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Article

Armus Is a Rac1 Effector that Inactivates Rab7 and Regulates E-Cadherin Degradation

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Summary

Background: Cell-cell adhesion and intracellular trafficking are regulated by signaling pathways from small GTPases of the Rho, Arf, and Rab subfamilies. How signaling from distinct small GTPases are integrated in a given process is poorly understood.

Results: We find that a TBC/RabGAP protein, Armus, integrates signaling between Arf6, Rac1, and Rab7 during junction disassembly. Armus binds specifically to activated Rac1 and its C-terminal TBC/RabGAP domain inactivates Rab7. Thus, Armus is a novel Rac1 effector and a bona fide GAP for Rab7 in vitro and in vivo, a unique and previously unreported combination. Arf6 activation efficiently disrupts cell-cell contacts and is known to activate Rac1 and Rab7. Arf6-induced E-cadherin degradation is efficiently blocked by expression of Armus C-terminal domain or after Armus RNAi. Coexpression of Arf6 with dominant-negative Rab7 or Rac1 also inhibits junction disassembly. Importantly, Armus RabGAP expression also prevents EGF-induced scattering in keratinocytes, a process shown here to require Arf6, Rac1, and Rab7 function. To our knowledge, this is the first report to demonstrate a molecular and functional link between Rac1 and Rab7.

Conclusions: Our data indicate that active Rac1 recruits Armus to locally inactivate Rab7 and facilitate E-cadherin degradation in lysosomes. Thus, the integration of Rac1 and Rab7 activities by Armus provides an important regulatory node for E-cadherin turnover and stability of cell-cell contacts.

Introduction

Small GTPases of the Rho, Arf, and Rab families are essential regulators of many important cellular processes. Although each of these families has a specific role, often their functions overlap. For example, Rho, Rab, and Arf small GTPases are known to regulate proliferation, intracellular trafficking, migration, and attachment to substrata or neighboring cells [1–6]. In spite of the importance of these cellular processes for homeostasis and disease, the molecular mechanisms of how signaling from different GTPases is coordinated are not fully understood.

Destabilization of cell-cell contacts in epithelial cells is one such process in which multiple small GTPases play a role. Cell-cell adhesion mediated by E-cadherin receptors is a key event in the regulation of epithelial morphogenesis, differentiation, and function [4, 7]. Perturbation of epithelial junctions results in acquisition of mesenchymal characteristics, with increased proliferation and motility [2, 4]. Thus, an appropriate control of E-cadherin adhesion and surface levels is important for many aspects of epithelial function in health and disease.

Activation of Rac1 and Arf6 destabilize E-cadherin receptors from junctions in different cell types, upon which cells detach from epithelial sheets [8, 9]. This phenotype is part of a wide range of Rac1 and Arf6 functions during tumor progression including hyperproliferation, rearrangement of the cytoskeleton, and invasion [2, 4]. Rac1 activation is sufficient to perturb cell-cell adhesion by itself or when activated by Ras and other oncogenes [10–12]. A number of reports suggest that Rac1 is activated downstream of Arf6 and is necessary to promote cytoskeletal changes and cell motility [2, 5, 13]. Yet, conflicting evidence exists as to whether Rac1 is required downstream of Arf6 to perturb cell-cell contacts. For example, Arf6 activation in MDCK cells results in a transient decrease in Rac1 activity, which correlates temporally with junction breakdown [14].

The molecular mechanisms leading to junction disassembly have not been fully elucidated. One possibility is the involvement of Rabs, which regulate the dynamics of internalization/recycling of adhesive receptors such as cadherins and integrins [1, 13]. Upon expression of oncogenes or growth factor stimulation, the function of Rab5 is required for cadherin internalization/recycling, thereby effectively reducing its surface levels and cell-cell adhesion [1]. Inappropriate activation of Rac1 leads to increased internalization of E-cadherin receptors and their accumulation in intracellular vesicles [10, 11]. However, the specific Rabs required downstream of Rac1 are not known.

Arf6-induced junction disassembly may also involve a cross-talk with Rab GTPases, even though Arf6 per se can regulate endocytosis and exocytosis [2]. In MDCK cells, v-Src-induced activation of Arf6 perturbs cadherin adhesion in a Rab5- and Rab7-dependent manner [15]. Consistent with the above, expression of active Arf6 [8] or its activation by v-Src [15] promotes accumulation of E-cadherin in intracellular vesicles [9, 15].

The existence of distinct pathways controlled by Arf6, Rac1, and Rab small GTPases in the regulation of cadherin adhesion cannot be formally excluded. However, most likely there are nodes of interaction between pathways regulated by these small GTPases to efficiently modulate cadherin surface levels. Mechanistically, these nodes could be indirect (1) through modulation of phosphoinositide production [2, 13] or (2)

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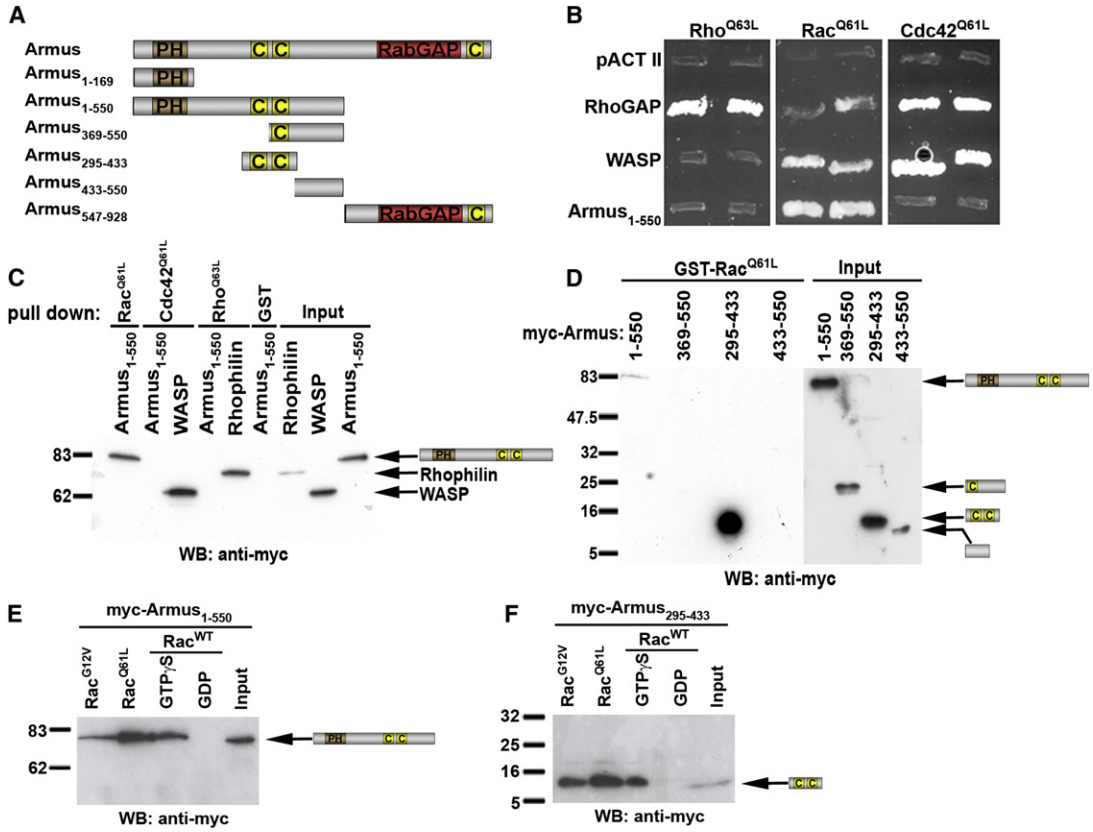

Figure 1. Armus Is a Novel Rac1 Binding Protein

(A) Representation of the domain structure of Armus and truncation mutants used in this study. Amino acid region of each mutant is shown in subscript and the domains contained in each fragment are also shown. The clone isolated in the yeast two-hybrid screen represents the first 550 amino acids of Armus ($Armus_{1-550}$). PH, Pleckstrin Homology domain; C, coiled-coil domains; RabGAP, TBC/RabGAP domain.

