Reggies/flotillins regulate E-cadherin–mediated cell contact formation by affecting EGFR trafficking

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ABSTRACT The reggie/flotillin proteins are implicated in membrane trafficking and, together with the cellular prion protein (PrP), in the recruitment of E-cadherin to cell contact sites. Here, we demonstrate that reggies, as well as PrP down-regulation, in epithelial A431 cells cause overlapping processes and abnormal formation of adherens junctions (AJs). This defect in cell adhesion results from reggie effects on Src tyrosine kinases and epidermal growth factor receptor (EGFR): loss of reggies reduces Src activation and EGFR phosphorylation at residues targeted by Src and c-cbl and leads to increased surface exposure of EGFR by blocking its internalization. The prolonged EGFR signaling at the plasma membrane enhances cell motility and macropinocytosis, by which junction-associated E-cadherin is internalized and recycled back to AJs. Accordingly, blockage of EGFR signaling or macropinocytosis in reggie-deficient cells restores normal AJ formation. Thus, by promoting EGFR internalization, reggies restrict the EGFR signaling involved in E-cadherin macropinocytosis and recycling and regulate AJ formation and dynamics and thereby cell adhesion.

INTRODUCTION

Adhesion between epithelial cells typically depends on the adhesion molecule E-cadherin and its linkage to the actin cytoskeleton through the intracellular ligands α-, β-, and p120-catenin (Nishimura and Takeichi, 2009). Disturbances in E-cadherin function can cause epithelial tumor progression to invasiveness and metastasis (Gavard and Gutkind, 2008). A major factor underlying impaired cell adhesion and thus cancer is elevation of epidermal growth factor (EGF) and EGF receptor (EGFR) signaling through which many crucial signal transduction molecules are (over-) activated (Gavard and Gutkind, 2008). This imbalanced signaling affects many cellular functions, including increase in cell motility and decrease in cell adhesion, by modifying regulators of the E-cadherin/catenin complex or its internalization and turnover (Mosesson et al., 2008). In intact tissues, the adhesion-disrupting influence of EGFR signaling is restricted by efficient EGFR internalization and down-regulation (Sorkin and Goh, 2008).

The spatiotemporally controlled internalization and turnover of the E-cadherin/catenin complex is necessary for the maintenance of cell adhesion, which prevents extensive cell overlap (Green et al., 2010). Thus, although seemingly stable, the adhesive contacts between cells are continually remodeled. Within cell contact sites, E-cadherin is typically concentrated in adherens junctions (AJs), which are highly dynamic structures and subject to a continuous basal-to-apical flow (Kametani and Takeichi, 2007). Concurrently, E-cadherin molecules in AJs are continually turned over (Hong et al., 2010). Thus, although seemingly stable, the adhesive contacts between cells are continually remodeled. Within cell contact sites, E-cadherin is typically concentrated in adherens junctions (AJs), which are highly dynamic structures and subject to a continuous basal-to-apical flow (Kametani and Takeichi, 2007). Concurrently, E-cadherin molecules in AJs are continually turned over (Hong et al., 2010). Bryant et al. (2007) demonstrated that E-cadherin at cell contact sites is internalized by macropinocytosis, recycled, and redelivered to the plasma membrane (PM) without degradation. It is not known, however, whether this process affects cell adhesion, and its regulation is still not well understood, but macropinocytosis apparently internalizes the E-cadherin/catenin complex as a whole. Macropinocytosis is an effective way to incorporate in a clathrin-independent manner segments of membrane that are redelivered to distinct sites of the cell (Falcone et al., 2006). Typical activators of...
macropinocytosis in epithelial cells are EGF, as well as phosphati-
didylinositol 3-kinase (PI3K) and Rac1, downstream of EGFR signaling (Falcone et al., 2006).

E-cadherin plays a major role during tissue reorganization and
migration in the developing embryo (Halbleib and Nelson, 2006). Only recently has it been recognized in zebrafish embryos that the
recruitment of E-cadherin from intracellular Rab11-positive vesicles
to cell contact sites depends on prion protein (PrP)–PrP trans-inter-
action and the ensuing signal transduction (Malaga-Trillo et al., 2009). Because PrP associates with reggie microdomains (Solis et al., 2010), these data raised the question of whether reggies would affect E-cadherin functions in mammalian epithelial cells.

The reggie proteins (also known as flotillins) are expressed in vir-
tually every cell type and across organisms as distant as fly and hu-
man (Stuermer, 2010). Although this suggests that they subserve
basic cellular functions, precisely which roles reggies play has re-
mained elusive. That reggies are crucial for membrane trafficking
and turnover came from evidence in neurons, where they are neces-
sary for growth cone elongation (Munderloh et al., 2009). Further-
more, participation of reggies in signal transduction has been re-
peatedly reported (Stuermer, 2010). Reggies interact with Src
 tyrosine kinases and adaptor proteins of the ponsin family. Together
they activate the ubiquitin ligase c-cbl and the GTPase TC10 and
control the exocyst-assisted translocation of membrane proteins
(Kioka et al., 2002). Evidence from the literature, together with re-
results from our own work, recently led to the hypothesis that reggies
regulate the recruitment and targeted delivery of specific mem-
brane proteins from intracellular compartments to specific sites of
the PM (Stuermer, 2010). This attributes to reggies a role in cargo
trafficking and accounts for the fact that reggies are essential for
growth cone elongation, neuronal differentiation, and axon regen-
eration (Munderloh et al., 2009). Of interest, reggies have been im-
plicated in a clathrin- and dynamin-independent endocytic route
of the glycosylphosphatidylinositol-anchored protein CDS9 (Glebov et al., 2006). Furthermore, reggies have also been shown to facili-
tate clathrin-dependent endocytosis of the amyloid precursor pro-
tein and the transporters of cholesterol and dopamine (Schneider et al., 2008; Cremona et al., 2011; Ge et al., 2011).

