# The Rac activator Tiam1 controls tight junction biogenesis in keratinocytes through binding to and activation of the Par polarity complex

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The GTPases Rac and Cdc42 play a pivotal role in the establishment of cell polarity by stimulating biogenesis of tight junctions (TJs). In this study, we show that the Rac-specific guanine nucleotide exchange factor Tiam1 (T-lymphoma invasion and metastasis) controls the cell polarity of epidermal keratinocytes. Similar to wild-type (WT) keratinocytes, Tiam1-deficient cells establish primordial E-cadherin–based adhesions, but subsequent junction maturation and membrane sealing are severely impaired. Tiam1 and V12Rac1 can rescue the TJ maturation defect in Tiam1-deficient cells, indicating that this defect is the result of impaired Tiam1–Rac signaling. Tiam1 interacts with Par3 and aPKCζ, which are two components of the conserved Par3–Par6–aPKC polarity complex, and triggers biogenesis of the TJ through the activation of Rac and aPKCζ, which is independent of Cdc42. Rac is activated upon the formation of primordial adhesions (PAs) in WT but not in Tiam1-deficient cells. Our data indicate that Tiam1-mediated activation of Rac in PAs controls TJ biogenesis and polarity in epithelial cells by association with and activation of the Par3–Par6–aPKC polarity complex.

## Introduction

In epithelial cells, apical–basal polarity is maintained through the formation of several intercellular adhesion systems consisting of adherens junctions (AJs), desmosomes, and tight junctions (TJs). The TJ consists of transmembrane proteins occludin, claudins, and junctional adhesion molecules (JAMs), which are organized in intramembranous strands and are linked to the F-actin cytoskeleton either directly or indirectly through members of the MAGUK (membrane-associated guanylate kinase) family of proteins ZO-1, -2, and -3. The TJ regulates paracellular diffusion and functionally segregates the plasma membrane into two compartments, which is a requirement for full polarization of epithelial cells (Tsukita et al., 2001).

In epithelial cells, Cdc42 and Rac1 control the formation and function of the AJ and TJ (Lozano et al., 2003). During formation of intercellular contacts, cadherins cluster at the plasma membrane to form spotlike structures, or puncta, at the end of thin actin cables that extend from the puncta toward the cortical F-actin cytoskeleton (Vasioukhin and Fuchs, 2001). These primordial adhesions (PAs), which contain components of both the AJ and TJ, are subsequently assembled in beltlike AJs and TJs. This process is accompanied by reorganization of the cortical actin cytoskeleton and establishment of cell polarity. In mammalian epithelia, the Par3–Par6–aPKC polarity complex is necessary for the establishment of cell polarity. It localizes to the TJ and regulates its formation and positioning with respect to basolateral and apical membrane domains (Macara, 2004). The polarity complex is recruited to PAs (Suzuki et al., 2002), where it is thought to be activated through binding of active Cdc42 and Rac1 to Par6 (Lin et al., 2000). The subsequent activation of aPKC (PKCζ or aPKCλ/ι) leads to the assembly of TJs, although the exact downstream events are still unknown. Through initial cell–cell contacts, cadherins together with nectins stimulate the activity of Cdc42 and Rac (Yap and Kovacs, 2003), which has been proposed as the cue that activates the polarity complex (Takai et al., 2003). We have previously identified the Rac activator Tiam1 (T-lymphoma invasion and metastasis), which stimulates the strength of intercellular adhesion (Malliri et al., 2004). In this study, we show that Tiam1-mediated Rac activation controls TJ biogenesis and cell polarity by association with and activation of the Par polarity complex in keratinocytes.

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Abbreviations used in this paper: AJ, adherens junction; DH, Dbl homology; FL, full length; GAP, GTPase-activating protein; JAM, junctional adhesion molecule; KO, knockout; MBP, myelin basic protein; PA, primordial adhesion; siRNA, small interference RNA; STEF, SIF and Tiam1-like exchange factor; Tiam, T-lymphoma invasion and metastasis; TJ, tight junction; WT, wild type.

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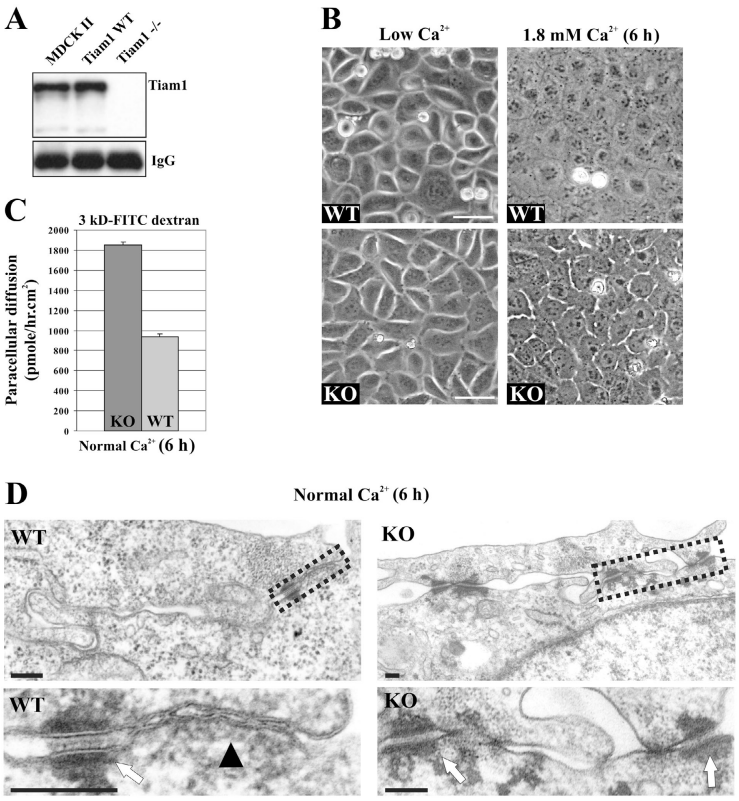


Figure 1. **Tiam1 is required for membrane sealing and TJ formation in epidermal keratinocytes.** (A) Tiam1 was immunoprecipitated from cell lysates using anti-Tiam1 antibody (C16). Western blotting was performed with anti-Tiam1 antibody (DH). (B–D) WT and Tiam1KO cells were grown to confluency and incubated with 1.8 mM $Ca^{2+}$ (normal $Ca^{2+}$) for 6 h. (B) Phase-contrast images show sealing of the paracellular space in WT but not in Tiam1KO cells. Bars, 20 μm. (C) Paracellular diffusion of 3 kD FITC-dextran through keratinocyte monolayers that were cultured for 6 h in 1.8 mM $Ca^{2+}$. Paracellular diffusion was measured for 2.5 h. Data are shown as means ± SD from three independent experiments. (D) EM images of the apical part of the lateral plasma membrane of keratinocyte monolayers. Arrowhead indicates TJ; arrows indicate desmosomes. Boxes with dotted lines indicate the area that is magnified in the bottom panel. Notice the membrane fusions that are typical of TJs in WT cells. Bars, 200 nm.

