The Role and Regulation of the Exchange Factor GEF-H1 in Tubular Cells

by

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Institute of Medical Science
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Abstract

The Rho family small GTPases are key regulators of the cytoskeleton, through which they impact and control many vital cellular functions, including growth, vesicle trafficking, intercellular junctions, transepithelial transport, migration, and gene transcription. Activation of Rho GTPases is induced by Guanine Nucleotide Exchange Factors (GEFs). We have previously shown that Tumour Necrosis Factor-α (TNF), plasma membrane depolarization, and immunosuppressive drugs activate RhoA through a specific exchange factor, GEF-H1. However, the question of whether other stimuli, such as hyperosmolarity, that activate RhoA, act through GEF-H1 and whether GEF-H1 activates other RhoGTPases was not known.

The overall objective of this research project has been to gain insights into the complex mechanism through which the Rho GTPases, Rac and RhoA, are regulated in tubular cells. Specifically, we wished to explore the role and pathway-specific regulation of GEF-H1 in hyperosmotic stress- and TNF-induced signalling in tubular cells.

In order to accomplish our goals, we optimized and used affinity precipitation assays to detect GEF-H1 activation (RhoA(G17A) and Rac(G15A)). We found that 1) GEF-H1 is activated by hyperosmotic stress and mediates the hyperosmolarity-induced RhoA activation, as well as nuclear translocation of the Myocardin-Related Transcription Factor (MRTF); 2)

TNF induces activation of both Rac and RhoA through GEF-H1, but via different mechanisms. Epidermal Growth Factor Receptor (EGFR)- and Extracellular signal Regulated Kinase (ERK)-dependent phosphorylation at the Thr678 site of GEF-H1 is a prerequisite for RhoA activation only, while both Rac and RhoA activation require GEF-H1 phosphorylation on Ser885. Interestingly, Rac is required for TNF-induced RhoA activation.

Together these findings highlight a role for GEF-H1 as an osmosensitive molecule that regulates cellular reprogramming through MRTF. Importantly, we have also uncovered a novel mechanism explaining hierarchical activation of Rac and RhoA by TNF. Such a mechanism could be key in coordinating GEF function and fine-tuning Rac and RhoA activation.

Acknowledgements

I dedicate this thesis to four people: my mother, Nazneen Waheed, for her unconditional love, support, and devotion throughout my life; my father, Dr. Mohammed Abdul Waheed, for making me believe, at the age of six, how amazing science is, and that I could become anything I wanted to be, including a Scientist; my husband, Fahad Anwar, who has given me great love and joy in the one year we have been married, and supported me unconditionally in the preparation of this thesis; and last, but not least, to my supervisor, Dr. Katalin Szászi, who has supported me through all the ups and downs of research, encouraged me every step of the way, and called my Western Blots beautiful.

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Contributions

Faiza Waheed (author) solely prepared this thesis. The author participated in all aspects of planning, execution, analysis, and writing of all original research and publications. The author received funding from the Li Ka Shing Scholarship, UofT Open Fellowship, and operating grants from Early Researcher Ontario Government award, CIHR, NSERC, and the Kidney Foundation of Canada.

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Dr. Katalin Szászi (Supervisor) – mentorship; laboratory resources; guidance and assistance in planning, execution, and analysis of experiments as well as manuscript and thesis preparation.

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Qinghong Dan – performed experiments in Figure 19D, 24C and 30A. Assisted in experiments for Figure 31A

Pam Speight – performed experiment in Figure 30B

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List of Abbreviations

ADAM	*a d*isintegrin *a*nd *m*etalloprotease
ADP	Adenosine diphosphate
AJ	Adherens Junction
Amp	Ampicillin
aPKC	atypical Protein Kinase C
Arp2/3	Actin-related protein 2/3
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
Cdk5	Cyclin-dependent kinase 5
Cdk1	Cyclin-dependent kinase 1
Crb	Crumbs
CsA	Cyclosporin A
DAPI	4′,6-diamidino-2-phenylindole
Dbl	Diffuse B-cell-lymphoma
Dbs	Dbl's big sister
DH	Dbl homology
Dlg	Disc Large
ECIS	Electric cell substrate impedance sensing
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELA	Ehrlich-Lettre ascites
EMT	Epithelial–mesenchymal transition
ERK	Extracellular signal Regulated Kinase
ERM	Ezrin, Radixin, and Moesin
FAK	Focal Adhesion Kinase
FGDY	Faciogenital Dysplasia
FRET	Fluorescence Resonance Energy Transfer
GAP	GTPase Activating Protein
GDI	Guanine Nucleotide Dissociation Inhibitor
GDP	Guanosine diphosphate
GEF	Guanine Nucleotide Exchange Factor
GPCR	G-protein-coupled receptor
GSH	Glutathione sepharose
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICAM-1	Cell-cell adhesion molecule-1
IGF-1	Insulin-like Growth Factor-1
IL-1	Interleukin-1
IPTG	Isopropyl B-D-thiogalactopyranoside

IQGAP	IQ motif containing GTPase activating protein
JAM	Junction Adhesion Molecule
JNK	c-Jun N-terminal kinase
LARG	Leukemia-associated Rho guanine nucleotide exchange factor
Lgl	Lethal giant larvae
LPA	Lysophophatidic acid
LPS	Lipopolysaccharide
MAPK	Mitogen Associated Protein Kinase
MBS	Myosin-binding subunit
mDia	Mammalian homolog of the Diaphanous formin
MHC	Myosin Heavy Chain
MLC	Myosin Light Chain
MLCK	Myosin light chain kinase
MRTF	Myocardin-related Transcription Factor
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear Factor κB
NR	Nonrelated
PAK	p21-activated kinase
PALS1	Protein Associated with Lin Seven 1
Par	Partitioning defective
Par1b	Polarity-regulating kinase Partitioning-defective 1b
PATJ	PALS-associated Tight Junction Protein
PBD	p21-binding domain
PH	Pleckstrin homology
PI3-K	Phosphatidylinositol 3- kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PIP5-K	Phosphatidylinositol-4-phosphate 5-kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKN	Protein Kinase N
PLD	Phospholipase D
RBD	Rho-binding domain
RGS	Regulator of G-protein signalling
Rho	Ras homology
ROK/ROCK	Rho kinase
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RTK	Receptor Tyrosine Kinase
Scrib	Scribble
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMA	α-Smooth Muscle Actin
Sos	Son of Sevenless

SRF	Serum Response Factor
SRL	Sirolimus
TACE	TNF-alpha Convertase enzyme
TAMP	Tight-junction-associated Marvel protein
TER	Transepithelial resistance
TGFβ	Transforming growth factor-β1
Tiam1	T-cell lymphoma invasion and metastasis-1
TJ	Tight Junction
TMPS	Triple Membrane Passing Signal
TNF	Tumour Necrosis Factor-α
WASP	Wiskott-Aldrich Syndrome Protein
WAVE	WASP Verprolin Homologous

List of Publications

1) **Waheed, F.,** Dan, Q., Zhang, Y., Tanimura, S., Speight, P., Kapus, A., and Szászi, K. (2013): Central role of the exchange factor GEF-H1 in TNF-α-induced sequential activation of Rac, ADAM17/TACE and RhoA in tubular epithelial cells. Molecular Biology of the Cell Apr;24(7):1068-82

2) Ly, D.L.*, **Waheed, F.***, Lodyga, M.*, Speight, P., Masszi, A., Nakano, H., Hersom, M., Pedersen, S.F., Szászi, K., and Kapus, A. (2013): Hyperosmotic stress regulates the distribution and stability of Myocardin-Related Transcription Factor, a key modulator of the cytoskeleton. Am J Physiol Cell Physiol. Jan 15;304(2):C115-27. *Co-first authors

3) **Waheed, F.,** Speight, P., Dan, Q., Garcia-Mata, R., and Szászi, K. (2012): Affinity precipitation of active Rho-GEFs using a GST-tagged mutant Rho protein (GST-RhoA(G17A)) from epithelial cell lysates. Journal of Visualized Experiments. J Vis Exp. Mar 31;(61). pii: 3932

4) Martin-Martin, N., Dan, Q., Amoozadeh, Y., **Waheed, F.**, McMorrow, T., Ryan, M.P. and Szászi, K. (2012): Rho and Rho kinase mediate sirolimus and cyclosporine A-induced barrier function alteration in renal proximal tubular cells. International Journal of Biochemistry and Cell Biology. 44(1):178-88

5) Malam, Z., Parodo, J., **Waheed, F.**, Szászi, K., Kapus, A., and Marshall, J.C. (2011): Pre-B Cell Colony-Enhancing Factor (PBEF/Nampt/Visfatin) Primes Neutrophils for Augmented Respiratory Burst Activity Through Partial Assembly of the NADPH Oxidase. Journal of Immunology, 186(11):6474-84

6) Kakiashvili, E., Dan, Q., Vandermeer, M., Zhang, J., **Waheed, F.,** Pham, M., and Szászi, K. (2011): The Epidermal Growth Factor Receptor mediates Tumour Necrosis Factor-α-induced activation of the ERK/GEF-H1/RhoA pathway in tubular epithelium. J Biol Chem. 289:9268-79

7) **Waheed, F.**, Speight, P., Kawai, G., Dan, Q., Kapus, A., and Szászi, K. (2010): Extracellular signal regulated kinase and GEF-H1 mediate depolarization-induced Rho activation and paracellular permeability increase. Am. J. Physiol. Cell Physiology. 298(6):C1376-1387

8) Kakiashvili, E., Speight, P., **Waheed, F.,** Seth, R., Lodyga, M., Rotstein, O.D., Kapus, A., and Szászi, K. (2009): GEF-H1 mediates TNF-α-induced Rho activation and myosin phosphorylation: role in the regulation of tubular paracellular permeability. J. Biol. Chem. 284 (17):11454-11466

CHAPTER 1 Introduction

1.1. Epithelial cells

1.1.1. Functions

Many vital organ systems (e.g., urogenital, the digestive tracts, and the respiratory) are lined with a monolayer of epithelial cells. This layer creates a barrier between the inside of the body and the environment. In addition, highly specialized epithelial cells make up glands. Well-described functions of the epithelium include transport processes, such as secretion and absorption, as well as protection and mechano-sensation.

Cells within an epithelial monolayer show a high degree of apico-basolateral polarization, where the apical side faces the lumen (external environment) and the basolateral side is towards the tissue. For example, the epithelial cells of the kidney proximal tubules face the tubular lumen on their apical side, and are responsible for reabsorption of ion, water, and nutrients (Terry et al. 2010). Additionally, the tubular epithelium also forms a protective barrier against toxins and microorganisms. Polarization of the tubular epithelium is vital for transport processes. During the course of my PhD, I have studied signal transduction mechanisms in the kidney tubular epithelium that regulate the cytoskeleton and the intercellular junctions, which are key determinants of transepithelial transport. Hence, in my introduction, I start by highlighting the intercellular junction protein complexes and the actin cytoskeleton of the cell that plays a critical role in regulating the junctions.

1.1.2. Junctional complexes

The apical and basolateral sides of polarized epithelia are equipped with unique transport proteins that carry out directional transport. Epithelial cells are connected to each other via

four distinct intercellular junctional complexes: tight junctions (TJ), adherens junctions (AJ), desmosomes, and gap junctions (Figure 1).

1.1.3. Properties of TJs

TJs, the apical-most of the epithelial junctional complexes, have two major functions: they act as "gates" and "fences". Their gate function involves sealing the intercellular pathway to allow for only selective paracellular transport. In their capacity as fences, TJs separate the apical and basolateral membranes. This contributes to the formation of apico-basolateral polarity, which as mentioned above, is an extremely important feature of epithelial cells that ascertains their function.

Proteins associated with TJs are either transmembrane or cytosolic proteins (Figure 2) (Furuse 2010). Claudins, Tight-junction-associated Marvel proteins (TAMPs), and single span proteins make up the transmembrane category. The cytosolic proteins are collectively termed the 'cytoplasmic plaque', and consist of various adapters, signalling proteins, transcription factors, and cytoskeletal components (reviewed in Shen et al. 2010).

1.1.3.1. Transmembrane proteins

Claudin family proteins are essential TJ components that determine 'permselectivity' (Van Itallie and Anderson 2004, Angelow et al. 2007). Claudins play a central role in the gate function of TJs, balancing the dual roles of maintaining barrier and permeability. Some members of the family create a barrier and prevent the free diffusion of solutes. Other claudins exhibit unique cation- or anion-selectivity, and generate the paracellular pore pathway (Colegio et al. 2002, Van Itallie and Anderson 2004, and reviewed in Szaszi and

Figure 1. Junctional complexes in epithelial cells

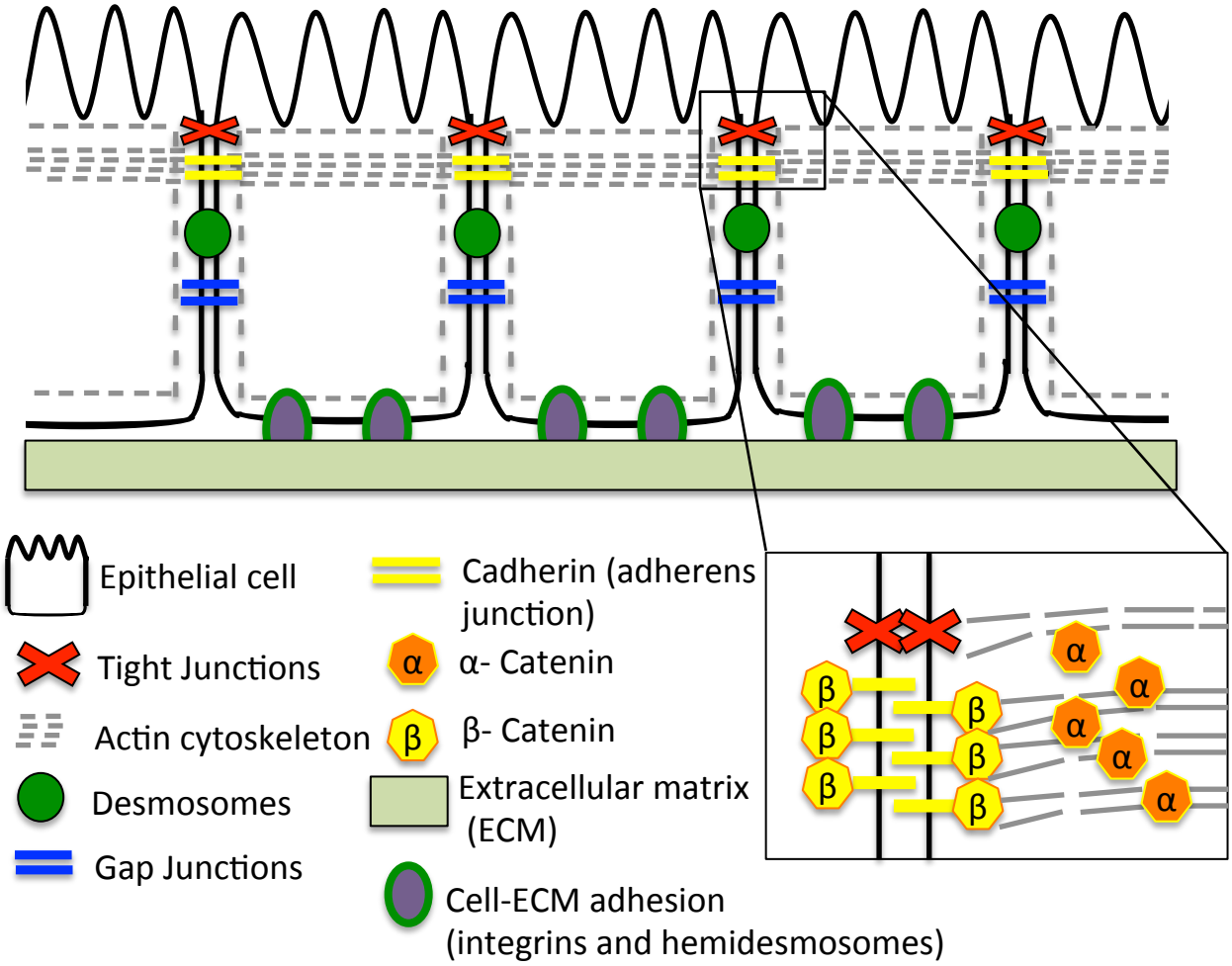


Figure 1. Schematic representation of the major junctional complexes linking epithelial cells. The tight junctions (TJs) form the apical-most complex, and have a *barrier* function as well as a *gate* function. The adherens junctions comprises of cadherin proteins linked to catenins (inset). Both junctions are linked to the circumferential acto-myosin belt. Desmosomes and Gap Junctions are also present on the apical side. Hemidesmosomes, along with integrins, make up the basolateral junctional complexes. Loss of these junctional complexes may prime the cells for epithelial mesenchymal transition (EMT). Based on Kokkinos et al. 2010.

Figure 2. Tight Junction (TJ) and associated proteins

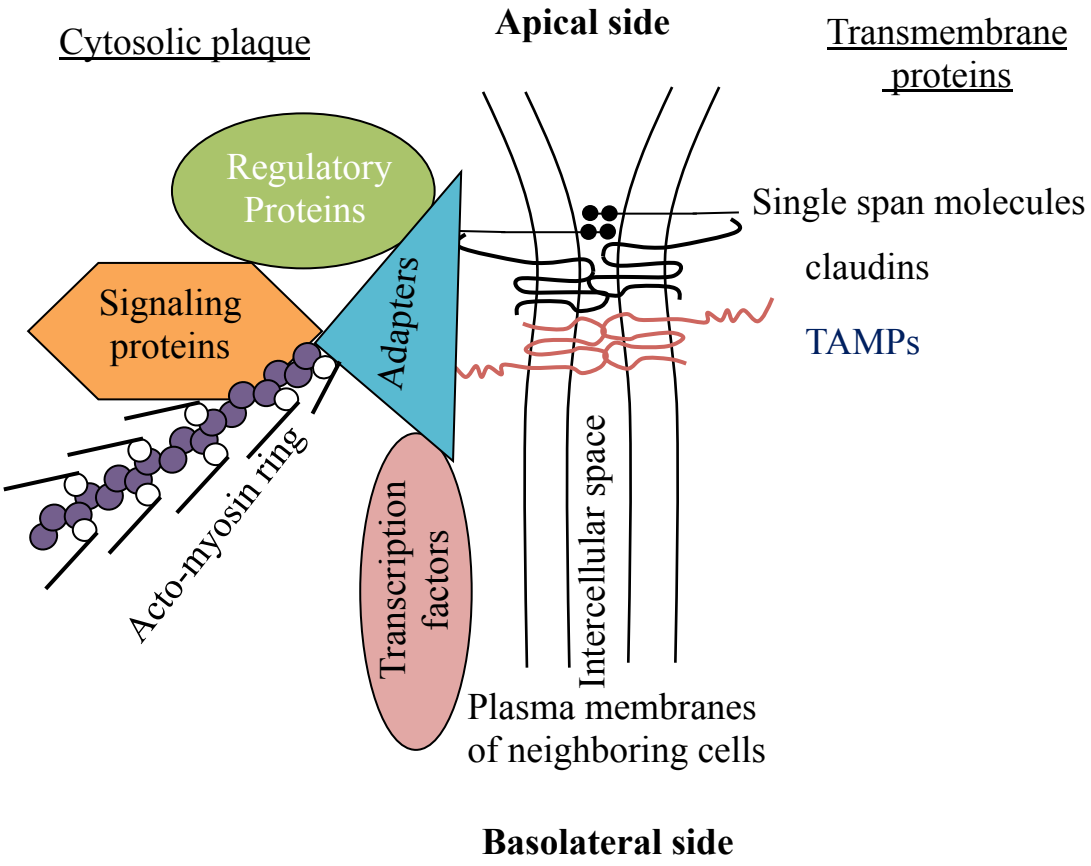


Figure 2. Schematic overview of the TJs and associated proteins. The transmembrane proteins belonging to three families (claudins, Tight junction-associated Marvel proteins (TAMP), such as occludin, and single span proteins, such as the Junctional adhesion molecules (JAM) are connected to a large number of cytosolic proteins (cytosolic plaque). The cytosolic plaque is a signalling center, and the adapter proteins found within the plaque generate multiprotein complexes. The plaque proteins control assembly/ disassembly and permeability of the junctions, regulate gene expression and provide input for small GTPase regulation, cell cycle control, differentiation, contact inhibition and apoptosis. Based on Szászi and Amoozadeh, Submitted to Int Rev Cell Mol Biol.

Amoozadeh, Submitted). Interestingly, epithelial layers express many different types of claudins, and the types of claudins present determine their permeability properties.

The TAMP family of proteins comprises of Occludin, Tricellulin, and MarvelD3 (Mariano et al. 2011), all of which are incorporated into the TJ strands. Although they have similarities in structure, they have unique functions and properties (Raleigh et al. 2010). ***Occludin*** has often been used as a TJ marker, due to its expression in all TJs (Cummins 2012). Despite findings pointing to a role for occludin in the formation and regulation of TJs, its specific function, is as yet, unknown (Furuse et al. 1996). Although some evidence suggests a role for occludin in permeability regulation, studies in occludin knockout mice suggest a likely role for occludin as a signal-organizing molecule (Saitou et al. 2000, Schulzke et al. 2005). Its overexpression has been shown to increase not only transepithelial resistance (TER), which indicates a reduction in ionic permeability, but also the paracellular leak of small molecular weight molecules (Balda et al. 1996, McCarthy et al. 1996). Additional functions of occludin might include roles in cell-cell adhesion, growth regulation, transmigration of neutrophils in inflammation, and regulation of epithelial sensitivity to cytokine-induced TJ remodelling (Van Itallie and Anderson 1997, Huber et al. 2000, Van Itallie et al. 2010, Runkle et al. 2011).

Uniquely localized at the tricellular junctions, ***Tricellulin*** has been linked to organization, and sealing of TJs, as well as the regulation of transport of macromolecular cargo across TJs (Ikenouchi et al. 2005, Ikenouchi et al. 2008, Krug et al. 2009). Tricellulin was also reported to form a heteromeric complex with occludin (Westphal et al. 2010). When occludin was silenced, tricellulin was found localized at the bicellular TJs (Ikenouchi et al. 2008).

The third TAMP family member, ***MarvelD3***, has been shown to localize to TJs alongside occludin (Raleigh et al. 2010). Downregulation of MarvelD3 is known to increase TER (Cording et al. 2013). Interestingly, MarvelD3 exhibits partial compensation for loss of occludin or tricellulin. However, complete recovery of functions was not observed, thereby affirming the unique roles of all three TAMP family members (Raleigh et al. 2010).

Divided into the immunoglobulin superfamily of proteins and other members, the ***Single-span transmembrane proteins of the TJs*** are not incorporated into TJ strands (reviewed in Szaszi and Amoozadeh, Submitted). One of the members of the immunoglobulin family are the Junction Adhesion Molecules (JAMs). There are three known JAMs, which play roles in the regulation of the TJ barrier formation and stability (Bazzoni et al. 2000, Liu et al. 2000, Mandell et al. 2005).

1.1.3.2. Cytosolic plaque proteins

Interactions of transmembrane TJ proteins with a wide variety of cytosolic plaque proteins are essential for the formation and regulation of TJs. The plaque is made up of several adapters that act as scaffolds and signalling proteins. Plaque proteins connect membrane proteins to the Rho family GTPases and the cytoskeleton, which is central in the regulation of TJs (Kapus and Szaszi 2006, Ivanov 2008, Rodgers and Fanning 2011). The best characterized cytoplasmic plaque adapter proteins are Zona Occludens (ZO)-1, 2, and 3 (Fanning and Anderson 2009). These proteins contain a PDZ-domain, which allows them to connect the proteins of the TJs to each other, as well as to the cytoskeleton, thereby providing indispensible support in the assembly and function of the TJs (reviewed in Fanning and Anderson 2009, Bauer et al. 2010, Paschoud et al. 2012, and Gonzalez-Mariscal et al. 2012).

1.1.4. Adherens junctions, desmosomes, and gap junctions

AJs are formed by cadherin proteins (e.g., E-cadherin) that bind cytosolically to catenins (Figure 1). Cadherins regulate the formation and organization of tissues by generating Ca^{++}-based adhesions between adjacent cells (Rudini and Dejana 2008). Like the TJs and desmosomes, cadherins are transmembrane proteins with extracellular domains and cytoplasmic tails. Cadherins have unique transmembrane domains, and associated cytosolic proteins that link them to the cytoskeleton (Szaszi et al. 2012, see next section). Their cytoplasmic tails are associated with the catenin proteins (α-catenin, p120 catenin, and β-catenin) that attach AJs to the actin cytoskeleton (Figure 1) (Harris and Tepass 2010).

Desmosomes provide stability to cells against mechanical forces by strengthening stable cell-cell adhesions (Delva et al. 2009). Gap junctions are made up of connexins that generate ion permeability pathways and direct connections between adjacent cell cytoplasms (Sohl and Willecke 2004).

1.1.5. Establishment of apico-basal polarity

Apico-basolateral polarization of the epithelium is the asymmetrical distribution of proteins and lipid molecules at the two distinct membrane compartments (Raleigh et al. 2010). Polarity is an integral feature of the epithelium and provides the basis for *directional transport*. Hence, the formation and maintenance of epithelial apico-basal polarity is critical for normal kidney development and functions (chapter 1.1.6).

In the last ten years, a greater understanding of the formation and maintenance of apico-basal polarity complexes has developed. Polarization requires interplay between three multi-protein complexes and intercellular junction proteins (Raleigh et al. 2010, Pieczynski and

Margolis 2011, Schluter and Margolis 2012). The three protein complexes are Crumbs, and Partitioning defective (Par), which are apically located, and the Scribble complex, which is laterally localized. Initially characterized in *C. elegans*, and *Drosophila*, these protein complexes are evolutionarily conserved and separate the apical from the basolateral membrane (Schluter and Margolis 2012). The polarity complex proteins also target and maintain junctional proteins at the border between the apical and basolateral sides to promote junction formation.

1.1.6. Proximal tubule epithelial cells

Proximal tubule epithelial cells in the kidney are highly polarized. This polarization is the basis of directional transport within these cells. Tubules have highly specific transport capabilities due to the presence of unique transporters located specifically on their apical or basolateral membranes. The proximal tubule is primarily responsible for re-absorption of NaCl, glucose and water. Transport functions in the proximal tubule can occur through transcellular or paracellular pathways.

Transcellular transport occurs through transporters present on the apical and basolateral surfaces of proximal tubule cells. Apical transporters include Na^+-glucose cotransporters (SGLT1 and SGLT2), Na^+-H^+ exchangers, aquaporin-1, and Na^+-cation cotransporters. The Na^+/K^+ ATPase on the basolateral membrane allows tubular cells to maintain a concentration gradient for Na^+ reabsorption. Glucose reabsorbed from the apical side is transported across the basolateral membrane via GLUT2 and GLUT1. Passive transport through the paracellular pathway in proximal tubule cells occurs through tight junctions, and also plays an important role in Na^+ and water reabsorption.

Tubular cells, hence, contribute to normal kidney function through their many specific transport abilities. The cytoskeleton of the cells maintains the high degree of polarization necessary for normal function. A family of proteins called Rho family GTPases are the major regulators of the cytoskeleton.

1.2. Rho family GTPases

Rho family GTPases regulate a wide array of functions, ranging from rearrangement of the actin cytoskeleton during cell polarization and migration to oncogenesis. However, their mechanisms of activation by various stimuli are still under investigation. Rho GTPases are a subgroup of the Ras superfamily of GTP hydrolases (Rho = Ras homology), and are found in all eukaryotic cells (Hall 2012). There are twenty-three mammalian genes that encode Rho GTPases, which are classified into six subfamilies: Rho, Rac, Cdc42, Rnd, RhoBTB and RhoT/Miro (reviewed in Bustelo et al. 2007) (Figure 3).

The focus of my research has been to elucidate the mechanism of activation of two Rho proteins, RhoA and Rac, by different stimuli and their regulator, the guanine nucleotide exchange factor, GEF-H1, in proximal tubule epithelial cells.

1.2.1. Structure

Similar to the majority of the Ras superfamily proteins, Rho/Rac GTPases are "molecular switches" that cycle between active GTP-bound, and inactive GDP-bound forms (Figure 4) (Jaffe and Hall 2005). The ~20 kDa Rho proteins have a core of 5 β-strands surrounded by 5 α-helices (Owen 2005). The β-strands are arranged in a combination of parallel and anti-

Figure 3. Rho GTPase family tree

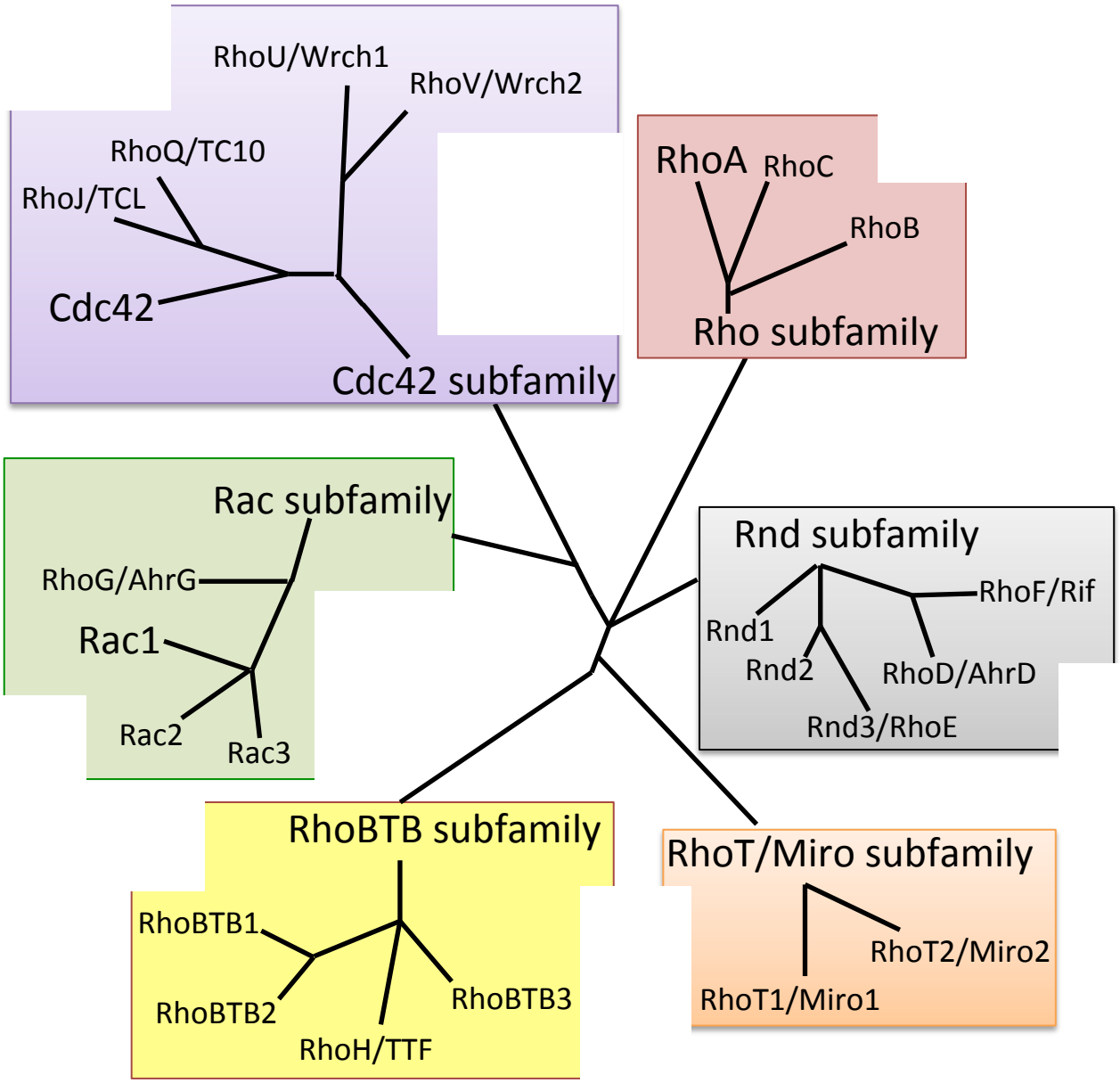


Figure 3. RhoGTPase superfamily is subdivided into six families; the Rho, Rac, Cdc42, Rnd, RhoBTB, and RhoT/Miro subfamilies. However, the three best characterized Rho GTPases are RhoA, Rac1, and Cdc42.

Figure 4. Rho GTPase activation/inactivation cycle

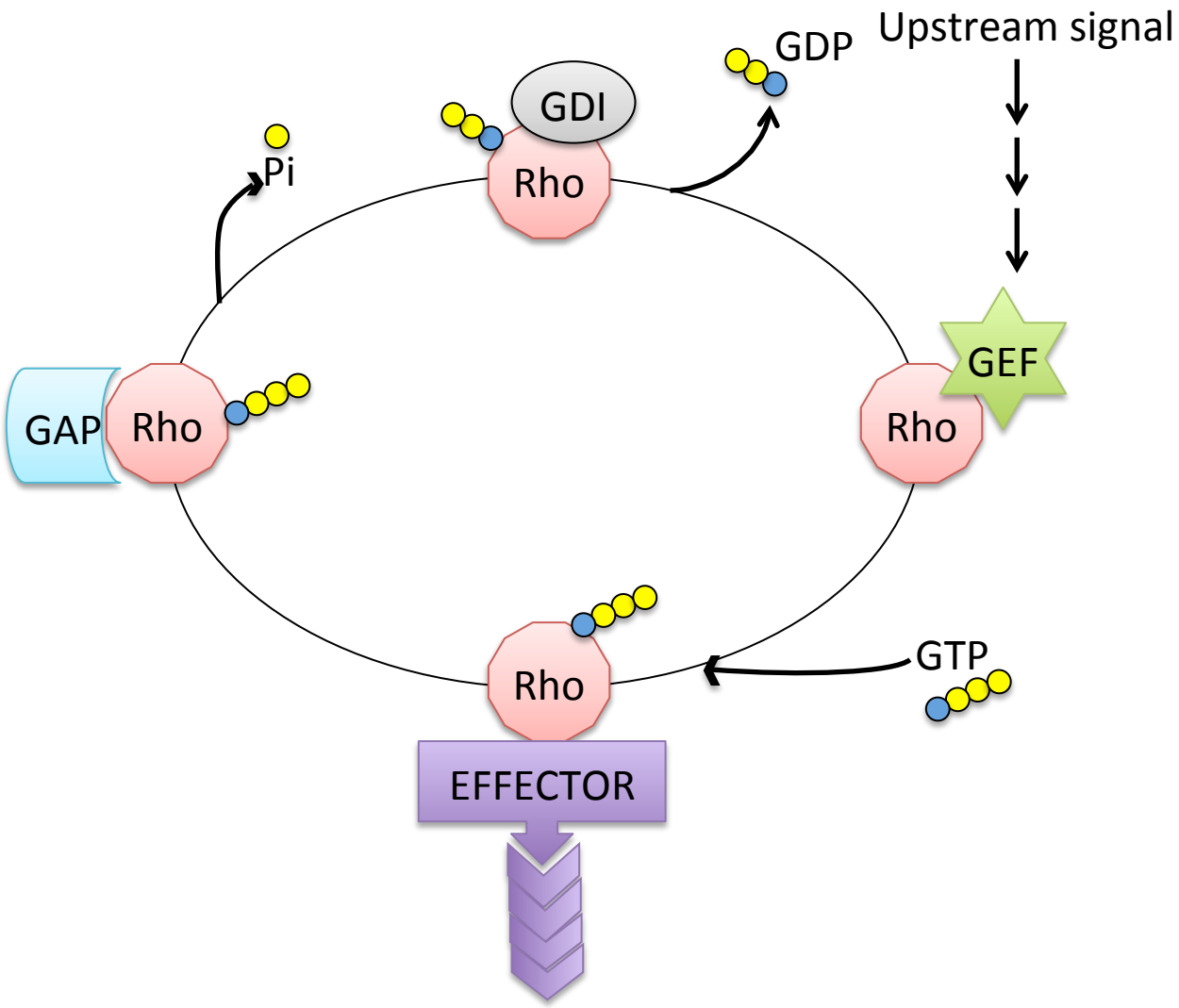


Figure 4. Activation of Rho GTPases. Rho proteins cycle between activated GTP-bound forms, and inactive GDP-bound forms. Their activation requires proteins called Guanine Nucleotide Exchange Factors (GEFs), and their inactivation is through GTPase Activating Proteins (GAPs). Guanine Dissociation Inhibitors (GDIs) bind to Rho proteins and sequester them in the cytosol, preventing their binding to GEFs. Activation of a GEF from an upstream signal induces its binding to the Rho protein, where it can catalyze the exchange of GDP for GTP and activate the Rho protein.

parallel β-sheets. The GDP- and GTP-bound forms of Rho proteins differ in the two loops, known as switch 1 (or the effector loop) and switch 2 (Figure 5a) (Owen 2005). Binding of GTP (or analogues) changes the conformation of these switches dramatically. Given that these switch regions are highly flexible, new interactions with GTP causes structural and dynamic changes in both the loops. This results in reorientation and conformational change in the effector loop that allows its downstream association with effector proteins (Figure 5b) (Owen 2005). Rho family proteins have distinct pockets for binding not just nucleotides, but also Mg^{2+}, which is essential for the high-affinity binding of guanine nucleotides (Rossman et al. 2005).

In the nucleotide-free state, Rho GTPases have the highest affinity towards binding an activated Guanine Nucleotide Exchange Factor (GEF) (discussed in chapter 1.2.3.1). GEFs catalyze the exchange of GDP for GTP. Rho GTPase intermediates that are nucleotide- (and Mg^{2+}) free can be preferentially loaded with GTP since cellular concentrations of GTP are substantially higher than those of GDP (Rossman et al. 2005). Rho GTPases perform their regulatory functions and bind to effectors to initiate signal transduction pathways only when they're in their active GTP-bound forms.

Posttranslational Modifications

Rho GTPases, like the Ras superfamily members, undergo posttranslational modifications, such as prenylation, and carboxymethylation of a conserved Cysteine residue at the C-terminal end (Adamson et al. 1992). Prenylation has been shown to be important for various functions of the Rho proteins, and allows for their targeting to membranes (Cox et al. 1992, Kreck et al. 1996, and reviewed in Cox and Der 1992). Rho GTPases may also be regulated

Figure 5a. Structure of RhoA

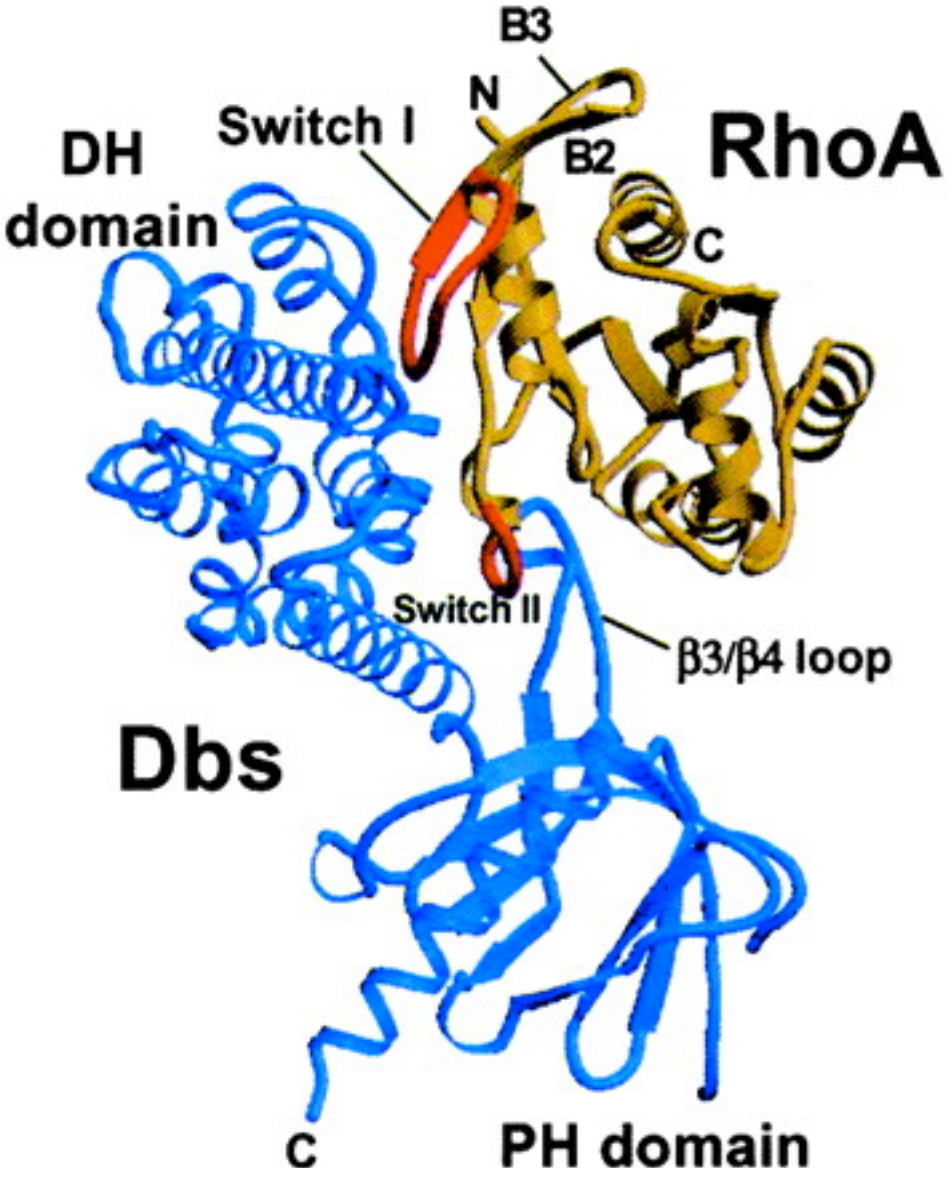


Figure 5a. Structure of RhoA complexed with a GEF, Dbs. The switch regions (I and II) are shown in red, and regulate specificity and interaction of Rho proteins with their regulators/effectors. The PH domain of Dbs, through its β3/β4 loop, makes direct contact with RhoA. From Hakoshima et al. 2003 (The Japanese Biochemical Society).

Figure 5b. Domain Structure of Rho GTPases

Figure 5b. Organization of the different domains in Rho family GTPases. RhoA, RhoB, RhoC, RhoD, RhoF, Rac1, Rac2, Rac3, RhoG, Cdc42, RhoJ/TCL, and RhoQ/TC10 have similar basic structures of the 'typical' Rho proteins. Adapted from Vega and Ridley, 2008

by phosphorylation. For example, phosphorylation of RhoA at Ser188 by Protein Kinase A (PKA) can inhibit RhoA activation (Schmidt et al. 1997).

1.2.2. Overview of functions of Rho GTPases

One of the highly conserved functions of the Rho, Rac, Cdc42, and Rnd subfamilies from yeast to humans is regulation of the actin cytoskeleton. The Rho GTPase signalling cascade promotes general cellular responses such as cytoskeletal remodelling, vesicle trafficking, cell polarity, paracellular permeability regulation, differentiation, microtubule dynamics, cell cycle progression, and gene transcription (reviewed in Etienne-Manneville and Hall 2002). A lot of these functions are related to their effects on the actin cytoskeleton, and will be discussed further in chapter 1.2.6.

The RhoBTB and RhoT/Miro subfamilies are considered atypical, since they differ from the other subfamilies in terms of structure, function, and regulation (Bustelo et al. 2007). In 2002 RhoBTB2 was found to be one of the genes deleted in breast cancer (Hamaguchi et al. 2002). Since then, RhoBTB proteins have been implicated in tumourigenesis as tumour suppressors, albeit their involvement in carcinogenic process is different from that of other Rho protein families (reviewed in Berthold et al. 2008). RhoBTB proteins are involved in targeting proteins to the proteasome, thereby maintaining a constant and strict level of proteins involved in cell cycle regulation and vesicular transport. Members of the other atypical family, the Miro GTPases are localized at the mitochondria, where they are essential for mitochondrial trafficking and biogenesis (Fransson et al. 2006).

To date, RhoA, Rac and Cdc42 are the three best-characterized Rho GTPases. As mentioned earlier, my research focused on the activation of two specific Rho proteins, RhoA and Rac, in epithelial cells. Hence, these proteins will be the primary focus of my thesis.

Classically, the functions of RhoA and Rac have been described to be the formation of contractile actomyosin filaments called stress fibers, and membrane protrusions called lamellipodia, respectively (reviewed in Jaffe and Hall 2005; chapter 1.2.6). Regulating actin dynamics is the major function of these two Rho proteins, through which they regulate many vital processes, such as migration, phagocytosis, endocytosis, morphogenesis, and cytokinesis (Hall 2012) (see chapter 1.2.5).

1.2.3. Regulation of Rho GTPases by GEFs, GAPs, and GDIs

There are three regulatory protein families that control the GDP-GTP cycling of Rho GTPases: Guanine Nucleotide Exchange Factors (GEFs), GTPase Activating Proteins (GAPs) and Guanine Nucleotide Dissociation Inhibitors (GDIs). GEFs catalyze exchange of GDP for GTP, thereby activating the Rho protein, whereas GAPs promote hydrolysis of the bound GTP, switching the protein back to its inactive state (Bustelo et al. 2007). GDIs inhibit disassociation of GDP from the Rho GTPase, thereby keeping it in its inactive state.

1.2.3.1. GEFs with a DH-domain structure

There are currently around 80 RhoGEFs, which can be divided into GEFs that have a DH domain, and those that don't.

In 1985 the first mammalian Rho GEF was identified from diffuse B-cell-lymphoma cells, and aptly named Dbl. Dbl was shown to catalyze the activation of Cdc42. Since their initial

discovery, 71 Rho family GEFs with a DH (Dbl homology) domain have been identified (Hall 2012). Most GEFs contain this ~200-residue DH domain, which catalyzes GEF activity by stabilizing GTP-free Rho intermediates. This then leads to the preferential loading of GTP, since GTP is present in higher concentrations in the cell than GDP (Buchsbaum 2007). The DH domain has a helical structure, comprising 10-15 α–helices and 3_{10}-helices (Rossman et al. 2005). These helices are arranged roughly into an oblong helical structure, whose shape is compared to that of a *chaise longue* where the 'seat back' of the chaise is made up by α-helices arranged in a U-shape (Figure 5a) (Worthylake et al. 2000). CR1-CR3 (the three conserved regions) form the core of the DH domains. Specifically, CR1, CR3, and conserved residues within the C-terminus form the bulk of the binding site for the Rho GTPase, and amino acid substitution within these regions leads to incapacitation of nucleotide exchange activity (Liu et al. 1998, Rossman et al. 2005). GEFs primarily differ in their conformations due to varying lengths and orientations of the C-terminal helix (Rossman et al. 2005). DH domains interact comprehensively with the switch regions in Rho GTPases (Figure 5a, see chapter 1.2.2.1.); switch 1 interacts with CR1 and CR3, and switch 2 with CR3 and parts of the C-terminal α-helix (Rossman et al. 2005). However, it is the 'seat back' of the GEF that confers its specificity towards GTPases. This portion is poorly conserved between the various GEF DH domains. Various mutation studies (Cheng et al. 2002, Snyder et al. 2002) that made single amino acid substitutions within the seat backs of DH domains found that this could change the specificity of a GEF from one Rho GTPase to another.

Adjacent and C-terminal to the DH domain, most GEFs also contain a PH (Pleckstrin homology) domain. This domain may be important for GEF localization, interactions with phospholipids and proteins (Buchsbaum 2007), and in some instances, collaborating with DH

domains to activate Rho GTPases (Liu et al. 1998). PH domains bind phosphatidylinositol-(4,5)-bisphosphate (PIP2) and have classically been shown to play a role in the targeting of proteins to different membranes. The role of PH domains in localizing GEFs to the plasma membrane, however, is not entirely clear, since there are reports with conflicting data. Whereas some studies found that deletion of PH domains interfered with GEF function, and substitution with a plasma membrane-targeting sequence restored function (Ron et al. 1991, Ferguson et al. 1995, Whitehead et al. 1995), others found no connection between PH domains and localization of the GEFs Sos1, Dbs, Tiam1, and Vav1 (Chen et al. 1997, Baumeister et al. 2003, Rossman et al. 2003). The interaction of PH domains with phosphoinositides in lipid bilayers, however, was shown to have several different roles in small GTPase activation. Lipid binding can expose the GTPase-binding domain within the DH domain, and enhance GEF activity due to conformational changes within the DH and PH domains (Rossman et al. 2005).

Another important function related to PH domains within GEFs is to serve as docking sites for proteins involved in signalling cascades downstream of activated Rho GTPases (Rossman et al. 2005). For example, Dbl binds the plasma membrane-actin cytoskeleton linker protein ezrin through its PH domain. Ezrin is then activated by Rho (Vanni et al. 2004). Another GEF, Dbl's big sister (Dbs), binds activated Rac through its PH domain. This binding appears to activate Dbs, which then promotes RhoA activation as evidenced by increased stress fiber formation and presence of RhoA.GTP (Cheng et al. 2004). This is an example of a GEF acting as an effector (through its PH domain) to one Rho GTPase (Rac1) that enables its activation towards another Rho GTPase (RhoA). Hence, PH domains (or PH-like

domains) adjacent to DH domains are essential to almost all GEFs for activation of Rho GTPases and downstream signalling cascades.

Mechanism of activation of GEFs

Most GEFs have low activity or are inactive under resting conditions. They are activated by various kinds of stimuli, acting through different signalling pathways. However, the context-specific activation of GEFs is not yet fully understood. For example, it is not known how the Rho GTPase specificity of GEFs is determined, and what kind of adjustments are involved whereby one GEF can activate two different Rho proteins.

The many described mechanisms controlling GEF activation, which may be interrelated inputs, are: relief of intramolecular inhibitory interactions, phosphorylation, change in intracellular localization, and binding to/ release from inhibitory proteins (Buchsbaum 2007). Truncation of N-termini of GEFs leads, in most cases, to constitutive activation (Rossman et al. 2005). For example, deletion of the N-terminus, including the Tyr174 residue, leads to constitutive activation of Vav1. This is due to the fact that the N-terminal region of the GEF Vav1 containing Tyr174 acts as an intramolecular negative regulator of its DH domain, by binding to the latter and preventing access by a Rho GTPase (Aghazadeh et al. 2000).

Phosphorylation of GEFs also controls their activation. There are examples of both negative and positive regulation by phosphorylation, depending on the site phosphorylated, and the kinase involved. A good example here is the Ras guanine nucleotide-releasing factor (Ras-GRF), which when phosphorylated by the kinase Src, activates Rac. However, upon phosphorylation by a different kinase, Cyclin-dependent kinase-5 (Cdk-5), its activation towards Rac is inhibited (Kiyono et al. 2000, Kesavapany et al. 2004). Our own studies show

that phosphorylation is also important for the regulation of GEF-H1, a RhoA and Rac GEF (Ren et al. 1998, Waheed et al. 2013, chapters 1.3 and 5). Interestingly, GEF-H1 is also a good example of a GEF that is regulated by intracellular localization. It has been shown to bind microtubules (see chapter 1.3.2), which leads to its inactivation. Stimuli (or mutations) that release GEF-H1 from microtubules activate it, followed by activation of its target Rho GTPase. T-cell lymphoma invasion and metastasis-1 (Tiam1) is another GEF whose activation requires its translocation from the cytosol to the plasma membrane where it is able to catalyze activation of Rac1 (Buchanan et al. 2000).

Interactions

There is an array of protein-protein interactions described for GEFs. In this section, I will attempt to highlight a few typical examples. In general, these interactions can be with activators, inactivators, effectors, and other regulators of Rho signalling.

Some effectors of Rho GTPases, such as p21-activated kinase (PAK), were shown to bind GEFs and use them as scaffolds. These GEF scaffolds may assemble large signalling complexes that can transmit chemotactic signals from upstream activators such as the G-protein-coupled receptors (GPCRs) (Figure 6a; chapter 1.2.4.1) (Li et al. 2003). PAK binds to an activated GPCR and the Cdc42 GEF, α-Pix, and enhances the activation of Cdc42. Upon activation, Cdc42 further activates its effector PAK and the downstream signalling cascade. However, whether such scaffolding functions of the GEFs are widespread remains to be established.

Regulator of G-protein signalling (RGS) domain-containing GEFs, such as p115RhoGEF and Leukemia-associated Rho guanine nucleotide exchange factor (LARG), directly connect

signals from GPCRs to downstream activation of Rho family GTPases (Wells et al. 2002, Rossman et al. 2005) (discussed in chapter 1.2.3). The RGS domain functions as a GAP for heterotrimeric G proteins that downregulates GPCR subunit activation. Through a classic negative feedback mechanism (GEF for Rho GTPase and GAP for G proteins associated with GPCRs), this dynamic ensures a tight control over how GPCR activation regulates downstream Rho GTPase activation. The binding of the GPCR to the GEF enhances catalytic exchange activity of the GEF towards the Rho GTPase (Wells et al. 2002).

Another example of a GEF that interacts with Rho GTPase activators, such as receptor tyrosine kinases (RTKs) (Figure 6a, 6b; see chapter 1.2.4) is Ephexin (Shamah et al. 2001). Ephexin is a GEF that binds directly to the RTK Eph4A through its DH-PH domain. Ephexin is able to activate RhoA, but can activate Rac and Cdc42 as well. However, upon activation, Eph4A clusters. This clustering strongly enhances RhoA activation and prevents Cdc42 and Rac activation. Hence, the authors hypothesize that this might be a mechanism by which a growing actin structure/appendage (Cdc42 and Rac activation) is dismantled (RhoA activation), a process that requires excellent spatio-temporal regulation of actin remodelling by Rho proteins through interactions with GEFs. Ras GTPase has also been shown to activate Rho proteins. For example, Tiam1, a Rac GEF, has a Ras-binding domain, and hence, when bound to active Ras, it is able to amplify the Rac signalling cascade and link Ras activation to downstream Rac activation (Lambert et al. 2002).

1.2.3.2. GEFs without a DH-domain

Distinct from DH domain GEFs in terms of their structure and the mode through which they activate Rho proteins, DOCK180 superfamily of proteins are newly discovered GEFs for

Rho GTPases (reviewed in Cote and Vuori 2007). DOCK family members have been shown to play a role in cell migration, morphogenesis, and phagocytosis. The DOCK180-related GEFs are eleven proteins that are divided into four subfamilies (DOCK A subfamily- DOCK D subfamily) (Brugnera et al. 2002, Cote and Vuori 2002, Meller et al. 2002). These GEFs are unique in that they lack the DH-domain. However, they contain two highly conserved regions called Dock-homology region-1 and -2 (DHR1 and DHR2) (Rossman et al. 2005). The DHR2, also known as Docker domain, is vital in Rho GTPase interaction and activation (catalysis of nucleotide exchange), for which DHR2 is both necessary and sufficient (Brugnera et al. 2002, Cote and Vuori 2002, Lu et al. 2005).

Some members of the DOCK superfamily have C2 domains for lipid-binding, and armadillo arrays that enable them to form suprahelical structures that interact with other proteins (Rossman et al. 2005). DOCK proteins may also have a Src-homology-3 domain (SH3) to bind polyproline regions in other proteins, or a PH domain. For example Dock9, also known as zizimin1, and SWAP70, are both DOCK proteins with PH domains (Rossman et al. 2005). Dock1 (also referred to as Dock180), Dock2, and Dock3 (also known as modifier of cell adhesion) are specific for Rac (Brugnera et al. 2002, Namekata et al. 2004), whereas zizimin1 has been shown to be a Cdc42 GEF (Meller et al. 2002). Interesting binding partners for at least four DOCK proteins are members of the evolutionarily conserved Engulfment and cell Motility (ELMO) protein family (ELMO 1-3). These proteins not only contain a PH-domain, but also a PxxP motif that binds to the SH3 domain of DOCK proteins. ELMO has three functions in Dock180-catalyzed activation of Rac GTPase: it targets Dock180 to the plasma membrane to enable access of the GEF to Rac; helps Dock180

stabilize Rac in its nucleotide-free transition state; and resolves a self-inhibitory Dock180 conformation state (reviewed in Lu et al. 2005, Lu and Ravichandran 2006).

1.2.3.3. Role of Dbl-family GEFs in disease

Altered expression or function caused by naturally occurring mutations in GEFs can lead to diseases. Here, I will discuss two major disorders in which GEFs are known to play a role: cancer, and developmental disorders of the nervous system.

Cancer

Since Rho GTPases participate in functions such as cell proliferation, it is not difficult to imagine that they also play a role in diseases with aberrant proliferation, such as cancer. Analysis of patient-derived samples revealed that several members of Dbl-family GEFs are overexpressed, or have gain-of-function mutations. These findings suggest a role for these GEFs in oncogenesis (reviewed in Boettner and Van Aelst 2002). As mentioned already, the founding member of the DH domain GEFs, Dbl, was isolated from human diffuse B-cell lymphoma (Srivastava et al. 1986). Another GEF overexpressed in cancer is Epithelial cell transforming sequence 2 (Ect2), which is primarily a RhoA activator, but can also activate Rac and Cdc42 (Tatsumoto et al. 1999, Solski et al. 2004). Ect2 mRNA and/or protein is overexpressed in several human tumour cell lines and tissues, such as lung and oesophageal squamous cell carcinomas (Salhia et al. 2008, Saito et al. 2003, Zhang et al. 2008), and is associated with poor outcomes (Sano et al. 2006, Hirata et al. 2009). P-Rex1 (phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1), a Rac-specific GEF, has been implicated in prostate cancer cell invasion (Qin et al. 2009). P-Rex1 gene and protein were most highly overexpressed in metastatic prostate cancer cell lines and P-Rex1 protein expression was highest in metastatic prostate tumour tissue. Additionally, P-Rex1

overexpression was correlated with concurrent activation of Extracellular Signal Regular Kinase (ERK) (a Mitogen Activated Protein Kinase (MAPK)) signalling in melanomas (Shields et al. 2007). GEF-H1 is overexpressed in a panel of 32 cancer cell lines with mutant p53 expression (Mizuarai et al. 2006). This overexpression was accompanied by increased accumulation of RhoA.GTP. GEF-H1 is also regulated by the oncogene human pituitary tumour-transforming gene 1 (hPTTG1) (Liao et al. 2012), that acts as a transcriptional activator of GEF-H1. Increased expression of hPTTG1 is known to be a breast cancer risk factor (Ogbagabriel et al. 2005), and hPTTG1 has been identified as a 'signature molecule' for metastasis (Ramaswamy et al. 2003). A recent study by Liao and others shows increased GEF-H1 expression in most of the eight studied breast cancer cell lines (Liao et al. 2012). The authors ascribe this effect to concurrent overexpression of hPTTG1.

