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Hajdo-Milašinovi, A.

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General introduction

Rho GTPases in cell motility and tumorigenesis

Amra Hajdo-Milašinović, Alexander E. Mertens, Irene H. L. Hamelers, John G. Collard

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RHO GTPASES IN CELL MOTILITY AND TUMORIGENESIS

Amra Hajdo-Milašinović, Alexander E. Mertens, Irene H. L. Hamelers, John G. Collard

The Netherlands Cancer Institute, Department of Cell Biology, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Abstract
Rho proteins are small regulatory molecules that belong to the Ras superfamily of proteins. They act as molecular switches, shuffling between an active, GTP-bound state, and inactive GDP-bound state. Upon activation, they interact with a multitude of downstream effectors. In this way Rho proteins regulate a broad range of cellular processes, including cell motility, growth, apoptosis, gene transcription and others. Therefore, it is not surprising that Rho proteins are shown to be involved in different aspects of tumorigenesis. In particular, as key regulators of cell motility, Rho GTPases are implicated in invasion and metastasis of a tumor. In this paper we will focus on the involvement of Rho proteins in cell migration and their involvement in the different steps of tumorigenesis.

Introduction
Similar to Ras proteins, Rho GTPases bind GTP/GDP and cycle between active GTP- and inactive GDP-bound state. In contrast to Ras oncogenes, Rho proteins have never been found mutated in human tumors; however, their involvement in cancer development has been clearly established and is an active area of research. Rho GTPases are ubiquitously expressed and so far 20 Rho proteins have been described in humans (1). Based on primary sequence and known functions, they can roughly be divided into 5 groups, being the Rho-like, Rac-like, Cdc42-like, Rnd, and
RhoBTB subfamilies (Figure 1). Of these 20 members, RhoA/B, Rac1/2 and Cdc42 are the most widely studied. Rho proteins are involved in the regulation of many cellular processes, including cytoskeletal organization, cell motility, cell cycle progression and growth, apoptosis, gene transcription and vesicle transport. Consequently, it is not surprising that Rho proteins were found to be involved in all the different steps of tumor development. In this chapter, we describe in more detail how the Rho proteins are regulated, what their role is in cell migration and maintenance of cell-cell adhesions. In addition, we summarize the data that implicate this protein family in cancer development.

**Figure 1**: Rho protein family tree. Based on sequence homology and function, the 20 Rho protein family members are divided into 5 groups: Rho-like, Rac-like, Cdc42-like, Rnd and RhoBTB.
1. Regulation of Rho GTPase activity

Rho proteins cycle between an active, GTP-bound conformation and an inactive, GDP-bound conformation. In the GTP-bound form, they interact with downstream target proteins to induce cellular responses. Rho proteins exchange nucleotide and hydrolyse GTP at slow rates. These reactions are catalysed by guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively (Figure 2).

![Figure 2: Regulation of RhoGTPase function. Rho GTPases shuttle between an active and an inactive state. RhoGDIs keep Rho proteins in a GDP-bound inactive state. GEFs and GAPs regulate the GDP release and the GTP hydrolysis, respectively.]

The guanine nucleotide exchange factors (GEFs) promote the exchange of GDP for GTP, in this fashion activating the GTPase. RhoGEFs are generally large, multidomain proteins that typically contain a Dbl homology (DH) domain (2) which catalyses GDP-GTP exchange. The DH domain is often flanked by pleckstrin homology (PH) domain. The DH-PH domain units show varying specificities for subsets of Rho family proteins (3). There are many more GEF proteins than there are Rho proteins; so far, about 60 GEFs have been identified in the mammalian genomes (4). This diversity probably represents the engagement in a wide variety of signaling pathways in different tissues. In addition, RhoGEFs are not just simply activators of Rho proteins, but they also serve as docking sites for adaptor proteins and
downstream effector proteins of Rho-like GTPases (reviewed in 5). This additional feature promotes RhoGEFs into tools for fine-tuned spatial and temporal regulation of Rho GTPases and their downstream signaling.

GTPase-activating proteins (GAPs) stimulate the rate of GTP hydrolysis, thereby converting GTPases to their inactive, GDP-bound form. So far, there are about 70 proteins identified that contain a RhoGAP domain (6). Apart from their function to inactivate Rho GTPases there is evidence that some GAPs also act as Rho effectors and transmit signals downstream from Rho proteins.

The third group of Rho family regulators is known as the guanine-nucleotide dissociation inhibitors (GDI). GDIs bind a subset of Rho proteins, inhibit nucleotide exchange and prevent the binding of these proteins to the membranes, where they are activated. To date, three RhoGDIs have been identified, and a few other molecules have been suggested to have RhoGDI activity. A large fraction of Rho proteins in the cell is likely to be bound to GDIs (7). How RhoGDIs interact with Rho proteins is known from structural and biochemical studies (8,9), but it is still unclear how this interaction is regulated. GDI phosphorylation and the binding of other proteins to GDI can stimulate release of Rho GTPases from GDIs. In this way, Rho proteins can be delivered by the GDIs to a particular site of action in the cell.

The localization of Rho proteins is regulated by lipid modifications. Rho GTPases are generally post-translationally modified at their C-termini by prenylation of a conserved cysteine, and this is a determinant for targeting these proteins to the plasma membranes (10).

