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THE FUNCTION OF TIAM1 IN TUMOR CELL BIOLOGY

TOMASZ P. RYGIEL

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THE FUNCTION OF TIAM1 IN TUMOR CELL BIOLOGY

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Chapter 1

General introduction and outline of the thesis

Chapter 1 - List of contents

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Physiological and pathological functions of Rho proteins
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Apical-baso-lateral cell polarity and epithelial-mesenchymal transition
Cell proliferation, apoptosis and tumor formation
Rho proteins in vivo – studies with GTPase knockout mice
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RHO GTPASES – BACKGROUND

Cancer is a disease characterized by uncontrolled cell proliferation which leads to tumor formation. Tumor cells may invade and destroy adjacent tissues, or even disseminate to distant organs. Nearly all cancers are caused by aberrations in the genetic material of the initiated cells. These genetic abnormalities typically involve two types of genes. The first type represents the cancer-promoting oncogenes, which are often found activated in cancer cells. Proteins encoded by these genes stimulate cell proliferation, protect against apoptosis and induce cell dedifferentiation. The second class of genes represents the tumor suppressor genes, the inactivation of which leads to inaccurate DNA replication, impaired cell cycle control and inhibition of apoptosis. Aberrant control of processes regulated by small GTPases of the Rho family may contribute to or even induce tumor formation.

Rho GTPases belong to the superfamily of Ras-like small GTPases. They are widely expressed and have diverse physiological functions. Rho GTPases control a large number of cellular processes including actin- and microtubule cytoskeleton reorganization, cell migration and invasion as well as gene transcription (Heasman and Ridley, 2008). In the past, most studies have concentrated on three members of the Rho family i.e. RhoA, Rac1, and Cdc42. However, the Rho GTPase family is much larger and according to protein sequence homology consists of 7 subfamilies: Rho, Rac, Cdc42, Rnd, RhoBTB, Wrch and RhoT/Miro (Fig. 1) (Ellenbroek and Collard, 2007). In the human genome twenty-three gene loci exist, which by alternative splicing encode at least twenty-six different proteins. For instance, Rac1b represents a splice variant of Rac1 (Bustelo et al., 2007). RhoBTB and RhoT are least homologous to the other members of the Rho family because of their structural distinction. All Rho GTPases, with exception of RhoBTB and RhoT, are small 200-250 amino acids proteins containing a GTPase catalytic domain. Amino acid sequences of the GTPase domains of Rho proteins share around 30% identity with Ras GTPases, and 40-95% identity within the Rho family (Wennerberg and Der, 2004). Rho proteins bind GTP or GDP which determines their conformation and activity. Rho GTPases in the GTP-bound form are active and can bind a large variety of downstream effector proteins. Each member of the Rho GTPase family has multiple downstream effectors. Interestingly, a number of effectors can be activated by different Rho GTPases. Binding to the effectors is frequently accommodated by scaffold proteins leading to a large variety of protein complexes and thereby a variety of downstream signaling. Many of these effectors are serine-threonine kinases that control downstream signaling by phosphorylating their target proteins. Although over 60 effector proteins are known for RhoA, Rac1 and Cdc42, surprisingly, no common sequence motifs in these effectors have been identified (Etienne-Manneville and Hall, 2002;Bustelo et al., 2007). This illustrates the complexity of the interactions of Rho proteins with effectors and thereby the intricacy of processes controlled by Rho proteins.

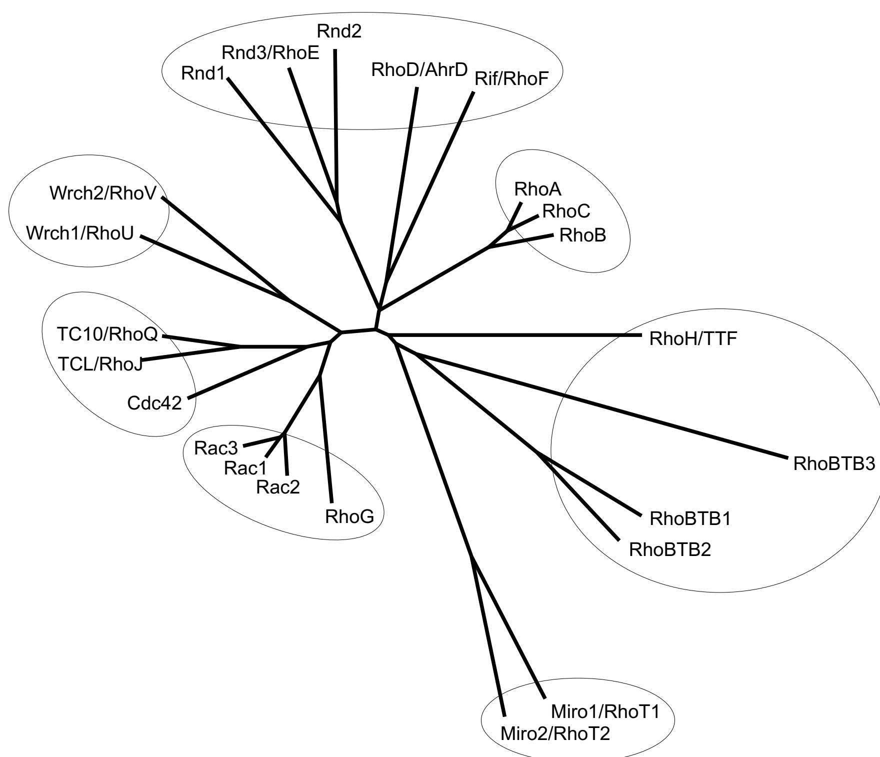


Figure 1. An unrooted phylogenetic tree depicting the relationship between Rho GTPases. The dendrogram demonstrates the relation between different Rho GTPase family members. Ovals indicate the seven closer related subfamilies: RhoA-related, Rnd proteins, Wrch subfamily, Cdc42-related, Rac-related, Miro proteins and the least clustered RhoBTB subfamilly. The tree was constructed using sequences of the human proteins with application of CLUSTALW multiple sequence alignment tool (http://align.genome.jp/) and the Treeview graphic program.

REGULATION OF RHO GTPASES

The activity of Rho proteins is controlled at several levels and includes the activation state of the GTPase, its intracellular localization, the transcription regulation and/or protein degradation. The most important regulatory mechanism of Rho proteins is the guanine nucleotide binding to its GTPase domain. This domain can bind either GTP or GDP which affects the protein conformation and thereby enables interaction with downstream effectors (Fig. 2). GTP-bound Rho proteins are thus in an active state and bind effectors proteins whereas GDP-bound Rho proteins are inactive. Rho proteins posses low intrinsic GTPase activity and can hydrolyze GTP to GDP thereby reverting the protein conformation and activation state. This mechanism ensures temporary activation of Rho proteins.

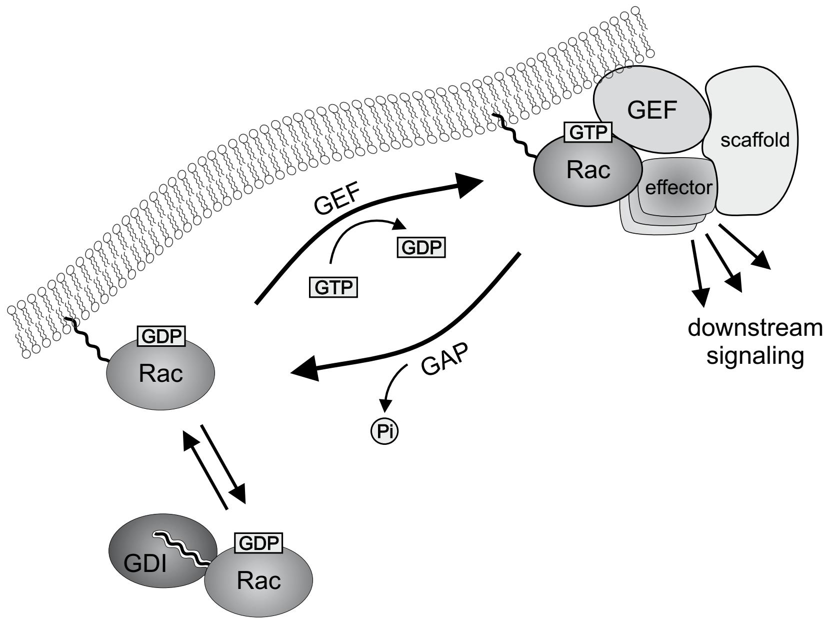
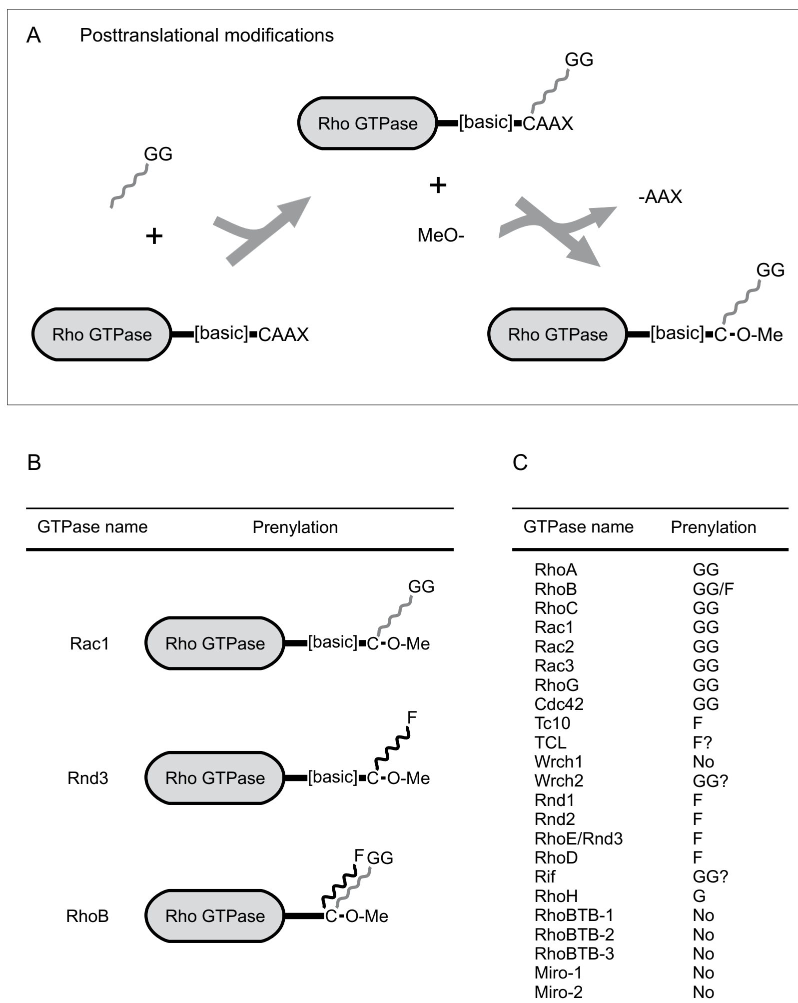


Figure 2. Regulation of Rho GTPase activity as depicted for the Rac GTPase. Inactive Rac, in the GDP bound state, can be sequestered in the cytoplasm by GDI proteins. GDI binding masks the isoprenyl chain and blocks Rac translocation from the cytoplasm to the plasma membrane. Rac released by GDI can bind to the plasma membrane where it can be activated. Rac can bind either GDP or GTP. The exchange of GDP for GTP is facilitated by GEFs and GAP proteins stimulate the hydrolysis of GTP. GEFs stimulate GTP binding and thereby activate Rac. GEFs may influence Rac downstream signaling by selective binding to Rac effectors as well (see also text).

Two groups of proteins control directly the Rho GTPase cycling between GTP and GDP binding. The first group consists of guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP for GTP, thereby activating Rho proteins. Members of the second group are GTPase activating proteins (GAPs). These proteins inactivate Rho GTPases by stimulating the intrinsic GTPase activity thus the hydrolysis of bound GTP to GDP. The family of Rho GEFs consists of around 70 members which all contain a Dbl homology (DH) catalytic GTPase domain (Rossman et al., 2005). Apart of these classical GEFs, a newly identified family of non-conventional GEFs exists. These so-called CDM proteins facilitate Rho GTPase activation by a DH-unrelated domain and Ced-5, Dock180, and Myoblast-city (CDM) are the most known members (Meller et al., 2005) (Rossman et al., 2005). Most of the Rho GTPases are also negatively regulated by guanidine nucleotide dissociation inhibitors (GDIs). GDIs interact with GDP bound Rho GTPases and reassure their inactive state by two means. Firstly, GDIs inhibit nucleotide exchange thereby locking GTPase in the GDP-bound inactive state. Secondly GDIs sequester Rho proteins in the cytoplasm preventing them from activation by plasma membrane associated GEFs. Most Rho GTPases require membrane association for their proper activation and function.



GTPase name	Prenylation
RhoA	GG
RhoB	GG/F
RhoC	GG
Rac1	GG
Rac2	GG
Rac3	GG
RhoG	GG
Cdc42	GG
Tc10	F
TCL	F?
Wrch1	No
Wrch2	GG?
Rnd1	F
Rnd2	F
RhoE/Rnd3	F
RhoD	F
Rif	GG?
RhoH	G
RhoBTB-1	No
RhoBTB-2	No
RhoBTB-3	No
Miro-1	No
Miro-2	No

Figure 3. Posttranslational modifications of Rho proteins. A. Rho GTPases are prenylated on the cystein in the CAAX C-terminal motif. This modification is followed by proteolysis of the last 3 amino acids and methylation of the terminal cystein. The polybasic domain is located just upstream of the CAAX motif. B. Examples of possible prenylations: geranyl-geranylation (GG), farnesylation (F). In the case of RhoB both modifications are possible. C. List of possible prenylation in the Rho GTPase family.

The subcellular localization of Rho GTPases is achieved by posttranslational modifications. The carboxy-terminal sequence of some of the Rho GTPases contains a so-called "CAAX box" motif. This conserved protein motif undergoes prenylation such as the attachment of a geranyl-geranyl or farnesyl group (Winter-Vann and Casey, 2005) (Fig. 3). Prenylation is followed by proteolytic removal of the last three amino acids of the C-terminal (AAX) motif and subsequent methylation of the C-terminal prenyl-cystein (Yamane et al.,

1991). Incorporation of farnesyl or geranyl-geranyl groups is required for proper membrane anchoring and biological activity of Rho GTPases. Interestingly, RhoB can be either farnesylated or geranyl-geranylated and these modifications localize the protein either in the plasma membrane or in endomembranes, respectively (Lebowitz et al., 1997). The subcellular localization of Rho GTPases is also affected by the polybasic domain located just upstream of the CAAX box. Small sequence variation, in the motif immediately upstream of the CAAX box - polybasic domain, determines intracellular localization and/or biological effects of very homologous proteins (Filippi et al., 2004). An example of this is the Rac subfamily where small changes in the polybasic region determine the different intracellular localization of Rac1, Rac2 Rac3, and RhoG (Prieto-Sanchez and Bustelo, 2003;Hajdo-Milasinovic et al., 2007).

Most of the Rho proteins are ubiquitously expressed but some show tissue or cell type specific expression. For instance, the expression of Rac2 is restricted to haematopoietic cells only. The expression of RhoG is induced during the G1 phase of cell cycle (Vincent et al., 1992) whereas Wrch1 is a Wnt responsive gene of which the expression is induced by activation of the Wnt signaling pathway (Tao et al., 2001). Additional level of Rho GTPase regulation is controlled by local changes in the protein stability or degradation. For instance, migrating cells show mutually exclusive local activation of RhoA and Rac1. It ensures that Rac1 and Cdc42 activity is restricted to protruding lamellae whereas RhoA is active in the retracting cell-tail. All this is achieved by local ubiquitination and degradation of RhoA at the cell front, a process which is dependent on Rac1 and Cdc42 activity (Wang et al., 2003).

PHYSIOLOGICAL AND PATHOLOGICAL FUNCTIONS OF RHO PROTEINS

Cytoskeleton remodeling, cell migration and tumor metastasis

RhoA, Rac1 and Cdc42 are the major regulators of dynamic changes of the actin cytoskeleton in response to receptor stimulation or matrix interactions. However, they have distinct or even counteractive functions. Rac1 induces actin-rich protrusions or lamellipodia at the leading edge of migrating cells whereas RhoA is involved in the generation of contractile forces essential for retraction of the cell body and tail (Worthylake et al., 2001). Cdc42 has a role in the formation of filopodia (Chen et al., 2000). Indeed, Cdc42-deficient mouse embryonic fibroblasts (MEFs) completely lack filopodia (Yang et al., 2006). The local activation of Rac in the front of migrating cells is necessary for cell polarization in many cell types e.g. epithelial cells and lymphocytes (Pegtel et al., 2007;Gerard et al., 2007). Actin polymerization is a coordinated process of assembly and disassembly of filaments. The actin polymerization process is controlled by the Arp2/3 complex and mDia whereas actin de-polymerization occurs by cofilin. Both Rac1 and Cdc42 induce peripheral actin

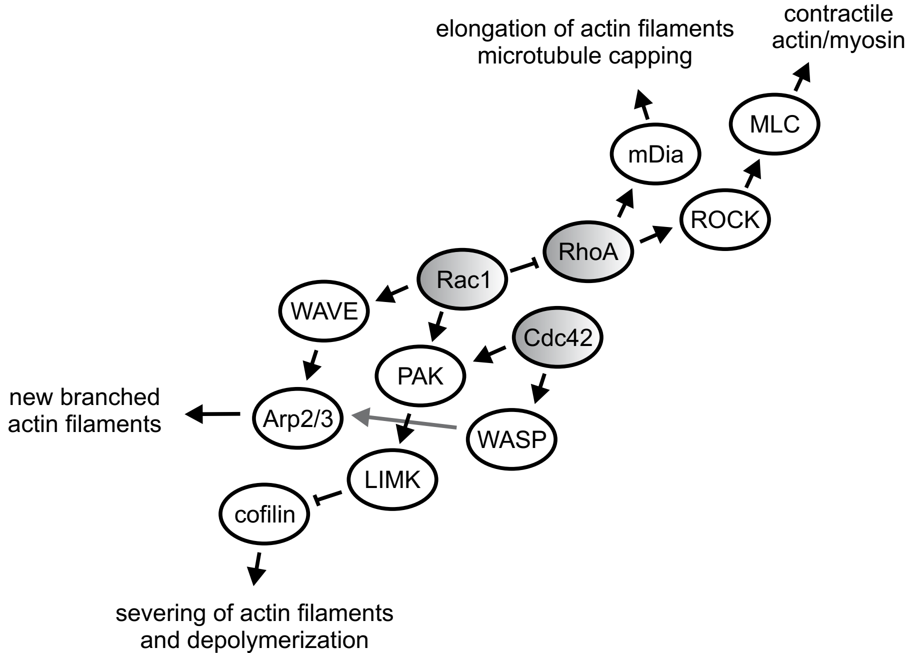


Figure 4. Actin-microtubule cytoskeleton is controlled by Rac1, Cdc42 and RhoA. Both Rac1 and Cdc42 induce actin polymerization by Arp2/3 activation. This process is mediated by their downstream effectors WAVE and WASP, respectively. Rac and Cdc42 through PAK stimulate LIM kinase (LIMK), a negative regulator of cofilin. Inhibition of cofilin stops actin filament fragmentation and depolymerization. Rac1 is able to inhibit RhoA activity and its downstream signaling. The main RhoA effector ROCK activates MLC-kinase and induces actin/myosin contractility. RhoA also stimulates actin filaments elongation by activation of mDia.

polymerization by induction of branched filament networks through activation of the Arp2/3 complex (Eden et al., 2002;Ho et al., 2001)(Fig. 4). Rac1 and Cdc42 independently activate Arp2/3 by their effectors WAVE and WASP, respectively. Another major mechanism of actin filament polymerization is dependent on RhoA and its effector mDia. MDia binds at barbed ends of the filaments and adds successive actin monomers (Zigmond, 2004). Both Rac1 and Cdc42 inhibit cofilin function by their downstream effector p21-activated kinase (PAK). PAK activates LIM kinase a negative cofilin regulator, which phosphorylates cofilin and thereby prevents actin filament severing (Pollard and Borisy, 2003;Svitkina and Borisy, 1999). LIM kinase-dependent cofilin phosphorylation is also induced by the RhoA/ ROCK pathway (Ohashi et al., 2000). These examples clearly show how Rac1 and to lesser extend Cdc42 oppose RhoA function and vice versa.

The actin filament network is only a part of a more intricate cellular cytoskeleton complex. Interaction of actin cytoskeleton with microtubule network is crucial for cell shape establishment and cell migration. Therefore, it is not surprising that Rho GTPases regulate the dynamics and organization of microtubules as well. Cdc42/Rac1-dependent

PAK activation induces elongation of microtubules (Daub et al., 2001). Rho GTPases control interaction of microtubule plus ends with proteins of the cell cortex. The RhoA effector mDia takes part in the stabilization of plus ends at the cell cortex (Wen et al., 2004). Furthermore, Rac1 and Cdc42 can induce microtubule capture in the cell cortex by stimulation of binding IQGAP to the capture protein CLIP-17 (Fukata et al., 2002). The cytoskeleton dynamics controlled by Rho GTPases affects also other cellular processes including vesicular trafficking, exo- and endoctyosis and phagocytosis (Ridley, 2006). Furthermore, Rho-mediated cytoskeleton dynamics control proper initiation and maintenance of cell adhesions (both cell-cell and cell-matrix adhesions) required for tissue organization and cell migration.

Apical-baso-lateral cell polarity and epithelial-mesenchymal transition

Sheets of epithelial cells delimit organs and separate them from the environment e.g. the skin and gut. Epithelial cells have an asymmetric morphology with specific junctional complexes such as adherens junctions (AJs) and tight junctions (TJs) that connect neighboring epithelial cells. AJs, typically consisting of classical cadherins, mediate cell-cell adhesion whereas TJs ensure a transepithelial barrier for molecule diffusion and separate the cell membrane components in epithelial cells into apical- and basolateral domains (Ebnet, 2008). Rho GTPases regulate the establishment and maintenance of cell junctions as well as the cross-talk between AJs and TJs (Yamanaka and Ohno, 2008). RhoA and mDia-driven actin polymerization are required for the formation and maintenance of AJs. On the other hand, signaling of the RhoA-effector ROCK induces disruption of AJs by enhanced cell contractility (Sahai and Marshall, 2002). Interestingly, Rac1 signaling can induce either stabilization or disassembly of AJs (Malliri et al., 2004;Braga et al., 2000). Thus, a reciprocal balance of Rac and Rho activities may steer cell characteristics into a more epithelial or mesenchymal phenotype (Sander et al., 1999). During contact formation of epithelial cells, the clustering of E-cadherin leads to the establishment of primordial cell-cell adhesions and the activation of Rac1 and Cdc42 (Nakagawa et al., 2001); (Kim et al., 2000). Subsequent maturation of these adhesions into AJs and TJs requires activation of the Par3 polarity complex including atypical PKC downstream of Rac1 (Yamanaka et al., 2001). In keratinocytes this process depends on the Rac1-GEF Tiam1 which couples Rac1 activation to the Par polarity complex (Mertens et al., 2005). Establishment of apical-basal polarity is negatively regulated by RhoA activity (Noren et al., 2001). However, RhoA and localized acto-myosin contractility is required during epithelial polarization (Ivanov et al., 2005). This illustrates the complexity of the function of Rho proteins in polarization processes, which is also cell type dependent.

As already mentioned, Rho GTPases regulate the assembly of TJs and cell polarity in conjunction with the Par polarity complex. Remarkably, Rho GTPases are involved in polarity signaling in different contexts in various cell types. In addition to a function in

the establishment of apical-basal polarity, the Rac activator Tiam1 and the Par polarity complex regulate front-rear polarity and directional cell migration in keratinocytes (Pegtel et al., 2007). Establishment of cellular asymmetry and axon specification during neuronal morphogenesis also involves collaboration between Rho GTPases and the Par polarity complex. Activation of the Ras-like GTPase Rap1 and Cdc42 is a necessary upstream event to activate the Par complex and subsequently Rac1 during axon specification (Schwamborn and Puschel, 2004). The Rac GEF Tiam1 is particularly involved in Rac-mediated selected axon outgrowth (Nishimura et al., 2005). Interestingly, a similar signaling pathway is activated during chemokine-induced lymphocyte polarization and migration. Chemokines activate Rap1 that activates Cdc42 that subsequently stimulates the Par complex which in turn activates Rac1 through the Rac-GEF Tiam1 (Gerard et al., 2007) (Fig. 5). The data described above underline the remarkable flexibility of the Par complex and Rho GTPases that control distinct types of cellular polarity.

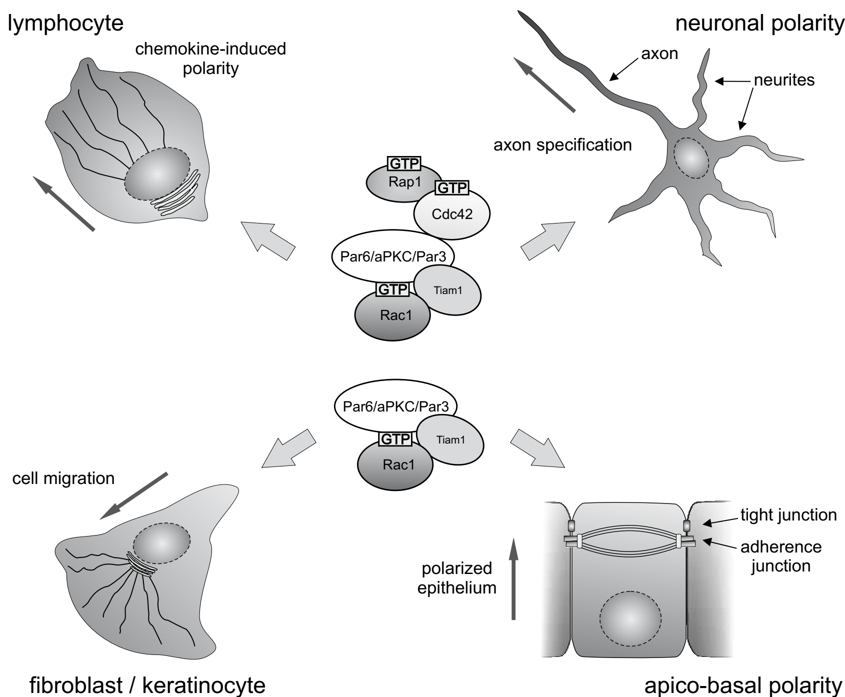


Figure 5. The Par polarity complex controls various cell polarity events. During axon specification in neuronal cells and in chemokine-induced T-cell polarization, Rap1 -through activation of Cdc42 - activates the Par-polarity complex leading to Tiam1-mediated Rac activation and cytoskeleton reorganizations required for cell polarization. During epithelial apico-basal cell polarization, Tiam1-mediated Rac1 activation triggers the Par complex and induces atypical PKC signaling leading to cytoskeleton reorganization required for polarization. Chemokine-induced migration of both T-cells and epithelial cells is also dependent on Cdc42

Epithelial-mesenchymal transition (EMT) is a developmental program characterized by loss of cell-cell adhesion, inhibition of E-cadherin expression, and increased cell motility. EMT is essential for many developmental processes such as neural tube formation. During EMT epithelial cells lose their cell-cell adhesions and apical-basal polarity and acquire a fibroblastoid morphology and increased migratory potential (Yang and Weinberg, 2008). Invasive or metastatic tumor cells also undergo EMT-like processes. Remarkably, inactivation of at least one of the genes controlling cell polarity (scrib, lgl, cdc42) combined with expression of oncogenic RasV12 leads to metastatic behavior of tumors, including loss of E-cadherin expression, migration, invasion, and secondary tumor formation (Pagliarini and Xu, 2003). This illustrates the potential function of polarity proteins in tumor progression. A potent EMT stimulus is TGF-β, which down-regulates the expression of polarity proteins such as Par3 and induces disassembly of TJs (Wang et al., 2008). Interestingly, TGF-β-induced EMT effects are conducted by inhibition of RhoA signaling (Ozdamar et al., 2005). Thus, not only AJ disassembly but also TJ disruption can lead to EMT and tumor invasion. Invasion of malignant skin- and intestinal tumors is enhanced by Tiam1-deficiency (Malliri et al., 2002). Indeed, Tiam1 down-regulation in MDCK cells promotes loss of epithelial polarity and EMT (Malliri et al., 2006). Other Rho proteins may also contribute to tumor progression. RhoA is required for trans-endothelial migration of monocytes (Worthylake et al., 2001) and RhoC has been implicated in metastasis of lung cancer cells (Ikoma et al., 2004).

Tumor invasion is a complex process that involves not only the acquisition of migratory capacity and release of cell-cell adhesions, but also proteolysis of extracellular matrix (ECM) components. Proteolysis of ECM enables invasive cells to penetrate the basal lamina and Rho GTPases are involved in ECM proteolysis as well. In normal human keratinocytes, Rac1 and RhoA induce MMP-1 expression by ERK1/2 activation whereas Cdc42 represses MMP-1 expression by activation of p38 (Deroanne et al., 2005). On the other hand, Rac1 can inhibit metalloproteinases by inducing the expression of their inhibitors. Active Rac1 and its GEF Tiam1 induce the expression of tissue inhibitors of metalloproteinase-1 TIMP-1 and TIMP-2 and thereby inhibit renal cell carcinoma invasion (Engers et al., 2001). Interestingly, Tiam1/Rac1 induced TIMP-1 expression is mediated by ROS production and ERK1/2 activation (Engers et al., 2006). The importance of Rac activity for cell migration was shown in *in vivo* experiments where wound re-epitheliation in skins of mice was impaired by lack of the Rac1-activator Tiam1 or the expression of a dominant-negative Rac1 (Hamelers et al., 2005;Tscharntke et al., 2007). Thus, many cellular processes controlled by Rho GTPases signaling may contribute to tumor invasion and progression.

Cell proliferation, apoptosis and tumor formation

In general activation of Rho GTPases increases the rate of cell proliferation. Inactivation of Rho GTPases by toxin treatment or expression of dominant negative mutants of RhoA,

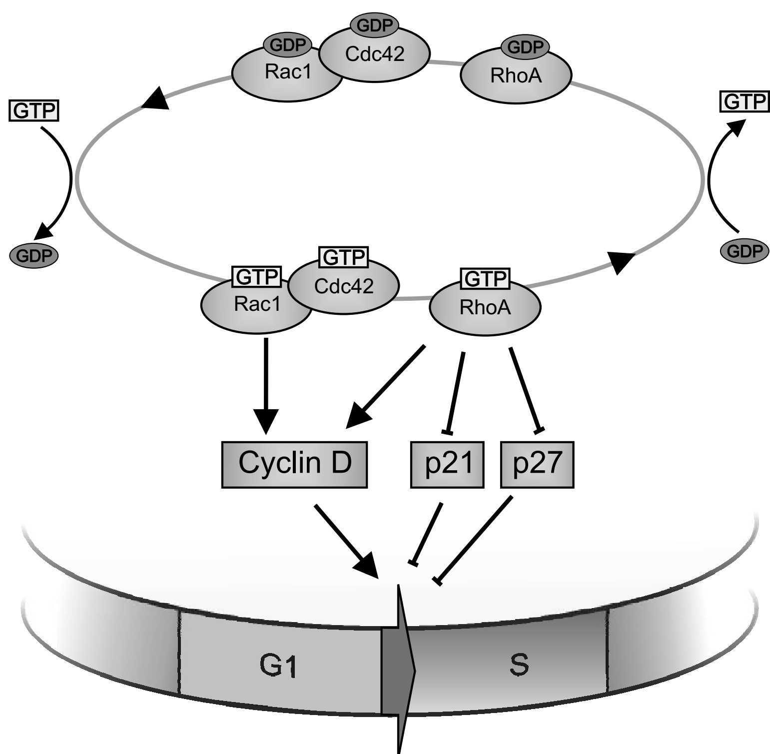


Figure 6. Diagram illustrating the effects of Rho GTPases on cell cycle progression. When activated all three GTPases Rac1, Cdc42 and RhoA can stimulate cyclin D expression. This leads to G1-S phase cell cycle progression. Furthermore, RhoA inhibits expression of the negative regulators of cell cycle progression, CDKIs p21 and p27, (see also text).

Rac1 and Cdc42 blocks G1-S phase transition (Yamamoto et al., 1993;Olson et al., 1995) (Fig. 6). Cell cycle progression is controlled by Cyclins, Cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs). One of the most studied cyclins is Cyclin D1, which expression is increased in normal proliferating cells and tumor cells (Diehl, 2002). Both Rac1 and RhoA are able to induce Cyclin D1 expression. Rac1 signals through either NFκB or PAK whereas RhoA signals through the activation of ROCK (Joyce et al., 1999;Cai et al., 2003;Croft and Olson, 2006). Furthermore, RhoA induces expression of Cyclin D1 in mid-G1-phase through sustained activation of ERK1/2. Rac1 and Cdc42 activities are also necessary for Cyclin D1 expression in the early G1-phase (Welsh et al., 2001). RhoA modulates CDKIs levels and downregulation of one of the CDKIs (p21) by RhoA is necessary for Ras induced cell cycle entry (Liberto et al., 2002). On the other hand, RhoA inhibits p21 expression by the transcription factor SP1 (Adnane et al., 1998). Also activated Rac1 inhibits p21 expression as shown in prostate cancer cells (Knight-Krajewski et al., 2004). Cell adhesion mediated degradation of p21 is dependent on Rac1 and Cdc42 activity (Bao

et al., 2002). The CDK inhibitor p27 is regulated by RhoA. Activation of RhoA inhibits p27 translation which is important for Ras induced cell transformation (Vidal et al., 2002). Thus, it seems that Rho GTPases contribute to cell cycle progression by different means, which could explain why high levels of Rho GTPases are frequently found in cancer cells. This makes them potential drug targets for the treatment of cancer.

Tumor initiation is enhanced by escaping the natural anti-tumor mechanisms such as apoptosis. Rho GTPases are involved in the regulation of cell survival both in pro- and anti-apoptotic signaling. Cdc42, Rac1 and RhoA have initially been implicated in the stimulation of NFκB, a major anti-apoptotic pathway (Perona et al., 1997) (Fig. 7). Rac-mediated generation of reactive oxygen species (ROS) is necessary for the transcriptional activity of NFκB (Kheradmand et al., 1998). However, these results have been questioned as other studies showed that NFκB is not regulated by ROS (Hayakawa et al., 2003). The ability of Rac1 to produce ROS is dependent on the protein motif called the insert region (residues 124-135) (Joneson and Bar-Sagi, 1998). Rac1 is a well-established activator of NADPH oxidase (Nox) in phagocytic (Abo et al., 1991) and nonphagocytic cells (Cheng

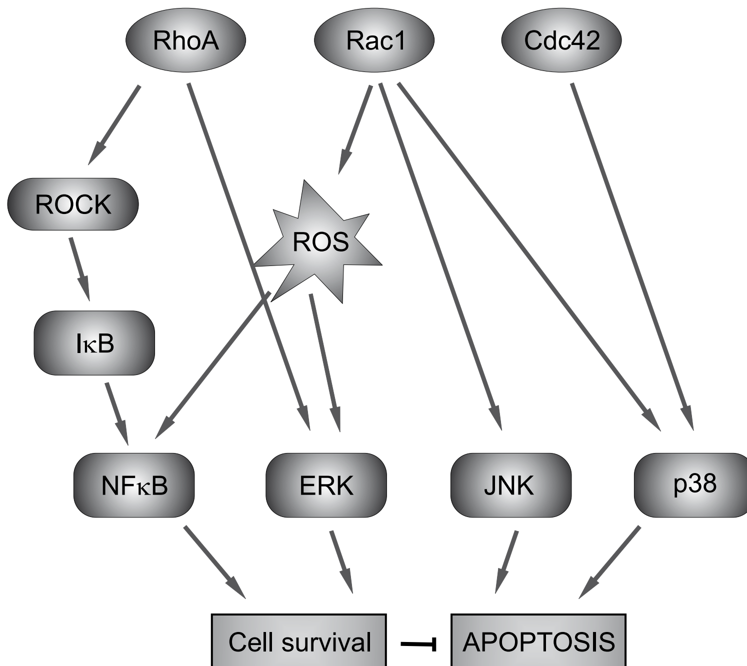


Figure 7. Involvement of Rho GTPases in the cell survival and apoptosis signaling. RhoA can activate NFκB by ROCK and IκB stimulation. Rac1 can also induce NFκB by ROS-production dependent mechanism. Furthermore, RhoA counteracts apoptosis by activation of ERK signaling. Rac1 can also stimulate ERK by a ROS-dependent pathway. Rac1 activates stress-induced kinases such as JNK and p38. Cdc42 can induce p38 activation as well.

et al., 2006;Ueyama et al., 2006). High concentration of ROS are pro-apoptotic but at low concentrations ROS is mitogenic and shows pro-survival effects (Arnold et al., 2001;Liu et al., 2005). Tiam1/Rac1 signaling is able to induce ROS-dependent ERK1/2 activation and promotes survival of keratinocytes (Rygiel et al., 2008). Furthermore, ROS has been implicated in Rac1-mediated inactivation of RhoA. By ROS production, Rac1 activates a RhoA-specific GAP, p190RhoGAP, which leads to the inactivation of RhoA (Nimnual et al., 2003). Interestingly, recent study showed that Rac1 induced ERK activation is Nox4 dependent (Wu et al., 2007). The use of intracellular ROS levels as a signaling molecule may be dangerous as too high levels of ROS can induce senescence or even apoptosis. An example of this comes from studies in primary embryonic fibroblasts, where Rac1 and its splice variant Rac1b dependent ROS production enhances cell senescence and genomic instability (Debidda et al., 2006). Rac1 and Cdc42, especially when constitutively active, may cooperate in the pro-apoptotic signaling by activating stress induced MAPKs p38 and JNK (Lee et al., 2001;Minden et al., 1995). Rac1 and its target protein POSH are involved in JNK-mediated induction of apoptosis (Xu et al., 2003). Activation of both p38 and JNK may thus induce apoptosis; however effects on cell survival and apoptosis seem to depend on the signaling context. Thus, high activity of Rho GTPases is generally beneficial for the survival of cancer-initiated cells and provides them with a selective advantage during tumor formation and growth.

Rho proteins in vivo – studies with GTPase knockout mice

The functions of several Rho GTPases have been investigated in gene knockout experiments. Rac1 deletion leads to embryonic lethality caused by gastrulation defects and increased apoptosis (Sugihara et al., 1998). However, the tissue-specific knockout of Rac1 enables to study its function in various situations *in vivo*. This approach proved to be very fruitful and so far established Rac1 function in several processes such as: neutrophil migration (Glogauer et al., 2003), hematopoietic (Gu et al., 2003) and epidermal stem cell homeostasis and hair development (Benitah et al., 2005;Chrostek et al., 2006). Furthermore, lack of Rac1 negatively affects the following physiological processes such as Nox activation in cardiomyocytes (Satoh et al., 2006), brain development (Chen et al., 2007) and formation of myelin sheets of the central nervous system (Nodari et al., 2007;Benninger et al., 2007) as well as B-cell development but only in the absence of Rac2 (Walmsley et al., 2003). Rac2-deficient mice have only hematopoietic defects such as impaired neutrophil chemotaxis and superoxide production (Roberts et al., 1999), consistent with the findings that the expression of Rac2 is restricted to lymphoid and myeloid cells. Rac3 knockout mice have slight motor coordination problems and enhanced learning abilities (Corbetta et al., 2005), confirming its importance in the neuronal tissue where it is mainly expressed. RhoG knock-out mice develop normally but have enhanced antigen responses of T and B cells (Vigorito et al., 2004). Cdc42 knock-out, similarly like Rac1 is early embryonic lethal

(Chen et al., 2000), whereas Cdc42-negative embryonic stem cells have defects in polarity and Golgi localization (Czuchra et al., 2005), which substantiates the established role of Cdc42 in cell polarization *in vitro*. Tissue specific knock-out of Cdc42 indicate it function in: hematopoietic stem cell homeostasis (Yang et al., 2007), brain development (Cappello et al., 2006; Chen et al., 2006), skin stem cells and hair development (Wu et al., 2006), Schwann cell proliferation and axono-genesis (Garvalov et al., 2007;Benninger et al., 2007). Thus, removal of Rac1 and Cdc42 affects similar processes and tissues, suggesting that both GTPases act partly in the same signaling pathways. Knock-out of Cdc42 in liver promotes hepatocellular carcinoma development (van Hengel et al., 2008).