## Results and discussion

To study the role of Tiam1 in the establishment of intercellular contacts and cell polarity, we used epidermal keratinocytes that were derived from wild-type (WT) and Tiam1 knockout (KO) mice. Cells were cultured in medium containing low $Ca^{2+}$ levels (0.02 mM) to prevent terminal differentiation. The expression profile of several differentiation markers reflected typical nondifferentiated cells of the basal layer of the epidermis (unpublished data). WT keratinocytes showed similar Tiam1 protein levels as MDCKII cells, whereas Tiam1KO keratinocytes lacked the protein (Fig. 1 A).

To gain insight into the function of Tiam1 in the formation and maturation of intercellular contacts, we raised $Ca^{2+}$ concentrations in the medium of confluent monolayers of WT and Tiam1KO keratinocytes to 1.8 mM (normal). Phase-contrast microscopy revealed that WT keratinocytes completely sealed the paracellular space within 6 h in normal $Ca^{2+}$, whereas this process was impaired in Tiam1KO cells (Fig. 1 B). The high paracellular diffusion of a nonionic molecular tracer through Tiam1KO monolayers (Fig. 1 C) suggested that incomplete sealing was associated with the absence of TJs. Indeed, ultrastructural studies showed that Tiam1KO keratinocytes lacked typical TJ structures after 6 h in normal $Ca^{2+}$, whereas TJs were clearly present in virtually all WT cells. No differences were found in the formation of desmosomes in both cell types (Fig. 1 D). From these data, we conclude that Tiam1KO cells are impaired in TJ formation and sealing of the paracellular space.

The aberrant phenotype of Tiam1KO cells could be the result of impaired formation of initial E-cadherin–dependent cell–cell contacts, as these PAs are considered to be a prerequisite for the formation and maturation of TJs (Lozano et al., 2003). PAs contain components of AJs, TJs (occludin, claudin-1, JAM-A, and ZO-1), and nectins, and these are functionally segregated during the subsequent polarization process (Suzuki et al., 2002). Therefore, we first analyzed the localization of components of both AJ and TJ on the induction of cell–cell contacts. After 30 min in normal $Ca^{2+}$, occludin, ZO-1, and E-cadherin colocalized at the ends of F-actin bundles at the interphase of neighboring cells in WT and Tiam1KO cells (Fig. 2 A, top). Also, nectin-2 and afadin were localized in these PAs (Fig. S1, A and B; available at http://www.jcb.org/cgi/content/full/jcb.200502129/DC1). All adhesion molecules were organized in zipperlike structures, as described in epithelial cells establishing initial cell–cell contacts (Vasioukhin and Fuchs, 2001). A similar pattern of localization was observed for β-catenin, claudin-1, and JAM-A (not depicted). Apparently, both WT and Tiam1KO keratinocytes form PAs equally well within 30 min.

WT cells had made fully matured intercellular junctions in which E-cadherin, occludin, ZO-1, nectin-2, and afadin perfectly aligned along the cortical actin cytoskeleton in a linear fashion 6 h after the $Ca^{2+}$ switch (Fig. 2 A and Fig. S1, A and B). In addition, confocal xz projections of these cells showed clear spatial separation of TJs and AJs at the lateral membrane (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200502129/DC1). In contrast, no maturation of junctions occurred in Tiam1KO cells, and adhesion zippers were still present in between all cells (Fig. 2 A, bottom). F-actin bundles were tightly packed in beltlike structures at the apical side

