Regulation of localization and function of the Rho family small GTPase, Rnd3

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ABSTRACT

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Regulation of localization and function of the Rho family small GTPase, Rnd3

(under the direction of Adrienne D. Cox)

The Rnd proteins (Rnd1, Rnd2, Rnd3/RhoE) form a distinct branch of the Rho family of small GTPases. Rnd3 decreases RhoA activity and regulates both cytoskeletal organization (to cause cell rounding, hence the name Rnd) and cell cycle progression. How Rnd3 itself, is regulated to cause these changes is still under investigation. One possible mechanism is spatiotemporal regulation of this constitutively activated GTPase by dynamically modulated post-translational modifications. In this work, I have shown that, upon protein kinase C (PKC) agonist stimulation, Rnd3 undergoes an electrophoretic mobility shift and becomes metabolically labeled with ^{32}P, and its subcellular localization becomes enriched at internal membranes. These changes are blocked by inhibition of conventional PKC isoforms and do not occur in PKCα-null cells or with a nonphosphorylatable mutant of Rnd3, indicating that Rnd3 is a target for PKCα-mediated phosphorylation. I have provided evidence that integrin engagement regulates the downstream signaling functions of Rnd3 by inducing PKCα-mediated phosphorylation. These processes result in increased downstream signaling to Rho-ROCK pathway targets such as cofilin and myosin light chain. Thus, integrin engagement is a physiological regulator of Rnd3 posttranslational modification by PKCα, and in turn is a mediator of Rnd3

subcellular localization and downstream signaling. Further, I have demonstrated that both localization and function of Rnd3 require post-prenyl processing. Rnd3 localization to the plasma membrane is inhibited both in cells devoid of the post-prenyl processing enzymes Ras converting enzyme1 (Rce1) and isoprenylcysteine carboxymethyltransferase (Icmt), and also upon mutation of the Rnd3 C-terminal tetrapeptide CAAX motif to one insensitive to Rce1/Icmt. The function of Rnd3 is also inhibited in the absence of post-prenyl processing. Ectopic expression of Rnd3 causes a smaller loss of stress fibers and less cell rounding in *Rce1-/-* and in *Icmt-/-* fibroblasts than in matched WT cells, and cells expressing the misprocessed Rnd3 CAAX mutant display more stress fibers than cells expressing WT Rnd3. Together, these results add an additional mechanism of Rnd3 regulation to those documented previously, and clarify how Rnd3 modulates Rho signaling to alter cytoskeletal organization.

DEDICATION

The work found in this dissertation is dedicated to the loving memories of my grandmother, Eugenia Madigan, and my friend, CPT Matthew J. August.

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In the immortal words of Frank Zappa: "you should be diggin' it while it's happening, 'cause it just might be a one-shot deal"…

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
BTB	Broad complex, Tramtrack and Bric-a-brac
Ca^{2+}	calcium ion
CAAX	CAAX motif: cysteine, aliphatic, aliphatic, X = any amino acid
cDNA	complementary DNA
CIP	calf intestinal phosphatase
C-terminal	carboxy-terminal
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle media
DN	dominant negative
ECM	extracellular matrix
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
F	farnesyl isoprenoid lipid
F-actin	filamentous actin
FLAG	FLAG epitope tag
FN	fibronectin
FPP	farnesylpyrophosphate
FTase	farnesyltransferase
FTI	farnesyltransferase inhibitor
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor

GDP	guanine nucleotide diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GG	geranylgeranyl isoprenoid lipid
GGTase I	geranylgeranyltransferase I
GGTI	geranylgeranyltransferase I inhibitor
GST	glutathione S-transferase
GTP	guanine nucleotide triphosphate
GTPase	guanosine nucleotide triphosphatase
HA	hemagglutinin
HEK	human embryonic kidney cells
HRP	horseradish peroxidase
Icmt	isoprenylcysteine-O-carboxyl methyltransferase
IRAK	interleukin-1 receptor-associated kinase
IRES	internal ribosome entry site
KD	kinase-deficient
LIMK	LIM kinase
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
MARCKS	myristoylated alanine rich C-kinase substrate
MDCK	Madin-Darby canine kidney cells
MDMA	3,4-methylenedioxymethamphetamine
MEF	mouse embryonic fibroblast

MgcRacGAP	male germ cell-RacGAP
MLC	myosin light chain
MLCP	myosin light chain phosphatase
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NF-kappaB	nuclear factor kappaB
NLS	nuclear localization signal
N-terminal	amino-terminal
^{32}P	phosphorus-32 isotope
PCR	polymerase chain reaction
PDE 6D	phosphodiesterase 6D
PDGF	platelet-derived growth factor
PH	pleckstrin homology
PKA	protein kinase A
PKC	protein kinase C
PKCα	protein kinase C, alpha isoform
PLD	phospholipase D
PM	plasma membrane
PMA	phorbol myristate acetate
PMSF	phenyl-methyl sulfonyl fluoride
Ral-GDS	Ral guanine nucleotide dissociation stimulator
RBD	Rho binding domain
Rce1	Ras converting enzyme 1

RIPA	radioimmunoprecipitation assay
RNA	ribodeoxynucleic acid
RNAi	RNA interference
ROCK	Rho kinase
S6K	ribosomal protein S6 kinase
SAAX	SAAX motif; CAAX motif with a cys to ser mutation
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
SRF	serum response factor
TAP	tandem affinity purification
TCA	trichloroacetic acid
TEV	tobacco etch virus
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRE	TPA response element
Tsc1/2	tuberous sclerosis proteins 1 and 2
WT	wild type

CHAPTER 1

INTRODUCTION

Introduction

The Ras and Rho branches of the Ras superfamily of small GTPases comprise important signaling molecules that become activated in response to a wide array of extracellular stimuli (1). These proteins are characterized as binary molecular switches by their ability to bind and hydrolyze GTP to GDP (2). When bound to GDP, these proteins are in an inactive state. When bound to GTP, they are active and are thus able to bind effector molecules and activate downstream signaling pathways. These downstream signaling pathways control gene expression and also regulate important cellular processes such as proliferation, differentiation, motility and survival (1). The importance of Ras can be seen in part by the large number of cancers that harbor activating mutations in Ras superfamily GTPases, particularly in the Ras proteins themselves (3).

The Rho family proteins constitute an important family of the larger Ras superfamily. Like the Ras proteins, Rho proteins are largely regulated by GDP/GTP cycling (4). Rho proteins have been shown to regulate numerous cellular functions such as cytoskeleton regulation, cell adhesion, cell polarity, endocytosis, vesicular trafficking, cell cycle progression, differentiation and gene transcription, and have also been shown to have a role in oncogenesis (5-8).

Much of the current information available on the Rho family of small GTPases has been gained through the three best characterized members: RhoA, Rac1 and Cdc42 (9, 10). Besides these well-studied, "classical" Rho GTPases, there exist several members of this family that are considered "atypical" (11). Members of the Rnd subfamily (Rnd1, Rnd2 and Rnd3/RhoE) are examples of atypical Rho proteins (12). The main function attributed to the Rnd proteins to date is to counteract the functions of RhoA (13, 14), a key regulator of stress fiber formation. The Rnd proteins can downregulate RhoA and cause cell rounding, hence the name, Rnd. Rnd proteins lack intrinsic GTPase activity (15) and are exclusively GTP-bound *in vivo* and are thus found constitutively in an "active" state (16). Therefore, additional mechanisms of regulation must exist for these proteins. Regulation at the transcriptional level and/or by post-translational mechanisms have been suggested (17). Characterizing additional modes of regulation for the GTPase-deficient, atypical Rho family GTPase protein, Rnd3, has been the major thrust of my dissertation research and will be discussed further below and in subsequent chapters.

The Ras superfamily of small GTPases

The Ras superfamily of small GTPases comprises over 150 members, divided into five major branches based on both sequence and functional similarity (1, 18, 19): Ras, Rho, Rab, Ran and Arf (Figure 1.1). Ras GTPases are the founding members of this superfamily. The small GTPase Ras is a central hub in numerous intracellular signaling pathways. Ras proteins receive signals from diverse upstream activators such as receptor tyrosine kinases, G-protein coupled receptors and integrins (20, 21). These signals are transmitted through Ras to numerous downstream effectors and their pathways (22, 23). The most characterized

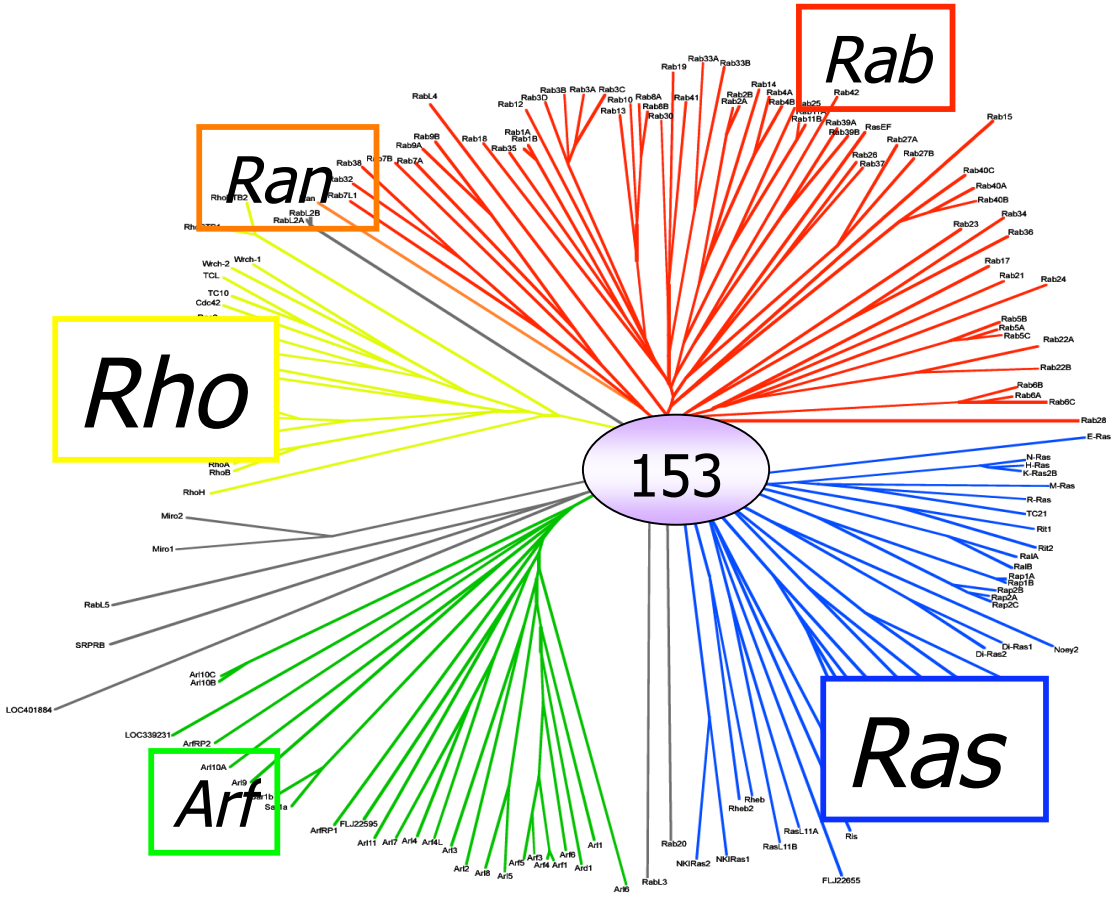


Figure 1.1: The Ras superfamily of small GTPases. This superfamily can be divided into five major branches based upon sequence and functional similarity. Adapted from Wennerberg, Rossman and Der, 2005, *J Cell Sci*, 118: 843-846.

effectors leading directly from Ras are the serine/threonine kinase Raf (24), phosphatidylinositol 3-kinase (PI3-K) (25) and the Ral-guanine nucleotide exchange factor (Ral-GEF) (26, 27). Signaling through Ras affects gene transcription to alter such cellular processes as survival, proliferation, differentiation, adhesion and motility.

Ras proteins were initially identified as viral oncogenes (28, 29). Subsequent studies discovered mutated versions of Ras proteins in human cancers (30-32). The Ras proteins are considered oncoproteins due to the large number of human cancers that contain mutated and activated Ras proteins (3). Three genes encode the four major human Ras proteins (H-Ras, N-Ras, K-Ras4A and K-Ras4B). Oncogenic mutations result in Ras proteins that are constitutively GTP-bound and are therefore chronically active. Typically, these mutations occur at the hot spots of codons 12, 13 and 61 (3). *Ras* is the most frequently mutated oncogene in human tumors, and mutational activation of Ras proteins occur in roughly 30% of human tumors, with the prevalence as high as 90% in pancreatic cancer and 50% in colon cancer (33-36). Furthermore, even in cancers that lack Ras mutations, increased Ras signaling can result from either *ras* gene amplification or activating mutations in growth factor receptors (21). With such a high prevalence of Ras mutations found in human cancer, intense efforts have been focused on inhibiting Ras-dependent signaling pathways as possible therapies in cancer treatments (37).

Other members of the Ras family include R-Ras, Rap1, Ral and Rheb proteins and their relatives. The full role of R-Ras (and the closely related isoforms R-Ras2/TC21 and M-Ras/R-Ras3) in cells is still under investigation. Currently, R-Ras has been shown to activate integrins (38) and increase Rho-ROCK activity (39) leading to cell migration. The Rap proteins (Rap1 and Rap2) are involved in several aspects of cell adhesion, including integrin-

mediated cell adhesion and cadherin-mediated cell junction formation (40). The Ral proteins (Ral A and B) constitute one of the three main effector arms of the Ras signal transduction pathway (41). They are involved in various cellular pathways such as vesicle sorting and gene expression and also have effects on the actin cytoskeleton (42). Recent work has shown that activation of RalA is critical for Ras-induced tumorigenesis (43). The Rheb (Ras homolog encriched in brain) (44) protein is largely known for its involvement in the insulin/mTOR/S6K signaling pathway. This pathway is involved in protein synthesis and has effects on both the cell cycle and cell size (45). Mutations in the GAP complex for Rheb (Tsc1/Tsc2) manifest in a genetic condition termed tuberous sclerosis leading to benign tumors and neurological disorders (46).

The Rab (Ras-like proteins in brain) family proteins are best known for their roles in regulating vesicular transport (47, 48). Studies have shown that these proteins are involved in the four major steps of vesicular membrane traffic: vesicle budding, vesicle delivery, vesicle tethering and fusion of the vesicle membrane with the membrane of the target compartment (49).

The Ran (Ras-like nuclear) protein is the only member of this Ras subfamily (50). Ran has been shown to be important in nucleocytoplasmic transport of RNA and proteins through its interaction with importin protein- and exportin protein-complexed cargo (51). Ran is also known to be important in mitotic control through mitotic spindle assembly and dynamics (52).

The Arf (ADP-ribosylation factor) subfamily, like the Rab subfamily, is involved in regulating vesicular transport. This subfamily is made up of six family members, with Arf1 and Arf6 being the best characterized (53, 54). Arf1, by recruiting coat proteins, regulates the

formation of vesicle coats for vesicular membrane formation in cargo sorting and release (55). Arf6 is known to be also involved in endocytic membrane trafficking (56) and actin remodeling (54).

The fifth Ras subfamily contains the Rho (Ras homologous) proteins (4). Much of the work I have performed has been centered around the Rho proteins, particularly a novel member of this family termed Rnd3. These proteins and their multiple modes of regulation will be described in detail below.

The Rho family of small GTPases

As indicated above, the Rho family of proteins comprises one of the 5 major branches of the Ras superfamily of small GTPases. This family includes 25 distinct proteins encoded by 22 genes (4). Rho proteins share approximately 30% amino acid identity with other Ras superfamily members and 40-95% identity within the family (4). Rho proteins have an additional "Rho insert" domain of 12-14 amino acids, located at residues ~123-137, between the fifth β strand and the fourth α helix in the GTPase domain (57). Based on criteria including sequence identity and biological function, Rho family proteins can be further divided into five main subfamilies: RhoA-related (RhoA, RhoB and RhoC), Rac1-related (Rac1, Rac1b, Rac2, Rac3 and RhoG), Cdc42-related (Cdc42, bCdc42, TC10, TCL, Wrch-1 and Chp/Wrch-2), RhoBTB (RhoBTB1 and RhoBTB2) and Rnd (Rnd1, Rnd2 and Rnd3/RhoE) (Figure 1.2). Several other Rho proteins, such as RhoD, Rif and RhoH/TTF, do not fall into any of the five Rho subfamilies. Additionally, another set of related proteins named the Miros (mitochondrial Rho) have been characterized (58). Although these proteins

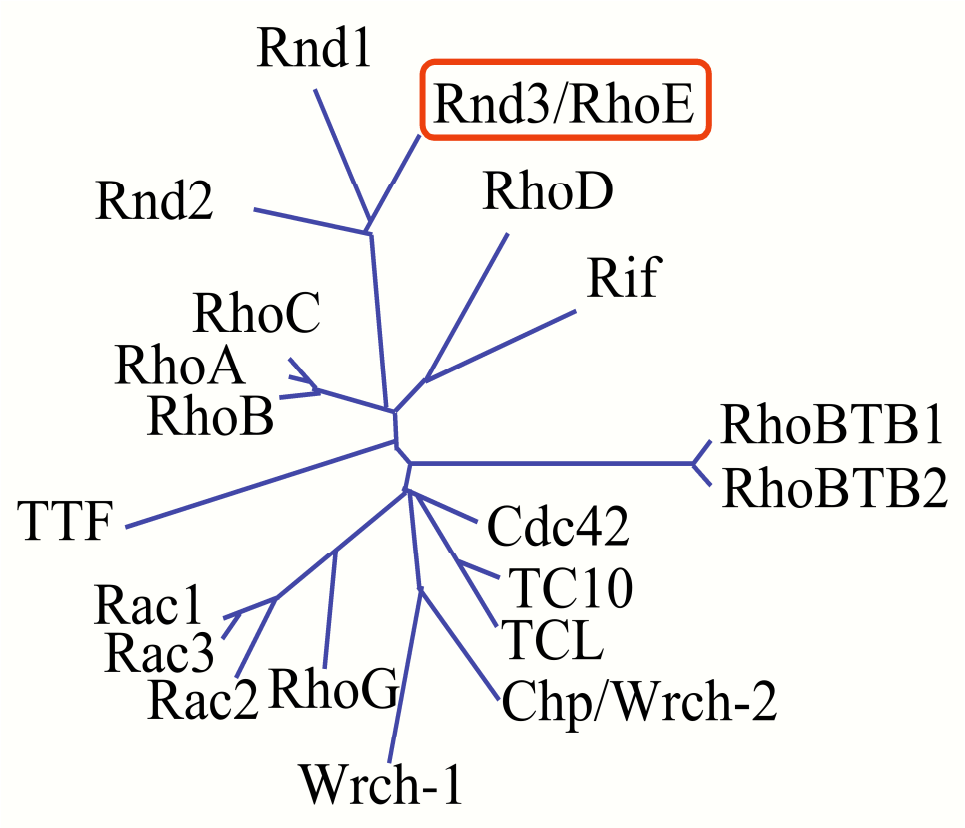


Figure 1.2: The Rho family of small GTPases. Rnd3/RhoE is a member of the Rnd subfamily, which also includes Rnd1 and Rnd2. Adapted from Wherlock and Mellor, 2002, *J Cell Sci*, 115: 239-240.

contain two GTPase domains, they show a high level of divergence and are thus not considered true members of the Rho family.

The canonical or classical Rho GTPases: RhoA, Rac1 and Cdc42, are the founding members of their respective subfamilies, and these proteins are very well characterized with respect to structure, regulation, interacting partners, subcellular localization and biological activity (4, 5, 9, 10, 59, 60). In contrast, although the "atypical" GTPases display well-defined GTP-binding domains, they possess unique N- and C-terminal extensions and often lack the ability to cycle GDP/GTP binding (11). Examples of atypical GTPases include RhoH/TTF (61, 62), RhoBTBs (63), Wrch-1 (64-66), Chp (67-69) and the Rnd proteins (12). Because these proteins do not GDP/GTP cycle and are constitutively GTP bound, additional mechanisms of regulation must exist for these proteins. Mechanisms of regulation at the transcriptional and/or post-translational levels have been explored; these will be discussed further for Rnd proteins below.

Much of our information on the Rho family of small GTPases has been gained through studies of the canonical, founding members of this family, RhoA, Rac1 and Cdc42 (4, 9, 10). Activation of RhoA leads to formation of stress fibers and focal adhesions (70), while activation of Rac1 and Cdc42 lead to the formation of lamellipodia and filopodia, respectively (71, 72) (Figure 1.3). These Rho proteins are well recognized for their role in each of the four major steps of cell migration: lamellipodium extension, formation of new adhesions, cell body contraction and tail detachment (73). Cell migration involves coordinated polarization and reorganization of the actin cytoskeleton. In a polarized migrating cell, Rho GTPases are regulated in a spatiotemporally controlled manner. Rac1 and Cdc42 activities are found primarily in the front of the cell to regulate actin

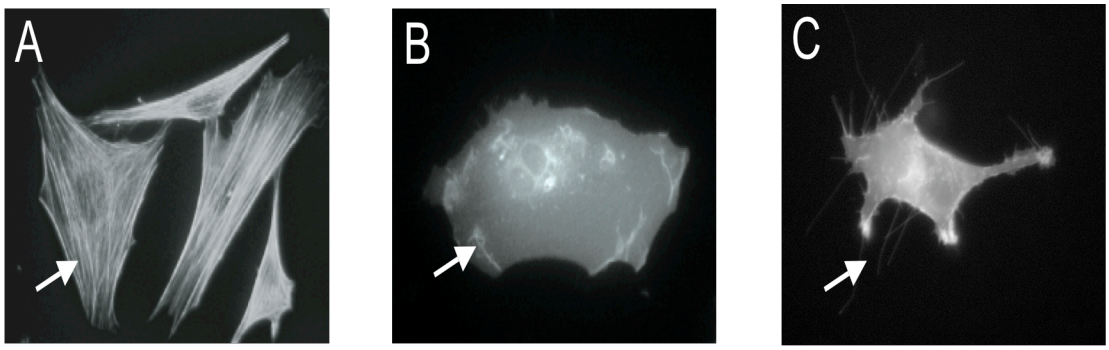


Figure 1.3: The effects of Rho protein expression on the actin cytoskeleton. A, Rho expression forms stress fibers. **B,** Rac1 expression forms lamellapodia. **C,** Cdc42 expression forms filopodia. Arrows indicate structures. Pictures courtesy of Patricia J. Keller.

polymerization to promote protrusion and to control direction in response to extracellular signals, respectively. RhoA was initially thought to be active primarily in the cell body to stimulate actin-myosin contractility to create cell tension (5). However, the use of biosensor probes to detect the small pools of GTP-bound and active Rho proteins has also identified active RhoA at the leading edge of randomly migrating but not growth-factor stimulated migratory cells (74), where it is tightly regulated, in some cases by at least one cancer-associated RhoGAP, DLC-1 (75).

Although no oncogenic mutations in Rho family proteins have been found in human cancers, overexpression of several Rho proteins, as well as dysregulation of their upstream activators (GEFs) and downstream negative regulators (GAPs, GDIs) leading to Rho hyperactivation has been documented. Rho proteins and their regulators, described in more detail below, have been demonstrated to play roles in cancer initiation, progression, invasion and metastasis (8, 76-80).

Regulation of Rho GDP/GTP cycling: GEFs, GAPs and GDIs

Like the Ras proteins, Rho family GTPases act as molecular switches, cycling between an inactive, GDP-bound state and an active, GTP-bound state. Two regions, switch I and switch II, undergo a conformational change when the protein becomes GTP-bound (2), thereby promoting interaction with downstream effectors (59). The intrinsic GDP/GTP exchange and GTP hydrolysis of most small GTPases is slow. Therefore, two major classes of proteins regulate the cycling of Rho GTPases: GEFs and GAPs (Figure 1.4).

GEFs (guanine nucleotide exchange factors) accelerate the intrinsic GDP-GTP exchange activity to favor formation of the active GTP-bound protein (81, 82). The majority

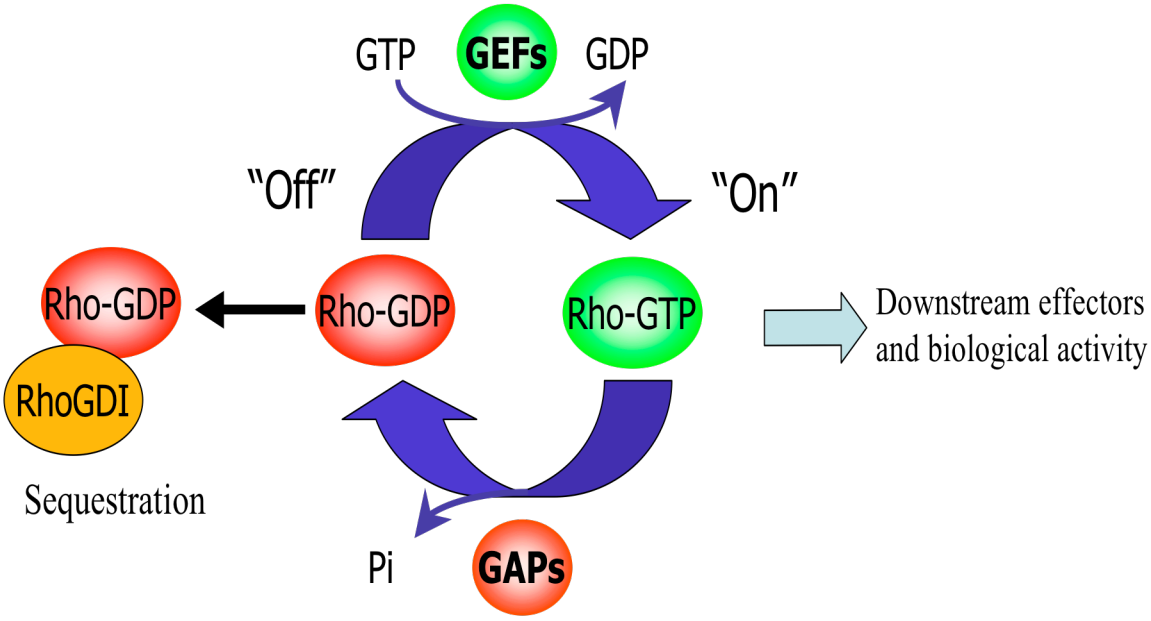


Figure 1.4: Rho proteins function as molecular switches. Rho-GDP is inactive, whereas Rho-GTP is active and can interact with downstream effectors to promote biological activity. GEFs activate Rho proteins by promoting the exchange of GDP for GTP. GAPs inactivate Rho proteins by accelerating their intrinsically slow GTPase activity, promoting the hydrolysis of GTP to GDP. RhoGDI sequesters Rho-GDP in the cytosol, preventing it from being activated.

of RhoGEFs belong to the Dbl family (83-85), which currently is known to comprise sixty-nine members, characterized by the presence of tandem DH (Dbl homology) and PH (pleckstrin homology) domains important for their exchange activity. Another, smaller family of RhoGEFs consists of the DOCK family proteins (86).

GAPs (GTPase-activating proteins) inactivate GTPases by enhancing their intrinsic GTPase activity, leading to increased GTP hydrolysis and formation of the inactive GDP-bound form (87, 88). GAPs, through the use of a commonly shared "arginine finger", stabilize the nucleotide state, from GTP-bound to nucleotide-free, and facilitate reloading with GDP (2). Approximately seventy Rho-specific GAPs have been identified to date (4). The large number of Rho-specific GEFs and GAPs is testimony to the multitude of signals that activate Rho proteins and to the need to tightly regulate their activity and their many downstream effects.

A third type of protein involved in regulating Rho but not Ras GTPases are the GDIs (guanine nucleotide dissociation inhibitors), of which there are three known human isoforms: RhoGDI-1, RhoGDI-2 (or D4/Ly-GDI) and RhoGDI-3, or α, β and γ. GDIs bind to GDP-bound Rho proteins and sequester them in the cytoplasm where they are unable to interact with GEFs, thus inhibiting GDP/GTP exchange (89-91). GDIs have also been shown to mask the C-terminal lipid modifications of Rho proteins and to shuttle them to the cytoplasm where they are unable to signal to downstream effectors (92, 93). Conversely, palmitoylation of the C-termini of TC10 and RhoB was shown to inhibit GDI binding (94). These lipid modifications are discussed in the following section.

Prenylation of small GTPases

Correct subcellular localization is required for proper biologial activity, and subcellular localization in turn of small GTPases is controlled by a combination of sequence information and post-translational modifications. Nearly all members of the Rho and Ras families terminate in a C-terminal CAAX motif (where C=cysteine, A=aliphatic residue and X=any amino acid) (95), a crucial signal for prenylation, a permanent post-translational modification required for correct GTPase subcellular localization and for biological activity. Proper localization relies in part on an ordered cascade of enzymatic reactions signaled by the CAAX motif, of which prenylation is the first and obligate step (96) (Figure 1.5). The first reaction is stimulated by either of two cytosolic prenyltransferase enzymes: farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I) (97, 98). The "X" in the CAAX motif determines the prenylation specificity of that protein (99-103). The classical Rho proteins terminate in X = L and are therefore substrates for GGTase I, which adds a C20 geranylgeranyl isoprenoid group to the cysteine of the CAAX motif, while Ras proteins (X = S or M) are substrates for FTase, which adds a C15 farnesyl isoprenoid group (95, 104, 105). However, many of the nonclassical Rho GTPases are also farnesylated (106), including the Rnd3 protein that is the subject of this dissertation (16).

It has been appreciated for some time that proper membrane targeting and transforming ability of the oncogenic Ras proteins requires processing through the prenylation pathway. Due to this observation, protein prenyltransferases, most notably FTase, have been targets of anti-cancer drug discovery efforts (107-109). To this end, FTase inhibitors (FTIs) have been developed and were shown to have promising preclinical anti-tumor results. Despite initial promising results, the anti-tumor efficacy of FTIs in the clinic

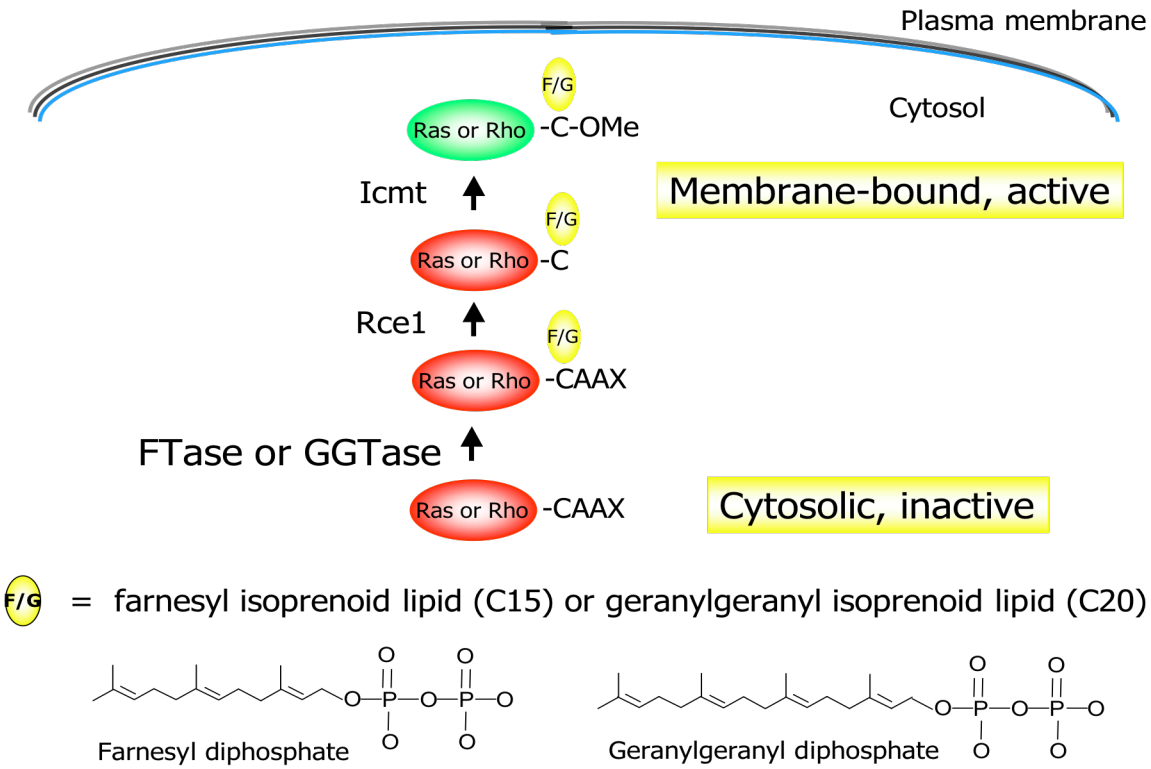


Figure 1.5: Proper membrane localization of Ras and Rho proteins involves a three step enzymatic cascade. Farnesyl transferase attaches a 15 carbon farnesyl isoprenoid group to the C-terminal cysteine of Ras proteins. Geranylgeranyl transferase attaches a 20 carbon geranylgeranyl isoprenoid group to Rho proteins. For both Ras and Rho proteins, Rce1 cleaves off the –AAX sequence and Icmt methylates the exposed prenylated cysteine residue.

has been very disappointing. One possible explanation may stem from the fact that K-Ras4B and N-Ras, the Ras isoforms most frequently mutated in cancers, undergoe a process termed alternative prenylation (110-112). Under normal circumstances, K-Ras4B and N-Ras undergoe only farnesylation, but when farnesylation is blocked, as with FTIs, K-Ras4B and N-Ras can also undergo geranylgeranylation. Alternative prenylation is likely a major contributor to blunting the therapeutic potential of FTIs in treatment of cancers that are driven by oncogenic Ras. Furthermore, it is thought that many of the observed cellular effects of FTIs may be due to inhibition of other CAAX motif-containing proteins, including farnesylated Rho GTPases (113). Additionally, with increasing evidence of misregulation of Rho proteins in cancer and other diseases (8, 76), inhibitors of GGTase I, termed GGTIs, are being tested and validated in preclinical studies (114, 115).

Post-prenyl processing of small GTPases

Prenylation by either a C15 farnesyl or C20 geranylgeranyl isoprenoid group is not sufficient for complete CAAX-signaled processing; two further processing steps, termed post-prenyl processing, are needed (116, 117). The first post-prenyl step involves proteolytic cleavage of the –AAX residues by an endoplasmic reticulum (ER)-localized protease termed Ras converting enzyme 1 (Rce1) (118, 119). The second step involves carboxymethylation of the newly prenylated terminal cysteine residue, catalyzed by another ER-localized enzyme termed Isoprenylcysteine carboxyl methyltransferase (Icmt) (118, 120). The end result of this enzymatic cascade involving both prenylation and post-prenyl processing is thought to make the carboxy-terminal domain of CAAX motif-containing proteins more hydrophilic, to better facilitate proper interactions with lipid-rich cell membranes (121).

Rce1 is the only enzyme found in vertebrates that facilitates –AAX proteolysis, and it acts on both farnesylated and geranylgeranylated proteins (122). Rce1 was first identified by its role in processing yeast Ras proteins (123). Rce1-mediated proteolysis of CAAX motif-containing proteins is essential in mouse development. Genetic disruption of Rce1 in mice caused an embryonic lethal phenotype at approximately embryonic day (E) 15.5 (119). The exact cause for this lethality is still unclear, but targeted deletion of the Rce1 gene in the mouse heart caused severe cardiac defects and led to death by age 10 months (124). Cells devoid of Rce1 were shown to contain mislocalized Ras proteins (119, 125, 126). Furthermore, it was shown that loss of Rce1-mediated –AAX proteolysis sensitized cells to FTI treatment (127).

Icmt was first identified in yeast due to its requirement in the processing of **a**-factor mating pheromone (128, 129). Similar to Rce1, Icmt is the only enzyme found in vertebrates to catalyze the C-terminal prenylcysteine methylation reaction (130, 131). Like Rce1, genetic ablation of Icmt causes embryonic lethality at E11.5. The basis for this greater lethality when compared to Rce1 loss is unknown, albeit a defect in liver development was detected (132, 133). One proposed explanation is loss of methylation of a subset of Rab proteins, which are not Rce1 substrates, and which are important in membrane trafficking (131, 134). Furthermore, unlike Rce1 ablation, which caused only minor disruption of oncogenesis, conditional ablation of Icmt caused a 50% decrease in the transformation of fibroblasts by the oncogenes K-ras4B and B-raf (135). In Icmt -/- mouse embryonic stem cells, a large fraction of GFP-tagged K-Ras4B was trapped in the cytoplasm, and fluorescence at the plasma membrane was reduced (131).

Beyond prenylation and the two post-prenyl processing steps, a second signal found in the C-terminus immediately upstream of the CAAX motif is needed for proper membrane localization of CAAX-terminating proteins. This second signal can be conferred either by palmitoylation of one or two cysteine residues or by the presence of a polybasic region (94, 136-140). Additionally, the presence of a "third signal" has been suggested, as demonstrated by the loss of membrane binding upon mutation of sequences around sites of palmitoylation that do not affect the lipid modifications (141).

These additional membrane targeting signals are found in the hypervariable region of Ras superfamily proteins, which consist of approximately 20 amino acids upstream of the CAAX motif. As its name implies, this region represents the largest degree of sequence variance between highly related family members. For example, RhoA, RhoB and RhoC are highly homologous throughout most of their primary sequence, but differ greatly in their hypervariable regions (142). This sequence difference is thought to provide specificity for differential subcellular targeting of highly related GTPases. Another function of the hypervariable region, as is the case with Ras proteins, is to contribute to interactions with downstream effectors and regulatory proteins (143-145).

