The function of Tiam1/Rac signaling in polarity and cancer
Ellenbroek, Saskia

Link to publication

Citation for published version (APA):
Ellenbroek, S. I. J. (2013). The function of Tiam1/Rac signaling in polarity and cancer

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
The function of Tiam1/Rac signaling in polarity and cancer

Saskia Ellenbroek

Uitnodiging
voor het bijwonen van de openbare verdediging van het proefschrift van
Saskia Ellenbroek
Vrijdag 26 april 2013
om 12.00 uur
Agnietenkapel
Universiteit van Amsterdam
Oudezijds Voorburgwal 231
Amsterdam

Receptie ter plaatse
na afloop van de promotie

Paranimfen:
Amra Hajdo-Milasinovic
amra.hajdomilasinovic@gmail.com
06-18365703

Daan Visser
jpdvisser@gmail.com
06-44282723
THE FUNCTION OF TIAM1/RAC SIGNALING IN POLARITY AND CANCER

Saskia Ellenbroek
The research described in this thesis was performed at the division of Cell Biology of the Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital (NKI-AvL), Amsterdam, The Netherlands

Cover: Puzzle of immunofluorescence image of astrocytes derived from Tiam1 knockout mice, illustrating the microtubule cytoskeleton (purple), centrosomes (lime) and nuclei (seagreen) of cells treated with a chemical Rac inhibitor for 6 hours following scratch-wounding.

Cover design: Maikel Jongsma

Printing of this thesis was financially supported by the Dutch Cancer Society (KWF), the Netherlands Cancer Institute and the University of Amsterdam

ISBN: 978-94-6108-420-0

Printed by Gildeprint Drukkerijen, the Netherlands

Copyright © 2013 by Saskia I.J. Ellenbroek. All rights reserved. No parts of this book may be reproduced, stored in a retrieval system or transmitted in any form or by any means, without prior permission of the author. The copyright of the publications remains with the publishers.
THE FUNCTION OF TIAM1/RAC SIGNALING
IN POLARITY AND CANCER

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op vrijdag 26 april 2013, te 12.00 uur

door

Saskia Inge Johanna Ellenbroek
geboren te Zwolle
PROMOTIECOMMISSIE

Promotor: Prof. dr. A.J.M. Berns
Co-promotor: Dr. J.G. Collard

Overige leden: Prof. dr. R. Versteeg
             Prof. dr. S.T. Pals
             Prof. dr. P.L. Hordijk
             Prof. dr. D.S. Peeper
             Dr. J. de Rooij

Faculteit der Geneeskunde
# TABLE OF CONTENTS

Chapter 1  
**Preface and scope of the thesis**  
General introduction and outline of the thesis

Chapter 2  
**Rho GTPases: functions and association with cancer**  
*Clinical and Experimental Metastasis*

Chapter 3  
**Cell polarity proteins and cancer**  
*Seminars in Cancer Biology*

Chapter 4  
**Rac1 and Rac3 have opposing functions in cell adhesion and differentiation of neuronal cells**  
*Journal of Cell Science*

Chapter 5  
**The Par-Tiam1 complex controls persistent migration by stabilizing microtubule-dependent front-rear polarity**  
*Current Biology*

Chapter 6  
**The Rac activator Tiam1 is required for astrocyte protrusional outgrowth but dispensable for orientation of the microtubule organizing centre**  
*Small GTPases*

Chapter 7  
**Summarizing discussion**

Nederlandse Samenvatting  
List of publications  
Dankwoord
Chapter 1

Preface and scope of the thesis

General introduction and outline of the thesis
Cancer is a complex and multistep disease. Many aspects of the molecular mechanisms underlying the different phases of tumorigenesis are currently unknown. In the studies presented in this thesis the focus is on unraveling mechanisms underlying Rho GTPase signaling and the regulating proteins in different modes of polarity and in tumor development and progression. We investigated different functions of Tiam1, a Rac-specific activator, and studied the differences between Rac1 and its close homologue Rac3.

Small GTPases

The Ras superfamily of small guanosine triphosphatases (GTPases) represents an abundant family of proteins that as binary signaling switches, cycling from an active GTP-bound form to an inactive GDP-bound form (see figure 1). The Ras superfamily currently comprises over 150 family members, divided over multiple subfamilies. Rho (for Ras homologous) GTPases represent a subfamily of the Ras superfamily and comprises over twenty proteins that all contain a Rho insert domain in their protein sequence (as discussed in detail in chapter 2). Together with their regulators and interacting proteins, Rho GTPases account for approximately one percent of the human genome. The activity and cellular outcome of these proteins is regulated by three classes of proteins. Guanine nucleotide exchange factors (GEFs) catalyze the release of GDP binding and subsequent GTP binding. GTPase-activating proteins (GAPs) on the other hand, stimulate the intrinsic ability of GTPases to hydrolyze GTP to GDP, hence promote the inactive state. The third class of GTPase regulating proteins is formed by guanine nucleotide dissociation inhibitors (GDIs) which bind to inactive GTPases, preventing their cycling and activation at the plasma membrane or they bind to active GTPases, preventing their hydrolysis and interaction with effector proteins. A wide variety of proteins had been identified as downstream effector proteins of Rho GTPases, which can be activated by stimulation of cell surface receptors. Active Rho proteins can subsequently bind effector proteins which in turn activate diverging signal transduction routes resulting in a great diversity of cellular outcomes ranging from gene transcription, vesicle trafficking and cytoskeleton reorganization, processes which affect growth, differentiation, adhesion, and migration of cells.

The best studied members of the Rho GTPase family are Cdc42, Rho and Rac. As a result of the wide range of biological activities involving Rho GTPase signaling, aberrant regulation of their activity contributes to the development and progression of several pathologic conditions, including tumorigenesis. Cancer is a multistep process which also involves migration when cell disseminate from the primary tumor and form metastases. Mutations or alterations in expression of GEFs and GAPs, compromising the regulation of GTPase signaling, have been shown to be involved in different steps of the oncogenic process. Mutations in RhoGTPases proteins however are rarely associated
Chapter 1

Metastases are the main cause of cancer-associated death. Many of the signaling cascades contributing to the different phases of this complex disease remain to be elucidated. Tiam1 (for T lymphoma invasion and metastasis) was identified as a protein that induced the invasive and metastatic potential of T lymphoma cells. Expression of Tiam1 is present in almost all healthy tissues and highest expression is found in brain and testis. The Tiam1 protein sequence harbors several distinct domains, including a Pleckstrin-homology (PH) domain at the amino-terminal half of the protein as well as a combination of a Dbl-homology (DH) domain followed by another PH domain (see figure 2). This combination of DH-PH domains is typical for a group of GEFs for small GTPases of the Rho family. Indeed, Tiam1 specifically catalyzes the exchange of GDP for GTP in the switch region of Rac, thereby activating this protein. Due to the different regulatory regions present in the protein sequence of Tiam1, it serves a scaffold function, which enables it to determine signaling downstream of Rac.

Over the past years Tiam1-mediated Rac activation has been shown to be involved in a variety of cellular processes, including regulation of the cytoskeleton, survival and apoptosis,

Figure 1: GTPases act as binary signaling switches. GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. Guanine nucleotide exchange factors (GEFs) enhance the binding of GTP, bringing the GTPase to its active conformation. GTPase activating proteins (GAPs) stimulate GTP hydrolysis, thereby returning the GTPase to its inactive state. Guanine nucleotide dissociation inhibitors (GDIs) prevent the cycling of nucleotides and binding to effector proteins.

The Rac-activator Tiam1

Metastases are the main cause of cancer-associated death. Many of the signaling cascades contributing to the different phases of this complex disease remain to be elucidated. Tiam1 (for T lymphoma invasion and metastasis) was identified as a protein that induced the invasive and metastatic potential of T lymphoma cells. Expression of Tiam1 is present in almost all healthy tissues and highest expression is found in brain and testis. The Tiam1 protein sequence harbors several distinct domains, including a Pleckstrin-homology (PH) domain at the amino-terminal half of the protein as well as a combination of a Dbl-homology (DH) domain followed by another PH domain (see figure 2). This combination of DH-PH domains is typical for a group of GEFs for small GTPases of the Rho family. Indeed, Tiam1 specifically catalyzes the exchange of GDP for GTP in the switch region of Rac, thereby activating this protein. Due to the different regulatory regions present in the protein sequence of Tiam1, it serves a scaffold function, which enables it to determine signaling downstream of Rac.

Over the past years Tiam1-mediated Rac activation has been shown to be involved in a variety of cellular processes, including regulation of the cytoskeleton, survival and apoptosis,
cell polarity (in different cell types and cellular contexts), and several studies link deregulation of Tiam1 signaling to cancer. Given its involvement in many cellular events contributing to several steps in cancer development and progression, it is essential to characterize the mechanisms underlying Tiam1’s function in the different processes. Eventually, information on the role of Tiam1/Rac signaling in cancer will contribute to the design and improvement of diagnostic tools or new targets for therapeutic intervention of specific tumors.

Figure 2: Tiam1 is a multi-domain protein. Schematic representation of the protein domains present in the Tiam1 protein. From the N-terminus Tiam1 contains: PEST sequences (P), an N-terminal Pleckstrin-homology domain (PH), a coiled-coil region (CC), an extended structure (Ex), a Ras-binding domain (RBD), a PSD-95/DlgA/ZO-1 (PDZ) domain, a Dbl-homology domain (DH) and a C-terminal PH domain. The combination of DH-PH domains is typical for GEFs for Rho GTPases and in Tiam1 this mediates Rac activation.

SCAPE OF THE THESIS

In Chapter 2 an overview is given of the current knowledge on the involvement of Rho GTPases in different aspects of cancer. Many cellular processes in development as well as tissue maintenance and pathological conditions involve cytoskeletal changes. Proteins belonging to the Rho family of small GTPases are the key proteins controlling these cytoskeletal changes. Cancer is a highly complex, multistep disease which involves cellular processes including cytoskeletal remodeling, proliferation and cell migration. Therefore, it is not surprising that aberrant Rho GTPase signaling contributes to the different steps of cancer development and progression. In chapter 2 we describe the relation between aberrant GTPase signaling at the level of alterations of regulators and downstream effectors as well as at the level of the Rho GTPases themselves.

In Chapter 3 the current knowledge on the relationship between polarity proteins and cancer is summarized. Most tissues are derived from epithelial cells and therefore loss of epithelial polarity in particular has been implicated in tumor development and progression. Polarity has been defined as the asymmetrical distribution of cellular functions or proteins within cells. During embryonic development correct polarization of cells is crucial, as well as for maintenance of tissue integrity and in the process of wound healing. Loss of polarity is considered a hallmark as well as a precondition for cancer. Several conserved proteins have been identified to signal in functional complexes that regulate different modes of polarization.
Chapter 4 describes a study to identify functional differences between two highly homologous Rho GTPases. Although Rac1 and Rac3 display 92% sequence homology we observed striking differences in both function or localization between these proteins. By exchanging different parts of the protein sequences we identified the protein region responsible for the observed differences.

In Chapter 5 the function of Tiam1 in polarization of freely migrating epithelial cells was investigated. We studied the persistence of cells as well as their capacity to migrate in the absence or presence of the Tiam1 protein. Furthermore, we investigated a potential link to signaling of the Par polarity complex, as we previously showed in contacting keratinocytes.

Chapter 6 describes the study in which we investigated the potential function of Tiam1 in the polarized outgrowth of astrocytes. Astrocytes are supporting cells of the central nervous system and neuronal guidance during development and recovery of the central nervous systems is one of their important functions. Previous studies showed the importance of both Rac activity and the Par polarity complex during protrusion formation of astrocytes. We demonstrated previously that Tiam1 can signal in conjunction with the Par complex. Therefore we investigated whether Tiam1 is the GEF responsible for the required Rac activity during this polarization process.

In Chapter 7 the different studies and results described in this thesis are summarized and discussed in relation to findings from other recent studies.
Chapter 2

Rho GTPases: functions and association with cancer

Saskia I.J. Ellenbroek and John G. Collard

ABSTRACT

Rho GTPases are small proteins that act as binary molecular switches in a wide range of signaling pathways upon stimulation of cell surface receptors. Three different classes of regulatory proteins control their activity. In the activated state small GTPases are able to bind a variety of effector proteins and initiate downstream signalling. Rho GTPases regulate important cellular processes ranging from cytoskeletal remodelling and gene expression to cell proliferation and membrane trafficking. Therefore it is not surprising that deregulated Rho signalling can contribute to disturbed cellular phenotypes in a wide range of diseases. The main focus of this review will be the diversity of functions of Rho GTPases and the effects of aberrant Rho GTPase signalling in various aspects of cancer.

The small GTPases

Small GTPases of the Ras superfamily are small (~21 kDa) proteins that function as binary switches in a wide variety of signalling pathways (reviewed in [1, 2]). These signaling cascades are initiated by the stimulation of cell surface receptors and particularly regulate gene transcription, vesicle trafficking and cytoskeleton reorganisation, processes which affect growth, differentiation, adhesion, and migration of cells. Small GTPases can be either in an active conformation (bound to GTP) or in an inactive conformation (bound to GDP) [3]. Only in the GTP-bound state these proteins are able to bind effector proteins and transduce signals from a large variety of membrane receptors including cytokine and growth factor receptors, adhesion receptors (such as integrins) and G-protein coupled receptors [4, 5].

The Rho (Ras homologous) proteins represent a subfamily of the Ras superfamily of small GTPases, which also includes the Ras family, the Arf family, the Rab family and the Ran family [6]. Rho proteins differ from other small GTPases because their sequences contain a Rho insert domain in the GTPase domain [7]. This insert domain has been suggested to be involved in the activation of downstream effector proteins. As depicted in Fig. 1, at the moment over twenty Rho GTPases have been identified which can be divided into six groups: the Rho proteins (RhoA, RhoB, RhoC), the Rac proteins (Rac1, Rac2, Rac3, RhoG), the Cdc42-like proteins (Cdc42, TC10, TCL, Wrch1, Chp), the Rnd proteins (Rnd1, Rnd2, Rnd3/RhoE), the RhoBTB proteins (RhoBTB1, RhoBTB2, RhoBTB3) and the Miro proteins (Miro1, Miro2). Some Rho proteins do not belong to any of these subgroups, including RhoD, Rif and RhoH/TTF (see Fig. 1). Some Rho proteins are classified as atypical GTPases because they are not regulated as the other, classical GTPases. The atypical Rho GTPases include the Rnd, Miro and RhoBTB subfamilies, RhoH, Wrch1 and Chp [8, 9]. Interestingly, the RhoBTB and Miro proteins are larger than 21 kDa, the average size of the classical GTPases. Miro1 and Miro2 (for mitochondrial Rho) are expressed in the mitochondria and lack the insert domain. They contain an additional C-terminal GTPase domain that is unrelated to the Rho GTPases [9].

Most of the information available on the function of Rho proteins comes
from studies on the best-characterised members Cdc42, Rac1 and RhoA. These GTPases have been shown to control a broad range of cellular processes ranging from the regulation of the actin and the microtubule cytoskeleton to gene transcription (reviewed in [3, 10–12]). In this review we will focus on the functions of specific Rho GTPases and the regulation of their activity. Moreover, we will discuss recent findings on deregulated signalling by Rho proteins and how this may contribute to various aspects of cancer.

Figure 1: The family of small Rho GTPases. Dendrogram of the family of Rho GTPases representing the relationship between the 23 members. Coloured shapes indicate the six different subfamilies: RhoA-related, Rac-related, Cdc42-related, Rnd proteins, RhoBTB proteins and Miro proteins. Alternative names are indicated between brackets. Phylogenetic analysis was performed using ClustalW with protein sequences obtained from the NCBI database and illustrated by TreeView.
Regulators of Rho GTPases

The activity of Rho-like GTPases in response to receptor stimulation is strictly controlled in order to stimulate, locally and temporally, specific downstream signaling pathways in cells. The regulators of the activity of Rho GTPases consist of three classes of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (see Fig. 2).

To date, over 70 Rho GEFs have been identified (reviewed in [13]). GEFs catalyze the release of GDP and subsequent GTP binding, which alters the conformation of the switch regions of GTPases, thereby increasing the binding affinity of effector proteins leading to downstream signalling. GEFs that catalyze the exchange of GDP for GTP of Rho GTPases can be classified in two distinct groups. One group is defined by the presence of two characteristic domains, which account for the catalytic activity. These GEFs contain a catalytic Dbl homology (DH) domain, which is almost invariably followed by a pleckstrin-homology (PH) domain (see Fig. 3).

![Figure 2: Regulation of GTPase cycle.](image)

GDP-bound inactive GTPases are mainly cytoplasmic, maintained there by GDIs masking the C-terminal tail required for plasma membrane localisation. Upon dissociation of the GDI, posttranslational modification can take place and GTPases translocate to the plasma membrane, where they can be activated by GEFs upon external stimuli from surface ligand-receptor systems such as adhesion receptors, G-protein coupled receptors (GPCRs) and receptor tyrosine kinases. Upon activation by GEFs, Rho GTPases can bind different effector proteins, selection of which can be mediated by GEFs and induce downstream signalling pathways. GAPs inactivate the Rho GTPases and switch off the downstream signalling.
PH domain interacts with phospholipids, which may activate the catalytic DH domain of GEFs and localise them to the plasma membrane [14, 15]. The second group of GEFs for Rho GTPases consists of proteins related to Dock180 (dedicator of cytokinesis 180). These proteins contain a Dock-homology region-2 (DHR2 or CZH2) domain, which renders these proteins catalytically active [16–18]. Besides promoting the exchange of nucleotides, GEFs contain various additional domains and are able to influence and determine the signalling route downstream of Rho GTPases by direct binding to different effector molecules or to serve as scaffold proteins that associate with components of downstream effector signalling pathways [14, 15]. In Fig. 3, examples of DH domain containing GEFs are shown, some displaying specificity for a single RhoGTPase, others are able to activate multiple Rho GTPases. GEFs specific for single Rho GTPases include p190RhoGEF which acts on RhoA [19], Tiam1 which activates Rac [14] and Tuba, which activates Cdc42 [20]. Trio contains two DH-PH domain units of which each functions as a catalytic unit: one specific for Rac and RhoG and one specific for RhoA (see Fig. 3) [21]. Vav proteins (Vav1-3) may activate Rac1, RhoA and Cdc42, although they are mostly considered to activate Rac in vivo [22, 23].

Over 60 Rho GAP proteins have been identified [24, 25]. These regulatory proteins enhance the intrinsic ability of small GTPases to hydrolyze bound GTP to GDP, which is intrinsically very low. Thus, GAPs promote inactivation and reverse effector binding, thereby shutting down the signalling pathway (see Fig. 2). Like GEFs, GAPs are usually multidomain proteins and able to associate with many other proteins [26]. It has been shown for example that p120-catenin at specific sites in cells (adherens junctions) to locally inhibit Rho activity to allow proper formation of adherens junctions [27]. Downregulation of RhoA activity by activation of p190RhoGAP can be regulated by Rac-mediated phosphorylation of p190RhoGAP [28].

With a few exceptions, Rho family members have a C-terminal sequence that ends with a CAAX motif [9, 29]. Post-translational modifications of Rho GTPases at the C-terminus, such as prenylation (farnesylation or geranylgeranylation) or palmitoylation, determine their intracellular localisation. They act as a lipid anchor and allow GTPases to localise (and attach) to the plasma membrane where they can be activated by GEFs. The regulatory GDI proteins, of which three mammalian members have been identified [30], are cytosolic proteins that form complexes with inactive, GDP-bound Rho GTPases. Herewith they prevent cycling of the GTPases between cytosol and the plasma membrane and therefore also activation of Rho GTPases by GEFs. Furthermore, GDIs are able to interact with active, GTP-bound GTPases, preventing hydrolysis and interaction with downstream effectors. Association with GDIs thus keeps Rho GTPases in the cytoplasm, inactive or unable to signal towards downstream effectors (see Fig. 2) [30–32]. Phosphorylation of Rho GDIs may lead to dissociation of the complex and allow Rho GTPases to translocate from the cytoplasm to the plasma membrane, where they can be activated by GEFs and bind effectors. P21-activated kinase1 (PAK1) for example has been shown to phosphorylate RhoGDI and cause dissociation of Rac1 from a complex with RhoGDI. Furthermore, phosphorylation of RhoGDIs by protein kinase C can occur in response to elevated intracellular calcium levels, as a result of receptor
activation, leading to translocation of Rac1 to the plasma membrane [33].

In normal conditions the activity of small GTPases is tightly regulated by a large number of GEFs and GAPs. The activation levels of these regulators are also strictly controlled. The activation of GTPases occurs in a cell type-dependent manner and in specific receptor signalling pathways only, as is underlined by the observation that there are many more GEFs, GAPs and effector proteins than there are Rho GTPase family members. The activation state of all Rho GTPases is dependent on the balance of the activities of their regulators (GEFs, GAPs and GDIs). Thus, the local amount of GTP-bound protein and the time during which the protein is active determines the downstream signalling at specific sites in cells. Because of the wide array of processes that are regulated by small Rho GTPases, disturbing the regulation of the activity of these proteins is expected to have severe consequences for a wide variety of cellular events.