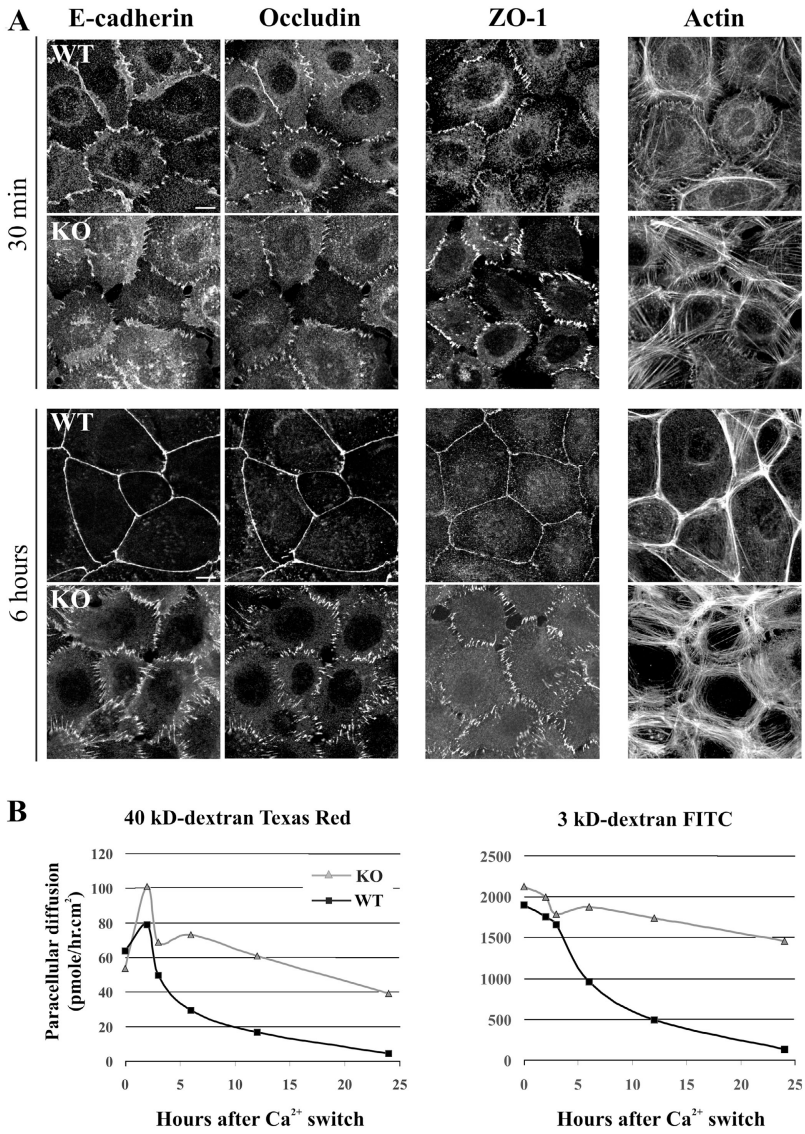


Figure 2. **Tiam1 deficiency impairs junction maturation and TJ barrier function.** (A) Tiam1 is not essential for the formation of PAs, but its absence impairs subsequent junction maturation. The concentration of $Ca^{2+}$ was increased to 1.8 mM in the culture medium of keratinocyte monolayers for the indicated times. Cells were double stained with anti–E-cadherin and antioccludin antibodies or stained with anti–ZO-1 antibody or phalloidin for F-actin. Bars, 10 μm. (B) Tiam1 deficiency impairs TJ barrier function. Paracellular diffusion of 3 kD FITC-dextran and 40 kD Texas red–dextran through keratinocyte monolayers, which were cultured in 1.8 mM $Ca^{2+}$ for the indicated time points. Paracellular diffusion was measured for 2.5 h. A representative example of three independent experiments is shown.

of the cell in conjunction with the adhesive structures 6 h after the $Ca^{2+}$ switch in WT cells. In Tiam1KO cells, actin bundles were not organized at the cell cortex, and many stress fibers spanned the entire cell (Fig. 2 A, bottom). The impaired junction maturation in Tiam1KO cells was seen even 24 h after the $Ca^{2+}$ switch (Fig. S1 C) and correlated with the absence of TJs. After 36–48 h, Tiam1KO cells were eventually able to form linearly organized adhesive structures with matured TJs and largely sealed the paracellular space (Fig. S1, C and D). At these later time points, the spatial separation of TJs and AJs as determined by ZO-1 and E-cadherin staining, respectively, was also seen in xz projections of the lateral membrane of Tiam1KO keratinocytes (Fig. S2). The impaired junction maturation was consistent with findings that paracellular diffusion of both 3- and 40-kD dextran through Tiam1KO monolayers was persistently high even 24 h after the $Ca^{2+}$ switch (Fig. 2 B). In WT cells, the diffusion of both tracers rapidly decreased within 6 h after the $Ca^{2+}$ switch. The fact that Tiam1KO cells were eventually able to form mature TJs could not be explained by up-regulation of the Tiam1-related guanidyl exchange factor STEF1 (SIF and Tiam1-like exchange factor), which is also termed Tiam2. STEF1 mRNA and protein were hardly expressed by keratinocytes, and no differences were found between the two genotypes (Fig. S2, B and C).

Our data indicate that Tiam1KO keratinocytes are able to form PAs with similar kinetics as WT cells. However, the maturation of these PAs into functional TJs is impaired, which is consistent with the high paracellular diffusion that was observed in Tiam1KO cells.

### Tiam1 regulates junction maturation in a Rac-dependent manner

To demonstrate that impaired TJ formation in Tiam1KO keratinocytes was caused by a lack of Tiam1, we restored Tiam1 and Rac1 activity in Tiam1KO cells. The re-expression of full-length (FL) Tiam1 or constitutively active V12Rac1 led to complete maturation of intercellular adhesions within 6 h after the $Ca^{2+}$ switch with similar kinetics as found in WT cells (Fig. 3, A and B). Moreover, a Tiam1 mutant with a short deletion in the catalytic Dbl homology (DH) domain (Tiam1ΔDH),