A recent study found that, whereas farnesylated Ras proteins require full post-prenyl processing for proper membrane localization, geranylgeranylated Rho proteins do not (126). This study employed activated versions of GTPases. Whether the WT proteins behave similarly would be interesting to determine. Furthermore, only the most characterized Rho proteins were studied. Whether the less-studied Rho proteins follow this trend would be interesting to determine as well. In Chapter 3 below, I have described my studies to

determine the role of post-prenyl processing in the proper subcellular localization and biological functions of the farnesylated Rho GTPase, Rnd3.

RhoA-related subfamily of Rho proteins: similar but different

The RhoA-related subfamily is comprised of three members (RhoA, RhoB and RhoC) (146, 147) that share roughly 85% identity at the amino acid level, with the most divergence at their C-terminal hypervariable regions (Figure 1.6). Despite the high level of sequence identity, the three RhoA-related proteins have distinct expression profiles, posttranslational modifications and subcellular localizations. These Rho proteins are expressed in all tissues tested, but their levels vary greatly depending on the tissue type (142). While RhoA and RhoC are expressed constitutively, RhoB is an immediate-early gene whose expression is induced by growth factor signaling (148) in a cell cycle-dependent manner (149) and by exposure to stressors including irradiation and chemical agents (150, 151).

RhoA, B and C all terminate in CAAX motifs and are thus post-translationally modified by isoprenylation (97). However, whereas RhoA and C are prenylated by a geranylgeranyl group, RhoB has been shown to be both geranylgeranylated and farnesylated (95). This is surprising, given that all of these CAAX motifs terminate in leucine; however, the RhoB C-terminal sequence of CCKVL uniquely dictates both modifications (152), and is an example of how sequences upstream of the terminal X residue can also influence prenylation specificity. In addition, the dual prenylation of RhoB confers distinct localization and functional properties on this otherwise highly related family member, as described below.

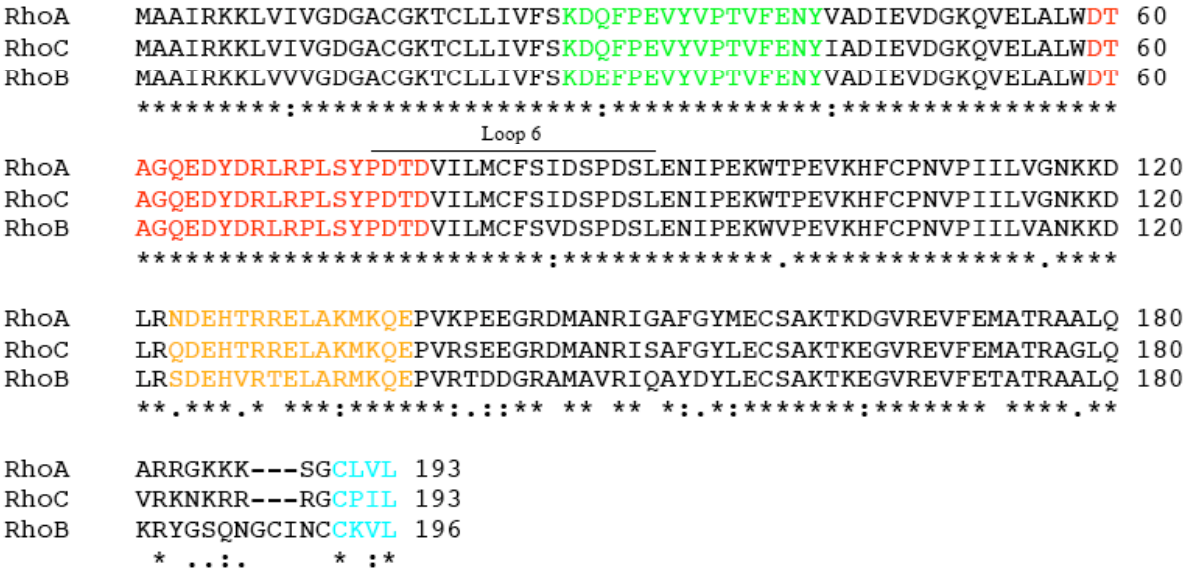


Figure 1.6: Sequence alignment of the RhoA family proteins. Highlighted are Switch I (green), Switch II (red), the Rho insert domain (orange) and the CAAX prenylation motif (cyan). Loop 6 is noted by a line above the corresponding sequence. "*****" indicates that the residues in that column are identical in all sequences in the alignment. "**:**" indicates that conserved substitutions have been observed. "**.**" indicates that semi-conserved substitutions are observed. Accession numbers: NP_001655 (RhoA), NP_004031 (RhoB) and NP_786886 (RhoC).

RhoA and RhoC have been shown to localize to the plasma membrane and the cytoplasm (142, 152), whereas RhoB is localized primarily on endosomes (94, 152, 153). When RhoB is exclusively geranylgeranylated upon blocking of farnesylation, it is found mainly on the plasma membrane rather than in endosomes (154). This observation argues that not only is RhoB distinct from RhoA and RhoC, but also that the two differently prenylated forms of RhoB have different subcellular localizations and possibly different functions from each other.

Variations in subcellular localization may explain some of the differences between the three Rho proteins in terms of biological functions. As mentioned previously, subcellular localization affects both effector binding patterns and GEF and GAP availabilities (81, 83, 87, 88). While these highly related Rho proteins share many of the same GEFs and GAPs, some differences have been reported. For example, XPLN has been shown to be a GEF for both RhoA and RhoB, but not for RhoC (155). Rho effector proteins will be discussed in a later section.

While the *rhoA* gene locus has not yet been targeted, mice lacking either *rhoB* or *rhoC* were found to be both viable and fertile. Using *rhoB* -/- MEFs, it was found that RhoB is important for cell motility, but not for adhesion or spreading. Furthermore, it was shown that *rhoB* -/- mice are more susceptible to developing tumors when tested in a skin carcinogenesis assay (156). Through the use of *rhoC* -/- mice, it was determined that loss of RhoC does not affect tumor development, but decreases tumor cell motility and metastatic cell survival leading to a drastic inhibition of metastasis (157). A study where the cardiac-specific activity of all three Rho proteins were abrogated by RhoGDI expression, led to embryonic lethality due to improper cardiac morphogenesis (158). The availability of a

rhoA null mouse would surely help to uncover its role in development and further discriminate unique roles for these three highly related proteins.

While RhoA has been shown to be required for Ras transformation (159, 160), RhoB has a growth inhibitory function (161-163). Furthermore, RhoB expression has been shown to be downregulated by Ras through a PI3K/Akt-dependent pathway (164). Whereas RhoC overexpression does not increase transformation, its expression promotes metastasis (165). One study comparing the gene expression profiles of non-metastatic and highly metastatic melanoma cell lines showed that the gene encoding RhoC was expressed at a significantly higher level in the metastatic line versus non-metastatic line (166). In the same study, the authors showed that overexpressed RhoC enhanced metastasis whereas dominant negative RhoC inhibited it (166). Expression in the same metastatic A375M cells of a p190RhoGAP-RhoC chimera, to selectively target the GAP activity to downregulate RhoC, reduced their migratory and invasive phenotypes, whereas chimeras made with RhoA and RhoB did not (167). Furthermore, RhoC expression increases as cells become more metastatic (168). In contrast to RhoC, RhoB overexpression has been shown to inhibit migration and invasion (164). Thus, a simplistic summary suggests that the primary functions of these closely related isoforms are motility and migration for RhoA, growth suppressing activity for RhoB, and invasion and metastasis for RhoC. Additional evidence suggesting greater complexity will be discussed below.

RhoA: effectors, downstream signaling and biological consequences

Throughout evolution, nature has increased the number of Rho proteins as organisms become more complex. Although the three members of the RhoA/B/C subfamily are highly

similar (~85% identical), it is clear that they are not functionally redundant. Their sequence divergence is thought to give these highly related proteins the diversity needed to perform distinct cellular functions, either by differential signaling to common effectors or by affording access to specialized subcellular locations for interaction with distinct effector proteins.

RhoA effectors

The small GTPase RhoA has been shown to be important in a wide array of cellular processes such as actin cytoskeletal organization, gene expression, cell cycle progression and transformation (5, 9, 169). This wide range of functions is made possible by preferential interaction of the GTP-bound form of RhoA with a multitude of specific effectors (59). In contrast to Ras, in which the effector binding domain consists simply of a core region representing residues 32-40 (Switch I), plus flanking residues from ~25-45, the picture in Rho is more complicated. By the use of chimeric proteins between RhoA and H-Ras, and between RhoA and Rac1, Hall and colleagues showed that, unlike Ras, the "effector binding region" of RhoA must require residues well outside the core effector domain of Switch I (170, 171). Rho family proteins contain an "insert domain" (residues ~123-137) not found in other Ras superfamily GTPases, and this insert region is required for the activation of specific RhoA effectors even if not for their binding (172). Finally, residues in loop 6 of Rho proteins (~75-92) are also required for the specificity of RhoA effector interactions (173, 174).

There are well over a dozen proteins known to interact with RhoA in a GTP-dependent manner (59, 60, 142) and that are therefore candidates to be true effector targets

(Figure 1.7). These include the Rho kinases, ROCK I and ROCK II (elaborated further on page 25) (175-179), the protein kinase N (PKN)-related kinases (180-183), the myosin-binding subunit of myosin light chain (MLC) phosphatase (MLCP) (184), Citron kinase (185), Rhophilin (182), Rhophilin-2 (186), Rhotekin (187), Kinectin (188, 189), p116RIP (190), phospholipase D (PLD) (191) and the diaphanous-related formins mDia1 and mDia2 (189, 192).

Sequence divergence in and around the Switch I effector binding region (Figure 1.6) adds to the diversity and selectivity of effector proteins for specific Rho isoforms. Most RhoA effectors are known to also interact with RhoB and/or RhoC, although their interaction intensities vary. For example, in interaction studies, the Rho effectors ROCK and Citron kinase both display a higher affinity for RhoC over RhoA (193). Furthermore, *in vivo*, RhoC has a greater ability to activate ROCK as compared to RhoA (194). These studies grew out of intensive efforts to understand the ways in which Rho proteins regulate the cytoskeleton in normal function and in disease states, especially oncogenesis.

RhoA signaling and downstream consequences: cytoskeletal organization

Initial studies with Rho proteins had hinted at a possible role in cytoskeletal control. In these studies, it was noted that p21 Rho protein was ADP-ribosylated by C3 exoenzyme *in vitro* and that this *in vitro* ADP-ribosylated Rho protein corresponded to the dominant C3 substrate of eukaryotic cells. It was also noted that treatment of cells with C3 exoenzyme caused the loss of microfilaments and rounding of cells (195). In another study, ADP-ribosylated Rho protein was microinjected into fibroblast cells and rounding up of the injected cells was noted (196). The authors from both studies suggested that the biological

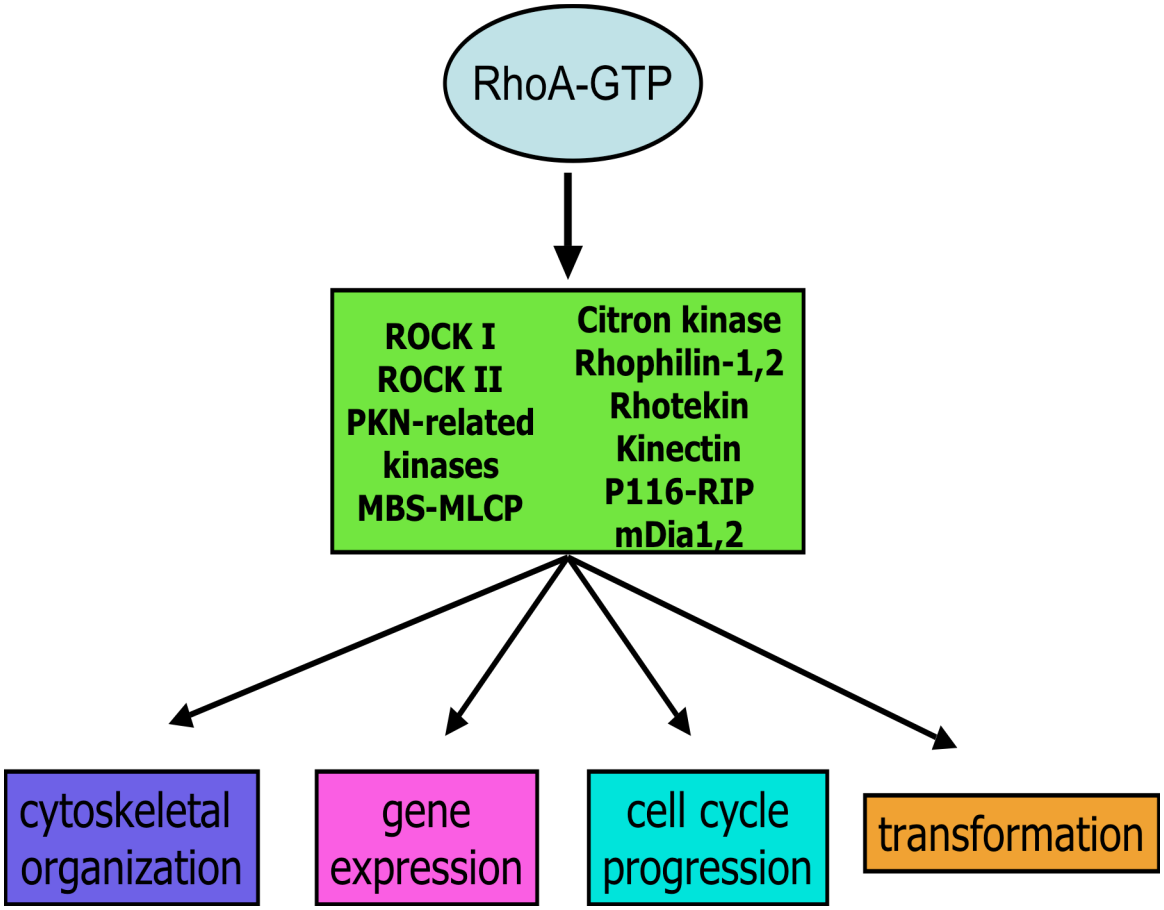


Figure 1.7: RhoA effectors and cellular processes. Well over a dozen proteins are known to interact with RhoA in a GTP-dependent manner, leading to a wide array of cellular processes such as actin cytoskeletal organization, gene expression, cell cycle progression and transformation.

role of Rho was to control some aspect of cytoskeletal organization. The connection between RhoA, extracellular signaling and cytoskeletal control was not directly made until the publication of a seminal paper from Anne Ridley and Alan Hall in 1992 (70). It was in this paper, the authors established that stress fibers and focal adhesions were regulated by RhoA. Stress fibers (197) are axial bundles of F-actin and myosin that traverse the cytoplasm and terminate at focal adhesions (198), which are cellular structures that link the extracellular matrix (ECM) on the outside of the cell, through integrin receptors, to the actin cytoskeleton inside the cell. It is thought that strong adhesions to the substratum mediated by integrin receptors and stimulation of contractility by soluble serum factors generate tension, resulting in alignment and bundling of actin filaments to form stress fibers, and resulting in clustering of integrins and associated proteins to form focal adhesions (198). Ridley and Hall found that lysophosphatidic acid (LPA) in serum induced the formation of these structures and that treatment with C3 exoezyme blocked their formation. They also established that RhoA lies in a signaling pathway downstream from growth factor receptors located on the cell surface (169).

At least two effectors, ROCK and mDia, appear to be required for Rho-induced assembly of stress fibers and subsequent focal adhesion formation (59) (Figure 1.8). The ROCK serine/threonine kinases, also called Rho kinases, were the first effectors of Rho to be discovered (199). Two ROCK isoforms have been identified (175, 176, 178, 200): ROCK I (also known as ROCK 1, ROKβ and p160ROCK) and ROCK II (also known as ROCK 2, ROKα and Rho kinase). Phosphorylation of three downstream targets have been shown to be important for the formation of stress fibers in cells. ROCK has been shown to phosphorylate the myosin-binding subunit of MLC phosphatase (184, 201, 202). MLC

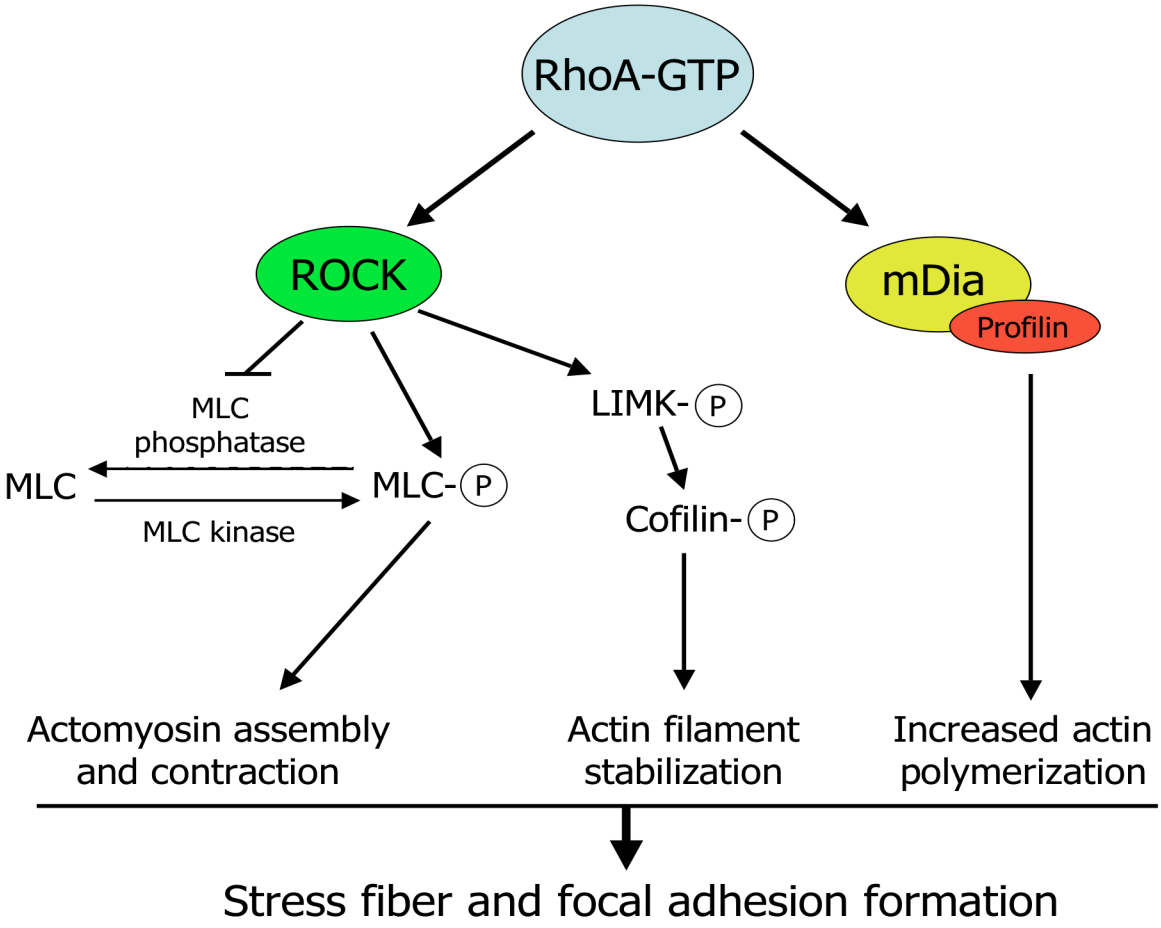


Figure 1.8: RhoA-GTP signal transduction pathways leading to stress fiber and focal adhesion formation. Active RhoA signals to the effector ROCK leading to phosphorylation of MLC phosphatase, which increases MLC phosphorylation. ROCK also phosphorylates MLC directly. The increased phosphorylation of MLC stimulates the ATPase activity of myosin II, which leads to actomyosin assembly and contractility. ROCK also phosphorylates and activates LIM kinases which, in turn, phosphorylate and inactivate the actin severing protein cofilin, leading to actin filament stabilization. Active RhoA also signals to the effector mDia who, through binding with the G-actin-binding protein Profilin, leads to increased actin polymerization. The final outcome of these signaling pathways results in stress fiber and focal adhesion formation. Adapted from Bishop and Hall, 2000, *Biochem. J.*, 348: 241-255.

phosphatase is inhibited by phosphorylation and this inhibition indirectly leads to an increase in phosphorylation of MLC itself (59). ROCK has also been shown to directly phosphorylate MLC (203, 204). Phosphorylation of MLC occurs at serine 19 and this phosphorylation stimulates the actin-activating ATPase activity of myosin II and promotes the assembly of actomyosin filaments (59). The third downstream target of ROCK involved in the formation of stress fibers is LIM kinase (LIMK1 and LIMK2). ROCK was shown to phosphorylate LIMK1 at threonine 508 (205) and LIMK2 at threonine 505 (206). Phosphorylation of both LIM kinases was shown to enhance their ability to phosphorylate cofilin (207). Cofilin is an actin severing protein, and phosphorylation on serine 3 by either LIM kinase inhibits its actin severing/depolymerization activity (208-210).

Expression of constitutively active ROCK does not induce correctly organized stress fibers (176, 179). Yet, inhibition of ROCK by Y-27632, a ROCK inhibitor (211), causes loss of serum- and activated Rho-induced stress fibers (212). These data suggest that ROCK is necessary, but not sufficient, for Rho-induced stress fiber assembly. A second RhoA effector, mDia, was shown to complement the activity of ROCK in stress fiber formation (213). mDia1 and 2 isoforms are mammalian homologs of the Drosophila diaphanous protein and are members of the formin-homology family of proteins involved in actin nucleation (214). Expression of activated forms of mDia promoted the assembly of thin stress fibers that appeared to be less bundled than those produced by activation of RhoA or by expression of an activated form of ROCK. Experimental titration of activated forms of both ROCK and mDia induced the formation of stress fibers that better resembled those found when RhoA is activated normally (213).

The exact role that mDia plays in cooperation with ROCK to form proper stress fibers has yet to be determined. Possible roles include its effects on microtubules (215) or by its interaction with profilin, which promotes actin polymerization (192). The sum effect of the actions of ROCK and mDia is to increase acto-myosin contractility. Increased Rho-stimulated acto-myosin contractility has been shown to lead to formation of stress fibers and subsequent focal adhesions (198, 216).

RhoA signaling and downstream consequences: cell cycle progression

In addition to its role in cytoskeletal organization, RhoA has also been shown to be functionally required for the G1-S phase transition in fibroblast cells. In one study, addition of C3 exoenzyme, an ADP-ribosyltransferase that inactivates Rho proteins (217), prevented serum starved Swiss 3T3 cells from entering S phase. The authors identified RhoA as the single protein that was ADP-ribosylated in the cells when C3 exoenzyme was added and clearly show that RhoA plays a critical role in the G1-S phase progression (218). In another study, the authors microinjected constitutively activated mutants of RhoA, Rac1 and Cdc42 into quiescent fibroblasts and observed cell cycle progression through G1 into S phase. They observed that microinjection of dominant negative forms of these Rho proteins or C3 exoenzyme into quiescent fibroblasts prevented serum-induced S phase entrance (219).

The requirement for RhoA in cell cycle progression may be due to two specific actions: regulation of gene transcription (specifically cyclin D1) and downregulation of cyclin dependent kinase inhibitors (220). Two main pathways have been described in support of RhoA involvement in gene transcription. First, RhoA (along with Rac1 and Cdc42) was shown to activate transcriptional activation by SRF. Serum response factor (SRF) is a

transcription factor, which regulates many immediate-early genes (221). Functional RhoA was shown to be required for serum- and LPA-induced activation of SRF. It was also shown that activated RhoA can activate SRF in the absence of external stimuli (222). Also, it was shown that RhoA can activate the SRF-related transcription factor MEF2C (223). This RhoA-dependent activation of MEF2C involves the RhoA effector PKN and activation of ERK6 (p38γ) (224), a member of the MAPK superfamily. The authors also provide evidence that activation of the ERK6 (p38γ) pathway is required for the ability of RhoA to subvert normal cell growth and induce cellular transformation (223). The SRF and MEF2C transcription factors lead to increased transcription of c-Fos and c-Jun, respectively (222, 223). c-Jun and c-Fos, along with related proteins, form the AP-1 transcription factor (225). AP-1 binds to palindromic DNA sequence termed the TRE, which is present in the regulatory regions of many genes, thus controlling their expression. Expression of c-Jun and c-Fos, to form the AP-1 transcription factor, play an important role in cell proliferation (226).

Second, it was shown that RhoA (along with Rac1 and Cdc42) efficiently induces the transcriptional activity of nuclear factor kappaB (NF-kappaB) by a mechanism that involves both phosphorylation of Ikappa Balpha and translocation of p50/p50 and p50/p65 dimers to the nucleus. The authors showed, through use of dominant negative mutants, that activation of NF-kappaB by TNFα depends on both RhoA and Cdc42 (227). The same authors, in an additional paper, show that the NF-kappaB and C/EBPβ transcription factors are accessory proteins for RhoA-linked regulation of the activity of SRF (228). The transcription of cyclin D1 has been shown to be controlled by the AP-1 (229) and NF-kappaB (230) transcription factors. Cyclin D1 has been shown to be crucial for cell proliferation and tumorigenesis as its expression increases as the cell enters the cell cycle and it has been shown to be

overexpressed in a variety of cancers (231). Thus, one example of how RhoA may contol cell cycle progression, and hence increased proliferation, would be regulation of cyclin-D1 expression through control of AP-1 and NF-kappaB.

Another example of how RhoA may control cell cycle progression is through the downregulation of cyclin-dependent kinase inhibitors p21-CIP1 and p27-KIP1 (232). p21-CIP1 (233) and p27-KIP1 (234) are cyclin-dependent kinase inhibitors that control cell cycle progression through G1 phase into S phase, a major checkpoint for proliferating cells. Downregulation of p21-CIP1 by active RhoA has been shown to be crucial for oncogenic Ras to promote cell cycle entry (235). Furthermore, it was shown that Ras-transformed cells contain high levels of RhoA-GTP, which functions to inhibit the expression of p21-CIP1. Further, the actions of RhoA-GTP are uncoupled from ROCK and stress fiber formation, as inhibition of ROCK by the inhibitor Y-27632 did not affect the levels of p21-CIP1 (236). Currently, the only clue as to how RhoA might regulate p21-CIP1 levels was provided by a report which showed that RhoA activity affects the phosphorylation of the SP1 transcription factors, which regulate the transcription from the p21-CIP1 promoter. The authors show that treatment with a GGTI upregulated levels of p21-CIP1. Furthermore, they show that both treatment with C3 exoenzyme and expression of a dominant negative RhoA increased the levels and activity of p21-CIP1 (237). Furthermore, it has also been shown, through the use of both dominant negative RhoA and C3 exoenzyme treatment, that RhoA activity is required for growth factor-dependent downregulation of p27-KIP1 and progression from G1 to S phase (238, 239).

Finally, several RhoGEFs, including Ect2 and GEF-H1, have been demonstrated to be critical for cytokinesis (240-242). Taken together, all of these results indicate that RhoA activity is required for proper cell proliferation.

RhoA signaling and downstream consequences: oncogenic transformation

Perhaps not surprisingly, given its roles in cytoskeletal organization and cell cycle control, there is also evidence that RhoA is involved in both morphological and growth transformation. In one study, using focus forming assays as the read-out , coexpression of an activated form of RhoA along with a weakly transforming Raf-1 mutant greatly enhanced transformation. Furthermore, coexpression of a dominant negative mutant of RhoA reduced oncogenic Ras transformation. The authors also showed that activated RhoA further enhanced oncogenic Ras-triggered morphologic transformation, as well as growth in soft agar and cell motility (159). In a different study, it was shown that activated RhoA strongly cooperates with constitutively active Raf in focus formation assays in NIH 3T3 cells. The authors showed that a dominant negative RhoA inhibited focus formation by both activated Ras and Raf. Furthermore, the authors showed that stable coexpression of dominant negative RhoA and activated Ras in Rat1 fibroblast cells reverted Ras transformation (160).

The central role of RhoA in regulating processes critical to cell morphology and growth suggests that, in addition to the proximal regulation of RhoA activity by GEFs, GAPs and GDIs, upstream signaling that impinges upon RhoA activity levels will also provide critical inputs to both cytoskeletal organization and cellular proliferation. One such upstream signal is represented by the atypical Rho-related protein, Rnd3, as described in the next section.

Rnd proteins: atypical Rho GTPases

Similarities to / differences from classical Rho GTPases

The Rnd proteins represent a subfamily of the Rho family of small GTPases. These proteins show a high degree of sequence similarity to RhoA (45-49%) but display distinct biochemical and functional properties (4, 12). The Rnd family consists of three members, which are ~90% identical in their GTPase domains: Rnd1/RhoS/Rho6/ARHS (chromosome location 12q12-q13), Rnd2/RhoN/Rho7/ARHN (chromosome location 17q21) and Rnd3/RhoE/Rho8/ARHE (chromosome location 2q23.3). Rnd proteins are found only in vertebrates and thus arose late in evolution (~500 million years ago). Rnd1 is expressed in the brain and liver, while Rnd2 is expressed in the brain, liver and testes. Although Rnd3 mRNA is ubiquitously expressed, Rnd3 protein is found basally at low levels but is induced by specific signaling as discussed later. All three Rnd proteins possess unique C-terminal extensions and both Rnd1 and Rnd3 possess novel N-terminal extensions, of 8 and 18 amino acids, respectively. These novel N-terminal extensions have been shown to be important for proper membrane localization. Addition of these N- and C-terminal extensions increase the size of the Rnd proteins (27-30 kD), as compared to other well studied members of the Rho family (21 kD). Therefore, Rnd proteins are migrate at a slower mobility when examined by SDS-PAGE. Furthermore, Rnd proteins have basic isoelectric points, in contrast to RhoA, which has an acidic isoelectric point (12), suggesting distinct microenvironments and signaling partners.

Rnd proteins display several unusual biochemical properties compared to other Rho proteins. First, the CAAX motifs of all three Rnd proteins terminate in a methionine, and are thus classified as potential substrates for prenylation by farnesyl transferase (99, 102). In

contrast to the classical Rho family proteins, which are geranylgeranylated, Rnd proteins have been shown to be farnesylated (15, 16, 106). In the case of Rnd3, farnesylation is required for membrane localization and for its ability to alter cytoskeletal organization (Singh et al., unpublished data).

In contrast to most Ras and Rho family proteins, which cycle through binding GDP and GTP, Rnd proteins are GTPase-deficient, resistant to GAP activity, and display a higher affinity for GTP over GDP (15, 16, 243). Thus, Rnd proteins are found predominantly bound to GTP in cells and are considered to be constitutively active. Although their core structure is very similar to that of GTP-bound RhoA, Rnd proteins contain natural substitutions at the Ras amino acid positions Gly12, Ala59 and Gln61 (Figure 1.9). Mutations of these residues in Ras result in an activated version that is unable to hydrolyze GTP, and is thus constitutively active and transforming. In Rnd3, residues 32, 79 and 81 (equivalent to Ras positions Gly12, Ala59 and Gln61; RhoA positions Gly14, Ala61 and Gln63) are all serines. Mutating serines 79 and 81 to the corresponding residues in WT RhoA resulted in a version of Rnd3 that was able to hydrolyze GTP at a level equal to WT RhoA. Mutating all three Rnd3 serines to the appropriate WT RhoA residues resulted in GTPase activity greater than that of WT RhoA (16).

The crystal structure of the core GTPase domain of Rnd3 has been solved (244, 245). From these studies, it was noted that the primary loss of GTPase activity is due to the replacement of the catalytically competent glutamine with a serine (Ser81). Glutamine lowers the energy of the transition state, thus enhancing the rate of hydrolysis (244). The side chain of serine 81, which corresponds to the catalytically active glutamine 63 in RhoA, is rotated away from the γ-phosphate and is unable to stabilize a nucleophilic water molecule

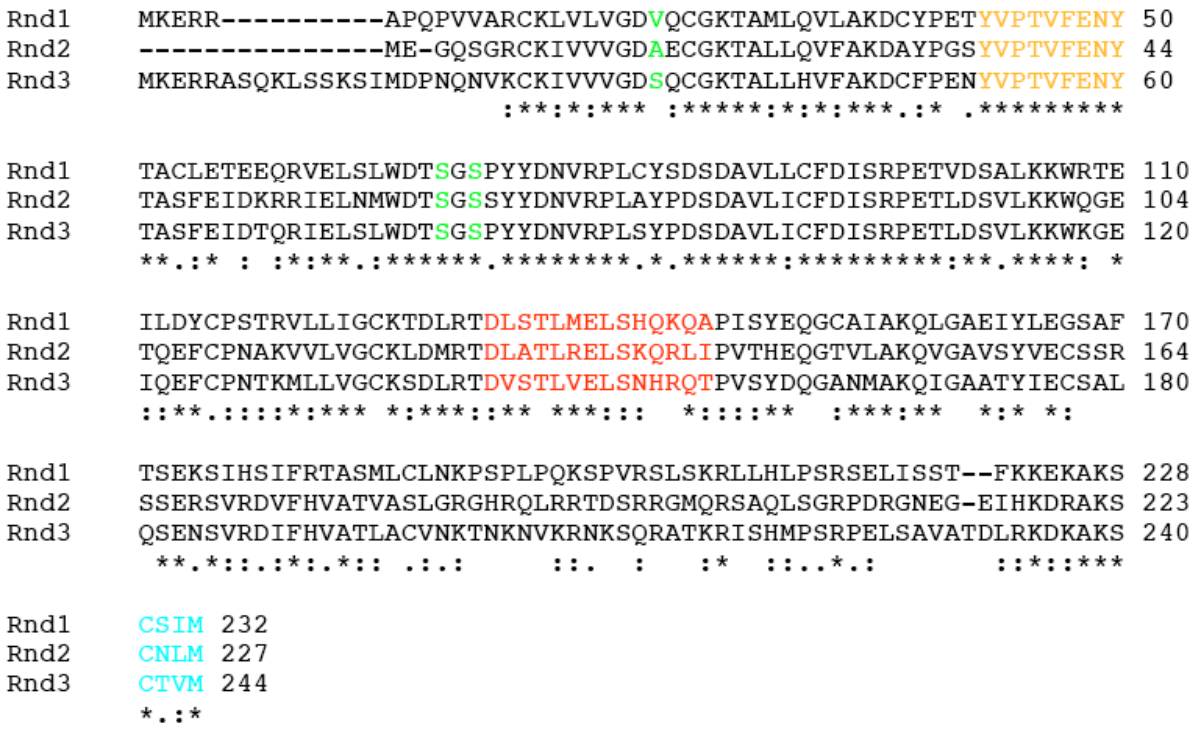


Figure 1.9: Sequence alignment of the Rnd family proteins. Highlighted are the core effector domain (orange), the Rho insert domain (Red) and CAAX prenylation motif (cyan). Residue substitutions involved in Rnd protein GTPase deficiency are highlighted in green. "*" indicates that the residues in that column are identical in all sequences in the alignment. "**:**" indicates that conserved substitutions have been observed. "**.**" indicates that semi-conserved substitutions are observed. Accession numbers: NP_055285 (Rnd1), NP_005431 (Rnd2) and NP_005159 (Rnd3).

for an in-line attack of the γ-phosphate (245). Multiple amino acid residue differences in the Rnd3 Switch I and Switch II regions compared to other small GTPases contribute to its stability in the GTP-bound state and its apparent inability to transition to a GDP-bound state (244). Further, it was noted that the presence of the serine residue at position 32 would cause a steric clash with the RhoGAP arginine finger (245). From these studies it was also shown that four of the six residues of RhoA that are involved in an extensive hydrogen-bonding network with RhoGAP deviate in Rnd3. Thus, these structural differences make a Rnd3-RhoGAP interaction unlikely (245). Given that Rnd proteins do not hydrolyze GTP and are resistant to GTPase activity and that the concentration of GTP in the cell is ten-fold higher than that of GDP, it also seems very unlikely that Rnd3 requires interaction with RhoGEFs in order to become GTP-bound and active (245). Taken together, all of these properties suggest that the activity of Rnd3 must be regulated in a manner distinct from GDP/GTP cycling.

Regulation of expression / activity of Rnd proteins

Since Rnd proteins do not GDP/GTP cycle, they are thought to be regulated in part at the level of expression. A wide variety of signals have been shown to induce the expression of Rnd proteins, including cytokines, growth factors, sex steroid hormones, neurotransmitters and genotoxic stressors. In the case of Rnd1, expression has been shown to be induced by promoter hypomethylation in gastric cancer (246) and upon inflammation in endothelial cells (247). Furthermore, Rnd1 expression was also increased in rat smooth muscle by the sex hormone steroids estradiol and progesterone (248). Rnd2 expression can be induced in monocytes activated by lipopolysaccharides (249). Rnd3 expression is induced in MDCK cells by the Ras/Raf/MEK/ERK pathway (250). In both Swiss 3T3 fibroblasts and bone

marrow-derived mesenchymal stem cells, Rnd3 expression was shown to be induced by platelet-derived growth factor (PDGF) (14, 251). Binding of factor VIIa to tissue factor on keratinocytes also induces Rnd3 expression (252). Furthermore, stress agents such as ultraviolet-B irradiation (253, 254) and the DNA-damaging agents cisplatin, mitomycin C, etoposide and camptothecin have also been shown to increase Rnd3 expression (255, 256), as does ethanol exposure in astrocytes (257).

Like Rnd1, Rnd3 expression is also regulated by sex hormones. For example, Rnd3 expression is increased in breast tissue of parous women as compared to nulliparous women (258). Several studies have shown that the expression of Rnd proteins is increased in the myometrium during pregnancy. Pregnancy-induced increases of Rnd1 in rat myometrium (259) and Rnd3 in rabbit myometrium (260) and an increase in Rnd1, Rnd2, and Rnd3 in both rat and human myometrium (261) have been documented. Studies of human myometrial samples in another study also showed an increase in Rnd2 and Rnd3 expression, but only a non-significant increase in Rnd1 expression was noted (262).