Global knockout of RhoB or RhoC have no obvious phenotype but RhoB-deficient mice are more susceptible for DMBA-induced skin tumors whereas oncogene-transformed RhoC-deficient cells show reduced metastatic potential (Liu et al., 2001;Hakem et al., 2005). RhoH knock-out mice show a defect in T-cell receptor signaling and thymocyte maturation (Dorn et al., 2007). Further studies on the *in vivo* function of the respective Rho GTPases and additional Rho proteins still needs to be done but such studies certainly will lead to novel insights into the essential function of Rho GTPases in various cellular processes and tissues *in vivo*.

Rho GTPases in tumorigenicity

Oncogenic transformation is a multi-step process which requires deregulation of cellular mechanisms responsible for the control of cell proliferation and survival (Fig. 8). This can be achieved by the activation of oncogenes often in combination with the inhibition of tumor suppressor genes. Mutations in various oncogenes are frequently found in human cancers, especially in the Ras genes, or in some of their downstream targets. Unlike in the Ras family proteins no point mutations in genes encoding Rho GTPases have been found in human tumors. An exception is RhoH as point mutations in RhoH are found in B-cell lymphomas and RhoH gene rearrangements are found in non-Hodgkin's lymphomas and multiple myelomas (Preudhomme et al., 2000;Pasqualucci et al., 2001). Overexpression of active mutants of RhoA, Rac1 and Wrch1 can induce morphological transformation of fibroblasts, whereas Cdc42 has weaker transforming activity (Zong et al., 2001;Westwick et al., 1997;Wu et al., 1998;Shutes et al., 2004). Overexpression of several Rho proteins (RhoA, RhoB, RhoC, Rac1, Rac1b, Rac2, Rac3, RhoG, Cdc42, Wrch2) or their regulators has been found in various types of tumors including breast, colorectal, and lung tumors (Ellenbroek and Collard, 2007). The increased expression of these proteins suggests a potential function of the activated proteins in the formation and/or progression of these tumors. Indeed, *in vitro* studies indicate that inhibitory mutants of RhoA, RhoG, Rac1, TC10 and

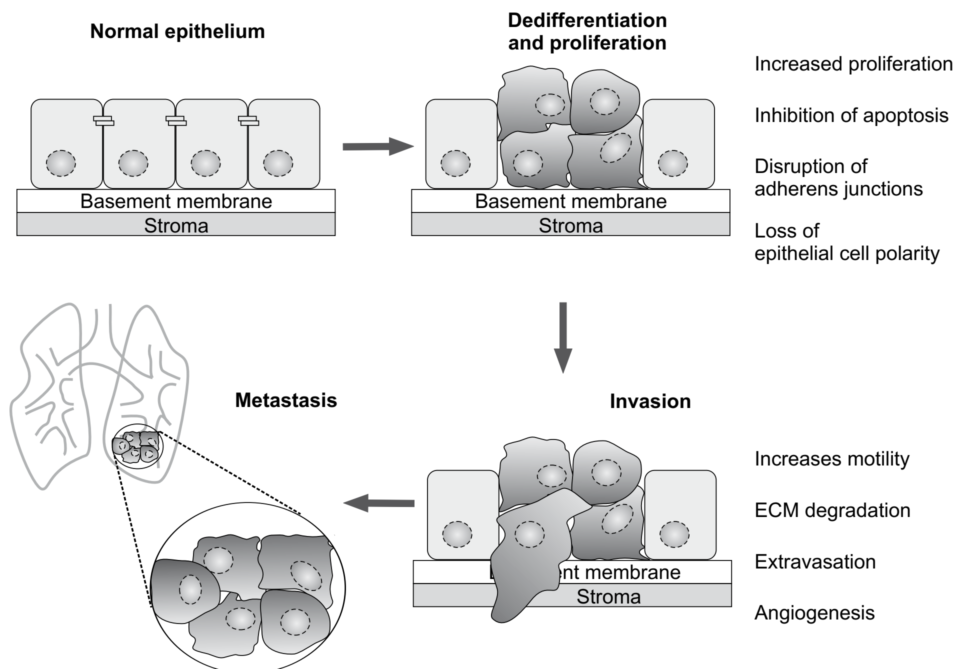


Figure 8. Involvement of Rho GTPases in tumor initiation and progression. Rho GTPases contribute to the early and late events in tumorigenicity. Upregulation of Rho GTPase signaling can frequently lead to increased cell proliferation and inhibition of apoptosis. Deregulation of Rho proteins signaling can enhance tumor progression by loss of cellular polarity and disruption of adherence junctions and EMT. In the later stage of tumor progression cells may acquire a migratory phenotype and the ability to degrade extracellular matrix components, both processes are controlled by Rho GTPases. Furthermore Rho proteins can influence angiogenesis, which is necessary for the growth of large tumors by facilitating supply of nutrients and oxygen. RhoGTPases have also been implicated in metastasis especially in processes such as extravasation and survival in the blood stream (see also text).

Cdc42 prevent transformation of fibroblasts by oncogenic Ras (Qiu et al., 1995b;Qiu et al., 1995a;Qiu et al., 1997;Roux et al., 1997;Murphy et al., 1999). This supports a model where Rho proteins collaborate with oncogenic Ras in the morphological transformation of cells.

The lack of oncogenic mutations found in genes encoding Rho GTPases could be due to the requirement of Rho proteins to cycle between an active and inactive confirmation. Activating mutations that cause constitutive activation of Rho GTPases do not provide the ability to cycle. Proper activation of Rho GTPases and the cycling mechanism is provided by overexpression of Rho GTPases or by activation of their GEFs or inhibition of their GAPs. This idea is supported by the findings that Rho GEFs are more efficient in morphological transformation of fibroblast than constitutively active mutants of Rho GTPases (Jaffe and Hall, 2002). Additionally, fast-cycling mutants of Rac1, RhoA and especially

Cdc42 appear to have higher transforming capacity than the constitutively active mutants of these GTPases (Lin et al., 1999;Fidyk et al., 2006). This suggests that efficient morphological transformation requires not only activated GTP-bound GTPases but particularly the cycling of the GTPases between the GDP and GTP bound forms. As discussed earlier, GEFs are also able to direct GTPase downstream signaling. This is achieved by the ability of GEFs to select and bind particular effector proteins for specific GTPase-downstream signaling (Mertens et al., 2003;Rossman et al., 2005). There are several examples of GEFs and GAP involved in tumorigenesis, reviewed by in (Ellenbroek and Collard, 2007). The Rac-specific GEF Tiam1 is mutated in renal-cell carcinoma (RCC) cell lines and in primary RCC (Engers et al., 2000). The RhoA-specific GEF LARG is involved in a chromosome translocation and creates a fusion protein with mixed linkage leukemia (MLL) found in leukemias (Kourlas et al., 2000). LARG cooperates also with activated Raf-1 in NIH3T3 cell transformation, indicating that LARG harbors oncogenic potential (Reuther et al., 2001). On the other hand, GAPs can function as tumor suppressor genes. For instance, the p190RhoGAP gene is frequently deleted in human gliomas whereas exogenous expression of p190RhoGAP suppresses glioma-like cellular phenotype (Wolf et al., 2003). Concerning the involvement of Rho GTPases in tumorigenesis, it must be noted that aberrant expression of their regulators may contribute to tumorigenesis as the activity of Rho proteins and their downstream signaling is the key of their oncogenic potential.

Tumors that are unable to attract new blood vessels are restricted in their growth as a result of lack of oxygen and essential nutrients. Some tumors can induce blood vessel growth (angiogenesis) by secreting various growth factors including Vascular Endothelial Growth Factor (VEGF). A significant body of evidence suggests that RhoA plays an essential role in angiogenesis downstream of VEGF receptors, reviewed in (Bryan and D'Amore, 2007). RhoA signaling is critical in regulating endothelial cells assembly into new blood vessels (Hoang et al., 2004). Also Rac1 has been implicated in the capillary tube assembly of endothelial cells (Connolly et al., 2002). Thus, Rho GTPases do not only contribute to tumor initiation and growth but also support angiogenesis, a hallmark of malignant progression. The data discussed above suggest that Rho GTPases do not act as true oncogenes but rather contribute to various aspects of oncogenic transformation, which includes tumor initiation, tumor growth, angiogenesis and metastasis.

Tiam1, a GEF that activates Rac

As discussed in the paragraph 2 of this chapter, Rho GTPase activity is tightly regulated by various mechanisms. The most important regulators are the GEFs that control the nucleotide exchange and thereby the activity of the GTPases. The importance of nucleotide exchange is emphasized by comparison of the transforming capacity of Rho GTPases and

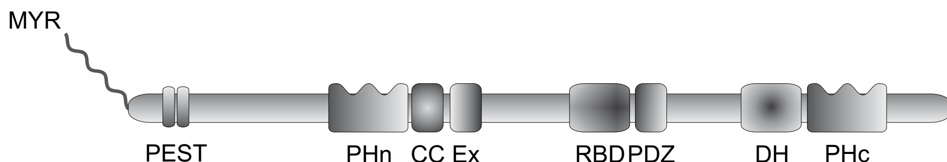


Figure 9. Schematic representation of the protein domains present in Tiam1. Abbreviations used: MYR-myristylation site, PEST motif - acts as a signal peptide for protein degradation, PHn and PHc N-terminal and C-terminal pleckstrin homology domains, CC (Coiled coil domain) and Ex (extension domain) are protein-protein interaction domains, RBD Ras binding domain, PDZ (post synaptic density protein (PSD95)) domain, DH – (Dbl homology domain, catalytical domain) (for description of domain function see the text).

their GEFs. Tiam1 represents a specific GEF for Rac1 but is also able to activate Rac2 and Rac3 (Michiels et al., 1995). Tiam1 was identified as a gene, which confers invasive capacity of otherwise non-invasive T lymphoma cells (Habets et al., 1994). The Tiam1 gene is highly conserved among mammals, and the major domains of the encoded protein are conserved between Drosophila and human. The gene is expressed in almost all tissues with the highest expression in the brain and testis (Habets et al., 1995). The Tiam1 gene encodes a protein consisting of 1591 amino acids, which harbors several domains (Fig. 9).

The Dbl-homology domain (DH) is located in the C-terminal part of the protein and functions as the catalytic domain that is responsible for Rac binding and guanine nucleotide exchange. Tiam1 harbors two Pleckstrin homology (PH) domains that can bind phosphatidylinositol lipids present in cell membranes. The N-terminally located PH domain (PHn) is followed by a coiled-coil region and an adjacent ex-motif (CC-Ex) which function as protein-protein interaction domains (Stam et al., 1997). Furthermore, N-terminus of Tiam1 contains two PEST domains that are involved in protein degradation. In the central region of the protein two additional domains are located: a Ras binding domain (RBD) (Lambert et al., 2002) and a PDZ domain (post synaptic density (PSD95) protein domain). PDZ domains represent protein-protein interaction domains but the specific function of the PDZ domain in Tiam1 is currently unknown. A myristoylation motif is present at the very N-terminus of Tiam1, this modification is required for plasma membrane localization. The PHn domain is also essential for the plasma membrane localization of Tiam1 (Michiels et al., 1997).

GEFs of Rho GTPases can be activated by various external an internal stimuli but it is not entirely clear yet by which stimuli or mechanisms Tiam1 is activated. It is well documented that N-terminally truncated Tiam1 (C1199) is more efficient in Rac1 activation when compared to the full-length protein (Michiels et al., 1997). C1199-Tiam1 is predominantly localized at the plasma membrane and shows increased protein stability. Association of Tiam1 with membrane phospholipids seems to affect the activity of Tiam1. The PHn domain of Tiam1 binds phosphoinositides including PI(4,5)P(2) and PI(3,4,5)

P(3) a product of PI3-kinase (Fleming et al., 2004;Ceccarelli et al., 2007). Phosphoinositides binding as well as membrane localization is crucial for the ability of Tiam1 to induce Rac1-mediated membrane ruffles and JNK activation (Michiels et al., 1995;Fleming et al., 2004). Phosphorylation may be another way to regulate the activity of Tiam1 towards Rac1. Several reports describe the phosphorylation and activation of Tiam1 by various kinases. Tiam1 is phosphorylated by Ca2+/calmodulin kinase II and PKC upon stimulation with PDGF and lysophosphatydic acid (LPA), respectively (Fleming et al., 1999;Fleming et al., 1997). In addition, activation of the LPA1 receptor induces Tiam1-dependent Rac1 activation (van Leeuwen et al., 2003). Oncogenic Src phosphorylates Tiam1 on multiple tyrosines and Tiam1 is claimed to cooperate with Src in Rac1activation and the formation of membrane ruffles (Servitja et al., 2003). Tiam1 is also phosphorylated on Tyr-829 by the TrkB upon BDNF stimulation (Miyamoto et al., 2006). TrkB is supposed to bind and activate Tiam1 by this phosphorylation leading to Rac1 activation.

Several reports have shown that Tiam1 interacts with a number of different proteins (Table 1). Although not all interactions have been confirmed and validated most of these

Table 1. Table representing proteins that have been claimed to associate with Tiam1. Interaction domains have been indicated as well as original publication in which the associations have been described.

Protein name	Tiam1 interacting domain	Function	Reference
CD44 (hyaluronic acid receptor)	CC-Ex	ND (debatable existence)	(Bourguignon et al., 2000b)
ankyrin (cytoskeletal protein)	CC-Ex	ND	(Bourguignon et al., 2000a)
JIP/IB2 (scaffold)	PHn-CC-Ex	p38 activation	(Buchsbaum et al., 2002)
Spinophilin (scaffold)	PHn-CC-Ex	p70-S6-kinase activation	(Buchsbaum et al., 2003)
Nm23H1 (tumor suppressor)	N-terminus	Inhibition of Rac1 activity (debatable existence)	(Otsuki et al., 2001) (Palacios et al., 2002)
ephrin and EphA2 (ephrin, ephrin receptor)	ND	neurite outgrowth	(Tanaka et al., 2004)
NMDAR (glutamate receptor)	ND	dendrites development	(Tolias et al., 2005)
Arp2/3 (actin polymerization complex)	PHn-CC	actin polymerization	(Ten Klooster et al., 2006)
Par3 (member of Par complex)	CC-Ex	Tight junction formation	(Nishimura et al., 2005) (Mertens et al., 2005)
Ras	RBD	Ras-Rac1 activation	(Lambert et al., 2002)
IRSp53 (scaffold)	PHn-CC-Ex	WAVE2	(Connolly et al., 2005)
Rap1	DH-PHc	Rap1-Rac1 activation	(Arthur et al., 2004)
TrkB (neuronal receptor)	PHn-CC-Ex	BDNF-Rac1 activation	(Miyamoto et al., 2006)
c-Myc	N-terminus	ND (debatable existence)	(Otsuki et al., 2003)

interactions have a regulatory effect by directing Rac1 activity towards particular downstream effectors. Three scaffold proteins JIP/IB2, Spinophilin and IRSp53 bind to the PHn-CC-Ex domain of Tiam1 (Buchsbaum et al., 2002;Buchsbaum et al., 2003;Connolly et al., 2005). JIP/IB2 binds the Rac1 effector protein MLK3, which is an upstream activator of p38 (Buchsbaum et al., 2003) and thereby JIP/IB2 couples Tiam1-mediated Rac1 activation to p38 signaling. The second scaffold protein, Spinophilin, binds and enhances activity of p70S6-kinase, another Rac1 effector. Co-expression of Spinophilin and Tiam1 enhances p70S6-kinase activity (Buchsbaum et al., 2003). By binding to the third scaffold, IRSp53, Tiam1 directs Rac1 activity towards lamellipodia formation and local actin polymerization (Connolly et al., 2005). Clustering of IRSp53 and Rac1 creates a link between Rac1 and Arp2/3 complex during Rac-induced actin polymerization in lamellipodium formation (Suetsugu et al., 2006). Moreover, Tiam1 binds directly to p21Arc, a subunit of the Arp2/3 complex (Ten Klooster et al., 2006), providing a direct connection between activated Rac and Arp2/3- mediated actin polymerization.

As mentioned earlier, Tiam1 has also been implicated in polarity signaling mediated by the Par complex. Tiam1 binds directly to Par3 one of the components of the Par complex. Intriguingly, Tiam1 functions upstream of the Par complex during tight junction formation and activates the Par complex (Mertens et al., 2005;Chen and Macara, 2005). However, during the establishment of neuronal cell polarity and T-cell polarization, Cdc42 activates the Par polarity complex and Tiam1 functions downstream of this complex to activate Rac required for the establishment of cell polarity (Nishimura et al., 2005;Gerard et al., 2007). Tiam1 is also involved in the control of cell-cell and cell-matrix interaction. Tiam1-mediated Rac1 activation stimulates cadherin-mediated cell-cell adhesion in mesenchymal-like Ras-transformed Madin-Darby canine kidney (MDCK) cells, in normal MDCK cells and in NIH3T3 fibroblasts (Hordijk et al., 1997;Sander et al., 1999;Malliri et al., 2004). Tiam1 also functions in the integrin-mediated cell-matrix adhesions. In mouse keratinocytes Tiam1 conducts $\alpha3\beta1$-mediated activation of Rac1 that is required for the production and secretion of Laminin-5 (Hamelers et al., 2005). This signaling affects also keratinocytes spreading and migration. The data above indicate that Tiam1 is involved in cell polarization processes as well as cell adhesion processes.

In earlier studies, Tiam1 has been implicated in neuronal cell physiology by studying Tiam1 expression pattern in the developing brain (Ehler et al., 1997). Later studies showed that Tiam1 controls neuronal morphology and in particular the regulation of neurite growth-cones (Kunda et al., 2001;Matsuo et al., 2003). Ephrins are signaling molecules involved in the establishment of neural networks by the induction of collapse and elongation of neurites. Tiam1 binds to membrane localized ephrin-B1 and ephrin receptor EphA2. Interaction of Tiam1 with ephrin-B1 and EphA2 induces Rac1 activation and mediates neurite outgrowth (Tanaka et al., 2004). Another neuronal regulatory protein that binds to Tiam1 is NMDA-type glutamate receptor. Tiam1 mediates effects of the NMDA

receptor during dendrite development by Rac1-dependent actin remodeling (Tolias et al., 2005). High expression of Tiam1 in neuronal tissue is thus correlated with its function in neurite development.

During dendritic spine morphogenesis, Par3 regulates neural spine formation by binding to Tiam1 and thereby leading to spatial restriction of Rac1 activation (Zhang and Macara, 2006). Tiam1-Par interaction is also crucial in T-cell function. Similarly as in the neuronal polarization, in Rap1- and chemokine-induced T cell polarization, Cdc42 functions upstream of the Par complex. Tiam1 binds both Rap1 and Par3, and stimulates Rac1-mediated actin remodeling required for the establishment of T cell polarity (Gerard et al., 2007). Another cellular process that depends on the establishment of cellular asymmetry/polarity is cell migration. Migrating cells need to polarize and reorganize the cytoskeleton to create a front and a rear. Indeed, Tiam1 together with the Par polarity complex stabilizes microtubules required for persistent, chemotactic migration of keratinocytes and lymphocytes (Pegtel et al., 2007;Gerard et al., 2007). The data summarized above illustrate that Tiam1 plays a key role in the control of cell polarity processes in different cellular contexts.

With respect to oncogenicity, it has been claimed that the N-terminus of Tiam1 binds to the tumor suppressor Nm23H1 (Otsuki et al., 2001). However, we and others were unable to confirm these data and it is unlikely that this proposed interaction has an effect on Rac1 activation *in vivo*. It has been shown that Rac1 is a necessary component in Ras-induced cell transformation (Qiu et al., 1995a). Ras influences cell morphology by PI3-kinase-dependent Rac1 activation (Rodriguez-Viciana et al., 1997). However, Ras may also activate Rac directly by binding to the RBD of Tiam1 and thereby activating Rac independently of PI3-kinase (Lambert et al., 2002). Tiam1 influences tumor development and progression in mouse tumor models. Tiam1-deficiency reduces tumor initiation and growth in skin tumors induced by activated Ras (Malliri et al., 2002). Furthermore, Tiam1-deficiency inhibits intestinal tumor development in APC Min/+ mice (Multiple intestinal neoplasia), similarly as seen in Ras-induced skin tumors (Malliri et al., 2006). Wnt canonical signaling stimulates expression of Tiam1 in human and mouse intestinal tumors (Malliri et al., 2006). Tiam1 contributes to Neu-induced mammary tumorigenesis in mice, but has no effect on the development of Myc induced mammary tumors (Strumane et al., 2008a). Similarly, Tiam1 has also no effect on the onset or progression of lymphomas in the mouse model of PI3-kinase driven lymphomas (Strumane et al., 2008b). Thus, Tiam1 seems to be an enhancer of tumor onset or progression in the presence of certain but not all oncogenes. Prevention of apoptosis enhances the process of tumor initiation. As described above Rac1 predominantly inhibits apoptosis and promotes cell survival, similarly Tiam1 is implicated in restraining apoptosis. However, as with Rac1, Tiam1 function seems to depend on the cellular and signaling context. Tiam1 is proposed to be a downstream mediator of bufalin-induced apoptosis (Kawazoe et al., 1999). On the other hand, during apoptosis

Tiam1 is a target of caspase proteolysis, suggesting an anti-apoptotic function (Qi et al., 2001). Indeed, in the mouse model of skin tumorigenesis, the basal layer of skins of Tiam1 knock-out animals contains increased numbers of apoptotic cells (Malliri et al., 2002). Furthermore, Tiam1-deficient keratinocytes show increased susceptibility for apoptosis after challenging with pro-apoptotic stimuli. The molecular mechanism of the Tiam1-dependent anti-apoptotic signaling involves Rac1-induced ROS production and ERK activation (Rygiel et al., 2008). The data summarized above demonstrate that Tiam1 may contribute to tumor initiation and progression by various means. Further investigations will unravel details of the function of Tiam1 in Rac-mediated signaling processes as well as aberrant signaling pathways that contribute to the formation and progression of tumors.

Outline of the thesis

As discussed in the first chapter, cellular signaling pathways controlled by Rho GTPases regulate various physiological cell processes and, when deregulated, they contribute to various diseases including cancer. In **chapter 1**, we have discussed the physiological function of Rho proteins and the various mechanisms by which the activity of these proteins is regulated. Furthermore, we discussed the pathological functions of Rho proteins including their role in various aspects of cancer. Finally, we have discussed the function of Tiam1, representing an activator of the Rho-like GTPase Rac, and of which the functions are analyzed in more detail as described in other chapters of this thesis.

In **chapter 2**, we describe investigation of the Tiam1 function in intestinal tumorigenicity in APC Min/+ mice. In previous studies, we found that Tiam1 is involved in various aspects of skin tumorigenicity induced by the activation of the Ras oncogene. Based on these findings we started to investigate the function of Tiam1 in tumorigenicity induced by different oncogenic pathways. APC Min/+ mice produce intestinal tumors as a result of constitutive activation of the oncogenic Wnt signaling pathway. We found that Tiam1 is a Wnt responsive gene and that the expression levels of Tiam1 are upregulated in both mouse and human intestinal tumors. Inhibition of Wnt signaling decreased the expression of Tiam1 in tumor cells cultured *in vitro*. To investigate in more detail the function of Tiam1 in Wnt-induced intestinal tumorigenicity we crossed APC Min/+ mice with Tiam1-deficient mice and found that Tiam1 influences both initiation and progression of Wnt-induced intestinal lesions similarly as found in Ras-induced skin tumors.

Chapter 3 describes studies on the involvement of Tiam1 in the development of mammary tumors. We used two different mammary tumor mouse models in which mammary tumors were induced by tissue-specific expression of either the Neu/Her-2 or the Myc oncogene. We crossed Tiam1-deficient mice with the mammary tumor prone mice and found that Tiam1-deficiency leads to decreased tumor initiation and metastasis in Neu/

Her2 driven tumors. In contrast, the initiation, growth and progression of Myc- induced mammary tumors were completely independent on the presence of Tiam1. These data illustrate that the effects of Tiam1 on tumor development and progression depends on the oncogenic pathway involved.

In **chapter 4**, we summarize the various methods to study the function of Tiam1 in tumorigenicity. We describe the DMBA/TPA chemical carcinogenesis protocol to study the function of Tiam1 in Ras-induced skin tumors. The two-stage carcinogenesis protocol allows studying various aspects of tumorigenicity including the initiation, promotion, and progression of tumors. In this chapter, we also discuss various methods to isolate different cell types from mice including embryonic fibroblasts, embryonic keratinocytes and tumor cells. In addition, we have discussed experimental procedures to investigate apoptosis sensitivity and transforming capacity of cells *in vitro*.

Chapter 5 presents investigation of the mechanisms by which Tiam1 supports cell survival and is able to counteract apoptosis. As a model for this study, we have used mouse keratinocytes derived from wild type and Tiam1-deficient mice. Our findings describe a new Tiam1/Rac1 signaling pathway, which assures cell survival downstream of Rac. It involves Rac-mediated production of reactive oxygen species (ROS) and stimulation of extracellular induced kinases (ERK), one of the most important signaling pathways, which protect cells from induced or spontaneous apoptosis. Inhibition of apoptosis may greatly enhance both tumor initiation and tumor growth.

Chapter 6 focuses on the role of Tiam1 in cell polarity in particular in the establishment of epithelial apical-basal cell polarity through the formation of tight junction. In this study, we make also use of mouse keratinocytes derived from WT and Tiam1-deficient mice. We found that Tiam1-deficient keratinocytes are impaired in the formation of tight junctions. Tiam1 binds to Par3, which is one of the components of Par polarity complex. Activation of the Par complex leads to PKCζ activation, which is required for tight junction formation and maturation. Through the activation of Rac, Tiam1 triggers the activation of the Par polarity complex and thereby the effector kinase PKCζ. Loss of epithelial apical-basal polarity is one of the hallmarks of cell de-differentiation observed during tumor progression. Thus, in this context Tiam1 may be seen as a repressor of tumor progression.

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Chapter 2

The Rac activator Tiam1 is a Wnt-responsive gene that modifies intestinal tumor development

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ABSTRACT

Mutations in the canonical Wnt signaling pathway, leading to its activation, are known to cause the majority of intestinal tumors. However, few genes targeted by this pathway have been demonstrated to affect tumor development *in vivo*. Here we show that *Tiam1*, a selective Rac GTPase activator, is a Wnt-responsive gene expressed in the base of intestinal crypts and up-regulated in mouse intestinal tumors and human colon adenomas. Moreover, by comparing tumor development in APC mutant multiple intestinal neoplasia (Min) mice expressing or lacking Tiam1, we found that Tiam1 deficiency significantly reduces the formation and growth of polyps *in vivo*. However, invasion of malignant intestinal tumors is enhanced by a lack of Tiam1. In line with this, knock-down of Tiam1 reduced the growth potential of human colorectal cancer (CRC) cells and their ability to form E-cadherin-based adhesions, a prerequisite for local invasion of tumor cells. Our data indicate a novel crosstalk between Tiam1-Rac and canonical Wnt-signaling pathways that influences intestinal tumor formation and progression.

INTRODUCTION

The lumen of the adult mammalian intestine is lined with an absorptive and secretory epithelium that undergoes continuous self-renewal. The epithelium is organized into two functional domains: one comprises proliferating progenitor cells located physically within crypts; the other, terminally-differentiated, specialized cells occupying predominantly the villi in the small intestine and the mucosal surface in the colon. In the adult intestinal mucosa, the canonical Wnt signaling pathway (reviewed in (Nelson and Nusse, 2004)) maintains the undifferentiated, proliferative crypt phenotype (van de Wetering et al., 2002), controls cell positioning along the crypt-villus axis (Batlle et al., 2002), and regulates aspects of Paneth cell maturation (van Es et al., 2005). The canonical Wnt signaling pathway is initiated by extracellular Wnt glycoproteins binding transmembrane receptors. Ligand binding induces a cascade of protein-protein interactions, the ultimate consequence of which is stabilization and accumulation of cytosolic β-catenin. This protein, known also for its role in cadherin-mediated intercellular adhesion, relays the signal to the nucleus, where, through direct binding to TCF family transcription factors, it induces target gene expression: TCFs provide DNA-binding sequence specificity; β-catenin essential transactivation domains (Nelson and Nusse, 2004).

Mice deficient for TCF4 die around birth and lack proliferative cells specifically in intestinal crypt precursor regions (Korinek et al., 1998). Forced expression in the mucosa of dickkopf1, a secreted inhibitor of canonical Wnt signaling, blocks crypt formation and self-renewal in adult mice and impairs cell positioning (Pinto et al., 2003;Kuhnert et al., 2004). In contrast, aberrant activation of the Wnt pathway results in intestinal tumor formation (reviewed in (Bienz and Clevers, 2000)). Germline mutations in humans abolishing the function of APC, a key inhibitor of the canonical Wnt pathway, are responsible for an inherited tumor syndrome referred to as familial adenomatous polyposis (FAP) (Kinzler et al., 1991). Carriers develop multiple benign colorectal adenomatous polyps, a fraction of which generally progress to adenocarcinomas. Similar mutations are found in the majority of sporadic colorectal tumors (Bienz and Clevers, 2000). In sporadic tumors with wild-type APC, β-catenin is frequently found to harbour activating gain-of-function mutations (Morin et al., 1997). A nonsense mutation in APC was also revealed to be responsible for the multiple intestinal neoplasia (Min) found in the mouse model of the same name (Su et al., 1992).

Tiam1 is a guanine nucleotide exchange factor (GEF) that selectively activates the Rho-like GTPase Rac (Michiels et al., 1995). In turn, Rac regulates actin polymerization, cell adhesion and motility, and also cell survival and cell cycle progression (reviewed in (Burridge and Wennerberg, 2004;Coleman et al., 2004)). We have previously shown that Tiam1 expression is transcriptionally regulated during epithelial to mesenchymal transitions (Malliri et al., 2004;Zondag et al., 2000). In addition, Tiam1 activity is regulated by

post-translational modifications and protein-protein interactions (reviewed in (Mertens et al., 2003)). Potentially oncoproteins could use these mechanisms to regulate Tiam1/Rac signaling. Tiam1 is a potent modifier of oncogenic Ras-induced skin tumor initiation, promotion and progression (Malliri et al., 2002). Given the clinical significance of human colorectal tumors and the need to identify genes that influence intestinal tumor development, we decided to examine the role of Tiam1 in intestinal tumorigenesis. Our findings indicate a novel crosstalk between Tiam1/Rac signaling and the canonical Wnt-signaling pathway, with *Tiam1* itself appearing to be a significant component of the Wnt-regulated genetic program implicated in intestinal tumorigenesis.

Materials and methods

Antibodies, immunohistochemistry and immunofluorescence

Immunohistochemistry was performed on paraffin-embedded tissue sections (4 μm) using an anti-DH Tiam1-specific rabbit polyclonal antibody (Habets et al., 1994), antibodies against lysozyme (DAKO), and Ki-67 (Novocastra) as previously described (Batlle et al., 2002;Malliri et al., 2002;van de Wetering et al., 2002). Periodic-acid Schiff (PAS) staining, to visualize goblet cells, was performed as standard (Pinto et al., 2003). Human colon tumors were collected from subtotal colectomy specimens of FAP patients and patients with sporadic colon adenomas. For immunofluorescence, cells grown on glass cover slips were processed as described previously (Malliri et al., 2004). Cells were stained for β-catenin using a monoclonal antibody obtained from BD Biosciences. Primary antibodies were visualized with fluorescein isothiocyanate-labeled secondary antibodies (Zymed Laboratories Inc.). Images were recorded with a Leica TCS-NT confocal laser scanning microscope.

Cell culture, constructs and transfection

DLD1 cells carrying a doxycycline-inducible dominant negative (DN)TCF4 or P44 isoform of TCF1 (*i.e.* TCF proteins lacking the β-catenin interaction domain) (van de Wetering et al., 2002) were cultured in RPMI with 5% FBS. The doxycycline-inducible siRNA system and sequences of Tiam1 siRNA oligonucleotides used to inducibly down-regulate Tiam1 were previously described (Malliri et al., 2004;van de Wetering et al., 2003). Rat intestinal epithelial (RIE) cells (from ATCC) were grown in DMEM with 5% FBS, supplemented with 0.1 IU/ml insulin. RIE cells were infected with a retroviral expression vector encoding Wnt1 or an oncogenic form of β -catenin (S33Y) and protein was harvested 48 hrs later.

Protein and RNA analysis

Tiam1 protein levels were analyzed by western blotting using anti-Tiam1 polyclonal antibodies either from Santa Cruz Biotechnology, Inc. or as previously described (19). North-

ern blot analysis was performed as previously described (Habets et al., 1994). Protein levels of Cyclin D1 were analyzed using an anti-Cyclin D1 monoclonal antibody (Upstate), Myc using the 9E10 anti-Myc monoclonal antibody, TCF1 using the anti-TCF1 7H3 monoclonal antibody (Upstate), TCF4 using the anti-TCF4 6H5-3 monoclonal antibody (Upstate) and Rac using an anti-Rac1 monoclonal antibody (BD Biosciences).

Growth curves

DLD1 cells were plated in triplicate in twelve-well plates at density 4 x 10^4 cells per well. Cells were grown in the presence or absence of doxycycline, which was refreshed every 48 hrs. Cell proliferation was determined from total protein amount in a dye-binding assay. Briefly, cells were fixed daily in 10% TCA and later stained with 1% bromophenyl blue (BPB). BPB was eluted with 10mM Tris and the absorbance was measured in a spectrophotometer at wavelength 590 nm. The readings were normalized for final concentration of BPB in control cells.

Mice

Tiam1$^{-/-}$ mice generated as described in (Malliri et al., 2002) on a C57BL/6 background as well as C57BL/6 *Tiam1*$^{+/+}$ mice were crossed with Balb/c Min/+ mice. Animals with a mixed C57BL/6//Balb/c background were used throughout the experiments. Mice were genotyped, and those carrying the Min allele were analyzed for intestinal tumor development (*Tiam1*$^{+/+}$//Min/+ n = 37, *Tiam1*$^{+/-}$//Min/+ n = 79, *Tiam1*$^{-/-}$//Min/+ n = 27). All mice analysed were sacrificed at week 14 after birth. The entire small intestine as well as the colon were coiled into a Swiss-roll like configuration, fixed in formalin and paraffin-embedded. Sections through the widest part of the roll were stained with haematoxylin/eosin, and a pathologist scored the number and grades of tumors blindly. For determining the size of lesions, a graticule was used to measure the largest dimension of all lesions from 8 randomly selected mice of each genotype (*Tiam1*$^{+/+}$//Min/+, *Tiam1*$^{+/-}$//Min/+, and *Tiam1*$^{-/-}$//Min/+) of the same gender and age at sacrifice. In total we measured the size of 315 lesions from *Tiam1*$^{+/+}$//Min/+ mice, 297 lesions from *Tiam1*$^{+/-}$//Min/+ mice and 134 lesions from *Tiam1*$^{-/-}$//Min/+ mice.

Results

Immunohistochemical staining of the intestine of wild-type mice revealed Tiam1 expression in cells at the base of small intestine crypts (Fig. 1*A*). The cells occupying these positions include Paneth cells and neighboring progenitor cells. The Paneth cells are terminally differentiated secretory cells that control the intestinal bacterial flora. Interestingly, both Paneth- and progenitor cells possess high levels of nuclear β-catenin (Batlle

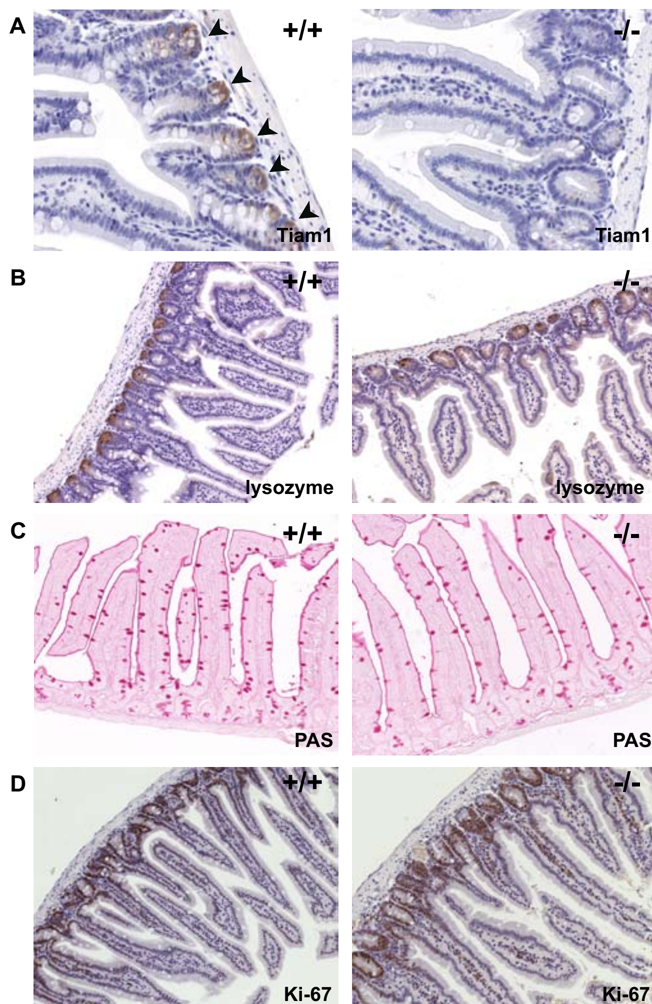


Figure 1. Tiam1 expression in intestinal mucosa and histology of intestines from adult wild type and *Tiam1*$^{-/-}$ mice. *A*, Immnuohistochemical staining revealed Tiam1 expression at the base of small intestine crypts (arrowheads) of *Tiam1*$^{+/+}$ mice, where Wnt signaling takes place. No Tiam1 expression was detected in intestines of *Tiam1*$^{-/-}$ mice. *B*, Lysozyme staining of Paneth cell granules. *C,* PAS staining of mucin production in goblet cells. *D*, Ki67 staining of proliferating cells. Sections in *A-D* are from adult wild-type and *Tiam1*$^{-/-}$ mice.

et al., 2002), a hallmark of active canonical Wnt signaling, suggesting that Tiam1 might be a Wnt responsive gene. As expected, no Tiam1 staining was observed in intestines from *Tiam1*$^{-/-}$ mice (Fig. 1*A*). We did not find abnormalities in the intestines of adult *Tiam1*$^{-/-}$ mice when compared with wild-type mice. Analysis of markers of differentiation (such as lysozyme staining of Paneth cell granules, PAS staining of mucin production in goblet cells, and alkaline phosphatase staining of enterocytes) and proliferation (Ki67

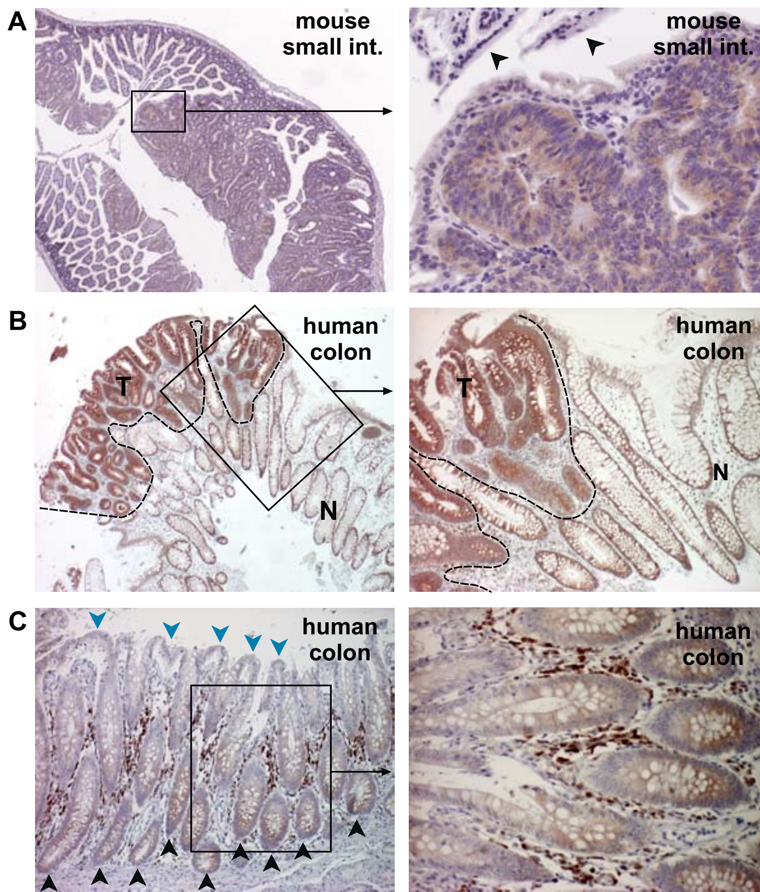


Figure 2. Tiam1 expression in mouse neoplastic intestinal mucosa and in human normal and neoplastic colonic mucosa. *A*, Tiam1 is uniformly expressed in adenoma cells of *Tiam1*$^{+/+}$//Min/+ mice. Note the absence of Tiam1 expression in normal tissue (arrowheads in right panel). *B*, Sections from human colon adenomas were immunohistochemically stained for Tiam1. In all cases studied and as shown here for a representative FAP polyp, Tiam1 expression was increased in dysplastic crypts compared to adjacent normal colonic mucosa. Dashed line depicts the boundary between normal (N) and tumour (T) tissues. *C*, In normal human colonic mucosa, the level of Tiam1 protein is highest within cells at the base of the crypt (black arrowheads) and decreases towards the luminal surface (blue arrowheads). Note strongly stained plasma cells in the stroma. (Boxed regions in *A* and *B* and *C* are magnified and shown in adjacent right panels).

expression) revealed no differences between wild-type and *Tiam1*$^{-/-}$ mice (Fig. 1*B*, *C*, *D* and data not shown). This indicates that Tiam1-deficiency does not visibly affect intestinal development or subsequent homeostasis.

