The transforming Rho family GTPase, Wrch-1, regulates epithelial cell morphogenesis through modulating cell junctions and actin cytoskeletal dynamics

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ABSTRACT

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The transforming Rho family GTPase, Wrch-1 regulates epithelial cell morphogenesis through modulating cell junctions and actin cytoskeletal dynamics
(Under the direction of Dr. Adrienne D. Cox)

Rho GTPases are members of the Ras superfamily of GTP binding proteins that function as molecular switches. In their GTP-bound, active state, Rho proteins are most well known for their ability to modulate the actin cytoskeletal network. In addition, Rho GTPases control signaling pathways that regulate diverse cellular functions such as cell shape, motility, and proliferation. When misregulated in cancer cells, Rho proteins influence aberrant growth, invasion and metastasis. Misregulation of Rho GTPases themselves or their regulators is associated with human cancers, making them attractive pharmacological targets for molecularly targeted cancer therapy. The atypical Rho GTPase Wrch-1 was initially discovered as a Wnt-1 responsive gene that, when activated, phenocopies Wnt-morphological transformation of mouse mammary epithelial cells. Like other Rho GTPases, Wrch-1 modulates actin cytoskeletal organization, but little is known about its downstream effectors and signaling pathways that may contribute to cellular transformation. Therefore, in this dissertation I discuss the characterization of a downstream effector of Wrch-1 that contributes to Wrch-1 normal biological function and its ability to mediate cellular transformation. Wrch-1 distributed along the apical and basolateral membranes in polarized MDCK epithelial cells and bound the cell polarity protein Par6 in a GTP-dependent manner. Activated Wrch-1 negatively regulated the kinetics of tight junction assembly during

epithelial cell polarization, without a detectable effect on overall cell polarity in confluent monolayers. Disruption of tight junction dynamics driven by activated Wrch-1 was accompanied by dramatic cytoskeletal reorganization and multilayering of MDCK cells grown in two-dimensional (2D) culture. Additionally, expression of constitutively active Wrch-1 disrupted cystogenesis of cells grown in three-dimensional (3D) culture, resulting in aberrant multilumenal structures. The loss of epithelial morphogenesis mediated by activated Wrch-1 correlated with the ability of Wrch-1 to promote cellular transformation. Finally, an effector domain mutation in activated Wrch-1 that prevents Par6 binding repressed the transforming activity and abrogated the ability of Wrch-1 to disrupt tight junction formation, actin organization, and epithelial morphogenesis. Thus, I potentially uncovered a mechanism by which activated Wrch-1 induces morphological and growth transformation that involves Par6-mediated disruption of tight junctions and actin organization.

DEDICATION

To my parents Rosalyn Brady and Donald Brady who have always supported me through my academic, athletic, and personal endeavors and encouraged me to go for my dreams. Your unconditional love has driven me to the best that I can be. To my twin brother Jonathan Brady for always being my biggest fan and for always having faith in me.

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PREFACE

The work presented in Chapters 2 and 3 of this dissertation have been submitted for publication in Molecular and Cellular Biology.

The transforming Rho family GTPase, Wrch-1, disrupts epithelial cell tight junctions and epithelial morphogenesis.

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2D	two-dimensional
3D	three-dimensional
A	alanine
AJ	adherens junction
AJC	apical junctional complex
APC	adenomatous polyposis colon protein
aPKC	atypical protein kinase C
Arf	ADP-ribosylation factor
ASIP	aPKC-specific interacting protein
BCL3	B cell lymphoma 3
C	cysteine
C-terminus	carboxyl-terminus
Ca	calcium
CAAX	cysteine-aliphatic-aliphatic-any amino acid
Cdc42	cell division cycle
CFP	cyan fluorescent protein
Chp	Cdc42 homologous protein
Crb	Crumbs
CRIB	Cdc42/Rac interactive binding
D	aspartic acid
Dbl	diffuse B-cell lymphoma
DH	Dbl homology

DHR	Dock homology region
DLC	deleted in liver cancer
Dlg	Discs large
DMEM-H	high glucose Dulbeco's modified Eagle medium
Ect2	epithelial cell-transforming sequence 2
EDM	effector domain mutant
EMK	ELKL (Glu-Leu-Lys-Leu) motif kinase
EVOM	epithelial voltohmmeter
FAK	focal adhesion kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FTase	farnesyltransferase
FRET	fluorescence resonance energy transfer
G	glycine
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanine triphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GGTase	geranylgeranyltransferase
Gly	glycine
GSK-3β	glycogen-synthase kinase-3β
GST	glutathione-*S*-transferase

GTP	guanine triphosphate
GTPase	guanosine triphosphatase
GTPγS	guanosine 5'-O-(gamma-thio)triphosphate
HA	hemagglutinin
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid
HRP	horseradish peroxidase
I	isoleucine
ICMT	isoprenylcystine carboxyl methyltransferase
IP	immunoprecipitate
JNK	c-Jun N-terminal kinase
K	lysine
kD	kilodalton
L	leucine
LARG	leukemia-associated Rho guanine exchange factor
LAZ3	lymphoma-associated zinc finger 3
LCM	low calcium media
Lgl	lethal giant larvae
LIMK	Lim kinase
M	methionine
MARK	microtubule-affinity-regulating-kinase
MDCK	Madin Darby canine kidney
MEM	minimum essential medium
Mg	magnesium

MLB	magnesium lysis buffer
MLC	myosin light chain
MLCK	myosin light chain kinase
MMEC	mouse mammary epithelial cell
MMTV	mouse mammary tumor virus
mRNA	messenger RNA
N	asparagine
N-terminus	amino-terminus
N-WASP	N-Wiskott-Aldrich syndrome protein
NCM	normal calcium media
NLS	nuclear localization signal
NP-40	Nonidet P-40
PAK	p21 activated-kinase
Pals1	protein asssociated with Lin Seven 1
Par1	partitioning defective gene 1
Par2	partitioning defective gene 2
Par3	partitioning defective gene 3
Par4	partitioning defective gene 4
Par5	partitioning defective gene 5
Par6	partitioning defective gene 6
PATJ	PALS1-associated TJ protein
PB1	phagocyte oxidase/Bem1
PCR	polymerase chain reaction

PDZ	PSD95/Discs Large/ZO-1
PH	Pleckstrin homology
PI3K	phosphatidylinositol 3 kinase
PtdIns(3,4)P_2	phoshatidiylinositol(4,5)bisphosphate
PtdIns(4,5)P_2	phoshatidiylinositol(4,5)bisphosphate
PtdIns(3,4,5)P_3	phoshatidiylinositol(3,4,5)triphosphate
PKC-3	protein kinase C-3
PKCλ	protein kinase C-lambda
PKCζ	protein kinase C-zeta
PM	plasma membrane
PPARγ	peroxisome proliferator-activated receptor gamma
P/S	penicillin/streptomycin
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene difluoride
Pyk2	proline-rich tyrosine kinase 2
Q	glutamine
R	arginine
Rab	Ras-like proteins in brain
Rac	Ras-related C3 substrate
Ran	Ras-like nuclear
Ras	rat sarcoma
Rce1	Ras converting enzyme 1, CAAX-specific protease
Rho	Ras homologous

RhoBTB	Rho Broad complex, Tramtrack and Bric à brac
Rho GDI	Rho GDP dissociation inhibitor
RING	really interesting new gene
RNAi	interfering RNA
Rnd	round
RRS	Ras recruitment system
RT-PCR	reverse transcriptase-polymerase chain reaction
S	serine
Scrib	Scribble
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	serine
SFRP1	secreted frizzled-related protein 1
SH3	Src homology 3
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMEM	supplemented minimum essential medium
SMURF	Smad ubiquitylation regulatory factor
STK11	serine/threonine kinase-11
T	threonine
TC10	teratocarcinoma 10
TCF	T cell factor
TCL	TC10-like
TER	transepithelial electrical resistance

TGF-β	transforming growth factor-beta
Thr	threonine
TIAM1	T-cell invasion and metastasis gene 1
TJ	tight junction
TNF-α	tumor necrosis factor-α
UBA	ubiquitin-associated
UNC-CH	University of North Carolina at Chapel Hill
V	valine
Val	valine
WASP	Wiskott-Aldrich syndrome protein
WIF	Wnt inhibitory factor
Wnt	Wingless/Int
Wrch-1	Wnt-regulated Cdc42 homolog 1
Y	tyrosine
YFP	yellow fluorescent protein
ZO-1	zonula occludens-1

CHAPTER 1

INTRODUCTION

1.1 Ras superfamily of small GTPases

Small GTPases of the Ras superfamily function as molecular switches to elicit a diverse range of cellular processes through numerous signaling pathways (Mitin et al., 2005; Wennerberg et al., 2005). These cellular processes are initiated by the release of a myriad of extracellular stimuli, such as hormones, growth factors and other signaling molecules, which bind to and activate cell surface receptors capable of transducing signals to promote multiple biological activities. Extensive research over the past several decades has implicated the Ras superfamily of small GTPases as central signaling nodes in regulating such processes as gene transcription, vesicle trafficking, cytoskeleton reorganization, cell survival, and cell cycle progression (Figure 1.1A). It is well appreciated that aberrant activation of Ras small GTPases contributes to multiple human diseases (Schubbert et al., 2007). However, Ras isoforms were originally characterized as viral oncogenes and subsequently discovered to be mutated in 30% of human cancers (Bos, 1989; Chien et al., 1979; Der et al., 1982; Shih et al., 1978). These oncogenic mutations render Ras proteins GTPase-deficient, resulting in constitutive activation and thus, leading to excessive activation of their downstream signaling pathways that promote many of the steps involved in cancer (Wennerberg et al., 2005).

The Ras superfamily of small GTPases includes over 150 GTP-binding proteins, based on their sequence identity and GTP-binding motifs (Wennerberg et al., 2005). Most small GTPases share this biochemical mechanism, allowing them to function as

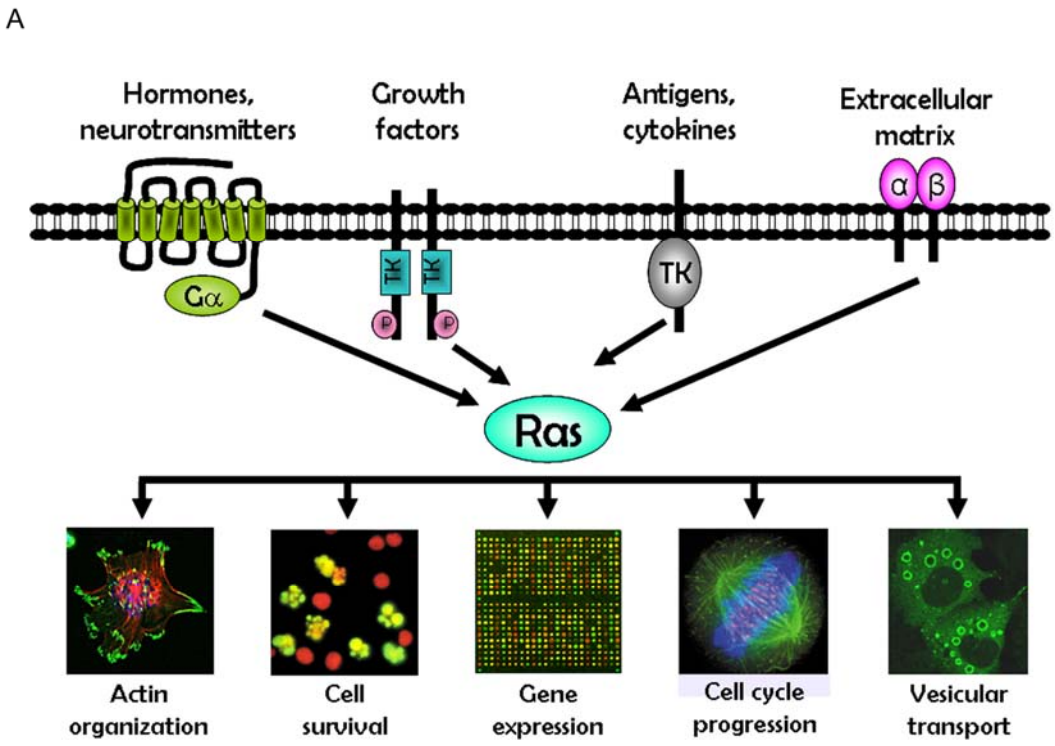


Figure 1.1A Ras superfamily and Rho subfamily of small GTPases.
A) Ras superfamily GTPases are activated by a myriad of extracellular stimuli to elicit diverse biological activities.

B

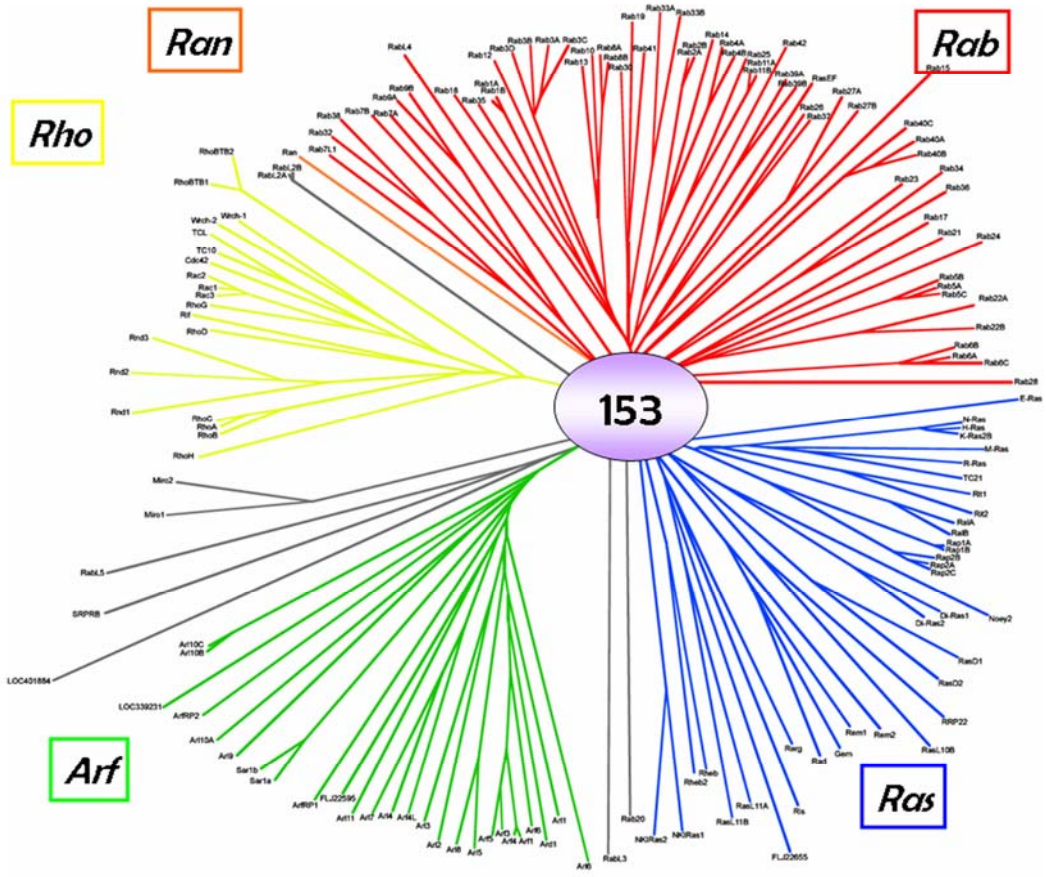
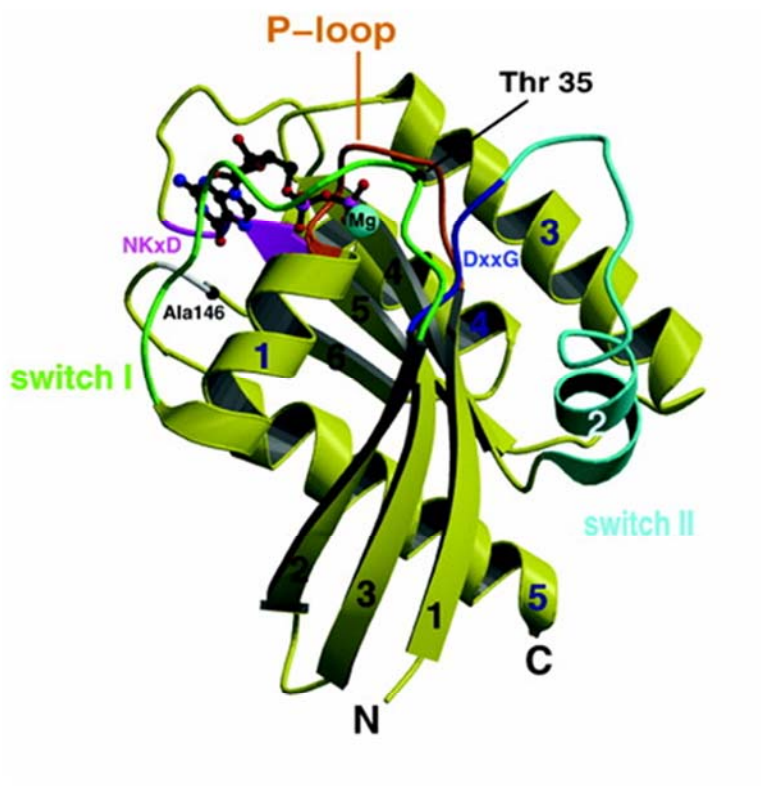


Figure 1.1B Ras superfamily and Rho subfamily of small GTPases.
B) The Ras superfamily of small GTPases can be divided into five major subfamilies (Wennerberg et al., 2005).

C



D

Figure 1.1C & D Ras superfamily and Rho subfamily of small GTPases.
C) 3D structure of small GTPase. Conserved sequence elements and switch regions are indicated by different colors. Mg^{2+} and guanine nucleotide are represented by ball-and stick (Vetter and Wittinghofer, 2001). D) Four conserved sequence elements are essential to GTP binding proteins interactions with nucleotides: GXXXXGKS/T (P-loop), Thr35 within Switch I, DXXG motif within Switch II, and N/TKXD motif. The functions of these motifs are described in the text.

E

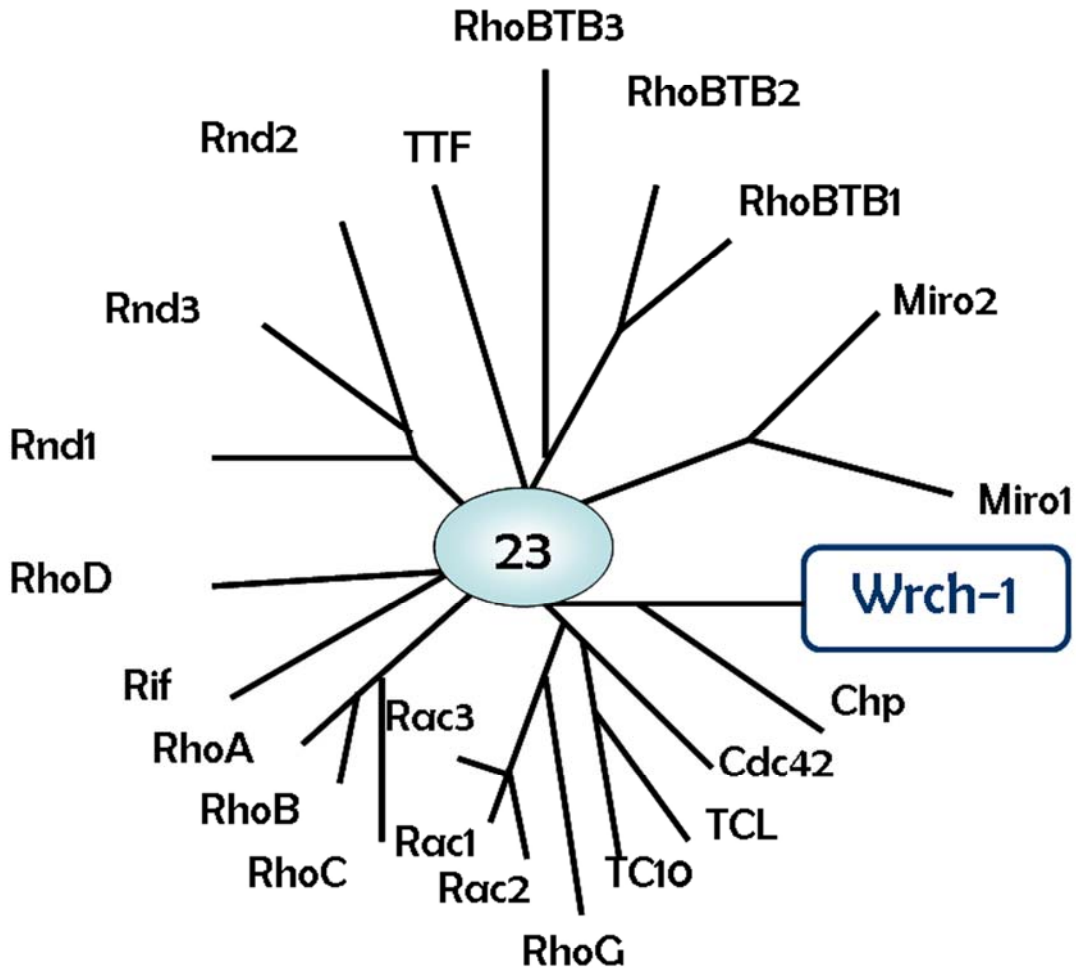


Figure 1.1E Ras superfamily and Rho subfamily of small GTPases.
E) The Rho subfamily of small GTPases dendrogram. Wrch-1 (blue box), an atypical member of the family, belongs to Cdc42 subgroup and is the focus of this dissertation. Adapted from (Ellenbroek and Collard, 2007).

molecular switches cycling between GTP- and GDP-bound states, a feature that is important for their regulation and function (Vetter and Wittinghofer, 2001). When in the GTP-bound state, small GTPases are in an active conformation that allows them to interact with their downstream effectors, which mediate their diverse biological activities in response to extracellular stimuli. In addition to conformational changes and protein regulators of GTP/GDP cycling, small GTPase function is also regulated by posttranslational modification by prenyl and/or palmitoyl lipid moieties. C-terminal modification of small GTPases by prenylation and/or palmitoylation helps them associate with cellular membranes where they can both be activated and associate with downstream effectors (Takai et al., 2001; Wennerberg et al., 2005).

The Ras superfamily of small GTPases can be divided into five subfamilies: Ras, Ran, Rab, Arf, and Rho, based on sequence and functional similarities (Figure 1.1B) (Wennerberg et al., 2005). Despite these similarities, variations in structure, divergent subcellular localizations, and different sets of protein regulators and effectors allow these small GTPases to be integral components in a multitude of cellular functions. The Rat sarcoma (Ras) oncoproteins are the most extensively studied of Ras subfamily members because of their involvement in human cancers, along with normal cell processes such as cell proliferation, differentiation, and survival (Mitin et al., 2005; Repasky et al., 2004). There is one Ras-like nuclear (Ran) protein, which regulates nucleocytoplasmic shuttling of RNA and protein and mediates mitotic spindle assembly, nuclear envelope assembly breakdown, and DNA replication in mammalian cells (Li et al., 2003; Weis, 2003). Ras-like proteins in brain (Rab) GTPases are well known for regulating vesicular transport and protein trafficking between intracellular organelles in the endocytic and secretory pathways (Schwartz et al.,

2007). The ADP-ribosylation factor (Arf) family proteins, like Rab proteins, modulate vesicular transport in the endocytic and exocytic pathways by helping with vesicular coat formation (Gillingham and Munro, 2007). The Ras homologous (Rho) subfamily of GTPases responds to various extracellular stimuli to promote multiple biological activities, but its members are best known for regulating the actin cytoskeleton (Etienne-Manneville and Hall, 2002; Hall, 1998). The Rho subfamily of small GTPases and one of its members, Wrch-1, will be described in detail in the following sections.

1.2 Rho family of small GTPases

Rho GTPases are a subfamily of Ras-related small GTPases that share 30% sequence identity with members of the Ras subfamily and 40-95% sequence identity within the Rho subfamily within their GTPase domain (Wennerberg and Der, 2004). The evolutionarily conserved inclusion of the Rho insert domain distinguishes the Rho family of small GTPases from the other members of the Ras superfamily (Valencia et al., 1991). Like Ras, classic Rho GTPases contain a GTPase domain and short N- and C- terminal extensions. However, many of the less studied Rho GTPases can be classified as atypical members of the family based on their divergent structure, function, and modes of regulation (Aspenstrom et al., 2007). The distinct regulation and biological function of atypical Rho GTPases will be discussed in detail later in this chapter.

Rho GTPases are structurally comprised of five α-helices and six-stranded β-sheets, which contain four or five highly conserved GTPase domain motifs (Figure 1.1C) (Bourne et al., 1991; Vetter and Wittinghofer, 2001). These conserved motifs are instrumental in nucleotide binding and hydrolysis. Specifically, the GXXXXGK(S/T) motif, also known as the P-loop, plays an integral role in binding to the phosphate group of GTP and GDP, while

the N/TKXD motif interacts with the nucleotide base of GTP and GDP and thus coordinates binding and recognition of these nucleotides (Figure 1.1C) (Saraste et al., 1990; Vetter and Wittinghofer, 2001).

Crystallization of small GTPases in the GTP-bound and GDP-bound state has unlocked structural insight into the molecular switch that occurs within the GTPase domain (Sprang, 1997). The two regions within the GTPase domain that undergo conformational change during GTP/GDP exchange and hydrolysis are called switch I (residues 30 to 38 in Ras/Rho) and II (residues 59 to 67 in Ras/Rho) (Milburn et al., 1990) (Figure 1.1C). In the GTP-bound conformation, conserved residues Thr and Gly (Thr35 and Gly60 in Ras) in the switch regions form hydrogen bonds with the γ-phosphate of GTP, to form what is known as the 'loaded spring conformation'. Upon hydrolysis of GTP, the hydrogen bonds are released, allowing the 'spring' to relax, thus forming the GDP-bound conformation. In addition, Thr35 interacts with and coordinates the Mg^{2+} ion that is known to enhance nucleotide binding, while Asp57 and Gly60, part of a conserved DXXG motif, aid in nucleotide specificity and binding (Figure 1.1D) (Vetter and Wittinghofer, 2001). Figure 1.1 contains a schematic diagram that details the domain architecture of Rho GTPases (Figure 1.1D). The intrinsic exchange and hydrolysis of GTPases is slow; therefore protein modulators of GTP/GDP cycling are necessary. Their functions will be discussed later in this chapter.

Further investigation of Rho GTPases has uncovered several other ways to regulate their activity in addition to modulation of GTP/GDP cycling. It is well appreciated that Rho GTPases associate with cellular membranes and that their distinct subcellular localizations dictate their association with downstream effectors and subsequent biological activities. Posttranslational modifications and sequences within the C-terminus of Rho GTPases are

essential to proper subcellular targeting (Michaelson et al., 2001; Wennerberg and Der, 2004). C-terminal modifications and sequences that contribute to the diversity of Rho GTPase localization and function will be highlighted later in this chapter.

The Rho GTPase family is comprised of 23 members that can be divided into six major branches based on sequence homology and function: RhoA-related (RhoA, RhoB, RhoC), Rac-related (Rac1, Rac2, Rac3, RhoG), Cdc42-related (Cdc42, TC10, TCL, Wrch-1, Wrhc-2/Chp), Rnd (Rnd1, Rnd2, Rnd3/RhoE), RhoBTB (RhoBTB1, RhoBTB2, RhoBTB3), and Miro (Miro1, Miro2) (Figure 1.1E). RhoD, Rif and RhoH/TTF are also Rho GTPases but are not stratified into one of the six subfamilies because their primary sequences are divergent. Alternative splicing of Rac1 and Cdc42 encode Rac1b and the brain isoform of Cdc42, respectively, bringing the protein family to 25 distinct proteins (Wennerberg and Der, 2004). As stated previously, some of the Rho GTPases have more divergent structure and function, including the Rnd and RhoBTB subfamilies, RhoH, Wrch-1, and Wrch-2/Chp. Together, these form a group of atypical Rho GTPases (Aspenstrom et al., 2007). The inclusion of the Miro subfamily is controversial in the GTPase field due to their divergence in structure and it has been suggested that they be considered another subfamily within the Ras superfamily. It is well appreciated that Rho GTPases orchestrate diverse cellular processes in variety of mammalian cell types as well as in yeast, flies, and worms (Boureux et al., 2007). Many of the biological functions associated with Rho GTPase activation, from cytoskeletal rearrangement to gene transcription, have been elucidated through studying the three classical family members: RhoA, Rac1, and Cdc42. Through investigation of other family members of Rho GTPases, such as Wrch-1, Rnd3, and RhoBTB, various other

signaling pathways and biological functions for this family of proteins could potentially be uncovered.

The founding members of the Rho subfamily of GTPases, RhoA, Rac1, and Cdc42, are activated in response to extracellular stimuli and subsequently induce dynamic rearrangement of the actin cytoskeleton to regulate cell morphology and polarity (Hall, 1998). Specifically, constitutive activation of RhoA leads to the formation of contractile actin-myosin filaments better known as stress fibers (Ridley and Hall, 1992). However, membrane ruffling and lamellipodia formation at the plasma membrane are characteristics of Rac1 activation (Ridley et al., 1992). Cdc42 activation induces filopodia, which are specific actin filament protrusions at the plasma membrane (Kozma et al., 1995; Nobes et al., 1995). These effects on the actin cytoskeleton are mediated by diverse signaling pathways upstream and downstream of each Rho family GTPase to promote cell polarity, cell motility and cell morphology (Nobes and Hall, 1995). Rho GTPase modulation of actin organization in different organisms and cell types will be highlighted later in this chapter.

More in-depth characterization of members of the Rho GTPase family within the past decade spurred the identification of several other signaling pathways regulated by Rho GTPases that are independent of their role in actin cytoskeleton regulation. Specifically, these GTPases regulate gene transcription, cell cycle progression, microtubule dynamics, vesicular transport, and a variety of enzymatic activities (Jaffe and Hall, 2005). Although these other biological activities will not be discussed here, the complexity of the biological activities that Rho GTPases elicit highlights the importance of the many branches of Rho GTPases in cell function. The Cdc42 branch of Rho GTPases will be highlighted here, given that Wrch-1, the focus of this dissertation, is one of the atypical members of this subgroup.

1.3 Regulators of Rho GTPase GTP/GDP cycling

Like other members of the Ras superfamily, Rho GTPases function as molecular switches, cycling between an active, GTP-bound state and inactive, GDP bound state. When GTP-bound, Rho GTPases elicit various biological functions through interaction with their downstream effectors (Figure 1.2). Their slow intrinsic GTPase activity turns Rho family proteins off to their GDP-bound state, thus terminating their signaling capabilities. However, members of the Rnd subfamily and RhoH are chronically GTP-bound in their wild type forms (Foster et al., 1996; Li et al., 2002; Wennerberg and Der, 2004). Since GTP-bound Rho GTPases mediate diverse cellular functions, tight regulation of their GTP/GDP cycling is necessary for normal cellular homeostasis.

Specific somatic and germline missense mutations in different Ras isoforms lock the GTPases in their active, GTP-bound state and promote human developmental disorders and cancer, further highlighting the importance of cellular mechanisms to inactive these proteins. In human cancers harboring Ras mutations, the affected codons are usually found at positions G12, G13, and Q61, while mutations at other codons have been discovered in developmental disorders (Bos, 1989; Schubbert et al., 2007). Point mutations of Ras genes found in 30% of human cancers disrupt their intrinsic GTPase activity and render these proteins insensitive to their protein regulators (Schubbert et al., 2007).

The discovery of oncogenic mutations in Ras has led to the use of analogous laboratory-generated mutants of Rho GTPases (G12V or Q61L in Ras, Rac1, RhoA, and Cdc42) to lock them in the GTP-bound state. Other mutations, such as G15A (nucleotide-free) and T17N (GDP-bound), cause the proteins to function as dominant negatives by

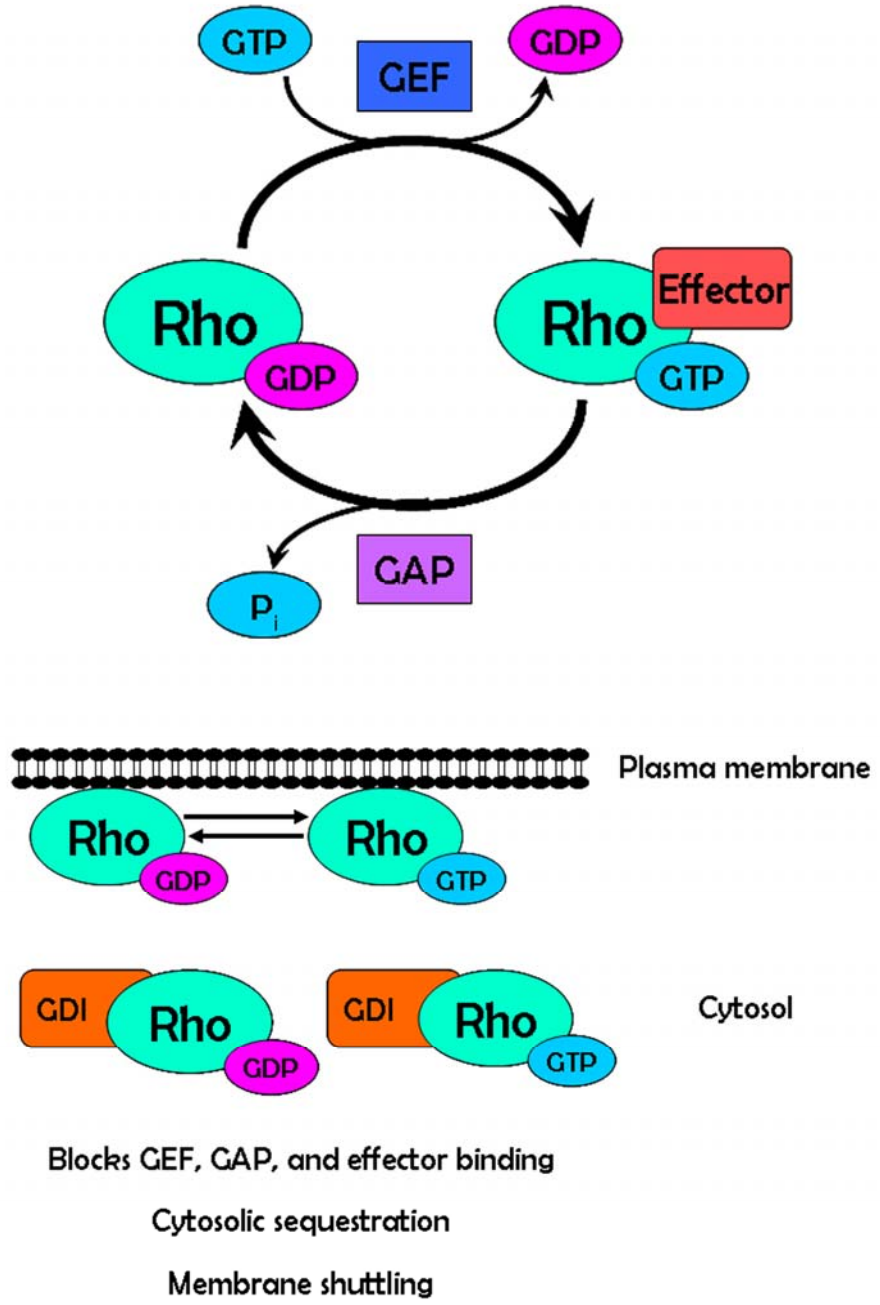


Figure 1.2 Rho GTPases function as molecular switches.
Rho GTPases function as molecular switches cycling between an active, GTP-bound state and inactive, GDP bound state. GEFs and GAPs tightly regulate the nucleotide status of GTPases. Rho GDIs negatively regulate Rho GTPase function by interfering with GEF, GAP, and effector binding or by shuttling Rho GTPases from the plasma membrane to cytosol or other internal membranes.

sequestering Rho GEFs, thereby inhibiting the activation of their endogenous wild type counterparts. These constitutively active and dominant negative mutants of Rho GTPases have been used to study the biological effects of Rho GTPase activation and inactivation. In my studies, I used mutations in Wrch-1 (Q107L and T63N) analogous to activating and dominant negative mutations in Cdc42 (Q61L and T17N) to help delineate the biological functions of Wrch-1. Naturally occurring activating mutations have yet to be identified in Rho GTPases. However, alterations in expression of Rho GTPases themselves or of their positive and negative regulators contribute to tumorigenesis (Sahai and Marshall, 2002).

