# Rac1 and Rac3 have opposing functions in cell adhesion and differentiation of neuronal cells

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## Summary

**Rac1 and Rac3 are highly homologous members of the Rho small GTPase family. Rac1 is ubiquitously expressed and regulates cell adhesion, migration and differentiation in various cell types. Rac3 is primarily expressed in brain and may therefore have a specific function in neuronal cells. We found that depletion of Rac1 by short interference RNA leads to decreased cell-matrix adhesions and cell rounding in neuronal N1E-115 cells. By contrast, depletion of Rac3 induces stronger cell adhesions and dramatically increases the outgrowth of neurite-like protrusions, suggesting opposite functions for Rac1 and Rac3 in neuronal cells. Consistent with this, overexpression of Rac1 induces cell spreading, whereas overexpression of Rac3 results in a contractile round morphology. Rac1 is mainly found at the plasma membrane, whereas Rac3 is predominantly localized in the perinuclear region. Residues 185-187, present in the variable polybasic rich region at the carboxyl terminus are responsible for the difference in phenotype induced by Rac1 and Rac3 as well as for their different intracellular localization. The Rac1-opposing function of Rac3 is not mediated by or dependent on components of the RhoA signaling pathway. It rather seems that Rac3 exerts its function through negatively affecting integrin-mediated cell-matrix adhesions. Together, our data reveal that Rac3 opposes Rac1 in the regulation of cell adhesion and differentiation of neuronal cells.**

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Key words: Rac1, Rac3, RhoA, Neurite outgrowth, Cell-matrix adhesions, Polybasic rich region

## Introduction

Rac proteins belong to the family of Rho-like GTPases. These are small regulatory proteins that act as binary switches, being inactive in a GDP bound state and active when GTP-bound. In their active state, small GTPases bind to various downstream effectors and thereby regulate a large variety of cellular processes, including cytoskeletal rearrangements, cell motility, cell-cell and cell-matrix adhesions, gene transcription and cell survival (Van Aelst and D'Souza-Schorey, 1997; Burridge and Wennerberg, 2004). Guanine nucleotide exchange factors (Fukuhara et al., 2001) stimulate the exchange of GDP for GTP, thereby activating Rho-like GTPases (Fukuhara et al., 2001; Rossman et al., 2005). GTPase-activating proteins inactivate small GTPases by stimulating their intrinsic GTPase activity (Diekmann et al., 1995), whereas GDP dissociation inhibitors (GDIs) prevent GDP dissociation.

In mammals, the Rac family is represented by ubiquitously expressed Rac1, myeloid-lineage-specific Rac2, and brain-enriched Rac3. We and others have shown that activated Rac1 is required for spreading and neurite outgrowth in neuroblastoma cells (Leeuwen et al., 1997; Sarner et al., 2000; Aoki et al., 2004) and primary hippocampal neurons (Schwamborn and Puschel, 2004). During the past decade, it has been shown that Rac1 affects neuritogenesis by regulating cytoskeletal rearrangements and neuronal polarity, stimulating axonal growth, growth cone stability and axon guidance, dendrite formation and spine morphogenesis (reviewed by Govek et al., 2005). Rac1 facilitates neuritogenesis by binding and activating several effector molecules. For instance, by activating Pak kinases (p21 activated kinases), Rac1 suppress myosin light chain (MLC) phosphorylation, thereby attenuating myosin contractility (Sander et al., 1999; Bokoch, 2003). Through Pak, Rac1 also phosphorylates myosin II heavy chain (Van Leeuwen et al., 1999), resulting in relaxation of cells and promotes actin polymerization via LIMK/cofilin pathway, a step necessary for lamellipodia formation and neurite outgrowth (Kuhn et al., 2000). Furthermore, Rac1 signaling stimulates cell-matrix adhesions via Git1/PIX/paxillin complex (Turner et al., 1999; Manabe et al., 2002; Paris et al., 2003). Also, Rac1 increases transcription of genes associated with neuronal differentiation by phosphorylating and activating c-terminal Jun kinase (JNK) (Teramoto et al., 1996; Kita et al., 1998).

Rac3 is a less well characterized and is abundantly expressed in mammalian brain (Haataja et al., 1997). Rac1 and Rac3 share 92% of amino acid sequence, diverging predominantly in the carboxyl terminus. The last three amino acids of the carboxyl terminus (CAAX box) are not conserved. This region determines the isoprenylation and thereby membrane targeting of the protein (Seabra, 1998). Rac1 and Rac3 also differ in the polybasic region (PBR) directly upstream of the CAAX box, which can serve as an additional binding site for effectors (van Hennik et al., 2003; Yamauchi et al., 2005) or GEFs (ten Klooster et al., 2006). The effector-binding regions (residues 26-45) are conserved in Rac1 and Rac3, as well as the switch I and II regions, responsible for nucleotide binding (Haeusler

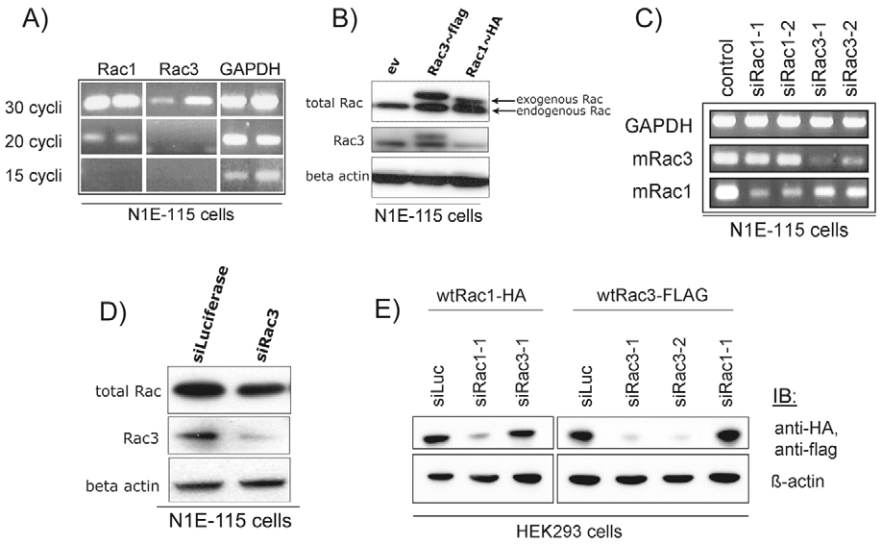


**Fig. 1.** Both Rac1 and Rac3 are endogenously expressed in N1E-115 cells, and are efficiently and specifically downregulated by shRNA. (A) Total RNA isolated from N1E-115 was used as template for cDNA synthesis. Primers specific for Rac1, Rac3 or GAPDH were used in semi-quantitative reverse-transcription PCR to show the relative expression levels of these genes. (B) Cells were transfected with pcDNA3.1 empty vector (ev), Rac3~FLAG or Rac1~HA, lysed and western blotted. Upper panel shows total Rac staining. Note that Rac3 antibody recognizes specifically Rac3 and not Rac1 (middle panel). β-actin staining was used as loading control. (C) RT-PCR showing the expression levels of GAPDH (control; top panel), endogenous Rac3 (middle panel) and endogenous Rac1 (lower panel) in cells in which either shRac1 (lane 2, 3) or shRac3 (lane 4, 5) has been expressed. Control cells (lane 1) were transfected with siLuc. (D) Western blot showing depletion of Rac3 protein upon expression of shRac3, visualized by total Rac antibody (upper panel) and Rac3-specific antibody (middle panel). Beta actin staining is shown as loading control. (E) Western blot showing depletion of either Rac1 or Rac3 by shRac1 or shRac3, respectively. pSuper constructs, containing different sequences specifically targeting Rac1, Rac3 (e.g. shRac1-1, shRac3-1 and shRac3-2), or luciferase, were co-transfected with pcDNA3/Rac1~HA or pcDNA3/Rac3~FLAG constructs in HEK293 cells.

et al., 2003). Therefore, it is not surprising that constitutively active Rac3 is able to activate Pak, JNK and phospholipase C beta (PLCβ), which are well-described Rac1 downstream effectors (Haataja et al., 1997; Snyder et al., 2003). Moreover, constitutively active Rac3 shows transforming capacity, and induces foci formation in fibroblasts (Joyce and Cox, 2003; Keller et al., 2005), similarly to constitutively active Rac1. Clearly, the conserved effector domain and overall similarity allow Rac3 to mimic Rac1 signaling when used in a constitutive active form.

The physiological function of Rac3 in mammalian neuronal cells is poorly understood. Rac1 and Rac3 are differentially distributed in developing mouse brain (Bolis et al., 2003), where expression of Rac3 peaks during the time of intensive synaptogenesis. The avian orthologue of mammalian Rac3, termed Rac1B, is specifically expressed in developing chicken nervous system (Malosio et al., 1997). Moreover, Rac1B overexpression induced pronounced neurite outgrowth in dissociated avian retinal neurons (Albertinazzi et al., 1998). Rac1-null mice die early during embryonic development (Sugihara et al., 1998). Rac3-null mice show no obvious developmental defects (Corbetta et al., 2005), but show motor coordination and motor learning superior to that of wild-type mice, suggesting a function of Rac3 in later events of neuronal development. The limited data available suggest a specific role of Rac3 in mammalian neuronal cells. However, it is currently unclear what this function might be and whether this function is different from that of Rac1.