In the present study we set out to clarify whether reggies might
contribute to the internalization, turnover, trafficking, and targeted
deployment of E-cadherin and whether they might regulate cell ad-
hesion in association with PrP. Our results show that reggie down-
regulation inhibits EGFR internalization by blocking the phosphory-
lation of two of its major tyrosine phosphorylation sites (Y1045 and
Y845) and disturbs cell adhesion and AJ formation and dynamics.
This defect in AJ formation and dynamics is caused by an imbal-
anced rate of the macropinoctotic uptake and redelivery of E-cadherin
to AJs. Accordingly, AJs can be rescued by amiloride, Rac1, and
PI3K inhibition, all known inhibitors of macropinocytosis. Reggies are
not required for macropinosome formation, but instead regulate, to-
together with PrP, the targeted recycling of E-cadherin to AJs.

RESULTS
The role of reggies in E-cadherin–mediated cell
contact formation

Human A431 epidermoid carcinoma cells naturally express E-cad-
herin and coaccumulate reggie-1, PrP, and E-cadherin at cell con-
tacts (Figure 1, A and B). As shown for other cell types (Langhorst
et al., 2008), reggie-1 also occurs at several types of vesicles. To
determine the role of reggie-1 and PrP in cell contact formation, we
generated permanently transfected A431 cell lines by using short
hairpin RNA (shRNA) vectors against reggie-1 and PrP (hereafter

shR1 and shPrP, respectively). Of interest, down-regulation of reg-
gie-1 not only affected the expression of reggie-2 (Solis et al., 2007),
but also decreased the protein levels of PrP (Supplemental Figure
S1, A and B). Conversely, PrP ablation seemed not to affect the lev-
els of both reggie-1 and -2 (Supplemental Figure S1, A and B). shR1

FIGURE 1: Reggies regulate cell contact inhibition and intercellular
adhesion in A431 cells. Immunostaining of endogenous PrP (A) and
reggie-1 (R1, B) showed that both proteins colocalized with
E-cadherin (E-cad) at cell contact sites in A431 cells. Scale bars,
10 μm. (C) Down-regulation of reggie-1 (shR1) or PrP (shPrP) induced
a significant increase in overlapping processes (yellow arrowheads)
as revealed by E-cad immunostaining. Scale bars, 10 μm.
(D) Quantification of overlapping areas (n = 3, ***p < 0.001, one-way
ANOVA, mean ± SEM). (E) Intercellular adhesion was analyzed using
the dispase-based dissociation assay. Whereas a low degree of
fragmentation of the cell carpets was observed in control shLuc cells
(left), increased levels of fragmentation were apparent in shR1
(middle) and shPrP (right) cells. (F) Quantification of the dispase assay
(n = 4, **p < 0.01, ***p < 0.001, one-way ANOVA, mean ± SEM).


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and shPrP cells were organized in small clusters and retained cell contacts like wild-type (WT) and shRNA control cells (hereafter shLuc; Supplemental Figure S2A). E-cadherin immunostaining of shRNA-transfected cells did not show any apparent abnormality. However, both shR1 and shPrP cells formed overlapping processes with neighboring cells of an area twice as large as shLuc cells (Figure 1, C and D). No other defects, such as in cell size, shape, or number, were observed in shR1 and shPrP cells (unpublished data). This result suggests that reggies and PrP are involved in E-cadherin-mediated contact formation in A431 cells, consistent with findings in zebrafish embryos (Malaga-Trillo et al., 2009). Similar phenotypes were observed in cell contacts of MCF-7 mammary epithelial cells and E-cadherin–enhanced green fluorescent protein (EGFP)–expressing HeLa cells treated with small interfering RNAs (siRNAs) against reggie-1 or PrP (Supplemental Figure S2, B and C).

To analyze whether the increase in overlapping processes observed in shR1 and shPrP cells revealed that the cell adhesion defects were not due to an impaired expression of E-cadherin, β-catenin, or p120-catenin nor of caveolin-1, as another lipid raft protein (Supplemental Figure S1A). Moreover, neither were reggie-1 and PrP required for the formation of the E-cadherin/β-catenin complex (Supplemental Figure S1C) nor was the cell surface E-cadherin expression significantly affected in shR1 and shPrP cells, as shown by biotinylation and trypsinization experiments (Supplemental Figure S1, D and E). Because a fraction of E-cadherin has been reported to associate with lipid rafts (Seveau et al., 2004) and reggies are raft components, we analyzed the E-cadherin raft distribution in A431 cells. However, the amount of E-cadherin in lipid raft fractions was very small in these cells and was not visibly affected upon down-regulation of reggies or PrP (Supplemental Figure S1F). Of interest, we observed approximately twofold increase in the tyrosine phosphorylation level of β-catenin (Supplemental Figure S1G) but not that of p120-catenin or E-cadherin in shR1 and shPrP cells (unpublished data). To test whether down-regulation of reggies or PrP would affect the level of E-cadherin endocytosis, surface proteins were biotinylated and cells incubated for 2 h at 37°C. After stripping of the residual surface biotinylated proteins, the internalized E-cadherin was determined. Western blots showed that the pool of internalized E-cadherin in shR1 and shPrP cells was not different from that in control cells (Supplemental Figure S1H). Thus reggies and PrP appear not to control the overall E-cadherin surface localization, its binding with the major catenins, or its raft association.

The role of reggies in the formation, organization, and dynamics of AJs

Given that down-regulation of reggies did not cause an overall reduction of E-cadherin at the PM, we reasoned that perhaps specific elements, the AJs, might be affected. A431 cells treated with 0.2% Triton X-100 before fixation selectively retain E-cadherin stabilized in AJs, which appear as streaks (Shewan et al., 2005). In WT and control shLuc cells, detergent-resistant E-cadherin staining appeared in prominent streaks at cell contacts, which colocalized with the ends of perijunctional actin bundles and partially with reggie-1 and PrP (Figure 2A and Supplemental Figure S3, A–C). However, in contrast to WT and shLuc cells, detergent-resistant AJs appeared strongly

![Image](308x285 to 542x735)

**FIGURE 2:** Disruption of AJ formation and dynamics after reggie-1 and PrP down-regulation. (A) Immunostainings of the detergent-resistant pools of E-cadherin (E-cad) and β-catenin (β-cat) showed the streaks typical of AJs at contact sites in control A431 cells (shLuc, first row) and revealed disorganized AJs in reggie-1 (shR1, second row) and PrP (shPrP, third row) knockdown cells (enlargement of boxed areas on the right). Scale bars, 10 μm. (B) E-cadherin–EGFP (E-cad-EGFP)–expressing cells were transfected with control siRNA (siGL2), siRNA against reggie-1 (siR1), or siRNA against PrP (siPrP) and AJ movements recorded for 20 min. In contrast to control cells (left), AJs were not well defined, and their basal-to-apical movements were significantly reduced in siR1 (middle) and siPrP (right) cells. Trajectories of individual AJs from basal (b) to apical (a) regions of overlapping cell contacts (outlined by black lines) are shown in color (bottom). Scale bars, 5 μm. (C, D) Quantifications showed a significant reduction of velocity (C) and number (D) of AJs in cells treated as in B (n = 5, *p < 0.05, ***p < 0.001, one-way ANOVA, mean ± SEM).

