The Par-Tiam1 Complex Controls Persistent Migration by Stabilizing Microtubule-Dependent Front-Rear Polarity

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Summary

Background: The establishment and maintenance of cell polarity is crucial for many biological functions and is regulated by conserved protein complexes. The Par polarity complex consisting of Par3, Par6, and PKCζ, in conjunction with Tiam1-mediated Rac signaling, controls apical-basal cell polarity in contacting epithelial cells. Here we tested the hypothesis that the Par complex, in conjunction with Tiam1, controls “front-rear” polarity during the persistent migration of freely migrating keratinocytes.

Results: Wild-type (WT) epidermal keratinocytes lacking cell-cell contacts are stably front-rear polarized and migrate persistently. In contrast, Tiam1-deficient (Tiam1 KO) and (si)Par3-depleted keratinocytes are generally unpolarized and migrate randomly because front-rear polarity is short lived. Immunoprecipitation experiments show that in migrating keratinocytes, Tiam1 associates with Par3, PKCζ, and Tiam1 proteins are enriched at the leading edges of polarized keratinocytes. Tiam1 KO keratinocytes are impaired in chemotactic migration toward growth factors, whereas haptotactic migration is similar to WT. Par3 depletion or the blocking of PKCζ signaling in WT keratinocytes impairs chemotaxis but has no additional effect on Tiam1 KO cells. The migratory and morphological defects in keratinocytes with impaired Par-Tiam1 function closely resemble cells with pharmacologically destabilized microtubules (MTs). Indeed, MTs in Tiam1 KO keratinocytes and WT cells treated with a PKCζ inhibitor are unstable, thereby negatively influencing directional but not random migration.

Conclusions: We conclude that the Par-Tiam1 complex stabilizes front-rear polarization of noncontacting migratory cells, thereby stimulating persistent and chemotactic migration, whereas in contacting keratinocytes, the same complex controls the establishment of long-lasting apical-basal polarity. These findings underscore a remarkable flexibility of the Par polarity complex that, depending on the biological context, controls distinct forms of cellular polarity.

Introduction

Coordinated or persistent directional cell migration of neural crest cells, hematopoietic stem cells, epithelial cells, and germ cells is essential for the early development of multicellular organisms [1]. In adult organisms, persistent cell migration plays an important role in wound healing [2] and immune function [3]. In pathological conditions, aberrant persistent cell migration leads to developmental disorders and altered immune function and can contribute to metastasis of cancer cells [4, 5].

At the molecular level, growth factors and chemokines, as well as integrin-extracellular-matrix interactions, provide the signals and tools required for cell motility, including persistent migration [6–8]. Most migrating cells must polarize initially by extending an actin-rich protrusion such as a lamellipodium in the direction of migration, resulting in an asymmetric cell with a distinct front-rear polarity [9]. It is generally believed that actin-dependent protrusion formation generates the tractional forces that are converted into cell displacement [6]. The microtubule (MT) network is also involved in cell migration, possibly by directing the sites of actin polymerization and lamellipodial protrusion [10] and/or by influencing adhesions [11]; however, the exact molecular mechanism(s) remain unclear.

The small Rho-like GTPases Rac1, RhoA, and Cdc42 control cytoskeletal rearrangements that lead to morphological changes, such as the conversion from a nonmigrating to a migrating cell, by inducing a polarized phenotype from a nonpolarized phenotype [12]. Besides their role in propelling migration, Rho GTPases also function in the establishment of apical-basal polarity in nonmigrating epithelial cells [13, 14], axon specification in neuronal cells [15], and T cell polarity [16]. Currently, it is becoming clear that various evolutionary conserved polarity protein complexes control the establishment of polarity together with Rho GTPases. The polarity proteins serve as spatial-temporal cues, locally activating Rho GTPases that subsequently act on the cytoskeleton to stimulate cell polarization and migration [6]. Because Rho GTPases regulate cell polarity, it is likely that specific regulators of Rho GTPases are also involved in the regulation of cell polarity, as shown for the Rac activator Tiam1 (T lymphoma invasion and metastasis) [16, 17].

We show here that the Par (partitioning defective) polarity complex and Tiam1 function together in controlling the persistence of migrating epidermal keratinocytes by stabilizing transient front-rear cell polarization via the microtubule system. This explains how signaling via the Par-Tiam1 complex can steer keratinocyte migration.

Results

Tiam1 Is Required for Persistent but Not Random Migration

To study motility behavior of freely migrating epidermal keratinocytes, we sparsely seeded epidermal
keratinocytes on exogenous Laminin5 (LN5) substrate in low calcium (0.02 mM) conditions. These cells acquire a distinct front-rear-polarized morphology, whereas Tiam1 deficient cells are irregularly shaped with variable number of protrusions.

Figure 1. Tiam1 Deficiency Impairs Directionally Persistent Migration

(A) Phase-contrast images of typical WT and Tiam1 KO freely migrating keratinocytes on a collagen IV substrate. Many WT cells are intrinsically polarized, showing a distinct front-rear cellular asymmetry, whereas Tiam1 deficient cells are irregularly shaped with variable number of protrusions.