Several other GEFs have been found to be overactive or mutated in different diseases. As mentioned earlier, LARG is an RGS domain GEF and hence, capable of transmitting and amplifying signals from upstream GPCRs to downstream Rho-dependent signalling (Fukuhara et al. 2000). LARG was found to be a fusion partner to Mixed Lineage Leukemia (MLL) gene identified in acute myeloid leukemia (Kourlas et al. 2000). Fusion of MLL to LARG renders LARG constitutively active, by deletion of N-terminal sequences upstream of its DH domain. In general, such a loss of N-terminal sequences in Dbl family proteins has been shown to lead to their constitutive activity (Rossman et al. 2005). This is also the case for GEF-H1 (see chapter 1.3).

Tiam1 is a Rac GEF that can act downstream of Ras. This GEF has a missense mutation in tumours and renal carcinoma cell lines (Engers et al. 2000). This Ala441Gly mutation in the N-terminus of the PH domain of Tiam1 is implicated in preventing its correct membrane

localization and functions. However, it still mediates overactivation of the Rac pathway. Mice deficient in Tiam1 have impaired Ras-induced induction of Squamous Cell Carcinomas, which might be due to the fact that Rac activity has been linked to the transforming activity of Ras (Malliri et al. 2002).

Developmental and Neurological Disorders

Several GEFs have been linked to developmental disorders. Faciogenital Dysplasia (FGDY) is an X-linked developmental disorder characterized by specific patterns of skeletal defects, which may also be accompanied by mental retardation (Pasteris et al. 1994). A Dbl-family GEF, *Fgd1*, has been implicated in FGDY. There are 16 distinct *Fgd1* mutations that are found in FGDY, including mutations in, or deletion of, the DH domain of *Fgd1* (Orrico et al. 2004). Two other GEFs associated with X-linked mental retardation (MRX) are α-Pix and ARHGEF6, GEFs for Cdc42 and Rac, respectively. Implicated α-Pix mutations include deletions in its N-terminus. ARHGEF6 is part of a signalling complex with the Rac effector PAK family proteins (Bagrodia et al. 1998, Manser et al. 1998), which are also implicated in MRX.

1.2.3.4. GAPs

Turning off Rho GTPase activity is an important requirement, and is carried out by GAPs. Rho proteins have very slow rates of inherent GTP hydrolysis, which is the step accelerated by the GAP protein family (reviewed in Csepanyi-Komi et al. 2012). There are about 70 known GAPs in the human genome. Similar to GEFs, GAP action is also specific to a certain subfamily of Rho GTPases; however, GAPs may act on several members within the subfamily (Csepanyi-Komi et al. 2012, Hall 2012). Although specific functional roles of

most GAPs are not as well studied as for the GEFs, the large number of different domains found in these proteins points to their involvement in a wide variety of signal transduction pathways in different tissues (Burridge and Wennerberg 2004).

GAP activity is confined to a 25-30 kDa region called the "GAP domain" (Csepanyi-Komi et al. 2012). Although there is variability within the catalytic mechanism of the family-specific GAP domains, in most cases GAPs provide a critical residue to the catalytic site. For GAPs acting on Ras and Rho family proteins, this critical residue is an arginine (Ahmadian et al. 1997, Rittinger et al. 1997, Nassar et al. 1998).

The native conformation of GAPs is a folded autoinhibited state (Moskwa et al. 2005, Colon-Gonzalez et al. 2008, Eberth et al. 2009). Like GEFs, GAPs are subject to various regulatory mechanisms, such as phosphorylation, lipid-binding, protein interaction, and degradation (reviewed in Bernards and Settleman 2005). Also like GEFs, the outcome of phosphorylation varies depending on the residue being phosphorylated and the kinase involved (Csepanyi-Komi et al. 2012). For example, p190RhoGAP, when phosphorylated on its serine residues at the far C-terminal end (S1472, S1476, S1483) by the kinase glycogen synthase-3-β, shows decreased GAP activity towards both its substrates, Rac and Rho (Jiang et al. 2008). However, phosphorylation of p190RhoGAP at another site, its Tyr1105 residue by c-Src enables its association with p120RasGAP. p190RhoGAP then modulates the RasGAP activity of p120RasGAP (Hu and Settleman 1997, Roof et al. 1998). Hence, the latter example of regulation by phosphorylation shows an interesting way of GAPs controlling the activities of each other (Yang et al. 2009). Although the significance of such crosstalk between GAPs is yet unclear, it might serve as a back-up mechanism ensuring the whole system does not shut down due to a single "off" signal (Yang et al. 2009).

Phosphorylation and interaction with phospholipids can switch GAP preference from one Rho GTPase to another. For example, p190RhoGAP, when associated with acidic phospholipids, reversibly switches from Rho to Rac as its substrate (Ligeti et al. 2004). However, upon phosphorylation by PKCα (in its polybasic region), it dissociates from the lipid bilayer, inducing a reverse switch, from Rac to Rho (Levay et al. 2009). Similarly, phosphorylation of another GAP, MgcRacGAP, by Aurora B kinase enables this RacGAP to act upon Rho (Minoshima et al. 2003).

Since the primary function of GAPs is to inactivate Rho GTPases, inhibition or deletion of some GAPs has been shown to result in overactivation of their targets (Csepanyi-Komi et al. 2012). For example, enhancement of superoxide production and phagocytosis of neutrophilic granulocytes were observed when the critical arginine of the RacGAP ARHGAP25 was mutated (Geiszt et al. 2001, Csepanyi-Komi et al. 2012). Also, defects in the RasGAP, Neurofibromatosis 1, led to excessive cell proliferation and tumour development (Basu et al. 1992, Bollag et al. 1996).

Hence, it can be speculated that constitutive GAP activity controls important biological processes and functions by downregulating small G protein activities (Csepanyi-Komi et al. 2012). A balance of Rho activation and inactivation, as well as correct spatio-temporal activation, depends on GAPs. Regulation of Rho GTPase inactivation by GAPs is a highly dynamic and well-coordinated process.

1.2.3.5. GDIs

There are three characterized mammalian GDIs acting on Rho family GTPases: RhoGDI1 (also known as RhoGDIα), RhoGDI2 (also known as RhoGDIβ, Ly/GDI, D4) and RhoGDI3

(RhoGDIγ) (Garcia-Mata et al. 2011). This family of proteins serves to maintain Rho GTPases in the cytosol in their inactive sequestered state in non-stimulated cells (Bustelo et al. 2007). Of the three, RhoGDI1 is the best characterized. It is ubiquitously expressed, and acts on Rho, Rac and Cdc42 (Fukumoto et al. 1990, Leonard et al. 1992). Although most highly expressed in hematopoietic cells, RhoGDI2 is also found in other tissues, as well as cancer cells (Lelias et al. 1993, Scherle et al. 1993). Of the three GDIs, RhoGDI3 is expressed at the lowest levels (Garcia-Mata et al. 2011). Expressed preferentially in the brain and pancreas (Lelias et al. 1993, Scherle et al. 1993), it shows the most sequence divergence due to a unique amino terminal extension that targets it to cellular membranes, including the Golgi complex (Brunet et al. 2002).

RhoGDIs bind with high affinity only prenylated Rho proteins (Ridley 2000, chapter 1.2.1.). Structure analyses of GDIs have revealed that they contain a hydrophobic cleft for binding isoprenes (Gosser et al. 1997, Ridley 2000). Hence, GDIs mask the prenyl-group on Rho proteins and keep them in the cytosol by disabling their binding to the membrane (Keep et al. 1997). The N-terminal domain of RhoGDI interacts with both the switch I and switch II domains of Rho proteins, limiting the spatial flexibility required for transition of Rho GTPases between different nucleotide-bound forms (Garcia-Mata et al. 2011). Hence, this interaction results in 'locking up' of the Rho protein.

Like GAPs, RhoGDIs were initially thought to be exclusively inhibitory molecules that prevented Rho protein activation by binding and preventing nucleotide exchange. The exact functions of RhoGDIs, however, proved more complex, since RhoGDIs were shown to act as Rho GTPase chaperones. RhoGDIs were observed to remove Rho proteins from membranes and contain them in a cytosolic complex away from membranes (Leonard et al. 1992, Garcia-

Mata et al. 2011). This explains why at any given time, 90-95% of Rho proteins are present in inactive complexes in the cytosol, and not at the membranes (Garcia-Mata et al. 2011, Boulter et al. 2012). Also, it has been shown that phosphorylation of RhoA at Ser188 by PKA increases affinity of RhoA for RhoGDI, which stabilizes it, and protects it from proteasomal degradation (Rolli-Derkinderen et al. 2005, Boulter et al. 2010). Hence, this chaperone-like function performed by RhoGDI stabilizes the large cytosolic pool of Rho that is not associated with the membranes, but can be rapidly activated upon a specific stimulus.

Rho GDI also interacts with Ezrin, Radixin, and Moesin (ERM) proteins, which associate with the transmembrane protein CD44, the cell-cell adhesion molecule (ICAM-1), and the actin cytoskeleton (Tsukita et al. 1997). ERM proteins can inhibit RhoGDI activity and release Rho from RhoGDI (Takahashi et al. 1997). Hence, stimuli that activate ERM proteins and release them from their 'closed' conformation can also dissociate Rho from GDI, leading to its activation (Ridley 2000). Thus GDIs are yet another important layer in Rho protein activity regulation.

1.2.4. Activators of RhoA and Rac

I will discuss two major activators of Rho family GTPases: 1) various cell-surface receptors (chapter 1.2.4.1), and 2) various cell stress stimuli (chapter 1.2.4.2). Cell surface receptors that activate Rho/Rac include receptor tyrosine kinases (RTKs), G-Protein-Coupled Receptors (GPCRs), cytokine receptors, and adhesion receptors (reviewed in Rossman et al. 2005) (Figure 6a). Stress stimuli that are known to activate these two proteins include Tumour Necrosis Factor-α (TNF), osmotic stress, plasma membrane depolarization,

Figure 6a. Activation of Rho GTPases

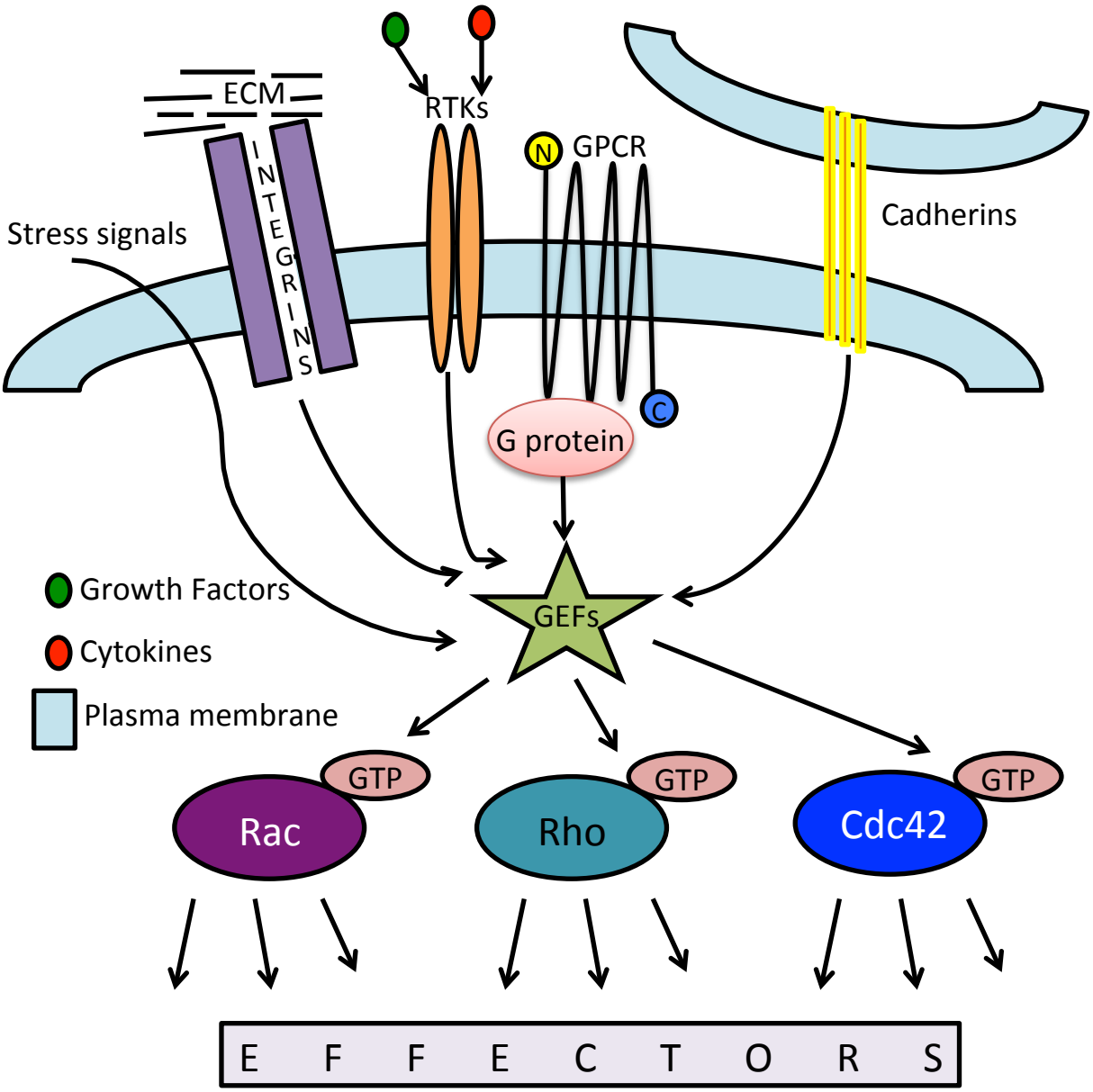


Figure 6a. Extracellular signals from G-protein Coupled Receptors (GPCRs)/G proteins, Receptor Tyrosine Kinases (RTKs), Stressors, Cadherins, and Integrins activate Rho GTPases through various GEFs. Once activated, Rho proteins bind to and specifically activate downstream effectors. Additionally, stimuli may also targets GAPs and inactivate them, thereby activating Rho proteins.

mechanical stress, and immunosuppressive drugs (Kakiashvili et al. 2009, Ly et al. 2013, Waheed et al. 2010, Guilluy et al. 2011, Martin-Martin et al. 2012).

1.2.4.1. Cell-surface receptors that activate Rho GTPases

Structure and overview of GPCRs and G proteins

The GPCR family comprises over 800 members, and is the largest membrane receptor family (reviewed in Liebmann 2011). Two important ways of GPCR activation of Rho GTPases are: directly through RGS-containing GEFs, or indirectly through RTKs.

GPCRs are characterized by seven transmembrane domains connected by an array of extracellular and intracellular loops (Dohlman et al. 1987, and reviewed in Whitehead et al. 2001). Ligand recognition and binding is through the extracellular N terminus, whereas the C terminus is intracellular and participates in binding to effectors and signal transduction mediators (Whitehead et al. 2001). Of the different domains, the transmembrane regions within the GPCR are the most highly conserved, comprising 20-25 amino acid stretches that form α-helices. Ligands of the GPCR family include neurotransmitters, hormones, phospholipids, photons, odorants and purine nucleotides (Dohlman et al. 1987, Whitehead et al. 2001). Binding and engagement of a ligand/agonist to its preferred GPCR leads to activation of an associated member of the heterotrimeric G protein family (Figure 6a, 6b) (Whitehead et al. 2001).

Anchored to the intracellular surface of the plasma membrane, GPCR-associated members of the heterotrimeric G protein family (G proteins) consist of an α subunit (Gα) and a βγ dimer subunit (Gβγ) (Bourne 1997, Whitehead et al. 2001). Gα binds to guanine nucleotides. The

Figure 6b. Activation of Rho proteins through ERK MAPK

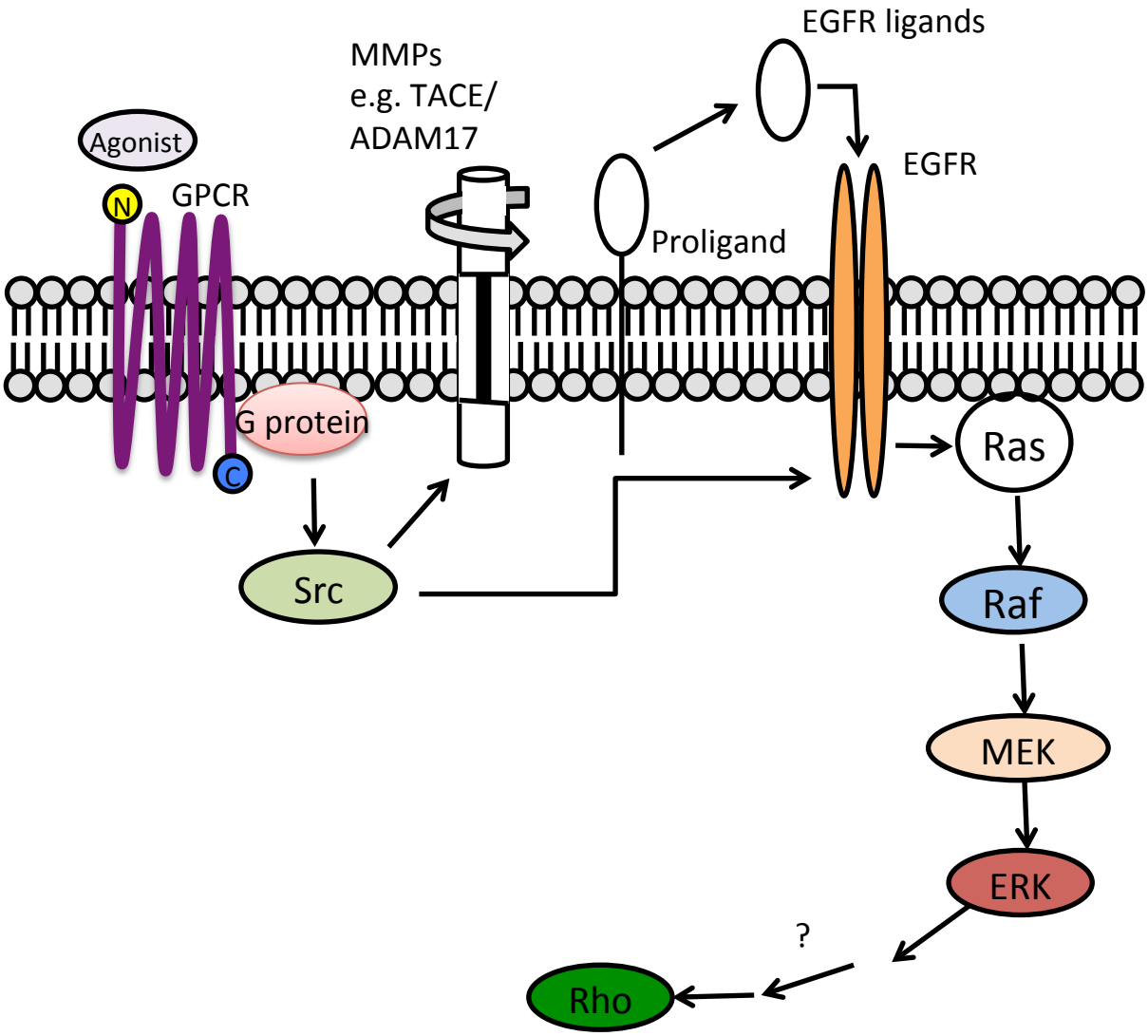


Figure 6b. Activation of ERK/Rho through GPCR-mediated transactivation of the RTK, Epidermal Growth Factor Receptor (EGFR). This pathway is called the Triple Membrane Passing Signal (TMPS) pathway. Binding of agonists activates the GPCR, which activates Matrix Metalloproteases (MMPs), such as a disintegrin and metalloprotease 17 (ADAM 17), via signalling through G proteins and Src kinase. MMPs cleave EGFR proligands such as Transforming Growth Factor-α (TGF-α) to their active forms. Binding of specific ligands activates the EGFR, which through the small GTPase Ras, activates the Ras/Raf/MEK/ERK pathway. Adapted from Liebmann (2011).

We have shown a similar pathway in our lab for the TNF-induced transactivation of the EGFR through ADAM17/TACE, leading to ERK activation. ERK then phosphorylates GEF-H1, which then activates Rho (Chapter 5).

kind of signalling events induced by a specific receptor depends upon the combination of the Gα and Gβγ units present, and availability of particular effectors and regulatory proteins (Whitehead et al. 2001).

Interaction of the GPCR molecule with the heterotrimeric G proteins has several contact points, the most crucial being residues within the third cytoplasmic loop, and the membrane proximal portion of the cytoplasmic end (Bourne 1997). In the inactive, ligand-less state, the GPCR-associated Gα subunit is bound to GDP. Active conformation of GPCR occurs upon ligand binding, enabling the GPCR to act as a GEF for the Gα subunit. Exchange of GDP for GTP on the Gα subunit allows it to disengage from the Gβγ dimer subunit, and permits signalling events to occur (Whitehead et al. 2001). At least four different major families of Gα subunits are currently known: $G_{12/13}$, $G_{i/o}$, G_s and $G_{q/11}$ (Kjoller and Hall 1999). These have different downstream effectors. Some can directly activate or inactivate enzymes (e.g., adenylate cyclase, phospholipase, or Rho family GEFs) and can also interact with ion channels (e.g., K^+ or Ca^{2+} channels).

The phospholipid Lysophophatidic acid (LPA), a potent mitogen, was the first agonist identified to act through one or more GPCRs, leading to activation of several Gα subunits, as well as the small GTPase, Rho (Ridley and Hall 1992). Other examples of agonists capable of activating G proteins and Rho GTPases through GPCRs are Sphingosine 1-phosphate (S1P), bombesin, thrombin, and endothelin (Kjoller and Hall 1999). Constitutively active versions of Gα12 and Gα13 induced the formation of stress fibers in Swiss 3T3 fibroblasts, as well as activation of downstream Rho effectors such as Serum Response Factor, and Phospholipase D (Buhl et al. 1995, Fromm et al. 1997, Gohla et al. 1998, Plonk et al. 1998).

LPA-induced Rho activation occurs through the Gα13 subunit, whereas Gα12 mediates thrombin-induced activation of Rho (Gohla et al. 1998, Gohla et al. 1999). Hence, Gα12 and Gα13 are G proteins that act through RGS-GEFs, e.g., p115RhoGEF and LARG (see below).

Direct activation of Rho GTPases through RGS-GEFs

One of the mechanisms directly linking Gα subunit activation to Rho activation is through GEFs containing a Regulator of G-protein signalling (RGS) domain (see chapter 1.2.3.1). These proteins act as GAPs for the G protein subunit, and are also Rho GEFs. For example, p115RhoGEF binds to both Gα12 and Gα13, but is preferentially activated towards Rho by Gα13 (Hart et al. 1998, Kozasa et al. 2011). Two other Rho GEFs, PDZ-RhoGEF and LARG, contain an RGS domain and are also activated by G proteins (Fukuhara et al. 2000).

Indirect activation of Rho GTPases by GPCRs through RTKs

Another means of linking GPCR and G protein activation to Rho GTPase activation is through transactivation of surface RTKs, such as the Epidermal Growth Factor Receptor (EGFR) (Figure 6a, 6b) (reviewed in Liebmann 2011). Liebmann describes the highly complex cross-talk that can occur between the RTKs and GPCRs, where the GPCRs use the RTKs as an effector signalling system, and the RTKs integrate signals from the GPCRs. This mechanism accounts for the proliferative signals initiated by GPCRs (Figure 6b). Tyrosine kinase inhibitors, as well as a dominant negative EGFR block LPA/Gα13-induced Rho activation (Gohla et al. 1998).

This process has been termed the transactivation of the EGFR by GPCRs through the Triple Membrane Passing Signal (TMPS) pathway (Figure 6b, reviewed in Prenzel et al. 2001,

Fischer et al. 2003, Liebmann 2011). Upon binding agonists, GPCRs are activated, and in turn activate the *a d*isintegrin *a*nd *m*etalloprotease (ADAM) family of proteases, which cleave and generate soluble ligands of the EGF family (reviewed in Ohtsu et al. 2006). These ligands then activate EGFR and its downstream signalling pathways.

Activation of Rho proteins by cytokines

The mechanism of EGFR transactivation, however, is not exclusive to GPCRs alone. In studies performed in our lab (Figure 7), we have shown a similar pathway: the cytokine TNF, through its receptor, is able to transactivate EGFR, leading to downstream activation of Rac, which is followed by Rho activation (see chapter 5, Kakiashvili et al. 2011, Waheed et al. 2013). Another cytokine receptor, the Interleukin-1 (IL-1) receptor also transduces IL-1-induced signals in Swiss 3T3 fibroblasts, leading to activation of Cdc42, followed by Rac and, finally, RhoA activation (Nobes and Hall 1995, Puls et al. 1999).

Activation of Rho GTPases by RTKs

Other growth factors such as Platelet-derived Growth Factor (PDGF), Epidermal Growth Factor (EGF), and insulin were shown to activate Rac, followed by a subsequent Rho activation (Burridge and Wennerberg 2004). Studies investigating growth factor-induced Rac activation discovered that Rac activation was linked to upstream Phosphatidylinositol 3-kinase (PI3-K) activation. Since PI3-K, a phospholipid kinase, is also known to be a Rac effector, it surprisingly seems to be acting both upstream and downstream of Rac activation (see chapter 1.2.5 and reviewed in Kjoller and Hall 1999). Phosphatidylinositol 3,4,5-triphosphate (PIP3), the lipid product of PI3-K that is associated with the plasma membrane,

binds to the PH domains of several GEFs, including Sos, Vav, and Tiam1, and leads to activation of RhoA, Rac and Cdc42 (Rameh et al. 1997, Han et al. 1998).

Another important proliferative signal propagated to the Rho family GTPases from GPCRs and RTKs, is through the small G protein Ras (Figure 6b) (Bar-Sagi and Hall 2000, Liebmann 2011). Proliferative signals from GPCRs lead to activation of the MAPK ERK (see chapter 1.2.4). ERK activation requires a series of steps (Figure 6b), starting from activation of the small G protein Ras through the exchange factor Son of Sevenless (SOS). Ras activation is induced by RTKs, such as the EGFR (reviewed in Liebmann 2011, Johnson and Chen 2012). Upon activation and binding to the kinase Raf, Ras initiates the Raf/MEK/ERK pathway, leading to survival. Once activated by the kinase MEK, ERK has been shown by others, as well as our own lab, to activate Rho by phosphorylating its exchange factor GEF-H1 (see Figure 6b) (Fujishiro et al. 2008, Waheed et al. 2010, Kakiashvili et al. 2011, Waheed et al. 2013).

Adhesion receptors and Rho GTPases

Another class of receptors that have an increasingly recognized impact on Rho GTPases are adhesion receptors. Since adhesion molecules have a distinct effect on organization of the cytoskeleton, this is not surprising (discussed in chapter 1.2.5). Integrins (DeMali et al. 2003), cadherins (Braga 2002), and Immunoglobulin (Ig) superfamily members (Thompson et al. 2002) are some of the recognized classes of cell adhesion molecules shown to activate Cdc42, Rac or Rho (Figure 6a) (Burridge and Wennerberg 2004). For example, it has long been known that Cdc42 and Rac are activated in cells plated on Fibronectin (FN), which

promotes integrin-mediated spreading on the matrix (Barry et al. 1997, Clark et al. 1998, Kjoller and Hall 1999).

A common feature of many adhesion receptors is that they act through positive feedback loops involving Rho proteins. Integrins and cadherins are two such examples. Integrin-induced activation of Rho GTPases requires activation and clustering of integrins. This leads to recruitment of proteins, such as the regulators of Rho GTPase activation (Hotchin and Hall 1995). However, Rho GTPase activation itself is a required step for the initial integrin clustering. Thus, once activated, integrins further promote Rho GTPase activation, thereby perpetuating the positive feedback. In a recent paper by the Burridge group, the Rho GEFs LARG and GEF-H1 have been identified as mediators of mechanical stimuli through integrins (Guilluy et al. 2011).

Cadherin engagement and subsequent activation of Rac and Cdc42 have also been shown to be a positive feedback mechanism (Noren et al. 2003). ICAM-1 is an Ig-family adhesion molecule. Cross-linking of ICAM-1 molecules leads to increased activation of Rho, and formation of stress fibers (Adamson et al. 1999). Thus, several different cell surface receptors, upon ligand binding/activation, lead to activation of Rho GTPases through various and highly complex signal transduction systems. The ensuing small GTPase activation then further enhances clustering, augmenting the effect.

1.2.4.2. Cell stress stimuli that activate RhoA signalling

In our lab, we have shown GEF-H1 and RhoA to be activated in kidney proximal tubule epithelial cells by various stimuli. TNF, depolarization of the plasma membrane, hyperosmotic shock, immunosuppressive drugs (Cyclosporine A and Sirolimus), and EGF

activate RhoA signalling (Kakiashvili et al. 2009, Waheed et al. 2013, Waheed et al. 2010, Kakiashvili et al. 2011, Ly et al. 2013, Martin-Martin et al. 2012). In the next sections, I will discuss activation of Rho signalling by the first three stimuli.

Tumour Necrosis Factor-α (TNF)

TNF is a pleiotropic pro-inflammatory peptide cytokine that is synthesized as a membrane protein in response to inflammation, infection, and injury (Baud and Karin 2001). Cleavage by the metalloprotease TNF-alpha Convertase enzyme (TACE, also known as ADAM17) releases a 17kDa soluble peptide (reviewed in Wajant et al. 2003). It acts on two receptors, the ubiquitous TNF receptor 1 (TNFR1), which is constitutively expressed, and the inducible TNFR2. Adapter proteins such as TNF receptor activating factors (TRAFs) and receptor interacting protein (RIP) mediate various signalling events that determine outcomes such as apoptosis, inflammation, and gene transcription (Lee and Lee 2002).

Increasingly, TNF has been shown to play a role in both acute renal injury and chronic diseases such as kidney fibrosis (reviewed in Vielhauer and Mayadas 2007). While TNF is almost undetectable in normal kidneys, elevated intrarenal, serum or urine concentrations were reported in various pathological states, including ischemia-reperfusion, acute transplant rejection, endotoxinaemia, treatment with the chemotherapeutic agent cisplatin, and diabetic nephropathy (Donnahoo et al. 2000, Donnahoo et al. 2001, Oliveira et al. 2002, Sato et al. 2004, Hribova et al. 2005, Ramesh et al. 2007). In animal models of these conditions, kidney injury was prevented or reduced by inhibition of TNF production, addition of TNF-neutralizing antibodies, and in TNF receptor knockout mice (Daemen et al. 1999, Donnahoo et al. 2000, Cunningham et al. 2002, Ramesh and Reeves 2002, Misseri et al. 2005, Vielhauer

and Mayadas 2007). TNF can be produced by resident kidney cells (fibroblasts, mesangial cells and the tubular epithelium), as well as by infiltrating macrophages and lymphocytes. Tubular TNF production was shown to be stimulated by reperfusion-injury, unilateral ureteral obstruction, and exposure to Lipopolysaccharide (LPS), IL-1-α or hypoxia (Jevnikar et al. 1991, Yard et al. 1992, Misseri et al. 2004, Pascher and Klupp 2005, Zager et al. 2005, Meldrum et al. 2006, Meldrum et al. 2007). Most of these stimuli also up-regulate TNF receptors (Vielhauer and Mayadas 2007). Hence, the central role of TNF in mediating kidney injury is well established; however, the underlying mechanisms are poorly defined.

Interestingly, TNF was suggested to contribute both to tissue injury and protection/repair. In our previous studies (Figure 7), we found that TNF, similar to GPCRs, also transactivates EGFR through ADAM17/TACE, an enzyme that releases EGFR ligands. TACE and EGFR then mediate activation of the MAPK ERK, which in turn induced RhoA activation through the exchange factor GEF-H1 (see chapter 5). Our studies, therefore, raise the intriguing possibility that TACE/ERK/GEF-H1/Rho pathway is a key regulator of the epithelial cytoskeleton and junctions. Coupling of a pro-inflammatory (TNF) and proliferative (EGFR) signalling pathway might also serve as a proliferation/apoptosis switch, and could play a major role during epithelial wound healing and repair. Interestingly, in our most recent studies we also found that TNF enhances wound healing (chapter 5). However, the upstream mechanisms that mediate TNF-induced TACE and EGFR activation remained unknown. Our latest findings addressing this question are discussed in Chapter 5.

Figure 7. Summary of previous findings

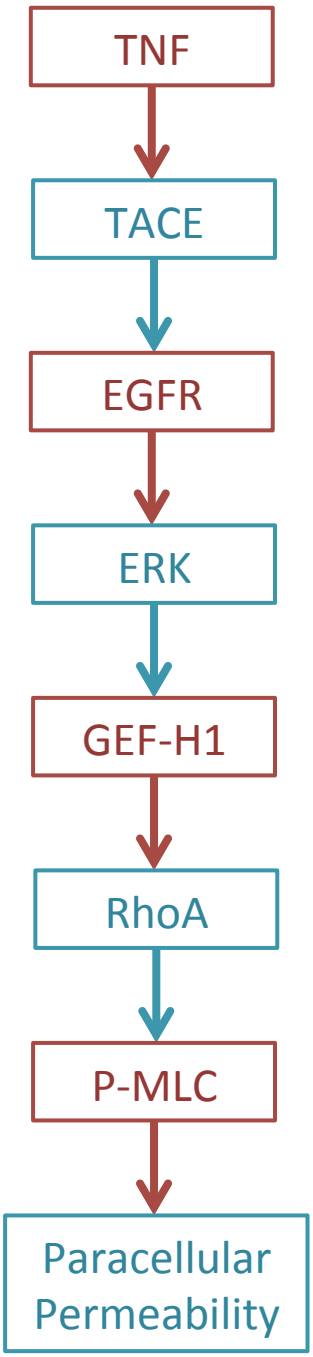


Figure 7. Summary of previous findings. Studies performed in our lab, prior to the work described in this thesis, have shown that TNF transactivates the EGFR through the metalloprotease TACE/ADAM17. EGFR is required for TNF-induced activation of ERK. ERK phosphorylates GEF-H1 on T678, enabling it to activate RhoA. GEF-H1 silencing also prevents TNF-induced phosphorylation of MLC. TNF increases paracellular permeability in tubular cells, which is prevented by a dominant negative phospho-mutant of MLC.

Depolarization of the plasma membrane

Resting plasma membrane potential contributes to the maintenance of cellular ionic homeostasis and provides the driving force for electrogenic ion transport. In nonexcitable cells, such as tubules, where the resting potential is relatively stable, physiological changes in potential can result from various electrogenic ion transport processes, such as activation of the Na^+-coupled amino acid and glucose cotransporters (Hoyer and Gogelein 1991) or stretch-induced channels (Sohn et al. 2000). In addition, pathological conditions, such as ATP depletion during hypoxia or metabolic substrate deprivation, and oxidative stress can also perturb the normal membrane potential (Haddad and Donnelly 1990, Balestrino 1995, Brzezinska et al. 2005).

Thus, while physiological potential changes signify an altered functional state of the cells, a large and sustained depolarization can be associated with injury. In all of these cases, adequate adaptive and protective cellular responses are needed. Therefore, it is not surprising that depolarization, similar to other physical factors, including anisoosmolarity (Koivusalo et al. 2009) or heat shock (Arya et al. 2007), was shown to activate various cellular signalling pathways.

Depolarization-induced calcium signalling is a well-described phenomenon. However, more recently, activation of other important signalling pathways initiated by depolarization have been described in neurons and neuron-like cells (Enslen et al. 1996, Egea et al. 1998). In the past years, however, previous work in our lab (Szaszi et al. 2005), as well as studies by others (Chifflet et al. 2003, Chifflet et al. 2004, Chifflet et al. 2005) have shown that depolarization-induced morphological changes occur in epithelia as well. For example, in

cultured eye epithelial cells depolarization causes reorganization of F-actin and microtubules and appearance of intercellular gaps (Chifflet et al. 2003, Chifflet et al. 2004). Chifflet and others (Chifflet et al. 2005) demonstrated that wounding of an epithelial monolayer results in depolarization of the plasma membrane in the cells bordering the wound. The ensuing actin rearrangement likely contributes to wound healing (Chifflet et al. 2005). In LLC-PK1 and Madin-Darby canine kidney (MDCK) kidney tubular cells, depolarization elevates phosphorylation of MLC through RhoA and its effector ROK (Szaszi et al. 2005). Further, we have shown that the exchange factor GEF-H1 is activated by depolarization and mediates RhoA activation (chapter 1.3.3).

Our lab also investigated the functional consequences of depolarization-induced myosin phosphorylation in tubular cells. The phosphorylation state of MLC is a key determinant of transepithelial and paracellular transport processes (Kapus and Szaszi 2006, Shen et al. 2006, Turner 2006, Ivanov 2008 and chapter 1.2.5). Since our previous studies (Szaszi et al. 2005) showed that depolarization, triggered by the electrogenic Na^+-alanine transporter, also induced myosin phosphorylation, we conceived that depolarization could serve as a coupling signal between electrogenic cotransporters and the paracellular pathway (Kapus and Szaszi 2006). Indeed, we found that depolarization elevates paracellular permeability, and this is mediated by phospho-MLC (Waheed et al. 2010).

Hyperosmotic stress

Cell volume is a key homeostatic parameter, and a disproportionate change in volume is a threat constantly faced by cells. Cell volume can change due to exposure to 'aniso-osmotic' environments. For example, such conditions are present in the renal medulla, which contains

the highest levels of interstitial NaCl and urea in the body (reviewed in Burg et al. 2007). Physiologic transport of solutes across the membrane can also alter intracellular osmolarity, which results in water movement and cell volume changes (shrinkage or swelling of the cells). Under such circumstances, cells try to either restore homeostasis by compensatory change in volume, and/or by strengthening their structure to withstand any imposed changes in cell shape/size (reviewed in Di Ciano-Oliveira et al. 2006). Hence, effecting these responses requires three primary cellular responses: regulation of transport processes to normalize cell volume, enhanced transcription of osmolyte transporters and enzymes involved in the response, and reorganization of the cytoskeleton to reinforce cell structure. This latter also contributes to the first two responses (Di Ciano-Oliveira et al. 2006).

The actin cytoskeleton has a role in cell volume regulation (Henson 1999, Pedersen and Hoffmann 2002). Actin is actively affected by volume changes and functionally interacts with several transport proteins and their regulators involved in volume-response (reviewed in Pedersen et al. 2001). Research in Ehrlich ascites tumour cells, HL-60 cells, and neutrophils has shown a net increase in peripheral F-actin content in response to hyperosmotic challenge (Hallows et al. 1996, Pedersen et al. 1999, Rizoli et al. 2000). Additionally, our lab has previously shown that in tubular epithelial cells, this peripheral accumulation of an actin belt is accompanied by disassembly of stress fibres in the center of the cell (Szaszi et al. 2000a, Szaszi et al. 2000b, Di Ciano et al. 2002). The mechanism surrounding such contrary, yet complementary effects, however, remains unsolved.

As major regulators of the actin cytoskeleton, it is not surprising, then, that Rho family GTPases were shown to participate in, and regulate, hypertonicity-induced reorganization of the cytoskeleton. Our lab, and others, have shown that Rac, Cdc42, and Rho are all activated

by hyperosmotic stress (Di Ciano et al. 2002, Lewis et al. 2002, Di Ciano-Oliveira et al. 2003). However, regulation of Rho proteins in such a scenario is incompletely understood. In our most recent study, one of the mysteries we sought to unravel is the identity of the GEF responsible for hyperosmotic stress-induced Rho activation (Ly et al. 2013) (chapter 4). As discussed in chapter 4, we found that GEF-H1 is activated by hyperosmotic stress, and contributes to the regulation of Myocardin-Related Transcription Factor (MRTF), a co-activator of SRF, that is known to drive expression of several cytoskeletal genes (Parmacek 2007) (chapter 1.2.5).

1.2.5. Effectors of Rho proteins

Upon activation and translocation to specific subcellular locations, RhoA and Rac are able to engage with proteins that are their downstream effectors in specific signalling cascades (reviewed in Bishop and Hall 2000, Jaffe and Hall 2005, Bustelo et al. 2007). Mainly using affinity chromatography and the yeast two-hybrid system, so far more than 70 effectors have been identified for Rho and Rac GTPases. Effector proteins interact specifically with the GTP-bound forms of Rho and Rac, and recognize and bind to specific residues within switch I and switch II, called docking/recognition sites (Bishop and Hall 2000, Bustelo et al. 2007). Different effectors are known to recognize and interact with different residues within the switch I region itself. Effector activation by Rho GTPases is most commonly through disruption of intramolecular autoinhibitory interactions within the effector proteins to uncover functional domains (Bishop and Hall 2000).

Effectors of RhoA

Some of the best characterized targets of RhoA are Rho-kinase (ROCK or ROK), the mammalian homolog of the *Diaphanous* formin (mDia), Protein Kinase N (PKN)/PRK1, rhotekin, rhophilin, citron kinase, Phosphatidylinositol-4-phosphate 5-kinase (PIP5-K), and Serum Response Factor (SRF) (Figure 8).

ROK, a Ser/Thr kinase, is a major effector activated by RhoA, and leads to activation of several signalling cascades (reviewed in Schwartz 2004). ROK has two isoforms, ROK1 and ROK2. The amino terminus of ROK hosts its kinase domain, whereas the carboxy-terminal end has a PH domain. The C-terminal end of a putative coiled-coil domain in the middle of ROK interacts with Rho.GTP, which then activates the phosphotransferase activity of ROK (Matsui et al. 1998, Kaibuchi et al. 1999). ROK regulates contractility of the actomyosin complex via two mechanisms; 1) by phosphorylating Myosin Light Chain (MLC) (Burridge and Chrzanowska-Wodnicka 1996, Ridley 1996); and 2) by phosphorylating and inhibiting the myosin-binding subunit (MBS) of myosin phosphatase (Amano et al. 2000). Expression of constitutively activated ROK, as well as Rho, leads to increased phosphorylation and activation of MLC (Burridge and Chrzanowska-Wodnicka 1996, Kureishi et al. 1997). ROK is essential for Rho-induced formation of stress fibers, since its activation of MLC stimulates both, association of actin filaments with myosin II, and the ATPase activity of myosin (Ridley 2000) (see chapter 1.2.5). Interestingly, ROK is necessary but not sufficient for correct assembly of stress fibers induced by Rho activation (see mDia below) (Ridley 2000). ROK also regulates junction assembly/disassembly, most likely through its effects on the actomyosin complex (Ridley 2000).

Figure 8. Some of the effectors and effects of RhoA

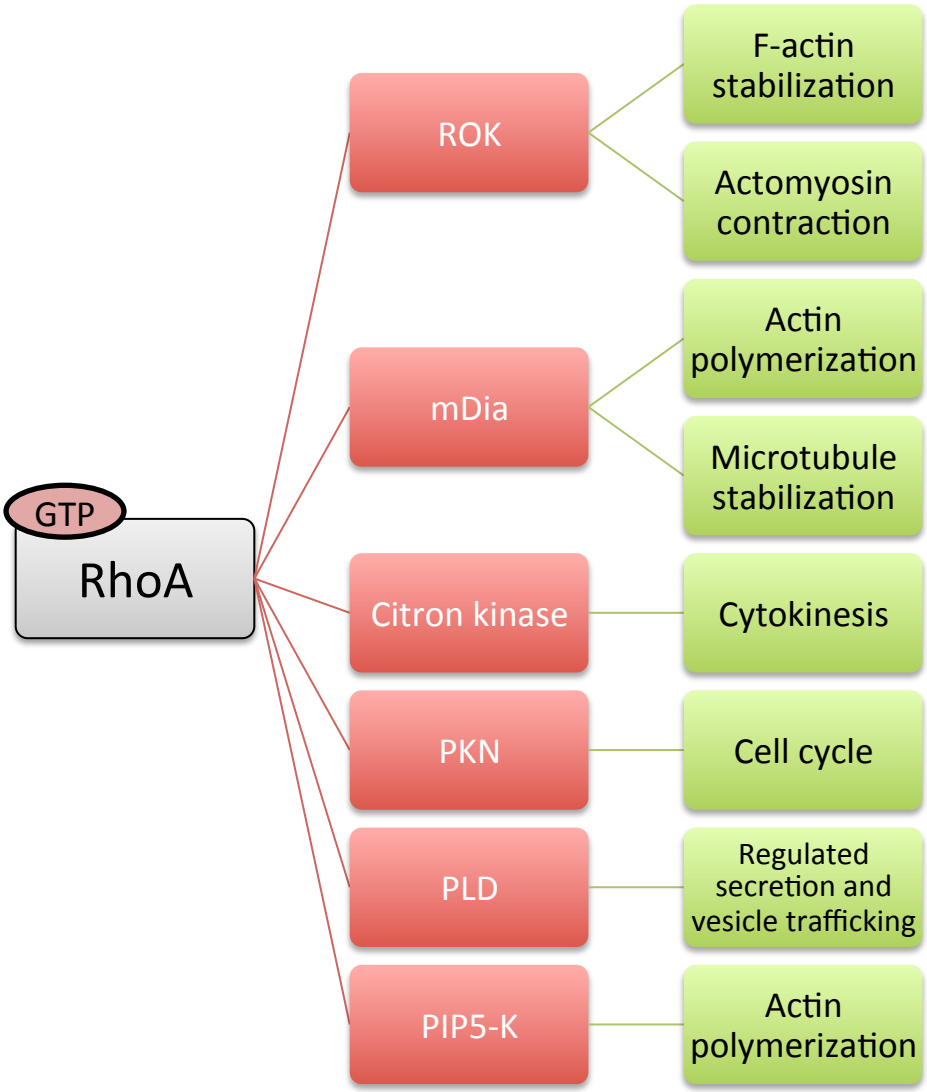


Figure 8. Some of the major effectors and effects of RhoA. Together, Rho kinase (ROK) and mDia carry out most of the effects of RhoA on the actin and microtubule cytoskeletons. Phosphatidylinositol-4-phosphate 5-kinase (PIP5-K) signals through the second messenger, phosphatidylinositol 4,5-bisphosphate (PIP2), to regulate among other things, actin polymerization. Citron kinase, Protein Kinase N (PKN), and Phospholipase D (PLD) and important in cytokinsesis, cell cycle regulation, and membrane remodelling events, respectively.

As mentioned above, MBS is a subunit of the myosin phosphatase. Myosin phosphatase binds to MLC via MBS and dephosphorylates and inactivates it (Kaibuchi et al. 1999). The C-terminus of MBS binds to Rho.GTP, whereby ROK is able to phosphorylate not just MLC, but also MBS (Kimura et al. 1996). Phosphorylation of MBS by ROK leads to its inactivation, and hence the prevention of MLC inactivation. MBS and ROK together participate in regulating levels of phosphorylated MLC, and hence are key regulators of actomyosin-based cell contractility.

In addition to MLC, ROK phosphorylates several other proteins that act further downstream in Rho signalling. LIMK is phosphorylated by ROK. LIMK then phosphorylates and inactivates the actin depolymerizing protein Cofilin, inhibiting its actin-severing ability. This regulates actin cytoskeleton reorganization (Miyazaki et al. 2006, Sato and Iiri 2006) (see chapter 1.2.5). ROK also phosphorylates ERM proteins, thereby enabling the linking of actin to the membrane (Matsui et al. 1998, Bretscher 1999). The ubiquitous Na^+/H^+ exchanger (NHE1) is also activated by ROK, which through (an) unknown mechanism(s) may also contribute to stress fiber and focal adhesion formation (Vexler et al. 1996, Tominaga and Barber 1998). A second effector kinase of Rho, **Citron kinase** directly phosphorylates MLC on two residues that leads to the activation of the latter, and drives the contraction of the actomyosin ring during cytokinesis (Madaule et al. 1998). Citron kinase localizes specifically to the cleavage furrow in HeLa cells, and drives the Rho-dependent process of cytokinesis (Birkenfeld et al. 2007).

mDia is recognized as the other major Rho effector that, together with ROK is important for stress fiber formation (Ridley 2000). mDia is a member of the formin-homology (FH) family

and contains two FH domains (Wasserman 1998). In its FH1 domain, mDia has several proline-rich motifs, which interact with Profilin, a G-actin-binding protein. This binding of mDia to profilin promotes actin polymerization, and formation of stress fibers (Wasserman 1998, Watanabe et al. 1999).

PKN/PRK1 is a kinase belonging to the Protein Kinase C (PKC) superfamily and is activated by phospholipids. It binds to Rho.GTP through its N-terminal regulator domain, which has three leucine zipper-like motifs (Watanabe et al. 1999, Amano et al. 2000). Binding to Rho.GTP activates the kinase activity of PKN. However, the physiological functions of PKN are unknown (Ridley 2000). An isoform of PKN/PRK1, PRK2, interacts with Rac.GTP and plays a role in actin cytoskeleton reorganization (Kaibuchi et al. 1999).

Two Rho effectors that have similar Rho-binding domains (RBDs) as MBS and PKN/PRK1, are **Rhotekin and Rhofilin** (Reid et al. 1996, Watanabe et al. 1996). Although both these proteins bind Rho.GTP, their functions are not very well known. However, an important, and widely utilized experimental significance of Rhotekin involves its Rho binding domain (RBD), which binds specifically and with high affinity to only Rho.GTP. Binding of Rhotekin to active Rho reduces the intrinsic catalytic rate of GTP hydrolysis of Rho. This makes the RBD motif of Rhotekin a valuable experimental tool for the isolation of Rho.GTP from cell lysates through the process of affinity purification (Ito et al. 2006).

Phosphatidylinositol 4,5-bisphosphate (PIP2) is the lipid product of **PIP5-K** and plays a role in actin cytoskeleton regulation through its interactions with different actin-binding proteins, such as profilin, α-actinin, gelsolin, and vinculin (Janmey 1994, Tapon and Hall 1997). Although a physical association between Rho/Rac and PIP5-K has been detected in Swiss

3T3 fibroblasts, some studies suggested that this interaction may be indirect (Ren et al. 1996, Bishop and Hall 2000). However, it has been shown that both Rac and Rho enhance the production of PIP2, which at the plasma membrane plays a role in actin filament turnover and re-arrangement (Tapon and Hall 1997). Rho and Rac have also been shown to regulate **Phospholipase D (PLD)** (Santy and Casanova 2001). This enzyme catalyzes hydrolysis of phosphatidylcholine, resulting in production of phosphatidic acid and choline. Regulation of phosphatidic acid levels is important, since phosphatidic acid is a second messenger that plays a role in crucial membrane remodelling events, such as regulated secretion and vesicle trafficking (Santy and Casanova 2001).

Rho and ROK also activate through actin the **Serum Response Factor (SRF)**, a transcription factor that binds to the Serum Response Element (SRE) in the promoters of target genes. Activation of these genes can lead to the regulation of cell cycle, cell growth, and differentiation (Settleman 2003). SRF-driven activation of smooth and skeletal muscle gene expression is dependent on its nuclear translocation, which is facilitated by Rho/ROK via their effects on actin polymerization (Liu et al. 2003). Myocardin-Related Transcription Factor (MRTF) is an actin-regulated co-activator of SRF, and a major link between regulation of the actin cytoskeleton and transcriptional control (see chapter 4) (Ly et al. 2013).

Effectors of Rac

Proteins identified as targets for Rac include PAKs, WASP (Wiskott-Aldrich Syndrome Protein) Verprolin Homologous (WAVE), IQ motif containing GTPase activating protein

(IQGAP), p67phox, and other kinases, such as c-Jun N-terminal kinase (JNK), p38 MAPK, and p42/p44 (ERK) (Figure 9).

PAKs are a family of Ser/Thr kinases, and divided into two groups: Group I PAKs (PAK1-3) bind Rho GTPases and are activated by them, whereas activation of Group II PAKs (PAK4-6) is independent of Rho and Rac, as they are activated by Cdc42 (Eswaran et al. 2008). An N-terminal regulatory and a highly conserved C-terminal catalytic domain are common to all PAKs. Group I PAKs have in their regulatory domains a Cdc42/Rac-interactive binding motif, and an autoinhibitory switch domain (AID) (Eswaran et al. 2008). Although PAKs act as effectors of both Rac and Cdc42, I will mostly discuss their roles as Rac effectors.

Once activated by Rac/Cdc42, PAK1 is a major regulator of the actin cytoskeleton. It induces formation of lamellipodia, filopodia, and membrane ruffles via activation of LIMK, mixed lineage kinase (MLK), and other regulators (Bokoch 2003, Arias-Romero and Chernoff 2008). PAK1 phosphorylates LIMK which through phosphorylation and inactivation of Cofilin, prevents depolymerization of actin filaments (Eswaran et al. 2008). PAK activity also regulates actomyosin contractility, since PAK phosphorylates and inactivates myosin light chain kinase (MLCK), thereby reducing MLC phosphorylation (Sanders et al. 1999) (see chapter 1.2.5). This, however, is controversial, since some studies report that PAK leads to an increase in MLC phosphorylation (reviewed in Bokoch 2003). PAK also binds filamin, a major actin binding protein found at the cell cortex that is concentrated in membrane ruffles (Burridge and Wennerberg 2004). Filamin plays a role as a scaffold protein for PAK, as well as in stabilizing membrane protrusions by cross-linking F-actin filaments (Burridge and Wennerberg 2004). Through PAK1, Rac has also been shown to regulate microtubule dynamics (Wittmann and Waterman-Storer 2001). PAK1 phosphorylates and inactivates the

Figure 9. Some of the effectors and effects of Rac

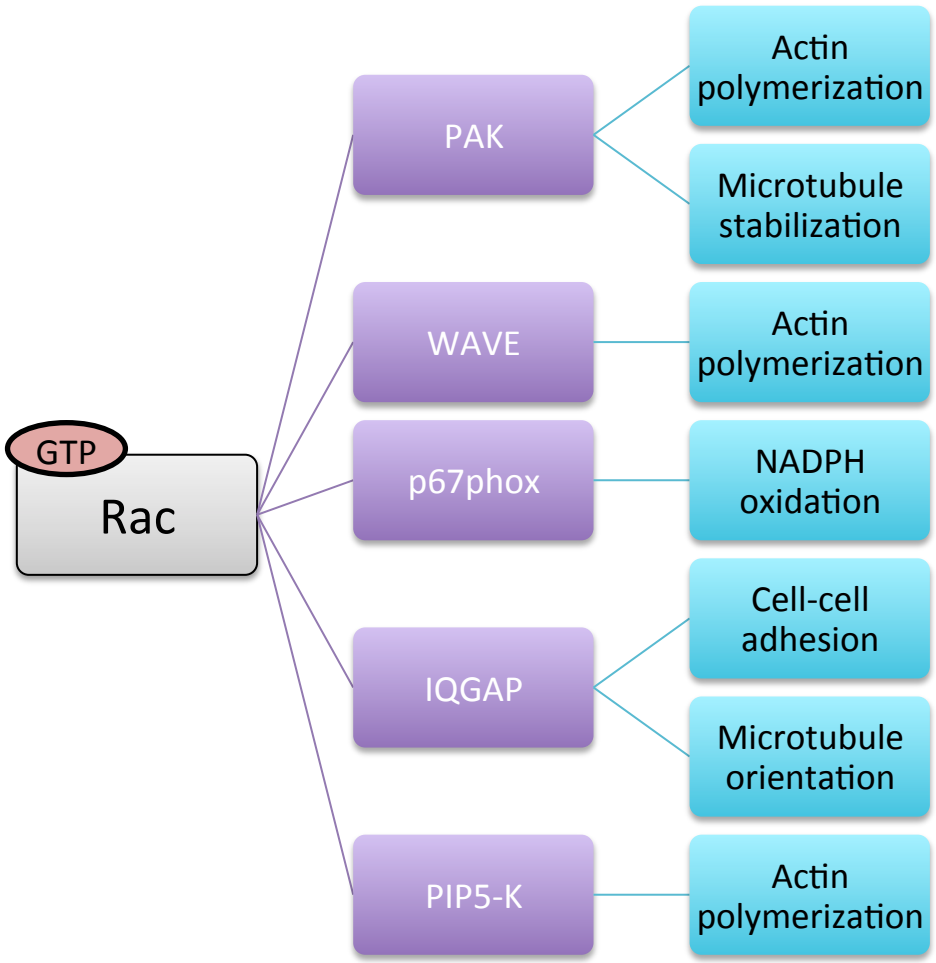


Figure 9. Some of the effectors and effects of Rac. The primary effectors of Rac are PAK1 and WAVE, through which the Rho GTPase mediates its effects on the actin and microtubule cytoskeletons. Through its effects on p67phox, Rac plays a role in superoxide production by the NADPH oxidase. IQGAPs localize to intercellular junctions, where they mediate cell-cell adhesion via crosslinking of F-actin. Like RhoA, Rac also regulates actin polymerization through another effector, PIP5-K.

microtubule destabilizing protein, Stathmin/Op18, thereby promoting microtubule stabilization. Other described interactions of PAKs are with the Rac GEF Pix, which is necessary for formation of lamellipodia by PAK (Manser et al. 1998). PAKs also contribute to regulation of gene expression through the JNK and p38 kinase pathways (see below) (Zhang et al. 1995, Bagrodia et al. 1998).

