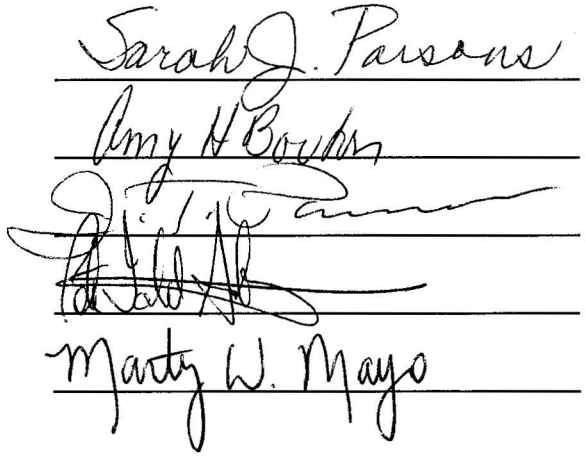
The Mitotic Regulation of p190RhoGAP and Its Role in Cytokinesis

Sergio Armando Sánchez Manchinelly
Tuxtla Gutiérrez, Chiapas, México

B.S., Brigham Young University, 2003

A Dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Microbiology
University of Virginia
May, 2010



ABSTRACT

Mitosis and cytokinesis are the final stages of the cell cycle where organelles and replicated DNA are equally separated to give rise to two daughter cells. Irreversible progression through these stages is mediated by proteasomal-dependent degradation of critical cell cycle regulatory proteins such as Cyclin A, Cyclin B, and Securin (Peters, 2002). In addition to proteasomal degradation, an important regulator of cytokinesis progression is the small GTPase RhoA (Glotzer, 2001), whose high activity is required for successful completion of cell division.

P190 is a RhoA-specific GTPase Activating Protein (GAP) that inhibits RhoA activity by stimulating the hydrolysis of Rho-bound GTP to GDP. Previous work in our laboratory defined a role for p190 in cytokinesis (Su et al., 2003), namely that (1) overexpression of p190 resulted in a multinucleation phenotype, indicative of cytokinesis failure, (2) p190 was localized to the cleavage furrow, where it colocalized with actin and opposed the action of the Rho Guanine Nucleotide Exchange Factor (GEF) Ect2 (Mikawa et al., 2008) and (3) p190 protein levels were observed to decrease drastically in cytokinesis through ubiquitin-mediated proteasomal degradation.

Results presented in this dissertation reveal that the GAP activity of p190 is responsible for the observed multinucleation phenotype and that mitotic p190 degradation, and the associated decrease in GAP activity, is required for successful cytokinesis completion, consistent with the requirement for high RhoGTP levels during this stage of the cell cycle. Furthermore, we discovered that four N-terminal lysine residues in p190

are required for its mitotic degradation. Preliminary results suggest the involvement of the APC/C^{cdc20} E3 ligase complex in mitotic p190 ubiquitination. Additionally, p190 was found to affect the localization of the phosphorylated form of myosin II, necessary for contractile activity at the cleavage furrow, likely through association with the molecular scaffold Anillin, which is critical for the organization and stabilization of the contractile ring. Intriguingly, the interaction between these two molecules was contractility dependent, providing an initial indication of a potential mechanosensing mechanism whereby cells can sense appropriate tension along the cleavage furrow and relay that information to RhoA, the master regulator of contractile ring dynamics. Finally, we report the GAP-dependent involvement of p190 in the alignment of metaphase chromosomes at the equator of a dividing cell. This process does not involve RhoA function but rather, appears to be regulated by the Rho family GTPase Cdc42. This observation suggests that p190 may act as a dual-specificity GAP with affinity for Cdc42 during mitosis and for RhoA affinity during cytokinesis. Together, the work presented in this dissertation increases and clarifies our understanding of the role p190 plays in the Rho family-mediated processes necessary for proper cell division.

TABLE OF CONTENTS

List of Figures

Chapter 1

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 2

Abbreviations

APC/C	anaphase promoting complex/cyclosome
Bcr	breakpoint cluster region
C3	Clostridium botulinum exotoxin C3
Cdc20	cell division cycle protein 20
Cdc42	cell division cycle protein 42
Cdh1	fizzy-related protein homolog isoform 1
CP	core particle
cycB/cdk1	cyclin B1/cyclin-dependent kinase 1
DAPI	4',6-diamidino-2-phenylindole
Dox	doxycycline
ECL	enhanced chemiluminescence
ECM	extra cellular matrix
EGF	epidermal growth factor
FRET	fluorescence resonance energy transfer
GAP	GTPase activating protein
GBD	GTP binding domain
GDP	guanosine diphosphate
GDI	guanosine diphosphate dissociation inhibitor
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein

GTP	guanosine triphosphate
GTPase	guanosine triphosphate hydrolase
HA	hemagglutinin
HRP	horseradish peroxidase
IF	immunofluorescence
IP	immunoprecipitation
kDa	kilodaltons
mAb	monoclonal antibody
MD	middle domain
mDia	mammalian Diaphanous
MLC	myosin regulatory light chain
MN	multinucleation
NOC	nocodazole
NP-40	Igepal CA-630
OA	okadaic Acid
p120	p120RasGAP
p190	p190RhoGAP
p190B	p190RhoGAP isoform B
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
RIPA	radioimmunoprecipitation assay

RNAi	RNA interference
ROCK	Rho-associated, coiled-coil containing protein kinase I
RTK	receptor tyrosine kinase
RP	regulatory particle
SCF	Skp1/Cdc53/Cullin/F-box receptor complex
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Ser/thr	serine or threonine
SFK	Src-family kinase
TnT	transcription and translation product
VEGFR	vascular endothelial growth factor receptor
WB	western blot

Acknowledgements

The nearly seven years I've spent as a graduate student have been the best and most challenging years of my academic life. Many people have made a tremendous difference in my life as I've gone through the joys and challenges associated with the completion of a doctoral degree. First I would like to thank the outstanding members of my dissertation committee, Tom Parsons, Todd Stukenberg, Amy Bouton and Marty Mayo. I am forever grateful for the way in which their scientific expertise has shaped the way I think about science. I admire them and their work and feel privileged to have had the opportunity to associate with them through the years.

I am particularly grateful to my mentor, Sally Parsons. I could not have been better matched with a mentor who would provide me with the right combination of steady guidance and intellectual freedom. I am grateful for the voice of experience and reason that Sally was to me and for the patience she had to allow me to figure things out on my own and blaze my own path. I am also grateful for the passion for learning and discovery that Sally has. I have inherited a portion of Sally's joy of learning and know that my life will be continually blessed because of it. Thank you Sally for shaping me into the scientist and person that I now am.

I am also grateful for the numerous members of the S. Parsons laboratory and for the help and support they have provided me through the years. I would like to thank Ling Su for her patience in teaching me all there is to know about p190RhoGAP. She certainly was the role model that I aspired to emulate during my scientific endeavors.

Little of the work presented in this dissertation would have been accomplished without the help and mentorship of Ling Su. Similarly, special thanks go to Masahito Mikawa. We joined the lab around the same time and shared many good times together. His expertise on the p190 project was also critical to my scientific development and the work presented in this dissertation. I am also grateful to Tsuyoshi Miyake for all the molecular biology help he gave me and for numerous hours of "Apple" talk. Thanks to John DaSilva for serving as a wonderful role model of scientific maturity and development as well as a good friend. Thanks to Jessica Pritchard and Kirsten Ludwig for being remarkable companions in the long and arduous road of graduate school. Thank you for your listening ears and quick-thinking minds, for your honesty and support, without all of which I would not be the scientist and person that I am now. Thanks to Eli Casarez, for always being a good friend and for sharing reagents, solutions and the bench with me for many years. To Bill Faust, for being one of the most helpful and reliable persons I have ever met. It has been a pleasure getting to know you and I look to emulate your example of how to be the one person that is always willing to help and get people in a good mood.

I also would like to thank Ann Beyer for helping me discover laboratory research and guiding me towards the path I now complete, a graduate degree in Microbiology at the University of Virginia. Within her laboratory, Yvonne, Sarah and Martha were also great influences and examples to me.

I am forever indebted to my mom and dad, Enrique and Maria Elena, a man with a vision and dream for his children and a woman with the strength and character to help make those dreams a reality. I have certainly come quite a long way from my humble

beginnings in southern Mexico to accomplish many great things. However, let it be known that I have become the person that I am and achieved the things I that have because of the great example my parents gave me and, above all, because of the opportunities they afforded me and for which they sacrificed dearly.

Finally, none of the wonderful events in the last nine years of my life would have been possible without the ever-loving companionship of my best friend, my wife Sarah. I can not imagine going through the long and arduous road of graduate school without the love, constant cheers and support that Sarah always had for me. Sarah, I truly admire you and love you deeply. You are the best and most wonderful blessing I have ever received and will continue to be so throughout time and all eternity. I love you with all my heart and feel blessed beyond comprehension for the privilege of walking through life with you, hand-in-hand.

Dedication

To my Children:

Elizabeth, Ethan, Kate, Sophie and those still to join our family.

Anything you can dream, you can achieve through hard work and dedication. Anything.

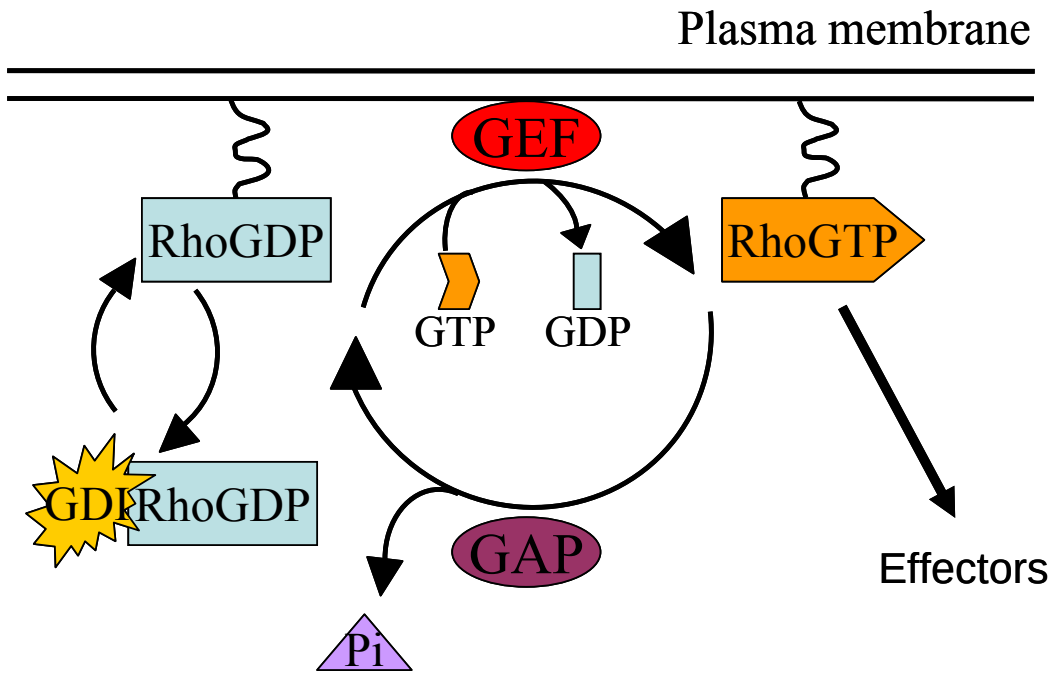
Chapter 1

Introduction

Many important cellular processes such as migration, adhesion, polarity, and cell division require the reorganization of the actin cytoskeleton. This structural system relies on numerous actin-associated and regulatory proteins for proper function. RhoGTPases have long been implicated as master regulators of actin cytoskeleton dynamics and, therefore, the modulation Rho activity is of vital importance in the cellular context. The protein that is the main focus of these studies is p190RhoGAP (p190), a negative regulator of Rho activity. Although much is known about p190 function in migration, adhesion, and cell morphology, processes that take part during the interphase stage of the cell cycle, little was known regarding the involvement of p190 in modulating RhoGTP signaling during mitosis until work from our laboratory was published in 2003. The results presented in this dissertation are the continuation of that original work and offer further clarity in our understanding of the role and regulation of p190RhoGAP during mitosis and cytokinesis.

RHO GTPASES

RhoGTPases are a family of small GTPases that are central to the regulation of actin cytoskeleton organization and dynamics. There are numerous RhoGTP family members which are divided into three subgroups, Rho, Rac and Cdc42, named after the best known GTPases in each group. The Rho subgroup is the largest of the three, comprising RhoA, RhoB, RhoC, RhoD, RhoE, RhoG, RhoH and the Rnd proteins. The Rac subgroup includes Rac1, Rac2 and Rac3 while the Cdc42 subgroup consists of Cdc42Hs, Tc10, and G25K (Aspenstrom, 1999). RhoGTPases are nucleotide binding



Adapted from Etienne-Manneville and Hall. 2002. Nature 420, 629-635

Figure 1. The Rho activation cycle

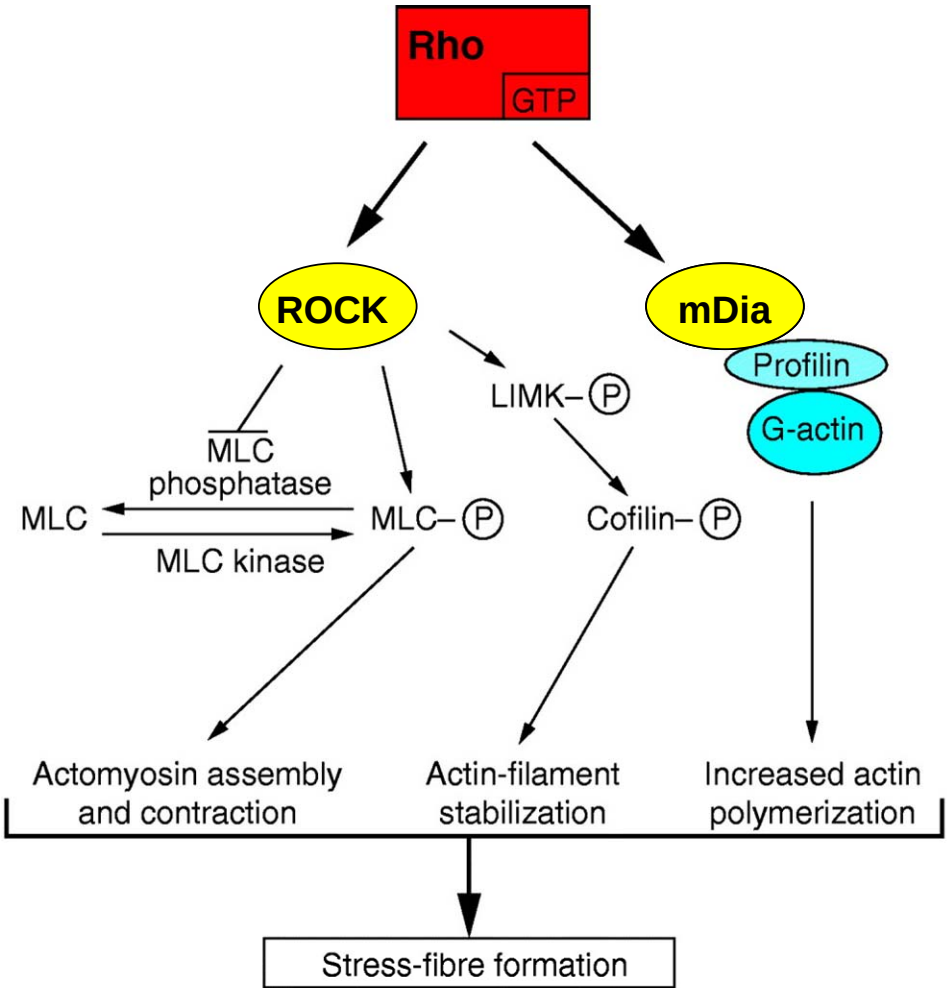
The activity of Rho family small GTPases is regulated by three classes of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDI). Rho binds GTP with the aid of GEFs, which exchange GDP with GTP. Rho-GTP is the active form of the protein. GAPs stimulate the intrinsic GTP hydrolysis activity of RhoGTPases, releasing inorganic phosphate. Rho-GDP is the inactive form of Rho. GDIs prevent GDP release, thereby maintaining Rho in the inactive state. Rho regulates actin cytoskeleton dynamics by means of several effector proteins.

proteins that function as molecular switches, given their ability to rapidly cycle between on and off states. Their activation status is dependent on nucleotide binding: when GTPases are bound to guanosine triphosphate (GTP) they are fully activated, whereas binding to guanosine diphosphate (GDP) results in their inactivation. Nucleotide binding status is affected by the actions of three GTPase regulatory proteins: Guanine nucleotide Exchange Factors (GEFs), GTPase Activating Proteins (GAPs) and Guanine nucleotide Dissociation Inhibitors (GDIs). GEFs promote nucleotide exchange of GTPase-bound GDP for GTP, resulting in RhoGTPase activation. GAPs stimulate the intrinsic GTP hydrolysis activity of GTPases, increasing the rates of bound-GTP conversion into GDP, and GDIs block spontaneous Rho activation by sequestering it away from the membrane (Figure 1). Due to the nature of Rho activation, rapidly cycling between active and inactive states, growth factor stimulation, integrin engagement and G-protein coupled receptors are known to activate both GEFs and GAPs (Hall and Nobes, 2000).

Although accepted as bona fide regulators of cellular actin structures and their dynamics, it was several years before researchers discerned the specificity of these proteins as studies clarified the different roles that RhoA, Rac1, and Cdc42 played in directing the dynamics and stability of different actin structures in the cell. Microinjection experiments that introduced constitutively active RhoA in serum-starved NIH3T3 cells revealed that RhoA activity resulted in the assembly of actin stress fiber formation (Nobes and Hall, 1995). In contrast, inhibition of RhoA activity by treatment with the C.*botulinum* exotoxin C3, which specifically ADP-ribosylates Rho and prevents its activation (Nemoto et al., 1991), resulted in the loss of stress fiber and lack of focal

adhesion formation (Ridley and Hall, 1992). Similar microinjection experiments revealed that Rac 1 activity directs lamellipodia formation at the edges of the cell (Ridley et al., 1992) and that Cdc42 activity resulted in the formation of finger-like protrusions of cell membrane called filopodia (Nobes and Hall, 1995;Hall, 1998). Because the work presented in this dissertation is focused on the regulation of RhoA activity and signaling during mitosis by p190RhoGAP, I will further discuss and elaborate on the downstream signaling specific to the GTPase RhoA.

As depicted in Figure 2, RhoA activity is the initiating signaling event that results in actin polymerization, particularly in stress fiber formation. RhoA directs the three major steps involved in stress fiber formation: actin polymerization, actin filament stabilization and actomyosin assembly/contraction (Bishop and Hall, 2000b). Rho directs actin polymerization through some of its most important effectors, the Diaphanous or mDia proteins. mDia proteins contain formin homology domains which are capable of nucleating actin filaments. Unlike the Arp2/3 complex, involved in the formation of branched filament networks, mDia functions to mediate the formation of straight actin filaments (Chang and Peter, 2002). These straight actin filaments can then be stabilized and bundled to form stress fibers. Rho mediates filament stabilization primarily through its effector Rho Kinase I, hereafter referred to as ROCK (Yoneda et al., 2005). ROCK activation results in the phosphorylation of LIM kinase which in turn phosphorylates and inactivates cofilin, an actin-binding protein. Cofilin disassembles actin filaments by severing them and increasing the off-rate for actin monomers at the barbed-end (Carlier et al., 1997;Ichetovkin et al., 2000). Rho-mediated inhibition of cofilin thus increases



Adapted from Bishop and Hall,
Biochem. J. (2000) 348, 241-255

Figure 2. Stress fiber regulation by RhoA

Rho regulates stress fiber formation through acto-myosin assembly and contraction, actin filament stabilization, and actin polymerization. Active Rho (RhoGTP) regulates these processes through Rho-associated kinase I (ROCK) and mDia proteins. ROCK mediates phosphorylation of myosin light chain (MLC) phosphatase, inhibiting its activity, and of MLC directly. Increased MLC phosphorylation results in actomyosin filament assembly and contraction. ROCK also phosphorylates LIM kinase (LIMK), which facilitates actin filament stabilization by inhibitory phosphorylation of cofilin. Rho directs increased actin polymerization through mDia proteins, actin nucleators that produce long, straight filaments. The association of mDia with Profilin, an actin-binding protein, promotes actin polymerization.

actin filament stability. Rho also directs actomyosin assembly and contraction through the actions of ROCK on the molecular motor myosin II. Discovered in 1978, myosin II is an actin-binding motor protein made up of two heavy chains, two essential light chains and two regulatory light chains (Pollard et al., 1978). Actin crosslinking by myosin II and the ensuing formation of stress fibers is regulated by Rho through ROCK. For myosin II to bind and crosslink actin, Threonine 18 of its myosin light chain (MLC) must be phosphorylated (Bresnick et al., 1995;Matsumura et al., 2001). Moreover, ROCK-mediated phosphorylation of MLC at Ser 19 results in the activation of the ATPase activity and motor function of myosin II (Hirano et al., 2003). Thus, myosin motor activity is controlled by phosphorylation of its regulatory light chains (MLC) at these two residues which are also targeted by other kinases, including Rho substrate citron and myosin light chain kinase among others (Hirano et al., 2003). To ensure full activation of myosin II, ROCK also phosphorylates the phosphatase in opposition to MLC phosphorylation, Myosin Light Chain Phosphatase. Phosphorylation of this phosphatase renders it inactive and allows for a tremendous increase in MLC phosphorylation and myosin II activation (Ito et al., 2004). Myosin phosphorylation and activation increases its actin-binding affinity, permitting the crosslinking of several actin fibers into a more stable and mechanically active actomyosin filament. Myosin II phosphorylation and activation also result in increased ATPase activity of this motor protein, allowing it to walk along crosslinked filaments and providing the mechanical force necessary for such cellular events as cell migration and cytokinesis (Jaffe and Hall, 2005).

The involvement of RhoA in cytokinesis, the last stage of the cellular life cycle, is

of particular relevance to the work presented in this dissertation. Though addressed in further detail later in this Introduction, I will briefly review the participation of RhoA in cytokinesis. Numerous laboratories have detected increases in RhoGTP levels during cytokinesis and reported that inhibition of Rho activity at this stage of the cell cycle results in cells failing to divide properly, giving rise to multinucleation (Kimura et al., 2000b;Maddox and Burridge, 2003;Yoshizaki et al., 2004;Su et al., 2009). In order for cells to divide properly during cytokinesis, an actomyosin ring must be formed at the midzone of the dividing cell, where it provides the mechanical forces that allow membrane furrowing and eventual separation of daughter cells. Activation and function of downstream Rho effectors ROCK and mDia, as described above, is crucial for actomyosin ring formation and contraction (Figure 6).

Central to the work in this dissertation is the negative regulation of cellular RhoGTP levels by a particular GAP, p190RhoGAP. Of all RhoGTPase family members, p190RhoGAP has greatest specificity for RhoA and is an important component of stress fiber and focal adhesion regulatory complexes (Ridley et al., 1992;Ridley et al., 1993;Nobes and Hall, 1995;Chang et al., 1995). This dissertation expands on the regulation and function of p190, particularly during cytokinesis, as we have previously observed that its expression levels are significantly reduced at a time when RhoGTP levels are greatest and reported that its overexpression causes multinucleation and cytokinesis failure. Included below is a broad review of the p190 literature that will provide readers with the proper perspective of my work, in light of the essential cellular role of RhoA, described above.

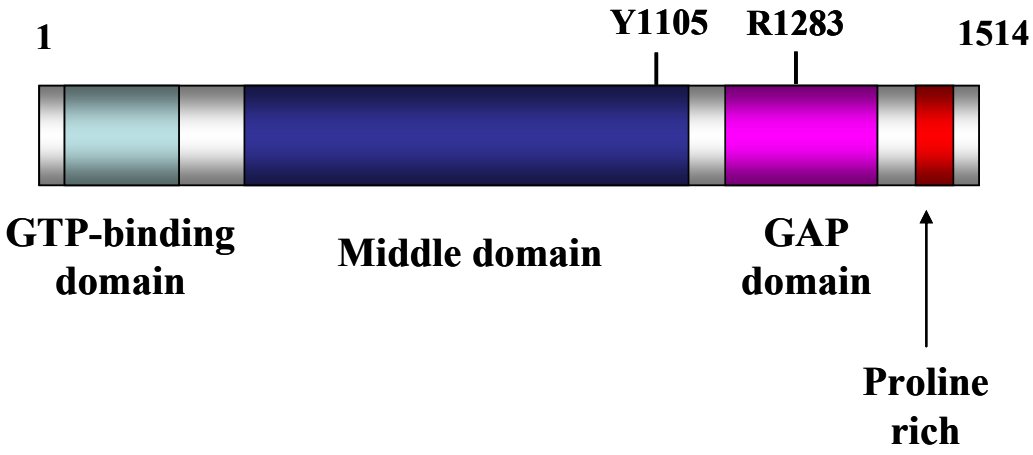

1
Y1105
R1283
1514
GTP-binding domain
Middle domain
GAP domain
Proline rich

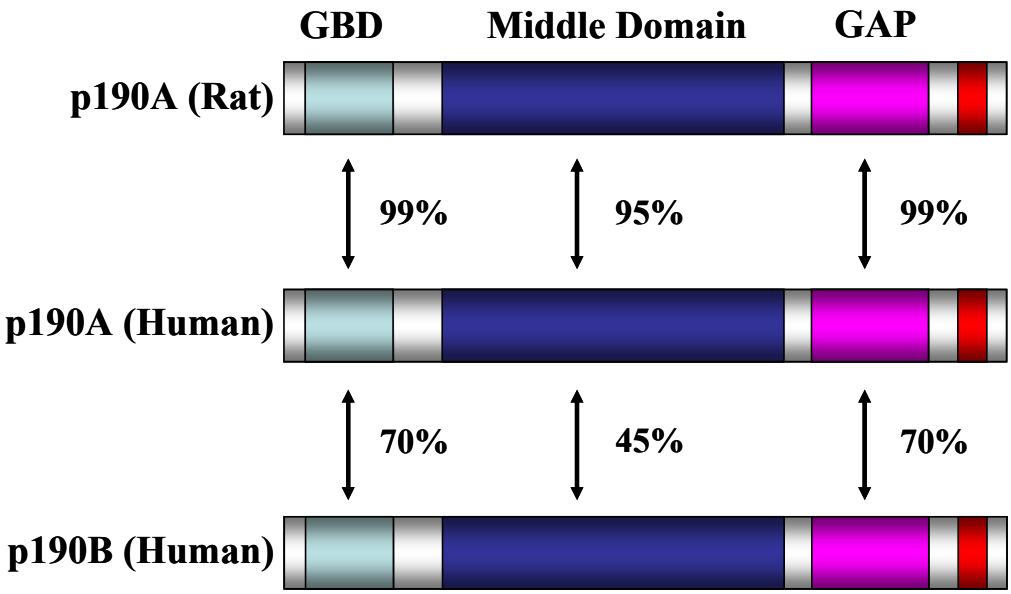
Figure 3. Schematic representation of p190RhoGAP

p190 is a RhoGTPase-activating protein (GAP) that consists of a N-terminal GTP-binding domain or GBD (indicated in light blue), a Middle Domain or MD (indicated in blue) and a GAP domain (indicated in purple). The GBD can bind GTP directly. The MD regulates p190 interactions and function through protein-protein interaction domains and phosphorylation sites. Phosphorylation of Y1105 by c-Src results in increased p190RhoGAP activity. The MD has been arbitrarily divided into 3 sections (Sect 1, Sect 2, and Sect 3). The GAP domain is specific for Rho family members, particularly RhoA. Arginine 1283 (R1283) mediates p190-RhoA interaction. Adjacent to the GAP domain is a proline rich region (indicated in orange).

P190RhoGAP

Historical background

p190RhoGAP-A, a Rho GTPase activating protein (hereafter referred to as p190), was first described in 1990 by Ellis and colleagues as a tyrosine phosphorylated protein that associated with p120RasGAP and p62Dok in cells that had been transformed by cytoplasmic and receptor-like tyrosine kinases such as v-src, v-fps and p56Lck as well as in cells stimulated with the epidermal growth factor (EGF) (Ellis et al., 1990;Ellis et al., 1991). A signaling pathway that directly linked the EGF and v-Src as upstream effectors of p190RhoGAP phosphorylation and its association with the p120RasGAP-p62Dok complex would be described shortly thereafter (Bouton et al., 1991b;Chang et al., 1993). In 1992, p190 was cloned and characterized by a group from MIT lead by Dr. Robert Weinberg (Settleman et al., 1992c). They analyzed several cDNAs encoding p190 which yielded an expected protein sequence containing two discrete domains that were homologous to others previously described. The N-terminus revealed motifs associated with all GTP binding proteins (GTPases) known at that time. The C-terminus included a GTPase Activating Protein (GAP) domain (Figure 3). Moderate progress in understanding the role of the N-terminal GTPase domain has been made since this initial study was published. In fact, a partial aim of the work presented in this dissertation was to shed further light on the biological role of the GTPase Binding Domain (GBD)-containing N-terminus of p190. The C-terminal GAP domain has dominated the interest of researchers working on p190 and much is known regarding its function and regulation. Further discussion regarding each of these individual domains and our current



Adapted from Tikoo et al. *Gene* (2000) 257:23-31.

Figure 4. Schematic representation of the homology between p190A and p190B

There is sequence conservation of the common regions of p190 among species and related proteins. The light blue region represents the GBD domain, the blue region represents the MD, and the purple region denotes the GAP domain. The percentages indicate the amount of identity between the sequences for the particular domain. The rat and human p190A proteins are nearly identical. Though structurally homologous, the amino acid sequences of p190A and p190B are significantly different, the greatest divergence being found within the MD.

understanding of their function and regulation will be undertaken shortly hereafter, as part of this Introduction.

Several years after the discovery of p190, an additional member of the p190RhoGAP family of proteins was described and designated p190RhoGAP-B, hereafter referred to as p190B (Burbelo et al., 1995). This study reported the cloning of a protein with 52% overall amino acid sequence homology to p190, with which it shared similar domain architecture. Just as with p190, the N-terminus included motifs associated with GTPases and the C-terminus included a GAP domain shown to have *in vitro* specificity for RhoA, Rac1 and Cdc42, in this hierarchical order. Results further showed that it localized diffusely in the cytoplasm of a variety of cells. Moreover, it colocalized in fibrillar patterns with α5β3 integrin, a receptor for fibronectin, and Rho in response to fibronectin treatment, providing a transmembrane link between integrins and Rho.

Although the nomenclature used to designate these two proteins could lead one to believe that these homologues are splice variants of one gene, p190 and p190B are encoded by separate genes located on completely different chromosomes. The p190 gene is found at chromosome location 19q13.3, which is reportedly deleted in some gliomas and other carcinomas (Tikoo et al., 2000). The p190B gene is located instead on chromosome 14q (Burbelo et al., 1998). Although encoded by different genes, the redundancy of these two molecules at the nucleotide and protein sequence levels is a good indication of their critical role and particular relevance on vital biological processes, to be discussed in following sections.

Structure and domains

The sequence of the p190 protein has been divided into three major regions or domains: an N-terminal GTP-binding domain (GBD), a Middle Domain (MD), and a C-terminal GTPase activating protein domain (GAP), as shown in Figure 3. There is a high degree of homology between human and rat p190 sequences throughout all domains. A comparison between p190 and p190B homologues yields significant divergence in their sequences, particularly in the Middle Domain region (Figure 4). P190 and p190B share approximately 70% homology in the N-terminus sequences of the protein, including the GTP binding domain (Figure 4).

N-terminal GTP Binding Domain

The N-terminus of p190 contains several motifs associated with GTPases including phosphate-, magnesium^{2+}-, and guanine-binding motifs (Settleman et al., 1992c). Together, they mediate direct binding of p190 to GTP and GDP in a saturatable manner that is time- and dose-dependent (Foster et al., 1994;Roof et al., 2000). Purified p190 protein was reported to be unable to hydrolyze GTP to GDP, making the biological significance of p190-GTP binding unclear. Interestingly, when the guanine binding motif present in p190 is mutated, precluding GTP binding, its ability to interact with p120RasGAP or function as a GAP remains unaffected, decoupling the functions of the two domains. A later study where GTP binding is reduced by mutation of a different residue, Ser 36 in the phosphate binding motif, reported that *in vivo* GAP activity toward Rho is decreased when GTP binding is disrupted, opposing previous findings (Tatsis et

al., 1998). Our studies, some reported here and others previously published by Su and colleagues (Su et al., 2003), support the initial claim that GAP activity is independent of GTP binding as several p190 deletion mutants missing portions or the complete GBD continue to negatively affect RhoGTP levels as evidenced by an increase in cytokinesis failure and multinucleation, a phenotype mediated through inactivation of downstream Rho signaling. The GBD can be phosphorylated at several sites by Src kinase, and this modification results in loss of GTP binding activity (Roof et al., 2000). Finally, Su and colleagues (2003) reported that the GBD, together with a portion of the middle domain, regulates p190 degradation during cytokinesis. Work described in chapter 3 of this dissertation suggests that four individual lysines at the N-terminus regulate mitotic p190 degradation.

Middle Domain

The Middle Domain (MD) is the largest of the three p190 domains and has been arbitrarily divided into sections 1, 2, and 3 for ease of study. The middle domain contains several protein-protein interaction motifs as well as numerous putative and confirmed tyrosine and serine/threonine phosphorylation sites that regulate p190 activity and interactions. The MD contains four FF domains, which consist of 50 amino acids flanked by two conserved phenylalanine (F) residues (Bedford and Leder, 1999). FF domains are present in proteins that bind to or interact with RNA-binding proteins, and the presence of four FF domains in both p190 and p190B suggest that they may have nuclear functions by interacting with RNA binding, processing, and transport proteins.

Recent reports have elucidated a transcriptional function for these p190 FF domains. Researchers reported an interaction, via FF domains, between p190 and the transcriptional regulator TFII-I wherein p190 sequesters TFII-I in the cytosol, preventing its translocation into the nucleus and the resulting transcription of serum inducible genes including c-fos (Jiang et al., 2005). PDGF-mediated phosphorylation of p190 FF domains results in TFII-I release and its transport into the nucleus, revealing a pathway by which mitogens may promote transcriptional responses. Furthermore, through FF domain-mediated interactions with TFII-I and its antagonistic transcription factor GATA2, p190 is involved in the regulation of vascular endothelial growth factor (VEGF) receptor 2 (VGFR2) gene expression, taking active part in mechanisms that control angiogenesis (Mammoto et al., 2009) by responding to mechanical as well as chemical cues. In addition to FF domains, several putative SH3 binding motifs, with the sequence PXXP, are found distributed throughout the middle domain. SH3 domains are found in proteins that regulate the actin cytoskeleton, as well as in Src kinase. Although interactions with numerous proteins are possible through this domain, to date, no reports of PXXP-mediated p190 interactions have been published.

Several confirmed and putative phosphorylation sites can be found throughout the MD. The most important phosphorylation event on p190 occurs at tyrosine 1105 (Y1105) in Section 3 of the MD. Phosphorylation of Y1105 is mediated by c-Src and several other Src family kinases (SFKs) (Roof et al., 1998;Liang et al., 2004;Shen et al., 2008;Wolf et al., 2001;Holinstat et al., 2006). Phosphorylation at Y1105 activates the GAP domain of p190 and allows its association with p120RasGAP (Roof et al., 1998).

Numerous serine and threonine (ser/thr) phosphorylation sites are also present in the MD of p190. It has been reported that during mitosis, tyrosine phosphorylation decreases while ser/thr phosphorylation increases, with an overall GAP inactivation effect (Maddox and Burridge, 2003). Numerous kinases are able to modify ser/thr residues in the MD of p190 and they are discussed in more detail in an upcoming section. As shown in Figure 4, p190 and p190B share only 45% sequence homology throughout the MD (Tikoo et al., 2000). Given that regulation of p190 activity is exercised through the MD, the decreased homology between p190 and p190B allows for different regulatory mechanisms to be in place for each of these proteins, promoting their participation in non-overlapping cellular responses.

GAP Domain

Rho family GTPases are active when bound to GTP and become inactive when the bound nucleotide is hydrolyzed and converted into GDP. Rho family GTPases are intrinsically able to hydrolyze bound GTP but do so with especially slow kinetics. A GAP protein, such as p190, contains a GTPase activating protein domain which increases the hydrolysis rate of Rho-bound GTP. The GAP domain of p190 is found at the C-terminus of the protein and it shares significant homology with other GAP domains found in proteins such as bcr (breakpoint cluster region) and n-chimerin (Settleman et al., 1992c), both of which have GAP activity toward Rho family GTPases. Shortly after its cloning, the GAP activity of p190 was confirmed to be RhoGTPase-specific, with greatest affinity toward Rho. Researchers tested the GAP activity of purified p190

against a panel of small GTPases from the Rho, Ras and Rab families (Settleman et al., 1992a). In their study, *in vitro* GAP assays revealed that the activity of p190 was specifically directed toward RhoA, Rac1 and Cdc42 and thus could be classified as a Rho family-specific GAP. Anne Ridley and colleagues further showed that when microinjected into Swiss 3T3 cells, the GAP domain of p190 had specific activity for Rho, as cells became more rounded and lost actin stress fiber structures. What is more, LPA-induced stress fiber formation was abolished in cells microinjected with the GAP domain p190 whereas PDGF treatment continued to induce Rac1-dependent membrane ruffles. These data establish the *in vivo* RhoA-specificity of p190.

The structural requirements of GAP-mediated GTP hydrolysis in RhoGTPases were described over ten years ago by Li and Zheng using RhoA-p190 interactions as a model for GAP function (Li et al., 1997). Using protein chimeras and point mutations, p190 residues important for RhoA interaction were exposed. Two residues located in the putative G-protein binding helix pocket of the GAP domain of p190, Arginine 1283 and Lysine 1321, were found to be crucial for the RhoA-p190 interaction. Mutation of Lysine 1321 resulted in loss of GAP activity and disruption of the p190-RhoA interaction. On the other hand, mutation of Arginine 1283 preserved p190-RhoA binding but ablated the GAP activity of p190. The latter construct is used in the research herein presented as a dominant negative form of p190. The authors conclude that the role of the GAP domain of p190 in the hydrolysis of Rho-bound GTP "is in part to supply active site residue Arginine 1283 for efficient catalysis". Similar to p190, the GAP activity of p190B exhibits *in vitro* specificity for Rho, Rac and Cdc42 (Burbelo et al., 1995) and *in*

vivo, has only been reported to act on RhoA. However, through interactions mediated by its MD, p190B is also able to interact with Rac1 (Bustos et al., 2008). P190 and p190B share around 70% sequence homology throughout the GAP domain (Tikoo et al., 2000).

Regulation of Activity

Through its ability to modulate RhoGTP levels, p190RhoGAP is a central player in the regulatory signaling pathways directing cytoskeletal dynamics and organization. Because of this vital cellular role, several regulatory mechanisms are in place to ensure proper and timely p190 activation and inactivation. Such mechanisms include phosphorylation, dephosphorylation, protein-protein interactions and ubiquitin-mediated degradation. Initial reports described p190 as a tyrosine phosphorylated protein that associated with p120RasGAP upon transformation by Src or treatment with EGF (Ellis et al., 1990;Bouton et al., 1991a). Because levels of phosphorylated tyrosine in p190 varied with the level of Src protein and not with EGF treatment, it was determined that p190 is a preferred substrate of Src (Chang et al., 1993;Chang et al., 1995;Roof et al., 1998;Brouns et al., 2001). Although tyrosine phosphorylation is observed throughout the molecule, the main phosphorylation site is at Y1105 (Roof et al., 1998). Y1105 plays a central role in the regulation of p190 activity, as studies have shown that its phosphorylation by Src results in an increase in binding to p120RasGAP and the activation of the GAP domain of p190 (Haskell et al., 2001). Several other kinases, including Src Family Kinases (SFKs), have also been reported to phosphorylate p190 at Y1105 and other residues, resulting in the activation of its GAP domain and implicating p190 in a number of important cellular

events. For example, Fyn-mediated p190 phosphorylation is involved in oligondendrocyte differentiation (Wolf et al., 2001), and Brk (Breast tumor kinase)-mediated p190 phosphorylation results in increased proliferation and migration of breast cancer cells (Shen et al., 2008). Other kinases, such a focal adhesion kinase (FAK), fgr, v-abl, and TEL-ARG are able to activate the RhoGAP activity of p190 by phosphorylation and have thus revealed the involvement of p190 in cell migration, adhesion pathways, vascular permeability and semaphorin signaling (Continolo et al., 2005;Holinstat et al., 2006;Hernandez et al., 2004;Shimizu et al., 2008;Palmi et al., 2006).

The relevance of tyrosine phosphorylation as a means of activating p190 has been cemented by additional reports where a number of signaling molecules, none of them tyrosine kinases, indirectly regulate the GAP activity of p190 by transiently increasing its overall tyrosine phosphorylation status. Some of the molecules that indirectly increase the tyrosine phosphorylation and activation of p190 include v-FMS (Trouliaris et al., 1995), NADPH oxidase (Nox-1) (Shinohara et al., 2007), angiotensin II (Rattan et al., 2003), $G\alpha_{13}$ (Bartolome et al., 2008), protein kinase C (PKC) (Brandt et al., 2002), and the p110Δ isoform of phosphatidylinositol 3-kinase (PI3K) (Papakonstanti et al., 2007). Tyrosine phosphorylation resulting from signaling initiated through these molecules results in modulation of cellular pathways that direct contractility, cell morphology, invasion, chemotaxis and proliferation. The detailed mechanisms by which these proteins activate p190 are still to be clarified.

How does phosphorylation of Y1105 result in an increase in the GAP activity of

p190? p190 was originally described as a tyrosine phosphorylated protein associated with p120RasGAP. This interaction has been further dissected and shown to result in increased p190RhoGAP activity (Chang et al., 1995;Haskell et al., 2001;Bradley et al., 2006;Shen et al., 2008). Two tyrosine residues in p190, Y1087 and Y1105, were originally reported as being required for p120 binding. It was proposed that the two tandem phosphorylated tyrosines served as binding sites for the two SH2 domains present in p120RasGAP (Bryant et al., 1995;Hu and Settleman, 1997). It was later reported that, in fact, a single tyrosine, Y1105, was the sole mediator of p190-p120 interaction (Roof et al., 1998). Point mutations of this residue result in inhibition of the interaction between p190 and p120RasGAP and ablation of its GAP activity, suggesting that binding of p120 is required for p190 activation. Other reports indicated that the association between these proteins allows the recruitment and localization of p190 to a detergent insoluble cytoskeletal fraction, where it is able to exert its inhibitory function on Rho (Chen et al., 2003;Shen et al., 2008). However, in some studies, p190 has been observed to remain active even when it is precluded from p120RasGAP association (Tsubouchi et al., 2002). Tsubouchi and colleagues report that paxillin, when phosphorylated at two critical residues, competes with p190 for binding to the SH2 domains of RasGAP. Interestingly, free p190 continued to perform its Rho inhibitory functions effectively. Moreover, in p120RasGAP-null fibroblasts, p190 is still tyrosine phosphorylated but at a decreased level, suggesting that p190 tyrosine phosphorylated and activated in the absence of p120RasGAP, possibly through the mitogen-activated protein kinase (MAPK) pathway (van der et al., 1997), illustrating that p190 may become and remain active after tyrosine

phosphorylation regardless of its association with RasGAP.

The interplay between p190 and p120 carries out further, as research has established that activation of one of these GAPs, by phosphorylation, results in the inactivation of the other (Moran et al., 1991;Settleman et al., 1992a;Haskell et al., 2001;Koehler and Moran, 2001;Sastry et al., 2006;Shen et al., 2008) establishing a mechanism by which Rho and Ras activation can be coupled and result in coordinated signaling pathways and cellular outcomes. Further details regarding the mechanism by which Ras regulates Rho activation have been clarified. Researchers have reported that p190 activation by phosphorylation is sensitive to the redox state of a cell, regulated by increased Ras activity (Shinohara et al., 2007). For example, in K-Ras transformed rat kidney cells, reactive oxygen species (ROS) generated by Nox-1 result in RhoA inactivation. This effect is mediated through oxidative inactivation of the low molecular weight-protein tyrosine phosphatase (LMW-PTP) and a concurrent increase in tyrosine phosphorylation of p190, the direct target of the phosphatase. Similarly, Nimnual and colleagues (2003) demonstrated that Rac activation also results in the production of reactive oxygen species (ROS) which, in turn, inactivate LMW-PTP, resulting in an increase in p190RhoGAP activity by preventing its dephosphorylation. Together, these studies propose an elegant mechanism by which one GTPase can negatively affect the function of another, thus coupling and coordinating signaling pathways and actin cytoskeleton-dependent cellular processes.

Given that tyrosine phosphorylation increases the GAP activity of p190, it is to be expected that tyrosine phosphatases have the opposite effect, as exemplified above by

LMW-PTP. A number of phosphatases with activity toward p190 have been identified. The tyrosine phosphatases PTP20 and Ptprz have been shown to mediate the dephosphorylation of Y1105, specifically. Overexpression of protein tyrosine phosphatase 20 (PTP20) induces retraction of the cell body with the extension of long, dendritic-like processes and decreased cell adhesion to the substratum which correlate with a reduction in the phosphotyrosine levels of p190 RhoGAP. Furthermore, overexpression of PTP20 reduced the formation of p190-p120 complexes (Shiota et al., 2003). Ptprz, predominantly expressed in the brain, has been implicated in memory formation through a ROCK-dependent pathway. A Ptprz-deficient mouse model demonstrated that Y1105 phosphorylation levels remained elevated when compared with wild-type mice. *In vitro* assays further confirmed the ability of Ptprz to directly decrease Y1105 phosphorylation (Tamura et al., 2006). Several other phosphatases have also been reported to decrease p190 tyrosine phosphorylation levels, although it is unclear whether they have specificity for any particular residue. As previously described, LMW-PTP decreases the tyrosine phosphorylation and activity of p190 in response to changes in the cell's redox state. The phosphatase PTP-PEST also acts upon p190 as a means of directing cell protrusion and retraction in migrating cells (Sastry et al., 2006). A decrease in the tyrosine phosphorylationof p190, however, is not solely mediated by phosphatases. Several other proteins have been shown to indirectly decrease p190 phosphorylation levels, by interfering with Src activation. Some of these proteins include Thy-1 (Barker et al., 2004), Src-associated in mitosis 68 (Sam68) (Huot et al., 2009), caveolin-1 (Grande-Garcia et al., 2007), and the receptor for activated protein kinase C (RACK1)

(Miller et al., 2004). Moreover, it has been shown that latent transforming growth factor-β (TGF-β) (Kim *et al*. 2006; Park *et al*. 2006) and tissue transflutaminase (tTG) (Janiak *et al*. 2006) can also lead to reduced p190 tyrosine phosphorylation levels.

In opposition to the activating effect of tyrosine phosphorylation on p190, ser/thr phosphorylation results in decreased RhoGAP activity. The negative effect of ser/thr phosphorylation was first described by Maddox and colleagues as a part of a study to determine the role of cortical retraction and rigidity during mitotic cell rounding (Maddox and Burridge, 2003). They found that increased RhoGTP levels are required for both cortical retraction and rigidity and looked further into the mechanism by which this increase is mediated. Their report revealed that tyrosine phosphorylation of p190 and its interaction with p120, hallmarks of p190 activation, are decreased during mitosis whereas, ser/thr phosphorylation of p190 is increased. Characterization of ser/thr phosphorylated p190 isolated from mitotic cell samples revealed that it had decreased GAP activity toward RhoA, as measured by *in vitro* GAP assays. Two ser/thr kinases, glycogen synthase kinase-3β (GSK-3β) and ROCK, have been shown to target and inactivate the GAP activity of p190. Researchers reported that p190 is required for polarized cell migration as a phosphoprylation substrate of GSK-3β, a critical regulator of cell polarity. This phosphorylation results in an *in vivo* decrease in p190 GAP activity (Jiang et al., 2008). Similarly, the RhoA downstream effector ROCK phosphorylates Ser 1150, near the GAP domain, rendering this domain inactive and creating a positive feedback loop that allows for prolonged RhoA activation, as observed in vasospasms (Mori et al., 2009).

Finally, degradation of nearly 70% of the cellular p190 pool during cytokinesis serves as an additional negative regulatory mechanism for p190 GAP activity during a clearly defined time period in the cell cycle. Su and others (2003) in our laboratory were first to report severely decreased p190 protein levels in late mitosis, and their work demonstrated that this decrease is mediated in a ubiquitin-proteasome pathway-dependent manner. They also showed that upon re-entry into the G1 phase of the cell cycle, the amount of p190 protein detected returned to normal levels. Overexpression of p190 resulted in a multinucleation phenotype, purportedly because p190 levels remained high during cytokinesis, preventing sufficient RhoA activation. In a later study, our laboratory showed that overexpression of p190 result in decreased RhoGTP levels at the cleavage furrow, as determined using a RhoGTP biosensor (Su et al., 2009). These series of results led us to hypothesize that a decrease in p190 levels was important and necessary in mediating the increase in RhoGTP levels necessary for cell division. Work described in Chapter 3 of this dissertation confirmed that p190 degradation is required for successful cytokinesis completion and identified four lysines necessary for its mitotic degradation.

