RECLIP (REVERSIBLE CROSS-LINK IMMUNO-PRECIPITATION) REVEALS A NOVEL INTERACTION BETWEEN P120-CATENIN AND P160 RHO KINASE

By

Andrew Leslie Smith

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Approved

Professor Alissa M. Weaver

Professor Robert J. Coffey

Professor David B. Friedman

Professor Steven K. Hanks

Professor Albert. B Reynolds

Professor Christopher S. Williams

ORIGNINAL PUBLICATIONS

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To all of my family, friends, mentors who helped me reach this goal.

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To all of those who have come before me, and those that will come after me.

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LIST OF ABBREVIATIONS

AIG	Anchorage Independent Growth
AJ	Adherens Junction
APC	Adenomatous Polyposis Coli
ARVCF	Armadillo Repeat protein deleted in Velo-Cardio-Facial syndrome
ATP	Adenosine Tri-Phosphate
Ca^{2+}	Calcium
CBD	Catenin Binding Domain
CK1ε	Casein Kinase 1 ε
CRD	Cysteine Rich Domain
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DSP	Dithiobis[succinimidyl propionate]
DTME	Dithio-bismaleimidoethane
DTT	Dithiothreitol
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
FBS	Fetal Bovine Serum
GAP	GTPase activating Protein
GDI	Guanosine Dissociation Inhibitor
GDP	Guanosine Di-Phosphate

GEF	GTP Exchange Factor
GFP	Green Fluorescent Protein
GOLGA4	Golgin A4
GSK3β	Glycogen Synthase Kinase 3 β
GTP	Guanosine Tri-Phosphate
IF	Immunofluorescence
IP	Immuno-Precipitation
JMD	Juxta-Membrane Domain
K	Lysine
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LCM	Low Calcium Media
LIMK	LIM Kinase
MDCK	Madin-Darby Canine Kidney cells
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
MSH2	Mut-S Homolog 2
N	Asparagine
p120i	p120-depleted (referencing cell lines)
PAK5	p21 Activated Kinase 5
PKC	Protein Kinase C
PBS	Phosphate Buffered Saline
PMA	Phorbol-12-Myristate-13-Acetate
pRS	pRetroSuper

ReCLIP	Reversible Cross-Link Immuno-Precipitation
RIPA	Radioimmunoprecipitation Assay Buffer
ROCK1	p160 Rho Kinase
ROCK2	Rho Kinase 2
ROCK1i	ROCK1-depleted (referencing cell lines)
RTK	Receptor Tyrosine Kinase
S	Serine
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
shRNA	Short hairpin Ribonucleic Acid
T	Threonine
TAP	Tandem-Affinity Purification
TBS(-T)	Tris Buffered Saline (-Tween 20)
WB	Western Blot
WCL	Whole Cell Lysate
WT	Wild Type
Y	Tyrosine

CHAPTER I

INTRODUCTION

An Introduction to p120-catenin

p120-catenin (hereafter p120) was originally identified as a highly tyrosine-phosphorylated protein in src-transformed chick embryo fibroblasts (Reynolds et al., 1989). p120 is the prototypical member of a family of armadillo-repeat proteins that includes δ-catenin, ARVCF, p0071, and plakophilins (Anastasiadis and Reynolds, 2000). p120 was later identified as a catenin that interacts with and stabilizes classical cadherins such as E-cadherin and N-cadherin (Davis et al., 2003; Ireton et al., 2002; Reynolds et al., 1994). Cadherins mediate cell-cell adhesion through homophilic interactions between adjacent cells (Takeichi, 1995). By regulating cadherin stability, p120 is an important regulator of cell-cell adhesion and tissue morphogenesis. p120 can also localize to the cytoplasm and nucleus, where its functions remain unclear. Recent studies, described below, have demonstrated that p120 also participates in a number of signaling pathways that are frequently altered in cancer including tyrosine kinase signaling, Rho GTPase signaling, transcriptional regulation, and Wnt signaling (Figure 1). Furthermore, downregulation and/or mis-localization of p120 has been detected in a number of cancers and often correlates with poor prognosis (van Hengel and Van Roy, 2007). Thus, p120 may serve as a tumor suppressor.

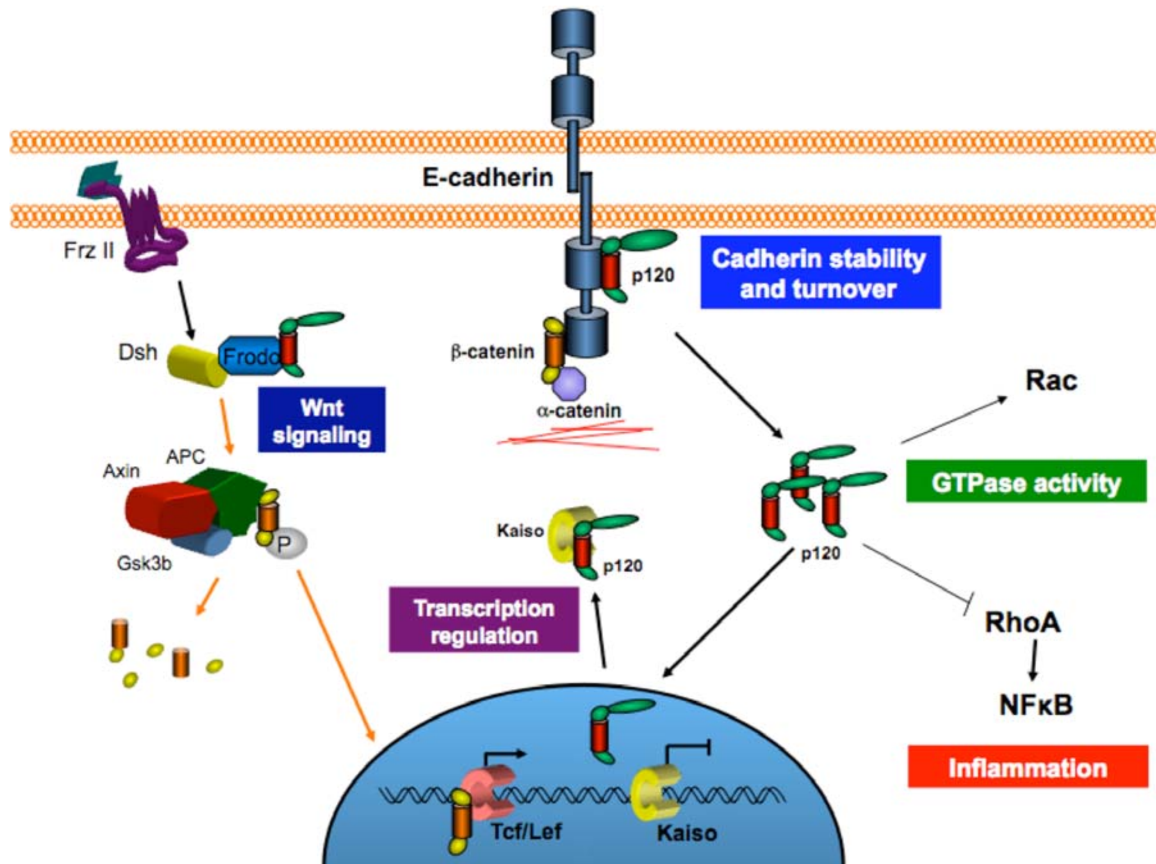

Figure 1: Localization and functions of p120. In epithelial cells, p120 is primarily localized to the cell membrane, where it binds to and stabilizes E-cadherin. p120 is also found in the cytoplasm where it may regulate Rho GTPases, and in the nucleus where its function remains unknown. p120 has also been implicated in Wnt signaling, which also involves β-catenin in a cadherin-independent signaling function.

Structure of p120-catenin

Among the most prominent features of p120 is a central armadillo-repeat domain. p120 has 9 42 amino acid armadillo repeats, with arm repeats 1-7 required for the interaction with classical cadherins (Ireton et al., 2002). The armadillo domain is flanked by the N-terminal regulatory domain and the C-terminal tail (Figure 2). The regulatory domain is extensively phosphorylated on tyrosine, serine, and threonine residues and features a 100 amino acid coiled-coil domain on the extreme N-terminus. The N-terminal regulatory domain can regulate p120's adhesive functions through a mechanism that may involve phosphorylation of the N-terminus (Aono et al., 1999). The C-terminal tail of p120 is relatively poorly understood, but does contain 3 known phosphorylation sites and has been implicated in cadherin trafficking (Liu et al., 2007).

The N-terminus of p120 contains four in-frame start codons, resulting in four possible p120 isoforms, designated isoforms 1-4 (Figure 2) (Keirsebilck et al., 1998). Isoform 1 encodes the full-length p120 protein, while isoforms 2-4 produce progressively truncated proteins, with isoform 4 lacking the entire N-terminus. Isoform 1 is expressed primarily in mesenchymal and motile cells such as fibroblasts, whereas isoform 3 is the predominant p120 isoform in epithelial cells (Mo and Reynolds, 1996). Isoform one appears to preferentially interact with mesenchymal cadherins (e.g. N-cadherin) while isoform 3 preferentially binds E-cadherin (Seidel et al., 2004). Isoform 4 is rarely observed at the protein level, but has been detected at the mRNA level. Interestingly, when expressed exogenously, isoform 4 can stabilize E-cadherin more efficiently than other p120 isoforms presumably because it is not subject to regulation via the N-terminus (Aono et al., 1999; Ireton et al., 2002).

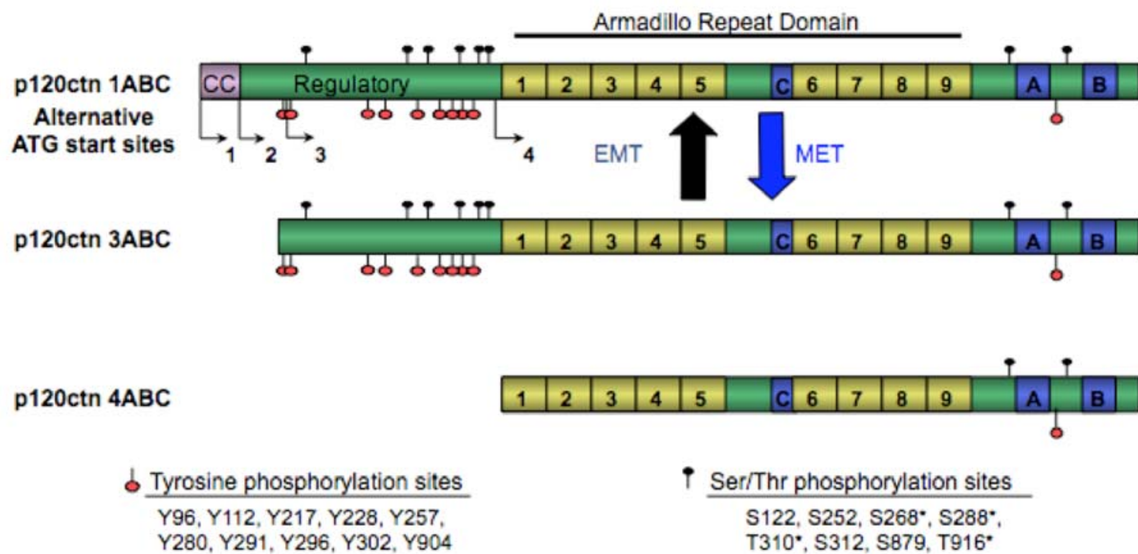

Figure 2: Structure of p120 isoforms. The schematic structure of full-length p120 1ABC and isoforms 3 and 4 are shown. Alternatively spliced exons (A, B, C) are indicated by blue boxes. Serine/threonine and tyrosine phosphorylation sites are indicated black and red balloons, respectively.

In addition to alternative start codons, p120 features 3 alternatively spliced exons, designated as exons A, B, and C. The role of these alternatively spliced exons remain unclear; however, exon B does contain a functional nuclear export signal (NES) (van Hengel et al., 1999). Thus it appears that p120 isoforms and exons may determine the specific function of a given p120 molecule; however, current reagents and assays have been unable to precisely determine the functional significance of p120 isoforms. It is worth noting that in the vast majority of studies in which p120 is exogenously expressed, only exon A is included. Thus, any functions associated with exons B and C have been excluded from these studies.

Phosphorylation of p120

The N-terminal regulatory domain and C-terminal tail of p120 contains ten tyrosine and eight serine/threonine phosphorylation sites that are regulated by a variety of signaling pathways (Figure 3). All of the tyrosine phosphorylation sites identified (Figure 2) can be phosphorylated by oncogenic src (Luo et al., 2008; Mariner, 2001; Mariner et al., 2001); however, other kinases including EGFR (Mariner et al., 2004), Fyn (Castaño et al., 2007), and Fer (Lee, 2005) have also been implicated in tyrosine phosphorylation of p120. Tyrosine phosphorylation appears to affect the ability of p120 to bind to cadherins (Ozawa and Ohkubo, 2001), providing a potential mechanism by which tyrosine kinases can destabilize cell-cell adhesion. However, simultaneous mutation of eight tyrosines to phenylalanine revealed no defects with regards to cell-cell adhesion and the actin cytoskeleton (Mariner et al., 2001). This suggests that tyrosine phosphorylation is

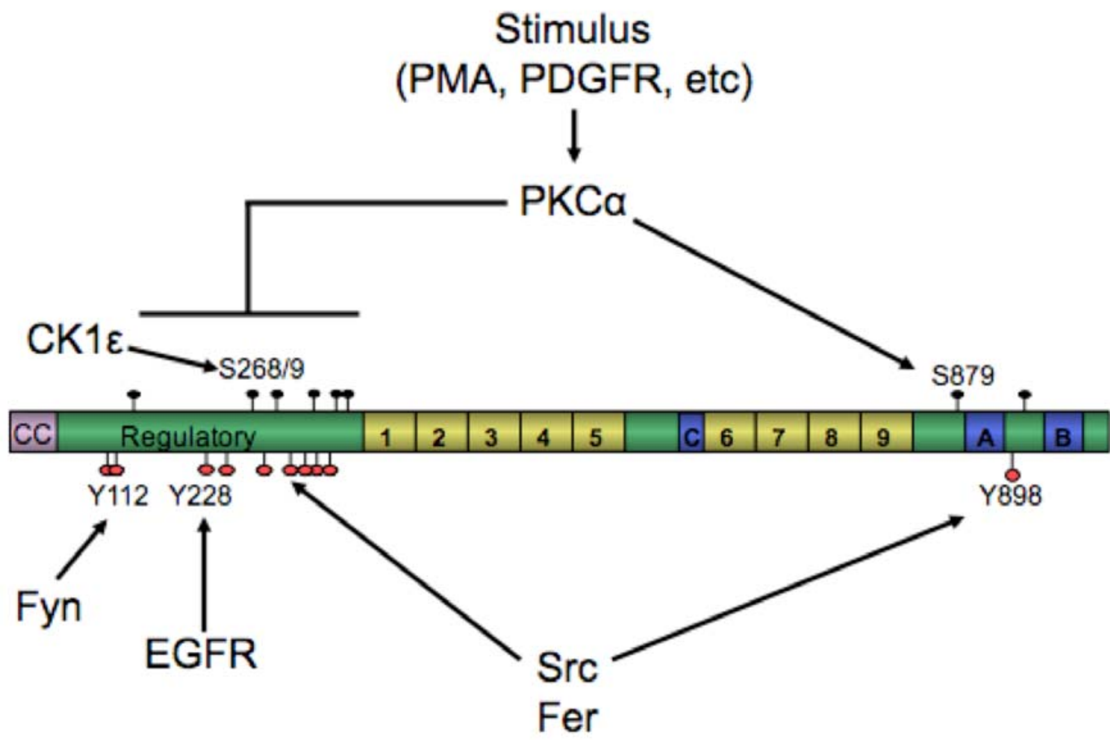

Figure 3: Reported mechanisms of p120 phosphorylation. PKCα activation by PMA or other stimuli induces dephosphorylation of the N-terminus of p120, and phosphorylation of S879 on the C-terminus. CK1ε can phosphorylate S268 and S279 in tandem. Src can phosphorylate each of the identified tyrosine phosphorylation sites. Fer, EGFR, and Fyn have also been implicated.

dispensable for the adhesive functions of p120, but may be involved in cadherin-independent functions.

The mechanisms governing serine/threonine phosphorylation of p120 remain unclear. Activation of PKC, specifically PKCα, causes dephosphorylation of serine/threonine residues within the regulatory domain and promotes the phosphorylation of serine 879 on the C-terminal tail (Brown et al., 2009; Xia et al., 2006; Xia et al., 2003). However, the phosphatases and kinases that act on p120 downstream of PKC remain unknown. Recent evidence indicates that serines 268 and 288 can be phosphorylated by CK1ε (Casagolda et al., 2010) and PAK5 (Wong et al., 2010), respectively. To date, few specific functions have been associated with p120 phosphorylation, and those that have been identified appear to depend on specific cellular contexts. As with the tyrosine residues, simultaneous mutation of all the serine/threonine phosphorylation sites to alanine did not induce any cell-cell adhesion or cytoskeletal defects in cells (Xia et al., 2006). Thus, the mechanisms and functional significance of p120 phosphorylation remains an area of active investigation.

An Introduction to the Cadherin Complex

The structure and function of the cadherin complex

Classical cadherins are single-pass transmembrane proteins that form homotypic interactions *in trans* with cadherins on adjacent cells to mediate cell-cell adhesion (Takeichi and Abe, 2005). Homophilic interactions are mediated by the extracellular

domain of cadherins in a calcium-dependant manner, adhesion is further strengthened by the lateral clustering of cadherin molecules, which is mediated in part by homophilic *cis* interactions between adjacent cadherins on the cell surface (Ozawa, 2002; Shan et al., 2000) in addition to interactions with the underlying actin cytoskeleton. This lateral clustering of cadherins at cell surface combined with the formation of adhesive trans-dimers between cadherins adjacent cells results in the formation of the adherens junction (AJ) (Figure 4).

Cadherins feature a large extracellular domain and a small cytoplasmic tail that interacts with catenins. In this cytoplasmic tail, p120 binds to the highly conserved Juxta-Membrane Domain (JMD) (Thoreson et al., 2000) while β-catenin and Plakoglobin bind to the C-terminal Catenin Binding Domain (CBD) (Gumbiner, 2005; Stappert and Kemler, 1994). β-catenin and/or Plakoglobin directly bind to α-catenin, which physically/and or functionally links cadherins to the actin-cytoskeleton (Rimm et al., 1995; Yamada and Nelson, 2007). E-cadherin (or another classical cadherin), p120, β-catenin, and α-catenin represent the core components of the cadherin complex, although many other proteins are known to associate with the complex to regulate adhesive strength, cadherin turnover, and downstream signaling events.

In addition to their structural role, cadherins participate in a number of cancer-relevant cell signaling pathways including receptor and non-receptor tyrosine kinase signaling (Pece et al., 2000; Calautti et al., 1998; McLachlan et al., 2007), activation of Rho family GTPases (Calautti et al., 2002; Fukuyama et al., 2006; Pece and Gutkind, 2000), and PI3K signaling (Woodfield et al., 2001). These mechanisms largely involve

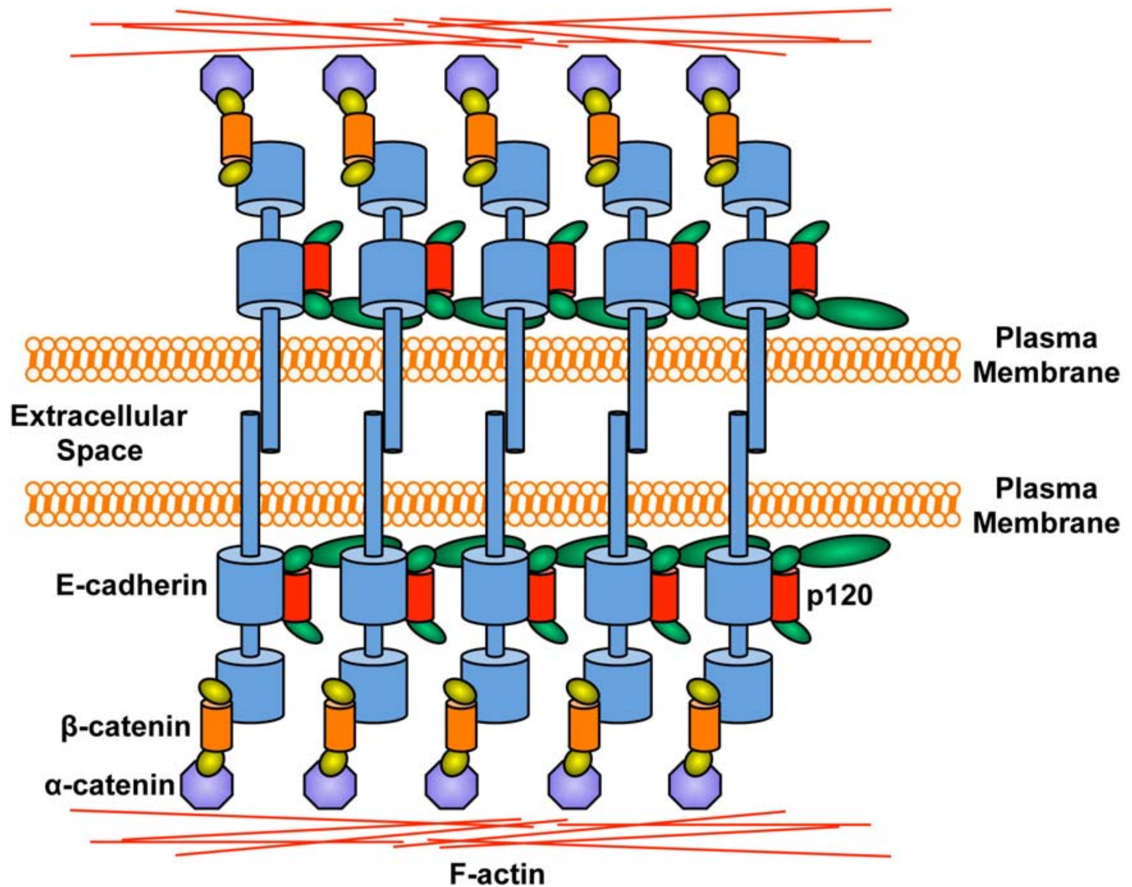

Figure 4: Schematic of the adherens junction. The core components of the cadherin complex are shown on each cadherin molecule. To show the catenins, the cytoplasmic tail of E-cadherin is enlarged relative to the extracellular domain in this schematic. Each cadherin is functionally linked to the F-actin cytoskeleton by α-catenin.

the recruitment of signaling molecules to the cadherin complex. For example, E-cadherin can physically interact with EGFR via the cadherin's extracellular domain and/or the cytoplasmic adaptor protein Merlin (Curto et al., 2007), and this interaction can inhibit ligand-dependent RTK signaling in confluent cells (Qian et al., 2004). Conversely, cadherin clustering has can activate EGFR in a ligand-independent manner by co-clustering of the receptor with cadherins.

E-cadherin status and cancer progression

As a tumor transitions to malignancy, tumor cells escape from the primary tumor to secondary sites of metastasis (Hanahan and Weinberg, 2000). This often involves individual cells detaching from adjacent cells and migrating away from the tumor, a process that involves the loss of cell-cell adhesion (Yilmaz and Christofori, 2010). Consistent with this, loss of E-cadherin has been observed as tumors progress to malignancy (Birchmeier and Behrens, 1994). The transition of epithelial cells from a sessile phenotype associated with normal tissue to a motile phenotype associated with malignancy is known as Epithelial-to-Mesenchymal Transition (EMT) (Micalizzi et al., 2010). EMT is characterized by a number of physical and genetic changes in cell adhesion and cellular signaling. One hallmark of EMT is the downregulation of E-cadherin and upregulation of N-cadherin (or other mesenchymal cadherins) via the transcription factors Snail and Slug (Batlle et al., 2000; Cano et al., 2000). Interestingly, this tumor progression is accompanied by altered p120 localization and/or switching of p120 isoforms from 1 to 3 (Bellovin et al., 2005; Sarrió et al., 2004). E-cadherin can also suppress tumorigenesis via sequestration of β-catenin at the membrane, thereby

suppressing Wnt signaling, which is frequently up-regulated in cancer (Gordon and Nusse, 2006).

Regulation of Cadherin Stability by p120

Physical Interaction between p120 and E-cadherin

p120 directly interacts with E-cadherin (and other classical cadherins) via the highly conserved Juxta-Membrane Domain (JMD) of the cadherin's cytoplasmic tail (Thoreson et al., 2000). A triple-alanine mutation within the JMD can effectively uncouple p120 from E-cadherin without affecting β-catenin binding and results in significantly weaker cell-cell adhesion. Conversely, p120 interacts with E-cadherin via its central armadillo-repeat domain, particularly arm repeats 1-5 (Ireton et al., 2002; Ishiyama et al., 2010). Deletion of any of these individual repeats produces a p120 that cannot stabilize cadherins.

Recently the crystal structure of the JMD in complex with p120 4A has been resolved, revealing key molecular details of the p120-E-cadhering interaction (Ishiyama et al., 2010). p120 features a basic arm that binds to the JMD within an N-terminal acidic region via several salt bridges and a triple-glycine motif. Residues K401 and K444 of p120 form salt bridges with the acidic region of the JMD, while N478 interacts with the triple-glycine motif of the JMD. Mutation of any one of these 3 amino acids in p120 can uncouple p120 from E-cadherin. In addition to the basic arm, p120 also has a hydrophobic pocket in which a C-terminal "anchor region" of the JMD rests. Interestingly, these binding sites on the JMD all correspond precisely with the triple-

alanine mutations of E-cadherin, which our lab had previously demonstrated to uncouple p120 and E-cadherin (Thoreson et al., 2000).

Stabilization of E-cadherin by p120

Given the association of p120 with E-cadherin and the dramatic disruption of cell-cell adhesion in src-transformed cells, it was hypothesized that p120 played an important role in regulating the cadherin complex. Using the p120-deficient SW48 cell line, it was demonstrated that p120 expression promoted E-cadherin stability. p120 expression did not upregulate E-cadherin mRNA levels, but did approximately double the half-life of E-cadherin protein (Ireton et al., 2002). Depletion of endogenous p120 in multiple cell lines using shRNA further confirmed these results, and indicated that in the absence of p120, cell-surface E-cadherin is rapidly internalized and degraded (Davis et al., 2003). These data are supported by structural analysis of the p120-JMD interaction. When bound to E-cadherins, p120 masks a dileucine motif within the JMD which, when unmasked, promotes internalization of E-cadherin (Ishiyama et al., 2010; Miranda et al., 2001; Miyashita and Ozawa, 2007). While p120-family members such as ARVCF can functionally substitute for p120, they are rarely expressed in most tissue, particularly in epithelial cells (Mariner et al., 2000). Thus, in most cell types, loss of p120 leads to loss of cadherins and cell-cell adhesion, a hallmark of metastatic cancer progression.

