Discovery of Small Molecule Modulators of Ras Superfamily Proteins – Studies of MgcRacGAP and Ras

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Doctoral Programme in Biomedicine (DPBM)

Discovery of small molecule modulators of Ras superfamily proteins
Studies of MgcRacGAP and Ras

Arjan J. van Adrichem

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Medicine of the University of Helsinki in Lecture hall 1, Haartman Institute, Haartmaninkatu 3, on Friday 9th of September 2016 at 12 o’clock.

Helsinki 2016
“It doesn’t matter if you try and try and try again, and fail.
It does matter if you try and fail, and fail to try again.”

— Charles F. Kettering

To you who I don’t know yet, but will forever love.
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### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Full name</th>
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<tbody>
<tr>
<td>Arf</td>
<td>ADP ribosylation factor</td>
</tr>
<tr>
<td>Arl</td>
<td>Arf-like</td>
</tr>
<tr>
<td>Arp</td>
<td>actin-related protein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCR</td>
<td>breakpoint cluster region protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cdc42</td>
<td>cell division cycle-42</td>
</tr>
<tr>
<td>CDK1</td>
<td>cyclin-dependent kinase 1</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology</td>
</tr>
<tr>
<td>DHR</td>
<td>Dock homology region</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EC50</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
<td>Ect2</td>
<td>epithelial cell transforming sequence 2</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle's minimum essential medium</td>
</tr>
<tr>
<td>Erk</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDF</td>
<td>GDI displacement factors</td>
</tr>
<tr>
<td>GDI</td>
<td>guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>guanosine triphosphatases</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HTS</td>
<td>high-throughput screening</td>
</tr>
<tr>
<td>IC50</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>IL-6 (or IL6)</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IL6R</td>
<td>interleukin-6 receptor</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen-activated protein</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase/Erk kinase</td>
</tr>
<tr>
<td>MgCrGAP</td>
<td>Male Germ Cell RacGAP</td>
</tr>
<tr>
<td>MINC1</td>
<td>MgcRacGAP Inhibitor Compound 1</td>
</tr>
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<td>Miro</td>
<td>mitochondrial Rho</td>
</tr>
<tr>
<td>MKLP1</td>
<td>mitotic kinesin-like protein 1</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate-oxidase</td>
</tr>
<tr>
<td>NF-1</td>
<td>neurofibromin</td>
</tr>
<tr>
<td>PAINS</td>
<td>pan-assay interference compounds</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PAR</td>
<td>partitioning defective homolog</td>
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<tr>
<td>PBD</td>
<td>p21 binding domain</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDEδ</td>
<td>delta subunit of retinal rod phosphodiesterase</td>
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<td>phosphatidylinositol-3 kinase</td>
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<tr>
<td>PKN</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKN</td>
<td>protein kinase N</td>
</tr>
<tr>
<td>Plk1</td>
<td>polo-like kinase 1</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PRC1</td>
<td>protein regulator of cytokinesis</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>QRET</td>
<td>quenching resonance energy transfer</td>
</tr>
<tr>
<td>Rab</td>
<td>Ras-related proteins in brain</td>
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<tr>
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<tr>
<td>Ran</td>
<td>Ras-like nuclear</td>
</tr>
<tr>
<td>Rap</td>
<td>Ras proximal</td>
</tr>
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<td>rat sarcoma</td>
</tr>
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<td>RBD</td>
<td>Ras binding domain</td>
</tr>
<tr>
<td>REM</td>
<td>Ras exchange motif</td>
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<tr>
<td>Rho</td>
<td>Ras homologous</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>Sar</td>
<td>secretion-associated and Ras-related</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td><strong>Amino Acid</strong></td>
<td><strong>3-Letter</strong></td>
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<td>----------------------</td>
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<tr>
<td>Alanine</td>
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<td>Proline</td>
<td>Pro</td>
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<tr>
<td>Tyrosine</td>
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</tr>
<tr>
<td>Valine</td>
<td>Val</td>
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Original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:


* These authors contributed equally to this work

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Abstract

Small guanosine triphosphatases (GTPases) are a family of low molecular weight guanosine diphosphate (GDP)-/guanosine triphosphate (GTP)-binding proteins that act as "molecular switches", regulating key cellular processes, such as cell proliferation and differentiation. The activity of small GTPases is controlled by guanine nucleotide exchange factors (GEFs), which facilitate nucleotide exchange from GDP to GTP, and GTPase activating proteins (GAPs), which stimulate intrinsic GTP hydrolysis of the GTPase. Complex, yet unresolved, mechanisms maintain the precise spatiotemporal regulation of these proteins, which is essential for accurate cellular signaling. Accordingly, aberrant function of small GTPases is associated with a large number of human diseases, such as cancer, neurodegenerative diseases and inflammatory disorders. The availability of small molecule modulators of GTPase activity would be highly valuable to improve the understanding of small GTPases of the Ras superfamily and their regulators. However, very few probes of this kind currently exist. To address this gap, I have focused on discovering different small GTPase-modulating probes through different screening approaches.

MgcRacGAP is a GAP protein for Rho family small G-proteins and a key regulator of cytokinesis. After almost two decades of research, the detailed functional role, its relevant target GTPase and its GAP activity in cytokinesis are not yet fully resolved. Furthermore, like many other regulators of GTPase activity, its overexpression has been linked to different malignant properties, such as epithelial-to-mesenchymal transition, cell polarity and (invasive) migration, as well as correlated to a poor clinical prognosis in many types of cancers. To be able to investigate the biological role of MgcRacGAP, we first addressed the lack of chemical tools to probe its function by developing a high throughput screening strategy to identify compounds inhibiting its GAP function. The discovery of MgcRacGAP Inhibitor Compound 1 (MINC1), a selective MgcRacGAP-Rac1 inhibitor, represents the first described selective small molecule inhibitor of a Rho GTPase activating protein and shows that development of inhibitors of small GTPase activating proteins is possible. We utilized MINC1 to study the function of MgcRacGAP in cell division as well as the
role in the events that regulate signal transducer and activator of transcription 3 (STAT3) signaling. Notably, we showed that MINC1-mediated inhibition of MgcRacGAP caused impaired mitotic spindle formation during the metaphase, which suggests that the GAP activity of MgcRacGAP plays an important role in mitosis. To address the role of MgcRacGAP in the events that regulate STAT3 phosphorylation and subsequent nuclear translocation, we used both MINC1 treatment and small interference RNA (siRNA)-mediated gene silencing of MgcRacGAP. With these complementary techniques we showed that inhibition of MgcRacGAP triggers STAT3 phosphorylation caused by a Rac1-PAR3-IL6-IL6R-JAK2 mediated autocrine/paracrine mechanism.

Due to their key role in different essential cellular processes, non-specific inhibition of GTPases or their regulators is expected to result in significant risk of side effects. Compounds that interfere with specific protein-protein interactions are expected to circumvent this problem, yet most current screening methods fail to detect these. To address this, we have developed a protein-protein interaction inhibitor screening strategy for the oncoprotein Ras and identified ten compounds that inhibited GTP hydrolysis in a concentration dependent manner. Four of these compounds could not be detected with the established method. Of the three compounds that showed efficacy in Ras signaling dependent cell lines, one compound had a direct effect on the activation status of Ras.

In summary, this thesis describes i) the discovery of the first RhoGAP inhibitor named MINC1, ii) the application of MINC1 to elucidate the role of MgcRacGAP in the activation and nuclear translocation events of STAT3 and iii) the development and exploration of a new screening strategy to discover Ras protein-protein inhibitors.
1. Introduction

With the advances in science in the last fifty years, and particularly in the field of molecular biology with the discovery of tools to analyze and manipulate genes and proteins, there has been a growing understanding of the underlying biology that drive diseases. The identification of so-called oncogenes and subsequent increasing understanding of their function at molecular level, has associated various members of the GTPase and kinase protein families with cancer and many other diseases, such as neurodegenerative diseases and inflammatory disorders. As a result, drug development has become more biology-driven, rather than based on serendipity. Kinases, with approximately 30 approved drugs for cancer treatment and more in clinical trails, have been successfully targeted in cancer drug discovery. However, less than a few clinically relevant GTPase targeting drugs have made it to the market to date. There are several reasons for this discrepancy. Despite both being nucleotide-binding proteins, small GTPases show a higher affinity for guanine nucleotides than kinases do for adenosine nucleotides, making it more difficult to compete against with a small molecule inhibitor. In contrast to kinases, there are no other obvious small molecule-binding pockets on small GTPases besides the nucleotide-binding pocket. Kinase inhibitor discovery has been enabled by high enzymatic activity of kinases, as it was it easier to develop sensitive biochemical assay that needed only low amount of protein. Last, while both small G-proteins and kinases tend to serve as nodes in signaling transduction, other domains within the protein itself regulate kinases, whereas up- and downstream regulatory proteins as well as scaffolding proteins direct GTPase activity and thereby determine the role of a GTPase in a wide range of different signaling pathways. As a result, targeting GTPases is expected to result in very broad unspecific inhibitory effect, leading to severe side effects. On the other hand, targeting GTPase-regulatory proteins is expected to result in specific modulation of select GTPase signaling pathways. In this thesis, my aim was to elucidate the function of GTPase-regulating proteins such as MgcRacGAP using small molecule modulators identified through novel screening assays.
2. Literature review

The Ras superfamily of small guanosine triphosphatases (GTPases) comprises over 150 members in humans that all share a common biochemical characteristic: Cycling between a guanosine triphosphate (GTP)-bound state and a guanosine diphosphate (GDP)-bound state, small GTPases act as molecular switches in a variety of cellular processes (Figure 1)\(^2,3,5\). Besides regulating normal physiological processes, small GTPases and their regulators have been shown to contribute to malignant tumor characteristics such as invasiveness, metastasis, inflammation and relapse-causing regeneration\(^4,6-9\). Because of this biological and therapeutic potential, small GTPases have been considered targets from a drug discovery perspective ever since their discovery. In this review, I will first introduce the small GTPases and their regulators, focusing mainly on Ras and Rho family proteins. Furthermore, I will discuss the drug discovery efforts in the field of small GTPases. And last, I will go in greater detail on the function of Male Germ Cell RacGAP (MgcRacGAP, gene name \textit{RACGAP1}), both in physiological and pathological processes and why there is a need for MgcRacGAP inhibitors.

Figure 1 \textit{The small GTPase cycle.} Most small GTPases cycle between what is commonly considered an inactive GDP-bound and an active GTP-bound form. \textit{In vivo}, their intrinsic activity is regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine–nucleotide-dissociation inhibitors (GDIs). Active GTPases interact with effector proteins to mediate a response. GDP, guanosine diphosphate; GTP, guanosine triphosphate P\(_i\), inorganic phosphate.
2.1 Small GTPases

2.1.1 General structure of small GTPases
Highly conserved from lower eukaryotes to mammals, the Ras superfamily of small GTPases is divided into five major families based on their genomic sequence and functional similarities: Ras, Rho, Rab, Arf and Ran (Figure 2A)\(^3,10\). All small GTPase proteins have a conserved ~20 kDa catalytic domain, built of a six-stranded \(\beta\)-sheet surrounded by five \(\alpha\)-helices, which includes the five conserved motifs (G1-G5) that recognize the \(\beta\)-phosphate and the magnesium ion or the guanine base (Figure 2B)\(^2,11,12\). Two highly flexible segments within this domain, switch I and II, facilitate the conformation change that distinguish the GTP- and GDP-bound forms\(^13\). Switch I region corresponds with the conserved G2 motif and is part of the so-called effector binding domain, whereas switch II region comprises the G3 and the c-terminal adjacent \(\alpha2\) helix\(^14\). The distinct conformational change resulting from the substitution of GDP by GTP causes the GTP-bound GTPase proteins to bind with great affinity to effector proteins, as was shown for Ras and Raf\(^{13,15}\).

In addition to the domains common for all small GTPases, there are structural features that are restricted to one or a few subfamilies (Figure 2B). Arf proteins have an amino-terminal extension, whereas Ran proteins contain a carboxy-terminal extension. In both cases, substitution of GDP by GTP causes a large conformational change in switch I, Figure 2 (next page) The mammalian Ras GTPase superfamily and GTPase functional domains. A) The Ras superfamily of small GTPases is divided into five subfamilies: Ras, Rho, Ran, Rab and Arf. Subfamilies, grouped according to structural similarity criteria in smaller highlighted areas, share the same color throughout the figure. The GTPase proteins studied in this thesis are depicted in increased font. B) Schematic representation of the protein domains of Ras superfamily members. The core G-domain, corresponding to Ras residues 4–166, contains the nucleotide binding domains (G1-5, gradient black), the effector binding domain (residues 32-40, pink), and the switch regions (S1 and SII). Ras, Rho and Rab proteins terminate in a hypervariable region (HV) that is essential for membrane binding. Arf and Ran family GTPases contain amino-terminal (N) and carboxyl-terminal extension (C), respectively. The Rho family specific domain (Rho insert, yellow triangle) is inserted between Ras residues 122 and 123. (Image adapted from Vega and Ridley\(^1\), Wennerberg, Rossman and Der\(^3\) and Vigil et al.\(^4\)).
A

**Ras family**
proliferation
differentiation
transcription

- K-Ras
- H-Ras
- N-Ras
- Rap2C
- Rap2A
- Rap1A
- Rap1B
- RaIA
- RaIB
- RhoBTB2/DBC-2
- RhoBTB1
- RhoH/TTF
- RhoU/Whr
- RhoV/Chp
- RhoJ/TCL
- RhoQ/TC10
- Cdc42
- Rac2
- Rac1
- Rac3
- RhoG
- RhoF/Rif
- RhoD
- RhoB
- RhoA

**Rho family**
actin cytoskeleton
adhesion
proliferation
transcription

- Rnd1/Rho6
- Rnd2/RhoN
- Rnd3/RhoE
- Rnd1/Rho6
- Rnd2/RhoN
- Rnd3/RhoE

**Others**
intracellular traffic
dermatocytosis
secretion

**Arf family**
nuclear-cytoplasmic
transport

**Ran family**
membrane trafficking

**B**

G domain (4-166)

- N
- G
- C
- I

= Farnesyl (C15) isoprenoid
= Geranylgeranyl (C20) isoprenoid
= Myristate (C14) fatty acid
= Palmitate (C16) fatty acid
repositioning the terminal extension and enabling regulator/effector interactions\textsuperscript{16-18}. A so-called Rho-specific insert domain of 9-12 residues distinguishes Rho proteins from the others\textsuperscript{19}. Ras, Rho and Rab small GTPases have carboxy-terminal hypervariable sequences, which terminate in case of most Ras and Rho GTPases with a CAAX motif and for Rab with cysteine-containing motifs (CC, CXC, CCX, CCXX, or CCXXX; C=cytsteine, A=aliphatic, X=any amino acid). These motifs enable post-translational modification with lipophilic groups by prenylation (farnesylation or geranylgeranylation) or palmitoylation, which enhances the small GTPase interaction with different cellular membranes (reviewed in [20,21]). It is through these post-translational modifications that small GTPase proteins gain their characteristic subcellular membrane compartment localization and can fulfill their physiological function by interacting with membrane-associated regulator and effector proteins.

2.1.2 Membrane targeting by lipid modification

In order to successfully localize to the membrane small GTPase proteins need to go through a series of posttranslational modifications after synthesis\textsuperscript{20}. In this maturation process, lipophilic groups are attached to the GTPase protein to provide an anchor for membrane association (Figure 2B). To illustrate, Ras proteins first receive by prenylation a 15-carbon atom farnesyl or the 20-carbon atom geranylgeranyl group on the cysteine residue in the CAAX-motif at the C-terminal end in the cytoplasm\textsuperscript{22,23}. Thereafter, in the endoplasmic reticulum\textsuperscript{24} the last three amino acids, the AAX sequence, are removed by proteolytic cleavage\textsuperscript{25}, and subsequently a carboxymethyl group is introduced on the C-terminal cysteine residue\textsuperscript{26}. This first series of posttranslational modifications provides the first signal and, together with the second signal coming from the polybasic positively-charged lysine residues upstream of the C- terminus in K-Ras 4B, it enables membrane association for this Ras isoform\textsuperscript{27}. For K-Ras 4A, H- and N-Ras, the second signal required for correct membrane association is provided by a 16-carbon atom palmitoyl fatty acid moiety that is added in the Golgi complex to an upstream C-terminal cysteine residue\textsuperscript{24,27}.

Generally, the proteins of the Ras superfamily are grouped on the basis of the abovementioned sequence homologies, however these distinct
structural features also determine subcellular localization and thereby their specific functions in the cell. Where Rab and Arf GTPases appear to be the regulating proteins in the stepwise process of vesicular transport between donor and acceptor membrane-bound compartments, Ras and Rho GTPases function as signaling hubs in a myriad of different pathways, conveying signals to different effector pathways depending on the signaling complex formed.

2.1.3 Subfamilies of the Ras superfamily

The Ras oncoproteins were discovered on the basis of the homology to the rat sarcoma virus genes\textsuperscript{28,29}, and are the founding members of the Ras superfamily of small GTPases that were discovered in later studies. Early studies on the viral \textit{HRAS} and \textit{KRAS} oncogenes showed that these genes encode for 21 kDa proteins\textsuperscript{30}, which can also be found in the normal genomic DNA of, and expressed by vertebrate cells\textsuperscript{31-33}. Both the viral encoded, as well as the endogenously expressed proteins were demonstrated to be membrane-associated\textsuperscript{34,35}, and able to bind guanine nucleotides\textsuperscript{34,36}. The preferential binding to GTP of viral Ras isoforms was shown to enable Ras overexpression-induced cellular transformation of the host cell\textsuperscript{36-38}, and that the guanine nucleotide-binding activity is common to the family members and regulate, among other things, cell proliferation and survival. Their aberrant function was discovered when the transforming genes in human cancer cells were identified as mutated \textit{HRAS} and \textit{KRAS}\textsuperscript{39-41}. To date, the Ras family contains 36 members in mammals\textsuperscript{3,42}. In addition to the Ras proteins, it contains two more major groups; Ras-like (Ral) proteins, which regulate vesicle sorting, cell morphology as well as proliferation\textsuperscript{43}, and Ras proximal (Rap) proteins, which are regulators of integrin activation and cell motility\textsuperscript{44}, as well as some less studied other groups (Figure 2A).

In their mature form, Ras proteins are activated in response to a large variety of extracellular signals, including many different receptor tyrosine kinases, various G-protein-linked receptors, as well as intracellular second messenger signals, such as calcium\textsuperscript{45}. While activated, Ras can interact with numerous effector proteins to initiate downstream signaling, of which best-characterized is the Raf kinase\textsuperscript{46-49}, leading to activation of the mitogen-activated protein (MAP, also known as Erk, extracellular signal-
regulated kinase) kinase pathway. Furthermore, Ras-GTP interacts with lipid kinases, like the phosphatidylinositol-3 kinase (PI3K), which results in the increase of phosphatidylinositol-3,4,5-trisphosphate and subsequent phosphorylation of protein kinase B (PKB, also known as Akt) by 3-phosphoinositide-dependent protein kinases. Additionally, Ras also interacts with regulatory proteins of other GTPase proteins. For instance, signaling from Ras GTPase family member Ral, and Rho GTPase family member Rac, is initiated through Ras effector proteins (Figure 3). Interestingly, whereas Ras binds to and signals through p110α subunit of PI3K, Rho GTPase family members Ras-related C3 botulinum toxin substrate (Rac) and cell division cycle-42 (Cdc42) regulate PI3K through the other regulatory subunit.