In the present study, we addressed this question and investigated the function of Rac1 and Rac3 in neuronal morphology and differentiation, using N1E-115 murine neuroblastoma cells as a model. Our data show that Rac3 has a specific, Rac1-opposing function with respect to cell-matrix adhesions and outgrowth of neurite-like protrusions. This Rac3 function depends on the variable polybasic-rich region located in the carboxyl terminus.

## Results

### Both Rac1 and Rac3 are endogenously expressed in N1E-115 cells

To investigate the functions of Rac1 and Rac3 in neuronal morphology, we selected neuroblastoma N1E-115 cells as a model. Firstly, we analyzed the expression of both Rac proteins in N1E-115 cells. We designed specific Rac1 and Rac3 primers and performed RT-PCR analysis, using total RNA isolated from serum-cultured N1E-115 cells as a template. As shown in Fig. 1A, both Rac1 and Rac3 transcripts are present in N1E-115 cells (lanes 1-4). In a semi-quantitative RT-PCR (Marone et al., 2001), by using different numbers of PCR cycles to titrate out the available template, we estimated that Rac3 accounts for about 30% of the total Rac transcript in N1E-115 cells (Fig. 1A and data not shown). Furthermore, we used a Rac3-specific antibody (Joyce and Cox, 2003) to demonstrate that N1E-115 cells contain significant amounts of Rac3 protein (Fig. 1B). Comparing the protein levels of Rac1, Rac3 and endogenous Rac3 before and after depletion with shRac3 (Fig. 1B,D), we estimate that Rac3 represents about 30% of the total Rac at protein level also in these cells.

### Specific downregulation of Rac1 and Rac3 by shRNA

We next investigated the loss-of-function effects of Rac1 and Rac3 in N1E-115 cells. To circumvent specificity issues associated with dominant negative mutants, we used a knockdown approach. We designed short interference RNA (siRNA) sequences targeting specifically mouse Rac1 or Rac3. Several target sequences per gene were used, which were cloned in pSuper or pRetroSuper (Brummelkamp et al., 2002), and designated as shRac1-1, shRac1-2, shRac3-1 and shRac3-2. To

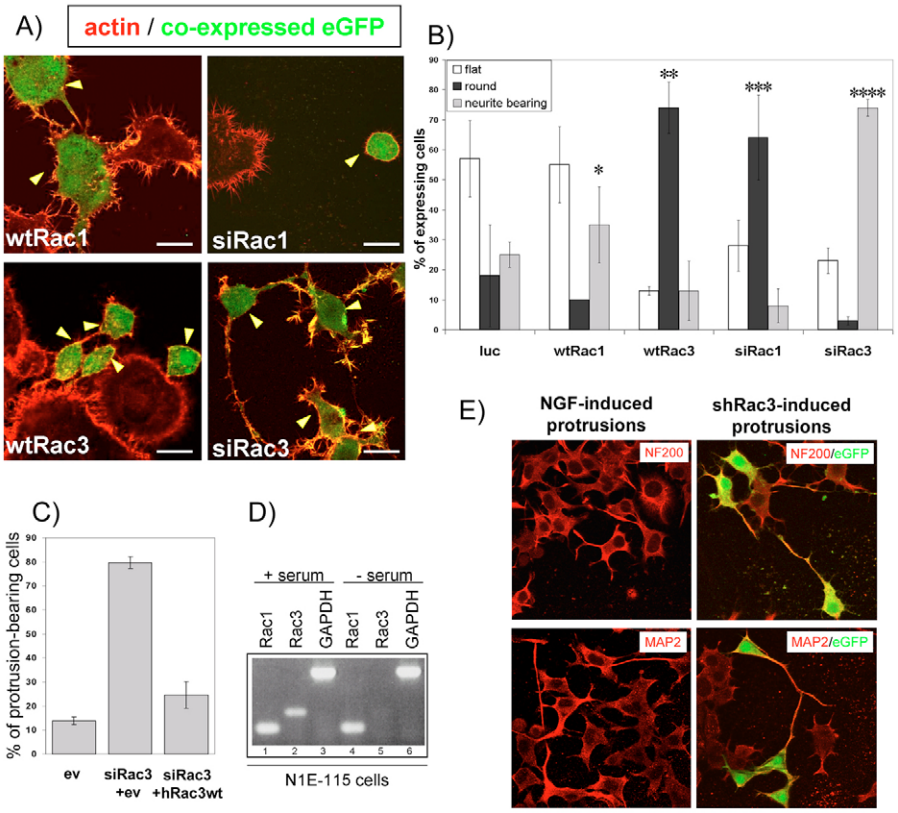


**Fig. 2.** Rac1 and Rac3 produce opposite effects on N1E-115 morphology. (A) Cells were transfected with pcDNA3/Rac1~HA, pcDNA3/Rac3~FLAG, pSuper/shRac1 or pSuper/shRac3, together with a green fluorescent protein vector (ratio 10:1). Subsequently, cells were serum-starved for 24 hours, fixed and stained with phalloidin to visualize filamentous actin. Bar, 25 μm. (B) Quantification of the changes in morphology as seen in A from at least three independent experiments (50-100 cells counted per experiment). Cells were scored as flat, round or protrusion bearing. *P* values: protrusions in Rac1 versus control **P*=0.2; rounded cells in Rac33 vs control ***P*=0.014; rounded cells siRac1 vs control ****P*=0.038; protrusion outgrowth in siRac3 vs control *****P*=0.003. (C) Cells were transfected with pcDNA3.1 empty vector (ev), mouse-specific shRac3 together with ev, or mouse-specific shRac3 combined with human Rac3 cDNA. Protrusion-bearing cells (where protrusion is >1× cell body) were counted and depicted in a bar graph. Note that the phenotype induced by mouse-specific shRac3 is prevented by co-expression of human Rac3. (D) Rac3 is downregulated in differentiated serum-starved N1E-115 cells. Serum-cultured and serum-starved N1E-115 cells were analyzed by RT-PCR for the amount of Rac1 and Rac3 mRNA. (E) Rac3 depletion induces differentiation of N1E-115 cells as shown by various differentiation markers. Parental N1E-115 cells were either transfected with shRac3 (together with eGFP in a ratio of 10:1, to track the transfected cells) and cultured under normal conditions (right panels) or differentiated by 24 hours serum-starvation and 4 hours NGF treatment (left panels). The cells were stained with either anti-neurofilament 200 (NF200) antibody (upper panels) or anti-microtubule-associated protein 2 antibody (lower panels).

demonstrate the specificity of the short hairpin RNA (shRNA) constructs, cells were firstly transfected with shRac1-1, shRac1-2, shRac3-1 or shRac3-2, and the RNA contents were subsequently analyzed by RT-PCR. As shown in Fig. 1C, endogenously expressed Rac1 was efficiently downregulated by both shRac1-1 and shRac1-2 constructs, but not by shRac3-1 or shRac3-2. The same holds true for endogenous Rac3, the mRNA levels of which were markedly decreased by the expression of shRac3-1 or shRac3-2, but not by the expression of shRac1 constructs. In further experiments we used shRac1-1 and shRac3-1 (unless indicated otherwise), and abbreviated the names to 'shRac1' and 'shRac3', respectively. In order to demonstrate that the expression of shRac3 also affects Rac3 protein levels, we analyzed the endogenous Rac3 protein levels in N1E-115 cells expressing shRac3 or short interference luciferase (siLuc). As shown in Fig. 1D, shRac3 efficiently depleted the endogenous Rac3 protein levels. To further demonstrate the specificity of the various shRNA constructs, shRac1-1, shRac3-1, shRac3-2 or the control short hairpin luciferase (shLuc) were co-expressed with cDNAs of either Rac1 or Rac3 in HEK293 cells (Fig. 1E). shRNAs specific for Rac1 or Rac3, but not shLuc, decreased dramatically the protein levels of overexpressed Rac1 or Rac3, respectively. No downregulation of Rac3 by shRac1 or vice versa was found (Fig. 1E). Together, these data demonstrate that shRac1 and shRac3 constructs efficiently and specifically downregulate their respective targets, Rac1 and Rac3, both at the mRNA and protein levels.