Exposure of mice to the drugs cocaine and MDMA (ecstasy) led to upregulation of Rnd3 message in the hippocampus, striatum and prefrontal cortex regions of the mouse brain (263, 264). Meanwhile, Rnd2 message was upregulated only in the prefrontal cortex after exposure to cocaine, whereas Rnd1 message was not affected after treatment with either drug (264).

Finally, in *Xenopus laevis* Rnd1 (XRnd1) was isolated in a screen for genes that perturbed secondary axis formation in *Xenopus* embryos. XRnd1 was shown to be transiently expressed during the time that certain tissues were undergoing morphogenic remodeling (265). These examples illustrate that Rnd protein expression is modulated by many different

cellular events. However, these expression changes are unlikely to be modulated rapidly enough to allow temporal regulation of Rnd protein activity in a manner appropriate to most normal signal transduction events.

In addition to regulation of Rnd activity by changes in expression, Rnd proteins may also be regulated by post-translational modifications that directly or indirectly alter its abundance and/or its localization. While this work was in progress, Ridley and colleagues demonstrated that Rnd3 binds to and becomes phosphorylated by the serine/threonine kinase and Rho effector, ROCK I (266). Coexpression of constitutively active ROCK I increased the half-life of Rnd3 by increasing protein stability, supporting the notion that phosphorylation can contribute to Rnd3 activity by modulating its expression.

Effectors and biological activities of Rnd proteins

Rnd proteins are largely known, indeed named, for their effects on the cytoskeleton. Expression of Rnd1 and Rnd3 but not Rnd2 in fibroblast and epithelial cells causes a loss of stress fibers and focal adhesions leading to rounding-up of cells (15, 243) (Figure 1.10). Because the phenotype resulting from Rnd activity (stress fiber disassembly) is the opposite of that seen with active RhoA (stress fiber assembly), Rnd proteins are thought to elicit these morphological changes by abrogating signaling from RhoA. Two possible models to explain mechanisms by which Rnd proteins may antagonize RhoA signaling have been put forth, and evidence has been provided in support of each model (Figure 1.11). In the one model, Rnd proteins (Rnd1 and Rnd3) bind and activate p190RhoGAP, which decreases the levels of active GTP-bound RhoA. In the other model, Rnd3, but not Rnd1, binds and sequesters

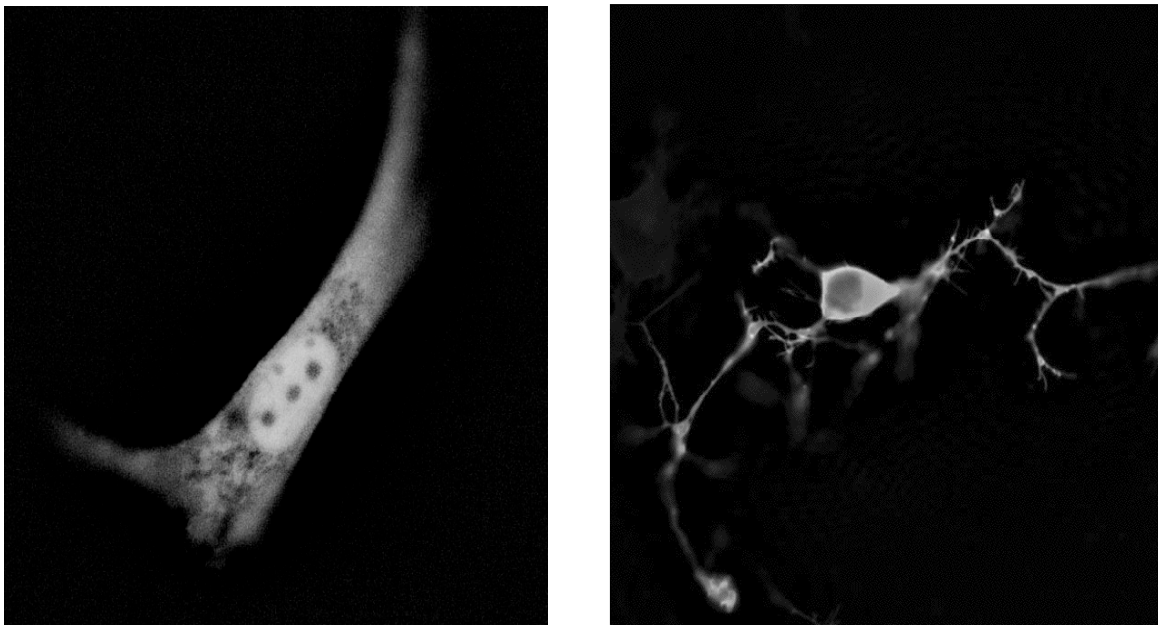


Figure 1.10: Effects of Rnd3 expression on the actin cytoskeleton. Expression of exogenous Rnd3 in NIH 3T3 cells results in a loss of stress fibers and focal adhesions, leading to rounding of the cell. Hence "Rnd" for rounding.

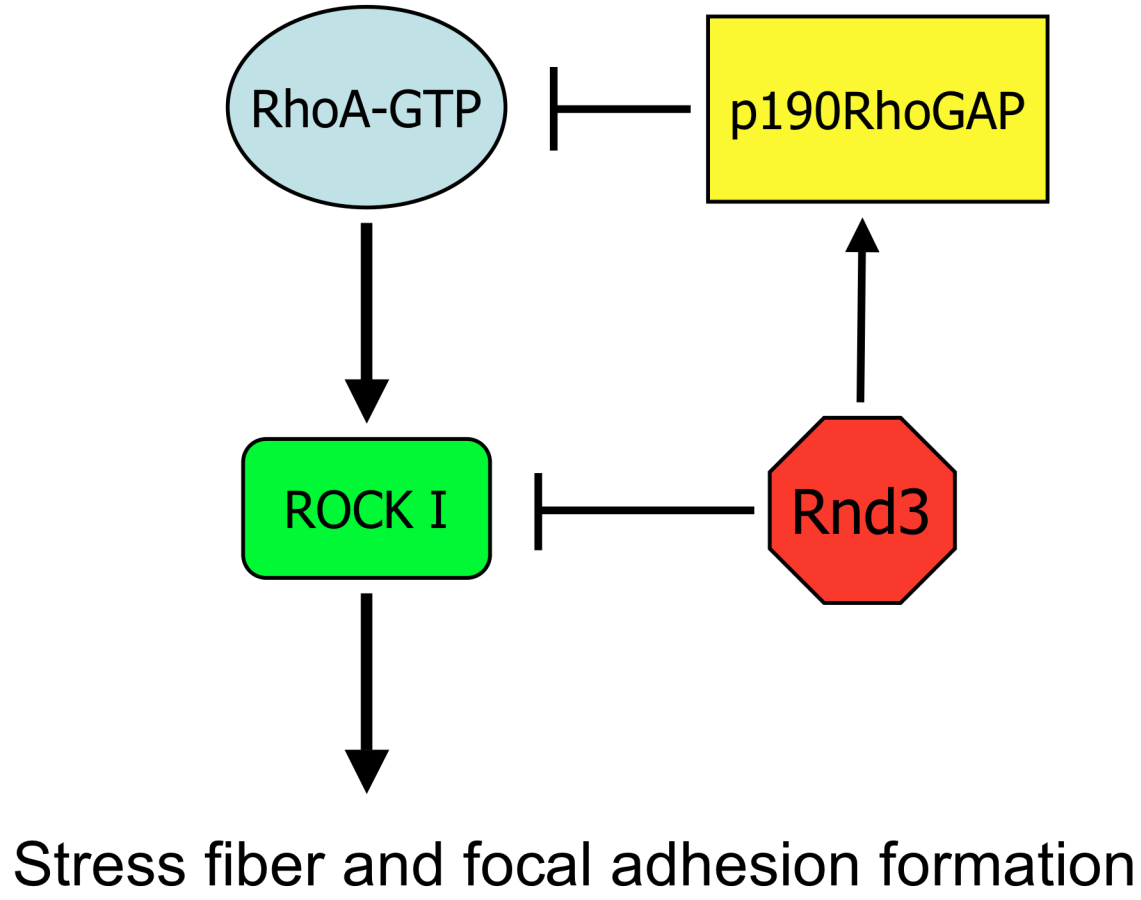


Figure 1.11: Rnd3 antagonizes the RhoA-ROCK I signaling pathway at two separate levels. Rnd3 has been shown to bind to and activate p190RhoGAP leading to reduced levels of RhoA-GTP. Rnd3 has also been shown to bind and inhibit the RhoA effector ROCK I. Overexpression of exogenous Rnd3 leads to loss of stress fibers and focal adhesions, leading to cell rounding.

ROCK I, a downstream effector necessary for stress fiber and focal adhesion formation. These models may not be mutually exclusive, however.

Rnd3 was first identified in a yeast two-hybrid screen using p190ARhoGAP as bait and called RhoE (16). Overexpression of either Rnd1 or Rnd3 in fibroblasts causes cell rounding due to stress fiber disassembly and focal adhesion turnover (15, 243), very similar to the effect seen with overexpression of p190RhoGAP (267). Therefore, a logical study would be to see if Rnd proteins function with p190RhoGAP to elicit cytoskeletal reorganization through downregulation of RhoA.

In support of the first model, Rnd proteins have been shown to bind to p190RhoGAP by yeast two-hybrid screen (13). In this report, the authors showed that the cellular effects of Rnd proteins are mediated through p190RhoGAP, the most abundant GAP for RhoA in cells (268). They further showed that a region of Rnd3 found within amino acids 16-93 is sufficient for interacting with p190RhoGAP. This region of Rnd3 contains the P loop along with both switch I and switch II. They also showed that two specific mutants of Rnd3 were unable to bind to p190RhoGAP. The first mutant was Rnd3(T37N), which is analogous to the persistently GDP-bound RhoA(T19N) mutant. The second mutant was Rnd3(T55A), which is analogous to the RhoA(T37A) mutant that shows impaired effector binding. Furthermore, they showed that both of these mutants had no effects on cell morphology, such as cell rounding. Using knockout 3T3 fibroblasts, it was shown that the rounding effects of Rnd1 and Rnd3 were substantially attenuated in both p190A-/- and p190B-/- cells, as compared to control WT 3T3 cells. The authors went on to show that, in *in vitro* GAP assays, addition of all three Rnd proteins increased p190A- and p190B-dependent GAP activity toward RhoA-GTP by approximately 2-fold. Furthermore, the Rnd3(T55A) mutant did not affect p190-

dependent GAP activity toward RhoA-GTP. Using Rhotekin RBD assays (269) and Tat-mediated protein transduction, the authors went on to show that in control 3T3 fibroblasts, transduction of WT Rnd3, but not Rnd3(T55A), substantially reduced RhoA-GTP levels. The authors in this study concluded that Rnd proteins inhibit RhoA signaling by increasing the GAP activity of p190RhoGAP towards RhoA-GTP.

In the second model, a study has shown that Rnd3 binds to and sequesters ROCK I, a downstream effector of RhoA (14). To determine how Rnd3 inhibits stress fiber formation, the authors investigated whether Rnd3 was able to bind to known RhoA targets involved in stress fiber formation. In a GST pull down experiment, GST-tagged Rnd3 selectively pulled down ROCK I from cells. Using truncation mutants of ROCK I in GST-Rnd3 pulldown experiments, the authors determined that Rnd3 binds to the N-terminus of ROCK I. They also determined that Rnd3 does not bind to the related ROCK II. They determined that the minimal region for Rnd3 binding is amino acids 1-420 of ROCK I, a region that includes the kinase domain. This region of ROCK I is different from the region that binds RhoA, which binds in the RBD found near the C-terminal end of ROCK I (amino acids 934-1015). The authors determined that the kinase activity of ROCK I was not needed for Rnd3 binding, as a kinase dead form of ROCK I was still able to interact with Rnd3. Using immunostaining, they determined that endogenous Rnd3 localized to the Golgi complex and the plasma membrane of COS-7 cells. Using co-staining with ROCK I and Rnd3 antibodies, the authors noted a significant degree of overlap of the two proteins in cells, especially the trans-Golgi network. The authors went on to show, through coimmunoprecipitation experiments, that exogenous Rnd3 and ROCK I directly interact. They further showed that the endogenous proteins also directly interact. Using a coexpression/GST-pull down assay in COS-7 cells,

the authors showed that Rnd3 and RhoA cannot bind to ROCK I simultaneously, even though they bind to different regions of ROCK I.

To test whether Rnd3 had any effect on the ability of ROCK I to phosphorylate downstream effectors, the authors investigated the effect of Rnd3 expression on phosphorylation of MLC phosphatase (MLCP), a major downstream target of ROCK I (199). Coexpression of Rnd3 in COS-7 cells reduced MLCP phosphorylation induced by both constitutively active and WT ROCK I, as well as reducing the background level of MLCP phosphorylation in the absence of exogenous ROCK I. These results indicate that Rnd3 inhibits stress fiber formation by preventing ROCK I from phosphorylating MLCP. Finally, the authors went on to show that cytoskeletal changes, mainly cell rounding, caused by treatment with a high concentration of PDGF coincided with increased Rnd3 protein expression. These results correlate well with the role of Rnd3 in negatively regulating stress fiber formation. The kinase domain of ROCK is highly inaccessible, as the C-terminal region can bind to the N-terminal region to form an autoinhibited structure (270, 271). The authors speculated that in this inactive conformation the Rnd3-binding site near the kinase domain would be masked. They went on to state that RhoA-GTP binding to ROCK I induces an open conformation and Rnd3 can bind once RhoA has dissociated.

It is quite obvious from all of the research to date that Rnd1 and Rnd3 affect RhoA-mediated cytoskeleton organization. Yet, the question remains whether either of the two models presented above is more physiologically relevant. I hypothesize that the effects of Rnd1 and Rnd3 on the actin cytoskeleton are largely attributable to p190RhoGAP-mediated activity, based on three lines of evidence. First, Rnd effects on the actin cytoskeleton are much less pronounced in p190RhoGAP -/- cells (13). Second, Rnd3 has been shown to bind

to only ROCK I (14) and not ROCK II (266). To date, no differences in function have been reported for these two highly related proteins (199), suggesting that ROCK II activity would compensate for loss of ROCK I. Third and most importantly, Rnd1 does not bind (or binds poorly) to ROCK I, but is quite efficient at inducing loss of stress fibers and focal adhesions (15). Therefore, I support the p190RhoGAP model. Naturally, this does not preclude a contribution from the ROCK model, or from additional unknown mechanisms.

The Rnd3-ROCK I model may still play a role in cellular activities. In Ras-transformed cells, there is a loss of stress fibers and focal adhesions, similar to what is seen in cells overexpressing Rnd3 (15, 243). Nevertheless, despite this, Ras-transformed cells often show an upregulation of active RhoA, which might be predicted to lead to an increase, not a decrease, in stress fiber formation. Raf activation in MDCK cells leads to both an increase in Rnd3 expression and to loss of stress fibers and focal adhesion (250). However, Ras-transformed cells still need upregulation of active RhoA for cell proliferation (236). Perhaps increased Rnd3 expression in Ras-transformed cells is involved in the uncoupling of active RhoA from ROCK signaling by sequestering away ROCK I, leading to promotion of proliferation but loss of stress fibers and focal adhesions. In this way, Rnd3 might contribute to the transformed phenotype and increased motility seen in Ras transformed cells.

A few other Rnd protein effectors have been described in addition to ROCK and p190RhoGAP (Figure 1.12). One example is the protein Socius (Latin for "partner"). Socius was pulled out of a yeast two-hybrid screen of a rat brain cDNA library using Rnd1 as bait (272). The report describing the cloning of Socius shows that it has an open reading frame that encodes a protein of 485 amino acids and has a predicted molecular mass of 54.7 kD. Analysis of the predicted amino acid sequence of Socius revealed a UBX domain in the C-

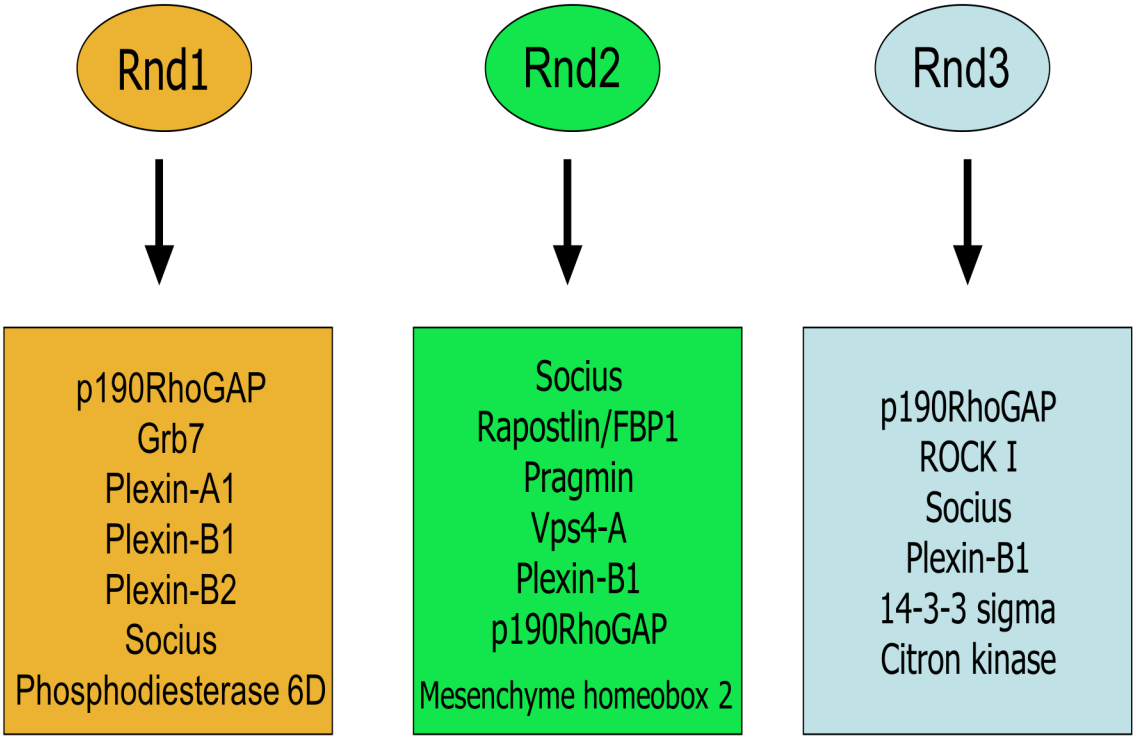


Figure 1.12: Effectors of Rnd proteins. Rnd proteins share similar effectors implying some conservation of function. Differential binding to other effectors dictates distinct biological functions.

terminal region. The UBX domain is an ~80 amino acid module of unknown function, structurally related to ubiquitin (273). Socius can bind to all three Rnd proteins. Furthermore, by using pulldown assays, the authors showed that Myc-tagged Socius expressed in COS-7 cells bound to Rnd1 but was unable to bind to GST-tagged RhoA, Rac1 or Cdc42, whether loaded with GTP-γS or GDP. In pulldown experiments, Rnd1(T27N), a putatively dominant negative mutant (equivalent to RhoA(T19N)) was unable to bind to GST-Socius, and a Rnd1(T45A) effector domain mutant (equivalent to RhoA(T37A)) was binding-impaired. The function of Socius is unknown, but it was shown to bind Rnd proteins through its C-terminal region and to colocalize with both Rnd1 and Rnd3 at the plasma membrane. In COS-7 cells, the authors showed that expression of either Rnd1 or Rnd3 caused the translocation of Socius to the cell periphery. Although expression of WT Socius in fibroblasts had little effect on the actin cytoskeleton, a constitutively membrane-targeted form of Socius with an artificial CAAX motif attached was shown to induce the disassembly of stress fibers but did not cause a branching phenotype. This suggests that the Rnd protein-induced disassembly of stress fibers and the Rnd branching phenotype are differentially regulated by at least two distict signaling pathways, and that Socius is involved only in the pathway leading to disassembly of stress fibers. The authors suggested that Socius may serve as an adapter or scaffold protein, linking Rnd proteins to cytoskeletal regulatory molecules that might bind to the UBX domain.

Rnd1 has also been shown to bind to the Grb7 adapter protein (274) and to phosphodiesterase 6D (275). The interaction between Rnd1 and Grb7 was shown by yeast two-hybrid, by *in vitro* binding and by pull down assays (274). Grb7 (276) belongs to the Grb7/10/14 family of adapter proteins (277), which are related to the prototypic Grb2 adapter

protein involved in Ras activation (278). The interaction between Rnd1 and Grb7 involved the switch II region of Rnd1 and an SH2 phosphotyrosine recognition domain of Grb7, although no tyrosine phosphorylation of Rnd1 was detectable. This interaction could be involved in stress fiber inhibition in lamellipodia, where Grb7 partially localizes (12).

The interaction of Rnd1 and phosphodiesterase 6D (PDE 6D) was shown by yeast two-hybrid assay (275). In this report, the authors showed that PDE 6D (279) (also referred to as the δ subunit of retinal rod phosphodiesterase) was able to extract Ras and Rap family members from the membranes of HeLa cells, an activity reminiscent of the well-characterized RhoGDIs (280) and RabGDIs (281). It should be noted that Rnd proteins do not associate with RhoGDI (15) and it was shown that RhoGDI does not extract Rnd3 from membranes (282). Although it was not determined in this report whether Rnd1 was extracted from membranes (275), given the structural similarities (279) between PDE6D and RhoGDIs, I speculate that perhaps PDE 6D regulates Rnd1 protein (along with Rnd2 and Rnd3) activity in a way similar to the manner in which RhoGDIs regulate canonical Rho GTPases (89-91).

Rnd2 binds a distinct set of putative effector proteins, including Rapostlin (283), Vacuolar protein sorting protein 4-A (284), Pragmin (285), MgcRacGAP (286), and Mesenchyme homeobox 2 protein (287). The same group that identified Socius (272) also identified three novel proteins using Rnd2 as bait in yeast two-hybrid screens. The first protein was Rapostlin (named for "apostle of Rnd2"), also known as forming binding protein 1 (FBP1) (283). In *in vitro* binding assays, Rapostlin specifically bound to Rnd2 among the Rho family of GTPases and did so in a GTP-dependent manner. The authors showed that Rapostlin binds to microtubules and that, in PC12 neuronal cells, Rapostlin induced neurite

branching in response to Rnd2. In a follow-up paper, the authors described numerous splice variants of Rapostlin and showed that all are able to bind to Rnd2 in a GTP-dependent manner, along with binding to the neural Wiskott-Aldrich syndrome protein (N-WASP) (288). In immunoprecipitation experiments, Rnd2 reduced the interaction of full length Rapostlin with N-WASP, but had no effect on the interaction of N-WASP with Rapostlin splice variants.

The second protein found to interact with Rnd2 was Vps4-A or Vacuolar protein sorting 4-A (284), a member of the AAA ATPase family and a central regulator of early endosomal trafficking. The authors showed interaction by *in vitro* binding and coimmunoprecipitation assays, with Vps4-A binding to both GTP- and GDP-loaded forms of Rnd2. When Rnd2 was co-expressed with an ATPase-deficient mutant of Vps4-A in HeLa cells, it was recruited to Vps4-A-bound early endosomes. The authors suggested that Rnd2 is involved in regulation of endosomal trafficking via direct binding to Vps4-A.

The third protein found to interact with Rnd2, via a yeast two-hybrid screen, was Pragmin (285). The authors showed through *in vitro* and *in vivo* binding assays that Pragmin specifically bound to Rnd2 and did so in a GTP-dependent manner. Surprisingly, Rnd2-bound Pragmin significantly stimulated RhoA activity and induced cell contraction through the RhoA-ROCK pathway in HeLa cells. In PC12 neural cells, Pragmin expression inhibited NGF-induced neurite outgrowth in response to Rnd2, whereas knockdown of Pragmin by siRNA enhanced neurite elongation. The authors suggested that Rnd2 regulates neurite outgrowth by functioning as a RhoA activator through Pragmin, in contrast to Rnd1 and Rnd3 which inhibit RhoA signaling. These results could also give clarification to why Rnd2 does not induce loss of stress fibers and focal adhesions when expressed in fibroblasts (15).

In a study using male germ cells, MgcRacGAP was shown to physically interact with Rnd2 by both GST-pulldown and by co-immunoprecipitation. Furthermore, these two proteins were shown to colocalize (286). MgcRacGAP is a RhoA-specific GAP that was shown to localize to the mitotic spindle in metaphase and to be condensed at the midbody during cytokinesis. Furthermore, using a GAP-inactive mutant, the GAP activity of MgcRacGAP was shown to be required for cytokinesis (289, 290). It was later shown that MgcRacGAP initiates cytokinesis through the controlled assembly of the contractile ring (291). The authors suggested that MgcRacGAP, and quite possibly other RhoGAPs, may participate in signaling pathways involving Rnd family proteins (286). Using a stringent, high-throughput yeast two-hybrid system, Rnd2 was shown to interact with the mesenchyme homeobox 2 protein (MEOX2) (287). The expression of the gene encoding MEOX2 was shown to be downregulated in vascular smooth muscle cells when quiescent cells were stimulated by mitogens to reenter the cell cycle. Conversely, this gene was shown to be upregulated when proliferating cells were starved of serum. Thus, MEOX2 may play a regulatory role in the cell cycle (292). What role the interaction of Rnd2 and MEOX2 may play in the cell is currently unknown.

Rnd2, like Rnd1 and Rnd3, also interacts with proteins important for axon growth. Axon guidance occurs by directional protrusion of a growth cone. Neurite extensions on the growth cone sense the environment in several directions. The neurite retracts in the respone to a repulsive signal and the cytoskeleton rearranges to orient the growth cone in another direction, so as to detect an attractive signal. Plexins are the receptors for the semaphorins, which are transmembrane or secreted proteins that guide cell migration or axon pathfinding. Semaphorin recognition leads to plexin activation and the retraction and collapse of the

growth cone due to RhoA-dependent actin contraction (12, 293). All three Rnd proteins have been shown to bind to plexin B1 (294). In one study, the authors examined the binding ability of the three Rnd proteins. They showed that all three Rnd proteins directly interact with the cytoplasmic domain of Plexin-B1. The main emphasis of the study was centered on Rnd1 and its direct interaction with Plexin-B1. The authors showed that, in COS-7 cells, coexpression of Rnd1 and Plexin-B1 induced cell contraction in response to semaphorin 4D, and the interaction between these proteins was required. Semaphorin 4D-induced contraction in Plexin-B1/Rnd1-expressing COS-7 cells was suppressed by dominant negative RhoA, a Rho-associated kinase inhibitor, a dominant negative form of PDZ-RhoGEF, or deletion of the carboxyl-terminal PDZ-RhoGEF-binding region of Plexin-B1, indicating that the PDZ-RhoGEF/RhoA/Rho-associated kinase pathway was involved in this morphological effect. They also found that Rnd1 promoted the interaction between Plexin-B1 and PDZ-RhoGEF and thereby dramatically potentiated the Plexin-B1-mediated RhoA activation. The authors suggested that Rnd1 plays an important role in the regulation of Plexin-B1 signaling, leading to RhoA activation during axon guidance and cell migration.

In an additional paper from the same group, they showed that Plexin-B1 directly stimulates the intrinsic GTPase activity of R-Ras (295). The authors showed that the GAP activity of Plexin-B1 required the interaction of Rnd1 and that down-regulation of R-Ras activity by the Plexin-B1-Rnd3 complex was essential for the Semaphorin 4D-induced growth cone collapse in hippocampal neurons. The authors speculated, and then demonstrated, that Plexin-B1 mediates Semaphorin 4D-induced repulsive axon guidance signaling by acting as a GAP for R-Ras. Rnd1 binding to Plexin-B1 opens the two R-Ras GAP domains of Plexin-B1 to allow full GAP activity towards R-Ras (296). The full role of

either Rnd2 or Rnd3 involvement in regulation of PDZ-RhoGEF-mediated Rho activation by Plexin-B1 or Plexin-B1-mediated R-Ras GAP activity remains to be determined.

In addition to its relatively well-studied effects on the cytoskeleton, there is evidence that Rnd3 may also be involved in regulation of the cell cycle. G1 cell cycle progression is controlled by the D-type and E-type cyclins (297). Past studies have shown that inhibition of RhoA blocks G1 progression (218, 219). RhoA was shown to be required for sustained ERK signaling which is associated with expression of cyclin D1 during mid-G1 phase (298). Furthermore, it was shown that cyclin D1 expression is controlled by RhoA/ROCK signaling through stress fiber-mediated integrin signaling that sustains ERK activation (299). Meanwhile, a recent report indicates that ectopic overexpression of Rnd3 blocks cell-cycle progression at G1 phase (255). The authors demonstrated that increased ectopic Rnd3 expression in fibroblast cells inhibited cell proliferation and prevented serum starved cells from entering the cell cycle in response to growth factor stimulation. This inhibition of cell proliferation was due to a lack of cyclin D1, a protein known to be important in cell cycle progression. Further, the authors demonstrated that increased Rnd3 expression negatively affected the translation of cyclin D1 mRNA and that expression of cyclin D1 could not rescue the growth arrest induced by Rnd3. They suggested that Rnd3 may also affect the translation of other mRNAs. With this in mind, perhaps Rnd3 physically interacts with components of the translational machinery, such as eIF4E (300).

In agreement with these findings, another study has found that expression of Rnd3, along with disrupting the actin cytoskeleton, inhibited U87 glioblastoma cell proliferation (301). In this study, the authors showed that Rnd3 expressing U87 cells showed a reduction in Rb phosphorylation and in cyclin D1 expression. Induction of cyclin D1 is one event

required for Rb phosphorylation (inactivation), leading to cell cycle progression (297). They further showed that Rnd3 expression in these cells inhibits ERK activation following serum stimulation in quiescent cells. The authors proposed that Rnd3 inhibits ERK activation, thereby decreasing cyclin D1 expression and leading to a reduction in Rb inactivation. They speculated that this mechanism is involved in the Rnd3-induced cell growth inhibition. They further went on to show that Rnd3 expression induced apoptosis in U87 cells along with colon carcinaoma and melanoma cells and stated that their results indicate that Rnd3 plays an important role in the regulation of cell proliferation and survival. They suggested that Rnd3 may be considered an "oncosuppressor" since it is able to induce apoptosis in several tumor cell lines. Furthermore, another study found that forced overexpression of Rnd3 in prostate cancer cells, devoid of Rnd3, resulted in G2/M cell cycle arrest. These authors found that the cell cycle arrest was due to the inhibition of expression of Cdc2 and cyclin B1, two proteins essential for G2/M phase transition. The authors suggested that Rnd3 is a tumor suppressor gene, which is downregulated early in the development of prostate cancer (302).

Rnd3 has also been shown to have pro-survival activities in response to genotoxic stress. Rnd3 has been shown to be a direct p53 transcriptional target gene (256). In this report, the authors showed that genotoxic stress triggered actin depolymerization, resulting in actin stress fiber disassembly through p53-dependent Rnd3 induction. They found that Rnd3 inhibited ROCK I activity during genotoxic stress and thereby suppressed apoptosis. Rnd3-specific knockdown resulted in stress fiber maintenance and a striking increase in apoptosis. The authors demonstrated that p53-mediated induction of Rnd3, in response to DNA damage, favors cell survival partly through inhibition of ROCK I-mediated apoptosis. In another report from the same group, they showed that Rnd3 protein levels increased upon exposure to

UVB in human keratinocyte cells (254). The authors showed that knockdown of Rnd3 by siRNA resulted in a significant increase in apoptosis and a reduction in protein levels of the pro-survival targets p21, JNK, p38 and cyclin D1, as well as an increase in reactive oxygen species levels. The authors suggested that Rnd3 is a pro-survival factor acting upstream of p21, JNK, p38 and cyclin D1. Through the use of siRNA to p53, the authors showed that Rnd3 functions independently of its known associates, p53 and ROCK I. The authors noted that targeted expression of Rnd3 in the epidermis of mice resulted in a significant reduction of the number of apoptotic cells following UVB irradiation. The authors stated that Rnd3 induction counteracts UVB-induced apoptosis and may serve as a novel target for the prevention of UVB-induced photodamage regardless of p53 status.

Rnd3 has also been shown to participate in the stimulation of the inflammatory response induced by ethanol in astrocytes (257). Expression of exogenous Rnd3 in astrocytes resulted in a disruption of the actin cytoskeleton and a decrease in the protein levels of RhoA and Rac1, along with a decrease in the phosphorylation of the RhoA/ROCK downstream target MLC phosphatase. The authors also showed that treatment of astrocytes with ethanol resulted in an increase in endogenous Rnd3 protein levels. Overexpression of Rnd3 in astrocytes also resulted in stimulation of the inflammatory response as seen by induction of the IRAK/ERK/NF-kappaB pathway and COX-2 expression. The authors showed that treatment of astrocytes resulted in the induction of the IRAK/ERK/NF-kappaB pathway and COX-2 expression, which coincided with increased Rnd3 protein expression. The authors stated that the above results strongly support the conclusion that Rnd3 has a role in the stimulation of the inflammatory pathway induced by alcohol.

One report has given evidence that Rnd3 is a key regulator of apical junction dynamics (303). In this report, the authors showed that, in rat mammary epithelial tumor cells, exogenously expressed Rnd3 colocalized with actin at cell periphery and induced the localization of the adherens junction protein β-catenin and the tight junction protein ZO-1 to sites of cell-cell contact. Tight junctions regulate the diffusion of solutes on the basis of size and charge through a paracellular pathway and restrict the lateral diffusion of lipids and membrane proteins between the apical and basolateral regions (304, 305). Adherens junctions are responsible for intercellular adhesion between neighboring cells (306). Furthermore, this expression of Rnd3 led to the formation of highly sealed tight junctions. Also, the authors showed that expression of Rnd3 was able to rescue the disruptive effects of constitutively active RhoA on apical junction organization. The authors suggested that the antagonism between RhoA and Rnd3 in mammary epithelial tumor cells plays a fundamental role in controlling apical junction architecture and the formation of tight junctions.

Rnd proteins and cancer

There is strong evidence that Rnd3 is dysregulated in human cancer, especially when the Raf pathway is activated. For example, increased Rnd3 expression has been found in pancreatic tumors (307), colon cancer cell lines (308) and melanomas (309, 310). Recently, using immunochemistry on tissue microarrays from 115 patients with non-small cell lung cancer, it was shown that patients with Rnd3-negative tumors had a substantially longer cancer-related survival than did patients with Rnd3-positive tumors (311). Rnd3 expression in prostate cancer is controversial. One report, using immunoblot analysis, showed that Rnd3 protein expression was seen in benign prostate cancer cells, but not in cancer cells, with a

similar distribution of both mRNA and protein in patient tissues (302). However, another report has shown that levels of Rnd3 expression are far higher in prostate cancer cell lines with high metastatic potential compared with those of low metastatic potential (312). Perhaps, prostate cancer progression requires initially low levels of Rnd3 for increased proliferation and then high levels of Rnd3 for increased metastatic invasiveness.

There are also conflicting results regarding Ras transformation and Rnd3. One group has shown that Rnd3 negatively regulates Ras- and Raf-induced fibroblast transformation. Yet, others have shown that Rnd3 acts in concert with Raf (250) and Ras (Singh et al., unpublished). Perhaps, like many signaling pathways, Rnd3 is regulated in a tissue-specific manner, to signal to different effectors and perform different tasks. Similarly, the stage of the tumor may influence whether Rnd3 is up- or downregulated. Currently there is insufficient information to determine whether this is the case.

In contrast to Rnd3, there is currently little information regarding Rnd1 and Rnd2 in human cancer. The gene encoding Rnd2 is the centromeric neighbor of the breast/ovarian cancer susceptibility gene encoding BRCA1, although in the opposite orientation; however, there is currently no information available regarding Rnd2 and breast and ovarian cancers.

In cancers, chronic upregulation of Rnd3 may serve to selectively regulate its downstream targets. In normal cells, however, the activity of Rnd3 must be tightly regulated. I have hypothesized that Rnd3 activity is regulated by PKC-mediated phosphorylation, and this work is discussed in detail in Chapter 2. Below I set forth my rationale for this hypothesis.

Dynamic membrane association by an electrostatic switch

Numerous examples exist in the literature in which phosphorylation of membrane-associated proteins results in loss of affinity for membranes. One example is ARNO, a GEF for the Arf family of small GTPases (313). Binding of ARNO to the plasma membrane is mediated by dual action of its pleckstrin homology (PH) domain, which binds to phosphoinositides, and its polybasic domain, which interacts with acidic membrane phospholipids (314, 315). ARNO is phosphorylated on serine 392 located within its C-terminal polybasic region (316). Introduction of a negative charge to this region reduces interaction of ARNO with the plasma membrane and has the functional consequence of inhibiting guanine nucleotide exchange. This phosphorylation-mediated regulation of nucleotide exchange is the result of a process termed a "PH domain-electrostatic switch" (317).

The regulation of ARNO by the PH domain-electrostatic switch is analogous to the regulation of the MARCKS (myristoylated alanine-rich C kinase substrate) protein by a "myristoyl-electrostatic switch". MARCKS association with the plasma membrane requires a myristoyl fatty acid moiety as well as electrostatic interaction of its basic effector domain with acidic phospholipids (318). Phosphorylation of serines in the basic effector domain of MARCKS neutralizes its electrostatic interaction with the plasma membrane and causes its displacement from the plasma membrane (319).

K-Ras4B was shown to be a substrate for PKC phosphorylation in its C-terminal polybasic region (320), and our lab has shown recently, in collaboration with Mark Philips and colleagues, that this phosphorylation influences both its subcellular localization and function (321). It has been postulated that the C-terminal phosphorylation combines with the

C-terminal farnesyl isoprenoid modification to create a "farnesyl-phosphate switch" to regulate K-Ras4B membrane association and function. Previous studies have demonstrated that proper K-Ras4B membrane interactions require both hydrophobic interaction of the farnesyl group with the lipid bilayer and electrostatic interaction of the polybasic region with acidic phospholipids (136, 138).