Three classes of regulatory proteins facilitate GTP/GDP cycling of Rho GTPases: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (Figure 1.2) (Wennerberg and Der, 2004). GEFs accelerate the release of GDP and subsequent binding of GTP to GTPases by inducing specific conformational changes within their switch regions (Figure 1.2). These changes allow for high affinity interaction of Rho GTPases with their effectors, leading to downstream signaling (Schmidt and Hall, 2002). To date there are two distinct groups of Rho GEFs. The largest group of Rho GEFs comprises the Dbl family, characterized by tandem Dbl homology (DH) and Pleckstrin homology (PH) domains that are important for their exchange activity. The central dogma of Dbl family activation of Rho GTPases is that the PH domain interacts with phospholipids, which promotes the proper localization and subsequent activation of the catalytic DH domain through allosteric mechanisms. There are 69 human Dbl family Rho GEFs that contain the tandem DH/PH cassette; these have divergent domain architecture in regions outside of this cassette that reflect the diversity of signals and cellular functions mediated by activated Rho GTPases (Rossman et al., 2005).

The second group of Rho GEFs consists of 11 proteins that form the Dock family (Cote and Vuori, 2007). Members of the Dock family of GEFS do not contain the catalytic DH domain but are comprised of two regions of high sequence conservation, Dock homology region-1 and -2 (DHR-1 and DHR-2). The DHR-2 domain alone or in combination with its partner protein Elmo catalyzes nucleotide exchange, while the DHR-1 domain is structurally similar to C2 domains known to interact with phospholipids (Meller et al., 2005). The vast number of Rho GEFs highlights the diversity of signaling inputs capable of activating Rho GTPases to elicit their biological activities.

As stated previously, the GTPase activity of GTP-binding proteins is intrinsically slow and thus mechanisms to accelerate their GTPase activity are necessary to terminate their signaling outputs. GAPs increase the slow intrinsic GTP hydrolysis of GTPases and thus promote the formation of the GDP-bound, inactive state (Figure 1.2) (Vetter and Wittinghofer, 2001). GAPs for Rho GTPases contain a major structural element, the 'arginine finger', which promotes their shared mechanism of action (Moon and Zheng, 2003). When GAPs interact with a GTP-bound GTPase, this 'arginine finger' present in the GAP is inserted into the active site of the GTPase to facilitate the transition from the GTP-bound state to a nucleotide-free state and subsequent reloading with GDP (Lamarche and Hall, 1994; Vetter and Wittinghofer, 2001). The human genome contains 160 potential GAPs for the Ras superfamily of GTPases, with roughly 70 of these identified proteins being GAPs for the Rho subfamily alone (Bernards, 2003). Like the numerous GEFs, the existence of such a copious number of GAPs for the small number of Rho GTPases points out the importance of regulating their activation and inactivation in response to diverse extracellular stimuli.

The third class of regulatory protein is that of the GDIs, which were first characterized as inhibitors of nucleotide exchange, thus blocking effector and GAP binding of GTP-bound Rho GTPases (Chuang et al., 1993; DerMardirossian and Bokoch, 2005; Fukumoto et al., 1990). In addition, Rho GDIs recognize prenyl groups, which enables them to regulate the membrane association and dissociation of prenylated Rho family members, effectively blocking their activity (Figure 1.2) (Michaelson et al., 2001). There is distinct binding specificity for different Rho GTPases to the three known human Rho GDIs (RhoGDIα/GDI1, Ly/D4GDIβ/GDI2, and RhoGDIγ/GDI3). Rho GDI-mediated masking of the prenyl group on Rho GTPases functions to sequester Rho proteins to the cytosol, preventing membrane association and downstream biological consequences (Dovas and Couchman, 2005; Hoffman et al., 2000).

Although these three distinct classes of regulators for Rho GTPases have proven to be fundamental in regulating their GTP/GDP cycling, several Rho GTPases are not regulated in this manner. As stated previously, these proteins fall into an atypical subgroup of Rho GTPases and include the Rnd and RhoBTB subfamilies, RhoH, Wrch-1, and Chp. Evidence for the existence of GEFs and GAPs for atypical Rho GTPases is lacking. In contrast to classic Rho GTPases that are regulated by GEFs, GAPs and GDIs, the atypical Rho GTPases are often regulated by their level of expression or additional domains involved in protein-protein interactions (Aspenstrom et al., 2007). RhoH, Rnd proteins, and RhoBTB proteins harbor "activating" amino acid substitutions at positions important for GTP hydrolysis, rendering these proteins GTPase-deficient (Aspenstrom et al., 2007; Chardin, 2006; Li et al., 2002). However, Wrch-1 and Chp, members of the Cdc42 subfamily of GTPases, do not contain activating mutations that render them GTPase-deficient. Although Wrch-1 possesses

a rapid rate of nucleotide exchange, it was shown to be predominantly in the GDP-bound conformation *in vivo* (Jordan et al., 1999; Saras et al., 2004; Shutes et al., 2004). Whether Chp, like Wrch-1, has an enhanced nucleotide exchange rate remains to be investigated since detailed biochemical analysis has not been done.

1.4 Regulation of Rho GTPase localization by C-terminal modification

GTPase function and activity can be regulated in several other ways. Specifically, Rho GTPase location within a cell is important for interactions with effectors, which is also dependent upon association with membranes (Cox and Der, 1992). Membrane association of GTPases is primarily regulated by posttranslational C-terminal lipid modification. Posttranslational lipid modifications of Rho GTPases occur within their C-terminal tetrapeptide CAAX motifs and/or hypervariable regions. These modifications are not exclusive to the Rho GTPases; Ras and Rab family proteins are also posttranslationally modified (Khosravi-Far et al., 1992). One lipid modification, prenylation, is made at the cysteine residue of the CAAX motif for most members of the Rho GTPase family. Some Rho GTPases are irreversibly modified by a farnesyl isoprenoid (C15), while others are modified by a geranylgeranyl isoprenoid (C20) (Figure 1.3A) (Fu and Casey, 1999; Wennerberg and Der, 2004). The amino acid at the X position within the CAAX motif dictates whether the Rho GTPase will be a substrate for isoprenylation by farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase I) (Figure 1.3B). For example, if the X amino acid of the CAAX motif is an L or F amino acid, then the GTPase is a substrate for GGTase I, as seen in Cdc42, Rac1, Rac2, Rac3, RhoA, RhoB, and RhoC (Cox, 1995). In contrast, termination of the CAAX motif in an S, M, Q, or A is indicative of modification by FTase as seen in Rnd proteins. However, some members of the Rho subfamily, such as

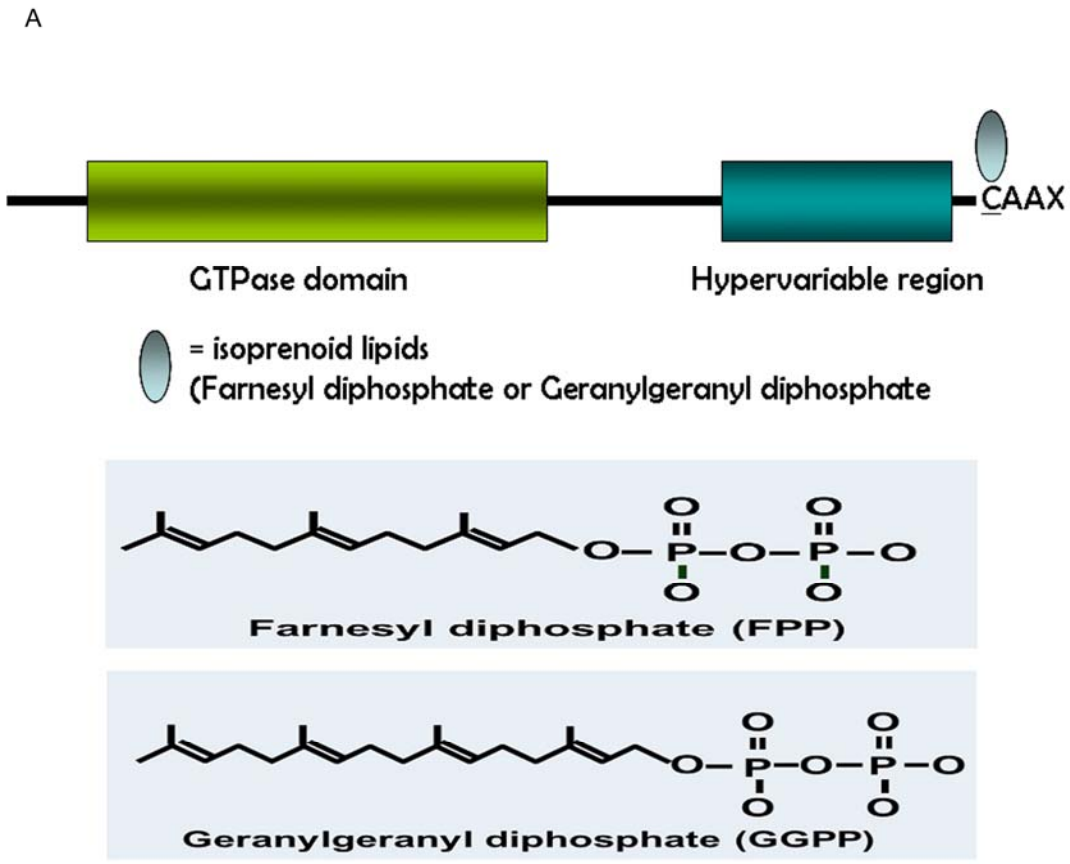


Figure 1.3A C-terminal modifications and sequences regulate Rho GTPase membrane targeting.

A) C-terminal CAAX motif and hypervariable region dictates membrane localization and function. The cysteine residue within CAAX motif is irreversibly modified by C15 farnesyl or C20 geranylgeranyl isoprenoid lipids.

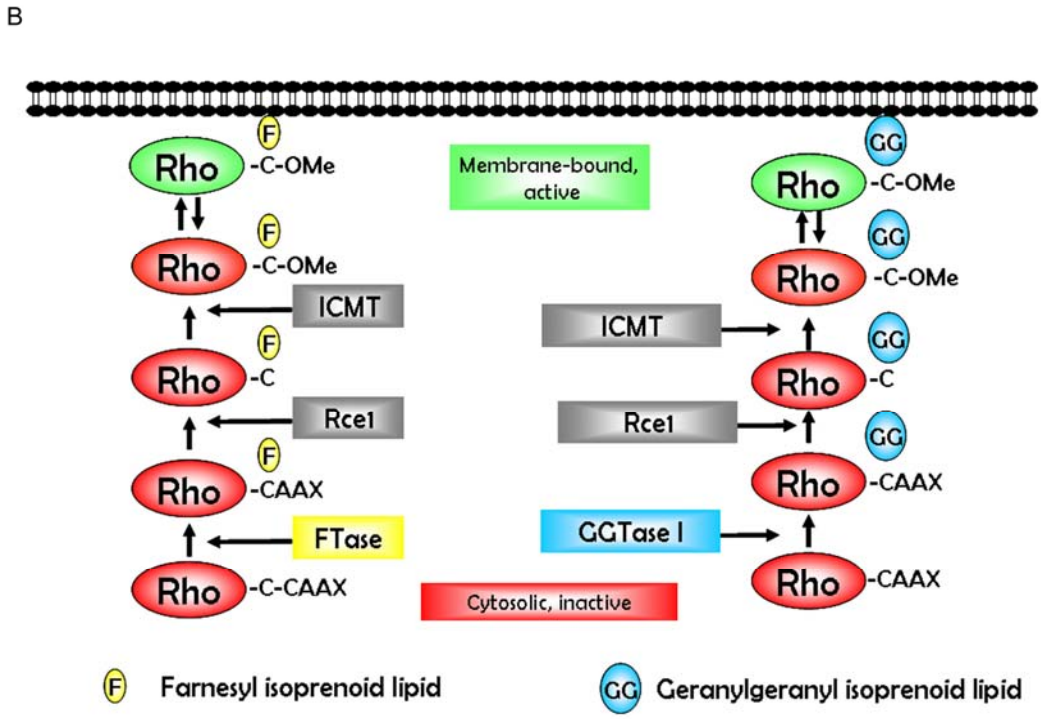


Figure 1.3B C-terminal modifications and sequences regulate Rho GTPase membrane targeting.

B) Most Rho GTPases are modified within their C-termini by FTase or GGTase that irreversibly attach farnesyl or geranylgeranyl isoprenoids. The –AAX within the CAAX motif is proteolytically cleaved by Rce1 and followed by methylation of cysteine residue by ICMT to promote proper membrane association.

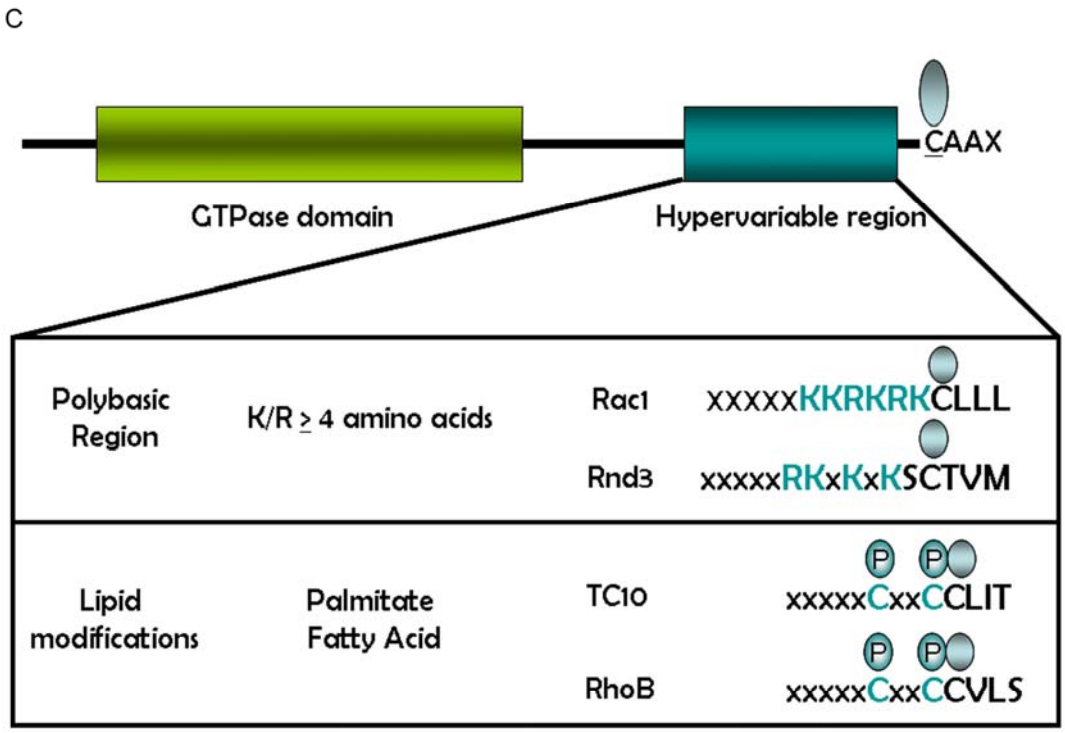


Figure 1.3C C-terminal modifications and sequences regulate Rho GTPase membrane targeting.

C) The hypervariable region contains secondary membrane signals that increase the membrane affinity of Rho GTPases: stretch of basic amino acids (lysine,K or arginine,R) or upstream cysteine residue that can be reversibly modified by palmitate fatty acid.

TC10 and TCL, terminate in alternate residues, making it difficult to predict whether the protein will be geranylgeranylated or farnesylated or are devoid of a functional CAAX motif altogether, as are RhoBTB1/2 and Miro subfamily proteins (Table 1.1) (Wennerberg and Der, 2004).

After prenylation, two subsequent enzymatic modifications of prenylated Rho GTPases are necessary for their proper localization and function. First, the –AAX of the CAAX motif is proteolytically cleaved by the protease Rce1, and next the cysteine residue of the CAAX motif is methylated by isoprenylcysteine carboxyl methyltransferase (ICMT) (Ashby et al., 1992; Otto et al., 1999). This prenylated, clipped, and methylated small GTPase is fully processed and can be inserted into cellular membranes where GTPases interact with their downstream effectors (Figure 1.3B). However, processing by Rce1 and ICMT has been reported to be necessary for proper localization of geranylgeranylated GTPases but not farnesylated GTPases (Michaelson et al., 2005). These experiments were completed with a subset of the Ras and Rho superfamily of GTPases; further investigation of the necessary posttranslational modifications for other members of the family remains to be determined.

The region of least sequence identity among the the Ras superfamily of proteins, found at their C-termini, is referred to as the hypervariable domain, and this region is both necessary and sufficient for membrane association (Hancock et al., 1990; Michaelson et al., 2001). The hypervariable region consists of approximately the last 20 amino acids, which includes the CAAX motif and a second membrane targeting signal, although it is longer in some of the atypical Rho GTPases. Rho GTPases vary significantly within their C-terminal hypervariable regions, and these divergent membrane targeting sequences account for their

GTPase	CAAX	F or GG	K/R(++) or P
RhoA	CLVL	GG	K/R(++)
RhoB	CKVL	F GG	P
RhoC	CPIL	GG	K/R(++)
Rac1	CLLL	GG	K/R(++)
Rac2	CSLL	GG	K/R(++)
Rac3	CTVF	GG	K/R(++)
RhoG	CILL	GG	K/R(++)
Cdc42	CVIL	GG	K/R(++)
TC10	CLIT	F	P
TCL	CSIT	?	P
Wrch-1	X	X	P K/R(++)
Chp	X	X	P K/R(++)
Rnd1	CSIM	F	K/R(++)
Rnd2	CNLM	F	K/R(++)
Rnd3/RhoE	CTVM	F	K/R(++)
RhoD	CVVT	GG	K/R(++)
Rif	CLLL	GG	K/R(++)
RhoH/TTF	CKIF	GG	K/R(++)

Table 1.1 C-terminal lipid modifications and sequences that contribute to Rho GTPase localization.
Most Rho GTPases terminate in a CAAX motif where the X=L, which indicates geranylgeranylation of the cysteine residues of the CAAX. The Rnd proteins terminate in X=M, which indicates farnesylation of the cysteine residue of the CAAX motif. TC10 is also a substrate for farnesylation, while RhoB can be alternatively prenylated by FTase or GGTaseI. The hypervariable region with in the C-termini of Rho GTPases usually contains a second signal for membrane association. This second signal for membrane targeting is either the addition of a palmitate to a cysteine upstream of the CAAX motif or a lysine/arginine rich sequence (polybasic region) upstream of the CAAX motif. Wrch-1 and Chp are not prenylated but are modified by palmitoylation and contain a polybasic region.

diverse subcellular localizations (Michaelson et al., 2001; Wennerberg and Der, 2004). For example, RhoA, RhoB, and RhoC have high sequence identity within their GTP and effector binding regions, but their divergent C-terminal sequences direct these GTPases to distinct subcellular localizations that allow them to use overlapping groups of effectors to mediate different cellular functions (Wennerberg and Der, 2004; Wheeler and Ridley, 2004). The second signal for membrane targeting is either the addition of a palmitate fatty acid to a cysteine upstream of the CAAX motif or a lysine/arginine rich sequence (polybasic region) upstream of the CAAX motif (Figure 1.3C). For example, TC10 and RhoB are known to be palmitoylated at upstream cysteines within their hypervariable regions in addition to being prenylated at the cysteine in their CAAX motifs. Addition of a palmitate prevents RhoGDI interaction, which also contributes to differences in localization (Michaelson et al., 2001). The polybasic region increases membrane affinity by altering electrostatic interactions. Mutation of the upstream palmitoylated cysteines of TC10 and RhoB to serine, or of the hypervariable region basic residues of Rac1 to alanine, prevents these Rho GTPases from associating with specific cellular membrane domains (Adamson et al., 1992; Michaelson et al., 2001; van Hennik et al., 2003; Watson et al., 2003). These data highlight the requirement of a second signal in addition to prenylation within the CAAX motif to direct Rho GTPases to their proper localization. Table 1.1 illustrates which members of the Rho GTPase family are prenylated (GG or F), palmitoylated, and/or contain a polybasic region, and highlights the complexity of Rho GTPase membrane association and subsequent subcellular localization. Interestingly, Wrch-1 and Chp are not prenylated but are instead substrates for acylation by C16 palmitoyl fatty acids (Berzat et al., 2005b; Chenette et al., 2005). The sequence

elements required for the membrane association of Cdc42 subfamily members will be discussed below.

The polybasic region of the Rho GTPase, Rac1, has been shown to promote its nuclear localization (Lanning et al., 2004). Several other Rho GTPases contain a canonical NLS consensus sequence, K/(K/R)X(K/R), but whether these proteins also localize to the nucleus remains to be determined. Wrch-1, the focus of this dissertation, contains a canonical NLS, and fully processed GFP-Wrch-1 has been visualized in the nucleus (Yoel Kloog, personal communication). However, the significance of Wrch-1 nuclear localization to its function in cellular processes remains to be determined.

1.5 Cdc42 subfamily of Rho GTPases

The founding member of the Cdc42 subfamily of Rho GTPases, Cdc42 was first identified in a screen for genes that regulate cell division, as a gene essential for normal bud site orientation in dividing *Saccharomyces cerevisiae* cells, hence "cell division cycle protein 42" (Adams et al., 1990; Johnson et al., 1987; Johnson and Pringle, 1990). The human homolog was first characterized as a protein downstream of receptor tyrosine kinase signaling (Hart et al., 1990; Shinjo et al., 1990). Further investigation into the function of the newly identified GTP-binding protein Cdc42 led to the discovery that activated Cdc42 drives cell polarity formation by remodeling the actin cytoskeleton in yeast and induces the formation of filopodia, actin microspikes, in mammalian cells (Kozma et al., 1995; Li et al., 1995). Interestingly, two isoforms of Cdc42 exist in both mice and humans, generated by alternative exon splicing (Marks and Kwiatkowski, 1996; Nicole et al., 1999). However, the most commonly studied isoform, Cdc42p, is ubiquitously expressed, whereas Cdc42b expression is restricted to the brain.

The Cdc42 subfamily of Rho GTPases also includes four other GTP-binding proteins, Teratocarcinoma 10 (TC10), TC10-like (TCL), Wnt-regulated Cdc42 homolog-1 (Wrch-1), and Cdc42 homolog protein (Chp), also known as Wrch-2 (Wennerberg and Der, 2004). All members of the Cdc42 subfamily of Rho GTPases are known to induce filopodia formation. Cdc42 subfamily regulation of filopodia formation is most likely through interaction with Wiscott-Aldrich syndrome proteins (WASPs) that modulate actin nucleation and polymerization through binding to the Arp2/3 complex (Miki et al., 1998; Symons et al., 1996). Interestingly, Wrch-1 does not interact with WASP proteins, although it has been shown to produce filopodia (Aspenstrom et al., 2004). This suggests that Wrch-1 utilizes a distinct set of effector proteins for this function and that identification of these effectors would be a fruitful avenue of future research. Further discussion of Wrch-1-mediated regulation of actin dynamics will be discussed in sections 1.6 and 1.10, and in Chapter 3. In addition to Cdc42 regulation of actin cytoskeleton and cell shape, GTP-bound Cdc42 modulates cell polarity, gene expression, vesicular trafficking, and cell cycle progression (Erickson and Cerione, 2001).

Interestingly, the loss of Cdc42 in fibroblastoid cells does not result in defects in filopodia formation, directed cell migration or cell polarization, suggesting that one or more other proteins compensate for the activity of Cdc42 (Czuchra et al., 2005). In addition, expression of dominant negative Cdc42 in *cdc42*-null fibroblastoid cells does not inhibit filopodia formation. These data highlight the importance of Cdc42-independent pathways to induce filopodia formation; these could include activation of the other Cdc42 subfamily members, TC10, TCL, Chp, and Wrch-1 by additional GEFs that are not sequestered by dominant negative Cdc42. In contrast, ablation of Cdc42 expression in *Drosophila*

hemocytes disrupted polarized cell migration and increased cell migration (Stramer et al., 2005). Expression of dominant negative Cdc42 in *Drosophila* hemocytes induces similar migratory defects as ablation of the *cdc42* gene in these same cells. These data suggest that *Drosophila* do not express another Rho GTPase gene capable of compensating for the loss of Cdc42 functions. These investigations potentially address the reason why genes encoding the other members of the Cdc42 family, TC10, TCL, Wrch-1 and Chp, were selected for in higher organisms: to regulate cellular processes by Cdc42-independent mechanisms in order to expand the repertoire of regulatory possibilities and to provide additional robustness to complex systems.

The five Cdc42 family GTPases share high sequence identity within their GTP- and effector binding regions but are divergent within their Rho insert and C-terminal hypervariable regions (Figure 1.4A). The effector binding region is responsible for interacting with downstream binding partners of Rho GTPases in their GTP-bound state and functions to elicit their biological activities. Since the effector binding region is highly homologous among the Cdc42 subfamily members, these proteins can interact with many of the same effectors (Figure 1.4B) (Aspenstrom et al., 2004).

Of the approximately 30 known effectors for Rho, Rac, and Cdc42, 20 of these proteins are predicted to interact with Cdc42 (Cotteret and Chernoff, 2002). The specificity of effector binding to Cdc42 subfamily members over other Rho GTPases is encoded in defined sequences within both the GTPase and the effector itself. These effector proteins have multiple functions in cells, from protein and lipid kinases to scaffolds that help elicit the numerous cellular processes modulated by activated Cdc42. One sequence shared among many of the Cdc42 effectors is the Cdc42/Rac interactive binding (CRIB) domain that is

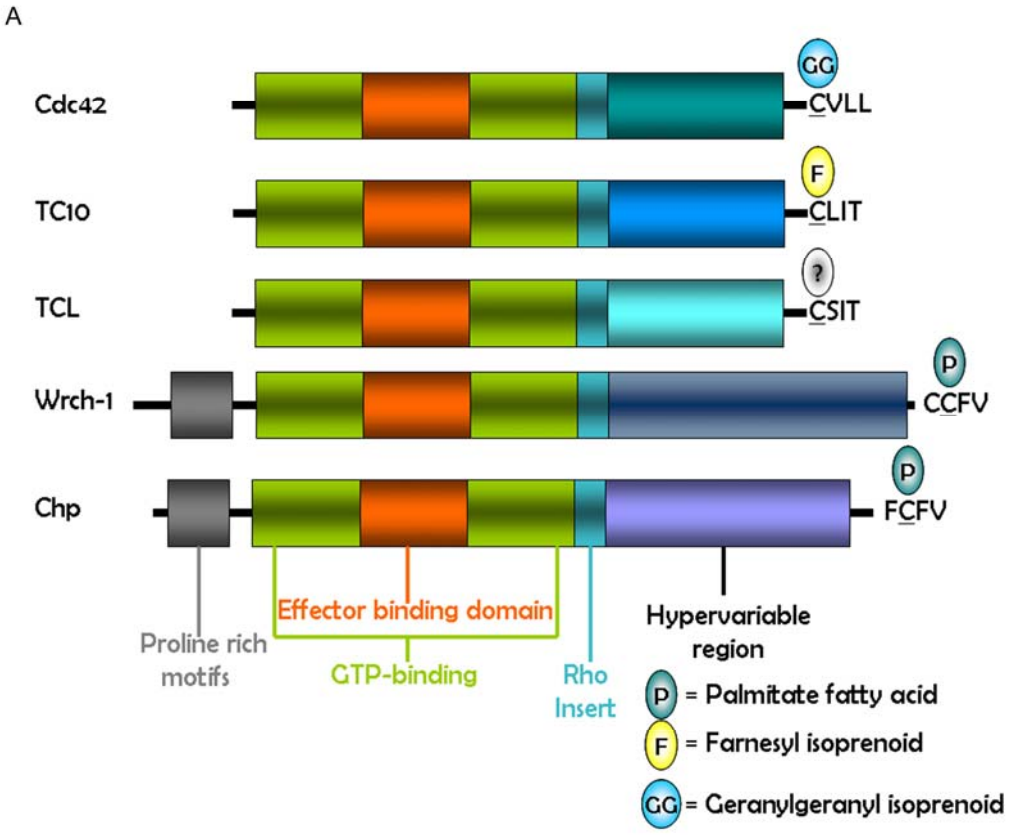


Figure 1.4A Cdc42-related proteins share sequence similarities and effector interactions with Cdc42.

A) Cdc42 subfamily of Rho GTPases consists of 5 GTP-binding proteins that are most similar within their GTP-binding region, effector domain, and Rho insert domain. Divergent sequences and modifications within their C-terminal hypervariable regions and CAAX motifs dictate different membrane localization and function.

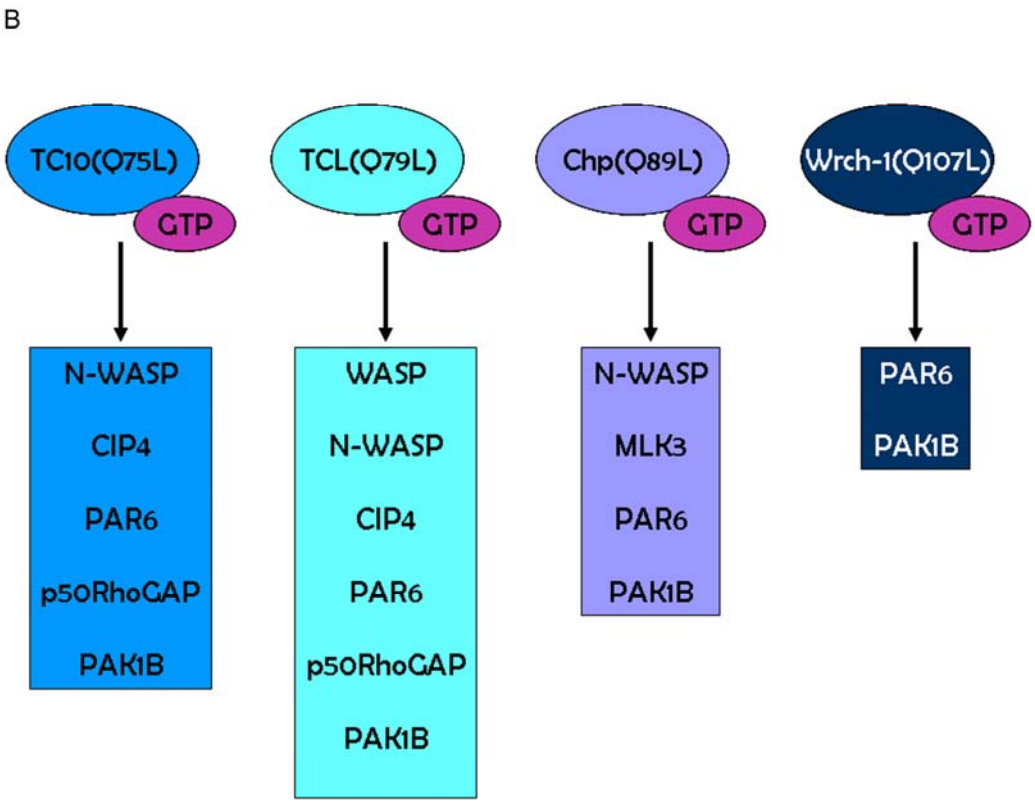


Figure 1.4B Cdc42-related proteins share sequence similarities and effector interactions with Cdc42.
B) Results of yeast two-hybrid assays for the interaction of Cdc42 subfamily members with known Cdc42-binding proteins suggest divergent functions for these highly related proteins (Aspenstrom et al., 2004).

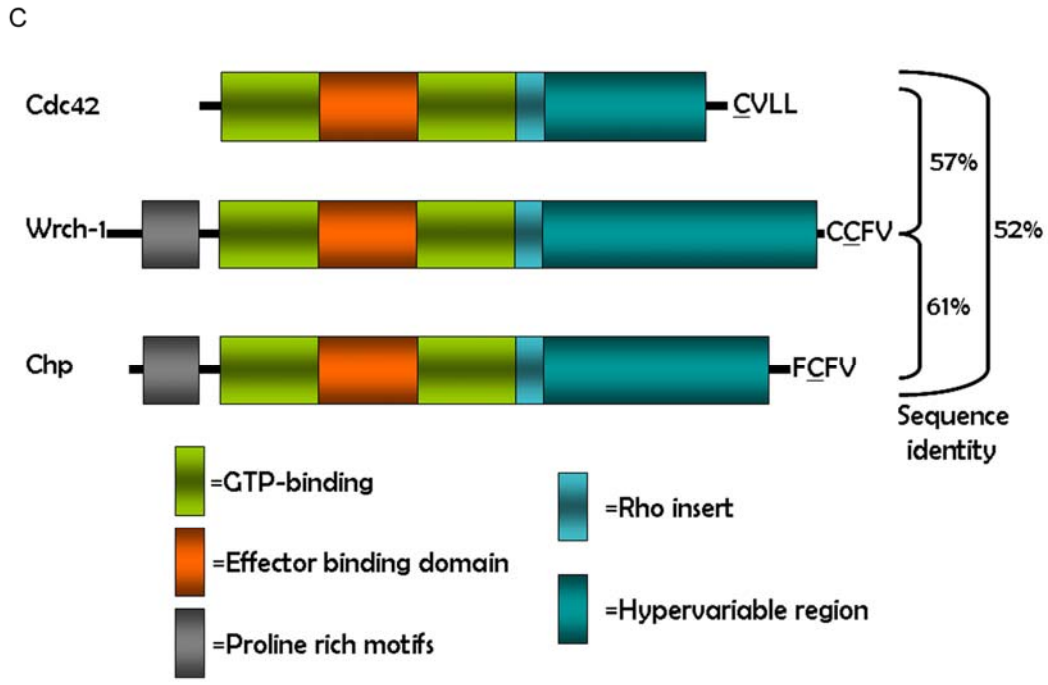


Figure 1.4C Cdc42-related proteins share sequence similarities and effector interactions with Cdc42.
C) Wrch-1 and Chp are homologous to Cdc42 but contain N-terminal proline rich motifs and long C-terminal extensions.

required for interacting with residues 26-48 of Cdc42 (Bishop and Hall, 2000; Cotteret and Chernoff, 2002). As indicated in Figure 1.4B, the other members of the Cdc42 subfamily exhibit differential association with many of the known Cdc42 effectors, as determined by yeast two hybrid screen (Aspenstrom et al., 2004) (Figure 1.4B). Obviously, these screens did not contain all known Cdc42 effectors and no functional studies were done. Therefore, future investigation to elucidate other binding partners for Cdc42 itself and other members of the Cdc42 subfamily may prove to be beneficial for deciphering their divergent biological functions. This introduction will focus on one specific CRIB domain-containing protein scaffold, Par6, and its biological functions and regulation by Rho GTPases. In addition, I will examine Rho GTPase regulation of the actin cytoskeleton, cell junctions, and epithelial morphogenesis and the downstream effectors and signaling pathways instrumental in these cellular processes in sections 1.9, 1.10, and 1.11. For my dissertation studies, I chose to investigate the GTP-dependent interaction between the atypical Rho GTPase, Wrch-1, and Par6, and the Wrch-1-mediated biological activities that require this association, as described in Chapters 2 and 3.