(B) Representative individual migration tracks of WT and Tiam1 KO keratinocytes migrating on LN5 substrate. The total track distance of all individual cells combined was similar for both cell types.

(C) Average persistence (D:T ratio) derived from the tracks depicted in (B). The shaded area indicates the persistence (D:T) value for random migration (RM) as determined by simulation (see the Supplemental Experimental Procedures), and a persistence of 1 indicates completely linear migration (LM).

(D) Quantification of average persistence of migrating WT and Tiam1 KO cell populations seeded on a collagen IV substrate measured from multiple (n = 3) independent movies.

(E) Average cell motility measured by average cell velocity of WT and Tiam1 KO cell populations on LN5 and collagen IV substrates. Most WT and Tiam1 KO keratinocytes in freely migrating populations migrate with velocities between 0 and 2 \( \mu \text{m/min} \) (Figure S2A).

Error bars indicate standard deviations, and asterisks indicate significant differences in a Student’s t test (p < 0.05 was considered significant) for persistence and/or velocity compared to WT. The scale bar represents 10 \( \mu \text{m} \).

keratinocytes on exogenous Laminin5 (LN5) substrate in low calcium (0.02 mM) conditions. These cells acquire a distinct front-rear-polarized morphology, whereas cells lacking the Rac-activator Tiam1 (Tiam1 KO [knockout]) are hardly polarized (Figure 1A and Figure S1A in the Supplemental Data available online). Video microscopy shows that wild-type (WT) keratinocytes migrate in a persistent (i.e., linear) fashion. Tiam1 KO keratinocytes migrate equally well, although their migration pattern appears more erratic, with cells often changing direction (Movies S1 and S2). Comparing the composed migratory paths of WT and Tiam1 KO keratinocytes confirms this finding because the migration tracks of Tiam1 KO cells are condensed (Figure 1B). The average D:T (direct distance:total distance) ratio, or persistence of WT cells on LN5, was 0.8, compared to 0.5 for Tiam1 KO cells (p < 0.025, Figure 1C). The combined total distance for WT and Tiam1 KO cells was similar (data not shown), suggesting similar average cell velocities.

We extended these studies to a collagen IV substrate (Figure 1D), combining multi-well, automated time-lapse imaging with an unbiased computational tracking analysis allowing for the simultaneous tracking of many migrating cells in various conditions (Figure S1B). We
found that WT keratinocytes migrate more persistently on collagen, as well, compared to Tiam1 KO cells (D:T ratio 0.68 and 0.45, respectively, p < 0.001) (Figure 1D).

To visualize overall cell motility, we constructed velocity histograms of freely migrating Tiam1 KO and WT cell populations by plotting cell velocity against the number of cells (Figure S2A). In both genotypes, over 95% of the cells migrate with (average) velocities between 0.2 and 4.0 μm/min, and no obvious subpopulations exist for either genotype. WT keratinocytes, however, migrate on average slightly faster than do Tiam1 KO cells (1.0 μm/min versus 0.72 μm/min on LN5, and 0.72 versus 0.62 μm/min on collagen IV [p < 0.05]) (Figure 1E). However, Tiam1 appears to be nonessential for determining cell velocity because cells of both genotypes share the same range of individual cell velocities. It is conceivable that more-frequent changes in direction of Tiam1 KO cells reduce the average cell velocity. These experiments indicate that Tiam1 controls migratory persistence independent of the cell substrate but does not control random migration because it is dispensable for propelling keratinocyte migration.

Tiam1-Deficient Keratinocytes Are Impaired in Chemotactic Migration

Growth factors (GFs) are strong external inducers of chemotactic migration. Cells of epithelial origin stabilize polarized lamellipodial protrusions and migrate directionally toward growth factors [18, 19].

We investigated the physiological migration toward a GF gradient (1ng/ml epidermal growth factor [EGF], 10ng/ml insulin growth factor [IGF], 5 μg hydrocortisone, and 10ng/ml prostaglandin) by using Boyden-chamber assays. WT epidermal keratinocytes migrated extremely well toward a GF gradient (Figure 2A and 2B), with a 6-fold increase (p < 0.0001 in a Student’s t test). In contrast, the migration rate of Tiam1 KO cells upon the application of a GF gradient rarely increased (1.4-fold, p = 0.16). Haptotactic migration (without a GF gradient) was similar between WT and Tiam1 KO cells (Figures 2A and 2B). Tiam1-mediated chemotactic migration was not substrate dependent because we found similar results on fibronectin (FN)- and vitronectin (VN)-coated Boyden chambers (Figure S2B). We conclude, therefore, that chemotactic but not haptotactic migration of keratinocytes is Tiam1 but not substrate dependent.

