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The Rac activator Tiam1 is required for polarized protrusional outgrowth of primary astrocytes by affecting the organization of the microtubule network

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Abbreviations: GEF, guanine nucleotide exchange factor; ko, knockout; Noc, nocodazole; SEM, standard error of the mean; SD, standard deviation; MEF, mouse embryonic fibroblast; wt, wild type

Polarized cell migration is a crucial process in the development and repair of tissues, as well as in pathological conditions, including cancer. Recent studies have elucidated important roles for Rho GTPases in the establishment and maintenance of polarity prior to and during cell migration. Here, we show that Tiam1, a specific activator of the small GTPase Rac, is required for the polarized outgrowth of protrusions in primary astrocytes during the initial phase of cell polarization after scratch-wounding monolayers of cells. Tiam1 deficiency delays closure of wounds in confluent monolayers. Lack of Tiam1 impairs adoption of an asymmetrical cell shape as well as microtubule organization within protrusions. Positioning of the centrosome and Golgi apparatus, however, are independent of Tiam1-Rac signaling. We speculate that the function of Tiam1 in polarized outgrowth of astrocyte protrusions involves regulation of microtubule organization, possibly by stabilizing the microtubule cytoskeleton. Our results add Tiam1 as a player to the growing list of proteins involved in polarized outgrowth of protrusions and further elucidate the signaling pathways leading to cell polarization.

Introduction

Cell polarization and migration are two important processes for development as well as maintenance of tissue integrity. In addition, they play pivotal roles during pathological conditions, including cancer and inflammatory diseases. Numerous studies over the past years have revealed that Rho GTPases are crucial for the signaling pathways underlying the establishment of polarity, including polarized outgrowth of protrusions that precedes cell migration.[1] Seminal work by Etienne-Manneville and colleagues has shown that astrocytes provide a specifically interesting model to study the signaling pathways underlying polarized outgrowth and migration of cells, since migration is slow and accompanied by evident morphological changes.[2-5] Responses observed in vitro using astrocyte cultures resemble what occurs in vivo in response to a wound.[2,6] Mechanical disruption of confluent cell monolayers induces local activation of integrins. This triggers signaling pathways leading to cytoskeletal rearrangement resulting in two important aspects of astrocyte polarization: (1) the adoption of asymmetrical cell shapes together with the formation of protrusions in the direction of migration and (2) reorientation of the centrosome and the Golgi apparatus toward the direction of migration.[2] For the establishment of many different modes of polarity, both actin and microtubule cytoskeletal rearrangements are fundamental processes that involve signaling via Rho GTPases.[1,7,8] It is well-accepted that actin polymerization and rearrangement depend on different Rho GTPases, including Rho, Rac and Cdc42.[9-11] The microtubule cytoskeleton plays an important role as well and provides directional guidance.[12] In addition, in migrating cells there is a balance between the activity of Rac and Cdc42 at the leading edge and RhoA activity at the trailing edge.[10,13-15]

Although the exact signaling pathways underlying astrocyte protrusion formation remain to be determined, it is clear that initial activation of integrins triggers an intracellular signaling cascade that involves numerous proteins, including Rho GTPases and polarity proteins.[2] Cdc42 is essential for the outgrowth of astrocyte protrusions and signaling downstream occurs via activation of aPKCζ and Par6. Subsequently, upon inactivation of GSK3β, APC together with Dlg1 regulates microtubule

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anchoring to control reorientation of the centrosome and Golgi apparatus.[2-4,16]

We and others have shown that Tiam1, a Rac-specific guanine nucleotide exchange factor (GEF), is important for several modes of polarization and signaling in conjunction with the Par complex in different cell types, including T- neuronal and epithelial cells.[17-23] These results, together with previous implications of Rac in polarized outgrowth of astrocyte protrusions, prompted us to investigate the possible role of Tiam1 in astrocyte protrusion formation.[2] In this study, we used primary mouse astrocytes and mouse embryonic fibroblasts, both lacking Tiam1 expression, to further define the mechanism underlying this process. We analyzed the ability of these cells to establish asymmetrical morphology, and to organize the cytoskeleton along the polarity axis. We found that Tiam1 is required for the adoption of asymmetrical cell shape in response to scratch-wounding of cell monolayers. In addition, Tiam1 deficiency delays closure of wounds in confluent monolayers. Lack of Tiam1 expression does not affect the reorientation of centrosome and Golgi, but instead results in disturbed organization of the microtubule cytoskeleton in protrusions. Together, these data delineate a function of Tiam1 in one of two separate processes that are involved in astrocyte polarization upon scratch-wounding.

Results

Protrusional outgrowth is dependent on Tiam1. To investigate the effect of Tiam1 expression on the polarized outgrowth of astrocyte protrusions, astrocytes were isolated from newborn wild-type (wt) and Tiam1 knockout (Tiam1 ko) mice.[24] In addition, we isolated mouse embryonic fibroblasts (MEFs) from wt and Tiam1 ko embryos at embryonic day 12.5. Western blot analysis confirmed Tiam1 expression in wt astrocytes and MEFs and absence of Tiam1 expression in cells isolated from Tiam1 ko mice (**Fig. 1A and B**). Upon isolation and purification of astrocytes or MEFs no pronounced differences in morphology were observed between wt and Tiam1 ko cells in semi-confluent conditions (**Fig. 1C and D**). Purity of the astrocyte population was determined by the expression of the astrocyte marker protein GFAP (at least 95% at the time of experiments). Protrusional outgrowth of cells was stimulated by applying a scratch-wound to monolayers of cells and subsequent protrusion formation as well as cell morphology was examined using phase-contrast microscopy and confocal microscopy after immunostaining of cells. Protrusions became apparent approximately 2 h after scratch-wounding in both wt and Tiam1 ko astrocytes and MEFs, and became more pronounced over time, specifically in the astrocytes. Phase contrast images show striking differences in morphology of the protrusion between wt cells and Tiam1-deficient cells (**Fig. 1E and F**). Wt cells facing the wounds showed elongated, narrow protrusions perpendicular to the direction of the scratch-wound, in contrast to Tiam1 ko cells, which had much shorter protrusions that in addition appeared wider, in both primary astrocytes and MEFs. A schematic representation emphasizes the observed morphological differences after scratching monolayers of cells (**Fig. 1G and H**).