## Rac1 and Rac3 display opposing functions in N1E-115 cells

When seeded on plastic and cultured in the presence of serum, N1E-115 cells show predominantly a contracted morphology and adhere weakly to a plastic or glass surface. After serum starvation for 24 hours, N1E-115 cells become more adhesive and start to differentiate, a process involving formation of one or more neurite-like protrusions. To determine the effect of downregulation of both Rac1 and Rac3 on the morphology of N1E-115 cells, we seeded the cells on an uncoated glass surface. Rac1- or Rac3-specific shRNAs were transiently expressed in N1E-115 cells, together with enhanced green fluorescent protein (eGFP) to visualize the transfected cells. Twenty-four hours after transfection, cells were deprived of serum for 16 hours and subsequently fixed, and analyzed by confocal microscopy. Rac1 depletion led to severe loss of cell-matrix adhesions and round morphology when compared to control cells (Fig. 2A, upper right panel). A subset of the affected cells even detached and underwent apoptosis in a later stage. Interestingly, downregulation of endogenous Rac3 induced a strikingly opposite morphology. The cells adhered well and showed prominent outgrowth of neurite-like protrusions (Fig. 2A, lower right panel). To exclude the possibility that the observed phenotypes were a consequence of unspecific shRNA effects, we repeated the experiments with different shRNA sequences targeting various regions of Rac3, with similar results (data not shown). In addition, co-

expression of mouse-specific shRac3 together with human Rac3 cDNA prevented the phenotypic changes induced by shRac3 (Fig. 2C), indicating that the outgrowth of neurite-like extensions is indeed caused by Rac3 depletion. We conclude that Rac3 depletion induces neurite-like protrusions in N1E-115 cells, in contrast to Rac1 depletion, which induces cell rounding and impaired cell-matrix adhesions.

We also examined the effect of overexpression of Rac1 and Rac3 on neuroblastoma morphology. As expected, overexpression of wild-type Rac1 induced a symmetrically flattened morphology, occasionally accompanied by neurite-like outgrowth (Fig. 2A, upper left panel). The phenotype was milder than the morphological changes observed upon overexpression of constitutively active $Rac1^{G12V}$, as we have described earlier (Leeuwen et al., 1997). By contrast, cells expressing exogenous wild-type Rac3 were small and round, and showed a tendency for clustering (Fig. 2A, lower left panel). They adhered relatively poorly to the matrix, but showed normal growth capabilities. In addition, we found that overexpressed Rac3 did not affect the activity of endogenous Rac1 (supplementary material Fig. S1), indicating that Rac3 does not induce cell rounding by inhibiting the activity of endogenous Rac1.

To quantify the observed phenotypes, the morphological changes induced by Rac1, Rac3, shRac1 and shRac3 were scored as flat, round or protrusion bearing, and depicted in a bar graph (Fig. 2B). Similar results were found when using other neuroblastoma cells such as N2A cells (data not shown).

To gain further insight into the function of Rac3 in cell differentiation, we analyzed whether differentiation induced by serum starvation influenced the expression of endogenous Rac3. As shown in Fig. 2D, Rac3 mRNA expression was completely lost in serum-starved, differentiated N1E-115 cells, whereas the Rac1 transcript was still present to the same extent as in proliferating cells. In addition, we analyzed Rac3 expression in cells differentiated in other fashions, e.g. by dibutyryl cyclic AMP treatment (Abe et al., 2003) or by DMSO treatment (Kranenburg et al., 1995; Leeuwen et al., 1997). RT-PCR showed similar results to those of differentiation induced by serum starvation, namely a loss of Rac3 transcript in differentiated cells (not shown). These data indicate that suppression of Rac3 mRNA levels is both required and sufficient for the differentiation and protrusion outgrowth in N1E-115 cells.

Since shRac3-induced neurite-like outgrowths are similar in length and morphology to those of fully differentiated N1E-115 cells, we also examined whether shRac3-induced protrusions were positive for differentiation markers, which are enriched in the protrusions of differentiated N1E-115 cells. To this end, N1E-115 cells were transfected with shRac3 (and eGFP) and compared with N1E-115 cells that were differentiated by serum starvation (24 hours) and subsequent NGF treatment (4 hours). As shown in Fig. 2E, analysis of cells for the expression of the differentiation markers neurofilament 160 (NF160), neurofilament 200 (NF200), (tyrosin) tubulin and microtubule-associated protein 2 (Harada et al., 2002; Pan et al., 2005; De Laurenzi et al., 2000) revealed that shRac3-expressing N1E-115 cells show equal enrichment of differentiation markers in neurite-like extensions as NGF-induced differentiated cells (Fig. 2E, and data not shown).

We conclude that Rac1 and Rac3 affect neuroblastoma morphology and differentiation in an opposite fashion.

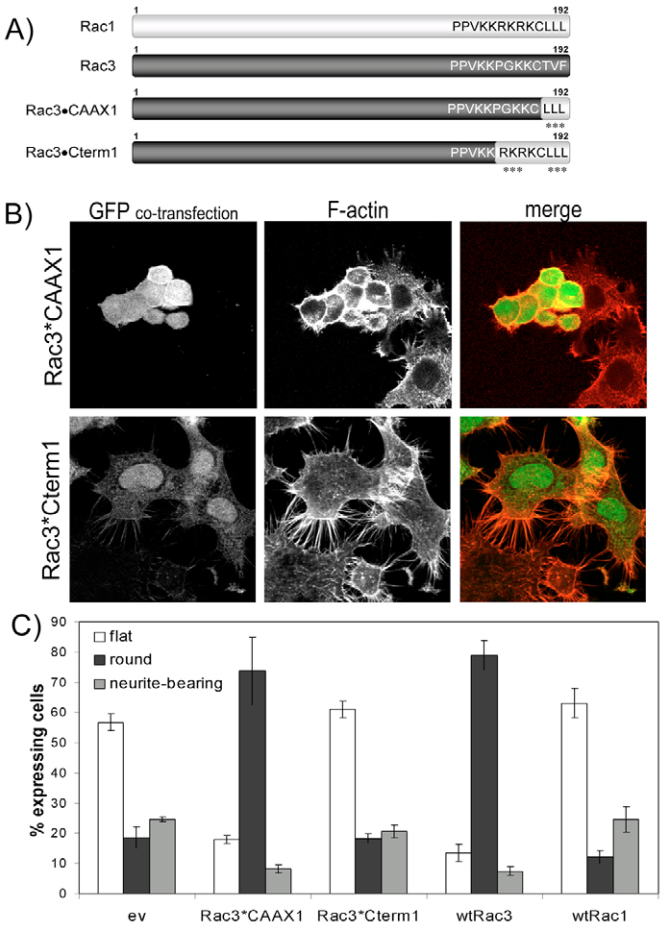


**Fig. 3.** Specific Rac3-induced cell rounding and intracellular localization are dependent on amino acid residues 185-187. (A) Schematic representation of chimeric Rac3/Rac1 mutants used in the experiments. The asterisks indicate divergent residues. (B) Transient expression of Rac3*CAAX1 and Rac3*Cterm1 mutants in N1E-115 cells. eGFP-containing construct was co-transfected in a 1:5 ratio, to visualize transfected cells. 24 hours after transfection, cells were serum-starved for 24 hours, stained with phalloidin to visualize F-actin and photographed. Bar, 25 μm. Note that Rac3*CAAX1-induced morphology is similar to the cell rounding induced by the parental Rac3 protein, whereas the Rac3*Cterm1 mutant induces cell relaxation and spreading similar to the effect of Rac1. (C) Morphologies observed in B were scored as flat, round or protrusion bearing in two independent experiments, and presented in a bar-diagram. Control cells (ev) were transfected with pcDNA3.1 empty vector combined with eGFP-containing construct (ratio 10:1).

Downregulation of Rac1 and overexpression of Rac3 induce cell rounding, whereas downregulation of Rac3 and overexpression of Rac1 induce cell spreading and outgrowth of neurite-like protrusions.