Activated Rac interacts with the **WAVE** complex, from which it releases activated WAVE (Eden et al. 2002). The Actin-related protein 2/3 (Arp2/3) complex is then initialized by WAVE, which induces actin polymerization and formation of lamellipodia (Soderling and Scott 2006). The Arp2/3 complex serves as a nucleation core from which polymerizing actin can branch into new filaments (Suetsugu et al. 2002). Arp2/3 also competes with actin capping proteins to bind the barbed end of an existing filament, from which it enables growth and polymerization of a branched actin filament (Aguda et al. 2005).

Although called GAPs, **IQGAP1** and IQGAP2 do not have GAP activity, but show homology to RasGAP (Hart et al. 1996). Rac and Cdc42 bind IQGAP1, an actin binding protein that is known to regulate cell-cell adhesion and orientation of microtubules (Hart et al. 1996). IQGAPs can oligomerize and cross-link F-actin, which they are found in complex with (Fukata et al. 1997). IQGAP1 is localized to intercellular junctions in epithelial cells, where it plays a role in actin organization (Kuroda et al. 1998). IQGAP1 also binds to Clip170, a protein found in microtubule tips, and facilitates the direction of cell polarization by ensnaring growing microtubules at the leading edge of migrating fibroblasts (Soderling and Scott 2006).

Rac also plays an important role as a regulator of **p67*phox*** (which it directly binds), an essential structural component of the Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, that produces reactive oxygen species (ROS) (Diekmann et al. 1994). Due to the toxicity of ROS, their production was generally thought to be restricted to phagosomes (Ridley 2000). However, more recent studies have shown the presence of oxidases in many cells. Produced by the mitochondria, or the various forms of the NADPH oxidase enzyme, low levels of ROS have signalling roles (reviewed in D'Autreaux and Toledano 2007). Also, since Rac is involved in the phagocytic process, its role in the production of ROS serves a dual purpose (Ridley 2000). Nuclear Factor κB (NFκB)-dependent gene expression is activated by the production of ROS. This activation of genes results in, among other things, effects on progression of cell cycle (Schwartz 2004).

Interestingly, PAK has been shown to phosphorylate p67phox on a site adjacent to its Rac-binding site (Ahmed et al. 1998). This could imply that Rac exerts its effects on the NADPH oxidase through its effector PAK. Other research describes the role of Rac as an allosteric regulator of the NADPH complex that induces a conformational change in the complex, thereby allowing catalytic activity of the NADPH oxidase complex (Nisimoto et al. 1997). More studies are needed to elucidate the exact roles of PAK and Rac in activation of the NADPH oxidase.

In a cell-type- and stimulus-specific manner, Rac and Cdc42 activate the kinases, **JNK**, **p38** MAPK, and p44/p42 **ERK** MAPK (Cancelas et al. 2005, Carstanjen et al. 2005, reviewed in Pai et al. 2010). JNKs are stress kinases (activated by inflammatory signals, changes in ROS levels, ultraviolet radiation, and so on) that, upon activation, enter the nucleus and activate transcription factors such as c-Jun, c-Fos, Elk1, and Elk4 (Kesavapany et al. 2004). p38

MAPKs (see chapter 5) are also stress-responsive kinases activated by stimuli such as cytokines, heat shock, osmotic shock, and UV radiation. Activation of p38 by Rac/Cdc42 is through the activation of PAK, which phosphorylates MAPK kinase kinase, leading to subsequent p38 activation (Kaur et al. 2005). ERK MAPKs are activated by growth factors and phorbol esters, such as Phorbol 12-Myristate 13-acetate (PMA). They participate primarily in regulation of cell growth and differentiation (Pearson et al. 2001). Rac can also activate ERK through a mechanism involving PAK-mediated phosphorylation of the kinases upstream of ERK (Sundberg-Smith et al. 2005).

1.2.6. Structures and functions regulated by Rho and Rac

Rho GTPases play a pivotal role in regulating actin dynamics through a series of well-defined signal transduction pathways, which lead to 1) effects on the polymerization and elongation of actin filaments, and 2) effects on myosin phosphorylation and contractility (Jaffe and Hall 2005).

1.2.6.1. Actin polymerization

Actin is a protein capable of forming long filaments that can be arranged into various structures that not only provide shape and support, but also the ability to generate force within the cell (Manser 2004). Actin exists as monomers (G-actin) that are able to polymerize into filaments (F-actin) (Figure 10a). Filaments can branch, and can also organize into highly ordered structures, e.g., through bundling. A component of the cytoskeleton, actin is a protein that is conserved from yeast to humans (Schmidt and Hall 1998). It has intrinsic ATPase activity within its ATP binding domain. When bound to ATP, actin monomers attach to the barbed/plus end of growing filaments (Manser 2004).

Figure 10. Acto-myosin

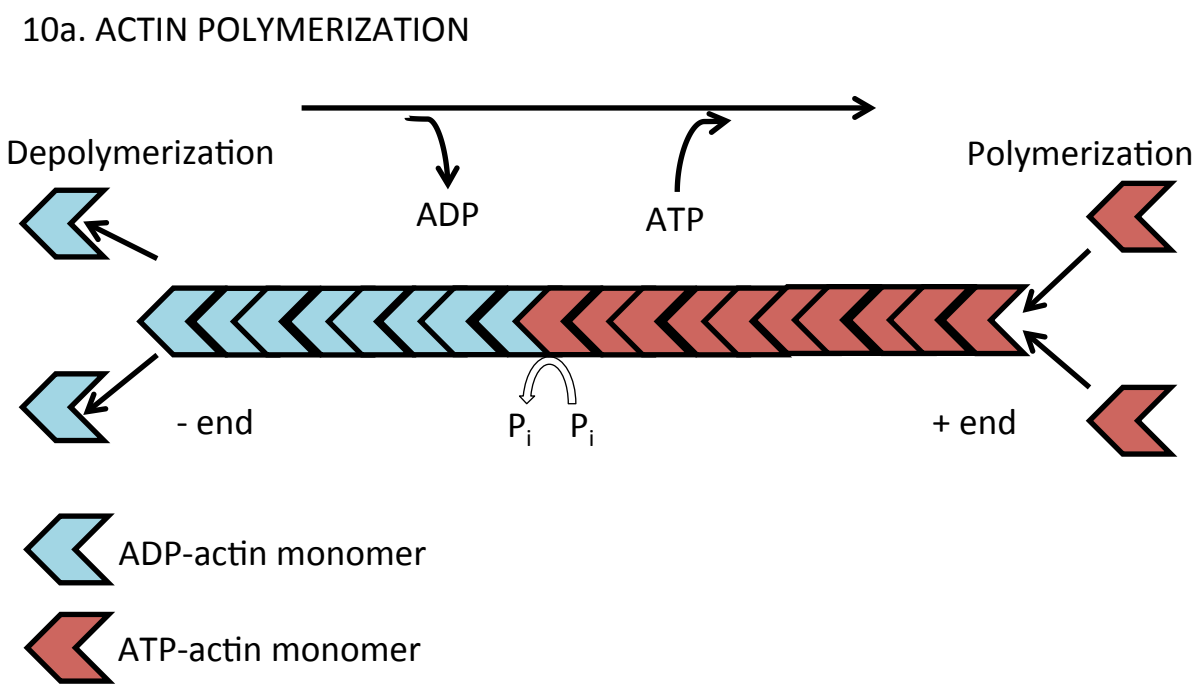


Figure 10. Organization of actin and myosin. (a) Polymerization of monomeric G actin into filamentous F actin occurs at the barbed '+' end, where ATP-bound actin monomers are added on.

The phenomenon of treadmilling occurs when monomeric actin is lost from both ends of a filament, without any change in the total length of the filament. Called Globular (G)-actin, monomers of actin bind to each other, and also to proteins such as profilin and cofilin, whereas filamentous (F)-actin binds to other actin binding proteins (Manser 2004). In the leading edge of migrating cells, actin filaments are arranged in a dense network, with the growing (plus) end oriented toward the plasma membrane (Svitkina and Borisy 1999). Here the polymerizing actin filaments provide a 'pushing force' to extend the plasma membrane at the leading edge of the cell outward. Myosin, on the other hand, provides the 'pulling' force at the different sites of cell attachment (Manser 2004).

Actin polymerization requires coordinated action between the two main polymerization proteins, the Arp2/3 complex and formins, and the filament severing and capping proteins. As mentioned above, activation of the Arp2/3 complex by Rac occurs indirectly through WAVE. Cdc42, through its effector WASP, is also a major activator of the Arp2/3 complex (Castellano et al. 1999). This complex acts as a site of *de novo* actin polymerization. It binds to the side of an existing actin filament, and enables the growth of a new filament at a 70° angle from the original filament (Manser 2004). This leads to branching of F-actin and further polymerization of the branches. WASP recruits profilin to actin polymerization sites. Another level of control of profilin is due to the augmentation of its function by PIP2 levels, which in turn are regulated by PIP5-K (see chapter 1.2.5). Hence, as mentioned earlier, regulation of PIP5-K by both Rho and Rac, enables them to govern actin polymerization in yet another way.

Rho stimulates actin polymerization in mammalian cells through its effector mDia, as well as the actin-severing protein cofilin (Jaffe and Hall 2005). mDia binds to the barbed/plus end of actin filaments and adds on actin monomers, thereby elongating the filaments. As discussed in chapter 1.2.5, mDia also binds the protein profilin, which promotes the addition of G-actin monomers to the barbed end of a growing actin filament (Manser 2004).

Actin disassembly is the rate-limiting step in actin dynamics at the leading edge (Manser 2004). Cofilin (or actin depolymerizing factor (ADF)) is an actin filament severing protein, which leads to the generation of new barbed ends that serve as sites for further actin polymerization and filament elongation (Ghosh et al. 2004). Cofilin is also important in actin filament disassembly, and together the processes of filament elongation and disassembly regulate the spatio-temporal generation of membrane protrusions (Pollard and Borisy 2003, DesMarais et al. 2005). Cofilin regulation is a tightly controlled process, achieved via phosphorylation by LIMK. As discussed earlier, LIMK in turn, is activated by Rac/Cdc42 effectors, PAKs, or by the Rho effector ROK. This phosphorylation-dependent inactivation of Cofilin by LIMK also plays an important role in the stabilization of actomyosin filaments (Ohashi et al. 2000).

1.2.6.2. Contractility generated by myosin

Stress fibers, generated by activated Rho, are contractile filaments present in the leading edge of cells, as well as within focal-adhesion complexes at the 'tail'/trailing edge of migrating cells (Galbraith and Sheetz 1997, Pelham and Wang 1999). In non-muscle cells, such as the epithelium, Rho/ROK, along with other effectors of Rho, assemble actin and myosin II into 'functional motor units' that are similar to those in muscle cells (Manser 2004). Non-muscle myosin comprises two myosin II heavy chains (MHCs) that are identical, and two regulatory

myosin light chains (MLCs) (Figure 10b). The MHCs are assembled into a 'head and tail structure', where the C-termini form the tail and the N-termini form the two globular heads. Wrapped around the neck region of the MHCs, are the regulatory MLCs, which control myosin activity via their phosphorylation state.

Stress fibers are assembled filaments of actin bridged by MHCs, where the heads of MHCs bind to F-actin in a specific manner (Manser 2004). Both MHC tails and F-actin are arranged antiparallel to other MHC tails and F-actin, respectively. These associated actin-myosin structures also assemble laterally the tail regions. Actomyosin contraction is dependent on the ability of the globular myosin head to transform energy from ATP hydrolysis into physical mechanical force. ATP-binding releases F-actin from the myosin head. Hydrolysis of the bound ATP on myosin, into ADP and phosphate, provides the energy needed for activation of the myosin head and its positioning into a 'high-energy, extended' state (Figure 11). This extended myosin head now binds to a new site on F-actin, generating a 'bridge' between the two components. Upon release of ADP and the phosphate, the myosin head settles into a 'low-energy' position, thereby pulling the bound actin filament along with it (called a 'power stroke'). Hence, once again, upon binding of ATP, the actin-myosin bond is destabilized, leading to the detachment of the myosin from F-actin. The cycle repeats itself when the ATP on the myosin head is hydrolyzed. Muscle contraction occurs when many myosin heads move actin filaments in unison and in the same direction, relative to myosin filaments.

In non-muscle cells contraction of myosin is regulated by phosphorylation of MLC, since only phosphorylated MLC enables a conformational change in the MHC heads ('active' conformation), allowing the binding of the MHC heads to F-actin (Figure 11).

Figure 10. Acto-myosin

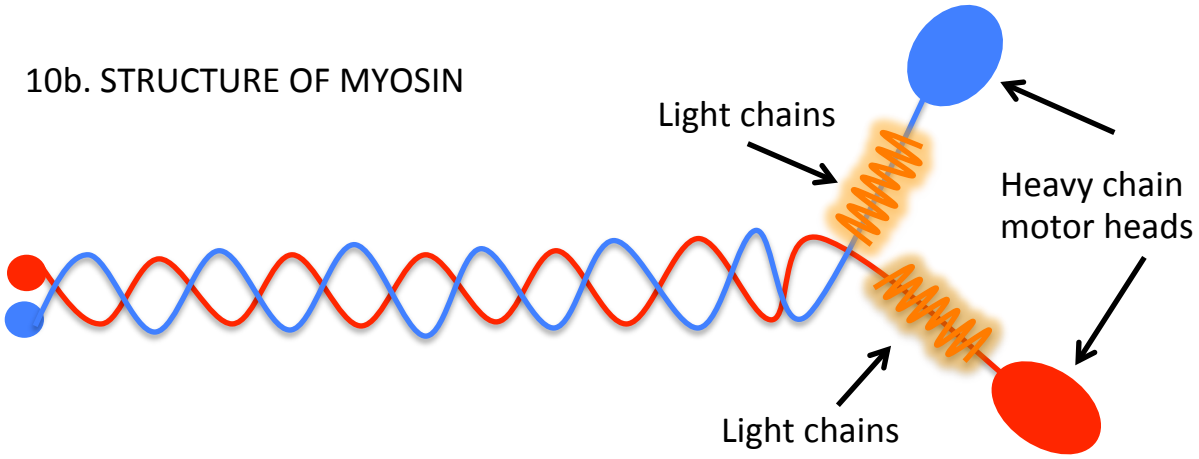


Figure 10. Organization of actin and myosin. (b) Conventional myosin in non-muscle cells is made up two identical myosin II heavy chains (MHCs), and two regulatory myosin light chains (MLCs) which are wrapped around the neck region of the MHCs. The MHC 'tail' are coiled-coil rods that can associate in an anti-parallel way with each other. The MHCs end in two globular heads in the N-terminal end. Inactivation of MLC phosphatase, leading to increased MLC phosphorylation, and/or direct phosphorylation of MLC by MLC Kinase or ROK, regulates myosin activity.

Figure 11. Acto-myosin-based contractility

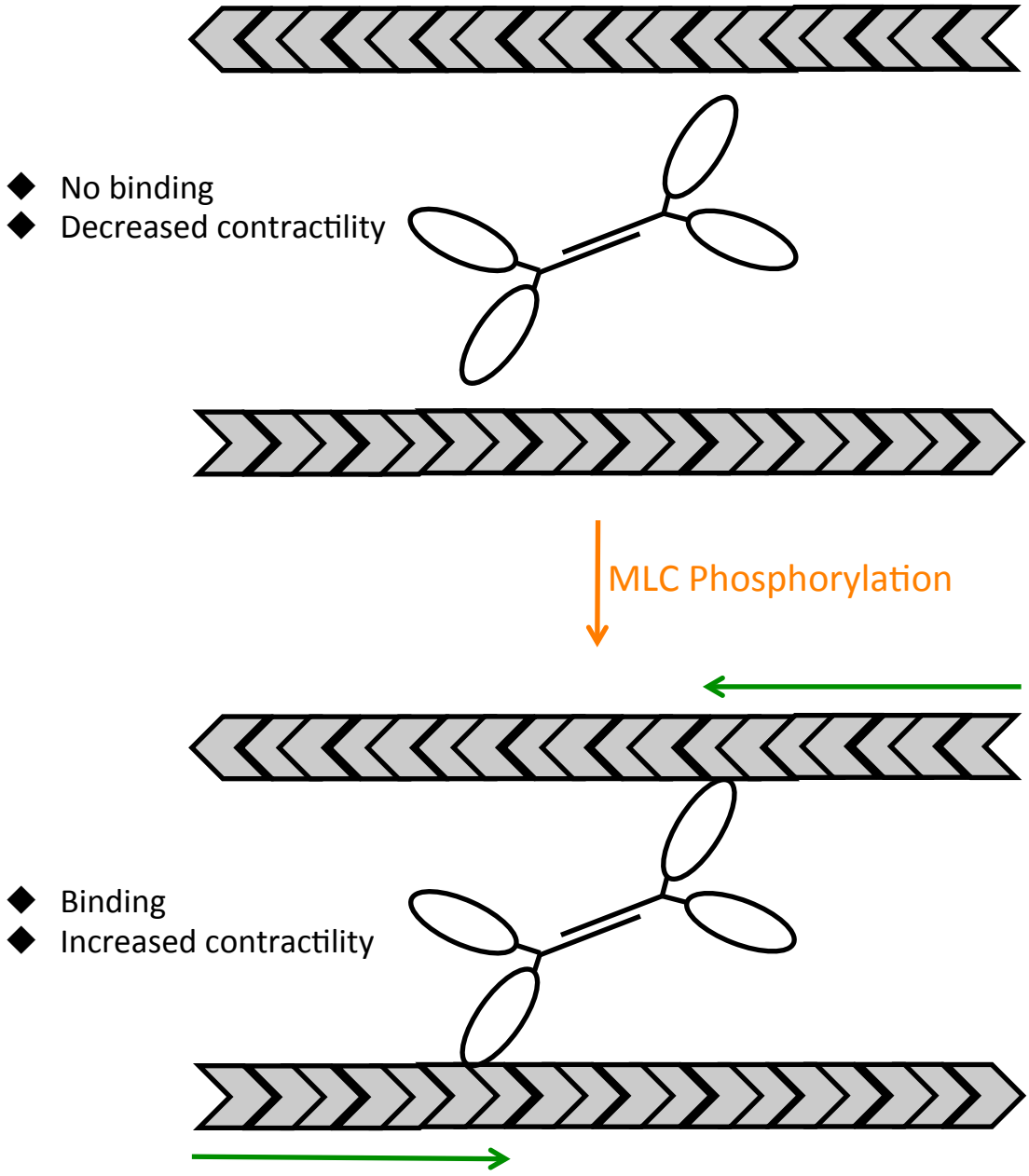


Figure 11. Regulation of actin-myosin contraction in non-muscle cells. Phosphorylation of MLC, enables a conformational change in the MHC heads making them 'active', and enabling them to bind to F-actin. Upon binding of ATP to the myosin head, it releases the F-actin. Hydrolysis of the ATP by the myosin head then gives it energy to pull back, thereby causing the F-actin to move, generating a contraction. Based on Wiggan et al. (2012).

Phosphorylation and activation of MLC occurs either through the so-called calcium sensitization pathway, or through the classical pathway. In the calcium sensitization pathway, which seems to be the major pathway in tubular epithelial cells, Rho regulates MLC phosphorylation through its effector ROK. As mentioned earlier, activated ROK works through two different mechanisms to phosphorylate MLC: 1) it inhibits the activity of the myosin phosphatase by binding to the MBS of myosin phosphatase and phosphorylating it, and 2) ROK directly phosphorylates MLC on the same residues as MLCK (in the classical pathway)- Ser19 and Thr18. The classical pathway involves the formation of an active calcium-calmodulin complex due to an increase in cytosolic calcium levels. This active complex then binds to MLCK and activates it. Activated MLCK then phosphorylates MLC, enabling the uncovering of active sites on the MHC heads and binding to F-actin, followed by actomyosin contraction (Kawano et al. 2005). However, the key event in some cells, as proposed by Riento and Ridley, is the ROK-dependent phosphorylation and inactivation of MLC phosphatase (Riento and Ridley 2003). As mentioned above, this leads to increased phosphorylation and activation of MLC, which then promotes the actin filament cross-linking activity of myosin II (reviewed in Jaffe and Hall 2005).

1.2.6.3. Focal Adhesion Complexes

Studies in fibroblasts show that Rho, Rac and Cdc42 are all involved in the development of integrin-containing focal adhesion complexes (Nobes and Hall 1995). Focal adhesions are multiprotein complexes assembled at the cytoplasmic domains of integrins, through which mechanical forces, as well as regulatory signals to and from the ECM are transmitted (Zaidel-Bar et al. 2004). Interaction of the actin cytoskeleton with the integrin complex results in intracellular tension and reinforces the formation of focal complexes, which are

also dependent on myosin II activation (Small et al. 1999). Vinculin, talin, and α-actinin are proteins that link actin filaments to the integrin complex (Manser 2004). The binding of vinculin to talin and α-actinin is a crucial step in the process of formation of focal complexes, and is facilitated by PIP2 (Gilmore and Burridge 1996). PIP2 levels are regulated by PIP5-K, which in turn is governed by Rho/ROK, and Rac/PAK (Manser et al. 1997, Oude Weernink et al. 2000).

1.2.6.4. Role of Rho and the actomyosin complex in junctions

Actomyosin-based contractility is a widely accepted mechanism by which Rho can regulate TJs (see chapter 1.1.3). A perijunctional actomyosin ring is closely associated with TJs (Figure 1) (Nusrat et al. 2000, Rodgers and Fanning 2011), where the phosphorylation status of MLC regulates contractility, and determines paracellular permeability (Turner 2000, Kapus and Szaszi 2006). Additionally, Rho, Rac and Cdc42 are essential not only in the development of TJs during establishment of polarized epithelial layers, but also in maintenance of TJs. As the central regulators of actin polymerization, and actomyosin-based contractility, these Rho proteins determine membrane trafficking of junction proteins, including their endocytosis (Ivanov et al. 2004, Samarin et al. 2007, Schwarz et al. 2007, Shen 2012).

TJs bind to the acto-myosin ring via many scaffolding proteins (Figure 2). The best-known TJ scaffold (adapter) proteins are ZO 1-3, and cingulin, which binds to myosin (Rodgers and Fanning 2011). Enhanced myosin activation (increased phosphorylation of MLC) leading to increased contractility of the actomyosin ring, alters TJs, leading to increased paracellular permeability of the epithelium (Figure 7) (reviewed in Turner 2000, Kapus and Szaszi 2006). Indeed, many stimuli that alter paracellular permeability do so by modulating myosin

phosphorylation and contractility. Our lab has worked with two such stimuli, TNF and depolarization of the plasma membrane (chapter 1.2.4.2) (Kakiashvili et al. 2009, Waheed et al. 2010, Kakiashvili et al. 2011). Both these stimuli provoke increased phosphorylation of MLC, leading to changes in contractility and increased paracellular permeability.

1.2.6.5. Cell Migration and wound healing

Cell migration is an important process in all multicellular organisms, during not just development, but throughout life. It plays key roles in processes such as immune responsiveness and wound healing (Raftopoulou and Hall 2004). Cells can migrate individually, as in leukocytes transmigrating through an endothelial layer in response to chemotactic stimuli released at the site of injury. In addition, cells can also migrate as a collective unit, where an entire monolayer of cells migrates as a whole. Wound repair in epithelial layers is an example of the latter process.

Situated at the interface of internal and external environments, epithelial cells are continuously exposed to harmful chemicals, toxins, mechanical stress, infectious agents, and other potentially injurious effects. Epithelial cells also have a high energy demand due to their numerous transport processes and, as a result, are sensitive to decrements in oxygen supply, or the presence of harmful toxins. Also, some epithelial layers, such as in the intestine, have high turnover rates, and cells in these layers have to be continually replaced (Szaszi et al. 2012). Epithelial cell migration is an important feature of the process of wound healing.

The two major types of wound healing mechanisms in the epithelium are purse-string closure, and sheet migration. The first type, as the name suggests, involves cells adjacent to a small wound contracting at their apical sides to come closer together to close the gap (Baur et

al. 1984). These cells generate a continual actin-myosin contractile belt that uses the force of contraction to pull the cells closer (reviewed in Garcia-Fernandez et al. 2009). The adherens junctions of the cells are crucial in transmitting this contractile force (Bement et al. 1993).

Epithelial sheet migration has some unique characteristics. During wound healing, these cells move as a multicellular unit (Poujade et al. 2007, Rorth 2009, Friedl and Wolf 2010, Khalil and Friedl 2010). The cells neighbouring the site of injury display specific morphological changes. They develop membrane protrusions and migrate into the site of injury to close the wound. However, cells at the leading edge maintain their cell-cell contacts with the cells behind them, and as a result, they pull the entire epithelial sheet forward. Also, remarkably, the cells at the back are not merely passively dragged forward, but also display active migration (Szaszi et al. 2012).

Cell migration, best characterized for individual migrating cells, is a process involving multiple steps that are complex and cyclical (Horwitz and Webb 2003, Petrie et al. 2009). Directional cell migration is similar in all cell types, with some variations in specific cells (Rikitake and Takai 2011). I will briefly discuss the process of epithelial sheet migration during the process of wound healing. Firstly, a stimulus is required to induce cytoskeletal remodelling and the necessary morphological changes needed for directed cell migration. During wound healing, this stimulus is cell injury, and the resulting exposure of extracellular matrix (ECM) proteins. Chemokines, cytokines and growth factors released due to injury act as directional migration cues (Sturm and Dignass 2008, Crosby and Waters 2010, Iizuka and Konno 2011). The exposed ECM proteins cause integrin clustering and activation at the leading edge adjacent to the wound, and this activation of integrins serves as a directional signal for migration of cells (Sturm and Dignass 2008, Crosby and Waters 2010, Iizuka and

Konno 2011). Second, disassembly of cell-cell contacts and junctions also stimulates migration, since cell-cell contacts have a strong inhibitory effect on cell migration (Mayor and Carmona-Fontaine 2010). This disassembly occurs, most likely, as a result of injury to adjacent cells at the wound edge.

Third, the cells, through localized activation of integrins and Rho GTPases (discussed below), acquire a front-rear polarity, since junction disassembly also results in a loss of apico-basal polarity (Etienne-Manneville 2008). Front-rear polarization is essential for *directed* migration. This polarization alters actin dynamics at the leading edge by enhancing actin polymerization and enabling the formation of membrane protrusions (Figure 12) (Szaszi et al. 2012). During this cell polarization, the microtubule network is also rearranged, allowing for altered vesicular transport, which potentiates delivery of necessary proteins to the leading edge.

Fourth, as discussed below, activation of two Rho GTPases, Rac and Cdc42, at the leading edge leads to increased actin polymerization and formation of characteristic membrane protrusions: lamellipodia and filopodia, respectively (Spiering and Hodgson 2011). The newly exposed integrin adhesion sites in the denuded area bind to these membrane protrusions, and exert a pulling force in the forward direction. However, the cells still maintain some degree of cell-cell contacts, as well as front-rear polarity, allowing a collective net movement in the forward direction (Farooqui and Fenteany 2005).

Rho GTPases in cell migration

Almost all cell migration processes require activation of Rho family GTPases. These proteins orchestrate remodelling of the cytoskeleton and the generation of front-rear polarity

Figure 12. Rho GTPases in cell migration

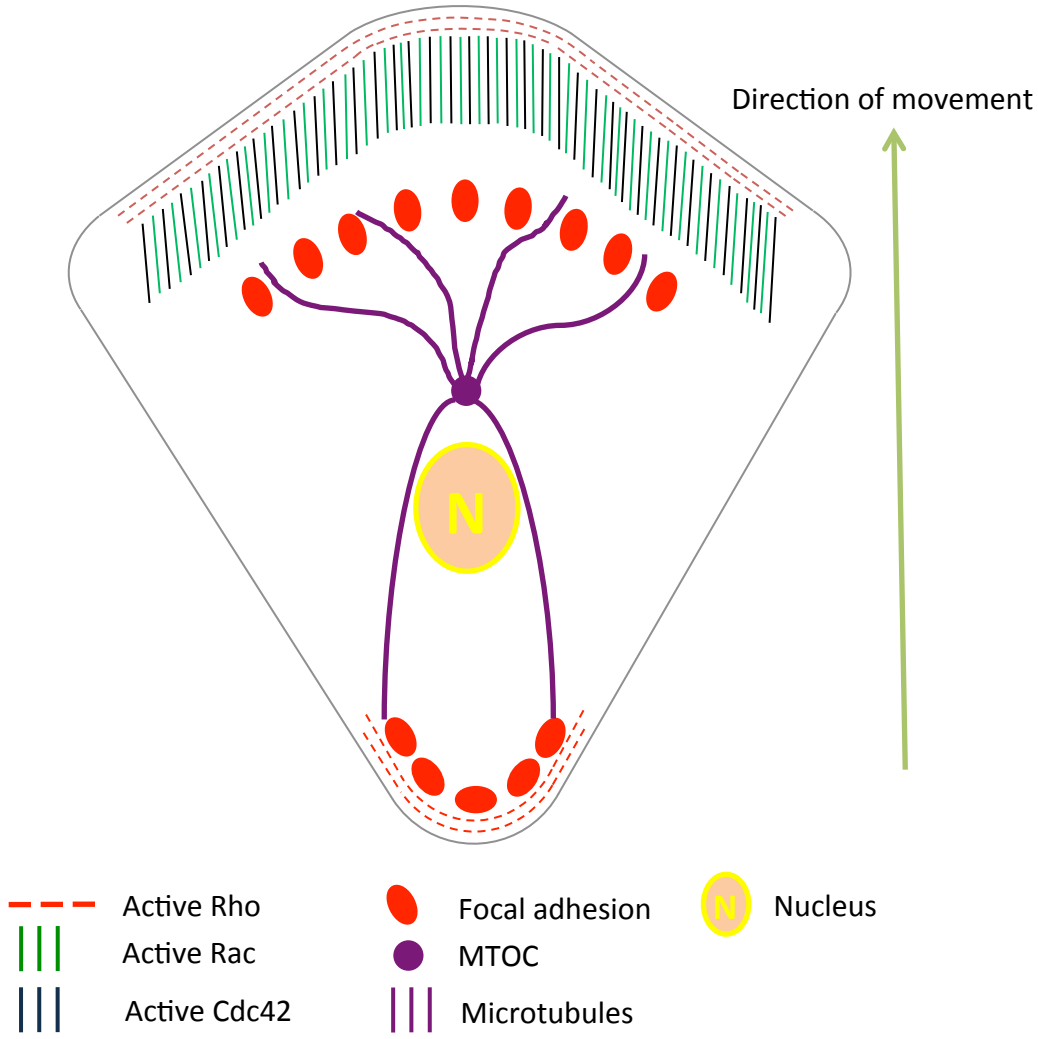


Figure 12. Localization of Rho GTPases during cell migration. Studies from Machacek et al. (2009) show an exciting and new picture of how the Rho proteins required for cell migration are activated spatially. The group show that RhoA activation is required not only at the rear of migrating cell for tail retraction, but also at the very edge (1.8 µm) of the leading edge. This could be due to the ability of RhoA to initiate 'spontaneous' actin polymerization, through its effector mDia, at the leading edge (Machacek et al. 2009). RhoA is also active in maturing focal adhesions. The Microtubule Organizing Center (MTOC) is thought to re-orient towards the leading edge allowing for a polarized microtubule array that aids migration. Adapted from Machacek et al. (2009).

necessary for directed movement in either an individual cell or an entire monolayer of interconnected cells that migrate together as a sheet. The simplistic understanding of the three best-studied Rho GTPases is that their activation is spatially restricted. Rho is important for the generation of contractility through phosphorylation and activation of MLC at the trailing edge of the cell, and hence necessary for tail retraction. Rac and Cdc42 are required for the formation of lamellipodia and finger-like filopodia, respectively, at the front (leading edge) (Jaffe and Hall 2005). Rac and Cdc42, through their effectors WAVE and WASP, respectively, activate the Arp2/3 complex, resulting in polymerization of actin. Rac initiates lamellipodia that extend the membrane forward as a sheath, whereas Cdc42 generates filopodia that sense the environment of the migrating cell. Cdc42 is the primary regulator of not just apico-basal polarity (as discussed in chapter 1.1.5), but also front-rear polarity, which is a key feature of migration of individual cells or cell clusters (Nelson 2003).

Actin structures are highly complex and dynamic. Actin polymerization necessary for migration has several requirements. For example, the actin polymerization machinery should be linked to external stimulatory or inhibitory cues. Since actin structures are dynamic, and the shape of the polymerizing actin meshwork has to be regulated, the actin cytoskeleton is constantly remodelled due to the availability of the monomers. An example of constant remodelling is polymerization of actin at the membrane, and depolymerization behind the membrane. A large number of regulators and modulators are involved in this process, some of which are signalling molecules or messengers, and others are structural proteins that give shape/organization to the actin cytoskeleton. PIP2 and PIP3 are membrane-derived signalling molecules that regulate activation of Rho GTPases at the leading edge. Rho proteins in turn regulate PIP2 and PIP3 levels through their respective lipid kinases. The two main kinase

effectors of Rho and Rac that are especially important for migration during wound healing are ROK and PAK (Zegers 2008, Narumiya et al. 2009, Ridley 2011). In addition, Rho GTPases have a key role in regulating the shape and kinetics of the polymerizing actin meshwork. This happens through several proteins regulating actin dynamics, such as polymerizations motors (formins), affinity modulators (profilin), severing proteins (cofilin), and various capping proteins (such as CapZ).

A great advance in our understanding of the role of Rho proteins came from the application of a new technology called Fluorescence Resonance Energy Transfer (FRET). Since this technology detects activity of the Rho GTPase in situ in live cells during migration, it changed the classical view about the very strict spatial distribution of Rho proteins. Through the use of FRET-based probes (Gardiner et al. 2002), and live biosensor molecules (Hodgson et al. 2010) a more complex picture is emerging, where both Rac.GTP and Rho.GTP are present at the front and rear of migrating cells (Machacek et al. 2009, Hall 2012) (Figure 12). It can be hypothesized that the role of active Rho at the front of the cell is different from its function at the rear. At the rear, Rho activation promotes actomyosin contractility, which leads to tail retraction (Figure 12) (Machacek et al. 2009, Hall 2012). At the front, active Rho, through its effector mDia, may stimulate activation of Rac (Machacek et al. 2009, Hall 2012). This interesting possibility stems from the observation that mDia can positively affect Rac activity. Rho is also necessary for formation of focal contacts at the base of the leading edge. Further, in addition to their role in actin polymerization, Rho GTPases might be important in regulating other processes. For example, it was also found that the presence of Rac.GTP in migrating cells, as seen during dorsal closure in *Drosophila*, also activates a MAP kinase pathway (Harden et al. 1999).

Hence, Rho GTPases are activated in a spatio-temporally controlled manner. Activation of Rho GTPases is in turn regulated by their interactions with different GEFs, GAPs, and GDIs, which suggests that these regulators must also be spatio-temporally controlled. This is an exciting area for future research. I have discussed this further in chapter 6.2. Another level of complexity is that the small GTPases have been shown to regulate each other. In their early studies Alan Hall and co-workers (Nobes and Hall 1995) showed that active mutants of Cdc42, Rac and Rho activate each other in a hierarchical manner in fibroblasts; Cdc42 activates Rac, which can activate Rho. The exact mechanism of such a hierarchical activation, however, remained unknown. With our work (chapter 5), we have tried to answer some of these questions.

1.3. GEF-H1

I have so far discussed several GEFs that have been shown to activate Rho GTPases. I will now focus on GEF-H1, a RhoA/Rac GEF (Ren et al. 1998, Benais-Pont et al. 2003), which has been the main focus of my research. GEF-H1 (and its murine homolog *lfc*) is an exchange factor that binds to and is regulated by microtubules and tight junctions (Figure 13) (Ren et al. 1998, Benais-Pont et al. 2003; reviewed in Birkenfeld et al. 2008). My research has centered primarily on mechanisms mediating activation of GEF-H1 by various stimuli.

1.3.1. Structure

Similar to most DH domain-containing GEFs, GEF-H1 has a typical DH-PH domain structure (Figure 13). GEF-H1 is one of only three known GEFs that localizes to microtubules. It has a C1 domain, which is a zinc-finger motif-containing region. The Cys53 residue within this domain is crucial for microtubule binding. Mutations in this conserved

Figure 13. Structure of GEF-H1

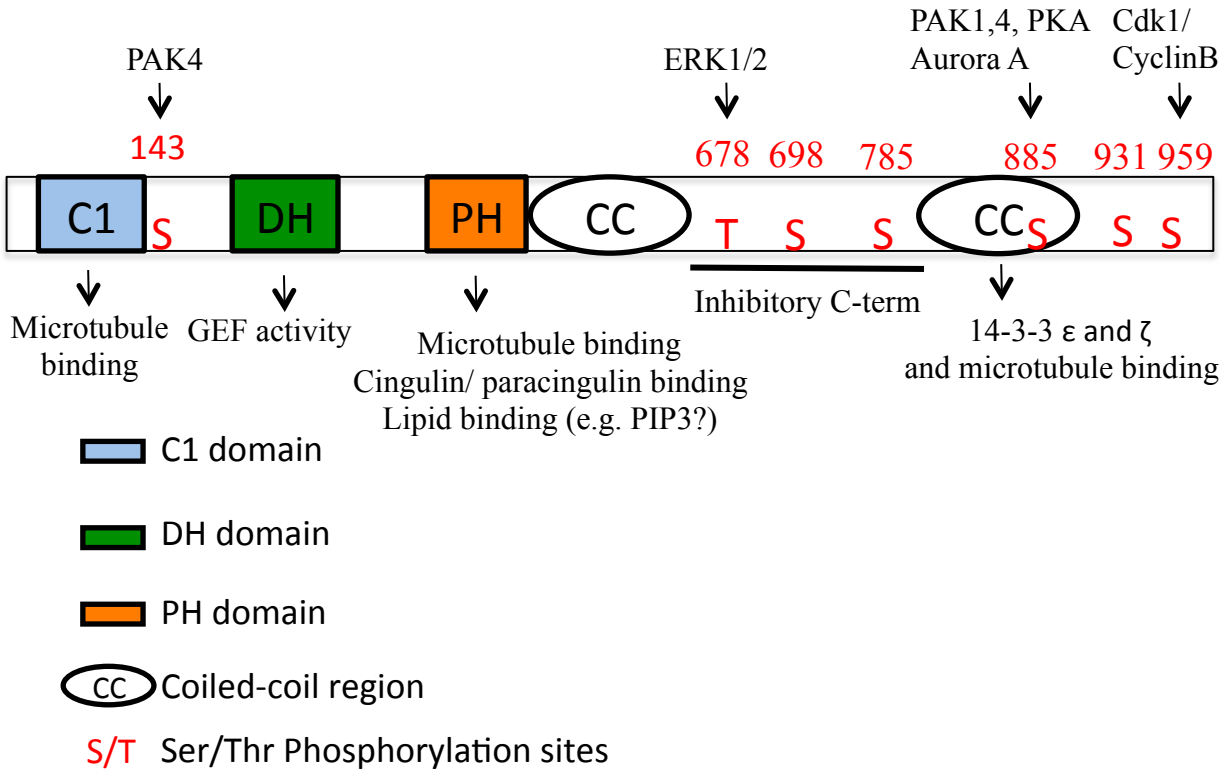


Figure 13. Structure of GEF-H1. GEF-H1 is a DH domain-containing GEF that binds microtubules and tight junctions. It is a Rac and RhoA GEF. GEF-H1 has several phosphorylation sites that are targeted by several different kinases. Our studies, as well as others, have pointed to a role of the MAPK ERK in the phosphorylation of T678, leading to RhoA activation. We have also shown the S885 site is important in regulating GEF-H1 activation towards Rac and RhoA (chapter 5). Abbreviations: PAK 1 and PAK 4, p21-activated kinase 1 and 4; PKA, Protein Kinase A; Cdk1, Cyclin-dependent kinase 1; PIP3, phosphatidylinositol-3,4,5-triphosphate.

residue, or deletion of the entire C1 domain abolishes the microtubule-binding capacity of GEF-H1, and increases its enzymatic activity *in vivo* (reviewed in Birkenfeld et al. 2008). The PH domain of GEF-H1 is also important for microtubule binding. This domain enables targeting of GEF-H1 to the plasma membrane and various subcellular compartments, such as TJs. The inhibitory C-terminus (containing the coiled-coil motif) region in GEF-H1 is also recognized for microtubule binding ability. The DH domain of GEF-H1 has been shown to physically interact with both Rac and RhoA (Ren et al. 1998, Gao et al. 2001). Mutations in highly conserved residues within the DH domain prevent its catalytic exchange activity towards both RhoA and Rac, and mitigate Rho protein activation-induced events, such as actin reorganization (Birkenfeld et al. 2008).

GEF-H1 has several phosphorylation sites that are targets of various kinases. Thr678 is phosphorylated by the MAPK ERK (Fujishiro et al. 2008, Kakiashvili et al. 2011). Ser142 and Ser885 are phosphorylated by the Cdc42 effector PAK4 in NIH-3T3 fibroblasts (Callow et al. 2005), or, according to another group, by the Rac/Cdc42 effector kinase PAK1 (Table 1) (Zenke et al. 2004). Other kinases shown to phosphorylate GEF-H1 include Aurora A kinase, Cyclin-dependent kinase 1 (Cdk1), Polarity-regulating kinase Partitioning-defective 1b (Par1b), and Protein Kinase A (PKA) (which phosphorylates the murine homolog *lfc*) (Birkenfeld et al. 2007, Meiri et al. 2009, Yamahashi et al. 2011).

1.3.2. Regulation of GEF-H1 by microtubules and tight junctions

Under basal conditions, most of GEF-H1 is bound to microtubules and is considered to be inactive. Stimuli that disrupt this binding activate the exchange factor and allow for downstream activation of the Rho GTPase (Tonami et al. 2011 and reviewed in Birkenfeld et

Table 1. Overview of regulation of GEF-H1 by phosphorylation and binding to Microtubules (MTs)

Year	Authors	Cell type	Kinase	MT-binding	Active for Rho?	Active for Rac?
2002	Krendel et al.	COS-1, HeLa	-	Used Noco	↑ Rho activity	Not active
2004	Zenke et al.	COS-1, HeLa	PAK1	Ⓟ at S885 ↑ MT-binding	↓ Rho activity	-
2005	Callow et al.	NIH-3T3	PAK4	Ⓟ at S885 ↓ MT-binding Ⓟ at S142	↓ stress fibers ↑ stress fibers	↑ lamellipodia -
2007	Birkenfeld et al.	HeLa	Aurora A kinase Cdk1/ CyclinB	Ⓟ at S885 Ⓟ at S959	↑ Rho activity ↓ Rho activity (maybe)	-
2008	Chang et al.	HeLa	-	Used Noco	↑ Rho activity, ↑ stress fibers, and ↑pMLC	-
2011	Tonami et al.	NIH-3T3	-	Inhibited CAPN6, released GEF-H1 from MTs	-	↑ Rac activity, GEF-H1 association with Rac in lamellipodia

Table 1. Regulation of GEF-H1 by phosphorylation and binding to Microtubules (MTs). GEF-H1 has been shown to be phosphorylated at its S885 residue by PAK1 and PAK4, which was shown both, to increases its binding to MTs (PAK1), or decrease its binding to MTs (PAK4) thereby releasing it into the cytosol to be activated. A couple of studies have used the MT depolymerizing drug Nocodazole (Noco) and observed increased Rho activation. Inhibition of the cysteine protease, Calpain-6 (CAPN6), also led to release of GEF-H1 from MTs. Cell types: COS-1- fibroblasts from monkey kidney; HeLa- immortalized (cervical) epithelial cells; NIH-3T3- fibroblasts.

al. 2008). Callow et al. (2005) noted that S142 phosphorylation of GEF-H1 promoted formation of stress fibers (Rho activation), whereas the S885 phosphorylation released GEF-H1 from the microtubules, prevented Rho activation, and promoted formation of lamellipodia (Rac activation) (Table 1). However, seemingly contradictory are results from the study in HeLa and COS-1 cells, published a year earlier (Zenke et al. 2004). This work shows that the Rac/Cdc42 effector PAK1 phosphorylates GEF-H1 on its S885 residue, enabling its binding to 14-3-3 proteins and microtubules, which inactivates it. The study by Zenke et al. does not show direct interaction of PAK1 with GEF-H1, as the Callow et al. study does with PAK4 and GEF-H1. Hence, PAK1 could act on GEF-H1 through other effectors or kinases. It is also possible that the difference in the findings of these two studies can be explained by cell-type and/or kinase-specific (in)activation, and the presence of other phosphorylation sites that were not explored. Both studies, however, hypothesize that this intricate regulation of GEF-H1 via binding to microtubules and phosphorylation could fine-tune its activity selectively towards RhoA or Rac in a spatio-temporal manner.

GEF-H1 has also been shown to be regulated by tight junctions. It has been shown to localize to epithelial TJs, where it binds the adaptor proteins cingulin and paracingulin that inhibit its activity (Aijaz et al. 2005, Chang et al. 2008). Downregulation of cingulin increases Rho activity, which appears to be due to release of GEF-H1 from junctions. Upon release from TJs, GEF-H1 activates the RhoA-ROK pathway, and induces myosin phosphorylation and cell proliferation (Aijaz et al. 2005, Chang et al. 2008).

1.3.3. Function

An overall picture is emerging that GEF-H1 is a major molecular link in relaying messages between the microtubules and the Rho GTPase-regulated actin cytoskeleton. GEF-H1 has been implicated in a wide range of functions in various cell types, including the regulation of cell growth, cytokinesis, migration, epithelial and endothelial barrier, dendritic spine morphology, antigen presentation, vesicular trafficking, and signalling by mechanical stimuli (Birkenfeld et al. 2007, Kang et al. 2009, Nalbant et al. 2009, Nie et al. 2009, Guilluy et al. 2011, Pathak and Dermardirossian 2013; reviewed in Birkenfeld et al. 2008). I will highlight a few of these functions.

1.3.3.1. Role in proliferation

In keeping with the fact that release from microtubules increases GEF-H1 activity, it was shown by Westwick et al that overexpression of C-terminally truncated *Lfc*, that does not bind to microtubules, induced expression of cyclin D1 in NIH-3T3 cells, and promoted cell cycle progression (Westwick et al. 1998). This truncated GEF-H1 mutant also induced oncogenic cellular transformation through hyperactivation of RhoA (Sahai and Marshall 2002). It was also shown that mutated p53 in many cancers elevates GEF-H1 expression, resulting in increased RhoA activation, which leads to accelerated proliferation in tumour cells (Mizuarai et al. 2006). A study by Birkenfeld and others also linked GEF-H1 to cytokinesis. The authors of this study show that depleting GEF-H1 in HeLa cells, leads to impaired cytokinesis, as evidenced by a significant increase in multinucleated cells (Birkenfeld et al. 2007).

1.3.3.2. Role in migration

Nalbant et al. have shown that GEF-H1 depleted HeLa cells have reduced directed migration, as well as reduced migration speed (Nalbant et al. 2009). These findings suggest that cross-talk mediated by GEF-H1 between microtubules and actin plays a critical role in directed cell migration. Using live cell biosensors to follow Rho activation, they also found that GEF-H1 was required at the leading edge in lamellipodia for localized Rho activation. Depletion of GEF-H1 in these cells significantly increased turnover of focal adhesions.

1.3.3.3. Role in permeability regulation

Regulation of barrier permeability is of prime importance for the normal functioning of both epithelial and endothelial cells. Paracellular permeability is determined, primarily, by TJs. Dysregulation of both epithelial and endothelial TJs has been implicated in a number of diseases. In endothelial cells increased permeability causes vascular leakage that has been shown to play a pathogenic role in many severe acute and chronic diseases, such as sepsis and atherosclerosis (Dudek and Garcia 2001, Wettschureck and Offermanns 2002, Birkenfeld et al. 2008). In addition, increased lung endothelial permeability plays a role in bronchial asthma and acute lung injury. Elevated epithelial permeability also plays a role in a number of pathological conditions, including inflammatory bowel disease and acute lung injury (Turner 2006).

RhoA-mediated cytoskeleton remodelling and microtubule dynamics have both been shown to play a role in the regulation of endothelial and epithelial permeability (Verin et al. 2001, Rolfe et al. 2005). One of the Rho exchange factors that has been implicated in junctions regulation is GEF-H1, which, as mentioned earlier, has been shown to localize to epithelial tight junctions (Benais-Pont et al. 2003, and reviewed in Terry et al. 2010). Several studies

have shown that GEF-H1 has a role in regulating epithelial and endothelial paracellular permeability. However, there is no agreement on its exact role. Here, I will highlight a few studies in which the authors downregulated GEF-H1 to assess its importance in junction regulation.

Benais-Pont and others show that in Madin-Darby canine kidney cells, downregulation of GEF-H1 does not affect TER. However, paracellular permeability of 4 kD FITC-dextran was reduced approximately by half in cells lacking GEF-H1 (Benais-Pont et al. 2003). In contrast, Birukova and others show that GEF-H1 depletion slightly reduces basal TER, and mitigates agonist-induced decrease in TER in endothelial cells (Birukova et al. 2006). In a different study where the authors assessed turnover of epithelial apical junction complexes (AJCs), Samarin and others showed that GEF-H1 downregulation did not prevent turnover of basal epithelial AJCs, but mitigated the Ca^{++}-removal-induced disassembly of AJCs (Samarin et al. 2007).

In contrast to the studies listed above, Terry et al. recently showed that Rho activation at the epithelial junctions was dependent not on GEF-H1, but on p114RhoGEF (Terry et al. 2011). Nevertheless, even in this study, GEF-H1 downregulation significantly reduced stress fiber formation, suggesting that GEF-H1 is a key regulator of the actin cytoskeleton, but not at the junctions. These results could imply that perhaps there are two different pools of Rho (at the junctions and in the cell center) that can be separately activated. Different GEFs might act on Rho in different cell compartments. In addition, GEFs might play a role as scaffolds, assembling various localized signalling complexes. Taken together, it is possible that, in some cells, GEF-H1 is an important mediator of central actin remodelling, but does not play a role in junctional actin regulation. Therefore, further studies are required to understand not

only the spatio-temporal activation of GEF-H1, but also its role in Rho activation at junctions.

1.3.3.4. Activation of GEF-H1 by depolarization of the plasma membrane

Work from our lab has demonstrated that depolarization activates RhoA. In search of the underlying mechanisms, we identified GEF-H1 as the exchange factor mediating the effects of depolarization on RhoA. Multiple studies (e.g., Pappenheimer 1993, Baldassa et al. 2003, Obara et al. 2007) have demonstrated that in neurons and PC12 pheochromocytoma cells, depolarization activates ERK, which in turn is known to phosphorylate and activate GEF-H1. This prompted us to ask whether depolarization could activate ERK in epithelial cells as well and, if so, whether ERK could mediate depolarization-induced Rho activation.

As discussed earlier, GEFs constitute a large family (Rossman et al. 2005). Upon undertaking of this research, Rho GEF(s) regulated by depolarization had not yet been identified. GEF-H1, a microtubule and junction-bound RhoGEF was a good candidate since 1) it is expressed in tubular cells, 2) it has been implicated in paracellular permeability control in tubular cells (Benais-Pont et al. 2003, Kakiashvili et al. 2009), and 3) it was shown to be regulated by ERK (Fujishiro et al. 2008, Kakiashvili et al. 2009). Interestingly, GEF-H1 was also recently found in complex with the AMPA receptor, which mediates fast synaptic potential in neurons (Kang et al. 2009). In light of these data, we examined the possible role of GEF-H1 in mediating depolarization-induced effects. We found that GEF-H1 was indeed activated by depolarization towards RhoA, and this activation required ERK (Waheed et al. 2010).

CHAPTER 2 Objectives and Hypotheses

Rationale:

Rho family GTPases are major regulators of the cytoskeleton, through which they impact and regulate vital cellular processes such as transepithelial transport. Also, activation of RhoA can lead to changes in gene expression, for example, through the RhoA effector SRF, and its co-activator, MRTF (see chapter 1.2.5).

In chapter 1.2.4.2, I have discussed three stimuli that have been shown in our lab to activate RhoA signalling, including hyperosmotic stress, and the inflammatory cytokine, TNF. However, despite their importance, the mechanisms underlying the activation/inactivation of Rho proteins through these stimuli are not fully understood. Specific members of the very large Rho GEF family mediate activation of Rho proteins in response to various extracellular stimuli (Rossman et al. 2005). We have previously shown that GEF-H1 is activated by TNF, and mediates RhoA activation in tubular cells. However, the question of how GEF-H1/RhoA are activated in a pathway- and context-specific manner remains to be established. Further, it was also not clear how specific GEF-H1 is to TNF signalling: can other stimuli activate RhoA through GEF-H1? Finally, although GEF-H1 has been implicated in many biological processes, the list of functions of GEF-H1 remained incomplete.

The overall objective of my research project has been to gain insights into the complex mechanism(s) through which Rho family small GTPases, Rac and RhoA, are regulated in tubular cells. Specifically, to identify the exchange factor that mediates small GTPase activation by various stimuli, and to explore the underlying mechanisms and the functional consequences.

Hypotheses:

My first overarching hypothesis is that GEF-H1 is a critical element linking stress signals to transcription factors that control cytoskeletal protein expression. Specifically, I hypothesize that hyperosmotic stress activates Rho GTPase through GEF-H1, which then mediates the nuclear translocation and activation of the transcription factor MRTF.

My second overarching hypothesis is that GEF-H1 is a dual exchange factor for Rac and RhoA, and its specificity is regulated by phosphorylation. Specifically, I hypothesize that the inflammatory cytokine TNF activates both Rac and RhoA in tubular cells through GEF-H1. The RhoA and Rac-specific activities of GEF-H1 are controlled by specific phosphorylation sites. Moreover, the two Rho proteins are activated in a hierarchical manner, where Rac is required for RhoA activation.

The following specific aims were formulated to test these general hypotheses:

Specific Aim 1. To define the role of the GEF-H1/Rho/ROK pathway in hyperosmotic stress-induced translocation of MRTF into the nucleus.

To accomplish this aim, I asked the following questions:

1) Does hyperosmotic stress activate RhoA in tubular cells through GEF-H1?
2) Does GEF-H1 regulate translocation of MRTF to the nucleus via RhoA and Rho kinase?

Specific Aim 2. To define the mechanism through which GEF-H1 mediates TNF-induced sequential activation of Rac and RhoA in tubular epithelial cells.

To achieve this aim, I asked the following questions:

1) Does TNF activate Rac in tubular cells, and if yes, is this activation through GEF-H1?
2) What is the mechanism of TNF-induced GEF-H1-mediated Rac activation, i.e., is it similar to RhoA activation?
3) Is specificity of GEF-H1 towards Rac and RhoA regulated by phosphorylation?

Materials and Methods

Reagents

TNF was from Sigma-Aldrich (St. Louis, MO), Bovine serum albumin (BSA) was from BioShop Canada (Burlington, Canada), Protein A/G agarose was from Santa Cruz Biotechnology (Santa Cruz, CA), and the glutathione-Sepharose beads were from GE Healthcare Life- sciences (Piscataway, NJ).

Primary Antibodies

Antibodies against the following proteins were used: RhoA, GEF-H1 (55B6), Rac1/2/3, p38, phospho-p38 (Thr-180/Tyr-182), GFP, EGFR, phospho-EGFR (Y845), and IκBα from Cell Signaling Technology (Danvers, MA); TACE and phospho–S885-GEF-H1 (ab94348) from Abcam (Cambridge, MA); phospho-p44/42 mitogen-activated protein kinase (MAPK; ERK1/2; Thr-202/Tyr-204), p44/42 MAPK (ERK1/2), myc, lamin A/C (N-18) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and p65 NFκB from Santa Cruz Biotechnology (Santa Cruz, CA); HA tag from Covance (Princeton, NJ); tubulin from Sigma-Aldrich; HA-tag antibody coupled to agarose beads from Santa Cruz Biotechnology; Histone (H1 + core proteins) from Millipore (Billerica, MA). The polyclonal MRTF antibody has been previously described (Sasazuki et al. 2002).

Secondary Antibodies

Peroxidase- and Cy3-labeled secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). 4′,6-diamidino-2-phenylindole (DAPI) nucleic acid stain was from Invitrogen (Burlington, Canada).

Inhibitors

PD98059, SB203580, Y-27632, AG1478, TAPI-1, calyculin A, and PP2 were from EMD Biosciences (Mississauga, Canada). Complete Mini protease inhibitor tablet and PhosSTOP phosphatase inhibitor tablet were from Roche Diagnostics (Laval, QC, Canada), and the BaculoGold protease inhibitor cocktail was from BD Pharmingen (Mississauga, ON, Canada).

Cell Culture and Treatment

LLC-PK_1 (or LLC-PK1) cell line, a kidney proximal tubule epithelial cell line, (clones 101 and 4; Kakiashvili et al. 2009) (American Type Culture Collection, Manassas, VA) was used. We have used these cells in our previous work, where we have shown the activation of the Rho/ROK/pMLC signalling pathway induced by different stimuli (Szászi et al. 2000a,b, Di Ciano et al. 2002, Di Ciano-Oliveira et al. 2003, Kakiashvili et al. 2009, Waheed et al. 2010, Kakiashvili et al. 2011). LLC-PK_1 cells also form polarized monolayers, displaying organelles (e.g., microvilli, primary cilium) and protein markers (e.g., occludin, E-cadherin) of polarized epithelial cells. LLC-PK_1 cells were maintained in low-glucose DMEM supplemented with 10% fetal bovine serum and 1% antibiotic suspension (penicillin and streptomycin; Invitrogen) in an atmosphere containing 5% CO_2. Confluent cells were serum depleted for at least 3 h in DMEM before the experiments.