C-terminal phosphorylation of Ras-related proteins and regulation of function

In addition to regulation by GDP/GTP cycling, there is growing evidence for regulation of GTPase function via stimulus-mediated posttranslational modifications. Several members of the Ras and Rho family of small GTPases have been shown to be substrates for phosphorylation at their C-terminal regions immediately upstream of the CAAX-containing membrane targeting motif (Figure 1.13). Furthermore, these phosphorylation events have been shown to have functional consequences. Rap1 was documented to be phosphorylated on serine 180, close to its C-terminus, by protein kinase A (PKA). This phosphorylation was shown to regulate its subcellular localization and association with effector molecules (322-324). Furthermore, RhoA was also documented to be phosphorylated close to its C-terminus on serine 188 by PKA (325). This phosphorylation event was shown to negatively regulate RhoA activity by translocation away from membranes, through enhanced interaction with RhoGDI (282, 326). Recently, it was shown that nerve growth factor (NGF) elicits PKA-dependent phosphorylation on serine 188 in PC12 neuronal cells (327). This phosphorylation event renders RhoA unable to bind to ROCK, which interacts with the C-terminus of RhoA. The authors suggested that

Protein	Phosph. site	Sequence (human)	Kinase
K-Ras4B	Serine 181	KKKKKKSKTK-----CVIM	PKC
Rap1-A	Serine 180	KKPKKKS--------CLLL	PKA
RhoA	Serine 188	RRGKKKSG-------CLVL	PKA
RalA	Serine 194	KKKKRKSLAKRIRERCCIL	Aurora-A
Rnd3	Serine 240	RKDKAKS--------CTVM	ROCK, PKC

Figure 1.13: C-terminal phosphorylation of small GTPases. Several small GTPases have been shown to be phosphorylated in their C-terminal region located within the polybasic region, just upstream of the CAAX motif. These phosphorylation events have been shown to have biological consequences and are believed to reduce the affinity of the proteins for the plasma membrane.

phosphorylation at serine 188 "may serve as a novel secondary switch of RhoA, capable of overriding GTP-binding-elicited effector activation to a subset of effector targets" (327).

As mentioned above, our group has recently shown that the previously appreciated phosphorylation of K-Ras4B (320) is directed by protein kinase C (PKC) at the C-terminal serine 181 (321). This phosphorylation event causes K-Ras4B to translocate from the plasma membrane to the mitochondria, resulting in enhanced apoptosis. Additionally, our group along with Chris Counter and colleagues has determined that Aurora-A kinase-mediated phosphorylation of the Ras-related small GTPase RalA (328) leads to loss of RalA plasma membrane localization and translocation to the cytosol and internal membranes (Lim et al., under revision).

I hypothesize that stimulus-mediated phosphorylation of Ras-related small GTPases may be a more common mode of regulation, distinct from GDP/GTP cycling, than has been appreciated. Rnd3 contains a putative PKC site at its extreme C-terminus at serine 240, and may therefore be regulated in a manner similar to those documented above for other Ras-related small GTPases (Figure 1.14). What effect PKC-mediated phosphorylation at serine 240 has on Rnd3 localization, and hence its function, has been a major area of my dissertation research and will be discussed further in detail in Chapter 2 below.

The protein kinase C (PKC) family of serine/threonine kinases

The PKC family of serine/threonine kinases is divided into three classes, grouped together on the basis of their sequence homology and modes of action (329) (Figure 1.15). Members of the conventional class of PKCs (α, βI, βII and γ) are Ca^{2+}-dependent and are activated by both phosphatidylserine (PS) and diacylglycerol (DAG). Members of the novel

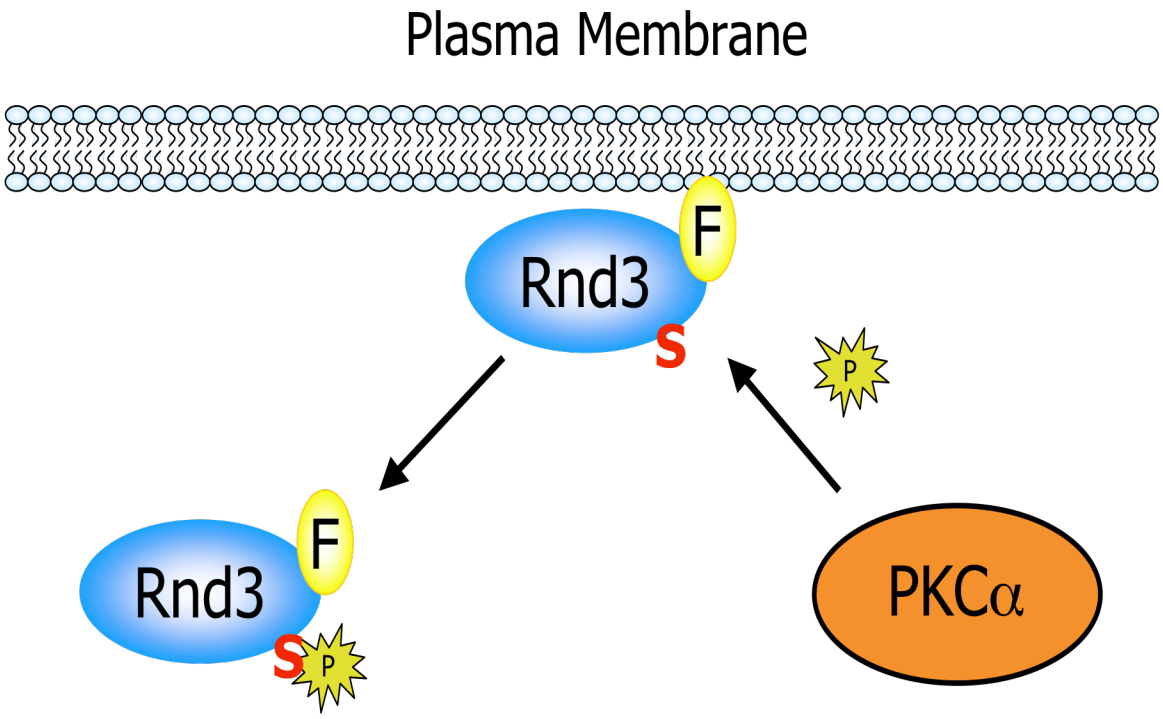


Figure 1.14: The proposed Rnd3 electrostatic switch. Rnd3 is associated with the plasma membrane due to hydrophobic interaction with the lipid bilayer and electrostatic interaction of the polybasic region with acidic phospholipids. Introduction of negative charges by phosphorylation reduces interaction of Rnd3 with the plasma membrane and causes its displacement from the plasma membrane.

Conventional PKCs	Novel PKCs	Atypical PKCs
α, βI, βII, γ	δ, ε, η, θ	δ, ι, λ
Ca^{2+}-dependent	Ca^{2+}-independent	Ca^{2+}-independent
Activated by PS and DAG	Activated by PS and DAG	Activated by PS, but not DAG

Figure 1.15: The PKC family of serine/threonine kinases. The PKC family of kinases is divided into three groups: conventional, novel and atypical. This grouping is based upon their sequence homology and modes of activation: PS is phosphatidylserine and DAG is diacylglycerol.

class of PKCs (δ, ε, η and θ) are Ca^{2+}-independent but are still activated by both PS and DAG. The atypical class of PKCs (ζ, ι and λ) is both Ca^{2+}- and DAG-independent. PKC is thought to reside in the cytosol in an inactive conformation and to translocate to the plasma membrane upon activation, where it regulates numerous cellular functions through its phosphorylation of target substrates (330, 331). Through the use of specific PKC inhibitors and genetically ablated mouse embryo fibroblasts, I have implicated PKCα, a conventional PKC, in the phosphorylation of Rnd3. I show, in Chapter 2 below, that PKCα-mediated phosphorylation of Rnd3 has an effect on Rnd3 localization and hence, on its function. I also demonstrate, in Chapter 3, that Rnd3 is subject to post-prenyl processing by Rce1 and Icmt, and that these post-translational modifications also regulate Rnd3 activity. Together, my dissertation research presents evidence for novel regulatory mechanisms for this atypical and interesting small GTPase of the Rho family.

CHAPTER 2

REGULATION OF RND3 LOCALIZATION AND FUNCTION BY PKCα-MEDIATED PHOSHORYLATION

Abstract

The Rnd proteins (Rnd1, Rnd2 and Rnd3/RhoE) form a distinct branch of the Rho family of small GTPases. Rnd3 decreases RhoA activity and regulates cytoskeletal organization and cell cycle progression. Since Rnd3 is a constitutively-activated GTPase, how Rnd3 itself is regulated to cause these changes is still under investigation. I have shown that, upon PKC agonist stimulation, Rnd3 undergoes an electrophoretic mobility shift and becomes metabolically labeled with ^{32}P, and its subcellular localization becomes enriched at internal membranes. These changes are blocked by inhibition of conventional, but not novel, PKC isoforms and do not occur in PKCα-null cells or with a nonphosphorylatable mutant Rnd3. I provide evidence that integrin engagement regulates downstream signaling functions of Rnd3 by inducing PKCα-mediated phosphorylation. This results in increased downstream signaling to Rho-ROCK pathway targets such as cofilin and myosin light chain (MLC). Thus, integrin engagement is a physiological regulator of Rnd3 posttranslational modification by PKCα, and this modification in turn is a mediator of Rnd3 subcellular

localization and downstream signaling. These results add a new mechanism of Rnd3 regulation, and clarify how Rnd3 modulates Rho signaling to alter cytoskeletal organization.

Introduction

Members of the Rho family of small GTPases are involved in the regulation of cell growth and survival as well as organization of the actin cytoskeleton to control cell shape and cell motility (5, 59, 332). These proteins act as molecular switches by cycling between an inactive GDP bound form and an active GTP bound form, the latter of which is then able to interact preferentially with effector molecules (1). This molecular switching is regulated by two classes of regulatory proteins: guanine nucleotide exchange factors (GEFs), that accelerates the intrinsic GDP-GTP exchange activity to favor formation of the active GTP-bound protein (81, 83), and GTPase activating proteins (GAPs), that inactivate G-proteins by enhancing their intrinsic GTPase activity to increase GTP hydrolysis and formation of the inactive GDP-bound form (87, 88). The most thoroughly characterized proteins of this family of small GTPases are RhoA, Rac1 and Cdc42 (4, 9, 10). Activation of RhoA leads to formation of stress fibers and focal adhesions (70), while activation of Rac1 and Cdc42 lead to the formation of lamellipodia and filopodia, respectively (71, 72).

The Rnd family of proteins (Rnd1, Rnd2 and Rnd3/RhoE, also known as Rho6, Rho7 and Rho8, respectively) form a unique branch of the Rho family (12). One striking difference between Rnd proteins and other members of the Rho family is their effect on the actin cytoskeleton. In contrast to RhoA, which upregulates stress fibers and focal adhesions in both epithelial cells and fibroblasts, expression of either Rnd1 or Rnd3 causes a decrease in stress fibers and the disappearance of focal adhesions, leading to cell rounding (hence “Rnd”

for rounding) (15, 243). Recently, multiple mechanisms by which Rnd proteins counteract the effects of RhoA have been brought to light. First, Rnd proteins were found to bind and activate p190RhoGAP, increasing its GAP activity toward GTP-bound RhoA. Expression of Rnd3 led to reduced cellular levels of GTP-bound RhoA by a p190RhoGAP-dependent mechanism (13). Second, Rnd3 was shown to bind directly to the RhoA effector ROCK1. Overexpression of Rnd3 inhibited ROCK1-induced stress fiber formation and phosphorylation of the ROCK1 target myosin light chain phosphatase (MLC-P) (14). Thus, Rnd proteins antagonize the effects of RhoA by multiple mechanisms.

Another striking difference from most other members of the Rho family is that Rnd proteins lack intrinsic GTPase activity (15) and their activity is not controlled by GEFs or GAPs. Because they are GTPase-deficient, Rnd proteins are bound to GTP *in vivo* and are constitutively in an "active" state (16) due to sequence divergence at highly conserved positions critical for normal GTP hydrolysis (15, 16). These results suggest that the activity of Rnd proteins is regulated not by GTP/GDP cycling, but at the level of expression and/or by post-translational modifications. Indeed, Rnd3 protein expression is induced upon growth factor addition (14), stress signaling (254, 256) and upon transformation by active Ras or Raf (250) and (Singh et al., unpublished). Rnd3 expression is also deregulated in human cancers (302, 307, 310-312). These changes in protein levels are mediated largely at the level of transcription, but may also be mediated at the level of translation or protein stability (255).

A majority of Ras and Rho family GTPases, including Rnd proteins, terminate in C-terminal CAAX tetrapeptide motifs (where C = cysteine, A = aliphatic residue and X = any amino acid) (95). The CAAX motif is a crucial signal needed for these proteins to be post-translationally modified by isoprenylation, proteolytic removal of the AAX residues, and

carboxylmethylation of the prenylated cysteine. The CAAX-signaled modifications are necessary but not sufficient for the correct subcellular localization, membrane association and biological activity of Rho GTPases (97, 333, 334). Additional C-terminal sequence elements, palmitoylated cysteines or polybasic residues, are also required to serve as a second subcellular targeting signal (136). Rnd3 terminates in a methionine in the "X" position and is thus farnesylated like Ras family proteins (16). Farnesylation of Rnd3 is required for membrane localization and for its ability to alter the cytoskeleton (Singh et al., unpublished data).

In addition to regulation by GDP/GTP cycling, there is growing evidence for posttranslational modification of Ras family GTPase function upon stimulus-mediated phosphorylation. Several small GTPases of the Ras and Rho families have been shown to be substrates for phosphorylation on serine residues at their C-terminal regions immediately upstream of the CAAX motif, and these phosphorylation events have been demonstrated to have functional consequences. Phosphorylation of Rap1 by protein kinase A (PKA) on S180, close to its C-terminus, has been shown to regulate its subcellular localization and its association with other proteins (322-324). Also, phosphorylation of RhoA by PKA on S188 close to its C-terminus has been shown to negatively regulate its activity by translocating RhoA from membranes, through enhanced interaction of RhoA with Rho-GDI (282, 325). Furthermore, we have shown recently that the previously appreciated phosphorylation of K-Ras4B (320) is directed by protein kinase C (PKC) at S181 of the C-terminus (321). This phosphorylation causes K-Ras4B to translocate from the plasma membrane to the mitochondria, resulting in the biological consequence of enhanced apoptosis (321). We reasoned that the location and function of Rnd proteins might also be regulated in a similar

manner by phosphorylation of a C-terminal serine residue (Figure 1.14). Studies undertaken to explore the role of phosphorylation in the regulation of localization, and hence function, are described in this chapter.

Materials and Methods

Antibodies and reagents

Anti-hemagglutinin (HA) (HA.11 clone 16B12) and -Myc (clone 9E10) antibodies were from Covance. Antibodies directed against β-actin (clone AC-74) and the FLAG epitope (M2), and the PKC agonist phorbol myristic acid (PMA), were from Sigma. Anti-green fluorescent protein (GFP) antibody (clone 3E6) was from Molecular Probes. Anti-PKCα antibody (clone 3) was from BD Biosciences. Anti-Rnd3 mouse monoclonal antibody (clone 4) was from Upstate Biotechnologies-Millipore. A rabbit polyclonal antibody directed against the N-terminus of Rnd3 has been described previously (250) and was kindly provided by Steen Hansen. Antibodies directed against total cofilin, phospho-cofilin (Ser 3), total MLC2, phospho-MLC2 (Ser 19), phospho-MARCKS (Ser152/156) and phospho-(Ser) PKC substrate were all from Cell Signaling Technology. Ionomycin and Y-27632 were from Calbiochem. Bryostatin-1, Gö-6976 and Rottlerin were from BIOMOL Research Laboratories. Calf intestinal phosphatase (CIP) was from New England Biolabs.

Molecular constructs

Mammalian expression constructs encoding HA-Rnd3 and GFP-Rnd3 were generated by inserting the full length human Rnd3 cDNA into the BamHI sites of pCGN-hygro (335) and pEGFP-C1 (Clontech), respectively. Site-directed mutagenesis to produce cDNA

sequences encoding the Rnd3-S240A, Rnd3-S240E, Rnd3-S7,11A and Rnd3-S7,11,240A mutant proteins was performed using the QuickChange Mutagenesis Kit (Stratagene), according to the manufacturer's instructions. Full length wild type and kinase-deficient (K368R) rat PKCα cDNAs (a generous gift from William Davis, University of North Carolina at Chapel Hill [UNC-CH]) were PCR-amplified and inserted into the XhoI and HindIII sites of both pEGFP-C1 and pCMV-3b, to generate GFP-PKCα and Myc-PKCα expression constructs, respectively. The FLAG-Rnd3 expression construct was generated by inserting full length human wild type Rnd3 cDNA into the EcoRI and XhoI sites of pHIT-FLAG3 (a generous gift from Yanping Zhang, UNC-CH). Generation of FLAG-Rnd3-S7A, S11A, S210A, T214A, S218A, S222A, S240A (a seven amino acid, phosphodeficient Rnd3; henceforth termed "Rnd3-All A") has been described previously (266). To generate the GFP-Rnd3-All A expression construct, the open reading frame from FLAG-Rnd3-All A was PCR amplified using a 5' primer containing a HindIII site and a 3' primer containing a SalI site. The PCR product was cut with HindIII and SalI restriction enzymes and ligated into the HindIII and SalI sites of pEGFP-C3. The tandem affinity purification (TAP) expression constructs were generated by inserting a DNA cassette encoding two protein-A modules, a tobacco etch virus (TEV) protease cleavage site and a FLAG tag in-frame into the BamHI and EcoRI sites of the plasmid pcDNA3.1(+) (Invitrogen) to produce a new vector termed pTAP-Go. A stop codon was inserted after the FLAG tag to produce pTAP-Stop. pTAP-Rnd3 was produced by inserting human Rnd3 cDNA into the EcoRI and XhoI sites in-frame behind the TAP tag cassette found in pTAP-Go. All sequences were verified by the Genome Analysis Facility at UNC-CH.

Cell culture and transfections

NIH 3T3 mouse fibroblasts were maintained in high glucose Dulbecco's modified Eagle medium (DMEM-H) (GIBCO-Invitrogen) containing 10% calf serum (Invitrogen) and penicillin-streptomycin (P/S, Invitrogen) at 37°C in a humidified atmosphere of 10% CO_2. Isolation of PKCα +/+ and -/- mouse embryonic fibroblasts has been described previously (336). These cells were cultured in DMEM-H without sodium pyruvate (Sigma) containing 10% fetal calf serum (FCS), glutamine and P/S (Invitrogen), and maintained at 37°C in a humidified atmosphere of 5% CO_2. HEK-293 cells were cultured in DMEM-H media containing 10% FCS and P/S, and maintained at 37°C in a humidified atmosphere of 5% CO_2. Expression vectors were transfected into NIH 3T3 and HEK-293 cells using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's instructions. Expression vectors were transfected into PKCα mouse embryo fibroblast cells using Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's instructions.

Calf intestinal phosphatase treatment assay

Equal amounts of lysate (devoid of phosphatase inhibitors) from NIH 3T3 cells expressing HA-Rnd3 (treated with or without 100 nM PMA for 10 min) were incubated in phosphatase buffer (100 mM NaCl, Tris-HCl pH 7.9, 10 mM $MgCl_2$ and 1 mM DTT) with or without 20 units of calf intestinal phosphatase at 37° C for 1 h. Lysates were resolved on 12% SDS-PAGE, transferred to Immobilon PVDF (Millipore) and immunoblotted with anti-HA antibody.

Fibronectin engagement assay

FN (20 μg/ml) was immobilized onto tissue culture dishes in PBS overnight at 4°C. Dishes were then rinsed twice with PBS and blocked with 0.5% delipidated bovine serum albumin (BSA) (Sigma) in DMEM for 1 h at 37°C. NIH 3T3 cells were serum-starved in DMEM containing 0.5% delipidated BSA for 6 h. Cells were then trypsinized with trypsin-EDTA (Cellgro) and neutralized in an equal amount of Trypsin Neutralizing Solution (Cambrex Bio Science). Cells were centrifuged, then resuspended in DMEM containing 0.5% delipidated BSA and kept in suspension on tissue culture plates coated with 1% agarose for 1 h at 37°C. After 1 h in suspension, cells were plated onto FN-coated tissue culture dishes for various times, onto plastic for 2 h, or kept in suspension for an additional 2 h before being lysed. Cell lysate proteins were precipitated in 12.5% trichloroacetic acid (TCA), washed twice with ice-cold acetone, dried and resuspended in Laemmli sample buffer. Samples were resolved on 12% SDS-PAGE, transferred to Immobilon PVDF (Millipore) and immunoblotted with appropriate antibodies.

Live cell imaging

To visualize the effects of PKC activation on Rnd3 localization in real time, NIH 3T3 cells were transiently transfected with GFP-Rnd3. After 24 h, cells were treated with either bryostatin-1 (100 nM) or PMA (100 nM) and ionomycin (500 μg/ml). Live cell images were captured on a Zeiss 510 LSM confocal microscope at 20X magnification and analyzed using LSM 5 Image browser software (Zeiss). To evaluate a role for different PKC isoforms in modulating Rnd3 localization, NIH 3T3 cells were transiently transfected with an expression

vector encoding GFP-Rnd3 as described above. After 24 h, cells were incubated with either DMSO vehicle or the PKC inhibitors Gö-6976 (2.5 μM) or Rottlerin (10 μM). After 3 h, cells were treated with PKC agonists PMA (100 nM) and ionomycin (500 μg/mL), and live cell images were captured by confocal microscopy at five min intervals as described above.

Western blot analysis

Cells were washed with PBS, lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate and supplemented with Complete Protease Inhibitor Cocktail tablets (Roche) along with phenyl-methyl sulfonyl fluoride (PMSF) and sodium pervanadate) and centrifuged to remove insoluble material. 2X Laemmli sample buffer was added to equivalent amounts of cellular lysates which were then resolved on 12% SDS-PAGE and transferred to Immobilon PVDF membranes. Membranes were blocked in 5% nonfat dry milk in TBS-Tween-20 and probed with appropriate primary antibodies, followed by anti-mouse or -rabbit IgG-horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences). Membranes were then incubated in SuperSignal West Dura Extended Duration substrate (Pierce) and the signal developed on HyBlot CL autoradiography film (Denville Scientific Inc.).

Results

Rnd3 subcellular localization is altered upon PKC activation. Our inspection of Rnd protein sequences revealed a consensus PKC site at serine 240 in the C-terminal hypervariable membrane targeting domain immediately upstream of the CAAX prenylation motif, similar to the arrangement seen in the C-terminus of K-Ras4B (Figure 2.1). We

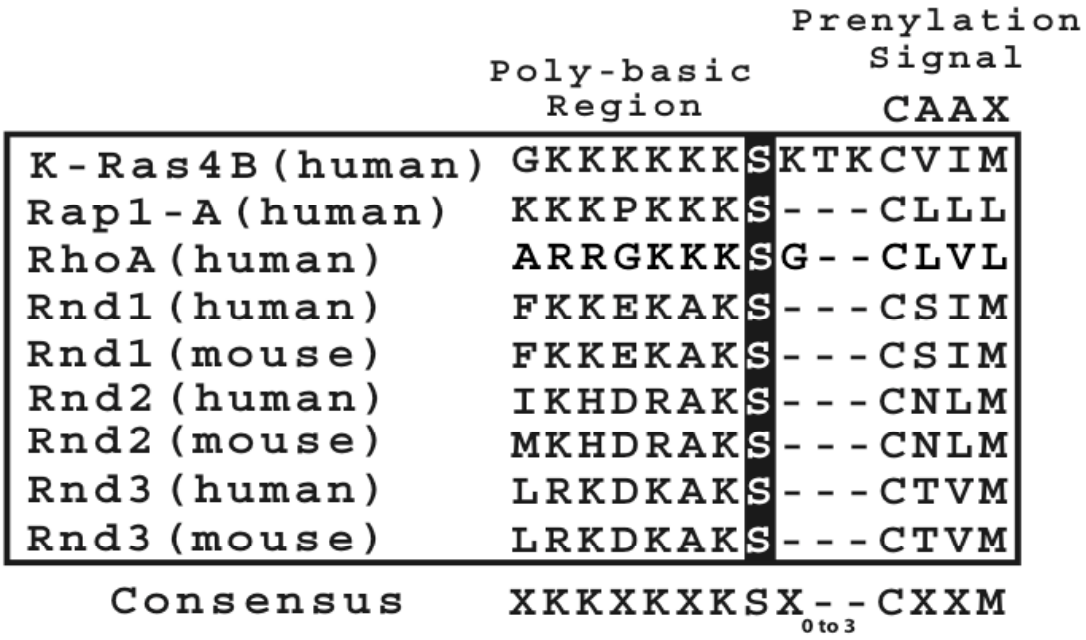
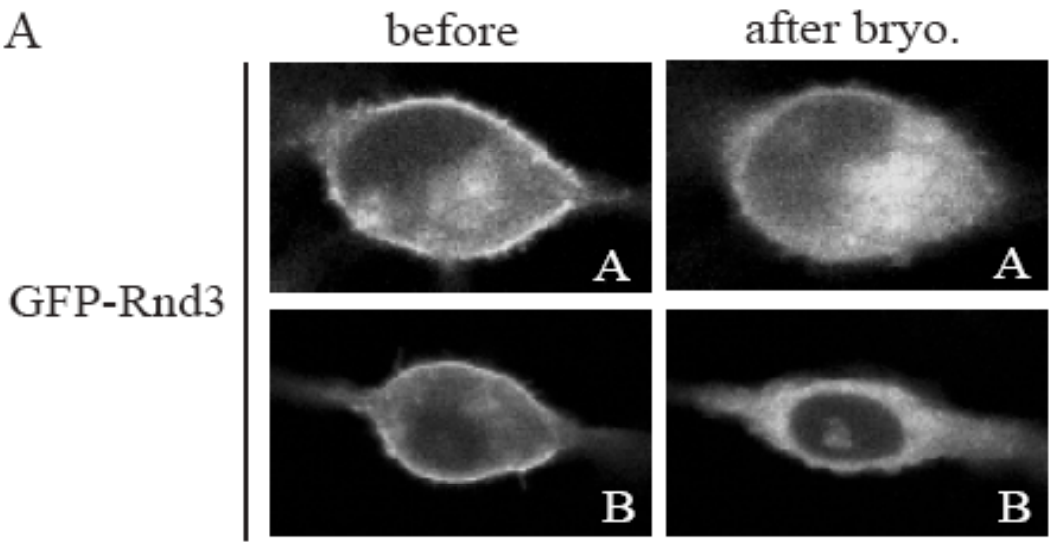


Figure 2.1: Amino acid sequence alignment of the C-terminal hypervariable membrane targeting regions of Ras superfamily small GTPases. Rnd proteins contain a conserved potentially phosphorylatable serine residue immediately upstream of the CAAX motif.

reasoned that the subcellular localization of Rnd3, like that of K-Ras4B, Rap1 and RhoA, might also be regulated by a combination of prenylation and C-terminal phosphorylation. We therefore used PKC agonists to test the effects of PKC activation on Rnd3 subcellular localization. To visualize these effects in living cells, we treated NIH 3T3 mouse fibroblast cells, transiently expressing GFP-tagged Rnd3, with the non-phorbol, PKC-specific agonist bryostatin-1. Live cell images were taken before and 10 min after treatment. As shown in Figure 2.2A, treatment with bryostatin-1 caused rapid loss of Rnd3 from the plasma membrane and enrichment in cytosol and internal membranes. To determine whether this response was unique to bryostatin-1 or a reproducible consequence of activating PKC, we also treated cells with PMA and ionomycin. PMA is a phorbol ester that specifically activates PKC, whereas ionomycin is a calcium ionophore that acts as a mobile ion carrier and activates the conventional forms of PKC by raising the intracellular levels of Ca^{2+}. NIH 3T3 cells expressing GFP-Rnd3 were treated with PMA + ionomycin, and live cell images were taken at 5-min increments. As shown in Figure 2.2B, concurrent treatment with PMA and ionomycin, like bryostatin-1, also caused loss of Rnd3 from the plasma membrane and enrichment at cytosol and internal membranes. The change in Rnd3 subcellular localization after treatment with distinct types of PKC agonists indicates that PKC activity is inversely correlated with Rnd3 plasma membrane binding.

The PKC family of serine/threonine kinases is divided into three classes, grouped together on the basis of their sequence homology and modes of activation (329). Most PKC family members are thought to reside in the cytosol in an inactive conformation and to translocate to the plasma membrane upon activation, where they regulate numerous cellular functions through phosphorylation of target substrates (330, 331). To begin to elucidate the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before
after bryo.
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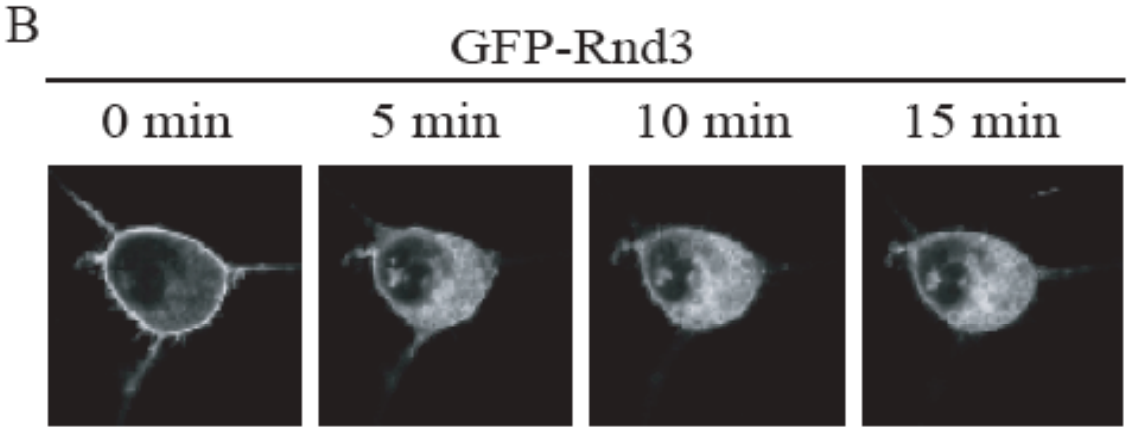
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GFP-Rnd3
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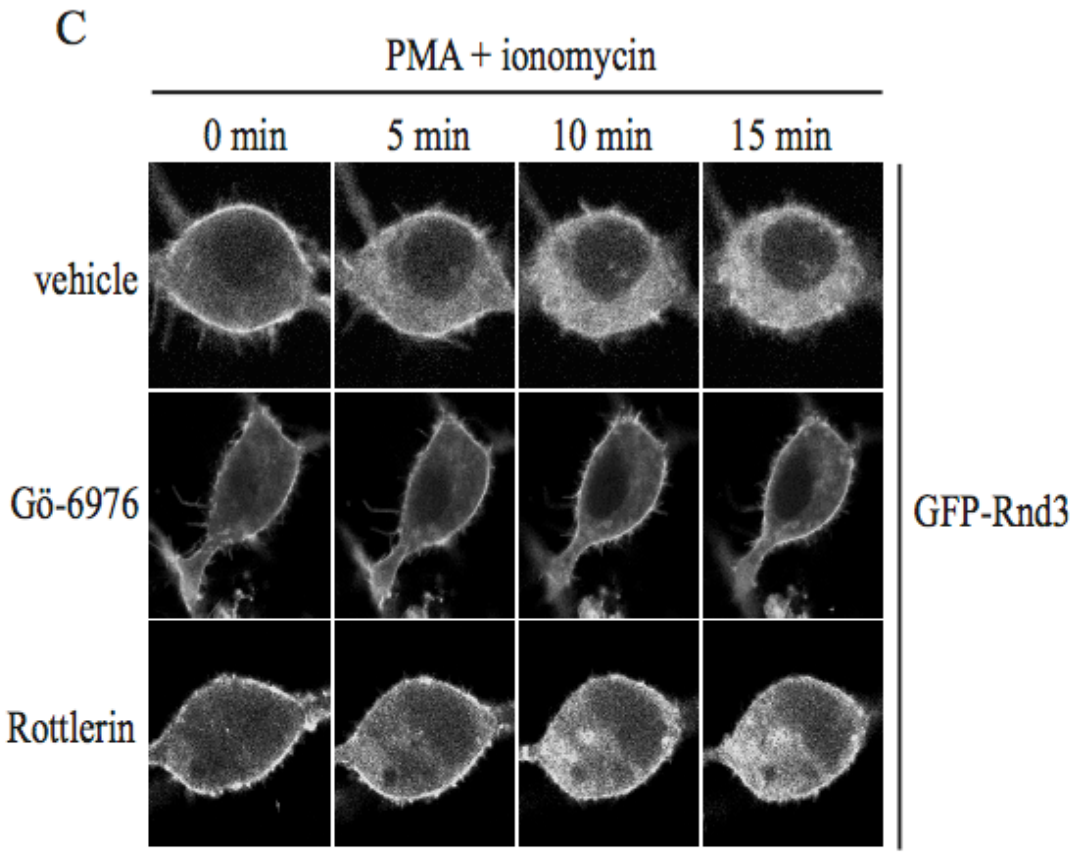
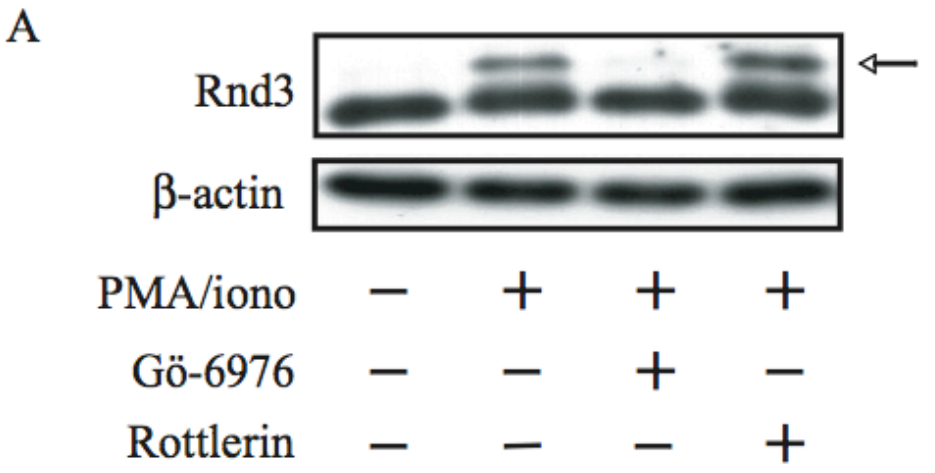
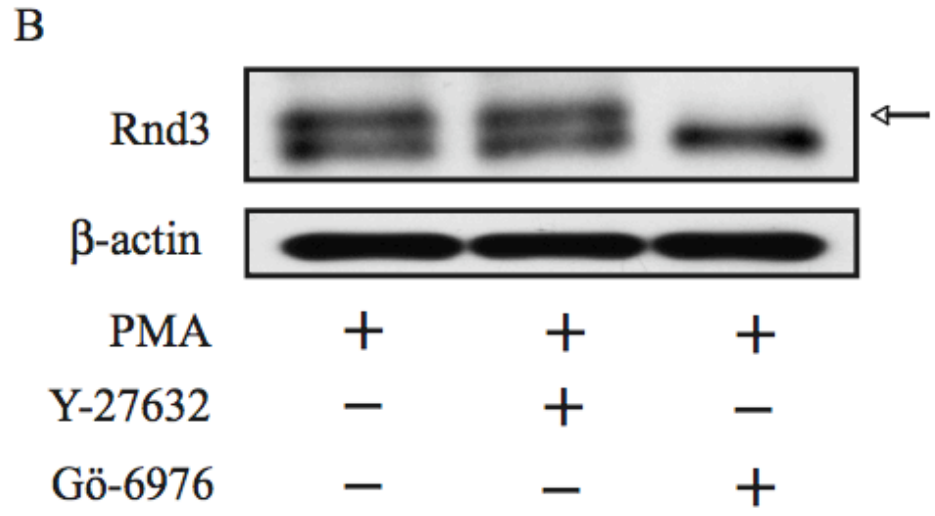


Figure 2.2: Activation of conventional, but not novel, PKCs causes Rnd3 translocation from the plasma membrane. A, PKC agonist bryostatin-1 causes loss of Rnd3 from the plasma membrane. NIH 3T3 cells transiently expressing GFP-Rnd3 were treated with bryostatin-1 (100 nM). Representative live images of two individual cells before (left panel) and 10 min after (right panel) addition of agonist are shown. **B,** Activation of PKC by using PMA + ionomycin also causes loss of Rnd3 from the plasma membrane. NIH 3T3 cells transiently expressing GFP-Rnd3 were treated with PMA (100 nM) + ionomycin (500 μg/ml). Live images are shown of a single cell visualized at 5 min increments. **C,** Inhibitor of conventional, but not novel, PKCs blocks Rnd3 translocation. NIH 3T3 cells transiently expressing GFP-Rnd3 were treated with DMSO vehicle, Gö-6976 (2.5 μM) to inhibit conventional PKCs, or Rottlerin (10 μM) to inhibit novel PKCδ, for 3 h prior to stimulation with PMA + ionomycin. Live images are shown of single cells visualized at 5 min increments. All images shown are representative of at least three independent experiments in which at least 50 cells were visualized.

identity of the specific PKC family member(s) responsible for the change in Rnd3 subcellular localization after stimulation with broad-based PKC activators, we employed the use of class-specific PKC inhibitors. First, we used Gö6976, an indocarbazole compound that has been shown to discriminate clearly between conventional and novel PKCs, selectively inhibiting conventional but not novel PKCs (337). In addition, we used Rottlerin, a naturally derived product that selectively inhibits the novel PKC isoform PKCδ (338). NIH 3T3 cells expressing GFP-Rnd3 as above were treated with either Gö6976, Rottlerin or DMSO vehicle alone for 3 h prior to stimulation with PMA + ionomycin. As shown in Figure 2.2C, the conventional PKC inhibitor Gö6976 but not the PKCδ-specific inhibitor Rottlerin or vehicle control blocked alterations in Rnd3 localization. This result indicates that at least one conventional PKC isoform is involved in the regulation of the subcellular location of Rnd3. Because NIH 3T3 cells express only the alpha isoform of conventional PKCs (339), it is likely that PKCα is the major isoform responsible for the effects seen on Rnd3 in these cells.