...disorganized in shR1 and shPrP cells: AJs were often not evident, and a diffuse E-cadherin distribution was observed along the entire contact sites (Figure 2A). By contrast, the formation and organization of desmosomes were not affected in shR1 and shPrP cells
generated a permanently transfected A431 cell line expressing E-cadherin–EGFP and transfected these cells with a specific siRNA against reggie-1. Time-lapse microscopy studies showed that, in fact, E-cadherin–labeled AJs moved from the basal to the apical aspect of cell contacts in a very well-organized manner in WT and control siRNA-transfected cells (Figure 2B and Supplemental Movie S1). As expected, AJs appeared strongly disorganized in cells transfected with siRNA against reggie-1: individual AJs were often not visible, and a rather homogeneous fluorescence was observed over the entire contact site (Figure 2B and Supplemental Movie S2). Accordingly, the amount of AJs visible at cell contacts was significantly reduced in these cells (Figure 2D). Of note, the AJs that did form were markedly impaired in their coordinated basal-to-apical movement (Figure 2B). Individual AJs normally move with an average velocity of 0.32 ± 0.05 μm/min in WT and 0.30 ± 0.04 μm/min in control transfected cells, which fell to 0.14 ± 0.02 μm/min in cells transfected with siRNA against reggie-1 (Figure 2C). A very similar phenotype was observed in cells treated with siRNA against PrP (Figure 2, B and D, and Supplemental Movie S3), confirming the functional association between reggie-1 and PrP.

Taken together, these data indicate that the expression of reggies (and PrP) is necessary for adhesive strength, the correct formation and organization of AJs, and the coordinated movement of AJs at contact sites.

The role of reggies in EGFR endocytosis and signaling

It is well known that cell adhesion is impaired as a consequence of increased EGFR activity (Gavard and Gutkind, 2008). Therefore we examined whether increased EGFR signaling might be responsible for the defects in AJs observed in shR1 cells. Of interest, EGFR immunostaining revealed its colocalization with E-cadherin–EGFP in AJs at cell contact sites (especially at the most apical region), suggesting a functional relationship between EGFR signaling and AJ formation (Supplemental Figure S4A). When control shLuc cells were exposed to 10 ng/ml EGF, AJ formation was impaired, mimicking the phenotype observed in shR1 cells (Figure 3, A–C, and Table 1). Whereas higher EGF concentrations showed stronger effects on AJ formation, lower concentrations only partially mimicked the shR1 phenotype (unpublished data). Of note, the defects in AJs observed in shR1 cells were fully rescued by incubation with the specific EGFR blockers tyrphostin AG-1478 and PD158780 (Figure 3, C and D, and Table 1), strongly indicating that reggies affect AJ formation by regulating EGFR signaling.

Because cell adhesion is often inversely correlated with cell motility, we examined whether ruffling activity and wound-closure...
Table 1: Quantification of AJs and Cell motility.

| Number of AJs (AJ/μm²) | Cell motility (μm/min) |
|------------------------|------------------------|
| shLuc                  | 0.170 ± 0.014          | 0.060 ± 0.000           |
| shLuc + EGF            | 0.073 ± 0.015***       | 0.108 ± 0.019*          |
| shR1                   | 0.043 ± 0.009***       | 0.138 ± 0.023*          |
| shR1 + PD158780        | 0.162 ± 0.013          | 0.060 ± 0.000           |
| shR1 + tyrophostin AG-1478 | 0.154 ± 0.010      | n.d.                    |

EGF stimulation of control shLuc cells mimicked the defect in AJ formation (number of AJs) and cell motility observed in shR1 cells. EGF inhibitors (PD158780 and tyrophostin AG-1478) rescued both AJ and cell motility defects in shR1 cells. n.d., Data not determined. Statistical differences related to shLuc cells. n = 3, *p < 0.05, **p < 0.001, one-way ANOVA, mean ± SEM.

EGFR activity is tightly controlled and regulated by tyrosine phosphorylation at multiple sites of the receptor. Therefore we examined whether reggies might contribute to the tyrosine phosphorylation state of EGFR upon EGF stimulation. Western blots were performed with antibodies specific for the phosphorylated EGFR tyrosine residues 845, 992, 1045, 1068, and 1148, respectively. In addition to the expected delay in EGFR degradation (Figure 5, A and B), the absence of reggies caused a strong reduction in the phosphorylation of Y845 and Y1045 and had milder effects on other tyrosine residues (Figure 5, A and C). Y1045 is the major autophosphorylation residue of EGFR and is involved in c-cbl-dependent EGFR ubiquitination and internalization (Sorkin and Goh, 2008). Consistently, we observed that shR1 cells showed reduced c-cbl immunostaining at the PM after 5 min of EGF stimulation (Supplemental Figure S4D). EGFR phosphorylation at the Y845 residue was reported to depend on Src activity (Leu and Maa, 2003). Therefore we analyzed the level of activated Src using specific antibodies against the activated forms of the kinases. Indeed, shR1 cells showed an overall reduced level in activated Src kinases compared with control shLuc cells (Figure 6, A and B).

Next we studied whether the impaired EGFR internalization observed in shR1 cells may affect downstream signaling molecules known to require EGFR signaling in endosomes (Murphy et al., 2009). Accordingly, during EGF stimulation the activation of Erk1/2, PI3K, and Akt was significantly reduced in shR1 cells, whereas p38 MAPK activation was enhanced (Figure 6, A and B). In addition, we observed that the levels of reggie-1 slightly decrease during EGF stimulation of control shLuc cells (Figures 5A and 6A); however, this effect was not significant.