Various tools have been employed to analyse Rho protein function. Two types of point mutants have been used extensively: activated mutants, which are constitutively GTP-bound (the GAP proteins can not bind anymore) and dominant-negative mutants, which generally have reduced affinity for nucleotides (11) and are titrating out GEFs (12). Bacterial toxins that inactivate Rho proteins have also been used to study Rho function (reviewed in 13). A prototypical agent is C3 exotransferase, produced by Clostridium botulinum. C3 (ADP-)ribosylates RhoA, B, and C leading to the inactivation of these proteins (14). More recently, the
downregulation of various Rho proteins by short interference RNA sequences have become a powerful tool to study the specific function of Rho proteins.

2. Function of Rho GTPases
The most intensively investigated function of Rho proteins is their involvement in the regulation of the cytoskeletal organization in response to extracellular signals. However, over the past few years it has been shown that Rho GTPases also play crucial roles in many other cellular events, such as membrane trafficking, transcriptional regulation, apoptosis, cell polarization, cell growth control, and cell differentiation. Within the scope of this chapter we will discuss in particular the involvement of Rho proteins in cell migration and cell adhesion, processes associated with the formation and progression of tumors.

2.1 Rho GTPases in migration
Cell migration is essential to normal development of multicellular organisms and plays a crucial role in morphogenesis throughout embryonic development. Cells migrate from epithelial layers to end up in locations where they start to differentiate and form specialized tissues and organs. Migration is also a prominent component of tissue repair and immune protection. It is not surprising that migration is a crucial factor in many pathological processes as well. Vascular diseases, osteoporosis, chronic inflammatory diseases, mental retardation and cancer correlate tightly with impaired, deregulated or intensified migratory capacity of cells. So, understanding the mechanisms underlying cell migration might potentially lead to more effective therapeutic approaches for treating disease.

Cells start to migrate in response to different extracellular cues (e.g., growth factors, signals on neighboring cells, or signals from the extracellular matrix) that act either as attractants or repellents. This evokes signaling cascades within the cell that lead to cytoskeletal changes, cell-substrate adhesions, loss of cell-cell adhesions and other processes that are required for forward movement.

Cell migration can be seen as a cyclic process, consisted of four mechanistically separated steps (15). Firstly, in response to migration-promoting agent, cells start to polarize and extend a leading edge protrusions or lamellipodium in the direction of migration. These protrusions are dependent on actin polymerisation. Secondly, the
new adhesions are being established at the front of the cell, serving also as traction sites for migration as the cell move over them. Subsequently, the contraction of the cell body occurs, and finally, the adhesions at the cell rear become detached.

During the migratory processes, Rho proteins regulate the changes in cell adhesion and the actin cytoskeletal changes during cell migration (Figure 3). In fibroblasts, Rho can be activated by the addition of extracellular ligands and activated Rho leads to the assembly of contractile actin-myosin filaments (stress fibers) and of associated focal adhesion complexes. Rac is activated by a distinct set of agonists (different growth factors, insulin), and activated Rac induces the assembly of a meshwork of actin filaments at the cell periphery to produce lamellipodia and membrane ruffles. Furthermore, activation of Cdc42 induces actin-rich surface protrusions called filopodia. Other processes regulated by Rho GTPases that are important for cell migration, are focal adhesion complex formation and turn-over, the establishment of cell polarity, microtubule dynamics, vesicular transport pathways and the signaling up- and downstream of integrins. Rather then exploring these functions one by one, we will discuss the various steps of migration together with the Rho protein function in every particular step.

**Figure 3:** Steps in cell migration. Cdc42, along with Par proteins and aPKC, are involved in the generation of polarity. The migration cycle begins with the formation of a protrusion (filopodia and lamellipodia). Rac and Cdc42 and other signaling pathways regulate the formation of actin branches. Protrusions are stabilized by the formation of adhesions. This process requires integrin activation, clustering, and the recruitment of structural and signaling components to nascent adhesions. At the cell rear, adhesions disassemble as the rear retracts. This process is mediated by Rho.
2.1.1 Cell polarization

In order to migrate, a cell must be polarized, meaning that different molecular processes must define the front and the rear of the cell. A key player in regulation of cell polarity is Cdc42. The GTPase Cdc42 is active toward the front of migrating cells (16), and both inhibition and over-activation of Cdc42 can disrupt directionality of migration (17). Cdc42 restricts the area where the lamellipodia can be formed (18), and it also affects polarity by localizing the microtubule-organizing center (MTOC) in front of the nucleus, oriented toward the leading edge (Figure 2) (17,19). The effects of Cdc42 on MTOC position appear to involve the Cdc42 effector PAR6, which forms a complex with PAR3 and an atypical protein kinase C (aPKC) (20,21). An additional role of Cdc42 in cell migration is its role in filopodia formation, by initiating the actin polymerisation. These protrusions are formed in the process of cell polarization and are required for the direction sensing in many different cell types (22,23). Cdc42 is able to stimulate actin polymerization via its interaction with WASp and N-WASp, leading to activation of the Arp2/3 complex that mediates actin filament assembly. For proper cell polarization, a tight regulation of the microtubule dynamics is needed, which involve not only Cdc42 (24,25) but also both Rac (24,25), and Rho (26-29).

2.1.2 Lamellipodia

Lamellipodia are protrusive structures generated at the leading edge of migrating cells. They consist of branched actin filament networks formed through the actin-nucleating activity of the Arp2/3 complex (30). Through localized activation of the Arp2/3 complex, the lamellipodia are induced to grow in a particular direction, providing the basis for directional migration. Activation of the Arp2/3 complex occurs by WASp/WAVE family members, which are downstream effectors of Cdc42 and Rac respectively (31).