Rnd3 is phosphorylated upon PKC activation. To evaluate the possible direct involvement of Rnd3 phosphorylation in modulating its location, we wished to determine whether Rnd3 itself becomes phosphorylated upon activation of PKC. NIH 3T3 cells transiently expressing HA-tagged Rnd3 were treated with PKC agonists as in Figure 2.2C. Cell lysates were collected, resolved on SDS-PAGE and immunoblotted with anti-HA antibody. As shown in Figure 2.3A, a slightly slower migrating band consistent with post-translationally modified HA-Rnd3 appeared in lysates of cells stimulated with PMA + ionomycin, but not with DMSO vehicle. This result is consistent with phosphorylation of Rnd3 upon PKC activation. Furthermore, Gö6976, but not Rottlerin, blocked this mobility


A
Rnd3
β-actin
PMA/iono − + + +
Gö-6976 − − + −
Rottlerin − − − +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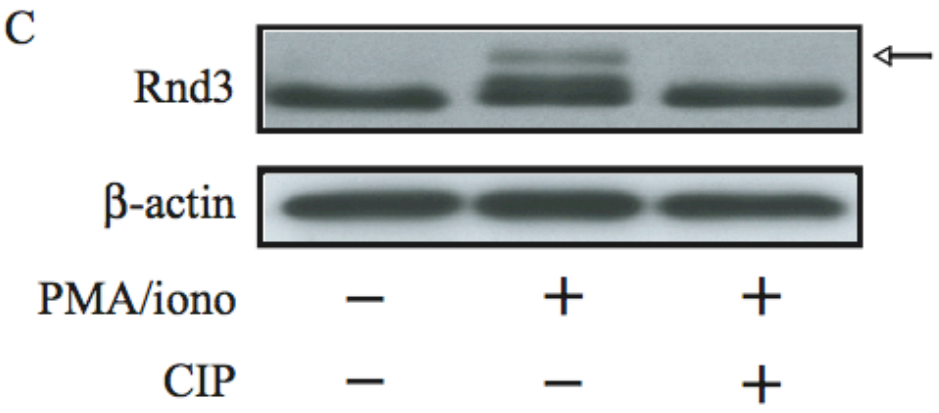
Y-27632 − + −
Gö-6976 − − +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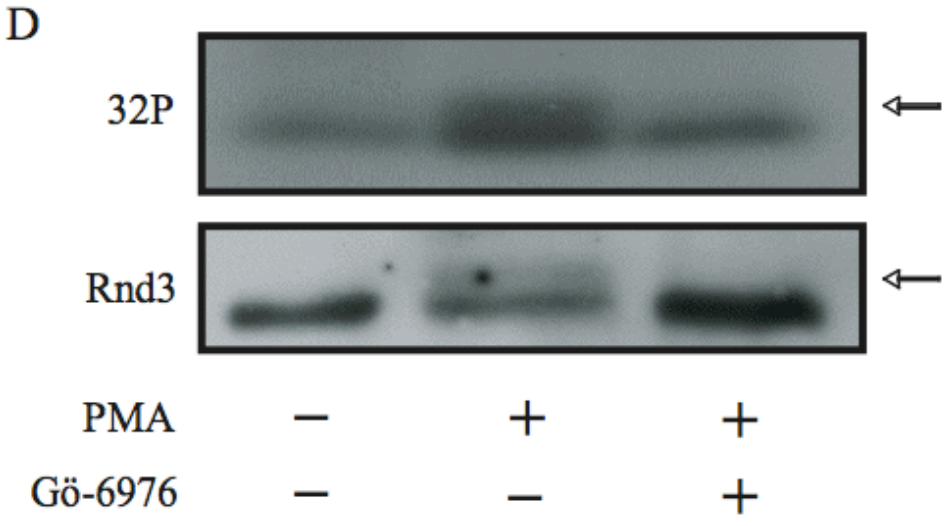
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Figure 2.3: Activation of conventional, but not novel, PKCs causes phosphorylation-dependent mobility shift of Rnd3 and incorporation of labeled phosphate. A, NIH 3T3 cells transiently expressing HA-Rnd3 were treated with PKC agonist and inhibitors as indicated. Cell lysates were resolved on SDS-PAGE and immunoblotted with anti-HA antibody. The slower migrating Rnd3 band is seen only in PKC agonist-stimulated cell lysates and is blocked by the PKC inhibitor Gö-6976. **B,** Pretreatment with the ROCK inhibitor, Y-27632, does not prevent the electrophoretic mobility shift of Rnd3. NIH 3T3 cells expressing HA-Rnd3 were pretreated for 3 h with either DMSO vehicle, Y-27632 (10 μM) or Gö-6976 (2.5 μM). Cells were then treated with PMA for 10 min as above, and cell lysates were resolved on SDS-PAGE. **C,** Calf intestinal phosphatase (CIP) treatment causes disappearance of the slower migrating band of Rnd3. NIH 3T3 cells transiently expressing HA-Rnd3 expression vector were treated with PMA + ionomycin. CIP was added to the cell lysate to reverse phosphorylation. Cell lysates were resolved on SDS-PAGE and immunoblotted with anti-HA antibody. **D,** Inducible but not basal Rnd3 phosphorylation is sensitive to inhibition of conventional PKCs. NIH 3T3 cells transiently expressing HA-Rnd3 were serum starved for 12 h, then cultured in phosphate-free media and metabolically labeled with ^{32}P-orthophosphate prior to treatment with PMA and/or Gö-6976. Anti-HA-immunoprecipitated proteins were resolved on SDS-PAGE and developed by autoradiography. Small portions of the immunoprecipitates were run on a duplicate gel, transferred to membrane and probed with anti-HA antibody to confirm equal amounts of immunoprecipitated proteins.

shift, suggesting that it is mediated by a conventional PKC. Recently, our collaborator, Anne Ridley, showed that Rnd3 is subject to ROCK-mediated phosphorylation (266). ROCK and PKCs share a common phosphorylation recognition sequence (340) and results from recent publications have demonstrated that the two families of kinases directly phosphorylate identical residues found on a number of protein substrates (341). We therefore investigated whether ROCK-mediated Rnd3 phosphorylation was stimulated by treatment with PMA. As seen in Figure 2.3B (lane 2), pretreatment of NIH 3T3 cells with the ROCK-specific inhibitor Y-27632 failed to prevent a mobility shift of HA-tagged Rnd3 when cells were treated with PMA. Once again, pretreatment with the conventional PKC inhibitor Gö6976 prevented the mobility shift of HA-tagged Rnd3 normally seen upon PMA treatment (lane 3). The results seen in Figure 2.3B suggest that the effects seen on Rnd3 when cells are treated with PMA (plasma membrane translocation and gel mobility shift) are due specifically to activation of PKC and not ROCK.

Next, to confirm that the slower migrating band represents a phosphorylation event, we treated cells with a PKC agonist followed by calf intestinal phosphatase (CIP) and immunoblotted with anti-HA antibody as before. CIP treatment (Figure 2.3C, lane 3) abrogated the appearance of the slower migrating band present in the cells treated with PKC agonist alone (Figure 2.3C, lane 2). Lastly, NIH 3T3 cells transiently expressing HA-Rnd3 were metabolically labeled with ^{32}P-orthophosphate and treated with PMA or DMSO vehicle. HA-tagged Rnd3 was then immunoprecipitated and resolved by SDS-PAGE. To confirm that equal amounts of HA-Rnd3 were present regardless of treatment, small portions of the HA immunoprecipitations were run on a separate gel and immunoblotted with anti-HA antibody. As shown in Figure 2.3D, the shifted form of HA-Rnd3 seen in the agonist-treated cells (lane

2) incorporated the radioactive phosphate label, indicating that the shifted form represents phosphorylated protein. Furthermore, pre-treatment with the conventional PKC inhibitor Gö6976 blocked the appearance of the radioactively labeled, slower migrating band (lane 3). Interestingly, Rnd3 is also basally phosphorylated, as shown by the incorporation of ^{32}P into the lower band regardless of PKC activation status. Taken together, these results are consistent with inducible phosphorylation of Rnd3 by activation of a conventional PKC.

Mutation of Rnd3 C-terminal S240 alone does not alter cell morphology, Rnd3 PKC sensitivity or Rnd3 localization. We had identified serine 240, just upstream of the CAAX motif, as a potential PKC phosphorylation site similar to those found in some other small GTPases that regulate their localization and function. To determine if phosphorylation of serine 240 is required for Rnd3 function, we used site-directed mutagenesis to mutate the serine to a nonphosphorylatable alanine residue, thereby generating a putatively phospho-deficient Rnd3 protein, termed S240A. After sequence confirmation, we transiently expressed both GFP-Rnd3-WT and GFP-Rnd3S240A in NIH 3T3 cells (Figure 2.4A). As anticipated, cells expressing empty GFP vector were flat and well spread, whereas cells expressing GFP-Rnd3-WT were rounded. Most of the GFP-Rnd3 was located at the plasma membrane, with additional cytosolic and perinuclear staining observed (Figure 2.4A). Surprisingly, the morphology of cells expressing either the putatively phospho-deficient S240A mutant or the phospho-mimetic S240E was indistinguishable from that of cells expressing GFP-Rnd3-WT, and GFP-Rnd3-S240A and GFP-Rnd3-S240E were localized similarly to that of GFP-Rnd3-WT (Figure 2.4A). In addition, staining with Texas Red phalloidin revealed no change in stress fibers in cells expressing the three different Rnd3

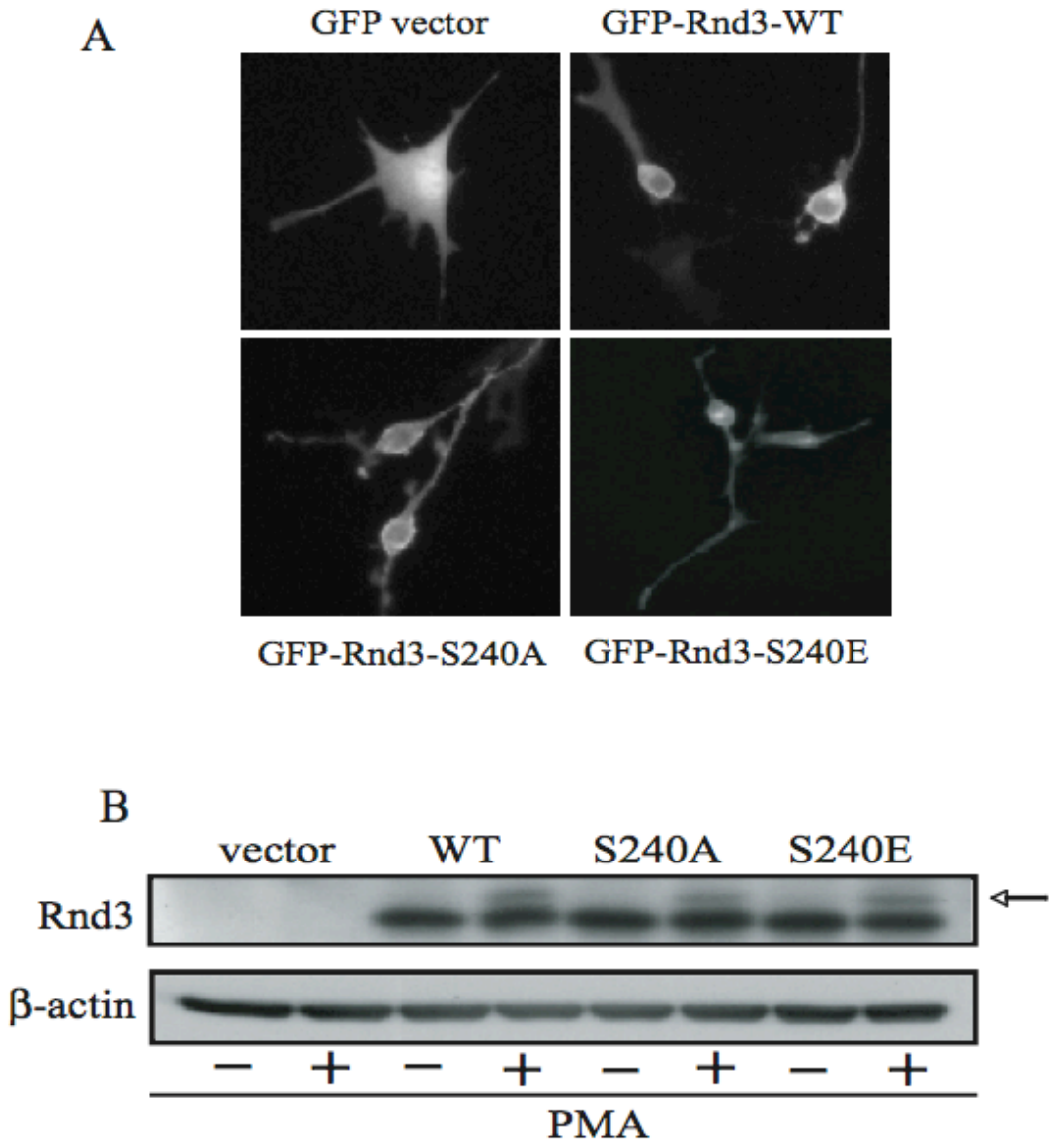
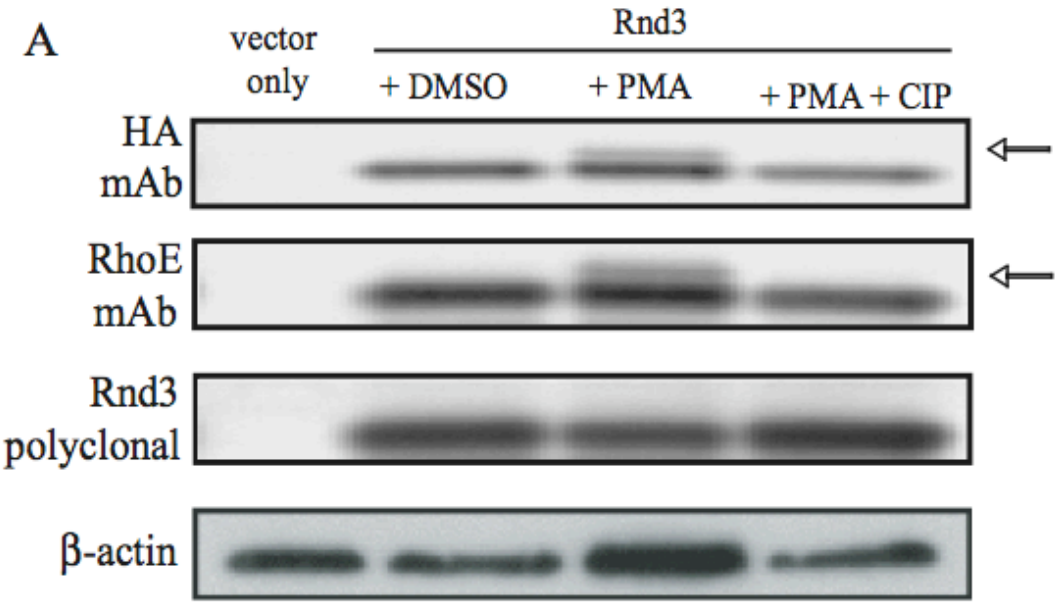


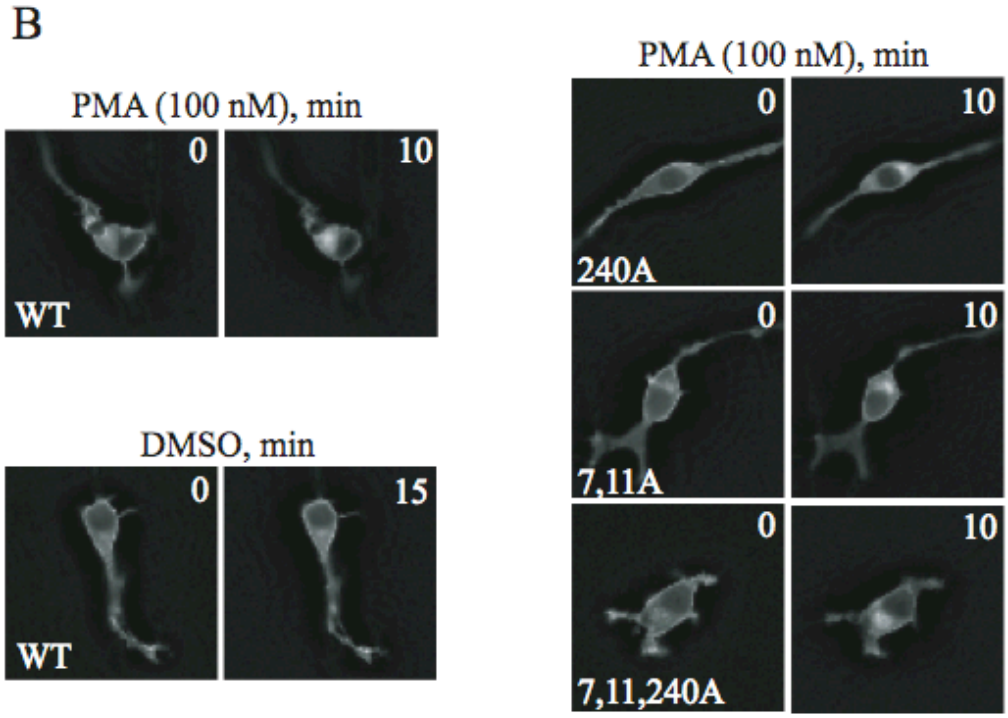
Figure 2.4: Phosphorylatable serine at residue 240 is not required for effects of PKC activation on Rnd3. A, Similar morphology of cells expressing GFP-Rnd3-WT, GFP-Rnd3-S240A and GFP-Rnd3-S240E. NIH 3T3 cells were transiently transfected with either GFP-vector, GFP-Rnd3-WT, GFP-Rnd3-S240A or GFP-Rnd3-S240E expression constructs. **B,** The phosphodeficient S240A mutant still displays a mobility shift. NIH 3T3 cells transiently expressing HA-Rnd3 proteins were treated with PMA for 10 min. Cell lysates were resolved on SDS-PAGE and immunoblotted with anti-HA antibody.

proteins (data not shown). These surprising results indicate that phosphorylation of S240 is not required for the ability of Rnd3 to cause cytoskeletal changes and cell rounding, and suggests that at least one other site in Rnd3 is also targeted by PKC.

To determine whether the S240A mutation rendered Rnd3 PKC-insensitive, we evaluated whether it retained or lost the PKC-induced mobility shift on SDS-PAGE. Contrary to our initial hypothesis, but consistent with the cell morphology data, both the WT and the S240A mutant forms of Rnd3 displayed the same mobility on SDS-PAGE in the absence or presence of PKC activation (Figure 2.4B). Taken together, we concluded that phosphorylation of Rnd3 at serine 240 alone is not sufficient to regulate Rnd3 subcellular localization or to produce the mobility-shifted form of Rnd3.

Additional sites of PKC-mediated phosphorylation in Rnd3. In optimizing the SDS-PAGE gel mobility-shift experiments using HA-tagged Rnd3, we employed a rabbit polyclonal antibody produced against the N-terminus of Rnd3 (250). Surprisingly, this antibody did not detect the mobility shift of HA-Rnd3 in lysates from NIH 3T3 cells that had been treated with PMA. Yet, this shift was seen reproducibly when immunoblotting with either an antibody directed against the HA epitope tag or a mouse monoclonal antibody directed against the entire Rnd3/RhoE protein (Figure 2.5A). We therefore postulated that the site or sites of phosphorylation responsible for the shifted form of Rnd3 must be located in the first 15 amino acids that were used in producing the Rnd3 polyclonal antibody. Visual inspection of the Rnd3 sequence, and the phosphorylation prediction program NetPhos 2.0 (342), revealed two additional consensus PKC phosphorylation sites at serines 7 and 11. Together with the previous data demonstrating a correlation between alteration of Rnd3


A
vector only
Rnd3
+ DMSO
+ PMA
+ PMA + CIP
HA mAb
RhoE mAb
Rnd3 polyclonal
β-actin


B
PMA (100 nM), min
0
10
WT
DMSO, min
0
15
WT
PMA (100 nM), min
0
10
240A
0
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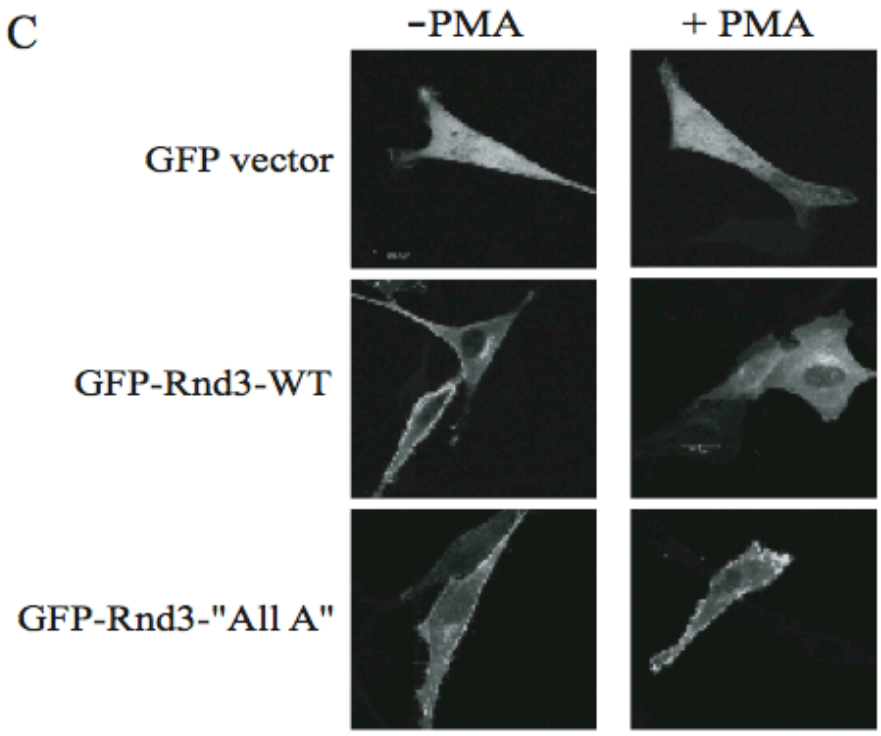
7,11A
0
10
7,11,240A


C
-PMA
+ PMA
GFP vector
GFP-Rnd3-WT
GFP-Rnd3-"All A"

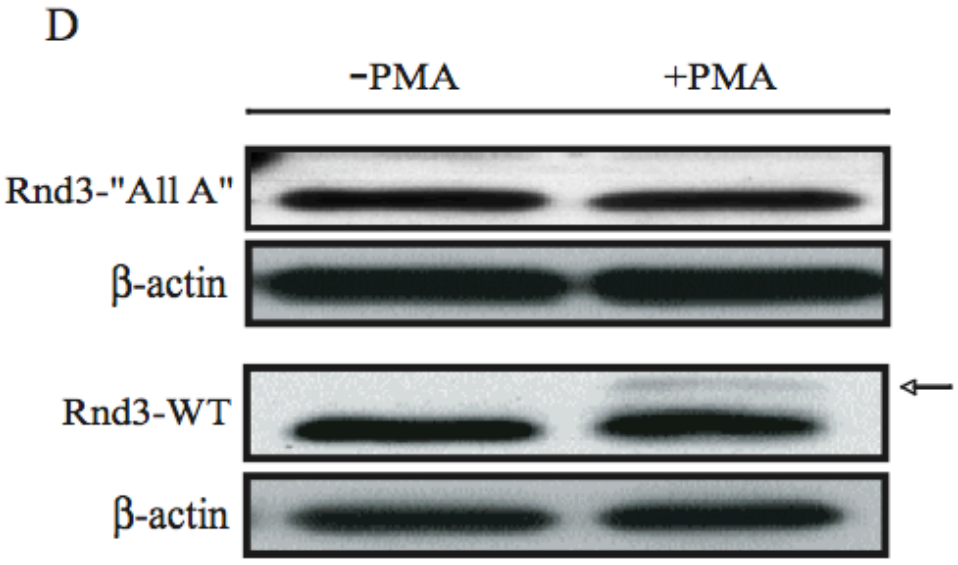

D
-PMA
+PMA
Rnd3-"All A"
β-actin
Rnd3-WT
β-actin

Figure 2.5: Multiple residues of Rnd3 are involved in its PKC-dependent translocation and electrophoretic mobility shift. A, An N-terminal peptide polyclonal antibody (aa 1-15) does not recognize the mobility-shifted form of Rnd3. NIH 3T3 cells transiently expressing HA-Rnd3 or empty vector were treated with either DMSO vehicle or PMA for 10 min +/- CIP treatment. Cell lysates were resolved on SDS-PAGE and immunoblotted initially with anti-HA antibody. The blot was then stripped and blotted sequentially with a specific anti-Rnd3/RhoE antibody and anti-Rnd3 antiserum. **B,** GFP-Rnd3 multiple phosphorylation mutants still translocate from the plasma membrane after PKC activation. NIH 3T3 cells were transiently transfected with either GFP-Rnd3-WT or GFP-Rnd3 phosphorylation mutants and treated with either vehicle or PMA for the indicated times. Live images were taken on a confocal microscope. **C,** GFP-Rnd3-WT, but not GFP-Rnd3-All A, translocate from the plasma membrane after PKC activation. NIH 3T3 cells were transiently transfected with either GFP-vector, GFP-Rnd3-WT or GFP-Rnd3-All A. Cells were treated with either vehicle or PMA for 10 min. Live cell images were taken on a confocal microscope. PMA treatment caused loss of GFP-WT-Rnd3 from the plasma membrane along with a corresponding flattened phenotype. A similar event was not seen in GFP-Rnd3-All A expressing cells. **D,** FLAG-Rnd3-WT, but not FLAG-Rnd3-All-A, displays an electrophoretic mobility shift after PKC activation. NIH 3T3 cells were transiently transfected with either FLAG-Rnd3-WT or FLAG-Rnd3-All-A and treated with either vehicle or PMA for 10 min. Lysates were resolved on SDS-PAGE and blotted with anti-FLAG antibody to visualize FLAG-tagged Rnd3 protein.

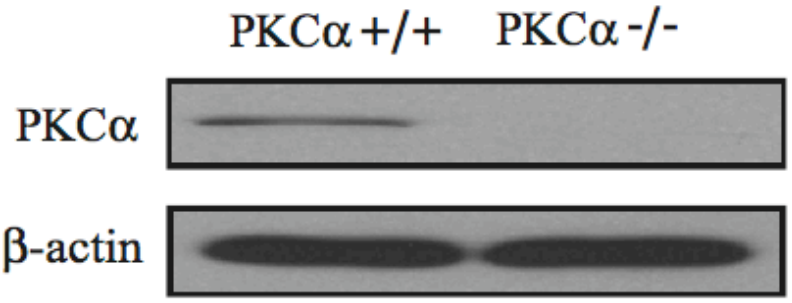
subcellular localization and the presence of a mobility-shifted form of Rnd3 on SDS-PAGE, we hypothesized that phosphorylation of Rnd3 within this unique N-terminal extension, which would introduce negative charges, could be responsible for the effects on Rnd3 seen after treatment with PKC agonist. We reasoned that phosphorylation of Rnd3 at serines 7 and 11 in the N-terminal extension, with or without phosphorylation at serine 240, may disrupt the polar interactions of Rnd3 with the plasma membrane. Site-directed mutagenesis was then used to generate GFP-tagged versions of Rnd3 that contained alanine substitutions at serines 7 and 11 along with serine 240, and these mutant GFP-Rnd3 constructs were expressed transiently in NIH 3T3 cells. However, as with GFP-Rnd3-S240A, the subcellular localization of both the double and triple serine mutants, GFP-Rnd3-S7,11A and GFP-Rnd3-S7,11,240A were also altered indistinguishably from that of GFP-Rnd3-WT after treatment with PMA (Figure 2.5B).

Possible explanations for the lack of effect of the triple mutant S7A, S11A, S240A is that phosphorylation at one or more other site(s) is the primary target of PKC or that Rnd3 can be phosphorylated on additional sites when the preffered sites are absent. To explore this possibility, numerous serine residues and a threonine (S7, S11, S210, T214, S220, S222 and S240) in Rnd3 were mutated to the corresponding phospho-deficient alanines to generate a nonphosphorylatable form of Rnd3 (Rnd3-All A). This nonphosphorylatable mutant was used in a PMA treatment translocation assay. As seen in Figure 2.5C, stimulation of NIH 3T3 cells with PMA caused the loss of GFP-tagged Rnd3 WT from the plasma membrane. Consistent with a requirement for Rnd3 to become phosphorylated in order for it to translocate upon PKC activation, PMA stimulation did not cause the loss of the nonphosphorylatable "All A" mutant Rnd3 from the plasma membrane. Furthermore, a

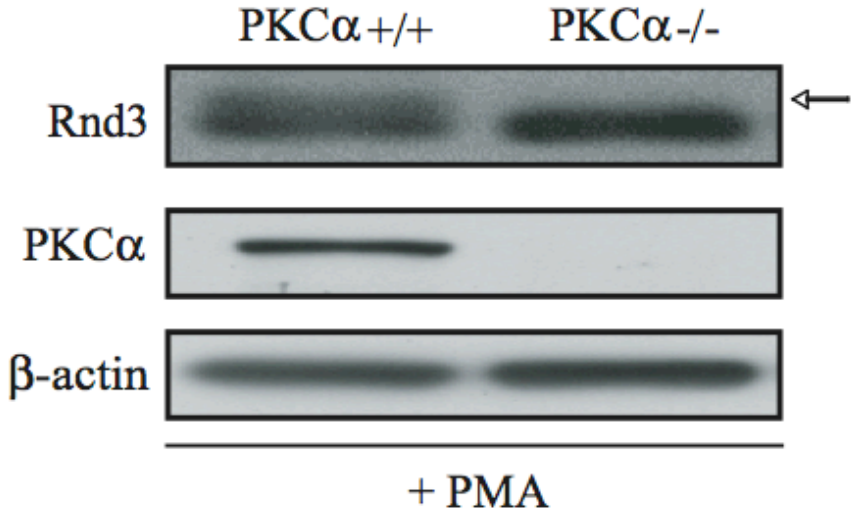
FLAG-tagged version of Rnd3-All A did not display a gel mobility shift when expressed in NIH 3T3 cells stimulated with PMA, as was seen with FLAG-Rnd3-WT (Figure 2.5D). Thus, while further work will be needed to identify the minimal number of PKC phosphorylation sites needed for membrane translocation, both the mobility shift and the translocation seen upon PKC activation require that Rnd3 itself be able to become phosphorylated.

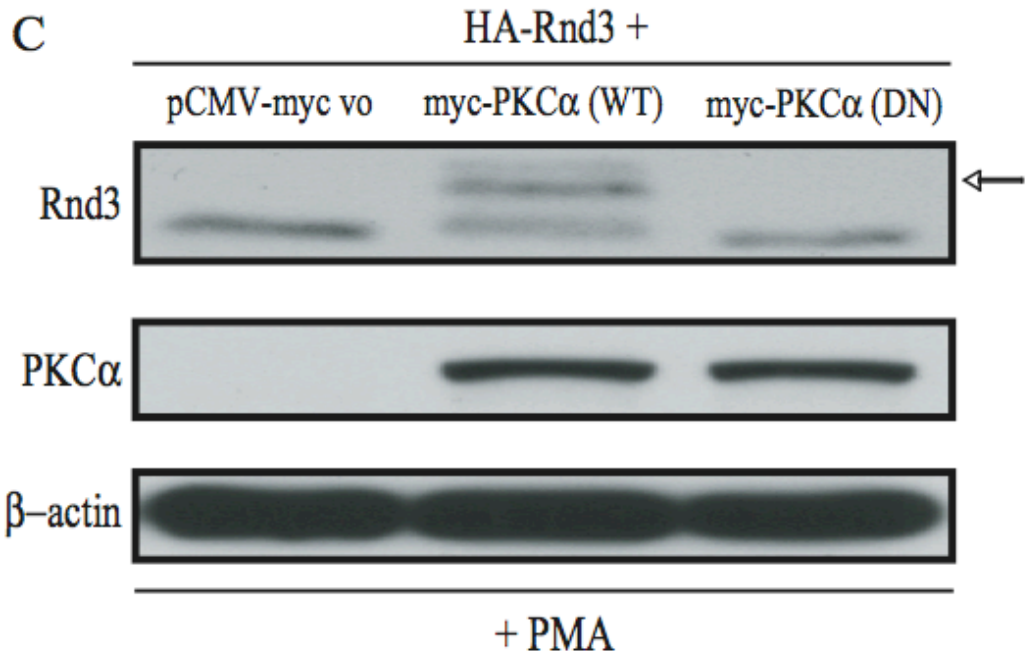
PKCα is the isoform responsible for Rnd3 phosphorylation. While the specific target residue(s) have not yet been determined, the exact identity of the PKC isoform(s) responsible for the phosphorylation and altered localization of Rnd3 upon PKC activation also remained to be uncovered. Based on the PKC inhibitor data shown previously (Figures 2.2C, 2.3A and 2.3B) and the fact that NIH 3T3 cells express only the alpha isoform of conventional PKCs (339), we hypothesized that PKCα was the isoform involved. Therefore, we performed additional studies in mouse embryo fibroblasts (MEFs) in which PKCα had been genetically ablated (336). Cell lysates from PKCα -/- and matched control WT MEFs were separated on SDS-PAGE and immunoblotted with an isoform-specific anti-PKCα antibody. As shown in Figure 2.6A, PKCα protein was undetectable in the -/- MEFs whereas it was easily detectable in the wild type matched control MEFs. We then tested whether PKCα is required for the electrophoretic mobility shift of Rnd3 seen upon stimulation with PKC agonists. PKCα -/- MEFs and WT control cells transiently expressing HA-Rnd3 were treated with the PKC agonist PMA. Lysates from these cells were resolved on SDS-PAGE and immunoblotted with anti-HA antibody. As shown in Figure 2.6B, the slower migrating band of Rnd3 was seen only in the WT MEF cells and not in the PKCα -/- MEF cells,