The best-studied Rho GTPases are the members of the Rho, Rac and Cdc42 subfamilies, which have primarily been studied in their function as regulators of cytoskeletal reorganization in response to extracellular signals. RhoA-C enable the formation of stress fibers and focal adhesion assembly by actin polymerization, whereas Rac1, Rac3 and RhoG are responsible for lamellipodium formation as well as membrane ruffling and the members of the Cdc42 subgroup direct filopodia formation through actin polymerization. In addition to cytoskeletal reorganization, Rho GTPase proteins have been implemented in numerous other cellular responses, such as the involvement of Rho in smooth muscle contraction, Rac in the generation of reactive oxygen species (ROS), and Cdc42 in the endo- and exocytosis of vesicles.

Like Ras, active Rho GTPases promote the activation of numerous effectors of which the major pathways have been included in Figure 3. Some of these effectors are GTPase specific; Rho-associated protein kinases (ROCKs) and protein kinase N (PKN) are Rho downstream effectors that promote formation of stress fibers and focal adhesions, Rac-specific ROS-generating NADPH oxidase, as well as WASP family verprolin-homologous (WAVE), and Wiskott-Aldrich syndrome protein (WASP) or N-WASP as Cdc42-specific targets. While the interaction of Rac1 and Cdc42 is specific with WAVE and WASP, respectively, both effectors interact directly with Actin-related protein-2/3 (Arp2/3) complex to promote actin polymerization. Other shared downstream pathways are the c-Jun NH2-terminal kinase (JNK) and p38 MAP kinase cascade as well.
as NF-κB, by which Rho GTPases regulate gene transcription\textsuperscript{87-90}. Furthermore, many other effectors are directly shared, such as p21-activated kinase (PAK)-family kinases for Rac and Cdc42\textsuperscript{91-93}

**Figure 3** **Snapshot of the Ras/ Rho GTPase signaling networks.** Extracellular stimuli act through cell surface receptors, such as G-protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) as well as integrins, to recruit guanine nucleotide exchange factors (GEF), which in turn activate GTPases. Once activated, GTPase proteins can stimulate a wide range of different downstream pathways. Note that the system is a highly integrated network, with several feed-forward and feedback loops at several layers. For example, EGF receptor-mediated activation of promiscuous Ras/Rho GEF Sos initiates Ras signaling\textsuperscript{94}. Activated Ras initiates several signaling cascades, among which two best characterized are the Raf-Mek-Erk pathway\textsuperscript{46-49}, leading into gene expression that mediates cell cycle progression, and PI3K-Akt pathway\textsuperscript{48,49}, promoting cell survival. Furthermore, Ras signaling branching to the Ral and Rho GTPase signaling network by activating several GEFs. Rho GTPase activation results in cytoskeletal rearrangements, enabling cell motility as well as cytokinesis. For example, Rac1 and Cdc42 stimulate actin polymerization through Arp2/3 complex. The Arp2/3 complex also acts as a scaffold protein that binds Tiam1, which in turn will activate the Rac1-WAVE pathway and increased activation of the Arp2/3 complex\textsuperscript{95}. Arp2/3, actin-related protein 2/3; Cdc42, cell division cycle-42; Erk, extracellular
(Figure 3 continue) signal-regulated kinase; JNK, Jun N-terminal kinase; Mek, mitogen-activated protein kinase/Erk kinase; mTor, mammalian target of rapamycin; NF-κB, nuclear factor-κB; PAK, p21-activated kinase; PI3K, phosphoinositide 3-kinase; PLD, phospholipase D; Rac, Ras-related C3 botulinum toxin substrate; Ral, Ras-like protein; RalBP1, Ral-binding protein-1; ROCK, Rho kinase; Sos, son of sevenless; TIAM1, T-cell lymphoma invasion and metastasis-1; WAVE, WASP family verprolin-homologous; WASP, Wiskott-Aldrich syndrome protein. (Figure is based on Berndt, Hamilton and Sebti96, Repasky, Chenette and Der97, Schwartz98, Karnoub and Weinberg99 as well as Biro, Munoz and Weninger100.)

There is notable cross-talk between the Ras and Rho GTPase subfamilies, a good example being the direct regulation of PI3K by Rac and Cdc4261,62, as well as the indirect regulation via RhoA-mediated ROCK activation and subsequent stimulation of phosphatase and tensin homolog (PTEN)101,102, a direct suppressor of PI3K activity103,104. In turn, PI3K can activate Rho GTPase regulatory proteins105,106, mediating the activation of PAK family kinases by Rho GTPases and subsequent phosphorylation of MAP kinase Raf107,108.

The ADP ribosylation factor (Arf) and Ras-related proteins in brain (Rab) families direct intracellular vesicular trafficking42,109-112. With over 30 and approximately 70 family members in the Arf and Rab subfamilies, respectively42, they comprise more than half of the small GTPase proteins. The minimal amount of Rab proteins essential for a viable eukaryotic cell is eleven113. The substantial number of different proteins is the result of expansion by gene duplication that led to segregation by specialization of function in higher eukaryotes114. Localization and function in different transport processes is dependent on the C-terminal sequences (different cysteine-containing motifs, see Figure 2B) and post-translational modifications. Substitution of different prenyl anchors on Rab GTPases is followed by incorrect localization to their characteristic organelle membrane115.

Like Rab proteins, Arf proteins have distinct cellular localization116. This distinct localization is important for downstream signaling, as in vitro binding assays have shown that many Arf effector proteins can interact with more than one Arf protein117-119. For example, it has been suggested that the role of Arf6 at plasma membrane is similar to the role of Arf1 at the Golgi, however by spatial separation each protein fulfills unique
functions. Where Arf6 regulates endocytic membrane trafficking and actin remodeling, Arf1 controls the formation of coat protein I-coated vesicles, recruitment of clathrin through adapter protein complexes as well as the assembly of other organelle structures\textsuperscript{111,112}. Arf proteins, as well as Arf subfamily members Arf-like (Arl) and more distantly related secretion-associated and Ras-related (Sar) proteins, regulate vesicle budding from donor compartments, while Rab subfamily member proteins regulate the subsequent steps in the endo- or exocytotic vesicles trafficking pathway, like the coupling of vesicles to motor proteins, regulating vesicle motility as well as the formation of large protein complexes required for tethering and subsequent docking and fusion of the vesicles with the acceptor compartments (reviewed in [109,120]).

The single family-member Ras-like nuclear (Ran) protein\textsuperscript{42}, presumably the most abundant small GTPase in the cell\textsuperscript{121}, has an essential role in the nucleocytoplasmic transport of macromolecules, such as ribonucleic acid (RNA) and proteins\textsuperscript{122,123}. In interphase cells, GTP-bound Ran is highly compartmentalized in the nucleus, whereas GDP-bound Ran resides in the cytoplasm\textsuperscript{124,125}. This spatial gradient of the GTP-bound Ran facilitates the directionality of nuclear import and export\textsuperscript{126,127}. To illustrate, in the cytoplasm, GDP-bound Ran binds to importins and their cargo and subsequently translocates to the nucleus. In the nucleus, Ran becomes GTP-bound by nucleotide exchange and as a result the complex dissociates. Exportins, on the other hand, need GTP-bound Ran to transport their cargo and will release this upon GTP hydrolysis in the cytoplasm. Furthermore, in addition to its function in interphase cells, Ran GDP/GTP cycling was demonstrated to be involved in mitotic spindle formation\textsuperscript{128-131}, as well as the induction of the nuclear envelope assembly\textsuperscript{132,133}.

\textbf{2.2 Regulators of small GTPases}

Most small GTPases have an intrinsic ability to exchange the bound nucleotide and to hydrolyze bound GTP, yet, \textit{in vivo}, this intrinsic ability is accelerated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively\textsuperscript{45,134}. Being able to modulate
GTPase activity, GEFs and GAPs are controlling GTPase-mediated signaling. So-called adaptor and scaffold proteins assist by attracting the proteins needed in the signaling pathway, localizing GTPase, GEF and GAP proteins to specific places at the cellular membrane and thereby determine which downstream pathways are activated. To illustrate, the activation of the RasGEF son of sevenless (Sos)\textsuperscript{135,136} through the epidermal growth factor (EGF) receptor tyrosine kinase mediates Ras signaling\textsuperscript{94} (Figure 3). Growth factor receptor-bound protein 2 (Grb2), an adaptor protein that is recruited to tyrosine-phosphorylated receptors, facilitates Ras activation by binding to the EGF receptor with its Src homology (SH) 2 domain, and Sos protein with its SH3 domain\textsuperscript{137}. In turn, Ras-GTP can bind RhoGEF T-lymphoma invasion and metastasis-inducing protein 1 (Tiam1)\textsuperscript{138}, which results in the activation of Rac signaling\textsuperscript{59,139}. However, in addition to a RasGEF domain, Sos contains another GEF domain that is Rac1 specific and, in complex with the scaffold proteins Abi1–Eps8 and PI3K, enables Rac signaling\textsuperscript{140,141}. The adaptor protein Grb2 and scaffold protein Abi1 interact with the same region of Sos, thereby facilitating the differential regulation of Ras versus Rac activity of Sos\textsuperscript{140}. While Sos is unique, having two GEF domains, similar complex dependent pathways have been described for other GEFs. For example, the interaction of the RhoGEF Tiam1 / Rac1 complex with insulin receptor substrate p53/p58 leads to actin polymerization\textsuperscript{142}, whereas the interaction of this complex with spinophilin activates p70 S6 kinase\textsuperscript{143}.

2.2.1 Guanine nucleotide exchange factors
Since GEFs are required for the rapid exchange of GDP for GTP, the modulation of their catalytic activity is considered the most common mechanism to alter GTPase-mediated signaling, thereby linking activated plasma membrane receptors to downstream GTPases as well as mediating the signaling from one GTPase pathway to the other (Figure 3). The predominantly membrane-associated GEF proteins catalyze the exchange of GDP by causing a conformational change of the small GTPase that lowers the affinity for the nucleotide as well as stabilize the nucleotide-free reaction intermediate\textsuperscript{144}. The mechanism by which GEF proteins catalyze the nucleotide exchange is depending on the 20-30 kDa family-specific catalytic domain. Although the GEFs are structurally unrelated, the
multistep GEF-dependent nucleotide exchange follows a common principle (Figure 4A), which was first shown with Ras/Sos\textsuperscript{145} and Arf/Sec7\textsuperscript{146}.

GEF-GTPase binding causes a conformational change of the small GTPase protein structure, where switch I region moves away from the nucleotide-binding site\textsuperscript{145,146}. Extensive interaction of the GEF with switch II region ensures that the nucleotide-free GTPase is stable\textsuperscript{145,146}, but also contributes to the family-specificity of the GEF-GTPase interaction that is observed for RhoGEFs\textsuperscript{147}. These conformational changes disturb the
interaction of the conserved lysine from the phosphate binding-loop with the β-phosphate of GDP. In the case of Ras/Sos, but not for Arf-Sec7, the resulting hydrophobic repulsion created towards the Mg$^{2+}$ ion, enhanced by the alternative positioning of the conserved alanine, further lowers the affinity towards the GDP$^{148}$. In the new configuration the phosphate groups of the nucleotide will no longer be stabilized and accordingly be released first, followed by the base$^{145,148}$. Small GTPases generally do not discriminate between GDP and GTP in terms of binding affinity, but, because of the excess of cellular GTP over GDP$^{149}$, the release of bound GDP is followed by subsequent binding of the more abundant GTP. The new nucleotide will bind base first to the nucleotide-free GTPase and, as a result weaken the affinity for the GEF, subsequently displacing it altogether.

For most GTPase families the number of GEF proteins is approximately the same as the number of GTPase proteins. However, the Rho subfamily forms an exception to this rule, as the GEFs outnumber the GTPase proteins 4:1$^{150}$. The most studied Ras- and RhoGEF proteins are characterized by the CDC25 homology domain, often in combination with a Ras exchange motif (REM), and the Dbl homology (DH) and pleckstrin homology (PH) domains, respectively, which together provide the minimal structural unit that is necessary for GEF activity and target GTPases recognition as well as cellular localization$^{136,151-154}$. In addition to the tandem DH-PH domain containing RhoGEFs, a second, structurally and mechanistically different, subfamily of RhoGEF exists that contains a Dock homology region (DHR) domain$^{155}$. This subfamily of RhoGEFs catalyzes the nucleotide exchange reaction of Rac and/or Cdc42, but not RhoA (reviewed in [156,157]).

In addition to the domains forming the minimal structural unit, GEF proteins typically contain protein-specific domains that define the unique cellular functions of the different family members by regulating their localization, formation of signaling complexes and/or activation$^{5,157}$. This essential diversity in domain structures is best illustrated by the promiscuity of the RhoGEFs; there is a large set of RhoGEFs that can activate RhoA, Rac1 and Cdc42 in vitro, yet in vivo each GEF activates their target GTPases in a pathway specific manners$^{157,158}$. For example, specific phosphorylation of Tiam1 by mitotic kinase cyclin-dependent
kinase 1 (Cdk1) primarily results in the Rac1-mediated activation of PAK1/2\textsuperscript{159}, whereas Rac1 activation by Vav primarily results in the activation of the JNK cascade\textsuperscript{160,161}.

These additional domains, as well as the PH domain in RhoGEFs, control activation by providing a mechanism for intramolecular inhibition as they fold around the catalytic domain and prevent interaction with the GTPase. To illustrate, the active site of nonphosphorylated Vav proteins\textsuperscript{162} is obstructed by its N-terminal CH-Ac region that is folded over catalytic core\textsuperscript{163}. Upon phosphorylation of tyrosine residue 174, by Src and Syk kinases\textsuperscript{164-166}, the inhibited structure is relieved\textsuperscript{163}. In fact, using NIH3T3 fibroblast transformation assays\textsuperscript{167}, most GEF proteins, e.g. Dbl\textsuperscript{168}, Vav\textsuperscript{169}, Tiam\textsuperscript{138} and epithelial cell transforming sequence 2 (Ect2)\textsuperscript{170}, were originally discovered from human tumor-derived DNA as amino-terminal truncated, activated versions of the normal gene product that were able to demonstrate transforming properties, such as the loss of density-dependent growth inhibition as well as form colonies in soft agar, and, therefore, dubbed oncogenes.

2.2.2 GTPase activating proteins
The intrinsic GTPase activity of small GTPases is generally slow and GAPs are required to stimulate the GTP-hydrolysis activity, which in turn inactivates small GTPase signaling\textsuperscript{5,134,171,172}. For Rho and Ras GTPase proteins, \textit{in vitro} GTP hydrolysis occurs at higher rates than nucleotide exchange. While the nucleotide exchange rate is increased by GEFs \textit{in vivo}, in resting cells, only low levels of GTP-bound small GTPases are found, which implies that GAPs actively maintain GTPase-mediated signaling\textsuperscript{173}.

GAPs can be seen as effectors, regulating signaling by specifically turning off pathways. Rac1-mediated activation of GIT1, an Arf GAP, results in the decrease of active Arf6 and subsequent recycling of clathrin-independent endosomes\textsuperscript{174}. By turning off pathways, competitive pathways can become active. Rac1 activity is suppressed by MgcRacGAP, which is shown to result in increased activity of RhoA driving invasive migration event\textsuperscript{175}. Furthermore, oncogenic Ras mutants not only have an impaired intrinsic GTP hydrolysis, the oncogenic transformation by
constitutive activation is mainly driven by the loss of GAP-mediated inactivation\textsuperscript{176}, illustrating that turning off pathways is as important as turning them on.

Like GEF proteins, GAP proteins are not conserved between different GTPase families. Accordingly, the reaction mechanism by which GAP proteins stimulate the GTP-hydrolysis reaction is different between families of small GTPases (reviewed in [134]). However, the tertiary structure as well as the mechanism of the GAP-assisted hydrolysis was found to be similar for Ras and Rho GTPases\textsuperscript{177}.

During the GAP-stimulated hydrolysis, the 20-50 kDa GAP domain provides an arginine residue, the so-called arginine finger\textsuperscript{178,179}. The arginine finger neutralizes the negative charge on the $\gamma$-phosphate during the transition state, as well as stabilizes the position of a conserved glutamine in the switch II domain of the GTPase (residue 61 in Ras and 63 in Rho) (Figure 4). The conserved glutamine serves as catalytic site for the in-line nucleophilic attack of a water molecule on the $\gamma$-phosphate of the GTP.

Whereas it has been shown that GEF-mediated nucleotide exchange is a multistep process, it is unclear whether GAP-GTPase association and arginine finger positioning are two separate steps. In case of p50RhoGAP and Cdc42, two different orientations of the arginine finger have been shown\textsuperscript{179,180}, which would indicate it may be a multistep process. While recent work addressing this issue could not answer this as yet, the derived data supports a general concept for GAP-mediated GTP hydrolysis in which any residue that can be moved into the binding pocket and drive out the water molecules would be able to stimulate the GTPase reaction\textsuperscript{172}.

The GAP domain alone is sufficient for both the binding to, as well as stimulating the GTPase activity of GTP-bound GTPase proteins\textsuperscript{181}, which implies that the multidomain GAPs are, like GEF proteins, regulated through the flanking domains. The majority of flanking domains is restricted to one or two subfamilies of GAP proteins, with the exception of the most common motifs, the SH3 domain and PH domain (reviewed in [171]). For each GTPase family, there are less or as many GAPs as
GTPase proteins, except for the Rho family, where the GAPs outnumber the GTPase proteins 3:1\textsuperscript{150}. Accordingly, the most diverse set of flanking domains is found in the RhoGAP family, suggesting a diverse variety in regulating mechanisms.

Flanking domains are involved in e.g. protein-protein interactions, protein-lipid binding and intramolecular inhibition, as well as provide protein kinase phosphorylation sites. For example, during metaphase the GAP activity of MgcRacGAP is sequestered while in complex with protein regulator of cytokinesis 1 (PRC1)\textsuperscript{182}. Phosphorylation of the N-terminal region of MgcRacGAP by Aurora B kinase results in the loss of the protein-protein mediated inhibition\textsuperscript{182}. A more complex mechanism of regulation has been found for β2-chimaerin\textsuperscript{183}, a RhoGAP consisting of an N-terminal SH2-domain that sterically blocks the adjacent C1-domain as well as the C-terminal GAP-domain. Membrane interaction leads to a large conformational change of the protein, which subsequently enables diacylglycerol (DAG), a second messenger signaling lipid, to compete with the N-terminal segment that is otherwise tightly bound by the C1-domain. Upon binding of DAG, the N-terminal segment is displaced and no longer inhibits Rac binding. Furthermore, it is believed that other GAPs, like GEFs, reside in a folded conformation that provides an intramolecular inhibition or reduces the GAP activity, though the mechanism of autoinhibition are much less well understood than for GEFs.

2.2.3 Guanine nucleotide dissociation inhibitors

Most small GTPases require association with cellular membranes in order to perform their biological functions. The addition of a lipid moiety is not only crucial for the membrane anchoring, but also provides the third mechanism of regulation (Figure 1). Guanine nucleotide dissociation inhibitors (GDIs) were initially thought to block spontaneous activation of small GTPases, thereby stabilizing the inactive form\textsuperscript{184}. However, GDIs have only been found in the Rho and Rab families of small GTPases\textsuperscript{134,150}. In addition to the Rho and Rab GDI proteins, another regulatory protein with a similar function was more recently found for Ras GTPases; the delta subunit of retinal rod phosphodiesterase (PDEδ), which can extract farnesylated Ras proteins from the membrane\textsuperscript{185,186}. Current knowledge suggests that these regulatory proteins actively extract prenylated
GTPases, either GTP- or GDP-bound, from the membrane by forming high-affinity complexes, thereby sequestering their target GTPases in the cytosol, which as such are unable to interact with their membrane bound effectors\cite{109,134,187,188}. More specifically, Rab GDIs function as regulating chaperones, extracting Rab proteins from the acceptor membrane, recycle them to the cytosol and delivering them back to the donor membrane\cite{189}.