Rac is required for lamellipodium extension induced by growth factors, cytokines and extracellular matrix components and inhibition of Rac activity impairs cell migration (22,23,32). Activated Rac is localized preferentially towards the front of migrating cells (33). A major protein that can activate Rac at the leading edge is phosphoinositide 3-kinase (PI 3-kinase) that is activated either via tyrosine kinases or
G-protein-coupled receptors (34-36). The products of PI 3-kinase, PtdIns (3,4,5) P3 / PtdIns (3,4) P2 (PIP3/PIP2), appear to be enriched at the leading edge of migrating cells (37-39). Tiam1 and Vav2 are PIP3-responsive GEF proteins localised at the leading edge of cells. They are likely candidates for regulating local Rac activity during directed cell migration (34,40,41).

A downstream effector of Rac important for cytoskeletal rearrangements and membrane ruffling is p21-activated kinase (PAK). Both Rac and Cdc42 activate PAK; its activation promotes formation of lamellipodia (42) and leads to the loss of stress fibers and focal adhesions (43). Via PAK, Rac has been reported to stimulate the activity of LIM-kinase (44,45), which phosphorylates and inactivates cofilin, a protein that promotes actin depolymerization (45). Thus Rac is able to inhibit cofilin-induced depolymerization.

In addition, myosins have been implicated in cell migration (46), and Rac can affect the phosphorylation of both myosin II heavy chain (MHC; (47)) and myosin light chain (MLC) via PAK (48-50). Phosphorylation of the myosin heavy chain by PAK inhibits myosin function and causes the disassembly of actomyosin structures (47). In addition to myosins, several other targets downstream of Rac and PAK have been implicated in actin reorganisation, including the actin binding protein filamin, the paxillin/Pix/PKL complex, formins and the adaptor protein Nck (reviewed in 13).

### 2.1.3 Cell-matrix adhesions

Prior to cell migration, a protrusion must extend which needs subsequently to be stabilized by adhesion to a substrate. Small focal complex structures are localized in the lamellipodia of most migrating cells, and are important in mediating the attachment of the extending lamellipodium to the extracellular matrix (ECM; 15). Although many different receptors are involved in the migration of different cell types, the integrins are the major migration-promoting receptors. Integrins act as the “feet” of a migrating cell by supporting adhesion to the ECM or other cells and by linking via adapters with the actin filaments inside of the cell.
Rac is required for focal complex assembly (51-53). In the case of Rho, integrin clustering is very pronounced and results from tension aggregating dispersed integrins, such that they align through their attachment with the ends of stress fibres in focal adhesions (54). In some cell lines, the inhibition of Rho decreases adhesion, causing a retraction of the lamellae and rounding of the cell body. The speed of cell migration is dependent on the substrate composition. Indeed, the relative levels of Rho, Rac and Cdc42 activation vary with extracellular matrix composition (55-57). There is continuous crosstalk between integrins and Rac to allow cells to respond to changes in extracellular matrix composition of growth factor receptors (58).

The turnover of focal complexes/adhesions is important for migration of cells. If they are persistently sustained, the cell cannot migrate because it cannot detach from the ECM. On the other hand, if they are properly disassembled but their assembly is deregulated, migration is again impaired because the cell does not have sufficient grip on EMC to move forward. Both Rac and Rho are directly and indirectly involved in the regulation of turnover of focal adhesions/complexes (48,59,60).

2.1.4 Cell body contraction
In order to migrate, after induction of forward protrusions, the cell needs to contract and retract the rear. Cell body contraction is dependent on actomyosin contractility (61), a process regulated by Rho. Rho acts via ROCKs (also known as Rho-kinases) to affect MLC phosphorylation, both by inhibiting MLC phosphatase and by direct phosphorylation of MLC (62,63). MLC phosphorylation is also regulated by MLC kinase (MLCK), which is activated by calcium, and stimulated by the ERK MAPKs (64). It is likely that ROCKs and MLCK act in concert to regulate different aspects of cell contractility. ROCKs are required for MLC phosphorylation associated with actin filaments in the cell body, whereas MLCK is required at the cell periphery (65). Rho promotes myosin contractility and the resulting tension drives the formation of stress fibers and focal adhesions. Thus, reducing Rho activity has two opposing effects: it promotes cell migration by lowering adhesion, but decreases it on the other hand by inhibiting cell body contraction.
2.2 Rho-GTPases as regulators of cell-cell adhesion in tumor progression

Invasion of surrounding tissue is one of the most important steps in tumor metastasis. It requires the release of tumor cells from the primary tumor. The major intercellular adhesion molecules expressed by epithelial cells belong to the family of the classical cadherins (E/P/N-cadherins), where E-cadherin is the most abundantly expressed isoform. In addition to cadherins, which are concentrated in the so-called adherens junction (AJ), epithelial cells form intercellular contacts through tight junctions (TJ) and desmosomes (Figure 4). Cadherin complexes and tight junctional components associate with the cortical F-actin cytoskeleton, whereas desmosomal cadherins are linked to intermediate filaments called cytokeratins.

2.2.1 Cadherin expression and function

Classical cadherins mediate intercellular adhesion in a homotypic and Ca2+-dependent manner through the formation of zipper-like molecular structures on opposing cells (66,67). Cadherins are stabilized on the plasma membrane through dimerisation (68) and the linkage of the cytoplasmic tails to the F-actin cytoskeleton.
via the Armadillo-family proteins β- and γ-catenin and the actin binding proteins α-actinin and vinculin (69,70).