Taken together, the present observations suggest that the impaired cell adhesion upon down-regulation of reggies seems to result from delayed EGFR endocytosis, which, in turn, might induce a sustained EGFR activity at the PM.

Reggies and macropinocytosis

Our results indicate that the defective AJ formation in shR1 cells is caused by elevated surface EGFR signaling without noticeable effects on the ratio of E-cadherin at the PM versus intracellular pools. Still, AJs are E-cadherin–dependent structures and are characterized by continuous E-cadherin turnover (Hong et al., 2010). It has been reported that in EGF-stimulated MCF-7 cells the level of surface-exposed E-cadherin is kept at steady state through its internalization by macropinocytosis and recycling (Bryant et al., 2007). Therefore we examined macropinosome formation and E-cadherin trafficking in shR1 and control shLuc cells and tested whether its macropinocytic uptake and deployment at AJs might be affected by reggies.

To determine whether reggies are involved in macropinocytosis, we applied EGF-rhodamine to reggie-1–EGFP–expressing A431 cells. By 5 min after stimulation, cells exhibited the typical ring-shaped macropinocytic structures containing both EGF and reggie-1 (Figure 7A). These macropinosomes also contained endogenous reggie-1, EGFR, and E-cadherin, together with β-catenin and p120, as well as PrP (Figure 7, B–D, and Supplemental Figure S5, A–D).

When we examined whether the absence of reggies affects macropinocytosis, we found that shR1 cells did exhibit macropinosomes after EGF application (Supplemental Figure S5, B–D). Thus reggies are apparently not necessary for macropinosome formation. By contrast, shR1 cells showed macropinosomes with an apparently higher frequency than shLuc cells (unpublished data), indicating that depletion of reggies promotes macropinosome formation. For a semiquantitative assessment, we stimulated shR1 and control shLuc cells with EGF in the presence of high–molecular...
weight Alexa-labeled dextran, which is typically endocytosed by macropinosomes (Kerr and Teasdale, 2009). A comparison between cells showed that shR1 cells internalized dextran more efficiently than did shLuc cells (Figure 7, E and F), implying that the absence of reggies promotes macropinocytic uptake. Accordingly, the same effect was observed in shPrP cells (Supplemental Figure S6, A and B). This increased macropinocytosis in shR1 and shPrP cells can be explained by the persistence of EGFR at the PM upon EGF stimulation. The fact that reggie-1 associates with but is not necessary for macropinosome formation suggests that reggies may play a role in the trafficking of macropinocytic cargo proteins.
whether a specific Rac1 inhibitor had the same rescuing effect as amiloride. Indeed, the Rac1 inhibitor increased AJ formation in shR1 cells (Figure 8B and Table 2). Because down-regulation of reggies induced higher levels of p38 kinase activation after EGF stimulation (Figure 6), we used a p38-specific inhibitor and rescued the defects in AJ formation (Table 2). On the other hand, no rescue effects were observed with inhibitors of other EGFR downstream signaling molecules, such as MEK1/2, PLC, and ROCK (Figure 8C and Table 2). Thus the EGFR signaling involved in macropinocytosis affects the reggie-dependent regulation of AJs.

Consistent with the well-characterized role of PI3K in the regulation of macropinocytosis in EGF-stimulated A431 cells (Araki et al., 2007), the AJ phenotype of shR1 cells was also rescued by a PI3K-specific inhibitor (Figure 8D and Table 2). This result appears incongruent with the reduced phosphorylation of the PI3K-regulatory subunits p85 and p55 observed in shR1 cells during EGF stimulation (Figure 6). However, the reduced PI3K activation is in agreement with the reduced downstream activation of Akt (Figure 6), since both kinases seem to require EGFR signaling in endosomes to complete activation (Murphy et al., 2009). To reconcile this issue, we analyzed the role of PI3K and Akt in the EGF-mediated macropinocytosis of shR1 cells using specific inhibitors. Indeed, the PI3K inhibitor blocked the increased uptake of dextran in shR1 cells after EGF stimulation, whereas the Akt inhibitors IV and VIII had no significant effects (Supplemental Figure S6, E and F). Together these results indicate that the EGF-mediated PI3K activation, but not its downstream signaling to Akt, is involved in the increased macropinocytosis of shR1 cells. Because the first step in the activation of PI3K is believed to occur at the PM (Sorkin and von Zastrow, 2009), we speculate that the delayed EGFR endocytosis in shR1 cells might induce a local increase in the PI3K activity at the cell surface. Therefore we analyzed the subcellular localization of the phosphorylated PI3K subunits p85 and p55 in shR1 and control shLuc cells during EGF stimulation. Immunostainings revealed a weak signal for the activated PI3K subunits in serum-starved cells, which clearly increased upon EGF stimulation (Figure 8E), confirming the specificity of the antibody. Of interest, we observed an accumulation of the phosphorylated PI3K subunits at the apical pole of cell contacts (Figure 8E). Conversely, EGF-stimulated shLuc cells displayed more widespread signals of the activated PI3K subunits at the apical and basal regions without accumulation at cell contacts (Figure 8E). In sum, our results indicate that AJ formation is impaired in shR1 cells because the delayed EGFR internalization enhances its signaling at the PM (probably at the apical region), which, in turn, increases macropinocytosis and recycling of E-cadherin to AJs.

Rescue of AJ formation

As mentioned, E-cadherin is internalized by macropinocytosis and recycled at steady state (Bryant et al., 2007). This observation, together with our finding that down-regulation of reggies raises the rate of macropinocytosis and disturbs AJ formation and dynamics, led to the hypothesis that these phenomena are causally linked. Thus blockage of macropinocytosis in shR1 cells might counterbalance the abnormal AJ phenotype. Therefore we monitored AJ formation in E-cadherin-EGFP–expressing shR1 cells after amiloride treatment, a classic blocker of macropinocytosis (Kerr and Teasdale, 2009). As expected, amiloride blocked the increased uptake of dextran in shR1 cells after EGF stimulation (Supplemental Figure S6, C and D). Of importance, AJ formation, which is severely disturbed in shR1 cells (Figure 3C), returned to near-normal rates upon amiloride treatment (Figure 8A and Table 2). Therefore we tested directly whether a specific Rac1 inhibitor had the same rescuing effect as amiloride. Indeed, the Rac1 inhibitor increased AJ formation in shR1 cells (Figure 8B and Table 2). Because down-regulation of reggies induced higher levels of p38 kinase activation after EGF stimulation (Figure 6), we used a p38-specific inhibitor and rescued the defects in AJ formation (Table 2). On the other hand, no rescue effects were observed with inhibitors of other EGFR downstream signaling molecules, such as MEK1/2, PLC, and ROCK (Figure 8C and Table 2). Thus the EGFR signaling involved in macropinocytosis affects the reggie-dependent regulation of AJs.