Rho GTPase signaling and p160 Rho Kinase

Rho Family GTPases

Rho (Ras Homologous) Family GTPases are members of the Ras superfamily of small proteins that function as binary switches in response to stimuli. In addition to the Rho family, the Ras superfamily includes the Ras, Ran, Rab, and Arf families of GTPases. Rho family proteins are distinguished from other Ras-related proteins by the presence of a Rho insert domain within the GTPase domain, which is involved in the activation of downstream effectors (Valencia et al., 1991). The Rho family of GTPases is itself divided into several groups, including the Rho proteins (RhoA, RhoB, and RhoC), the Rac proteins (Rac1, Rac2, Rac3, and RhoG), the Cdc42-like proteins (cdc42, TC10, TCL, Wrch1, Chp), the Rnd proteins (Rnd1, Rnd2, and Rnd3/RhoE), the RhoBTB proteins (RhoBTB1, RhoBTB2, RhoBTB3), and the Miro proteins (Miro1, Miro2) (Grise et al., 2009). In particular RhoA and Rac1 have been functionally linked to p120 and cadherin function.

Rho GTPases exist in either an active conformation with GTP bound or in an inactive conformation with GDP bound. In the active GTP-bound state, the GTPase can bind to effector proteins to initiate downstream signaling events (Jaffe and Hall, 2005) (Figure 5). Activation of GTPases is catalyzed by Guanine Exchange Factors (GEFs) that promote the release of bound GDP and subsequent GTP binding, inducing a conformational change that permits effector binding and downstream signaling (Schmidt and Hall, 2002). Inactivation occurs when the GTPase hydrolyzes GTP to GDP, catalyzed by GTPase Activating Proteins (GAPs), switching the GTPase back to the

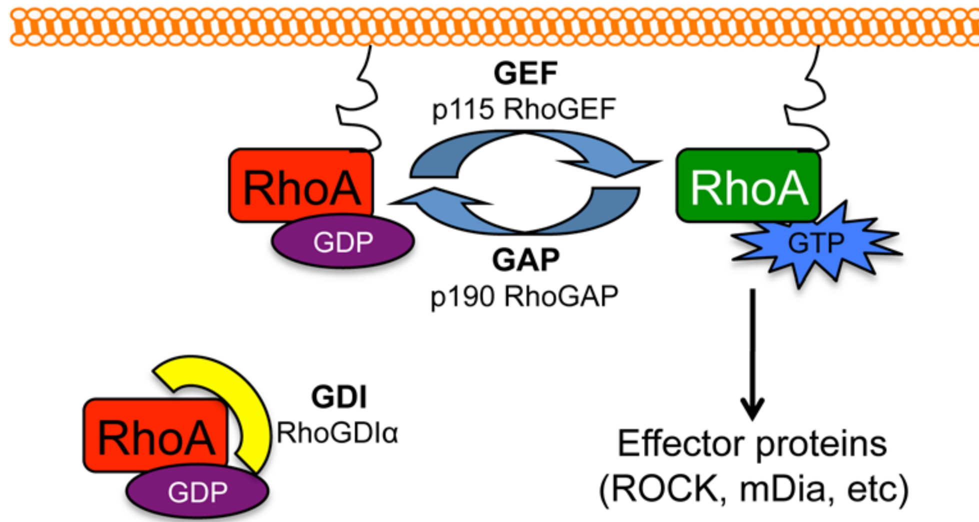

Figure 5: Regulation of Rho GTPases. A schematic illustrating the mechanisms of RhoA regulation. RhoA is activated by GEFs (e.g. p115 RhoGEF), and activated RhoA can then activate downstream effector proteins. RhoA activity is suppressed by GAPs (e.g. p190 RhoGAP). GDP-bound Rho is maintained in the inactive state by GDIs (e.g. RhoGDIα) that sequester Rho in the cytoplasm and prevent membrane anchoring.

inactive state and causing dissociation of effector proteins (Bernards, 2003). GTPase signaling can also be suppressed by Guanine Dissociation Inhibitors (GDIs), which bind to GDP-bound GTPases and sequester them from GEFs and effector proteins (Olofsson, 1999). Binding to a GDI also protects GTPases from proteosomal degradation (Boulter et al., 2010). Typically, active GTPases are localized to the cell membrane by a C-terminal CAAX motif while inactive GTPases are found in the cytoplasm.

RhoA and Rac1 Signaling

The RhoA and Rac1 GTPases are among the most well characterized members of the Rho family of GTPases. These GTPases play an important role in the regulation of the actin cytoskeleton downstream of adhesion and receptor-mediated signaling during diverse processes such as motility, adhesion, and cell growth (Burridge and Wennerberg, 2004). RhoA promotes contractility through its effector proteins such as Rho Kinase, which activates Myosin, and LIMK, which inhibits cofilin to promote the assembly of actin stress fibers. Rac1 activation leads to pronounced cell spreading, membrane ruffling, and migration in part through activation of the PAK family of serine/threonine kinases (Hall, 2005). However, both RhoA and Rac1 are also involved in the formation of adhesion complexes (both focal adhesions and adherens junctions) (Braga et al., 1997; Hotchin and Hall, 1995), protein trafficking (Garnacho et al., 2008; Matas et al., 2005), transcriptional regulation, and proliferation (Hall, 2005).

Balance between RhoA and Rac1 activities is maintained, in part, by the antagonistic relationship between the two GTPases. Specifically, activation of Rac1 leads to the generation of Reactive Oxygen Species and the downstream activation of p190

RhoGAP, a Rho-specific GAP, leading to the suppression of RhoA signaling (Nimnual et al., 2003). In epithelial cells, this antagonism is particularly important for the maintenance of proper cell morphology and is disrupted in transformed cells (Zondag et al., 2000). Previous work from our lab has demonstrated that p120 plays a role in this pathway of Rac-mediated inhibition of RhoA by recruiting p190 RhoGAP to the cadherin complex (Wildenberg et al., 2006). On the other hand, RhoA can inhibit Rac1 activity through Rho Kinase (Tsuji et al., 2002; Yamaguchi et al., 2001), although the precise mechanisms remain unclear.

Rho Kinases

A major function of RhoA is to promote acto-myosin contractility, which is accomplished in part through activation of Rho Kinase (ROCK). Rho Kinase, the first identified effector of RhoA, is a serine/threonine kinase that is related to myotonic dystrophy kinase (DMPK), DMPK-related cdc42 binding kinase (MRCK), and citron kinase (Riento and Ridley, 2003). Two isoforms of ROCK exist; p160 Rho Kinase/ROCK1/ROKβ and ROCK2/ROKα. The two isoforms share 65% overall sequence identity, and 92% similarity within their kinase domains (Nakagawa et al., 1996) (Figure 6a). Both isoforms are expressed ubiquitously, although the relative expression levels of ROCK1 and ROCK2 vary among different tissues. ROCK1 is the predominate isoform in the liver, spleen, and kidney, while ROCK2 is expression is highest in muscle and brain tissue. There are a number of redundant functions shared between ROCK1 and ROCK2, but a number of isoform-specific functions have been

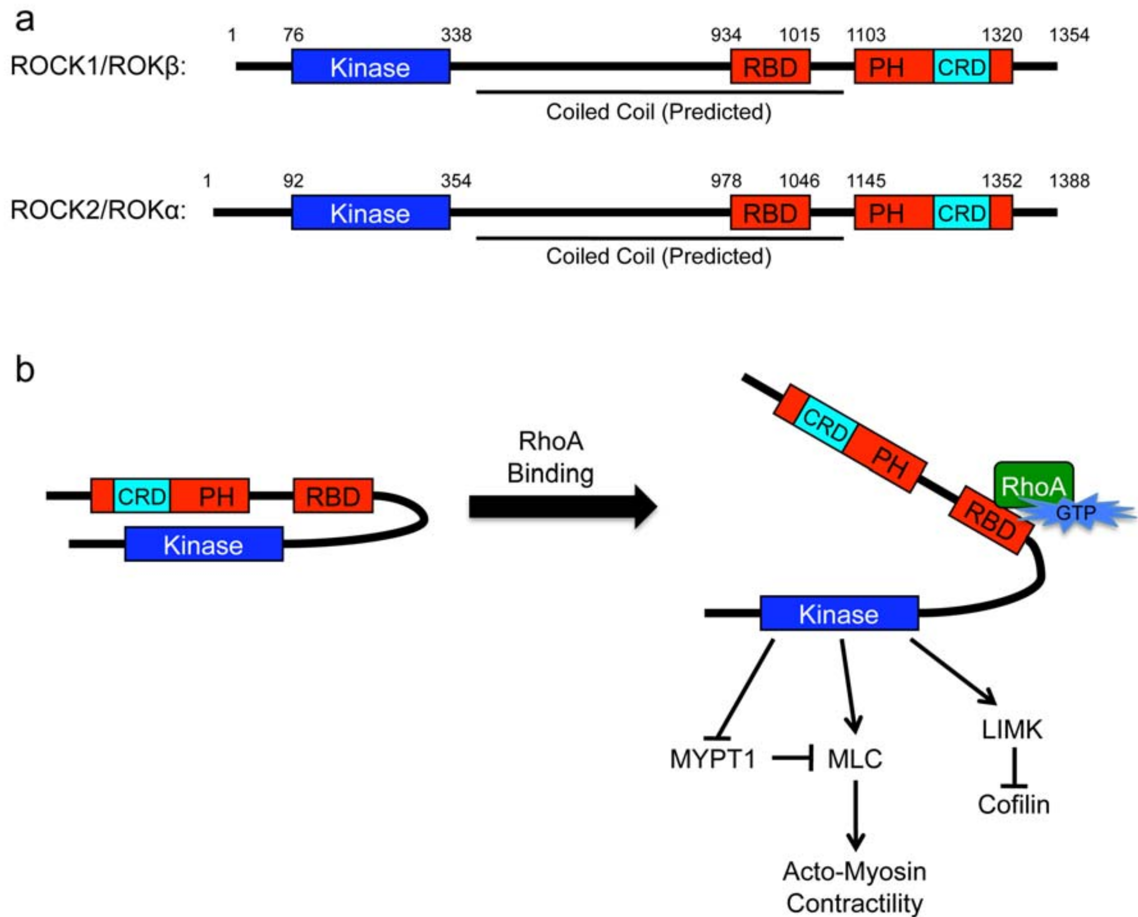

Figure 6: Isoforms and regulation of p160 Rho Kinases. (a) A schematic of ROCK1 and ROCK2. The region between the kinase domain and the Pleckstrin Homology (PH) domain, including the Rho Binding Domain (RBD) is predicted to form a coiled-coil. The PH domain also contains a Cysteine Rich Domain (CRD). **(b)** Schematic for activation of ROCK. In the inactive state, the C-terminus of ROCK is folded over to mask the kinase domain. Rho binding induces a conformational change that unmasks the kinase domain.

identified (Lock and Hotchin, 2009). In particular, ROCK2 plays a role in the disassembly of epithelial apical junctions (Samarin et al., 2007). Rho kinases are activated by RhoA binding, which induces a conformational change that unmasks the kinase domain of ROCK, allowing it to phosphorylate its substrates (Figure 6b).

The major substrates of ROCK are proteins that regulate acto-myosin contractility. ROCK can promote myosin activity by directly phosphorylating the regulatory light chain of myosin II on serine 19 (Amano et al., 1996; Totsukawa et al., 2000), which stimulates the ATPase activity of Myosin (Somlyo and Somlyo, 2000). Alternatively, ROCK1 can indirectly promote myosin activity by phosphorylating and thereby inhibiting Myosin Phosphatase. Myosin Phosphatase is responsible for dephosphorylating serine 19 of myosin light chain, thereby suppressing the motor activity (Kawano et al., 1999; Kimura et al., 1996). In addition to the myosin pathway, ROCK can phosphorylate LIM-Kinase (LIMK), which in-turn phosphorylates and inhibits the actin-severing protein cofilin (Maekawa et al., 1999). This results in the stabilization of actin cables, another requirement for contractility. Thus, cellular processes that require acto-myosin contractility such as motility, adhesion, and polarity, are all dependent on ROCK activity.

Rho Kinases phosphorylate a number of additional substrates besides Myosin and LIMK. ROCK can phosphorylate and activate Ezrin/Radixin/Moesin (ERM) proteins, a family of proteins that crosslink actin filaments and membrane proteins and are involved in epithelial polarity and migration (Matsui et al., 1998; McClatchey and Fehon, 2009). ROCK can also regulate the formation of intermediate filaments by phosphorylating vimentin, glial fibrillary acid protein (GFAP), and nuerofilament L protein (NF-L),

leading to disassembly of intermediate filaments (Goto et al., 1998; Hashimoto et al., 1998; Kosako et al., 1997). ROCK1 can also phosphorylate the translation co-factor Elongation Factor 1α (EF-1α), which also functions as an actin bundling protein (Izawa et al., 2000).

RhoA and Rho Kinases in cancer

Given their importance in regulating the actin cytoskeleton, adhesion, and motility, it is not surprising that RhoA and ROCK have been implicated in cancer progression. RhoA is required for transformation by oncogenic Ras (Qiu et al., 1995), indicating that cross-talk between GTPases plays an important role in cancer progression. Furthermore, increased RhoA expression has been detected in a number of epithelial cancers (Ellenbroek and Collard, 2007). Unlike Ras, which is frequently mutated in cancer, no mutations in RhoA have been detected in human cancers. It appears that mutation of Rho proteins is not tolerated, however mutation of regulatory and effector proteins have been detected in cancers.

Recently, 3 unique activating mutations in ROCK1 have been identified in malignant human cancers (Lochhead et al., 2010). In addition, elevated levels of ROCK1 and ROCK2 have been detected in late-stage testicular cancer (Kamai et al., 2004). Furthermore, ROCK1 expression was found to be significantly higher in human mammary tumors, and both ROCK isoforms contributed to breast cancer cell invasion (Lane et al., 2008). Interestingly, ROCK activity is suppressed in Ras-transformed cells, and over-expression of ROCK can reverse morphological transformation (Izawa et al., 1998), suggesting that inhibition of ROCK signaling is required for tumorigenicity. In our

own studies, p120 loss in v-src and Rac-transformed MDCK cells blocks growth in soft agar, and growth can be rescued ROCK inhibition using Y-27632 (Dohn et al., 2009). Thus p120 appears to be directly involved in the suppression of ROCK1 in transformed cells.

Regulation of Rho GTPases by p120

Over-expression of p120 using CMV-driven expression vectors led to dramatic alterations in cell morphology, suggesting that p120 regulates the actin cytoskeleton. Initial studies suggested that p120 behaved as a RhoGDI (Anastasiadis et al., 2000), directly binding and sequestering RhoA. In *Drosophila melanogaster*, a p120 homolog can directly bind Rho1 (Magie and Parkhurst, 2002), supporting a GDI-like function. However, further study revealed that the *Drosophila* p120 homolog might be distinct from mammalian p120 with respect to cell-cell adhesions and Rho function (Fox, 2005; Myster, 2003). Recent studies in mammalian cells have identified potential binding sites for a direct p120-RhoA interaction, Y112 and amino acids 622-8 (Castaño et al., 2007; Yanagisawa et al., 2008). p120 can also suppress RhoA indirectly by recruiting p190-RhoGAP to the cadherin complex (Wildenberg et al., 2006), suggesting that there are multiple mechanisms by which p120 can suppress RhoA activity.

While the majority of work has focused on the relationship between p120 and RhoA, recent evidence points to an equally important relationship with Rac1. Early studies indicated p120 over-expression leads to activation of Rac1 by Vav2 (Noren et al., 2000). In addition, cadherin-dependent activation of Rac1 requires the binding of p120 to E-cadherin (Goodwin et al., 2003). Similarly to its interaction with RhoA, p120 can

physically interact with Rac1b (Orlichenko et al., 2010), a constitutively active splice-variant or Rac1 found in tumor cells (Matos, 2003). This interaction appears to involve the same residues of p120 that mediate the p120-RhoA interaction, suggesting that p120 regulates Rac1b using a similar mechanism as RhoA. Furthermore, activation of Rac1 by p120 is essential for the growth and survival of tumor cells through a mechanism involving the Ras/Mek/ERK pathway (Soto et al., 2008). However in non-tumorigenic cells, p120 suppresses Rac1 signaling, thereby suppressing growth. These data indicate that p120 can affect Rac1 signaling differently depending on cellular context. In highly motile cells (e.g. fibroblasts and metastatic cancer cells), p120 promotes Rac1 activity and cell survival, possibly through stabilization of mesenchymal cadherins (Yanagisawa and Anastasiadis, 2006). On the other hand, in normal epithelial cells, p120 suppresses Rac1 signaling and cell growth, in part through stabilization of E-cadherin.

p120's physical and functional interaction with both RhoA and Rac1 is particularly interesting because of the antagonistic relationship between the two small GTPases. In NIH-3T3 cells, Rac1-mediated inhibition of RhoA requires p120 to recruit p190 RhoGAP to the cadherin complex. This process appears to be required in contact inhibition of cell growth in NIH-3T3 cells. Presumably, this pathway is intact in Rac-transformed MDCK cells grown in soft agar, since p120 is required to suppress RhoA and support anchorage independent growth (AIG) (Dohn et al., 2009). Thus p120 appears to be important for the cross-talk between Rac1 and RhoA, keeping the activities of each GTPase in check.

p120 Binding Partners

p120 primarily interacts with classical cadherins, but recent work has demonstrated that p120 can interact with a variety of proteins and complexes. However, by analogy to β-catenin, a structurally and functionally similar protein, relatively few p120 binding partners have been characterized (Figure 7). Importantly, many of these β-catenin binding partners, identified through protein-protein interaction screens, mediate the cadherin-independent Wnt signaling functions of β-catenin, which play an important role in cancer progression. A comprehensive list of binding partners is provided in Table 1. In particular, p120 interacts with kinases and phosphatases, RhoA regulatory complexes, and with transcriptional regulators.

Interactions with Kinases and Phosphatases

p120 can associate with a number of kinases and phosphatases. In particular, p120 has been shown to interact with a number of tyrosine phosphatases including SHP-1 (Keilhack et al., 2000), RPTPμ (Zondag et al., 2000), and DEP1 (Holsinger et al., 2002). p120 can also interact with the src-family kinase Fyn and the non-src family tyrosine kinase Fer (Piedra et al., 2003). Phosphorylation of p120 by Fyn appears to regulate binding of RhoA (Castano et al., 2007). However, no specific physiological function has been assigned to the other interactions between p120 and tyrosine kinases and phosphatases, although it has been proposed that modulation of p120 phosphorylation by kinases and phosphatases regulates p120's association with cadherins (Ozawa et al., 2001, Aono et al., 1999), and thereby regulates cadherin stability and cell-cell adhesion.

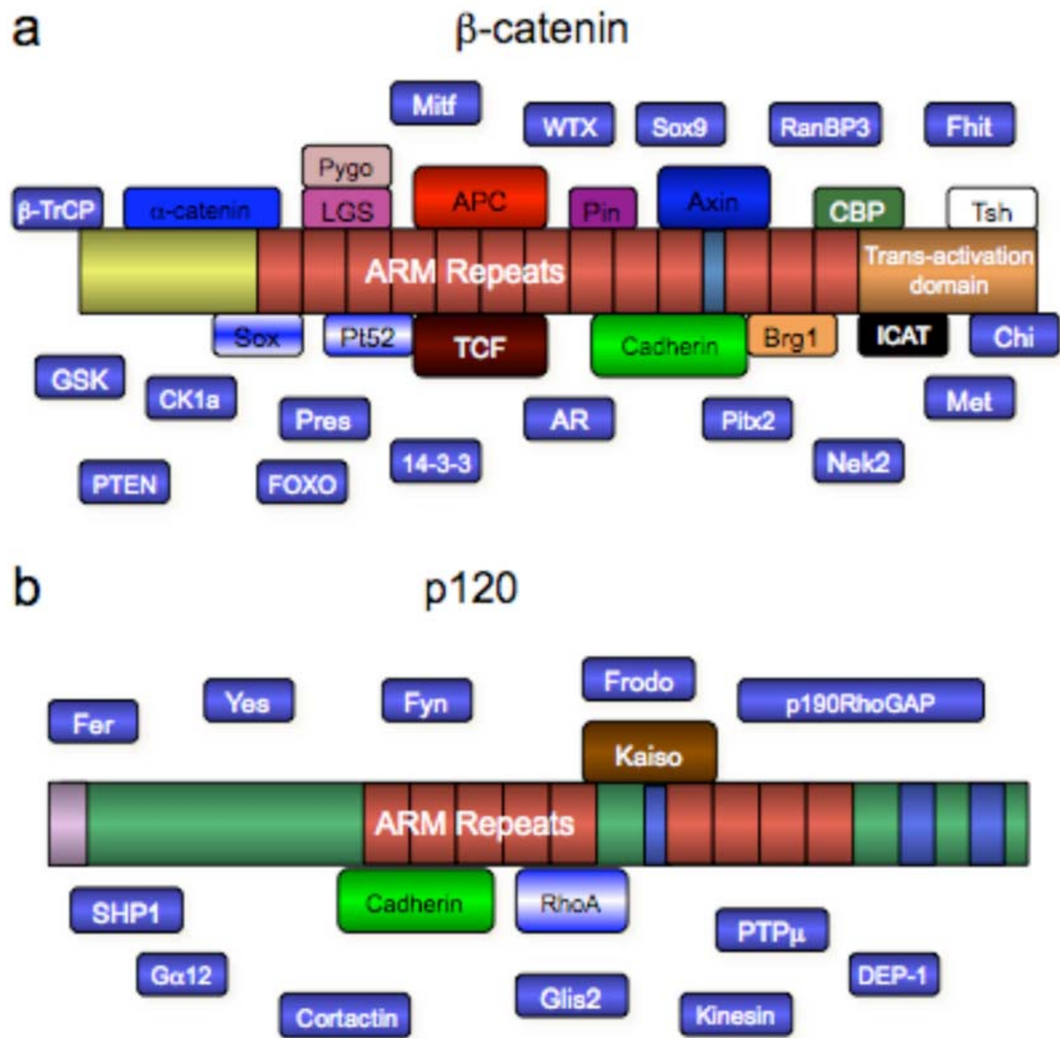

Figure 7: A schematic comparison of known β-catenin and p120 partners.

Table 1: Reported binding partners of p120. When available, the reported binding site on p120 is provided for each protein. N/A indicates no binding site has been identified.

Binding Partner	***Binding Site (p120)***	***Proposed Function of the Interaction***	***Reference***
Kaiso	ARMs 1-6	Transcriptional Regulation	Daniel and Reynolds, 1999
MUC1	N/A	Promotes p120 nuclear localization	Li and Kufe, 2001
Kinesin	N-terminus	Cadherin transport/recycling	Chen et al., 2003
p190 RhoGAP	N/A	Rho Inhibition and Contact Inhibition	Wildenberg et al., 2006
RhoA	N-terminus & 622-628	Rho Inhibition (GDI-like mechanism)	Anastasiadis et al., 2000
Rac1b	N-terminus & 622-628	Directed cell migration	Orlichenko et al., 2010
Cortactin	N/A	Lamellopdial dynamics	Boguslavsky et al., 2007
Fer	N/A	Tyrosine phosphorylates p120, suppresses neurite branching	Lee et al., 2005
Fyn	N/A	Tyrosine phosphorylates p120 (Y112), regulates RhoA binding	Piedra et al., 2003
DEP1	N/A	Dephosphorylation of p120	Holsinger et al., 2002
SHP1	N/A	Dephosphorylation of p120	Keilhack et al., 2000
RPTPμ	N-terminus	Dephosphorylation of p120	Zondag et al., 2000
Glis2	N/A	Transcriptional Regulation	Hosking et al., 2007
ϒ-secretase	N/A	Receptor proteolysis	Kiss et al., 2008
Frodo	N/A	Stabilizes p120 in response to Wnt signaling	Park et al., 2006
Nanos-1	N/A	Suppression of Rho inhibition	Strumane et al., 2006
Cdk2	N/A	Cell cycle regulation	Chartier et al., 2007
Desmoglein 3	N/A	Desmosome assembly	Kanno et al., 2008
CagA	N/A	Suppresses p120 phosphorylation and cell-invasive phenotype	Oliviera et al., 2009
PLEKHA7	N-terminus	Anchorage of microtubules to the adherens junction	Meng et al., 2008
Pak5	N/A	Phosphorylates p120 on S288	Wong et al., 2010
Casein Kinase 1ε (CK1ε)	N/A	Phosphorylation Serine 268 and 269, mediates a role in Wnt signaling	Casagolda et al., 2010
GSK-3β	N-terminus	Mediates degradation of p120 by the APC destruction complex	Hong et al., 2010

Although p120 is extensively phosphorylated on serine and threonine, no phosphatases that bind and act on p120 have been identified. Recently, two serine/threonine kinases have been identified to interact with p120. Casein Kinase 1ε (CK1ε) binds and phosphorylates p120 on S268 following Wnt stimulation in SW480 cells (Casagolda et al., 2010). In this system, p120 is required for the formation of the Wnt signalasome and subsequent β-catenin activation. The Rac/cdc42 effector PAK5 can bind to p120 and phosphorylate it on S288 (Wong et al., 2010), suggesting that effector of Rho GTPases can signal to p120. p120 may also associate with GSK3β within the destruction complex to regulate p120 levels in a manner similar to β-catenin (Hong et al., 2010). Future studies using phospho-specific p120 antibodies could identify the kinases and phosphatases that directly bind and regulate p120, and elucidate the function of these phosphorylation events.

