P120-CATENIN AND P190RHOGAP REGULATE CELL-CELL ADHESION BY COORDINATING ANTAGONISM BETWEEN RAC AND RHO

By

Gregg Anthony Wildenberg

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology

May, 2007

Nashville, Tennessee

Approved:

Professor Albert B. Reynolds

Professor Lynn Matrisian

Professor Vito Quaranta

Asst. Professor Alissa Weaver

Asst. Professor Anne Kenworthy

ORIGINAL PUBLICATIONS

Wildenberg, G.A., Dohn, M.R., Carnahan, R.H., Davis, M.A., Lobdell, N.A., Settleman, J., Reynolds, A.B. (2006). p120-catenin and p190RhoGAP regulate cell-cell adhesion by coordinating antagonism between Rac and Rho. *Cell*, 127, 1027-39.

Xia, X., Carnahan, R.H., Vaughan, M.H., Wildenberg, G.A., Reynolds, A.B. (2006). p120 serine and threonine phosphorylation is controlled by multiple ligand-receptor pathways but not cadherin ligation. *ECR* 312, 3336-48.

DEDICATION

To truth, understanding,

&

the humility to know the difference

between the two

ACKNOWLEDGMENT

Life is defined by moments of sadness and joy, moments of successes and failures, moments of wrong and right. If moments provide definition, then it is the people around us that give us purpose. My graduate work has been no exception to this. While the complete number of people and the extent of their influence is beyond words and limit, there are a few that need to be acknowledged.

First, I would like to thank my mentor, Al Reynolds. He has taught me as much about being a scientist as he has being a person. Al has been by my side not as an instructor or overseer, but rather as one who praises abilities and encourages to challenge weaknesses. In that simple guidance, I have learned an immeasurable skill in the necessity to challenge oneself before challenging a scientific question. The extent of his influence on me will go far beyond graduate school and display itself to a far greater circumference than my scientific career to which I can offer my thanks and appreciation.

I also need to thank my wife, Mary. She is the love of my life, and has supported me through so many days of frustration, stress, anxiety, and exuberance. Through her thoughtfulness and sincerity, I have acquired balance in my life that everyone needs to be successful with any endeavor. She challenges me to be a better person, and to continually grow with each day. Without her, not only would I have little to show for my accomplishments, but I would be without my best friend to share all of my happiness with.

Lastly, I would like to thank the support of my committee, Lynn Matrisian, Vito Quaranta, Alissa Weaver, and Anne Kenworthy. Although our interactions were few, they have served as models of success in science. I have paid careful attention and understood how they have each applied their own perspectives and unique talents to their respective passions. From them, I have learned a great deal on how to apply my perspectives and my passions to my future in science.

My graduate work has been supported by the Training Program in Breast Cancer Research, and p120 Cell-Cell Adhesion NIH RO1.

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LIST OF ABBREVIATIONS

AJ	Adherens junction
ASF	Actin stress fiber
DCR	Dorsal circular ruffles
EC	Extracellular domain
ECM	Extracellular matrix
EMT	Epithelial-mesynchemal transistion
FA	Focal adhesion
GAP	GTPase activating protein
GEF	Guanine exchange factor
GDI	Guanine nucleotide dissociation inhibitor
GCPR	G-coupled protein receptor
HGF	Hepatocyte growth factor
LMW-PTP	Low molecular weight protein tyrosine phosphatase
LPA	Lysophosphatidic acid
MAPK	Mitogen activated protein kinase
MEF	Murine embryonic fibroblasts
p120	p120-catenin
p190	p190RhoGAP
PAK	p21-activated kinase
PDGFR	Platelet derived growth factor receptor
Rac	Rac1

Rho	RhoA
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase

CHAPTER I

INTRODUCTION

Molecular Progression of Cancer

Tumorigenesis is a multistep process that involves genetic variations that drive the transformation of a normal cell to malignancy (Hanahan and Weinberg 2000). During this process tumor cells undergo clonal expansion whereby cells harboring certain genetic changes are selected for their ability to grow in an unregulated fashion. Eventually, selected populations of cells invade the surrounding basement membrane and enter the vasculature where they metastasize systemically. Meanwhile, the primary tumor also develops its own vascular system to supply itself with the necessary nutrients for continued growth. Although there are numerous distinct types of cancer, they are all governed by six general principles. These are self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, immortalization, angiogenesis, and invasion/ metastasis (Hanahan and Weinberg 2000). Conversely, the tight regulation of these processes is essential for normal cellular function.

The cancer phenotype is generally represented by a misregulation in growth factor and cell adhesion (cell-cell and cell-extracellular matrix (ECM)) signaling. Historically, a reductionist approach has been taken to isolate the individual contributions of different receptors in mediating the biological responses essential to normal cellular function. Through this approach, major contributions have been made in eliciting key mechanisms in the regulation of growth and motility. However, it has become apparent that biological

responses are not simply products of individual and/or parallel signaling pathways relayed from multiple receptors. Rather, receptor systems mediate cross-talk in order to carry out the multiple events that are simultaneously necessary for cellular motility, growth, and adhesion. For example. in suspended cells (i.e. without cell-ECM adhesion), activation of Rac1 (Rac) by growth factors is unable to activate its downstream effector, p21-activated kinase (PAK), suggesting that integrins are necessary to properly localize the Rac activity that comes from receptor tyrosine kinase (RTK) signaling (del Pozo, Price et al. 2000). Similarly, E-cadherin-mediated activation of Rac and mitogen activated protein kinase (MAPK) depends on a functional epidermal growth factor receptor (EGFR) (Pece and Gutkind 2000; Betson, Lozano et al. 2002). Finally, Src-induced deregulation of adherens junctions (AJs) requires integrin assembly of focal adhesion complexes (Avizienyte, Wyke et al. 2002). Thus, a cellular event such as AJ formation does not occur autonomously through cadherin clustering, but rather requires the rearrangement and cooperation of multiple receptor systems.

RhoGTPase Signaling

The small Rho family GTPases Rac and RhoA (Rho) play critical roles in coupling growth factor and adhesion receptor signaling to rearrangements in the actin cytoskeleton during motility, adhesion, and growth (Burridge and Wennerberg 2004). RhoGTPases cycle from the inactive, GDP bound form, to the active, GTP bound form, and localize from the cytoplasm to the cell membrane, respectively (Hall 1992). The activity of RhoGTPases is tightly controlled by three groups of proteins. These are Guanine Exchange Factors (GEFs), GTPase Activating Proteins (GAPs), and Guanine nucleotide

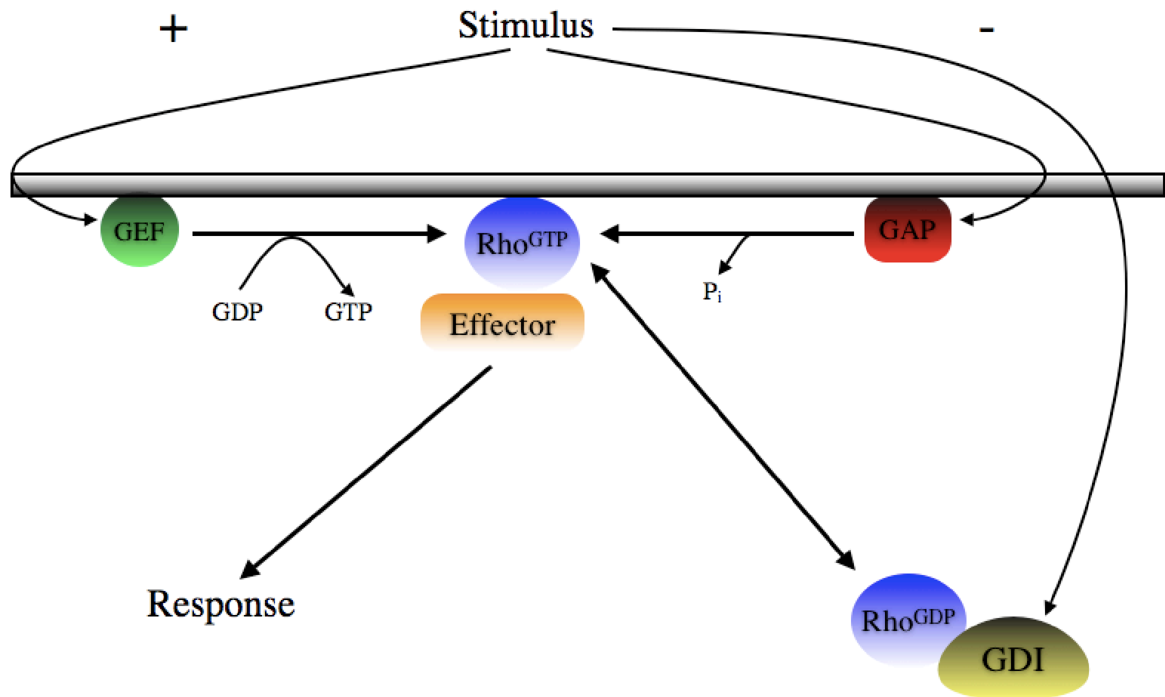


Figure 1. Schematic of RhoGTPase signaling.
RhoGTPases (*e.g.* Rho) are regulated by three groups of proteins-GEFs, GAPs, and GDIs. The relative amount of activity from positive regulators (GEFs) and negative regulators (GAPs, GDIs) determines the overall activity of the RhoGTPase itself. If the net activity results in the activation of Rho (Rho^{GTP}), Rho binds to and activates its effectors, which elicit a biological response (*e.g.* actin stress fibers in the case of Rho activation).

Dissociation Inhibitors (GDIs) . Each group of regulatory proteins is activated by up-stream signals received from receptors such as RTKs, cadherins, integrins, and G-coupled protein receptors (GCPRs) (Nobes and Hall 1994; Kjoller and Hall 1999). The relative amount of Rho or Rac activity is not determined by the solitary activation of a GEF, GAP, or GDI, but rather by the relative balance of activities from all three groups of proteins (Fig. 1).

RhoGTPases are best known for their activities on the actin cytoskeleton. Rac activation enhances cell spreading, migration, and membrane ruffling, and Rho activity stimulates focal adhesion (FA) formation and cell contractility via assembly of actin stress fibers (Jaffe and Hall 2005). However, Rac and Rho function in a wide variety of cellular events that include regulation of AJ formation, cell-ECM attachment, motility, invasion, transcriptional regulation, and proliferation (Evers, Zondag et al. 2000; Schwartz 2004). The role of Rac and Rho in these processes is not entirely clear, but generally involves the activation of downstream effectors, which in turn elicit effects on the actin cytoskeleton, gene transcription, and the cell cycle machinery. Misregulation of Rac and Rho activities has also been linked to tumorigenesis, and expression of dominant active mutants have been shown to be sufficient to induce transformation (Jaffe and Hall 2002).

In many cell types, Rho activity is negatively regulated by Rac, and the balance between these antagonistic activities is critical for coordination of cell motility with cell-cell adhesion and specification of cell morphology (Evers, Zondag et al. 2000). Activation of Rac by either platelet derived growth factor receptor (PDGFR) or integrins inhibits Rho via generation of reactive oxygen species (ROS), inhibition of low molecular weight pro-

tein tyrosine phosphatase (LMW-PTP), and tyrosine phosphorylation (activation) of p190RhoGAP (p190), a major GAP for Rho (Sander, ten Klooster et al. 1999; Nimnual, Taylor et al. 2003). In the case of PDGFR, this pathway mediates rapid disassembly of actin stress fibers (ASFs) and formation of dorsal circular ruffles (DCRs). Additionally, Rac inhibition of Rho induces “epithelialization” of cells by promoting AJ assembly and suppression of stress fiber-induced contractility (Sander, ten Klooster et al. 1999; Malliri, van Es et al. 2004; Wojciak-Stothard, Tsang et al. 2005).

p190RhoGAP

p190 is a ubiquitously expressed GAP that exhibits specificity for Rho. It was originally discovered as a binding partner of p120RasGAP (RasGAP) whose association is induced by tyrosine phosphorylation (Bernards and Settleman 2005). Although association of p190 with RasGAP does not directly affect the catalytic GAP activity of p190, recent evidence suggests that it may affect the subcellular localization of p190 (Bradley, Hernandez et al. 2006). In addition to a GAP domain, p190 contains a N-terminal GTP binding domain. The function of this domain is not known, but the ability of p190 to bind GTP has been shown to be required for its activity on Rho (Foster, Hu et al. 1994; Tatsis, Lannigan et al. 1998; Roof, Dukes et al. 2000).

Activation of p190 is mediated by RTKs, integrins, and cadherins (Ellis, Moran et al. 1990; Arthur and Burridge 2001; Noren, Arthur et al. 2003). Upon activation, p190 becomes phosphorylated and translocates from the cytoplasm to membranes and/or actin cytoskeletal structures where it can access activated Rho (Sharma 1998; Arthur and Burridge 2001; Sordella, Jiang et al. 2003). Thus, both p190 tyrosine phosphorylation and

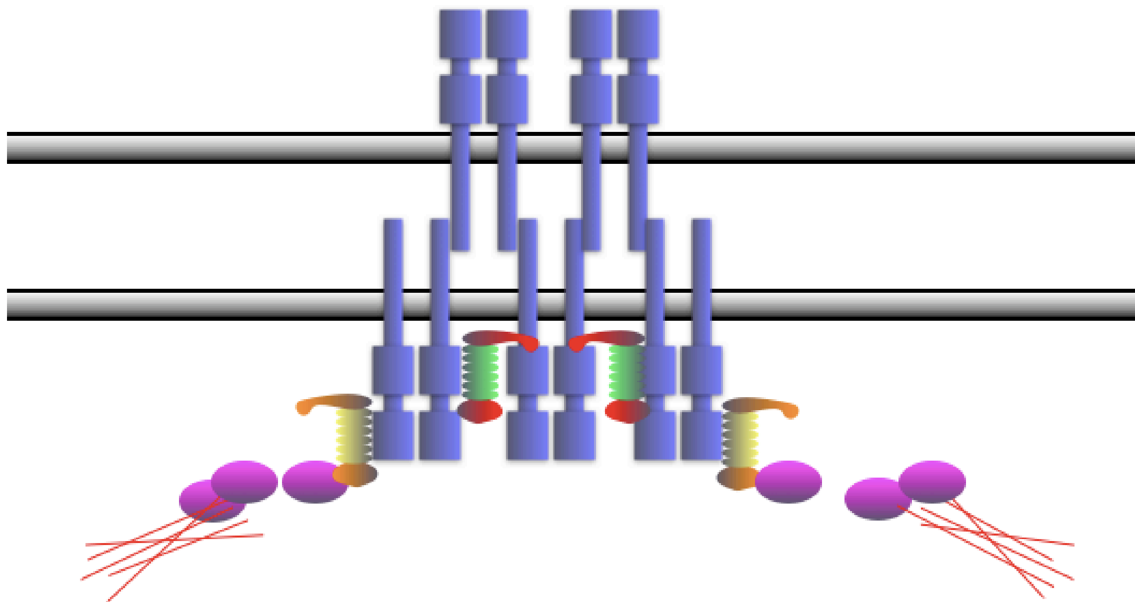


Figure 2. Schematic of the Cadherin complex.
The extracellular domain of cadherins (blue) mediate both homophilic *cis* interactions with adjacent cadherin molecules and *trans* interactions with cadherins on neighboring cells. p120 (red/green) interacts with the juxta-membrane domain, and β-catenin (orange/yellow) interacts with the catenin-binding domain of cadherins. α-catenin (purple) either interacts with β-catenin or F-actin.

translocation are necessary for proper p190 function. p190 has been localized to focal adhesions and lipid rafts, but has not been associated with any other cellular structures (Sharma 1998; Sordella, Jiang et al. 2003). p190 has been shown to function in integrin-mediated spreading, neuron outgrowth, and fasciculation, but the contribution of p190 to RTK and cadherin signaling remains largely unexplored (Arthur and Burridge 2001; Brouns, Matheson et al. 2001).

Cadherin Signaling

Cadherin function

Cadherins comprise a superfamily of transmembrane adhesion receptors that mediate Ca^{+2}-dependent cell-cell adhesion. Classical cadherins (i.e. E-, N-, VE-, and R-cadherin) are the most widely studied of the cadherin superfamily, and are often associated with various forms of AJs. Cadherins play critical roles in development, motility, growth suppression, polarization, and tissue morphogenesis (Yap, Brieher et al. 1997). Cadherins form lateral dimers, which then mediate homophilic interactions through repeating extracellular (EC) domains with cadherins of a nearby cell (Gumbiner 2005). Although cell-cell adhesion is mediated by the extracellular domain of cadherins, it is not sufficient to induce the strong adhesion observed by cadherin clustering (Yap, Brieher et al. 1997; Yap, Niessen et al. 1998). Instead, the catenins (α, β, and p120-catenin), which directly associate with the cadherin cytoplasmic tail, are thought to regulate AJ assembly through functional but poorly understood interactions with RhoGTPases and the actin cytoskeleton (Yap, Niessen et al. 1998; Braga 2002) (Fig 2).

Cadherin Regulation

Regulation of AJ assembly is a dynamic process that involves post-translational modification of the cadherin and associated catenins. Though poorly understood, cellular signals such as those from growth factors, transiently influence the ability of cadherins to form stable AJs. Phosphorylation of the catenins by RTKs or non-receptor tyrosine kinases such as Src, Fyn, or Abl have been implicated in this process as phosphorylation of catenins have been correlated to either a loss or strengthening of cell-cell adhesion (Roura, Miravet et al. 1999; Piedra, Miravet et al. 2003). Likewise, phosphatases have been linked to this process (Xu, Craig et al. 2004; Sallee, Wittchen et al. 2006). Activity by kinases or phosphatases on catenins is associated with changes in the ability of the cadherins to cluster, though the mechanisms are not known.

Loss of E-cadherin expression in epithelial-derived tumors is closely correlated with the transition to metastasis. Restoration of E-cadherin expression has been shown to reduce or block metastasis both *in vitro* and *in vivo* (Frixen, Behrens et al. 1991; Oka, Shiozaki et al. 1993). In addition to the direct loss of E-cadherin expression, cell-cell adhesion can be dysregulated through posttranslational mechanisms such as phosphorylation. Aberrant signaling from oncogenes such as Src, or different growth factors such as hepatocyte growth factor (HGF), and platelet derived growth factor (PDGF) can negatively affect the ability to form stable AJs (Matsuyoshi, Hamaguchi et al. 1992; Watabe, Matsumoto et al. 1993; Piedra, Miravet et al. 2003; Uglow, Slater et al. 2003). Alterations in these signaling pathways in transformed cells, therefore, could result in a loss of cadherin-dependent adhesion and increased invasiveness. Thus, misregulation of the

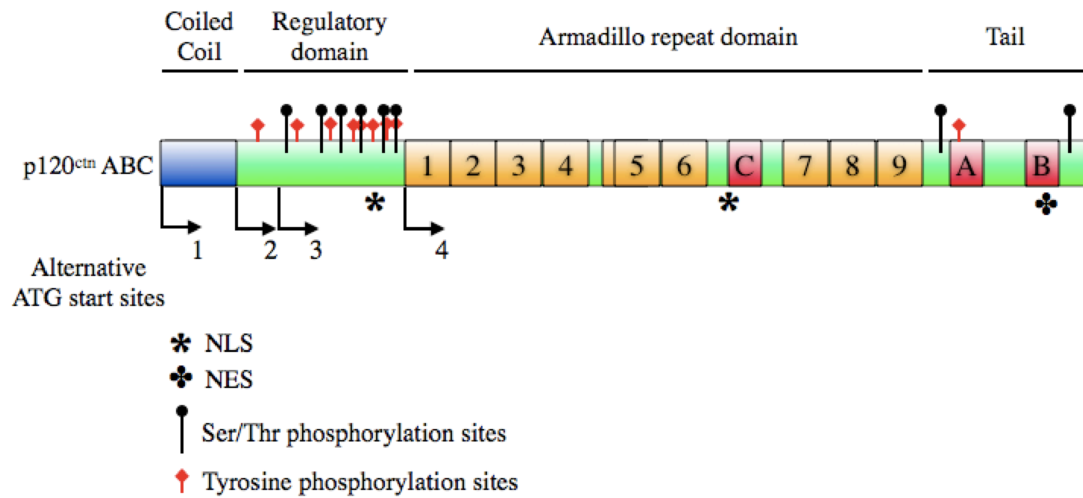


Figure 3. Schematic of p120.
p120 contains a central Armadillo repeat domain containing nine Arm repeats. Several different isoforms of p120 are expressed due to four alternative start codons in the N-terminus, and three C-terminal exons. Isoform 1 possesses a coiled-coil domain, which mediates protein-protein interactions. Tyrosine and serine/threonine phosphorylation sites are concentrated in the N-terminal regulatory domain. p120 also possess nuclear localization sequences (NLS) and a nuclear export sequence (NES).

cadherin function can occur through a variety of means, and represents a major signaling defect during tumor progression.

p120-catenin

p120 family and localization

p120-catenin (p120) was originally identified as a Src substrate, and is the prototypic member of an Arm domain subfamily that includes ARVCF, δ-catenin, and p0071. All family members are ubiqutiously expressed with the exception of δ-catenin, which is expressed specifically in neuronal cells. Structurally, all of these proteins contain an N-terminal coiled-coiled motif, and all but p120 have a C-terminal PDZ-binding motif. The internal Armadillo domain contains nine Arm repeats, which mediates protein-protein interactions (Anastasiadis and Reynolds 2000) (Fig. 3). In contrast to β-catenin which binds to the catenin binding domain (CBD) of cadherins, p120 binds to the juxtamembrane domain (JMD) (Thoreson, Anastasiadis et al. 2000). Since p120 does not physically link cadherins to the actin cytoskeleton, p120 appears to modulate assembly and disassembly of AJs by an indirect mechanism. Additionally, unlike β-catenin, which is rapidly degraded when sequestered in the cytoplasm, cytoplasmic p120 is stable. It has been proposed that cytoplasmic p120 inhibits Rho, but the mechanism is not known (see below) (Anastasiadis, Moon et al. 2000; Noren, Liu et al. 2000; Grosheva, Shtutman et al. 2001). Lastly, p120 can localize to the nucleus where it negatively regulates the transcriptional repressor, Kaiso (Daniel and Reynolds 1999; Daniel, Spring et al. 2002; Spring, Kelly et al. 2005).

p120 isoforms

p120 expresses multiple isoforms, which are derived by alternative splicing of a single gene. Splicing occurs at the N-terminus due to different start codons (1, 2, 3), and C-terminally spliced at sites of 3 exons (A, B, C) (Reynolds, Daniel et al. 1994; Keirsebilck, Bonne et al. 1998). Interestingly, fibroblasts predominantly express the full length 1A isoform, whereas epithelial cells express the slightly shorter, 3A isoform. These isoforms differ by the presence in isoform 1A of an N-terminal coiled-coiled domain, which typically mediate protein-protein interactions (Fig. 3). The N-terminus has been linked to a role in promoting motility and invasion (Cozzolino, Stagni et al. 2003; Yanagisawa and Anastasiadis 2006). However, in many tumor cell lines, p120 isoform expression is quite heterogeneous (Mo and Reynolds 1996), which may reflect a role for isoform switching promoting tumor progression.

p120 phosphorylation

Phosphorylation undoubtedly regulates the function of p120 most likely by connecting various signaling pathways to the dynamic regulation of cell-cell adhesion. However, due to the multiple phosphorylation sites (both tyrosine, and serine/threonine) on p120, and the multiple kinases that can phosphorylate p120, it has been difficult to determine the role of p120 phosphorylation (Reynolds, Roesel et al. 1989; Downing and Reynolds 1991; Mariner, Anastasiadis et al. 2001). Probably, individual phosphorylation sites serve as substrates for specific kinases, and the net phosphorylation of p120 received by multiple signals ultimately regulates the the adhesive strength of a cell. Interestingly,

expression of isoform 4A, which lacks almost all of the tyrosine and serine/threonine sites, in the SW48 cell line strengthens adhesion better than isoform 1A or 3A, which contain all of the phosphorylation sites (Ireton, Davis et al. 2002). This indicates that phosphorylation is not necessary for p120 to stabilize cadherins, but perhaps it is necessary for negative regulation of adhesion.

p120 function

We and others have recently demonstrated that p120 is required for cadherin stability. Using the p120-deficient colon carcinoma cell line, SW48, it was found that re-expression of p120 increased E-cadherin levels and restored an epithelial morphology (Ireton, Davis et al. 2002). Structure-function analysis revealed that restoration of E-cadherin levels requires direct p120-cadherin interaction. Further studies demonstrated that p120 mediates cadherin stability, thus dynamically regulating the amount of cadherin available at the cell surface for adhesion (Xiao, Allison et al. 2003). Follow up studies using siRNA-mediated depletion of p120 showed that this is indeed a general mechanism for p120 in stabilizing all classical cadherin types in different cell lines (Davis, Ireton et al. 2003). *In vivo*, p120-targeted ablation in the salivary gland also leads to about a 50% reduction in E-cadherin protein levels, and is associated with severe alterations in epithelial morphology (Davis and Reynolds 2006). These observations suggest that p120 mediates cadherin turnover, and that adhesion may be controlled by phosphorylation events that impact p120 to dynamically regulate AJ assembly and disassembly.