Biological Function

By virtue of its ability to regulate RhoGTP levels and downstream actin cytoskeleton dynamics, p190 is involved in numerous critical cellular events. In this section, I will give a brief overview of the varied functions attributed to p190 and the data supporting its participation.

Adhesion, Migration and Invasion

Integrins are transmembrane receptors that mediate and regulate adhesion between a cell and the extracellular matrix (ECM). The first link between the p190RhoGAP family of proteins and adhesion became evident during the characterization of p190B, as it was reported to colocalize with α5 integrin subunits and Rho after cells were stimulated with fibronectin-coated beads (an integrin activator) (Burbelo et al., 1995). Further research described a direct link between integrins and p190 through experiments showing engagement of the β1 integrin subunit as a means to stimulate tyrosine phosphorylation of p190 (Nakahara et al., 1998). The increased phosphorylation of p190 correlated well with increased invasion through ECM and with p190 localization to membrane protrusions, where it colocalized with actin. Microinjection of p190 blocking antibodies resulted in decreased ECM degradation and decreased invasive potential. A decade later, Bass and colleagues (2008) brought together the findings of Burbelo and Nakahara into a mechanistic model as they reported that p190 is the convergence point of adhesion signals from the integrin heterodimer receptor α5β1 and syndecan-4, a single transmembrane domain protein that acts as a co-receptor for G protein-coupled receptors (GPCRs). Their results confirmed that matrix-induced tyrosine phosphorylation of p190 is stimulated solely by engagement of α5β1 integrin. Parallel engagement of syndecan-4 causes redistribution of the tyrosine-phosphorylated (active) pool of p190 to the membrane, where it affects RhoA activity, through direct activation of protein kinase C alpha by syndecan-4 (Bass et al., 2008). Activation of both pathways is necessary for the efficient regulation of RhoA and focal

adhesion formation. Arthur and colleagues (2000) provided further mechanistic insights regarding p190 phosphorylation resulting from integrin engagement and corroborated the important role of p190 GAP activity in lowering RhoA activity levels. Their study showed that integrin engagement results in c-Src-mediated tyrosine phosphorylation of p190 and a direct decrease in RhoGTP levels, which facilitates cell spreading and migration by altering stress fiber dynamics. Keeping with previously established descriptions of p190 action, only Rho was affected and not Rac1 or Cdc42. Furthermore, a p190 dominant-negative GAP mutant (R1283A) inhibited migration, suggesting that p190-mediated Rho inactivation is necessary for migration (Arthur et al., 2000). Similar observations were made in rat ovarian granulosa cells which, when treated with follicle-stimulating hormone, presented with increased p190 tyrosine phosphorylation and decreased RhoA activation, resulting in decreased cell substratum adhesion and increased migratory potential (Shiota et al., 2003).

Cadherins are an additional family of transmembrane receptors that mediate cell adhesion and migration and do so by affecting p190 and Rho activity levels. Cadherin engagement induces Src-dependent tyrosine phosphorylation of p190, increases its ability to interact with p120RasGAP and results in decreased RhoGTP levels (Noren et al., 2003). Cadherin signaling has also been shown to suppress RhoA activity while Rac1 is activated. A recent report described roles for p120-catenin and p190RhoGAP that coordinate crosstalk between these systems and regulate cadherin function (Wildenberg et al., 2006). This report suggests that receptor-induced Rac activity causes translocation of p190 to adherens junctions (AJs), where it couples to the cadherin complex via

interaction with p120-catenin. AJ formation is dependent on this p120catenin-p190RhoGAP interaction and fails altogether if either of these proteins is compromised. The proposed mechanism couples Rac activation to localized inhibition of Rho, essential for AJ formation. Rac is activated by receptor tyrosine kinases (RTKs), integrins, and cadherins, receptors that can also activate p190. p120-catenin and p190RhoGAP use RhoGTPases to mediate crosstalk between a wide variety of receptors in order to coordinate cadherin function with other activities that direct cell-substrate adhesion, cell motility, and proliferation.

Regulation of cell-cell and cell-substratum adhesion is not limited to transmembrane receptors and their ability to directly affect p190RhoGAP activity and RhoGTP levels, as there is an ever increasing list of cytoskeletal-related proteins reported to participate in focal adhesion and stress fiber regulation through modulation of p190 phosphotyrosine levels. For example, focal adhesion kinase (FAK) is a well established regulator of cell adhesion and has been shown to exert direct influence on the p190-RhoA pathway. Inhibition of FAK decreases tyrosine phosphorylation of p190RhoGAP and elevates the level of GTP-bound Rho (Playford et al., 2008). Similarly, Thy-1, a GPI-anchored glycoprotein, regulates migratory and adhesive events such as focal adhesion formation and stress fiber disassembly in a Src-dependent manner through phosphorylation of p190 and the ensuing inhibition of Rho activity (Barker et al., 2004).

Neuronal Differentiation/Development

p190A is ubiquitously expressed in all fetal and adult tissues (Settleman et al.,

1992c;Tikoo et al., 2000). Additional studies demonstrated that p190A was highly expressed in brain, spinal cord and eyes of developing mouse embryos, a finding that was the first to bring attention to the involvement of p190 in neuronal development (Brouns et al., 2000). As a part of this study, Brouns and colleagues created transgenic mice with a targeted disruption of p190 in order to study the effect of the partial deletion of p190. It is important to note that the transgenic mice produced an enzymatically active form of p190 since the resulting protein product, an N-terminal deletion, still contained intact Middle and GAP domains. Transgenic mice with disrupted p190 were defective in neural development, displayed aberrant tissue morphogenesis and abnormalities in forebrain hemisphere fusion, ventricle shape, optic cup formation, neural tube closure, and layering of cerebral cortex cells through excessive actin polymerization in cells of the neural tube floor plate. Ninety-five percent of mice died within the first two days after birth and the rest within the first three weeks (Brouns et al., 2001). Mice also exhibited defects in axon guidance and fasciculation. Improper axon guidance may result in inappropriate sensing of chemoattractant gradients and axons "wandering" into the wrong compartment of the brain. Fasciculation defects prevent proper bundling or grouping of myelinated or unmyelinated axons.

It is unclear why the authors of these studies decided to create transgenic mice with gene disruptions that would yield N-terminal disrupted protein products while leaving the enzymatically active GAP domain undisturbed. As discussed earlier in the introduction, the GAP domain and its activity are the means by which p190 mediates its effects on Rho and the actin cytoskeleton, with little known functionality being attributed

to the N-terminal GTP binding domain. It was therefore surprising and puzzling to see such marked phenotypes in these transgenic mice, unless the N-terminus is able to regulate GAP domain function. Previously published results from work in our laboratory (Su et al., 2003;Mikawa et al., 2008;Su et al., 2009) and results included in this dissertation may provide an explanation for such striking phenotypes. Our results show that the N-terminus of p190 is important in mediating its ubiquitination and degradation during cytokinesis and that failure to decrease p190 levels during cytokinesis results in multinucleation and apoptosis (K. Ludwig, personal communications). Furthermore, it is possible that accumulation of p190 in cells that escape either phenotype results in decreased RhoGTP levels and aberrant migration patterns leading to excessive actin polymerization as well as incomplete closure of the optic fissure and neural tube, reported by Brouns and colleagues. Nevertheless, the drastic phenotypes observed point to the importance of appropriate p190 regulation and activity for proper neuronal development.

Vascular Permeability and Angiogenesis

The thin layer of cells that line the interior surface of blood vessels is known as the vascular endothelium. It forms the interface between circulating blood in the lumen and the rest of the vessel wall. Endothelial cells interact with ECM proteins at focal adhesions, contributing to the integrity of the vascular endothelial barrier through regulation of cell junctions. Angiogenesis requires the modification of these junctions to allow new vessel growth. In lung injury patients, increased endothelial permeability gives rise to serious conditions including alveolar flooding, hypoxemia, and tissue

leukocyte infiltration, all of which trigger inflammatory cytokine production leading to increased morbidity and mortality. FAK activation is required for barrier function recovery after thrombin-mediated increases in endothelial junction permeability and does so through a mechanism of action that requires p190. *In vitro* studies showed that thrombin causes a transient activation of RhoA followed by prolonged activation of FAK and recovery of barrier function. Moreover, thrombin induced increased tyrosine phosphorylation of p190, along with a decrease in RhoA activity. Under thrombin-treatment conditions, FAK was found associated with p190, and recombinant FAK phosphorylated p190RhoGAP *in vitro* (Holinstat et al., 2006). Holinstat and colleagues then took their findings into the lung microcirculation where they recapitulated their *in vitro* findings in an *in vivo* system. Thrombin-induced permeability is also attenuated through a separate, yet similar, pathway requiring Hepatocyte Growth Factor (HGF) activation of the GEF Tiam1 and of Rac1. As previously described in this introduction, activation of Rac1 results in the inactivation of Rho through an increase in p190 tyrosine phosphorylation, leading to greater GAP activity toward RhoA, in a Tiam1-Rac1 dependent fashion (Birukova et al., 2007;Herbrand and Ahmadian, 2006). In conclusion, these studies show that FAK- and Tiam1-induced down-modulation of RhoA activity, via p190RhoGAP, is a crucial step in signaling endothelial barrier restoration after increases in permeability.

Recently, a study reported finding a role for p190 in angiogenesis. Quite interestingly, p190 regulates angiogenesis at the transcriptional level, rather than through a mechanism requiring its GAP activity toward RhoA. Mammoto and colleagues

cleverly discovered that p190 controls angiogenesis through its FF domains, which are spread throughout the MD and mediate association with RNA-binding proteins and transcription factors such as TFII-I and GATA2 (Mammoto et al., 2009). These two transcription factors have antagonistic regulatory functions on Vascular Endothelial Growth Factor receptor (VEGFR) expression, crucial in the initiation of the angiogenic signaling cascade. TFII-I decreases VEGFR transcription, whereas GATA2 increases it, and p190 was found to bind both transcription factors and sequester them in the cytosol, preventing them from entering the nucleus. Through a series of elegant experiments, Mammoto and colleagues demonstrated that p190 acted as a mechanosensing transcriptional switch, given that variations in ECM elasticity directed p190 binding to either TFII-I or GATA2 and thus either inhibited or stimulated VEGFR expression and directed capillary network formation in human microvascular endothelial cells *in vitro* and during retinal angiogenesis *in vivo*. The authors propose that "this is the first known functional cross-antagonism between transcription factors that controls tissue morphogenesis, and that responds to both mechanical and chemical cues."

Cell Death

Study of p190-related cell death began in our laboratory after researchers were unsuccessful in their attempts to create stable cell lines overexpressing p190 (L. Su, personal communication, B. Dukes thesis). Overexpression of p190 in both epithelial and fibroblast cells resulted in shrunken cells and chromatin condensation, originally determined by DAPI staining. More recent work by Kirsten Ludwig, also in our

laboratory, has shown that p190 overexpression in both epithelial cells and fibroblasts induces three different phenotypes: multinucleation, dendrite-like morphology and apoptosis. Her work suggests that multinucleation and acquisition of dendrite-like morphology represent intermediate steps in a pathway to apoptotic cell death. Expression of dominant-negative p190 (R1283A) and constitutively active Rho prevent cells from becoming apoptotic. Furthermore, her work has also revealed that p190-induced apoptosis is a caspase-dependent event, given that the pan-caspase inhibitor Z-VAD drastically decreases the amount of apoptosis observed. Finally, her observations revealed that greater endogenous p190 expression levels in breast cancer cell lines correlate with increased sensitivity to docetaxel-induced apoptosis. Conversely, cell lines producing lower amounts of endogenous p190 prove to be less sensitive to the drug. Together, these results propose an important role for p190 in Rho-dependent mechanisms regulating cell death. The implication that increased p190 levels enhance the effect of taxanes as chemotherapeutic agents is of particular relevance since docetaxel is commonly used in the clinic to treat breast, ovarian and non-small cell lung cancers. One could envision that, as part of personalized treatment regimens, identification of patients with tumors expressing increased p190 levels would classify them as prime candidates for docetaxel treatment given their potential increased sensitivity to the drug.

Cell Cycle Progression

Work in our laboratory has defined a role for p190 in cell cycle progression, as it regulates the completion of cytokinesis, the last stage in mitosis (a more detailed

description of cell cycle stages is given in the following section). In an attempt to characterize the role that p190 plays in regulating the actin cytoskeleton and determine the reason behind their inability to prepare stable cell lines overexpressing p190, Su and colleagues (Su et al., 2003) discovered that p190 protein levels remained constant throughout the cell cycle yet decreased drastically during the later stages of mitosis. The decrease in protein levels observed through Western blot was nearly 70% of interphase levels. Furthermore, the decrease in p190 levels was shown to be mediated through the ubiquitin-proteasome pathway, in a similar fashion as other important cell cycle regulators driving mitotic progression, such as cyclinB, securin, and polo-like kinase. This was discovered through the use of the proteasome inhibitor MG132 as well as through direct *in vitro* ubiquitination of p190 in mitotic cell lysates. To understand the relevance of such a marked decrease in p190 levels, Su and colleagues overexpressed p190 in MDA-MB-468 cells (468) and assessed its effects by imunofluorescence microscopy. To their surprise, they noticed a striking increase in multinucleated cells, a phenotype indicative of cytokinesis failure. Structure-function analysis of p190 revealed that the C-terminus of the protein, containing the GAP domain, several phosphorylation sites and a poly-proline domain, was required for the observed multinucleation phenotype. Moreover, immunofluorescence microscopy also revealed that p190 colocalized with actin at the cleavage furrow, the site at the midzone of a dividing cell where furrowing occurs, placing p190 at the site where the most dramatic and important actin reorganization events of mitosis occur. Further studies in our laboratory have shown that overexpression of p190 results in a decrease in RhoGTP levels at the cleavage

furrow of dividing cells, as measured by a Fluorescence Resonance Energy Transfer (FRET)-based biosensor (Su et al., 2009), and that the role of p190 during cytokinesis is to function in direct opposition to the RhoGEF Ect2, a well characterized mitotic activator of RhoA signaling (Mikawa et al., 2008). Mikawa and colleagues also reported that p190 was found in a mitotic complex with Ect2, further implicating p190 as a critical participant in the important processes necessary for progression through mitosis and into G1.

Results reported in this dissertation are a continuation of the aforementioned studies as they provide further details regarding the role of p190 in mitosis. Briefly, results show that mitotic p190 degradation is required for cytokinesis completion and avoidance of the multinucleation phenotype observed in p190 overexpressing cells. Additionally, four lysines are identified as critical residues directing the mitotic degradation of p190. Results also indicate that p190 overexpression disturbs cytokinesis by decreasing ROCK-dependent phosphorylation of the regulatory light chain of myosin II, inhibiting contractility of the cleavage furrow necessary for cell abscission.

MITOSIS AND CYTOKINESIS

For ease of scientific study, the cellular life cycle has been divided into four periods: G1, S, G2, and M phases. The following section is a brief overview of the cell cycle, with particular emphasis placed on mitosis, as the studies herein contain attempts to explain p190 regulation and function during this particular phase. During G1 and G2, the gap phases, cell growth and metabolite accumulation occurs in preparation for the

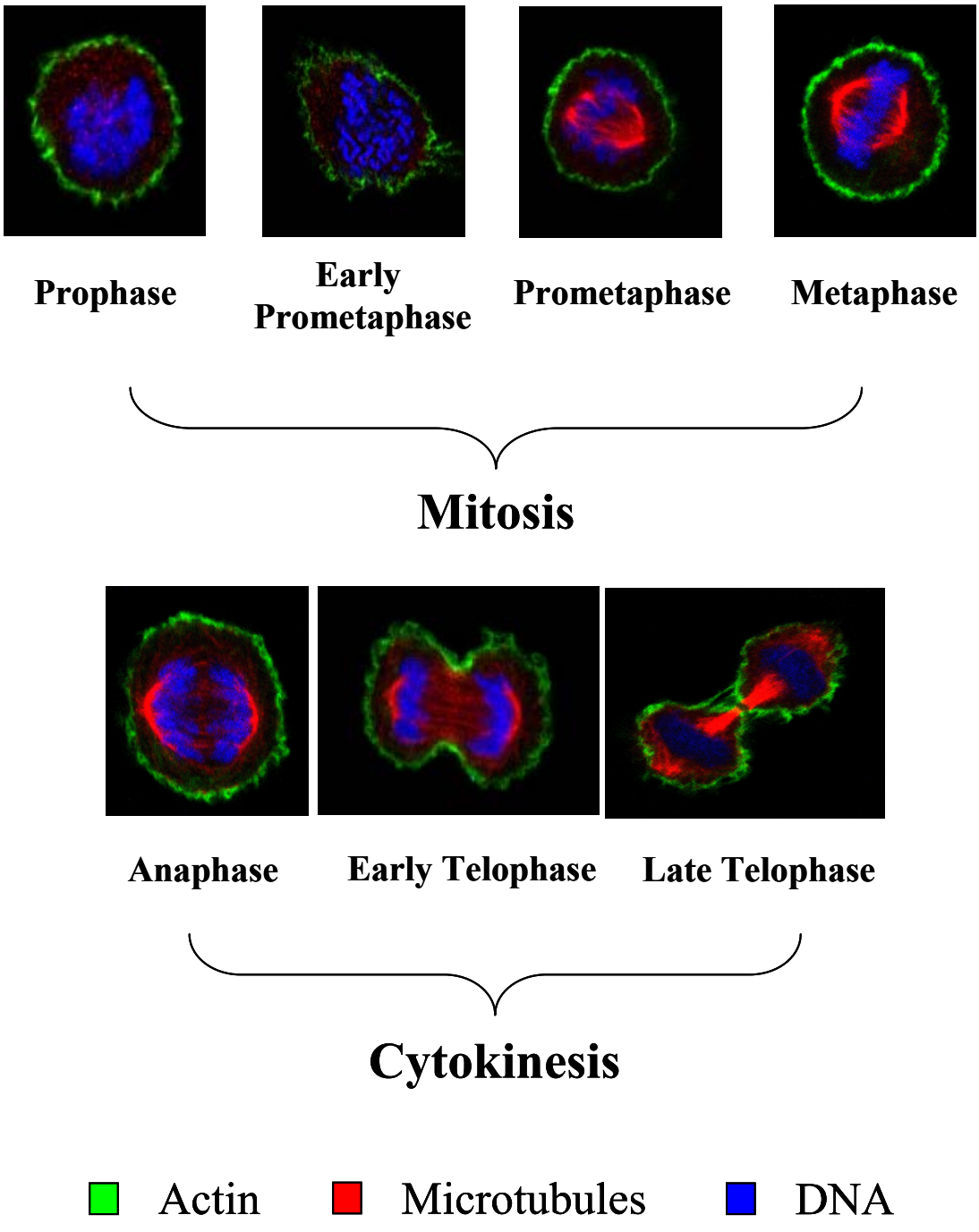

Prophase
Early Prometaphase
Prometaphase
Metaphase
Mitosis
Anaphase
Early Telophase
Late Telophase
Cytokinesis
Actin
Microtubules
DNA

Figure 5. The different stages of mitosis and cytokinesis

Mitosis and cytokinesis are divided into several stages. The first stage of mitosis is prophase when chromosome condensation and nuclear envelope breakdown begin. Duplicated centrosomes begin to migrate to different poles of the cell. During prometaphase, chromosome condensation is completed and the nuclear envelope disappears, releasing chromosomes into the cytosol where they are captured by microtubules emanating from centrosomes at each pole of the cell. In metaphase, a fully-functional microtubule spindle has captured all chromosomes and aligned them at the metaphase plate, in preparation for sister chromatid separation, which occurs during anaphase. An actomyosin contractile ring forms at the cleavage furrow at this stage and begins to contract. During the final stage, telophase, the nuclear envelopes have reformed around the separated nuclei and a midbody is formed by the contracting cleavage furrow. Ultimately, cells pinch apart at the midbody and separate into two daughter cells, in a process called abscission. The images here presented obtained using HeLa cells.

more active S and M phases. During S phase, the synthesis phase, nuclear DNA is replicated. During M phase, mitosis, the cell divides into two daughter cells with each receiving equal amounts of the replicated DNA, cellular organelles and cytosolic contents. Mitosis is divided into several discrete phases which include prophase, prometaphase, metaphase, anaphase and telophase, as illustrated in Figure 5. During the first stage, prophase, nuclear DNA condenses and organizes into chromosomes. Centrosomes, duplicated in S phase, separate to opposite sides of the nucleus and the nuclear envelope begins to break down. In prometaphase, nuclear envelope breakdown is completed, releasing chromosomes into the cytosol. Microtubules begin to emanate from centrosomes, and a mitotic microtubule spindle begins to form, capturing chromosomes at structures called kinetochores. Interactions with microtubules cause initial movement of chromosomes toward the center of the cell. During metaphase, chromosomes captured by the mitotic spindle are aligned at the center of the cell, forming the metaphase plate. As cells transition from metaphase into the next stage, anaphase, chromosomes separate into the individual sister chromatids which are segregated to opposite poles. Chromosome alignment and segregation are absolutely dependent on the microtubule mitotic spindle. Also during anaphase, a cleavage furrow formation site is defined, in alignment with the previous location of the metaphase plate and equidistant from the poles. It is at the cleavage furrow formation site that an actomyosin ring is formed, its constriction providing the mechanical forces required for membrane invagination and later on, furrowing. As the cleavage furrow deepens, the cell enters the final mitotic stage, telophase. During telophase the cleavage furrow proceeds until it effectively

separates daughter cells, creating a microtubule-rich and highly dense structure called the midbody. To complete cell separation, the midbody needs to be resolved and cells "pinch off" in a process called abscission (Glotzer, 2001). Many in the field view the first three stages of mitosis as preparatory whereas the last two effectively execute cell division. Because of these clear functional differences, the first three stages are referred to, collectively, as mitosis and the last two stages as cytokinesis. Because it is particularly germane to this dissertation, a more detailed review of the RhoA-dependent mechanisms regulating cytokinesis is included below.

A critical regulator and driver of cytokinesis is the small GTPase RhoA. Studies done during the 1990's using RhoA inhibitors clearly showed the need for activated Rho during cytokinesis. Early reports used several means to inactivate RhoA, including RhoGDI and C3 toxin, during Xenopus cell division (Kishi et al., 1993). Under these treatments, mitosis proceeded normally but no membrane furrowing (cytokinesis) could be detected. Likewise, microinjection of Rho inhibitors into Xenopus embryos prevented cell division, through a mechanism that included inhibition of the actomyosin contractile ring at the cleavage furrow. A similar phenotype was observed in sand dollar eggs (Mabuchi et al., 1993), where an actomyosin ring did not form after treatment with C3 toxin. In addition, C3 toxin caused the regression of previously formed actomyosin rings, thus demonstrating the need for sustained Rho activity for successful cytokinesis.

Several studies, including those reported in this dissertation, have shown localization of RhoA during cytokinesis at the cleavage furrow formation site (Takaishi et al., 1995;Nishimura et al., 1998a) where, in fact, it is one of the first proteins to arrive

and orchestrate the recruitment of numerous cytokinesis proteins to the cleavage furrow and the organization of ultrastructures such as the contractile ring. More recent studies propose that RhoA localization to the cleavage furrow must be accompanied by an increase in RhoGTP levels. Initial inquiries on the necessity of increased RhoGTP levels reported the need for a global increase in Rho activation to ensure cytokinesis completion (Kimura et al., 2000b;Maddox and Burridge, 2003;Yoshizaki et al., 2004). As technology improved, more complex inquiries as to the timing and spatial regulation of increased RhoGTP levels were possible and resulted in the discovery of discrete RhoGTP activity zones along the equator of the bipolar dividing cell (Bement et al., 2006b;Su et al., 2009). These studies showed that these Rho activity zones are a prerequisite for cleavage furrow formation and function, and therefore crucial for cytokinesis.

Localization of RhoA to the cleavage furrow seems to be directed by the microtubule spindle. Bement, Benik and von Dassow (2005) published an elegant set of experiments that revealed the role of microtubules in RhoA localization during cytokinesis. Four dimensional microscopy and a RhoGTP biosensor were employed to reveal a precisely bounded zone of RhoA localization that is independent of actin polymerization. Their approach allowed them to see how Nocodazole-induced disruption of microtubules resulted in loss of RhoGTP zones. Furthermore, focusing and localization of the Rho zones were dependent on microtubules, as illustrated by their physical manipulations of the spindle and the ensuing relocalization of RhoGTP zones (Bement et al., 2005).

A substantial number of studies point to the need for increased RhoGTP levels

during cytokinesis. The question then becomes, how is Rho activated in cytokinesis? Initial studies identified a GEF called Ect2 as the primary nucleotide exchange factor for RhoA during mitosis. Ect2 localizes to the nucleus during interphase and to the cleavage furrow and midbody during cytokinesis. Additionally, its inhibition or constitutive activation results in cytokinesis failure and multinucleation (Tatsumoto et al., 1999). Shortly after Tatsumoto's findings, Kimura and colleagues (2000) reported that the observed increase in mitotic RhoGTP levels, which peak during cytokinesis, was mediated by the activity of Ect2. Yet, the localization of Ect2 to the cleavage furrow remained unclear. Later, it was shown that Ect2 associated with the centralspindlin complex, explaining how it is localized to the cleavage furrow, where it can activate Rho (Zhao and Fang, 2005b). The centralspindlin complex, consisting of a plus-end directed kinesin and a Rho family GAP, associates with the mitotic spindle and, via its plus-end directed kinesin motor, walks towards the cell equator where it is enriched during cytokinesis. Through its association with the centralspindlin complex, Ect2 is captured from the cytosol and literally carried to the cellular region where its GEF activity is needed.

Due to its cyclical activation, a decrease in RhoGAP activity could also mediate the observed mitotic RhoGTP increase. As previously discussed, inactivation of p190 in mitosis by ser/thr phosphorylation could lead to increased RhoGTP levels (Maddox and Burridge, 2003). The kinases responsible for this phosphorylation increase are still unidentified but there are significant clues that cdk1 and Aurora B are involved in the process (A.S. Maddox, L. Su personal communication; personal observations). This

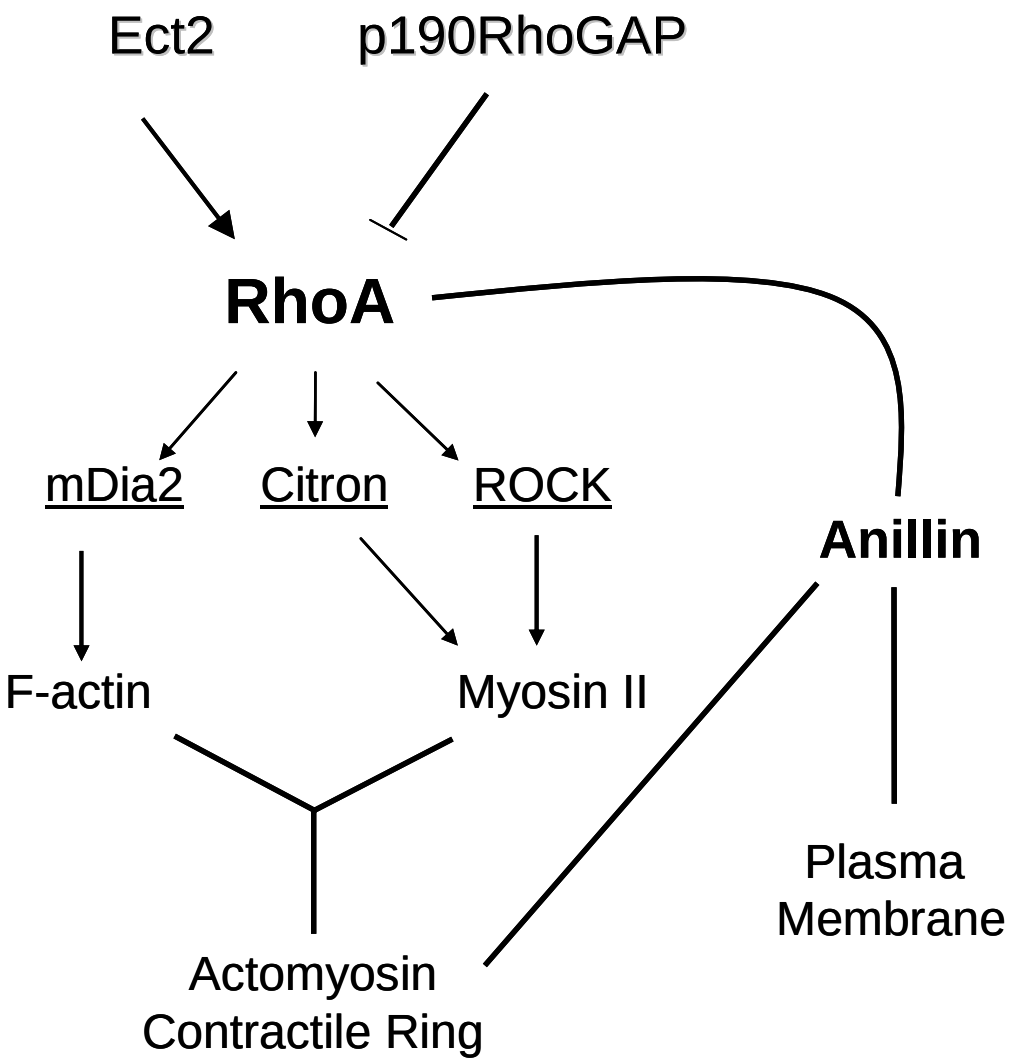

Ect2
p190RhoGAP
RhoA
mDia2
Citron
ROCK
Anillin
F-actin
Myosin II
Plasma
Membrane
Actomyosin
Contractile Ring

Figure 6. Schematic of Rho signaling pathways during cytokinesis

Rho signaling during mitosis, which directs the polymerization and contraction of an actomyosin ring, is in many ways similar to what was previously described for stress fiber formation. Rho activity is regulated by the mitotic GEF Ect2 and p190RhoGAP. When active, Rho signals the formation of actin bundles by mDia 2. These bundles are crosslinked by myosin II after its activation by kinases, giving rise to an actomyosin contractile ring. Contraction is also mediated by Citron- and ROCK I- dependent phosphorylation of myosin II. The contractile ring is anchored to the plasma membrane at the equatorial midzone by means of its interaction with Anillin, a molecular scaffold. Anillin binds RhoA, actin, myosin II and other molecules, allowing for precise monitoring and regulation of cleavage furrow dynamics.

dissertation and previously published work in our laboratory proposes that an additional mechanism by which RhoGTP levels decrease is the marked ubiquitin-mediated degradation of p190 during cytokinesis. The robust nature of RhoA regulatory pathways during cytokinesis is a clear indication of the critical role RhoA plays in the process.

In many ways, RhoA activation and its role in stress fiber formation during interphase is repeated during contractile ring formation in cytokinesis. Although several major differences exist between the resulting structures (organization and localization of many stress fibers versus organization and localization of the contractile ring, for example) the signaling directing their organization is virtually identical. For instance, once the cleavage furrow formation site is established through a RhoGTP activity zone, Rho directs contractile ring formation and ingression primarily through its effectors mDia, ROCK and Citron kinase (See Figure 6). Similar to stress fibers, the contractile ring is a structure made of actin filaments bundled together and crosslinked by myosin II. Actin nucleation is initiated by RhoGTP through its downstream effector mDia2. mDia proteins are actin nucleators that promote the polymerization of long and unbranched filaments. A recent study reported that depletion of mDia 2, not mDia 1 or 3, resulted in multinucleation of NIH3T3 cells. Additionally, its depletion resulted in the loss of an actin ring along the equator of the cell after anaphase onset as well as in the mislocalization of a number of cytokinesis proteins associated with the contractile ring such as RhoA, myosin II and Anillin (Watanabe et al., 2008). Actin filament crosslinking by myosin II and, consequently, the formation of an actomyosin ring structure is regulated by Rho, given that two primary Rho effectors, Citron kinase and ROCK, are

able to phosphorylate MLC (Glotzer, 2001). Once activated, myosin "walks" along crosslinked actin filaments, providing the mechanical force necessary for actomyosin ring contraction and membrane furrowing, which proceeds until cells are separated by abscission (Matsumura et al., 2001).

The actomyosin ring is assembled on a network of cytoskeletal proteins that act as a scaffold and connect the contractile ring to the plasma membrane. Among these cytoskeletal components, a particularly important protein is the molecular scaffold Anillin. Similar to Ect2, Anillin is a nuclear protein during interphase that is released into the cytoplasm after nuclear envelope breakdown and accumulates at the cleavage furrow formation site during cytokinesis (Field and Alberts, 1995), where it colocalizes with RhoA and actin. There, it organizes and stabilizes contractile ring components and regulators through its scaffolding function in a RhoGTP-dependent manner (Figure 6). Through numerous protein interaction domains at its N-terminus, Anillin has been shown to associate with actin, myosin II, and RhoA, bringing together the structural components of the contractile ring and its regulators (Field and Alberts, 1995;Oegema et al., 2000;Straight et al., 2005;Piekny and Glotzer, 2008). Anillin also contains C-terminal PH domains that mediate its association with the membrane, serving as a membrane anchor for the contractile ring. Anillin was shown to be critical for cytokinesis completion when, upon its depletion, cleavage furrow ingression is unable to proceed to completion and, during late cytokinesis, it causes rapid myosin-based oscillatory movement of the loosely associated contractile ring components (Zhao and Fang, 2005a). Thus, by means of its scaffolding function and anchoring to the membrane, Anillin is a

crucial participant of cytokinesis. Work presented in chapter five of this dissertation expands on the critical role that Anillin plays during cytokinesis as we discovered that it is associated with p190 during cytokinesis, perhaps recruiting or maintaining p190 at the cleavage furrow, in close proximity to its target, RhoA. Furthermore, we show that the interaction between p190 and Anillin is contractility-dependent, providing initial clues of a potential mechanism whereby cells can assess sufficient tension across the contractile ring and membrane and, through p190, relay the information to the master regulator of actin-based contractility, RhoA.

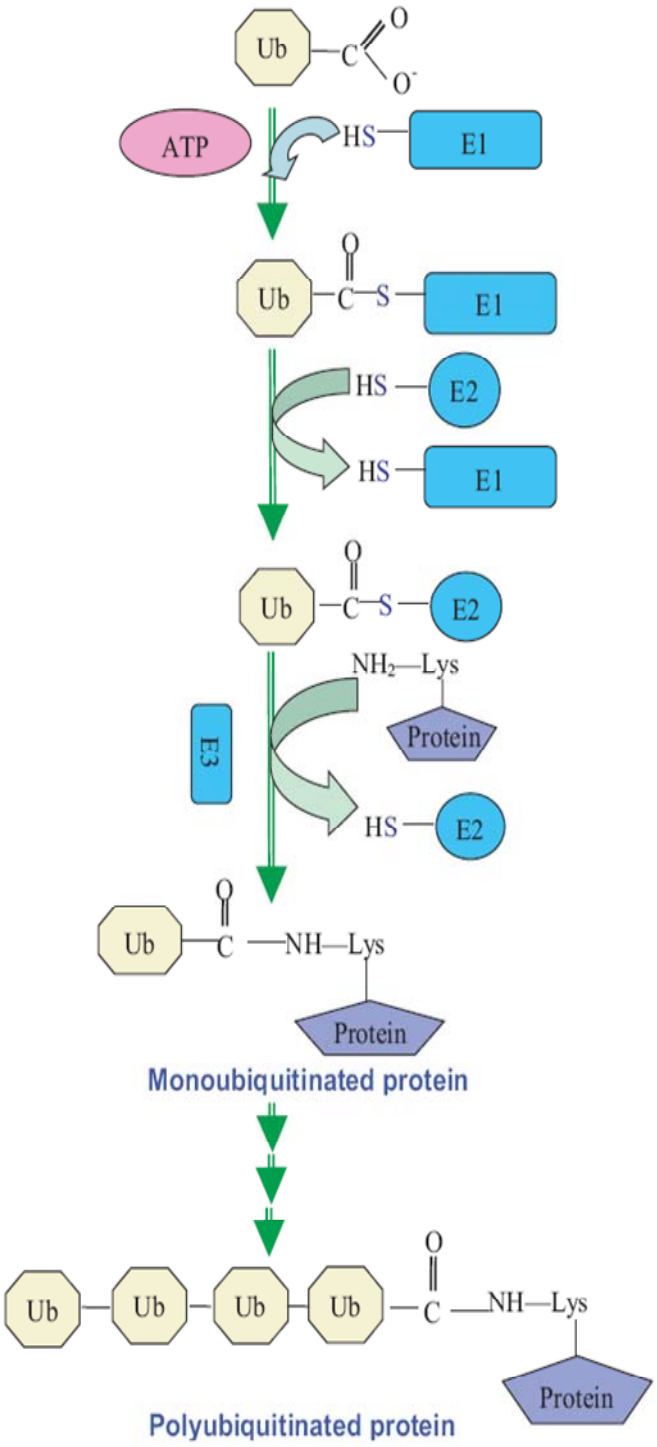
PROTEIN UBIQUITINATION

Progression through the different stages of the cell cycle is an important process that needs to occur in an organized, efficient and unidirectional manner, always promoting advancement into the next stage. The cell accomplishes this unidirectional, forward progress by degrading important cell cycle regulatory proteins. For example, cyclinB is the regulatory component of the two member protein kinase complex cycB/cdk1. As its name implies, the cyclical expression of cyclinB is important for regulation of the kinase activity of cdk1 and for cell cycle progression (Glotzer et al., 1991). In order for cells to advance through mitosis, cyclinB must be degraded during metaphase to inactivate cdk1 kinase activity and allow entry into cytokinesis. Several other important regulatory factors are also degraded during mitosis including Securin, CyclinA and Polo-like kinase (Murray, 2004). Degradation of these and numerous other proteins is mediated through a complex and tightly regulated process based on post-

translational modifications involving the 8.5 KDa protein Ubiquitin (Wilkinson et al., 1980).

Ubiquitin is a small protein, 76 amino acids in length, that is present in all eukaryotic cells. It can be conjugated through a multistep-multienzyme process to other proteins by means of an isopeptide bond, formed between an amine group in the side chain of a lysine in a "target" protein and the C-terminus of ubiquitin. Additional ubiquitin molecules can be added to some of the seven lysines in the ubiquitin first conjugated to the substrate, giving rise to polyubiquitin chains (Pickart, 2001a). Polyubiquitin chains linked through lysines 11, 29 and 48 of ubiquitin, target proteins for proteasome-mediated degradation whereas polyubiquitin chains linked through lysine 6 or 36 function instead as a reversible modification regulating protein activity, localization or trafficking (Nandi et al., 2006). Similarly, monoubiquitination directs protein activity, localization or trafficking. The EGFR, for example, is monoubiquitinated, internalized and thus targeted to endosomes where it will either be recycled and sent back to the membrane or directed to the lysosomes for non-proteasomal degradation (Glickman and Ciechanover, 2002).

Work by Avram Hershko, Aaron Chiechanover and Irwin Rose to determine the mechanism of ubiquitination resulted in the 2004 Nobel Prize in Chemistry. Their work described what is now called the classical ubiquitination pathway involving three enzymes called E1, E2 and E3 (Figure 7). Below is a brief description of this pathway. The E1 enzyme serves as an ubiquitin activator which, in an ATP-dependent process, creates an ubiquitin C-terminal adenylate. Ubiquitin adenylate then becomes an



Adapted from Nandi et al J. Biosc. 2006 March; 31(1): 137-155

Figure 7. The classical ubiquitination pathway

Ubiquitination employs the activities of several enzymes that are necessary for the activation and specificity of ubiquitin conjugation onto substrates. Briefly, an E1 or activating enzyme contains an active site cysteine (denoted as SH) which covalently binds ubiquitin in an ATP-dependent reaction. The same covalent bonds are formed with the E2 or conjugating enzyme and ubiquitin. The E3 ligase typically binds to the substrate protein and gives specificity to the transfer of the ubiquitin from the E2 enzyme to the epsilon amino group (ε-NH_2) of a lysine on the target protein. In many cases, this lysine is near an ubiquitination motif. Additional ubiquitin molecules can be added to the initial ubiquitin to create polyubiquitin chains, targeting a protein for proteasomal degradation.

enzyme-bound substrate for the formation of an E1-ubiquitin thiol esther bond with the sulfur on a cysteine side chain in the active site of E1. Once this is accomplished, and through a similar reaction as just described, ubiquitin is then transferred to the cysteine in the active site of the conjugating enzyme E2. Finally, ubiquitin is conjugated to a lysine on a substrate in an E3 enzyme-dependent reaction. Multiple rounds of this process may result in the polyubiquitination of proteins, with each additional ubiquitin being added to the previous one, and not to the substrate itself, to form a polyubiquitin chain. Polyubiquitinated proteins are recognized by the 26S proteasome and targeted for degradation. Fascinatingly, during the degradation process of the polyubiquitinated substrate, ubiquitin itself is spared from enzymatic digestion and recycled back into the cytosol.

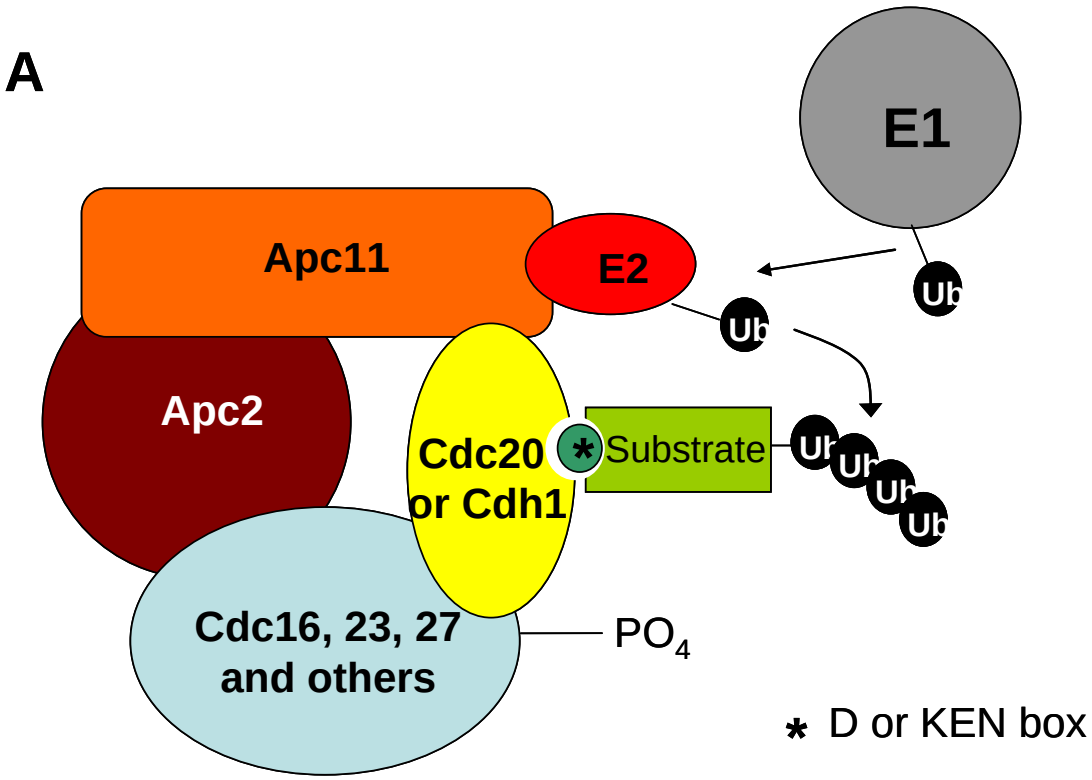
Ubiquitination reactions are initiated by the action of one major E1 activating enzyme present in cells. E1 can relay activated ubiquitin to more than twenty E2 enzymes which in turn can interact with hundreds of existing E3 enzymes or ligases, thus providing the specificity required for the ubiquitination of thousands of cellular proteins. Substrate recognition for ubiquitination is mediated by E3 ligases and regulated by various mechanisms. Phosphorylation is one such mechanism, as exemplified by the cdk inhibitor p27 (Montagnoli et al., 1999). Phosphorylation of a degradation motif in p27 creates a "phospho-degron" and allows its recognition and ubiquitination by the Skip2/Cullin/Fbox (SCF) complex. Phosphorylation of the ligase has also been observed as a mechanism for regulating substrate ubiquitination. For example, the Anaphase Promoting Complex/Cyclosome (APC/C), the most important E3 ligase active during

mitosis, needs to be phosphorylated by either cdk1 or polo-like kinase before it can become activated and recognize ubiquitination substrates (Lahav-Baratz et al., 1995;Shteinberg et al., 1999;Golan et al., 2002). Phosphorylation of both E3 ligase and substrate has also been observed in certain cases such as with the E3 ligase c-Cbl and its substrate, the EGFR (Galisteo et al., 1995;Grovdal et al., 2004). Finally, association with ancillary proteins may be required for substrate recognition by an E3 ligase, as is the case with the E6-AP protein, a ligase which associates with the E6 protein from Human Papiloma Virus (HPV) and targets p53 for ubiquitination (Talis et al., 1998).

Also important in the recognition of ubiquitination substrates by E3 ligases are specific degradation sequences present in potential substrates. Various degradation motifs or "degrons" have been identified and include the D, KEN, A, and O boxes, among others. D and KEN boxes are recognized by the APC/C and are of particular importance to the work presented here, given that p190 contains these degrons in abundance and throughout its protein sequence. D boxes, first identified in cyclins, are motifs with the following amino acid sequence RXXLXXXN/E (Glotzer et al., 1991;King et al., 1996;Fang et al., 1998) and are present in numerous proteins degraded during mitosis. KEN boxes, with the sequence KENXXXD/N are also present in proteins degraded in mitosis (Pfleger and Kirschner, 2000). It is important to emphasize that these motifs act simply as E3 recognition sequences and are not themselves modified. Rather, lysines lying in near proximity are modified and serve as final ubiquitin acceptors. Now that several degrons have been identified, these sequences give helpful initial hints for the identification of the E3 ligase that directs the degradation of a protein.

The aforementioned E3 ligases, SCF and APC/C, are the major ligases driving progression through the cell cycle. The SCF drives progression into S, through G2 and to M phase (Ang and Wade, 2005). The APC/C is active during mitosis and through the end of G1 (Peters, 2002). Both ligases, the SCF and APC/C, are RING domain-dependent multiprotein complexes. This means that they contain a RING finger-containing protein subunit within the complex which mediates interaction with an E2 enzyme and ubiquitin transfer to substrates. A separate protein is employed in substrate binding/recognition. Given that the APC/C is the dominant E3 ligase during mitosis, when p190 is ubiquitinated and degraded, further details on the mechanisms regulating its activity are provided (Figure 8A). The APC/C has two requirements for full E3 ligase activation: it must be phosphorylated by polo kinase and cdk1 (Golan et al., 2002) and bound by one of its two known activators, cdc20 and cdh1. The function of these activator proteins is to bind ubiquitination substrates by means of the degrons present in substrate sequences. Only one such activator may be bound to the APC/C at one time. During the initial stages of mitosis (prometaphase to anaphase) the APC/C is bound to the activator cdc20 (See Figure 8B). The APC/C^{cdc20} ubiquitination complex recognizes and binds D box-containing proteins. The APC/C binds to cdh1 later, during cytokinesis and into G1.