FIGURE 5: Biochemical analysis of EGFR phosphorylation. (A–C) Control shLuc and shR1 cells were exposed to 10 ng/ml EGF between 0 and 120 min, and the total EGFR and the phosphorylation (P) of specific tyrosine residues of EGFR were determined with specific antibodies as indicated. Reggie-1 (R1) and α-tubulin (α-tub) served as loading control. The quantification of the differences between shLuc and shR1 cells of total EGFR as well as each of the five tyrosine residues normalized to total EGFR are shown in B and C, respectively (n = 4, *p < 0.05, **p < 0.01, ***p < 0.001, paired t test).
vesicles were tracked and quantified at the focal plane of newly forming AJs (basal pole of cell contacts; Hong et al., 2010). Indeed, the amount of E-cadherin–positive vesicles traveling in the basal region of cell contacts was significantly increased in shR1 cells compared with shLuc cells (Figure 8, F and G, and Supplemental Movies S4 and S5). This result shows that down-regulation of reggies results in an increase in E-cadherin vesicle trafficking during EGF stimulation. Finally, we analyzed whether reggies might be involved in the targeting of E-cadherin–loaded vesicles to AJs. EGF treatment of A431 cells cotransfected with E-cadherin–EGFP and reggie-1–monomeric red fluorescent protein (mRFP) showed the expected colocalization of both proteins in macropinosomes (Figure 8H). In addition, we observed double-labeled vesicles and vesiculotubular structures (Figure 8H). Then we recorded vesicle trafficking at the focal plane of the AJs upon EGF stimulation. The vast majority of vesicles carried both E-cadherin and reggie-1 (Supplemental Figure S6G). Of importance, double-labeled vesicles and highly dynamic vesiculotubular structures were observed to move toward as well as from AJs (Supplemental Figure S6G and Supplemental Movie S6). These data suggest that reggies may be involved in the trafficking of E-cadherin and its targeted delivery to AJs.

DISCUSSION

The results of this study show that down-regulation of reggies and PrP impaired cell adhesion by disrupting the spatiotemporal pattern of AJs, which normally undergo a basal-to-apical flow at the cell contact sites: AJs stalled or failed to form in shR1 and shPrP cells. Dissection of the underlying events uncovered that down-regulation of reggies led on the one hand to the reduction of activated Src tyrosine kinases and of phosphorylation of the tyrosine residue (Y805) on EGFR, which is the target of Src, and on the other hand had a major effect on tyrosine phosphorylation of the EGFR residue (Y1045) relevant for c-cbl-dependent EGFR internalization (Sorkin and Goh, 2008). Delayed internalization prolonged EGFR signaling at the PM, and this, in turn, increased the efficacy of E-cadherin macropinocytosis and negatively affected AJ formation (Figure 9). Indeed, AJ malformation correlated with and probably arose from defects in the trafficking of E-cadherin vesicles at the level of the AJs in shR1 cells. Thus reggies apparently coordinate the trafficking of specific cargo vesicles involved in membrane protein recycling and redelivery in epithelial cells.

Reggies, Src tyrosine kinase, and c-cbl

The two effects of reggies (regulation of Src activity and c-cbl-dependent events) are in line with earlier evidence showing that reggies interact with several Src tyrosine kinases in many different cells (Stuermer, 2010) and that reggies communicate with c-cbl through CAP (c-cbl–associated protein; Kioka et al., 2002). The functional association between EGFR and Src might connect EGFR to PrP- and reggie-mediated signaling, since both reggie and PrP
associate with and trigger signals via Src-family tyrosine kinases (Stuermer, 2010). Our results are in agreement with the view that PrP trans-interactions in reggie microdomains activate Src kinases (Solis et al., 2010; Stuermer, 2010), thereby affecting EGFR phosphorylation and endocytosis. This might explain previous results showing that Erk1/2 activation induced by antibody cross-linking of PrP is mediated by EGFR signaling (Monnet et al., 2004) and that Src kinase activity increases EGFR endocytosis during EGF stimulation (Ware et al., 1997). Furthermore, our results suggest that the activity of c-cbl, which is crucial for EGFR down-regulation (Sorkin and Goh, 2008), depends on reggies. c-cbl has been involved in the regulation of a reggie-dependent signaling cascade leading to the activation of the small GTPase TC10 (Kioka et al., 2002) and in the targeted recycling of specific cell surface receptors (Baldys et al., 2009). This suggests a function of the reggie/c-cbl interaction in guiding E-cadherin to the forming AJs.

Reggies and macropinocytosis

Our results indicate that the prolonged surface exposure of EGFR in shR1 cells influences EGFR signaling and the ensuing activation at the PM of PI3K and Rac1, which, in turn, regulate macropinocytosis (Kerr and Teasdale, 2009). Thus, through their effect on EGFR phosphorylation and internalization, reggies restrict the efficacy of macropinocytosis. The fact that macropinocytosis was increased in the absence of reggies implies that they are not required for processes such as formation of the macropinocytic cup or its budding into the cell. Reggies assembled at macropinocytic structures containing E-cadherin, EGFR, and PrP and, according to live observations, might control the trafficking of E-cadherin vesicles at the level of AJs. Thus reggies seem to guarantee a well-controlled macropinocytic uptake of E-cadherin (and EGFR) and the targeted recycling of E-cadherin (and probably a fraction of EGFR) to the forming AJs. We observed that the total amount of E-cadherin at the PM versus intracellular compartments was unchanged in shR1 and shPrP cells. This is expected if macropinocytic uptake of E-cadherin is counterbalanced by its recycling back to the PM, as previously reported (Bryant et al., 2007).