In contrast to the restricted expression of Tiam1 at the base of small intestine crypts of mice, the protein was expressed uniformly in adenomatous polyps arisen in Min/+ mice

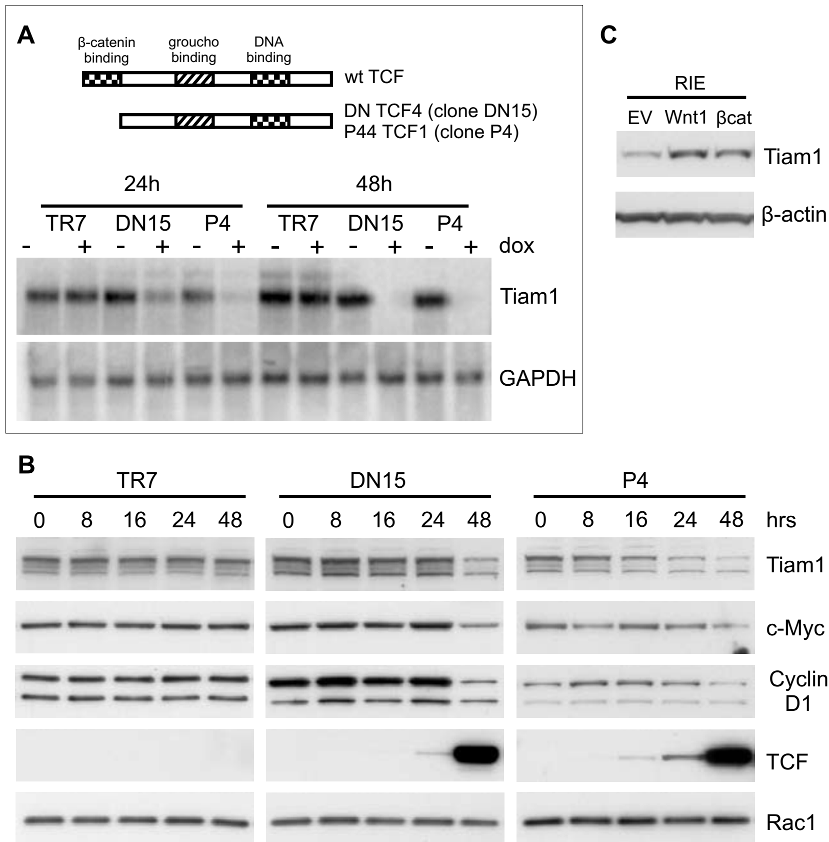


Figure 3. *Tiam1* is a Wnt-responsive gene. *A*, DLD1 human colon carcinoma cells inducibly overexpressing DN TCF4 (DN15) or P44 TCF1 (P4), as well as control DLD1 cells expressing the tetracycline repressor plasmid alone (TR7), were cultured in the presence (+) or absence (-) of doxycycline for either 24 or 48 hrs. RNA was isolated and Northern blot analysis performed for Tiam1 and GAPDH mRNA levels. *B*, TR7, DN15 and P4 cells were incubated with (+) or without (-) doxycycline for the indicated times before protein lysates were isolated and immunoblotting performed for Tiam1, c-Myc, cyclin D1, TCF, and Rac. *C*, Rat intestinal epithelial (RIE) cells were infected with either empty vector, a retroviral vector encoding for Wnt1 or a retroviral vector encoding for a dominant active form of β-catenin. Protein was extracted 48h later and immunoblotting for Tiam1 and β-actin (loading control) performed.

(Fig. 2*A*). These mice carry a nonsense mutation in the APC gene and produce intestinal tumors as a result of increased Wnt signaling (Su et al., 1992). In addition, immuno-histochemical staining of human colon adenomas from both FAP patients (n=38) and sporadic cases (n=13) revealed strong and uniform Tiam1 protein expression in all cases examined. The protein levels exceeded the levels in adjacent normal mucosa as found in mice (for example see Fig. 2*B*). Additionally, in normal human colonic mucosa, we observed a gradient of Tiam1 protein decreasing from cells at the base of the crypt to the luminal surface (Fig. 2*C*). This expression pattern parallels the gradient of Wnt pathway

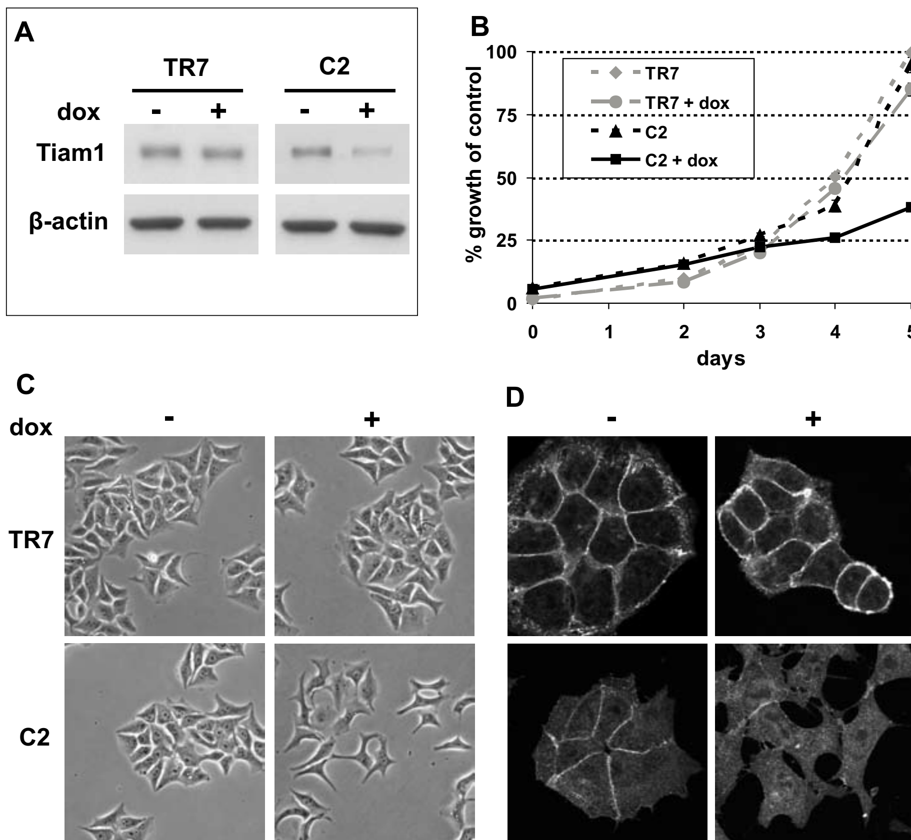


Figure 4. Tiam1 influences human CRC cell growth and cell–cell adhesion. *A*, Tiam1 protein levels in a representative clone of DLD1 cells inducibly expressing Tiam1 siRNA (clone C2) as compared to control TR7 cells. Protein was harvested 48 hrs after plating in the presence (+) or absence (-) of doxycycline and immunoblotted for Tiam1 or β-actin (loading control). *B*, C2 cells induced with doxycycline grow slower than untreated C2 cells or control TR7 cells in the presence or absence of doxycycline. *C*, Phase contrast images of control TR7 cells or C2 cells 48 hrs after plating in the presence (+) or absence (-) of doxycycline. Control cells grow in tightly packed colonies even in the presence of doxycycline. C2 cells display reduced colony formation specifically in the presence of doxycycline. *D*, Confocal images of colonies of either control TR7 cells or C2 cells fixed and stained for β-catenin 48 hrs after plating in the presence (+) or absence (-) of doxycycline. Tiam1 siRNA induction leads to reduced β-catenin at sites of cell–cell contact.

activity as documented in the colon (van de Wetering et al, 2002). Since Paneth cells are absent in the colon, positive cells within the base of the human crypt should represent progenitor cells.

The specific expression of Tiam1 at sites of endogenous canonical Wnt signaling and its apparent up-regulation in neoplastic lesions resulting from excessive Wnt signaling prompted us to evaluate further whether *Tiam1* is a Wnt responsive gene. For this we used a human CRC cell line, DLD1, engineered to express inhibitory forms of TCF transcription factors upon addition of doxycycline (van de Wetering et al, 2002). Abrogation

of Wnt signaling by the induced expression of the inhibitory forms of TCF resulted in a complete loss of Tiam1 messenger RNA by 48 hours (Fig. 3*A*). Protein expression was also significantly reduced in this interval (Fig. 3*B*). Down-regulation of Tiam1 was temporally correlated with appearance of the inhibitory forms of TCF and occurred with similar kinetics to c-Myc and Cyclin D1 (Fig. *3B*), both considered direct TCF targets (He et al., 1998;Tetsu and McCormick, 1999). Together these findings indicate that Tiam1 is a Wnt-responsive gene and potentially a direct TCF target. To further substantiate these findings, we overexpressed Wnt1 and an oncogenic form of β-catenin (lacking the Ser 33 regulatory phosphorylation site) in rat intestinal epithelial (RIE) cells in which the Wnt pathway is not activated. Exogenous expression of both Wnt1 and β-catenin in RIE cells resulted in a significant up-regulation of Tiam1 protein levels (Fig. 3*C*), consistent with the canonical Wnt pathway controlling levels of expression of Tiam1.

To further study the consequences of changes in the levels of Tiam1 in colon tumor cells, we engineered the human DLD1 cells to express small interfering (si) RNA designed to knock-down Tiam1 upon addition of doxycycline. In earlier studies, we have successfully used the Tiam1-specific siRNA sequence to downregulate Tiam1 protein levels in other cell types (Malliri et al., 2004). We generated several independent DLD1 cell clones in which doxycycline addition induced a selective reduction in Tiam1 expression. The induced expression of siRNA of Tiam1 significantly reduced Tiam1 levels when compared to parental control TR7 cells as shown for clone C2 (Fig. 4*A*). Compared to TR7 control cells, all individual clones (as well as a population of mixed clones) showed decreased growth potential in the presence of doxycycline (see Fig. 4*B* for clone C2). This suggests that reduction of Tiam1 protein levels negatively affects cell growth in DLD1 cells. Knock-down of Tiam1 also influenced the growth pattern and morphological appearance of DLD1 cells. DLD1 cells normally grow in regular tessellated colonies and display an epithelioid phenotype with characteristic cadherin-based cell–cell adhesions (Fig. 4*C* and *D*). Knock-down of Tiam1 resulted in diffuse colonies containing scattered, fibroblast-like cells (Fig. 4*C*) and release of beta-catenin from the plasma membrane (Fig. 4*D*). These findings are consistent with the earlier reported requirement of Tiam1 in the formation and maintenance of cadherin-based cell adhesions (Hordijk et al., 1997;Malliri et al., 2004). From these *in vitro* studies we conclude that Tiam1 is a Wnt responsive gene, which influences the growth rate of colorectal cancer cells as well as the strength of cadherin-based adhesions between these cells. Reduced cell–cell adhesion is frequently associated with progression of epithelial tumors.

To address whether Tiam1 expression contributes to the formation and progression of Wnt-induced intestinal tumors *in vivo*, we examined tumor formation in Min/+ mice in different *Tiam1* backgrounds. In comparison to *Tiam1*$^{+/+}$//Min/+ mice, *Tiam1*$^{-/-}$//Min/+ mice demonstrated a 50% reduction ($p<0.005$, t-test) in intestinal tumor numbers (Fig. 5*A*). A similar proportional reduction in numbers was observed across the spectrum of tu-

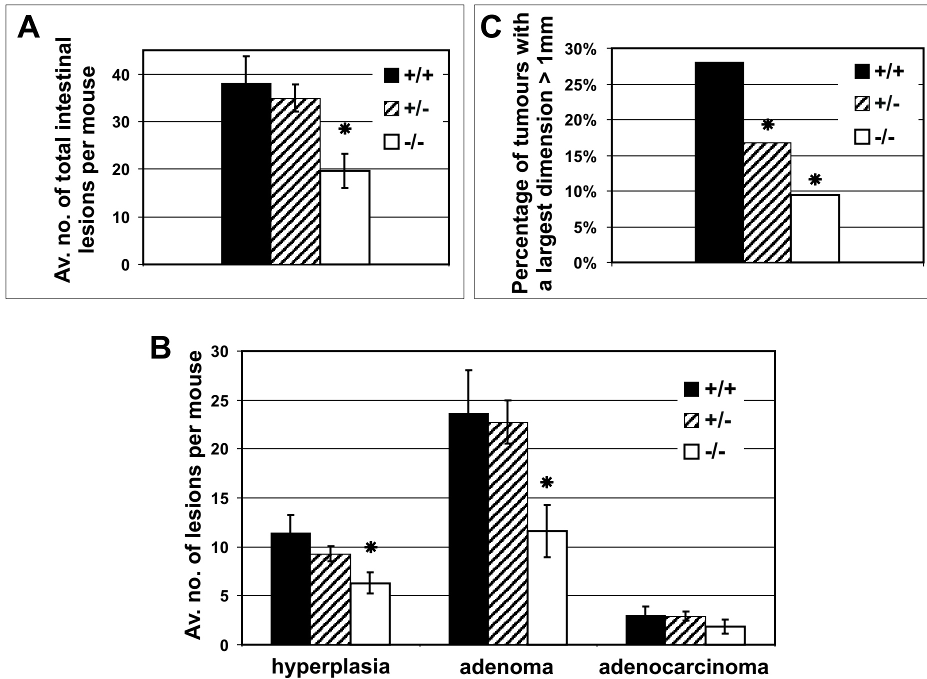
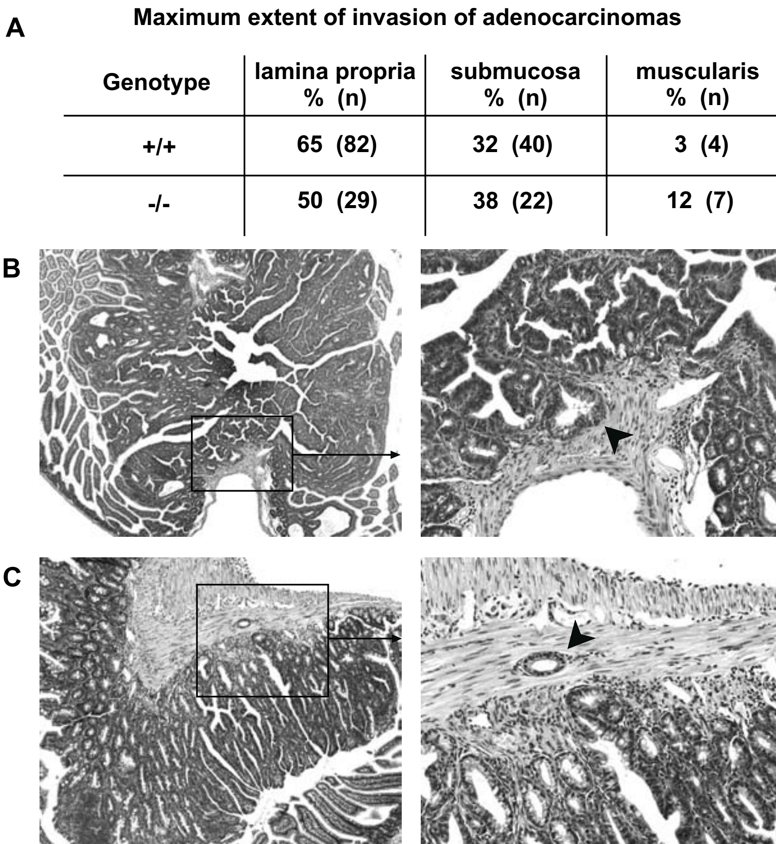


Figure 5. Absence of Tiam1 impairs intestinal tumour formation in Min/+ mice. *A*, Average number of total intestinal lesions per mouse found in *Tiam1*$^{+/+}$//Min/+, *Tiam1*$^{+/-}$//Min/+ and *Tiam1*$^{-/-}$//Min/+ mice. Y-error bars show standard error of the mean. *The 50% reduction in lesions in *Tiam1*$^{-/-}$//Min/+ mice compared to *Tiam1*$^{+/+}$//Min/+ mice is statistically highly significant ($p<0.005$; student t-test). *B*, Average number of intestinal hyperplasias, adenomas, and adenocarcinomas per mouse is depicted for each genotype. Y-error bars show standard error of the mean. *The reduction in hyperplasias and adenomas in *Tiam1*$^{-/-}$//Min/+ mice compared to *Tiam1*$^{+/+}$//Min/+ mice is statistically highly significant ($p<0.005$; student t-test). *C*, Percentage of tumours with a largest dimension bigger than 1mm. *The reduction in tumour size in both *Tiam1*$^{+/-}$// Min/+ and *Tiam1*$^{-/-}$//Min/+ mice is statistically highly significant ($p<0.005$; ANOVA).

mor types including hyperplasias, adenomas, and adenocarcinomas (see Fig. 5*B*). Further analysis of the various tumors revealed that their growth was also significantly reduced in heterozygous *Tiam1*$^{+/-}$//Min/+ mice and was reduced further in *Tiam1*$^{-/-}$//Min/+ mice (Fig. 5*C*). The intermediate phenotype of the heterozygous mice indicates that the observed differences depend on the level of Tiam1 expression. From these studies we conclude that Tiam1 is a Wnt responsive gene, which is implicated in the formation and growth of Wnt-induced intestinal tumors.

We also attempted to determine the rate of malignant progression of intestinal tumors in *Tiam1*$^{+/+}$ or *Tiam1*$^{-/-}$ backgrounds. However, malignant progression is infrequently observed in Min/+ mice, since they are typically sacrificed at a young age due to morbidity arising from bowel obstruction and anemia. Malignant progression in the small intestine is defined as tumor invasion into the lamina propria or beyond. Upon sacrificing mice at 14 weeks, we observed a similar fraction of adenocarcinomas (approximately 8% of all le-



Maximum extent of invasion of adenocarcinomas

Genotype	lamina propria % (n)	submucosa % (n)	muscularis % (n)
+/+	65 (82)	32 (40)	3 (4)
-/-	50 (29)	38 (22)	12 (7)

Figure 6. Absence of Tiam1 seems to increase invasiveness of malignant tumours in Min/+ mice. *A*, Table summarizing the maximum extent of invasion of intestinal adenocarcinomas found in Min/+ mice of *Tiam1*$^{+/+}$ or *Tiam1*$^{-/-}$ backgrounds. The increased percentage of tumours invading into both the submucosa and muscularis propria in *Tiam1*$^{-/-}$//Min/+ mice is statistically significant ($p<0.05$; chi-squared test). *B*, Section of an adenocarcinoma showing invasion in the submucosa, stained with haematoxylin and eosin. *C*, Section of an adenocarcinoma showing invasion in the submucosa and muscularis propria, stained with haematoxylin and eosin. (Boxed areas in *B* and *C* are magnified (right panels) and tumour cells invading the submucosa and muscularis propria respectively are indicated by arrowheads).

sions) independently of *Tiam1* status, suggesting that Tiam1-deficiency does not affect the progression from adenomas to adenocarcinomas. However, when examining the extent of tumor invasion, we found a significantly larger fraction ($p<0.05$; chi-squared test) of malignant tumors to have invaded into the submucosa and muscularis propria in *Tiam1*$^{-/-}$//Min/+ mice compared to *Tiam1*$^{+/+}$//Min/+ mice (Fig. 6*A*). Representative examples of invasion of malignant tumors into the submucosa and muscularis propria are shown in Fig. 6*B* and Fig. 6*C* respectively. These data suggest that adenocarcinomas arisen in Tiam1-deficient mice are more aggressive than those arisen in Tiam1 wild type.

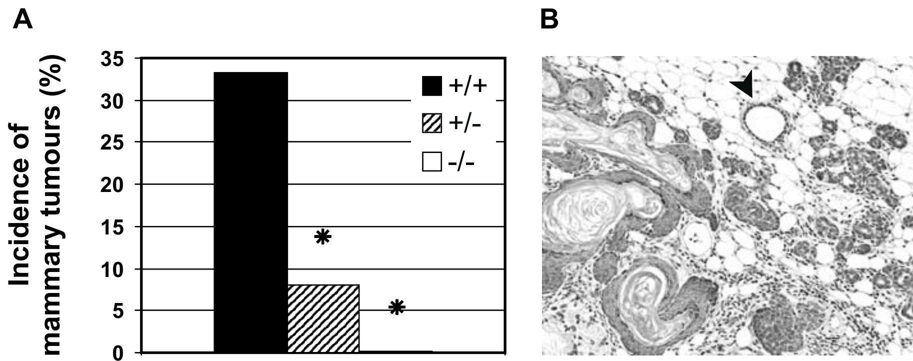


Figure 7. Absence of Tiam1 impairs mammary tumour formation in Min/+ mice. *A*, Percentage of female mice with one or more mammary tumours. The reduction in incidence in both *Tiam1*$^{+/-}$//Min/+ (n=34) and *Tiam1*$^{-/-}$//Min/+ (n=12) mice is statistically significant when compared to *Tiam1*$^{+/+}$//Min/+ (n=23) mice ($p<0.05$; chi-squared test). *B*, Tiam1 immunohistochemical staining in a representative mammary tumour found in a *Tiam1*$^{+/+}$//Min/+ mouse. Arrowhead points to a normal mammary gland which is negative for Tiam1.

Female Min/+ mice also develop mammary tumors at low incidence, which are likewise attributed to aberrant Wnt signaling. Interestingly, in addition to the effect of Tiam1 depletion on intestinal tumorigenicity, we also observed a dramatic reduction in the incidence of mammary tumors in *Tiam1*$^{+/-}$//Min/+ mice that was even more apparent in *Tiam1*$^{-/-}$//Min/+ mice (Fig. 7*A*). Mammary tumors in *Tiam1*$^{+/+}$//Min/+ mice were invariably squamous cell carcinomas and, when immunohistochemically stained for Tiam1, displayed Tiam1 up-regulation compared to normal mammary tissue (Fig. 7*B*). These findings further support our conclusions that *Tiam1* is a Wnt-responsive gene that is implicated in the formation of Wnt-induced tumors including intestinal and mammary tumors.

Discussion

Due to its central role in intestinal tumorigenesis, several groups have concentrated on the identification of genes that are regulated by the canonical Wnt signaling pathway, and especially those up-regulated in CRC cells (Fujita et al., 2001;He et al., 1999;Mann et al., 1999;Sansom et al., 2004;van de Wetering et al., 2002). Certain of these targets have been demonstrated to regulate intestinal homeostasis, including notably c-Myb and EphB family members. However, few Wnt target genes have as yet been demonstrated to influence intestinal tumorigenesis *in vivo* (Park et al., 2001;Wilson et al., 1997;Zhu et al., 2004;Batlle et al., 2005). Our data presented here indicate that Tiam1 is a Wnt target gene involved in Wnt-induced tumorigenicity. Additional confirmation was recently provided by a gene-profiling screen that identified *Tiam1* as a gene up-regulated in the intestines

of *APC* conditional mutant mice (Sansom et al., 2004). The effects of Wnt signaling on Tiam1 levels could be either direct through TCF, or indirect through another regulator that is itself a direct TCF target. The similar kinetics of downregulation of Tiam1, c-Myc and Cyclin D1 by the inhibitory forms of TCF suggest that Tiam1 is a direct target of TCF. Using bioinformatics, we have identified two consensus TCF binding sites directly upstream of the transcription start of Tiam1. These sites were located at –206 and –902 bp upstream of the transcription start in the human genome. Moreover, an additional six potential TCF sites were found in the region 2 to 5 kb downstream of the transcription start of human Tiam1. These sites might allow TCF to influence directly Tiam1 expression.

Our data implicate Tiam1 in intestinal tumorigenesis. We find that Min/+ mice lacking Tiam1 have a significantly reduced susceptibility to develop Wnt-induced intestinal and mammary tumors. Moreover, the growth of these tumors is also significantly impaired. Our *in vitro* experiments using a human CRC-derived cell line in which Tiam1 was downregulated confirmed the requirement for Tiam1 for optimal growth. Moreover, we observed increased Tiam1 expression in human colorectal tumors, suggesting that this gene is implicated in both mouse and human intestinal tumorigenesis.

We previously observed that susceptibility to develop Ras-induced skin tumors, following application of a two-step chemical carcinogenesis protocol, was significantly reduced in *Tiam1*$^{-/-}$ mice (Malliri et al., 2002). Tiam1 physically associates with the activated Ras-GTPase and this association stimulates Rac activity (Lambert et al., 2002). The Tiam1/Rac signaling module thus appears to be selectively recruited by at least two independent oncogenic signaling pathways of major clinical significance, albeit by distinct mechanisms. This suggests that Tiam1-mediated Rac activation is a significant modifier of tumor development, and therefore a potentially interesting therapeutic target. The requirement for Tiam1 for optimal tumor cell growth is a consistent finding in both skin and intestinal tumor models, and was also recently demonstrated for MDCK cells, an immortalized kidney epithelial cell line (Malliri et al., 2004). Our data are also consistent with a previously published study showing that targeted expression of a dominant-active mutant of Rac1 to the mouse intestine resulted in enlarged crypts and increased proliferation (Malliri et al., 2004;Stappenbeck and Gordon, 2001). It is possible that Tiam1-deficiency results in reduced intestinal tumor numbers by increasing susceptibility to apoptosis of initiated intestinal epithelial cells, although this is very difficult to address in this model. However, TUNEL labeling did not reveal increased apoptosis in polyps arising in *Tiam1*$^{-/-}$//Min/+ mice (data not shown).

Knock-down of Tiam1 in DLD1 cells by siRNA reduced their ability to form cadherin-mediated cell–cell adhesions, consistent with earlier *in vitro* studies showing that Tiam1 is critical for the formation and maintenance of cell–cell adhesions and for suppressing epithelial cell motility and invasion (Hordijk et al., 1997;Malliri et al., 2004). *In vivo,* and particularly with Ras-induced skin tumors, it appears that loss of Tiam1 expression

facilitates malignant progression, presumably by its effect on cadherin based cell adhesions. The observed increased invasive growth in the intestinal tumors could be due to the same phenomena, as we found reduced cadherin-based adhesions in DLD1 cells in which Tiam1 was downregulated by siRNA. In a recent study overexpression of Tiam1 was found to increase the *in vitro* migration of SW480 colorectal carcinoma cells as well as their ability to metastasize in an orthotopic nude mouse model (Minard et al., 2005). The apparent discrepancy between our present data and the above study could potentially be attributable to the specific nature of the selected SW480 cells, in which Tiam1 overexpression does not promote cadherin-based cell–cell adhesions. Earlier studies have indicated that the effect of Tiam1 on invasion is cell type specific and dependent on its capacity to influence E-cadherin-based adhesions (Sander et al., 1998). Intriguingly, another Rac-specific exchange factor, Asef, expressed in CRC-derived cells, is able to antagonize cadherin-mediated adhesion and to promote cell motility *in vitro* (Kawasaki et al., 2003). Asef is hyperactivated by truncated, mutant APC, found in the majority of CRC cells. Knock-down of Asef impaired CRC cell motility. Thus despite both molecules activating Rac, Asef and Tiam1 promote different biological outcomes, consistent with the concept that GEFs not only activate Rho-like molecules, but also direct that activity to particular downstream signaling and biological ends (Malliri and Collard, 2003).

Taken together, our data indicate a crosstalk between the canonical Wnt and Tiam1/Rac signaling pathways that influences the initiation, growth and progression of intestinal tumors.

Acknowledgments

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Chapter 3

Tiam1-deficiency impairs mammary tumor formation in MMTV-c-*neu* but not in MMTV-c-*myc* mice

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ABSTRACT

Rho-like small GTPases, including RhoA, Rac1 and Cdc42, are crucial for the regulation of a large variety of biological processes such as the cytoskeletal organization and gene transcription. The activities of Rho GTPases are predominantly controlled by guanine nucleotide exchange factors (GEFs), which activate GTPases by catalyzing the exchange of bound GDP for GTP. Earlier, we have identified the *Tiam1* gene as an invasion-inducing gene that encodes a specific activator (GEF) of the Rac GTPase. We found that Tiam1-mediated Rac signaling functions in various aspects of tumorigenicity including the formation and progression of Ras-induced skin tumors and Wnt-induced intestinal tumors. Here, we further distinguish the oncogenic pathways that depend on Tiam1 signaling in the mammary gland.

We crossed Tiam1 knockout mice with MMTV-c-*myc* and MMTV-c-*neu* transgenic mice, in which the expression of both oncogenes is targeted to the mammary gland leading to mammary tumorigenesis. We found Tiam1 important for Neu-induced tumor formation and progression but not for Myc-induced tumors. Tiam1-deficiency delayed Neu-induced tumor initiation and reduced metastasis but had no effect on the growth of the MMTV-c-*neu* tumors.

Our data indicate that the Rac activator Tiam1 contributes to tumorigenicity induced by specific oncogenic signaling pathways only.

INTRODUCTION

The activity of Rho-like GTPases in response to receptor stimulation is strictly controlled 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). To date, over 70 Rho GEFs have been identified (Schmidt and Hall, 2002). In earlier studies, we have identified the *Tiam1* gene (T-cell invasion and metastasis gene 1), which encodes a specific GEF and thus activator of the Rho-like GTPase Rac (Habets et al., 1994;Michiels et al., 1995). Rho GTPases are best characterized for their regulation of actin cytoskeleton dynamics, but they also control various other processes including apoptosis, cell proliferation and gene transcription (Bishop and Hall, 2000). It is therefore not surprising that Rho GTPases and their regulators may contribute to various aspects of tumorigenicity (Malliri et al., 2002a;Sahai and Marshall, 2002).

The activators of Rho GTPases not only catalyze GDP/GTP exchange but also contribute to Rho GTPase downstream signaling by connecting active GTPases to specific scaffold and effector proteins (Mertens et al., 2003;Rossman et al., 2005). Scaffold proteins that complex Tiam1 with components of specific Rac effector pathways include IB2 and spinophilin, which direct Tiam1-mediated Rac activation towards the p38 MAPK and p70S6 K cascades implicated in transcription and translation, respectively (Buchsbaum et al., 2003;Buchsbaum et al., 2002). Tiam1 also binds to different components of the Par polarity complex and thereby regulates polarity processes in various cell types. Tiam1 associates with Par3 and PKC and connects Tiam1-mediated Rac signaling to the establishment of apical–basal cell polarity in contacting epithelial cells as well as front–rear polarity in freely migrating epithelial cells (Chen and Macara, 2005;Mertens et al., 2005;Pegtel et al., 2007). Tiam1 also associates with activated Rap1 and in conjunction with the Par polarity complex controls chemokine-induced T-cell polarization (Gerard et al., 2007). The association of Tiam1 with IRSp53 and p21Arc provides a direct link between Tiam1-mediated Rac activation and Arp2/3 complex-controlled actin polarization, which is required for cytoskeletal dynamics during cell migration and cell polarization (Connolly et al., 2005;Ten Klooster et al., 2006). In receptor signaling, activated Ras may activate Rac by direct binding to Tiam1 (Lambert et al., 2002) or indirectly by activation of phosphoinositide 3-kinase (PI3-kinase) that may recruit and activate Tiam1 and thereby Rac downstream of Ras ((Fleming et al., 2000;Zondag et al., 2000).

Tiam1 is expressed in human and rodent tumor cells of different tissue origin and has been shown to affect various aspects of tumorigenicity (Habets et al., 1995;Minard et al., 2004). The influence of Tiam1 on different stages of tumor development is illustrated in previous studies using mouse tumor model systems and Tiam1 knockout (*Tiam1* $^{-/-}$) mice. Tiam1-deficiency inhibits Ras-induced mouse skin tumor initiation and growth in a two-

stage DMBA/TPA carcinogenesis model (Malliri et al., 2002b). These skin tumors arise by DMBA-induced Ras mutations in keratinocytes of the epidermis. *Tiam1* $^{-/-}$ mice are more resistant to the Ras-induced skin tumor development because epidermal keratinocytes are more susceptible for Ras-induced apoptosis during tumor initiation. Although the few Ras-induced skin tumors that do occur in *Tiam1* $^{-/-}$ mice grow much slower than wild type tumors, they convert more frequently to a malignant phenotype, presumably as a result of the function of Tiam1 in maintenance of E-cadherin-based cell–cell adhesions (Malliri et al., 2002b;Malliri et al., 2004). Such a bifunctional effect of Tiam1-deficiency on tumor formation and progression was also found for intestinal tumors in APC Min (multiple intestinal neoplasia) mutant mice (Malliri et al., 2006). Tiam1 is a Wnt-responsive gene in colon cells and its deficiency reduces the formation and growth of polyps in APC Min mutant mice but promotes invasion of progressed malignant intestinal tumors (Malliri et al., 2006). These *in vivo* data indicate that Tiam1 functions downstream of at least two independent oncogenic signaling pathways, i.e., the Ras and the Wnt pathway.

Tiam1 is thus a potential therapeutic target, and chemical inhibitors have been developed to inhibit the function of Tiam1 and Rac in tumors *in vivo* (Gao et al., 2004;Shutes et al., 2007). In this context, it is important to decipher the specificity of Tiam1 as a modifier of tumor development and progression in the context of different oncogenic signaling pathways and of tumor cell types. Therefore, we investigated the function of Tiam1 in mammary tumorigenesis induced by two alternative oncogenic signaling pathways. We crossed *Tiam1* $^{-/-}$ mice with two strains of breast cancer prone transgenic mice that express oncogenic Myc or Neu under the control of the mouse mammary tumor virus (MMTV) promoter. Interestingly, we found that Tiam1-deficiency did not influence Myc-induced tumorigenesis but specifically impaired c-*neu* induced mammary tumor formation in mice, illustrating that Tiam1-mediated Rac signaling is required for only specific oncogenic signaling pathways that lead to tumorigenesis.

Materials and methods

Mice

A congenic line of FVB/*Tiam1* $^{-/-}$ mice was used, which was generated as described earlier (Malliri et al., 2002b). Transgenic MMTV-c-*neu*, line TG.NK (Muller et al., 1988) and MMTV-c-*myc* (Stewart et al., 1984). All transgenic mice were on FVB background. Transgenic male mice were crossed with *Tiam1* $^{-/-}$ females. The resulting *Tiam1* $^{\pm}$/MMTV-oncogene males were backcrossed with female *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice. From their offspring, *Tiam1* $^{+/+}$/MMTV-oncogene and *Tiam1* $^{\pm}$/MMTV-oncogene males were backcrossed to *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ females, respectively, to yield the experimental groups: *Tiam1* $^{+/+}$/MMTV-oncogene, *Tiam1* $^{\pm}$/MMTV-oncogene, *Tiam1* $^{-/-}$/MMTV-oncogene. Only

females were used for subsequent analyses. MMTV-c-*neu* females were kept as virgins throughout the entire observation period. MMTV-c-*myc* females underwent forced breeding for two pregnancies to promote tumorigenesis. Mice were monitored by palpation for tumors and killed when they harbored a mammary tumor that reached a size of 10 mm. Local ethics committee for animal experiments approved the mouse experiments according to the Dutch law that implements the European guideline 86/609/EEG.

Histology and immunohistochemistry

H&E stainings and immunohistochemistry were performed on 4 µm paraffin-embedded tissue sections as described (Strumane et al., 2005). Antibodies used are as follows: an anti-DH Tiam1-specific rabbit polyclonal antibody (Habets et al., 1994), polyclonal anti-Keratin 1 (Covance/Babco, 1:250), anti-Keratin 8 (troma-1; University of Iowa, Department of Biological Sciences, Iowa City, Iowa USA; 1:400) and anti-Keratin 14 (Covance/Babco; 1:10,000).

Whole mounts of mammary glands

Inguinal mammary fat pads were excised from euthanized mice and stretched on a glass slide for fixation in methanol:chloroform:acidic acid (6:3:1) for at least 24 h. After washing in 70% ethanol for 1 h, the slides were rinsed in water and stained in carmine for 24 h. All incubations were performed at room temperature (RT). The fat pads were dehydrated in a graded series of alcohols and kept in methyl salicylate to make photographic images.

Isolation of mammary epithelial cells

The left and right mammary fat pads 2 and 3 were excised from euthanized mice at 16 weeks of age. The isolated tissues were washed three times in 70% ethanol and transferred to L15 medium (Gibco BRL) with 10% fetal calf serum (FCS). The fat pads were chopped using a scalpel and digested in a 0.3% collagenase–0.15% trypsin mix in serum-free L15 medium at 37°C for 1 h with periodic shaking. Cells were pelleted and washed four times with L15 medium with serum and subsequently incubated in DMEM containing 10% FCS, 2 mM L-glutamine (L-Gln) and 100 U/ml penicillin/100 µg/ml streptomycin (P/S) at 37°C, 5% CO_2, 5% O_2 to remove contaminating fibroblasts. After 1 h, the epithelial cells were still floating in the medium and could be easily separated from the fibroblasts that had attached to the bottom of the culture flasks. The pelleted epithelial cells were resuspended and cultured for maximal 3 days in DMEM:F12 (1:1; Gibco-BRL) medium containing 10% FCS and P/S and supplemented with 5 µg/ml insulin, 5 ng/ml cholera toxin and 5 ng/ml EGF.

Western blotting

Lysates were prepared using standard SDS or RIPA lysis buffers as indicated. Cultured cells were washed with PBS and scraped in lysis buffer. Snap frozen tumor material was first grinded in a mortar and than lysed. Proteins were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). After blocking with 5% skimmed milk, the blots are probed using the indicated antibodies. Primary antibodies used were anti-DH (Habets et al., 1994) 1/500 and C-16 (sc-872, Santa Cruz, Venendaal, The Netherlands; 1/1.000) against Tiam1, anti-α-tubulin (Clone B-5-1-2, Sigma, 1/5.000), anti-c-erbB2/HER-2/neu (Ab-17, Neomarkers, 1/2.000) and anti-Rac1 (clone 23A8, Upstate Biotechnology, Venendaal, The Netherlands; 1/1.000). As secondary antibodies, peroxidase-conjugated IgGs were used followed by enhanced chemiluminiscence (ECL) detection (Amersham).