The Rho and Rab GDI proteins are unrelated, but both function through a two-site interface with the target GTPase\cite{134,187}, which is illustrated with the following RhoGTPase example. The N-terminal domain of the RhoGDI recognizes the switch region of the GTPase, which affects the GDP–GTP cycling. The C-terminal domain interacts with the prenylated C-terminus of the GTPase, binding the lipid tail in its hydrophobic pocket and thereby regulating the membrane / cytosol distribution. Unlike the Rho and Rab GDI proteins, PDEδ lacks a GTPase binding domain\cite{134,185}. Accordingly, binding of Ras GTPases by PDEδ occurs through a single domain that is structurally related to the RhoGDI lipid-binding domain\cite{134,185}. As a result, PDEδ is less specific than Rho and Rab GDI proteins, and has been reported to be able to interact with non-prenylated proteins such as Arf GTPase Arl3\cite{185}.

While the lipid moiety is docked in the hydrophobic pocket, it is believed that the more diverse C-terminus of the GTPase is not completely masked\cite{190}. With the exposed part, the GTPase is able to interact with adaptor proteins in the cytosol or at the plasma membrane\cite{190}. As a result, Rho and Rab GDI proteins can be specifically regulated by kinase-mediated phosphorylation of the GDI and GDI displacement factors (GDFs)\cite{188,190}. To illustrate the first, phosphorylation of RhoGDI by PAK1 results in the release of Rac1, but not RhoA or Cdc42\cite{191}. In the second case, GDFs possess, like GDIs, a hydrophobic pocket that can bind a lipid moiety. When the Rab-GDI complex is back at the donor membrane, where the GDFs are located, the Rab prenyl tail will transfer from the GDI to the GDF, leaving Rab at the membrane in complex with GDF\cite{189}. In both cases, the GTPase will dissociate from the GDI, generally resulting in GEF-mediated activation of the GTPase and initiation of downstream signaling.
2.2.4 GTPase regulation by kinases and phosphatases

GEF and GAP proteins are regulated through phosphorylation and subsequent dephosphorylation by kinases and phosphatases, respectively. However, it has been shown that also GTPases are regulated by phosphorylation/ dephosphorylation, with examples found in almost all GTPase families, such as RhoA\textsuperscript{192,193} and Cdc42\textsuperscript{194}, Rab24\textsuperscript{195}, Ran\textsuperscript{196} as well as R-Ras\textsuperscript{197} and Ras\textsuperscript{198-200}. Paradoxically, Ras-mediated Raf signaling is terminated by the GAP-stimulated hydrolysis, while Raf binds to Ras with a nanomolar affinity\textsuperscript{15}, many fold greater than that of RasGAPs p120RasGAP and neurofibromin (NF-1) (0.1-10 µM)\textsuperscript{201}. Phosphorylation of Ras Y36 enables the GAP to compete off the Raf and shutting down Ras signaling\textsuperscript{198}. Vice versa, dephosphorylation of Ras by tyrosine-protein phosphatase non-receptor type 11 (also known as SHP-2) enhances Ras association with Raf and enables downstream signaling\textsuperscript{199}. While similar regulatory mechanisms have been found for most other small GTPase families\textsuperscript{194-197}, it is still too early to conclude whether this is a small GTPase wide mechanism that should be incorporated in the traditional GTPases cycle (Figure 1) as regulatory node. For example, the phosphorylation of RhoA S188 seems to represent an extra switch that dedicates what specific downstream signaling pathways to activate\textsuperscript{193}, rather than controlling the on/off switch.

GTPases, together with GEFs, GAPs and GDIs, function as the signaling nodes for many pivotal regulatory pathways. These pathways are not parallel sequential cascades, but rather highly integrated signaling networks, where, under tight temporal control and distinct spatial distribution, different pathways can function separately, but also interact with one and other. Thus, a defect in the GTPase regulatory system can be compensated by redundancy, but for key nodes, alterations can have devastating results.

2.3 Small GTPases in cancer

Due to the role of small GTPases in a variety of essential cellular processes, aberrant function and/or regulation has long been associated with multiple human diseases, like various forms of cancer,
neurodegenerative diseases, cardiovascular diseases, endocrine diseases and inflammatory disorders\textsuperscript{1,4,8,150,202-206}. In the majority of cases, uncontrolled activation of the GTPase causes aberrant downstream signaling. There are four mechanisms that cause an accumulation of activated GTP-bound GTPase; i. increased GEF activity, ii. decreased GAP activity, iii. mutations in the small GTPase causing an impaired intrinsic GTP hydrolysis ability (Figure 1), and iv. overexpression of the GTPase.

Advances in genome sequencing have made it possible to identify and map all occurring sequence abnormalities and mutations in cancer. The most commonly found driver mutations, found in approximately 30% of all human cancers, result in the mutationally activated variants of the Ras GTPases\textsuperscript{207,208}. In the three RAS genes, two hot spots have been identified at codon positions 12-13 and 61, coding for the tandem glycine glycine in the P-loop and the conserved glutamine in switch region II of the Ras proteins, respectively (Figure 5A). During the GAP-mediated hydrolysis reaction, the arginine finger is inserted into the GTPase along side of glycine residue 12\textsuperscript{178}. Since glycine is the smallest amino acid, any mutation will introduce a more bulky amino acid, generating a mutant that is insensitive to hydrolysis because the arginine finger can no longer be inserted. The glutamine at residue 61 is also involved in GTP hydrolysis, where it serves as the catalytic site for the in-line nucleophilic attack\textsuperscript{178,179}. Accordingly, oncogenic mutations of codon 61 reduce the intrinsic GTP hydrolysis rate and, like the oncogenic mutations at position 12-13, result in a constitutively active Ras protein (Figure 5B).

Interestingly, the incidence of RAS mutations is not only spread differentially over the isoforms, each isoform has a distinctive codon mutation signature as well as tissue-specific associations\textsuperscript{207,208}. For example, in Ras-driven cancers the KRAS isoform is most frequently mutated (>85%), followed by NRAS (~10%) and HRAS (<5%)\textsuperscript{209} (Figure 5C). Mutations in the KRAS gene occur predominately at codon 12 and only a few mutations are observed at codon 61, whereas mutations in NRAS are found twice as often at codon 61 compared to codon 12\textsuperscript{9,208,209} (Figure 5C). Last, generally only a single mutant isoform is observed in different types of cancer tissue, e.g. pancreatic ductal adenocarcinoma seems exclusively driven by the KRAS mutations, while malignant melanoma is predominately driven by NRAS mutations\textsuperscript{207,208}. 

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Figure 5 Oncogenic mutations of Ras and isoform specific mutation bias. A) The three key oncogenic mutations, at codon 12, 13 and 61, occur in the region where the Ras isoforms are identical. Visualized in the schematic representation of the Ras protein are; the nucleotide binding domains (G1-5, gradient black), the effector binding domain (residues 32-40, pink), the switch regions (S1 and SII) and the hypervariable region (HV, blue). Below is given the isoform sequence similarity percentage between the Ras isoforms with in subscript the corresponding regions by amino acid numbering. B) The oncogenic mutation at codon G12, G13 and Q61 impair GAP-stimulated GTP hydrolysis resulting in a constitutively active Ras protein. C) The approximate frequencies of Ras mutations by isoform (bigger circle more mutations found) and by codon (colors of the pie chart). (Image adapted from Cox and Der\textsuperscript{209} and Prior, Lewis and Mattos\textsuperscript{208}.)

In contrast to the high oncogenic mutation rates in RAS encoding genes, RHO genes are rarely mutated in cancer\textsuperscript{210}. Only two mutational hotspots have been found and, remarkably, they are at different locations of the GTPase protein than the Ras oncoproteins; one causing a so-called fast-cycling mutant of Rac1 (P29S) in melanoma\textsuperscript{211,212} and the other inducing a dominant negative loss-of-function for RhoA (G17V) in gastric cancer\textsuperscript{213,214}, as well as in angioimmunoblastic T cell lymphomas\textsuperscript{215,216}. Instead, recent reviews have shown that Rho GTPase activation is generally a result of deregulated gene expression\textsuperscript{1,210}, and/or (mutationally) altered RhoGEF and/or RhoGAP activity\textsuperscript{4,158}. Of note, in addition to mutational activation, upstream deregulation also happens for
Ras GTPases, where loss-of-function mutations in the p120RasGAP and NF-1 RasGAPs, as well as gain-of-function mutations in RasGEF Sos1 cause Ras hyperactivation in different Ras-related diseases\textsuperscript{8,9,134}.

Mouse models have shown that the deregulation of Rho GTPases in embryonic development leads to severe developmental defects, with Rac1 and Cdc42 as well as regulators Sos1, Trio and Ect2 deficient mice being embryonic lethal\textsuperscript{158,217}. It is clear that in development these proteins are essential for normal physiology, but aberrant expression later in life results in reactivation of small GTPase-regulated developmental pathways, like Wnt\textsuperscript{218}, Notch\textsuperscript{219}, and Hedgehog\textsuperscript{220,221}.

A recent study identified chromosome 3q26 to be frequently amplified, estimating that copy number gains occur in about 20\% of human tumors\textsuperscript{222}. Located on chromosome 3q26 are, amongst others, genes that encode for Ect2, p110\(\alpha\) subunit of PI3K, protein kinase C iota (PKCi) and transcription factor SOX-2. Interestingly, most of these proteins have a role in different Rac GTPase signal transduction pathways. GTP-bound Ras binds to and signals through p110\(\alpha\) subunit of PI3K\textsuperscript{53,54}, whereas PI3K activates Rho GTPase regulatory proteins\textsuperscript{105,106}, and as such mediates activation of Rac1. Work from different labs has shown that Rac1 is required for Ras induced tumorigenesis in different type of cells\textsuperscript{223-225}. Moreover, it was shown that the complex of PKCi and partitioning defective homolog (PAR) 6\(\alpha\) regulates the cytoplasmic localization of Ect2, also leading to Rac1 activation and subsequently inducing cellular transformation of non-small cell lung cancer\textsuperscript{226}. These findings are largely in line with earlier observations describing that Ect2 cytoplasmic mislocalization results in the activation of Rho GTPase signaling, though RhoA was predominately activated, and leads to malignant transformation of NIH 3T3 cells\textsuperscript{227}. Consistent with the latter results is the report that cytoplasmic Ect2 preferentially activates RhoA\textsuperscript{228}. On the other hand, this same study showed that not cytoplasmic Ect2, but the localization of phosphorylated Ect2 to the nucleus and the subsequent activation of Rac1 drives ovarian cancer cell transformation. Of note, in interphase the Rac1 specific GAP MgcRacGAP is also localized in the nucleus\textsuperscript{229}. Furthermore, it was demonstrated that inappropriate activation of PKCi resulted in the activation of pro-survival transcription factor proteins, such as STAT3 and transcription factor p65 (also known as NF-\(\kappa\)B). Interestingly, interactions
between MgcRacGAP, GTP-bound Rac1 and STAT3 have been described, but I will return to this in section 2.4.

Phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger (P-Rex) 1 and 2 are RhoGEFs that primarily activate Rac1, thereby mediating Rac signaling downstream of G protein-coupled receptors and PI3K. P-Rex1 overexpression has been reported in melanoma cell lines as well as human tissue, driving invasion in a Rac-dependent manner. Another study identified P-Rex2 to be frequently mutated in human melanoma and, to demonstrate the functional relevance, missense and truncation mutants were expressed in TERT-immortalized NRAS mutant human melanocytes, transplanted into immunodeficient mice and shown to increase the rate of tumor formation. Given that Rac1 mutants are found exclusively in melanoma, it underscores the important driver function of Rho GTPase mediated signaling in cancer.

2.4 Targeting small GTPase signaling as drug discovery approach

With the profound role of GTPase in biology and uncontrolled GTPase activation driving aberrant downstream signaling, it is interesting to be able to control GTPase signaling. In the following section I present an overview of small molecule inhibitors that have been developed to target different nodes of small GTPases regulation (Figure 6). Furthermore, I aim to address the wide range of screening methods used and different mechanisms of inhibitory activity that have been discovered (see Table 1). The overview is primarily focused on compounds modulating Ras and Rho GTPase signaling.

2.4.1 Drug discovery approaches

In general, there are two major strategies for drug discovery. Most of the compounds that will be discussed have been discovered through so-called “bottom-up” approaches, starting at the protein level. Through drug discovery history these bottom-up approaches have been immensely
Table 1 Overview GTPase activity modulating compounds discussed in this thesis. N.a., not applicable;

<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
<th>Assay type</th>
<th>Ref.</th>
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<td>EHop-016</td>
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<tr>
<td>AZA1</td>
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<td>Rational design based on NSC23766</td>
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</tr>
<tr>
<td>AZA197</td>
<td>Cdc42-GEF</td>
<td>Rational design based on NSC23766</td>
<td>249,250</td>
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<tr>
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<td>Docking-based virtual screening</td>
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<td>Fragment-based using NMR</td>
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</tr>
<tr>
<td>DCAI (DCIE)</td>
<td>Ras-Sos</td>
<td>Fragment-based using NMR</td>
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<tr>
<td>N.a.</td>
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<td>Phenotypical screening assay</td>
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<td>Compound screening in canine model</td>
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<td>N.a.</td>
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<tr>
<td>deltarasin</td>
<td>PDEδ</td>
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a. Multiple compounds were identified in this screen, but not given names.
b. Tipifarnib was developed by Johnson & Johnson, no reports are available in scientific literature describing the initial discovery and the methods used.
Figure 6 *Nodes in the small GTPase cycle targeted for drug discovery.* There are several regulatory nodes in the GTPase cycle that have been exploited for small molecule-mediated modulation; 1) lock GTPase in GDP-bound state, 2) block GEF-mediated nucleotide exchange, 3) compete with GTP for nucleotide binding pocket, 4) disrupt GTPase-effector binding, 5) inhibit downstream effector signaling, 6) target posttranslational modification, e.g. the addition of a farnesyl group by farnesyl transferase (FTase) and 7) interfere with the regulatory function of GDIs. GAP, GTPase activating protein; GDI, guanine–nucleotide-dissociation inhibitor; GDP, guanosine diphosphate; GEF, guanine exchange factor; GTP, guanosine triphosphate; P<sub>i</sub>, inorganic phosphate.

useful in identifying potent and selective compounds, however they have always suffered from three distinct problems; i. they are limited to measurable functions of a protein, for example enzymatic activity, ii. *in vitro* successes do not guarantee *in vivo* activity as a compound can exhibit poor cell permeability and iii. unexpected off-target cellular effects might not be easily diagnosed.

The opposite strategy, the “top-down” approach, starts at the phenotypic level and circumvents these challenges. However, phenotypic screening generally experiences challenges in distinguishing observable phenotypes. In case of the Rhodblock compounds, a screening strategy was used that was analogous to a genetic modifier screen; cells were sensitized using RNA interference (RNAi) to impair signaling through the Rho pathway and subsequently treated with small molecules. As a result of
the partial depletion of Rho, cytokinesis was successful only in half of the cases. Compounds that suppressed or enhanced the phenotype, meaning either increased success or failure of cytokinesis, respectively, were selected for follow-up secondary assays and target identification. By this screening approach nine so-called Rhodblock inhibitors were discovered that have different targets within the Rho pathway in cytokinesis. One compound, Rhodblock 6, directly interfered with the Rho pathway activity by inhibiting Rho kinase. The other compounds indirectly interfered. For example, Rhodblocks 1a and 3 disrupted the important scaffolding functions of Rho-regulated proteins, such as Anillin and the septins, resulting in the mislocalization of several important Rho pathway proteins and decreased Rho pathway activity. While these compounds can be used to gain insight in the Rho pathway, the exact targets for most are unknown, making it more challenging to improve them to clinically relevant compounds.

2.4.2 Nucleotide binding inhibitors

The crucial role of GTP-bound GTPases in oncogenic transformation marked them as potential therapeutic target in cancer. Accordingly, early studies aimed to inhibit GTP binding by nucleotide-competitive small molecules, in a similar way that protein kinase inhibitors were designed to compete for binding with adenosine triphosphate (ATP). In contrast to protein kinases, which typically show micromolar binding affinities toward ATP, most small GTPases have picomolar affinity toward guanine nucleotides, making them challenging targets for nucleotide-competitive small molecule inhibitors.12

Nevertheless, two Rho family inhibitors that interfere with the nucleotide binding have been identified. Through an NIH Molecular Libraries Program screening campaign (PubChem AID 1772), using a multiplex flow cytometry bead-based assay, several lead compounds were identified that interfered with GTPase nucleotide binding.236-240 In particular, one class of these modulators was identified as selective Rho family GTPase Cdc42 inhibitors, with compound ML141 triggering nucleotide release and locked Cdc42 GTPase in an inactive confirmation, thus interfering with Cdc42-dependent cellular processes such as formation of filopodia.238,240 Furthermore, originally found as an inhibitor of β-amyloid processing,
EHT-1864 was shown to block the Rac1 signaling pathways\cite{241,242}. A subsequent study of the mechanism of action revealed that, as a nucleotide binding inhibitor of all Rac GTPases isoforms, EHT-1864 blocked GEF-mediated nucleotide exchange, preventing downstream effector binding and thereby inhibited Rac-dependent processes in cells\cite{275}. To-date, due to the high affinity of small GTPases towards their nucleotides, no clinically relevant nucleotide-competitive small molecules have been developed.

### 2.4.3 Inhibitors of GTPase-GEF interaction

As a result of the high affinity of small GTPases towards their nucleotide, GEF proteins are required to accelerate the slow intrinsic exchange reaction in vivo\cite{5,134}. Accordingly, a common strategy to inhibit GTPase activation is to interfere with the small GTPase-GEF interaction. However, Ras GTPases are frequently mutationally activated (reviewed in \cite{207,208}) and, as a result, impaired in their ability to hydrolyze GTP allowing these mutants to remain in active GTP-bound state even in the presence of a GAP\cite{178}. Inhibiting GEF-mediated activation is only interesting, if the target GTPase has the ability to hydrolyze GTP. Rho GTPases are rarely mutated, and their activation is often a result of deregulated expression and/or upstream activity of (mutated) regulators\cite{1,158,210,276}. Therefore, I will largely focus on inhibitors of GTPase-GEF interaction concerning the Rho GTPase family and to a lesser extent on the Ras GTPase family.

Rho GTPase proteins contain a ridge between two shallow pockets within the GEF binding area that determines the unique GEF specificity (F56 of Cdc42, W56 in Rac1)\cite{277,278}. The importance of the surface groove was exploited for a series of virtual screening campaigns, which identified Rac1-GEF interaction inhibitors NSC23766\cite{243} and ZINC69391\cite{244} as well as Cdc42-GEF specific inhibitor ZCL278\cite{251}. NSC23766 dose-dependently inhibited Rac1 activation by the Rac1 W56-interacting Rac-selective GEFs Trio and Tiam1\cite{243}, yet had no effect on nucleotide exchange activity stimulated by the promiscuous GEF Vav1, which does not interact with W56\cite{277}. The interaction between other Rho proteins, such as RhoA and Cdc42, and their GEFs or the interaction between Rac1 and the GAP breakpoint cluster region protein (BCR) was also not affected by
NSC23766. ZINC69391 was identified and served as lead inhibitor for the subsequent rational design of analog 1A-116\textsuperscript{244}. 1A-166 was shown to inhibit Rac-mediated cell proliferation in normal full growth media condition as well as to reduce of lung colonization in mice. ZCL278 is a low micromolar inhibitor of the interaction between Cdc42 and its GEF intersectin\textsuperscript{251}. In vitro, ZCL278 inhibits Cdc42-mediated microspike formation, reduces the amount of active Cdc42 in fibroblast cell cultures as well as suppresses actin-based motility and wound healing in a metastatic prostate cell line. A similar recognition motif is present around residue W58 of RhoA and is only shared with close relative isoforms RhoB and RhoC\textsuperscript{252}. A virtual screen that was designed to identify compounds binding around residue W58 identified Rho-specific inhibitor Rhosin. In cellular assays Rhosin inhibited Rho-specific downstream signaling, but did not affect Rac1 or Cdc42 downstream signaling.