In addition to its role in stabilizing cadherins, overexpression of p120 causes extensive, atypical branching through the inhibition of Rho (Anastasiadis, Moon et al. 2000;

Noren, Liu et al. 2000; Grosheva, Shtutman et al. 2001). These data indicated that p120 inhibits Rho in the cytoplasm, and a GDI-like mechanism was proposed based on the ability of purified p120 to stabilize the GDP-bound form of Rho *in vitro* (Anastasiadis, Moon et al. 2000). Developmental studies in *Xenopus lavis* as well as conditional knock-out studies done in the epidermis of mice confirm a functional relationship between p120 and Rho, but the mechanism for this interaction is not known (Fang, Ji et al. 2004; Perez-Moreno, Davis et al. 2006) . Furthermore, the Drosophila homologue of p120 interacts with the GDP-bound form of Rho1 (Drosophilia RhoA) (Magie, Pinto-Santini et al. 2002). Recently, work done by Yanagisawa et al in the invasive adenocarcinoma cell line MDA-MB-231, demonstrated that p120 can promote Rac activation through interaction with cadherin-11, and Rho inhibition in a cadherin-independent manner (Yanagisawa and Anastasiadis 2006), however, no mechanism was defined. Although little is known regarding the mechanism or functional consequence, p120 clearly regulates the activity of Rho.

Presently, the roles of p120 in adhesion and Rho inhibition appear to be separate functions. It has been postulated that the relative amount of p120 bound to a cadherin may regulate the degree of cytoplasmic p120 available to inhibit Rho. Alternatively, p120 may function to deliver inactive Rho from the cytoplasm to the plasma membrane where it becomes activated locally by a GEF. Phosphorylation of p120 would ultimately serve then to regulate the ratio of cadherin bound/cytoplasmic p120 and thus adhesion and Rho inhibition. These models suggests that the role of p120 in cadherin-stabilization and Rho-inhibition are mutually exclusive. However, it has not been ruled out that these two processes are functionally linked. Indeed, RhoGTPases play critical roles in AJ as-

sembly and disassembly (Kuroda, Fukata et al. 1997; Sander, van Delft et al. 1998; Lozano, Betson et al. 2003). The contribution of either Rac or Rho to AJ formation is complex, and apparently depends on the maturity of cell-cell contacts. For example, Rac localizes to and is necessary for nascent cell-cell adhesions, but does not localize to more mature contacts (Braga, Del Maschio et al. 1999; Ehrlich, Hansen et al. 2002). As fore mentioned, Rac can "epithelialize" fibroblasts by stabilizing N-cadherin-based AJs suggesting that Rac drives the formation and stabilization of cell-cell adhesions. Indeed, other reports have suggested a similar role for Rac in epithelial cells (Kuroda, Fukata et al. 1997; Takaishi, Sasaki et al. 1997). On the other hand, Rho activity has been associated with a more fibroblastic phenotype that is associated with poor cell-cell adhesions (Zhong, Kinch et al. 1997). Although the activities of Rac and Rho clearly affect AJ formation, how these activities are regulated at the cadherin complex remains unknown. Given the affect p120 has on both cell-cell adhesion and Rho activity, it is likely that p120 may coordinate these two processes.

Hypothesis

My working hypothesis is that p120 inhibition of Rho functions in the regulation of AJ assembly.

CHAPTER II

MATERIALS AND METHODS

Cell Culture and plasmids

NIH3T3 and p190A-/- MEF cells were cultured in DMEM/High Glucose, 10% Fetal Bovine Serum (FBS), and 1% penicillin/streptomycin. Clonal p120-knockdown cell lines were generated by serial dilution. Rac1 and C3 constructs were PCR amplified from PcDNA3 and subcloned into LZRS-ms-GFP. p190-A cDNAs were subcloned from Rc-HAp190-A or MFG into LZRS-ms-GFP or LZRS-ms-neo. LZRS-ms-neo/N-cadherin was a gift from Dr. Margaret Wheelock. p190A constructs and null MEFs were a gift from Jeff Settleman. Retroviral vectors and siRNA for p120 and N-cadherin have been described (Davis, Ireton et al. 2003).

Antibodies

Secondary antibodies were Alexa-Fluor conjugates from Molecular Probes. mAbs 15D2, KT3, and 8D11 were generated in our lab. mAbs pp120 (cat # 610136), N-cadherin (610921), Rac1 (610650), p190 (610150), and anti-phosphotyrosine (PY20) (610000) were from BD Biosciences. mAb anti-RhoA (sc-418) and pab anti-N-cadherin (sc-7939) was from Santa Cruz. Y-27632 (Y0503), fibronectin (F2006), H_20_2 (H1009), and mAbs anti-p120RasGAP (B4F8), α-tubulin (T5168), and vinculin (V4139) were from Sigma-Aldrich. mAbs p-p38 (9216), p38 (9212), MAPK (9102), and p-MAPK (9106)

were from Cell Signaling. PDGF-BB (GF018) was from Chemicon, and PDGFRβ (97A) antibody was from Dr. Andrius Kazlauskas.

PDGFR Signaling

Immunoflourescent (IF) methods have been described (Davis, Ireton et al. 2003). Cells were fixed/permeabilized in 3% paraformaldehyde/0.2% Triton X-100, and stained for 30 min. For Dorsal Circular Ruffle formation, cells were serum starved for 24 hrs (0.2% FBS), and stimulated with 20-50 ng/ml of PDGF-BB for 7 min. Immunoprecipitation (IP) and Western Blot (WB) methods were as described (Davis, Ireton et al. 2003). Briefly, cells were lysed in RIPA buffer, protein concentrations equilibrated by BCA assay. For IP, cells were lysed, nutated at 4°C for 5 min. in a 1.5 ml eppendorf tube, and then passed through a 20 1.1/2 gauge syringe 15 times. Cell debris was pelleted by centrifugation, and supernatant was transferred to a new eppendorf tube. Equal amounts of protein were IP'ed with indicated antibodies overnight at 4°C. Complexes were collected with Protein G-Sepharose, for 1.5 hrs, transferred to a new eppendorf tube, washed, and processed for WB. For p38 analysis, serum starved cells were stimulated with 50ng/ml of PDGF-BB for 7 min. PDGFRβ was immunoprecipitated from serum starved cells after stimulation with 50 ng/ml of PDGF-BB for 7 min.

Rac and Rho Activation

Rhotekin/ PAK Assay and ROCK inhibition

Rhotekin and PAK assays were performed as described (Anastasiadis, Moon et al. 2000). Briefly, serum starved cells were stimulated with 20 ng/ml of PDGF-BB for 3-5 min or 5μM of LPA for 15 min. Cells were lysed in Lysis Buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500mM NaCL, 10mM $MgCl_2$, 1mM PMSF, 5 μg/ml leupeptin, and 2 μg/ml aprotinin), 15 μl of lysate was used for analysis of total protein levels, and remaining lysate was incubated with 30 μg of GST-RBD or GST-PBD (Cytoskeleton Inc.) to IP activated forms of RhoA or Rac1. IPs were washed three times in Wash Buffer (50mM Tris, pH 7.2, 1% Triton X-100, 150 mM NaCl, 10mM $MgCl_2$, 1mM PMSF, 5 μg/ml leupeptin, and 2 μg/ml aprotinin). Levels of total and activated RhoA or Rac1 were analyzed by WB. To inhibit ROCK, 10 μM Y-27632 was added to cells for 30 min.

RhoA affects on integrin signaling

In spreading assays, cells were serum starved, trypsinized, washed, and replated on uncoated or coated glass coverslips (10 μg/ml of Fibronectin) for 20 min. For wound assays, confluent cells were scratched with a plastic pipette tip, and washed in PBS. Normal growth media was added and cells were incubated under normal growth conditions. Images of multiple wound regions were taken every 12 hours, and the distance of each wound was calculated, and averaged together for statistical significance. 10 μg/ml Mitomycin C (MMC) was added for 3 hours prior to wounding, and present throughout.

Proliferation, and Secondary Foci Formation Assays

To characterize morphology of subconfluent and contact inhibited cells, cells were grown at indicated density and bright field images captured. For proliferation assays, cells were seeded at equal numbers, grown for 24 hours, and then cultured in medium with or without FBS. At indicated times, cells were trypsinized and counted. For secondary focus formation assays, p120i cells were co-cultured with WT cells at a ratio of 1:100 (300 p120i-GFP: 30,000 WT), and seeded into 35 mm dishes. Cells were cultured for 10 days changing media every 3 days, washed in PBS, fixed in 3% PFA, and visualized for GFP expression. WT cells were also cultured alone, or with p120i cells at ratios of 1:6 and 1:30, or with p120i cells infected with LZRS-ms-GFP, LZRS-ms-GFP/C3, LZRS-ms-neo, or LZRS-ms-neo/N-cadherin at a 1:30 ratio. After 10 days, cells were fixed, stained with 0.5% crystal violet / 70% EtOH, and washed with PBS.

Rac to Rho Signaling

<u>Rac inhibition of Rho</u>

For Rac inhibition of Rho, cells were infected with LZRS-ms-GFP or LZRS-ms-GFP/DA-Rac and visualized with indicated antibodies. For ROS generation, serum starved cells were treated with 1mM of H_20_2 for 10 min. For p190 analysis, cells were infected with LZRS-ms-GFP or LZRS-ms-GFP/p190A, serum starved, and visualized for actin. For p190 analysis, cells were infected with LZRS-ms-GFP or LZRS-ms-GFP/DA-

Rac, and serum starved. Cells were then lysed in RIPA buffer, and p190 immunoprecipitates were subjected to western blot with indicated antibodies.

p190 -/- MEFS analyses

For analysis of AJ formation, p190A -/- cells were infected with LZRS-ms-neo p190A constructs, and then reinfected with LZRS-ms-GFP/DA-Rac1. Cells were then either incubated with control or 10 μM Y-27632 for 30 min, and visualized with indicated antibodies. Adherens junctions were quantified by scoring the number of cells forming obvious junction formation out of 200 total cells, and expressed as a ratio of positive cells to total cells.

CHAPTER III

P120 REDUCTION CAUSES CONSTITUTIVE RHO ACTIVATION

Introduction

In addition to its role in stabilizing cadherins, p120 can regulate Rho activity. Overexpression of p120 in NIH3T3 fibroblasts has been shown to cause extensive branching through the inhibition of Rho (Anastasiadis, Moon et al. 2000; Noren, Liu et al. 2000; Grosheva, Shtutman et al. 2001). *In vitro* analysis suggested that p120 may function as a GDI by binding to and sequestering the inactive form of Rho in the cytoplasm (Anastasiadis, Moon et al. 2000). Additionally, p120-induced branching was suppressed by co-expression of C-cadherin, which further suggests a cytoplasmic role for p120 in Rho inhibition (Noren, Liu et al. 2000). Overall, these observations suggested a model in which the degree of Rho inhibition by p120 is ultimately controlled by the ratio of cadherin bound and cytoplasmic p120.

From these data, it is clear that p120 is able to inhibit Rho both *in vivo* (in cells) and *in vitro*. However, the mechanism and physiological context of p120-mediated Rho inhibition is unclear. Overexpression bypasses the spatial and temporal constraints that are required for normal signal transduction, and makes it difficult to interpret the physiological context in which an event takes place. When p120 localization was analyzed under conditions of increasing density in MDCK cells, p120 redistributed from the cytoplasm to AJs as density increased (Grosheva, Shtutman et al. 2001). Thus, the model derived from overexpression data would predict that upon increasing density, more p120 is sequestered

from the cytoplasm to the cadherin, resulting in the activation of Rho. However, when Rho activity was monitored in MDCK cells at increasing densities, Rho activity was significantly lower when cells were at a higher density (Noren, Niessen et al. 2001). Thus, the current model of p120-mediated Rho inhibition is incomplete, and is lacking physiological context and mechanism.

The permanent or transient loss of cadherin expression is often correlated with increased invasion of numerous cancer types (Birchmeier and Behrens 1994). When this occurs, p120 becomes exclusively localized to the cytoplasm where it presumably inhibits Rho. Invasion of cancer cells has been linked in part to a misregulation of RhoGTPase signaling (Sahai and Marshall 2002; Lozano, Betson et al. 2003), and recent evidence has suggested that cytoplasmic p120 in the breast adenocarcinoma cell line, MDA-MD-231, promotes invasion through the inhibition of Rho (Yanagisawa and Anastasiadis 2006). Thus, loss of cadherin expression during tumor progression could promote invasion and metastases by sequestering p120 in the cytoplasm. Alternatively, if p120 regulates Rho at the cadherin complex, loss of cadherin expression would result in Rho activation. In either regard, the effects of cadherin loss observed in cancer is likely due in part to the misregulation of p120 signaling to Rho.

Recently, siRNA technology has allowed for the targeted degradation of mRNA (Fire, Xu et al. 1998) in cell culture. This allows for the analysis of the functional consequences of loss rather than gain of a specific protein, thereby eliminating the artifactual products of overexpression. Additionally, siRNA allows for a cell culture-based, epigenetic analysis of signaling pathways, which is often difficult to do in knock-out animal studies. Since overexpression of p120 leads to Rho inhibition, it is predicted that reduc-

tion of p120 will result in the activation of Rho, which will have effects on signaling pathways that inhibit Rho in a p120-dependent manner.

Results

p120 loss induces potent stress fiber assembly and blocks PDGFR-mediated actin rearrangements

To investigate the effect of p120 on Rho signaling, we induced stable siRNA-mediated knockdown of p120 in NIH3T3 fibroblasts (hereafter "p120i" cells) (Fig. 4). Three different siRNA sequences to murine p120 as well as a control mismatched siRNA (pRs-hp120) (Davis, Ireton et al. 2003) were tested to ensure that the effects observed were due to specific targeting of p120. Upon initial analysis, p120i cells contained enhanced actin stress fibers (ASFs), and the effects of PDGF stimulation on actin remodeling were completely blocked (Fig. 5). Specifically, ASFs and DCRs failed to form in the absence of p120.

We next sought to determine if the PDGFR was nonfunctional in the p120i cells. However, both PDGFR and p38 activation (as measured by tyrosine phosphorylation) in response to PDGF was identical in WT and p120i cells (Fig. 6). Lastly, PDGFR-mediated DCR formation has been shown to be mediated by a Rac-dependent mechanism (Hooshmand-Rad, Claesson-Welsh et al. 1997; Sander, ten Klooster et al. 1999; Nimnual, Taylor et al. 2003). Therefore, p120 loss could affect the ability of the PDGFR to activate Rac. To test this, PAK assays were performed to determine the ability of PDGF to

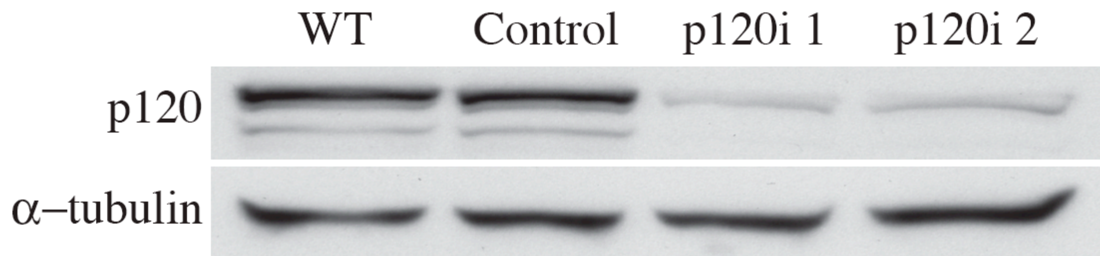


Figure 4. siRNA-mediated knock-down of p120 in NIH3T3.
p120 levels were analyzed by Western blotting in WT cells (WT), or in independently derived cell lines expressing control or p120 specific siRNAs (p120i 1 and p120i 2), as indicated.

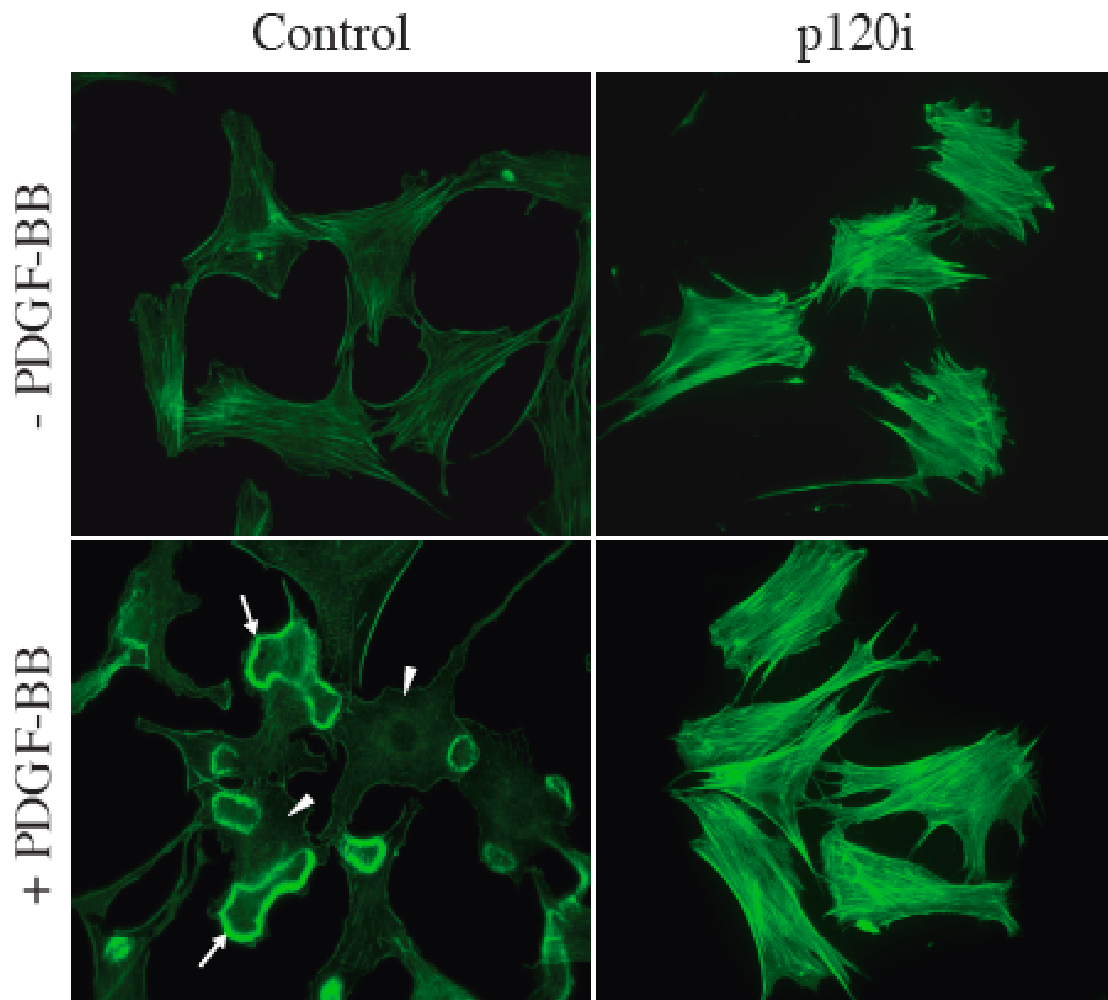


Figure 5. p120 depletion results in enhanced stress fibers, and blocks PDGF-induced actin remodleing.
NIH3T3 cells expressing irrelevant siRNA (control) or p120 specific siRNA (p120i) were serum starved, stimulated with PDGF-BB for 7 minutes, and stained for F-actin. Arrows denote PDGF-induced dorsal circular ruffles; arrowheads show regions of simultaneous disassembly of actin stress fibers (ASFs). Note unusually prominent ASFs in p120i cells, and the complete insensitivity of ASFs to PDGF.

activate Rac in the absence of p120. Interestingly, total Rac levels were elevated in p120i cells because the p120-depleted cells failed to growth arrest during serum starvation (see Fig.10). Nevertheless, in four separate experiments, Rac activation by PDGF stimulation was equal in WT and p120i cells (Fig. 6). Thus, activation of the PDGFR and Rac activation occurs normally in p120i cells and cannot explain the failure to remodel actin.

p120 loss results in constitutive Rho activation and blocks integrin signaling

Increased ASFs in p120i cells suggested that Rho was activated. To test this, levels of activated Rho were assayed by Rhotekin with and without addition of a known activator of Rho, lysophosphatidic acid (LPA) or to PDGF, which is known to inhibit Rho through the activation of Rac (Sander, ten Klooster et al. 1999). As shown in Figure 7, Rho activity was increased by LPA and decreased by PDGF in WT cells. However, Rho activity in resting p120i cells was higher than Rho levels in LPA-treated WT cells, and unaffected by PDGF. Thus, in the absence of p120, Rho is both activated and insensitive to PDGF.

Rho also functions in integrin-based focal adhesion assembly, cell spreading, and motility (Nobes and Hall 1995; Chrzanowska-Wodnicka and Burridge 1996; Rottner, Hall et al. 1999). Indeed p120i cells exhibit an increase in the number and size of focal adhesions as shown by vinculin staining (Fig. 8A). Additionally, p120i cells were able to spread in an integrin-independent manner as demonstrated by their ability to spread on glass as efficiently as on fibronectin (Fig. 8B). Lastly, p120i cells failed to migrate into a wound as compared to WT cells (Fig. 9). Overall, p120-ablation results in constitutive Rho activation, and uncouples Rho from regulation by both RTK- and integrin-based

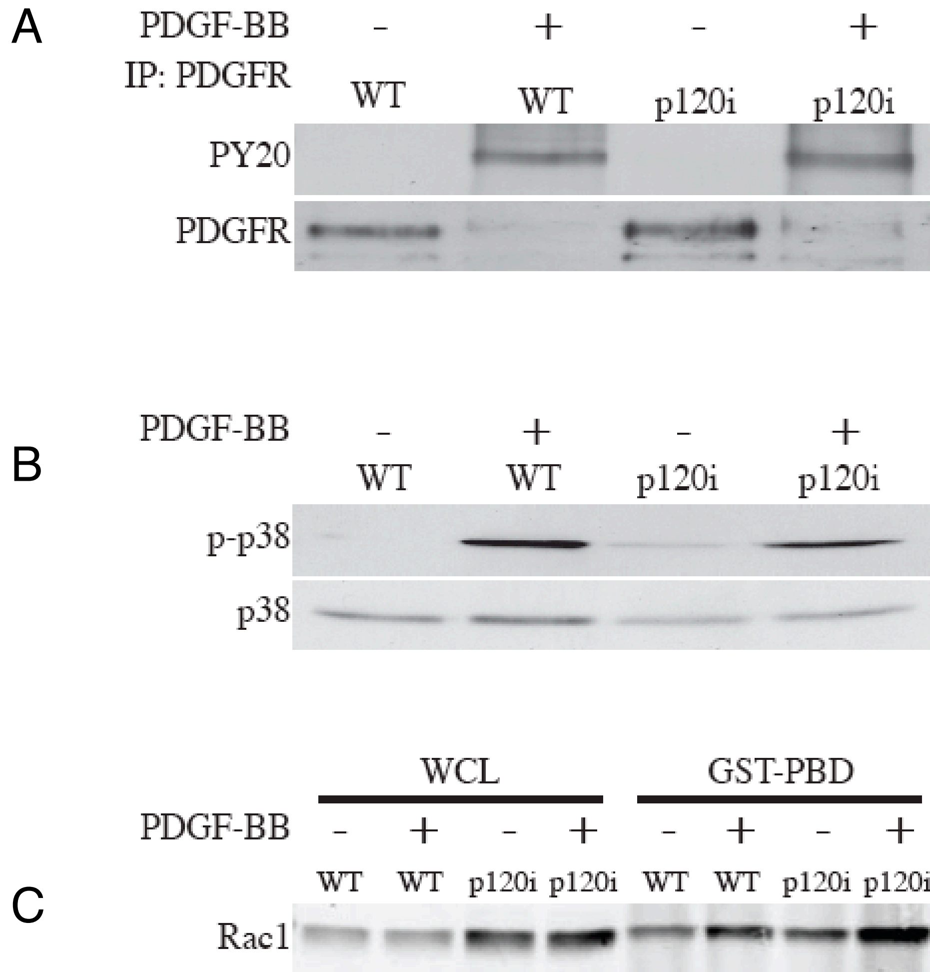


Figure 6. PDGFR activation, and downstream signaling to MAPK and Rac is unchanged in p120i cells.
(A) Serum starved WT and p120i cells were treated with PDGF as indicated. PDGFR immunoprecipitates were analyzed by Western blotting with antibodies to phosphotyrosine (PY20) or PDGFR.
(B) Whole cell lysates were Western blotted with antibodies to phospho-p38 (p-p38) or p38.
(C) To assess Rac activation by PDGF, PAK assays were performed (ie, GST-PBD pulldowns) to selectively isolate active Rac, and levels of total Rac(WCL), and active Rac (GST-PBD) were analyzed by Western blotting.