We observed that the tyrosine phosphorylation level of β-catenin was increased in our shR1 and shPrP cells without an apparent effect on E-cadherin/catenin complex formation. Phosphorylation of β-catenin by EGFR, Src, and various other kinases is believed to negatively affect E-cadherin/catenin interactions and adhesion (Lilien and Balsamo, 2005). This view, however, has been challenged in an elegant paper using β-catenin/plakoglobin double-null F9 cells (Tominaga et al., 2008). That study uncovered the phosphorylation of various tyrosine residues that had no effect on E-cadherin/catenin complex formation and cell adhesion. The identification of the tyrosine residue(s) responsible for the increased β-catenin phosphorylation in our shR1 and shPrP cells and its/their potential role in E-cadherin macropinocytosis await further analysis.

The role of PrP in E-cadherin turnover and recruitment

Aside from the fact that reggie depletion led to down-regulation of PrP cells in which only PrP was down-regulated mimicked the reggie phenotype in several respects. That PrP colocalized with reggies, E-cadherin, and EGFR not only at cell contact sites, but also in the macropinosomes, suggests that PrP might be functionally involved

FIGURE 7: Formation of macropinosomes in A431 cells treated with EGF. (A) Reggie-1-EGFP (R1-EGFP)–transfected cells showed colocalization of reggie-1 and EGF-rhodamine (EGF-rhod) in the forming macropinosome after 5 min of EGF treatment (boxed area enlarged in inserts). (B) R1-EGFP colocalized with EGFR in a macropinosome. (C, D) Immunostaining with antibodies against E-cadherin (E-cad) and EGFR, as well as with antibodies against PrP and EGFR, showed colocalization of the protein pairs in macropinosomes. Scale bars, 10 μm. (E, F) Addition of Alexa-dextran (dextran) showed a significantly higher amount of internalization in shR1 cells compared with control shLuc cells (n = 3, ***p < 0.001, t test, mean ± SEM). Scale bars, 10 μm.
FIGURE 8: Macropinocytosis blockers rescue AJ formation in reggie-knockdown cells. (A–D) E-cadherin-EGFP (E-cad-EGFP)–transfected shR1 cells showed a recovery of AJ formation when treated with the macropinocytosis blockers amiloride (A), Rac1 (B), and PI3K (LY294002, D) but not when treated with the PLC blocker U-73122 (C). Selected areas showed cell contacts between E-cad–EGFP expressing and nontransfected cells for better visualization. Scale bars, 5 μm. (E) shLuc and shR1 cells were either nonstimulated (EGF 0 min) or stimulated with 10 ng/ml EGF for 20 min and immunostained with an antibody against the phosphorylated PI3K subunits p85 and p55 (P-PI3K). The weak staining of nonstimulated cells contrasts with the stronger signals after EGF stimulation, particularly in shLuc cells. Phosphorylated PI3K subunits accumulated at cell contacts in the apical pole of shR1 cells (boxed area), as opposed to a more widespread distribution in shLuc cells at apical and basal regions. Scale bars, 20 μm. (F) E-cad-EGFP–labeled vesicles of EGF-stimulated control shLuc and shR1 cells were monitored by time-lapse recordings at the focal plane of AJs for 1 min. The vesicle tracks were traced as indicated by the lines (right). Scale bars, 5 μm. (G) The quantification of E-cadherin vesicles emerging into the focal plane of AJs showed a significant increase in shR1 over control shLuc cells (n = 10, **p < 0.001, t test, mean ± SEM). (H) E-cad-EGFP– and reggie-1-mRFP (R1-mRFP)–cotransfected A431 cells were stimulated with EGF for 15 min. A selected frame (boxed areas) showed that both proteins were colocalized at the same vesicles and tubulovesicular structures. Scale bars: 10 μm.
in the macropinocytic uptake and recycling of E-cadherin to AJs. The detailed analysis of PrP's affect on the generation of AJs awaits further experimentation. However, the contribution of PrP to these events might consist in the recruitment of E-cadherin from the vesicles to cell contacts as in the zebrafish embryo (Malaga-Trillo et al., 2009). In the embryo, recruitment of E-cadherin to contact sites required PrP trans-interactions and might be needed for the recruitment of E-cadherin from macropinosytic vesicles back to AJs. PrP may exert this function by interacting with reggies and its associated signaling molecules. Together they could form a landmark for E-cadherin deployment in epithelial cells (Stuermer, 2010).

**Reggies and clathrin-mediated endocytosis**

We showed here that EGFR phosphorylation and internalization are impaired in shR1 cells. However, we did not directly examine which events might consist in the recruitment of E-cadherin from the vesicles to cell contacts as in the zebrafish embryo. This clathrin-coated pit pathway is perhaps the most important pathway of EGFR internalization (Sorkin and Goh, 2008). It would imply that reggies facilitate the clathrin-mediated endocytosis of EGFR. Accordingly, it was recently demonstrated that reggies can modulate the clathrin-dependent uptake of the amyloid precursor protein (Schneider et al., 2008), the dopamine transporter (Cremona et al., 2011), and a cholesterol transporter (Ge et al., 2011). The modulation of the clathrin-dependent endocytosis via reggies might involve an interaction with the adapter AP2 (Ge et al., 2011), although reggies were previously not observed at clathrin-coated pits (Langhorst et al., 2008; Schneider et al., 2008).

During the revision of this paper, an article was published showing that siRNA-mediated down-regulation of reggie-2 in HeLa cells reduced the EGF-mediated phosphorylation of EGFR (only at Y1173) and Erk1/2 but had no effect on EGFR endocytosis and the PI3K/Akt pathway (Amaddii et al., 2012). Our results, however, show that reggie-1 down-regulation inhibits EGFR phosphorylation at various residues (particularly at Y845 and Y1045), impairs EGFR endocytosis and degradation, and affects the downstream targets PI3K/Akt and Erk1/2, which are known to require receptor endocytosis (Murphy et al., 2009; Sorkin and von Zastrow, 2009). Thus the different effects on the EGFR pathway induced by siRNAs against reggie-2 might result from the high residual levels of reggie-1 observed under these conditions (Amaddii et al., 2012).