Apoptosis quantification

MDA-MB-361 cells were cultured in DMEM:F12 (1:1; Gibco-BRL) medium supplemented with P/S, 10% FCS, 10 ng/ml EGF and 10 μg/ml insulin. The Tiam1-specific siRNA oligo GCGAAGGAGCAGGTTTTCT (Malliri et al., 2004) was transfected into the MDA-MB-361 cells using the Dharmafect-1 reagent (Dharmacon). A scrambled sequence was used as nonspecific control siRNA oligo (si*CONTROL* non-targeting siRNA, Dharmacon). Six hours after transfection, the transfection mix was replaced by culture medium, which was re-freshed again 24 h after transfection and apoptosis was quantified 72 h after transfection. Both floating and adherent cells were lysed together and apoptosis was analyzed using the Cell Death Detection ELISA kit (Roche) according to manufactures instructions.

Results

Tiam1 expression in the mammary gland is increased in tumor tissue

We started our analyses by determining Tiam1 expression in the mammary glands and mammary tumors of transgenic and non-transgenic female mice. Tiam1 is expressed in the normal mammary gland as shown by Western blot analysis of primary cells isolated from 16-week-old wild type mice (Fig. 1A, left lane). At this age, we found increased Tiam1 expression levels in the precancerous mammary gland of MMTV-c-*neu* transgenic mice (Fig. 1A, right lane). Western blot analysis revealed Tiam1 expression in both MMTV-c-*myc* and MMTV-c-*neu* tumors (Fig. 1B). Consistent with these data, others also found that Tiam1 was increased in MMTV-c-*neu* tumors compared to normal tissue at the mRNA level (Landis et al., 2005). Tiam1 expression in tumors was confirmed by immunohistochemical staining of mammary tumors isolated from *Tiam1* $^{+/+}$ mice (Fig. 1C). These data demonstrate that Tiam1 is expressed in the mammary gland and that its expression is increased in Neu-induced and Myc-induced mammary tumors.

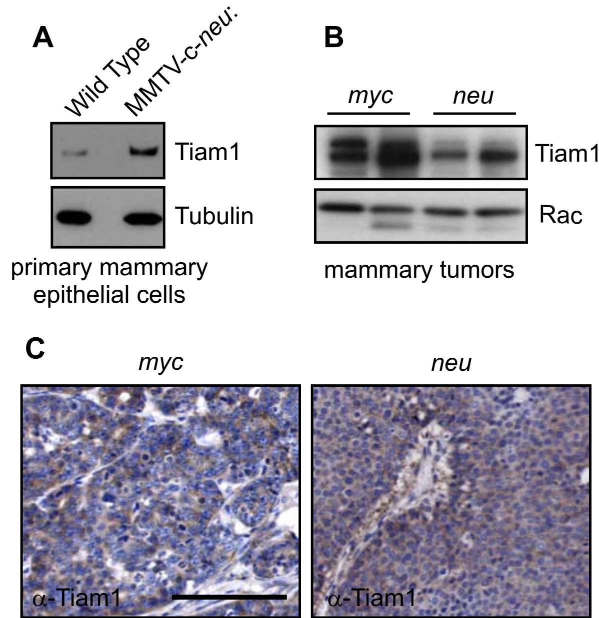

Figure1. Tiam1 expression in mouse mammary tissue and tumors. A Tiam1 is detected (C16 antibody) by Western blotting in primary epithelial cells isolated from precancerous mammary glands of 16-week-old mice. Tiam1 expression is increased in the mammary gland of MMTV-c-*neu* mice compared to wild type mice. The same blot was probed for the detection of α-tubulin for loading control. **B** Western blot analysis of Tiam1 protein levels in tumor samples derived from MMTV-c-*myc* and MMTV-c-*neu* mice (C16 antibody). Two SDS lysates of snap-frozen tumors isolated from different mice are shown for each transgenic line. The same blot was probed with a Rac antibody and used as a loading control. **C** Expression of Tiam1 in histological tumor slides. Mammary carcinomas from MMTV-c-*myc*;*Tiam1* $^{+/+}$ and MMTV-c-*neu*;*Tiam1* $^{+/+}$ are shown probed for Tiam1 (anti-DH antibody). The *scale bar* indicates 100 μm

Normal mammary gland development in Tiam1-deficient mice

Tiam1 knockout mice are viable and do not show any obvious aberrant phenotype (Malliri et al., 2002b). Also the mammary glands of *Tiam1* $^{-/-}$ mice are functionally normal as *Tiam1* $^{-/-}$ females are able to suckle their offspring. To exclude that tumorigenesis is influenced by morphological differences in mammary gland development, we compared in detail the appearance of the inguinal mammary glands of virgin *Tiam1* $^{-/-}$ female mice with that of *Tiam1* $^{+/+}$ mice in nontransgenic and transgenic animals at different ages (Fig. 2). The appearance of *Tiam1* $^{-/-}$ mammary glands was identical to that of wild type glands in adult mice (not shown). However, we found a delay in the early development of the mammary gland in *Tiam1* $^{-/-}$ when compared to wild type mice. The outgrowth of the inguinal mammary glands is determined by how far the glands extend beyond the inguinal lymph node (to the right side in Fig. 2). The mammary ductal system was significantly less far proliferated within the adipose stroma in 4-week-old and 6-week-old *Tiam1* $^{-/-}$

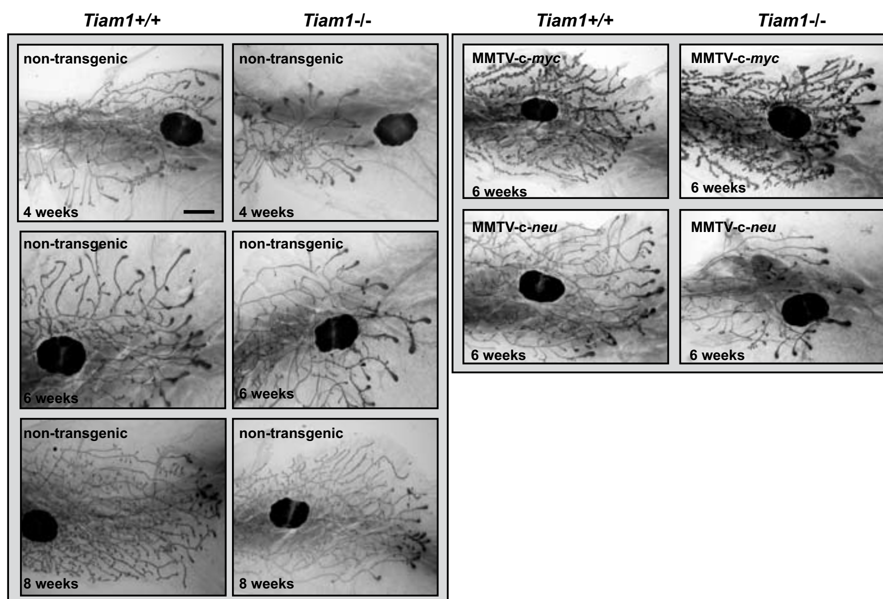

Figure 2. Analysis of mammary gland development. Inguinal mammary glands from 4-, 6-, and 8-week-old *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ virgin female mice were compared within the nontransgenic mice (*left panel*) and from 6-week-old *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice in the MMTV-c-*myc* and MMTV-c-*neu* transgenics (*right panel*). All mammary glands are positioned in the same orientation so that the outgrowth of the mammary glands is presented from left to right. The distance towards or beyond the inguinal lymph node is a measure for the outgrowth of the mammary gland. Representative images show the delay in elongation of the system of mammary ducts in the *Tiam1* $^{-/-}$ mice compared to *Tiam1* $^{+/+}$ mice in the different strains. Mammary whole mounts were stained with carmine red. The *scale bar* indicates 1.5 mm

mice when compared to age-matched wild type mice (Fig. 2 left panel). At 8 weeks, the appearance of the mammary gland in Tiam1$^{+/+}$ and Tiam1$^{-/-}$ mice was indistinguishable, indicating that development was delayed but not impaired (Fig. 2 left panel). This delay in early mammary gland elongation during puberty in *Tiam1* $^{-/-}$ mice was also observed for both transgenic strains in a *Tiam1* $^{-/-}$ background as exemplified in 6-week-old mice (Fig. 2 right panel). By analyzing mammary glands at different time points, we found that the delay in outgrowth of the mammary gland was most apparent at 4 weeks of age, declined within the subsequent weeks and by 8 weeks of age we could not discriminate anymore between wild type and Tiam1 knockout mammary glands. As the first tumors in MMTV-oncogene mice became detectable only by 17 weeks of age, we conclude that it is unlikely that the delay in early normal mammary gland development affects mammary tumorigenesis in the mouse mammary tumor models used.

Tiam1 is involved in mammary tumorigenesis induced by Neu and not by Myc

To address whether Tiam1 is involved in specific oncogenic pathways, we examined mammary tumorigenesis in MMTV-c-*neu* and MMTV-c-*myc* female mice in *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ backgrounds. Weekly palpation of the mice revealed that Tiam1-deficiency strongly delayed the appearance of tumors in MMTV-c-*neu* transgenic mice, whereas the latency of mammary tumors induced by Myc was not affected by the loss of Tiam1 (Fig. 3). MMTV-c-*myc* mice developed mammary tumors with the same latency in *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice (Fig. 3A). Although all except one of the MMTV-c-*neu* mice developed palpable tumors over an observation period of 1 year (Table 1), the latency was significantly longer in the Tiam1-deficient mice ($P < 0.001$) (Fig. 3B). At the time when tumors were detected in 100% of the MMTV-c-*neu*;*Tiam*1$^{+/+}$ mice (age 29 weeks), detectable tumors were found in only 19% of the MMTV-c-*neu*;*Tiam*1$^{-/-}$ mice. This delay in the rate of tumor initiation is also reflected in the T_{50} that denotes the age at which 50% of the populations possess at least one palpable tumor. The T_{50} was 23.5 weeks for MMTV-c-*neu*;*Tiam*1$^{+/+}$

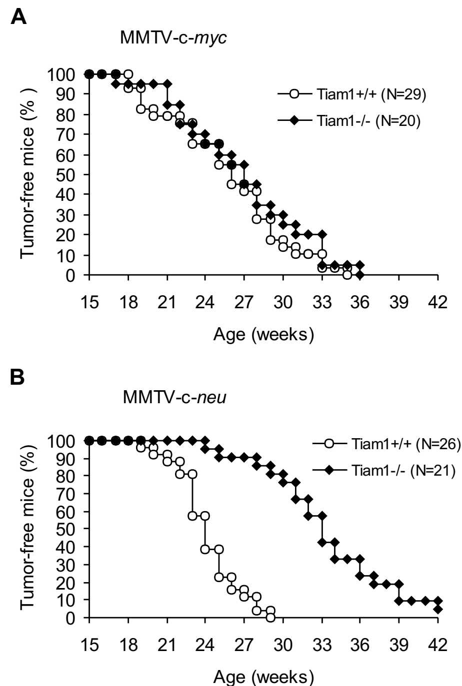

Figure 3. Kinetics of oncogene-induced mammary tumor initiation in *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice. The age when a palpable mammary tumor first appears represents the latency of tumor initiation. **A** Tumor initiation in MMTV-*myc*;*Tiam1* $^{-/-}$ ($n = 20$) compared to MMTV-*myc*;*Tiam1* $^{+/+}$ ($n = 29$) is not different ($P = 0.41$, Student's *t* test). **B** The longer latency in MMTV-*neu*;*Tiam1* $^{-/-}$ ($n = 21$) compared to MMTV-*neu*;*Tiam1* $^{+/+}$ ($n = 26$) is statistically significant ($P < 0.001$, Student's *t* test). *N* is the number of mice analyzed

Table 1. Incidence of mammary tumors in transgenic mice

	Tiam1$^{+/+}$	**Tiam1$^{-/-}$**
MMTV-c-*myc*	29/29	20/20
MMTV-c-*neu*	26/26	20/21

The presented numbers are the ratios of females displaying mammary tumors to the total number of females observed over a period of 52 weeks

mice and 32.5 weeks for MMTV-c-*neu*;*Tiam*1$^{-/-}$ mice. Together, these data indicate that, while Tiam1 is not required for the induction of mammary tumors by Myc, it does play a critical role in the initiation of mammary tumors induced by Neu. More specifically, Tiam1-deficiency extends the latency of Neu-induced mammary tumor initiation.

Tiam1-deficiency does not influence the type of tumor differentiation in MMTV-c-*neu* and MMTV-c-*myc* mice

We analyzed a possible relation between the involvement of Tiam1 and the tumor characteristics. The MMTV-c-*myc* and c-*neu* tumors were defined as adenocarcinomas based on histopathological analysis of hematoxylin and eosin (H&E) stainings (Fig. 4A). To further analyze the differentiation status of the tumors in our analysis, we performed additional stainings for Keratins. The MMTV-c-*myc* and c-*Neu* tumors were all positive for Keratin 8 in both *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice (Fig. 4B, a–d), indicating that they all had a glandular character, although this was less evidently suggested by the H&E staining in the case of the MMTV-c-*neu* tumors. Keratin 1 is normally not expressed in the mammary gland and is used as a marker for epidermal differentiation in mammary tumors. All tumors were negative for Keratin 1 (Fig. 4b, e–h), while the adjacent skin tissue served as an intrinsic positive control (not shown). Although the Keratin 1 staining suggested that none of the tumors contained epidermal characteristics, MMTV-c-*myc* tumors showed a positive Keratin 14 staining indicating squamous metaplasia (Fig. 4B, i, j) in both *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice. However, the MMTV-c-*neu* tumors were completely negative for Keratin 14 (Fig. 4B, k, l). From these immunohistological analyses, we conclude that the presence or lack of Tiam1 has no effect on the differentiation type of the MMTV-c-*myc* and MMTV-c-*neu* mammary tumors. The MMTV-c-*neu* tumors show homogeneously a glandular differentiation (Keratin 8-positive), whereas MMTV-c-*myc* tumors consist of different components with glandular (Keratin 8-positive) and squamous (Keratin 14 positive) differentiation. This is in agreement with the notion that MMTV-c-*neu* mice uniformly develop mammary tumors (Bargmann et al., 1986;Muller et al., 1988), whereas MMTV-c-*myc* mice develop mammary tumors in a stochastic fashion. As reported earlier, c-*myc* is necessary but not sufficient for tumorigenesis, indicating that additional events are required for mammary tumor development in these mice (Li et al., 2000;Stewart et al., 1984;Tsukamoto et al., 1988). Taken together, MMTV-c-*neu* tumors can be discriminated

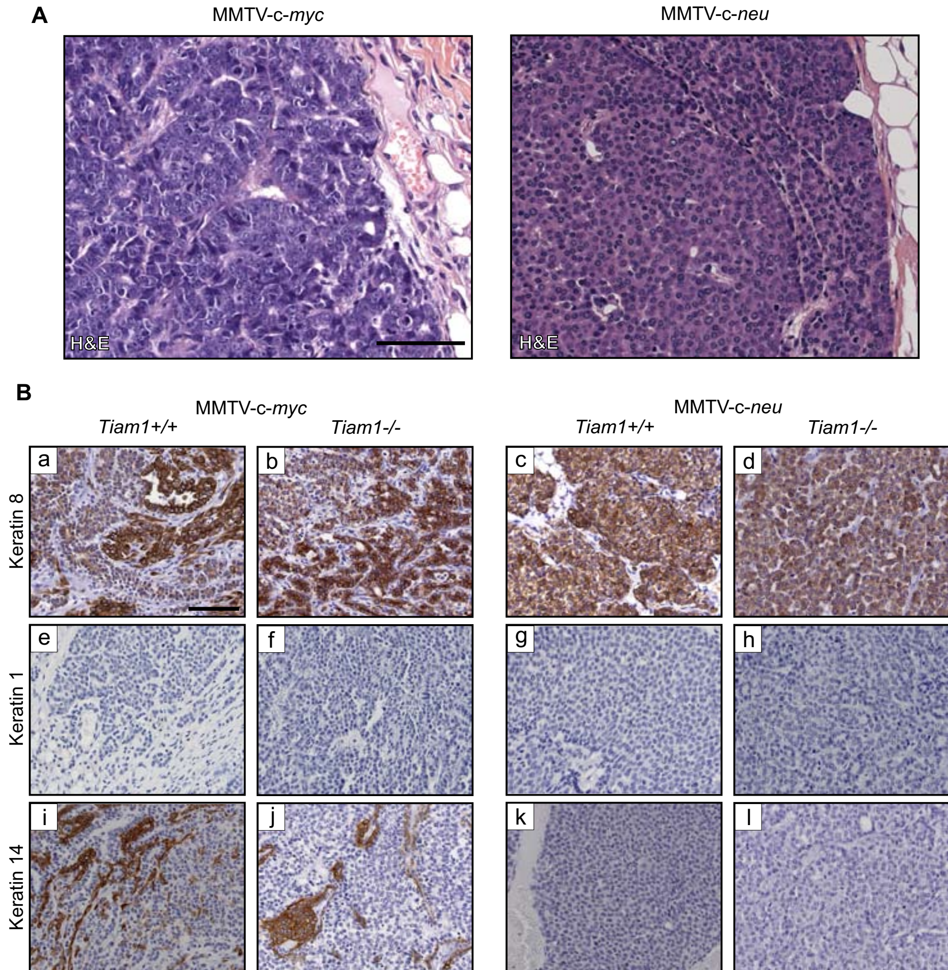

Figure 4. Tiam1 does not affect differentiation of mammary tumors in MMTV-c-myc and MMTV-c-neu mice. A Sections of mammary tumors from the indicated transgenic mice, stained with hematoxylin and eosin (H&E). The *scale bar* indicates 100 µm. **B** Immunohistochemical stainings of MMTV-c-*myc* and MMTV-c-*neu* tumors in *Tiam* $^{+/+}$ and *Tiam1* $^{-/-}$ mice. All tumors are positive for Keratin 8, which is specific for glandular characteristics. All tumors are negative for the epidermal marker Keratin 1. Keratin 14 staining shows squamous differentiation in MMTV-c-*myc* tumors, while MMTV-c-*neu* tumors are negative for Keratin 14. The *scale bar* indicates 100 µm

from MMTV-c-*myc* tumors by the fact that they show glandular differentiation only and no squamous characteristics. Furthermore, the presence or absence of Tiam1 does not influence the histological type of tumors raised by Myc or Neu expression.

Tiam1-deficiency affects initiation but not growth of *neu*-induced mammary tumors

Western blot analysis of mammary tumors in *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice showed that disruption of Tiam1 expression did not alter the expression levels of the Neu protein (Fig. 5A). This excludes the possibility that the delay in initiation of Neu-induced mammary tumors in *Tiam1* $^{-/-}$ mice was caused by an inadequate expression of the *neu* transgene in the mammary glands of these mice. The latency of MMTV-c-*neu*-induced tumors in the *Tiam1* $^{\pm}$ group was intermediate between that in the *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ groups (Fig. 5B) suggesting a dose-dependent effect of Tiam1 on the initiation of Neu-induced mammary tumors. We found earlier a similar dose-dependent effect of Tiam1 on Ras-induced skin tumors (Malliri et al., 2002b).

The decreased susceptibility to Neu-induced tumors in Tiam1-deficient mice was also reflected by a decreased number of tumors per mouse (Fig. 5C). Mice were euthanized for dissection when they harbored at least one mammary tumor that reached a size of 10 mm. The median number of tumors with a size >4 mm per mouse is four in *Tiam1* $^{+/+}$ mice, three tumors per mouse in *Tiam1* $^{\pm}$ and two tumors per mouse in *Tiam1* $^{-/-}$ mice, again suggesting a dose-dependent effect of Tiam1 on tumor initiation (Fig. 5C). Tumor growth was determined by the time between detection of the first palpable tumor and necropsy, i.e., when the tumor had grown out to a size that reached 10 mm. We found that the average growth of individual MMTV-c-*neu* tumors was not significantly different in *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ backgrounds (Fig. 5D). This indicates that once a tumor is initiated in the *Tiam1* $^{-/-}$ mice, it is able to grow as fast as in the *Tiam1* $^{+/+}$ mice. Tiam1-deficiency also did not affect mammary tumor growth in the MMTV-c-*myc* model in which tumor initiation was not affected (not shown). Together, these data indicate that the delayed latency and the fewer tumors in *Tiam1* $^{-/-}$ mice compared to *Tiam1* $^{+/+}$ mice are a consequence of a specific function of Tiam1 in Neu-induced tumor initiation rather than an involvement of Tiam1 in the growth of the mammary tumors.

Tiam1 provides survival signaling in Neu-induced mammary tumor cells

Prevention of apoptosis is a necessary step during the process of tumor initiation. The increased susceptibility for apoptosis was found to be the underlying mechanism of decreased skin tumor incidence in *Tiam1* $^{-/-}$ mice (Malliri et al., 2002b). As in Ras-induced skin tumors, it is possible that Tiam1-deficiency results in a reduced number of tumors in the MMTV-c-*neu* mouse model by increasing the susceptibility to apoptosis of the targeted mammary epithelial cells. Attempts to analyze apoptosis *in vivo* in established tumors did not discriminate Neu-induced tumors between Tiam1$^{+/+}$ and Tiam1$^{-/-}$ mice. Therefore, we analyzed the dependency of the survival of Neu-expressing breast cancer cells on Tiam1 *in vitro*. As a model, we used the human breast cancer cells MDA-MB-361, which are characterized by high Neu expression. Western blot analysis showed that MDA-MB-361

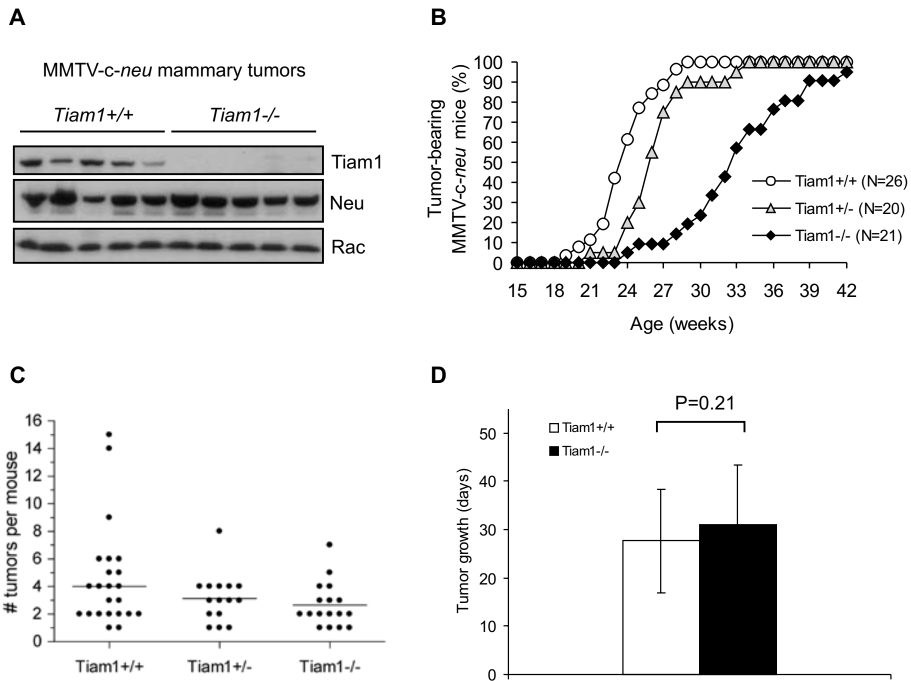

Figure 5. Tiam1 affects number of neu-induced mammary tumors. A Western blot analyses. SDS lysates of snap frozen tumors isolated from *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ MMTV-c-*neu* mice were blotted and probed for detection of endogenous Tiam1 and transgenic Neu. The same blot was probed for Rac1 and used as loading control. **B** Kinetics of the tumor susceptibility in MMTV-*neu*;*Tiam1* $^{\pm}$ mice ($N = 20$) is intermediate between MMTV-*neu*;*Tiam1* $^{+/+}$ ($N = 26$) and MMTV-*neu*;*Tiam1* $^{-/-}$ ($N = 21$) mice. The percentage of mice with a palpable mammary tumor is presented in time. *N* is the number of mice analyzed. **C** Plot of the total number of tumors with a size >4 mm per mouse in MMTV-c-*neu*;*Tiam1* $^{-/-}$, MMTV-c-*neu*;*Tiam1* $^{\pm}$ and MMTV-c-*neu*;*Tiam1* $^{+/+}$ mice at the time of necropsy. The median number of tumors per mouse is indicated as a *horizontal line* and is 4 in *Tiam1* $^{+/+}$ mice ($N = 23$; range 1–15 tumors per mouse), 3 in *Tiam1* $^{\pm}$ mice (N = 15; range 1–8 tumors per mouse) and only 2 in *Tiam1* $^{-/-}$ mice ($N = 17$; range 1–7 tumors per mouse). *N* is the number of mice analyzed. **D** Tumor growth is similar in *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice. The average time for a tumor to grow out from just palpable to a diameter that reached 10 mm in the *Tiam1* $^{-/-}$ mice (N = 14) compared to *Tiam1* $^{-/-}$ mice (N = 23) is not significantly changed ($P = 0.21$, Student's *t* test). *N* is the number of mice analyzed

cells express Tiam1 that can be downregulated by Tiam1-specific siRNA (Fig. 6A). The susceptibility to apoptosis was measured using a cell death detection kit that quantitatively determines cytoplasmic histone-associated DNA fragments by ELISA (enzyme-linked immunosorbent assay). Interestingly, the number of apoptotic cells is increased upon downregulation of Tiam1 when compared to cells that were transfected with a nonspecific control siRNA (Fig. 6B), indicating that Tiam1 also controls apoptosis in Neu-expressing tumor cells.

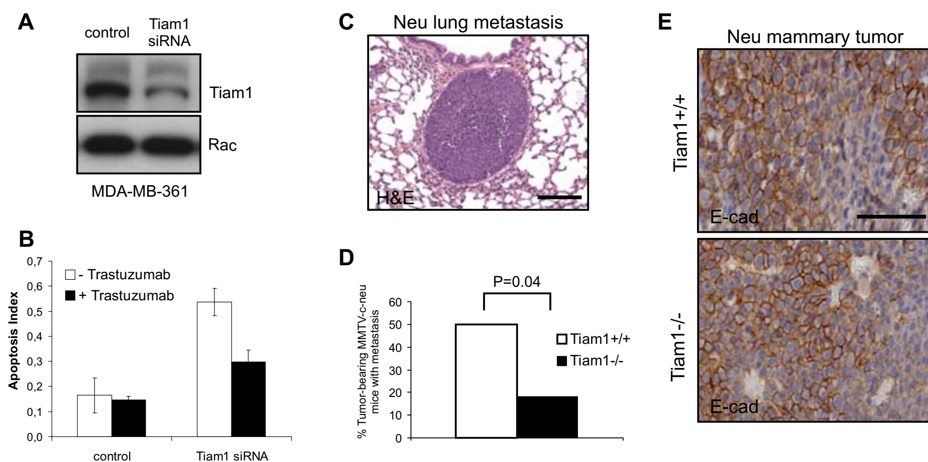

Figure 6. Tiam1 affects apoptosis and metastasis in the cells with high Neu expression. A, **B** Apoptosis downstream of Neu signaling is Tiam1-dependent. **A** Western blot analysis showing Tiam1 downregulation in MDA–MB-361 breast cancer cells 3 days after transfection of Tiam1-specific siRNA. As a control, a nonspecific scrambled siRNA sequence was used. The same blot was probed with a Rac antibody and used as a loading control. **B** Tiam1 downregulation increased apoptosis as determined by the measurement of the amount of cytoplasmic histone-associated DNA fragments. Apoptosis was measured 3 days after siRNA transfection. *Bars* show the average of three independent experiments. **C–E** Metastasis of Neu-induced tumors was lower in *Tiam* $^{-/-}$ than in *Tiam* $^{+/+}$ mice. **C** Representative image of an H&E-stained slide showing a typical nonextravasating metastatic embolus of mammary adenocarcinoma cells in the lung of a MMTV-c-*neu* mice. The *scale bar* indicates 100 µm. **D** Mammary tumor-bearing *Tiam1* $^{+/+}$ mice show pulmonary metastases in 50% of the animals ($N = 22$), while metastatic emboli in the lungs were found for only 18% of the *Tiam* $^{-/-}$ mice ($N = 17$). *N* is the number of mice analyzed. **E** E-cadherin expression in mammary tumors in MMTV-c-*neu*;*Tiam1* $^{+/+}$ and MMTV-c-*neu*;*Tiam1* $^{-/-}$ mice. The *scale bar* indicates 100 µm

Tiam1-deficiency affects metastatic potential of Neu-induced mammary tumors

We also analyzed the MMTV-c-*neu* tumor-bearing mice for the presence of metastases by histopathological analysis. We found metastases mainly in the lungs and occasionally in the heart. This is consistent with earlier observations that MMTV-c-*neu*-induced mammary tumors metastasize to the lung with high frequency (Guy et al., 1992;Siegel et al., 2003;Taverna et al., 2005). Most of the observed lung lesions were intravascular metastases representing tumor cells that remain fully contained within a pulmonary vessel without extravasations as shown by an H&E staining of lung tissue (Fig. 6C). The percentage of mice with lung micrometastases was found to be significantly lower in MMTV-c-*neu*;*Tiam1* $^{-/-}$ mice compared to MMTV-c-*neu*;*Tiam1* $^{+/+}$ mice. We detected lung metastases in 50% of the MMTV-c-*neu*;*Tiam1* $^{+/+}$ mice and only in 18% of the MMTV-c-*neu*;*Tiam1* $^{-/-}$ mice (Fig. 6D). Tiam1 has been shown to be involved in strengthening of E-cadherin-based cell–cell adhesions, which influences metastatic capacities of tumor cells (Malliri et al., 2004). However, E-cadherin staining of MMTV-c-*neu* mammary tumors showed fields of E-cadherin-negative cells within a majority of E-cadherin-positive tumor cells in both *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice (Fig. 6E), suggesting that Tiam1-deficiency

did not affect the metastatic capacity of Neu-induced mammary tumors by affecting the E-cadherin-mediated cell–cell adhesions. Presumably, the lower incidence of metastases in the *Tiam1* $^{-/-}$ mice is a direct consequence of the lower number of mammary tumors produced per mouse in *Tiam1* $^{-/-}$ mice.

Discussion

We crossed *Tiam1* $^{-/-}$ mice with MMTV-c-*myc* and MMTV-c-*neu* transgenic mice to study the consequences of Tiam1-deficiency in Myc-induced and Neu-induced mammary tumorigenesis. Our analyses revealed that Tiam1 is required for oncogenic signaling induced by Neu but not by Myc. More specifically, we found that Tiam1-deficiency delays the initiation of Neu-induced mammary tumors but does not affect the growth of these tumors. Initiation and growth of Myc-induced mammary tumors was independent of the expression of Tiam1.

Besides the dramatic delay in the onset of the first detectable Neu-induced mammary tumors, also the total number of tumors per mouse was lower in *Tiam1* $^{-/-}$ mice than in *Tiam1* $^{+/+}$ animals. This is consistent with the findings in skin and intestinal tumors, where the latency of tumor onset and the number of tumors per mouse were dramatically decreased in a Tiam1-deficient background (Malliri et al., 2002b;Malliri et al., 2006). In the skin tumor model, we found that *Tiam1* $^{-/-}$ mice produced less Ras-induced tumors, because keratinocytes in the basal layer of the epidermis are more susceptible to apoptosis (Malliri et al., 2002b). Recently, we found that the Tiam1/Rac-mediated survival pathway in keratinocytes acts through ROS-mediated activation of the ERK pathway (Rygiel et al., 2008). Also in APC Min mice, the decreased initiation of intestinal tumors by aberrant β-catenin signaling in Tiam1-deficient mice compared to wild type mice was attributed to increased apoptosis susceptibility (Malliri et al., 2006). Consistent with this, Tiam1 expression levels correlate with apoptosis susceptibility in human colon tumor cells (Minard et al., 2006).

It is likely that Tiam1-deficiency in the MMTV-c-*neu* model affects tumor initiation by increasing the susceptibility to apoptosis of the targeted mammary epithelial cells. We have attempted to study apoptosis sensitivity *in vivo*, but we could not find significant differences in apoptosis between established Neu-induced tumors produced in *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice (data not shown). Analysis of apoptosis in tumor samples of MMTV-c-*neu* tumors by TUNEL and Caspase 3 stainings appeared difficult because of the heterogeneity of these tumors. Moreover, apoptosis resistance is most likely essential during the initiating events of tumorigenicity, which is difficult to study *in vivo*. Once tumors have been formed, differences in apoptosis sensitivity in *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ tumors are presumably not detectable anymore, as Tiam1-independent events have rescued the tumor-

initiating cells from apoptosis. We performed therefore *in vitro* studies using MDA-MB-361 breast cancer cells with high Neu expression and found that the survival signaling of these tumor cells is dependent on the presence of Tiam1. Similarly, as found in DMBA-induced skin tumors and β-catenin-induced intestinal tumors, the presence of Tiam1 seems to be required to prevent apoptosis during initiation of mammary tumors by the Neu oncogene. Tiam1-mediated Rac-signaling might prevent apoptosis by activating various well-known survival signaling pathways including the NFκB and ERK pathways (Joneson and Bar-Sagi, 1999;Rygiel et al., 2008;Zahir et al., 2003).

Although less mammary tumors were produced in *Tiam1* $^{-/-}$ mice, the growth of the tumors in *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice was the same once they were formed. In contrast, we showed in skin and intestinal tumors a function of Tiam1 in both initiation and growth of these tumors (Malliri et al., 2002b;Malliri et al., 2006). Interestingly, in DMBA (Ras)-induced skin tumors, decreased growth of tumors in *Tiam1* $^{-/-}$ mice was found only when proliferation of tumors was promoted by TPA treatment. Skin tumors that were generated by treatment with DMBA only grew equally well in the presence or absence of Tiam1 (Malliri et al., 2002b), indicating that TPA-induced but not DMBA-induced proliferation depends on Tiam1-mediated Rac activation. TPA is able to induce Cyclin D1 expression, a regulator of cell proliferation (Yan and Wenner, 2001), suggesting that Tiam1 is required for TPA-induced proliferation by influencing Cyclin D1 levels. Moreover, both the Ras and Neu oncogenes are absolutely dependent on Cyclin D1 expression for mammary tumor formation in MMTV-*ras* and MMTV-c-*neu* transgenic mice (Yu et al., 2001). Cyclin D1-deficient mice are resistant to Ras-induced and Neu-induced mammary tumors, while they remain fully sensitive to other oncogenic pathways (Yu et al., 2001). However, the fact that the proliferation of Neu-induced tumors is independent of Tiam1 suggests that Tiam1 does not regulate Cyclin D1 levels in mammary tumors. Indeed, tumor lysates from MMTV-c-*neu*;*Tiam1* $^{+/+}$ and MMTV-c-*neu*;*Tiam1* $^{-/-}$ mice revealed a large variation in Cyclin D1 levels independent of the presence of Tiam1 (data not shown). As Neu predominantly signals through Ras and the growth of DMBA-only treated skin tumors is independent of Tiam1, it is unlikely that Tiam1–Rac signaling contributes to Ras-controlled proliferation of tumors. Interestingly, it has been shown that Neu-mediated protection from apoptosis is dependent on its association with the Par polarity complex, while Neu-mediated proliferation is not (Aranda et al., 2006). Tiam1 associates with the Par polarity complex and is able to activate this complex (Mertens et al., 2006), providing a possible mechanism by which Tiam1 could interfere in initiation but not growth of Neu-induced mammary tumors.

A higher number of metastases was found in the MMTV-c-*neu*;*Tiam1* $^{+/+}$ mice when compared to MMTV-c-*neu*;*Tiam1* $^{-/-}$ mice, suggesting that Tiam1 promotes metastasis of breast tumors. Studies in human tumors also show a positive correlation between Tiam1 expression and progression and invasiveness of mammary, colon and prostate tumors (Adam et al., 2001;Engers et al., 2006;Liu et al., 2007;Minard et al., 2005;Minard et al.,

2006). This is in contrast to the findings in skin and intestinal tumors, where progression was associated with loss of Tiam1 (Malliri et al., 2002b;Malliri et al., 2006). The latter could be explained by a function of Tiam1 in the formation and maintenance of intercellular adhesions (Engers et al., 2001;Hordijk et al., 1997;Mertens et al., 2005;Uhlenbrock et al., 2004). In the MMTV-c-*neu* mice, the metastases appear in pulmonary blood vessels as tight tumor emboli that are thought to arise from circulating cell clumps that get stocked in the veins of the lungs. As in human inflammatory breast cancers (IBC), such circulating tumor emboli might benefit from strong E-cadherin-mediated cell–cell interactions favoring passive dissemination in distinct organs (Kleer et al., 2001;Tomlinson et al., 2001). However, we could not find significant differences in E-cadherin expression between Neu-induced tumors in *Tiam1* $^{+/+}$ and *Tiam1* $^{-/-}$ mice that could support such a mechanism. Alternatively, the higher number of metastases found in the MMTV-c-*neu*;*Tiam1* $^{+/+}$ mice could be the result of the increased number of tumors found in these mice.

In conclusion, the effects of Tiam1-mediated Rac signaling on tumorigenesis appear oncogene-dependent and tumor cell type-dependent and either positively or negatively correlate with tumor progression. As Tiam1-mediated Rac activation controls different signaling pathways that may influence initiation, growth and progression of tumors, the cellular outcome of altered Tiam1 expression may depend on a balance between factors that promote or inhibit the formation and progression of tumors.

Acknowledgments

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Chapter 4

The Rac Activator Tiam1 and Ras-induced oncogenesis

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Abstract

The *Tiam1* gene encodes a guanine nucleotide exchange factor (GEF) that specifically activates the Rho-like GTPase Rac. *In vitro* studies indicate that Tiam1 localizes to adherens junctions and plays a role in the formation and maintenance of cadherin-based cell adhesions thereby regulating migration of epithelial cells. *In vivo* studies implicate Tiam1 in various aspects of tumorigenesis. In this chapter we discuss the use of the DMBA/TPA chemical carcinogenesis protocol in Tiam1-deficient mice to study the role of Tiam1 in Ras-induced skin tumors. This two-stage carcinogenesis protocol allows to study initiation, promotion and progression of tumors in a Tiam1-positive and -negative background. Moreover, we describe methods to study the role of Tiam1 in: susceptibility to apoptosis, cell growth, and Ras transformation by *in vivo* and *in vitro* experiments. The latter make use of tumor cells and primary embryonic fibroblasts and keratinocytes isolated from mice.

INTRODUCTION

Mouse genetic engineering technologies provide powerful means of elucidating the multistage process of tumorigenesis *in vivo*. Switch on/off systems for regulatable reversible gene expression include interferon, tetracycline and tamoxifen, possibly in combination with Cre-Lox or Flp-FRT recombinase conditional technologies (Jonkers and Berns, 2002;Hirst and Balmain, 2004). Mice with somatic expression of an oncogene or somatic inactivation of a tumor-suppressor gene mimic human sporadic tumor formation and are very useful to study the importance of particular genes in specific tumor types. Moreover, bioluminescence imaging allows quantification of tumor growth and metastasis in time. This method has been used initially in luciferase-expressing transplanted tumors (Edinger et al., 1999) but is now applicable for somatic tumors in Cre/loxP mouse models when crossed with a conditional reporter line for Cre-dependent luciferase expression (Lyons et al., 2003).