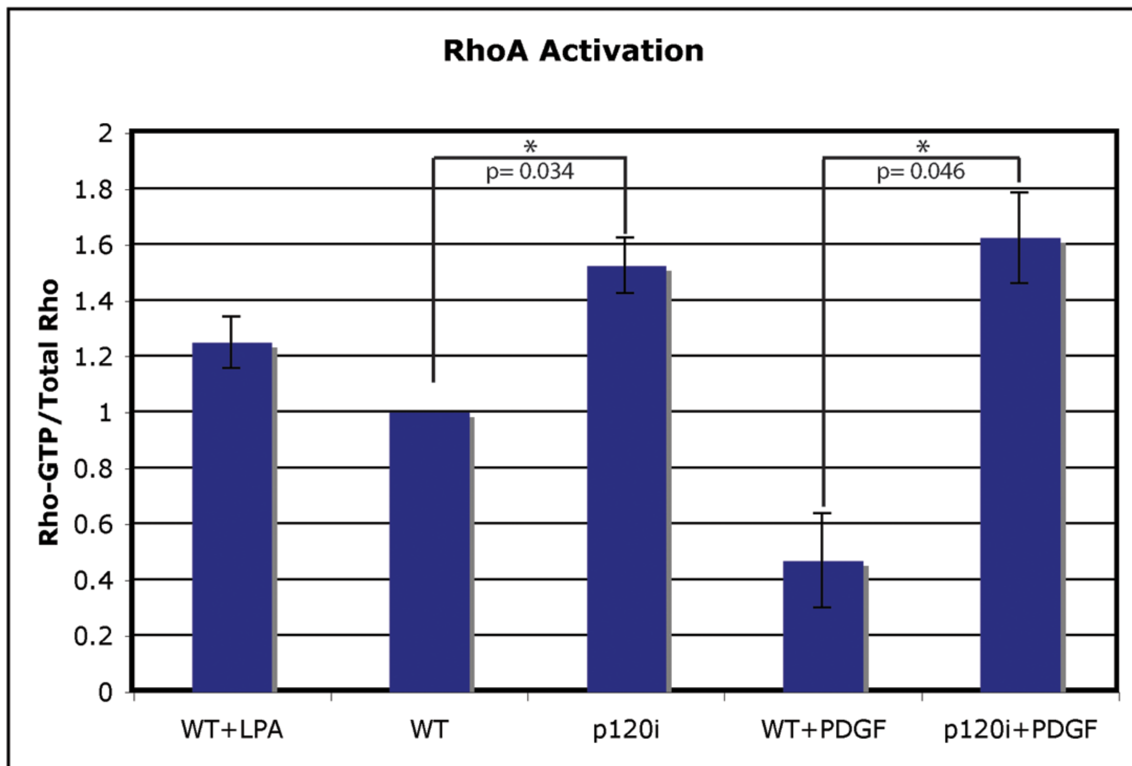


Figure 7. Rho is constitutively active and unaffected by PDGF in p120i cells. Serum starved cells were treated as indicated and assayed directly for Rho activity by GST-Rhotekin pulldown. Results are expressed as the ratio of activated to total Rho. Results were expressed as a ratio of WT control (unstimulated WT NIH3T3). Error bars represent standard error of the mean. Student's T-test was performed between WT and p120i cells, and WT+PDGF and p120i+PDGF to analyze statistical difference within the two groups (n = 3 independent experiments).

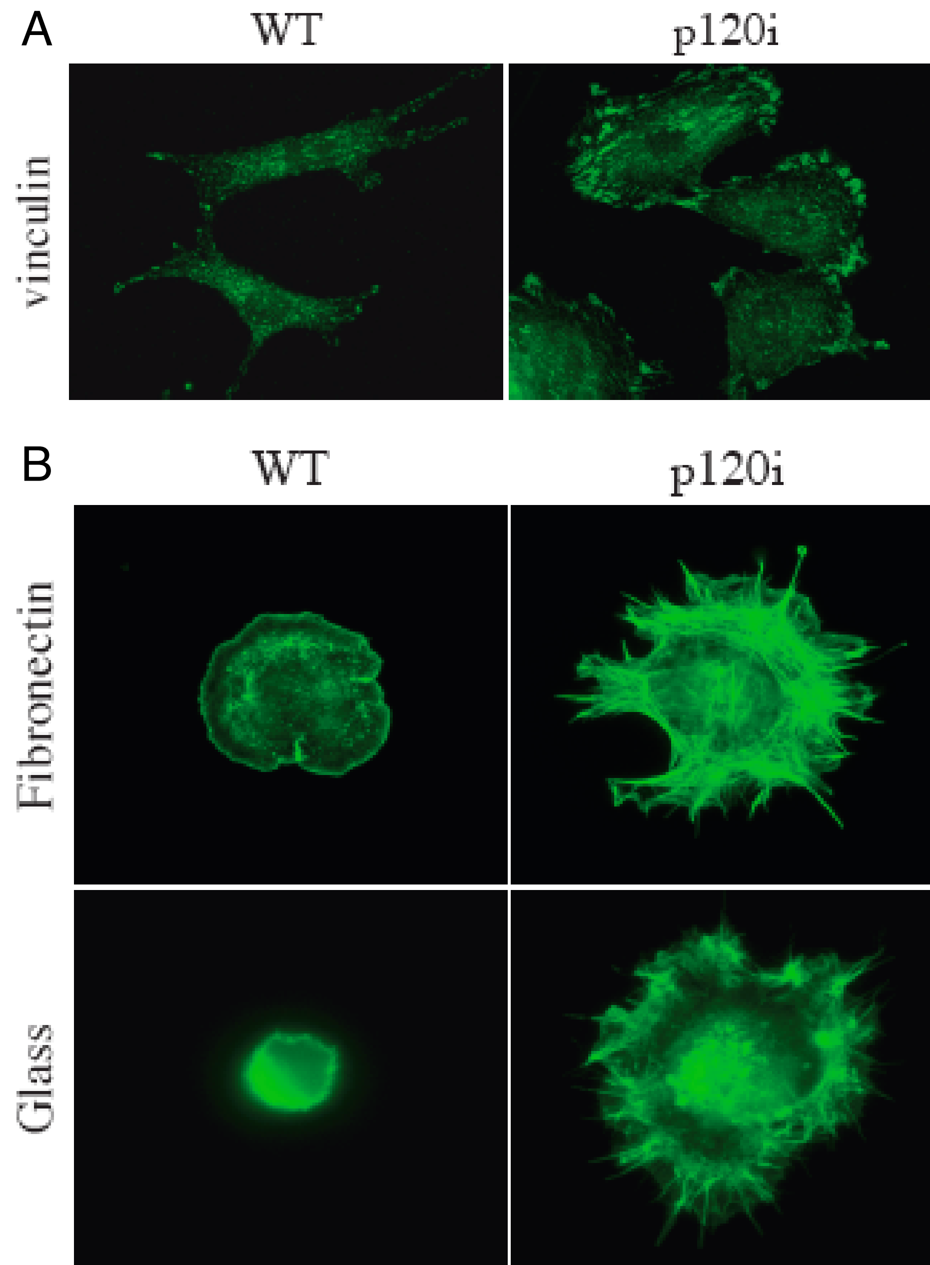


Figure 8. p120i cells have enhanced focal adhesions and spread in an integrin-independent manner.
(A) WT and p120i cells were stained with antibodies to the focal adhesion marker vinculin.
(B) Serum starved cells were trypsinized, plated for exactly 20 minutes on either fibronectin or glass, and stained for actin.

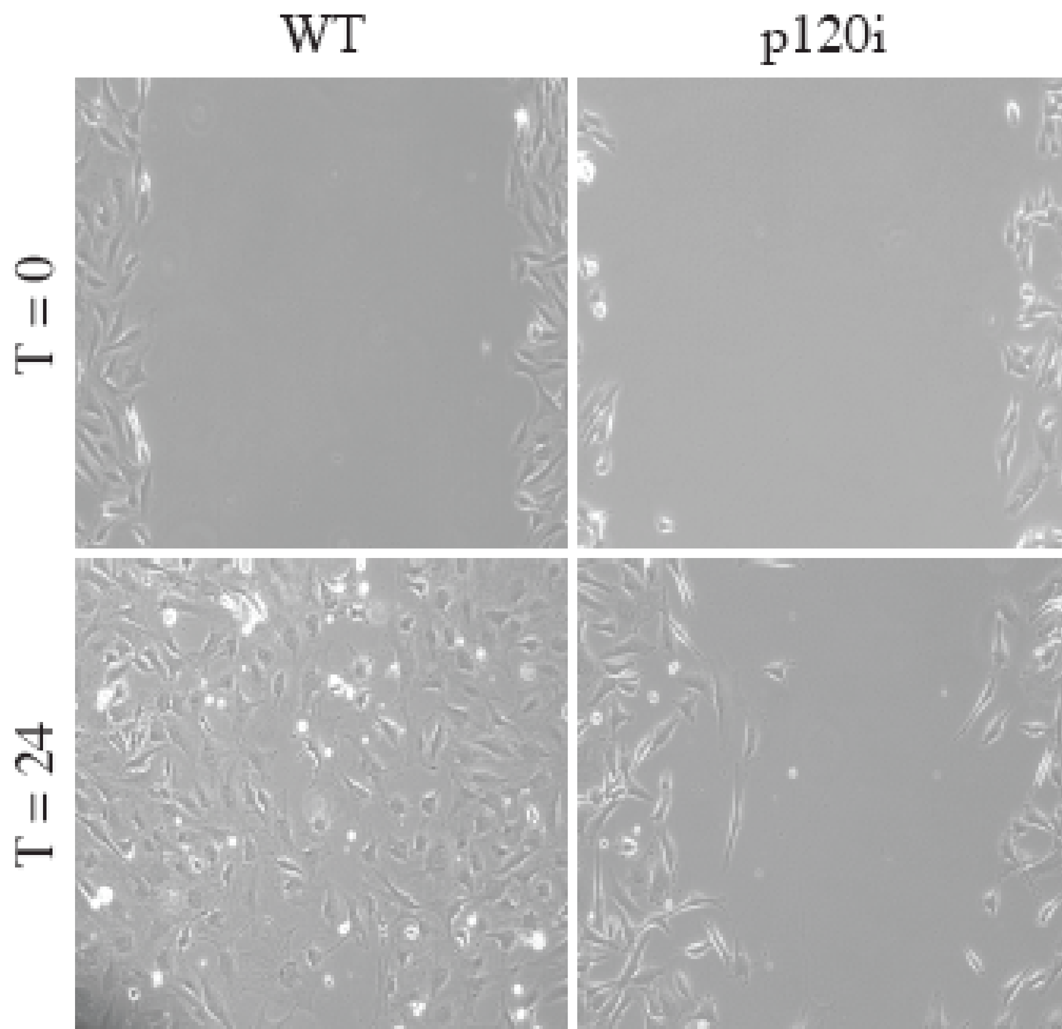


Figure 9. p120i cells fail to migrate into a wound.
WT and p120i cells were ‘wounded’ by scraping with a plastic pipette tip and photographed immediately (T=0) or after 24 hrs (T=24).

mechanisms.

p120i cells are partially transformed

Increased Rho activation has also been associated with cell transformation and changes in cell proliferation (Qiu, Chen et al. 1995; del Peso, Hernandez-Alcoceba et al. 1997; Fritz, Just et al. 1999; Jaffe and Hall 2002). Because Rho was constitutively activated in p120i cells, we examined if these cells were transformed. Interestingly, whereas WT cells growth arrested normally at high density, p120i cells continued to proliferate to extremely high density (Fig. 10) until they rolled off the plate as a single sheet of viable cells (not shown). Consistent with this, p120i cells grew to 4 fold higher density than WT cells when grown in the presence of serum. Surprisingly, when grown in the absence of serum, WT cells died after 7 days, but p120i cells continued to grow, albeit slower than normal (Fig. 11). p120i cells were also able to induce foci in secondary foci formation assays (Fig. 12). p120i-induced foci was blocked by expression of the Rho-inhibitor C3, and suppressed (but not blocked) by expression of N-cadherin. Overall, these data are consistent with the effects of activating Rho mutants or Rho-GEF oncogenes, which also induce foci formation and loss of contact inhibition (Jaffe and Hall 2002).

Discussion

p120 regulates RTK- and integrin-dependent Rho inhibition

These data demonstrate that ablation of p120 in NIH3T3 results in the constitutive activation of Rho. Furthermore, upstream signals from RTKs (i.e. PDGFR) and integrins

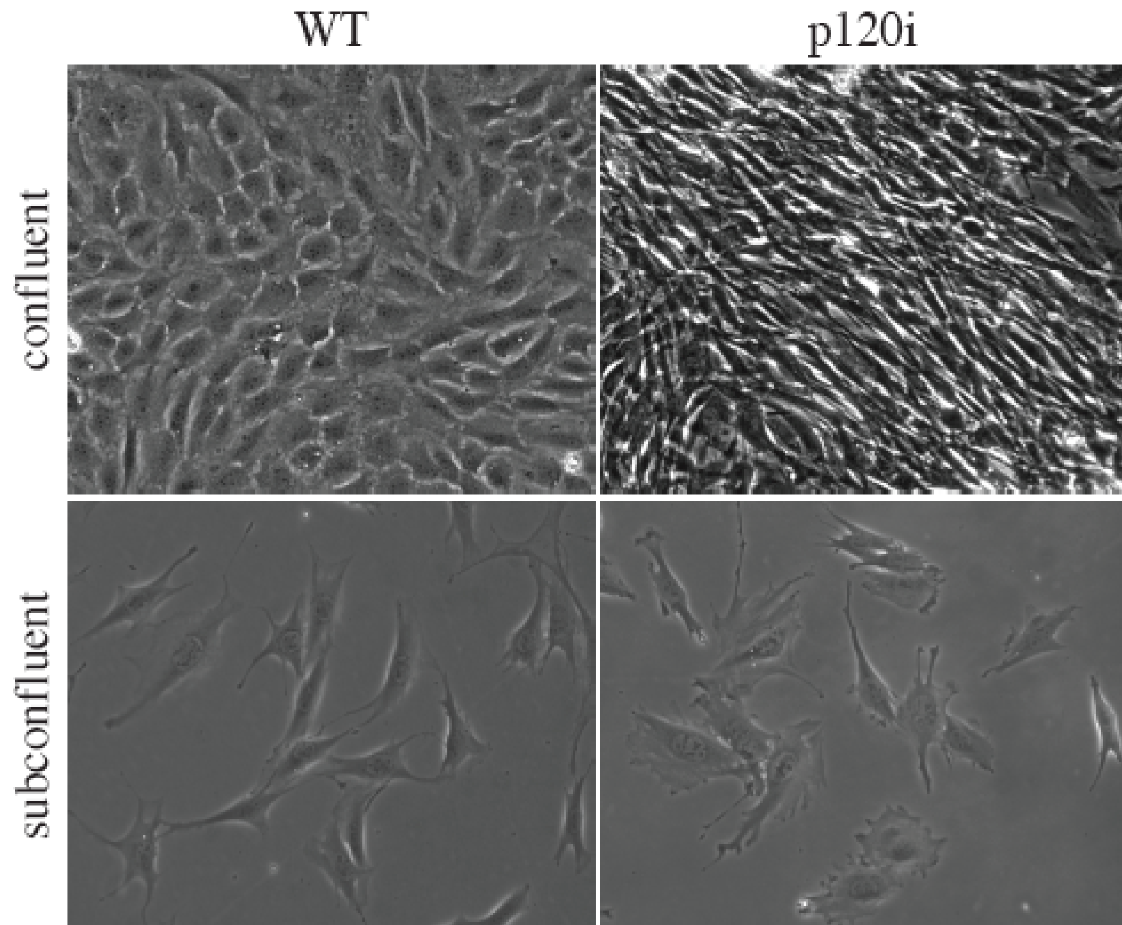
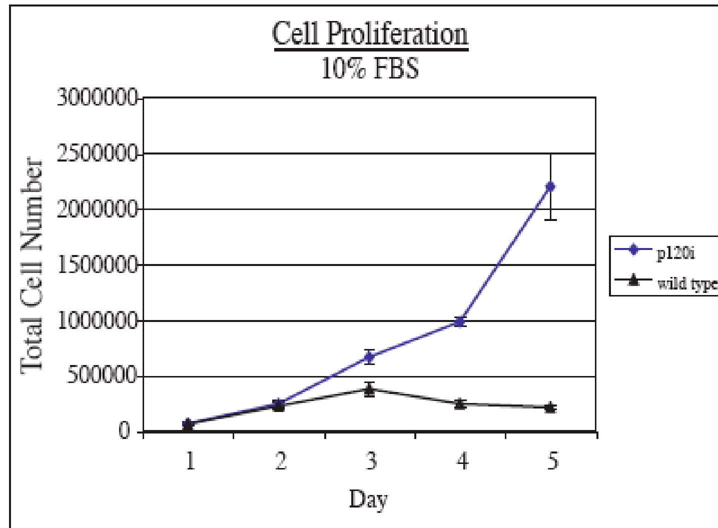


Figure 10. p120i cells grow to high density and fail to contact inhibit.
WT and p120i cells were photographed at low density (subconfluent), or two days following density-dependant growth arrest of WT cells (confluent). WT cells persist for weeks in this state, but p120i cells continue to proliferate and then rolled off the plate as a single sheet of viable cells.



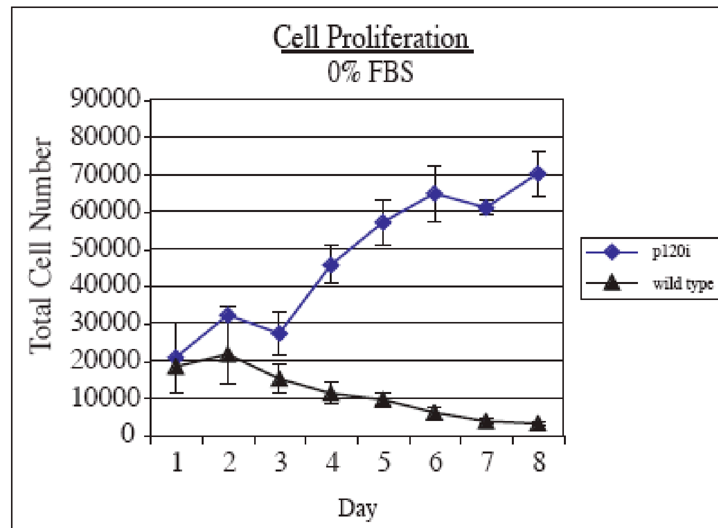


Figure 11. p120i cells grow to higher density in 10% FBS, and proliferate in the absence of serum.
Cells were plated at equal densities, cultured in the presence (upper panel) or absence (lower panel) of serum, and counted at 24 hour intervals (n = 3 independent experiments). Note that normal cells growth arrest within 48 hrs if serum is present (upper panel), but p120i cells grow to over 4 fold higher density before rolling off the plate. p120i cells grow in the absence of serum (lower panel), whereas WT cells do not.

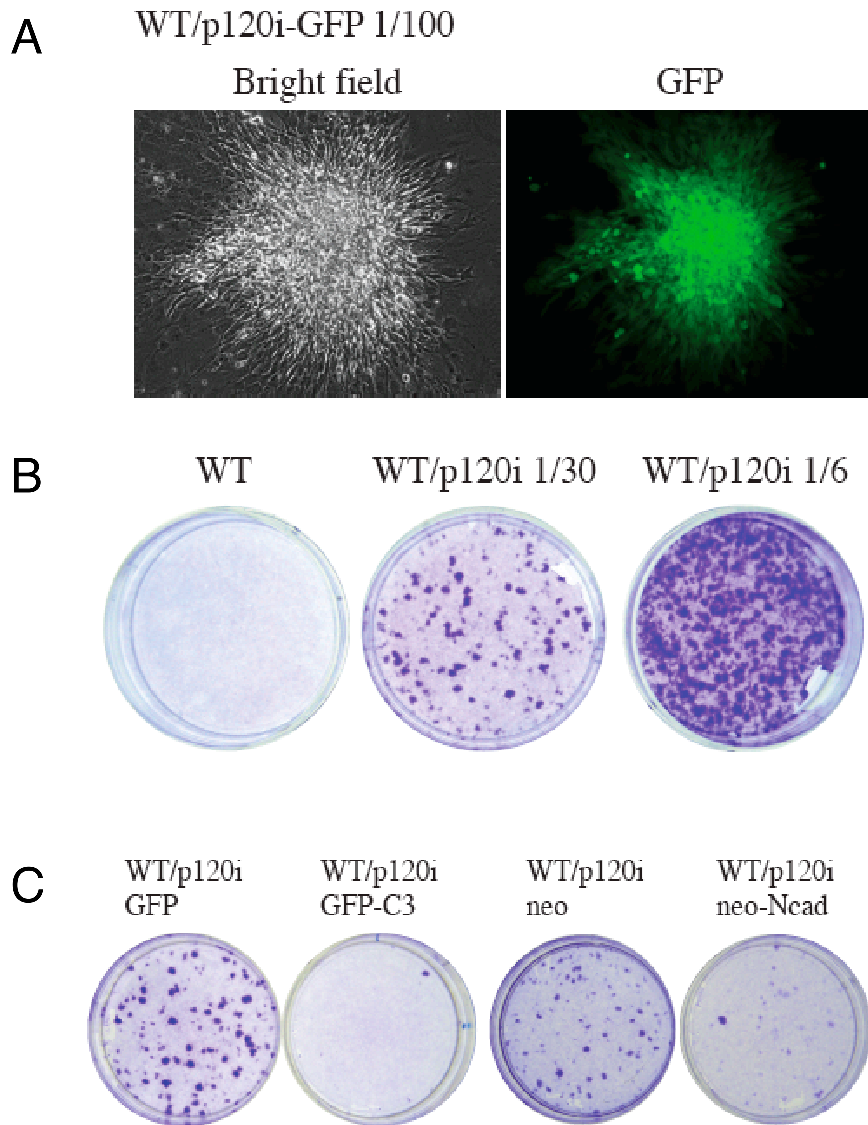


Figure 12. p120i cells induce foci formation.
(A) High magnification photographs of foci generated by GFP-marked p120i cells reveal that foci consist of concentrations of overgrown p120i cells.
(B) Contact inhibition was assessed by focus formation. p120i cells were mixed at the indicated ratios with WT cells, co-cultured until confluent, and then cultured an additional 10 days. Foci were detected by crystal violet staining.
(C) Focus formation by p120i cells is blocked by expression of the Rho inhibitor C3, and reduced by expression of N-cadherin.

are unable to inhibit Rho activity in p120-depleted cells. This is particularly surprising given that p120 interacts exclusively with N-cadherin, and is not known to be involved in RTK or integrin signaling. This suggests that RTKs or integrins either utilize p120 in a cadherin-independent manner, or that p120 mediates crosstalk between these receptors and the cadherin complex to regulate Rho. p120 is tyrosine phosphorylated by various growth factors (Kanner, Reynolds et al. 1991), and these data suggest that signaling to p120 is essential for Rho inactivation.

Interestingly, PDGFR-mediated DCRs have been shown to be formed through Rac inhibition of Rho. p120 reduction does not affect Rac activation by the PDGFR, but blocks signaling to Rho. Therefore, it appears that p120 may function in relaying Rac activity to Rho inhibition. Because Rac activation by PDGFR is unaffected in p120i cells, it also suggests that Rac activity alone is insufficient in mediating changes on the actin cytoskeleton (e.g. membrane ruffles). Rather, it is the combined effect of Rac activation and Rho inhibition that drives this process. Local Rac activation and Rho inhibition has been documented in a variety of processes such as RTK signaling, EMT, integrin spreading, motility, and contact inhibition, and thus represents a major arm of Rac signaling (Sander, van Delft et al. 1998; Ren, Kiosses et al. 1999; Zondag, Evers et al. 2000; Noren, Niessen et al. 2001; Wojciak-Stothard, Potempa et al. 2001; Nimnual, Taylor et al. 2003). The extraordinary level of Rho activation caused by p120-depletion is consistent with a prominent role for p120 in regulating the overall activity of Rho. Presumably, GEFs, GDIs, and GAPs should be able to intertact with Rho in p120i cells. It is surprising, therefore, that these alternative mechanisms are apparently insufficient to compensate for the reduction of p120. Nevertheless, the fact that multiple signaling pathways

(i.e. RTKs, integrins) apparently must signal through p120 to inhibit Rho may explain the potency of p120 loss. Interestingly, the increase in the resting level of Rho activity in p120i cells reinforces the concept that the activity of RhoGTPases is determined by the balance of positive and negative signals (see Fig. 1). Therefore, depletion of p120 presumably uncouples a major negative stimulus and shifts the balance towards Rho activation.

Initial observations of p120 depletion confirms that p120 plays a major role in regulation of Rho that previous overexpression experiments demonstrated. This approach has also revealed a surprising and novel role for p120 in controlling RTK and integrin-dependent inhibition of Rho. An outstanding question is how p120 coordinates signals from RTKs and integrins to inhibit Rho. Presently, it is difficult to interpret this data since p120 has never been functionally or physically associated with either system. It also remains to be determined whether the effects seen on Rho activity in p120-depleted cells is cadherin-dependent or independent. Nevertheless, it is clear from these data that p120 plays a major role in regulating the overall activity of Rho, and appears to globally affect signaling from multiple receptor systems to Rho.

p120-depletion partially transforms NIH3T3

Along with its role in actin stress fiber assembly, Rho is a critical regulator of the cell cycle. Overexpression of RhoGEFs or activating mutants of Rho lead to oncogenic transformation in many cell types (Jaffe and Hall 2002). Therefore, the affects on growth in the p120i cells are not surprising given that Rho is constitutively active. However, it is surprising that this is caused by the loss of p120 expression alone. These data suggest a

major role for p120 as a tumor suppressor through the regulation of Rho. Foci formation assays were previously utilized to determine if overexpressing certain genes could function as oncogenes by assaying for their ability to form foci and transform NIH3T3. However, with the advent of siRNA, a similar approach in unveiling tumor supressor capabilities can be utilized.