Interactions with Rho Proteins

As discussed above, p120 plays an important role in the regulation of Rho GTPases through interactions with the Rho proteins themselves as well as with Rho regulatory proteins. p120 can directly bind to both RhoA (Castano et al., 2007) and Rac1b (Orlichenko et al., 2010), a constitutively active splice variant of Rac1. In both cases, this interaction is mediated by phosphorylation of Y112 on the N-terminus and amino acids 622-8. p120 also interacts with p190 RhoGAP to localize Rho suppression to the cadherin complex (Wildenberg et al., 2006). In addition, the zinc-finger protein Nanos1 regulates RhoA activity. Nanos1 interacts with and promotes p120 translocation to the cytoplasm, and suppress the Rho-inhibitory activity of p120 (Strumane et al.,

2006). One intriguing hypothesis is that p120 is part of a Rho-regulatory complex, allowing for the rapid regulation of Rho activity at the cell membrane (e.g. the cadherin complex) or the cytoplasm.

Interaction with Transcription Factors

The observed nuclear localization of p120 has led to speculation that p120 plays a role in gene regulation. This idea is supported by the interaction of p120 with two transcription factors; Kaiso (Daniel and Reynolds, 1999) and Glis2 (Hosking et al., 2007). Kaiso was first identified in a Yeast-Two-Hybrid screen and was later identified as a transcriptional repressor that may link p120 to Wnt signaling (Kim et al., 2004). The nature of the Kaiso-p120 interaction remains unclear, but it has been proposed that p120 can sequester Kaiso in the cytoplasm, relieving Kaiso's transcriptional repression and thereby promoting gene transcription (Daniel, 2007). Glis2 is also a transcriptional repressor that is involved in neuronal differentiation. p120 promotes cleavage of Glis2, which can suppress transcriptional activity. p120 can therefore suppress the activity of two distinct transcriptional repressors, thereby promoting the transcription of their specific gene targets.

Additional p120 Complexes

In addition to the binding partners already discussed, other p120 binding partners have been reported. In most cases, the precise functions of these interactions are poorly understood. p120 nuclear localization is promoted by an interaction with DF3/MUC1, a glycoprotein that is highly expressed in cancer cells (Li and Kufe, 2001). p120 may

regulate cell migration and adhesion via an interaction with another src-substrate, cortactin (Boguslavsky et al., 2007). p120 has been implicated in the processes of cytokinesis via its interactions with the cdk2/cyclin E complex (Chartier et al., 2007) as well as with microtubules (Franz and Ridley, 2004). At the cell membrane, p120 can interact with desmoglein 3 (Kanno et al., 2008) and the Gamma-Secretase complex (Kiss et al., 2008), facilitating crosstalk between cadherins and other adhesion receptors and/or membrane proteins. Thus, it appears there are a number of p120 functions mediated by protein-protein interactions that have yet to be understood.

Hypothesis

The Wnt signaling functions of β-catenin were initially identified through protein interaction screens. By analogy, p120 binding partner studies have lagged behind, due in large part to the labile nature of p120 complexes. I hypothesize that novel p120-binding partners can be identified using in-cell crosslinking to stabilize otherwise-labile complexes followed by mass spectrometry analysis. Herein, I describe a novel approach (ReCLIP) to identify p120 binding partners, through which I have identified a novel physical and functional interaction between p120 and p160 Rho Kinase (ROCK1).

CHAPTER II

MATERIALS AND METHODS

Cell Lines and Cell Culture

Cell lines and media

Phoenix 293 cells were a kind gift from Dr. Linda Sealy. A431 and A431D epidermoid cervical carcinoma cell lines were obtained from Dr. Margaret Wheelock (University of Nebraska Medical Center). A431D cells expressing wild type (WT) or 764AAA E-cadherin (Thoreson et al., 2000) were generated using the LZRS-MS-neo retroviral vector as described previously (Ireton et al., 2002), (Xia et al., 2006). A431, A431D, MCF-7, Caco-2, HCA-7 and MDCK II cells were cultured in DMEM (Gibco/Invitrogen) supplemented with 10% FBS (Hyclone) and 1% penicillin-streptomycin (Gibco/Invitrogen). Phoenix 293 and 293T cells were cultured in DMEM supplemented with 10% Heat-Inactivated FBS and 1% penicillin-streptomycin.

Drug treatments

Phorbol-12-Myrsitate-13-Acetate (PMA) (524400) and Y-27632 (688000) were purchased from EMD Biosciences. Prior to treatment, A431 or MCF-7 cells were washed twice with PBS and serum starved overnight with DMEM supplemented with 0.1% FBS. The next day, cells were treated with DMSO vehicle or 200 nM PMA for 30 minutes

unless otherwise indicated. For Y-27632 treatments, cells were treated with inhibitor or DMSO vehicle alone for 24 hours unless otherwise indicated.

<u>Calcium switch assay</u>

A431 cells were plated onto glass coverslips (for immunofluorescence analysis) in standard DMEM growth media. Approximately 24 hours after plating, cells were serum starved overnight. The next day, starvation media was removed and replaced with Low Calcium Media (LCM) (Calcium-Free DMEM supplemented with 5.0 μM $CaCl_2$). Cells were incubated in LCM for 2 hours, and 1.8 mM $CaCl_2$ was added directly to cells for the indicated time intervals prior to processing. For control cells, 1.8 mM $CaCl_2$ was added immediately to LCM to prevent calcium depletion. For cadherin-blocking experiments, cells were incubated with 5 μg/mL HECD1 (anti-E-cadherin mAb) and 2 μg/mL 6A9 (anti-P-cadherin mAb) for 30 minutes prior to calcium restoration.

Retroviral and Lentiviral Transduction

<u>Retrovirus and lentivirus production and infection</u>

To generate retrovirus particles, the Phoenix 293 cells were transfected using the calcium phosphate method as described previously (Davis et al., 2003). Retrovirus constructs used were based on the LZRS-MS-neo and pRetro-Super (pRS) shRNA vectors described previously (Ireton et al., 2002, Davis et al., 2003). Virus was harvested 48 hours post-transfection by passing the cell-culture media through a 0.45 μm filter. Target cells were transduced by incubation with retrovirus-containing media containing 4

μg/mL Polybrene. Approximately 48 hours post-infection, infected cells were selected using either G418 (for LZRS-neo transductions) or Puromycin (for pRS transductions).

To generate lentiviral particles, 293T cells were co-transfected with the pLKO.1 shRNA plasmid of interest, pCMV-dR7.74psPAX2 packaging plasmid, and pMD2.G envelope plasmid using the calcium phosphate method as described previously (Brown et al., 2009). Lentivirus was harvested 48 hours post-transfection and target cells were transduced as described above. Approximately 48 hours post-infection, infected cells were selected using Puromycin.

Plasmids

The LZRS-MS-neo (LZRS-neo) vector was used for exogenous expression of p120, E-cadherin, and ROCK1. p120 and E-cadherin constructs used are as follows: LZRS-neo mp120 1A, LZRS-neo mp120 3A, LZRS-neo mp120 4A, LZRS-neo mp120 3A Δarm1 (Ireton et al., 2002), LZRS-neo mp120 3A Δarm1 CAAX (Xia et al., 2006), LZRS-neo mp120 1A Δ622-8 (Anastasiadis et al., 2000), LZRS-neo E-cadherin, and LZRS-neo 764AAA E-cadherin. p120 cDNAs were first cloned into the pMS shuttle vector prior to ligation into LZRS-neo. All point mutations were generated by site-directed mutagenesis as described previously (Xia et al., 2003).

ROCK1 cDNA was obtained from Dr. Shuh Narumiya (Kyoto University) in the pCMX expression vector. To generate LZRS-neo ROCK1-GFP, the ROCK1 ORF was PCR amplified with the stop codon removed and subsequently ligated in pENTR 3C to generate pENTR-ROCK1 Δstop. pENTR-ROCK1 Δstop was recombined with LZRS-neo

GW-GFP using the Gateway Cloning system (Invitrogen) to generate LZRS-neo ROCK1-GFP.

For shRNA mediated knockdown of p120, pRS-hp120 was employed as described previously (Davis et al., 2003). For ROCK1 knockdown, Non-Target or ROCK1 shRNA constructs in the pLKO.1 lentivirus vector were purchased from Sigma. For all ROCK1-knockdown experiments described herein, cells transduced with ROCK1 shRNA TRCN0000121094 are shown. Similar results were obtained using ROCK1 shRNA TRCN0000002160.

Antibodies

The generation of monoclonal and polyclonal antibodies for p120 (pp120, 15D2, 8D11, F1aSH) has been described (Wu et al., 1998). Of note, mAb 15D2 was used for all p120 immunoprecipitations unless otherwise noted, while mAb 8D11 is used as a control IgG because it does not recognize human p120. The generation of anti-phospho-S268, phosho-S288, phosho-T310, and phospho-T916, and phospho-S879 monoclonal antibodies has been previously described (Xia et al., 2004; Vaughan et al., 2007). Other antibodies used include anti-E-cadherin monoclonal antibody (BD Transduction), anti-α-catenin rabbit polyclonal antibody (C-2081, Sigma), anti-β-catenin rabbit polyclonal antibody (C-2206 Sigma), anti-p42/44 MAPK (ERK1/2) rabbit polyclonal antibody (Cell Signaling), and anti-Focal Adhesion Kinase (FAK) rabbit polyclonal antibody (C-20, Santa Cruz), anti-ROCK1 rabbit polyclonal antibody (Chemicon), Ezrin monoclonal antibody (BD Transduction), anti-GFP monoclonal antibodies (Roche), and anti-tubulin monoclonal antibody (clone DM1A, Sigma). Anti-cd98 monoclonal antibody 4F2 was a

kind gift from Dr. Roy Zent (Haynes et al., 1981), (Zent et al., 2000). Anti-E-cadherin and anti-P-cadherin monoclonal antibodies HECD1 and 6A9, respectively, were kind gifts from Dr. Margaret Wheelock. Secondary antibodies for western blot analysis include anti-mouse AlexaFluor 680 (Molecular Probes) and anti-rabbit IRdye 800 (Rockland Immunochemicals). Secondary antibodies used for immunofluorescence analysis include anti-mouse IgG, anti-mouse IgG2a and anti-rabbit IgG or conjugated to AlexaFluor 488 or 594 (Molecular Probes).

Crosslinking and Immunoprecipitation

In-cell crosslinking

In-cell cross-linking was performed using Dithiobis[succinimidyl propionate] (DSP) and Dithio-bismaleimidoethane (DTME) (Pierce/Thermo Scientific). For each experiment, cross-linkers were freshly prepared as a 20 mM solution in Dimethyl Sulfoxide (DMSO) and diluted to the indicated final working concentrations in Phosphate-buffered saline, pH 7.4 (PBS, Fisher Scientific). Cells were washed twice with PBS at room temperature to remove all traces of media and incubated with the cross-linker solution for 30 minutes at room temperature. After removal of the cross-linker solution, cells were incubated at room temperature for 10 minutes with quenching solution (20 mM Tris-Cl pH 7.4, 5 mM L-Cysteine). Quenching solution was then removed and cell lysates were prepared as described below.

Bead preparation

To prepare magnetic beads for immunoprecipitation, Protein G Dynabeads (Dynal/Invitrogen) were washed with Citrate Phosphate buffer pH 5.0 (25 mM citric acid, 50 mM dibasic sodium phosphate) and incubated with either 15D2 or 8D11 monoclonal antibodies for 2 hours at room temperature with end-over-end rotation. Bead-antibody complexes were washed with citrate phosphate buffer, followed by two washes with 0.2 M Triethanolamine (TEA) pH 8.2. Antibodies were covalently bound to Protein G beads by incubation 20 mM Dimethyl Pimelimidate (Sigma) in TEA for 30 minutes at room temperature with end-over-end rotation, followed by incubation for 15 minutes with 50 mM Tris-Cl pH 7.5 to quench the crosslinking reaction. Subsequently, beads were washed three times with PBS-Tween. After washing with 0.1 M Glycine, pH 2.5 to remove non-covalently bound antibodies, beads where washed again with PBS-Tween and stored at 4°C.

Lysate preparation, conventional immunoprecipitation, and western blot analysis

Lysis, immunoprecipitation, and western blot methods have been described previously (Xia et al., 2006). Briefly, cells were lysed in Radioimmunoprecipitation Assay (RIPA) buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) or Digitonin buffer (20 mM Tris pH 7.5, 150mM NaCl, 1% Digitonin) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 5 mg/mL Leupeptin, 2 mg/mL Aprotinin, 1 mM EDTA, 50 mM NaF, and 1 mM $NaVO_4$). Lysates were cleared by centrifugation and total protein concentrations were determined by BCA assay (Pierce/Thermo Scientific). For immunoprecipitation, the specified antibody was

added to the clarified lysate for 2 hours at 4°C with end-over-end rotation, followed by incubation with Protein G sepharose (GE Healthcare) for an additional hour at 4°C. Beads were washed with lysis buffer, resuspended in 2x Laemmli Sample Buffer (LSB), and boiled for 5 minutes. Lysates were prepared in LSB or non-reducing sample buffer (50 mM Tris pH 6.8, 4% Glycerol, 1% SDS, 0.004% Bromophenol Blue) as indicated. Cross-linked lysates were incubated with 50 mM DTT for 15 minutes prior to boiling to ensure cleavage of disulfide bonds within the cross-linkers.

Immunoprecipitations and whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Whatman) for western blotting. Non-specific binding to membranes was blocked with 3% nonfat milk or 5% BSA (phospho-antibodies only) in TBS (10 mM Tris pH 7.4, 150 mM NaCl), and membranes were incubated with primary antibody in milk overnight at 4°C. Membranes were incubated with secondary antibody in Odyssey blocking buffer (Li-Cor) for 1 hour at room temperature. Antibodies were detected using the Odyssey infrared imaging system (Li-Cor).

Reversible Cross-Link ImmunoPrecipitation (ReCLIP) procedure

Four 15 cm dishes of 90% confluent A431, MCF-7, MCF-10A, Caco-2, or HCA-7 cells (approximately 1×10^8 cells) were used for each experiment. RIPA lysis buffer was prepared fresh and filter-sterilized the day before cells were lysed. Cells were washed twice with freshly-prepared PBS pH 7.4 to remove all traces of media. Following removal of PBS, 10 mL of a 0.5 mM crosslinker solution in PBS (as described above) was added to the each plate. Cells were incubated with crosslinkers for 30 minutes at room temperature, with occasional agitation. Crosslinker solution was then removed, and

10 mL quenching solution was added to each plate for an additional 10 minutes. Following quenching, plates were placed on an ice-water bath and washed once more with chilled PBS, and lysed with freshly-prepared RIPA buffer plus protease and phosphatase inhibitors (1 mL RIPA buffer per dish). Lysates were homogenized using a 23-gauge needle and cleared by centrifugation. Equal volumes of clarified lysate were incubated with either 15D2 (p120) or 8D11 (control) bound Protein G Dynabeads for 3 hours at 4°C with end-over-end rotation. The beads were then washed 5 times with 1 mL RIPA buffer supplemented with protease and phosphatase inhibitors. p120 binding partners were eluted by incubating the beads with RIPA buffer supplemented with 50 mM DTT in for 30 minutes at 37°C with end-over-end rotation.

For mass spectrometry analysis, eluates were boiled in freshly prepared LSB, separated by SDS-PAGE on Nu-PAGE 4-12% Bis-Tris gels (Novex/Invitrogen) and stained with “Blue Silver” colloidal coomassie stain (Candiano et al., 2004). The entire lane was excised and processed for shotgun analysis using single-dimension liquid-chromatography tandem-mass spectrometry (LC-MS/MS) by the Vanderbilt University Medical Center Proteomics Laboratory (according to procedures described below). For silver stain analysis, 10% of the eluate was separated by SDS-PAGE and protein was visualized using Silver Stain Plus (Bio-Rad), according to the manufacturer’s protocols. Following staining, gels were imaged using the FluorChem-8900 Gel Documentation System (Alpha Innotech).

Mass spectrometry and protein identification

Proteins were resolved by SDS-PAGE, visualized with colloidal coomassie stain, and protein bands of interest were excised and cut into 1 mm cubes and equilibrated in 50 mM NH_4HCO_3. Proteins were then reduced within the gel pieces with DTT (3 mM in 100 mM NH_4HCO_3, 37°C for 15 min) followed by alkylation with iodoacetamide (6 mM in 50 mM NH_4HCO_3 for 15 min). The gel pieces were then dehydrated with acetonitrile and rehydrated with 15 mL 12.5 mM NH_4HCO_3 containing 0.01 mg/mL trypsin (Trypsin Gold, Promega), and trypsin digestion was carried out for >2 h at 37°C. Peptides were extracted with 60% acetonitrile, 0.1% formic acid, dried by vacuum centrifugation and reconstituted in 15 μL 0.1% formic acid. 5 μL of peptide hydrosylate were analyzed by C18 reverse-phase LC-MS/MS using a Thermo LTQ ion trap mass spectrometer equipped with a Thermo MicroAS autosampler and Thermo Surveyor HPLC pump, nanospray source, and Xcalibur 2.0 instrument control using standard triple-play methods. Tandem MS data were analyzed with the Sequest algorithm to search a human subset of the UniRef100 database (Jan 23 2007, 223514 entries) using Xcorr cutoffs of $\geqq 1.8$ for $[M+2H]^{2+}/2$ ions and $\geqq 2.5$ for $[M+3H]^{3+}/3$ ions . In addition, the database contained a concatenated reverse decoy database to estimate false-discovery rates, which were at 5% or below

Immunofluorescence Microscopy

Cells were plated on glass coverslips 2 days before treatment and processing for immunofluorescence staining. Briefly, cells were fixed in 3% Paraformaldehyde for 30 minutes, and permeabilized in PBS/0.2% Triton X-100 for 5 minutes. Cells were blocked

with PBS containing 5% BSA for 10 minutes. Cells were incubated with the indicated primary antibodies diluted in 5% BSA for 30 minutes, followed by secondary antibodies for another 30 minutes. To stain actin, AlexaFluor 488-conjugated Phalloidin was used in place of secondary antibody. Cells were stained with 0.5 μg/mL Hoechst dye for one minute to stain nuclei. Coverslips with stained cells were mounted onto glass slides using Prolong Gold anti-fade reagent (Invitrogen) and imaged using a Zeiss Axiovert fluorescence microscope with a 63x objective. Images were acquired and processed using Metamorph software (Molecular Devices). To quantify cell-cell contact localization of ROCK1, four distinct regions of the coverslip were imaged using a 20x objective. Total cells were quantified using Hoechst-dye nuclei staining, and cells with ROCK1 localized to junctions were manually counted using ImageJ. The percent of cells with ROCK1 localized to cell-cell contacts in each field was calculated and averaged. Statistical analysis was performed using a 2-tailed t-test.

***In-vitro* ROCK1 Kinase Assay**

A431 cells were grown to 90% confluence and serum-starved overnight with DMEM supplemented with 0.1% FBS. The next day, cells were lysed in RIPA buffer and clarified lysates were incubated with anti-p120 mAb 15D2 for 1 hour, followed by incubation with Protein-G Sepharose beads for an additional hour. Beads were washed three times with RIPA buffer, followed by an additional 2 washes with ROCK1 Kinase Buffer (10 mM MOPS, 0.2 mM EDTA, 10 mM MgAc) without ATP. Following washing beads were incubated with 200 ng constitutively active ROCK1 (amino acids 17-535) (Millipore) in ROCK1 kinase buffer with 0.1 mM ATP for 15 minutes. Kinase reactions

were stopped by addition of 2x LSB and boiling samples for 5 minutes. p120 phosphorylation was assessed by western blot using phospho-specific p120 monoclonal antibodies and anti-p120 pAb F1αSH to detect total p120.

CHAPTER III

RECLIP (REVERSIBLE CROSS-LINK IMMUNO-PRECIPITATION): AN EFFICIENT METHOD FOR INTERROGATION OF LABILE PROTEIN COMPLEXES

Introduction

Identifying functionally relevant protein-protein interactions remains a significant problem in discovery-based research. Affinity purification coupled with Mass Spectrometry (MS) analysis is a rapid, sensitive, and unbiased method for identifying novel protein-protein interactions. While ongoing technical advances have dramatically improved the sensitivity and efficiency of mass spectrometry instruments and methods, most experiments are limited by the quality of the sample itself. Current methods represent a compromise where recovery is sacrificed for specificity or vise versa. Conventional co-immunoprecipitation by itself is invariably accompanied by unacceptable background. A common solution is to add a second affinity purification step. This Tandem-Affinity-Purification (TAP-tag) approach, however, minimizes background at the expense of transient and/or weak interactions that are lost because of the additional processing (Rigaut et al., 1999), (Puig et al., 2001).

Here, I have used p120-catenin (hereafter p120) and the E-cadherin complex as a model to develop an approach that captures labile interactions without sacrificing specificity. Whereas β- and γ-catenins bind cadherins with high affinity under a variety of conditions, the p120 interaction is relatively labile. In RIPA buffer, for example, p120 is

almost undetectable in cadherin immunoprecipitates, whereas the other catenins are efficiently recovered. Gentler detergents (i.e. NP-40) improve recovery, but are nonetheless relatively inefficient (Reynolds et al., 1994). Digitonin can effectively preserve p120 binding in some cell types, but appears to act selectively on soluble (as opposed to cytoskeleton tethered) complexes (Reynolds et al., 1994), (Kiss et al., 2008) and previous attempts using TAP methods have been unsuccessful due to extremely low recovery of p120 complexes (unpublished observations).

Chemical crosslinkers have been employed to stabilize protein-protein interactions for structural studies (Studdert and Parkinson, 2007), or to demonstrate interaction between already suspected binding partners (Vretou et al., 2008). For example, it has been used successfully to capture transient dimerization of the Epidermal Growth Factor Receptor in response to ligand (Zhou et al., 1993). In particular, the cell-permeable, lysine-reactive crosslinker Dithiobis[succinimidyl propionate] (DSP, also called Lamont's Reagent) has been successfully used to facilitate co-immunoprecipitation of weakly interacting binding partners (Zhang et al., 2007). Recently, DSP-crosslinking has been combined with affinity-purification and mass spectrometry to identify novel binding partners (Salazar et al., 2009), (Humphries et al., 2009), suggesting that in-cell crosslinking can be used to characterize weak and transient complexes by mass spectrometry.

Here, using p120 and the cadherin complex as a model system, we describe an efficient approach that employs cell-permeable, thiol-cleavable crosslinkers to stabilize normally labile interactions (i.e. the p120 - E-cadherin interaction) *in vivo* prior to cell lysis and affinity purification. In our model, p120 was directly immunoprecipitated under

stringent conditions and binding partners were selectively eluted from the p120 "bait" by chemical cleavage of the crosslinker. Unlike other approaches, this elution scheme removes the target protein along with the beads and antibody from the final sample, resulting in very low background. Western blot and MS analyses revealed that all core components of the cadherin complex were efficiently recovered along with several novel candidates for direct or indirect p120 binding partners. This approach, which we have termed ReCLIP (Reversible Cross-Link Immuno-Precipitation) is simple and produced remarkably clean preparations of p120 binding partners for proteomic analyses. These results suggest that ReCLIP provides high sensitivity without sacrificing specificity, and therefore provides a robust alternative to other affinity-purification methods.

Results

Determination of optimal crosslinker concentrations

We initially identified candidate crosslinkers and evaluated conditions for use. Two specific crosslinkers, Dithiobis[succinimidyl propionate] (DSP) and Dithio-bismaleimidoethane (DTME), were chosen based on their distinct chemical properties. DSP reacts with primary amines and has a spacer-arm of 12 Å (Figure 8a), forming crosslinks between lysine residues of interacting proteins. DSP has been commonly used in a variety protein-interaction studies (Appenzeller et al., 1999), (Studdert and Parkinson, 2007) due in part to the high abundance of lysine residues in proteins. DTME reacts with sulfhydryl groups and has a spacer arm of 13.3 Å (Figure 8b), forming crosslinks between cysteine residues of interacting proteins. DTME would be expected to produce fewer crosslinks, however it may capture interactions that DSP cannot. While

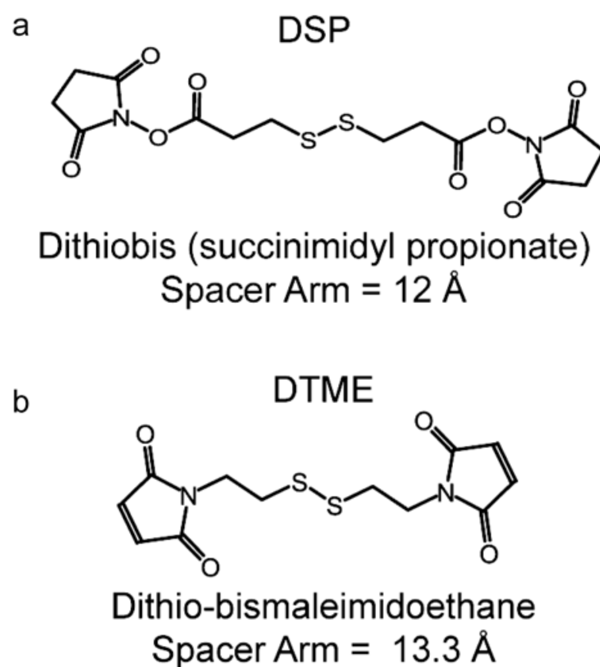

Figure 8: Chemical structures of ReCLIP crosslinkers DSP and DTME. The chemical structures of DSP **(a)** and DTME **(b)** are shown, images were constructed with the DrawIt application in KnowItAll Informatics System v. 4.1 (Bio-Rad). DSP features NHS-ester reactive groups at both ends (a) while DTME features meleimide reactive groups (b). Note the central disulfide bond in each molecule, allowing for cleavage by reducing agents.

not commonly used, DTME has been successfully applied to protein-protein interactions studies (Casula et al., 2009). Importantly, both compounds are cell-permeable, allowing for in-cell crosslinking of endogenous complexes prior to cell-lysis. Additionally, both compounds are thiol-cleavable, allowing for “reversal” of the crosslinks via chemical cleavage by a reducing agent (i.e. DTT).

Optimal crosslinker conditions were determined using A431 epidermoid carcinoma cells, a human epithelial cell line that has been used for a number of cell-cell adhesion studies (Norvell and Green, 1998), (Davis et al., 2003). A431 cells were washed with PBS and exposed for 30 minutes to increasing concentrations of DSP or DTME in PBS, pH 7.4. Cells were then lysed at 4˚C in RIPA and the lysates treated for 15 min with DTT (reducing, panels b and d) or not (nonreducing, panels a and c), as indicated. Samples were then analyzed by SDS-PAGE, followed by Western blotting for E-cadherin (top panels) or p120 (bottom panels).

