Characterization of Rho GTPase GAP/GEF modules in the ascomycete *Neurospora crassa*

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I hereby confirm that this thesis has been written independently and with no other sources and aids than quoted.

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Systematic Rho GAP analysis in *Neurospora crassa.*

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List of Abbreviations

aa	amino acid
A. gossypii	*Ashbya gossypii*
A. nidulans	*Aspergillus nidulans*
A. fumigates	*Aspergillus fumigatus*
ATP	adenosine triphosphate
BD	DNA-binding domain
bp	base pair
C. albicans	*Candida albicans*
CAR	actin/myosin-based contractile ring
Cdk	cyclin dependent kinase
cDNA	complementary DNA
C. purpurea	*Claviceps purpurea*
co-IP	co-immunoprecipitation
DH	Dbl homology
DHR	Dock homology region
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOCK	Dedicator of cytokinesis
DTT	dithiotreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELMO	Engulfment and Cell Motility
FGSC	Fungal Genetic Stock Center
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
HA	hemaagglutinin
het	heterokaryon
his	histidine
hyg	hygromycin
IP	immunoprecipitation
IPTG	isopropyl β-D-thiogalactopyranoside
kD	kiloDalton
LB	Luria Broth
M. grisea	*Magnaporthe grisea*
M. oryzae	*Magnaporthe oryzae*
MW	molecular weight
NCBI	National Center for Biotechnology Information
N. crassa	*Neurospora crassa*
nic	nicotinamide
NP-40	Nonidet P-4
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P. marneffei	*Penicillium marneffei*
PH	Pleckstrin homology
Rho	Ras homologue
RNA	ribonucleic acid
S. cerevisiae	*Saccharomyces cerevisiae*

SD	Synthetic Defined
SDS	sodium dodecyl sulfate
S. pombe	*Schizosaccharomyces pombe*
TEMED	tetramethylethylenediamine
T. reesei	*Trichoderma reesei*
trp	tryptophane
ts	temperature-sensitive
U. maydis	*Ustilago maydis*
VMM	Vogel's Minimal Medium
WB	Western blot
YEPD	Yeast Extract Peptone Dextrose

1 Summary

Rho (Ras homologue) GTPases are members of the Ras superfamily and known as key players in highly conserved signaling pathways regulating cellular processes like metabolism, survival, differentiation, vesicle transport and morphogenesis. The cycling between active and inactive states is essential for full signaling activity of the Rho GTPase (Barale *et al.*, 2006, Vanni *et al.*, 2005). Regulation of Rho GTPases is achieved by GTPase activating proteins (GAPs) leading to an inactive Rho GTPase and guanine nucleotide exchange factors (GEFs) that activate the small Rho GTPase (Jaffe & Hall, 2005b). Their molecular function during polar tip growth in the model mold *Neurospora crassa* is poorly understood.
The filamentous fungi *N. crassa* encodes six Rho GTPases named RHO-1 to RHO-4, CDC-42 and RAC. Previous phylogenetic and domain structure analyses identified ten potential GAPs in *N. crassa*. Since LRG-1 has already been described as Rho1 specific GAP (Vogt & Seiler, 2008), the nine remaining GAPs were further analyzed. *In vitro* GAP activity assays determined target specificity of four GAPs (RGA-1 to RGA-4) towards specific Rho GTPases. *In vitro* GAP activity assays depicted dual specificity of RGA-1 to RHO-1 and RHO-4. While *in vitro* pull-down assays confirmed the RGA-1 interaction with RHO-4, further *in vivo* RGA-1-GFP microscopy studies revealed a localization pattern at the forming septa, comparable to the localization of GFP-RHO-1. Together with the Δ*rga-1* defect in septation (irregular clusters and curved septa), which defects phenocopy those of the dominant-active Rho4, these data imply a role of RGA-1 during septation as dual specific GAP of RHO-4 and RHO-1 in *N. crassa*. In this study, a comparative phenotypic characterization of nine GAPs was elaborated, which show predominantly marginal phenotypes. Furthermore, any of the GAPs revealed in stress tests hypersensitivity to Congo Red, Calcoflour White (interfering cell wall assembly) and latrunculin A (actin polymerization inhibitor) a preliminary hint to their potential function in the regulation of the actin cytoskeleton and/or proper function of the cell wall. RGA-2, RGA-3 and RGA-4 were assigned to be involved in CDC-42, RAC and RHO-3 regulation. To investigate functional relations three double deletion mutants were generated. The double deletion of Δ*rga-2*;Δ*rga-3* leads to a phenotype resembling the Δ*rga-2* mutant, whereas the Δ*rga-3*;Δ*rga-4* double deletion phenocopied the Δ*rga-4* deletion mutant and in both strains no additional morphological characteristics were identified. However, the Δ*rga-2*;Δ*rga-4* double deletion was characterized by more severe morphological defects than the single deletion mutants. These results suggest overlapping or partially redundant functions for RGA-2 and RGA-4 in regulation of Rho GTPases in relation to maintain polar growth in *N. crassa.*
Dock180 and Elmo1 were first described in mammals as bipartite GEF of Rac1 (Cote & Vuori, 2002, Jaffe & Hall, 2005b). So far, in *N. crassa* only CDC-24 was described to function as dual GEF of the RAC-1-CDC-42 module (Araujo-Palomares *et al.*, 2011). Interaction studies verified that ELMO and DOCK form a complex and both proteins interact with RAC, but not with CDC-42 in *N. crassa*. Phenotypical characterization of ∆*dock* revealed a bulgy hyperbranched phenotype, whereas the ∆*elmo* phenotype was reminiscent to the ∆*dock* phenotype, but not identical. The defects of the ∆*dock* mutant were phenocopied by the ∆*dock*;∆*elmo* double deletion. *In vivo* microscopy studies identified subapical GFP-DOCK localization in a patchy membrane associated manner. The CRIB-GFP reporter construct revealed additional subapical membrane associated localization in ∆*dock*. These results indicate a role of DOCK in polar growth in *N. crassa*.
Taken together, this analysis in *N. crassa* will establish a more comprehensive understanding how important the spatio-temporal regulation of Rho GTPases is.

2 Zusammenfassung

Rho (Ras Homolog) GTPasen sind Mitglieder der Ras-Superfamilie und bekannt als Schlüsselfiguren bei hoch konservierten Signalwegen, indem sie zelluläre Prozesse wie Stoffwechsel, Überleben, Differenzierung, Vesikeltransport und Morphogenese regulieren. Der Wechsel zwischen aktiven und inaktiven Zuständen der Rho GTPasen ist essentiell für ihre volle Aktivität in Signalwegen (Barale *et al*., 2006, Vanni *et al*., 2005). Rho GTPasen werden mittels GTPase-aktivierenden Proteinen (GAPs) inaktiviert und Rho-Guaninnukleotid-Austauschfaktoren (GEFs) aktiviert (Jaffe & Hall, 2005b). Über ihre Funktion während des polaren Hyphenwachstums ist im Modellorganismus *Neurospora crassa* jedoch nur wenig bekannt.

In dem filamentösen Pilz *Neurospora crassa* werden sechs Rho GTPasen RHO-1 bis RHO-4, CDC-42 und RAC kodiert. Vorherige phylogenetischen und strukturellen Analysen identifizierten zehn potentielle GAPs in *N. crassa*. Da LRG-1 schon als Rho-1 spezifisches GAP beschrieben ist (Vogt & Seiler, 2008), wurden weiterhin die restlichen neun GAPs analysiert. Vier GAPs (RGA-1 bis RGA-4) wurden mittels *in vitro* GAP-Aktivitätstests bestimmt und spezifischen Rho GTPasen zugeordnet. In-vitro-GAP Aktivitätstests zeigten eine duale Spezifität von RGA-1 für RHO-1 und RHO-4. Während *in vitro* Pulldown assays die RGA-1 Interaktion mit RHO-4 bestätigten, zeigten weitere *in vivo* Mikroskopiestudien mit RGA-1-GFP eine Lokalisation an der Septe, vergleichbar mit der Lokalisation von GFP-RHO-1. Zusammen mit dem *Δrga-1* Septierungsdefekt (unregelmäßige Cluster und gekrümmte Septen), welche phänotypische Kopien des dominant aktiven RHO-4 darstellten, implizierten diese Daten eine Rolle von RGA-1 während der Septierung als dual spezifisches GAP von RHO-1 und RHO-4 in *N. crassa*. In dieser Studie wurde ein vergleichende phänotypische Charakterisierung der neun GAPs ausgearbeitet, diese zeigte jedoch vorwiegend marginal Phänotypen. Weiterhin zeigten einige der GAPs eine Überempfindlichkeit gegenüber Kongorot, Calcoflour White (stören die Zellwand) und Latrunkulin A (Aktinpolymerisations-Inhibitor) welches einen vorläufigen Hinweis auf ihre potentielle Funktion bei der Regulation des Aktin-Zytoskeletts und / oder die ordnungsgemäße Funktion der Zellwand lieferten. RGA-2, RGA-3 und RGA-4 sind an der Regulation von CDC-42, RAC und RHO-3 beteiligt. Um einen funktionelle Zusammenhang zu ermitteln wurden drei Doppelmutanten erzeugt. Die Δrga-2; Δrga-3 Doppelmutante führte zu einem Phänotyp ähnlich dem der Δrga-2 Mutante, während die Δrga-3; Δrga-4 Doppelmutante eine phänotypische Kopie der Δrga-4-Deletionsmutante war und in beiden Stämmen wurden keine zusätzlichen morphologischen Eigenschaften identifiziert. Jedoch zeigte die Δrga-2; Δrga-4 Doppelmutante schwerwiegendere morphologischen Defekte, als die beiden Einzelmutanten. Diese Ergebnisse legten eine überlappende oder teilweise redundante Funktionen für RGA-2 und RGA-4 bei der Regulierung der Rho GTPasen in Bezug auf polares Wachstum in *N. crassa* nahe.

DOCK180 und ELMO1 wurden erstmals in Säugetieren als zweiteilige GEF von Rac1 beschrieben (Cote & Vouri, 2002; Jaffe & Hall, 2005). Bisher wurde in *Neurospora crassa* nur CDC-24 als funktionelles duales GEF des RAC-CDC-42 Moduls beschrieben (Araujo-Palomares *et al*, 2011). Interaktionsstudien bestätigten, dass ELMO und DOCK einen Komplex bilden und beide Proteine interagieren mit RAC, aber nicht mit CDC-42 in *N. crassa*. Die phänotypische Charakterisierung von ∆dock ergab einen bauchigen hyperverzweigten Phänotyp, während der ∆elmo Phänotyp an den der ∆ dock Deletionsmutante erinnerte, jedoch nicht identisch war. Die Δ*elmo*Δ*dock* Doppelmutante gleicht phänotypisch der Δ*dock* Einzelmutante. In vivo Mikroskopiestudien identifizierten eine subapikale unregelmässige membran assoziierte GFP-DOCK Lokalisation. Das CRIB-GFP Reporter Konstrukt zeigte eine zusätzliche subapicale Membran assoziierte Lokalisierung in ∆dock. Diese Ergebnisse weisen auf eine Rolle von DOCK in polaren Wachstums in *N. crassa* hin.

Zusammengenommen werden diese Analysen in *N. crassa* zu einem umfassenderen Verständnis beitragen wie wichtig die räumlich-zeitliche Regulation von Rho-GTPasen ist.

3 Introduction

3.1 Rho GTPases: key regulators in signaling pathways

The first genes encoding Ras homologues guanosine triphosphatases (Rho GTPases) were identified about thirty years ago (Madaule & Axel, 1985). Initially, small GTPases were characterized as regulators of the actin cytoskeleton, polarity and cell morphology in mammals and yeast (Adams *et al.*, 1990, Johnson & Pringle, 1990, Kozma *et al.*, 1995, Ridley & Hall, 1992a, Ridley & Hall, 1992b) . Rho GTPases are key components participating in regulation of multiple cellular processes, for example cell polarity and cell morphology. The asymmetry of cells is important for unicellular and multicellular organisms, because directed cell growth is one of the most fundamental prerequisite in development. Polar growth is triggered by a wealth of internal and/or external factors (Perez & Rincon, 2010). In order to ensure polarity of the cell, cytoskeletal and secretory elements, plasma membrane proteins, cell wall proteins and extra cellular matrix constituents are arranged (Drubin & Nelson, 1996).

Rho GTPases function as "molecular switches" that cycle between an active GTP-bound and an inactive GDP-bound form (Figure 1). Transition between these two forms is achieved through GTPase-activating proteins (GAPs) leading to the inactive form, guanine nucleotide exchange factors (GEFs) that activate the small G-protein and guanine nucleotide dissociation inhibitors (GDIs) which block spontaneous activation (Jaffe & Hall, 2005; (Olofsson, 1999). In addition to the high number of regulatory proteins, many effector proteins exist, coupling the activated Rho GTPase to downstream signaling events (Assemat *et al.*, 2008, Iden & Collard, 2008).

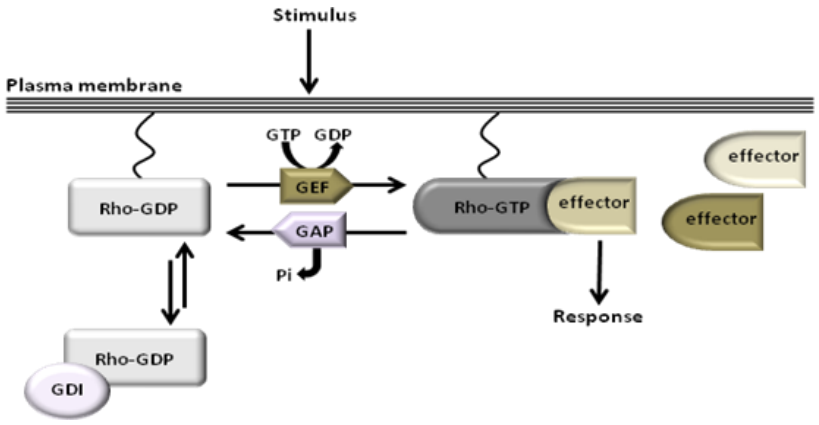


FIGURE 1: Schema of the Rho GTPase "switching" regulation mechanism with GAPs, GEFs and GDIs. The Rho GTPase is bound to the plasma membrane and depending on activation or inactivation state bound to GDP or GTP. Only the GTP bound form can bind to various effectors, which in turn triggers a specific response (modified to Perez & Rincon, 2010).

3.2 Rho GTPases in fungal development

Studies based on high throughput molecular methods suggest, that around 5.1 million fungal species exist (Blackwell, 2011). Many of them grow apically and form filamentous multicellular hypha, which are separated by septa (Boyce & Andrianopoulos, 2007, Momany, 2002, Wendland & Walther, 2005). This polar growth mode together with the ability to explore ecological niches is certainly a reason for their evolutionary success (Morris *et al.*, 2007, Pringle & Taylor, 2002). Moreover, fungi cause severe problems of plants and animals. Understanding the polarity processes by molecular analysis could point the way to targets for antifungal drugs. In addition, fungi are used in various processes and products (Peberdy, 1994). Nevertheless, it is still a big challenge to understand the mechanisms behind hyphal growth.

Polarity is determined by selecting a position where the single spore breaks dormancy. After a period of isotropic expansion the cells switch to polar growth and polarity is "established". Next, the machinery and the material to prepare new membrane and cell wall are provided, a germ tube emerges and polarity is "maintained". Finally, the germ tube extends apically (Figure 2). In filamentous fungi multiple polarity axes are spawned simultaneously out of the hyphae and the mycelium is created (d'Enfert & Fontaine, 1997, Momany, 2005). The switch to polar growth is permanent in filamentous fungi, whereas in budding yeast *S. cerevisiae* phases of isotropic and polar growth rotate (Momany, 2002).

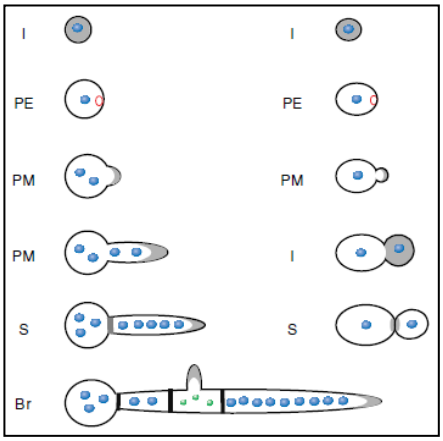


FIGURE 2: Polarity in filamentous fungi and budding yeast. Growth areas are highlighted in grey. I- isotropic expansion; PE- polarity establishment; PM- polarity maintenance; S- septation; Br- branching. Blue ovals represent interphase nuclei; green dots the mitotic nuclei; small open red circles the cortical markers. For details see text (Momany, 2002).

An important feature of filamentous fungi is the rapid growth of up to ≥ 1 µm per second (Seiler & Plamann, 2003). Early studies proposed three models to enable directed and polar hyphal tip growth. First the "amoeboid model" where hyphal tips expand by the force of the cytoskeleton, while the cell wall determines hyphal shape (Heath & Steinberg, 1999). Second the "steady-state model", which predicted that turgor pressure is able to enlarge new and still flexible cell wall material at the hyphal

apex (Wessels, 1986). And the third, the “vesicle supply center” (VCS) regulates growth by a controlled gradient of exocytosis of enzymes. In combination with turgor pressure, hyphal elongation and shape were determined (Bartnicki-Garcia, 1989). Thus, a possible explanation of hyphal growth could be a combination of the three models (Steinberg, 2007).

Fungi are exposed to a multitude of external factors during development and it is essential for their survival to maintain extreme environmental changes. Yeasts and other fungi solve this problem by cell stabilization by rigid cell walls (Levin, 2005). However, the apically formed new cell wall fibers (chitin or glucan chains) are still flexible and not yet cross linked. Due to hyphal elongation, subapical chitin crystallized, is covalent bound to ß-1,3 glucans and the cell walls become solid . Still controversial is the theory of powering tip growth by increasing the cell wall via pressure through the cytoplasm (Steinberg, 2007).

The characteristic tubular shape of filamentous fungi is achieved by concentrating processes like cell wall synthesis at the hyphal apex (Bartnicki-Garcia, 1969). Polarized growth requires a constant stream of secretory vesicles delivered to the fungal tip. Synthases are transported by secretory vesicles from distal regions of the hypha to the apical dome, where they then fuse to the apical plasma membrane and synthesize their respective polysaccharides (Drgonova *et al.*, 1996, Lopez-Romero *et al.*, 1978, Qadota *et al.*, 1996). Recent studies revealed the accumulation of chitin synthases (CHS) at the core of the SPK in *N. crassa* (Riquelme *et al.*, 2007) and in several other fungi at the hyphal tip (Weber *et al.*, 2006, Takeshita *et al.*, 2005). The involved components, the ß-1,3- glucan synthase complex and several members of the chitin synthase family, localized at the hyphal tip (Beauvais *et al.*, 2001a, Riquelme et al., 2007, Takeshita et al., 2005, Weber et al., 2006). In yeast and filamentous fungi ß-1,3 glucan is synthesized by the ß-1,3-glucan synthase, a multiprotein complex consisting at least of the catalytic subunit Fks1 and the regulatory component the Rho GTPase Rho1 (Kang & Cabib, 1986).

Behind the apical cap an apical body exist, called the Spitzenkörper (SPK), which was originally identified in active growing hypha (Girbardt, 1957). The SPK is a multivesicular structure, involved in organization of vesicles at the growing tip, which are estimated to fuse with the apical plasma membrane (Borkovich *et al.*, 2004, Riquelme & Sanchez-Leon, 2014, Riquelme *et al.*, 2011). The SPK appears as dynamic apical complex consisting of multiple components, changing in composition and pattern between different fungal species (Lopez-Franco *et al.*; 1994). Microscopy studies investigated the SPK as center of vesicle accumulation with ribosomes, actin microfilaments and some undefined granular material (Howard, 1981, Grove & Bracker, 1970, Girbardt, 1969). An important aspect is that the SPK itself moves forward, thought to determine direction and rate of hyphal growth (Riquelme *et al.*, 2002). The position of the SPK is influencing hyphal growth and a (reversible) SPK dislocation from the apical dome has been reported to cause a decrease in hyphal elongation rate, resulting in a rounded and increased diameter of the apex (Bartnicki-Garcia *et al.*, 1995). The SPK was also identified

to correlate with growth direction, while a trajectory shift from the existing cell axis triggered a permanent change in growth direction (Riquelme *et al.*, 1998).

The SPK, the polarisome and the exocyst are elements of the tip growth machinery (Harris, 2006, Harris & Momany, 2004, Sudbery, 2011). The exocyst is a conserved octameric complex and was first identified in *S. cerevisiae* (Novick & Schekman, 1979). The exocyst components were described to be hydrophilic cytosolic proteins, which are able to associate with membranes (TerBush & Novick, 1995) and their concept is to tether vesicles to sites of exocytosis. In filamentous fungi exocytosis is steadily focused to the hyphal apex, where the SPK collects the secretory vesicles and finally they are delivered to the apical plasma membrane (Bartnicki-Garcia, 1989). In *N. crassa* two exocyst components, EXO-70 and EXO-84 were identified as part of the outer layer of the SPK, whereas SEC-3, SEC-5, SEC6, SEC-8 and SEC-15 were found at the apical plasma membrane (Riquelme *et al.*, 2014). The presence of this complex is necessary in *N. crassa* for the formation of a functional SPK and the maintenance of regular hyphal growth (Riquelme *et al*, 2014). A connection between the exocyst complex and the Rho GTPases Rho4 was quite recently described in *S. pombe* (Perez *et al.*, 2015).

However, multiple cellular processes are regulated by Rho GTPases and aside from polarity they participate in numerous conserved mechanisms, for example septation. Many proteins involved in polar growth localize apically and to the septum. A possible reason could be that the septum formation requires localized cell wall and membrane generation, which is alike to polar growth (Araujo-Palomares et al., 2011, Richthammer *et al.*, 2012, Sudbery, 2011). Cellular division is tightly regulated and crucial for cell development in mammals and fungi (Nanninga, 2001). Cytokinesis requires precise spatial and temporal regulation to ensure genetic stability. Every organism undergoing this process pass three main stages: First the site selection where cell division should occur, second the positioning of protein complexes involved in division and third the formation and constriction of the contractile ring and septum construction (Walther & Wendland, 2003). Common upon fungal division is that the actin/myosin based contractile ring is assembled at the selected site of division and the septum is formed through membrane invagination. However, the general mechanism is conserved but requires the coordination of several key events and the involved proteins vary within fungi (Adams, 2004, Cabib, 2004, Latge, 2007, Sipiczki, 2007). Many of these events are controlled by Rho GTPases and their regulators (Hall, 1998, Hall, 2012). Within the hyphal compartments central at the septum, the septal pore exist, maintaining intercellular communication, transport of organelles and cytoplasm through the hyphal compartments. Damaged hypha are able to close this septal pore via Woronin bodies (Jedd & Chua, 2000, Lai *et al.*, 2012, Tenney *et al.*, 2000, Yuan *et al.*, 2003).

3.3 Rho GTPases: structure and regulation mechanisms

Efficient activation of specific GTPases requires not only the coordination of the “on” switch, because cycling between active and inactive states is essential for full signaling activity of the small GTPase.

Missregulation of Rho GTPases leads to dominant-active or dominant-negative Rho GTPases, affecting multiple cellular processes like cell wall formation, polarity, branching and conidiation in *P. marneffei*, *A. nidulans*, *C. purpurea* and *N. crassa* (Araujo-Palomares *et al*., 2011, Boyce *et al*., 2001, Boyce *et al*., 2003, Scheffer *et al*., 2005, Virag *et al*., 2007).