Cadherins play a critical role in tissue morphogenesis and homeostasis of tissue architecture and their functional elimination represents a key step in the acquisition of the invasive phenotype for many epithelial tumors. In vitro, a strong correlation has been found between the loss of E-cadherin and the loss of the epithelial phenotype whereas overexpression of E-cadherin in mesenchymal cells induces a mesenchymal-epithelial transition (71,72,72). The progression from benign to malignant epithelial tumors and the dedifferentiation of tumor cells in vivo is strongly correlated with the loss of function of E-cadherin (73).

The loss of function of E-cadherin in epithelial tumors can have multiple causes (reviewed in 74-76). Since loss-of-function mutations in the E-cadherin gene are sporadic in human tumors and re-expression of E-cadherin in distant metastases is often observed, epigenetic regulation of cadherin expression is probably most relevant in tumor progression. Downregulation at the RNA level can be established through regulation of transcription factors such as SNAIL, Slug, and SIP1. Other mechanisms of gene silencing involve hypermethylation of the E-cadherin promoter. At the post-translational level, cadherins can be regulated by enhanced turnover and degradation, processes that can be triggered by several oncogenic signaling pathways and importantly by Rho GTPases.

2.2.2 Rho-GTPases regulate cadherin-based intercellular adhesions
Rho GTPases are actively involved in the formation and maintenance of intercellular adhesions. Conceivably, F-actin dynamics mediated by Rho-GTPases could influence the assembly, stability and function of the adherens junction and the tight junction. However, the effects of Rho GTPases on cell-cell adhesion complexes are not all mediated through rearrangement of the actin cytoskeleton. Although the involvement of Rho proteins in the formation and maintenance of cell-cell contacts are well studies, contradictory results make it difficult to form a clear consensus for their roles in these processes (reviewed in 77-79).
Initially, Rac1, Cdc42 and RhoA where found to affect cadherin accumulation in intercellular adhesions of MDCK cells and primary human keratinocytes. Inactivation of RhoA and Rac1 inhibit the formation of and break down already established adherens junctions in MDCK cells (80-82). RhoA was implicated in clustering of cadherin receptors (82), whereas Rac probably mediates actin recruitment to primary contacts, thereby stimulating stabilization and additional binding of cadherins to the cortical actin cytoskeleton (81,82).

In contrast, both Rac1 and RhoA have also been described to disassemble cadherin-based adhesions in human epidermal keratinocytes (83-85). Rac activity stimulates disassembly of the adherens junction in a dose and time dependent manner (86), possibly via enhanced endocytosis of cadherins (84).

In transformed cells, different oncogenic signaling pathways regulate the stability of the adherens junction via Rho GTPases. Again, GTPase signaling can either contribute to disruption or stabilization of the AJ. Tiam1, a previously mentioned GEF for Rac, induces a mesenchymal-to-epithelial reversion of Ras transformed MDCK cells (34,87). Over-expression of Tiam1 blocks cell-cell dissociation (or scattering) by hepatocyte growth factor (HGF/SF) in MDCK cells (87) and it is required for maintenance of the AJ upon overexpression of the viral oncogene E1A (88).

The role of RhoA in support of the transformed phenotype is more unequivocal; Ras-transformation of epithelial cells is often associated with a more contractile fibroblastic phenotype. Enhanced RhoA activity, generating contractile force through the formation of stress fibers, was found to contribute to this phenotype (89,90). The migratory potential of Ras-transformed cells furthermore is associated with low Rac activity and high Rho activity (89,91) the activity of RhoA cooperates with HGF and TGF in the disruption of cadherin-based adhesions (92,93). From all these studies it is clear that RhoA and Rac1 may differentially regulate cadherin-based cell-cell adhesions, but the outcome of their activities depends on the cellular context and whether or not they are targets of oncogenic signaling pathways.
Several downstream effectors of Rho GTPases were identified that affect cadherins either directly or via the actin cytoskeleton (reviewed in 78). One important target of both Rac1 and Cdc42 that regulates E-cadherin stability is the actin-binding protein IQGAP1 (94,95). IQGAP1 hampers the stable association of E-cadherin to the actin cytoskeleton. Recent data suggest that IQGAP1 is involved in a positive feedback-loop in which it activates Rac1 downstream of E-cadherin (96). Two downstream effectors of Rho signaling, ROCK and mDia, differentially affect the AJ. Whereas ROCK mediated actin-myosin contraction downstream of RhoC and to a lesser extend RhoA disrupts AJ formation, actin polymerization downstream of mDia is required for maintenance and formation of the AJ (91).

2.2.3 Cadherins signal towards Rho-GTPases
Cadherin molecules do not only glue cells but are also considered as signaling complexes that mediate outside-in signaling. The cytoplasmic domain of E-cadherin binds several molecules involved in intracellular signaling towards differentiation-, growth- and survival pathways. Interestingly, classical cadherins signal in an adhesion dependent fashion manner directly or indirectly to the Rho-GTPases RhoA, Cdc42 and in particular to Rac1.

Early work described a correlation between E-cadherin ligation, the activation of Rac1 (97,98) and the downregulation of RhoA activity (97) in differentiated epithelial cells. By the use of planar surfaces coated with recombinant cadherin-specific adhesive ligands, Rho-GTPase signaling has been studied as a direct consequence of cadherin ligation. In cadherin expressing cells that adhered to substrates coated with Xenopus C-cadherin (97) or human E-cadherin (99,100) Rac1 was activated in minutes upon binding to the substrate. Long term adhesion to C-cadherin (97) or adhesion of mouse C2C12 cells to chick N-cadherin (101) led to lower or higher RhoA activity respectively, which indicates that the outcome of cadherin signaling might differ in different cell-types or with different cadherin isoforms.