The Ras proto-oncogene is mutationally activated in many human cancers. Activated Ras induces multiple signaling pathways that are mediated by different effector proteins that bind to activated Ras. These effectors include Raf protein kinases, phosphoinositide-3 kinases, and guanine nucleotide exchange factors (GEFs) for the small GTPases Ral and Rac (Bar-Sagi and Hall, 2000;Repasky et al., 2004). The effector molecules act either in parallel or synergistically in oncogenic signaling downstream of active Ras. The Rac-specific GEF Tiam1 provides a direct link between Ras and Rac by activating Rac upon binding to activated Ras (Lambert et al., 2002). The *Tiam1* gene was initially identified in our laboratory by retroviral insertional mutagenesis in combination with *in vitro* selection for invasive T-lymphoma cells (Habets et al., 1994). Tiam1 binds to Ras via a conserved Ras-binding domain (RBD) (Fig. 1). The 1591 aa Tiam1 protein is further characterized by a C-terminal

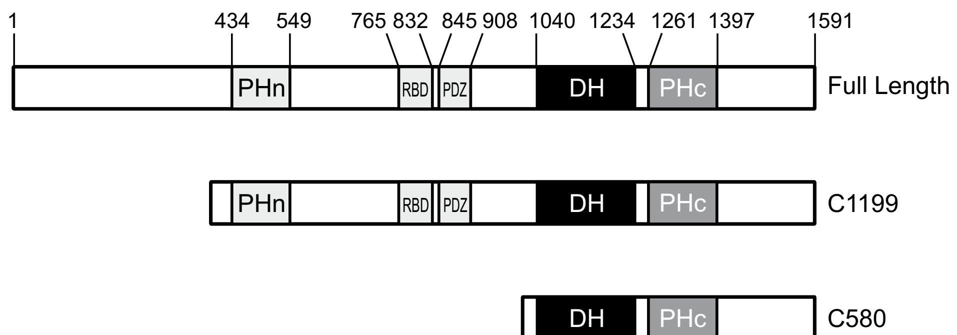

Figure 1. Schematic representation of the protein domains in Tiam1. Numbers indicate the amino acid positions. Catalytic Guanine nucleotide exchange (GEF)-activity resides in the Dbl-homology (DH) domain that is always flanked by a Pleckstrin homology (PH) domain. C1199 and C580 are amino-terminal truncated mutant versions of Tiam1. PHn and PHc: amino- and carboxy-terminal Pleckstrin Homology domain respectively; RBD: Ras-binding domain; PDZ: PSD-95 Discs large/ZO-1 homology domain.

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catalytic Dbl homology (DH) domain flanked by a Pleckstrin homology (PH) domain. This DH-PH unit is characteristic for Dbl-like guanine nucleotide exchange factors. Unique for Tiam1 is an additional NH_2-terminal PH domain, through which Tiam1 is localized to the membrane (Michiels et al., 1997). Further functional analysis of Tiam1 has shown that Tiam1-mediated activation of Rac promotes invasiveness of T-lymphoma cells *in vitro* and metastasis *in vivo* (Michiels et al., 1995). However, in epithelial cells Tiam1/Rac signaling prevents invasiveness by increasing the strength of E-cadherin-based cell-cell adhesions *in vitro* (Hordijk et al., 1997;Zondag et al., 2000). To elucidate Tiam1 functions *in vivo* we generated Tiam1 knockout mice (Malliri et al., 2002). In this chapter we describe the use of the Tiam1-deficient mice and cells derived from these mice to study the role of Tiam1 in Ras-mediated tumorigenesis in particular.

Tiam1-deficiency and mouse tumor models

Tiam1 knockout mice

To investigate the role of Tiam1 in Ras-mediated oncogenicity, mice deficient in Tiam1 ($Tiam1^{-/-}$) were used. A targeting vector was generated in which a promoterless GEO cassette (LacZ-Neo-polyA) was fused in frame with the translation initiation codon in the second exon of Tiam1. Insertion of a reporter gene such as β-galactosidase or green fluorescent protein (GFP) allows a rapid assessment of which cell types normally support the expression of that gene. $Tiam1^{-/-}$ mice are fertile and no major defects are observed in any of the organs analyzed. Based on LacZ activity found in the engineered mice, Tiam1 is widely expressed in most tissues but expression is most prominent in brain and testis. In the skin, Tiam1 is present in basal and suprabasal keratinocytes of the interfollicular epidermis and in hair follicles, where it is predominantly expressed in the infundibular portion. As Tiam1 is dispensable for development, tumors can be induced in various tissues in the Tiam1-deficient mice. In addition, Tiam1-deficient mice can be crossed with tumor-prone transgenic mice to study the involvement of Tiam1 in different oncogenic signaling pathways in specific cells and tissues.

Ras-induced skin carcinogenesis

Mutational activation of Ras is frequently found in skin cancers. Skin squamous tumors can be induced in mice by a well-established quantitative two-stage DMBA/TPA chemical carcinogenesis protocol (Quintanilla et al., 1986;Yuspa, 1994). This method is ideal to study the timing of qualitative and quantitative alterations which take place during the mechanistically distinct stages of chemical carcinogenesis, allowing analysis of the events that lead to the transitions from initiation to promotion and finally to malignant conversion and progression to carcinomas.

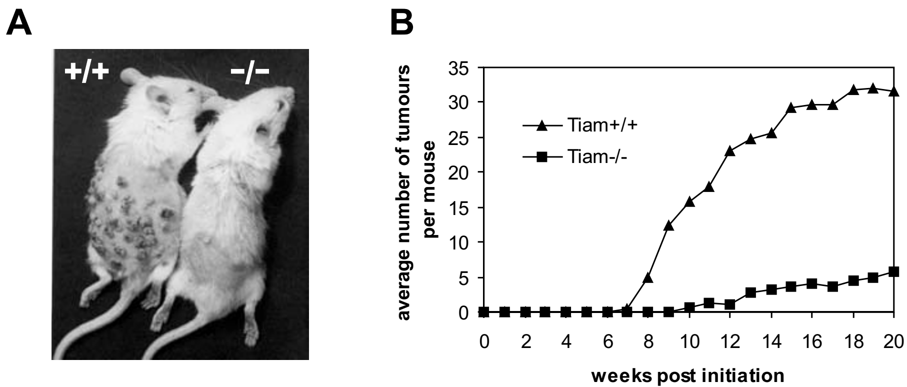

Figure 2. Analysis of Tiam1 in DMBA/TPA-mediated skin carcinogenesis. (A) Eighteen weeks after treatment, papillomas developed on the back of wild type mice (+/+) but not of Tiam1 knockout mice (-/-). (B) Tiam1$^{-/-}$ mice developed much less tumours than wild type mice.

Treatment of the skin with DMBA (7,12-dimethylbenz(a)anthracene) almost invariably introduces oncogenic mutations of the Ha-Ras gene in epidermal keratinocytes. A to T transversions in the Ras codon 61 are the most frequent mutations found in this protocol (Finch et al., 1996). Subsequent repeated TPA (12-*O*-tetradecanoylphorbol-13-acetate) treatment for twenty weeks lead to tumor promotion that causes the selective clonal out-growth of the initiated cells targeted by Ras mutation to produce benign lesions (papillomas). Approximately 20% of the produced papillomas undergo malignant progression into squamous cell carcinomas, which can undergo epithelial to mesenchymal transition (EMT) to spindle cell carcinomas. The incidence of carcinomas can be substantially enhanced by treating papilloma-bearing mice with mutagens such as urethane, nitroquinoline-N-oxide or cisplatinum suggesting that distinct additional genetic events are responsible for malignant conversion.

To dissect the different stages of the carcinogenesis process, variations on the two stage carcinogenesis applications can be performed. Increasing concentrations of DMBA and/or TPA during the treatments result in an increasing number of lesions per mouse. When the repeated TPA treatments are stopped, existing papillomas may regress or reduce in size. Mice can also be repeatedly treated with DMBA alone. This complete carcinogenesis protocol leads directly to the formation of predominantly squamous cell carcinomas. To study effects on growth and survival of epidermal keratinocytes, skin hyperplasia can be induced by TPA treatment with or without previous DMBA treatment. The genetic background of treated mice can influence the carcinogenesis process, i.e. the number of tumors produced or the progression of these tumors. Although DMBA/TPA-induced tumors have been described in Black 6 mice, we have better experience when using this protocol on FVB mice.

We found that Tiam1$^{-/-}$ mice are resistant to the development of skin tumors induced by DMBA/TPA treatment (Malliri et al., 2002) (Fig. 2). Moreover, the few tumors produced in Tiam1$^{-/-}$ mice grew much slower than did tumors in wild type mice. Tiam1-deficiency was associated with increased apoptosis in the basal layer of the epidermis during initiation and with impeded proliferation during promotion. Although the number of tumors in Tiam1$^{-/-}$ was small, a greater proportion progressed to malignancy, suggesting that Tiam1-deficiency promotes malignant conversion. Tiam1 is required for the proper formation and maintenance of E-cadherin-mediated cell-cell adhesion, of which the loss is associated with tumor progression (Zondag et al., 2000;Malliri et al., 2004). Therefore, Tiam1-deficiency might favour malignant progression by reduced strength of E-cadherin-mediated adhesion *in vivo*.

Genetic ablation of other potential Ras effectors has also been shown to reduce tumor formation in the DMBA/TPA skin carcinogenesis model. As we found for Tiam1, deficiency of both RalGDS and Phospholipase Cε (PLCε) leads to reduced initiation and growth of skin tumors (Gonzalez-Garcia et al., 2005;Bai et al., 2004). However, while papillomas in Tiam1$^{-/-}$ mice progressed more frequently to malignant carcinomas than in wild type mice, RalGDS and PLCε deficient mice show a lower percentage of malignant tumors as compared to control tumors in wild type mice. Apparently, different Ras effectors cooperate with Ras in the oncogenic process by regulating different aspects of tumorigenicity.

Alternative to the DMBA/TPA protocol, Ras activation in mouse models can be achieved using transgenetic approaches. In the case of oncogenic Ras, constitutive expression cannot be used to study tumorigenesis as these animals show early developmental defects and malformations. Even in conditional systems, a minor leakage of oncogenic Ras expression often results in severe phenotypes. Examples of models with conditional mutant Ras expression are v-Ha-ras expression targeted to the mammary gland by MMTV promoter for mammary tumorigenesis (Sinn et al., 1987), expression of mutant Ras from different keratin promoters directing expression to specific cells in the skin (Bailleul et al., 1990;Brown et al., 1998), and Cre-mediated somatic induction of Ras in the lungs, a model for non-small cell lung cancer (Meuwissen et al., 2001). A tetracycline-inducible system for K-Ras expression in epidermal stem cells has been described to study the promotion of squamous cell carcinomas (Vitale-Cross et al., 2004). In a 4-hydroxytamoxifen (4OHT)-regulated system expressing Ras under control of the keratin 14 promoter, Ras reversibly induced massive cutaneous hyperplasia and suppressed differentiation (Tarutani et al., 2003;Perez-Mancera and Tuveson, 2006)

Protocols for *in vivo* studies

Protocol for two-stage DMBA/TPA carcinogenesis

The backs of 8-week-old mice are shaved using a hair clipper. The next day initiation is carried out by a single application of 25 μg DMBA (Sigma) in 200 μl acetone topically on the shaved area of the dorsal skin. Control mice receive acetone only. A week after DMBA initiation, mice are skin painted with 200 μl of a 10^{-4}M solution TPA (Sigma) in acetone twice weekly at the site of DMBA application for 20 weeks. For complete carcinogenesis mice are treated biweekly with 5 μg DMBA alone in 200 μl acetone for 20 weeks. Mice are visually examined twice weekly for tumor formation and number and size of tumors is determined. Mice are killed when moribund, if any individual tumor reaches a diameter of 1 cm, or at the termination of the experiment at 30 weeks. Tumors and organs can be isolated and fixed for histochemical examination. Cells from tumors can be isolated as described below (II-1-1) for *in vitro* studies.

Proliferation and Apoptosis assays *in vivo*

In proliferating cells, exogenous 5-bromo-2-deoxyuridine (BrdU) is incorporated into genomic DNA during DNA replication in the S-phase of the cell cycle. Therefore, BrdU incorporation can be used to detect cycling cells. To measure the number of proliferating cells in tumors and organs, mice are injected intraperitoneal with BrdU (Sigma) at 50 mg/kg in 200 μl PBS, usually 2-4 hrs before sacrificing the mice. Alternatively, BrdU can be applied in the drinking water of the mice at 0.8 mg/ml. BrdU incorporation in proliferating cells is detected on paraffin sections using an anti-BrdU antibody (1/50; DAKO) according to the immunohistochemistry protocol as described below. Alternatively to the BrdU protocol, sections are stained for the proliferation marker PCNA by immunohistochemistry (CS-56 antibody 1/500; Santa Cruz).

Apoptosis, or programmed cell death, is associated with changes in several cellular processes. For example, it alters plasma membrane asymmetry, cleaves cellular DNA into histone-associated DNA fragments, and activates ICE-like proteases. In the TUNEL (terminal deoxynucleotidyl transferase(TdT)-mediated dUTP nick end labeling) assay, apoptotic cells are labeled *in situ* by a TdT reaction tailing labeled nucleotides into DNA strand breaks that occur during early apoptosis. For detection of apoptotic cells in paraffin-embedded tissue sections we use the *in situ* cell death detection kit, POD (Boehringer). For pre-treatment of the sections we refer to the immunohistochemical protocol as described below with the exception that antigen retrieval is performed by a proteinase K (20 μg/ml) treatment for 15 min at room temperature (RT). Using the reagents of the kit, the TdT-mediated incorporation of fluorescein-dUTP is performed for 1 hour at 37°C. TUNEL POD solution, containing an anti-fluorescein antibody conjugated with peroxidase (POD), is

applied for 30 min at 37°C. After washing in PBS, DAB detection is performed as described below in the protocol for immunohistochemistry.

Histology and Immunohistochemistry

Tumors and organs are dissected and embedded in Tissue-Tek® OCT™ compound (Sakura) and frozen or fixed in 10% buffered formalin for 24-48 hours at room temperature as required. Paraffin-embedded tissues sections (3 μm) are prepared using a semi-automatic microtome (Leica, 2255) and captured on Superfrost Plus object glasses in a water bath. Sections of the tumors and tissues are stained with haematoxylin and eosin for histological classification.

Standard ABC (Avidin-Biotin Complex) techniques are used for antigen-specific immunohistochemical detection (Malliri et al., 2002). The principle is based on the irreversible high affinity binding of avidin to biotin (Hsu et al., 1981). Avidin has four binding sites for biotin and is used to complex biotinylated horseradish peroxidase (HRP) to biotin-conjugated secondary antibody used for immunohistochemistry. De-paraffinized slides are cooked for 20 min in 0.1M citrate buffer pH 6.0 for antigen retrieval. After cooling down, endogenous peroxidase is blocked in 3% H_2O_2 in methanol at RT for 10 min. Slides are pre-incubated for 30 min at RT in 5% normal goat serum in PBS with 1% BSA. Primary antibodies are applied overnight at 4°C. Subsequent slides are incubated for 1 hour at RT with the appropriate anti-mouse or anti-rabbit biotin-conjugated secondary antibodies (DAKO). After washing, slides are incubated for 30 min at RT with preformed Avidin and Biotinylated HRP Complex (ABC; DAKO) in PBS/BSA. For detection we use the HRP substrate DAB (3,3-diaminobenzidine tetrahydrochloride) which produces an alcohol-insoluble brown precipitate in the sections. Slides are rinsed in 0.05M Tris/HCl pH 7.6 and treated for 5 min at RT with substrate buffer (0.05% DAB (Sigma) 0.01% H_2O_2 in 0.05M Tris/HCl-0.1M imidazole pH 7.6). After counterstaining with Haematoxylin slides are rinsed with tap water for 10 min, subsequently rinsed in raising concentrations alcohol and xylene and embedded in DePeX mounting medium (Gurr; BDH laboratory supplies). For Tiam1 staining we use different dilutions (1/500-1/1.500) of C16 (Santa Cruz) and a house-made anti-DH polyclonal antibody (Habets et al., 1994).

Protocol for β-Gal detection

Freeze coupes made from dissected mouse organs or whole embryos are fixed for 5 min at RT in 2% paraformaldehyde; 0.2% glutaraldehyde. Tissues are washed in PBS and stained overnight at 37°C in X-Gal solution (0.1M sodium phosphate pH 7.3, 2 mM $MgCl_2$, 5mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml X-Gal (dissolved in DMSO; Invitrogen)). After washing in PBS, tissues are counterstained using Nuclear Fast Red, dehydrated and mounted in non-aqueous DePeX mounting medium (Gurr; BDH laboratory supplies).

In vitro analysis of Tiam1/Rac signaling

Overexpression or downregulation of gene of interest in cells cultured *in vitro* are ideal tools to study the function of the respective gene. Although we found that Tiam1 is expressed at low levels in most murine tissues, some established human and rodent cell lines do not show detectable expression of Tiam1 at the RNA (Habets et al., 1995) or protein level (Sander et al., 1999). Ectopic expression of Tiam1 in cells has been used extensively for functional studies. Besides full-length Tiam1 expression constructs, we have used more stable N-terminally truncated versions of Tiam1 (Fig. 1). C1199 Tiam1, referring to the C-terminal 1199 amino acids, resembles an activated mutant of Tiam1 that is encoded by a truncated Tiam1 transcript as found after proviral insertion (Habets et al., 1994). The C580 Tiam1 mutant contains the minimal DH-PHc catalytic GEF domains only. By overexpression studies we have investigated the role of Tiam1-mediated Rac activation in invasiveness of lymphoid cells (Habets et al., 1994;Michiels et al., 1995), in lamellar spreading and neurite formation in neuronal cells (Leeuwen et al., 1997), in inhibition of invasion by upregulation of the E-cadherin/catenin-complex in epithelial cells (Hordijk et al., 1997;Sander et al., 1998), and in epithelial to mesenchymal transition (EMT) (Sander et al., 1999;Zondag et al., 2000).

For functional studies we have downregulated Tiam1 to circumvent Tiam1-independent side effects in artificial overexpression. Efficient dominant-negative versions of Tiam1 are not available but recently we have made use of the siRNA technique (Brummelkamp et al., 2002) to downregulate endogenous Tiam1. We cloned different Tiam1 RNA targeting oligonucleotides containing a 9-bp hairpin loop in pSuper and pRetrosuper for transfection and retroviral infection experiments respectively. Infection of MDCK cells with canine Tiam1 siRNA results in a knock-down of Tiam1 protein levels of at least 50%, which results in a transition to a mesenchymal morphology resembling EMT and in dramatic effects on the migratory behaviour of the cells (Malliri et al., 2004). The siRNA technique is highly specific and can be used for transient and stable gene silencing. However, the knock-down efficiency for a given gene may vary considerably and different constructs have to be tested for each gene. Silencing by siRNA also creates highly variable cell populations with different knock-down levels and complete downregulation of the protein of interest is never achieved.

To ensure complete knock-out of Tiam1 in homogeneous cell populations we have isolated cells from Tiam1$^{-/-}$ mice in which the *Tiam1* gene has been ablated by homologous recombination. Mouse embryonic fibroblasts (MEFs) can be easily isolated (see II-1-2) and can be cultured for prolonged times after immortalization. Wild type (WT) MEFs grow in tightly packed colonies with few scattered cells at the periphery while Tiam1$^{-/-}$ MEFs grow in irregular colonies that frequently show dispersed cells at the edges. Tiam1$^{-/-}$ MEFs show reduced levels of active Rac when compared to WT MEFs (Malliri et al., 2004;van Leeu-

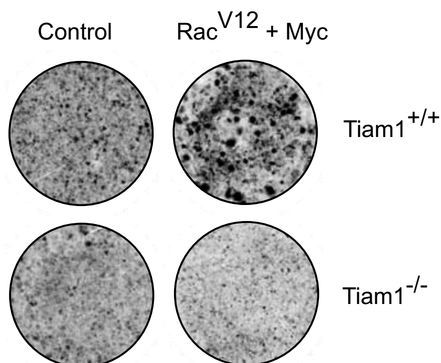

Figure 3. Focus formation assay in primary embryonic fibroblasts. Focus formation induced by co-expression of Ras^{V12} and Myc in primary embryonic fibroblasts (MEFs) derived from wild type and Tiam1$^{-/-}$ mice. The number of foci is strongly reduced in Tiam1$^{-/-}$ MEFs when compared to wild type MEFs.

wen et al., 2003) and fail to undergo E1A-induced mesenchymal to epithelial transition (MET), a process dependent on Tiam1 (Malliri et al., 2004). Primary WT and Tiam1$^{-/-}$ MEFs have also been used in focus formation assays (see II-1-4). Co-expression of dominant active Ras^{V12} and Myc in MEFs induces much more foci in WT than in Tiam1$^{-/-}$ cells (Fig. 3), illustrating the importance of Tiam1 in Ras-mediated transformation of cells (Malliri et al., 2002;Lambert et al., 2002). Besides MEFs many other primary cell types can be isolated from mice such as neuronal cells, endothelial cells, lymphoid cells, and epithelial cells from skin, lung or intestine. In addition, tumors induced in recombinant mice can be source of cells (see II-1-1). Keratinocytes, derived from the epidermis of newborn Tiam1$^{-/-}$ mouse, provide an excellent tool for *in vitro* studies on epithelial cells. Primary keratinocytes have to be immortalized (e.g. by SV40 large T antigen) to prevent apoptosis or terminal differentiation (see II-1-3). We are using Tiam1$^{-/-}$ keratinocytes to study the role of Tiam1 in Rac-mediated cell-cell and cell-matrix interactions as well as in cell polarization, cell migration and apoptosis susceptibility.

Protocols for *in vitro* studies

Isolation of skin tumor cells

We isolated skin tumor cells from lesions that are formed in mice treated with the DMBA/TPA protocol as described above. Tumors are excised and washed in PBS. The tissue is chopped using a scalpel and subsequently incubated for 1 hour at RT in a digestion solution (3 mg/ml collagenase (Sigma) and 1.5 mg/ml trypsin (Difco) in DMEM) to dissociate cell-cell and cell–matrix adhesions. The cell suspension is filtered through a 70 µm nylon cell strainer, centrifuged and resuspended in DMEM supplemented with 10% FCS, 100 U/

ml penicillin and 100 μg/ml streptomycin (P/S) before plating in standard tissue culture dishes.

Isolation of mouse embryonic fibroblasts (MEFs)

To isolate MEFs female mice are sacrificed at day 12.5 of pregnancy. The peritoneum is opened and the uterus is removed and cut along the upper side. The embryos are taken out and placed in separate wells. Embryos are decapitated and soft tissue is removed. Embryo carcasses are minced and transferred to cold PBS. After centrifugation, cells derived form each embryo are resuspended in 5 ml PBS containing 50 μg/ml trypsin, 50 μM EDTA and P/S, and incubated overnight at 4°C. Trypsin is blocked by addition of complete culture medium (DMEM, 10% FCS, 0.1 mM β-mercaptoethanol, P/S). The suspension of cells and tissue debris is allowed to settle down for 2 min and supernatant is transferred to a culture flask. Primary MEFs can be efficiently immortalized by transfection with large T antigen. Alternatively, continuous passaging of the cells will eventually lead to spontaneous immortalization.

Isolation of Keratinocytes

To isolate primary keratinocytes newborn (1-3 days old) mice are decapitated and washed in water and 70% ethanol. Limbs and tail are amputated with scissors under sterile conditions. The skin of the mice is cut on the dorsal side all along the length of the body and carefully separated from the rest of the carcase. The skin is washed in PBS (supplemented with P/S) and remaining fat tissue and blood vessels are removed. The isolated skin is stretched with the dermal side faced down on a sterile Whatmann paper that is soaked with trypsin (2.5 mg/ml, EDTA free) and incubated overnight at 4°C. The next day the dermis is separated from the epidermis, and both are minced with tweezers and scissors. Suspensions of epidermis and dermis are incubated separately for 1 hour at 4°C in DMEM (supplemented with 10% FBS and P/S) under gentle stirring. Subsequently, cell suspensions are filtered through a 70 μm cell strainer, centrifuged at 900 rpm for 5 min. and resuspended in DMEM containing 10% FBS, P/S. Dermal and epidermal cells are plated separately on 6 well plates coated with collagen I. The following day culture medium is replaced by serum-free keratinocyte medium supplemented with growth factors (Cascade Biologics) and $CaCl_2$ (0.02 mM). Keratinocytes can be immortalized by introduction of SV40 large T antigen.

Focus formation assay

Fibroblasts (NIH3T3) or MEFs, grown to a density of 40-50%, are infected with retroviruses carrying the desired oncogenes (e.g. *Ras*^{V12} and/or c-*Myc*). After 24-48 hours when cells reach confluency, the medium is refreshed with DMEM containing 2% FBS (for MEFs) or 5% NCS (for NIH-3T3). Cells are cultured for 14 days with medium refreshments every

three days. At the end of the experiment cells are fixed with methanol and stained with 1% crystal violet.

Apoptosis assay on *in vitro* cultured cells

Apoptosis can be induced in MEFs or keratinocytes by several means including growth factor deprivation, TNF-alpha and TRAF treatment, UV and gamma-irradiation, surface detachment (anoikis), hyperosmotic conditions or heat shock (43-45°C). Cells that undergo apoptosis expose phosphatidylserine on the external side of the cell membrane. Annexin-V is a protein that specifically binds phosphatidylserine. Therefore, apoptotic cells can be specifically stained with annexin-V protein conjugated to a fluorochrome like APC. In the case of growth factor starvation-induced apoptosis in keratinocytes, normal growth medium is replaced for growth factor-free medium and the degree of apoptosis is analyzed after 24 hours. For this, the cells are trypsinized, washed twice with cold PBS and are resuspended in annexin-V-binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). 2-4 µl APC-labeled annexin-V (Becton Dickinson) is added to each sample containing $1x10^5$ to $1x10^6$ cells and incubated on ice for 15 min protected from light. Subsequently, 400 µl of annexin-binding buffer containing 1.25 µg/ml propidium iodide is added to the samples and cells are analyzed by flow cytometry. Unstained and single stained samples are used for proper calibration of the Flow cytometer.

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Chapter 5

The Rac activator Tiam1 prevents keratinocyte apoptosis by controlling ROS-mediated ERK phosphorylation

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ABSTRACT

Tiam1 is a ubiquitously expressed activator of the small GTPase Rac. Previously we found that Tiam1 knockout (KO) mice are resistant to DMBA-induced skin tumorigenicity, which correlated with increased apoptosis in keratinocytes of the skin epidermis. Here we have studied the mechanisms by which Tiam1 protects against apoptosis. We found that Tiam1-KO keratinocytes show increased apoptosis in response to apoptotic stimuli including growth factor deprivation and heat shock treatment. Expression of catalytically active Tiam1, but not inactive Tiam1, rescues apoptosis susceptibility of Tiam1-KO keratinocytes, indicating that this defect is caused by impaired Tiam1-mediated Rac activation. Apoptosis induced by growth factor starvation correlates with impaired ERK phosphorylation in Tiam1-KO keratinocytes. Moreover, Tiam1-KO keratinocytes contain lower levels of intracellular reactive oxygen species (ROS) when compared to wild type (WT) cells. The ROS content of keratinocytes is dependent on both Tiam1 and the activity of NADPH oxidase (Nox) and is required for ERK-mediated survival signaling. Indeed, Tiam1 deficiency or inhibition of intracellular ROS production blocks ERK phosphorylation and sensitizes WT keratinocytes to apoptotic stimuli. Our results indicate that the Rac activator Tiam1 controls the intracellular redox balance by Nox-mediated ROS production, which regulates ERK phosphorylation and the susceptibility of keratinocytes to apoptotic signaling.

INTRODUCTION

In earlier studies we have identified the Tiam1 gene which encodes an activator of the Rho-like Rac GTPase (Habets et al., 1994;Habets et al., 1995;Michiels et al., 1995). Tiam1 regulates various Rac-mediated signaling pathways including integrin-mediated cell-matrix adhesions (Hamelers et al., 2005), E-cadherin-mediated cell-cell adhesions (Hordijk et al., 1997;Sander et al., 1998;Malliri et al., 2004), and cell polarity processes (Mertens et al., 2005;Chen and Macara, 2005;Gerard et al., 2007;Pegtel et al., 2007). Tiam1-mediated Rac activation also plays a role in various aspects of tumorigenicity (Malliri et al., 2002;Malliri et al., 2006). Interestingly, the decreased tumor initiation observed in Tiam1-kockout (KO) mice upon DMBA treatment was accompanied by increased apoptosis observed in keratinocytes of the basal layer of the epidermis, suggesting that the DMBA-induced apoptosis prevents the initiation of skin tumors in Tiam1-deficient mice.

Apoptosis is an intrinsic mechanism for the induction of controlled and necessary cell death, for instance during development or viral infection. Prevention of apoptosis by oncogenic agents is a crucial step in the process of tumor initiation. The intrinsic apoptotic pathway is controlled by the family of Bcl-2 proteins that regulates mitochondria integrity and in particular Cytochrome c release. Two Bcl-2 subfamilies, consisting of pro-apoptotic proteins that include Bad, Bax, and Bak, and anti-apoptotic proteins that include Bcl-2, Bcl-xL, and Mlc-1, counteract each other in the stimulation or inhibition of mitochondrial Cytochrome c release (Daniel et al., 2003). The expression and phosphorylation of Bcl-2 family proteins is controlled by survival signaling pathways that are predominantly regulated by the PI3K-PKB/Akt- (phosphoinositide-3 kinase – protein kinase B) and the MAPK (mitogen activated protein kinases) pathways. Various extracellular stimuli can activate both pathways including: serum components, growth factors (GF) such as EGF and IGF-1 (Henson and Gibson, 2006;Bernal et al., 2006;Kooijman, 2006), integrins (Cordes, 2006) and small molecule secondary messengers such as Ca^{2+} or reactive oxygen species (ROS) (Martindale and Holbrook, 2002).

The level of reactive oxygen species (ROS) in normal and tumor cells play an important role in cell survival and apoptosis. Different ROS levels may have opposite effects in the same type of cells. Low ROS concentrations (up to 10-20 µM) are mitogenic and anti-apoptotic (Irani et al., 1997;Arnold et al., 2001;Liu et al., 2005), whereas high ROS levels (above 50-100 µM) induce growth arrest or apoptosis (Stone and Yang, 2006). Physiological levels of ROS are maintained by growth factors such as PDGF or EGF that signal primarily via the NADPH oxidase family proteins Nox 1-5 (Sundaresan et al., 1995;Bae et al., 1997;Lambeth, 2004). The activity of the Nox1-3 enzymes is regulated by the Rho-like GTPase Rac, which activity determines to a large extend the intracellular ROS production (Sauer et al., 2001). Little is known, however, about the activators of Rac that function upstream of Nox.

As decreased skin tumor initiation observed in Tiam1-kockout (KO) mice was accompanied by increased apoptosis in keratinocytes of the basal layer of the epidermis (Malliri et al., 2002), we have investigated the mechanisms by which Tiam1 could control survival signaling in keratinocytes. To this end, we isolated keratinocytes from the skins of wild type and Tiam1-KO mice and studied differences in apoptotic and survival signaling in these cells. We found that the susceptibility of keratinocytes for apoptosis induced by GF starvation was dependent on Tiam1/Rac and ERK activation. The ERK-mediated survival pathway was dependent on the presence of intracellular ROS. Tiam1-KO cells are deficient in Rac-dependent activation of Nox and show therefore lower ROS production. As ROS stimulates the ERK survival pathway, Tiam1-deficient cells lack this survival-signaling pathway and therefore are more susceptible to apoptotic stimuli.

Materials and methods

Cell culture

Keratinocytes were isolated from the skin of newborn FVB mice and immortalized by SV40 large T antigen expression (Mertens et al., 2005). Cells were cultured in plastic dishes coated with collagen IV (Becton Dickinson) in EpiLife keratinocyte medium (Cascade Biologics) supplemented with 20 μM $CaCl_2$ and EpiLife Defined Growth Supplement (GF), which consisted of EGF, insulin-like growth factor, hydrocortisone, prostaglandin and transferrin (Cascade Biologics). For the suspension assay ($5x10^5$) cells were trypsinized and incubated in an ultra-low cluster (ULC) six-well cell culture dishes (Costar). Were indicated, cells were treated with MEK inhibitor-PD98059 (Calbiochem), antioxidant N-Acetyl-Cysteine (NAC) (Sigma-Aldrich) or the NADPH oxidase inhibitors: diphenyleneiodonium chloride (DPI) (Calbiochem) and Apocynin (Calbiochem).

Expression vectors, cell transfection and retroviral transduction

Keratinocytes were transfected, using FuGENE (Roche) with pSuper vectors containing short hairpin RNA (shRNA) targeting Tiam1 or luciferase as a control (Malliri et al., 2004). Full length Tiam1 and catalytically inactive Tiam1-ΔDH coding sequences (Michiels et al., 1997) were cloned into the LZRS-IRES-blasticidin retroviral vector (Michiels et al., 2000). Retroviral constructs were transfected into Phoenix ecotropic packaging cells, and retrovirus containing supernatants were collected and used for infections, as previously described (Michiels et al., 2000).

Apoptosis assays

Apoptosis was induced in cell cultures at 60-80% confluent. Growth factor (GF) starvation was done for 24 hours or as otherwise indicated in the legends to the figures. In heat shock experiments, cells were incubated for 90 minutes at 43˚C followed by 6 hours culturing at 37˚C. Subsequently, cells were harvested for quantification of apoptosis.

For DNA profile analysis, cells were harvested by trypsinization, fixed with cold 70% ethanol, washed with PBS and stained with propidium iodine (PI) followed by flow cytometric analysis using a FACScallibur (Becton Dickinson). For annexin-V/PI staining, cells were harvested by trypsinization, washed twice with cold PBS, and resuspended in annexin binding buffer (10 mM Hepes/NaHO, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) in a final concentration of $1x10^6$-$1x10^7$ cells/ml. Subsequently, 200µl aliquots were stained with annexin-V-APC (Becton Dickinson) and propidium iodine and analyzed using a FACScallibur (Becton Dickinson).

Free nucleosome quantification was performed with the use of Cell Death Detection ELISA kit (Roche) according to manufacturer's instructions. For the measurement of PARP cleavage, floating cells were collected by centrifugation and lysed together with the adherent cells in RIPA buffer. Lysates were further used for protein content determination by the BCA protein assay kit (Pierce) and for western blotting using PARP specific antibody.

Rac activity assay

Rac activity was determined as described previously (Malliri et al., 2004). GTP-bound, active Rac (Rac-GTP) was precipitated from cell lysates using a biotinylated Rac1 binding domain of PAK1. Total Rac was used as a control.

ROS visualization and quantification

The amount of intracellular reactive oxygen species (ROS) was visualized by dichlorofluorescein diacetate (DFC) (Calbiochem). The cleavage product of DCF, 2',7'-dichlorofluorescein, is fluorescent upon oxidation by reactive oxygen species (Rosenkranz et al., 1992). Briefly, cells were cultured in 12-well plates. The culture medium was removed and replaced with PBS supplemented with 10 µM DCF for 10 minutes. Subsequently cells were washed with PBS and images were acquired from three or more randomly chosen fields using an epifluorescence microscope (Zeiss, Axiovert 25) equipped with a digital camera AxioCam MRc (Zeiss) and software MRGrab 1.0.0.4 (Carl Zeiss Vision GmbH). Quantification of the total amount of intracellular ROS was performed in a similar way. Adherent cells were washed with PBS and loaded with DCF 10 µM for 10 minutes. After washing, the cells were lysed in buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 300 mM sucrose, 1% Triton, pH 7,4) and the fluorescence of the lysates was determined in a Wallac 1420 Victor2 multipliable reader (PerkinElmer) with excitation wavelength 490 nm and emission wavelength 530 nm. The readouts were normalized for total protein con-

tent as determined with the BCA protein assay kit (Pierce) and the ROS levels of wild type cells was set at 1. When the amount of ROS per cell was determined by FACS analysis, cells were first trypsinized and subsequently loaded with DCF in PBS for 10 min, washed, and analyzed using a FACScallibur (Becton Dickinson) in FL1 channel.

Western blotting and antibodies

For Western blotting, cells were washed with cold PBS and lysed in RIPA buffer (150 mM NaCl, 10 mM Tris·HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with 1 mM NaF, 2 mM Na_3VO_4 and a protease inhibitor cocktail (Sigma). The BCA protein assay kit (Pierce) was used to determine protein contents. Equal amounts of protein were resuspended in 1 x NuPAGE LDS sample buffer (Invitrogen) and separated on NuPage 4-12% polyacrylamide gels (Invitrogen). Proteins were transferred to PVDF membranes and stained with primary antibody. Subsequently, proteins were visualized by staining with appropriate anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) and by using an enhanced chemiluminescence kit (Pierce). To remove bound antibodies, membranes were stripped by incubating them in a low pH glycine solution (0.2 M glycine, 1% SDS, pH 2.0) for 15 minutes. Immunoblottings were performed with the following primary antibodies: Tiam1 (C16), Nox1 (Mox1, H-75), p47-phox (H-195), (Santa Cruz), p22-phox (MW-1843) (Sanquin), β-actin (AC-15, Sigma), phospho-IκB ($pSpS^{32/34}$) (Biosource), E-cadherin (clone-36), Rac1 (23A8), ERK2 (clone-33) (BD Transduction Laboratories), phospho-ERK1/2 p44/42 MAP Kinase (Thr202/Tyr204), total ERK1/2 (137F5), phospho-Akt (Thr308), total Akt and PARP (all Cell Signaling). Picture densitometry quantification was performed with Image J software (National Institutes of Health).