The APC/C^{cdh1} complex is more versatile in its substrate recognition capacities as it recognizes not only D box-containing proteins but also KEN box-containing substrates. In order to facilitate a smooth transition from mitosis into cytokinesis, between APC/C^{cdc20} and APC/C^{cdh1} complex function, cdc20 (itself a KEN box-containing protein)



Adapted from Peters, JM. (2006) Nat. Rev. Mol. Cell Biol. 7, 644-656

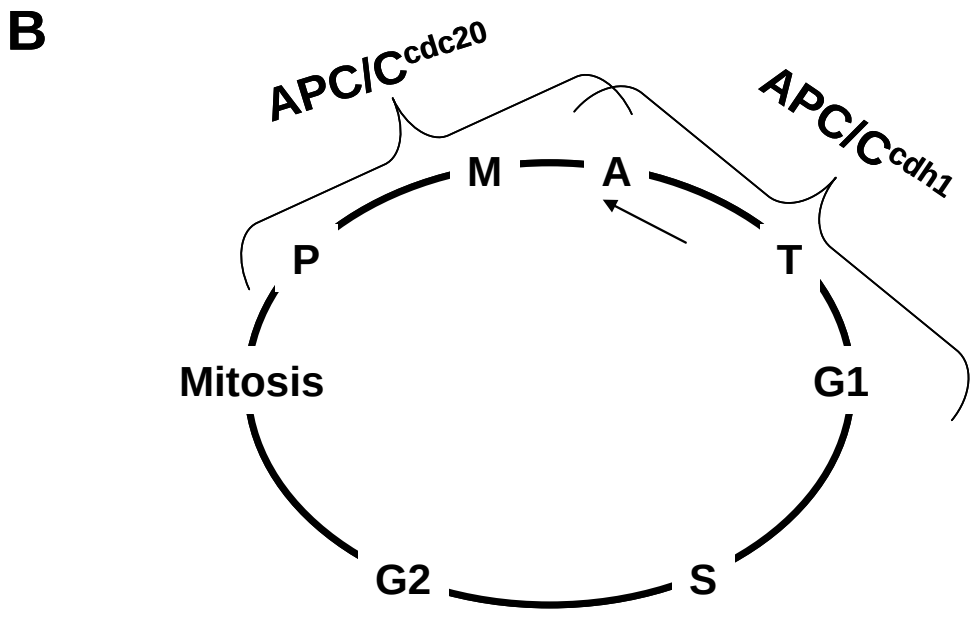


Figure 8. A schematic of APC/C-dependent ubiquitination and activity

A) The APC is an E3 ligase consisting of approximately 12-15 subunits. A schematic of the core complex is depicted here. The core structure of the APC/C consists of a RING finger protein (Apc11) that mediates interactions with the E2 enzyme. The structural protein Apc2 serves as a scaffold stabilizing the Apc11 and the region for substrate recognition. Other proteins (Cdc16, 23 and 27) stabilize interactions with other APC/C complex proteins and, together with Apc2 bind APC/C activators Cdc20 or Cdh1. Through interactions with either Cdc20 or Cdh1, substrates are recognized and recruited to the APC/C. Ubiquitination of APC/C substrates begins with E1, which then conjugates activated ubiquitin to an E2, associated with the APC/C. Substrate recognition by APC/C activators then brings E2 and substrate into close proximity, where ubiquitin is then transferred from E2 to substrate.

B) The APC/C^{cdc20} complex is active in mitosis, prophase through anaphase, whereas the APC/C^{cdh1} complex is active from anaphase until the end of G1. Interestingly, cdc20 itself is an APC/C^{cdh1} substrate. Its degradation allows for full APC/C^{cdh1} activity.

is a cdh1 substrate and becomes ubiquitinated/degraded as APC/C^{cdh1} activity increases (Peters, 2006). As previously reported by Su et al, (2003) p190 is ubiquitinated and degraded during mitosis. Work presented in chapter three of this dissertation identifies four lysine residues at the N-terminus of the protein which are necessary for p190 degradation, putative primary ubiquitination sites. Furthermore, p190 contains numerous D and KEN boxes along the length of its sequence making it a suitable candidate for APC/C-dependent ubiquitin-mediated degradation. Results from our studies and attempts to identify the E3 ligase that recognizes and ubiquitinates p190 are included in chapter four of this dissertation.

The 26S proteasome

Once proteins are ubiquitinated and labeled for degradation, they must be recognized and processed by the 26S proteasome, the recycling center of cells. The proteasome is a massive multiprotein structure of nearly 2.5 MDa and contains several different components with varied protease activity. The proteasome is a barrel-shaped structure with top and bottom "lids" that make it resemble a trash can (Figure 9). The barrel-shaped portion of the proteasome is the 20S core particle (CP) and the "lids" are 19S regulatory particles (RP). The CP is made up of four rings of seven subunits each which are stacked on top of one another. The top and bottom rings are structural in nature and are made up of α subunits, while the middle two rings, made up of β subunits, contain the catalytic activity of the proteasome. The catalytic activity of these inner β rings is limited to the inside of the barrel and is made up of chymotrypsin-like,.

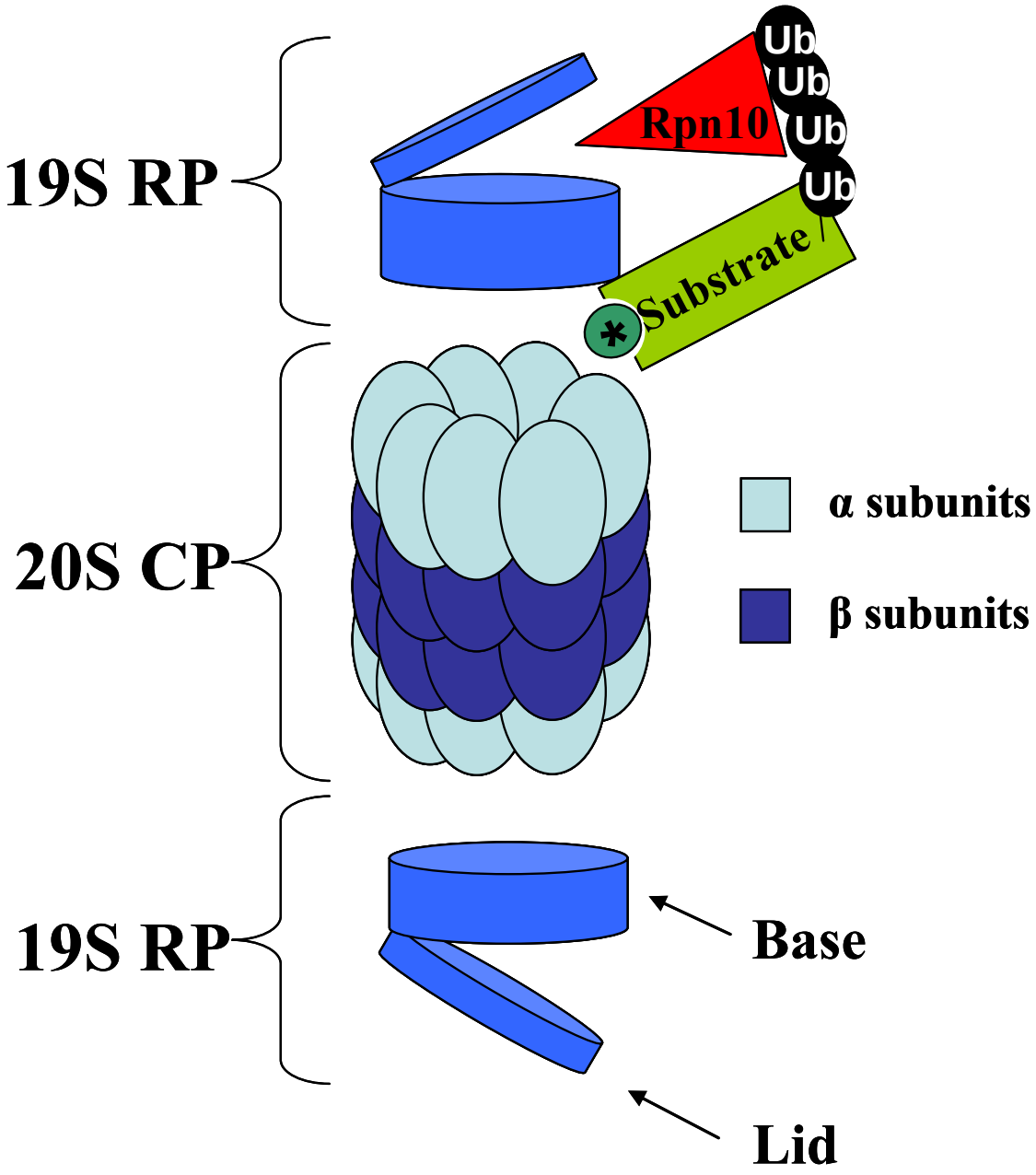

19S RP
Ub
Ub
Ub
Ub
Rpn10
Substrate
*
20S CP
α subunits
β subunits
19S RP
Base
Lid

Figure 9. Schematic representation of the 26S proteasome

The 26S proteasome is a multi-protein complex that degrades polyubiquitinated proteins. It is composed of two 19S regulatory particle (RP) and a 20S core particle (CP). The RP consists of structures referred to as the lid and the base. The lid is necessary for protein degradation. Rpn10 stabilizes the interaction between the lid and the base and directly binds polyubiquitin chains on the protein targeted for degradation. The base contains several ATPases that are thought to unfold and translocate proteins through a gated channel into the CP. The CP is made up of four rings, two outer rings (in light blue) made up of seven α subunits and two inner rings (in dark blue) made up of seven β subunits. The β-subunits contain the catalytic activity of the proteasome.

trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing activities (Heinemeyer et al., 1997). Regulatory particles are divided into "lid" (distal to the CP) and "base" (proximal to the CP) substructures. The 26S holoenzyme is formed when one CP and two RP are associated. ATPase activity residing in the base portion of the RP is necessary to unfold ubiquitinated proteins and to translocate them into the inside of the CP for degradation. The mechanism by which proteins are recruited to the proteasome is still unclear. However, the RP is able to interact with ubiquitin binding domain-containing proteins, such as Rpn10, chaperone proteins and other components of the ubiquitin pathway and thus participates in the capture and presentation of ubiquitinated proteins to the CP (Elsasser and Finley, 2005). Ubiquitinated proteins processed by the proteasome are broken down into short-lived, small peptides ranging usually from 7-9 amino acids in length, though peptides ranging from 4-25 amino acids are also possible. These small peptides are further processed in the cytosol and converted into single amino acids which can then be recycled during new protein synthesis (Voges et al., 1999).

Perspectives

The regulation of p190 expression levels and its role in the modulation of RhoA activity during cytokinesis has been described in this Introduction as it is currently found in the scientific literature. In summary, previous data have demonstrated that mitotic p190 degradation is mediated by the ubiquitin-proteasome pathway with the express purpose of allowing increased RhoGTP levels during cytokinesis. Increased RhoGTP levels result in greater signaling to downstream effectors, formation of a stable

actomyosin ring and contraction leading to cytokinesis completion and cell abscission. In this dissertation, further details regarding the mitotic regulation of p190 emerge and an increased understanding of its role in cytokinesis becomes evident. Our findings (Chapter 3) show that p190 degradation is required for proper completion of cytokinesis given our observation that, in addition to overexpression, sustained endogenous p190 levels during mitosis result in an increase in multinucleated cells, indicative of cytokinesis failure. Preliminary data (Chapter 4) regarding the identity of the E3 ubiquitin ligase that targets p190 during mitosis, though inconclusive, suggest that the APC/C may be involved. Furthermore, we establish that p190 degradation is mediated through four N-terminal lysine residues (Chapter 3). We also show that p190 overexpression during cytokinesis or sustained endogenous levels of p190 affect cytokinesis primarily through mislocalization of a phosphorylated form of myosin II, known to regulate myosin motor activity, and propose that an observed interaction between p190 and the molecular scaffold Anillin could be part of a mechanosensing switch that regulates RhoA activity as a function of effective contractility across the cleavage furrow (Chapter 5). Finally, we also discover a potential new role for p190 during cytokinesis, as a regulator of microtubule-kinetochore interactions during early mitosis (Chapter 6). Further experimentation will answer important questions regarding this most interesting observation, including whether p190 can function as a Cdc42-specific GAP at earlier times during mitosis. As a whole, these studies have more definitely described the mechanism of p190 degradation and increased our understanding of p190's regulatory functions during cytokinesis.

Chapter 2

Material and Methods

Reagents- The following chemicals were used throughout this study: Thymidine, Nocodazole, MG132, DAPI (Sigma), Lactacystin, Blebbistatin, Okadaic Acid (Calbiochem) and Doxycycline (Clontech). All monoclonal and polyclonal antibodies used in the work presented in this dissertation are identified in Tables I and II of this chapter.

Cell culture and synchronization- MDA-MB-468 Tet-on p190, ΔGBD or vector only inducible human breast cancer cells (Su et al., 2003) and HeLa human cervical carcinoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin and streptomycin (Invitrogen). Growth media used for MDA-MB-468 cells also contained puromycin (130ng/mL) and geneticin (G418, 400μg/mL) (Sigma). Cells were synchronized by treatment with 2mM thymidine for 14-16 hrs, released for 8 hrs, and subsequently treated with 50ng/mL nocodazole for 14-16 hrs. Cells were released from the nocodazole block and harvested by mitotic shake off at indicated times. Either lactacystin (10 μM) or MG132 (20mM) were added during the time of release as indicated.

Plasmid constructs- The following p190 constructs used in this study were previously described in Su et al. (2003): ΔGBD, ΔGAP (Δ C-terminus), GBD, Section 1 (S1), and GAP. The Y1105A, R1283A, 4KR and GBDS1 4KR mutants were generated by using the QuickChangeII Site-Directed Mutagenesis kit (Stratagene) and expressed in the triple-HA-tagged vector pKH3 (kind gift from I. Macara, University of Virginia). Other cDNA fragments of p190 used in this study were also cloned into the pKH3 vector –at BamHI and EcoRI sites- after generation by PCR using the following primer pairs:

GBDIVS, 5'-GCCAGGATCCATGATGATGGCAAGAAAG-3' and 5'-GCGCGAATTCTCACCACTTTAAGAACTCTGGC-3'; for **GBDS1**, 5'-GCCAGGATCCATGATGATGGCAAGAAAG-3' and 5'-AATTGAATTCCTGAAACGGCAACATTCCCC-3'; for **IVS/S1**, 5'-GTCAGGATCCACAAGCAAAGGACAAG-3' and 5'-AATTGAATTCCTGAAACGGCAACATTCCCC-3'; for **S2S3GAP**, 5'-GTGAGGATCCCCTGTGAACTCTTTCCAGA-3' and 5'- GGCCGAATTCCTGATAAGAAGACAAC-3'. All mutants obtained by PCR were confirmed by sequencing prior to use. Constructs for cdh1 and cdc20 were a kind gift from the M.W. Kirschner laboratory and were cloned in to the pCS2+ cloning vector (designed by David Turner and Ralph Rupp at the Fred Hutchinson Cancer Center in 1993). All plasmid constructs were transfected into HeLa cells using the Polyfect reagent from Qiagen, as per manufacturer's instructions optimized for this cell line.

In vitro ubiquitination assay- In vitro ubiquitination reactions were performed as described by Cockman et al. (Cockman et al., 2000). Extracts were prepared from mitotic MDA-MB-468 cells which provided all enzymes for the ubiquitination reaction. Briefly, cells were swollen in a hypotonic buffer (20 mM Tris, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT), homogenized, and centrifuged at 10,000 x g for 10 min at 4^0C. Aliquots of supernatants were stored at -70^0C. Ubiquitination reactions were performed at 30^0C for 4 hr in a total volume of 40 μL, which contained 2.5 μL of programmed reticulocyte lysate, 81 μg hypotonic extract, 1X Energy-Regenerating System (Boston Biochem), 20 μg ubiquitin (Sigma), 3 μM ubiquitin aldehyde (Boston

Biochem), and 20 μM MG132. All constructs were *in vitro*-transcribed and translated in the presence of 20 μCi [^{35} S] methionine (1,175 Ci mmol^{-1}; Amersham Biosiences) using TnT Quick Coupled Transcription/Translation System (Promega). Products of the reaction were analyzed by 7% SDS-PAGE and phosphoimager scanning (Molecular Dynamics Storm Scanner from Molecular Dynamics, Inc.). Densitometric analysis was performed using the ImageQuant software (Molecular Dynamics, Inc.). Quantitation of the extent of ubiquitination of each construct was determined by densitometric scanning of the area above the *in vitro* translated product ("U" lanes in Fig. 14B) in samples that contained the complete ubiquitination reaction and relating that value to the area above the luciferase control. These normalized values were then related to the picomoles of [^{35}S] methionine-labeled TnT product present in the reaction mix. Picomoles of TnT product were calculated on the basis of the total moles of methionine present (hot and cold) in the translation reaction, the incorporation of [^{35} S] methionine in the product, its molecular weight, and the number of methionines it contained. Values obtained from the Luc control were set at 1, and values of other samples were compared to it for generation of fold ubiquitination/picomoles of protein.

In vivo degradation assay- HeLa cells cultured in duplicate 100mm dishes were transfected with the various p190 plasmids listed in the figures. Twenty-four hours post-transfection, one dish was synchronized as described, and cells were released from prometaphase arrest for 40 min. The control dish was maintained as an asynchronous cell population. Cells were lysed at 4^{0}C in RIPA buffer (50 mM Tris pH 7.2, 150 mM NaCl, 0.25% deoxycholate, 1% NP40) supplemented with 0.5% aprotinin, 1 mM sodium

orthovanadate, 12.5 μg/mL leupeptin, and 1 mM PMSF. Protein concentration was determined by the BCA protein assay (Pierce), and 100 μg cell extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blotting. Densitometric analysis was done using the software AlphaEase (AlphaInnotech Corp.).

In vitro ubiquitination assay with Anaphase Promoting Complex- This assay was performed by Bing Li, a member of Hongtao Yu's laboratory in the Pharmacology Department of the University of Texas Southwestern Medical Center as described in (Bembenek and Yu, 2001). Briefly, interphase APC/C was immunopurified from cells by using α-APC3 (Cdc27) beads. These beads were then incubated for 1 h at room temperature in the presence or absence of hCdh1. After incubation, the APC beads were washed twice with XB and used in ubiquitination assays. Each assay was performed in a volume of 5 μl and the reaction mixture contained an energy-regenerating system, 150 μm bovine ubiquitin, 5 μm human E1, 2 μmUbcH10, 2 μl of the APC beads and the radiolabeled substrate. The reactions were incubated at room temperature for 1 h, quenched with SDS sample buffer, and analyzed by SDS-PAGE and phosphoimager scanning.

RNAi reconstitution assay- The RNAi reconstitution assay was performed in MDA-MB-468 Tet-on ΔGBD p190 cells, in MDA-MB-468 Tet-on wt p190 cells, and in HeLa cells. The RNAi oligos were double stranded and custom made by Dharmacon to silence the expression of human p190-A protein by targeting a unique sequence at the N-terminus of the protein. The sequence of the double stranded oligos was 5' AAG AUG CAC AUU GUG GAG CAG 3'. MDA-MB-468 cells were treated with the p190 siRNA

oligos (60 pmol) using the Oligofectamine reagent (Invitrogen), as per the manufacturer's instructions. After 24 hrs, cells were transfected with a second round of siRNA, and doxycycline was added (1μg/ml) to induce the expression of stably transfected ΔGBD or wt p190. Twenty-four hours later, cells were harvested for Western blot analysis or fixed for multinucleation assays, described below. HeLa cells were treated with the siRNA using Oligofectamine reagent and, 24 hrs post-siRNA treatment, transfected with the pKH3-ΔGBD plasmid using the Polyfect reagent (Qiagen) as per the manufacturer's instructions. After an additional 24 hrs, cells were harvested for Western blot analysis or fixed for use in multinucleation assays.

Multinucleation assay- Treated MDA-MB-468 and HeLa cells were fixed by treatment with 4% paraformaldehyde for 20 min, washed three times with PBS, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 20% goat serum at room temperature for 1 hr. After blocking, both cell lines were incubated with HA-11 Ab (1:1000 dil) for 1 hour and with Alexa 594-conjugated anti-mouse Ab (1:1000) for 30 min. Nuclear DNA was stained with 2μg/mL DAPI in PBS for 3 min. Coverslips were mounted on microscope slides with VectaShield, and cells were viewed on a Leica microscope fitted for epifluroescence. The presence of multinucleated cells was determined by phase and DAPI staining.

Imaging Flow Cytometry: Cells used for this analysis were prepared similarly as cells analyzed by confocal microscopy with two major exceptions. First, after Nocodazole release, cells were mechanically detached from dishes and transferred to 15 ml conical tubes where they were fixed by incubation in 4% paraformaldehyde (10%

TCA for Rho staining). After fixation, cells were permeabilized with 0.2% Triton X-100 and blocked with 20% goat serum at room temperature for 1 hr. After blocking, samples were incubated with appropriate primary and secondary antibodies, as described in table I. As a last preparatory step, cells were resuspended in a 2μg/mL DAPI solution and stored at 4˚C until analysis was performed. Imaging flow cytometry experiments were performed by Mr. Benjamin Kim, an undergraduate researcher, using the Amnis Imagestream instrument available at the University of Virginia Flow Cytometry Core Facility.

Table I: Antibodies for Immunofluorescence Imaging

Antibody	Company	Type	Dilution
P190 (Clone 30)	BD Transd. Lab	Mouse mAb	IF 1:200
P190-B (Clone 54)	BD Transd. Lab	Mouse mAb	IF 1:200
Rho (26C4)	SCBT	Mouse mAb	IF: 1/100
Anillin (H-300)	SCBT	Rabbit pAb	IF: 1:250
AuroraB (AIM-1)	BD Transd. Lab	Mouse mAb	IF 1:200
α-Tubulin (DM1A) FITC-Conjugate	Sigma	Mouse mAb	IF: 1:100
Myosin Light Chain (MY-21)	Sigma	Mouse Ascites Fluid	IF: 1:100
Phospho MLC2	Cell Signaling	Rabbit pAb	IF 1:50
HA.11	Bethyl Labs	Rabbit pAb	IF 1:200
AF 594 Conj GαM	Molecular Probes	Goat	1:2000
AF 594 Conj GαR	Molecular Probes	Goat	1:2000
AF 647 Conj GαM	Molecular Probes	Goat	1:2000
AF 647 Conj GαR	Molecular Probes	Goat	1:2000
AF 488 Conj GαM	Molecular Probes	Goat	1:2000
AF 488 Conj GαR	Molecular Probes	Goat	1:2000

Confocal Microscopy- HeLa or MDA-MB-468 cells were grown to 60% confluency in normal growth media, treated with Nocodazole for 16-20 hours to induce mitotic arrest and released, for 40 minutes, onto poly-L-lysine coated coverslips in culture dishes supplemented with fresh media. After release, cells were fixed for 20 min with 4% paraformaldehyde, permeabilized for 5 min with 0.2% Triton X-100 and blocked with 20% goat serum at room temperature for 1 hr. After blocking, samples were incubated with appropriate primary and secondary antibodies (See table below) for 1.5 hrs at RT. Cells were mounted onto glass microscope slides using ProLong® Gold antifade reagent with DAPI as the mounting medium. After overnight drying at 4˚C, cells were analyzed in a Zeiss LSM510 confocal head (Carl Zeiss MicroImaging GmbH) mounted onto an Axiovert 200 wide-field fluorescence microscope, fitted with a purple diode laser for 405 nm excitation; an argon laser with 458, 477, 514, and 488 nm lines; a 543 nm HeNe laser; and a 633 nm HeNe laser. Images were captured using the accompanying LSM software (Carl Zeiss MicroImaging GmbH).

Immunoprecipitation- Cell lysates were prepared as described above and incubated with 5-10μg of the appropriate immunoprecipitating antibody for 2 hrs to overnight (depending on which protein is being targeted for capture). After primary antibody incubation, immunecomplexes are precipitated by protein A or G agarose (Invitrogen) incubation and separated from the rest of the lysate by centrifugation at 5,000 rpm for 1 minute. After several cold PBS washes, 60 μl of 2X SDS sample buffer is added to each sample, then used for Western blotting analysis, as described below.

Western blotting- Cell lysates were prepared as described above and analyzed by

8% SDS-PAGE. Proteins in the gel were wet-transferred to a nitrocellulose membrane and immunoblotted for the different proteins as described on individual figures and listed in Table II in this Chapter. Immune complexes were detected with the corresponding HRP-conjugated secondary antibodies, at a 1:5000 dilution using ECL reagent.

Table II: Antibodies for Western Immunoblotting and Immunoprecipitations

Antibody	Company	Type	Dilution
P190 (Clone 30)	BD Transd. Lab	Mouse mAb	1:500 IP: 5µg
P190 (8C10)	S. Parsons lab	Mouse mAb	1 µg/mL
P190 (P27)	S. Parsons lab	Rabbit	1 µg/mL
P190-B (Clone 54)	BD Transd. Lab	Mouse mAb	1/250
Cyclin A (H-432)	SCBT	Rabbit pAb	1:2000
Cyclin B1	SCBT	Rabbit pAb	1:1000
Cdh1 (DCS-266)	Sigma	Mouse mAb	1 µg/mL IP: 10µg
Cdc20 (C-19)	SCBT	Rabbit pAb	1:200 IP: 10µg
Phospho-Ser (4A4)	Upstate	Mouse mAb	1:500
Cdc27 (AF3.1)	SCBT	Mouse mAb	1:1000 IP: 5µg
β-actin (AC-15)	Sigma	Mouse mAb	1:20,000
Anillin (H-300)	SCBT	Rabbit pAb	1:1000 IP: 5µg
HA.11	Covance	Mouse mAb	1:1000 IP: 5 µg
HA.11	Bethyl Labs	Rabbit pAb	1:1000
Myc (71D10)	SCBT	Mouse mAb	1:1000
12CA5	M. Weber Lab	Mouse mAb	1:1000
MAPK (B3B9)	M. Weber Lab	Mouse mAb	1:10,000

Chapter 3

Mitotic Downregulation of P190RhoGAP Is Required for the Successful Completion of Cytokinesis

INTRODUCTION

Mitosis is the final stage of the cell cycle where a cell's cytoplasm, organelles and replicated DNA are equally separated to give rise to two daughter cells. Mitosis is divided into several, well-defined, stages: Prophase, Prometaphase, Metaphase, Anaphase, and Telophase. Cytokinesis, encompassing Anaphase and Telophase, begins shortly after sister chromatid separation with the formation of a cleavage furrow and proceeds until cell abscission is completed (Glotzer, 2001). Irreversible progression through the different mitotic stages depends largely on the proteasomal-dependent degradation of cell cycle regulatory proteins such as Cyclin A, Cyclin B, Securin, and Plk1 (Peters, 2002).

In addition to proteasomal degradation, an important driver of cytokinesis is the small GTPase RhoA (Balasubramanian et al., 2004). Rho family GTPases are regarded as molecular switches that cycle between the active (GTP bound) and inactive (GDP bound) states. Rho activation states are regulated by three classes of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and GDP dissociation inhibitors (GDIs). Multiple lines of evidence show that RhoA regulates furrow formation and actomyosin ring contraction during cytokinesis (Barr and Gruneberg, 2007). For example, inhibition of RhoA activity prevents cleavage furrow formation (Kishi et al., 1993;Mabuchi et al., 1993) and RhoA and its activator, the GEF ECT2, localize to the cleavage furrow and midbody. These findings and others strongly support RhoA involvement in cytokinesis (Takaishi et al., 1995;Prokopenko et al., 1999;Tatsumoto et al., 1999).

Opposing Ect2's GEF activity is p190RhoGAP-A (hereafter referred to only as p190)

which provides a negative component of RhoA regulation (Mikawa et al., 2008). P190 is a member of the p190RhoGAP family of proteins which also includes p190B. Both p190 and p190B have an N-terminal GTP binding domain (GBD), a Middle Domain (MD) that contains multiple protein interaction motifs, and a C-terminal GTPase Activating Protein (GAP) domain. P190 and p190B have C-terminal GAP domains with great specificity for the Rho family of GTPases, particularly Rho A (Settleman et al., 1992c;Ridley et al., 1993). By means of their GAP activity, p190 and p190B play vital roles in the regulation of actin cytoskeleton-dependent cellular processes. Although both RhoGAPs target Rho and are involved in similar pathways, recent data suggests that there are differences in the functions of each of these proteins (Ludwig et al., 2009). P190 mediates cell–substratum interactions (Bass et al., 2008), cell–cell adhesion (Playford et al., 2008;Wildenberg et al., 2006), cell motility and migration (Barker et al., 2004;Jiang et al., 2008), vascular permeability (Holinstat et al., 2006;Mammoto et al., 2007), cell-cycle progression (Maddox and Burridge, 2003), death (K. Ludwig and S. J. Parsons, unpublished observations), and neuronal differentiation and development (Billuart et al., 2001;Brouns et al., 2000;Sfakianos et al., 2007;Zhang and Macara, 2008). p190B regulates cell and organism size via modulation of CREB (cAMP response element binding) activity (Sordella et al., 2002), cell-fate determination (Sordella et al., 2003), mammary bud development and ductal morphogenesis (Chakravarty et al., 2003;Heckman et al., 2007;Vargo-Gogola et al., 2006), neural development (Brouns et al., 2001;Matheson et al., 2006), and angiogenesis (Guegan et al., 2008).

Whereas the role of p190B during mitosis or cytokinesis remains unexplored, our

laboratory identified an additional, cell cycle-associated function of p190 (Su et al., 2003). These studies demonstrated that overexpression of p190 results in multinucleation, a phenotype indicative of cytokinesis failure. This event is dependent on the C-terminal region of p190 which contains the RhoGAP domain, a poly-proline domain, and several kinase recognition sites (Jiang et al., 2008). Immunofluorescence microscopy of mitotic cells revealed that endogenous p190 localizes to the cleavage furrow along with actin, and upon overexpression interferes with the normal positioning and contraction of the furrow. Furthermore, p190 protein levels were observed to transiently decrease in late mitosis, a decrease that was mediated by the ubiquitin-proteasome pathway. These results raised the questions of whether the cell cycle-linked downregulation of p190 is required for successful completion of cytokinesis, how mitotic p190 degradation is regulated and whether p190B is also involved in mitotic regulation.

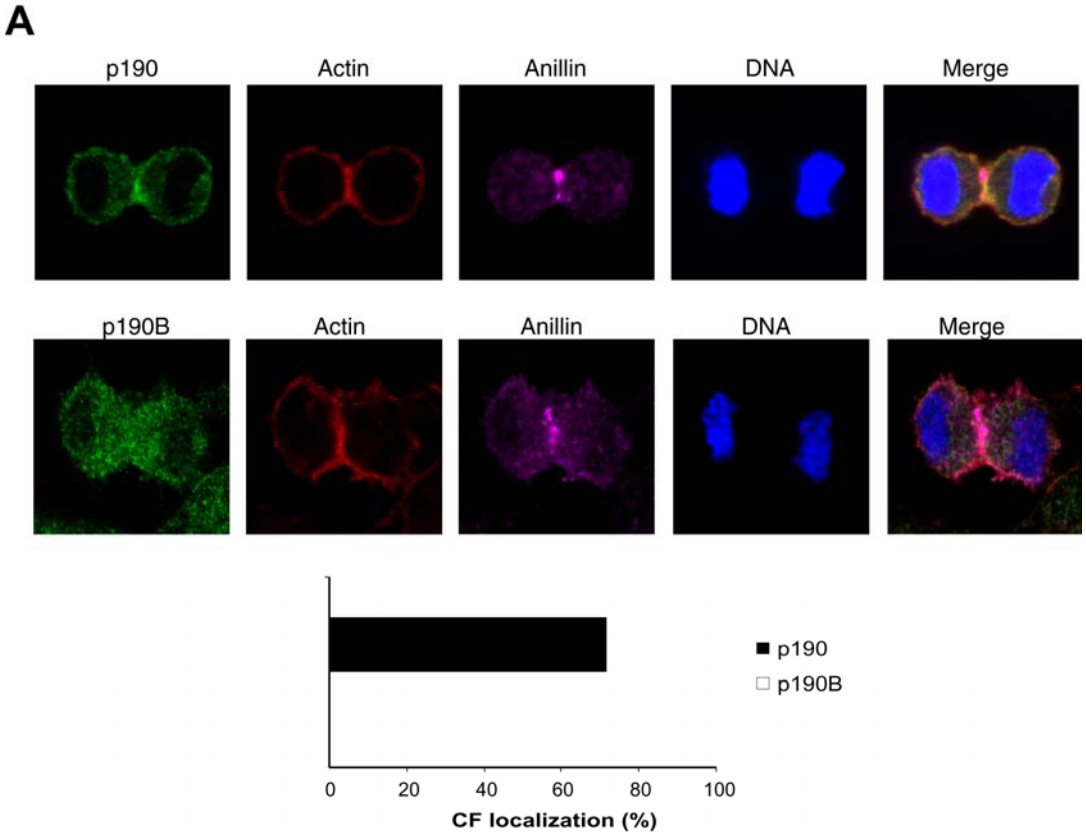
Here, we demonstrate that p190 and p190B have differing roles in mitosis as overexpression of p190, and not p190B, induce multinucleation. Furthermore, only p190, and not p190B, localized to the cleavage furrow during cytokinesis. We then focus on p190 and show that its decrease in expression levels during mitosis is required for successful completion of cytokinesis and that when it fails to occur, cell division is blocked and multinucleation ensues. The minimal region on p190 required for significant ubiquitination was mapped to the GBDS1 domain. Moreover, four lysine residues near the extreme N-terminus were identified as necessary for mitotic p190 degradation. Using point mutations, we further demonstrate that the multinucleated phenotype is dependent on the RhoGAP activity of p190, and therefore, on aberrant Rho signaling. Together,

these findings indicate that downregulation of p190 is necessary for the completion of cytokinesis and avoidance of mitotic catastrophe. They also define the mechanism by which cells achieve the well characterized increase in RhoGTP levels during cytokinesis.

RESULTS

P190, and not its B isoform, localizes to the cleavage furrow during cytokinesis and when overexpressed, causes multinucleation.

The p190RhoGAP family of proteins consists of two closely related homologues, p190 and p190B. Although both target the Rho signaling pathway and have certain overlapping functions, recent research suggests that they are also involved in cellular events that are exclusive to each. Previously published observations (Su et al., 2003) from our laboratory showed that p190 plays an important role during cytokinesis, localizing to the cleavage furrow and causing multinucleation when overexpressed. We questioned whether these observations were specific to p190 and whether p190B had similar mitotic localization and phenotype. To answer this question we compared the localization patterns of endogenous p190 and p190B during HeLa cell cytokinesis by confocal immunofluorescence microscopy. P190 was observed at the cleavage furrow during cytokinesis and colocalized with actin and the contractile ring scaffolding protein Anillin in over 70% of analyzed cells (Figure 10A). In contrast, p190B retained a diffuse cytosolic localization pattern and was not observed at the cleavage furrow or anywhere else on the membrane during cytokinesis (Figure 10A). To determine whether p190B overexpression could mirror p190 action and lead to multinucleation, HeLa cells were transfected with equal amounts of either p190 or p190B cDNAs and their multinucleation


A
p190
Actin
Anillin
DNA
Merge
p190B
Actin
Anillin
DNA
Merge
p190
p190B
0
20
40
60
80
100
CF localization (%)

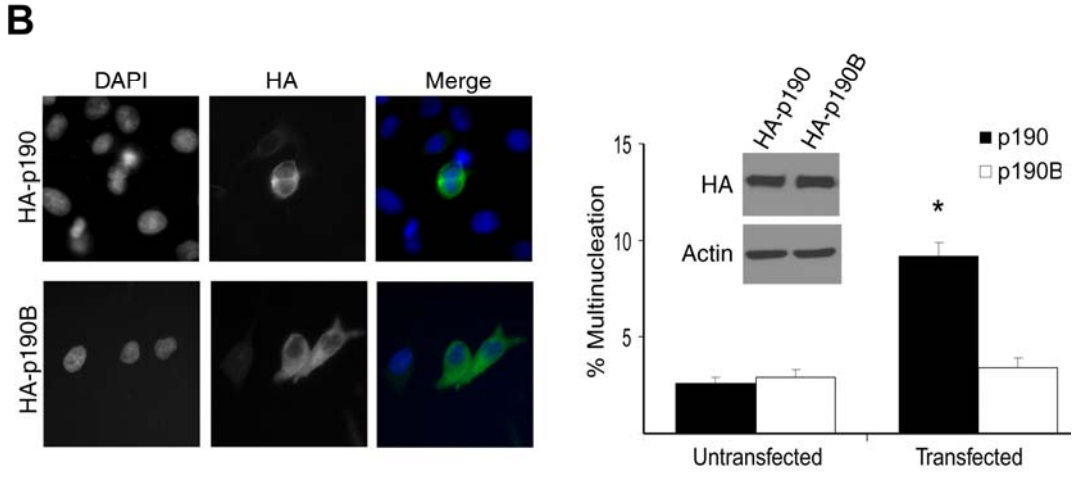

B
DAPI
HA
Merge
HA-p190
HA-p190B
HA-p190
HA-p190B
HA
Actin
p190
p190B
% Multinucleation
15
10
5
*
Untransfected
Transfected

Figure 10. P190, and not its B isoform, localizes to the cleavage furrow during cytokinesis and when overexpressed, causes multinucleation

A) Comparison of p190A and B localization to the cleavage furrow. Endogenous p190A and B proteins were detected in asynchronous cell populations by immmunofluorescence staining and analyzed by confocal microscopy. Graph shows the quantification of the observed phenotype as a percentage of the total number of mitotic cells counted ($n \geq 30$).

B) Overexpression of p190B does not induce multinucleation. HeLa cells were transfected with 2μg of HA-tagged p190A or B plasmids and incubated for 48 hrs. After incubation, cells were analyzed by fluorescence microscopy in a multinucleation assay, as described in Material and Methods (Chapter 2). One half of the samples were prepared for the multinucleation assay and the other half for WB analysis to confirm expression of transfected constructs. The left panel shows a typical fluorescence micrograph from a multinucleation assay. Quantification of multiple experiments is expressed as the ratio of % MN vs. control $\pm$ SEM ($N \geq 100$). * represents $p<0.02$ comparing p190 overexpressing cells to nontransfected control cells. Insert: 100μg of whole cell lysate was analyzed by Western blotting with HA or actin antibodies.

status was assessed by immunofluorescence microscopy. Whereas p190 overexpression elicited the expected multinucleation phenotype, cells transfected with p190B cDNA had no significant increase in multinucleation as compared to non-transfected controls (Figure10B). These data suggest the role of p190 in cytokinesis may be an event specific to this homologue and that p190B may have a separate, and at present undefined, role during cytokinesis.

A decrease in p190 levels during mitosis is required for successful completion of cytokinesis.

We previously reported that p190 protein levels transiently decrease in late mitosis, an event mediated by ubiquitination and proteasomal degradation (Su et al., 2003). Those results raised the question of whether the cell cycle-linked downregulation of p190 is an event required for successful completion of cytokinesis.

To test this hypothesis, we used an RNA interference (RNAi) reconstitution approach, whereby endogenous p190 protein was silenced by transfection of a p190-specific siRNA and then rescued by the expression of an N-terminal deletion mutant (ΔGBD). We previously identified this mutant as resistant to mitotic degradation (Su et al., 2003). Furthermore, the ΔGBD mutant also lacked the sequences targeted by the siRNA (Figure 11A). We utilized MDA-MB-468 cells stably transfected with a tetracycline inducible (tet-on) construct that, in response to regulated doxycycline treatment, expressed either ΔGBD p190 or wt p190 at levels similar to the endogenous p190 protein (Figure 11B and 11E). Western blotting analysis (Figure 11B) showed that

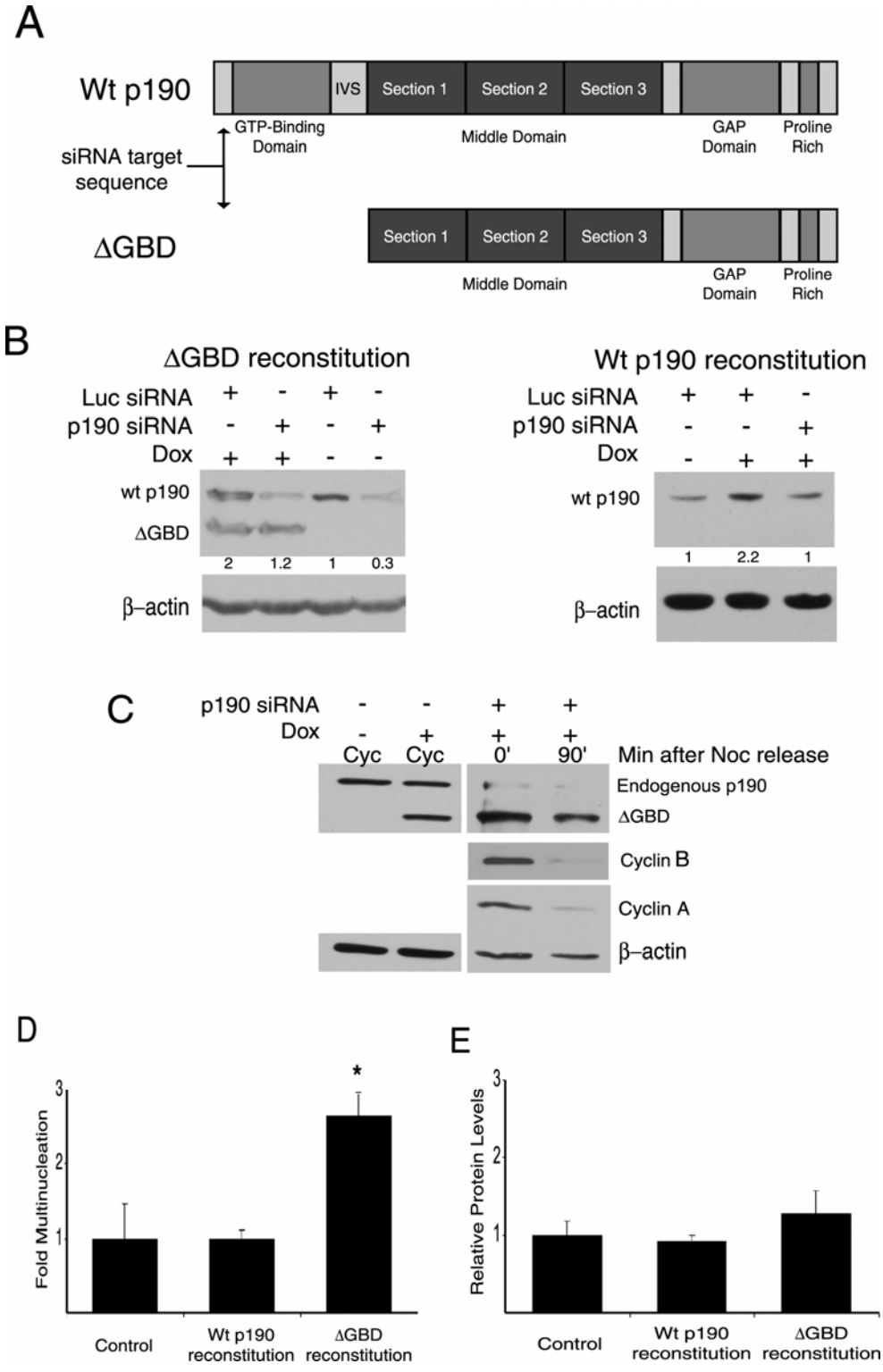

A
Wt p190
IVS
Section 1
Section 2
Section 3
GTP-Binding Domain
Middle Domain
GAP Domain
Proline Rich
siRNA target sequence
ΔGBD
Section 1
Section 2
Section 3
Middle Domain
GAP Domain
Proline Rich
B
ΔGBD reconstitution
Luc siRNA + - + -
p190 siRNA - + - +
Dox + + - -
wt p190
ΔGBD
2 1.2 1 0.3
β–actin
Wt p190 reconstitution
Luc siRNA + + -
p190 siRNA - - +
Dox - + +
wt p190
1 2.2 1
β–actin
C
p190 siRNA - - + +
Dox - + + +
Cyc Cyc 0' 90'
Min after Noc release
Endogenous p190
ΔGBD
Cyclin B
Cyclin A
β–actin
D
Fold Multinucleation
3
2
1
*
Control
Wt p190 reconstitution
ΔGBD reconstitution
E
Relative Protein Levels
3
2
1
Control
Wt p190 reconstitution
ΔGBD reconstitution

Figure 11. A decrease in p190 levels during mitosis is required for successful completion of cytokinesis in Dox-inducible MDA-MB-468 cells

A) Experimental constructs for the RNAi reconstitution.

B) Representative Western blot of a reconstitution experiment. Dox-inducible expression of ΔGBD and wt p190 in MDA-MB-468 cells is shown after 48hr siRNA and 24 hr doxycycline treatments. 100μg of whole cell lysate from each sample was analyzed by SDS-PAGE and immunoblotted as indicated. Fold expression of total p190 protein (full length and ΔGBD) is indicated between the gel images.

C) The ΔGBD mutant is expressed in mitosis. A reconstitution experiment was performed and Cyclin A, B1 expression monitored as a marker for mitotic progression.

D) Persistent expression of p190 in mitosis increases the frequency of cytokinesis failure and multinucleation. RNAi reconstitution, followed by multinucleation assays, was performed as described in Experimental Procedures. Quantification of multiple experiments is expressed as the ratio of % MN vs. control $\pm$ SEM (N=5). * represents $p<0.02$ comparing ΔGBD reconstitution to control or wt p190 reconstitution.

E) Relative expression levels of wt or ΔGBD p190. Quantification is based on densitometric analysis of Western blots from numerous reconstitution experiments and is expressed as the fold multinucleation vs. control $\pm$ SEM (N=5), control values = 1.

endogenous p190 protein levels in p190 siRNA- treated cells were reduced to approximately 30% of control siRNA-treated cells and that upon treatment with doxycycline, ΔGBD protein was expressed at levels similar to those of the endogenous p190 protein. Induction of the wt p190 protein in MDA-MB-468 cells after p190 siRNA treatment resulted in overall sustained levels of p190 that were similar to the controls. Figure 11C shows that the ΔGBD mutant was expressed and protected from cellular degradation and RNAi in mitosis, when Cyclin A and B1 levels, used as a marker for mitotic progression, were reduced.

After siRNA reconstitution, cells were analyzed by immunofluorescence (IF) microscopy for extent of multinucleation. Figure 11D demonstrates that cells reconstituted with the non-degradable ΔGBD mutant displayed a 2.5-fold increase in multinucleation over control cells (Luc siRNA, no Dox) and cells reconstituted with wt p190. When total p190 protein levels were compared among the various treatment groups, expression levels detected in both wt p190 and ΔGBD reconstituted cells were similar to that of control cells and showed no statistically significant difference (Figure 11E).

HeLa cells were also employed to test the requirement for a decrease in p190 levels for successful cytokinesis (Figure 12). siRNA-mediated silencing of p190 was followed by transient introduction of the ΔGBD construct and assessment of multinucleation. Silencing approached 90% (Panel A) and the amount of ΔGBD plasmid transfected was titrated to yield levels of ΔGBD expression equal to endogenous p190 (Figure 12B and C). Figure 12D shows that the number of multinucleated cells among

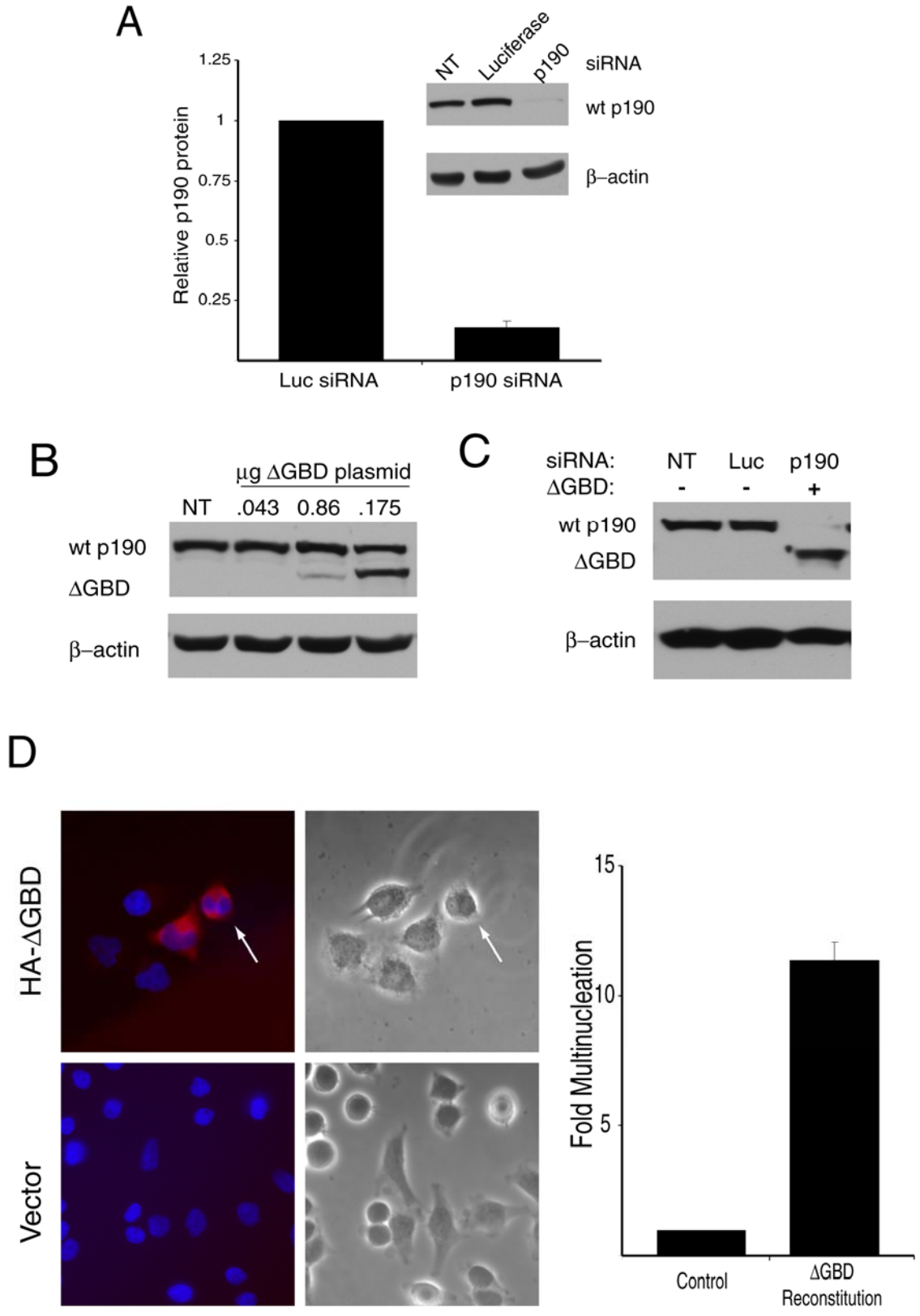

A
1.25
1
0.75
0.5
0.25
Relative p190 protein
Luc siRNA
p190 siRNA
NT
Luciferase
p190
siRNA
wt p190
β–actin
B
μg ΔGBD plasmid
NT
.043
0.86
.175
wt p190
ΔGBD
β–actin
C
siRNA:
ΔGBD:
NT
Luc
p190
-
-
+
wt p190
ΔGBD
β–actin
D
HA-ΔGBD
Vector
15
10
5
Fold Multinucleation
Control
ΔGBD
Reconstitution

Figure 12. A decrease in p190 levels during mitosis is required for successful completion of cytokinesis in transiently transfected HeLa cells

A) p190siRNA silences p190 expression in HeLa cells. Quantification of multiple experiments (N=3) is represented in the graph. Insert: 100μg of whole cell lysate was analyzed by Western blotting after HeLa cells were treated with 60 pmol of p190 siRNA for 48 hrs.