(B) Yeast two-hybrid binding assay with $Armus_{1-550}$ and activated forms of RhoA (Rho^{Q63L}), Rac1 (Rac^{Q61L}), Cdc42 ($Cdc42^{Q61L}$), and empty vector (pACTII). RhoGAP and WASP were used as positive controls.

(C) Pull-down assay with GST-fusion proteins (activated Rac^{Q61L}, $Cdc42^{Q61L}$, or Rho^{Q63L}) and in vitro translated (IVT) myc-$Armus_{1-550}$, myc-WASP, and myc-Rhophilin (positive controls for Cdc42 and RhoA binding, respectively).

(D) GST-Rac^{Q61L} was incubated with different IVT Armus fragments as described on the top of the panel.

(E and F) Activated GST-Rac1 ($Rac1^{Q61L}$ or $Rac1^{G12V}$ mutations) or wild-type (Rac^{WT}) loaded with GTPγS or GDP were incubated with IVT myc-$Armus_{1-550}$ (E) or myc-$Armus_{295-433}$ (F). Data representative of three independent experiments, thereafter referred to as n = 3. Input represents 10% of amount used in pull-downs. See also Figure S1.

due to the presence of specific domains that recruit Rac activity to vesicles/plasma membrane (i.e., p85, BAR, and PH domains found in upstream regulators) [16]. Alternatively, nodes to integrate signaling by different small GTPases may be direct, via multiple domains in a single protein. This single protein would have the ability to interact with a particular small GTPase in a GTP-dependent manner and have domains to activate or downregulate a distinct small GTPase. For example, ALS2/alsin can interact with active Rac1 and induce Rab5 activation [17, 18]. Other examples are ARAP (contains ArfGAP and RhoGAP domains—GAP, GTPase activating protein), p50RhoGAP (interacts with Rab11 and inactivates Rho) [19], Arfaptin (binds to active Arf1/Arf6 and inactive Rac1), and ARGHGAP10 (interacts with active Arf1 and is a Cdc42 GAP) [5, 16]. Yet, the vast numbers of small GTPases and their overlapping cellular functions imply that further nodes of interconnection between their activities must exist.

Thus, although a cross-talk between signaling from Rac1, Rab, and Arf6 may occur in different cellular processes, the precise molecular mechanisms have not yet been unravelled. Here we provide new mechanistic insights into a cross-talk between these small GTPases during disassembly of cell-cell contacts. We identify the function of an uncharacterized protein, Armus, a member of the TBC/RabGAP family (Tre2/Bub2/Cdc16; TBC domain) [20]. Armus contains an unusual and previously unreported combination of domains: it interacts with activated Rac1 via a novel motif and at the C terminus contains a TBC/RabGAP domain. Importantly, Armus plays a key role in EGF-induced scattering and Arf6-dependent disassembly of junctions, by integrating Rac1 activation with Rab7 function to promote E-cadherin degradation.

Results

Identification and Characterization of a Novel Rac-Binding Protein

By using activated Rac1 as bait, we have identified a novel Rac1 binding protein after a yeast two-hybrid screen with a normal human keratinocyte cDNA library (Figure 1A). The yeast two-hybrid clone specifically bound to activated Rac1,

but not active RhoA or Cdc42 (Figure 1B). A single 3.9 kb mRNA species was identified in keratinocyte RNA samples, but multiple bands were observed in epithelia-containing organs in human multitissue northern blots (Figures S1A and S1B available online). The full-length cDNA was cloned with RT-PCR and normal keratinocyte total RNA: it includes a predicted PH domain, coiled-coil domains in the middle and at the C terminus, and a putative TBC/RabGAP domain [16, 20] (hereafter referred to as the RabGAP domain; Figure 1A).

The isolated clone (928 amino acids, hereafter referred to as aa) is homologous to a member of TBC/RabGAP family, TBC1D2 isoform A (860 aa), and identical to its variant c (928 aa), but contains polymorphisms at three different sites (Figure S1C). Armus is also highly homologous to PARIS-1 (917 aa), a protein overexpressed in prostate cancers that contains a deletion of 11 amino acids [21], suggesting that the deletion could be specific for prostate tumors. Consistent with a number of sequences containing TBC/RabGAP domains, additional homologous EST sequences may be isoforms or truncation mutants isolated from different organs or tumor samples (V.M.M.B., unpublished data). Because of the widespread Armus distribution in different tissues (Figure S1B) and the distinct, more complete sequence than PARIS-1, we named the protein Armus (meaning "hinge, linker" in Latin [armus] and ancient Greek [αρμυσ]). According to the well-accepted function-based approach to name proteins, the name reflects the novel function of Armus as a linker between active Rac1 and Rab signaling pathways via the RabGAP domain [16].

The ability of Armus to interact specifically with activated Rac1 was confirmed in vitro by pull downs with in vitro translated Armus and immobilized active Rac1, RhoA, or Cdc42 (Figure 1C). The binding site for Rac1 is present at Armus N-terminal region, central coiled-coil domains ($Armus_{295-433}$, Figure 1D), which contains no sequences similar to CRIB motifs or other Rac-binding domains [22]. Armus interaction with wild-type Rac1 occurred in a GTP-dependent manner (Figures 1E and 1F). The isolated coiled-coiled domains bound more efficiently to active Rac when compared to $Armus_{1-550}$ (Figures 1E and 1F), consistent with the binding properties of other Rac targets [22].

Armus intracellular localization was quantified accordingly to its presence/absence at keratinocyte junctions (Figure 2). A proportion of exogenous PH domain ($Armus_{1-169}$) localized at cell-cell contacts in 73% of expressing cells, whereas the C-terminal region localized in the cytoplasm and less efficiently at junctions ($Armus_{547-928}$; Figures 2A and 2B). In contrast, although some faint junctional localization was observed, $Armus_{1-550}$ was mostly found at enlarged vesicles in the cytoplasm that did not contain E-cadherin (Figures 2A and 2B). It appeared that Armus is sequestered away from junctions in cells in which vesicles are present. Interestingly, distinct pools of full-length Armus ($Armus_{1-928}$) were seen at all intracellular localizations: cell-cell contacts, vesicles, and cytoplasm (Figures 2A and 2B), suggesting that no spurious localization was observed upon expression of truncation mutants.