Several molecular mechanisms have been proposed by which cadherins signal to Rho-GTPases. One candidate molecule that is supposed to control Rac1 signaling is
type 1A PI3 kinase, of which the lipid products are potent activators of several Rac-GEFs. Cadherin dependent adhesion recruits and activates PI3 kinase (100,102) and the p85 regulatory subunit of PI3 kinase is a direct binding partner for β-catenin (103).

Rac1, Cdc42 and RhoA activity can also be regulated via p120-catenin, the armadillo-family protein that binds the membrane proximal region of the cadherin cytoplasmic tail (104-107). p120 can shuttle between the plasma membrane (where it binds cadherins) and the cytoplasm where it can inactivate RhoA (104), but activates Rac1 (107) and Cdc42 (106), possibly via a direct interaction of p120 with the Rac-GEF Vav2 (105). The cytoplasmic pool of p120 seems to enhance the migratory capacity of epithelial cells in a Rac1/Cdc42 dependent manner, whereas cadherins sequester p120 to the plasma membrane, thereby suppressing its function in cell motility (106). Functional loss of cadherins in epithelial tumors could therefore enhance the migratory potential of these cells via p120 and Rho-GTPase signaling, as recently shown for invasive breast cancer cells (108).

Loss of expression of E-cadherin is often correlated with the enhanced expression of other types of cadherins, a process called the ‘cadherin switch’. This switch is often associated with the loosening of cell-cell contacts and a transition to a migratory phenotype. Since N-cadherin was shown to activate several Rho-GTPases (101,109) it is likely that GTPase signaling downstream of N-cadherin affects cell migration and epithelial to mesenchymal transition of tumor cells, although no direct evidence for this is currently available. Recently, R-cadherin, a classical cadherin with high homology to N-cadherin, was shown to increase the steady state activity of Rac1 and Cdc42 resulting in increased motility of different R-cadherin expressing cell-lines (110). A potential role of R-cadherin in tumorigenesis has however not been investigated yet.

In summary, Rho GTPases are required for the formation and maintenance of cadherin-based adhesions. However, Rho-GTPase signaling downstream of cadherins can also positively or negatively affect the migratory capacity of cells by (local) feedback signaling towards the cadherins and the tight junctions. Interestingly,
different types of cadherins, especially the ones overexpressed in epithelial tumors, might differentially regulate the steady state levels of Rho-GTPases and could thereby enhance cell migration rather than promote stable intercellular adhesion.

2.2.4 Rho-GTPases regulate tight junctions and apicobasal polarity

Loss of cell polarity is one of the important hallmarks of epithelial cancer cells that become invasive. The spatial and functional separation of the apical and baso-lateral plasma membrane of epithelial cells (apicobasal polarity) is established through asymmetric distribution of junctional intercellular adhesion complexes that are linked to the actin cytoskeleton. At the most apical region of the plasma membrane of endothelial and epithelial cells the tight junction creates a barrier for paracellular diffusion of small soluble molecules and it restricts free intramembrane diffusion of integral components of the plasma membrane. Loss of tight junction function occurs at multiple stages in tumor metastasis, between tumor cells themselves but also between cells of the vascular endothelium through which tumor cells can invade the underlying tissue (111). Moreover, leakiness of epithelial tight junctions allows luminal growth factors to reach their receptors at the baso-lateral membrane, causing changes in epithelial cell proliferation and survival. (30,112).

The integral membrane proteins occludin, JAMs and claudins, that make up the tight junction are connected to the F-actin cytoskeleton (113). Therefore, Rho-mediated cortical actin dynamics influence the assembly, stability and function in regulation of paracellular permeability of the tight junction (114-116). Recent studies indicate that tight junctions can also function as signaling platforms, regulating cell morphology and gene expression (117) and RhoGTPase signaling (118).

Cdc42 plays a direct role in the formation of the tight junction and the establishment of apicobasal polarity in epithelial cells. Activated Cdc42, as a binding partner for the adaptor molecule PAR6, was implicated in the recruitment of the polarity complex (consisting of PAR6, PAR3 and an atypical PKC) to the plasma membrane (119,120) at regions where initial cell-cell contacts are formed. This signaling complex is of pivotal importance for the sequential formation of the tight junction and full maturation of the initial cell-cell contacts into the cortical adhesion belt (121,122). Initial cell-cell
contacts mediated by cadherins and nectins activate Cdc42 (123) and recruit the polarity complex and stimulate the assembly of the tight junction.

3. Rho GTPases in in vivo transformation and metastasis

3.1 Rho proteins in human cancer
The first findings that implied a role for Rho GTPases in cancer-development came from in vitro foci formation assays. These assays can be used as a model for growth factor- and anchorage-independent tumor cell growth. Foci-formation assays identified many N-terminal-truncated RhoGEFs, such as Dbl, Ect2, Lfc and Vav as proto-oncogenes (reviewed in 124,125). Furthermore, activating mutants of RhoA, RhoG, Rac1, TC10 and Cdc42 were shown to induce foci formation, albeit weakly compared to constitutively active Ras (V12Ras) (126,127). Inhibitory mutants of Rho proteins prevent Ras-induced transformation in soft-agar assays and tumor formation in nude mice (128-132), and V12Rac1 cooperates with constitutively active Raf (RafCAAX) in focus formation (130,133). These observations suggest that Rho proteins act downstream of Ras in oncogenic transformation of cells.