The structural core element of Rho GTPases is a 20 kD specific domain, called the G domain. The G domain provides an universal structure- and switch-mechanism and is responsible for nucleotide binding and hydrolysis (Vetter & Wittinghofer, 2001). The structure was first observed in Ras (Milburn *et al.*, 1990) and Rho differs only by an extra α helical insertion of 13 amino acids (Hirshberg *et al.*, 1997).

Another important prerequisite for functionality of Rho GTPases is their subcellular membrane localization. By a series of posttranslational modifications this attachment is triggered by a prenylation motif at their C-terminus, named the CaaX box (C= Cystein; a= aliphatic and X= any amino acid) (Bustelo *et al.*, 2007).

Effector proteins only bind to active GTP-bound GTPases, such as p21-activated kinases (PAKs) and mitogen-activated protein kinases (MAPKs), which promotes in turn multiple cellular processes like actin patch polarization, septin ring formation, actin cable polarization and assembly, exocytosis and cell fusion (Park & Bi, 2007, Perez & Rincon, 2010). The Rho GTPase effector interaction is mediated by several domains increasing the interaction specificity (Dvorsky *et al.*, 2004). As an exception, several Cdc42 and/or Rac effectors possess a conserved Cdc42/Rac-interactive binding domain, abbreviated CRIB, which is involved in GTPase binding (Karnoub *et al.*, 2004). The interaction of Rho GTPases with different effector proteins allows the control of multiple biochemical pathways and therefore important cellular processes (Etienne-Manneville, 2004, Jaffe & Hall, 2005b). In *S. cerevisiae* the PAK - like protein kinase Cla4p is a downstream effector protein of the Cdc42p GTPase-module and is involved in budding and cytokinesis (Cvrckova *et al.*, 1995, Versele & Thorner, 2004). In *A. gossypii* Cla4 was found to be involved in septation, because its deletion results in mutants severely impaired in actin and chitin ring formation (Ayad-Durieux *et al.*, 2000) and in *U. maydis* Cla4 is described to be a Rac1 effector (Lovely & Perlin, 2011). PAKs belong to the group of Ste20-related kinases, which are involved in different cellular and developmental processes such as morphogenesis, cell cycle regulation, apoptosis and act as potential regulators of the MAP kinase cascades (Dan *et al.*, 2001).

3.4 Rho GTPases in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*

Budding yeast *S. cerevisiae* and fission yeast *S. pombe* encode six Rho GTPases Rho1(p) to Rho5(p) and Cdc42(p) and the number of their regulators exceed those of the GTPases. Family members of Rho GTPases participate as key proteins in various cellular activities including establishment and maintenance of polarity and cytokinesis (Harris & Momany, 2004, Howell & Lew, 2012, Park & Bi,

2007, Perez & Rincon, 2010, Wang *et al.*, 2014). In earlier times was reported that Rac is not present in budding yeast *S. cerevisiae*, however, recent studies of phylogenetic and functional analyses provides the possibility that Rho5p might be a Rac ortholog (Elias & Klimes, 2012, Harris, 2011, Singh *et al.*, 2008). In this case, *S. pombe* would be the only organism without a homolog of Rac (Elias & Klimes, 2012).

The front of cells is often defined by accumulation of active GTP-bound GTPases at the cellular cortex. GTPases seems to appear to be stable concentrated at the front of cells but studies in budding yeast *S. cerevisiae* identified a dynamic polar cap. The Cdc42p molecules exchanged rapidly between the cytosol and the polar cap and they stay just for a few seconds at the front of the cells (Wedlich-Soldner *et al.*, 2004). Nevertheless, the Rho GTPase Cdc42p is key regulator of polarity and involved in budding, mating and filamentous growth (Perez & Rincon, 2010) and additionally involved in actin organization and septation (Adams et al., 1990, Atkins *et al.*, 2013, Caviston *et al.*, 2003, Howell & Lew, 2012, Johnson & Pringle, 1990, Onishi *et al.*, 2013, Rincon *et al.*, 2007). Rho1p is identified to be master regulator of cell wall formation, cell wall integrity and the actin cytoskeleton (Drgonova et al., 1996, Kohno *et al.*, 1996, Madaule *et al.*, 1987). The function of Rho2 is only poorly defined and so far budding yeast Rho2p was reported to be involved in organization of the actin cytoskeleton and cell wall (Manning *et al.*, 1997, Marcoux *et al.*, 2000). Rho3p is described to act in polar cell growth via exocytosis and formin activation (Adamo *et al.*, 1999, Doignon *et al.*, 1999, Imai *et al.*, 1996, Matsui & Toh-e, 1992, Robinson *et al.*, 1999, Wu *et al.*, 2010). The Rho4p deletion depicted no defect, whereas the double deletion of Δ*rho3*Δ*rho4* was characterized by a severe growth phenotype (Dong *et al.*, 2003, Matsui & Toh-e, 1992, Roumanie *et al.*, 2005). So, Rho3p and Rho4p have partially redundant functions and were reported to be involved in cell polarity and regulation of exocytosis in *S. cerevisiae*, whereas Rho5p mediates stress response (Schmitz *et al.*, 2002, Singh et al., 2008).

In *S. pombe* Cdc42 in essential for viability and the loss of Cdc42 function resulted in cell polarity defects (Estravis *et al.*, 2011, Etienne-Manneville, 2004, Johnson, 1999) Rho5, suggested to be a Rho1p homologue in *S. pombe,* controls the actin cytoskeleton organization and the cell wall synthesis (Nakano *et al.*, 2005, Rincon *et al.*, 2006). Studies implicated fission yeast Rho2 in cell wall synthesis by regulation of α-glucan synthase (Calonge *et al.*, 2000). Rho3 functions in polarized growth and is modulating the exocyst complex in order to cell separation (Nakano *et al.*, 2002, Wang *et al.*, 2003). Rho4 localized to the septum during mitosis and is reported to be involved in septation and the cell wall integrity pathway. Deletion strains of *rho4* are still viable in *S. pombe* but have cell separation defects at higher temperatures caused by their inability to target glucanases for degradation to the primary septum (Santos *et al.*, 2005, Santos *et al.*, 2003, Nakano *et al.*, 2003). Additionally Rho4 is required for the proper localization of the exocyst (and septins) at higher temperatures. Further interaction studies demonstrated that Rho4 is interacting with exocyst subunits (Sec8 and Exo70),

whereas Sec8 preferentially binds to activated GTP-Rho4, suggesting that Sec8 could be an effector of Rho4 (Perez *et al*, 2015).

3.5 Rho GTPases in filamentous fungi

Different pathways assure the complex property of polar growth: GTPases of the Ras super family, cAMP dependent protein kinases (PKA), mitogen-activated protein kinases (MAPK) and nuclear Dbf2-related (NDR) kinases are involved in fungal hyphal polarity, development and pathogenicity. Many studies concentrate on Rho GTPases modules and their regulation (Borkovich et al., 2004, Harris, 2006, Wendland & Philippsen, 2001).

Rac was considered to be the founding member of the Rho GTPase family where Cdc42 and the Rho proteins descended from (Boureux *et al.*, 2007). Both proteins, Rac and its close relative Cdc42, were described to affect polarized growth (Boyce et al., 2001, Boyce et al., 2003, Boyce *et al.*, 2005, Chen & Dickman, 2004, Mahlert *et al.*, 2006, Virag et al., 2007, Weinzierl *et al.*, 2002). In the basidiomycete *Ustilago maydis* Rac1 plays a crucial role in cell polarity and is necessary to induce filament formation and apical tip growth, while Cdc42 regulates cytokinesis (Etienne-Manneville, 2004, Mahlert et al., 2006). In the dimorphic fungus *Penicillium marneffei* Cdc42 and Rac regulate together the maintenance of polarized growth (Boyce *et al*., 2005). The RAC-CDC-42 module is responsible for polarized growth and hyphal morphogenesis in *Neurospora crassa*. The Δ*rac* deletion leads to dichotomous tip splitting and an apical hyperbranching phenotype, while the Δ*cdc42* phenotype is related to this, but is observed in a lower extent (Araujo-Palomares *et al*, 2011).

Rho1 was described in various studies as key regulator of hyphal growth and polarity. In *Aspergillus fumigatus* Rho1 is described as part of the β−1,3-glucan synthase complex and localized apically in active growing cells (Beauvais *et al.*, 2001b) while conditional *rho1* mutants showed cell lysis at hyphal tips, reduced radial growth and conidiation under suppressive conditions (Dichtl *et al.*, 2010, Dichtl *et al.*, 2012). *Aspergillus nidulans* RhoA was described to be implicated in cell wall synthesis, polar growth and branching (Guest *et al.*, 2004). A similar role was suggested for Rho1 in *Ashbya gossypii*. The deletion of *rho1* resulted in reduced filamentous growth and cell lysis. In *N. crassa rho-1* mutants revealed the function of RHO-1 in cell polarization and maintenance of cell wall integrity (CWI) pathway in *N. crassa*. Additionally, RHO-1 was described to function as regulatory subunit of the glucan synthase. Conditional *rho-1, rho-2* double deletion mutants determined cell polarity and growth defects (Richthammer *et al*, 2012).

In filamentous fungi there are only a few studies on the function of Rho2. RhoB of *A. niger* might play a role in cell wall integrity (Kwon *et al.*, 2011) and AgRho2 appears to have a role in regulation of branching, mainly at the hyphal tip (Nordmann *et al.*, 2014). In *N. crassa* was RHO-2 proposed to function in a partially redundant manner with RHO-1 in regulating cell wall homeostasis in *N. crassa* (Vogt & Seiler, 2008; Richthammer *et al*, 2012).

Until now, the function of Rho3 is not entirely understood. In the mesophilic filamentous fungi *Trichoderma reesei* Rho3 is acting in exocytosis but not essential (Vasara *et al.*, 2001a, Vasara *et al.*, 2001b). AgRHO-3 is involved in hyphal tip growth and *rho3* mutants displayed apically defects in *A. gossypii* (Wendland & Philippsen, 2000). However, the *Magnaporthe grisea rho3* mutant had no defects morphology and vegetative growth (Zheng *et al.*, 2007). No data are available regarding to the function of Rho3 in *N. crassa*.

In different organisms Rho4 was associated with cytokinesis and septum formation and their mutants were described by abnormal morphology, cell wall defects and altered septation pattern. In *C. albicans* Rho4 is involved in septum construction. This fact was accompanied by its localization to septation sites and cells lacking *rho4* which depict a defect in septation (Dunkler & Wendland, 2007). On the contrary to this, the *rho4* deletion showed no or only minor defects in *S. cerevisiae* and *A. gossypii* (Matsui & Toh-e, 1992, Wendland & Philippsen, 2000). In *N. crassa* the *rho4* mutant was slow in growth, aseptated and showed abnormal hyphal morphology. Activated *rho-4* alleles suggested a role of *rho-4* in the CWI pathway (cell wall defect and lysis at hyphal tips), similar to the *S. pombe rho4* and *rhoA* of *A. nidulans* (Rasmussen & Glass, 2005).

Studies in filamentous fungi revealed the important role of Rho GTPases in hyphal development, but their general biological functions and their relative importance differ widely between the species. These diverse Rho GTPase signaling is regulated by their interaction with GAPs, GEFs and GDIs (Jaffe & Hall, 2005).

3.6 Regulation of Rho GTPases via GTPase-activating proteins (GAPs) and Guanine nucleotide exchange factors (GEFs)

It is obvious that Rho GTPases are involved in numerous important developmental processes. The key for deciphering the temporal and spatial activity of Rho GTPases is to understand the “fine-tuned” regulation by their GAPs and GEFs. Noteworthy is that the number of GAPs present in genomes is exceeding those of the GEFs (Adams *et al.*, 2000, Borkovich et al., 2004, Goffeau *et al.*, 1996, Jaffe & Hall, 2005a) in turn exceeding those of the small GTPases. This fact indicates that the “fine-tuning” of Rho GTPase activity by the "off" switch is of great importance for providing the specificity for the Rho GTPase modules. In addition, GAPs and GEFs are involved in regulation of several output pathways and many act as scaffolds to link upstream and downstream components of signaling cascades, the cross-talk to other Rho modules or other cellular pathways (Rossman *et al.*, 2005, Rossman & Sondek, 2005, Tcherkezian & Lamarche-Vane, 2007).

GAPs and GEFs are multidomain proteins implying typically domains used for lipid and/or protein interaction. In principle, the domain organization provides a first indication of possible regulatory mechanisms and the domains are thought to integrate signals for effective crosstalk between several

signal transduction pathways (Cote & Vuori, 2007, Tcherkezian & Lamarche-Vane, 2007, Yarwood *et al.*, 2006).

Many GAP proteins contain several different structural motifs, but the the common motifs are: First the Src homology domain 3 domain (SH3) binding to proline-containing peptides and second the phosphoinositide-binding pleckstrin homology (PH) domain. The interaction of GAPs with Rho GTPases is important in order to increase their hydrolysis reaction by several orders of magnitude, since their intrinsic GTP hydrolysis reaction is very slow (Bos *et al.*, 2007, Symons & Settleman, 2000). The residues required for nucleotide binding and hydrolysis are typically present in the G-domain of GTPases and the hydrolysis reaction has been well studied in small GTPases for example Ras (Bos et al., 2007, Inoue & Randazzo, 2007, Vetter & Wittinghofer, 2001). Thus, an efficient GTP hydrolysis reaction requires catalytic residues supplied *in cis* by the G-domain and *in trans* by the GAP protein. An arginine residue within the GAP domain, the so-called “arginine finger”, together with a conserved catalytic Gln from the GTPase stabilize this mechanism (Hakoshima *et al.*, 2003). However, intriguingly different mechanisms are operative in GTPases (Anand *et al.*, 2013).

Two families of guanine exchange factors (GEFs), Dbl and Dock, are described to be responsible for spatio-temporal activation of Rho GTPases and downstream signaling events in mammals (Laurin & Cote, 2014). They are important in embryonic development and their miss-regulation leads to a variety of diseases (Alan & Lundquist, 2013, Bryan *et al.*, 2005a, Bryan *et al.*, 2005b, Cancelas & Williams, 2009, Hall & Lalli, 2010). Dbl GEF proteins have at least two domains: the DbI homology domain and the pleckstrin homology domain, which together promote full GEF activity (Schmidt & Hall, 2002, Zheng, 2001). Structural studies in mammals revealed a conserved nucleotide exchange mechanism catalyzed by GEFs (Rossman *et al.*, 2002, Snyder *et al.*, 2002, Worthylake *et al.*, 2000). The family of Dock GEFs is described by two conserved domains: First the lipid binding Dock homology region 1 (DHR-1) and second the GEF DHR-2 module (Laurin & Cote, 2014). The DHR-2 domain of Dock180-related GEFs catalyzes the guanine nucleotide exchange on Rho GTPases (Yang *et al.*, 2009). The GEF Dock180 is described to possess additional autoinhibitory domains. Dock180 is auto-inhibited by binding of the SH3 domain to the *in cis* DHR-2 domain preventing Dock180 from interaction with Rac1. Until after the PxxP region of Elmo1 is bound to the SH3 domain of Dock180, the autoinhibition dissolves and Rac1 interaction and GEF catalysis of the DHR-2 domain is possible (Cote & Vuori, 2007, Patel *et al.*, 2010).

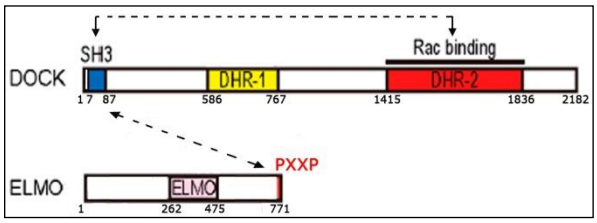


Figure 3: Domain structure of the proteins DOCK and ELMO in *N. crassa* modified to (Hanawa-Suetsugu *et al.*, 2012). The domains involved in interaction and autoinhibition were described in mammals and are indicated by dashed arrows (Cote & Vouri, 2007).

Further, an autoinhibitory switch is suggested for the adapter protein Elmo1 in mammals: the N-terminal ELMO inhibitory domain (EID; defined by an HEAT domain followed by four ARR) binds to the C- terminal Elmo1 autoregulatory domain (EAD; resembling the DAD in formin proteins) (Lammers *et al.*, 2005, Nezami *et al.*, 2006, Patel et al., 2010). These additional mechanisms are important features in specific regulation of Rho GTPases (Figure 3).

3.7 Aims of this work

Rho GTPases are key players involved in signal transduction pathways that regulate multiple fundamental cellular processes including morphogenesis, differentiation, survival, metabolism and vesicle transport. Rho GTPases function as molecular switches between an active and inactive state regulated by GAPs and GEFs. Their molecular functions during the polar tip growth in the filamentous fungi N. crassa are poorly understood. The ascomycete *Neurospora crassa* encodes six Rho GTPases and numerous putative regulators and many of them were not assigned to Rho GTPases and are still uncharacterized. The aim of this study was to further investigate nine predicted GAPs and the potential GEFs DOCK and ELMO. Taken together, this analysis will assist in establishing a more comprehensive understanding of the Rho GTPase signaling during hyphal growth of filamentous fungi.

4 Materials and methods

4.1 Strains, media and growth conditions

General handling procedures and media used in this study to cultivate *N. crassa* have been described by (Davis, 1970) or are available at the Fungal Genetic Stock Center (www.fgsc.net). Strains were grown in liquid or solid (supplemented with 2% agar) Vogel's minimal media (VMM) with 2% (w/v) sucrose. *N. crassa* was crossed on 2% corn meal agar and 0.1% glucose (Sigma, St. Louis, USA).

Chemicals and culture media components, as well as all supplements in stress tests, were used from AppliChem GmbH, Carl Roth GmbH & Co. KG, Invitrogen GmbH, Merck KGaA, Roche Diagnostics GmbH and Sigma-Aldrich Chemie GmbH. To restrict radial growth on VMM supplement plates, sucrose was exchanged by 2% L-sorbose, 0.05% glucose and 0.05% fructose (Mishra & Tatum, 1972, Taft *et al.*, 1991). 1mg/ml Congo Red, 5% NaCl, 0.3µM Benomyl, 0.01% SDS, 5mg/ml Lysing enzyme, 0.5µM Caspofungin, 2mg/ml Calcoflour and 2nM Latrunculin A were added to the growth medium to analyse the sensitivities of the deletion mutants.

Media for auxotrophic strains were supplemented with 8µg/ml nicotinamide, 200µg/ml tryptophane or 150µg/ml histidine. The concentration of hygromycin B (InvivoGen, USA) was adjusted to 200µg/ml to select transformants.

Escherichia coli DH5α were grown in liquid LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) or solid medium (with 1.5% agar) modified from (Bertani, 1951) and were incubated at 37°C. 100µg/ml of ampicillin or kanamycin was used for selection (all from Sigma-Aldrich, Taufkirchen, Germany).

4.2 Transformation

Saccharomyces cerevisiae cultures were grown under non-selective conditions at 30°C in Yeast Extract Peptone Dextrose (YEPD) medium (2% peptone, 1% yeast extract, 2% glucose, for solid medium 2% agar was added). Furthermore, Synthetic Defined (Remy *et al.*) minimal medium lacking different amino acids was used for selection of plasmid expression or interaction in the yeast two-hybrid assay (0.17% yeast nitrogen base (w/o amino acids, w/o ammonium sulphate), 0.5% ammonium sulphate, 10mg L-adenine sulphate, 10mg L-argenine, 10mg L-histidine, 15mg L-isoleucine, 50mg L-leucine, 15mg L-lysine, 10mg L-methionine, 25mg L-phenylalanine, 10mg L-tryptophane , 100mg L-threonine, 15mg L-tyrosine, 10mg uracil and 75mg L-valine, 2% glucose, 1,5% agar).

For amplification of plasmids the *Escherichia coli* strain DH5α [F–, Φ80dΔ(*lacZ*)M15-1, Δ(*lacZYA-argF*)U169, *recA1*, *endA1*, *hsdR17* (rK–, mK+), *supE44*, λ–, *thi1*, *gyrA96*, *relA1*] (Woodcock *et al.*, 1989) was used.

Yeast two-hybrid analyses were performed with the *Saccharomyces cerevisiae strain* AH109 [*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2:: GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ*] (James *et al.*, 1996)(Clontech , USA).

4.3 Plasmid construction

4.3.1 General cloning procedures

The cloning procedures were planned and documented with the DNASTAR® SeqBuilder (Version 8.0.3(1); DNASTAR, Inc., USA) software. Gene sequences were obtained from the Broad *Neurospora crassa* Database www.broadinstitute.org.

DNA fragments were amplified with Q5® High-Fidelity DNA Polymerase (NEB, Ipswich, USA) by polymerase chain reaction (PCR) and subcloned into the pJET1.2 blunt vector of the CloneJET™ PCR Cloning Kit (Fermentas GmbH, Germany). Subsequently, plasmids were digested and after ligation of insert into the respective final vector, plasmids were confirmed by restriction patterns and sequencing. Alternatively, Gibson assembly was performed as developed by (Gibson, 2009). Therefore, the Gibson master mix contained: 80µl 5x isothermal reaction buffer (1M TrisHCL pH 7.5; 1M $MgCl_2$; 100mM dATP; 100mM dCTP; 100mM dGTP; 100mM CTP;1M DTT; 250mg PEG8000; 100mM NAD; 1ml H_20), 40µl Taq ligase (40u/µl); 0,16µl T5 exonuclease (1u/µl), 5µl Q5® High-Fidelity DNA Polymerase (2u/µl) and H_20 is added up to 300µl (NEB, Ipswich, USA). 15µl aliquots were stored at -20°C. Equal amounts of DNA (amplificated insert and the linearized vector) were added to the Gibson master mix, incubated for 1h at 50°C and then directly transformed into *E. coli* DH5α cells.

4.3.2 Plasmids for yeast two hybrid analysis

As template for PCR amplification a cDNA library (www.FGSC.net) was used. Proteins were expressed as N-terminal fusion constructs with SV40 NLS (nuclear localization signal)-GAL4 activation domain from pGADT7 or with GAL4 DNA binding domain from pGBKT7 (both from Clontech). Full length DOCK (NCU09492), ELMO (NCU03264) and the DOCK DHR domain (1290aa-1846aa) cDNAs were amplified with Q5® High-Fidelity DNA Polymerase and primers SL209 and SL210; SL193 and SL194; SL201 and SL202 were afterwards inserted into pGADT7. Additionally, cDNAs were amplified for pGBKT7 with primers SL193 and SL198; SL195 and SL200; SL201 and SL206.

4.3.3 Constructs of HA-epitope-tagged fusion proteins for co-immunoprecipitation experiments

N-terminally HA-tagged versions of *elmo*, *rac-1* and *cdc-42* were amplified by PCR from genomic wild type DNA with the respective primer pairs SL172for and SL192rev; SL133for and SL134rev; SL135for and SL136rev. The vector was digested with the respective restriction enzyme (indicated in Table 5) and the correct insertion of the fragment was verified by sequencing. After transformation of the linearized HA-tagged fusion proteins via electroporation, expression levels were checked by Western hybridization and immunodetection with an anti-HA antibody.

4.3.4 GFP plasmids for localization studies and co- immunoprecipitation experiments

Constructs for GFP-tagged fusion proteins were generated by amplification of the ORFs as annotated in the *N. crassa* database (http://www.broad.mit.edu). Different unique restriction sites were added to the ends of the primers to introduce the DNA fragments into the final vector. For the N-terminal GFP-tagged constructs of dock, elmo and NCU00196, the primer pairs SL156for and SL156rev, SL92for and SL96rev, as well as SL59for and SL60rev were used, respectively.

A C-terminal GFP-tagged NCU00196 construct was amplificated with wild type DNA as template and the primer pair SL215for and SL216rev. The accordant fragment was subcloned into the pJet1.2 blunt vector. Correct insertion of the fragment was verified by sequencing, the vector was digested with the respective restriction enzyme (indicated in Table 5) and the PCR fragments were introduced into the final vector pCCG::N-GFP or pMF272ATGtoATC.