Results

Tiam1-deficiency increases susceptibility to apoptosis

Tiam1-knockout (KO) mice are resistant to DMBA/TPA-induced skin tumorigenesis due to increased apoptosis of keratinocytes present in the basal layer of the epidermis (Malliri et al., 2002). To analyze the mechanism by which Tiam1 controls survival and apoptosis in keratinocytes, we used keratinocytes isolated from skins of newborn wild type (WT) and Tiam1-KO FVB mice (Mertens et al., 2005). In keratinocyte culture medium, which does not contain serum but various keratinocyte growth factor supplements, isolated WT and Tiam1-KO keratinocytes exhibited a similar morphology and a very low percentage of apoptotic cells (Fig. 1A, upper panels). However, when cells were treated with apoptotic stimuli such as heat shock treatment, profound morphological changes occurred in the cells lacking Tiam1, whereas WT cells showed no effect (Fig 1A, lower panels). We

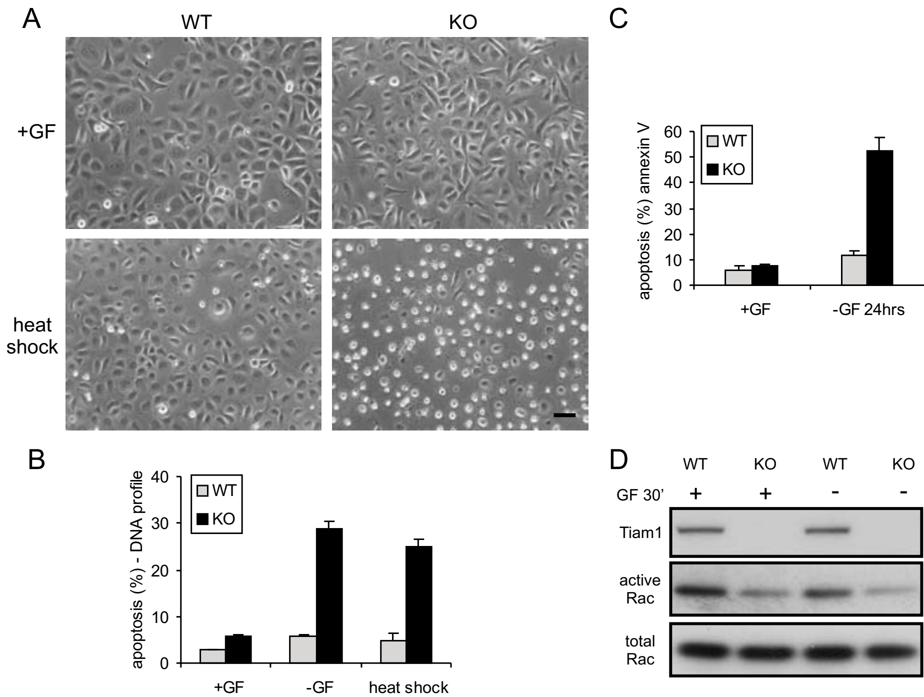

Figure 1. Tiam1-KO keratinocytes are more susceptible for apoptosis than wild type keratinocytes. A. Phase-contrast photographs of wild type (WT) and Tiam1-deficient keratinocytes (KO) cultured on collagen IV-coated tissue culture plates. Cells were grown in normal culture conditions or heat shock treated for 90 minutes at 43°C, which was followed by 6 hours recovery at 37°C. Bar represents 50 μm. B. Quantification of apoptosis induced by GF deprivation (24 hours) or heat shock treatment in WT and Tiam1-KO keratinocytes. Both adherent and non-adherent cells were collected and subjected to DNA profile analysis. Cells with sub-G1 DNA content were considered as apoptotic. Error bars represent standard deviation from triplicate samples. C. Quantification of apoptosis induced by GF deprivation (24 hours) in WT and Tiam1-KO keratinocytes. Both adherent and non-adherent cells were collected, stained with annexin-V and the DNA stain propidium iodide (PI), and analyzed with a FACS. Annexin-V-positive cells were considered as apoptotic cells. Error bars represent standard deviation from 3 independent experiments. D. Rac activity assay performed in lysates from WT and Tiam1-KO keratinocytes. Cells were GF starved for 30 min (GF-). Tiam1 and Rac were used as expression and loading control, respectively. The data presented is a representative example of four independent experiments.

found that heat shock treatment or growth factor (GF) deprivation resulted in a strong increase of apoptosis in Tiam1-KO cells when compared to WT cells (Figs. 1 B and C). The degree of apoptosis was quantified by various means including DNA content analysis (Fig. 1B) and annexin-V staining (Fig. 1C). We conclude that Tiam1-KO keratinocytes are more susceptible to apoptotic stimuli than WT cells, consistent with the observed increase in apoptosis in the basal layer of the epidermis of Tiam1-KO mice upon DMBA treatment (Malliri et al., 2002). Tiam1 is a specific activator of Rac and Rac signaling has been implicated in apoptosis resistance (Ruggieri et al., 2001;Le et al., 2005). We therefore analyzed the Rac activity state of WT and Tiam1-KO keratinocytes. Both in normal culture conditions and in the absence of growth factors, Tiam1-KO keratinocytes showed

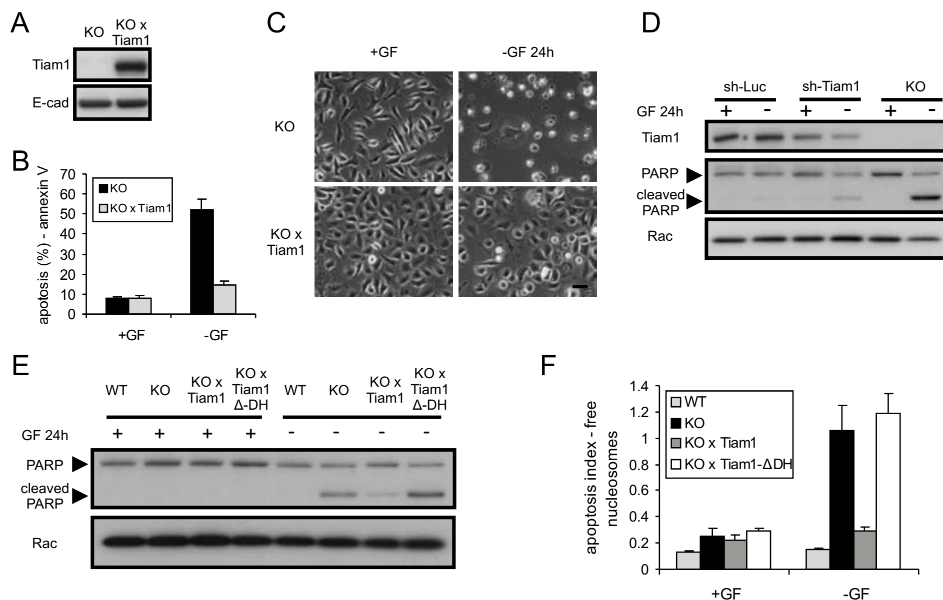

Figure 2. Expression of Tiam1 rescues apoptotic sensitivity of Tiam1-KO keratinocytes. A. Immunoblot showing the expression of exogenous full length Tiam1 in Tiam1-KO cells. E-cadherin was used as loading control. B. Quantification of apoptosis upon GF deprivation for 24 hours by annexin-V staining and FACS analysis of Tiam1-KO keratinocytes expressing empty vector or exogenous Tiam1. Error bars represent standard deviation from three independent experiments. C. Phase-contrast photographs of Tiam1-KO keratinocytes expressing exogenous Tiam1 in GF-contaning medium and upon GF starvation for 24 hours. Bar represents 50 μm. D. GF starvation (24 hrs) induced apoptosis in WT keratinocytes transfected with short hairpin RNA constructs or Tiam1-KO cells. Luciferase targeting sequence was used as a control short hairpin RNA. Tiam1 downregulation was approximately 50%. Apoptosis was monitored by PARP cleavage and seen only in GF-starved cells expressing Tiam1-specific siRNA or Tiam1-KO cells. E. Western-blot showing keratinocytes apoptosis (PARP cleavage) induced by GF starvation in WT, Tiam1-KO and Tiam1-KO with exogenous expression of either full length Tiam1 or an inactive mutant of Tiam1 (Tiam1-ΔDH). Rac expression was used as loading control. F. Apoptosis estimation by quantification of free nucleosomes bound to DNA in WT, Tiam1-KO, and Tiam1-KO keratinocytes with exogenous expression of full length Tiam1 or Tiam1-ΔDH. Only expression of full-length Tiam1 rescued apoptosis induced in Tiam1-KO keratinocytes by GF starvation. The presented data is a representative example of three independently performed experiments.

a decreased Rac activity (Fig. 1D), consistent with the function of Tiam1 in the activation of Rac (Michiels et al., 1995).

In order to demonstrate that the increased sensitivity to apoptotic stimuli of Tiam1-KO keratinocytes was due to a lack of Tiam1, we introduced full length Tiam1 into Tiam1-KO keratinocytes (Fig. 2A). Retroviral transduction of exogenous Tiam1 into Tiam1-KO keratinocytes resulted in a complete rescue of the susceptibility to apoptotic stimuli such as GF deprivation as demonstrated by annexin-V staining (Fig. 2B) and aberrant cell morphology (Fig. 2C). Tiam1-KO keratinocytes that expressed exogenous Tiam1 became also insensitive to heat shock treatment and showed similar low percentages of apoptotic cells as wild type cells (not shown).

We also demonstrated apoptosis biochemically by using caspase-dependent cleavage of specific proteins. Poly ADP-ribose polymerase (PARP) is one of the caspase targets and caspase-mediated proteolysis generates 89 kDa cleavage fragment of PARP. Additionally we monitored apoptosis biochemically by detection of histone-associated DNA fragments in mono- and oligonucleosomes. Both methods were used to further substantiate the function of Tiam1 in prevention of apoptosis. Downregulation of Tiam1 to approximately 50 percent of the levels found in control cells sensitized already WT cells for apoptosis upon GF starvation, as demonstrated by enhanced PARP cleavage in shTiam1-expressing cells (Fig. 2D). Apoptosis is more pronounced in Tiam1 KO cells, suggesting that apoptosis sensitivity is dependent on the dosage of Tiam1. Indeed, tumor incidence, which is related to apoptosis sensitivity is also dependent on the dosage of Tiam1 as observed in wild type, Tiam1-deficient and Tiam1 heterozygous mice (Malliri et al., 2002;Malliri et al., 2006). This indicates that decreased levels or lack of Tiam1 are responsible for the increased apoptosis sensitivity found in Tiam1-KO cells.

Exogenous expression of full length Tiam1 in Tiam1-KO keratinocytes rescued apoptotic sensitivity, as measured by two apoptotic markers e.g. PARP cleavage (Fig. 2E) and free nucleosome appearance (Fig. 2F). However, expression of Tiam1-ΔDH, which contains a mutation in the Rac activation domain (Michiels et al., 1997), did not rescue apoptosis susceptibility (Figs. 2E and F), indicating that the impaired Tiam1-mediated Rac activation is responsible for the increased apoptosis sensitivity of Tiam1-KO cells. From these data we conclude that the increased susceptibility to apoptosis of Tiam1-deficient cells is due to a lack of Tiam1 and Rac downstream signaling. Apparently, Tiam1-mediated Rac activation is required for cell survival upon apoptotic stimuli.

Tiam1 is required for ERK-mediated survival signaling upon GF starvation

In order to study the mechanisms by which Tiam1 influences apoptosis, we focused on the induction of apoptosis by growth factor deprivation. The growth factor (GF) supplement (Cascade Biologics) that is added to keratinocyte medium contains EGF, insulin-like growth factor, hydrocortisone, prostaglandin and transferrin. To determine whether EGF or insulin could rescue the apoptotic effect of GF deprivation, we analyzed apoptosis in keratinocytes grown for 24 hours in medium with and without complete growth factor supplement or medium with EGF or insulin only. As shown in Fig 3A, both EGF and insulin were able to prevent apoptosis of GF-starved Tiam1-KO cells. This indicates that EGF and insulin GF signaling is normal in Tiam1-deficient cells and that these survival-signaling pathways are not dependent on Tiam1-mediated Rac activation.

To determine the kinetics of apoptosis induced by GF deprivation, we analyzed PARP cleavage in WT and Tiam1-KO keratinocytes at various time points after GF starvation (Fig. 3B, upper panel). Apoptosis in the cell cultures was monitored by the appearance of caspase-truncated PARP. Accumulation of the cleaved PARP product was observed in

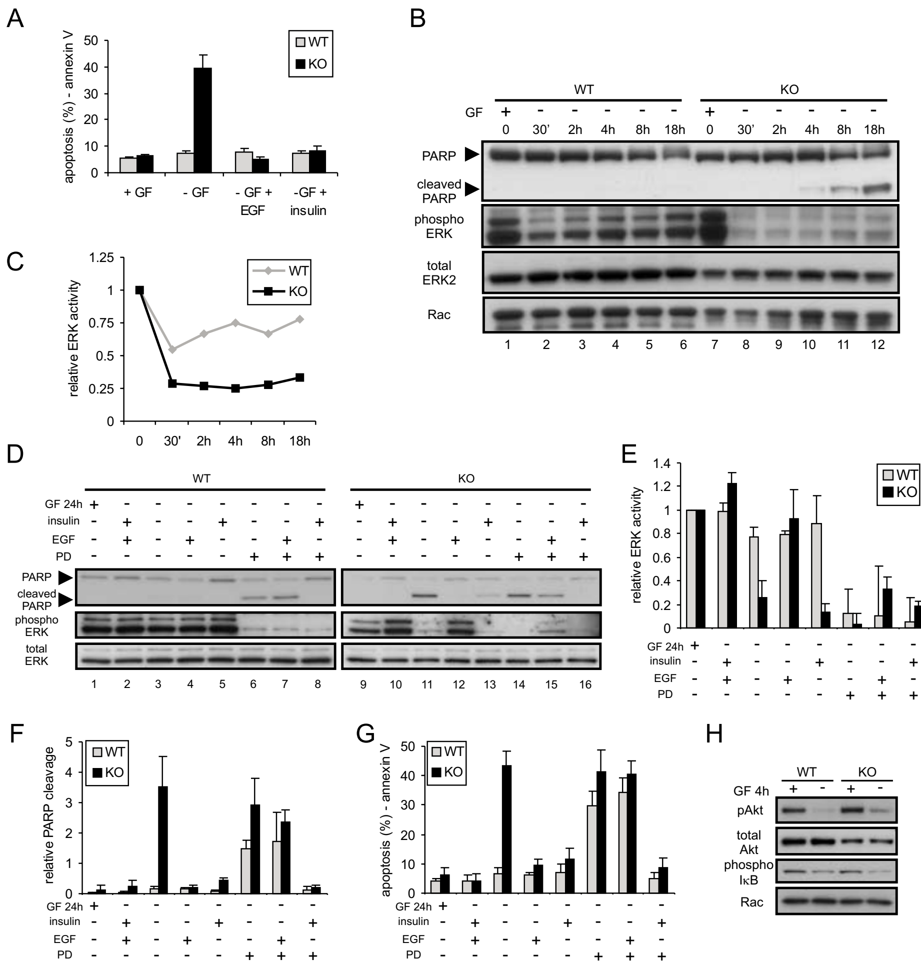

Figure 3. Tiam1-KO keratinocytes have impaired ERK phosphorylation - a necessary survival signaling. A. Quantification of apoptotic cells by annexin-V staining and subsequent FACS analysis. WT and Tiam1-KO keratinocytes were deprived of complete GF supplement and treated with EGF (20 ng/ml) or insulin (10 µg/ml) where indicated. Replacement of complete GF supplement by EGF or insulin only rescued apoptosis. B. Time-dependent induction of apoptosis and ERK1/2 phosphorylation upon GF starvation. Immunoblot shows PARP cleavage and ERK1/2 phosphorylation while Rac and total ERK are shown as loading controls. C. Quantification of the data presented in Fig 3B and normalized to total ERK2. D. ERK signaling is required for survival upon GF starvation whereas insulin provides ERK1/2-independent survival signaling. WT and Tiam1-KO keratinocytes were cultured in absence of GF for 24 hours with or without EGF (20 ng/ml), insulin (10 µg/ml) or the MEK inhibitor PD-98059 (10 µM). The immunoblots shown are representative examples of three independent experiments. E. Quantification of ERK1/2 phosphorylation from three independent experiments with the conditions as given in figure 3D. Values were normalized for total ERK and readouts corresponding to GF-supplemented conditions were set to 1. Error bars represent standard deviation. F. Quantification of PARP cleaved product from three independent experiments with the conditions as in the figure 3D, values were normalized for total ERK. Error bars represent standard deviation. G. Quantification of apoptosis induced by GF starvation by annexin-V staining and FACS analysis. Similarly as for the experiment shown in Fig 3D, WT and Tiam1-KO keratinocytes were cultured in absence of GF for 24 hours with or without EGF (20 ng/ml), Insulin (10 µg/ml) or the MEK inhibitor PD-98059 (10 µM). Error bars represent standard deviation from three independent experiments. H. Western blot depicting phosphorylation of Akt and IκB in wild type and Tiam1-KO keratinocytes in control and GF-deprived conditions (4 hours). Total Akt and Rac are presented as loading controls.

Tiam1-KO keratinocytes starting from 4 hours after GF deprivation and increased during the next hours (8 and 18 hrs). In contrast, WT keratinocytes did not show any PARP cleavage within the observed time frame (Fig. 3B, upper panel), consistent with the absence of apoptosis in WT cells as determined by annexin-V staining (see Figs. 1 and 2). Stress- and growth factor-induced ERK activation represents an important survival signal pathways in the presence of apoptotic stimuli (Henson and Gibson, 2006). We analyzed therefore the phosphorylation status of ERK upon GF starvation in WT and Tiam1-KO keratinocytes. Phosphorylation of ERK was high in both WT and Tiam1-KO cells in the presence of GF (compare lanes 1 and 7 in Fig. 3B), confirming our earlier conclusion that GF signaling was normal in Tiam1-KO cells. Strikingly, upon GF deprivation an initial drop in ERK-phosphorylation was seen, which readily recovered in WT- but not in Tiam1-KO keratinocytes (Fig. 3B). Total levels or ERK and Rac1 were measured as loading controls and data were quantified as shown in Figure 3C. These findings suggest that Tiam1-deficient cells are sensitive for apoptosis induced by GF deprivation due to an impaired GF-independent ERK survival signaling.

In order to substantiate the findings that a lack of ERK activation is causally related to increased apoptosis sensitivity of Tiam1-KO cells, we treated cells with the chemical ERK inhibitor PD-098059. The PD inhibitor blocks ERK specific kinase (MEK1) and prevents ERK1/2 phosphorylation (Alessi et al., 1995). The PD inhibitor thus prevents survival signals through the ERK pathway (e.g. EGF signaling; stress-induced signaling) but has no effect on survival signals mediated by other pathways such as the PI3-kinase-Akt pathway (e.g. insulin signaling). As shown in Figure 3D and G, EGF or a mixture of insulin and EGF or total growth factors resulted in ERK activation and prevented apoptosis in both Tiam1-KO and WT cells as determined by the cleavage of PARP (Fig. 3D, lanes 1-5 and 9-13) and annexin-V staining (Fig 3G). Both ERK phosphorylation and PARP cleavage have been quantified in Figs 3E and 3F respectively. Addition of the PD inhibitor impaired GF-independent ERK activation in WT cells after GF starvation leading to increased apoptosis as measured by PARP cleavage (Fig. 3D, compare lanes 3 and 6). The PD inhibitor had little effect on Tiam1-KO cells since no GF-independent ERK activation was seen in these cells (Fig. 3D, compare lanes 11 and 14). PD inhibited also EGF-induced ERK activation leading to apoptosis in both WT and Tiam1-KO cells cultured in EGF containing medium (Fig. 3D, lanes 4,7 and 12,15). Interestingly, PD inhibited GF-independent ERK activation in insulin-treated WT cells but did not inhibit insulin-induced survival signals in both WT and Tiam1-KO cells (Fig. 3D, lanes 8 and 16). This is consistent with the findings that insulin-induced survival signaling is mediated through the PI3-kinase/Akt pathway rather than the ERK pathway (Zaka et al., 2005). Insulin is therefore able to prevent apoptosis in the presence of the PD inhibitor (Fig. 3D, lanes 8 and 16). From these studies we conclude that ERK activation is impaired in Tiam1-KO cells upon GF starvation and that this impaired ERK signaling is responsible for the increased susceptibility to apoptosis of

Tiam1-deficient cells. Consistent with this we found no major differences in Akt- or IκB phosphorylation between WT and KO cells (Fig. 3H), suggesting that the PI3K-Akt and NFκB survival signaling pathways are not responsible for the differences in apoptosis upon GF starvation.

Tiam1 induces ERK phosphorylation and cell survival by regulating ROS production

Reactive oxygen species (ROS) such as H_2O_2 are known to induce ERK phosphorylation and activate the ERK pathway (Guyton et al., 1996). It has been shown that Rac GTPases have an indispensable role in activating ROS-producing enzymes such as the NADPH oxidase, Nox. (Miyano et al., 2006;Cheng et al., 2006;Joneson and Bar-Sagi, 1998). As Tiam1 is a specific activator of Rac, we investigated whether Tiam1 could regulate ERK phosphorylation through the production of ROS. Indeed, intracellular ROS levels were significantly lower in Tiam1-KO keratinocytes than in WT cells as determined by 2',7'-dichlorofluorescin (DCF) fluorescence measurements in intact cells (Fig. 4A) and cell lysates (Fig. 4B). In WT cells, ROS levels dropped slightly within the first 30 minutes of GF starvation and subsequently recovered to the levels found in cells grown in the presence of GF. These data correlate with the drop and recovery of ERK phosphorylation in WT cells upon GF deprivation (Fig. 3B). Similar results were found by FACS analysis of DCF stained cells. WT cells contained on average higher ROS levels than Tiam1-KO cells (Fig. 4C). These data suggests that Tiam1 is involved in ROS production leading to ERK activation. Indeed, expression of full length Tiam1 in Tiam1-KO keratinocytes (Fig. 4D) increased the level of intracellular ROS in Tiam1-KO cells to that of WT cells level (Fig. 4E). Expression of Tiam1-ΔDH, which is unable to activate Rac (Michiels et al., 1997), did not increase the levels of ROS (Fig. 4E), indicating that Tiam1-mediated Rac activation is required for the ROS production. Moreover, downregulation of Tiam1 in WT cells by shRNA to approximately 50% of the WT levels inhibited partly intracellular ROS production in WT cells (Fig. 4F). Tiam1 downregulation also resulted in inhibition of ERK phosphorylation in GF starved conditions (Fig. 4G). Together these data suggest that Tiam1-mediated Rac activation is responsible for the control of intracellular ROS and ERK phosphorylation in the absence of GF.

Tiam1 controls Nox activity that regulates ROS production and ERK phosphorylation

To further substantiate a function of Tiam1 in ROS-mediated ERK phosphorylation and survival signaling, WT keratinocytes were starved in the presence of increasing concentrations of the oxygen scavenger NAC (N-acetylcysteine). Interestingly, treatment of the WT cells with NAC resulted in decreased ERK phosphorylation (Fig. 5A) accompanied by increased sensitivity to apoptotic stimuli as shown by PARP cleavage (Fig. 5B) and cell

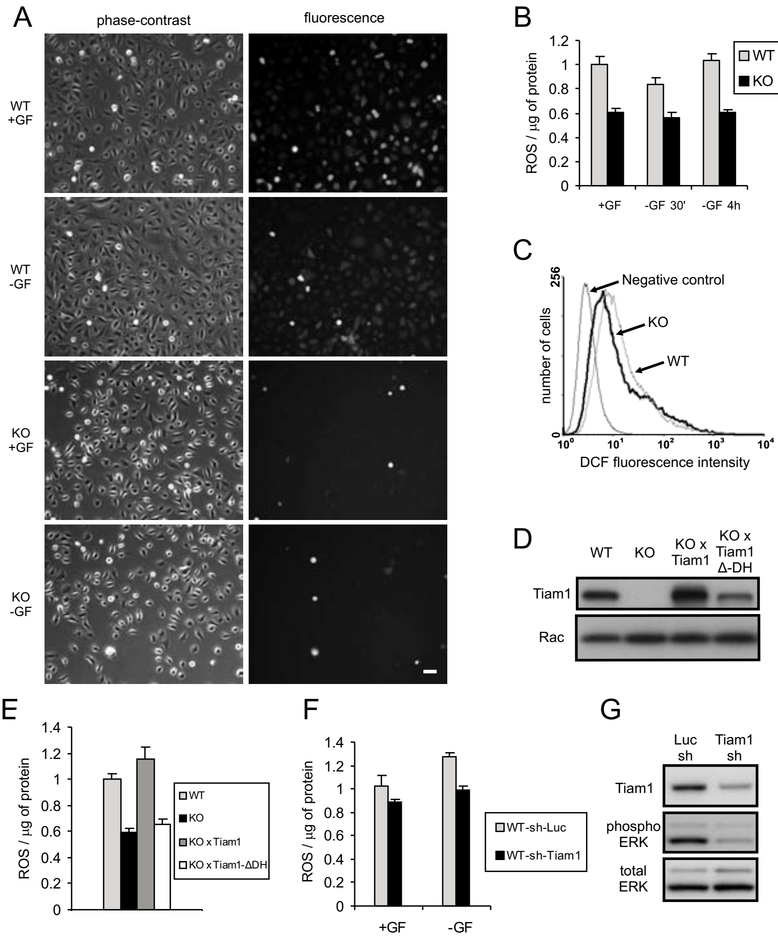

Figure 4. Tiam1-KO keratinocytes have impaired ROS production. A. The level of intracellular ROS in WT and Tiam1-KO keratinocytes was visualized by DCF staining. The cells were starved for GF for 4 hours. Images were taken using an epifluorescence microscope. Bar represents 50 µm. B. Quantification of intracellular ROS in WT and Tiam1-KO keratinocytes upon GF starvation for 30 minutes and 4 hours. Cells were loaded with DCF, lysed and DCF-fluorescence was measured in the lysates. Fluorescence values were normalized for the amount of total protein present in the lysates. The fluorescence value of WT cells was set to 1. Error bars represent standard deviation from triplicate measurements. The result shown is a representative example of four independent experiments. C. Quantification of ROS levels at a single cell level in WT and Tiam1-KO keratinocytes by DCF staining and FACS analysis. D. Western blot showing the exogenous expression of full length and the deletion mutant of Tiam1 (Tiam1-ΔDH) in Tiam1-KO keratinocytes. Expression of Rac functions as a loading control. E. Exogenous expression of full length Tiam1 but not Tiam1-ΔDH restores ROS production in Tiam1-KO keratinocytes. Intracellular ROS was measured as described in figure 4B. F. Partial downregulation of Tiam1 reduces ROS production in WT keratinocytes, both in the presence and absence of GF. Intracellular ROS content was measured as described in figure 4B. The data is a representative example of three independent experiments. G. Immunoblot showing Tiam1 expression in wild type keratinocytes upon short hairpin-RNA expression. Partial downregulation of Tiam1 (~50%) inhibits ERK1/2 phosphorylation (in GF starved conditions) when compared to control cells. Tiam1 total ERK were used as expression and loading controls respectively.

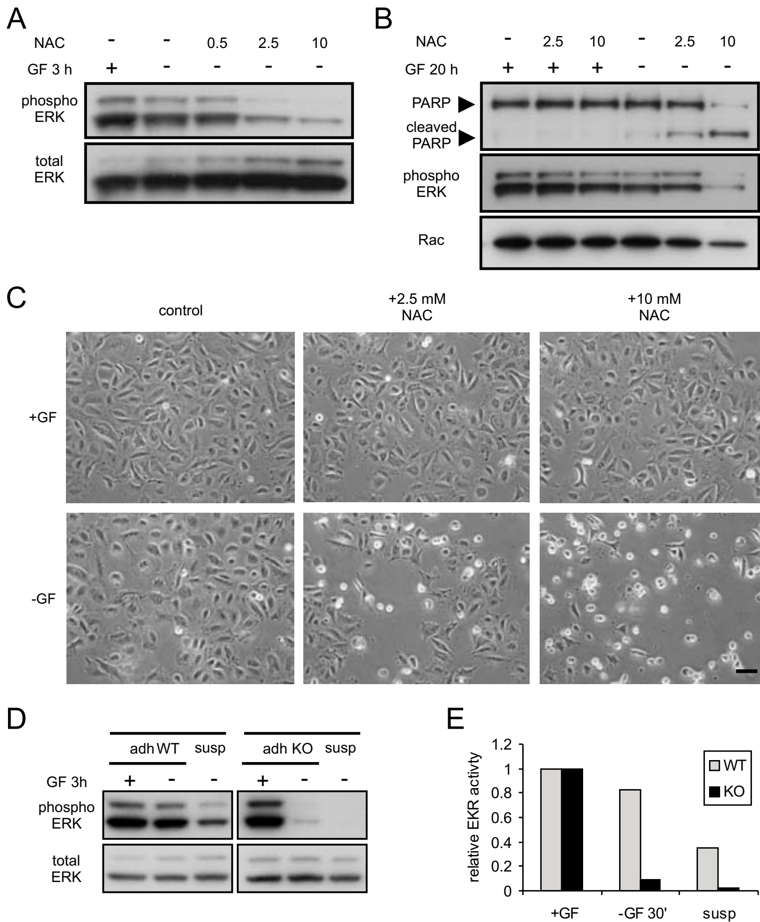

Figure 5. ROS and cell-matrix adhesions stimulate ERK phosphorylation in the absence of GF. A. Western blot depicting that ERK phosphorylation in GF-starved WT keratinocytes is dependent on the presence of ROS and negatively correlates with increasing concentrations of antioxidant (NAC). The cells were starved for 3 hours in the presence of different concentrations of the ROS scavenger NAC. Total ERK expression was used as loading control. B. Immunoblot showing PARP cleavage and ERK phosphorylation in WT keratinocytes. The cells were cultured in the presence or absence of GF and NAC, as indicated. PARP cleavage correlates with prolonged (20 hours) GF-starvation combined with NAC treatment. Rac was used as loading control. NAC was added at the beginning of the experiment. C. Phase-contrast images of WT keratinocytes subjected to GF starvation and/or NAC treatment. The images correspond to the conditions in Fig. 5B. Bar represents 50 µm. The data presented are representative examples of three independent experiments. D. Western blot showing ERK phosphorylation in wild type and Tiam1-KO cells in adhesive control, GF-starved (3 hours) and suspension conditions. Total ERK serves as a loading control. Results are typical example of three independent experiments. E. Quantification of ERK activity from figure 5E, normalized for total ERK expression and readouts corresponding to GF-supplemented conditions were set to 1.

morphology changes (Fig. 5C). Note that in the presence of GF, ROS titration had no effect on ERK phosphorylation or apoptosis (Fig 5B), indicating that GF-induced survival signaling is mediated by ROS-independent ERK phosphorylation. This is consistent with our

conclusion that the GF-induced survival-signaling pathway acts in parallel to the Tiam1/Rac-dependent ERK survival-signaling pathway. In the absence of GF, ERK phosphorylation is fully dependent on Tiam1-mediated Rac activation that controls ROS production. Tiam1-KO keratinocytes therefore fail to survive because of the impaired ROS-mediated ERK phosphorylation and survival signaling. We wondered what signals could be involved in ERK phosphorylation upon GF starvation. Either cell-cell or cell-matrix interactions could function in ERK-mediated survival signaling in growth factor-deprived conditions. As keratinocytes are cultured in low-Ca^{2+} medium in which no E-cadherin-mediated cell-cell contacts are formed, we concentrated on cell-matrix adhesions. To address this question, attached and suspended WT cells were GF-starved. Interestingly we found that ERK activation upon GF starvation is largely impaired in suspended WT cells (Figs. 5D and E), suggesting that integrin-mediated cell-matrix adhesions play a role in ERK-mediated survival signaling.

To investigate the source of ROS that in wild type cells supports ERK activation and cell survival we focused our attention on NADPH oxidases (Nox). Nox enzymes are known for their inducible ROS production and dependency on Rac activity. To test potential Nox involvement in the Tiam1-mediated production of intracellular ROS we used a Nox specific inhibitor diphenyleneiodonium chloride (DPI). Treatment of keratinocytes with DPI resulted in dramatic inhibition of ROS production in WT cells whereas ROS production in Tiam1-KO cells was hardly affected (Fig. 6A). This suggests that WT keratinocytes have a much higher fraction of ROS that is produced by Nox and dependent on Tiam1. Indeed, a general antioxidant like NAC decreased intracellular ROS levels similarly in both WT and KO keratinocytes (Fig. 6B). To further substantiate a function of Nox in ROS production, we treated WT and KO cells with another Nox-specific inhibitor (Apocinin). Addition of Apocinin inhibited ROS production in WT cells and not in KO cells (Fig. 6C) similarly as found for DPI (Fig. 6A), further substantiating a function of Nox in Tiam1-mediated ROS production in keratinocytes. To exclude the possibility that lack of Tiam1 influences the expression of Nox enzyme components, we analyzed the degree of expression of Nox1 and various components of the Nox pathway by Western blotting. We found no indications that Nox enzymes or Nox components were differently expressed in wild type and Tiam1 KO cells (Fig 6D). Similarly, micro array analyses of wild type and Tiam1-KO keratinocytes did not reveal any indication of differences in expression of Nox- and Nox-associated genes in both genotypes (not shown). These data support our conclusion that lack of Tiam1 decreases ROS production by impaired Rac activation rather than by impaired expression of ROS-producing enzymes.

Treatment of cells with DPI or NAC had an inhibitory effect on ERK phosphorylation in the absence but not in the presence of GF (Fig. 6E), consistently with our earlier conclusion that GF-induced ERK phosphorylation is independent of ROS (Fig. 5B). Similar results were found upon induction of apoptosis by heat shock treatment (Fig 6F). From these

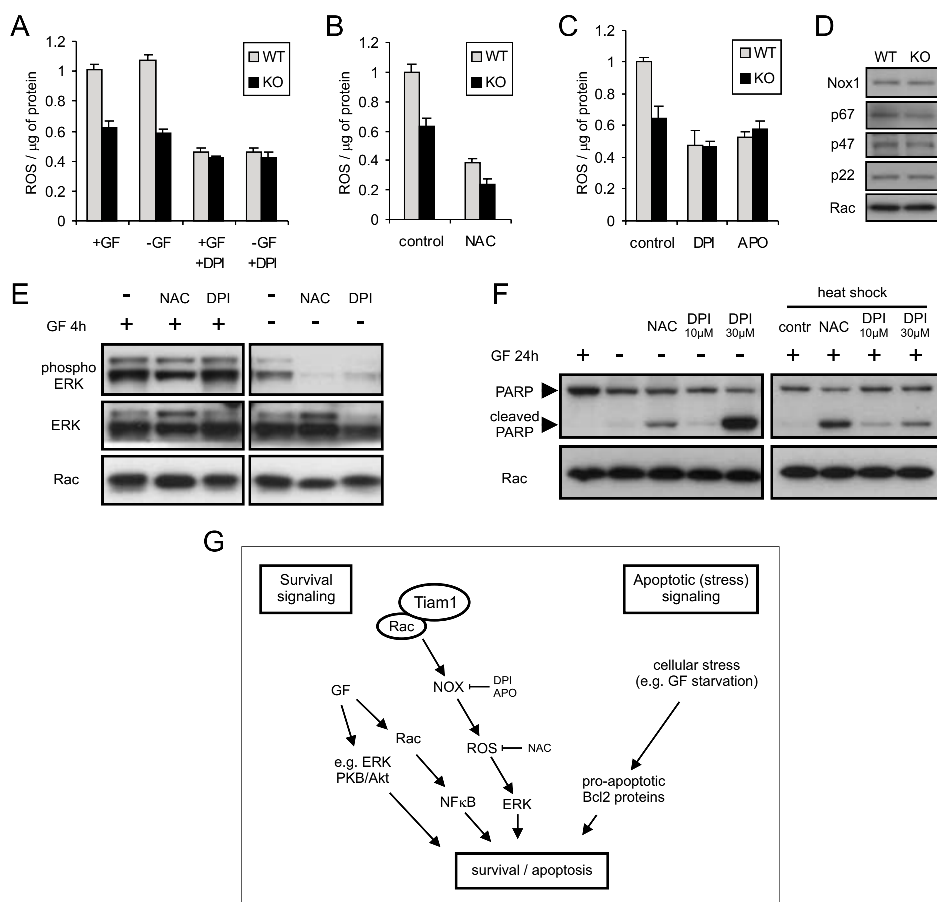

Figure 6. Nox dependent ROS production controls ERK phosphorylation and survival. A. The levels of intracellular ROS were quantified by DFC fluorescence measurement in cell lysates of WT and Tiam1-KO keratinocytes. WT and Tiam1-KO keratinocytes were cultured in the presence or absence of GF for 4 hours and treated with the Nox inhibitor DPI (15 mM) in the last 30 minutes. Measurements were conducted as described in figure 4B. B. Quantification of intracellular ROS levels in WT and Tiam1-KO keratinocytes treated with or without the antioxidant NAC (10 mM). Pretreatment was carried out for 30 minutes prior to analysis. C. Quantification of intracellular ROS levels in WT and Tiam1-KO keratinocytes treated with or without the Nox inhibitor DPI (15 mM) or Apcynin (APO)(10 mg/ml) in the last 30 minutes. D. Western blot showing expression of various Nox components (Nox1-Mox1, p67-phox, p47-phox and p22-phox) in wild type and Tiam1-KO cells, Rac expression serves as loading control. E. Immunoblot showing ERK phosphorylation in WT keratinocytes in control or in GF-deprived conditions (4 hours) in the presence or absence of NAC (10 mM) or DPI (15 µM). Total ERK and Rac expression was used as a loading control. The data are a representative example of two independently performed experiments. F. Westernblot showing PARP cleavage in WT keratinocytes GF-starved for 24 hours or heat shock treated (90 minutes at 43°C). Cells were treated with NAC or DPI at the start of the experiment. Rac expression serves as a loading control. The figure is a representative example of four independently performed experiments. G. Model depicting regulation of cellular fate by survival and proapoptotic signaling. Apoptotic stimuli e.g. GF starvation induces the activation of Bcl-2 pro-apoptotic proteins. Tiam1 acts in the survival pathway by controlling Rac-dependent Nox-mediated ROS production and ERK activation. Growth factors may provide survival signals independent of Tiam1-Rac signaling, leading to ERK and/or Akt or NFκB activation.

results we conclude that the susceptibility of Tiam1-KO cells for apoptotic stimuli such as GF-deprivation and heat shock treatment is largely caused by impaired Nox-mediated ROS production that is required for ERK-mediated survival signaling (see Fig. 6G).

Discussion

In the present study we show that Tiam1 is involved in cell survival signaling during stress-induced apoptosis. The presence of Tiam1 protects keratinocytes from apoptosis induced by GF starvation and heat shock treatment. We found that the Tiam1/Rac-mediated survival pathway acts through activation of the ERK pathway. In the absence of growth factors, survival of keratinocytes is completely dependent on Tiam1-mediated ERK activation, which is regulated by intracellular ROS levels and dependent on cell matrix adhesions. Tiam1-KO cells have lower intracellular ROS levels when compared to WT cells. Exogenous expression of catalytically active Tiam1 in Tiam1-KO keratinocytes restores ROS levels to the amounts found in WT cells, and thereby reduces the susceptibility to apoptosis. Inhibition of ROS production in WT cells by Nox inhibitors or an antioxidant such as NAC reduces both ROS production and ERK phosphorylation and simultaneously increases the sensitivity for apoptotic signals. From these studies we conclude that Tiam1 is necessary for Rac-mediated activation of Nox, which leads to increased ROS production that is required for ERK-mediated survival signaling upon stress signaling such as GF-deprivation or heat shock treatment (Fig 6F).

The activity of Rac has been associated with survival signaling in various model systems. Conditional knockout of both Rac1 and Rac2 in the B-cell lineage results in increased apoptosis during B-cell development (Walmsley et al., 2003). Earlier studies have shown that interleukin-1β-mediated activation of NF-κB is dependent on Rac activity and the presence of ROS (Sulciner et al., 1996). Rac is able to prevent anoikis by activation of NF-κB downstream of the α6β4 integrin in mammary epithelial cells (Zahir et al., 2003). Rac activity is also required in β1 integrin mediated survival signaling and inhibition of anoikis (Hirsch et al., 2002). Interestingly, β1-mutated mouse embryonic fibroblasts have impaired Rac-dependent ERK nuclear translocation, suggesting an involvement of Rac in ERK signaling and survival (Hirsch et al., 2002). Also in neuronal cells Rac seems to play a crucial role in survival signaling as inhibition of Rac activity induces apoptosis by stabilizing the Bim protein (Le et al., 2005). Bim is one of the pro-apoptotic Bcl2 homologues of the BH3 only family and its stability is controlled by ERK phosphorylation (Loucks et al., 2006). ERK phosphorylates Bim on serine 69 and thereby promotes its degradation (Luciano et al., 2003). Furthermore, B cell antigen receptor (BCR)-mediated apoptotic signaling is inhibited by ERK-dependent phosphorylation and degradation of Bim (Craxton et al., 2005). Our data are consistent with reports showing that apoptosis induced by GF-starvation is

associated with inhibition of ERK phosphorylation (Manohar et al., 2004). Interestingly, we found that Tiam1-deficient keratinocytes are impaired in Rac activation upon α3β1-mediated adhesion to a Laminin 5, a cell substrate which is produced by keratinocytes (Hamelers et al., 2005). As survival signaling upon GF starvation is reduced in suspended keratinocytes, it is tempting to speculate that the ERK activation is derived from α3β1 integrin-mediated cell-matrix adhesions. Tiam1 could thus control Rac-mediated Nox/ROS/ERK pathway downstream of α3β1-mediated interactions with Laminin 5.

Reactive oxygen species (ROS) are generally seen as harmful to biomolecules and organisms (HARMAN, 1956). However, besides negative effects, ROS are also actively produced and required for many physiological functions in cells (Stone and Yang, 2006;Voeikov, 2006). In addition to the role of ROS in pathogen killing and angiogenesis (Segal, 2005;Ushio-Fukai, 2006), the production of ROS has also been reported to be important in cellular stress conditions. Stimuli that induce production of free radicals are known to have anti- or pro-apoptotic effects, depending on the duration of the stress (Liu et al., 2005;Reinehr et al., 2006;Papaiahgari et al., 2006). One of the ROS downstream signaling targets is ERK. ROS-mediated ERK activation has either pro-survival or pro-apoptotic effects depending on the concentration of oxidative radicals and cell types tested (Guyton et al., 1996;Wang et al., 1998;Chamulitrat et al., 2003;Zhuang and Schnellmann, 2006). However, in most instances ERK activation has a pro-survival function (Henson and Gibson, 2006). We found that ROS-mediated ERK activation in Tiam1-deficient keratinocytes is necessary to prevent apoptosis induced by GF starvation. Interestingly, Tiam1/Rac-mediated ERK activation and survival signaling is not required in normal growth conditions when various growth factors are providing survival signals. Tiam1/Rac-mediated survival signaling, however, becomes apparent in stress situation e.g. upon GF starvation.