The sequence divergences found in the Rho insert regions and C-terminal hypervariable regions of Cdc42 subfamily members dictate divergent membrane localization (Figure 1.4A) (Michaelson et al., 2001). The divergent membrane localization of Cdc42-related proteins in turn suggests their use of distinct effectors due to differential availability at different membrane sites and consequently distinct biological activities for these otherwise highly related proteins. Specifically, Cdc42p and Cdc42b are found in the cytosol, and associate with the Golgi and perinuclear regions, and weakly localize to the plasma membrane. In contrast, TC10 and TCL localize to internal membrane vesicles and the

plasma membrane (Aspenstrom et al., 2004; Michaelson et al., 2001). The explanation for the distinct membrane localizations of these proteins could be two-fold, based on their C-terminal lipid modifications. First, Cdc42 is exclusively geranylgeranylated, while TC10 is farnesylated. Second, the incorporation of a palmitoyl group at a cysteine found upstream from the CAAX motif in TC10 but not in Cdc42 prevents association with Rho GDI and subsequent cytosolic sequestration (Michaelson et al., 2001). To date it is not known whether TCL is prenylated by FTase or GGTase I, although its CAAX motif (CSII) suggests that it may be a substrate for both.

Chp differs from other Rho GTPases because it lacks a conventional C-terminal CAAX motif (FCFV) and is not prenylated, but like TC10 and TCL it associates with both the plasma membrane and internal membranes. Chp membrane localization is dependent upon reversible C-terminal palmitoylation at the C-terminal cysteine residue (Chenette et al., 2005). Since palmitoylation is a reversible process it may serve as a potential mechanism to prevent chronic membrane association of Chp and TC10, analogous to Rho GDI sequestration of Cdc42. An in-depth description of Wrch-1 C-terminal modifications that dictate its subcellular localization and that regulate its function will be discussed in the following section of this chapter.

TC10 and TCL are highly related members of the Cdc42 subfamily of Rho GTPases that have overlapping function with the small GTPase Cdc42. However, the regulators, membrane localization and effector molecules for TC10 and TCL are distinct from those of Cdc42 (Murphy et al., 1999; Neudauer et al., 1998; Vignal et al., 2000). No known Rho GEFs of the Dbl family have yet been identified that regulate exchange of GDP for GTP on either TC10 or TCL. Interestingly, the Rap GEF C3G has been shown to activate TC10 in

response to insulin signaling in adipocytes (Chiang et al., 2001). This activation of TC10 promotes TC10-mediated translocation of GLUT-4 to the plasma membrane and drives adipocyte differentiation (Chiang et al., 2001; Kanzaki and Pessin, 2001). Like TC10, TCL is also involved in adipocyte differentiation through activation of PPARγ signaling pathway (Nishizuka et al., 2003). In addition to their roles in adipocyte differentiation, TC10 and TCL both induce neurite outgrowth and are upregulated during neurite differentiation and regeneration (Abe et al., 2003; Tanabe et al., 2000).

The most recently identified members of the Cdc42 subfamily of Rho GTPases, Wrch-1 and Chp/Wrch-2, contain N-terminal and C-terminal extensions when compared to Cdc42 (Figure 1.4C). Wrch-1 and Chp respectively share 57% and 52% sequence identity with Cdc42, and 61% identity with each other (Aronheim et al., 1998; Tao et al., 2001). Chp was first identified in a novel two hybrid system, the Ras recruitment system (RRS), for proteins that interact with the serine/threonine kinase p21 activated kinase 2 Pak2 (Aronheim et al., 1998). Like Cdc42, activated Chp interacts with Pak1 and N-WASP, induces filopodia formation, and transforms NIH 3T3 cells (Aronheim et al., 1998; Aspenstrom et al., 2004; Chenette et al., 2005). Further analysis of Chp activation revealed that TNF-α activates Chp only at the endosomal compartment, suggesting that spatial regulation of Chp-stimulated JNK activation is required (Chenette et al., 2006). However, the cellular processes downstream of TNF-α-mediated activation of Chp at endosomes and subsequent JNK activation remain to be determined. In addition, the GEFs, GAPs, and other upstream signals and downstream effectors that help Chp elicit its biological functions have not been elucidated and warrant further investigation. In the following section, I will discuss the regulation, binding partners, membrane association, and biological functions for the other

atypical member of the Cdc42 family, Wrch-1, and highlight what is yet to be determined about Wrch-1 to address why the work in this dissertation was undertaken.

1.6 Wrch-1, an atypical member of the Cdc42 subfamily of Rho GTPases

Wrch-1 was initially discovered in a screen for Wnt-1 responsive genes that may be relevant in Wnt-1 signaling and in Wnt-1-mediated cellular transformation and tumor formation. Specifically, Wrch-1 expression was rapidly induced upon Wnt-1 signaling and was upregulated in both Wnt-1-mediated morphologically transformed C57MG mammary epithelial cells and MMTV-Wnt-1 transgenic mouse mammary tumors. In addition, constitutively activated Wrch-1(Q107L), a GTPase-deficient mutant analogous to Cdc42(Q61L), phenocopied Wnt-1-mediated morphological transformation of C57MG mammary epithelial cells (Tao et al., 2001). This suggests that Wrch-1 expression and activity may contribute to Wnt-mediated morphological transformation, but this has not been further investigated.

As stated previously, Wrch-1 is a member of the Cdc42 subfamily of Rho GTPases and like other members of this protein family it induces filopodia formation (Aspenstrom et al., 2004; Tao et al., 2001). Wrch-1 is most closely related to Chp/Wrch-2, sharing 61% sequence identity with Chp and 57% with Cdc42 (Figure 1.4C). Like that of Chp, Wrch-1 mRNA is ubiquitous in human tissues with higher levels of expression in brain, heart, skeletal muscle, liver, placenta, and lung (Tao et al., 2001). In addition, homologs to Wrch-1 have been identified in many species including mouse, dog, chicken, the nematode *Caenorhabditis elegans*, the sea slug *Ciona intestinalis* and the parasitic trematode *Schistosoma japonicum*. Recently, investigators examined the expression of Rho subfamily members in 26 eukaryotic genomes and identified their emergence during evolution

(Boureux et al., 2007). Their data suggest that although Rac1, Cdc42, and RhoA are the founding members of the Rho GTPase family, the other members are also evolutionarily conserved and potentially necessary for many physiological processes yet to be identified. Interestingly, Wrch-1/Chp homologs, which they characterized as the RhoU/RhoV subfamily, emerged in coelomates (*Drosophila melanogaster* and *Caenorhabditis elegans)* and are found in all other species thereafter, also suggesting that Wrch-1 and Chp have evolutionarily conserved functions yet to be determined (Boureux et al., 2007).

Despite the sequence identity between Wrch-1 and Cdc42, Wrch-1 has unique characteristics that suggest divergent regulation and biological functions. As indicated in Figure 1.4C, Wrch-1 contains long N- and C-terminal extensions when compared to the sequence of Cdc42 or of other members of the Rho GTPase subfamily. The 46 amino acid N-terminal extension present in Wrch-1 contains poly-proline PxxP binding motifs for Src homology 3 (SH3) domain-containing proteins such as Grb2, NCKβ, and PLCγ (Saras et al., 2004; Shutes et al., 2004). Interestingly, truncation of this N-terminal extension of Wrch-1 increases its ability to activate Pak1 and induce cellular transformation (Shutes et al., 2004). Truncation of the N-terminus of Chp, a close relative of Wrch-1, has a similar effect on cellular transformation, suggesting a conserved mechanism for the N-terminal extension of these proteins to negatively regulate their activity (Chenette et al., 2005). However, recent findings suggest that the N-terminus of Wrch-1 is necessary for its ability to induce stress fiber dissolution and filopodia formation through binding to the nonreceptor tyrosine kinases Pyk2 and Src (Ruusala and Aspenstrom, 2007). This potentially suggests that the N-terminal extension of Wrch-1 has opposing effects on cytoskeletal dynamics and on cellular

transformation and that the binding partners necessary for these biological functions are distinct. This question is explored in my dissertation studies described in Chapters 2 and 3.

As stated previously, the C-terminal hypervariable regions and CAAX motifs of Rho GTPases dictate their subcellular localization and subsequent biological function. The C-terminus of Wrch-1 resembles that of Cdc42 in that it contains a hypervariable region and terminates in a putative CAAX motif, CCFV (Figure 1.4C). Unlike Cdc42, however, neither of the C-terminal cysteines of the CCFV motif within Wrch-1 is isoprenylated. Instead, Wrch-1 is modified by the fatty acid lipid, palmitate, at the second cysteine within the CCFV tetrapeptide, and this modification promotes its subcellular localization to the plasma membrane and internal membranes in fibroblasts (Table 1.1). Mutation of the tetrapeptide CCFV sequence in Wrch-1 to CSFV, thus preventing palmitoylation, disrupts Wrch-1 membrane localization and cellular transformation (Berzat et al., 2005b). Thus, the C-terminal lipid modification motif of Wrch-1 is the tripeptide "CFV", like that of Chp (Chenette, 2005), and like those of *Arabidopsis* type II Rho GTPases (Ivanchenko et al., 2000; Lavy et al., 2002) .

As has been seen in other palmitoylated Rho GTPases such as TC10, RhoB and Chp (Chenette et al., 2006; Michaelson et al., 2001), but in contrast to Cdc42, I determined that Wrch-1 does not interact with Rho GDIα and that its localization to the plasma membrane and internal membranes is not affected by Rho GDIα (Berzat et al., 2005a). This suggests that the reversibility of protein palmitoylation within the C-terminus of Wrch-1 may substitute functionally for Rho GDI-mediated sequestration as a mode to dynamically regulate Wrch-1 biological activities. In addition, an intact C-terminus is necessary for Wrch-1 localization to focal adhesions, which has been visualized in both fibroblasts and

osteoclasts (Chuang et al., 2007; Ory et al., 2007; Saras et al., 2004). The potential role of Wrch-1 localization to focal adhesions in its biological functions of cell migration and adhesion will be discussed later in this chapter.

In addition to the distinct sequence elements, subcellular localizations, and posttranslational modifications of Wrch-1 when compared to Cdc42, Wrch-1 displays unique biochemical properties. Wrch-1 exhibits a fast intrinsic nucleotide exchange rate, while its intrinsic GTPase activity is comparable to that of Cdc42 (Saras et al., 2004; Shutes et al., 2004). The rapid exchange rate could suggest that Wrch-1 may not be activated by GEFs but in another manner. One proposed mechanism is release of the auto-inhibitory N-terminal extension of Wrch-1 upon specific protein-protein interactions, that then allows for enhanced effector interactions (Shutes et al., 2004). However, expression of a putative dominant negative mutant of Wrch-1 (T63N) inhibits Wrch-1-mediated changes in cytoskeletal organization (Ruusala and Aspenstrom, 2007; Saras et al., 2004; Tao et al., 2001). By analogy to Ras it has been proposed that the GDP-bound (T17N, Cdc42 numbering) and nucleotide free (G15A, Cdc42 numbering) mutants of Rho GTPases bind preferentially to GEFs and lead to GEF sequestration and their inability to activate the endogenous proteins. The assumption that Rho family dominant negatives function by this mechanism suggests that Wrch-1 does in fact have GEFs but that they are yet to be identified.

GAPs for Wrch-1 have not been identified either but are likely to exist. The only two biochemical characterizations of Wrch-1 GTPase activity are in contrast. One study showed that Wrch-1 has no detectable intrinsic GTPase activity (Saras et al., 2004), while another study showed that Wrch-1 has normal intrinsic and stimulated GTPase activity that can be revealed by suitable nucleotide loading techniques (Shutes et al., 2004). GTPase-deficient

Rho GTPases such as the Rnd proteins, RhoBTB proteins, and RhoH have unusual amino acids in conserved positions required for GTP hydrolysis, but that is not true for Wrch-1 (Wennerberg and Der, 2004). Further, virtually all the biochemical and biological studies of Wrch-1 demonstrate that a putatively GTPase-deficient mutant of Wrch-1 (Q107L) is more efficient at effector binding, has stronger downstream signaling and induces more transformation than wild type Wrch-1, consistent with GTPase deficiency. Therefore, it is likely that GAPs capable of accelerating Wrch-1 intrinsic GTP hydrolysis do exist, and that their identification will be instrumental in elucidating Wrch-1 regulation and function.

Although some of the biochemical characteristics, subcellular localization and membrane association of Wrch-1 have been identified, full identification of Wrch-1 downstream effectors, regulators, biological functions, and contributions to cancer development remain elusive. Over the past few years our lab and others have uncovered some Wrch-1 functions and signaling pathways. The first characterization of Wrch-1 showed that constitutively activated Wrch-1(Q107L) activates Pak1 and JNK and promotes filopodia formation, stress fiber dissolution, and morphological transformation (Tao et al., 2001). A recent study set out to identify signaling pathways upstream and downstream of Wrch-1 that mediate its cytoskeletal effects. Interestingly, this study identified the non-receptor tyrosine kinase Pyk2 as an effector for Wrch-1. The interaction between Pyk2 and Wrch-1 required an intact N-terminal extension and effector domain in Wrch-1 (Ruusala and Aspenstrom, 2007). Pyk2 is a non-receptor tyrosine kinase that is highly related to focal adhesion associated kinase (FAK) and both of these proteins are involved in regulating cell adhesion and focal adhesion assembly through modulation of the actin cytoskeleton (Avraham et al., 2000; Wozniak et al., 2004). Pyk2 expression was required for Wrch-1-

mediated filopodia formation and stress fiber dissolution, because RNAi-mediated ablation of Pyk2 expression abrogated Wrch-1 induced cytoskeletal reorganization. In addition, the interaction and biological functions downstream of the Wrch-1-Pyk2 complex were dependent on expression and activity of the non-receptor tyrosine kinase Src. This suggests a potential mechanism by which Wrch-1 functions downstream of Src in a complex with Pyk2 to regulate cytoskeletal reorganization to promote cell migration (Ruusala and Aspenstrom, 2007). Our lab has been investigating Src-mediated phosphorylation of tyrosine residues within the C-terminal membrane targeting domain of Wrch-1 and the consequences of that phosphorylation to Wrch-1 biological function. Whether the Wrch-1/Src interactions uncovered by the two groups are related is not currently known.

Recent studies have highlighted an additional role for Wrch-1 in the regulation of cell migration through mechanisms clearly distinct from those of Cdc42, such as modulating focal adhesion turnover. Two independent studies determined that Wrch-1 localized to focal adhesions and that expression of constitutively activated Wrch-1(Q107L) decreased the number of focal adhesions per cell and increased cell migration (Chuang et al., 2007; Ory et al., 2007). Chuang et al. demonstrated that RNAi-mediated depletion of Wrch-1 leads to decreases in MLC phosphorylation and fibroblast cell migration. They propose that the potential mechanism for Wrch-1-mediated regulation of focal adhesions and subsequent cell migration is dependent on Wrch-1 regulation of MLCK activity downstream of Pak1 (Chuang et al., 2007). However, the physiological relevance and context for Wrch-1 regulation of cell migration remains to be determined.

Current research efforts have focused on Wrch-1 as major modulator of cell migration, but as stated previously Wrch-1 was first identified as a protein capable of

inducing morphological transformation of mouse mammary epithelial cells (Tao et al., 2001). In addition, our lab went on to show that expression of constitutively activated Wrch-1(Q107L) in NIH 3T3 cells promotes anchorage-independent growth in soft agar (Berzat et al., 2005b; Shutes et al., 2004), a hallmark of growth transformation and a frequent predictor of tumorigenic ability. The identification of Wrch-1 as a gene whose expression is regulated by Wnt-1 signaling in mouse mammary epithelial cells and its ability to phenocopy Wnt-1 mediated transformation, along with aberrant regulation of Wrch-1 expression in various cancers, suggest the possibility that Wrch-1 may play a role in human tumorigenesis. Indeed, Wrch-1 expression is altered in several types of cancers including colon, gastric, breast and ovarian (Kirikoshi and Katoh, 2002). Although Wrch-1 activation promotes cellular transformation in model systems and Wrch-1 expression is altered in human cancers, the mechanisms underlying Wrch-1-mediated cellular transformation remain to be elucidated.

When the studies in this dissertation were undertaken there were only two candidate effectors for Wrch-1: Pak1 and Par6 (Figure 1.4B). It has been shown previously that transformation mediated by aberrant activation of Cdc42 or Rac can be potentiated by the Par6-PKCζ complex (Qiu et al., 2000). These findings support a link between contact-inhibited growth and the regulation of cell polarity by Rho GTPases through the Par6-PKCζ complex. Therefore, I set out to determine whether Wrch-1 interacted with the scaffold protein, Par6, in a GTP-dependent manner, and what the biological consequences of an interaction between Wrch-1 and Par6 would be. More importantly, I wanted to determine whether this interaction contributed to Wrch-1-mediated cellular transformation. Discussion of the known biological functions of Par6 and regulation of Par6 by Rho family small GTPases can be found in the following sections.

1.7 Par6-Par3-aPKC complex overview

Model organisms such as *C. elegans* and *Drosophila* have been powerful tools to elucidate genes and signaling pathways involved in developmental biology. In the early 1980s, both *C. elegans* and *Drosophila* began to be used to identify genes involved in many developmental processes such as embryonic development (Nusslein-Volhard and Wieschaus, 1980; Strome and Wood, 1982). Specifically, one group interested in identifying genes involved in the earliest stages of *C. elegans* embryonic development performed a screen for maternal embryonic lethal mutants (Kemphues, 2000; Kemphues et al., 1988). Normally during *C. elegans* development the one-cell embryo divides asymmetrically into two unequally sized daughter cells. In order for asymmetric cell division to occur properly, certain proteins are polarized to the anterior pole or posterior pole of the one-cell embryo to function as spatial cues necessary for segregating cell-fate determinants. In addition, these proteins coordinate spindle orientation, which is also required for proper asymmetric cell division (Bowerman and Shelton, 1999; Rose and Kemphues, 1998). Kemphues and colleagues were able to identify many genes involved in early embryogenesis that can be classified into two separate groups: cell fate-determining genes and cell polarity genes. During the initial screen for embryonic lethal mutants an interesting phenotype was discovered, in which the one-cell embryo underwent an equal and synchronous cell division. This suggested to the researchers that partitioning of cytoplasmic components necessary for normal cell division was disrupted (Kemphues et al., 1988). The cell polarity genes with the strongest defects during embryogenesis are also called the *par* genes, for par titioning defective genes.

Altogether six *par* genes were identified in the maternal embryonic lethal mutant screen briefly described above (Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Hung and Kemphues, 1999; Kemphues, 2000; Levitan et al., 1994; Morton et al., 2002; Watts et al., 1996). The six *par* genes encode proteins with distinct cellular functions in many organisms (Table 1.2). These core *par* genes form an integrated signaling network instrumental in regulating cell polarity in multiple cell types from worms to humans (Goldstein and Macara, 2007). The partitioning defective genes consist of Par3 and Par6 (protein scaffolds), Par1 and Par4 (serine/threonine kinases), Par2 (a RING finger protein), and Par5 (a 14-3-3 protein). The serine/threonine kinase atypical PKC was not originally identified as a member of the polarity genes in *C. elegans*. It wasn't until the mammalian homolog of Par3 was shown to associate with aPKC in epithelial cells and to display asymmetric apicobasal localization that the *C. elegans* homolog of aPKC, PKC-3, was discovered (Izumi et al., 1998). Like mutants of Par3 and Par6, the *C. elegans* aPKC homolog caused a partitioning phenotype in the *C. elegans* zygote, suggesting that these proteins function in a parallel fashion to regulate polarization and spindle orientation in the one-cell embryo (Tabuse et al., 1998). In the *C. elegans* one-cell embryo, Par3, Par6, and aPKC are segregated to the anterior pole, whereas Par1 and Par2 are enriched in the posterior pole. The exact mechanism by which the *par* proteins are segregated to distinct cellular poles remains to be elucidated in *C. elegans,* but advancements in *Drosophila* and vertebrate cell systems have paved the way for understanding the dynamic interplay between these proteins (Hurov et al., 2004; Kusakabe and Nishida, 2004; Suzuki et al., 2004). Although regulation of the molecular interactions between all the polarity proteins is fundamental to

C. elegans	*Drosophila*	Mammals	Domains
Par1	Par1	MARK/Par1/CTAK/KP78,EMK	Ser/Thr kinase,UBA
Par2	Not identified	Not identified	Zing finger
Par3	Bazooka	Par3,Par3L	CR1, PDZ, aPKCBD
Par4	Lkb1	Lkb1/STK11	Ser/Thr kinase
Par5	14-3-3ε	14-3-3	Phosphoserine binding
Par6	Par6	Par6A to Par6D	PDZ, semi-CRIB, PB1
PKC-3	aPKC	PKCζ,PKCλ	Ser/Thr kinase, PB1
Cdc42	Cdc42	Cdc42	GTP-binding, prenylation

Table 1.2 Polarity proteins involved in polarization of multiple cell systems in *C.elegans, Drosophila,* and mammals.
Conservation of polarity proteins involved in asymmetric cell divisions of the *C.elegans* zygote in *Drosophila* and mammals. (Macara, 2004)

their function in many organisms, I will focus on the evolutionarily conserved Par6-Par3-aPKC complex and its biological functions and regulation.

Until 1998, there was only a genetic link between the *par* proteins, and these studies were restricted to the regulation of cell polarity in the *C. elegans* zygote. However, in 1998 Kuchinke et al. cloned the *Drosophila* homolog to Par3, Bazooka, and determined that Bazooka expression was required for proper coordination of the axis of apical-basal cell polarity in the *Drosophila* embryo (Kuchinke et al., 1998). At the same time another group cloned the mammalian homolog to Par3, ASIP, while searching for binding partners for aPKC and determined that both ASIP and aPKC are asymmetrically localized in mammalian epithelial cells (Izumi et al., 1998). Once Par6 was found to be an interacting protein for the small GTPase Cdc42, the evolutionarily conserved interaction between Par6, Par3, and aPKC was characterized (Joberty et al., 2000; Johansson et al., 2000; Lin et al., 2000). The intermolecular interactions between small GTPases and the Par6-Par3-aPKC cassette will be illustrated in section 1.9. These groundbreaking studies suggested that this protein complex was evolutionarily conserved from worms to vertebrates and plays a fundamental role in establishment and maintenance of cell polarity in multiple cell types, from a *C. elegans* zygote to a mammalian differentiated epithelial cell.

The establishment and maintenance of cell polarity is critical for the development and function of organisms (Wodarz, 2002). Cell polarity regulates the segregation of apical and basolateral membranes during epithelial morphogenesis, cell fate determinants during asymmetric cell division and development of axons and dendrites during neuron specialization (Nelson, 2003). The evolutionarily conserved Par6-Par3-aPKC protein cassette is asymmetrically segregated in multiple cell types and regulates the development of

cell polarity. Specifically, this conserved multiprotein complex is integral in the establishment and maintenance of cell polarity during several developmental stages and cellular processes such as the *C. elegans* one-cell stage embryo; *Drosophila* oocyte development; epithelial polarization in *C.elegans*, *Drosophila* and mammals; neuronal development in flies and mammals; and polarized cell migration (Figure 1.5A) (Goldstein and Macara, 2007). Although the Par6-Par3-aPKC complex is highly conserved and functions to regulate cell polarity in diverse cellular contexts, I will address only how the Par6-Par3-aPKC protein complex functions to regulate epithelial polarization in mammalian cell systems through dynamic regulation of intercellular junctions and the cytoskeleton.

1.8 Regulation of epithelial cell polarity and tight junctions by Par6-Par3-aPKC complex regulation of epithelial cell polarity

Epithelial cells are highly polarized structures with respect to their distinct apical and basolateral membranes, cytoskeletal organization, organized protein sorting and intercellular junctional complexes (Rodriguez-Boulan and Nelson, 1989). In multicellular organisms epithelial cells form physiological and mechanical barriers and control tissue architecture. The polarity of epithelial cells is regulated and maintained by the asymmetric distribution of evolutionarily-conserved signaling complexes, such as the Par6-Par3-aPKC complex, which function to integrate organizational cues from both cell-cell and cell-extracellular matrix adhesions. Their dynamic regulation and modulation of cell-cell contacts mediated by cellular junctions, such as adherens and tight junctions in vertebrates, is essential for proper epithelial cell polarity (Nelson, 2003). In mammalian epithelial cells, three multiprotein complexes (Crb-Pals1-PATJ, Par6-Par3-aPKC, and Dlg-Scrib-Lgl) are instrumental to the establishment and maintenance of epithelial cell polarity by regulating the segregation of distinct apical and basolateral membrane domains through protein phosphorylation (Figure

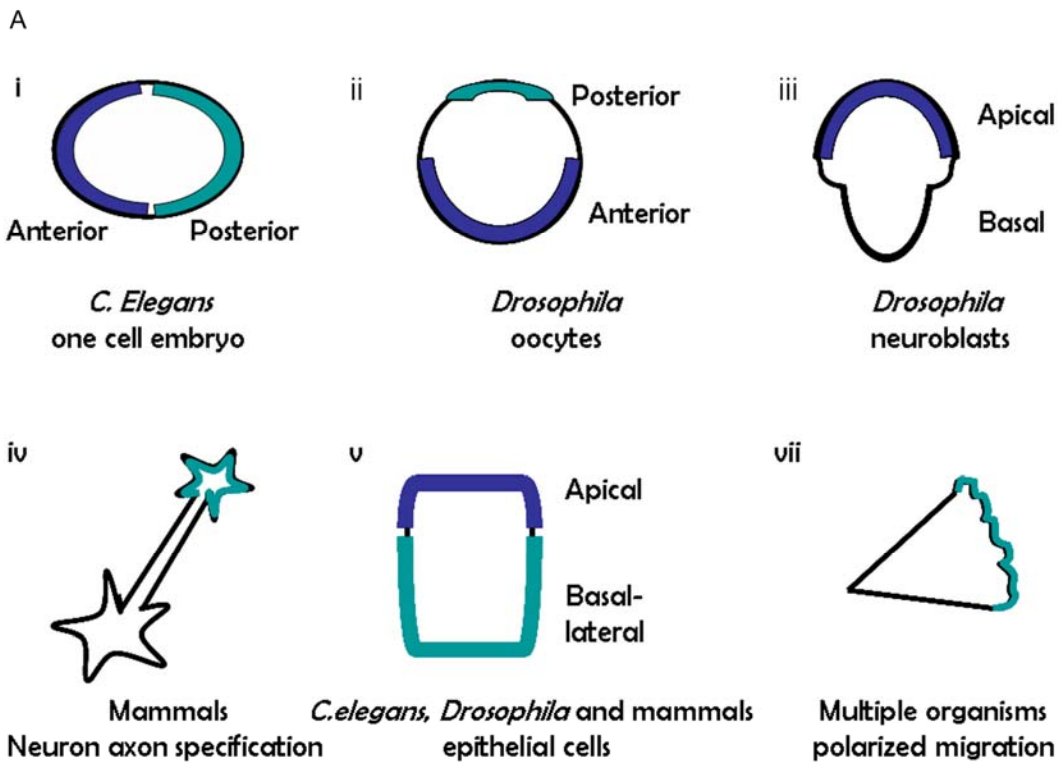


Figure 1.5A Localization of polarity protein complexes during development and mammalian epithelial cell polarization.
A) Opposing distribution of Par6-Par3-aPKC complex (blue) and Par1 polarity protein (green) in multiple systems. Adapted from (Goldstein and Macara, 2007)

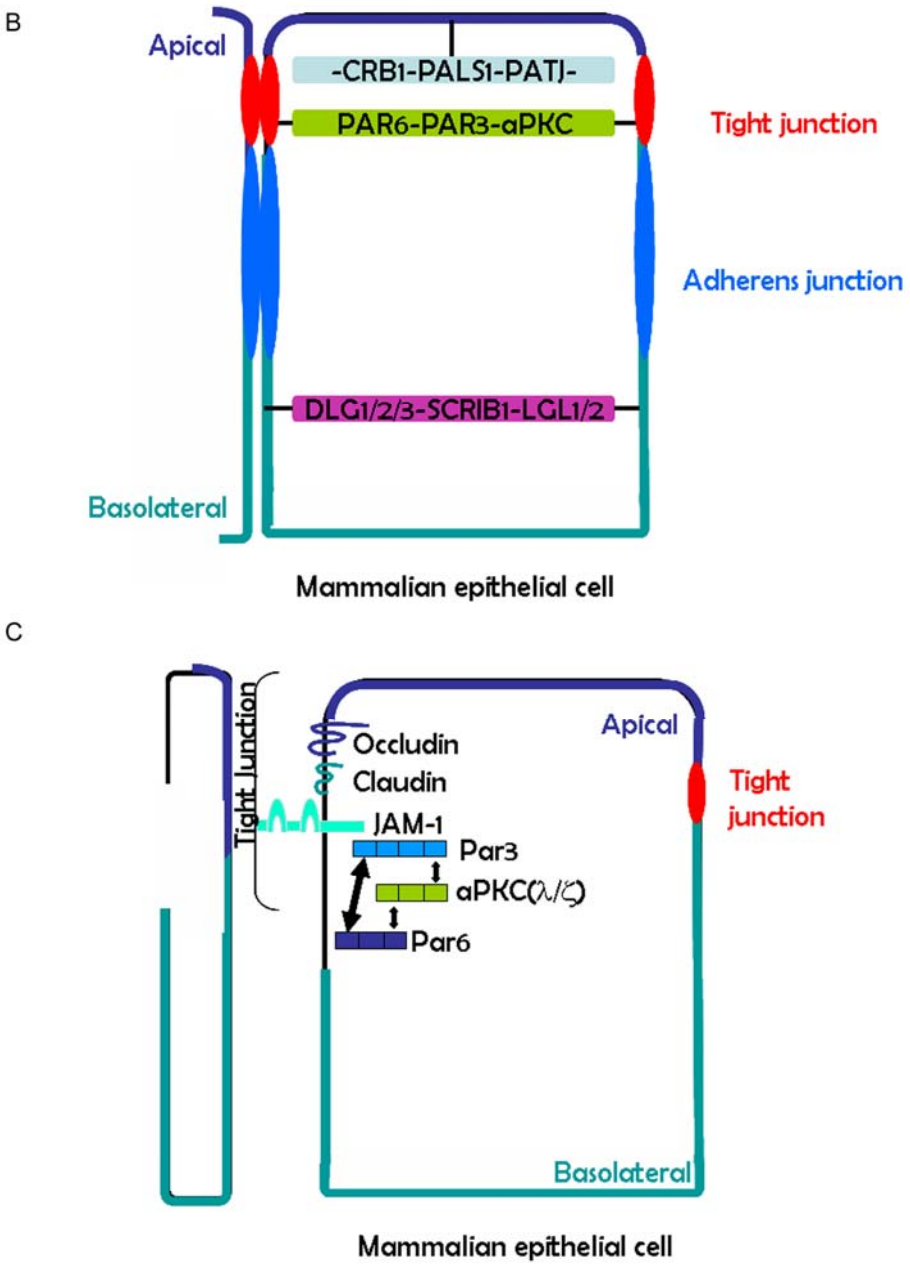


Figure 1.5B & C Localization of polarity protein complexes during development and mammalian epithelial cell polarization.
B) Membrane localization of CRB1-PALS1-PATJ (blue, apical), Par6-Par3-aPKC (green, tight junctions), and DLG-SCRIB1-LGL (purple, basolateral) polarity complexes in mammalian epithelial cells. C) Schematic diagram of localization to Par6-Par3-aPKC protein complex tight junctions. Molecular interactions between the members of the complex and tight junction-associated proteins are indicated by arrows.

1.5B) (Macara, 2004). In recent years direct interactions between these evolutionarily conserved multiprotein complexes have been characterized in mammalian epithelial cells and highlight the dynamic crosstalk between the complexes necessary to achieve epithelial cell polarization (Hurd et al., 2003; Plant et al., 2003; Yamanaka et al., 2003). Studies in *Drosophila* and in mammalian epithelial cell systems have given great insight into the functional interactions between the polarity complexes, namely that the multiprotein complexes antagonize one another and mutually exclude one another from the same region, leading to the formation of distinct membrane domains with defined compositions (Figure 1.5B) (Bilder et al., 2003). However, as stated above, this dissertation will focus specifically on the Par6-Par3-aPKC ternary complex and the roles that complex plays in regulating tight junction biogenesis.

Tight junctions contribute to the maintenance of cell polarity by forming a barrier that prevents the diffusion of lipids within the membrane and of proteins between the apical and basolateral membranes, and by forming a sheet impermeable to ions and solutes. Tight junctions are composed of: 1) integral membrane proteins that constitute tight junction strands; 2) cytoplasmic proteins that organize the integral membrane proteins and connect them to actin filaments and other cytoplasmic proteins; and 3) signaling proteins that may be involved in junction assembly (Shin et al., 2006). The first indication that the Par6-Par3-aPKC complex played a role in the modulation of epithelial cell polarity through regulation of tight junction formation was the discovery that Par3 and aPKC co-localized at tight junctions at the apical-lateral membrane boundary (Izumi et al., 1998). The third member of the multiprotein complex, Par6, was also shown later to associate with tight junctions in polarized MDCKII cells (Johansson et al., 2000). It has been proposed that the targeting of

the Par6-Par3-aPKC complex to tight junctions is due to the interaction between Par3 and the tight junctional integral membrane protein JAM, thus targeting Par3 to primordial junction complexes where it functions as scaffold to recruit Par6 and aPKC (Figure 1.5C) (Ebnet et al., 2001; Itoh et al., 2001). As in *C. elegans* and *Drosophila*, members of the mammalian Par6-Par3-aPKC complex require intermolecular interactions for their proper membrane localization in epithelial cells, while regulation of the kinase activity of aPKC isoforms, λ and ζ, is required for the establishment of epithelial cell polarity (Chen and Macara, 2005; Gao et al., 2002; Suzuki et al., 2002; Suzuki et al., 2001; Yamanaka et al., 2001).

Interestingly, the independent characterization of these proteins as tight junction-associated proteins in mammalian epithelial cells led to many elegant studies investigating the ability of Par6, Par3, and aPKC to modulate tight junction dynamics. Aberrant activation of any member of this complex leads to inhibition of tight junction formation by mislocalizing tight junction-associated proteins and by increasing cellular permeability to ions and nonionic molecules. Specifically, overexpression of dominant negative aPKC disrupts the localization of the cytoplasmic tight junction-associated protein, ZO-1, and the development of a membrane barrier as measured by transepithelial resistance (TER) to ion flow across a cell monolayer and paracellular diffusion of a nonionic solute (Suzuki et al., 2002; Suzuki et al., 2001; Yamanaka et al., 2001).