## The hypervariable polybasic region is responsible for the functional difference between Rac1 and Rac3

Since Rac1 and Rac3 are highly homologous proteins, we investigated which of the few variable regions are responsible for the remarkable difference in their function. The carboxyl terminus harbors most of the non-conserved residues in the Rac

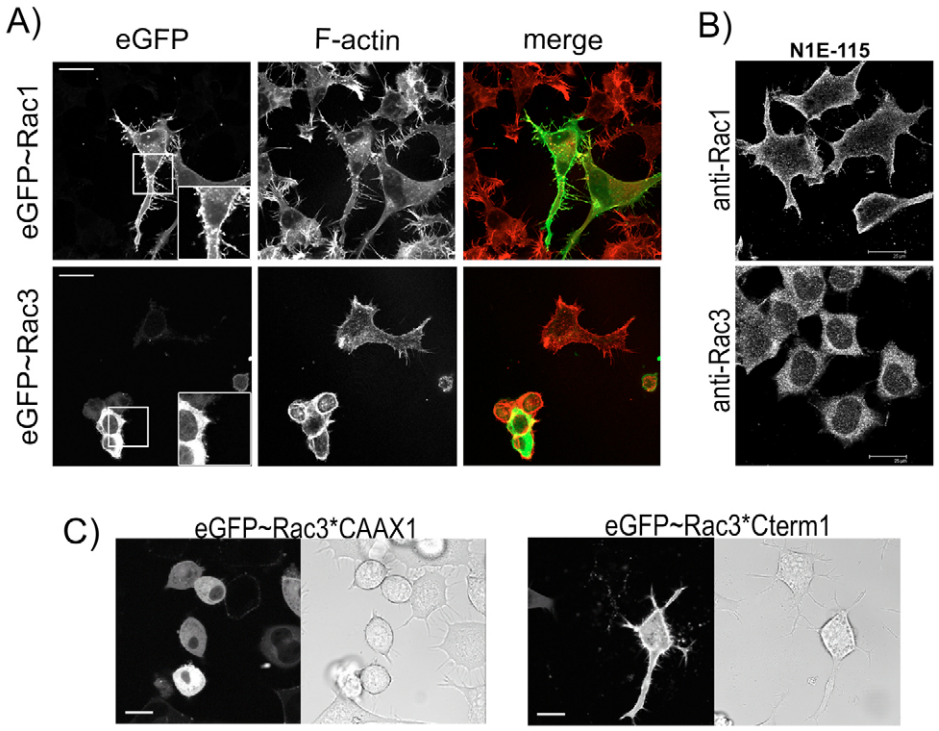


**Fig. 4.** Both endogenous and exogenous Rac1 and Rac3 localize to different intracellular compartments. (A) N-terminally eGFP-coupled Rac1 or -Rac3 was expressed transiently in N1E-115 cells. Cells were subsequently serum-starved for 8 hours, fixed and stained with phalloidin to visualize filamentous actin. Bar, 25 μm. (B) N1E-115 cells cultured under normal conditions were fixed and stained with anti-Rac1 or anti-Rac3 antibodies. (C) N-terminal eGFP-coupled Rac3*CAAX1 and Rac3*Cterm1 mutants were expressed in N1E-115 cells to monitor their intracellular localization.

proteins (Fig. 3A). The last four amino acids of the carboxyl terminus form the CAAX box, a motif that is subject to posttranslational modification, isoprenylation, which facilitates the insertion of the protein into membranes (Cox and Der, 1992). Furthermore, Rac1 and Rac3 harbor an additional three amino acid difference in the polybasic region (PBR) adjacent to the CAAX box (Fig. 3A). This variable PBR has been suggested to represent a binding site for downstream effectors and exchange factors (van Hennik et al., 2003; Yamauchi et al., 2005; ten Klooster et al., 2006).

We analyzed the contribution of both regions in the Rac3-induced phenotype. For this, we substituted the CAAX box (residues 189-192) or the entire variable PBR including the CAAX box (residues 180-192) of Rac3 for their Rac1 counterparts thereby generating mutant Rac3*CAAX1 and Rac3*Cterm1, respectively (Fig. 3A). These mutants were transiently expressed in N1E-115 cells together with eGFP. Expression of the Rac3*CAAX1 mutant induced a round morphology and weak cell-matrix adhesions (Fig. 3B), similarly to cells expressing wild-type Rac3 protein (Fig. 2A). By contrast, expression of the Rac3*Cterm1 mutant induced cell spreading (Fig. 3B) and, when serum starvation was extended to 48 hours, promoted protrusion outgrowth (not shown), fully resembling the phenotype induced by Rac1. The morphological changes were scored as flat, round or protrusion bearing and depicted in a bar graph (Fig. 3C). Notably, the Rac3*CAAX1 and Rac3*Cterm1 mutants differ only in residues 185-187 (Fig. 3A), and yet they induce fully opposing phenotypes, suggesting that residues 185-187, and not the CAAX motif on its own, are responsible for the difference in Rac1 and Rac3 function.

## Rac1 and Rac3 are differently localized in N1E-115 cells

Since the function of small GTPases greatly depends on their location in the cell, we analyzed the intracellular location of Rac1 and Rac3 in N1E-115 cells. Firstly, we tagged the N terminus of both proteins with eGFP and expressed them transiently in N1E-115 cells. The tagged proteins induced a phenotype similar to untagged proteins. Rac1 is principally targeted to the plasma membrane and cell protrusions (Fig. 4A, upper panels). By contrast, Rac3 is predominantly localized in the perinuclear region (Fig. 4A, lower panels), suggesting that Rac1 and Rac3 are targeted to different cellular compartments in neuroblastoma cells. Although the phenotype induced by eGFP-tagged proteins seems to be somewhat more dramatic, especially in the case of Rac1, they nonetheless cause similar morphologies as the non-tagged proteins. Moreover, we investigated the location of endogenous Rac1 and Rac3 proteins in N1E-115 cells. As shown in Fig. 4B, staining with an anti-Rac3-specific antibody revealed endogenous Rac3 to be in the perinuclear region, confirming the data found with expressed eGFP-tagged Rac3 protein. In contrast to Rac3, endogenous Rac1 was found at the plasma membrane (Fig. 4B), consistent with the data obtained with eGFP-tagged Rac1 protein.

To determine whether the intracellular localization of Rac3 also depends on aa residues 185-187 in the carboxyl terminus, as was found for Rac3 function, we tagged the carboxyl terminus mutants of Rac3 with eGFP to visualize the localization of these mutant proteins. The Rac3*CAAX1 mutant was found to be predominantly in the perinuclear region, comparable to the distribution of the parental Rac3 protein (Fig. 4A,C). By contrast, the Rac3*Cterm1 mutant is targeted to the plasma membrane (Fig. 4C), similarly to Rac1 protein (Fig. 4A).

From these data we conclude that endogenous Rac1 and Rac3 are targeted to different cell compartments (plasma membrane and perinuclear region, respectively) and that this difference in localization (and function) is dependent on the amino acid residues 185-187 in the carboxyl terminus.

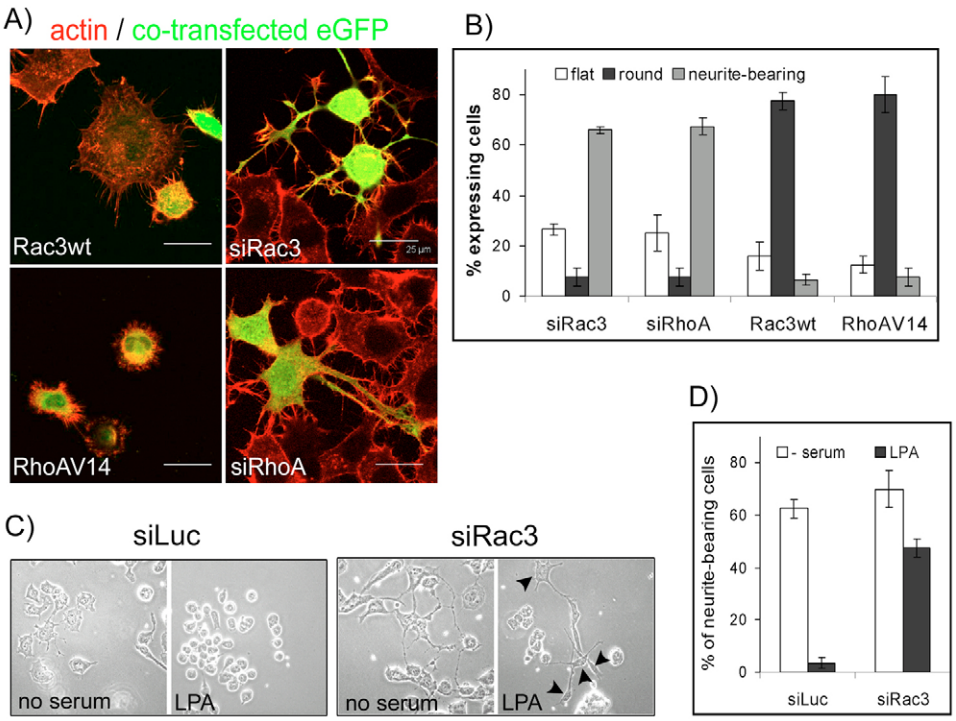


**Fig. 5.** Rac3 phenocopies RhoA in N1E-115 cells. (A) Cells were transfected with pcDNA3.1/Rac3, pcDNA3.1/RhoA$^{G14V}$, pSuper/shRac3 or pSuper/shRhoA, together with 10:1 eGFP. Subsequently, the cells were fixed and stained with phalloidin to visualize F-actin. Bar, 25 μm. Note that both Rac3 and RhoA overexpression induce cell rounding, whereas the depletion of either protein leads to protrusion formation. (B) Morphologies were scored as flat, round or protrusion bearing and shown here in a bar graph. At least 200 cells from two independent experiments were counted. (C) Neurite-like protrusions induced by shRac3 are not responsive to LPA. N1E-115 cells were transfected with either shLuc or shRac3 together with 5:1 eGFP to visualize the transfected cells. Cells were either stimulated or not with 5 μM LPA for 15 minutes and subsequently photographed. (D) Quantification of the results shown in (C). Protrusions-bearing cells were counted in three independent experiments and represented in a bar graph.