Instead of screening, using previously published molecules as lead compound and subsequently improve biochemical properties has proven to be feasible. The optimization of NSC23766 by rational design led to the identification of EHop-016, an inhibitor that binds the effector domain of Rac1 and inhibits Rac1 activity at physiologically relevant concentrations of 1 µM\textsuperscript{247,248}. Unlike NSC23766, EHop-016 reduces the interaction of RhoGEF Vav2 with Rac1, with only modest activity towards Cdc42 and none towards RhoA\textsuperscript{247}. Furthermore, EHop-016 was shown to significantly reduce mammary fat pad tumor growth, metastasis, and angiogenesis in a nude mouse model of experimental metastasis\textsuperscript{248}.

While rational design using NSC23766 as lead compound has generated a Rac-specific inhibitor EHop-016, similarly derived close related analogs AZA1 and AZA197 showed altered selectivity\textsuperscript{249,250}. AZA1 was shown to inhibit the GEF-mediated activation of both Cdc42 and Rac1, but not RhoA, in dose-dependent matter\textsuperscript{250}. Notably, AZA1 restrained proliferation and cellular migration of prostate cancer cells as well as reduced growth of human xenografts in mice. On the other hand, AZA197 was shown to be selective towards Cdc42\textsuperscript{249}. Using colon cancer cells, it was demonstrated that AZA197 specifically inhibited nucleotide exchange of Cdc42, without inhibition of Rac1 or RhoA GTPases. Like AZA1, AZA197 treatment inhibited cancer cell proliferation and migration as well as
reduced downstream signaling though PAK1 *in vitro* and significantly increased mouse survival in tumor xenografts models.

ITX3, a specific inhibitor of Rac1 activation by Trio, was identified through a screening procedure using a yeast exchange assay to select inhibitors that specifically block the activation of RhoG by TrioN, the N-terminal GEF domain of the multidomain protein Trio^{245,246}. *In vitro*, ITX3 inhibited nucleotide exchange on RhoG and Rac1, as well as nerve growth factor-induced neurite outgrowth in PC12 cells, a process that is mediated by the N-terminal RhoG and Rac1-targeting domain of Trio. However, the compound is only effective at high >50 µM concentrations.

Two independent studies using different NMR-based fragment library screening approaches, identified the same novel hydrophobic pocket between switch regions I and II of Ras^{256,257}. One of the hit compounds, DCAI, was shown to bind to Ras and inhibit Sos-mediated nucleotide exchange by blocking the interaction between Sos and Ras^{257}. The binding to Ras did not affect the intrinsic nucleotide exchange by Ras. Notably, half maximal effective concentration (EC_{50}) values derived from cell-based experiments, *e.g.*, EGF-stimulated Ras activation as well as subsequent recruitment of Raf to the cytoplasmic membrane, were a full log-fold more potent than the biochemical assays, raising concerns about possible off-target driven effects.

### 2.4.4 Locking the complex of macromolecules by interfacial inhibition.

The concept of interfacial inhibition utilizes the dynamic behavior of protein complexes and the ability of small molecule compounds to bind at such interfaces with high selectivity locking the complex in one state. The natural compound Brefeldin A inhibits the activity of the Arf GEF Sec7 by stabilizing the complex between Sec7 and Arf1, thereby blocking nucleotide exchange^{253,254}. Brefeldin A is a prototypical interfacial inhibitor, locking the complex of macromolecules in a nonproductive transition state that is unable to fulfill its biological function^{279,280}. This mode of inhibition is distinct from the previous compounds, which inhibit the interaction between small GTPase and GEF.
Though efforts have been made, only a handful of these compounds have been described to date. In case of Ras GTPase, a fragment screening campaign using X-ray crystallography was performed and three distinct small molecule binding sites were identified at the interface between H-Ras and Sos\textsuperscript{255}. However, the ligands identified to bind reversibly to the Ras-Sos complex were not sufficiently potent to show measurable stabilization of the complex. While the interfacial inhibitory compounds identified were not sufficiently potent, the reactive compounds that covalently bound to residue C118, were shown to completely inhibit K-Ras-Sos functional activity\textsuperscript{255}.

2.4.5 Inhibition by covalent modification
Inhibition by covalent modification is successfully used by nature to inhibit GTPase signaling, exemplified by the bacterial toxin C3 transferase that inactivates RhoA by ADP-ribosylation\textsuperscript{281}. C3 transferase is commonly used to study the role of RhoA in cellular systems. Covalent inhibitors SML-8-73-1 and SML-10-70-1 were discovered by using a design based on the GDP scaffold, targeting the nucleotide binding site of K-Ras G12C and block binding to GTP\textsuperscript{263,264}. Docking of the compound in the nucleotide binding pocket brings the reactive moiety of the compound close to the neighboring mutant-specific cysteine residue and subsequently forms a covalent bond with the cysteine residue. In the resulting conformation, which resembles the inactive GDP-bound state, Ras is unable to interact with effector proteins\textsuperscript{263}.

A novel approach in both attempting to block mutant Ras signaling as well as screening for small GTPase inhibitors was applied in the discovery of the covalent inhibitor 4M22\textsuperscript{261}. First, the screening was based on a method called tethering\textsuperscript{282}, utilizing the cysteine residue of non-small-cell lung cancer specific \textit{KRAS} mutant (K-Ras G12C) to irreversibly bind small molecule compounds to the target protein. Using a K-Ras construct lacking other cysteine residues (K-Ras G12C/C51S/C80L/C118S), a chemical starting point was identified from a small library of 480 ‘tethering compounds’ and subsequently exploited to generate analogs through X-ray structures of co-complexes. Second, 4M22 and analogs are compounds that alter the nucleotide preference of Ras GTPase to favor GDP over GTP, thereby impairing binding to Raf. The ability of 4M22 to
lock K-Ras in GDP-bound state is achieved by targeting a recently discovered allostERIC pocket under the Switch II loop region that is exposed exclusively in GDP-bound state.

4M22 is very specific, as it was demonstrated to reduce cell proliferation and induce apoptosis in cell lines containing G12C mutations compared to cell lines lacking this mutation\textsuperscript{281}. However, further characterization of 4M22’s mechanism of action revealed low covalent engagement in heterozygous K-Ras G12C NCI-H358 cells\textsuperscript{262}. Subsequent iterative structure-based design of covalent ligands targeting the Switch II pocket of K-Ras G12C led to the development of ARS-853, which showed a near complete inhibition of Ras-Raf interaction and downstream signaling through both the MAPK and PI3K signaling pathways at 1 µM\textsuperscript{262}. Moreover, ARS-853 was demonstrated to inhibit oncogenic transformation in cellular assays at similar concentration. Of note, data provided by this study showed that K-Ras G12C activity levels are responsive to growth factor stimulation as well as inhibitory signals, strengthen the model that aberrant signaling of K-Ras G12C happens by “hyperexcitation”, rapidly cycling nucleotides in response to upstream signaling inputs. This warrants that aberrant signaling of Rho GTPases could be caused by a deregulated expression of both GEFs and GAPs.

2.4.6 Inhibitors of GTPase–effector interactions

While the inhibition of GTPase-GEF activity might not be beneficial in the treatment of many Ras driven cancers, where different mutations prevent GTP hydrolysis, and interfacial inhibitors yet to be found, interfering with GTPase-effector binding presents an excellent opportunity to abrogate the downstream signaling events.

The non-steroidal anti-inflammatory prodrug sulindac was used in cancer prevention and therapy\textsuperscript{283}, before Müller and coworkers discovered that its metabolite, sulindac sulfide, binds to Ras and impairs downstream signaling\textsuperscript{284}. Using a sulindac-derived compound library in a phenotypical screening assay based on the ability of H-Ras oncogene to transform Madine–Darby canine kidney cells, more potent analogs were identified\textsuperscript{258,285}, and utilized to show that these compounds bind to Ras in a non-covalent manner near the switch I region, thereby inhibiting the
formation of the Ras-Raf complex. Rigosertib (ON01910) was initially discovered following the synthesis of family of non-ATP competitive small molecule kinase inhibitors, which were screened for anti-proliferative properties on tumor cells and subsequently selected for potent inhibitory effect of polo-like kinase 1 (Plk1) activity. Additional research has demonstrated that rigosertib is in fact a Ras-mimetic, binding to the Ras binding domain (RBD) commonly found in many Ras effector proteins. Binding of rigosertib to the RBD of the effector proteins resulted in an inability to bind to activated Ras, and thereby inhibited multiple Ras-driven signaling pathways, including both PI3K, as well as Raf. In vivo studies, using xenograft models of human colorectal and lung cancers as well as genetically modified mouse model of pancreatic cancer containing the K-Ras G12D mutation, showed that rigosertib is a potent inhibitor of tumor growth. Furthermore, subgroups of myelodysplastic syndrome patients having cytogenetic abnormalities that are associated with Ras activation (monosomy 7 and trisomy 8) have responded best to treatment with rigosertib in a phase 3 randomized trial.

Other Ras-effector binding inhibitors were discovered through targeted screening approaches. Through a yeast two-hybrid screen another set of Ras-Raf inhibitor compounds were discovered. This study, as well as the follow-up studies from different groups, showed that MCP compounds, notably MCP1, MCP53 and MCP110, inhibited Ras-induced Raf-1 activation, reduced invasiveness and anchorage-independent growth in cells harboring K-Ras mutation, but not constitutively active Raf-1, and inhibited growth in human xenograft models for MCP110 in particular.

After solving the first complete tertiary structure of H-Ras, it became apparent that in GTP-bound state, Ras does possess well-defined surface pockets suitable for drug binding. Subsequent in silico screening and biochemical characterization has identified Kobe0065 and its analog Kobe2602 to efficiently inhibit, both in vitro and in vivo, the binding of activated GTP-bound Ras with Ras-effector proteins.

With the possibilities to target GTPase-GEF activity, the need for Rho GTPase-effector inhibitors to perturb signaling might appear to be not as a necessity as for Ras GTPase proteins. Nevertheless, substantial effort
has been put in the discovery of Rho effector inhibitors, most notably Rho kinase and PAK inhibitors (reviewed in [297,298]). Interestingly, two ROCK inhibitors, fasudil and ripasudil, have been approved for clinical use in Japan and/or China, whereas one PAK inhibitor, PF-3758309, made it to phase I clinical trials. Fasudil, HA1077, was discovered for having a potent vasodilator effect in a canine model, completely reversing the induced cerebral vasospasm\textsuperscript{266}. While it was hypothesized the target would be a kinase\textsuperscript{265}, it was not until a decade later that it was shown that fasudil was in fact a Rho kinase inhibitor\textsuperscript{299}. Where fasudil demonstrated about equal potency towards both ROCK isoforms, the more recently discovered ripasudil displayed a slightly higher inhibitory effect towards ROCK2\textsuperscript{267}. Results of preceding screening campaigns were used to rational design ATP-competitive, group II PAK inhibitor PF-3758309\textsuperscript{268}. PF-3758309 inhibits group II PAKs as well as group I member PAK1 at low nM concentration, whereas inhibition for other group I PAKs was observed in submicromolar range. Although significant tumor growth inhibition was observed in a panel of human xenograft models, clinical development of PF-3758309 has been put on hold as no tumor responses were observed in a phase I clinical trial\textsuperscript{300}.

Following the success of Rac inhibitor NCS23766, \textit{in silico} screening has successfully been applied for Rho GTPase effectors as well\textsuperscript{269}. Phox-I1 was discovered in an effort to identify inhibitors targeting the binding of the Rac1/2-p67\textsubscript{phox}, a pivotal event in the activation of NADPH oxidase, which is responsible for ROS production in neutrophils. In particular, Phox-I1 and analogs displayed similar cellular ROS inhibitory activity in cultured as well as primary cells, without affecting the Rac-mediated actin cytoskeleton structure.

\textbf{2.4.7 Exploiting the need for post-translational modification}

The only GTPase-modulating compounds that have made it to clinical trails to date are the farnesyltransferase inhibitors lonafarnib\textsuperscript{270,271} and tipifarnib\textsuperscript{301,302}. After synthesis, cytosolic Ras proteins are processed at the endoplasmic reticulum by prenyltransferases, which is needed for the trafficking to and association with the plasma membrane. Because of alternative prenylation by related geranylgeranyltransferases, the loss of membrane anchoring activity can be restored for N- and K-Ras, and
initially farnesyltransferase inhibitor treatment failed in clinical trails\textsuperscript{96}. The increased understanding of the Ras isoform specific posttranslational modification process and the lack of prenylation redundancy for H-Ras has reignited the exploration of tipifarnib as H-Ras specific inhibitor in clinical trials (\textit{e.g.} NCT02535650 and NCT02383927)\textsuperscript{303,304}.

When prenylated, Ras and Rap GTPase membrane association is regulated by Ras GTPase GDI-like solubilizing factor PDEδ\textsuperscript{185}. Specifically, PDEδ activity enables Ras signaling by enriching Ras GTPase at the plasma membrane, whereas down-regulation of PDEδ results in a random distribution to all cellular membranes and thereby suppresses both wild-type as constitutive oncogenic Ras signaling\textsuperscript{186}. Deltarasin, a small molecule that bind to the farnesyl-binding pocket of PDEδ, disrupts the formation of the Ras-PDEδ complex\textsuperscript{271,272}. The lead inhibitor was discovered through a high-throughput AlphaScreen using a farnesylated K-Ras4B peptide and PDEδ. Subsequent structure-guided design based on the crystal structure of the initial hit led to development of deltarsarin\textsuperscript{272,273}. Deltarasin is able to impair the accumulation of K-Ras at the plasma membrane at low micromolar concentration, thus inhibiting RAS signaling. Accordingly, it was shown to suppress proliferation of oncogenic K-Ras dependent human pancreatic ductal adenocarcinoma cells \textit{in vitro} and \textit{in vivo}.

Despite the efforts put in the discovery and development of small GTPase-modulating tool compounds, a relatively short list of compounds has been described, of which only a few have made it to clinical applications to date. Therefore, GTPase signaling inhibition efforts have resorted to indirect modulators, targeting Rho downstream pathways with prior discussed PAK and ROCK inhibitors. In similar fashion, Ras downstream pathways are targeted with kinase inhibitors, which are discussed in depth in review [207]. However, in case of mutationally activated Ras, the compensatory signaling cascades, such as the crosstalk between and the feedback loops within the MAPK and PI3K pathways, have reduced the overall durability and potency of such approaches, and the clinical outcome has often been disappointing\textsuperscript{305}. Due to the better understanding that GTPase signaling is mediated through protein-protein interactions, with an essential role for regulator proteins GEFs and GAPs, opens the possibility to inhibit GTPase signaling by interfacial inhibitors, exemplified
by the natural compound Brefeldin A. However, to facilitate and enable larger scale screening for interfacial inhibitors, current assay methods needs to be adapted to be able to detect interfacial inhibition.

2.4 MgcRacGAP

MgcRacGAP is a 632 amino acid long Rho GTPase GAP protein with three well-defined domains; a N-terminal coiled-coil, a mid-protein C1-domain and a C-terminal Rho-GAP domain (Figure 7). MgcRacGAP was first isolated through a two-hybrid cloning procedure using Rac2 (Q61L) as bait almost two decades ago, but the target for its GAP activity is still controversial. After the isolation, the C-terminal GAP domain was cloned and it was shown that in vitro MgcRacGAP strongly stimulated GTPase activity of the Rho GTPases Rac1 and Cdc42, but was almost inactive towards RhoA. In a parallel study it was shown that MgcRacGAP was expressed at high levels in replicating cells, yet not expressed in terminally differentiated cells, which suggested that MgcRacGAP played role in cell division through the regulation of Rac-related process. Of note, the authors also implicated that MgcRacGAP could play a role in other processes regulated by Rac, such as tumor cell invasion and metastasis. A third study confirmed that the MgcRacGAP GAP domain was more active towards Rac and Cdc42 than towards RhoA in vitro, however, at the same time also demonstrated using RNAi that only RhoA was required for cytokinesis, and, therefore, concluded that RhoA may be the critical target of MgcRacGAP in vivo.

MgcRacGAP mRNA expression levels were found to be cell cycle dependent, peaking at the G2/M phase. Detailed immunohistochemical studies showed that MgcRacGAP colocalized with the mitotic spindle in metaphase, binding directly to microtubules via its N-terminal coiled-coil-like domain, transferred to the midzone in anaphase and telophase, and thereafter moved to the midbody in cytokinesis. During metaphase, the activity of MgcRacGAP is down-regulated by PRC1, which binds to the GAP domain of MgcRacGAP and thereby prevents phosphorylation-mediated GAP activity. At the end of anaphase, MgcRacGAP, together with Mitotic
Kinesin-Like Protein 1 (MKLP1) form the centralspindlin, an evolutionarily conserved hetero-tetrameric complex, and accumulates at the cell equator on the overlapping antiparallel microtubules. In telophase, Aurora B kinase phosphorylates MKLP1, whereas MgcRacGAP is phosphorylated by Plk1, creating a docking site for the RhoGEF Ect2. As a result, Ect2 localizes to the central spindle to form the MgcRacGAP/MKLP1/Ect2 complex that stimulates and controls the activation of RhoA. Premature binding of Ect2 with the centralspindlin is prevented by CDK-1 phosphorylation of Ect2, which inactivates a membrane-binding motif within Ect2 and thereby inhibits the Ect2–MgcRacGAP interaction during metaphase. At the terminal phase of cytokinesis, the centralspindlin complex of MgcRacGAP, which is membrane bound through its C1 domain, and MKLP1, which is connected to the microtubules, connects the plasma membrane to the intercellular bridge, providing the connection between the cleavage furrow and the mitotic spindle needed to complete the abscission. Interestingly, while the interaction between MgcRacGAP and MKLP1 is evolutionarily conserved, the primary structure of the N-terminal region of MgcRacGAP, through which the binding to MKLP1 occurs, is not well conserved.

Figure 7 Schematic representation of MgcRacGAP. Visualized in the schematic representation of MgcRacGAP are from N-terminus to C-terminus; the coiled coil domain, the protein kinase C conserved region 1 (C1, also known as cysteine-rich zinc-finger domain), and Rho-GAP domain, with in subscript the corresponding regions by amino acid numbering. MgcRacGAP interacts through its N-terminal domain with Mitotic Kinesin-Like Protein 1 (MKLP1) and Ect2, and through its C-terminal domain with Rho GTPases as well as protein regulator of cytokinesis 1 (PRC1). The red lines correspond with the two Polo-like kinase 1 (Plk1) phosphorylation sites (Ser157 and Ser164).
Cytokinesis is a well-studied process and the role of Ect2’s GEF activity in activating RhoA at the equatorial cortex is well established\textsuperscript{319,320}. The detailed functional role of MgcRacGAP, however, is not yet fully resolved\textsuperscript{321-323}. The relevant target GTPase in cytokinesis, as well as its GAP activity as such, is being debated.

First, the controversy between the \textit{in vivo} and \textit{in vitro} data; early RNAi results obtained with \textit{C. elegans} demonstrated that RhoA, in contradiction to Rac1 or Cdc42, was required for cytokinesis, and thus RhoA had to be the \textit{in vivo} target of MgcRacGAP’s GAP activity\textsuperscript{309}. In agreement with this hypothesis were the results obtained with \textit{Xenopus} embryos showing that the GAP activity of MgcRacGAP was needed to inactivate RhoA creating a “GTPase flux,” the rapid cycling of RhoA between the GTP- and GDP-bound forms\textsuperscript{322}. Other studies suggest that MgcRacGAP’s GAP activity contributes to GEF activation, and thereby indirectly contribute to activation of RhoA\textsuperscript{324,325}. However, numerous studies have demonstrated that \textit{in vitro} MgcRacGAP has little to no GAP activity towards RhoA\textsuperscript{307,309,326,327}, and these results are consistent with observations in \textit{C. elegans} and HeLa cells, suggesting that MgcRacGAP’s GAP activity is directed to Rac1 or Cdc42\textsuperscript{321,326,328}. The observation that MgcRacGAP colocalized with Aurora B and RhoA, but not Rac1 or Cdc42, seemed to shed light to the matter, as it was reported that Aurora B phosphorylation of MgcRacGAP serine residue 387 converted latent GAP activity toward RhoA to active \textit{in vitro}\textsuperscript{329}. However, recent studies have shown that GAP activity was not converted by phosphorylation at serine 387\textsuperscript{326,330}. Instead, phosphorylation at serine 387 was demonstrated to cause a GAP dead protein\textsuperscript{330}. Strengthening the hypothesis that Rac1 is the relevant target GTPase and not RhoA is the recent identification of Trio as a mitotic GEF of Rac1, counteracting MgcRacGAP’s function in cytokinesis\textsuperscript{331}.