Interestingly, GF-starved WT and Tiam1 KO keratinocytes equally induced P42/44 MAPK (mitogen-activated protein kinase) phosphorylation upon EGF stimulation (Figure 2C). Also, keratinocytes migrated well to a gradient of EGF alone (Figure S2C), suggesting that impaired chemotaxis of Tiam1 KO cells is not due to a defect in MAPK-dependent GF signaling. To validate that the lack of Tiam1 expression was causal for impaired chemotactic migration, we re-expressed full-length Tiam1 protein in Tiam1 KO cells (Figure 2D, third lane). The re-expression of Tiam1 restored the defect in chemotactic migration (Figure 2E, third bar), whereas the expression of a green fluorescent protein (GFP)-vector control had no effect (Figure 2E, fourth bar), confirming that Tiam1 expression is required for the chemotactic migration of epidermal keratinocytes.

To investigate whether the function of Tiam1 in directional migration applies to epithelial cells in general, we analyzed the chemotactic migration of MDCK (Madin Darby canine kidney) cells to serum in low calcium conditions. We found that the depletion of Tiam1 significantly reduced the chemotaxis of MDCK cells in the absence of cell-cell contacts (Figure 2F). In standard calcium (1.8 mM) conditions, MDCK cells form strong cell-cell adhesions [20], and Tiam1 depletion by siRNA in these conditions stimulates cell migration to serum because of a loss of cell-cell adhesions and apical-basal polarity [21]. These studies show that Tiam1 is required for chemotactic migration of epithelial cells independent of substrate and highlight the context-dependent role of Tiam1 in (epithelial) cell migration.

Persistent and Chemotactic Migration Is Regulated by the Par-Tiam1 Polarity Complex

Tiam1 deficiency impairs directional migration but not random migration, even though Rac activity is reduced in migrating keratinocytes lacking Tiam1 (Figure S3D). The expression of RacV12 in Tiam1 KO cells did not rescue polarization but led to increased spread “pancake”-like cells that do not migrate (Figure S3C). Nevertheless, because Tiam1 KO cells are motile cells, it is unlikely that Tiam1 controls keratinocyte migration by activating the Rac-dependent migration machinery [8].

Because Tiam1 has been shown to associate with the Par complex and to regulate the long-lasting apical-basal cell polarity of nonmigrating keratinocytes [13], we tested the possibility that Tiam1 stimulates directional migration by regulating Par-mediated front-rear cell polarity. Consistent with earlier findings [13, 22, 23], we confirmed the existence of the regulatory Par-Tiam1 complex in persistently migrating keratinocytes. Tiam1 associates with both endogenous Par3 and PKCζ proteins in migrating keratinocytes, and PKCζ is phosphorylated (activated) when Tiam1 is associated with the Par complex (Figure 3A).

To investigate whether Par3 controls persistence, we depleted endogenous Par3 by using small interfering RNA (siRNA) to approximately 30% in WT and Tiam1 KO keratinocytes (Figure 3B). Persistence was reduced in freely migrating Par3-depleted WT keratinocytes (D:T ratio WT 0.68 versus WT siPar3 0.55, p < 0.001), even though Par3 depletion was not complete in all cells. In contrast, Par3 depletion did not further reduce the persistence of Tiam1 KO cells, suggesting that the functions of Par3 and Tiam1 overlap in the regulation of directional persistence (Figure 3C). Consistent with this finding, the depletion of either Par3 or Tiam1 by siRNA-reduced chemotactic migration toward growth factors 2-fold to 3-fold compared to control (siLuc) cells (Figures 3D and E), indicating that these proteins also functionally overlap in regulating chemotaxis. As expected, the depletion of Tiam1 or Par3 by siRNA did not reduce persistence or chemotaxis completely to the level observed in Tiam1 KO cells (Figures 3C–3E) because the downregulation of these proteins was not 100% and varied between cells.

The activation of the Par complex leads to activation of PKCζ, the main effector of the conserved Par polarity complex [13, 16, 24]. To investigate whether activation of the Par polarity complex is required for chemotaxis, we stably expressed a kinase-dead (kd) mutant of
PKCζ in WT keratinocytes. The expression of this mutant strongly inhibited chemotaxis (Figure 3F). We also inhibited downstream PKCζ signaling in WT and Tiam1 KO keratinocytes by using a myristoylated inhibitory peptide (PKCζ inh) that abrogates PKCζ function in cell polarity [16, 25]. Concordantly, blocking PKCζ signaling in WT keratinocytes chemically reduced persistence (Figure S3F). In contrast, chemically blocking PI3K signaling with LY-294002 blocked chemotaxis but had no effect on persistence (Figures S2E and S3F). Although the inhibition of downstream PKCζ signaling strongly reduced chemotaxis of WT cells, PKCζ inhibition had no significant additional effect on the chemotaxis of Tiam1 KO keratinocytes (Figure 3F), suggesting that the PKCζ activity for chemotaxis is Tiam1 dependent.

Although PKCζ has multiple biological functions, including an established role in Par-mediated cell polarity, our experiments support the conclusion that PKCζ functions together with Tiam1 and Par3 in both chemotaxis and persistence.