Tiam1 deficiency delays wound closure of confluent monolayers of primary astrocytes and MEFs. Scratch-wounds in confluent monolayers were closed by wt MEFs after approximately 21 h. Tiam1 ko MEFs showed a delay in the closure of scratch wounds (**Fig. 2A**, right panels, **and 2B**). The difference in wound closure was significant from 2.5 h after scratching until total closure of the wound area. This difference in migration is dependent on the presence of serum factors, and absent when cells are scratched in medium without serum (**Fig. 2A**, left panels). In the latter condition, without serum factors, neither wt nor Tiam1 ko MEFs close the wounds within the average time frame. However, the observed difference in morphology of wound edge cells (elongated protrusions in wt MEFs vs. more symmetrical cell shape in Tiam1 ko MEFs) was maintained in serum-free conditions (**Fig. 2A**, left panels). We also studied wound closure in astrocytes, which migrate considerably slower than MEFs and on average will cover wound areas only after 36–48 h. As observed for Tiam1 ko MEFs, Tiam1 ko astrocytes showed significantly delayed coverage of the wound area from 2.5 h after scratching (**Fig. 2C**). In both MEFs and astrocytes the proliferation rates of wt and Tiam1 ko cells were equal, excluding the possibility of altered growth rates to be causal for the observed differences. For both cell types, the initial coverage of a wound area after scratching was caused by elongation of the cells at the front row of the monolayer by protrusional outgrowth. Afterwards, cells slowly migrated into the wound area, increasing wound coverage.

Tiam1 is required for asymmetrical cell shape and microtubule organization upon induction of polarization. Astrocytes, because of their extremely elongated protrusions, provide an excellent model to study potential cytoskeletal differences. Immunostainings were performed to visualize both the actin and the microtubule cytoskeleton of protruding cells. These stainings revealed that the microtubule cytoskeleton of wt astrocytes was organized in elongated bundles perpendicular to the scratch-wound, reaching to the outer tip of the protrusion (**Fig. 3A**, left panels), whereas this organization of the microtubule cytoskeleton was severely impaired in Tiam1 ko astrocytes (**Fig. 3A**, right panels). In Tiam1 ko cells, microtubule bundles were not aligned parallel to the direction of migration and did not form bundles directed straight to the tip of the protrusion, but filled the protrusion in a rather disorganized manner (**Fig. 3A**, right panels). The morphology of the actin cytoskeleton was comparable between wt and Tiam1 ko astrocytes (**Fig. 3A**, upper panels). Both showed very low actin density in the tip of protrusions. A dense line of actin fibers was visible at the center of the cell body, approximately in front of the nucleus. To further analyze the polarized morphology (cellular asymmetry), both length and width of protrusions of primary astrocytes at the leading edge were quantified. Starting from about 2 h after wounding confluent monolayers, the length of protrusions of Tiam1 ko astrocytes was significantly reduced compared with wt astrocytes (**Fig. 3B**). This difference increased over time. Width of the cells was significantly different between wt and Tiam1 ko astrocytes at 7 h post-wounding, when Tiam1 ko protrusions were wider than wt (**Fig. 3C**). Additionally, the ratio of length to

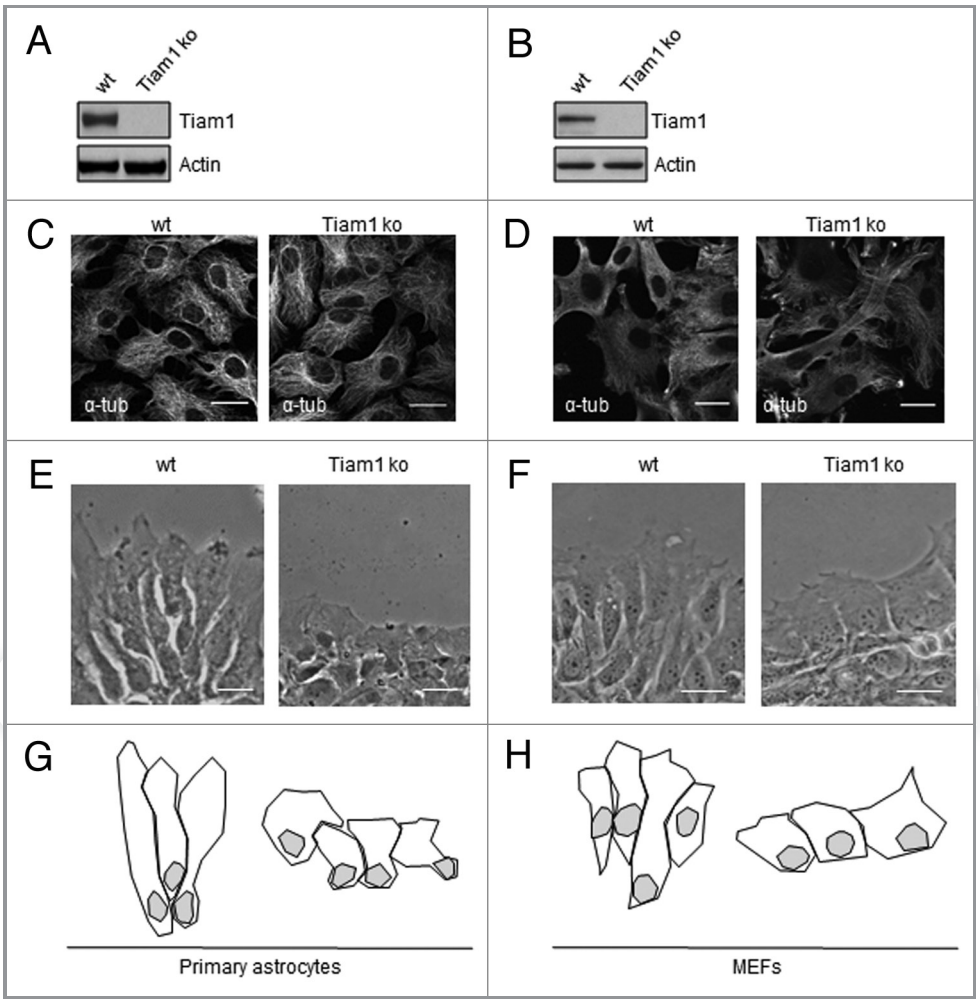

Figure 1. Tiam1 is required for proper protrusion formation in primary astrocytes and MEFs after scratch-wounding of monolayers. (A and B) Western blot analysis of Tiam1 expression in primary astrocytes (A) and embryonic fibroblasts (B) derived from wt and Tiam1 ko mice. Actin is used as loading control. (C and D) Wt and Tiam1 ko semi-confluent astrocytes and MEFs were fixed and normal morphology was analyzed using α-tubulin staining after fixation. (E and F) Phase-contrast images of protrusions formed 8 h after scratching monolayers of wt and Tiam1 ko primary astrocytes (E) and 4.5 h after scratching monolayers of MEFs (F). (G and H) Schematic representation of images in (E and F) to illustrate morphological differences observed in primary astrocytes (G) and MEFs (H). Bar, 25 μm.

width was determined to analyze the development of asymmetrical cell shape. The development of asymmetrical cell morphology was clearly hampered in Tiam1 ko astrocytes (**Fig. 3D**). Finally, we scored cells as polarized when the length of the protrusion exceeded the width of the protrusion at least four times (**Fig. 3E**). This quantification clearly showed the reduced ability of Tiam1 ko astrocytes to acquire a polarized, asymmetrical morphology compared with wt astrocytes. From these results, we conclude that Tiam1 is required for the protrusional outgrowth of astrocytes and the induction of polarized cell shape at the onset of migration into a scratch-wound.