Second, in contradiction to the many studies showing that MgcRacGAP is essential for cytokinesis, two studies have reported that, in \textit{Drosophila} neuroblasts and chicken B cells, MgcRacGAP’s GAP activity is not required at all for cell division\textsuperscript{323,332}. However, these two models systems are used to study asymmetric cell division and the development of non-adherent B cells, respectively, whereas previous results have been obtained with adherent mammalian cell lines undergoing symmetrical cell division.
Soon after the discovery of MgcRacGAP it was suggested that, besides having function in cytokinesis, MgcRacGAP could play a role in other processes regulated by GTPases and the link was made to Rac-mediated tumor cell invasion and metastasis. A recent study focusing on the Rho GTPase signaling transcriptome revealed high RNA expression of several RhoGAP genes in basal-like breast cancer tumors. Further examination on the role of several RhoGAP, including MgcRacGAP, in promoting oncogenesis led the authors to propose that RhoGAPs can act as oncogenes in cancer. Indeed, many other papers have shown that high MgcRacGAP mRNA expression is not only associated with poor disease-free survival, it also has a prognostic significance in numerous types of cancers, including breast cancer, as well as colorectal cancer, hepatocellular carcinoma, gastric cancer, non-small cell lung cancer, melanoma, squamous cell carcinomas, and uterine carcinosarcoma. Likewise, the expression of the other complex members MKLP1 and Ect2 has been demonstrated to be upregulated in cancer, and the occurrence of relapses correlates with upregulation in several human malignancies including colorectal, ovarian, pancreatic, and non-small cell lung cancer as well as hepatocellular carcinoma and glioblastomas. However, it is poorly understood how and if the complex, or its individual components, contribute to the malignancy.

Several mechanisms have been brought forward to how MgcRacGAP, MKLP1 and Ect2 could contribute to malignancy. In both cancer and development, when the MgcRacGAP/MKLP1/Ect2 complex proteins are expressed at high levels, their functions have been linked to controlling polarity, and driving epithelial-to-mesenchymal transition. According to some recent reports, MgcRacGAP is localized at cell junctions of cultured epithelial cells and locally regulates the activity of Rac. However, there are controversies on the precise subcellular location and role of MgcRacGAP; it was shown that MgcRacGAP, together with MKLP1, regulates Rho signaling at the adherens junctions of interphase epithelial cells, whereas another study demonstrated that MgcRacGAP specifically colocalizes at the tight junctions while Ect2 is distributed heterogeneously at the adherens junctions. A third study showed that the phosphorylation of MgcRacGAP by Akt on threonine residue 249 results in the recruitment of MgcRacGAP to invasive pseudopods, where it locally
suppresses the activity of Rac1 resulting in increased activity of RhoA, promoting invasive migration in a RhoA dependent manner.\textsuperscript{175}

MgcRacGAP, together with Ect2, Rac1 and Cdc42, is required for centromere maintenance by stabilizing newly incorporated CENP-A during G1 phase.\textsuperscript{356} CENP-A is an essential histone H3 variant located in the centromeres, where it directs kinetochore assembly during mitosis. Thus, improper functioning of MgcRacGAP could lead to inappropriate CENP-A incorporation needed for successful chromosome segregation and, therefore, result in genomic instability. Moreover, the MgcRacGAP/MKLP1/Ect2 complex is required for initiation and progression of cytokinesis, but needs to be inactivated to complete it. Sustained Aurora B activity was demonstrated to lead to persistent MKLP1 phosphorylation, delaying abscission.\textsuperscript{357} Accordingly, overexpression of the centralspindlin could lead to an increased frequency of cell division failure, resulting in aneuploidy that is presumed to be an early step in cancer formation.\textsuperscript{229}

After cytokinesis, the complex becomes part of the midbody, which remains with the daughter cell carrying the older centrosome.\textsuperscript{358} Midbody loss is associated with stem-cell differentiation, whereas the gain of it leads to reprogramming of induced pluripotent stem cells and increased tumor characteristics of cancer cells \textit{in vitro}.\textsuperscript{358} Overexpression, as such, is expected to lead to midbody enrichment and increasing tumorigenicity of cancer cells. Additionally, MgcRacGAP was reported to be essential for the nuclear translocation of STAT factors, including the oncoprotein STAT3, which is well known to regulate cancer drug resistance and stemness.\textsuperscript{359-361} In these studies is described the seemingly contradictory interaction between MgcRacGAP, GTP-bound Rac1 and STAT family members 3 and 5A, implicating that the MgcRacGAP/Rac-GTP complex functions both as a mediator of STAT tyrosine phosphorylation and as a chaperone for nuclear translocation of STAT transcription factors.\textsuperscript{361}

Taken together, it is clear that MgcRacGAP is involved in a multitude of different processes that, when deregulated, all could contribute to human oncogenesis, such as sustained proliferation, promoting genetic instability, activating invasion and metastasis.\textsuperscript{362} Like in cytokinesis, the role of MgcRacGAP in the processes related to malignancy is under debate. The conflicting results on the role of MgcRacGAP, and its GAP
activity, could very well arise from the cellular context\textsuperscript{326,363}. However, it was shown that experiments which manipulate a single protein, \textit{e.g.} by overexpression of an exogenous Rho GTPase, did not only affect the endogenous target protein, but affected the levels and activity of other Rho GTPases due to competition for binding\textsuperscript{364}. Therefore, it should also be considered that the varying results are likely an outcome of studying a highly temporally controlled process by using molecular biology tools that are not temporally controlled nor can easily be dosed.

In summary, research of the previous decades has shown that GTPases and their regulators function as essential signaling nodes for many pivotal regulatory pathways. These pathways form a highly integrated signaling network of regulatory events controlling normal cell physiology as well as aberrant function. Small molecule modulators of GTPase activity would be highly valuable to further delineate these pathways, but very few effective probes of this kind currently exist. To address this gap, we have focused on discovering different small GTPase-modulating probes through different screening approaches.
3. Aims of the study

The overall aim of this doctoral study was to identify and utilize new small molecule modulators of GTPase activity, focusing on the previously undrugged GAP proteins, and thereby improve the understanding of the Ras superfamily of small GTPases and their regulators.

The specific aims were

1. Develop small molecule inhibitors of MgcRacGAP that can be used to study MgcRacGAP and evaluate it as a potential therapeutic target.

2. Study the role of MgcRacGAP as mediator of phosphorylation and chaperone for nuclear translocation of STAT proteins.

3. Develop a high throughput screening (HTS) compatible method to allow identification and characterization of novel small molecule inhibitors of the Ras GTPase cycle.
4. Material and methods

The materials and methods used in the presented thesis have been described in great detail in the original publications, but a brief general description of the techniques used are also described here. The following tables summarize the fundamental experimental procedures, reagents and cell culture details.

Table 2 List of methods used during this thesis

<table>
<thead>
<tr>
<th>Method</th>
<th>Study</th>
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<tbody>
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<td>Protein expression and purification</td>
<td>I</td>
</tr>
<tr>
<td>Biochemical GTPase assays</td>
<td>I, III</td>
</tr>
<tr>
<td>Synthesis of MINC1</td>
<td>I</td>
</tr>
<tr>
<td>HTS data analysis</td>
<td>I, III</td>
</tr>
<tr>
<td>Protein-protein interaction dynamics</td>
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</tr>
<tr>
<td>Cell culture and transfection methods</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Cytotoxicity and cell proliferation assays</td>
<td>I, III</td>
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<tr>
<td>Active GTPase pull-down assay</td>
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<tr>
<td>Immunofluorescence analysis</td>
<td>I, II</td>
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<tr>
<td>Gel electrophoresis and western blot analysis</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Luciferase reporter assays</td>
<td>II</td>
</tr>
<tr>
<td>Small molecule library screening</td>
<td>I, III</td>
</tr>
</tbody>
</table>

Table 3 List of reagents and compounds used during this thesis

<table>
<thead>
<tr>
<th>Reagents and compounds</th>
<th>Study</th>
</tr>
</thead>
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</tr>
<tr>
<td>Small interference RNA</td>
<td>I, II</td>
</tr>
<tr>
<td>MINC1 (CID 744230)</td>
<td>I, II</td>
</tr>
<tr>
<td>Stattic (CID 2779853; Tocris Bioscience, 2798)</td>
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<tr>
<td>Ruxolitinib (CID 25126798; ChemiTek, CT-INCB)</td>
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<tr>
<td>Interleukin-6 (AH Diagnostics 14-8069-62)</td>
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<tr>
<td>Interferon-α (Immuno diagnostic Oy, 11350-1)</td>
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<tr>
<td>pGL4.47[Luc2P/SIE/Hygro] (Promega)</td>
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</tr>
<tr>
<td>pGL4[Luc2P/STAT5/Hygro] (Promega)</td>
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</tr>
<tr>
<td>pBABEpuro MgcRacGAP</td>
<td>I</td>
</tr>
</tbody>
</table>
4.1 Protein expression and purification

Protein expression vectors (Table 4) were constructed by amplifying the desired part of the DNA by polymerase chain reaction (PCR) procedure, adding EcoRI and XhoI restriction enzyme recognition sites to both ends of the amplified DNA. Subsequently, the DNA was ligated between the EcoRI and XhoI restriction sites of pGEX-4T-1 (GE Healthcare). The BCR and p50RhoGAP GAP domain expression vectors were analogous to constructs that have been described previously. The human Rho GTPase fast-cycling variants were produced by PCR-based Phusion Site-Directed Mutagenesis (Thermo Scientific) and subcloned into the bacterial expression vector pGEX-4T-1. All DNA constructs were confirmed by DNA sequencing.

Recombinant proteins were expressed and purified as described. In short, DH5α or BL-21 bacterial cultures were grown up to 1 liter and subsequently induced with a final concentration of 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) to express the fusion proteins. After an overnight induction at room temperature, the bacterial pellet was resuspended in ice-cold lysis buffer (150 mM NaCl, 20 mM HEPES pH 7.5, 1 mM MgCl₂, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, 10 µM GDP), sonicated, cleared by centrifugation and soluble fusion proteins were captured with glutathione-sepharose or Ni-NTA-sepharose. Protein-bound beads were washed two times with lysis buffer, followed by two times with wash buffer (150 mM NaCl, 20 mM HEPES pH 7.5, 1 mM MgCl₂, 1 mM DTT, 10 µM GDP). Wash and elution buffers for the GAP domain constructs lacked MgCl₂ and GDP. The GST fusion proteins were eluted from the beads with elution buffer (10 mM Glutathione, 50 mM Tris pH 8.0, 1 mM MgCl₂), whereas 6xHis fusion proteins were further washed with 1 mM and 5 mM imidazole supplemented wash buffer and subsequently eluted with 150 mM NaCl, 20 mM HEPES pH 7.5 and 80 mM imidazole. Protein concentrations were determined using Bradford protein assays, pooled when necessary and immediately snap frozen and stored at -80°C. SDS-PAGE and Coomassie Blue staining were used to analyze protein purities.
4.2 Biochemical GTPase assays

In short, all GAP assays, except for the GTP hydrolysis by wild type Rac1, were performed in a buffer containing 15 mM HEPES (pH 7.5), 20 mM NaCl, 1 mM EGTA, 0.02% Tween 20, 0.1 mg/mL bovine serum albumin, 2% dimethyl sulfoxide (DMSO) and 150 μM GTP at room temperature for 2 h. After optimization, the assays were carried out using 600 nM Rac1 (F28L) and 2 nM MgcRacGAP in the primary and orthogonal screen, which was substituted for either 200 nM BCR or 10-50 nM p50RhoGAP (depending on the batch) in the counter screen assays. To mimic GAP activity inhibition, GAPs were omitted from the assay mix. The amount of hydrolysis could effectively be determined by measuring both GDP and inorganic phosphate, using the ADP Hunter Plus assay kit (primary screen) and malachite green assay (orthogonal screen), respectively. A prototype kit of the now-published GTPase/GAP/GEF-Glo Bioluminescent Assay System was used to measure GTP hydrolysis by wild type Rac1368.

Table 4 Recombinant proteins. Proteins of which the NCBI accession is marked with asterisk have a mutation inserted in the original NCBI reference sequence. The mutation is given in the preceding column with the protein name. All proteins except for those referenced have been constructed for the work described in this thesis. DH5α or BL-21 bacterial strains were used to express the recombinant proteins. PBD, p21 binding domain; RBD, Ras binding domain.

<table>
<thead>
<tr>
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<td>NP 001035957</td>
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The GTPase nucleotide exchange assays were performed in a buffer containing 50 mM Tris (pH 8.0), 10 mM MgCl₂, 3 mg/mL Triton-X 100 and 0.1 mg/mL γ-globulins. Equal molarities of GTPase/GEF (200 nM for Ras and Sos, 500 nM for RhoA and Ect2) were incubated with 10 nM Eu³⁺-GTP and 22 µM Quench II. The competitive GTP detection assays were conducted in a buffer composed of 20 mM HEPES (pH 7.5), 1 mM NaCl, 1 mM MgCl₂, 0.01% (w/v) Triton-X 100 and 0.005% (w/v) γ-globulins. GTP hydrolysis was facilitated by 500 nM GTPase, 300 nM GEF, 900 nM GAP, 700 nM GTP, 350 nM GDP, 7.5 nM Eu³⁺-GTP, 12 nM 2A4GTP and 2.5 µM Quench III. The primary screening was conducted with H-Ras, RasGAP p120RasGAP and RasGEF Sos, but was substituted in the follow-up screens by K-Ras and RhoA (with Ect2 as GEF). After 20 min incubation, the time-resolved luminescence (TRL) signals were monitored.

4.3 Small molecule library screening

For the small molecule library screening, compounds were transferred by Echo acoustic dispensers from stock solutions plates into the appropriate destination assay plates for single dose testing. The 20 480 screened small molecule compounds for the MgcRacGAP inhibitor study were from a chemical diversity collection from ChemDiv, whereas 1 280 compounds from a ChemBridge chemical diversity collection were used for the Ras inhibitor screening assay. Dose response testing at the concentration range of 0.1-100 µM was done for compounds identified in single dose screening using the primary assay conditions as well as homologue proteins. Analogous to the MgcRacGAP inhibitor study at FIMM, an additional 342 046 compounds from the NIH Molecular Libraries Small Molecule Repository were screened at Southern Research Institute (Birmingham, AL, USA; See PubChem AID 624330 for complete protocol).
4.4 Synthesis of MINC1 and analog

MINC1 has been re-synthesized by Annika Fagerholm in the group of Ari Koskinen at the Aalto University. See Figure 8 for the synthesis of MINC1. A short list of analog compounds have been synthesized to obtain structure-activity relationship (SAR) data for further development of potent inhibitors analogs as well as to be used to study the function of MgcRacGAP in cells.

4.5 HTS data analysis

Positive and negative control samples were used to determine the Z’-factor values for each HTS assay plate, whereafter plates with Z’-factor values lower than 0.5 were excluded from further analysis\textsuperscript{377}. Next, the controls were normalized as 100% and 0% inhibition and used to calculate the normalized inhibition for each compound. The cutoff values for each assay are given in Table 5. GraphPad Prism was used to fit the four-parameter dose-response curves constrained between 0% inhibition at the bottom and 100% inhibition at the top of the curve.

Table 5 Overview of cutoff values used for the HTS assays. SD, standard deviation.

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<th>Assay</th>
<th>Threshold</th>
<th>Study</th>
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<td>Primary GAP assay</td>
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<tr>
<td>Orthogonal assay</td>
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</tr>
<tr>
<td>Counter screen assay</td>
<td>≥50% MgcRacGAP ≤15% BCR GAP</td>
<td>I</td>
</tr>
<tr>
<td>GTP hydrolysis assay</td>
<td>≥25% inhibition</td>
<td>III</td>
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<tr>
<td>GTP association assay</td>
<td>≥25% inhibition</td>
<td>III</td>
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</table>
Figure 8 Synthesis of MINC1 analogs, general conditions and results. A) Summary of MINC1 and analog synthesis. X represents an oxygen, sulfur or nitrogen atom and n represents 1 or 2 carbon atoms, specified in lower table. Nd, not determined. B) In the previous conditions, entries 3 and 5 did not yield any product. Therefore, a different method to synthesize those was needed. The salt formulations made the molecules more soluble. (Image kindly provided by Annika Fagerholm.)
4.6 Protein-protein interaction dynamics

Protein-protein interaction between MgcRacGAP and Rac1 Q61L was confirmed with a pull-down assay using immobilized GST-MgcRacGAP and soluble 6xHis-Rac1 (Q61L). In brief, 25 µM of Rac1 (Q61L) was incubated with equal molar glutathione sepharose bead-bound MgcRacGAP for 2 h at 4°C, using a buffer containing 15 mM HEPES (pH 7.5), 20 mM NaCl, 1 mM EGTA, 0.02% Tween 20, 0.1 mg/mL bovine serum albumin. The same buffer with 0.1% Tween 20 was used to wash the beads three times and subsequently was washed an additional three times with reaction buffer (containing 0.02% Tween 20). Equal volume of 2x Laemmlı buffer was added and the sample was processed as a western blot sample (described in section 4.11 of this thesis) and immunoblotted for Rac1.

Dissociation of 6xHis-Rac1 (Q61L) from immobilized GST-MgcRacGAP was analyzed by bio-layer interferometry using an Octet Red 384 (ForteBio). MgcRacGAP was immobilized by dipping anti-GST probes in 2 µM GST-MgcRacGAP, 1x phosphate-buffered saline (PBS) solution with 2% DMSO. Rac1 (Q61L) was bound to MgcRacGAP by transferring the probes to 3.5 µM 6xHis-Rac1 (Q61L), 1x PBS solution with 12.5-50 µM MINC1 or 2% DMSO. In the last step, the probes were transferred to a 1x PBS solution with 12.5-50 µM MINC1 or 2% DMSO to determine the dissociation of Rac1 (Q61L) from MgcRacGAP.

4.7 Cell culture and transfection methods

The cell lines used in the studies throughout this thesis were cultured in specified growth medium (Table 6) supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin. When indicated by provider, selection antibiotics were used in expansion stage as well. Subsequently, the cells were frozen down in small aliquots. This allowed us to perform and repeat all experiments with cells in the same passage number.

We used different transfection methods to specifically enhance or inhibit gene expression. In brief, to overexpress protein or to introduce a reporter plasmid, cells were transiently transfected with plasmid DNA
using either Lipofectamine 2000 or FuGENE HD. Gene silencing was achieved with small interference RNA, which were introduced into the cells with Lipofectamine 2000 for bulk transfections or RNAiMAX in case of multi-well screening. Details on specific cellular assays, e.g. cell cycle synchronization, cellular movement and wound healing assays as well as the cell cycle reporter assay, are described in study I.

Table 6 Overview of the cell lines and culture conditions used in the studies for the presented thesis. DMEM; Dulbecco’s Modified Eagle Medium, EMEM; Eagle’s Minimum Essential Medium, v/v; volume/volume, FBS; fetal bovine serum, and RPMI; Roswell Park Memorial Institute.