Unlike Ras, there are no reports of mutated, constitutively active forms of Rho proteins in human tumors. The only Rho protein known to be altered genetically in human tumors is RhoH, which is frequently rearranged in lymphomas (148) (Table 1) or to have mutations in the 5' untranslated region (149), which are predicted to affect RhoH expression. Many reports have shown that other members of the Rho GTPase family are over-expressed in human cancer (see table) and that, in some cases, this correlates with clinical outcome. In breast cancer and testicular germ-cell cancer RhoA expression levels correlate positively with progression of the disease (134,140). RhoC is overexpressed in pancreatic ductal adenocarcinoma and inflammatory breast cancer (141-143).

Two reports describe a role for Rho-GEFs in human tumorigenesis (150-152). Tiam1, a Rac-specific GEF was found to have a point mutation in the PH-domain of one of
Table 1: Aberrant regulation of Rho proteins and their regulators in human cancer

<table>
<thead>
<tr>
<th>Gene</th>
<th>GTPase specificity</th>
<th>Type of deregulation</th>
<th>Tumor types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdc42</td>
<td></td>
<td>Overexpression</td>
<td>Breast cancer (134)</td>
</tr>
<tr>
<td>Rac1</td>
<td></td>
<td>Overexpression</td>
<td>Breast &amp; gastric cancer (135)</td>
</tr>
<tr>
<td>Rac1B</td>
<td></td>
<td>Alternative splicing</td>
<td>Colon &amp; breast cancer (136,137)</td>
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<tr>
<td>Rac2</td>
<td></td>
<td>Overexpression</td>
<td>Head and neck squamous carcinoma (138)</td>
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<tr>
<td>Rac3</td>
<td></td>
<td>Hyperactive (majority of Rac3 in GTP-bound state)</td>
<td>Breast cancer (139)</td>
</tr>
<tr>
<td>RhoA</td>
<td></td>
<td>Overexpression</td>
<td>Colon, breast &amp; lung cancer (134) Head and neck squamous carcinoma (138) Gastric cancer (135)</td>
</tr>
<tr>
<td>RhoC</td>
<td></td>
<td>Overexpression</td>
<td>Inflammatory breast cancer (141,142) Pancreatic ductal adeno-carcinoma (143) Non-small cell lung carcinoma (144) Ovarian cancer (145) Gastric cancer (146) Hepatocellular carcinoma (147)</td>
</tr>
<tr>
<td>Tiam1</td>
<td>Rac</td>
<td>Point mutation</td>
<td>Renal-cell carcinoma (150,151)</td>
</tr>
<tr>
<td>LARG</td>
<td>Rho</td>
<td>5’ End of MLL gene fused to the 3’ end of LARG</td>
<td>Acute myeloid leukemia (152)</td>
</tr>
</tbody>
</table>
the two alleles, in around 10% (4 of 35) of the examined renal cell carcinoma samples. The mutation results in an alanine-glycine substitution at amino acid 441, and was suggested to be a dominant gain-of-function mutation. However, in three of five renal cell carcinoma cell lines a decrease in the Tiam1 expression was found, including two that contained the A441G mutation (150,151). The significance of this in light of the gain-of-function activity of this mutation is unclear. Another RhoGEF, leukemia-associated Rho guanine nucleotide exchange factor (LARG) has been isolated as a fusion partner of the mixed-lineage leukemia (MLL) gene in a patient with acute myeloid leukemia. The fusion protein contained the N-terminal part of MLL in frame with the C-terminal part of LARG, which includes the DH/PH domain (important for GEF function), a domain with homology to the Rho GEF Lsc, and a nuclear localization signal (152). It remains to be established whether the MLL-LARG fusion protein is sufficient to induce transformation in myeloid cells and how frequently the locus is affected in human cancer.

Several lines of evidence indicate that the cycling of Rho GTPases between GTP- and GDP-bound states might be important for transformation. In vitro studies have shown that Rho-GEFs are more potent oncogenes than GTPases defective Rho proteins, and that a fast GTP-GDP cycling mutant of Cdc42 has a greater transforming capacity than a GTPase defective mutant (132,153). Furthermore, the splice variant of Rac1, Rac1B, which has an increased GTP-GDP cycling rate in vitro is highly overexpressed in breast and colon cancer (136). The requirement for cycling between GDP- and GTP-bound states might reflect the cyclic nature of the processes that are regulated by Rho proteins in tumorigenesis, and might provide an explanation for the fact that GTPase defective mutants –analogous to those in oncogenic Ras- have not been identified in tumors.

3.2 Participation of Rho GTPases in different stages of tumorigenesis

3.2.1 Rho proteins in growth and apoptosis
The growth rate of tumors is determined by the difference between cell growth and cell death. Rho proteins have been implicated in the (de)regulation of both processes
in normal and tumor cells. Rho proteins regulate cell growth via multiple pathways, including regulation of the expression of cell cycle proteins like cyclin D1 and the CDKIs p21Waf1 and p27Kip1 (reviewed in 91). In addition, the Rho proteins can modulate the activity of growth-factor-regulated pathways by regulating the transport and turnover of growth-factor receptors. RhoB and its effector PKC-related kinase 1 (PRK1), affect the kinetics of the epidermal growth-factor receptor (EGFR) internalization after ligand stimulation (154), by coordinately regulating the movement of vesicles along microfilament networks (155). However, many of the studies that led to the conclusions outlined above have been done in vitro using fibroblasts, which are cells that rarely become cancerous. Further work is needed to determine via which pathways Rho proteins regulate cell proliferation in vivo.