Tiam1 is dispensable for the reorientation of the centrosome and Golgi. Besides the formation of protrusions in the direction of migration and the adoption of asymmetrical cell shape, cytoskeletal rearrangements are a second hallmark of polarized cell migration. Upon the induction of migratory signaling cascades, both the centrosome and the Golgi apparatus are reoriented, resulting in a position in front of the nucleus, facing the direction of migration.[2] Given the observation that Tiam1 is required for protrusional outgrowth and that lack of Tiam1 expression disturbs the microtubule cytoskeleton, we next examined if Tiam1 had an effect on centrosome reorientation. Both in astrocytes and MEFs, the majority of wt and Tiam1 ko cells at the wound edge showed localization of the centrosome in a polarized fashion, in the quadrant facing the scratch 6 h after scratch-wounding (**Fig. 4A and C**, upper panels). Similar results, with no difference between reorientation in wt and Tiam1 ko cells, were observed with immunostainings to detect the Golgi apparatus (**Fig. 4A and C**, lower panels). Quantification of polarized localization of the centrosome revealed no significant differences in orientation between wt and Tiam1 ko astrocytes and MEFs (**Fig. 4B and D**). Also at early time points centrosomes of Tiam1 ko astrocytes and MEFs were localized in a polarized fashion to the same extent as in wt cells (data not shown). Therefore, we conclude that

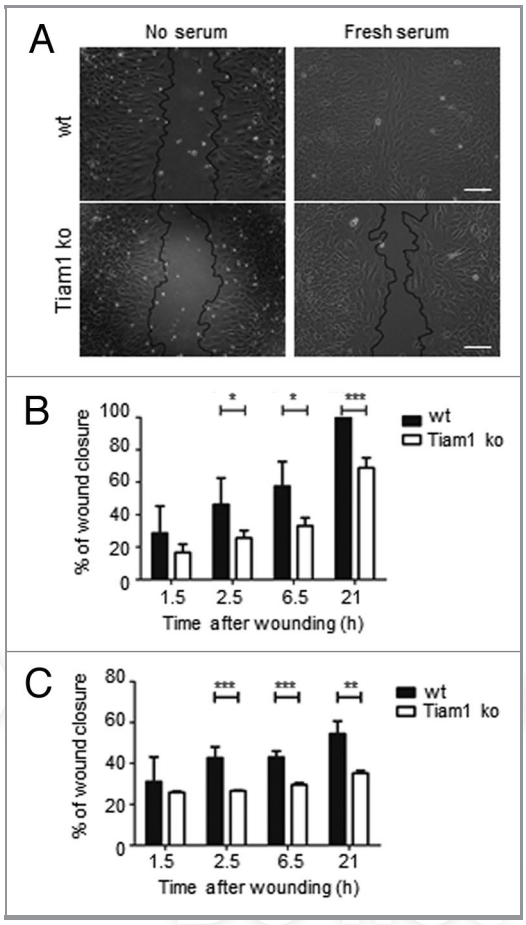

Figure 2. Tiam1 deficiency delays wound closure in MEFs and primary astrocytes. (A) Phase-contrast images of wt and Tiam1 ko MEFs at 21 h post-wounding in the absence or presence of serum. (B) Quantification of wound closure of wt and Tiam1 ko MEFs. (C) Quantification of wound closure of wt and Tiam1 astrocytes. Data represent average ± SD
*p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.

Tiam1 is dispensable for the reorientation of the centrosome and Golgi.

Tiam1-mediated Rac activity is responsible for polarized outgrowth of protrusions. Since Tiam1 is a Rac-specific GEF, we hypothesized that reduced Rac activation due to absence of Tiam1 expression resulted in the observed impaired protrusion formation. Analysis of pull-down assays using the Cdc42-Rac1 interactive binding (CRIB) domain of PAK1 confirmed decreased levels of active, GTP-bound Rac in both astrocytes and MEFs lacking Tiam1 expression compared with wt cells (**Fig. 5A and B**). To investigate whether Rac signaling is involved in the observed differences in polarized cell shape, we used two compounds shown to inhibit signaling downstream of Rac.[25-27] NSC23766 disrupts the interaction between Rac and its GEFs Trio and Tiam1, whereas EHT1864 promotes the loss of bound nucleotide as well as inhibition of GEF activity of Tiam1. Treatment with the inhibitors greatly affected the morphology of wt astrocyte protrusions, resembling that of untreated Tiam1 ko protrusions with impaired length and disturbed microtubule organization (**Fig. 5C**, lower panels, **and D**). There was no significant effect on the morphology of Tiam1 ko astrocytes. Quantification of the cellular asymmetry confirmed that wt astrocytes had lost their ability to adopt an asymmetrical cell shape due to pharmacological Rac inhibition (**Fig. 5D**). The ratio of length to width of wt protrusions after treatment with the Rac inhibitors was significantly reduced and comparable to that observed in untreated Tiam1 ko astrocytes, whereas the effect of Rac inhibitors on Tiam1 ko astrocyte protrusions was minimal. Analysis of centrosome reorientation after treatment with the inhibitors are in line with previous studies by others, which showed that Rac activity is not required for centrosome reorientation.[2]