The function of Rac proteins in regulation of Nox enzymes and ROS production has been described earlier. Hematopoietic cells like neutrophils and macrophages use Rac1 and especially Rac2 (the hematopoietic-specific Rac isoform) for the activation of gp-91phox (Nox2) and the delivery of the oxidative burst (Minakami and Sumimotoa, 2006)). In non-hematopoietic cell types, both Rac1 and Rac3 may control ROS production as demonstrated in primary mouse embryonic fibroblasts (Dolado et al., 2007). Rac1 binds the Nox activator Noxa1/p67^{phox} and activated GTP-loaded Rac1 is necessary for Nox1 and Nox3 activation (Cheng et al., 2006;Ueyama et al., 2006). However, little is known about the activators of Rac which function in Rac-mediated Nox activation. Here we show that Tiam1 is involved in the Rac1/Nox-dependent ROS production and ERK-mediated survival signaling in keratinocytes independent of normal growth factor signaling. In human neutrophils, Rac2 activation and Nox2 complex formation leading to ROS production, was shown to correlate with phosphorylation of Vav1, suggesting a function of Vav1 in ROS production in neurtrophils (Zhao et al., 2003). In Caco-2 and HEK293T cells, growth factor-induced ROS production (PDGF and EGF) was dependent on βPix-mediated Rac-

Nox activity (Park et al., 2004), whereas Vav2 was shown to be involved in Nox dependent ROS production in kidney mesengila cells (Chen et al., 2007). Apparently, specific Rac-GEFs can contribute to the activation of Nox and thereby ROS production. In the COS-phox cells with overexpression of all components of Nox2 a hematopoietic-specific GEF, Vav1 was more efficient in inducing ROS production than other GEFs such as Vav2 or Tiam1 (Price et al., 2002).

The prevention of apoptosis is a necessary step in tumor initiation. Tiam1-deficient mice are resistant to the initiation of skin tumors induced by oncogenic Ras and intestinal tumors induced by the canonical Wnt signaling pathway (Malliri et al., 2002;Malliri et al., 2006). Earlier we found an inverse correlation between Tiam1-dosage and apoptosis in the epidermal keratinocytes of mice treated with DMBA (Malliri et al., 2002). These findings are consistent with the present data that Tiam1 functions in survival signaling mediated by Nox-controlled ROS production in a dosage-dependent manner. Oncogenic Ras requires Rac for efficient cell transformation (Qiu et al., 1995) and induces ROS production by a Rac-dependent mechanism (Irani et al., 1997). Tiam1 can bind activated Ras directly (Lambert et al., 2002) and thereby may regulate Ras-mediated Rac activation required for ROS-controlled survival signaling. Thus Tiam1 may act in survival signaling pathways that prevent apoptosis in response to both oncogenic and stress signals.

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Chapter 6

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

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ABSTRACT

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

INTRODUCTION

In epithelial cells, apical-basal polarity is maintained through the formation of several intercellular adhesion systems, consisting of Adherens Junctions (AJ), Desmosomes and Tight Junctions (TJ). The TJ consists of transmembrane proteins Occludin, Claudins, and Junctional Adhesion Molecules (JAMs), which are organized in intra-membraneous strands and are linked to the F-actin cytoskeleton either directly or indirectly through members of the MAGUK-family of proteins ZO-1/2/3. The TJ regulates paracellular diffusion and functionally segregates the plasma membrane in two compartments, a requirement for full polarization of epithelial cells (Tsukita et al., 2001a).

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

MATERIALS AND METHODS

Keratinocyte isolation and culturing

Keratinocytes were isolated from newborn WT and Tiam1KO mice (Malliri et al., 2002). Skins were removed and trypsinized (0.25% Trypsin, EDTA-free) for 16 h at 4°C to separate epidermis from the dermis. Both fractions were minced and cells were detached by stirring on ice for 1 h. Cell suspension were filtered and seeded on dishes coated with

collagen IV (10 μg/cm^2, Becton Dickinson) and cultured in Epilife keratinocyte medium (Cascade Biologics) supplemented with 20 μM $CaCl_2$ and Epilife defined growth supplement. Epidermal keratinocytes were immortalized with SV40 Large T. Primary cultures as well as populations of immortalized epidermal keratinocytes at low passage numbers (< 20) were used in our studies. Keratinocytes were grown on collagen IV-coated glass cover slips or plastic. For Ca^{2+}-switch assays, cells were grown to confluency at low Ca^{2+} (0.02mM) and $CaCl_2$ was added to the medium to a final concentration of 1.8 mM.

Antibodies

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

Immunoblotting, immunoprecipitation and PKCζ kinase assay

Total cell lysates were prepared in hot SDS lysis buffer (1% SDS, 10mM EDTA and protease inhibitors). For immunoprecipitation, lysates of COS-7 cells and keratinocytes were prepared in standard RIPA buffer. Extracts were clarified by centrifugation and precleared with γ-bind Prot G-sephasose (Amersham Biosciences) for 1 h at 4°C. Pre-cleared lysates were incubated with antibodies preabsorbed on Protein G-sephasose, 16 h at 4°C. Immunocomplexes were washed (3x) and denatured with SDS and separated by SDS-PAGE.

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

Rac and Cdc42-activity assays

Rac and Cdc42-activity were determined as described earlier (Malliri et al., 2004) using a biotinylated CRIB-peptide of PAK1. Densitometric analysis was done with Image J.

Microscopy, immunofluorescence, and EM

Cells were examined with a Zeiss Axiovert 25 phase contrast microscope and photographed using a digital camera. For immunofluorescence, cells grown on glass cover slips were fixed in 4% PFA, permeabilized in 0.1% Saponin (Sigma-Aldrich), blocked in 5% skimmed milk, and incubated with the primary antibodies (1.5 h in PBS/0.1% saponin/1% BSA). Cells were incubated (1 h) with secondary antibodies conjugated to Alexa488 or

Alexa594 (Molecular Probes), washed and mounted with Mowiol-DABCO. Filamentous actin was labeled with 0.2 μM Alexa 568-phalloidin (Molecular Probes). Images were taken with a Leica TCS SP2 confocal microscope and were arranged and resized using Corel Graphics Suite 12.

For electron microscopy, cells were grown on Thermanox Plastic (Nunc), fixed in 2.5% glutaraldehyde, post fixed in 1% OsO_4 and stained en bloc with uranylacetate and were flat embedded. Samples were examined with a Philips CM 10 electron microscope.

Paracellular diffusion of non-ionic molecular tracers

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

Occludin extracellular loop peptide

An occludin inhibitory peptide was synthesized spanning Gly^{194}-Gln^{241} of the 2nd extracellular loop of mouse Occludin, as described for chicken Occludin (Wong and Gumbiner, 1997). Inhibition was optimal at 5 μM.

Expression vectors

pmt2SM-Myc-Par3 was a gift from Ian Macara. Myc-V12Rac1, -N17Cdc42, -L28, -V12Cdc42 and Tiam1-coding sequences were cloned into the retroviral vector LZRS-IRES-blasticidin (Michiels et al., 2000). LZRSbsd-VSV-Occludin was constructed by subcloning FL VSV-tagged Occludin from pCB6 (a gift from C.M. van Itallie) into the XbaI and SnaBI sites of LZRS-IRES-blasticidin. pSuper-siRNA-Tiam1 and pSuper-siRNA-Luciferase have been described (Malliri et al., 2004). pmtSM-myc-PKCζ-WT and pmtSM-myc-PKCζ-K281W were provided by W.J. van Blitterswijk. Myc-PKCζ-WT was subcloned into the Swa1 and Not1 sites of LZRSbsd. pBabe-SV40 LargeT was a gift from R. Bernards. pEGFP-N1 was obtained from Invitrogen. The pCDNA3-STEF1 and pmt2SM-HA-Rap1GAP plasmids were gifts from M. Hoshino and J.L. Bos respectively.

Cell transfection and retroviral transduction

Retroviral vectors (LZRS and pBabe) were transduced to keratinocytes as described (Michiels et al., 2000). All other plasmids were transiently transfected in semi confluent keratinocytes or COS7 cells using the lipofectamine FuGENE 6, according to the manufacturers protocol (Roche Diagnostics).

Results and discussion

To study the role of Tiam1 in the establishment of intercellular contacts and cell-polarity, we used epidermal keratinocytes derived from WT and Tiam1 knockout (Tiam1KO) mice. Cells were cultured in medium containing low Ca^{2+} levels (0.02 mM) to prevent terminal differentiation. The expression profile of several differentiation markers reflected typical non-differentiated cells of the basal layer of the epidermis (not shown). WT keratinocytes showed similar Tiam1 protein levels as MDCKII cells, whereas Tiam1KO keratinocytes lacked the protein (Fig 1A).

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

The aberrant phenotype of Tiam1KO cells could be the result of impaired formation of initial E-cadherin-dependent cell-cell contacts, as these PAs are considered a prerequisite for the formation and maturation of TJs (Lozano et al., 2003). PAs contain components of AJs, TJs (Occludin, Claudin-1, JAM-A and ZO-1) and Nectins and these are functionally segregated during the subsequent polarization process (Suzuki et al., 2002). We analyzed therefore first the localization of components of both AJ and TJ upon induction of cell-cell contacts. After 30 min in normal Ca^{2+}, Occludin, ZO-1 and E-cadherin (co-) localized at the ends of F-actin bundles at the interphase of neighboring cells in WT and Tiam1KO cells (Fig 2A, upper panels). Also Nectin 2 and Afadin, were localized in these PAs (Fig S1A&B). All adhesion molecules were organized in 'zipper'-like structures, as described in epithelial cells establishing initial cell-cell contacts (Vasioukhin and Fuchs, 2001). A similar pattern of localization was observed for β-catenin, Claudin-1, and JAM-A (not shown). Apparently, both WT and Tiam1KO keratinocytes form PAs equally well within 30 minutes.

WT cells had made fully matured intercellular junctions in which E-cadherin, Occludin, ZO-1, Nectin 2 and Afadin perfectly aligned along the cortical actin cytoskeleton in a linear fashion, 6 h after the Ca^{2+} switch (Fig 2A & Fig S1A&B). In addition, confocal *xz*-projections of these cells showed clear spatial separation of TJs and AJs at the lateral membrane (Fig S2A). In contrast, no maturation of junctions occurred in Tiam1KO cells

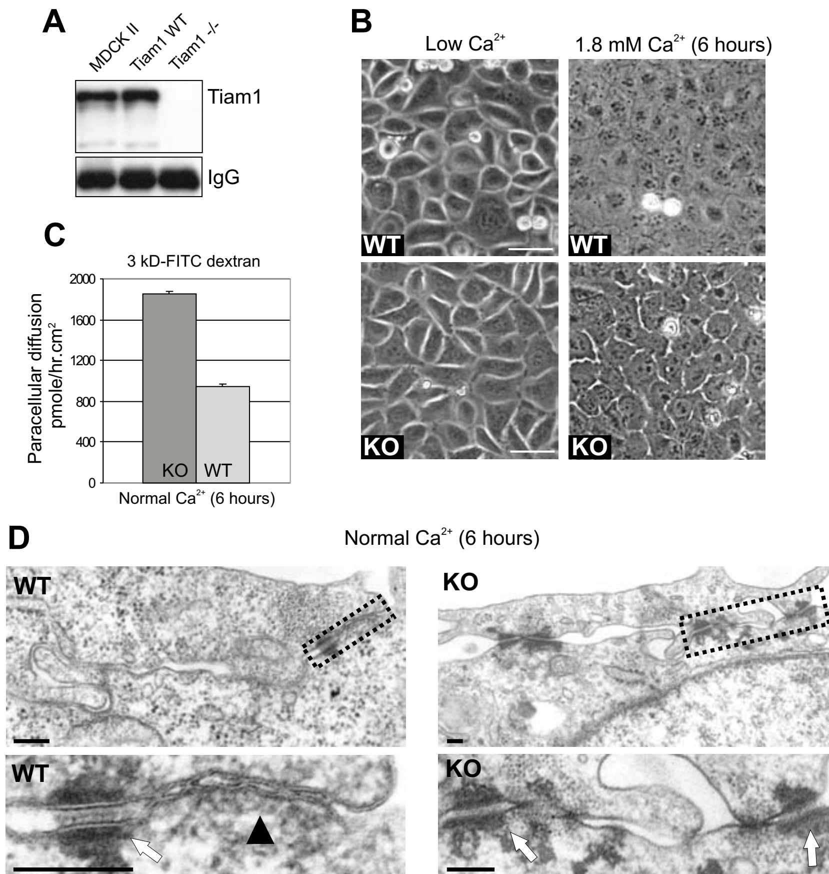

Figure 1. Tiam1 is required for membrane sealing and tight junction formation in epidermal keratinocytes. A Tiam1 was immunoprecipitated from cell lysates using anti-Tiam1 Ab (C16). Western blotting was performed with anti-Tiam1 Ab (DH). **B, C** and **D,** WT and Tiam1KO cells were grown to confluency and then incubated with 1.8 mM Ca^{2+} (normal Ca^{2+}) for 6 h. **B** Phase contrast images show sealing of the paracellular space in WT- but not in Tiam1KO cells. Bar = 20 µm. **C** Paracellular diffusion of 3 kD-FITC dextran through keratinocyte monolayers cultured for 6 h in 1.8 mM Ca^{2+}. Paracellular diffusion was measured for 2.5 h. The data are shown as mean ± SD from 3 independent experiments. **D** EM images of the apical part of the lateral plasma membrane of keratinocyte monolayers. Arrowhead indicates TJ; Arrows indicate Desmosomes. Notice the 'membrane fusions' typical of TJs in WT cells. Bar = 200 nm.

and adhesion zippers were still present in between all cells (Fig 2A, lower panels). F-actin bundles were tightly packed in belt-like structures at the apical side of the cell in conjunction with the adhesive structures, 6 h after the Ca^{2+} switch in WT cells. In Tiam1KO cells, actin bundles were not organized at the cell cortex and many stress fibers spanned the entire cell (Fig 2A, lower panels). The impaired junction maturation in Tiam1KO cells was seen even 24 h after the Ca^{2+} switch (Fig S1C) and correlated with the absence of TJs.

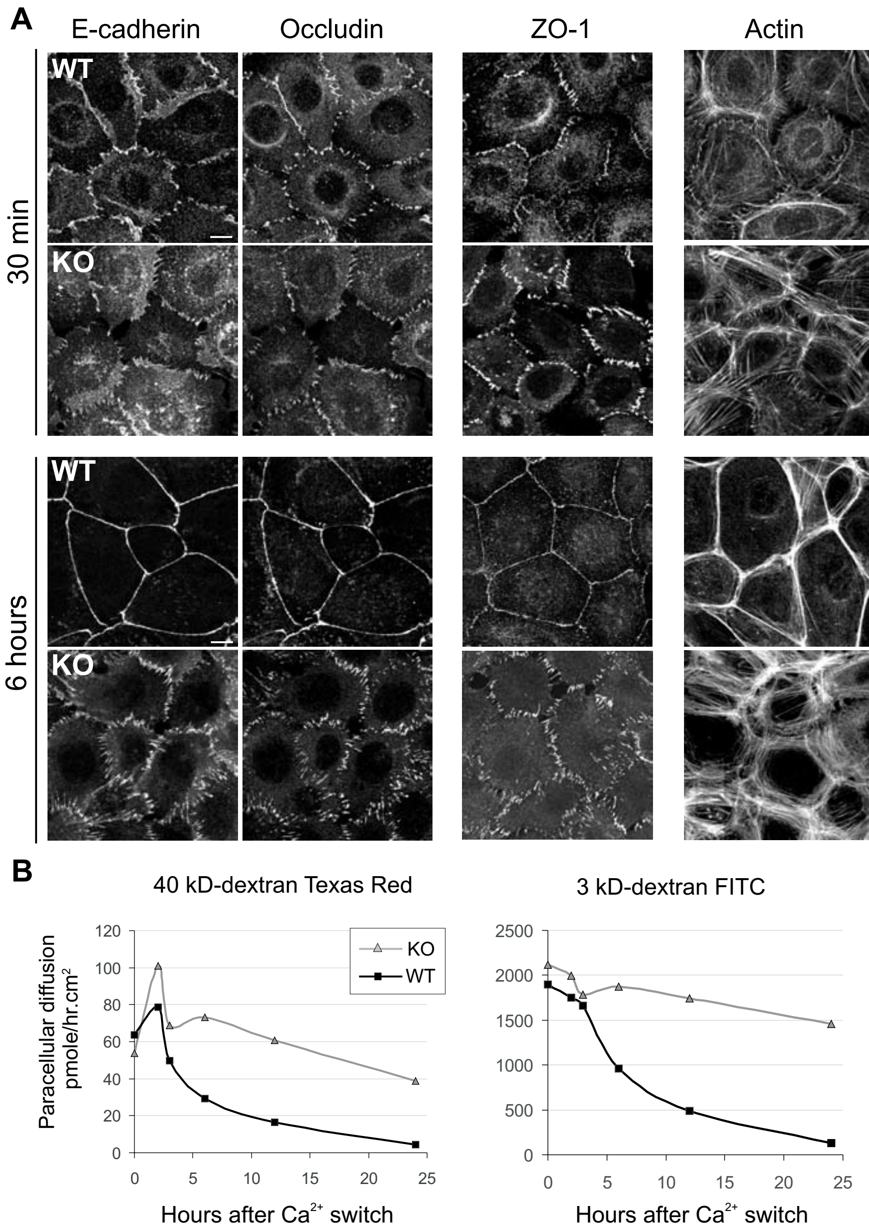

Figure 2. Tiam1-deficiency impairs junction maturation and TJ barrier function. A Tiam1 is not essential for the formation of primordial adhesions but its absence impairs subsequent junction maturation. Concentration of Ca^{2+} was increased to 1.8 mM in medium of keratinocyte monolayers for the indicated times. Cells were double-stained with anti-E-cadherin Ab and anti-Occludin Ab, or stained with anti-ZO-1 Ab or phalloidin for F-actin. Bar = 10 µm. **B** Tiam1-deficiency impairs TJ barrier function. Paracellular diffusion of 3 kD-FITC and 40 kD-Texas Red dextran through keratinocyte monolayers, which were cultured in 1.8 mM Ca^{2+}, for the indicated time points. Paracellular diffusion was measured for 2.5 h. Shown is a representative example of three independent experiments.

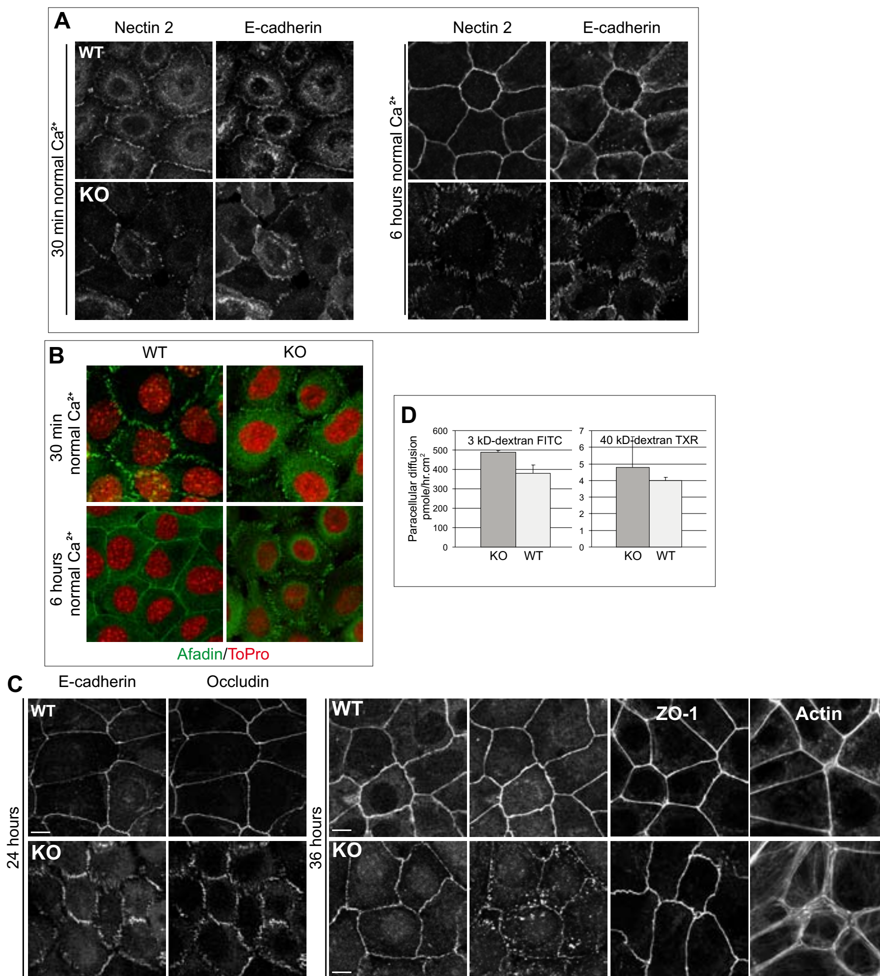

Figure S1. Tiam1-deficiency impairs junction maturation and TJ barrier function. Time course upon a Ca^{2+}switch (up to 36 h) using WT and Tiam1KO keratinocytes, which demonstrates the delay in TJ maturation in Tiam1KO cells. (A and B) Nectin-2 and afadin are present in primordial adhesions of WT and Tiam1-deficient keratinocytes. As already described for AJ and TJ markers (Fig. 2 A), nectin-2 and afadin (which links nectin-2 to the F-actin cytoskeleton) -mediated primordial adhesions form similarly in WT and Tiam1-deficient (Tiam1KO) keratinocytes but fail to mature in Tiam1KO keratinocytes. Keratinocyte monolayers were switched to normal Ca^{2+} for the indicated time periods, and cells were fixed with 4% PFA and double stained with rat antinectin-2 and mouse anti-E-cadherin antibodies (A) or mouse antiafadin antibody (B; gifts from Y. Takai, Osaka University, Osaka, Japan). Nuclei were stained with ToPro3 (Invitrogen). (C and D) Impaired TJ formation in Tiam1-deficient keratinocytes. Junction maturation is still impaired in Tiam1KO keratinocytes 24 h after a Ca^{2+} switch. Tiam1KO cells eventually showed partially maturated intercellular junctions 36 h after the Ca^{2+} switch. (C) Keratinocyte monolayers were switched to normal Ca^{2+} for the indicated time periods, and cells were fixed with 4% PFA and double stained with anti-E-cadherin and antioccludin antibodies or stained with anti-ZO-1 antibody. F-actin was stained with phalloidin. Bars, 10 µm. (D) 36 h after the Ca^{2+} switch, Tiam1KO keratinocytes largely restored TJ barrier function. Paracellular diffusion was determined as described in Paracellular diffusion tracers: TXR and Texas red. Error bars represent SD.

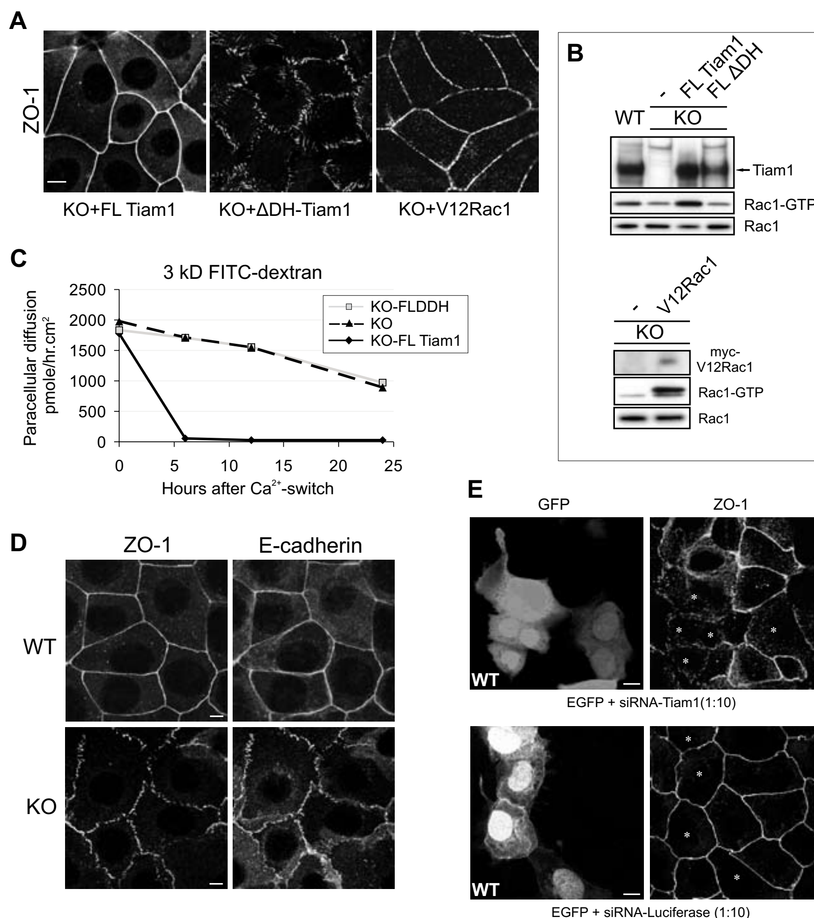

Figure 3. Tiam1 signaling to Rac is required for junction maturation and TJ formation. A Expression of V12Rac1 or FL-Tiam1, but not of catalytically inactive Tiam1 (ΔDH-Tiam1), in Tiam1-/- cells restores junction maturation within 6 h after a Ca^{2+} switch. **B** Western blot analysis and Rac activity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

After 36 to 48 h, Tiam1KO cells were eventually able to form linearly organized adhesive structures with matured TJs and largely sealed the paracellular space (Fig S1C&D). At these later time points, the spatial separation of TJs and AJs as determined by ZO-1 and E-cadherin staining respectively, was also seen in *xz*-projections of the lateral membrane of Tiam1KO keratinocytes (Fig S2). The impaired junction maturation was consistent with the

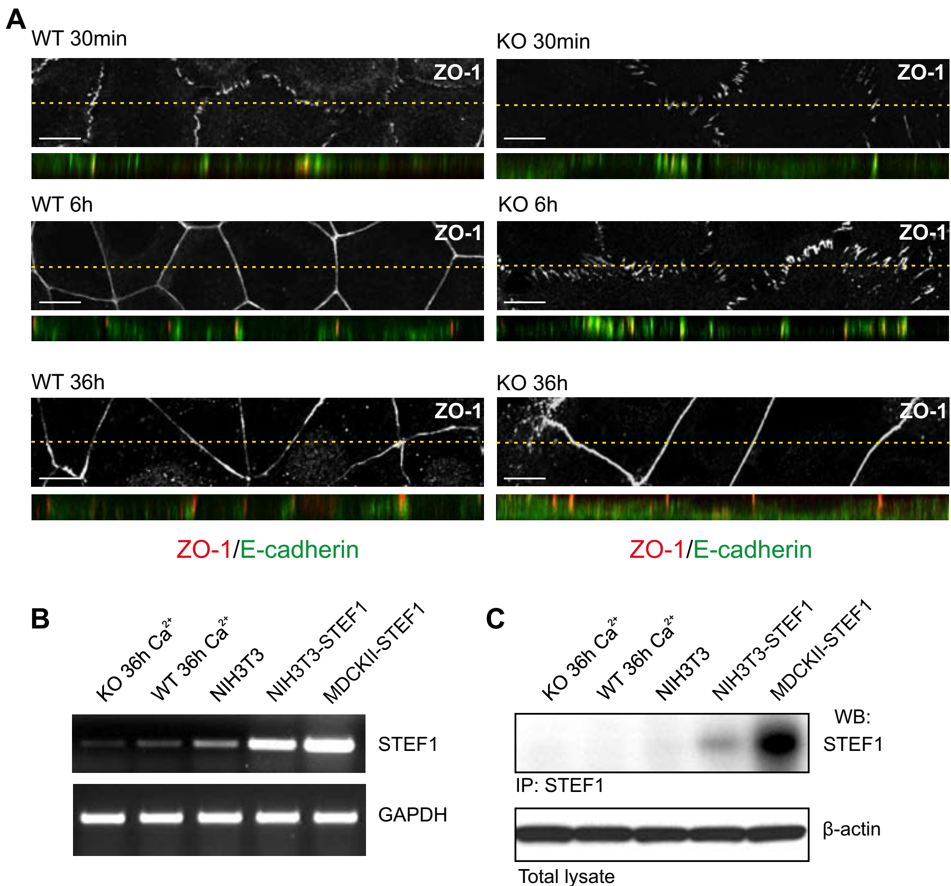

Figure S2. Tiam1-deficiency impairs junction maturation. The full polarization of Tiam1KO cells after 36 h in 1.8 mM Ca^{2+}, as shown by the segregation of TJ and AJ markers, is not a consequence of STEF1 upregulation. (A) Impaired segregation of junctional components in Tiam1-deficient keratinocytes. E-cadherin and ZO-1 labeling overlaps in adhesion zippers in Tiam1KO keratinocytes up to 6 h in normal Ca^{2+}, which is indicative of a defective segregation of the AJ from the TJ. In contrast, WT keratinocytes express ZO-1 separately from E-cadherin at the apical side of the lateral plasma membrane, which suggests a full segregation of the two junctions. This is only observed after 36 h in Tiam1KO keratinocytes. Keratinocyte monolayers were switched to normal Ca^{2+} for the indicated time periods, and cells were fixed with 4% PFA and double stained with anti-E-cadherin and anti-ZO-1 antibodies. Cells were examined with a confocal microscope (model TCS SP2; Leica), and representative xy and xz image stacks are shown. Dashed lines indicate the xz projectional axis. Bars, 10 µm. (B and C) STEF1 does not compensate for Tiam1 deficiency in Tiam1KO keratinocytes. The restoration of junction maturation in Tiam1KO cells 36 h after the Ca^{2+}switch (Fig. S1, C and D) is not mediated by STEF1 upregulation at the transscriptional or translational level. As a positive control for STEF1 expression, NIH3T3 and MDCKII cells were transiently transfected with pCDNA3-STEF1, selected for 48 h on G418, and processed for RNA extraction or immunoprecipitation. Wild-type (WT) and Tiam1KO (KO) keratinocytes were grown to confluency and were switched for 36 h to 1.8 mM Ca^{2+}. (B) Total cellular RNA was isolated using RNAzol B (Campro Scientific), and cDNA was synthesized by RT-PCR carried out on 1 Ìg RNA with oligo dT by using the Thermoscript RT-PCR system kit (Invitrogen). Using the standard PCR protocol for the Platinum Taq PCRx DNA polymerase kit (Invitrogen), specific transcripts were amplified with the following primers (Sigma Genosys): mouse STEF1 (forward, 5′-GGAGGGCCTGGCGGAGTTTC-3′; and reverse, 5′-GTGACGAGTCTGCGCCTTGAGC-3′) and GAPDH (forward, 5′-ACCACAGTCCATGCCATCAC-3′; and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′). (C) STEF1 was immunoprecipitated (IP) from 500 µg of total protein using STEF1 polyclonal antibody. A fraction of 10% of the IP was stained for STEF1. ß-actin was stained as a loading control.

findings that paracellular diffusion of both 3 kDa- and 40 kDa-dextran through Tiam1KO monolayers was persistently high even 24 hours after the Ca^{2+} switch (Fig 2B). In WT cells, the diffusion of both tracers rapidly decreased within 6 h after the Ca^{2+} switch. The fact that Tiam1KO cells eventually were able to form mature TJs could not be explained by upregulation of the Tiam1-related GEF, called STEF1/Tiam2. STEF1 mRNA and protein were hardly expressed by keratinocytes and no differences were found between the two genotypes (Fig S2B&C).

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

Tiam1 regulates junction maturation in a Rac dependent manner

To demonstrate that impaired TJ formation in Tiam1KO keratinocytes was caused by a lack of Tiam1, we restored Tiam1 and Rac1 activity in Tiam1KO cells. Re-expression of full length (FL-) Tiam1 or constitutively active V12Rac1 led to complete maturation of intercellular adhesions within 6 h after the Ca^{2+} switch, with similar kinetics as found in WT cells (Fig 3A,B). Moreover, a Tiam1 mutant with a short deletion in the catalytic Dbl-homology domain (Tiam1ΔDH), which is unable to activate Rac (Fig 3B), could not rescue the impaired TJ formation in Tiam1KO cells. It indicates that capacity of Tiam1 to activate Rac is required for TJ maturation. These morphological data were confirmed by analyzing paracellular diffusion in Tiam1KO cells expressing various Tiam1 constructs (Fig 3C). The impaired TJ maturation was also seen in non-immortalized, primary Tiam1KO keratinocytes, 4 days post-isolation (Fig 3D). Conversely, down-regulation of endogenous Tiam1 in WT keratinocytes by RNA interference resulted in impaired junction maturation whereas control luciferase siRNA had no effect (Fig 3E). This confirms that the observed phenotype depends on the presence or absence of Tiam1. From these data we conclude that the capacity of Tiam1 to activate Rac is required for proper maturation of intercellular junctions in keratinocytes.

Tiam1-Rac signaling influences the expression of TJ molecules

Impaired TJ formation could be a consequence of decreased expression of TJ molecules in Tiam1KO cells, as the number of TJ strands in cells correlates with the expression level of Occludin and Claudins (Tsukita et al., 2001b). In low Ca^{2+} medium (in which cells do not form TJs) Tiam1KO cells showed lower levels of mRNA (not shown) and of protein (Fig 4A) of the TJ- molecules Occludin, ZO-1, and Claudin-1 when compared to WT cells. No differences were found in the expression levels of E-cadherin (Fig 4A), nor of - and β-catenin (not shown). FL-Tiam1, but not Tiam1ΔDH (Fig 3B), could fully restore the TJ protein levels in Tiam1KO cells (Fig 4A), indicating that Tiam1-mediated activation of Rac

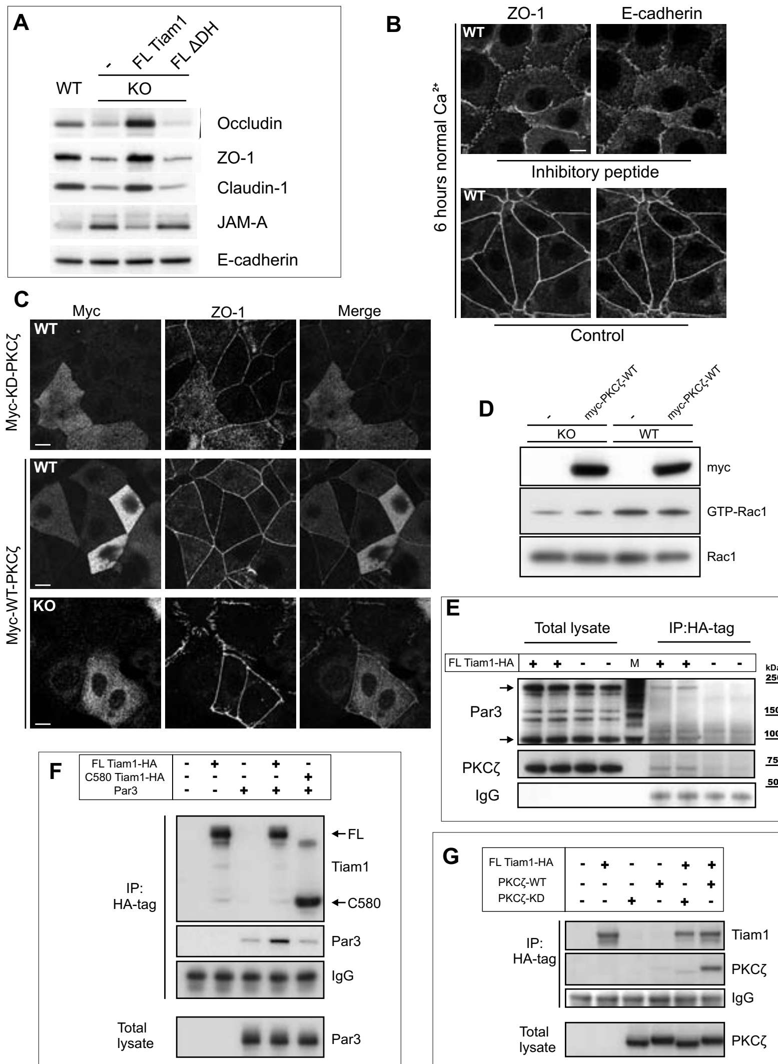

Figure 4. Tiam1 regulates expression of TJ molecules and interacts with the Par3-Par6-PKCζ polarity complex. A Tiam1-Rac signalling differentially regulates the expression of TJ molecules. Lysates of the keratinocytes cultured in low Ca^{2+} medium were immunoblotted for the indicated junctional proteins. **B** Membrane associated Occludin facilitates junction maturation. WT cells were switched to 1.8 mM Ca^{2+} for 6 h in the presence or absence (DMSO Ctrl) of an Occludin inhibitory peptide. **C** WT and Tiam1KO keratinocytes were transiently transfected with myc-tagged WT- or kinase dead PKCζ-K281W, switched to normal Ca^{2+} for 6 h, fixed and double-stained for Myc and ZO-1. PKCζ-K281W disturbs the junctional localization of ZO-1 in WT keratinocytes whereas WT-PKCζ has no effect. WT-PKCζ fully restores junction maturation in Tiam1KO keratinocytes. Bar = 10 μm. **D** Rac1 activity assay on WT and Tiam1KO cells stably expressing myc-PKCζ-WT. HA-tagged Tiam1 was expressed in Tiam1KO keratinocytes **E** or in COS7 cells **(F and G)** by retroviral transduction and immunoprecipitated using anti-HA Ab. **E** FL-Tiam1 co-immunoprecipitates endogenous Par3 and aPKCζ from two independent infected populations of Tiam1KO cells that expressed HA-FLTiam1 and were cultured for 6 h at 1.8 mM Ca^{2+}. Empty-vector infected Tiam1KO cells were used as a control. **F** FL-Tiam1, but not a C-terminal Tiam1 mutant (C580), containing the GEF domain only, co-immunoprecipitates Par3 from COS7 cell lysates. **G** FL-Tiam1 co-immunoprecipitates exogenous WT- but not PKCζ-K281W from COS7 cell lysates. Immunocomplexes were immunoblotted using the indicated Abs.

controls the amount of TJ molecules, even under conditions where no TJs are formed. Interestingly, the expression of JAM-A was enhanced in Tiam1KO cells (Fig 4A). JAM-A triggers TJ biogenesis and membrane sealing (Liu et al., 2000), suggesting that Tiam1KO keratinocytes attempt to compensate the lack of Tiam1 by upregulating JAM-A.

Since Occludin and Claudins possess adhesive properties and might be involved in maturation of TJs upon their recruitment to primordial adhesions, we inhibited Occludin at the cell surface using an inhibitory peptide (Wong and Gumbiner, 1997). WT cells treated with the peptide for 6 h during the Ca^{2+} switch, showed still disorganized cell-cell contacts (Fig 4B) similarly as seen in Tiam1KO cells, suggesting that the maturation of "adhesion zippers" depends on the formation of TJs. Both ZO-1 and E-cadherin were distributed in a zipper-like manner in contrast to the normal linear organization in WT control cells. The reduced amounts of Occludin observed in Tiam1KO cells could thus be a limiting factor for junction maturation. However, overexpression of Occludin (Fig S3A), or of both Occludin and ZO1 (Fig S3C), did not lead to enhanced maturation of TJs in Tiam1KO cells. Apparently, Occludin (or an associated protein) is required for proper junction maturation, but its reduced expression nor that of ZO-1, is the cause of impaired TJ formation in Tiam1KO cells.