Figure 9a shows a dose-dependent reduction in monomeric E-cadherin (top panel, arrowhead) and the simultaneous appearance of crosslinked complexes across the top of the gel that are too large to resolve (arrow). Note that the monomeric E-cadherin (and p120, lower panel) is decreased at 0.5 mM DSP and almost absent at 1.0 mM, indicating that the vast majority of E-cadherin and p120 is crosslinked into high molecular complexes at these concentrations. Figure 9b shows that monomeric protein is efficiently recovered by addition of DTT. Note that at 0.5 mM DSP, virtually all of the monomeric E-cadherin and over half of the monomeric p120 are recovered (compare lanes 6 in a and b, upper and lower panels respectively), and that the high molecular weight bands are no longer present. It is not entirely clear why the recovery of p120 in whole cell lysates is

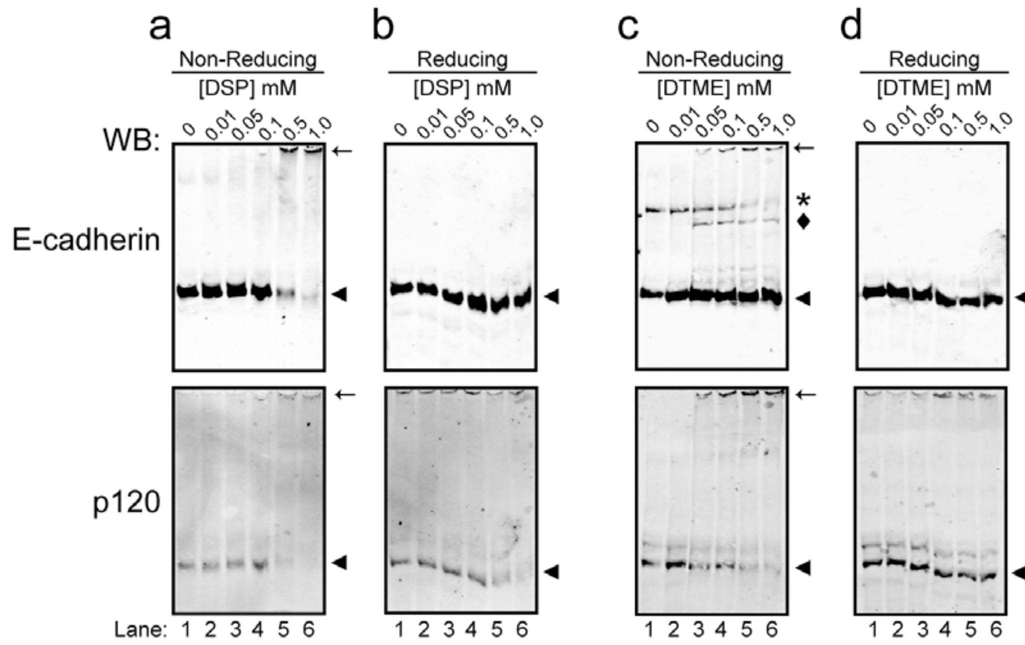

Figure 9: Titration of intracellular cross-linking of p120 and E-cadherin. Western blot analysis of E-cadherin (top panels) and p120 (bottom panels) in whole cell lysates of A431 cells treated with the indicated concentrations of DSP **(a, b)** or DTME **(c, d)** between 0.01 mM and 1.0 mM. Samples were prepared under non-reducing (a, c) and reducing conditions (b, d) as indicated. Arrowheads indicate monomeric E-cadherin and p120, large cross-linked species are indicated with arrows, smaller crosslinked E-cadherin species are indicated with a diamond (u), and a possible cysteine-induced E-cadherin dimer is indicated with an asterisk (*).

less efficient than that for E-cadherin. The difference, however, is not generally observed in immunoprecipitates, suggesting that the phenomenon may reflect competition for reducing agent among the large number of crosslinked proteins present in the whole cell lysate.

Crosslinking with DTME was less efficient, as evidenced by the relatively high levels of monomeric E-cadherin remaining at the 1.0 mM dose (panel c, compare lanes 1 and 6). This is consistent with the lower abundance of cysteine residues relative to lysine. Nonetheless, the appearance of progressively larger E-cadherin-containing complexes with increasing DTME indicates the presence of crosslinked species. The faster migrating band (Figure 9c, diamond) probably represents a partial complex. The exact content is not known, but p120 is clearly absent. Further crosslinking generates p120-containing higher order complexes, which are too large to resolve by SDS-PAGE (arrow). In these non-reduced samples, an additional E-cadherin band is present even in the absence of cross-linker (asterisk). The precise identity of this E-cadherin complex is unclear, but it may represent cadherin dimers caused by the addition of cysteine to quench the DTME crosslinking reaction, as dimerization is induced, in part, by cysteine mediated disulfide bonds within the extracellular domain (Boggon et al., 2002), (Troyanovsky et al., 2003). Interestingly, for reasons not entirely clear, DTME appears to crosslink p120 more efficiently than E-cadherin, as evidenced by significant loss of monomeric p120 (panel c, compare lanes 1 through 6).

Based on these data, we chose 0.5 mM DSP and 0.5 mM DTME as optimal concentrations for subsequent experiments. In the case of DSP, 1.0 mM was more effective than 0.5 mM, but I chose the lesser of the two to limit nonspecific capture. For

DTME, there was no apparent difference between 1.0 and 0.5 mM so the lesser amount was used.

Efficacy, efficiency, and specificity of crosslinking with DSP and DTME

Next, I used the E-cadherin – p120 interaction as a model to assess the efficacy of DSP and DTME under the above conditions. The amount of E-cadherin co-immunoprecipitating with p120 was determined after in-cell crosslinking with DSP or DTME (Figure 10a). Cell lysis in a digitonin-containing buffer (without crosslinking) was used as a reference (Figure 10a, lane 1), because it is relatively effective in A431 cells at preserving the p120 – E-cadherin interaction (Kiss et al., 2008). In contrast, the remaining samples were treated with DSP, DTME, or vehicle alone (DMSO), as above, and lysed in RIPA buffer. p120 was then immunoprecipitated from all samples, eluted in reducing LSB, and analyzed by SDS-PAGE and Western blotting.

Figure 10a shows that E-cadherin recovery from p120 immunoprecipitates after DSP crosslinking was as good, if not better, than that obtained from the digitonin lysate (compare lanes 1 and 3). DTME was less efficient (lane 4), whereas no E-cadherin was recovered in the absence of crosslinker (i.e. DMSO, lane 2). Thus, E-cadherin was not recovered in RIPA alone, but crosslinking with DSP preserved the interaction. Moreover, irrelevant cytoplasmic (i.e. ERK1/2) and membrane-associated (i.e. Focal Adhesion Kinase) proteins were absent from the p120 immunoprecipitates but clearly present in whole cell lysates. Thus, DSP and DTME crosslinking appears to be quite specific under these conditions.

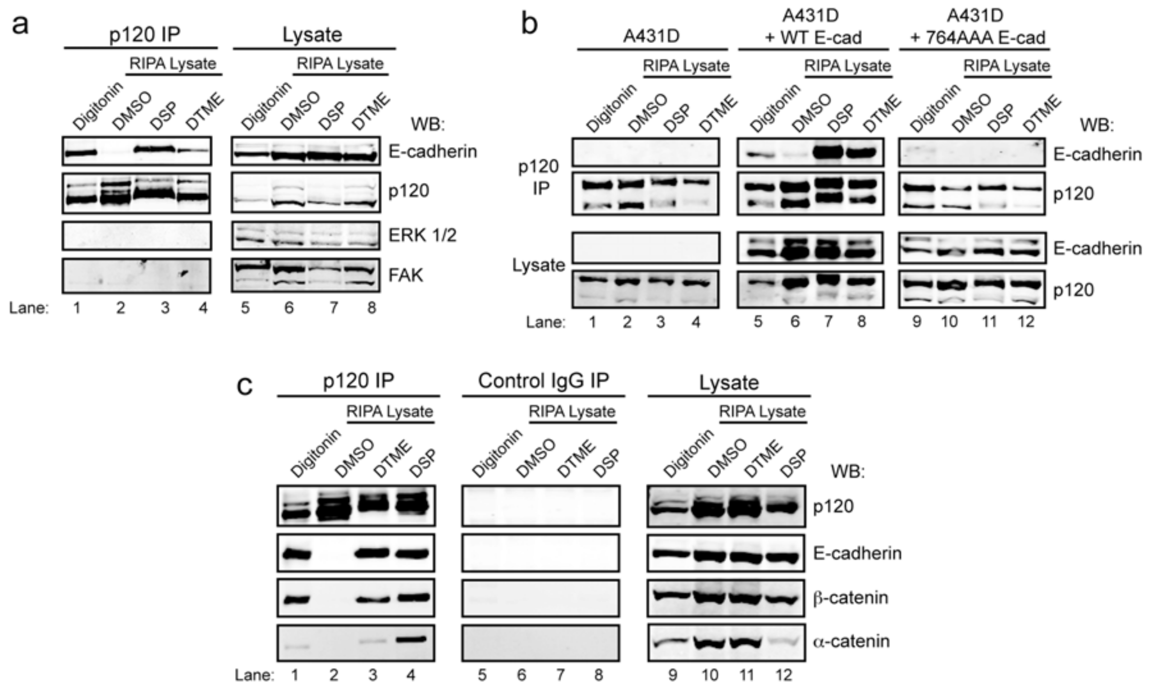

Figure 10: In-cell cross-linking preserves the interaction of p120 and E-cadherin and is specific for interacting proteins. (a) Western blot analysis of p120, E-cadherin, FAK, and p42/44 MAPK in p120 immunoprecipitates and lysates from A431 cells lysed in 1% digitonin or RIPA buffer following treatment with DMSO vehicle or 0.5 mM cross-linker as indicated. **(b)** Cadherin-negative A431-D cells, and A431-D cells stably expressing wild type (WT E-cad) or p120-uncoupled (764 E-cad) E-cadherin were prepared and analyzed as in A. **(c)** Western blot analysis of p120, E-cadherin, β-catenin, and α-catenin in p120, control IgG immunoprecipitates, and lysates from A431 cells treated as in panel a.

To further assess specificity, I asked whether E-cadherin and p120 could be crosslinked under conditions where physical interaction is selectively uncoupled (Figure 2b). Our lab has previously described a minimal E-cadherin mutant (E-cad 764AAA) that is physically uncoupled from p120 but nonetheless forms cell-cell junctions and interacts normally with β-catenin (Thoreson et al., 2000). In Figure 10b, I introduced WT E-cadherins (lanes 5-8) or 764AAA E-cadherin (lanes 9-12) into the A431D cell line, a cadherin-negative A431 variant. The absence of E-cad 764AAA in p120 immunoprecipitations (lanes 9-12, top panel) shows clearly that this mutant is not crosslinked to p120, implying that direct physical interaction is indeed essential. In contrast, WT E-cadherin is efficiently crosslinked (lanes 5-8, top panel).

To further test the efficacy of crosslinking, I extended the analysis to α- and β-catenins, which form a tertiary (indirect) complex with p120 via E-cadherin (Figure 10c). Interestingly, the entire complex is efficiently crosslinked by DSP (lane 4). α-catenin, in particular, was easily recovered relative to the DTME or digitonin methods. The middle panels (lanes 5-8) show that negative-control immunoprecipitation with a p120 monoclonal antibody that does not recognize human p120 (control IgG, mAb 8D11) under conditions identical to the first panel (lanes 1-4) does not bring down members of the cadherin complex.

Reversible Cross-Linking Immuno-Precipitation (ReCLIP) for Mass Spectrometry

Figure 11 illustrates the procedure I have developed for rapid and clean isolation of binding partners for MS analysis. The schematic (panel a) shows immunoprecipitation of a crosslinked p120 complex followed by selective elution of the

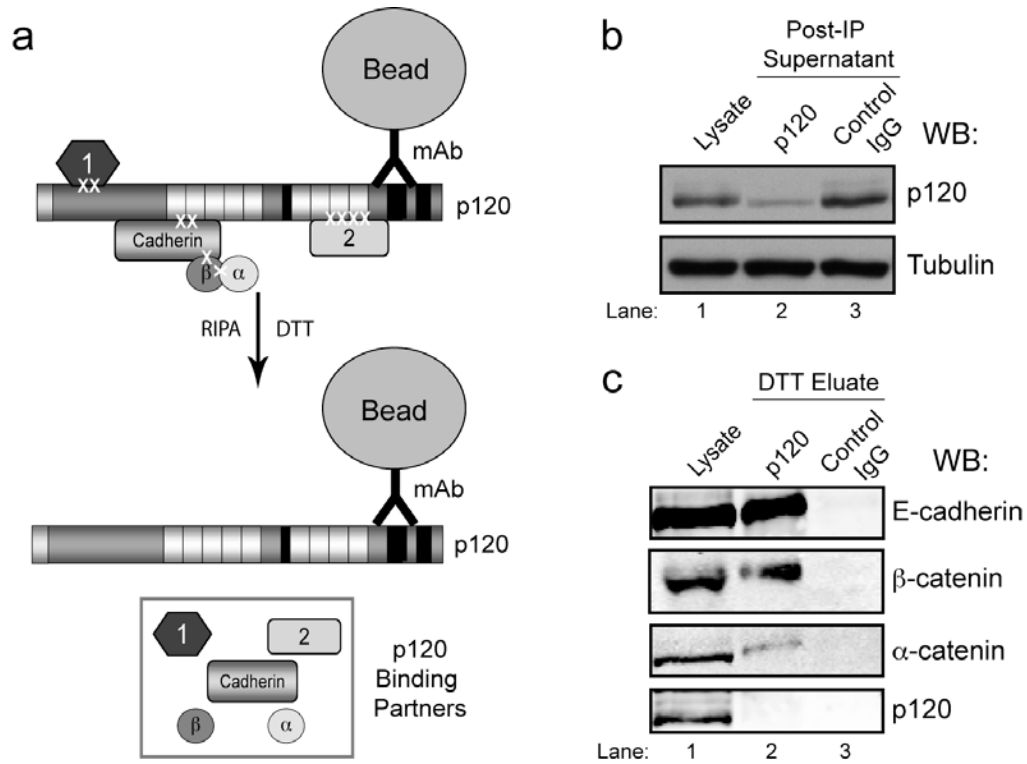

Figure 11: Elution of binding partners from p120. (a) A schematic of the elution strategy. Following immunoprecipitation and washing of cross-linked complexes on p120 mAb beads, binding partners are released by incubation with DTT in RIPA buffer, cleaving the cross-links and releasing interacting proteins from p120. **(b)** A representative western blot demonstrating depletion of p120 from A431 cell lysates following immunoprecipitation with p120 mAb beads of control IgG beads. Tubulin is shown as a loading control. **(c)** Elution of known binding partners, but not p120, from p120 mAb beads. Whole cell lysate is shown as a control, and 10% of the DTT eluate was analyzed for E-cadherin, β-catenin, α-catenin, and p120 by Western blot.

individual components. Binding partners are efficiently recovered by breaking the crosslinks with reducing agent, essentially reversing the procedure. With the antibody covalently bound to the bead (see Bead preparation in chapter two), DTT releases crosslinked binding partners only. The most abundant protein 'contaminants', mAb 15D2 and mAb-bound p120 itself (the bait) are discarded along with the beads, resulting in a highly purified mixture of eluted binding partners. Panel b illustrates the efficiency of the immunoprecipitation, as evidenced by depletion of p120 from the supernatant (panel b, compare lanes 1 and 2). Panel c shows that the coimmunoprecipitated E-cadherin is efficiently recovered by DTT elution (panel c, top panel, compare lanes 1 and 2) while p120 is essentially absent, having been discarded with the beads (panel c, bottom panel, lane 2). Furthermore, immunoprecipitation using control IgG (mAb 8D11) does not deplete p120 from the lysate (panel b, lane 3) and E-cadherin and associated catenins are not detected in the DTT eluate (panel c, lane 3).

Efficacy of ReCLIP

To test the efficacy of ReCLIP, p120 and control elutions from A431 cells crosslinked with DSP were subjected to shotgun analysis by single-dimension liquid chromatography tandem mass spectrometry (LC-MS/MS). Relative protein abundance was measured using the total number of peptides detected for each protein (spectral counts). Core p120 binding partners were easily identified, as evidenced by high spectral counts for E-cadherin and the catenins (Table 2). Note, however, that spectral counts are only partly indicative of protein abundance. For example, E-cadherin is consistently under-represented relative to its size, which is similar to the catenins. Importantly,

cadherin proteins are not detected control pull downs (i.e. zero peptides), as shown in Table 2. Nonspecific background (i.e., proteins detected in both experimental and control samples) was remarkably low, consisting primarily of common artifacts such as chaperones, metabolic proteins, and highly abundant cytoskeletal proteins, as illustrated in Table 3.

Table 2: Recovery and identification of core p120 binding partners using ReCLIP. Average spectral totals for E-cadherin, α-catenin, β-catenin, and Plakoglobin from 3 independent ReCLIP experiments from A431 cells treated with DSP. No peptides for these proteins were identified in the corresponding control samples.

Protein	*UniProt Accession*	*Average spectral count in p120 IP*	*Standard Error*	*Average spectral count in control IP*
E-cadherin	IPI00000513.1	9	2.11	0
α-catenin	IPI00215948.4	38	9.17	0
β-catenin	IPI00017292.1	24	6.07	0
Plakoglobin	IPI00554711.2	12	5.08	0

Effects of simultaneous DSP and DTME crosslinking

Next, I asked whether use of DSP and DTME together is more efficient than either one alone. Figure 12a shows the number of distinct peptides (per protein) of cadherin complex proteins detected using individual or combined crosslinkers. For E-cadherin and α-catenin, combining DSP and DTME was clearly more efficient than individual usage, whereas no little or no improvement was observed for β-catenin and Plakoglobin. The same result is illustrated by Western blotting (Figure 12b) using E-cadherin as the readout. In the experiment shown, the DSP + DTME combination was highly effective (compare lanes 7 and 8), whereas each compound by itself was less efficient (compare lane 1 and 2, and lane 4 and 5).

The efficacy of ReCLIP under three crosslinking conditions was further evaluated by SDS-PAGE and silver staining (figure 12c). The data indicate an excellent signal to

Table 3: Common background proteins detected by ReCLIP in A431 cells. Proteins detected in both p120 and control eluates are listed. The highest single spectral count detected for each in p120 and control eluates across all experiments in figures 11 and 12 are shown.

Protein	***UniProt Accession***	***p120***	***Control***
Alpha-actinin-4	IPI00013808.1	8	78
D-3-phosphoglycerate dehydrogenase	IPI00219018.7	3	6
Elongation Factor 1 alpha 2	IPI00014424.1	6	4
Elongation Factor 2	IPI00186290.6	6	3
TUBA1C	IPI00166768.2	4	6
heat shock protein 90kDa alpha (cytosolic), class A member	IPI00382470.3	6	5
14-3-3 protein epsilon	IPI00000816.1	9	3
61 kDa protein	IPI00472102.3	3	5
Fatty acid synthase	IPI00026781.2	11	9
Isoform 1 of L-lactate dehydrogenase A chain	IPI00217966.7	2	3
Isoform 1 of Protein-L-isoaspartate(D-aspartate) O-methyltransferase	IPI00411680.8	7	2
Protein disulfide-isomerase A3	IPI00025252.1	3	6
Isoform M1 of Pyruvate kinase isozymes M1/M2	IPI00220644.8	8	9
Isoform alpha-enolase of Alpha-enolase	IPI00465248.5	4	2
Peroxiredoxin-1	IPI00000874.1	6	8
Phosphoglycerate kinase 1	IPI00169383.3	3	5
Src substrate cortactin	IPI00029601.4	2	15
Heat shock 70 kDa protein 1L	IPI00301277.1	3	3
Isoform 1 of LIM and SH3 domain protein 1	IPI00000861.1	2	5
Isoform Short of RNA-binding protein FUS	IPI00221354.1	2	6
Transketolase	IPI00643920.2	6	8

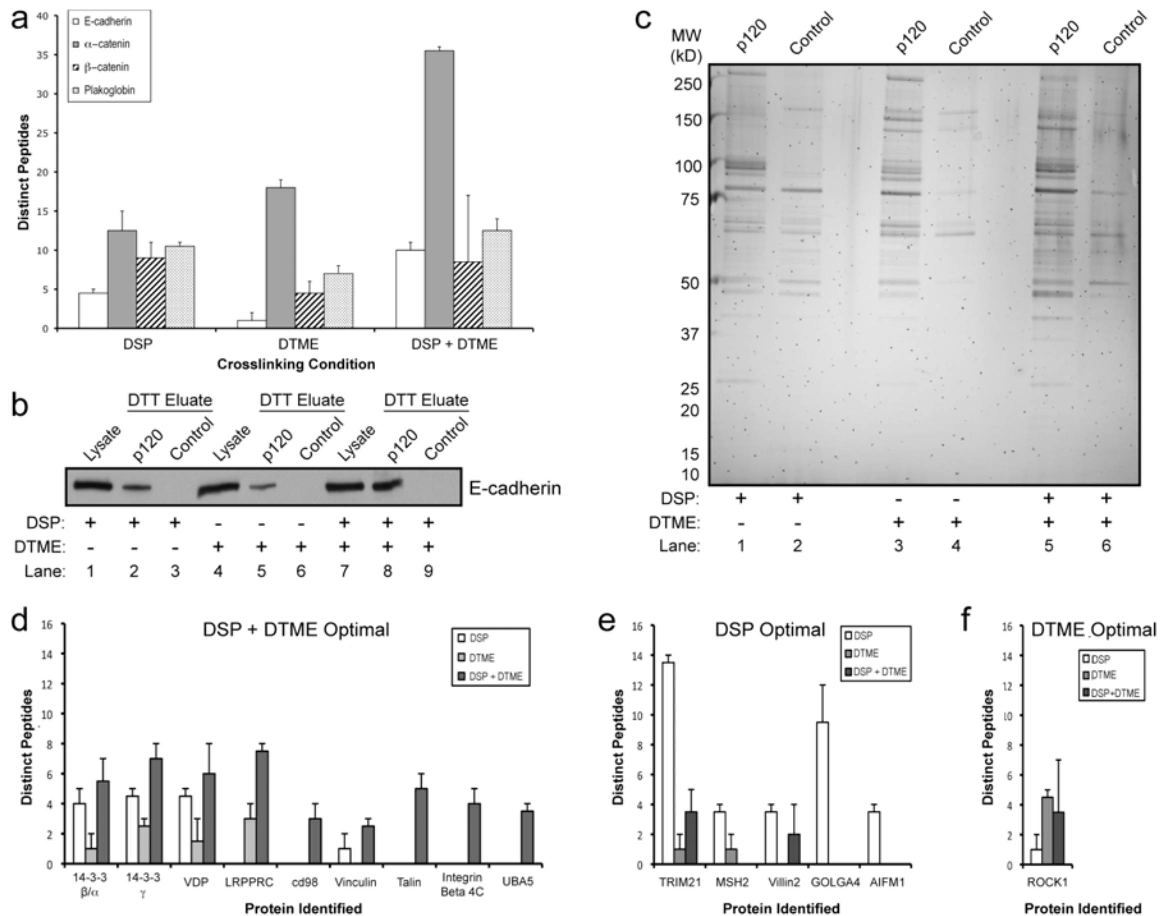

Figure 12: Cross-linkers can be combined to enhance complex recovery. (a) Average number of distinct peptides identified in 2 LC-MS/MS runs for E-cadherin, α-catenin, β-catenin, and plakoglobin from A431 cells treated with DSP, DTME, or both compounds simultaneously (DSP + DTME). Error bars represent standard error of the mean. Background levels were similar across all conditions. **(b)** Western blot analysis of E-cadherin levels in lysates (Lysate), p120 eluates (p120), and control IgG eluates (Control) from A431 cells treated with the indicated cross-linkers. **(c)** Silver stain analysis of total protein recovery from p120 and control IgG eluates from each condition (DSP, DTME, or DSP + DTME). **(d-f)** Average distinct peptide recovery of 15 additional putative p120 binding partners under each cross-linking condition. Proteins were grouped based on whether more peptides were detected using the combination of DSP and DTME (d), DSP alone (e) or DTME alone (f).

noise ratio across all three conditions, with very few bands detectable in control IgG lanes (lanes 2, 4, and 6). For each condition, some of the bands were unique, as expected. Notably, combining DSP and DTME captured most of the individual bands observed with either crosslinker alone (compare lane 5 with lanes 1 and 3) while background remained remarkably low (compare lanes 5 and 6). These data indicate that regardless of the crosslinker used, ReCLIP provides robust recovery with very low background.

In addition to the core components of the cadherin complex, I identified at least 15 unique candidate p120 binding partners in MS analysis, and grouped them according to the condition that resulted in the highest number of peptide hits (Figure 12 d-f). For example, figure 12d contains the candidates for which DSP and DTME together yielded more hits than DSP (e) or DTME (f) alone. The cutoff for inclusion was a minimum of two hits against a background of zero, although the majority exceeded these criteria. As expected, the highest number of peptide hits for most of the candidates was obtained when DSP and DTME were combined (panel d). However, for five of the candidates, the highest number of hits was obtained using DSP alone (e), whereas DTME was optimal for only one protein (f). Interestingly, five of the candidates were captured only when DSP and DTME were used together. On the other hand, combining DSP and DTME prevented capture of three candidates (MSH2, GOLGA4, and AIFM1). In general, the use of both DSP and DTME together was most effective in that the majority of candidates (12/15) were detected and only three were missed. With DSP or DTME alone, just over half of the candidates (8/15) were missed. Overall, these data suggest that the most effective approach is to combine DSP and DTME, but this approach may not be

ideal for all proteins. Thus, it is recommended that investigators test each crosslinker individually and in combination in order to determine the appropriate ReCLIP condition for a given target protein.

p120 interacts with cd98 indirectly through E-cadherin

Mass spectrometry analysis identified cd98 in p120 eluates, consistent with an earlier report that cd98 could be recruited to cell-cell junctions by E-cadherin (Nakamura *et al.* 1999). To study the relationship between p120, cd98, and E-cadherin, cd98 localization was analyzed by immunofluorescence in A431 and cadherin-negative A431-D cells expressing wild type or p120-uncoupled E-cadherin. In A431 cells, cd98 localizes prominently at cell-cell junctions along with p120 (figure 13a). In the absence of E-cadherin, cd98 is diffusely localized throughout the cell, with no detectable co-localization with p120 (figure 13b, top row). In the presence of wild-type E-cadherin, both cd98 and p120 are efficiently recruited to cell-cell junctions (b, middle row, arrows). Expression of the p120-uncoupled 764AAA E-cadherin also recruits cd98 to cell-cell junctions (c, lower row, arrows), while p120 remains diffusely localized in the cytoplasm. This result indicates that p120 is not necessary to recruit cd98 to the cadherin complex. These data suggests that cd98 binds to E-cadherin (or another component of the complex), but not p120.