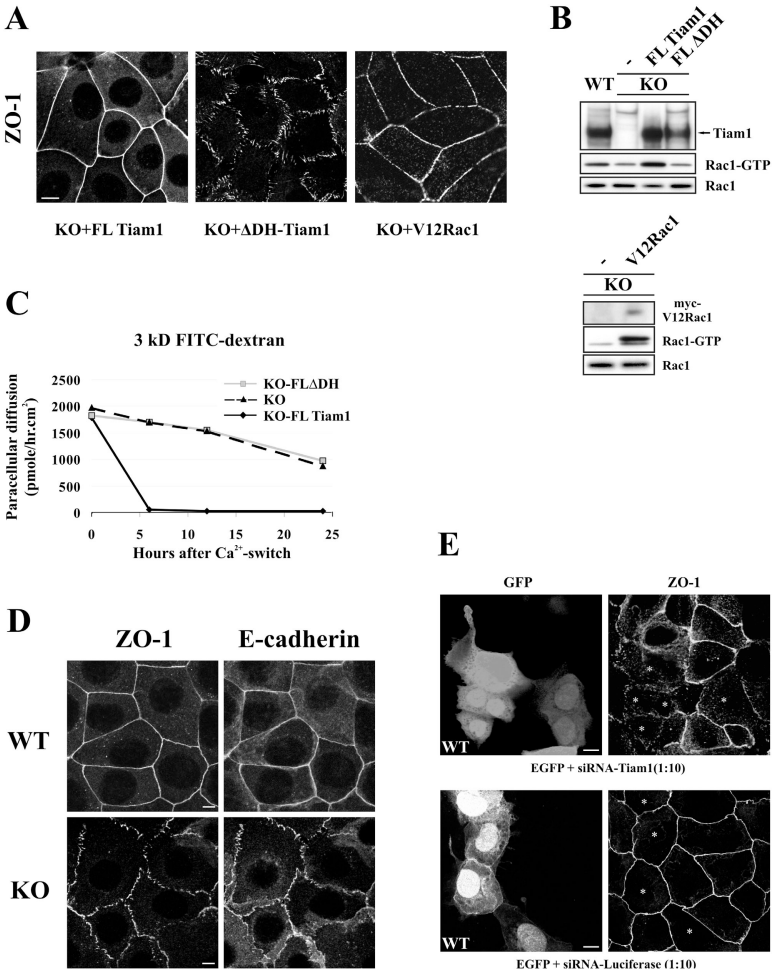


Figure 3. **Tiam1 signaling to Rac is required for junction maturation and TJ formation.** (A) Expression of V12Rac1 or FL-Tiam1, but not of catalytically inactive Tiam1 (Tiam1ΔDH), in Tiam1−/− cells restores junction maturation within 6 h after a $Ca^{2+}$ switch. (B) Western blot analysis and Rac activity assays on total lysates from Tiam1KO cells stably expressing the indicated Tiam1 constructs and myc-tagged V12Rac1. (C) Expression of FL-Tiam1, but not Tiam1ΔDH, restores TJ barrier function in Tiam1KO keratinocytes. Paracellular diffusion was measured at the indicated time points after a $Ca^{2+}$ switch. (D) Primary WT and Tiam1KO keratinocytes (4 d after isolation) that were switched to normal $Ca^{2+}$ for 6 h display a similar phenotype as immortilized keratinocytes. Cells were double stained for ZO-1 and E-cadherin. (E) Down-regulation of Tiam1 disturbs junction maturation in WT keratinocytes. EGFP was cotransfected with pSUPER-siRNA-Tiam1 or with siRNA-luciferase (control) into WT keratinocytes in a molar ratio of 1:10. After 48 h, cells were stained for ZO-1 (6 h after the $Ca^{2+}$ switch). Asterisks indicate GFP-positive (transfected) cells, as shown on the left. Bars, 10 μm.

which is unable to activate Rac (Fig. 3 B), could not rescue the impaired TJ formation in Tiam1KO cells, indicating that the capacity of Tiam1 to activate Rac is required for TJ maturation. These morphological data were confirmed by analyzing paracellular diffusion in Tiam1KO cells expressing various Tiam1 constructs (Fig. 3 C). Impaired TJ maturation was also seen in nonimmortalized primary Tiam1KO keratinocytes 4 d after isolation (Fig. 3 D). Conversely, down-regulation of endogenous Tiam1 in WT keratinocytes by small interference RNA (siRNA) resulted in impaired junction maturation, whereas control luciferase siRNA had no effect (Fig. 3 E). This confirms that the observed phenotype depends on the presence or absence of Tiam1. From these data, we conclude that the capacity of Tiam1 to activate Rac is required for proper maturation of intercellular junctions in keratinocytes.

### Tiam1-Rac signaling influences the expression of TJ molecules

Impaired TJ formation could be a consequence of decreased expression of TJ molecules in Tiam1KO cells, as the number of TJ strands in cells correlates with the expression level of occludin and claudins (Tsukita et al., 2001). In low $Ca^{2+}$ medium (in which cells do not form TJs), Tiam1KO cells showed lower levels of mRNA (not depicted) and protein (Fig. 4 A) of the TJ molecules occludin, ZO-1, and claudin-1 when compared with WT cells. No differences were found in the expression levels of E-cadherin (Fig. 4 A) or α- and β-catenin (not depicted). FL-Tiam1, but not Tiam1ΔDH (Fig. 3 B), could fully restore TJ protein levels in Tiam1KO cells (Fig. 4 A), indicating that the Tiam1-mediated activation of Rac controls the amount of TJ molecules even under conditions in which no TJs are formed. Interestingly, the expression of JAM-A was enhanced in Tiam1KO cells (Fig. 4 A). JAM-A triggers TJ biogenesis and membrane sealing (Liu et al., 2000), suggesting that Tiam1KO keratinocytes attempt to compensate for the lack of Tiam1 by up-regulating JAM-A.

Because occludin and claudins possess adhesive properties and might be involved in the maturation of TJs upon their recruitment to PAs, we inhibited occludin at the cell surface by using an inhibitory peptide (Wong and Gumbiner, 1997). WT cells that were treated with the peptide for 6 h during the $Ca^{2+}$ switch still showed disorganized cell–cell contacts (Fig. 4 B) that were similar to those seen in Tiam1KO cells, suggesting that the maturation of "adhesion zippers" depends on the formation of TJs. Both ZO-1 and E-cadherin were distributed in a zipperlike manner, which is in contrast to the normal linear organization in WT control cells. Thus, the reduced amounts of occludin that were observed in Tiam1KO cells could be a limiting factor for junction maturation. However, the overexpression of occludin (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200502129/