Apoptosis is a counterbalance to mechanisms of cell proliferation and is critically important in regulation of the immune system, development, and normal tissue homeostasis. Cancer cells frequently show enhanced sensitivity to pro-apoptotic stimuli (156), but often become resistant to pro-apoptotic anti cancer therapies over time (157). Rho proteins have been implicated in both pro- and anti-apoptotic signaling, and in the apoptotic process itself (reviewed in (158). More significantly, they are involved in the decision to commit to apoptosis; ectopic expression of active Rac1 can provide a survival signal to protect tumor cell lines or transformed fibroblasts from apoptosis (159-161). Other mechanisms that link Rho proteins to cell survival have been described in non-transformed haematopoetic cells: Rac2 is required for the activation of the pro-survival kinase AKT in mast cells (162), and Rho function prevents p53-dependent apoptosis during T-cell development (163). In other contexts, Rho proteins might promote pro-apoptotic signaling. RhoB is required for the induction of apoptosis by DNA-damaging agents (164) and farnesyltransferase inhibitors, but not other cytotoxic treatments (165), and Rac is required for FAS-induced apoptosis (166,167).

3.2.2 Rho proteins in invasion and metastasis
In the tumor progression phase, tumor cells have altered morphological characteristics and, in the case of metastasis, acquire the ability to traverse tissue
boundaries. Given the role of Rho proteins in the regulation of cell motility in normal cells, and their aberrant regulation in tumor cells, it is likely that they are involved in the invasive phenotype of tumor cells.

Rho proteins are involved in the loss of epithelial polarity that is evident even in benign tumors, and are also important in the epithelial to mesenchymal transition (EMT) that is sometimes observed in more aggressive epithelial tumors (see previous paragraphs on Rho protein signaling in cell-cell contacts). In addition to the disruption of cell polarity and cell–cell junctions, increased motility and the ability to remodel the ECM is required for tumor cells to become locally invasive. RhoA and Rac1 can regulate the function of ezrin, moesin and radixin: these related proteins promote cell motility by linking the actin cytoskeleton to the plasma membrane through the membrane-spanning ECM receptor CD44. RhoA can promote phosphorylation of ezrin by ROCK, leading to its increased association with the cytoskeleton (168), whereas Rac1 promotes the phosphorylation and inhibition of the ezrin antagonist neurofibromatosis 2 (NF2) (169,170). Several lines of evidence indicate that this is an important event in tumor progression: ezrin and CD44 are frequently overexpressed in metastatic tumor cells, and NF2 is a tumor-suppressor gene (170-174), the deletion of which gives rise to highly metastatic tumors (175). In addition, RhoA and Rac1 can modulate the degradation and remodeling of the ECM either by regulating the levels of matrix metalloproteinases (MMPs) that degrade the ECM or by regulating the levels of their antagonists, tissue inhibitors of metalloproteinases (TIMPs) (151,176-178).

The ability to enter either the blood or the lymphatic vasculature is required for tumor cells to metastasize to distant sites. RhoA and ROCK are required in both the endothelial and the migrating cells for them to cross the vascular endothelium (179,180). Overexpression of RhoC leads to increased expression of angiogenic factors in breast epithelial cells (142), which could lead to increased vascularization of the tumor and an increased likelihood of tumor cells entering the blood stream. RhoC overexpression also promotes the ability of melanoma cells to exit the blood and colonize the lungs (181), and interfering mutants — which sequester RhoGEFs
away from endogenous Rho — of RhoA and Cdc42 prevent T-cell hybridomas from exiting the blood and colonizing the liver (182).

### 3.3 Rho protein signaling in mouse models

Since Rho proteins play a major role in regulating cell growth apoptosis and cell motility in normal cells, it is not surprising that they are involved in the development and progression of tumors. However, it has proven difficult to extrapolate the studies in normal cells to the tumor environment. The literature on Rho protein function in tumor progression is confusing, mainly because different studies have indicated contradictory roles for the Rho proteins. However, upon closer inspection the findings may be explained if Rho proteins have different functions at different stages of tumor development. Recent studies, in which recombinant mice were used, directly implicate Rho proteins in all stages of tumorigenesis, and reveal both tumor-promoting and –suppressing functions.

#### 3.3.1 Rho protein signaling in Ras-induced skin carcinogenesis

To gain insight in the role of small GTPases in the physiological functions, two groups examined the consequences of functional deletion of members of Rho GTPase family in the mouse: Tiam1, a GEF for Rac (183), and RhoB (164). The knockout of either gene did not negatively affect mouse development, fertility or wound healing. However, these models did show that deregulated Rho protein signaling could influence various processes involved in tumorigenesis. Both studies applied a two-stage chemical skin carcinogenesis protocol, which induces oncogenic activation of the c-Ha-Ras gene in the basal layer of the epidermis followed by the induction of the outgrowth and progression of the initiated keratinocytes.

In the RhoB knockout mice the treatment resulted in the development of increased numbers of skin tumors compared with wild-type mice (164). Moreover, DNA-damaging agents were found to induce less apoptosis in Ras- and E1A-transformed RhoB-deficient primary mouse embryonic fibroblasts. This suggests that RhoB normally suppresses tumorigenesis by promoting apoptosis following cellular stress.
Application of the same protocol to Tiam1-deficient mice had a different result: these mice had much fewer and smaller tumors compared to the wild-type mice (183). Malliri et al. showed that Tiam1-deficiency was associated with more apoptotic cells in the tumors during initiation and with impeded proliferation during tumor promotion. These results are consistent with in vitro studies showing that Tiam/Rac-signaling is required for Ras-induced tumorigenesis through the stimulation of cell growth and enhancing cell survival subsequent to cellular stress.