Discussion

Here, I have used reversible in-cell crosslinking to develop an extremely efficient method (ReCLIP) for studying protein complexes by mass spectrometry. The component

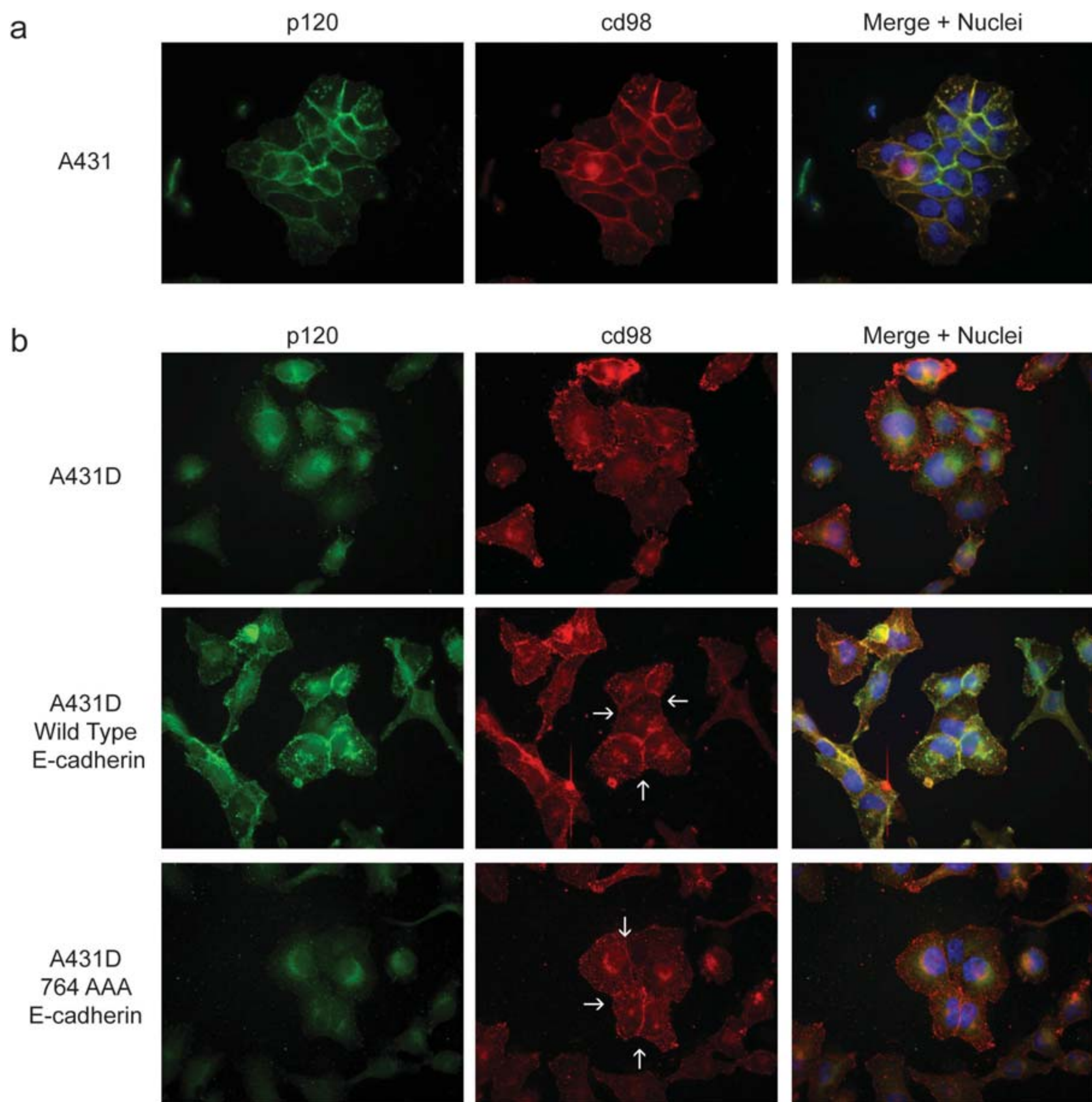

Figure 13: Indirect association of p120 and cd98. (a) Immunofluorescence analysis of endogenous p120 (green) and cd98 (red) in paraformaldehyde fixed A431 cells. Co-localization and nuclei (blue) is shown in the merged images. **(b)** Immunofluorescence analysis of endogenous p120 (green) and cd98 (red) in paraformaldehyde fixed parental A431D cells and A431D cells expressing wild type (Wild Type E-cadherin) or p120-uncoupled (764 AAA E-cadherin) E-cadherin. Arrows indicate junction-localized cd98.

techniques by themselves are not necessarily novel, but they are uniquely combined and optimized to generate a powerful method for studying labile complexes. The single immunoprecipitation approach minimizes sample loss, a common problem in TAP methods. Furthermore, covalent crosslinking preserves relevant interactions despite stringent lysis and washing conditions that reduce background. Thus ReCLIP appears to be particularly powerful for studying labile protein interactions that in principle could be lost using TAP approaches.

Among the several optimized parameters of the ReCLIP method, two in particular turn out to be critical. First, in-cell crosslinking covalently stabilizes endogenous interactions (as they occur *in vivo*). Thus, weak or transient interactions are captured in situ and retained, regardless of subsequent lysis and washing conditions, until the very end of the procedure when the product is eluted. Second, the elution method itself is both gentle and highly selective. A major difference between ReCLIP and other methods is that only putative binding partners are eluted when the crosslinks are cleaved (see Figure 11a). Thus, beads, antibody, and other components of the solid phase, including the bait itself (in this case, p120) are completely absent from final sample. The removal of bait and immunoprecipitating antibodies from the sample is important because these are by far the most abundant protein contaminants present in most methods.

A potential consideration when using ReCLIP in conjunction with MS is that some of the recovered peptides are covalently bound by a cleaved crosslinker. After cleavage by reducing agent, half of each crosslinker remains attached to a target residue in the crosslinked protein. In addition, bound crosslinker may alter proteolytic cleavage patterns, as has been demonstrated for other lysine modifications (Cameron et al., 1985).

In addition The cleaved crosslinker alters peptide mass and can prevent recognition by standard MS algorithms (Baldwin, 2004). Both events (mass-shift and reduced cleavage) can reduce the number of peptides generated and/or detected. Such complications are not likely to affect the data significantly because crosslinked peptides represent only a small fraction of the total number generated following digestion of the sample with trypsin. The effect is further limited by using the minimal effective concentration of the crosslinker, as determined by preliminary titration experiments. It is also possible to identify modified peptides by re-analyzing the spectra using a subset database that allows for the extra mass (105.16 Da per Lysine for DSP and 159.21 Da per Cysteine for DTME) produced by the cleaved crosslinker (Nesvizhskii et al., 2006).

One potential drawback to ReCLIP is that very low molecular weight proteins might be missed because there are fewer available sites for crosslinking, and fewer tryptic peptides to detect. For example, if a protein is crosslinked and contains only two tryptic peptides, one will be missed due to the crosslink modification. Such proteins would be overlooked because the score (one peptide against zero background) is below the cutoff for positive identification. Thus, small proteins (e.g. small GTPases such as RhoA) may be overlooked, because few unmodified peptides are available. Thus, it is important to consider protein size and peptide coverage when assessing proteins with relatively low peptide scores (e.g. two peptides against zero background).

ReCLIP has been optimized to study endogenous complexes using a monoclonal antibody. By design, this allows physiologically relevant complexes to be recovered with a relatively high degree of specificity. However, ReCLIP can still be used in conjunction with epitope-tags (e.g., Flag, Myc, HA epitope tags) in cases where specific antibodies

are not available. Protein over-expression, however, may increase nonspecific interactions. For example, we have noticed that components of the proteosome are selectively identified under such conditions. Presumably, the cell is targeting the excess protein for degradation and we are then crosslinking it to components of the proteosome. It is possible that we missed certain previously identified p120 binding partners (e.g. Kinesin Heavy Chain (Chen et al., 2003), (Yanagisawa et al., 2004)) for this reason, as the interaction between p120 and Kinesin Heavy Chain is more efficiently detected under conditions of p120 over-expression. Alternatively, the interaction may be different or absent in A431 cells.

Surprisingly, Kaiso was not identified as a p120 binding partner by ReCLIP. However, Kaiso is a relatively low abundance transcriptional repressor found primarily in the nucleus in cultured cells (Daniel and Reynolds, 1999). Thus, it is possible that spatial separation, low Kaiso expression, low interaction stoichiometry, or any combination thereof ultimately limits the sensitivity of ReCLIP. Of note, p120 and Kaiso can be detected by conventional co-immunoprecipitation in gentle detergent buffers (Daniel and Reynolds, 1999), suggesting that low abundance of Kaiso is not by itself the limiting factor. Instead, cell lysis without prior crosslinking may actually facilitate such interactions by permitting the mixing of proteins from otherwise spatially separate pools (e.g. nuclear Kaiso and cytoplasmic p120). With ReCLIP, protein complexes are crosslinked in situ and then lysed in RIPA, a stringent buffer designed expressly to be compatible with antibody-antigen interactions while preventing nonspecific and/or weak interactions. Thus, some events that occur post-lysis (e.g. the p120-Kaiso interaction) will undoubtedly be prevented by the ReCLIP lysis and washing conditions. On the other

hand, this feature of ReCLIP may allow one to selectively capture physiological complexes under a defined condition and time interval, potentially identifying interactions that occur transiently in response to a stimulus.

In addition to the cadherin complex, I identified several candidate p120 binding partners (Figure 4 c-e) including p160 Rho Kinase (ROCK1). ROCK1 is a prominent effector of RhoA that regulates the acto-myosin machinery and other signaling pathways (Riento and Ridley, 2003). This novel interaction, which will be described in chapter five, is consistent with other known roles of p120. For example, p120 regulates the activity of RhoA (Anastasiadis et al., 2000) and can associate with p190 RhoGAP at the adherens junction (Wildenberg et al., 2006). ROCK1 has not been linked to p120 by other methods (e.g. conventional immunoprecipitation and TAP-Tag), consistent with the apparent increased sensitivity of ReCLIP. Interestingly, no Rho-family GTPases were detected using ReCLIP, including RhoA, which has been reported to directly interact with p120 (Magie et al., 2002), (Castaño et al., 2007). A potential explanation for this result is the inherent bias of mass-spectrometry against small proteins. Nonetheless, the recovery of ROCK1 along with its substrate Villin-2/Ezrin suggests that a functional Rho complex associates with p120.

Another candidate binding partner, cd98 (also known as 4F2 Heavy Chain), appears to reflect capture of a tertiary interaction. In general, tertiary (as apposed to direct) interactions are considerably more difficult to capture by conventional methods, but in principle could be significantly stabilized by limited crosslinking. cd98 is an integral membrane protein that forms a heterodimer with the LAT-2 amino-acid transporter (also known as 4F2 Light Chain) (Nakamura et al., 1999). cd98 also regulates

β1-integrin clustering (Henderson et al., 2004), (Cai, 2005), (Kim and Hahn, 2008) and heterotypic cell-cell interactions (Nguyen et al., 2008). Interestingly, a previous study suggested the recruitment of cd98 to cadherin-based cell-cell junctions (Nakamura et al., 1999). Consistent with this report, I find that cd98 co-localizes precisely with E-cadherin and p120 in A431 cells (see Figure 13a). However, in E-cadherin reconstitution experiments in A431D cells, cd98 is also recruited to both wild type and p120 uncoupled E-cadherin complexes, indicating that the direct interaction is not with p120 itself, but instead to some other member of the E-cadherin complex (see Figure 13b). As with ROCK1, I have not detected cd98 by other methods. Importantly, the indirect association of p120 with cd98 provides additional evidence that ReCLIP can routinely capture tertiary interactions that would otherwise be lost, making it attractive for interactome mapping studies.

In summary, I have developed ReCLIP (Reversible Cross-Link Immuno-Precipitation), an approach designed expressly to retain weak interactions without sacrificing specificity and/or sensitivity. The procedure is relatively simple and yet generates excellent signal-to-noise ratios in MS analyses. Although I have focused on the cadherin complex as a model system, the method should be broadly applicable, provided users optimize crosslinkers and immunoprecipitation conditions for their own targets. Overall, ReCLIP offers a potentially powerful alternative to previously described affinity-purification approaches and appears to be particularly suitable for interrogating labile protein complexes.

CHAPTER IV

ROCK1 PHYSICALLY ASSOCIATES WITH THE CADHERIN COMPLEX IN A P120-DEPENDENT MANNER

Introduction

Establishment and maintenance of cell-cell adhesion requires the coordination of a wide array of signaling events. Modulation of the actin cytoskeleton is particularly important to maintain strong cell-cell adhesion. As discussed in Chapter one, the cadherin complex is functionally linked to the actin-cytoskeleton via α-catenin, and disruption of the actin cytoskeleton dramatically reduces cell-cell adhesion (Fischer and Quinlan, 1998). Coordination of the actin-cytoskeleton and cell-cell adhesion is important for the integrity of epithelial sheets with a network of apical stress fibers connected across multiple adjacent cells via adherens junctions (Vaezi et al., 2002). The precise physical interactions between cadherins and the acto-myosin network remain unclear. Although α-catenin physically interacts with both actin and the cadherin complex, recent work has suggested that α-catenin cannot interact with both actin and cadherins simultaneously (Yamada et al., 2005).

While the physical links between the acto-myosin network and the cadherin complex remain poorly understood, the functional links between them are more apparent. One of the major signaling pathways involved is the RhoA pathway. Early studies using dominant-active and dominant-negative RhoA mutants demonstrated that both excess activation and inhibition of Rho signaling can ablate cell-cell adhesion, presumably by

disrupting normal cytoskeletal organization (Braga et al., 1997), and junction maturation and cell-type can influence the precise effects of these GTPases (Braga et al., 1999). Furthermore, cadherins themselves can regulate the activity of Rho GTPases (Betson et al., 2002; Fukuyama et al., 2006).

Rho kinase, a major effector of RhoA, can regulate cadherin function in both a positive and negative manner. For example, ROCK promotes vascular permeability by disrupting VE-cadherin based junctions (Wójciak-Stothard et al., 2001). In epithelial cells, ROCK activity disrupts adherens junctions downstream of RhoA, while another RhoA effector, mDia, promotes adherens junction assembly (Sahai and Marshall, 2002). Furthermore, ROCK activity is required for the loss of E-cadherin during TFG-β induced Epithelial-to-Mesenchymal Transition (Bhowmick et al., 2001). In contrast, inhibition of ROCK leads to a failure of epithelial polarization and blocks the formation of new tight junctions and adherens junctions in a calcium switch assay (Walsh et al., 2001). Recently, studies in human embryonic stem cells have demonstrated that ROCK activity is required for tight cell-cell adhesion (Harb et al., 2008). Similarly, suppression of RhoA and ROCK can reduce cellular aggregation and N-cadherin expression in differentiating neurons (Laplante et al., 2004). Furthermore, recent work has demonstrated that Myosin II, a direct target of ROCK, is essential for cell-cell adhesion and cadherin clustering (Li et al., 2010; Shewan et al., 2005; Smutny et al., 2011).

As discussed above, several lines of evidence point to a functional relationship between cadherins in ROCK signaling. Given its role in regulating both Rho GTPases and the cadherin complex, p120 is an attractive candidate to mediate this functional interaction. In chapter three, I identified p160 Rho Kinase (ROCK1) as a candidate p120

binding partner using ReCLIP. This result suggests that ROCK1 may physically associate with p120 and the cadherin complex in order to regulate cell-cell adhesion.

Results

Identification of p160 Rho Kinase as a p120 binding partner

In p120 ReCLIP samples from A431 cells, 15 distinct peptides of ROCK1 were recovered, covering 12.8% of the total amino-acid sequence. No ROCK1 peptides were detected in the control pulldowns with an irrelevant IgG. Sequence alignment analysis revealed that all but two peptides (mapped to the highly conserved kinase domain) were specific to ROCK1 rather than ROCK2 (Table 4). As Figure 14a illustrates, Peptides from multiple distinct regions of ROCK1 were detected, with the majority (11/15) representing the predicted coiled-coil domain. Additionally, ROCK1 was detected in immunoprecipitations from Caco-2 colorectal adenocarcinoma, MCF-7 mammary adenocarcinoma, and MCF-10A mammary epithelial cells (Table 5, Appendix A), suggesting this interaction is relevant in other epithelial cell types.

ROCK1 physically associates with p120 at cell-cell junctions

The identification of ROCK1 as a p120 binding partner was confirmed in A431 cells by crosslink immunoprecipitation experiments. As figure 14b illustrates, ROCK1 can be co-immunoprecipitated with p120 from DSP-crosslinked A431 cells using 2 separate p120 antibodies (pp120 and 15D2, lanes 2 and 3), but not with an antibody that does not recognize human p120 (IgG, lane 4)). This interaction appears to have relatively

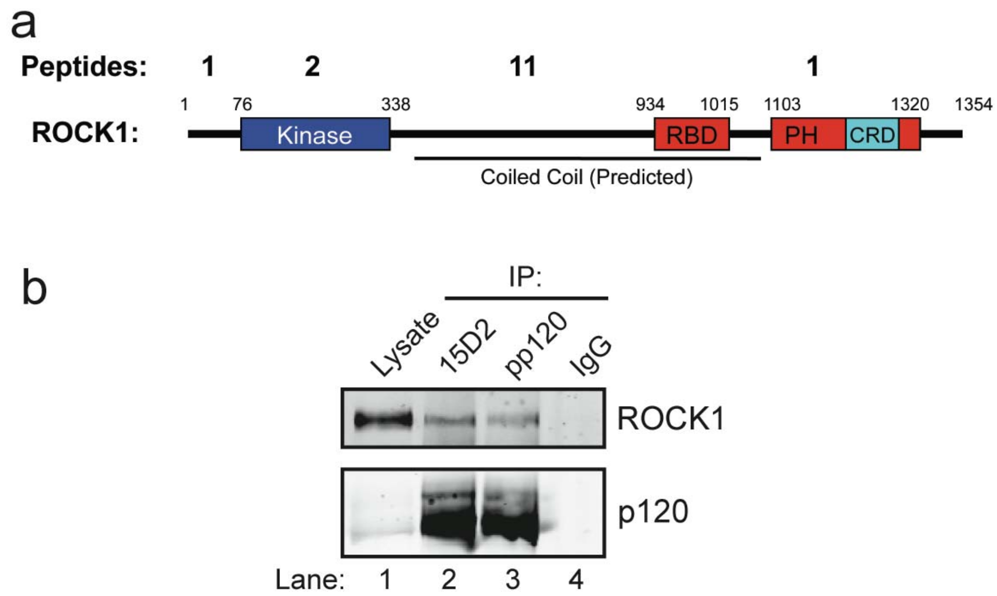

Figure 14: Identification of p160 Rho Kinase (ROCK1) as a p120 binding partner. **(a)** A schematic of ROCK1, with the distribution of unique peptides for each region of the protein. **(b)** Western blot analysis of ROCK1 and p120 in a whole cell lysate, p120, and control immunoprecipitates from DSP-crosslinked A431 cells. ROCK1 is pulled down with 2 separate p120 monoclonal antibodies (15D2 and pp120) but not a control IgG.

Table 4: ROCK1 peptides detected in p120 ReCLIP samples. The identified sequence and specific location ROCK1 peptides identified by mass spectrometry in p120 ReCLIP samples. Identified sequences were compared to the full sequence of ROCK1 and ROCK2.

Peptide	*Location*	*ROCK Domain*	*ROCK1*	*ROCK2*	*Detection in control*
AESEQLAR	899 - 906	Coiled-coil	+	-	-
GAFGEVQLVR	85 - 94	Kinase	+	+	-
IEGWLSVPNR	1121 -30	PH	+	-	-
LLEFELAQLTK	820 - 830	Coiled-coil	+	-	-
NIDNFLSR	51 - 58	N-terminus	+	-	-
SLQESLQK	423 - 430	Coiled-coil	+	-	-
YLSSANPNDNR	405 – 415	Coiled-coil	+	-	-
INEYQR	495 - 500	Coiled-coil	+	-	-
ITSLQEEVK	631 - 639	Coiled-coil	+	-	-
LLLQNELK	784 - 791	Coiled-coil	+	-	-
GLLEEQYFELTQESK	907 - 921	Coiled-coil	+	-	-
NLESTVSQIEKEK	476 - 488	Coiled-coil	+	-	-
NVENEVSTLKDQLEDLKK	511 - 528	Coiled-coil	+	-	-
SDSAFFWEER	116 – 125	Kinase	+	+	-
YLSSANPNDNR	405 – 415	Coiled-coil	+	-	-

low stoichiometry, with only a low level of ROCK1 co-immunoprecipitating with a relatively large amount of p120 (lane 2 and 3). Nonetheless, coupled with the mass spectrometry-based identification of ROCK1, these data suggest that ROCK1 physically associates with p120.

The localization of endogenous ROCK1 was analyzed by immunofluorescence microscopy in A431 and MCF-7 epithelial cells (Figure 15a, upper row). Cells were plated on glass coverslips and stained with an anti-ROCK1 polyclonal antibody and anti-p120 monoclonal antibody. In A431 cells ROCK1 co-localized precisely with p120 at cell-cell junctions and in cytoplasmic vesicle structures (arrows). In MCF-7 mammary

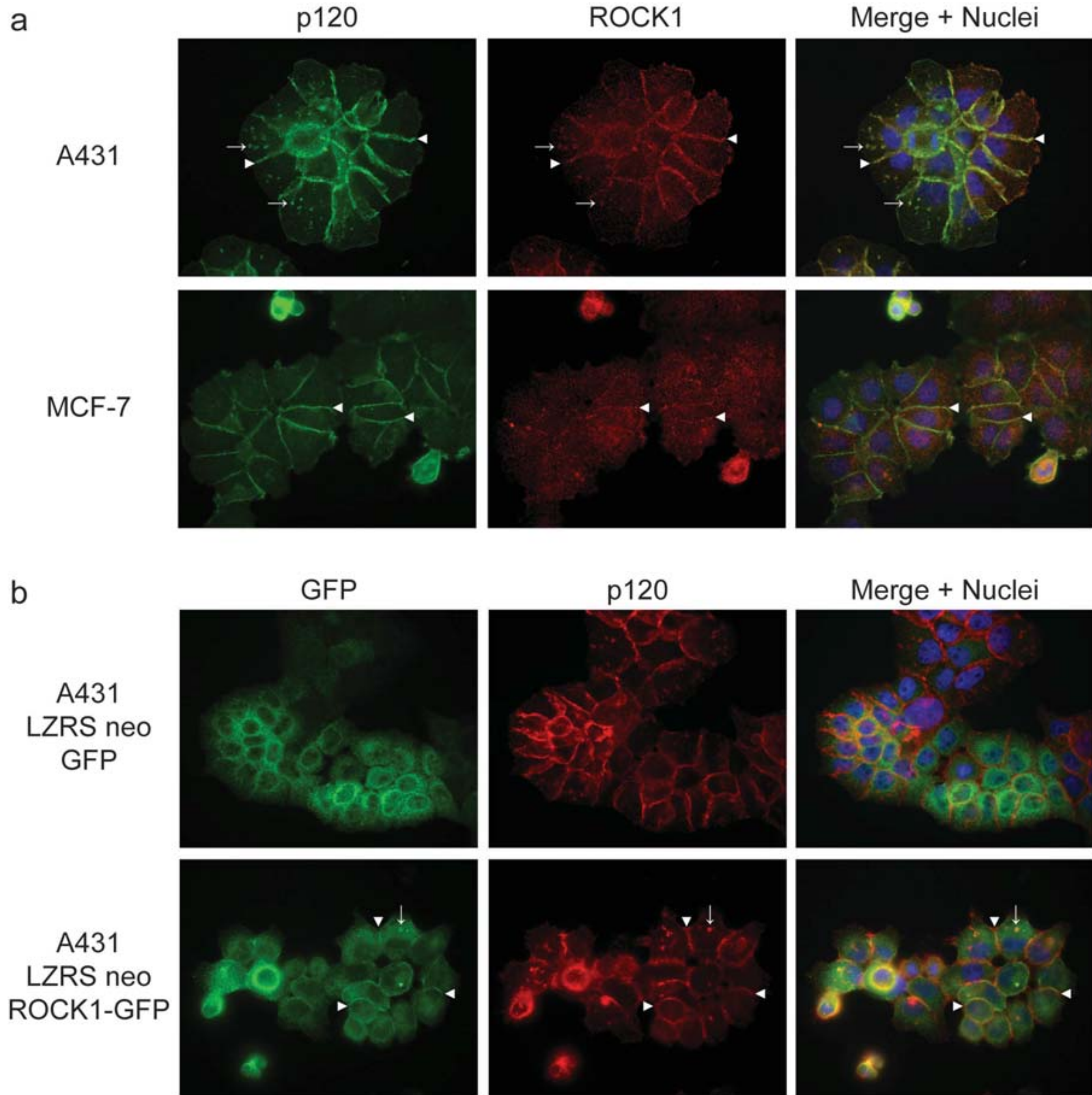

Figure 15: ROCK1 co-localizes with p120 at cell-cell junctions. (a) Immunofluorescence analysis of endogenous p120 and ROCK1 in A431 (upper panels) and MCF7 cells (lower panels). Arrowheads indicate co-localization of ROCK1 (red) with p120 (green) at cell-cell junctions. Arrows indicate co-localization of ROCK1 and p120 in cytoplasmic vesicles. **(b)** Immunofluorescence analysis of GFP alone or ROCK1-GFP (green) and p120 (red) in A431 cells. Arrowheads indicate co-localization of GFP and p120 at cell-cell junctions.

adenocarcinoma cells (Figure 15a, lower row), ROCK1 also co-localized with p120 at cell-cell junctions. Interestingly, ROCK1 is primarily diffuse with limited cell-cell junction staining (arrow heads) in MCF-7 cells, making the association difficult to detect. Nonetheless, using ReCLIP and mass spectrometry I was able to detect an interaction between p120 and ROCK1 in MCF-7 cells (as reported in Appendix A). Thus, although the p120-ROCK1 association in MCF-7 cells is difficult to discern by traditional approaches (i.e. immunoprecipitation and immunofluorescence), it is readily detectable using ReCLIP.