Potential role for Tiam1 in regulation of microtubule stabilization involved in cytoskeletal organization during protrusional outgrowth of astrocytes. Previous studies implicated Tiam1 in stabilization of polarization in both keratinocytes and T cells, presumably by stabilization of the microtubule network.[17,21] Stabilized microtubules are enriched with tubulins containing different types of post-translational modifications, including acetylation and detyrosination (also referred to as Glu-tubulin, since a glutamate residue is exposed after removal of the C-terminal tyrosine) (reviewed by Westermann and Weber).[28] To investigate if the observed differences in morphology of the protrusions were due to effects of Tiam1 on microtubule stability, we analyzed the presence and localization of these post-translationally modified tubulin pools. In monolayers of MEFs we observed that distribution of detyrosinated tubulin was different (**Fig. 6A**). In wt MEFs, this pool was mainly found around the nucleus, whereas Tiam1 ko MEFs displayed pronounced staining of detyrosinated tubulin on cytoplasmic extensions, referred to as tails, spreading over the monolayer. This morphology closely resembles that observed in CLASP2 knockout MEFs and is indicative of defects in cell polarity.[29] In scratched monolayers of wt MEFs detyrosinated tubulin was clearly present in the majority of wound-edge cells (**Fig. 6B**). In Tiam1 ko MEFs, however, detyrosinated tubulin was visible in the protrusions of only few cells (**Fig. 6B**). In scratch-wounded wt astrocytes a pool of detyrosinated tubulin was clearly present throughout the entire elongated protrusions, oriented toward the scratched area (**Fig. 6C**, lower left panel). In Tiam1 ko astrocyte protrusions, detyrosinated tubulin seemed to be restricted to the area close to the nucleus and did not spread toward the tip of the protrusion (**Fig. 6C**, lower right panel). These data suggest that both Tiam1 ko astrocytes and Tiam1 ko MEFs contain less stable microtubules, particularly in the front of the protrusions.

Lysates from protruding astrocytes were used to further examine microtubule stability in our cells. We investigated the cellular responses to the microtubule destabilizing agent nocodazole in terms of levels of post-translational modifications in tubulin pools. Western blot analysis revealed a slight but consistent decrease in the levels of detyrosinated tubulin in untreated Tiam1 ko astrocytes compared with wt cells (**Fig. 6D and E**). However, levels of post-translationally modified tubulin seem to decrease to a similar extent upon incubation with nocodazole in both wt and Tiam1 ko astrocytes (**Fig. 6D and E**). These results suggest that Tiam1, as previously shown for keratinocytes,[21] may function in microtubule stabilization in MEFs and

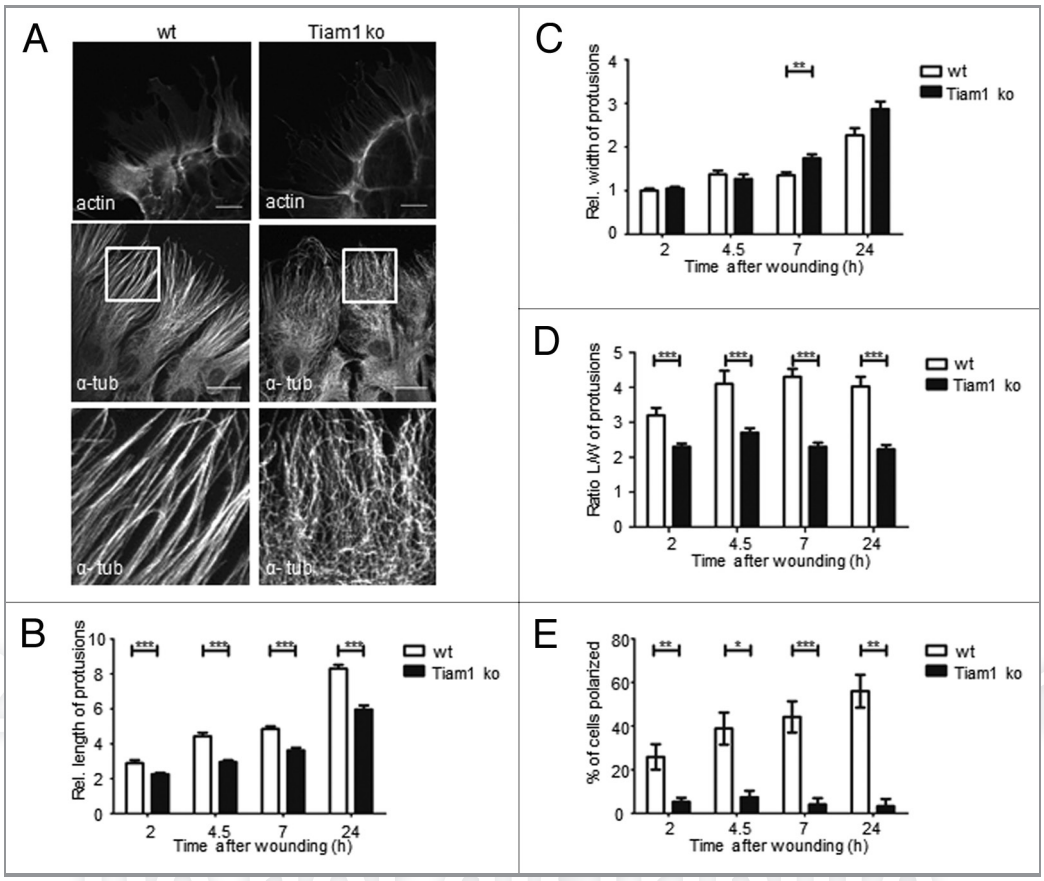

Figure 3. Tiam1 is required to obtain pronounced cellular asymmetry and organization of the microtubule cytoskeleton after scratch-wounding of primary astrocytes. (A) The actin and microtubule cytoskeletons were visualized by fixing wt and Tiam1 ko primary astrocytes and staining with Alexa Fluor-568-coupled phalloidin and anti-α-tubulin antibody 24 h after scratching monolayers of astrocytes. The boxed areas are enlarged below the corresponding panels. Bar, 25 μm. (B–E) Quantification of morphology of protrusions formed at different time points after scratching monolayers, showing length (B), width (C), ratio of length to width (D) and the number of cells with polarized morphology (E). Cells were determined polarized if length of the protrusion exceeded the width at least four times. Over 100 cells were analyzed in three separate experiments. Data represent average ± SEM. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$.

astrocytes Tiam1 by affecting levels of post-translationally modified tubulin.

Taken together, our results demonstrate that Tiam1, through its Rac-GEF function, is required for the polarized outgrowth of protrusions and subsequent wound closure in primary astrocytes and MEFs. Tiam1 expression is dispensable for the polarized distribution of organelles as centrosome and Golgi apparatus. Our data indicate that lack of Tiam1 disturbs the microtubule cytoskeleton within the protrusions of wound-edge cells, suggesting a role for Tiam1 in microtubule organization and/or stabilization.