CHAPTER IV

P120 IS ESSENTIAL FOR RAC TO RHO SIGNALING

Introduction

p120 depletion in NIH3T3 prevents PDGFR-induced ASF disassembly and formation of DCRs. DCR formation has been previously shown to be a Rac-dependent mechanism (”the Bar-Sagi pathway”) described by Bar-Sagi and colleagues (Nimnual, Taylor et al. 2003). Briefly, Rac activation results in the production of reactive oxygen species (ROS), which inhibits low-molecular weight protein tyrosine phosphatase (LMW-PTP). Inhibition of LMW-PTP results in tyrosine phosphorylation (activation) of p190, which in turn inhibits Rho (Fig. 13). The coordination of Rac and Rho activities is an essential process in ensuring the proper balance of actinomyosin contractility that is necessary in the regulation of cell shape, motility, adhesion, and growth. The Bar-Sagi pathway is utilized by both RTK and integrin signaling to rearrange the actin cytoskeleton (Sander, ten Klooster et al. 1999; Nimnual, Taylor et al. 2003), and has also been shown to function in the maintainance of both E-cadherin and VE-cadherin based AJs (Zondag, Evers et al. 2000; van Wetering, van Buul et al. 2002; Malliri, van Es et al. 2004; Wojciak-Stothard, Tsang et al. 2005). p190 is an ubiquitously expressed GAP with specific activity towards Rho (Hall 1992; Lamarche and Hall 1994). p190 functions in a wide variety of processes that include neuronal outgrowth and fasciculation, integrin-dependent adhesion, cytokinesis, and adipogenesis (Nakahara, Mueller et al. 1998; Billuart, Winter et al. 2001; Brouns, Matheson et al. 2001; Sordella, Jiang et al. 2003; Su, Agati et al. 2003;

Figure 13. Schematic of the 'Bar-Sagi' pathway showing previously described molecular events controlling Rac-mediated inhibition of Rho.

Barberis, Casazza et al. 2005). p190 contains a C-terminal GAP domain, which catalyzes the hydrolysis of GTP bound to Rho. The N-terminal end of p190 contains a GTP binding domain that is necessary for its activity on Rho, though the mechanism is not known (Tatsis, Lannigan et al. 1998; Roof, Dukes et al. 2000). p190 is tyrosine phosphorylated by numerous kinases such as EGFR, Src family members, and Arg/Abl (Chang, Gill et al. 1995; Haskell, Nickles et al. 2001; Wolf, Wilkes et al. 2001; Hernandez, Settleman et al. 2004). Upon tyrosine phosphorylation, p190 translocates from the cytoplasm to membranes and/or cytoskeletal structures such as ruffles where it can access pools of activated Rho (Arthur and Burridge 2001) (Sharma 1998). Thus, p190 phosphorylation and membrane translocation are tightly linked and are both necessary for its subsequent roles in cell signaling and Rho inhibition. Lastly, p190 forms a low level, constitutive complex with p120RasGAP (RasGAP), which increases upon tyrosine phosphorylation of p190. The role of RasGAP in p190 signaling is not clear, but studies suggest that RasGAP is necessary for phosphorylation of p190, and localization of p190 at focal adhesions. The mechanisms are not well understood (DeClue, Vass et al. 1993; Ellis, Measday et al. 1995; van der Geer, Henkemeyer et al. 1997; Fincham, Chudleigh et al. 1999).

Cadherin engagement has also been shown to induce src-dependent phosphorylation of p190 (Noren, Arthur et al. 2003). The functional consequence of this event, or where p190 localizes upon cadherin ligation, however, is not known. Several lines of evidence suggest that alleviation of contractility through the inhibition of Rho is necessary for proper AJ formation, and the reciprocal disassembly of AJs may be due to increased contractility through Rho activation (Dudek and Garcia 2001; Garcia, Liu et al. 2001; Wojciak-Stothard, Potempa et al. 2001). p190, therefore, may be involved in alle-

viating contractility through Rho inhibition during AJ formation. Interestingly, growth factor and integrin signaling, through regulation of Rac and Rho, can promote either AJ assembly or disassembly (Ridley, Comoglio et al. 1995; Monier-Gavelle and Duband 1997; Potempa and Ridley 1998; Chen and Gumbiner 2006). Although how regulation of RhoGTPases by RTKs or integrins is translated into AJ assembly/disassembly is not known, p120 is likely to be involved in this process due to its dual role in cell-cell adhesion and Rho regulation. In chapter three, we demonstrated that depletion of p120 blocks both RTK and integrin signaling at the level of Rho. Therefore, since both receptor systems utilize the Bar-sagi pathway to activate Rac and inhibit Rho, p120 may play a role in this pathway in relaying crosstalk between RTKs, integrins, and AJs.

Results

Rac needs p120 to inhibit Rho

We have previously shown in chapter III that p120-depletion in NIH3T3 results in the constitutive activation of Rho, which surprisingly blocks both RTK- and integrin-mediated rearrangements in the actin cytoskeleton. To determine the function behind these observations, we carried out a series of experiments to isolate the precise mechanism of p120-dependent Rho inhibition. To ensure that elevated ASFs in p120i cells was due to aberrant Rho signaling, we inhibited ROCK, a downstream effector of Rho that functions in stress fiber assembly (Leung, Chen et al. 1996). As seen in Figure 14, inhibition of ROCK with the specific inhibitor, Y-27632, caused the complete loss of ASFs in both WT and p120i cells. Thus, effects of p120 loss occur upstream of Rho and ROCK,

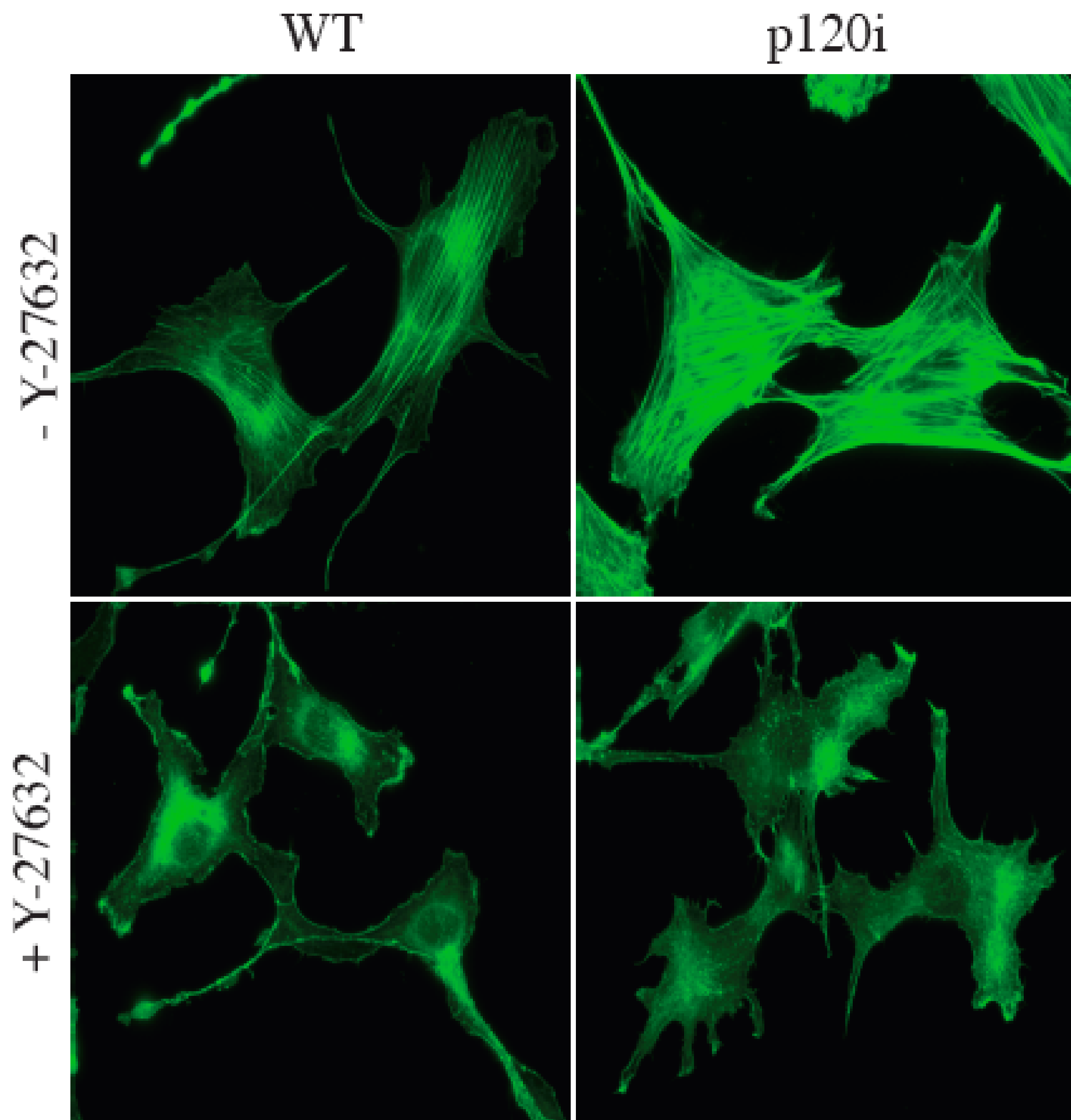


Figure 14. The effects of p120-depletion on actin stress fibers is upstream of ROCK. WT and p120i cells were treated as indicated with Y-27632, a selective inhibitor of ROCK, and stained for actin.

and are dependent on the effects of Rho activation. In contrast, expression of DA-Rac (Fig. 15) and ROS treatment (Fig. 16) disrupted ASFs in WT cells efficiently, but this activity was completely blocked in p120i cells. Therefore, p120 functions downstream of Rac-mediated ROS generation, but upstream of Rho.

To determine whether p120 functions upstream or downstream of p190, DA-Rac was expressed in WT and p120i cells and the activation of p190 was analyzed. Surprisingly, p190 tyrosine phosphorylation and association with p120RasGAP were increased by DA-Rac expression equally in the presence or absence of p120 (Fig. 17A). Additionally, overexpression of p190 was able to inhibit ASFs equally in WT and p120i cells (Fig. 17B). This suggests that Rac signaling to p190 is intact in p120i cells, and that p120 is not a necessary cofactor for p190 to couple to Rho when p190 is overexpressed. However, the striking failure of Rac to inhibit Rho in p120i cells suggests a previously unidentified functional interaction between p120 and p190.

Rac induces p190 localization to N-cadherin complexes

Upon tyrosine phosphorylation, p190 translocates to the plasma membrane were is can access active pools of Rho (Moon and Zheng 2003; Sordella, Jiang et al. 2003). Since p120 depletion does not affect Rac-induced tyrosine phosphorylation of p190, the inability of Rac to inhibit Rho in p120i cells may be due instead to a failure to properly localize p190 to the plasma membrane. To test this, we re-analyzed PDGF-induced DCR formation. Interestingly, PDGF treatment caused the precise colocalization of p190 and p120 at DCRs (Fig 18). p120 is dependent on cadherin association for localization to membranes (Thoreson, Anastasiadis et al. 2000) and indeed, N-cadherin also colocalized

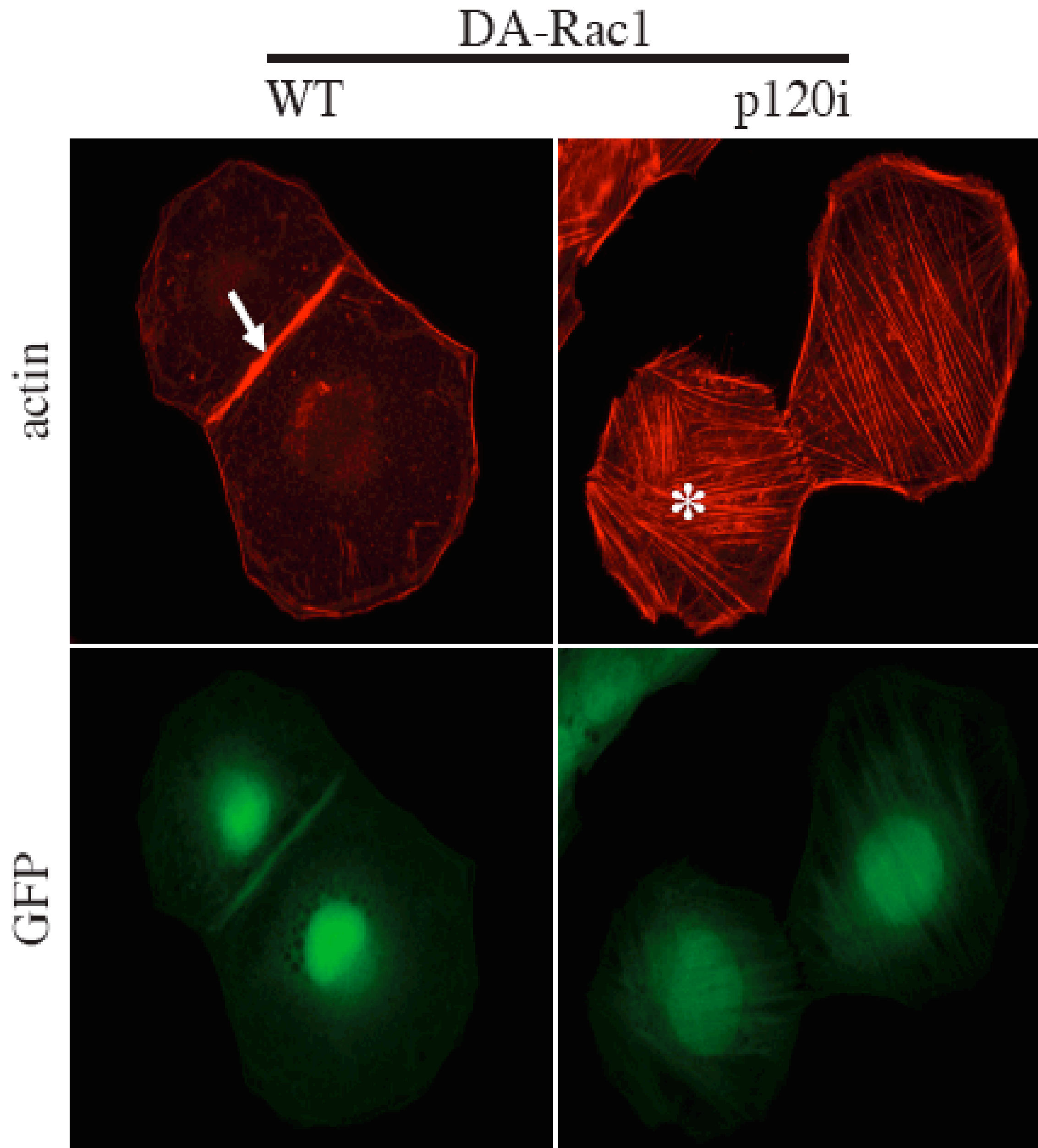


Figure 15. p120-depletion blocks DA-Rac-induced disassembly of actin stress fibers. To bypass the PDGFR, WT and p120i cells were infected by a retrovirus expressing DA-Rac (and an IRES-linked GFP marker), serum starved, and stained for actin. Note enhanced AJs (arrow) and loss of central ASFs in WT cells. Conversely, AJs fail in p120i cells, which exhibit excessive central ASFs (asterisk).

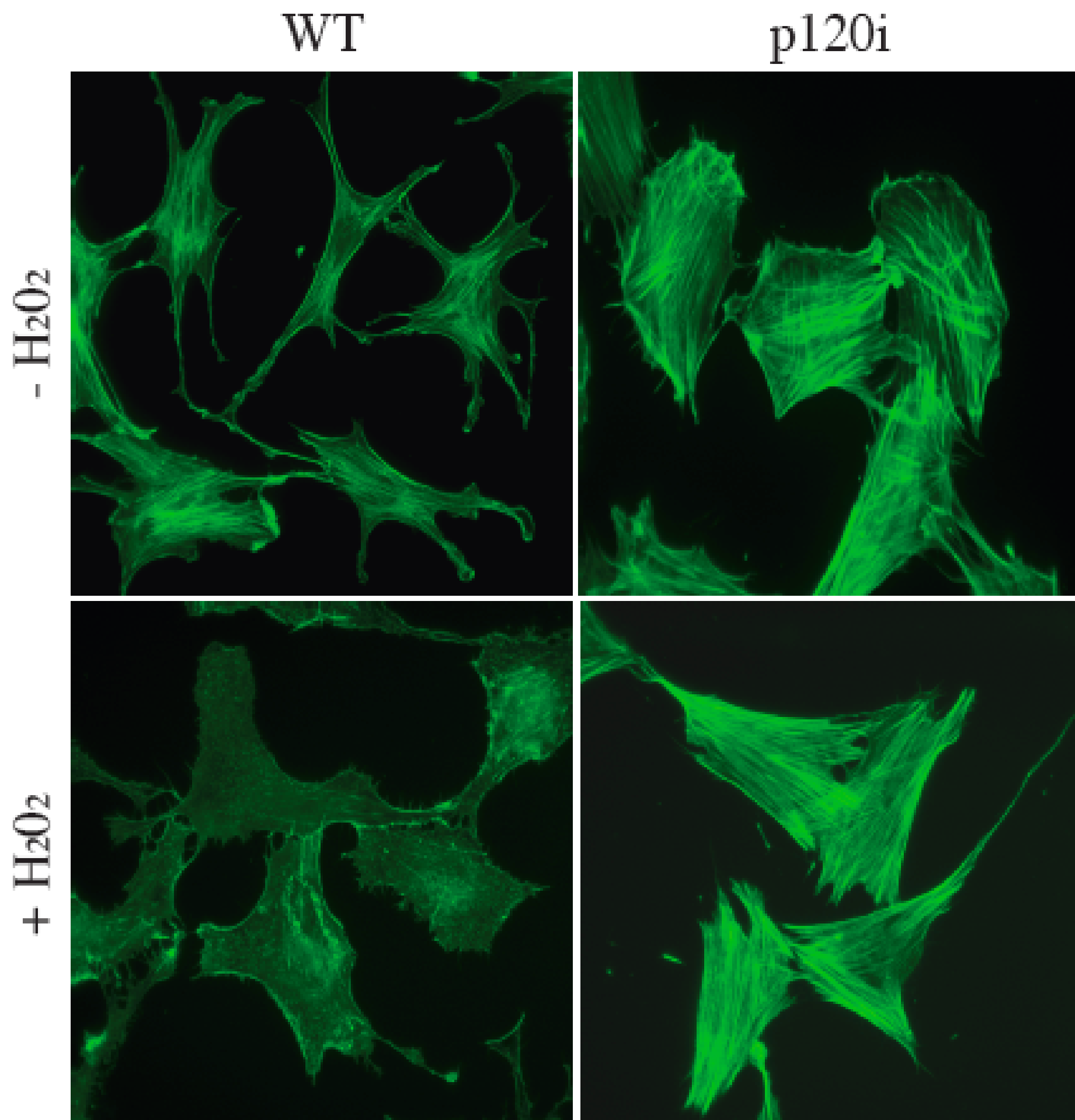


Figure 16. p120-depletion blocks ROS-induced stress fiber disassembly.
To bypass Rac1, WT or p120i cells were H_2O_2 treated (ROS) as indicated and stained for actin. Note that p120i cells are unresponsive.

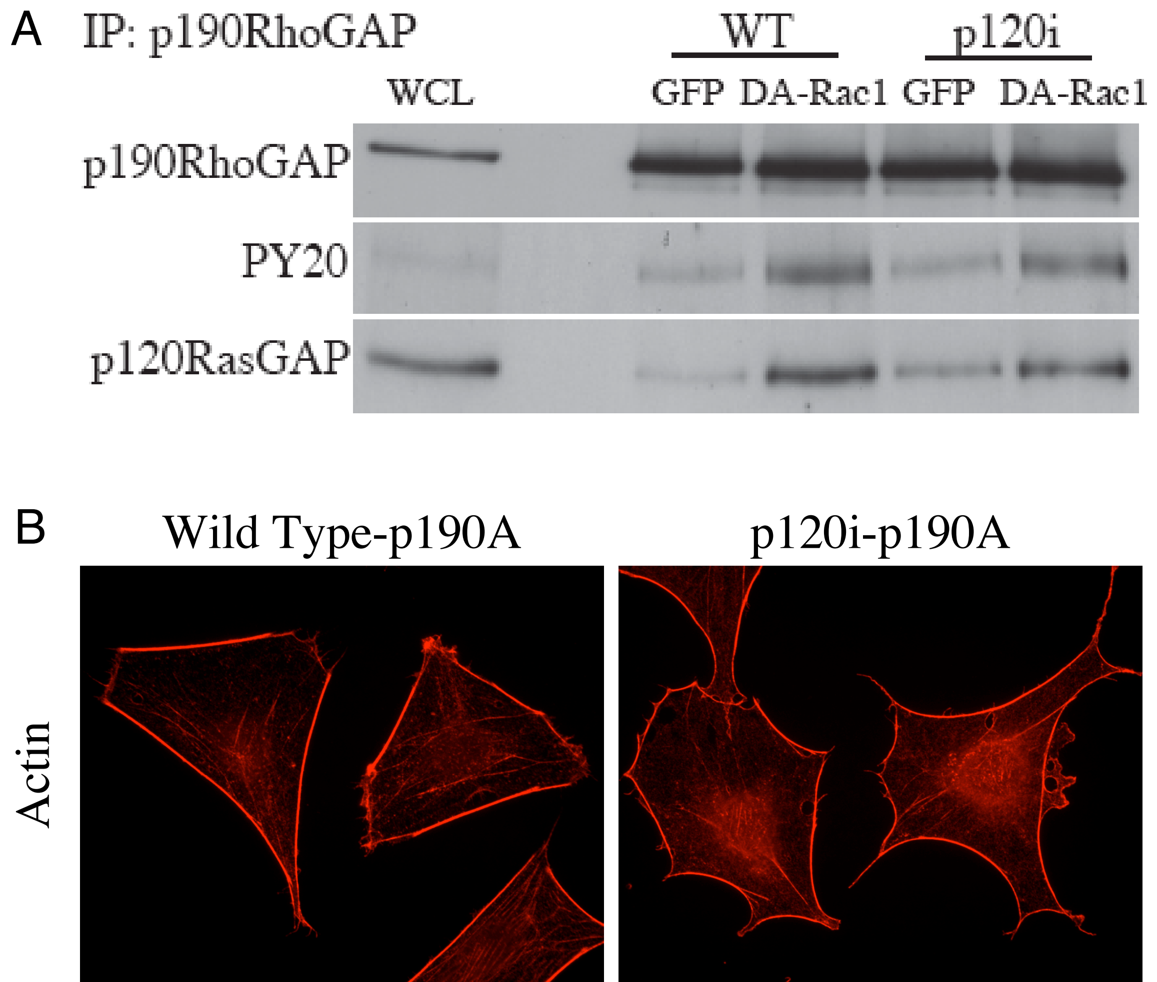


Figure 17. p120 functions either downstream or upstream of p190.
(A) WT and p120i cells were infected with retrovirus expressing GFP alone (GFP) or GFP and DA-Rac (DA-Rac1). p190 activation was assessed by immunoprecipitation of p190 and Western blotting for p190, phosphotyrosine (PY20), or p120RasGAP. Note that p190 is activated by DA-Rac in both WT and p120i cells.
(B) WT and p120i cells were infected with retrovirus expressing WT-p190RhoGAP-A (p190A), serum starved, and stained for actin.

with p120 and p190 in DCRs (Fig 18). Additionally, the obligatory role for p120 in PDGFR signaling to Rho implies tight functional and perhaps physical interaction between PDGFR and N-cadherin complexes. Consistent with this, activated PDGFR also co-localized with N-cadherin in DCRs (Fig. 19). Thus DCRs are N-cadherin-based structures that form in response to growth factor signaling (e.g. PDGFR). As predicted, siRNA-mediated ablation of N-cadherin abolishes DCR formation by PDGF (Fig. 20). These data demonstrates that PDGFR signals through Rac to cause the recruitment of p190 to N-cadherin-based DCRs.

Rac-induced recruitment of p190 to N-cadherin is dependent on p120

Rac activation also results in the "epithelialization" of NIH3T3 and the assembly of stable AJs via a pathway involving the inhibition of Rho (Sander, ten Klooster et al. 1999). To determine if Rac-induced AJs behaved in a similar manner to DCRs, we directly examined the effects of Rac signaling on AJ formation in WT and p120i cells. In both WT and p120i resting cells, p190 localization was restricted to the cytoplasm (Fig. 21). However, expression of DA-Rac in WT caused the striking translocation of p190 from the cytoplasm to AJs where it colocalized with N-cadherin (Fig. 22). However, translocation of p190 to AJs was completely blocked in p120i cells, and junctions failed to completely assemble into a tight linear array (Fig. 22). DA-Rac-induced translocation of p190 to AJ was also observed in the epithelial cell line, A431 (Fig. 23). Interestingly, in the MCF-10A mammary epithelial cell line, p190 localized to AJs basally (Fig. 24). Thus localization of p190 to AJs is not limited to fibroblasts.