ROCK1 localization was further assessed using exogenous ROCK1 fused to GFP (ROCK1-GFP) (Figure 15b). A431 cells were transduced with GFP alone (LZRS neo GFP) or ROCK1-GFP (LZRS neo ROCK1-GFP) and analyzed by immunofluorescence for GFP and p120. ROCK1-GFP was primarily localized in the cytoplasm, however a distinct pool of ROCK1-GFP was localized to cell-cell junctions (arrow heads). In some cells, ROCK1-GFP co-localized with p120 in cytoplasmic vesicles (arrows), similar to endogenous ROCK1. In cells expressing GFP alone, GFP was localized diffusely throughout the cytoplasm and nucleus, but was not detectable cell-cell junctions. These data suggest that ROCK1 associates with p120 as part of the cadherin complex.

ROCK1 is recruited to the cadherin complex

A calcium switch assay was preformed to confirm that ROCK is associated with the cadherin complex. A431 cells were incubated in low calcium media (LCM) to deplete extracellular calcium levels and $CaCl_2$ was added directly to the media to restore

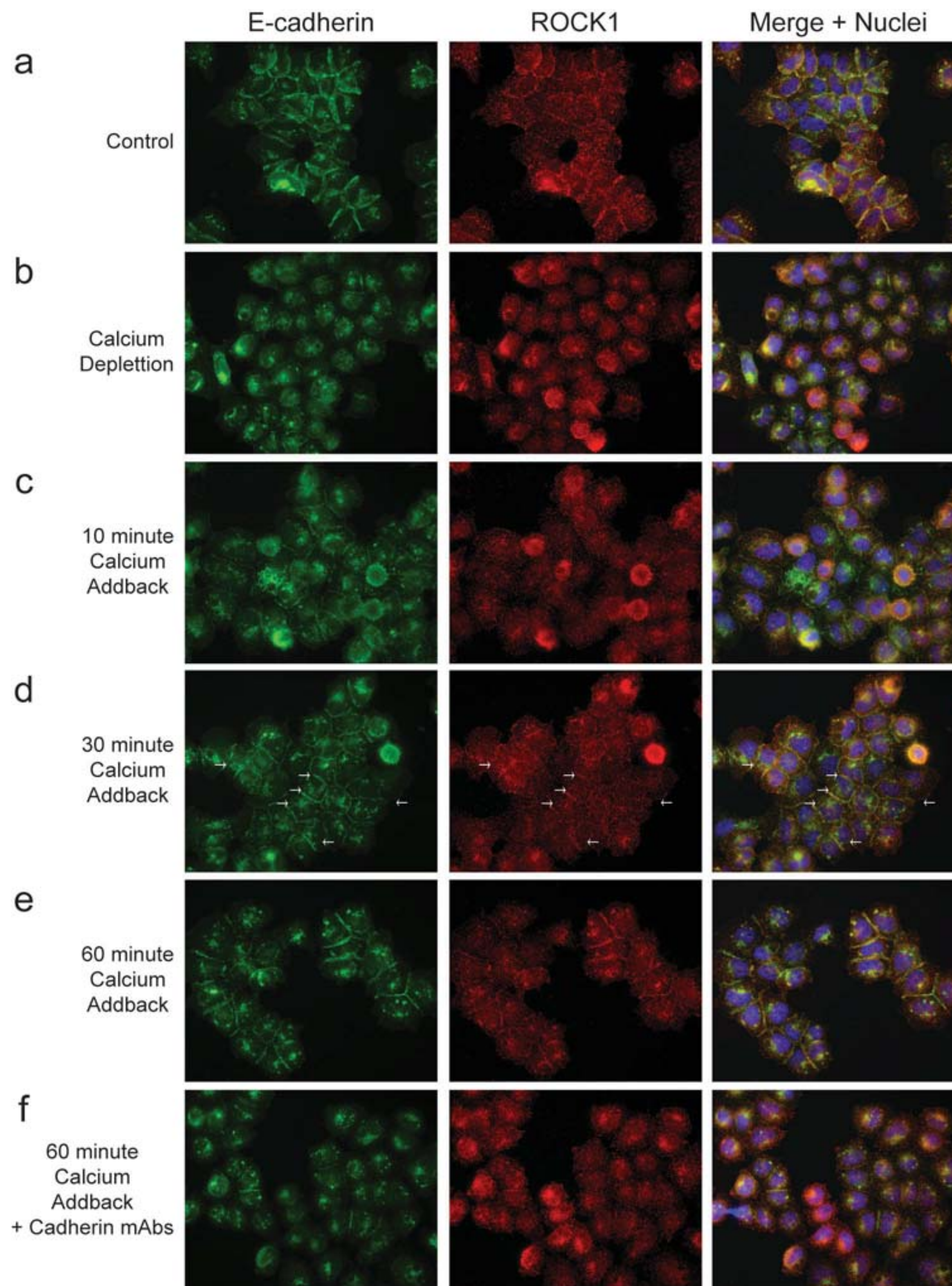

Figure 16: ROCK1 is recruited to cell-cell junctions by cadherins. Immunofluorescence analysis of E-cadherin (green) and ROCK1 (red) A431 cells during a calcium switch assay. Cells were fixed and stained for the following conditions: **(a)** no calcium depletion (control), **(b)** depletion of calcium using low calcium media, **(c)** 10 minutes post-calcium addback, **(d)** 30 minutes post calcium addback (white arrows indicate low levels of ROCK1 at cell junctions), **(e)** 60 minutes post-calcium addback, **(f)** 60 minutes post calcium addback with incubation with E-cadherin and P-cadherin blocking antibodies (HECD-1 and 6A9).

physiological calcium levels (1.8 mM $CaCl_2$) prior to immunofluorescence analysis for E-cadherin and ROCK1. As Figure 16 illustrates, depletion of extracellular calcium destabilized the adherens junction (b) and eliminated the pronounced cell-junction localization of ROCK1 observed under normal calcium levels (a). When cadherin contacts were initially established (i.e. the 10 minute time point, c), little to no ROCK1 was localized to the nascent adherens junction. By 30 minutes post-calcium addback, ROCK1 began to concentrate at the maturing junction (d, arrows). By 60 minutes post-calcium addback, ROCK1 was highly concentrated at cell junctions (e), similar to cells under normal calcium concentrations. ROCK1 recruitment correlated with the increased clustering of E-cadherin at cell-cell junctions (as assessed by E-cadherin staining). Thus it appears that ROCK1 recruitment does not occur during the initial stages of cell-cell adhesion, but rather during the clustering of cadherins following initial cell-cell contact as cadherins are engaged. Furthermore, inhibition of cadherin function using blocking antibodies against E-cadherin and P-cadherin prevented ROCK1 concentration at cell-cell contacts (f), confirming that this a cadherin-dependent event.

<u>ROCK1 physically interacts with E-cadherin in a p120-dependent manner</u>

To determine if ROCK1 is physically associated with the cadherin complex, crosslink-immunoprecipitation experiments were preformed in cadherin-negative A431D cells and A431D cells expressing either wild type or p120-uncoupled 764AAA E cadherin (Thoreson et al., 2000). Cells were pretreated with crosslinkers, lysed in RIPA buffer, and immunoprecipitations for p120 (Figure 17a lanes 2, 6, 10) , E-cadherin (lanes 3, 7, 11) an irrelevant control antibody (IgG lanes 4, 8, 12) were carried out. Recovery

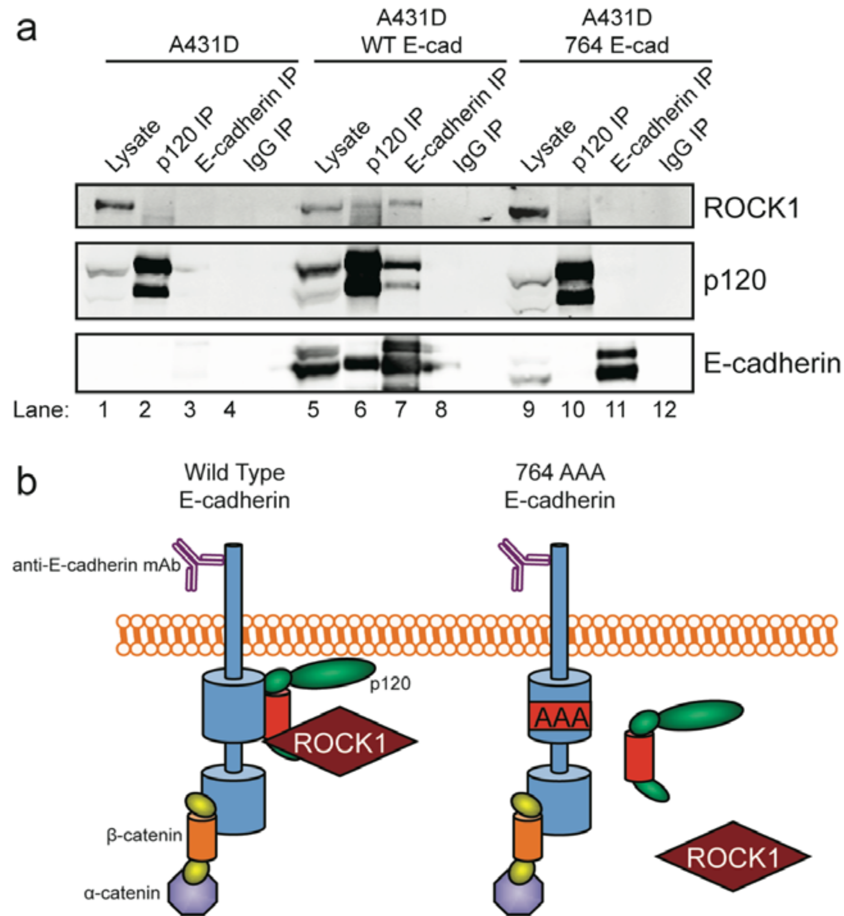

Figure 17: **ROCK1 physically associates with the cadherin complex in a p120-dependent manner. (a)** Western blot analysis of lysates and p120, E-cadherin, or control immunoprecipitations from DSP-crosslinked A431D cells and A431D cells expressing wild type or 764AAA E-cadherin. **(b)** A model depicting p120-dependent association of ROCK1 with the cadherin complex, as detected by immunoprecipitation in (a).

of ROCK1, p120, and E-cadherin was assessed by western blot. ROCK1 co-immunoprecipitated with wild-type E-cadherin (Figure 17a, top panel, lane 7), indicating that ROCK1 physically associates with the cadherin complex. However, ROCK1 did not co-immunoprecipitate with p120 uncoupled 764AAA E-cadherin (Figure 17a, top panel, lane 11), suggesting that p120 is required for ROCK1 to associate with the cadherin complex. Furthermore, ROCK1 was co-immunoprecipitated with p120 in the presence of wild-type E-cadherin, but not in the presence of 764AAA E-cadherin or in the total absence of cadherins (Figure 17a, top panel, compare lane 6 to lanes 2 and 10). This result is illustrated schematically in figure 17b.

Immunofluorescence analysis of E-cadherin and ROCK in the A431D cells demonstrated that ROCK1 localized efficiently to adherens junctions in the presence of wild-type cadherin (Figure 18a, arrows). However, In the presence of 764AAA E-cadherin, ROCK1 was significantly less abundant at cell junctions, relative to cells expressing wild-type E-cadherin (Figure 18a, b). These data suggest the presence of a complex consisting of E-cadherin, p120, and ROCK1 as part of the adherens junction. Importantly, ROCK1 is only physically associated with p120-bound E-cadherin.

p120 associates with ROCK1 at the plasma membrane

A p120 knockdown-reconstitution system (Davis et al., 2003) was used to assess the functional relationship between p120 and ROCK1 (Figure 19). Briefly, endogenous p120 was stably depleted using human-specific shRNA, and p120 was then reconstituted by expressing murine p120, which is unaffected by the shRNA. Endogenous ROCK1

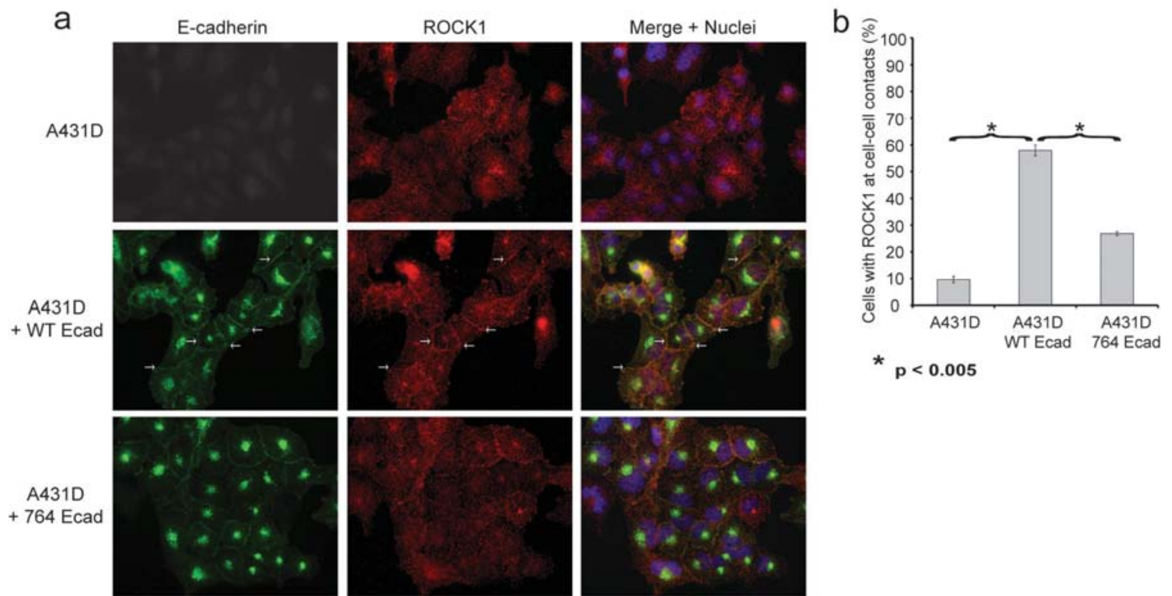

Figure 18: ROCK1 localization in E-cadherin-reconstituted A431D cells. (a) Immunofluorescence analysis of E-cadherin (green) and ROCK1 (red) in A431D cells expressing wild type or 764AAA E-cadherin. White arrows indicate cell-cell contact localized ROCK1. **(b)** Quantification of cell-cell junction localization of ROCK1 junction localization in A431D cells. Bars represent the average percentage of cells in 4 20x fields with ROCK1 localized to cell-cell contacts. Error bars represent standard error of the mean.

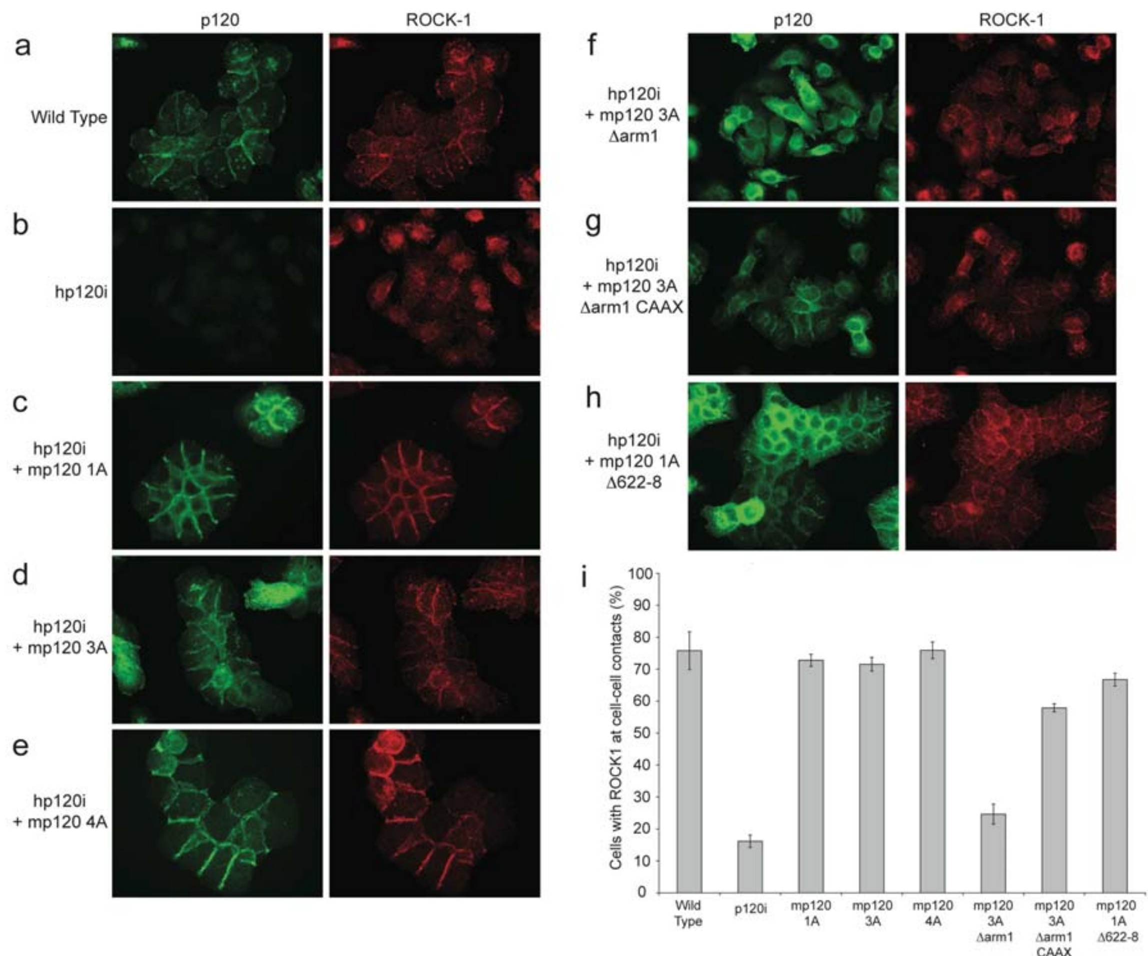

Figure 19: p120 can regulate ROCK1 localization at cell-cell junctions. Immunofluorescence analysis of endogenous ROCK1 in wild type A431 cells **(a)**, p120-depleted A431 cells (hp120i) **(b)**, or hp120i cells expressing **(c)** mp120 1A, **(d)** mp120 3A, **(e)** mp120 4A, **(f)** mp120 3A Δarm1, **(g)** mp120 3A Δarm1 CAAX, or **(h)** mp120 1A Δ622-8. **(i)** Quantification of ROCK1 localization at cell-cell contacts in the analyzed A431 cells lines. Bars represent the average percentage of cells in 4 20x fields with ROCK1 localized to cell-cell contacts. Error bars represent standard error of the mean.

localization was assessed by immunofluorescence in wild type A431 cells and p120-depleted cells, and p120-reconstituted cells. Depletion of p120 leads to degradation of the cadherin complex and destabilization of cell-cell junctions. Consistent with this, ROCK1 was lost from cell-cell contacts following p120 depletion (Figure 19b). p120 status did not affect ROCK1 protein expression levels as assessed by western blot (data not shown). Reconstitution of wild-type p120 efficiently restored cell-cell junctions and ROCK1 localization at the cell junction (Figure 19c-e), regardless of the isoform (mp120 1A, 3A, or 4A) used for reconstitution. In contrast, addback of a mutant that does not bind to cadherins (mp120 3A Δarm1 Figure 19f) did not restore ROCK1 cell-junction localization. However, membrane targeting of the Δarm1 mutant using a C-terminal CAAX motif (Xia et al., 2006; Wildenberg et al., 2006) restored co-localization of p120 and ROCK1 (Figure 19g), suggesting that these two proteins can interact in the absence of the cadherin complex. These data indicate that p120 can regulate ROCK1 localization to cell-cell contacts when it p120 is membrane associated (e.g. cadherin-bound). A Rho-uncoupled p120 mutant (mp120 1A Δ622-8) (Anastasiadis et al., 2000) (Figure 19h) also co-localized with ROCK1 at cell-cell contacts. These data suggest that the association of ROCK1 with p120 is involved in the function and/or stabilization of the cadherin complex, rather than direct regulation of RhoA activity by p120.

ROCK1 depletion affects the cadherin complex

The functional relationship between ROCK1 and p120 was further assessed by shRNA-mediated depletion of ROCK1 in A431 cells (Figure 20). I was able to

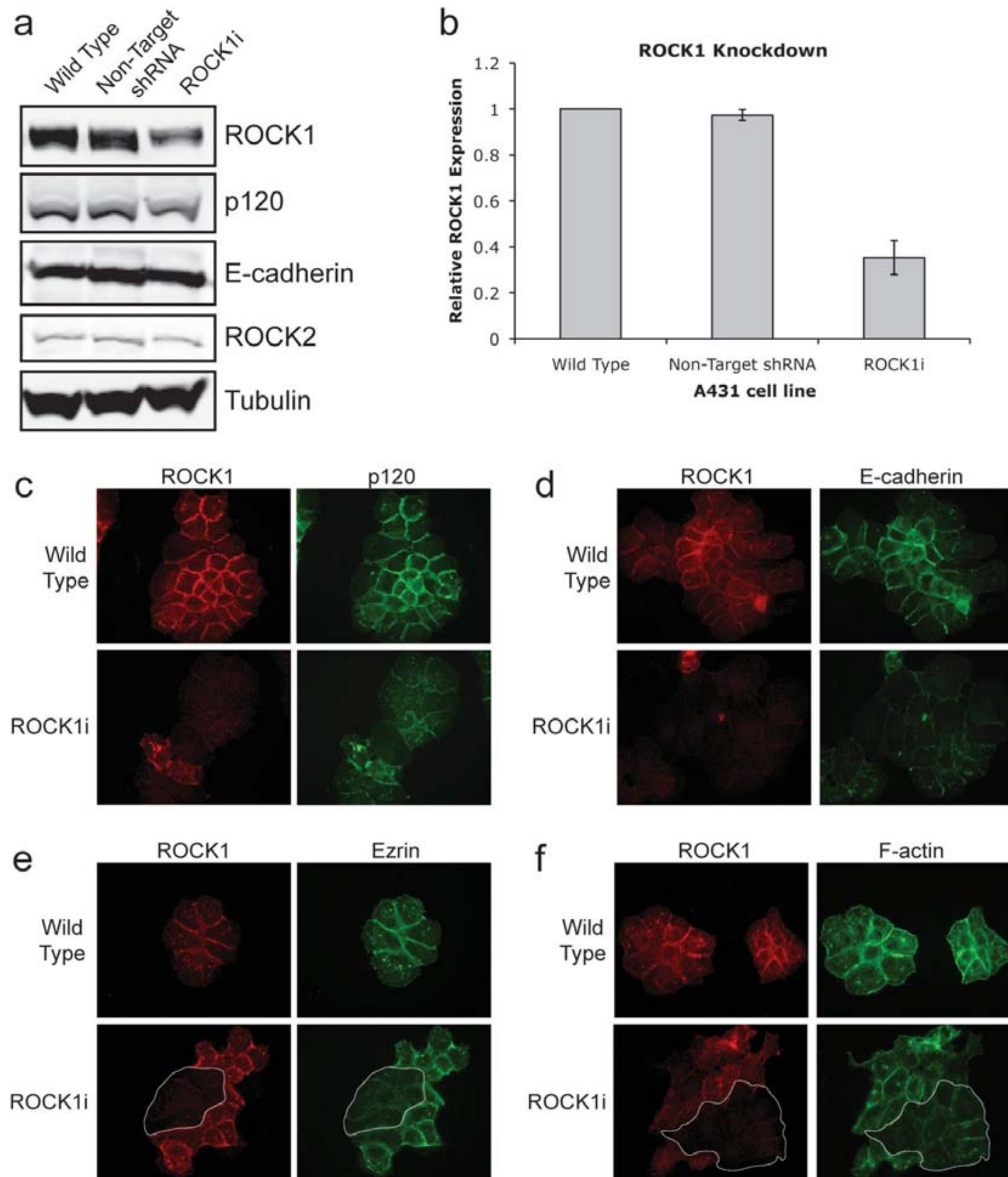

Figure 20: Knockdown of ROCK1 affects cell-cell adhesion. (a) Western blot analysis of wild type A431 cells and A431 cells expressing Non-target or ROCK1 shRNA. **(b)** Quantification of ROCK1 levels as detected by western blot. Bars represent the relative ROCK1 levels normalized to tubulin, averaged from 3 experiments. Error bars represent standard error of the mean. **(c-f)** Immunofluorescence analysis of ROCK1 (red) and p120 **(c)**, E-cadherin **(d)**, Ezrin **(e)**, and F-actin **(f)** in wild type A431 cells and A431 cells expressing ROCK1 shRNA. Where applicable, ROCK1-knockdown cells are outlined in white.

achieve approximately 65% depletion of endogenous ROCK1 in A431 cells, with no change in ROCK2 levels as assessed by western blot (Figure 20a-b). Stable knockdown of ROCK1 did not affect the protein levels of p120 and E-cadherin. Immunofluorescence analysis indicated that knockdown of ROCK1 was mosaic, with a small population of cells expressing little to no ROCK1 (ROCK1-knockdown cells), and a larger population of cells expressing wild-type ROCK1 levels. ROCK1-knockdown cells were larger, suggesting reduced contractility in the absence of ROCK1. Strikingly, distribution of p120 and E-cadherin was dramatically altered in the ROCK1-knockdown cells, particularly at cell-cell junctions (Figure 20c and d, respectively). Based on p120 and E-cadherin staining, cell-cell junctions were disorganized and weaker relative to cells with normal ROCK1 levels. The same effect was observed for α-catenin, β-catenin, Plakoglobin, and P-cadherin (Figure 21), suggesting that ROCK1 depletion affects the integrity of the entire cadherin complex. Membrane localization of Ezrin was also disrupted (Figure 20e), indicating that ROCK activity was deficient in ROCK1-knockdown cells. Analysis of the actin cytoskeleton using phalloidin revealed that the junctional actin network of ROCK1i cells was disrupted (Figure 20f), which could account for the disruption of the cadherin complex. Similar results were observed in ROCK1 depleted MCF-7 cells depleted of ROCK1 (Figure 22).