Antisera were raised against three different peptides from Armus sequence and recognized a protein band of 105 KDa from keratinocyte lysates (Figures S2A and S2B), which corresponds to the predicted size of the full-length protein. Staining of endogenous Armus with affinity-purified antibodies (IC2) was faint, but clearly disappeared when antibodies were preincubated with the peptide antigen (Figure 2C). A pool of endogenous Armus localized at punctate structures in cytoplasm and at junctions in most keratinocytes (Figure 2C). Interestingly, endogenous Armus was specifically coimmunoprecipitated with endogenous E-cadherin, but not α3 integrins (Figure 2D and data not shown). As control, GIT1 [5], which indirectly associates with Rac1, could not be coprecipitated with E-cadherin complexes under these conditions (Figure 2D). Surprizingly, Armus PH domain mediated the association with cadherin complexes (Figure S2C). This result is consistent with the preferential localization of Armus PH domain to junctions (Figures 2A and 2B) and its inability to interact with lipids (data not shown). Our results indicate that a direct or indirect interaction with cadherin complexes may mediate Armus localization at cell-cell contacts.

Armus Is a Bona Fide GAP for Rab7

To directly test whether Armus RabGAP domain is functional, an in vitro GAP assay was performed on different Rabs (Figure 3A; Figure S3). Armus RabGAP domain potently enhanced the intrinsic GTP hydrolysis of Rab7, a Rab that regulates late endosomal transport and lysosomal biogenesis [6]. Deletion of the last C-terminal amino acids of Rab7 ($Rab7^{\Delta 22}$) had no effect on the ability of Armus to potentiate GTP hydrolysis on Rab7. Because no increase in hydrolysis was observed on other Rabs tested (Figure 3A), the above results strongly suggest that Armus RabGAP may be a GAP specific for Rab7. A caveat of the above assay (10 min time point) is that fast-hydrolyzing GTPases such as Rab5 cannot be formally excluded as substrate for Armus. Clearly, further work should extend the repertoire of Rab proteins to test for additional substrates of Armus RabGAP.

We next measured the activity of Rab7 in vivo by using the ability of its effector RILP to specifically interact with Rab7.GTP [23]. Expression of Armus C terminus significantly reduced the amount of active GFP-$Rab7^{WT}$ precipitated by GST-RILP (Figure 3B), suggesting that Armus inactivates Rab7 in vivo. Conversely, inhibition of Rac1 by dominant-negative Rac^{T17N} elevated the active levels of endogenous Rab7 (Figure 3C), indicating that Rac regulates endogenous Rab7 activation, a previously unreported function of Rac1. Taken together, our data convincingly argue that Armus RabGAP is able to inactivate Rab7 in vitro and in vivo. However, participation of Armus in other trafficking events cannot be formally excluded at this stage (see below).

Armus Is Required for Arf6-Induced Junction Disassembly

Armus association with cadherin complexes and localization at junctions suggests a putative function at cell-cell contacts (Figure 2). We investigated Arf6-induced destabilization of E-cadherin adhesion, because it is known to activate Rac1 and Rab7 [2, 15], two small GTPases functionally linked to Armus. Expression of activated Arf6 potently removed E-cadherin from keratinocyte cell-cell contacts and induced the accumulation of junctional components at a perinuclear localization (Figure 4; see also Figures 5A, 6A, and 6B). Similar events were observed with slower kinetics when a fast-cycling Arf6 mutant [24] was expressed (Figures S4A–S4C). Upon active Arf6 expression, endogenous Armus disappeared from junctions and a pool colocalized with internalized E-cadherin at the perinuclear compartment (Figure 4, see also Figure S4D for exogenous Armus). In contrast, the lysosomal markers LAMP1 and CD63 were recruited to, but remained adjacent to, sites where E-cadherin accumulated (Figure 4).

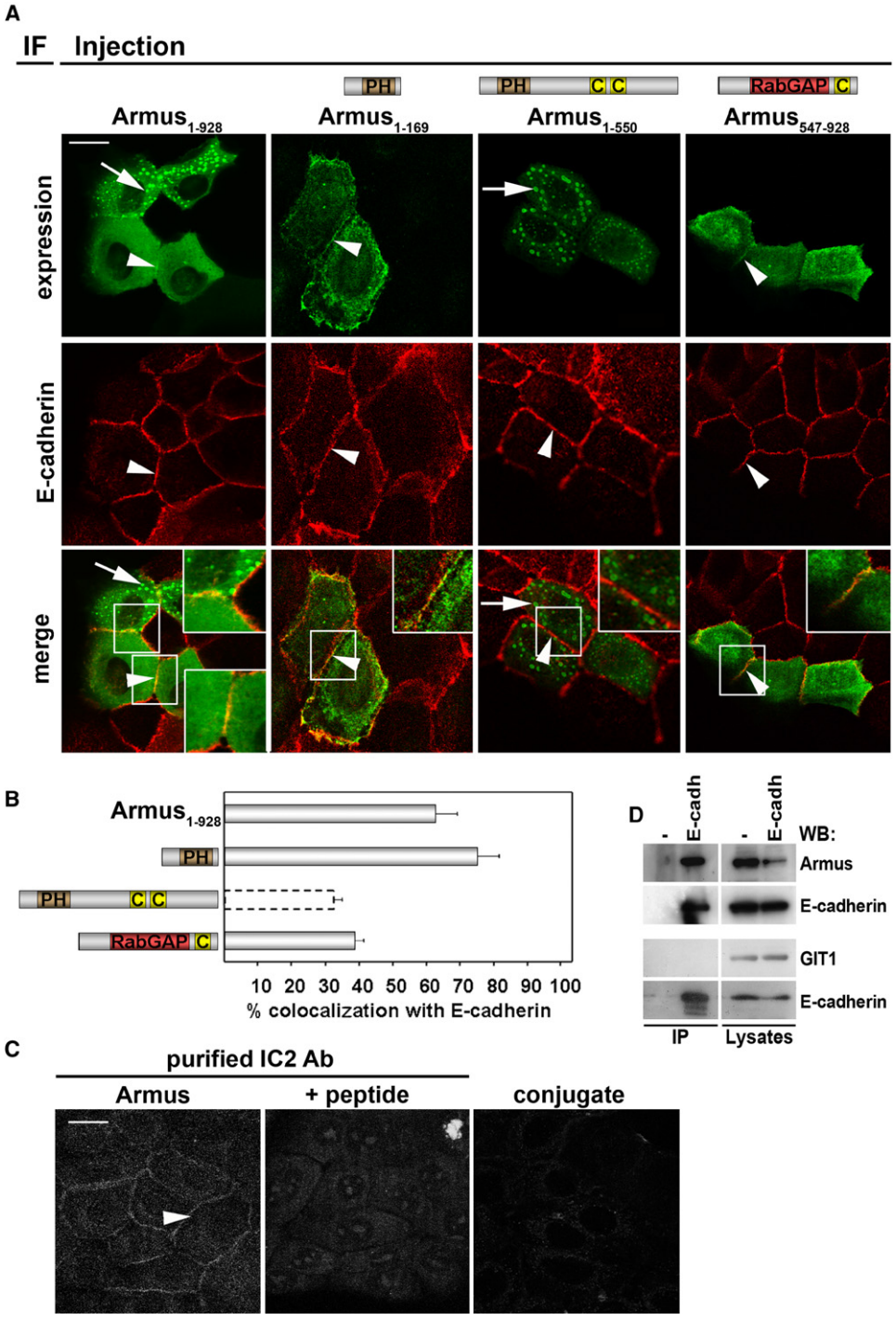

Figure 2. Intracellular Localization of Armus

(A) Armus full-length ($Armus_{1-928}$) and different truncation mutants were expressed in keratinocytes and their localization assessed by staining for endogenous E-cadherin and tagged constructs. Merged images are shown in the bottom panels. Inserts show amplification of the boxed areas.