The assembly and function of tight junctions is also negatively regulated by the overexpression of full length Par6 or an active Par6 fragment that does not contain the N-terminal aPKC binding domain (Gao et al., 2002; Joberty et al., 2000; Yamanaka et al., 2001). The exact mechanism by which ΔN-Par6 inhibits tight junction assembly is not well known but biochemical studies suggest that the N-terminus of Par6 binds with high affinity

to the N-terminal regulatory domains of PKCζ and PKCλ and stimulates their kinase activity. However, full length Par6 is incapable of activating aPKC because the CRIB-PDZ cassette of Par6 has an auto-inhibitory effect (Gao et al., 2002; Garrard et al., 2003; Yamanaka et al., 2001). The molecular interactions necessary to alleviate the auto-inhibition mediated by the CRIB-PDZ cassette of Par6, that in turn promote its ability to activate aPKC kinase activity, will be discussed in section 1.9.

When the effect of Par3 on tight junction formation and function was first assessed, overexpression of Par3 was seen to induce only minor defects in tight junction dynamics (Hirose et al., 2002; Mishima et al., 2002). A recent study that used RNAi-mediated knockdown of Par3 in MDCKII cells was able to overcome the previous inability to examine Par3 as an integral part of tight junction biogenesis. Chen et al. determined that ablation of Par3 protein from MDCKII cells disrupted tight junction formation and cell polarization (Chen and Macara, 2005). Interestingly, activation or inactivation of any member of the Par6-Par3-aPKC has been shown to disrupt the establishment of tight junctions during cell-cell contact initiation, while these proteins do not disrupt tight junctions in monolayers at steady state, suggesting that other cellular mechanisms and complexes are necessary to maintain epithelial cell polarity once established.

Although a clear link between tight junction formation and cell polarity regulation by the Par6-Par3-aPKC has been well documented, the exact mechanism by which these proteins interact and regulate other cellular components to coordinate cell polarization and cell junction formation has only recently begun to surface. One cellular structure that is perturbed by the aberrant activation of Par6, Par3, and aPKC is the actin cytoskeletal network, which is intimately connected to epithelial cell junction formation and cell

polarization (Munro, 2006). It is well appreciated that small GTPases, such as Cdc42 and Rac1, along with the Rho GEFs, Tiam1 and Ect2, are capable of activating the Par6-Par3-aPKC complex to regulate tight junction formation (Chen and Macara, 2005; Joberty et al., 2000; Lin et al., 2000; Yamanaka et al., 2001). This link between the Rho GTPase subfamily and the evolutionarily conserved Par6-Par3-aPKC polarity complex to regulate tight junction formation and possibly actin cytoskeletal dynamics is not surprising, given that tight coordination of cellular movements by Rho GTPases is necessary for proper cell polarization. In the next two sections I will discuss how Rho GTPases themselves and their regulators regulate Par6-Par3-aPKC to mediate diverse cellular functions in mammalian epithelial cells.

1.9 Regulation of the Par6-Par3-aPKC complex by Rho GTPases

In 2000, four independent groups reported that the Rho family GTPases, Cdc42 and Rac1, bind to the cell polarity protein Par6 in a GTP-dependent manner, facilitating the formation of a quaternary complex between the small GTPase, Par6, Par3, and aPKC (Joberty et al., 2000; Johansson et al., 2000; Lin et al., 2000; Qiu et al., 2000). Supporting this, Par6 contains an incomplete Cdc42/Rac-interactive-binding (CRIB) domain, a short conserved sequence shared within some downstream effectors of Cdc42 and Rac, adjacent to the N-terminus of its PSD95/Discs Large/ZO-1 (PDZ) domain (Figure 1.6A) (Joberty et al., 2000). The CRIB domain within Par6 is missing two critical conserved histidine residues that are normally present in CRIB domain-containing proteins. Therefore, the CRIB domain in Par6 is often considered a semi-CRIB domain. Like Cdc42 and Rac1, the Rho GTPases TC10, Wrch-1, Chp, and Rac3 have been shown to interact with Par6 in yeast two hybrid

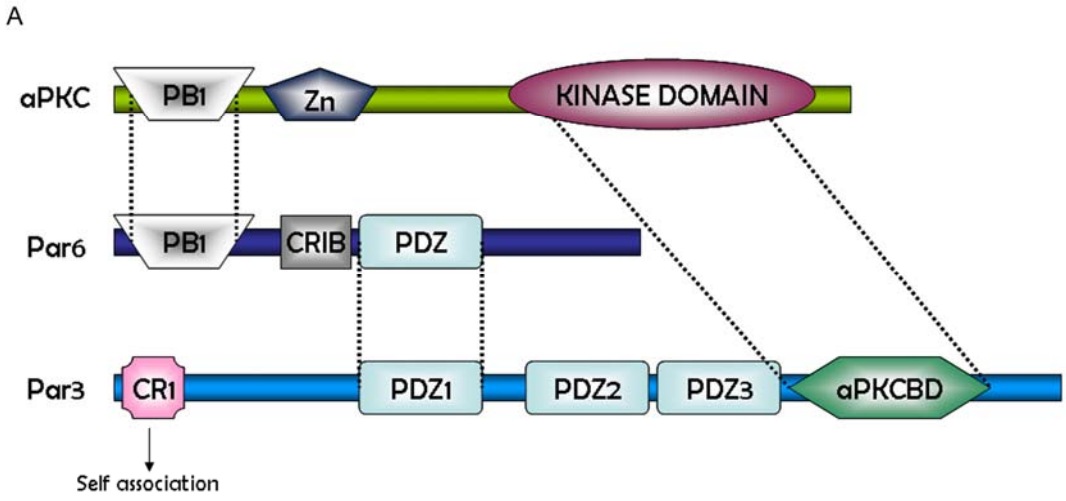


Figure 1.6A Molecular interactions between Par6-Par3-aPKC complex and the small Rho family of GTPases.

A) Domain architecture and molecular interactions of Par6, Par3, aPKC. Connecting lines indicate regions of the proteins that interact with one another. Biological consequences of these interactions are discussed in text. Adapted from (Macara, 2004).

B

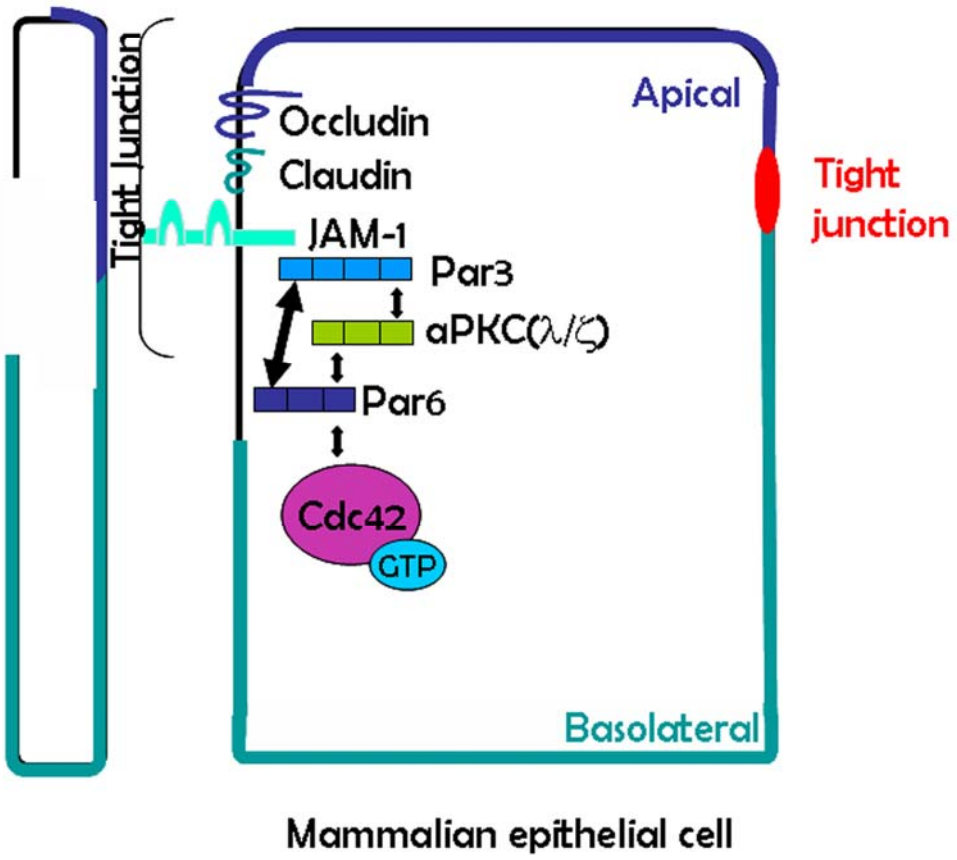


Figure 1.6B Molecular interactions between Par6-Par3-aPKC complex and the small Rho family of GTPases.

B) Schematic representation of Cdc42 regulation of Par6-Par3-aPKC at apical membrane domain of mammalian epithelial cells to regulation tight junction formation during polarization.

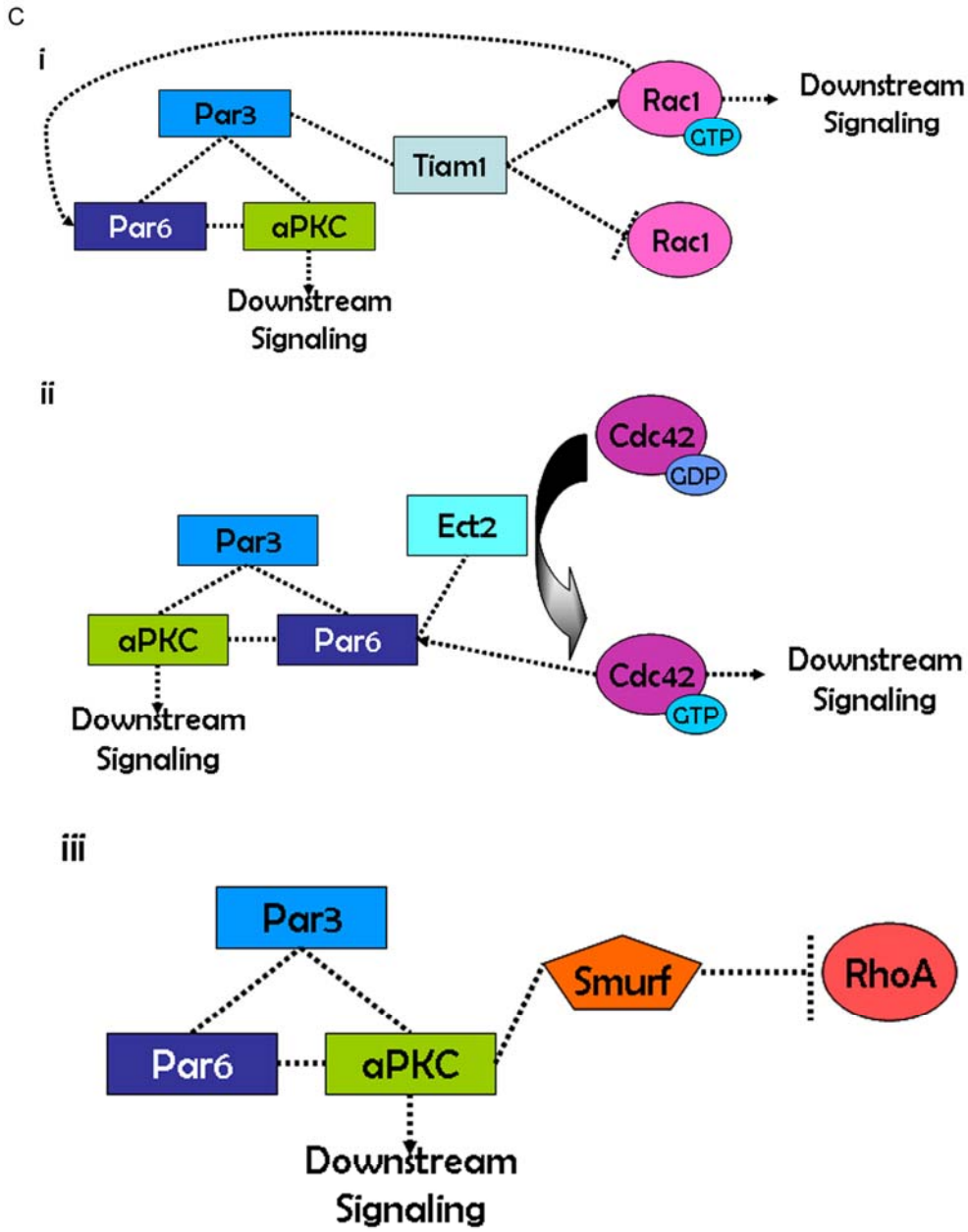


Figure 1.6C Molecular interactions between Par6-Par3-aPKC complex and the small Rho family of GTPases.

C) Par protein regulation of Rho family GTPases. i. Par3 recruitment of Tiam1 activates Rac1, which can in turn bind and activate Par6-aPKC cassette. In other contexts, Par3 sequesters or inhibits Tiam1 mediate activation of Rac1. ii. Par6 association with Ect2 may localize Ect2 to tight junctions and lead to subsequent activation of Cdc42 and Par6-aPKC cassette forming a positive feedback loop. iii. aPKC association with E3 ligase Smurf promotes localized degradation of RhoA. Adapted from (Munro, 2006).

screens and/or biochemical assays (Aspenstrom et al., 2004; Joberty et al., 2000; Keller et al., 2005).

The C-terminal PDZ domain of Par6 heterodimerizes with the first PDZ domain of Par3 (Lin et al., 2000). PDZ domains are protein-protein interaction domains that recognize C-terminal motifs (Thr/Ser-X-Val where X is any amino acid) in their binding partners or that heterodimerize with other PDZ domains. PDZ domain-containing proteins often serve as scaffolds or adaptors for the establishment of multiprotein complexes at distinct subcellular localizations and are often associated with cell polarization (Roh and Margolis, 2003). The extreme N-terminus of Par6 contains a Phagocyte oxidase/Bem1 (PB1) domain that functions as a protein-protein interaction module by associating with other PB1 domains, such as the PB1 domain within the N-termini of the aPKCs, λ and ζ. As stated earlier, truncation of the N-terminal PB1 domain of Par6 disrupts the association with aPKC, allowing it to function as a dominant negative protein. An illustration of the domain architecture and molecular interactions of Par6, Par3, and aPKC can be found in Figure 1.6A.

In addition to functioning to couple Par6 to Par3, the Par6 C-terminal PDZ domain, in conjunction with the CRIB domain, promotes Par6 association with Cdc42 (Joberty et al., 2000; Lin et al., 2000). Interestingly, expression of the CRIB-PDZ cassette negatively regulates tight junction formation in MDCKII cells (Garrard et al., 2003). Based on the crystal structure of GTP-Cdc42 bound to Par6 and extensive fluorescence resonance energy transfer (FRET) experiments, a model can be postulated for the ability of small GTPases to bind and release the auto-inhibited conformation of Par6 that subsequently leads to the activation of aPKC. In this model, Par6 and aPKC are constitutively bound in an inactive confirmation due to the ability of the CRIB-PDZ cassette of Par6 to inhibit the N-terminal

PB1 domain of Par6 from stimulating the kinase activity of aPKC. However. in response to cell-cell contact or other extracellular stimuli, Cdc42 is activated and binds to the CRIB-PDZ cassette of Par6 and induces a slight conformational change within Par6, which relieves this inhibitory effect and results in an increase in aPKC kinase activity (Garrard et al., 2003; Yamanaka et al., 2001).

Therefore, the identification of small GTPases as the missing link to activating the signaling capacity of the Par6-Par3-aPKC complex in mammalian epithelial cells was instrumental in establishing this multiprotein complex as an integral part in regulating epithelial cell polarity in diverse cellular contexts. In epithelial cells it is believed that Par3 functions as a scaffold at tight junctions to recruit Par6 and aPKCs to the complex, while GTP-bound Cdc42 regulates the signaling capabilities to promote tight junction formation and cell polarity (Figure 1.6B). These findings correlate with data from the overexpression or inactivation of Par3, Par6, aPKC and Cdc42 mutants, showing that they all negatively regulate the initial formation of tight junctions but do not affect tight junction maintenance (Chen and Macara, 2005; Gao et al., 2002; Hirose et al., 2002; Mishima et al., 2002; Rojas et al., 2001; Suzuki et al., 2002; Suzuki et al., 2001; Yamanaka et al., 2001).

Cdc42 and Rac1 regulation of the Par6-Par3-aPKC complex is well characterized, along with the biological functions downstream of these proteins. Briefly, Cdc42 activation of the signaling capabilities of the Par6-Par3-aPKC complex is associated with: oriented migration of mammalian astrocytes; apical-basal polarization of *Drosophila* and mammalian epithelial cells; axon differentiation of mammalian hippocampal neurons; asymmetric cell division of *Drosophila* sensory-organ precursor cells and neuroblasts; and anterior-posterior axis specification of the *C. elegans* zygote (Macara, 2004). However, the exact mechanisms

for each of these biological activities downstream of the Cdc42-Par6-Par3-aPKC complex remain to be determined in these model systems for studying cell polarization, but are most likely linked to modulation of the actin and microtubule cytoskeletons.

Interestingly, several recent papers have suggested that the Par6-Par3-aPKC complex is capable of regulating the activity of small GTPases. In 2005, three independent studies in different cell systems characterized an association between Par3 and the Rac-specific GEFs, Tiam1 and Tiam2, that leads to regulation of Rac activity. In neuroblastoma cells and hippocampal neurons, Par3 binds Tiam2 to stimulate Rac activation. Rac activation downstream of Par3-Tiam2 interaction stimulates lamellipodia formation, axon outgrowth and neuronal polarization (Nishimura et al., 2005). However, the association between Par3 and Tiam1 in mammalian epithelial cells has an opposing effect on Rac activity. Loss of Par3 in MDCKII cells disrupts tight junction assembly and apical actomyosin cytoskeletal organization. These biological outcomes elicited by the loss of Par3 are accompanied by Rac activation and can be rescued by expressing dominant negative Rac or by Tiam1 knockdown. This suggest that, unlike in neurons, Par3 binding to Tiam1 may normally inhibits its ability to activate Rac (Chen and Macara, 2005). Macara and colleagues have suggested that Par3 association with Tiam1 is independent of Par6-aPKC cassette. In mouse keratinocytes, Tiam1 loss disrupts actomyosin organization and tight junction formation, but this is overcome by expressing constitutively active Rac, suggesting that Rac activation is required for tight junction formation and modulation of the actin cytoskeleton (Mertens et al., 2005). The reasons for the differences in the ability of Par3 to regulate Rac activity upon associating with Tiam1 are unknown. However, these papers have uncovered an interesting paradigm in which Rac can activate the Par6-Par3-aPKC complex and can be subsequently activated or

inactivated downstream of the polarity complex depending on the cellular context (Figure 1.6Ci).

Rac is not the only small GTPase that has been shown to be regulated by the Par complex. In MDCKII cells, the Rho-GEF Ect2 binds to Par6 and leads to the activation of Cdc42, but the significance of this activation has not been determined (Liu et al., 2004b). It is possible that Ect2 associates with tight junctions through its interaction with Par6 and is able to recruit and activate Cdc42 in a spatially restricted manner to regulate tight junction formation and cell polarity through the Par6-Par3-aPKC complex (Figure 1.6Cii). In addition to regulating and being activated by the Par6-Par3-aPKC complex, Cdc42 and Rac may subsequently downregulate the small GTPase, RhoA. There is evidence that aPKC binds to the E3 ubiquitin ligase Smurf-1, that promotes ubiquitination and degradation of Rho in motile cells and downstream of TGF-β in mammalian epithelial cells (Figure 1.6Ciii) (Ozdamar et al., 2005; Wang et al., 2003). All of these reports suggest that regulation of Rho GTPase function downstream of the Par protein complex is instrumental in Rho GTPase activation and localized regulation of the cytoskeleton (i.e., tight junctions of mammalian cells or the leading edge of fibroblasts), which is necessary for many of the biological activities elicited by the Par6-Par3-aPKC complex (Figure 1.6C).

Although this model for Cdc42 and Rac1-mediated activation and regulation of the Par6-Par3-aPKC cassette has been thoroughly examined, the ability of other Rho GTPases such as TC10, Wrch-1, Chp, and Rac3 to regulate the complex in a similar fashion to elicit analogous or divergent biological functions is unknown. Therefore I set out to determine if the interaction between Wrch-1 and Par6 previously identified by yeast two-hybrid screen could be detected in mammalian cells and if the interaction between Wrch-1 and Par6 elicited

changes in tight junction dynamics, actin cytoskeleton organization, and epithelial morphogenesis. I also wanted to investigate whether the interaction between Wrch-1 and Par6 was able to contribute to Wrch-1-mediated cellular transformation.

1.10 Rho GTPase regulation of tight junctions and actin cytoskeletal organization in epithelial cells

Tight cell-cell adhesions, cytoskeletal organization, polarized vesicular traffic, cuboidal morphology, and distinct apical and basolateral membranes characterize the polarized morphology of epithelial cells. Cell junctions and the cytoskeletal network, important structural components of epithelial cells, regulate these characteristics that contribute to the formation and maintenance of normal cell morphology. Normal epithelial cell morphology is maintained by two types of intercellular adhesive junction: adherens junctions and tight junctions that form the apical junctional complex (Figure 1.7A). Adherens junctions are adhesive structures formed through calcium-dependent interactions between cadherin molecules on adjacent epithelial cells. Cadherin molecules, such as E-cadherin, form membrane anchor sites for the actin cytoskeleton through interactions with catenins, β-catenin and α-catenin. The association of adherens junctions with the cytoskeleton provides strength and stability to cell-cell contacts (Yap et al., 1997). The architecture of mammalian epithelial cell adherens junctions is illustrated in Figure 1.7B. Interestingly, the formation of adherens junctions is a prerequisite to the formation of other cellular junctions such as tight junctions and gap junctions (Gumbiner et al., 1988).

Tight junctions are the most apical members of the apical junctional complex and they form a continuous, circumferential, belt-like structure. Functionally, tight junctions form both a paracellular diffusion barrier, or gate, that regulates permeability of the epithelial

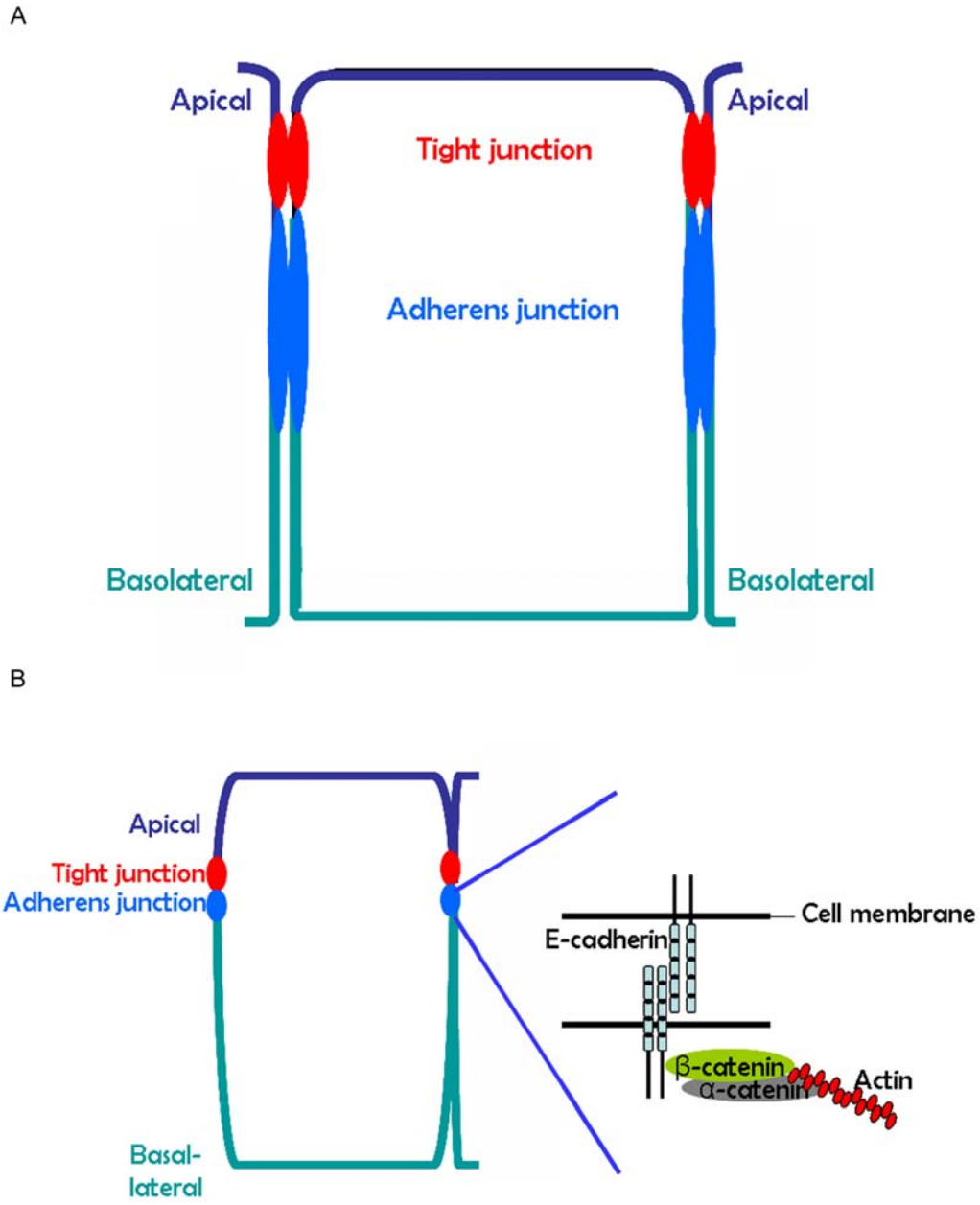


Figure 1.7A & B Polarized epithelial cell junctional complexes and actin organization. A) Diagram of adherens junction and tight junction localization in mammalian epithelial cells. B) Adherens junctions form calcium dependent adhesive structures compote of integral membrane cadherins that are linked to the actin cytoskeleton through α- and β-catenin.

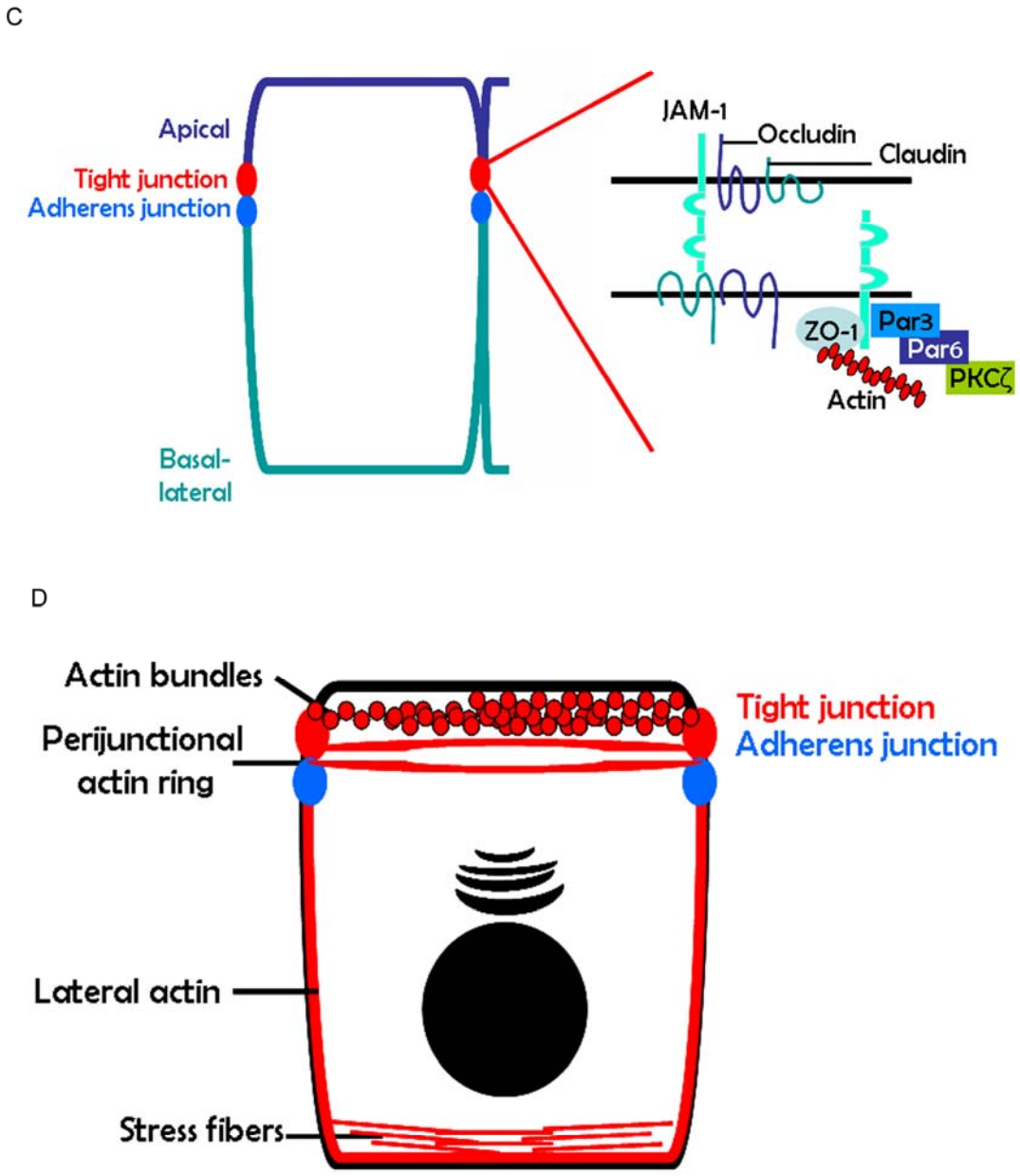


Figure 1.7C & D Polarized epithelial cell junctional complexes and actin organization. C) Tight junctions form a barrier to the diffusion of proteins and lipids within membrane, along with a selective permeability barrier to solutes and ions. D) The actin cytoskeleton forms a brush border consisting of actin bundles. An actin rich ring structure is anchored to cell junctional complexes. Actin filaments line the lateral membrane, while actin stress fibers are organized along base of the cells.

monolayer to ions and solutes, and also an intramembrane diffusion barrier, or fence, which prevents the diffusion of lipids and proteins between the apical and basolateral membranes.

As stated previously, tight junctions are composed of integral membrane proteins, cytoplasmic proteins, and signaling proteins that function together to orchestrate the formation of polarized epithelial cells. Three types of integral membrane proteins are found within tight junction strands in epithelial cells: occludins, claudins, and junctional adhesion molecules (JAMs). The claudins are thought to constitute the intramembrane and paracellular diffusion barriers found within epithelia, while the cytoplasmic plaque of protein scaffolds and signaling molecules regulates their function. The protein scaffolds within tight junctions are thought to bind to the integral membrane proteins and form peripheral membrane binding sites for the recruitment of signaling molecules such as protein kinases, small GTPases, and transcription factors. In addition to recruiting signaling molecules capable of regulating tight junction formation and maintenance, many of the integral membrane proteins and protein scaffolds function as linkers to interact with the actin cytoskeleton (Matter and Balda, 2003b; Shin et al., 2006). There is significant evidence to suggest an intimate link between dynamic regulation of the actin cytoskeleton and tight junction assembly (Turner, 2000). The architecture of mammalian epithelial cell tight junctions is illustrated in Figure 1.7C.

Many different experimental systems have been used in order to investigate intracellular signaling pathways involved in the assembly and function of tight junctions. Most of these experimental systems involve the plating of epithelial cells on a permeable substrate that allows for the measurement of characteristics unique to tight junctions such as TER, which indicates ionic conductivity that can be used to determine integrity and ion

selectivity of tight junctions, and paracellular permeability of hydrophilic tracers, which allows for the quantification of diffusion across tight junctions and determination of size selectivity of the paracellular diffusion barrier (Matter and Balda, 2003a). The most widely used approach to manipulate cell junctions is the 'calcium switch' assay in MDCK cells. In this assay, cell-cell adhesion mediated by calcium-dependent E-cadherin interactions on neighboring cells is disrupted by plating epithelial cells in culture medium containing a low concentration of calcium. Upon the addition of a normal concentration of calcium to the medium, E-cadherin-dependent intercellular adhesion then promotes the assembly of primordial junctions that contain adherens junction- and tight junction-associated proteins. The proper localization and function of these components leads to the formation and establishment of mature adherens junctions and tight junctions. Many signaling pathways and the proteins such as small GTPases that are involved in tight junction formation have been identified by the use of this calcium switch assay (Matter and Balda, 2003b). I have used this assay in my disssertation studies described in Chapter 2.

Members of the Ras, Rab, and Rho small GTPase families have all been linked to the assembly and function of tight junctions. This observation was first made in a calcium switch assay in the presence of non-hydrolyzable GTPγS, which inhibits the ability of proteins that bind and hydrolyze GTP to be turned 'off'. The addition of GTPγS to MDCK cells during calcium switch disrupted the assembly and sealing of tight junctions (Balda et al., 1991). RhoA was the first Rho subfamily member shown to be involved in tight junction assembly. Microinjection of C3 transferase, a *Clostridium botulinum* toxin that inactivates RhoA, RhoB, and RhoC, or the use of *Clostridium difficile*, whose toxins also inactivate Rho, inhibited the assembly of both adherens junctions and tight junctions (Nusrat et al., 1995;

Nusrat et al., 2001). However, most of the experimental evidence for the involvement of Rho GTPases in tight junction formation has been elucidated in MDCK cells that express constitutively active or dominant negative mutants of RhoA, Rac1, or Cdc42 during cell polarization induced by calcium switch.

Specifically, the expression of constitutively active or dominant negative mutants of RhoA, Rac1 or Cdc42 has distinct effects on tight junction dynamics. Interestingly, usually either constitutive activation or inactivation of these Rho GTPases leads to disruption of tight junctions as measured by increases in paracellular permeability (decrease in TER and increase in paracellular flux of nonionic tracers) and the mislocalization of tight junction-associated proteins (Jou et al., 1998; Rojas et al., 2001). Furthermore, another group (Bruewer et al., 2004) compared constitutive activation and inactivation of RhoA, Rac1, and Cdc42 in parallel with the use of a tetracycline-repressible system to tightly regulate the expression of constitutively active and dominant negative mutants of these three Rho GTPases in MDCK cells, in hopes of resolving ambiguities in the literature about the effects of Rho proteins on tight junction barrier function.