Wound Migration on Exogenous Substrate

Directional (epithelial) migration is commonly examined in scratch-wound assays [2]. A restriction of these assays is that the wound space is devoid of substrate and is not compensated for by serum in the case of keratinocytes. To solve this, we designed a method using
cell-tracker dye to follow WT- and Tiam1-keratinocyte migration out of a monolayer into substrate-covered (collagen and LN5) wounds (see schematic in Figure 4A). Overlay images from starting (0 hr) and end (24 hr) points illustrate that WT keratinocytes (red) migrated well out of the monolayer, covering a large part of the initial wound area, whereas Tiam1 KO cells (green) hardly migrated into the wound space (Figure 4A). In mixed cultures, we observed essentially the same (Figure 4B) and noticed that at early time points, WT cells (red) migrating out of the border are front-rear polarized, in contrast to Tiam1 KO cells (unstained), which remain unpolarized in the border area (Figure 4C). These results substantiate that Tiam1 is required for the directional migration of keratinocytes into a wound and suggest that Tiam1-mediated front-rear polarization is required for efficient wound healing.

The Par-Tiam1 Complex Is Asymmetrically Enriched in Front-Rear Polarized Keratinocytes

To investigate whether the Par-Tiam1 complex controls front-rear polarization of keratinocytes, we quantified polarized morphology in freely migrating WT and Tiam1 KO keratinocytes measured in sparse cultures on collagen IV, with or without reduced Par3 expression.

Figure 3. The Par Complex Controls Persistence and Chemotactic Migration

(A) FL-Tiam1 coimmunoprecipitates endogenous Par3 (upper band in top panel) and phospho-PKCζ from a population of freely migrating Tiam1 KO cells that express HA-tagged full-length Tiam1. Untransfected Tiam1 KO cells were used as a control. Total lysates show equal loading of Par3 and PKCζ proteins.

(B) Immunoblots of total lysates from WT and Tiam1 KO keratinocytes with and without Par3 knockdown by retroviral siRNA. Visible are the 180 and 100 kDa isoforms. Total Rac was used as a loading control.

(C) Persistence (D:T ratio) of WT and Tiam1 KO keratinocytes measured in sparse cultures on collagen IV, with or without reduced Par3 expression.

(D) Chemotactic (Boyden chamber) migration assay toward GFs. Representative images of the bottom membranes containing migrated WT, (si)Par3-, (si)Luc control-, and (si)Tiam1-depleted WT keratinocytes are shown.

(E) Quantification of experiment shown in (D).

(F) Results of chemotactic migration assays of WT cells, WT cells expressing a kinase-dead mutant of PKCζ, and Tiam1 KO cells with and without PKCζ inhibitor. Asterisks indicate significant (p < 0.05) differences between WT (control) cells in a Student’s t test, and error bars represent standard deviations.
Par3 is able to associate with Tiam1 (Figure 3A) and is able to function together with Tiam1 in apical-basal polarity [17, 22] and persistent migration (this study). To gain further support for a regulatory role of Par-Tiam1 signaling in front-rear polarization, we performed intracellular localization studies for Par polarity proteins and Tiam1-GFP by using confocal imaging. Endogenous Par3 protein was consistently enriched at the leading edge of migrating keratinocytes (Figure 4E, white arrowheads). Par3 was not enriched at leading edges of nonpolarized WT and Tiam1 KO cells (Red arrowheads) (50% versus 10%, p < 0.05) (Figure 4E). Endogenous PKCζ had an asymmetric enrichment at leading edges of polarized cells analogous to, albeit less consistent than, that of Par3. In addition, colocalization with Par3 was detected in WT cells (Figure 4E, small images). Endogenous Tiam1 was difficult to visualize by antibody staining; therefore, we expressed GFP-tagged Tiam1 in keratinocytes that localized to the leading edge (Figure 4F). We were unable to detect Tiam1-GFP at the leading edge of nonpolarized cells (Red arrowheads).