(B) Quantification of the localization of exogenous Armus at sites of cell-cell contacts (presence/absence). Diagrams on the top of immunofluorescence pictures (A) and at the side of the graph (B) show the domain structure of constructs used. Dashed lines represent faint junctional staining of $Armus_{1-550}$.

(C) Affinity-purified IC2 antibodies (see also Figure S2) were used to stain endogenous Armus. IC2 preincubated with the antigen peptide (+peptide) or conjugate alone (conjugate) are shown as controls.

(D) Endogenous E-cadherin was immunoprecipitated from keratinocyte lysates, and the presence of endogenous Armus and GIT1 was assessed by western blots.

Arrowheads show junctional localization; arrows point to enlarged vesicles. IF, immunofluorescence. Error bars represent standard error. n = 3 (except for $Armus_{1-550}$ where n = 8). Scale bar represents 16 μm or 32 μm for insets.

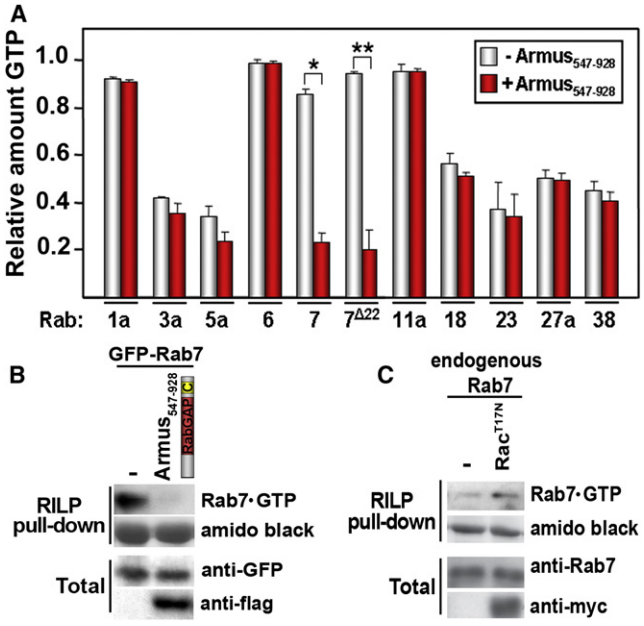

Figure 3. Armus RabGAP Has Activity toward Rab7 In Vitro and In Vivo

(A) GTP hydrolysis of wild-type versions of different Rabs was quantified in the absence (intrinsic activity, – $Armus_{547-928}$) or presence of Armus C-terminal domain (+ $Armus_{547-928}$). Values are expressed as relative GTP content [(GTP)/(GTP + GDP)]. For fusion proteins used, refer to Figure S3.

(B and C) Determination of the activation status of Rab7 in vivo. Lysates were prepared after expression of wild-type Rab7 (GFP-$Rab7^{WT}$) alone or in combination with Armus C-terminal region (flag-$Armus_{547-928}$) (B) or after inhibition of Rac1 (myc-$Rac1^{T17N}$, C). Active Rab7 was quantified by pull-down with GST-RILP (RILP pull-down). Expression of constructs or endogenous proteins was assessed with the antibodies shown on the right of each panel. GST-RILP was detected by amido-black staining. Error bars represent standard error. n = 3. *p < 0.00002; **p < 0.007.

Further characterization of this compartment will occur as a separate study.

Interestingly, coexpression of activated Arf6 with Armus C-terminal domain (WT) significantly prevented disruption of cell-cell adhesion and changes in keratinocyte morphology (Figures 5A and 5B), whereas an inactive Armus RabGAP mutant (RE) was not effective (Figure 5B; Figure S5A). PAK is a serine-threonine kinase that participates in many Rac functions [3, 22]. In contrast to Armus RabGAP expression, inhibition of PAK did not rescue the destabilization of cadherins induced by active Arf6 (PAK^{AID}; Figure 5C). It is unlikely that Armus C-terminal fragment could interfere with junction perturbation by titrating out active small GTPases: it was not able to interact with Rac1 or active Arf6 (Figure S5B and data not shown). The above results suggest that Armus is downstream of Arf6 activation and is specifically required for junction destabilization.

To substantiate the above result, endogenous Armus was efficiently depleted by RNAi (Figure 5D). After transfection of active Arf6 and Armus depletion, the amount of intact junctions increased 2-fold (Figure 5E; Figure S5C). The rescue obtained after Armus siRNA was partial and smaller than that obtained by expression of Armus C-terminal domain (Figure 5B). One explanation is that all keratinocytes expressing Arf6 were considered in the quantification. Thus, some cells might have residual Armus and show junction disruption, thereby lowering the rescue levels observed.

Furthermore, depletion of Armus protein is predicted to (1) remove the localized regulation/inactivation of Rab7 at vesicles being targeted to lysosomes and (2) increase Rab7 activation overall (because a GAP specific for Rab7 is not present). We interpret that the partial rescue effect of Armus depletion on junctions may result from the balance of these two responses. Whereas Armus RNAi may prevent the localized regulation of Rab7 cycling, Armus RabGAP expression is predicted to block the increase in Rab7.GTP levels induced by Arf6. Yet, both approaches can significantly rescue the stability of cell-cell contacts.

Our results suggest that Armus may specifically modulate E-cadherin transport to lysosomes by regulating Rab7 cycling. Total and endocytosed levels of E-cadherin and β1-integrin were determined after expression of different constructs (Figures 5F and 5G; Figure S6). Levels of internalized proteins were assessed by biotinylation of surface proteins on ice (30 min) after 3.5 hr of transfection of the different constructs (Figure 5G). After incubation at 37°C for different time points (1–5 hr), biotinylated surface proteins were stripped and lysates prepared. Biotinylated internalized proteins were precipitated with streptavidin-agarose and amount assessed by blots (see Figure S6).

Active Arf6 potently induced degradation of E-cadherin receptors (as shown by the reduction in total levels, Figure 5F; Figure S6A), yet total levels of β1-integrin were not affected (Figure S6C). Interestingly, coexpression of $Arf6^{Q67L}$ and Armus RabGAP prevented E-cadherin degradation and the observed increase in E-cadherin internalization (Figures 5F and 5G; Figure S6B). As controls, expression of GFP or Armus C-terminal region containing the RabGAP domain did not interfere with total levels or the internalized pool of E-cadherin (Figures 5F and 5G). Expression of Armus RabGAP or GFP did not affect β1-integrin internalization pattern (Figure S6D), similarly to what observed with E-cadherin. We surmise that a potential mechanism for the efficient rescue of Arf6-dependent junction disruption by $Armus_{547-928}$ may be the inhibition of cadherin degradation via inactivation of Rab7.