To determine if the effects of ROCK1 depletion on the cadherin complex are the result of disruption of the acto-myosin pathway, I used chemical inhibitors to ablate ROCK and myosin II activity in wild type A431 cells and analyzed the cells by immunofluorescence (Figure 23). Wild-type A431 cells were serum starved and treated

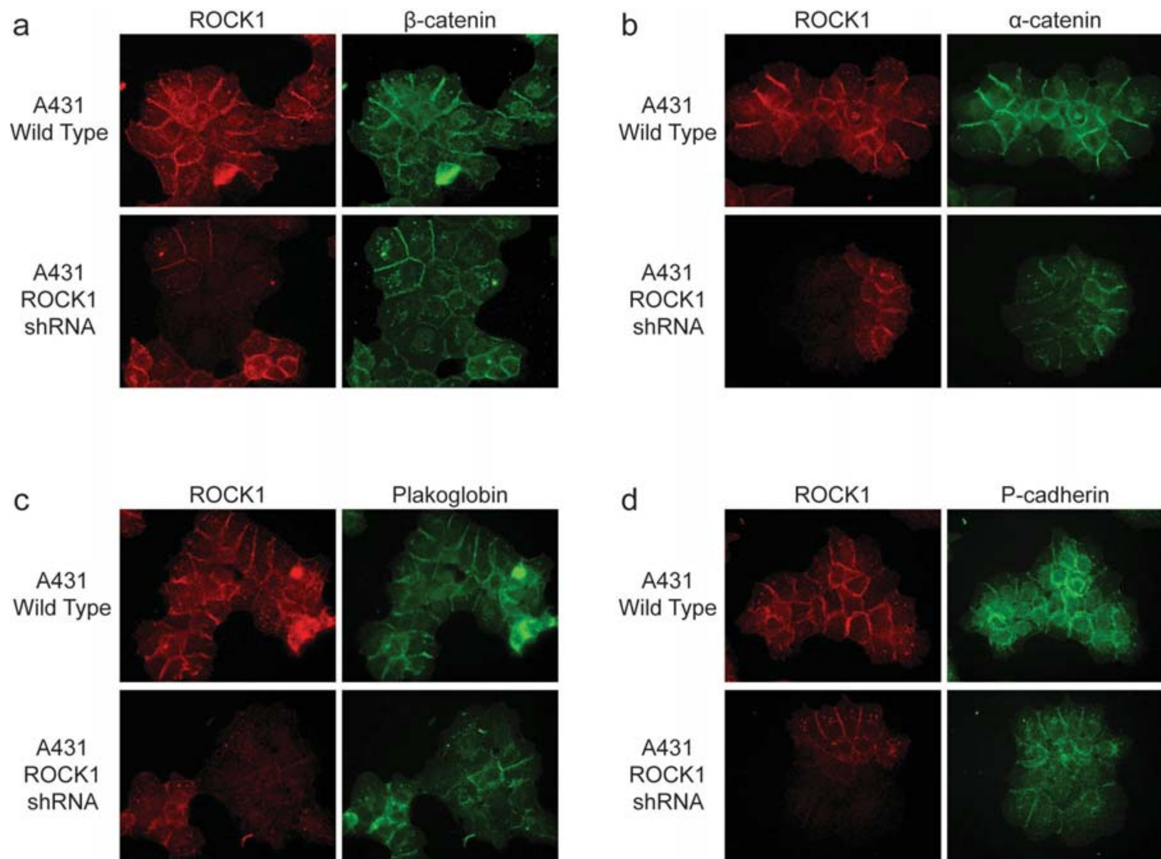

Figure 21: ROCK1 depletion affects the entire cadherin complex.
Immunofluorescence analysis of ROCK1 (red) and β-catenin **(a)**, α-catenin **(b)**, Plakoglobin **(c)**, and P-cadherin **(d)** in wild type A431 cells and A431 cells expressing ROCK1 shRNA.

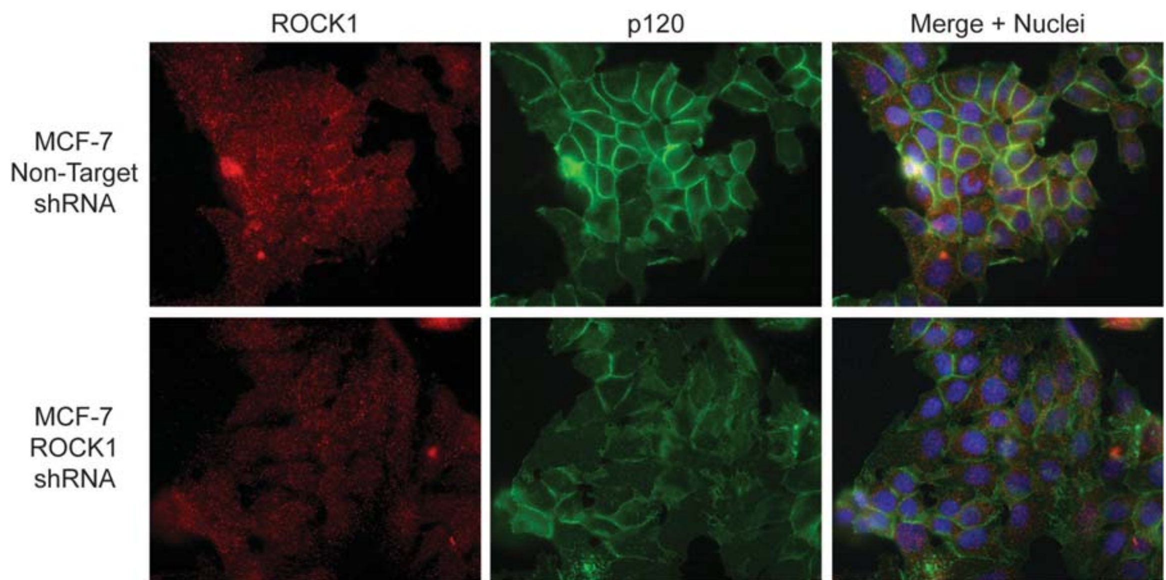

Figure 22: ROCK1 depletion in MCF-7 cells affects p120 distribution. Immunofluorescence analysis of ROCK1 (red) and p120 (green) in MCF-7 cells expressing Non-Target shRNA or ROCK1 shRNA

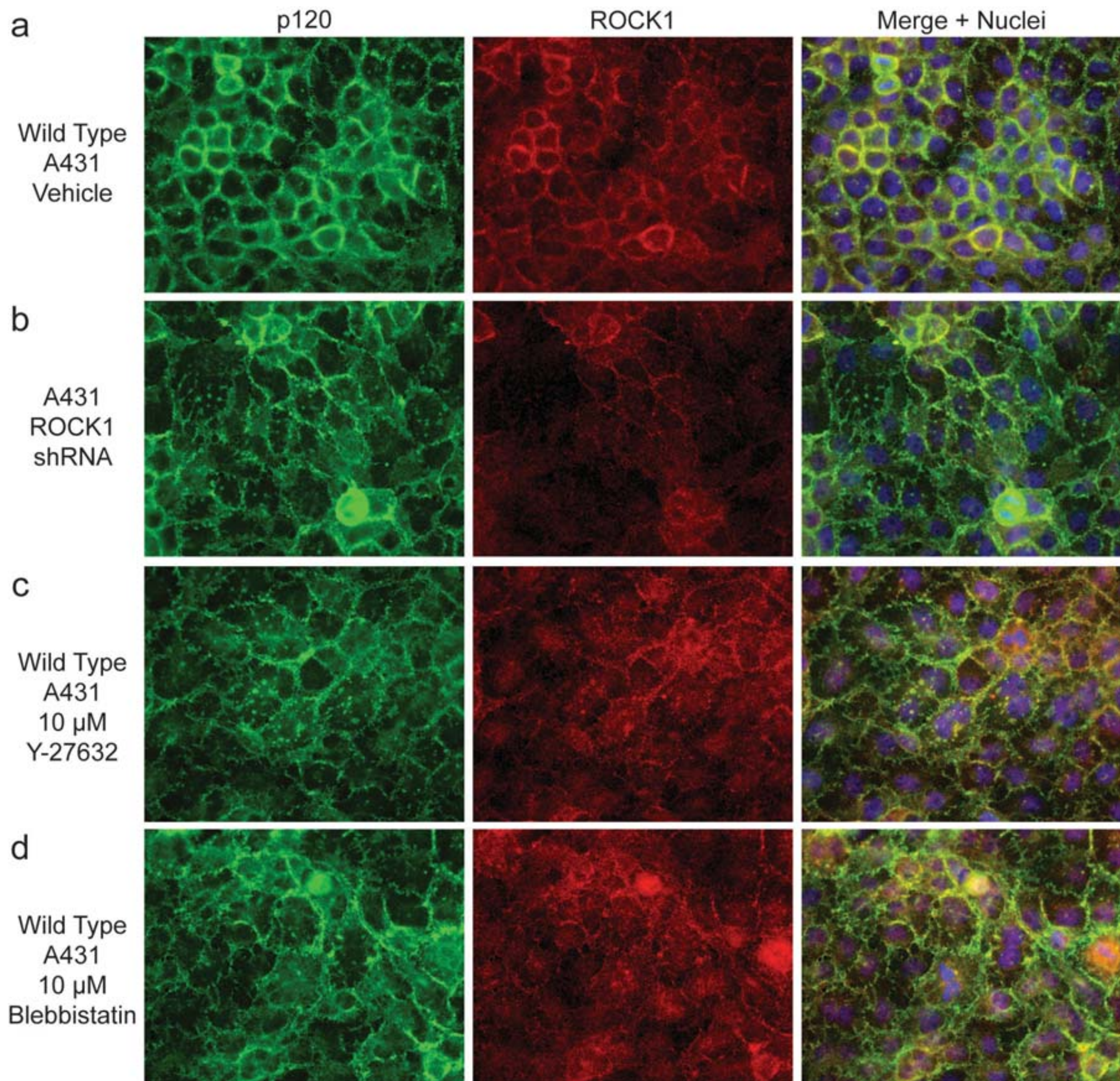

Figure 23: Inhibition of ROCK and Myosin II activity mimic the effects of ROCK1 depletion. Immunofluorescence analysis p120 (green) and ROCK1 (red) of vehicle treated wild-type A431 cells **(a)**, A431 cells expressing ROCK1 shRNA **(b)**, and wild type A431 cells treated with 10 μM Y-27632 **(c)** or 10 μM Blebbistatin **(d)** for 24 hours.

with DMSO vehicle alone (a), 10 μM Y-27632 (c), or 10 μM Blebbistatin (d) for 24 hours, then processed for immunofluorescence analysis to detect p120 and ROCK1. For comparison, ROCK1 knockdown cells were serum starved and analyzed in parallel (b). Similar to ROCK1 depletion, Y-27632 and Blebbistatin resulted in large cells with disorganized adherens junctions, as assessed by p120 staining. Thus the effects of ROCK1 depletion are comparable to the effects of global ROCK inhibition. Furthermore, inhibition of myosin II produces the same phenotype of ROCK1 depletion. These results suggest that ROCK1's role in the cadherin complex is to maintain the local acto-myosin network, thereby stabilizing cell-cell adhesion.

ROCK1 phosphorylates p120

p120 has several serine/threonine phosphorylation sites, but few direct kinases that phosphorylate p120 are known. The ability of ROCK1 to phosphorylate p120 was tested using an *in vitro* kinase assay using immunoprecipitated p120 from A431 cells as a substrate for constitutively active ROCK1. p120 was immunoprecipitated from A431 cells, and subsequently incubated with purified active ROCK1 (amino acids 17-535) in the presence of absence of 10 μM Y-27632. As Figure 24a shows, p120 is highly phosphorylated on Serine 268 (S268) and weakly phosphorylated on Threonine 916 (T916), but not on any other residue assayed (serines 288 and 879, and threonine 310, b-d). Phosphorylation of S268 does not occur in the presence of Y-27632 or in the absence of ATP (data not shown), indicating this is a ROCK-specific phosphorylation event.

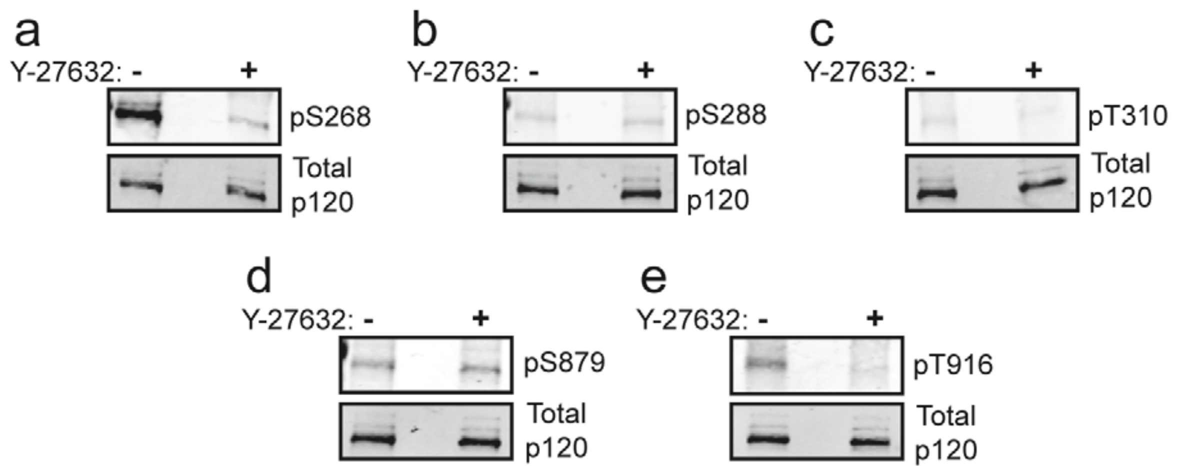

Figure 24: ROCK1 can phosphorylate p120 *in vitro*. Western blot analysis of ROCK1 kinase assays using immunoprecipitated p120 from A431 as a substrate for constitutively active ROCK1 in the presence or absence of Y-27632. Reactions were analyzed for total p120 (lower panels) and **(a)** pS268, **(b)** pS288, **(c)** pT310, **(d)** pS879, and **(e)** pT916.

Interestingly, p120 S268 does not match the reported ROCK consensus sequence of R/K-X-X-S/T, where "X" represents any amino acid (Riento and Ridley, 2003). Upstream sequence analysis reveals that S268 is within a highly conserved motif of R-V-G-G-S, which resembles the classical ROCK consensus sequence with V265 representing an extra residue upstream of the two variable residues. This suggests that there may be more variability of ROCK1 substrates than originally described.

Discussion

Modulation of the actin cytoskeleton by Rho GTPases plays an important role in cell-cell adhesion, however precise mechanisms remain unclear. In particular, the ROCK-Myosin pathway has been implicated in this process (Shewan et al., 2005). While most reports discuss Rho kinases as a cytoplasmic proteins, ROCK1 has previously been detected at the cell membrane and at cell-cell contacts (Walsh et al., 2001; Nishimura and Takeichi, 2008), however the function of this pool of ROCK1, and how it associates with components of cell-cell junctions, have remained unclear. Here, I have demonstrated for the first time that p160 Rho Kinase, a major RhoA effector, physically associates with the cadherin complex through p120.

Using a calcium switch assay, I have found that ROCK1 is recruited to nascent adherens junctions in a cadherin-dependent manner. ROCK1 recruitment occurs after the initial cadherin contacts are made, thus ROCK1 is not involved in the initial formation of the adherens junctions. Instead, ROCK1 is recruited at later time points, suggesting that ROCK1 is involved in the maintenance and/or maturation of the cadherin complex. The time-course of ROCK1 recruitment to the cadherin complex is consistent with

establishment of a junctional actin network (Zhang et al., 2005). This pool of junctional actin is not necessary for the establishment of cell-cell contacts, but is involved in the stabilization of clustered cadherins. Consistent with this, ROCK1 knockdown does not ablate cell-cell adhesion but does disrupt the normal organization of p120, E-cadherin, and F-actin at cell-cell junctions. These data suggest that ROCK1 is required for the maintenance of strong cell-cell adhesion in part through the modulation of a junction-localized pool of F-actin. It is likely that additional ROCK1 targets are recruited to the cadherin complex at this later stage and contribute to cell-cell adhesion. ReCLIP analysis of E-cadherin complexes following initial cadherin engagement (i.e. 5 minutes post-calcium addback) and during maturation of junctions (i.e. 30 minutes post-calcium addback) could identify proteins that participate in the ROCK1 cell-cell adhesion function.

Importantly, the association of ROCK1 with the cadherin complex is dependent on p120, as a p120-uncoupled E-cadherin does not co-immunoprecipitate ROCK1. Immunofluorescence analysis of A431D cells illustrates a low level of ROCK1 can still localize to the cell membrane in the absence of cadherins or in the presence of the 764AAA E-cadherin, which does not bind p120. However, although this pool of ROCK1 is still membrane-bound, it can’t physically interact with cadherins without p120. p120-dependent recruitment of ROCK1 to the cadherin complex may explain the altered cytoskeletal organization of cells expressing 764AAA E-cadherin (Thoreson et al., 2000).

The mechanism that recruits ROCK1 to the membrane in these cells is unclear, but may be attributed to the C-terminal Pleckstrin Homology (PH) domain of ROCK1, which binds to lipids and targets proteins to cell membranes (Lemmon and Ferguson,

2000). Alternatively, ROCK1 may be recruited to the membrane by Shroom3 (Nishimura and Takeichi, 2008), which can recruit Rho Kinases to apical junctions. Additionally, it appears that p120 does not interact with ROCK1 unless it is recruited to the membrane by E-cadherin or another mechanism (i.e. use of a CAAX box). Taken together, these data indicate that p120 can recruit membrane-associated ROCK1 to the cadherin complex.

While this is the first report of a physical link interaction between ROCK1 and the cadherin complex, a functional relationship has been suggested in a number of studies. In some cases, ROCK1 can negatively regulate cell-cell adhesion, due largely to excess acto-myosin contractility (Wójciak-Stothard et al., 2001). While apparently contradictory to our results in A431 cells, these studies clearly support an important functional relationship between ROCK and the cadherin complex. It is likely that this relationship varies depending on cellular context. Importantly, most of these studies have relied on the expression of dominant-active/negative mutants and over-expression approaches, whereas I have found a physical interaction between p120 and ROCK1 under endogenous, physiological conditions. Excessive activation/inhibition of ROCK signaling in past studies may account for the differences between previous reports and our own results in A431 cells.

Our studies support several lines of evidence indicating a requirement for ROCK activity in cadherin function. Recent studies have indicated that acto-myosin contractility, downstream of ROCK1, is necessary for the establishment and maturation of adherens junctions (Shewan et al., 2005; Smutny et al., 2010). In these studies the ROCK appeared to be acting through a pool of junction-localized myosin IIA. Similarly, I observe that global inhibition of either ROCK with Y-27632 or of Myosin II with blebbistatin mimics

the effects of ROCK1 knockdown, leading to disorganized adherens junctions. By binding to p120, ROCK1 could act directly on the cadherin complex and/or the pool of myosin IIA localized at cell-cell junctions. Furthermore, a physical interaction with the cadherin complex would allow rapid, localized induction of contractility without affecting other cellular compartments. Additionally, ROCK1 can phosphorylate p120 *in vitro*, suggesting that ROCK1 may be acting directly on p120 in addition to activating myosin IIA at the adherens junction.

ROCK1 knockdown and inhibition of ROCK dramatically effects p120 distribution in A431 and MCF-7 cells. Similarly, inhibition of Myosin IIA using either the myosin inhibitor blebbistatin or shRNA led to dramatic loss of E-cadherin in human embryonic stem cells due to downregulation of p120 (Li et al., 2010). A similar effect has been observed using the ROCK inhibitor Y-27632 (Harb et al., 2008). Interestingly, inhibition of ROCK activity promoted self-renewal of stem cells in the absence of cell-cell adhesion and feeder fibroblasts. These data are consistent with our own work in A431 cells, as loss of ROCK1, which acts directly on Myosin II, also disrupts p120, although to a much lesser degree. Protein levels of p120 and E-cadherin are not affected by ROCK1 depletion, suggesting that the interaction of p120 and E-cadherin remains intact in these cells. In A431 cells, it appears p120 and the rest of the cadherin complex is mislocalized or modified rather than degraded. This may be due to differences in the model systems used in these studies. Li et al. used human embryonic stem cells, while our studies employed the A431 epidermoid carcinoma cell line. It is possible that p120 and the cadherin complex may be more dependent on acto-myosin contractility in

pluripotent stem cells, as opposed to an immortalized cell line derived from fully differentiated tissue.

A functional relationship between p120 and RhoA, upstream of ROCK1, has been suggested by several earlier studies. In particular, it has been suggested that p120 can directly bind to RhoA and act as a Guanine Dissociation Inhibitor (GDI), thereby preventing RhoA activation (Anastasiadis et al., 2000; Castano et al., 2007; Yanagisawa et al., 2008). Our lab has previously demonstrated that p120 can associate with p190 RhoGAP and recruit it to the cadherin complex, (Wildenberg et al., 2006), making p120 and the cadherin complex an important point of regulation for RhoA. Here, I report that ROCK1, a major RhoA effector, associates with p120 at the adherens junction. Thus, in addition to suppressing RhoA through p190 RhoGAP and/or a GDI function, p120 can also recruit a major effector of RhoA, which would allow p120 to promote downstream signaling. Taken together, these data point to a dynamic RhoA complex within the adherens junction, with Rho effectors (ROCK1) and Rho suppressors (p190 RhoGAP and p120 itself) forming a functional unit. In this scenario, when contractility is needed at the adherens junction, ROCK1 is readily available to initiate signaling to Myosin II. Once the optimal levels of contractility are achieved, RhoA can be rapidly suppressed by p190 RhoGAP and/or p120's GDI function. Thus allowing rapid cycling of RhoA activity and downstream signaling at the cadherin complex.

In an *in vitro* kinase assay, I have found that ROCK1 can specifically phosphorylate p120 on serine 268. Modulation of p120 serine/threonine phosphorylation appears to be quite complex. Activation of PKC induces dephosphorylation of all known serine/threonine phosphorylation sites on the N-terminus of p120 (Xia et al., 2003) while

also inducing dramatic phosphorylation of S879 on the C-terminus (Brown et al., 2009). To date very few specific kinases and phosphatases that act directly on p120 have been identified. Identifying *in vivo* kinases has been difficult in part because S268, along with the majority of other serine/threonine sites, are basally phosphorylated. Further complicating our analysis, S268, along with the rest of the N-terminal serine/threonine phosphorylation sites appear to be dephosphorylated in response to Dominant Active RhoA expression (Xia et al., 2006). Thus under some conditions, Rho signaling may actually suppress S268 phosphorylation even through ROCK1 can directly phosphorylate this site. It should be noted however, that expression of dominant active Rho constructs has been known to introduce a number of artifacts in experimental systems (Boulter et al., 2010).

Recent work has shown that in SW480 cells, S268 and S269 can be phosphorylated by CK1ε following Wnt stimulation (Casagolda et al., 2010). In A431 cells, Wnt 3a and Wnt 5a cultured media did not modify S268 phosphorylation status (unpublished observations), suggesting that S268 is differentially modulated depending on cellular context. ROCK activation downstream of Wnt signaling is an attractive hypothesis, but other possibilities such as cadherin-based signaling must also be considered. Interestingly, S268 is highly phosphorylated in the presence of wild-type, but not p120-uncoupled 764AAA E-cadherin (Xia et al., 2006), and ROCK1 is physically associated with wild-type but not 764AAA E-cadherin. Thus cadherin binding and/or membrane association appears to play some role in S268 phosphorylation. The significance of S268 phosphorylation by ROCK1 remains unclear. A431 cells expressing S268 and S268/9 phospho-deficient and phospho-mimetic mutants are indistinguishable

from wild-type controls with respect to cell-cell adhesion (unpublished observations). Additional functional assays are necessary to determine if these mutants induce a measurable phenotype. Future experiments aimed at identifying the conditions under which ROCK1 phosphorylates p120 in cells should help clarify the function of this phosphorylation event.

I have illustrated a novel physical and functional relationship between p120-catenin and ROCK1. Importantly, p120 physically links ROCK1 to the cadherin complex. p120-dependent recruitment of ROCK1 to the cadherin complex may explain many recent findings regarding the role of acto-myosin contractility in cadherin function (Harb et al., 2008; Li et al., 2010; Smutny et al, 2010) and collective migration (Hidalgo-Carcedo et al., 2011). Furthermore, p120 can be phosphorylated by ROCK1 on S268, suggesting that ROCK1 function at the cadherin complex involves phosphorylation of p120. I was unable to map the interaction between p120 and ROCK1 using a Yeast-2-Hybrid approach, possibly due to an unknown phosphorylation event mediating the interaction. Future work focused on mapping this interaction and the generation of minimal uncoupling mutants will be necessary to determine more precisely how p120 and ROCK1 work together to co-ordinate acto-myosin contractility and cell-cell adhesion.

CHAPTER V

FUTURE DIRECTIONS

Introduction

In ReCLIP, I have developed a powerful tool to study labile protein complexes. Using this approach, I have identified several novel putative p120 binding partners. Among these, I have found that cd98 associates with p120 through E-cadherin, and ROCK1 associates with E-cadherin in a p120-dependent manner. Furthermore, I have demonstrated that p120 can be directly phosphorylated by ROCK1 on S268 *in vitro*, suggesting that this interaction may control p120 phosphorylation. While these findings have provided new insights into the function of p120 and the cadherin complex, a number of questions remain. Furthermore, the full potential of ReCLIP has yet to be explored.

Generation of a p120 interactome

A particularly exciting application of ReCLIP is the development of a comprehensive p120 interactome. A functional interaction network could be generated by first determining the binding partners of p120 and then reiterating the process on p120's direct binding partners. Proteins that are commonly detected across all the samples could be included the functional network. For example, E-cadherin and β-catenin ReCLIP eluates could be analyzed along side p120 eluates, common proteins in each sample could be incorporated into a protein interaction network for the cadherin complex. The cadherin interaction network could then be expanded by performing ReCLIP using some of these

common proteins as bait. Similarly, p120 eluates could be compared to eluates from Kaiso pull-downs to provide further insight into the functional significance of the p120-Kaiso interaction. The generation of such extensive interaction maps would require considerable effort and optimization, but would provide a large number of new functional insights into the function and regulation p120 and it's associated complexes. Furthermore, such studies were not feasible using prior to the development of ReCLIP.