B) Titration of ΔGBD expression in HeLa cells. HeLa cells were transfected with increasing amounts of the ΔGBD plasmid, as indicated, for 48hrs before harvesting. 100μg of whole cell lysate was analyzed by SDS-PAGE and then immunoblotted as indicated.

C) RNAi reconstitution after transient transfection. Representative experiment combining approaches described in A and B.

D) Persistent expression of the ΔGBD p190 mutant in HeLa cells results in increased multinucleation. Multinucleation assays were performed after cells were treated as described in A and B. Arrows indicate multinucleated cells. Control cells displayed the multinucleation phenotype in approximately 3% of the population. Quantification of multiple experiments is expressed as the fold multinucleation vs. control $\pm$ SEM (N=4), control values = 1.

ΔGBD transfectants was ~11-fold higher than vector control, an increase from ~3% to over 33% in a 48 hr period. Taken together, these data indicate that failure to down-regulate p190 in mitosis leads to an increase in cytokinesis defects, supporting the conclusion that degradation of p190 is required for successful cell division.

The GAP activity of p190 is necessary and sufficient to induce the multinucleation phenotype.

To better understand the mechanism by which improperly sustained p190 levels in mitosis resulted in multinucleation, we tested whether the GAP domain alone and its Rho-directed activity were necessary and sufficient for the effect. To this end, HeLa cells were transiently transfected with a panel of HA-tagged p190 mutants (depicted in Figure 13A) and assessed for multinucleation (Figures 13A, 13C). Relative levels of expression were determined by Western blotting with α-HA antibodies (Figure 13 B). Figure 13C shows that upon transfection of wt p190, approximately 10% of expressing cells became multinucleated. Similar levels of mitotic failure were noted in cells expressing the ΔGBD deletion mutant, and control levels were observed in ΔGAP expressing cells, as previously reported (Su et al., 2003). The isolated GAP domain induced approximately 16% multinucleation, eight times greater than induced by vector alone, confirming that the GAP domain of p190 was sufficient for disruption of cytokinesis. To further examine the requirement for GAP activity in this event, two additional mutants were tested, Y1105F and R1283A. Both point mutants render p190 enzymatically inactive. Tyrosine 1105 is phosphorylated by Src, and this phosphorylation results in an increased z

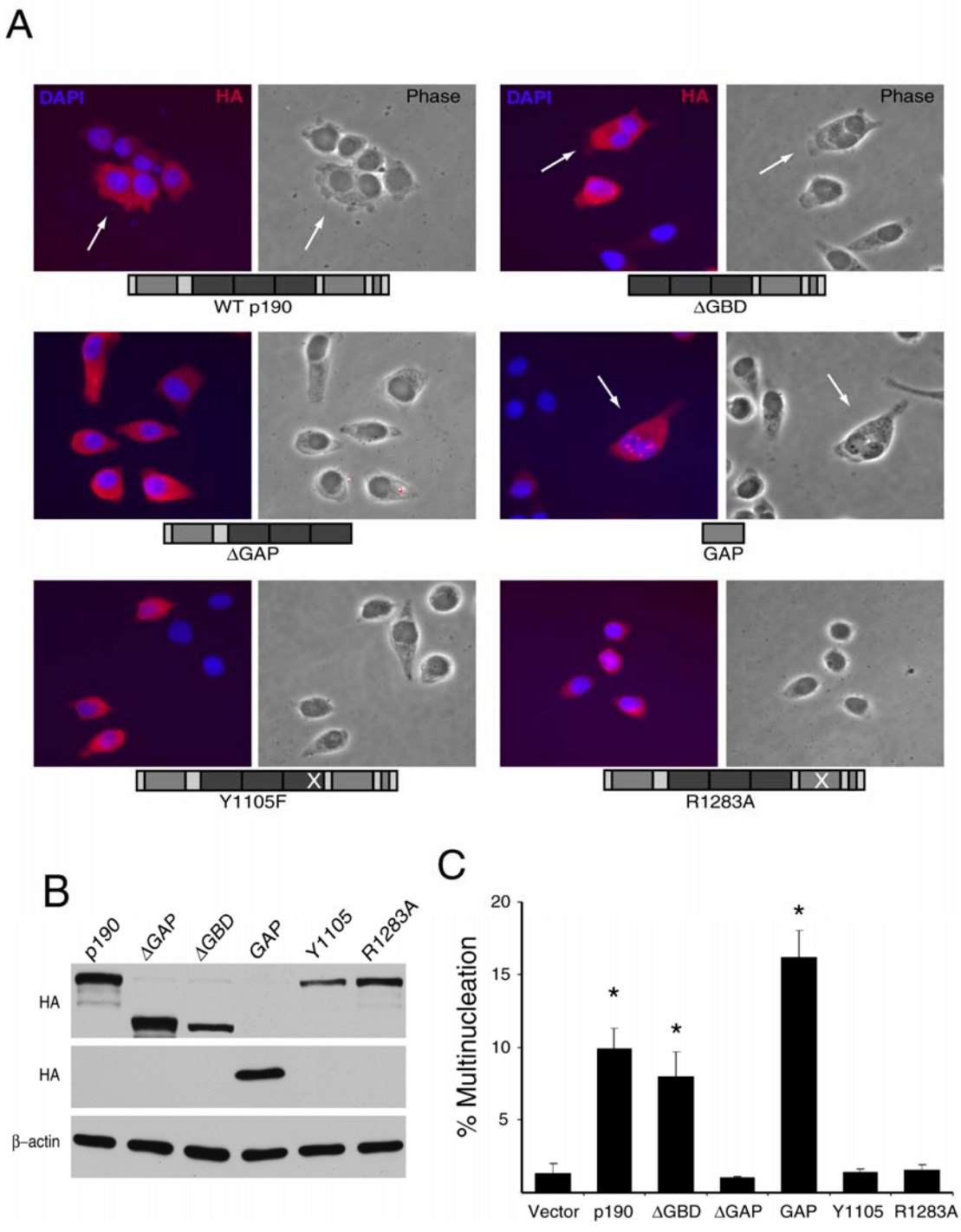

A
DAPI
HA
Phase
WT p190
ΔGBD
ΔGAP
GAP
Y1105F
R1283A
B
p190
ΔGAP
ΔGBD
GAP
Y1105
R1283A
HA
HA
β–actin
C
% Multinucleation
20
15
10
5
Vector
p190
ΔGBD
ΔGAP
GAP
Y1105
R1283A

Figure 13. The GAP activity of p190 is necessary and sufficient to induce the multinucleation phenotype

A) Immunofluorescence microscopy of HeLa cells transfected with the indicated constructs of p190. HeLa cells were transfected with 2μg each of the different p190 constructs depicted. Cells were prepared for immunofluorescence microscopy 48 hrs post-transfection. Arrows indicate multinucleated cells. Representative images are shown.

B) Western blot analysis of the expression of p190 mutants. 50μg whole cell lysate of transfected cells was analyzed by SDS-PAGE and immunoblotting as indicated.

C) Multinucleation analysis of transfected cells shown in Panel A. Quantification of multiple experiments was analysed by ANOVA, and statistical significance was determined by the Dunnett's test. Values are expressed as the mean % MN cells $\pm$ SEM (N=4). * represents $p < 0.02$ comparing transfected mutants to vector.

indicate that the GAP activity of p190 is not only sufficient but also required for the multinucleation phenotype observed in p190 overexpressing cells and that c-Src-mediated phosphorylation of the protein is also required for this event.

The N-terminal GBDS1 region of p190RhoGAP is sufficient for p190 ubiquitination.

To determine the domains of p190 required for its mitotic ubiquitination, a panel of deletion mutants was prepared and tested for their ability to be ubiquitinated in an *in vitro* ubiquitination assay (Figure 14A). Figures 14B and C show that full length wt p190 protein was ubiquitinated 5-fold above control. Only two of the deletion mutants tested, ΔGAP and GBDS1, showed ubiquitination levels similar to full length p190. The high levels of ubiquitination observed with the ΔGAP mutant suggested that the C-terminus of p190 is dispensable for its ubiquitination. Similarly, high ubiquitination levels of the GBDS1 mutant suggest that most p190 ubiquitination occurs at the N-terminus of the protein, indicating that sequences within the GBD domain and Section 1 (S1) of the Middle Domain are sufficient for p190 ubiquitination and may be important in mediating p190's mitotic degradation.

When the different sub-domains found in the ΔGAP and GBDS1 mutants were separately tested for in vitro ubiquitination, a significant overall decrease in ubiquitination was observed. For example, testing of the isolated GBD domain yielded levels similar to those of control reactions, while the isolated S1 domain was modified more readily than control reactions but to a significantly lesser extent than the full length protein. Other N-terminal deletion mutants, ΔGBD, IVS/S1, and S2S3GAP, exhibited

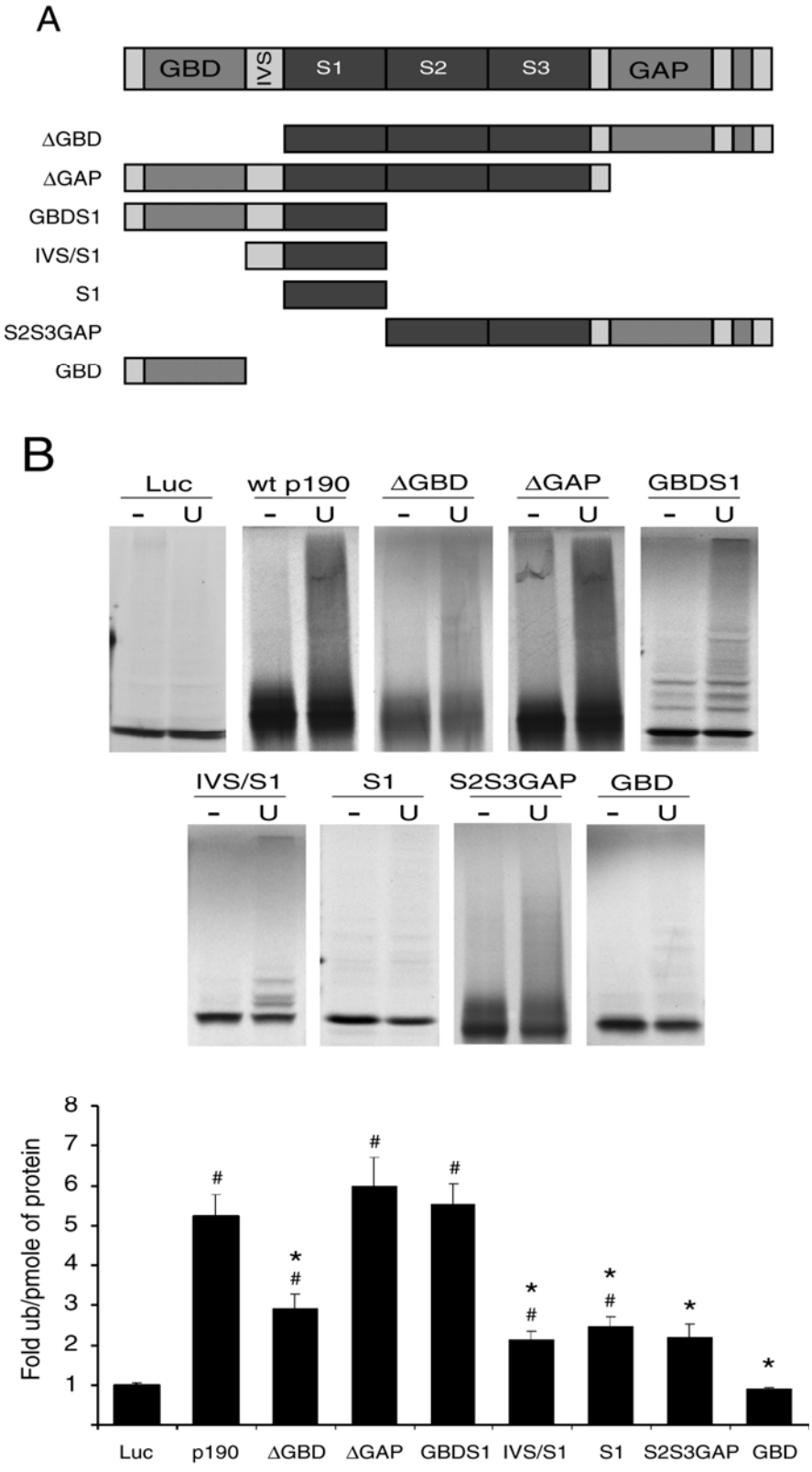
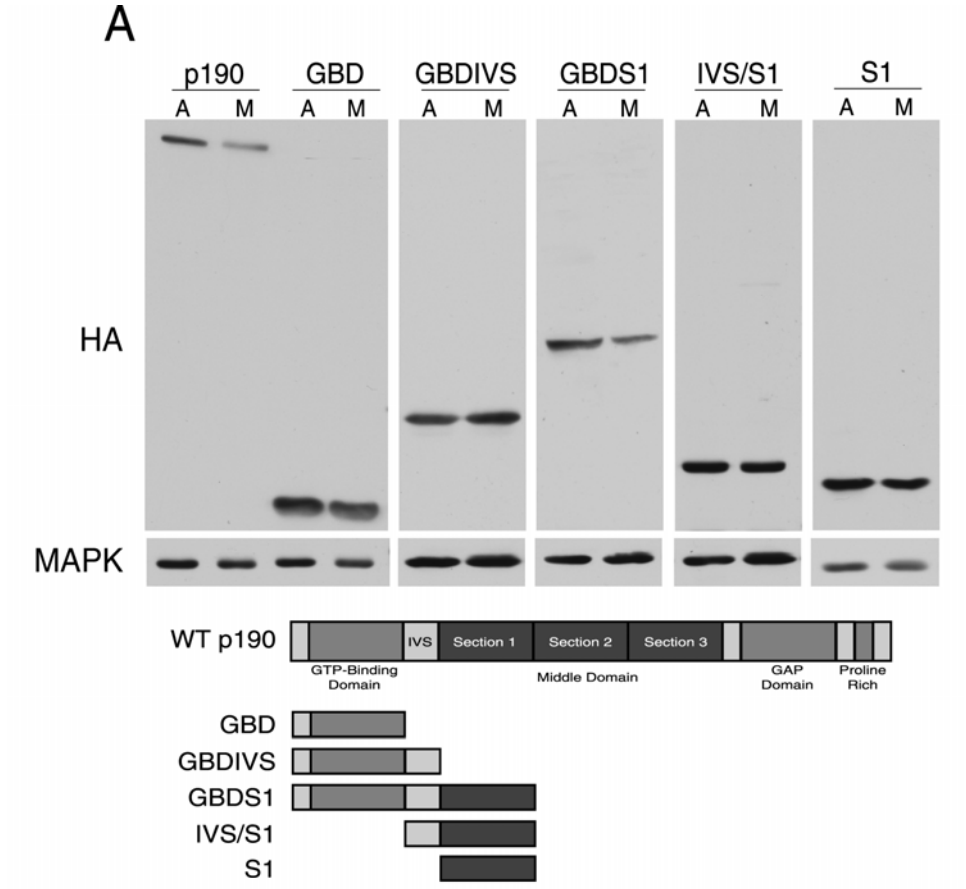

A
GBD
IVS
S1
S2
S3
GAP
ΔGBD
ΔGAP
GBDS1
IVS/S1
S1
S2S3GAP
GBD
B
Luc
wt p190
ΔGBD
ΔGAP
GBDS1
IVS/S1
S1
S2S3GAP
GBD
- U
C
Fold ub/pmole of protein
Luc
p190
ΔGBD
ΔGAP
GBDS1
IVS/S1
S1
S2S3GAP
GBD

Figure 14. The N-terminal GBDS1 region of p190RhoGAP is sufficient and necessary for its ubiquitination

(A) p190 plasmid constructs used in the *in vitro* ubiquitination assay. IVS: Intervening sequence. (B) *In vitro* ubiquitination of p190 deletion mutants. Plasmids were transcribed, translated and [^{35}S]-labeled *in vitro*, then, subjected to *in vitro* ubiquitination as described in Experimental Procedures. A luciferase (Luc) construct was used as a negative control for the ubiquitination reactions. (–) lanes represent reactions performed in the absence of exogenous ubiquitin. (U) lanes represent reactions performed in the presence of exogenous ubiquitin. (C) Quantitation of ubiquitination assays. ANOVA analysis and the Dunnet's test were employed to determine significant variances of fold ubiquitination per pmoles p190 mutants and Luc control. Values are expressed as the mean ± SEM (N= 3-5). # represents $p<0.02$, comparing Luc to p190 mutant; ∗ represents $p<0.02$, comparing mutant to wt p190. Data for this figure was contributed by Dr. Joyce Agati Miller


A
p190
GBD
GBDIVS
GBDS1
IVS/S1
S1
A M A M A M A M A M A M
HA
MAPK
WT p190
IVS
Section 1
Section 2
Section 3
GTP-Binding Domain
Middle Domain
GAP Domain
Proline Rich
GBD
GBDIVS
GBDS1
IVS/S1
S1

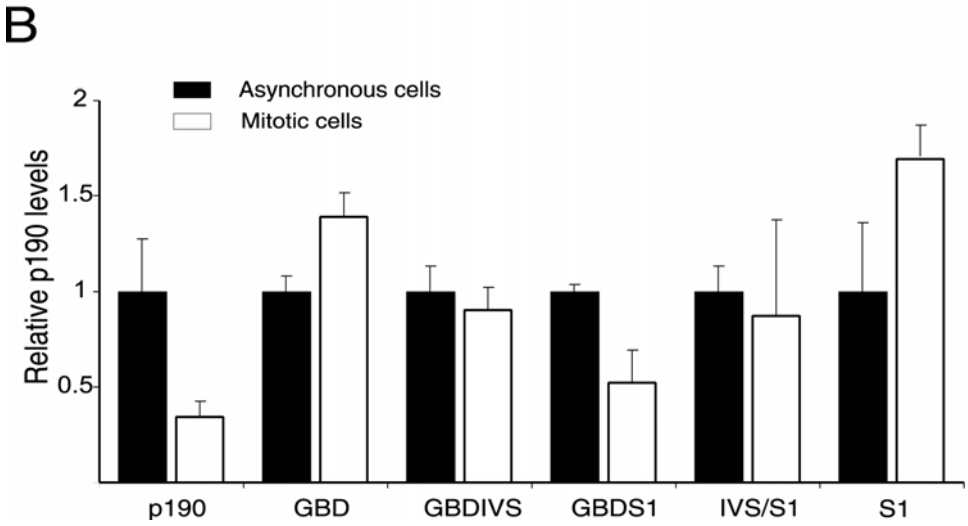

B
Asynchronous cells
Mitotic cells
Relative p190 levels
2
1.5
1
0.5
p190
GBD
GBDIVS
GBDS1
IVS/S1
S1

Figure 15. The GBDS1 region is necessary and sufficient for mitotic degradation of p190

A) Degradation of N-terminal p190 fragments in HeLa cells. HeLa cells were transiently transfected with various p190 expression plasmids, as indicated. At 24 hrs post-transfection, cells were either maintained as an asynchronous population (A) or synchronized with nocodazole (M) and subsequently released for 40 min. 100μg whole cell lysate was subjected to SDS-PAGE followed by Western blotting with indicated antibodies.

B) Quantification of p190 protein levels in asynchronous and mitotic cell populations. Levels of HA-p190 were quantified by densitometric analysis of Western blots. The ratio of p190 to MAPK for the "Asynchronous" lanes of each mutant group was arbitrarily set to 1, and the corresponding "Mitotic" lanes (also corrected for amount of MAPK) were further normalized to that value. Values represent the normalized mean $\pm$SEM (N=3-5). *$p<0.02$ relative to asynchronous cells expressing the corresponding p190 fragment. Data for this figure was contributed by Dr. Joyce Agati Miller

levels of ubiquitination that were comparable to those of S1 alone. Together, these data indicate that the combined GBDS1 domains at the N-terminus of p190 are sufficient and necessary for directing ubiquitination *in vitro*, although other regions can be poorly ubiquitinated in its absence.

The GBDS1 region is necessary and sufficient for mitotic p190RhoGAP degradation.

Because the N-terminus of p190 was necessary and sufficient for ubiquitination (Figure 14), we next examined whether the GBD and S1 domains acted in a cooperative fashion to regulate the mitotic degradation of p190 and whether they were necessary and/or sufficient for degradation. Therefore, we tested smaller N-terminal deletion mutants to determine the minimal region of p190 that supported degradation. Figure 15 shows that neither the GBD nor S1 alone were sufficient for degradation. However, the combined GBD and S1 domains (GBDS1) were degraded, indicating that this region is both sufficient and necessary for mitotic degradation of p190.

N-terminal lysine residues are required for p190 degradation.

Because p190 degradation is a necessary mitotic event, we attempted to further define a small region within the molecule or a set of amino acid residues involved in regulating its mitotic stability. To do so, the N-terminal GBD domain was deleted by a nested set of one or two 83 amino acid lengths (leaving two-thirds or one-third of the GBD in the context of the wt p190) and these constructs, designated Δ83 and Δ160, were tested in degradation assays. Figure 18A shows that both mutants were protected from

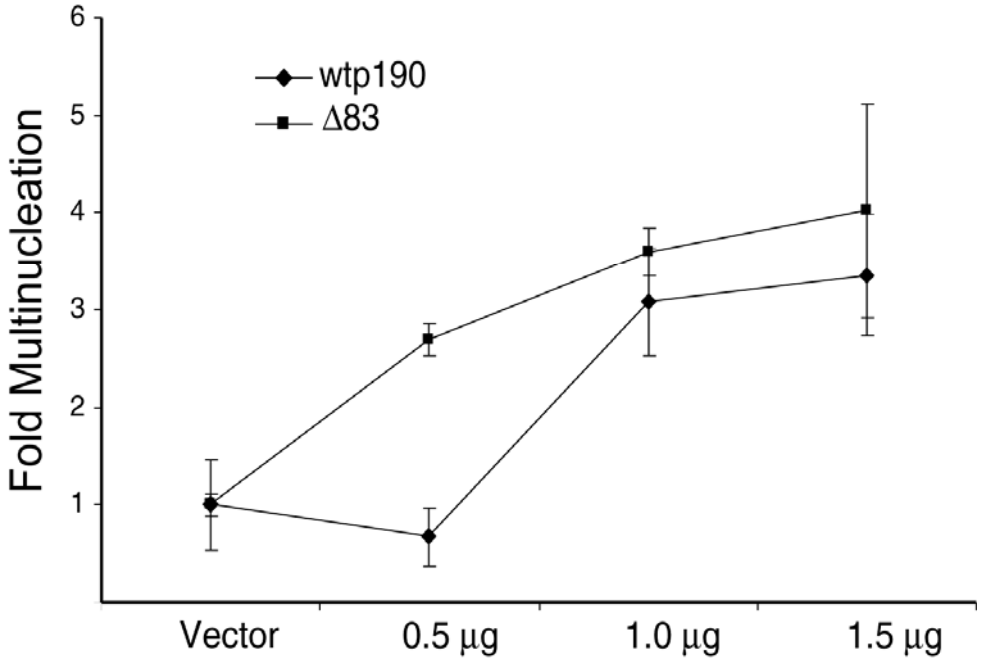

Fold Multinucleation
6
5
4
3
2
1
wtp190
Δ83
Vector
0.5 µg
1.0 µg
1.5 µg

Figure 16. Overexpression of the Δ83 p190 mutant increases multinucleation levels

HeLa cells were transfected with increasing amounts of either wt p190 or the Δ83 mutant and incubated for 24 hrs. Samples were then prepared for immunofluorescence microscopy and visualization of multinucleation (MN) was performed as described in Experimental Procedures. Quantification of multiple experiments is expressed as the ratio of % MN vs. control $\pm$ SEM (N=5), where control values = 1. Data for this figure was contributed by Dr. Ling Su.

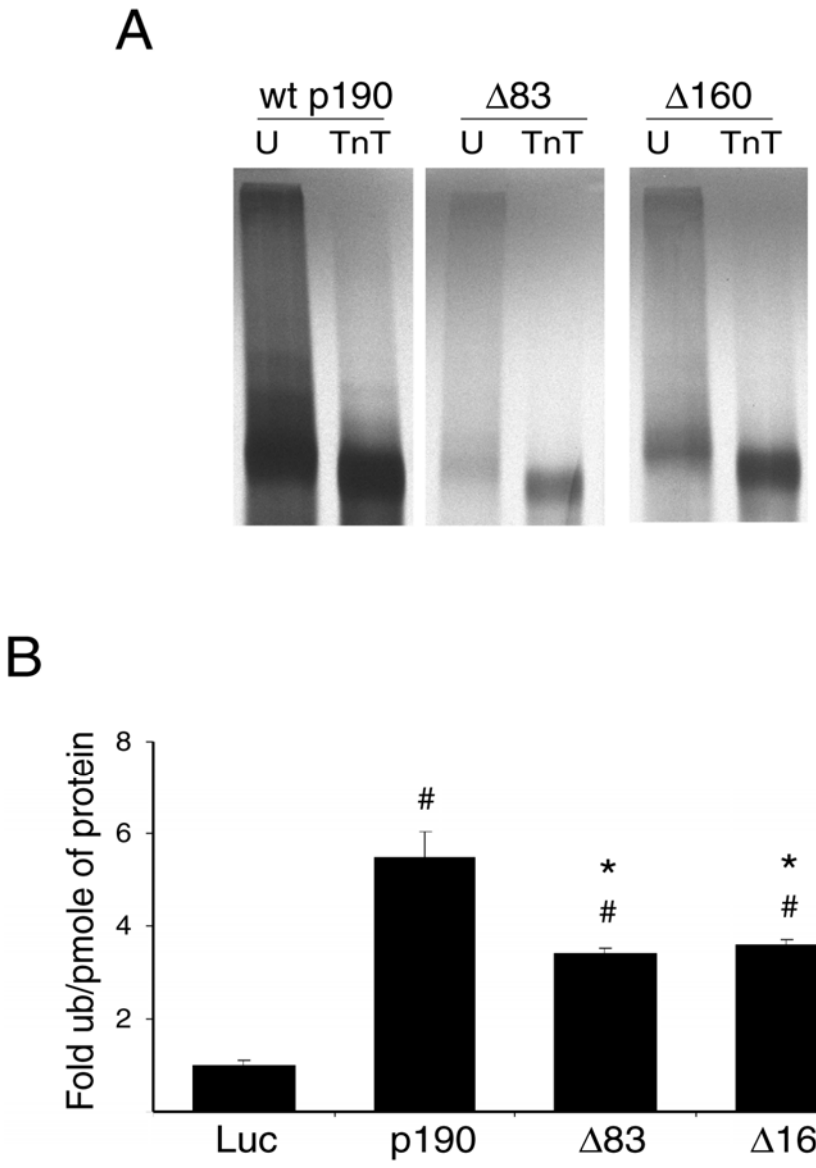

A
wt p190
Δ83
Δ160
U
TnT
U
TnT
U
TnT
B
Fold ub/pmole of protein
8
6
4
2
#
*
#
*
#
Luc
p190
Δ83
Δ160

Figure 17. Ubiquitination of wt p190 and the Δ83, Δ160 N-terminal mutants

A) Wild-type p190, Δ83 p190, and Δ160 p190 were subjected to *in vitro* ubiquitination assays. The assays and their quantification were performed as described in Experimental Procedures. The acronym TnT stands for *in vitro* transcribed and translated protein product.

B) Quantification of multiple experiments is expressed as the mean $\pm$ SEM (N=3-5), where the luciferase control value was arbitrarily set at 1. # represents $p<0.02$ comparing Luc to p190 constructs; * represents $p<0.02$ comparing wt p190 to Δ83 and Δ160 mutants. Data for this figure was contributed by Dr. Joyce Agati Miller

degradation, as no significant changes in protein levels were observed, indicating that residues within the first 83 amino acids of p190 are critical for regulating its mitotic stability. Interestingly, multinucleation assays after introducing increasing amounts of either wt or Δ83 p190 by transient transfection revealed that at lower concentrations, the non-degradable form of p190 (Δ83) was more than twice as efficient in causing the multinucleation phenotype (Figure 16). Yet, at greater concentrations, such differences disappear. Furthermore, ubiquitination assays comparing wt p190 to both Δ83 and Δ160 showed decreased ubiquitination levels for both mutants (Figure 17) suggesting that regions or specific amino acids important to the ubiquitination and mitotic degradation of p190 were, in fact, to be found within the first 83 amino acids of the protein.

After examination of the amino acid sequence of the N-terminal GBDS1 region, the minimal fragment of p190 that supports both mitotic ubiquitination and degradation, we noticed that although nearly 60 lysines are found in this fragment, only four are located within the first 83 amino acids (Figure 18B). We hypothesized that these lysines, K6, K26, K28, and K35, had the potential to be involved in the ubiquitin-proteasome pathway-dependent degradation of the GBDS1. We therefore, tested whether point mutants, lysine to arginine substitutions, of these N-terminal residues would have an effect on mitotic GBDS1 stability. The resulting mutant with all four substitutions, designated GBDS1 4KR, was then tested in degradation assays. Although the degradation of endogenous p190 protein, shown as a control, continues to be observed in the mitotic cell samples, the GBDS1 4KR mutant is spared from mitotic degradation (Figure 18B).

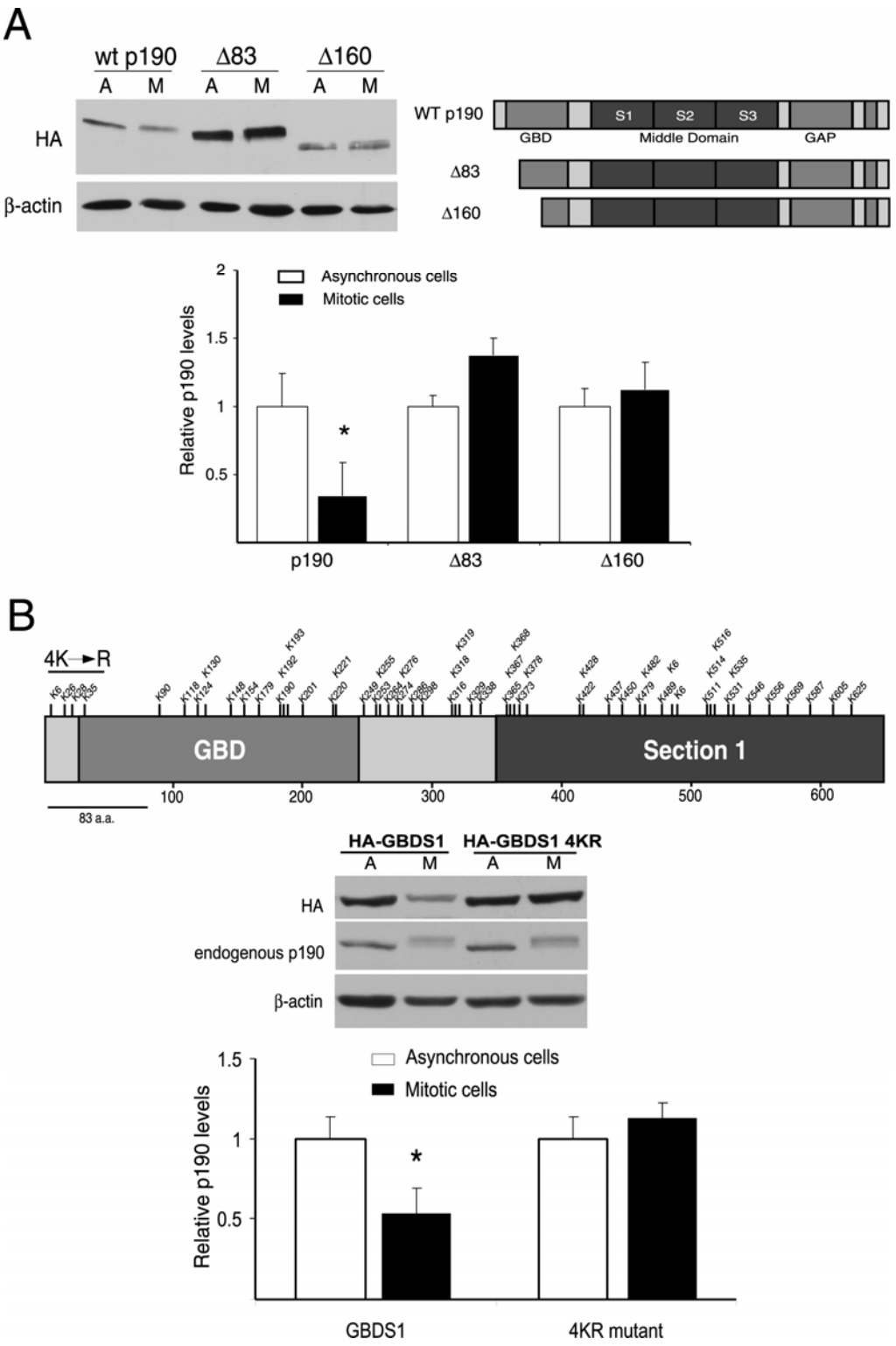
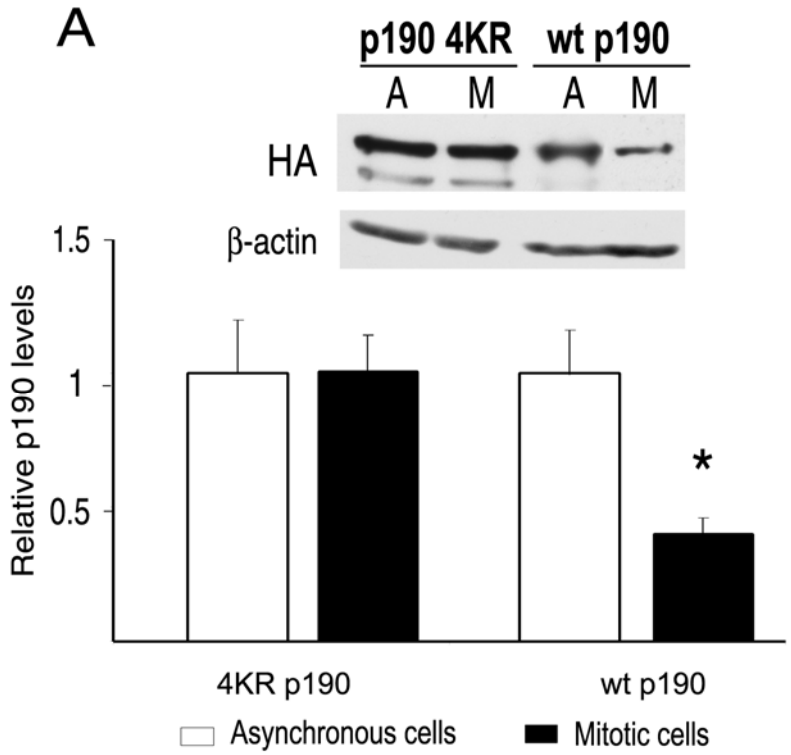

A
wt p190
Δ83
Δ160
A M A M A M
HA
β-actin
WT p190
GBD
S1
S2
S3
Middle Domain
GAP
Δ83
Δ160
Asynchronous cells
Mitotic cells
Relative p190 levels
2
1.5
1
0.5
*
p190
Δ83
Δ160
B
4K→R
GBD
Section 1
100
200
300
400
500
600
83 a.a.
HA-GBDS1
HA-GBDS1 4KR
A M A M
HA
endogenous p190
β-actin
Asynchronous cells
Mitotic cells
Relative p190 levels
1.5
1
0.5
*
GBDS1
4KR mutant

Figure 18. N-terminal lysine residues direct the degradation of p190 in HeLa cells

A) N-terminal deletion mutants of p190 (Δ83 and Δ160) are insensitive to mitotic degradation. HeLa cells were transiently transfected with the indicated p190 expression plasmids and subjected to degradation assays, SDS-PAGE analysis and Western blotting. HA-tagged p190 variants were immunoblotted with the HA.11 antibody. A representative gel image is shown. Levels of Δ83 and Δ160 were quantified by densitometric analysis of Western blots. The ratio of mutant to β-actin for the "Asynchronous" lanes of each mutant group was arbitrarily set to 1, and the corresponding "Mitotic" lanes (also corrected for amount of β-actin) were normalized to that value. Values represent the normalized mean $\pm$SEM (N=3-5). *$p<0.02$ relative to asynchronous cells expressing the corresponding N-terminal mutant.

B) The GBDS1 4KR mutant is insensitive to mitotic degradation. Diagram of the rationale for the generation of the GBDS1 4KR mutant. HeLa cells were transiently transfected with the indicated GBDS1 expression plasmids and subjected to a degradation assay and analysis as described in panel A. A representative gel image is shown. Quantification represents the normalized mean $\pm$SEM (N=3). *$p<0.02$ relative to asynchronous cells expressing the corresponding N-terminal mutant.


A
p190 4KR
wt p190
A
M
A
M
HA
β-actin
1.5
1
0.5
Relative p190 levels
*
4KR p190
wt p190
Asynchronous cells
Mitotic cells

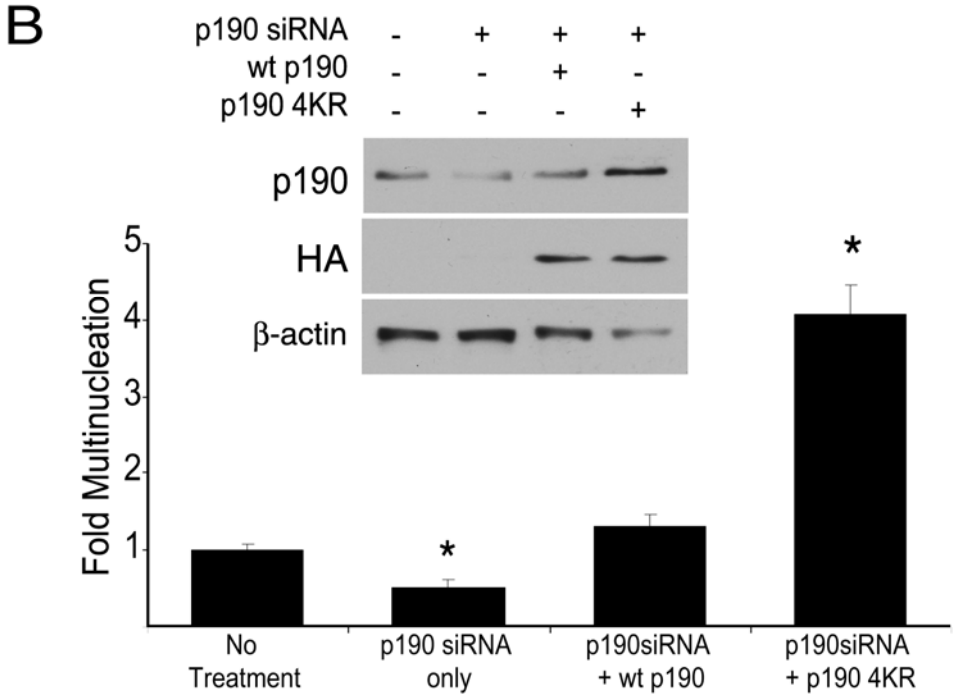

B
p190 siRNA - + + +
wt p190 - - + -
p190 4KR - - - +
p190
HA
β-actin
5
4
3
2
1
Fold Multinucleation
*
*
No Treatment
p190 siRNA only
p190siRNA + wt p190
p190siRNA + p190 4KR

Figure 19. N-terminal lysine residues direct the degradation of p190 in HeLa cells A) The full length p190 4KR mutant is insensitive to mitotic degradation. HeLa cells were transiently transfected with the indicated pkH3 p190 expression plasmids and subjected to degradation assays. A representative gel image is shown. Quantification represents the normalized mean $\pm$SEM (N=3-5). *$p<0.02$ relative to asynchronous cells expressing the corresponding N-terminal mutant. B) Persistent expression of the p190 4KR mutant in HeLa cells results in increased multinucleation. Multinucleation assays were performed after RNAi reconstitution, where cells were treated with human-specific p190 siRNA for 24 hrs, transfected with either wt p190 or p190 4KR rat cDNA and allowed to incubate for 24 more hours. Samples were then prepared for immunofluorescence microscopy. A representative gel image of knock-down and reconstitution with mutant and wild-type p190 levels is shown as an insert. Quantification of multiple experiments is expressed as the fold multinucleation vs. Mock treated $\pm$ SEM (N=3), where Mock treatment values were set at 1. * represents $p<0.02$ when comparing to Mock or wt p190 reconstitution.

To evaluate the relevance of these four N-terminal lysines in the context of the full length molecule, a full length p190 construct containing the four lysine to arginine substitutions (p190 4KR) was generated, transfected into HeLa cells, and its mitotic stability compared against wild-type p190 protein in degradation assays. Figure 19A shows that whereas wild-type p190 is degraded in mitosis, the p190 4KR mutant is resistant to mitotic degradation, confirming the relevance of these four N-terminal residues to the mitotic degradation of p190. Finally, to test whether the degradation-resistant p190 4KR mutant would yield an increase in the multinucleation phenotype similar to the one caused by the degradation-resistant ΔGBD mutant used in earlier assays (Figures 11 and 12), a siRNA reconstitution, using p190 4KR, followed by a multinucleation assay were performed. The siRNA oligo used was specific to endogenous human p190, thus allowing the introduction and expression of rat p190 constructs (wild-type p190 and p190 4KR) in siRNA treated Hela cells. Results confirmed our previous findings showing that, after silencing of the endogenous p190 pool, expression of the degradation-resistant p190 4KR mutant resulted in a four-fold increase in observed multinucleation whereas expression of the wild-type p190 caused no significant difference in multinucleation, as compared to the Luciferase siRNA control sample (Figure 19B) further establishing the relevance of these four lysine residues in the regulation of mitotic p190 degradation.

DISCUSSION

Mitotic progression is inextricably dependent on the proteasomal degradation of

important regulatory molecules such as A- and B-type cyclins, Securin and Plk1, among others (Peters, 2002). Furthermore, progression and completion of cytokinesis is also largely dependent on RhoA activity, which regulates the positioning as well as the establishment and contraction of the actomyosin ring at the cleavage furrow (Balasubramanian et al., 2004). Work in our laboratory provides a link between these two mitotic regulatory mechanisms. We previously reported that the expression levels of p190RhoGAP, a negative regulator of RhoA signaling, are cell cycle-regulated and strikingly decreased in late mitosis (Su et al., 2003). Results presented in this chapter, reveal that regulation of cytokinesis is clearly associated with p190 and not its closely related homologue, p190B. Results further demonstrate that a decrease in p190 levels during mitosis is required for completion of cytokinesis, cell abscission, and avoidance of mitotic catastrophe. The timing of p190 degradation correlates with the detection of active Rho in the cleavage furrow (Yoshizaki et al., 2004;Su et al., 2009) suggesting that a key event that must occur in late mitosis is a decrease in RhoGAP activity. We confirm that the unregulated GAP activity of p190 is responsible for the observed multinucleation phenotype and report that four N-terminal lysine residues are required for the mitotic degradation of p190. Together, these findings suggest that p190 is a critical regulator of cytokinesis.

Existing models ascertain the critical role that RhoA plays in regulating events in mitosis and cytokinesis. Several reports indicate that RhoA activity increases in late mitosis (Maddox and Burridge, 2003;Yoshizaki et al., 2004), and it is widely accepted that this increased activation is necessary for successful completion of cytokinesis. How

are increased RhoGTP levels attained in cytokinesis? Numerous studies have revealed the important role that the RhoGEF Ect2 plays in this activation event (Kimura et al., 2000a;Yuce et al., 2005;Nishimura and Yonemura, 2006;Chalamalasetty et al., 2006). However, a recent report (Mikawa et al., 2008) has shown that Ect2 and p190 have counteracting roles in regulating cytokinesis and demonstrates not only that they associate in mitosis, but also colocalize at the cleavage furrow, where RhoA is found (Nishimura et al., 1998b). The present study describes an additional molecular event that must occur to achieve the increased RhoGTP levels needed for cytokinesis. Here we show that a decrease in p190 levels during mitosis is required to avoid cytokinesis failure and multinucleation. We demonstrate that when cells are unable to lower mitotic levels of p190 protein, as is the case with the non-degradable ΔGBD, Δ83 and 4KR mutants (Figs. 11, 12, 16 & 19B, respectively), cytokinesis failure increases significantly. This is true not only in overexpression settings but also when the non-degradable protein is expressed at what appears to be similar levels as the endogenous p190 protein, as is the case for the ΔGBD and 4KR mutants. Based on these results, we envision a model where the activities of p190 and Ect2 negatively and positively, respectively, regulate Rho activity levels until late in mitosis when increased RhoGTP is required for directing actomyosin ring positioning, establishment, and contraction. At that time, p190, the negative regulator of RhoA, is degraded and its levels decreased sufficiently to allow full activation of RhoA to occur.

Current models of contractile ring formation posit that its organization is regulated by “RhoGTP activity zones” (Piekny et al., 2005;Bement et al., 2006a;Bement

et al., 2005). These Rho zones are dynamic due to the cyclical nature of Rho activation and spatially confined to a narrow region of the plasma membrane which serves as a signal for recruitment/organization of a contractile ring (Miller et al., 2008). In a recent study (Su et al., 2009), our group showed that p190 participates in the establishment and stability of such Rho zones. Elevated levels of p190 during cytokinesis were shown to result in multiple cycles of abnormal contractile ring organization and site selection. When furrows were able to form, they were unstable, giving way to cytokinesis failure. Moreover, through FRET analysis, it was determined that the Rho activity zone in these cells is reduced significantly, giving rise to the aforementioned phenotypes. Altogether, one can conclude that a decrease in endogenous p190 levels during cytokinesis is required not only to increase RhoGTP levels in general but also to properly focus and stabilize the Rho activity zone which will signal the organization and activation of the contractile ring.

Our results suggest that the mitotic role of p190 is specific to the A homologue and not to p190B. Although both function as GTPase-activating proteins (GAPs) for RhoA, share 50% sequence identity and are structurally analogous, they are reported to have differing functions in various cellular settings (Sordella et al., 2002;Sordella et al., 2003;Chakravarty et al., 2003;Heckman et al., 2007). The experimental data suggest that p190B fails to induce multinucleation when overexpressed indicating that p190B may not have a direct role in regulating cytokinesis. The dispersed cytosolic localization of endogenous p190B during mitosis, not enriched in the membrane and cleavage furrow, further supports that interpretation. It is likely that functional and localization differences

between p190 and p190B are due to the significant variance existing in the middle domain of both proteins. The numerous phosphorylation sites and protein interaction domains contained in this region are unique to each homologue. Further experimentation will be necessary to understand how each of these molecules is differentially regulated and to identify the post-translational modifications or protein-protein interactions behind such differences.