Discussion

To study a potential role of Tiam1 in polarized outgrowth of protrusions, we used murine primary astrocytes and fibroblasts with and without Tiam1. Wounding monolayers of primary astrocytes in particular allowed the investigation of protrusion formation at the wound edge, as well as changes of the cytoskeleton due to the induction of polarization.[2-5] Cells lacking Tiam1 showed a remarkable decrease in length of protrusions formed after wounding. At later time points, the width of protrusions also significantly differed between wt and Tiam1 ko astrocytes. Quantification of cellular asymmetry showed a significant defect of Tiam1 ko astrocytes to adopt an asymmetrical shape after wound-induced outgrowth. Tiam1 ko cells failed to form long and narrow protrusions that are typical for polarized astrocytes. Also in primary fibroblasts this difference was clearly visible although less pronounced. For both cell types studied, Tiam1 deficiency resulted in delayed closure of scratch-induced wounds in monolayers of cells. Despite impaired protrusional outgrowth due to lack of Tiam1, the organization of actin cytoskeleton within protrusions was rather similar in wt and Tiam1-deficient astrocytes. Interestingly however, the organization of the microtubule cytoskeleton differed greatly. Whereas protrusions of wt astrocytes contained elongated parallel bundles of microtubules that followed the protrusion all the way to the tip, protrusions of Tiam1 ko astrocytes comprised rather disorganized microtubules throughout the cell (illustrated in **Fig. 7A**). These differences in the microtubule cytoskeleton were not due to and did not result in altered re-positioning of the centrosome and Golgi apparatus,

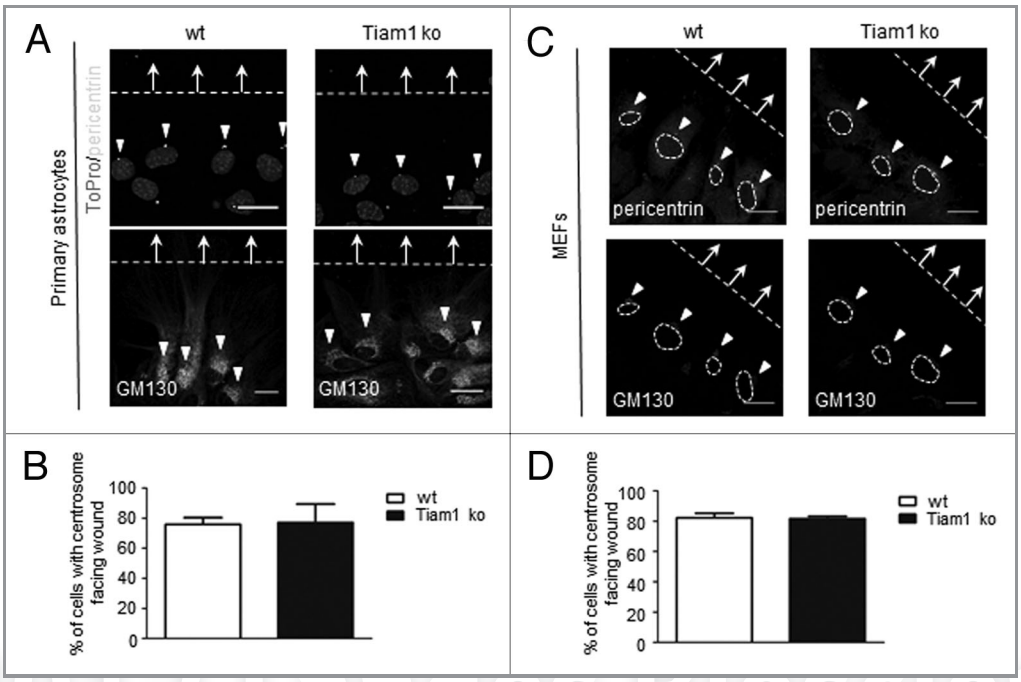

Figure 4. Tiam1 is dispensable for reorientation of centrosome and Golgi apparatus after induction of polarized outgrowth of protrusions by scratching monolayers of primary astrocytes and MEFs. (A) Positions of centrosome (green) and nucleus (blue) were visualized in primary astrocytes fixed 4.5 h after wounding using anti-pericentrin antibody and ToPro3 respectively (upper panels). Golgi apparatus was visualized using GM-130 antibody (lower panels) Bar, 25 μm. (B) Quantification of centrosome reorientation in front-row wt and Tiam1 ko primary astrocytes 4.5 h after wounding. (C) Centrosome (green, upper panels) and Golgi (blue, lower panels) reorientation were visualized in MEFs using anti-pericentrin antibody and GM-130 antibody respectively (4 h after wounding). Bar, 25 μm. Dotted lines indicate the position of the nuclei. (D) Quantification of centrosome reorientation in front-row wt and Tiam1 ko MEFs. Centrosomes were scored as correctly reoriented when localized in the quadrant facing the wound area. Arrowheads indicate the positions of centrosomes and Golgi, arrows indicate the direction of migration, perpendicular to the wound. Over 80 cells were analyzed in three separate experiments. Data represent average ± SEM.

as both organelles were localized in a polarized fashion within the quadrant of the cell facing the wound area in the majority of both wt and Tiam1 ko astrocytes and MEFs.

Tiam1 was identified as a Rac-specific GEF and it is well accepted that the Rho GTPase Rac functions in many cellular processes, including actin and microtubule cytoskeletal organization as well as cell-matrix adhesions.[1,10,30] Since these processes are all required for astrocyte protrusion formation, it was tempting to speculate that impaired Rac activation was causal for the observed morphological differences in Tiam1 ko cells. Indeed, Rac-GTP levels were decreased in Tiam1 ko astrocytes and MEFs compared with their wt counterparts. In addition, using pharmacological inhibitors we found that Rac inhibition in wt astrocytes impaired protrusional outgrowth and induced cytoskeletal characteristics that resembled Tiam1 ko astrocyte protrusions. This is in line with previous reports that suggest that Rac activity is essential for protrusional outgrowth, since dominant-negative Rac constructs interfere with protrusion formation.[2,31,32]

Since Tiam1 has been shown to signal in conjunction with the Par3/Par6/aPKCζ-polarity complex in different cellular polarization processes, we investigated a possible interplay in astrocyte protrusion formation.[1,17-23] Interestingly, we found that Tiam1 was dispensable for centrosome reorientation, as has been previously shown for cells expressing dominant-negative Rac.[2] This suggests that Tiam1 does not regulate the function of the Par6/aPKCζ complex in MTOC reorientation, previously ascribed to Cdc42 in rat astrocytes.[2-4] We did not find differences in levels of phosphorylated aPKCζ and known downstream targets of the Par complex in astrocyte protrusion formation, such as GSK3β, between polarized protruding wt and Tiam1 astrocytes and MEFs (unpublished data). This indicates that Tiam1-mediated Rac activation does not activate aPKCζ in protruding astrocytes, as has been shown previously during tight junction formation in keratinocytes.[19] Moreover, levels of β-catenin, regulated through GSK3β, were similar between protruding wt and Tiam1 ko astrocytes and MEFs. This further suggests that Tiam1 does not signal in conjunction with the Par6/aPKCζ complex in the described downstream signaling toward GSK3β. Apparently, Tiam1 acts in parallel with the Par6/aPKCζ complex in protrusion outgrowth (**Fig. 7B**).