The following media were used in the experiments exploring the effects of depolarization and hyperosmolarity: Na^+ medium: 130 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4); K^+ medium: 130 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and 20 mM

HEPES (pH 7.4). Hyperosmolarity was induced by adding 150 mM NaCl to the isotonic Na+ medium, or in a subset of experiments, instead of NaCl, equiosmotic sucrose was added with identical results. Cells were incubated in the isotonic Na^+ medium for 15 minutes before treatment with the high K^+ (depolarization) medium or the hyperosmotic medium (high NaCl).

Vectors and Transfection

The vectors used were kind gifts from the following investigators: cDNAs encoding for the GST-RBD portion of Rhotekin, the GST-PBD portion of Pak, GST-RhoA(G17A), and GST-Rac(G15A) (Garcia-Mata et al. 2006) from K. Burridge (University of North Carolina, Chapel Hill, NC); active pCMV-FLAG p38-α (Flag-p38) from R. J. Davis (University of Massachusetts, Worcester, MA; (Raingeaud et al. 1995)); myc-RacT17N, a dominant-negative Rac (DN-Rac), and pCMV5-HA3-WT-GEF-H1 (HA-GEF-H1) were from G. Bokoch (Scripps Institute, La Jolla, CA; (Zhang et al. 1995, Zenke et al. 2004)); and HA-ERK2 and GFP-tagged wild-type and T678A mutant GEF-H1 were from M. Kohno (Nagasaki University, Nagasaki, Japan; (Fujishiro et al. 2008)). The GEF-H1 point mutants GEF-H1S885A and GEF-H1T678D were generated by Jenny Zhang from the WT-GFP-GEF-H1 construct using PCR-based mutagenesis with the following primers: for GEF-H1S885A, 5′-GTGGATCCTCGGCGGCGCGCCCTCCCCGCAGGCGATG-3′ and 5′-CATCGCCTGCGGGGAGGGCGCGCCGCCGAGGATCCAC-3′; and for GEF-H1T678D, 5′-AACTGCTCTTGGATCCCCGAGAGCCAGCC-3′ and 5′-GGCTGGCTCTCGGGGATCCAAGAGCAGTTC-3′. The GEF-H1S885A/T678D double mutant was prepared by introducing the S-to-A mutation into GEF-H1T678D. The AA-MLC

vector (Di Ciano-Oliveira et al. 2005) was from H. Hosoya (Dept. Biological Sciences, Hiroshima University).

LLC-PK_1 cells were transiently transfected with DNA vectors using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN; or Promega, Madison, WI) or jetPRIME transfection reagent (Polyplus-Transfection, New York, NY), according to the manufacturers' instructions. Unless otherwise indicated, experiments were performed 48 h after transfection. The following DNA concentrations were used for transfecting 10-cm dishes using FuGENE 6: 2 μg of HA-ERK with or without 5 μg of DN-Rac or active p38; or 6 μg of WT or mutant GFP-GEF-H1; or 5 μg of HA-GEF-H1. For expression of DNA vectors along with silencing of endogenous GEF-H1, two different protocols were used, which allowed efficient silencing and protein expression without significant cell toxicity. In a sequential transfection protocol, LLC-PK_1 cells were transfected with the porcine-specific GEF-H1 siRNA #2 using Lipofectamine RNAiMAX, as described. Twenty-four hours later, cells were transfected with human GFP-GEF-H1S885A or GFP-GEF-H1T678A along with HA-ERK2 using FuGENE 6, as described, and experiments were performed 1 d later. In some experiments a cotransfection protocol was followed. The jetPRIME transfection reagent was used to cotransfect siRNA and DNA vectors, as well as the shRNA vector and other DNA-based vectors. The following shRNA and DNA concentrations were used for 6-cm dishes: 3 μg of empty pRNAT vector or GEF-H1–specific shRNA along with 1.0 μg of HA-ERK-2.

Gene silencing using siRNA

The following porcine sequences were targeted by the siRNAs.

GEF-H1: #1, AACAAGAGCATCACAGCCAAG (Kakiashvili et al. 2009, Waheed et al. 2010), and #2, AACGGGCATCTCTTCACCACC (porcine specific).

Rac 1/2: #1, AAATACCTGGAGTGCTCGGCG, and #2, UCGAGAAACUGAAGGAGAA.

TACE: #1, GGUGAAAGGCACUACAAUAUU, and #2, UAUUGUAGUGCCUUUCACCUU.

RhoA, AAAGCAGGTAGAGTTGGCTTT (Waheed et al. 2013).

The siRNAs were obtained from Applied Biosystems/Ambion (Austin, TX) or ThermoScientific/Dharmacon (Lafayette, CO). All experiments using Rac, GEF-H1, and TACE silencing in LLC-PK1 cells were performed with two different siRNAs, and the data obtained were pooled. TACE in NRK cells was silenced using a predesigned and validated ON-TARGETplus siRNA from ThermoScientific/Dharmacon. Cells were transfected with 100 nM siRNA oligonucleotide using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. Control cells were transfected with 100 nM Silencer siRNA negative control # 2 (NR siRNA; Applied Biosystems/Ambion).

For the porcine-specific shRNA plasmid, two complementary oligonucleotides were generated: the porcine GEF-H1–specific sequence GCTATACCAACGGGCATCT and the hairpin loop sequence TTCAAGAGA and restriction site overhangs to allow directional cloning into the BamH1 and Xho1 sites of the pRNAT-CMV3.2 expression vector (GenScript, Piscataway, NJ). The two strands were annealed and ligated to the cut and

purified vector. Positive clones were purified and sequenced. Empty pRNAT vector was used for control.

Preparation of GST-fusion proteins

Described in detail in chapter 3. Preparation of GST-RBD (amino acids 7–89 of Rhotekin) and GST-PDB (p21-binding domain of PAK1), GST-RhoA(G17A), and GST-Rac(G15A) has been described (Waheed et al. 2012). Protein bound to the beads was estimated by SDS–PAGE, followed by Coomassie blue staining, and the beads were kept at 4°C for immediate use or stored frozen in the presence of glycerol.

Rac and RhoA activity assays

Active (GTP-bound) Rac and RhoA were captured using GST-PBD or GST-RBD, respectively, as described (Sebe et al. 2008, Kakiashvili et al. 2009). Briefly, confluent LLC-PK1 cells grown on 6- or 10-cm dishes were treated as indicated in the respective figure legends. Cells were lysed with ice-cold buffer. The Rac assay buffer contained 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM NaF, and protease inhibitors. The RhoA assay buffer contained 100 mM NaCl, 50 mM Tris base (pH 7.6), 20 mM NaF, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 1 mM Na3VO4, and protease inhibitors. After centrifugation, aliquots for determination of total Rac or RhoA were removed. The remaining supernatants were incubated at 4°C for 45 min with 20–25 μg of GST-RBD or GST-PBD beads, followed by extensive washing. Total cell lysates and the RBD- or PDB-captured proteins were analyzed by Western blotting using Rac1/2/3 or RhoA antibody. Results were quantified by densitometry.

Affinity precipitation of activated GEFs

See chapter 3. In short, active GEFs were affinity precipitated from cell lysates using the Rac(G15A) or RhoA(G17A) mutant, which cannot bind nucleotide and therefore has high affinity for activated GEFs (Garcia-Mata et al. 2006), as in our earlier work (Kakiashvili et al. 2009, Waheed et al. 2010). This method is described in a video protocol (Waheed et al. 2012). GEF-H1 in the precipitates was detected by Western blotting. Precipitation with glutathione–Sepharose beads containing no fusion proteins resulted in no GEF-H1 precipitation (Figure 15a). GEF-H1 in total cell lysates was also detected for each sample (total GEF-H1). Precipitated (active) and total GEF-H1 were quantified by densitometry.

Immunoprecipitation

To assess phosphorylation of HA-ERK2, we transfected LLC-PK1 cells in 10-cm dishes with HA-ERK2 with or without cotransfections, as described for the specific experiments. Forty-eight hours later the cells were serum depleted and treated as indicated in the corresponding figure legends. Cells were lysed with the lysis buffer used for preparing Western blotting samples, and HA-tagged ERK was precipitated using 20 μl of HA antibody coupled to agarose beads for 1 h at 4°C. The precipitates were washed and eluted in sample buffer, then subjected to Western blot analysis using anti–phospho-ERK and anti-HA. Control experiments in which lysates from nontransfected cells were used verified the specificity of the immunoprecipitation. For exploring the phosphorylation of HA-GEF-H1 the lysis buffer was also supplemented with 10 nM calyculin (Kakiashvili et al. 2009). HA-GEF-H1 transfection and precipitation was done as for HA-ERK. Phosphorylation of S885 of the precipitated protein was tested using anti–phospho-S885 GEF-H1.

Preparation of nuclear extracts

Nuclear extracts were prepared from confluent layers of LLC-PK1 cells grown on 6-cm dishes, as described previously (Masszi et al. 2010), using the NE-PER Nuclear Extraction Kit from Pierce Biotechnology (Rockford, IL) according to the manufacturer's recommendation. The nuclear extracts were collected, and their protein concentration was determined. Samples containing 10 μg protein were analyzed by Western blotting. Antibodies against histones or lamin were used as markers of nuclear fraction.

Western blotting

After treatment, cells were lysed on ice with cold lysis buffer containing 100 mM NaCl, 30 mM HEPES (pH 7.5), 20 mM NaF, 1 mM ethylene glycol tetraacetic acid, and 1% Triton X-100, supplemented with 1 mM Na_3VO_4, 1 mM PMSF, and Mini Protease Inhibitor Tablet (Roche Diagnostic). For the detection of phosphoproteins the lysis buffer was also supplemented with PhosSTOP phosphatase inhibitor (Roche Diagnostic). Protein concentration was determined by the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL) with BSA used as standard. SDS–PAGE and Western blotting was performed as in Kakiashvili et al. (2009). Blots were blocked in Tris-buffered saline containing 3% BSA and incubated with the primary antibody overnight. Antibody binding was visualized with the corresponding peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence method (kit from GE Healthcare Lifesciences, Piscataway, NJ). Where indicated, blots were stripped and reprobed to demonstrate equal loading or detect levels of down-regulated proteins. Because the phospho-ERK (pERK) antibody proved difficult to

strip, those blots were first developed using total ERK antibody, followed by reprobing with pERK. Data were quantified using densitometry.

Densitometry

Films with nonsaturated exposures were scanned and densitometry analysis performed using a GS-800 calibrated densitometer and the "band analysis" option of Quantity One software (Bio-Rad, Hercules, CA) as described previously (Waheed et al. 2010). In each assay the amount of the investigated protein species was normalized to the appropriate control (e.g., active RhoA to total RhoA, active GEF-H1 to total GEF-H1, pERK to total ERK protein, etc.). Because the basal levels of many investigated proteins were often either undetectable or just slightly above the background, to achieve accurate and stringent comparison, signals were expressed relative to the response detected in stimulated cells, taken as 100%, as described in the figure legends.

ECIS-based wound-healing assay

Wound healing was quantified using the Electric Cell-substrate Impedance Sensing (ECIS) Ztheta system (Applied Biophysics, Troy, NY), as in Szaszi et al. (2012). LLC-PK1 cells were plated in wells of an 8W1E array (2 × 105 cells/well in 400 μl of culture medium). In experiments in which GEF-H1 was silenced, LLC-PK1 cells were transfected with the NR or GEF-H1–specific siRNA using Lipofectamine RNAiMAX, as described, and 24 h later the cells were trypsinized, counted, and plated on the electrode. In all experiments, after plating on the electrode, the cells were grown for 20–24 h to reach confluence, as indicated by the drop in C measured at 32 kHz. Next a wound was generated in the monolayer by applying a 3-mA, 32-kHz voltage pulse for 20 s, and recovery of the layer was monitored by measuring

C at 32 kHz. To quantify and compare wound healing, the half–recovery time was calculated for each curve, as in Szaszi et al. (2012). Briefly, the difference in the C values at the last time point before wounding and the first time point after wounding was calculated and taken as the total wounding (100%). Next the recovery percentage was calculated at each time point from the highest C value (taken as 0% recovery) and plotted against the time from wounding (taken as 0 h). The 50% recovery time for each curve was determined, and expressed as fold from the control, taken as 1.

Immunofluorescence microscopy

Confluent cells grown on coverslips were treated as indicated in the corresponding figure legend and fixed with 4% paraformaldehyde. Immunofluorescence staining was carried out as in Kakiashvili et al. (2009). Briefly, after permeabilization with 0.1% Triton X-100, the coverslips were blocked with 3% BSA in phosphate-buffered saline. Next cells were incubated with anti-p65 NFκB (1:100). Bound antibody was detected using the corresponding fluorescent secondary antibody (1:1000), which also contained DAPI to counterstain nuclei. All samples were viewed using an Olympus IX81 microscope (Melville, NY) coupled to an Evolution QEi Monochrome camera (Media Cybernetics, Bethesda, MD).

Statistical analysis

All shown blots are representatives of at least three similar experiments. Data are presented as mean ± SE of the number of experiments indicated (n). Statistical significance was assessed by one-way analysis of variance with Newman–Keuls posttesting or Student's t test, as appropriate, using Prism (GraphPad Software, La Jolla, CA). For clarity on the figures

only the most important significant differences are indicated: *p < 0.05, **p < 0.01, ***p < 0.001, ns, nonsignificant.

CHAPTER 3 Affinity precipitation of active Rho-GEFs using a GST-tagged mutant Rho protein (GST-RhoA (G17A)) from epithelial cell lysates

This chapter has been modified from the following:

Waheed, F., Speight, P., Dan, Q., Garcia-Mata, R., and Szászi, K. (2012): Affinity precipitation of active Rho-GEFs using a GST-tagged mutant Rho protein (GST-RhoA(G17A)) from epithelial cell lysates. Journal of Visualized Experiments. J Vis Exp. Mar 31;(61). pii: 3932

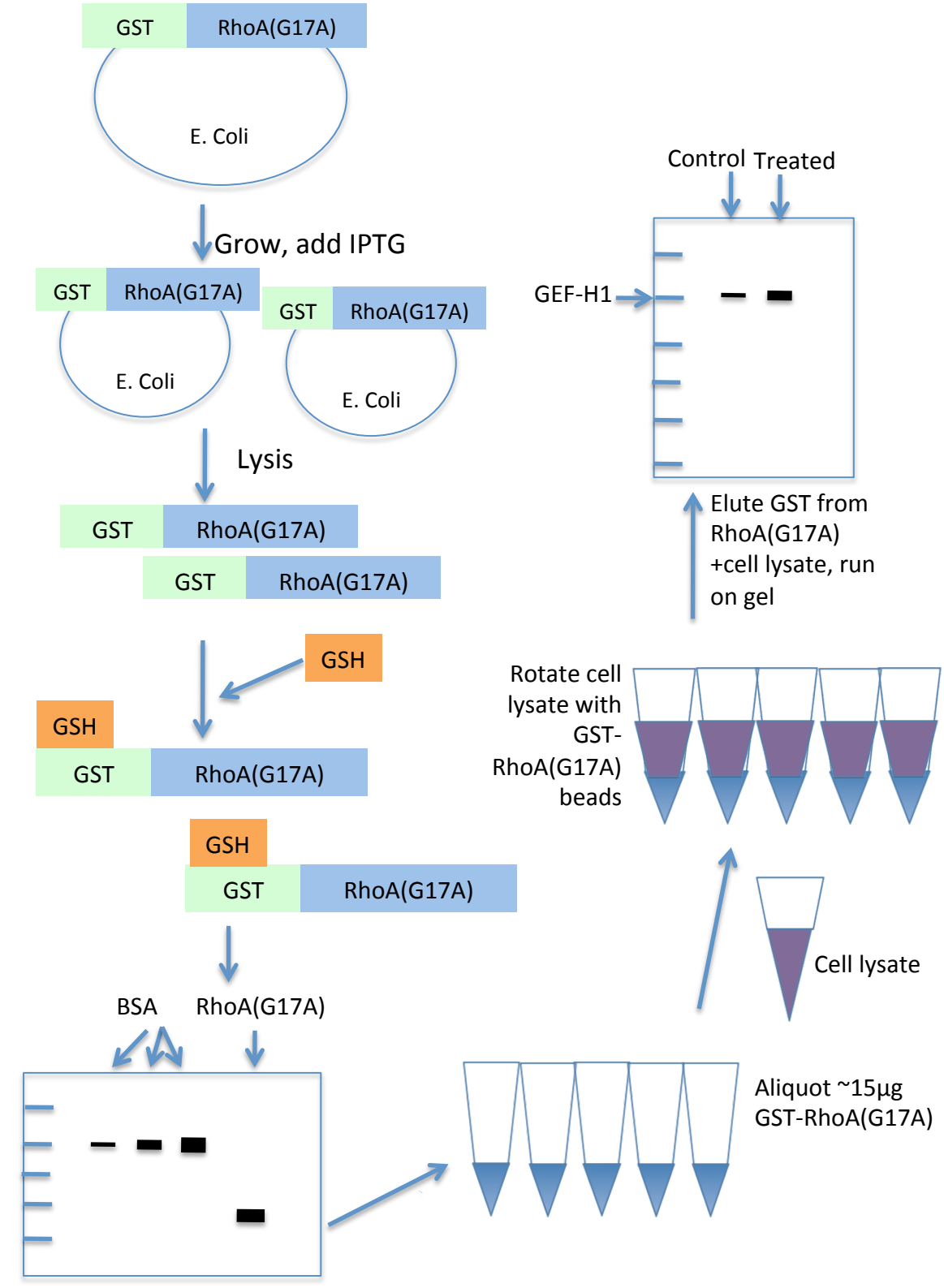
3.1. Summary

In order to follow activation of exchange factors towards Rac and RhoA, we adapted and extensively used an affinity precipitation method. The method presented here describes the assay to follow activation of RhoA-specific GEFs in cultured cells by making use of a mutant RhoA GST fusion protein that has high affinity for activated GEFs. GEFs are precipitated from cell lysates, detected by Western blotting and quantified by densitometry. This assay is not widely used yet, and forms the basis of my work. We adapted and validated it for optimal detection of GEF activation in tubular cells challenged by different stimuli. Since the assay is of interest to many researchers, we also presented a detailed protocol and video demonstration.

3.2. Introduction

Activators of Rho proteins, Rho-GEFs constitute a large family, with overlapping specificities (Rossman et al. 2005). Although a lot of progress has been made in identifying the GEFs activated by specific signals, there are still many questions remaining regarding the pathway-specific regulation of these proteins. The number of Rho-GEFs exceeds 70, and each cell expresses more than one GEF protein. In addition, many of these proteins activate not only Rho, but other members of the family (see chapter 5), contributing further to the complexity of the regulatory networks. Importantly, exploring how GEFs are regulated requires a method to follow the active pool of individual GEFs in cells activated by different stimuli. Here we provide a step-by-step protocol (Figure 14) for a method used to assess and quantify the available active Rho-specific GEFs using an affinity precipitation assay. This

Figure 14 Schematic summary of the active GEF affinity precipitation assay



assay has been adapted in our lab to be successfully used in kidney tubular cell lines (Kakiashvili et al. 2009, Waheed et al. 2010, Kakiashvili et al. 2011).

The assay takes advantage of a "nucleotide free" mutant RhoA, with a high affinity for active GEFs. The mutation (G17A) renders the protein unable to bind GDP or GTP and this state mimics the intermediate state that is bound to the GEF. A GST-tagged version of this mutant protein is expressed and purified from *E. coli*, bound to glutathione sepharose beads and used to precipitate active GEFs from lysates of untreated and stimulated cells (Arthur et al. 2002, Garcia-Mata et al. 2006). As most GEFs are activated via posttranslational modifications or release from inhibitory bindings, their active state is preserved in cell lysates, and they can be detected by this assay (Garcia-Mata and Burridge 2007). Captured proteins can be probed for known GEFs by detection with specific antibodies using Western blotting, or analyzed by Mass Spectrometry to identify unknown GEFs activated by certain stimuli.

3.3. Protocol

1.Transformation of *E. coli* with the pGEX-RhoA(G17A) Construct

1.1. Prepare LB-Agar by dissolving 2.5 g LB and 1.5 g Agar in 100 ml dH_2O. Autoclave and cool to an estimated 50-55°C, which as a rule of thumb, is when the flask can be held comfortably.

1.2. Prepare Ampicillin (Amp) stock by dissolving 50 mg/ ml in dH_2O. Syringe filter and freeze unused aliquots. Add 100 µl of Amp stock (final concentration 50 µg/ml) to the LB-Agar from 1.1. Swirl to mix and pour into 10 cm bacterial dishes (15-20 ml/dish).

Allow it to solidify (15-30 min.) and store unused plates inverted at 4°C for 2-3 weeks.

1.3. To transform *E. Coli*, quickly thaw an aliquot of DH5α competent cells in an ice bath. Add 1 µl of pGEXRhoA(G17A) DNA diluted to 25-50 ng/µl. Flick the tube to mix and incubate on ice for 30 minutes. Heat shock at 42°C for 45 seconds and place back on ice for 2 minutes. Add 900 µl SOC medium and grow for one hour at 37°C with shaking.

1.4. Spread 50-100 µl of the transformed bacteria on an LB-Agar-Amp plate using a bent sterile Pasteur pipette. Incubate the plate right side up in a 37°C incubator for 5 minutes and then invert and grow overnight.

1.5. A single colony will be picked from the plate for preparation of the GST-tagged protein (step 2.1). For future use, wrap and store plates inverted at 4°C for about 3 weeks. In addition, bacterial stocks can be prepared for more prolonged storage by growing individual colonies in 2 ml sterile LB-Amp overnight at 37°C with shaking. Mix an aliquot with sterile 80% glycerol in a 1:1 ratio and freeze at -80°C.

2. Preparation of GST-RhoA(G17A) Beads

1.1. Prepare LB by adding 25 g LB to 1 L dH20 and autoclaving. When cool, add 50 µl Amp from stock to 50 ml LB (50 µg/ml final concentration). Inoculate with a well isolated colony of transformed bacteria and grow overnight at 37°C with agitation. When at full density (OD600 > 1.0) dilute with 450 ml LB-Amp and grow for an additional 30 minutes at 37°C.

1.2. Prepare a 100 mM stock solution of Isopropyl B-D-thiogalactopyranoside (IPTG) by dissolving 0.238 g in 10 ml dH2O. Store in aliquots at -20°C. Induce bacteria to

produce Rho protein by adding 500 µl 100 mM IPTG to 500 ml culture (a final concentration of 100 µM). Reduce temperature to 22-24°C and grow for ~16 h hours.

1.3. Spin culture at 3600 g for 10 minutes at 4°C. If needed, the 500 ml culture can be divided into 50 ml tubes for centrifugation. Freeze pellet(s) for at least 1 hour (or preferably overnight) at -80°C.

1.4. Prepare 200 ml lysis buffer containing 20 mM HEPES (0.95 g)/ pH 7.5; 150 mM NaCl (1.75 g); 5 mM $MgCl_2$ (0.203 g); 1% TX-100 (2 ml). Prepare stock solutions of 1M DTT (1.542 g in 10 ml dH2O) and 100 mM PMSF (0.174 g/10 ml EtOH). To prepare lysis buffer +, supplement 10 ml with 1mM DTT (10 µl of stock) and 1 mM PMSF (100 µl of stock) and one Complete Mini Protease Inhibitor tablet.

1.5. Working on ice, add 10 ml lysis buffer+ to the pellets from step 2.3. Resuspend thoroughly by gentle vortexing and pipetting. Avoid foaming. Sonicate on ice for 1 minute at setting 4 with 50% pulse. Spin the sonicated lysate at 15,000-20,000 g for 15 minutes at 4°C, and remove the clarified sonicate (supernatant) to a sterile capped 15 ml tube.

1.6. Prepare the Glutathione Sepharose by gently mixing the original tube containing a 75% slurry and transfer 335 µl into a 15 ml tube. Use a wide bore tip to pipette beads. Add 10 ml cold PBS, and spin 500 g for 5 minutes at 4°C. Discard the supernatant, add 1 ml lysis buffer+ to the beads and spin as for previous wash. Discard the supernatant and add lysis buffer+ to make a 50% slurry.

1.7. Add 250 µl of equilibrated bead slurry to the supernatant from step 2.5. Rotate at 4°C for 45 minutes.

1.8. Prepare 500 ml HBS containing 20 mM HEPES (2.38 g)/pH 7.5 and 150 mM NaCl (4.38 g) in dH_2O. Prepare stock solutions of 1M $MgCl_2$ (0.952 g in 10 ml dH2O) and 1M DTT (1.542 g in 10 ml dH_2O). To prepare HBS+, supplement 100 ml just before use with 5 mM $MgCl_2$ (50 µl from stock) and 1 mM DTT (100 µl from stock).

1.9. Spin the beads from step 2.7 at 500 g for 5 minutes at 4°C. Discard the supernatant and wash beads 2x with 10 ml lysis buffer+, and 2x with 10 ml HBS+. After the final wash, make a 50% slurry by resuspending the beads in HBS+ supplemented with BD BaculoGold protease inhibitor (20 µl of 50x BD BaculoGold/ml).

1.10. Dilute 10 µl of the final beads preparation with 2x Laemmli sample buffer containing β-mercaptoethanol. Make Bovine Serum Albumin (BSA) standards. Use a 2 mg/ml stock (0.02 g of BSA in 10 ml dH_2O). Mix 10 µl of stock with 10 µl Laemmli (20 µg final); 5 µl of stock with 5 µl of dH_2O and 10 µl Laemmli (10 µg final); and 2.5 µl stock with 7.5 µl dH_2O and 10 µl Laemmli (5 µg final). Boil all samples (5 min). Spin the bead sample and run supernatant with BSA standards and molecular weight markers on a 10% SDS-polyacrylamide gel.

1.11. Prepare the Comassie Blue stain (0.1 g in 10 ml Acetic Acid, 40 ml Methanol and 50 ml dH_2O) and the destain solution (500 ml dH_2O, 400 ml methanol and 100 ml acetic acid). Store at room temperature. Stain the gel for 20-30 minutes, remove the dye (it can be reused multiple times) and rinse with destain solution twice. Continue to destain with gentle shaking for several hours until bands are clearly visible.

1.12. Estimate the concentration of GST-RhoA(G17A) coupled to the beads using the BSA standards as a reference (Figure 15). Aliquot an equal volume of beads

Figure 15. Demonstration of purified protein from the RhoA(G17A) bead preparation protocol

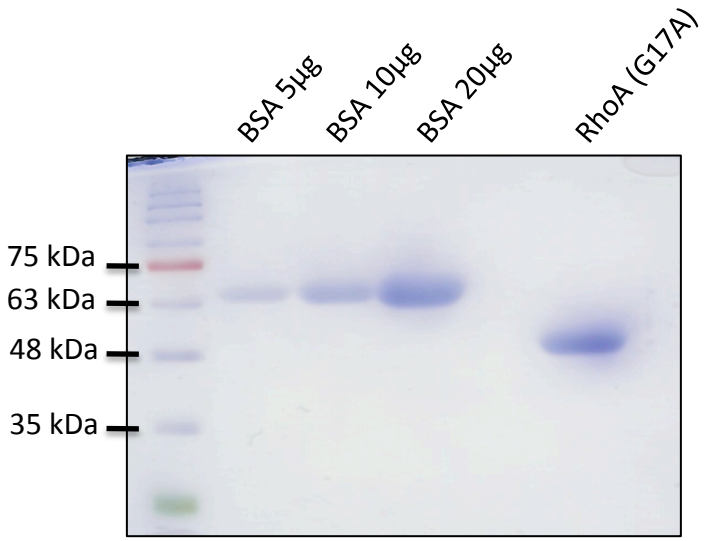


Figure 15. *Demonstration of purified protein from the RhoA(G17A) bead preparation protocol*. A sample of the RhoA (G17A) bead preparation (10 µL), along with BSA (used as standard- 10 µL each) were run on a gel and stained with Coomassie dye and subsequently destained. The result will resemble the sample gel shown above. This shows a bead preparation that has roughly yielded about 15 µg protein (compared to the BSA standards) in 10 µL of slurry. The molecular weight of the protein is the combined weight of RhoA+GST (~50 kDa).

containing ~10-15 µg protein into 1.5 ml micro centrifuge tubes. Store beads at 4°C to use within a day. Freeze at -80°C in HBS+/glycerol in a 3:1 ratio to use within a few days.

3. GEF Pulldown Assay with Nucleotide Free RhoA(G17A) Beads

1.1. Grow cells in 10 cm dishes to confluence. Serum deprive for at least 3 hours and treat as required.

1.2. Prepare lysis buffer+ as in step 2.4. Prepare enough lysis buffer for 700 µl/dish plus some extra amount to allow for pipetting errors. Add the protease inhibitors just before use.

1.3. Working on ice, remove culture medium from the dishes and wash with ice-cold HBS. Remove all the HBS and add 700 µl lysis buffer+ to each dish. Swirl plates to cover all areas, scrape and collect lysates into numbered 1.5 ml tubes. Spin at 15,000 g for 1 min at 4°C. The supernatant will be used for the assay.

1.4. If your cell number is equivalent in all dishes being tested, you can omit doing a protein assay, and move to step 3.5. Otherwise measure the protein concentration of each supernatant using Bio-Rad quick protein assay and equalize the supernatant for volume and concentration. The amount of total protein depends on the cell types used (typically 1-1.5 mg protein for LLC-PK1 cells).

1.5. Remove 30 µl of each supernatant and mix with 30 µl 2x reducing Laemmli sample buffer, boil and set aside for step 3.7. Add remaining supernatants to aliquots of the GST-RhoA(G17A) beads from step 2.12. Rotate for 45 minutes at 4°C.

1.6. Spin beads at 6800 g for 10 seconds at 4°C. Discard the supernatant and wash the beads 3x with lysis buffer, spinning in the same way between washes. Completely

remove the final wash using a 1 cc syringe fitted with a 30 G needle and add 20 µl 2x reducing Laemmli sample buffer. Boil for 5 min. Spin to pellet beads and either run the supernatant immediately (preferable) or store it at -80°C for later analysis.

1.7. Run 20 µl total cell lysates and all of the precipitated protein samples on the appropriate percentage SDS-polyacrylamide gel for the size of GEF you are studying. Detect your GEF of choice by Western blotting using a specific antibody.

3.4. Representative Results

Part 1 and 2 of the protocol describes preparation of GST-RhoA(G17A) coupled to GSH-sepharose beads and its testing by SDS-PAGE (see outline of protocol on Figure 14). A typical Coomassie stained gel is shown on Figure 15. The sample with the eluted protein should contain a single band at approximately 50 kDa (Figure 15, lane 6). The concentration of the protein can be estimated using the BSA reference samples. In the example on Figure 15, the concentration of RhoA(G17A) is estimated to be 15 µg/10 µl. Thus, aliquots of 10 µl/tube were prepared.

Part 3 of the protocol describes the affinity precipitation assay. A successful GEF assay detecting activation of the exchange factor GEF-H1 is shown on Figure 16A, and B. The RhoA(G17A) protein captured some GEF-H1 from the control (untreated) cell lysates, suggesting that GEF-H1 has basal activity. The amount precipitated however increases in cells treated with the inflammatory cytokine TNF, consistent with the notion that TNF activates GEF-H1 (Kakiashvili et al. 2009, Kakiashvili et al. 2011). As a negative control when cell lysates are incubated with glutathione beads alone, there is no precipitation of GEF (Figure 16A). Importantly, the total cell lysates show similar amounts of GEF-H1 in the

Figure 16. RhoA(G17A) beads can specifically capture active GEF-H1

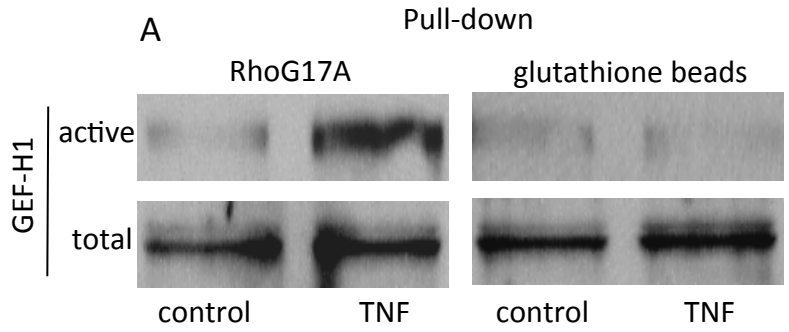


Figure 16A. LLC-PK1 cells were treated with 10 ng/ml TNF for 5 min, lysed and subjected to precipitation with either GST-RhoG17A (left) or empty glutathione beads (right). GEF-H1 in the precipitates (active) and the lysates (total) was detected on Western blots.

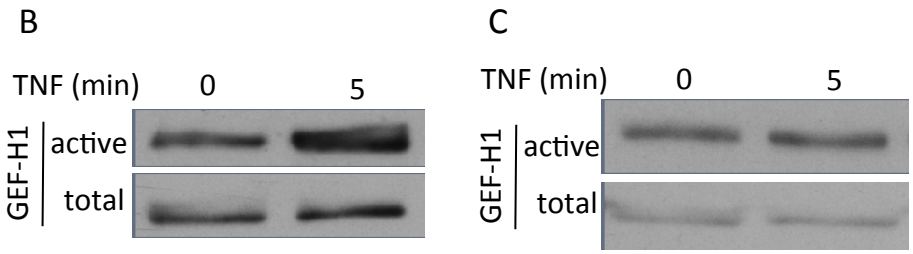


Figure 16B. *RhoA(G17A) beads can specifically capture active GEF-H1.* TNF-induced activation of a specific GEF, GEF-H1 (detected by Western Blotting using specific antibody against it) towards RhoA. Activation is seen since there is more GEF-H1 precipitating with the beads with TNF when compared to the control (0 min). C. An example of an unsuccessful experiment shows no difference between untreated and TNF treated samples, and is likely due to the fact that the TNF may not have caused an activation in these cells. See 'Troubleshooting' for tips.

control and the treated sample, suggesting that the treatment did not alter GEF-H1 levels and the input used in the assay is equal.

3.5. Discussion

The method presented here is the only available non-radioactive activation assay for GEFs that can follow the active pool of GEFs in cells. The assay is similar to the precipitation assays used for following activation of small GTPases as well as GEFs against Rac and Cdc42. Those assays use different GST-tagged proteins and have slight differences from the one described here, however the basic steps are the same. Thus, this protocol can easily be adapted for other small GTPase and GEF activation assays.

The presented GEF assay was recently modified for application for nuclear fractions (Dubash et al. 2011, Guilluy et al. 2011). With further modifications, testing of GEF activation in other subcellular compartments might also be possible.

We use the presented method to study activation of GEFs in epithelial cell lines (Kakiashvili et al. 2009, Waheed et al. 2010, Kakiashvili et al. 2011). With some optimization, this assay should be adequate to detect GEFs from any cell line. When adapting to a specific cell type, finding the optimal cell number, lysis buffer volume, and detection method for the GEF to be tested (a good antibody for Western blotting is important) is essential. For initial setup of the assay it is advisable to use a stimulus that is known to activate the GEF of interest. When using an unknown stimulus, always use a positive control to verify that the assay is working.

This assay can be used to detect activation of known GEFs by Western blotting. However, it is also adequate to identify unknown GEFs. For this, captured GEFs from control and

stimulated samples should be analyzed on a Coomassie-stained gel. Bands that appear only in stimulated samples might contain activated GEFs and can be sent for identification by Mass Spectrometry (e.g., Kakiashvili et al. 2009).

Critical steps in the protocol:

Colonies of transformed bacteria should be picked from fresh, properly prepared plates to ensure adequate selection by Amp, good outgrowth and yield. Transformation conditions for competent cells obtained from other sources may vary and should be consulted.

All steps of the protein preparation protocol (from step 2.3) and the assay (from step 3.2) should be performed at 4°C with cooled solutions and centrifuges.

Bacterial lysis (step 2.5) should be thorough and complete in order to obtain a homogeneous suspension. When lysing the bacteria, vortex and pipette the lysate alternately, while maintaining it at 4°C and ensure sonication is done on ice to prevent denaturing the protein. If using a different model of sonicator, conditions may need to be adjusted. Incubation of sonicate with the beads should always be done at 4^{o}C on a rotator to ensure sufficient binding, and care should be taken to keep the timing consistent.

GST-Rho mutants are somewhat unstable when expressed in bacteria, so it is best to use prepared beads right away or within a few days.

The precipitation assay (Part 3) is time and temperature sensitive, as active GEFs can be easily lost from the cell lysate, so steps should be performed as quickly as possible.

Trouble-shooting tips:

No or very low amount of mutant Rho protein in the final bead preparation: this may be caused by inefficient induction, insufficient lysis of the bacteria, or a loss of the protein during the preparation process or storage. To help troubleshoot some of these possibilities, samples of bacteria can be analyzed before and after induction. If there is poor induction of the protein repeat the process using a colony from a freshly streaked plate or from re-transformed competent cells. Different IPTG concentrations and induction times should also be tested. If the lysis is insufficient (i.e., the protein remains in the pellet instead of the supernatant) varying salt and detergent concentrations in the lysis buffer can be tried. Alternate sonication times and settings should be considered, and samples before and after sonication can be checked by microscopy to determine efficiency of lysis.

No precipitated GEF, even though the GST-protein is present on the beads: this may be due to technical issues during the precipitation assay, or due to a real absence of activation of the studied GEF using the stimulus applied. Always use a known stimulus as a positive control to verify that your assay works. Use the prepared beads within a few days. If the precipitation assay captures undetectable amounts of the GEF studied in all conditions, verify that your GEF is present and is well detectable in the supernatant after centrifugation that is to be used for the assay (step 3.3). Make sure all buffers and protease inhibitors are fresh, and perform all steps on ice as fast as possible. Increase the amount of input protein (e.g., by using lysates from 2 plates/sample).

If the precipitation assay shows basal precipitation of your GEF, but no difference is seen between the control and stimulated samples (Figure 16C), start troubleshooting by verifying

that the applied stimulus worked using other known effects (e.g., by detecting activation of other signalling pathways). Consider changing the treatment conditions and/or concentrations. Rely on data from the literature reporting how your stimulus activates Rho or other signalling to predict likely times and concentrations. When optimizing treatment time, use both short and long time points, as GEF activation might be best detectable at a time point prior to well detectable Rho activation. Finally, the same stimulus could result in a variable degree of activation due to a change in cell responsiveness caused by passage number, cell confluency, etc.

Table 2. List of Reagents and equipment used in the RhoA (G17A) bead prep and precipitation assay protocol

Name of reagent	**Company**	**Catalogue number**	**Comments (optional)**
LB	BioShop	LBL407.1	Keep sterile after autoclaving
Glycerol	BioShop	GLY002.1	
Ampicillin	BioShop	AMP201.25	Store stock sol. at -20°C
Agar	BioShop	AGR001.500	
IPTG	Calbiochem	420322	Store stock sol. at -20°C
Glutathione Sepharose beads	GE Healthcare	17-0756-01	
PBS 10x	SIGMA	D1408	
Hepes	SIGMA	H4034	
NaCl	BioShop	SOD001.10	
MgCl2	SIGMA	M-9272	Add just before use
TX-100	SIGMA-ALDRICH	X100	
DTT	OmniPur EMD	3860	Add just before use
PMSF	SIGMA	P-7626	Add just before use
Protease Inhibitor 50x	BD BaculoGold	51-21426Z	Add just before use
Complete Mini, EDTA-free 10x	Roche	11 836 170 001	Add just before use
Laemmli Sample Buffer 2x	Bio-Rad	161-0737	

β-mercaptoethanol	SIGMA	M7154	Add just before use
Coomassie Brilliant Blue	Bio-Rad	R-250	
Acetic Acid	CALEDON	1000-1	
Methanol	CALEDON	6701-7	
Bio-Rad Protein Assay	Bio-Rad	500-0114	
BLUelf prestained protein ladder	FroggaBio	PM008-0500	

Name of equipment	**Company**	**Catalogue number**	**Comments (optional)**
RC-5B centrifuge	Sorvall	SS-34 Rotor	Use at 4°C
Centrifuge	Sorvall-Thermo Scientific	ST-16R	Use at 4°C
Micro centrifuge	Eppendorf	5417R	Use at 4°C
Bacterial shaker	INFORS HT Ecotron	AG CH-1403 Bottmingen	Use at 37°C or at 22°C
Sonicator	Branson	Sonifier-450	Use at RT
Rotator	Glas-Col	099A CR4012	Use at 4°C

CHAPTER 4 The GEF-H1/RhoA/ROK pathway mediates hyperosmolarity-induced nuclear translocation of MRTF

This chapter has been modified from the following:

Ly, D.L.*, **Waheed, F.***, Lodyga, M.*, Speight, P., Masszi, A., Nakano, H., Hersom, M., Pedersen, S.F., Szászi, K., and Kapus, A. (2013) Hyperosmotic stress regulates the distribution and stability of Myocardin-Related Transcription Factor, a key modulator of the cytoskeleton. Am J Physiol Cell Physiol. Jan 15; 304(2):C115-27. *Co-first authors

4.1. Summary

Our lab has shown that hyperosmotic stress regulates the distribution and stability of the myocardin-related transcription factor (MRTF), a key modulator of the cytoskeleton. MRTF is a known co-activator of serum response factor (SRF) (chapter 1.2.4), and we wanted to investigate the mechanism of hyperosmotic stress-induced MRTF regulation. We found that the RhoA/ROK pathway mediates hyperosmolarity-induced MRTF translocation to the nucleus. Moreover, GEF-H1 is activated towards RhoA by hyperosmotic stress, and is required for the nuclear translocation of MRTF.

4.2. Introduction

Osmotically challenged cells mobilize a set of adaptive responses, which include activation of volume-correcting transport systems (Hoffmann et al. 2009), expression of osmoprotective genes (Burg et al. 2007), and remodelling of the cytoskeleton (Di Ciano-Oliveira et al. 2006). Hyperosmotic stress-induced cytoskeletal restructuring manifests in enhanced peripheral actin polymerization (Hallows et al. 1996, Pedersen et al. 1999, Rizoli et al. 2000, Di Ciano et al. 2002), formation of a polygonal actin-myosin lattice (Malek et al. 2007), and enhanced cell contractility (Pedersen and Hoffmann 2002, Di Ciano-Oliveira et al. 2003, Di Ciano-Oliveira et al. 2005). These responses are thought to reinforce the cell, enabling it to withstand shrinkage-provoked mechanical trauma. Regarding the underlying mechanisms, we and others have shown that hyperosmolarity activates several Rho family GTPases, which in turn are central mediators of the ensuing cytoskeletal effects (Lewis et al. 2002, Di Ciano-Oliveira et al. 2003, Uhlik et al. 2003, Rasmussen et al. 2008, Zhou et al. 2011). For

example, RhoA/Rho kinase-mediated cofilin phosphorylation is a key contributor to shrinkage-induced increase in F-actin (Thirone et al. 2009).

While crucial for structural reinforcement, hyperosmotic activation of Rho family GTPases and the consequent cytoskeleton remodelling itself may fulfill other roles too. Namely, the Rho family and the cytoskeleton have emerged as important regulators of gene expression (Olson and Nordheim 2010). One key link between the state of the actin skeleton and transcriptional control is MRTF, the two isoforms of which, MRTF-A (also known a MAL or MKL1) and MRTF-B (MKL2), are ubiquitously expressed. MRTF is an actin-regulated transcriptional co-activator, which, when stimulated, forms a complex with SRF thereby driving the expression of a variety of cytoskeletal genes (Posern and Treisman 2006, Parmacek 2007). Under resting conditions, MRTF is bound to monomeric (G) actin and resides in the cytosol. According to the current view, upon enhanced actin polymerization, G-actin may be "stolen away" from MRTF, which consequently unmasks its nuclear localization sequence thereby promoting its nuclear accumulation (Miralles et al. 2003, Vartiainen et al. 2007, Mouilleron et al. 2008). This scenario raises the intriguing possibility that MRTF might be an osmosensitive molecule whose nuclear transport is affected by cell shrinkage.

We also investigated the hitherto largely unexplored upstream mechanism for RhoA activation under hyperosmotic stress. Our results suggest that osmolarity regulates MRTF at multiple levels in tubular cells. Modest hyperosmolarity induces nuclear MRTF accumulation through the GEF-H1/RhoA/ROK pathway.

4.3. Results

4.3.1. The RhoA/Rho kinase pathway mediates hyperosmolarity-induced nuclear translocation of MRTF

We wished to determine the signalling pathway responsible for MRTF translocation. Hypertonicity has been shown to activate various Rho family GTPases (RhoA, Rac, Cdc42) with varying magnitude and kinetics in different cell types (Lewis et al. 2002, Di Ciano-Oliveira et al. 2003, Uhlik et al. 2003, Rasmussen et al. 2008, Thirone et al. 2009). Both RhoA and Rac have been shown to impact MRTF nuclear translocation (Miralles et al. 2003, Busche et al. 2008, Sebe et al. 2008) and both are osmotically sensitive. In LLC-PK1 cells hypertonicity elicits only a small and transient (<1 min) Rac activation followed by suppression (Thirone et al. 2009). RhoA, however, shows a sizable and sustained hyperosmolarity-induced activation (Figure 17A). To test this possibility, we used nonrelated and RhoA-specific siRNA, the latter of which essentially eliminated RhoA expression in the monolayer as verified by Western blotting (Figure 17C). RhoA siRNA did not change MRTF distribution under isotonic conditions, but it completely abolished the osmotically induced nuclear uptake of MRTF (Figure 17B). Since the Rho effector ROK was implicated in osmotically induced actin polymerization (Thirone et al. 2009) and MRTF can undergo Rho-dependent phosphorylation (Miralles et al. 2003), we tested the potential involvement of ROK in MRTF redistribution. The potent ROK inhibitor Y-27632 fully prevented the hypertonicity-induced nuclear accumulation of MRTF, as demonstrated both by immunostaining (Figure 17D) and Western blotting of nuclear extracts (Figure 17E).

Figure 17. Hyperosmotic stress induces Rho-mediated MRTF nuclear translocation

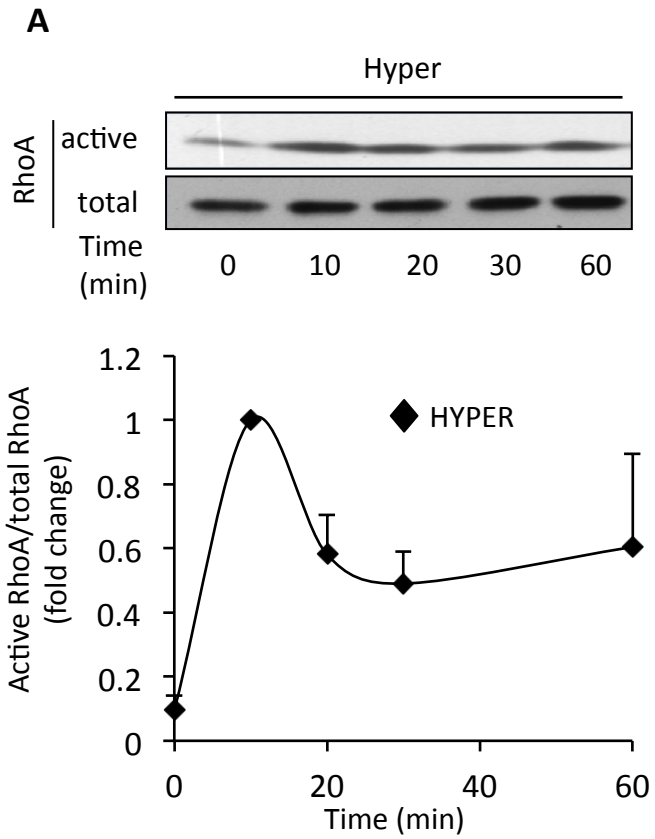


Figure 17. A. LLC-PK1 cells were incubated in hyperosmotic medium for the indicated times. At the end of the treatment the cells were lysed. Active RhoA was measured using the GST-RBD pulldown assay. RhoA in the pulldowns and in the total cell lysates was detected by Western blotting and quantified using densitometry. The graphs show normalized amounts of active RhoA expressed as fold changes from the 10 min hyperosmolarity-treated sample, taken as unity.

Figure 17. Hyperosmotic stress induces Rho/ROK-mediated MRTF nuclear translocation

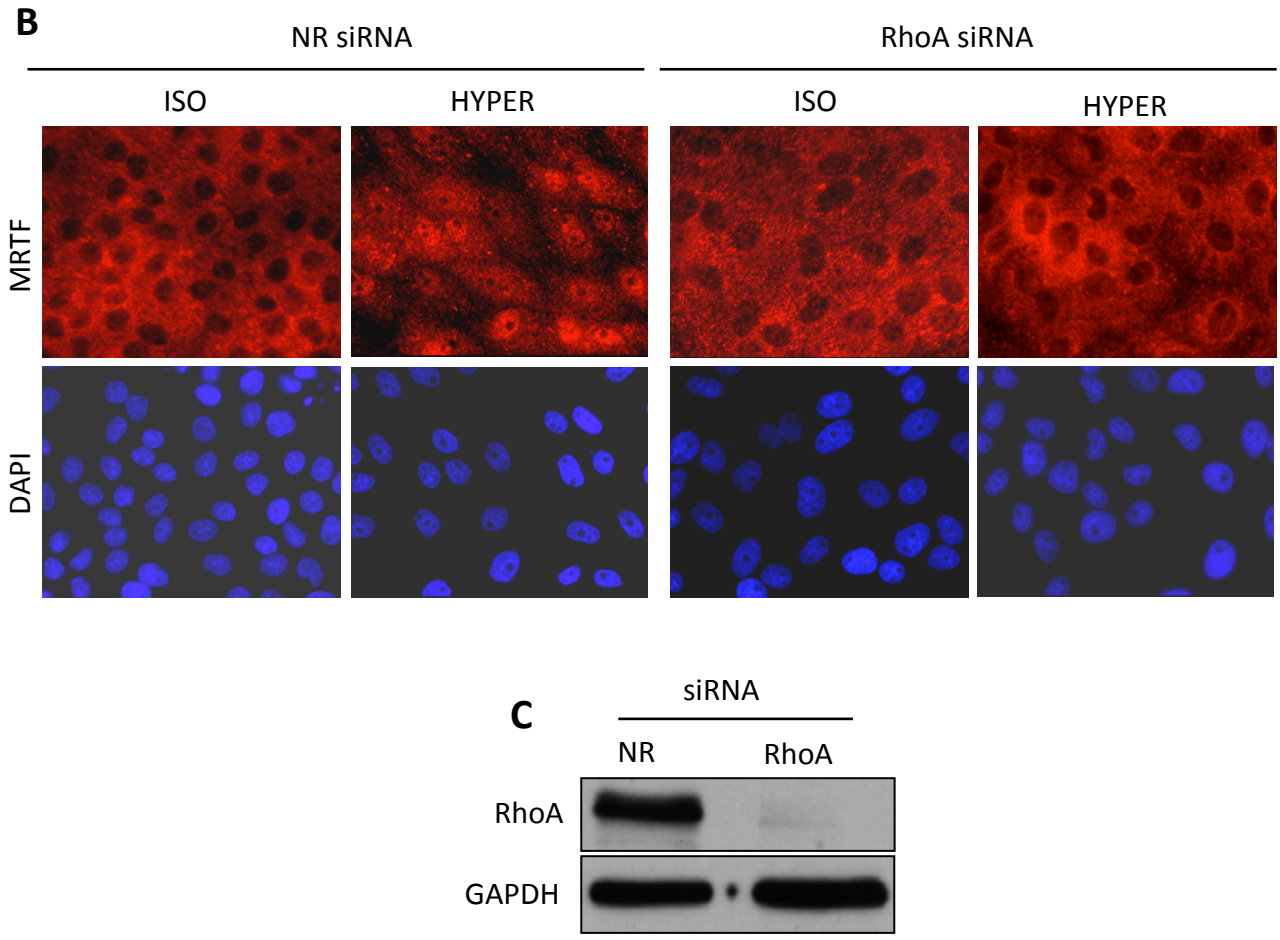


Figure 17. *Hyperosmolarity-induced MRTF translocation is mediated by RhoA and Rho kinase*. *B,C*. LLC-PK cells were transfected with control (non-related, NR) or RhoA-specific siRNA, as indicated. In B, 48 h post-transfection cells were treated with isotonic Na^+-medium or hyperosmotic medium for 20 min, then fixed and stained to visualize MRTF (top images) and the nuclei in the corresponding fields (DAPI, bottom images). Please note, that nuclear MRTF staining is absent in cells transfected with RhoA siRNA. In C 48 h post-transfection cells were lysed and levels of RhoA and GAPDH (loading control) were assessed in cell lysates using Western blotting to demonstrate effective downregulation of RhoA by the siRNA.

Figure 17. Hyperosmotic stress induces Rho/ROK-mediated MRTF nuclear translocation

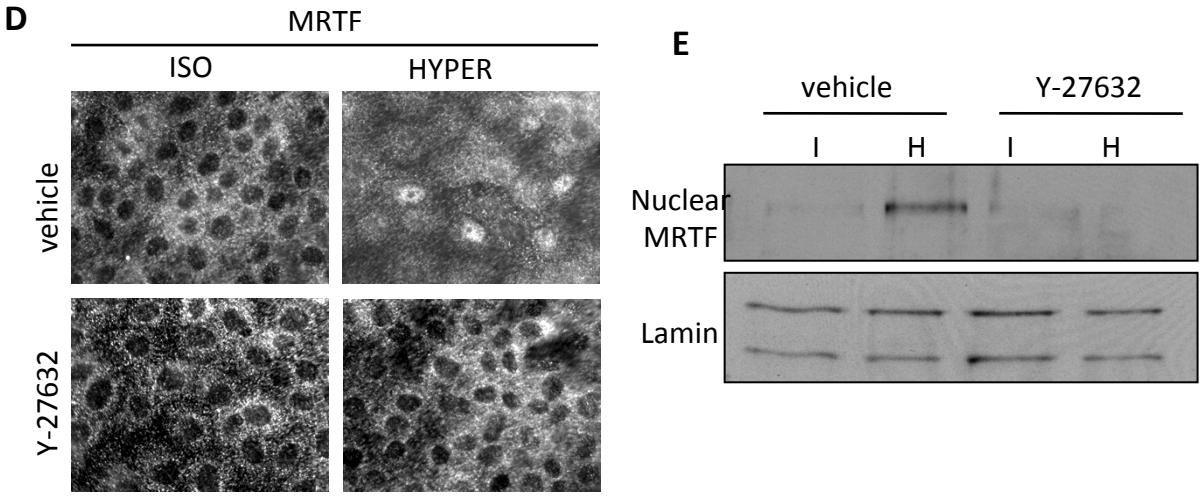


Figure 17. *D*. Cells were pretreated with 20 µM Y27632 for 30 min, followed by the addition of hyperosmotic medium in the presence of Y27632 for 20 min. MRTF was visualized by immunofluorescence (left pictures). The blot on the right shows MRTF and lamin in nuclear fractions prepared from cells after the indicated treatments.

Together, these data imply that the RhoA/ROK pathway is indispensable for the hypertonicity-induced nuclear translocation of MRTF.