**Effector proteins of Rho GTPases**

Over 70 effector proteins have been described binding to the family of Rho GTPases [34, 35]. Each Rho GTPase has binding affinity for multiple effector proteins and some effector proteins are recognised by multiple Rho GTPases, a selection of which is shown in Fig. 3. Many effector proteins of Rho GTPases are serine/threonine kinases and by phosphorylating downstream target proteins they are able to initiate various signalling cascades, regulating different cellular processes. Best-characterised effector proteins for Rac1 and Cdc42 are p21-activated kinases (PAKs). PAKs are a highly conserved family of proteins with six human members, PAK1-6, all serine/threonine kinases, some of which are able to bind active Rac1 (PAK1-3), and all of which can bind active Cdc42 (PAK1-6) [36]. PAKs are important mediators of the organization of the cytoskeleton of cells (see below). Rho-associated coiled-coil-containing protein kinases (ROCK-I and ROCK-II), also serine/threonine kinases, represent the best-characterised effector proteins that bind active RhoA. The activation of ROCK may lead to different cellular events that regulate particularly cytoskeletal changes affecting cell–cell or cell–substrate adhesions and cell migration. These effects are mediated via actomyosin-mediated cell contraction [37]. Furthermore, RhoA, through the activation of ROCK, is able to stimulate cell cycle progression by affecting the transcription levels of several cell cycle regulating genes (see below) [38].

Other non-kinase effector proteins promote downstream signalling by functioning as scaffold proteins, transferring signals from Rho GTPases via interactions with other proteins. For example, N-WASP is a Cdc42-specific effector protein that upon activation by Cdc42 subsequently binds other proteins, such as the Arp2/3 complex to regulate actin polymerisation [39].
Understanding the regulatory possibilities of Rho GTPase signalling becomes more complicated with evidence that small GTPases can regulate each other’s activity via crosstalk, providing an extra tool for fine tuning of signalling events. This regulation can occur between different small GTPase subfamilies as well as within subfamilies. Crosstalk between Ras and Rho proteins is observed in several biological processes, including cell transformation, cell migration and epithelial-mesenchymal transition (EMT) (reviewed in [1]). Oncogenic Ras signals towards Raf and Rac1 and the activation of both is required for oncogenic transformation of cells [40]. It has been shown that Tiam1, a Rac-specific GEF, can link Ras and Rac signalling by binding to activated Ras and subsequent activation of Rac [41].

The balance between Rac1 and RhoA activity, so between two small GTPases of one family, is crucial for several cellular processes such as cell–cell and cell–matrix adhesion, cell migration and EMT [42, 43]. A mechanism by which Rac1 can control RhoA activity is through Rac-mediated production of reactive oxygen species (ROS). Increased ROS levels inhibit the low-molecular-weight protein tyrosine phosphatase (LMW-PTP) leading to increased tyrosine phosphorylation and activation of its target, p190RhoGAP. Rac-mediated activation of p190RhoGAP may thus lead to decreased Rho activity at specific intracellular sites in cells [28].

Figure 3: Rho GTPases and interacting proteins. Selected overview of GEFs that can activate RhoA, Rac1 and/or Cdc42 and allow interaction with various downstream effector proteins, resulting in diverse cellular responses. Domain structures of GEFs are schematically represented; adjacent DH-PH domains are a common feature of Rho GEFs, except for Tuba, a Cdc42-GEF, which contains a BAR domain instead of a PH domain. In general, GEFs contain different additional domains, which allow them to associate with other proteins such as adapter and effector proteins. BAR, Bin1/amphiphysin/ Rvs167; CC, coiled-coil region; CR, cysteine-rich zink-butterfly motif; DH, Dbl-homology; Ex, extended structure; Ig, immunoglobulin-like domain; KIN, serine/threonine protein kinase domain; L-rich, leucine-rich region; P, PEST-sequence; PDZ, PSD-95/DlgA/ZO-1 domain; PH, pleckstrin homology; RBD, Ras-binding domain; s, spectrin repeats; SH3, Src-homology; Zn, zinc finger motif.

Cross-talk by small GTPases
FUNCTIONS OF RHO GTPASES

Regulation of the actin cytoskeleton

The Rho family of GTPases have been best characterized for their particular function in the regulation of the actin cytoskeleton in response to receptor signalling. All Rho proteins play an important role in organizing the actin filament system, except for the RhoBTB and Miro proteins. The activation of Miro proteins influences the mitochondrial network, but not the actin filament system [44, 45]. RhoA regulates the formation of contractile actomyosin bundles (stress fibres) and focal adhesions, Rac1 regulates the formation of actin-rich protrusions (lamellipodia) as well as membrane ruffling and Cdc42 regulates the formation of filopodia [46]. These actin dynamics are regulated by coordinated activation of different signalling pathways downstream of the small GTPases. RhoA can interact with its effector protein ROCK (as described above), which can subsequently activate myosin light chain kinase, leading to activation of myosin (by phosphorylation), increased contractility and formation of stress fibres. Furthermore, RhoA can stimulate actin polymerisation via its effector proteins mDia1 and mDia2, which catalyse F-actin assembly in filopodia and lamellae [47, 48]. Cdc42 can signal to the Arp2/3 complex via its effector N-WASP, which results in actin polymerisation and the formation of filopodia [39, 49]. Rac1 regulates actin organisation by activating WAVE (for example via IRSp53) or PAK, resulting in altered actin nucleation activity of the Arp2/3 complex [50, 51]. Furthermore, the Rac-activator Tiam1 can bind IRSp53 [52] and p21Arc [51], one of the components of the Arp2/3 complex, providing a mechanism to directly regulate Tiam1/Rac-mediated actin polymerisation processes by the Arp2/3 complex.

Regulation of the microtubule cytoskeleton

Besides regulating the actin cytoskeleton, small Rho GTPases are also important regulators of the microtubule cytoskeleton, via regulation of activity of several downstream effector proteins. Interestingly, mDia is a downstream effector protein of RhoA, which is involved in both the regulation of the actin cytoskeleton as well as the microtubule cytoskeleton. RhoA can promote the formation of stable and aligned microtubules via signalling through mDia [53, 54]. Both Rac1 and Cdc42 can influence microtubule stability by mediating PAK signalling to stathmin, an important microtubule destabilizing protein [55]. Furthermore, Rac1 and Cdc42 are able to promote microtubule capture at the cell cortex (leading edge of migrating cells) by stimulating binding of their effector protein IQGAP to the capture protein CLIP-170 [56]. Microtubule capture is necessary for the stabilisation of microtubules, which is essential for polarisation of cells. Cdc42 plays another crucial role concerning microtubule organisation, during polarisation and directional cell migration. It binds to one of its effector proteins, Par6, which forms a polarity complex with atypical PKC and together transduce signals to downstream targets resulting for example in the reorientation of the Golgi apparatus and microtubule organizing centre (MTOC) [57]. The Rac-GEF Tiam1 also associates with proteins of the Par polarity complex (Par3, Par6 and atypical PKC) and
promotes microtubule stability, thereby allowing cells to stably polarise and to migrate in a persistent fashion [58].

Rho GTPases and cytoskeleton-dependent processes

Because of their function in cytoskeletal organisation, small Rho GTPases regulate various cytoskeleton-dependent processes such as changes in cell shape, cell adhesion, cell spreading, cell migration and cell polarity. Adhesive structures such as tight junctions (TJs), adherens junctions (AJs) and desmosomes are linked to the cytoskeleton and determine epithelial morphology and functionality and therefore play an essential role in the maintenance of tissue architecture [59]. Rho GTPases have been shown to regulate the formation and maintenance of these adhesive structures (AJs and TJs) [60–62]. Interestingly, Rho GTPase signalling can contribute not only to stabilisation but also to disassembly of AJs leading to EMT [63, 64]. The reciprocal balance between Rac and Rho activity determines the epithelial or mesenchymal phenotype of epithelial cells [42]. Downstream effector proteins regulate the effect of activation of RhoA on cell adhesion. RhoA signalling via mDia and subsequent actin polymerisation is required for formation and maintenance of AJs, whereas RhoA signalling through ROCK results in disruption of AJs caused by increased contractility [65]. In vitro studies have shown that Tiam1-mediated Rac activity in conjunction with the Par polarity complex is essential for the establishment of apical-basal polarity of epithelial cells and interference with either Tiam1 or the Par complex facilitates EMT and migration of cells [66]. Interestingly, the same Par-Tiam1 complex also regulates front-rear polarity and directional migration in dissociated migratory epithelial cells [58]. This underscores a remarkable flexibility of the Par complex and the Rac GTPase that depending on the biological context, controls distinct forms of cellular polarity in the same cell type.

Rho GTPases also function in polarisation processes in other cell types. A Par/Tiam1/Rac complex in conjunction with Cdc42 plays an essential role in chemokine-induced cell polarisation and chemotactic migration of T cells [67]. Rac is also required for the polarisation and chemotactic migration of neutrophils [68, 69]. In neuronal cells, signalling pathways involving Cdc42 and the Par/Tiam1/Rac complex are essential for axon specification. Localised polarity signalling determines which neurite grows out to be a future axon [70]. The outcome of signalling of the Par complex in conjunction with Rho GTPases is clearly context and cell type dependent.

Rho GTPase signalling is required for the regulation of vesicular trafficking, including exocytosis, endocytosis and phagocytosis, processes which are dependent on actin and microtubule dynamics and essential for establishment of asymmetrical distribution of proteins in polarised cells [31, 71, 72].

Rho GTPases and regulation of transcription

Besides these well-described functions related to cytoskeletal regulation, Rho proteins have also been implicated in the control of gene transcription. RhoA controls the activation of the nuclear transcription factor serum response factor (SRF), by which it can regulate the transcription of many genes [11, 73]. Furthermore, RhoA, Rac1 and Cdc42 regulate cell cycle progression and growth as well as apoptosis, by regulating
transcription of specific genes, including cyclin D1 [74, 75]. Cyclin D1 belongs to a family of proteins that regulate cell cycle progression by stimulating G1 to S phase transition. The transcription of cyclin D1 is controlled by transcription factors like ETS, AP-1 and NFkB of which activity is regulated by Rho GTPases including RhoA, Rac1 and Cdc42 [76]. These transcription factors may also control anti-apoptotic survival signalling. Rac promotes cell survival by activating the NFkB pathway [77, 78] or by the production of ROS that promotes ROS-dependent Erk-mediated survival signalling (Rygiel et al., unpublished results). Rac1 also stimulates survival signalling through activation of the phosphatidylinositol 3-kinase (PI3K)-Akt signalling pathway [79].

Currently very little is known about the function of the RhoBTB proteins. As mentioned above, these proteins do not seem to function as cytoskeletal organisers and they are expressed in vesicular structures [44]. The presence of BTB (Broad complex/Tramtrack/Bric a brac) domains hints for a role in transcriptional regulation, since these domains are found in proteins that function in this process [80]. RhoBTB2 has been suggested to be involved in protein degradation by binding of its BTB domain to a ubiquitin ligase scaffold protein [81]. However, the localisation of RhoBTB proteins in cytoskeletal vesicular structures suggests a role in endocytosis or related processes. The atypical GTPases Miro1 and Miro2 function in mitochondrial trafficking and via the interaction with kinesin-binding proteins might serve as a link between mitochondria and microtubule trafficking [9].

The necessity of tightly regulated Rho GTPase signalling is illustrated by the embryonic lethality of mice after knockout of for example Rac1 or Cdc42 [82, 83]. Because of their regulatory function in various signalling cascades, it is not surprising that aberrant signalling by Rho GTPases has been found to be involved in disturbed cellular phenotypes in a wide range of diseases, including neurodegenerative disorders and cancer [84, 85].

**RHO GTPASES AND CANCER**

The knowledge that Ras proteins are mutated in 30% of human cancers of different origins [86, 87], suggested that the same might hold true for the Rho family of small GTPases. Unexpectedly, to date, no mutations have been found in Rho proteins. Only one member of the Rho family of small GTPases has been reported to be genetically altered in human cancer (see below). Considering the cellular functions of Rho proteins in the regulation of polarisation, migration, proliferation and survival of cells, processes required for tumour formation and progression, it was expected that mutational activation of Rho proteins could deregulate these processes. Apparently, mutational activation or inactivation of Rho proteins is not favourable for the initiation or progression of tumours. Since mutations in Rho proteins have not been found, deregulation of Rho GTPase signalling could occur at the level of expression or activation of Rho GTPases, accomplished by the level of expression or activation of their regulators or downstream effectors.

Indeed, numerous in vitro and in vivo studies using tumour-derived cell lines, primary tumours and mouse models in which cancer development
and progression has been studied clearly indicate that deregulated signalling of small Rho GTPases plays an important role in the initiation as well as the progression of (human) cancer. The results from these studies and the lack of mutations in Rho proteins strongly suggest that disturbance of Rho GTPase signalling in diseases, including cancer, are caused by alterations at the level of their regulators, rather than by mutations at the level of the small Rho GTPases.

**Alteration of Rho proteins in cancer**

As mentioned above, only one human Rho GTPase has been found to be genetically altered in human cancers. Rearrangement of the gene RhoH (also known as TTF) was found in non-Hodgkin’s lymphomas and multiple myeloma [88], as well as mutations in the 50 UTR in diffuse large-cell lymphomas [89]. The exact contribution of these alterations in the RhoH gene to pathology has not been elucidated yet. In contrast to the low incidence of genetic alterations of Rho GTPases, several Rho GTPases have been reported to be aberrantly expressed or activated in human cancers or cancer-derived cell lines. An overview of a selection of observed aberrations in small Rho GTPase activity or expression levels in human cancers is given in Table 1, of which a few examples are highlighted below in more detail. Rho GTPases found to be aberrantly expressed in human cancers include RhoA, RhoB, RhoC, Rac1 Rac2, Rac3, RhoG, Cdc42 and Rnd3/RhoE. Altered expression can take place at the mRNA level or at the protein level and for some of these GTPases expression levels have been linked to prognosis and development of diseases (for references see Table 1).

Overexpression of RhoA for example, has been observed in breast, colon, lung [90, 91] and gastric cancer [92], as well as in head and neck squamous cell carcinoma (HNSCC) [93], bladder [94] and testicular cancer [95]. In vitro studies have shown that as a consequence of elevated expression, RhoA is localised to the plasma membrane where it can be activated and promote invasion by signalling towards ROCK and actomyosin [96].

Studies in which malignant breast tissue was compared to benign tissue revealed that the Rac1 protein levels were increased in malignant breast tissue, suggesting that increased Rac activity promotes breast cancer development in vivo [90, 91]. Interestingly, a Rac1 splice variant, designated Rac1b, which is highly active through accelerated GDP/GTP exchange [97] and is unable to interact with RhoGDI [98] was found overexpressed in breast and colon cancer [97, 99]. Rac1b is able to promote cellular transformation in human breast and colon carcinoma cells [100]. In vitro studies using mouse fibroblasts indicate that the effect of Rac1b signalling might involve increased stimulation of survival signalling via NFƙB [101]. Interestingly, a study using mouse mammary epithelial cells revealed that expression levels of Rac1b can be altered by stromelysin-1/matrix metalloproteinase-3 (MMP-3), an enzyme that is upregulated in many breast tumours. This stromal enzyme induces EMT as well as the expression of Rac1b and therewith stimulates an increase in ROS, causing DNA damage [102]. In contrast to Rac1 deficiency which leads to embryonic lethality [82], Rac3 deficiency does not impair embryonic development [103]. An important function of Rac3 in cancer progression was shown in a mouse leukaemia model. Mice lacking Rac3 were found to be protected against
lymphoblastic leukaemia induced by crossing with mice expressing the BCR-ABL fusion oncogene [104]. This suggests that interference with Rac3 function may provide a strategy for therapeutic intervention of B-cell lymphomas. Elevated levels of hyperactive Rac3 were found in highly proliferative human breast cancer-derived cell-lines and tumour tissue. Although these data have been questioned, Rac3 may cause persistent activity of its effector, PAK, which contributes to DNA synthesis of highly proliferative cells [105].

**Alteration of regulators of Rho GTPases in cancer**

**GEFs**

Mutations in or altered expression of regulatory proteins of GTPase activity will also result in deregulated signalling downstream of Rho proteins (see Table 1). The Rac-specific GEF Tiam1 controls the establishment and maintenance of E-cadherin based cell–cell adhesions and as a consequence loss of Tiam1 leads to EMT [66, 106]. Indeed, Tiam1-deficiency promotes invasiveness of Ras-induced epithelial skin tumours as well as β-catenin/TCF-induced intestinal tumours in mouse tumour models [107, 108]. Moreover, mutations in the N-terminal PH domain of Tiam1, which could affect the intracellular localisation of this GEF, have been found in 10% of analyzed samples from human renal-cell carcinomas [109]. Tiam1 protein levels were also found to be increased and correlated positively with disease prognosis in human prostate carcinomas [110]. This positive correlation was also found in studies examining breast cancer, where Tiam1 expression correlated with invasiveness of human breast tumour cells and degree of progression of breast tumours [111, 112]. Intriguingly, the effects of Tiam1-mediated Rac signalling on tumour formation and progression seem tumour type dependent and either positively or negatively correlate with tumour progression. As Tiam1 may influence tumour initiation, tumour growth as well as tumour progression by activating different signalling pathways [107], the cellular outcome of altered expression of Tiam1 may depend on a balance between factors that are regulated by Tiam1- mediated Rac activation and that influence tumourigenicity. For instance, the effects of Tiam1 on E-cadherin-based cell–cell adhesions, apoptosis, cell proliferation and polarised directed cell migration may affect the formation and progression of tumours in multiple ways.

Dock180 can activate Rac only when it is bound to the ELMO1 protein (engulfment and cell motility 1) [16]. In human glioma samples it was shown that expression of this bipartite Rac GEF was upregulated, only in the invasive areas of the tumours. Furthermore, in vitro overexpression of the Dock180-ELMO1 complex increased the invasive and migratory capacity of cells [113]. A GEF that specifically activates RhoA, termed LARG (for leukaemia-associated Rho GEF), was isolated from a patient with acute myeloid leukaemia (AML), as a fusion partner of a gene frequently rearranged in both AML and acute lymphocytic leukaemia (ALL) [114]. This fusion of the mixed lineage leukaemia (MLL) gene to LARG causes truncation of LARG upon chromosomal translocation. Fusion of these genes is suggested to contribute to the development of leukaemia by activating RhoA signalling pathways [115]. A RhoA-specific GEF, GEF-H1, has been shown to accelerate tumour cell proliferation upon transcriptional activation by expression
of mutant p53 proteins. The expression levels of GEF-H1 correlate with the mutation status of p53 in several cancer cell lines [116]. Increased expression of GEF-H1 results in increased activation of RhoA and might therefore contribute to enhanced invasion and metastasis as well as growth of p53 mutant tumours.

**GDIs**

Also alterations in expression of GDIs have been reported to promote developmental stages of cancer. RhoGDI (RhoGDI1 or RhoGDIα), which is able to bind RhoA, RhoB, Rac1, Rac2 and Cdc42 [117], is overexpressed in invasive ovarian cancers [118]. RhoGDI may also influence breast cancer cell motility through enhanced transcription levels of estrogen receptors (ER) a and b [119]. D4-GDI (RhoGDI2 or RhoGDIβ) is expressed in a number of breast cancer cell lines and expression levels correlate with invasiveness of these cell lines. D4-GDI is not expressed in benign-derived mammary epithelial cells [120]. In contrast, downregulated expression of D4-GDI was found to stimulate development of metastatic bladder cancer [121]. These findings suggest that deregulation of these Rho signalling pathways may promote or inhibit tumour progression in multiple ways. The mechanisms by which expression levels of GDIs influence Rho GTPase signalling pathways and tumour progression remain to be elucidated. An important aspect of this area of research will be the determination of the specificity of RhoGDIs for the various Rho GTPases in vivo, which has not been demonstrated yet.

**GAPs**

Expression levels of different Rho GAPs have also been linked to tumourigenesis, including DLC-1 and DLC-2 (deleted in liver cancer 1 and 2). Genomic deletion of DLC-1, a Rho GAP for RhoA and Cdc42, was found in primary breast tumours [122], whereas DLC-2 expression was downregulated in hepatocellular carcinomas [123]. Deletion or downregulation of these GAPs leads to increased levels of GTP-bound RhoA and thus enhanced GTPase activity and downstream signalling, which could contribute to tumourigenesis. ARHGAP8 is a Rho GAP which has been found to be upregulated in the majority of colon tumours [124].