In most migrating cells, the front is characterized by rapid actin polymerization and adhesion turnover.[33] Interestingly, although the importance of Rac in the regulation of the actin cytoskeleton is well described, this function seems less important in astrocyte protrusion formation.[9,14] Very little actin is present in these protrusions, and we and others have found that protrusions can still be formed in the presence of agents that alter the polymerization status of actin (ref. 2 and unpublished data). It seems that the particular function of Tiam1-mediated Rac activity identified in this study involves the microtubule cytoskeleton

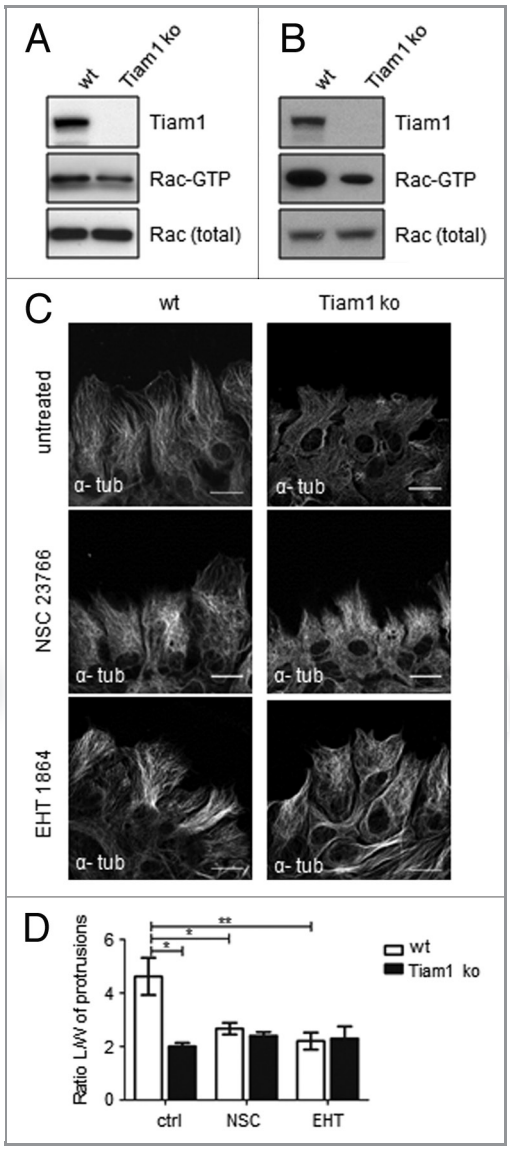

Figure 5. Tiam1-mediated Rac activity is required for asymmetrical cell shape after scratch-wounding of monolayers. (A and B) Western blot analysis of Rac-GTP levels in lysates of wt and Tiam1 ko astrocytes (A) and MEFs (B). Total levels of Rac were used as loading control. (C) Immunofluorescence staining with α-tubulin antibody visualized the microtubule cytoskeleton of protrusions formed in untreated wt and Tiam1 ko astrocytes (upper panels) and astrocytes treated with 10 μM NSC23766 or 50 μM EHT1864 for 6 h immediately after scratching (lower panels). Bar, 25 μm. (B) Quantification of cellular asymmetry of astrocytes treated with Rac inhibitors compared with untreated astrocytes, represented as the average ratio of length to width ± SEM, 6 h after scratch-wounding. *p ≤ 0.05, **p ≤ 0.01.

rather than the actin cytoskeleton. In the leading edge of migrating cells microtubules are stabilized toward the direction of migration.[34] Tiam1 can regulate microtubule stability in single migrating keratinocytes and in absence of Tiam1 this disturbed process affects persistent migration of these cells.[21] Furthermore, downregulation of Tiam1 expression can lead to reduced levels of acetylated tubulin, suggesting a role in stabilization of microtubules in ES cells.[35] In line with this, we found that protrusions of astrocytes lacking Tiam1 contain reduced pools of stabilized, detyrosinated tubulin, compared with wt astrocyte protrusions. Moreover, we observed similarities between MEFs lacking Tiam1 expression and results from studies using CLASP2-deficient MEFs.[29] CLASP2 has been shown to be involved in the stabilization of microtubules during migration, as well as crosslinking with the cell cortex.[29,36] Together with our findings that the organization of microtubule cytoskeleton is disturbed in Tiam1 ko astrocytes, our data suggest that Tiam1 influences this microtubule organization in astrocytes by effecting microtubule stability.

The exact mechanism by which Tiam1 affects microtubule organization remains to be established. Rac, via the activation of PAK, has been shown to be involved in the regulation of microtubule dynamics at the leading edge of migration cells. However, previous reports also suggested that PAK alone is insufficient to promote microtubule growth downstream of Rac.[37,38] It is likely that interactions between the actin and the microtubule cytoskeleton play an important role in regulating cytoskeletal dynamics in leading edges.[7,39] IQGAP is an actin-binding effector protein of both Rac and Cdc42 and together these proteins have been shown to contribute to microtubule capture.[40] Furthermore, IQGAP can interact with APC and CLIP170 and thereby provides a link between actin and microtubule dynamics.[40,41] Tiam1 has previously been shown to associate with microtubules during axon formation.[42] In addition, indirect binding of Tiam1 to microtubules has been demonstrated in cultured hippocampal neurons via interaction with MAP1B, which can bind both microtubules and actin.[43] Also for other GEFs it has been shown that interaction with microtubules directly, or indirectly via for example microtubule plus end binding proteins, can regulate their activation. Asef, another Rac-specific GEF has been shown to interact with the microtubule plus end binding protein APC, which enhances its GEF activity and is involved in migration of cells.[44,45] GEF-H1 is a GEF for multiple RhoGTPases, including Rac1, and mediates crosstalk between the microtubule and actin cytoskeleton by association with microtubules which is regulated by phosphorylation by Par1b/MARK2.[46-48] In T- and B-cells the Rac/Rho GEF Vav has been shown to associate with tubulin.[49,50] Rac activation by the GEF DOCK7 triggers microtubule stabilization in developing axons of hippocampal neurons by PAK-mediated phosphorylation of stathmin.[38,51,52] Phoshorylation of stathmin reduces its depolymerizing activity. Further study on the potential direct or indirect binding of Tiam1 to microtubules will be of interest to reveal the function of this protein in protrusion formation that precedes migration of MEFs and primary astrocytes.