Table 5: Primer used in this study. Restriction enzyme recognition sites are underlined.

Primer name	Sequence 5`-3`
SL59 NGFP196 for AscI	ggc gcg cca ATG ACG TCC GCC GCC GC
SL65 NGFP196 rev PacI	tta att aaT TAG TCA TCT AGA TCC ATG TGC
SL215 pMF196 for SpeI	act agt ATG ACG TCC GCC GCC GCT G
SL216 pMF196 rev PacI	tta att aaG TCA TCT AGA TCC ATG TGC CTT
SL92 NGFP3264 for AscI	ggc gcg cca ATG GAT CAG GCC GAT ATA CCG
SL96 NGFP3264 rev PacI	tta att aaT CAG ATC TCA TAG TAA TAA TC
SL155 NGFP9492 for PacI	tta att aaa ATG CCC TGG CAA CCA CTG C
SL156 NGFP9492 rev XbaI	tct aga CTA TTC CTC ATC CAG TGC CC
SL133 pHAN1Rac for SpeI	tta att aaa ATG CCC TGG CAA CCA CTG C
SL134 pHAN1Rac rev PacI	tta att aaT TAG AGG ATA GTG CAC TTG GAC
SL135 pHAN1 Cdc4 SpeI	act agt ATG GTG ACG GGA ACT ATC AAG
SL136 pHAN1 Cdc42 PacI	tta att aaT CAC AGA ATC AAG CAC TTC TTG
SL172 pHAN13264 for SpeI	act agt ATG GAT CAG GCC GAT ATA CCG
SL192 pHAN1 3264 rev PacI	tta att aaT CAG ATC TCA TAG TAA TAA TC
SL193 pGAD3264 for NdeI	cat atg ATG GAT CAG GCC GATA
SL194 pGAD3264 rev EcoRI	gaa ttc TCA GAT CTC ATA GTA ATA A
SL201 pGAD 9492 DHR for NdeI	cat atg ATG CCC TGG CAA CCA CTG CC
SL202 pGAD DHR rev NdeI	cat atg CTA TTC CTC ATC CAG TGC CC
SL209 pGAD9492 for NdeI	cat atg ATG CCC TGG CAA CCA CTG CC
SL210 pGAD9492 rev NdeI	cat atg CTA TTC CTC ATC CAG TGC CCC

SL198 pGBK3264 rev EcoRI	gaa ttc TCA GAT CTC ATA GTA ATA A
SL206 pGBK 9492 DHR rev NotI	gcg gcc gcG GGT GGT GGT GTG AAG
SL195 pGAD9492 for NdeI	cat atg ATG CCC TGG CAA CCA C
SL200 pGBK 9492 rev NotI	gcg gcc gcC TAT TCC TCA T

Table 4: Plasmids used and generated in this study.

Plasmid	Short description	Source
pJet1.2 blunt	Cloning vector for subcloning of PCR fragments	Fermentas GmbH, Germany
pGBKT7	Yeast two-hybrid vector for expression of N-terminal GAL4 DNA binding domain fusion proteins under control of truncated P_{ADH1}; carrying *TRP1*	Clontech, USA
pGBKT7-53	pGBKT7; murine $p53_{aa72-390}$cDNA	Clontech, USA
pGBKT7-Lam	pGBKT7; human lamin C cDNA	Clontech, USA
pGADT7	Yeast two-hybrid vector for expression of N-terminal GAL4 activation domain fusion proteins under control of full-length P_{ADH1}; carrying *LEU2*	Clontech, USA
pGADT7-T	pGADT7; SV40 large T-$antigen_{aa86-708}$cDNA	Clontech, USA
pHAN1	Fungal expression vector for N-terminal HA fusion proteins under control of P_{ccg-1}; target to *his-3* locus	(Kawabata, 2007)
pMF272ATGtoATC	Fungal expression vector for C-terminal GFP fusion proteins under control of P_{ccg-1}; target to *his-3* locus; start codon ATG was mutated to ATC	(Freitag *et al.*, 2004) modified by A. Dettmann
pCCG::N-GFP	Fungal expression vector for N-terminal GFP fusion proteins under control of Pccg-1; target to *his-3* locus	(Honda & Selker, 2009)
pHAN1-6454	pHAN1; NCU06454 (*cdc-42*)	this study
pHAN1-2160	pHAN1; NCU02160 (*rac-1*)	this study
pHAN1-3264	pHAN1; NCU03264 (*elmo*)	this study
pCCG::N-GFP-9492	pCCG::N-GFP, NCU09492 (*dock*)	this study
pCCG::N-GFP-3264	pCCG::N-GFP, NCU03264 (*elmo*)	this study
pCCG::N-GFP-0196	pCCG::N-GFP, NCU0196 (*rga-1*)	this study

pMF272ATGtoATC-0196	pMF272ATGtoATC; NCU0196 (*rga-1*)	this study
pAL1-CRIB^{CLA-4}	pCCG::CRIB^{cla-4-}gfp::bar+	(Lichius *et al*, 2014)
pCCG::N-GFP-CRIB	pCCG::N-GFP, CRIB^{CLA-4}	this study
pGBKT7-9492	pGBKT7; NCU09492 (*dock*) cDNA	this study
pGBKT7-3264	pGBKT7; NCU03264 (*elmo*) cDNA	this study
pGBKT7-DHR	pGBKT7; NCU09492$^{1290-1846aa}$ (*dock*) cDNA	this study
pGBKT7-6454	pGBKT7; NCU06454 (*cdc-42*) cDNA	kind gift of S. Maerz
pGBKT7-2160	pGBKT7; NCU02160 (*rac*) cDNA	kind gift of S. Maerz
pGADT7-9492	pGADT7; NCU09492 (*dock*) cDNA	this study
pGADT7-3264	pGADT7; NCU03264 (*elmo*) cDNA	this study
pGADT7-DHR	pGADT7; NCU09492$^{1290-1846aa}$ (*dock*) cDNA	this study
pGADT7-6454	pGADT7; NCU06454 (*cdc-42*) cDNA	kind gift of S. Maerz
pGADT7-2160	pGADT7; NCU02160 (*rac*) cDNA	kind gift of S. Maerz
pNV72	pMal-c2x; changed multiple cloning site, expression plasmid for MalE fusions	(Vogt & Seiler, 2008)
pMalc2xL_Cdc42	pNV72; *cdc-42* cDNA	(Vogt & Seiler, 2008)
pMalc2xL_Rac	pNV72; *rac-1* cDNA	(Vogt & Seiler, 2008)
pMalc2xL_Rho1	pNV72; *rho-1* cDNA	(Vogt & Seiler, 2008)
pMalc2xL_Rho2	pNV72; *rho-2* cDNA	(Vogt & Seiler, 2008)
pMalc2xL_Rho3	pNV72; *rho-3* cDNA	(Vogt & Seiler, 2008)
pMalc2xL_Rho4	pNV72; *rho-4* cDNA	(Vogt & Seiler, 2008)
pMalc2xL_196	pNV72; NCU00196 (255-455aa) cDNA	kind gift of S. Maerz
pMalc2xL_553	pNV72; NCU0553 (531-742aa) cDNA	kind gift of S. Maerz
pMalc2xL_1472	pNV72; NCU01472 (88-308aa) cDNA	kind gift of S. Maerz
pMalc2xL_2524	pNV72; NCU02524 (1120-1317) cDNA	kind gift of S. Maerz
pMalc2xL_2915	pNV72; NCU02915 (441-678) cDNA	kind gift of S. Maerz
pMalc2xL_7622	pNV72; NCU07622 (110-626) cDNA	kind gift of S. Maerz
pMalc2xL_7688	pNV72; NCU07688 (972-1196) cDNA	kind gift of S. Maerz
pMalc2xL_9537	pNV72; NCU09537 (435-669aa) cDNA	kind gift of S. Maerz
pMalc2xL_10647	pNV72; NCU10647 (369-572aa) cDNA	kind gift of S. Maerz

4.4 Strains

N. crassa strains used in this study are depicted in Table 3. Some of the strains were obtained from the Fungal Genetic Stock Center (FGSC) at the University of Missouri, USA. In point, characterized single deletion strains in this study have been generated within the framework of the *Neurospora* genome

project hosted at Dartmouth Medical School, Great Britain, following the procedure described in (Dunlap *et al.*, 2007). Detailed descriptions are available at http://dartmouth.edu/~neurosporagenome/protocols.html. A hygromycin resistance cassette was used to disrupt the full-length open reading frames. All resulting strains were verified by Southern hybridization.

Most of the *N. crassa* strains in this study were produced via transformation applying plasmids aiming the *his-3* locus. The expression cassette is flanked by the region for homologous recombination, the *his-3* locus, and restores the functional *his-3* allele (Margolin, 1997, Aramayo & Metzenberg, 1996). Transformants expressing the desired protein were selected on minimal medium and further analyzed by Western hybridization and immunodetection with the respective antibodies.

Functionality of the expressed fusion protein is proven if the expression strain was crossed with the respective deletion mutant. The resulting strain is hygromycin resistant and the phenotypic defects were suppressed.

Co-immunoprecipitation studies with HA- and GFP-tagged fusion proteins were generated by transformation of the plasmids in auxotrophic strains *trp-3; his-3* and *nic-3; his-3*. Histidine-prototrophic transformants with expression of the fusion proteins of interest were selected. Fusion of a Nic$^-$ with a Trp$^-$ strain on VMM is necessary to generate prototrophic heterokaryotic strains, expressing both fusion proteins (Kawabata, 2007).

Table 5: Strains used in this study.

Strain	Genotype	Source
wild type *A*	74-OR23-1V *A*	FGSC#987
wild type *a*	ORS-SL6 *a*	FGSC#4200
his-3 A	*his-3$^-$A*	FGSC#6103
his-3 a	*his-3$^-$a*	FGSC #718
trp-1, his-3	*trp-1$^-$, his-3$^-$*	(Maerz *et al.*, 2009)
nic-3, his-3	*nic-3$^-$, his-3$^-$*	(Maerz *et al*, 2009)
3xHA-03264nic	*nic-3$^-$his-3$^+$::Pccg-1-3xHA*-NCU03264$^+$	this study
3xHA-03264trp	*trp-1$^-$his-3$^+$::Pccg-1-3xHA*-NCU03264$^+$	this study
3xHA-02160nic	*nic-3$^-$his-3$^+$::Pccg-1-3xHA*-NCU02160$^+$	this study
3xHA-02160trp	*trp-1$^-$his-3$^+$::Pccg-1-3xHA*-NCU02160$^+$	this study
3xHA-06454nic	*nic-3$^-$his-3$^+$::Pccg-1-3xHA*-NCU06454$^+$	this study
3xHA-06454trp	*trp-1$^-$his-3$^+$::Pccg-1-3xHA*-NCU06454$^+$	this study
3264-sgfp	*his-3$^+$::Pccg-1-NCU03264$^+$-sgfp*	this study
0196-sgfp	*his-3$^+$::Pccg-1-NCU00196$^+$-sgfp*	this study
gfp-9492	*Pccg-1-sgfp-9492::his-3*	this study

gfp-3264	*Pccg-1-sgfp-3264::his-3*	this study
gfp-196	*Pccg-1-sgfp-196::his-3*	this study
gfp-6454	*Pccg-1-sgfp-6454::his-3*	gift of S. März
gfp-2160	*Pccg-1-sgfp-2160::his-3*	gift of S. März
gfp-9492 (compl.)	*ΔNCU09492::hph^{R}his-3^{+}::Pccg-1-sgfp-NCU09492^{+}*	this study
0196-sgfp (compl.)	*ΔNCU00196::hph^{R}his-3^{+}::Pccg-1-NCU00196^{+}-sgfp*	this study
wt CRIB $^{CLA-4}$-GFP	*Pccg-1::crib $^{cla-4}$ –gfp::bar^{+}*	(Lichius *et al*, 2014)
gfp-CRIB	*Pccg-1-sgfp-CRIB^{CLA-4}::his-3*	this study
gfp-Rho1_3'UTR	*Pccg-1-sgfp-1484_3´UTR::his-3*	kind gift of M. Enseleit
ΔNCU03264	*hph::NCU03264*Δ a	FGSC #14076
ΔNCU03264	*hph::NCU03264*Δ A	FGSC #14077
ΔNCU09492	*hph::NCU09492*Δ a	FGSC #14138
ΔNCU09492	*hph::NCU09492*Δ A	FGSC #14139
ΔNCU03264ΔNCU09492	*hph::NCU03264*Δ;*hph::NCU9492*Δ	this study
ΔNCU00196	*hph::NCU00196*Δ a	FGSC #11454
ΔNCU00553	*hph::NCU00553*Δ	FGSC #11328
ΔNCU01472	*hph::NCU01472*Δ	FGSC #13621
ΔNCU02524	*hph::NCU02524*Δ	FGSC #11747
ΔNCU02915	*hph::NCU02915*Δ	FGSC #11443
ΔNCU07622	*hph::NCU07622*Δ	FGSC #11341
ΔNCU07688	*hph::NCU07688*Δ	FGSC #14423
ΔNCU09537	*hph::NCU09537*Δ	FGSC #13829
ΔNCU10647	*hph::NCU10647*Δ	FGSC #18643
NCU00196Δ his-3-	*hph::NCU00196*Δ *his-3-*	FGSC #11454 x FGSC #6103
NCU09492Δ his-3-	*hph::NCU09492*Δ *his-3-*	FGSC #14138 x FGSC #6103
ΔNCU02915ΔNCU07622	*hph::NCU02915*Δ;*hph::NCU7622*Δ	this study
ΔNCU00553ΔNCU07688	*hph::NCU00553*Δ*hph::NCU07688*Δ	this study
ΔNCU02524ΔNCU00553	*hph::NCU02524*Δ*hph::NCU00553*Δ	this study
ΔNCU02524ΔNCU07688	*hph::NCU02524*Δ*hph::NCU07688*Δ	this study
ΔNCU00196ΔNCU10647	*hph::NCU00196*Δ*hph::NCU10647*Δ	this study

4.5 General molecular biological methods

Standard molecular methods were performed as described in (Sambrook, 2001, Ausubel, 2002) and were partially modified or adapted from manufacturer's manuals.

4.5.1 Genomic DNA preparation

Preparation of genomic DNA isolation from *N. crassa* strains was performed as described in (Weiland, 1997). Liquid cultures were filtered through a Büchner funnel and ground with liquid nitrogen. Pulverized mycelium was homogenized in extraction buffer (50mM Tris, pH 8.0, 50mM ethylenediaminetetraacetic acid (EDTA), 3% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol) and subsequently incubated for 2 hours at 65°C. Three extraction steps with Roti®-Phenol /chloroform, isoamyl alcohol (25/24/1) (Carl Roth GmbH &Co KG, Germany) and in addition one with chloroform were performed. Thus, the DNA was precipitated in 0.7 volumes of Isopropanol. 1xTE buffer (10mM Tris, pH8.0, 1mM EDTA) and 0.1µg/ml RNaseA (Fermentas GmbH, Germany) solved the DNA and digested the RNA, respectively. Another precipitation step was done with 1/10 volume of 3M sodium acetate and 2 volumes of 100% ethanol. Nucleic acid was washed once with 70% ethanol, then air-dried, redissolved in 100µl H_2O and then stored at 4°C.

4.5.2 Polymerase chain reaction (PCR)

Amplification of DNA by polymerase chain reaction was performed with Q5® High-Fidelity DNA Polymerase in accordance with standard protocols (Ausubel *et al.*, 2002) or as recommended in manufacturer's manuals. PCR reactions contained an initial template denaturation step (2 min, 98°C) followed by 25-35 cycles, each cycle consisting of 10s denaturation at 98°C, primer annealing (10s-30s, 50-60°C depending on the designed primers), a product elongation step at 72°C (20s-30min/1kb template length), and a final elongation step of 2min at 72°C. Only analytical and control PCRs were performed with *Taq* DNA polymerase. For Taq PCR reaction an initial denaturation step (30s at 98°C) and a product elongation step (at 72°C and 1min/1kb) was used.

Primers were appointed from Eurofins MWG Operon (Ebersberg, Germany) or Invitrogen GmbH (Karlsruhe, Germany). Genomic DNA, cDNA or plasmids were used as template for PCR. Colony PCRs (used for the identification of positive *E.coli* transformants) were performed with *E. coli* cells as template (Zon *et al.*, 1989).

4.5.3 DNA agarose gel electrophoresis and isolation

Plasmids, PCR products and DNA fragments were analysed by horizontal agarose gel electrophoresis using the Mini Sub-Cell System (Bio-Rad Laboratories GmbH, Germany).The DNA was stained with ethidiumbromide (Carl Roth GmbH & Co. KG) and visualized by the Molecular Imager Gel Doc XR System (Bio-Rad Laboratories GmbH, Germany). As DNA molecular weight marker, the Gene Ruler™

1kb DNA ladder (Fermentas GmbH, Germany) was used. Sliced DNA was extracted out of the gel using the peqGOLD Gel Extraction Kit (PeqLab GmbH, Erlangen, Germany). Plasmid DNA from *E. coli* was prepared using peqGOLD Plasmid Miniprep Kit I (PeqLab GmbH, Erlangen, Germany) according to manufacturer's manual.

4.5.4 Enzymatic digestion of DNA

Restriction enzymes and buffers were ordered from Fermentas GmbH (Germany) and New England Biolabs GmbH (United States of America) and used in accordance to product manuals, respectively.

4.5.5 Ligation

Ligation reactions were performed in 20µl volumes with an overall concentration of vector plus insert of 1-10 µg/ml for efficient ligation, using T4 DNA ligase (Fermentas GmbH, Germany) in a 2 fold concentrated quick ligation buffer (50mM HEPES pH7.6, 10mM $MgCl_2$, 2mM DTT, 2mM ATP and 7% (v/v) PEG4000) for 30 min at room temperature (RT) or over night (ON) at 4°C.

4.5.6 DNA Transformation

DNA transformation in chemically competent *E. coli* cells was performed as described in (Inoue *et al.*, 1990). *N. crassa* conidia were harvested after 9 to 11 days and washed with 1M sorbitol. The electroporation was performed in cuvettes obtained from PEQLAB Biotechnologie GmbH (Germany) with the following settings (voltage 1.5kV; capacitance: 50µF; resistance: 200Ω). Afterwards, conidia were resuspended in 1M sorbitol and plated on VMM. For selection of transformants with dominant markers, e.g. hygromycin B, conidia were resuspended in VMM, incubated for 3h at RT and plated on selective medium. DNA was electroporated as mentioned in (Margolin *et al.*, 1997) with minor modifications.

4.5.7 Sequence analysis

The DNA was sequenced by the Göttingen Genomics Laboratory at the Institute of Microbiology and Genetics, University of Göttingen (G2L, Göttingen, Germany) and the GATC Biotech AG (Germany). Sequences were analysed using 4Peaks (version 1.7.2; Mekentosj B.V., The Netherland), Lasergene (DNASTAR, Inc., Madison, USA) and GATCViewerTM (GATC Biotech AG, Germany) software. BLAST searches at NCBI (http://www.ncbi.nlm.nih.gov/) were performed for alignments of sequences.

4.6 Biochemical and immunological techniques

4.6.1 Protein isolation of *N. crassa*

N. crassa strains were grown in liquid VMM, harvested by filtration using a Büchner funnel and ground in liquid nitrogen. Then, the pulverized mycelium was homogenized with protein extraction buffer

(50mM Tris pH7.5, 100mM KCl, 10mM $MgCl_2$, 0.1% NP40; freshly added 2mM benzamidine, 2mM DTT, 1mM Pefabloc SC), centrifuged (16000g at 4°C for 10 min) and mixed with 3x Laemmli sample buffer (10% glycerol, 5% β-mercaptoethanol, 15% SDS, 12,5% upper-buffer (0,5M Tris-HCl pH6.8), 0,75% bromophenol blue, 3M urea; modified from (Laemmli, 1970). Finally, the samples were boiled at 98°C for 10 min.

4.6.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western hybridization

Protein samples were boiled at 98°C for 10min and loaded on a vertical discontinuous polyacrylamide gel electrophoresis PAGE (Ornstein, 1964, Davis, 1964) in the attendance of sodium dodecyl sulfate (SDS) (Laemmli, 1970) by using the Mini-Protean® 3 Cell System (Bio-Rad Laboratories GmbH, Germany). Polyacrylamide gels were submerged in running buffer (2.5mM Tris base, 19.2mM glycine and 0.1% SDS). PageRuler™ Prestained Protein Ladder (Fermentas GmbH, Germany) was loaded as marker for molecular weight determination of proteins of interest and electrophoretic separation was performed with 15mA per polyacrylamide gel. Separated proteins were visualized by staining with Coomassie Brilliant Blue (0.1% Coomassie Brilliant Blue, 40% methanol, 10% acetic acid; (Merril, 1990)).

Alternatively, western hybridization was performed. Proteins were transferred electrophoretically from polyacrylamide gels to Protran® nitrocellulose membrane (Whatman GmbH, Germany) in Mini Trans-Blot® Cells (Bio-Rad Laboratories GmbH, Germany). After electroblotting at 100V for 1h, the nitrocellulose membrane was stained reversible with Ponceau S (0.1% Ponceau S in 5% acetic acid; (Salinovich & Montelaro, 1986). The immunological detection (western hybridization) was based on the method described by (Towbin *et al.*, 1979). An initial blocking step (of the membrane) with 5% Sucofin milk powder (TSI GmbH & Co. KG, Germany) in PBS solution (10mM sodium phosphate, 150mM NaCl, pH 7.4) was performed. Mouse monoclonal Anti-GFP (B-2) (Santa Cruz Biotechnology, Heidelberg, Germany) or Anti-HA (clone HA-7) were used as primary antibody (incubation 1hour) and were detected by peroxidase-coupled goat anti-mouse IgG (Invitrogen GmbH, Karlsruhe, Germany). Detection was performed using Immobilon™ Chemiluminescent Western HRP Substrate (Millipore, USA) and the FUSION SL™ Advance System, equipped with a cooled CCD camera with 4.2/10.0 megapixel resolution (PeqLab GmbH, Erlangen, Germany).

4.6.3 (Co-) Immunoprecipitation analysis

N. crassa strains were grown in liquid minimal medium, harvested by filtration and ground in liquid nitrogen. All buffers used for immunoprecipitation contain the following additives: 25mM β-glycerophosphate, 10ng/µl leupeptine, 10ng/µl aprotinine, 2ng/µl Pepstatin A, 2mM DTT, 1mM Pefabloc SC, 2mM benzamidine, 5mM NaF and 1mM Na_3VO_4. The pulverized mycelium was

homogenized in IP buffer (50mM Tris pH7.5, 100mM KCl, 10mM $MgCl_2$, 0.1% NP40) and centrifuged two times at 4°C (15min at 4500g, then second centrifugation step for 45minh at 16000g). The clear supernatant was incubated on a rotation device for two hours with GFP-Trap beads (ChromoTek, USA) or 2µl/ml lysate monoclonal mouse Anti-HA antibody and together with 5mg/ml lysate Protein-A-Sepharose beads (GE Healthcare Life Sciences, USA) for an additional hour at 4°C. This mixture was centrifuged for 1min at 4500g and washed twice with IP buffer. Immunoprecipitated proteins were recovered by boiling of the sepharose beads for 10min at 98°C in 3x Laemmli buffer and stored at -20°C.

