAGGacaacttggcgaacagcTCTCTG AAGctgtttccgaagtgtgctCG (bottom) were inserted into the RNAi-Ready pSIREN-RetroQ vector (Clontech, Mountain View, CA) to produce Rab35 shRNA1. Bold letters correspond to oligonucleotides 255–273 of the shRNA1 sequence. Luciferase shRNA was used as control (Fortier et al., 2008). Retrovirus production in Phoenix cells, infection, and selection were performed as described (Fortier et al., 2008). Cells were grown continuously in 1 μg/ml puromycin. Different clones were isolated by limited dilution. Rab35 silencing was assessed in 11 random clones and a pool of Rab35 shRNA1 pSIREN-RetroQ C2C12 myoblasts and in 10 random clones and a pool of Rab35 shRNA2 pSIREN-RetroQ C2C12 myoblasts.
All presented experiments were performed with at least the pool and two random clones in triplicate.

**Gel electrophoresis and immunoblotting**

Cells cultured in 100-mm dishes were rinsed in cold phosphate-buffered saline (PBS) and lysed in 10 mM piperezine-N,N′-bis(2-ethanesulfonic acid), pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 0.5% NP40, 1 mM EDTA, 1 mM orthovanadate, and protease inhibitor cocktail (Sigma-Aldrich). Then 30- to 50-μg protein extracts were resolved on polyacrylamide gels (8, 12, and 15%) and transferred onto Immobilon-FL membranes. Membranes were incubated with monoclonal antibodies against N-cadherin (1:1000), β-catenin (1:500), p120 catenin (1:1000), and Rab11 (1:1000; BD Transduction Laboratories, Lexington, KY), troponin T (1:500), α-tubulin (1:500), and myosin (1:2000; Sigma-Aldrich), fascin (1:500; Abcam, Cambridge, MA), and M-cadherin (1:200; Nanotools, Munich, Germany) or with polyclonal antibodies against Rab35 (1:800; gift from A. Echard), myogenin (Santa Cruz Biotechnology, Santa Cruz, CA), GFP (1:800; Rockland Immunochemicals, Gilbertsville, PA), and actin (1:1000; Sigma-Aldrich). Antibodies were revealed with Alexa Fluor 546–conjugated, Alexa Fluor 488–conjugated, or Alexa Fluor 633–conjugated, or affinity-purified anti–M-cadherin and N-cadherin (1:50), anti–adaptin-γ (1:200), anti–E-cadherin (1:1000), anti–clathrin heavy chain (1:100) and anti–adaptin-α (AP-2, 1:100; all from BD Transduction Laboratories) or affinity-purified anti–M-cadherin and N-cadherin (1:50) antibodies. Antibodies were revealed with Alexa Fluor 546–conjugated, Alexa Fluor 488–conjugated, or Alexa Fluor 633–conjugated goat anti–mouse or rabbit antibodies (Molecular Probes, Eugene, OR).

For transferrin uptake, cells were incubated in DMEM at 37°C for 30 min. Tetramethylrhodamine–labeled transferrin (20 μg/ml in medium containing 1% BSA; Molecular Probes) was internalized at 37°C for 45 min to reach the turnover steady state, and then cells were rinsed twice before fixation and cadherin expression analysis by immunofluorescence. For P(4,5)P2 detection, an anti-P(4,5)P2 monoclonal antibody (2C11; Santa Cruz Biotechnology) was used according to the protocol described in Hammond et al. (2009).

Images were taken either with a Zeiss LSM Meta 510 confocal microscope or with a MetaMorph-driven (Molecular Devices, Sunnyvale, CA) wide-field Axiosimager Z2 fluorescence microscope (Zeiss, Jena, Germany) with a PL APO 63× objective (numerical aperture 1.32; Leica, Melville, NY) and a CoolSNAP HQ camera (Photometrics, Woburn, MA). Images were processed using Photoshop and Illustrator (Adobe, San Jose, CA).

**Deconvolution**

Sixteen-bit images were captured and epifluorescence images were first restored with Huygens (Scientific Volume Imaging, Hilversum, Netherlands; Ponti et al., 2007). Huygens is an iterative program that can reassign light, after encoding as gray levels, to its sources in the stack with a very high probability using a point spread function. This process removes the fuzziness contained in the stack while keeping the three-dimensional information. In the present study the maximum likelihood estimation algorithm was used throughout. Restored stacks were then further processed with Imaris (Bitplane, Zurich, Switzerland). Colocalization was analyzed with the Imaris Colocalization module.

**Time-lapse experiments**

Parental and Rab35 shRNA C2C12 myoblasts were grown to confluence before analysis by time-lapse microscopy. Alternatively, parental and Rab35 shRNA C2C12 myoblasts were transfected with N-cadherin–GFP/RFP, M-cadherin–GFP, Rab35WT-RFP, or Rab35S22N-RFP. Time-lapse confocal microscopy was performed using a spinning Nipkow disk. Images were saved as .tiff files and further compiled into QuickTime movies (Apple, Cupertino, CA) using the MetaMorph program.