Figure 4. The Par-Tiam1 Complex Controls Front-Rear Polarity in Persistent Migration
(A) A schematic representation of a hole-migration assay is on the right. A monolayer of keratinocytes, grown under low-calcium conditions, is formed around an inert rod (black). The removal of the rod leaves a substrate-covered circular space devoid of cells (dotted line). Subsequently, keratinocytes at the border are able to migrate into the hole. Pictures represent overlay images of a hole-migration assay with cell-tracker dye-stained WT keratinocytes (red) or KO keratinocytes (green) 0 hr and 24 hr after removal of the rod.
(B) Image of a hole-migration assay after 24 hr with a mixed culture of (1:1) WT (red) and Tiam1 KO (green) cells.
(C) Images showing polarized migrating WT cells (red) into the open space (asterisks). Tiam1 KO cells are not stained. These experiments were performed on both LN5 and collagen IV substrates, and results were comparable.
(D) Images and quantification (bar diagram) showing an estimation of the percentage of front-rear polarized keratinocytes in large populations (n > 100). Represented are control (si)Luc WT keratinocytes, (si)Par3-depleted WT keratinocytes, WT keratinocytes treated with PKCζ inhibitor (INH), and Tiam1 KO cells.
(E) Confocal images of endogenous Par3 and PKCζ in migrating keratinocytes. White arrowheads indicate enrichment at the leading edge of protruding lamellae, whereas red arrowheads indicate lack of enrichment. White arrows indicate the direction of migration. The bar diagram shows the percentage of cells with Par3 enrichment at leading edges in polarized versus unpolarized cells. The top two right small images show endogenous Par3 and PKCζ colocalization at leading edge.
(F) Tiam1-GFP localization to the leading edge in WT keratinocytes (white arrowhead) but not in (si)Par3-depleted cells (red arrowhead).
(G) Colocalization (yellow/orange) of Tiam1-GFP with endogenous Par3 and/or dynamic F-actin visualized by phalloidin staining. The scale bar represents ~10 μm. Arrows indicate direction of migration.
leading edges of siPar3 keratinocytes (Figure 4F), suggesting that lowering the levels of endogenous Par3 prevents efficient Tiam1 localization to this area. Dynamic F-actin is highly enriched at the leading edge of migrating epithelial cells [26], including front-rear polarized WT and Tiam1 KO keratinocytes (Figure S2F). We observed the colocalization of Tiam1-GFP with F-actin and Par3 in the leading edge of polarized cells (Figure 4G). A GFP-control vector showed aspecific localization (Figure S2F). We propose that enrichment at the leading edge of Par3, Tiam1 and PKCζ proteins support a functional role for these proteins in cellular asymmetry, as observed in front-rear-polarized keratinocytes.

The Par-Tiam1 Complex Controls Stability of Front-Rear Polarity

Close examination of sequential time-lapse images showed that persistently migrating WT keratinocytes remain (stably) polarized for relatively long periods (Figure 5A). Although Par3-depleted and Tiam1 KO cells
can polarize, polarization in these cells is much more transient, explaining the low percentage of polarization in a population (Figure 4D).

We quantified the time period of constitutive polarization of WT (si)Par3-depleted and Tiam1-deficient keratinocytes before depolarization (Figure 5A, bar diagram). Approximately 60% of WT cells remained constitutively polarized longer than 120 min, whereas less than 20% of migrating (si)Par3-depleted or Tiam1 KO cells remained polarized for such a time-period. The average time WT cells remained front-rear polarized before depolarizing was 120 min, compared to 40 and 45 min for Par3-depleted and Tiam1 KO cells, respectively (p < 0.01 for both siPar3 and Tiam1 KO cells, n > 70). These results indicate that the Par-Tiam1 complex regulates constitutive front-rear polarity of keratinocytes and that impaired Par-Tiam1 function causes rapid depolarization.

The actin cytoskeleton is essential for establishing and maintaining polarized cell morphology during cell migration [6], whereas MTs play an important role in directional migration [11, 27, 28]. To investigate the influence of MTs on front-rear polarity in keratinocytes, we treated WT cells with various concentrations (0.5–5 μM) of the MT antagonist nocodazole. Intriguingly, cells treated with as low as 1 μM of nocodazole also rapidly lose front-rear polarity (Figure 5A) and migrate, similar to keratinocytes with impaired Par signaling, rapidly projecting and retracting protrusions (Movies S3 and S4). Treatment with cytochalasin B, so that actin polymerization could be prevented, blocked both protrusion formation and retraction, thereby abrogating cell motility altogether (data not shown).

Together, these studies indicate that the MT network is essential for persistent but not random migration of epidermal keratinocytes. These experiments suggest that the Par-Tiam1 complex controls the stability of front-rear keratinocyte polarity by acting on the MT network.

**Par-Tiam1 Controls Microtubule Stability**

We analyzed whether the MT network is specifically oriented toward the direction of migration in persistently migrating polarized keratinocytes. We initially incubated keratinocytes with a specific centrosome antibody pericentrin, but did not discover any correlation between orientation of the centrosome and the nucleus, in persistently versus randomly migrating cells (data not shown).

To investigate whether the Par-Tiam1 complex affects the MT network itself, we compared WT with Tiam1 KO cells for potential differences in length or abundance of MT filaments by immunofluorescence staining for α-tubulin. We found by Western blotting that WT keratinocytes contain significantly more stable, acetylated MTs compared to Tiam1 KO cells and siPar3 keratinocytes (Figure 5B), suggesting that Tiam1 and Par3 are associated with the presence of stabilized MTs in keratinocytes.

We next investigated whether Tiam1 might be involved in MT stability directly by using a nocodazole-resistance assay, and found that shortly (5–10 min) after adding nocodazole, the architecture of MT filaments visualized by α-tubulin staining was severely disrupted in the peripheral lamellae of Tiam1 KO cells and consisted mostly of monomers indicated by the “fuzzy” or diffuse staining (Figure 5C, bar diagram). In contrast, subsets of intact MT filaments are visible and appear to be intact in most (70%) nocodazole-treated WT cells but not in Tiam1 KO cells (p < 0.05). Lastly, we found that treatment with 1 μM PKCζ inhibitor, besides impairing front-rear polarity, also affected the stability of MTs, leading to a similar disruption of MT filaments (diffuse α-tubulin staining) as did nocodazole treatment (Figure 5C).