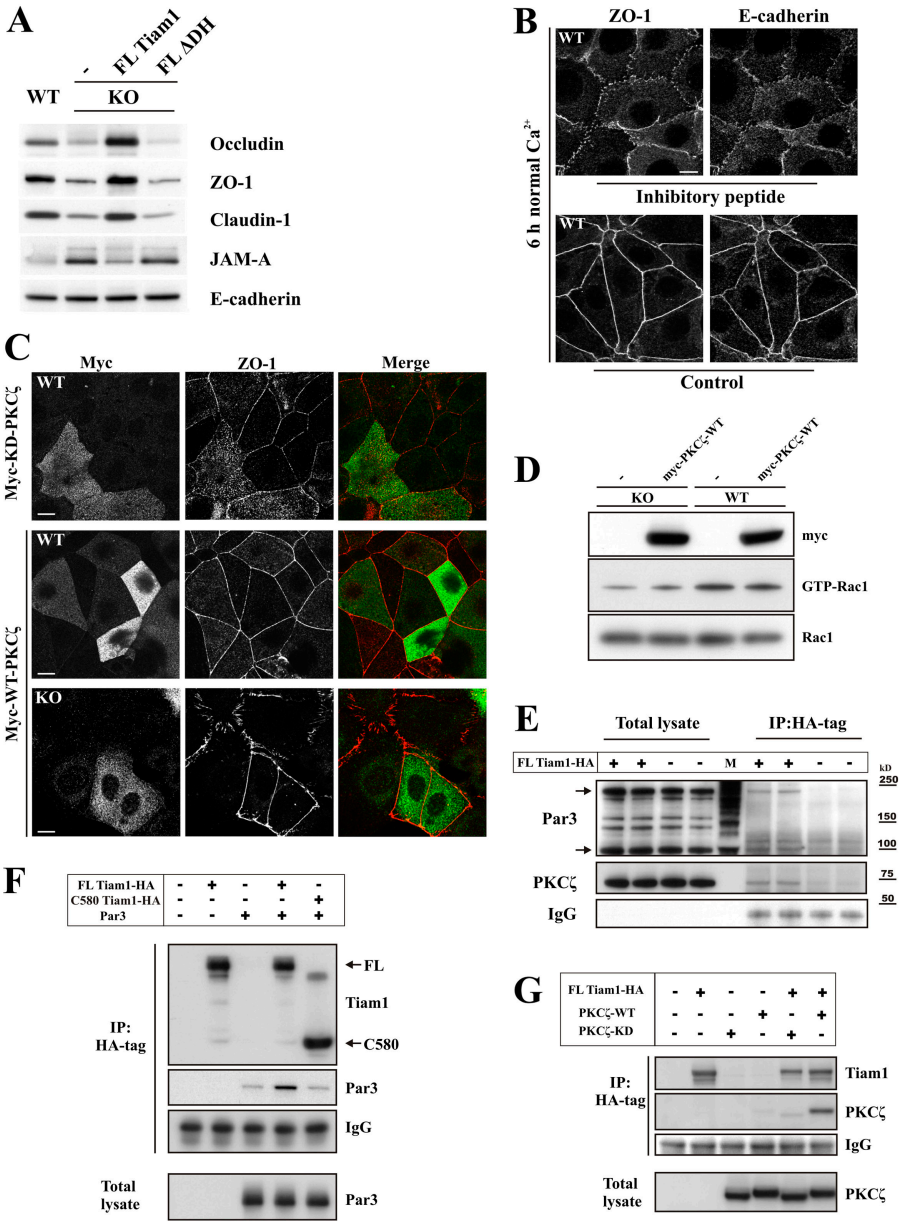


Figure 4. **Tiam1 regulates the expression of TJ molecules and interacts with the Par3–Par6–PKCζ polarity complex.** (A) Tiam1–Rac signaling differentially regulates the expression of TJ molecules. Lysates of keratinocytes cultured in low $Ca^{2+}$ medium were immunoblotted for the indicated junctional proteins. (B) Membrane-associated occludin facilitates junction maturation. WT cells were switched to 1.8 mM $Ca^{2+}$ for 6 h in the presence or absence (DMSO control) of an occludin inhibitory peptide. (C) WT and Tiam1KO keratinocytes were transiently transfected with myc-tagged WT- or kinase-dead PKCζ-K281W, switched to normal $Ca^{2+}$ for 6 h, fixed, and double stained for myc and ZO-1. PKCζ-K281W disturbs the junctional localization of ZO-1 in WT keratinocytes, whereas WT-PKCζ has no effect. WT-PKCζ fully restores junction maturation in Tiam1KO keratinocytes. Bars, 10 μm. (D) Rac1 activity assay on WT and Tiam1KO cells stably expressing myc–WT-PKCζ. (E–G) HA-tagged Tiam1 was expressed in Tiam1KO keratinocytes (E) or in COS-7 cells (F and G) by retroviral transduction and immunoprecipitated using anti-HA antibody. (E) FL-Tiam1 coimmunoprecipitates endogenous Par3 and aPKCζ from two independent infected populations of Tiam1KO cells that expressed HA–FL-Tiam1 and were cultured for 6 h at 1.8 mM $Ca^{2+}$. Empty vector–infected Tiam1KO cells were used as a control. M, molecular mass marker. (F) FL-Tiam1, but not a COOH-terminal Tiam1 mutant (C580), containing the guanine nucleotide exchange factor domain only, coimmunoprecipitates Par3 from COS-7 cell lysates. (G) FL-Tiam1 coimmunoprecipitates exogenous WT- but not PKCζ-K281W from COS-7 cell lysates. Immunocomplexes were immunoblotted using the indicated antibodies.

DC1) or of both occludin and ZO-1 (Fig. S3 C) did not lead to enhanced maturation of TJs in Tiam1KO cells. Apparently, occludin (or an associated protein) is required for proper junction maturation, but its reduced expression or that of ZO-1 is not the cause of impaired TJ formation in Tiam1KO cells.

### Tiam1 binds components of the polarity complex and activates Rac upon the formation of PAs

The polarity complex, which consists of Par3, Par6, and aPKCζ/λ, has been shown to drive the biogenesis of TJs in a Rac1- or Cdc42-dependent manner, making it a likely candidate to control junction maturation by Tiam1. Indeed, transient expression of kinase-dead PKCζ (K281W) inhibited junction maturation in WT keratinocytes (Fig. 4 C), which is consistent with earlier data that PKCλ (K281W) causes delayed junction maturation in epithelial cells (Suzuki et al., 2002). Interestingly, PKCζ-WT completely restored junction maturation in Tiam1KO keratinocytes, as seen by the linear organization of ZO-1 within 6 h (Fig. 4 C, bottom). These findings indicate that Tiam1 acts upstream of PKCζ in TJ formation that is regulated by the polarity complex. To investigate the possibility that Rac is also activated downstream of PKCζ to control TJ formation, we measured Rac activity in WT and Tiam1KO cells stably expressing PKCζ-WT. Rac activity was unaffected in both cell types (Fig. 4 D), again suggesting that Rac controls cell polarity through the activation of PKCζ.

We also investigated the possibility that Tiam1 interacts with components of the polarity complex. FL-Tiam1 that was expressed in Tiam1KO cells was immunoprecipitated, and endogenous PKCζ and Par3, but not Par6, coimmunoprecipitated with Tiam1 (Fig. 4 E and not depicted). In addition, we expressed Tiam1 mutants with either Par3 or various mutants of PKCζ in COS-7 cells. FL-Tiam1, but not C580-Tiam1,