A previous study by Su et al (Su et al., 2003) showed that the C-terminus of p190 was required for mediating the multinucleation phenotype after p190 overexpression. While that analysis defined a region containing the GAP domain as important for p190-induced multinucleation, it did not define p190 RhoGAP activity as critical to the event. In addition to the GAP domain, other important protein interaction domains are also located at the C-terminus, such as the poly-proline domain and numerous phosphorylation sites (Jiang et al., 2008) that could potentially play a role in mediating the multinucleation phenotype. By using finer site-directed and deletional mutagenesis we report that it is, in fact, the Rho GAP activity which is responsible for the multinucleation phenotype (Figure 13). Mutation of Y1105 and of R1283 in p190 (the GAP activating, c-Src-dependent phosphorylation site and a GAP domain point mutant, respectively) prevent the multinucleation phenotype, while overexpression of the GAP domain alone elicits a strong phenotype, stronger than that of the full length protein (Figure 13). This result further supports the idea that the downregulation of p190 is necessary for increased RhoGTP levels and appropriate Rho zone focusing. It also suggests a role for c-Src tyrosine kinase through tyrosine 1105 phosphorylation in this

process. c-Src involvement in regulating mitosis has been documented for over two decades. The tyrosine kinase activity of c-Src increases in M phase (Chackalaparampil and Shalloway, 1988;Kuga et al., 2007), specifically during metaphase progression and cytokinesis, and this increase can be blocked by treatment with Src family kinase (SFK) inhibitors (Moasser et al., 1999;Ng et al., 2005;Kasahara et al., 2007). Thus, regulation of p190RhoGAP in mitosis may be a new mechanism of mitotic regulation by c-Src and SFKs.

Structure-function analysis of regions of the molecule regulating p190 degradation directed our interest to the GBDS1 N-terminal fragment of the protein, as it was heavily ubiquitinated *in vitro* and required for mitotic degradation (Figs 14 and 15). Results indicated that only the combined GBDS1 and not either of the individual domains (GBD or S1) were sufficient for ubiquitination and degradation. Furthermore, deletion of the first 83 amino acids of p190 resulted in protection of the fragment from mitotic degradation (Figure 18A) as well as a decrease in ubiquitination (Figure 17). Together, these results suggested that a degradation motif or sequence might exist in the first 83 amino acids, yet none were found. However, in contrast to the rest of the GBDS1 which is laden with lysine residues, only four isolated lysines are found within the first 83 residues of the p190 sequence. Alanine substitution of these lysines, in the context of both the GBDS1 fragment and the full length protein, stabilized p190 during mitosis (Fig 18B and 19A). Thus we define these residues as the minimal region of p190 regulating its degradation. From these data, we envision a mechanism where there are sequences spread between the GBD and S1 regions that are critical for mediating p190 degradation.

For example, a sequence in S1 could function as a recognition site for the ubiquitination machinery, whereas lysines in the GBD domain, potentially K6, K26, K28 and K35, could act as ubiquitin-accepting residues. It is also possible that in the native conformation of the protein, intramolecular interactions between these two regions are necessary to expose ubiquitin accepting or recognition sites elsewhere in the protein. Moreover, it is also possible that sequences along the GBD or S1 may be further modified or involved in protein-protein interactions that, in combination, are required for degradation so that individual domains are found to be stable.

Our results propose that the degradation of p190 in mitosis is a specific and carefully regulated event. In a process in which energy efficiency and conservation are paramount, the systematic degradation of p190 in late mitosis is indicative of its critical role as a negative regulator of RhoA activation and, therefore, of cytokinesis.

Chapter 4

A Preliminary Study on Mitosis-Specific Post-translational Modifications of p190RhoGAP

INTRODUCTION

As a negative regulator of Rho activity, p190 participates in a number of important cellular processes including cell adhesion, migration, invasion, cell death and cell cycle progression, among others. Through a C-terminal GAP domain, p190 can exert its inhibitory effect on RhoGTP levels by accelerating Rho's intrinsic ability to hydrolyze GTP (Hall and Nobes, 2000). Given its crucial role in modulating critical cellular processes, the regulation of p190 GAP activity is managed carefully and closely. The GAP activity of p190 toward RhoA is in large part regulated through post-translational modifications including phosphorylation and ubiquitination.

Tyrosine phosphorylation of p190, particularly at Y1105, results in activation of its GAP activity and downmodulation of Rho signaling (Chang et al., 1995;Roof et al., 1998). A large number of kinases, predominantly c-Src and other Src family kinases, have been reported to mediate tyrosine phosphorylation of p190. Conversely, tyrosine dephosphorylation by several tyrosine phosphatases inactivates p190 RhoGAP function (Nimnual et al., 2003;Shiota et al., 2003;Tamura et al., 2006). An additional mechanism that decreases p190 GAP activity is its phosphorylation at various ser/thr residues by several different kinases (Maddox and Burridge, 2003;Jiang et al., 2008;Mori et al., 2009). To date, three ser/thr kinases have been confirmed to act on p190, though involvement of other kinases is likely. Ser/thr phosphorylation levels of p190 are particularly high during mitosis whereas its tyrosine phosphorylation is decreased at that time (Maddox and Burridge, 2003). Given that increased RhoGTP levels are necessary for proper cell division, reports of changes in p190 phosphorylation patterns propose a

viable mechanism for achieving increased RhoA activity in mitosis. However, ser/thr phosphorylation is not the only mechanism available to a cell for the inactivation of p190 GAP activity during mitosis.

Work in our laboratory has shown that p190 is ubiquitinated during mitosis (Su et al., 2003). This modification leads to a marked decrease in p190 protein levels during cytokinesis. Degradation of p190 has been shown to occur by the ubiquitin-proteasome pathway. Su and colleagues reported that the N-terminus of p190 was important for its ubiquitination and degradation. Work in chapter three of this dissertation further defined the GBDS1 region of p190 as necessary and sufficient for its ubiquitination (Figure 14). Additionally, four N-terminal lysine residues, at positions 6, 26, 28 and 35, were identified as necessary for p190 degradation, suggesting a potential role for these residues as primary ubiquitin accepting lysines (Figures 18, 19). A decrease in p190 levels by ubiquitin-mediated proteasomal degradation and further reduction of its associated GAP activity during cytokinesis is an additional mechanism by which cells can attain elevated RhoGTP levels, necessary for cell division.

Protein ubiquitination is mediated through an enzymatic cascade including enzymes E1, E2 and E3 (Pickart, 2001b). Briefly, E1 activates ubiquitin in an ATP-dependent process and transfers it to a conjugating enzyme, E2. Most eukaryotic cells have a single E1 and nearly twenty five E2 enzymes. The exquisite specificity of this process is provided by the availability of hundreds of E3 enzymes, which mediate interactions between substrates and the ubiquitination machinery. The specificity of E3 enzymes, also called ubiquitin ligases, comes from their ability to bind substrates through

defined recognition sequences commonly referred as degrons. Among the most important ubiquitin ligases, the Anaphase Promoting Complex/Cyclosome (APC/C) is of particular relevance to this work as it has been identified as the ligase mediating ubiquitination of several cell cycle regulators during mitosis, when p190 is also ubiquitinated (Peters, 2002). The APC/C is a multisubunit complex that interacts with substrates by means of two "activator" proteins: Cdc20 and Cdh1. These activators have the ability to recognize particular degrons present in important mitotic regulators such as cyclinB, Securin and polo-like kinase. Cdc20 is active earlier during mitosis where it recognizes degrons known as Destruction or D boxes, which consist of the sequence RXXLXXXN/E. Cdh1, active later during cytokinesis, is more flexible in its binding specificity as it is able to recognize and bind D and KEN boxes in substrates. KEN boxes are defined by the sequence KENXXXD/N (Burton et al., 2005;Kraft et al., 2005). Because D and KEN boxes are recognized exclusively by the APC/C, the presence of either of these destruction motifs in any given ubiquitinated protein is a good initial indication that it may be an APC/C substrate.

In this chapter, I present a collection of data on mitotic post-translational modifications of p190, such as ubiquitination and ser/thr phosphorylation. Both of these can result in reduced p190 GAP activity. Efforts to identify the ligase that mediates p190 ubiquitination in mitosis focused on testing the involvement of the APC/C in the process. Though inconclusive, results provide clues regarding the identity of the E3 ligase that ubiquitinates p190. Also included in this chapter is a preliminary study on the mitotic ser/thr phosphorylation of p190 and the effects of several mitotic kinase inhibitors on its

phosphorylation, thus providing initial evidence regarding the identity of the kinase(s) that accomplishes these modifications.

RESULTS

To better understand the mechanism regulating the mitotic downregulation of p190, an attempt was made to identify the ubiquitin ligase that mediates mitotic p190 ubiquitination. Analysis of the p190 protein sequence revealed that it contained numerous amino acid motifs throughout the molecule that conformed to sequences described for D and KEN boxes (Burton et al., 2005;Kraft et al., 2005). As depicted in Figure 20, six D box-like and two KEN box-like motifs can be found in the p190 sequence. The presence and high concentration of these unique motifs on p190 was an initial indicator to us that the APC/C may be involved in p190 ubiquitination, since these degrons are not utilized by other E3 ligases.

P190 associates with APC/C activators cdc20 and cdh1

The APC/C is an E3 ligase that "recruits" substrates for ubiquitination by an E2 enzyme. It does so by associating with "activators" cdc20 and cdh1, which in turn, bind D and KEN box-containing substrates. To verify the potential involvement of the APC/C in p190 ubiquitination, co-immunoprecipitation assays of mitotic HeLa cell lysates were performed. The goal was to determine whether p190 could associate with either cdc20 or cdh1 (Figure 21). Discovery of such associations would provide supporting evidence for p190 being an APC/C substrate. To increase the probability of

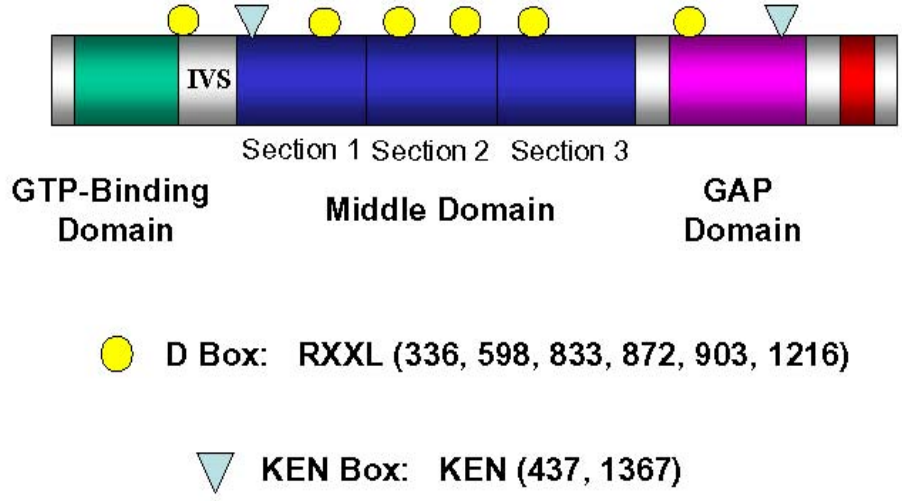
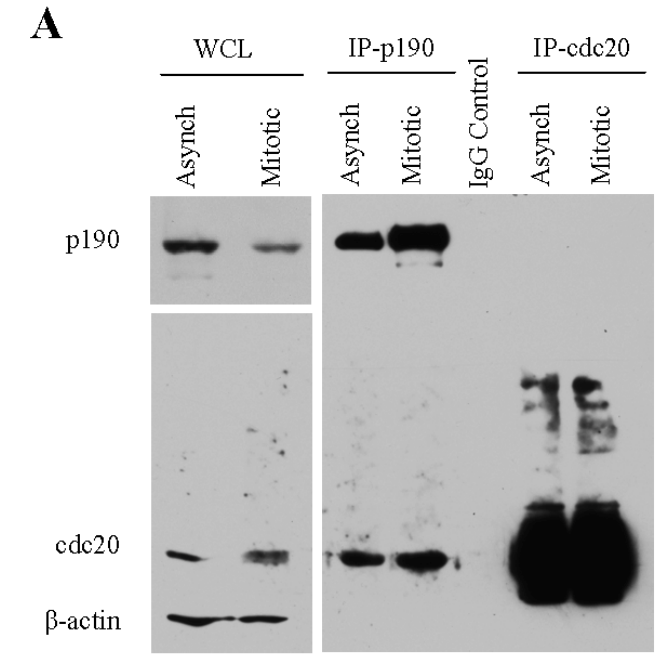

IVS
Section 1 Section 2 Section 3
GTP-Binding Domain
Middle Domain
GAP Domain
D Box: RXXL (336, 598, 833, 872, 903, 1216)
KEN Box: KEN (437, 1367)

Figure 20. Schematic representation of p190RhoGAP and its putative destruction motifs

The different D and KEN boxes present on the p190 sequence are illustrated. Their location is identified by the amino acid position number of the first residue in the motif. D boxes are degrons recognized by the APC/C^{cdc20} and the APC/C^{cdh1} E3 ligase complexes. KEN boxes are recognized exclusively by the APC/C^{cdh1} E3 ligase complex.


A
WCL
IP-p190
IP-cdc20
Asynch
Mitotic
Asynch
Mitotic
IgG Control
Asynch
Mitotic
p190
cdc20
β-actin

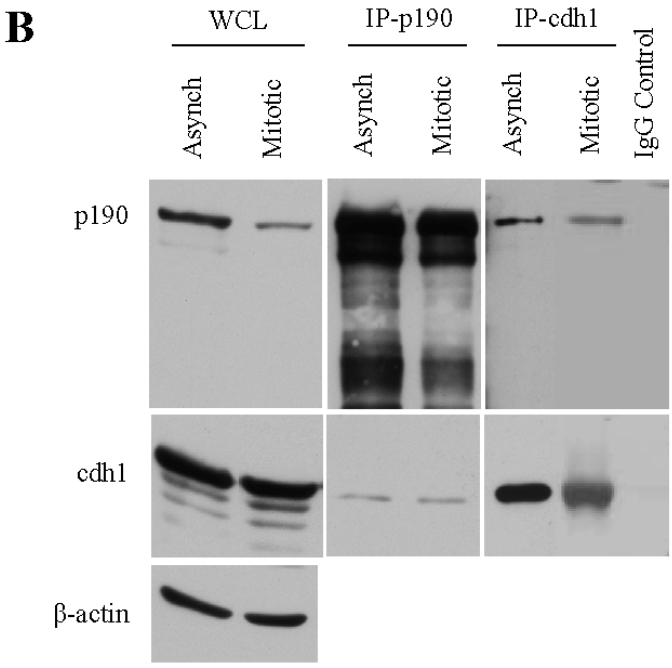

B
WCL
IP-p190
IP-cdh1
Asynch
Mitotic
Asynch
Mitotic
Asynch
Mitotic
IgG Control
p190
cdh1
β-actin

Figure 21. P190 associates with APC/C activators cdc20 and cdh1 when overexpressed

A) p190 and cdc20 co-associate when overexpressed. Reciprocal co-immunoprecipitations using anti-p190 and anti-cdc20 antibodies were performed after HeLa cells, grown in 100 mm culture dishes, were co transfected with 5µg of p190 and 3µg of cdc20 plasmid. Twenty-four hour post-transfection, one cell sample was enriched for mitotic cells by Nocodazole treatment while the other was left as an asynchronous cell population. Different exposures are denoted by breaks in blot images. Immunoprecipitations were performed as described in Material and Methods. Anti-mouse IgG antibodies were used, at a similar concentration as immunoprecipitating antibodies, as negative antibody controls. ($N \geq 5$)

B) p190 and cdh1 co-associate when overexpressed. Cell samples and immunoprecipitations were performed as described above. Different exposures are denoted by breaks in blot images. Anti-rabbit IgG antibodies were used, at a similar concentration as immunoprecipitating antibodies, as negative antibody controls. ($N \geq 5$)

detecting potential interactions, initial experiments were performed in cells co-overexpressing of p190 and either of the APC/C activator proteins. As shown in Figure 21, anti-p190 antibody coimmunoprecipitated both APC/C activators along with p190 in both mitotic and asynchronous cell populations, but more efficiently from mitotic cells. However, p190 immunoprecipitations consistently displayed a greater amount of associated cdc20 than cdh1 (Fig 2A and B, 3A and B). Reciprocal immunoprecipitations using antibodies targeting cdc20 or cdh1 resulted in efficient immunoprecipitation of both APC/C activators, but only cdh1, not cdc20, associated with p190. This was an interesting result given that p190 immunoprecipitations pulled down significant amounts of associated cdc20. The possibility that endogenous proteins could be found in a complex was then tested. Mitotic cell lysates were prepared with synchronized and untransfected HeLa cells and probed with antibodies specific for p190 and the APC/C activator proteins. No consistent interactions between these molecules were seen when cdc20 or cdh1 antibodies were used for immunoprecipitations. In contrast, when p190 antibodies were used, associations of p190 with both cdc20 and cdh1 were detected (Figure 22A, B). As before, association with cdc20 was more readily observed than association with cdh1, the latter being observed only in longer exposures of Western blotting samples (Figure 22B only). Together, these data indicated that p190 could interact with both APC/C activators and therefore could have the potential for interaction with the APC/C during mitosis. Furthermore, cdc20 appeared to be more abundantly associated with p190 than with cdh1.

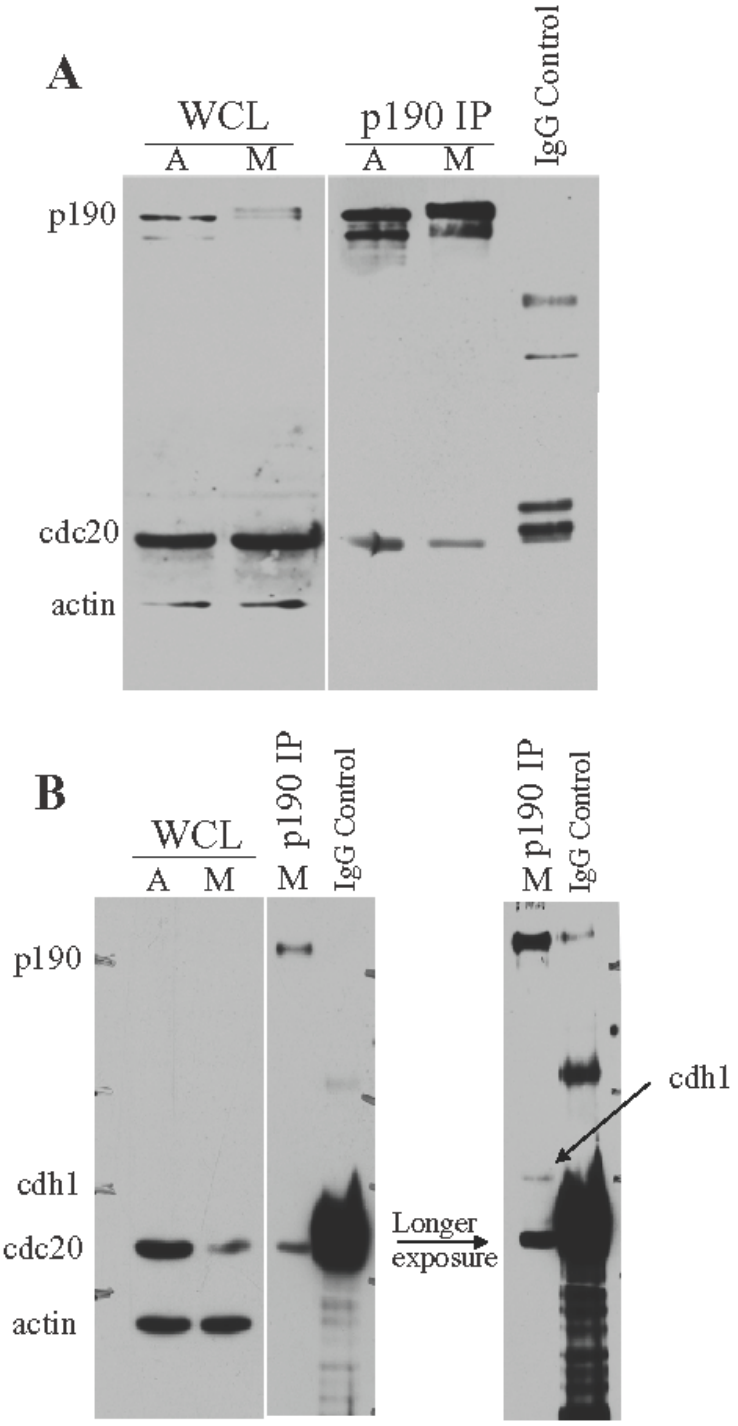
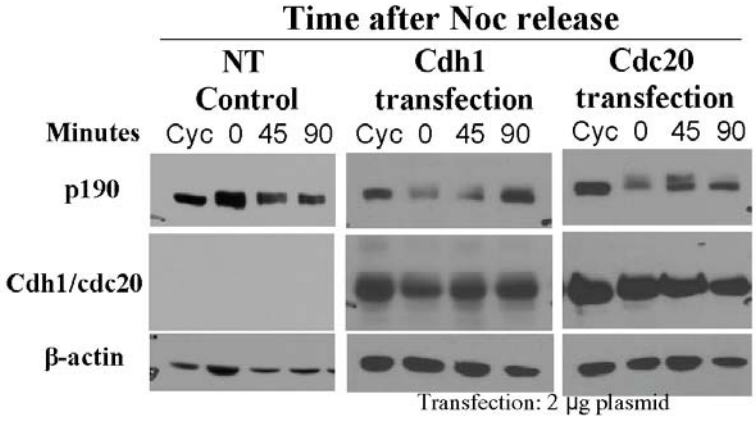

A
WCL
p190 IP
IgG Control
A
M
A
M
p190
cdc20
actin
B
WCL
p190 IP
IgG Control
A
M
M
p190
cdh1
cdc20
actin
Longer exposure
p190 IP
IgG Control
M
cdh1

Figure 22. Endogenous cdc20 and cdh1 proteins are associated with p190 only in immunoprecipitations with p190-specific antibodies

A) Endogenous p190 and cdc20 co-associate in p190-specific immunoprecipitations. Reciprocal co-immunoprecipitations using anti-p190 and anti-cdc20 antibodies were performed in untransfected HeLa cells. Twenty-four hour after seeding duplicate cell samples, one was enriched for mitotic cells by Nocodazole treatment while the other was left as an asynchronous cell population. Immunoprecipitations were performed as described in Material and Methods. Different exposures are denoted by breaks in blot images. Anti-mouse IgG antibodies were used, at a similar concentration as immunoprecipitating antibodies, as negative antibody controls. ($N \geq 5$)

B) Endogenous p190 and cdh1 co-associate in p190-specific immunoprecipitations. Cell samples and immunoprecipitations were performed as described above. Anti-rabbit IgG antibodies were used, at a similar concentration as immunoprecipitating antibodies, as negative antibody controls. ($N \geq 5$)


Time after Noc release
NT Control
Cdh1 transfection
Cdc20 transfection
Minutes
Cyc 0 45 90
Cyc 0 45 90
Cyc 0 45 90
p190
Cdh1/cdc20
β-actin
Transfection: 2 μg plasmid

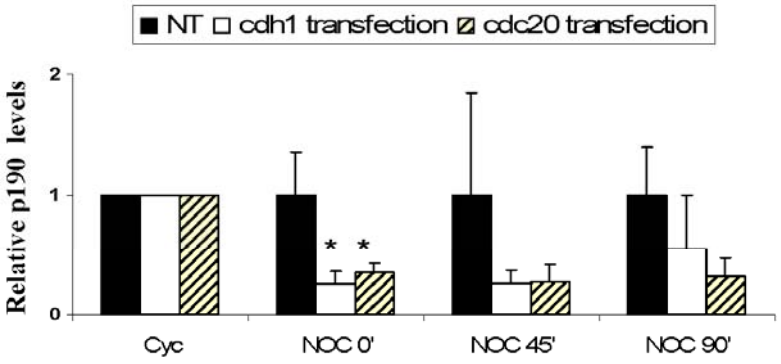

NT
cdh1 transfection
cdc20 transfection
Relative p190 levels
2
1
0
*
*
Cyc
NOC 0'
NOC 45'
NOC 90'

Figure 23. Overexpression of APC/C activators results in an earlier reduction of mitotic p190 levels

HeLa cells were transfected with 2μg of either cdc20 or cdh1 plasmids and the mitotic cell population enriched by Nocodazole treatment as previously described and detailed in Material and Methods and released into fresh media at the indicated times. Cells were harvested with 0.5% NP-40 lysis buffer and 100μg of whole cell lysates analyzed by SDS-PAGE. P190 levels were quantified by densitometric analysis of Western blots. The ratio of p190 to β-actin for the non-treated control samples was arbitrarily set to 1, and samples transfected with APC/C activators (also corrected for amount of β-actin) were normalized to that value. Values represent the normalized mean $\pm$SEM (N=4). * equals $p<0.05$ relative to non-transfected controls.

Variations in the expression of APC/C activators, particularly cdc20, have a negative effect on mitotic p190 protein levels

The next approach was to test whether variations in the expression levels of cdc20 and cdh1 proteins (i.e. overexpression) could have an effect on p190 protein levels. We hypothesized that increased amounts of APC/C activators might result in faster and/or greater degradation of APC/C substrates, including p190. To test this hypothesis, two separate experiments were designed. The first experiment tested whether p190 levels would decrease at earlier time points when cdc20 and cdh1 were equally and separately overexpressed. The second experiment was designed to test wether increased amounts of APC/C activators would be capable of mediating greater p190 degradation. In the first experiment HeLa cells were transfected with cdc20 or cdh1 plasmids, synchronized with Nocodazole and released from arrest for different amounts of time (Figure 23). Quantification of several experiments (n=4) revealed that even before cells were released from Nocodazole treatment and at a much earlier time point (0') than usually observed, p190 levels were significantly decreased in cdc20 and cdh1 transfected samples, whereas nontransfected control samples retained higher p190 levels. In fact, p190 levels remained low in samples overexpressing APC/C activators at all times tested. Unfortunately, because of the large variation in the amount of p190 degradation observed in nontransfected control samples during these experiments, no statistically significant differences were found between control and cdc20 or cdh1 overexpressing samples released from Nocodazole treatment for 45 and 90 minutes, although the overall trend, as

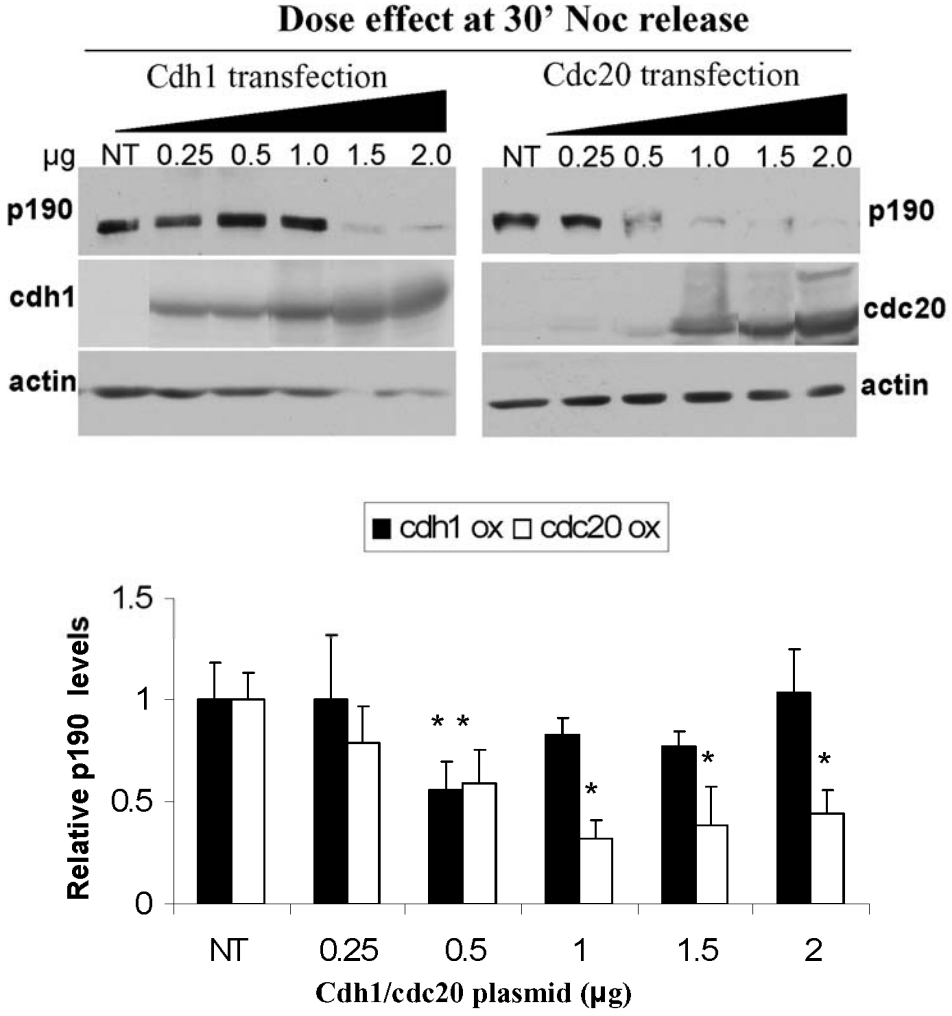

Dose effect at 30' Noc release
Cdh1 transfection
Cdc20 transfection
µg NT 0.25 0.5 1.0 1.5 2.0
NT 0.25 0.5 1.0 1.5 2.0
p190
cdh1
actin
p190
cdc20
actin
cdh1 ox cdc20 ox
Relative p190 levels
1.5
1
0.5
0
NT
0.25
0.5
1
1.5
2
Cdh1/cdc20 plasmid (µg)

Figure 24. Overexpression of increasing amounts of cdc20, but not of cdh1, result in a more dramatic and persistent reduction of mitotic p190 levels

HeLa cells were transfected with the indicated increasing amounts of cdc20 or cdh1 plasmids and the cell population was enriched for mitotic cells by Nocodazole treatment as previously described. After Nocodazole treatment, cells were released into fresh media for thirty minutes. After release, cells were harvested with 0.5% NP-40 lysis buffer and 100μg of whole cell lysates analyzed by SDS-PAGE. P190 levels were quantified by densitometric analysis of Western blots. The ratio of p190 to β-actin for the non-treated control samples was arbitrarily set to 1, and samples transfected with APC/C activators (also corrected for amount of β-actin) were normalized to that value. Values represent the normalized mean $\pm$SEM (N=3). * equals $p<0.05$ relative to non-transfected controls.

seen in the graph on Figure 23, showed lowered p190 levels when cdc20 or cdh1 were overexpressed.

In the second experiment, HeLa cells were transfected with increasing amounts of cdc20 or cdh1 plasmid, synchronized with Nocodazole and released for 30 minutes. The hypothesis being tested was that the extent of p190 degradation would increase with increasing amounts of cdc20 and/or cdh1. Figure 24 shows that transfected samples displayed a significant decrease in p190 levels starting at plasmid transfection levels of 0.5μg and sustained or further reduced this decrease with greater levels of cdc20. In contrast, in cdh1 transfected cells only the sample transfected with 0.5μg of cdh1 plasmid displayed any significant difference in p190 levels from nontransfected control, making interpretation of this data set particularly difficult. From these experiments we can tentatively conclude that increasing amounts of cdc20 result in a corresponding decrease in p190 levels whereas increasing amounts of cdh1 do not. However, lack of proper controls to evaluate mitotic progression in these experiments makes even the cdc20 result difficult to interpret in the greater context of mitosis and cytokinesis (see Discussion).

In vitro transcribed and translated p190 is not a suitable target for purified APC/C ubiquitination assays

In an effort to more definitively and directly address the question of whether p190 is an APC substrate, *in vitro* ubiquitination assays using immunopurified APC/C and radioactively labeled, *in vitro* transcribed and translated p190 were performed. Technical difficulties and our lack of expertise in this kind of assay prevented us from answering

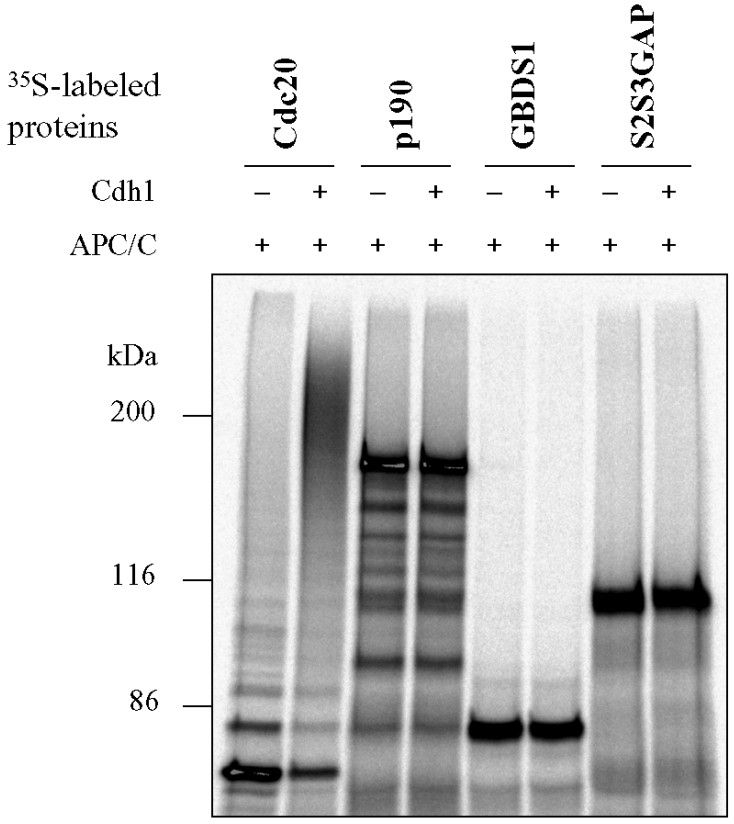

35S-labeled proteins
Cdc20
p190
GBDS1
S2S3GAP
Cdh1
APC/C
kDa
200
116
86

Figure 25. *In vitro* transcribed and translated p190 is not a suitable target for purified APC/C-mediated ubiquitination assays

This in vitro ubiquitination assay was performed by staff in Dr. Hoingtao Yu's laboratory at UT Southwestern Medical Center. Briefly, full length p190 and the deletion mutants GBDS1 and S2S3GAP were *in vitro* transcribed and translated, labeled with [^{35}S]-Methionine and incubated with immunopurified APC/C^{cdh1} E3 ligase complex. After reactions were completed, samples were analyzed by SDS-PAGE and radiodetection of substrates was performed using a phosphoimager scanner. Cdc20 is used here not as an APC/C activator but as a positive control for APC/C ubiquitination. Further details regarding this assay are included in Materials and Methods.

this question through experimentation in our laboratory. As a result, we set up a collaboration with an expert in the field of mitotic ubiquitination, Dr. Hongtao Yu at UT Southwestern Medical Center in Houston, Texas. Using full length p190 plasmid and purified cdh1 and APC/C, staff in Dr. Yu's laboratory tested whether p190 was an *in vitro* APC/C substrate (see Materials and Methods, Chapter 2). Additional plasmids provided to Dr. Yu and used as controls for this assay included the GBDS1 and S2S3GAP deletion mutants of p190. These mutants functioned as positive and negative controls, respectively, based on results described in Chapter 3 which showed that the N-terminal GBDS1 mutant is ubiquitinated by mitotic extracts of HeLa cells to similar levels as wild-type p190, whereas the S2S3GAP is minimally ubiquitinated (See Figure 14). Results from Dr. Yu's laboratory shown in Figure 25, revealed that neither full length p190 nor GBDS1 are ubiquitinated above levels in "control" samples (No cdh1 lanes) when purified APC/C and cdh1 are used. Ubiquitination of cdc20, used as a positive control, was evident in the cdh1-containing lane and observed as a decrease in intensity of the bands near its native molecular weight of ~55 kDa and a concomitant increase in the darkness of the lane at higher molecular weights (near the 200 kDa marker), indicative of polyubiquitination. We concluded from these experiments that, although ubiquitinated in mitotic cell extracts (see Figure 14), *in vitro* transcribed and translated p190 is not a suitable target for purified APC/C-mediated ubiquitination.

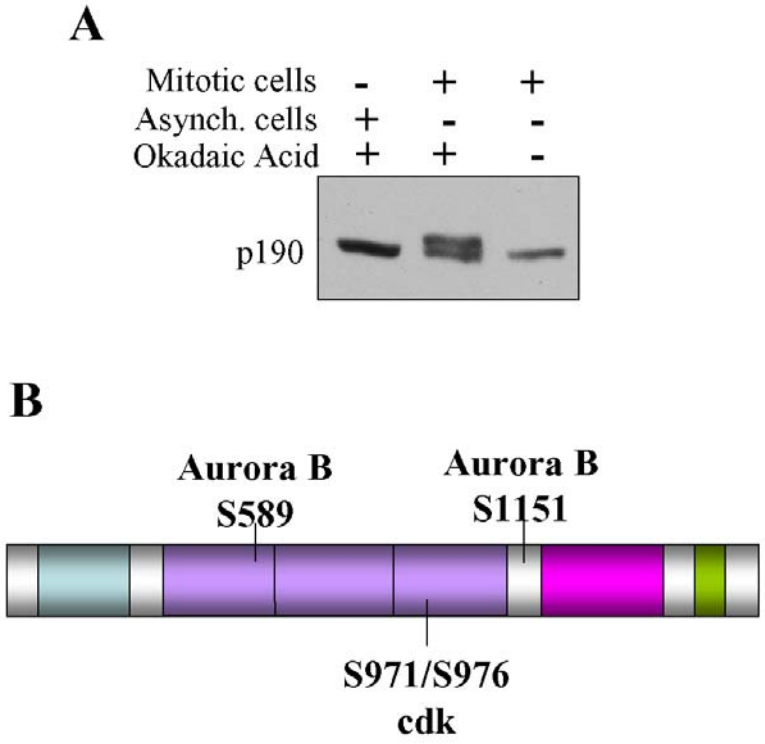

A
Mitotic cells - + +
Asynch. cells + - -
Okadaic Acid + + -
p190
B
Aurora B
S589
Aurora B
S1151
S971/S976
cdk
C
IP-HA
IgG control IP
M
A
pSer
83
MW marker lane
Empty lane
HA-MD
83

Figure 26. p190 is phosphorylated by a ser/thr kinase in mitosis

A) Treatment with Okadaic Acid retards the electrophoretic mobility of mitotic p190. HeLa cells were treated with Nocodazole to enrich the mitotic population. Thirty minutes before harvesting these cells, one sample from the mitotic cell population was treated with the ser/thr phosphatase inhibitor Okadaic Acid. Cells were then harvested with 0.5% NP-40 lysis buffer supplemented with protease inhibitors +/- Okadaic Acid. 100μg of mitotic whole cell lysates were analyzed by SDS-PAGE and immunoblotted as indicated.

B) A schematic representation of the putative cdk1 and AuroraB phosphorylation sites in the MD of p190. This information was collected through a mass spectrometry analysis undertaken to identify mitosis-specific phosphorylation sites on p190 and was performed by Dr. Ling Su.

C) The MD of p190 is ser/thr phosphorylated. Cell lysates from mitotic-enriched and asynchronous cell populations transfected with 5μg of HA-tagged Middle Domain plasmids were analyzed by immunoprecipitation using HA-11 antibody. Purified immunecomplexes were then analyzed by SDS-PAGE and immunoblotted as indicated. Anti-rabbit IgG antibodies were used as negative antibody controls and added to samples at similar concentrations as the immunoprecipitating antibodies.

p190 is phosphorylated by a ser/thr kinase in mitosis

We then turned our attention to a different post-translational modification observed in p190 during mitosis, ser/thr phosphorylation. Mitotic ser/thr phosphorylation of p190 has been shown to decrease its GAP activity and has been offered as a mechanism by which increased RhoGTP levels are attained during cytokinesis (Maddox and Burridge, 2003). The rationale for undertaking these studies was the observation that, in contrast to the usual single band, p190 forms two electrophoretically distinct bands during mitosis when lysates are prepared using buffer containing ser/thr phosphatase inhibitors. Changes in electrophoresis mobility have been associated with ser/thr phosphorylation; therefore, this possibility was further investigated by comparing interphase cell lysates with mitotic cell lysates that had been treated or non-treated with Okadaic Acid (OA), a ser/thr phosphatase inhibitor. Figure 26A shows that a p190 doublet is observed when OA is added to mitotic cell samples and that removal of said inhibitor restores gel electrophoresis mobility to levels similar to those observed in interphase lysates. We conclude that p190 is ser/thr phosphorylated during mitosis.

A concurrent research project conducted by Dr. Ling Su, at the time a post-doctoral fellow in our laboratory, was seeking to identify mitosis-specific phosphorylation sites in p190 by mass spectrometry. Her efforts resulted in the identification of four phosphorylation sites with strong mitotic specificity. The sequence in these putative phosphorylation sites conformed to the established consensus recognition sequences of mitotic kinases Aurora B and cdk1, as illustrated in Figure 26B. All four mitosis-specific phosphorylation sites identified were found within the Middle

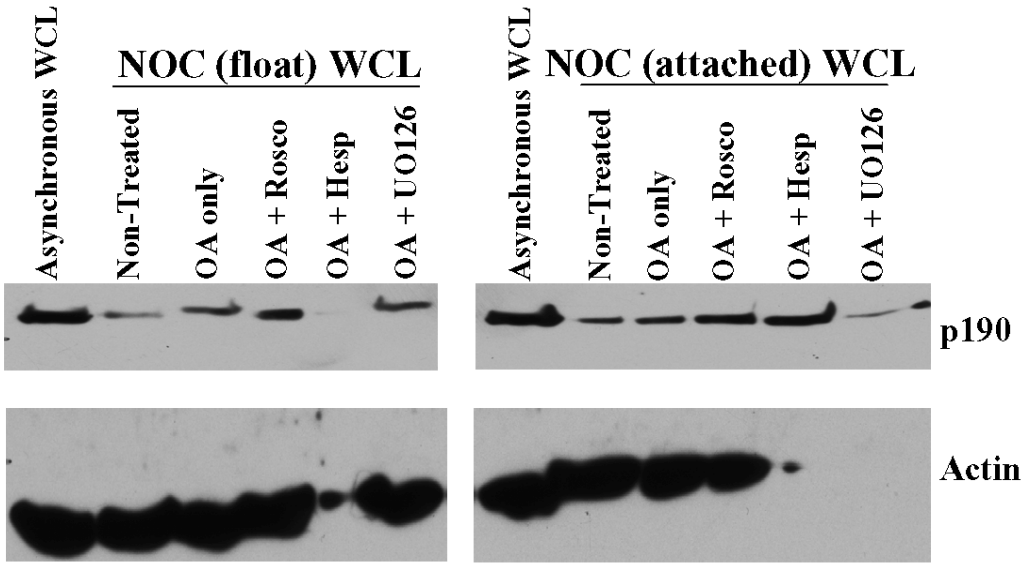

Asynchronous WCL
NOC (float) WCL
Non-Treated
OA only
OA + Rosco
OA + Hesp
OA + UO126
Asynchronous WCL
NOC (attached) WCL
Non-Treated
OA only
OA + Rosco
OA + Hesp
OA + UO126
p190
Actin

Figure 27. Inhibitors of the mitotic ser/thr kinases cdk1 and Aurora B restore p190's electrophoretic mobility

HeLa cells were treated with Nocodazole for 16-18 hours and treated with the indicated kinase inhibitors two hours before harvesting. Lysates were prepared using 0.5% NP-40 lysis buffer supplemented with protease inhibitors and the indicated kinase inhibitors. 100μg of whole cell lysates was analyzed by SDS-PAGE and immunoblotted as indicated (N=3).

Domain (MD) of p190, and thus we tested whether a MD fragment of p190 could be phosphorylated during mitosis when transfected into HeLa cells. This was accomplished by transfection of a triple HA-tagged MD plasmid into HeLa cells, which was then immunoprecipitated from asynchronous and mitotic lysates by means of an anti-HA antibody and analyzed by Western blotting using a phospho Serine (pSer) antibody (Figure 26C). A pSer band was visible after immunoprecipitation of the MD from both mitotic and asynchronous cell lysates, confirming our hypothesis that the MD of p190 can be ser/thr phosphorylated in mitosis. However, whether the phosphorylations in mitosis were distinct from those in interphase was not known.

Inhibitors of the mitotic ser/thr kinases cdk1 and Aurora B restore p190's electrophoretic mobility

An attempt was then made to identify the kinase responsible for p190 phosphorylation during mitosis by treatment of mitotic lysates with kinase inhibitors specific to cdk1 (Roscovitin), AuroraB (Hesperadin) and MAPK (UO126) (Figure 27). Briefly, cells were treated with Nocodazole and then, one hour after harvesting, treated with each of the kinase inhibitors and OA. After all treatments had been completed, floating mitotic cells were collected by mechanical shake off. In addition to this sample, Nocodazole treated cells that remained attached to the dish and had not yet rounded up and entered mitosis were also collected. This assay would determine whether there was any effect of specific kinase inhibition by changes in the electrophoretic mobility of p190. P190 in lysates collected from asynchronous cell samples was used as a reference

point for gel mobility comparisons. Within the mitotic cell samples, labeled Noc(float) in Figure 27, a p190 band in the non-treated sample with nearly the same mobility as interphase p190 can be seen. Addition of OA resulted in a visibly higher p190 band which decreased to non-treated levels when samples receive a combination of OA+Roscovitine. The OA+Hesperadin treatment appeared to be toxic to mitotic cells, as lower protein levels were consistently collected and analyzed. Although in shorter exposures no p190 band is detected in OA+Hesperadin samples, longer exposures such as the one shown in Figure 27 revealed a faint p190 band at the same level as that observed in the OA+Roscovitine sample, lower than the band in the OA-only sample. Inhibition of MAPK by UO126 in the OA+UO126 sample had no effect on p190 band mobility, showing a band at the same level as that of the OA only sample. The same experiment was performed on non-mitotic cell lysates, labeled NOC(att) in Figure 27, where treatment with the inhibitors caused no observable differences in p190 band gel mobility; all p190 bands migrated similarly to that from the control. Together, these results appear to indicate that cdk1 and Aurora B may play a role in the mitotic phosphorylation of p190. Further and more in depth analysis involving in vitro kinase assays and point-mutations is required before more concrete assertions on this matter can be made.

DISCUSSION

Post-translational modifications are an important mechanism by which protein function and stability are regulated. In the case of p190, post-translational modifications such as ubiquitination and ser/thr phosphorylation are known to decrease its GAP activity

and thus allow for increased RhoGTP levels necessary for successful completion of cytokinesis (Maddox and Burridge, 2003;Su et al., 2003). Presented here are preliminary results obtained in an effort to better understand the means by which p190 is ubiquitinated and ser/thr phosphorylated during mitosis. The results, though incomplete, suggest a potential role by the E3 ligase APC/C as well as mitotic kinases cyclin B and Aurora B in the ubiquitination and phosphorylation of p190 during mitosis.

Results in Figures 21 and 22 show that p190 can associate with APC/C activators cdh1 and cdc20 in co-immunoprecipitation assays of mitotic cell lysates. Complex formation between p190 and APC/C activators could be interpreted as supporting evidence of p190 being an APC/C substrate. Association between p190 and cdc20 was preferred, as larger amounts of cdc20, overexpressed or at endogenous levels, were consistently found in p190 precipitates. Interestingly, Su and colleagues (2003) reported that p190 ubiquitination was observed in the early stages of mitosis, the same time cdc20 is associated with the APC/C and actively participating in substrate recognition and protein ubiquitination. Rather confusingly, however, when reciprocal immunoprecipitations were performed with antibodies targeting APC/C activators, only cdh1 was found in complex with p190. The cdc20 immunoprecipitations showed no interaction with p190 even in overnight exposures. Should the interaction between p190 and cdc20 be a bona fide cellular association, it is possible that antibody binding competes with p190 for cdc20 binding or that it causes steric hindrance that blocks D-box recognition sequences in cdc20 so that p190 and other substrates can not be bound. Inclusion of other corroborated APC/C^{cdc20} substrates such as cyclin A or cyclin B in this

immunoprecipitation assay would help clarify these results and, more importantly, validate the approach undertaken.