**Reggies drive macropinocytic membrane turnover and redelivery**

Our work on EGFR indicates that its internalization is impaired in shR1 cells. This enhances the macropinocytosis through which E-cadherin is redelivered to cell contact sites. Yet redelivery might be imprecise in shR1 cells, which correlates with increased and, by inference, error-prone E-cadherin vesicle trafficking. The latter role of reggies—macropinocytic recycling and targeted redelivery of the macropinocytic cargo—could explain results from other reports and indicates which function reggies might generally subserve. For example, in neurons reggies are enriched in the growth cone and are indispensable for axon elongation (Stuermer, 2010). In addition, reggies were implicated in the turnover of semaphorins in the growth cone (Carcea et al., 2010). This fits the notion that neurons use a highly efficient macropinocytic turnover mechanism of membrane and associated proteins during growth cone elongation and navigation (Tojiama et al., 2011). This mechanism is known to be regulated by neurotrophin receptor tyrosine kinases (Valdez et al., 2007). Of interest, neutrophils derived from a reggie-2/flotillin-1 knockout mouse had problems with chemotactic movements in Matrigel, which correlates with the accumulation of reggies at the uropod of these cells (Ludwig et al., 2010). Migratory cells depend, like growth cones, on macropinocytic membrane turnover at their leading and trailing edges, and migration is also triggered by stimulation of receptor tyrosine kinases.

Thus, if reggies generally regulate the macropinocytic turnover mediated by receptor tyrosine kinases in polarized cells, this would account for the fact that reggies are present in virtually every cell type and why they have been implicated in seemingly different functions.

**MATERIALS AND METHODS**

**Reagents, antibodies, and plasmids**

Cell culture reagents were purchased from Life Technologies (Darmstadt, Germany). Antibodies and their distributors were as...
follows: monoclonal antibodies (mAbs) against E-cadherin, p120, β-catenin, α-catenin, ESA/reggie-1, flotillin-1/reggie-2, EGFR, and caveolin-1 (BD Biosciences, Heidelberg, Germany); anti-PrP (6H4; Pionrics, Schlieren-Switzerland, Zurich, Switzerland; desmoplakin 1/2 (Progen, Heidelberg, Germany); mAb against phosphotyrosine (P-Tyr100) and the polyclonal antibodies (pAbs) against E-cadherin, EGFR, c-cbl, phospho-EGFR Y845, Y992, Y1045, Y1068, and Y1148, Src, phospho-Src Y416, Akt, phospho-Akt Thr-308, phospho-Akt Ser-473, p38, phospho-p38 Thr-180/Tyr-182, PI3K p85, phospho-PI3K (p85 Y458/p55 Y199), Erk1/2, and phospho-Erk1/2 Thr-202/204 (Cell Signaling, Frankfurt am Main, Germany); and pAb anti-α-tubulin (Abcam, Cambridge, MA). Secondary antibodies for immunostaining and Western blot analyses were from Jackson ImmunoResearch Laboratories (West Grove, PA). Phalloidin–Alexa 548 was from Invitrogen (Carlsbad, CA). The following inhibitors were used: EGFR inhibitors PD158780 (Sigma-Aldrich, St. Louis, MO) and tyrphostin AG-1478 (Cell Signaling); PI3K inhibitor LY294002 and MEK1/2 inhibitor U0126 (Cell Signaling); Rac1-inhibitor and Akt inhibitors IV and VIII (Calbiochem, La Jolla, CA); and amiloride, p38 kinase inhibitor SB 202190, PLC inhibitor U-73122, and ROCK inhibitor Y-27632 (Sigma-Aldrich). The reggie-1–EGFP rescue construct and the caveolin-1–EGFP vector were described previously (Langhorst et al., 2008; Munderloh et al., 2009). The human E-cadherin–EGFP vector was generously provided by Vann Bennett (Duke University Medical Center, Durham, NC).

**Cell culture and cell lines**

A431, MCF-7, and HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum, l-glutamine, and penicillin/streptomycin. A431 cells were grown until ∼90% of confluency (hereafter indicated as “near confluency”) to avoid the formation of multiple cell layers (Van Itallie et al., 1995). Vector transfection was carried out using FugeneHD (Roche, Mannheim, Germany), following the manufacturer’s protocol, and siRNA transfection using Nanofectin siRNA transfection reagent (PAA, Linz, Austria). Alexa Fluor 546–labeled siRNA duplexes against reggie-1 (R1.0) and PrP were obtained from Qiagen (Valencia, CA). The following inhibitors were used: EGFR inhibitors PD158780, G418. The reggie-1–EGFP rescue construct and the caveolin-1–EGFP vector were described previously (Langhorst et al., 2008; Munderloh et al., 2009). The human E-cadherin–EGFP vector was generously provided by Vann Bennett (Duke University Medical Center, Durham, NC).

**Immunofluorescence and microscopy**

A431, MCF-7, and HeLa cells were fixed and stained as previously described (Langhorst et al., 2008). Cells were analyzed with an α-Plan-Apochromat 63x/1.4 objective at a confocal microscope (LSM510 Meta) and/or Axioplan2 equipped with an AxioCam HRm (all from Zeiss, Jena, Germany). Visualization of Triton-resistant AJs in A431 cell lines was performed according to Shewan et al. (2005). Briefly, cells were grown on poly-l-lysine (pLys)–coated coverslips to near confluency, immersed for 10 s in ice-cold Triton buffer (10 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, 0.2% Triton X-100), fixed, and immunostained as described.

**Quantification of overlapping cell contacts**

A431 cell lines were grown on pLys–coated coverslips until near confluency, fixed, and stained for E-cadherin and 4′,6-diamidino-2-phenylindole. Widefield images were acquired in an Axioplan2 microscope, and the overlapping area of ∼400 cell contacts was marked and measured using AxioVision 4.8 software (Zeiss). The mean area covered by cell contacts of shRNA control cells was set to 100%, and statistical analysis was performed from three independent experiments using a one-way analysis of variance (ANOVA) test.

**Dispase-based dissociation assay**

The dispase-based dissociation assay has been previously described (Huen et al., 2002). Briefly, shRNA stably transfected A431 cells were grown in duplicate on 60-mm plates until confluency was entirely reached. Cells were washed with PBS and incubated with a 2.4 U/ml dispase (Roche) solution for 1 h at 37°C, and the released cell carpets were carefully washed and transferred to 15-ml tubes. Tubes were inverted 60 times on a rocker, and cell fragments were counted. Quantification was done from four replicates of three independent experiments and statistical analyses using a one-way ANOVA test.