For most observations on the various regulators (GEFs, GAPs and GDIs) of Rho GTPases in cancer it is unclear what the possible effect is on the activity of Rho GTPases in tumours. Moreover, the specificity of most of the regulators of Rho proteins in vivo is still unknown which makes such studies difficult. Maybe deregulation of Rho signalling as such is sufficient for their contribution to tumourigenicity. Nevertheless, collectively all data documented so far suggest that Rho GTPase family members contribute to the various stages of tumourigenesis and may act differently during the initiation and the progression of tumours. The apparent ability of regulators of Rho proteins to modulate Rho-mediated signalling pathways in tumours may lend themselves as targets for small molecule therapeutic agents against cancer.
<table>
<thead>
<tr>
<th>Rho GTPase</th>
<th>Alteration</th>
<th>Tumour type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RhoA</td>
<td>Overexpression</td>
<td>Breast, colon, lung, gastric, HNSCCa, bladder &amp; testicular cancer</td>
<td>Fritz et al. [90, 91] (breast/colon/lung), Pan et al. [92] (gastric), Abraham et al. [93] (HNSCC), Kamai et al. [94, 95] (bladder/testicular)</td>
</tr>
<tr>
<td>RhoB</td>
<td>Overexpression or downregulation</td>
<td>Breast (overexpression), lung &amp; HNSCCa (downregulation)</td>
<td>Fritz et al. [91] (breast), Mazieres et al. [147] (lung), Adnane et al. [148] (HNSCC)</td>
</tr>
<tr>
<td>RhoC</td>
<td>Overexpression</td>
<td>(Inflammatory) breast &amp; metastatic gastric cancer, pancreatic ductal adenocarcinoma, bladder cancer, NSCLCa, &amp; HNSCCa</td>
<td>Van Golen et al. [144] (breast), Liu et al. [149] (gastric), Suwa et al. [150] (pancreas), Kamai et al. [94] (bladder), Shikada et al. [151] (NSCLC), Kleer et al. [152] (HNSCC)</td>
</tr>
<tr>
<td>Rac1</td>
<td>Overexpression</td>
<td>Breast, gastric &amp; testicular cancer OSCCa</td>
<td>Fritz et al. [90, 91] (breast/colon/lung), Pan et al. [92] (gastric), Kamai et al. [95] (testicular), Liu et al. [153] (OSCC)</td>
</tr>
<tr>
<td>Rac1b</td>
<td>Alternative splicing (19 aa insert)</td>
<td>Breast &amp; colon cancer</td>
<td>Schneler et al. [97] (breast), Jordan et al. [99] (colon)</td>
</tr>
<tr>
<td>Rac2</td>
<td>Overexpression</td>
<td>HNSCCa</td>
<td>Abraham et al. [93]</td>
</tr>
<tr>
<td>Rac3</td>
<td>Hyperactive or overexpression</td>
<td>Breast cancer</td>
<td>Mira et al. [105]</td>
</tr>
<tr>
<td>RhoG</td>
<td>Overexpression</td>
<td>Breast cancer</td>
<td>Jiang et al. [154]</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Overexpression</td>
<td>Breast &amp; testicular cancer</td>
<td>Fritze [90, 91] (breast), Kamai et al. [95] (testicular)</td>
</tr>
<tr>
<td>RhoH/TTF</td>
<td>Rearrangement and mutations (5’UTR)</td>
<td>Non-Hodgkin’s lymphoma &amp; multiple myeloma (rearrangement) &amp; diffuse large B-cell lymphoma (mutation)</td>
<td>Preudhomme et al. [88] (Hodgkin/multiple myeloma), Pasqualucci et al. [89] (diffuse large B-cell lymphoma)</td>
</tr>
<tr>
<td>Rnd3/RhoE</td>
<td>Overexpression or downregulation</td>
<td>NSCLCa (overexpression) prostate cancer (downregulation)</td>
<td>Zhang et al. [155] (NSCLC), Bektic et al. [156] (prostate)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regulator of Rho GTPase</th>
<th>Alteration</th>
<th>Tumour type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiam 1</td>
<td>Point mutation or overexpression</td>
<td>Renal-cell carcinoma (point mutation), prostate carcinoma &amp; breast cancer (overexpression) AML</td>
<td>Engers et al. [109] (renal cell), Engers et al. [110] (prostate), Adam et al. [111], Minard et al. [112] (breast)</td>
</tr>
<tr>
<td>LARG</td>
<td>Fusion to MLL</td>
<td>Glioma</td>
<td>Kourlas et al. [114]</td>
</tr>
<tr>
<td>Dock180-ELMO1</td>
<td>Ectopic expression</td>
<td>Pancreatic adenocarcinoma, neuroblastoma</td>
<td>Fernandez-Zapico et al. [157] (pancreas), Hornstein et al. [158] (neuroblastoma)</td>
</tr>
<tr>
<td>Vav1</td>
<td>Overexpression</td>
<td>Breast cancer</td>
<td>Jarzymska et al. [113]</td>
</tr>
<tr>
<td>β-PIX</td>
<td>Overexpression or downregulation</td>
<td>Invasive ovarian (overexpression) &amp; breast cancer (overexpression or downregulation)</td>
<td>Jones et al. [118] (Ovarian), Fritz et al. [91] (breast, overexpression), Jiang et al. [154] (breast, downregulation)</td>
</tr>
<tr>
<td>Rho GDlo</td>
<td>Overexpression or downregulation</td>
<td>Breast cancer &amp; ovarian adenocarcinoma (overexpression), bladder cancer (downregulation)</td>
<td>Zhang et al. [120] (breast), Tapper et al. [160] (ovarian), Theodorescu et al. [121] (bladder)</td>
</tr>
<tr>
<td>D4-GDI</td>
<td>Overexpression or downregulation</td>
<td>Breast cancer</td>
<td>Jiang et al. [154]</td>
</tr>
<tr>
<td>Rho GDly</td>
<td>Downregulation</td>
<td>Breast cancer (deletion), hepatocellular carcinoma (deletion &amp; methylation)</td>
<td>Yuan et al. [122] (breast), Wong et al. [161] (hepatocellular carcinoma)</td>
</tr>
<tr>
<td>DLC-1</td>
<td>Overexpression</td>
<td>Colon cancer</td>
<td>Ching et al. [123]</td>
</tr>
</tbody>
</table>

*a HNSCC: head and neck squamous cell carcinoma
*b NSCLC: non-small cell lung cancer
*c OSCC: oral squamous cell carcinoma
Chapter 2

Alteration of downstream effectors of Rho proteins and cancer

Besides disturbed signalling at the regulator or GTPase level, Rho GTPase signalling may also contribute to pathologic development via alterations at the level of the effector proteins. Expression of PAK for example (effector of both Rac1 and Cdc42) is upregulated in some breast cancers [125]. This upregulation of PAK induces anchorage-independent growth as well as abnormal organisation of mitotic spindles in experiments with human epithelial breast cancer cells (MCF-7) [126]. Furthermore, elevated expression levels of ROCK-I and II have been associated with higher stages of testicular cancer [95]. ROCK expression might affect migratory capacities of testicular tumour cells by interfering with tightly regulated activation of actomyosin and therefore may contribute to metastatic spread and progression of this particular type of cancer.

TUMOUR INITIATION: CONTRIBUTION OF RHO GTPASE SIGNALLING TO TUMOUR INITIATION AND GROWTH STUDIED IN VIVO

The first evidence that Rho proteins could be linked to the initiation of cancer came from in vitro studies showing the requirement of signalling via Rho GTPases for Ras oncoproteins to induce oncogenic transformation [127, 128]. Oncogenic Ras induces transformation and allows fibroblasts to form foci, groups of cells with a refractile, spindle shaped morphology that exhibit growth factor- and anchorage-independent growth in vitro. Constitutive active mutants of RhoA, Rac1 and Cdc42 contribute to Ras-induced morphological transformation, whereas dominant-negative mutants of these GTPases prevent transformation. Furthermore, activated Rac1 has been shown to suppress apoptosis induced by oncogenic Ras via activation of NFkB [129], thereby moderating the growth advantage for Ras transformed cells. These observations suggest that oncogenic Ras is able to activate or to collaborate with specific Rho family proteins, which in turn activate a spectrum of functions that contribute to full (malignant) Ras transforming activity [130].

The in vitro observations that Rho proteins can contribute to oncogenic transformation have been confirmed by in vivo studies. Mice lacking the Rac-activator Tiam1 are protected from initial development of Ras-induced skin cancers. In the few skin tumours that did develop in Tiam1-deficient mice, growth was slow but malignant progression was promoted by the absence of Tiam1 [107]. These results indicate that Tiam1-Rac signalling may contribute to both initiation and progression of skin cancer and acts synergistically with Ras during tumour initiation. The inhibitory effect on tumour initiation by a lack of Tiam1-mediated Rac activation was caused by an increase in apoptosis of the epidermal keratinocytes. Apparently, Tiam1-mediated Rac activation suppresses Ras-induced apoptosis and thereby promotes cell survival and subsequent tumour initiation and growth. Similar experiments were performed using RhoB-knockout mice, with opposing results compared to the Tiam1-knockout mice. Interestingly, in RhoB-deficient mice the number of Ras-induced tumours increased compared to
wild-type mice, most likely also by a RhoB-dependent function in apoptosis [131]. This means that in conditions of cellular stress RhoB might function to suppress tumorigenesis by stimulating apoptosis, thereby antagonizing the effects of Rac that acts in anti-apoptotic survival signalling.

Considering the effects of Rho GTPases on cell cycle progression through cyclin D1, it is of interest that breast cancer mouse models revealed that the Neu- and Ras-oncogenic pathways are dependent on cyclin D1 whereas c-myc and Wnt-1 oncogenic pathways are not [132]. Mice lacking cyclin D1 show resistance to breast cancer induced by the Neu and the Ras oncogenes, while remaining sensitive to other oncogenic pathways driven by c-myc and Wnt-1. Mice lacking Tiam1 also show reduced initiation of breast tumours induced by Neu (Strumane et al., unpublished results) and of intestinal tumours induced by Wnt [108]. Apparently, different oncogenic pathways require different additional signalling pathways for efficient initiation of tumours.

TUMOUR PROGRESSION: CONTRIBUTION OF RHO GTPASE SIGNALLING TO INVASION AND MIGRATION OF TUMOURS

Cadherin proteins are major players in the establishment and maintenance of AJs between epithelial cells (with E-cadherin most abundantly expressed). Loss or deregulation of E-cadherin mediated cell adhesions leads to EMT [133] and is correlated with progression of epithelial tumours [134]. Rho GTPases regulate both disassembly as well as stabilisation of E-cadherin based adhesions. It is therefore not surprising that Rho proteins have been implicated in the alteration of cell–cell adhesion status of tumour cells [135]. In vitro studies revealed that Tiam1-mediated Rac activation is required for proper cell junction formation [66]. Accordingly, loss of Tiam1 promotes EMT in vitro and the progression of mouse skin tumours in vivo [64, 107]. Results from several in vitro studies also suggest that the activation status of Rac or Rho can be positively or negatively affected downstream of cadherin signalling [136, 137]. For instance, cadherin-mediated cell adhesions inhibit RhoA via p190RhoGAP [27, 137], providing a possible mechanism through which elevated cadherin expression can enhance cell migration rather than promote stable intercellular adhesion of epithelial tumours. Comparable to this, Tiam1 promotes apical-basal cell polarisation in epithelial cells but also promotes directional migration in freely migrating epithelial cells [58, 66]. Cell substrate interactions may determine which of these pathways are operational [138].

Invasive tumour cells require adaptation of cell–matrix adhesions in order to be able to cross tissue boundaries and spread to distal parts of the body (illustrated in Fig. 4). Cross-talk between different small Rho GTPases is very important for tight regulation of this process. RhoA and Rac1 are able to control the levels of matrix metalloproteinases (MMPs), which degrade the extracellular matrix (ECM) (reviewed in [135]). In addition to the regulation of levels of MMPs, Rho GTPases can influence the remodelling of the ECM by regulating tissue inhibitors of metalloproteinases (TIMPs). The Rac-GEF Tiam1 upregulates the transcriptional levels of TIMP-1 and posttranslational levels of TIMP-2 in renal-cell carcinomas, relating Tiam1 expression to the invasive
Figure 4: Contribution of Rho GTPase signalling to different steps of cancer development. (a) Deregulated Rho GTPase signalling can contribute to tumour initiation by influencing apoptosis and survival. (b) Once a tumour is initiated, Rho proteins can contribute to tumour development by stimulating growth and loss of cell polarity. (c) By altering cell-cell and cell-matrix adhesion, Rho proteins allow tumour cells to become invasive and (d) migrate to distal parts of the body where metastases are formed and angiogenesis is stimulated.
capacities of this tumour cell type [139].
After EMT and adaptation of cell–matrix adhesion tumour cells need to acquire migratory capacities in order to invade and migrate towards distal parts of the body, as illustrated in Fig. 4. This migration can occur as single cells or collectively, when cells move as a sheet. Simpson and colleagues showed that RhoA and RhoC serve opposing functions in invasive breast carcinoma [140]. Similar results were obtained in studies concerning colon carcinoma, which showed that RhoA activation was decreased during EMT, whereas RhoC activity was induced. Apparently, RhoA inhibits post-EMT migration of cells, in contrast to RhoC which promotes cellular migration after EMT in colon carcinoma [141]. Furthermore, RhoC was found to be required for metastasis of melanoma cells in vivo and in vitro experiments indicate that elevated expression levels correlate with increased metastatic potential of this tumour type [142]. A RhoC-deficient mouse model confirmed a role for RhoC in tumour cell motility and metastasis. It also demonstrated that RhoC is not required for the initiation of tumours but rather for tumour progression and metastasis [143]. An additional mechanism by which RhoC might contribute to tumour progression, besides influencing cell migration, is the induction of angiogenic factors which promote tumour vascularisation, as has been shown in breast cancer [144]. In vivo studies using mice with reduced Rac1 activity due to knockout of Tiam1 demonstrated the requirement of properly regulated Rac1 signalling for re-epithelialisation of skin wounds, a process involving the migration and proliferation of keratinocytes [145]. Similar studies using transgenic mice expressing a dominant-negative mutant of Rac1 confirmed these data [146], further supporting that epidermal wound healing is reduced as a result of Rac-dependent proliferation and migration of keratinocytes. Together, these in vivo examples illustrate the importance of tight regulation of Rho GTPase signalling and demonstrate that aberrant Rho signalling may contribute the formation and progression of tumours.

CONCLUSIONS

We have discussed here examples of in vivo and in vitro studies illustrating the function of small Rho GTPases and their contribution to different stages of cancer development. The effect of altered signalling from Rho GTPases is cell type and cellular context dependent. An important aspect of initiation of cancer is the coordination of uncontrolled proliferation (growth) and the prevention of apoptosis. Since Rho GTPases are involved in regulation of both processes, they play a pivotal role in the initiation of tumour formation. Rho proteins also function in the formation and disassembly of E-cadherin-mediated cell–cell adhesions and can therefore contribute to EMT and metastasis. Moreover, the regulatory functions of Rho GTPases on cytoskeleton remodelling allow these proteins to influence cancer-related processes such as the invasion, migration and metastasis of cells. Elucidation of the mechanisms by which regulators of Rho proteins modulate Rho-mediated signalling pathways in tumours may provide novel targets for small molecule therapeutic agents to treat cancer.
ABBREVIATIONS

AJ, Adherens junction; Arp, Actin-related protein; DH, Dbl homology; DOCK, Dedicator of cytokinesis; EMT, Epithelial-mesenchymal transition; ERK, Extracellular signal-regulated kinase; GAP, GTPase-activating protein; GDI, Guanine nucleotide dissociation inhibitor; GEF, Guanine nucleotide exchange factor; MMP, Matrix metalloproteinase; NFkB, Nuclear factor kB; NWASP, Neural Wiskott-Aldrich-syndrome protein; PAK, p21-activated kinase; PH, Pleckstrin homology; PKC, Protein kinase C; ROCK, Rho-associated coiled-coil containing protein kinase; ROS, Reactive oxygen species; SRF, Serum response factor; Tiam1, T-lymphoma invasion and metastasis 1; TJ, Tight junction; WAVE, WASP-like verprolin-homologous protein.

REFERENCES


35. Bustelo XR, Sauzeau V, Berenjeno IM
Chapter 2


expression and mutation analyses and correlation with clinical parameters. Br J Cancer 87:635–644


108. Malliri A, Rygiel TP, van der Kammen


growth suppressor function and is underexpressed in hepatocellular carcinoma. J Biol Chem 278:10824–10830


155. Zhang C, Zhou F, Li N, Shi S, Feng X,


Chapter 3

Cell polarity proteins and cancer

Saskia I.J. Ellenbroek, Sandra Iden and John G. Collard

Chapter 3

ABSTRACT

Cell polarity is essential in many biological processes and required for development as well as maintenance of tissue integrity. Loss of polarity is considered both a hallmark and precondition for human cancer. Three conserved polarity protein complexes regulate different modes of polarity that are conserved throughout numerous cell types and species. These complexes are the Crumbs, Par and Scribble complex. Given the importance of cell polarity for normal tissue homeostasis, aberrant polarity signaling is suggested to contribute to the multistep processes of human cancer. Most human cancers are formed from epithelial cells. Evidence confirming the roles for polarity proteins in different phases of the oncogenic trajectory comes from functional studies using mammalian cells as well as Drosophila and zebrafish models. Furthermore, several reports have revealed aberrant expression and localization of polarity proteins in different human tumors. In this review we will give an overview on the current data available that couple polarity signaling to tumorigenesis, particularly in epithelial cells.

Polarity processes

Cell polarity is a crucial phenomenon in many biological processes that contribute to normal tissue integrity and development and is defined as an asymmetrical cell shape, and/or asymmetrical distribution of proteins and functions in cells (1). The fact that cell polarization is an absolute requirement for development and that maintenance of proper tissue integrity depends on proper cell polarity becomes clear from a wide variety of polarization processes present throughout numerous cellular actions and cell types. In both embryonic and adult state, asymmetric cell divisions are important for development and tissue homeostasis. To create cells with different functions and cell fates, two non-identical daughter cells have to be produced during cell division. Similarly, in order to maintain controlled populations of stem cells, asymmetric cell divisions need to be regulated accurately (2;3). In T cells, polarization is required for important T-cell specific features to ensure functionality, including transendothelial migration and responses to chemokines and antigens (4). Neuronal cells depend on strictly regulated polarity signaling for processes such as axon specification and dendritic spine formation (5). Cell polarity is essential for migration of cells, for example in tissues that are subject to continuous renewal such as intestine and skin. In addition, front-rear polarity is required in migration processes during development and in wound healing processes. Finally, correctly regulated polarity signaling is crucial for the maintenance of apical-basal polarity of epithelial cells ensuring tissue integrity and altered polarity might contribute to processes involved in developmental or pathologic epithelial-to-mesenchymal transition (EMT) (6). In epithelial cells in particular, evidence is accumulating that loss of cell polarity is a precondition and a hallmark for cancer.
Molecular regulators of cell polarity

All of the above-described forms of cell polarity are thought to be regulated by a few conserved proteins, which have been shown to regulate different modes of polarity in different tissues and organisms. These proteins are clustered in three polarity protein complexes, termed the Par, Crumbs, and Scribble complex.

The Partitioning defective (Par) complex consists of Par3, Par6 and an atypical protein kinase C (Fig. 1A). These proteins have been identified in Caenorhabditis elegans where knockdown resulted in aberrant symmetrical division of the fertilized zygote (7). Interestingly, recently it has become clear that the binding of the Par3 protein to aPKC/Par6 is dynamic and not necessarily required for all polarity processes. aPKC-dependent phosphorylation can expel Par3 from the aPKC/Par6 module (8;9).

The Crumbs complex has been identified in Drosophila melanogaster. This complex consists of Crumbs, Pals1 (Protein associated with Lin seven 1) and Pals1-associated tight junction protein (PATJ) (Fig. 1A). The Par complex is localized apically in epithelial cells, and together with the Crumbs complex regulates apical domain maintenance. These two complexes act in a mutually antagonistic fashion with the third complex, the so-called Scribble complex, which also has been identified in Drosophila (Fig. 1A). The Scribble complex consists of Scribble, Discs large (Dlg) and Lethal giant larvae (Lgl). In epithelial cells this complex is located basolaterally and necessary for maintenance of the basolateral membrane and basal protein restriction (Fig.1A).

After initial identification of the polarity proteins, numerous subsequent studies revealed diverging roles for the proteins of these complexes to polarization of several cell types, conserved in different species (reviewed in (10)). Signaling between these three polarity protein complexes is interconnected via interactions between members of the different complexes (Fig.1B). For example, Lgl1 and 2 can compete with Par3 for binding to a module of Par6 and aPKC. The latter protein can phosphorylate Lgl, thereby releasing it from the Par6/aPKC dimer, resulting in localization of Lgl to the basolateral side of cells. This allows the formation of an active Par complex comprising aPKC/Par6/Par3. This competition of binding has been observed in both Drosophila (polarized neuroblast) and mammalian polarizing migrating cells (11;12). This process of mutual exclusion is crucial for spatial restriction of these proteins required for proper polarization of cells. In addition, aPKC is able to bind both PATJ and Crumbs, the latter being a phosphorylation target (13).