Tiam1 binds components of the polarity complex and activates Rac upon the formation of primordial adhesions

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

We investigated also the possibility that Tiam1 interacts with components of the polarity complex. FL-Tiam1 expressed in Tiam1KO cells was immunoprecipitated and endogenous PKCζ and Par3, but not Par6, co-immunoprecipitated with Tiam1 (Fig 4E and data not shown). In addition, we expressed Tiam1 mutants with either Par3 or various mutants of PKCζ in COS7-cells. FL-Tiam1, but not C580-Tiam1, co-immunoprecipitated withPar3 (Fig 4F). C580-Tiam1 lacks the putative protein interaction domain, consisting of the N-terminal PH domain and the flanking coiled-coil domain (Mertens et al., 2003), sug-

gesting that Tiam1 interacts with Par3 through one or more of these domains. Indeed, recently it was shown that Tiam1 directly interacts with Par3 through its PHn-CC domains (Nishimura et al., 2005). Tiam1 also co-immunoprecipitated PKCζ-WT, but not kinase-dead PKCζ(K281W), which contains a single mutation in the ATP binding site (Fig 4G). These data substantiate the specificity of the protein interactions and suggest that Tiam1 is preferentially associated with the active form of the polarity complex.

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

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

Tiam1-dependent activation of Rac in WT keratinocytes, followed the same kinetics as the formation of PAs (see Fig 2), suggesting that Tiam1 is a downstream target of Cadherins/Nectins to activate Rac and thereby to activate the polarity complex. Since activation of the polarity complex leads to activation of PKCζ, we immunoprecipitated PKCζ from keratinocyte lysates and determined its activation. As shown in Figure 5D, Tiam1KO cells showed less endogenous PKCζ-activity than WT cells as determined by auto-phosphorylation of PKCζ and the phosphorylation of Myelin Basic Protein (MBP). Moreover, introduction of FL Tiam1, V12Rac1 and V12Cdc42 into Tiam1KO cells restored TJ formation (Figs 3&5B) and also increased PKCζ-activity to the level equal or higher than found in WT cells (Fig 5D). To control for the specificity of the *in vitro* kinase assay and the PKCζ antibody used for the immunoprecipitation, we stably overexpressed

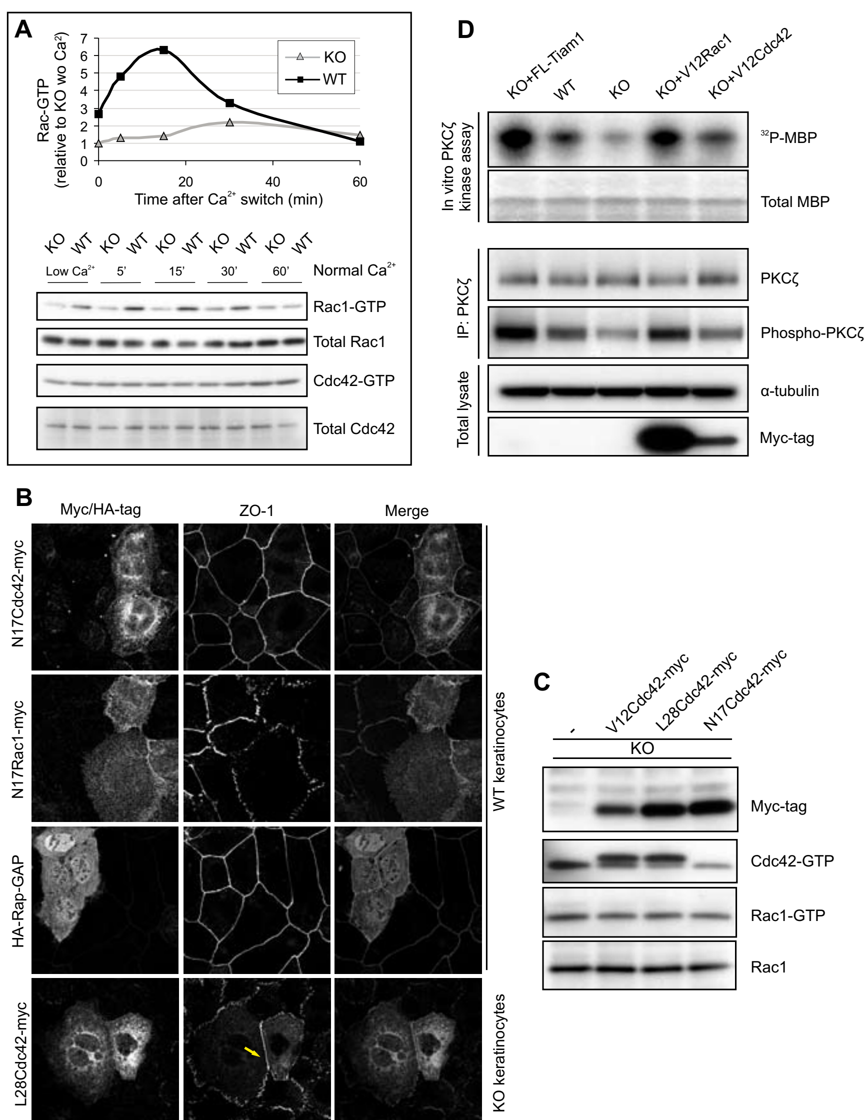

Figure 5. Tiam1 and Rac1, but not Cdc42 or Rap1, control the activity of the polarity complex and TJ formation. A Cadherin signaling transiently activates Rac in a Tiam1 dependentmanner. Rac and Cdc42 activity assays from WT and Tiam1KO keratinocytes after a Ca^{2+} switch for the indicated times. The graph represents the level of activated Rac (corrected for total Rac), relative to its activity in Tiam1KO cells cultured in low Ca^{2+}. **B** WT keratinocytes were transiently transfected with myc-N17Cdc42, -N17Rac1 or HA-Rap1GAP. Tiam1KO keratinocytes were transfected with myc-L28Cdc42. 48 h after transfection, cells were switched to normal Ca^{2+} for 6 h, fixed and double-stained for Myc or HA and ZO-1. Arrow indicates linearization of the TJ. **C** Cdc42 activity assay in Tiam1KO cells stably expressing myc-tagged L28, V12 or N17Cdc42 mutants. **D** Tiam1, Rac and Cdc42 can regulate PKCζ activity. Endogenous PKCζ was immunoprecipitated from WT, Tiam1KO and Tiam1KO cells expressing FL-Tiam1, V12Rac1 or L28Cdc42. PKCζ kinase activity was assayed *in vitro* using MBP as a substrate. Thr410-phosphorylation in the activation loop of PKCζ was analyzed as an alternative way to measure its activity. α-tubulin was used as a loading control. Representative example of 4 experiments.

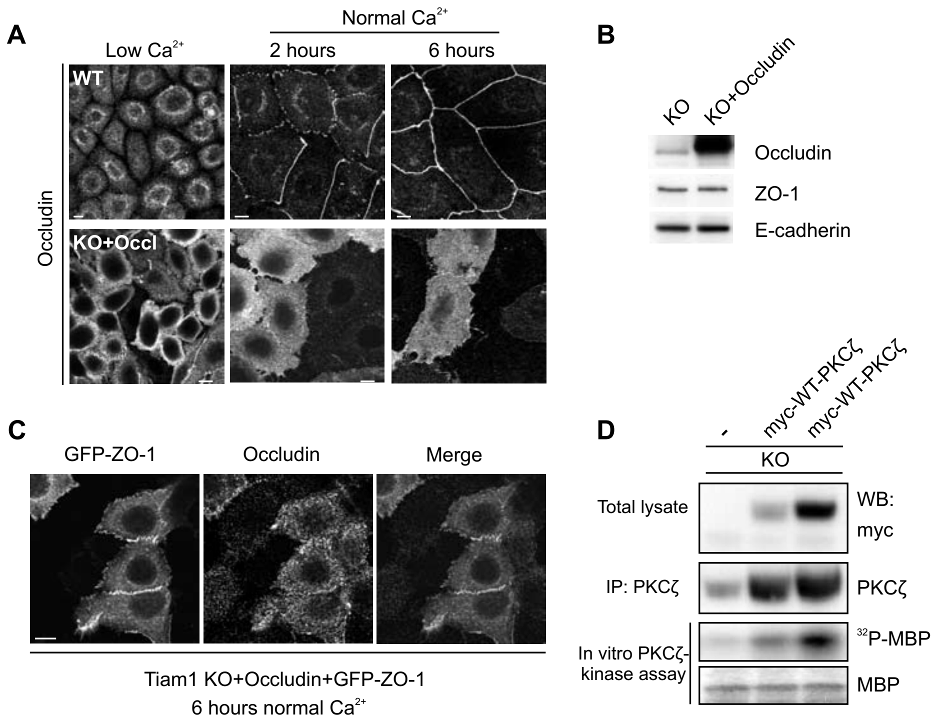

Figure S3. The expression of occludin and ZO-1 in Tiam1KO cells does not restore their capacity to form mature TJs 6 h after a Ca2+ switch. (A-C) Occludin and ZO-1 do not restore junction maturation in Tiam1KO keratinocytes. (A) Overexpression of occludin in Tiam1KO cells (blasticidin selected population) does not restore junction maturation. Cells were switched to normal 1.8 mM Ca^{2+} for the indicated time points and were stained for occludin. Bars, 10 µm. (B) Western blot showing the stable expression of occludin in TiamKO cells. In comparison, no differences were found in E-cadherin and ZO-1 expression. (C) Tiam1KO keratinocytes stably expressing occludin (as in A) were transiently supertransfected with pmt2SM-GFP-ZO1. Cells were switched to normal 1.8 mM Ca^{2+} for 6 h and were fixed and stained for occludin. The expression of both exogenous occludin and ZO-1 did not change the kinetics of junction maturation in Tiam1KO cells, as demonstrated by the presence of adhesion zippers, 6 h after the Ca^{2+} switch. Bar, 10 µm. (D) aPKCζ selectively phosphorylates MBP in vitro. In vitro phosphorylation of MBP clearly depends on the amount of PKC that is immunoprecipitated from kerinocyte cells lysates, which is indicative of the specificity of the assay. Tiam1KO keratinocyte cell lines stably expressing different levels of myc-tagged WT-PKCζ and the parental cell line were lysed and used for the in vitro kinase assay (as described in Immunoblotting assay).

different amounts of PKCζ-WT in Tiam1 KO keratinocytes. Indeed, MBP-phosphorylation turned out to be PKCζ-dose dependent (Fig S3D). Together, these data indicate that upon the formation of PAs, Tiam1-mediated Rac activation is required for activation of the polarity complex, leading to TJ maturation. Although Cdc42 and Rac1 differentially regulate the actin cytoskeleton, our data suggest that both pathways independently can converge to activate the polarity complex, presumably via Par6. Tiam1-Rac signaling, rather than Cdc42 activity, however predominantly regulates cell polarity in keratinocytes.

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

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

In conclusion, our data indicate that Tiam1-mediated activation of Rac - and not inactivation of Rac - is required for TJ formation in epithelial keratinocytes and that the concept that Tiam1-controlled activation of Rac is required for proper cell polarization holds true for both epithelial and neuronal cells. In addition, our data support a model in which Tiam1 and Rac function upstream of the polarity complex independently of Cdc42. Rac

is not activated in Tiam1KO cells that show impaired formation of TJs, suggesting that Tiam1 is required for local Rac activation upon the formation of PAs. This Rac activity is necessary for the activation of the polarity complex, which leads to activation of PKCζ and subsequently to TJ biogenesis.

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Summary and conclusions

SUMMARY AND CONCLUSIONS

The family of Rho GTPases consists of several small proteins that function as molecular switches regulating a wide variety of cellular signaling pathways. They control important cellular processes including cytoskeletal dynamics, cell migration, cell-cycle progression and gene expression. It is therefore not surprising that aberrant regulation of Rho GTPases can contribute to a wide range of diseases including cancer. The activity of Rho GTPases is controlled by three types of regulatory proteins that determine whether Rho proteins are bound to GDP or GTP. In the active form, when bound to GTP, Rho proteins can bind to their effector proteins and initiate downstream signaling. In the **first chapter** of this thesis Rho GTPases and their regulators are introduced. Furthermore, this chapter focuses on the involvement of Rho GTPases in the onset and progression of cancer. Most tumors are caused by genetic alterations such as the accumulation of DNA mutations which often lead to uncontrolled cell growth. Mutations are particularly found in oncogenes and tumor suppressor genes. So far no mutations have been found in the Rho GTPases suggesting that they do not play an essential role in tumor initiating events. Moreover, activating mutations in Rho GTPases are less effective in cell transformation than mutations found in their regulators. This suggests that Rho GTPases have to cycle between the GDP and GTP state in order to function efficiently in cell transformation. Recent studies indicate that Rho GTPases may contribute to various aspects of tumorigenicity including tumor initiation and progression. In particular, the expression levels of Rho GTPases may vary in tumors leading to increased or decreased Rho downstream signaling.

The best characterized members of the Rho GTPase family are: RhoA, Rac1 and Cdc42. They regulate a broad variety of cellular processes ranging from actin and microtubule cytoskeleton dynamics to related processes such as cell polarization, cell adhesion, cell migration, and vesicle transport. In addition, these proteins are involved in the control of gene transcription leading to cell proliferation or differentiation. One of the specific activators of Rac1 is Tiam1. Like other GEF proteins, Tiam1 stimulates the exchange of GDP for GTP, thereby activating Rac1. Activated Rac1 binds to downstream effectors generally serine/threonine kinases that trigger phosphorylation of other proteins or kinases, which exert than specific functions. However, the activation of Rac1 is not the only function of Tiam1, as GEFs may also associate with specific effector proteins or scaffold proteins thereby channeling the activation of Rac1 towards specific downstream signaling pathways. Thus, a specific GEF such as Tiam1 not only activates Rac1 but also determines the Rac1 downstream signaling.

Earlier *in vitro* studies established a role for Rac1 in Ras-induced transformation of fibroblasts. Rac1 is required for Ras-induced morphological transformation of cells and oncogenic Ras is able to activate Rac1. To test this hypothesis *in vivo*, we have studied Ras-induced skin tumorigenicity in Tiam1-deficient mice (Malliri et al *Nature* 417:867-871). Tumors were initiated with the Ras-activating carcinogen DMBA and subsequently tumor

growth was promoted by repeated treatments with the tumor promoter TPA. Interestingly, the involvement of Tiam1 in tumor onset and progression was more complex than initially anticipated. Tiam1-deficient mice produced much less skin tumors than control wild type mice and these tumors also showed low growth potential. However, the few tumors that did arise in the Tiam1-deficient mice appeared to be more malignant than tumors from wild type mice. Tiam1-Rac1 signaling seems to function in survival signaling which prevents apoptosis during tumor initiation. Tumor initiation is thus reduced in Tiam1-deficient mice as a result of increased apoptosis in the tumor initiated cells. With respect to tumor cell proliferation, we found that tumors formed in Tiam1-deficient mice and promoted with TPA were much smaller that tumors induced in wild type controls. This growth difference was not observed when tumor growth was promoted by multiple DMBA applications. Apparently TPA-induced tumor growth requires Tiam1. TPA induces Cyclin D1 expression (Yan et al *J Cell Physiol* 186:338-349) a cell-cycle promoting protein. Indeed, we found increased levels of Cyclin D1 in wild type tumors suggesting that Tiam1 is required for efficient TPA-induced Cyclin D1 production required for optimal cell proliferation. As discussed below, the increase in tumor progression by the lack of Tiam1 is most likely due to the fact that Tiam1 plays an essential role in the formation and maintenance of apico-basal cell polarity in epithelial cells. Lack of Tiam1 would thus lead to reduced cell-cell adhesions, loss of cell polarity and increased epithelial-mesenchymal transition, which are all hallmarks of tumor progression.

As Tiam1 appeared to play a role in various aspects of Ras-induced tumorigenicity, we wondered whether other oncogenic pathways required Tiam1-mediated Rac1-signaling. As most of the familiar and spontaneous intestinal tumors are driven by the activation of Wnt canonical signaling pathway we also addressed the possible involvement of Tiam1 in Wnt-induced intestinal tumorigenicity in mice. As discussed in **chapter two,** APC Min/+ mice, which are prone to develop intestinal tumors, were crossed with Tiam1-deficient mice. Similarly to what we found for Ras-induced skin tumors, Tiam1-deficient mice with an APC mutation developed fewer tumors with decreased growth potential when compared with control animals. In addition, these tumors showed increased invasion into tissues underlying the intestinal epithelium. Consistent with these *in vivo* observations, knock-down of Tiam1 in colon tumor cells resulted in inhibition of cell proliferation and disorganization of epithelial morphology by loss of E-cadherin-based cell-cell adhesions. We found that Tiam1 represents a Wnt responsive gene and that the expression of Tiam1 is increased in human and mouse intestinal tumors. The dependency of Tiam1 on Wnt signaling was demonstrated by inhibition of Wnt transcriptional activity, which led to a similar block in Tiam1 expression as found for other Wnt target genes such as c-Myc and Cyclin D1. This indicates that Tiam1 is indeed one of the genes controlled by the Wnt canonical signaling and Tcf/β-catenin transcriptional complex.

Thus, tumor initiation and progression induced by two completely different oncogenic pathways are affected by the presence or absence of Tiam1, but the mechanism by which Tiam1 contributes to tumorigenicity is completely different in these two tumor models. As mentioned above, Wnt signaling enchances Tiam1 expression whereas oncogenic Ras can activate Tiam1 by two independent pathways. The Ras-PI3-kinase pathway promotes Tiam1 activity, and Tiam1 in turn activates Rac1. In addition, activated Ras can bind Tiam1 directly; thereby connecting activated Ras via Tiam1 to the activation of Rac1.

We subsequently investigated the function of Tiam1 in tumorigenesis induced by additional oncogenic signaling pathways. As described in **chapter three,** we have analyzed the function of Tiam1 in mammary tumorigenesis induced by either the Neu/Her2 or the c-Myc oncogenes. In these studies, mice were used in which Neu/Her2 or c-Myc were expressed in the mouse mammary gland under the control of the Mouse Mammary Tumor Virus (MMTV) promoter. Intriguingly, Tiam1 affected Neu-induced tumorigenicity but not Myc-induced tumors. The effect of Tiam1 on the Neu-induced tumors was similar to the effects of Ras- and Wnt-driven tumors, where lack of Tiam1 decreased tumor initiation. However, in contrast to the Ras and Wnt tumors, Tiam1 deficiency did not affect tumor growth in Neu-induced tumors. As found in Ras- and Wnt-induced tumors, Tiam1-deficiency increased the sensitivity to apoptosis in the Neu-expressing tumors, thereby most likely influencing tumor initiation. Indeed, upon downregulation of Tiam1 we measured increased apoptosis sensitivity in human mammary tumor cells with high Neu expression.

Myc-induced mammary tumors appeared to be completely independent on the presence or absence of Tiam1 with respect to tumor initiation and tumor growth. Apparently, Myc-induced tumors do not require Tiam1/Rac1-mediated survival signaling to prevent apoptosis or Rac1-induced Cyclin D1 production to promote proliferation. Thus Tiam1 contributes to tumorigenicity induced by different but not all oncogenic signaling pathways.

The use of Tiam1-deficient mice in combination with mouse tumor models proved to be a powerful technique to investigate the function of Tiam1 in various aspects of tumorigenicity. **Chapter four** describes detailed experimental methods that can be used to study the function of specific genes in tumorigenesis. Focus is on the chemical carcinogenesis protocol in the skin in which the tumor initiator DMBA and the tumor promoter TPA are used. This approach allows studying different aspects of tumorigenicity including the initiation, promotion, and progression of tumors. In addition, we described methods to isolate cells from solid mouse tumors as well as healthy tissues; these tissues include embryonic fibroblasts and primary keratinocytes. Furthermore, we discussed experimental protocols to investigate the transformation and apoptosis sensitivity of cells *in vitro*.

As described above, Tiam1 is involved in the regulation of cell survival and apoptosis sensitivity especially in the context of cell transformation. **Chapter five** describes the molecular mechanisms by which Tiam1 influences apoptosis sensitivity. We have made use of keratinocytes isolated from wild type and Tiam1 knock-out mice. We found that

Tiam1-deficient keratinocytes showed increased sensitivity to apoptosis induced by stress conditions such as growth factor deprivation and heat shock treatment. Decreased ERK activation (phosphorylation) in Tiam1-deficient cells was responsible for the increased apoptosis sensitivity. Interestingly, ERK activation was dependent on cellular ROS production. Tiam1 regulates the sensitivity of keratinocytes for apoptosis by targeting Rac1 activity towards ROS production and ERK activation. Rac1 can stimulate ROS production by activation of Nox family enzymes. This Rac-ROS-ERK-mediated anti-apoptotic pathway could thus support cell survival during tumor initiation. Deregulation of cellular signaling caused by oncogenic activation often triggers apoptosis and inhibition of apoptosis in tumor initiated cells may therefore promote tumor formation. Importantly, a similar signaling cascade was recently described for the viral protein Tat, another stress factor which triggers an anti apoptotic pathway (Wu et al *J Biol Chem* 282:37412-37419). Tat induces activation of Rac1 downstream of Nox4 and ROS production which in turn induces ERK activation and survival of endothelial cells.

De-differentiation of epithelial cells into migratory mesenchymal cells is part of normal organogenesis and is called Epithelial Mesenchymal Transition (EMT). During tumor progression, tumor cells may also undergo EMT which is characterized by the loss of apico-basal cell polarity, cell-cell contacts and the acquisition of migratory potential. Thus, proteins that regulate cellular polarity may also control EMT of tumor cells. **Chapter six** of this thesis describes the involvement of Tiam1 in the establishment of epithelial cell polarity by controlling tight junction (TJ) formation. We found that loss of Tiam1 impairs the formation of functional TJs. Tiam1 binds to Par3, one of the components of the Par-polarity complex. By activation of Rac1, Tiam1 triggers the Par complex leading to the activation of PKCζ, the effector protein of the Par-complex. Indeed, Tiam1-deficiency leads to weakened E-cadherin-based cell-cell adhesions and promotes EMT and cell migration. Tiam1, in conjunction with the Par-polarity complex, controls also other aspects of cell polarity such as front-rear polarity and hence persistent migration in lymphoid and epithelial cells (Gerard et al., *J Cell Biol* 176:863-875, 2007)(Pegtel et al., *Curr Biol* 17:1623-1634, 2007). The presence of Tiam1 may thus promote apical basal cell polarity and thereby prevent EMT and tumor cell migration. On the other hand, in single epithelial and lymphoid cells, Tiam1 promotes front-rear cell polarity and thereby enhances persistent migration of these cells.

In conclusion, we found that specific oncogenic signaling pathways collaborate with the Tiam1/Rac1 signaling pathway in various aspects of tumorigenicity including tumor initiation, tumor growth, and tumor progression. Tumor initiation is influenced by the effects of Tiam1/Rac1 signaling on apoptosis sensitivity, whereas tumor growth is influenced by the effects of Tiam1/Rac1 signaling on cell proliferation through Cyclin D1 production. Tumor progression can be influenced by the function of Tiam1/Rac1 signaling in various aspects of cell polarization including apical basal and front-rear polarization. The latter may influence EMT and cell migration and thereby tumor progression and metastasis.

Appendices

SAMENVATTING

De Rho GTPases omvatten een familie van kleine eiwitten die functioneren als moleculaire “schakelaars”. Zij kunnen verschillende signaaltransductieroutes in de cel activeren en regelen belangrijke cellulaire processen waaronder de dynamiek van het cytoskelet, hechting, migratie, proliferatie en ook de expressie van genen in cellen. Omdat Rho GTPases zoveel cellulaire processen reguleren is het niet verwonderlijk dat defecten in de regeling van de activiteit van deze eiwitten kunnen bijdragen aan het ontstaan van uiteenlopende ziektes, waaronder kanker.

In het **eerste hoofdstuk** van dit proefschrift wordt een introductie gegeven over de Rho GTPases en hun regulatoren. Hierbij concentreren we ons op literatuurgegevens over de rol van deze eiwitten bij het ontstaan en de progressie van kanker. De best gekarakteriseerde leden van de familie van Rho GTPases zijn drie eiwitten: RhoA, Rac1 en Cdc42. Het invasie-inducerende gen Tiam1 codeert voor een specifieke activator van het Rac1 eiwit. Net als andere activators van Rho eiwitten stimuleert Tiam1 de uitwisseling van GDP voor GTP, waardoor Rac1 geactiveerd wordt. In de geactiveerde status kunnen weer andere eiwitten aan Rac1 binden (effector eiwitten) waarmee de signaaltransductieroute wordt aangezet.

In eerdere studies is gevonden dat Rac1 een rol speelt bij de cellulaire transformatie door het Ras oncogen. Rac1 is nodig voor Ras-geïnduceerde morfologische transformatie van cellen en het oncogen Ras is in staat Rac1 te activeren. Tiam1 speelt een prominente rol in de initiatie en progressie van Ras-geïnduceerde huidtumoren in de muis. Naar aanleiding van deze bevinding vroegen wij ons af of Tiam1, door middel van Rac signalering, dezelfde effecten heeft op de tumorgenese geïnduceerd door verschillende andere oncogenen.

Ongeremde activiteit van de Wnt-signaaltransductieroute is een van de grootse oorzaken van familiaire en spontane darmtumoren in de mens. Om een eventuele functie van Tiam1 in Wnt-geïnduceerde tumorgenese te bestuderen maakten wij gebruik van APC^{Min} muizen, welke een verhoogde gevoeligheid hebben voor de vorming van darmtumoren door een continue activiteit van de Wnt signaaltransductieroute. Zoals bediscussieerd in **hoofdstuk twee** hebben we gevonden dat Tiam1-knockout muizen, onder invloed van verhoogde Wnt-signalering, zowel minder tumoren als tumoren met een verminderde groeipotentiaal vertonen in vergelijking met controle muizen. Echter, de Tiam1-knockout tumoren infiltreerde in onderliggende weefsels van het darmepitheel, in tegenstelling tot de tumoren in controlemuizen. Tiam1 kan dus verschillende stadia van de tumorgenese beïnvloeden. Daarnaast bleek de expressie van het Tiam1-gen direct (en positief) te worden beïnvloed door de activatie van de Wnt signaaltransductieroute.

In **hoofdstuk drie** beschrijven we de functie van Tiam1 in het ontstaan en de verdere ontwikkeling van borsttumoren onder invloed van de oncogenen Neu/Her2 en c-Myc.

Voor deze studies gebruikten wij transgene muizen, welke Neu/Her2 of c-Myc specifiek in de borstklier tot expressie brengen. Hieruit bleek Tiam1 wel een effect op Neu- maar niet op c-Myc-geïnduceerde tumoren te hebben. Dit effect was gedeeltelijk vergelijkbaar met voorgaande studies van Ras- en Wnt-geïnduceerde tumoren (respectievelijk in de huid en de dunne darm), waarin de afwezigheid van Tiam1 zowel tumorinitiatie als -groei remde. Zoals in Ras- en Wnt-geïnduceerde tumoren leidde de afwezigheid van Tiam1 in Neu-geïnduceerde tumoren tot een verhoogde gevoeligheid voor gereguleerde celdood (apoptose). We vonden echter geen defect in de groei van deze Tiam1-deficiënte Neu-tumoren, dit in tegenstelling tot de Ras- en Wnt-modellen. De verhoogde gevoeligheid voor apoptose in Tiam1-knockout cellen hangt nauw samen met het defect in tumorinitiatie, zoals we die in de verscheidene muismodellen hebben geconstateerd. Opmerkelijk was verder de bevinding dat initiatie, groei en progressie van c-Myc geïnduceerde borsttumoren volledig onafhankelijk is van Tiam1. Kortom, sommige maar niet alle oncogene signaaltransductieroutes werken samen met Tiam1 en Rac1.

Het gebruik van knock-out muizen in combinatie met muis-tumormodellen maakt het mogelijk om de verschillende aspecten van tumorgenese te bestuderen. In **hoofdstuk vier** worden experimentele technieken beschreven die gebruikt kunnen worden om de functies van specifieke genen tijdens tumorgenese te onderzoeken. De nadruk ligt hierbij op een protocol waarin carcinogenese op een chemische manier in de huid geïnduceerd wordt, door gebruik te maken van de tumorinitiërende stof DMBA en de tumorstimulerende stof TPA. Daarnaast zijn methodes beschreven om zowel tumorcellen te isoleren uit tumoren in de muis, als normale specifieke celtypen.

In **hoofdstuk vijf** worden de moleculaire mechanismen beschreven waarmee Tiam1 de gevoeligheid voor apoptose, en daarmee tumorinitiatie, beïnvloedt. We hebben ontdekt dat keratinocyten zonder expressie van Tiam1 een verhoogde gevoeligheid voor apoptose vertonen, wanneer deze geïnduceerd wordt door stress omstandigheden, zoals groeifactor deprivatie en thermische schok. Deze toename in gevoeligheid voor apoptose werd veroorzaakt door een afname van ERK activering (fosforylering) in deze Tiam1-deficiënte cellen. Opmerkelijk is dat de stress-geïnduceerde activatie van ERK afhankelijk is van Tiam1-Rac gemedieerde productie van Reactive Oxygen Species (ROS) in cellen. Tiam1 reguleert dus de apoptose-gevoeligheid van keratinocyten door Rac1 activiteit te richten op ROS-gemedieerde ERK activering, welke als overlevingssignaal fungeert tijdens condities van stress.

De-differentiatie van epitheliale cellen tot migrerende mesenchymale cellen wordt Epitheliale naar Mesenchymale Transitie (EMT) genoemd en is noodzakelijk voor ontwikkeling en organogenese. Epitheliale tumorcellen ondergaan een soortgelijke EMT tijdens tumorprogressie en metastasering. EMT van tumorcellen wordt gekarakteriseerd door het verlies van cel-cel contacten en van apicale-basale celpolariteit. Tegelijkertijd ontwikkelen deze cellen capaciteiten om te migreren. Eiwitten die celpolariteit reguleren kunnen dus

ook EMT van tumorcellen reguleren. In **hoofdstuk zes** van dit proefschrift wordt beschreven hoe we de rol van Tiam1 hebben onderzocht in de regulatie van bepaalde cel-cel contacten (Tight Junctions (TJs)) bij het ontstaan van epitheliale celpolariteit. We zagen dat verlies van Tiam1-expressie de formatie van functionele TJs en Adherens Junctions (AJs) in epitheliale cellen beperkt. Tiam1 kan binden aan Par3, een van de componenten van het Par-polariteitscomplex. Tiam1-gemedieerde Rac1 activiteit is noodzakelijk om het Par complex te activeren door PKCζ te activeren, het effector eiwit van het Par complex. Onderbreking van deze polariteitsignalering door verstoorde Tiam1/Rac1 signalering kan de verbinding tussen epitheliale tumorcellen verzwakken en daardoor EMT veroorzaken en dus ook tumorprogressie.

Samenvattend hebben we gevonden dat specifieke oncogene signaleringsroutes samenwerken met de Tiam1/Rac1 signaleringsroute in processen als tumorinitiatie en tumorgroei, als gevolg van Tiam1/Rac1 gestuurde effecten op respectievelijk gevoeligheid voor apoptose en celproliferatie. Tiam1 beïnvloedt tumorprogressie door zijn rol in de verschillende aspecten van polariteitsignalering. Door te binden aan het Par complex kan Tiam1/Rac1 signalering apicale-basale polariteit van cellen met onderlinge celcontacten alsmede asymmetrie tussen voor- en achterzijde van migrerende cellen reguleren. Beiden processen kunnen EMT en celmigratie beïnvloeden en daardoor tumorprogressie en metastasering van tumorcellen.

Streszczenie

Rodzina małych Rho GTPaz złożona jest z kilku białek, które spełniają role molekularnych przełączników w komórkowych szlakach sygnalnych. Rho GTPazy kontrolują tak różnorodne procesy komórkowe jak: dynamikę cytoszkieletu, migracje, cykl komórkowy czy ekspresje genów. Nieprawidłowa kontrola aktywności Rho GTPaz może przyczynić się do szeregu chorób łącznie z nowotworami.

W **pierwszym rozdziale** tej pracy przedstawione zostały podstawowe informacje o Rho GTPazach w procesie powstawania i rozwoju nowotworów. Najlepiej scharakteryzowanymi przedstawicielami Rho GTPaz są trzej członkowie rodziny: RhoA, Rac1 i Cdc42. Tiam1 jest specyficznym aktywatorem malej GTPazy Rac1. Podobnie jak inne aktywatory Rac1 dokonuje tego przed wymianę GDP na GTP, zmieniając strukturę i stan aktywności Rac1, co z kolei prowadzi do aktywacji kaskady efektorowych-białek zależnych od Rac1.

W naszych wcześniejszych badaniach dowiedliśmy roli Rac1 w transformacji komórkowej zależnej od Ras. Rac1 jest wymagany do morfologicznych zmian komórek wywoływanych przez Ras, ponadto zmutowana i aktywna forma Ras może stymulować Rac1. W badaniach in vivo stwierdziliśmy, że Tiam1 odgrywa rolę zarówno podczas indukcji jak i rozrostu nowotworów zainicjowanych przed aktywacje Ras. W związku z tym, postanowiliśmy zbadać czy zależne od Tiam1 sygnały komórkowe kontrolowane przez Rac1 wpływają na rozwój nowotworowy zapoczątkowany przez inne onkogeny. Aktywacja ścieżek sygnalnych zależnych od Wnt jest jedną z głównych przyczyn powstawania wrodzonych bądź spontanicznych nowotworów jelita. Żeby zbadać role Tiam1 w nowotworzeniu zależnym od Wnt, użyliśmy szczepu myszy APC Min/+. Z powodu konstutywnej aktywacji ścieżki sygnalnej Wnt, szczep ten charakteryzuje się wrodzoną tendencją do rozwoju nowotworów jelita. W **rozdziale drugim** tej pracy przedstawiamy dowody na to, że myszy pozbawione genu Tiam1, a posiadające aktywna ścieżkę sygnalna Wnt, rozwijają mniej wolno-rosnących nowotworów jelitowych w porównaniu do kontrolnych myszy. Jednakże, nowotwory powstałe w myszach bez Tiam1 odznaczały się większym potencjałem inwazyjności do przyległego nabłonka jelitowego. Ponadto, ekspresja Tiam1 pozytywnie korelowała z wysoką aktywnością ścieżki sygnalnej Wnt. Podsumowując, ekspresja Tiam1 regulowana jest przez ścieżkę sygnalną Wnt, co wpływa na różne aspekty nowotworzenia.

Rozdział trzeci przedstawia analizę funkcji Tiam1 w procesie rozwoju nowotworów piersi aktywnie indukowanych przez onkogeny Her2/Neu lub Myc. W tych eksperymentach aktywne formy Her2/Neu lub Myc zostały eksprymowane w mysim gruczole piersiowym. Brak Tiam1 zmniejszył liczbę nowotworów powstałych przez ekspresje Her2/Neu, co jest zgodne z zaobserwowanym wcześniej wpływem na nowotwory indukowane przez Ras i Wnt. Jednakże w przeciwieństwie do Ras i Wnt brak Tiam1 nie wpłynął na proliferacje komórek nowotworów indukowanych przez Neu, co za tym idzie rozrost nowotworów. Jednakże, tak jak wcześniej zanotowano dla Ras i Wnt, brak Tiam1 zwiększył podatność komórek z Neu na apoptozę. Co więcej, downregulacja Tiam1 w komórkach raka piersi z wysoką ekspresją Her2, zwiększyła liczbę apoptotycznych komórek. Nowotwory indukowany przez ekspresję Myc wydają się być niezależne od obecności Tiam1.

Appendixes

Użycie mysich knock-out'ów, np.: myszy bez genu Tiam1, w połączeniu z mysimi modelami nowotworowymi, jest cenną techniką w badaniach nad funkcją specyficznych genów w procesie nowotworzenia. **Rozdział czwarty** opisuje w szczegółach techniki, które mogą być zastosowane w badaniach konkretnych genów w kontekście nowotworzenia. Nacisk został położony na procedurę chemicznego indukowania nowotworów skóry przy użyciu związków chemicznych DMBA i TPA. Ponadto, opisaliśmy także metody izolacji komórek nowotworowych oraz normalnych komórek z tkanek mysich.

Jak wspomniano powyżej, Tiam1 bierze udział w regulacji podatności komórek na apoptozę, w szczególności w kontekście nowotworowej transformacji komórkowej. **Rozdział piąty** opisuje molekularne mechanizmy, dzięki którym Tiam1 kontroluje wrażliwość komórek na apoptozę. Zaobserwowaliśmy, że keranocyty pozbawione Tiam1 wykazują zwiększoną podatność na apoptozę powodowaną przez stres komórkowy jak: pozbawianie czynników wzrostu czy szok termiczny. Za zwiększoną apoptozę komórek pozbawionych Tiam1, odpowiedzialny jest niski poziom aktywacji ERK. Co ciekawe, odkryliśmy, że aktywacja ERK jest zależna od wewnątrzkomórkowej produkcji wolnych rodników tlenowych (ROS). Zatem Tiam1 reguluje podatność keranocytów na apoptozę przez ukierunkowanie aktywności Rac1 na produkcje ROS i aktywacje ERK.

Odróżnicowanie się komórek nabłonkowych w migrujące komórki mezenchymalne jest częścią normalnego płodowego procesu rozwojowego i nazywane jest tranzycją epitelialno-mezenchymalną (EMT). Podczas rozwoju nowotworów o pochodzeniu nabłonkowym, komórki przechodzą podobny proces jak EMT. Charakteryzuje się on utratą polaryzacji komórkowej i zanikiem kontaktów międzykomórkowych, w połączeniu z nabyciem zdolności do migracji. Zatem białka regulujące polaryzację komórek mogą też wpływać na EMT komórek nowotworowych. **Rozdział szósty** opisuje udział Tiam1 w regulacji polaryzacji komórek nabłonkowych a szczególności tworzenia ścisłych połączeń międzykomórkowych (TJ). Zaobserwowaliśmy, że utrata Tiam1 hamuje tworzenie funkcjonalnych TJ. Tiam1 wiąże się do Par3, jednego z komponentów białkowego kompleksu polaryzacji-Par. Przez aktywację Par3 Tiam1 stymuluje kompleks-Par, co prowadzi do aktywacji kinazy PKCζ, kontrolowanej przez ten kompleks.

Podsumowując, wykazaliśmy, że niektóre onkogenne szlaki sygnalne współdziałają ze szlakiem Tiam1/Rac1 w procesie powstawania i rozwoju nowotworów. Dzieje się to poprzez modyfikacje podatności na apoptozę a także zwiększenie proliferacji komórek. Tiam1 może mieć również wpływ na rozwój, już zapoczątkowanych nowotworów przez swoją role w procesie polaryzacji komórkowej. To z kolei może odbić się na EMT, migracji komórek a co za tym idzie przerzutach nowotworowych.

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Curriculum vitae

Tomasz Rygiel was born on 19th of October 1978 in Jasło, Poland. From 1992 to 1997 he attended the Mikołaj Kopernik High School in Krosno. In 1996 and 1997, he took part in the National Biology Olympiad and in 1997 he was laureate of the XXVI National Biology Olympiad. In 1997, he started his studies at the Faculty of Biology at the Warsaw University. As a student he was a member of Molecular Biology Students Association and the Field Biology Students Association. He was engaged in the organization of scientific workshop during the Warsaw Science Festival in 2000 and 2001. In 2001, he also organized the IX OAK – International Workshop in the Scientific Presentation in Murzynowo. In 2002, thanks to the Erasmus Student Exchange Program, he conducted a 5-month research project at the University of Amsterdam at the Swammerdam Institute for Life Sciences. Under the supervision of Dr. Hans van der Spek he was involved in research concerning the structure of yeast mitochondrial chaperons Phb1 and Phb2. The same year he graduated at the Warsaw University with specialization in molecular biology. In December 2002, he started his graduate research project at the Division of Cell Biology in the Netherlands Cancer Institute. Under the supervision of Dr. J. G. Collard and Prof. Dr. A. J. M. Berns he conducted research focused on the role of the Rac1-activator Tiam1 in the regulation of cell signaling and in tumor onset and progression. In 2007, he joined the group of Prof. Dr. Linde Meyaard at the Department of Immunology at University Medical Center Utrecht, for his postdoctoral research. His current research is focused on the immune-regulation during anti-viral and anti-tumor immunological responses.

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