These researchers found that constitutively active and dominant negative mutants of RhoA, Rac1, and Cdc42 disrupted tight junction gate function as measured by decrease in TER development and increase in paracellular flux of FITC-dextran. Constitutively active forms of these proteins resulted in the mislocalization of tight junction-associated proteins such as ZO-1, claudin, and occludin, while their dominant negative counterparts had no effect on the localization of tight junction components. These functional and structural alterations observed in MDCK cells expressing constitutively active and dominant negative RhoA, Rac1, and Cdc42 resulted in subsequent increases in detergent solubility of tight

junction proteins, which suggests that these proteins are internalized upon expression of these mutants (Bruewer et al., 2004). Together, these data highlight the importance of tightly regulating the GTP-cycling of the small GTPases RhoA, Rac1, and Cdc42 during cell polarization in order to form structurally functional tight junctions. Since examination of the regulation of tight junction barrier function by Rho GTPases has been limited to the classic proteins RhoA, Rac1, and Cdc42, it would be important in the future to determine if other Rho GTPases including Wrch-1 are involved in tight junction dynamics to regulate epithelial cell morphology. Therefore, I investigated the functional consequences to tight junctions upon the expression of constitutively active Wrch-1. The results of these studies can be found in Chapter 2.

When Bruewer et al. investigated the ability of the Rho GTPases RhoA, Rac1, and Cdc42 to regulate tight junction dynamics; they observed that disruption of tight junction assembly and function was accompanied by alterations in the F-actin cytoskeleton and cell morphology (Bruewer et al., 2004). This is not a surprising result since Rho GTPases are best known for their ability to regulate the actin cytoskeleton to alter cell shape (Hall, 1998). In polarized epithelial cells there are three distinct pools of F-actin organization. These pools encompass an actin-rich apical brush border pool, a circumferential band of F-actin encircling cells at the perijunctional level that is associated with the apical junctional complex, and a pool of stress fibers adjacent to the basal membrane that mediate attachment to the extracellular matrix (Figure 1.7D). Upon expression of constitutively active or dominant negative forms of RhoA, Rac1, or Cdc42 there are dramatic alterations in these distinct pools of actin, depending on the small GTPase whose activity is improperly regulated (Bruewer et

al., 2004). However, the ability of the lesser known Rho GTPases to regulate actin organization in epithelial cells has not been investigated.

As stated previously, the expression of activated, GTP-bound Wrch-1 was found to induce the dissolution of actin stress fibers and filopodia formation in fibroblasts (Aspenstrom et al., 2004; Saras et al., 2004; Tao et al., 2001). In addition, Wrch-1 expression regulates focal adhesion turnover and cell migration (Chuang et al., 2007; Ory et al., 2007; Ruusala and Aspenstrom, 2007). However, the ability of Wrch-1 to regulate cytoskeletal dynamics of epithelial cells to regulate epithelial cell morphology and function remained to be determined and will be addressed in Chapter 3 of this dissertation.

In epithelial cells, there is an intimate relationship between the actin cytoskeleton and cell junctions. As stated above, the actin cytoskeleton is enriched at the apical surface and forms a contractile ring anchored to adherens junctions and tight junctions which is required to maintain proper epithelial cell shape and function. The contraction of both this actin ring and an array of basal stress fibers are active during cell polarization and epithelial morphogenesis (Jamora and Fuchs, 2002). Anchoring of the actin cytoskeleton to cell junctions integrates cell-cell contacts with changes in cell morphology and with morphogenetic movements of epithelial cells that occur during organogenesis (Lecuit and Lenne, 2007). In addition, dynamic reorganization of F-actin is closely correlated with changes in cell structure and the regulation of junction assembly. A functional link between tight junctions and the perijunctional actomyosin ring was initially described in studies using compounds capable of disrupting actin filament formation and severing formed actin filaments (Turner, 2000). Specifically, the treatment of epithelial monolayers with cytochalasin, which severs actin filaments, results in disruption of the formation and

contraction of perijunctional actomyosin ring and subsequent increases in paracellular permeability (decreased TER and increased paracellular flux of nonionic solutes) in multiple epithelial cell types (Bentzel et al., 1980; Madara et al., 1986; Stevenson and Begg, 1994). Under normal physiological conditions it is thought that dynamic relaxation and contraction of the apical perijunctional actomyosin ring regulates tight junction permeability. When the actomyosin ring is relaxed there is less tension on tight junctions, resulting in decreased tight junction permeability, while contraction of the actomyosin ring increases tension of the tight junction and leads to an increase in tight junction permeability (Turner, 2000). In addition to regulating tight junction barrier function, the actin cytoskeleton is instrumental in the assembly of tight junctions at proper membrane sites during cell polarization. During the process of polarization induced by calcium switch, apical actin is associated with a perinuclear ring structure that redistributes to the plasma membrane during junction assembly and polarization, and is believed to mediate the movement of junctional proteins from internal vesicles to the periphery of the cells (Ando-Akatsuka et al., 1999; Shen and Turner, 2005).

Since Rho GTPase function is required for regulation of tight junction dynamics and integrity and Rho proteins are best known for their ability to reorganize the actin cytoskeleton, it is not hard to believe that Rho GTPases regulate tight junctions by modulating the actin cytoskeletal dynamics which are essential for tight junction assembly and function. To this end, a few effectors and signaling pathways downstream of Rho GTPases that involve the regulation of myosin contraction and actin filament formation have recently been implicated in regulation of tight junction formation and function (Chen and Macara, 2006; Shen et al., 2006; Turner et al., 1997). Interestingly, expression of the

constitutively active form of myosin light chain kinase (MLCK) leads to increased phosphorylation of myosin light chain (MLC), increased tight junction permeability, mislocalization of tight junction-associated proteins and reorganization of perijunctional F-actin (Shen et al., 2006).

In addition, Chen et al. recently identified a link between the cell polarity protein Par3 and regulation of actin dynamics important for proper tight junction assembly, in which Par3 binds to and inhibits LIMK activity, leading to a subsequent decrease in cofilin activity (Chen and Macara, 2005). Interestingly, two of the known Wrch-1 effector proteins, PAK1 and Par6, are known to associate with MLCK or Par3, suggesting that Wrch-1 may potentially function in a parallel pathway in epithelial cells through these signaling pathways to regulate actin organization and subsequent tight junction dynamics. Therefore, I set out to determine if the atypical Rho GTPase, Wrch-1, is capable of regulating tight junction dynamics during cell polarization. More importantly, I wanted to address whether any effects on tight junction assembly and function could be explained by disruption of the actin cytoskeleton. The results of my experiments in MDCK cells stably expressing constitutively activated Wrch-1 will be addressed in Chapters 2 and 3.

1.11 Rho GTPase regulation of epithelial morphogenesis

The organization of single cells into multicellular tissues requires spatiotemporal coordination of cell proliferation, migration, cytoskeletal organization, adhesion, polarization, differentiation and death. The most prominent organization of cells within higher organisms is that of epithelia that are comprised of coherent sheets of cells that form a physical barrier between the body interior and the external environment. Some epithelia cover the outside of an organism and contain multiple layers (i.e. skin) in order to protect

vital internal organs. In contrast, internal organs are usually lined by a monolayer of epithelial cells (i.e. kidney, mammary gland, and lung) and typically consist of two types of epithelial structures: spherical cysts and cylindrical tubules. Both epithelial cysts and tubules enclose a central lumen with their apical cell surface facing the hollow lumenal center and with their basal surface making critical contacts with the basement membrane that drives polarization (Figure 1.8A). However, the ability to study the complexity of epithelial morphogenesis outside of model organisms was difficult until the recent development of three-dimensional (3D) culture systems. In addition, the use of 3D culture systems for studying epithelial morphogenesis *in vitro* with model cell lines has begun to aid in the understanding of diseases in which normal epithelial architecture is lost, such as polycystic kidney disease, cancer, atherosclerotic heart disease, and faciogenital dysplasia (O'Brien et al., 2002; Zegers et al., 2003).

Until recent years, examination of epithelial cell processes was restricted to two dimensional (2D) cultures of epithelial cells on permeable supports. Although 2D culture systems have led to the discovery of many proteins and signaling pathways involved in different cellular processes essential for the formation of normal epithelial cell morphology, 2D culture systems do not fully mimic physiological conditions. Recent studies have highlighted the superiority of investigating effects on epithelial cell polarity and morphology in 3D culture systems, because they better mimic the *in vivo* formation of epithelial structures (O'Brien et al., 2002). Bissell and colleagues pioneered the plating of epithelial cells into 3D extracellular matrix to produce *in vivo*-like epithelial cysts and tubules (Hagios et al., 1998). When grown in 2D, MDCK cells are known to form highly polarized epithelial monolayers that, when embedded in a collagen type I matrix, form cysts composed of highly polarized

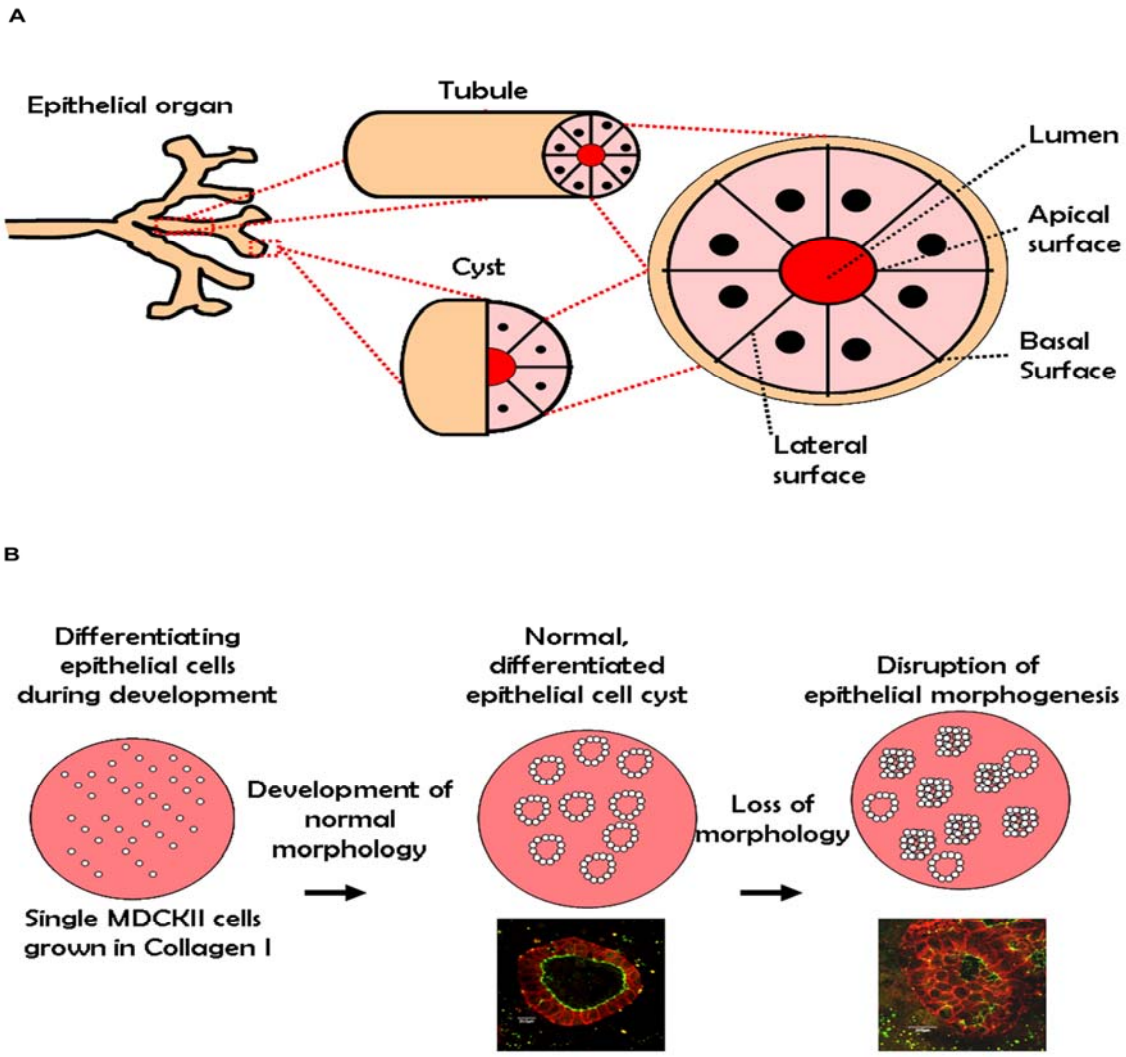


Figure 1.8 Epithelial structures in the human organs and 3D culture systems.
A) Organs in the human body many consists of highly organized epithelia structures. epithelia. Epithelia within these organs form tubules and cysts. In cross section, tubules and cysts enclose a central lumen with their apical cell surface facing the hollow lumenal center, lateral membrane domain facing neighboring cells, and their basal surface making critical contacts with the basement membrane. Adapted from (O'Brien et al., 2002). B) When grown in 3D extracellular matrix such as collagen I, monodisperesed MDCK cells undergo cystogenesis to form a complex epithelial structure (cyst). Visualization of immunostained 3D cultures with confocal microscopy allows for the labeling of the apical (ZO-1,green) and basolateral surface (E-cadherin, red) of cysts formed by MDCK cells. Loss of epithelial morphogenesis can be inferred with the use of confocal analysis of immunostained 3D cultures (bottom right; ZO-1,green and E-Cadherin , red).

monolayers that enclose a lumen and are encircled by a basement membrane (O'Brien et al., 2006; Pollack et al., 1998). In the last ten years, Mostov and colleagues have begun to use confocal analysis of immunostained 3D cultures as a powerful tool for understanding MDCK cyst and tubule morphogenesis (O'Brien et al., 2006; Pollack et al., 1998). The development of this groundbreaking technique that allows for the staining and imaging of intact 3D epithelial cultures has allowed researchers to begin to determine the mechanistic basis of cellular behavior during cystogenesis (Figure 1.8B).

Due to their roles in actin cytoskeletal organization, vesicular trafficking, and cell polarity, Rho GTPases have emerged as essential players in the development of epithelial morphogenesis in many model organisms (Van Aelst and Symons, 2002). Two of the structural elements (cell junctions and actin cytoskeleton) involved in the development of epithelial morphology regulated by Rho GTPases in 2D culture have been discussed above. However, the recent development of *in situ* confocal analysis of immunostained 3D culture systems mentioned above has allowed the investigation of Rho GTPases themselves and their regulators in a more physiological context of epithelial morphogenesis (O'Brien et al., 2006).

To date, the function of two Rho GTPases, Rac1 and Cdc42, in epithelial morphogenesis has been investigated in MDCK cells grown in collagen I matrix. O'Brien et al. determined that the Rho GTPase, Rac1, is involved in the regulation of apical pole development in MDCK cysts formed in collagen I matrix. Normally, apically localized proteins such as GP135 are found facing the hollow lumen of MDCK cysts embedded in collagen. In contrast, stable expression of dominant negative Rac1(T17N) in MDCK cells resulted in cysts with inverted polarization (apical markers facing the cyst periphery). These researchers determined that dominant negative Rac1(T17N) expression inhibited assembly of

the basement membrane component laminin. Thus, Rac1 is involved in proper laminin assembly and localization in order to orient the apical pole during epithelial morphogenesis (O'Brien et al., 2001).

Further investigation by this same group allowed for the elucidation of a signaling pathway in which β1 integrin engagement orients the apical pole of polarized cysts by activating the Rac1 that organizes laminin along the basement membrane (Yu et al., 2005). Interestingly, Rac1 uses the PI3K/AKT signaling pathway downstream of β1 integrin engagement to regulate the axis of polarity during cystogenesis (Liu et al., 2007). These data highlight the importance of evaluating the ability of Rho GTPases to contribute to epithelial morphogenesis by using 3D culture systems, rather than 2D culture systems in which the contribution of extracellular matrix to tissue orientation is absent.

More recent studies have only begun to scratch the surface of the involvement of Rho GTPases in the development of epithelial morphogenesis. Another report investigating the involvement of phosphoinositides in identifying distinct apical basolateral domains during cystogenesis determined that enrichment of PtdIns(4,5)P2 at the apical pole downstream of apically localized Phosphatase and Tensin homolog (PTEN) activity leads to the recruitment of the Cdc42-specific Rho GEF, Annexin2 (Martin-Belmonte et al., 2007). The apical restriction of the phosphoinositide, PtdIns(4,5)P2, serves as a signal to promote the association of Annexin2 via its PH domain to specific membrane regions, and thereby promotes the spatially restricted recruitment and activation of Cdc42 at the apical pole of developing MDCK cysts. Interestingly, ablation of Cdc42 during cystogenesis prevents the formation of a central lumen structure due to the inability of these MDCK cysts to form an apical membrane. Annexin2-mediated recruitment of Cdc42 to the apical membrane results

in accumulation of the Cdc42 effector Par6 and its binding partner aPKC to the apical membrane of MDCK cysts (Martin-Belmonte et al., 2007). Inactivation of aPKC activity with a pseudosubstrate inhibitor results in defects in cystogenesis similar to those found upon ablation of Cdc42 expression or expression of dominant negative form of Par6 incapable of activating aPKC (Kim et al., 2007; Martin-Belmonte et al., 2007). Together these data suggest that restriction of PtdIns(4,5)P2 at the apical surface by the lipid phosphatase PTEN results in the recruitment of the Cdc42-specific GEF, Annexin2, that binds and activates Cdc42 along the apical membrane. GTP-bound, active Cdc42 then recruits the cell polarity complex Par6-aPKC to regulate the formation of the apical surface and lumenal structure.

Although the Rho GTPases Rac1 and Cdc42 and their downstream signaling pathways have been shown to be master regulators of the development of cell polarity during epithelial morphogenesis in 3D collagen culture systems, little is known about the involvement of the other members of the Rho GTPase family. In many other cellular processes there is a requirement for many of the other members of the Rho GTPase family to fine tune the signaling events downstream of the activation to promote diverse biological activities. Therefore, I set out to determine if the atypical Rho GTPase Wrch-1 contributes to epithelial morphogenesis in 3D culture. I also used MDCK cystogenesis as a readout for the ability of constitutively activated Wrch-1 to promote the loss of normal morphology that is associated with the transformed phenotype.

1.12 Rho GTPases in cancer

Normal, differentiated epithelial cells display a cuboidal polarized morphology. During tumor progression, they lose this polarized morphology, a process that involves the loss of cell-cell adhesions, loss of cell polarity, and cytoskeletal reorganization that promotes

dedifferentiation, invasion, and metastasis. In addition to these morphological changes, cancer cells exhibit excessive cell proliferation and inhibition of normal apoptosis programs that promote cell survival. Rho GTPases are instrumental in regulating many of the normal cellular processes that are misregulated during cancer progression, suggesting that, like members of the Ras superfamily, Rho GTPases may also be mutationally activated in human cancers. However, unlike Ras proteins that are oncogenically mutated in 30% of human cancers, no naturally occurring mutations in Rho GTPases have been found, with the exception of RhoH/TTF (Pasqualucci et al., 2001; Preudhomme et al., 2000). The absence of mutationally activated Rho GTPases in human cancers suggests that GTP/GDP cycling of these proteins may be required for their function. However, Rho GTPases contribute to tumorigenesis through overexpression of the GTPases themselves or of their positive regulators, or through activating mutations or overexpression of upstream signaling pathway components. In addition, downregulation of Rho GTPase negative regulators such as GAPs is associated with tumor progression. Misregulation of Rho GTPases in cancer leads to aberrant growth, dedifferentiation, invasion and metastasis (Figure 1.9) (Ellenbroek and Collard, 2007; Sahai and Marshall, 2002).

As stated above, RhoH is the only Rho GTPase that has been found to be genetically altered in human cancers. The RhoH gene has been found to be rearranged in non-Hodgkin's lymphomas and multiple myeloma, along with mutations in the 5'UTR in diffuse large cell lymphomas (Pasqualucci et al., 2001; Preudhomme et al., 2000). The rearrangement found in these hematopoietic cancers is caused by a t(3;4)(q27;p11-13) chromosomal translocation resulting in a gene fusion with the BCL3/LAZ3 oncogene (Dallery-Prudhomme et al., 1997). However, it is unknown whether or how these RhoH translocations and hypermutations

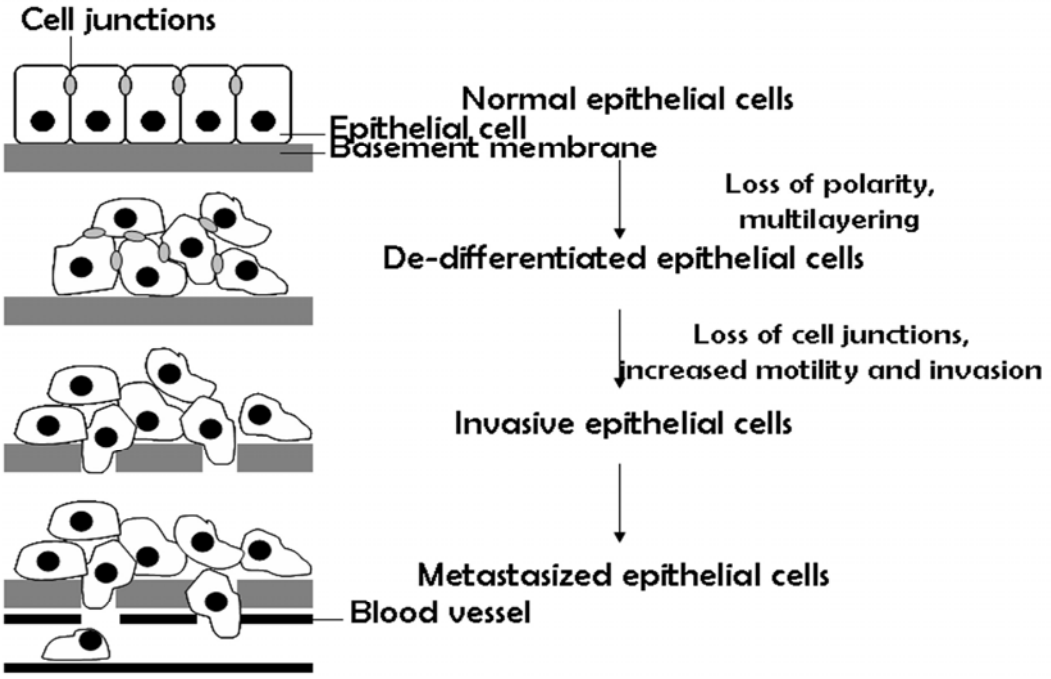


Figure 1.9 Rho GTPases contribute to multiple steps of tumorigenesis.
Misregulation of Rho GTPases participates in at least 3 stages of tumor progression: de-differentiation, invasion and metastasis through the loss of cell polarity, loss of cell junctions, increased motility, intravasation, and vascularization. Adapted from (Sahai and Marshall, 2002).

contribute to the pathology of these cancers. Although other Rho GTPases have not been found to be genetically altered in cancer, the expression and activation of several of the Rho GTPases have been reported to be misregulated in human cancers. Altered expression of RhoA, RhoB, RhoC, Rac1, Rac1b, Rac2, Rac3, RhoG, Cdc42, RhoH/TTF, and Rnd3/RhoE have been reported in various human cancers, including breast, colon, lung, gastric, lymphoma, melanoma, and pancreatic (Ellenbroek and Collard, 2007). Specifically, upregulation of RhoC is associated with aggressive cancers such as pancreatic ductal adenocarcinoma, metastatic gastric carcinoma and melanoma, inflammatory breast cancer, and nonsmall cell lung cancer (Liu et al., 2004a; Shikada et al., 2003; Suwa et al., 1998; van Golen et al., 2002). Expression of the Rac1 splice variant Rac1b, that is known to be constitutively active due to accelerated GDP/GTP exchange, is elevated in colorectal and breast tumors (Jordan et al., 1999; Schnelzer et al., 2000). These are a few examples of altered expression of Rho GTPases; their specific contributions to tumorigenesis on an individual basis still need to be investigated.

Interestingly, misregulated expression of Rho GTPase regulators resulting in deregulated signaling downstream of Rho proteins is also associated with various human cancers. Several Rho family GEFs, positive regulators of Rho family activation, are found either overexpressed or truncated in human tumors, leading to constitutive activation of Rho GTPase-mediated signaling pathways and tumorigenesis. In leukemia, chromosomal translocations result in BCR-ABL and MLL-LARG chimeras that contribute to tumorigenesis (Kin et al., 2001; Kourlas et al., 2000). In addition, genomic deletion and promoter methylation results in downregulation of the RhoGAPs, DLC-1 and DLC-2, in breast cancer and in hepatocellular carcinoma (Ching et al., 2003; Wong et al., 2003; Yuan et

al., 2003a; Yuan et al., 2004; Yuan et al., 2003b). Overexpression and downregulation of each of the RhoGDIs has been reported in human cancers of different origins, but the role and mechanism by which this may contribute to tumor progression remains to be determined (Dovas and Couchman, 2005; Zhang and Zhang, 2006). Together, these reports of alterations in Rho GTPases themselves and of their positive and negative regulators in human cancers illustrates the importance of validating these proteins as drug targets for cancer treatment, and of discovering pharmacologic inhibitors of the validated proteins to combat cancer development and progression.

As stated previously in Section 1.6, the atypical Rho GTPase Wrch-1 was initially discovered as a Wnt-1 responsive gene that, when activated, phenocopied Wnt-1-mediated morphological transformation of C57MG mammary epithelial cells (Tao et al., 2001). Wrch-1 message levels are regulated by Wnt1 in a β-catenin-independent manner, and are reported to be altered in several types of cancers including colon, gastric, breast and ovarian (Kirikoshi and Katoh, 2002; Tao et al., 2001). Further investigation into the ability of Wrch-1 to induce transformation confirmed that constitutively activated Wrch-1 promotes anchorage-independent growth of NIH 3T3 fibroblasts (Berzat et al., 2005b; Shutes et al., 2004). However, the mechanisms by which Wrch-1 promotes or contributes to transformed phenotype and human cancer remained to be determined. To that end, during my dissertation research I set out to investigate the biological activities and downstream effectors of activated Wrch-1 that could potentially contribute to Wrch-1-mediated cellular transformation. Therefore much of my research has been focused on determining mechanisms for Wrch-1 regulation of normal epithelial cell morphology by modulating epithelial tight junctions and the actin cytoskeleton through the cell polarity cassette formed

by Par6-aPKC, and how the loss of epithelial cell morphology contributes to cancer biology (Figure 1.10 and 1.11). The results of my research project will be addressed in the following chapters of this dissertation, entitled "The Rho family GTPase, Wrch-1, disrupts epithelial tight junction dynamics through interaction with Par6" (Chapter 2) and "The transforming, Rho family GTPase, Wrch-1, disrupts epithelial cytoskeletal organization and epithelial morphogenesis" (Chapter 3).

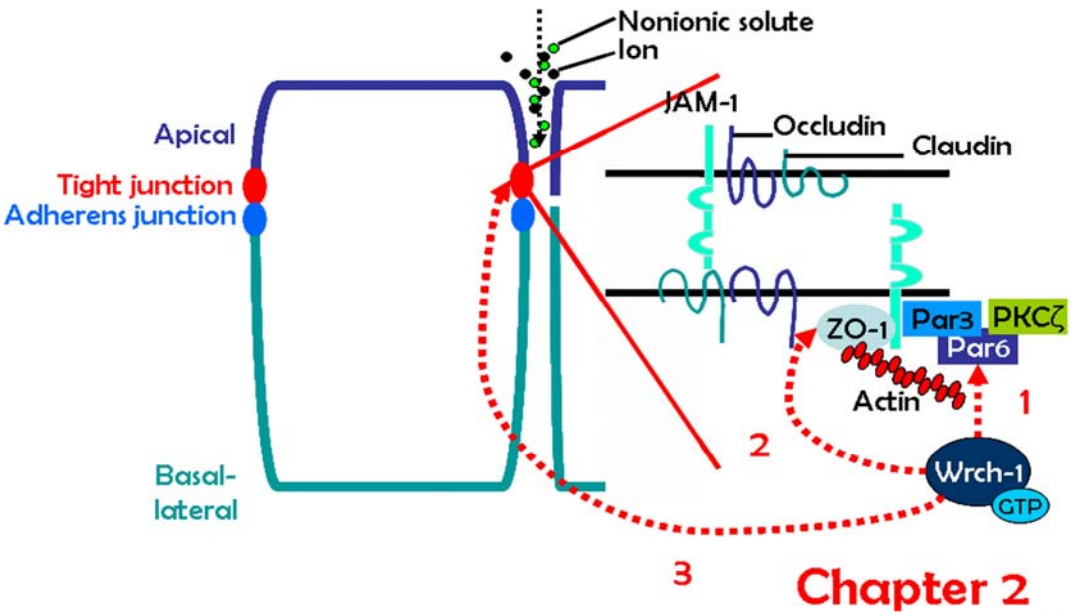


Figure 1.10 Evaluating Wrch-1 association with cell polarity protein Par6 and regulation of epithelial cell tight junction dynamics during cell polarization.
Schematic of dissertation Chapter 2. Chapter 2 describes: (1) the use of biochemical methods to determine whether Wrch-1 interacts with the cell polarity protein Par6 in a GTP-dependent manner; the use of MDCK cells as an experimental model system to determine whether activated Wrch-1 disrupts tight junction formation measured by ZO-1 localization (2) and/or tight junction integrity measured paracellular permeability of ions and nonionic solutes (3) during epithelial cell polarization.

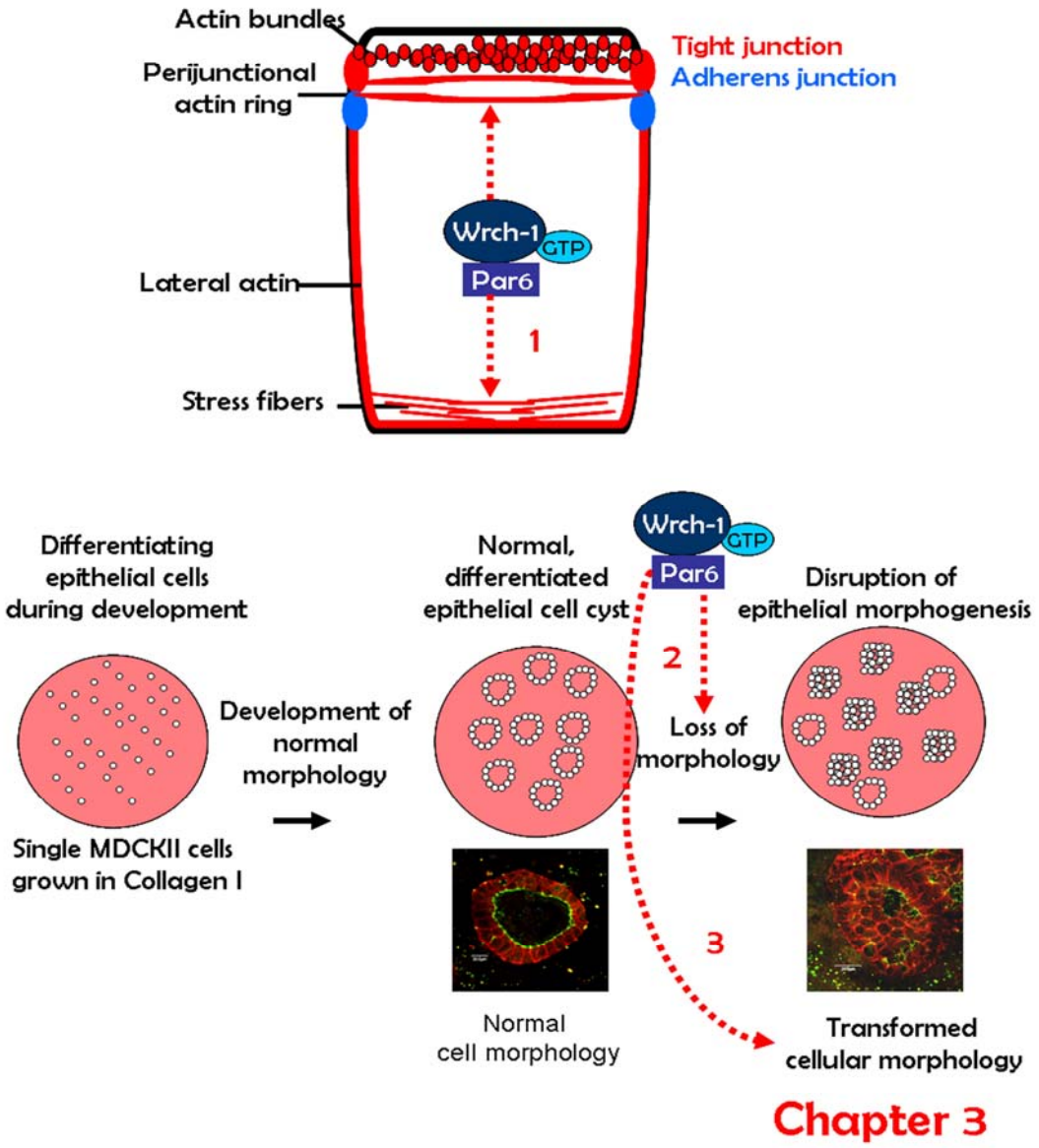


Figure 1.11 Evaluating Wrch-1 regulation of epithelial cell actin organization and morphogenesis that may potentially promote Wrch-1-mediated cellular transformation. Schematic of dissertation Chapter 3. Chapter 3 describes: (1) the use MDCK cells as an experimental model system to determine whether activated Wrch-1 disrupts actin organization; (2) the use of confocal analysis of immunostained of 3D collagen I culture systems to determine whether activated Wrch-1 disrupts MDCK cell cystogenesis; (3) the use of soft agar assays to determine whether activated Wrch-1 expression promotes anchorage independent growth of MDCK cells.

CHAPTER 2

THE RHO FAMILY GTPASE, WRCH-1, DISRUPTS EPITHELIAL TIGHT JUNCTION DYNAMICS THROUGH INTERACTION WITH PAR6

2.1 Abstract

Tight cell-cell adhesions, cuboidal morphology, and distinct apical and basolateral membranes characterize the polarized morphology of epithelial cells. Tight junctions and adherens junctions, important structural components of epithelial cells, regulate these characteristics that contribute to the maintenance of cell polarity and morphology. The Rho subfamily of GTPases function as molecular switches to regulate diverse cellular functions such as cell-cell adhesion, cell polarity, motility, and proliferation through interaction with their downstream effectors. Wrch-1, an atypical and transforming Rho GTPase, regulates cellular activities including proliferation and actin organization, but its functions and effectors remain poorly characterized. We show here that Wrch-1 distributes to tight junctions and adherens junctions along the apical and basolateral membranes in MDCK cells and binds the cell polarity protein Par6 in a GTP-dependent manner. Activated Wrch-1 negatively regulates the kinetics of tight junction assembly and the integrity of tight junctions during epithelial cell polarization, but has no detectable effect on overall cell polarity in confluent monolayers. An effector domain mutant of activated Wrch-1 that inhibits Par6 binding abrogates the ability of Wrch-1 to disrupt TJ formation and function. We hypothesize that one of the normal biological functions for Wrch-1 may be to regulate tight junction dynamics in order to maintain proper cell morphology in part through interacting

with Par6-PKCζ complex. Whether constitutively active Wrch-1-mediated cellular transformation occurs in part through a mechanism involving disruption of tight junction integrity will be important to elucidate in the future.

2.2 Introduction

Rho family small GTPases are Ras-related proteins that regulate many normal cellular properties such as cell shape, cell motility and migration, gene transcription and cell proliferation (Etienne-Manneville and Hall, 2002). Like other members of the Ras superfamily, Rho GTPases function as molecular switches cycling between an active GTP-bound state and inactive GDP-bound state (Symons and Settleman, 2000). When GTP-bound and active, they elicit biological functions through interactions with their downstream effectors (Jaffe and Hall, 2005).