## Rac3 phenocopies the effect of RhoA in N1E-115 cells

Previous studies have demonstrated the involvement of various Rho GTPases in neuritogenesis (reviewed by Govek et al., 2005). The small GTPase RhoA counteracts the Rac1 action in neuronal and most other cell types (Sander et al., 1999; Burridge and Wennerberg, 2004), by modulating actomyosin dynamics to achieve contractility (Amano et al., 1996; Leeuwen et al., 1997; Kranenburg et al., 1997). Since our experiments show that Rac3 opposes the function of Rac1 in neuroblastoma cells, we investigated whether Rac3 is using the RhoA signaling pathway that leads to cell rounding and protrusion retraction.

As shown in Fig. 5A, depletion of endogenous RhoA leads to neurite-like outgrowth, whereas overexpression of constitutively active RhoA$^{G14V}$ induces cell rounding and protrusion retraction, similar to effects of Rac3 depletion and Rac3 overexpression. We scored the phenotypes as flat, round or protrusion bearing and the combined data from three independent experiments are represented in a bar graph (Fig. 5B). From these data we conclude that the morphological changes induced by overexpression or downregulation of RhoA and Rac3 in N1E-115 cells are remarkably similar.

To investigate whether Rac3 is acting in the same signaling pathway as RhoA, we tested whether the activation of RhoA could overcome shRac3-induced neurite-like outgrowth. Upon stimulation with lysophosphatidic acid (LPA), RhoA and its downstream effector Rho-associated kinase (ROCK) are activated, leading to phosphorylation of MLC (Amano et al., 1996; Leeuwen et al., 1997; Kranenburg et al., 1997), which initiates the actomyosin contractile forces. N1E-115 cells were transfected with shLuc or shRac3, together with eGFP to track the expressing cells. As shown in Fig. 5C,D, serum-starved, protrusion-bearing N1E-115 cells retracted their neurite-like protrusions and rounded up in response to LPA treatment. By contrast, protrusion-bearing cells expressing shRac3 (indicated with arrows in Fig. 5C) did not show this response. The cell body was slightly retracted, but the neurite-like protrusions were sustained (Fig. 5C,D). Moreover, co-expression of constitutive active RhoA$^{G14V}$ was unable to counteract the outgrowth-promoting effect of shRac3 (not shown). Obviously, the signals delivered by LPA/RhoA are not sufficient to prevent the protrusion outgrowth process induced by depletion of Rac3. This suggests that Rac3 affects contractility either by acting in a RhoA-independent pathway or by interfering in the RhoA pathway downstream of RhoA.

## Rac3-induced rounding is independent of ROCK

Functional ROCK is essential for RhoA-induced cell rounding, and inhibition of ROCK induces spreading and neurite-like outgrowth in N1E-115 cells (Kranenburg et al., 1997; Amano et al., 1998). Therefore, we investigated whether Rac3-induced cell rounding requires the activity of ROCK. Treatment of rounded RhoA$^{V14}$-expressing cells with the ROCK inhibitor Y27632 led to cell spreading and neurite-like outgrowth (Fig. 6A, lower panels), similar to the empty vector-expressing cells (Fig. 6A, upper panels), indicating that, in both cases, rounding of the cells is dependent on LPA/RhoA signaling. By contrast, cells expressing Rac3 did not respond to ROCK inhibition by spreading (Fig. 6A, middle panels). Prolonging the incubation time with inhibitor did not have significant effects (data not shown). The phenotypes were scored as flat, round or protrusion bearing, and the percentage of round cells of two independent experiments is presented in the bar graph (Fig. 6B). These data show that, in contrast to RhoA-induced cell rounding, Rac3-mediated cell rounding does not depend on ROCK activity.

### Rac3-induced cell rounding does not depend on MLC phosphorylation

RhoA-induced rounding of cells is dependent on elevated levels of MLC phosphorylation by ROCK and MLC kinase (MLCK). Therefore, we examined whether Rac3-induced rounding of N1E-115 cells is accompanied by/dependent on increased MLC phosphorylation. N1E-115 cells, stably expressing Rac1, Rac3 or control vector, were cultured in the presence of serum. Under these conditions, control cells (ev) were rounded and showed MLC phosphorylation (Fig. 6C, lane 1). To induce maximum MLC phosphorylation, control cells were starved for 4 hours and subsequently stimulated with 5 μM of LPA (Fig. 6C, lane 4). Rac1-expressing cells showed suppressed MLC phosphorylation (Fig. 6C, lane 2) associated with the flat morphology of these cells (Fig. 2A). Interestingly, Rac3-expressing cells, that are round and exhibit a similar phenotype to LPA-stimulated cells, showed hardly any MLC phosphorylation, comparable to that of Rac1-expressing cells. These data indicate that that Rac3-induced cell rounding is not mediated by MLC phosphorylation in N1E-115 cells. As MLC is phosphorylated by MLCK, we also investigated the effects of inhibition of MLCK on the morphology of Rac3-expressing cells, using ML-7, a specific inhibitor of MLCK. As shown in Fig. 6A (right panels), control cells and $RhoA^{G14V}$-expressing cells responded to MLCK inhibition by spreading. By contrast, the contractile morphology of Rac3-expressing cells remained unaffected by ML-7 treatment. Apparently, Rac3-induced rounding is not depended on MLC phosphorylation levels or the activity of MLCK.

Taken together, we conclude that the RhoA/ROCK/MLC-P signaling pathway does not mediate Rac3-induced cell rounding.

### Rac3-expressing cells show defect cell-matrix adhesions

In N1E-115 cells, serum deprivation triggers an increase in cell-matrix adhesions, whereupon neurite-like outgrowth takes place. Various agents, including growth factors and kinase inhibitors, can also trigger this process (Leeuwen et al., 1997; van Horck et al., 2002). However, rounded Rac3-expressing cells were unable to spread or differentiate, regardless of whether differentiation was triggered by serum withdrawal, nerve growth factor (Estrach et al., 2002), dimethyl sulfoxide [DMSO (Kranenburg et al., 1995); 623/id (Leeuwen et al., 1997)], ROCK inhibitor or cyclic AMP (Abe et al., 2003) (Figs 2, 3, 5 and 6, and data not shown). We also found that coating the surface with laminin-1, collagen, poly-L-lysine or fibronectin did not stimulate spreading of Rac3-expressing cells when compared to an uncoated surface (data not shown). Moreover, depletion of endogenous Rac3 induced neurite-like outgrowth and differentiation, even in conditions that normally do not support differentiation (e.g. presence of LPA or serum; see Fig. 5C,D). Since neuronal differentiation and neurite outgrowth depend largely on proper cell-matrix adhesions (Govek et al., 2005), we hypothesized that Rac3 protein might interfere with the formation of cell-matrix adhesions in neuroblastoma cells.

We addressed this issue by examining the distribution of paxillin, one of the key components of adhesion structures such as focal adhesions (FA) and focal complexes (FC). Upon serum starvation, Rac1-expressing cells or control cells showed abundant focal adhesion structures at the periphery of cells