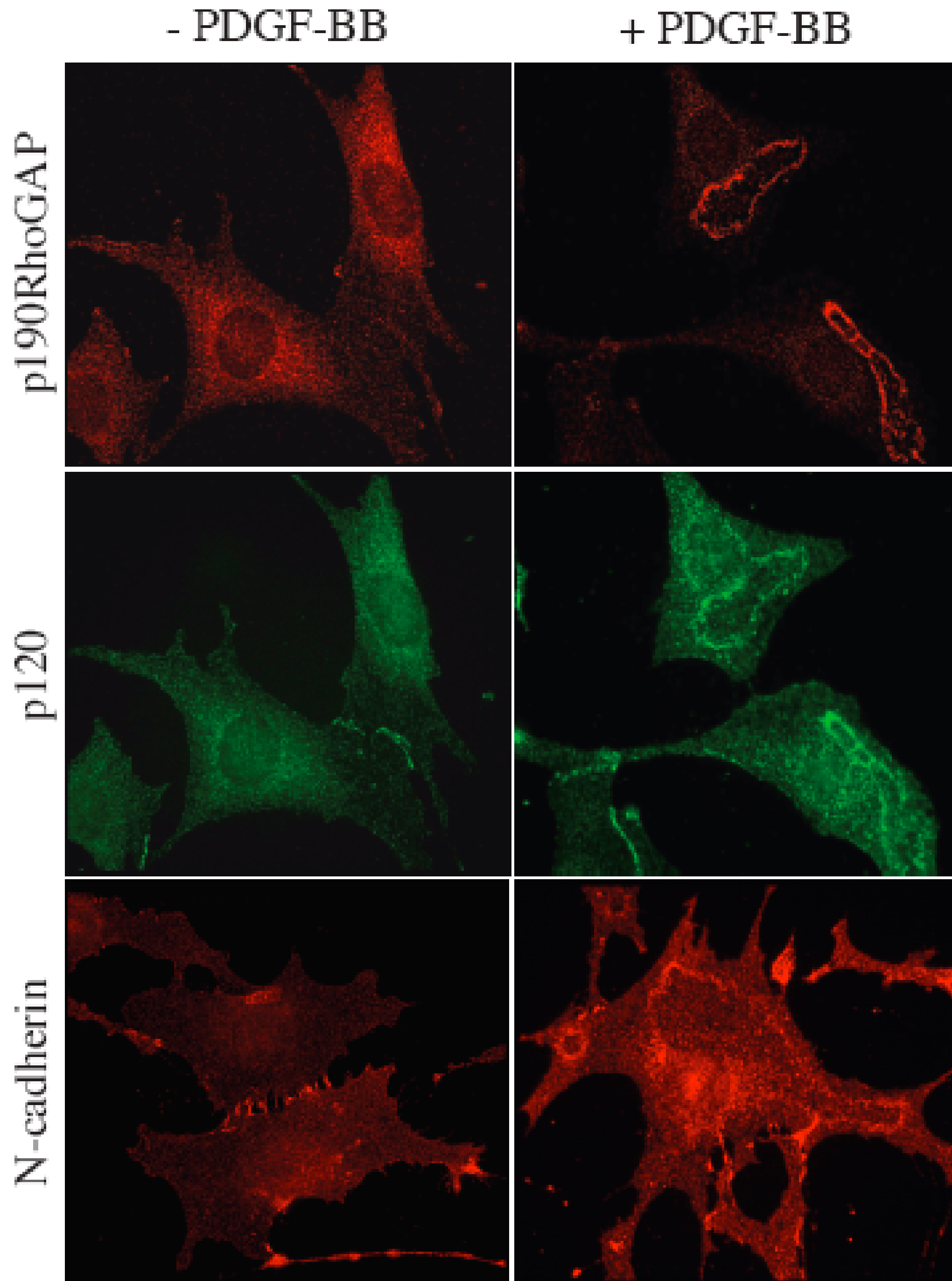


Figure 18. PDGF induces the colocalization of p190, p120, and N-cadherin in DCRs. Serum starved cells were mock stimulated (-PDGF-BB) or PDGF-stimulated (+ PDGF-BB) for 7 minutes and processed for coimmunofluorescence with antibodies to p190RhoGAP, and p120, or N-cadherin alone.

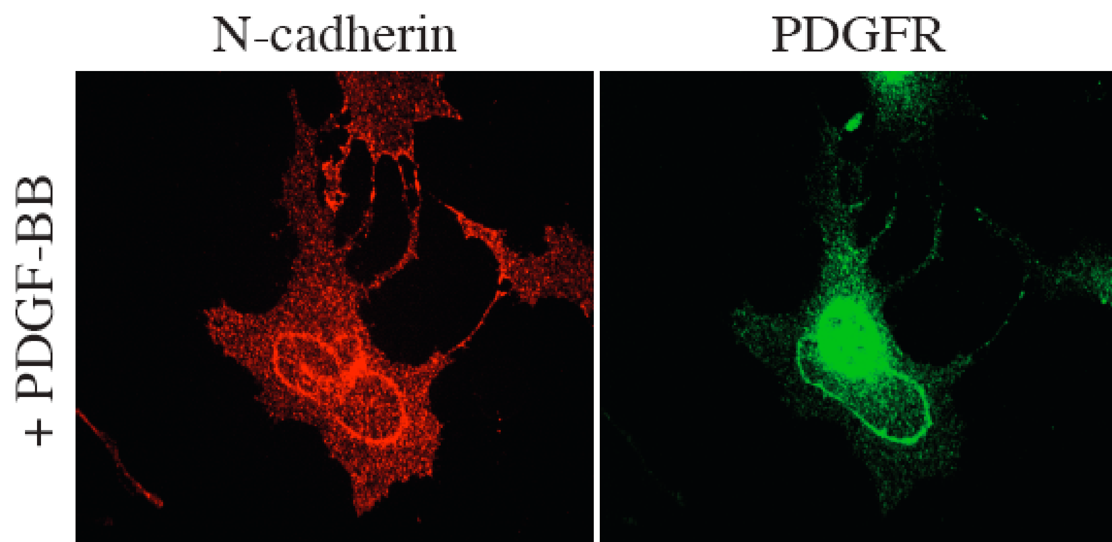


Figure 19. PDGFR and N-cadherin colocalize in PDGF-induced DCRs.
Cells were serum starved, PDGF-stimulated, and costained for N-cadherin and PDGFR.

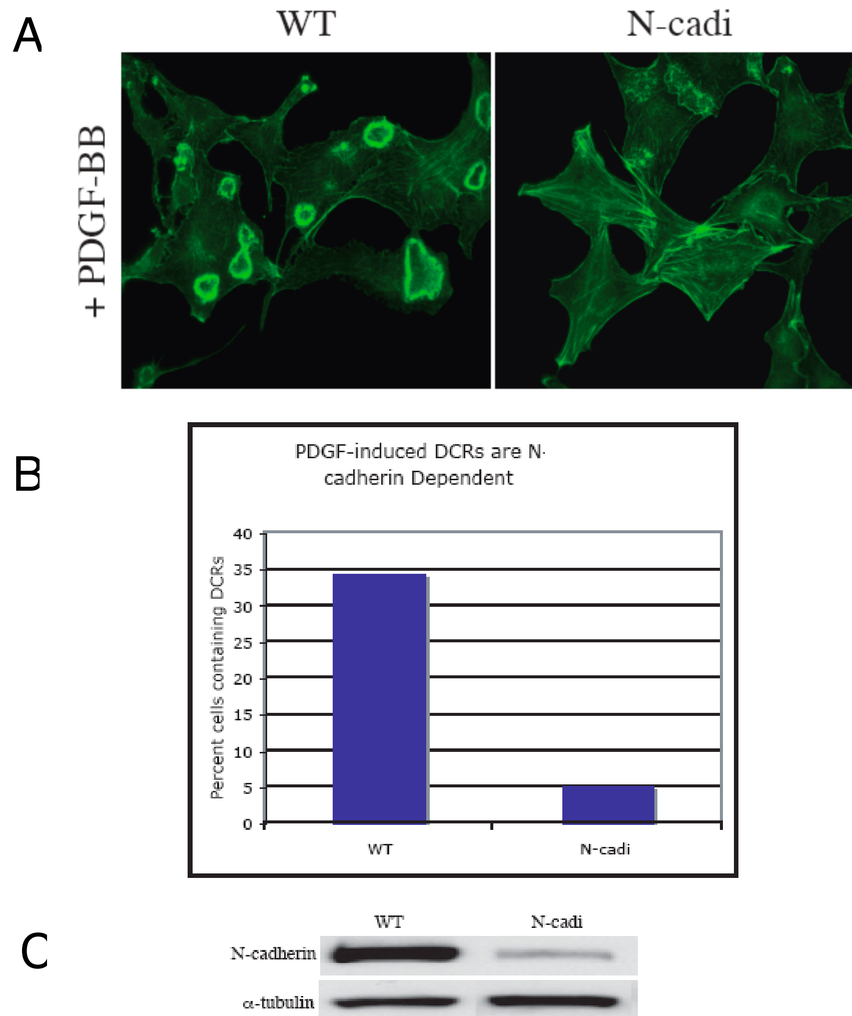


Figure 20. PDGF-induced DCRs are N-cadherin-dependent structures.
(A) WT and N-cadherin knockdown cells (N-cadi) were PDGF stimulated as previously described and stained for actin.
(B) Quantification of PDGF-induced DCRs in WT and N-cadherin knockdown cells. 350 cells were randomly selected and scored for the presence or absence of dorsal circular ruffles after PDGF stimulation in wild type (WT) and N-cadherin knockdown (N-cadi) cells.
(**C**) Analysis of N-cadherin expression by Western blotting in WT and N-cadherin siRNA (N-cadi) expressing NIH3T3 cells.

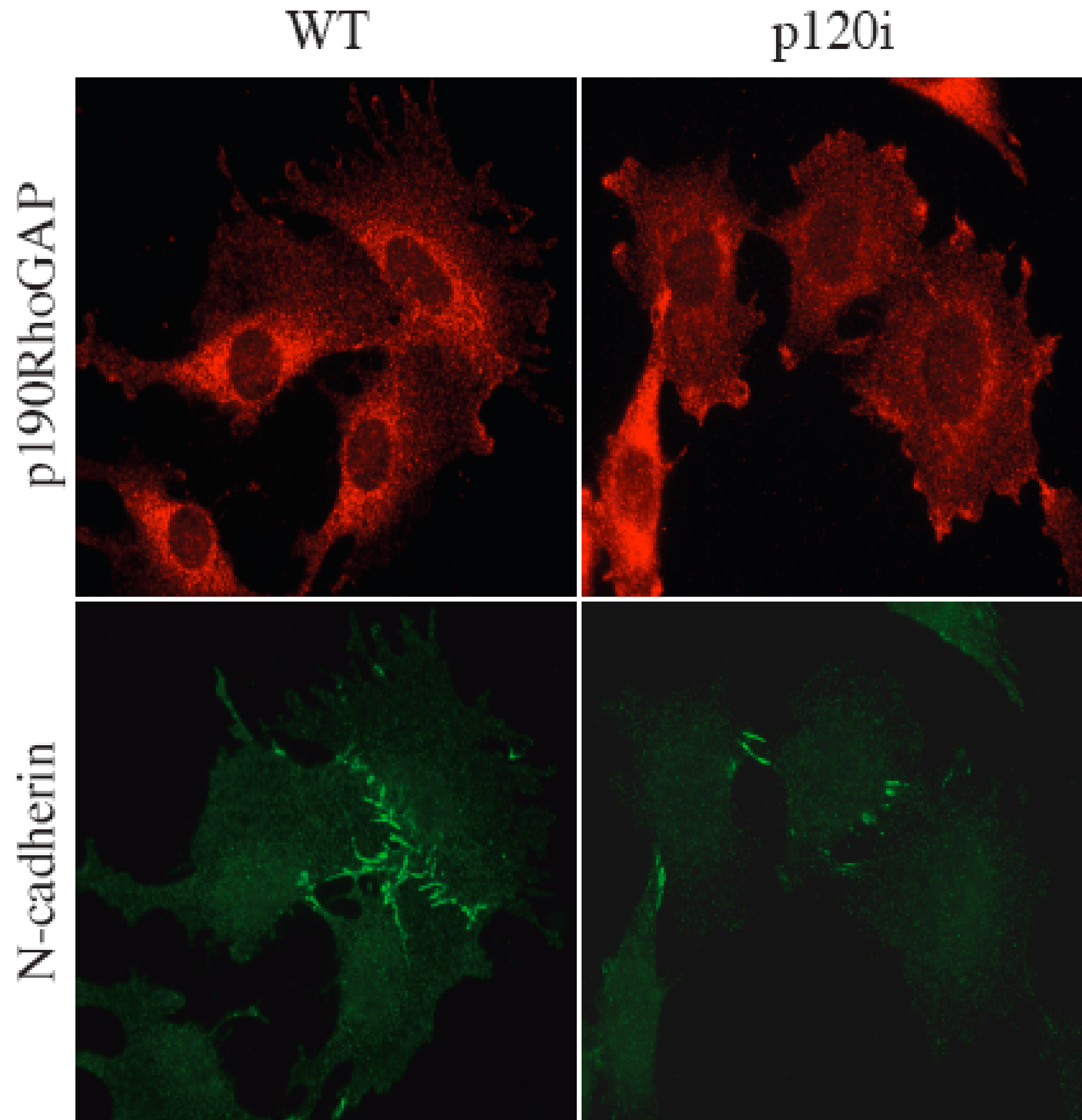


Figure 21. p190 predominantly localizes in the cytoplasm in WT and p120i cells. WT and p120i cells were costained with antibodies to p190 and N-cadherin.

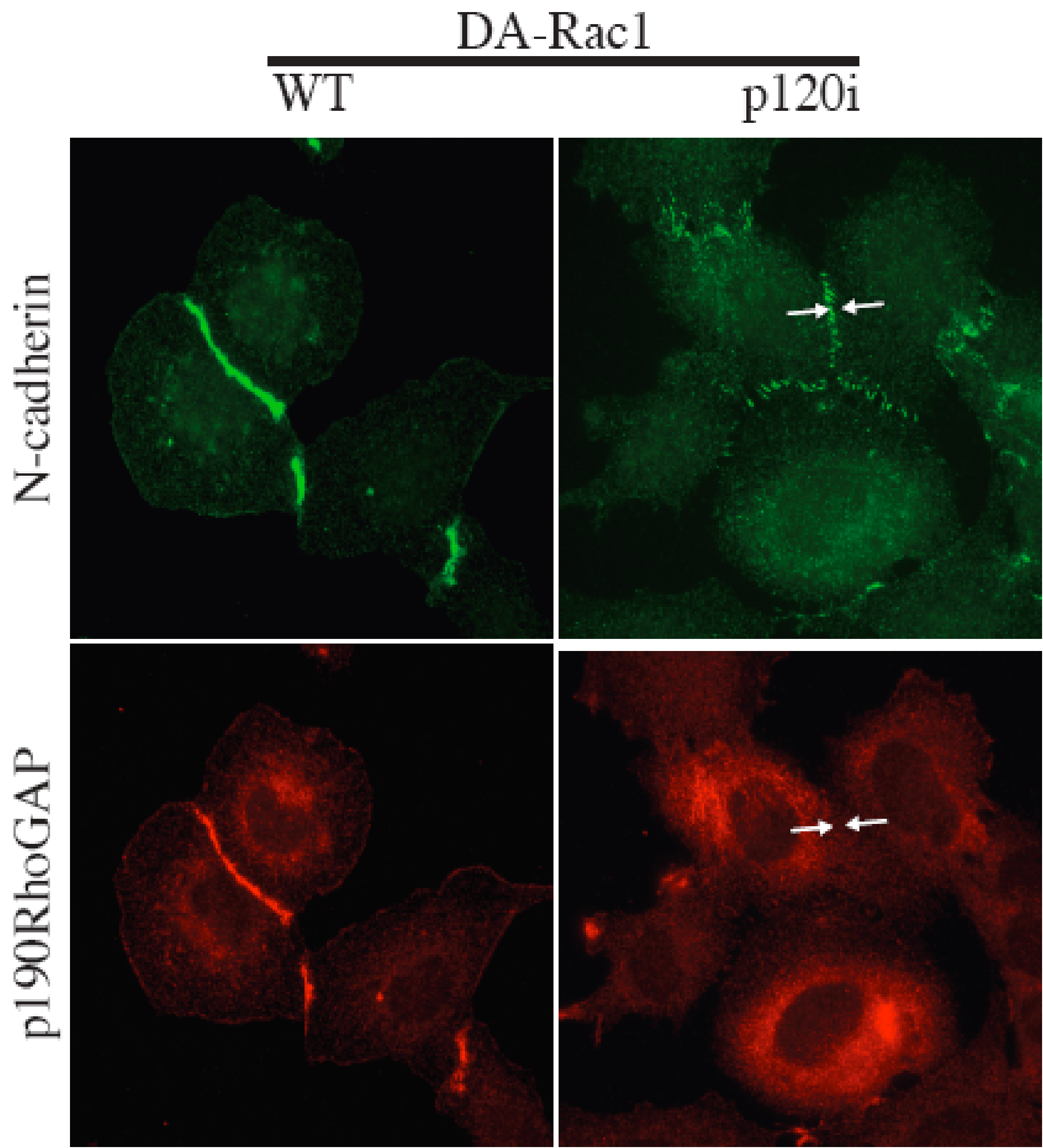


Figure 22. DA-Rac causes the colocalization of p190 with N-cadherin in WT, but not in p120i cells.
WT and p120i cells were infected by a retrovirus expressing DA-Rac1, serum starved, and stained for N-cadherin and p190. Arrows indicate compromised AJs and absence of p190 staining in p120i cells.

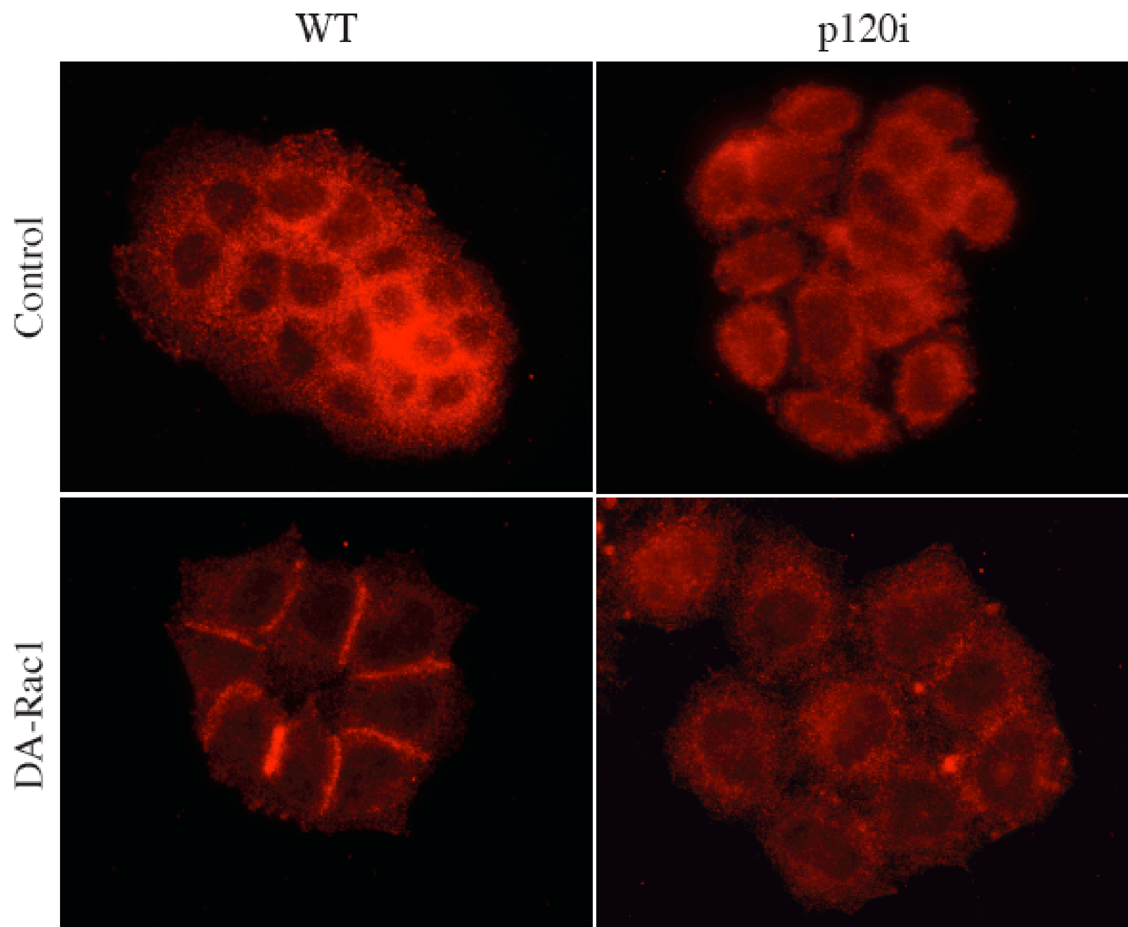


Figure 23. DA-Rac induces p120-dependent association of p190RhoGAP with adherens junctions in the epithelial cell line A431.
WT and p120i A431 cells were either infected with control retrovirus or with DA-Rac expressing retrovirus, and visualized for p190. Note that DA-Rac drives p190 to AJs in WT cells, but not in p120i cells.

p190 and p120 interact

The dependency on p120 for p190 recruitment to AJs suggests that the two physically interact. Therefore, we performed coimmunoprecipitation experiments with p120 and other core components of the cadherin complex (Fig. 25A). p190 coimmunoprecipitated efficiently with p120, but not with β-catenin or N-cadherin, suggesting that p190 directly interacts with p120, and indirectly with the remaining cadherin complex. To clarify this, a p120 mutant was generated that interacts constitutively with the plasma membrane, but lacks an Armadillo repeat necessary for binding to cadherins (i.e. p1201A-ΔArm1-CAAX). As shown in Figure 25B, this p120 mutant localized to the plasma membrane, and efficiently recruited p190 but not N-cadherin. Therefore, p120 is able to recruit p190 independent of N-cadherin or other cadherin complex components. These data suggests that Rac-induced binding of p190 to cadherin complexes in both DCRs and AJs is mediated by association with p120.

p190 is necessary for Rac-induced adherens junction formation

Although p190 does not localize to AJs under normal detection methods, the above data suggests a functional role for p190 in cell-cell adhesion. To address this further, p190A -/- MEFs were analyzed for their ability to form proper AJs by expression of DA-Rac. AJs in the absence of p190A were not able to form after DA-Rac expression as shown by mislocalized p120 and N-cadherin (Fig. 26). To confirm that the defect was due to the absence of p190A, DA-Rac-induced AJs were rescued by expression of WT-p190A. In contrast, expression of p190A mutants defective in N-terminal GTP binding (M5) or RhoGAP activity (30-1) were completely unable to rescue AJs (Fig. 26). Both

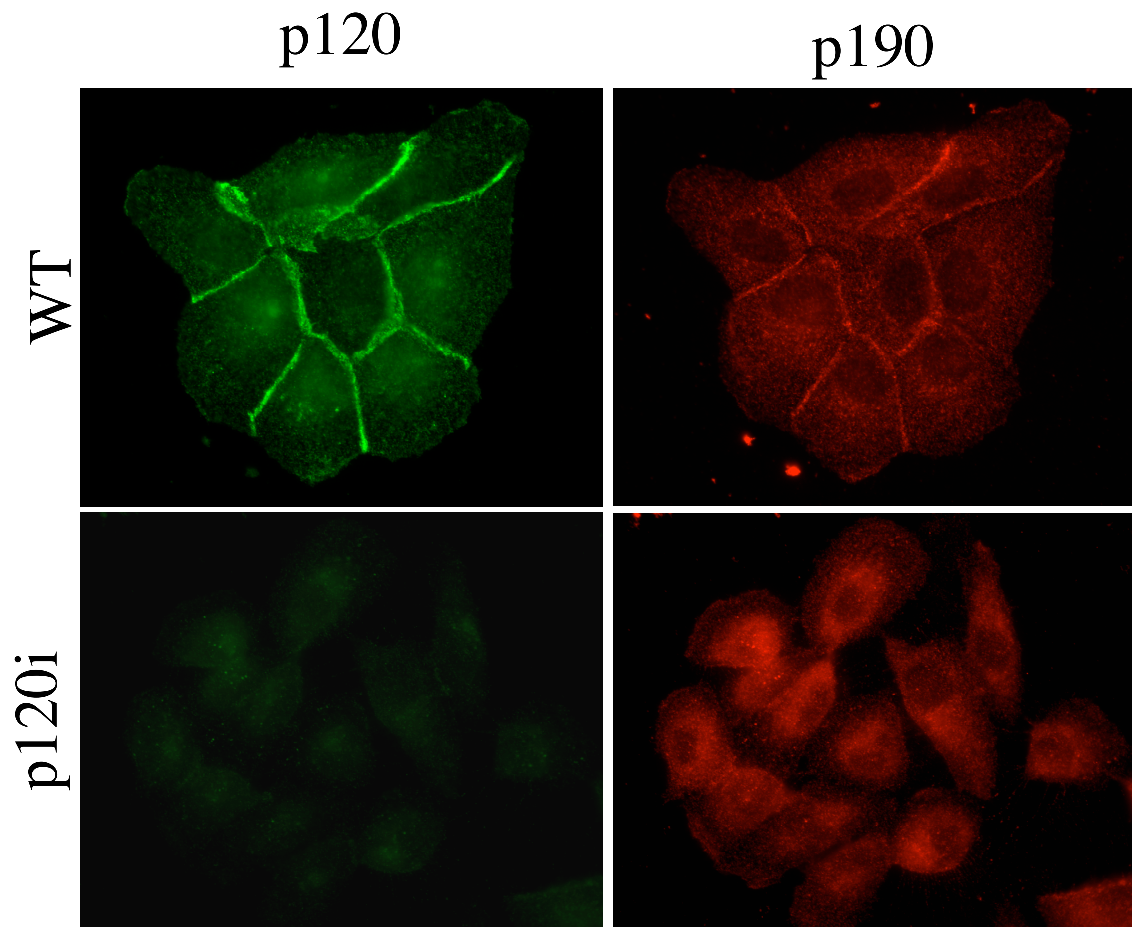


Figure 24. p190 localizes at AJs in a p120-dependent manner in MCF-10A cells. WT and p120i MCF-10A cells were co-stained with antibodies to p120 and p190.