Armus Mediates a Crosstalk between Rac1 and Rab7

We reasoned that Armus may bring together signaling molecules that are relevant for perturbation of cell-cell adhesion induced by Arf6 (i.e., Rac1 and Rab7). In contrast to MDCK cells [14], when Arf6 was activated in keratinocytes, attenuation of Rac1 signaling efficiently prevented the disassembly of cadherin-dependent junctions (Figures 6A and 6C). Inhibition of Rab7 ($Rab7^{T22N}$) per se did not alter E-cadherin stability, but significantly interfered with junction disruption when coexpressed with active Arf6 (Figures 6B and 6D). In contrast, inhibition of Rab1 was unable to prevent junction disruption by Arf6 (Figure 6E), indicating the specificity for Rab7. Therefore, preventing activation of Rab7 (by dominant-negative $Rab7^{T22N}$) or forcibly inactivating Rab7 (by Armus) potently interfered with Arf6-dependent E-cadherin degradation.

In addition, Armus RabGAP expression or inhibition of Rac1 or Rab7 partially reversed the accumulation of E-cadherin at the perinuclear region induced by active Arf6 (Figure 6F). It is formally possible that Armus may also interfere with other trafficking events. For example, expression of Armus RabGAP redistributed vesicles induced by active Rab5 to the cell periphery (Figure S7). Thus, although Armus RabGAP did not appear to inactivate Rab5 (Figure 4A), it was clearly able to modulate the distribution of Rab5-induced vesicles. The

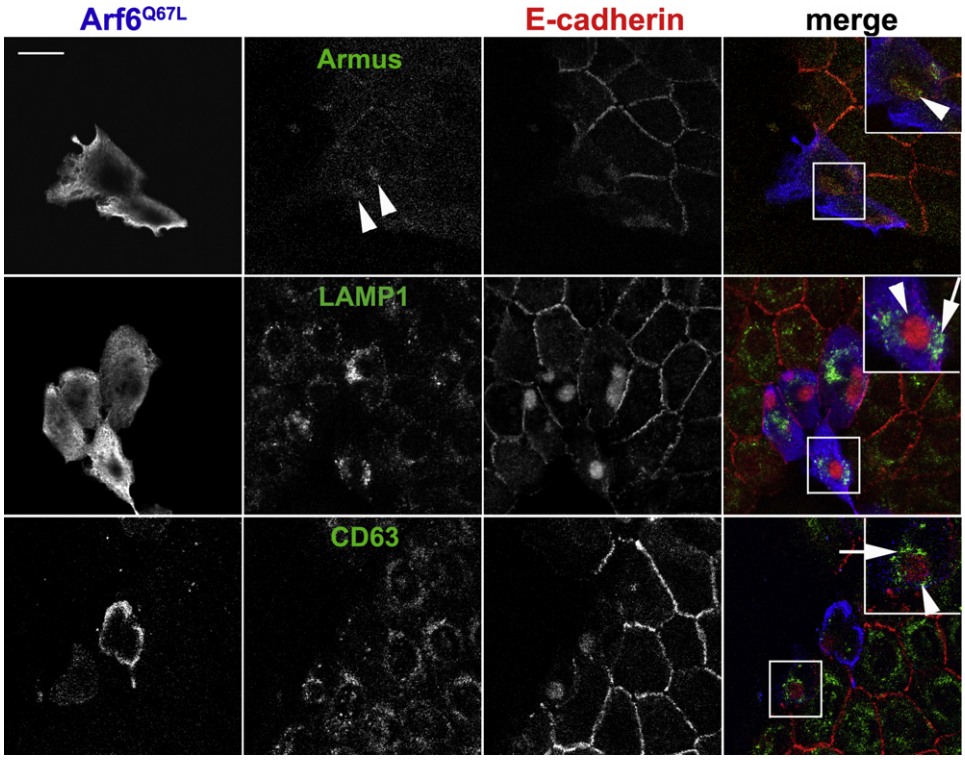

Figure 4. Armus Accumulates at a Perinuclear Region when Active Arf6 Is Expressed

Activated Arf6 (HA-Arf6^{Q67L}, 0.1 mg/ml) was expressed in keratinocytes for 3 hr. Cells were triple-labeled for HA-tag, E-cadherin, and endogenous Armus, LAMP1, or CD63 as markers of lysosomes. Insets show enlargement of the boxed area. Arrowheads indicate vesicular compartment with accumulated E-cadherin/Armus; arrows show recruited LAMP1 or CD63 staining. n = 3. Scale bar represents 16 μm or 32 μm for insets. See Figure S4 for supporting data.

precise mechanisms for this effect remain to be established in future work. Nevertheless, our results point to the key function of Rac1 and Rab7 in the regulation of cadherin turnover downstream of Arf6.

To address whether Armus also participates in cadherin degradation after a physiological stimulus, EGF-induced scattering was performed in HaCaTs, a nontransformed keratinocyte cell line. Expression of Armus C-terminal region (WT) reduced the ability to scatter in response to EGF, but not when mutated to impair its GAP activity (RE; Figures 7A and 7B). EGF treatment induced activation of Rab7 (Rab7.GTP), yet no changes were detected in Rab7 protein levels (Figure 7C). EGF-dependent activation of Rab7 was counteracted by expression of Armus C-terminal domain, but not the catalytic-deficient mutant (Figure 7C). Interestingly, blocking Arf6, Rac1, or Rab7 activation by dominant-negative mutants also significantly prevented EGF scattering (Figure 7D). Our data strongly support the participation of Armus and its functional partners in scattering induced by EGF. Furthermore, our results indicate that a coordination of signaling between Rac1 activation, its effector Armus, and Rab7 cycling is required to ensure efficient destabilization of E-cadherin adhesion and receptor degradation.

Discussion

A fundamental issue in signaling is how different small GTPases cross-talk to each other in the regulation of a single cellular process. Here, we characterize Armus, a TBC/RabGAP-containing protein [16], that provides a potential mechanism via which Rac1 can directly couple to Rab7 signaling and intracellular trafficking. The relevance of our findings is 3-fold: (1) we show that Rac1 is able to regulate Rab7 function, a previously unknown cellular role for Rac; (2) Armus is a key molecule to relay signals from active Rac1 to modulate Rab7 cycling; and (3) importantly, we have identified a function for Armus in the regulation of E-cadherin turnover.

There are about 52 coding sequences predicted to contain TBC/RabGAP domains in human databases [16]. The TCB/RabGAP family has generated much interest [25, 26], because the upstream regulation of Rab function is poorly understood [6]. Moreover, RabGAP proteins have been implicated in diseases such as Warburg microsyndrome [27] and different types of cancers [21, 28, 29]. Apart from three polymorphic sites, Armus is identical to the TBC1D2 isoform A variant c and contains extra amino acids than PARIS-1, an isoform overexpressed in prostate cancer [16, 21]. However, the function of these proteins is not known nor have their Rab substrates and interacting partners been identified.

Armus has a unique combination of domains with the possibility to integrate the function of different small GTPases. Armus PH domain mediates localization at cell-cell contacts and coprecipitates with cadherin complexes. The central coiled-coil domains interact specifically with active Rac1 but do not contain CRIB motifs, consistent with a subset of effectors that bind exclusively to Rac [22]. Finally, a C-terminal TBC/RabGAP domain contains a predicted arginine finger responsible for catalysis [16, 30] and potently inactivates Rab7, an important regulator of lysosomal biogenesis, in vivo and in vitro. Although Armus does not appear to inactivate different Rabs tested here, it is possible that further studies may unravel additional Rab substrates.