**Scratch assay**

A431 cells were grown to near confluency on a 12-well plate, and a scratch was performed with a tip of a micropipette. Cells were washed twice with PBS and cultured normally. Images of the wound were taken after indicated time points and the empty (wound) area was measured. The percentage of wound closure was calculated by comparing the initial wound area with the wound after 24 h. Quantification was done from four replicates of three independent experiments and statistical analyses using a paired t test.

**Electron microscopy analyses**

Electron micrographs taken randomly at 20,000 primary magnifications were enlarged to 80,000 times for morphometric analyses of AJs. The apparent length of structures fulfilling the criteria of AJs in A431 cells, as exemplified by Troyanovsky et al. (2006), was measured where they occurred in strictly perpendicular section.

**Biochemical analyses**

A431 cells grown on plates to near confluency were lysed with ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5 100 mM NaCl, 5 mM MgCl₂, 2 mM EDTA, 1% Triton X-100, 10% glycerin) supplemented with protease and phosphatase inhibitor cocktails (ThermoFisher Scientific, Waltham, MA). Extracts were cleared by centrifugation and boiled at 95°C for 5 min or used for immunoprecipitation analyses. Briefly, lysates were incubated with 1 μg of antibody against E-cadherin, p120, or β-catenin for 1 h at 4°C. Then 20 μl of protein G agarose (Roche) was added and incubated overnight at
Quantification of cell surface E-cadherin and E-cadherin endocytosis

shRNA permanently transfected A431 cells grown on plates to near confluency were incubated on ice with 1 mg/ml sulfo-NHS-SS-biotin (Pierce, ThermoFisher Scientific) in PBS for 30 min. Free sulfo-NHS-SS-biotin was quenched by two washes with ice-cold 50 mM NH₄Cl in PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂ and several washes in PBS. Cell extracts and E-cadherin immunoprecipitation were done as described. Biotinylated E-cadherin was analyzed by Western blots using ExtrAvidin-peroxidase (Sigma-Aldrich) and anti-E-cadherin mAb.

For E-cadherin trypsinization, A431 cells were grown on plates to near confluency and incubated in HEPES-buffered saline supplemented with 0.01% trypsin for 10 min at 37°C in the presence of 2 mM Ca²⁺ or 1 mM ethylene glycol tetraacetic acid. Cells were immediately boiled in Laemmli buffer, and E-cadherin was detected by Western blots.

Endocytosis of E-cadherin was studied as previously described (Le et al., 1999). Briefly, A431 cells grown on plates to near confluency were biotinylated as described. Cells were incubated for 2 h at 37°C in normal media supplemented with 10 μM cycloheximide to stop protein synthesis. Surface biotin was removed by incubation with three 20-min washes with ice-cold glutathione solution (60 mM glutathione, 75 mM NaCl, 75 mM NaOH, and 1% bovine serum albumin). Biotinylated proteins from cleared cell extracts (lysed as described earlier) were collected with 10 μl of streptavidin beads overnight at 4°C and finally analyzed by Western blot against E-cadherin.

EGF stimulation

Transfected and nontransfected shRNA A431 cells were serum starved for 4 h, stimulated with 10 ng/ml EGF (Sigma-Aldrich) in DMEM-HEPES, pH 7.4, supplemented with 10 μM cycloheximide for the time indicated in the corresponding figures, and prepared for immunostaining, biotinylation, and biochemical analyses as described. Statistical analyses were done using a one-way ANOVA test or a paired t-test from at least three independent experiments.

EGF-rhodamine and dextran uptake

A431 cells were prepared as described, stimulated with 20 ng/ml EGF-rhodamine (Molecular Probes, Invitrogen) or with 10 ng/ml EGF in the presence of 0.5 mg/ml dextran–Alexa Fluor 488 (10,000 MW; Molecular Probes, Invitrogen) for the times indicated in the corresponding figures, fixed, and mounted for confocal microscopy analysis. For EGF-rhodamine uptake, cells were washed three times with ice-cold acidic buffer (0.2 M acetic acid, 0.5 M NaCl) to remove surface-bound EGF-rhodamine before fixation. For dextran uptake experiments, cells were alternatively pretreated for 30 min with dimethyl sulfoxide, 1 mM amiloride, 50 μM LY294002, 1 μM Akt inhibitor IV, or 10 μM Akt inhibitor VIII before EGF stimulation. The LSM Image Browser (Zeiss) was used for fluorescence quantification of ~300 cells per each time point from at least three independent experiments. Statistical analyses were done using a one-way ANOVA test or a paired t-test.

Live-cell imaging

A431 cells were transfected for 48–72 h on plys-coated coverslips. Cells were recorded using a Colibri Cell Observer SD imaging system equipped with an α-Plan Fluar 100×/1.45 objective and an AxioCam HRm (Zeiss). Cells were maintained in medium on 37°C preheated incubator and objective, and images were acquired with 100% light-emitting diode power. Images for the analysis of AJ's in A431 cells expressing E-cadherin–EGFP were acquired every 2 min for 20-min periods as described. Images were further analyzed using ImageJ and AxioVision 4.8 (Zeiss). Total amount of AJ's of 10–20 cell contacts and the distance covered by 25 AJ's from five randomly selected cell contacts were measured. Alternatively, cells were stimulated with 10 ng/ml EGF or treated with 50 nM PD158780, 100 nM tyrphostin AG-1478, 1 mM amiloride, 50 μM LY294002, 20 μM U0126, 100 μM Rac1-inhibitor, 20 μM SB 202190, 20 μM U-73122, or 20 μM Y-27632 each for 1 h at 37°C before live imaging and analyzed as described. For kymograph analyses, region at the cell contacts were selected, and kymographs were produced using ImageJ; the speed of cell movement was calculated using the plug-in Kymo Line ROI (Elisa May, University of Konstanz). For vesicle trafficking, E-cadherin–EGFP–transfected and R1-mRFP–cotransfected A431 cells were serum starved for 4 h and stimulated with 10 ng/ml EGF during the recording. Images were acquired every 0.5 s for 5 min as described. Cell contacts of 10–20 cells were measured and analyzed using AxioVision 4.8 and Imaris software (Bitplane Scientific Software, Zurich, Switzerland). Statistical analysis was performed using a one-way ANOVA test or a paired t-test.

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