Analysis of results from Figure 24, where APC/C activators were overexpressed in increasing amounts and the corresponding decrease in p190 protein measured, correlate well with results from co-immunoprecipitation assays in Figures 21 and 22. Quantification of several independent experiments showed that when increasing amounts of cdc20 (0.5µg-2µg plasmid) were transfected into cells, a significant and steady decrease in p190 levels resulted, whereas no significant decrease in p190 levels was observed after cdh1 overexpression, except for the 0.5µg cdh1 plasmid transfection samples. On the other hand, these results are internally inconsistent with those in Figure 23 where 2µg of either cdc20 or cdh1 were transfected, and significant differences in p190 levels between treatments were observed before Nocodazole release. The lack of statistical significance at the later time points may have more to do with technical deficiencies in the execution of these experiments given that throughout this dissertation, dramatic decreases in p190 levels are observed at 40 minutes after Nocodazole release. Also, inconsistencies in measured time points (45 min after Nocodazole release in Figure 23 and 30 minutes after Nocodazole release in Figure 24) make results from this section difficult to compare. In addition to these internal inconsistencies, the lack of controls for mitotic progression such as cyclin A or cyclin B degradation prevents us from accurately relating the time points measured to specific mitotic stages. This is particularly relevant in light of the fact that overexpression of APC/C activators often results in shortened mitosis and cytokinesis, since mitotic checkpoints are quickly bypassed by uncontrolled

degradation of regulatory proteins (Todd Stuckenberg, personal communication). Together, these data do not provide definitive answers to questions regarding the effects of APC/C activator overexpression on p190 degradation but only provide clues regarding those effects.

The *in vitro* ubiquitination assays with immunopurified APC/C in Figure 25 showed that p190 is not an *in vitro* substrate of the purified APC/C^{cdh1} complex. Several reasons make this finding difficult to interpret and translate into the cellular context. First, due to limited ubiquitination activity of the reconstituted APC^{cdc20} complex in vitro, the assay is routinely performed by our collaborators using cdh1 and not cdc20 as the activator. This is likely due to the requirement for additional phosphorylations on varied APC/C subunits, or their targets, required for activity of the APC^{cdc20} complex and unavailable in this *in vitro* system. Given the timing of p190 degradation in early mitosis and the data presented earlier in this chapter regarding p190-cdc20 association, we believe that the APC/C^{cdc20} complex is preferred for p190 ubiquitination. Again, given technical limitations inherent to this assay, such a combination was not attempted. Furthermore, this assay relies on the use of APC/C immunopurified from interphase cells. The regulation of mitotic and interphase APC/C could be sufficiently different such that mitotic substrates may not be recognized and ubiquitinated by interphase APC/C. As described above, mitosis-specific phosphorylation of several APC/C subunits is required for mitotic function whereas such modifications are not needed during interphase (Peters, 2002). Furthermore, p190 may need "priming" post-translational modifications exclusive to mitosis which do not occur in the *in vitro* expression system used to generate the p190

protein tested in this *in vitro* assay or in the purified APC/C preparation. As shown in this chapter (Figure 26B), mitotic-specific phosphorylation has been detected and may play a crucial role in regulating not only the GAP activity of p190 during mitosis but also its stability. Additionally, it may be possible that other E3 ligases active in mitosis, such as Chfr or the Cul3/KLHL9/KLHL13 complex, may be involved in p190 ubiquitination (Chaturvedi et al., 2002;Sumara et al., 2007). An appropriate initial strategy to test this possibility could include coimmunoprecipitations assays with Chfr, Cul3 or p190-specific antibodies, especially because reports available in the literature indicate detection of physical association between these ligases and their identified mitotic substrates. Moreover, shRNA-mediated silencing of these ligases could be employed to determine their *in vivo* involvement in p190 ubiquitination.

The changes in p190 gel mobility shown in Figure 26 corroborate previously described findings of mitotic ser/thr phosphorylation of p190 by Maddox and colleagues. Previous work in our laboratory showed that there are four putative, mitotic-specific phosphorylation sites in p190, all contained within the MD, and we confirm that this domain of p190 can be phosphorylated during mitosis. Point mutations of residues identified by mass spectrometry as putative phosphorylation sites will be necessary to confirm their *in vivo* usage by cellular kinases. We did not test whether the isolated GBD or GAP domains could also be phosphorylated during mitosis. Furthermore, our use of mitotic kinase inhibitors yielded only initial information regarding the involvement of mitotic kinases in p190 phosphorylation, largely because the readout chosen, changes in p190 mobility, was not a direct measurement of phosphorylation. However, the data

suggest the involvement of cdk1 and Aurora B kinases, as their inhibition resulted in a lower mobility p190 band. These results warrant further study. Additional approaches to addressing the question of mitotic serine phosphorylation of p190 would include p190 immunoprecipitation followed by Western blotting and detection of associated phosphoproteins with pSer or kinase specific antibodies. In vitro kinase assays would more specifically determine the potential of each kinase to phosphorylate p190.

The results presented in this Chapter are preliminary and do not support the drawing of any final conclusions regarding the identity of the E3 ligase involved in mitotic p190 ubiquitination or of the kinase(s) involved in mitotic p190 phosphorylation. However, the data do provide leads as to appropriate follow-up experimentation, particularly with regard to the potential requirement for phosphorylation of p190 for ubiquitination and the subsequent identification of the E3 ligase mediating this modification. Given the advances made in other concurrent projects and the difficulties encountered with this line of experimentation, we focused efforts and attention to endeavors presented in Chapters 5 and 6.

Chapter 5

The Role of P190RhoGAP in Cytokinesis

INTRODUCTION

Cytokinesis is the final step in cell division where ingression of a cleavage furrow separates daughter cells. It initiates in anaphase and continues throughout telophase when membrane invagination at the equatorial cell cortex occurs. Cytokinesis is completed when intercellular membrane abscission separates daughter cells (Glotzer, 2001). The contractile forces required for furrow ingression are provided by a ring of filamentous actin and myosin II that is juxtaposed to the cell membrane at the equator of the dividing cell. Assembly and proper regulation of this contractile ring is critical for achieving proper cell division and is under the control of the small GTPase RhoA (Piekny et al., 2005).

RhoA is a molecular switch that cycles between active (GTP-bound) and inactive (GDP-bound) states. Transitions between activation states are facilitated by Guanine nucleotide Exchange Factors or GEFs (activators), GTPase Activating Proteins or GAPs (inactivators), and GDP Dissociation Inhibitors or GDIs (inactivators). RhoA localizes to the cleavage furrow formation site (Takaishi et al., 1995;Nishimura et al., 1998b) and the inhibition of RhoA activity prevents cleavage furrow formation (Kishi et al., 1993;Mabuchi et al., 1993). Increased RhoGTP levels during the latter stages of mitosis have been observed in multiple independent studies (Kimura et al., 2000b;Maddox and Burridge, 2003;Yoshizaki et al., 2004). Furthermore, RhoGTP activity zones at the contractile ring formation site have more recently been described as a prerequisite for cleavage furrow formation and function (Bement et al., 2005;Bement et al., 2006b;Su et al., 2009), suggesting that increased Rho activity, localized to the equatorial midzone

during cytokinesis, is required for cell division.

Active RhoA mediates contractile ring assembly through its downstream effector mDia2, an actin nucleator that induces the polymerization of long, unbranched actin filaments (Watanabe et al., 2008). Binding of Myosin II, a mechanoenzyme, crosslinks newly created actin filaments to form a fully functional contractile ring (Wu et al., 2006;Vavylonis et al., 2008). Phosphorylation of the Regulatory Light Chain (MLC2) of myosin II triggers its motor activity and provides the mechanical force required for furrow contraction and ingression (Bresnick et al., 1995). This phosphorylation event is regulated by several Rho effectors including Rho kinase, Citron kinase, and Myosin Light Chain kinase (Kosako et al., 2000;Yamashiro et al., 2003;Poperechnaya et al., 2000).

Actomyosin filaments are assembled on a network of cytoskeletal proteins at the cell cortex that act as a scaffold and connect the filaments to the plasma membrane. Anillin, an actin-binding protein, is a crucial component of this scaffold and is required for cytokinesis (Oegema et al., 2000;Zhao and Fang, 2005a). Anillin has been shown to interact with actin, myosin II and RhoA (Piekny and Glotzer, 2008) thus playing a vital role in linking the structural components of the contractile ring to the signaling proteins and events that regulate cytokinesis.

Additionally, a number of other proteins are involved in the regulation of cytokinesis. Microtubules form the mitotic spindle that provides the framework for chromosome segregation and the signals for the positioning of the cleavage furrow formation site at the equatorial midzone and, as a consequence, direct contractile ring formation (Bement et al., 2005). Aurora B, as a member of the chromosomal passenger

complex (CPC), is essential for assembly and stability of the bipolar mitotic spindle, chromosome-kinetochore attachment and chromosome segregation through its regulation of the spindle assembly checkpoint (SAC) (Ruchaud et al., 2007). Moreover, through its interactions with the centralspindlin complex, AuroraB mediates the recruitment of the RhoGEF Ect2 to the cleavage furrow where it activates RhoA, initiating the downstream signaling pathways that direct contractile ring establishment and function (Zhao and Fang, 2005b).

In opposition to Ect2 GEF activity at the cleavage furrow is p190RhoGAP (p190), a negative regulator of RhoGTP activity (Ludwig et al., 2009). p190 localizes to the cleavage furrow during cytokinesis where it associates with Ect2 (Mikawa et al., 2008). Because of the cyclical nature of RhoA activation, this paradoxical association between the Rho activating and inactivating enzymes is important for the careful regulation of RhoGTP levels needed for cell division. Reports of proteasome-dependent degradation of p190 in late mitosis provide an additional mechanism by which the necessary increase in RhoGTP levels is achieved (Su et al., 2003). Overexpression of p190 results in decreased RhoGTP levels at the cleavage furrow and multinucleation, a phenotype indicative of cytokinesis failure (Su et al., 2009).

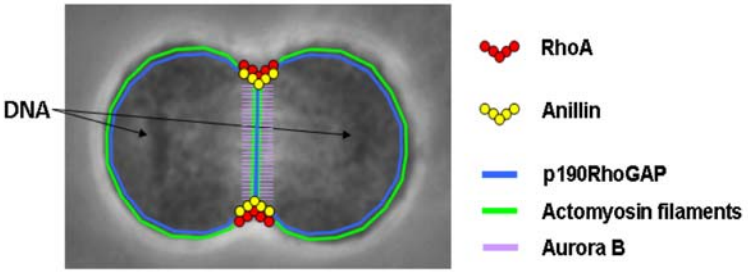
A greater understanding of RhoA regulation and its effect on downstream targets is required before the complex nature of cytokinesis is clearly revealed. Here, we use a p190 overexpression approach to attest its effect on cytokinesis and to identify affected downstream Rho effectors or pathways. In this study, we show that p190 overexpression results in mislocalization of a phosphorylated form of myosin II from the cleavage

furrow. Phosphorylation of Ser19 of the regulatory light chain (pMLC2) of myosin II is targeted by several Rho effector kinases and is necessary for myosin motor activity. Its mislocalization upon p190 overexpression may be indicative of decreased contractility at the cleavage furrow. Our results also show that p190 overexpression interferes with proper Anillin localization to the cleavage furrow in a small fraction of cells. Moreover, we also report that endogenous p190 interacts with endogenous Anillin in mitotic cell extracts in a contractility-dependent fashion, providing clues of their potential involvement in a mechanosensing pathway feeding back to RhoA and regulating its activation based on contractile ring functionality.

RESULTS

Overexpression of p190 affects mitotic myosin II activation but not its localization or the localization of RhoA, Aurora B and Microtubules.

Successful completion of cytokinesis requires the proper localization and activation of several proteins which are critical for cell division, including the major cytokinesis regulators RhoA, Anillin, Actin, Myosin II, Aurora B and microtubules (Figure 28). Endogenous p190 localizes to the cell cortex throughout mitosis and co-localizes with actin at the cleavage furrow during cytokinesis (Figure 29) as previously reported (Su et al., 2003). To determine the mechanism by which p190 overexpression causes cytokinesis failure and induces the multinucleation phenotype, HeLa cells were either mock transfected or transfected with p190 cDNA and the effect of this treatment on the localization of the aforementioned proteins was assessed by confocal



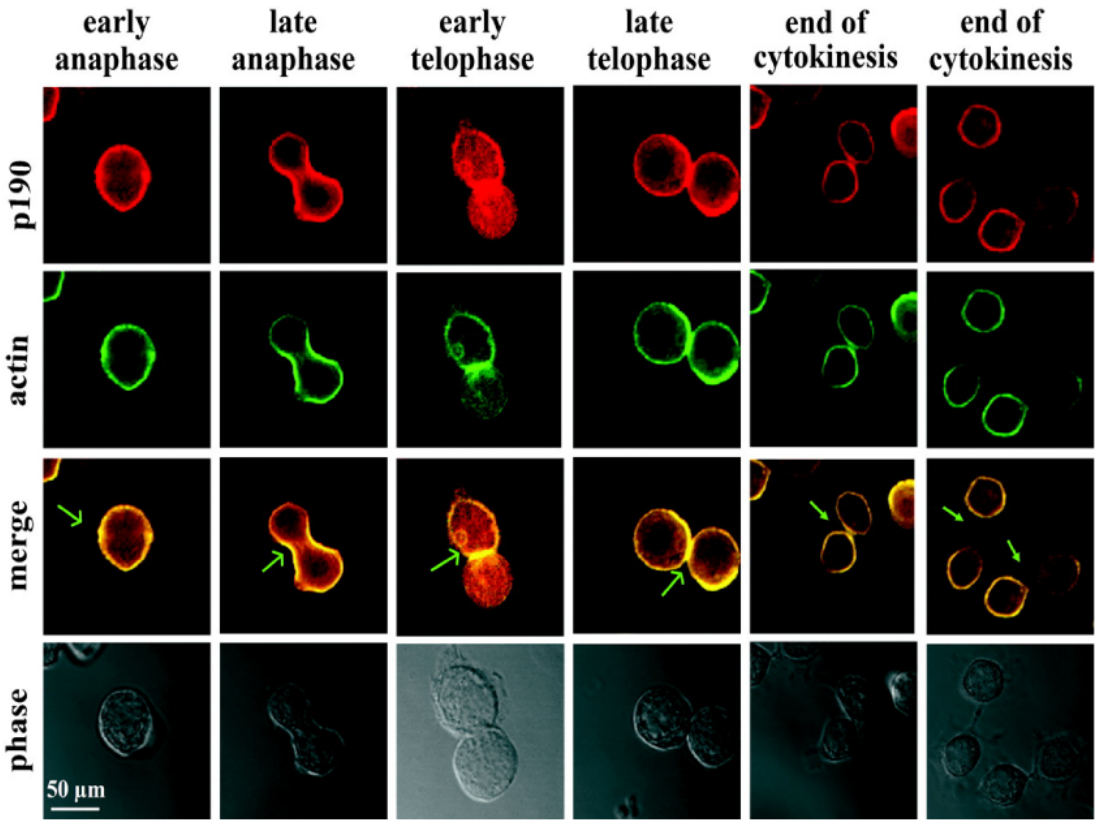
Protein	Localization	Function
RhoA	CF, CR	Regulates signals from anaphase to assemble and ingress CR
p190	CF, CR	Negative regulator of Rho activity through its GAP domain
Anillin	CF, CR	Scaffold; binds Rho, Actin & Myosin; req'd for Myosin II activation
Aurora B	Chr, Midzone	Kinase, spindle assembly checkpoint, MT-kinetochore interactions, Rho activation at CF
Myosin II	CC, CR	Molecular motor; provides force needed for CR contraction
Actin	CC, CR	Forms the filaments, together with myosin II, that make up the CR
Microtubules	Mitotic Spindle	Provides tension for chromatid separation, signals CF formation site

CC: Cell Cortex, CF: Cleavage Furrow, CR: Contractile Ring, Chr: Chromosomes

Figure 28. Diagram of the normal localization of mitotic proteins tested in this study

The function and localization information reported in this figure was gathered from several published review and research articles referenced in the text.

A



Published in Su, et al. 2003. JBC 163 (3), 2003

B

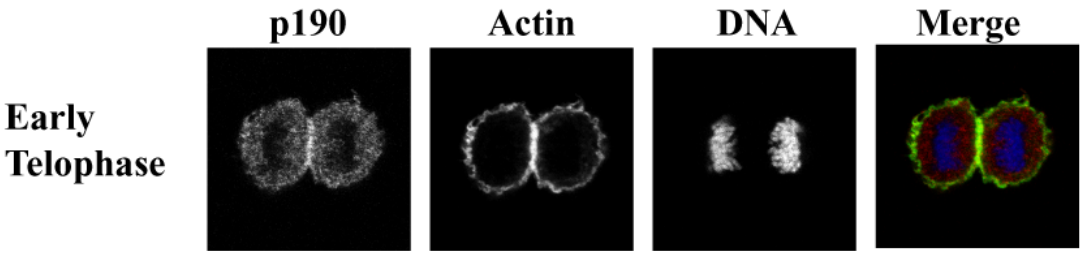


Figure 29. The localization patterns of mitotic p190 in MDA-MB-468 and HeLa cells

A) The mitotic localization of endogenous p190 in MDA-MD-468 cells. The data presented in this panel show the localization of endogenous p190 as detected by the p27 antibody, developed in the Parsons Laboratory (Chang et al, 1995), and has been previously published as referenced at the bottom of the figure.

B) The mitotic localization of endogenous p190 in HeLa cells. p190 was detected using a p190-specific antibody available through BD Transduction Labs. Further details regarding this antibody and the experimental procedure are given in Chapter 2 of this dissertation.

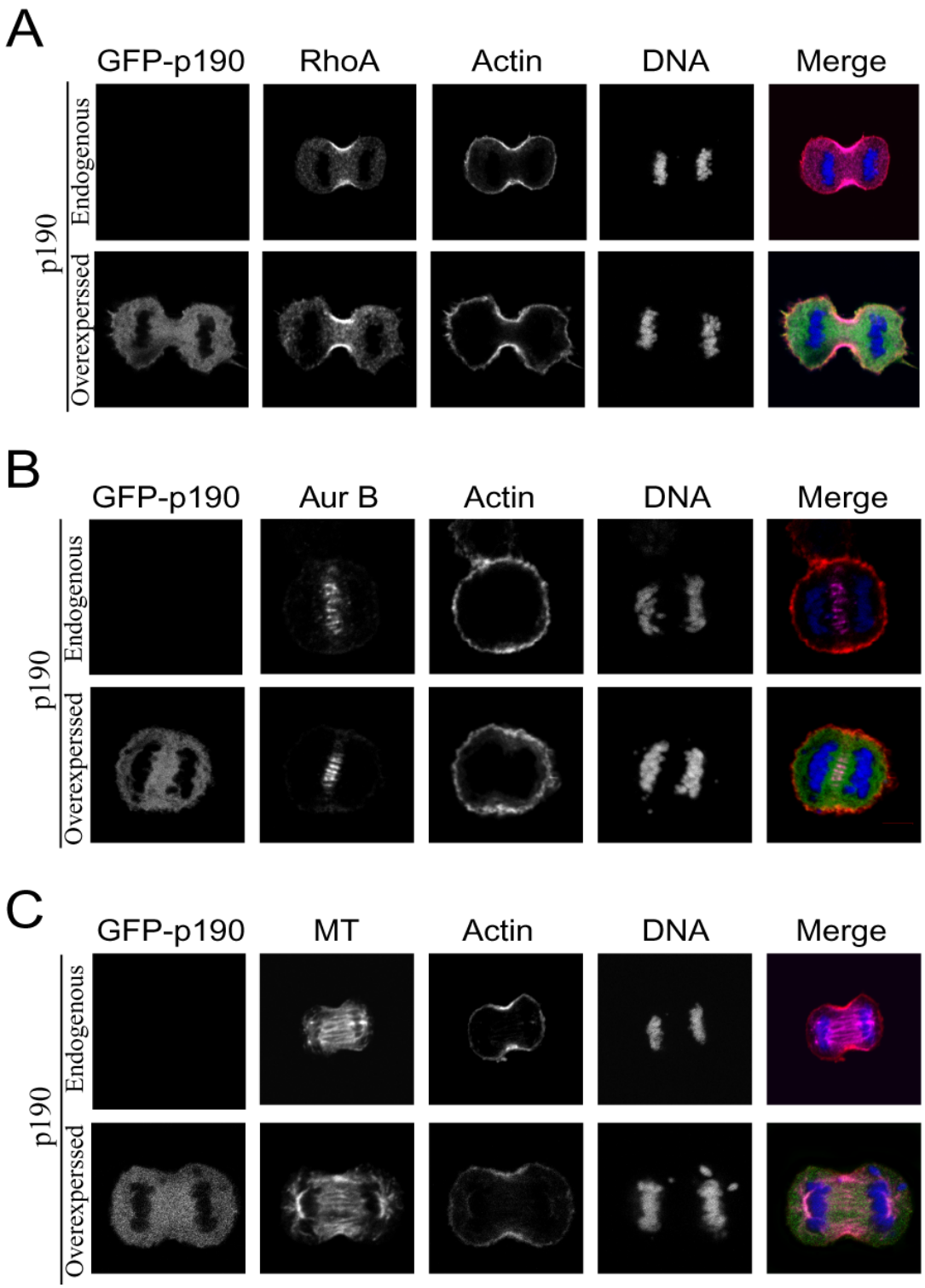

A
GFP-p190
RhoA
Actin
DNA
Merge
p190
Endogenous
Overexperssed
B
GFP-p190
Aur B
Actin
DNA
Merge
p190
Endogenous
Overexperssed
C
GFP-p190
MT
Actin
DNA
Merge
p190
Endogenous
Overexperssed

Figure 30. p190 overexpression does not affect the mitotic localization of RhoA, Aurora B or Microtubules

A) p190 overexpression does not affect the mitotic localization of RhoA. Cell preparation and immunofluorescence staining are detailed in Chapter 2 of this dissertation. The upper set of micrographs is representative of a cell from non-transfected samples. The lower set is representative of a cell from p190-transfected samples. For both samples images are representative of $N \geq 30$ cells.

B) p190 overexpression does not affect the mitotic localization of Aurora B. Cell preparation and immunofluorescence staining are detailed in Chapter 2 of this dissertation. The upper set of micrographs is representative of a cell from non-transfected samples. The lower set is representative of a cell from p190-transfected samples. For both samples images are representative of $N \geq 30$ cells.

C) p190 overexpression does not affect the mitotic localization of Microtubules. Cell preparation and immunofluorescence staining are detailed in Chapter 2 of this dissertation. The upper set of micrographs is representative of a cell from non-transfected samples. The lower set is representative of a cell from p190-transfected samples. For both samples images are representative of $N \geq 30$ cells.

immunofluorescence microscopy. Results revealed that after p190 overexpression, several cytokinesis regulators continued to localize properly, at the cleavage furrow or the mitotic spindle (Figure 30A, B, C). RhoA continued to localize to the cell cortex at the cleavage furrow, where it is normally observed after anaphase onset (Figure 30A). Likewise, Aurora B localization followed its usual pattern as a chromosomal passenger complex protein: it was found associated with metaphase plate chromosomes (not shown) and remained at the equator of the cell after anaphase onset, aligned with the spindle midzone, the future site of cleavage furrow formation (Figure 30B). Microtubules were also able to form functional mitotic spindles and appeared unaffected by p190 overexpression treatments (Figure 30C).

Myosin II localization to the cleavage furrow also remained largely unaffected (Figure 31A). However, the phosphorylated form of myosin II regulatory light chain, which directs the ATPase activity of myosin II, appears to be mislocalized after p190 overexpression (Figure 31B). Whereas a Myosin Light Chain 2 phosphorylation signal (pMLC2) is observed throughout the cell cortex of an untreated cell, and particularly enriched at the cleavage furrow, p190 overexpression resulted in a loss of pMLC2 signal at those sites and a corresponding increase of such signal in the cytosol. Quantification of the phenotype indicated that over half (53%) of p190 overexpressing cells lost pMLC2 signal at the cleavage furrow. These results suggest that p190 overexpression may cause cytokinesis failure and a multinucleation phenotype by affecting the localization of pMLC2 at the cleavage furrow. Because pMLC2 is necessary for myosin II motor activity, it is possible that this mislocalization is associated with inactivation of myosin II

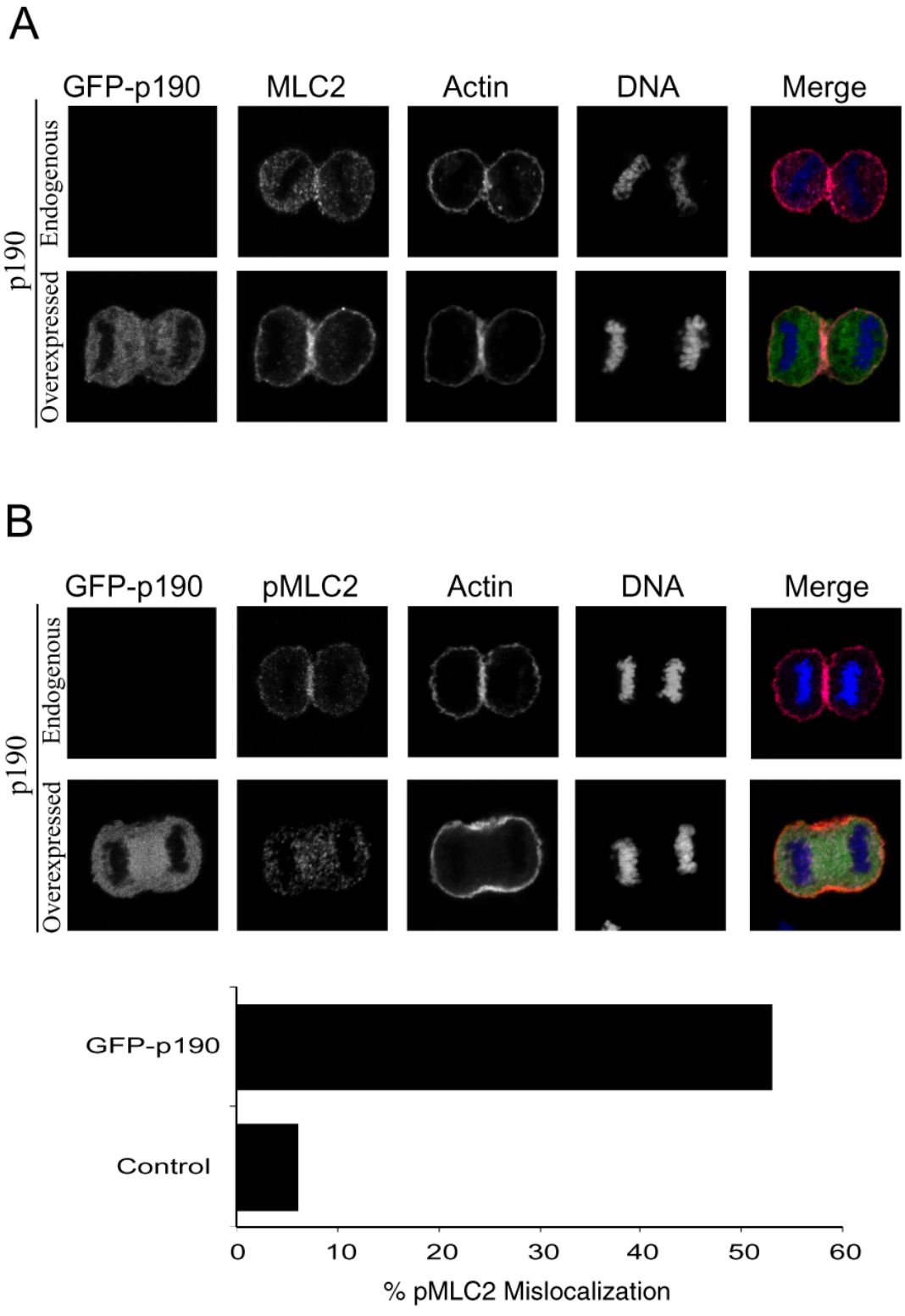

A
GFP-p190
MLC2
Actin
DNA
Merge
p190
Endogenous
Overexpressed
B
GFP-p190
pMLC2
Actin
DNA
Merge
p190
Endogenous
Overexpressed
GFP-p190
Control
0
10
20
30
40
50
60
% pMLC2 Mislocalization

Figure 31. p190 overexpression does not affect the mitotic localization of Myosin II but decreases its activation

A) p190 overexpression does not affect the mitotic localization of Myosin II. Cell preparation and immunofluorescence staining are detailed in Chapter 2 of this dissertation. The upper set of micrographs is representative of a cell from non-transfected samples. The lower set is representative of a cell from p190-transfected samples. For both samples images are representative of $N \geq 30$ cells.

B) p190 overexpression decreases pMLC2 levels at the cleavage furrow during cytokinesis. Cell preparation and immunofluorescence staining are detailed in Chapter 2 of this dissertation. The upper set of micrographs is representative of a cell from non-transfected samples. The lower set is representative of a cell from p190-transfected samples. Loss of pMLC2 signal was determined by a lack of distinct cortical and contractile ring-associated signal after incubation with a pMLC2-specific antibody. Quantification of the observed phenotype was expressed as the percentage of cells having lost pMLC2 signal at the contrac from the total number of observed p190-positive cells in cytokinesis. For both samples images are representative of $N \geq 30$ cells.

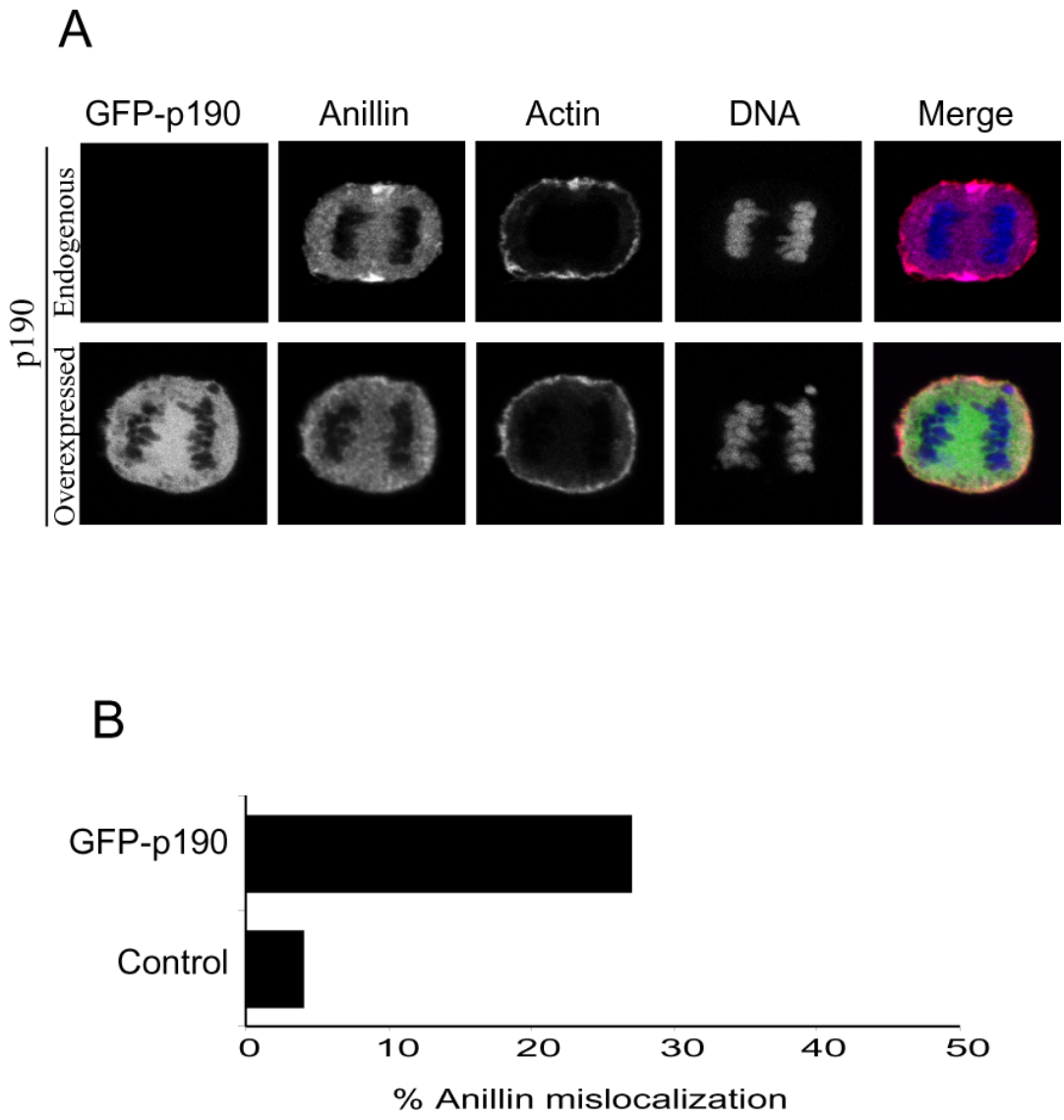

A
GFP-p190
Anillin
Actin
DNA
Merge
p190
Endogenous
Overexpressed
B
GFP-p190
Control
0
10
20
30
40
50
% Anillin mislocalization

Figure 32. p190 overexpression has a modest effect on the localization of Anillin to the cleavage furrow

A) p190 overexpression has a modest effect on the localization of Anillin to the cleavage furrow. Cell preparation and immunofluorescence staining are detailed in Chapter 2 of this dissertation. The upper set of micrographs is representative of a cell from non-transfected samples. The lower set is representative of a cell from p190-transfected samples. Anillin mislocalization was determined by a lack of cortical associated fluorescence signal at the equator of the cells, otherwise known as the cleavage furrow formation site, after incubation with an Anillin-specific antibody. For both samples images are representative of $N \geq 30$ cells.

B) Quantification of the mislocalization of Anillin after p190 overexpression. Quantification of the observed phenotype was expressed as the percentage of cells having lost Anillin signal from the total number of observed p190-positive cells in cytokinesis, as exemplified in A.

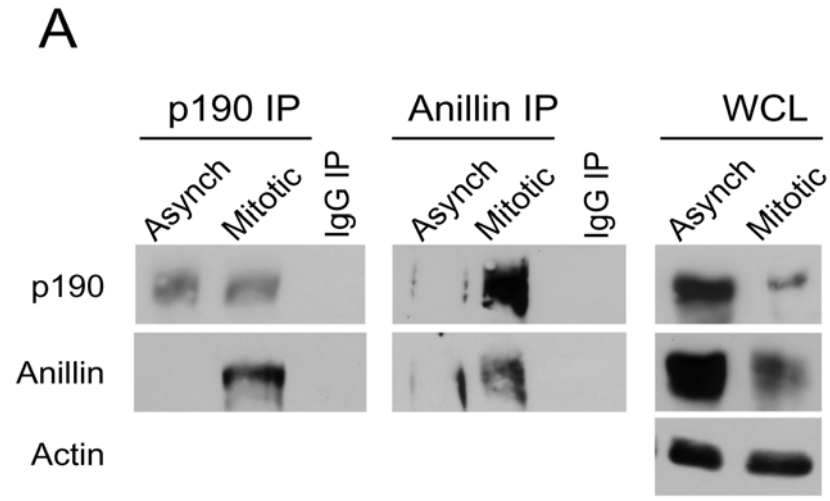
and the inability cells to initiate and/or sustain contractility at the cleavage furrow.

Overexpression of p190 affects Anillin localization

Anillin is a molecular scaffold crucial to the successful completion of cytokinesis. It is involved in the establishment of the contractile ring and mediates cleavage furrow stability and activity. Because Anillin performs such an essential role in cytokinesis we examined whether p190 overexpression in HeLa cells affected Anillin localization to the cleavage furrow. Confocal microscopy imaging revealed that in fact, when p190 is overexpressed, Anillin localization to the cleavage furrow is lost (Figure 32) in a portion of treated cells. However, the frequency of such a mislocalization event was significantly lower than that of pMLC2 mislocalization (Figure 31B). Quantification of Anillin mislocalization revealed that only 27% of cells exhibited that phenotype. These results suggest that although modest in effect, p190 interferes with Anillin localization to the cleavage furrow. With Anillin playing a central role during cytokinesis, this event has the potential for significant effects on cytokinesis.

p190 and Anillin co-associate during mitosis and their interaction is contractility-dependent.

As a molecular scaffold, Anillin binds several structural and regulatory molecules involved in contractile ring assembly and function, creating a molecular complex where effector and substrate proteins are placed within close proximity (Piekny and Glotzer, 2008). We hypothesized that the effect of p190 on Anillin localization, as well as the


A
p190 IP
Anillin IP
WCL
Asynch
Mitotic
IgG IP
Asynch
Mitotic
IgG IP
Asynch
Mitotic
p190
Anillin
Actin

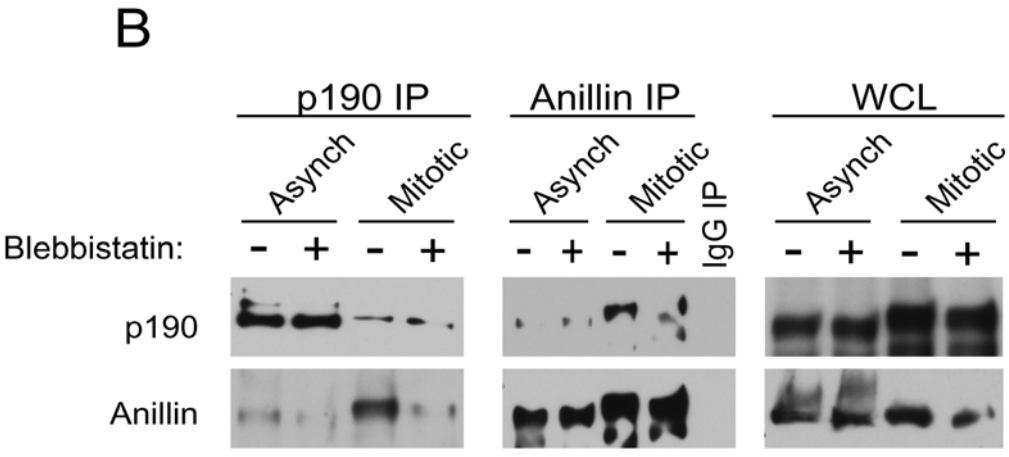

B
p190 IP
Anillin IP
WCL
Asynch
Mitotic
Asynch
Mitotic
IgG IP
Asynch
Mitotic
Blebbistatin:
- + - +
- + - +
- + - +
p190
Anillin

Figure 33. p190 and Anillin co-associate during mitosis and their interaction is contractility-dependent.

A) p190 and Anillin co-associate during mitosis. Non-transfected HeLa cells treated with Nocodazole or left untreated, were used as the source of whole cell lysates for reciprocal co-immunoprecipitations with p190 and Anillin specific antibodies. Twenty-four hour after seeding duplicate cell samples, one was enriched for mitotic cells by Nocodazole treatment while the other was left as an asynchronous cell population. Immunoprecipitations were performed as described in Material and Methods. Anti-rabbit IgG antibodies were used as negative antibody controls at a similar concentration as immunoprecipitating antibodies and incubated in a mixture of mitotic and asynchronous cell lysates. (N = 3)

B) The mitotic association between p190 and Anillin is contractility-dependent. In addition to Nocodazole treatment, mitotic and asynchronous cells were also treated with Blebbistatin, an inhibitor of myosin II-dependent contractility for 30 minutes before cell harvesting. Immunoprecipitations were performed as described above. Anti-rabbit IgG antibodies were used, at a similar concentration as immunoprecipitating antibodies, as negative antibody controls and incubated in a mixture of mitotic and asynchronous cell lysates. (N = 3)

anchoring of p190 at the cleavage furrow formation site, could be mediated through a potential interaction between the two proteins. We tested whether p190 and Anillin were able to interact in synchronized mitotic HeLa cell extracts by co-immunoprecipitation. Figure 33A shows that an association between p190 and Anillin can be biochemically detected at endogenous protein levels and that this interaction was limited to mitotic cell samples, supporting the hypothesis.

Biochemical signals provided by increased RhoGTP levels initiate the mechanical deformation of the cleavage furrow that permits progression through cytokinesis and eventual cell division. To ensure that membrane furrowing is proceeding appropriately, feedback mechanisms allowing correction of errors that may occur during cleavage furrow contraction are required. Such feedback mechanisms are proposed to follow changes in cell shape and contractility at the cleavage furrow and involve myosin II (Effler et al., 2007). Anillin acts as a bridge between contractile ring structural components and the biochemical signal transducer that regulates them and hence is well positioned to acts as a convergence point between both biochemical and mechanosensitive inputs. Because p190 negatively regulates RhoA and myosin II activity at the cleavage furrow (Figure 31B) and endogenously associates with Anillin during mitosis (Figure 33A), we hypothesized that the p190-Anillin interaction could be involved in a mechanosensing feedback loop and therefore, could be sensitive to contractility perturbations. To test our hypothesis we disrupted the mechanical forces required for cytokinesis by treating synchronized HeLa cells with the myosin II inhibitor blebbistatin. We then assessed the effect of this disturbance on the p190-Anillin complex

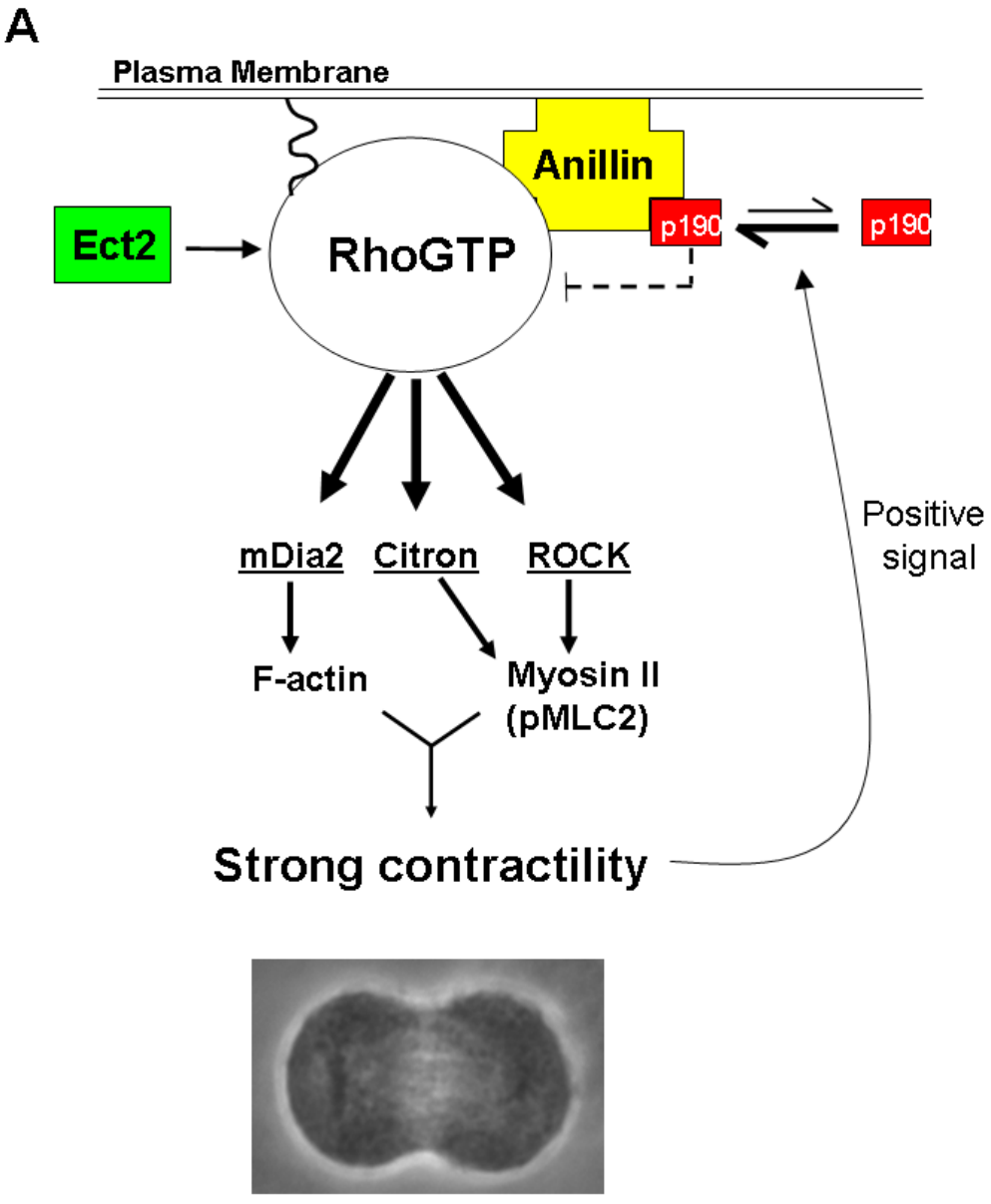
previously detected. Results confirmed the mitotic interaction between Anillin and p190 described in Figure 33A in untreated samples yet, showed that this interaction was disrupted in blebbistatin-treated cells (Figure 33B). Together, these results show that p190 overexpression is mildly disruptive of Anillin localization and that this may occur because of an endogenous p190-Anillin association specific to mitosis. Furthermore, this association is sensitive to blebbistatin inhibition which we hypothesize may be a part of a feedback mechanism to regulate the mechanical strength of the contractile ring through the regulation of RhoGTP levels and myosin II activation at the cleavage furrow.

DISCUSSION

Cytokinesis is a critical process in the cell cycle. As such, it is regulated by numerous proteins whose mechanism of action and associations are deliberately complex to ensure successful cell division. The results outlined in this report are focused on understanding the role that one such regulator, p190RhoGAP, plays in the process of cytokinesis. Here we report that overexpression of p190 results in the loss of pMLC2 localization to the cleavage furrow, suggestive of a potential decrease in myosin II activity associated with the cleavage furrow. We also show that p190 overexpression affects Anillin localization to the cleavage furrow during cytokinesis. We further demonstrate that endogenous p190 and Anillin associate during mitosis and that their interaction is contractility-dependent.

Previous studies have shown that p190 negatively affects cytokinesis in a RhoGAP-dependent fashion (Su et al., 2003;Mikawa et al., 2008;Sanchez Manchinelly et

al., 2010). Throughout the years, numerous studies have confirmed the critical involvement of RhoA in cytokinesis and shown that its inhibition results in cytokinesis failure (Mabuchi et al., 1993;Kishi et al., 1993). Many of those studies have made use of dominant negative mutants, inhibitors, and RNAi to ablate RhoA activity and analyze its role in cytokinesis (Drechsel et al., 1997;O'Connell et al., 1999;Jantsch-Plunger et al., 2000). The present study also employs a Rho-inhibitory approach but with a significant difference. First, it utilizes a naturally occurring negative regulator of Rho, p190, and second, rather than a complete inactivation, an attenuation of Rho activity is achieved by p190 overexpression. Previous work in our laboratory has demonstrated, by FRET analysis, that overexpression of p190 results in decreased RhoGTP levels at the cleavage furrow during cytokinesis (Su et al., 2009) not a complete inactivation of Rho signaling, as observed in C3 exotoxin- and Rho siRNA-treated cells. This partial decrease affords us an opportunity to ask further questions regarding Rho signaling in cytokinesis including which, if any, Rho downstream effector is more sensitive to decreased RhoGTP levels at the cleavage furrow. The primary effectors of Rho activation in cytokinesis are mDia proteins and Rho-activated kinases ROCK and Citron (Narumiya and Yasuda, 2006). mDia proteins direct actin nucleation needed for contractile ring formation, while ROCK and Citron kinases phosphorylate MLC2, among other substrates, and thus stimulate myosin II-dependent contractility, as shown in figure 34. Our results consistently showed normal actin localization and enrichment at the cleavage furrow in p190 overexpression settings (Figure 30A-C), suggesting that mDia function was not significantly dampened by a decrease in RhoGTP levels during cytokinesis. However,


A
Plasma Membrane
Anillin
Ect2
RhoGTP
p190
p190
mDia2
Citron
ROCK
F-actin
Myosin II
(pMLC2)
Positive
signal
Strong contractility

B

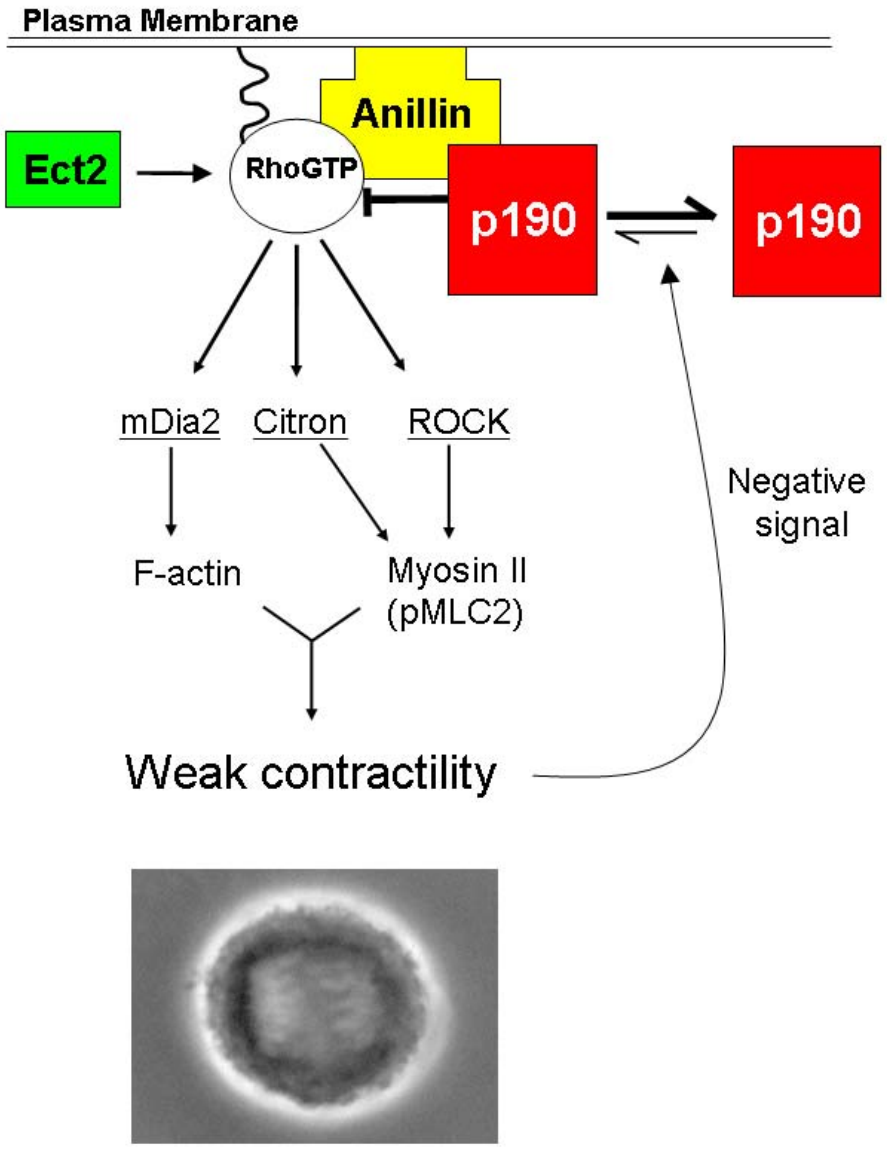

Plasma Membrane
Anillin
Ect2
RhoGTP
p190
p190
mDia2
Citron
ROCK
Negative signal
F-actin
Myosin II (pMLC2)
Weak contractility

Figure 34. Model of proposed p190 function at the cleavage furrow during cytokinesis

A) Under normal conditions, p190 is degraded in mitosis (Chapter 3) and only a small pool persists. Remaining p190 is localized to the cleavage furrow and found in association with the molecular scaffold Anillin (Chapter 5). Decreased p190 GAP activity allows for increased RhoGTP levels and activation of downstream signaling pathways, resulting in the formation of an actomyosin ring and cleavage furrow contractility.