**ACKNOWLEDGMENTS**

We are grateful to Genaro Patino-Lopez (National Institutes of Health) for providing various plasmids encoding the RFP- and GFP-tagged Rab35 constructs. We thank the Montpellier RIO Imaging Facility. This work was supported by the Ligue Nationale contre le Cancer (Equipe labellisée), the Institut National du Cancer, and the Association Française contre les Myopathies. C.G.R. was supported by the Institut National de la Santé et de la Recherche Médicale. A.E. was supported by grants from the Institut Pasteur, the Center National de la Recherche Scientifique, and the Schlumberger Foundation for Education and Research.

**REFERENCES**

Akiyama C, Shinozaki-Narikawa N, Kitazawa T, Hamakubo T, Kodama T, Shibasaki Y (2005). Phosphatidylinositol-4-phosphate 5-kinase gamma is associated with cell-cell junction in A431 epithelial cells. Cell Biol Int 29, 514–520.

Allaire PD, Marat AL, Dall’Armi C, Di Paolo G, McPherson PS, Ritter B (2010). The Connexin DENN domain: a GEF for Rab35 mediating cargo-specific exit from early endosomes. Mol Cell 37, 370–382.

Bach AS, Enjalbert S, Comunale F, Bodin S, Vitale N, Charrasse S, Gauthier-Rouvire C (2010). ADP-ribosylation factor 6 regulates mammalian myoblast fusion through phospholipase D1 and phosphatidylinositol 4,5-bisphosphate signaling pathways. Mol Biol Cell 21, 2412–2424.

Baum B, Georgiou M (2011). Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. J Cell Biol 192, 907–917.

Bhuin T, Roy JK (2009). Rab11 is required for myoblast fusion in Drosophila. Cell Tissue Res 336, 489–499.

Braga VM, Betson M, Li X, Lamarche-Vane N (2000). Activation of the small GTPase Rac is sufficient to disrupt cadherin-dependent cell-cell adhesion in normal human keratinocytes. Mol Biol Cell 11, 3703–3721.
Vol. 24, February 1, 2013

Rab35 regulates adherens junctions

Laux T, Fukami K, Thelen M, Golub T, Frey D, Caroni P (2000). GAP43, Kouranti I, Sachse M, Arouche N, Goud B, Echard A (2011). Rab35 GTPase and OCRL phosphatase remodel lipids and F-actin for successful cytokinesis. Nat Cell Biol 13, 981–988.

Davis MA, Iretin RC, Reynolds AB (2003). A core function for p120-catenin in cadherin turnover. J Cell Biol 163, 523–534.

Delva E, Kowalczyk AP (2009). Regulation of cadherin trafficking. Traffic 10, 259–267.

Griffin HD, Hawthorne JN (1978). Calcium-activated hydrolysis of phosphatidylinositol-4,5-bisphosphate by PIP5Kgamma regulates gelsolin, actin assembly, and adhesion strength of N-cadherin junctions. Mol Biol Cell 18, 3026–3038.

Fortier M, Comunale F, Kucharzak J, Blangiardi A, Charrasse S, Meriane M, Comunale F, Blangy A, Gauthier-Rouviere C (2002). MICAL-L1 is a tubular endosomal membrane hub that connects Rab35 and Arf6 with Rab8a. Traffic 13, 82–93.

Roeth JF, Sawyer JK, Wiener DA, Peifer M (2009). Rab11 helps maintain apical crumbs and adherens junctions in the Drosophila embryonic ectoderm. PLoS One 4, e7634.

Schnitzer MA, Lawless JA, Buckwalter ML, Anderson RA (2009). Out, in and back again: PtdIns(4,5)P2 regulates cadherin trafficking in epithelial morphogenesis. Biochem J 418, 247–260.

Shim J, Lee SM, Lee MS, Yoon J, Kweon HS, Kim YJ (2010). Rab35 mediates transport of Cdc42 and Rac1 to the plasma membrane during phagocytosis. Mol Cell Biol 30, 1421–1433.

Stennicke HR (2009). Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10, 513–525.

Takeichi M (1995). Morphogenetic roles of classic cadherins. Curr Opin Cell Biol 7, 619–627.

Tautel N, Comunale F, Favard C, Charrasse S, Bodin S, Gauthier-Rouviere C (2009). N-cadherin/p120 catenin association at cell-cell contacts occurs in cholesterol-rich membrane domains and is required for RhoA activation and myogenensis. J Biol Chem 284, 23137–23145.

Troyanovsky S (2005). Cadherin dimers in cell-cell adhesion. Eur J Cell Biol 84, 225–233.

Varnai P, Ballo T (1998). Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools. J Cell Biol 143, 501–510.

Xiao K, Allison DF, Buckley KM, Kotte MD, Vincent PA, Faundez V, Kowalczyk AP (2003). Cellular signals at adherens junctions. Cell 112, 535–548.

Yap AS, Crampton MS, Hardin J (2007). Making and breaking contacts: the cellular biology of cadherin regulation. Curr Opin Cell Biol 19, 508–514.

Zerial M, McBride H (2001). Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 2, 107–117.

Zhang J, Fonovic M, Suyama K, Bogoy M, Scott MP (2009). Rab35 controls actin bundling by recruiting fascin as an effector protein. Science 325, 1250–1254.