4.6.4 Protein expression and purification from *E. coli*

RAC, CDC-42, RHO-1 to RHO-4 GTPases and the indicated GAP domains were expressed as fusion proteins with an N-terminal maltose binding protein (MBP) tag (see table 2). The purification was performed as described by (modified from Vogt & Seiler, 2008). The respective MBP-Rho GTPase fusion proteins or the MBP-GAP domain fusion proteins (pNV plasmids) were transformed into E.coli BL21 (DE3) cells (Novagen, belongs to Merck KGaA, Germany) plated on LB+ medium (1% NaCl, 0.8% yeast extract, 1.8% peptone, 2% glucose, 2% agar) with chloramphenicol/ampicillin and incubated ON at 37°C. A single colony was transferred into 50ml preculture, grown to an OD600 of 1.0-1.5 at 37°C and then stored ON at 4°C. 250ml main culture was inoculated with 2-4ml preculture to an OD600 of 0.02 and grown to an OD600 of 1.0-1.5. Fusion protein expression was established by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2mM. After 2h, cells were harvested by centrifugation and the pellet was resuspended in 15ml lysis buffer (50mM Tris, pH7.4, 125mM NaCl, 5 mM MgCl2, 10% glycerol, 0.02% NP-40, 2mM DTT, 1mM PMSF, 0.35 mg/ml benzamidine, 10µM GTP). Samples were stored at -20°C. The suspension was incubated with a spatula of lysozyme on a rotating wheel at 4°C for 1 hour, centrifuged (16000g, 20min, 4°C) and the supernatant was incubated with pre-equilibrated Amylose Resin (New England Biolabs, USA) on a rotation device for 2 hours at 4°C. The Resin was washed twice with washing buffer (lysis buffer with 250mM $NaCl_2$). A small proportion of the Resin was separated, mixed with elution buffer (50mM Tris, pH7.4, 200mM $NaCl_2$, 5mM $MgCl_2$, 10% glycerol, 0.02% Nonidet-P40, 2mM DTT, 20mM maltose) and the first protein-rich fraction was pooled. The eluated samples were prepared for SDS PAGE and the purification of fusion proteins were controlled by Coomassie staining.

4.6.5 *In vitro* pull down assays

After protein expression and purification in E.coli as in section 4.6.4 the remaining Resin was incubated with GTP reaction buffer (20mM Hepes, pH 7.6; 25mM $NaCl_2$; 2mM EDTA; 1mg/ml BSA; 0,5mM DTT; 10mM GTP) for 25min at RT while shaking. During this incubation step, the MBP-Rho GTPase fusion proteins were loaded with the same amount of GTP. The reaction was stopped by adding 0.5M $MgCl_2$.

The suspension was centrifuged (4500g, 5min, 4°C) and the supernatant thrown away. Meanwhile, pulverized mycelium of *N. crassa* was homogenized in *N. crassa* IP buffer and centrifuged two times at 4°C. About 12ml of the *N. crassa* extract (*196-gfp*) were combined with the pelleted Amylose Resin, incubated on the rotation device for 30min and washed twice with IP buffer. Precipitated GFP-tagged proteins were recovered by boiling of the sepharose beads for 10min at 98°C in 3x Laemmli buffer and stored at -20°C.

4.6.6 *In vitro* Rho GAP activity assays

Rho GTPase assays were performed as described (modified by Vogt & Seiler, 2008). Pre-equilibration of the RHO proteins was performed for 15 minutes at 25°C in 30 µl buffer A (20 mM HEPES, pH 7.6, 25 mM sodium chloride, 2 mM EDTA, 1 mg/ml BSA, 0.5 mM DTT, 0.005% cholic acid) including 0.5 µM GTPase and 5 µCi (0.17 µM) [-32P]-GTP. Adding of 1 µl 0.5 M $MgCl_2$ and putting the samples on ice stopped the GTP loading. 5 µl GAP was added to start the reaction (final concentration: 20 mM HEPES, pH 7.6, 1 mg/ml BSA, 0.1 mM DTT, 1 mM GTP and 4 µM MBP::GAP) at 25°C. 5 µl samples of the reaction were stopped in 1 ml ice-cold wash buffer (50 mM Tris, pH 7.5, 50 mM sodium chloride, 5 mM MgCl2) and filtered through BA85 nitrocellulose membranes (Whatman, Maidstone, Great Britain). The filters were washed with 6 ml wash buffer, dried and measured by scintillation counting in a QuantaSmart scintillation counter (Perkin Elmer, Waltham, USA).

4.7 Yeast two-hybrid studies

The Matchmaker™ Two-Hybrid System 3 (Clontech, USA) was used following to manufacturer's manuals for yeast two hybrid analyses according to (Fields and Song, 1989). The pGADT7plasmid and the pGBKT7 plasmid were co-transformed into the *S. cerevisiae* strain AH109 as described in the protocols of Schiestl and Gietz, 1989. The transformants were selected on SD medium lacking leucine and tryptophane by their restored ability to grow. Positive interaction of fusion proteins was shown by their ability to activate the reporter genes *HIS3* and *ADE2*. For a yeast drop test, single colonies were picked, suspended in water and 5µl of serial dilutions were dropped on SD medium. To exclude autoactivation of the fusion proteins, each plasmid was tested with the empty vector of the counterpart.

4.8 Microscopy

For the analysis of colonial and hyphal morphology, an Olympus SZX16 (Olympus, Tokyo, Japan) stereomicroscope equipped with an Olympus SDF PLAPO 1xPF objective was used (März et al., 2010). Images were aquired with an Olympus ColorView III camera operated by the program Cell^{D}analySIS Image Processing (Olympus SoftImaging Solutions GmbH, Germany). Spinning disc confocal microscopy was performed as described (Dettmann *et al*., 2014) using an inverted Axio Observer Z1 microscope

(Carl Zeiss AG, Oberkochen, Germany) equipped with a CSU-22 confocal scanner unit and a CCD camera (AxiocamMRm Rev.3). ZEN Blue 2012 software (Carl Zeiss AG, Oberkochen, Germany) was used for image/video acquisition and image analysis. Cell wall and plasma membrane were stained with Calcofluor White (2µg/ml dissolved in water; Sigma-Aldrich, Taufkirchen, Germany) and FM4-64 (1mg/ml dissolved in water; Invitrogen GmbH, Germany), respectively. Time-lapse imaging was performed at capture intervals of 0.5 ms to 3s for periods up to 5.20 min using the water immersion objective 40x/1.1 or 63x/1.2.

4.9 Mass spectrometry and database analysis

For protein identification by mass spectrometry, peptides of in-gel trypsinated proteins were extracted of Commassie-stained gel slices. Peptides of 5 µl sample solution were trapped and washed with 0.05% trifluoroacetic acid on an Acclaim® PepMap 100 column (75µm x 2cm, C18, 3µm, 100Å, P/N164535 Thermo Scientific) at a flow rate of 4µl/min for 12min. Analytical peptide separation by reverse phase chromatography was performed on an Acclaim® PepMap RSLC column (75 µm x 15 cm, C18, 3 µm, 100 Å, P/N164534 Thermo Scientific) running a gradient from 96% solvent A (0.1% formic acid) and 4% solvent B (acetonitrile, 0.1% formic acid) to 50% solvent B within 25min at a flow rate of 250ml/min (solvents and chemicals: Fisher Chemicals). Peptides eluted from the chromatographic column were on-line ionized by nano-electrospray using the Nanospray Flex Ion Source (Thermo Scientific) and transferred into the mass spectrometer. Full scans within m/z of 300-1850 were recorded by the Orbitrap-FT analyzer at a resolution of 60.000 at m/z 400. Each sample was analyzed using two different fragmentation techniques applying a data dependent top 5 experiment: Collision-induced decay with multistage activation and readout in the LTQ Velos Pro linear ion trap, and higher energy collision dissociation and subsequent readout in the Orbitrap-FT analyzer. LC/MS method programming and data acquisition was performed with the XCalibur 2.2 software (Thermo Fisher). Orbitrap raw files were analyzed with the Proteome Discoverer 1.3 software (Thermo Scientific) using the Mascot and Sequest search engines against the *N. crassa* protein database with the following criteria: Peptide mass tolerance 10ppm, MS/MS ion mass tolerance 0.8Da, and up to two missed cleavages allowed.

5 Results

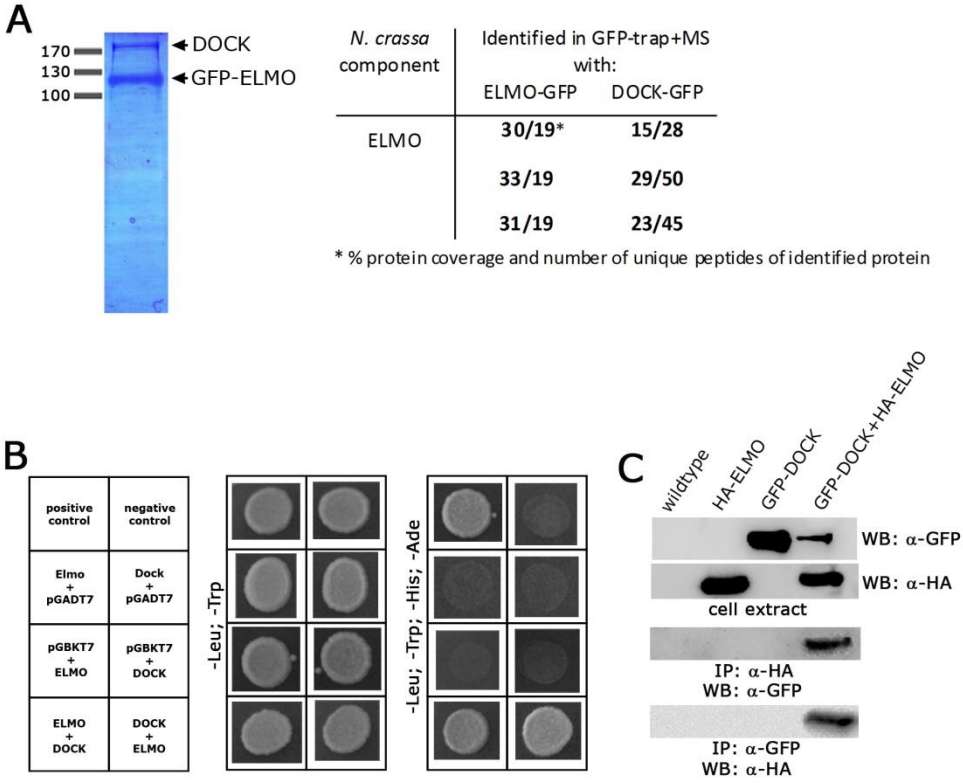
5.1 Analysis of the guanine nucleotide exchange factors DOCK and ELMO

The DOCK180 (dedicator of cytokinesis) protein and its adapter protein ELMO1 (engulfment and cell motility) were first described in mammals (Brugnera *et al.*, 2002, Cote & Vuori, 2007, Lu *et al.*, 2005, Patel *et al.*, 2011). Both proteins form a complex and act as bipartite GEF of the Rho GTPase RAC1 (Cote & Vouri, 2007). In the human opportunistic pathogen *C. albicans*, RAC1 and its activator DCK1 are responsible for the transition to filamentous growth. RAC1 and DCK1 together with LMO1, a protein with sequence similarity to the human ELMO1, are involved in cell wall integrity pathway (Hope *et al.*, 2010). Quite recently, Schmitz et al identified Dck1p and Lmo1p acting upstream of the RhoGTPase Rho5p in *S. cerevisiae* (Schmitz *et al.*, 2015).

5.1.1 ELMO and DOCK form a complex in *N. crassa*

Based on predicted structural domains NCU09492/DOCK and NCU03264/ELMO were identified in *N. crassa*. Previous *in vitro* GDP-GTP exchange assays with the NCU09492 domain revealed no GEF induced stimulation of any Rho GTPases (Richthammer *et al*, 2011) and so far both proteins remained uncharacterized in *N. crassa*. To clarify the role of ELMO and DOCK in regulation of Rho GTPases in *N. crassa* further studies were performed.

In order to identify interacting components of ELMO a N-terminal GFP-tagged ELMO construct was generated. Thus, GFP-trap affinity purification experiments coupled with mass spectrometry analysis were performed with GFP-tagged ELMO and it was possible to co-purify DOCK (Figure 4A). Yeast two hybrid assays with the generated full length proteins confirmed the interaction of DOCK and ELMO (Figure 3B). *In vivo* co-immunoprecipitation studies were performed to verify the ELMO-DOCK interaction in *N. crassa*. A forced heterokaryon coexpressing HA-tagged ELMO and GFP-tagged DOCK was generated and the GFP-DOCK precipitation allowed detection of the HA-ELMO and vice versa, suggesting a stable interaction of these proteins (Figure 4C).



N. crassa component	Identified in GFP-trap+MS with: ELMO-GFP	DOCK-GFP
ELMO	**30/19***	**15/28**
	33/19	**29/50**
	31/19	**23/45**

* % protein coverage and number of unique peptides of identified protein

Figure 4: Interaction studies of DOCK and ELMO.
(A).GFP-trap affinity purification experiments coupled with mass spectrometry analysis identified DOCK interacting with ELMO. Details in table on the right (n=3).
(B) Yeast two hybrid analysis between ELMO and DOCK showed an interaction.
(C) Co-immunoprecipitation studies verified an interaction between ELMO and DOCK in *N. crassa*.

5.1.2 ELMO and DOCK interact with the Rho GTPase RAC, but not with CDC-42

To explore if DOCK and/or ELMO interact with the Rho GTPase RAC or CDC-42 *in vivo* co-immunoprecipitation studies were performed in *N. crassa*. To investigate a potential interaction forced heterokaryons, which were able to grow on minimal medium by complementation of the individual auxotrophies, were used for the co-immunoprecipitation studies and a strain with HA-tagged RhoGTPase (RAC/NCU2160 or CDC-42/NCU6454) and GFP-tagged GEF (ELMO or DOCK) was generated. ELMO and DOCK interact with RAC but not with CDC-42 (Figure 5A,B).

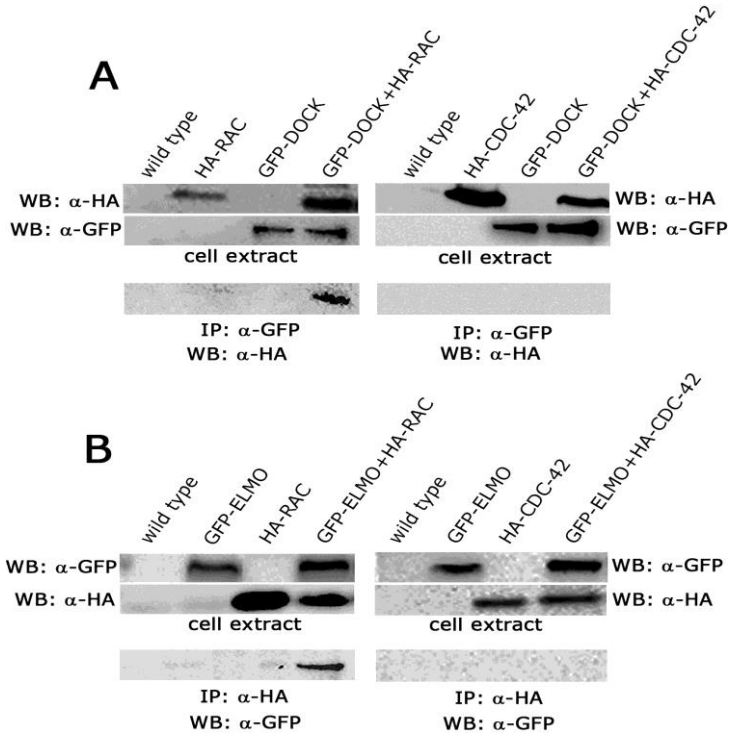


Figure 5: Interaction studies of DOCK and ELMO with RAC and CDC-42.
(A) *In vivo* co-immunoprecipitation studies identified DOCK interaction with RAC, but not with CDC-42.
(B) Interaction of ELMO with RAC, but not with CDC-42, verified by *in vivo* co- immunoprecipitation studies.

5.1.3 Phenotypic characterization of Δ*dock* and Δ*elmo* mutants

In contrast to the tubular shaped wild type hyphal morphology the Δ*dock* deletion mutant exhibited an irregular bulgy hyphal morphology, while the Δ*elmo* mutant morphology was reminiscent to the Δ*dock* mutant, but not identical (Figure 6A). The relative growth of the Δ*dock* (83% +/-1.9%) deletion mutant was decreased, whereas the growth of the Δ*elmo* (103% +/- 2.2%) deletion mutant was similar to the wild type growth (100% +/-1.1%) (Figure 6B). Wild type aerial mycelium formation and conidiation were comparable to the Δ*dock* deletion mutant. Yet, the Δ*elmo* aerial mycelium formation was slightly reduced (Figure 5A). In comparison to wild type a 5-fold increased branching pattern was noted for the Δ*dock* deletion mutant and the branching pattern of the Δ*elmo* mutant was 1.8-fold increased (counted per 600nm from the hyphal tip) (Figure 6C). To further analyze the functional relationship between DOCK and ELMO, a Δ*dock*;Δ*elmo* double mutant was generated. The double deletion Δ*dock*;Δ*elmo* resembled the Δ*dock* deletion phenotype in the bulgy hyphal morphology, aerial mycelium formation, relative growth rate (83% +/-2.2) and 5-fold increased branching pattern (Figure 5A,B,C).

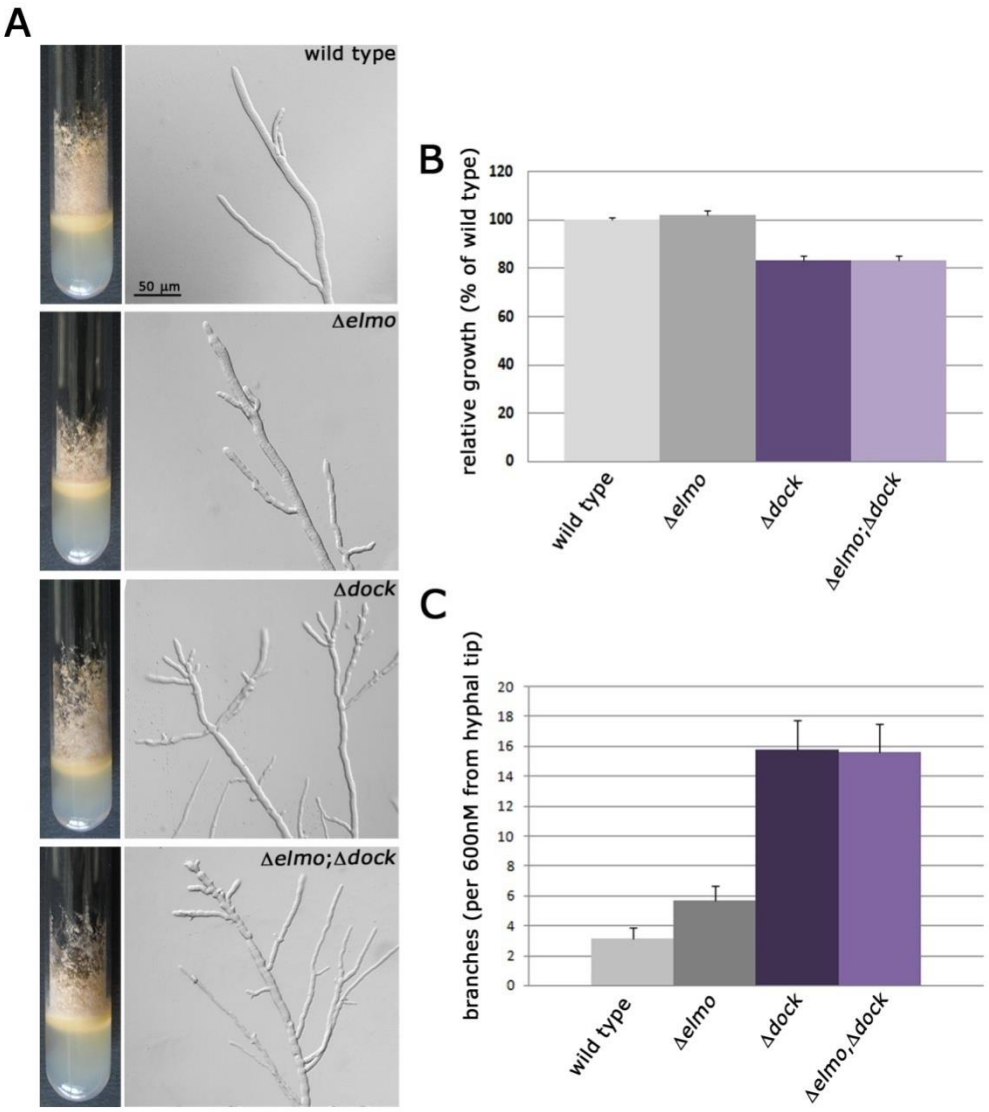


Figure 6: DOCK is involved in polar growth.
(A) Comparison of wild type, Δ*dock,* Δ*elmo* and Δ*dock;*Δ*elmo* morphology. The Δ*dock* revealed an irregular, bulgy hyphal morphology and the Δ*elmo* deletion mutant was reminiscent to the Δ*dock* mutant, but not identical. However, the double deletion resembled the Δ*dock* phenotype (growth for 2 days on minimal medium, bar=50µm). In comparison to wild type the aerial mycelium is slightly decreased in Δ*elmo* (growth for 5 days on minimal medium).
(B) Δ*dock* and Δ*dock;*Δ*elmo* displayed phenotypes of slower growth compared to wild type.
(C) Δ*dock* and Δ*dock;*Δ*elmo* depicted an 5-fold increased branching pattern (calculated per 600nm from hyphal tip).

5.1.4 DOCK is involved in regulation of polar growth

In order to investigate the altered hyphal morphology and the decreased hyphal elongation rate further studies with wild type and Δ*dock* were done.

The wild type is permanent tubular shaped and the hyphal elongation rate was constant and polar. The hypha of the Δ*dock* deletion mutant stopped growth, whereupon the hyphal apex is rounded and increased in diameter (Figure 7A). Periods of apolar growth alternated with periods of polar growth in Δ*dock*. To further investigate the apolar hyphal growth of the Δ*dock* deletion mutant, the hyphal

elongation of wild type in comparison to Δ*dock* was monitored (growth in µm per 2s). The hyphal elongation of wild type was in regular intervals (from 0,53 µm/2s up to 0,63 µm/2s), whereas the Δ*dock* mutant depicted strong fluctuation (0 µm/2s up to 0,61 µm/2s) (Figure 7B). This discrepancy in polar growth indicates a role of DOCK during constant hyphal elongation and polar growth.