Identification of cytoplasmic and nuclear p120 binding partners

To date, p120 ReCLIP studies have been preformed exclusively in epithelial cell lines. In epithelial cells, it has been suggested that the vast majority of the total cellular p120 is present at the membrane, bound to E-cadherin (Kiss et al., 2008). Thus the binding partners detected in these experiments most likely represent cadherin-based functions of p120, as appears to be the case with ROCK1. Furthermore, the large abundance of cadherin-bound p120 may mask any cadherin-independent p120 binding partners in mass spectrometry analysis. As a result, the currently reported ReCLIP experiments do not provide a complete picture of p120 binding partners. Cytoplasmic p120 complexes are under-represented in MS analysis relative to the highly abundant cadherin-based complexes and nuclear complexes are lost due to inefficient recovery of nuclear proteins using standard lysis methods.

Determining the binding partners of cytoplasmic p120 is particularly interesting because the transition to metastasis is often associated with increased cytoplasmic localization of p120 (Sarrio et al., 2004). One approach to study cytoplasmic p120 is to perform ReCLIP using a cell line that lacks classical cadherins, such as the A431D or

MDA-MD-231 cell lines. In these cells, endogenous p120 is mostly cytoplasmic due to the lack of cadherins. In this context, any proteins identified would represent candidate binding partners for cytoplasmic p120. Furthermore, wild-type E-cadherin can be exogenously expressed in these cells to recruit p120 to the membrane, away from cytoplasmic binding partners. This could provide a useful experimental control, as real cytoplasmic p120 binding partners would not be detected in ReCLIP samples from cells expressing exogenous E-cadherin. A potential drawback of this approach is that the abnormally high levels of cytoplasmic p120 in these cells may lead to non-physiological interactions. Nonetheless, cadherin-negative cell lines should provide a good starting point, since the established experimental procedures should require little alteration in these systems.

Most standard lysis conditions do not efficiently recover nuclear proteins, thus nuclear p120 binding partners would likely be lost even in cadherin-negative cells. To overcome this issue, subcellular fractionation could be carried out following crosslinking, so the cytosolic and nuclear p120 complexes can be purified in isolation from each other. Furthermore, subcellular fractionation could be carried out using epithelial cell lines such as A431 or MCF-7 cells rather than cadherin-negative cell lines. Regardless of the cell line used, the starting material will likely have to be scaled up considerably to account for the low abundance of nuclear p120, and crosslinking conditions may need to be further optimized to ensure nuclear protein complexes are efficiently crosslinked.

Structure-Function Analysis of the p120-ROCK1 Interaction

Although I have shown that ROCK1 and p120 co-immunoprecipitate, and that

ROCK1 associates with E-cadherin in a p120-dependent manner, I have been unable to determine if p120 and ROCK1 directly interact with each other. Future experiments are aimed at determining this as well as mapping the reciprocal binding sites between p120 and ROCK1. A potential approach is to use purified protein fragments fused to either Maltose Binding Protein (MBP) and Glutathione-S-Transferase (GST) for *in vitro* binding assays. Distinct binding sites can then be mapped using site-directed mutagenesis to determine the specific amino acids required for the interaction.

If a direct interaction between p120 and ROCK1 can be identified and mapped, then minimal uncoupling mutants can be generated. Such mutants would uncouple p120 from ROCK1 without affecting its other binding partners, and vice-versa. Expression of these mutants in cells could help identify the specific function of the p120-ROCK1 interaction. For example, do the cell-cell adhesion functions of ROCK1 require the interaction with p120? Does expression of p120-uncoupled ROCK1 rescue the effects of ROCK1 knockdown or ROCK inhibition? Furthermore, does expression of a ROCK1-uncoupled p120 mimic ROCK inhibition with respect to the cell-cell adhesion? Generation of minimal uncoupling mutants should allow these questions to be answered *in vivo*.

Physiological mechanisms of ROCK1-dependent p120 phosphorylation

Using an *in vitro* kinase assay with a constitutively active ROCK1 fragment, I have demonstrated that ROCK1 can phosphorylate p120 on serine 268. However, the physiological mechanism by which ROCK1 phosphorylates p120 remains unknown. A recent report indicates that Wnt stimulation can induce S268 phosphorylation and this is

required for Wnt signaling (Casagolda et al., 2010). In A431 cells, Wnt 3a and 5a cultured media does not affect S268 phosphorylation, suggesting that this pathway may not be intact in all cell-types.

One approach to identify stimuli that phosphorylate p120 in a ROCK-dependant manner is to use an In-Cell Western (ICW) assay to simultaneously screen multiple agonists in the presence and absence of the ROCK inhibitor Y-27632. This assay has previously been used to evaluate phosphorylation of p120 on S879. The 96-well format of the ICW assay should significantly accelerate the process of screening agonists. One appealing hypothesis is that S268 is phosphorylated by ROCK1 in response to cadherin engagement and clustering. Our calcium switch experiments indicate that ROCK1 is recruited to the newly established adherens junctions, and A431-D and p120-addback experiments suggest p120 only associates with ROCK1 when it is localized to the cadherin complex or the cell membrane. Furthermore, serine 268 is highly phosphorylated when it is cadherin or membrane-associated (Xia et al., 2006). Preliminary experiments using calcium switch and antibody-mediated cadherin clustering assays (Betson et al., 2002) should provide important clues as to whether cadherin clustering involves ROCK1-mediated phosphorylation of p120.

Functional Significance of S268 Phosphorylation

Although I have found that ROCK1 depletion or inhibition effects p120 and the cadherin complex, I have not been able to identify a specific function for S268 phosphorylation. Previous studies indicate that S268 phosphorylation is not required for cadherin stability (Xia et al., 2006). Furthermore, mutation of all serine/threonine

phosphorylation sites to alanine appears to have no effect on cadherin stability. Thus, it is unlikely that p120 phosphorylation is essential to cadherin engagement and clustering.

If S268 phosphorylation is ROCK1-dependent, then assays based on ROCK1 function rather than p120 function may prove more fruitful. For example, is S268 phosphorylation involved in the ability of ROCK to regulate cell migration (Worthylake et al., 2001)? Additionally, the pS268 mAb and ReCLIP could be used to identify binding partners that are specific to serine-268 phosphorylated p120. Novel binding partners or complexes identified in these experiments could provide insight into the function of S268 phosphorylation, and help direct future functional experiments.

CHAPTER VI

CONCLUDING REMARKS

This work has generated an important new tool to facilitate the study of p120 and the cadherin complex. ReCLIP has been optimized for the cadherin complex and has proven highly effective for these studies, but the method is also applicable to other targets, provided the necessary optimizations (i.e. crosslinker concentrations and treatment times, antibody concentrations) are carried out prior to large scale experiments. Every protein and complex is different, thus different conditions may be required for different targets. In order to fully exploit the potential of ReCLIP, more in depth studies will be necessary to determine the limitations of the method. In particular, the efficiency of ReCLIP to study over-expressed proteins has yet to be determined. Nonetheless, we anticipate that ReCLIP could become a widely used approach for mass spectrometry-based protein interaction studies. Indeed, it has already been used by a number of researchers throughout Vanderbilt University.

Using ReCLIP, we have overcome a significant problem in the p120 field, specifically the labile nature of p120 interactions. Our initial attempts using a TAP-tag method proved unsuccessful, with efficient recovery of p120 itself but few binding partners detected, including the cadherin complex. With ReCLIP, however, I not only dramatically improved cadherin complex recovery over previous attempts, but also identified several putative p120 binding partners in a clean single affinity-purification approach. Future experiments aimed at studying p120 interactions under a variety of

conditions should provide even further insight into p120 function. Thus ReCLIP will facilitate more sophisticated and comprehensive p120 studies than we have been able to do in the past.

With the identification of ROCK1 as a p120 binding partner, I have shed new light onto the function and regulation of the actin cytoskeleton at the cadherin complex. It is clear that that the cadherin complex is far more than a structural module, and plays an active role in coordinating adhesion, motility, and cell growth. ROCK1 almost certainly fits into this role, given the variety of its substrates and the diversity of its own cellular functions. Importantly, the p120-dependent recruitment of ROCK1 to the cadherin complex suggests that p120 can play a role in potentiating Rho signaling, as well as suppressing Rho signaling as has been previously reported. This adds a new layer of complexity to the functions of p120 with regards to both cadherin stabilization and Rho regulation. The full significance of this work remains to be determined, but we are in a good position to elucidate both broad p120 functions and the evolving relationship between p120 and the Rho/ROCK/acto-myosin pathway.

APPENDIX A

ADDITIONAL APPLICATIONS OF RECLIP TO IDENTIFY P120 BINDING PARTNERS

Introduction

As illustrated in chapter three, I have developed a highly efficient method for isolating and identifying p120 binding partners. Using ReCLIP, I identified several novel candidate p120 binding partners in A431 cells under standard growth conditions. Because ReCLIP uses a monoclonal antibody to recover target complexes, this approach affords a great deal of versatility. For example, it is possible to efficiently compare endogenous binding partners across multiple cell types (i.e. breast, colon, fibroblast cells) to identify tissue-specific binding partners and potential functions. In addition, complexes can be studied in the presence and absence of specific stimuli that induce p120 phosphorylation. Signaling through p120 likely involves the transient recruitment of protein to p120 in response to phosphorylation. Utilizing ReCLIP, phospho-specific complexes can be immunoprecipitated with phospho-specific mAb beads and analyzed by mass spectroscopy. This approach could not only identify the kinases and phosphatases that directly modify p120, but also identify downstream functional interactions that may be too weak or transient to detect without cross-linking.

The preliminary studies described here represent an initial attempt to exploit ReCLIP in the above manner. While the results are not conclusive, they do provide a good starting point for future comprehensive studies of p120 binding partners across

multiple cell lines and conditions that could yield more valuable results. Furthermore, these preliminary studies highlight important considerations that should be included in the planning of future ReCLIP experiments.

Results

Comparison of ReCLIP results across epithelial cell lines

To determine if variability between cell lines affects ReCLIP efficacy, I performed ReCLIP in A431 epidermoid carcinoma cells, MCF-7 mammary adenocarcinoma cells, MCF-10A non-transformed mammary epithelial cells, Caco-2 colorectal adenocarcinoma cells, and HCA-7 colonic adenocarcinoma cells. Crosslinking in all cell lines was carried out using the DSP and DTME combination. p120 was immunoprecipitated with mAb 15D2, while mAb 8D11 was used as a negative control. Western blot analysis of 10% of each eluate confirmed that ReCLIP successfully recovered E-cadherin in all cell lines using mAb 15D2, but not 8D11 (Figure 25). HCA-7 cells express very low levels of p120 and E-cadherin, thus the recovery of E-cadherin was not detectable in the fraction of eluate analyzed by western blot. However, subsequent MS analysis shows that a small amount of E-cadherin and associated catenins were recovered (Table 5).

Following western blot analysis, the remaining ReCLIP eluates were analyzed by single dimension LC-MS/MS. Background was comparable across all cell lines, and only proteins with 0 spectral counts detected in control eluates were considered valid

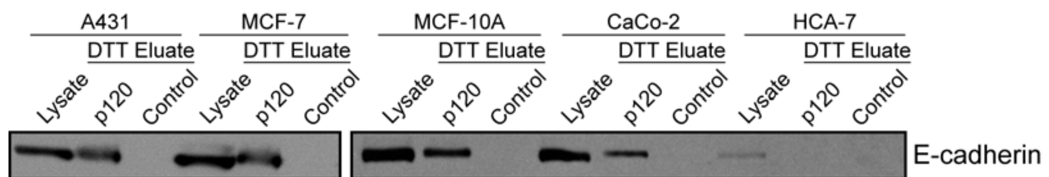

Figure 25: E-cadherin recovery in ReCLIP eluates from epithelial cell lines. Western blot analysis of E-cadherin in whole cell lysates, p120, and control IgG ReCLIP eluates from A431, MCF-7, MCF-10A, Caco-2, and HCA-7 cells. Due to the number of samples, two separate blots were used.

candidates. Proteins identified across 2 or more cell lines are listed in table 5. As Table 5 shows, the core components of the cadherin complex were recovered across all cell lines, with particularly robust recovery from MCF-7 cells. A comparison of proteins detected in 2 or more cell lines showed that several candidate binding partners previously recovered from A431 cells were also recovered from the other cell lines tested including ROCK1, cd98, VDP, and vinculin. In this particular preliminary experiment, ROCK1 and vinculin were not detected in A431 cells, but were detected in other cell lines. Furthermore, the polarity protein scribble was recovered exclusively in the breast epithelial cell lines (MCF-7 and MCF-10A). These preliminary results indicate that the efficacy of ReCLIP is consistent across multiple epithelial cell lines. Furthermore, these results show some

Table 5: Common proteins detected across multiple cell lines by ReCLIP. Total spectral counts detected for each indicated protein from p120 ReCLIP eluates. For all proteins listed, zero peptides were detected in control samples.

Protein	***UniProt Accession***	***A431***	***MCF7***	***MCF 10A***	***Caco-2***	***HCA-7***
cd98 (4F2 Heavy Chain)	IPI00027493.1	6	4	0	7	0
E-cadherin	IPI00000513.1	6	12	12	10	4
alpha1-catenin	IPI00215948.4	15	30	22	18	8
beta1-catenin	IPI00017292.1	16	22	20	17	3
Junction Plakoglobin	IPI00554711.2	14	20	9	19	4
TAP/VDP/p115	IPI00031583.4	5	2	7	4	4
ROCK1	IPI00022542.1	0	10	12	4	0
Isoform 1 of L-lactate dehydrogenase A chain	IPI00217966.7	0	2	0	2	3
Scribble (Isoform 3 of Protein LAP4)	IPI00410666.1	0	3	2	0	0
Na/K-transporting ATPase subunit α-1	IPI00006482.1	4	3	0	5	0
Na/K-transporting ATPase subunit a-2	IPI00003021.1	3	3	0	3	0
Isoform 1 of Vinculin	IPI00291175.7	0	0	5	5	3
EpCAM (Tumor-associated calcium signal transducer 1)	IPI00296215.1	0	3	2	2	0
Transferrin receptor protein 1	IPI00022462.2	0	2	2	0	0

complexes may associate with p120 more prevalently in some cell types (i.e. scribble in mammary epithelial cells), underscoring the importance of testing multiple cell lines in binding partner studies.

Identification of binding partners in PMA stimulated cells

Prior to ReCLIP analysis of phosphorylated p120, it was necessary to determine if in-cell crosslinking could affect p120 phosphorylation status. A431 cells were serum starved and treated with vehicle or PMA for 30 minutes to activate PKC signaling and induce phosphorylation on p120 S879. Following PMA treatment, in-cell crosslinking was carried out using DMSO vehicle or the DSP and DTME combination as described previously. Whole cell lysates were then analyzed by western blot to assess p120 S879 phosphorylation. As Figure 26a illustrates, PMA treatment induces robust phosphorylation of S879 in both the presence and absence of crosslinker. These data suggest that p120 phosphorylation is not affected by the crosslinking process.

The MCF-7 cell line was selected for large-scale ReCLIP analysis of p120 binding partners in PMA-stimulated cells, due to the improved binding partner recovery I observed in previous experiments. I confirmed that similar to A431 cells, p120 phosphorylation status in MCF-7 cells was unaffected by crosslinking (data not shown). MCF-7 cells were serum starved overnight, and treated with either vehicle alone or PMA for 30 minutes, then processed for ReCLIP analysis the DSP and DTME combination crosslinking condition. Immunoprecipitations were carried out using mAb 15D2 for total p120, anti-phospho-S879 mAb for phosphorylated p120 alone, or mAb 8D11 as a negative control. Western blot analysis of ReCLIP eluates demonstrates that E-cadherin

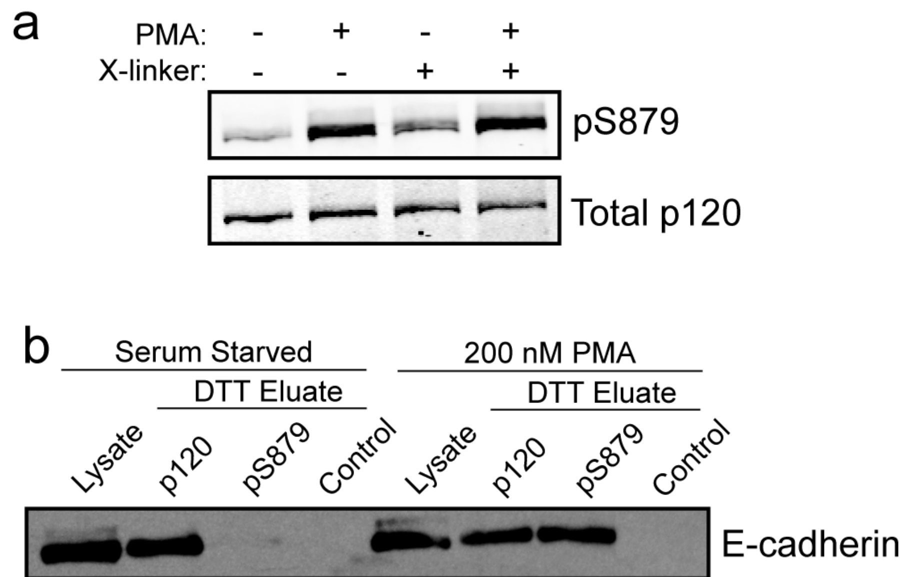

Figure 26: Application of ReCLIP to study p120 phosphorylation. (a) Western blot analysis of phospho-S879 (pS879) and total p120 in A431 cells in the presence of absence of PMA and crosslinkers (DSP and DTME combined). **(b)** Western blot analysis of E-cadherin whole cell lysates and p120, pS879, and control IgG ReCLIP eluates from serum starved and PMA-treated MCF-7 cells.

is efficiently recovered in 15D2 immunoprecipitations from both serum starved and PMA treated cells (Figure 26b). Using the S879 mAb, E-cadherin was only recovered from PMA-treated cells, consistent with phosphorylation of S879 in response to PMA. ReCLIP samples were analyzed by single dimension LC-MS/MS to identify putative binding partners. As expected, the core components of the cadherin complex were recovered efficiently using mAb 15D2 under both serum starvation and PMA conditions (Table 6). In addition, multiple binding partners previously identified in other cell lines were detected including cd98, VDP, GOLGA4, ROCK1, and Scribble. In addition, p190 RhoGAP was identified in 15D2 ReCLIP eluates, consistent with our earlier studies (Wildenberg et al., 2006). Using the anti-pS879 mAb, the cadherin complex was recovered under both serum starvation, consistent with the low level of basal S879 phosphorylation observed in western blot analysis. Under PMA treated conditions, recovery of E-cadherin was more efficient in PMA treated cells as assessed by spectral counts.

In addition to the cadherin complex, pS879 ReCLIP also captured Kinesin Heavy Chain (KHC), which has been previously identified as a p120 binding partner (Chen et al., 2003). Surprisingly, these eluates also contained snd1, a component of the RNA-Induced Silencing Complex (RISC) that is up-regulated in colon carcinogenesis and may regulate E-cadherin and APC (Tsuchiya et al., 2007). Recovery of both KHC and snd1 were equivalent between serum starved and PMA treated cells, as assessed by spectral counts, unlike the cadherin complex. Furthermore neither protein was detected in 15D2 eluates It is possible that KHC and snd1 are basally associated with p120, but cannot be recovered by 15D2 due to epitope masking.

Table 6: Proteins identified in serum starved and PMA-treated MCF-7 cells. Total spectral counts for the identified proteins identified in 15D2 and pS879 ReCLIP samples from serum starved of PMA-stimulated cells. No peptides of the indicated proteins were detected in control samples.

		Serum Starved		***PMA***	
Protein	***UniProt Accession***	***15D2***	***pS879***	***15D2***	***pS879***
cd98 (4F2 heavy chain)	IPI00027493.1	7	3	5	13
E-cadherin	IPI00000513.1	16	5	10	21
alpha1-catenin	IPI00215948.4	38	18	26	56
beta1-catenin	IPI00017292.1	23	18	15	36
Junction Plakoglobin	IPI00554711.2	22	11	21	32
52 kDa Ro protein (TRIM21)	IPI00018971.7	20	29	13	28
TAP/VDP/p115	IPI00031583.4	10	0	11	0
ROCK1	IPI00022542.1	46	0	32	0
GOLGA4 (Isoform 1 of Golgin Subfamily A member 4)	IPI00013272.1	54	0	34	0
Epiplakin	IPI00010951.2	5	0	3	0
Scribble (Isoform 3 of Protein LAP4)	IPI00410666.1	4	0	0	4
Isoform 3 of Septin-9	IPI00455033.5	2	4	3	5
Septin-2	IPI00014177	2	3	0	5
Septin-11	IPI00019376.6	0	5	0	4
Isoform 1 of Rho GEF 7	IPI00449906.3	3	0	2	0
Rab GDP dissociation inhibitor beta	IPI00031461.1	2	0	0	3
Ras-related protein Rab-11B	IPI00020436.4	0	0	0	2
p190 RhoGAP (Isoform 1 of Glucocorticoid receptor DNA-binding factor 1)	IPI00334715.3	6	0	9	0
Kinesin heavy chain	IPI00012837.1	0	3	0	2
Snd1 (staphylococcal nuclease and tudor domain containing 1)	IPI00140420.4	0	18	0	12
adaptor-related protein complex 2, beta 1 subunit	IPI00784156	3	2	2	0
adaptor-related protein complex 1, gamma 1 subunit isoform	IPI00293396.5	3	0	2	0
Coatomer subunit alpha	IPI00295857.6	5	0	3	0
Isoform 2 of Coiled-coil domain-containing protein C6orf97	IPI00216412.8	0	9	0	3
Sodium/potassium-transporting ATPase subunit α-2	IPI00003021.1	0	0	0	2
Sodium/potassium-transporting ATPase subunit α-1	IPI00006482.1	0	0	2	4
Myosin-9	IPI00019502.3	0	0	0	2
Myosin-Ic	IPI00010418.4	0	0	0	3

Discussion

Chapter three described the extensive optimization and validation of ReCLIP in A431 cells under standard growth conditions. To exploit the potential of ReCLIP, I sought to characterize p120 binding partners in a wider range of cell types and conditions. The experiments described here are only preliminary, but do provide evidence of the efficacy of ReCLIP across multiple cell-types and conditions and highlight important considerations for future applications of ReCLIP. The methodology described here should facilitate more comprehensive studies to identify binding partners via ReCLIP.

ReCLIP results across multiple human epithelial cell lines were remarkably consistent with respect to the cadherin complex. The core components of the cadherin complex (E-cadherin and α-catenin, β-catenin, and Plakoglobin) were all efficiently recovered with similar spectral counts across four of the five cell lines tested. Recovery was less robust in HCA-7 cells, as this cell line expresses less p120 and E-cadherin as indicated in western blot experiments. The recovery of candidate binding partners such as cd98 and ROCK1 in other cell lines besides A431 cells provides further support for the significance of these interactions. Interestingly, the polarity protein scribble was detectable in mammary epithelial cell lines (MCF-7 and MCF-10A), but not in other cell lines. This suggests that the components of p120 complexes may vary between different tissue types, and highlights the value of screening multiple cell lines in binding partner studies. If only one cell line is studied, it is likely that a number of important interactions would be missed because they may not be prevalent in the specific cell line used. A

comprehensive ReCLIP screen of cell lines covering multiple tissue types would be extremely informative and provide a broader view of p120 complexes and functions.

Because ReCLIP has been optimized for use with monoclonal antibodies, phospho-specific antibodies can be used with ReCLIP to specifically study binding partners associated with the phosphorylated form of a target protein. To that end, I performed ReCLIP in PMA-stimulated or unstimulated (serum starved) cells using both a total p120 (15D2) and a phospho-S879 mAb for immunoprecipitation. The physiological significance of PKC-mediated phosphorylation of S879, and dephosphorylation of the N-terminus remains unknown, despite extensive study (Xia et al., 2006, Brown et al., 2009). Unfortunately, aside from the cadherin complex no change in binding partner recovery with the pS879 mAb was observed following PMA treatment. It is possible that any phospho-specific transient binding partners had already dissociated from p120 by the 30 minute treatment time-point at which crosslinking was initiated, as this represents the peak of p120 phosphorylation following PMA treatment. Crosslinking at earlier time-points may capture transient interactions before they dissociate. Thus, preliminary time-course experiments should be carried out to determine the shortest treatment time at which crosslinking can be initiated to minimize the loss of transient binding partners.

Interestingly, some binding partners that were recovered abundantly using mAb 15D2, such as ROCK1 and GOLGA4, were not recovered at all using the pS879 mAb, suggesting that these proteins may not associate with p120 when S879 is phosphorylated. It is possible that differential localization of phosphorylated versus unphosphorylated p120 may explain this (e.g. p120 may not be phosphorylated when associated with GOLGA4 in the Golgi Apparatus). Alternatively, these protein complexes may mask the

pS879 epitope, such that only p120 that is not associated with these complexes is recovered by the pS879 mAb. Similarly, KHC and snd1 were detected in pS879 but not 15D2 eluates. Thus it is clear that different monoclonal antibodies can recover unique sets of binding partners. It would be particularly interesting to compare ReCLIP results using multiple p120 monoclonal antibodies, as this experiment could reveal novel binding partners that are not detectable using 15D2 alone.

Taken together, these experiments indicate that ReCLIP is effective across multiple cell types and conditions. However, the differences between recovered proteins across the different cell lines highlights the importance of evaluating multiple cell lines prior to more complex ReCLIP experiments. While PMA-treatment did not yield any phospho-specific novel binding partners in our experiments, further optimization of treatment times and crosslinking conditions may yield novel phospho-specific transient binding partners that could not be captured at the 30 minute time-point used here. Similar experiments could be carried out using different combinations of stimuli and phospho-specific p120 mAbs. Alternatively, total p120 could be isolated from a cell line treated with a panel of stimulants (EGF, PMA, LPA, Wnt 3a, etc) to identify changes in p120 complexes under each condition. However, each condition will require optimization to determine the minimal treatment time prior to carrying out large-scale ReCLIP experiments.

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