Wrch-1 is an atypical member of the Cdc42 subgroup of Rho GTPases that are perhaps best known for inducing the formation of actin microspikes and filopodia. Wrch-1 shares 57% sequence identity with Cdc42 and 61% sequence identity with its close relative, Chp/Wrch-2 (Aronheim et al., 1998; Tao et al., 2001). Despite this high sequence identity, Wrch-1 has unique characteristics that suggest regulation and biological functions divergent from those of each of these relatives. For example, Wrch-1 was initially discovered as a Wnt1-responsive gene that, when mutationally activated (Q107L, analogous to the Q61L mutation in Ras and Cdc42), phenocopied Wnt-1 morphological transformation (Tao et al., 2001). Recent studies have highlighted an additional role for Wrch-1 in the regulation of cell migration through mechanisms clearly distinct from those of Cdc42, such as modulating focal adhesion turnover (Chuang et al., 2007; Ory et al., 2007). In addition, Wrch-1 contains a 46 amino acid N-terminal extension not found in Cdc42. This extension contains poly-

proline PxxP binding motifs for Src homology 3 (SH3) domain-containing proteins such as Grb2, PLCγ and NCKβ (Saras et al., 2004). We and others have shown that expression of activated Wrch-1 leads to activation of PAK1 and JNK, formation of filopodia and cellular transformation of NIH 3T3 fibroblasts (Berzat et al., 2005b; Saras et al., 2004). Although some of the biochemical characteristics, subcellular localization and membrane association properties of Wrch-1 have been identified, full identification and characterization of Wrch-1 downstream effectors, regulators, biological functions, and potential contributions to cancer remain elusive.

A yeast two-hybrid screen using Wrch-1 as bait identified two proteins as potential interacting partners: Par6 and PAK1b (Aspenstrom et al., 2004). Par6 is known to form an evolutionarily conserved complex with PKCζ and Par3 that is instrumental in establishing epithelial cell polarity and in regulating tight junction (TJ) formation (Shin et al., 2006). This suggested the possibility that Wrch-1 might have similar functions, and thus share a critical role in many aspects of normal cell and tissue homeostasis. Classic Rho GTPases, including Rac1 and Cdc42, are known to participate in the formation of TJs through interactions with the cell polarity proteins, Par6 and Par3. Aberrant activation of these GTPases is known to disrupt TJs, cell polarity and to induce epithelial cell transformation (Chen and Macara, 2005; Joberty et al., 2000; Johansson et al., 2000; Qiu et al., 2000; Rojas et al., 2001). Whether Wrch-1 or other atypical Cdc42-related proteins also participate in TJ regulation remained to be tested.

In the present study, we sought to determine whether Wrch-1 regulates epithelial cell morphology through modulating TJs. Specifically, we investigated a potential interaction between Wrch-1 and the cell polarity complex Par6-PKCζ, and we used polarized epithelial

cells to elucidate whether expression of Wrch-1 affects epithelial cell TJ assembly and integrity. We demonstrate that activated Wrch-1 has dramatic effects on TJ dynamics in part through a GTP-dependent interaction with Par6. Together these data reveal a potential mechanism by which Wrch-1 may control normal cellular morphology, and highlight the importance of identifying cellular mechanisms that tightly regulate the GTP-cycling of Wrch-1 so that normal cellular and tissue homeostasis is maintained.

2.3 Results

2.3.1 Wrch-1 interacts with both the cell polarity protein Par6 and the atypical protein kinase PKCζ in a GTP-dependent manner

The function of Rho GTPases is dictated by their subcellular localization and interaction with downstream effectors. Therefore, the observation that active GTP-bound Wrch-1 interacts with the cell polarity protein Par6 in a yeast two-hybrid screen (Aspenstrom et al., 2004), suggested a possible role for Wrch-1 in regulating cell polarity. To determine whether GTP-Wrch-1 and Par6 interact *in vitro* and whether the interaction is nucleotide-dependent, we expressed Par6 as a GST fusion protein in *E. coli* and performed GST-Par6 pulldowns from lysates of COS-7 cells expressing Wrch-1 proteins. As predicted by the yeast two-hybrid screen, we found that both wild type (WT) and constitutively activated Wrch-1(Q107L) interacted with GST-Par6 (Figure 2.1A) but not with GST-alone controls (data not shown), whereas GDP-bound Wrch-1(T63N) did not interact. We also observed that N-terminally truncated ΔN-Wrch-1(Q107L) still bound to Par6 (Figure 2.1A), indicating that this interaction does not require the polyproline-rich N-terminal extension. To further investigate this interaction, we co-expressed the HA-tagged Wrch proteins with Myc-tagged Par6 in COS-7 cells, and then assessed their interaction by immunoprecipitation. As predicted by our *in vitro* GST pulldowns, GTP-bound Wrch-1(WT or Q107L) but not GDP-

bound Wrch-1(T63N) associated with immunoprecipitated Par-6 (Figure 2.1B). These data show that the cell polarity protein, Par6, associates with Wrch-1 both *in vitro* and *in vivo* in a GTP-dependent manner, supporting the notion that it could be a physiological effector of Wrch-1 function.

Par6 is normally associated with a ternary complex that includes Par3 and the atypical PKC, PKCζ (Joberty et al., 2000). To determine whether Wrch-1 also interacts with this complex, we used GST-pulldowns (Figure 2.2A) and immunoprecipitation (Figure 2.2B) to examine the interaction of Wrch-1 with PKCζ. We observed that both Wrch-1(WT) and (Q107L) interacted with endogenous PKCζ *in vitro* and *in vivo*. These observations indicate that polarity protein complexes containing Par6 and PKCζ may be physiologically relevant binding partners of Wrch-1.

2.3.2 Wrch-1 localizes to cell-cell junctions in polarized MDCKII cells

The Par6-PKCζ complex regulates the formation of epithelial cell junctions that are required for normal epithelial cell polarity and morphology (Suzuki et al., 2001; Yamanaka et al., 2001), and the interaction of Wrch-1 with this complex suggested that Wrch-1, like Par6 and PKCζ, may also localize at these junctions in polarized cells. Therefore we used confocal microscopy to visualize Wrch-1 localization in polarized MDCKII epithelial cells stably expressing HA-tagged Wrch-1 proteins. Both wild type and activated Wrch-1 localized to the plasma membrane (PM) and microvilli at the apical surface, but were associated predominantly with the basolateral PM (Figures 2.3A).

We then attempted to determine whether Wrch-1 colocalizes with components of the polarity complex. Due to the lack of commercially available antibodies that can effectively detect Par6 by immunofluorescence (IF), we used antibodies against Par3, which is known to

associate with both Par6 and PKCζ at TJ in polarized MDCKII cells (Izumi et al., 1998). In empty vector control MDCKII cells, Par3 was restricted to a defined band at cell-cell contact regions known as the apical junctional complex (AJC) (Figure 2.3B, XZ scans). As described above, both wild type and activated Wrch-1 were distributed all along the lateral cell contacts, but clearly overlapped with endogenous Par3 at the AJC (Figure 2.3B). Similar results were obtained for PKCζ (Figure 2.3C) supporting the idea that Wrch-1 may use endogenous Par3-Par6-PKCζ as a *bona fide* downstream effector complex. This partial overlap is not unexpected, because we anticipate that Wrch-1 has multiple functions in epithelial cells, not all of which are linked to its interactions with Par3, Par6 and PKCζ.

The distribution of Wrch-1 predominantly to basolateral membranes is consistent with its potential association with cell-cell junctions, such as TJs and adherens junctions (AJs). TJs are the most apical of cell junctions, and TJ proteins such as occludin and ZO-1, like Par6, are generally restricted to a tight band that circumscribes the AJC. AJs are adhesive structures formed through calcium-dependent interactions between cadherin molecules on adjacent epithelial cells, and AJ proteins such as E-cadherin and β-catenin are distributed to the AJC and along the entire lateral PM. To investigate the possibility that Wrch-1 localizes to both TJs and AJs in polarized epithelial cells, we examined MDCKII cells stably expressing HA-Wrch-1(WT). Wrch-1 localized at the lateral PM overlapped with the TJ transmembrane protein, occludin, and with the TJ peripheral protein, ZO-1, but did not localize exclusively to TJs (Figures 2.4A & B, top panels). The partial overlap between these proteins at cell-cell contact regions (Figures 2.4A & B, XZ) suggests that the spatial distribution of Wrch-1 may allow it to regulate TJs.

We also used IF to investigate the distribution of Wrch-1 in conjunction with the AJ-associated proteins, β-catenin and E-cadherin. These proteins overlapped with Wrch-1 along the basolateral membrane (Figures 2.4C & D, bottom XZ panels) and along the PM at the apical and basal margins of the lateral membrane (Figure 2.4C &D, top Apical and Basal panels). These data suggest a potential role for Wrch-1 in regulating structure and function of AJC by modulating cell junctions (TJs and AJs), which are required for proper epithelial cell morphology.

2.3.3 Activated Wrch-1 disrupts tight junction formation during cell polarization

Rho GTPases regulate the formation and maintenance of both TJs and AJs (Fujita and Braga, 2005; Shin et al., 2006). Wrch-1 distribution to cell-cell junctions and association with the TJ-associated Par6-PKCζ complex suggest that Wrch-1 could regulate TJ formation and function. Therefore, we generated a large panel of MDCKII epithelial cells stably expressing HA-tagged Wrch-1 proteins (Figure 2.5A) and used confocal microscopy to investigate the localization of TJ proteins during calcium-induced cell polarization in the presence or absence of activated Wrch-1. Briefly, culturing cells in calcium-depleted medium disrupts cell-cell contacts, and junction proteins are internalized in cytosolic vesicles. Restoration of calcium (calcium switch) triggers TJ assembly and cell polarization in a series of kinetically regulated steps. In the absence of calcium (Figure 2.5B, 0 h), the TJ-associated protein ZO-1 was normally localized to a diffuse perinuclear ring structure enriched in actin filaments (Stevenson and Begg, 1994). Following calcium switch, ZO-1 was redistributed to the lateral surface of the newly formed cell-cell contacts (Figure 2.5B, 12 h) in both control cells and cell expressing Wrch-1(WT). This redistribution of ZO-1 is consistent with the formation of normal TJs.

Strikingly, in cells expressing activated Wrch-1(Q107L), the diffuse perinuclear accumulation of ZO-1 and F-actin was missing in the absence of cell-cell contacts (Figure 2.5B, 0 h). Furthermore, following re-addition of calcium the movement of ZO-1 to the AJC was significantly delayed, and the distribution of ZO-1 within the AJC at later time points (Figure 2.5B, 12 h) was disorganized relative to cells expressing vector only or Wrch-1(WT). This mislocalization of ZO-1 is consistent with a delay in proper TJ formation. However, ZO-1 eventually localized properly to TJs in these monolayers after 5 days (data not shown), suggesting that it is the kinetics of TJ formation, and not maintenance of TJ structure in polarized cells, that is altered by activated Wrch-1.

2.3.4 Activated Wrch-1 disrupts tight junction integrity during cell polarization

The physiological function of TJs, known as the "gate" function (Matter and Balda, 2003b), is to form a barrier to the movement of ions and solutes between cells. The formation of this barrier over time can be measured by the development of transepithelial resistance (TER), and is a functional measure of TJ assembly. To investigate the role of Wrch-1 in normal TJ "gate" function, we measured TER development in MDCKII cells after calcium switch. In cells expressing empty vector or Wrch-1(WT), TER increased linearly, reaching an initial peak value at ~18 h before falling to steady state levels. In contrast, cells expressing activated Wrch-1(Q107L or ΔN-Q107L) were delayed in initial TER development, displaying a 3-fold difference in measured resistance (Figure 2.5C). Expression of an activated form of Wrch-1 that is mislocalized to the cytosol due to mutational loss of its C-terminal palmitoylation site, Wrch-1(Q107L/SSFV), did not delay TER development (Figure 2.5C), suggesting that proper membrane localization of Wrch-1 is required for its ability to modulate the TJ "gate" function.

Significantly, expression of active, GTP-bound Wrch-1 did not prevent the eventual formation of a functional TJ seal by ~80 h, as indicated by resistance measures above 800 Ω x cm^2. This is in contrast to constitutively active Ras, which is known to disrupt cell junctions entirely (Mullin et al., 2005) (Figure 2.5C). These data show that enhanced Wrch-1 activity disrupts TJ "gate" function only during early TJ assembly, whereas steady state TJ permeability is unaffected.

The "gate" formed by TJs is not only selectively permeable to ion flow, but also regulates the paracellular flux of hydrophilic uncharged molecules. Thus, we measured the paracellular diffusion of FITC-conjugated Dextran tracer molecules of 4,000 kD and 40,000 kD across cell monolayers. Tracer diffusion was evaluated 6 h after induction of cell-cell contacts by the addition of calcium to the apical surface of the confluent cell monolayers grown on filters. As expected, there was minimal difference in the observed fluorescence from either tracer in cells expressing Wrch-1(WT) compared to empty vector control cells. In contrast, we observed a significant enhancement in the diffusion of both dextrans in cells expressing activated Wrch-1 or Ras (Figure 2.5D). As in the TER experiments, the nonpalmitoylated cytosolic mutant of activated Wrch-1(Q107L/SSFV) did not produce an increase in paracellular flux (Figure 2.5D). Similarly, dextran flux in cells expressing activated Wrch-1 was ultimately normal at ~18 h after calcium switch (Figure 2.5D). Taken together, these data suggest that aberrant activation of Wrch-1 leads to disruption of early events in TJ assembly.

2.3.5 Effector domain mutant of Wrch-1 incapable of binding Par6 abrogates its ability to disrupt tight junction formation and integrity, during cell polarization

To determine if the ability of Wrch-1 to disrupt TJ dynamics was linked to the GTP-dependent interaction between Wrch-1 and Par6, we performed site-directed mutagenesis

within the effector domain of Wrch-1 to create a mutant incapable of binding Par6. Previous mutational analysis of Cdc42 identified a single point mutation at Tyr40 in the effector domain that was capable of blocking the interaction of active Cdc42-GTP with the CRIB-PDZ cassette of Par6 (Garrard et al., 2003). Therefore, we constructed a point mutation at the analogous residue of Wrch-1, F86C, to test whether this mutation would abrogate the GTP-dependent interaction of Wrch-1 and Par6. Unlike the HA-tagged parental Wrch-1(Q107L), the effector domain mutant (EDM) Wrch-1(Q107L/F86C) did not interact with GST-Par6 or immunoprecipitated Myc-Par6 (Figures 2.6A & B), as predicted by analogy to the Cdc42 EDM.

We then generated MDCKII cells stably expressing the Wrch-1 EDM (Figure 2.6C) to test the requirement for Par6 or other CRIB domain containing proteins in Wrch-1-mediated modulation of TJ assembly and cytoskeletal dynamics. We observed that the Wrch-1 EDM did not interfere with correct localization of ZO-1 during calcium-induced cell polarization (Figure 2.6D). Expression of the activated Wrch-1 EDM was also unable to delay initial TER development (Figures 2.6E, respectively). These data suggest that the interaction between Wrch-1 and Par6 may contribute to the localization of active GTP-bound Wrch-1 and the regulation of TJ assembly.

2.4 Discussion

The critical influence of the classic activated Rho GTPases RhoA, Rac1 and Cdc42 on such functions as actin cytoskeletal organization, cell polarity, cell adhesion and microtubule dynamics is well recognized (Etienne-Manneville and Hall, 2002), but the roles of the atypical family GTPases in these processes are less well understood. Recent studies have described roles for the atypical Rho GTPase Wrch-1 in actin cytoskeletal organization (Saras

et al., 2004), focal adhesion formation, and cell motility in non-epithelial cells (Chuang et al., 2007; Ory et al., 2007). In the present study, we have identified a novel biological function for Wrch-1 in regulating the kinetics of TJ assembly and integrity of TJs. We hypothesize that the disruption of TJ dynamics by excessive Wrch-1 activity may contribute to the ability of constitutively active, GTP-locked Wrch-1 to induce transformation of epithelial cells.

Since the effector targets of Rho GTPases help to dictate their specific biological functions, identification of the downstream effectors of GTP-Wrch-1 is essential to elucidating the contributions Wrch-1 makes to normal tissue homeostasis and human cancers. We identified the cell polarity protein, Par6, as a GTP-dependent binding partner for Wrch-1. This led us to investigate whether, like Par6, Wrch-1 regulates TJ formation in epithelial cells (Gao et al., 2002; Suzuki et al., 2002; Suzuki et al., 2001; Yamanaka et al., 2001). We found that expression of constitutively activated, GTP-locked Wrch-1 disrupts TJ formation and integrity during cell polarization as determined by delayed ZO-1 localization and increased permeability. However, TJs do form eventually in cells expressing activated Wrch-1, based on the localization of ZO-1, the resistance formed, and normal paracellular flux at steady state, suggesting that regulation of Wrch-1 activation is necessary only for early TJ dynamics. These findings are strikingly similar to exogenous expression of a dominant negative mutant of Par6 that results in a kinetic delay in TJs. TJs form eventually but the initial steps, like ZO-1 localization, absence of the initial peak in TER developments, and increase in paracellular permeability are significantly delayed (Gao et al., 2002; Yamanaka et al., 2001). Therefore, it is possible that active Wrch-1 functions in a pathway parallel to the Par6-aPKC complex to regulate TJ assembly.

Our data suggest that Par6 may be one important effector of activated Wrch-1 in regulating TJ dynamics. This hypothesis is supported in part by the observation that an effector domain mutant of activated Wrch-1 that cannot bind Par6 is unable to disrupt TJ formation and function in MDCKII cells. Interestingly, the effects of activated Wrch-1 are also similar to those found after RNAi-mediated knockdown of several proteins within polarity complexes, such as PALS1 and PATJ (Shin et al., 2005; Sourisseau et al., 2006; Straight et al., 2004). These observations suggest that Wrch-1 may act more generally through interaction with the entire polarity complex. This hypothesis is supported by our observation that Wrch-1 binds polarity complex proteins. Like activated Wrch-1, dominant negative aPKC expression in MDCKII has similar negative effects on TJs (Suzuki et al., 2002; Suzuki et al., 2001).

Although we have linked Wrch-1 to the cell polarity complex formed by Par6 and PKCζ as a possible downstream pathway involved in activated Wrch-1-mediated disruption of TJ dynamics, the use of EDM of Wrch-1 does not negate the involvement of other Wrch-1 effectors like Pak1. Pak1 is an inhibitor of myosin light chain kinase (Sanders et al., 1999), which is a potent regulator of cytoskeletal dynamics. Chuang et al. demonstrated that RNAi-mediated depletion of Wrch-1 leads to decreases in MLC phosphorylation and fibroblast cell migration. Interestingly, in other studies (Shen et al., 2006), expression of the constitutively active form of MLCK led to increased phosphorylation of MLC, increased TJ permeability, mislocalization of TJ-associated proteins and reorganization of perijunctional F-actin. These findings are identical to the alterations in TJ dynamics that we observed upon expression of activated Wrch-1. Together, these data suggest that Wrch-1 regulation of MLCK activity

downstream of PAK1 could be another possible molecular mechanism for the Wrch-1 biological functions we evaluated here.

Regulation of actin cytoskeleton dynamics by Rho GTPases has been proposed as a general mechanism to regulate TJ assembly during cell polarization (Bruewer et al., 2004; Nusrat et al., 2001). Interestingly, we observed that the actin-associated perinuclear ring structure to which ZO-1 normally localizes is absent in MDCK cells expressing constitutively activated Wrch-1. This actin-associated perinuclear ring is also absent in ZO-1 knockdown MDCK cells and this structure is proposed to be involved in TJ assembly (McNeil et al., 2006). Therefore, future investigation into actin cytoskeletal dynamics during cell polarization in activated Wrch-1-expressing MDCK cells will help identify actin-based structures that may be perturbed and affect the normal formation of TJs.

Our observations that Wrch-1 can interact with the Par6-PKCζ polarity complex and can disrupt epithelial TJs in a manner that can be abrogated by blocking Par6 interaction suggest a potential mechanism for at least one normal biological function mediated by Wrch-1. Wrch-1 is expressed ubiquitously in human tissues, with highest levels of expression in brain, heart, skeletal muscle, liver, placenta, and lung (Tao et al., 2001). Future investigation of the roles that Wrch-1 may play in normal cellular physiology is of great interest, and may best be examined in epithelial tissues in which tight regulation of TJ dynamics is necessary. More importantly, this discovery highlights that aberrant activation of Wrch-1 promotes the disruption of TJs, an alteration in normal cellular architecture that is associated with cellular transformation. Further determination of the normal biological functions and downstream effectors of the small GTPase Wrch-1 will provide great insight into the mechanisms behind Wrch-1-mediated cellular transformation.

2.5 Materials and methods

2.5.1 Cell culture, transfection, and retroviral infection

COS-7 cells were grown in DMEM (GIBCO/Invitrogen) supplemented with 10% FBS (Sigma) and 1% penicillin-streptomycin (P/S), and maintained in 5% CO_2 at 37^{o}C. Cells were transfected using TransIT-LT1 (Mirus) according to the manufacturer's instructions. MDCKII cells, generously provided by Robert Nicholas (UNC-CH), were grown as above and supplemented with 1% non-essential amino acids (NEAA, Invitrogen) ("complete medium"). Stable MDCKII cell lines were generated by retroviral infection. Production of retrovirus was obtained by $CaCl_2$-mediated transfection of pBabe-HAII-puro, pVPack-Gag/Pol, and pVPack-Ampho (Stratagene) vectors into 293T cells. Cells were infected by exposure to retroviral supernant containing 8 μg/ml of Polybrene (American Bioanalytical) and maintained in puromycin for 10 days, after which colonies were pooled for use.

2.5.2 Molecular constructs

Human Wrch-1 (WT, Q107L, ΔN-WT, ΔN-Q107L and Q107L/C255S/C256S) were generated as described previously (Berzat et al., 2005b). For HA epitope-tagged Wrch-1, 5' and 3' BamHI sites were introduced by PCR, for ligation into the BamHI site of pCGN-hygro or pBabe-HAII-puro retroviral expression vectors. Human Wrch-1(Q107L/F86C) was generated by site-directed mutagenesis and subcloned as described above. To generate Myc- and GST-tagged human Par6, PCR was used to introduce 5' BamHI and 3' EcoRI sites into a pBabe-T7-Par6C construct (gift of Channing Der, UNC-CH) for subcloning into pCMV3B(Myc) and pGEX4T(GST), respectively. All sequences were verified by the Genome Analysis Facility at UNC-CH.

2.5.3 Western blot analysis

Cells were lysed in Magnesium Lysis Buffer (MLB) [25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, 10% glycerol, 10 mM $MgCl_2$ and complete protease inhibitor tablet (Roche)] or 1% Triton X-100 containing 1X protease inhibitor cocktail (Roche). Cell lysates were cleared by centrifugation and protein concentrations were determined using the DC Lowry protein assay (BIO-RAD). Samples were prepared in 5X sample buffer and 20 μg of protein for each sample was resolved on 12% or 8% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF, Millipore), blocked overnight in 3% fish gelatin (Sigma), incubated with anti-HA (HA.11, Covance), anti-Myc (9E10, Covance), anti-GST (Covance), anti-β-actin (Sigma), or anti-PKCζ antibody (C-20, Santa Cruz). Washed membranes were incubated in anti-mouse or anti-rabbit IgG-HRP (Amersham Biosciences), or anti-mouse kappa light chain-HRP (Zymed), washed again, and developed using SuperSignal West Dura extended duration substrate (Pierce).

2.5.4 GST-pulldown assays and immunoprecipitation

COS-7 cells were lysed 24 h after transfection in MLB and the lysates pre-cleared with GST-Sepharose beads. Pre-cleared lysates were incubated with GST or GST-Par6C beads. GST-Par6 was purified as described previously (Garrard et al., 2003). Beads were collected and washed with MLB and resuspended in sample buffer. SDS-PAGE analysis and immunoblot were performed as described above.

COS-7 cells were lysed 48 h after transfection in MLB. Pre-cleared lysates were incubated with mouse anti-HA or anti-Myc antibody, and the immunoprecipitates were collected with Protein G PLUS-Sepharose beads (Zymed). SDS-PAGE analysis and immunoblot were performed as described above.

2.5.5 Calcium switch

MDCKII cells were plated on 12-mm Transwell filters (Corning-Costar) for 4 h in normal calcium medium (NCM, 1.8 mM Ca^{2++}). After attachment, cells were rinsed gently with SMEM and then incubated in low calcium medium (LCM, 5 μM Ca^{2++}) overnight to disrupt cell-cell contacts. After 18 h, calcium was restored (calcium switch) by replacement of LCM with NCM.

2.5.6 TER and paracellular permeability assays

To measure TER, MDCKII cells were plated on 12-mm Transwell filters at 5.0 x 10^5 cells per cm^2 filter and subjected to calcium switch. TER (Ω x cm^2) was measured using an Epithelial Voltohmmeter (EVOM, World Precision Instruments). Three separate filters were used for each cell line and the mean resistance was calculated after subtraction of the background resistance from a filter containing only culture medium.

Paracellular flux was measured in MDCKII cells subjected to calcium switch. After incubation in LCM, NCM containing 2 mg/ml of 4,000 kD FITC-Dextran or 40,000 kD FITC-Dextran tracer (Sigma) was placed in the apical chamber and NCM without tracer in the basal chamber. Monolayers were incubated in NCM for 6 hr or 18 h at 37°C, and the basal chamber media was collected. FITC-Dextran tracer was measured with a microplate spectrofluorometer (excitation: 492 nm; emission: 520 nm; SpectraMax Gemini).

2.5.7 Immunofluorescence

IF of cells grown on filters was performed as described previously (Wolff et al., 2005). Briefly, fixed and permeabilized MDCKII cells were incubated overnight in antibodies to HA (mouse, Covance), ZO-1 (rabbit, Zymed), β-catenin (rabbit, Sigma), Occludin (rabbit, Zymed), E-cadherin (rat, Sigma), PKCζ (rabbit, Santa Cruz), and/or Par3

(rabbit, Upstate). Cells were then washed and incubated overnight in secondary anti-mouse, anti-rabbit or anti-rat antibodies conjugated to Alexa488, Alexa594 or Alexa647, respectively. After antibody treatment, filters were mounted on glass slides for imaging.

Confocal microscopy was performed on an Olympus Fluoview 300 laser scanning confocal imaging system configured with an IX70 fluorescence microscope fitted with a PlanApo X60 oil objective. Images were acquired with the use of Olympus Fluoview software and subsequently resized in Adobe Photoshop. Multiple XY and XZ scans were acquired for each monolayer of cells.

2.6 Acknowledgements

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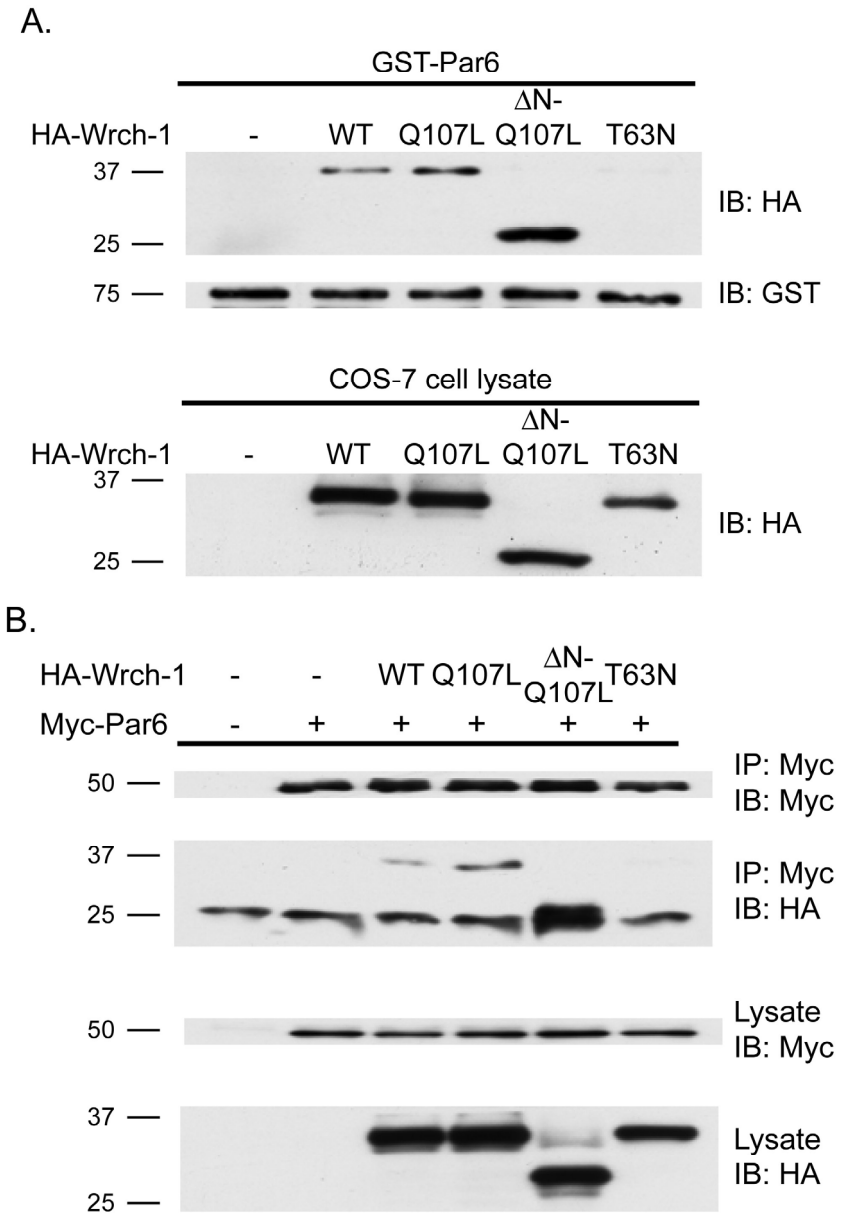

Figure 2.1 GTP-bound Wrch-1 interacts with Par6 *in vitro* and *in vivo*.
A) COS-7 cell lysates expressing empty vector or HA-tagged Wrch-1 constructs were incubated with GST alone or GST-Par6, and the presence of Wrch-1 in the pulldown probed by western blot with anti-HA antibody. Pulldown of GST-Par6 was confirmed with anti-GST antibody. B) COS-7 cells were co-transfected with empty vector or HA-tagged Wrch-1 along with Myc-tagged Par6. Par6 was immunoprecipitated (IP) with anti-Myc antibody and IPs probed for Wrch-1 by immunoblotting (IB) with anti-HA antibody.

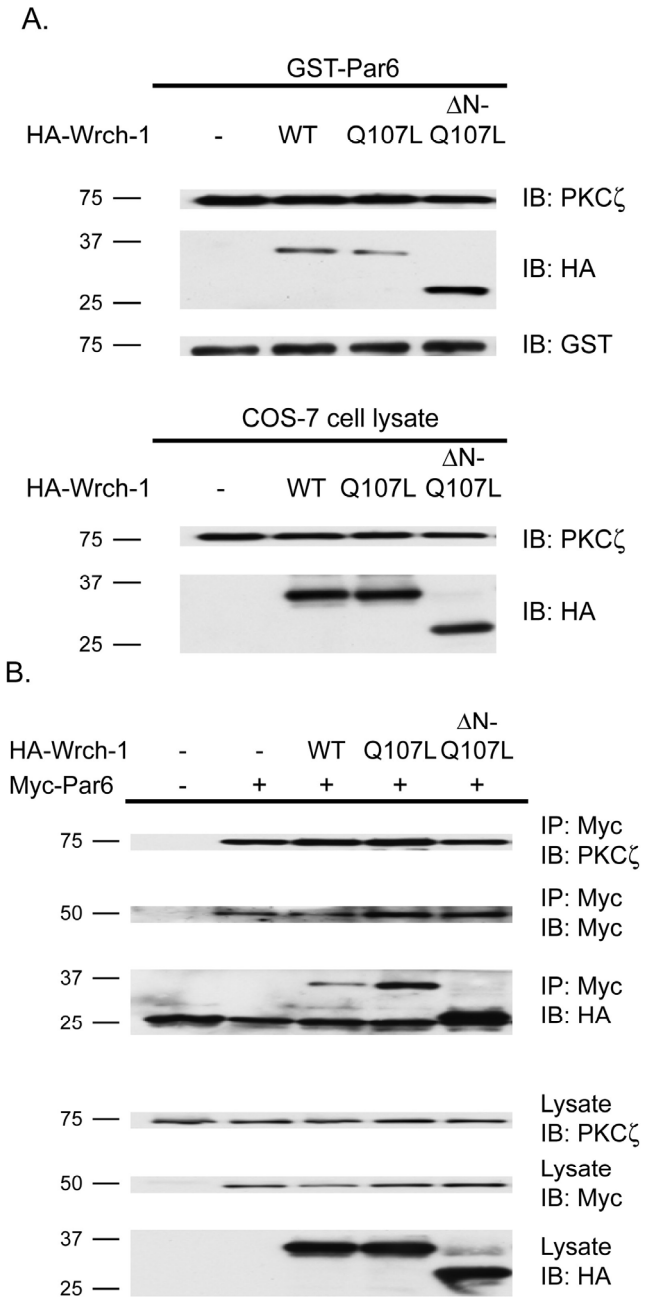

Figure 2.2 GTP-bound Wrch-1 interacts with both Par6 and PKCζ *in vitro* and *in vivo*. A) COS-7 cell lysates expressing empty vector or the indicated HA-tagged Wrch-1 constructs were incubated with GST alone or GST-Par6. Binding to HA-Wrch-1 and endogenous PKCζ was determined by western blotting with anti-HA and anti-PKCζ antibodies. Pulldown of GST-Par6 was confirmed with anti-GST antibody. B) COS-7 cells were co-transfected HA-Wrch-1 and Myc-tagged Par6. Myc-Par6 was immunoprecipitated with mouse anti-Myc antibody and the immunoprecipitates probed as above with mouse anti-HA and rabbit anti-PKCζ antibodies.