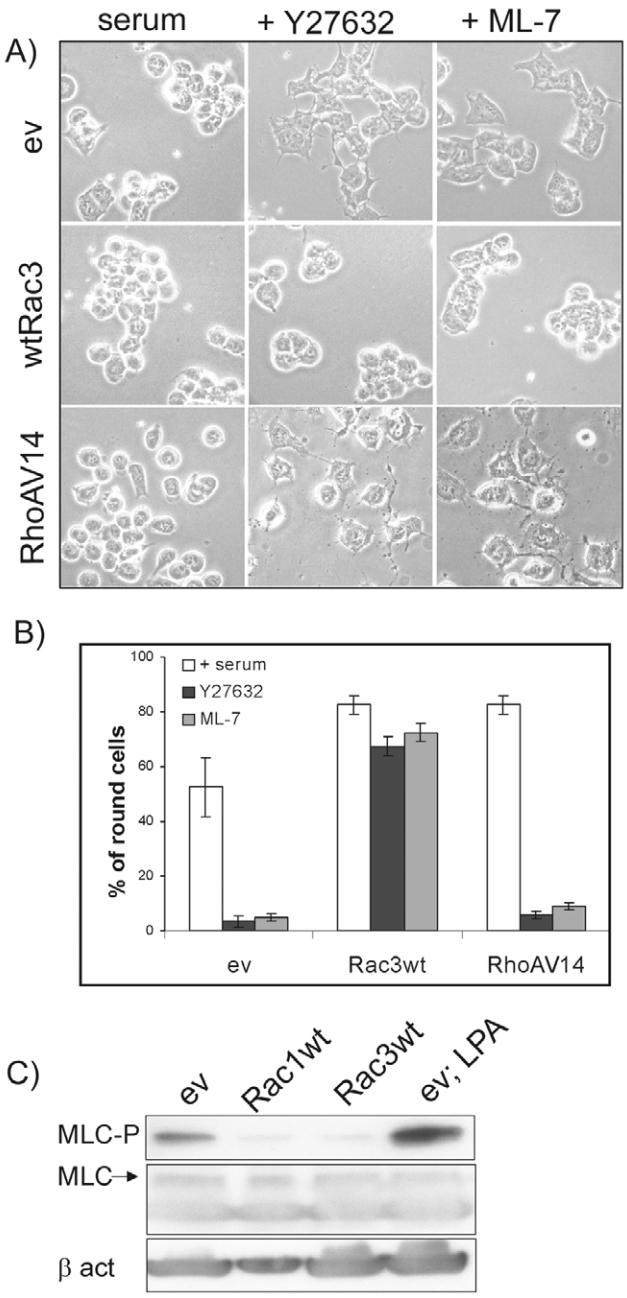


**Fig. 6.** Inhibition of ROCK or MLCK impairs RhoA- but not Rac3-induced cell rounding. (A) N1E-115 cells were transfected with control vector (empty vector pcDNA3.1; ev), pcDNA3.1/Rac3 or with pcDNA3/$RhoA^{G14V}$ construct. After being cultured for 48 hours in the presence of 2% serum, cells were treated with the ROCK inhibitor Y-27632 (10 μM) or MLCK inhibitor ML-7 (2 μM) for 4 hours and subsequently photographed. (B) Quantification of the results shown in (A). The round cells were counted (per 100 cells) in at least two different experiments and depicted in a bar graph. (C) In contrast to LPA/RhoA-induced rounding, Rac3-triggered rounding does not involve an increase in MLC phosphorylation (MLC-P). Western blot of N1E-115 cells expressing empty vector, Rac1 or Rac3 that were cultured in 2% serum (lanes 1, 2 and 3). As positive control (Lom et al., 1993), N1E-115 cells were starved during 4 hours and subsequently stimulated with 5 μM LPA for 15 min to induce maximal MLC phosphorylation. As a loading control, both β-actin and total MLC levels are shown.

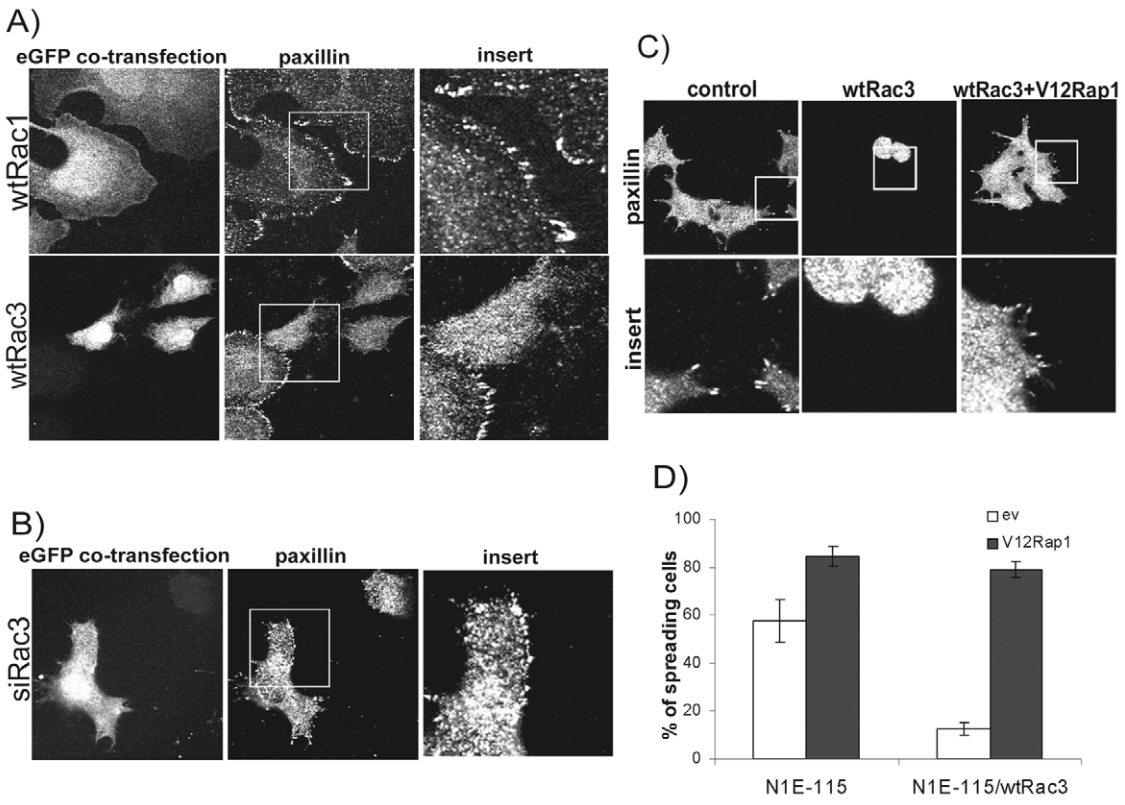


**Fig. 7.** Rac3-expressing cells show defective cell-matrix adhesions, which can be restored by direct activation of integrins by $Rap^{G12V}$. (A) Fluorescent images showing paxillin distribution in N1E-115 cells expressing Rac1 or Rac3. Cells were transiently transfected, cultured in serum-containing medium for 24 hours and subsequently serum-starved for 12 hours to trigger increase in cell-matrix adhesions, which precedes the outgrowth of protrusions and differentiation. Panels on the right show enlargements of the boxed regions in the middle panels. Note the absence of paxillin-rich structures in Rac3-expressing cells (middle and right panel). (B) Depletion of Rac3 is accompanied with increased cell-matrix adhesion and paxillin staining. N1E-115 cells were transfected with shRac3 (together with 5:1 eGFP), cultured for 24 hours and stained for paxillin. (C) Parental N1E-115 cells and N1E-115 cells stably expressing Rac3 were transiently transfected with control vector or pcDNA3.1/ $Rap^{G12V}$. Cells were subsequently starved for 1 hour, and stained for paxillin. Note the restoration of the paxillin-rich structures in N1E-115/Rac3 cells that express $Rap^{G12V}$ (middle and right panels). (D) Bar graph representing the quantification of the experiment shown in C. At least 100 cells were counted per sample, derived from two independent experiments.

(Fig. 7A, upper panels). These also structures stained positive for other FC markers, such as vinculin or phosphorylated tyrosine (data not shown). By contrast, Rac3-expressing cells did not show the formation of the focal complexes (Fig. 7A, lower panels), not even during prolonged serum deprivation or NGF stimulation (data not shown). In addition, Rac3-depleted cells showed increased number of FCs at the onset of the protrusion formation. These focal complexes were distributed not only at the tip of lamellae but also along the whole surface of the spreading cell (Fig. 7B). Moreover, FCs in shRac3-expressing cells appeared shorter than the FCs in control cells, possibly indicating a faster turnover of the FCs in Rac3-depleted cells (Bershadsky et al., 2006).

Since the formation of focal complex is dependent on integrin activation and clustering, we investigated whether the adhesion defect in Rac3-expressing cells could be overcome by robust and direct stimulation of integrin signaling/clustering. Rap1 is a small GTPase of the Ras superfamily, shown to potently activate integrins in various cellular systems (reviewed by Bos, 2005). We, therefore, expressed an active Rap1 mutant ($Rap^{G12V}$) in N1E-115/Rac3 cells. $Rap^{G12V}$ expression abrogated Rac3-induced cell rounding and resulted in cell relaxation and spreading, both in the presence or absence of serum (Fig. 7C,D). Moreover, $Rap^{G12V}$-induced spreading in N1E-115/Rac3 cells is accompanied by the formation of FC structures, similar to those observed in N1E-115 cells transfected with $Rap^{G12V}$ (data not shown). These results suggest that Rac3 interferes in integrin-mediated cell-matrix adhesions by an unknown mechanism and that this blockade can be overcome by direct stimulation of integrin signaling by expression of constitutively active Rap1.

## Discussion

We show here that Rac1 and Rac3 have distinct and opposite functions in mammalian neuronal cells. Depletion of Rac1 in N1E-115 murine neuroblastoma cells induces loss of cell-matrix adhesions, rounding and detachment of cells. This is consistent with a number of studies that have implicated Rac1 in cell-matrix adhesions in various cell types, including cell-matrix adhesions and neurite outgrowth in neuronal cells (Leeuwen et al., 1997; Sarner et al., 2000; Aoki et al., 2004; Schwamborn and Puschel, 2004). Strikingly, depletion of endogenous Rac3 leads to cell spreading and pronounced

neurite-like outgrowth. This is in agreement with overexpression experiments showing that Rac3 induces cell rounding and attenuates cell-matrix adhesions, whereas Rac1 induces cell spreading, followed by outgrowth of protrusions. Also the intracellular localization Rac1 and Rac3 differs in N1E-115 cells. Rac1 is predominantly targeted to the plasma membrane, whereas Rac3 is mainly found in the perinuclear region. We have pinpointed residues 185-187 in the polybasic-rich region of the carboxyl terminus as responsible for the differences in function and localization of Rac1 and Rac3. Furthermore, our experiments show that Rac3-mediated cell rounding is not dependent on components of the RhoA signaling pathway. By contrast, our data indicate that Rac3 induces cell rounding and prevents differentiation by attenuating proper formation of cell-matrix adhesions.