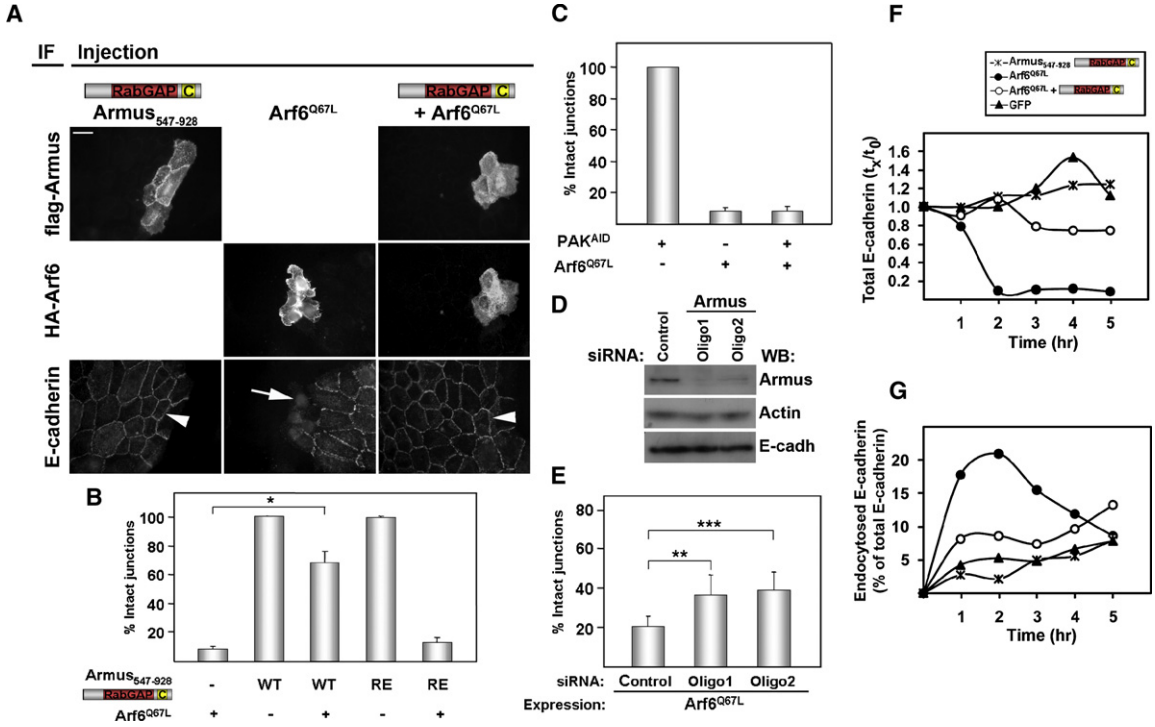

Figure 5. Armus Functions Downstream of Arf6 during Disassembly of Cell-Cell Contacts

(A) $Armus_{547-928}$ was expressed by itself or in combination with active $Arf6^{Q67L}$. Keratinocytes were fixed and stained for the respective tags and E-cadherin. Arrowheads indicate intact junctions; arrow shows accumulation of intracellular E-cadherin. Scale bar represents 50 μm.

(B and C) Percentage of intact junctions in neighboring cells expressing $Arf6^{Q67L}$ alone or in combination with $Armus_{547-928}$ (wild-type or RE mutant, B) or PAK autoinhibitory domain (PAK^{AID}, C). Armus RE mutant images are shown in Figure S5A.

(D) Keratinocytes were treated with different siRNA oligos, lysates prepared, and probed with antibodies shown on the left of panels.

(E) After Armus RNAi, cells were transfected with HA-$Arf6^{Q67L}$ and the percentage of intact junctions between neighboring, Arf6-expressing cells was quantified as described in Experimental Procedures. Immunofluorescence images are shown in Figure S5C.

(F and G) After transfection of $Arf6^{Q67L}$ alone or in combination with $Armus_{547-928}$ for 3.5 hr, cells were biotinylated and the total amount (F) and the internalized pool (G) of E-cadherin receptors were determined. Time is shown in hours after biotinylation. Expression of GFP was used as controls. Values were normalized to the amount quantified at time 0 (t_0); t_X is the expression level observed in time X. Corrensponding western blots are shown in Figure S6. Error bars represent standard error. n = 3. *p < 0.006; **p < 0.03; ***p < 0.014.

Our hypothesis is that Armus may facilitate delivery to lysosomes because the release of a Rab from acceptor membranes requires its inactivation [6]. To our knowledge, Rac1 has not been previously implicated in Rab7 and lysosomal function. It has been proposed that Rac1 participates in intracellular trafficking indirectly, via reorganization of the cortical cytoskeleton or actin-dependent movement of vesicles [13]. However, our data indicate that Rac1 signaling may interfere with trafficking more directly by regulating Rab/Arf localization and function, as shown previously [17, 18, 31]. Indeed, we show here that inhibition of Rac1 can activate endogenous Rab7. We suggest that Armus potentially modulates Rab7 activity at sites in which active Rac1 is present (Figure 7E).

Rac1 is essential for Arf6-induced breakdown of epithelial colonies, motility, and cytoskeletal rearrangements [8, 14, 24, 32]. Arf6 requires Rac1 activation for disruption of keratinocyte junction (Figure 7E), in line with the ability of Arf6 to activate Rac1 by different mechanisms [2]. However, our results differ from MDCK cells because, in the latter, disassembly of cell-cell contacts by Arf6 correlates with Rac1 downregulation [14, 15]. This difference most likely reflects cell type-specific regulation of junctions [4, 10, 12, 33].

We demonstrate that Armus is a key molecule to coordinate Rac1 and Rab7 activities in the pathway Arf6 > Rac1 > Armus > Rab7 (Figure 7E). In support of our model, junction destabilization induced by active Arf6 is efficiently rescued upon interfering with the function of endogenous Rac1, Armus, or Rab7 (Figure 7F). Our data suggest that Armus function on cadherin adhesion is specific for Rab7 inactivation and as a Rac1 effector: Armus does not sequester active Rac1 or Arf6, and inhibition of Rab1 or PAK1, a Rac effector, cannot block junction disassembly triggered by Arf6.

We envisage three potential mechanisms via which Armus RabGAP expression maintains stable junctions after Arf6 activation (Figure 7F). First, coexpression of Armus RabGAP with active Arf6 reduces the levels of internalized E-cadherin considerably. However, at steady-state Armus RabGAP expression does not perturb E-cadherin levels or localization at junctions. Moreover, Armus does not appear to inactivate Rab5 or Rab11, Rabs that regulate endocytic/recycling transport [6, 13]. Yet, Armus RabGAP redistributes active Rab5-positive vesicles to cell periphery, suggesting a potential regulation of early trafficking events and/or cadherin endocytosis more directly (Figure 7F).