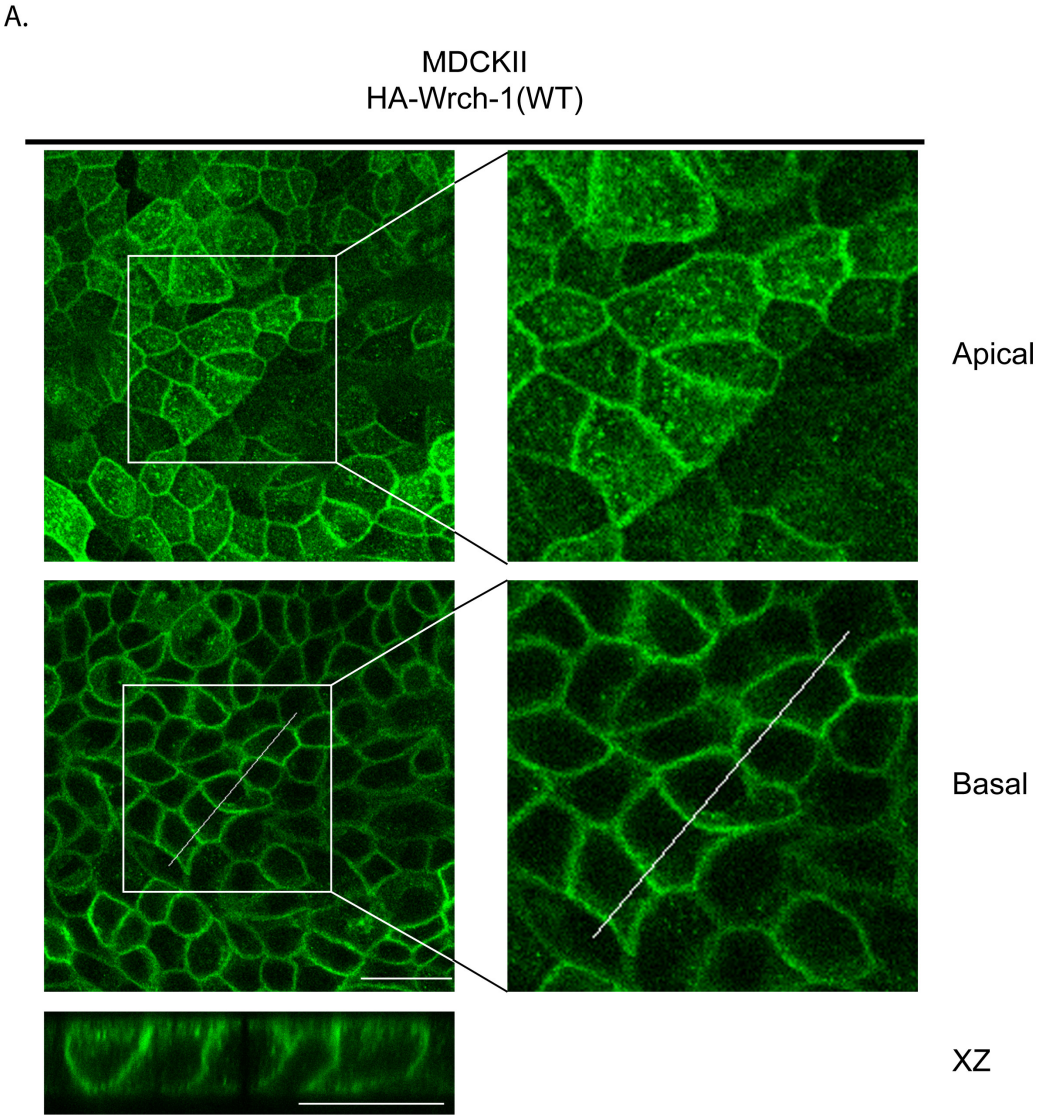

Figure 2.3A Wrch-1 displays overlapping localization with cell polarity proteins Par3 and PKCζ in polarized MDCKII cells.
A) Cells stably expressing empty vector or HA-tagged Wrch-1 were grown to confluency on 12-mm Transwell filters, then fixed and stained using primary antibodies against HA epitope tag (green). IF staining was visualized using a confocal microscope. Square panels: XY sections; rectangular panels: XZ sections (top to bottom). Scale bars = 20 μm.

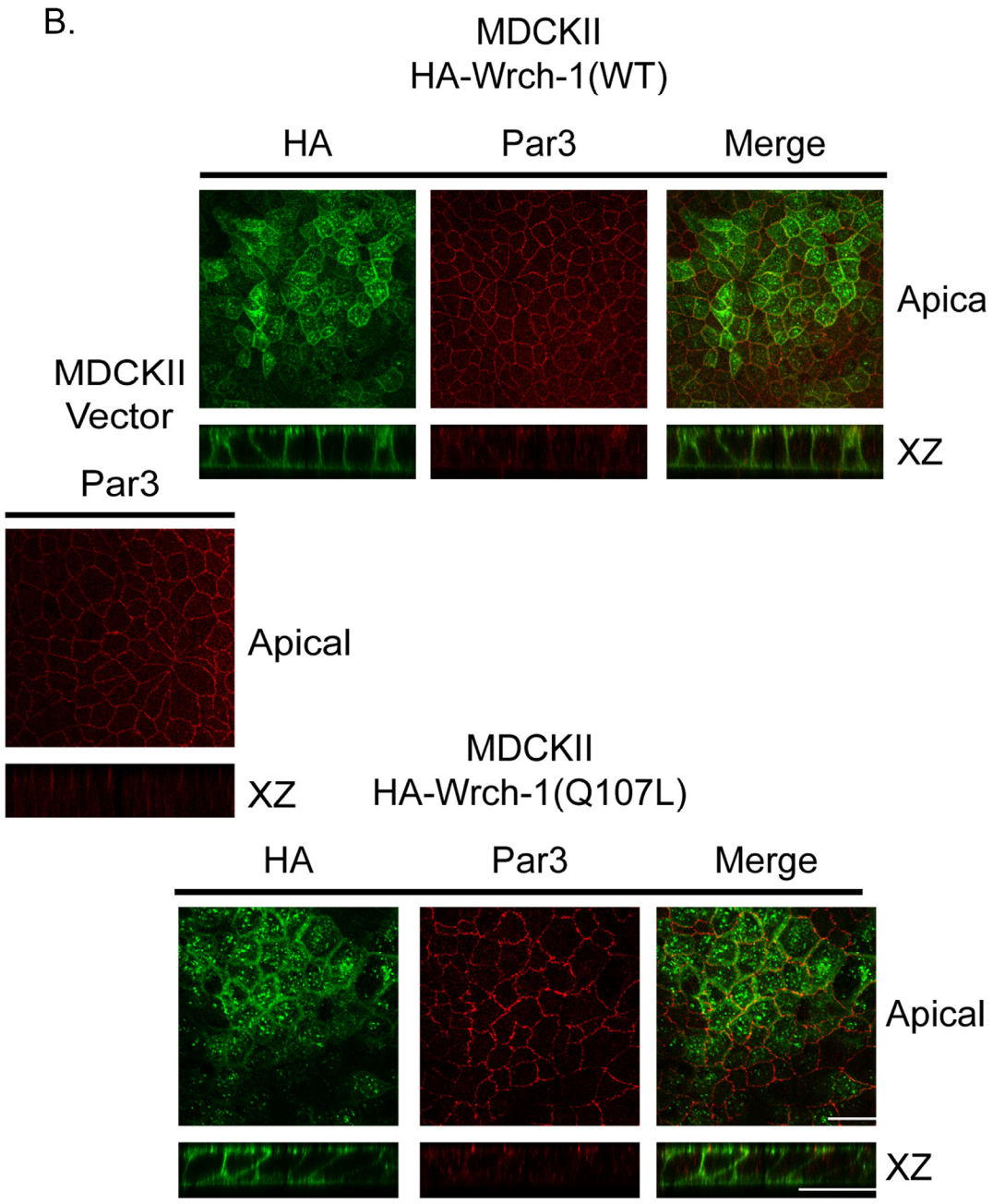

Figure 2.3B Wrch-1 displays overlapping localization with cell polarity proteins Par3 and PKCζ in polarized MDCKII cells.
B) Cells stably expressing empty vector or HA-tagged Wrch-1 were grown to confluency on 12-mm Transwell filters, then fixed and stained using primary antibodies against HA epitope tag (green) and Par3 (red). IF staining was visualized using a confocal microscope. Square panels: XY sections; rectangular panels: XZ sections (top to bottom). Scale bars = 20 μm.

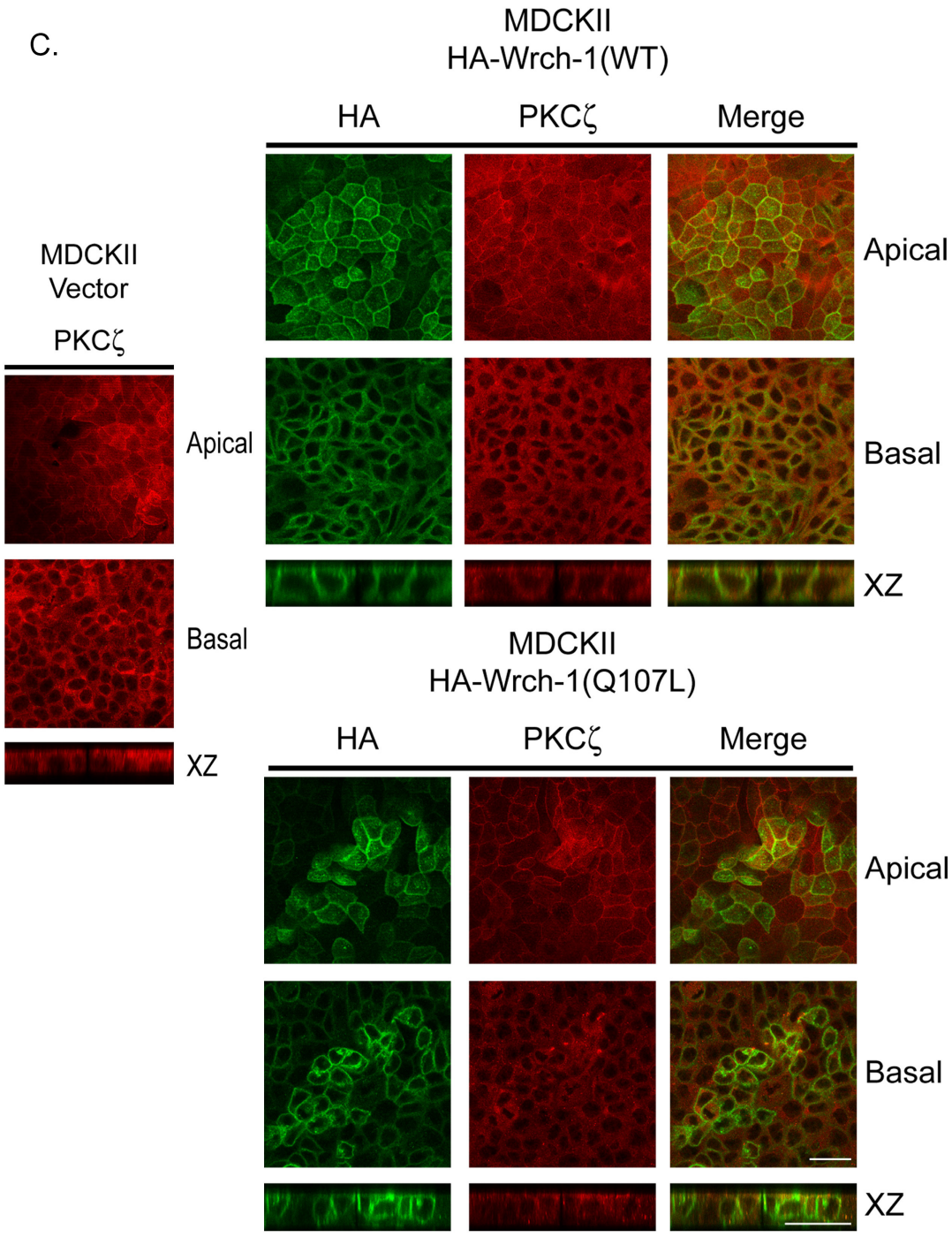

Figure 2.3C Wrch-1 displays overlapping localization with cell polarity proteins Par3 and PKCζ in polarized MDCKII cells.
C) Cells stably expressing empty vector or HA-tagged Wrch-1 were grown to confluency on 12-mm Transwell filters, then fixed and stained using primary antibodies against HA epitope tag (green) and PKCζ . IF staining was visualized using a confocal microscope. Square panels: XY sections; rectangular panels: XZ sections (top to bottom). Scale bars = 20 μm.

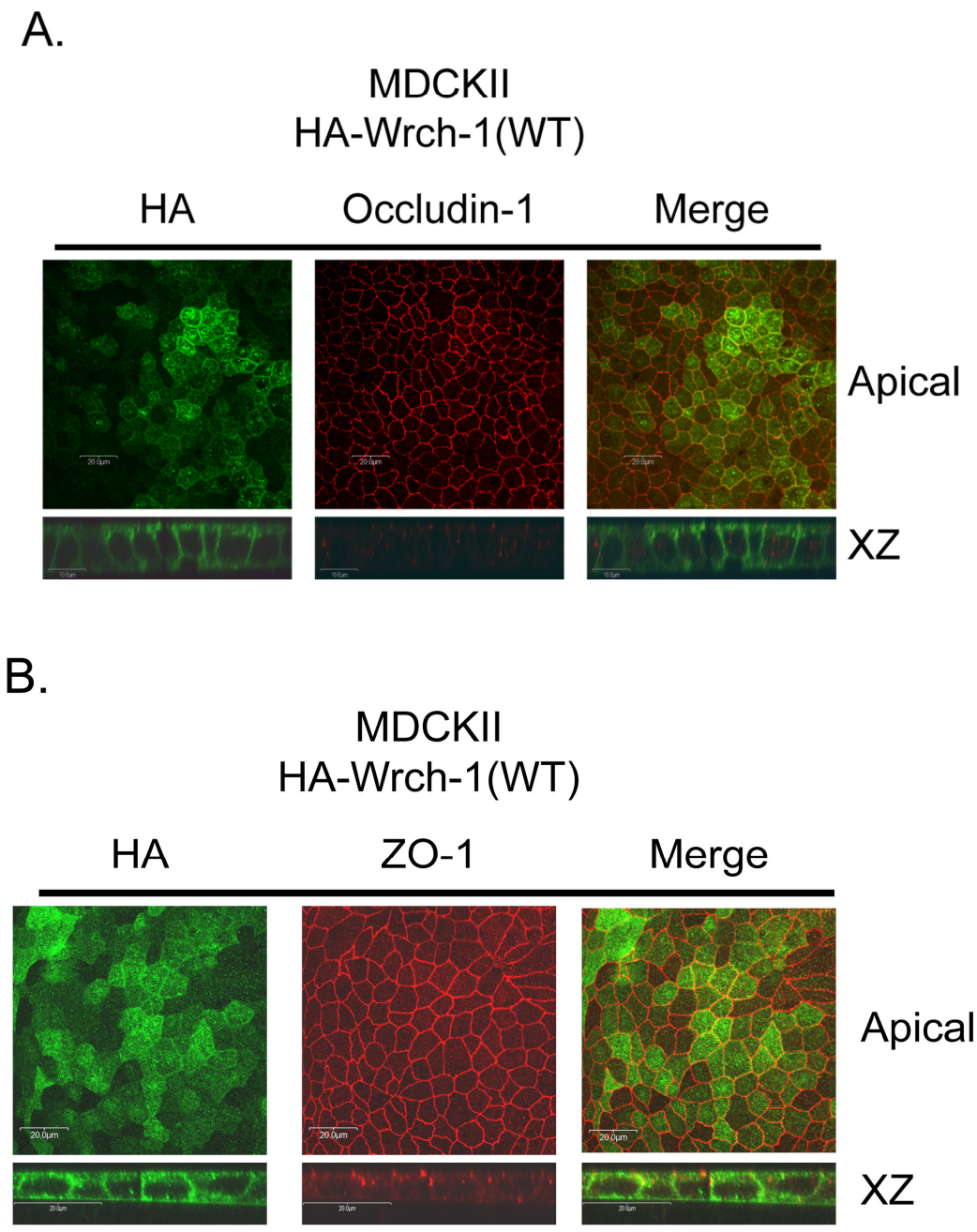

Figure 2.4A & B Wrch-1 localizes to cell junctions in polarized MDCK cells.
Cells stably expressing empty vector or HA-tagged Wrch-1 were grown to confluency on 12-mm Transwell filters, then fixed and stained using primary antibodies against HA epitope tag (green) and either A) occludin (red) or B) ZO-1 (red). Square panels: XY sections; rectangular panels: XZ sections (top to bottom). Scale bars = 20 μm.

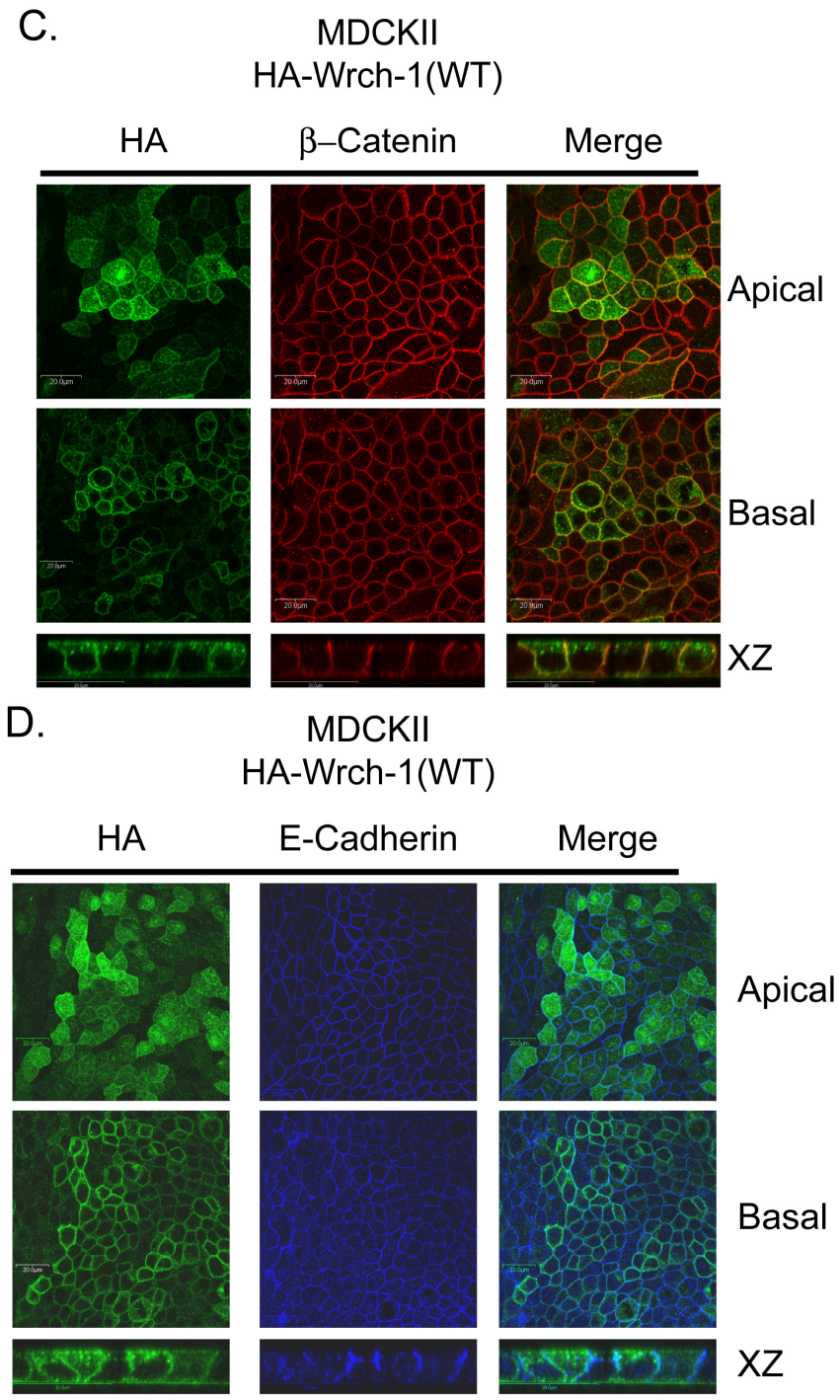

Figure 2.4C & D Wrch-1 localizes to cell junctions in polarized MDCK cells.
Cells stably expressing empty vector or HA-tagged Wrch-1 were grown to confluency on 12-mm Transwell filters, then fixed and stained using primary antibodies against HA epitope tag (green) and either C) β-catenin (red) or D) E-cadherin (blue). Square panels: XY sections; rectangular panels: XZ sections (top to bottom). Scale bars = 20 μm.

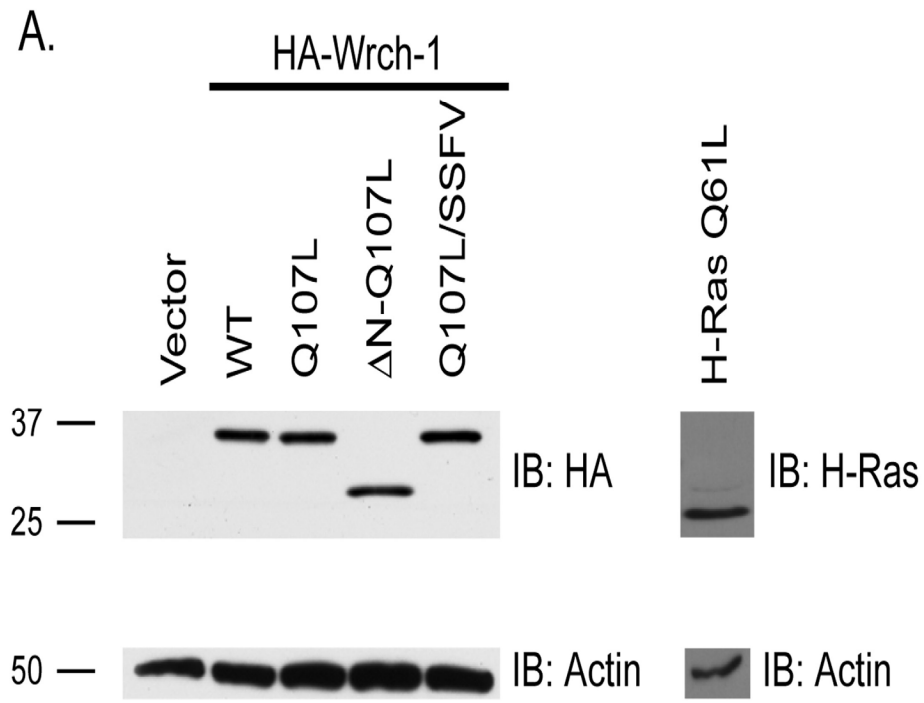

Figure 2.5A Activated Wrch-1 disrupts tight junction formation and integrity during cell polarization.

A) Equivalent expression in MDCKII cells of indicated HA-tagged Wrch-1 constructs and H-Ras(Q61L) shown by immunoblot with anti-HA and anti-Ras antibodies. Loading control, anti-β-actin.

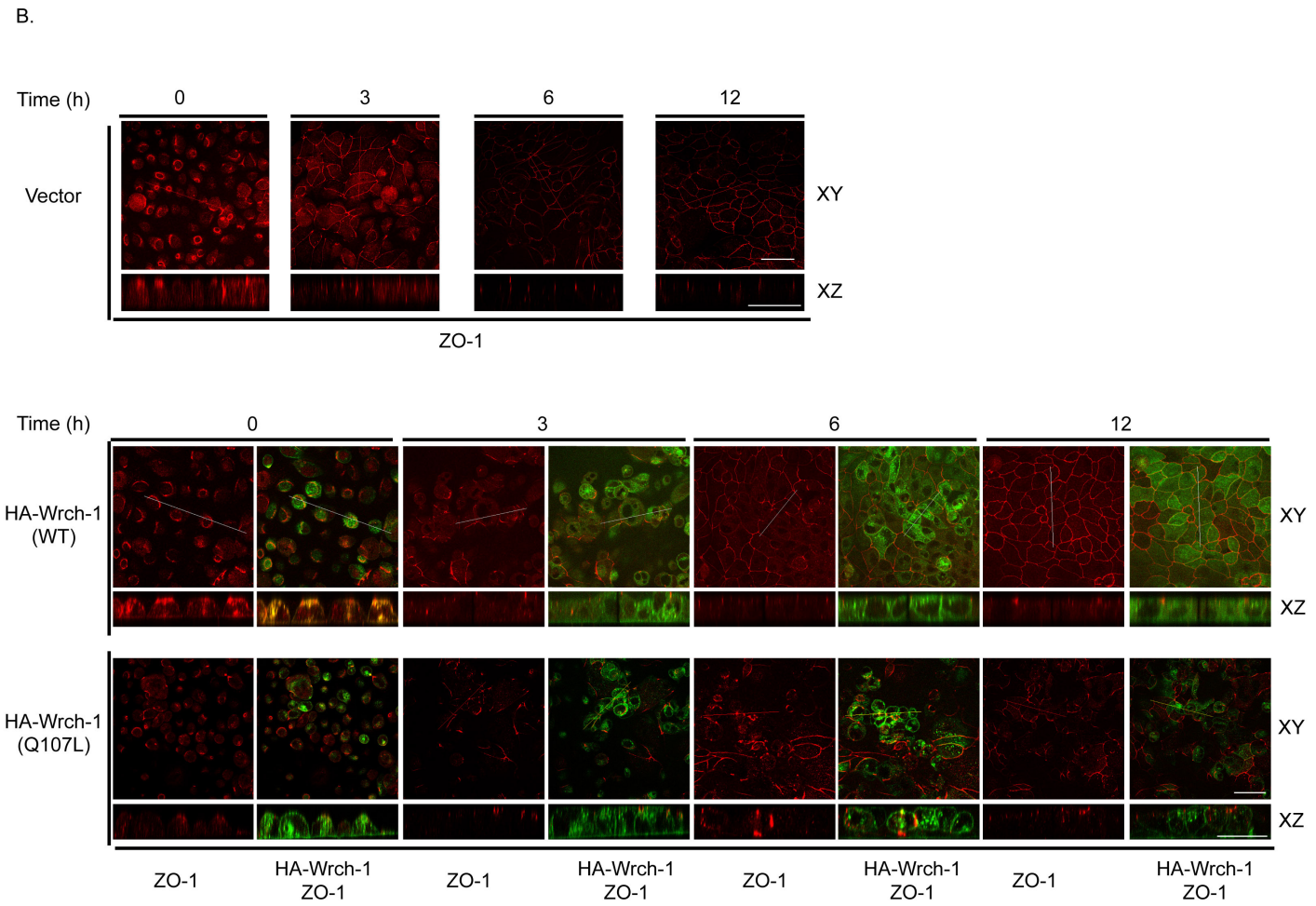

Figure 2.5B Activated Wrch-1 disrupts tight junction formation and integrity during cell polarization.
B) MDCKII cells stably expressing empty vector or HA-tagged Wrch-1 were grown on 12-mm Transwell filters and subjected to calcium switch. Cells were fixed at indicated intervals and stained using primary antibodies against HA epitope tag (green) and ZO-1 (red). Square panels: XY sections; rectangular panels: XZ sections (top to bottom). Scale bars = 20 μm.

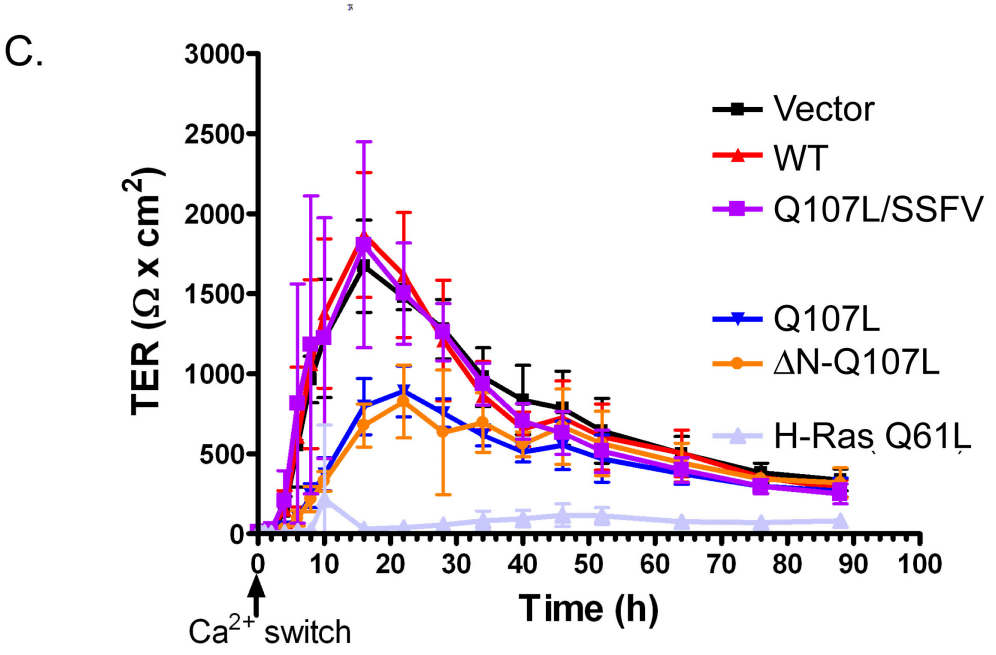

Figure 2.5C Activated Wrch-1 disrupts tight junction formation and integrity during cell polarization.

C) MDCKII cells stably expressing empty vector or various HA-tagged Wrch-1 constructs were grown on 12-mm Transwell filters and subjected to calcium switch. At indicated time points after calcium readdition, TER (Ω x cm2) was measured using an epithelial voltohmmeter (EVOM).

D.

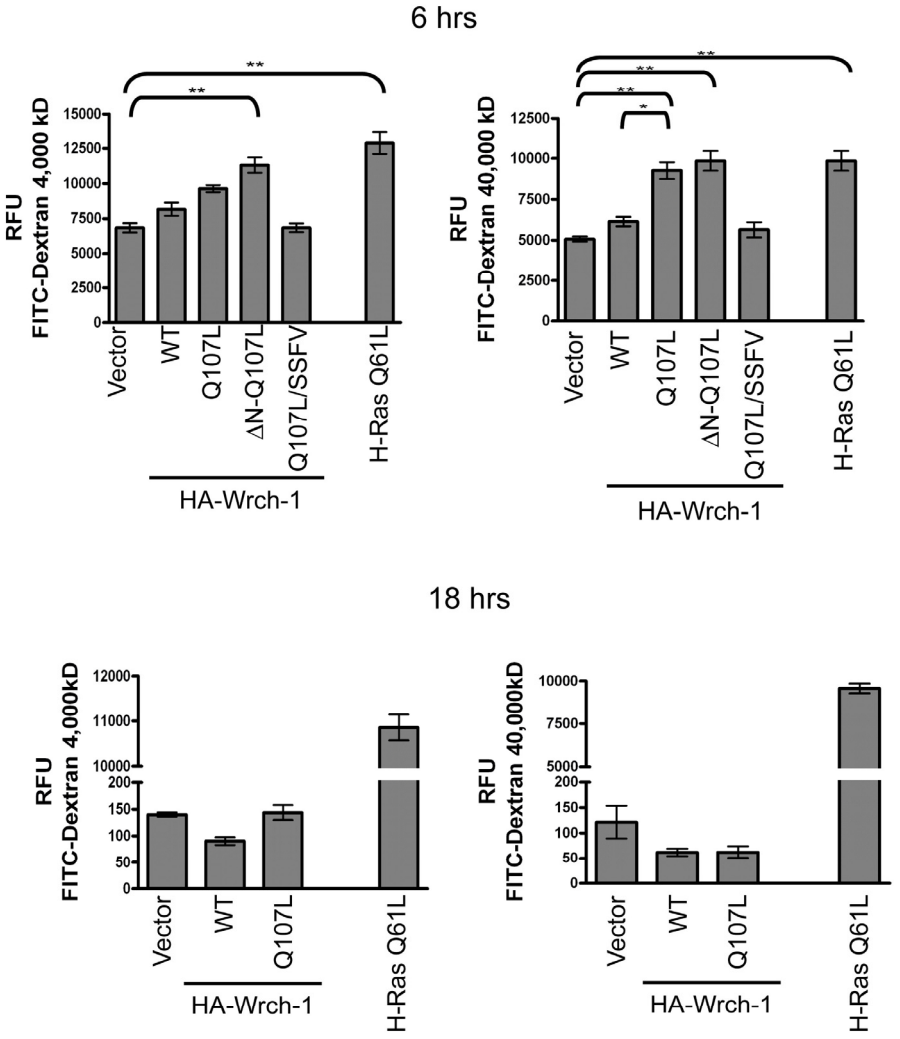

Figure 2.5D Activated Wrch-1 disrupts tight junction formation and integrity during cell polarization.

D) MDCKII cells stably expressing empty vector or various HA-tagged Wrch-1 constructs were grown on 12-mm Transwell filters and subjected to calcium switch. Paracellular flux of FITC-Dextran 4,000 kD (FD-4) or FITC-Dextran 40,000 kD (FD-40) tracer was examined 6 h and 18h after calcium switch. Bar graphs represent an average of three independent experiments carried out in triplicate for each cell line, +/- SEM. Significant p values of < 0.01 or < 0.001 are indicated by * or **, respectively. Tukey's multiple comparison test was used to determine significance between cell lines.

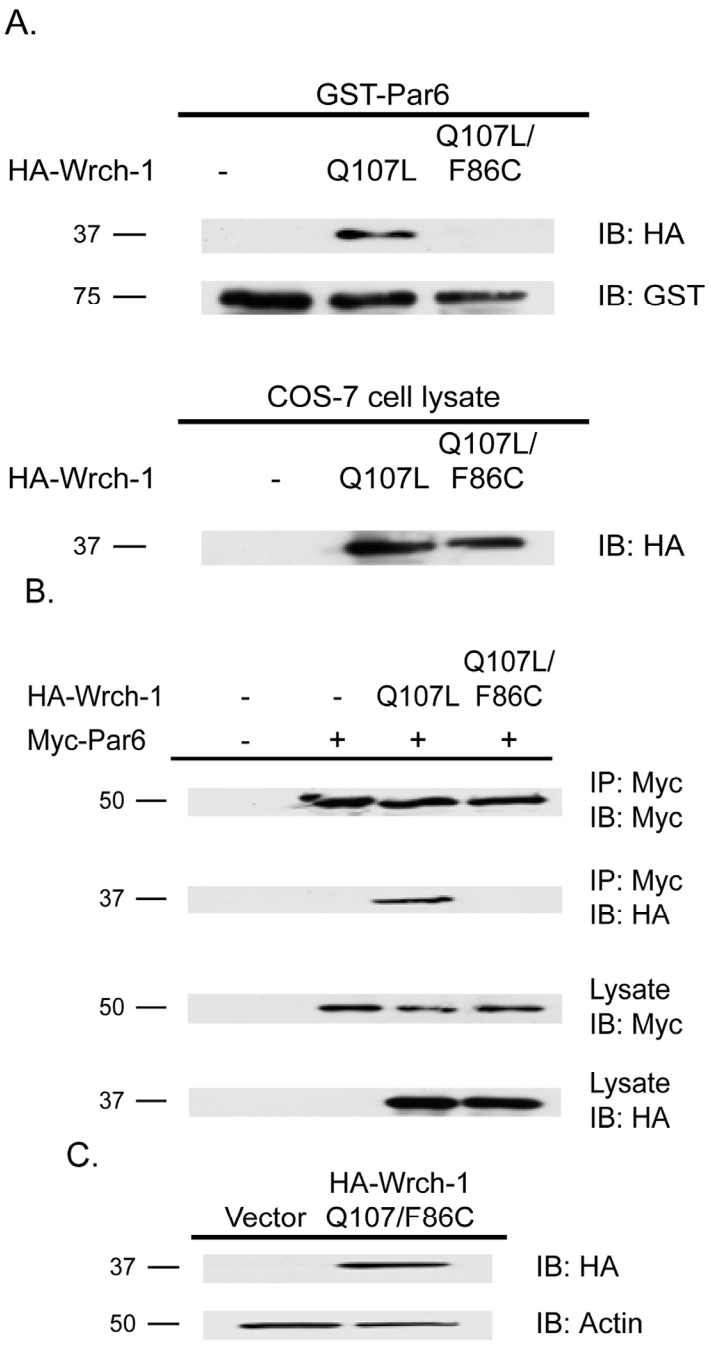

Figure 2.6A, B, & C An effector domain mutation in Wrch-1 abrogates Par6-binding and its ability to disrupt tight junction formation during cell polarization.
A) Lysates from COS-7 cells transiently expressing the indicated constructs including the effector domain mutant Wrch-1(Q107L/F86C) were subjected to GST pulldowns as in Figure 2.1A. B) Lysates from COS-7 cells expressing the indicated constructs were subjected to co-immunoprecipitation and immunoblotting as in Figure 2.1B. C) Stable expression in MDCKII cells of HA-tagged Wrch-1(Q107L/F86C) was detected by immunoblotting with anti-HA antibody. Anti-β-actin was used as a loading control.