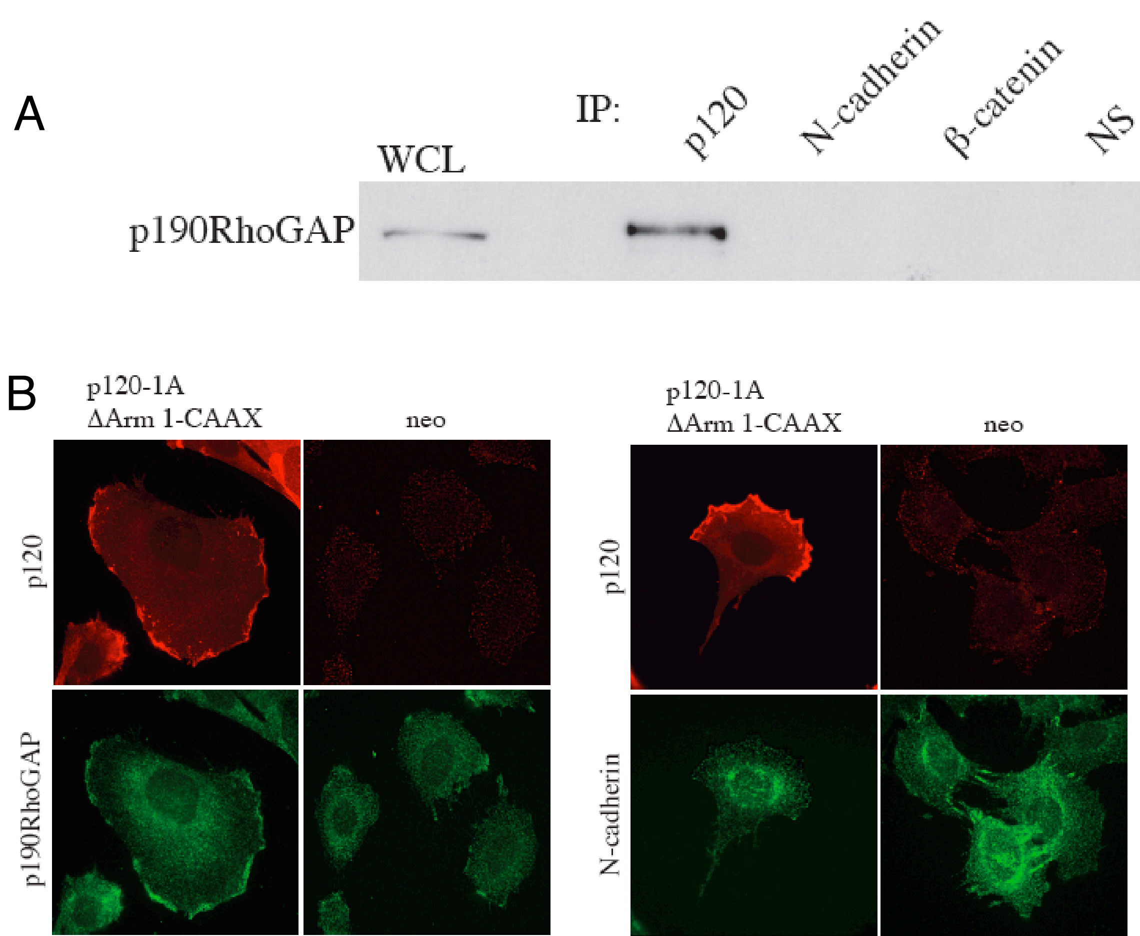


Figure 25. p120 interacts with p190, and is sufficient to induce p190 translocation independently of the cadherin complex.

(A) Immunoprecipitation with p120, N-cadherin, β-catenin, or irrelevant control (NS) antibodies was followed by Western blotting with antibodies to p190. Whole cell lysate (WCL, left) was included as a marker for p190.

(B) A murine p120 mutant (mp120-1A-ΔArm1-CAAX) was generated that cannot bind to cadherins but localizes to membranes by virtue of a fused CAAX box. The construct contains a silent mutation within the siRNA targeted region of p120 to allow its expression in the murine p120 siRNA background. p120i cells expressing the p120 mutant (mp120-1A-ΔArm 1-CAAX), or neomycin alone (neo), were costained with antibodies to p120 and p190 **(left panels)** or p120 and N-cadherin **(right panels)**.

mutations are unable to inhibit Rho through different mechanisms, which suggests that the activity on Rho by p190 is essential in this process. These mutants, however, were able to efficiently coimmunoprecipitate with p120, and therefore do not fail to permit AJ formation through a failure to bind p120 (Fig. 27).

These data show that p120 and p190 are essential in the formation of stable AJs. Without either p120 or p190, there is a failure to effectively inactivate Rho thereby preventing AJ formation. To understand the relationship between Rho signaling and AJ assembly further, ROCK was inhibited in DA-Rac expressing p190A -/- cell lines. ROCK inhibition in all cell lines was able to partially rescue AJ formation as shown in Figure 28. Thus, it is likely that Rac coordinates AJ assembly by promoting the recruitment of p190 to N-cadherin through p120 binding. There, p190 is able to inhibit Rho and suppress the contractility required for AJ formation.

Discussion

Rac requires p120 to inhibit Rho

Here we utilized an epistasis approach to identify where p120 lies in the Bar-Sagi pathway. To this end, we were able to identify p120 as an essential regulator of p190 localization at the plasma membrane in Rac-mediated inhibition of Rho. Although p120 was not necessary for phosphorylation of p190 by Rac, p120 was absolutely required to localize p190 to the cell surface where it could access activated pools of Rho. Because p120 only localizes to the plasma membrane through interacting with N-cadherin (or E-cadherin in A431 and MCF-10A cells), the activation of p190 is concentrated at cadherin

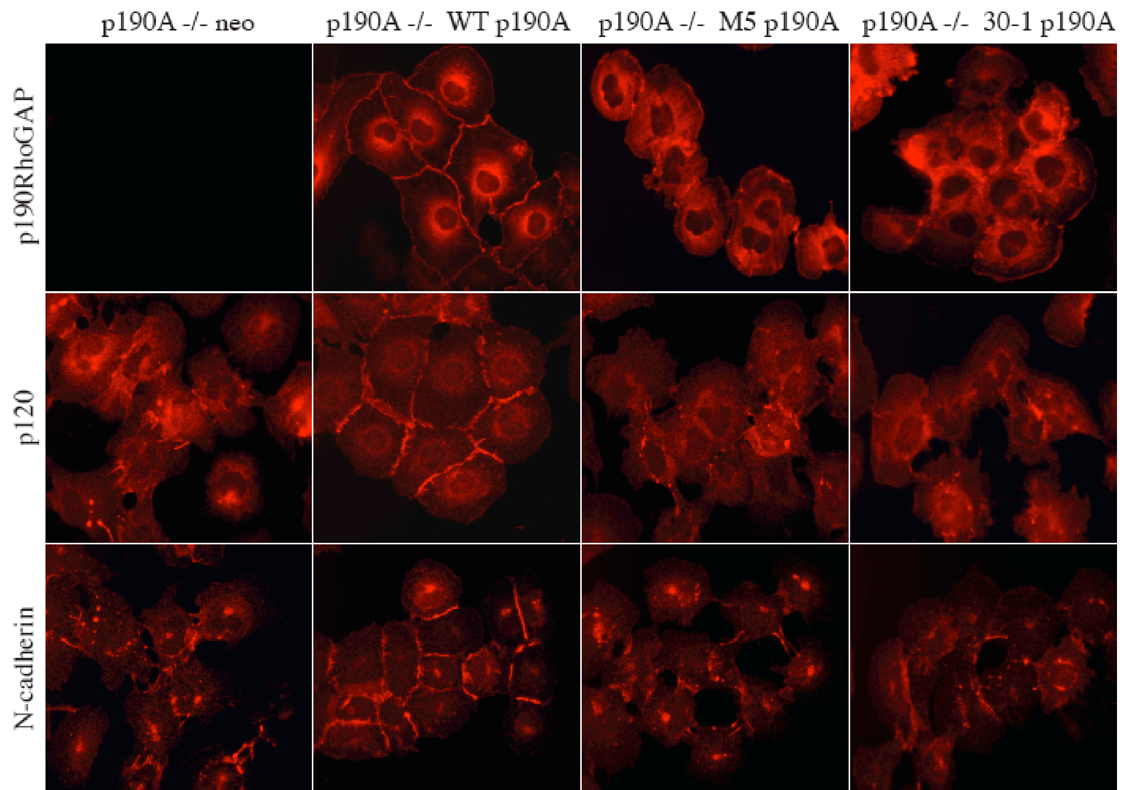


Figure 26. p190 is required for AJ formation.
p190A-null MEFS (p190A -/-) expressing empty vector (neo), WT p190-A (WT p190A), or functionally dead p190-A mutants (M5 p190A or 30-1 p190A) were infected with DA-Rac retrovirus and stained with antibodies to p190-A, p120, or N-cadherin, as indicated. The presence of DA-Rac was confirmed by concomitant GFP expression (not shown). Note that AJs do not form in the absence of p190-A (first column), and both p120 and N-cadherin are internalized. p120 and N-cadherin levels are not affected (not shown).

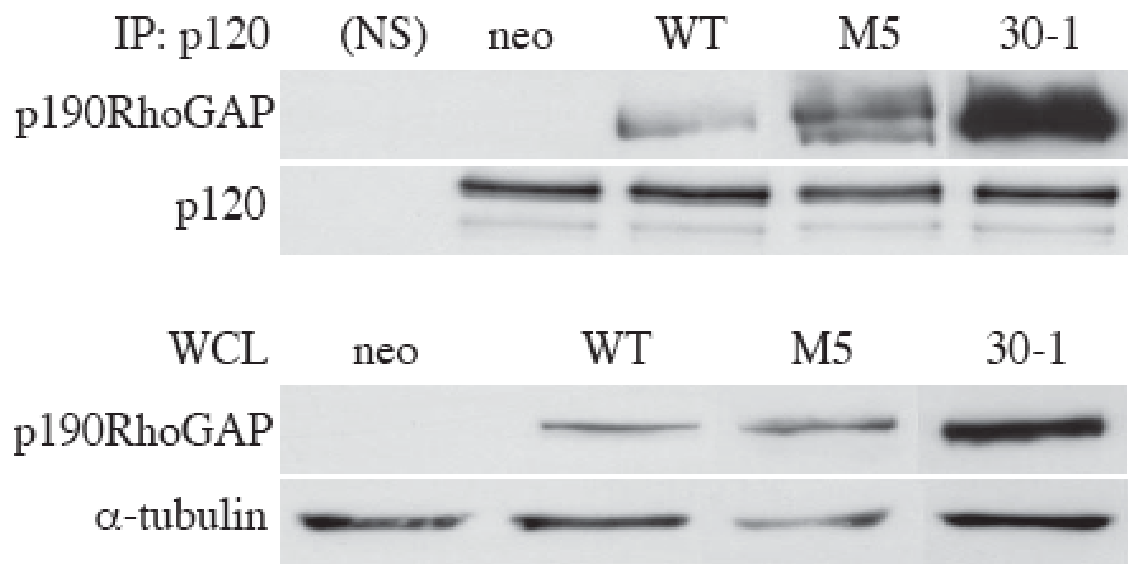
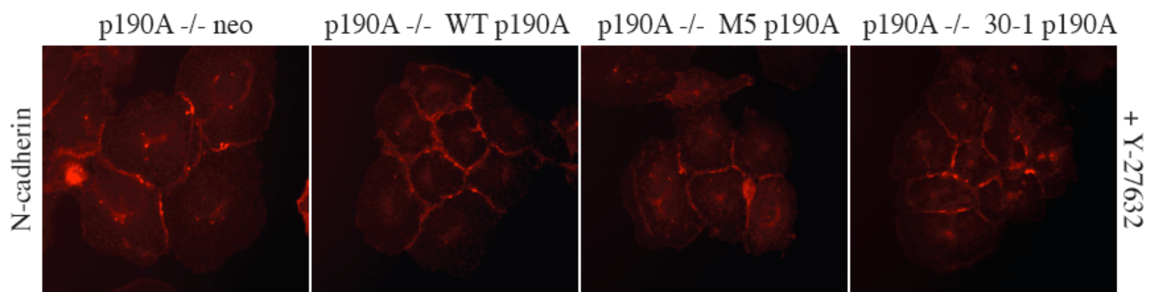


Figure 27. p190A mutants interact with p120.
p120 was immunoprecipitated from RIPA cell lysates generated from the above cell lines and then Western blotted with antibodies to p190 or p120 as indicated. (NS) reflects immunoprecipitation with a nonspecific isotype matched control monoclonal antibody. The lower panel shows p190 expression levels in whole cell lysate
(WCL) from the same samples used for coimmunoprecipitation. Tubulin is a loading control.



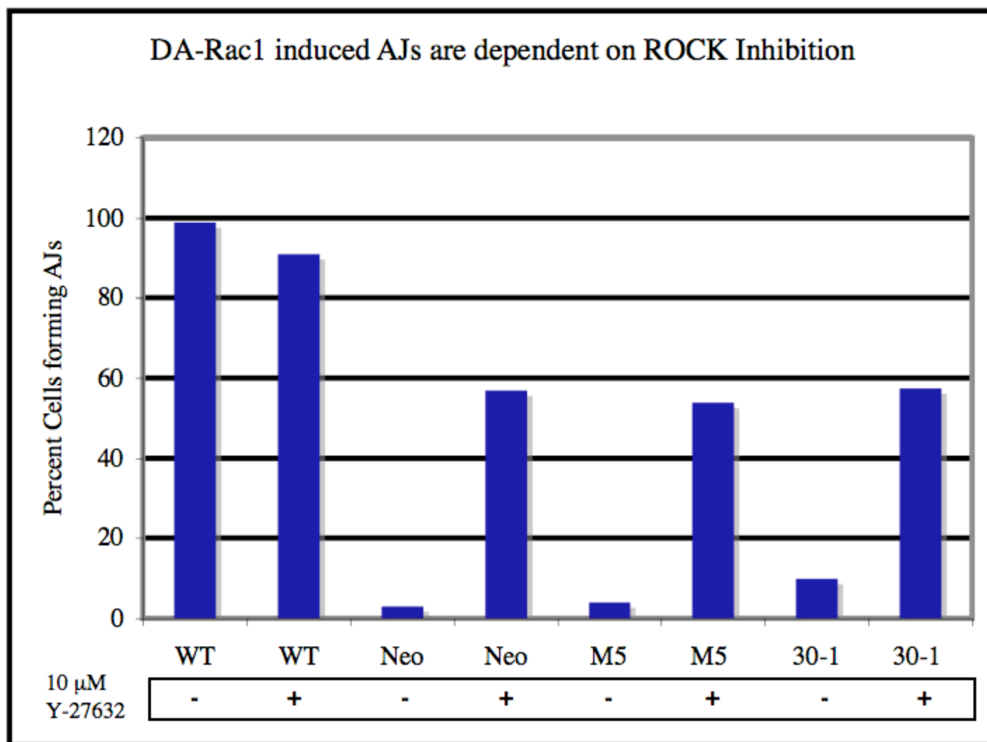


Figure 28. Inhibition of ROCK partially restores DA-Rac-induced AJs in p190A-/- cell lines.
p190A-/- MEFs expressing various p190A mutants were infected with retrovirus expressing DA-Rac. Cells were incubated with the ROCK inhibitor, Y-27632, fixed, and stained for N-cadherin. To quantify the data, 200 cells were selected at random, and cells with obvious AJ rescue were scored as rescued, whereas cells lacking this phenotype were scored negative, and data expressed as the ratio of these events (see table). ROCK inhibition restored AJs in about 50% of DA-Rac expressing cells. Rescue was essentially absent without the inhibitor.

complexes. Although we have not ruled out the possibility that p190 can translocate to the membrane through p120-independent means, it is apparent that p120 and the cadherin complex is necessary for Rac to couple to Rho. Likewise, these data do not rule out other means for negatively regulating Rho such as through inhibition of a GEF or activation of a GDI. However, given that the Bar-Sagi pathway represents a major pathway in a variety of several receptor types, and that cells become transformed without proper Rac to Rho signaling, the translocation of p190 to the cadherin complex represents a major aspect of p190 function and overall regulation of Rho. Recently, it was reported that p190 localizes to focal adhesions in a RasGAP-dependent manner (Bradley, Hernandez et al. 2006). Since p120 does not localize to focal adhesions, it is possible, therefore, that RasGAP and p120 could compete for p190 localization to focal adhesion or AJs, respectively. However, integrins fail to regulate Rho in p120i cells, which suggests that p190 must translocate to the cadherin complex for integrins to properly inhibit Rho. Thus it is tempting to speculate that proper focal adhesion formation depends on both organization of integrins and cadherins.

p120/p190 and the cadherin complex regulates overall inhibition of Rho

The complete failure of Rac to inhibit Rho in p120i cells establishes p120 and the cadherin complex as obligatory intermediates in Rac to Rho signaling (i.e. the Bar-Sagi pathway). By localizing the Bar-Sagi pathway to cadherin complexes, a cellular context is established for Rac to Rho signaling. Given that the Bar-Sagi pathway is utilized by multiple receptor systems (e.g. RTKs, integrins), these data define the cadherin complex as a major gateway through which signals must be relayed to rearrange the actin cyto-

skeleton. Our initial observations postulated that the effects of p120 depletion on RTK and integrin signaling could be through a cadherin independent or dependent mechanism (see chapter III). These data now favor the latter interpretation since inhibition of Rho by p120 appears to occur when p120 and p190 are localized to N-cadherin. Furthermore, PDGFR-induced DCRs are cadherin based structures that cannot form in N-cadherin-depleted cells. This highlights a surprising and novel role for p120 in not only organizing Rho activity at the cadherin complex, but also being the rate limiting step in the ability of RTKs and integrins to inhibit Rho. Alternatively, Rho may be so highly activated in p120i cells that other systems simply cannot suppress Rho enough to elicit their respective biological response. It is admittedly difficult to determine absolutely whether RTKs, integrins, and cadherins regulate Rho through separate pathways, or if all signaling to Rho is channeled through the cadherin complex. However, functionally placing p120 in the Bar-Sagi pathway, and the complete failure of RTKs and integrins to reorganize the actin cytoskeleton in p120i cells suggests that p120 and the cadherin complex plays a far greater role in coordinating Rac-mediated inhibition of Rho in general.

Our model also predicts that the loss of cadherin expression would result in a similar Rho-dependent phenotype seen by the loss of p120 (e.g. p120i cells). Since p190 translocation to the plasma membrane depends on the cadherin complex, both p120 and cadherin expression are theoretically rate limiting for functionally coupling of Rac to Rho. Though Rho activity was not directly tested by Rhotekin assay, N-cadherin knockdown NIH3T3 (N-cadi) did not appear to have prominent ASFs as seen in p120i cells. siRNA-mediated knockdown efficiency for N-cadherin was not as high as for p120, and therefore the remaining levels of N-cadherin may have been sufficient to regulate Rho

activity by some reduced measure. It has recently been shown that in the E-cadherin deficient cell line, MBA-MD-231, p120 inhibits Rho in the cytoplasm (Yanagisawa and Anastasiadis 2006). This suggests that p120 can regulate Rho both at the plasma membrane when associated with cadherins, or in the cytoplasm when cadherin expression is lost. Therefore, in N-cadi cells, cytoplasmic p120 may still inhibit Rho by another unknown mechanism. Drosophila p120 has been shown to directly interact with GDP-bound Rho, and others have shown a weak interaction in mammalian cell lines (Magie, Pinto-Santini et al. 2002; Bellovin, Bates et al. 2005). Therefore, in N-cadi cells, p120 may still inhibit Rho, but in a Rac-independent manner by physically interacting with and sequestering Rho in the cytoplasm. Thus Rac-dependent mechanisms such as PDGFR-induced DCRs would still be blocked by N-cadherin loss, but inhibition of Rho by cytoplasmic p120 would be sufficient to suppress ASFs.

Likewise, p190A -/- MEFs would be predicted to have elevated Rho activity. However, this cell line also does not exhibit phenotypes of increased Rho activity such as strong ASFs. This cell line does express the p190A homologue, p190B, which also functions as a GAP for Rho. p190B is also necessary for AJ formation by Rac as p190B -/- MEFs do not form AJs upon DA-Rac expresssion (data not shown). Therefore, both forms of p190 are necessary for coupling Rac to Rho, but the presence of one gene may be sufficient to partially suppress Rho levels to a degree in which, at basal levels, elevated ASFs are not observed. Alternatively, p190A -/- or p190B -/- cell lines may have evolved mechanisms during clonal expansion and immortalization to suppress Rho activity to compensate for the lack of these genes. Nevertheless, p190A -/- or p190B -/- MEFs are functionally uncoupled from signaling events that require Rho inhibition as shown by

our data, and several other reports that have demonstrated in p190A-/- or B-/- MEFs, the requirement of p190-dependent inhibition of Rho in various processes such as adipogenesis, neuronal fasciculation, and semaphorin-mediated axon guiding (Brouns, Matheson et al. 2001; Sordella, Jiang et al. 2003; Barberis, Casazza et al. 2005).

Cadherin-bound p120 stabilizes AJs by inhibiting Rho

Our lab has shown previously that p120 is necessary for the stablization of cadherin levels (Ireton, Davis et al. 2002). This mechanism requires direct p120-cadherin interaction, which is consistent with the hypothesis that p120 and p190 coordinately regulate cadherin and AJ stability by localizing and controlling Rho-mediated contractility. Likewise, control of Rac activity by RTKs and integrins is likely to be a major mechanism in regulating AJ assembly. Although the functional interactions between RTKs and cadherins are not well understood, post-translational regulation of cadherin stability and AJ assembly by RTK activity is well documented (Wrobel, Debnath et al. 2004). Thus, it is exciting to speculate that regulation of AJ assembly/disassembly by growth factor signaling occurs through Rac-inhibition of Rho and the p120/p190 complex. Although not extensively tested, this appears to be particularly important in MCF-10A cells in which p190 localizes to AJs basally. Thus, MCF-10A may be an ideal epithelial cell line to investigate the role of p120 and p190 in epithelial cells.

This data suggests that the major function of p120 is to inhibit Rho at the site of the cadherin complex. This is contrary to our original hypothesis, which suggested that p120 predominantly inhibits Rho in the cytoplasm. Indeed, recent evidence has suggested that p120 regulates Rac through a cadherin-dependent manner and Rho through a cadherin-

independent mechanism (Yanagisawa and Anastasiadis 2006). Though, no mechanism was identified, their observations suggests that p120-depletion by siRNA induces Rho activation, and can be rescued by a p120 mutant that cannot associate with cadherins. However, a cytoplasmic role for p120-mediated inhibition of Rho would predict that as more AJs are formed and hence more p120 is recruited from the cytoplasm to the cadherin complex, there would be a porportional elevation in the amount of activated Rho. This is contrary to data that demonstrates upon high cell density, Rho activity is suppressed by cadherin engagement (Noren, Niessen et al. 2001). Furthermore, the cell line utilized in this study is the highly invasive MDA-MB-231 breast adenocarcinoma cell line, which has lost normal E-cadherin expression and undergone epithelial to mesenchymal transition (EMT). Therefore, it is difficult to determine if normal signaling mechanisms are intact in their model system. Attempts to re-express E-cadherin in MD-MB-231 and examine DA-Rac-induced localization of p190 were not successful. Similarily, expression of E-cadherin in other cadherin-deficient tumorigenic cell lines also failed to localize p190 by DA-Rac expression (data not shown). However, as noted above, cell lines which have an intact cadherin complex (e.g. A431, MCF-10A) are able to efficiently localize p190 in a p120-dependent manner. Therefore, it is possible that cell lines that have lost cadherin expression have also lost critical signaling pathways that regulate p190 translocation to the cadherin complex, which is consistent with a role for p190 in regulating AJ assembly. We do not eliminate the possibility that there is an additional mechanism for Rho regulation by cytoplasmic p120, but our data strongly suggest a prominent role for p120 in regulating Rho at the cadherin complex. The discrepancies between our data and those of another lab raise the possibility of different roles for p120

in the inhibition of Rho depending on whether or not the cell has undergone oncogenic transformation. This highlights an interesting paradigm in p120 signaling that requires further exploration to appreciate the role of p120 during tumor progression.

p120 is a tumor suppressor

The striking loss of contact inhibition and acquired ability to grow without serum in p120i cells suggests that p120 is a tumor suppressor. Here we demonstrate that loss of p120 expression results in the concomitant downregulation of cadherin expression and constitutive activation of Rho. E-cadherin is frequently downregulated in epithelial tumors (Birchmeier and Behrens 1994), and loss of E-cadherin expression has been observed in both early and late stages of tumor progression indicating a role as both a tumor and invasion suppressor (Berx, Nollet et al. 1998; Berx and Van Roy 2001). This concept has been widely tested both *in vitro* and *in vivo* demonstrating that E-cadherin expression and function is critical in suppressing tumor progression (Derksen, Liu et al. 2006; Olmeda, Jorda et al. 2006; Zhang, Alt-Holland et al. 2006). Although our research describes the effects of p120 loss in fibroblasts (*i.e*. N-cadherin expressing cells), accumulating evidence in our lab suggests that the mechanism described here is conserved in epithelial cell lines. The Rip1Tag2 mouse model for beta-cell carcinogenesis has been used to demonstrate the E-cadherin loss is the rate-limiting step in progression from adenoma to carcinoma (Perl, Wilgenbus et al. 1998). Interestingly, it was recently shown that the effects on E-cadherin loss in tumor progression of the Rip1Tag2 mouse are not due to β-catenin-mediated Wnt signaling. Furthermore, tumor progression in this model is not affected by loss of Tcf-1, a major target of β-catenin/Wnt signaling (Herzig, Sa-

varese et al. 2006). Alternatively, the effects of E-cadherin loss on tumor progression could be due to misregulation of p120-dependent signaling processes including regulation of Rho.