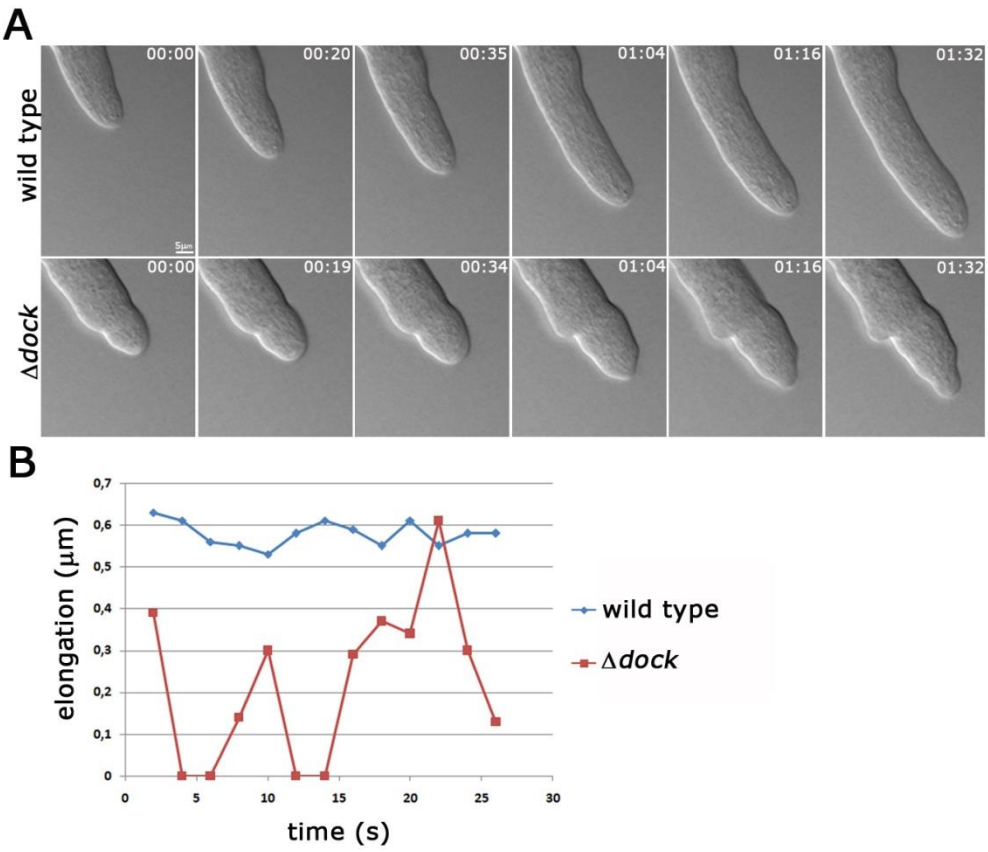


Figure 7: Δ*dock* is impaired in constant and polar tip growth.
(A) Growth of wild type (upper panel) was polar and constant, while the Δ*dock* deletion mutant exhibited polar and apolar growth periods (lower panel). Δ*dock* hyphal elongation stopped and the apex was rounded and increased in diameter (growth on minimal medium, bar=5µm).
(B) Hyphal elongation in µm per 2s (growth on minimal medium).
Time is given in min:sec.

In filamentous fungi the Spitzenkörper (SPK) is present in active growing tips (Dijksterhuis, 2003, Lopez-Franco *et al.*, 1994, Virag & Harris, 2006). To investigate a potential connection between the SPK and the irregular bulgy Δ*dock* phenotype *in vivo* time course experiments with wild type and Δ*dock* stained with FM4-64 (marker of endocytosis, vacuolar membranes, plasma membrane and SPK) were performed.

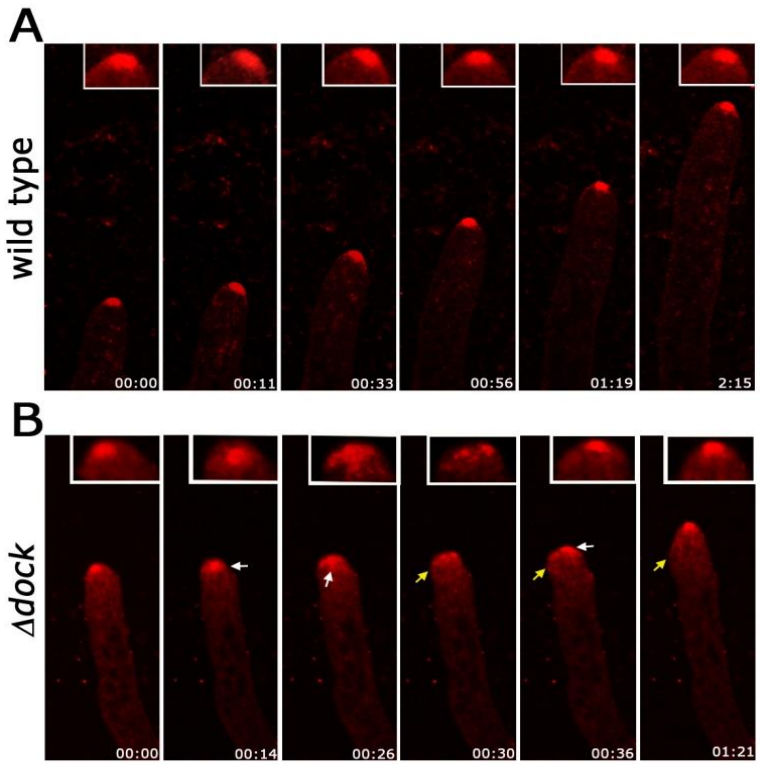


Figure 8: TiFF series of wild type and Δ*dock*.
(A) Wild type depicted constant polar growth with the SPK at the apical pole.
(B) The SPK of Δ*dock* deletion mutant dissociates from the apical pole (indicated by white arrows). The SPK collapsed and the hypha bulged (indicated by yellow arrows). As the SPK returned to the apical pole hyphal elongation was resumed. The SPK is stained with FM4-64 and time is given in min:sec.

In wild type the SPK was constant round and present at the apical pole, whereas the SPK of the Δ*dock* deletion mutant dissociated from the apical pole along the hyphal axis (Figure 8A,B). The outline of the SPK became undistinguishable, collapsed and an alteration in cell shape was observed in Δ*dock*. When the SPK of Δ*dock* returned to the apical pole, the common wild type tubular shape and hyphal cell elongation were restored. This suggested an association between the transient SPK disturbance and the permanent bulged cell shape of the Δ*dock* deletion mutant.

To further clarify the Δ*dock* hyperbranching phenotype, FM4-64 was used to visualize the SPK and to determine initiation of branching. *In vivo* time course experiments were performed and representative TiFF series were shown in Figure 9A,B. The Δ*dock* deletion mutant revealed subapical multiple SPK aggregates in contrast to wild type.

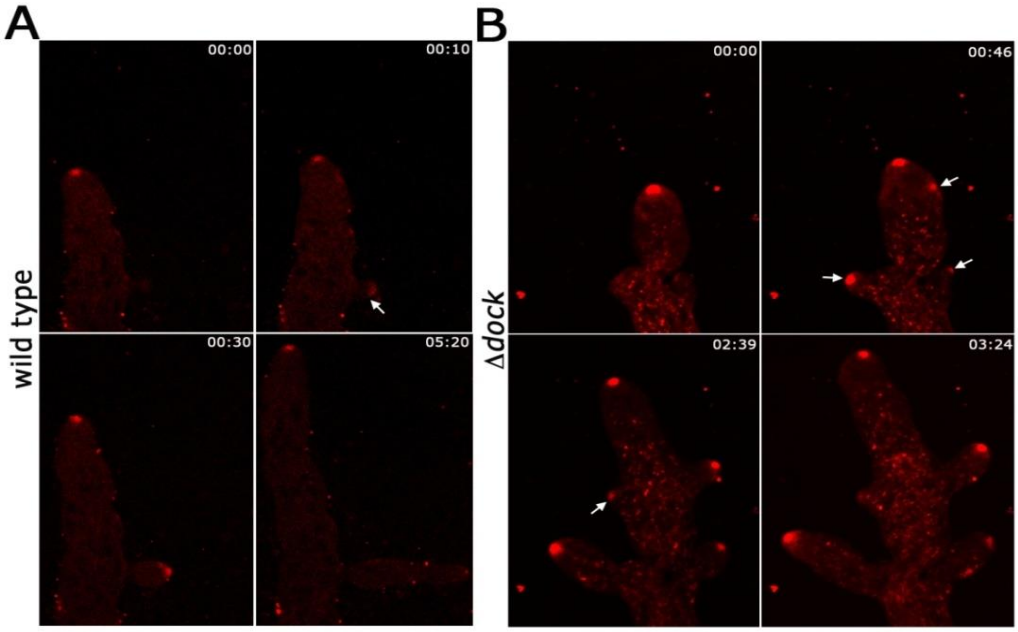


Figure 9: TiFF series of wild type hyphal branches in comparison to Δ*dock*.
(A) Regular hyphal branching is monitored at different timepoints of wild type.
(B) Subapical hyperbranching is identified in the Δ*dock* deletion mutant (indicated by white arrows).
The SPK is stained with FM4-64 and time is given in min:sec.

5.1.5 GFP-DOCK localized patchy to the subapical membrane

To investigate the cellular distribution of DOCK, a GFP-DOCK fusion construct was generated and targeted to the *his-3* locus under the control of the *ccg-1* promoter in wild type.

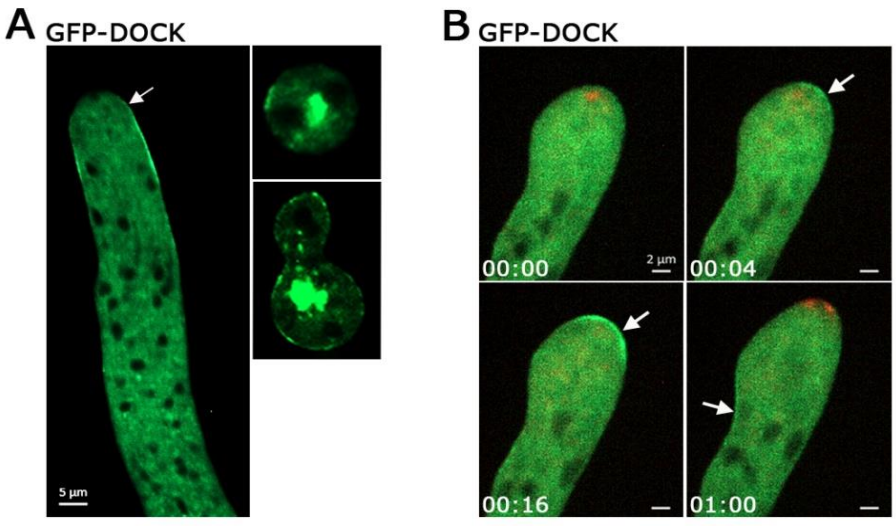


Figure 10: Localization studies of GFP-DOCK.
(A) GFP-DOCK localizes patchy, subapical and membrane associated (indicated by arrow) in mature hypha. In conidia and germlings a spotty localization was found.
(B) The GFP-tagged DOCK localizes subapical, but if the hyphal growth arrested an apical GFP-DOCK crescent was found (indicated by arrows). The SPK was stained with FM4-64. Scale bars as indicated.

GFP-DOCK localized spotty at the cortex in conidia and germlings. In mature hypha GFP-DOCK was identified to localize patchy to the subapical membrane (Figure 10A). When hyphal elongation arrested an additional GFP-DOCK localization was observed as apical crescent (Figure 10B).

5.1.6 DOCK may silence additional points of growth

The GEF CDC-24 is dual specific and regulates RAC and CDC-42 in *N. crassa* (Araujo-Palomares *et al*, 2011). Previous experiments suggested that DOCK only interacts with the Rho GTPase RAC. To determine the relation between RAC and its GEF DOCK the Cdc42-Rac-interactive-binding reporter was used for further localization studies. It was shown that the CRIB reporter labeled exclusively active GTP-bound GTPases and the *N. crassa* reporter was used as robust marker to discriminate between inactive and active RhoGTPases during polarized growth (Lichius *et al.*, 2014).

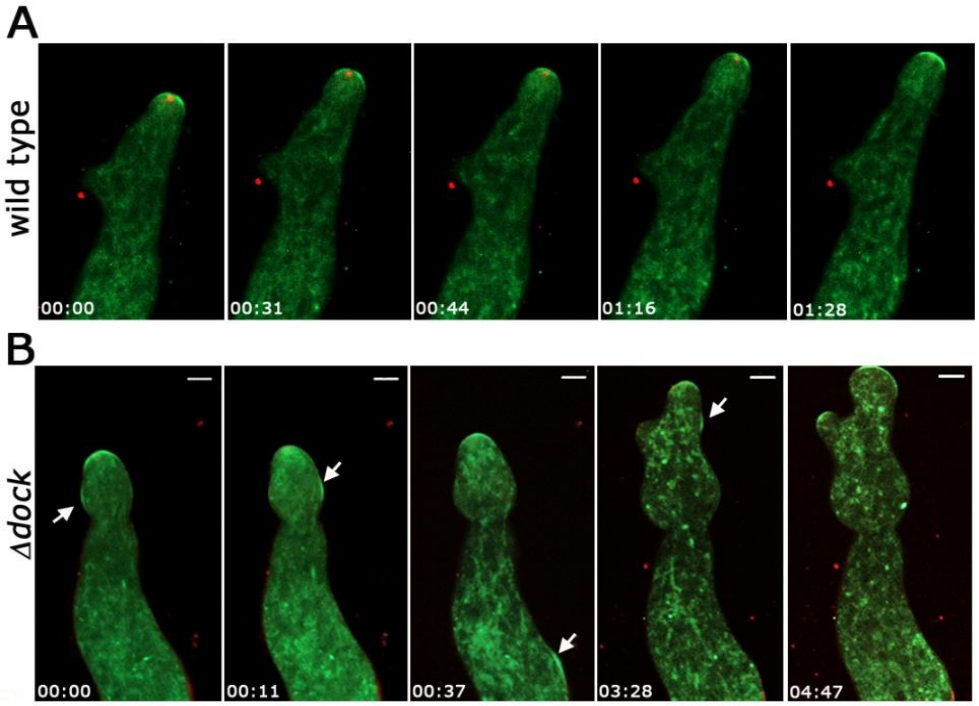


Figure 11: Localization of GFP-CRIB in wild type and the Δ*dock* deletion background.
(A) GFP-CRIB localized as apical cap in wild type (indicated by arrows).
(B) GFP-CRIB localized as apical cap and in addition subapical membrane associated (indicated by arrows)
The SPK was stained with FM4-64. Scale bars as indicated. Time is given in min:sec.

In vivo time course experiments with the GFP-CRIB reporter in wild type and the Δ*dock* deletion background were performed and representative TiFF series were shown in Figure 11A,B. The GFP-CRIB reporter was localized as membrane associated apical crescent in wild type, whereas in the Δ*dock* deletion background it was also associated to the subapical membrane (Figure 11B). These results suggested a role of DOCK in Rho GTPase regulation.

5.2 Characterization of the putative Rho GTPase-activating proteins (GAPs) in *N. crassa*

5.2.1 Four GAPs were assigned towards GTPases by *in vitro* GAP assays

Rho GTPases are highly conserved key components in signal transduction pathways from yeast to mammals (Jaffe & Hall, 2005; Park & Bi, 2007). Rho GTPases function as molecular switches and are regulated by GTPase-activating proteins (GAPs) leading to an inactive GTPase.

Phylogenetic and domain structure analysis identified previously ten GAP proteins in *N. crassa* (C. Richthammer, 2011). So far the only characterized GAP is LRG-1, dedicated to be essential for apical tip extension and restriction of subapical branch formation (Vogt & Seiler, 2008).

NCU number/name	GAP domains	RhoGTPase
NCU00196 /RGA-1	224-455 aa	RHO-1 RHO-4
NCU00553 /RGA-2	531-742 aa	RAC CDC-42
NCU01472 /GPL-1	88-308 aa	inactive
NCU02524 /RGA-3	1120-1317 aa	RAC CDC-42 RHO-3
NCU02915 /GPL-2	441-678 aa	inactiv
NCU07622 /GPL-3	110-626 aa	inactive
NCU07688 /RGA-4	972-1196 aa	RAC CDC-42 RHO-3
NCU09537 /GPL-4	453-669 aa	inactive
NCU10647 /GPL-5	369-572 aa	inactive

Table 4: Predicted GAP proteins in *N. crassa*. NCU number of the potencial GAPs with name, the amino acids fused to MBP protein (pMAL vector) and the specificity to Rho GTPases (identified in Figure 11) are indicated.

In vitro GTPase assays with the nine remaining GAPs were performed to determine GAP specificity towards the six RhoGTPases. The addition of the MBP-tagged GAP domains (for details see Table 4) revealed that four of the GAPs caused significant changes in RHO activity and the GAPs were assigned towards specific Rho GTPases (Figure 12). The NCU00196/RGA-1 protein was dual specific for RHO-1 and RHO-4, while NCU00553/RGA-2, NCU02524/RGA-3 and NCU07688/RGA-4 depicted dual specificity to RAC and CDC-42. In addition RGA-3 and RGA-4 were assigned to RHO-3 (Figure 11). Five proteins NCU01472/GPL-1, NCU02915/GPL-2, NCU07622/GPL-3, NCU09537/GPL-4 and NCU10647/GPL-5 remained inactive towards the six Rho GTPases.

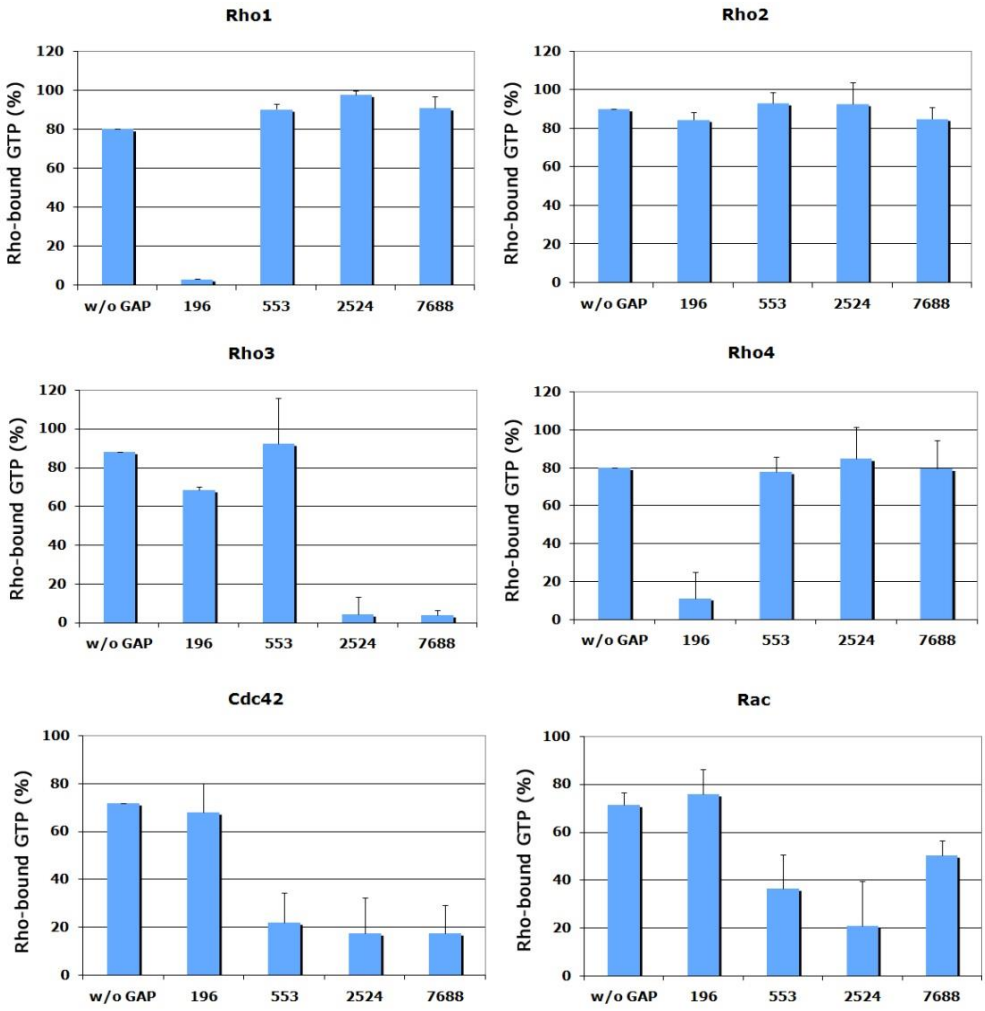


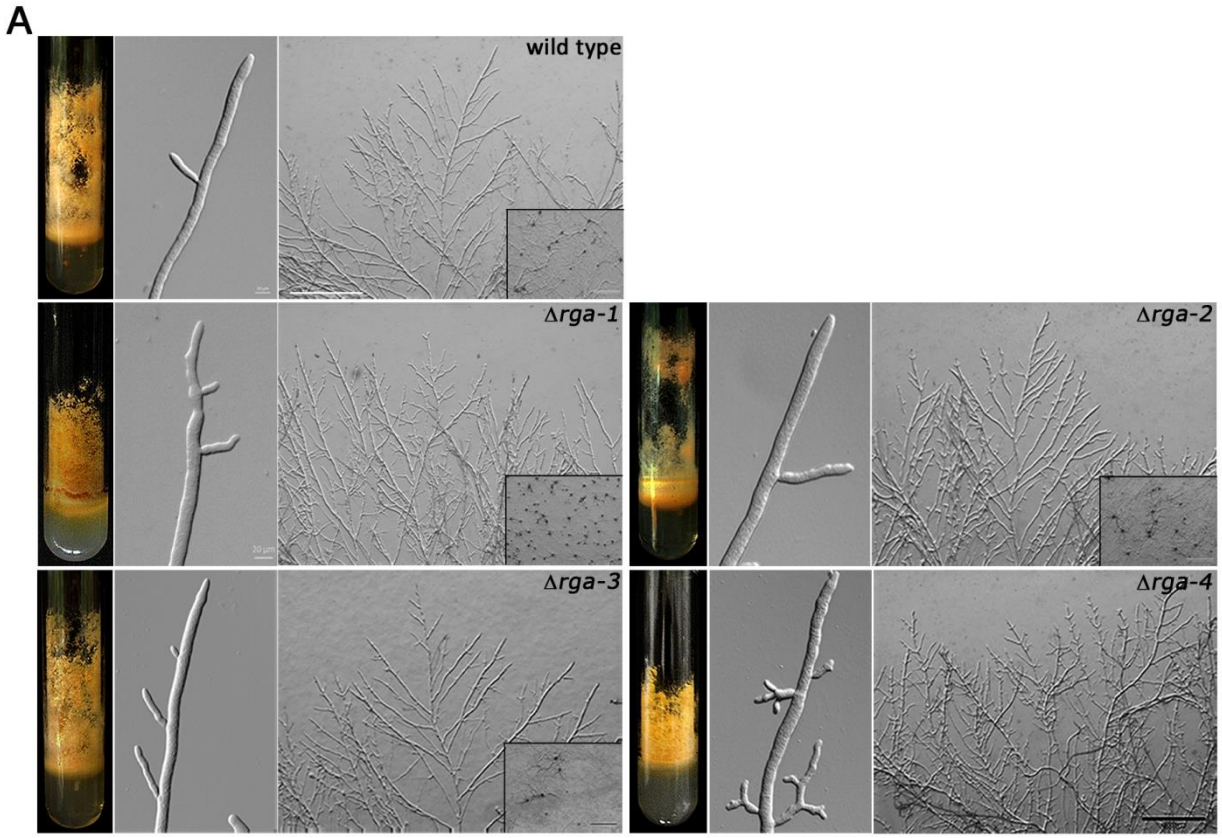
Figure 12: Intrinsic and MBP-RGA-1$^{224-455}$/NCU00196, MBP-RGA-2$^{531-742}$/NCU00553, MBP- RGA-3$^{1120-1317}$/NCU02524, MBP-RGA-4$^{972-1196}$/NCU07688 stimulated GTPase activities of the six RhoGTPase proteins. These results indicated RGA-1 as specific GAP of RHO-1 and RHO-4. RGA-2, RGA-3 and RGA-4 depicted dual specificity to RAC and CDC-42. In addition, RGA-3 and RGA-4 were specific to RHO-3.

5.2.2 Phenotypical characterization of the nine GAPs in *N. crassa*

To further describe the four active (RGA-1 to RGA-4) and five inactive (GPL-1 to GPL-5) GAP proteins, a comparative phenotypical characterization of the mutants, which were available as part of the *Neurospora* genome project, was established (Colot *et al.*, 2006). Vegetative growth, asexual and sexual development as well as drug sensitivity assays were performed in comparison to wild type development.