Another interesting observation in the Tiam1-knockout mice was that although the mice develop less tumors, a larger portion of these tumors progresses to a more malignant stage than in wild-type mice (183). This implicates that Tiam1-deficiency is favorable in malignant conversion. This hypothesis was confirmed by the observation that the tumors in wild-type mice gradually lost Tiam1-expression progressing from benign papillomas to highly invasive spindle cell carcinomas (183).

### 3.3.2 Rho protein signaling in lymphoid tumors

RhoH has been found to be frequently translocated or mutated in human lymphoid tumors (148,149). However, the potential function of RhoH in the formation of lymphoid tumors remains to be established. In mouse models, a role for Rho proteins in the development of lymphomas has been elucidated. In transgenic mice, in which the expression of C3 toxin is driven by the thymocyte-specific Lck promoter, aggressive malignant thymic lymphomas were found (184). Because C3 toxin inactivates RhoA, -B and -C, it is not clear which of the inhibited Rho proteins play a role in promoting tumorigenesis.

In search of genes involved in the process of tumor cell invasion and metastasis, the Tiam-1 gene was identified by retroviral insertional mutagenesis in combination with in vitro selection of invasive T-lymphoma variants. Furthermore, it was shown that cell clones, which were invasive in vitro, also caused metastases when injected in nude mice (185). Later, Tiam1 was found to be activated by retroviral insertions in T lymphomas induced by Moloney murine leukaemia virus infection of transgenic E mu-Pim1 mice, thereby inducing an accelerated onset of T-cell lymphomas. This indicates that Tiam1 can cooperate with Pim1 in in vivo lymphomagenesis (186).
3.3.3 Rho proteins in Wnt/APC signaling

Wnt proteins constitute a large family of secreted signaling molecules that play central roles in animal development (187). Well-studied examples of Wnt regulation of embryogenesis involve the canonical β-catenin signaling pathway, which in Xenopus induces dorsal axis formation and plays a key role in human carcinogenesis (188). In the Wnt/β-catenin pathway, signals are initiated that result in the transcription of genes that regulate cell growth and differentiation. β-catenin participates in this pathway as a heterodimer with T-cell factor (TCF) transcription factors. In the absence of a Wnt signal, cytosolic β-catenin is degraded through a pathway that is dependent upon adenomatous polyposis coli (APC). However, upon stimulation of the Wnt pathway, cytosolic β-catenin is stabilized. The Wnt pathway is tightly regulated in normal cells, but its regulation is often disrupted during tumorigenesis (reviewed in 189).

Recently, Rho proteins have been implicated in Wnt-signaling pathways. In embryonic development of Drosophila and Xenopus, Rac and Rho have been implicated in Wnt/Fz signaling in regulating cell polarity and movements: a Wnt-Fz-Dvl-Daam1-Rho-ROCK pathway and a Wnt-Fz-Dvl-Rac-JNK pathway (190-193). A common Wnt-Fz-Dvl signaling cassette branches into these two pathways downstream of Dvl, most likely via distinct Dvl-Rac and Dvl-Rho complexes. The Rho-ROCK pathway regulates the phosphorylation of non-muscle myosin regulatory light chain and thus the assembly of actin filaments, whereas the Rac-JNK pathway regulates cytoskeletal or nuclear events (190).

Furthermore, preliminary results from our laboratory indicate that Tiam1 is a Wnt-responsive gene. Moreover, its deficiency impairs the development of intestinal tumors in APC mutant mice (A. Malliri et al., unpublished data), similarly as shown before for Ras-induced skin tumors. These data suggest that Tiam1 and Rac play a role in the oncogenic Wnt signaling pathway.

Also another recently identified Wnt-transcriptional target is a member of the Rho GTPases family: Wrch-1, a novel Cdc42-like GTPase. Like Wnt-1, Wrch-1 is not normally expressed in the mouse mammary gland, but it could be detected in Wnt-1
induced mouse mammary tumors. This finding is consistent with the involvement of Wrch-1 in Wnt-1-induced transformation and tumor formation, and is further supported by the observation that Wrch-1 can mimic the effect of Wnt-1 in morphological transformation of breast cancer cells (194).

**Concluding remarks**

The single most notable feature of Rho GTPases is their participation in so many aspects of cell biology, ranging from the fundamental to the highly specialized. The analysis of individual signal transduction pathways controlled by Rho GTPases still poses many problems, and with so many GEFs, GAPs and targets it has proved difficult to link specific receptors all the way through to a biological response. Recently, many efforts have been directed towards the understanding of how spatially localized activation of GTPases is achieved or, when activated, how GTPases are able to 'choose' the correct target(s) from the large number of possibilities present in the same cell. The proposed models involve positive feedback loops, cooperative signaling pathways, scaffold proteins and intracellular compartmentalization.

It is now clear that Rho GTPases are involved in almost every stage of tumorigenesis. The lack of a general consensus as to the relative importance of the various mechanisms that link Rho proteins to tumorigenesis means that the challenge is now to determine the crucial pathways that link Rho proteins to cancer. The use of in vivo tumorigenesis models and mouse genetics, together with studies on human tumors, should help to identify the important connections in vivo. Such studies can prove the idea that Rho proteins or their effectors are valid anti-tumor targets; the next step will be to develop pharmacological agents to interfere with Rho-protein function. Hopefully, these will be useful leads in the development of therapies that can be taken into the clinic.
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