Likewise, alterations in Rho signaling have been implicated in various processes of tumor progression including increased proliferation, de-differentiation, invasion, migration, and metastasis(Lozano, Betson et al. 2003). Although there are few cancers where Rho is directly mutated, aberrant Rho signaling can also result from either increased expression of Rho, or increased GTP loading (activation). There are numerous examples of both instances in various tumors, although the latter is more readily observed (Lozano, Betson et al. 2003). For example, chromosomal rearrangements to the gene locus of p190A has been documented in a number of tumors including pancreatic carcinomas and gliomas (Tikoo, Czekay et al. 2000). Loss of p190A function in turn results in the unbalanced activation of Rho. Likewise, mutational activation of various RTKs results in the upregulation of Rho signaling (Boettner and Van Aelst 2002). Lastly, Rho activity is necessary for cellular transformation by other oncogenes including Ras (Qiu, Chen et al. 1995). Thus, although Rho is not directly mutated in cancers, the abilities of an oncogenic signal to transform cells appears to depend in part on Rho activation.

Since both cadherins and Rho both play important roles in tumor progression, p120 loss would result in the misregulation of two protiens involved in tumorigenesis. p120 loss in human tumors has been documented in a variety of cancers including colon, gastric, and lung carcinoma (Thoreson and Reynolds 2002). Therefore, based on the effects observed in this thesis, p120 loss would significantly contribute to tumor progression through the downregulation of cadherin expression, and aberrant Rho activation. The

acquired capabilities outlined by Hanahan and Weinberg highlight six basic principles that are characteristic to most cancers. These are evading apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasions and metastasis, limitless replicative potential, and sustained angiogenesis (Hanahan and Weinberg 2000). We observe in our data, that p120-depletion results in at least three of the six oncogenic characteristics. Namely, insensitivity to anti-growth signals (i.e. failure to contact inhibit), and self-sufficiency in growth signals (i.e. growth without serum).

In addition to the affects of direct loss on tumor progression, the role of p120 in coordinating crosstalk between different receptors suggests that p120 represents a major signaling nexus that may be affected by various receptors that are often causal in tumor progression. Our data demonstrates that p120 regulates the dynamic assembly/ disassembly of AJs by integrating signals from multiple receptors (e.g. RTKs, integrins) to Rho regulation. Misregulation of cell-cell adhesion is a critical step in promoting tumorigenesis and invasion, and in addition to direct loss of cadherin expression, AJ formation may be downregulated by aberrant signaling. Many RTKs as well as different types of integrins are missexpressed or mutated in almost all cancers (Kolibaba and Druker 1997; Porter and Vaillancourt 1998; Blume-Jensen and Hunter 2001), and promote the disassembly of AJs (Birchmeier, Birchmeier et al. 2003; Mareel and Leroy 2003). Mechanistically, this may reflect a misregulation of p120-dependent Rac inhibition of Rho. For example, HGF and TGF-B-induced disruption of AJs in epithelial cells correlates with Rho activation. Likewise, in Ras-transformed MDCK cells, elevated Rho activity results in the breakdown of cell-cell adhesions (Takaishi, Sasaki et al. 1994; Zondag, Evers et al. 2000; Bhowmick, Ghiassi et al. 2001). Similarily, inhibition of Rac

signaling through transcriptional downregulation of the Rac-GEF, Tiam1, promotes disassembly of AJs (Sander, van Delft et al. 1998). Lastly, Src-mediated disruption of AJs requires FAK and integrin signaling (Avizienyte, Wyke et al. 2002). Interestingly, p120 is tyrosine phosphorylated by numerous RTKs including EGFR, PDGFR, and CSFR (Reynolds, Roesel et al. 1989) indicating that growth factor-induced phosphorylation of p120 may ultimately regulate its ability to interact with p190. Thus, the misregulation of cell-cell adhesion often observed in tumor cell lines may be due to aberrant signaling from a mutated RTK or integrin that affects the ability of p120 to properly regulate Rho and AJ assembly. Although not directly tested, our data suggests that p120 function may be relevant to other characteristics of transformation outlined by Hanahan and Weinberg. For example, there is a functional, but poorly understood relationship between the pro-angiogenic Eph receptors and cadherin signaling (Zantek, Azimi et al. 1999; Orsulic and Kemler 2000) that suggests a role for p120 and the cadherin complex in angiogenesis. Our data sheds new light on the role of the cadherin complex in being a critical intermediate between various receptors and their biological responses, and provides new avenues to explore through which p120 and the cadherin complex may affect tumor progression far beyond cell-cell adhesion.

p120 cooperates with β-catenin and α-catenin to regulate cell-cell adhesion

Overall, our data demonstrates that p120 functions to regulate cell-cell adhesion through both stabilization and inhibition of Rho. In addition, the other core components of the cadherin complex, β-catenin and α-catenin, play important roles in the regulation of cell-cell adhesion. The catenin binding domain (CBD) of cadherins mediates direct

interaction with β-catenin. β-catenin then associates with α-catenin. The role of α-catenin in adherens junction stability is not known since binding of α-catenin to β-catenin or to the actin cytoskeleton is mutually exclusive (Yamada, Pokutta et al. 2005; Drees, Pokutta et al. 2005). Yamada *et al*. demonstrated that β-catenin/α-catenin and α-catenin/F-actin complexes are mutually exclusive suggesting that α-catenin does not physically link E-cadherin to the actin cytoskeleton (Yamada, Pokutta et al. 2005). α-catenin has been subsequently shown to bind to and recruit the actin nucleator, formin 1, and compete with the Arp 2/3 complex for F-actin binding (Kobielak, Pasolli et al. 2004; Drees, Pokutta et al. 2005). Therefore, α-catenin may ultimately regulate the balance between branched actin networks (Arp 2/3) and actin bundles (formins) to promote the proper actin arrangement that is ideally suited for AJ formation.

AJ assembly can also be regulated through β-catenin signaling. Most notably, Rac has been shown to promote the β-catenin/α-catenin interaction by inhibiting IQGAP, which otherwise binds to β-catenin and displaces α-catenin (Kuroda, Fukata et al. 1998). In addition to Rac signaling, phosphorylation of β-catenin can affects its affinity for cadherin binding. Overexpression of kinases such as Src and Fer results in disassembly of AJs which has been correlated with increased phosphorylation of β-catenin (Lilien, Balsamo et al. 2002). Activation of the β-catenin-directed phosphatase PTP1b by Fer has also been shown to promote AJ assembly suggesting that dephosphorylation of β-catenin is essential in maintaining cell-cell adhesions (Xu, Craig et al. 2004). However, p120 is also a substrate of Fer, and therefore it is unclear what the relative contributions of β-catenin dephosphorylation and p120 phosphorylation are to the observed affects on AJ formation. Overall, each catenin functions to regulate cell-cell adhesion, and each is sub-

ject to regulation by multiple signaling events. Interestingly, many of these signaling events appear to simultaneously impact the function of all three catenins. For example, Rac activation promotes the formation of both the β-catenin/α-catenin and the p120/p190 complex. Therefore, Rac may promote AJ assembly by globally arranging all of the catenins under the cadherin complex. However, the relative contribution of each catenin in cell-cell adhesion is not well appreciated, which is primarily due to the fact that the precise role of each catenin in regulating cell-cell adhesion is just now being elucidated. As in the case with Rac signaling, all three catenins are essential to the dynamic process of AJ assembly and disassembly. Misregulation of one catenin is likely to disrupt AJs by its specific contribution to cell-cell adhesion.

Spatial versus global regulation of Rho

We conclude that cadherin complexes are an obligatory intermediate in the Bar-Sagi pathway, which is essential in regulation of the actin cytoskeleton by RTKs, integrins, and cadherins (Noren, Niessen et al. 2001; Nimnual, Taylor et al. 2003) (Fig. 29). In fibroblasts, AJ formation is blocked by strong inhibition of Rho (Sander, ten Klooster et al. 1999). On the other hand, too much Rho activity also disrupts AJs and leads to cell transformation (Jaffe and Hall 2002), as illustrated here in cells lacking p120. Thus, exquisite control of Rho activity is essential for proper cell-cell adhesion and to prevent uncontrolled cell growth. The transient targeting of otherwise cytoplasmic p190 to cadherin complexes is ideally suited for this role, and provides an extremely sensitive means of regulating local, and perhaps overall Rho activity in cells. RhoGTPase-mediated cadherin internalization and/or turnover allows for rapid modulation of cell-cell adhesion by post-

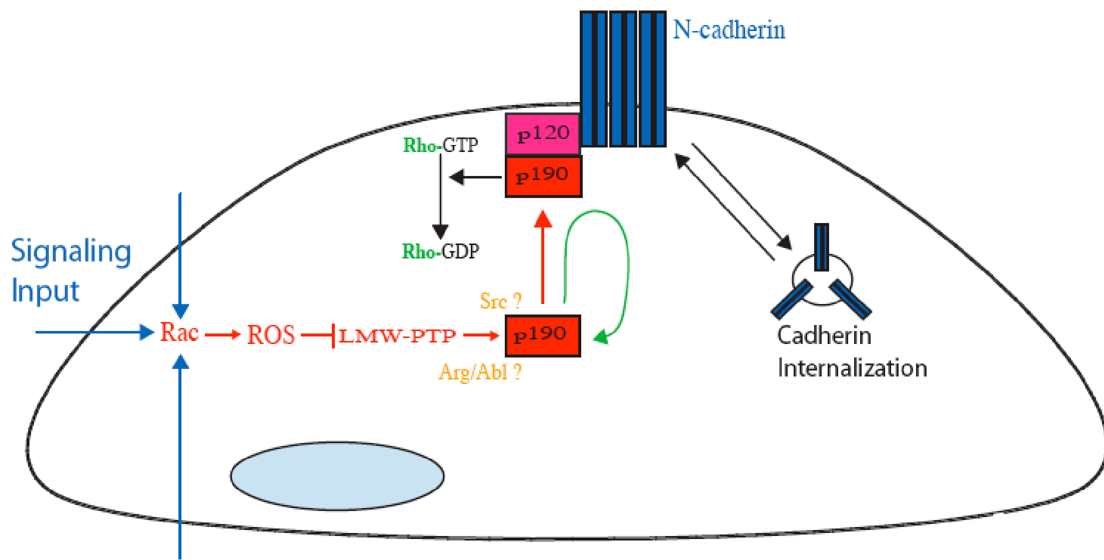


Figure 29. Model for the role of p120 and p190 in regulation of cadherin function. Signaling input to Rac occurs through multiple receptor types, including RTKs, integrins, and cadherins. Rac activity induces translocation of cytoplasmic p190 to membranes via a pathway involving generation of reactive oxygen species (ROS), inhibition of LMW-PTP, and subsequent activation/tyrosine phosphorylation of p190 by associated tyrosine kinases (probably Src and/or Abl family kinases). p190 interacts transiently with cadherin-bound p120, which in turn targets Rho inactivation to membrane domains involved in cadherin activity. Organization of the pathway around the cadherin complex reflects the importance of linked Rac and Rho activities for successful AJ formation.

transcriptional signaling mechanisms, as would be required during wound healing, changes in endothelial cell permeability, and many growth factor-induced alterations in cell behavior. Thus, the novel mechanisms described herein may be essential for many morphologic and adhesive changes associated with the activation of receptor systems.

The dependancy of the cadherin complex on actin remodeling by other receptors suggests that these receptors are functionally connected. How they are functionally connected remains an open question. One possibility is that different receptor types physically and transiently couple to one another in response to some stimulus (Fig. 30). Alternatively, the local activation of one receptor transmits a signal across the cell to a different receptor type (Fig. 31). In p120i NIH3T3, Rho is uncoupled from multiple receptors including RTKs and integrins. PDGFR and N-cadherin co-localize in DCRs upon stimulation with PDGF-BB, and PGFR-induced actin rearrangements depend on a functional cadherin complex (see Fig. 19-20). Likewise, others have reported that E-cadherin and activated EGFR co-localize at AJs (Pece and Gutkind 2000; Qian, Karpova et al. 2004). Certain types of integrins have also been reported to co-localize with cadherins, although this concept is currently under debate (Chattopadhyay, Wang et al. 2003) (personal communication, Vito Quaranta). Lastly, RTKs have also been shown to co-localize and are functionally coupled with integrin receptors (Brunton, MacPherson et al. 2004). This evidence suggests that crosstalk between apparently distant receptors occurs through transient signaling events that bring these receptors together spatially. Thus the global affects of p120 depletion on Rho activation would be either primarily due to the fact that multiple receptors must transiently couple with the cadherin complex to regulate Rho or a lack of inhibition in the cytoplasm. Depending on which receptor pairs with the cadherin

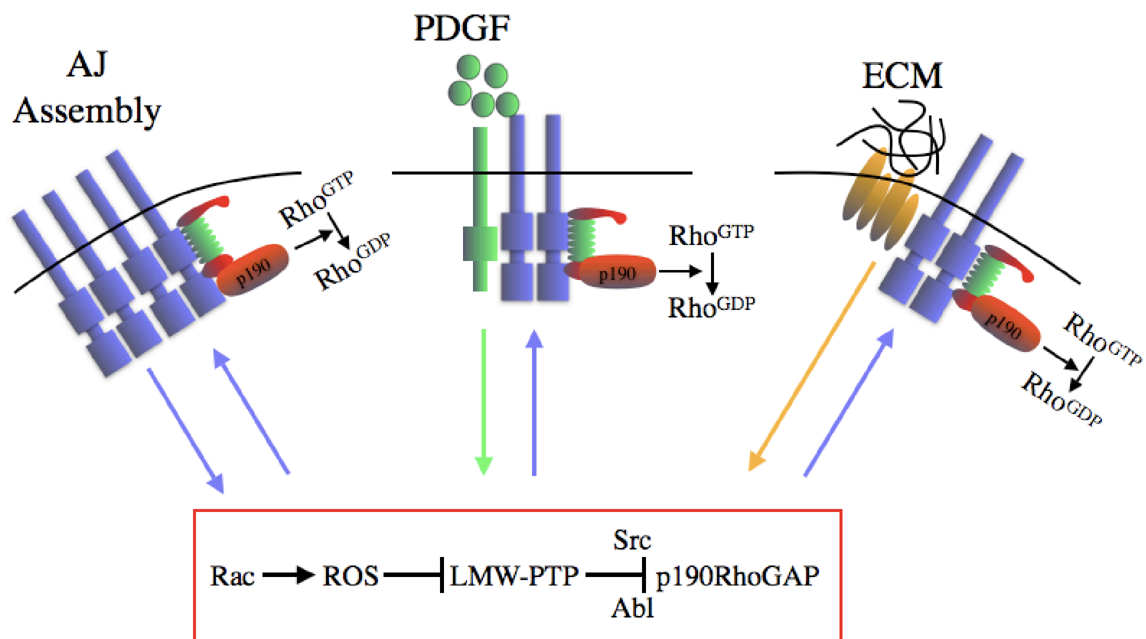


Figure 30. Global regulation of Rac-mediated inhibition of Rho through transient interactions between different receptor types.
Upon activation by various stimuli (e.g. AJ assembly, PDGF, ECM), receptors transiently interact with the cadherin complex to activate Rac and inhibit Rho.

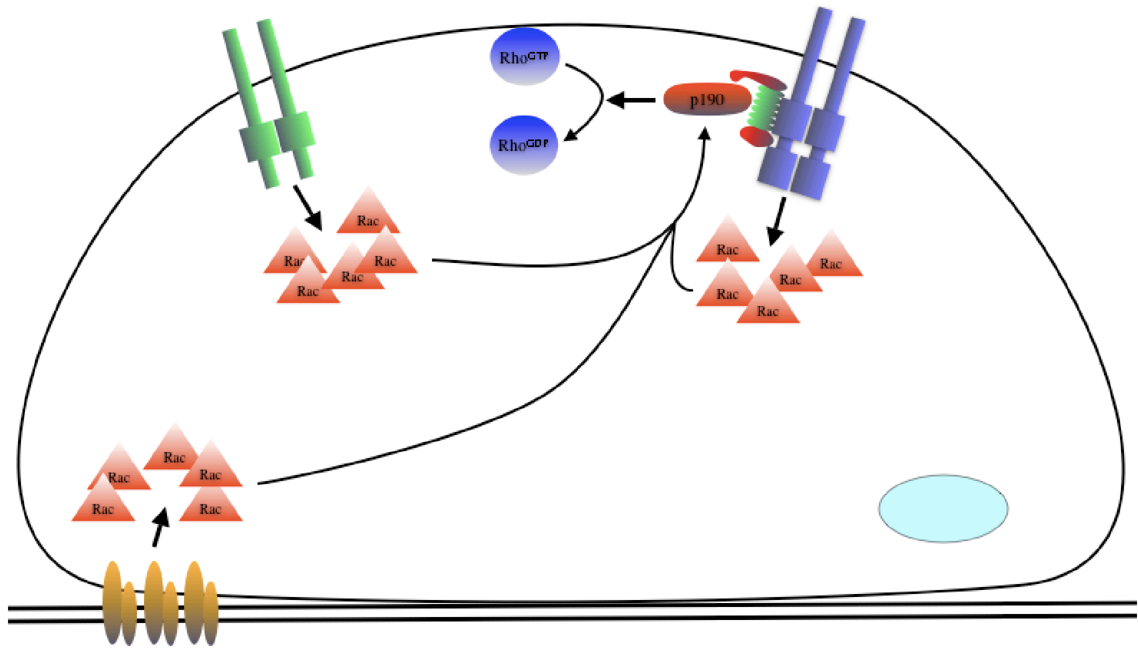


Figure 31. Global regulation of Rho is mediated by localizing Rac activity to the cadherin complex.
Activation of different receptor types transmits the local activation of Rac to the cadherin complex to recruit p190 where it inhibits Rho.

complex, a different biological response is elicited. For example, RTK coupling with the cadherin complex mediates DCRs and integrin coupling permits cell spreading on ECM.

On the other hand, the distinct localization, but functional dependency of different receptor types suggests that certain signals may be transmitted over distances within the cell. Our data shows that the regulation of Rho by integrins, RTKs, and cadherins are functionally linked through the p120/p190 complex. Indeed, p120i cells fail to migrate into a wound reflecting a putative inability of integrins to properly regulate Rho. Therefore, focal adhesions on the basal surface must transmit signals to laterally localized cadherins to inhibit Rho to permit motility. Thus, it is difficult to imagine transient interactions between integrins and cadherins when they are localized to distinct regions within the cell. Actin-based contractility extends throughout the entire cell, and globally affects both integrin- and cadherin-based complexes in an apparent antagonistic manner (D'Souza-Schorey 2005; Bershadsky, Ballestrem et al. 2006). Increased contractility promotes integrin-mediated focal adhesions, and the disassembly of AJs. On the other hand, a reduction in contractility results in the opposite effect. Perhaps integrin signaling locally activates Rac which then initiates low level changes in contractility. Once initiated by focal adhesions, changes in contractility could permit cadherin clustering and p120-dependent Rac-inhibition of Rho at sites of cell-cell adhesions. In this scenario determining the relative contribution to integrin-dependent and cadherin-dependent activation of Rac would be critical in understanding how these receptors mediate cross-talk. Likewise, when a cell is globally stimulated with a growth factor such as PDGF, cadherin-dependent DCRs form in spatially distinct regions. Local activation of Rac by PDGFR may initiate the changes in contractility to permit cadherin-dependent Rho inac-

tivation and organization of the cadherin complex. How different receptors communicate to one another remains an open question. Regardless of how cross-talk is mediated, our data highlights the biological significance of these cross-talk events, and suggests that interactions between different receptor types represents a major component of general cellular signaling.

CHAPTER V

ROLE OF P120 AND P190 IN CONTACT INHIBITION OF GROWTH AND CELLULAR TRANSFORMATION

Introduction

Contact inhibition is a process in which increased cell density negatively regulates cell proliferation. Loss of contact inhibition of growth is a hallmark of most cancers, but the mechanisms that regulate this process are not understood. Contact inhibition is mediated by cadherin-based cell-cell adhesions, and loss of contact inhibition is paralleled with a disruption in cell-cell adhesion (Takahashi and Suzuki 1996; Bracke, Depypere et al. 1997; St Croix, Sheehan et al. 1998). Specific cadherin ligation between sparsely plated fibroblasts and latex beads coated with the extracellular domain of N-cadherin is sufficient to induce cell cycle arrest involving regulation of the cell cycle kinase inhibitors (CKIs) p21 and p27 (Levenberg, Yarden et al. 1999; Gavard, Marthiens et al. 2004). Although the mechanism is unknown, these observations suggest that signaling through N-cadherin alone is sufficient to inhibit proliferation.

In addition to their role in regulating the actin cytoskeleton, Rac and Rho have important roles in regulating cell proliferation. Rac can stimulate the expression of cyclin D1 in a stress fiber independent manner whereas Rho negatively regulates p21 and p27 in a stress fiber dependent pathway (Olson, Paterson et al. 1998; Roovers and Assoian 2003). Activation of Erk signaling by Rho was shown to be dependent on stress fiber formation and integrin clustering (Roovers and Assoian 2003) suggesting that regulation of cell adhesion and growth by Rho are functionally related. On the other hand, too much

Rho activity can transform cells since overexpression of RhoGEFs or dominant-active mutants of Rho are able to cause partial transformation and a complete loss of contact inhibition (Jaffe and Hall 2002). Therefore, maintaining proper levels of Rho activity is crucial for both promoting and inhibiting cell growth. The parallels between cadherin-dependent regulation of the cell cycle and RhoGTPase signaling, however, remains largely unexplored.

Given the duel role for p120 in stabilizing cadherins and regulating Rho, p120 is a likely candidate for mediating contact inhibition of growth. As shown in chapter III, reduction of p120 expression results in the loss of contact inhibition due to the constitutive activation of Rho (Fig. 10-12). Furthermore, our data localizes p120-mediated inhibition of Rho at sites of cadherin engagement suggesting that AJ assembly and Rho inhibition by p120 are functionally related. Therefore, it is likely that as cell density increases, there would be a concomitant increase in Rho inhibition by p120.

Interestingly, both p120 and p190 are prominent Src substrates. Transformation by Src results in a loss of contact inhibition as well as dramatic alterations to the actin cytoskeleton. The mechanism of Src transformation is largely unknown, but involves both misregulation of adhesion (cell-cell and cell-ECM) and RhoGTPase signaling (Matsuyoshi, Hamaguchi et al. 1992; Fincham, Wyke et al. 1995; Avizienyte, Wyke et al. 2002; Frame, Fincham et al. 2002). Given the requirement of p120 and the cadherin complex to recruit p190 to the plasma membrane to inhibit Rho, our hypothesis is that as cell density increases, so does the number of cell-cell contacts, which in turn recruits more p190 to the cell surface. This functionally translates into a reduction of Rho activ-

ity to levels low enough to suppress growth. Lastly, the role of the p120/p190 complex may have an important role in Src-induced misregulation of cell-cell adhesion.

Results

p120i cells have increased MAPK signaling

Rho induces cell proliferation through activation of Erk signaling pathways (Roovers and Assoian 2003; Roovers, Klein et al. 2003). Therefore, we analyzed MAPK activities to determine if the growth abnormalities observed in p120i cells are due to elevated Rho signaling. Indeed, basal levels of Erk1/2 phosphorylation is increased in p120i cells as compared to WT (Fig. 32). This observation is consistent with constitutively active Rho driving uncontrolled proliferation in p120i cells.