B) Upon overexpression, increased p190 levels result in decreased RhoGTP levels, decreased activation of downstream effectors and insufficient myosin II-based contractility at the cleavage furrow. To correct this defect, the involvement of a feedback loop is proposed. When contractility at the cleavage furrow is insufficient, the association between p190 and Anillin is disrupted, releasing p190 from the cleavage furrow regulatory complex. This, in turn, releases Rho from its p190-mediated inhibition and allows for increased activation of downstream signaling pathways inducing cleavage furrow contraction and successful cell division.

the observation that a decrease in pMLC2 levels at the contractile ring occurs after p190 overexpression (Figure 31) suggests that events downstream of the RhoA-activated kinases, Citron and ROCK, are more susceptible to disturbances in RhoGTP levels at the cleavage furrow. Both ROCK and Citron kinase target the same residue, Ser19, in MLC2 and therefore we are unable to identify which of these two effectors is most affected upon p190 overexpression. Additionally, mislocalization of either of these kinases, or both, could explain the observed decrease in MLC phosphorylation.

Although suggestive of myosin II activation defects, the results here presented come short of providing a definitive explanation on the mechanism by which p190 overexpression yields multinucleation. Because separate cell populations were employed to analyze the localization of myosin II and its activation status, it is impossible to ascertain whether the loss of pMLC2 signal at the cleavage furrow is due to a concurrent loss of myosin II localization or to actual myosin II inactivation. To address this question more directly, an experiment in which both myosin II localization and activation are analyzed in the same cell population is needed. Furthermore, rescue of the multinucleation phenotype by co-overexpression of p190 and a constitutively active phosphomimetic MLC2 construct would assert the importance of myosin II activation more definitively Therefore, the data here presented is insufficient to distinguish or identify whether myosin inactivation or its mislocalization serve as the mechanism of multinucleation induction.

During cytokinesis, Anillin concentrates at the equatorial cell cortex (Figure 32), where it colocalizes with Rho, Ect2, actin, myosin and numerous other proteins. Our

results indicate that overexpression of p190 affects Anillin localization in nearly a third of transfected cells (Figure 32). These results are in accordance with, and further support, previous studies reporting that a pool of active RhoA is necessary for proper Anillin localization to the equatorial cell cortex (Piekny and Glotzer, 2008). Furthermore, other reports have shown that the silencing of Ect2 prevents proper Anillin localization because it results in a complete loss of RhoGTP at the cleavage furrow (Piekny and Glotzer, 2008). As previously noted, p190 overexpression does not ablate RhoGTP levels but instead attenuates them. Fitting with the current model of Anillin localization, the overexpression of p190 mediates a moderate decrease in RhoGTP levels during early cytokinesis which in turn results in a modest decrease in the number of observed Anillin mislocalization events.

We hypothesized that p190 could be interacting with Anillin because they both localize to the cleavage furrow during cytokinesis and are associated with Rho. Furthermore, we believed that an interaction between the two proteins was likely because Anillin associates with numerous other cytokinesis-related proteins and serves as an anchor for the contractile ring machinery. Our results show that these proteins do interact, as is revealed by co-immunoprecipitation experiments (Figure 33A). Their interaction is limited to mitosis, when both proteins localize to the cleavage furrow, as observed by immunofluorescence microscopy, and the interaction is detected when the proteins are expressed at endogenous levels. It is possible that this interaction is the means by which p190 is localized and "anchored" at the equatorial cortex where it is then well placed to carry out its regulatory functions toward RhoGTP enriched in that zone.

The finding that the p190-Anillin interaction was sensitive to blebbistatin treatment was further intriguing. If Anillin functions as the anchor for p190 localization to the cleavage furrow, and the interaction is sensitive to a contractility inhibitor, then their association could be considered as a means to detect proper or sufficient contractility of the actomyosin ring at the cleavage furrow and therefore, the p190-Anillin axis could act as a mechanosensing switch. We envision a model in which optimal RhoGTP levels are ensured by the mechanosensing nature of the p190-Anillin complex (Figure 34A and B). When contraction of the CR is strong, p190 remains associated with Anillin to prevent further RhoGTP increases, overstimulation of the system and cytokinesis failure. If contraction at the cleavage furrow is weak, p190 dissociates from the Anillin-Rho complex allowing for greater Rho activation and resulting in increased contractility of the cleavage furrow.

Together, our results increase our understanding of regulatory and downstream events involving RhoA during cytokinesis. We propose that, during cytokinesis, decreased p190 levels allow for RhoA-dependent localization or activation of myosin II at the cleavage furrow, inducing furrow contraction and facilitating the interaction between p190 and Anillin at the equatorial cortex. This interaction potentially allows p190 to exert its inhibitory activity toward RhoA and prevent uncontrolled Rho activation, mediated by Ect2RhoGEF. Association of Anillin with p190 ensures that Rho activity is kept at an optimal level, as it is well known that either constitutive activation or inhibition of the Rho signaling pathway results in cytokinesis failure.

Chapter 6

A New Role for P190RhoGAP in Mitosis: Regulation of Chromosomal Alignment at the Metaphase Plate

INTRODUCTION

The Rho family of GTPases is comprised of Rho, Rac, Cdc42 and several other poorly characterized GTPases. Increasing evidence supports the involvement of Rho family GTPases in numerous cellular functions including cell polarity and migration, cell cycle progression, morphogenesis, gene expression, lipid metabolism and ROS generation. The abundant literature on RhoGTPases and their cellular functions has been well reviewed (Bishop and Hall, 2000a;Etienne-Manneville and Hall, 2002;Jaffe and Hall, 2005). Rho GTPases act as molecular switches that cycle between two conformational states: GTP-bound (active) and GDP-bound (inactive). Their nucleotide binding status is mediated by regulatory proteins: Guanine nucleotide Exchange Factors (GEFs) mediate Rho GTPase activation whereas GTPase Activating Proteins (GAPs) mediate their inactivation. When GTP-bound, Rho family GTPases stimulate downstream effector proteins that in turn act on substrates to perform specific cellular functions.

Many of the cellular events regulated by Rho GTPases involve their ability to control actin and microtubule cytoskeleton dynamics (Jaffe and Hall, 2005). Through downstream effectors such as mDia proteins, the ARP2/3 complex and cofilin, RhoGTPases direct actin polymerization. Actin filament organization is regulated by Rho Kinase (ROCK) and its ability to induce myosin light chain phosphorylation as well as by actin bundling proteins such as fascin. Microtubule dynamics have also been reported to be under the control of several RhoGTPase substrates including the Op18/stathmin family which promotes disassembly of microtubules and inhibits their

further polymerization. In addition to their role as actin nucleating factors, the members of the mDia family of proteins are involved in the stabilization of microtubules by increasing cellular levels of detyrosinated tubulin, leading to less dynamic microtubules. Furthermore, mDia and IQGAP have been implicated in the regulation of microtubule plus-end capture.

RhoGTPases are important throughout the cell cycle and the particular relevance of RhoA activity during mitosis has been appreciated for many years. During cytokinesis, the final stage of mitosis, RhoA mediates cell division by directing actomyosin ring positioning, formation and contraction at the equatorial midzone and does so through effector proteins mDia and ROCK, as described previously. Less appreciated is the recently described mitotic role that Cdc42 plays during metaphase (Yasuda et al., 2004). Cdc42 and its downstream effector mDia3 are implicated in the bi-orientation and stabilization of spindle microtubule attachment to kinetochores and in the regulation of chromosome alignment during metaphase. When either Cdc42 or mDia3 are inactivated, chromosome-kinetochore attachment fails and multinucleation ensues. Interestingly, the activation of Cdc42 during metaphase is reported to be mediated by Ect2 and MgcRacGAP (Tatsumoto et al., 1999;Oceguera-Yanez et al., 2005;Minoshima et al., 2003) which are also known to regulate Rho activity levels during cytokinesis, providing a link between the regulation of the early and late stages of mitosis through modulation of separate RhoGTPases. This also provides evidence of GTPase-specificity switching at different mitotic stages by both GEFs and GAPs.

Work in our laboratory is focused on understanding the role that a particular

protein, p190RhoGAP (p190), plays during mitosis. Studies from our group have shown that p190 protein levels are decreased in cytokinesis by a ubiquitin-proteasome dependent mechanism and that its overexpression results in a multinucleation phenotype (Su et al., 2003). We have also shown that p190 GAP activity stands in opposition to Ect2 GEF activity during cytokinesis (Mikawa et al., 2008;Su et al., 2009). Together, these results suggest that, similarly to the Cdc42 GTPase activating protein MgcRacGAP, p190 plays an important role during cytokinesis by regulating RhoGTP levels and countering the RhoGEF activity of Ect2. Soon after its initial discovery, the GAP activity of p190 was confirmed to be specific for RhoA (Settleman et al., 1992b;Ridley et al., 1993) through *in vitro* and *in vivo* experimentation. Further details stemming from these initial reports confirmed that, whereas p190 displayed no Rac or Cdc42 GAP activity in interphase cells, it showed hierarchical specificity during *in vitro* experimentation, highest for RhoA, followed by Rac and then Cdc42.

Here we report that low level overexpression of p190 in a breast epithelial cancer cell line, MDA-MB-468 (hereafter referred to simply as 468 cells), results in the misalignment of chromosomes during metaphase, a defect similar to that observed in cells where the activity of Cdc42, or its downstream effector mDia3, has been inhibited. Results further confirm that the observed metaphase defect is mediated through the GAP domain of p190. Currently, no metaphase function has been ascribed to RhoA, therefore, there exists a possibility that p190 may mediate the observed metaphase defect through regulation of Cdc42 activity levels. The results reported herein mark the beginning of a larger study that will seek to further characterize the means by which p190 regulates not

only actin cytoskeleton dynamics in cytokinesis but also the microtubule network during earlier mitotic stages.

RESULTS

We originally set out to study p190 overexpression in 468 cells to confirm findings reported in chapter five of this dissertation. We were attempting to replicate the mislocalization and inactivation of Anillin and Myosin II observed in HeLa cells after p190 overexpression in a different system. One considerable difference exists between these two systems: whereas in HeLa cells p190 cDNA is transiently transfected using liposome-based reagents, in 468 cells the p190 gene has been incorporated into the genome of this cell line and its expression is under the control of a doxycycline inducible promoter, as part of a tet-on system. When this cell line is treated with doxycycline, nearly all cells express the exogenous p190 at levels comparable to that of the endogenous protein (Su et al., 2003). This tet-on system was employed to overexpress p190 in 468 cells and then assess its effects on cytokinesis proteins. Cells were synchronized with Nocodazole to enrich the mitotic population, released for 30 minutes, fixed and prepared for examination by confocal microscopy. Surprisingly, and in contrast to results obtained in HeLa cells, there was marginal effect of p190 overexpression on the localization and activation of the cytokinesis proteins tested. Instead, two different phenotypes related to nuclear division became apparent.

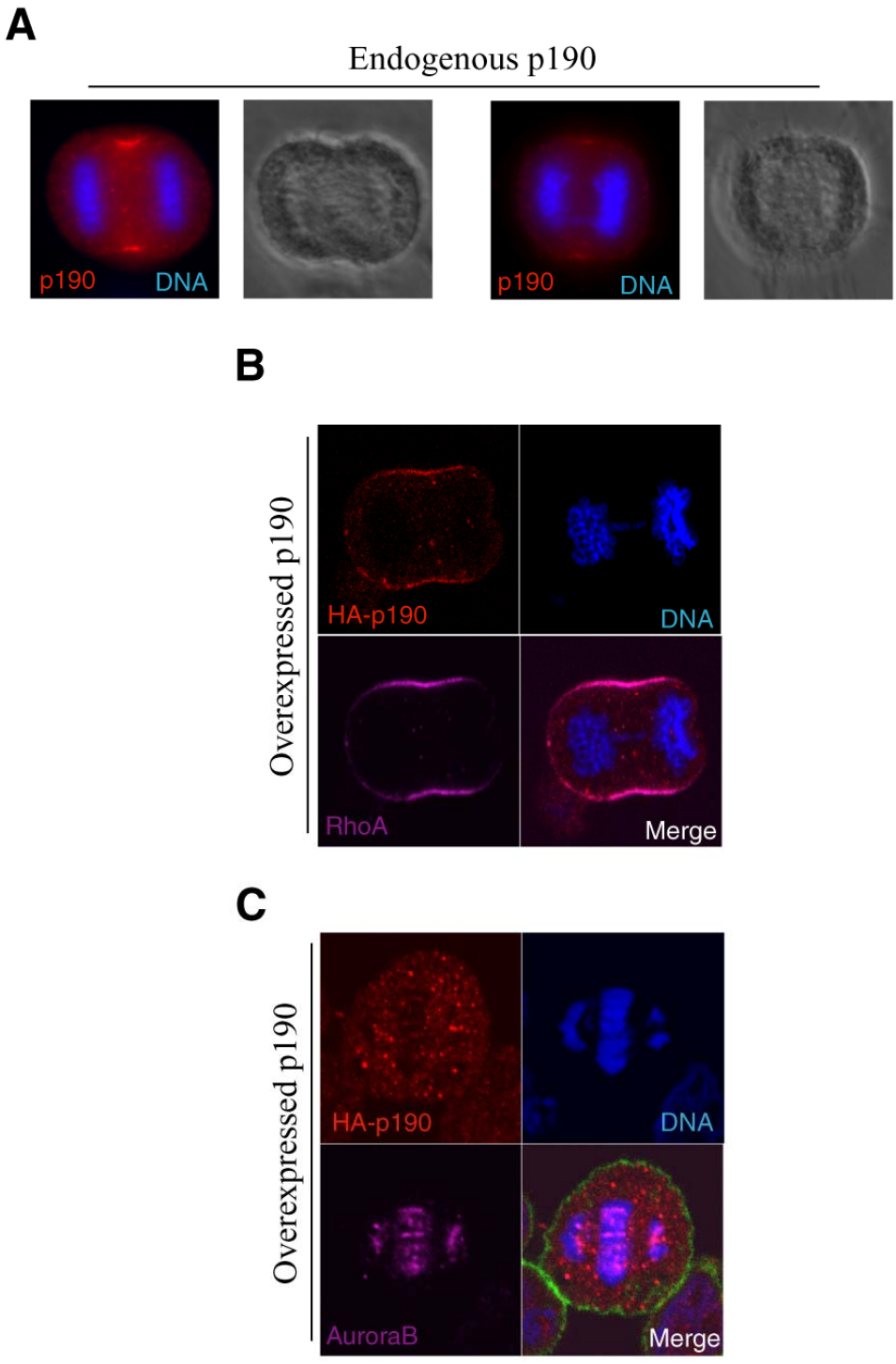

A
Endogenous p190
p190
DNA
p190
DNA
B
Overexpressed p190
HA-p190
DNA
RhoA
Merge
C
Overexpressed p190
HA-p190
DNA
AuroraB
Merge

Figure 35. Overexpression of p190 in 468 cells gives rise to a different mitotic defect than in HeLa cells

A) Chromosome segregation in parental 468 cells. Twenty four hours after seeding, parental 468 cells were fixed and stained for confocal microscopy analysis, as detailed in Chapter 2. Both sets of images shown represent untreated parental 468 cells. The set on the right shows a lagging chromosome whereas the one of the left has successfully segregated its chromosomes. Image shown is representative of a larger population analyzed in these experiments ($N \geq 30$).

B) p190 overexpression increases the number of lagging chromosomes. Dox-inducible 468 cells were treated with 1μg/ml Doxycycline for twenty-four hours to induce expression of full length p190. Cells were then prepared for confocal microscopy analysis as previously described. A lagging chromosome is observed in the DAPI (DNA) stain panel. Image shown is representative of a larger population analyzed in these experiments ($N \geq 30$).

C) p190 overexpression disrupts chromosome alignment during metaphase. Dox-inducible 468 cells were treated with 1μg/ml Doxycycline for twenty-four hours to induce expression of full length p190. Cells were then prepared for confocal microscopy analysis as previously described. Abnormal chromosomal alignment is observed in the DAPI (DNA) stain panel. Image shown is representative of a larger population analyzed in these experiments ($N \geq 30$).

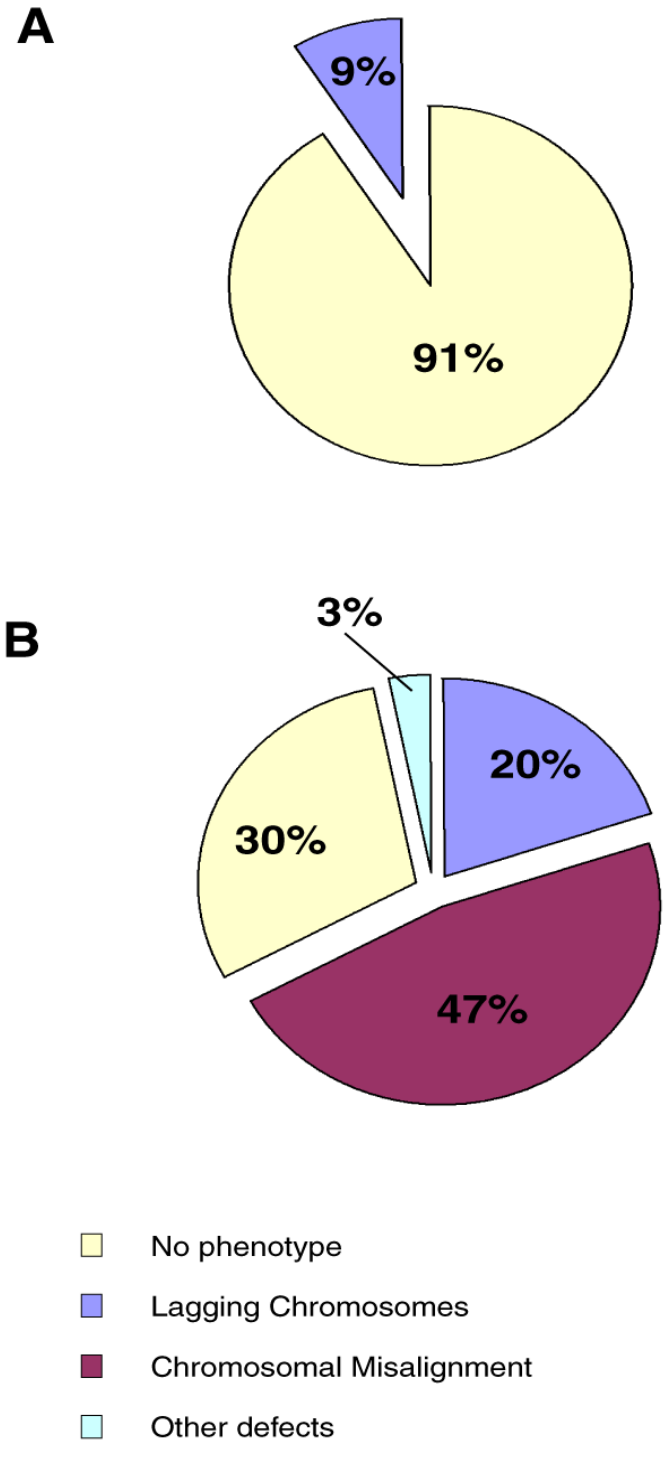

A
9%
91%
B
3%
20%
30%
47%
No phenotype
Lagging Chromosomes
Chromosomal Misalignment
Other defects

Figure 36. Quantification of the mitotic defects observed after p190 overexpression

A) Phenotype quantification in parental 468 cells. Quantification is expressed as the percent of cells, out of all mitotic cells detected, that exhibit either normal or lagging chromosome phenotypes, as shown in Figure 34A ($N \geq 30$).

B) Phenotype quantification in Dox-inducible full length p190 overexpressing 468 cells. Quantification is expressed as the percent of cells, out of all mitotic cells detected, displaying either normal or abnormal chromosome alignment after Doxycycline treatment for twenty four hours to induce full length p190 overexpression, as shown in Figure 34B and C ($N \geq 30$).

Overexpression of p190 in 468 cells gives rise to a different mitotic defec than in HeLa cells

The first phenotype observed was a doubling in the number of anaphase and telophase cells with lagging chromosomes (Figure 34A and B) in cells overexpressing p190 compared to control samples of parental 468 cells. In control samples, only 9 percent of cells had lagging chromosomes whereas in p190-overexpressing cells, 20 percent displayed that phenotype (Figure 35A and B). The second phenotype observed was characterized by more sever nuclear division abnormalities than observed in controls. This cell population had an increased number of cells with a clearly defined set of chromosomes aligned at the metaphase plate as well as a small number of chromosomes which remained away from the midzone, near the poles, and altogether disassociated from the chromosomal metaphase plate (Figure 34C). This phenotype was designated a chromosomal misalignment abnormality. Nearly half of the p190-overexpressing cells displayed this phenotype, in varying degrees of severity, in contrast with the complete absence of such chromosomal misalignment abnormality in control populations of parental 468 cells (Figure 35A and B). This second phenotype caused a large population of cells to be seemingly arrested in metaphase as indirectly measured by p190, RhoA and Aurora B localization. Whereas both p190 and RhoA localize to the equatorial cell cortex only during anaphase and telophase, clearly seen in p190-overexpressing cells with lagging chromosomes (Figure 34B), no such localization was observed in p190-overexpressing cells with chromosome alignment abnormalities (Figure 34C and not shown), suggesting that the latter population of cells were in metaphase and had not

entered into cytokinesis. Association of Aurora B with chromosomes is observed during metaphase when it ensures proper capture of chromosomes by microtubules, correcting any instances where improper attachment occurs (Vader and Lens, 2008). As shown in chapter five, after anaphase onset and chromosome segregation Aurora B localizes to the midzone and in telophase it is found at the midbody. Control cells continued to localize Aurora B properly whereas in p190 overexpressing cells with the chromosome congression phenotype, Aurora B remained associated with all chromosomes regardless of their proper or improper alignment at the metaphase plate (Figure 34C), suggesting that the Spindle Assembly Checkpoint (SAC) had not been satisfied and cells had not progressed into cytokinesis.

P190-induced defects in chromosomal alignment are dependent on p190 GAP activity.

To determine whether the chromosomal misalignment phenotype was related to p190 GAP activity, tet-on, dox-inducible 468 cell lines expressing wild-type p190 (wt p190), a N-terminus deletion mutant (ΔGBD), a C-terminus deletion mutant (ΔGAP) or empty vector were treated as described above and analyzed not by confocal microscopy but by imaging flow cytometry. Results showed a marked increase of the chromosome congression defect in doxycycline-treated wt p190 and ΔGBD 468 cell lines when compared to their non-treated counterparts, nearly doubling the incidence of the phenotype (Figure 36). Both constructs have an intact GAP domain and the further increased response observed in the ΔGBD cell line is likely related to its insensitivity to mitotic degradation, as reported earlier in this dissertation. In contrast is the response of

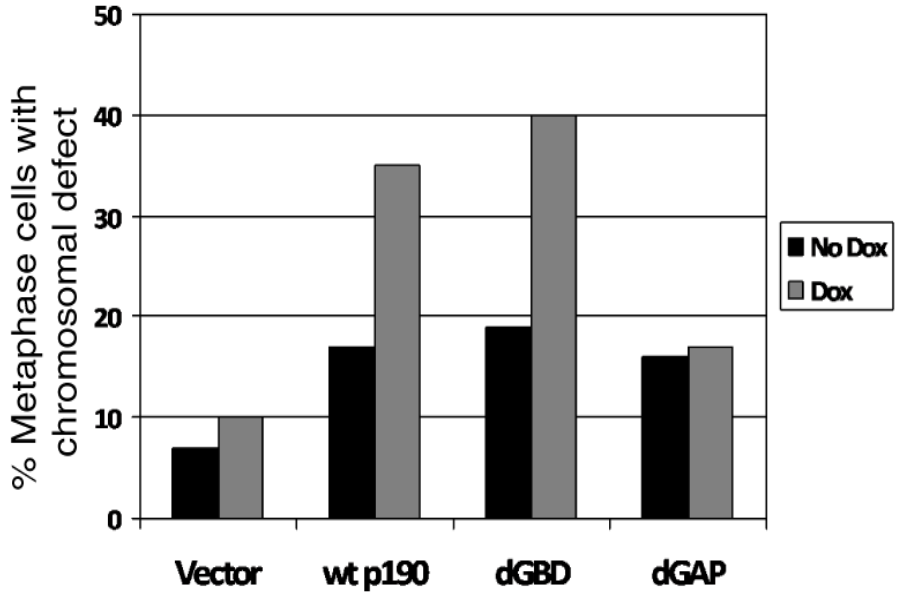

% Metaphase cells with chromosomal defect
50
40
30
20
10
0
Vector
wt p190
dGBD
dGAP
No Dox
Dox

Figure 37. P190-induced defects in chromosomal alignment are dependent on p190's GAP activity

Four different MDA-MB-468 cell lines -vector only, wild type p190, ΔGBD, and ΔGAP- were prepared, as described in Chapter 2, for imaging flow cytometry at the University of Virginia Flow Cytometry Core Facility. The instrument used was the Imagestream by Amnis (Seattle, WA). Cells of interest ($N \geq 200$) were detected by various criteria including 4N DNA content, round shape, cellular localization of DNA signal. This experiment was performed and analyzed with the assistance of an undergraduate researcher, Mr. Ben Kim.

the ΔGAP mutant, a p190 deletion mutant devoid of GAP activity, after Doxycycline induction. No significant difference is recorded between Doxycycline-treated and untreated samples, suggesting that the GAP activity of p190 is important in mediating the abnormal chromosomal congression phenotype.

DISCUSSION

The involvement of RhoA in cell division has been well established for many years. However, although other RhoGTPases such as Cdc42 and Rac are also important regulators of the actin cytoskeleton, neither was thought to play an important role in mitosis, until recently. The reported participation of Cdc42 in the regulation of bipolar attachment of spindle microtubules to the kinetochore (Yasuda et al., 2004) has changed that perspective while at the same time increasing the complexity of the biochemical signals necessary for proper cell division as well as the regulation of such signals. Yasuda *et al.* report that when Cdc42 or its downstream effector mDia3 are inactivated, chromosome capture by microtubules fails and the metaphase plate does not form. This results in cells arresting in metaphase and then going back into interphase without proceeding through cytokinesis, giving rise to multinucleated cells with aberrant tetraploid nuclei. Results shown in this chapter indicate that nearly half of p190-overexpressing 468 cells exhibit severe chromosomal misalignment abnormalities in a phenotype that closely resembles that which was described by Yasuda and colleagues after inhibition of Cdc42 GTPase activity. Moreover, our results also show that this phenotype is mediated through the GAP activity of p190, as expression of a C-terminal

deletion mutant of p190 lacking the GAP domain was unable to elicit significantly greater responses than non-treated cells.

The results from these initial exploratory experiments may be explained by several separate theories. The first would require RhoA to play a previously undescribed role in the regulation of microtubule-kinetochore interactions during metaphase that mirrors the role of Cdc42. The second theory would require p190 to be an *in vivo* dual-specificity GAP, able to act on both RhoA and Cdc42. A third theory could involve Rho function during early mitotic stages as a regulator of centrosome separation and astral microtubule attachment to the cell cortex. Because of the preliminary nature of this project and results, we are unable to rule out any of these theories. However, the literature provides numerous studies that make the latter two theories more plausible than the first.

Yasuda et al. provide evidence that RhoA is not involved in microtubule-kinetochore interactions. Their study showed that a pan-RhoGTPase inhibitor, C. *difficile* Toxin B, as well as expression of either constitutively active or dominant negative Cdc42 prevented microtubule-chromosome interactions during metaphase. In contrast, expression of constitutively active or dominant negative RhoA did not result in metaphase related phenotypes. Instead, RhoA mutants produced expected cytokinesis defects. Constitutively active or dominant negative Rac1 expression had no discernable effect on mitosis, further confirming the specificity of their results. Together, these results establish a clear case against the theory of RhoA involvement in microtubule-chromosome attachment as a means to explain the participation of p190 in the process.

Evidence supporting the theory of a double-specificity p190RhoGAP is currently unavailable, yet several dual-specificity GTPase activating proteins, including MgcRacGAP, have been previously described. Interestingly, several comparisons can be made between these two GAPs that suggest the possibility that p190, like MgcRacGAP, may be able to act as a dual-specifity GAP in living cells. In addition to being able to regulate RhoGTP levels during cytokinesis, both proteins are able to associate with Ect2 during mitosis and their activity is in direct opposition to Ect2 (Zhao and Fang, 2005b;Mikawa et al., 2008), both are required for successful cytokinesis and improper regulation of their activity results in multinucleation (Zhao and Fang, 2005b;Su et al., 2003), and both associate with kinesins (Mishima et al., 2002). Furthermore, the specificity of MgcRacGAP for Cdc42 in metaphase reported by Yasuda and colleagues is switched to RhoGAP-specific activity by phosphorylation at Ser 387, mediated by Aurora B (Minoshima et al., 2003) during mitosis, hence proposing a mechanism by which the GTPase specificity of other GAPs, including p190, may be regulated. Unpublished results from a phosphoproteomic analysis performed in our laboratory to differentiate interphase from mitotic phosphorylation events in p190 have identified several putative ser/thr phosphorylation sites in the Middle Domain of p190 that are preferentially modified in mitosis. Intriguingly, several of these sites correspond to Aurora B consensus phosphorylation sequences. Other putative phosphorylation sites correspond to sequences modified by the cyclin B-cdk1 complex that serves as a major regulator of mitosis. The effect of post-translational modifications at each of these putative mitotic p190 phosphorylation sites is at present unknown, although an overall increase in Ser/Thr

phosphorylation in p190 has been reported (Maddox and Burridge, 2003) resulting in decreased p190RhoGAP activity. Whether increased p190 ser/thr phosphorylation results in greater affinity of p190 for Cdc42 is a fascinating possibility that merits further testing. Finally, in support of p190 functioning as a GAP for other GTPases in addition to RhoA, Settleman and colleagues reported nearly twenty years ago that, *in vitro*, p190 can function as a GAP for all three major RhoGTPases, RhoA, Rac and Cdc42 (Settleman et al., 1992a).

Numerous additional experiments are necessary to confirm or refute whether p190RhoGAP is a dual-specificity GAP in mitosis. Initial experiments could include Cdc42GTP-binding assays to detect active Cdc42 in mitotic samples after overexpression of either wild type p190 or the GAP inactive R1283A mutant. Additionally, co-overexpression of constitutively active Cdc42 and wt p190, in an attempt to rescue the chromosome congression abnormalities, would confirm whether p190 mediates the phenotype through modulation of Cdc42GTP levels. Furthermore, immunoprecipitation or binding assays between p190 and Cdc42 loaded with the non-hydolyzable γ-S GTP would offer additional information regarding the potential direct binding and effect of p190 specific to Cdc42. Together, the numerous similarities recounted between the function and regulation of p190 and MgcRacGAP provide precedent for the hypothesis that p190 may act as a dual-specificity GAP throughout the different stages of mitosis and provide a potential mechanism by which our experimental observations here reported may be explained (Figure 38B).

Initial reports have implicated Rho in the process of centrosome separation and

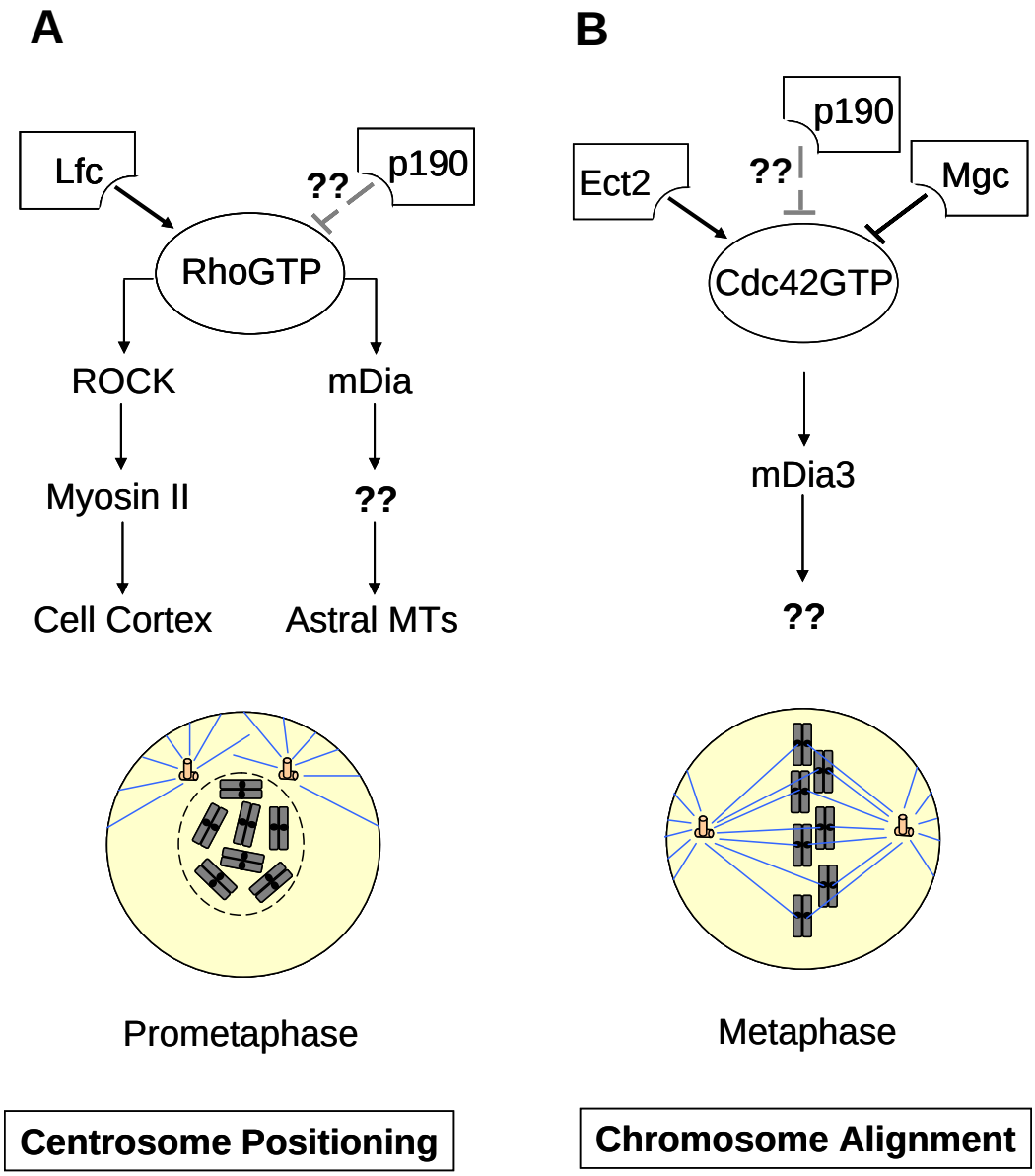

A
Lfc
??
p190
RhoGTP
ROCK
mDia
Myosin II
??
Cell Cortex
Astral MTs
Prometaphase
Centrosome Positioning
B
p190
Ect2
??
Mgc
Cdc42GTP
mDia3
??
Metaphase
Chromosome Alignment

Figure 38. A representation of the different cell division pathways which p190 may affect to induce cytokinesis failure in MDA-MB-468 cells

A) Rho is involved in astral microtubule interactions with the cell cortex in prometaphase. Lfc is a Rho GEF known to activate Rho in prometaphase. At that stage, Rho may target ROCK to phosphorylate and activate myosin II at the cell cortex and mDia at astral microtubules to direct proper centrosome separation and spindle orientation. p190 overexpression could negatively affect RhoGTP levels in prophase, disrupting the activation of downstream Rho effectors and resulting in improper centrosome separation. Thus, the phenotype observed in MBA-MD-468 cells could also be mediated in a Rho-specific manner.

B) Cdc42 is required for microtubule-kinetochore interactions and chromosomal alignment. Ect2, a dual-specificity GEF, activates Cdc42 in metaphase and Rho in telophase. Upon Ect2 activation during metaphase, Cdc42 acts on mDia3 at the kinetochores to facilitate biorientation and stabilization of attachment of the spindle microtubules (MTs) to the kinetochore. Bioriented and stabilized microtubule-kinetochore attachment ensures correct chromosomal alignment at the metaphase plate and segregation in anaphase. p190 overexpression could negatively affect Cdc42GTP levels in metaphase, disrupting the activation of downstream Rho effectors and resulting in improper chromosomal alignment and segregation. Thus, the phenotype observed in MBA-MD-468 cells may be mediated in a Cdc42-specific manner.

astral microtubule attachment to the cell cortex. Centrosome separation first occurs around the nuclear envelope at the onset of nuclear envelope breakdown and is dependent upon aster microtubule attachment to the cell cortex. A 2004 study examined signaling events downstream of Rho and their involvement in centrosome separation (Rosenblatt et al., 2004) and reported that prometaphase disruption of myosin II function by drugs such as blebbistatin or the ROCK inhibitor Y-27632 interferes with normal spindle assembly and positioning. Time-lapse movies revealed that these treatments block the separation and positioning of duplicated centrosomes after nuclear envelope breakdown, thereby preventing the migration of the microtubule asters to opposite sides of chromosomes. Defective spindles exhibited two main phenotypes, a "lopsided" phenotype in which chromosomes remain on one side of the asters and an "at the pole" phenotype where unaligned chromosomes are seen at one or both poles, similar to the phenotype we observe upon p190 overexpression in 468 cells. Immobilization of cortical movement produces similar spindle defects as myosin II disruption and dampens astral microtubule attachment to the cell cortex. This suggests that the previously reported Rho-dependent activity of myosin II (Maddox and Burridge, 2003) is required early in mitosis not only for cell rounding but also for centrosome separation and positioning. A proposed model of Rho function in centrosome separation is presented in Figure 38A.

To understand whether the mechanism by which p190 overexpression causes the chromosome misalignment phenotype in 468 cells by disrupting centrosome separation, it is necessary to perform time-lapse microscopy experiments where cells overexpressing fluorescently labeled p190 are followed through mitosis until phenotype onset. If the

phenotype is mediated by improper centrosome separation, microinjection of a constitutively active form of MLC2 could be performed to rescue or prevent the chromosomal misalignment phenotypes.

Interestingly, this phenotype was not observed in HeLa cells but was described in kangaroo rat kidney cells. Similarly, we did not observe the chromosome misalignment phenotype in HeLa cells, only in 468 breast cancer cells. The stark differences between phenotypes in the two cell lines we tested, HeLa and 468, invite questions regarding the inherent differences between the systems. It is unclear why such contrasting phenotypes are observed. However, when taking into consideration that both cell lines are aneuploid, transformed, tumorigenic and developed from primary cancer cells, it is certainly plausible that any of a number of mitotic checkpoint proteins may have altered expression or activity patterns that result in the particular ability of each cell line to present with vastly different phenotypes that highlight the separate mitotic events in which p190 participates. For this reason we find those inherent differences between cell lines to be quite useful as we endeavor to understand the mitotic role of p190RhoGAP.

Chapter 7

Summary and Perspectives

SUMMARY

Cell division, or cytokinesis, is the process by which the replicated DNA and organelles of a cell are equally divided to give rise to two daughter cells. Cytokinesis is observed in all cellular-based life, and thus it is an essential biological process. A cell that fails to divide and successfully complete this process becomes a binucelated, tetraploid cell, having twice the normal DNA content. At this junction, cells follow one of two potential fates: they activate appropriate checkpoints, become apoptotic and die or they begin down the pathway of tumorigenesis. In many human carcinomas, tetraploid cells arise as an early tumorigenic population and precede the emergence of aneuploid cells (Margolis et al., 2003). Aneuploidy and chromosomal instability are characteristic of the great majority of human cancers and are linked to the progressive development of high-grade, invasive tumors (Cahill et al., 1999). The mechanism by which tumor cells proceed through a precancerous tetraploid intermediate to aneuploidy is therefore of central scientific importance. Given its crucial role in the propagation and continuation of life, as well as in the prevention of tumorigenesis, a robust regulatory system has evolved to ensure the fidelity and reliability of cytokinesis. The work presented in this dissertation is designed to increase our understanding of said cytokinesis regulatory systems by investigating the modulatory events that direct the activity of the master regulator and orchestrator of cytokinesis, the small GTPase RhoA.

The main focus of this dissertation is to understand the important role that p190RhoGAP, a negative regulator of RhoA, plays during cytokinesis and how its proper regulation is crucial to successful cell division. The results herein presented are part of a

research effort that began several years ago when failed attempts to make stable-cell lines overexpressing p190 revealed that increased amounts of p190 were toxic to these cells. Further experimentation showed that, whereas p190 protein levels are consistent throughout most of the cell cycle, they are dramatically decreased during mitosis and this decrease is mediated by the ubiquitin-proteasome pathway. Moreover, overexpression of p190 results in cytokinesis failure, as determined by the emergence of binucleated, tetraploid cells (Su et al., 2003). These results were well aligned with cytokinesis models calling for increased RhoGTP levels during cytokinesis, the time when its negative regulator, p190, is degraded. Although p190 had been well characterized in its regulation of Rho activity in processes such as migration, adhesion and cell polarity, this report by Su and colleagues was the first to describe p190 as a negative regulator of cytokinesis. In fact, immunofluorescence analysis of p190 localization during cytokinesis placed it at the cleavage furrow, the central hub for cytokinesis events, where it colocalized with RhoA and actin. More recently, research from our laboratory demonstrated that the role of p190 in cytokinesis is in direct opposition to the function of the mitotic-specific RhoGEF Ect2 (Mikawa et al., 2008), specifically, negatively regulating levels of active Rho in the cleavage furrow during cytokinesis, ensuring successful cell division (Su et al., 2009).

The work in this dissertation provides further important details regarding the regulation of p190RhoGAP during cytokinesis. First, our findings show that p190 degradation is required for proper completion of cytokinesis given our observation that sustained endogenous p190 levels during mitosis result in an increase in multinucleated cells, indicative of cytokinesis failure. Preliminary data regarding the identity of the E3

ubiquitin ligase that targets p190 during mitosis suggests that the APC/C may be involved. Furthermore, we establish that p190 degradation is mediated through four N-terminal lysine residues, which could act as primary ubiquitin acceptors in a cellular context. We also show that increased p190 levels potentially affect cytokinesis through inactivation or mislocalization of myosin II normally found at the cleavage furrow and propose that an observed interaction between p190 and the molecular scaffold Anillin could be part of a mechanosensing switch regulating RhoA activity as a function of the contractile force generated across the cleavage furrow. Finally, we also discover a potential new role for p190 during cytokinesis, as a regulator of microtubule-chromosome interactions during early mitosis.

PERSPECTIVES

Clarification and further details on the regulation of p190 during cytokinesis

Work presented in this dissertation expands our understanding of p190 regulation by showing that binucleation and tetraploidy are not just results of the overexpression of p190 but occur due to inappropriately sustained levels of p190 at a point in the cell cycle when degradation is required. siRNA reconstitution experiments showed that when cells are unable to properly regulate and decrease p190 levels during mitosis, as was the case with cells reconstituted with the non-degradable ΔGBD p190 deletion mutant, they had a greater propensity to fail cytokinesis and become binucleated. Cells reconstituted with wild type p190, expressed at nearly endogenous levels, did not have this increased propensity for binucleation and were able to avoid cytokinesis failure. Results further

confirmed that it is the GAP activity of p190 which drives the multinucleation phenotype, as overexpression of the isolated GAP domain of p190 proved to be a strong inducer of muntinucleation while expression of a full length p190 molecule lacking GAP activity had no effect on cytokinesis failure. Together, these results confirmed and clarified the role that p190 regulation plays during cell division.

Several reports indicate that an increase in RhoA activity is necessary during cytokinesis (Maddox and Burridge, 2003;Yoshizaki et al., 2004). Interestingly, the literature has been focused on describing the role that the RhoGEF ECT2 plays in this activation event (Kimura et al., 2000a;Yuce et al., 2005;Nishimura and Yonemura, 2006;Chalamalasetty et al., 2006), paying little attention to the cyclical nature of Rho activity which requires the coordinated action of both GEFs and GAPs. Our experiments identify an additional mechanism to ensure that increased RhoGTP levels are achieved during cytokinesis, namely, by drastically decreasing the GAP levels of p190, a well known and powerful negative regulator of RhoGTP levels.

Further studies into the mechanisms of p190 regulation have identified four N-terminal lysines that are crucial for p190 degradation. Lysines play a central role as ubiquitin acceptors and, though shown to be central to p190 degradation, we have not confirmed their role as the primary ubiquitination sites of p190. In fact, using ubiquitination studies of deletion mutants missing the first N-terminal 83 amino acids of p190, we can attribute 40% of total p190 ubiquitination to these four N-terminal lysines. Though it would be important to assess the ability of these lysines to be ubiquitinated, such an experiment is fraught with complications because, to our present knowledge,

most of the N-terminal half of p190 is required for degradation and at the same time heavily ubiquitinated in vitro, making any interpretations of potential results nearly impossible.

The identification of the E3 ligase mediating the ubiquitination of p190 and the role of recognition sequences found in p190 would have further clarified the mechanism by which p190 is recognized and degraded during mitosis. Preliminary data outlined in Chapter 4 suggested that APC/C might function in that capacity. However, we also need to consider and test the possibility that other mitotic E3 ligases, such as Chfr or the Cul3/KLHL9/KLHL13 complex, may be involved in p190 degradation (Chaturvedi et al., 2002;Sumara et al., 2007). The initial set of experiments performed to test this possibility could include coimmunoprecipitation assays with Chfr, Cul3 or p190-specific antibodies. If p190 is a substrate for either of these ligases, this approach may prove effective, especially in light of published reports in the literature showing physical association between these ligases and their identified mitotic substrates. Moreover, shRNA-mediated silencing of these ligases could be employed to determine their *in vivo* involvement in p190 ubiquitination. *In vitro* testing could involve direct ubiquitination assays with purified components and radiolabeled p190. These assays would have a much higher likelihood of success because, unlike the APC/C complex which contains approximately 15 subunits, the Chfr is a single molecule ligase and the Cul3/KLHL9/KLHL13 consists of only three components. Then, it would be important to understand whether additional post-translational modifications of p190, such as ser/thr phosphorylation, are required for its ubiquitination. To this end, point mutations of the

putative cyclin B and Aurora B phosphorylation sites could be expressed in cells to test their involvement in mitotic p190 degradation. The process of E3 ligase identification is a challenging undertaking, and these suggestions could prove useful and important.