Crumbs has been shown to compete with Par3 for binding to Par6 in a Drosophila epithelial model (8). Furthermore, Par6 has been shown to interact with Pals1 via its PDZ-domain (14). Clearly, the three conserved polarity protein complexes have many potential ways of signaling interplay, which might affect each other’s function (Fig. 1B). The interactions of the proteins and their downstream targets are highly cell type and context dependent.

Cytoskeletal changes are required for all different modes of cell polarity and numerous studies indicate a link between signaling of small GTPases of the Rho family and polarity proteins (reviewed in (10)). Small RhoGTPases, including Cdc42, Rac1 and RhoA, control the cytoskeletal changes in cells by switching between an active GTP-bound state and an inactive GDP-bound state.
Signaling by these proteins is initiated by the activation of cell surface receptors and triggers various processes including gene transcription, differentiation, adhesion and migration, all subject to intensive cytoskeletal reorganization.

Figure 1: Polarity protein complexes. Three conserved polarity protein complexes i.e. the Par, the Crumbs and the Scribble complex, regulate different modes of cell polarity. (A) In contacting epithelial cells the Par complex is located apically in region where the tight junctions (TJs) are present and consists of Par3, Par6 and aPKC. Also apically localized is the Crumbs complex, consisting of Crb3, Pals1 and PATJ. Mutual exclusion of these apical polarity proteins and the basolaterally localized Scribble complex (comprising Scrib, Dlg1, Lgl1/2) confers apical–basal polarity. (B) Interplay between polarity proteins of the different complexes is possible in multiple ways, including competition of binding of Lgl1/2 and Par3 to Par6/aPKC (I), binding of proteins from different complexes (II), phosphorylation of Par3, Lgl1/2 and Crb3 by aPKC (III) and mutual exclusion of the Scribble complex and the apically located Par and Crumbs complex (IV). See also text.
The polarity protein Par3 can bind the Rac-activator Tiam1 linking Rac activation to polarity signaling of the Par complex. Tiam1-mediated Rac activity has been shown to be able to activate the Par complex during epithelial tight junction (TJ) maturation (15). Alternatively, Par6/Par3 has been shown to regulate Cdc42-mediated Rac1 activation through Tiam1 in neuronal cells (16). Both Rac and Cdc42 can activate aPKC by binding to Par6 (17;18). Binding of these small GTPases releases Par6-induced inhibition of aPKC and results in aPKC-mediated downstream signaling (19). Rho-kinase (effector protein of RhoA) on the other hand, can suppress the activity of the Par complex by phosphorylating Par3 (20). These studies indicate that cross-talk between small GTPases and specific polarity proteins during various polarization processes exists and as a consequence that abnormal GTPase signaling could contribute to aberrant polarity signaling. In the following paragraphs we will give an overview of studies investigating the contribution of (aberrant) polarity signaling to the initiation and progression of tumors.

Polarity proteins in cancer

Since cell polarity is crucial for the development of tissue integrity and tissue homeostasis, loss of cell polarity is suggested to result in tissue disorganization and to facilitate both initiation and progression of cancer. Most human cancers are formed from epithelial cells, a cell type in which polarity proteins play crucial roles in maintaining epithelial structures (21). Tumorigenesis is a multistep process in which genetic aberrations may cause initial benign overproliferation, accompanied by inhibition of apoptosis and enhanced survival. The transition of benign cells to fully transformed malignant tumor cells includes altered cell-matrix adhesion, invasion and finally migration to distal parts where tumor cells extravasate and metastasize. Dissemination of epithelial tumor cells is accompanied by EMT, a process initiated by different transcriptional repressors, including Snail and ZEB1 (22). Loss of apical-basal polarity and cell-cell adhesions causes EMT and is associated with metastasis.

Deregulated signaling of each the three polarity protein complexes has been suggested to contribute to all separate steps of human tumorigenesis. In this review we have combined current knowledge on aberrant expression of the polarity proteins as well as functional evidence from different experimental models, including Drosophila and zebrafish. This review concentrates on the three different polarity complexes: The Crumbs, the Par and the Scribble complex.

Crumbs complex proteins and cancer

The Crumbs polarity complex, consisting of Crumbs (Crb1-3 in mammals), Pals1 (Protein associated with Lin seven 1) (Pals1 and 2 and MPP1-4 and -7 in mammals) and Pals1-associated tight junction protein (PATJ) (PATJ and MUPP1 in mammals), was identified in Drosophila epithelial cells and shown to regulate a common pathway essential for induction and maintenance of proper apical-basal
polarity (23-25). This protein complex plays a crucial role in apical domain specification of the plasma membrane. Although evidence is accumulating on the involvement of deregulated polarity signaling to tumorigenesis, little is currently known about the possible contribution of deregulated signaling from the Crumbs complex. However, the Crb3 protein (expressed in epithelial cells) has been shown to suppress progression of mammalian epithelial tumors in functional studies. Analysis of gene expression in mouse kidney epithelium cells (iBMK cells) selected for the ability to induce tumor growth in vivo, revealed inhibited expression of Crb3 in cells with tumorigenic potential. This repression of Crb3 occurs concomitantly with increased expression of vimentin and reduced expression of E-cadherin, two important hallmarks of EMT. In addition, expression negatively correlates with both migratory and metastatic capacity of cells (26). Inhibition of Crb3 expression impairs TJ formation and disrupts organization of epithelium in 3D cultures. Crb3 is suggested to control growth arrest by contact inhibition and maintenance of apical-basal polarity (26). Other indications that disruption of the Crumbs complex makes cells more susceptible to EMT come from studies using mouse mammary epithelial cells. Varelas et al. show that assembly of the Crumbs complex results in phosphorylation of TAZ/YAP, transcriptional regulators controlled by the Hippo pathway, which sense cell density and regulate tissue growth. Phosphorylation of TAZ/YAP upon Crumbs complex formation leads to suppression of TGFβ-SMAD signaling that normally promotes EMT (27). In addition, recently Crumbs has been shown to act as a tumor suppressor in Drosophila imaginal disc epithelium. The same study revealed that Crumbs can bind Expanded, an apical membrane associated protein known to regulate the Hippo pathway (28). Repression of Crumbs can be mediated by the proteins ZEB1 and Snail by direct binding to the protein (29-31). ZEB1 and Snail also regulate the expression of the cell-cell adhesion molecule E-cadherin and thereby control EMT. Both ZEB1 and Snail expression levels are increased in different human cancers, correlating with dedifferentiation and invasion of tumor cells (32-34). These findings link known regulators of EMT to polarity protein signaling through the Crumbs protein (22). Recently it has been shown that Crumbs can antagonize Rac1 in *Drosophila* embryos by inhibiting the positive feedback loop between this small GTPase and PI3K. Rac1 functions in Par complex-mediated signaling, which is also important to maintain epithelial tissue integrity. Disturbance of the balance between Crumbs and Rac1 results in loss of epithelial organization (35). Together, the described data suggest that loss of polarity due to altered Crumbs expression can contribute to tumorigenesis. Crumbs apparently not only has tumor suppressive potential in *Drosophila*, but also in mammalian cells.

Multiple polarity proteins have been identified to be targets of viral oncoproteins (36). The human papilloma virus (HPV) is the primary risk factor for development of cervical cancer. Functional studies revealed that PATJ, another member of the Crumbs complex, is targeted for proteasomal degradation by E6 oncoproteins in cervical cancer-derived cell lines. This might represent a mechanism by which loss of polarity can be induced since PATJ has been shown to be required for proper TJ formation (37;38). In the human epithelial colorectal adenocarcinoma-derived Caco-2 cell line, PATJ is required for stability
of the Crumbs complex, and to localize TJ proteins and organize apical and lateral domains (39). Downregulation of PATJ by viral oncoproteins could thus contribute to the progression of cancer, due to disturbed TJ formation and loss of apical-basal cell polarity.

In mice, tissue specific inactivation of Pals1 suggests a function of the protein in survival signaling, whereas complete loss of Pals1 results in embryonic lethality. Loss of Pals1 in developing mammalian cerebral cortex blocks survival signals, resulting in almost complete absence of the entire cortex. This inhibition of survival signaling is linked to decreased mTor signaling observed in these conditional Pals1 mice. Progenitor cell divisions and cell fate determination are deregulated. In addition, loss of Pals1 disrupts the composition of the Crumbs complex, resulting in mislocalized aPKC, a component of the Par polarity complex, and induction of TJ and polarity defects (40;41). This again illustrates the interplay between the different polarity complexes. To date, there is no direct evidence for altered Pals1 signaling in cancer. The above described studies indicate possible pro-survival signaling mediated by Pals1 which upon alteration of protein expression might influence tumorigenesis. In addition, altered expression of Pals1 could perhaps affect tumor progression through negative effects on epithelial tissue integrity. However, conditional knockout studies combined with mouse models of cancer are required to prove the potential function of the Pals1 protein in tumorigenesis. So far this function has only been suggested in development and homeostasis.

Taken together, studies on the Crumbs complex in relation to cancer reveal that proteins of this polarity complex may act as a tumor suppressor by its effects on TJ formation.

Par complex proteins and cancer

The Par polarity complex consists of Par3 (Par3A and B in mammals), Par6 (Par6A, B and G in mammals) and aPKC (aPKCζ or aPKCλ (mice)/ι (human). For aPKC evidence is accumulating that this kinase is involved in the progression of human cancer (see also (42)). In many human cancers, including human non-small cell lung cancer (NSCLC), aPKCι is overexpressed and correlates with poor prognosis (43). Similarly, in human ovarian cancer overexpression and mislocalization of the protein contributes to poor prognosis. The overexpression of aPKC correlates with overexpression of Cyclin E and loss of apical-basal polarity. Interestingly, comparable results were observed in Drosophila epithelial tissue (imaginal disc) where overexpression of active aPKC also resulted in defects in apical-basal cell polarity, increased Cyclin E expression and enhanced proliferation (44). Although the exact relation between aPKC and Cyclin E expression remains to be determined, it is interesting to note that Cyclin E levels have been linked to multiple human cancers (45). Further evidence for the involvement of aPKC in human cancer comes from studies where overexpression of (cytoplasmic) aPKCι was observed in invasive ductal breast carcinomas, which also correlates with poor prognosis (46). Studies in Drosophila revealed that cortical localization of aPKC results in overgrowth of neuroblasts, through release of the membrane localization of Lgl (47). In addition to the above mentioned observed upregulation of aPKCι expression in NSCLC, mouse studies further stress the importance of aPKC in tumor formation,
Table 1: Aberrant polarity protein signaling in human cancer. Examples of aberrant expression or localization of polarity proteins detected in human cancer.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Alteration</th>
<th>Tumor type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPKC</td>
<td>Overexpression</td>
<td>NSCLC</td>
<td>Regala et al. (43)</td>
</tr>
<tr>
<td>aPKC</td>
<td>Overexpression, mislocalization</td>
<td>Ovarian cancer</td>
<td>Eder et al. (44)</td>
</tr>
<tr>
<td>aPKC</td>
<td>Overexpression (cytoplasmic)</td>
<td>Invasive ductal breast carcinoma</td>
<td>Kojima et al. (46)</td>
</tr>
<tr>
<td>Par6</td>
<td>Overexpression</td>
<td>Breast cancer</td>
<td>Nolan et al. (64)</td>
</tr>
<tr>
<td>Scrib</td>
<td>Downregulation, mislocalization</td>
<td>(Invasive) cervical cancer, Colon adenocarcinoma, Endometrial cancer</td>
<td>Nakagawa et al. (73), Gardiol et al. (74)</td>
</tr>
<tr>
<td>Dlg1</td>
<td>Downregulation, mislocalization</td>
<td>Invasive cervical carcinoma, colon and kidney carcinoma</td>
<td>Ouyang et al. (75), Pearson et al. (76)</td>
</tr>
<tr>
<td>Lgl1</td>
<td>Downregulation</td>
<td>Malignant melanoma</td>
<td>Cavatorta et al. (77)</td>
</tr>
<tr>
<td>Lgl2</td>
<td>Downregulation, Mislocalization</td>
<td>Lung, ovarian, prostate, breast and colon carcinoma</td>
<td>Grifoni et al. (95)</td>
</tr>
<tr>
<td></td>
<td>Gastric dysplasia</td>
<td>Gastric dysplasia</td>
<td>Lisovsky et al. (97,98)</td>
</tr>
<tr>
<td></td>
<td>Colorectal adenoma</td>
<td>Colorectal adenoma</td>
<td>Schimanski et al. (99)</td>
</tr>
</tbody>
</table>

since loss of the protein inhibits the formation of Ras-induced lung tumors (48). While examining the Par3 gene in cell lines derived from human esophageal squamous cell carcinomas homozygous loss of the gene was observed as well as reduced protein expression (49). Total loss of Par3 expression in mice causes embryonic lethality due to epicardial defects (50). During development of the mammalian neocortex both Par3 and Par6 play a role in asymmetric cell division and cell fate specification (51;52). Affecting asymmetrical division could result in increasing numbers of tumor initiating cells and dedifferentiation, according to the cancer stem cell theory (53). TGFβ plays a key role in the dissociation of TJs and downregulates Par3 expression in rat epithelial cells, revealing a mechanism by which TGFβ could mediate loss of apical-basal cell polarity and induction of EMT (54;55). Loss of Par3 expression in epidermal layers of the mouse skin does not result in spontaneous tumors, but instead results in reduced numbers and growth of papillomas in a Ras-driven tumor model (Iden et al. unpublished results), similar to deletion of Tiam1 and Rac1 in this model (56;57). Not only do these observations stress the conjunctural action of these proteins, they also provide evidence for a pro-oncogenic function. From the same study however, evidence for a tumor suppressive function of Par3 became apparent as well. Loss of epidermal Par3 expression results in increased incidence of a Ras-induced cutaneous tumor type, called keratoacanthoma. These tumors, arising from different compartments of the epidermis, display reduced Par3 expression, indicating a tumor suppressive function of Par3 in this tumor type. In these tumor-promoting and suppressing functions of Par3, the protein likely serves to localize aPKC to cell-cell contacts (Iden et al. unpublished results).

The resemblance between the effect on papilloma formation in Par3-deficient, Rac-deficient and Tiam1-deficient mice indicates that Par3 acts at the level of Tiam1-Rac signaling. Additional evidence for conjunctural activity of these proteins comes from results on the regulation of polarizing keratinocytes, where Par3 and Tiam1 act in conjunction to regulate polarity of both single persistently migrating keratinocytes and apical-basal polarized contacting keratinocytes (15;58;59). PI3kinase (PI3K) might be an important candidate to connect signaling from Ras and Par3. Par3 can interact with PI3K as well as PTEN and phosphoinositides as has been shown in axonal studies and membrane polarity studies (60;61). Further evidence for a pivotal role for PI3K in integration of the signaling from Ras and polarity proteins
Chapter 3

comes from *Drosophila* screening studies. Loss of PI3K (or Akt) in cells expressing RasV12 as well as interfering RNAs for Dlg blocks cell cycle progression in a tumor model, whereas the effect on normal tissue is only minor (62). Ras has previously been shown to exert its effects on proliferation, survival and polarity by affecting Raf/MEK/ERK, PI3K/Akt and Tiam1/Rac- signaling pathways (63).

Par6 has been shown to play a promoting role in tumor initiation and progression through induction of proliferation in cultured human mammary epithelial cells. This function involves conjunctival signaling between Par6, Cdc42 and αPKC, but not Par3. Furthermore, overexpression of Par6 is found in human breast cancer where it is suggested to be important for tumor initiation and progression (64). Functional in vitro studies indicate that Par6 could contribute to EMT, since it interacts with the TGFβ receptor. For the TGFβ-induced dissolution of TJs Par6 has to be phosphorylated and preventing this event maintains stable TJs upon stimulation with TGFβ (65). More recently, it has been suggested that this interaction contributes to breast cancer progression based on results from breast cancer models in mice. In mammary fat pad assays blocking the TGFβ-dependent phosphorylation of Par6 reduces metastasis to the lungs and highlights the importance of loss of polarity signaling for EMT and metastasis (66). Interestingly, increased Par6 expression is detected in stromal tissue derived from NSCLC and correlates to improved prognosis (67). This observation stresses the importance of thorough investigation of the influence of the tumor microenvironment (TME) that comprises immune cells, stromal cells and extracellular factors. The TME likely contributes to the ability of transforming cells to acquire/accumulate all typical features of cancer (68-70).

Together, studies on the Par polarity complex indicate that the proteins can serve both tumor suppressive (Par3) as well as oncogenic (αPKC, Par3, Par6) functions in various human cancers. Aberrant signaling is suggested to intervene at the level of tumor initiation as well as tumor progression.

**Scribble complex proteins and cancer**

Already over 30 years ago, both Lgl (Lgl1 and 2 in mammals) and Dlg (Dlg1-4 in mammals and hDlg5 in human) have been described as tumor suppressor proteins in *Drosophila* (71). Later also Scribble (Scrib) was identified as a fly tumor suppressor and shown to act in a common pathway with Dlg and Lgl to regulate cell growth and polarity. These three proteins are therefore referred to as ‘the Scribble complex’ (72). Currently, concerning the role of polarity proteins in initiation and development of human tumors, most is known about the proteins of the Scribble complex.

The Scribble protein was found both mislocalized and downregulated in spontaneous tumors in human and mice. In humans, mislocalized Scribble is observed in cervical cancer, colon adenocarcinoma, endometrial and prostate cancer (73-76). Both Scribble and Dlg1 have been associated with cervical cancer in which reduced protein expression is observed. Moreover, expression of both proteins is specifically decreased in invasive cervical carcinoma (73;77). Targeted degradation by oncoproteins from HPV is suggested to be associated with the decreased
Chapter 3

Table 2: Tumor promoting or suppressive function of different polarity proteins in various tumor model systems. Examples of the evidence for tumor promoting or suppressing roles of the various polarity proteins in cancer based on functional studies in various tumor model systems. Note that proteins of the Par complex may act both as tumor promoter and tumor suppressor. For details see text.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tumor promoting or suppressing function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crumbs</td>
<td>Dros. imaginal disc epithelium</td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>Crb3</td>
<td>Mouse kidney epithelial cells</td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>Mouse mammary epithelial cells</td>
<td>Tumor suppressor</td>
<td>Varelas et al. (27)</td>
</tr>
<tr>
<td>aPKC</td>
<td>Dros. imaginal disc epithelium</td>
<td>Tumor promoter</td>
</tr>
<tr>
<td>Dros. neuroblast</td>
<td>Tumor promoter</td>
<td>Grigioni et al. (47)</td>
</tr>
<tr>
<td>Mouse lung tumor formation</td>
<td>Tumor promoter</td>
<td>Regalia et al. (46)</td>
</tr>
<tr>
<td>Mouse skin papilloma formation</td>
<td>Tumor promoter</td>
<td>Iden et al. (unpubl.)</td>
</tr>
<tr>
<td>Mouse skin keratoacanthoma formation</td>
<td>Tumor suppressor</td>
<td>Iden et al. (unpubl.)</td>
</tr>
<tr>
<td>Par6</td>
<td>Human esophageal squamous cell carcinoma cells</td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>Mouse mammary epithelial cells</td>
<td>Tumor suppressor</td>
<td>Vitoria-Peitto et al. (66)</td>
</tr>
<tr>
<td>Scribble</td>
<td>Tumor suppressor</td>
<td>Nolan et al. (64)</td>
</tr>
<tr>
<td>Mouse mammary fat pad</td>
<td>Tumor suppressor</td>
<td>Zhan et al. (86)</td>
</tr>
<tr>
<td>Mouse prostate tumor formation</td>
<td>Tumor suppressor</td>
<td>Pearson et al. (76)</td>
</tr>
<tr>
<td>Human epithelial cells</td>
<td>Tumor suppressor</td>
<td>Dow et al. (89)</td>
</tr>
<tr>
<td>Dlg</td>
<td>Dros. multiple tissues</td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>Lgl1</td>
<td>Knockout mouse</td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>Lgl2</td>
<td>Zebral fish epidermis</td>
<td>Tumor suppressor</td>
</tr>
</tbody>
</table>

Targets of viral oncoproteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tumor promoting or suppressing function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PATJ</td>
<td>Human cervical cancer cells</td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>Scribble</td>
<td>MDCK cells</td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>Baby rat epithelial cells</td>
<td>Tumor suppressor</td>
<td>Thomas et al. (80)</td>
</tr>
<tr>
<td>Dlg1</td>
<td>Human 293 cells</td>
<td>Tumor suppressor</td>
</tr>
</tbody>
</table>

levels of these tumor suppressors and consequent poor prognosis (78;79). In MDCK cells and primary epithelial cells HPV-derived E6 oncoproteins promote ubiquitin-mediated degradation of Scribble (80). Expression levels of both Scribble and Dlg1 are correlated with loss of tissue architecture in the colon (74). In the human colon-derived epithelial cell line SK-CO15 downregulation of Scribble inhibits TJ reassembly, independently of Lgl1 and Dlg1 (81).