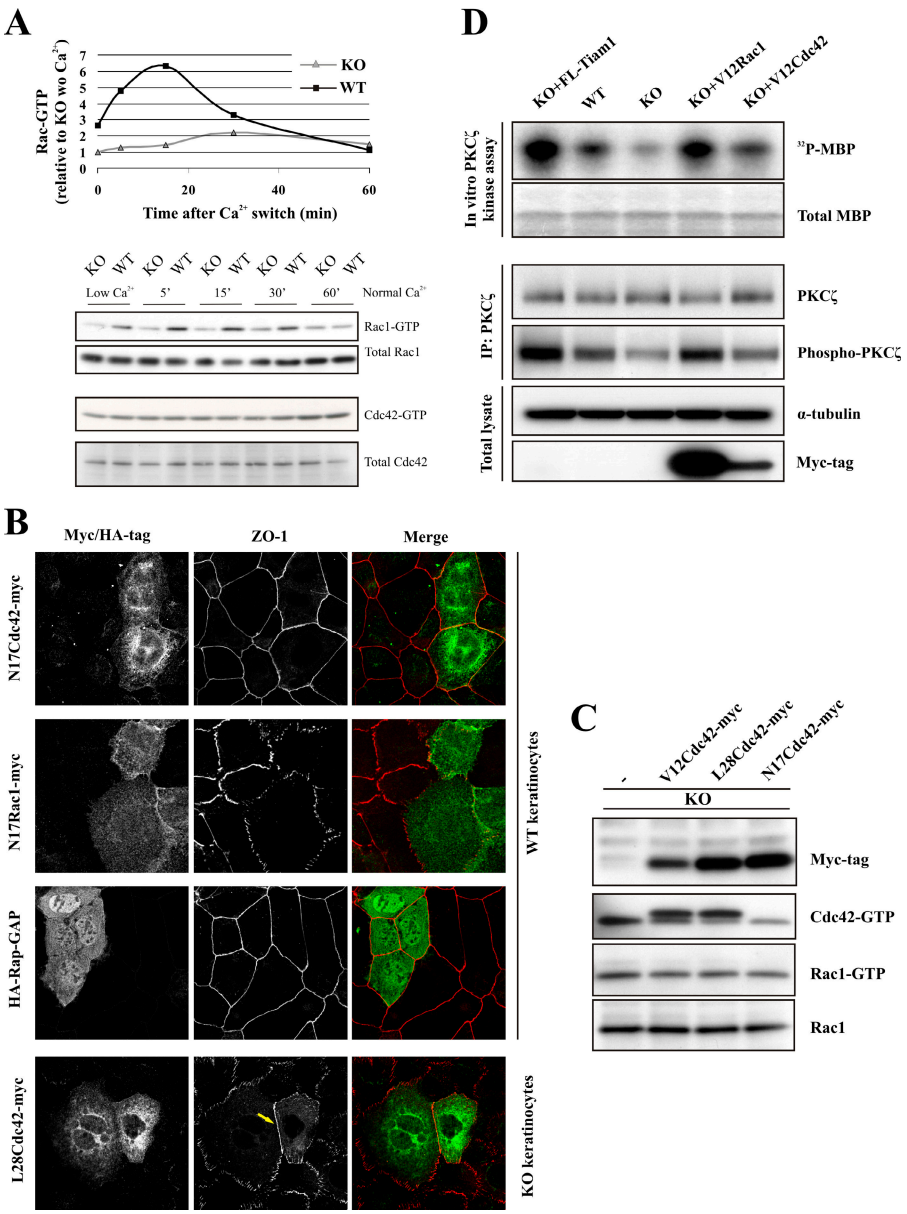


Figure 5. **Tiam1 and Rac1, but not Cdc42 or Rap1, control the activity of the polarity complex and TJ formation.** (A) Cadherin signaling transiently activates Rac in a Tiam1-dependent manner. Rac and Cdc42 activity assays from WT and Tiam1KO keratinocytes after a $Ca^{2+}$ switch for the indicated times. The graph represents the level of activated Rac (corrected for total Rac) relative to its activity in Tiam1KO cells cultured in low $Ca^{2+}$. (B) WT keratinocytes were transiently transfected with myc-N17Cdc42, -N17Rac1, or HA-Rap1GAP. Tiam1KO keratinocytes were transfected with myc-L28Cdc42. 48 h after transfection, cells were switched to normal $Ca^{2+}$ for 6 h, fixed, and double stained for myc or HA and ZO-1. Arrow indicates linearization of the TJ. (C) Tiam1, Rac, and Cdc42 can regulate PKCζ activity. Endogenous PKCζ was immunoprecipitated from WT, Tiam1KO, and Tiam1KO cells expressing FL-Tiam1, V12Rac1, or L28Cdc42. PKCζ kinase activity was assayed in vitro by using MBP as a substrate. Thr410 phosphorylation in the activation loop of PKCζ was analyzed as an alternative way to measure its activity. α-Tubulin was used as a loading control. Representative example of four experiments. (D) Cdc42 and Rac activity assay in Tiam1KO cells stably expressing myc-tagged L28, V12, or N17Cdc42 mutants.

coimmunoprecipitated with Par3 (Fig. 4 F). C580-Tiam1 lacks the putative protein interaction domain, which consists of the $NH_2$-terminal pleckstrin homology domain and the flanking coiled coil domain (Mertens et al., 2003), suggesting that Tiam1 interacts with Par3 through one or more of these domains. Indeed, it was recently shown that Tiam1 directly interacts with Par3 through these domains (Nishimura et al., 2005). Tiam1 also coimmunoprecipitated PKCζ-WT but not kinase-dead PKCζ, which contains a single mutation in the ATP-binding site (Fig. 4 G). These data substantiate the specificity of protein interactions and suggest that Tiam1 is preferentially associated with the active form of the polarity complex.

The docking of the polarity complex to PAs (Suzuki et al., 2002) suggests that its activation at these sites triggers TJ assembly. Cdc42 and Rac1 have both been shown to activate the polarity complex through Par6 (Lin et al., 2000). Local activation of these GTPases at PAs, presumably as a consequence of combined signaling of nectins and cadherins, could, therefore, activate the polarity complex. To study whether Tiam1 is required for the activation of Rac upon formation of PAs, we analyzed Rac activation in WT and Tiam1KO cells upon a $Ca^{2+}$ switch. In WT keratinocytes, Rac activity was stimulated within 15–30 min and recessed to basal levels within 1 h (Fig. 5 A), whereas Tiam1KO cells hardly activated Rac, suggesting that Tiam1 is required for the activation of Rac that leads to TJ biogenesis. No activation of Cdc42 was found upon the formation of PAs in both genotypes (Fig. 5 A), suggesting that TJ maturation in keratinocytes is not dependent on Cdc42 activity. Indeed, the expression of dominant-negative N17Cdc42 in WT keratinocytes did not affect the formation of PAs and subsequent maturation of TJs in WT cells, whereas N17Rac1 did (Fig. 5 B). Because Rap1 activity is thought to act upstream of Cdc42 to control Par6-mediated neuronal cell polarity (Schwamborn and Puschel, 2004), we also inhibited Rap1 signaling by expressing

Rap GAP (GTPase-activating protein ) in WT cells. This did not have any effect on the formation of TJs upon the $Ca^{2+}$ switch (Fig. 5 B), which is in line with our conclusion that the activation of Cdc42 is not required for the formation of PAs and TJs in keratinocytes.

Similar to V12Rac1, the expression of constitutively active L28Cdc42 in Tiam1KO cells rescued impaired TJ formation in these cells (Fig. 5 B). Rac activity was not affected by the expression of Cdc42 mutants in keratinocytes (Fig. 5 D), indicating that Cdc42 can activate the polarity complex independently of Tiam1 and Rac.