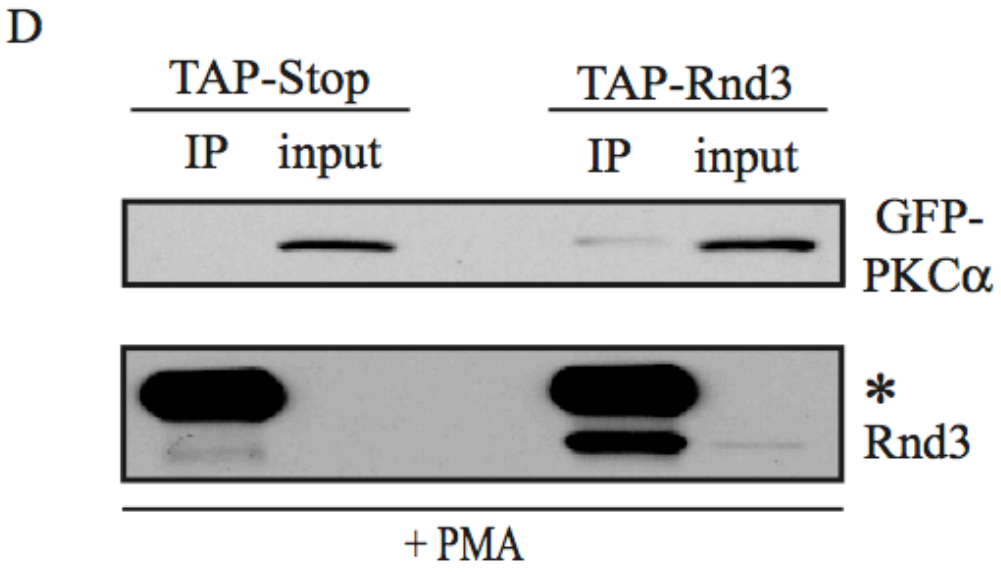
A



B




C
HA-Rnd3 +
pCMV-myc vo
myc-PKCα (WT)
myc-PKCα (DN)
Rnd3
PKCα
β–actin
+ PMA


D
TAP-Stop
TAP-Rnd3
IP
input
IP
input
GFP-
PKCα
*
Rnd3
+ PMA

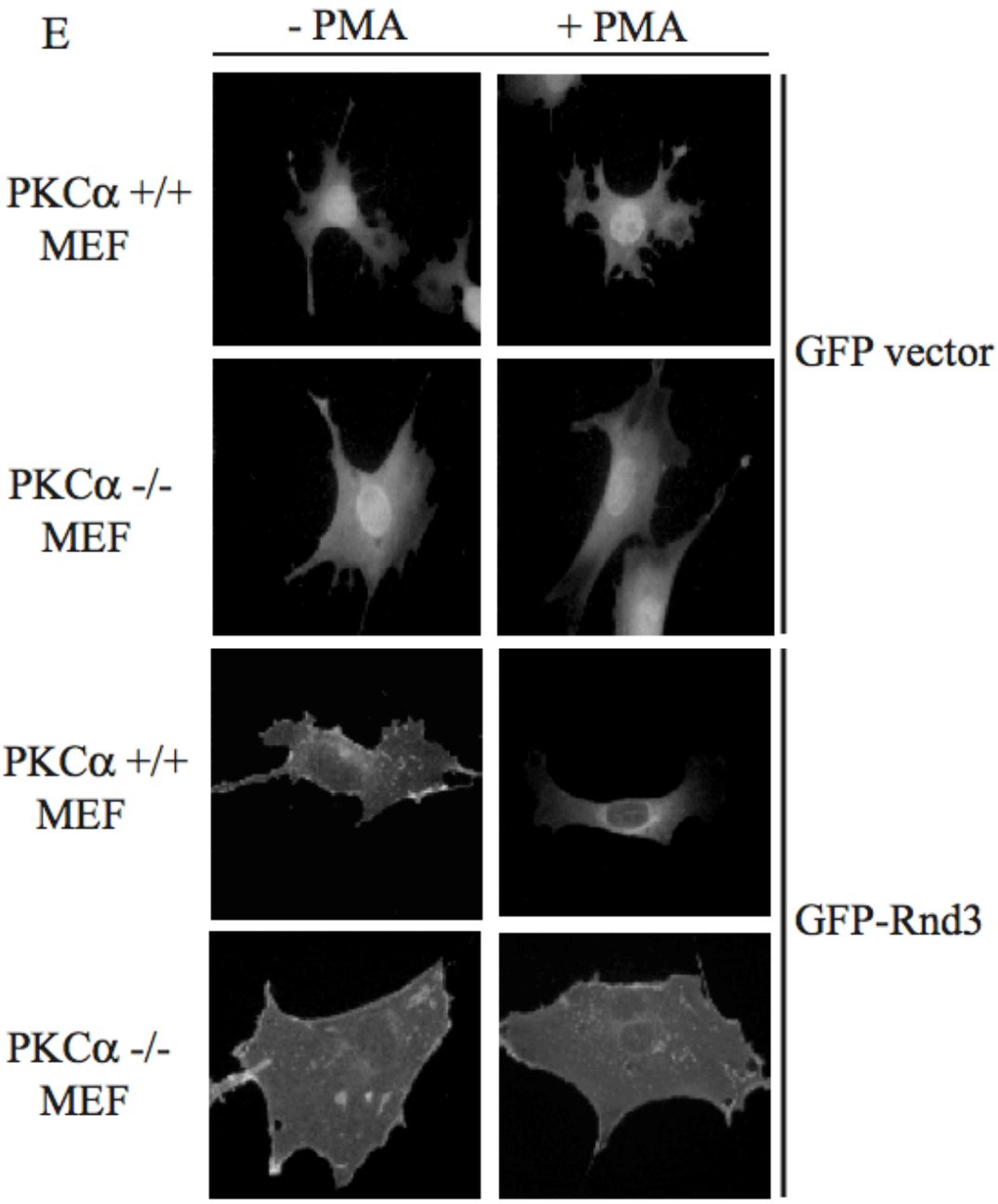

E
- PMA
+ PMA
PKCα +/+ MEF
PKCα -/- MEF
PKCα +/+ MEF
PKCα -/- MEF
GFP vector
GFP-Rnd3

Figure 2.6: PKCα is the PKC isozyme responsible for Rnd3 phosphorylation. A, PKCα null MEF cells do not express any detectable PKCα protein. Cell lysates from PKCα -/- MEFs and matched WT control MEFs were separated by SDS-PAGE and probed with an anti-PKCα antibody. **B,** PMA stimulation causes a gel mobility-shift of Rnd3 in WT but not in matched PKCα null MEFs. Cells transiently expressing HA-Rnd3 were treated with PKC agonist PMA for 10 min. Cell lysates were resolved on SDS-PAGE and blotted with anti-HA antibody. **C,** Reintroduction of PKCα-WT, but not of dominant negative (DN) PKCα-DN, into PKCα null MEFS causes a mobility shift of Rnd3 when cells are treated with PKC agonist PMA. Cells transiently expressing HA-Rnd3 along with either pCMV-vector, Myc-PKCα-WT or Myc-PKCα-DN were treated with PKC agonist PMA for 10 min. Cell lysates were resolved on SDS-PAGE and probed with anti-HA antibody. **D,** Rnd3 and PKCα interact *in vivo*. HEK 293 cells, stably expressing either empty pTAP2 vector only or Rnd3 fused to the TAP tag, were transiently transfected with a GFP-PKCα expression construct and were treated with PMA for 10 min. Lysates from these cells were immunoprecipitated with FLAG antibody. Immunoprecipitates, along with cell lysate inputs, were resolved on SDS-PAGE and probed with anti-GFP and anti-FLAG antibodies (* = IgG heavy chain). **E,** PMA stimulation causes loss of GFP-Rnd3 from the plasma membrane in matched WT control MEFs, but not in PKCα -/- MEFs. Cells transiently expressing either GFP vector only or GFP-Rnd3 were treated with PKC agonist PMA for 10 min. Images were taken before and after PMA treatment.

demonstrating that the PKCα isoform is required for the mobility-shifted form that we confirmed previously to represent PKC-mediated phosphorylated Rnd3. To confirm that the kinase activity of PKCα is required for its effects on Rnd3 phosphorylation, we reintroduced either WT or kinase-deficient (KD) PKCα into PKCα -/- MEFs and looked for restoration of the appearance of the slower migrating form. The K368R PKCα mutant is considered kinase-deficient as it abolishes ATP binding ability (343). PKCα -/- MEFs, transiently expressing HA-Rnd3 along with either empty Myc-vector only, Myc-PKCα-WT or Myc-PKCα-KD were treated with the PKC agonist PMA, and cell lysates were resolved on SDS-PAGE and immunoblotted with anti-HA antibody. As shown in Figure 2.6C, an electrophoretic mobility shift of HA-Rnd3 was seen only upon reintroduction of PKCα-WT but not kinase-deficient PKCα. Thus, the kinase activity of PKCα is required for Rnd3 phosphorylation.

Rnd3 and PKCα physically interact. We next investigated the possibility of a direct physical interaction between Rnd3 and PKCα *in vivo*. To this end, HEK-293 cells stably expressing either Rnd3 fused to a tandem affinity purification (TAP) tag (pTAP-Rnd3) or the pTAP2 tag-only empty vector negative control (pTAP-Stop) were transfected along with a GFP-PKCα expression construct. The TAP tag used in this experiment employs two Protein-A modules fused in frame upstream of a FLAG tag (see Materials and Methods). Cells were stimulated with PMA, lysates were collected and TAP-tagged Rnd3 was immunoprecipitated using FLAG antibody and then resolved on SDS-PAGE. As seen in Figure 2.6D, GFP-tagged PKCα was co-immunoprecipitated in lysates where TAP-tagged Rnd3 was expressed and not in the TAP-tag only control lysates. GFP-PKCα did not co-immunoprecipitate with

TAP-Rnd3 in the absence of the PKC agonist PMA (data not shown). The data in Figure 2.6D thus point to a direct interaction of Rnd3 with PKCα in cells that have been stimulated to activate PKCα, and further bolster the results of previous experiments where both the PMA-mediated translocation and electrophoretic mobility shift of Rnd3 were abrogated by the presence of the conventional PKC inhibitor Gö6976 (Figures 2.2C, 2.3A and 2.3B).

Rnd3 does not translocate from the plasma membrane in PKCα null MEFs after PKC activation. Next, we transiently expressed GFP vector only and GFP-Rnd3 in both PKCα -/- and matched control WT MEFs. Cells were treated with the PKC agonist PMA and images were taken before and after treatment. As seen in Figure 2.6E, PMA treatment has no effect on the cellular localization of GFP alone in either PKCα -/- or the matched control WT MEFs. However, PMA treatment in WT MEFs caused the loss of GFP-Rnd3 from the plasma membrane. In contrast, PMA treatment did not cause the loss of GFP-Rnd3 from the plasma membrane in PKCα -/- MEFs. Thus, the data presented (the use of a specific conventional PKC inhibitor and PKCα -/- MEFs, along with the direct interaction between Rnd3 and PKCα) suggests that PKCα is the isoform responsible for Rnd3 phosphorylation.

Fibronectin engagement results in PKCα-mediated phosphorylation of Rnd3. We speculated that physiologically important pathways involving both alterations in cytoskeletal organization and activation of PKCα may lead to phosphorylation of Rnd3. PKCα was one of the first signaling molecules detected within focal adhesions (344), and evidence now suggests that integrins and the transmembrane heparan sulphate proteoglycan syndecan-4 can act cooperatively to activate PKCα, to then generate the adhesion-mediated signals necessary

for the assembly of stress fibers and focal adhesions (345). Consistent with this, pharmacological inhibitors of PKCα and transient expression of DN PKCα suppress focal adhesion formation and cell migration mediated by α5β1 integrins in cells plated on FN (346). In addition, the RhoA-selective activator p115RhoGEF is a substrate for and is stimulated by PKCα (347). Finally, RhoGDI is a direct phosphorylation target of PKCα, leading to disruption of RhoGDI-RhoA binding, increased RhoA membrane association, and upregulation of RhoA signaling (348). Taken together, we envisioned a model in which inhibiting restraints placed on RhoA by Rnd3 could be eliminated by FN engagement-mediated phosphorylation and cytoplasmic sequestration of Rnd3 by PKCα. In this scenario, outlined in the model presented in Figure 2.7, Rnd3 is a key mediator of this cascade.

We therefore investigated the effects of fibronectin engagement on cell spreading and PKCα-mediated phosphorylation of Rnd3. To this end, NIH 3T3 cells were serum-starved for 6 h in culture medium containing 0.5% delipidated-BSA, to avoid signals from normal serum lipids such as lysophosphatidic acid (LPA) that are known to stimulate Rho activity. After serum starvation, cells were kept in suspension for 1 h and then either plated on FN-coated tissue culture plates for various times or kept in suspension for a further 2 h, as decribed in Experimental Procedures. Serum-starved cells attached and spread within 20 min when plated on FN, but attached poorly and did not spread even at 120 min when plated on plastic (Figure 2.8A).

We then investigated whether FN engagement-mediated activation of PKCα leads to phosphorylation of Rnd3. Lysates from cells transiently expressing FLAG-Rnd3, treated as indicated above, were probed with anti-FLAG antibody to confirm that equal amounts of FLAG-Rnd3 protein were present in each sample. Surprisingly, despite the unequivocal

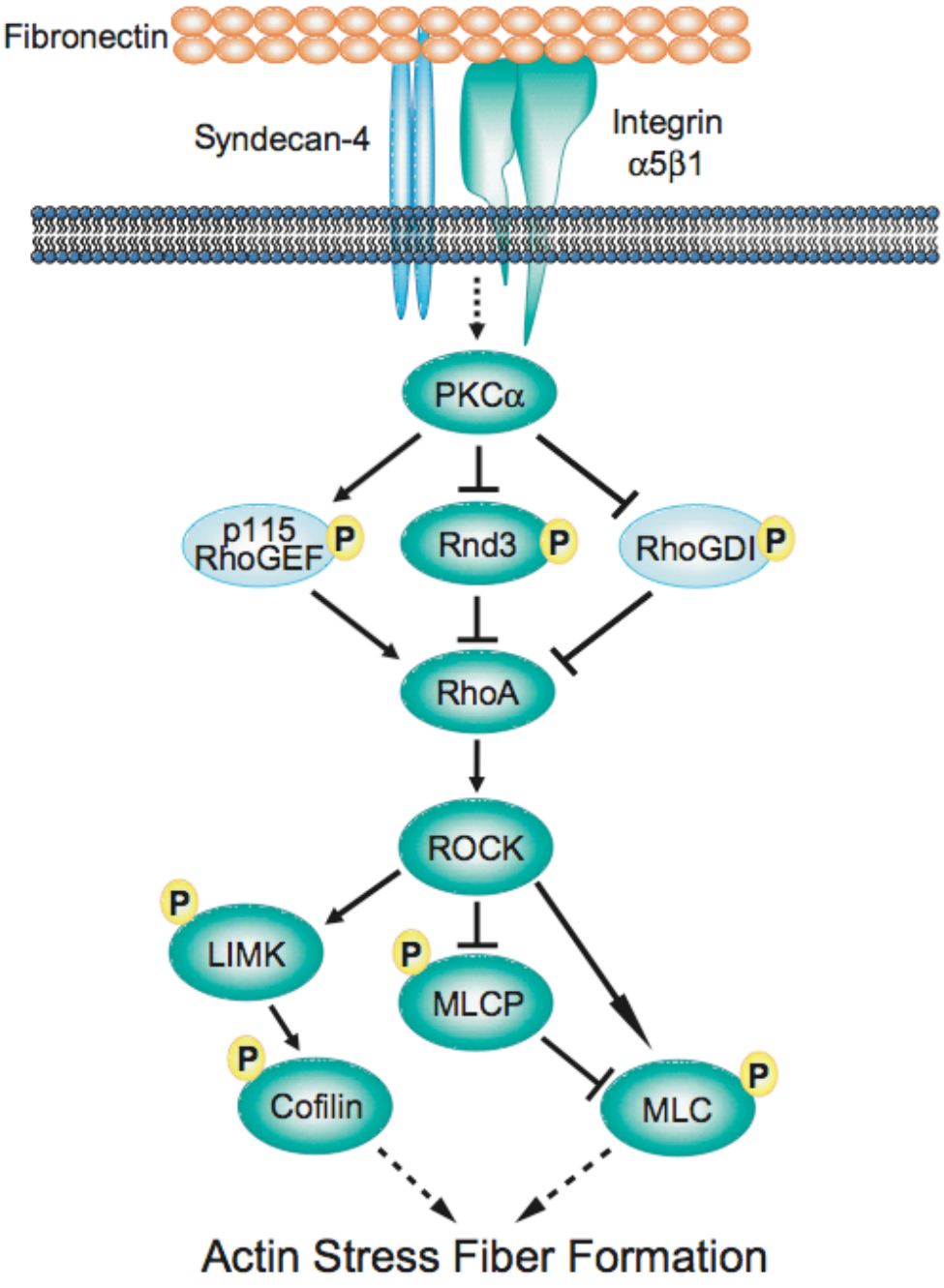
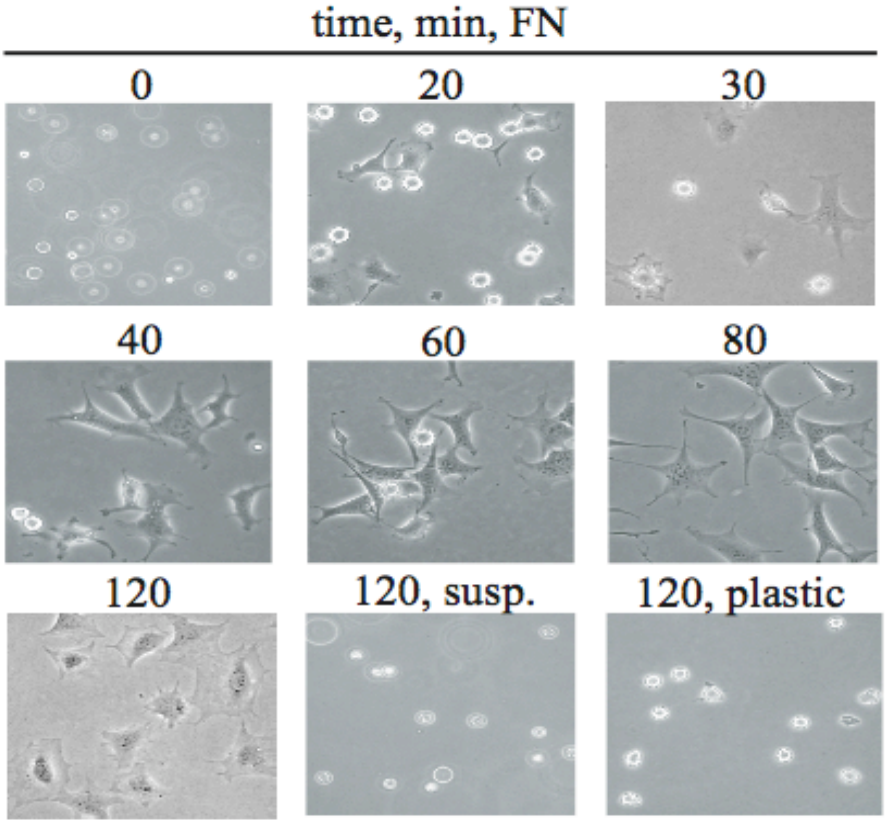


Figure 2.7: Model: Cellular engagement of fibronectin causes activation of PKCα through α5β1 integrin – syndecan-4 and leads to signaling through the Rho-ROCK pathway.

A



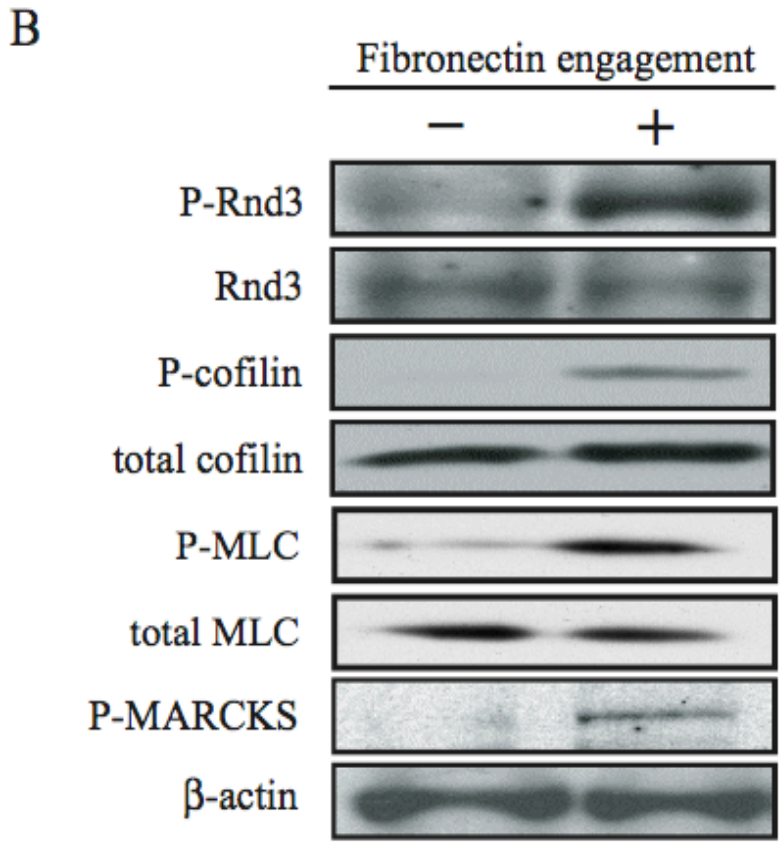


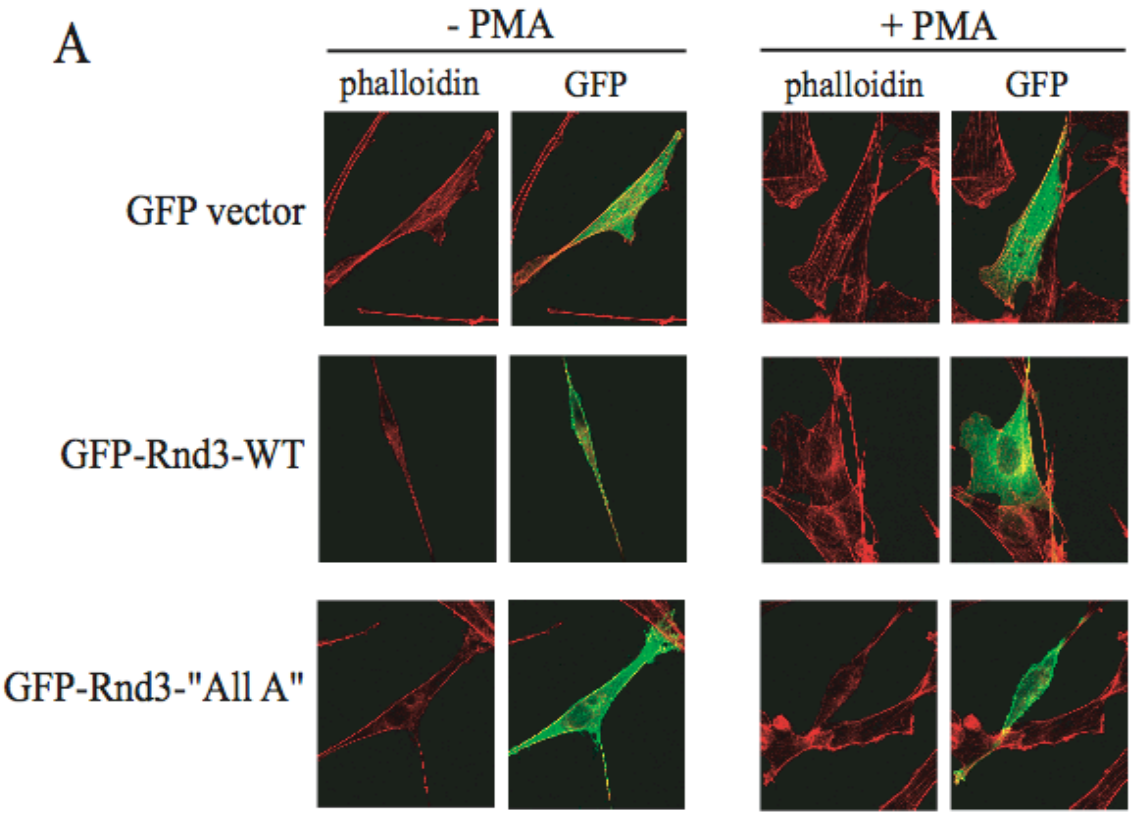
Figure 2.8: Fibronectin engagement results in PKCα-mediated phosphorylation of Rnd3. A, Serum-starved cells attached and spread within 20 min when plated on FN, but attached poorly and did not spread even at 120 min when plated on plastic. NIH 3T3 cells were serum-starved in 0.5% delipidated BSA and plated on FN for various times, held in suspension or plated on plastic, as decribed in Materials and Methods, then visualized by using a light microscope. **B,** Fibronectin engagement leads to increased phosphorylation of exogenous FLAG-tagged Rnd3. Cells transiently expressing FLAG-Rnd3 were treated as above. Cell lysates were separated by SDS-PAGE and probed with anti-FLAG antibody to detect the presence of exogenous Rnd3 protein. The blot was then stripped and re-probed with an anti-phospho-serine PKC substrate antibody. Lysates were also probed with anti-P-MARCKS antibody to detect PKCα-dependent phosphorylation, and with anti-P-cofilin and anti-P-MLC antibodies to detect activity of the Rho-ROCK signaling pathway.

activation of PKCα as shown by increased levels of phosphorylated MARCKS protein, no electrophoretic mobility shift of Rnd3 was seen in lysates from cells plated on FN. Phosphorylation of MARCKS (myristoylated alanine-rich C kinase substrate) is a reliable marker for activation of PKCα (349-352). We then considered the possibility that activation of PKC upon integrin engagement might produce a weaker signal than that produced upon stimulation with the PKC agonists bryostatin-1 or PMA, the latter of which could lead to phosphorylation of additional sites responsible for the mobility-shifted form of Rnd3. We therefore probed the same blot with an antibody that specifically detects phosphorylated serines in PKC substrate proteins (353). Using this antibody, phosphorylated FLAG-Rnd3 protein was easily detectable in cells plated on FN, but not in cells kept in suspension (Figure 2.8B). Thus, FN engagement leads to phosphorylation of FLAG-Rnd3, which coincides with phosphorylation of MARCKS protein.

Further, under the same conditions, engagement of FN led to an increase in phospho-cofilin and phospho-MLC. Phosphorylation of the actin-severing protein cofilin is a readout for the activity of ROCK, an immediate downstream effector of RhoA (207, 208), specifically when α5β1 integrins are engaged with the ECM protein fibronectin (354). MLC, which is a downstream target of the Rho-ROCK signaling pathway (via LIM kinase) involved in actomyosin contractility (184, 203, 204), is also phosphorylated in response to plating of cells onto FN (269). Our results are therefore consistent with the model proposed in Figure 2.7 in which FN engagement signals through a linear pathway of PKCα to Rnd3, leading to its translocation from the plasma membrane and thus preventing disruption of signals from the Rho-ROCK pathway leading to changes in the actin cytoskeleton.

PKCα-dependent Rnd3 phosphorylation downregulates Rnd3 inhibitory activity and leads to increased signaling through the Rho-ROCK pathway. As mentioned earlier, Rnd3 exerts its biological activity at least in part by counteracting the effects of RhoA signaling. Because our model predicts that PKC-mediated phosphorylation decreases Rnd3 activity, we investigated whether Rnd3 phosphorylation leads to an increase in signaling through the Rho-ROCK pathway. To this end, NIH 3T3 cells were transiently transfected with either GFP only, GFP-Rnd3-WT or GFP-Rnd3-All A expression constructs. Transfected cells were treated with either DMSO vehicle or PMA and then fixed and stained with Rhodamine-conjugated phalloidin to mark actin. As seen in Figure 2.9A, PMA treatment caused not only translocation of GFP-Rnd3-WT from the plasma membrane but also the restoration of stress fibers, along with greater spreading and a flattened appearance of the cells. None of these changes were seen when cells were treated with only DMSO vehicle. In direct opposition to the results seen with GFP-Rnd-WT, PMA treatment had no effect on the plasma membrane localization of the PKC-insensitive mutant GFP-Rnd3-All A; stress fibers were not restored, and the cells did not flatten and spread. Thus, phosphorylation of Rnd3 is required for the reappearance of stress fibers upon PMA treatment, and the non-phosphorylated All A mutant can act as a dominant negative to overcome the ability of endogenous Rnd3 to restore stress fibers.

To uncover a possible molecular mechanism for the restoration of stress fibers and cell spreading in PMA-treated cells expressing Rnd3-WT but not Rnd3-All A, lysates from PMA-treated cells were resolved on SDS-PAGE and immunoblotted for phospho-MLC. As seen in Figure 2.9B, in Rnd3-WT expressing cells, the levels of phospho-MLC were higher after treatment with PMA, as compared to treatment with DMSO vehicle only. In contrast,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+ PMA
phalloidin
GFP
phalloidin
GFP
GFP vector
GFP-Rnd3-WT
GFP-Rnd3-"All A"

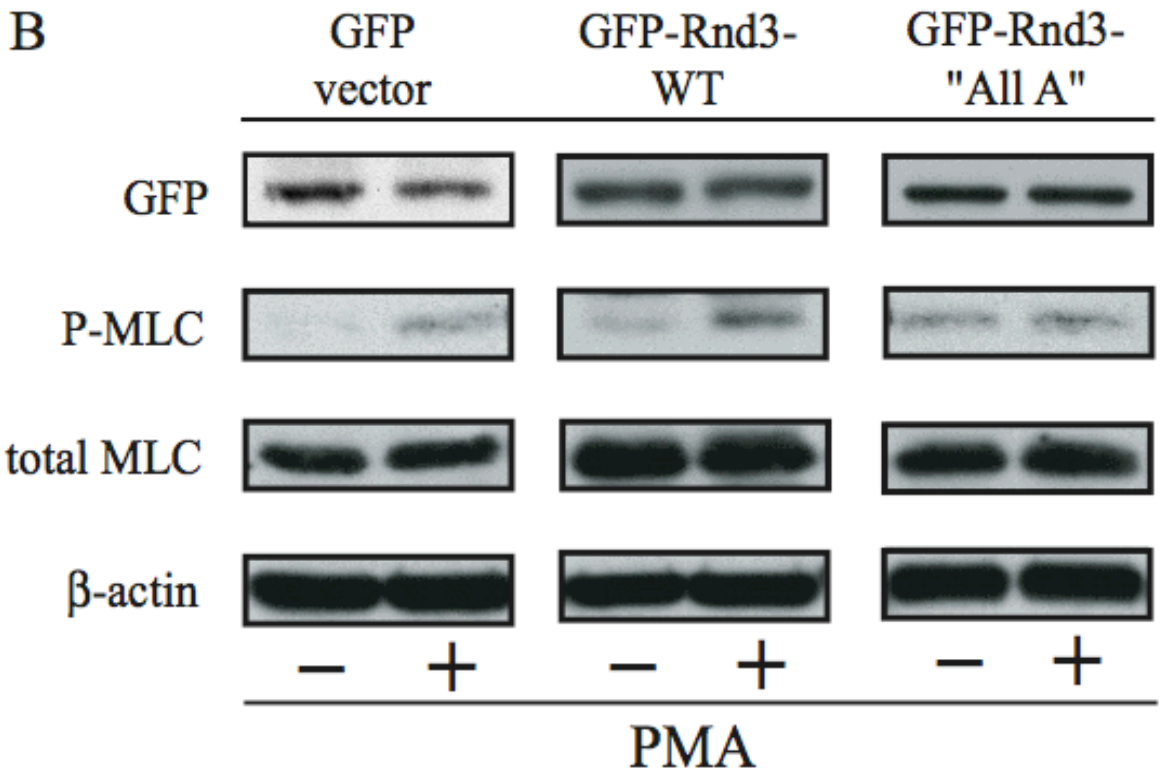
B
GFP vector
GFP-Rnd3-WT
GFP-Rnd3-"All A"
GFP
P-MLC
total MLC
β-actin
– + – + – +
PMA

Figure 2.9: PKCα-dependent Rnd3 phosphorylation downregulates Rnd3 inhibitory activity and leads to increased signaling through the Rho-ROCK pathway. A, Treatment with PMA causes re-formation of stress fibers in GFP-Rnd3-WT but not in GFP-Rnd3-All A-expressing cells. NIH 3T3 cells were transiently transfected with either GFP-vector, GFP-Rnd3-WT or the nonphosphorylatable GFP-Rnd3-All A mutant and then treated with either DMSO vehicle or PMA for 10 min. Live images were taken on a confocal microscope. Actin structures were visualized with Texas Red-phalloidin. **B,** PKCα-dependent phosphorylation leads to increased signaling through the Rho-ROCK pathway. Cells were transfected and treated as above. Lysates were separated by SDS-PAGE and probed with anti-GFP-antibody to visualize equal expression of the GFP-fusion proteins. Additionally, lysates were probed with anti-P-MLC antibody to detect signaling from the Rho-ROCK pathway.

Rnd3-All A effectively acted as a dominant negative in this pathway, abrogating the ability of PKC activation to increase phospho-MLC. The data are thus consistent with a model in which Rnd3 located at the plasma membrane is able to disrupt signals from the Rho-ROCK pathway that are involved in stress fiber formation/maintenance. We envision that, when Rnd3 becomes phosphorylated after PKC activation (PMA treatment, FN engagement) and is translocated from the plasma membrane, it is no longer able to disrupt the Rho-ROCK signaling pathway. The Rnd3-All A mutant, which is no longer subject to PKC-mediated phosphorylation, is still located on the plasma membrane, even after PKC activation. Hence, in Rnd3-All A expressing cells, signals coming from the Rho-ROCK pathway at the plasma membrane can still be disrupted by this PKC-insensitive mutant Rnd3. These results demonstrate that PKCα phosphorylation of Rnd3 represents an important negative feedback loop that may be critical to restoration of signaling through Rho-ROCK following transient activity of the GTPase-insensitive Rho family protein Rnd3.

Discussion

It has long been appreciated that Rnd3/RhoE is constitutively GTP-bound (16), and therefore that its activity must be regulated by means other than GTP-GDP cycling. Rnd3 protein expression is known to be tightly regulated and responsive to both internal and external cues. Although Rnd3 protein is found in low abundance at steady state, its mRNA is ubiquitously expressed (15). Previous studies have shown that Ras or Raf activation caused upregulated Rnd3 gene and protein expression in MDCK and other epithelial cells (250) (Singh et al., unpublished data). Also, platelet-derived growth factor (PDGF) stimulation of Swiss 3T3 fibroblasts caused cell rounding and branching, which coincided with

upregulation of Rnd3 protein expression (255). Recently, our collaborators found that Rnd3 gene transcription was also associated with ERK MAPK activation in melanomas (310). However, modulation of expression is a relatively slow process compared to many signaling activities mediated by small GTPases. Rapid modulation of Rnd3 activity by other means might be required to regulate dynamic signaling processes. We recently showed that Rnd3/RhoE is a direct target of the serine/threonine kinase ROCK, altering Rnd3 protein degradation (266). Here we have presented evidence that post-translational regulation of Rnd3 activity can also be accomplished via differential subcellular localization due to PKCα-mediated phosphorylation. Our results thus add an additional mechanism of regulation to those documented previously, and clarify how Rnd3 modulates Rho signaling to alter cytoskeletal organization.

Several closely related proteins have been shown previously to be substrates for phosphorylation by PKC. In particular, K-Ras4B was shown to be a substrate for PKC phosphorylation in its C-terminal polybasic region (320, 355, 356), and we have shown recently that this phosphorylation influences both its subcellular localization and its function, in part by altering effector interactions due to this differential localization (321). We reasoned that Rnd3, which is also a farnesylated protein with a polybasic domain, might be regulated in a manner similar to that of K-Ras4B.

Our inspection of the Rnd3 amino acid sequence revealed a potential PKC phosphorylation site at residue S240, just upstream of the CAAX motif in the C-terminal hypervariable membrane-targeting domain. Here we demonstrate that Rnd3 is phosphorylated upon PKC activation, and that inhibition of conventional PKC isoforms abrogates this phosphorylation. However, we have determined that phosphorylation at S240

was not solely responsible for the effects seen on Rnd3 due to PKC activation. Rather, multiple additional residues may be necessary.

Both Rnd3 and Rnd1 can induce inhibition of actin stress fibers and focal adhesions upon transfection into fibroblasts (15). Both of these Rnd proteins contain an extra N-terminal extension that is not found in other Rho proteins, including in Rnd2, which does not induce disruption of actin stress fibers. Furthermore, these extensions are needed for proper plasma membrane localization and function (15). When the first 6 amino acids were deleted from Rnd1, this N-terminal truncation mutant failed to localize correctly and was unable to block stress fiber formation (15). Whether Rnd1 is also a target of PKC-mediated phosphorylation is presently unknown. Similarly to the effects of Rnd1 N-terminal truncation, the original clone of Rnd3, which was missing the first 15 N-terminal residues, also lacked the ability to down regulate stress fibers (16). It could be envisioned that phosphorylation of residues located in both the N- and C-terminal extensions would reduce plasma membrane affinity. Further, when the crystal structure of Rnd3 was solved, only the core GTP-binding domain was amenable to crystallization, as the N- and C-terminal extensions were subject to proteolysis (244, 245). These N- and C-terminal extensions are highly disorganized and attempts have been made unsuccessfully to plot their possible structure by current protein structure computer programs. Thus, although it seems likely that these extensions are involved in Rnd3 membrane interactions, structural information on these extensions is lacking. Therefore, it is currently impossible to predict potential effects of phosphorylation of these residues on Rnd3 secondary structure that may influence such interactions.

The exact phosphorylation sites contained within these extensions necessary for loss of Rnd3 from the plasma membrane and translocation to the cytosol still remain to be

deciphered. We concluded that phosphorylation of Rnd3 at serine 240 alone is not sufficient to regulate Rnd3 subcellular localization or to produce the mobility-shifted form of Rnd3. Our preliminary mutagenesis analyses of other candidate phosphorylation sites suggests that multiple PKC sites are likely to be involved.

We have also shown in this report that the phosphorylation state of Rnd3 has direct consequences on its cellular location, with phosphorylation causing loss of plasma membrane localization and translocation to the cytosol. We have also determined that Aurora-A-mediated phosphorylation of the small GTPase RalA (328) leads to loss of plasma membrane localization and translocation to the cytosol (Lim et al., under revision). Previous studies on the localization and functional consequences of PKA-mediated phosphorylation of Rap and RhoA (324, 326, 357), as well as our lab's recent observations on PKC-mediated phosphorylation of K-Ras4B (321), indicate that these posttranslational modifications are important modulators of both localization and biological function. Taken together, we suggest that stimulus-mediated phosphorylation of Ras family small GTPases may be a more common mechanism of their regulation, distinct from GTP/GDP cycling, than has been appreciated. Indeed, as similar results have been documented for the ARF nucleotide exchange factor ARNO (314, 316, 317), even GTP/GDP cycling itself may be regulated in a similar manner under some circumstances.

After demonstrating, through several lines of evidence, that the conventional PKC responsible for Rnd3 phosphorylation is PKCα, we sought out an extracellular stimulus-mediated mechanism of PKCα activation and regulation of Rnd3 (358). Our observations support a mechanism wherein fibronectin-mediated integrin activation, of PKCα results in PKCα-mediated phosphorylation of Rnd3. Initial studies in fibroblasts revealed that

engagement of the α5β1 integrin by the cell-binding domain of fibronectin (FN) was insufficient for cells to form focal adhesions (359). It was later discovered that an additional interaction composed of the heparin sulfate proteoglycan syndecan-4 binding to the heparin-binding domain of FN was necessary to trigger focal adhesion formation (360). However, this requirement could be bypassed by activation of PKCα (361), demonstrating not only that PKCα is present in focal adhesions, but that its activity contributes to their formation. In a series of studies by Oh, Couchman and colleagues, it was revealed that a ternary complex is formed, at sites of focal adhesion assembly, consisting of the cytoplasmic domain of syndecan-4 interacting with the catalytic subunit of PKCα in combination with PIP_2 to yield a sustained, higher level of PKCα activity (362-364). Use of pharmacological inhibitors of PKCα and transient expression of dominant negative PKCα suppressed focal adhesion formation and cell migration mediated by α5β1 integrins in melanoma cells, which also express syndecan-4, when plated on FN (346). It has also been shown that PKCα can be activated in cells by adhesion to FN (352). Previous studies have shown that RhoA is activated in cells when plated on FN (269) and that syndecan-4 acts cooperatively with integrins in a Rho-dependent fashion in the assembly of focal adhesions and actin stress fibers (345), although the mechanism was not delineated. We have presented evidence here that PKCα-mediated phosphorylation of Rnd3 leads to increased signaling through the Rho-ROCK signaling pathway.