<table>
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4.8 Cytotoxicity and cell proliferation assays

Cells were exposed to hit compounds to identify those compounds that were directly toxic to cells. While small details varied from assay to assay, in essence all compounds were tested in dose response (0.1 - 100 µM), using vehicle (DMSO or water) and 20 µM benzethonium chloride as negative and positive controls for cell death, respectively. To determine
toxicity, the cell culture growth medium was supplemented with CellToxGreen reagent according to manufacturer’s recommendations. CellToxGreen is stable at 37°C for a prolonged period of time, which allows not only to obtain end-point data, but also kinetic data when followed by live cell imaging using an IncuCyte FLR microscope (Essen Bioscience). Cell proliferation was determined by either phase contrast confluency or CellTiter-Glo Luminescent Cell Viability Assay (used according to manufacturer’s recommendations).

4.9 Active GTPase pull-down assay

To assess whether a hit compound had an effect on the GTPase activation status, GTP-GTPase levels were determined by pull-down assay using immobilized PAK1 PBD and Raf1 RBD for Rac1 and Ras, respectively. In brief, cells were treated with either compound or vehicle and after an incubation period lysed in lysis buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton, 1 mM MgCl₂) supplemented with protease inhibitor cocktail (Millipore #539134). The concentration-equalized lysates were incubated with appropriate immobilized protein binding domain before being processed analogous to a western blot sample (described in section 4.11 this thesis).

4.10 Immunofluorescence analysis

For immunofluorescence (IF) analysis, cells were fixed with ice-cold methanol or 4% formaldehyde in 1x PBS and subsequently blocked with 5% (weight/volume; w/v) bovine serum albumin (BSA) in 1x PBS to prevent unspecific binding of the antibodies. In case of formaldehyde fixation, the blocking buffer was supplemented with 0.2% (w/v) Triton X-100 and 0.05% (w/v) Tween 20 to permeabilize the cells. Primary and secondary antibodies (see Table 7) were diluted in same solutions, unless stated otherwise by the manufactures. After each antibody
Table 7 **Antibodies used in this thesis.** IF, immunofluorescence; n.a., not applicable; WB, western blot.

### Primary antibodies

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### Secondary antibodies

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### Dyes

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incubation step, excess of antibody was removed by extensive washing with PBS. Coverslips were mounted with medium containing DAPI when appropriate. Slides were imaged using a Nikon 9i fluorescence microscope with 10x or 60x objective and NIS elements AR software, whereas plates were imaged using ScanR with 20x objective and associated software. Afterwards, the color balance was normalized for all images using ImageJ. For the quantification of MINC1’s effects on cell division arrest, eight randomly chosen fields per condition were manually scored for the number of normal (mononuclear), mitotic (rounded up), multinuclear and dead cells by eye. To evaluate the effect of MINC1 on STAT3 phosphorylation, ImageJ was used to quantify the relative signal intensities of phospho- and total STAT3 in randomly selected images of different conditions.

4.11 Gel electrophoresis and western blot analysis

Protein concentrations of purified recombinant protein solutions were determined using the Bradford protein assay, whereas protein concentrations of cell lysates were determined with DC protein assay. After recombinant protein solutions and concentration-equalized lysates were denaturized by heating for 5 min at 95°C with Laemmli buffer containing 0.05 mM DTT, the samples were loaded and separated on 10-15% SDS-PAGE gel. Coomassie Blue staining was applied for the recombinant protein samples to analysis protein purities, whereas cell lysate samples were transferred to PVDF (polyvinylidene fluoride) membrane and stained with appropriate antibodies to semi-quantify protein expression using a Li-Cor Odyssey infrared imaging system.

4.12 Luciferase reporter assays

The exact details of each luciferase reporter assay can be found in study II as well as in the literature, but generally cells were plated such that at the day of measurement cells had reached a near-confluent state.
HEK293 GloResponse SIE Luc2P Hygro (HEK293 SIE-Luc) cells, which stably express a STAT3 firefly luciferase reporter, were used for the majority of experiments. In other cases, reporter plasmids were transiently transfected into HEK293 or HeLa cells using either Lipofectamine 2000 or FuGENE HD. If required, cells were starved using growth medium containing 0.5% (v/v) FBS 6 h before measurement and/or stimulated with appropriate cytokine or vehicle 3 h before measurement. Luciferase activity was measured using One-Glo luciferase detection reagent (Promega) according to the manufacturer’s recommendations.
5. Results and discussion

5.1 The MgcRacGAP inhibitor (I - II)

5.1.1 Development of the MgcRacGAP biochemical assay (I)

To address the lack of chemical tools to probe MgcRacGAP’s function, we set out to identify an MgcRacGAP inhibitor that could help to provide insight into the biological role of MgcRacGAP. The first challenge was to overcome the very high affinity of small GTP-binding proteins for the nucleotide\(^1\). Without a GEF in the assay solution, the nucleotide exchange step will be the rate-limiting step restricting a biochemical assay using wild type small GTPases to a near-single turnover assay (Figure 9A). Previously, it was shown that the GDP dissociation rate of \(G_{\alpha_{1}}\) GTPase subunit of heterotrimeric G-proteins increased if a mutation was introduced in the conserved TCAT loop\(^{380}\). Using this mutant protein and the GAP RGS4 (Regulators of G-protein Signaling 4), multiple rounds of turnover of GTP to GDP were measured. In similar fashion, for the so-called fast-cycling mutant small GTPase proteins, which were shown to display a lowered nucleotide affinity, the GDP dissociates from the GTPase in the absence of a GEF\(^{381}\). In a GTP-rich environment, this results in a continuous GTPase cycle with the GTP hydrolysis step as the rate-limiting factor, making it possible to measure GAP activity alterations (Figure 9B). Using fast-cycling mutants of several Rho GTPases, we determined for each the GAP-stimulated GTPase activity with different GAPs and established what GAP concentrations to use to remain in linear response range (Figure 10, unpublished). Consistent with previous studies suggesting that MgcRacGAP is a GTPase activating protein for Rac1 and not RhoA\(^{326}\), MgcRacGAP did not exhibit GAP activity toward RhoA. In case of our primary GTPase and GAP pair, Rac1 and MgcRacGAP, using the fast-cycling protein allowed for a three- to fourfold signal window between intrinsic and GAP-stimulated GTPase activities, while remaining in a linear response range of GAP concentrations (I; Figure 1C).
Figure 9 Design of an HTS-compatible assay using fast cycling GTPase mutants to measure the biochemical activity of GAPs. A) Wild type GTPase cycles between GDP- and GTP-bound forms. Due to a very high affinity towards the guanine nucleotides, the nucleotide exchange step is highly rate limiting (red arrow). B) Fast-cycling mutant GTPase (GTPase*) exhibit reduced affinity for nucleotide, which results in a higher rate of nucleotide exchange compared to wild type GTPase. With an excess of GTP, this system will continuously cycle with the GTP hydrolysis step as rate limiting factor (red arrow). In this thesis fast-cycling mutant GTPases Rac1 (F28L), as well as Rac3 (F28L), Cdc42 (F28L), RhoA (F30L) and RhoG (F28L) were used in combination with the GAP domains of MgcRacGAP (345-618), p50RhoGAP (205-439) and BCR (1010-1271).

The GAP-stimulated GTPase reaction can be monitored by the increase of inorganic phosphate as well as GDP or the decrease of GTP (Figure 9). We decided to monitor the production of inorganic phosphate and GDP, which could be measured effectively using malachite green and the ADP Hunter Plus assay kit from DiscoveRx, respectively. In addition to detecting different analytes, these methods are based on different detection readouts (absorbance vs. fluorescence). This makes the combination of these assays ideal for the use of a primary and secondary orthogonal validation screening strategy. Since the ADP Hunter Plus assay gave higher and more consistent Z'-factors than the malachite green assay, we decided to use the ADP Hunter Plus assay as the primary detection method and the malachite green assay as an orthogonal validation method.
Figure 10 **GAP activity is GTPase dependent.** Each GAP has a different GAP activity for different small GTPase proteins. The response range for each GAP-GTPase pair was determined by titration. The GAP concentrations used in the screen were adjusted to obtain similar GAP-stimulated GTPase activity in all assays, yet remain in linear response range. Error bars represent SD (n=3).
Next, to make the MgcRacGAP-Rac1 assay HTS compatible, we optimized protein and GTP concentrations as well as incubation times in a 96-well format and subsequently miniaturized to 384- and 1536-well formats using automated liquid handling (I; Table 1). MgcRacGAP was omitted from the assay mix to mimic 100% inhibition of the GAP activity. In addition to omitting the GAP, including a GAP insensitive mutant like Rac1 (Q61L) appears to an ideal negative control for the in vitro specificity assays. However, the GAP insensitive mutant Rac1 (Q61L) also has an impaired intrinsic GTPase activity. Therefore, it will be unlikely to detect any signal of GDP-release.

5.1.2 Discovery of MINC1 through high throughput screening (I)

For this study we performed two high throughput screens. In-house, we performed a 20 480 compound screen using a chemical diversity set from ChemDiv. In addition, we screened 342 046 compounds from the NIH Molecular Libraries Small Molecule Repository chemical collection (PubChem AID 624330). As expected, we observed that the majority of the analyzed compounds showed little or no GAP inhibition. Predefined thresholds (Table 5) were applied to identify active compounds eligible for verification by follow-up screening. In case of the screening campaign with NIH, a computational filter was used after the primary screen to reduce the number of false positive compounds by cross-referencing our data with data from previous NIH screens using the ADP Hunter assay. The implementation of the filter increased the confirmation ratio in the orthogonal assay from 13% in the in-house screen to 23% in the NIH screen.

| Table 8 Results identification of an MgcRacGAP inhibitor through a novel type of screening assay. N.a., not applicable; SRI, Southern Research Institute. |
|---------------------------------|---------|---------|
| FIMM                            | SRI-NIH |
| Original number of compounds    | 20 480  | 342 046 |
| Primary screen: ADP Hunter Plus assay | 245     | 4,243   |
| Computational filter for false positive | n.a.    | 2,362   |
| Secondary screen: Malachite green assay | 37      | 557     |
| Manual filter for promiscuity   | n.a.    | 13      |
| Tertiary screen: MgcRacGAP vs. BCR | 9       | n.a.    |
| Dose response test vs. different GAPs | 2       | 1       |
| Confirmation screen alternate source | 1       | 1       |
After systematically executing the primary screens and complementary assays (Table 8), we were able to identify and confirm two compounds that showed selective biochemical inhibition of MgcRacGAP in vitro. The first compound, which we named MINC1 (MgcRacGAP Inhibitor Compound 1; Figure 11A), showed an MgcRacGAP-selective dose response curves with an half maximal inhibitory concentration (IC$_{50}$) of 15±5 μM and no detectable activity towards BCR and p50RhoGAP (Figure 11B). When we analyzed the selectivity of MINC1 towards MgcRacGAP using different GTPases, we found that MINC1 showed selectivity towards MgcRacGAP and Rac1 (Figure 11C, unpublished). New structural insight into Rac1 recognition of MgcRacGAP may explain why MINC1 is Rac1-MgcRacGAP specific. First, compared to other RhoGAPs, the arginine finger of MgcRacGAP is aligned differently. Second, like other Rho GTPase and RhoGAP pairs, the surface charges are complementary; a generally positive charged surface area of MgcRacGAP interacts with the opposing largely negative charged surface of Rac1. However, because of the exceptional conformation of MgcRacGAP’s arginine finger, the space between MgcRacGAP and Rac1 at the interactive area is twice as large as compared to other Rho GTPase and RhoGAP pairs. In our in silico modeling experiments we could not dock MINC1, or any of the other inhibitors, to either the surface of MgcRacGAP or Rac1. However, with this new knowledge of a different binding mode, it would be interesting to perform a docking study using both MgcRacGAP and Rac1 and test whether MINC1 would be able to dock to the MgcRacGAP-Rac1 specific groove.

Using the publicly available data from PubChem, a public repository for biological activities of small molecules, we found that MINC1 (CID 744230) had been included in 23 reported bioassays to date and was only reported active in one of these assays against an unrelated target, showing marginal activity. To gain more insight in the functional groups of MINC1, we set out to explore the structure-activity relationship of MINC1 with MgcRacGAP-Rac1. However, no close structural analogs of MINC1 were present in our compound collection or commercially available. Without knowledge about possible docking sites, rational structure-based compound design was not possible. Thus, we synthesized a short list of analogs with mostly single moiety or functional group substitutions, but
none of these compounds appeared to be more active in the biochemical screen than the lead compound (Figure 12).

**Figure 11** MINC1 shows selectivity for MgcRacGAP and Rac1 in vitro. A) Molecular structure of MINC1, CID 744230. B) Dose response analyses (0.1-100 μM) of MINC1 against MgcRacGAP (red), p50RhoGAP (dotted black line) and BCR GAP (solid black line) with Rac1 F28L. MINC1 selectively inhibits MgcRacGAP IC_{50} 15±5 μM. Error bars represent SD (n=8). C) Dose response analyses (0.1-100 μM) of MINC1 against Rac1 F28L (red), Rac3 F28L (dotted black line) and Cdc42 F28L (solid black line) with MgcRacGAP. MINC1 exhibits selective inhibition for Rac1 IC_{50} 11±5 μM. Error bars represent SD (n=6).
Three MINC1 analogs showed modest activity in vitro. A short list of MINC1 structural analogs, designed to have mostly single moiety (2-5) or functional group substitutions (6-9), were synthesized. The compounds were tested in dose response analyses (0.1-100 μM) against MgcRacGAP with the primary assay using MINC1 as control (red). Analogs 2 (blue), 6 (black) and 7 (green) showed modest activity, whereas most of the newly synthesized compounds displayed no activity. In addition, compound 7 (green) showed similar activity versus p50RhoGAP and BCR GAP. Error bars represent SD (n≥3).

The second hit compound, named MINC2, exhibited selectivity for MgcRacGAP over BCR and p50RhoGAP, with an IC50 of 18±7 μM (I; Fig. 3B/D). While there was no significant difference between the IC50’s in the initial biochemical data, subsequent cell-based assays indicated that MINC2 had no efficacy in cells and, therefore, we continued with MINC1 for follow-up studies.

In order to reduce the error caused by different incubation times, we preplated the compounds and re-dissolved these in assay buffer before adding the rest of the assay components. Lastly, the reaction was
initiated by adding GTP. We found that when GTP addition was delayed, and thus the proteins were given the time to pre-incubate with MINC1, the dose response curves shifted and the IC$_{50}$ decreased almost one order of magnitude to 2±1 μM (Figure 13A). When we analyzed this phenomenon more closely, we found that the shift only occurred when Rac1 was included in the pre-incubation mix, while presence of MgcRacGAP in the mixture had no additive effect on the inhibition (Figure 13B) nor did we observe any effects on GTP hydrolysis when MgcRacGAP was substituted with p50RhoGAP or left out.

Next, we set out to determine whether MINC1 was able to inhibit the GAP function of MgcRacGAP on wild type Rac1. Up till this point all the biochemical assay had been performed using fast cycling mutant Rac1 (F28L). To be able to measure GAP-stimulated GTPase activity of wild type Rac1, we used GTP-loaded Rac1. We found that there was no effect on the GAP activity by addition of MINC1 to a single turnover assay (I; Fig. S1B). When we replaced the fast-cycling mutant for wild type Rac1 protein, making the nucleotide exchange the rate-limiting factor (Figure 9A), we did not detect any notable inhibition of MgcRacGAP-stimulated activity by MINC1. These data suggest that, while MINC1 acts selectively on the Rac1-MgcRacGAP complex, the binding to Rac1 is slow and at the same time the interaction with MgcRacGAP is fast.

Based on these data, we hypothesized that MINC1 stabilized the Rac1-MgcRacGAP complex and as such inhibits GAP-catalyzed GTPase activity. To test this hypothesis we measured the real-time protein-protein interaction dynamics between MgcRacGAP and Rac1 in the presence or absence of MINC1 by bio-layer interferometry. In case the complex was treated with MINC1, we observed a dose dependent decrease of dissociation (Figure 14). Taken together, these data suggest that MINC1 stabilizes the Rac1-MgcRacGAP complex rather than inhibits binding.

5.1.3 MINC1 treated cells fail to complete cell division (I)

Previous data have shown that MgcRacGAP is required to complete cytokinesis, but also to play an important role in centromere maintenance$^{321,326,356}$. We predicted that if MINC1 would work as an
**Figure 13 MINC1 acts on the Rac1-MgcRacGAP complex through Rac1.** A) Using the primary assay conditions, a 2 h pre-incubation of MINC1 with the proteins before GTP addition results in a shift of the dose response curve by almost an order of magnitude; IC$_{50}$, no incubation 15±5 μM, IC$_{50}$, incubation 2±1 μM. Error bars represent SD (n=8). B) A 2 h pre-incubation of Rac1 with MINC1 (solid red line) results in the same dose response curves shift as pre-incubation of the complex with MINC1 (solid black line). The presence of MgcRacGAP in the mixture (dotted black line) had no effect on the inhibition. Inc/w, incubation with. Error bars represent SD (n=3).
MINC1 stabilizes the Rac1-MgcRacGAP complex. Real-time protein-protein interaction dynamics between GST-MgcRacGAP and 6xHis-Rac1 (Q61L) in the presence or absence of MINC1 were analyzed by bio-layer interferometry. MgcRacGAP was immobilized on an anti-GST probe, whereafter Rac1 (Q61L) was bound to MgcRacGAP in the presence of 12.5-50 μM MINC1 or 2% DMSO. The dissociation of Rac1 (Q61L) from MgcRacGAP was determined in the presence of 12.5-50 μM MINC1 or 2% DMSO. Pre-incubation of MINC1 with the complex slowed down the dissociation process in dose dependent manner. Error bars not presented (n=2).

Figure 15 MgcRacGAP inhibitor in cells, it would have a significant effect on cell growth. Next, we proceeded to investigate whether MINC1 had an effect on cells by monitoring cell proliferation and cell death using live-cell imaging. We found that treatment with MINC1 significantly, and dose dependently, slowed down cell proliferation (Figure 15). The predicted IC₅₀ in the cell-based assay (~20 μM) was surprisingly low, being in the same order of magnitude as the IC₅₀ of the initial biochemical data (~15 μM). Unusual kinetic behaviors such as slow binding behavior and/or slow off rates have been correlated with disproportionately potent cell-based activity. Taken together with the observed near one-order of magnitude shift of the IC₅₀ in the biochemical assay after pre-incubation, the potent cell-based activity strengthens the hypothesis that MINC1 stabilizes the Rac1-MgcRacGAP complex.

Cell death did not occur directly upon treatment with MINC1, but only with prolonged high dose-treatment of MINC1 (Figure 15). We noticed that MINC1-treated cells rounded up, as expected during division of adherent cells, but seemingly failed to complete cell division. Prolonged
Figure 15 **MINC1 treatment dose-dependently reduced proliferation rate of human tumor-derived cell without causing direct toxicity.** MDA-MB-231 cells were treated with 10-80 μM MINC1, DMSO (black) or 20 μM benzethonium chloride (BzCl, red) and followed by live cell imaging for 70h for proliferation (upper panel, determined by confluency) or cell toxicity (lower panel, determined by fluorescence intensity). Significance was assessed at three different time points (12 - 36 - 60h) using two-way ANOVA followed by Dunnett’s multiple comparisons test (confluence control DMSO and death control BzCl). *, P<0.05; **, P<0.01; ***, P<0.001, ****, P<0.0001; n.a. not applicable; ns; not significant. Error bars represent SD (n=4).
mitotic arrest cell death is associated with apoptosis\textsuperscript{385,386}. We investigated whether MINC1 treatment prevented the cell from entering mitosis (M-phase). Using both non-synchronized as well as synchronized cell cultures, we found that MINC1 treated cells fail to pass from G2 to M-phase (\textit{I}; \textbf{Fig 5}). Interestingly, cells in a non-synchronized cells culture did not phenotypically respond until after >3 h of MINC1 exposure, providing support to the slow kinetic behavior of MINC1.