The Δ*rga-1*, Δ*rga-2* and Δ*rga-4* deletion mutants depicted an increased branching pattern and together with the Δ*rga-3* deletion mutant the four GAP mutants had frequently short branches. In contrast to wild type the hyphal morphology of the Δ*gpl-3* and *gpl-5* deletion mutants were thin and bumpy. Both deletion mutants revealed decreased side branches at a modified angle. An irregular zig-zag growth and tip splitting were identified in the Δ*rga-4* deletion mutant (Figure 12A). Δ*rga-1*, Δ*rga-4*, Δ*gpl-3* and Δ*gpl-5* revealed reduced hyphal extension rates in comparison to wild type (see table 5).

Only the Δ*gpl-1* deletion mutant was characterized by an increased mycelium extension rate. The germination rate was decreased in Δ*rga-3*, Δ*gpl-2*, Δ*gpl-3* and Δ*gpl-5* (table 5). Calcoflour White was used to stain chitin of fungal septa and to investigate the septation frequency in wild type and the nine GAP deletion mutants. An irregular septation pattern was observed in the Δ*rga-1* (cluster, short spirals, slightly increased) and Δ*gpl-5* (increased in distal regions). The aerial mycelium formation was reduced for Δ*rga-1*, Δ*gpl-3* and Δ*gpl-5* and on the contrary an increased height of aerial mycelium was found in Δ*rga-2* and Δ*gpl-1* (Figure 13A,B). Furthermore the conidia were counted and the Δ*rga-2*, Δ*rga-3*, Δ*gpl-2* and Δ*gpl-3* revealed decreased conidiation (Table 5). The sexual development structures protoperithecia and perithecia (ΔxΔ) were reduced in their formation in the Δ*rga-3* deletion mutant, while a few protoperithecia and perithecia were observed in the Δ*rga-4*, Δ*gpl-3* and Δ*gpl-5* deletion mutants (Figure 13A,B). The Δ*gpl-1* (ΔxΔ crosses) revealed thinner and longer ascospores in comparison to wild type (Figure 13C).



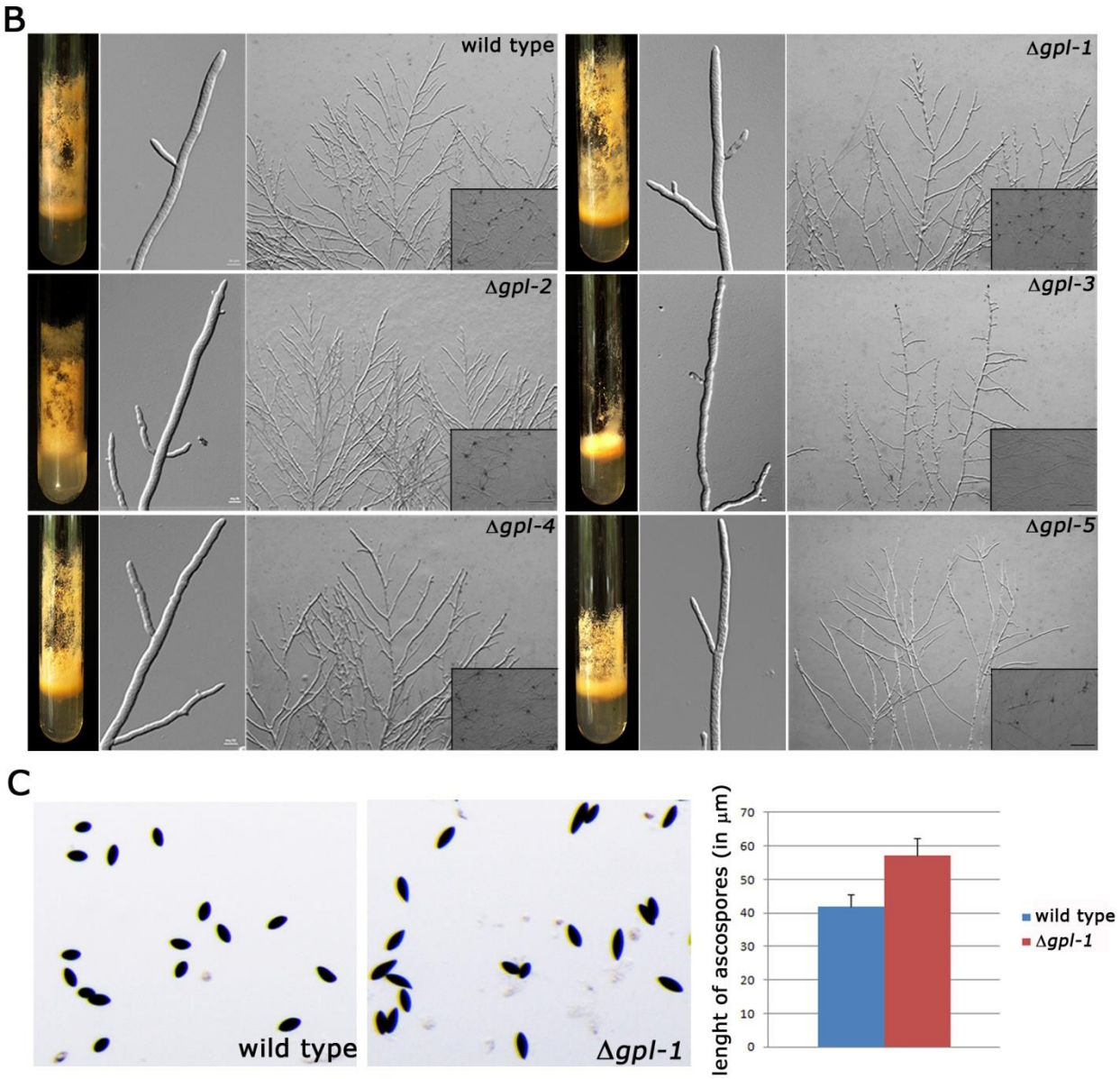


Figure 13: Phenotypical characterization of wild type, Δ*rga-1* to Δ*rga-4* and Δ*gpl-1* to Δ*gpl-5* deletion mutants.
(A) Comparison of wild type and Δ*rga-1* to Δ*rga-4* morphology. Aerial mycelium with conidia (asexual spores), hyphal morphology and protoperithecia (female sexual structure) formation were analyzed (for details see text and table5; growth for 2 days on minimal medium, bar=50µm).
(B) Comparison of wild type and Δ*gpl-1* to Δ*gpl-5* morphology. Aerial mycelium formation with conidia (asexual spores), hyphal morphology and protoperithecia (female sexual structure) formation were analyzed (for details see text and table5; growth for 2 days on minimal medium, bar=50µm).
(C) Δ*gpl-1* revealed abnormal ascospores. In contrast to wtxwt crosses with normal ascospores, the ΔxΔ crosses of the Δ*gpl-1* mutant displayed longer and thinner ascospores.

In a next step, stress sensitivity assays of wild type and the nine GAPs were performed to find indications for molecular functions. The conidia (asexual spores) were harvested and counted, the amount was calculated and serial dilutions (from $1{,}5x10^6$ to 1,5x10 conidia per 5µl) were dropped on minimal medium. Several specific drugs were added to the medium interfering processes like cell wall assembly, actin polymerization, glucan synthase, microtubule depolymerization or induced osmotic and salt stress.

Alteration in the actin cytoskeleton was suggested by the hypersensitivity to latrunculin A of the Δ*rga-1 to* Δ*rga-3,* Δ*gpl-1,* Δ*gpl-2*, Δ*gpl-4* and Δ*gpl-5* deletion mutants. Osmotic stress influenced growth of the Δ*gpl-2*, Δ*gpl-*, Δ*gpl-5* and Δ*rga-4* deletion mutants. The hypersensitivity to Congo Red (affected Δ*rga-1,* Δ*rga-4,* Δ*gpl-4*), Calcoflour White (impaired Δ*rga-2,* Δ*rga-4,* Δ*rga-3,* Δ*gpl-4,* Δ*gpl-5*), SDS (influenced Δ*gpl-4* and Δ*gpl-5*) and lysing enzyme (had an impact on Δ*rga-1* and Δ*rga-4)* suggested that these proteins are involved in proper cell wall function. No effect is observed with Caspofungin and Benomyl (Figure 14).

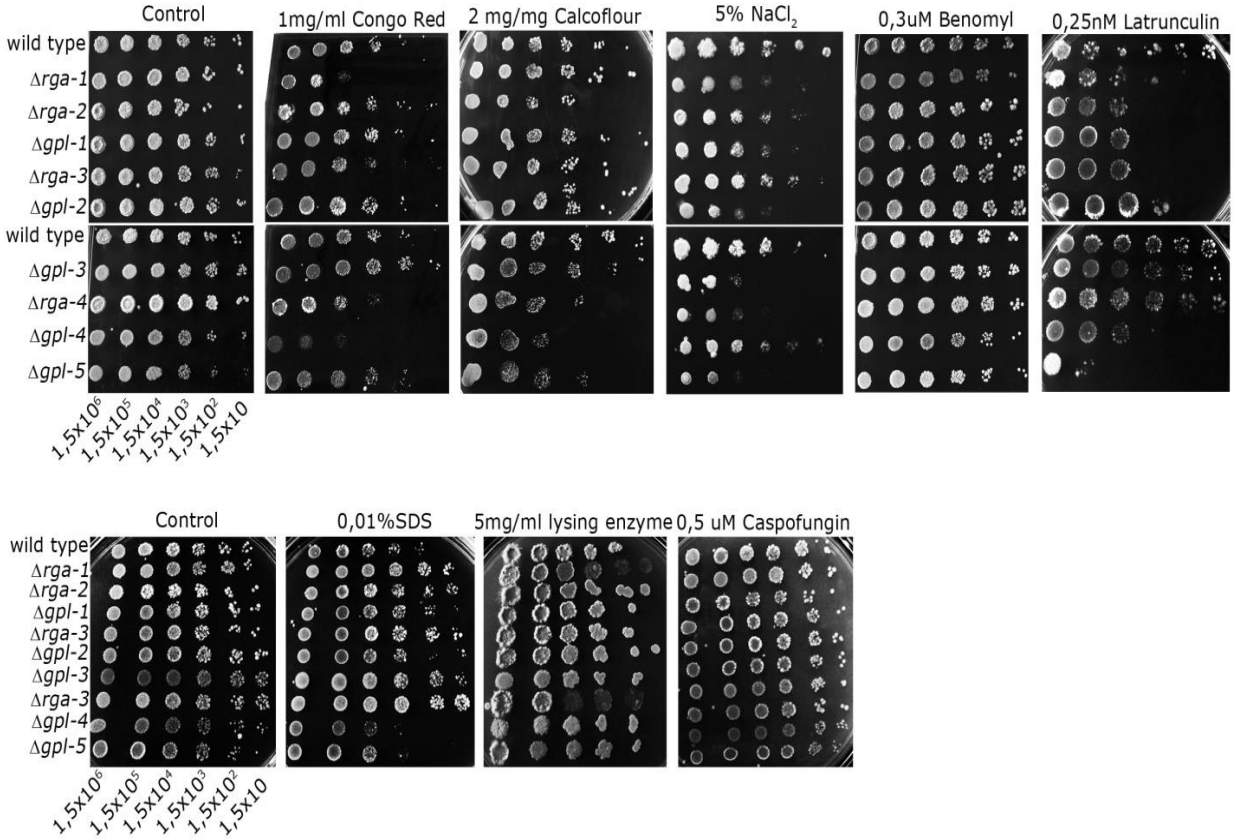


Figure 14: Sensitivity assays with wild type and the nine GAP deletion mutants.
Growth of serial dilutions of conidia of wild type, Δ*rga-1* to Δ*rga-4* and Δ*gpl-1* to Δ*gpl-5* on minimal medium supplemented with the indicated additives. 1% sorbose was added to restrict radial growth. Different additives were used as indicated.

To sum up, the stress sensitivity assays suggests a function of GAP proteins in the regulation of the actin cytoskeleton and/or proper function of the cell wall, yet the results were too diverse to identify one common feature.

Strain	Vegetative growth			Asexual development			Sexual development		Sensitivity
	Germination rate (after 5 h; n = 300)	**Mycelial extension rate (n = 6)**	**Hyphal morphology**	**Septation frequency**	**Aerial hyphae**	**Conidia (n = 3)**	**Protoperithecia (7 d; n = 80)**	**Perithecia and ascospores (ΔxΔ crosses)**	
wild type	100%	100%	wt	wt	wt	wt	100%	wt	
NCU00196 (*rga-1*)	+/- wt	reduced (85±2%)	increased branching; frequently short branches	increased; cluster and short spirals	reduced height	+/- wt	+/- wt	+/- wt	Congo Red
NCU00553 (*rga-2*)	+/- wt	+/- wt	increased branching	+/- wt	increased height	reduced (23±5%)	+/- wt	+/- wt	Latrunculin A Calcoflour
NCU02524 (*rga-3*)	reduced (70±5%)	+/- wt	frequently short branches	+/- wt	+/- wt	reduced (85±3%)	reduced (60±5%)	reduced	Latrunculin A
NCU07688 (*rga-4*)	+/- wt	reduced (85±4%)	increased short branches, hyphae thick and bumpy, irregular zigzag, tip splitting,	+/- wt	+/- wt	+/- wt	few (3±1%)	few	NaCl Calcoflour
NCU01472 (*gpl-1*)	+/- wt	increased (115±4%)	+/- wt	+/- wt	increased height	+/- wt	+/- wt	+/- wt; ascospores thin and pointed	Latrunculin A
NCU02915 (*gpl-2*)	reduced (70±3%)	+/- wt	+/- wt	+/- wt	+/- wt	reduced (40±3%)	+/- wt	+/- wt	Latrunculin A NaCl
NCU07622 (*gpl-3*)	reduced (60±5%)	reduced (75±3%)	hyphae thin and bumpy, long side branches at altered angle	+/- wt	reduced height	reduced (48±4%)	few	few	NaCl
NCU09537 (*gpl-4*)	+/- wt	+/- wt	+/- wt	+/- wt	+/- wt	+/- wt	+/- wt	+/- wt	Latrunculin A SDS Congo Rot Calcoflour
NCU10647 (*gpl-5*)	reduced (70±3%)	reduced (60±3%)	hyphae thin and bumpy, long side branches altered angle	increased in distal regions	reduced height	+/- wt	few (15±4%)	few	Latrunculin A SDSNaCL Calcoflour

Table5: Comparative phenotypical characterization of wild type and the nine deletion mutant strains.
Vegetative growth, asexual and sexual development and sensitivity assays of all strains was investigated. Freshly harvested conidia were plated on minimal medium, incubated and after 5 hours the number of germinated conidia was counted in comparison to wild type (n=300). Hyphal morphology was observed and in comparison to wild type described. The mycelium extension rate is calculated after 24h (n=6). Cell wall and septa were labeled with Calcofluor White and the septation pattern investigated. The aerial hyphae formation was verified in growth tubes. The amount of protoperithecia (after 7d, n=80) and perithecia (ΔxΔ crosses) in comparison to wild type was monitored. Sensitivities of the mutants were described as indicated (for details see Figure 13).

5.2.3 RGA-1 may act as dual specific GAP of RHO-1 and RHO-4 in *N. crassa*

To determine the RGA-1 specificity towards RHO-1 and/or RHO-4, *in vitro* pull down assays were performed. The six RhoGTPases fused to MBP were purified from *E. coli* and *N. crassa* RGA-1-GFP crude extract was added. A strain expressing a RGA-1 C-terminal GFP-tagged fusion protein was generated in *N. crassa*. The construct was expressed under the control of the *ccg-1* promoter and targeted of the *his-3* locus in the respective deletion strain and confirmed functionality of the fusion protein.

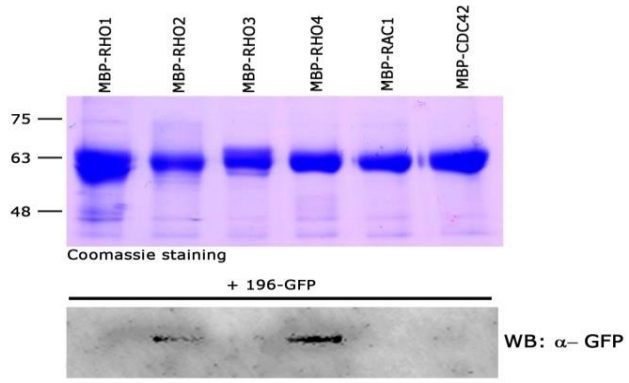


Figure 15: RGA-1-GFP interacts physically with RHO-2 and RHO-4.
GFP-RGA-1 was copurified with MBP fusions of RHO-2 and RHO-4. *E. coli* lysates of cells expressing the MBP-RHO fusion proteins were affinity purified. Copurified RGA-1-GFP was detected by Western blot using an α-GFP antibody (lower panel) and purified MBP-fusion proteins were visualized by Coomassie staining (upper panel).

A stable RGA-1-RHO-4 interaction was confirmed by western blot and immunodetection with a GFP-specific antibody. A weak interaction was detected with RHO-2 and no interaction could be identified between RGA-1 and RHO-1 (Figure 15).

To investigate the cellular distribution of the RGA-1 protein localization studies were performed in *N. crassa* (Figure 16A). RGA-1 and RHO-1 displayed identical localization patterns associated with the complete septum plate, while RHO-4 remained associated with the septal pore after completion of the septation process (Figure 13B). These results indicate a connection between RGA-1 and RHO-1. In addition RGA-1, RHO-1 and RHO-4 localized spotty near the septa in a membrane associated manner (Figure 16B).

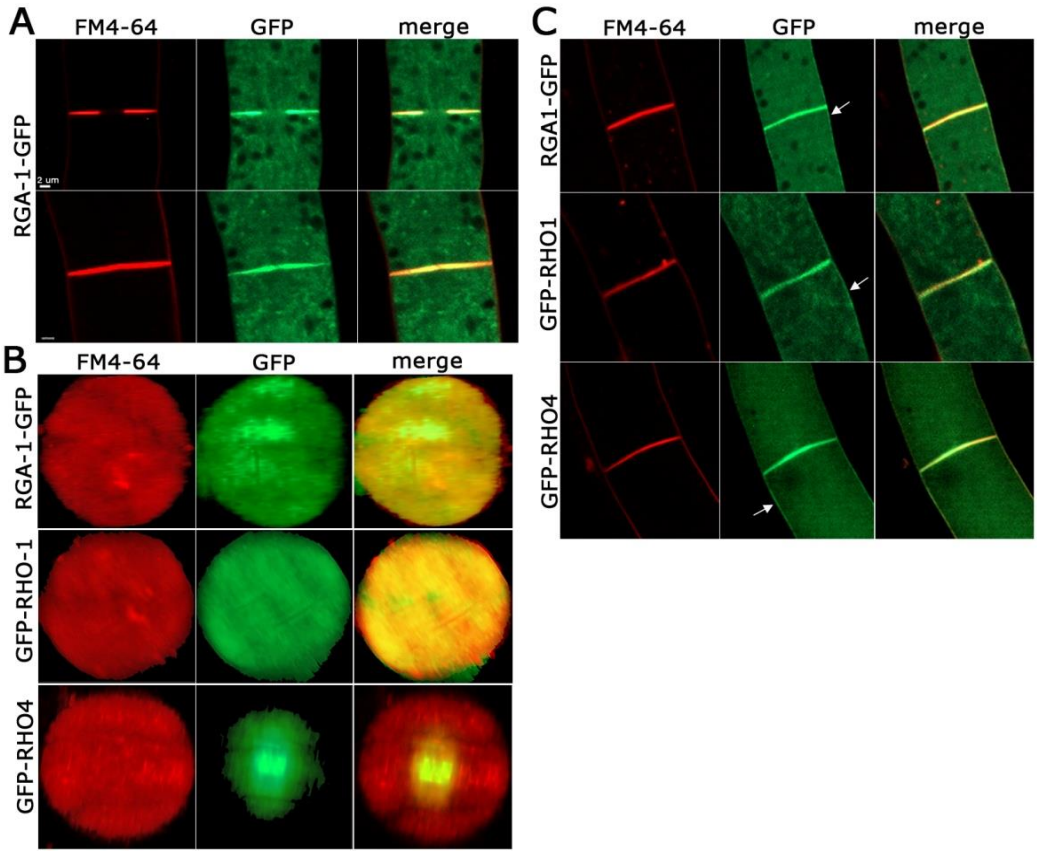


Figure 16: Localization studies of RGA-1-GFP in comparison to GFP-RHO-1 and GFP-RHO4.
(A) RGA-1-GFP localized at the forming septum.
(B) 3D reconstruction of z-stacks illustrated RGA-1-GFP localization at the complete septum and resembled the GFP-RHO-1 localization, while GFP-RHO-4 associated at the septal pore.
(C) Additional membrane associated localization of RGA-1-GFP, GFP-RHO-1 and GFP-RHO-4.
The plasma membrane and the septa were stained with FM4-64.

5.2.4 Phenotypical characterization of the Δ*rga-1* deletion mutant

To investigate the role of RGA-1 in septation, wild type and the Δ*rga-1* deletion mutant were stained with Calcoflour White. The Δ*rga-1* deletion mutant showed several septation defects (clusters of closely positioned septa and curved connected septa) in comparison to wild type (Figure 17A). The Δ*rga-1* deletion phenotype suggests RGA-1 acting as negative regulator of septation.

In vitro pull down assays and *in vitro* GAP assays showed RGA-1 interacting with RHO-4 and affected RHO-4 activity. The mutated *rho-4*(*D126A*) version was previous described mimicking an activated allele by increased GDP dissociation and therefore leading to more GTP-bound protein (Rasmussen & Glass, 2005). Thus, *rho-4*(*D126A*) and the Δ*rga-1* mutant were monitored related to septation. The identified clusters of closely positioned septa of *rho-4*(*D126A*) were comparable to the Δ*rga-1* mutant (Figure 17A). Subapical irregular septation resulted in decreased hyphal compartment lengths in both Δ*rga-1* and *rho-4*(*D126A*) (Figure 17A,B). However, curved septa were less frequent in *rho-4*(*D126A*) in comparison to the Δ*rga-1* mutant (Figure 17A). These results suggest that RGA-1 is involved in septation and negatively regulates RHO-4.

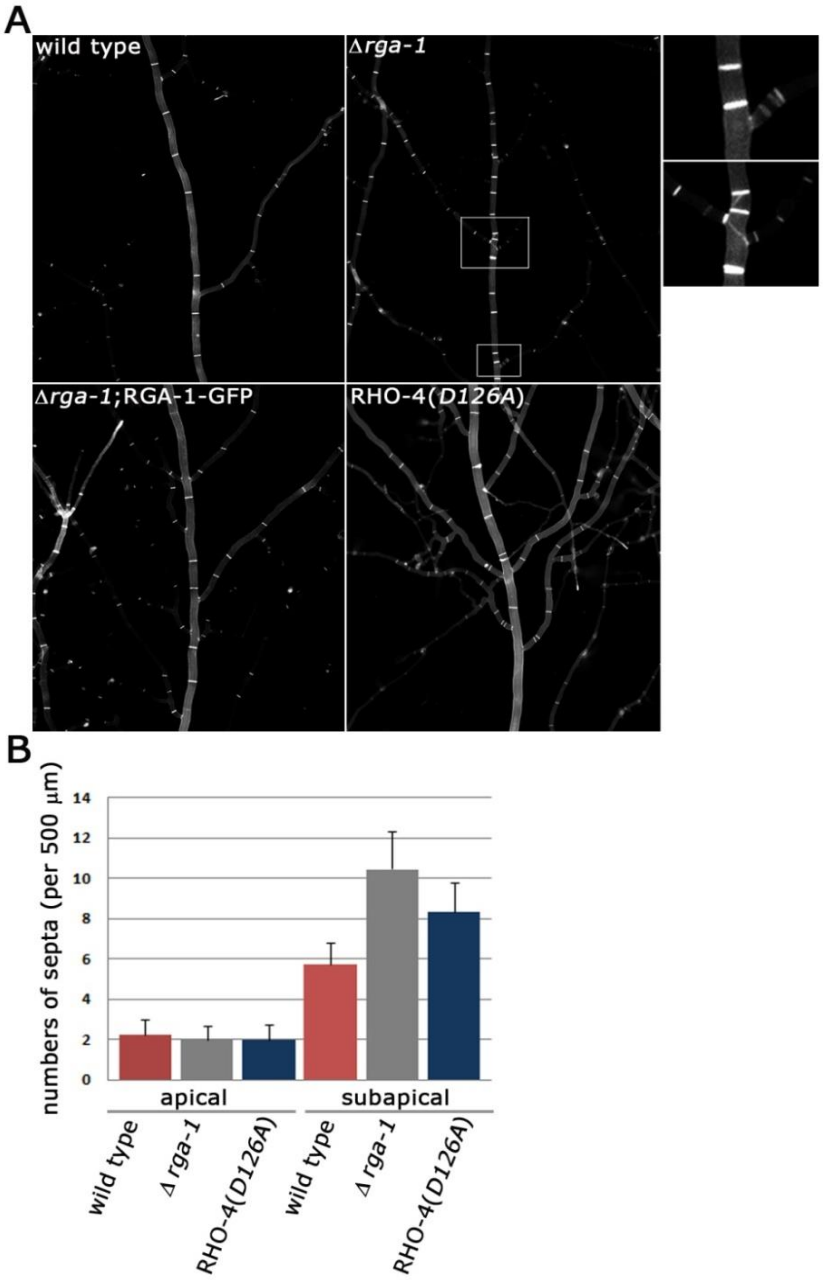


Figure 17: Comparison of septum formation and -pattern of wild type, *Δrga-1, Δrga-1;* GFP-RGA-1 and *rho-4* (D126A).