4.3.2. GEF-H1 is required for hyperosmolarity-induced RhoA activation

Little is known about the direct upstream mediators of osmotic RhoA activation. Our previous studies have proposed that ezrin-mediated inhibition of Rho-GDI might be involved in this process in Ehrlich-Lettre ascites (ELA) cells (Rasmussen et al. 2008), but no osmotically regulated GEF (i.e., direct RhoA activator) has been so far identified. We considered GEF-H1 as a good candidate, because this key RhoA-activating molecule has been shown to be regulated by mechanical (shear) stress and other physical factors (Birukova et al. 2010, Waheed et al. 2010, Guilluy et al. 2011, and chapter 1.4), and it can be associated with intercellular contacts (Benais-Pont et al. 2003), which have been proposed to transmit volume-dependent signals (Hoffmann et al. 2009). To address the potential role of GEF-H1, first we asked whether it is activated by hyperosmolarity. To this end we performed affinity pull-down assays, using a RhoA(G17A) mutant-GST fusion protein, which mimics nucleotide-free RhoA, and therefore specifically binds to activated GEFs (chapter 3, Garcia-Mata et al. 2006, Kakiashvili et al. 2009, Waheed et al. 2012). GEFs in lysates obtained from iso- and hypertonically treated cells were precipitated with RhoA(G17A)-GST-covered beads, and the precipitates were immunoblotted with an anti-GEF-H1 antibody. Hyperosmolarity induced a rapid increase in the amount of captured GEF-H1, which peaked after 2 min at 2.5-fold over the isotonic level (Figure 18A). To test the functional significance of the observed GEF-H1 activation, we treated the cells with nonrelated or GEF-H1-specific siRNA and performed RhoA activation assays after iso- and hypertonic exposure

Figure 18. *Hyperosmolarity activates RhoA and induces MRTF translocation though GEF-H1*

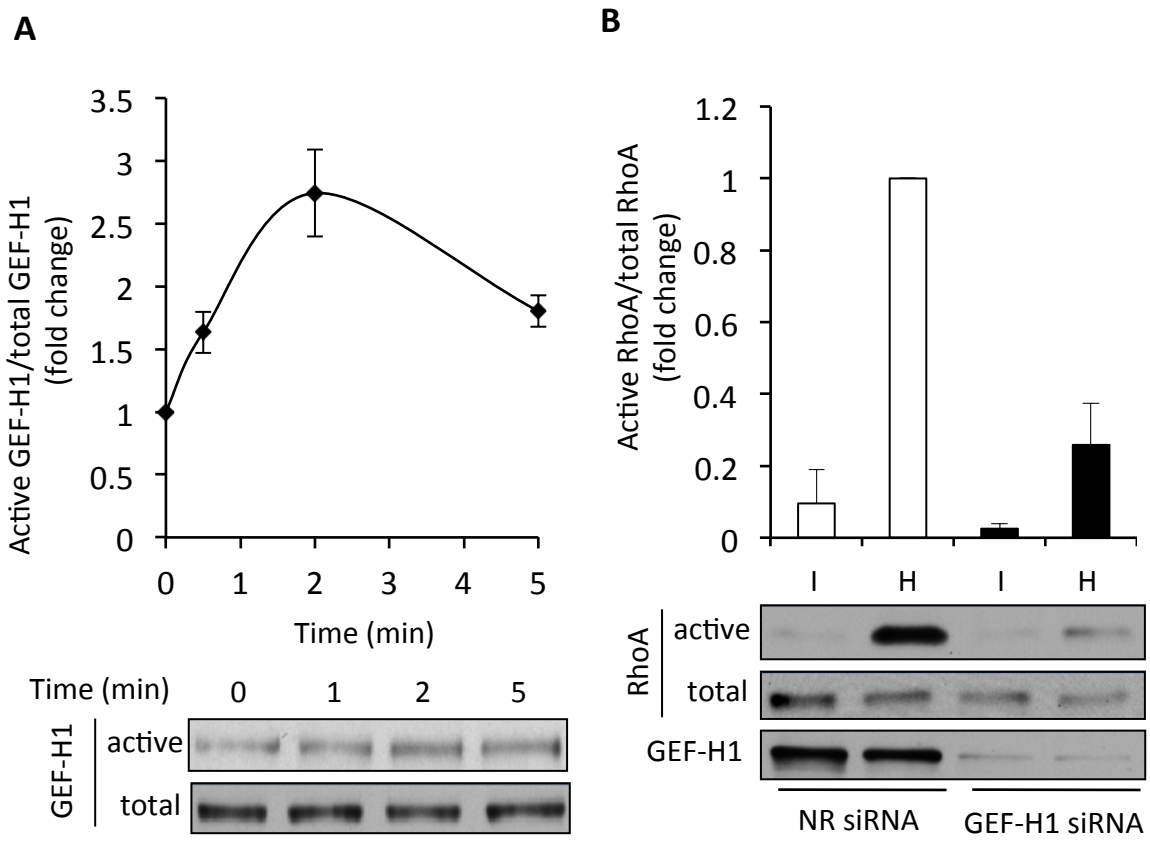


Figure 18. *Hyperosmolarity activates RhoA and induces MRTF translocation though GEF-H1. A*. LLC-PK1 cells were treated with isotonic Na^+ or hyperosmotic medium for the indicated times. At the end of the treatment the cells were lysed, and active GEFs were precipitated using GST-RhoA(G17A). GEF-H1 in the precipitates (active) and input cell lysates (total) was detected by Western blotting. The graph shows densitometric quantification. The amount of precipitated GEF-H1 was normalized to the corresponding total GEF-H1, and the normalized active GEF-H1 was expressed in each experiment as fold of the control taken as unity. The graph shows mean +/- S.E.M from n=3. *B.* LLC-PK1 cells were transfected with non-related (NR) or GEF-H1-specific siRNA, and forty eight hours later exposed to isotonic Na^+ (I) or hyperosmotic medium (H) for 5 min. In B active RhoA was precipitated from the cell lysates using GST-RBD, and RhoA in precipitate (active) and cell lysates (total) was detected using Western blotting. In B GEF-H1 in total cell lysates was also detected to demonstrate effectiveness of the siRNA. In each experiment, the normalized RhoA activity measured in the 5-min hypertonic samples was taken as 1, and all other values are expressed accordingly. The graph above the blot shows the cumulative data (means ± SE) for n=4 separate experiments. While the difference between the NR and GEF-H1 siRNA-treated isotonic samples is not significant ($P > 0.4$), the difference between the hypertonic NR and GEF-H1 siRNA-treated samples is highly significant ($P < 0.001$).

(Figure 18B). GEF-H1 siRNA induced a strong reduction in GEF-H1 expression and concomitantly suppressed basal RhoA activity and mitigated its rise over the isotonic (basal) level. The residual RhoA activation in the presence of GEF-H1 siRNA is likely due to the remaining GEF-H1 expression in a small portion of the cell population and might also reflect the contribution of other RhoA-activating pathways.

4.3.3. GEF-H1 mediates hypertonicity-induced MRTF translocation to the nucleus

Having seen that GEF-H1 is a key component of osmotically-induced RhoA activation, we tested its impact on MRTF translocation. As expected, GEF-H1 knockdown strongly mitigated the hypertonicity-triggered MRTF translocation, as detected both by immunofluorescence microscopy (Figure 18C) and Western blotting of nuclear extracts (Figure 18D).

Taken together, these results imply that the RhoA-ROK pathway is indispensable for the hypertonicity-induced MRTF translocation and that GEF-H1 is activated by hyperosmolarity and significantly contributes to the ensuing RhoA activation and consequent MRTF redistribution.

4.4. Discussion

Cytoskeletal remodelling is an immediate response to osmotic stress, which helps the cell withstand the ensuing mechanical trauma (Kuwayama et al. 1996, Di Ciano-Oliveira et al. 2006). Our current studies show that hyperosmolarity-induced, cytoskeleton-regulating signalling pathways and the consequent cytoskeletal changes themselves are not only

Figure 18. Hyperosmolarity activates RhoA and induces MRTF translocation though GEF-H1

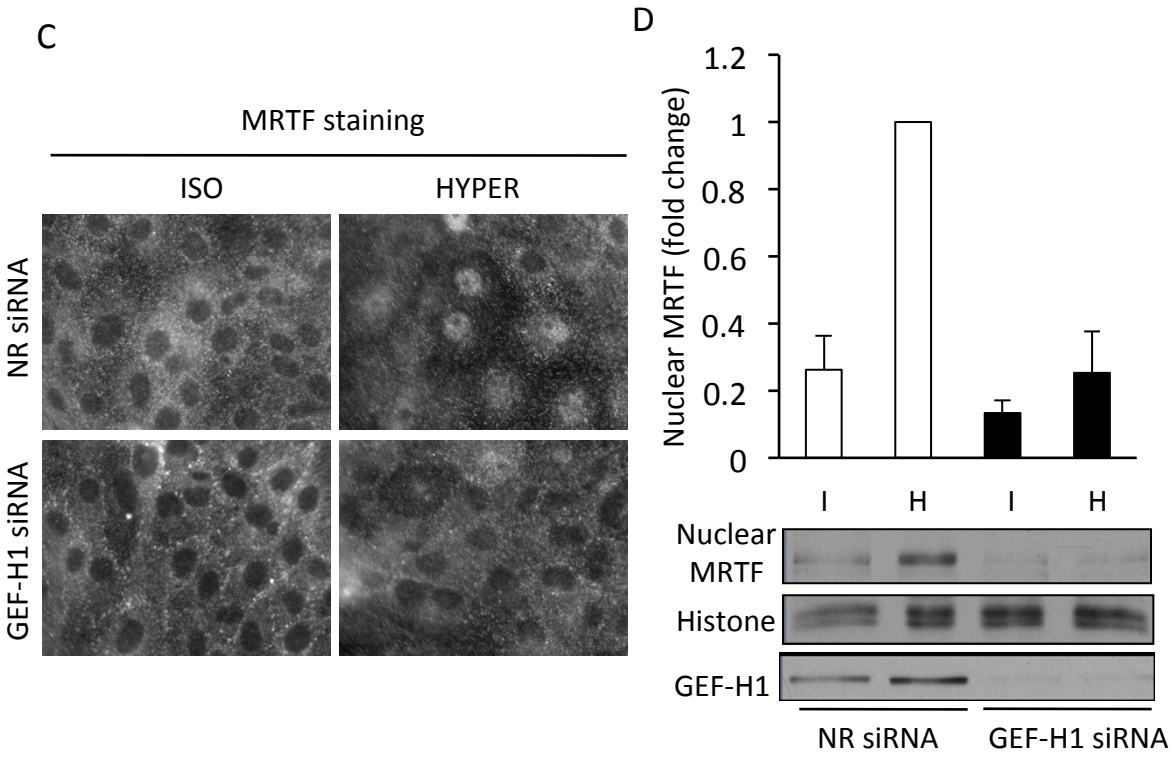


Figure 18*. Hyperosmolarity activates RhoA and induces MRTF translocation though GEF-H1. C, D.* LLC-PK1 cells were transfected with non-related (NR) or GEF-H1-specific siRNA, and forty eight hours later exposed to isotonic Na^+ (I) or hyperosmotic medium (H) for 10 min (C, D). In C MRTF translocation was detected by immunofluorescence and in D cells were lysed and MRTF was detected in nuclear fractions. Histones were used as a marker of the nuclear fraction. In D GEF-H1 in total cell lysates was also detected to demonstrate effectiveness of the siRNA. The amount of precipitated MRTF (D) was normalized to the corresponding histones and expressed as fold change from the maximal effect (hyperosmolarity-treated sample) taken as 1. The graph shows data for n = 6 experiments. $P < 0.001$ for the difference between the NR and GEF-H1 siRNA-treated hypertonic samples.

responsible for the acute structural adaptation, but they also mobilize transcription factors that can impact the expression of cytoskeletal genes. Our recent studies have shown that SRF is phosphorylated and activated upon hyperosmotic stimulation in ELA cells (Gorbatenko et al. 2011). However, SRF is a dual-function transcription factor that can drive both proliferation/survival-promoting early genes and cytoskeleton/muscle differentiation-specific genes (Miano 2003). Moreover, these two functional modalities were found to be competitive (toggle-switch mechanism), and the selection or ratio between them is governed by the interaction of SRF with distinct transcriptional co-activators, namely with components of the ternary complex (for proliferation) and MRTF (for cytoskeletal control) (Miano 2003, Buchwalter et al. 2004). Therefore, we sought to determine whether hyperosmolarity can directly impact the cytoskeletal arm, i.e., MRTF, the activity of which is predominantly regulated through its localization. Our results show that MRTF is an osmosensitive molecule that is rapidly translocated into the nucleus upon hypertonic treatment in a RhoA- and ROK-dependent manner.

Consistent with the possibility that the RhoA/ROK pathway acts primarily by inducing net F-actin polymerization, which is a key regulator of MRTF localization, we have shown earlier that the activation of ROK is an important contributor to the osmotically induced rise in F-actin, presumably because ROK mediates cofilin phosphorylation, which in turn reduces the F-actin severing activity of this protein (chapter 1.2.4) (Thirone et al. 2009). In addition, ROK (directly or indirectly) might induce MRTF phosphorylation as well. This possibility stems from the observations that MRTF undergoes RhoA-dependent phosphorylation (Miralles et al. 2003) and Y-27632 prevents both the stimulus-induced shift in the molecular mass of MRTF (Sebe et al. 2008) and its concomitant translocation. Nonetheless, the role of

this phosphorylation in the transport or activity of MRTF remains to be clarified. Finally, cell contractility (increased MLC phosphorylation) has also been shown to potentiate MRTF accumulation (Fan et al 2007). Since hyperosmolarity provokes RhoA/ROK-dependent MLC phosphorylation in tubular cells (Di Ciano et al. 2002), this mechanism may also facilitate nuclear MRTF accumulation under hyperosmolar conditions.

Our studies also provide insight into the hitherto unknown upstream mechanisms responsible for hypertonicity-induced RhoA activation. Based on our findings that hyperosmotic stress activates the exchange factor GEF-H1 and that GEF-H1 downregulation reduces the osmotically provoked RhoA activation and MRTF translocation, we conclude that GEF-H1 is an osmotically sensitive signal transducer and the GEF-H1/RhoA/ROK pathway is a major mediator of the hypertonicity- triggered MRTF translocation. This conclusion is in accord with a recent report showing that GEF-H1 can regulate SRF-dependent transcription (Smooth Muscle Actin (SMA) expression) in TGF-β-stimulated retinal pigment cells (Tsapara et al. 2010).

Furthermore, GEF-H1 has been recently shown to be activated by stretch (Birukova et al. 2010), extracellular matrix stiffening (Heck et al. 2012), and depolarization (Waheed et al. 2010), which, together with its osmosensitivity documented herein, implies that this molecule is a key mechanotransducer coupling physical changes to RhoA activation (see chapter 6.1). However, GEF-H1 silencing did not completely eliminate RhoA activation and MRTF translocation. While this could be due to some residual GEF-H1 activity, it is likely that GEF-H1 is not the only link between osmotic stress and RhoA. In this regard, previous studies have revealed that ezrin is activated by hyperosmotic stress and its downregulation mitigates the shrinkage-induced RhoA activation in ELA cells (Rasmussen et al. 2008). Since

active ezrin counteracts the RhoA-sequestering capacity of Rho-GDI (Takahashi et al. 1997), this mechanism may represent a significant permissive input. Another intriguing possibility comes from the elegant studies of Guilluy et al. (Guilluy et al. 2011), who showed that integrin-mediated force transduction activates two GEFs, GEF-H1 and LARG, in parallel and each of these is responsible for approximately half of the ensuing RhoA activation. LARG was activated via integrin-mediated stimulation of the Src-family kinase Fyn. These findings point to LARG as a candidate in the regulation of osmotic RhoA activation as well, because integrins were proposed to transmit cell volume-dependent signals (Schliess and Haussinger 2007) and previous studies by us (Kapus et al. 1999, Kapus et al. 2000) and others (Cantore et al. 2011) have shown that hyperosmotic stress selectively activates Fyn (and in certain cell types Yes) but not p60 Src, the third ubiquitous member of the family.

Although investigation of upstream mechanisms that connect the osmotic insult (or other mechanical stimuli) to GEF-H1 activation is beyond the scope of the current work, considering some potential mechanisms may facilitate future studies in this direction. GEF-H1 can be stimulated by its release from microtubules or TJs (Aijaz et al. 2005, Chang et al. 2008) (either due to microtubule disassembly or possibly due to GEF-H1 phosphorylation; Zenke et al. 2004, Callow et al. 2005) and by enhancing its intrinsic activity (again via phosphorylation) (Table 1). A variety of kinases (FAK, ERK, PAK family members, etc.) have been implicated in GEF-H1 regulation (Zenke et al. 2004, Fujishiro et al. 2008, Kakiashvili et al. 2009, Guilluy et al. 2011), and several of these are also affected by osmotic shock (Hoffmann et al. 2009). Future studies are warranted to test their involvement (see chapter 6.2.1).

In summary, we have identified the GEF-H1/RhoA/ROK/MRTF pathway as a tightly controlled osmosensitive and osmoprotective mechanism that provides a link between the osmotic environment and transcriptional control of the cytoskeleton.

CHAPTER 5 Site-specific phosphorylation of GEF-H1 mediates its sequential activation of Rac and RhoA

This chapter has been modified from the following:

Waheed, F., Dan, Q., Zhang, Y., Tanimura, S., Speight, P., Kapus, A., and Szászi, K. (2013): Central role of the exchange factor GEF-H1 in TNF-α-induced sequential activation of Rac, ADAM17/TACE and RhoA in tubular epithelial cells. Molecular Biology of the Cell Apr;24(7):1068-82

5.1. Summary

Our lab has previously shown in kidney tubular cells that TNF activates the Rho family small GTPases, RhoA and Rac, via the same exchange factor GEF-H1. This induces cytoskeleton remodelling through the pathway described on the scheme in Figure 3. In Waheed et al. (2013), our lab shows that TNF stimulates the enzyme TACE/ADAM17, leading to activation of the Epidermal Growth Factor Receptor (EGFR)/ERK pathway. TACE activation requires the MAP kinase p38, which is activated through the small GTPase Rac. Interestingly, TNF-induced activation of Rac and RhoA through GEF-H1 is via different mechanisms. EGFR- and ERK-dependent phosphorylation at the Thr678 site of GEF-H1 is a prerequisite for RhoA activation only, while both Rac and RhoA activation require GEF-H1 phosphorylation on Ser885. Also, GEF-H1-mediated Rac activation is upstream from the TACE/EGFR/ERK pathway, and regulates T678 phosphorylation. We also show that TNF enhances epithelial wound healing through TACE, ERK and GEF-H1.

5.2. Introduction

TNF activates RhoA through GEF-H1. EGFR and the MAPK ERK also participate in this signalling cascade, whereby transactivation of the EGFR through the enzyme TACE (Figure 6b, and Figure 13) is required for phosphorylation and activation of ERK. We and others (Fujishiro et al. 2008, Kakiashvili et al. 2009) have shown that ERK phosphorylates GEF-H1 on its Thr678 residue. This phosphorylation of GEF-H1 is essential for its activation towards RhoA. More recently, our lab has shown that the MAPK p38 is required for TNF-induced activation of TACE, and hence, ERK (Waheed et al. 2013). Since the small GTPase Rac has previously been shown to play a role in p38 activation via its effector PAK1 (Zhang et al.

1995), we sought to further understand the role of Rac in this signalling cascade. Moreover, we wanted to investigate whether GEF-H1 might also be involved in the activation of Rac, since when first described, GEF-H1 was also shown to be an exchange factor for Rac (Ren et al. 1998, Gao et al. 2001). Subsequent studies, however, have emphasized the role of GEF-H1 in Rho activation.

Hence, we were interested in exploring the relationship between GEF-H1 and Rho/Rac, interconnected in a signalling cascade alongside the MAPKs ERK and p38. These questions investigate how growth/cell proliferation signals via the EGFR/ERK are transmitted to two of the central regulators of the cytoskeleton, RhoA and Rac.

5.3. Results

5.3.1. TNF-induced Rac activation mediates p38 and ERK activation

To test whether TNF activates Rac, we used an affinity precipitation assay with glutathione S-transferase (GST)-p21-binding domain (PBD)-coupled beads. The antibody that we used to visualize precipitated Rac is able to detect all three Rac isoforms. Our results revealed that TNF induced Rac activation as early as after 0.5 min of stimulation (Figure 19A), with some further increase at the 5-min time point. To test the role of Rac in mediating the effects of TNF, we used a specific siRNA against Rac 1 and 2. As shown in Figure 19B, the siRNA induced a marked decrease in Rac expression. Importantly, Rac silencing prevented both TNF-induced p38 (Figure 19B) and ERK activation (Figure 19C).

We verified this finding by using a dominant-negative Rac (RacT17A, DN-Rac), which was cotransfected with HA-tagged ERK2. After treatment with TNF, HA-ERK was immunoprecipitated, and its phosphorylation status was evaluated by Western blotting with a

Figure 19. Rac is activated by TNF and mediates p38 and ERK activation

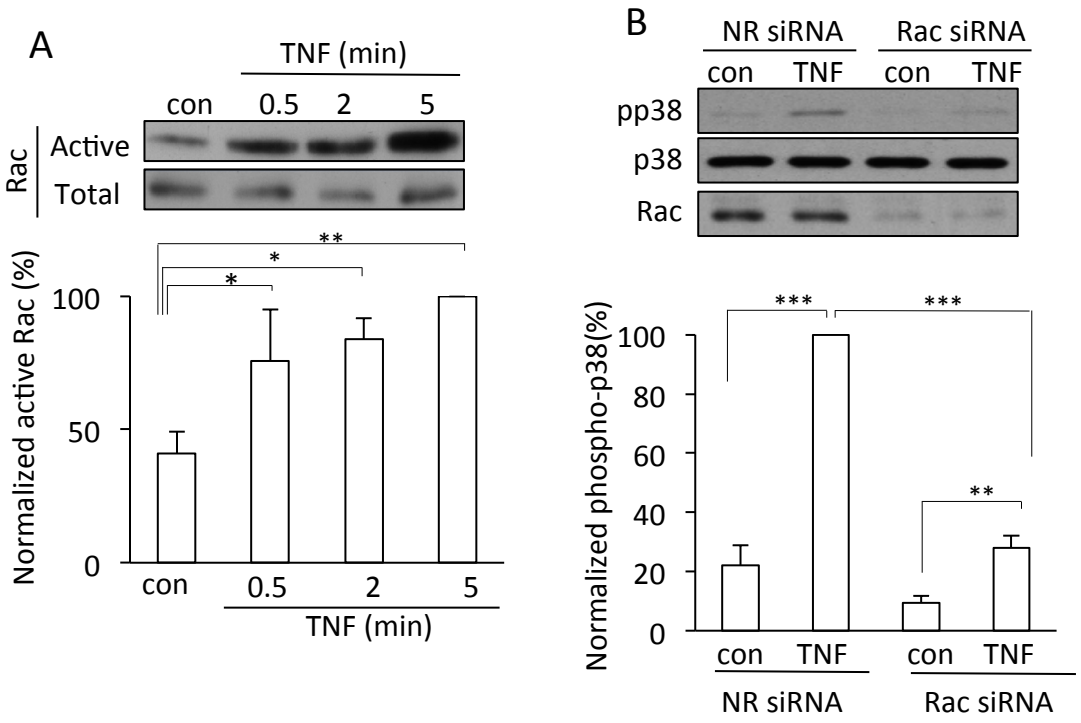


Figure 19. *Rac is activated by TNF and mediates p38 and ERK activation. (A) TNF activates Rac*. LLC-PK$_1$ cells were treated with 10 ng/ml TNF for the indicated times. Cells were lysed and active Rac was precipitated using GST-PBD. Rac in the precipitates and total cell lysates (active and total, respectively) was detected by Western blotting, and quantified by densitometry. The amount of active Rac in each sample was normalized to the corresponding total Rac. The data obtained in each experiment were expressed as % compared to the level of the 5 min TNF-treated sample that was taken as 100%.

(*B)* LLC-PK$_1$ cells were transfected with NR or porcine Rac1/2-specific siRNA. Forty-eight hours later the cells were treated with10 ng/ml TNF for 5 min. Total cell lysates were probed on Western blots with antibodies against phospho-p38, p38, Rac. The blots were quantified and phospho-p38 normalized with p38 in the same samples. The graphs in the figure show mean ± S.E. from n=5 (A); 8 (B) independent experiments.

Figure 19. Rac is activated by TNF and mediates p38 and ERK activation

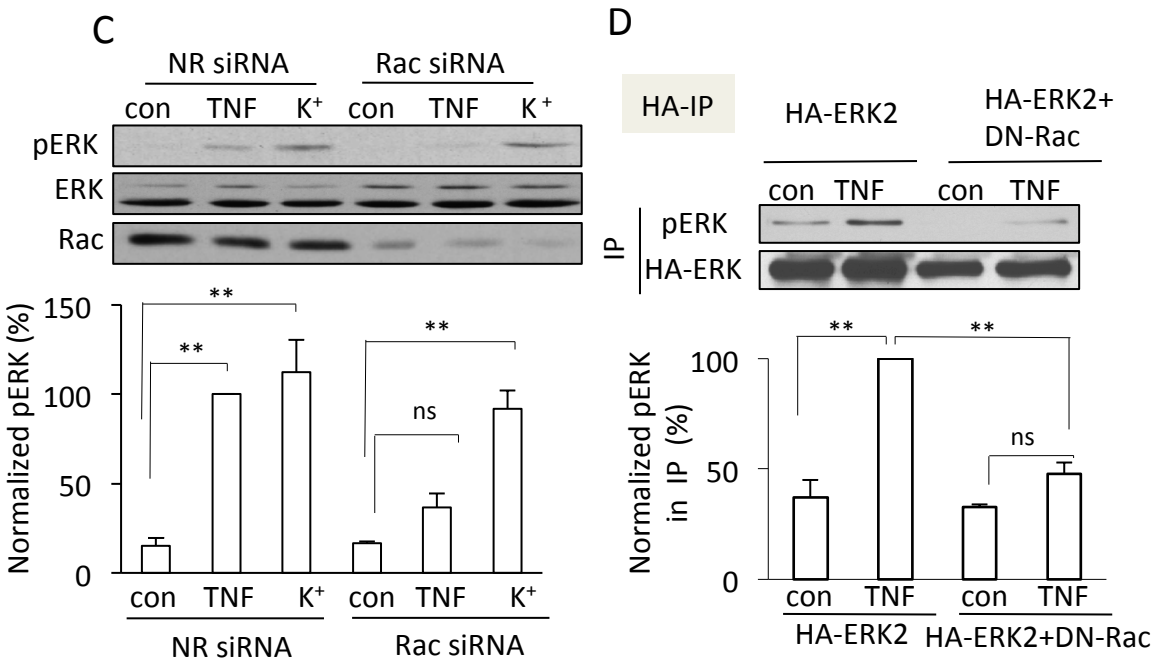


Figure 19. (*C)* LLC-PK$_1$ cells were transfected with NR siRNA or porcine Rac1/2 siRNA. Forty-eight hours later the cells were incubated in Na^+ medium for 15 min, followed by the addition of 10 ng/ml TNF in Na^+ medium, or exchange of the medium for K^+ medium (5 min). pERK was detected and quantified by normalizing the amount of phospho-ERK to total ERK in the corresponding cell lysates. The blot was stripped and reprobed with anti-Rac. (*D)* Cells were transfected with HA-ERK2 with or without cotransfection of DN-Rac, and 48 hours later treated with TNF (5 min). HA-ERK was precipitated and its phosphorylation detected. The graphs in the figure show mean ± S.E. from n=3 independent experiments.

pERK-specific antibody. This method allowed us to study ERK activation exclusively in the transfected cells. As shown in Figure 19D, whereas TNF induced phosphorylation of HA-ERK in cells transfected with HA-ERK alone, this was prevented by the coexpression of DN-Rac.

To determine whether the requirement for Rac is specific for TNF-induced ERK activation, we compared the effect of Rac silencing on ERK activation induced by TNF and plasma membrane depolarization. Depolarization activates RhoA through an ERK- and GEF-H1-dependent mechanism (see chapter 1.4.2; Waheed et al. 2010). As expected, depolarization induced by 130 mM KCl potently stimulated ERK phosphorylation in LLC-PK1 cells (Figure 19C). In contrast to the TNF-induced ERK activation, Rac silencing did not affect depolarization-induced ERK activation, suggesting that Rac does not mediate ERK activation by all stimuli but is specific for the TNF-induced pathway. Rac silencing, however, did not interfere with TNF-induced activation of NFκB (Figure 20, A and B), verifying that it did not cause an overall inhibition of all TNF-induced signalling.

5.3.2. Rac is required for TNF-induced RhoA activation

ERK activation is necessary for TNF-induced RhoA activation, suggesting that Rac might also be required for RhoA activation induced by this cytokine. To test this assumption, we explored RhoA activation using the Rho-binding domain (RBD)–GST precipitation assay after Rac silencing. TNF induced a readily-detectable RhoA activation in LLC-PK1 cells transfected with a nonrelated control siRNA. In contrast, RhoA was not activated in cells transfected with Rac siRNA (Figure 21). These findings suggest that in tubular epithelial

Figure 20. Rac silencing does not affect TNF-induced activation of NFκB

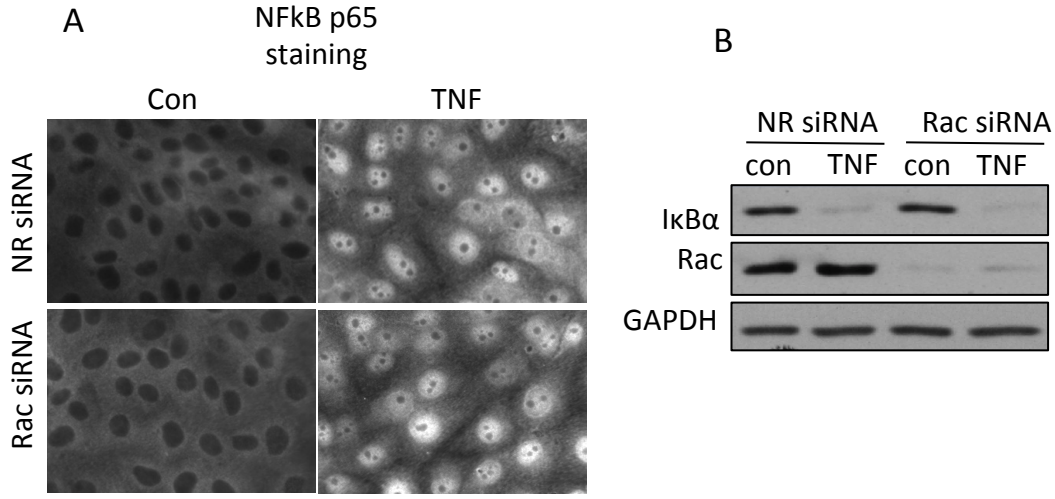


Figure 20. (*A, B) Rac silencing does not affect TNF-induced activation of NFκB.* Cells were transfected with NR or Rac-specific siRNA and 48 h later treated with TNF (30 min). In (A) cells were fixed, permeabilized and the p65 of NFκB was detected by immunostaining. In (B) cell were exposed to TNF for 15 min, lysed and IκB-α, Rac and GAPDH were detected in the cell lysates by Western blotting.

Figure 21. Rac mediates RhoA activation induced by TNF

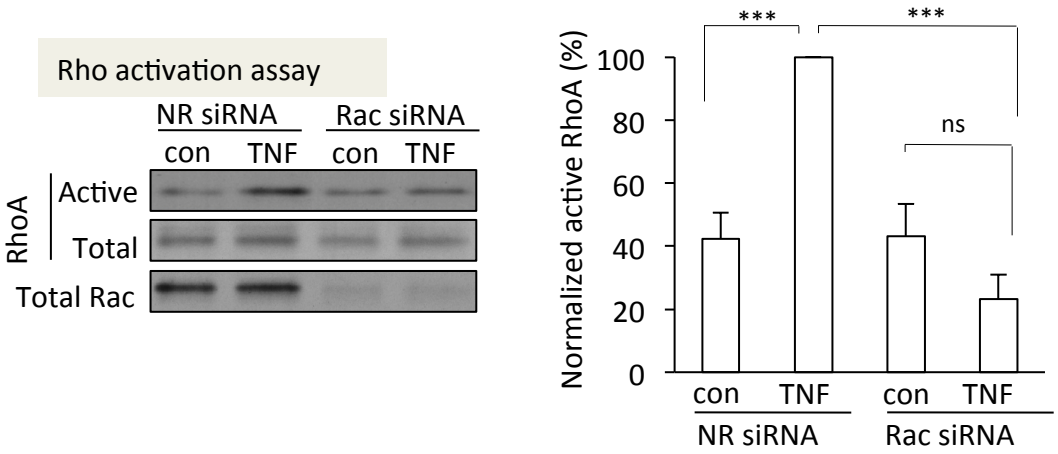


Figure 21*. Rac mediates RhoA activation induced by TNF.* A. LLC-PK$_1$ cells were transfected with NR siRNA or porcine Rac1/2-specific siRNA for 48 hours. Cells were treated with TNF (5 min) and active RhoA was precipitated with GST-RBD, and quantified as described for Rac in Fig19. The graph in the figure shows mean ± S.E. from n=5 independent experiments.

cells TNF-induced RhoA activation depends on Rac and that activation of the two small GTPases occurs as a sequential event with a hierarchy between Rac and RhoA.

5.3.3. TNF activates Rac in a GEF-H1-dependent manner

We next wished to identify whether GEF-H1, the exchange factor required for TNF-induced RhoA activation, was also found to have Rac exchange activity (Ren et al. 1998). This prompted us to explore its potential role in TNF-induced activation of Rac. Activated Rac GEFs were precipitated from control and TNF-treated cell lysates using the nucleotide-free Rac(G15A) mutant, and the presence of GEF-H1 was tested by Western blotting. Figure 5A shows that only a small amount of GEF-H1 was captured by GST-Rac(G15A) from untreated cells. Importantly, when cells were stimulated with TNF, the Rac(G15A)-associated GEF-H1 was significantly enhanced. Moreover, this was detectable as early as 0.5 min after the addition of TNF, similar to the rapid activation of Rac (Figure 22A). These data suggest that GEF-H1 is activated toward Rac. To substantiate that GEF-H1 indeed mediates TNF-induced Rac activation, we silenced it using a specific siRNA. The siRNA transfection achieved ≥90% reduction in GEF-H1 protein expression (Figure 22B). No change in the small basal activity of Rac was evident in cells transfected with the GEF-H1-specific siRNA. However, TNF-induced Rac activation was prevented by GEF-H1 silencing. In fact, Rac activity in TNF-treated and GEF-H1-downregulated cells was consistently lower than the control level. We verified that GEF-H1 silencing did not prevent TNF-induced activation of NFκB, suggesting that it did not prevent activation of the TNF receptors (Figure 23; Kakiashvili et al. 2009). Taken together, these data suggest that GEF-H1 mediates not only the TNF-induced activation of RhoA but also that of Rac.

Figure 22. TNF activates Rac through GEF-H1

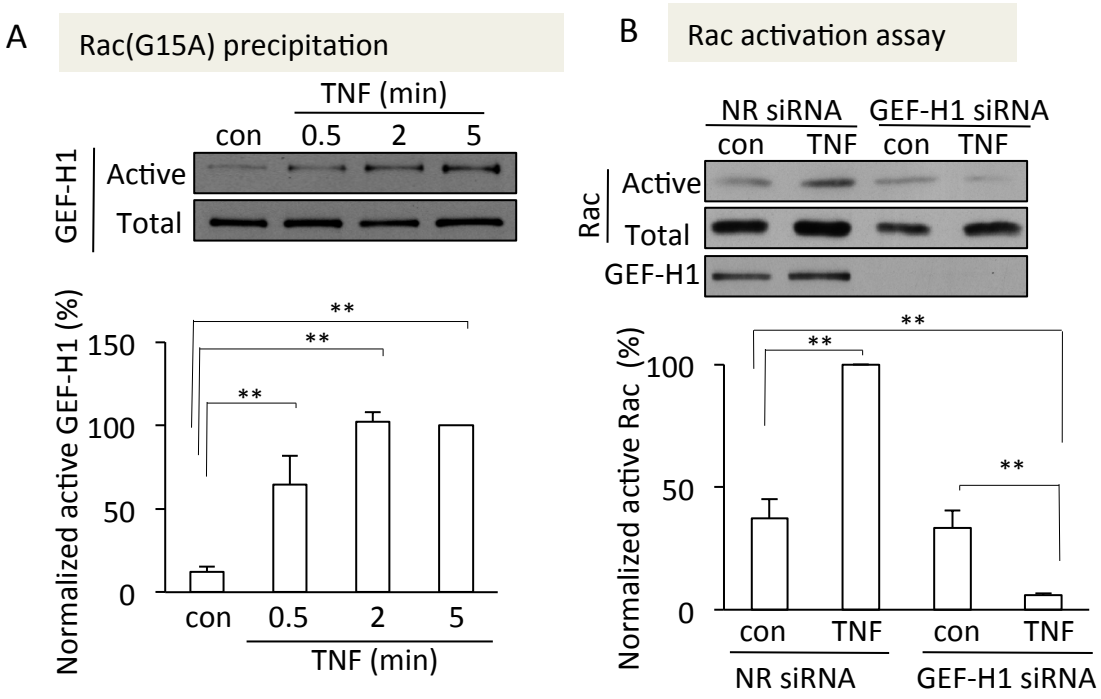


Figure 22. *TNF activates Rac through GEF-H1.* A. LLC-PK1 cells were treated with 10 ng/ml TNF for the indicated times. Active GEF-H1 was precipitated using GST-Rac(G15A). GEF-H1 in the precipitates and total cell lysates (active and total, respectively) was detected by Western blotting. The blots were quantified as described earlier for Rac. B. LLC-PK1 cells were transfected with NR siRNA or GEF-H1-specific siRNA. Forty-eight hours later cells were treated with 10 ng/ml TNF for 5 min. In B, active Rac was detected and quantified as in Fig 19A.

Figure 23. GEF-H1 silencing does not affect TNF-induced activation of NFκB

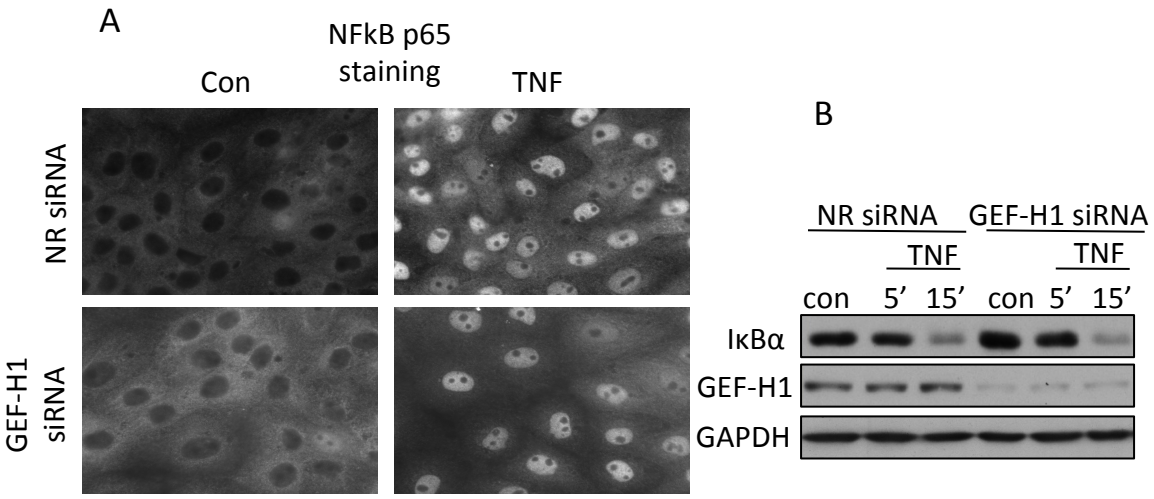


Figure 23. (*A, B)* GEF-H1 *silencing does not affect TNF-induced activation of NFκB.* Cells were transfected with NR or GEF-H1-specific siRNA and 48 h later treated with TNF (30 min). In (A) cells were fixed, permeabilized and the p65 of NFκB was detected by immunostaining. In (*B*) Cells were transfected with NR or GEF-H1-specific siRNA and 48 h later treated with TNF for 5 or 15 min as indicated. Cells were lysed and IκB-α, GEF-H1 and GAPDH were detected in the cell lysates by Western blotting.

5.3.4. TNF activates p38 and ERK through GEF-H1

We next sought to ascertain whether GEF-H1 is a mediator of TNF-induced activation of the p38/TACE/ERK pathway, as anticipated from its role in Rac activation. GEF-H1 silencing indeed reduced TNF-induced activation of ERK and p38 (Figure 24, A and B) and prevented TACE activation (data not shown). These effects were similar to those observed with Rac down-regulation (Figure 19, B and C).

To verify that p38 activation is indeed an effector of GEF-H1 in mediating ERK activation, we asked whether the inhibition of TNF-induced ERK activation observed when GEF-H1 was silenced can be overcome by overexpressing p38. First, we verified the effectiveness of GEF-H1 silencing in cells cotransfected with GEF-H1 siRNA and HA-ERK with or without active p38. As shown in Figure 24C (left), GEF-H1 was potently downregulated, and this abolished TNF-induced HA-ERK phosphorylation. Figure 24C (right) demonstrates that coexpression of an active p38 construct together with the nonrelated (NR) siRNA enhanced HA-ERK phosphorylation. FLAG-p38-induced ERK phosphorylation was not prevented by GEF-H1 silencing, suggesting that p38 is downstream from GEF-H1.

Since GEF-H1 also mediates TNF-induced RhoA activation, we next asked whether RhoA contributes to stimulation of ERK. Interestingly, silencing of RhoA using a specific siRNA also reduced TNF-induced ERK activation, although to a lesser extent than Rac silencing (Figure 24D). Importantly, we found that in cells transfected with RhoA siRNA, GEF-H1 levels were also reduced, which could partly explain this finding (see Discussion, and chapter 6.2).

Figure 24. TNF activates ERK and p38 through GEF-H1

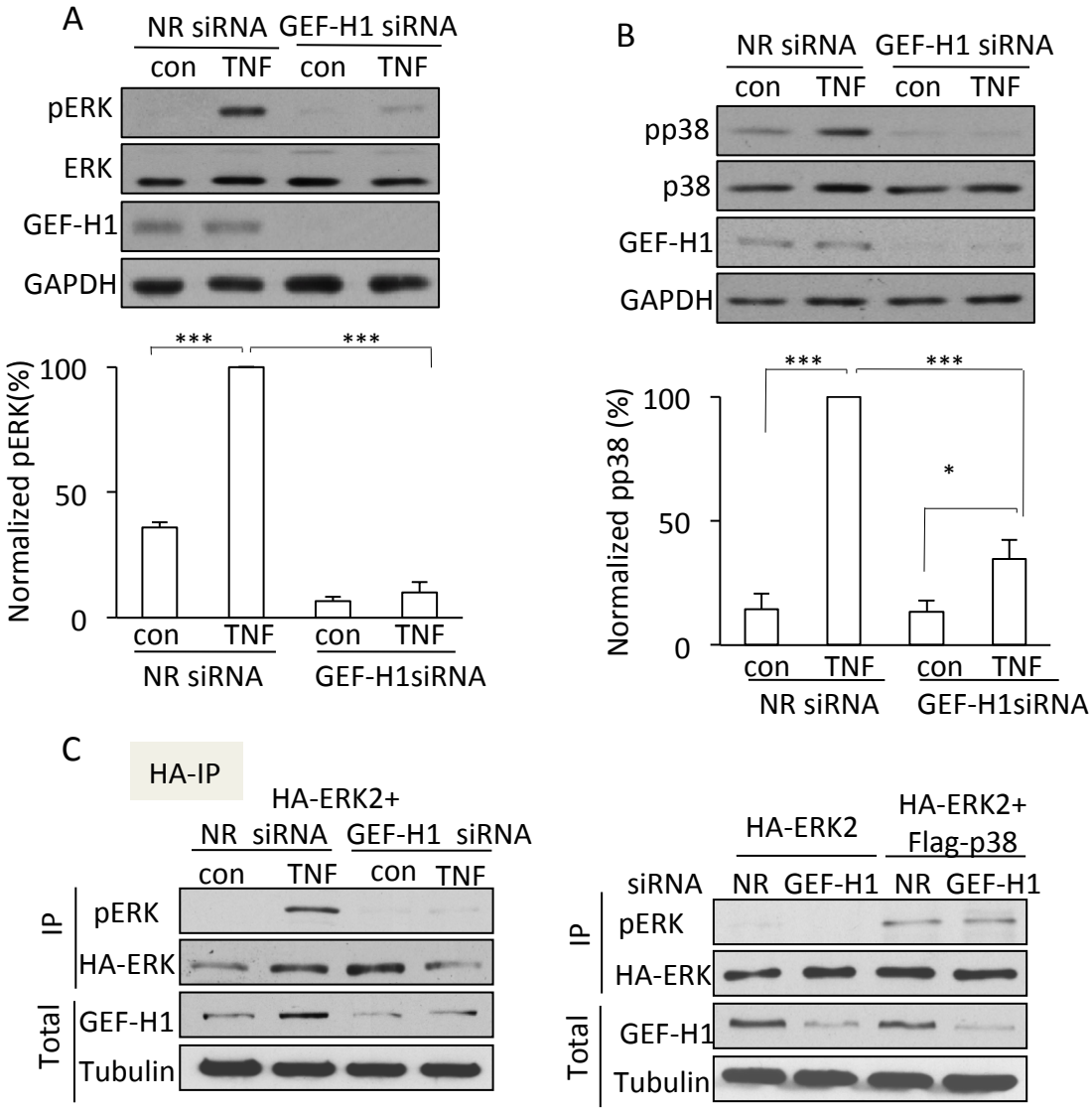


Figure 24. *TNF activates ERK and p38 through GEF-H1.* LLC-PK1 cells were transfected with NR siRNA or GEF-H1-specific siRNA. Forty-eight hours later the cells were treated with 10 ng/ml TNF for 5 min. *A* and B, pERK, ERK, phospho-p38, p38, GEF-H1 and GAPDH were detected by Western blotting. For all blots quantification was done using densitometry as described earlier. The data for phospho-p38, and pERK were normalized to the corresponding total levels of these proteins. C. LLC-PK1 cells were transfected with HA-ERK2 with cotransfection of NR siRNA, GEF-H1 siRNA or Flag-p38, as indicated. Where indicated, cells were treated with TNF for 5 min. HA-ERK was precipitated, and its phosphorylation assessed using a pERK antibody. The top two blots show the immunoprecipitated pERK and HA signals (IP), and the bottom two blots demonstrate GEF-H1 and tubulin in the corresponding total cells lysates. The graphs in the figure show mean ± S.E. from n=8 (A, B) independent experiments.

Figure 24D RhoA silencing reduces TNF-induced activation of ERK

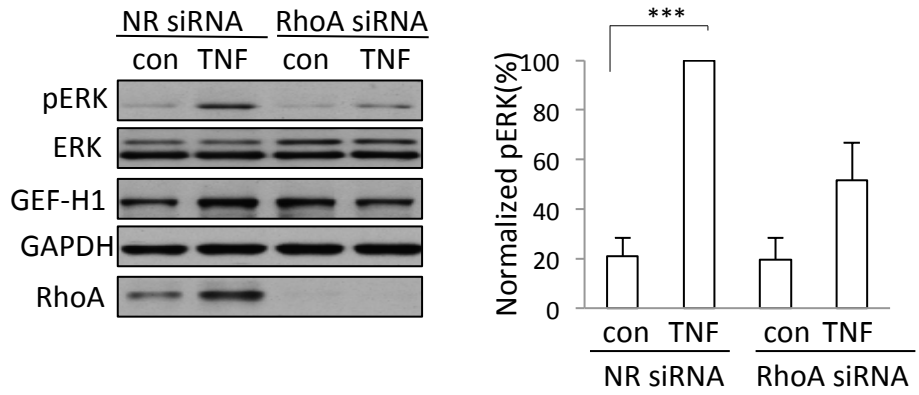


Figure 24D. *RhoA silencing reduces TNF-induced activation of ERK*. LLC-PK_1 cells were transfected with NR or RhoA-specific siRNA and 48 h later treated with TNF for 5 min. At the end of the treatment cells were lysed and pERK, ERK, GEF-H1, RhoA and GAPDH were detected by Western Blotting. The levels of pERK were quantified using densitometry, as described earlier. The graphs in the figure show mean ± S.E. from n=9.

5.3.5. TNF transactivates EGFR through GEF-H1

Having seen that TACE activation was regulated by GEF-H1 (not shown), we also sought to verify that GEF-H1 indeed regulates EGFR activation. Therefore we explored how GEF-H1 silencing affects TNF-induced EGFR activation. TNF-induced phosphorylation of EGFR was detected using an antibody against the phosphorylated Y845 site. TNF induced a well-detectable increase in phospho-EGFR in cells transfected with the control siRNA (Figure 25). GEF-H1 down-regulation prevented this increase. Surprisingly, GEF-H1 silencing also induced a significant drop in the levels of total EGFR protein. Normalizing EGFR phosphorylation to the total EGFR levels, however, revealed that GEF-H1 silencing also prevented TNF-induced phosphorylation of the remaining EGFR. Taken together, these data verify that GEF-H1 regulates EGFR activation induced by TNF. In addition, GEF-H1 silencing also reduces EGFR expression.

5.3.6. Rac activation and GEF-H1 stimulation towards Rac are independent of EGFR and ERK

In previous work we showed that TNF-induced, GEF-H1-dependent RhoA activation was mediated by EGFR and ERK. Our data presented so far, however, suggest that in contrast to RhoA activation, GEF-H1-dependent Rac activation is upstream of EGFR and ERK. To substantiate this notion, we explored how inhibition of EGFR and ERK affected Rac activation and stimulation of GEF-H1 toward Rac. RhoA activation was inhibited by the MEK1/2 inhibitor PD98059 and the EGFR inhibitor AG1478 (Kakiashvili et al. 2011). In contrast, neither of these inhibitors prevented TNF-induced Rac activation nor

Figure 25. TNF transactivates EGFR through GEF-H1

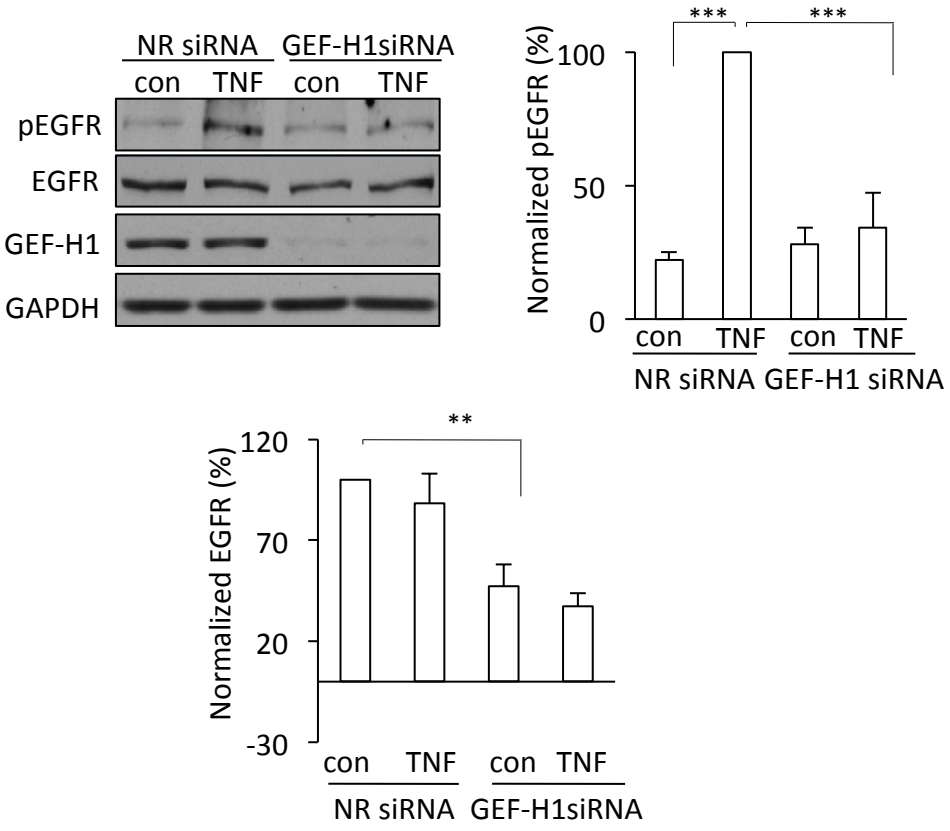


Figure 25. *TNF transactivates EGFR through GEF-H1*. LLC-PK$_1$ cells were transfected with NR siRNA or GEF-H1-specific siRNA. Forty-eight hours later cells were treated with 10 ng/ml TNF for 5 min. pEGFR was detected using an antibody against phospho-Y845 EGFR. For all blots quantification was done using densitometry as described earlier. pEGFR were normalized to the corresponding total EGFR, and the data for EGFR were normalized using GAPDH. The graphs in the figure show mean ± S.E. from n=3 independent experiments.

stimulation of GEF-H1 toward Rac (Figure 26, A–D). These data suggest that the Rac- and RhoA-specific exchange activities of GEF-H1 are indeed differentially regulated.

5.3.7. TNF-induced Rac activation and GEF-H1 stimulation towards Rac do not require phosphorylation on T678

A possible mechanism for the differential regulation of GEF-H1 is through specific phosphorylation sites. RhoA activation requires phosphorylation on T678 (Fujishiro et al. 2008, Kakiashvili et al. 2009). Therefore, we tested the role of this site in the differential regulation of GEF-H1 toward Rac and RhoA by comparing how a point mutant GEF-H1 that lacks the ERK-target T678 is activated toward Rac and RhoA. LLC-PK1 cells were transiently transfected with a GFP-tagged wild-type or GEF-H1T678A point-mutant protein. RhoA and Rac GEFs were precipitated using GST-tagged RhoA(G17A) or Rac(G15A), respectively, and the presence of GFP-tagged WT or mutant GEF-H1 was detected using an antibody against GFP. TNF enhanced the association of WT-GEF-H1 with both nucleotide-free small GTPases (Figure 27, A and B). Consistent with our previously reported findings, elimination of the T678 site prevented TNF-induced activation of GEF-H1 toward RhoA (Figure 27A). In contrast, TNF stimulated association of GEF-H1T678A with Rac(G15A) to a similar extent as the WT protein. Taken together, these data support the role of differential phosphorylation in GEF-H1 activation toward Rac and RhoA. Whereas GEF-H1 activation toward RhoA is mediated by EGFR- and ERK-dependent phosphorylation on T678, its activation toward Rac does not require this phosphorylation.

Figure 26. TNF-induced Rac activation and stimulation of GEF-H1 towards Rac do not require EGFR and ERK

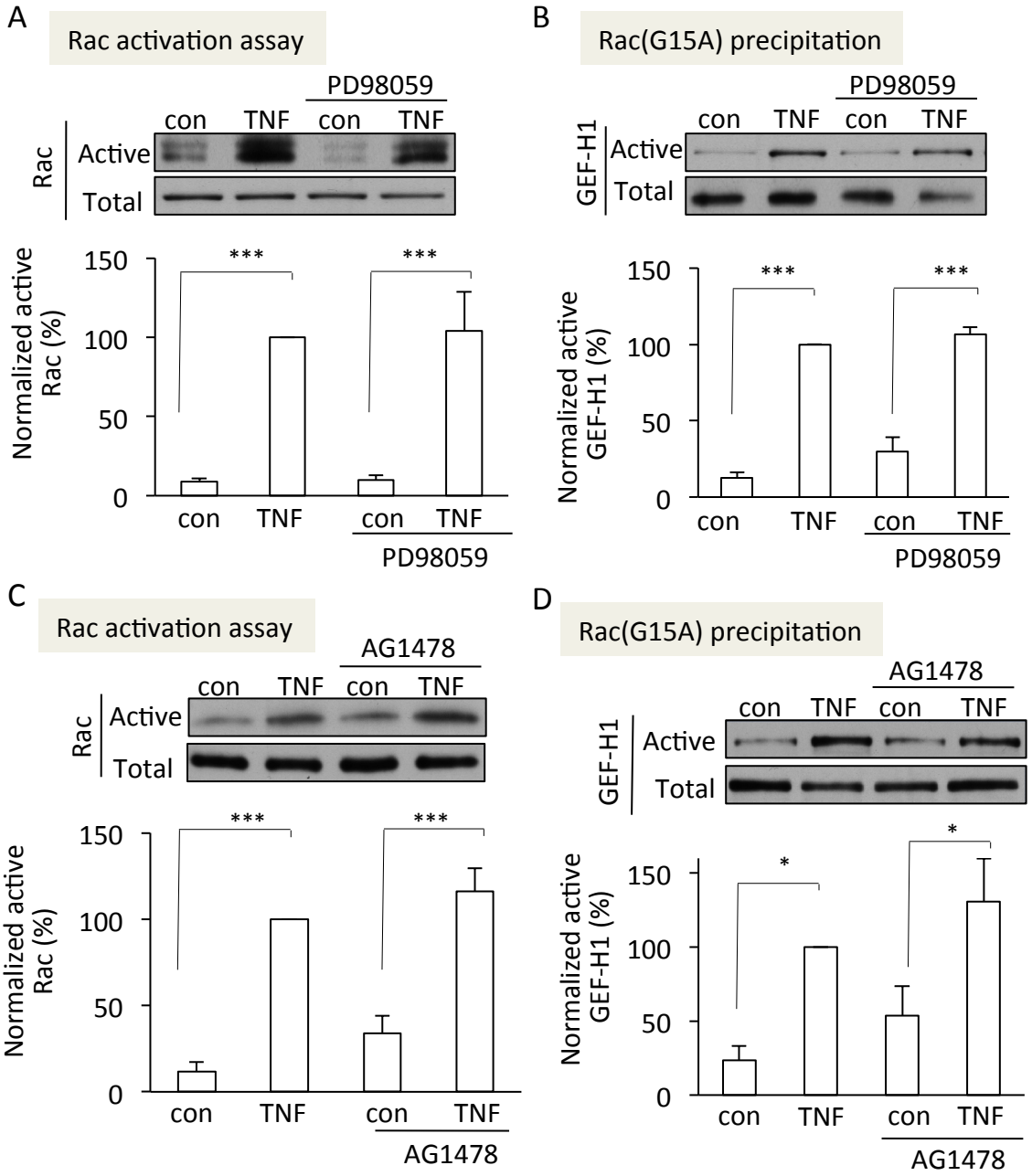


Figure 26. *TNF-induced Rac activation and stimulation of GEF-H1 towards Rac do not require EGFR and ERK*. LLC-PK$_1$ cells were treated with 20µM PD98059 (A, B) or 10 µM AG1478 (C, D) for 15 min, followed by addition of 10 ng/mL TNF for 5 min (A, C) or 2 min (B, D). In (A and C) active Rac was precipitated using GST-PBD. In (B and D) active GEFs were precipitated using GST-Rac(G15A), and GEF-H1 was detected by Western blotting. Densitometric analysis was done as described above. The graphs in the figure show mean ± S.E. from n=4 (A, B and C) or 3 (D) independent experiments.