Despite the fact that we failed to detect changes in overall Rac1 GTP levels upon MINC1 treatment, which is not unexpected as Rac1 will be mostly GDP-bound and only locally activated, these data are in line with what can be expected from an MgcRacGAP inhibitor. Further research could utilize Förster resonance energy transfer (FRET)-based biosensor for GTPases, \textit{e.g.} the Raichu biosensors\textsuperscript{387-389}, to measure the effect of MINC1 on the activation of Rac1 or other GTPases with high spatial and temporal resolution.

To obtain more insight in the cell division arrest, several different cell lines were treated with MINC1 for up to 48 h and fixed at different time points for immunofluorescence staining and image analysis. MINC1-treated cells showed a lower endpoint confluence as their counterparts when plated at similar densities, consistent with the earlier observation of reduced proliferation, as well as considerable amount of multinucleated cells and round-up cells with abnormal positioning of the nuclear DNA (\textit{I}; \textbf{Fig. 6C/F}). To characterize the effect of MINC1 on cell cycle progression and failed cytokinesis, we quantified the number of normal, mitotic, multinucleated and dead cells (Figure \textbf{16}). We observed a peak of the mitotic phenotype in the first 9 h after initiation of the treatment, whereas the number of multinucleated cells increased continuously, quadrupling in 24 h.

A subpopulation of the MINC1-treated cells displayed abnormal mitotic positioning of the nuclear DNA (\textit{I}; \textbf{Fig. 6D}). A similar monopolar spindle phenotype is reported for cells depleted of Plk1\textsuperscript{390-392}. Plk1 is an essential serine/threonine kinase in multiple early mitotic processes and directly regulates MgcRacGAP\textsuperscript{312-314}. Moreover, inhibition of Plk1 has been demonstrated to induce a loss of interaction between Ect2 and
MINC1 treatment impeded the cells from progressing mitosis. A549 cells were treated with DMSO (0 h control) or 25 μM MINC1 and fixed at different time points. The cells were subsequently stained to distinguish the membrane/cytoskeleton and nucleus. Immunofluorescence images were quantified for the amount of normal, mitotic, multinucleated and death cells per time point and results were plotted as a bar graph. Error bars represent SD (n>230 cells ‘24 h MINC1 treated’ condition and n>320 all other conditions).

MgcRacGAP in anaphase, preventing initiation of cytokinesis. Based on the similarities we decided to follow-up with high-magnification imaging and found extensive membrane protrusions (Figure 17). Cell morphology is determined by the balance of Rac and Rho activity, and these extensive membrane protrusions found in the MINC1-treated cells may suggest a loss of cortical actin rigidity that has been linked to deregulated Rac/Rho activity. Additionally, using a constitutively active MgcRacGAP mutant it was demonstrated that inhibition of MgcRacGAP is important for the correct formation of the mitotic spindle during the metaphase. Altogether, these data suggest that the GAP activity of MgcRacGAP plays an important role not only in cytokinesis, but already in mitosis and may be regulated by Plk1.

Alternatively, the abnormal mitotic positioning of the nuclear DNA may result from other MINC1-induced effects on the cell division machinery through different targets. Like the Plk1-inhibited phenotype, the Eg5-inhibited phenotype resembles a monopolar arrest, yet with a subtle morphological difference; in case of Plk1 inhibition, the chromosomes are unevenly spaced from the spindle pole, whereas the Eg5-inhibited phenotype is characterized by evenly spaced chromosomes. In study I
Figure 17 Abnormal spindle morphology in the metaphase in MINC1 treated cells. Representative 60x immunofluorescence images of mitotic and cytokinetic HeLa cells. Cells were treated for 48 h with DMSO (A/C/E/G) or 25 μM MINC1 (B/D/F/H). DNA (A/B, blue G/H) was visualized with Hoechst stain and α-tubulin (C/D, green G/H) and MgcRacGAP (E/F, red G/H) stained with antibodies. The characteristic metaphase spindles originating from opposite poles are lacking in MINC1 treated cells, instead they have a collapse spindle morphology (C-D, indicated by arrow). Rounded-up MINC1 treatment cells displayed extensive membrane protrusion (D, indicated by round ended arrow). Scale bar, 10 μm.
we reported that the chromosomes were unevenly spaced from the pole, similar to the Plk1-inhibited phenotype, but retrospectively it is arguable the chromosomes have been evenly spaced from the pole (Figure 17). Interestingly, a study using Tiam1-depleted cells as well as Rac1-deficient epithelial cells showed that these cells could escape an Eg5-induced mitotic arrest. Specifically, it was shown that Tiam1-Rac signaling is required to antagonize the Eg5-induced forces during bipolar spindle assembly. With Rac1 suppression shown to be essential for cytokinesis in both HeLa and RatA1 cells, and observed membrane protrusions suggesting a high Rac1/low RhoA activity in MINC1-treated cells, we hypothesize that MgcRacGAP-mediated suppression of the Rac1-PAK pathway may play a pivotal role in the cytokinesis. Accordingly, a recent study showed that depletion of Rac1 substrates PAK1/2 resulted in resistance to Eg5-induced mitotic arrest. Since Tiam1 is a RhoGEF, it acts as an antagonist of the RhoGAP. Thus, in both the Tiam1-induced as well as the MgcRacGAP-inhibited situation, Rac1 will be locally activated. To determine whether the GEF-induced and GAP-inhibited indeed result in a similar phenotype, it would be interesting to test if depletion of PAK1/2 can also rescue of MINC1-induced phenotype.

5.1.4 MgcRacGAP inhibition causes STAT3 transcriptional activity (II)

STAT proteins are well established for their ability to transmit specific signals from the plasma membrane to target genes in the nucleus, albeit all are activated by phosphorylation of a single tyrosine, predominantly by JAK family kinases. The co-regulatory events, such as nuclear import as well as alternative activation pathways appear to play important roles in the specificity of the signal.

Previous studies have described results linking Rac1 to STAT3 activity. For example, Rac1 activation was shown to induce autocrine interleukin-6 (IL-6) secretion stimulating STAT3 activity. Another study showed that activated Rac1 could form a complex with STAT3. Accordingly, the authors hypothesized that Rac1-mediated recruitment presents an alternative method to direct STAT3 to the kinase signaling complexes at the membrane. Finally, a series of studies described an interaction between MgcRacGAP, activated Rac1 and STAT family members 3 and 5A. Specifically, the authors proposed that MgcRacGAP/Rac-GTP
form a stable complex, which functions both as a mediator of STAT tyrosine phosphorylation as well as a chaperone for nuclear translocation of STAT proteins. Despite largely complementing previously proposed model, the results are seemingly contradictory, as the MgcRacGAP-Rac1 interaction will initiate MgcRacGAP-mediated GTP hydrolysis and, therefore, it is unlikely to have a long-lived MgcRacGAP/Rac-GTP complex.

Since previous reports proposed that the MgcRacGAP-Rac1 complex plays a key role in STAT activation and nuclear translocation, we decided to examine whether targeting the MgcRacGAP-Rac1 complex with MINC1 could interfere with STAT3 signaling. Surprisingly, we found that treatment with MINC1 resulted in a dose dependent increased STAT3 signaling for both starved and IL-6 stimulated cells (Figure 18). Previous results had been obtained with gene silencing by siRNA and, therefore, we also performed a siRNA-mediated knock down experiment. However, using a similar setup, we could not reproduce the previous results (Figure 19). Instead, under these conditions we observed that MgcRacGAP knockdown caused an increase in STAT3 transcription activity that resembled those obtained with MINC1. To verify whether the results were applicable to other cell lines as well as different assay models, we used transient transfections of STAT3 and STAT5 reporter plasmids in both HEK293 and HeLa cells. Also in these assay systems STAT transcriptional activation was dose dependently stimulated by MgcRacGAP-Rac1 inhibition (II; Fig. 1D-E).

Considering the role of MgcRacGAP in the highly regulated Rac1 GDP-GTP cycle, inhibition of the GAP function of MgcRacGAP is expected to lead to increased downstream signaling activity of Rac1. This downstream signaling activity of Rac1 can be mimicked using constitutively active Rac1 and work of others has shown that active Rac1 (G12V) induces STAT3 phosphorylation. Vice versa, we and others have shown by using siRNA mediated knockdown of Rac1 (II; Fig. S1) and a dominant negative Rac1 (T17N), respectively, that the inability of activating Rac1 results in the loss of STAT3 transcriptional signal. Based on these results we concluded that the GAP activity of MgcRacGAP controls Rac1 stimulated STAT3 transcriptional activity.
MINC1-mediated inhibition of MgcRacGAP induces a dose dependent increase of STAT3 transcriptional activity. Serum starved HEK293 SIE-Luc cells were, in the presence of vehicle (DMSO), 10 µM Stattic or increasing concentrations of the MgcRacGAP inhibitor MINC1, induced with or without IL-6. MINC-1 treatment significantly increased the STAT3 transcriptional activity of IL-6 induced HEK293 SIE-Luc cells (p≤0.0001, Sidak's multiple comparisons test). Error bars represent SD (n=3).

Increased STAT3 transcriptional activity by siRNA-mediated knockdown of MgcRacGAP. Gene silencing by siRNA targeting MgcRacGAP in HEK293 SIE-Luc cells showed activation of STAT3 for serum starved and IL-6 induced conditions. Sidak's multiple comparisons test was used to assess significance of the siRNA-mediated knockdown of MgcRacGAP (p≤0.01). Error bars represent SD (n=3).
5.1.5 MgcRacGAP inhibition causes phosphorylation of STAT3 (II)

To clarify the mechanism leading to STAT3 transcriptional activation, we decided to assess STAT3 phosphorylation status as well as cellular localization. We used lysates of HEK293 SIE-Luc cells and demonstrated that both siRNA-mediated and MINC1-mediated inactivation of MgcRacGAP resulted in an increase of STAT3 phosphorylation signal (II; Fig. 1C). Immunofluorescence image analysis of fixed HEK SIE-Luc cells revealed an upregulation of phospho-STAT3 signal in MINC1-treated cells compared to untreated counterparts, whereas no differences in total STAT3 were observed (Figure 20). Of note, despite the increased STAT3 transcriptional activity measured in the preceding experiments, the phospho-STAT3 signal appeared to be relatively uniformly upregulated through the MINC1-treated cells and not localized in the nucleus as might be anticipated (Figure 21A-B). Subsequent analysis with IL-6 stimulated cells showed that inhibition of MgcRacGAP did not affect the nuclear translocation of phospho-STAT3 (Figure 21C-D). Similar results have been published, showing that dominant negative Rac1 (T17N) does not affect nuclear transport of STAT3405. Together with the data showing that activated Rac1 induced STAT3 phosphorylation402-404, we hypothesize that the GAP function of MgcRacGAP is required for the termination of the GTPase signaling, but not for nuclear translocation.

Figure 20 MINC1-mediated MgcRacGAP inhibition induces tyrosine phosphorylation of STAT3. HEK293 SIE-Luc cells were treated for 6 h with either DMSO vehicle or 25 µM MINC1, fixed and immunostained with anti-STAT3 and anti-phospho-STAT3. The relative intensities of the STAT3 and phospho-STAT3 signals were determined from randomly selected immunofluorescence images and normalized for each antibody separately. Significance was assessed using Wilcoxon matched-pairs signed rank test. Error bars represent SD (n=8; *, P ≤ 0.05).
Figure 21  Treatment with MgcRacGAP inhibitor MINC1 induces tyrosine phosphorylation of STAT3, but does not block nuclear translocation of tyrosine phosphorylated STAT3. Representative immunofluorescence images of HEK293 SIE-Luc cells that were induced with IL-6 for 3 h (C-D) in the presence of either DMSO vehicle (A/C) or 25 µM MINC1 (B/D), were fixed and immunostained with Hoechst dye (1), anti-phospho-STAT3 (2) and anti-STAT3 (3). In non-IL-6 induced cell, MINC1 treated cells showed more intense phospho-STAT3 staining than control cells, whereas inhibition of MgcRacGAP by MINC1 did not lead to an inhibition of nuclear translocation of phospho-STAT3 in IL-6 induced cells. Scale bar, 25 µm.
MINC1-induced STAT3 activation is suppressed by inhibition of JAK/STAT. HEK293 SIE-Luc cells were treated with different concentrations of MINC1 and subsequently induced with IL-6 in the presence of vehicle (DMSO), 300 nM ruxolitinib (JAK1/2 inhibitor) or 10 µM Stattic (STAT3 inhibitor). Bonferroni post-hoc results indicated that significantly less STAT3 activation was detected when cells were treated with ruxolitinib or Stattic (p≤0.01). Error bars represent SD (n=6).

While STAT proteins can be activated by several different regulators, they are predominantly phosphorylated by JAK family kinases. It is well-established that STAT3 is activated by JAK family kinases upon IL-6 stimulation and it has also been demonstrated that Rac1-driven STAT3 activity is dependent on JAK2. To further examine the role of MgcRacGAP in the activation of STAT3, we turned to the JAK/STAT signaling pathway and investigated if inhibition of JAK, with JAK1/2 inhibitor ruxolitinib, or STAT3, with STAT3 inhibitor Stattic, would prevent MgcRacGAP inhibition-mediated STAT3 activation. We found that both ruxolitinib and Stattic treatment effectively blocked MINC1-mediated STAT3 activation, which indicates that the role of MgcRacGAP in the activation of STAT3 is upstream of JAKs (Figure 22).

In our immortalized, non-cancerous cell model STAT3 phosphorylation levels were inversely correlated with cell confluency, whereas the opposite has been reported for cancer cell lines. Moreover, persistent activation of STAT3 in colon cancer has been shown to be required for tumor growth, with inhibition ceasing cell proliferation as well as inducing apoptosis. Based on these observations, we hypothesized that STAT activity is upregulated in cancer cells to evade...
cell cycle arrest as well as gain the ability to migrate from the confluent site. A recent publication described a mechanism for the induction of IL-6 production through the activation of PAR3 signaling. PAR3 is a scaffold protein that binds the highly selective Rac GEF Tiam1, thereby directly regulating Rac1 activity. Another study demonstrated that PAR3 activates STAT3 through Rac1 and that reduced tumor-invasive and metastatic properties were observed if impaired PAR3 activity was reconstituted. Accordingly, we examined whether MINC1-mediated STAT3 activation was the result of PAR3-induced IL6 production and subsequent activation of the IL-6 receptor (IL6R)-JAK-STAT signaling axis. We found that MINC1-induced STAT3 activation was impaired by the knockdown of PAR3, JAK2 and IL6R (Figure 23). These results place Rac1, and its regulation by MgcRacGAP, upstream of Par3 as been shown before, and suggest that the STAT3 activation is dependent on a paracrine or autocrine IL-6 signaling feedback mechanism. In line with this hypothesis, STAT3 activation in cancer cells might be the result of a loss of cell-to-cell integrity, causing elevated levels of phospho-STAT3, which allow the cells to continuously replicate. It will be interesting to test whether IL-6 neutralizing antibodies can attenuate this effect uncontrolled proliferation.

Figure 23 MINC1-induced STAT3 activation is dependent on a PAR3-IL6-IL6R-JAK2 feedback loop. HEK293 SIE-Luc cells were transiently transfected with siRNA targeting PAR3, IL-6 receptor (IL6R), JAK2, STAT3 or scramble siRNA. Subsequently the HEK293 SIE-Luc cells were left untreated or treated with MINC1. In the PAR3, IL6R and JAK2 KD cells MINC1 treatment resulted in a significant loss of STAT3 transcriptional activity in comparison to the control cells (p≤0.05, Dunnett’s multiple comparisons test). Bars represent the average fold change for the non-treated and treated siRNAs pairs. Error bars represent SD (n=3).
Altogether, using both small molecule inhibitors and siRNAs, we established that inhibition of MgcRacGAP results in the activation of STAT3 and subsequent transcriptional activity. We found no evidence that MgcRacGAP has a function in nuclear translocation of STAT proteins, which is in contrast with earlier proposed models, established with a similar model system. Our results argue for a non-direct relationship between Rac1 and STAT3, mediated through the PAR3-IL6-IL6R-JAK2 signaling axis. However, direct interaction between Rac1 and STAT3 cannot be ruled out, as work of others has demonstrated that both models exist and, depending on cell type, even co-exist\textsuperscript{420}. With the growing awareness of the complexity of the highly integrated signaling network within cell, where small GTPases and their regulators direct and connect different pathways, it is not unlikely that the relationship between MgcRacGAP and STAT transcription factors is context-dependent and needs to be determined case-by-case.
5.2 The Ras inhibitors (III)

5.2.1 Development of Ras biochemical assays (III)

Drug discovery efforts targeting GTPases and their regulators have been hampered by a lack of suitable screening methodologies. Due to the high enzymatic activity, kinase assay have relative low protein consumption, whereas GTPase assays tend to use relative high concentrations of protein. This is particularly the case when using insensitive fluorescence-based methods, such as Mant\textsuperscript{421} and BODIPY\textsuperscript{422,423}. Measurements have been limited to GTPase or GEF activity, which can be achieved with incorporation or displacement of a fluorescently labeled analog. On the other hand, filtration binding assays using radioactive H-3 or P-32 γ-phosphate-labeled GTP are sensitive, and can be used to measure GAP activity as well\textsuperscript{424,425}. Activity is determined by measuring either residual radioactive GTP or inorganic phosphate, which is hazardous and, therefore, relatively expensive as well as not easily adapted to high throughput assays. Recently developed NMR methods use safe isotopes like C-13 and N-15 and can be applied real-time, however, they require substantial amount of isotope labeled protein\textsuperscript{426}.

A GTP-specific Fab fragment, 2A4\textsubscript{GTP} Fab, has been described and used to develop a heterogeneous assay to monitor GTP hydrolysis \textit{in vitro}\textsuperscript{427}. The Fab fragment has been developed to improve the quenching resonance energy transfer (QRET) method\textsuperscript{376}, which could only be used to monitor GTPase nucleotide exchange. The QRET method is based on the energy transfer between an Eu\textsuperscript{3+}-conjugated GTP and soluble quencher (Figure 24A). When the Eu\textsuperscript{3+}-GTP molecule is sterically protected, for example by binding to the GTPase or 2A4\textsubscript{GTP} Fab, the quencher no longer inhibits the lanthanide-based resonance energy transfer, resulting in an increase of signal. Like most other methods that monitor GTPase nucleotide exchange\textsuperscript{240,428,429}, the QRET method could not be used to detect GTPase-GEF complex conformation-trapping or non-competitive interfacial inhibitors\textsuperscript{279}. However, with the introduction of the Fab fragment, the full GTPase reaction can be monitored; the decrease of GTP, which as a result of conversion to GDP is effectively replaced by the nonhydrolyzable Eu\textsuperscript{3+}-GTP from the 2A4\textsubscript{GTP} Fab, results in an increase of signal because the bound 2A4\textsubscript{GTP} Fab is protected from the soluble quencher (Figure 24).
Figure 24 *Inhibitor screening strategy using competitive 2A4<sup>GTP</sup> Fab GTP hydrolysis detection.* A) In the presence of a relative high concentration of GTP ($T_1$), GTP will out compete Eu<sup>3+</sup>-GTP for binding to the GTP-specific Fab fragment, 2A4GTP Fab. While in solution, quencher (Q) will prevent energy transfer to the lanthanide and inhibit the luminescence signal. When less GTP is present ($T_2$), Eu<sup>3+</sup>-GTP will bind to 2A4GTP Fab and be sterically protected from the quencher, resulting in energy transfer and luminescence signal. B) Assay setup, where in the presence of GEF and GAP proteins, as well as an excess amount of GTP, GTPases will continuously hydrolyze GTP to GDP. C) Initially, high concentrations of GTP efficiently block Eu<sup>3+</sup>-GTP binding to the GTP-specific binding fragment (2A4<sup>GTP</sup> Fab). As a result, most of the Eu<sup>3+</sup>-GTP is in solution with the soluble quencher (Q). As long as the GTPlases continuously hydrolyzes GTP to GDP, GTP is converted to GDP and thus the nonhydrolyzable Eu<sup>3+</sup>-GTP will replace the GTP bound to the 2A4<sup>GTP</sup> Fab fragment. The 2A4<sup>GTP</sup> Fab fragment bound Eu<sup>3+</sup>-GTP is protected from the soluble quencher, resulting in an increase in time-resolved luminescence (TRL) signal over time. The reaction can be monitored real-time or by a two-step protocol (for which Eu<sup>3+</sup>-GTP and quencher are added after incubation period).
Because of the heterogeneous nature of the method, the GTP hydrolysis assay was not suitable for HTS. To create a homogenous assay suitable for HTS, we first optimized the salt concentrations. Previous results obtained with high salt concentration suggested that salt bridge formation potentially interfered with GTP and 2A4_{GTP} binding, resulting in a suboptimal signal\cite{427}. Indeed, we determined that with low salt concentrations the selectivity for GTP over GDP increased by an additional four-fold, establishing 220-fold selectivity for GTP over GDP (III; Fig. 1B-C and Table S-1). Next, different protein and nucleotide concentrations were tested to determine reproducibility as well as best controls (III; Fig. 1E). We mimicked 100% inhibition in the same manner as with the MgcRacGAP assay. However, excluding GAP from the reaction mixture resulted in a slightly elevated background signal compared to omitting either GEF or GTPase. We hypothesized that the GEF-facilitated dissociation of GDP enabled Ras to bind GTP and, by intrinsic GTP hydrolysis, subsequently converted to GDP. While this reaction will be significantly slower than the GAP-catalyzed reaction, we decided that omitting the GEF from the reaction mixture would serve as better positive control.