Confluency regulates Rho activity

Upon contact inhibition, cadherins become maximally engaged to suppress cell growth (St Croix, Sheehan et al. 1998). p120i cells fail to contact inhibit due to the constitutive activation of Rho, which may reflect a failure to recruit p190 to the plasma membrane upon increasing cell density. Rho activity was first analyzed under conditions of contact inhibition to confirm that Rho activity was suppressed when WT NIH3T3 cells underwent contact inhibition. Indeed, contact inhibited cells displayed about a 2-fold decrease in Rho activity as compared to, subconfluent, proliferating cells (Fig. 33). Interestingly, when p190 localization was analyzed, there was a noticeable shift from the cytoplasm in dense, proliferating cells to the plasma membrane in contact inhibited cells

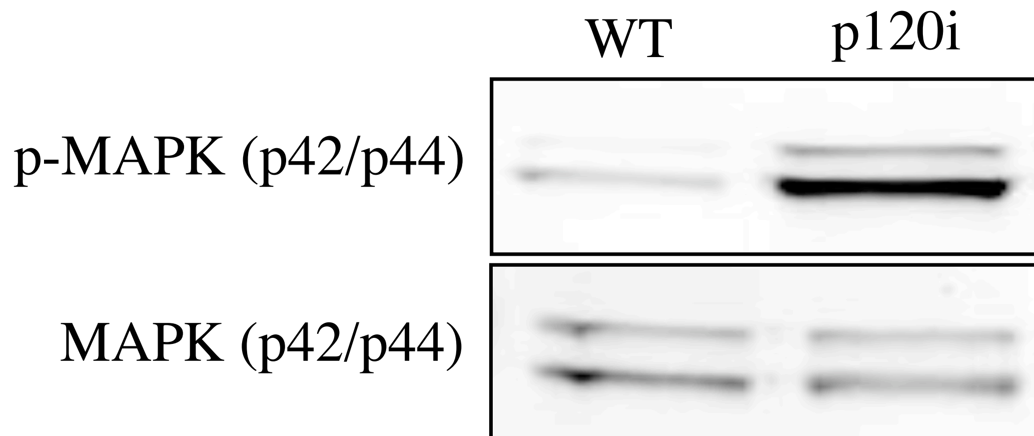


Figure 32. p120i cells have increased Erk activation.
Whole cell lysates of WT and p120i cells were Western blotted with antibodies for phospho-MAPK (p-MAPK) or total MAPK.

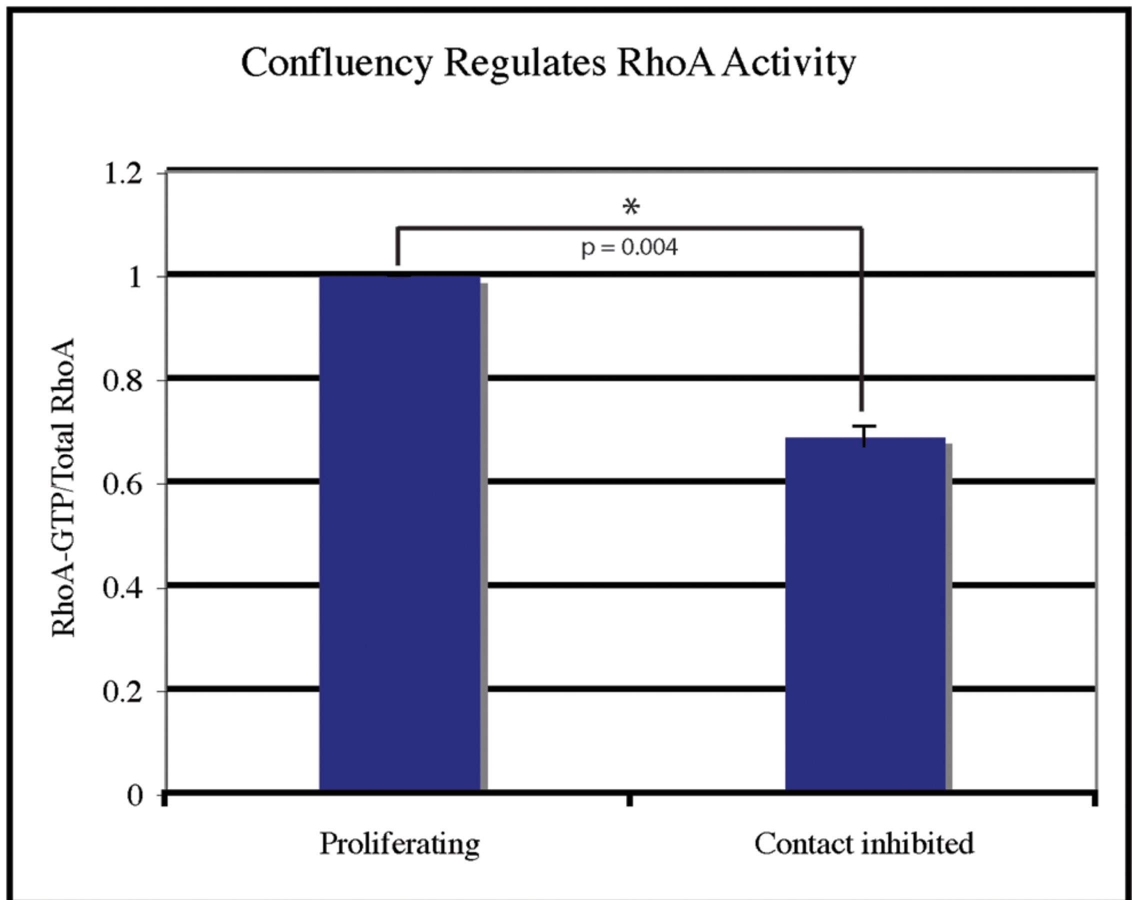


Figure 33. Contacted inhibited NIH3T3 have reduced Rho activity.
Rho activity was assayed from proliferating and contact inhibited cells by GST-Rhotekin pulldown assays. Results are expressed as the ratio of activated to total Rho. Results were expressed as a ratio of WT control (unstimulated WT NIH3T3). Error bars represent standard error of the mean. Error bars represent standard error of the mean, and student's T-test was performed for statistical analysis (n = 3 independent experiments).

(Fig. 34A). In dense cultures, p120 localized to ruffles as well as points of cell-cell contact, but p190 seemed to predominantly localize within the cytoplasm. Upon contact inhibition, both p120 and p190 localized mostly at cell-cell contacts. Furthermore, co-immunoprecipitation of p120 and p190 in proliferating or contact inhibited cells revealed that about three-fold more p190 interacted with p120 in contact inhibited cells (Fig. 34B). This data demonstrates that upon contact inhibition, membrane localized p190 increases through its association with p120.

Src regulates the interaction between p120 and p190

A prominent hallmark of Src transformation is the dramatic disruption in AJ assembly. Since p120 and p190 are both Src substrates, and are essential in AJ assembly, we sought to determine the functional contribution of the p120/p190 complex in Src transformation. To this end, a constitutively active mutant of Src (527F-Src) was expressed in WT NIH3T3 cells and co-immunoprecipitation experiments between p120 and p190 were performed. As seen in Figure 35, expression of 527F-Src almost completely abolished the ability of p120 and p190 to interact. Therefore, Src-induced misregulation of AJs may be due to disruption of the p120/p190 complex.

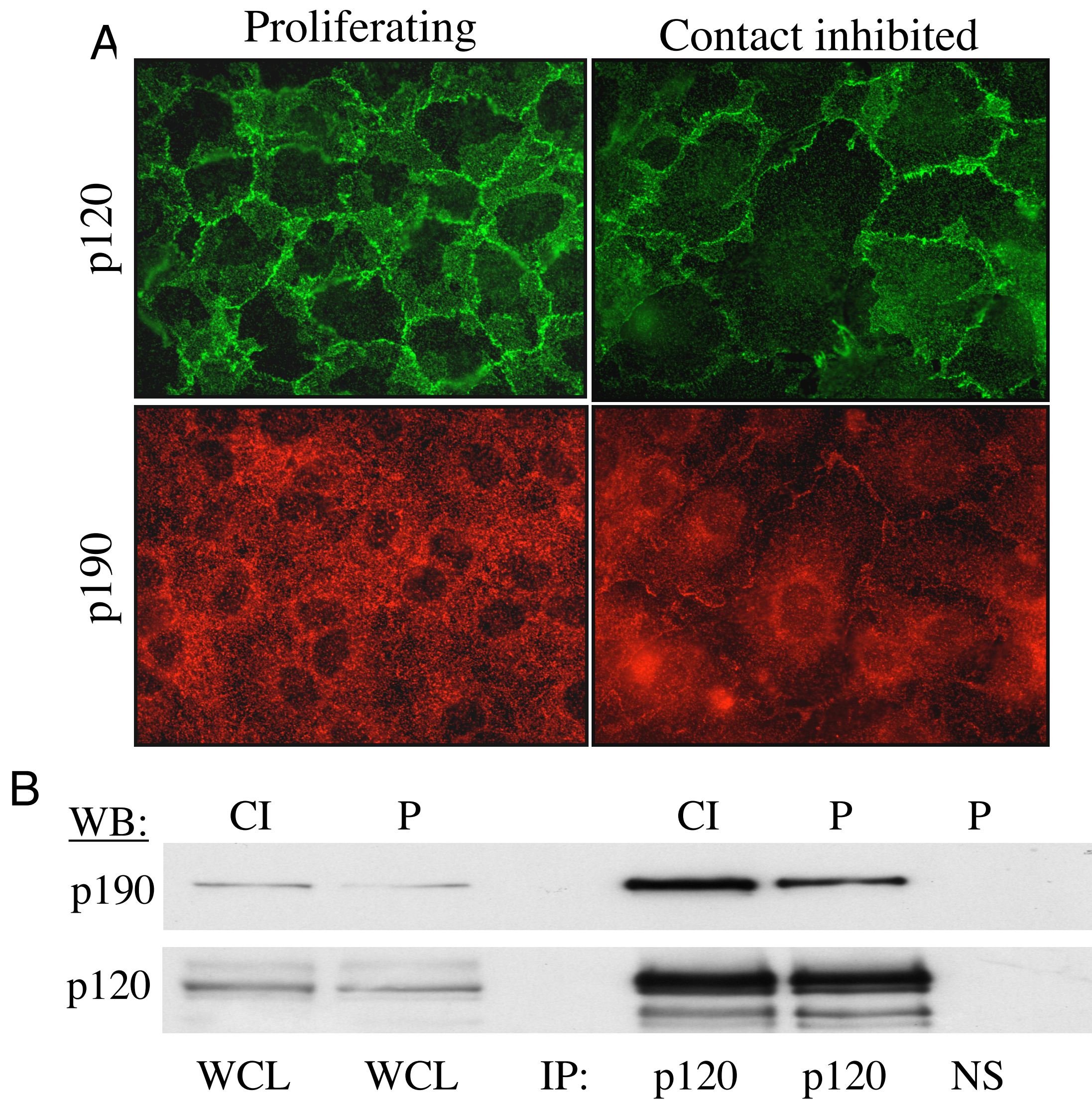


Figure 34. p190 colocalizes with and increases it association with p120 upon contact inhibition.
(A) WT NIH3T3 cells were allowed to grow to either high density (proliferating) or contact inhibition, and visualized for p120 and p190.
(B) Lysates from proliferating (P) or contact inhibited (CI) cells were immunoprecipitated with antibodies to p120 or a non-specific control (NS), and Western blotted with antibodies to p120 and p190.

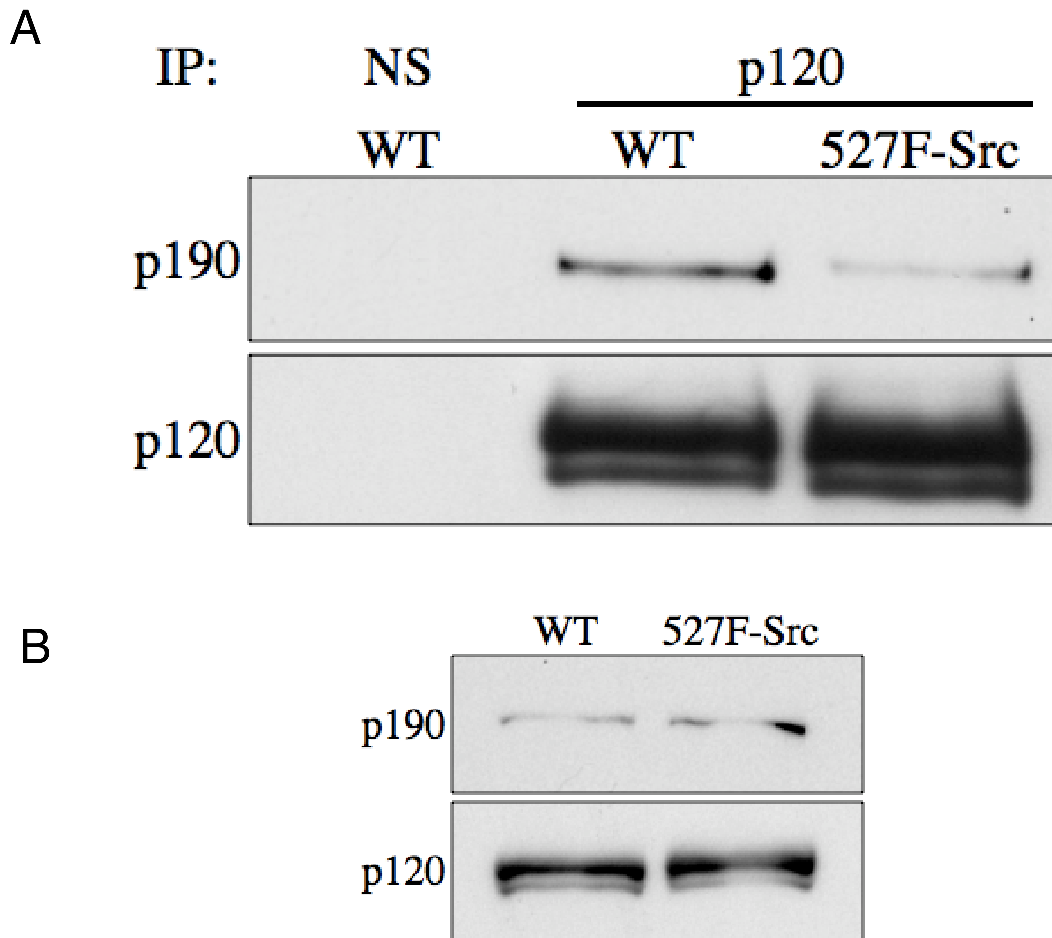


Figure 35. 527F-Src disrupts binding of p120 and p190.
(A) Immunoprecipitation with p120 or irrelevant antibodies (NS) from WT or 527F-Src expressing NIH3T3 lysates was followed by Western blotting with antibodies to p190 and p120.
(B) Whole cell lysates from WT or 527F-Src NIH3T3 were analyzed by Western blotting with antibodies to p190 and p120.

Discussion

p120/p190 interaction increases upon contact inhibition

As shown in chapter III, siRNA-mediated depletion of p120 caused the dramatic loss of contact inhibition of growth, which could be rescued by inhibiting Rho through expression of C3 (see Fig. 12). This suggests that the ability of p120 to suppress Rho activity is critical in maintaining contact inhibition. We have traced the mechanism of p120 inhibition of Rho to the p120-dependent recruitment of p190 to the cadherin complex under conditions that induce AJ formation. Therefore, if this model is correct, more AJs should translate into more membrane bound p190. Indeed, upon contact inhibition, more p190 is observed to localize to the cell surface, as well as about a three-fold increase in the amount of p190 bound to p120. When p120 and p190 were localized in dense cultures, p120 was enriched at both sites of cell-cell junctions but also within lamellipodia, whereas p190 was cytoplasmic. However, in contact inhibited cells, AJs are maximally engaged, and a majority of p120 is recruited along with p190 to cell-cell contacts. Thus cells in the dense, proliferating population appear to be smaller due to the more diffuse p120 staining relative to the contact inhibited cells in which most of the p120 is localized to precise cell-cell junctions. Overlapping lamellipodia from neighboring cells has been shown to be precursors to AJs (Ehrlich, Hansen et al. 2002). This suggests that p120 may have a role in establishing new AJs, and that although cells in dense cultures appear to have stable AJs, they are continually establishing new cell-cell contacts until the cell population undergoes contact inhibition. Interestingly, strong localization of p190 to the cell surface was only observed when cells became contact inhibited as opposed to merely

crowded. This observation demonstrates an intriguing correlation between the amount of p190 that localizes to the cadherin complex and the ability to suppress growth. As shown in previous chapters, p190 association with p120 at the cadherin complex stabilizes AJs through the inhibition of Rho. Thus our working model is that during a proliferative state, p190 translocation to AJs (and AJ assembly) must be transient to allow for the proper level of Rho activity to promote cell growth. Upon contact inhibition, AJs become stabilized and more p190 is recruited to the cell surface to suppress Rho and proliferation. Therefore, this model predicts that stabilization of AJs through the p120/p190 complex and stabilization of cell growth is a functionally identical process. Moreover, the degree of cellular proliferation is ultimately controlled by the regulation of p190 recruitment to the cadherin complex by either RTK, integrin, or direct cadherin signaling.

These data also suggest that AJ formation alone is sufficient to both recruit and activate p190. However, we do not rule out the possibility that upon contact inhibition another receptor system cross-talks with the cadherin complex to mediate the activation of p190, and induce translocation of p190 to p120 and the cadherin complex. Crosstalk between RTKs and cadherins is an important process in contact inhibition, but it is not clear how these two systems regulate each other upon increasing density (Pece and Gutkind 2000; Qian, Karpova et al. 2004). Adhesion of C-cadherin expressing cells to a surface coated with the extracellular domain of C-cadherin was sufficient to activate p190 (Noren, Arthur et al. 2003). Therefore, cadherins are indeed sufficient to both activate and recruit p190 to the cadherin complex, however, understanding the relationship between RTK- and cadherin-mediated p190 activation under condition of increasing density will be necessary to put these observations in the context of contact inhibition.

p120/p190 complex is regulated by Src

Expression of 527F-Src prevents the p120/p190 interaction, which presents a potentially exciting role for p120 and p190 in Src transformation. Furthermore, both p120 and p190 are prominent Src substrates, which suggests that tyrosine phosphorylation regulates the interaction between p120 and p190. This would provide for a highly sensitive means for regulating AJ assembly during processes such as wound healing and cell permeability. Identifying the phosphorylation sites on p120 and p190 necessary for regulating their interaction will be useful in determining their contribution to Src signaling.

Overall, these data demonstrate that the p120/p190 complex may have important roles in translating increasing cell density to inhibition of growth. Furthermore, regulation of the p120/p190 complex by tyrosine phosphorylation allows for the fine tuning of AJs that is critical in many biological processes.

CHAPTER VI

FUTURE DIRECTIONS

Introduction

Illuminating a possible mechanism for the inhibition of Rho by p120 has raised as many questions as it has answered. Targeting p120-mediated inhibition of Rho as a function of AJ assembly provides a general model that unifies the dual roles of p120 in Rho regulation and cadherin-based adhesion. This model establishes a mechanistic platform from which several questions can be tested. For example, there is wealth of literature on p190 signaling that now must be subjected to scrutiny from the perspective of cell-cell adhesion. Furthermore, since both p120 and p190 are prominent Src substrates, our data reveals a new avenue in exploring the role of p120 in Src signaling.

However, there are specific questions that must first be addressed directly. One of the surprising elements of this work is that p190 has not previously been localized to cadherin complexes. With the exception of MCF-10A cells, p190 does not obviously localize to the cadherin complex unless driven there by expression of DA-Rac. Additionally, determining the binding domains of p120 and p190 and selectively uncoupling the two proteins will be vital to a more detailed analysis of the role of this interaction in cellular signaling.

p190 localization in live cells

Our model for p120 and p190 signaling predicts that the association between p190 and p120 at the cadherin complex must be highly regulated and transient in order to maintain the proper level of Rho activity at any given time. Therefore, the reason why we do not detect p190 at the cadherin complex may be due to the fact that static measurements fail to capture this highly dynamic process. Since simple static images cannot represent this event, the most direct way to test this model is through live cell imaging of p190. Expression of GFP or RFP tagged genes have yielded new insights to cellular signaling networks with the increasing capacity of live cell imaging microscopy techniques. Through these approaches, an increasing appreciation for the roles of spatial and temporal constraints in signal transduction has emerged. Time lapse movies of fluorescently-tagged p190 under conditions of cadherin signaling such as wound repair, growth factor-induced cell scattering, and increasing cell density may reveal novel insights to the role of p190 in cadherin signaling.

Determine the binding sites of p120 and p190

Mapping the binding domains between p120 and p190 will be critical in moving forward with this project. Selectively uncoupling p120 and p190 will not only allow us to confirm some of our previous observations, but it will provide an essential tool for future investigations in p120 and Rho signaling. There are several approaches that can be used to map the binding sites between two proteins. Some of these include shotgun yeast two-hybrid, *in vitro* binding, and “mislocalization assays”. Briefly, shotgun yeast two-hybrid involves screening the full length cDNA of one gene against a library of randomly

sheared cDNA of another gene. By comparing the overlapping sequences of the gene fragments that interact with the full length cDNA, a discrete binding region can be identified. *in vitro* binding assays involve purification of recombinant proteins containing sequential deletions and assaying for binding. Lastly, “mislocalization assays” target one protein to the plasma membrane through a C-terminal CAAX fusion, and then assay for the co-localization of another protein. This approach was effective in demonstrating that p120 is able to co-localize with p190 independently of the cadherin complex (see Fig. 24). By introducing small mutations in this p120 mutant and assaying for p190 co-localization, the regions of p120 necessary for binding p190 will be identified.

<u>Role of p120/p190 complex in Src transformation</u>

Both p120 and p190 are major Src substrates. The function of tyrosine phosphorylation of p120 by Src remains unknown, and Src phosphorylation of p190 has been shown to both activate and inactivate p190 (Chang, Gill et al. 1995; Billuart, Winter et al. 2001). The interaction between p120 and p190 raises a new and exciting possibility for understanding the role of p120 in Src signaling both under physiological conditions and in Src transformation.

As shown in chapter V, expression of 527F-Src disrupts the interaction between p120 and p190. Interestingly, a 527F-Src mutant that cannot translocate to the plasma membrane (2A/527F-Src) is non-transforming. Additionally, this mutant retains its ability to phosphorylate many of its substrates. However, one of the substrates that is not phosphorylated by this Src mutant is p120. Co-expression of 2A/527F-Src with the N-terminus of RasGAP restores the transforming capabilities of this Src mutant (DeClue,

Vass et al. 1993). This N-terminal fragment of RasGAP encodes the SH2-SH3-SH2 (2-3-2) motif that mediates binding between RasGAP and p190. Expression of 2-3-2 has been shown subsequently to affect the activity of p190 (McGlade, Brunkhorst et al. 1993; Bradley, Hernandez et al. 2006). Therefore, the synergistic effects between 2A/527F-Src and the N-terminus of RasGAP could be due regulation of the p120/p190 complex that 527F-Src is able to mediate alone. Analysis of binding between p120 and p190 in the presence of 2A/527F-Src and 2A/527F-Src with the 2-3-2 peptide could help determine if this is so. Lastly, a mutational analysis of tyrosine residues in p120 and p190 to block the Src-induced disruption in p120/p190 binding should determine the functional significance of the p120/p190 complex in Src transformation.

Role of AJ formation in Ras signaling

p190 was first characterized by its constitutive association with p120RasGAP (RasGAP), a major GAP for Ras (Ellis, Moran et al. 1990). RasGAP is among three known GAPs for Ras, but its contribution to Ras signaling is not well understood. Activation of p190 by tyrosine phosphorylation increases the association between p190 and RasGAP, and is necessary for overall p190 function (Bradley, Hernandez et al. 2006). The novel role for p190 in AJ assembly described here in chapter IV establishes an exciting model for examining the relationships between RasGAP signaling and AJ dynamics.

Recent evidence regarding a role for RasGAP in anchoring p190 at focal adhesions bares a striking resemblance to our observations for p120 and AJ assembly (Bradley, Hernandez et al. 2006). It is therefore tempting to speculate antagonistic roles for p120 and RasGAP in competing for p190 recruitment to AJs and focal adhesions, respectively.

This would suggest that antagonism between integrin-mediated motility and cadherin-mediated AJ assembly may be functionally regulated by p190 recruitment. Thus it would be necessary to determine the different signals that induces p190 translocation to these two sites. Alternatively, p120-dependent recruitment of p190 to AJs may also cause RasGAP to localize to AJs. Localization of RasGAP at sites of AJ assembly would demonstrate a novel role for p120 in the negative regulation of Ras signaling. Implementation of several tools to uncouple RasGAP from p190 will help determine the role of RasGAP in p190 recruitment to AJs.

CHAPTER VII

CONCLUDING REMARKS

These data make a significant step forward in understanding the role of p120 in the regulation of Rho and AJ assembly. Additionally, they suggest a much broader role for p120 in communicating signaling cues from other receptor systems (e.g. RTKs, integrins) to the cadherin complex as they pertain to growth, motility and adhesion. Indeed it is difficult to build adequate models for these cellular functions without considering the signals that come simultaneously from RTKs, integrins, and cadherin suggesting that these receptors are functionally inseparable. Our model predicts that the ability of one receptor to regulate any of these processes depends on signaling through the cadherin complex, and that the cadherin complex is central in coordinating overall Rho activity (see Fig. 29). Furthermore, AJ assembly/disassembly is not merely an endpoint of RTK or integrin signaling, but rather crosstalk involves signaling <u>through</u> the cadherin complex to inhibit Rho. It is also likely that there is a reciprocal dependency of the cadherin complex on integrin and RTK signaling, and the relative balance of activity from each receptor drives the cell into a state of cell-cell adhesion, motility, or growth. This model dramatically simplifies how multiple signaling events (i.e. changes in cell-cell and cell-ECM adhesion, growth factor signaling) must coordinate their activities on the actin cytoskeleton for one unified event to occur (i.e. motility). Determining the relative role of p120 in coordinating these events both spatially and temporally will be an exciting advancement in under-

standing crosstalk between these receptor systems as it pertains to normal physiology as well as cancer.

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