Clarification and further details on the role that p190 plays during cytokinesis

Subsequent work showed that the A isoform of p190, and not p190B, is involved in cytokinesis. We showed that p190B is not localized to the contractile ring during cytokinesis but rather remains cytosolic and dissociated from the membrane. Furthermore, the overexpression of p190B does not result in multinucleation. Though identical in domain structure, p190 proteins are only 50% homologous at the amino acid level with the lowest homology percentage associated with the Middle Domain which functions as the regulatory center for p190 function. A comparative deletional and chimeric study of p190A and B function, particularly through analysis and comparison of their Middle Domains, would provide a wealth of useful information as we try to understand the differences between two very similar proteins.

The mechanism by which p190 causes multinucleation has also been addressed by the work presented in this dissertation. Chapter 5 shows that myosin II activity at the cleavage furrow is decreased, either by loss of the activating phosphorylation or by myosin mislocalization, through p190-mediated decreases in Rho activity. Determining which scenario occurs would more definitively establish the role of myosin II in p190-induced multinucleation and studies have been proposed in chapter 5 to resolve this uncertainty. Some of those experiments include immunofluorescence microscopy

analysis of myosin II localization and activation in the same cell population. Furthermore, rescue of the multinucleation phenotype by co-overexpression of p190 and a constitutively active phosphomimetic MLC2 construct would assert the importance of myosin II activation more definitively.

Interestingly, these studies found little evidence for impaired actin organization at the cleavage furrow. It would be important to fill in the gaps in our understanding of this mechanism, given that multiple separate pathways initiate with Rho and end with the activation or inactivation of myosin II. For example, myosin II can be activated through ROCK by direct phosphorylation of its regulatory light chains. Myosin II can also be activated through direct phosphorylation by Citron kinase, a Rho effector, or by ROCK-dependent inactivation of Myosin Phosphatase. Therefore, assessment of the activation levels of these kinases after p190 overexpression would reveal interesting clues regarding the signaling pathways downstream of RhoA which are more sensitive to disturbances in RhoGTP levels. A potential initial approach to address this question would be to assess the mitotic localization of these proteins and measure the phosphorylation levels of several of their mitotic substrates. Additionally, the use of constitutively active forms of Citron and ROCK to rescue p190-induced multinucleation could reveal whether one or both kinases are involved and affected by decreased RhoGTP levels. Likewise, expression of a dominant negative form of myosin phosphatase could be used to analyze its contribution to the multinucleation phenotype.

The observed association between p190 and Anillin has proven to be a fascinating discovery, particularly in light of the sensitivity of this interaction to inhibition of

contractility by blebbistatin treatment. The data in Chapter 5 suggest that the interaction between p190 and the molecular scaffold Anillin may be the means by which p190 is localized and "anchored" at the cleavage furrow, where it is then well placed to carry out its regulatory functions toward RhoGTP enriched in that zone. Further details of this association could be gleaned from deletional analysis. For example, coimmunoprecipitation assays after the overexpression of various deletion mutants of Anillin together with full length p190, and vice versa, could identify the domains that are important in mediating the interaction between these proteins. Then, Anillin deletion mutants with impaired binding to p190 could be expressed in cells to asses whether the p190-Anillin interaction is the means by which p190 localizes to the cleavage furrow during cytokinesis. Furthermore, the sensitivity of this interaction to inhibition of myosin contractililty is also of very exciting consequences. If Anillin functions as the anchor for p190 localization to the cleavage furrow, and the interaction is sensitive to a contractility inhibitor, then their association could function as a means of detecting whether sufficient contractile force exists for the actomyosin ring to proceed with closure. Such a process could be ongoing until contraction is complete. Therefore, the p190-Anillin axis could act as a mechanosensing switch. Specifically, optimal RhoGTP levels are ensured by the mechanosensing nature of the p190-Anillin complex. When contraction of the CR is strong, p190 remains associated with Anillin to prevent further RhoGTP increases, overstimulation of the system and cytokinesis failure. If contraction at the cleavage furrow is weak, p190 dissociates from the Anillin-Rho complex allowing for greater Rho activation and resulting in increased contractility of the cleavage furrow.

Identification of a new mitotic role for p190 in chromosome alignment

Results described in Chapter 6 revealed that low level, inducible overexpression of p190 in a breast epithelial cancer cell line, MDA-MB-468, results in the incomplete alignment of chromosomes at the metaphase plate and that this phenotype is largely dependent on the GAP activity of p190. A similar defect has been observed in cells where the activity of Cdc42, or its downstream effector mDia3, has been inhibited (Yasuda et al., 2004). Because no metaphase function has been currently ascribed to RhoA and the GAP–induced, p190 overexpression phenotype in 468 cells is limited to metaphase, there exists a possibility that p190 may mediate the misalignment of metaphase chromosomes through regulation of Cdc42 activity levels.

Evidence supporting the theory of a double-specificity p190RhoGAP is currently unavailable, yet several dual-specificity GTPase activating proteins, including MgcRacGAP in mitosis, have been previously described. Intriguingly, the dual-specificity of MgcRacGAP is regulated by Aurora B phosphorylation (Minoshima et al., 2003). As described in Chapter 4, there are two putative Aurora B phosphorylation sites that are mitotic-specific. Given several other similarities between p190 and MgcRacGAP, described in Chapter 5, it is reasonable to imagine that a similar mechanism regulating p190 specificity may be in place.

Several experiments are required to test this "dual-specificity GAP" hypothesis and to test whether specificity is regulated by mitotic-specific phosphorylation effects. To test whether p190 has Cdc42GAP activity in mitosis, initial experiments could include the cotransfection of p190 and constitutively active Cdc42 in an effort to rescue the p190-

induced chromosome misalignment phenotype. Furthermore, the use of a Fluorescence Resonance Energy Transfer (FRET) biosensor to monitor CDC42 activity levels during metaphase could reveal whether Cdc42GTP levels are affected at a specific time and place by overexpression of wild type or dominant negative (R1283) p190RhoGAP. In addition, Cdc42GTP-binding assays to detect its activity levels across a synchronized cell population after overexpression of either wild type or dominant negative (R1283) p190RhoGAP could be used to measure the effect of the GAP activity of p190 on Cdc42GTP levels. Should any changes in Cdc42 activity be attributed to p190 overexpression, then the mechanism set in place to regulate its dual-specificity would be addressed. A natural place to begin this exploration would be to test whether Aurora B, or even cdk1, phosphorylation had any effect on the overall chromosome misalignment phenotype or in any of the specific assays described above. Because RhoGTPase function is highly compartmentalized during mitosis and cytokinesis, fluorescence confocal microscopy could be used to assess potential Cdc42 and p190 colocalization during metaphase using either microphotographs or live cell imaging.

An additional explanation for the chromosomal misalignment phenotype described in this dissertation was recently presented in initial reports that described the potential role of RhoA in centrosome separation. In these studies, when Rho-dependent myosin II activity was inhibited, centrosomes failed to separate properly and chromosomal misalignment ensued (Rosenblatt et al., 2004). Inhibition of myosin II activity yielded phenotypes where chromosomes remained on one side of the centrosomes or unaligned at one or both poles, in a similar manner as observed upon

p190 overexpression in 468 cells. To further test the possibility that p190 acts through Rho during prometaphase to induce the chromosomal misalignment phenotype, it is first necessary to ensure that the onset of this phenotype occurs early on during mitosis and that centrosome separation is impaired. This could be achieved through time-lapse microscopy of cells overexpressing fluorescently labeled p190. If the phenotype in fact occurs during early mitosis, microinjection of constitutively active forms of Rho, ROCK or myosin II into p190 overexpressing cells to prevent or rescue chromosomal misalignment could be carried out.

Together, the results presented in this dissertation increase our understanding of the regulatory processes necessary for proper mitosis and cytokinesis by clarifying the ways in which p190 is regulated and functions during this critical stage of the cell cycle. Furthermore, this work provides an exciting and new direction for future p190 research by describing its involvement in chromosome alignment during early mitosis and its potential role as a dual-specificity GAP during mitosis.

References

1. Ang, X.L. and H.J.Wade. 2005. SCF-mediated protein degradation and cell cycle control. *Oncogene* 24:2860-2870.

2. Arthur, W.T., L.A.Petch, and K.Burridge. 2000. Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism. *Curr. Biol.* 10:719-722.

3. Aspenstrom, P. 1999. Effectors for the Rho GTPases. *Curr. Opin. Cell Biol.* 11:95-102.

4. Balasubramanian, M.K., E.Bi, and M.Glotzer. 2004. Comparative analysis of cytokinesis in budding yeast, fission yeast and animal cells. *Curr. Biol.* 14:R806-R818.

5. Barker, T.H., H.E.Grenett, M.W.MacEwen, S.G.Tilden, G.M.Fuller, J.Settleman, A.Woods, J.Murphy-Ullrich, and J.S.Hagood. 2004. Thy-1 regulates fibroblast focal adhesions, cytoskeletal organization and migration through modulation of p190 RhoGAP and Rho GTPase activity. *Exp. Cell Res.* 295:488-496.

6. Barr, F.A. and U.Gruneberg. 2007. Cytokinesis: placing and making the final cut. *Cell* 131:847-860.

7. Bartolome, R.A., N.Wright, I.Molina-Ortiz, F.J.Sanchez-Luque, and J.Teixido. 2008. Activated G(alpha)13 impairs cell invasiveness through p190RhoGAP-mediated inhibition of RhoA activity. *Cancer Res.* 68:8221-8230.

8. Bass, M.D., M.R.Morgan, K.A.Roach, J.Settleman, A.B.Goryachev, and M.J.Humphries. 2008. p190RhoGAP is the convergence point of adhesion signals from alpha 5 beta 1 integrin and syndecan-4. *J Cell Biol.* 181:1013-1026.

9. Bedford, M.T. and P.Leder. 1999. The FF domain: a novel motif that often accompanies WW domains. *Trends Biochem. Sci* 24:264-265.

10. Bembenek, J. and H.Yu. 2001. Regulation of the anaphase-promoting complex by the dual specificity phosphatase human Cdc14a. *J Biol. Chem.* 276:48237-48242.

11. Bement, W.M., H.A.Benink, and D.G.von. 2005. A microtubule-dependent zone of active RhoA during cleavage plane specification. *J Cell Biol.* 170:91-101.

12. Bement, W.M., A.L.Miller, and D.G.von. 2006a. Rho GTPase activity zones and transient contractile arrays. *Bioessays* 28:983-993.

13. Bement, W.M., A.L.Miller, and D.G.von. 2006b. Rho GTPase activity zones and transient contractile arrays. *Bioessays* 28:983-993.

14. Billuart, P., C.G.Winter, A.Maresh, X.Zhao, and L.Luo. 2001. Regulating axon branch stability: the role of p190 RhoGAP in repressing a retraction signaling pathway. *Cell* 107:195-207.

15. Birukova, A.A., E.Alekseeva, A.Mikaelyan, and K.G.Birukov. 2007. HGF attenuates thrombin-induced endothelial permeability by Tiam1-mediated activation of the Rac pathway and by Tiam1/Rac-dependent inhibition of the Rho pathway. *FASEB J* 21:2776-2786.

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

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

18. Bouton, A.H., S.B.Kanner, R.R.Vines, and J.T.Parsons. 1991a. Tyrosine phosphorylation of three cellular proteins correlates with transformation of rat 1 cells by pp60src. *Mol. Carcinog.* 4:145-152.

19. Bouton, A.H., S.B.Kanner, R.R.Vines, H.C.Wang, J.B.Gibbs, and J.T.Parsons. 1991b. Transformation by pp60src or stimulation of cells with epidermal growth factor induces the stable association of tyrosine-phosphorylated cellular proteins with GTPase-activating protein. *Mol. Cell Biol.* 11:945-953.

20. Bradley, W.D., S.E.Hernandez, J.Settleman, and A.J.Koleske. 2006. Integrin signaling through Arg activates p190RhoGAP by promoting its binding to p120RasGAP and recruitment to the membrane. *Mol. Biol. Cell* 17:4827-4836.

21. Brandt, D., M.Gimona, M.Hillmann, H.Haller, and H.Mischak. 2002. Protein kinase C induces actin reorganization via a Src- and Rho-dependent pathway. *J Biol. Chem.* 277:20903-20910.

22. Bresnick, A.R., V.L.Wolff-Long, O.Baumann, and T.D.Pollard. 1995. Phosphorylation on threonine-18 of the regulatory light chain dissociates the ATPase and motor properties of smooth muscle myosin II. *Biochemistry* 34:12576-12583.

23. Brouns, M.R., S.F.Matheson, K.Q.Hu, I.Delalle, V.S.Caviness, J.Silver, R.T.Bronson, and J.Settleman. 2000. The adhesion signaling molecule p190 RhoGAP is required for morphogenetic processes in neural development. *Development* 127:4891-4903.

24. Brouns, M.R., S.F.Matheson, and J.Settleman. 2001. p190 RhoGAP is the principal Src substrate in brain and regulates axon outgrowth, guidance and fasciculation. *Nat. Cell Biol.* 3:361-367.

25. Bryant, S.S., S.Briggs, T.E.Smithgall, G.A.Martin, F.McCormick, J.H.Chang, S.J.Parsons, and R.Jove. 1995. Two SH2 domains of p120 Ras GTPase-activating protein bind synergistically to tyrosine phosphorylated p190 Rho GTPase-activating protein. *J Biol. Chem.* 270:17947-17952.

26. Burbelo, P.D., A.A.Finegold, C.A.Kozak, Y.Yamada, and H.Takami. 1998. Cloning, genomic organization and chromosomal assignment of the mouse p190-B gene. *Biochim. Biophys. Acta* 1443:203-210.

27. Burbelo, P.D., S.Miyamoto, A.Utani, S.Brill, K.M.Yamada, A.Hall, and Y.Yamada. 1995. p190-B, a new member of the Rho GAP family, and Rho are induced to cluster after integrin cross-linking. *J. Biol. Chem.* 270:30919-30926.

28. Burton, J.L., V.Tsakraklides, and M.J.Solomon. 2005. Assembly of an APC-Cdh1-substrate complex is stimulated by engagement of a destruction box. *Mol. Cell* 18:533-542.

29. Bustos, R.I., M.A.Forget, J.E.Settleman, and S.H.Hansen. 2008. Coordination of Rho and Rac GTPase function via p190B RhoGAP. *Curr. Biol.* 18:1606-1611.

30. Cahill, D.P., K.W.Kinzler, B.Vogelstein, and C.Lengauer. 1999. Genetic instability and darwinian selection in tumours. *Trends Cell Biol.* 9:M57-M60.

31. Carlier, M.F., V.Laurent, J.Santolini, R.Melki, D.Didry, G.X.Xia, Y.Hong, N.H.Chua, and D.Pantaloni. 1997. Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *J Cell Biol.* 136:1307-1322.

32. Chackalaparampil, I. and D.Shalloway. 1988. Altered phosphorylation and activation of pp60c-src during fibroblast mitosis. *Cell* 52:801-810.

33. Chakravarty, G., D.Hadsell, W.Buitrago, J.Settleman, and J.M.Rosen. 2003. p190-B RhoGAP regulates mammary ductal morphogenesis. *Mol. Endocrinol.* 17:1054-1065.

34. Chalamalasetty, R.B., S.Hummer, E.A.Nigg, and H.H.Sillje. 2006. Influence of human Ect2 depletion and overexpression on cleavage furrow formation and abscission. *J Cell Sci* 119:3008-3019.

35. Chang, F. and M.Peter. 2002. Cell biology. Formins set the record straight. *Science* 297:531-532.

36. Chang, J.H., S.Gill, J.Settleman, and S.J.Parsons. 1995. c-Src regulates the simultaneous rearrangement of actin cytoskeleton, p190RhoGAP, and p120RasGAP following epidermal growth factor stimulation. *J Cell Biol.* 130:355-368.

37. Chang, J.H., L.K.Wilson, J.S.Moyers, K.Zhang, and S.J.Parsons. 1993. Increased levels of p21ras-GTP and enhanced DNA synthesis accompany elevated tyrosyl phosphorylation of GAP-associated proteins, p190 and p62, in c-src overexpressors. *Oncogene* 8:959-967.

38. Chaturvedi, P., V.Sudakin, M.L.Bobiak, P.W.Fisher, M.R.Mattern, S.A.Jablonski, M.R.Hurle, Y.Zhu, T.J.Yen, and B.B.Zhou. 2002. Chfr regulates a mitotic stress pathway through its RING-finger domain with ubiquitin ligase activity. *Cancer Res.* 62:1797-1801.

39. Chen, J.C., S.Zhuang, T.H.Nguyen, G.R.Boss, and R.B.Pilz. 2003. Oncogenic Ras leads to Rho activation by activating the mitogen-activated protein kinase pathway and decreasing Rho-GTPase-activating protein activity. *J Biol. Chem.* 278:2807-2818.

40. Cockman, M.E., N.Masson, D.R.Mole, P.Jaakkola, G.W.Chang, S.C.Clifford, E.R.Maher, C.W.Pugh, P.J.Ratcliffe, and P.H.Maxwell. 2000. Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol. Chem.* 275:25733-25741.

41. Continolo, S., A.Baruzzi, M.Majeed, E.Caveggion, L.Fumagalli, C.A.Lowell, and G.Berton. 2005. The proto-oncogene Fgr regulates cell migration and this requires its plasma membrane localization. *Exp. Cell Res.* 302:253-269.

42. Drechsel, D.N., A.A.Hyman, A.Hall, and M.Glotzer. 1997. A requirement for Rho and Cdc42 during cytokinesis in Xenopus embryos. *Curr. Biol.* 7:12-23.

43. Effler, J.C., P.A.Iglesias, and D.N.Robinson. 2007. A mechanosensory system controls cell shape changes during mitosis. *Cell Cycle* 6:30-35.

44. Ellis, C., X.Q.Liu, D.Anderson, N.Abraham, A.Veillette, and T.Pawson. 1991. Tyrosine phosphorylation of GAP and GAP-associated proteins in lymphoid and fibroblast cells expressing lck. *Oncogene* 6:895-901.

45. Ellis, C., M.Moran, F.McCormick, and T.Pawson. 1990. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. *Nature* 343:377-381.

46. Elsasser, S. and D.Finley. 2005. Delivery of ubiquitinated substrates to protein-unfolding machines. *Nat. Cell Biol.* 7:742-749.

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

48. Fang, G., H.Yu, and M.W.Kirschner. 1998. Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. *Mol. Cell* 2:163-171.

49. Field, C.M. and B.M.Alberts. 1995. Anillin, a contractile ring protein that cycles from the nucleus to the cell cortex. *J Cell Biol.* 131:165-178.

50. Foster, R., K.Q.Hu, D.A.Shaywitz, and J.Settleman. 1994. p190 RhoGAP, the major RasGAP-associated protein, binds GTP directly. *Mol. Cell Biol.* 14:7173-7181.

51. Galisteo, M.L., I.Dikic, A.G.Batzer, W.Y.Langdon, and J.Schlessinger. 1995. Tyrosine phosphorylation of the c-cbl proto-oncogene protein product and association with epidermal growth factor (EGF) receptor upon EGF stimulation. *J Biol. Chem.* 270:20242-20245.

52. Glickman, M.H. and A.Ciechanover. 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev.* 82:373-428.

53. Glotzer, M. 2001. Animal cell cytokinesis. *Annu. Rev. Cell Dev. Biol.* 17:351-386.

54. Glotzer, M., A.W.Murray, and M.W.Kirschner. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature* 349:132-138.

55. Golan, A., Y.Yudkovsky, and A.Hershko. 2002. The cyclin-ubiquitin ligase activity of cyclosome/APC is jointly activated by protein kinases Cdk1-cyclin B and Plk. *J Biol. Chem.* 277:15552-15557.

56. Grande-Garcia, A., A.Echarri, R.J.de, N.B.Alderson, C.M.Waterman-Storer, J.M.Valdivielso, and M.A.del Pozo. 2007. Caveolin-1 regulates cell polarization and directional migration through Src kinase and Rho GTPases. *J Cell Biol.* 177:683-694.

57. Grovdal, L.M., E.Stang, A.Sorkin, and I.H.Madshus. 2004. Direct interaction of Cbl with pTyr 1045 of the EGF receptor (EGFR) is required to sort the EGFR to lysosomes for degradation. *Exp. Cell Res.* 300:388-395.

58. Guegan, F., F.Tatin, T.Leste-Lasserre, G.Drutel, E.Genot, and V.Moreau. 2008. p190B RhoGAP regulates endothelial-cell-associated proteolysis through MT1-MMP and MMP2. *J Cell Sci* 121:2054-2061.

59. Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science* 279:509-514.

60. Hall, A. and C.D.Nobes. 2000. Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. *Philos. Trans. R. Soc. Lond B Biol. Sci* 355:965-970.

61. Haskell, M.D., A.L.Nickles, J.M.Agati, L.Su, B.D.Dukes, and S.J.Parsons. 2001. Phosphorylation of p190 on Tyr1105 by c-Src is necessary but not sufficient for EGF-induced actin disassembly in C3H10T1/2 fibroblasts. *J Cell Sci* 114:1699-1708.

62. Heckman, B.M., G.Chakravarty, T.Vargo-Gogola, M.Gonzales-Rimbau, D.L.Hadsell, A.V.Lee, J.Settleman, and J.M.Rosen. 2007. Crosstalk between the p190-B RhoGAP and IGF signaling pathways is required for embryonic mammary bud development. *Dev. Biol.* 309:137-149.

63. Heinemeyer, W., M.Fischer, T.Krimmer, U.Stachon, and D.H.Wolf. 1997. The active sites of the eukaryotic 20 S proteasome and their involvement in subunit precursor processing. *J Biol. Chem.* 272:25200-25209.

64. Herbrand, U. and M.R.Ahmadian. 2006. p190-RhoGAP as an integral component of the Tiam1/Rac1-induced downregulation of Rho. *Biol. Chem.* 387:311-317.

65. Hernandez, S.E., J.Settleman, and A.J.Koleske. 2004. Adhesion-dependent regulation of p190RhoGAP in the developing brain by the Abl-related gene tyrosine kinase. *Curr. Biol.* 14:691-696.

66. Hirano, K., D.N.Derkach, M.Hirano, J.Nishimura, and H.Kanaide. 2003. Protein kinase network in the regulation of phosphorylation and dephosphorylation of smooth muscle myosin light chain. *Mol. Cell Biochem.* 248:105-114.

67. Holinstat, M., N.Knezevic, M.Broman, A.M.Samarel, A.B.Malik, and D.Mehta. 2006. Suppression of RhoA activity by focal adhesion kinase-induced activation of p190RhoGAP: role in regulation of endothelial permeability. *J Biol. Chem.* 281:2296-2305.

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

69. Huot, M.E., C.M.Brown, N.Lamarche-Vane, and S.Richard. 2009. An adaptor role for cytoplasmic Sam68 in modulating Src activity during cell polarization. *Mol. Cell Biol.* 29:1933-1943.

70. Ichetovkin, I., J.Han, K.M.Pang, D.A.Knecht, and J.S.Condeelis. 2000. Actin filaments are severed by both native and recombinant dictyostelium cofilin but to different extents. *Cell Motil. Cytoskeleton* 45:293-306.

71. Ito, M., T.Nakano, F.Erdodi, and D.J.Hartshorne. 2004. Myosin phosphatase: structure, regulation and function. *Mol. Cell Biochem.* 259:197-209.

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

73. Jantsch-Plunger, V., P.Gonczy, A.Romano, H.Schnabel, D.Hamill, R.Schnabel, A.A.Hyman, and M.Glotzer. 2000. CYK-4: A Rho family gtpase activating protein (GAP) required for central spindle formation and cytokinesis. *J Cell Biol.* 149:1391-1404.

74. Jiang, W., M.Betson, R.Mulloy, R.Foster, M.Levay, E.Ligeti, and J.Settleman. 2008. p190A RhoGAP Is a Glycogen Synthase Kinase-3-{beta} Substrate Required for Polarized Cell Migration. *J Biol. Chem.* 283:20978-20988.

75. Jiang, W., R.Sordella, G.C.Chen, S.Hakre, A.L.Roy, and J.Settleman. 2005. An FF domain-dependent protein interaction mediates a signaling pathway for growth factor-induced gene expression. *Mol. Cell* 17:23-35.

76. Kasahara, K., Y.Nakayama, Y.Nakazato, K.Ikeda, T.Kuga, and N.Yamaguchi. 2007. Src signaling regulates completion of abscission in cytokinesis through ERK/MAPK activation at the midbody. *J Biol. Chem.* 282:5327-5339.

77. Kimura, K., T.Tsuji, Y.Takada, T.Miki, and S.Narumiya. 2000b. Accumulation of GTP-bound RhoA during cytokinesis and a critical role of ECT2 in this accumulation. *J Biol. Chem.* 275:17233-17236.

78. Kimura, K., T.Tsuji, Y.Takada, T.Miki, and S.Narumiya. 2000a. Accumulation of GTP-bound RhoA during cytokinesis and a critical role of ECT2 in this accumulation. *J Biol. Chem.* 275:17233-17236.

79. King, R.W., M.Glotzer, and M.W.Kirschner. 1996. Mutagenic analysis of the destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates. *Mol. Biol. Cell* 7:1343-1357.

80. Kishi, K., T.Sasaki, S.Kuroda, T.Itoh, and Y.Takai. 1993. Regulation of cytoplasmic division of Xenopus embryo by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI). *J Cell Biol.* 120:1187-1195.

81. Koehler, J.A. and M.F.Moran. 2001. Regulation of extracellular signal-regulated kinase activity by p120 RasGAP does not involve its pleckstrin homology or calcium-dependent lipid binding domains but does require these domains to regulate cell proliferation. *Cell Growth Differ.* 12:551-561.

82. Kosako, H., T.Yoshida, F.Matsumura, T.Ishizaki, S.Narumiya, and M.Inagaki. 2000. Rho-kinase/ROCK is involved in cytokinesis through the phosphorylation of myosin light chain and not ezrin/radixin/moesin proteins at the cleavage furrow. *Oncogene* 19:6059-6064.

83. Kraft, C., H.C.Vodermaier, S.Maurer-Stroh, F.Eisenhaber, and J.M.Peters. 2005. The WD40 propeller domain of Cdh1 functions as a destruction box receptor for APC/C substrates. *Mol. Cell* 18:543-553.

84. Kuga, T., Y.Nakayama, M.Hoshino, Y.Higashiyama, Y.Obata, D.Matsuda, K.Kasahara, Y.Fukumoto, and N.Yamaguchi. 2007. Differential mitotic activation of endogenous c-Src, c-Yes, and Lyn in HeLa cells. *Arch. Biochem. Biophys.* 466:116-124.

85. Lahav-Baratz, S., V.Sudakin, J.V.Ruderman, and A.Hershko. 1995. Reversible phosphorylation controls the activity of cyclosome-associated cyclin-ubiquitin ligase. *Proc. Natl. Acad. Sci U. S. A* 92:9303-9307.

86. Li, R., B.Zhang, and Y.Zheng. 1997. Structural determinants required for the interaction between Rho GTPase and the GTPase-activating domain of p190. *J Biol. Chem.* 272:32830-32835.

87. Liang, X., N.A.Draghi, and M.D.Resh. 2004. Signaling from integrins to Fyn to Rho family GTPases regulates morphologic differentiation of oligodendrocytes. *J Neurosci.* 24:7140-7149.

88. Ludwig,K., S.A.Sanchez Manchinelly, L.Su, M.Mikawa, and S.J.Parsons. p190RhoGAP-A. UCSD-Nature Molecule Pages . 2009. UCSD and Nature Publishing Group.
Ref Type: Electronic Citation

89. Mabuchi, I., Y.Hamaguchi, H.Fujimoto, N.Morii, M.Mishima, and S.Narumiya. 1993. A rho-like protein is involved in the organisation of the contractile ring in dividing sand dollar eggs. *Zygote.* 1:325-331.

90. Maddox, A.S. and K.Burridge. 2003. RhoA is required for cortical retraction and rigidity during mitotic cell rounding. *J Cell Biol.* 160:255-265.

91. Mammoto, A., K.M.Connor, T.Mammoto, C.W.Yung, D.Huh, C.M.Aderman, G.Mostoslavsky, L.E.Smith, and D.E.Ingber. 2009. A mechanosensitive transcriptional mechanism that controls angiogenesis. *Nature* 457:1103-1108.

92. Mammoto, A., S.Huang, and D.E.Ingber. 2007. Filamin links cell shape and cytoskeletal structure to Rho regulation by controlling accumulation of p190RhoGAP in lipid rafts. *J Cell Sci* 120:456-467.

93. Margolis, R.L., O.D.Lohez, and P.R.Andreassen. 2003. G1 tetraploidy checkpoint and the suppression of tumorigenesis. *J Cell Biochem.* 88:673-683.

94. Matheson, S.F., K.Q.Hu, M.R.Brouns, R.Sordella, J.D.VanderHeide, and J.Settleman. 2006. Distinct but overlapping functions for the closely related p190 RhoGAPs in neural development. *Dev. Neurosci.* 28:538-550.

95. Matsumura, F., G.Totsukawa, Y.Yamakita, and S.Yamashiro. 2001. Role of myosin light chain phosphorylation in the regulation of cytokinesis. *Cell Struct. Funct.* 26:639-644.

96. Mikawa, M., L.Su, and S.J.Parsons. 2008. Opposing roles of p190RhoGAP and Ect2 RhoGEF in regulating cytokinesis. *Cell Cycle* 7:2003-2012.

97. Miller, A.L., D.G.von, and W.M.Bement. 2008. Control of the cytokinetic apparatus by flux of the Rho GTPases. *Biochem. Soc. Trans.* 36:378-380.

98. Miller, L.D., K.C.Lee, D.Mochly-Rosen, and C.A.Cartwright. 2004. RACK1 regulates Src-mediated Sam68 and p190RhoGAP signaling. *Oncogene* 23:5682-5686.

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

100. Mishima, M., S.Kaitna, and M.Glotzer. 2002. Central spindle assembly and cytokinesis require a kinesin-like protein/RhoGAP complex with microtubule bundling activity. *Dev. Cell* 2:41-54.

101. Moasser, M.M., M.Srethapakdi, K.S.Sachar, A.J.Kraker, and N.Rosen. 1999. Inhibition of Src kinases by a selective tyrosine kinase inhibitor causes mitotic arrest. *Cancer Res.* 59:6145-6152.

102. Montagnoli, A., F.Fiore, E.Eytan, A.C.Carrano, G.F.Draetta, A.Hershko, and M.Pagano. 1999. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. *Genes Dev.* 13:1181-1189.

103. Moran, M.F., P.Polakis, F.McCormick, T.Pawson, and C.Ellis. 1991. Protein-tyrosine kinases regulate the phosphorylation, protein interactions, subcellular distribution, and activity of p21ras GTPase-activating protein. *Mol. Cell Biol.* 11:1804-1812.

104. Mori, K., M.Amano, M.Takefuji, K.Kato, Y.Morita, T.Nishioka, Y.Matsuura, T.Murohara, and K.Kaibuchi. 2009. Rho-kinase contributes to sustained RhoA

activation through phosphorylation of p190A RhoGAP. *J Biol. Chem.* 284:5067-5076.

105. Murray, A.W. 2004. Recycling the cell cycle: cyclins revisited. *Cell* 116:221-234.

106. Nakahara, H., S.C.Mueller, M.Nomizu, Y.Yamada, Y.Yeh, and W.T.Chen. 1998. Activation of beta1 integrin signaling stimulates tyrosine phosphorylation of p190RhoGAP and membrane-protrusive activities at invadopodia. *J Biol. Chem.* 273:9-12.

107. Nandi, D., P.Tahiliani, A.Kumar, and D.Chandu. 2006. The ubiquitin-proteasome system. *J Biosci.* 31:137-155.

108. Narumiya, S. and S.Yasuda. 2006. Rho GTPases in animal cell mitosis. *Curr. Opin. Cell Biol.* 18:199-205.

109. Nemoto, Y., T.Namba, S.Kozaki, and S.Narumiya. 1991. Clostridium botulinum C3 ADP-ribosyltransferase gene. Cloning, sequencing, and expression of a functional protein in Escherichia coli. *J Biol. Chem.* 266:19312-19319.

110. Ng, M.M., F.Chang, and D.R.Burgess. 2005. Movement of membrane domains and requirement of membrane signaling molecules for cytokinesis. *Dev. Cell* 9:781-790.

111. Nimnual, A.S., L.J.Taylor, and D.Bar-Sagi. 2003. Redox-dependent downregulation of Rho by Rac. *Nat. Cell Biol.* 5:236-241.

112. Nishimura, Y., K.Nakano, and I.Mabuchi. 1998b. Localization of Rho GTPase in sea urchin eggs. *FEBS Lett.* 441:121-126.

113. Nishimura, Y., K.Nakano, and I.Mabuchi. 1998a. Localization of Rho GTPase in sea urchin eggs. *FEBS Lett.* 441:121-126.

114. Nishimura, Y. and S.Yonemura. 2006. Centralspindlin regulates ECT2 and RhoA accumulation at the equatorial cortex during cytokinesis. *J Cell Sci* 119:104-114.

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

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

117. O'Connell, C.B., S.P.Wheatley, S.Ahmed, and Y.L.Wang. 1999. The small GTP-binding protein rho regulates cortical activities in cultured cells during division. *J Cell Biol.* 144:305-313.

118. Oceguera-Yanez, F., K.Kimura, S.Yasuda, C.Higashida, T.Kitamura, Y.Hiraoka, T.Haraguchi, and S.Narumiya. 2005. Ect2 and MgcRacGAP regulate the activation and function of Cdc42 in mitosis. *J Cell Biol.* 168:221-232.

119. Oegema, K., M.S.Savoian, T.J.Mitchison, and C.M.Field. 2000. Functional analysis of a human homologue of the Drosophila actin binding protein anillin suggests a role in cytokinesis. *J Cell Biol.* 150:539-552.

120. Palmi, C., G.Fazio, A.Cassetti, A.Aloisi, A.Villa, A.Biondi, and G.Cazzaniga. 2006. TEL/ARG induces cytoskeletal abnormalities in 293T cells. *Cancer Lett.* 241:79-86.

121. Papakonstanti, E.A., A.J.Ridley, and B.Vanhaesebroeck. 2007. The p110delta isoform of PI 3-kinase negatively controls RhoA and PTEN. *EMBO J* 26:3050-3061.

122. Peters, J.M. 2002. The anaphase-promoting complex: proteolysis in mitosis and beyond. *Mol. Cell* 9:931-943.

123. Peters, J.M. 2006. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat. Rev. Mol. Cell Biol.* 7:644-656.

124. Pfleger, C.M. and M.W.Kirschner. 2000. The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes Dev.* 14:655-665.

125. Pickart, C.M. 2001a. Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* 70:503-533.

126. Pickart, C.M. 2001b. Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* 70:503-533.

127. Piekny, A., M.Werner, and M.Glotzer. 2005. Cytokinesis: welcome to the Rho zone. *Trends Cell Biol.* 15:651-658.

128. Piekny, A.J. and M.Glotzer. 2008. Anillin is a scaffold protein that links RhoA, actin, and myosin during cytokinesis. *Curr. Biol.* 18:30-36.

129. Playford, M.P., K.Vadali, X.Cai, K.Burridge, and M.D.Schaller. 2008. Focal adhesion kinase regulates cell-cell contact formation in epithelial cells via modulation of Rho. *Exp. Cell Res.* 314:3187-3197.

130. Pollard, T.D., W.F.Stafford, and M.E.Porter. 1978. Characterization of a second myosin from Acanthamoeba castellanii. *J Biol. Chem.* 253:4798-4808.

131. Poperechnaya, A., O.Varlamova, P.J.Lin, J.T.Stull, and A.R.Bresnick. 2000. Localization and activity of myosin light chain kinase isoforms during the cell cycle. *J Cell Biol.* 151:697-708.

132. Prokopenko, S.N., A.Brumby, L.O'Keefe, L.Prior, Y.He, R.Saint, and H.J.Bellen. 1999. A putative exchange factor for Rho1 GTPase is required for initiation of cytokinesis in Drosophila. *Genes Dev.* 13:2301-2314.

133. Rattan, S., R.N.Puri, and Y.P.Fan. 2003. Involvement of rho and rho-associated kinase in sphincteric smooth muscle contraction by angiotensin II. *Exp. Biol. Med. (Maywood.)* 228:972-981.

134. Ridley, A.J. and A.Hall. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70:389-399.

135. Ridley, A.J., H.F.Paterson, C.L.Johnston, D.Diekmann, and A.Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 70:401-410.

136. Ridley, A.J., A.J.Self, F.Kasmi, H.F.Paterson, A.Hall, C.J.Marshall, and C.Ellis. 1993. rho family GTPase activating proteins p190, bcr and rhoGAP show distinct specificities in vitro and in vivo. *EMBO J* 12:5151-5160.

137. Roof, R.W., B.D.Dukes, J.H.Chang, and S.J.Parsons. 2000. Phosphorylation of the p190 RhoGAP N-terminal domain by c-Src results in a loss of GTP binding activity. *FEBS Lett.* 472:117-121.

138. Roof, R.W., M.D.Haskell, B.D.Dukes, N.Sherman, M.Kinter, and S.J.Parsons. 1998. Phosphotyrosine (p-Tyr)-dependent and -independent mechanisms of p190 RhoGAP-p120 RasGAP interaction: Tyr 1105 of p190, a substrate for c-Src, is the sole p-Tyr mediator of complex formation. *Mol. Cell Biol.* 18:7052-7063.

139. Rosenblatt, J., L.P.Cramer, B.Baum, and K.M.McGee. 2004. Myosin II-dependent cortical movement is required for centrosome separation and positioning during mitotic spindle assembly. *Cell* 117:361-372.

140. Ruchaud, S., M.Carmena, and W.C.Earnshaw. 2007. Chromosomal passengers: conducting cell division. *Nat. Rev. Mol. Cell Biol.* 8:798-812.

141. Sanchez Manchinelly,S.A., J.Agati Miller, L.Su, M.Mikawa, and S.J.Parsons. 2010. Mitotic Downregulation of p190RhoGAP Is Required for the Successful Completion of Cytokinesis. *Submitted*.

142. Sastry, S.K., Z.Rajfur, B.P.Liu, J.F.Cote, M.L.Tremblay, and K.Burridge. 2006. PTP-PEST couples membrane protrusion and tail retraction via VAV2 and p190RhoGAP. *J Biol. Chem.* 281:11627-11636.

143. Settleman, J., C.F.Albright, L.C.Foster, and R.A.Weinberg. 1992b. Association between GTPase activators for Rho and Ras families. *Nature* 359:153-154.

144. Settleman, J., C.F.Albright, L.C.Foster, and R.A.Weinberg. 1992a. Association between GTPase activators for Rho and Ras families. *Nature* 359:153-154.

145. Settleman, J., V.Narasimhan, L.C.Foster, and R.A.Weinberg. 1992c. Molecular cloning of cDNAs encoding the GAP-associated protein p190: implications for a signaling pathway from ras to the nucleus. *Cell* 69:539-549.

146. Sfakianos, M.K., A.Eisman, S.L.Gourley, W.D.Bradley, A.J.Scheetz, J.Settleman, J.R.Taylor, C.A.Greer, A.Williamson, and A.J.Koleske. 2007. Inhibition of Rho via Arg and p190RhoGAP in the postnatal mouse hippocampus regulates dendritic spine maturation, synapse and dendrite stability, and behavior. *J Neurosci.* 27:10982-10992.

147. Shen, C.H., H.Y.Chen, M.S.Lin, F.Y.Li, C.C.Chang, M.L.Kuo, J.Settleman, and R.H.Chen. 2008. Breast tumor kinase phosphorylates p190RhoGAP to regulate rho and ras and promote breast carcinoma growth, migration, and invasion. *Cancer Res.* 68:7779-7787.

148. Shimizu, A., A.Mammoto, J.E.Italiano, Jr., E.Pravda, A.C.Dudley, D.E.Ingber, and M.Klagsbrun. 2008. ABL2/ARG tyrosine kinase mediates SEMA3F-induced RhoA inactivation and cytoskeleton collapse in human glioma cells. *J Biol. Chem.* 283:27230-27238.

149. Shinohara, M., W.H.Shang, M.Kubodera, S.Harada, J.Mitsushita, M.Kato, H.Miyazaki, H.Sumimoto, and T.Kamata. 2007. Nox1 redox signaling mediates oncogenic Ras-induced disruption of stress fibers and focal adhesions by down-regulating Rho. *J Biol. Chem.* 282:17640-17648.

150. Shiota, M., T.Tanihiro, Y.Nakagawa, N.Aoki, N.Ishida, K.Miyazaki, A.Ullrich, and H.Miyazaki. 2003. Protein tyrosine phosphatase PTP20 induces actin cytoskeleton reorganization by dephosphorylating p190 RhoGAP in rat ovarian granulosa cells stimulated with follicle-stimulating hormone. *Mol. Endocrinol.* 17:534-549.

151. Shteinberg, M., Y.Protopopov, T.Listovsky, M.Brandeis, and A.Hershko. 1999. Phosphorylation of the cyclosome is required for its stimulation by Fizzy/cdc20. *Biochem. Biophys. Res. Commun.* 260:193-198.

152. Sordella, R., M.Classon, K.Q.Hu, S.F.Matheson, M.R.Brouns, B.Fine, L.Zhang, H.Takami, Y.Yamada, and J.Settleman. 2002. Modulation of CREB activity by the Rho GTPase regulates cell and organism size during mouse embryonic development. *Dev. Cell* 2:553-565.

153. Sordella, R., W.Jiang, G.C.Chen, M.Curto, and J.Settleman. 2003. Modulation of Rho GTPase signaling regulates a switch between adipogenesis and myogenesis. *Cell* 113:147-158.

154. Straight, A.F., C.M.Field, and T.J.Mitchison. 2005. Anillin binds nonmuscle myosin II and regulates the contractile ring. *Mol. Biol. Cell* 16:193-201.

155. Su, L., J.M.Agati, and S.J.Parsons. 2003. p190RhoGAP is cell cycle regulated and affects cytokinesis. *J Cell Biol.* 163:571-582.

156. Su, L., O.Pertz, M.Mikawa, K.Hahn, and S.J.Parsons. 2009. p190RhoGAP negatively regulates Rho activity at the cleavage furrow of mitotic cells. *Exp. Cell Res.*

157. Sumara, I., M.Quadroni, C.Frei, M.H.Olma, G.Sumara, R.Ricci, and M.Peter. 2007. A Cul3-based E3 ligase removes Aurora B from mitotic chromosomes, regulating mitotic progression and completion of cytokinesis in human cells. *Dev. Cell* 12:887-900.

158. Takaishi, K., T.Sasaki, T.Kameyama, S.Tsukita, S.Tsukita, and Y.Takai. 1995. Translocation of activated Rho from the cytoplasm to membrane ruffling area, cell-cell adhesion sites and cleavage furrows. *Oncogene* 11:39-48.

159. Talis, A.L., J.M.Huibregtse, and P.M.Howley. 1998. The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. *J Biol. Chem.* 273:6439-6445.

160. Tamura, H., M.Fukada, A.Fujikawa, and M.Noda. 2006. Protein tyrosine phosphatase receptor type Z is involved in hippocampus-dependent memory formation through dephosphorylation at Y1105 on p190 RhoGAP. *Neurosci. Lett.* 399:33-38.

161. Tatsis, N., D.A.Lannigan, and I.G.Macara. 1998. The function of the p190 Rho GTPase-activating protein is controlled by its N-terminal GTP binding domain. *J Biol. Chem.* 273:34631-34638.

162. Tatsumoto, T., X.Xie, R.Blumenthal, I.Okamoto, and T.Miki. 1999. Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. *J Cell Biol.* 147:921-928.

163. Tikoo, A., S.Czekay, C.Viars, S.White, J.K.Heath, K.Arden, and H.Maruta. 2000. p190-A, a human tumor suppressor gene, maps to the chromosomal region 19q13.3 that is reportedly deleted in some gliomas. *Gene* 257:23-31.

164. Trouliaris, S., U.Smola, J.H.Chang, S.J.Parsons, H.Niemann, and T.Tamura. 1995. Tyrosine 807 of the v-Fms oncogene product controls cell morphology and association with p120RasGAP. *J Virol.* 69:6010-6020.

165. Tsubouchi, A., J.Sakakura, R.Yagi, Y.Mazaki, E.Schaefer, H.Yano, and H.Sabe. 2002. Localized suppression of RhoA activity by Tyr31/118-phosphorylated paxillin in cell adhesion and migration. *J Cell Biol.* 159:673-683.

166. Vader, G. and S.M.Lens. 2008. The Aurora kinase family in cell division and cancer. *Biochim. Biophys. Acta* 1786:60-72.

167. van der, G.P., M.Henkemeyer, T.Jacks, and T.Pawson. 1997. Aberrant Ras regulation and reduced p190 tyrosine phosphorylation in cells lacking p120-Gap. *Mol. Cell Biol.* 17:1840-1847.

168. Vargo-Gogola, T., B.M.Heckman, E.J.Gunther, L.A.Chodosh, and J.M.Rosen. 2006. P190-B Rho GTPase-activating protein overexpression disrupts ductal morphogenesis and induces hyperplastic lesions in the developing mammary gland. *Mol. Endocrinol.* 20:1391-1405.

169. Vavylonis, D., J.Q.Wu, S.Hao, B.O'Shaughnessy, and T.D.Pollard. 2008. Assembly mechanism of the contractile ring for cytokinesis by fission yeast. *Science* 319:97-100.

170. Voges, D., P.Zwickl, and W.Baumeister. 1999. The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu. Rev. Biochem.* 68:1015-1068.

171. Watanabe, S., Y.Ando, S.Yasuda, H.Hosoya, N.Watanabe, T.Ishizaki, and S.Narumiya. 2008. mDia2 Induces the Actin Scaffold for the Contractile Ring and Stabilizes Its Position during Cytokinesis in NIH 3T3 Cells. *Mol. Biol. Cell* 19:2328-2338.

172. Wildenberg, G.A., M.R.Dohn, R.H.Carnahan, M.A.Davis, N.A.Lobdell, J.Settleman, and A.B.Reynolds. 2006. p120-catenin and p190RhoGAP regulate cell-cell adhesion by coordinating antagonism between Rac and Rho. *Cell* 127:1027-1039.

173. Wilkinson, K.D., M.K.Urban, and A.L.Haas. 1980. Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. *J Biol. Chem.* 255:7529-7532.

174. Wolf, R.M., J.J.Wilkes, M.V.Chao, and M.D.Resh. 2001. Tyrosine phosphorylation of p190 RhoGAP by Fyn regulates oligodendrocyte differentiation. *J Neurobiol.* 49:62-78.

175. Wu, J.Q., V.Sirotkin, D.R.Kovar, M.Lord, C.C.Beltzner, J.R.Kuhn, and T.D.Pollard. 2006. Assembly of the cytokinetic contractile ring from a broad band of nodes in fission yeast. *J Cell Biol.* 174:391-402.

176. Yamashiro, S., G.Totsukawa, Y.Yamakita, Y.Sasaki, P.Madaule, T.Ishizaki, S.Narumiya, and F.Matsumura. 2003. Citron kinase, a Rho-dependent kinase, induces di-phosphorylation of regulatory light chain of myosin II. *Mol. Biol. Cell* 14:1745-1756.

177. Yasuda, S., F.Oceguera-Yanez, T.Kato, M.Okamoto, S.Yonemura, Y.Terada, T.Ishizaki, and S.Narumiya. 2004. Cdc42 and mDia3 regulate microtubule attachment to kinetochores. *Nature* 428:767-771.

178. Yoneda, A., H.A.Multhaupt, and J.R.Couchman. 2005. The Rho kinases I and II regulate different aspects of myosin II activity. *J Cell Biol.* 170:443-453.

179. Yoshizaki, H., Y.Ohba, M.C.Parrini, N.G.Dulyaninova, A.R.Bresnick, N.Mochizuki, and M.Matsuda. 2004. Cell type-specific regulation of RhoA activity during cytokinesis. *J. Biol. Chem.* 279:44756-44762.

180. Yuce, O., A.Piekny, and M.Glotzer. 2005. An ECT2-centralspindlin complex regulates the localization and function of RhoA. *J Cell Biol.* 170:571-582.

181. Zhang, H. and I.G.Macara. 2008. The PAR-6 polarity protein regulates dendritic spine morphogenesis through p190 RhoGAP and the Rho GTPase. *Dev. Cell* 14:216-226.

182. Zhao, W.M. and G.Fang. 2005a. Anillin is a substrate of anaphase-promoting complex/cyclosome (APC/C) that controls spatial contractility of myosin during late cytokinesis. *J Biol. Chem.* 280:33516-33524.

183. Zhao, W.M. and G.Fang. 2005b. MgcRacGAP controls the assembly of the contractile ring and the initiation of cytokinesis. *Proc. Natl. Acad. Sci U. S. A* 102:13158-13163.