It is interesting to note that viral oncoproteins target multiple proteins of one polarity complex that perform complementary functions, indicating their importance in maintenance of cell polarity. For example, the oncoprotein Tax, from the human T-cell leukemia virus type 1 (HTLV-1) can interact with both Scribble and Dlg1 affecting their localization, activity and function (82-85). In addition, Dlg1 localization is altered in colon and kidney carcinomas that are not associated with HPV (77). Additional evidence that polarity proteins from the Scribble complex function as tumor suppressors in tumorigenesis comes from functional studies, using tumor models with total or tissue-specific gene alteration. Mouse mammary fat pad transplantation assays suggest that Scribble acts as a tumor suppressor in mammalian epithelial cells as well (86).

Dysfunction or mal-expression of polarity proteins alone is not always sufficient to initiate tumor formation and/or progression in human pathology. Although mutations in Ras proteins are common (~30%) in human cancer, hardly ever is a mutation in this pathway sufficient to result
in tumor development (87;88). In human epithelial cells that express oncogenic Ras or Raf, loss of Scribble stimulates invasion of cells through the extracellular matrix. Scribble suppresses MAPK signaling in this system, which could be a mechanism involved in the above mentioned studies on invasive human cancers in which decreased Scribble expression was observed (89). Recently it was shown that targeted prostate-specific bi-allelic loss of Scribble predisposes mice to prostate intraepithelial neoplasia, whereas heterozygous loss induces prostate hyperplasia. Signaling of the MAPK-pathway is elevated in these neoplasias, confirming the suppressive capacity of Scribble on MAPK-signaling. Combining loss of Scribble with expression of oncogenic Ras in mouse prostate results in disease progression, and epithelial neoplasia are promoted (76). Interestingly, loss of Scribble has also been shown to cooperate with the c-myc oncogene and by blocking apoptosis epithelial cells can be transformed and mammary tumor formation is induced (86). Furthermore, screening studies in Drosophila revealed that mutations in Scribble result in impaired growth. However, combining mutations in Scribble with overexpression of oncogenic Ras results in loss of apical-basal polarity, neoplastic overgrowth and invasive and metastatic capacity (90;91). In Drosophila Lgl was found to require myc to stimulate clonal malignancy (92).

Interestingly, the two genetic aberrations not necessarily have to be in one and the same cell. Synergy can also occur in a paracrine manner when the two mutations are individually present in neighboring cells (93). Cytokines derived from Scribble mutant cells activate the JAK/STAT pathway in RasV12-expressing neighboring cells (93). These observations illustrate that cancer is a heterogeneous disease, with differences between tumors as well as between cells within a tumor. Furthermore, these observations stress the importance of the tumor microenvironment. Apparently, polarity proteins also collaborate with the tumor microenvironment to promote tumor progression. Lgl1 expression is significantly decreased in human malignant melanoma and loss of its expression correlates with progression (94). In addition, reduced expression of Lgl1 is detected in solid tumors from different origins, including lung, ovarian, prostate, breast and colon carcinoma (95). Mice lacking Lgl1 expression are born with hydrocephalus and die neonatally. Lgl1-deficiency results in failure of asymmetric cell divisions and severe brain dysplasias that contain structures resembling those observed in human primitive neuroectodermal tumors (96).

In human tissue, Lgl2 protein expression is lost and mislocalized in gastric dysplasia and adenocarcinoma (97;98). In colorectal cancer, reduced expression of human Lgl2 has been shown to contribute to the progression of these tumors. Moreover, loss of Lgl2 expression is linked to advanced stage and lymph node metastasis (99). Studies in zebrafish contribute to reveal the mechanisms underlying human cancer, where for example Lgl2 has been found to perform tumor suppressive functions (100). Lgl2-mutant cells display neoplasia and EMT, two important characteristics of human pathology.

In conclusion, there is increasing evidence that Scribble proteins which were originally identified as fly tumor suppressor genes serve a suppressive role in mammalian tumorigenesis as well. Studies analyzing expression and localization of the Scribble complex
proteins in human tumors suggest that both downregulation and mislocalization play a role during tumor initiation and progression.

CONCLUSIONS

Taken together, evidence is accumulating that signaling via and (de)regulation of proteins of all three conserved polarity complexes in various ways contribute to different steps of (epithelial) tumor formation and progression. For the Crumbs complex tumor suppressive potential is likely in mammalian cells, although poorly understood. Proteins of the Par complex are suggested to have both tumor suppressive potential (shown for Par3 and Par6) as well as tumor promoting functions (aPKC, Par3 and Par6). So far the best studied complex in human tumorigenesis is the Scribble complex. Similar to their original identification in flies, they serve tumor suppressive functions in mammalian cells. For all polarity protein complexes tightly regulated localization appears crucial for proper functioning in normal tissue. The ample possibilities of interplay between the three polarity complexes provide additional possibilities for involvement in cancer. Extending our understanding of the molecular pathways and interactions underlying polarity processes will help to exploit the possibilities of therapeutic intervention of polarity signaling for cancer diagnosis and treatment.

ACKNOWLEDGEMENTS

This work has been supported by grants from the Dutch Cancer Society and the EC (TuMIC) awarded to J.G. Collard. SI is currently supported by funds of CECAD Cologne and the German Research Foundation (CRC829, CRC832).

REFERENCES


71. Gateff, E. 1978. Malignant neoplasms
Chapter 3


Chapter 3

signaling in the zebrafish epidermis.
PLoS. Genet. 5:e1000720.
Chapter 4

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

Amra Hajdo-Milasinovic, Saskia I.J. Ellenbroek, Saskia van Es, Babet van der Vaart and John G. Collard

ABSTRACT

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 integrinmediated cell-matrix adhesions. Together, our data reveal that Rac3 opposes Rac1 in the regulation of cell adhesion and differentiation of neuronal cells.

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 brainenriched 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 suppresses 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 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 N1E115 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 N1E115 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 demonstrate the specificity of the short hairpin RNA (shRNA) constructs, cells were firstly transfected with shRac1-1, shRac12, 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 N1E115 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 Rac1G12V, 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 (supplemental figure 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). RTPCR 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.
Figure 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 Rac3 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 x 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).
Since shRac3-induced neurite-like outgrowths are similar in length and morphology to those of fully differentiated N1E115 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 shRac3expressing 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. 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 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 Rac3induced 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.
Chapter 4

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, 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.

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<sup>G14V</sup> 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.
Figure 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 cotransfected 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).
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

Figure 5: Rac3 phenocopies RhoA in N1E-115 cells. (A) Cells were transfected with pcDNA3.1/Rac3, pcDNA3.1/RhoAG14v, 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.
Figure 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.
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 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<sup>G14V</sup>-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; (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. 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 Rap\(^{G12V}\) cells. 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 supplemental figure S2). However, when using wildtype 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 tripletts; 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 1.5x10^4 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 NH2-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 NH2-terminally tagged with Myc and HA sequence, respectively, were obtained from UMR cDNA Resource Center, University of Missouri-Rolla, MO. Rac3 was NH2-terminally tagged with FLAG sequence and cloned into pcDNA3.1, as were constitutively active mutants Rac1^{G12V} (Leeuwen et al., 1997) and Rac3^{G12V}. NH2-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^{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
Chapter 4

**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 BglII/EcoRI site of pSuper vector (Brummelkamp et al., 2002). Primer sequences were as follows:

**Rac1-1 shRNA sense primer**
5’- gatccccTTTGCTTTTCCCTTGTGAGttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaTTTGCTTTTCCCTTGTGAGtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**Rac1-1 shRNA antisense primer**
5’- gatccccGAGAAAATGCCTGCTGTTGttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaGAGAAAATGCCTGCTGTTGtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**Rac1-2 shRNA sense primer**
5’- gatccccGTGGTACCCAGAGGTGCGGttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaGTGGTACCCAGAGGTGCGGtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**Rac1-2 shRNA antisense primer**
5’- gatccccGACAGTGTTCGACGAGGCCttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaGACAGTGTTCGACGAGGCCtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**Rac3-1 shRNA sense primer**
5’- gatccccGTGGTACCCAGAGGTGCGGttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaGTGGTACCCAGAGGTGCGGtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**Rac3-1 shRNA antisense primer**
5’- gatccccGACAGTGTTCGACGAGGCCttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaGACAGTGTTCGACGAGGCCtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**Rac3-2 shRNA sense primer**
5’- gatccccGTGGTACCCAGAGGTGCGGttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaGTGGTACCCAGAGGTGCGGtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**Rac3-2 shRNA antisense primer**
5’- gatccccGACAGTGTTCGACGAGGCCttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaGACAGTGTTCGACGAGGCCtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**RhoA-1 shRNA sense primer**
5’- gatccccTGGACTCCAGAAGTCAAGCttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaTGGACTCCAGAAGTCAAGCtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**RhoA-1 shRNA antisense primer**
5’- gatccccGCAGGTAGAGTTGGCTTTGttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaGCAGGTAGAGTTGGCTTTGtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**RhoA-2 shRNA sense primer**
5’- gatccccGCAGGTAGAGTTGGCTTTGttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaGCAGGTAGAGTTGGCTTTGtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**RhoA-2 shRNA antisense primer**
5’- gatccccGCAGGTAGAGTTGGCTTTGttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaGCAGGTAGAGTTGGCTTTGtcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**luciferase shRNA sense primer**
5’- gatccccCGTACGCGGAATACTTCGAttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaaCGTACGCGGAATACTTCGAATcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’;

**luciferase shRNA antisense primer**
5’- gatccccCGTACGCGGAATACTTCGA ttcaagagatcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAtttttggaaa3’;
5’- agcttttccaaaaa CGTACGCGGAATACTTCGA ATcttcgtggagaaCTCAACAGCGGCAAAAAGGAAAaggg3’.

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 7.5x10^5. After 24 hours, RNA was isolated using RNeasy (Qiagen) 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:

- **Rac1** (forward, 5’-ctgaaggagaagaagctgac-3’; and reverse, 5’-tcgtc-aaacactgtcttgag-3’),
- **Rac3** (forward, 5’-gacgacaaggacaccatgta-3’; and reverse, 5’-cctcgtc-aaacactgtcttc-3’),
- **glyceraldehyde-3-phosphate dehydrogenase** (forward, 5’-accaca-gtcatcctgtgac-3’; and reverse, 5’-tcaccacactggaggtc-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); antiMAP2, 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-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 containing 1% Nonidet P40 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.
ACKNOWLEDGEMENTS

We thank Adrienne Cox for providing the Rac3-specific antibody, colleagues from the Division of Cell Biology for stimulating discussions and I. Hamelers, E. Danen and T. Rygiel for critical reading of the manuscript. This work is supported by grants from the Dutch Cancer Society to J.G.C.

REFERENCES


SUPPLEMENTAL DATA

Supplemental figure S1: Expression of Rac3 does not affect the activation of endogenous Rac1. (A) Rac activity assay was performed in ev (pcDNA3.1) or Rac3 expressing cells and the GTP-bound active Rac samples (middle panel) were compared to total lysates of the same transfected cells (upper panel).

Supplemental figure S2: Constitutively active Rac1 and Rac3 mutants induce similar morphological changes, in contrast to their wild-type counterparts. Cells were transfected with Rac1G12V and Rac3G12V (together with 5:1 eGFP). After 24 hours, the cells were fixed and stained with phalloidin to visualize filamentous actin. Note that both constitutively active mutants induce a similar morphology including cell spreading; note also the formation of peripheral ruffles at the lamellipodia.
The Par-Tiam1 complex controls persistent migration by stabilizing microtubule-dependent front-rear polarity

D. Michiel Pegtel, Saskia I.J. Ellenbroek, Alexander E.E. Mertens, Rob A. van der Kammen, Johan de Rooij and John G. Collard

Current Biology (2007) 17:1623-34
ABSTRACT

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

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

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

INTRODUCTION

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

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

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

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

**RESULTS**

**Tiam1 is required for persistent but not random migration.**

To study motility behavior of freely migrating epidermal keratinocytes we sparsely seeded epidermal keratinocytes on exogenous Laminin5 (LN5) substrate in low calcium (0.02 mM) conditions. These cells acquire a distinct front-rear polarized morphology while cells lacking the Rac-activator Tiam1 (Tiam1-KO) are hardly polarized (Figure 1A and S1A in the Supplemental Data). Video microscopy shows that wild-type (WT) keratinocytes migrate in a persistent (i.e. linear) fashion. Tiam1 KO keratinocytes migrate equally well, albeit their migration pattern appears more erratic, with cells often changing direction (movies S1 and S2).

Comparing composed migratory paths of WT and Tiam1 KO keratinocytes confirms this finding since the migration tracks of Tiam1 KO cells are condensed (Figure 1B). The average D/T (direct distance/total distance) ratio, or persistence of WT cells on LN5 was 0.8 compared to 0.5 for Tiam1 KO cells (p < 0.025, Figure 1C). The combined total distance for WT and Tiam1 KO cells was similar (not shown) suggesting similar average cell velocities.

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

To visualize overall cell motility we constructed velocity histograms of freely migrating Tiam1 KO and WT cell populations by plotting cell velocity against the number of cells (Figure S2A). In both genotypes over 95% of the cells migrate with (average) velocities between 0.2 and 4.0 µm/min and no obvious subpopulations exist for either genotype. WT keratinocytes however migrate on average slightly faster than Tiam1 KO cells (1.0 µm/min vs. 0.72 µm/min on LN5.

Figure 1: Tiam1 deficiency impairs directionally persistent migration. (A) Phase contrast images of typical WT and Tiam1 KO freely migrating keratinocytes on a collagen IV substrate. Many WT cells are intrinsically polarized, showing a distinct front-rear cellular asymmetry, while Tiam1 deficient cells are irregularly shaped with variable number of protrusions. (B) Representative individual migration tracks of WT and Tiam1 KO keratinocytes migrating on LN5 substrate. The total track distance of all individual cells combined was similar for both cell types. (C) Average persistence (D/T ratio) derived from the tracks depicted in (B). The shaded area indicates persistence (D/T) value for random migration (RM) as determined by simulation (see Supplemental Experimental Procedures) and a persistence of 1 indicates completely linear migration (LM). (D) Quantification of average persistence of migrating WT and Tiam1 KO cell populations seeded on a collagen IV substrate measured from multiple (n=3) independent movies. (E) Average cell motility measured by average cell velocity of WT and Tiam1 KO cell populations on LN5 and collagen IV substrates. Most WT and Tiam1 KO keratinocytes in freely migrating populations migrate with velocities between 0 and 2µm/min (Figure S2A). Error-bars indicate standard deviations and asterisks indicate significant differences in a Student’s t test (p < 0.05 was considered significant) for persistence and/or velocity compared to WT. The scale bar represents 10µm.
and 0.72 vs. 0.62 µm/min on collagen IV (p < 0.05) (Figure 1E). However, Tiam1 appears not essential for determining cell velocity since cells of both genotypes share the same range of individual cell velocities. It is conceivable that more frequent changes in direction of Tiam1 KO cells reduce average cell velocity. These experiments indicate that Tiam1 controls migratory persistence independent of the cell substrate but does not control random migration since it is dispensable for propelling keratinocyte migration.

**Tiam1-deficient keratinocytes are impaired in chemotactic migration**

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

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

Interestingly, GF-starved WT and Tiam1 KO keratinocytes equally induced P42/44 MAPK phosphorylation upon EGF stimulation (Figure 2C). Also keratinocytes migrated well to a gradient of EGF alone (Figure S2C) suggesting that impaired chemotaxis of Tiam1 KO cells is not due to a defect in MAPK-dependent GF signaling. To validate that lack of Tiam1 expression was causal for impaired chemotactic migration, we re-expressed full-length Tiam1 protein in Tiam1 KO cells (Figure 2D, 3rd lane). Re-expression of Tiam1 restored the defect in chemotactic migration (Figure 2E, 3rd bar), while expression of a GFP-vector control had no effect (Figure 2E, 4th bar), confirming that Tiam1 expression is required for chemotactic migration of epidermal keratinocytes.

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

**Persistent and chemotactic migration is regulated by the Par/Tiam1 polarity complex**

Tiam1 deficiency impairs directional migration, but not random migration even though Rac activity is reduced in migrating...
keratinocytes lacking Tiam1 (Figure S3D). Expression of RacV12 in Tiam1 KO cells did not rescue polarization but led to increased spread ‘pan-cake’ like cells that do not migrate (Figure S3C). Nevertheless, since Tiam1 KO cells are motile cells, it is unlikely that Tiam1 controls keratinocyte migration by activating the Rac-dependent migration machinery [6].

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

To investigate whether Par3 controls persistence we depleted endogenous Par3 using siRNA to approximately 30% in WT and Tiam1 KO keratinocytes (Figure 3B). Persistence was reduced in freely migrating Par3-depleted WT keratinocytes (D/T ratio WT 0.68 vs. WT siPar3 0.55, p<0.001) even though Par3 depletion was not complete in all cells. In contrast, Par3 depletion did not further reduce persistence of Tiam1 KO cells, suggesting that the function of Par3 and Tiam1 overlap in the regulation of directional persistence (Figure 3C). Consistent with this finding, depletion of either Par3 or Tiam1 by siRNA reduced chemotactic migration towards growth factors 2-3 fold compared to control (siLuc) cells (Figures 3D and E), indicating these proteins

Figure 3: The Par complex controls persistence and chemotactic migration. (A) FL-Tiam1 co-immunoprecipitates endogenous Par3 (upper band in top panel) and phospho-PKCζ from a population of freely migrating Tiam1 KO cells that express HA–tagged full-length Tiam1. Untransfected Tiam1 KO cells were used as a control. Total lysates show equal loading of Par3 and PKCζ proteins. (B) Immunoblots of total lysates from WT and Tiam1 KO keratinocytes with and without Par3 knockdown by retroviral siRNA. Visible are the 180 and 100 kDa isoforms. Total Rac was used as a loading control. (C) Persistence (D/T ratio) of WT and Tiam1 KO keratinocytes measured in sparse cultures on collagen IV, with or without reduced Par3 expression. (D) Chemotactic (Boyden-chamber) migration assay towards GFs. Representative images of the bottom membranes containing migrated WT, (si)Par3, (si)Luc control and (si)Tiam1-depleted WT keratinocytes are shown. (E) Quantification of experiment shown in D. (F) Results of chemotactic migration assays of WT cells, WT cells expressing a kinase-dead mutant of PKCζ and Tiam1 KO cells with and without PKCζ inhibitor. Asterisks indicate significant (p < 0.05) differences between WT (control) cells in a Student’s t test and error bars represent standard deviations.
also functionally overlap in regulating chemotaxis. As expected, depletion of Tiam1 or Par3 by siRNA did not reduce persistence nor chemotaxis completely to the level observed in Tiam1 KO cells (Figure 3C-E) because downregulation of these proteins was not 100 percent and varied between cells.

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

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

Wound migration on exogenous substrate

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

The Par/Tiam1 complex is asymmetrically enriched in front-rear polarized keratinocytes

To investigate whether the Par-Tiam1 complex controls front-rear polarization of keratinocytes, we quantified polarized morphology in freely migrating WT and Tiam1 KO cell populations. At any given moment ~30% of WT keratinocytes in a population (n > 60) are front-rear polarized, whereas less than 5% of Tiam1 KO cells or (si)Par3 depleted cells
Chapter 5

Figure 4: The Par/Tiam1 complex controls front-rear polarity in persistent migration.

(A) A Schematic representation of a hole-migration assay is on the right. A monolayer of keratinocytes, grown under low-calcium conditions, is formed around an inert rod (black). Removal of the rod leaves a substrate-covered circular space devoid of cells (dotted line). Subsequently, keratinocytes at the border are able to migrate into the hole. Pictures represent overlay images of a hole-migration assay with cell-tracker dye-stained WT keratinocytes (red) or KO keratinocytes (green) 0 hours and 24 hours after removal of the rod.

(B) Image of a hole-migration assay after 24hr with a mixed culture of (1:1) WT (red) and Tiam1 KO (green) cells.

(C) Images showing polarized migrating WT cells (red) into the open space (asterisks). Tiam1-KO cells are not stained. These experiments were performed on both LN5 and collagen IV substrates and results were comparable.

(D) Images and quantification (bar diagram) showing estimation of the percentage of front-rear polarized keratinocytes in large populations (n>100). Represented are control (si) Luc WT keratinocytes, (si)Par3-depleted WT keratinocytes, WT keratinocytes treated with PKCζ inhibitor (INH) and Tiam1 KO cells.

(E) Confocal images of endogenous Par3 and PKCζ in migrating keratinocytes. White arrowheads indicate enrichment at the leading edge of protruding lamellae, whereas red arrowheads indicate lack of enrichment. White arrows indicate the direction of migration. The bar diagram shows the percentage of cells with Par3 enrichment at leading edges in polarized vs. unpolarized cells. The top two right small images show endogenous Par3 and PKCζ co-localization at leading edge. (F) Tiam1-GFP localization to the leading edge in WT keratinocytes (white arrowhead) but not in (si)Par3 depleted cells (red arrowhead).

(G) Colocalization (yellow/orange) of Tiam1-GFP with endogenous Par3 and/or dynamic F-actin visualized by phalloidin staining. The scale bar represents ~10 μm. Arrows indicate direction of migration.
are morphologically polarized (Figure 4D). Accordingly, WT cells incubated with the PKCζ inhibitor show similar low percentage in front-rear polarization.