To demonstrate that the decrease of MT stability in Tiam1 KO cells affects persistence, we analyzed persistent migration of nocodazole-treated WT and Tiam1-deficient keratinocytes. The treatment of WT cells with 1.25 μM nocodazole diminished directional persistence substantially (D:T ratio of 0.54 ± standard deviation [SD] 0.02 versus 0.72 ± SD 0.014), comparable to the level of untreated Tiam1 KO cells (D:T ratio WT 0.54 versus KO 0.55, p = 0.7). This finding suggests that MT stability is reduced in Tiam1 KO cells, thereby reducing migratory persistence. In contrast, treatment of 1.25 μM nocodazole has a much smaller effect on persistence in Tiam1 KO cells (Figure 5D). Collectively, these experiments indicate that Tiam1, Par3, and downstream PKCζ signaling play a role in the stability of MT filaments of migrating keratinocytes, thereby affecting persistent migration.

**Discussion**

Our results indicate that the Par polarity complex and Tiam1 control the persistent migration of epidermal keratinocytes by affecting the stability of MTs. Impaired Par and/or Tiam1 signaling reduces the stability of front-rear polarization, thereby decreasing persistence of the migrating cell. Immunoprecipitation and immunolocalization studies indicate that Tiam1, Par3, and PKCζ function together in maintaining cellular asymmetry. Indeed, Tiam1 deficiency, Par3 depletion by siRNA, and the chemical blocking of PKCζ signaling impair the stability of front-rear polarization and reduces persistence and chemotactic migration. The depletion of Par3 and/or the blocking of PKCζ signaling has no additional effect on Tiam1-deficient cells, supporting the idea that these proteins function together. Lastly, we show that Par3 depletion and Tiam1 deficiency reduce MT stability measured by acetylation. Conversely, the pharmacological disturbance of MTs impairs front-rear polarization and reduces persistence.

Our data thus indicate a functional role of the evolutionary conserved Par complex in persistent migration by controlling front-rear polarization of epithelial cells. Although Par polarity signaling in mammalian cells is best characterized in controlling the protrusion outgrowth of astrocytes or axon outgrowth of neuronal cells [23, 24], an active role in actual cell migration has not yet been demonstrated. In fact, Par polarity signaling in epithelial cells is perhaps better known for its role in the establishment of lasting apical-basal cell polarity, thereby contributing to tissue integrity [13, 22]. The loss of Par3 [22] or Par6 [29] or the overexpression of Par3 [30] leads to the dissolution of cell-cell junctions, enabling cell migration but not propelling migration per se. The Par polarity complex thus regulates two distinct cell polarity processes in keratinocytes, the first being persistent
clen cell-polarity processes, characterized by apical-basal polarity, and second being transient cell polarity, characterized by front-rear polarization during cell migration. The most concrete evidence thus far that Par polarity proteins might actively function in any form of migration in epithelial cells comes from experiments in the Drosophila ovary, where the depletion of Par-6, Par-3, or Bazooka impairs the migration of border cells [31].

Because the Par proteins remained asymmetrically localized, the authors concluded that apical-basal polarity is retained during border cell migration. An alternative explanation, based on our results, is that the asymmetrical distribution of Par proteins reflects (re-)localization to the leading edge of front-rear polarized cells, thereby controlling persistent border cell migration toward chemotactic cues of in the developing Drosophila ovary [32].

The depletion of Par3 by siRNA and the blocking of PKCζ signaling with a specific inhibitor reduces persistent and chemotactic migration. The lack of the Rac activator Tiam1 has identical effects on persistent and chemotactic migration, suggesting that these proteins function together in controlling migration. Indeed, Tiam1 acts in conjunction with the Par complex in other polarity processes [17]. To demonstrate that not all migratory pathways function via front-rear polarization mechanisms, we blocked the Rho-ROCK and PI3K migratory pathways by using specific chemical inhibitors. Blocking PI3K signaling did not result in impaired front-rear polarization and persistence, but it essentially blocked chemotactic migration in Boyden-chamber assays. ROCK inhibition with Y-27632 did not impair chemotactic migration, yet directional migration and front-rear polarity are affected (Figures S2D and S2E). As listed in Table 1, we found that Par3, Tiam1, and PKCζ have similar roles in front-rear polarity, chemotactic, persistent, and random migration, but differ from the role of PI3K and Rho-ROCK. These data suggest that the mechanism by which the Par-Tiam1 complex regulates migration is distinct from the PI3K and the Rho-ROCK pathways.