Together, available data from literature and our present results make it tempting to speculate that in protruding astrocytes Tiam1 is involved in the interaction between the actin and the microtubule cytoskeleton, or perhaps in the anchoring or capturing of microtubule tips.

Materials and Methods

Cell isolation and culture. Astrocytes were isolated from newborn (postnatal day 1–3) wt and Tiam1 ko mice on FVB

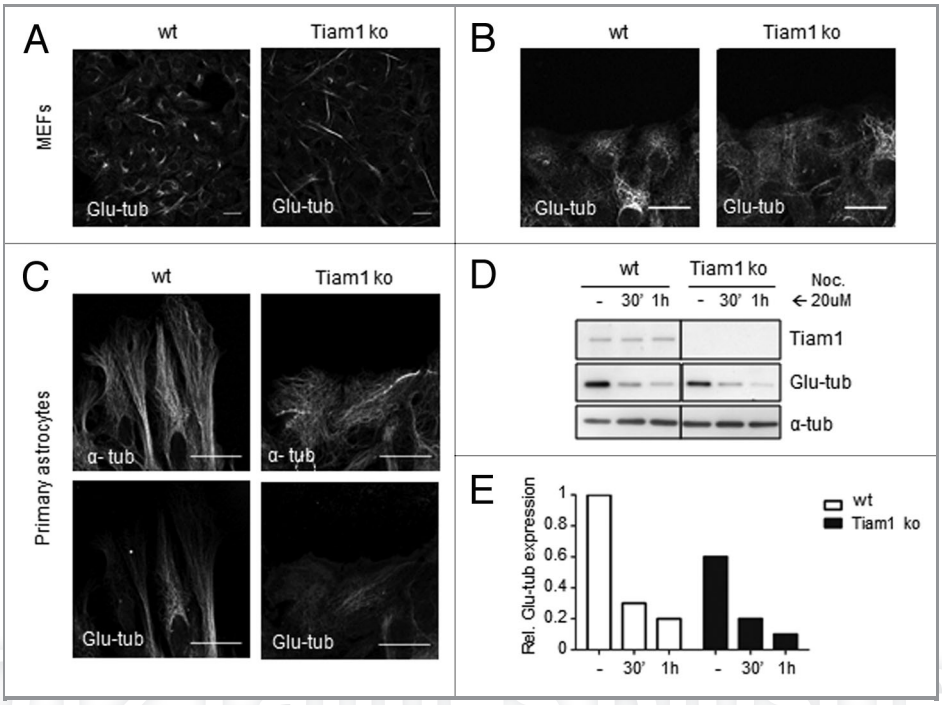

Figure 6. Role for Tiam1 in regulation of organization of microtubule cytoskeleton. (A) Confluent monolayer of wt and Tiam1 ko MEFs were stained for detyrosinated tubulin (Glu-tub). Bar, 25 μm. (B) Scratched monolayers of wt and Tiam1 ko MEFs were stained for detyrosinated tubulin 6 h after wounding. Bar, 25 μm. (C) Microtubule cytoskeleton of scratched wt and Tiam1 ko astrocytes was visualized 4 h after scratch-wounding using α-tubulin antibody (upper panels) and antibody to detect detyrosinated tubulin (lower panels). Bar, 25 μm. (D) Western blot analysis on lysates of sparsely seeded wt and Tiam1 ko primary astrocytes treated with nocodazole (Noc., 20 μM) for indicated times. Levels of post-translationally modified tubulin in primary astrocytes were determined, using an antibody detecting detyrosinated tubulin. α-tubulin was used as loading control. Results shown are representative of at least three independent experiments. (E) Quantification of D, expression levels of detyrosinated tubulin relative to corresponding α-tubulin levels.

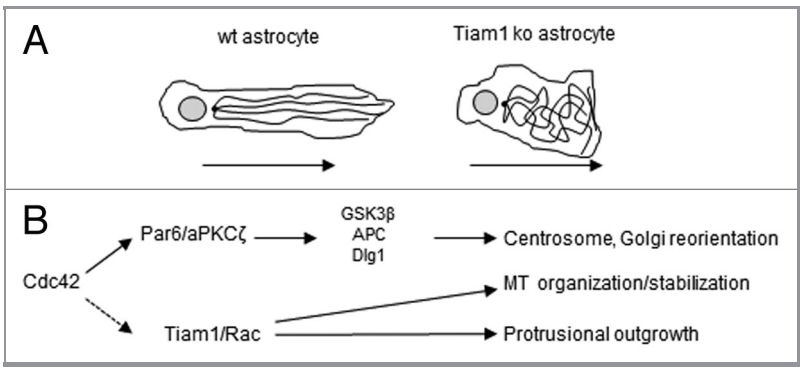

Figure 7. Model showing the involvement of Tiam1/Rac signaling in the outgrowth of astrocyte protrusions as well as the organization of the microtubule cytoskeleton. (A) Schematic representation of the effect of absence of Tiam1 expression on astrocyte protrusion outgrowth. Protrusional length is impaired and the integrity of the microtubule cytoskeleton is disturbed. Centrosome positioning is independent of Tiam1/Rac signaling. Arrow indicates the direction of protrusion formation. (B) Scratching monolayers of astrocytes induces activation of Cdc42, which in turn regulates a signaling pathway involving proteins such as GSK3β, APC and Dlg1, controling centrosome and Golgi reorientation.[2-4] In this study, we have shown that protrusional outgrowth on the other hand is regulated by Tiam1-mediated Rac activation. In addition, we demonstrated that lack of Tiam1 impairs protrusional outgrowth and the adoption of asymmetrical cell shape. Furthermore, in a pathway parallel to the Par6/aPKCζ pathway, Tiam1 functions in the regulation of the microtubule organization, perhaps through effects on microtubule stabilization.

background as described in a protocol generously provided by N.E. Savaskan.[24] Briefly, newborns were decapitated and after removal of skin from the head the skull was opened. Brain was transferred into a dish with Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin and 100 μg/ml streptomycin (P/S, Invitrogen). Brainstem and cerebellum were dissevered and meninges were removed. The cortex was transferred to a solution of 0.05% trypsin and 0.02% EDTA (Invitrogen), shattered and incubated for 4 min in a 37°C water bath. Subsequently, trypsin was inhibited by the addition of an equal volume of DMEM supplemented with 10% FCS and P/S. Cells were centrifuged at 800 rpm for 10 min at 4°C, resuspended and grown in DMEM with 10% FCS and P/S on culture plastic coated with poly-L-ornithine (0.5 mg/ml, Sigma). Microglia depletion was achieved by washing with pre-warmed PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ every third day. Purity of astrocyte cultures was confirmed (over 95%) by immunofluorescence staining using an antibody detecting the astrocyte marker protein glial fibrillary acidic protein (GFAP, Sigma). Cells used for the

described experiments were between in vitro days 9 and 20 and passaged maximally once.