Par3 is able to associate with Tiam1 (Figure 3A) and function together in apical-basal polarity [17,22] and persistent migration (this study). To gain further support for a regulatory role of Par/Tiam1 signaling in front-rear polarization, we performed intracellular localization studies for Par polarity proteins and Tiam1-GFP by using confocal imaging. Endogenous Par3 protein was consistently enriched at the leading edge of migrating keratinocytes (Figure 4E, white arrow heads). Par3 was not enriched at leading edges of non-polarized WT and Tiam1 KO cells (Red arrowheads) (50% vs. 10%, p < 0.05) (Figure 4E). Endogenous PKCζ had an asymmetric enrichment at leading edges of polarized cells analogous to, albeit less consistent than, that of Par3. In addition, co-localization with Par3 was detected in WT cells (Figure 4E small images). Endogenous Tiam1 was difficult to visualize by antibody staining; therefore, we expressed GFP-tagged Tiam1 in keratinocytes that localized to the leading edge (Figure 4F). We were unable to detect Tiam1-GFP at the leading edges of siPar3 keratinocytes (Figure 4F) suggesting that lowering the levels of endogenous Par3 prevents efficient Tiam1 localization to this area. Dynamic F-actin is highly enriched at the leading edge of migrating epithelial cells [26], including front-rear polarized WT and Tiam1 KO keratinocytes (Figure S2F). We observed the co-localization of Tiam1-GFP with F-actin and Par3 in the leading edge of polarized cells (Figure 4G). A GFP-control vector showed aspecific localization (Figure S2F). We propose that enrichment at the leading edge of Par3, Tiam1 and PKCζ proteins support a functional role for these proteins in cellular asymmetry, as observed in front-rear polarized keratinocytes.

The Par/Tiam1 complex controls stability of front-rear polarity

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

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

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

Together these studies indicate that the MT network is essential for persistent but not random migration of epidermal cells. Figure 5: Impaired Par-Tiam1 signaling affects duration of front-rear polarity and microtubule stability. (A) Consecutive time-lapse images of migrating WT, (si)Par3-depleted, Tiam1 KO, and nocodazole incubated (1 μM) keratinocytes at 40 minutes intervals. WT cells (n>20) remain front-rear polarized for more than 120 minutes in one direction (white arrows). An estimated 60% of the WT cells are constitutively polarized longer than 120 minutes compared to 15% for siPar3, Tiam1-deficient and nocodazole treated cells (bar diagram). (B) Microtubule (MT) organization in polarized WT and Tiam1 KO cells is similar (visualized by confocal imaging with α-tubulin monoclonal antibody). Western blotting with an antibody specific for stable (acetylated) MTs shows that populations of migrating WT cells contain much more stable MTs than do (si)Par3-depleted and Tiam1-deficient keratinocytes. Rac is a loading control. (C) Confocal Images of WT and Tiam1 KO cells incubated with α-tubulin antibody after 5 min. of nocodazole (1μM) treatment. Undisrupted MT filaments are visible (blow up) in the peripheral lamellae of ~70% of WT cells compared to ~30% of Tiam1 KO cells after nocodazole treatment (bar diagram). Treatment of WT cells with the specific PKCζ inhibitor (lower panel) impairs front-rear polarity and disrupts MTs similar to nocodazole treatment. (D) Average persistence of migrating WT cells (filled) bars and Tiam1 KO cells (open bars) treated with 2.5 and 1.25 μM nocodazole compared to untreated cells on collagen IV substrate. Asterisks indicate significant differences (p<0.05) compared to WT in a Student's t test, and error bars indicate standard deviations. The scale bar represents 10 μm. Arrows indicate direction of migration.
Chapter 5

DISCUSSION

Our results indicate that the Par polarity complex and Tiam1 control persistent migration of epidermal keratinocytes by affecting the stability of MTs. Impaired Par and/or Tiam1 signaling reduces the stability of front-rear polarization, thereby decreasing persistence of the migrating cell. Immunoprecipitation and

keratinocytes. These experiments suggest that the Par-Tiam1 complex controls the stability of front-rear keratinocyte polarity by acting on the MT network.

Par/Tiam1 controls microtubule stability

We analyzed whether the MT network is specifically oriented towards the direction of migration in persistently migrating polarized keratinocytes. We initially incubated keratinocytes with a specific centrosome antibody pericentrin, but did not discover any correlation between orientation of the centrosome and the nucleus, in persistently versus randomly migrating cells (data not shown). To investigate whether the Par-Tiam1 complex affects the MT network itself, we compared WT with Tiam1 KO cells for potential differences in length or abundance of MT filaments by immunofluorescence staining for α-tubulin but did not detect significant differences under standard conditions (Figure 5B). However we found by western blotting that WT keratinocytes contain significantly more stable, acetylated MTs compared to Tiam1 KO cells and siPar3 keratinocytes (Figure 5B), suggesting that Tiam1 and Par3 are associated with the presence of stabilized MTs in keratinocytes.

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

To demonstrate that the decrease of MT stability in Tiam1 KO cells affects persistence, we analyzed persistent migration of nocodazole-treated WT and Tiam1-deficent keratinocytes. Treatment of WT cells with 1.25 µM nocodazole diminished directional persistence substantially (D/T ratio of 0.54 +/- SD 0.02 vs. 0.72 +/- SD 0.014) comparable to the level of untreated Tiam1 KO cells (D/T ratio WT 0.54 vs KO 0.55, p = 0.7). This finding suggests that MT stability is reduced in Tiam1 KO cells thereby reducing migratory persistence. In contrast, treatment of 1.25 µM nocodazole has a much smaller effect on persistence in Tiam1 KO cells (Figure 5D). Collectively, these experiments indicate that Tiam1, Par3 and downstream PKCζ signaling play a role in the stability of MT filaments of migrating keratinocytes thereby affecting persistent migration.
immunolocalization studies indicate that Tiam1, Par3 and PKCζ function together in maintaining cellular asymmetry. Indeed, Tiam1 deficiency, Par3 depletion by siRNA and the chemical blocking of PKCζ signaling, impair the stability of front-rear polarization and reduce persistence and chemotactic migration. Depletion of Par3 and/or the blocking of PKCζ signaling has no additional effect on Tiam1-deficient cells, supporting the idea that these proteins function together. Lastly, we show that Par3 depletion and Tiam1 deficiency reduce MT stability measured by acetylation. Conversely, pharmacologically disturbance of MTs impairs front-rear polarization and reduces persistence.

Our data thus indicate a functional role of the evolutionary conserved Par complex in persistent migration by controlling front-rear polarization of epithelial cells. While Par polarity signaling in mammalian cells is best characterized in controlling the protrusion outgrowth of astrocytes or axon outgrowth of neuronal cells [23,24] an active role in actual cell migration has not yet been demonstrated. In fact, Par polarity signaling in epithelial cells is perhaps better known for its role in the establishment of lasting apical-basal cell polarity, thereby contributing to tissue integrity [13,22]. Loss of Par3 [22], Par6 [29] or overexpression of Par3 [30] leads to the dissolution of cell-cell junctions, enabling cell migration but not propelling migration per se. The Par polarity complex thus regulates two distinct cell polarity processes in keratinocytes, the first being permanent cell polarity processes, characterized by apical-basal polarity and second being transient cell polarity, characterized by front-rear polarization during cell migration. The most concrete evidence thus far that Par polarity proteins may actively function in any form of migration in epithelial cells, comes from experiments in the Drosophila ovary, where the depletion of Par-6, Par-3/Bazooka impairs the migration of border cells [31]. Because the Par proteins remained asymmetrically localized, the authors concluded that apical-basal polarity is retained during border cell migration. An alternative explanation, based on our results, is that the asymmetrical distribution of Par proteins reflects (re-)localization to the leading edge of front-rear polarized cells, thereby controlling persistent border cell migration towards chemotactic cues of in the developing Drosophila ovary [32].

The depletion of Par3 by siRNA and the blocking of PKCζ signaling with a specific inhibitor reduces persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration. The lack of the Rac-activator Tiam1 has identical effects on persistent and chemotactic migration.

### Table 1. Summary of Effects of Modulators of Cell polarity

<table>
<thead>
<tr>
<th>role in Front-Rear</th>
<th>role in Chemotactic Migration</th>
<th>role in Persistent Migration</th>
<th>role in Random Migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Par3</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Tiam1</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PKCζ</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PI3K</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>ROCK</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Racodazole</td>
<td>Yes</td>
<td>nd</td>
<td>Yes</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>-</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*This table shows an overview of the effects of each modulator and gene manipulation on migration characteristics and front-rear polarity. *--- indicates not determined. *--- indicates that cells do not migrate at all.*
To demonstrate that not all migratory pathways function via front-rear polarization mechanisms, we blocked the Rho-ROCK and PI3K migratory pathways, using specific chemical inhibitors. Blocking PI3K signaling did not result in impaired front-rear polarization and persistence, but essentially blocked chemotactic migration in Boyden chamber assays. ROCK inhibition with Y-27632, did not impair chemotactic migration, yet directional migration and front-rear polarity are affected (Figures S2D and S2E). As listed in Table 1, we found that Par3, Tiam1 and PKCζ have similar roles in front-rear polarity, chemotactic-, persistent- and random migration but differ from the role of PI3K and Rho-ROCK. These data suggest that the mechanism by which the Par-Tiam1 complex regulates migration is distinct from the PI3K and the Rho-ROCK pathways.

Figure 6: Model of the function of Par-Tiam1 polarity signaling in contacting and migrating keratinocytes. Impaired Par-Tiam1 polarity signaling interferes with Tight Junction (TJ) formation by preventing maturation of a zipper-like adhesive state (primordial adhesion) into mature tight junctions during the membrane-sealing process. Impaired Par-Tiam1 function interferes with the establishment of permanent (apical-basal) cell polarity [13]. In migrating conditions, (untreated) keratinocytes display transient front-rear polarization but migrate relatively linearly compared to keratinocytes with impaired Par-Tiam1 signaling. Such cells polarize only briefly and project multiple protrusions in different directions, polarize again and migrate in alternate direction before depolarizing again, resulting in random migration and decreasing persistent migration. The Par-Tiam1 complex thus extends the duration of transient cell polarity.

We found that WT keratinocytes contain MTs that are relatively resistant to nocodazole treatment and contain stable acetylated tubulin filaments, whereas Tiam1 KO cells show less stable filaments. In fact, keratinocytes with impaired Par-Tiam1 signaling have reduced MT stability that is required for stable front-rear polarization and migratory persistence. This finding is supported by migration studies in fibroblasts that lack the MT stabilizing (plus-end-associating) protein Clasp2. Claps2 KO fibroblasts have reduced persistence in wound-healing assays [27], though a role for Clasp2 in front-rear polarity has not been studied. In addition, blocking the post-translational modification of MTs compromises their stability, and reduces chemotactic migration [33].

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

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

We propose that the Par polarity complex and Tiam1-mediated Rac signaling regulate the persistent migration of keratinocytes by stabilizing MT-dependent transient front-rear polarity. We show that this has biological consequences, since impaired Par and/or Tiam1 signaling reduces chemotactic [16] and wound migration (this study) on exogenous substrates in vitro and skin wound healing in vivo [40]. Although a complex crosstalk exists between actin- and microtubule networks in migrating cells [10,41], we find that Par-Tiam1 polarity signaling affects MT stability, which directly influences persistent and chemotactic migration. On a larger scale, our findings suggest a model in which when cell-cell junctions are present, as in normal epithelium, the default function of Par-Tiam1 signaling, is to stimulate the establishment of long-lasting apical-basal cell polarity [13,22] thereby contributing to tissue integrity. However, when cell-cell contacts are absent such as in lymphoid cells [16,42] or perturbed as in epidermal wounds [40] and epithelial cancers, Par-Tiam1 signaling promotes cell migration by stabilizing transient front-rear polarity (Figure 6).

**MATERIALS AND METHODS**

**Cell culture**

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

Antibodies

PKC (C-20; Santa Cruz Biotechnology, Inc.), HA tag (hybridoma 12CA5, own production), Tiam1 (C16; Santa Cruz Biotechnology, Inc.), Tiam1-DH, (Malliri et al., 2002), Rac1 and Par3 (Upstate Biotechnology), phospho-MAPK and phospho-PKC (Thr410/403; Cell Signaling) were used. Furthermore, we have used total MAPK (a non-commercial rabbit polyclonal), Phalloidin-alexa 568, acetylated-tubulin, alpha-tubulin and β-actin (Sigma).

Coating culture dishes with ECM molecules

We coated culture dishes overnight at 4°C with recombinant extracellular matrix (ECM) proteins (except laminin-5) at the following concentrations: 10 mg/ml fibronectin (FN) (isolated from human plasma); 10 mg/ml Laminin1 (LN1) (Becton Dickinson, San Jose, CA); 10 mg/ml vitronectin (VN) (Sigma-Aldrich); 20mg/ml Collagen I (Vitrogen, The Netherlands); 25mg/ml Collagen IV (Becton Dickinson). LN5-matrices were obtained by culturing Rac-11P cells to confluency, after which cells were detached with 10 mM ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS), containing a mix of protease inhibitors (Complete TM-protease inhibitor cocktail tablets, Roche Applied Science, Germany) at 4°C. Before use, the dishes were washed twice with PBS.

Gene and siRNA transfer into keratinocytes by retroviral transduction

Full length, HA-tagged Tiam1 and kinase-dead PKCζ were cloned into PMX-blasticidin (Bsd.) or hygromycin (Hyg.) retroviral vectors. Retroviral constructs were transfected into Phoenix ecotropic packaging cells, and fresh viral supernatants were collected and used for multiple infections and antibiotic selection as described [13]. siRNA constructs against Tiam1 [21] and three self-designed shRNAs against Par3 were cloned into selectable pRetroSuper constructs (see supplemental data). Other siRNA sequences used for Par3 and siLUC and 3siTiam1 were described previously [21,24]. MDCK siPar3 and siLUC cells were generated by infection with blasticidin and hygromycin selectable retroviral constructs carrying the siRNA against Par3. Expression levels of exogenous proteins and downregulated endogenous proteins were determined by immunoblot analysis.

Western blotting and immunoprecipitation

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

followed. For immunoprecipitation, lysates of migrating keratinocytes were prepared in standard radioimmunoprecipitation assay buffer. Extracts were precleared with washed protein G–Sepharose beads (GE Healthcare) for 1 h at 4°C. Precleared lysates were incubated with the HA-specific antibody 12CA5 that was preabsorbed on protein G–Sepharose beads for 16 h at 4°C. Immunocomplexes were washed three times, denatured with SDS, and separated by SDS-PAGE.

Microscopy and immunofluorescent staining

For phase-contrast microscopy, cells were seeded for 24 hours onto plastic coated with LN5 or collagen IV or onto collagen IV-coated glass coverslips. For immunofluorescence staining, keratinocytes were seeded on collagen IV-coated glass coverslips. Typically, cells were fixed after 16 hours of culture with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 2% BSA in PBS. Filamentous actin was labeled with 0.2 mM Alexa 568-phalloidin (Molecular Probes). Primary antibodies were visualized with appropriate FITC- or Alexa568-labeled secondary antibodies (Zymed Laboratories Inc. or Molecular Probes). Confocal images were obtained on a Leica TCS NT microscope operated by Leica-imaging software using 40x and 60x objectives. Size bars in images typically indicate 10μm.

Rac activity assay

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

Hole migration assay

WT and Tiam1 KO cells were stained with cell tracker dye (Molecular Probes) according to manufacturer’s protocol. A lid-device with inert rods was carefully placed on the culture plate such that all 24 wells contain 1 metal rod covering the center of each well. The pre-stained keratinocytes were added to coated 24-wells micro plates (collagen IV (25 mg/ml), or LN5, secreted by cultured Rac-11P cells (as described above). After overnight culture the device was carefully removed leaving an uncovered circular space in the center of each well. Non-adherent cells were removed by washing with PBS, and subsequently cells were allowed to migrate under normal culturing conditions for various time-points up to 24 hours and images were taken.

Time-lapse imaging of cell migration and computational analysis

A detailed explanation is provided in the supplemental data. In brief, cells were followed automatically by live-imaging and multiple time-lapse movies that were analyzed for migratory properties using home-made cell-tracking software.

Chemotactic migration assays

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

We would like to thank Audrey Gérard and Tomek Rygiel for providing reagents used in these studies. We also thank Lenny Brocks and Lauran Oomen for excellent technical support with the time-lapse and confocal imaging. This work is supported by grants from the Dutch Cancer Society to John G. Collard.

ABBREVIATIONS

GEF, Guanine Nucleotide Exchange Factor; Tiam1, T lymphoma invasion and metastasis; Par, Partitioning defective; WT, wild type; KO, Knock out; GF, growth factor; MT, microtubules; LN5, Laminin 5; Coll IV, collagen IV; VN, Vitronectin; FN, Fibronectin; Par-Tiam1 complex: complex consisting of the core “Par polarity complex” (Par3-Par6-PKCζ) physically and functionally associated with the Rac1-activator Tiam1.

REFERENCES

Chapter 5


SUPPLEMENTAL DATA

Supplemental Experimental Procedures

Time-lapse Imaging and Tracking software

Approximately 7000 WT, Tiam1 KO or WT (si)Par3 keratinocytes were seeded in triplicate wells (48 well format) pre-coated with collagen IV (in standard keratinocyte conditions) and migration was analyzed the following day. Images were acquired using a 10x 0.5 NA Plan objective and a 0.5 NA ELWD condenser with a Zeiss Axiocam camera on a Zeiss Axiovert 200M microscope in climate-controlled incubator. A robotic stage (Zeiss MCU 28) was used to collect images at different stage positions. All electronic microscope functions were controlled using Axiovision software (Zeiss). Typically, images of >60 cells per frame (3 frames per well, Figure S1B) were taken every 3 minutes. We initially used a small number of randomly picked cells cultured on LN5 from a number of movies and tracked these using the ‘track objects’ function in MetaMorph (Universal Imaging Corp.) To determine cell trajectories, centroids of the cell-nuclei were followed. To automate this and allow unbiased analysis of many cells in multiple time-lapses, a program was written in Matlab (Mathworks) see also [ref S1], that determines the presence and of nuclei (Figure S1B) and tracks nuclei in consecutive frames using a neural network algorithm. Detection fidelity in our experiments was usually around 90% (Figure S1B), as confirmed by eye. Cell persistence (D/T ratio) was calculated as follows: For every cell in each consecutive frame (n), the net displacement (D) over a 16 frame-interval (n - n+16) was divided by the cumulative displacement (T) over the 8 2-frame intervals (n - n+2, n+2 - n+4, ... n+14 - n+16) spanned by this 16-frame interval. 2-frame intervals were used to minimize overestimation due to frame-to-frame nuclear movements. Cells migrating at less than 50% of the average velocity were automatically excluded from persistence calculation. A (hypothetical) D/T ratio of 1 implies completely linear migration (LM) while computer simulation indicated that completely random migrating (RM) cells have an expected D/T ratio of 0.36 (shaded area) or less. Persistence per frame is the average of the persistence value of all cells included in this calculation. Average persistence was calculated from three independent wells typically by averaging the persistence values of at least 80 frames. Persistence values did not change significantly by changing time intervals and was stable during the analysis period. To evaluate the meaning of the persistence values measured in our experiments, we generated random tracks of 80 frames simulating increasing persistence. To simulate fully random migration we used a random distribution of angles between the displacement vectors from frame n – (n+1) and (n+1) – (n+2) ranging between –180° and 180°. To simulate increasing directionality, we decreased the borders of this random distribution stepwise, by 30°. To simulate occasional change of direction, which occur even in very directional cells, a random number from a Gaussian distribution (-90° and -90°) was added to each angle (Figure S1C for results of track-generation). For each step we generated 2000 tracks. We used these randomized
tracks to determine average persistence values identically to our calculation from experimentally derived tracks. We determined that the persistence value for a randomly migrating population of cells is 0.365 ± 0.06 thus ~0.36 (Figure S1D). We never observed D/T ratios exceeding 0.75 (WT keratinocytes in standard conditions) in our experiments.

**Chemotactic migration assays**

GF starved epithelial cells were trypsinized and resuspended to 10⁶ cells per ml. of which 100 μl was seeded into porous (8μm), collagen IV (top+bottom) coated transwells and allowed to adhere for 1 hour. The top well contained either GF media (no gradient) or GF free media (gradient), while the bottom compartment always contained GF-containing media. Transwells were incubated overnight. The following day non-migrated cells that remained on the top membrane were removed with cotton swaps and the migrated cells on the bottom were fixed and stained with crystal violet. Cells were counted in three separate fields in duplicate or triplicate. An unpaired t-test was used to assess significance in potential difference in average migration. In addition, chemotactic assays with WT keratinocytes were performed in the presence of a cell-permeable PKCζ pseudosubstrate inhibitor (Calbiochem). At the indicated concentrations, no significant cell death was observed after 24h of treatment, and video microscopy showed that WT cells treated with 1 μM of inhibitor migrated with an average velocity similar to that of Tiam1 KO cells (0.7 μm/min).