Second, rescue of E-cadherin junctional levels by Armus RabGAP expression may result indirectly from preventing lysosomal targeting, thereby increasing E-cadherin recycling to the surface (Figure 7F). Indeed, impairment of a trafficking route can redirect proteins to different intracellular compartments, i.e., surface levels/degradation of other receptors can

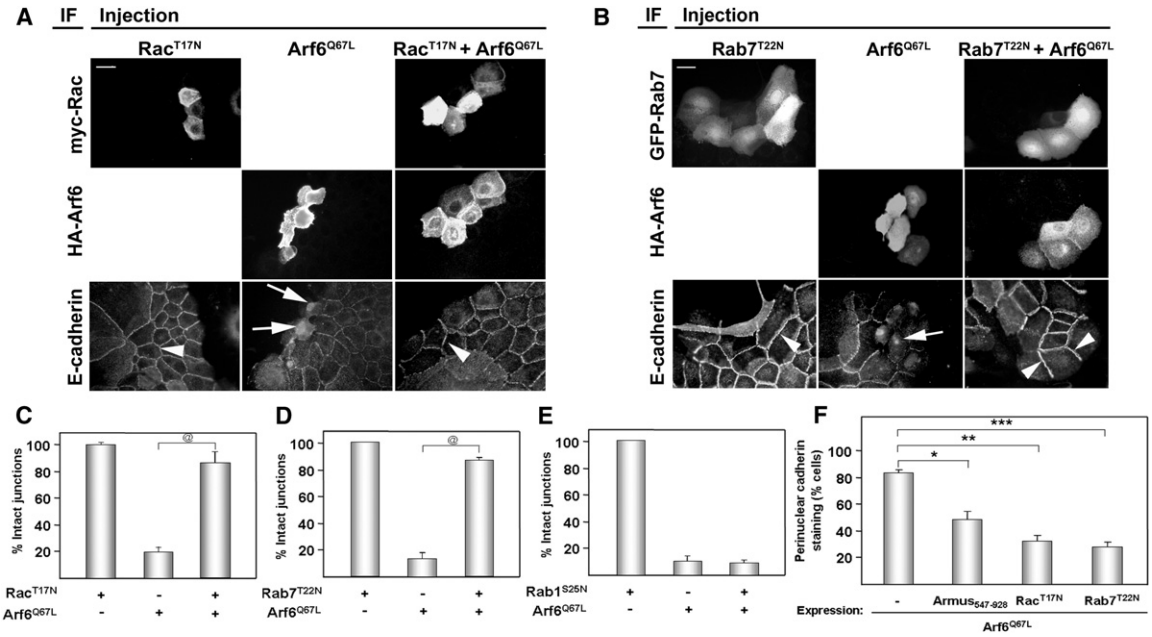

Figure 6. Rac1 and Rab7 Are Required Downstream of Arf6 to Perturb Cadherin Adhesion

(A and B) Expression of activated Arf6 (HA-Arf6^{Q67L}), dominant-negative Rac1 (myc-Rac^{T17N}), or Rab7 (0.05 mg/ml, Rab7^{T22N}) alone or in combination was performed for 3 hr (A and B), after which cells were fixed and stained for the tags and E-cadherin. Rac1 signaling was attenuated, but not to sufficient levels to disrupt cell-cell contacts [11]. Arrows point to accumulation of cadherin at perinuclear region; arrowheads show intact junctions. Scale bars represent 50 μm. See Figure S6 for effects of Armus RabGAP on Rab5-induced vesicles.

(C–E) Quantification of the percentage of cells containing intact junctions and expressing Arf6 alone or in combination with the dominant-negative constructs: Rac^{T17N} (C), Rab7^{T22N} (D), or Rab1^{S25N} as control (E).

(F) Percentage of coexpressing cells containing perinuclear accumulation of E-cadherin after microinjection of Arf6^{Q67L} alone or in combination with Armus$_{547-928}$, Rac^{T17N}, or Rab7^{T22N}. Error bars represent standard error. n ≥ 3. $^{@}p < 0.0004$; *p < 0.0009; **p < 0.00001; ***p < 0.00002.

also be regulated by manipulation of Rab7 activity [34, 35]. Our interpretation is supported by two lines of evidence: (1) when Armus is coexpressed with Arf6, cadherin receptors are not degraded and accumulation of E-cadherin at the perinuclear region is reduced; and (2) Rab7 inhibition per se is sufficient to rescue dissociation of junctions very efficiently.

Third, it is reasonable that, by inhibiting Rab7 cycling and lysosomal targeting, Armus RabGAP may induce Arf6-dependent recycling of internalized cadherin receptors from perinuclear compartment back to junctions. Arf6 is known to regulate both endocytosis and exocytosis [2] and has functional links with Rab11, one of the Rabs responsible for recycling from the perinuclear compartment [6]. Thus in our model, when Armus RabGAP is expressed or Rab7 inhibited, Arf6 signaling could be diverted to Rab11 to recycle receptors to the cell surface (Figure 7F).

Regulation of early trafficking events and lysosomal delivery downstream of Arf6 are not mutually exclusive possibilities. In fact, they may be consistent with the localization of Armus at junctions and perinuclear compartment where E-cadherin accumulates. We surmise that a potential interference on early trafficking events by Armus RabGAP may result from: (1) direct inactivation of additional Rab family members, (2) indirectly overloading intracellular transport by inhibition of lysosomal function, or (3) alteration of Arf6 signaling favoring recycling. Irrespective of the relative contribution of lysosomal delivery versus increased endocytosis, our data strongly demonstrate the involvement of Rac1, Armus, and Rab7 in cadherin turnover downstream of Arf6.

Interestingly, the pathway Arf6 > Rac1 > Armus > Rab7 also operates during EGF-induced scattering of keratinocytes. EGF is an essential growth factor during epithelial morphogenesis and homeostasis. Conversely, increased EGFR signaling occurs in many different epithelia-derived tumors leading to scattering, invasion, and poor prognosis [36]. Yet, the signaling events downstream of EGF receptors that mediate cell scattering are comparatively less understood than HGF-induced scattering, for example. We demonstrate for the first time the requirement of Arf6, Rac, and Rab7 as well as Armus in EGF-dependent epithelial scattering. Our findings point out to the potential widespread importance and physiological significance of the pathway.

Our data underscore the complexity of the interplay between signaling by different small GTPases and how dissecting the coordination of their activities at the molecular level can further our knowledge. The participation of Arf6 [2, 37] and Rac1 activation [4] in different aspects of epithelial tumorigenesis highlights the need to understand the molecular mechanisms involved and identify suitable therapeutic targets. Interestingly, Rab7 is overexpressed in adenomas [38] and interfering with Rab7 function has been suggested as a potential cancer therapy [39]. Armus-related proteins are upregulated in different epithelial tumors (PARIS-1 [21] and other truncated versions; data not shown), although their precise role (and consequence of deletions) remains to be established. Taken together with our data, there is a strong case that Armus-regulated pathway(s) may be important in E-cadherin degradation to facilitate metastasis during tumorigenesis. Because stable junctional E-cadherin is essential for epithelial homeostasis, our data have significant implications for tumor dedifferentiation and progression. Armus may be a good candidate to block epithelial dedifferentiation during