Mouse embryonic fibroblasts (MEFs) were isolated as described previously.[53] Briefly, embryos were removed from the uterus of wt and Tiam1 ko mice at day 12.5 of pregnancy. Embryos were decapitated, soft tissues were removed and carcasses were minced and transferred to cold PBS. Cells were centrifuged at 800 rpm for 5 min at 4°C, washed with PBS, centrifuged again and incubated with 0.05% trypsin and 0.02% EDTA, supplemented with 200 U/ml penicillin and 200 μg/ml streptomycin (2xP/S) ovenight at 4°C. The next day cells were resuspended in DMEM containing 10% FCS, 2xP/S and 0.1 mM β-mercapto-ethanol. After reaching confluency cells were split and subsequently grown in DMEM with 10% FCS, P/S and 0.1 mM β-mercapto-ethanol. All cells were incubated at 37°C in 5% CO_2.

Antibodies. The following antibodies were used for immunoblotting and immunostaining: anti-Rac1 (Upstate); anti-Tiam1 (C16, sc-872, Santa Cruz); anti-α-tubulin (Sigma); anti-detyrosinated tubulin (Chemicon), anti-pericentrin (Novus Biologicals), anti-GM130 (BD transduction laboratories). Filamentous actin was labeled with AlexaFluor568-phalloidin (Invitrogen). Nuclei were stained with Topro3 (Molecular Probes). Alexa Fluor-conjugated secondary antibodies were from Invitrogen.

Scratch-wound assay. Cells were seeded at equal concentrations, dependent on the size of tissue culture plate, and grown to confluency on glass coverslips (coated with 0.5 mg/ml poly-L-ornithine for astrocytes (Sigma) overnight at 4°C or 6 h at room temperature). Wounds were created by scraping cells off the monolayers with a yellow pipet tip. After scratching, floating cells were removed by either replacing the medium or filtering the medium cells were cultured in. Protrusional outgrowth was monitored using phase contrast microscopy (Axiovert 25; Carl Zeiss MicroImaging, Inc.) and photographed using a digital camera (model DSC-S85; Sony). Morphology of cells was further analyzed afterwards using immunofluorescence stainings and subsequent confocal microscopy. Wound closure was determined by measurement of the coverage of the scratched area at different time points in different experiments using ImageJ software (National Institutes of Health).

Rac activity assay. The level of GTP-loaded Rac was determined as described previously.[54] Briefly, cells were put on ice, washed with ice-cold PBS (containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and lysed with a lysisbuffer containing 0.5% Nonidet P-40, 10 mM Tris, 150 mM NaCl, 50 mM MgCl2, protease inhibitors and 2 μg of a biotinylated Cdc42-Rac1 interactive binding domain of PAK1. Cleared supernatants were added to washed streptavidin beads and rotated for 30 min at 4°C. Afterwards beads were washed and bound proteins were taken up in sample buffer (NuPage, Invitrogen) and subsequently used for Western blot analysis. After blocking in Tris-buffered saline containing Tween-20 (TBST) containing 5% skimmed milk, GTP-loaded Rac was detected with a Rac-specific antibody (Upstate).

Western blot analysis. Cells were washed with ice-cold PBS, lysed and scraped in either SDS lysis buffer (1% SDS, 10 mM EDTA, 1 mM sodium fluoride, 2 mM sodium orthovanadate and protease inhibitors) or RIPA lysis buffer (10 mM Tris pH 7.4, 100 mM sodium chloride, 1 mM EDTA and EGTA, 1 mM sodium fluoride, 2 mM sodium orthovanadate, 0.1% SDS, 0.5% sodium deoxycholate, 1% triton X-100, 10% glycerol and protease inhibitors). RIPA protein lysates were cleared of cellular debris by centrifugation at 4°C for 10 min at 12,000 rpm. For investigation of post-translationally modified tubulin pools cells were washed in pre-warmed PBS (37°C) before lysis. Equal amounts of protein were determined by BCA protein assay kit (Pierce) and separated by SDS-PAGE using pre-cast gradient gels (4–12% Nu-Page Bis-Tris, Invitrogen) and transferred to PVDF membranes. Aspecific binding of proteins was blocked by incubation in either 5% skimmed milk or 5% bovine serum albumin (BSA) in TBST. Proteins were detected by 1 h incubation of primary antibody at room temperature or overnight incubation at 4°C, followed by 1 h incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and subsequent chemiluminescence, using Western Lightning chemiluminescent substrate (PerkinElmer).

Immunofluorescence staining. Astrocytes were grown to confluency on glass coverslips coated with poly-l-ornithine (0.5 mg/ml). For the determination of microtubule stability MEFs were serum starved for 24 h by culturing in DMEM with P/S but without FCS. Induction of protrusional outgrowth was stimulated in these cells by the addition of fresh medium containing 10% FCS after scratching. After scratch-wound assays, cells were fixed at different time points in methanol, glutaraldehyde or 4% paraformaldehyde and permeabilized using 0.1% Triton X-100 in PBS. For visualization of stable pools of microtubules, cells were washed in pre-warmed PBS (37°C) before fixation in 4%PFA (also pre-warmed to 37°C). After blocking for 1 h with 2% BSA in PBS cells were incubated with primary antibodies for 1 h. Subsequently, cells were washed with 1% BSA in PBS and incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 1 h, washed and mounted in Mowiol-DABCO. Images were taken with a confocal microscope (model TCS SP2; Leica) and were analyzed and resized using Image J software.

Quantification of protrusion dimensions and centrosome localization. For the quantification of protrusional length and width only front row cells were taken into account. Confocal images of fixed and α-tubulin-stained cells were used to analyze different dimensions of protrusions using Image J software. Length was determined as distance from back of the nucleus to the tip of the protrusion. Width was determined at the tip of the protrusion. Cells were considered polarized if length exceeded width at least four times. Centrosome localization was determined by staining cells fixed with 4% paraformaldehyde (in PBS) with an anti-pericentrin antibody. The centrosomes were considered polarized when localized in the quadrant perpendicular to the direction of the wound. Cells in which the centrosome polarization was hard to interpret, because of localization too far from or on top of the nucleus where not taken into account, as well as cells that where located behind the front row cells.

Pharmacological Rac inhibition. Compounds used to interfere with Rac activity were NSC23766 (Merck) and EHT1864 (Sigma), used at concentrations of 10 and 50 μM, respectively.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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