We suggest that phosphorylation of Rnd3 leads to relocalization away from plasma membrane sites where it can antagonize signaling from the Rho-ROCK pathway, thus leading to remodeling of the actin cytoskeleton. Such a model would be consistent with a study from Larsson and colleagues, who demonstrated that the Rnd3 target p190RhoGAP

was present in membrane ruffles and neurite outgrowths, where it was modulated by, but did not interact directly with, the epsilon isoform of PKC (365). During the course of our studies, Couchman and colleagues described observations that further support our model (366). They described a mechanism where cell attachment on FN through syndecan-4 led to PKCα-dependent activation of Rho for the formation and maintenance of stress fibers. We offer here compelling evidence that Rnd3 may represent an important link directly connecting PKCα with the Rho-ROCK pathway and the myriad of cell responses they control through cytoskeletal organization via actomyosin contractility. Determining exactly how PKCα-mediated alterations in Rnd3 localization affect its ability to modulate Rho-ROCK will likely require a fuller characterization of Rnd3-interacting proteins.

CHAPTER 3

ROLE OF POST-PRENYL PROCESSING IN RND3 LOCALIZATION AND FUNCTION

(Some material appearing in Roberts, PJ et al., 2008, JBC)

Abstract

The Rho GTPases comprise a major branch of the Ras superfamily of small GTPases. Studies have demonstrated that distinct functions of the different Rho GTPases are dependent upon proper subcellular localization to diverse compartments where they are able to interact with discrete upstream regulators and downstream effectors. These studies have demonstrated that proper subcellular localization, and hence biological activity, is dependent upon a series of post-translational modifications governed by the C-terminal CAAX motif. These modifications involve prenylation of the CAAX motif followed by two post-prenyl processing steps. A recent study (94) found that, unlike farnesylated members of the Ras GTPases, the geranylgeranylated classical members of the Rho GTPases are not dependent upon post-prenyl processing for proper membrane association and hence biological activity. Rnd3/RhoE, a member of the Rnd subfamily of Rho GTPase, is subject to prenylation by a farnesyl isoprenoid. Hence, studies were undertaken to determine if Rnd3 is dependent upon post-prenyl processing for proper membrane association and biological function. Specific

structural mutations in the CAAX motif of Rnd3, along with the use of mouse embryonic fibroblasts genetically ablated for each of the two enzymes involved in post-prenyl processing, allowed us to determine that post-prenyl processing is necessary for proper membrane localization of Rnd3 and its biological function in stress fiber disassembly.

Introduction

The Rho family of small GTPases represents a major branch of the Ras superfamily of small GTPases (4, 5, 19, 59). These proteins act as molecular switches by cycling between an inactive GDP-bound form and an active GTP-bound form, the latter of which is then able to interact preferentially with effector molecules (1). This molecular switching is made possible by two classes of enzymes: guanine nucleotide exchange factors (GEFs), which activate G-proteins by catalyzing GDP-GTP exchange (81, 83), and GTPase activating proteins (GAPs), that inactivate G-proteins by hydrolyzing GTP to GDP (87, 88). The most thoroughly characterized members of this family are RhoA, Rac1 and Cdc42 (4, 9, 10). A major function of the Rho proteins is their regulation of the actin cytoskeleton (332, 367). Activation of Rho leads to stress fiber and focal adhesion formation (70), while activation of Rac1 and Cdc42 lead to lamellipodia and filopodia formation, respectively (71, 72). In addition to their effects on actin cytoskeleton organization, Rho proteins are involved in many critical processes necessary for numerous signal transduction pathways as well as transcriptional regulation and growth control (5).

Essentially all members of the Rho family, along with the Ras family, terminate in a CA_1A_2X motif (where C=cysteine, A=aliphatic residue and X=any amino acid) (95). This motif is a crucial signal needed for these proteins to be post-translationally modified by

prenylation, a permanent post-translational modification required for correct subcellular localization and for biological activity (97). Proper localization of prenylated, membrane associated proteins relies on an ordered cascade of enzymatic reactions (116). The first reaction in this enzymatic cascade is stimulated by either of two cytosolic prenyl transferase enzymes: farnesyl transferase (FTase) or geranylgeranyl tranferase I (GGTase I) (98). The "X" in the CAAX motif determines the prenylation specificity of that protein (99, 100, 102, 103). A large number of Rho proteins terminate in X = L and are therefore substrates for GGTase I, which adds a C20 geranylgeranyl isoprenoid group to the cysteine of the CAAX motif, while Ras proteins (X = S or M) are substrates for FTase, which adds a C15 farnesyl isoprenoid group (95).

Prenylation by either a C15 farnesyl or C20 geranylgeranyl isoprenoid group is not sufficient to complete the CAAX-signaled modifications; two further processing steps, termed post-prenyl processing, are needed (116, 117). The first step involves the proteolytic cleavage of the –AAX residues from the prenylated cysteine by an endoplasmic reticulum (ER)-localized protease termed Ras converting enzyme 1 (Rce1). The second step involves the methylation of the now-terminal prenylated cysteine residue catalyzed by another ER-localized enzyme termed Isoprenylcysteine carboxyl methyltransferase (Icmt). It is crucial to note that prenylation is an obligate prior step for both Rce1 and Icmt. The end result of this enzymatic cascade, involving both prenylation and post-prenyl processing, is thought to make the carboxy-terminal domain of CAAX motif-containing proteins more hydrophobic (121). Furthermore, a second signal, beyond CAAX-signaled prenylation and the two post-prenyl processing steps, is needed for proper membrane association. This second signal is

either palmitoylation of one or two cysteine residues or the presence of a polybasic region in the hypervariable domain immediately upstream of the CAAX motif (136, 138).

Much of the information we have on the Rho proteins has been gleaned from studies involving the three classical members of this family (RhoA, Rac1 and Cdc42) (9, 10, 332). Recent work has revealed that other members of this family have diverse cellular functions beyond those of the classical Rho family members (4, 11). It is believed that these varied cellular functions are made possible in part due to their localization to distinct subcellular compartments (94, 368). The Rnd family of proteins (Rnd1, Rnd2 and Rnd3/RhoE) form a unique branch of the Rho family (12). Unlike the other members of the Rho family that switch between an active and inactive state regulated by their GTP-binding status, Rnd proteins lack the ability to hydrolyze GTP and are resistant to GAP activity (15, 16). They are predominantly found bound to GTP *in vivo* and are constitutively in an "active" state (16) due to amino acid substitutions at highly conserved positions critical for normal GTP hydrolysis (15, 16). A distinctive action of the Rnd proteins is their effect on the actin cytoskeleton. In contrast to RhoA, which upregulates stress fibers and focal adhesions in both epithelial cells and fibroblasts, ectopic expression of either Rnd1 and Rnd3 causes stress fiber disassembly and the disappearance of focal adhesions, leading to cell rounding (hence "Rnd" for round) (15, 243). The CAAX motif of Rnd3 ends in a methionine and thus is predicted to be farnesylated. *In vivo*, Rnd3 was indeed shown to be a substrate for farnesylation (16). Furthermore, Rnd3 has been shown to be effectively mislocalized in cells after treatment with FTI (Singh et al., unpublished data). Additionally, Rnd3 contains a polybasic region just upstream of the CAAX motif, similar to the Ras protein K-Ras4B (140). Previous studies have shown that K-Ras4B activity is partially dependent upon post-

prenyl processing (369). Given that Rnd3 is both farnesylated and contains a polybasic region, this atypical Rho family small GTPase may also depend upon post-prenyl processing for proper membrane association and thus, for biological activity. Studies aimed at determining the role of post-prenyl processing in the regulation of Rnd3 localization and function are presented here in this chapter.

Materials and Methods

Molecular constructs

GFP-Rnd3 expression plasmids were generated by inserting the full length human Rnd3 cDNA into the BamHI site of pEGFP-C1 (Clontech). To generate GFP-Rnd3-(STVM), -(CTYM) and -(CTVR), mutagenic 3' primers were used in a PCR reaction to change the corresponding CAAX motif residues. GFP-Rnd3 (WT) vector was used as the template in these PCR reactions. Multiple bacterial colonies were screened and sequenced to confirm proper mutagenesis had occurred. The FLAG-Rnd3 WT expression construct was generated by inserting full length human wild type Rnd3 cDNA into the EcoRI and XhoI sites of pHIT-FLAG3 (a generous gift from Yanping Zhang, UNC-CH). Generation of FLAG-Rnd3-S7A, S11A, S210A, T214A, S218A, S222A, S240A (a seven amino acid, phosphodeficient Rnd3; henceforth termed "Rnd3-All A") has been described previously (266) and was a generous gift from Anne Ridley (King's College London). To generate the GFP-Rnd3-All A expression construct, the open reading frame from FLAG-Rnd3-All A was PCR amplified using a 5' primer containing a HindIII site and a 3' primer containing a SalI site. The PCR product was cut with HindIII and SalI restriction enzymes and ligated into the HindIII and SalI sites of pEGFP-C3. To generate the GFP-Rnd3-All A expression construct,

the open reading frame from FLAG-Rnd3-All A was inserted into the HindIII and SalI sites of pEGFP-C3. To generate GFP-Rnd3-All A-(STVM) and -(CTYM), mutagenic 3' primers were used in a PCR reaction to change the corresponding CAAX motif residues. GFP-Rnd3-All A vector was used in these PCR reactions as the template. Multiple bacterial colonies were screened and sequenced to confirm proper mutagenesis had occurred. All sequences were verified by the Genome Analysis Facility at UNC-CH.

Antibodies and reagents

FLAG tag antibody (M2) was from Sigma. Texas Red-phalloidin was from Invitrogen.

Cell culture

NIH 3T3 mouse fibroblasts were maintained in high glucose Dulbecco's modified Eagle medium (DMEM-H) (GIBCO-Invitrogen) containing 10% calf serum (Invitrogen) and penicillin-streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 10% CO_2. Spontaneously immortalized mouse embryo fibroblasts (MEFs) were originally prepared from *Rce1*-/- and *Icmt*-/- mouse embryos (133), along with control fibroblasts (*Rce1*+/+ and *Icmt*+/+) from littermate embryos, and were kindly provided by Stephen G. Young (University of California at Los Angeles). These MEFs were maintained in DMEM-H (GIBCO-Invitrogen) containing 15% fetal bovine serum (FBS) along with penicillin-streptomycin, nonessential amino acids and L-glutamine at 37°C in a humidified atmosphere of 5% CO_2.

Transfections, immunofluorescence and live cell microscopy

Expression vectors were transfected into NIH 3T3 cells using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's instructions. Expression vectors were transfected into MEFs using Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's instructions.

For immunofluorescence, NIH-3T3 cells were transiently transfected with GFP-tagged Rnd3 fusion constructs. Cells were either imaged live or fixed 24 hours later with 3.7% formaldehyde, permeabilized with Triton X-100, stained with Texas Red-phalloidin and mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI). Cells were visualized and imaged with a Zeiss Axioskop fluorescent microscope equipped with both FITC and TRITC filters. MetaMorph imaging software (Universal Imaging) was used for the manipulation and capture of images. Brightness and contrast of the JPEG images were adjusted using Adobe Photoshop software.

For live cell microscopy of MEFs, cells were plated, transfected with expression vectors for GFP-tagged proteins, and imaged in a 35-mm culture dish that was coated with poly-D-lysine and incorporated a No. 1.5 glass coverslip (MatTek, Ashland, MA). Cells were viewed using an inverted laser scanning confocal microscope (Zeiss 510 LSM) equipped with an oil immersion 63X NA 1.4 objective. Images were captured by scanning with the 488 nM spectral line of an argon-ion laser using the LP 505 emission filter. Brightness and contrast of the JPEG images were adjusted using Adobe Photoshop software.

Western blot analysis

Cells were washed with PBS, lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate and supplemented with Complete Protease Inhibitor Cocktail tablets (Roche) along with phenyl-methyl sulfonyl fluoride (PMSF) and sodium pervanadate) and centrifuged to remove insoluble material. Laemmli sample buffer (2X) was added to equivalent volumes of cellular lysates which were then resolved on 12% SDS-PAGE and transferred to Immobilon PVDF membranes. Membranes were blocked in 5% nonfat dry milk in TBS-Tween-20 and probed with appropriate primary antibodies, followed by anti-mouse or -rabbit IgG-horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences). Membranes were then incubated in SuperSignal West Dura Extended Duration substrate (Pierce) and the signal was developed on HyBlot CL autoradiography film (Denville Scientific Inc.).

Results

Specific CAAX motif mutations result in misprocessed Rnd3 proteins. To study the effect of abrogating post-prenyl processing on the localization and function of Rnd3, three different mutations were made in the Rnd3 CAAX motif. The first CAAX mutant changed the cysteine residue of the wild type CAAX motif, CVTM, to a serine residue (CTVM → STVM). This SAAX mutation is commonly used to generate a completely unprocessed protein, as the cysteine residue needed for farnesylation is lacking. The second CAAX mutant made changed the X residue from a methionine to an arginine (CTVM → CTVR). Farnesyltransferase does not utilize substrate proteins terminating in X = R (103); the specificity pockets of both FTase and GGTase discriminate against bulky amino acids

and arginine cannot be accommodated without disrupting the extended conformation of the CAAX motif. Therefore, this mutant protein will not be farnesylated and will therefore also not undergo any post-prenyl processing step. The third CAAX mutant made changed the A_2 residue from a valine to a tyrosine (CTVM → CTYM). Previous work has shown that this mutation in the CAAX motif of K-Ras4B results in a protein that is still farnesylated, but does not undergo either –AAX proteolysis or carboxy methylation (369).

FLAG-tagged Rnd3 CAAX motif mutants mentioned above, along with WT Rnd3, were transiently expressed in NIH 3T3 cells. Cell lysates were collected, resolved on SDS-PAGE and immunoblotted with anti-FLAG antibody. Previous studies have shown that unprocessed and partially-processed CAAX motif-containing proteins, including K-Ras4B, have slower apparent mobilities on SDS-PAGE, as compared to WT protein (369). As seen in Figure 3.1, all three Rnd3 CAAX motif mutants migrated at a slower mobility on SDS-PAGE as compared to WT Rnd3, which is indicative of improperly processed forms of Rnd3.

Expression of GFP-tagged versions of Rho GTPases has been used extensively to monitor their lipid modification status, because the localization of these proteins reflects their CAAX-signaled processing (65, 370-372). GFP contains a putative nuclear localization signal (NLS), and expression of GFP alone results in a diffuse cytoplasmic and nuclear accumulation pattern (373). Attachment of the GFP tag to either a Ras or Rho small GTPase sequence results in its nuclear exclusion (374), whereas complete inhibition of the lipid modifications of these GTPases results in a subcellular localization similar to that of GFP alone (65, 374). The localization of these GFP-small GTPase fusion proteins has been shown to reflect accurately the subcellular localization of the endogenous protein (94).

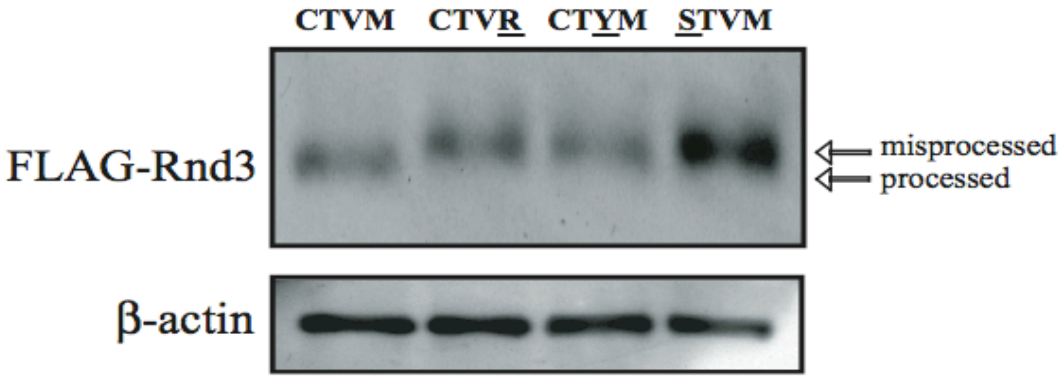

Figure 3.1: Rnd3 CAAX motif mutants are misprocessed as compared to WT Rnd3. Lysates from NIH 3T3 cells transiently expressing FLAG-tagged Rnd3 CAAX mutants or WT Rnd3 were resolved on SDS-PAGE and blotted with anti-FLAG antibody. Rnd3 proteins with CAAX motif mutations run at a slower mobility as compared to WT Rnd3 protein, which is fully processed.

Therefore, to visualize whether disrupting Rnd3 post-prenyl processing also disrupted its subcellular localization in living cells, GFP-tagged Rnd3 CAAX mutants were expressed in NIH-3T3 cells, along with GFP vector only and GFP-Rnd3 WT. As seen in Figure 3.2, GFP-tagged Rnd3 WT was found localized on the plasma membrane and perinuclear structures, while GFP alone was cytosolic with nuclear accumulation. Both the SAAX and the CTVR mutants displayed a localization that is very similar to GFP alone, consistent with the localization of completely unprocessed GTPases. In contrast, the A_2 mutant CTYM retained some plasma membrane localization, consistent with the prediction that this A_2 mutant is farnesylated, but neither –AAX proteolyzed nor carboxymethylated. Morphologically, cells expressing GFP-Rnd3 WT were rounded and poorly spread compared to cells expressing empty vector or to cells expressing all three Rnd3 CAAX motif mutants, which had the same flat and spread appearance. These results are consistent with a requirement for full CAAX-signaled processing in order for Rnd3 to be able to promote stress fiber disassembly.

Both membrane localization and function of Rnd3 depend on Rce1- and Icmt-mediated processing. To confirm that both post-prenyl processing steps are necessary for proper localization and function of Rnd3, these properties were examined in mouse embryonic fibroblasts (MEFs) genetically ablated for either *Rce1* and *Icmt* (133). First, FLAG-tagged Rnd3 WT was ectopically expressed in both *Rce1-/-* and *Icmt-/-* MEFs, or in matched control WT MEFs, and its expression was confirmed by western blotting for the FLAG tag. As seen in Figure 3.3, FLAG-tagged Rnd3 expressed in either Rce1-/- or Icmt-/- MEFs displayed a slower electrophoretic mobility on SDS-PAGE as compared to expression

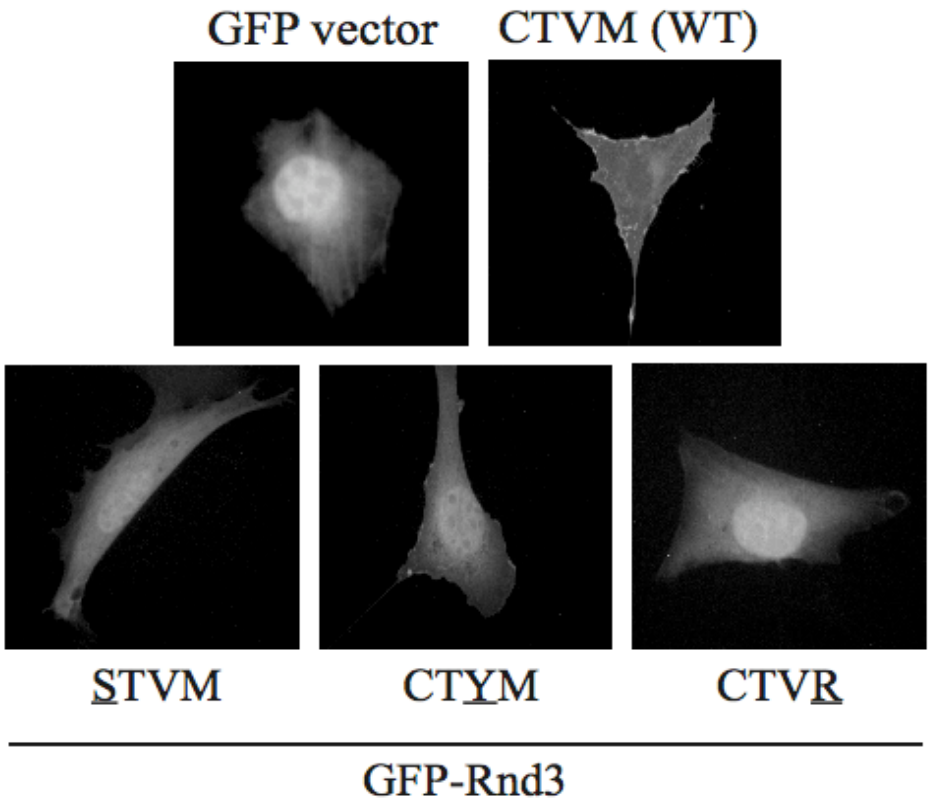

Figure 3.2: Misprocessing of GFP-tagged Rnd3 CAAX motif mutants results in Rnd3 cytosolic and nuclear accumulation. NIH 3T3 cells transiently expressing GFP vector only, GFP-Rnd3 WT or GFP-Rnd3 with CAAX motif mutations were visualized using fluorescent microscopy. Rnd3 proteins with CAAX motif mutations are localized to the cytosol and nucleus, similar to GFP alone. WT Rnd3 is found on the plasma membrane and endomembranes.

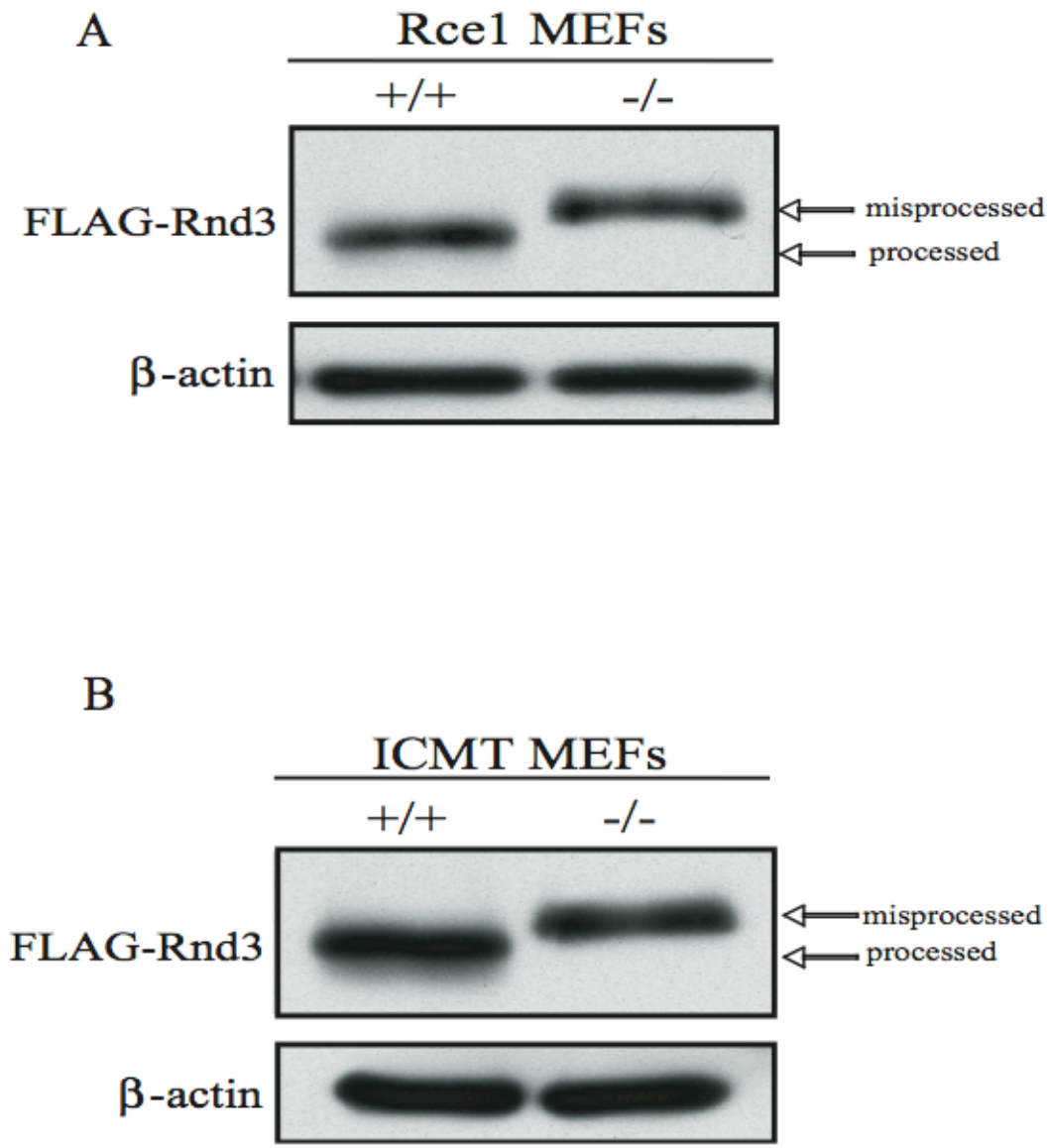

Figure 3.3: Rnd3 is misprocessed in both *Rce1-/-* and *Icmt-/-* MEFs. A, Lysates from *Rce1*+/+ and *Rce1*-/- and **B,** *Icmt*+/+ and *Icmt*-/- MEFS expressing FLAG-tagged Rnd3 were resolved on SDS-PAGE and blotted with anti-FLAG antibody. FLAG-Rnd3 protein from Rce1-/- and Icmt-/- MEF lysates runs at a slower mobility, as compared to the WT matched control MEFs, owing to misprocessing due to loss of –AAX proteolysis and prenylated terminal cysteine methylation, respectively.

in their matched control WT MEFs. This indicates that lack of either Rce1 or Icmt result in a processing defect of Rnd3. We next investigated whether Rnd3 is also mislocalized in these cells. As seen in Figure 3.4, GFP-tagged Rnd3 was mislocalized away from the plasma membrane in both *Rce1-/-* and *Icmt-/-* cells, as compared to their matched control WT MEFs. Furthermore, the rounding phenotype seen in WT MEFs expressing GFP-tagged Rnd3 was lost in both *Rce1-/-* and *Icmt-/-* cells. The rounding phenotype was only seen when GFP-tagged Rnd3 was found on the plasma membrane, indicating that Rnd3 disruption of RhoA-mediated stress fiber assembly requires that specific subcellular localization.

Specific CAAX motif mutations abrogate the gain-of-function phenotype of the All A mutant of Rnd3. As shown previously in Chapter 2, the "All A" phosphodeficient mutant of Rnd3 was found predominantly on the plasma membrane and, as expected, was resistant to translocation upon treatment with PKC agonists (Figures 2.5C and 2.9A). This All A mutant showed a gain-of-function phenotype as compared to WT Rnd3. Cells expressing this All A mutant were rounded, due to lack of stress fibers, even after treatment with PMA (Figure 2.9). To test whether post-prenyl processing is also required in the context of this PKC-resistant phosphodeficient Rnd3, similar CAAX motif mutants were constructed in the All A background. As seen in Figure 3.5, GFP-Rnd3-All A, with a WT CAAX motif, was localized on the plasma membrane as expected. The completely unprocessed SAAX mutant was localized to the cytoplasm and accumulated in the nucleus. While a large majority of the prenylated but not clipped or methylated Rnd3-All A-CTYM mutant was similarly localized, some was retained on the plasma membrane. This result indicates that

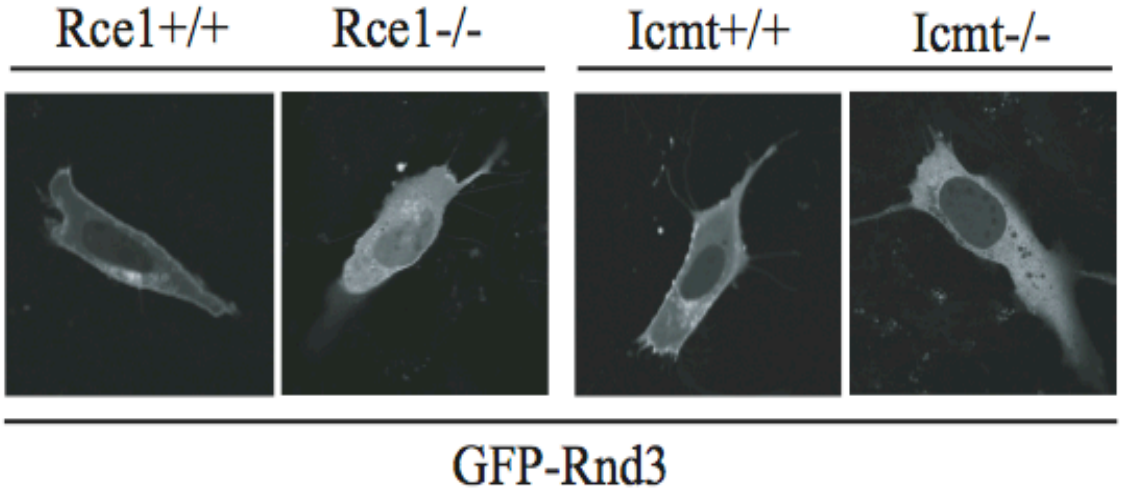

Figure 3.4: Rnd3 is mislocalized in both *Rce1-/-* and *Icmt-/-* MEFs. Live Rce1-/- and Icmt-/- MEFs, along with WT matched control MEFs, transiently expressing GFP-Rnd3, were visualized using confocal microscopy. In *Rce1-/-* MEFs, GFP-Rnd3 showed a significant decrease in plasma membrane localization, accompanied by substantial cytosolic distribution and some nuclear accumulation. In *Icmt-/-* MEFs, GFP-Rnd3 exhibited a complete loss of plasma membrane and endomembrane localization that was accompanied by increased cytosolic, but not nuclear, localization.

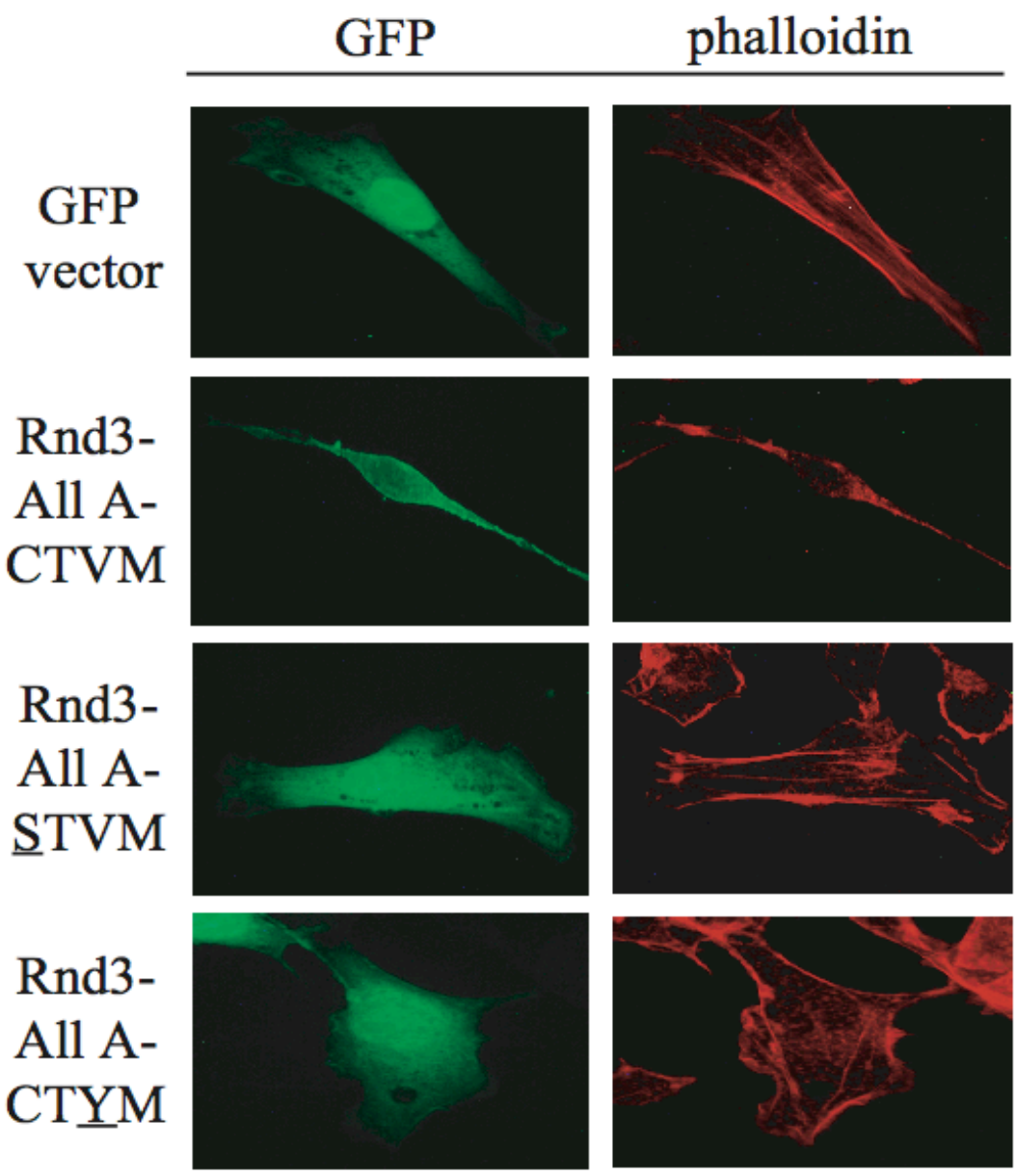

Figure 3.5: CAAX motif mutants abrogate the dominant phenotype of the nonphosphorylatable Rnd3-All A mutant. NIH 3T3 cells expressing GFP only, GFP-Rnd3-All A or GFP-Rnd3-All with CAAX motif mutants were fixed and labeled with Texas Red phalloidin to mark stress fibers. Cells were visualized by fluorescent microscopy. Expression of GFP-Rnd3-All A resulted in cell rounding and a complete loss of stress fibers. Both CAAX motif mutants displayed localization similar to GFP only. Cells expressing these mutants were flat, similar to cells expressing GFP only. The STVM mutant expressing cells displayed levels of stress fibers similar to cells expressing GFP only. Cells expressing the CTYM mutant had fewer stress fibers as compared to cells expressing GFP only.

the processing status of Rnd3 affects its localization, but that the PKC-sensitivity status of Rnd3 does not affect its location in the absence of PKC stimulation.

To test the functional capabilities of these mutants, cells expressing them were labeled with Texas Red-phalloidin to visualize stress fibers. As seen in Figure 3.5, many stress fibers were present in control cells expressing GFP vector only. In direct opposition to this, there were no stress fibers present in cells expressing GFP-Rnd3-All A with a WT CAAX motif. There were many stress fibers present in the completely unprocessed "SAAX" mutant of GFP-Rnd3-All A, highlighting biological consequences of its inability to localize correctly, while an intermediate number of stress fibers was present in the prenylated but not clipped or methylated CTYM CAAX motif mutant. This observation is consistent with the hypothesis that only Rnd3 localized on the plasma membrane is able to signal properly to downregulate Rho-ROCK signaling. Hence, proper post-prenyl processing is required for both Rnd3 localization and functional activity.

Discussion

In this chapter, I have used several approaches to study the role of post-prenyl processing in the regulation of localization and function of the novel Rho family member Rnd3. In contrast to the classical Rho family members, I have found that post-prenyl processing dictated by the CAAX motif, including the steps of proteolysis by Rce1 and carboxymethylation of the prenylated cysteine by Icmt, is necessary for proper Rnd3 localization and function. Interference with proper post-prenyl processing (either by CAAX motif mutations or expression in *Rce1* or *Icmt* null MEFs) resulted in a slower mobility of Rnd3 protein on SDS-PAGE, consistent with its partial processing. In addition, CAAX motif

mutations of Rnd3 that interfered with post-prenyl processing resulted in mislocalization of GFP-Rnd3 away from the plasma membrane and accumulation in the cytoplasm and nucleus in NIH 3T3 cells. GFP-tagged Rnd3 WT was similarly mislocalized when expressed in *Rce1-/-* and *Icmt-/-* MEFs in which post-prenyl processing does not occur due to the lack of relevant processing enzymes.

In the course of this study, I observed that mislocalized Rnd3 is also impaired in function. Not only was GFP-tagged Rnd3 mislocalized in *Rce1-/-* and *Icmt-/-* MEFs, but it also produced consistently less rounding in these cells as compared to matched WT control cells. Furthermore, I observed that the GFP-Rnd3-All A CTYM CAAX motif mutant, which undergoes prenylation but not post-prenyl processing, was not completely restricted from plasma membrane association and resulted in an amount of stress fibers intermediate between that of completely processed Rnd3 WT and the completely unprocessed Rnd3 SAAX mutant. Thus, while overwhelmingly mislocalized, the small pool of this partially processed form of Rnd3 (CTYM) may still provide sufficient signal from the plasma membrane to cause some stress fiber disassembly. I speculate that, when Rnd3 is mislocalized, it is unable to engage downstream effectors such as p190RhoGAP or ROCK I to abrogate the effects of RhoA-ROCK signaling. With the increasing evidence that Rnd3 is overexpressed in cancers with increased metastatic potential (307, 310, 311), Rnd3 may be a target of drugs that block either prenylation (108) or post-prenyl processing (116). It would be interesting to determine the role of Rnd3 in the observed cellular effects of FTIs and of current and future inhibitors of Rce1 and Icmt.