Previous studies have shown either no or small functional difference between Rac1 and Rac3. However, these conclusions were based on studies that used constitutively active and dominant negative mutants of Rac1 and Rac3 (Joyce and Cox, 2003; Keller et al., 2005; Haataja et al., 1997; Mira et al., 2000). Dominant negative Rac mutants are highly promiscuous in binding and sequestering various exchange factors (Feig, 1999), making it difficult to address the precise function of these highly homologous proteins. We therefore used the shRNA approach, which is a highly specific and efficient tool for loss-of-function studies.

Since the effector domains of Rac1 and Rac3 are 100% homologous (Haataja et al., 1997; Malosio et al., 1997), it is plausible that constitutively active Rac3 mutant is also able to bind and signal to most if not all Rac1 effectors, thereby circumventing the spatiotemporal regulation of these GTPases. Indeed, we found that overexpression of constitutive active mutants of Rac1 and Rac3 induce a similar spreading and aberrant migratory phenotype in N1E-115 cells (see supplementary material Fig. S2). However, when using wild-type Rac1 and Rac3 proteins, which are activated by endogenous GEFs (Fukuhara et al., 2001), we found that Rac1 and Rac3 cause remarkably different phenotypes. Rac1 induces cell spreading and differentiation, whereas Rac3 induces a rounded, poorly adherent phenotype in N1E-115 cells.

Expression of Rac3 impairs the formation of proper cell-matrix adhesion in neuroblastoma cells. Serum-deprived Rac3-expressing cells adhere to the matrix, but are unable to spread and differentiate. Apparently, Rac3 negatively affects spreading and/or differentiation. Spreading and neurite outgrowth are largely dependent on functional integrin signaling (Ridley et al., 2003; Govek et al., 2005). Growth factor- and extracellular matrix-triggered signaling leads to integrin clustering and the formation of focal complexes (FC) or focal adhesions (FA) (Ridley et al., 2003; Brown and Turner, 2004). Our immunofluorescence studies show that, in Rac3-expressing cells, paxillin is diffusely distributed and not localized at the tips at the periphery of the cell as in spreading control cells or Rac1-expressing cells. We propose that Rac3 disturbs the proper formation of the focal complex/focal adhesions by yet unknown mechanism and, as a consequence, prevents cell spreading and neurite-like outgrowth.

Carboxyl termini of Rac1 and Rac3 harbor two variable triplets; one in the polybasic-rich region (PBR) and one in the motif that is subjected to posttranslational modification of prenylation, termed the CAAX box. It is known that different prenylation may result in localization of proteins to either the plasma membrane or different endomembranes (Silvius, 2002; Cox and Der, 1992). We found that neither the specific Rac3-induced phenotype nor its typical localization in the perinuclear region, are dependent on the CAAX box sequence alone. As a matter of fact, our data implicate residues 185-187 in the PBR region of Rac3 as a motif responsible for the induction of the Rac3 phenotype. The substitution of these residues for their Rac1 counterparts reversed the phenotype from round to adherent and flat. Notably, mutation of residues 185-187 of Rac3 also altered the intracellular localization of the protein. Rac3 with the carboxyl terminus of Rac1 is targeted to the plasma membrane, as is Rac1. This PBR has recently been suggested to represent an additional effector-binding site (van Hennik et al., 2003; Yamauchi et al., 2005) and GEF binding site (ten Klooster et al., 2006). Additionally, PBR could also contribute to binding of transferases that facilitate the insertion of an isoprenoid tail, as has been shown for Ras small GTPases (James et al., 1995; Long et al., 2000). Furthermore, differences in PBR could diminish the electrostatic interactions between the protein and the membrane, resulting in reduced docking capacity of the small GTPase (Williams, 2003). Based on these data, it is conceivable that the Rac3-induced cell rounding and decrease in cell-matrix adhesions, as well as its localization to the perinuclear region, are specified by protein interactions that depend on the PBR (residues 185-187).

Our data show that Rac3 functions in a Rac1-opposing fashion in neuroblastoma cell. Previous studies have demonstrated the involvement of different Rho GTPases in the neuritogenesis and implied that Rac1 and RhoA antagonize each other while fulfilling their function. The relative strength of their signals determines the neuronal morphology (Govek et al., 2005). Small GTPase RhoA counteracts the Rac1 action in neuronal and most other cells (Sander et al., 1999; Burridge and Wennerberg, 2004), by modulating actomyosin dynamics to achieve contractility (Amano et al., 1996; Leeuwen et al., 1997; Kranenburg et al., 1997). Rho proteins often share upstream regulators or downstream effectors (Van Aelst and D'Souza-Schorey, 1997; Burridge, 1999). We were intrigued by the similarity between the Rac3- and RhoA-induced phenotypes, and investigated whether Rac3 counteracts cell spreading and differentiation by influencing the RhoA pathway. Our experiments clearly demonstrate that neither ROCK nor MLCK activity nor MLC phosphorylation function in the Rac3 pathway. The Rac3 pathway seems to act independently of the RhoA pathway.

The avian homologue of Rac3 (Rac1B) induced pronounced neurite outgrowth in dissociated avian neurons (Albertinazzi et al., 1998). The apparent opposing effects of mammalian Rac3 (this study) and its avian homologue are presumably due to species-specific effects of these Rac proteins. Studies in various models indicate that Rho GTPases may have quite differential effects on neurite formation depending on species, cell types, or even age of cells or organisms of which primary cells were isolated (Govek et al., 2005). For instance, inhibition of Rac1 promotes the neurite outgrowth in chick primary neurons (Fournier et al., 2003), but decreases neurite extension in mammalian hippocampal neurons (Schwamborn and Puschel, 2004).

Since Rac3 is highly expressed in brain (Haataja et al.,

1997; Bolis et al., 2003), it is plausible that Rac3 exerts a specific biological function in neuronal cells. Recently, a Rac3 knockout mouse has been generated that showed no apparent developmental defects (Corbetta et al., 2005). However, Rac3-null mice appeared to be superior to their wild-type littermates in motor coordination and motor learning, indicating a possible function of Rac3 in development of the functional nervous system. Our data show that Rac3 depletion induces pronounced neurite-like outgrowth and promotes the differentiation process. It is tempting to speculate that Rac3 depletion in the circuitry in regions that facilitate the motor learning and coordination leads to enhanced neuritogenesis and arborization, facilitating stronger and more effective circuitry.

In summary, we found that endogenous mammalian Rac1 and Rac3 are both required for normal morphology of neuroblastoma cells and that, in spite of high sequence homology, these two proteins exert different and opposing functions. Overexpression of Rac3 induces defects in cell-matrix adhesions, cell rounding and inability to differentiate. This function is dependent on residues 185-187 in the carboxyl terminus of the protein. Concomitantly, Rac3 depletion leads to pronounced neurite-like outgrowth and irreversible differentiation. Although Rac3 phenocopies RhoA in N1E-115 cells, we show that the Rac3 pathway is not mediated by or dependent on RhoA signaling. Finally, we found that the Rac3-induced phenotype is characterized by defect in integrin-mediated cell-matrix adhesions and that forced activation of integrin signaling can rescue the adhesion defect in Rac3-expressing neuroblastoma cells.

## Materials and Methods

### Cell culture and transfections

N1E-115 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (both from Invitrogen) and antibiotics. To induce differentiation, cells were washed with PBS and cultured for 16 hours in serum-free DMEM supplemented with antibiotics. Where indicated, the following chemicals were used: neurite growth factor (mNGF, Alomone labs); ROCK inhibitor Y27632 (Calbiochem); MLCK inhibitor ML-7 (Calbiochem); lysophosphatidic acid (LPA; Sigma).

For transient transfection assays, N1E-115 cells were seeded on glass coverslips in 12-wells dishes at a density of $1.5\times10^4$ cells. The next day, plasmids, as indicated, were mixed in a 10:1 ratio with pcDNA3/eGFP, and cells were transfected using FuGENE 6 transfection reagent (Roche Diagnostics), following the protocol as provided by the supplier.

To obtain cell lines stably expressing Rac1 or Rac3, cDNA encoding wild-type $NH_2$-terminally FLAG-tagged Rac1 or hemagglutinin (HA)-tagged Rac3 was cloned into a LZRS-IRES-zeo retroviral vector. The retroviral construct was transfected into Phoenix ecotropic packaging cells, and fresh viral supernatant was collected and used for infections of N1E-115 cells as described previously (Michiels et al., 2000). Cells were subsequently selected in medium supplemented with 25 μg/ml bleomycin (Sigma, St Louis, MO) for 48 hours.