Tiam1-dependent activation of Rac in WT keratinocytes followed the same kinetics as the formation of PAs (Fig. 2), suggesting that Tiam1 is a downstream target of cadherins/nectins to activate Rac and, thereby, to activate the polarity complex. Because activation of the polarity complex leads to activation of PKCζ, we immunoprecipitated PKCζ from keratinocyte lysates and determined its activation. As shown in Fig. 5 C, Tiam1KO cells showed less endogenous PKCζ activity than WT cells, as determined by auto-phosphorylation of PKCζ and the phosphorylation of myelin basic protein (MBP). Moreover, the introduction of FL-Tiam1, V12Rac1, and V12Cdc42 into Tiam1KO cells restored TJ formation (Figs. 3 and 5 B) and also increased PKCζ activity to a level equal to or higher than that found in WT cells (Fig. 5 C). To control for the specificity of the in vitro kinase assay and PKCζ antibody that was used for immunoprecipitation, we stably overexpressed different amounts of PKCζ-WT in Tiam1KO keratinocytes. Indeed, MBP phosphorylation turned out to be PKCζ dose–dependent (Fig. S3 D). Together, these data indicate that upon the formation of PAs, Tiam1-mediated Rac activation is required for activation of the polarity complex, leading to TJ maturation. Although Cdc42 and Rac1 differentially regulate the actin cytoskeleton, our data suggest that both pathways can independently converge to activate the polarity complex, presumably via Par6. Tiam1–Rac signaling, rather than Cdc42 activity, however, predominantly regulates cell polarity in keratinocytes.

We demonstrate that Tiam1-mediated Rac activation plays a key role at an early stage of intercellular adhesion to trigger the formation of TJs and, as a consequence, to trigger the polarization of epidermal keratinocytes. Cell polarization requires the formation of primordial intercellular contacts before the assembly of TJs. Several TJ molecules, which constitute PAs together with E-cadherin and nectins, are present in these intercellular contacts. The similar formation of PAs in WT and Tiam1KO keratinocytes suggests that Tiam1 is not essential for the formation of these initial intercellular contacts but regulates the subsequent assembly of TJs. The recruitment of the polarity complex to PAs is presumably caused by the direct binding of Par3 to the PDZ domain of JAM-A (Itoh et al., 2001). Thus, Tiam1 could be recruited to these sites as it associates with Par3 and PKCζ. Recently, JAM-A has been shown to colocalize with and promote the activity of the small GTPase Rap1 (Mandell et al., 2005). Rap1 acts upstream of Cdc42 (Schwamborn and Puschel, 2004) and has been reported to associate with Tiam1 (Arthur et al., 2004). However, we could not find any inhibition of TJ formation in WT or Tiam1KO keratinocytes when interfering with Rap or Cdc42 activity, excluding a role for these genes in TJ formation in keratinocytes. These conclusions are consistent with studies that show the activity of Cdc42 is dispensable for cell polarization in epithelial MDCK cells and epidermal keratinocytes (Gao et al., 2002; Tunggal et al., 2005), although another study has implicated Cdc42 in TJ biogenesis (Fukuhara et al., 2003).

During the evaluation of this manuscript, two reports were published that showed conflicting data on the role of Tiam1 and Rac in cell polarization. One study implicated Tiam1-mediated Rac activation in neuronal cell polarization (Nishimura et al., 2005), whereas another study reported that a Par3-mediated inactivation of Tiam1 and Rac is required for the polarization of epithelial MDCK cells (Chen and Macara, 2005). In the latter study, the effect of Tiam1–Rac signaling on TJ formation was investigated in MDCK cells in which Par3 was down-regulated using siRNA, which might explain why Chen and Macara's (2005) and our results are different. Tiam1 is known to stimulate either the association or dissociation of cell–cell adhesions in MDCK cells, which is dependent on its site of activation (i.e., intercellular adhesions or lamellipodia, respectively; Sander et al., 1998). Down-regulation of Par3 in MDCK cells, as studied by Chen and Macara (2005), might prevent the recruitment of Tiam1 to cell–cell contacts and, thereby, promote Tiam1-mediated Rac activation at the cell periphery, leading to the destabilization of cell–cell adhesions and the inhibition of TJ formation. We found earlier that down-regulation of Tiam1 by siRNA leads to impaired junction formation in MDCK cells (Malliri et al., 2004), which is consistent with this study of keratinocytes.

In conclusion, our data indicate that the Tiam1-mediated activation of Rac (and not the inactivation of Rac) is required for TJ formation in epithelial keratinocytes. The concept that the Tiam1-controlled activation of Rac is required for proper cell polarization holds true for both epithelial and neuronal cells. In addition, our data support a model in which Tiam1 and Rac function upstream of the polarity complex independently of Cdc42. Rac is not activated in Tiam1KO cells that show impaired formation of TJs, suggesting that Tiam1 is required for local Rac activation upon the formation of PAs. This Rac activity is necessary for activation of the polarity complex, which leads to the activation of PKCζ and, subsequently, to TJ biogenesis.

## Materials and methods

### Keratinocyte isolation and culturing

Keratinocytes were isolated from newborn WT and Tiam1KO mice (Malliri et al., 2002). Skins were removed and trypsinized (EDTA-free 0.25% trypsin) for 16 h at 4°C to separate the epidermis from the dermis. Both fractions were minced, and cells were detached by stirring on ice for 1 h. Cell suspensions were filtered and seeded on dishes coated with 10 μg/$cm^2$ collagen IV (Becton Dickinson) and cultured in Epilife keratinocyte medium (Cascade Biologics, Inc.) supplemented with 20 μM $CaCl_2$ and Epilife defined growth supplement. Epidermal keratinocytes were immortalized with SV40 large T antigen. Primary cultures and populations of immortalized epidermal keratinocytes at low passage numbers (<20) were used in our studies. Keratinocytes were grown on collagen IV–coated glass coverslips or plastic. For $Ca^{2+}$ switch assays, cells were grown to confluency at low $Ca^{2+}$ (0.02 mM), and $CaCl_2$ was added to the medium to a final concentration of 1.8 mM.