(A) Clusters of closely spaced septa and abnormal curved septa were shown in detail (right panel). Irregular septation was suppressed by the GFP-RGA-1 protein. Also dominant active *rho-4*(D126A) cells produced multiple and closely spaced septa.

(B) The number of septa was counted in wild type, *Δrga-1* and *rho-4*(D126A) apical and subapical.

Cell wall and septa were labeled with Calcofluor White.

5.2.5 The Δ*rga-1* and Δ*gpl-5* act independent in septation

Two GAP proteins (Δ*rga-1* and Δ*gpl-5*) depicted defects in their septation frequency (Figure 17A; Figure 18B). To further analyze a potential functional relationship between both proteins, a Δ*rga-1*;Δ*gpl-5* double mutant was generated by crossing of the single deletion mutants. The double deletion strain resembled the defects in regard to hyphal morphology, growth rate and sexual development of the Δ*gpl-5* deletion mutant (Figure 18A,C).

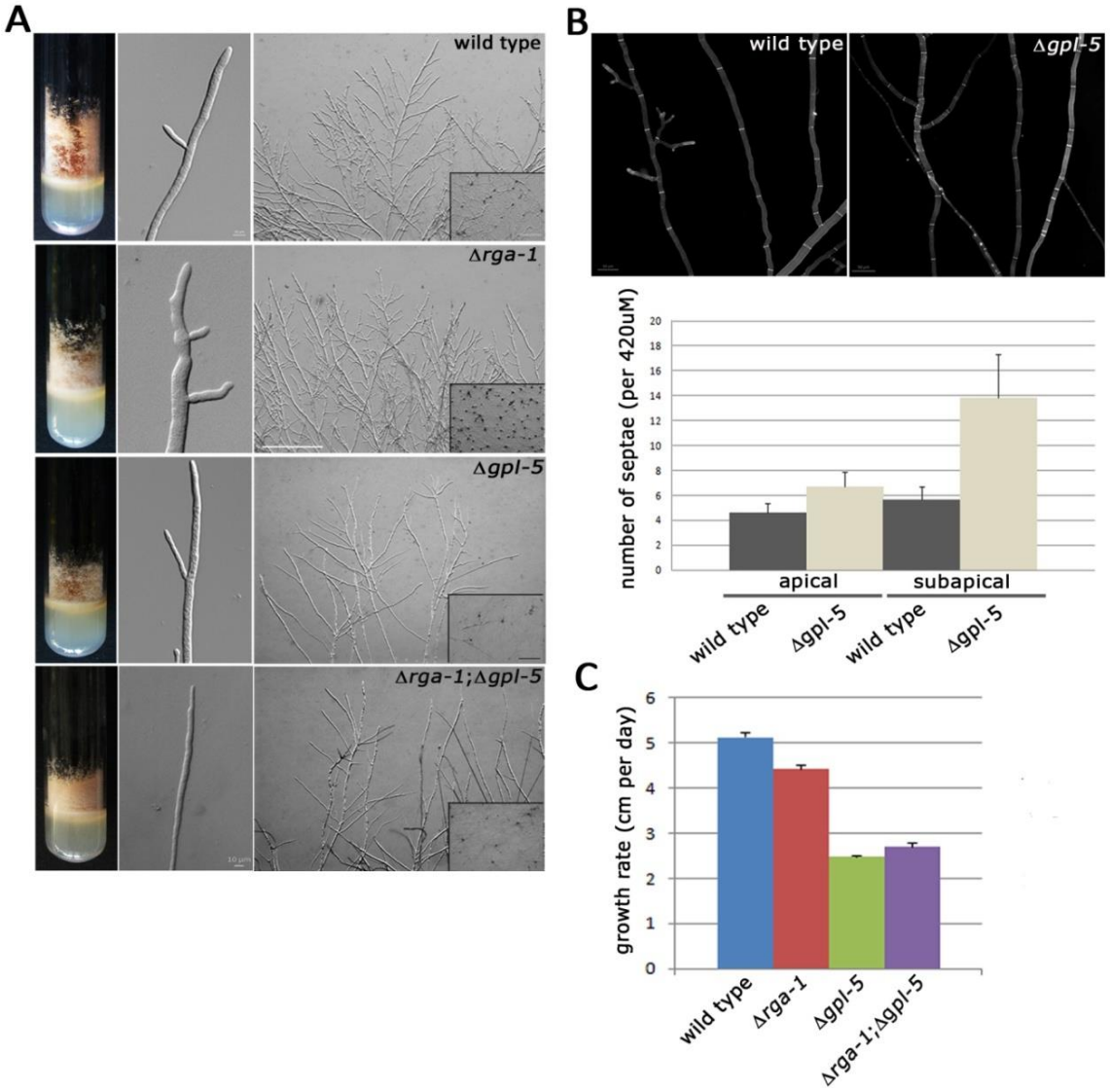


Figure 18: RGA-1 and GPL-5 are involved in septation but acting independent of each other.
(A) Comparison of wild type, Δ*rga-1,* Δ*gpl-5* and Δ*rga-1;*Δ*gpl-5* morphology. The Δ*rga-1* mutant revealed knobbed hypha with frequent short branches. In contrast the Δ*gpl-5* deletion mutant depicted thin hypha with long branches at an irregular angle. Whereas the double deletion resembled the Δ*gpl-5* phenotype (growth for 2 days on minimal medium, bar=50µm). In comparison to wild type the aerial mycelium is decreased in Δ*gpl-5* and the Δ*rga-1;*Δ*gpl-5* double deletion mutant (growth for 5 days on minimal medium). Protoperithecia (female sexual structure) formation is decreased in Δ*gpl-5* and the Δ*rga-1;*Δ*gpl-5* (right corner).
(B) The number of septa was apical and subapical counted in wild type and *Δgpl-5*. Cell wall and septa were labeled with Calcofluor White.
(C) Δ*gpl-5* and Δ*rga-1;*Δ*gpl-5* displayed phenotypes of slower growth compared to wild type.

The double-knockout Δ*rga-1;*Δ*gpl-5* strain revealed subapical increased septation comparable to the Δ*gpl-5* deletion mutant and curved connected septa of the Δ*rga-1* deletion mutant (Figure 18A,C). The Δ*rga-1;*Δ*gpl-5* double deletion phenotype was characterized by the sum of the single mutants and no additional effects were observed, suggesting that Δ*gpl-5* and Δ*rga-1* act independent from each other in septation.

5.3 The deletion of *gpl-2* leads to suppressed *gpl-3* deletion mutant defects

Previous phylogenetic and domain structure analysis identified two proteins, NCU02915/GPL-2 and NCU07622/GPL-3, based on their GAP domains to be potential GAP proteins in *N. crassa*.

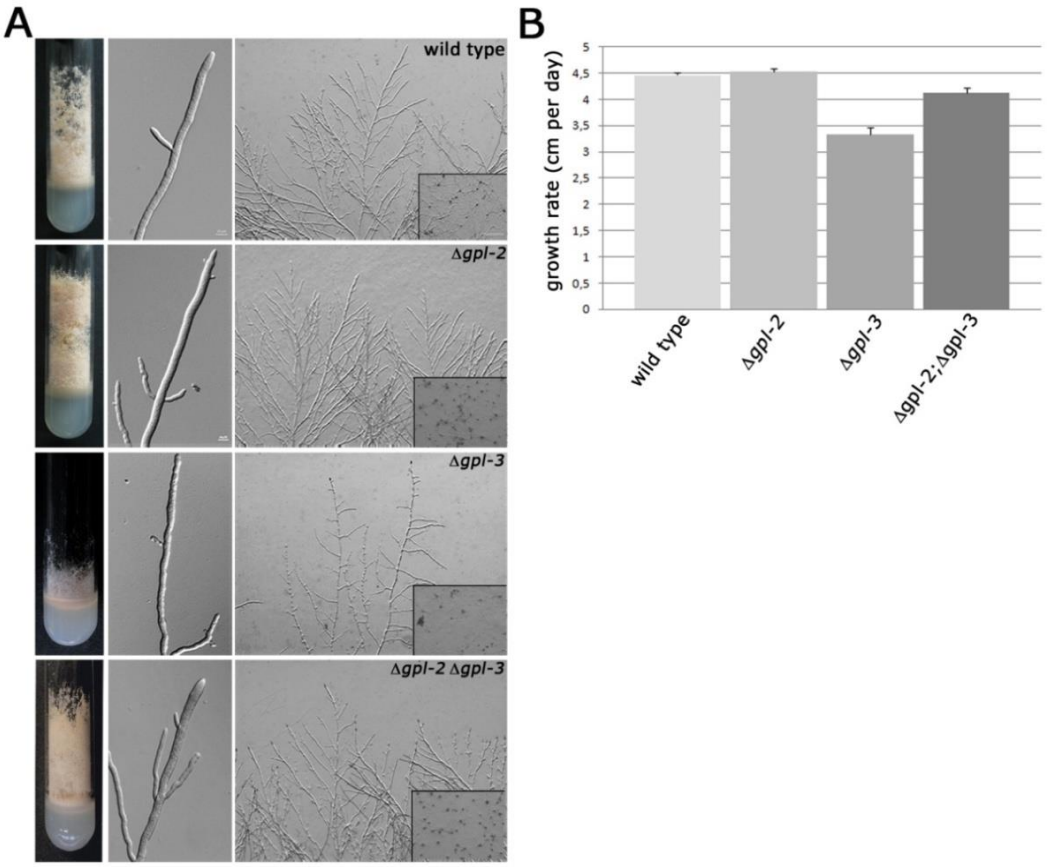


Figure 19: Deletion of *gpl-2* suppressed the *gpl-3* deletion defects.
(A) Comparison of wild type, Δ*gpl-2,* Δ*gpl-3* and Δ*gpl-2;*Δ*gpl-3* morphology. The Δ*gpl-2* mutant revealed wild type morphology. In contrast the Δ*gpl-3* deletion mutant depicted thin hypha with long branches at an irregular angle. Whereas the double deletion resembled the Δ*gpl-2* phenotype (growth for 2 days on minimal medium, bar=50µm) and defects of Δ*gpl-2* were suppressed. In comparison to wild type the aerial mycelium is decreased in Δ*gpl-3*, while the Δ*rga-1;*Δ*gpl-5* double deletion mutant restored wild type aerial hyphae formation (growth for 5 days on minimal medium). In addition protoperithecia formation was restored in the Δ*rga-1;*Δ*gpl-5* double deletion mutant.
(B) The Δ*gpl-2;*Δ*gpl-3* double deletion had an intermediate growth rate of the single deletion mutants.

In both proteins no additional domains were noted. To further analyze a potential functional relationship, a double mutant was generated. The Δ*gpl-2;*Δ*gpl-3* double mutant resulted in progeny

resembling the Δ*gpl-2* deletion phenotype related to hyphal morphology, aerial mycelium formation and sexual development. In comparison to the single deletion mutants, the Δ*gpl-2;*Δ*gpl-3* double deletion had an intermediate growth rate but no additional effects in the double deletion strain were identified (Figure 19A,B). These results indicate the Δ*gpl-2* mutant suppressed the Δ*gpl-3* mutant defects.

5.3.1 RGA-2, RGA-3 and RGA-4 are involved regulation of RAC, CDC-42 and RHO-3- phenotypical characterization of double deletion mutants

In vitro GAP assays assigned four GAPs to RhoGTPases and RGA-2, RGA-3 and RGA-4 depicted dual specificity to RAC and CDC-42. Thus, RGA-3 and RGA-4 were specific to RHO-3.

The *N. crassa* Δ*rga-2* single deletion mutant showed no defects, while Δ*rga-3 and* Δ*rga-4* were affected in protoperithecia formation and Δ*rga-4* depicted an increased branching pattern with tip splitting.

To further investigate a functional relationship between the proteins several *N. crassa* double mutants were generated. The Δ*rga-2;*Δ*rga-3* double deletion resembled the Δ*rga-2* single deletion phenotype in growth rate, aerial mycelium formation and protoperithecia formation. No additional effects in the double deletion strain were identified (Figure 20A,B).

The Δ*rga-3;*Δ*rga-4* double deletion revealed the Δ*rga-4* single deletion in growth rate, aerial mycelium formation, protoperithecia formation and hyphal morphology and branching. A phenotype exhibiting the single mutant was found and no additional characteristics were identified.

Only the Δ*rga-2;*Δ*rga-4* double deletion mutant depicted more severe characteristics than the single deletion mutants since growth rate and aerial mycelium formation were reduced, while sexual development was completely blocked. The hyphal morphology was affected and branching was increased (Figure 20A,B). These results suggest overlapping or partially redundant functions for Δ*rga-2* and Δ*rga-4* affecting polar growth in *N. crassa*.

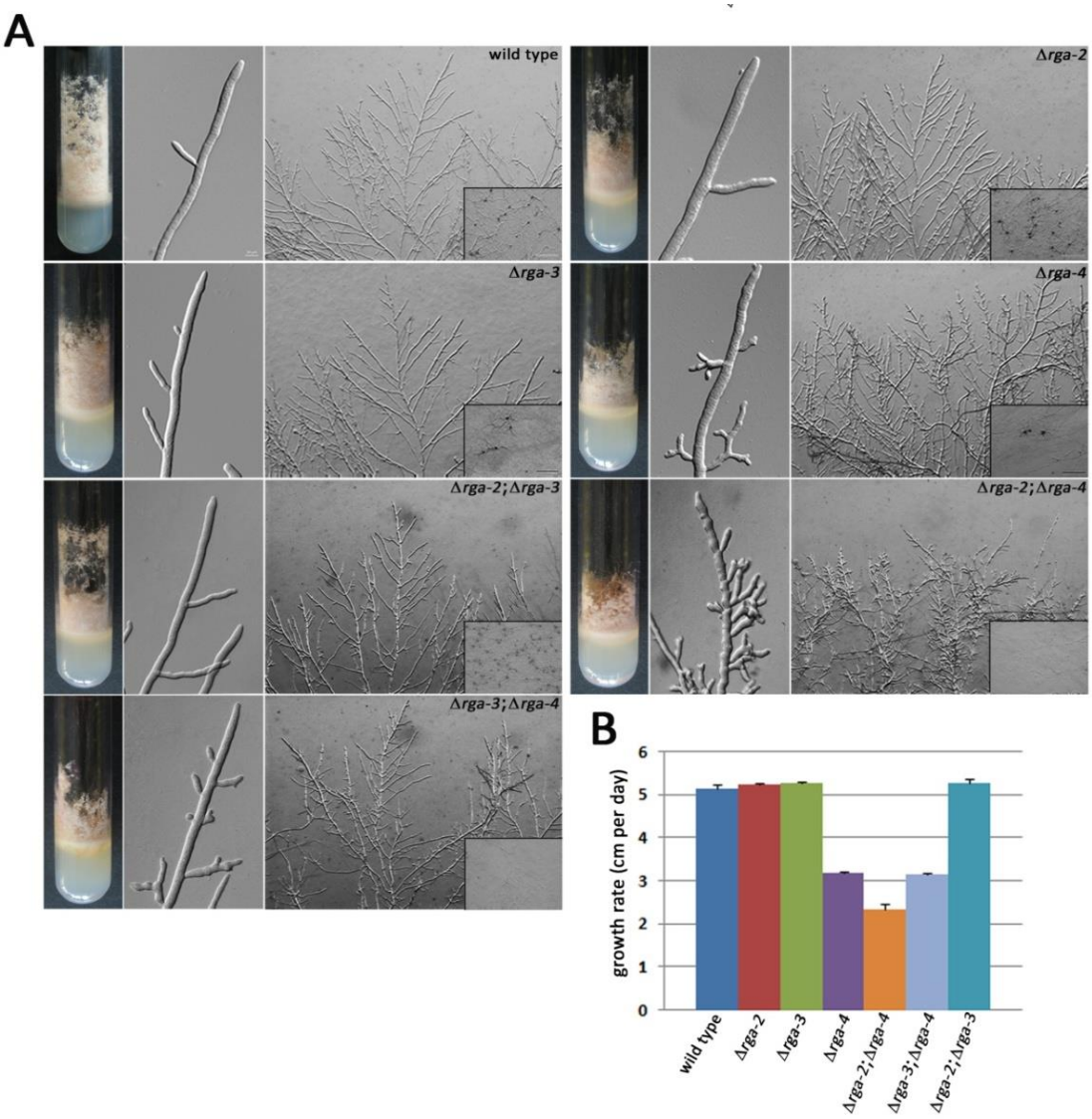


Figure 20: Comparative characterization of the double deletions Δ*rga-2;*Δ*rga-3*, Δ*rga-2;*Δ*rga-4* and Δ*rga-3;*Δ*rga-4* - active GAPs of CDC-42, RAC and RHO-3.

(A) Comparison of wild type, single and double deletion mutant morphology (Aerial mycelium formation, hyphal morphology and sexual development. For details see text).

(B) Growth rate of Δ*rga-2;*Δ*rga-4* was reduced in comparison to wild type and single deletion mutants, while Δ*rga-3;*Δ*rga-4* resembled Δ*rga-4* growth and Δ*rga-2;*Δ*rga-3* was comparable to wild type and the single deletion strains.

6 Discussion

6.1 ELMO and DOCK form a complex and are specific for the Rho GTPase RAC

At the moment only CDC-24 is described to act as dual specific GEF of the CDC-42-RAC module, which is required for polarized growth and hyphal morphogenesis in *N. crassa.* The GEF specificity was approved by loss of function studies and *in vitro* GDP-GTP exchange assays (Araujo-Palomares *et al*, 2011). Nevertheless, the Dock family GEFs emerged as interesting candidate because studies in mammals and other fungi revealed a role of Dock180 (and its adapter Elmo1) in regulation of Rac1 (Cote & Vuori, 2007, Herrmann *et al.*, 2014, Hope *et al.*, 2008, Hope et al., 2010). Rho GTPase regulation is important in embryonic development because their missregulation leads to a variety of diseases (Bryan *et al*, 2005; Cancelas & Williams, 2009; Hall & Lalli, 2010; Alan & Lundquist, 2013).

As mentioned in the introduction, numerous regulatory components of Rho GTPases had not been described in the filamentous fungus *Neurospora crassa*. Previous *in vitro* GEF activity assays, with the DOCK DHR domain, determined DOCK to be inactive towards the six Rho GTPases (Richthammer, 2011). This led to two possible assumptions: Either Dock is a non-functional GEF or the adapter Elmo is required for activity (Brugnera *et al*, 2002; Lu *et al*, 2005). *N. crassa* possesses homologues of ELMO/NCU03264 and DOCK/NCU09492 and both proteins were further analyzed to determine their function. First, GFP-trap affinity purification experiments coupled with mass spectrometry were performed to investigate proteins interacting with ELMO. However, by this analysis it was possible to identify DOCK as interaction partner (Figure 4A). Yeast to hybrid analysis verified that full length proteins of DOCK and ELMO interact (Figure 4B). Moreover, a strain containing HA-tagged ELMO and GFP-tagged DOCK was generated and a stable interaction of both was detected by *in vivo* co-immunoprecipitation experiments (Figure 4C). These results confirmed the assumption that ELMO and DOCK form a complex.

In *C. albicans* the Dck1 DHR domain was reported to bind Rac1, preferably the nucleotide-free form Rac1 (T17N) (Hope *et al*, 2008). Yeast two hybrid studies with the DOCK DHR-2 domain (1290aa-1846aa) with full length RAC and CDC-42 (and ELMO) showed no interaction (see Supplement Material 1). At the moment it is not clear yet, which region of DOCK is responsible for their interaction in *N. crassa*. Further experiments with truncated proteins of ELMO (SH3 domain) and DOCK (DHR-1 domain or DHR-2 domain plus X amino acids) could identify the domains involved in their interaction.

In *C. albicans* a complex of Dck1 and Lmo1 was proposed to regulate Rac function in invasive filamentous growth and cell wall integrity (Hope *et al*., 2008; Hope *et al*, 2010). To further elucidate this hypothesis in *N. crassa*, co-immunoprecipitation experiments of tagged proteins were performed

and exhibited weak interactions of ELMO and DOCK with RAC, but not with CDC-42 (Figure 4A,B). This supports the hypothesis of a bipartite GEF, specific for RAC activation. But surprisingly, interactions between these proteins could not be observed by yeast two-hybrid studies, suggesting that interactions of DOCK and ELMO with RAC might be indirect. Or in order to achieve a stable interaction between them, all three components are necessary. Additional experiments, such as yeast three hybrid experiments or pull down assays with tagged versions of the putative ternary complex could clear this point. Maybe the autoinhibitory domains of ELMO and DOCK play a significant role in "fine-tuning" of Rho GTPase regulation. The SH3 domain of Dock180 directly binds to the DHR-2 domain in mammals. Thus, Dock180 is autoinhibited until Elmo1 is bound and then is the DHR-2 domain exposed to Rac1 (Lu *et al*, 2005).

However, there was evidence that additional components are required to enable Rac signaling. In *C. purpurea* CpDock180 interact with CpBem1 and CpCla4, suggesting CpBem1 to be scaffold mediating activation of CpRac by CpDock180, whereas the effector CpCla4 possibly presenting a feedback to the CpCla4-CpCdc24 system. Interaction, localization and activation data assumed a Dock180-Rac-Cla4 complex involved in stress response (Herrmann *et al*, 2014). A ternary complex, consisting of Bem1-Rac-Cla4 has also been reported in *U. maydis* (Frieser S, 2011, Herrmann *et al*., 2014).

6.2 DOCK is involved in polar growth

The Rho GTPases CDC-42 and its close relative RAC were reported to affect polarized growth and the double deletion of both proteins led to a growth and polarity defect. Polarity was established in the absence of CDC-42 or RAC, but hyphal extension was highly decreased. *rac* mutants were characterized by dichotomous tip splitting and massive apical hyperbranching, also observed to a lower extent in *cdc-42* mutants. Nevertheless, their most prominent feature was the emergence of numerous subapical branches. So far, CDC-24 was characterized as dual GEF specific for RAC and CDC42 and the deletion of *cdc-24* phenocopied the Rho GTPase defects (Araujo-Palomares *et al*, 2011).