**Sequences of shPar3**

Par3-1: 5'-ATCCCCGGATCCAAAATCTAGTATTCAAGAGATCCAGTAGTTTGGATCCTTTTTGGAAA-3'
Par3-2: 5'-GATCCCCCCTGCAGGAAAGATTCAAGAGATCAGTTTCTTCGTCGCAGGTTTTTGGAAA-3'
Par3-3: 5'-GATCCCCACGAAATCAAAAAGCATTTCAAGAGAATGCAGTTTTGATTTTCGTGTTTTTGGAAA-3'

**Movies**

Supplemental movies are available at: http://www.current-biology.com/cgi/content/full/17/19/1623/DC1/H

- Supplemental Movie 1: Migrating WT keratinocytes in standard conditions on collagen IV;
- Supplemental Movie 2: Migrating Tiam1 KO keratinocytes in standard conditions on collagen IV;
- Supplemental Movie 3: Migrating siPar3 keratinocytes in standard conditions on collagen IV;
- Supplemental Movie 4: Migrating nocodazole treated keratinocytes (1μM in standard conditions on collagen IV);
- Supplemental Movie 5: Migrating PKCζ inhibitor treated keratinocytes (0.5 μM in standard conditions on collagen IV).

Movies are set to 15 frames/second each, frame-rate 3 min.

**Supplemental reference**

Supplemental figures

Figure S1: Monitoring and quantifying cell migration. (A) Typical images of WT and Tiam1 KO keratinocytes under normal growth conditions when seeded on dishes coated with either collagen IV or LN5. The bar diagrams represent an estimation of the percentage front-rear polarized cells of WT (closed bars) and Tiam1 KO (open bars) cultures, at any given time (n>60) indicating that front-rear polarity is independent on cell substrate. (B) Typical image frames of freely migrating keratinocytes (n>100) seeded on a collagen IV substrate and treated with PKCζ inhibitor. Right image depicts cells that have been assigned a tracking number to follow the individual cells over time. Note that the software recognizes over 90% of all cells. (C) Representative examples of tracks generated for the simulation of increasingly persistent migration (see materials and methods). Note that when the maximal allowed turn-angle is 0 we observe linear migration with a persistence value of 1, whereas changing the maximal turn-angle to 30° we see a reduction in persistence to 0.7, which is much more realistic for migrating WT keratinocytes in
culture. (D) We used these randomized tracks with increasing persistence (see material and methods) to determine average persistence values identically to our calculation from experimentally derived tracks. When the maximal turn-angle between subsequent displacements exceeds 90° the simulated persistence value does not drop any further, indicating that persistence values below the cut-off of 0.36 (± 0.06) represent random migration.
Figure S2: Analyses of substrate and growth-factor dependency of migration. (A) Velocity histogram of migrating WT (purple diamonds) and Tiam1 KO keratinocytes (pink squares) on a Collagen IV substrate. The cell velocity (x axis) is plotted against the number of cells (y axis) with that velocity. This graph shows that populations WT and Tiam1 KO cells have a similar distribution in velocities. Most Tiam1 KO and WT cells (>90%) migrate between 0 and 2 μm/min, the fastest cells reaching up to 4-5 μm/min. WT cells migrate on average slightly faster than KO cells. (B) Chemotactic migration towards GFs of WT and Tiam1 KO cells on fibronectin and vitronectin substrates. (C) Chemotactic migration of WT keratinocytes towards GFs (keratinocyte growth factor supplement, EDGS) or epidermal growth factor (EGF) alone (5ng/ml). (D) Images of WT keratinocytes treated with chemical inhibitors. The ROCK inhibitor (10μM) has profound effects on morphology (tail retraction is abrogated), whereas PI3K inhibitor has little affect on a polarized morphology (20μM). (E) Chemotactic migration of WT keratinocytes towards growth factors can be blocked by 20μM of PI3K inhibitor (LY) but not with 10μM of the Rho kinase inhibitor (Y). (F) F-actin expression visualized by Alexa-phalloidin staining in a polarized WT and a Tiam1 KO cell. Both cell types have F-actin enrichment at the leading edge. Similar localization is observed with GFP-tagged Tiam1 in keratinocytes (Figure 4F) but the GFP-vector control expressing keratinocytes show no GFP enrichment at the actin-rich leading edge. The scale bar represents, 10 μm.
Chapter 5

Figure S3. Analyses of signaling pathways involved in migration of epithelial cells. (A) Confocal images of endogenous Par3 in MDCK cells infected with (si)Par3 siLuc retroviral constructs. (si)Par3-depleted MDCK cells have less bright staining under similar settings, indicating the effective downregulation of Par3 expression not seen in siLuc cells. (B) Immunoblotting of MDCK lysates using the same antibody as in (A), confirming that si(Par3) depletion is specific and applies to all three Par3 isoforms. We used various Par3 specific siRNA sequences (see Supplemental Experimental Procedures) depicted as 1, 2 and 3 as well as two control constructs (siTiam1 and siLuc). (C) Bright-field images of Tiam1 KO cells and Tiam1 KO cells expressing a retroviral construct carrying constitutively active Rac1 (V12 mutation) that leads to broadly spread and immobilized cells and is therefore unable to rescue the impaired polarized morphology. (D) Rac assay showing that freely migrating Tiam1 KO keratinocytes have lower Rac activity than do their WT counterparts. (E) Dose-dependent inhibition of chemotaxis to GFs of WT keratinocytes with 1 µM PKCζ inhibitor (asterisks indicate significant differences compared to control in a Student’s t test). (F) Influence on average persistence after treatment with 1 µM PKCζ specific inhibitor and 20 µM LY (PI3K) inhibitor compared to WT cells.
Chapter 6

The Rac activator Tiam1 is required for astrocyte protrusional outgrowth but dispensable for orientation of the microtubule organizing centre.

Saskia I.J. Ellenbroek, Sandra Iden and John G. Collard

Small GTPases (2012) 3:4-14
ABSTRACT

Polarized cell migration is a crucial process in 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 scratching-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. 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. Responses observed in vitro using astrocyte cultures resemble what occurs in vivo in response to a wound. 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 towards the direction of migration. For the establishment of many different modes of polarity, both actin and microtubule cytoskeletal rearrangements are fundamental processes that involve signaling via Rho GTPases. It is well-accepted that actin polymerization and rearrangement depend on different Rho GTPases, including Rho, Rac and Cdc42. The microtubule cytoskeleton plays an important role as well and provides directional guidance. 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.\textsuperscript{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.\textsuperscript{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 anchoring to control reorientation of the centrosome and Golgi apparatus.\textsuperscript{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 cells, neuronal cells and epithelial cells.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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
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 hours after scratching monolayers of wt and Tiam1 ko primary astrocytes (E) and 4.5 hours 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.

using phase-contrast microscopy and confocal microscopy after immunostaining of cells. Protrusions became apparent approximately 2 hours 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 hours. Tiam1 ko MEFs showed a delay in the closure of scratch wounds (Fig. 2A right panels and B). The difference in wound closure was significant from 2.5 hours 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 versus 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 hours. As observed for Tiam1 ko MEFs, Tiam1 ko astrocytes showed significantly delayed coverage of the wound area from 2.5 hours 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.

![Figure 2: Tiam1 deficiency delays wound closure in MEFs and primary astrocytes.](image)

(A) Phase-contrast images of wt and Tiam1 ko MEFs at 21 hours 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 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

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 hours 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 4 times. Over 100 cells were analyzed in three separate experiments. Data represent average ± SEM. * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.0005.
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 two hours after wounding confluent monolayers, the length of protrusions of Tiam1 ko astrocytes was significantly reduced compared to wt astrocytes (Fig. 3B). This difference increased over time. Width of the cells was significantly different between wt and Tiam1 ko astrocytes at 7 hours post-wounding, when Tiam1 ko protrusions were wider than wt (Fig. 3C). Additionally, the ratio of length to 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 to 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. 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 hours 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 cells at the wound edge showed localization of the centrosome in a polarized fashion, in the quadrant facing the scratch 6 hours after scratch-wounding (Fig. 4A and C, upper 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 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 to 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. 27 NSC23766 disrupts the interaction between Rac and its GEFs Trio and Tiam1, whereas EHT1864 promotes the loss of bound nucleotide as well as 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

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 hours 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 hours 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 hours 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.
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. Analyses 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

Figure 5: Tiam1-mediated Rac activity is required for asymmetrical cell shape after scratch-wounding of monolayers. (A, 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 hours immediately after scratching (lower panels). Bar, 25 μm. (B) Quantification of cellular asymmetry of astrocytes treated with Rac inhibitors compared to untreated astrocytes, represented as the average ratio of length to width ± SEM, 6 hours after scratch-wounding. * p ≤ 0.05, ** p ≤ 0.01.
closely resembles that observed in CLASP2 knockout MEFs and is indicative of defects in cell polarity. 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 towards 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 towards 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 to 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, may function in microtubule stabilization in MEFs and 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. Cells lacking Tiam1 showed a remarkable decrease in length of protrusions formed after wounding. At later time points also width of protrusions 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 altered re-positioning of the centrosome and Golgi apparatus, 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.\textsuperscript{1,10,30} Since these processes are all required for astrocyte protrusion formation, it was

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 hours after wounding. Bar, 25 μm. (C) Microtubule cytoskeleton of scratched wt and Tiam1 ko astrocytes was visualized 4 hours 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 3 independent experiments. (E) Quantification of D, expression levels of detyrosinated tubulin relative to corresponding α-tubulin levels.
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 to 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 beta-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 towards GSK3β. Apparently, Tiam1 acts in parallel with the Par6/aPKCζ complex in

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, controlling 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.
protrusion outgrowth (Fig. 7B).

In most migrating cells, the front is characterized by rapid actin polymerization and adhesion turnover. 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. 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 rather than the actin cytoskeleton. In the leading edge of migrating cells microtubules are stabilized towards the direction of migration. Tiam1 can regulate microtubule stability in single migrating keratinocytes and in absence of Tiam1 this disturbed process affects persistent migration of these cells. Furthermore, downregulation of Tiam1 expression can lead to reduced levels of acetylated tubulin, suggesting a role in stabilization of microtubules in ES cells. In line with this, we found that protrusions of astrocytes lacking Tiam1 contain reduced pools of stabilized, detyrosinated tubulin, compared to wt astrocyte protrusions. Moreover, we observed similarities between MEFs lacking Tiam1 expression and results from studies using CLASP2-deficient MEFs. CLASP2 has been shown to be involved in the stabilization of microtubules during migration as well as crosslinking with the cell cortex. 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. It is likely that interactions between the actin and the microtubule cytoskeleton play an important role in regulating cytoskeletal dynamics in leading edges. IQGAP is an actin-binding effector protein of both Rac and Cdc42 and together these proteins have been shown to contribute to microtubule capture. Furthermore, IQGAP can interact with APC and CLIP170 and thereby provides a link between actin and microtubule dynamics. Tiam1 has previously been shown to associate with microtubules during axon formation. 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. 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. Furthermore, IQGAP can interact with APC and CLIP170 and thereby provides a link between actin and microtubule dynamics. Tiam1 has previously been shown to associate with microtubules during axon formation. 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. 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. Furthermore, IQGAP can interact with APC and CLIP170 and thereby provides a link between actin and microtubule dynamics. Tiam1 has previously been shown to associate with microtubules during axon formation. 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. 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.
Phosphorylation 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 (post-natal day 1-3) wt and Tiam1 ko mice on FVB background as described in a protocol generously provided by N.E. Savaskan. 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 minutes in 37°C water bath. Subsequently, trypsin was inhibited by addition of an equal volume of DMEM supplemented with 10% FCS and P/S. Cells were centrifuged at 800 rpm for 10 minutes 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 day in vitro 9 and 20 and passaged maximally once. Mouse embryonic fibroblasts (MEFs) were isolated as described previously. 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 minutes 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) overnight 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 hours 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 Microlmaging, 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. Briefly, cells were put on ice, washed with ice-cold PBS (containing 1mM CaCl₂ and 0.5mM MgCl₂) and lysed with a lysisbuffer containing 0.5% Nonidet P-40, 10mM 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 minutes 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 pH7.4, 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 minutes 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 measurement of the coverage of the scratched area at different time points in different experiments using ImageJ software (National Institutes of Health).
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 hours 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 hour with 2% BSA in PBS cells were incubated with primary antibodies for 1 hour. Subsequently, cells were washed with 1% BSA in PBS and incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 1 hour, 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 4 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 μM and 50 μM, respectively.

**ACKNOWLEDGEMENTS**

We would like to thank Ronny Schäfer and Rob van der Kammen for technical assistance. This work has been supported by grants from the Dutch Cancer Society and the EC (TuMIC) awarded to J.G. Collard.
Chapter 6

ABBREVIATIONS

GEF, guanine nucleotide exchange factor; ko, knock out; Noc, nocodazole; SEM, standard error of the mean; SD, standard deviation

REFERENCES


16. Manneville, J. B., Jehanno, M. &


Even-Ram, S. et al. Myosin IIA regulates
54. Malliri, A., van, E. S., Huveneers, S. & Collard, J. G. The Rac exchange factor
Chapter 7

Summarizing discussion
SUMMARIZING DISCUSSION

After the initial identification the Ras oncogene around the end of the 20th century, many homologous proteins were identified. They represent a family of small guanosine triphosphatases (GTPases) that all act as binary signaling switches, cycling from an active GTP-bound form to an inactive GDP-bound form. From then, research into the functional diversification of the different members flourished rapidly and as a consequence our understanding of their mechanisms of action and regulation expanded. That this area of research led to many interesting discoveries comes as no surprise if one realizes that the Ras superfamily currently comprises over 150 family members, divided over multiple subfamilies. Besides the GTPases many additional proteins were identified that act as regulators of these proteins as guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). The identification of numerous downstream effector proteins further complements the abundance of the area of research into the fundamental understanding of the importance of small GTPases. Stimulation of cell surface receptors leads to the activation of specific small GTPases which in turn activate diverging signal transduction routes resulting in a great diversity of cellular outcomes ranging from gene transcription, vesicle trafficking and cytoskeleton reorganization, processes which affect growth, differentiation, adhesion, and migration of cells. As a result of the wide range of biological activities involving GTPase signaling, aberrant regulation of their activity contributes to the development and progression of several pathologic conditions, including tumorigenesis.

In the studies described in this thesis the main focus was on the role of two proteins of the Rho subfamily of small GTPases (Rac1 and Rac3) and a Rac-specific GEF, Tiam1 (for T lymphoma invasion and metastasis), in different modes of polarity and cancer.

Rho GTPases and cancer

In chapter two the function and regulation of the family of Rho GTPases is introduced. At the time this manuscript was prepared, 23 Rho GTPases were identified that were divided into 6 groups. Currently the assignment of Miro proteins to the Rho family of GTPases is disputed, since they are also regarded to present a distinct family of the Ras superfamily, equally homologous to the Rab and Rho families. In addition, the evidence for involvement of the Rho GTPase proteins in different aspects of carcinogenesis is summarized in this chapter.

Mutations in the different isoforms of the Ras oncogene are detected in over 30% of all human cancers, and K-Ras mutations are even found in 90% of all pancreatic tumors. In contrast, mutations in Rho GTPases are rarely found. Interestingly, recently a recurrent gain-of-function point mutation in melanoma was identified in the Rac1 protein, Rac1P29S. However, in contrast to oncogenic Ras mutations which mostly result in elimination of GTPase activity (disabling the proteins to return to their inactive GDP-bound state), this specific
mutation in Rac1 preserves the capacity of the protein to hydrolyse GTP and cycle between the active GTP-bound state and the inactive GDP-bound state. Interestingly, as described in chapter two, deregulation of signaling downstream of Rho GTPases seems to occur mostly via alteration of their expression levels or via their regulators (GEFs, GAPs and GDIs).

Since cancer is a multistep disease, which involves processes such as growth but also cytoskeletal remodeling and migration to facilitate metastasis, it is not surprising that aberrant Rho GTPase signaling contributes to the different steps of cancer development and progression. Therefore, elucidation of the mechanisms by which perturbed regulation of Rho GTPase proteins contribute to tumorigenesis will provide valuable information for the development of new therapeutic targets to interfere in the pathological process.

Cell polarity proteins and cancer

Polarity is defined as cellular asymmetry, either in cell shape and/or distribution of proteins and functions, and is crucial for both embryonic development and maintenance of tissue integrity. Protein complexes consisting of conserved proteins regulate different modes of polarity, including asymmetric cell division, T cell polarity, neuronal axon specification, front-rear polarity during migration of cells and epithelial apical-basal cell polarity. Loss of the latter mode of polarity in particular has been implicated in tumor development and progression and is considered both a hallmark and a prerequisite of cancer.

In chapter three I discuss the evidence that is accumulating from studies using model organisms as well as data obtained from investigation of human tumor tissue, which confirms the implication of polarity proteins in different phases of cancer development. There are several clear links between proteins regulating polarity and (regulators of) GTPase signaling. Polarity proteins form integrate protein complexes with intercommunicate in order to regulate different modes of polarity. One of these protein complexes is the Par polarity complex consisting of Par3, Par6 and aPKCzeta. Tiam1 provides a link between GTPase signaling and different polarization processes by interacting with Par3. Furthermore, both Par3 and Par6 have been linked to Tiam1-mediated Rac activation in neuronal cells. Rac on the other hand, has been shown to activate aPKCzeta via binding to Par6. In addition to the evidence for a role of polarity proteins in several aspects of tumorigenesis as summarized in chapter three, three recent studies confirm a role for Par3 in tumor development. Par3 was shown to serve both tumor suppressive and tumor-promoting functions, in skin cancer. Furthermore, in breast cancer Par3 was shown to suppress tumorigenesis and metastasis. Mutations in Tiam1 were recently identified in whole-sequence genome analysis of late-stage neuroblastoma. Interestingly, alterations were found in six GEFs for Rac and 5 GAPs for Rho, implicating an important function of GTPase signaling in the progression of neuroblastoma. Therefore, investigating the function of Tiam1/Rac signaling and interactions with signaling pathways controlling polarity will help to reveal the mechanisms of cell polarity in cancer.
aberrant signaling contributing to cancer, which ultimately will contribute to cancer diagnosis and treatment.

**Minor differences in protein sequence result in opposing functions of Rac1 and Rac3**

As described in chapter four, RhoGTPases Rac1 and Rac3 (which can both be activated by Tiam1) are highly homologous but serve different functions in neuronal cells. Although sharing 92% sequence homology, we observed significantly different cellular outcomes of the activation of either protein. Other groups studying Rac1 and Rac3 reported the absence of functional differences. However, these studies relied on overexpression of active or dominant negative mutants, which disable spatiotemporal regulation of the GTPases, crucial for the outcome of signaling via effector proteins which may be the same.

In our study we made use of wild-type Rac1 and Rac3 constructs which can be activated by endogenous GEFs. We investigated functional differences between Rac1 and Rac3 by altering the expression of either protein using overexpression as well as downregulation in neuronal cells. Enhanced expression of Rac1 resulted in cell spreading and outgrowth of protrusions, in contrast to exogenous Rac3 which caused rounding of cells and attenuated adherence. Whereas Rac1 suppression using siRNA lead to rounding of cells and severe loss of cell-matrix adhesion, downregulation of Rac3 resulted in differentiation displayed by formation of neurite-like protrusion in neuroblastoma cells.

The minor differences in their protein sequence are not located in the effector loop where effector proteins bind, indicating that Rac1 and Rac3 can establish protein-protein interactions with the same set of effector proteins. The carboxyl terminus of the protein sequences on the other hand exhibit minor differences, suggesting that this region may be responsible for the functional differences observed between the two GTPases. Indeed, we identified the polybasic region as the region responsible for the differential outcome of activation of Rac1 and Rac3. Upon switching of the distinctive C-terminal amino acids located directly in front of the CAAX sequence at the end, we were able to convert the phenotypic responses upon overexpression of the proteins. Interestingly, we observed that the fully opposing phenotypes were dependent on only three variable amino acids.

Not only functional differences were caused by the three different amino acids in the polybasic region, also the localization of Rac1 and Rac3 within neuronal cells was found to depend on this region. Whereas Rac1 is localized mainly to the plasma membrane and cell protrusions of neuronal cells, Rac3 resides mainly the perinuclear region in the cytoplasm. Also this localization could be reverted by exchanging the three distinctive amino acids. Another triplet of amino acids that differs between the two proteins is located within the CAAX sequence, but switching of this region did not result in phenotypic or localization changes.