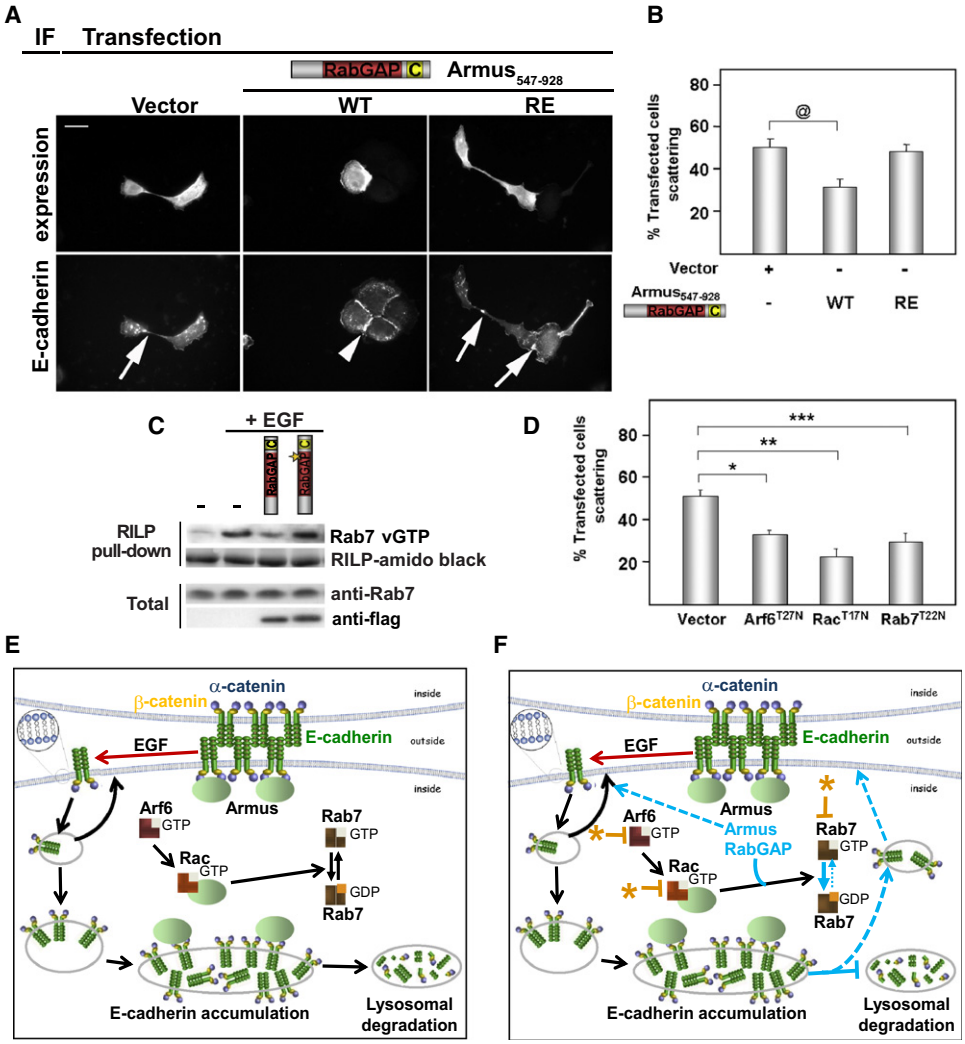

Figure 7. EGF-Induced Scattering Requires Armus Function in Keratinocytes

(A) HaCaT cells were transfected with Armus C-terminal domain ($Armus_{547-928}$, wild-type [WT], or catalytic inactive mutant [RE]), induced to scatter by treatment with EGF, fixed, and stained for the tag (expression) and E-cadherin. Arrows indicate a reduction of E-cadherin staining in cells beginning to scatter; arrowhead points to increased E-cadherin at junctions comparable to control nonexpressing cells. Scale bar represents 50 μm.

(B) Quantification of the number of scattered HaCaTs after EGF treatment as described in (A).

(C) HaCaT cells were treated as described in (A), and lysates were prepared to determine active Rab7 levels via RILP pull-down. Endogenous Rab7 and $Armus_{547-928}$ expression were detected with antibodies shown on the right of the panel; GST-RILP was detected with amido black staining.

(D) Quantification of the number of scattered HaCaTs after treatment with EGF and expression of dominant-negative $Arf6^{T27N}$, Rac^{T17N}, $Rab7^{T22N}$ constructs.

(E and F) Diagram summarizing our results.

(E) Expression of activated Arf6 or treatment with EGF in keratinocytes destabilizes E-cadherin adhesion and Arf6 promotes cadherin accumulation at a perinuclear region. Armus localizes at junctions and, after Arf6 activation, also accumulates at the perinuclear compartment. Active Arf6 induces Rac1 activation, Armus recruitment, and modulation of Rab7 cycling to allow degradation of E-cadherin in lysosomes.

(F) Expression of Armus RabGAP, inhibition of Arf6, Rac1, or Rab7 (respective dominant-negative constructs, shown by asterisk) prevents EGF-dependent degradation of E-cadherin in lysosomes. We hypothesize that, by forcibly inactivating Rab7, Armus RabGAP perturbs lysosomal targeting of cadherins and favors recycling of the receptors to cell surface/junctions or divert Arf6-dependent signaling to recycling (dashed lines). Error bars represent standard error. $n \geq 3$. $^{@}p < 0.008$; $^{*}p < 0.0007$; $^{**}p < 0.0004$; $^{***}p < 0.002$.

malignancy because of its efficient and specific inhibition of cadherin degradation.

Experimental Procedures

Cells

Normal human keratinocytes isolated from neonatal foreskin were cultured as described previously [40]. For RNAi experiments, keratinocytes were grown in low calcium medium [40]. HaCaT (immortalized keratinocytes) and COS-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma) containing 10% FCS and 5 mM glutamine. For scattering assays, HaCaTs were seeded in coverslips (1×10^4 cells) and cultured until small colonies were formed (4–8 cells). After transfection and expression for 24 hr, HaCaT cells were stimulated with 100 nM EGF in serum-free media containing 0.1% fatty acid-free BSA and incubated for 24 hr. For biotinylation assays, normal keratinocytes were seeded on 24-well plates and grown until 70% confluence.

Determination of Rab7 Activity Levels In Vivo

After expression of different constructs for 24 hr, HaCaT cells were lysed in lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 1% NP-40, 2.5 mM $MgCl_2$,

and 1 mM of each protease inhibitor PMSF, leupeptin, pepstatin, and pefabloc). Lysates were cleared by centrifugation at 14,000 rpm at 4°C for 5 min and incubated for 1 hr with RILP-GST beads at 4°C for 1 hr. After washing three times with washing buffer (same composition as above but without NP-40), beads were collected and proteins attached to beads were probed by western blotting. To detect endogenous Rab7 activation, cells were transfected with active HA-Arf6 or treated with EGF (100 mM in same media) for 24 hr in the presence or absence of different Armus constructs, then lysed and processed as above.

Quantifications

Quantification of the localization of Armus constructs at cadherin-dependent cell-cell contacts was performed with the following criteria. Cells were scored positive if exogenous Armus colocalized with cadherins and calculated relative to the total number of expressing cells (arbitrarily set as 100%). For quantification of junction disruption, only cell-cell contacts between two expressing cells were considered. Arf6-induced junction disruption was assessed by scoring cell-cell contacts between two expressing cells as intact (when cadherin staining was present). Values were shown as a percentage of total junctions between expressing cells. For scattering assays, only expressing cells at the periphery of epithelial colonies were counted (not single, isolated cells). Cells were scored as scattering if E-cadherin was absent from junctions, a reduction in the levels of junctional E-cadherin was observed, or cell shape was fusiform when compared to controls. Error bars represent standard error of the means. Statistics were calculated by Student's t test.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at doi:10.1016/j.cub.2009.12.053.

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