In this study the absence of DOCK leads to a growth and polarity defect, whereas the loss of ELMO caused less severe morphological defects and establishment of the primary axis is still possible in both strains. Cells without DOCK revealed an irregular bulgy hyphal morphology, whereas lack of ELMO leads to a milder affected hyphal morphology (Figure 6A,B). This notion was rather unexpected since studies in *C. albicans* suggested identical phenotypes for both proteins (Hope *et al*, 2010). However, strains deficient in DOCK are characterized by the emergence of multiple subapical branches, also observed in a lower extent in mutants affected in ELMO function (Figure 6C). In order to determine the genetic relationship between DOCK and ELMO a double mutant was generated which phenocopied defects of the Δ*dock* mutant. This dominant morphological defect supported the idea that DOCK has a more prominent role in *N. crassa* than ELMO. In this context two contrasting

models were proposed: On the one hand the bipartite GEF model, where Elmo1 and Dock180 together are essential for Rac1 activation (Brugnera *et al*, 2002) and on the other hand studies representing an alternative mechanism where ELMO is not necessary for the activation of Rac1 (Cote & Vouri, 2002; Cote & Vouri, 2007; Komander *et al*, 2008). However, further studies with the adapter protein ELMO have to elucidate the importance and function in *N. crassa*. To test this hypothesis *in vitro* GEF assays in presence and absence of ELMO could be a possibility in relation to RAC activity. Alternatively localization experiments of CRIB-GFP in the corresponding deletion backgrounds in comparison to the double deletion background could help to assess the role of ELMO in RAC activation.

The loss of DOCK leads to an apical polarity defect characterized by periods of apolar growth. Hyphal elongation completely stopped and thereupon the apex is rounded and increased in diameter (Figure 7A). During this apolar growth period no hyphal elongation takes place, whereas the wild type depicted the characteristic tubular shape and continuously hyphal elongation (Figure 7B). These results indicate that the loss of DOCK leads to deficits in determining constant polar tip growth in *N. crassa*. Consistent with this result was the defect in polarity in *A. gossypii* Δ*dck1* deletion mutants. Polar defects occurred during formation of germ tubes and in mature hypha. Interestingly, the hyphae of the Δ*dck1* mutant appeared to be wavy and had no consistent growth direction (Nordmann *et al*, 2014). The GEF Dck1 is supposed to be important for matrix- embedded dependent filamentous growth, and mutants were unable to form filaments and formed instead smooth colonies, identical to the *rac* deletion mutants in *C. albicans* (Hope *et al*, 2008).

A more detailed analysis of tip growth of cells without DOCK was done with FM4-64. Studies with live imaging and molecular tools reported the importance of the SPK in fungal development (Fischer-Parton S, 2000). The SPK is not defined as classical organelle, it is rather a multicomponent structure keeping all components together while moving within the apical dome, determining growth direction and hyphal morphology (Bartnicki-Garcia et al., 1995, Riquelme et al., 1998, Riquelme & Sanchez-Leon, 2014, Fischer-Parton S, 2000). The persistent bulge in hyphal shape appeared simultaneously with the SPK dissociation in the Δ*dock* mutant (Figure 8A,B). The *dock* deletion leads to an increase in tip diameter, instead of hyphal tip elongation and the apical still flexible cell wall allow enlargement of the hyphal tip. After re- association of the SPK to the apical dome hyphal elongation is resumed.

Furthermore these studies revealed subapical multiple diffuse SPK aggregates inducing multiple branches at small distance to each other (Figure 9A,B). *A. niger* and *P. marnefeii rac* deletion was characterized by apical hyperbranching and a potential explanation could be the inability to focus actin polymerization and the result could be multiple axes of polarity (Boyce *et al*., 2003; Kwon *et al*., 2010). But so far it is not clear, which process is responsible for this event.

6.3 DOCK localized subapical in a patchy membrane associated manner

In vivo localization studies revealed distinct patterns of CDC-42 as apical membrane associated crescent, of RAC as membrane associated ring (excluding regions of CDC-42) and of CDC-24 as membrane associated crescent and at the apical cytosol without the SPK (Araujo-Palomares et al, 2011). This is supported by localization studies of Cdc42 and Rac homologues in other fungi, observed in apical growing hypha of *P. marneffeii*, *A. nidulans*, *A. niger* and *C. albicans* (Bassilana *et al.*, 2003, Boyce et al., 2003, Boyce et al., 2005, Kwon et al., 2011, Virag et al., 2007). Different localization studies in *N. crassa*, *P. marneffei* and *C. albicans* suggested a function of Cdc42 and Rac in septation, this is supported by the *cflB* deletion mutant, which revealed septation and growth defects in *P. marneffei* (Boyce *et al*., 2003; Boyce *et al*, 2005; Bassilana *et al*., 2005; Araujo-Palomares *et al*, 2011). Furthermore, Cdc42 is responsible for cell separation control in *U. maydis* (Böhmer et al, 2008). It should also be mentioned that the GEFs CDC-24 and RGF-1 also localized to the septum in *N. crassa*, suggesting the formation of GEF-GTPase modules at the septum (Araujo-Palomares *et al*, 2011; Richthammer *et al*, 2012).

Ectopically expressed DOCK-GFP complemented the hyphal elongation defect of the Δ*dock* deletion mutant and DOCK-GFP localized subapical in a patchy membrane associated manner as well as to the septum (Figure 10A; Supplemental Figure 2). This result was unexpected and leaves the question open why DOCK-GFP localized subapical, because many proteins important for polar growth were found apically (Sudbery, 2011). However, an example of subapical membrane associated DOCK was identified in *Claviceps purpurea*. CpDock180 localized along the plasma membrane and in small vesicle like patterns along the hypha, however, resembling the CpRac localization in this organism (Herrmann *et al*, 2014). The rapid exchange of GTPase molecules between the cytosol and the polar cap reported in *S. cerevisiae*, could be a possible explanation for the absence of DOCK at the polar tip (Wedlich-Soldner *et al*, 2014). Interestingly, apical GFP-DOCK was identified apical if hyphal elongation arrested and in this case GFP-DOCK localized as apical crescent (Figure 10B). Therefore a possible speculation could be that DOCK is responsible to maintain constant polar hyphal elongation.

The interaction approaches together with the localization studies suggested RAC activation by CDC-24 and DOCK, both GEFs acting in spatially separated areas in parallel entry points, affecting the same linear pathway with similar phenotypic readouts. The identification of additional involved proteins as scaffolds and studies under endogenous expression conditions will be the purpose of future investigations.

In *S. cerevisiae*, *S. pombe* and *C. albicans* CDC-42-RAC-interactive-binding reporter were used to investigate polar growth. In *N. crassa* the CRIB reporter (derived from Cla4, member of the PAK family) was reported as useful tool to discriminate between inactive and active Rho GTPases during polar growth (Lichius *et al*, 2014). The CRIB reporter labeled local activated GTP-bound GTPases and

allows selectively analysis of the spatio-temporal dynamics of local activated CDC-42 and RAC-1 in wild type versus cells without DOCK. CRIB-GFP localized at the cells front where polarity occurred but in cells without DOCK additional subapical membrane associated areas were CRIB-GFP localized were identified. This altered localization pattern leads to the assumption that DOCK is involved in regulation of Rho GTPases in *N. crassa*.

6.4 *In vitro* GAP assays determined target specificity towards Rho GTPases

Rho GTPases are important as key regulators in polar signaling and their regulation is essential for their correct function. Previous phylogenetic and structure analysis in comparison to the yeasts identified ten proteins with distinctive GAP domains in *N. crassa* (Richthammer, 2011). The RHO-1 specific GAP LRG-1 was already characterized in *N. crassa* (Vogt & Seiler, 2008) but the nine remaining GAP proteins were investigated in this study. As a biochemical approach, *in vitro* GAP assays with the nine putative GAPs towards the six Rho GTPases, determined four of the GAPs to be active (Figure 12). This is the first study in *N. crassa* within four GAP proteins, RGA-1/NCU00196, RGA-2/NCU00533, RGA-3/NCU02524 and RGA-4/07688, were assigned towards their specific Rho GTPases. However, an alignment clarified diverse reasons why the five GAPs proteins were inactive. GPL-1/NCU01472 was insoluble, whereas GPL-2/NCU02915 and GPL-3/NCU07622 lack several residues, especially those required for GTPase binding and residues necessary for their activity. Two GAP proteins, GPL-4/NCU09537 and GPL-5/10647, were inactive of unknown reason.

In this study, the four active GAP proteins were named RGA-1/NCU00196, RGA-2/NCU00533, RGA-3/NCU02524 and RGA-4/07688. RGA-1 was identified to act as dual specific GAP of RHO-1 and RHO-4 in *N. crassa*. This is in contrast to the closest homologues in *S. cerevisiae* Sac7p, Bac7p and Rga5 in *S. pombe*. Interestingly, budding yeast Sac7p, Bag7p and fission yeast Rga5 were found to be Rho1(p) specific GAPs. Rga5 is described to regulate the interaction with Pck1 and cytokinesis, whereas Bag7p (homologue of Sac7p) interacts *in vivo* and *in vitro* with Rho1p (Fitch *et al.*, 2004, Lorberg *et al.*, 2001, Marquitz *et al.*, 2002, Schmidt *et al.*, 2002).

The GAP protein RGA-2 was identified as dual specific for CDC-42 and RAC in *N. crassa*. Even its close relatives are dual specific in both yeasts they regulate different modules. *S. pombe* Rga7 was characterized to negatively regulate the MAPK cell integrity pathway and dual specific to Rho2 and Rho4 (Soto *et al.*, 2010). In *S. cerevisiae* Rgd1p participates in areas of polarized and is stimulating Rho3p and Rho4p (Lefèbvre F, 2012).

N. crassa RGA-3 was determined to function as RAC, CDC-42 and RHO-3 specific GAP. Bem3p shares the specificity for Cdc42p in *S. cerevisiae*, but in contrast the *S. pombe* Rga2 was Rho2-specific and dedicated to be involved in morphogenesis and CWI MAPK pathway (Villar-Tajadura MA, 2008).

Moreover, the GAP protein RGA-4 was identified to be specific for the Rho GTPases RAC, CDC-42 and RHO-3. In *S. pombe* Rga4 and *S. cerevisiae* Rga1p and Rga2p were found as Cdc42 (p) specific GAPs.

Rga4 negatively regulates the cell MAPK integrity pathway (Soto *et al*, 2010), whereas Rga1p, Rga2p and Bem3p as Cdc42p specific GAPs were necessary for septin ring formation (Caviston *et al*, 2003).
To sum up, based on their conserved GAP domain it was possible to assign four of nine GAP proteins to their specific Rho GTPases. Furthermore, the GAP specificity revealed not only similarities but also differences in comparison to the yeasts and further studies are necessary to understand the GAP proteins in *N. crassa*.

6.5 Phenotypical characterization of the nine putative GAPs

In a next step, a detailed phenotypic mutant characterization of the nine GAP proteins was performed and the vegetative, asexual and sexual development was monitored in numerous different stages. The detailed morphological examination provided no clear results to organize or to subgroup the GAP proteins into categories because of their defects (Table 5). Furthermore, the hypersensitivity to Congo Red/Calcoflour White (interfering cell wall assembly) and latrunculin A (actin polymerization inhibitor) was a preliminary hint to their function in regulation of the actin cytoskeleton and/or proper function of the cell wall.
Interestingly, similar results were observed in the rice blast fungus *M. oryzae* where most of the Rho GAPs deletion mutants revealed only marginal phenotypes. Eight Rho GAP proteins identified and a comparative phenotypical characterization of the deletion mutants was performed in *M. oryzae*. The deletion of Molrg1 caused reduced conidiation and appressorium formation by germ tubes and the loss of pathogenicity, while cells lacking MoRga1 resulted in larger or gherkin-shaped conidia and caused a slight decrease in conidiation. Both GAPs interacted with constitutive active versions of MoRac1 and MoCdc42. As mentioned above, the remaining proteins MoRga2 to MoRga7 were dispensable for conidiation, vegetative growth, appressorial formation and pathogenicity, suggesting that these Rho GAPs function redundantly during fungal development (Ye *et al.*, 2014).
Based on previous phylogenetic and structural analysis GPL-2 / NCU02915 and GPL-3 / NCU07622 were identified to be GAP proteins (Richthammer, 2011). To investigate the genetic relationship in *N. crassa*, double mutants were generated. Surprisingly, the Δ*gpl-2*;Δ*gpl-3* double mutant revealed in relation to hyphal morphology and sexual development the Δ*gpl-2* mutant phenotype, but an intermediate growth rate of the single deletion phenotypes. This result suggested that the deletion of *gpl-2* suppressed the *gpl-3* deletion phenotype (Figure 19A,B). However, further experiments should be investigated to clarify the putative connections.
In vitro GAP assays determined RGA-2, RGA-3 and RGA-4 to be GAP proteins with specificity to RAC and CDC-42. The two GAPs RGA-3 and RGA-4 were additionally involved in regulation of RHO-3. In order to determine the genetic relationship, three double deletion mutants were generated in *N. crassa*. Two of the double deletions revealed with regard to their morphology, hyphal elongation and sexual development, one of the single deletion phenotypes. Only the Δ*rga-2*;Δ*rga-4* double deletion

mutant showed a more severe phenotype than the single deletion mutants. The hyphal morphology was affected (knobby), the hyphal elongation reduced and an increased branching pattern was found (Figure 20A,B). This leads to the assumption that both proteins have overlapping or partially redundant functions and the results suggested RGA-2 and RGA-4 to be involved in regulation of the same Rho GTPase in *N. crassa*. Nevertheless, a triple mutant of the three GAPs would be interesting for further investigations and localization studies with their respective Rho GTPases could identify further connections.

6.6 RGA-1 is a putative dual GAP of RHO-1 and RHO-4 in *N. crassa*

However, the comparative phenotypical characterization was used as starting point for further experiments. As described above, *in vitro* GAP assays revealed RGA-1 to be a RHO-1 and RHO-4 dual specific GAP. In contrast the related GAP proteins in both yeasts were described to be Rho1 specific in both yeasts (Marquitz *et al*, 2002; Schmidt *et al*, 2002). In *N. crassa* the deletion of *rga-1* depicted defects in septation (irregular septation, clusters of septa, curved septa) (Figure 17A). Ectopically expressed RGA-1-GFP complemented the defects of the Δ*rga-1* deletion mutant and localized to the forming septum and to the subapical membrane near the septa (Figure 16A,C). The defects of Δ*rga-1* are similar to the defects caused by miss-regulation of Rho4 (Rasmussen & Glass, 2005, Rasmussen & Glass, 2007). Rho4 was first identified in *S. pombe* and described to be involved in septation (Nakano *et al.*, 2003; Santos *et al.*, 2003). Further work in *N. crassa* has been shown that RHO-4 accumulates prior to actin ring formation at sites of septation and is essential for septum formation. Three dominant active *rho-4* versions have been previously described and all of them displayed defects in septation, but varied in their manifestation (Rasmussen & Glass, 2005, Rasmussen & Glass, 2007). Septation pattern and frequency revealed similarities of the *DA rho-4*(D126) and the deletion of *rga-1*. Due to the similar phenotypes of *rga-1* and *DA rho-4*(D126A) a connection of RGA-1 and RHO-4 was assumed (Figure 17A,B). It would be interesting to compare the phenotypical defects to *DA rho-1*, but the dominant active *rho-1*(G15V) allele expressed in wild type died within 2d with swollen and lysed hyphae (Vogt & Seiler, 2008). Early studies in *S. pombe* described the expression of constitutively active Rho1 mutants resulting in cell inviability and the formation of abnormal multiseptated cells (Nakano K, 1997). In contrast hyphae of the dominant *rho-A(E40I)* allele in *A. nidulans* were clearly compartmentalized, but did not have a regular branching pattern (Guest *et al*, 2004). However, no further data are available and further experiments with *DA rho1* alleles should be performed to address this question.

The RGA-1 RHO-4 interaction was strengthened by additional *in vitro* pull down experiments (Figure 15A). A stable interaction between RGA-1 and RHO-4 was identified. A possible reason for the stable interaction between RGA-1 and RHO-4 could be the turnover rate or the sufficient protein amount. However, rather unexpected was the RGA-1 RHO-2 interaction. Previous studies in *N. crassa* revealed

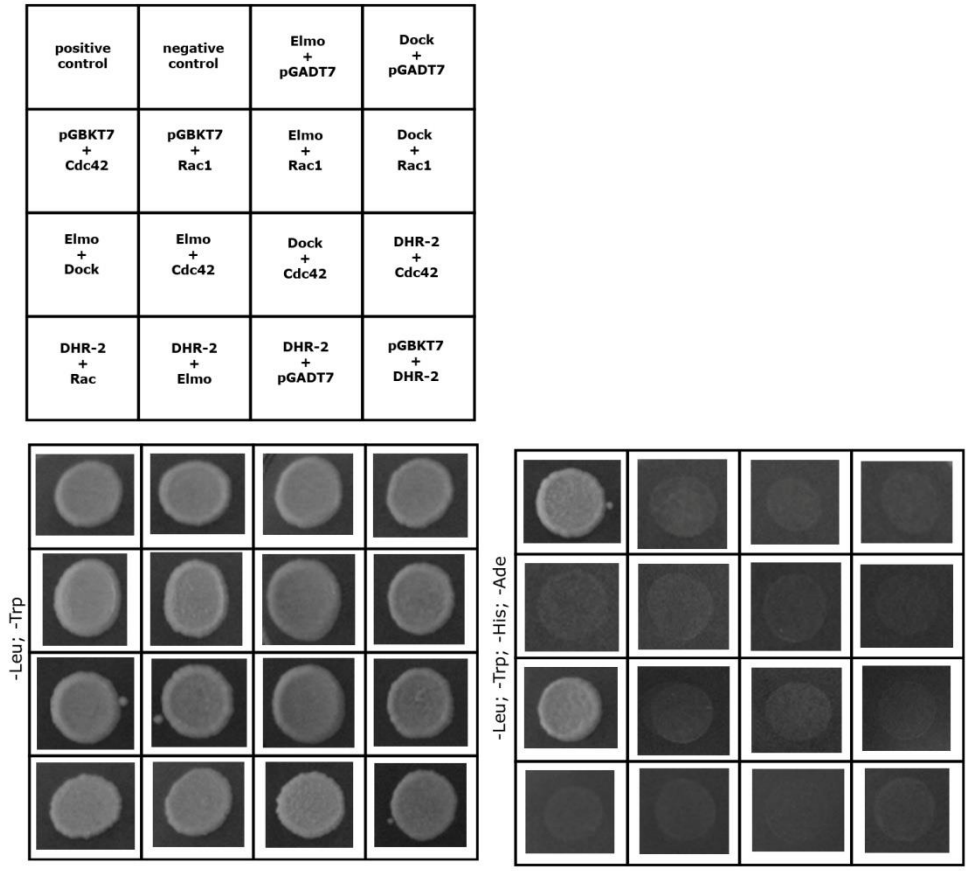
low expression of RHO-2 and thus it was speculated that RHO-2 is mainly regulated by protein turnover than specific activation by an exchange factor (Richthammer *et al*, 2012). These assumptions were supported by the current *in vivo* GAP assays and the previous reported *in vitro* GAP/GEF assays (Figure 12, Vogt & Seiler, 2008). Furthermore, Rho1p and Rho2p have partly overlapping functions during establishment of polar growth in budding yeast (Park & Bi, 2007). However, in *N. crassa* is known that RHO-2 has 64% amino acid identity with RHO-1 and seems to function in a partly redundant manner in regulation of cell wall dependent processes (Richthammer *et al*, 2012). Although the pull down experiments confirmed no RGA-1 RHO-1 interaction a possible explanation could be a transient interaction and the complex dissociated during the assay. In this context, it will be interesting to investigate whether RGA-1 and RHO-1 or RHO-2 physically interact by co-immunoprecipitation experiments.

Ectopically expressed RGA-1-GFP associated to the forming septum and the localization was comparable to GFP-RHO-1 (Figure 16B). Both Rho GTPases localized to the septum, but not identical. All three proteins were additionally found to localize at the plasma membrane (Figure 16C). RHO4 is assiciated to future septation sites, formed constricting rings upon septation and persisted around the septal pore after septum completion, whereas RHO-1 accumulated at the complete septum. Homologues of Rho1 localized apically and to the forming septum in *N. crassa* and *A. fumigatus* (Dichtl *et al*., 2010, Richthammer *et al*, 2012) this provides an indication of their role in polar growth and cell wall regulation. Maybe this fact could be the link between the three components.

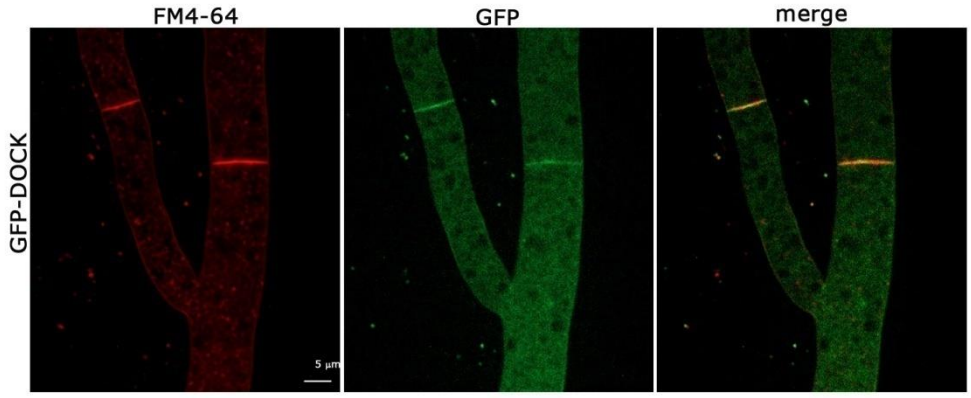
The phenotypical characterization of *rga-1* and *gpl-5* identified irregular septation in both deletion mutants (Table 5). The *rga-1* deletion leads to clusters and curved septa (Figure 17A), whereas *gpl-5* revealed an increased subapical septation pattern (Figure 18B). In addition, a double deletion strain of *rga-1* and *gpl-5* was generated to determine a putative functional relationship of these genes in order to septation. The Δ*rga-1;* Δ*gpl-5* deletion strain showed hyphal morphology of the *gpl-5* deletion strain but in relation to septation both septation defects were identified (curved septa, increased subapical septa; data not shown). In conclusion, both proteins are involved in septation, but potentially act in parallel pathways affecting septation.

In conclusion, these results suggest RGA-1 may act as dual specific GAP of RHO-1 and RHO-4 and is involved in regulation of septation in *N. crassa*.

7 Supplemental material



Supplemental Figure 1: Interaction studies of ELMO, DOCK and the DOCK DHR-2 domain with full length RAC andCDC-42. Yeast two hybrid analysis between ELMO and DOCK showed an interaction, no other interaction was identified.



Supplemental Figure 2: Localization studies of GFP-DOCK.
GFP-DOCK localized to the septa. The plasma membrane was stained with FM4-64. Scale bars as indicated.

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Studium:	2003 bis 2011	Biologie Studium (Diplom) an der Georg-August-Universität Göttingen
Hochschulabschluss:	Diplom-Biologin	
	Titel der Diplomarbeit: "Construction of a *Afu vosA* deletion mutant in *Aspergillus fumigatus* and characterization of the *Afu vosA* gene“	
Promotion:	2011 bis 2015	Promotion an der Georg-August Universität Göttingen im Rahmen des Promotionsprogramms "Molecular Biology of Cells" der Göttinger Graduate School for Neurosciences and Molecular Biosciences (GGNB)
	Titel der Doktorarbeit:	"Characterization of Rho GTPase GAP/GEF modules in the ascomycete *Neurospora crassa*”

Göttingen, den 31. März 2015