Figure 27. Differential role of GEF-H1 T678 phosphorylation sites in GEF-H1 activation towards Rac and RhoA

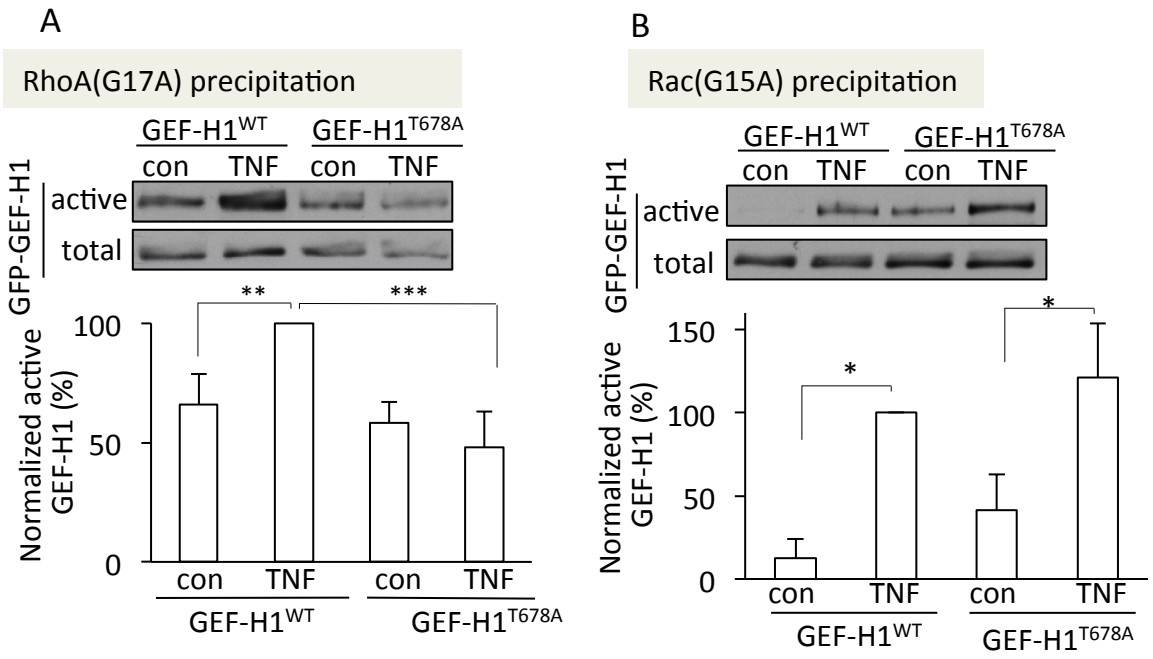


Figure 27. *Differential role of GEF-H1 T678 phosphorylation sites in GEF-H1 activation towards Rac and RhoA.* LLC-PK_1 cells were transfected with GFP-tagged wild type GEF-H1 (GEF-$H1^{WT}$), or the non-phosphorylatable point mutant GEF-$H1^{T678A}$ as indicated. 48 hours post-transfection cells were treated with 10 ng/ml TNF (5 min), and activated GEFs were precipitated using RhoA(G17A) (A) or Rac(G15A) (B). GFP-tagged GEF-H1 protein was detected by Western blotting using anti-GFP. Blots were quantified as described earlier. The graphs in the figure show mean ± S.E. from n=4 (A) or 3 (B) independent experiments

5.3.8. TNF-induced GEF-H1 activation toward Rac requires phosphorylation on Ser885

Next, we wished to gain insight into mechanisms that mediate TNF-induced activation of GEF-H1 toward Rac. In a mass spectrometry analysis of phosphorylated amino acids in GEF-H1 precipitated from TNF-stimulated cells (Kakiashvili et al. 2009), we found the S885 site to be phosphorylated. This site is the target of numerous kinases and has been implicated in GEF-H1 regulation (see Table 1, chapter 1.3; reviewed in Birkenfeld et al. 2008). Therefore we explored the role of this site in the TNF-induced effects. First, using an HA-tagged GEF-H1, we investigated basal and TNF-induced phosphorylation of S885. HA-tagged GEF-H1 was precipitated from control and TNF-treated cells, and its phosphorylation was tested using an antibody specific for phospho-S885 GEF-H1. In most (but not all) experiments, we found a trend for increased phosphorylation in the TNF-treated samples (Figure 28).

To gain further insight into the role of S885, we generated a point mutant lacking this phosphorylation site (GFP-GEF-H1S885A). Using the GST-Rac(G15A) precipitation assay, we tested whether this mutant can be activated toward Rac. As shown in Figure 29A and B, in contrast to GEF-H1T678A, GFP-GEF-H1S885A showed significantly reduced TNF-induced activation toward Rac. To further substantiate the differential role of the T678 and S885 sites in Rac and RhoA activation, we tested the effect of the phosphorylation-incompetent point mutant GEF-H1 molecules on ERK activation.

To eliminate the confounding effect of endogenous GEF-H1 in these experiments, we silenced GEF-H1 using either a porcine-specific siRNA (Figure 30A) or a DNA vector–based short hairpin RNA (shRNA; Figure 30B). Both approaches efficiently prevented TNF-

Figure 28. TNF induces elevation in GEF-H1 S885 phosphorylation

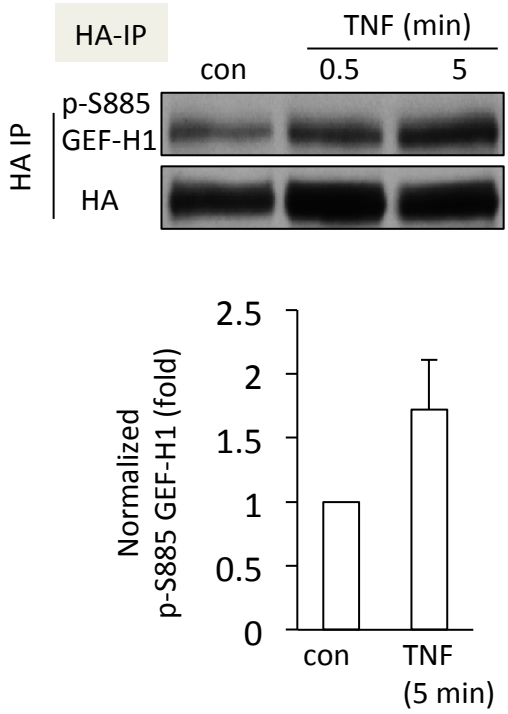


Figure 28. *TNF induces elevation in GEF-H1 S885 phosphorylation.* LLC-PK1 cells were transfected with an HA-tagged GEF-H1 for 48h. Cells were serum depleted overnight, and treated with TNF for 2 min or 5 min as indicated. At the end of treatment, cells were lysed in a lysis buffer supplemented with phosphatase inhibitors and 10 nM Calyculin A. HA-GEF-H1 was immunoprecipitated as described in Materials and Methods. Precipitated proteins were subjected to Western blotting using anti-phospho-S885 (p-S885) GEF-H1, and redeveloped using anti-HA. Blots were analysed by densitometry. The HA signal was used to normalize corresponding p-S885 signal. Normalized values from control were taken as 1 and values from treated samples were expressed as fold increase. The graph shows data from n=3 experiments.

Figure 29. Role of GEF-H1 S885 phosphorylation site in GEF-H1 activation towards Rac and RhoA

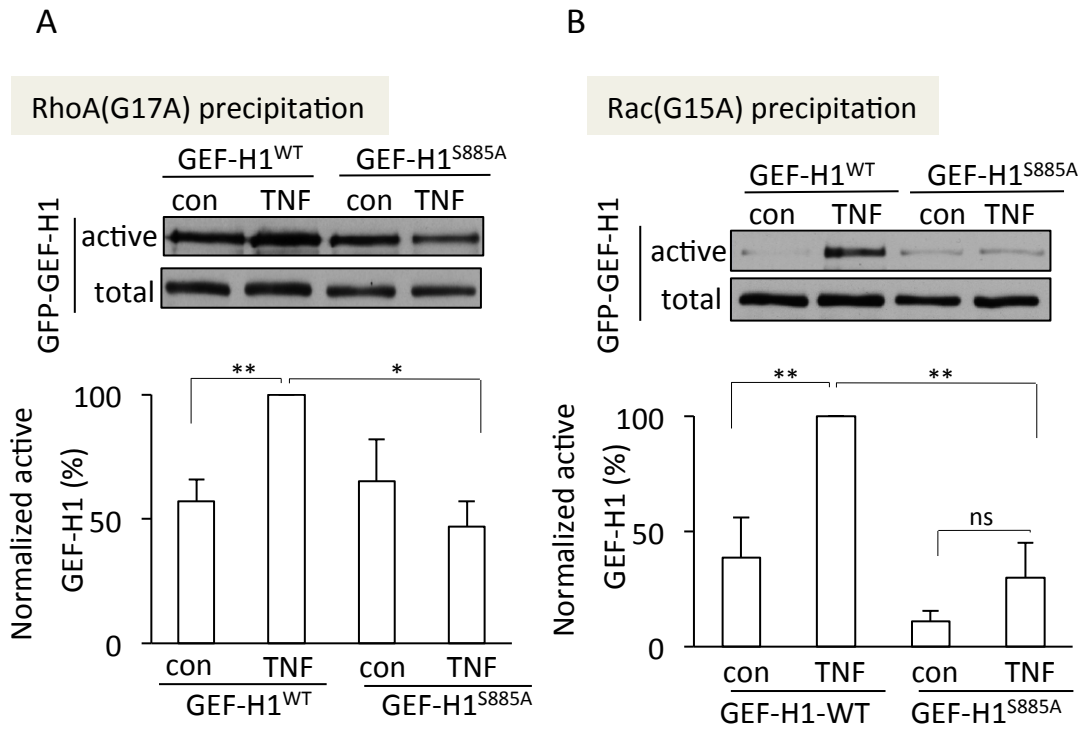


Figure 29. *Role of GEF-H1 S885 phosphorylation site in GEF-H1 activation towards Rac and RhoA.* LLC-PK$_1$ cells were transfected with GFP-tagged wild type GEF-H1 (GEF-H1^{WT}), or the non-phosphorylatable point mutant GEF-H1^{S885A} as indicated. 48 hours post-transfection cells were treated with 10 ng/ml TNF (5 min), and activated GEFs were precipitated using RhoA(G17A) (A) or Rac(G15A) (B). GFP-tagged GEF-H1 protein was detected by Western blotting using anti-GFP. The blots were quantified as described earlier. The graphs in the figure show mean ± S.E. from n=3 (A) or 4 (B) independent experiments

Figure 30. Differential role of T678 and S885 in TNF-induced ERK activation

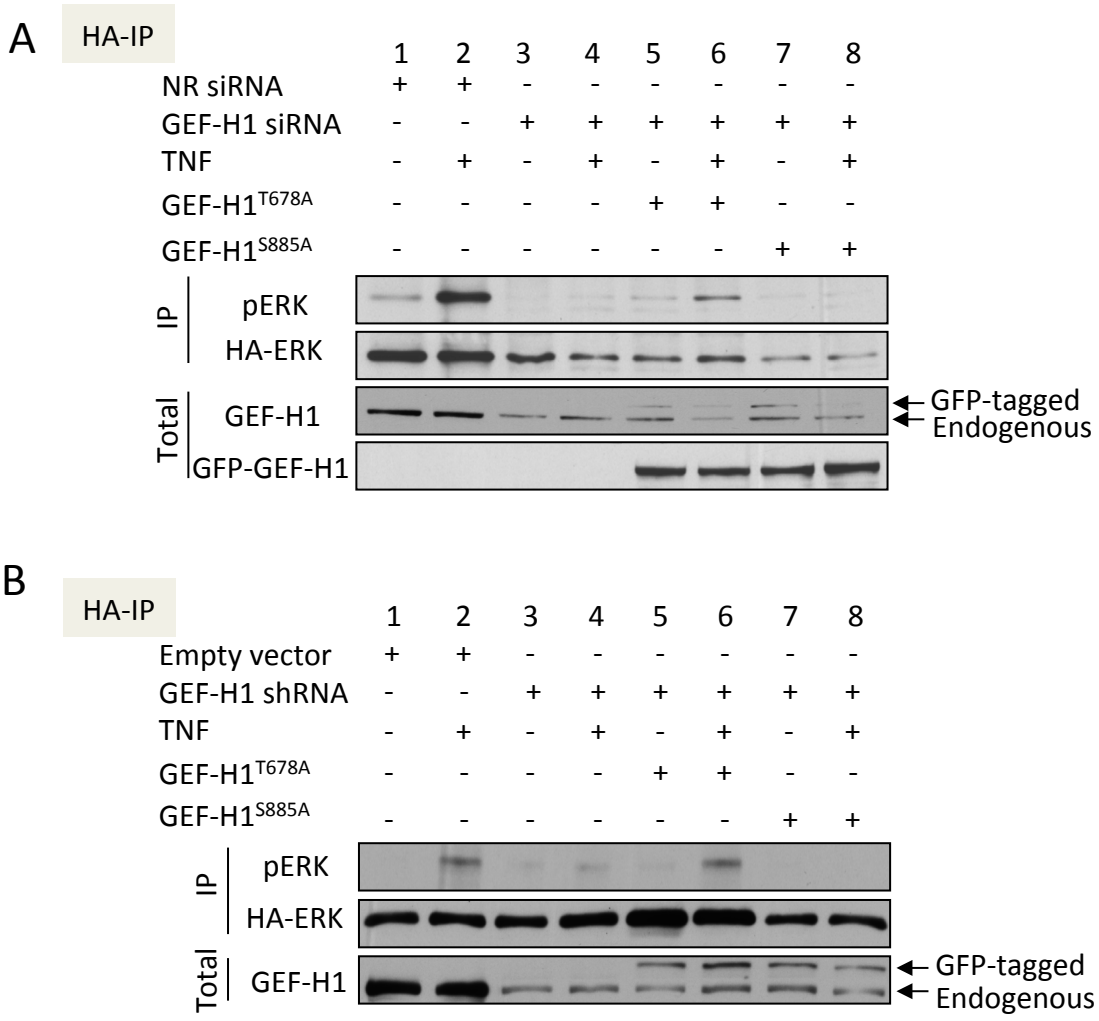


Figure 30. *(A and B) Differential role of T678 and S885 in TNF-induced ERK activation.* In (A) LLC-PK_1 cells grown in 6 cm dishes were transfected with 100nM NR or GEF-H1-specific siRNA and 24hours later with GFP-GEF-$H1^{T678A}$ or GFP- GEF-$H1^{TS885A}$ along with HA-ERK2. In (B) cells were transfected with GEF-H1 shRNA along with HA-ERK with or without GFP- GEF-$H1^{T678A}$ or GFP- GEF-$H1^{TS885A}$. Details of the transfection are described under the Materials and Methods section. Cells were treated with 10 ng/ml TNF, as indicated, and HA-ERK was immunoprecipitated and its phosphorylation detected using Western blotting as described earlier. GEF-H1 and GFP were also detected in the cell lysates to assess downregulation of endogenous GEF-H1 and expression of the GFP-tagged mutants.

induced ERK activation (Figure 30, A and B, compare lanes 1–4). Expression of the human (siRNA resistant) GFP-GEF-H1T678A in cells in which endogenous GEF-H1 was silenced resulted in restoration of TNF-induced ERK phosphorylation (Figure 30, A and B, lanes 5 and 6). In contrast, expression of GFP-GEF-H1S885A did not promote TNF-induced ERK activation (Figure 30, A and B, lanes 7 and 8). Taken together, the data verify that phosphorylation of S885 but not T678 plays a key role in TNF-induced GEF-H1 and Rac-dependent ERK activation.

5.3.9. S885 in GEF-H1 is required for TNF-induced GEF-H1 activation toward RhoA

Previous studies implicated the S885 site in regulation of GEF-H1–induced RhoA activation (see chapter 1.3; Zenke et al. 2004, Callow et al. 2005, Birkenfeld et al. 2007, Meiri et al. 2009, Yamahashi et al. 2011). Our data described so far suggest that the S885 site could affect RhoA activation indirectly through regulation of ERK activation and subsequent T678 phosphorylation. However, it is conceivable that S885 phosphorylation is also a direct regulator of activity of GEF-H1 toward RhoA. To test this possibility, we first asked whether absence of the S885 phosphorylation site affects TNF-induced GEF-H1 activation toward RhoA. As shown in Figure 29B GFP-GEF-H1S885A showed no TNF-induced enhanced association with GST-RhoA(G17A). To further substantiate a potential direct effect of S885 on RhoA activation, we induced RhoA-specific GEF-H1 activation by overexpressing FLAG-p38. As described earlier, active p38 induces ERK activation even when GEF-H1 is silenced (Figures 24C). As expected, GEF-H1wt was activated by coexpression of p38 (Figure 31A). In contrast, activation of GFP-GEF-H1S885A by p38 was much reduced

Figure 31. Role of S885 in GEF-H1 activation towards RhoA

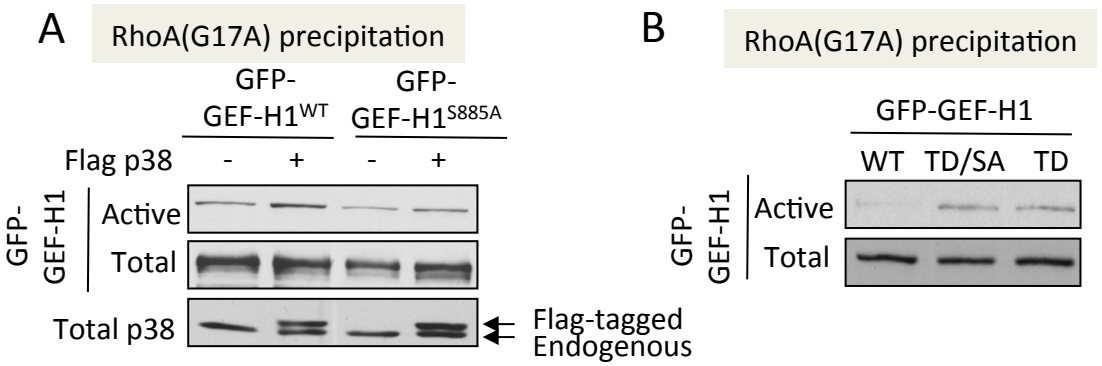


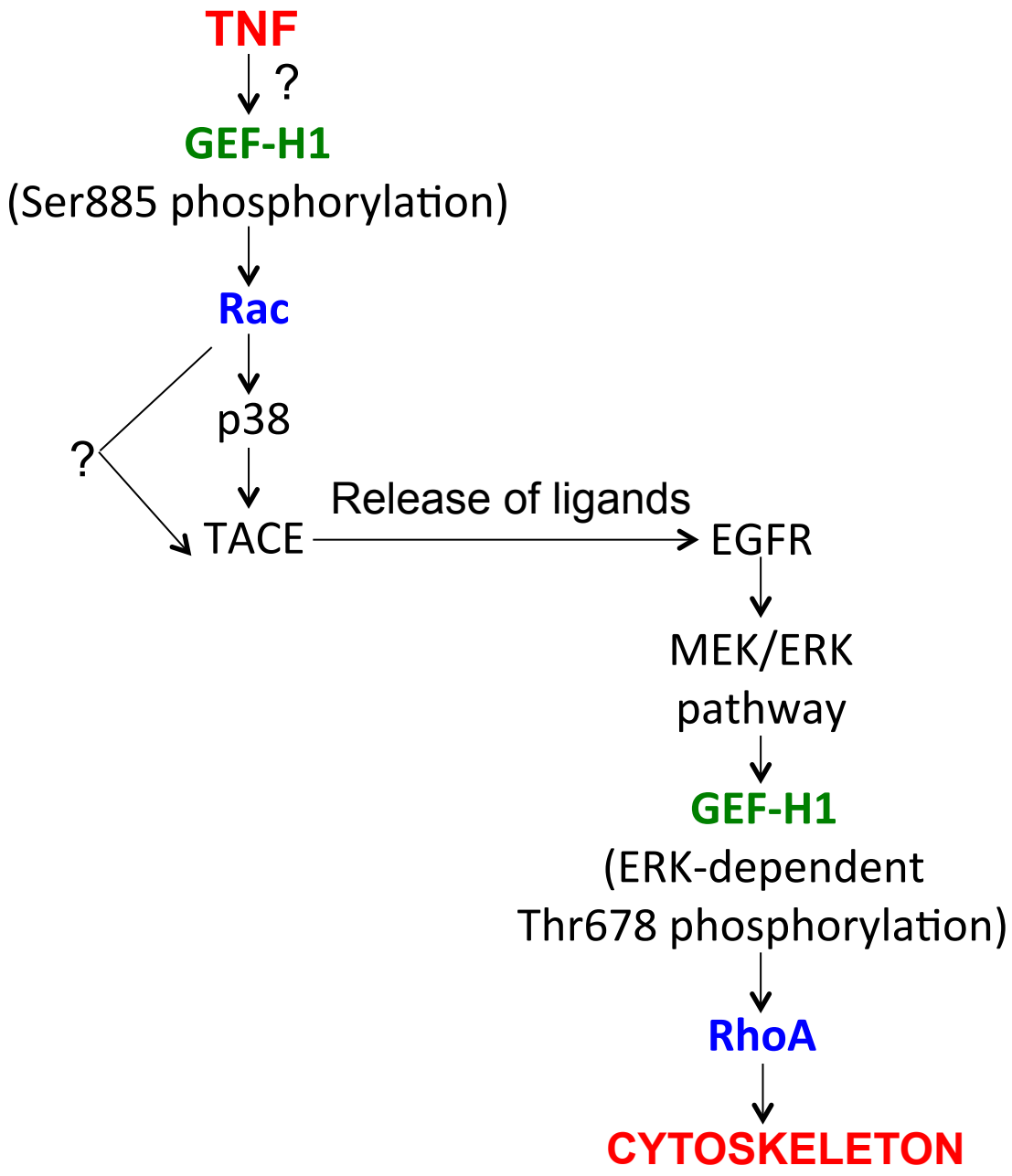
Figure 31. *(A and B) Role of S885 in GEF-H1 activation towards RhoA.* LLC-PK$_1$ cells were transfected with GFP-GEF-H1^{WT} or GFP-GEF-H1^{S885A} or GFP-GEF-H1^{T678D} (labelled as TD) or GFP-GEF-H1$^{T678D/S885A}$ (labelled as TD/SA) as indicated. Activated GFP-GEF-H1 was precipitated using RhoA(G17A) and detected by Western blotting with anti-GFP, as described earlier. In (A) p38 in the cell lysates was also detected. Please note that the transfected Flag-tagged p38 is visualized as an additional, higher band, (see arrows). Throughout the figure, representative blots of 3 independent experiments are shown.

(Figure 31A, lane 4). This finding suggests that S885 phosphorylation might directly regulate activation of GEF-H1 toward RhoA. An alternative possibility, however, is that effective phosphorylation of T678 (and thus activation toward RhoA) requires S885 phosphorylation even in the presence of active ERK. To test this possibility we generated a GEF-H1 molecule with a phosphomimetic mutation at T678 (GEF-H1T678D). As expected, this mutant showed enhanced precipitation with RhoA(G17A) compared with WT (Figure 31B), verifying that phosphorylation of this site mediates activation of GEF-H1 toward RhoA. Elimination of the S885 site by introducing an S885A mutation did not seem to alter this enhanced activity, as indicated by comparable precipitation of the T678D single and the T678D/S885A double mutants by RhoA(G17A). Taken together, our data suggest that S885 phosphorylation regulates TNF-induced GEF-H1 activation toward RhoA possibly through both direct and indirect effect(s) (see Discussion and Figure 32).

5.4. Discussion

TNF-induced EGFR transactivation in the tubular epithelium mediates ERK and RhoA activation, required for cellular responses, including junction remodelling and proliferation (Kakiashvili et al. 2011). The aim of this work was to explore mechanisms of TNF-induced EGFR transactivation. Our major findings are the following: 1) GEF-H1 and Rac are central regulators of TACE (not shown) and are essential for TNF-induced, p38-mediated activation of the TACE/EGFR/ERK pathway. 2) GEF-H1 mediates TNF-induced activation of both Rac and RhoA, but through different mechanisms. EGFR- and ERK-dependent phosphorylation of T678 is necessary only for GEF-H1 activation toward RhoA, whereas phosphorylation at the S885 site is necessary for activation toward both Rac and RhoA. Of interest, Rac and

Figure 32. Proposed mechanism of TNF-induced Rac and subsequent TACE/EGFR/ERK/GEF-H1/RhoA activation



RhoA are activated in a hierarchical manner because GEF-H1-stimulated Rac activation is a prerequisite for ERK-mediated GEF-H1 phosphorylation, which in turn is necessary for RhoA activation. Figure 32 summarizes the proposed mechanism of TNF-induced signalling toward Rac and RhoA. 3) TNF enhances epithelial migration in a wound-healing assay through GEF-H1, TACE, and ERK (not shown).

Many stimuli were shown to transactivate EGFR through ADAM family enzymes that release EGFR ligands, including HB-EGF, transforming growth factor-α, and amphiregulin (Liebmann 2011). Here we show that TNF activates ADAM17/TACE in tubular cells. Because the substrates of TACE include pro-TNF and the TNF receptors (Black et al. 1997, Moss et al. 1997; reviewed in Wajant et al. 2003), TNF-induced activation of this enzyme could represent a significant feedback step. TACE is believed to be regulated by translocation to the membrane, where it cleaves substrates (Schlondorff et al. 2000, Soond et al. 2005), and through phosphorylation by ERK, PDK1, Src and p38 (Diaz-Rodriguez et al. 2002, Soond et al. 2005, Zhang et al. 2006, Xu and Derynck 2010, Scott et al. 2011). Here, we show that GEF-H1 and Rac regulate TACE through p38. Rac is also a major regulator of NADPH oxidase (Miyano and Sumimoto 2007) and could potentially affect TACE through TNF-induced reactive oxygen species generation; however, this remains to be tested.

Interestingly, we found that RhoA silencing also reduced TACE and ERK activity. Although this could be partly due to reduced GEF-H1 expression caused by RhoA silencing, p38 activation under these conditions was only slightly decreased (unpublished data), suggesting that Rac and RhoA might regulate TACE and ERK through different mechanisms. RhoA might exert its effect through the cytoskeleton or by regulating translocation of the enzyme.

In colonic epithelial cells TACE conveys TNF-induced survival signals (Hilliard et al. 2011). TACE activation in these cells is MEK dependent but p38 independent (Liebmann 2011). The exchange factor mediating TNF-induced Rac activation might also be cell type dependent: in fibroblasts, TNF-induced Rac and cdc42 activation were shown to be mediated by Vav (Kant et al. 2011).

GEF-H1 is activated by physical stimuli, including mechanical force and hyperosmolarity (see chapter 6.1.2.1) (Birukova et al. 2010, Waheed et al. 2010, Guilluy et al. 2011, Heck et al. 2012, Nie et al. 2012, Ly et al. 2013), and its overexpression leads to cell transformation (Mizuarai et al. 2006) and promotes migration (Nalbant et al. 2009, Liao et al. 2012). When the results are taken together, it is conceivable that GEF-H1 is a central signalling hub for EGFR transactivation induced by a variety of stimuli. Such a role for GEF-H1 warrants further exploration and is discussed in chapter 6.2.

TNF traditionally was viewed as a proinjury cytokine, but a more complex picture is starting to emerge. In many epithelial cells, TNF promotes survival and proliferation, possibly due to transactivation of ErbB family receptors (Argast et al. 2004, Yamaoka et al. 2008, Hilliard et al. 2011, Kakiashvili et al. 2011). Our lab also shows that TNF enhances epithelial migration in a wound-healing assay. Importantly, this effect requires GEF-H1, as well as TACE and ERK. Thus, GEF-H1 might affect cell migration both as a regulator of TACE and EGFR transactivation and through ERK-dependent RhoA activation. In line with a central role of EGFR, its deletion in the proximal tubules was shown to delay recovery from acute kidney injury (Chen et al. 2012a, Chen et al. 2012b). However, EGFR overactivation can also contribute to nephropathies. Angiotensin II, a well-established fibrogenic factor, exerts some of its effects through TACE and EGFR (Chen et al. 2006, Shah and Catt 2006), and sustained

EGFR activation enhanced expression of transforming growth factor-β1 (TGFβ), a major inducer of epithelial–mesenchymal transition (EMT) and fibrosis (Chen et al. 2012). Additionally, RhoA and GEF-H1 were also shown to regulate expression of smooth muscle actin, a hallmark of EMT (Masszi et al. 2003, Fan et al. 2007, Tsapara et al. 2010, Ly et al. 2013) (chapter 6).

An important finding of this study is that GEF-H1 mediates both TNF-induced RhoA and Rac activation. Although most of the recent studies focus on RhoA activation by GEF-H1, earlier it was also shown to exert Rac-GEF activity (Ren et al. 1998). Further, Tonami et al. (2011) recently showed that knockdown of calpain-6 resulted in GEF-H1-dependent Rac activation. Our study provides the first example of a signalling pathway in which GEF-H1 can act as an activator of both Rac and RhoA, depending on its phosphorylation state. EGFR- and ERK-dependent phosphorylation of T678, required for GEF-H1-mediated RhoA activation, is not needed for Rac activation.

In contrast, surprisingly, Rac is upstream from the T678 phosphorylation. S885 phosphorylation is a prerequisite for both TNF-induced Rac and RhoA activation. Mass spectrometry analysis, as well as Western blotting with a phospho-S885-specific antibody, revealed that S885 is phosphorylated both in unstimulated and TNF-stimulated cells, with a trend for enhanced S885 phosphorylation in TNF-treated cells. Of importance, a nonphosphorylatable mutant of S885 no longer showed enhanced association with the nucleotide-free Rac(G15A) upon TNF stimulation. This mutant was also not activated toward RhoA by TNF and showed reduced activation upon p38-induced stimulation of the ERK pathway, which is independent of Rac. These data imply that the S885 site might have a direct role in GEF-H1 activation toward RhoA. Additionally, we found that introducing an

S885A mutation into an active GEF-H1 containing a phosphomimetic mutation at T678 (GEF-H1T678D/S885A) did not reduce its activity toward RhoA. Thus, it is likely that natural phosphorylation of T678 depends upon the S885 site not only because of the demonstrated indirect effect (through ERK), but also through an additional (direct) effect. Our future work will address this.

When the results are taken together, the S885 site seems to play a central role in GEF-H1 activation. Indeed, this site was shown to be phosphorylated by many kinases, including PAK1, PAK4, Aurora A, Par1b/MARK2, and PKA (Table 1) (Zenke et al. 2004, Callow et al. 2005, Birkenfeld et al. 2007, Meiri et al. 2009, Yamahashi et al. 2011). It was also suggested to regulate binding to microtubules (Zenke et al. 2004, Callow et al. 2005). In line with our present findings, Callow et al (2005) showed that expression of a phosphorylation-incompetent mutant of the S810 site in the short splice variant GEF-H1M (analogous to S885 in the full protein) reduced the abundance of stress fibers in fibroblasts. Further, a phosphomimetic S885D mutant showed enhanced RhoA activation (Birkenfeld et al. 2007). However, S885 phosphorylation is likely not the only switch turning on the protein. S959 (Birkenfeld et al. 2007), S142, and S3 (Callow et al. 2005, Yoshimura and Miki 2011) were also implicated in GEF-H1 regulation. Interestingly, single S885 mutants seem to show opposite effects to those of double mutants of S885 and S959, suggesting collaboration between these sites (Birkenfeld et al. 2007, Yamahashi et al. 2011). Overall, it is likely that differential single or double phosphorylation/dephosphorylation of these sites and T678 can fine-tune the RhoA and Rac exchange activities of GEF-H1. The potential role of other serine sites and kinases targeting them in TNF-induced GEF-H1 activation remains to be established.

Finally, our study demonstrates a hierarchical relationship between TNF-induced Rac and RhoA activation in tubular cells. This concept is in line with the pioneering studies of Alan Hall and his group showing that active Rac in fibroblasts stimulates RhoA (Ridley et al. 1992, Nobes and Hall 1995). Our findings provide a possible mechanism for a hierarchy between Rac and RhoA: GEF-H1-dependent Rac activation regulates the RhoA exchange activity of GEF-H1 by controlling its ERK-mediated T678 phosphorylation (Figure 32). In many cells Rac and RhoA activities were reported as mutually antagonistic or spatially restricted (e.g., Rac in the front, RhoA in the back of migrating cells). However, a more complex picture is emerging, suggesting that activation of the two GTPases can spatiotemporally coexist, for example, at the lamellipodium (Figure 11) (Kurokawa et al. 2005, Pertz et al. 2006, Pertz 2011). Such context-dependent fine-tuning requires tight pathway-specific control of regulators. I will discuss this further in chapter 6.1.4.

This research provides a prominent example of a mechanism that can achieve differential regulation, coordination, and coupling of activities of a single GEF toward Rac and RhoA, likely contributing to complex functions such as epithelial sheet migration. Understanding such mechanisms could help in the development of strategies to selectively affect Rac- or RhoA-specific activation of GEFs.

CHAPTER 6 Overall Conclusions, General Discussion and Future Directions

6.1. Conclusions and Discussion

The aim of this thesis was to investigate the mechanism through which the small GTPases, Rac and RhoA, are regulated in tubular cells, and to explore the role and specific regulation of GEF-H1 in hyperosmotic stress- and TNF-induced signalling in tubular cells. To this purpose, we designed studies that addressed the following questions: a) Does hyperosmotic stress activate GEF-H1, and if yes, is GEF-H1 required for the hypertonicity-induced, and Rho/ROK-mediated, nuclear translocation of MRTF? b) Is GEF-H1 required for TNF-induced Rac activation? c) How is the specificity of GEF-H1 activation towards Rac/RhoA regulated?

In summary, our results demonstrate that GEF-H1 is indeed activated by hyperosmotic stress, and is required for hypertonicity-induced RhoA activation. Additionally, GEF-H1 is necessary for hyperosmotic stress-induced translocation of MRTF to the nucleus. We also found that GEF-H1 is a TNF-activated exchange factor for both Rac and RhoA. Phosphorylation of GEF-H1 on different sites mediates its specificity towards the small GTPases; T678 phosphorylation is required for TNF-induced GEF-H1 activation towards RhoA, whereas the S885 phosphorylation is necessary for both the Rac and RhoA activation. Hence, our studies reveal a hierarchy in small GTPase activation, where Rac is required for TNF-induced RhoA activation.

Limitations of experimental approach and results

There are two major limitations of our studies that I would like to discuss. First, a significant portion of my work utilizes affinity precipitation assays to detect activation of RhoA, Rac, or GEF-H1. This assay, as any other experimental technique, has limitations. Firstly, the activation assay may not be sensitive enough to detect subtle overall activation of

RhoA/Rac/GEF-H1. Furthermore, it cannot detect localization-specific activation of these proteins in cells. Secondly, as the protocol requires centrifugation of cell lysates, this step excludes activated proteins bound to the cytoskeleton (which is sedimented). Thirdly, the bead preparation (e.g., GST-RhoA(G17A)) used to capture activated proteins in the cell lysate has to compete with endogenous proteins that also bind activated RhoA/Rac/GEF-H1. Fourthly, activation of target proteins is detected in cell lysates and not live cells.

The second important point that needs to be made is that our experiments have elucidated an important signalling pathway using a tubular cell line as a model. These studies have provided important new knowledge about molecular mechanisms involved in cytoskeleton and junction regulation. Activation of pathways will also have to be verified in in vivo models. Kidney injury animal models will allow the verification of the importance of our findings in kidney disease.

6.1.1. GEF-H1 as a central stress-sensor molecule

This thesis has so far described various conditions under which GEF-H1 is activated, and discussed the underlying pathways (as shown by our lab). These stimuli can be classified as 1) physical stressors, e.g., hyperosmotic shock, and plasma membrane depolarization, and 2) chemical stressors, e.g., TNF, and immunosuppressive drugs. Hence, we propose that GEF-H1 is a stress-sensor molecule that is able to convey danger signals received by the cell to Rho proteins. This then enables Rho GTPases to induce cytoskeleton remodelling, which can be adaptive or disruptive. Several examples support the role of GEF-H1 as a stress-sensor. The following sections will discuss these.

6.1.1.1. Activation of GEF-H1 by physical stress

Hyperosmotic stress as an activator of GEF-H1

As discussed in chapter 4, cytoskeleton remodelling is an immediate response to osmotic stress that allows the cell to cope with the resulting mechanical trauma. Our lab has previously shown that hyperosmotic stress activates the Rho/ROK/pMLC pathway. However, the mechanism of this activation was unknown. We have now successfully demonstrated that GEF-H1 is activated by osmotic stress, and mediates activation of the Rho pathway. Moreover, GEF-H1 also mediates Rho-dependent translocation of MRTF to the nucleus. As discussed earlier, MRTF is a transcriptional coactivator of SRF, which together drive expression of several cytoskeletal genes. Hence, this suggests a further role for GEF-H1 in transcriptional reprogramming of the cytoskeleton.

Activation of GEF-H1 by plasma membrane depolarization

Depolarization of the plasma membrane is yet another stress stimulus that has been shown to induce changes in cytoskeleton organization (reviewed in Chifflet and Hernandez 2012). Our own lab has demonstrated that plasma membrane depolarization induces activation of Rho/ROK, leading to phosphorylation of MLC. Increased pMLC enhances paracellular permeability (chapter 1.2.4.2). Importantly, we identified GEF-H1 as the exchange factor that is activated by depolarization, leading to Rho activation. Depolarization-induced activation of GEF-H1/Rho/ROK is in turn mediated by the MAPK ERK. Indeed, GEF-H1 has previously been shown (by others as well as our lab) to bind to ERK, which phosphorylates GEF-H1 on Thr678 and activates it (Fujishiro et al. 2008, Kakiashvili et al. 2009). Thus, our work has so far uncovered two examples of stimuli that activate GEF-H1 through ERK. Hence, the ERK-mediated activation of GEF-H1 might be a central and general mechanism

for Rho activation. Further, since both the ERK and Rho pathways have been implicated in regulation of gene expression and cell cycle, it can be hypothesized that some of the proliferative effects of ERK might be due to its impact on Rho (reviewed in Vega and Ridley 2008).

Activation of GEF-H1 by mechanical force through integrins

Further substantiating the role of GEF-H1 as a stress-sensor, recently Guilluy and others showed that GEF-H1 and LARG were both activated by mechanical force (Guilluy et al. 2011). Application of force on integrins triggers rearrangement of the cytoskeleton, leading to an increase in cellular stiffness (Wang et al. 1993, Matthews et al. 2006). Although Rho has been shown to be involved in cellular stiffness, its mechanism of activation was unknown (Matthews et al. 2006). Guilluy et al. showed that inhibition of both GEF-H1 and LARG using siRNA prevented the force-induced Rho activation. Moreover, ERK inhibition prevented force-induced GEF-H1 activation, providing one more example for ERK being a regulator of GEF-H1. Interestingly, activation of LARG was dependent not on ERK, but the Src family tyrosine kinase Fyn. Hence, the ERK/GEF-H1/Rho pathway is emerging as a signalling cascade that connects stress signals to cytoskeletal remodelling.

6.1.1.2. Activation of GEF-H1 by Chemical stress

TNF

Inflammatory cytokines such as TNF cause chemical stress to cells. As discussed in chapter 1.4.1, TNF has been known to alter barrier properties. To this effect, our lab has previously shown that TNF activates the Rho/ROK/pMLC pathway through GEF-H1, leading to an

increase in paracellular permeability. We have also shown that TNF enhances migration of tubular epithelial cells (Szaszi et al. 2012). Additionally, we have also investigated the pro-survival and proliferative effects of TNF, showing that TNF transactivates EGFR, which appears to promote cell migration and wound healing in tubular cells. Adding to the proliferative effects of TNF, our most recent studies also show that TNF enhances wound healing in tubular cells (Waheed et al. 2013) (chapter 5). Downregulation of GEF-H1, or inhibition of ERK diminishes the effect of TNF on wound healing in tubular cells, showing the importance of ERK/GEF-H1 activation in wound healing/cell migration. Hence, we hypothesized that EGFR transactivation that induces ERK activation could be the switch between the pro-apoptotic and pro-survival TNF pathways. Further studies are needed to verify this and to explore the exact role of the crosstalk between the TNF receptor and EGFR signalling pathways. I will discuss the role of GEF-H1 in EGFR transactivation in chapter 6.1.3.

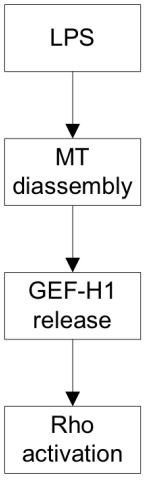
Immunosuppressants

The immunosuppressive drugs, Cyclosporin A (CsA) and Sirolimus (SRL), when used together, suppress transplant rejection and are widely used after kidney transplantation. However, this combination of drugs also has adverse side effects, which includes nephrotoxicity. Martin-Martin et al. have previously shown that CsA and SRL decrease paracellular permeability in kidney tubular cells (Martin-Martin et al. 2010). This might explain inadequate tubular re-absorption and magnesium wasting, as shown in a rat model (Andoh et al. 1996, Clarke and Ryan 1999). Disturbance of Mg^{2+} homeostasis has been described to be an important element of nephrotoxicity induced by CsA treatment (Clarke and Ryan 1999). More recently, our lab has shown that CsA and SRL activate the Rho/ROK

pathway (Martin-Martin et al. 2012). CsA and SRL also lead to phosphorylation (and hence inactivation) of the actin depolymerizing protein cofilin. Cofilin phosphorylation has been shown to be downstream of Rho/ROK activation, and leads to enhanced actin polymerization. Importantly, we showed that GEF-H1 contributes to the CsA and SRL-induced Rho activation, as well as phosphorylation of cofilin. GEF-H1 downregulation significantly decreased the appearance of stress fibers and cofilin phosphorylation. Hence, these results further solidify the role of GEF-H1 as a stress sensor molecule that links stress signals to cytoskeletal regulation.

Reactive oxygen species (ROS) and bacterial endotoxins

Oxidative stress caused by bacterial endotoxins, such as LPS, has been shown to cause pathological activation of endothelial cells, as well as barrier dysfunction. In endothelial cells, LPS treatment leads to increased ROS production and oxidative stress that causes cytoskeletal remodelling, the formation of paracellular gaps, and increased cellular permeability. ROS trigger activation of various cellular pathways, including Rho, p38 stress kinase, and NFκB. These signalling cascades are responsible for barrier dysfunction in endothelial cells.



Kratzer et al. recently investigated the effects of LPS-induced oxidative stress on Rho activation and microtubule dynamics (Kratzer et al. 2012). They noted that LPS induced disassembly of microtubules, which led to release of GEF-H1. Further, GEF-H1 was required for LPS-induced activation of Rho signalling, which led to barrier dysfunction in pulmonary endothelial cells, and aggravation of inflammation.

Further evidence of oxidative stress-induced GEF-H1 regulation comes from Guo et al. in Human umbilical vein endothelial cells (HUVECs) (Guo et al. 2012, Guo et al. 2012). In brief, these studies show that LPS increases expression of GEF-H1, and that GEF-H1/Rho signalling is required for LPS-induced activation of the transcription factor NFκB. NFκB is a central regulator of inflammatory response to tissue injury and infection (Yasumoto et al. 1992; chapter 1.2.4).

Fukazawa et al. investigated the effect of the enteroinvasive pathogenic bacteria *Shigella flexneri* on the intestinal barrier (Fukazawa et al. 2008). They found that cell invasion by *Shigella* required GEF-H1-mediated activation of Rho. Moreover, like Guo et al., Fukazawa et al. also noted GEF-H1 was essential for the activation of NFκB induced by *Shigella* infection. Hence, the authors concluded that GEF-H1 is an important player in cellular defense during pathogen invasion.

In contrast to the findings described above, we have found that GEF-H1 does not mediate the TNF-induced activation of NFκB. This conclusion is based on experiments showing that GEF-H1 silencing does not affect the translocation of p65 to the nucleus or the degradation of the inhibitory protein IκBα; phenomena that are associated with NFκB activation (Kakiashvili et al. 2009, Waheed et al. 2013). This might be explained as a stimulus-specific effect, as Fukazawa et al. also showed that in HEK293 cells, GEF-H1 downregulation had no effect on the TNF-induced activity of an NFκB-dependent promoter. Hence, these observations might imply that while LPS- and *Shigella*-induce NFκB activation in a GEF-H1-dependent mechanism, TNF can activate NFκB independent of GEF-H1/Rho signalling, as observed by our lab.

Transforming growth factor β1 (TGFβ)

Both our own findings and data from literature implicate GEF-H1 in fibrogenesis and regulation of EMT. TGFβ is a key fibrogenic and EMT-inducing cytokine that promotes cell differentiation. It has been implicated in cancer, immunity, heart disease, diabetes, kidney fibrosis, etc. (reviewed in Massague 2012). Tsapara et al. reported that in the retinal pigment epithelium (RPE), TGFβ contributes to retinal dysfunction and fibrosis. These effects are due to upregulation of GEF-H1 levels, leading to Rho activation (Tsapara et al. 2010). TGFβ in RPE also enhanced α-smooth muscle actin (SMA) expression, cell migration and de-differentiation. GEF-H1 inhibition prevented the α-SMA upregulation and enhanced cell migration. Additionally, migratory RPE cells had increased GEF-H1 levels, as demonstrated in samples obtained from patients. This suggests a role for GEF-H1 in fibrotic processes of the retina in vivo. Hence, the authors propose that not only is GEF-H1 a target of TGFβ, but it might also be a TGFβ effector that leads to Rho signalling and regulation of gene expression. These results are in line with our own findings that implicate GEF-H1 as a mediator of hyperosmotic stress-induced MRTF nuclear translocation. Thus, we see a solidifying picture of GEF-H1 as a critical cellular reprogrammer in response to stress stimuli.

6.1.2. GEF-H1 as a major regulator of cross-talk between inflammatory stimuli and the proliferative pathway

Transactivation of the EGFR

As discussed in chapter 1.3, GEF-H1 has been implicated in various diseases including hypertension, cancer, and fibrosis (reviewed in Birkenfeld et al. 2008), making it a possible

therapeutic target. As discussed above, the fibro-proliferative effects of GEF-H1 likely involve its role in MRTF nuclear translocation. Further, it is activated by EMT-inducing stimuli, including TGFβ. Transactivation of EGFR by TNF and subsequent ERK activation, however, might be the major pathway linking inflammatory signals and cell proliferation through GEF-H1 (see Figure 6b). Hence, we propose that GEF-H1 is a central regulator of TNF-induced EGFR transactivation events, whereby it connects TNF-induced cell stress response to EGFR/ERK-mediated proliferation.

Our studies explored the functional significance of TNF-induced EGFR transactivation. Using two methods to follow cell proliferation, an ECIS-based assay and the classical BrdU incorporation assay, our lab has shown that TNF enhances proliferation in tubular cells (Kakiashvili et al. 2011). Moreover, this effect is directly dependent on the transactivation of EGFR, since blocking EGFR activation prevents the increase in proliferation. Our recent findings also indicate that EGFR transactivation mediates TNF-induced stimulation of wound healing (Waheed et al. 2013).

EGFR activation might help wound healing upon injury, enhancing recovery. However, a tilted balance leading to EGFR overactivation could also contribute to the detrimental effects of various mediators (Chen et al. 2012b). TNF, like Angiotensin II was also implicated in the development of chronic kidney disease (Vielhauer and Mayadas 2007). Therefore, our finding that TNF can also exert some effects through EGFR in kidney cells raises the possibility that EGFR activation is a common key step in kidney diseases. Indeed, it is now acknowledged that EGFR signalling exerts a dual effect in the kidney. While it promotes wound healing in short exposures, its long-term effects are probably deleterious, at least

partly because it can promote pro-fibrotic events, such as EMT (e.g. Smith et al. 2009, Chen et al. 2012b, and see above).

As discussed in Chapter 5, GEF-H1 is required not only for TNF-induced transactivation of the EGFR, but silencing GEF-H1 decreases EGFR levels. This might be due to an effect on EGFR trafficking. Indeed, GEF-H1 has been shown to regulate membrane trafficking via the exocyst complex (Pathak et al. 2012, Pathak and Dermardirossian 2013). Therefore, downregulation of GEF-H1 might prevent recycling of the EGFR to the cell surface, leading to its degradation. This not only provides GEF-H1 with another level of control over the EGFR pathway, but also raises the possibility that elevated GEF-H1 levels found in various pathophysiological conditions could also lead to increased EGFR levels. This, however, remains to be verified. Overexpression of EGFR (and/or its ligands) is widely acknowledged to have a critical role in cancer development and metastasis (reviewed in Liebmann 2011). Therapies targeting EGFR, hence, could also target proteins involved in transactivation of the EGFR, such as GEF-H1.

Epidermal Growth Factor (EGF)

As demonstrated by our lab, the fact that EGF activates GEF-H1 in tubular cells is important since this shows direct activation of GEF-H1 by a growth factor. GEF-H1 is also required for the EGF-induced activation of Rho. This result is in line with the finding that TNF activates RhoA by transactivating the EGFR, and that GEF-H1/RhoA signalling plays a role in proliferation and cell cycle regulation (Bakal et al. 2005, Birkenfeld et al. 2007; chapter 1.3). Thus, taken together, our findings suggest that various stimuli can activate GEF-H1, leading to cytoskeletal regulation, proliferative signalling and cellular reprogramming.

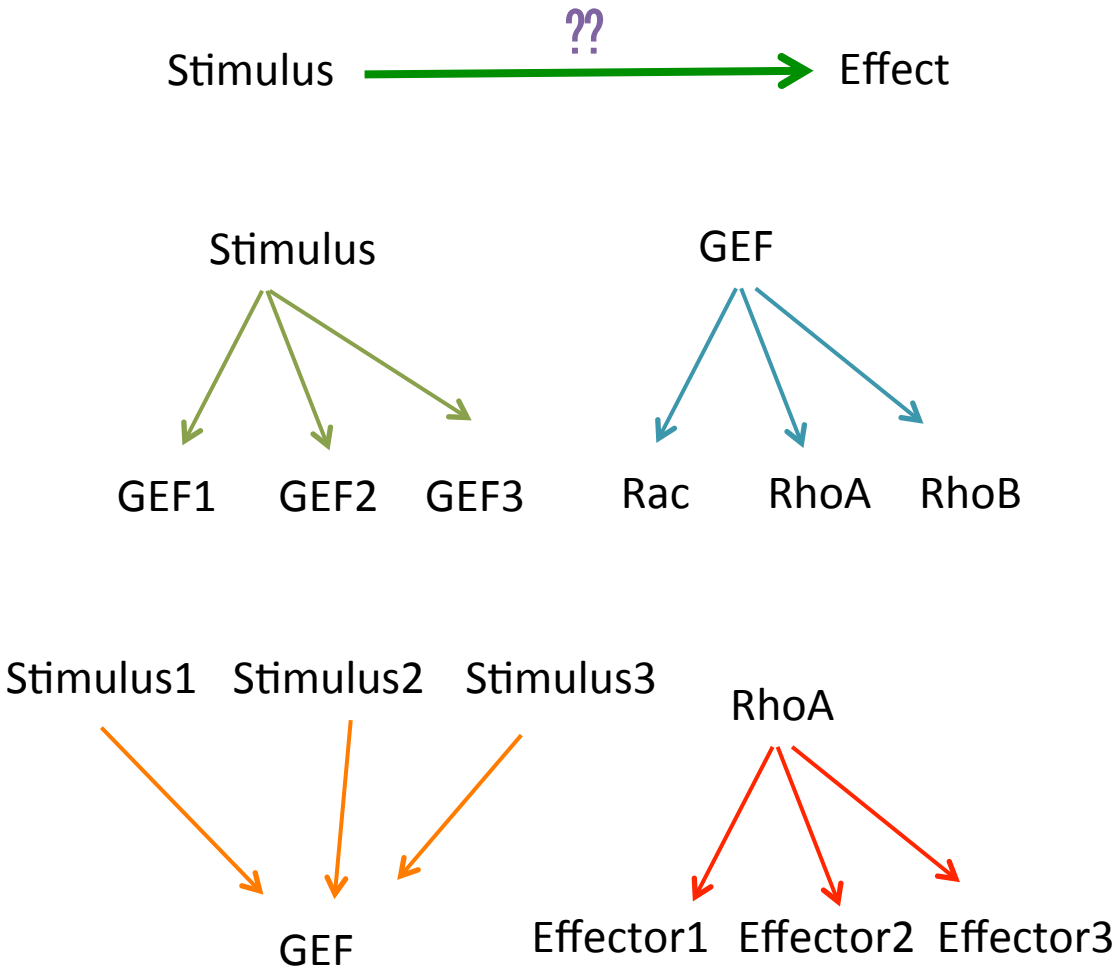
6.1.3. Regulation of the Rho pathway: Role of the many GEFs

6.1.3.1 Challenges in understanding Rho regulation

Understanding of the complex network of Rho regulators, effectors and Rho proteins themselves is incomplete. The most important question that arises from our own work and that of others is, how is specificity in the signalling pathway (stimulus/GEF/RhoGTPase) achieved? There are over 70 GEFs that have been described. Any given stimulus could activate several different GEFs, and conversely, several different stimuli can activate any given GEF (as in the case of GEF-H1) (Figure 33). Also, one GEF can show specificity towards more than one Rho GTPase; GEF-H1 can activate both Rac and Rho in tubular cells. Additionally, a single Rho GTPase, such as Rho, can activate several different effectors. The questions that stand out from these observations are several. We need to ask not only how specificity is achieved, but also whether the function of a GEF is specific, or are GEFs redundant. That is, for a GEF in question, is it only that specific GEF that can perform a particular function, or will another closely related GEF suffice? Also, what determines how a GEF is regulated in a context- and stimulus-specific manner?

The GEF-H1 activation assays that we have adapted and optimized in our lab allowed us to ask questions about the context- and stimulus-specific activation of GEF-H1 towards Rho and/or Rac. Surprisingly, we found that GEF-H1 is able to regulate its own activation. Specifically, S885 phosphorylation of GEF-H1 induced by TNF, is required for the subsequent activation of Rac, and the downstream transactivation of EGFR. This then promotes ERK activation, which is required for T678 phosphorylation of GEF-H1 that is necessary for TNF-induced Rho activation. Hence, GEF-H1 acts both upstream and

Figure 33. Context- and stimulus-specific activation of Rho GTPases, GEFs, and downstream effectors



downstream of ERK activation. This signalling pathway described by us highlights how GEF-H1 can control Rho activation at multiple levels by connecting different types of signalling.

We have also discovered GEF-H1 to be a critical player in Rho activation induced by different stimuli. As discussed earlier (also in chapter 1.3), we and others have placed GEF-H1 in a potentially non-redundant role in vital processes such as cell migration, endothelial permeability regulation, cellular reprogramming and regulation of the cytoskeleton. Hence, understanding the mechanism of activation of this protein by various stimuli is an important future project.

6.1.3.2. Hierarchy in Rho GTPase activation

Although GEF-H1 was initially described as a GEF for both RhoA and Rac (Ren et al. 1998, Gao et al. 2001), later studies emphasized it primarily as a Rho GEF, whose activity could be inhibited by Rac-induced phosphorylation. However, as shown in chapter 5, our lab discovered that TNF activates GEF-H1 towards both RhoA and Rac. This observation propelled us to ask how the RhoA and Rac activation of GEF-H1 was coordinated. We found not only that site-specific phosphorylation played a crucial role in ascertaining GEF-H1 activation towards Rac and RhoA, but also that there was a hierarchy in Rho GTPase activation by TNF. Downregulating Rac in tubular cells prevented TNF-induced Rho activation. These findings are indeed in line with the initial studies by Alan Hall and coworkers (Nobes and Hall 1995) that described a hierarchical activation of the three best-characterized Rho GTPases (discussed in chapter 1.2.6). In fibroblasts, active mutants of Cdc42, Rac and RhoA activated each other in a hierarchical manner. Our findings now

provide a possible mechanism that could explain how RhoA activation is regulated by Rac activity. Our work places GEF-H1 in the center of such a hierarchical activation.

Interestingly, more recent studies emphasize a primarily antagonistic relationship between Rho and Rac. In a recent review by the Burridge group, Guilluy et al highlight the different mechanisms that interconnect the Rho family proteins (Guilluy et al. 2011). RhoA and Rac1 have both been shown to inhibit each other. There are, however, mechanisms via which a positive regulation between RhoA and Rac1 exists. For example, the Rho effector mDia has been shown to activate Rac1. This is in contrast to the effects of ROK, another major Rho effector, which inhibits Rac1 activity (Tsuji et al. 2002). The role of Rac in the regulation of GEF-H1 could also represent a positive regulation mechanism between Rac and RhoA. Taken together, these observations suggest an intricate and essential coordination and crosstalk between Rho proteins. One function in which such a subtle coordination of the small GTPases has a critical role is cell migration where the Rho GTPases Cdc42, Rac, and Rho are essential in executing efficient movement.

6.2. Future Directions

My major findings, as described in this thesis, also opened up several new avenues of future studies with lots of new and exciting questions. The main one that jumps out is the question of the upstream mechanisms through which GEF-H1 is activated in a stimulus- and context-specific manner. Although our work and that of other labs has demonstrated mechanisms through which some stimuli affect GEF-H1, this question still remains largely unanswered. It would be interesting to find out whether phosphorylation and altered microtubule binding is a general mechanism for GEF-H1 activation by various activating stimuli. And finally, exploring the functional role and significance of GEF-H1 and its phosphorylation in animal disease models is an important future step.

6.2.1. Regulation of GEF-H1 activation

Our studies have shown that GEF-H1 is activated by various stress stimuli. However, the mechanism of this activation has not been fully worked out. As discussed in chapter 5.4, GEF-H1 activation can occur upon phosphorylation, or its release from microtubules or TJs. Although our studies have defined a key role for GEF-H1 phosphorylation, the responsible kinases and kinetics of the combinatory phosphorylation remain to be clarified. Future studies can focus on the role of both these factors on GEF-H1 activation.

Upstream kinase phosphorylating GEF-H1 on Ser885

In search for the kinase targeting Ser885, we have investigated the role of kinases known to phosphorylate GEF-H1, such as PAK1, PAK4, and PKC. However, we did not find any of these kinases to be upstream of S885 phosphorylation of GEF-H1 (results not shown). The

PKA inhibitor, H89, however, decreased the TNF-induced GEF-H1 activation towards both Rac and RhoA (Figure 34). Future studies using siRNA against PKA should verify these findings. Further studies should also investigate other kinases such as Focal Adhesion Kinase (FAK), which is activated by TNF and hyperosmotic stress, and is known to be involved in cytoskeletal regulation. Inhibition of FAK has been shown to prevent mechanical force on integrin-induced GEF-H1 activation towards RhoA (Guilluy et al. 2011). Hence, it would be interesting to find out if FAK regulates TNF-induced activation of Rac (and RhoA).

Role of microtubule binding in GEF-H1 activation

As mentioned earlier, GEF-H1 binds to and is regulated by the microtubules (Ren et al. 1998; reviewed in Birkenfeld et al. 2008). The polymerization state of microtubules appears to control GEF-H1 binding to this structure. Thus, microtubule depolymerization, induced by drugs such as Nocodazole, releases GEF-H1 into the cytosol, leading to its activation towards RhoA (Krendel et al. 2002, Chang et al. 2008). Conversely, using a microtubule co-sedimentation assay, Tonami et al. have demonstrated that the microtubule-stabilizing drug, Taxol, increases the amount of GEF-H1 that is bound to microtubules (Tonami et al. 2011).