We found that WT keratinocytes contain MTs that are relatively resistant to nocodazole treatment and contain stable acetylated tubulin filaments, whereas Tiam1 KO cells show less stable filaments. In fact, keratinocytes with impaired Par-Tiam1 signaling have a reduced MT stability that is required for stable front-rear polarization and migratory persistence. This finding is supported by migration studies in fibroblasts that lack the MT stabilizing (plus-end associating) protein Clasp2. Clasp2 KO fibroblasts have reduced persistence in wound-healing assays [27], though a role for Clasp2 in front-rear polarity has not been studied. In addition, blocking the post-translational modification of MTs compromises their stability and reduces chemotactic migration [33].

Our studies support the idea that Par polarity signaling regulates directional persistence together with Tiam1 by controlling MT stability. This might prove to be a general mechanism in directing cell migration and protrusion formation, even though the upstream pathways could be different. In the polarized outgrowth of astrocytes, for instance, Par signaling controls adenomatous polyposis coli (APC) localization that is associated with MT stability [34, 35]. However, a role for Tiam1 and/or Par3 on MT stability in astrocytes is unknown. Par3 has been found to associate with APC and the plus-end-directed microtubule motor protein KIF3A, which localizes APC to the tips of MTs [25, 36, 37]. Because we and others find that Tiam1 binds to Par3 [13, 23], it is tempting to speculate that Par3-Tiam1 might control MT plus-end stabilization at the leading edge of polarized keratinocytes, possibly by association with MT plus-end proteins.

Besides the emerging function of Tiam1 in controlling cell polarity, Tiam1 is in fact mostly recognized as a Rac GTPase activator [38]. We find that Tiam1 KO cells have lower overall Rac activity in keratinocytes. Interestingly, lowering the overall Rac activity in fibroblasts increases directional persistence [39], whereas the lack of Tiam1 reduces persistence in keratinocytes, suggesting different mechanisms. Indeed, because not all fibroblasts migrate similarly [9, 39], comparisons between studies that use different cell types should be interpreted with caution. Nevertheless, a fundamental role for MT stability in migratory persistence is likely [11, 27, 28].

We propose that the Par polarity complex and Tiam1-mediated Rac signaling regulate the persistent migration of keratinocytes by stabilizing MT-dependent transient front-rear polarity. We show that this has biological consequences because impaired Par and/or Tiam1 signaling reduces chemotactic [16] and wound migration (this study) on exogenous substrates in vitro and skin wound healing in vivo [40]. Although a complex cross-talk exists between actin and microtubule networks in migrating cells [10, 41], we find that Par-Tiam1 polarity signaling affects MT stability, which directly influences persistent and chemotactic migration. On a larger scale, our findings suggest a model in which when cell-cell junctions are present, as in normal epithelium, the default function of Par-Tiam1 signaling is to stimulate the

Table 1. Summary of Effects of Modulators of Cell Polarity

<table>
<thead>
<tr>
<th>Role in Front-Rear Polarity</th>
<th>Role in Chemotactic Migration</th>
<th>Role in Persistent Migration</th>
<th>Role in Random Migration</th>
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<td>Par3</td>
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</table>

This table shows an overview of the effects of chemical inhibitors and gene manipulation on migration characteristics and front-rear polarity. “n.d.” indicates not determined; “*” indicates that cells do not migrate or protrude.
impairment of apical-basal cell polarity

Figure 6. Model of the Function of Par-Tiam1 Polarity Signaling in Contacting and Migrating Keratinocytes

Impaired Par-Tiam1 polarity signaling interferes with tight junction (TJ) formation by preventing the maturation of a zipper-like adhesive state (primordial adhesion) into mature tight junctions during the membrane-sealing process. Impaired Par-Tiam1 function interferes with the establishment of permanent (apical-basal) cell polarity [13]. In migrating conditions, (untreated) keratinocytes display transient front-rear polarization but migrate relatively linearly compared to keratinocytes with impaired Par-Tiam1 signaling. Such cells polarize only briefly and project multiple protrusions in different directions, polarize again, and migrate in alternate direction before depolarizing again, resulting in random migration and decreasing persistent migration. The Par-Tiam1 complex thus extends the duration of transient cell polarity.

establishment of long-lasting apical-basal cell polarity [13, 22], thereby contributing to tissue integrity. However, when cell-cell contacts are absent, such as in lymphoid cells [16, 42], or perturbed, such as in epidermal wounds [40] and epithelial cancers, Par-Tiam1 signaling promotes cell migration by stabilizing transient front-rear polarity (Figure 6).

Experimental Procedures

Cell Culture

Keratinocyte isolation and cell culture were performed as described previously [40]. In brief, primary keratinocytes were isolated from newborn WT and Tiam1−/− mice [43] and cultured in medium (Epilife) containing 0.02 mM CaCl2 and defined growth supplement and 100 IU/ml penicillin and streptomycin (PS) (standard keratinocyte culture conditions). WT and Tiam1−/− keratinocytes were immortalized by transduction with pBabe puro-SV-40 Large T antigen viruses. Keratinocytes were typically maintained until 20 passages on collagen IV substrate. Rac-11P and MDCII cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine calf serum and PS.