A recent study suggested that members of the classical Rho family of small GTPases are not dependent on post-prenyl processing for proper localization (126). The grand scope

of a larger overall study by our lab in collaboration with Channing Der's lab (for which some of the Rnd3 data was collected) (106) was to examine the dependency of post-prenyl processing of all Rho family GTPase members. We have found, in opposition to the aforementioned study, that the majority of Rho family members, including geranylgeranylated members, are differentially dependent upon post-prenyl processing through Rce1 and Icmt for proper localization and/or function. Past work has demonstated that both H-Ras and K-Ras-mediated transformation is impaired when either *Rce1* or *Icmt* are disrupted (127, 135). It was also noted that transformation by B-Raf was inhibited by loss of Icmt activity, even though B-Raf itself is not an Icmt substrate. Therefore, some other target or targets of Icmt activity must be responsible for the Icmt deficiency-induced inhibition of Raf.

With the information gained in this study, it is plausible that Icmt deficiency-induced inhibition of Rho proteins could be responsible for the effects seen with Icmt functional loss. With this in mind, RhoA may represent one possible target of Icmt inhibitors. In the study mentioned above (106), we found that RhoA subcellular localization was sensitive to loss of Icmt function. A previous report noted that nonmethylated RhoA is less stable than the methylated form (375). In support of this, Bergo and colleagues have shown that the steady-state levels of GTP-bound RhoA and total RhoA were decreased in *Icmt* targeted fibroblasts transformed with K-Ras, due to accelerated protein turnover (135). Surprisingly, the authors saw an increase in the stability of Ras proteins in these cells. They also noted an upregulation in the level of p21-CIP1, a cyclin-dependent kinase inhibitor which blocks cell cycle progression. Past studies have shown p21-CIP1 levels are upregulated by activated Ras and that this upregulation can be antagonized by RhoA (235, 236). To test whether p21-

CIP1 was important for the effects seen with loss of Icmt function, Bergo and colleagues inactivated Icmt in K-Ras-transformed cells deleted for the gene encoding p21-CIP1. The authors observed that the *Icmt* inactivation in these cells had no measurable effect on cell growth on plastic plates or on the growth of colonies in soft agar. They concluded that the effect of *Icmt* inactivation on K-Ras transformation may have been a consequence of its effects on Rho and p21-CIP1, rather than being due to a direct effect on the intrinsic properties of K-Ras itself. In support of a role for RhoA in the effects seen with inhibition of Icmt function, Lu and colleagues have noted that an Icmt inhibitor decreased both RhoA methylation and activity along with endothelial monolayer permeability, a RhoA-dependent property (376). Furthermore, a change in the organization of intercellular junctions was noted. The authors suggest that carboxymethylation of RhoA may be critical for its ability to modulate endothelial barrier function. In a follow-up experiment the same authors noted that treatment of endothelial cells with an Icmt inhibitor decreased levels of RhoA protein (377). This decrease in RhoA protein levels coincided with a loss of GRP94, a protein that is a component of the unfolded protein response. The decrease in GRP94 protein levels caused apoptosis, possibly through dysfunction in the unfolded protein response. The authors suggest a novel link between RhoA and the unfolded protein response. With its documented role in cellular processes such as actin cytoskeletal organization, gene expression, cell cycle progression and transformation (169), perhaps RhoA may be a functionally important target for Icmt-dependent inhibitors.

Icmt knock out mice died earlier in development as compared to *Rce1* knock out mice, despite the fact that prior Rce1-mediated cleavage is necessary for Icmt-mediated processing to take place at the now-terminal farnesylated cysteine of its substrate proteins.

Several explanations could be offered for this observed disparity in phenotypes. First, there may be additional proteins in the cell that are reliant on Icmt function, but are not reliant on Rce1 function. Second, perhaps prenylated proteins are more affected by the presence of the exposed C-terminal cysteine than by the presence of the –AAX extension (378, 379). Finally, with the observations mentioned above regarding protein stability in *Icmt*-targeted cells, perhaps loss of Icmt function has a differential effect on the ability of proteins to avoid degradation in the cell.

Development of inhibitors of both Rce1 and Icmt is still currently in its infancy. In contrast to FTIs and GTIs, there is limited documentation in the literature of studies based on inhibiting the two post-prenyl processing enzymes. One example of an Icmt-specific inhibitory compound is the indole-based small molecule cysmethynil. Casey and colleagues have documented that cysmethynil treatment results in inhibition of cell growth in an Icmt-dependent fashion. They showed that treatment of cancer cells with cysmethynil resulted in mislocalization of Ras, impairment of EGF signaling and blockage of anchorage-independent growth, which could be reversed by overexpression of Icmt (380). Furthermore, inhibition of Icmt function has been shown to be a critical component of the antiproliferative effect of the antifolate methotrexate, a drug commonly used in chemotherapy (381). Recently, the first natural product inhibitor of Icmt named spermatinamine was described. This product, from the Australian marine sponge, was discovered in a natural product high-throughput screening of conducted to discover Icmt inhibitors (382). In addition, compound libraries are being screened and both peptidic and non-peptidic compounds are being designed and tested for use as Rce1-specific inhibitors (383-385).

While development of inhibitors of post-prenyl processing has been limited (116), perhaps the information gained here regarding sensitivity of a large number of Rho proteins to loss of post-prenyl processing will spur further interest in development of Rce1- and Icmt-specific inhibitors. It should be noted that while inhibitors of farnesyl transferase (FTIs) were initially designed as "anti-Ras" drugs; it is now generally accepted that Ras proteins are not the only targets of FTIs (107). Numerous Rho family proteins are currently being investigated as possible targets of FTIs and thus responsible for some of the effects seen with FTI treatment (113). It could be envisioned that, in the future, certain cancers that are dependent upon post-prenylated proteins (not only Ras family proteins, but also Rho family proteins) could possibly be treated with either Rce1 or Icmt inhibitors (80).

CHAPTER 4

CONCLUDING REMARKS, FUTURE DIRECTIONS AND SUMMARY

Concluding Remarks

Rho proteins act as molecular switches, cycling between an inactive GDP-bound form and an active GTP-bound form. This activated GTP-bound form is capable of interacting with downstream effectors, resulting in a myriad of cellular outcomes. Rnd3 is a member of the Rnd family of proteins, which form a distinct branch of the Rho family of small GTPases. Rnd3 has been shown to be GTPase-deficient and constitutively bound to GTP, and is considered to be constitutively active (15, 16). Rnd3 is largely known for its role in counteracting signaling from the RhoA-ROCK signaling pathway (13-15, 243). Because it does not undergo GDP/GTP cycling, other modes of regulation for Rnd3 have been proposed (12, 17). Regulation by expression has been suggested and numerous reports have indeed provided evidence that Rnd3 expression is modulated by various biological stimuli (14, 250, 254, 256). Because regulation by expression is a relatively slow process, more rapid and efficient modes of regulation for Rnd3 must exist. To this end, I decided to explore the possibility that Rnd3 may be regulated in a more dynamic fashion through post-translational mechanisms. Several Ras-related small GTPases have been shown to be phosphorylated at sites within the polybasic regions of their C-terminal hypervariable domains, just upstream of the CAAX membrane-targeting motif (282, 321, 323-325, 328). These phosphorylation

events have been shown to have biological consequences and are believed to reduce the affinity of these proteins for the plasma membrane, a subcellular location important for effector signaling. Inspection of Rnd protein sequences revealed a potentially phosphorylatable serine residue at position 240 in the hypervariable region, located between the polybasic region and the CAAX motif. This serine fits the consensus PKC phosphorylation motif. Therefore, I sought to determine if PKC-dependent phosphorylation at serine 240 occurred, and if so, whether it had any effect on Rnd3 localization and function. Through my research outlined in Chapter 2, I found that upon PKC activation, Rnd3 became phosphorylated and its subcellular localization was altered. I found that PKC activation caused rapid loss of Rnd3 from the plasma membrane and enrichment in the cytosol and on internal membranes. I also found that serine 240 alone is not responsible for the effects of PKC activation on Rnd3. Rather, through the use of specific mutants including a nonphosphorylatable mutant of Rnd3, I found that multiple sites of phosphorylation exist in Rnd3. By use of both pharmacologic and genetic analyses, I demonstrated a requirement for PKCα. I also provided evidence that integrin engagement regulates downstream signaling functions of Rnd3 by inducing PKCα-mediated phosphorylation. Furthermore, I have presented evidence that PKCα-mediated signaling, through phosphorylation of Rnd3, leads to increased signaling through the RhoA-ROCK signaling pathway. A recent study described a mechanism where FN engagement led to PKCα-dependent activation for the formation and maintenance of stress fibers; although no direct connection between PKCα and RhoA was shown (366). My observations support a mechanism wherein FN-mediated integrin activation of PKCα results in PKCα-mediated phosphorylation of Rnd3. I have offered compelling evidence that Rnd3 may represent an important link directly connecting PKCα

with the RhoA-ROCK signaling pathway. The increase in Rnd3 expression in metastatic cancers (307, 310, 312, 386) suggests that Rnd3 might be selected for due its role in counteracting signaling from the RhoA-ROCK pathway. Rnd3 function may contribute to the more motile phenotype seen in metastatic cancers and phosphorylation may represent one mode of regulation to keep its signaling functions in check. The results I have presented further suggest that stimulus-mediated phosphorylation of Ras-related small GTPases may be a more common mode of regulation, distinct from GDP/GTP cycling, than has been appreciated.

In the other major aspect of my work, I investigated a role for other post-translational mechanisms of regulation of Rnd3. Proper plasma membrane localization of Ras-related small GTPases is mediated by a three-step enzymatic pathway involving prenylation of the cysteine found in the CAAX motif by either 15 carbon farnesyl or 20 carbon geranylgeranyl isoprenoids followed by two post-prenyl processing steps: -AAX proteolysis and methylation of the terminal prenylated cysteine (96, 97, 105, 116). The end result of this enzymatic cascade, involving both prenylation and post-prenyl processing, is thought to make the C-terminal domain of CAAX motif-containing proteins more hydrophobic, to better facilitate proper interactions with membranes (121). Although the classical Rho proteins such as RhoA, Rac1 and Cdc42 have been well studied (4, 9), little is known about the less-studied Rho proteins, especially the importance of CAAX-mediated signaling modifications for their subcellular locations and functions (95). A recent study found that, while farnesylated Ras proteins require full post-prenyl processing for proper membrane localization, geranylgeranylated Rho proteins do not (126). As discussed in Chapter 3, I investigated Rnd3 as part of a large collaborative effort evaluating the importance of Rce1- and Icmt-

mediated post-prenyl processing for the membrane association and function of nonclassical Rho proteins. Through the use of CAAX-specific mutations I found that full processing (prenylation, along with –AAX cleavage and carboxyl methylation) is required for both proper membrane localization and function of Rnd3. I have also provided evidence that there is a direct correlation between Rnd3 plasma membrane localization and the ability to disrupt stress fiber formation/maintenance. Further, Rnd3 requires Rce1-mediated -AAX proteolysis and Icmt-mediated carboxyl methylation of the isoprenylcysteine for proper subcellular localization, as shown by the use of *Rce1-/-*, *Icmt-/-* and WT control MEFs. In contrast to control WT MEFs where GFP-Rnd3 was mainly plasma membrane localized along with some endomembrane localization, GFP-Rnd3 expressed in *Rce1-/-* MEFs showed a significant decrease in plasma membrane association, accompanied by substantial cytosolic distribution and some nuclear accumulation. GFP-Rnd3 expressed in Icmt-/- MEFs exhibited a complete loss of plasma membrane and endomembrane localization that was accompanied by increased cytosolic, but not nuclear, localization. My colleagues also showed that alternative prenylation in Rho proteins is a rare event, implying that most Rho GTPase targets of FTIs will likely be sensitive to FTI alone. Rnd3 in particular is solely farnesylated and thus may represent an important Rho target of FTIs. Further, we showed that, in contrast to the aforementioned study (126), RhoA was highly sensitive to Icmt loss and RhoB was highly sensitive to Rce1 loss. Also, we showed that the majority of Rho proteins were dependent on both Rce1 and Icmt for proper subcellular localization and/or function. These results provide further validation for continued work on developing pharmacological inhibitors of these two enzymes for use as possible cancer therapeutics.

Future Directions: Identifying novel interacting proteins of Rnd3.

Signal transduction pathways rely on an ordered cascade of protein-protein interactions. When, where and with what partners a specific protein interacts have particular significance for the biological functions of that protein. As previously mentioned, several known interaction partners have been identified for Rnd3, but how its activity is regulated, the specific signaling pathways in which it participates, and the full range of effectors it uses to achieve its biological effects remain poorly understood. Indeed, the normal roles of Rnd3 remain incompletely characterized. As described above, there is currently some evidence for the involvement of Rnd3 in at least three biological processes: regulation of cytoskeletal organization, cell cycle progression, and tumor suppressor activity. Screens for additional interacting partners that identify proteins with roles in each of these activities may provide support for the physiological relevance of these pathways to Rnd3 function. Identification of additional interacting partners would facilitate understanding of these and other processes.

Several yeast two-hybrid assay screens to identify Rnd3 effectors have been performed by others. In addition, a tandem affinity purification technique called TAP-tag has been applied for unbiased biochemical identification of interacting proteins. However, it is clear that more work remains to be done. A logical future direction for my studies is to identify and validate novel interacting protein partners for Rnd3, both to enhance understanding of effector targets and to uncover additional biological processes for which Rnd3 may be important.

Interactions of Rnd3 with both p190RhoGAP and ROCK I (13, 14) have been shown to facilitate the mechanisms by which Rnd proteins counteract the effects of RhoA on the actin cytoskeleton. A third protein shown to bind to Rnd3 is Socius, (272). The normal

function of Socius is unknown, but a membrane-targeted mutant of Socius disrupts the actin cytoskeleton similarly to the Rnd proteins. This suggests that, like p190RhoGAP and ROCK I, Socius may also participate in Rnd protein-mediated signal transduction pathways involved in the reorganization of the actin cytoskeleton. Given the competing models of Rnd3 modulation of RhoA activity described previously in this document, it seems entirely likely that additional Rnd3 effectors of these pathways exist, and a major future direction will be to identify them.

In contrast to the proteins above, which result in downregulation of RhoA activity, Rnd3 has also been shown in a yeast two-hybrid screen to interact with the Plexin-B1 protein (294), an activator of RhoA via PDZ-RhoGEF. As described in the Introduction above, the functional consequences of this interaction were not determined, but Rnd1/Plexin-B1 interaction was shown to mediate Semaphorin D-mediated growth cone collapse by activating RhoA and inactivating R-Ras. It will be important to validate whether this or other functional consequences also occur as a result of the Rnd3/Plexin-B1 interaction.

In a study using the tandem affinity purification (TAP) tag technique (described in detail below), Rnd3 was documented to interact with the 14-3-3-sigma protein (387). 14-3-3 proteins act as phosphoserine/phosphothreonine binding proteins involved in translocating phosphorylated proteins to and from specific subcellular compartments (388-390). As yet, the exact role for this interaction remains to be determined, but perhaps 14-3-3-sigma is involved in the shuttling of phosphorylated Rnd3 from the plasma membrane, which I described in Chapter 2.

In addition to its relatively well-studied effects on the cytoskeleton, there is evidence that Rnd3 may also be involved in regulation of the cell cycle, although there is disagreement

about its role. One report (255) suggested that this inhibition of cell proliferation was due to negative effects of Rnd3 on the translation of cyclin D1 mRNA. Perhaps Rnd3 physically interacts with components of the translational machinery. Thus, one possible direction for my long-term future studies could be to attempt to identify such components.

Lastly, Rnd3 may be involved in regulating the process of cytokinesis. In support of this possibility, RhoA and several of its regulators and effectors have been shown to be critical for this process. Initiation of cytokinesis requires the establishment of the cleavage plane, the assembly of the contractile ring and the ingression of the cleavage furrow (391). RhoA is recruited to the cleavage furrow during cytokinesis (392) and is needed for proper execution of cytokinesis (393, 394). Furthermore, mDia, citron kinase and ROCK, downstream effectors of Rho, were found to accumulate at the cleavage furrow during cytokenesis (192, 395, 396). It was shown that ROCK is involved in the progression of cytokinesis through the phosphorylation of several proteins including myosin light chain at the cleavage furrow (397). Finally, the RhoA-specific GAP MgcRacGAP/CYK-4 was shown to localize to the mitotic spindle in metaphase and to be condensed at the midbody during cytokinesis, and its GAP activity was shown to be required for initiation of cytokinesis (289, 290) through the controlled assembly of the contractile ring (291). MgcRacGAP has been shown to physically interact with Rnd2 both by GST-pulldown and by co-immunoprecipitation, and to colocalize (286). While neither Rnd1 or Rnd3 was investigated in this study, it could be envisioned that Rnd3 might interact with RhoA GAPs that specifically regulate RhoA during cytokinesis or bind ROCK and inhibit its downstream signaling. With p190RhoGAP being one of the most important effectors of Rnd3, this is plausible. Whether Rnd3 is localized to the mitotic apparatus still remains to be tested. What

other proteins interact with Rnd3 to regulate cell-cycle progression and possibly cytokinesis, in addition to actin cytoskeletal regulation, still remain to be identified.

To help elucidate the identities of novel interacting proteins of Rnd3 in an unbiased manner, I would use the TAP tag method to retrieve Rnd3-interacting proteins and then identify those associated proteins via mass-spectrometry. The TAP tag was originally developed for identification of protein complexes in yeast cells (398). This purification method involves fusion of the TAP tag (either N- or C-terminal) to the target protein followed by expression in the cell system of choice. Cell extracts are prepared and the fusion protein is recovered, along with known and potential novel binding partners, by two specific affinity purification and elution steps enabled by properties of the tag. The original TAP tag consists of two IgG binding domains of *Staphylococcus aureus* Protein A and a calmodulin binding peptide (CBP), separated by a tobacco etch virus (TEV) protease cleavage site. Purification of the target protein and its associated proteins involves incubation of cell extracts with an IgG matrix (which binds tightly to the double Protein A module affinity tag), followed by stringent washing and incubation with TEV protease. Incubation with TEV protease results in cleavage at the TEV cleavage site and removal of the first affinity tag. The partially enriched extract is then incubated with calmodulin beads in the presence of Ca^{2+} to bind to the CBP affinity tag, followed by stringent washing and a final release of the target protein and associated proteins with an elution buffer containing the Ca^{2+} chelating agent EDTA. This double (or tandem) affinity purification technique promotes the purification of a highly enriched sample containing the protein of interest along with both known and potentially novel interacting proteins that can then be concentrated and analyzed by mass spectrometry for positive identification (399).

The TAP tag method, in combination with mass spectrometry, could be a valid method of uncovering the identities of novel interactors of Rnd3. A report from Liu and colleagues described their successful use of a variation of the TAP tag method to uncover multiple novel protein interactors of the signaling molecule SMAD3 (400). The variation to the original TAP tag came in the form of a FLAG tag, rather than use of a calmodulin binding peptide as the second affinity tag. The authors indicated that the CBP affinity tag was problematic because so many cellular proteins are regulated by calmodulin. With this in mind, I proceeded through multiple cloning rounds to produce my own variant TAP tag similar to the one used by Liu and colleagues, in that its first affinity tag is a tandem Protein A-binding module, followed by a TEV protease site and ending in a FLAG tag to be used as the second affinity tag (Figure 4.1A). I then proceeded to clone this new TAP tag into the expression vector pcDNA3.1(+), making two variants. One variant is a read-through, which will allow fusion of the TAP tag to the N-terminus of the protein of interest, and the second variant is a negative control that has a stop codon immediately following the FLAG tag to produce an empty vector expressing only the TAP tag. I named the read-through variant "pTAP-Go" and the control variant which has the incorporated stop codon "pTAP-Stop". I inserted both TAP tag variants into the BamHI-EcoRI sites of pcDNA3.1(+). I then inserted into the newly produced pTAP-Go vector, by use of the EcoRI-XhoI sites, the coding sequence of Rnd3 with an added stop codon. I have named this construct "pTAP-Rnd3".

Both the pTAP-Rnd3 and the control pTAP-Stop variant vectors expressed quite well in both mouse NIH 3T3 cells and human HEK-293 cells (Figure 4.1B). Immunoblot analysis with anti-FLAG antibody of cell lysates expressing each construct showed that neither of the fusion proteins (TAP tag control or the TAP-Rnd3) had undergone significant

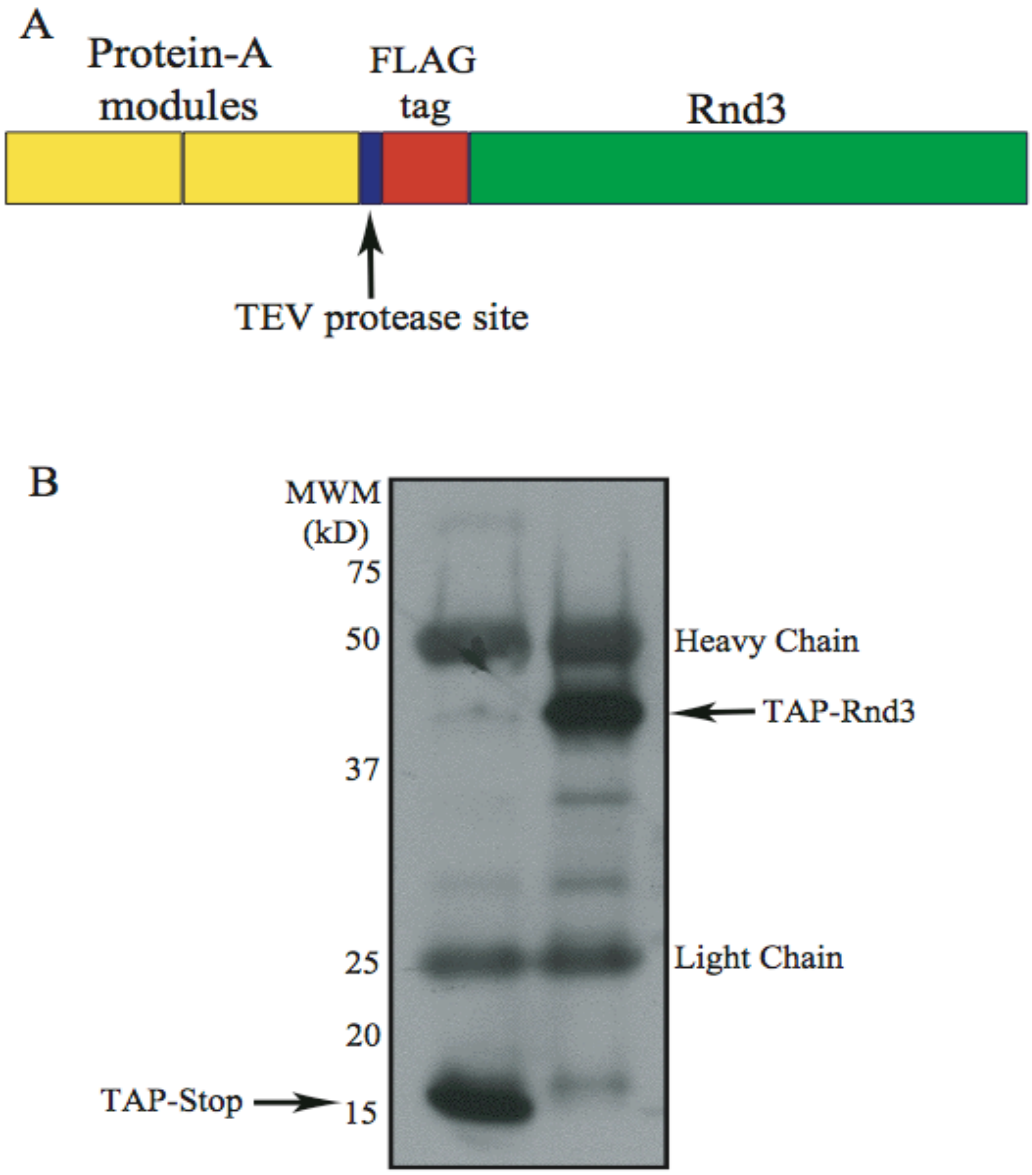

Figure 4.1: Design and validation of a novel TAP tag system. A, I have developed a novel TAP tag vector employing two Protein-A modules, a TEV protease site and a FLAG tag. Rnd3 was inserted in-frame behind the TAP tag cassette. **B,** Both the control TAP-Stop and TAP-Rnd3 express in NIH 3T3 cells. The expressed proteins are of the correct size and show no unwanted proteolysis.

proteolysis. Both fusion proteins could be purified using either IgG or FLAG antibody conjugated beads, and using recombinant TEV protease, I also demonstrated that the TEV protease site incorporated into the new TAP tag vector can be successfully cut, with no unwanted cleavage elsewhere (data not shown). This cleavage step is critical to avoid nonspecific binding of detector antibodies to the Protein A module. Finally, I have generated HEK-293 cell lines that stably express either the control TAP tag or the TAP-Rnd3 fusion.

If future attempts were successful in identifying bands obtained by interaction with TAP-Rnd3 but not TAP-stop, I would first need to confirm the interaction between Rnd3 and these newly identified proteins. To accomplish this, I would perform co-immunoprecipitation experiments in both directions - protein X binding to Rnd3 and Rnd3 binding to protein X. Also, I would perform colocalization experiments to visualize whether Rnd3 and protein X display overlapping cellular localizations.

To determine which of the newly identified interacting proteins is a potential Rnd3 effector, as distinct from a Rnd3 regulatory protein or one lacking in functional consequences, I would first determine if the newly identified proteins could still interact with Rnd3 effector domain mutants. Specifically, I would expect to see impaired binding to a Rnd3(T55A) mutant, which is analogous to the RhoA(T37A) mutant that shows impaired effector binding to multiple effectors. Binding of Rnd3 to p190RhoGAP was eliminated when this mutant was employed (13). I would also use other effector domain mutants that have been successfully used in studies of RhoA, since the core effector domains of Rnd3 and RhoA are identical (4, 401, 402).

As mentioned previously, the region encompassing Rnd3 residues 16-93 was shown to be the minimal region of Rnd3 required to bind to p190RhoGAP (13), consistent with

studies showing that RhoA binding to the ROCKs and PKN-related kinases requires not only the Switch I effector domain region but also loop 6 residues (amino acids 75-92, just C-terminal to Switch II) (174). Therefore, I would also determine whether mutations to the region of Rnd3 homologous to the loop 6 region of RhoA impair binding to candidate Rnd3 effectors. In addition, the insert region of RhoA has been shown to be important for regulation of ROCK activation (172). I would test whether mutations in the Rho insert region of Rnd3 (residues 142-155) also conferred loss of binding.

In addition, although Rnd3 is GTPase-deficient and thus constitutively GTP bound, I would like to determine whether the binding of Rnd3 to any of these newly identified proteins is GTP-dependent, which is an important criterion for a true effector protein. Therefore, I would use a Rnd3(T37N) mutant which is analogous to the persistently GDP-bound RhoA(T19N) mutant. As with Rnd3(T55A), binding of Rnd3 with p190RhoGAP was also eliminated when the Rnd3(T37N) mutant was employed. Hence, GTP binding is necessary for Rnd3 to bind to p190RhoGAP, and interaction of any novel interacting protein(s) that are true Rnd3 effectors would also require that Rnd3 be bound to GTP.

The next step would be to map out, by use of truncation mutants, which regions of Rnd3 and the newly identified candidate effector protein(s) are important for their interaction. Finally, it would be necessary to validate which of these proteins has a functional consequence for Rnd3 biological activity. If the candidates have known functions in any of the three biological activities cited above, whether cytoskeletal organization, cell cycle progression or cytokinesis, the functional assays would be chosen accordingly. Alternatively, if they have known functions in other biological processes, different assays would be appropriate, and could provide insight into other possible roles for Rnd3 in cellular

activities. Interacting proteins with no known biological functions would be lowest on the priority list for evaluation.

Overall, I am optimistic that, by using my TAP tag vectors, it will be possible to identify novel protein-protein interactors and effectors of Rnd3. Identifying novel interacting proteins of Rnd3 will surely help in clarifying its role in currently known cellular activities and possibly uncover previously unknown functions of Rnd3.

Future Directions: The role of Rnd3 in pancreatic cancer.

Pancreatic cancer is a devastating disease with an overall 5-year survival of only 4% (403). Currently the only opportunity for improved survival continues to be surgical resection for those with localized disease. Yet, this is only achievable for fewer than 15% of patients diagnosed, due to the fact that most patients diagnosed with pancreatic cancer already have metastatic disease (404). Even for patients who are able to undergo surgical resection, median survival is only 17 months (404). Clearly, it is important to identify new and better targets for intervention in this disease.

Expression profiling has identified upregulation of Rnd3 in pancreatic cancer compared to normal pancreas (307) (D. Billadeau, personal communication). In further support of Rnd3 expression increasing during cancer progression, Rnd3 has been recently shown to be upregulated in metastatic melanoma versus primary melanoma (310) and to be involved in Raf-dependent metastasis of melanoma cells (386). I would like to determine whether Rnd3 upregulation is merely correlated with or is functionally important in this progression.

To determine appropriate model cell lines to test my hypothesis that Rnd3 is functionally important in pancreatic cancer progression, I will first screen a panel of pancreatic cell lines for the abundance of Rnd3 protein. I expect that comparing Capan-1 and Capan-2 cell lines will be of interest. Like nearly all pancreatic cancers, both cell lines possess a mutant K-ras allele. Further, a prior study, from our group and the Counter group at Duke University, has shown that Capan-1 cells have a high level of K-Ras-GTP as compared to Capan-2 cells (43). A previous study has shown that Rnd3 expression is upregulated in Raf-induced transformed MDCK cells (250), and there is evidence that Rnd3 is upregulated by Ras/Raf activity in a MEK/ERK-dependent manner (Singh et al., unpublished data). It would be interesting to know if there is a direct correlation between levels of K-Ras-GTP and Rnd3 levels. Once I have identified levels of Rnd3 protein in this panel, I will use these cells to determine whether Rnd3 activity is important for their transformed properties.

As part of my goal to determine a possible role for Rnd3 in pancreatic cancer progression and metastasis, I have validated Rnd3-specific shRNA sequences capable of knocking down expression of Rnd3. As seen in Figure 4.2A, Rnd3 protein was greatly reduced in HEK-293 cells that stably express Rnd3-specific shRNA targeted to two distinct sequences of Rnd3 mRNA, compared to HEK-293 cells expressing an empty shRNA vector. I will next use these shRNAs to try to knockdown Rnd3 expression in my panel of pancreatic cancer cell lines.

Recently, I attempted this process unsuccessfully in two metastatic melanoma cell lines, both by transfection and by infection (Figure 4.2B). Because Rnd3 is highly upregulated in metastatic versus primary melanoma (310), I reasoned that there might be

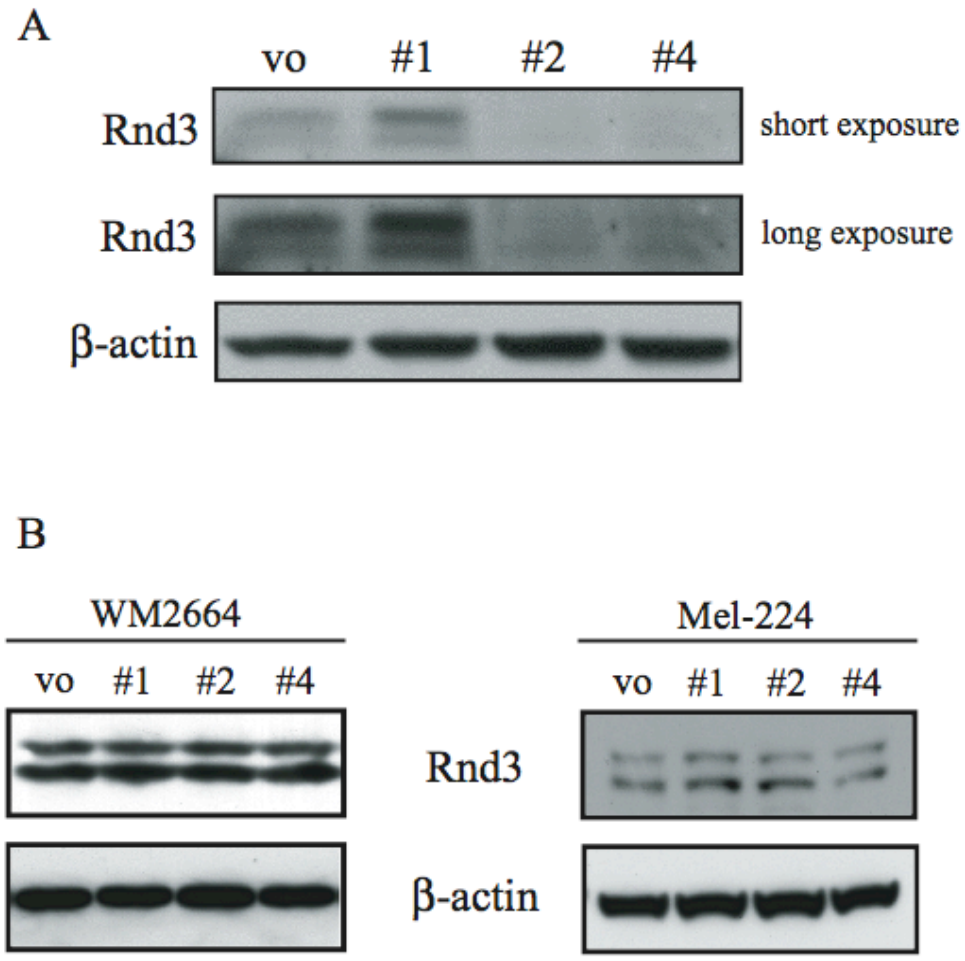

Figure 4.2: Rnd3 protein is knocked down by specific shRNA sequences. A, HEK-293 cells stably expressing Rnd3-specific shRNA display significant knock-down of Rnd3 protein in 2 out of 3 targeting vectors. **B,** None of the targeting vectors show knock-down of Rnd3 protein in two stably-selected metastatic melanoma cell lines.

selective pressure against cells lacking Rnd3. Perhaps metastatic melanoma cells "need" Rnd3 and the viability of these cells decreased when the levels of Rnd3 dropped below a certain threshold for a certain period of time. To test this theory, I intended to knockdown Rnd3 transiently. Unfortunately, Andrew Aplin and colleagues recently published results very similar to my planned experiments (386). This group showed that knockdown of Rnd3 via Rnd3-specific siRNA, in metastatic melanoma cells, resulted in an increase in signaling from the RhoA/ROCK/LIM kinase pathway leading to increased cofilin phosphorylation, stress fiber formation and reduced cell invasion. These results are consistent with my hypothesis that Rnd3 is required for proper RhoA signaling and subsequent cytoskeletal reorganization and transformation properties. Perhaps Aplin and collegues used siRNA oligonucleotides transiently because they too were unsuccessful at making stable metastatic melanoma cell lines having reduced Rnd3 expression by using vector-based shRNAs. Therefore, if I am unsuccessful at making stable pancreatic cancer cell lines using my validated Rnd3-specific shRNAs, as an alternative approach I will perform targeted knockdown of Rnd3 in these cells using validated Rnd3-specific siRNA oligonucleotides.

After achieving successful knockdown of Rnd3 in pancreatic cancer cells, I will perform assays looking at colony formation in soft agar, scratch-wound motility and invasion through Matrigel. I predict that cells in which Rnd3 is knocked down will display either fewer or smaller colonies in agar as well as decreased motility and invasion.

To validate that any functional impairments in Rnd3 knockdown cells are due to loss of Rnd3, I will force express an RNAi-insensitive version of Rnd3 and confirm restoration to endogenous levels of Rnd3. If the functional impairments in the knockdowns are based on decreased levels of Rnd3 protein, then the RNAi-insensitive Rnd3 will be able to restore

colony formation, motility and invasion even in the presence of knocked down endogenous Rnd3.

Lastly, using these stable knockdown cells I plan to look at tumor growth and metastasis in a pancreatic orthotopic mouse model. I plan to make stable cell lines that express both the Rnd3-specific shRNA and GFP to enable me to track tumor growth and metastasis. This will be done by using either an expression vector that has an IRES or an expression vector that contains two promoters that allows insertion and expression of two separate sequences (Rnd3 and GFP). These stable cell lines will be surgically orthotopically implanted into the pancreas of nude mice. Whole body fluorescent optical imaging will be used to visualize, in real time, tumor growth and metastasis *in vivo* (405-407). I will determine whether a reduction of Rnd3 in these cells has any effect on their metastatic potential *in vivo*. As mentioned previously, a recent study suggested that Rnd3 promotes melanoma invasion (386). In regards to pancreatic cancer, I predict that a reduction of Rnd3 protein, by use of RNAi technology, will have a negative effect on the potential of pancreatic cancer cells to invade and metastasize in the orthotopic pancreatic cancer mouse model. If this turns out to be the case, it would further support the importance of targeting Rnd3 for pancreatic cancer treatment.

Summary

Through the research outlined in this dissertation, I have shown that the GTPase-deficient Rho family protein Rnd3 can be regulated by mechanisms other than GDP/GTP cycling. The main theme that I have observed throughout my research is that localization of Rnd3 dictates its function. I have shown that PKCα-mediated phosphorylation of Rnd3

regulates both of these processes. Phosphorylated Rnd3 loses its affinity for the plasma membrane and is impaired in its ability to disassemble stress fibers and thereby cause cell rounding. Integrin-mediated signaling upon cell attachment causes PKCα-mediated phosphorylation of Rnd3 that is required for its ability to cause stress fiber disassembly and spreading, I suggest that stimulus-mediated phosphorylation of Ras-related small GTPases may be a more common mode of regulation, distinct from GDP/GTP cycling, than has been appreciated. Furthermore I have shown that Rnd3 is sensitive to loss of Rce1- and Icmt-mediated post-prenyl processing modifications. Mislocalization due to improper post-prenyl processing also abrogates Rnd3 function in regulating the actin cytoskeleton. The discovery that the localization and function of Rnd3, along with those of most Rho proteins, is sensitive to loss of post-prenyl processing provides validation that both Rce1 and Icmt may represent potentially important drug targets for use as cancer therapeutics.

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