However, whether binding to microtubules has a differential role in regulating GEF-H1 towards Rac or RhoA is not known. As evident from Table 1, there is conflicting data on how the microtubule-binding status of GEF-H1 correlates with its activation towards RhoA (Zenke et al. 2004, Callow et al. 2005). Zenke et al. showed that phosphorylation of GEF-H1 at S885 increases microtubule binding of GEF-H1, and decreases Rho activation. However, according to Callow et al., phosphorylation at the same site by PAK4, decreases microtubule binding of GEF-H1 and the formation of stress fibers (sign of Rho activity), but increases

Figure 34. PKA may mediate GEF-H1 activation towards Rac and RhoA

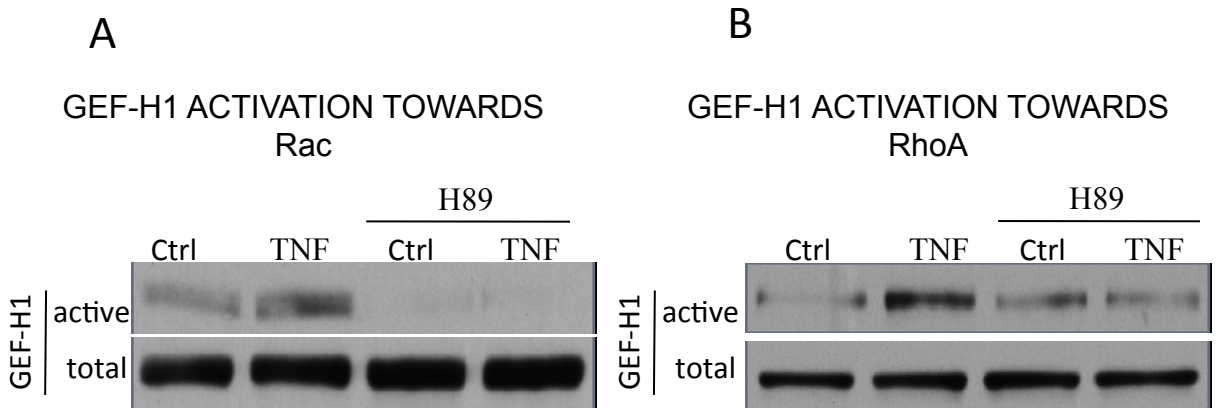


Figure 34. *PKA may mediate GEF-H1 activation towards Rac and RhoA.* LLC-PK_1 cells grown in 6 cm dishes were serum-starved for at least 4 hours upon confluency. They were pre-treated for 30 min with 10 μM H89 followed by 10 ng/mL TNF treatment for 2 min. Active GEF-H1 was precipitated using GST-Rac (G15A) (A) and GST-Rho(G17A) (B) and normalized to total GEF-H1 in the total cell lysates. Representative blots are shown.

formation of lamellipodia (sign of Rac activation). The reason for these discrepancies is not clear. One possibility is that other sites that are differentially phosphorylated and have not been looked at in these studies also affect activation towards Rac and Rho. One such site, based on our findings, could be T678.

Future studies in our lab could address this question. We can utilize Taxol and Nocodazole, microtubule stabilizing and depolymerizing drugs, respectively, to investigate how the microtubule polymerization state induces GEF-H1 activation towards not only RhoA, but also Rac. This can be achieved by using GEF-H1 activation assays (RacG15A and RhoG17A pull-downs). Additionally, Nocodazole-induced RhoA or Rac activation, respectively, can also be assessed by performing RhoA/Rac activation assays. The role of GEF-H1 in these studies can be demonstrated by using siRNA against it. Results from such studies would indicate directly whether microtubule dynamics affect actin dynamics through not just Rho, but also Rac.

Surprisingly, in preliminary results, we have found that stabilizing microtubules with Taxol may enhance basal GEF-H1 activation towards Rac (Figure 35). This finding is really interesting, since it makes us wonder whether GEF-H1 activation towards Rac is dependent on its binding to microtubules, and hence different from Rho activation. In contrast, however, two other studies show that release of GEF-H1 from microtubules leads to enhanced Rac activation (Table 1, pp.73) (Callow et al. 2005, Tonami et al. 2011). Follow-up studies should shed more light on this puzzle.

An interesting question that arises from the above line of questioning is whether various stimuli activate GEF-H1 by altering its microtubule binding. Although microtubules can

Figure 35. Microtubule stabilization may mediate GEF-H1 activity towards Rac

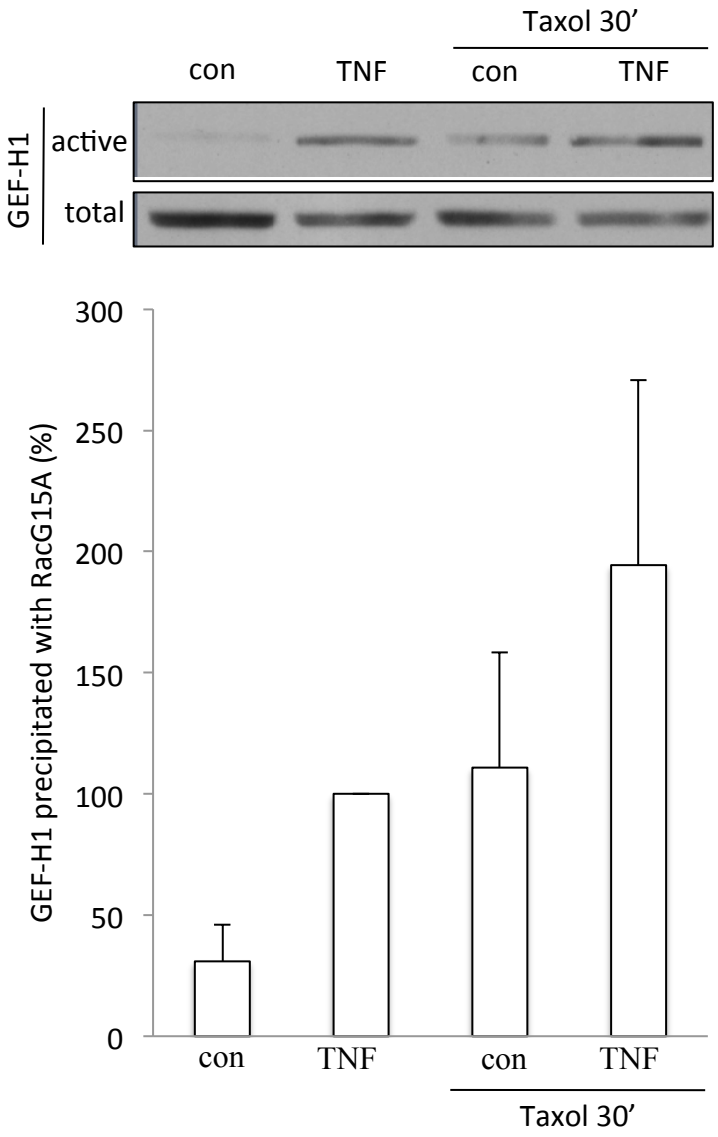


Figure 35. *Microtubule stabilization may mediate GEF-H1 activity towards Rac.* LLC-PK_1 cells grown in 6 cm dishes were serum-starved for at least 4 hours upon confluency. They were pre-treated for 30 min with 2 μM Taxol followed by TNF treatment for 2 min. Active GEF-H1 was precipitated using GST-Rac (G15A) and normalized to total GEF-H1 in total cell lysates. Densitometric analysis was done as described in the methods. The graphs show mean ± S.E. from n=4 independent experiments.

regulate GEF-H1 in some unstimulated cells, it is not clear whether they are involved in mediating the effects of various activating stimuli as well. Thus, it can be tested whether different stimuli activate GEF-H1 by enabling its release from microtubules. Microtubule binding of GEF-H1 can be tested biochemically, by using the microtubule co-sedimentation described in Tonami et al. (2011). A second way of studying association of GEF-H1 with microtubules in live cells is through the use of Fluorescence Recovery After Photobleaching (FRAP). FRAP is a single cell, live imaging technique that allows measurement of the diffusion of a fluorescently-tagged molecule using live imaging with a fluorescence microscope. Using high intensity illumination fluorescence within a small area of the cell is bleached, leaving a dark spot (Axelrod et al. 1976). Replacement by still-fluorescing molecules from the surrounding areas is then followed. This rate of motion depends on the freedom of the diffusing molecules. If, for example, these molecules are bound to microtubules, their rate of diffusion will be slowed. Using either the microtubule binding assay or FRAP, we can explore whether stimuli such as TNF, EGF, hyperosmolarity, or plasma membrane depolarization, alter GEF-H1 binding to microtubules. For FRAP, cells will be transfected with GFP-GEF-H1, and FRAP will be performed in control cells and cells treated with GEF-H1 activating stimuli. We can assess whether any of the stimuli induce a higher rate of diffusion of GFP-GEF-H1 compared to the non-treated control, which would suggest release from microtubules. Taxol-induced microtubule stabilization can be used as a negative control, and Nocodazole-induced microtubule disruption can serve as a positive control.

A further step can be to assess whether the phosphorylation status of GEF-H1 at T678 and S885 affects its microtubule binding. We have thus far seen evidence that phosphorylation

status of GEF-H1 determines its activity towards Rac or RhoA (chapter 5), and microtubule association has been shown to play different roles in the activation of these two Rho GTPases (Table 1). Similar FRAP experiments to those described above, with transfected wt as well as phospho-mutants of GEF-H1, can determine whether any of the mutations affect the rate of diffusion as compared to wt. A slowed rate of diffusion would suggest that the mutation positively regulates GEF-H1 binding to the microtubules and vice-versa.

Regulation of TACE/ERK activation by RhoA

As described in chapter 5.4, (Figure 24D) we found that RhoA downregulation also partially prevented ERK and TACE activation. However, the activation of p38 was not affected. We hypothesized that this might be due to the fact that RhoA appears to regulate GEF-H1 levels. We found that downregulating RhoA reduces GEF-H1 expression. An additional hypothesis is that Rho downregulation might prevent SRF-dependent transcription of proliferation/survival-promoting early genes, and hence impact TACE/ERK activation (Miano 2003). Hence, further studies looking into whether RhoA is able to regulate its own activation through a positive feedback mechanism via ERK are important. After transfection of a constitutively active RhoA mutant we can follow Rac activation using the Rac activation assay, and ERK activation by Western Blotting with a pERK antibody. Results showing that active Rho promotes Rac and ERK activation would suggest a positive feedback loop.

6.2.2. TNF-activated multiple RhoGEFs in tubular cells

As shown in Figure 31, cells express several GEFs, but the regulation and coordination of these in a signal- and context-specific manner is incompletely understood. Although we have previously ruled out Vav2 as a GEF activated by TNF in tubular cells (Kakiashvili et al.

2009), preliminary studies in our lab found that TNF also activates an RGS RhoGEF, p115RhoGEF. This raises the possibility that other RGS GEFs, LARG and PDZ-RhoGEF, might also be activated by TNF. This should be tested in future studies using the GEF activation assay with GST-RhoG17A, performed in control and TNF-treated cells. The presence of p115RhoGEF, LARG, and PDZ-RhoGEF in the precipitates can be detected by Western Blotting using antibodies specific to these proteins. It will be interesting to explore the specific roles of these GEFs and ask whether their activation follows a pathway similar to that of GEF-H1 activation.

6.2.3. Functional role of GEF-H1

Transactivation of EGFR

TNF-induced transactivation of EGFR that is mediated by GEF-H1 is an important phenomenon (Figure 25). Given that GEF-H1 can be activated by many different stress stimuli, some of which are also known to activate the EGFR, it would be exciting to find out whether GEF-H1 is a general mediator of EGFR transactivation. In these studies, EGFR transactivation can be followed by Western Blotting with a phospho-EGFR specific antibody in lysates of cells exposed to various stimuli (chapter 5). The role of GEF-H1 can be verified by downregulation.

Functional role of differential phosphorylation of GEF-H1

Both Rac and RhoA activation are required for cell migration. Whether GEF-H1 activity towards both GTPases is required is not known. Thus, it would be interesting to know whether the T678 and S885 phosphorylation sites control the role of GEF-H1 in migration,

and hence, have functional significance. Using the (Electric Cell-substrate Impedance Sensing) ECIS wound healing assay (see Methods section), we can find out whether GEF-H1 that cannot be phosphorylated on these sites delays wound healing. For this, the non-phosphorylatable mutants described in chapter 5 can be used. Further, since these mutants show reduced activation towards Rac and/or RhoA, they can help us detemine whether both the Rac- or RhoA-specific activities of GEF-H1 are required. The phosphomimetic mutants, by promoting increased Rac/RhoA activation, might accelerate wound healing independent of TNF. Additionally, the use of scratch-wounding assays in conjunction with the ECIS would allow us to visually follow changes in subcellular localization of the GFP-tagged mutants during wound healing.

To separate the Rac- and RhoA-specific activities of GEF-H1, we will use double GEF-H1 mutants that will allow activation of only one of the GTPases. S885A-T678D would show activation towards RhoA only and S885D-T678A would be active towards Rac alone. After silencing endogenous GEF-H1 using siRNA, a viral transfection system (see below) can be used to transfect wt or mutant GEF-H1. Following treatment with or without TNF, ECIS measurements and scratch wound assays can be performed. If either mutant shows reduced wound healing, it would suggest that GEF-H1 activation towards Rac/RhoA, respectively, is necessary for wound healing.

GFP-GEF-H1 wt and mutant Viral Vector preparation

Although our current transfection methods achieve efficient transfection in an epithelial monolayer, the experiments described above might require even further enhanced transfection efficiency. Therefore, we are in the process of generating adenoviral vectors that

will help us achieve high efficiency transfection of GFP-tagged wt or mutant T678 or S885 GEF-H1. This will enable us to study the molecular and functional effects of replacing endogenous GEF-H1 with either wt or mutant T678 or S885 GEF-H1. We will use the AdEasyTM Vector System (QBIOgene Inc.).

GEF-H1 activation in kidney disease models

Our work has made big strides towards understanding the molecular signalling of GEF-H1 activation by various stimuli. An important next step is to examine the role of GEF-H1 and its differential phosphorylation in animal models of kidney disease. We can study the activity and expression levels of GEF-H1 (and other GEFs), as well as its phosphorylation in various stages of kidney disease. This could be achieved by performing GEF-H1 precipitation assays (see chapter 3) on kidney tissue samples from kidney disease models, including acute kidney injury and various chronic kidney disease models. The latter should include diabetic nephropathy models, since this disease is known to involve the proximal tubules, and kidney fibrosis models, since GEF-H1 can contribute to EMT and fibrosis. Additionally, we could perform immunohistochemistry to follow changes in localization, expression and phosphorylation levels of GEF-H1. Knowledge gained from such studies would allow us to better understand the role and regulation of GEF-H1 in disease, and bring about intervention ideas that will help minimize disease progression.

Hence, the proposed future studies allow us to utilize methods and tools that our lab is well versed with, as well as newer methodologies, to gain novel and essential knowledge in Rho GTPase activation via GEF-H1 and other GEFs.

REFERENCES

Adamson, P., S. Etienne, P. O. Couraud, V. Calder and J. Greenwood (1999). "Lymphocyte migration through brain endothelial cell monolayers involves signaling through endothelial ICAM-1 via a rho-dependent pathway." J Immunol **162**(5): 2964-2973.

Adamson, P., C. J. Marshall, A. Hall and P. A. Tilbrook (1992). "Post-translational modifications of p21rho proteins." J Biol Chem **267**(28): 20033-20038.

Aghazadeh, B., W. E. Lowry, X. Y. Huang and M. K. Rosen (2000). "Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation." Cell **102**(5): 625-633.

Aguda, A. H., L. D. Burtnick and R. C. Robinson (2005). "The state of the filament." EMBO Rep **6**(3): 220-226.

Ahmadian, M. R., P. Stege, K. Scheffzek and A. Wittinghofer (1997). "Confirmation of the arginine-finger hypothesis for the GAP-stimulated GTP-hydrolysis reaction of Ras." Nat Struct Biol **4**(9): 686-689.

Ahmed, S., E. Prigmore, S. Govind, C. Veryard, R. Kozma, F. B. Wientjes, A. W. Segal and L. Lim (1998). "Cryptic Rac-binding and p21(Cdc42Hs/Rac)-activated kinase phosphorylation sites of NADPH oxidase component p67(phox)." J Biol Chem **273**(25): 15693-15701.

Aijaz, S., F. D'Atri, S. Citi, M. S. Balda and K. Matter (2005). "Binding of GEF-H1 to the tight junction-associated adaptor cingulin results in inhibition of Rho signaling and G1/S phase transition." Dev Cell **8**(5): 777-786.

Amano, M., Y. Fukata and K. Kaibuchi (2000). "Regulation and functions of Rho-associated kinase." Exp Cell Res **261**(1): 44-51.

Andoh, T. F., J. Lindsley, N. Franceschini and W. M. Bennett (1996). "Synergistic effects of cyclosporine and rapamycin in a chronic nephrotoxicity model." Transplantation **62**(3): 311-316.

Angelow, S., E. E. Schneeberger and A. S. Yu (2007). "Claudin-8 expression in renal epithelial cells augments the paracellular barrier by replacing endogenous claudin-2." J Membr Biol **215**(2-3): 147-159.

Argast, G. M., J. S. Campbell, J. T. Brooling and N. Fausto (2004). "Epidermal growth factor receptor transactivation mediates tumor necrosis factor-induced hepatocyte replication." J Biol Chem **279**(33): 34530-34536.

Arias-Romero, L. E. and J. Chernoff (2008). "A tale of two Paks." Biol Cell **100**(2): 97-108.

Arthur, W. T., S. M. Ellerbroek, C. J. Der, K. Burridge and K. Wennerberg (2002). "XPLN, a guanine nucleotide exchange factor for RhoA and RhoB, but not RhoC." J Biol Chem **277**(45): 42964-42972.

Arya, R., M. Mallik and S. C. Lakhotia (2007). "Heat shock genes - integrating cell survival and death." J Biosci **32**(3): 595-610.

Axelrod, D., D. E. Koppel, J. Schlessinger, E. Elson and W. W. Webb (1976). "Mobility measurement by analysis of fluorescence photobleaching recovery kinetics." Biophys J **16**(9): 1055-1069.

Bagrodia, S., S. J. Taylor, K. A. Jordon, L. Van Aelst and R. A. Cerione (1998). "A novel regulator of p21-activated kinases." J Biol Chem **273**(37): 23633-23636.

Bakal, C. J., D. Finan, J. LaRose, C. D. Wells, G. Gish, S. Kulkarni, P. DeSepulveda, A. Wilde and R. Rottapel (2005). "The Rho GTP exchange factor Lfc promotes spindle assembly in early mitosis." Proc Natl Acad Sci U S A **102**(27): 9529-9534.

Balda, M. S., J. A. Whitney, C. Flores, S. Gonzalez, M. Cereijido and K. Matter (1996). "Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein." J Cell Biol **134**(4): 1031-1049.

Baldassa, S., R. Zippel and E. Sturani (2003). "Depolarization-induced signaling to Ras, Rap1 and MAPKs in cortical neurons." Brain Res Mol Brain Res **119**(1): 111-122.

Balestrino, M. (1995). "Pathophysiology of anoxic depolarization: new findings and a working hypothesis." J Neurosci Methods **59**(1): 99-103.

Bar-Sagi, D. and A. Hall (2000). "Ras and Rho GTPases: a family reunion." Cell **103**(2): 227-238.

Barry, S. T., H. M. Flinn, M. J. Humphries, D. R. Critchley and A. J. Ridley (1997). "Requirement for Rho in integrin signalling." Cell Adhes Commun **4**(6): 387-398.

Basu, T. N., D. H. Gutmann, J. A. Fletcher, T. W. Glover, F. S. Collins and J. Downward (1992). "Aberrant regulation of ras proteins in malignant tumour cells from type 1 neurofibromatosis patients." Nature **356**(6371): 713-715.

Baud, V. and M. Karin (2001). "Signal transduction by tumor necrosis factor and its relatives." Trends Cell Biol **11**(9): 372-377.

Bauer, H., J. Zweimueller-Mayer, P. Steinbacher, A. Lametschwandtner and H. C. Bauer (2010). "The dual role of zonula occludens (ZO) proteins." J Biomed Biotechnol **2010**: 402593.

Baumeister, M. A., L. Martinu, K. L. Rossman, J. Sondek, M. A. Lemmon and M. M. Chou (2003). "Loss of phosphatidylinositol 3-phosphate binding by the C-terminal Tiam-1 pleckstrin homology domain prevents in vivo Rac1 activation without affecting membrane targeting." J Biol Chem **278**(13): 11457-11464.

Baur, P. S., Jr., D. H. Parks and J. D. Hudson (1984). "Epithelial mediated wound contraction in experimental wounds--the purse-string effect." J Trauma **24**(8): 713-720.

Bazzoni, G., O. M. Martinez-Estrada, F. Orsenigo, M. Cordenonsi, S. Citi and E. Dejana (2000). "Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin." J Biol Chem **275**(27): 20520-20526.

Bement, W. M., P. Forscher and M. S. Mooseker (1993). "A novel cytoskeletal structure involved in purse string wound closure and cell polarity maintenance." J Cell Biol **121**(3): 565-578.

Benais-Pont, G., A. Punn, C. Flores-Maldonado, J. Eckert, G. Raposo, T. P. Fleming, M. Cereijido, M. S. Balda and K. Matter (2003). "Identification of a tight junction-associated guanine nucleotide exchange factor that activates Rho and regulates paracellular permeability." J Cell Biol **160**(5): 729-740.

Bernards, A. and J. Settleman (2005). "GAPs in growth factor signalling." Growth Factors **23**(2): 143-149.

Berthold, J., K. Schenkova and F. Rivero (2008). "Rho GTPases of the RhoBTB subfamily and tumorigenesis." Acta Pharmacol Sin **29**(3): 285-295.

Birkenfeld, J., P. Nalbant, B. P. Bohl, O. Pertz, K. M. Hahn and G. M. Bokoch (2007). "GEF-H1 modulates localized RhoA activation during cytokinesis under the control of mitotic kinases." Dev Cell **12**(5): 699-712.

Birkenfeld, J., P. Nalbant, S. H. Yoon and G. M. Bokoch (2008). "Cellular functions of GEF-H1, a microtubule-regulated Rho-GEF: is altered GEF-H1 activity a crucial determinant of disease pathogenesis?" Trends Cell Biol **18**(5): 210-219.

Birukova, A. A., D. Adyshev, B. Gorshkov, G. M. Bokoch, K. G. Birukov and A. D. Verin (2006). "GEF-H1 is involved in agonist-induced human pulmonary endothelial barrier dysfunction." Am J Physiol Lung Cell Mol Physiol **290**(3): L540-548.

Birukova, A. A., P. Fu, J. Xing, B. Yakubov, I. Cokic and K. G. Birukov (2010). "Mechanotransduction by GEF-H1 as a novel mechanism of ventilator-induced vascular endothelial permeability." Am J Physiol Lung Cell Mol Physiol **298**(6): L837-848.

Bishop, A. L. and A. Hall (2000). "Rho GTPases and their effector proteins." Biochem J **348 Pt 2**: 241-255.

Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K. A. Schooley, M. Gerhart, R. Davis, J. N. Fitzner, R. S. Johnson, R. J. Paxton, C. J. March and D. P. Cerretti (1997). "A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells." Nature **385**(6618): 729-733.

Boettner, B. and L. Van Aelst (2002). "The role of Rho GTPases in disease development." Gene **286**(2): 155-174.

Bokoch, G. M. (2003). "Biology of the p21-activated kinases." Annu Rev Biochem **72**: 743-781.

Bollag, G., D. W. Clapp, S. Shih, F. Adler, Y. Y. Zhang, P. Thompson, B. J. Lange, M. H. Freedman, F. McCormick, T. Jacks and K. Shannon (1996). "Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells." Nat Genet **12**(2): 144-148.

Boulter, E., S. Estrach, R. Garcia-Mata and C. C. Feral (2012). "Off the beaten paths: alternative and crosstalk regulation of Rho GTPases." Faseb J **26**(2): 469-479.

Boulter, E., R. Garcia-Mata, C. Guilluy, A. Dubash, G. Rossi, P. J. Brennwald and K. Burridge (2010). "Regulation of Rho GTPase crosstalk, degradation and activity by RhoGDI1." Nat Cell Biol **12**(5): 477-483.

Bourne, H. R. (1997). "G proteins. The arginine finger strikes again." Nature **389**(6652): 673-674.

Braga, V. M. (2002). "Cell-cell adhesion and signalling." Curr Opin Cell Biol **14**(5): 546-556.

Bretscher, A. (1999). "Regulation of cortical structure by the ezrin-radixin-moesin protein family." Curr Opin Cell Biol **11**(1): 109-116.

Brugnera, E., L. Haney, C. Grimsley, M. Lu, S. F. Walk, A. C. Tosello-Trampont, I. G. Macara, H. Madhani, G. R. Fink and K. S. Ravichandran (2002). "Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex." Nat Cell Biol **4**(8): 574-582.

Brunet, N., A. Morin and B. Olofsson (2002). "RhoGDI-3 regulates RhoG and targets this protein to the Golgi complex through its unique N-terminal domain." Traffic **3**(5): 342-357.

Brzezinska, A. K., N. Lohr and W. M. Chilian (2005). "Electrophysiological effects of O2*-on the plasma membrane in vascular endothelial cells." Am J Physiol Heart Circ Physiol **289**(6): H2379-2386.

Buchanan, F. G., C. M. Elliot, M. Gibbs and J. H. Exton (2000). "Translocation of the Rac1 guanine nucleotide exchange factor Tiam1 induced by platelet-derived growth factor and lysophosphatidic acid." J Biol Chem **275**(13): 9742-9748.

Buchsbaum, R. J. (2007). "Rho activation at a glance." J Cell Sci **120**(Pt 7): 1149-1152.

Buchwalter, G., C. Gross and B. Wasylyk (2004). "Ets ternary complex transcription factors." Gene **324**: 1-14.

Buhl, A. M., N. L. Johnson, N. Dhanasekaran and G. L. Johnson (1995). "G alpha 12 and G alpha 13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly." J Biol Chem **270**(42): 24631-24634.

Burg, M. B., J. D. Ferraris and N. I. Dmitrieva (2007). "Cellular response to hyperosmotic stresses." Physiol Rev **87**(4): 1441-1474.

Burridge, K. and M. Chrzanowska-Wodnicka (1996). "Focal adhesions, contractility, and signaling." Annu Rev Cell Dev Biol **12**: 463-518.

Burridge, K. and K. Wennerberg (2004). "Rho and Rac take center stage." Cell **116**(2): 167-179.

Busche, S., A. Descot, S. Julien, H. Genth and G. Posern (2008). "Epithelial cell-cell contacts regulate SRF-mediated transcription via Rac-actin-MAL signalling." J Cell Sci **121**(Pt 7): 1025-1035.

Bustelo, X. R., V. Sauzeau and I. M. Berenjeno (2007). "GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo." Bioessays **29**(4): 356-370.

Callow, M. G., S. Zozulya, M. L. Gishizky, B. Jallal and T. Smeal (2005). "PAK4 mediates morphological changes through the regulation of GEF-H1." J Cell Sci **118**(Pt 9): 1861-1872.

Cancelas, J. A., A. W. Lee, R. Prabhakar, K. F. Stringer, Y. Zheng and D. A. Williams (2005). "Rac GTPases differentially integrate signals regulating hematopoietic stem cell localization." Nat Med **11**(8): 886-891.

Cantore, M., R. Reinehr, A. Sommerfeld, M. Becker and D. Haussinger (2011). "The Src family kinase Fyn mediates hyperosmolarity-induced Mrp2 and Bsep retrieval from canalicular membrane." J Biol Chem **286**(52): 45014-45029.

Carstanjen, D., A. Yamauchi, A. Koornneef, H. Zang, M. D. Filippi, C. Harris, J. Towe, S. Atkinson, Y. Zheng, M. C. Dinauer and D. A. Williams (2005). "Rac2 regulates neutrophil chemotaxis, superoxide production, and myeloid colony formation through multiple distinct effector pathways." J Immunol **174**(8): 4613-4620.

Castellano, F., P. Montcourrier, J. C. Guillemot, E. Gouin, L. Machesky, P. Cossart and P. Chavrier (1999). "Inducible recruitment of Cdc42 or WASP to a cell-surface receptor triggers actin polymerization and filopodium formation." Curr Biol **9**(7): 351-360.

Chang, Y. C., P. Nalbant, J. Birkenfeld, Z. F. Chang and G. M. Bokoch (2008). "GEF-H1 couples nocodazole-induced microtubule disassembly to cell contractility via RhoA." Mol Biol Cell **19**(5): 2147-2153.

Chen, J., J. K. Chen and R. C. Harris (2012a). "Deletion of the epidermal growth factor receptor in renal proximal tubule epithelial cells delays recovery from acute kidney injury." Kidney Int **82**(1): 45-52.

Chen, J., J. K. Chen, K. Nagai, D. Plieth, M. Tan, T. C. Lee, D. W. Threadgill, E. G. Neilson and R. C. Harris (2012b). "EGFR signaling promotes TGFbeta-dependent renal fibrosis." J Am Soc Nephrol **23**(2): 215-224.

Chen, J., J. K. Chen, E. G. Neilson and R. C. Harris (2006). "Role of EGF receptor activation in angiotensin II-induced renal epithelial cell hypertrophy." J Am Soc Nephrol **17**(6): 1615-1623.

Chen, R. H., S. Corbalan-Garcia and D. Bar-Sagi (1997). "The role of the PH domain in the signal-dependent membrane targeting of Sos." Embo J **16**(6): 1351-1359.

Cheng, L., G. M. Mahon, E. V. Kostenko and I. P. Whitehead (2004). "Pleckstrin homology domain-mediated activation of the rho-specific guanine nucleotide exchange factor Dbs by Rac1." J Biol Chem **279**(13): 12786-12793.

Cheng, L., K. L. Rossman, G. M. Mahon, D. K. Worthylake, M. Korus, J. Sondek and I. P. Whitehead (2002). "RhoGEF specificity mutants implicate RhoA as a target for Dbs transforming activity." Mol Cell Biol **22**(19): 6895-6905.

Chifflet, S., V. Correa, V. Nin, C. Justet and J. A. Hernandez (2004). "Effect of membrane potential depolarization on the organization of the actin cytoskeleton of eye epithelia. The role of adherens junctions." Exp Eye Res **79**(6): 769-777.

Chifflet, S. and J. A. Hernandez (2012). "The plasma membrane potential and the organization of the actin cytoskeleton of epithelial cells." Int J Cell Biol **2012**: 121424.

Chifflet, S., J. A. Hernandez and S. Grasso (2005). "A possible role for membrane depolarization in epithelial wound healing." Am J Physiol Cell Physiol **288**(6): C1420-1430.

Chifflet, S., J. A. Hernandez, S. Grasso and A. Cirillo (2003). "Nonspecific depolarization of the plasma membrane potential induces cytoskeletal modifications of bovine corneal endothelial cells in culture." Exp Cell Res **282**(1): 1-13.

Clark, E. A., W. G. King, J. S. Brugge, M. Symons and R. O. Hynes (1998). "Integrin-mediated signals regulated by members of the rho family of GTPases." J Cell Biol **142**(2): 573-586.

Clarke, H. and M. P. Ryan (1999). "Cyclosporine A-induced alterations in magnesium homeostasis in the rat." Life Sci **64**(15): 1295-1306.

Colegio, O. R., C. M. Van Itallie, H. J. McCrea, C. Rahner and J. M. Anderson (2002). "Claudins create charge-selective channels in the paracellular pathway between epithelial cells." Am J Physiol Cell Physiol **283**(1): C142-147.

Colon-Gonzalez, F., F. C. Leskow and M. G. Kazanietz (2008). "Identification of an autoinhibitory mechanism that restricts C1 domain-mediated activation of the Rac-GAP alpha2-chimaerin." J Biol Chem **283**(50): 35247-35257.

Cording, J., J. Berg, N. Kading, C. Bellmann, C. Tscheik, J. K. Westphal, S. Milatz, D. Gunzel, H. Wolburg, J. Piontek, O. Huber and I. E. Blasig (2013). "In tight junctions, claudins regulate the interactions between occludin, tricellulin and marvelD3, which, inversely, modulate claudin oligomerization." J Cell Sci **126**(Pt 2): 554-564.

Cote, J. F. and K. Vuori (2002). "Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity." J Cell Sci **115**(Pt 24): 4901-4913.

Cote, J. F. and K. Vuori (2007). "GEF what? Dock180 and related proteins help Rac to polarize cells in new ways." Trends Cell Biol **17**(8): 383-393.

Cox, A. D. and C. J. Der (1992). "Protein prenylation: more than just glue?" Curr Opin Cell Biol **4**(6): 1008-1016.

Cox, A. D., M. M. Hisaka, J. E. Buss and C. J. Der (1992). "Specific isoprenoid modification is required for function of normal, but not oncogenic, Ras protein." Mol Cell Biol **12**(6): 2606-2615.

Crosby, L. M. and C. M. Waters (2010). "Epithelial repair mechanisms in the lung." Am J Physiol Lung Cell Mol Physiol **298**(6): L715-731.

Csepanyi-Komi, R., M. Levay and E. Ligeti (2012). "Small G proteins and their regulators in cellular signalling." Mol Cell Endocrinol **353**(1-2): 10-20.

Csepanyi-Komi, R., G. Sirokmany, M. Geiszt and E. Ligeti (2012). "ARHGAP25, a novel Rac GTPase-activating protein, regulates phagocytosis in human neutrophilic granulocytes." Blood **119**(2): 573-582.

Cummins, P. M. (2012). "Occludin: one protein, many forms." Mol Cell Biol **32**(2): 242-250.

Cunningham, P. N., H. M. Dyanov, P. Park, J. Wang, K. A. Newell and R. J. Quigg (2002). "Acute renal failure in endotoxemia is caused by TNF acting directly on TNF receptor-1 in kidney." J Immunol **168**(11): 5817-5823.

D'Autreaux, B. and M. B. Toledano (2007). "ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis." Nat Rev Mol Cell Biol **8**(10): 813-824.

Daemen, M. A., M. W. van de Ven, E. Heineman and W. A. Buurman (1999). "Involvement of endogenous interleukin-10 and tumor necrosis factor-alpha in renal ischemia-reperfusion injury." Transplantation **67**(6): 792-800.

Delva, E., D. K. Tucker and A. P. Kowalczyk (2009). "The desmosome." Cold Spring Harb Perspect Biol **1**(2): a002543.

DeMali, K. A., K. Wennerberg and K. Burridge (2003). "Integrin signaling to the actin cytoskeleton." Curr Opin Cell Biol **15**(5): 572-582.

DesMarais, V., M. Ghosh, R. Eddy and J. Condeelis (2005). "Cofilin takes the lead." J Cell Sci **118**(Pt 1): 19-26.

Di Ciano, C., Z. Nie, K. Szaszi, A. Lewis, T. Uruno, X. Zhan, O. D. Rotstein, A. Mak and A. Kapus (2002). "Osmotic stress-induced remodeling of the cortical cytoskeleton." Am J Physiol Cell Physiol **283**(3): C850-865.

Di Ciano-Oliveira, C., M. Lodyga, L. Fan, K. Szaszi, H. Hosoya, O. D. Rotstein and A. Kapus (2005). "Is myosin light-chain phosphorylation a regulatory signal for the osmotic activation of the Na+-K+-2Cl- cotransporter?" Am J Physiol Cell Physiol **289**(1): C68-81.

Di Ciano-Oliveira, C., G. Sirokmany, K. Szaszi, W. T. Arthur, A. Masszi, M. Peterson, O. D. Rotstein and A. Kapus (2003). "Hyperosmotic stress activates Rho: differential involvement in Rho kinase-dependent MLC phosphorylation and NKCC activation." Am J Physiol Cell Physiol **285**(3): C555-566.

Di Ciano-Oliveira, C., A. C. Thirone, K. Szaszi and A. Kapus (2006). "Osmotic stress and the cytoskeleton: the R(h)ole of Rho GTPases." Acta Physiol (Oxf) **187**(1-2): 257-272.

Diaz-Rodriguez, E., J. C. Montero, A. Esparis-Ogando, L. Yuste and A. Pandiella (2002). "Extracellular signal-regulated kinase phosphorylates tumor necrosis factor alpha-converting enzyme at threonine 735: a potential role in regulated shedding." Mol Biol Cell **13**(6): 2031-2044.

Diekmann, D., A. Abo, C. Johnston, A. W. Segal and A. Hall (1994). "Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity." Science **265**(5171): 531-533.

Dohlman, H. G., M. G. Caron and R. J. Lefkowitz (1987). "A family of receptors coupled to guanine nucleotide regulatory proteins." Biochemistry **26**(10): 2657-2664.

Donnahoo, K. K., D. R. Meldrum, R. Shenkar, C. S. Chung, E. Abraham and A. H. Harken (2000). "Early renal ischemia, with or without reperfusion, activates NFkappaB and increases TNF-alpha bioactivity in the kidney." J Urol **163**(4): 1328-1332.

Donnahoo, K. K., X. Meng, L. Ao, A. Ayala, B. D. Shames, M. P. Cain, A. H. Harken and D. R. Meldrum (2001). "Differential cellular immunolocalization of renal tumour necrosis factor-alpha production during ischaemia versus endotoxaemia." Immunology **102**(1): 53-58.

Dubash, A. D., C. Guilluy, M. C. Srougi, E. Boulter, K. Burridge and R. Garcia-Mata (2011). "The small GTPase RhoA localizes to the nucleus and is activated by Net1 and DNA damage signals." PLoS ONE **6**(2): e17380.

Dudek, S. M. and J. G. Garcia (2001). "Cytoskeletal regulation of pulmonary vascular permeability." J Appl Physiol **91**(4): 1487-1500.

Eberth, A., R. Lundmark, L. Gremer, R. Dvorsky, K. T. Koessmeier, H. T. McMahon and M. R. Ahmadian (2009). "A BAR domain-mediated autoinhibitory mechanism for RhoGAPs of the GRAF family." Biochem J **417**(1): 371-377.

Eden, S., R. Rohatgi, A. V. Podtelejnikov, M. Mann and M. W. Kirschner (2002). "Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck." Nature **418**(6899): 790-793.

Egea, J., C. Espinet and J. X. Comella (1998). "Calmodulin modulates mitogen-activated protein kinase activation in response to membrane depolarization in PC12 cells." J Neurochem **70**(6): 2554-2564.

Engers, R., T. P. Zwaka, L. Gohr, A. Weber, C. D. Gerharz and H. E. Gabbert (2000). "Tiam1 mutations in human renal-cell carcinomas." Int J Cancer **88**(3): 369-376.

Enslen, H., H. Tokumitsu, P. J. Stork, R. J. Davis and T. R. Soderling (1996). "Regulation of mitogen-activated protein kinases by a calcium/calmodulin-dependent protein kinase cascade." Proc Natl Acad Sci U S A **93**(20): 10803-10808.

Eswaran, J., M. Soundararajan, R. Kumar and S. Knapp (2008). "UnPAKing the class differences among p21-activated kinases." Trends Biochem Sci **33**(8): 394-403.

Etienne-Manneville, S. (2008). "Polarity proteins in migration and invasion." Oncogene **27**(55): 6970-6980.

Etienne-Manneville, S. and A. Hall (2002). "Rho GTPases in cell biology." Nature **420**(6916): 629-635.

Fan, L., A. Sebe, Z. Peterfi, A. Masszi, A. C. Thirone, O. D. Rotstein, H. Nakano, C. A. McCulloch, K. Szaszi, I. Mucsi and A. Kapus (2007). "Cell contact-dependent regulation of epithelial-myofibroblast transition via the rho-rho kinase-phospho-myosin pathway." Mol Biol Cell **18**(3): 1083-1097.

Fanning, A. S. and J. M. Anderson (2009). "Zonula occludens-1 and -2 are cytosolic scaffolds that regulate the assembly of cellular junctions." Ann N Y Acad Sci **1165**: 113-120.

Farooqui, R. and G. Fenteany (2005). "Multiple rows of cells behind an epithelial wound edge extend cryptic lamellipodia to collectively drive cell-sheet movement." J Cell Sci **118**(Pt 1): 51-63.

Ferguson, K. M., M. A. Lemmon, P. B. Sigler and J. Schlessinger (1995). "Scratching the surface with the PH domain." Nat Struct Biol **2**(9): 715-718.

Fischer, O. M., S. Hart, A. Gschwind and A. Ullrich (2003). "EGFR signal transactivation in cancer cells." Biochem Soc Trans **31**(Pt 6): 1203-1208.

Fransson, S., A. Ruusala and P. Aspenstrom (2006). "The atypical Rho GTPases Miro-1 and Miro-2 have essential roles in mitochondrial trafficking." Biochem Biophys Res Commun **344**(2): 500-510.

Friedl, P. and K. Wolf (2010). "Plasticity of cell migration: a multiscale tuning model." J Cell Biol **188**(1): 11-19.

Fromm, C., O. A. Coso, S. Montaner, N. Xu and J. S. Gutkind (1997). "The small GTP-binding protein Rho links G protein-coupled receptors and Galpha12 to the serum response element and to cellular transformation." Proc Natl Acad Sci U S A **94**(19): 10098-10103.

Fujishiro, S. H., S. Tanimura, S. Mure, Y. Kashimoto, K. Watanabe and M. Kohno (2008). "ERK1/2 phosphorylate GEF-H1 to enhance its guanine nucleotide exchange activity toward RhoA." Biochem Biophys Res Commun **368**(1): 162-167.

Fukata, M., S. Kuroda, K. Fujii, T. Nakamura, I. Shoji, Y. Matsuura, K. Okawa, A. Iwamatsu, A. Kikuchi and K. Kaibuchi (1997). "Regulation of cross-linking of actin filament by IQGAP1, a target for Cdc42." J Biol Chem **272**(47): 29579-29583.

Fukazawa, A., C. Alonso, K. Kurachi, S. Gupta, C. F. Lesser, B. A. McCormick and H. C. Reinecker (2008). "GEF-H1 mediated control of NOD1 dependent NF-kappaB activation by Shigella effectors." PLoS Pathog **4**(11): e1000228.

Fukuhara, S., H. Chikumi and J. S. Gutkind (2000). "Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G(12) family to Rho." FEBS Lett **485**(2-3): 183-188.

Fukumoto, Y., K. Kaibuchi, Y. Hori, H. Fujioka, S. Araki, T. Ueda, A. Kikuchi and Y. Takai (1990). "Molecular cloning and characterization of a novel type of regulatory protein (GDI) for the rho proteins, ras p21-like small GTP-binding proteins." Oncogene **5**(9): 1321-1328.

Furuse, M. (2010). "Molecular basis of the core structure of tight junctions." Cold Spring Harb Perspect Biol **2**(1): a002907.

Furuse, M., K. Fujimoto, N. Sato, T. Hirase, S. Tsukita and S. Tsukita (1996). "Overexpression of occludin, a tight junction-associated integral membrane protein, induces the formation of intracellular multilamellar bodies bearing tight junction-like structures." J Cell Sci **109 (Pt 2)**: 429-435.

Galbraith, C. G. and M. P. Sheetz (1997). "A micromachined device provides a new bend on fibroblast traction forces." Proc Natl Acad Sci U S A **94**(17): 9114-9118.

Gao, Y., J. Xing, M. Streuli, T. L. Leto and Y. Zheng (2001). "Trp(56) of rac1 specifies interaction with a subset of guanine nucleotide exchange factors." J Biol Chem **276**(50): 47530-47541.

Garcia-Fernandez, B., I. Campos, J. Geiger, A. C. Santos and A. Jacinto (2009). "Epithelial resealing." Int J Dev Biol **53**(8-10): 1549-1556.

Garcia-Mata, R., E. Boulter and K. Burridge (2011). "The 'invisible hand': regulation of RHO GTPases by RHOGDIs." Nat Rev Mol Cell Biol **12**(8): 493-504.

Garcia-Mata, R. and K. Burridge (2007). "Catching a GEF by its tail." Trends Cell Biol **17**(1): 36-43.

Garcia-Mata, R., K. Wennerberg, W. T. Arthur, N. K. Noren, S. M. Ellerbroek and K. Burridge (2006). "Analysis of activated GAPs and GEFs in cell lysates." Methods Enzymol **406**: 425-437.

Gardiner, E. M., K. N. Pestonjamasp, B. P. Bohl, C. Chamberlain, K. M. Hahn and G. M. Bokoch (2002). "Spatial and temporal analysis of Rac activation during live neutrophil chemotaxis." Curr Biol **12**(23): 2029-2034.

Geiszt, M., M. C. Dagher, G. Molnar, A. Havasi, J. Faure, M. H. Paclet, F. Morel and E. Ligeti (2001). "Characterization of membrane-localized and cytosolic Rac-GTPase-activating

proteins in human neutrophil granulocytes: contribution to the regulation of NADPH oxidase." Biochem J **355**(Pt 3): 851-858.

Ghosh, M., X. Song, G. Mouneimne, M. Sidani, D. S. Lawrence and J. S. Condeelis (2004). "Cofilin promotes actin polymerization and defines the direction of cell motility." Science **304**(5671): 743-746.

Gilmore, A. P. and K. Burridge (1996). "Molecular mechanisms for focal adhesion assembly through regulation of protein-protein interactions." Structure **4**(6): 647-651.

Gohla, A., R. Harhammer and G. Schultz (1998). "The G-protein G13 but not G12 mediates signaling from lysophosphatidic acid receptor via epidermal growth factor receptor to Rho." J Biol Chem **273**(8): 4653-4659.

Gohla, A., S. Offermanns, T. M. Wilkie and G. Schultz (1999). "Differential involvement of Galpha12 and Galpha13 in receptor-mediated stress fiber formation." J Biol Chem **274**(25): 17901-17907.

Gonzalez-Mariscal, L., P. Bautista, S. Lechuga and M. Quiros (2012). "ZO-2, a tight junction scaffold protein involved in the regulation of cell proliferation and apoptosis." Ann N Y Acad Sci **1257**: 133-141.

Gorbatenko, A., M. Wiwel, H. Klingberg, A. B. Nielsen, A. Kapus and S. F. Pedersen (2011). "Hyperosmotic stress strongly potentiates serum response factor (SRF)-dependent transcriptional activity in Ehrlich Lettre Ascites cells through a mechanism involving p38 mitogen-activated protein kinase." J Cell Physiol **226**(11): 2857-2868.

Gosser, Y. Q., T. K. Nomanbhoy, B. Aghazadeh, D. Manor, C. Combs, R. A. Cerione and M. K. Rosen (1997). "C-terminal binding domain of Rho GDP-dissociation inhibitor directs N-terminal inhibitory peptide to GTPases." Nature **387**(6635): 814-819.

Guilluy, C., R. Garcia-Mata and K. Burridge (2011). "Rho protein crosstalk: another social network?" Trends Cell Biol.

Guilluy, C., V. Swaminathan, R. Garcia-Mata, E. T. O'Brien, R. Superfine and K. Burridge (2011). "The Rho GEFs LARG and GEF-H1 regulate the mechanical response to force on integrins." Nat Cell Biol **13**(6): 722-727.

Guo, F., Y. Xing, Z. Zhou, Y. Dou, J. Tang, C. Gao and J. Huan (2012). "Guanine-nucleotide exchange factor H1 mediates lipopolysaccharide-induced interleukin 6 and tumor necrosis factor alpha expression in endothelial cells via activation of nuclear factor kappaB." Shock **37**(5): 531-538.

Guo, F., Z. Zhou, Y. Dou, J. Tang, C. Gao and J. Huan (2012). "GEF-H1/RhoA signalling pathway mediates lipopolysaccharide-induced intercellular adhesion molecular-1 expression in endothelial cells via activation of p38 and NF-kappaB." Cytokine **57**(3): 417-428.

Haddad, G. G. and D. F. Donnelly (1990). "O2 deprivation induces a major depolarization in brain stem neurons in the adult but not in the neonatal rat." J Physiol **429**: 411-428.

Hall, A. (2012). "Rho family GTPases." Biochem Soc Trans **40**(6): 1378-1382.

Hallows, K. R., F. Y. Law, C. H. Packman and P. A. Knauf (1996). "Changes in cytoskeletal actin content, F-actin distribution, and surface morphology during HL-60 cell volume regulation." J Cell Physiol **167**(1): 60-71.

Hamaguchi, M., J. L. Meth, C. von Klitzing, W. Wei, D. Esposito, L. Rodgers, T. Walsh, P. Welcsh, M. C. King and M. H. Wigler (2002). "DBC2, a candidate for a tumor suppressor gene involved in breast cancer." Proc Natl Acad Sci U S A **99**(21): 13647-13652.

Han, J., K. Luby-Phelps, B. Das, X. Shu, Y. Xia, R. D. Mosteller, U. M. Krishna, J. R. Falck, M. A. White and D. Broek (1998). "Role of substrates and products of PI 3-kinase in

regulating activation of Rac-related guanosine triphosphatases by Vav." Science **279**(5350): 558-560.

Harris, T. J. and U. Tepass (2010). "Adherens junctions: from molecules to morphogenesis." Nat Rev Mol Cell Biol **11**(7): 502-514.

Hart, M. J., M. G. Callow, B. Souza and P. Polakis (1996). "IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs." Embo J **15**(12): 2997-3005.

Hart, M. J., X. Jiang, T. Kozasa, W. Roscoe, W. D. Singer, A. G. Gilman, P. C. Sternweis and G. Bollag (1998). "Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13." Science **280**(5372): 2112-2114.

Heck, J. N., S. M. Ponik, M. G. Garcia-Mendoza, C. A. Pehlke, D. R. Inman, K. W. Eliceiri and P. J. Keely (2012). "Microtubules regulate GEF-H1 in response to extracellular matrix stiffness." Mol Biol Cell **23**(13): 2583-2592.

Henson, J. H. (1999). "Relationships between the actin cytoskeleton and cell volume regulation." Microsc Res Tech **47**(2): 155-162.

Hilliard, V. C., M. R. Frey, P. J. Dempsey, R. M. Peek, Jr. and D. B. Polk (2011). "TNF-{alpha} converting enzyme-mediated ErbB4 transactivation by TNF promotes colonic epithelial cell survival." Am J Physiol Gastrointest Liver Physiol **301**(2): G338-346.

Hirata, D., T. Yamabuki, D. Miki, T. Ito, E. Tsuchiya, M. Fujita, M. Hosokawa, K. Chayama, Y. Nakamura and Y. Daigo (2009). "Involvement of epithelial cell transforming sequence-2 oncoantigen in lung and esophageal cancer progression." Clin Cancer Res **15**(1): 256-266.

Hodgson, L., F. Shen and K. Hahn (2010). "Biosensors for characterizing the dynamics of rho family GTPases in living cells." Curr Protoc Cell Biol **Chapter 14**: Unit 14 11 11-26.

Hoffmann, E. K., I. H. Lambert and S. F. Pedersen (2009). "Physiology of cell volume regulation in vertebrates." Physiol Rev **89**(1): 193-277.

Horwitz, R. and D. Webb (2003). "Cell migration." Curr Biol **13**(19): R756-759.

Hotchin, N. A. and A. Hall (1995). "The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases." J Cell Biol **131**(6 Pt 2): 1857-1865.

Hoyer, J. and H. Gogelein (1991). "Sodium-alanine cotransport in renal proximal tubule cells investigated by whole-cell current recording." J Gen Physiol **97**(5): 1073-1094.

Hribova, P., K. Kotsch, I. Brabcova, S. Vitko, H. D. Volk and J. Lacha (2005). "Cytokines and chemokine gene expression in human kidney transplantation." Transplant Proc **37**(2): 760-763.

Hu, K. Q. and J. Settleman (1997). "Tandem SH2 binding sites mediate the RasGAP-RhoGAP interaction: a conformational mechanism for SH3 domain regulation." Embo J **16**(3): 473-483.

Huber, D., M. S. Balda and K. Matter (2000). "Occludin modulates transepithelial migration of neutrophils." J Biol Chem **275**(8): 5773-5778.

Iizuka, M. and S. Konno (2011). "Wound healing of intestinal epithelial cells." World J Gastroenterol **17**(17): 2161-2171.

Ikenouchi, J., M. Furuse, K. Furuse, H. Sasaki, S. Tsukita and S. Tsukita (2005). "Tricellulin constitutes a novel barrier at tricellular contacts of epithelial cells." J Cell Biol **171**(6): 939-945.

Ikenouchi, J., H. Sasaki, S. Tsukita, M. Furuse and S. Tsukita (2008). "Loss of occludin affects tricellular localization of tricellulin." Mol Biol Cell **19**(11): 4687-4693.

Ito, H., I. Iwamoto, K. Mizutani, R. Morishita, T. Deguchi, Y. Nozawa, T. Asano and K. Nagata (2006). "Possible interaction of a Rho effector, Rhotekin, with a PDZ-protein, PIST, at synapses of hippocampal neurons." Neurosci Res **56**(2): 165-171.

Ivanov, A. I. (2008). "Actin motors that drive formation and disassembly of epithelial apical junctions." Front Biosci **13**: 6662-6681.

Ivanov, A. I., A. Nusrat and C. A. Parkos (2004). "The epithelium in inflammatory bowel disease: potential role of endocytosis of junctional proteins in barrier disruption." Novartis Found Symp **263**: 115-124; discussion 124-132, 211-118.

Jaffe, A. B. and A. Hall (2005). "Rho GTPases: biochemistry and biology." Annu Rev Cell Dev Biol **21**: 247-269.

Janmey, P. A. (1994). "Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly." Annu Rev Physiol **56**: 169-191.

Jevnikar, A. M., D. C. Brennan, G. G. Singer, J. E. Heng, W. Maslinski, R. P. Wuthrich, L. H. Glimcher and V. E. Kelley (1991). "Stimulated kidney tubular epithelial cells express membrane associated and secreted TNF alpha." Kidney Int **40**(2): 203-211.

Jiang, W. G., T. A. Martin, J. M. Lewis-Russell, A. Douglas-Jones, L. Ye and R. E. Mansel (2008). "Eplin-alpha expression in human breast cancer, the impact on cellular migration and clinical outcome." Mol Cancer **7**: 71.

Johnson, D. S. and Y. H. Chen (2012). "Ras family of small GTPases in immunity and inflammation." Curr Opin Pharmacol **12**(4): 458-463.

Kaibuchi, K., S. Kuroda and M. Amano (1999). "Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells." Annu Rev Biochem **68**: 459-486.

Kakiashvili, E., Q. Dan, M. Vandermeer, Y. Zhang, F. Waheed, M. Pham and K. Szaszi (2011). "The epidermal growth factor receptor mediates tumor necrosis factor-alpha-induced activation of the ERK/GEF-H1/RhoA pathway in tubular epithelium." J Biol Chem **286**(11): 9268-9279.

Kakiashvili, E., P. Speight, F. Waheed, R. Seth, M. Lodyga, S. Tanimura, M. Kohno, O. D. Rotstein, A. Kapus and K. Szaszi (2009). "GEF-H1 Mediates Tumor Necrosis Factor-{alpha}-induced Rho Activation and Myosin Phosphorylation: role in the regulation of tubular paracellular permeability." J Biol Chem **284**(17): 11454-11466.

Kang, M. G., Y. Guo and R. L. Huganir (2009). "AMPA receptor and GEF-H1/Lfc complex regulates dendritic spine development through RhoA signaling cascade." Proc Natl Acad Sci U S A **106**(9): 3549-3554.

Kant, S., W. Swat, S. Zhang, Z. Y. Zhang, B. G. Neel, R. A. Flavell and R. J. Davis (2011). "TNF-stimulated MAP kinase activation mediated by a Rho family GTPase signaling pathway." Genes Dev **25**(19): 2069-2078.

Kapus, A., C. Di Ciano, J. Sun, X. Zhan, L. Kim, T. W. Wong and O. D. Rotstein (2000). "Cell volume-dependent phosphorylation of proteins of the cortical cytoskeleton and cell-cell contact sites. The role of Fyn and FER kinases." J Biol Chem **275**(41): 32289-32298.

Kapus, A. and K. Szaszi (2006). "Coupling between apical and paracellular transport processes." Biochem Cell Biol **84**(6): 870-880.

Kapus, A., K. Szaszi, J. Sun, S. Rizoli and O. D. Rotstein (1999). "Cell shrinkage regulates Src kinases and induces tyrosine phosphorylation of cortactin, independent of the osmotic regulation of Na+/H+ exchangers." J Biol Chem **274**(12): 8093-8102.

Kaur, R., X. Liu, O. Gjoerup, A. Zhang, X. Yuan, S. P. Balk, M. C. Schneider and M. L. Lu (2005). "Activation of p21-activated kinase 6 by MAP kinase kinase 6 and p38 MAP kinase." J Biol Chem **280**(5): 3323-3330.

Kawano, Y., T. Kaneko-Kawano, T. Yoshimura, S. Kawabata and K. Kaibuchi (2005). RHOA/C and the actin cytoskeleton. RHO family GTPases. E. Manser. 3300 AA Dordrecht, The Netherlands., Springer**:** 113-136.

Keep, N. H., M. Barnes, I. Barsukov, R. Badii, L. Y. Lian, A. W. Segal, P. C. Moody and G. C. Roberts (1997). "A modulator of rho family G proteins, rhoGDI, binds these G proteins via an immunoglobulin-like domain and a flexible N-terminal arm." Structure **5**(5): 623-633.

Kesavapany, S., N. Amin, Y. L. Zheng, R. Nijhara, H. Jaffe, R. Sihag, J. S. Gutkind, S. Takahashi, A. Kulkarni, P. Grant and H. C. Pant (2004). "p35/cyclin-dependent kinase 5 phosphorylation of ras guanine nucleotide releasing factor 2 (RasGRF2) mediates Rac-dependent Extracellular Signal-regulated kinase 1/2 activity, altering RasGRF2 and microtubule-associated protein 1b distribution in neurons." J Neurosci **24**(18): 4421-4431.