Antibodies

PKC (C-20; Santa Cruz Biotechnology), HA tag (hybridoma 12CA5, own production), Tiam1 (C16; Santa Cruz Biotechnology), Tiam1-DH [43], Rac1 and Par3 (Upstate Biotechnology), phospho-MAPK, and phospho-PKC (Thr410/403; Cell Signaling) were used. Furthermore, we have used total MAPK (a noncommercial rabbit polyclonal antibody), Phalloidin-alexa 568, acetylated-tubulin, alpha-tubulin and β-actin (all from Sigma).

Coating Culture Dishes with ECM Molecules

We coated culture dishes overnight at 4 °C with recombinant extra-cellular matrix (ECM) proteins (except laminin5) at the following concentrations: 10 mg/ml fibronectin (FN) (isolated from human plasma); 10 mg/ml Laminin1 (LN1) (Becton Dickinson, San Jose, CA); 10 mg/ml vitronectin (VN) (Sigma-Aldrich); 25 mg/ml Collagen I (Vitrogen [The Netherlands]); and 25 mg/ml Collagen IV (Becton Dickinson). LN5 matrices were obtained by the culturing of Rac-11P cells to confluency, after which cells were detached with 10 mM ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS) containing a mix of protease inhibitors (Complete TM-protease inhibitor cocktail tablets, Roche Applied Science [Germany]) at 4 °C. Before use, the dishes were washed twice with PBS.

Gene and siRNA Transfer into Keratinocytes by Retroviral Transduction

Full-length, HA-tagged Tiam1 and kd PKCζ were cloned into PMX-plasticidin (BSd.) or hygromycin (Hyg.) retroviral vectors. Retroviral constructs were transfected into Phoenix ecotropic packaging cells, and fresh viral supernatants were collected and used for multiple infections and antibiotic selection as described [13]. siRNA constructs against Tiam1 [21] and three self-designed short hairpin RNAs (shRNAs) against Par3 were cloned into selectable pRetroSuper constructs (see Supplemental Data). Other siRNA sequences used for Par3 and siLUC and siTiam1 were described previously [21, 23]. MDCII siPar3 and siLUC cells were generated by infection with blasticidin and hygromycin selectable retroviral constructs carrying the siRNA against Par3. Expression levels of exogenous proteins and downregulated endogenous proteins were determined by immunoblot analysis.

Western Blotting and Immunoprecipitation

For western blotting, cell lysates (1% sodium dodecyl sulfate [SDS]) 10 mM EDTA) or samples of precipitated proteins were boiled for 5 min and resolved by SDS-PAGE (polyacrylamide gel electrophoresis). Proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad), blocked with bovine serum albumin (BSA) or skimmed milk, and probed with the indicated antibodies. Specific binding was detected with a secondary peroxidase-conjugated antibody (Amersham Biosciences), and chemiluminescence followed.

For immunoprecipitation, lysates of migrating keratinocytes were prepared in standard radioimmunoprecipitation assay buffer. Extracts were precleared with washed protein G Sepharose beads (Zymed) and probed with the indicated antibodies. Secondary peroxidase-conjugated antibodies were used. Immunoprecipitates were separated by SDS-PAGE and visualized using the appropriate peroxidase-conjugated secondary antibody (Zymed Laboratories or Molecular Probes). Confocal images were obtained on a Leica TCS NT microscope operated by Leica-imaging software with 40× and 60× objectives. Size bars in images typically indicate 10 μm.

Rac Activity Assay

Rac activity was determined as described previously [13] with a biotinylated Rac1 interactive binding motif peptide of PAK1.

Hole Migration Assay

WT and Tiam1 KO cells were stained with cell tracker dye (Molecular Probes) according to manufacturer’s protocol. A lid device with inert metal rods covering the center of each well. The pre-rod was carefully placed on the culture plate such that all 24 wells contained one metal rod covering the center of each well. The pre-stained keratinocytes were added to coated 24-well microplates (collagen IV [25 mg/ml], or LN5, secreted by cultured Rac-11P cells [as described above]). After overnight culture, the device was carefully removed, leaving an uncovered circular space in the center of each well. Nonadherent cells were removed by washing with PBS, and subsequently cells were allowed to migrate under normal culturing conditions for various time points up to 24 hr, and images were taken.
Time-Lapse Imaging of Cell Migration and Computational Analysis

A detailed explanation is provided in the Supplemental Data available online. In brief, cells were followed automatically by live imaging and multiple time-lapse movies were analyzed for migratory properties with homemade cell-tracking software.

Chemoattractant Migration Assays

Details are provided in the Supplemental Experimental Procedures. In brief, cells of various genotypes were seeded on precoated transwells (Costar) and assayed for migration with and without growth factors and/or chemical inhibitors.

Supplemental Data

Experimental Procedures and three figures are available at http://www.current-biology.com/cgi/content/full/17/19/1623/DC1/.

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References