### Antibodies

Immunoblotting and immunofluorescent stainings were performed with primary antibodies against ZO-1, occludin, claudin-1, and JAM-A (Zymed Laboratories), c-myc (A-14) and PKCζ (C-20; Santa Cruz Biotechnology, Inc.), E-cadherin (C36; Becton Dickinson), HA tag (hybridoma 12CA5), Tiam1 (C16; Santa Cruz Biotechnology, Inc.), α-DH, (Malliri et al., 2002), Rac1 and Par3 (Upstate Biotechnology), and phospho-PKCζ/λ (Thr410/403; Cell Signaling). Nectin-2 and afadin antibodies were gifts from Y. Takai (Osaka University, Osaka, Japan). STEF polyclonal antibody was a gift from M. Hoshino (Kyoto University, Kyoto, Japan).

### Immunoblotting, immunoprecipitation, and PKCζ kinase assay

Total cell lysates were prepared in hot SDS lysis buffer (1% SDS, 10 mM EDTA, and protease inhibitors). For immunoprecipitation, lysates of COS-7 cells and keratinocytes were prepared in standard radioimmunoprecipitation assay buffer. Extracts were clarified by centrifugation and precleared with γ-binding protein G–Sepharose beads (GE Healthcare) for 1 h at 4°C. Precleared lysates were incubated with antibodies that were preabsorbed on protein G–Sepharose beads for 16 h at 4°C. Immunocomplexes were washed three times, denatured with SDS, and separated by SDS-PAGE.

For in vitro PKCζ kinase assay, endogenous PKCζ was immunoprecipitated from keratinocyte lysates. Beads were washed and incubated in kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 μM ATP, 1 mM DTT, and 1 μCi γ-[$^{32}$P]ATP) with 10 μg MBP (Sigma-Aldrich) for 15 min at 30°C.

### Rac and Cdc42 activity assays

Rac and Cdc42 activity was determined as described previously (Malliri et al., 2004) using a biotinylated Cdc42–Rac1 interactive binding motif peptide of PAK1. Densitometric analysis was performed with Image J software (National Institutes of Health).

### Microscopy, immunofluorescence, and EM

Cells were examined with a phase-contrast microscope (Axiovert 25; Carl Zeiss MicroImaging, Inc.) and photographed using a digital camera (model DSC-S85; Sony). For immunofluorescence, cells grown on glass coverslips were fixed in 4% PFA, permeabilized in 0.1% saponin (Sigma-Aldrich), blocked in 5% skimmed milk, and incubated with primary antibodies (1.5 h in PBS/0.1% saponin/1% BSA). Cells were incubated for 1 h with secondary antibodies that were conjugated to AlexaFluor488 or AlexaFluor594 (Invitrogen), washed, and mounted with Mowiol-DABCO. Filamentous actin was labeled with 0.2 μM AlexaFluor568-phalloidin (Invitrogen). Images were taken with a confocal microscope (model TCS SP2; Leica) and were arranged and resized using CorelDRAW Graphics Suite 12 (Corel).

For EM, cells were grown on Thermanox plastic (Nunc), fixed in 2.5% glutaraldehyde, postfixed in 1% $OsO_4$, stained en bloc with uranyl-acetate, and flat embedded. Samples were examined with an electron microscope (model CM 10; Philips).

### Paracellular diffusion of nonionic molecular tracers

Paracellular flux assays were performed as described previously (Jou et al., 1998) using 0.2 mg/ml FITC-dextran (mol wt of 3.000 g/mol) and 0.5 mg/ml Texas red–dextran (mol wt of 40.000 g/mol) as tracers (Invitrogen). In brief, at various time points after the $Ca^{2+}$ switch, the assay was started through the addition of tracers to the apical compartment. After 2.5 h at 37°C, medium from the basal compartment was collected, and fluorescence was measured with a fluorimeter (Wallac Victor MLR; Perkin-Elmer). The amounts of diffused FITC- and Texas red–dextran were calculated from a titration curve.

### Occludin extracellular loop peptide

An occludin inhibitory peptide was synthesized spanning $Gly^{194}$–$Gln^{241}$ of the second extracellular loop of mouse occludin as described previously for chicken occludin (Wong and Gumbiner, 1997). Inhibition was optimal at 5 μM.

### Expression vectors

pmt2SM-myc-Par3 was a gift from I. Macara (University of Virginia, Charlottesville, VA). Myc-V12Rac1, -N17Cdc42, -L28, -V12Cdc42, and Tiam1-coding sequences were cloned into the retroviral vector LZRS-IRES-blasticidin (Michiels et al., 2000). LZRSbsd-VSV-occludin was constructed by subcloning FL VSV-tagged occludin from pCB6 (a gift from C.M. van Itallie, University of North Carolina, Chapel Hill, NC) into the XbaI and SnaBI sites of LZRS-IRES-blasticidin. pSuper-siRNA-Tiam1 and pSuper-siRNA-luciferase have been described previously (Malliri et al., 2004). pmtSM-myc–PKCζ-WT and pmtSM-myc–PKCζ-K281W were provided by W.J. van Blitterswijk (Netherlands Cancer Institute, Amsterdam, Netherlands). Myc–PKCζ-WT was subcloned into the Swa1 and Not1 sites of LZRSbsd. pBabe-SV40 largeT antigen was a gift from R. Bernards (Netherlands Cancer Institute). pEGFP-N1 was obtained from Invitrogen. pCDNA3-STEF1 and pmt2SM-HA-Rap1GAP plasmids were gifts from M. Hoshino and J.L. Bos (University of Utrecht, Utrecht, Netherlands), respectively.

### Cell transfection and retroviral transduction

Retroviral vectors (LZRS and pBabe) were transduced to keratinocytes as described previously (Michiels et al., 2000). All other plasmids were transiently transfected in semiconfluent keratinocytes or COS-7 cells using LipofectAMINE FuGENE 6 according to the manufacturer's protocol (Roche Diagnostics).

### Online supplemental material

Fig. S1 shows that nectin-2 and afadin are present in PAs of WT and Tiam1KO cells (A and B) and that Tiam1KO cells functionally restore their TJs 24–36 h after the $Ca^{2+}$ switch (C and D). Fig. S2 shows that segregation of the AJ and TJ is impaired in Tiam1KO cells, as shown by xz projections of E-cadherin and ZO-1 (A), and also shows the STEF1 expression profile in WT and Tiam1KO keratinocytes (B and C). Fig. S3 shows that occludin and ZO-1 do not restore TJ formation upon their expression in Tiam1KO cells (A–C) and shows an in vitro PKCζ kinase assay, demonstrating the selectivity of the assay (D). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200502129/DC1.

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