Khalil, A. A. and P. Friedl (2010). "Determinants of leader cells in collective cell migration." Integr Biol (Camb) **2**(11-12): 568-574.

Kimura, K., M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, A. Iwamatsu and K. Kaibuchi (1996). "Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase)." Science **273**(5272): 245-248.

Kiyono, M., Y. Kaziro and T. Satoh (2000). "Induction of rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25(Mm) following phosphorylation by the nonreceptor tyrosine kinase Src." J Biol Chem **275**(8): 5441-5446.

Kjoller, L. and A. Hall (1999). "Signaling to Rho GTPases." Exp Cell Res **253**(1): 166-179.

Koivusalo, M., A. Kapus and S. Grinstein (2009). "Sensors, transducers, and effectors that regulate cell size and shape." J Biol Chem **284**(11): 6595-6599.

Kourlas, P. J., M. P. Strout, B. Becknell, M. L. Veronese, C. M. Croce, K. S. Theil, R. Krahe, T. Ruutu, S. Knuutila, C. D. Bloomfield and M. A. Caligiuri (2000). "Identification of a gene at 11q23 encoding a guanine nucleotide exchange factor: evidence for its fusion with MLL in acute myeloid leukemia." Proc Natl Acad Sci U S A **97**(5): 2145-2150.

Kozasa, T., N. Hajicek, C. Chow and N. Suzuki (2011). "Signaling mechanisms of RhoGTPase regulation by the heterotrimeric G proteins G12 and G13." J Biochem.

Kratzer, E., Y. Tian, N. Sarich, T. Wu, A. Meliton, A. Leff and A. A. Birukova (2012). "Oxidative stress contributes to lung injury and barrier dysfunction via microtubule destabilization." Am J Respir Cell Mol Biol **47**(5): 688-697.

Kreck, M. L., J. L. Freeman, A. Abo and J. D. Lambeth (1996). "Membrane association of Rac is required for high activity of the respiratory burst oxidase." Biochemistry **35**(49): 15683-15692.

Krendel, M., F. T. Zenke and G. M. Bokoch (2002). "Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton." Nat Cell Biol **4**(4): 294-301.

Krug, S. M., S. Amasheh, J. F. Richter, S. Milatz, D. Gunzel, J. K. Westphal, O. Huber, J. D. Schulzke and M. Fromm (2009). "Tricellulin forms a barrier to macromolecules in tricellular tight junctions without affecting ion permeability." Mol Biol Cell **20**(16): 3713-3724.

Kureishi, Y., S. Kobayashi, M. Amano, K. Kimura, H. Kanaide, T. Nakano, K. Kaibuchi and M. Ito (1997). "Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation." J Biol Chem **272**(19): 12257-12260.

Kuroda, S., M. Fukata, M. Nakagawa, K. Fujii, T. Nakamura, T. Ookubo, I. Izawa, T. Nagase, N. Nomura, H. Tani, I. Shoji, Y. Matsuura, S. Yonehara and K. Kaibuchi (1998). "Role of IQGAP1, a target of the small GTPases Cdc42 and Rac1, in regulation of E-cadherin- mediated cell-cell adhesion." Science **281**(5378): 832-835.

Kurokawa, K., T. Nakamura, K. Aoki and M. Matsuda (2005). "Mechanism and role of localized activation of Rho-family GTPases in growth factor-stimulated fibroblasts and neuronal cells." Biochem Soc Trans **33**(Pt 4): 631-634.

Kuwayama, H., M. Ecke, G. Gerisch and P. J. Van Haastert (1996). "Protection against osmotic stress by cGMP-mediated myosin phosphorylation." Science **271**(5246): 207-209.

Lambert, J. M., Q. T. Lambert, G. W. Reuther, A. Malliri, D. P. Siderovski, J. Sondek, J. G. Collard and C. J. Der (2002). "Tiam1 mediates Ras activation of Rac by a PI(3)K-independent mechanism." Nat Cell Biol **4**(8): 621-625.

Lee, N. K. and S. Y. Lee (2002). "Modulation of life and death by the tumor necrosis factor receptor-associated factors (TRAFs)." J Biochem Mol Biol **35**(1): 61-66.

Lelias, J. M., C. N. Adra, G. M. Wulf, J. C. Guillemot, M. Khagad, D. Caput and B. Lim (1993). "cDNA cloning of a human mRNA preferentially expressed in hematopoietic cells and with homology to a GDP-dissociation inhibitor for the rho GTP-binding proteins." Proc Natl Acad Sci U S A **90**(4): 1479-1483.

Leonard, D., M. J. Hart, J. V. Platko, A. Eva, W. Henzel, T. Evans and R. A. Cerione (1992). "The identification and characterization of a GDP-dissociation inhibitor (GDI) for the CDC42Hs protein." J Biol Chem **267**(32): 22860-22868.

Levay, M., J. Settleman and E. Ligeti (2009). "Regulation of the substrate preference of p190RhoGAP by protein kinase C-mediated phosphorylation of a phospholipid binding site." Biochemistry **48**(36): 8615-8623.

Lewis, A., C. Di Ciano, O. D. Rotstein and A. Kapus (2002). "Osmotic stress activates Rac and Cdc42 in neutrophils: role in hypertonicity-induced actin polymerization." Am J Physiol Cell Physiol **282**(2): C271-279.

Li, Y., J. Yang, C. Dai, C. Wu and Y. Liu (2003). "Role for integrin-linked kinase in mediating tubular epithelial to mesenchymal transition and renal interstitial fibrogenesis." J Clin Invest **112**(4): 503-516.

Liao, Y. C., J. W. Ruan, I. Lua, M. H. Li, W. L. Chen, J. R. Wang, R. H. Kao and J. H. Chen (2012). "Overexpressed hPTTG1 promotes breast cancer cell invasion and metastasis by regulating GEF-H1/RhoA signalling." Oncogene **31**(25): 3086-3097.

Liebmann, C. (2011). "EGF receptor activation by GPCRs: an universal pathway reveals different versions." Mol Cell Endocrinol **331**(2): 222-231.

Ligeti, E., M. C. Dagher, S. E. Hernandez, A. J. Koleske and J. Settleman (2004). "Phospholipids can switch the GTPase substrate preference of a GTPase-activating protein." J Biol Chem **279**(7): 5055-5058.

Liu, H. W., A. J. Halayko, D. J. Fernandes, G. S. Harmon, J. A. McCauley, P. Kocieniewski, J. McConville, Y. Fu, S. M. Forsythe, P. Kogut, S. Bellam, M. Dowell, J. Churchill, H. Lesso, K. Kassiri, R. W. Mitchell, M. B. Hershenson, B. Camoretti-Mercado and J. Solway (2003). "The RhoA/Rho kinase pathway regulates nuclear localization of serum response factor." Am J Respir Cell Mol Biol **29**(1): 39-47.

Liu, J., J. Tian, M. Haas, J. I. Shapiro, A. Askari and Z. Xie (2000). "Ouabain interaction with cardiac Na+/K+-ATPase initiates signal cascades independent of changes in intracellular Na+ and Ca2+ concentrations." J Biol Chem **275**(36): 27838-27844.

Liu, X., H. Wang, M. Eberstadt, A. Schnuchel, E. T. Olejniczak, R. P. Meadows, J. M. Schkeryantz, D. A. Janowick, J. E. Harlan, E. A. Harris, D. E. Staunton and S. W. Fesik (1998). "NMR structure and mutagenesis of the N-terminal Dbl homology domain of the nucleotide exchange factor Trio." Cell **95**(2): 269-277.

Lu, M., J. M. Kinchen, K. L. Rossman, C. Grimsley, M. Hall, J. Sondek, M. O. Hengartner, V. Yajnik and K. S. Ravichandran (2005). "A Steric-inhibition model for regulation of nucleotide exchange via the Dock180 family of GEFs." Curr Biol **15**(4): 371-377.

Lu, M. and K. S. Ravichandran (2006). "Dock180-ELMO cooperation in Rac activation." Methods Enzymol **406**: 388-402.

Ly, D. L., F. Waheed, M. Lodyga, P. Speight, A. Masszi, H. Nakano, M. Hersom, S. F. Pedersen, K. Szaszi and A. Kapus (2013). "Hyperosmotic stress regulates the distribution and stability of myocardin-related transcription factor, a key modulator of the cytoskeleton." Am J Physiol Cell Physiol **304**(2): C115-127.

Machacek, M., L. Hodgson, C. Welch, H. Elliott, O. Pertz, P. Nalbant, A. Abell, G. L. Johnson, K. M. Hahn and G. Danuser (2009). "Coordination of Rho GTPase activities during cell protrusion." Nature **461**(7260): 99-103.

Madaule, P., M. Eda, N. Watanabe, K. Fujisawa, T. Matsuoka, H. Bito, T. Ishizaki and S. Narumiya (1998). "Role of citron kinase as a target of the small GTPase Rho in cytokinesis." Nature **394**(6692): 491-494.

Malek, A. M., C. Xu, E. S. Kim and S. L. Alper (2007). "Hypertonicity triggers RhoA-dependent assembly of myosin-containing striated polygonal actin networks in endothelial cells." Am J Physiol Cell Physiol **292**(5): C1645-1659.

Malliri, A., R. A. van der Kammen, K. Clark, M. van der Valk, F. Michiels and J. G. Collard (2002). "Mice deficient in the Rac activator Tiam1 are resistant to Ras-induced skin tumours." Nature **417**(6891): 867-871.

Mandell, K. J., B. A. Babbin, A. Nusrat and C. A. Parkos (2005). "Junctional adhesion molecule 1 regulates epithelial cell morphology through effects on beta1 integrins and Rap1 activity." J Biol Chem **280**(12): 11665-11674.

Manser, E. (2004). Rho GTPases in the organization of the actin cytoskeleton. Rho GTPases. M. Symons. New York, USA, Landes Bioscience/Kluwer Academic**:** 107-114.

Manser, E., H. Y. Huang, T. H. Loo, X. Q. Chen, J. M. Dong, T. Leung and L. Lim (1997). "Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes." Mol Cell Biol **17**(3): 1129-1143.

Manser, E., T. H. Loo, C. G. Koh, Z. S. Zhao, X. Q. Chen, L. Tan, I. Tan, T. Leung and L. Lim (1998). "PAK kinases are directly coupled to the PIX family of nucleotide exchange factors." Mol Cell **1**(2): 183-192.

Mariano, C., H. Sasaki, D. Brites and M. A. Brito (2011). "A look at tricellulin and its role in tight junction formation and maintenance." Eur J Cell Biol **90**(10): 787-796.

Martin-Martin, N., Q. Dan, Y. Amoozadeh, F. Waheed, T. McMorrow, M. P. Ryan and K. Szaszi (2012). "RhoA and Rho kinase mediate cyclosporine A and sirolimus-induced barrier tightening in renal proximal tubular cells." Int J Biochem Cell Biol **44**(1): 178-188.

Martin-Martin, N., G. Ryan, T. McMorrow and M. P. Ryan (2010). "Sirolimus and cyclosporine A alter barrier function in renal proximal tubular cells through stimulation of ERK1/2 signaling and claudin-1 expression." Am J Physiol Renal Physiol **298**(3): F672-682.

Massague, J. (2012). "TGF-beta signaling in development and disease." FEBS Lett **586**(14): 1833.

Masszi, A., C. Di Ciano, G. Sirokmany, W. T. Arthur, O. D. Rotstein, J. Wang, C. A. McCulloch, L. Rosivall, I. Mucsi and A. Kapus (2003). "Central role for Rho in TGF-beta1-induced alpha-smooth muscle actin expression during epithelial-mesenchymal transition." Am J Physiol Renal Physiol **284**(5): F911-924.

Masszi, A., P. Speight, E. Charbonney, M. Lodyga, H. Nakano, K. Szaszi and A. Kapus (2010). "Fate-determining mechanisms in epithelial-myofibroblast transition: major inhibitory role for Smad3." J Cell Biol **188**(3): 383-399.

Matsui, T., M. Maeda, Y. Doi, S. Yonemura, M. Amano, K. Kaibuchi, S. Tsukita and S. Tsukita (1998). "Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association." J Cell Biol **140**(3): 647-657.

Matthews, B. D., D. R. Overby, R. Mannix and D. E. Ingber (2006). "Cellular adaptation to mechanical stress: role of integrins, Rho, cytoskeletal tension and mechanosensitive ion channels." J Cell Sci **119**(Pt 3): 508-518.

Mayor, R. and C. Carmona-Fontaine (2010). "Keeping in touch with contact inhibition of locomotion." Trends Cell Biol **20**(6): 319-328.

McCarthy, K. M., I. B. Skare, M. C. Stankewich, M. Furuse, S. Tsukita, R. A. Rogers, R. D. Lynch and E. E. Schneeberger (1996). "Occludin is a functional component of the tight junction." J Cell Sci **109 (Pt 9)**: 2287-2298.

Meiri, D., M. A. Greeve, A. Brunet, D. Finan, C. D. Wells, J. LaRose and R. Rottapel (2009). "Modulation of Rho guanine exchange factor Lfc activity by protein kinase A-mediated phosphorylation." Mol Cell Biol **29**(21): 5963-5973.

Meldrum, K. K., P. Metcalfe, J. A. Leslie, R. Misseri, K. L. Hile and D. R. Meldrum (2006). "TNF-alpha neutralization decreases nuclear factor-kappaB activation and apoptosis during renal obstruction." J Surg Res **131**(2): 182-188.

Meldrum, K. K., R. Misseri, P. Metcalfe, C. A. Dinarello, K. L. Hile and D. R. Meldrum (2007). "TNF-alpha neutralization ameliorates obstruction-induced renal fibrosis and dysfunction." Am J Physiol Regul Integr Comp Physiol **292**(4): R1456-1464.

Meller, N., M. Irani-Tehrani, W. B. Kiosses, M. A. Del Pozo and M. A. Schwartz (2002). "Zizimin1, a novel Cdc42 activator, reveals a new GEF domain for Rho proteins." Nat Cell Biol **4**(9): 639-647.

Miano, J. M. (2003). "Serum response factor: toggling between disparate programs of gene expression." J Mol Cell Cardiol **35**(6): 577-593.

Minoshima, Y., T. Kawashima, K. Hirose, Y. Tonozuka, A. Kawajiri, Y. C. Bao, X. Deng, M. Tatsuka, S. Narumiya, W. S. May, Jr., T. Nosaka, K. Semba, T. Inoue, T. Satoh, M. Inagaki and T. Kitamura (2003). "Phosphorylation by aurora B converts MgcRacGAP to a RhoGAP during cytokinesis." Dev Cell **4**(4): 549-560.

Miralles, F., G. Posern, A. I. Zaromytidou and R. Treisman (2003). "Actin dynamics control SRF activity by regulation of its coactivator MAL." Cell **113**(3): 329-342.

Misseri, R., D. R. Meldrum, P. Dagher, K. Hile, R. C. Rink and K. K. Meldrum (2004). "Unilateral ureteral obstruction induces renal tubular cell production of tumor necrosis factor-alpha independent of inflammatory cell infiltration." J Urol **172**(4 Pt 2): 1595-1599; discussion 1599.

Misseri, R., D. R. Meldrum, C. A. Dinarello, P. Dagher, K. L. Hile, R. C. Rink and K. K. Meldrum (2005). "TNF-alpha mediates obstruction-induced renal tubular cell apoptosis and proapoptotic signaling." Am J Physiol Renal Physiol **288**(2): F406-411.

Miyano, K. and H. Sumimoto (2007). "Role of the small GTPase Rac in p22phox-dependent NADPH oxidases." Biochimie **89**(9): 1133-1144.

Miyazaki, K., S. Komatsu and M. Ikebe (2006). "Dynamics of RhoA and ROKalpha translocation in single living cells." Cell Biochem Biophys **45**(3): 243-254.

Mizuarai, S., K. Yamanaka and H. Kotani (2006). "Mutant p53 induces the GEF-H1 oncogene, a guanine nucleotide exchange factor-H1 for RhoA, resulting in accelerated cell proliferation in tumor cells." Cancer Res **66**(12): 6319-6326.

Moskwa, P., M. H. Paclet, M. C. Dagher and E. Ligeti (2005). "Autoinhibition of p50 Rho GTPase-activating protein (GAP) is released by prenylated small GTPases." J Biol Chem **280**(8): 6716-6720.

Moss, M. L., S. L. Jin, J. D. Becherer, D. M. Bickett, W. Burkhart, W. J. Chen, D. Hassler, M. T. Leesnitzer, G. McGeehan, M. Milla, M. Moyer, W. Rocque, T. Seaton, F. Schoenen, J. Warner and D. Willard (1997). "Structural features and biochemical properties of TNF-alpha converting enzyme (TACE)." J Neuroimmunol **72**(2): 127-129.

Mouilleron, S., S. Guettler, C. A. Langer, R. Treisman and N. Q. McDonald (2008). "Molecular basis for G-actin binding to RPEL motifs from the serum response factor coactivator MAL." Embo J **27**(23): 3198-3208.

Nalbant, P., Y. C. Chang, J. Birkenfeld, Z. F. Chang and G. M. Bokoch (2009). "Guanine nucleotide exchange factor-H1 regulates cell migration via localized activation of RhoA at the leading edge." Mol Biol Cell **20**(18): 4070-4082.

Namekata, K., Y. Enokido, K. Iwasawa and H. Kimura (2004). "MOCA induces membrane spreading by activating Rac1." J Biol Chem **279**(14): 14331-14337.

Narumiya, S., M. Tanji and T. Ishizaki (2009). "Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion." Cancer Metastasis Rev **28**(1-2): 65-76.

Nassar, N., G. R. Hoffman, D. Manor, J. C. Clardy and R. A. Cerione (1998). "Structures of Cdc42 bound to the active and catalytically compromised forms of Cdc42GAP." Nat Struct Biol **5**(12): 1047-1052.

Nelson, W. J. (2003). "Epithelial cell polarity from the outside looking in." News Physiol Sci **18**: 143-146.

Nie, M., S. Aijaz, I. V. Leefa Chong San, M. S. Balda and K. Matter (2009). "The Y-box factor ZONAB/DbpA associates with GEF-H1/Lfc and mediates Rho-stimulated transcription." EMBO Rep **10**(10): 1125-1131.

Nie, Z., A. D. Fryer and D. B. Jacoby (2012). "beta2-Agonists inhibit TNF-alpha-induced ICAM-1 expression in human airway parasympathetic neurons." PLoS ONE **7**(9): e44780.

Nisimoto, Y., J. L. Freeman, S. A. Motalebi, M. Hirshberg and J. D. Lambeth (1997). "Rac binding to p67(phox). Structural basis for interactions of the Rac1 effector region and insert region with components of the respiratory burst oxidase." J Biol Chem **272**(30): 18834-18841.

Nobes, C. D. and A. Hall (1995). "Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia." Cell **81**(1): 53-62.

Noren, N. K., W. T. Arthur and K. Burridge (2003). "Cadherin engagement inhibits RhoA via p190RhoGAP." J Biol Chem **278**(16): 13615-13618.

Nusrat, A., J. R. Turner and J. L. Madara (2000). "Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli:

nutrients, cytokines, and immune cells." Am J Physiol Gastrointest Liver Physiol **279**(5): G851-857.

Obara, Y., A. M. Horgan and P. J. Stork (2007). "The requirement of Ras and Rap1 for the activation of ERKs by cAMP, PACAP, and KCl in cerebellar granule cells." J Neurochem **101**(2): 470-482.

Ogbagabriel, S., M. Fernando, F. M. Waldman, S. Bose and A. P. Heaney (2005). "Securin is overexpressed in breast cancer." Mod Pathol **18**(7): 985-990.

Ohashi, K., K. Nagata, M. Maekawa, T. Ishizaki, S. Narumiya and K. Mizuno (2000). "Rho-associated kinase ROCK activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop." J Biol Chem **275**(5): 3577-3582.

Ohtsu, H., P. J. Dempsey and S. Eguchi (2006). "ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors." Am J Physiol Cell Physiol **291**(1): C1-10.

Oliveira, S. G., I. B. Oliveira, H. Abensur, M. R. Araujo, J. E. Romao and I. L. Noronha (2002). "Differential expression of cytokines, growth factors, and alpha-smooth muscle actin in renal allograft biopsies." Transplant Proc **34**(2): 479-481.

Olson, E. N. and A. Nordheim (2010). "Linking actin dynamics and gene transcription to drive cellular motile functions." Nat Rev Mol Cell Biol **11**(5): 353-365.

Orrico, A., L. Galli, M. L. Cavaliere, L. Garavelli, J. P. Fryns, E. Crushell, M. M. Rinaldi, A. Medeira and V. Sorrentino (2004). "Phenotypic and molecular characterisation of the Aarskog-Scott syndrome: a survey of the clinical variability in light of FGD1 mutation analysis in 46 patients." Eur J Hum Genet **12**(1): 16-23.

Oude Weernink, P. A., P. Schulte, Y. Guo, J. Wetzel, M. Amano, K. Kaibuchi, S. Haverland, M. Voss, M. Schmidt, G. W. Mayr and K. H. Jakobs (2000). "Stimulation of phosphatidylinositol-4-phosphate 5-kinase by Rho-kinase." J Biol Chem **275**(14): 10168-10174.

Owen, D. a. M., H.R. (2005). Structural Analysis of Rho protein complexes. Small GTPases: RHO family. E. Manser, Springer Netherlands**:** 31-72.

Pai, S. Y., C. Kim and D. A. Williams (2010). "Rac GTPases in human diseases." Dis Markers **29**(3-4): 177-187.

Pappenheimer, J. R. (1993). "On the coupling of membrane digestion with intestinal absorption of sugars and amino acids." Am J Physiol **265**(3 Pt 1): G409-417.

Parmacek, M. S. (2007). "Myocardin-related transcription factors: critical coactivators regulating cardiovascular development and adaptation." Circ Res **100**(5): 633-644.

Pascher, A. and J. Klupp (2005). "Biologics in the treatment of transplant rejection and ischemia/reperfusion injury: new applications for TNFalpha inhibitors?" BioDrugs **19**(4): 211-231.

Paschoud, S., L. Guillemot and S. Citi (2012). "Distinct domains of paracingulin are involved in its targeting to the actin cytoskeleton and regulation of apical junction assembly." J Biol Chem **287**(16): 13159-13169.

Pasteris, N. G., A. Cadle, L. J. Logie, M. E. Porteous, C. E. Schwartz, R. E. Stevenson, T. W. Glover, R. S. Wilroy and J. L. Gorski (1994). "Isolation and characterization of the faciogenital dysplasia (Aarskog-Scott syndrome) gene: a putative Rho/Rac guanine nucleotide exchange factor." Cell **79**(4): 669-678.

Pathak, R., V. D. Delorme-Walker, M. C. Howell, A. N. Anselmo, M. A. White, G. M. Bokoch and C. Dermardirossian (2012). "The microtubule-associated Rho activating factor GEF-H1 interacts with exocyst complex to regulate vesicle traffic." Dev Cell **23**(2): 397-411.

Pathak, R. and C. Dermardirossian (2013). "GEF-H1: Orchestrating the interplay between cytoskeleton and vesicle trafficking." Small GTPases **4**(3).

Pearson, G., F. Robinson, T. Beers Gibson, B. E. Xu, M. Karandikar, K. Berman and M. H. Cobb (2001). "Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions." Endocr Rev **22**(2): 153-183.

Pedersen, S. F. and E. K. Hoffmann (2002). "Possible interrelationship between changes in F-actin and myosin II, protein phosphorylation, and cell volume regulation in Ehrlich ascites tumor cells." Exp Cell Res **277**(1): 57-73.

Pedersen, S. F., E. K. Hoffmann and J. W. Mills (2001). "The cytoskeleton and cell volume regulation." Comp Biochem Physiol A Mol Integr Physiol **130**(3): 385-399.

Pedersen, S. F., J. W. Mills and E. K. Hoffmann (1999). "Role of the F-actin cytoskeleton in the RVD and RVI processes in Ehrlich ascites tumor cells." Exp Cell Res **252**(1): 63-74.

Pelham, R. J., Jr. and Y. Wang (1999). "High resolution detection of mechanical forces exerted by locomoting fibroblasts on the substrate." Mol Biol Cell **10**(4): 935-945.

Pertz, O. (2011). "Filopodia: Nanodevices that sense nanotopographic ECM cues to orient neurite outgrowth." Commun Integr Biol **4**(4): 436-439.

Pertz, O., L. Hodgson, R. L. Klemke and K. M. Hahn (2006). "Spatiotemporal dynamics of RhoA activity in migrating cells." Nature **440**(7087): 1069-1072.

Petrie, R. J., A. D. Doyle and K. M. Yamada (2009). "Random versus directionally persistent cell migration." Nat Rev Mol Cell Biol **10**(8): 538-549.

Pieczynski, J. and B. Margolis (2011). "Protein complexes that control renal epithelial polarity." Am J Physiol Renal Physiol **300**(3): F589-601.

Plonk, S. G., S. K. Park and J. H. Exton (1998). "The alpha-subunit of the heterotrimeric G protein G13 activates a phospholipase D isozyme by a pathway requiring Rho family GTPases." J Biol Chem **273**(9): 4823-4826.

Pollard, T. D. and G. G. Borisy (2003). "Cellular motility driven by assembly and disassembly of actin filaments." Cell **112**(4): 453-465.

Posern, G. and R. Treisman (2006). "Actin' together: serum response factor, its cofactors and the link to signal transduction." Trends Cell Biol **16**(11): 588-596.

Poujade, M., E. Grasland-Mongrain, A. Hertzog, J. Jouanneau, P. Chavrier, B. Ladoux, A. Buguin and P. Silberzan (2007). "Collective migration of an epithelial monolayer in response to a model wound." Proc Natl Acad Sci U S A **104**(41): 15988-15993.

Prenzel, N., O. M. Fischer, S. Streit, S. Hart and A. Ullrich (2001). "The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification." Endocr Relat Cancer **8**(1): 11-31.

Puls, A., A. G. Eliopoulos, C. D. Nobes, T. Bridges, L. S. Young and A. Hall (1999). "Activation of the small GTPase Cdc42 by the inflammatory cytokines TNF(alpha) and IL-1, and by the Epstein-Barr virus transforming protein LMP1." J Cell Sci **112 (Pt 17)**: 2983-2992.

Qin, J., Y. Xie, B. Wang, M. Hoshino, D. W. Wolff, J. Zhao, M. A. Scofield, F. J. Dowd, M. F. Lin and Y. Tu (2009). "Upregulation of PIP3-dependent Rac exchanger 1 (P-Rex1) promotes prostate cancer metastasis." Oncogene **28**(16): 1853-1863.

Raftopoulou, M. and A. Hall (2004). "Cell migration: Rho GTPases lead the way." Dev Biol **265**(1): 23-32.

Raingeaud, J., S. Gupta, J. S. Rogers, M. Dickens, J. Han, R. J. Ulevitch and R. J. Davis (1995). "Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine." J Biol Chem **270**(13): 7420-7426.

Raleigh, D. R., A. M. Marchiando, Y. Zhang, L. Shen, H. Sasaki, Y. Wang, M. Long and J. R. Turner (2010). "Tight junction-associated MARVEL proteins marveld3, tricellulin, and occludin have distinct but overlapping functions." Mol Biol Cell **21**(7): 1200-1213.

Ramaswamy, S., K. N. Ross, E. S. Lander and T. R. Golub (2003). "A molecular signature of metastasis in primary solid tumors." Nat Genet **33**(1): 49-54.

Rameh, L. E., A. Arvidsson, K. L. Carraway, 3rd, A. D. Couvillon, G. Rathbun, A. Crompton, B. VanRenterghem, M. P. Czech, K. S. Ravichandran, S. J. Burakoff, D. S. Wang, C. S. Chen and L. C. Cantley (1997). "A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains." J Biol Chem **272**(35): 22059-22066.

Ramesh, G. and W. B. Reeves (2002). "TNF-alpha mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity." J Clin Invest **110**(6): 835-842.

Ramesh, G., B. Zhang, S. Uematsu, S. Akira and W. B. Reeves (2007). "Endotoxin and cisplatin synergistically induce renal dysfunction and cytokine production in mice." Am J Physiol Renal Physiol **293**(1): F325-332.

Rasmussen, M., R. T. Alexander, B. V. Darborg, N. Mobjerg, E. K. Hoffmann, A. Kapus and S. F. Pedersen (2008). "Osmotic cell shrinkage activates ezrin/radixin/moesin (ERM) proteins: activation mechanisms and physiological implications." Am J Physiol Cell Physiol **294**(1): C197-212.

Reid, T., T. Furuyashiki, T. Ishizaki, G. Watanabe, N. Watanabe, K. Fujisawa, N. Morii, P. Madaule and S. Narumiya (1996). "Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the rho-binding domain." J Biol Chem **271**(23): 13556-13560.

Ren, X. D., G. M. Bokoch, A. Traynor-Kaplan, G. H. Jenkins, R. A. Anderson and M. A. Schwartz (1996). "Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells." Mol Biol Cell **7**(3): 435-442.

Ren, Y., R. Li, Y. Zheng and H. Busch (1998). "Cloning and characterization of GEF-H1, a microtubule-associated guanine nucleotide exchange factor for Rac and Rho GTPases." J Biol Chem **273**(52): 34954-34960.

Ridley, A. J. (1996). "Rho: theme and variations." Curr Biol **6**(10): 1256-1264.

Ridley, A. J. (2000). Rho. GTPases. A. Hall. New York, USA, Oxford University Press**:** 89-136.

Ridley, A. J. (2011). "Life at the leading edge." Cell **145**(7): 1012-1022.

Ridley, A. J. and A. Hall (1992). "The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors." Cell **70**(3): 389-399.

Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann and A. Hall (1992). "The small GTP-binding protein rac regulates growth factor-induced membrane ruffling." Cell **70**(3): 401-410.

Riento, K. and A. J. Ridley (2003). "Rocks: multifunctional kinases in cell behaviour." Nat Rev Mol Cell Biol **4**(6): 446-456.

Rikitake, Y. and Y. Takai (2011). "Directional cell migration regulation by small G proteins, nectin-like molecule-5, and afadin." Int Rev Cell Mol Biol **287**: 97-143.

Rittinger, K., P. A. Walker, J. F. Eccleston, K. Nurmahomed, D. Owen, E. Laue, S. J. Gamblin and S. J. Smerdon (1997). "Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP." Nature **388**(6643): 693-697.

Rizoli, S. B., O. D. Rotstein, J. Parodo, M. J. Phillips and A. Kapus (2000). "Hypertonic inhibition of exocytosis in neutrophils: central role for osmotic actin skeleton remodeling." Am J Physiol Cell Physiol **279**(3): C619-633.

Rodgers, L. S. and A. S. Fanning (2011). "Regulation of epithelial permeability by the actin cytoskeleton." Cytoskeleton (Hoboken) **68**(12): 653-660.

Rolfe, B. E., N. F. Worth, C. J. World, J. H. Campbell and G. R. Campbell (2005). "Rho and vascular disease." Atherosclerosis **183**(1): 1-16.

Rolli-Derkinderen, M., V. Sauzeau, L. Boyer, E. Lemichez, C. Baron, D. Henrion, G. Loirand and P. Pacaud (2005). "Phosphorylation of serine 188 protects RhoA from ubiquitin/proteasome-mediated degradation in vascular smooth muscle cells." Circ Res **96**(11): 1152-1160.

Ron, D., M. Zannini, M. Lewis, R. B. Wickner, L. T. Hunt, G. Graziani, S. R. Tronick, S. A. Aaronson and A. Eva (1991). "A region of proto-dbl essential for its transforming activity shows sequence similarity to a yeast cell cycle gene, CDC24, and the human breakpoint cluster gene, bcr." New Biol **3**(4): 372-379.

Roof, R. W., M. D. Haskell, B. D. Dukes, N. Sherman, M. Kinter and S. J. Parsons (1998). "Phosphotyrosine (p-Tyr)-dependent and -independent mechanisms of p190 RhoGAP-p120 RasGAP interaction: Tyr 1105 of p190, a substrate for c-Src, is the sole p-Tyr mediator of complex formation." Mol Cell Biol **18**(12): 7052-7063.

Rorth, P. (2009). "Collective cell migration." Annu Rev Cell Dev Biol **25**: 407-429.

Rossman, K. L., L. Cheng, G. M. Mahon, R. J. Rojas, J. T. Snyder, I. P. Whitehead and J. Sondek (2003). "Multifunctional roles for the PH domain of Dbs in regulating Rho GTPase activation." J Biol Chem **278**(20): 18393-18400.

Rossman, K. L., C. J. Der and J. Sondek (2005). "GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors." Nat Rev Mol Cell Biol **6**(2): 167-180.

Rudini, N. and E. Dejana (2008). "Adherens junctions." Curr Biol **18**(23): R1080-1082.

Runkle, E. A., J. M. Sundstrom, K. B. Runkle, X. Liu and D. A. Antonetti (2011). "Occludin localizes to centrosomes and modifies mitotic entry." J Biol Chem **286**(35): 30847-30858.

Sahai, E. and C. J. Marshall (2002). "RHO-GTPases and cancer." Nat Rev Cancer **2**(2): 133-142.

Saito, S., T. Tatsumoto, M. V. Lorenzi, M. Chedid, V. Kapoor, H. Sakata, J. Rubin and T. Miki (2003). "Rho exchange factor ECT2 is induced by growth factors and regulates cytokinesis through the N-terminal cell cycle regulator-related domains." J Cell Biochem **90**(4): 819-836.

Saitou, M., M. Furuse, H. Sasaki, J. D. Schulzke, M. Fromm, H. Takano, T. Noda and S. Tsukita (2000). "Complex phenotype of mice lacking occludin, a component of tight junction strands." Mol Biol Cell **11**(12): 4131-4142.

Salhia, B., N. L. Tran, A. Chan, A. Wolf, M. Nakada, F. Rutka, M. Ennis, W. S. McDonough, M. E. Berens, M. Symons and J. T. Rutka (2008). "The guanine nucleotide exchange factors trio, Ect2, and Vav3 mediate the invasive behavior of glioblastoma." Am J Pathol **173**(6): 1828-1838.

Samarin, S. N., A. I. Ivanov, G. Flatau, C. A. Parkos and A. Nusrat (2007). "Rho/ROCK-II Signaling Mediates Disassembly of Epithelial Apical Junctions." Mol Biol Cell.

Sanders, L. C., F. Matsumura, G. M. Bokoch and P. de Lanerolle (1999). "Inhibition of myosin light chain kinase by p21-activated kinase." Science **283**(5410): 2083-2085.

Sano, M., N. Genkai, N. Yajima, N. Tsuchiya, J. Homma, R. Tanaka, T. Miki and R. Yamanaka (2006). "Expression level of ECT2 proto-oncogene correlates with prognosis in glioma patients." Oncol Rep **16**(5): 1093-1098.

Santy, L. C. and J. E. Casanova (2001). "Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D." J Cell Biol **154**(3): 599-610.

Sasazuki, T., T. Sawada, S. Sakon, T. Kitamura, T. Kishi, T. Okazaki, M. Katano, M. Tanaka, M. Watanabe, H. Yagita, K. Okumura and H. Nakano (2002). "Identification of a novel transcriptional activator, BSAC, by a functional cloning to inhibit tumor necrosis factor-induced cell death." J Biol Chem **277**(32): 28853-28860.

Sato, H., T. Tanaka, T. Kita, H. Yamaguchi and N. Tanaka (2004). "The role of tumour necrosis factor-alpha in renal dysfunction following mild haemorrhage in rats." Int J Exp Pathol **85**(6): 345-353.

Sato, J. and T. Iiri (2006). "[Rho and Rho-kinase]." Nihon Rinsho **64 Suppl 5**: 197-202.

Scherle, P., T. Behrens and L. M. Staudt (1993). "Ly-GDI, a GDP-dissociation inhibitor of the RhoA GTP-binding protein, is expressed preferentially in lymphocytes." Proc Natl Acad Sci U S A **90**(16): 7568-7572.

Schliess, F. and D. Haussinger (2007). "Osmosensing by integrins in rat liver." Methods Enzymol **428**: 129-144.

Schlondorff, J., J. D. Becherer and C. P. Blobel (2000). "Intracellular maturation and localization of the tumour necrosis factor alpha convertase (TACE)." Biochem J **347 Pt 1**: 131-138.

Schluter, M. A. and B. Margolis (2012). "Apicobasal polarity in the kidney." Exp Cell Res **318**(9): 1033-1039.

Schmidt, A. and M. N. Hall (1998). "Signaling to the actin cytoskeleton." Annu Rev Cell Dev Biol **14**: 305-338.

Schmidt, G., P. Sehr, M. Wilm, J. Selzer, M. Mann and K. Aktories (1997). "Gln 63 of Rho is deamidated by Escherichia coli cytotoxic necrotizing factor-1." Nature **387**(6634): 725-729.

Schulzke, J. D., A. H. Gitter, J. Mankertz, S. Spiegel, U. Seidler, S. Amasheh, M. Saitou, S. Tsukita and M. Fromm (2005). "Epithelial transport and barrier function in occludin-deficient mice." Biochim Biophys Acta **1669**(1): 34-42.

Schwartz, M. (2004). "Rho signalling at a glance." J Cell Sci **117**(Pt 23): 5457-5458.

Schwarz, B. T., F. Wang, L. Shen, D. R. Clayburgh, L. Su, Y. Wang, Y. X. Fu and J. R. Turner (2007). "LIGHT signals directly to intestinal epithelia to cause barrier dysfunction via cytoskeletal and endocytic mechanisms." Gastroenterology **132**(7): 2383-2394.

Scott, A. J., K. P. O'Dea, D. O'Callaghan, L. Williams, J. O. Dokpesi, L. Tatton, J. M. Handy, P. J. Hogg and M. Takata (2011). "Reactive oxygen species and p38 mitogen-activated protein kinase mediate tumor necrosis factor alpha-converting enzyme (TACE/ADAM-17) activation in primary human monocytes." J Biol Chem **286**(41): 35466-35476.

Sebe, A., A. Masszi, M. Zulys, T. Yeung, P. Speight, O. D. Rotstein, H. Nakano, I. Mucsi, K. Szaszi and A. Kapus (2008). "Rac, PAK and p38 regulate cell contact-dependent nuclear translocation of myocardin-related transcription factor." FEBS Lett **582**(2): 291-298.

Settleman, J. (2003). "A nuclear MAL-function links Rho to SRF." Mol Cell **11**(5): 1121-1123.

Shah, B. H. and K. J. Catt (2006). "TACE-dependent EGF receptor activation in angiotensin-II-induced kidney disease." Trends Pharmacol Sci **27**(5): 235-237.

Shamah, S. M., M. Z. Lin, J. L. Goldberg, S. Estrach, M. Sahin, L. Hu, M. Bazalakova, R. L. Neve, G. Corfas, A. Debant and M. E. Greenberg (2001). "EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin." Cell **105**(2): 233-244.

Shen, L. (2012). "Tight junctions on the move: molecular mechanisms for epithelial barrier regulation." Ann N Y Acad Sci **1258**: 9-18.

Shen, L., E. D. Black, E. D. Witkowski, W. I. Lencer, V. Guerriero, E. E. Schneeberger and J. R. Turner (2006). "Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure." J Cell Sci **119**(Pt 10): 2095-2106.

Shen, L., C. R. Weber, D. R. Raleigh, D. Yu and J. R. Turner (2010). "Tight Junction Pore and Leak Pathways: A Dynamic Duo." Annu Rev Physiol.

Shields, J. M., N. E. Thomas, M. Cregger, A. J. Berger, M. Leslie, C. Torrice, H. Hao, S. Penland, J. Arbiser, G. Scott, T. Zhou, M. Bar-Eli, J. E. Bear, C. J. Der, W. K. Kaufmann, D. L. Rimm and N. E. Sharpless (2007). "Lack of extracellular signal-regulated kinase mitogen-activated protein kinase signaling shows a new type of melanoma." Cancer Res **67**(4): 1502-1512.

Small, J. V., K. Rottner and I. Kaverina (1999). "Functional design in the actin cytoskeleton." Curr Opin Cell Biol **11**(1): 54-60.

Smith, P. C., J. Guerrero, N. Tobar, M. Caceres, M. J. Gonzalez and J. Martinez (2009). "Tumor necrosis factor-alpha-stimulated membrane type 1-matrix metalloproteinase production is modulated by epidermal growth factor receptor signaling in human gingival fibroblasts." J Periodontal Res **44**(1): 73-80.

Snyder, J. T., D. K. Worthylake, K. L. Rossman, L. Betts, W. M. Pruitt, D. P. Siderovski, C. J. Der and J. Sondek (2002). "Structural basis for the selective activation of Rho GTPases by Dbl exchange factors." Nat Struct Biol **9**(6): 468-475.

Soderling, S. H. and J. D. Scott (2006). "WAVE signalling: from biochemistry to biology." Biochem Soc Trans **34**(Pt 1): 73-76.

Sohl, G. and K. Willecke (2004). "Gap junctions and the connexin protein family." Cardiovasc Res **62**(2): 228-232.

Sohn, H. Y., M. Keller, T. Gloe, H. Morawietz, U. Rueckschloss and U. Pohl (2000). "The small G-protein Rac mediates depolarization-induced superoxide formation in human endothelial cells." J Biol Chem **275**(25): 18745-18750.

Solski, P. A., R. S. Wilder, K. L. Rossman, J. Sondek, A. D. Cox, S. L. Campbell and C. J. Der (2004). "Requirement for C-terminal sequences in regulation of Ect2 guanine nucleotide exchange specificity and transformation." J Biol Chem **279**(24): 25226-25233.

Soond, S. M., B. Everson, D. W. Riches and G. Murphy (2005). "ERK-mediated phosphorylation of Thr735 in TNFalpha-converting enzyme and its potential role in TACE protein trafficking." J Cell Sci **118**(Pt 11): 2371-2380.

Spiering, D. and L. Hodgson (2011). "Dynamics of the Rho-family small GTPases in actin regulation and motility." Cell Adh Migr **5**(2): 170-180.

Srivastava, S. K., R. H. Wheelock, S. A. Aaronson and A. Eva (1986). "Identification of the protein encoded by the human diffuse B-cell lymphoma (dbl) oncogene." Proc Natl Acad Sci U S A **83**(23): 8868-8872.

Sturm, A. and A. U. Dignass (2008). "Epithelial restitution and wound healing in inflammatory bowel disease." World J Gastroenterol **14**(3): 348-353.

Suetsugu, S., H. Miki and T. Takenawa (2002). "Spatial and temporal regulation of actin polymerization for cytoskeleton formation through Arp2/3 complex and WASP/WAVE proteins." Cell Motil Cytoskeleton **51**(3): 113-122.

Sundberg-Smith, L. J., J. T. Doherty, C. P. Mack and J. M. Taylor (2005). "Adhesion stimulates direct PAK1/ERK2 association and leads to ERK-dependent PAK1 Thr212 phosphorylation." J Biol Chem **280**(3): 2055-2064.

Svitkina, T. M. and G. G. Borisy (1999). "Progress in protrusion: the tell-tale scar." Trends Biochem Sci **24**(11): 432-436.

Szaszi, K. and Y. Amoozadeh (Submitted). "Functions, regulation and pathological roles of tight junctions in the kidney tubular epithelium." Int Rev Cell Mol Biol.

Szaszi, K., S. Grinstein, J. Orlowski and A. Kapus (2000a). "Regulation of the epithelial Na(+) /H(+) exchanger isoform by the cytoskeleton." Cell Physiol Biochem **10**(5-6): 265-272.

Szaszi, K., K. Kurashima, A. Kapus, A. Paulsen, K. Kaibuchi, S. Grinstein and J. Orlowski (2000b). "RhoA and rho kinase regulate the epithelial Na+/H+ exchanger NHE3. Role of myosin light chain phosphorylation." J Biol Chem **275**(37): 28599-28606.

Szaszi, K., G. Sirokmany, C. Di Ciano-Oliveira, O. D. Rotstein and A. Kapus (2005). "Depolarization induces Rho-Rho kinase-mediated myosin light chain phosphorylation in kidney tubular cells." Am J Physiol Cell Physiol **289**(3): C673-685.

Szaszi, K., M. Vandermeer and Y. Amoozadeh (2012). Epithelial wound healing and the effects of cytokines investigated by ECIS. Electric Cell-Substrate Impedance Sensing in Cancer and Cancer Metastasis Research. W. G. Jiang, Springer Verlag. **17:** 131-176.

Takahashi, K., T. Sasaki, A. Mammoto, K. Takaishi, T. Kameyama, S. Tsukita and Y. Takai (1997). "Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein." J Biol Chem **272**(37): 23371-23375.

Tapon, N. and A. Hall (1997). "Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton." Curr Opin Cell Biol **9**(1): 86-92.

Tatsumoto, T., X. Xie, R. Blumenthal, I. Okamoto and T. Miki (1999). "Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis." J Cell Biol **147**(5): 921-928.

Terry, S., M. Nie, K. Matter and M. S. Balda (2010). "Rho signaling and tight junction functions." Physiology (Bethesda) **25**(1): 16-26.

Terry, S. J., C. Zihni, A. Elbediwy, E. Vitiello, I. V. Leefa Chong San, M. S. Balda and K. Matter (2011). "Spatially restricted activation of RhoA signalling at epithelial junctions by p114RhoGEF drives junction formation and morphogenesis." Nat Cell Biol **13**(2): 159-166.

Thirone, A. C., P. Speight, M. Zulys, O. D. Rotstein, K. Szaszi, S. F. Pedersen and A. Kapus (2009). "Hyperosmotic stress induces Rho/Rho kinase/LIM kinase-mediated cofilin phosphorylation in tubular cells: key role in the osmotically triggered F-actin response." Am J Physiol Cell Physiol **296**(3): C463-475.

Thompson, P. W., A. M. Randi and A. J. Ridley (2002). "Intercellular adhesion molecule (ICAM)-1, but not ICAM-2, activates RhoA and stimulates c-fos and rhoA transcription in endothelial cells." J Immunol **169**(2): 1007-1013.

Tominaga, T. and D. L. Barber (1998). "Na-H exchange acts downstream of RhoA to regulate integrin-induced cell adhesion and spreading." Mol Biol Cell **9**(8): 2287-2303.

Tonami, K., Y. Kurihara, S. Arima, K. Nishiyama, Y. Uchijima, T. Asano, H. Sorimachi and H. Kurihara (2011). "Calpain-6, a microtubule-stabilizing protein, regulates Rac1 activity and cell motility through interaction with GEF-H1." J Cell Sci **124**(Pt 8): 1214-1223.

Tsapara, A., P. Luthert, J. Greenwood, C. S. Hill, K. Matter and M. S. Balda (2010). "The RhoA Activator GEF-H1/Lfc Is a TGF-{beta} Target Gene and Effector that Regulates {alpha}-Smooth Muscle Actin Expression and Cell Migration." Mol Biol Cell.

Tsuji, T., T. Ishizaki, M. Okamoto, C. Higashida, K. Kimura, T. Furuyashiki, Y. Arakawa, R. B. Birge, T. Nakamoto, H. Hirai and S. Narumiya (2002). "ROCK and mDia1 antagonize in Rho-dependent Rac activation in Swiss 3T3 fibroblasts." J Cell Biol **157**(5): 819-830.

Tsukita, S., S. Yonemura and S. Tsukita (1997). "ERM proteins: head-to-tail regulation of actin-plasma membrane interaction." Trends Biochem Sci **22**(2): 53-58.

Turner, J. R. (2000). "'Putting the squeeze' on the tight junction: understanding cytoskeletal regulation." Semin Cell Dev Biol **11**(4): 301-308.

Turner, J. R. (2006). "Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application." Am J Pathol **169**(6): 1901-1909.

Uhlik, M. T., A. N. Abell, N. L. Johnson, W. Sun, B. D. Cuevas, K. E. Lobel-Rice, E. A. Horne, M. L. Dell'Acqua and G. L. Johnson (2003). "Rac-MEKK3-MKK3 scaffolding for p38 MAPK activation during hyperosmotic shock." Nat Cell Biol **5**(12): 1104-1110.

Van Itallie, C. M. and J. M. Anderson (1997). "Occludin confers adhesiveness when expressed in fibroblasts." J Cell Sci **110 (Pt 9)**: 1113-1121.

Van Itallie, C. M. and J. M. Anderson (2004). "The molecular physiology of tight junction pores." Physiology (Bethesda) **19**: 331-338.

Van Itallie, C. M. and J. M. Anderson (2004). "The role of claudins in determining paracellular charge selectivity." Proc Am Thorac Soc **1**(1): 38-41.

Van Itallie, C. M., A. S. Fanning, J. Holmes and J. M. Anderson (2010). "Occludin is required for cytokine-induced regulation of tight junction barriers." J Cell Sci **123**(Pt 16): 2844-2852.

Vanni, C., A. Parodi, P. Mancini, V. Visco, C. Ottaviano, M. R. Torrisi and A. Eva (2004). "Phosphorylation-independent membrane relocalization of ezrin following association with Dbl in vivo." Oncogene **23**(23): 4098-4106.

Vartiainen, M. K., S. Guettler, B. Larijani and R. Treisman (2007). "Nuclear actin regulates dynamic subcellular localization and activity of the SRF cofactor MAL." Science **316**(5832): 1749-1752.

Vega, F. M. and A. J. Ridley (2008). "Rho GTPases in cancer cell biology." FEBS Lett **582**(14): 2093-2101.

Verin, A. D., A. Birukova, P. Wang, F. Liu, P. Becker, K. Birukov and J. G. Garcia (2001). "Microtubule disassembly increases endothelial cell barrier dysfunction: role of MLC phosphorylation." Am J Physiol Lung Cell Mol Physiol **281**(3): L565-574.

Vexler, Z. S., M. Symons and D. L. Barber (1996). "Activation of Na+-H+ exchange is necessary for RhoA-induced stress fiber formation." J Biol Chem **271**(37): 22281-22284.

Vielhauer, V. and T. N. Mayadas (2007). "Functions of TNF and its receptors in renal disease: distinct roles in inflammatory tissue injury and immune regulation." Semin Nephrol **27**(3): 286-308.

Waheed, F., Q. Dan, Y. Amoozadeh, Y. Zhang, S. Tanimura, P. Speight, A. Kapus and K. Szaszi (2013). "Central role of the exchange factor GEF-H1 in TNF-alpha-induced sequential activation of Rac, ADAM17/TACE, and RhoA in tubular epithelial cells." Mol Biol Cell **24**(7): 1068-1082.

Waheed, F., P. Speight, Q. Dan, R. Garcia-Mata and K. Szaszi (2012). "Affinity precipitation of active Rho-GEFs using a GST-tagged mutant Rho protein (GST-RhoA(G17A)) from epithelial cell lysates." J Vis Exp(61).

Waheed, F., P. Speight, G. Kawai, Q. Dan, A. Kapus and K. Szaszi (2010). "Extracellular signal-regulated kinase and GEF-H1 mediate depolarization-induced Rho activation and paracellular permeability increase." Am J Physiol Cell Physiol **298**(6): C1376-1387.

Wajant, H., K. Pfizenmaier and P. Scheurich (2003). "Tumor necrosis factor signaling." Cell Death Differ **10**(1): 45-65.

Wang, N., J. P. Butler and D. E. Ingber (1993). "Mechanotransduction across the cell surface and through the cytoskeleton." Science **260**(5111): 1124-1127.

Wasserman, S. (1998). "FH proteins as cytoskeletal organizers." Trends Cell Biol **8**(3): 111-115.

Watanabe, G., Y. Saito, P. Madaule, T. Ishizaki, K. Fujisawa, N. Morii, H. Mukai, Y. Ono, A. Kakizuka and S. Narumiya (1996). "Protein kinase N (PKN) and PKN-related protein rhophilin as targets of small GTPase Rho." Science **271**(5249): 645-648.

Watanabe, N., T. Kato, A. Fujita, T. Ishizaki and S. Narumiya (1999). "Cooperation between mDia1 and ROCK in Rho-induced actin reorganization." Nat Cell Biol **1**(3): 136-143.

Wells, C. D., M. Y. Liu, M. Jackson, S. Gutowski, P. M. Sternweis, J. D. Rothstein, T. Kozasa and P. C. Sternweis (2002). "Mechanisms for reversible regulation between G13 and Rho exchange factors." J Biol Chem **277**(2): 1174-1181.

Westphal, J. K., M. J. Dorfel, S. M. Krug, J. D. Cording, J. Piontek, I. E. Blasig, R. Tauber, M. Fromm and O. Huber (2010). "Tricellulin forms homomeric and heteromeric tight junctional complexes." Cell Mol Life Sci **67**(12): 2057-2068.

Westwick, J. K., R. J. Lee, Q. T. Lambert, M. Symons, R. G. Pestell, C. J. Der and I. P. Whitehead (1998). "Transforming potential of Dbl family proteins correlates with transcription from the cyclin D1 promoter but not with activation of Jun NH2-terminal kinase, p38/Mpk2, serum response factor, or c-Jun." J Biol Chem **273**(27): 16739-16747.

Wettschureck, N. and S. Offermanns (2002). "Rho/Rho-kinase mediated signaling in physiology and pathophysiology." J Mol Med (Berl) **80**(10): 629-638.

Whitehead, I., H. Kirk, C. Tognon, G. Trigo-Gonzalez and R. Kay (1995). "Expression cloning of lfc, a novel oncogene with structural similarities to guanine nucleotide exchange factors and to the regulatory region of protein kinase C." J Biol Chem **270**(31): 18388-18395.

Whitehead, I. P., I. E. Zohn and C. J. Der (2001). "Rho GTPase-dependent transformation by G protein-coupled receptors." Oncogene **20**(13): 1547-1555.

Wittmann, T. and C. M. Waterman-Storer (2001). "Cell motility: can Rho GTPases and microtubules point the way?" J Cell Sci **114**(Pt 21): 3795-3803.

Worthylake, D. K., K. L. Rossman and J. Sondek (2000). "Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1." Nature **408**(6813): 682-688.

Xu, P. and R. Derynck (2010). "Direct activation of TACE-mediated ectodomain shedding by p38 MAP kinase regulates EGF receptor-dependent cell proliferation." Mol Cell **37**(4): 551-566.

Yamahashi, Y., Y. Saito, N. Murata-Kamiya and M. Hatakeyama (2011). "Polarity-regulating Kinase Partitioning-defective 1b (PAR1b) Phosphorylates Guanine Nucleotide Exchange Factor H1 (GEF-H1) to Regulate RhoA-dependent Actin Cytoskeletal Reorganization." J Biol Chem **286**(52): 44576-44584.

Yamaoka, T., F. Yan, H. Cao, S. S. Hobbs, R. S. Dise, W. Tong and D. B. Polk (2008). "Transactivation of EGF receptor and ErbB2 protects intestinal epithelial cells from TNF-induced apoptosis." Proc Natl Acad Sci U S A **105**(33): 11772-11777.

Yang, X. Y., M. Guan, D. Vigil, C. J. Der, D. R. Lowy and N. C. Popescu (2009). "p120Ras-GAP binds the DLC1 Rho-GAP tumor suppressor protein and inhibits its RhoA GTPase and growth-suppressing activities." Oncogene **28**(11): 1401-1409.

Yard, B. A., M. R. Daha, M. Kooymans-Couthino, J. A. Bruijn, M. E. Paape, E. Schrama, L. A. van Es and F. J. van der Woude (1992). "IL-1 alpha stimulated TNF alpha production by cultured human proximal tubular epithelial cells." Kidney Int **42**(2): 383-389.

Yasumoto, K., S. Okamoto, N. Mukaida, S. Murakami, M. Mai and K. Matsushima (1992). "Tumor necrosis factor alpha and interferon gamma synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-kB-like binding sites of the interleukin 8 gene." J Biol Chem **267**(31): 22506-22511.

Yoshimura, Y. and H. Miki (2011). "Dynamic regulation of GEF-H1 localization at microtubules by Par1b/MARK2." Biochem Biophys Res Commun **408**(2): 322-328.

Zager, R. A., A. C. Johnson, S. Y. Hanson and S. Lund (2005). "Ischemic proximal tubular injury primes mice to endotoxin-induced TNF-alpha generation and systemic release." Am J Physiol Renal Physiol **289**(2): F289-297.

Zaidel-Bar, R., M. Cohen, L. Addadi and B. Geiger (2004). "Hierarchical assembly of cell-matrix adhesion complexes." Biochem Soc Trans **32**(Pt3): 416-420.

Zegers, M. (2008). "Roles of P21-activated kinases and associated proteins in epithelial wound healing." Int Rev Cell Mol Biol **267**: 253-298.

Zenke, F. T., M. Krendel, C. DerMardirossian, C. C. King, B. P. Bohl and G. M. Bokoch (2004). "p21-activated kinase 1 phosphorylates and regulates 14-3-3 binding to GEF-H1, a microtubule-localized Rho exchange factor." J Biol Chem **279**(18): 18392-18400.

Zhang, M. L., S. Lu, L. Zhou and S. S. Zheng (2008). "Correlation between ECT2 gene expression and methylation change of ECT2 promoter region in pancreatic cancer." Hepatobiliary Pancreat Dis Int **7**(5): 533-538.

Zhang, Q., S. M. Thomas, V. W. Lui, S. Xi, J. M. Siegfried, H. Fan, T. E. Smithgall, G. B. Mills and J. R. Grandis (2006). "Phosphorylation of TNF-alpha converting enzyme by gastrin-releasing peptide induces amphiregulin release and EGF receptor activation." Proc Natl Acad Sci U S A **103**(18): 6901-6906.

Zhang, S., J. Han, M. A. Sells, J. Chernoff, U. G. Knaus, R. J. Ulevitch and G. M. Bokoch (1995). "Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1." J Biol Chem **270**(41): 23934-23936.

Zhou, Q., C. Gensch and J. K. Liao (2011). "Rho-associated coiled-coil-forming kinases (ROCKs): potential targets for the treatment of atherosclerosis and vascular disease." Trends Pharmacol Sci **32**(3): 167-173.