5.2.2 Identification and validation of Ras hydrolysis inhibitors (III)
In order to compare the performance of the GTP hydrolysis assay to a standard association assay, we miniaturized the QRET nucleotide exchange method\cite{376}, and subsequently assayed two identical sets of 1 280 chemically diverse compounds. The average Z'-factors ranged from 0.64 to 0.88. To identify active compounds eligible for verification by follow-up screening, we applied to both methods the same, predefined, thresholds (Table 5). After the first round of single dose testing, we identified 22 hits; eight compounds were found with both methods, nine exclusively with the GTPase cycling assay and five only with the nucleotide exchange assay (Figure 25 and III; Fig. 1F/H-I). From these 22 compounds, ten compounds were confirmed when tested as triplicates (III; Table 1 and Supplementary Table S-2 and Fig. S-2). Six compounds were identified with both the nucleotide exchange assay and the GTPase cycling assay. In addition, we identified a set of four compounds exclusively with the GTP hydrolysis assay. In subsequent dose response testing, all appeared to inhibit GTPase cycling or nucleotide
association in a concentration dependent manner. Of note, for most of the hit compounds there was only limited amount of bioactivity data available on PubChem. While most compounds with additional public bioactivity data were inactive in those cases, three compounds (QT-13, QT-113 and QT-317) had tested active in some bioassays, but none of these compounds were previously presented in a context of GTPases. Based on a pan-assay interference compounds filter\textsuperscript{430}, three compounds (QT-13, QT-26 and QT-113) were identified as containing substructural features that frequently cause false positive activity in a broad range of assays.

The primary screening identified a set of five compounds that were only active in the nucleotide exchange assay. Since the GTPase cycling assay should detect both GTP hydrolysis inhibitors and nucleotide exchange inhibitors, we did not expect to identify such a group, unless they were false positive hits. Confirmatory testing showed, like expected, that none of these five compounds had activity in the nucleotide exchange assay.

![Diagram](image)

Figure 25 The GTPase cycling assay identifies compounds that would go undetected by nucleotide exchange assay screening. A total of 22 compounds were identified in the primary screening round; nine were exclusively detected with the GTPase cycling assay (blue circle), five with the nucleotide exchange assay (yellow circle) and eight were found with both methods (overlap; in green). When the hit compounds were retested in triplicate, 10 of the 22 compounds confirmed; six exhibited inhibitory activity in both assays, suggesting that they affected nucleotide binding or exchange, whereas four inhibited only the GTPase cycling assay, suggesting that they were inhibitors of GTP hydrolysis or GAP activity.
Figure 26 **Three compounds show efficacy in cell-based assays.** Three lung cancer cell lines dependent on the K-Ras signaling pathway were subjected to dose response treatments of QT-43 (upper panels), QT-47 (middle panels) and QT-113 (lower panels). NCI-H292 (KRAS<sub>wt</sub>) (left), A549 (KRAS<sub>G12S</sub>) (middle) and H460 (KRAS<sub>Q61H</sub>) (right) were treated for 72 h, whereafter cell death (red) and cell survival (black) were determined by the multiplexed CellTox-Green cytotoxicity and CellTiter-Glo cell viability assay. Error bars represent SD (n=3).

To address the specificity of the identified compounds, we changed RasGAP p120RasGAP for NF-1 in the GTP hydrolysis assay (**III; Table 1**). It has been shown that NF-1 and p120RasGAP have different kinetic and thermodynamic characteristics when interacting with Ras, as well as form strikingly different complexes with Ras<sup>201,431</sup>. Changing the GAP did not result in a different inhibition pattern. Therefore, we hypothesized that these inhibitors target either the Ras or the Ras-Sos interface. To test this we used the related and unrelated GTPase proteins, K-Ras and RhoA (with RhoGEF Ect2), respectively, in the nucleotide association assay (**III; Table 1**). The interaction between GTPase and regulators is, in the majority of cases, family specific. We found that with the same threshold, five out of six compounds inhibited also the K-Ras/Sos interaction, whereas Rho/Ect2 nucleotide exchange was not affected. These data
strengthen the hypothesis that these inhibitors target Ras or the Ras-Sos interface. However, since all assays have been performed with the same detection technique, it could be possible that the compounds interfere with the QRET method. Though unlikely for a lanthanide-based assay to cause false positive detection due to autofluorescence, altering kinetics of nucleotide hydrolysis and/ or exchange could also be affected by the detection method. The well-established Mant-GTP assay, commonly used to measure nucleotide exchange, has recently been shown to have effects on GTPase reaction kinetics with the Mant-moiety. Future research should address the lack of orthogonal screening by testing these hits using the Mant-GTP assay, which should be used with caution, and/ or the new GTPase/GAP/GEF-Glo Bioluminescent Assay System. Another option is to validate the GTPase system and detecting method using the NMR-based assay that was used to interrogate the Mant-GTP assay (reviewed in [426]).

5.2.3 Ras hydrolysis inhibitors cause distinct cellular phenotypes (III)

Recent data have shown that K-Ras-driven cancers are dependent on wild-type H- and N-Ras. Thus, to determine whether the hit compounds had an efficacy in cells, we selected three cell lines that appear to be dependent on K-Ras signaling, since inhibition of the MAPK pathway effectively inhibits their cell proliferation. Two of the cell lines had activating KRAS point mutations; A549 (KRASG12S) and H460 (KRASQ61H) while the third, NCI-H292, did not carry KRAS mutations but was addicted to EGFR signaling through the Ras-MAPK pathway. We observed that three compounds displayed inhibitory effects on cell proliferation; QT-113 selectively reduced cell growth of the wild-type KRAS cells, whereas QT-43 and QT-46 inhibited the growth of all three cell lines, with the most prominent effect in cells possessing KRAS mutations (Figure 26).

In order to determine whether the compounds that demonstrated dose-dependent activity in the cell-based assays had a direct effect on cellular Ras protein, we examined their effect on nucleotide loading of Ras using serum starved EGF-stimulated HeLa cells. We found that a short incubation of QT-43 resulted in a moderate inhibition of EGF-induced GTP-loading, while a prolonged incubation with both QT-43 and QT-113 strongly reduced EGF-induced GTP-loading (Figure 27). Interestingly, we
noted that QT-43 not only effectively reduced Ras activation, there also seemed a baseline decrease total Ras. To rule out the possibility that the loss of Ras-GTP signal of prolonged QT-43 treatment was due to decreased input, presumably caused by decreased cell viability, we assessed protein expression and phosphorylation status using western blot analysis, investigating the effect of compound treatment on the Ras effector signaling pathways, MAPK and Akt (Figure 27). As before, HeLa cells were starved overnight and subsequently stimulated with EGF to induce Ras signaling. In line with the hypothesis that a decreased input caused the loss of signal, we did not observe any change in the two main downstream signaling pathways, MAPK and Akt, for compound QT-43. If any change was observed, it was an increase after 1 h of induction. A similar initial increase of signaling was observed for QT-113, with a substantial increase of pMEK both in baseline and EGF-stimulated sample. On the other hand, overnight treatment with QT-113 resulted in a

Figure 27 Long-term compound treatment induces loss of Ras effector signaling through MAPK pathways. To investigate the effect of compound treatment on the Ras effector signaling pathways, HeLa cells were serum deprived for 18 h in the presence of 100 µM compound and subsequently stimulated with 200 ng/mL epidermal growth factor (EGF) to induce Ras signaling. No change was observed for compound QT-43 in the two main downstream signaling pathways of Ras, whereas compound QT-113 induced a reduction of pAkt and a near complete loss of MAPK signaling.
reduction of pAkt and a near complete loss of phosphorylation of the MAPK signal pathway proteins.

Next we assessed the time point the effects of QT-113 treatment became apparent. Since biochemical inhibition of Ras cycling is expected to happen almost instantly, but the morphological changes that are depended on downstream signaling changes on might take some time, we focused on the first 8 h, including an overnight treatment as long-term control. We analyzed the cell morphology by live-cell imaging as well as collected cell lysates for phospho-protein analysis. Within the first 30 min, we observed a strong phenotypic response such that the cells subjected to QT-113 showed enhanced membrane ruffling, suggesting a rapid response of some kind (Figure 28). This effect was restricted only to the serum starved, QT-113 treated cells, as the QT-113 treated cells in complete medium did not display any immediate effect. Subsequent western blot analysis of the two main Ras effector signaling pathways revealed no immediate inhibitory response. On the contrary, long-term treatment of QT-113 led to a reduction of pAkt as well as potent decrease of MAPK pathway phosphorylation, whereas the short-term treatment initially moderately increased phosphorylation levels. Analysis of the live-cell imaging data revealed that the long-term treated samples had over the course of the experiment gradually built up a distinct phenotype. The majority of cells displayed extensive amount of vacuole formation close to the nucleus (Figure 28). Strikingly, this phenotypic effect was again only observed in the serum starved QT-113 treated cells, as cells treated with QT-133 in complete media or vehicle in starvation medium did not accumulate these vacuoles.

Taken together, QT-113 does induce a potent biological effect in NCI-H292 and HeLa cells, cells that carry RAS wild type genes. We did not observe growth inhibition using RAS mutant cell lines. Considering that the biochemical assay is designed with the intent to identify small molecule modulators of Ras GTPase cycling, GAP-insensitive mutant and cycling independent mutant cells will not be affected as much as RAS wild type cells. However, there are reasons to expect that the responses in NCI-H292 and HeLa cells are not linked to the effects seen in the biochemical Ras cycling assays. First, except for the fast, distinct
Figure 28 QT-113 treatment induces strong phenotypic response. Representative 20x phase-contrast images of HeLa cells at different time points of the treatment. Cells were conditioned for 6 h in complete medium (left column) or starvation medium (middle and right columns). Subsequently, cells were treated with 80 µM QT-113 (left and middle columns) or DMSO (right column). Within 15 min after compound addition (middle row), serum starved QT-113-treated cells exhibited ruffling of the membrane (middle panel), which was not observed in the complete medium counterpart or in either of the DMSO controls (left and right panel). Long-term exposure to QT-113 (>8 h) in starvation medium resulted in extensive amount of vacuole formation (last row, middle panel), while this phenotype was absent in both the complete medium counterpart and either of the DMSO controls (last row, left and right panel). Scale bar, 50 µm.
membrane ruffling (Figure 28), the overall response in cells occurred only after an extended incubation time with the compound, which is not consistent with biochemical inhibition of Ras cycling. Instead, the need for a long-term incubation argues that the reduction in Ras activation and downstream signaling comes as a result of the cells being severely affected by the long-term presence of the compound and, therefore, less responsive to external stimuli. In agreement with this conclusion we find, prior to the loss of response to EGF, that the compound-treated cells have accumulated large amounts of intracellular vacuoles. This distinct phenotype of extreme vacuolization has been associated with dysfunctional endocytosis and an unique form of non-apoptotic cell death in cell cultures called methuosis (reviewed in [435]), but is unclear whether this can occur under normal physiological conditions. Second, QT-113 is a compound flagged as a pan-assay interference compounds (PAINS)\(^{430}\). Its chemical structure is based on a fused tetra hydroquinoline backbone, which is a prime suspect for exhibiting false positive results in biochemical assays as well as complex effects in cell-based assays. Accordingly, QT-113 analogs deposited in PubChem that have been through thorough bioassay testing, appear as frequent positive results in diverse biochemical assays. One of these, Golgicide A\(^{436}\), is a compound affecting the Golgi apparatus. The limited biochemical and cell-based data precludes us from knowing whether QT-113 would be a true false positive or whether it will have effects on the Golgi in cells, but it is likely to have complex and polypharmacological effects in cells. Therefore, if QT-113 should be followed up on, it should be explored using analogs that do not contain the PAINS-flagging elements.

Despite the small number of compounds used in the proof-of-concept screen, we successfully used both the GTP hydrolysis assay as well as the nucleotide exchange assay to identify Ras cycle inhibitory molecules. Moreover, three of these compounds inhibited cell proliferation of Ras signaling dependent cell lines, of which one compound arguably showed to affect Ras GTP-loading. While none of the inhibitors exclusively identified with the GTP hydrolysis assay had any efficacy in the cell-based assays, the discovery of this unique set demonstrates the potential of this type of biochemical screening assays over the traditional assays that are designed to screen for inhibitors blocking a specific interaction of the Ras cycle. Given that this study was done with only a very small number of
compounds, it is likely that larger screens with greater chemical diversity will increase the probability of identifying cell active compounds.
6. Conclusions and future perspectives

The advances in the field of molecular biology over the past fifty years have contributed to an increased understanding of the encoding role of genes and executive task of proteins, and that aberrant function is the molecular basis of human disease. Small GTPases and their regulators form highly integrated signaling networks that, under tight temporal and spatial control, regulate a myriad of different cellular pathways. It is not surprising that various members of the GTPase protein families are associated with multiple human diseases, like cancer, neurodegenerative diseases, and inflammatory disorders. However, to date, despite the rational, biology-driven drug development only very few small molecule modulators of GTPase activity exist. In this thesis, the aim was to identify new modulators by the development of new biochemical assays, specifically targeting the previously undrugged GTPase-regulatory proteins as their modulation is expected to result in specific inhibition of selected GTPase signaling pathways.

Prior to these studies, RhoGAPs had not been successfully targeted in chemical biology or drug discovery. With the identification of MINC1 as a specific Rac1 - MgcRacGAP inhibitor, we showed in study I that RhoGAPs are both screenable and druggable small molecule targets. However, as MINC1 was discovered from a small molecule screen of over 360 000 compounds and with optimization studies still unsuccessful, it shows that RhoGAPs are challenging small molecule targets. The reason why MINC1 specifically inhibits Rac1-MgcRacGAP in vitro remains unknown. The recent observation that MgcRacGAP and Rac1 interact differently than other Rho GTPase and GAP pairs\textsuperscript{382}, leaving a twice as large space between them, could explain the specificity and, more importantly, could assist in future in silico modeling to identify compounds with higher affinity and faster binding kinetics. We expect that analogs with increased potency will help to improve the understanding of the function of MgcRacGAP in both normal and malignant cellular processes. Further, to address the high potency in cellular systems in the immediate future, without the help of more potent analogs, it would be interesting to study MINC1 in GTPase FRET-based biosensor reporter systems. If functional changes will be observed, this could lead to next questions such as...
whether local MgcRacGAP-mediated suppression of the Rac1-PAK pathway leads to the upregulation of RhoA within the division plane during cytokinesis. While both Rac and RhoA biosensor are available, the current binding kinetics of MINC1 are expected to be the limiting factor here.

Until more potent analogs have been identified, MINC1 is a valuable tool to study the function of MgcRacGAP in cells, as we showed in study II. In this study we tested the hypothesis that had been put forward regarding the nuclear translocation of STAT3, which was supposedly facilitated by MgcRacGAP as both a mediator of STAT tyrosine phosphorylation and as a vital chaperone for nuclear translocation of STAT transcription factors. To explore the mechanism of MgcRacGAP controlling the activity of oncoprotein STAT3, we used MINC1 and siRNA-mediated gene silencing. Contradictory to published observations, we observed that inhibition of MgcRacGAP led to the transcriptional activation of STAT3. We found that the links between MgcRacGAP, Rac1 and STAT3 were highly complex; in the model system we used, MINC1-induced STAT3 transcriptional activity appeared to be due to increased STAT3 phosphorylation caused by a Rac1-PAR3-IL6-IL6R-JAK2 mediated autocrine/paracrine mechanism rather than a direct interaction between Rac1 and STAT3. However, direct interaction between Rac1 and STAT3 have shown to exist and, depending on cell type and status, even to co-exist with indirect interaction mechanisms. Considering the role of STAT3 in cancer, it would be interesting to determine what factors regulate this apparent model and context dependency. Subsequently, disease specific models could be used for drug screening and eventually lead to improved drug therapies.

Finally, in study III we presented a novel type of HTS-compatible biochemical assay that can be used to screen for both competitive as well as interfacial inhibitors for the Ras GTPase cycle. We utilized the dynamic behavior of GTPase signaling, designing an assay that can screen for small molecule compounds that bind to the Ras-GEF or Ras-GAP protein complex interface with high selectivity and locking the complex in one state. With a small proof-of-concept screen, we successfully identified compounds that affected H-Ras GTPase cycling in vitro. It remains to be studied whether the effects caused by QT-113 treatment in cells are direct or secondary, off-target effects. For instance, while QT-113 treatment induced effects are restricted to RAS wild type cells, the effects
appear only after long-term incubation, which caused extensive amount of vacuole formation. Given that QT-113 is flagged as a possible pan-assay interference compound, there is a likelihood that QT-113 interferes not only with the Ras cycle proteins, but with other proteins as well. Nevertheless, since this extreme vacuolization has been associated with dysfunctional endocytosis caused by aberrant signaling from H-Ras\textsuperscript{435}, it would be interesting to study the effect of analog compounds that do not contain the substructural features that marked QT-113 as possible pan-assay interference compound. Furthermore, it remains to be seen whether a screen using an increased number of compounds will allow for discoveries of interfacial inhibitors.

When describing the literature about Ras family proteins and their regulators, it became apparent that drug-targeting efforts have mostly been disappointing. With recently discovered covalent inhibitors it has been shown that a small GTPase is directly drugable\textsuperscript{261,264}, however their use is limited to the Ras proteins harboring the G12C mutation. Furthermore, while Ras-mimetic small molecule kinase inhibitor rigosertib has shown to be a potent inhibitor of tumor growth in several disease models\textsuperscript{287,288}, in clinical setting only subgroups of patients have responded to treatment with rigosertib in a randomized trial\textsuperscript{293}. It is not unlikely that this limited response is due to compensatory signaling cascades reducing the overall durability and potency of the treatment. With the emerging evidence that GAPs are oncogenic\textsuperscript{333}, I anticipate that in the future more research will be focused on GAPs as it has been on GTPases and GEFs as well as downstream targets. The discovery of MINC1 demonstrates that RhoGAPs are druggable, and the assays and methodologies described in these studies could be used to design future studies in the largely unexplored field of targeting other small GTPase GAPs.
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Arjan
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Discovery of Small Molecule Modulators of Ras Superfamily Proteins – Studies of MgcRacGAP and Ras

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