### Plasmids

Human cDNAs of wild-type (wt) Rac1 and Rac3, cloned in vector pcDNA3.1 (Invitrogen) and $NH_2$-terminally tagged with Myc and HA sequence, respectively, were obtained from UMR cDNA Resource Center, University of Missouri-Rolla, MO. Rac3 was $NH_2$-terminally tagged with FLAG sequence and cloned into pcDNA3.1, as were constitutively active mutants $Rac1^{G12V}$ (Leeuwen et al., 1997) and $Rac3^{G12V}$. $NH_2$-terminally (eGFP)-tagged Rac1 and Rac3 proteins were obtained by cloning wild-type cDNAs into pEGFP-N1 expression vector (Clontech Laboratories). Mutants of Rac3 that contained either the C terminus of only the CAAX box of Rac1 (termed Rac3*Cterm1 and Rac3*CAAX1, respectively) were generated by site-directed mutagenesis. As a template, pcDNA3.1/Rac3 construct was used. Combining the universal T7 forward primer (Invitrogen), and mutation-containing reverse primers (sequence 5′-TGACTCGAGCTACAAGAGGAGGCACTTCCTCTTCCGCTTCTTCACTGGGGGCGGG-3′ and 5′-TGACTCGAGCTACAAGAGGAGGCACTTCTTCCCCGG-3′, for Rac3*Cterm1 and Rac3*CAAX1 mutants, respectively; purchased from Sigma-Genosys), mutated full-length cDNAs were obtained. Subsequently, these mutant sequences were cloned back into the pcDNA3.1 expression vector.

### shRNA preparations

The oligonucleotides all contained a 9-nucleotide hairpin loop and targeted different Rac1, Rac3 and luciferase RNA sequences. The oligonucleotides were annealed and cloned into the *Bgl*II/*Eco*RI site of pSuper vector (Brummelkamp et al., 2002). Primer sequences were as follows:

Rac1-1 shRNA sense primer, 5′gatccccTTTGCTTTTCCCTTGTGAG*ttcaagaga*CTCACAAGGGAAAAGCAAAttttggaaa3′;

Rac1-1 shRNA antisense primer, 5′agcttttccaaaaaTTTGCTTTTCCCTTGTGAG*tctcttgaa*CTCACAAGGGAAAAGCAAAggg-3′;

Rac1-2 shRNA sense primer, 5′gatccccGAGAAAATGCCTGCTGTTG*ttcaagaga*CAACAGCAGGCATTTTCTCttttggaaa3′;

Rac1-2 shRNA antisense primer, 5′agcttttccaaaaaGAGAAAATGCCTGCTGTTG*tctcttgaa*CAACAGCAGGCATTTTCTCggg3′;

Rac3-1 shRNA sense primer, 5′gatccccGTGGTACCCAGAGGTGCGG*ttcaagaga*CCGCACCTCTGGGTACCACttttggaaa3′;

Rac3-1 shRNA antisense primer, 5′agcttttccaaaaaGTGGTACCCAGAGGTGCGG*tctcttgaa*CCGCACCTCTGGGTACCACggg3′;

Rac3-2 shRNA sense primer, 5′gatccccGACAGTGTTCGACGAGGCC*ttcaagaga*GGCCTCGTCGAACACTGTCttttggaaa3′;

Rac3-2 shRNA antisense primer, 5′agcttttccaaaaaGACAGTGTTCGACGAGGCC*tctcttgaa*GGCCTCGTCGAACACTGTCggg3′;

RhoA-1 shRNA sense primer, 5′gatccccTGGACTCCAGAAGTCAAGC*ttcaagaga*GCTTGACTTCTGGAGTCCAttttggaaa3′;

RhoA-1 shRNA antisense primer, 5′ agcttttccaaaaaTGGACTCCAGAAGTCAAGC*tctcttgaa*GCTTGACTTCTGGAGTCCAggg3′;

RhoA-2 shRNA sense primer, 5′ gatccccGCAGGTAGAGTTGGCTTTG*ttcaagaga*CAAAGCCAACTCTACCTGCttttggaaa3′;

RhoA-2 shRNA antisense primer, 5′ agcttttccaaaaaGCAGGTAGAGTTGGCTTTG*tctcttgaa*CAAAGCCAACTCTACCTGCggg3′;

luciferase shRNA sense primer, 5′gatccccCGTACGCGGAATACTTCGA*ttcaagaga*TCGAAGTATTCCGCGTACGttttggaaa3′; and

luciferase shRNA antisense primer, 5′agcttttccaaaaaCGTACGCGGAATACTTCGA*tctcttgaa*TCGAAGTATTCCGCGTACGggg3′.

Capital letters indicate mRNA targeting sequences; italics indicate the hairpin loop.

### mRNA isolation and RT-PCR

For total cellular RNA isolation, N1E-115 cells were seeded in 10 cm dishes at a density of $7.5\times10^5$. After 24 hours, RNA was isolated using RNAzol B (Campro Scientific) and cDNA was synthesized by RT-PCR performed on 1 μg RNA using the Thermoscript RT-PCR system kit (Invitrogen). Specific transcripts were amplified with the following primers (Sigma-Genosys): Rac1 (forward, 5′-ctgaaggagaagaagctgac-3′; and reverse, 5′-tcgtcaaacactgtcttgag-3′), Rac3 (forward, 5′-gacgacaaggacaccattga-3′, and reverse, 5′-cctcgtcaaacactgtcttc-3′) and glyceraldehyde-3-phosphate dehydrogenase (forward, 5′-accacagtccatgccatcac-3′; and reverse, 5′-tccaccaccctgttgctgta-3′) using the Taq DNA polymerase kit (Invitrogen). The PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining.

### Phase-contrast microscopy, immunofluorescence and confocal microscopy

For phase-contrast microscopy, N1E-115 cells were seeded for 24 hours in 12-well dishes (either on plastic or glass coverslips). After 24 hours, cells were treated with appropriate reagents for the indicated time period, examined under a microscope (Axiovert 25; Carl Zeiss MicroImaging, Inc.), and photographed. The morphology of single eGFP-positive cells was scored as round, flat, or protrusion bearing. Cells with at least one process larger than one cell diameter were considered protrusion bearing. On average, 100 cells were counted per well and the values presented are the mean percentages (± s.e.m.) of at least two independent transfections.

For immunofluorescent staining, N1E-115 cells were seeded onto glass coverslips and transfected as described. 24 hours post-transfection, the medium was refreshed with DMEM containing 10% fetal calf serum, or DMEM only (serum starvation). After 16 hours incubation, cells were washed with PBS, fixed in 4% paraformaldehyde for 15 minutes, permeabilized with 0.2% Triton X-100 for 5 minutes, and blocked with 2% BSA in PBS. F-actin was visualized by incubating the cells with 0.2 μM Alexa Fluor 568-phalloidin (Invitrogen) for 45 minutes. Other antibodies used in immunofluorescence experiments were: anti-Rac1, 1:800 (Transduction Laboratories); anti-Rac3, 1:400 (gift from Adrienne D. Cox, University of North Carolina, Chapel Hill, NC); anti-neurofilament 160, 1:300 (Sigma, clone NN18); anti-neurofilament 200, 1:400 (Sigma, clone N52); anti-MAP2, 1:500 (Sigma, clone HM-2); mouse monoclonal anti-Y-tubulin, 1:700 (Sigma, clone TUB-1A2); mouse monoclonal anti-paxillin, 1:500 (BD Transduction Laboratories). Images were obtained by confocal microscopy (model TCS NT; Leica).

### Western blotting and Rac-activity assay

For western blotting, cell lysates were boiled for 5 minutes and separated by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories), blocked with BSA or skimmed milk, and probed using the indicated antibodies. Specific binding was detected using a secondary peroxidase-conjugated antibody (GE Healthcare) followed by chemiluminescence. For detection of Rac1-HA and Rac3-FLAG primary antibodies against HA tag (hybridoma 12CA5) or FLAG tag (M2; Sigma) were used. Anti-β-actin was purchased from Sigma-Aldrich. Phosphorylated MLC and MLC were detected with polyclonal antibody against phospho-MLC (Ser19; Cell Signaling Technology) and monoclonal anti-MLC mouse ascites fluid (clone MY-21; Sigma), respectively. Specific anti-Rac3 rabbit polyclonal antibody was a kind gift from Adrienne D. Cox, University of North Carolina, Chapel Hill, NC.

RacGTPase activity was assayed as previously described. Briefly, cells were gradually cooled on ice, washed with cold PBS+/+ and lysed with a 1% Nonidet P-40 buffer containing 2 μg/ml PAK-CRIB peptide (Price et al., 2003). Cell lysates were cleared by centrifugation, and active Rac1 and Rac3 were precipitated with streptavidin-agarose beads (Sigma-Aldrich) and solubilized in SDS sample buffer.

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