Whereas Rac1 is ubiquitously expressed, Rac3 expression is mainly restricted to the brain. From our
studies in neuronal cells, in which we observed that Rac3 expression is almost absent in differentiated cells and neurites are formed upon depletion of Rac3, we conclude that Rac3 may serve a function in suppressing differentiation and neuritogenesis by attenuating proper formation of cell-matrix adhesions. Since the polybasic region has been suggested to represent an additional binding site for effectors and GEFs21-23, follow-up studies were performed in our lab to investigate whether binding to different downstream effectors may explain the differential outcome in neuronal cells. GIT was identified as an effector protein that interacts with both Rac1 and Rac3 but in different manners24. Whereas interaction of GIT1 with (mostly GDP-bound) Rac1 depends on beta-Pix binding to the polybasic region (which differs from Rac3), Rac3-GTP interacts with GIT1 independent of beta-Pix. Rac3 mediated cell rounding and precluded differentiation was found to result from perturbed GIT1-paxillin interaction and subsequent distribution of paxillin as well as GIT1-mediated reduction of Arf6 activity.

Rac3 null mice have been generated and were viable without obvious developmental defects25,26. However, these mice showed enhanced motor coordination and motor learning. In contrast to the opposing functions that we observed, more recently Rac1 and Rac3 were both shown to be required during interneuron development, where they are suggested to act synergistically to regulate migration and differentiation of these cells27. Synergistic action of the two close homologues was recently also observed in the development of the inner ear, where they were found to contribute to processes regulating epithelial polarization, growth and survival28. The results from our studies and these of other groups stress that the cellular outcomes of activation of Rho GTPases are highly context and tissue specific and strictly regulated in both spatial and temporal manners.

Tiam1 and the Par complex regulate directional migration of keratinocytes

Chapter five describes the molecular mechanisms by which Tiam1 functions in migration of keratinocytes when apical-basal polarity is lost in these epithelial cells. Epithelial cells normally form tight layers of cells, with defined apical and basal sites separating luminal and extracellular spaces from underlying tissue. The cell-cell contacts between epithelial cells, mediated by tight junctions, serve a dual function, as they prevent the random distribution of proteins over the membrane thereby creating a functional division of the plasma membrane into apical and basolateral domains and they regulate paracellular transport. Most tumors arise from epithelial tissue. During tumorigenesis cell migration contributes to the metastatic process. Cells may detach from the primary tumor, invade the surrounding tissue, intravasate into the blood circulation and after extravasation migration precedes proliferation at secondary sites29. Different modes of migration have been identified, depending on multiple structural and molecular determinants defined by both the cellular context and extracellular cues30. Previously, our lab has shown that Tiam1-Rac signaling in conjunction with the Par complex, is required for maturation of tight junctions and membrane sealing8.
Furthermore, we have shown that Tiam1 is implicated in migration of keratinocytes31. Chapter five describes the study in which we investigated the potential partnership of Tiam1 and the Par complex during polarized migration of keratinocytes. Non-contacting keratinocytes migrate in a directional fashion, with a defined front and rear. We found that absence of Tiam1 expression hampers this directionality and induces random migration of keratinocytes. In addition, chemotactic migration was attenuated in Tiam1 knockout (Tiam1 KO) cells compared to wild-type control cells. Similarly, when cells were depleted of Par3 using siRNA we observed impaired directionality of migration as well as impaired chemotaxis. As we have shown in contacting cells, also in single non-contacting keratinocytes Tiam1 was found to associate with Par3 and aPKCzeta (members of the Par polarity complex). Interestingly, Par3 depletion or pharmacological inhibition of aPKCzeta did not result in additional effects in cells lacking Tiam1 expression. Therefore we concluded that Tiam1/Rac signals in conjunction with the Par complex not only in apical-basal polarity (to stimulate tight junction maturation), but also in front-rear polarity of migrating keratinocytes (to stimulate persistent and chemotactic migration).

Morphological differences between stably polarized migrating wild-type keratinocytes and Tiam1 KO cells which displayed short-lived front-rear polarity, suggested a role for Tiam1/Par in microtubule stabilization. We found Tiam1, Par3 and aPKCzeta localized at the leading edge of migrating cells. Recently, the group of Kaibuchi showed that this localization may represent specific adhesion structures in the front of migrating cells, which are dependent on talin32. They suggest an signaling cascade where integrin signaling via talin is propagated via direct binding to Tiam1 which in conjunction with the Par complex contributes to spreading and turnover of adhesions of migrating cells. It will be interesting to investigate whether this signaling cascade is linked to the observed effects induced by Tiam1/Par signaling in our keratinocyte model.

Tiam1 mediates several, but not all, aspects of astrocyte polarization

Chapter six describes the function of Tiam1 in another polarity setting, namely the polarization of astrocytes. Astrocytes are supporting cells of the nervous system, belonging to the glial cells. Besides a function in nutrient supply they also support neuronal outgrowth during development and during damage repair33. Here we show that Tiam1 is required for several steps involved in the polarized outgrowth of astrocytes in response to wounding but dispensable for some step in the polarization process. Astrocytes provide an excellent model to study polarization since induction of polarization by wounding of monolayers is accompanied by major morphological changes, including changes of the cytoskeleton and localization of specific cellular components, including the Golgi apparatus and the microtubule organizing center (MTOC). We made use of primary astrocytes derived from wild-type mice and mice lacking Tiam1 (Tiam1 KO). In addition we studied the role of Tiam1 in fibroblast derived from mouse embryos.
When we induced a wound in confluent monolayers of cells, we observed the typical phenotypic changes including the formation of length protrusions in the direction of the wound in wild-type cells, whereas cells lacking Tiam1 expression failed to form these long protrusions. In addition, in wild-type cells the microtubule cytoskeleton was aligned in the direction of the formed protrusion, with parallel bundles of microtubules reaching the tip of the protrusion. In Tiam1 KO cells however, the cytoskeleton was less organized and the microtubules that filled the shorter and wider protrusions were not nicely aligned. Furthermore, we showed that Tiam1 is dispensable for another important aspect of astrocyte polarization, the reorientation of the Golgi apparatus and MTOC. In both wild-type and Tiam1 KO cells Golgi and MTOC of wound-edge cells were oriented towards the wound area, located in front of the nucleus. Interestingly, this process has been shown to be regulated by Par6/aPKCzeta34-36. Together with our observation that Tiam1/Rac does not activate aPKCzeta in this process (in contrast to the process of tight junction maturation in keratinocytes8) indicates that in this polarization process Tiam1 does not signal in conjunction with the Par complex, but rather signals in a parallel pathway to regulate microtubule organization.

An intriguing question that remains to be answered is the exact mechanism underlying Tiam1’s importance in microtubule organization during astrocytes polarization. Involvement of Tiam1 in polarization processes has already been described in a number of other cellular systems, including, epithelial and T cells37. The polarization machineries used in the different cellular contexts all include the Par complex proteins and Tiam1. Our observation of the perturbed morphology of the microtubule cytoskeleton in astrocytes, together with our study on the investigation of the role of Tiam1 in keratinocyte polarization (described in chapter five) suggest that Tiam1 may influence the microtubule organization by stabilization of the microtubules, in a pathway parallel to the Par complex. A recent study supports this idea, in which was shown that Tiam1 can interact with a domain in the protein microtubule-associated protein 1B (MAP1B)38. This protein is known for its involvement in microtubule stabilization in neuronal cells. Interestingly, we observed some morphological aberrations in our Tiam1 KO cells that are similar to those observed in CLASP2 KO cells39. Active Rac has been shown to promote microtubule growth into lamellipodia via modulation of CLASP2, which binds to the plus-end of microtubules in the leading edge40,41. CLASP2 was shown to attach microtubule plus ends to the cell cortex and regulate microtubule stabilization and persistent migration39,42. Alternatively, Tiam1 may also be required for microtubule guidance. This suggestion is supported by a recent study on a close homologue of Tiam1, STEF (for Sif- and Tiam1-like exchange factor)43. In this study STEF was shown to regulate focal adhesion assembly as well as microtubule-dependent Rac activation. STEF-mediated Rac activity was required for microtubule targeting to focal adhesions. During astrocyte protrusion formation Tiam1/Rac signaling may mediate microtubule growth towards specific sites in the cell, which may explain the disturbed cytoskeletal organization observed in Tiam1 KO cells.
Overall, the studies presented in this thesis stress the highly context dependent outcome of signaling via Tiam1/Rac and polarity proteins and their involvement in a wide variety of cellular processes that all require strict regulation in order to prevent adverse effects that may contribute to cancer development and progression.

REFERENCES

Chapter 7


29. Ritsma, L. et al. Intravital


Metastasering (uitzaaiing) van tumorcellen is vaak de doodsoorzaak van kankerpatiënten. Weinig is bekend over de cellulaire signaleringsroutes die ten grondslag liggen aan het metastaseren van tumorcellen. Tiam1 werd ontdekt in een zoektocht naar invasie-stimulerende genen die betrokken zijn bij de uitzaaiing van tumorcellen. Het eiwit heeft verschillende functies in de regulatie van cellulaire processen die uiteindelijk het invasieve en migratoire karakter van cellen kunnen bepalen. Het is daarom van belang de effecten van Tiam1 op het gedrag van zowel gezonde cellen als tumorcellen te bestuderen. Uiteindelijk kan de verzamelde informatie, met name de kennis over de rol van Tiam1 in kanker, leiden tot betere diagnostische tools en nieuwe targets voor de bestrijding van specifieke tumoren.

Tiam1 kan Rac activeren, één van de eiwitten uit de familie van Rho GTPases. Deze eiwitten zijn in een actieve staat gebonden aan GTP en in inactieve staat gebonden aan GDP. Tiam1 activeert Rac door de wisseling naar de GTP-gebonden staat te stimuleren. Signaleringsroutes waarbij Rho GTPases betrokken zijn spelen een rol bij tal van processen binnen de cel, zoals de transcriptie van genen, organisatie van het cytoskelet, maar ook bij groei, differentiatie, adhesie en migratie van cellen.

De focus van de studies beschreven in dit proefschrift ligt op het bestuderen van de mechanismen die ten grondslag liggen aan signalering via Rho GTPases en de eiwitten die de activiteit van deze ‘schakelaars’ reguleren. Daarbij is in het bijzonder gekeken naar de functie van Tiam1 en de betrokken signaleringsroutes tijdens polarisatie van cellen en in de ontwikkeling van kanker. Daarnaast is gekeken naar de verschillende effecten van twee eiwitten die door Tiam1 geactiveerd kunnen worden, namelijk Rac1 en Rac3.

Veranderingen van het cytoskelet zijn noodzakelijk bij vele processen die plaatsvinden tijdens de embryonale ontwikkeling maar ook naderhand, voor het behoud van gezonde, intacte weefsels alsmede bij herstelprocessen, bijvoorbeeld na verwonding. De eiwitten behorend tot de Rho-familie van GTPases spelen een belangrijke rol bij het regelen van deze veranderingen van het cytoskelet. In Hoofdstuk 2 wordt een overzicht gegeven van onze kennis over de betrokkenheid van RhoGTPases bij de verschillende aspecten van kanker. Aangezien kanker een uiterst complexe ziekte is waarbij tal van cellulaire processen een rol spelen, waaronder reorganisatie van het cytoskelet, celdeling en migratie, is het niet verwonderlijk dat verstoring van deze signalering via Rho GTPases kan bijdragen aan de verschillende stappen van zowel het ontstaan als de progressie van kanker. In dit hoofdstuk wordt de relatie tussen verstoorde signalering van GTPases omschreven, die plaatsvindt op zowel het niveau van veranderingen in regulatoren en eiwitten waarin GTPases kunnen binden (effectoren) als op het niveau van de GTPase eiwitten zelf. Ras GTPases vertonen grote mate van homologie met Rho GTPases, maar terwijl mutaties in Ras GTPases worden gevonden in 30% van alle gevallen van humane kanker komen mutaties in Rho GTPases nauwelijks voor. Misregulatie van Rho GTPases lijkt voornamelijk
Celpolariteit wordt omschreven als de asymmetrische verdeling van eiwitten of functies in cellen en is cruciaal tijdens de embryonale ontwikkeling. Ook tijdens het normale behoud van de integriteit van weefsels en tijdens wondhealing is polariteit van bepaalde cellen essentieel. Onderzoek heeft aangetoond dat er een selecte groep van eiwitten bestaat die behouden is gebleven gedurende de evolutie en aanwezig zijn in vrijwel alle soorten organismen. Deze eiwitten vormen verschillende complexen die samen meerdere vormen van celpolariteit reguleren. In Hoofdstuk 3 hebben we de huidige kennis omtrent de relatie tussen deze polariteitseiwitten en kanker samengevat. De meeste vormen van kanker ontstaan uit epitheliaal weefsel. Verlies van polariteit in epitheliaal cellen wordt daarom gezien als een kenmerk en als een voorwaarde voor het ontstaan van kanker. Er zijn verschillende aanwijzingen die de signalering van polariteitseiwitten koppelen aan (de regulatoren van) Rho GTPases. Tiam1-gemedieerde Rac activatie is betrokken bij verschillende vormen van celpolariteit en deze signalering hangt samen met signalering van één van de polariteitscomplexen, het Par complex (bestaande uit Par3, Par6 en aPKCzeta). Het onderzoek van de functie van Tiam1/Rac signalering en de interactie met polariteitsmechanismen kan daarom bijdragen aan onze kennis over de verstoorde mechanismen die ten grondslag liggen aan tumorgenese.

In Hoofdstuk 4 wordt beschreven hoe de mogelijke verschillen tussen twee sterk gelijkende eiwitten uit de Rho GTPase familie hebben bestudeerd. Rac1 en Rac3 hebben een eiwitsequentie die voor 92% gelijk is, desondanks vonden we grote verschillen als gevolg van veranderde expressieniveaus in neuronale cellen, die beide eiwitten endogene tot expressie brengen. Van Rac1 is bekend dat het betrokken is bij tal van processen, waaronder celspreiding en adhesie van cellen aan een matrix. Rac3 bleek een tegenovergestelde functie te hebben en verhoogde expressie van dit eiwit resulteerde in afronding van cellen en vermindere adhesie. Uit de resultaten van dit onderzoek concludeerden we dat Rac3 waarschijnlijk een rol speelt bij de onderdrukking van differentiatie en de vorming van neurieten door cel-matrix adhesies te verhinderen. Aangezien we vonden dat de expressie van Rac3 in gedifferentieerde neuronale cellen verlaagd is, bestaat er waarschijnlijk een intrinsiek mechanisme om Rac3 expressie te onderdrukken tijdens differentiatie. We vonden ook dat Rac1 en Rac3 naar verschillende delen van de cel lokaliseert in neuronale cellen. Rac1 lokaliseert voornamelijk aan het plasmamembraan terwijl Rac3 voornamelijk wordt gevonden in de regio rondom de nucleus in het cytoplasma. Tot slot hebben we de regio binnen de eiwitsequentie geïdentificeerd die verantwoordelijk is voor de geobserveerde verschillen. Ondanks de hoge mate van homologie blijken drie aminozuren in het carboxyl-terminale deel van de eiwitsequentie verantwoordelijk te zijn voor de tegenstrijdigheid in zowel functies als lokalisatie van beide eiwitten.

In Hoofdstuk 5 hebben we de functie van Tiam1 bestudeerd in losliggende epitheliaal cellen. In gezond weefsel maken epitheliaal cellen onderling hechte contacten die van belang zijn om polariteit te behouden, waarbij een duidelijk onderscheid is tussen de onder- en de bovenkant van de cellen. Deze vorm van polariteit (apicaal-basale polariteit) gaat verloren op het moment dat de cel-cel contacten opgeheven worden of de
activiteit van bepaalde eiwitten die nodig zijn voor het behoud ervan veranderen. Een andere vorm van polariteit kan dan geïnitieerd worden, waardoor cellen een duidelijke voor- en achterkant krijgen en cellen directioneel (in een bepaalde richting) kunnen migreren. Afwezigheid van het Tiam1 eiwit verhindert de directionaliteit van migrerende cellen en hetzelfde effect is zichtbaar na onderdrukking van expressie van Par3 of inhibitie van aPKCzeta (beiden behorend tot het Par complex van polariteitseiwitten). Aangezien gecombineerde onderdrukking van Tiam1 en Par3 of Tiam1 en aPKCzeta geen additioneel effect oplevert, concludeerden we dat Tiam1 en het Par complex in dezelfde signaleringscascade functioneren. Beiden zijn nodig voor het stabiliseren van een voor- en achterkant van een cel om daarmee migratie in een rechte lijn mogelijk te maken. Het verlies aan directionaliteit kan verklaard worden door een verlies aan stabiliteit van het cytoskelet van microtubuli welke werd waargenomen in cellen zonder Tiam1 expressie. Eerder hebben we aangetoond dat in epitheliale cellen - die onderling contact maken - Tiam1 ook samenwerkt met het Par complex om de vorming en maturatie van cel-cel contacten te reguleren en daarmee apicale-basale polariteit in stand te houden. Uit onze onderzoeken blijkt dat de biologische context van groot belang is voor de regulatie van de verschillende vormen van celpolariteit. Tiam1 en het Par complex blijken samen te werken in de vorming van apicale-basale polariteit in cellen die onderling contact maken en in de vorming en stabiliteit van voor-achterkant-polariteit in losliggende migrerende cellen.

In Hoofdstuk 6 werd de Rac-specifieke activator Tiam1 geïdentificeerd als de activator van Rac die verantwoordelijk is voor gepolariseerde astrocyten. Astrocyten behoren tot de 'gliacellen' en zijn de ondersteunende cellen van het centraal zenuwstelsel. Ze spelen onder andere een cruciale rol bij het doorgeven van elektrische signalen tussen neuronen. Hun uitlopers, waarlangs neuronen kunnen groeien, zijn belangrijk bij de ontwikkeling en bij herstel van beschadiging van het centraal zenuwstelsel. Zodra cellen die contact maken dit contact verliezen door - bijvoorbeeld door verwonding - zullen die cellen dramatische veranderingen ondergaan wat betreft hun morfologie en hun cytoskelet dat bestaat uit actine en microtubuli. Onze studie toont aan dat Tiam1 een functie heeft bij deze morfologische verandering die voorafgaat aan de migratie van zowel astrocyten als embryonale fibroblasten uit muizen (MEFs) als gevolg van het induceren van 'wonden' in monolagen van cellen. Door gebruik te maken van cellen zonder Tiam1 expressie (Tiam1 KO cellen) toonden we aan dat Tiam1-gemedieerde Rac activatie nodig is voor de veranderingen in celvorm en de vorming van uitlopers, die significant verminderd zijn in afwezigheid van het Tiam1 eiwit. Naast verminderde uitgroei van uitlopers observeerden we ook grote verschillen in de interne organisatie van deze uitlopers wat betreft het cytoskelet van microtubuli. Tiam1 blijkt niet noodzakelijk voor alle facetten van polarisatie van astrocyten. Een tweede belangrijke verandering die optreedt tijdens deze vorm van polariteit is namelijk de lokalisatie van het Golgi apparaat en het centrosoom (waar de vezels van het microtubule-cytoskelet worden aangemaakt). Eerdere studies door andere onderzoeksgroepen toonden aan dat deze verandering afhankelijk is van Cdc42 activatie en signalering via Par6 en aPKCzeta. In cellen zonder Tiam1 is de lokalisatie van het Golgi apparaat
Nederlandse samenvatting

en het centrosoom niet anders dan in cellen met normale Tiam1 expressie. We concludeerden we dat Tiam1 nodig is voor de gepolariseerde uitgroei van uitlopers en deze functie waarschijnlijk vervult door in te grijpen op de organisatie van het cytoskelet van microtubuli. Het exacte mechanisme waarmee Tiam1 de lange uitlopers veroorzaakt hebben we tijdens ons onderzoek niet volledig achterhaald. We hebben wel aanwijzingen verzameld dat Tiam1 betrokken is bij de stabilisatie van lange parallelle bundels van microtubuli die uitgestrekt worden tot de uiterste punt van de uitlopers richting de wondrand. In tegenstelling tot onze bevindingen in onderzoek naar epitheliale polariteit lijkt deze functie van Tiam1 niet vervuld te worden in samenwerking met het Par complex maar is er waarschijnlijk sprake van een parallelle signaleringsroute die leidt tot Rac activatie. Deze resultaten geven nieuwe inzichten in de mogelijke mechanismen die celpolarisatie en cellmigratie reguleren en demonstreren dat meerdere signaleringsroutes waarbij zowel Tiam1/Rac als het Par complex, samen en afzonderlijk, betrokken kunnen zijn bij deze processen.

Tot slot wordt in Hoofdstuk 7 een samenvatting gegeven van de behaalde resultaten en worden deze resultaten bediscussieerd aan de hand van recente studies.

Samenvattend, de studies die in dit proefschrift beschreven staan tonen aan dat Rho GTPases en hun regulatoren op meerdere manieren kunnen bijdragen aan het ontstaan en de progressie van verschillende soorten kanker. Verder blijkt er een grote mate van verbondenheid tussen de signaleringsroutes die betrokken bij zijn bij polarisatie van cellen en Tiam1/Rac signalering. Daarnaast is het duidelijk geworden dat de uitkomst van signalering via Tiam1/Rac afhing van de cellulaire context en Tiam1/Rac signalering betrokken is bij tal van strikt gereguleerde processen.
LIST OF PUBLICATIONS