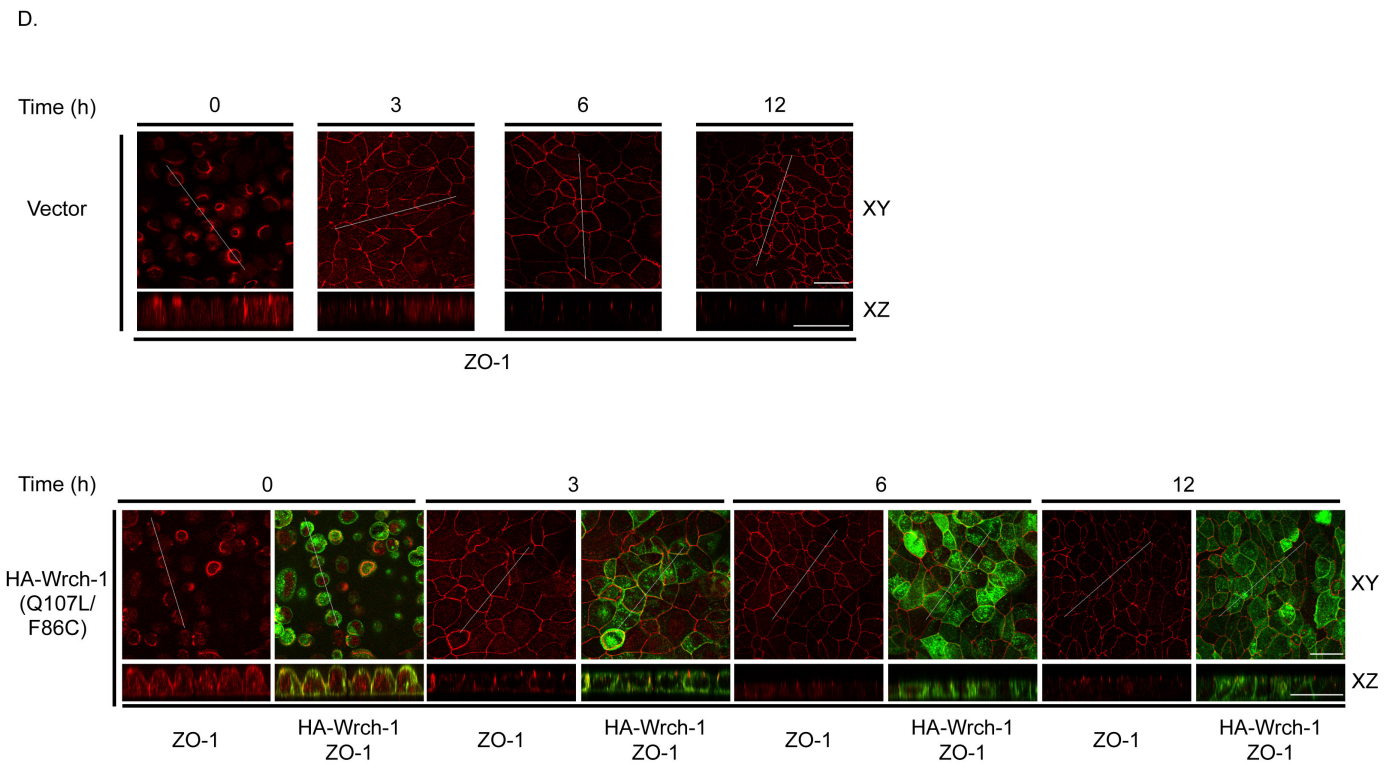

Figure 2.6D An effector domain mutation in Wrch-1 abrogates Par6-binding and its ability to disrupt tight junction formation during cell polarization.

D) MDCKII cells stably expressing empty vector or Wrch-1(Q107L/F86C) were subjected to calcium switch, and the localization of Wrch-1 (green) and ZO-1 (red) was evaluated as in Figure 2.5B. Square panels: XY sections; rectangular panels: XZ sections (top to bottom). Scale bars = 20 μm.

CHAPTER 3

THE TRANSFORMING RHO FAMILY GTPASE, WRCH-1, DISRUPTS EPITHELIAL CYTOSKELETAL ORGANIZATION AND EPITHELIAL MORPHOGENESIS

3.1 Abstract

There is increasing evidence that Wrch-1, an atypical and transforming Rho GTPase, dynamically modulates several distinct aspects of actin cytoskeletal organization that contribute to environmental sensing and cell motility, including the formation and turnover of filopodia and focal adhesions. We have previously shown that Wrch-1 negatively regulates tight junction (TJ) assembly in MDCKII cells during epithelial polarization, in part through a GTP-dependent interaction with the cell polarity protein, Par6. However, its role in epithelial cell cytoskeletal architecture and morphogenesis and how this may contribute to Wrch-1-mediated cellular transformation has not been examined. This study showed that Wrch-1 caused dramatic cytoskeletal reorganization and multilayering in cells grown in two-dimensional culture, and its constitutive activation disrupted cystogenesis of cells grown in three-dimensional (3D) culture, resulting in aberrant multilumenal structures. Finally, an effector domain mutation in activated Wrch-1 that prevents Par6 binding repressed transforming activity in MDCKII cells and abrogated the ability of Wrch-1 to disrupt actin organization and epithelial morphogenesis. Thus, the mechanisms of Wrch-1-induced morphological and growth transformation may include Par6-mediated disruption of TJs and actin organization.

3.2 Introduction

The Rho family of GTP-binding proteins consists of 25 distinct proteins that control signaling pathways that regulate diverse cellular functions such as cell shape, motility, and proliferation (Etienne-Manneville and Hall, 2002). When misregulated in cancer cells, Rho proteins influence aberrant growth, invasion and metastasis (Ellenbroek and Collard, 2007). However the involvement of atypical members of the Rho GTPase family in normal cellular functions and human diseases has only recently begun to be investigated (Aspenstrom et al., 2007).

Wrch-1 (Wnt-regulated Cdc42-homolog-1) is an atypical Rho family member that shares 57% sequence identity with Cdc42 and 61% sequence identity with its closest relative Wrch-2/Chp (Aronheim et al., 1998; Tao et al., 2001). Like Cdc42, Wrch-1 activation promotes actin microspikes and filopodia formation in fibroblasts and osteoclasts (Aspenstrom et al., 2004; Ory et al., 2007; Saras et al., 2004), but the effector pathways utilized by activated Wrch-1 to induce these cytoskeletal rearrangements remained elusive until recently. A very recent study identified the non-receptor tyrosine kinase Pyk2 as a relevant effector downstream of activated Wrch-1 instrumental in regulating Wrch-1-mediated filopodia formation and stress fiber dissolution in fibroblasts (Ruusala and Aspenstrom, 2007). Two other independent studies have highlighted an additional role for Wrch-1 in the regulation of cell migration through mechanisms clearly distinct from those of Cdc42, such as modulating focal adhesion turnover (Chuang et al., 2007; Ory et al., 2007). One proposed mechanism downstream of Wrch-1 in regulation of cell migration is activation of the serine/threonine kinase Pak1 that regulates myosin light chain phosphorylation and subsequently increases cell migration (Chuang et al., 2007). Together these reports highlight

a normal function for Wrch-1 as a master regulator of cell migration and cytoskeletal dynamics.

Although some of the normal functions, biochemical characteristics, subcellular localization and membrane association properties of Wrch-1 have been identified, most of these studies were not undertaken in epithelial cells. Wrch-1 was initially discovered as a Wnt1-responsive gene that, when mutationally activated (Q107L, analogous to the Q61L mutation in Ras and Cdc42), phenocopied Wnt-1 morphological transformation of C57MG mouse mammary epithelial cells (Tao et al., 2001). Wrch-1 message is differentially expressed in a variety of human tumors (Kirikoshi and Katoh, 2002), suggesting that its aberrant function may also contribute to oncogenesis. Whether Wrch-1 expression or activation contributes to tumorigenesis is unknown.

We set out to identify Wrch-1 normal functions and effectors in epithelial cells that, when misregulated, could potentially contribute to Wrch-1-mediated cellular transformation. We previously showed that Wrch-1 distributes to tight junctions (TJs) and adherens junctions (AJs) along the apical and basolateral membranes in MDCK cells and binds the cell polarity protein Par6 in a GTP-dependent manner (Chapter 2). Par6 is known to form an evolutionarily conserved complex with PKCζ and Par3 that is instrumental in establishing epithelial cell polarity and in regulating TJ formation (Shin et al., 2006). We determined that activated Wrch-1 disrupts TJ formation and function during cell polarization, in part through its GTP-dependent interaction with Par6 (Chapter 2). However, the cellular mechanism by which Wrch-1 negatively regulates TJs remained to be determined.

The ability of Rho GTPases to regulate actin cytoskeletal dynamics is another factor that regulates TJ dynamics and integrity (Bruewer et al., 2004; Nusrat et al., 2001). In

epithelial cells, the actin cytoskeleton is enriched at the apical surface and forms a contractile ring. anchored to adherens junctions (AJs) and TJs. which is required to maintain proper epithelial cell shape and function. The contraction of both this actin ring and an array of basal stress fibers are active during cell polarization and epithelial morphogenesis. Anchoring of the actin cytoskeleton to cell junctions integrates cell-cell contacts with changes in cell morphology and with morphogenetic movements of epithelial cells that occur during organogenesis (Lecuit and Lenne, 2007). Whether Wrch-1 regulation of the actin cytoskeleton is a potential mechanism for modulating epithelial cell morphology remains to be determined.

One of the earliest steps of tumor initiation and progression in epithelial cells is the loss of their polarized morphology, which is a process that involves the loss of cell-cell adhesions, loss of cell polarization and reorganization of the cytoskeleton. Together, these changes promote de-differentiation and are also required for later invasion and metastasis (Sahai and Marshall, 2002). Misregulation of Rho GTPases has been shown to lead to aberrant growth, de-differentiation, invasion and metastasis (Ellenbroek and Collard, 2007). As stated above, whether Wrch-1 expression and/or activation contribute to human cancer remains to be determined.

In the present study, we sought to determine whether Wrch-1 regulates TJs through modulating the actin cytoskeleton and subsequently regulating epithelial morphology, and whether the loss of normal cell morphology contributes to Wrch-1-mediated transformation. Specifically, we investigated whether activated Wrch-1 modulated actin cytoskeletal organization during cell polarization and at steady state to regulate the cellular morphology of MDCKII cells. We also investigated whether any such effects are connected with

anchorage-independent growth, a hallmark of transformation, and with loss of 3D morphogenesis, a hallmark of de-differentiation that is necessary for invasion and metastasis. We demonstrate that activated Wrch-1 has dramatic effects on cytoskeletal organization, epithelial morphogenesis, and these effects reveal a potential mechanism by which Wrch-1 may control normal cell morphology and contribute to Wrch-1-mediated transformation of epithelial cells.

3.3 Results

3.3.1 Activated Wrch-1 disrupts actin organization during cell polarization and overall cell morphology of MDCKII cells

The two best characterized functions for Wrch-1 in epithelial cells are induction of a transformed phenotype and disruption of TJ dynamics (Chapter 2) (Tao et al., 2001). There is an intimate relationship between the actin cytoskeleton, cell junctions and epithelial morphogenesis. The dynamic reorganization of F actin is closely correlated with changes in cell structure and the regulation of junction assembly. Our previous observation that constitutively activated Wrch-1 disruption of TJ dynamics was accompanied by a loss of ZO-1-associated perinuclear actin filaments during early junction assembly (Chapter 2, Figure 2.5) led us to hypothesize that Wrch-1 may regulate actin dynamics during polarization of epithelial cells. To address this hypothesis, we examined actin organization in monolayers of cells stably expressing empty vector, Wrch-1(WT) or Wrch-1(Q107L) following calcium switch. Briefly, culturing cells in calcium-depleted medium disrupts cell-cell contacts, and junction proteins are internalized in cytosolic vesicles. Restoration of calcium (calcium switch) triggers TJ and AJ assembly and cell polarization in a series of kinetically regulated steps. In the absence of cell-cell contact we observed a diffuse cytosolic ring of actin filaments at the apical surface of MDCKII cells expressing vector or Wrch-1(WT) (Figure

3.1A, 0 h). This structure was absent in cells expressing activated Wrch-1(Q107L), and the actin staining was distributed diffusely throughout the cytosol (Figure 3.1A, 0 h). After addition of calcium, the perinuclear actin ring redistributed towards the plasma membrane (PM) in cells expressing vector or Wrch-1(WT) (Figure 3.1A, 15 min), and subsequently accumulated at cell-cell contacts, as evident by the intense actin staining between cells (Figure 3.1A, 30 min). In contrast, cells expressing activated Wrch-1(Q107L) still lacked an actin-rich ring structure, and displayed an apparent decreased density of F-actin at the center and periphery (Figure 3.1A, 15 and 30 min). Furthermore, F-actin accumulation at cell-cell contacts was delayed. These results suggest that proper cycling of Wrch-1 is required to regulate cytoskeletal dynamics during junction assembly in epithelia.

These observations led us to examine whether activated Wrch-1 disrupts normal epithelial cell morphology in monolayer culture. Normal monolayers are composed of cuboidal, columnar epithelial cells with actin-rich microvilli, a contractile ring of F-actin on the apical surface, a cortical array of short actin filaments on the lateral domain, and actin stress fibers at the basal surface. Epithelial cells expressing vector or Wrch-1(WT) formed normal monolayers of tightly packed cuboidal cells, and F-actin accumulated in basal stress fibers and within the AJC. In contrast, cells expressing activated Wrch-1 were squamous, multilayered, and in general more disorganized within the monolayer (Figure 3.1B). This loss of normal cell morphology was accompanied by the disruption of perijunctional actin bundles normally associated with the AJC (Figure 3.1B, Apical), and by disruption of the basal stress fibers (Figure 3.1B, Basal). Together, these data show that expression of activated Wrch-1 disrupts normal epithelial cell morphogenesis and that this loss of normal

cell morphology could be mediated by Wrch-1 modulation of the actin cytoskeleton and subsequent breakdown in TJ dynamics.

Next we set out to investigate a potential molecular mechanism to connect Wrch-1-mediated disruption of actin cytoskeletal organization and TJ dynamics. We previously identified the cell polarity protein Par6 as an effector downstream of activated Wrch-1 that may contribute to Wrch-1-mediated disruption of TJ assembly and function (Chapter 2, Figure 2.1). To that end we characterized an effector domain mutation within Wrch-1 that renders Wrch-1(Q107L) incapable of interacting with Par6 (Chapter 2, Figure 2.6). Importantly, introduction of this effector domain mutation rendered activated Wrch-1 unable to disrupt TJ formation; to the contrary, TJs formed normally in MDCKII cells stably expressing the effector domain mutant, suggesting that Wrch-1 association with Par6 or another CRIB domain-containing protein is essential for Wrch-1-mediated disruption of TJ dynamics (Chapter 2, Figure 2.6). Therefore, we generated MDCKII cells stably expressing a Wrch-1 effector domain mutant (EDM, Wrch-1(Q107L/F86C)), that prevents binding to Par6. We then used these cells to test the requirement for Par6 or other CRIB domain-containing proteins in Wrch-1-mediated modulation of cytoskeletal dynamics (Chapter 2, Figure 2.6C). We observed that calcium-induced redistribution of F-actin from the perinuclear network to the AJC was indistinguishable in cells expressing the Wrch-1 EDM from that seen in cells expressing Wrch-1 WT (Figure 3.1C). Together these data suggest that a potential mechanism for Wrch-1-mediated disruption of TJ dynamics is through modulation of the actin cytoskeletal network in epithelial cells, in part through an interaction with the cell polarity protein, Par6.

3.3.2 Activated Wrch-1 disrupts epithelial morphogenesis and promotes anchorage independent growth of MDCKII cells

Recent studies have highlighted the superiority of investigating effects on epithelial cell polarity and morphology in 3D culture systems, because they mimic the *in vivo* formation of epithelial structures (Pollack et al., 1998; Zegers et al., 2003). For example, MDCK cells grown in extracellular matrix form highly polarized structures known as "cysts", composed of polarized cells whose apical domains surround a hollow center representing the lumen.

To investigate the role of Wrch-1 proteins in epithelial morphogenesis, we grew MDCKII cells in a matrix of collagen I. Monodispersed cells were grown for 10 days until they developed into cysts. These cysts were examined using immunofluorescence (IF) directed against apical and basal polarity determinants such as ZO-1 and E-cadherin to determine whether apico-basal polarity was established. Cells expressing empty vector or Wrch-1(WT) formed multicellular cysts with a single lumenal space and with polarized membrane domains in which ZO-1 was sequestered at the apical membrane and E-cadherin along the lateral membrane (Figure 3.2A). In contrast, cysts formed by cells expressing activated Wrch-1 contained either multiple mini-lumens (asterisks) or no lumen at all. However, individual cells within these highly disorganized cysts nevertheless retained proper apico-basal polarity, with ZO-1 sequestered towards the center and E-cadherin localized to the outside of the cysts. Importantly, expression of the active Wrch-1 mutant that lacks proper membrane localization did not disrupt normal epithelial cell polarity. These data suggest that proper epithelial morphogenesis in 3D requires proper regulation of Wrch-1 localization and activity.

We next attempted to quantify the degree of cyst lumen development. In normal cysts, cortical actin staining outlines the cell periphery (Figure 3.2A). Cysts expressing activated Wrch-1 did not contain defects in the formation of the cortical actin cytoskeleton. However,

the absence of a lumenal structure or the formation of multiple lumens (asterisks) was easily visualized upon staining these cysts with Texas-Red Phalloidin (Figure 3.2A). We determined that the formation of cysts with a single lumenal space was abrogated by 2 to 3-fold upon expression of activated Wrch-1, and that proper membrane localization was required for this observation (Figure 3.2A). These data highlight that proper regulation of Wrch-1 activity is necessary for normal epithelial morphogenesis in 3D and outline a potential mechanism for Wrch-1-mediated transformation.

The loss of epithelial cell morphogenesis due to the disruption of cell junctions is a characteristic of cellular transformation. Therefore we next examined the ability of Wrch-1 to induce anchorage-independent growth, another hallmark of transformation, by assessing the ability of MDCKII cells stably expressing Wrch-1 proteins to form colonies in soft agar. We observed that Wrch-1(WT) induced twice as many colonies as empty vector (Figure 3.2B). As predicted, activated full length and N-terminally truncated Wrch-1 were more potently transforming than WT, inducing colony numbers 4-fold over empty vector controls (Figure 3.2B), and those colonies were substantially larger in size than those induced by Wrch-1(WT). These data demonstrate that Wrch-1 activity promotes anchorage-independent growth. We hypothesize that the difference in colony size observed between wild type and constitutively activated forms of Wrch-1 is due to disruption of actin organization and cell-cell contacts by the latter.

3.3.3 Effector domain mutant of Wrch-1 incapable of binding Par6 abrogates Wrch-1-mediated disruption of epithelial morphogenesis and promotion of anchorage-independent growth of MDCKII cells

We hypothesized that the Par6-binding deficient EDM of Wrch-1 that failed to disrupt actin organization or perturb TJs would also be incapable of disrupting epithelial

morphogenesis. In cysts grown in 3D collagen from cells expressing vector or Wrch-1(Q107L/F86C), epithelial morphogenesis was intact (Figure 3.3A), and ZO-1 was sequestered at the apical surface facing the hollow lumenal structure in the center, while E-cadherin was localized to the basolateral membrane surface. These results indicate that Wrch-1-mediated disruption of epithelial morphogenesis (e.g., Figure 3.2A) requires the binding of a CRIB domain-containing effector such as Par6.

The inability of the EDM to misregulate epithelial morphogenesis led us to investigate whether it retained or lost the ability to induce anchorage-independent growth. Interestingly, this EDM was only partially defective in growth transformation. Cells expressing Wrch-1(Q107L/F86C) formed small and large colonies (Figure 3.3B) to an extent similar to that of wild type but not activated Wrch-1. These data suggest that formation of the large colonies induced by expression of active, constitutively GTP-bound Wrch-1 requires another effector whose binding is not perturbed by the F86C mutation that impairs Par6 interaction.

3.4 Discussion

The role of the atypical family of Rho GTPases in cellular functions such as actin cytoskeletal organization, cell polarity, cell adhesion and microtubule dynamics otherwise attributed to the classic Rho GTPases RhoA, Rac1 and Cdc42 is not well understood (Aspenstrom et al., 2007; Etienne-Manneville and Hall, 2002). Recent studies have described roles for the atypical Rho GTPase Wrch-1 in actin cytoskeletal organization (Saras et al., 2004), and in focal adhesion formation and cell motility in non-epithelial cells (Chuang et al., 2007; Ory et al., 2007). We recently discovered that Wrch-1 regulates TJ dynamics in part through interaction with the cell polarity protein Par6. In the present study, we have

identified a potential mechanism for Wrch-1 in regulating the kinetics of TJ assembly and integrity of TJs through disrupting actin cytoskeletal organization during cell polarization. In addition, we have determined that Wrch-1 regulation of actin cytoskeletal reorganization is necessary for proper epithelial morphogenesis. We hypothesize that the loss of epithelial morphogenesis seen here in the presence of excessive Wrch-1 activity contributes to the ability of constitutively active, GTP-locked Wrch-1 to induce transformation of epithelial cells.

Epithelial morphogenesis is a multi-step process that requires regulated adhesion and coordinated cell shape changes within an epithelium. Proper modulation of the actin cytoskeleton is a critical component of these morphogenetic processes. It was previously demonstrated that expression of activated Wrch-1 mediates actin stress fiber dissolution and cell rounding in fibroblasts (Saras et al., 2004). We have demonstrated here that activated Wrch-1 disrupts several distinct pools of F-actin during calcium-mediated polarization of MDCKII cells. First, we observed that the actin-rich perinuclear ring present in cells devoid of contact is missing in cells expressing activated Wrch-1. This structure redistributes to the PM during junction assembly and polarization, and is believed to mediate the movement of junctional proteins from internal vesicles to the periphery of the cells (Ando-Akatsuka et al., 1999; Shen and Turner, 2005). Therefore, we hypothesize that it is the disruption of this cytoskeletal domain by activated Wrch-1 that disrupts early TJ assembly in MDCKII cells.

Activated Wrch-1 also perturbed cytoskeletal organization in confluent monolayers. The sharp, linear band of actin associated with AJC was highly disorganized in MDCKII cells expressing activated Wrch-1, and basal stress fibers were disorganized. These changes were accompanied by a dramatic alteration in the organization of these cells within the

monolayer, and by defects in cystogenesis of cells grown in 3D collagen matrix. These observations suggest that Wrch-1 may regulate cytoskeletal dynamics that underlie tissue morphogenesis in epithelia.

Our data suggest that Par6 may be one important effector of activated Wrch-1 in epithelial morphogenesis. This hypothesis is supported in part by the observation that an effector domain mutant of activated Wrch-1 that cannot bind Par6 is unable to disrupt cell architecture and cyst formation in MDCKII cells. It is also supported by the previously published observations that Par6 disruption has many of the same effects on epithelial morphogenesis as activated Wrch-1. For example, overexpression of Par6 delays TJ assembly in MDCKII cells (Gao et al., 2002), and expression of a dominant negative form of Par6 in MDCK cells grown in collagen disrupts cystogenesis (Kim et al., 2007). Interestingly, the effects of activated Wrch-1 are also similar to those found after RNAi-mediated knockdown of several proteins within polarity complexes, such as PALS1 and PATJ (Shin et al., 2005; Sourisseau et al., 2006; Straight et al., 2004). These observations suggest that Wrch-1 may act more generally through interaction with the entire polarity complex. This hypothesis is supported by our observation with Wrch-1 also binds other polarity complex proteins like PKCζ.

Indeed, we had initially speculated that the identification of the polarity complex protein Par6 as a GTP-dependent binding partner of Wrch-1 suggested a possible role for Wrch-1 in modulating cell polarity. The evolutionarily conserved protein complex formed by Par3, Par6, and PKCζ is instrumental in establishing and maintaining cell polarity by regulating TJ formation (Goldstein and Macara, 2007; Shin et al., 2006). However, expression of activated Wrch-1 does not affect cell polarity, suggesting that Wrch-1

interaction with the Par3-Par6-PKCζ complex plays a role distinct from the previously described regulation of this polarity complex by Cdc42. It is well established in both model organisms and mammalian epithelial cells that Cdc42 plays an integral part in proper localization of the polarity complex to specific membrane domains and in subsequent activation of PKCζ kinase activity that is necessary for epithelial polarization (Goldstein and Macara, 2007; Shin et al., 2006). We previously showed that Cdc42 and Wrch-1 have distinct localizations in fibroblasts (Berzat et al., 2005b), and our current work also suggests that these related GTPases have divergent localizations in epithelial cells. Recently, Cdc42 was shown to localize to the apical surface of MDCK cysts grown in 3D, and suppression of Cdc42 expression disrupted normal apico-basal polarity in 3D culture (Martin-Belmonte et al., 2007). This is distinct from our observed predominant basolateral PM localization of Wrch-1 and the ability of activated Wrch-1 to disrupt cell morphology and cystogenesis without perturbing apico-basal polarity. Therefore, we hypothesize that both Cdc42 and Wrch-1 regulate the Par3-Par6-PKCζ complex, but at different subcellular locations to promulgate their distinct cellular functions.

Regulation of actin cytoskeleton dynamics by Rho GTPases has been proposed as a general mechanism to regulate TJ assembly during cell polarization (Bruewer et al., 2004; Nusrat et al., 1995; Nusrat et al., 2001). Expression of constitutively activated, GTP-locked Wrch-1 disrupts TJ formation and integrity during cell polarization as determined by delayed ZO-1 localization and increased permeability. However, TJs do form eventually in cells expressing activated Wrch-1, based on normal ZO-1 localization, transepithelial resistance and paracellular flux at steady state, suggesting that initial regulation of Wrch-1 activation is

necessary only for early TJ dynamics and not for the regulation of epithelial permeability (Chapter 2).

The most pressing question, then, is how the Wrch-1/Par6 effector complex regulates cytoskeletal dynamics. Chen et al. recently identified a link between the cell polarity protein Par3 and regulation of actin dynamics important for proper TJ assembly, in which Par3 binds to and inhibits LIMK activity, leading to a subsequent decrease in cofilin activity (Chen and Macara, 2005). Therefore, we predict that the association between active Wrch-1 and the Par3-Par6-PKCζ complex may ultimately alter actin dynamics through known Rho GTPase effectors such as LIMK. This suggests that we have identified a novel mechanism by which Wrch-1 modulates the actin cytoskeleton to regulate both TJ dynamics and epithelial cell morphology through one or more effectors such as Par6.

Despite the similarity in the epithelial phenotypes generated by Par-6 and Wrch-1 mutations, it is still possible that other Wrch-1 effectors may be involved. At present, the only other proteins identified to date whose properties are consistent with an effector function for Wrch-1 are PAK1 (Chuang et al., 2007; Saras et al., 2004; Tao et al., 2001) and the tyrosine kinase Pyk2 (Ruusala and Aspenstrom, 2007). Pyk2 is a Wrch-1 effector capable of mediating Wrch-1 effects on cytoskeletal dynamics, such as filopodia and stress fibers. Pyk2 could represent a relevant effector for Wrch-1-mediated defects in cystogenesis, because the single F86C point mutation in Wrch-1 was sufficient to block its interaction with Pyk2 or to abolish Wrch-1-mediated loss of stress fibers or formation of filopodia (Ruusala and Aspenstrom, 2007). Understanding the exact mechanisms whereby Wrch-1 regulates each of these distinct processes will likely require further investigation of known Wrch-1 effector targets as well as identification of novel Wrch-1 effectors.

PAK1 is an inhibitor of myosin light chain kinase (Sanders et al., 1999), which is a potent regulator of cytoskeletal dynamics. Chuang et al. demonstrated that RNAi-mediated depletion of Wrch-1 leads to decreases in MLC phosphorylation and fibroblast cell migration (Chuang et al., 2007). Interestingly, in other studies (Shen et al., 2006), expression of the constitutively active form of MLCK led to increased phosphorylation of MLC, increased TJ permeability, mislocalization of TJ-associated proteins and reorganization of perijunctional F-actin. These findings are identical to the alterations in epithelial cell architecture and TJ dynamics that we observed upon expression of activated Wrch-1. Together, these data suggest that Wrch-1 regulation of MLCK activity downstream of PAK1 could be another possible molecular mechanism for the Wrch-1 biological functions we evaluated here.

Recent studies have also identified a role for Wrch-1 regulation of focal adhesion dynamics in fibroblasts (Chuang et al., 2007; Ruusala and Aspenstrom, 2007) and osteoclasts (Ory et al., 2007) and it is possible that a similar mechanism could account for the defects in cystogenesis mediated by constitutively activated Wrch-1. However, the same EDM of activated Wrch-1 that rescued cyst formation (Q107L/F86C) was unable to localize to focal adhesions (Ory et al., 2007). This suggests that different effector proteins are necessary for these two Wrch-1 biological functions.

The loss of epithelial cell morphology is characteristic of de-differentiated cancer cells that lose normal cell polarity and cell-cell contacts. Previous studies in this laboratory demonstrated that activated Wrch-1 promotes anchorage-independent growth of NIH 3T3 fibroblasts, a hallmark of oncogenic transformation (Berzat et al., 2005b; Shutes et al., 2004), and we have shown here that stable expression of activated Wrch-1 also promotes anchorage-independent growth of MDCKII epithelial cells. Recent studies have highlighted the ability

of oncogenes to hijack cell polarity protein complexes to promote de-differentiation in the early stages of epithelial cell carcinogenesis. The Par6-PKCζ protein complex has been identified as a downstream player in the loss of cell polarity, in epithelial-mesenchymal transition, and in increased cell proliferation mediated by receptor tyrosine kinases, TGFβ and ErbB2, all of which promote cellular transformation and migration (Aranda et al., 2006; Ozdamar et al., 2005). However, the exact mechanisms by which misregulation of cell polarity proteins leads to de-differentiation and transformation remain to be determined. It has been shown previously that transformation mediated by aberrant activation of Cdc42 or Rac can be potentiated by the Par6-PKCζ complex (Qiu et al., 2000). These findings further support a link between the regulation of cell polarity by Rho GTPases and contact-inhibited growth.

Our observations that Wrch-1 can disrupt epithelial cell morphology and cell junctions in a manner that can be abrogated by blocking Par6 binding suggest a potential mechanism for Wrch-1-mediated transformation. The identification of Wrch-1 as a gene whose expression is regulated by Wnt-1 signaling in mouse mammary epithelial cells and its ability to phenocopy Wnt-1 mediated transformation, along with aberrant regulation of Wrch-1 expression in various cancers suggest the possibility that Wrch-1 may play a role in human tumorigenesis. Although naturally occurring activating mutations that lock Wrch-1 in the GTP-bound state have not been identified, the considerable evidence for a role for other Rho family GTPases in pathological states also does not include the discovery of activating mutations but instead involves aberrant activity of their regulators, the GEFs and GAPs that modulate GTP/GDP cycling. Thus, identification of its upstream activators, downstream

negative regulators and effector targets will be critical for a fuller understanding of the molecular mechanisms and role for Wrch-1 in transformation.

3.5 Materials and methods

3.5.1 Cell culture, transfection, and retroviral infection

COS-7 cells were grown in DMEM (GIBCO/Invitrogen) supplemented with 10% FBS (Sigma) and 1% penicillin-streptomycin (P/S), and maintained in 5% CO_2 at 37^{o}C. Cells were transfected using TransIT-LT1 (Mirus) according to the manufacturer's instructions.

MDCKII cells, generously provided by Robert Nicholas (UNC-CH), were grown as above and supplemented with 1% non-essential amino acids (NEAA, Invitrogen) ("complete medium").

Stable MDCKII cell lines were generated by retroviral infection. Production of retrovirus was obtained by $CaCl_2$-mediated transfection of pBabe-HAII-puro, pVPack-Gag/Pol, and pVPack-Ampho (Stratagene) vectors into 293T cells. Cells were infected by exposure to retroviral supernant containing 8 μg/ml of Polybrene (American Bioanalytical) and maintained in puromycin for 10 days, after which colonies were pooled for use.

Cystogenic growth in 3D collagen I gels was performed as described previously (O'Brien et al., 2001; O'Brien et al., 2006).

3.5.2 Molecular constructs

Human Wrch-1 (WT, Q107L, ΔN-WT, ΔN-Q107L and Q107L/C255S/C256S) were generated as described previously (Berzat et al., 2005b). For HA epitope-tagged Wrch-1, 5' and 3' BamHI sites were introduced by PCR, for ligation into the BamHI site of pCGN-hygro or pBabe-HAII-puro retroviral expression vectors. Human Wrch-1(Q107L/F86C) was

generated by site-directed mutagenesis and subcloned as described above. All sequences were verified by the Genome Analysis Facility at UNC-CH.

3.5.3 Calcium switch

MDCKII cells were plated on 12-mm Transwell filters (Corning-Costar) for 4 h in normal calcium medium (NCM, 1.8 mM Ca^{2++}). After attachment, cells were rinsed gently with SMEM and then incubated in low calcium medium (LCM, 5 μM Ca^{2++}) overnight to disrupt cell-cell contacts. After 18 h, calcium was restored (calcium switch) by replacement of LCM with NCM.

3.5.4 Immunofluorescence

IF of cells grown on filters was performed as described previously (Wolff et al., 2005). Briefly, fixed and permeabilized MDCKII cells were incubated overnight in antibodies to HA (mouse, Covance), ZO-1 (rabbit, Zymed), and/or E-cadherin (rat, Sigma). Cells were then washed and incubated overnight in secondary anti-mouse, anti-rabbit or anti-rat antibodies conjugated to Alexa488, Alexa594 or Alexa647, respectively and/or with Texas Red-Phalloidin (Molecular Probes). After antibody treatment, filters were mounted on glass slides for imaging.

Staining of MDCKII cysts grown in 3D collagen was performed as described previously (O'Brien et al., 2006; Roh et al., 2003). Briefly, collagen gels containing MDCKII cells were treated with collagenase type VII (Sigma C-2399), fixed and permeabilized, then incubated in antibodies and mounted for imaging as described above.

Confocal microscopy was performed on an Olympus Fluoview 300 laser scanning confocal imaging system configured with an IX70 fluorescence microscope fitted with a PlanApo X60 oil objective. Images were acquired with the use of Olympus Fluoview

software and subsequently resized in Adobe Photoshop. Multiple XY and XZ scans were acquired for each monolayer of cells or 3D collagen gel.

3.5.5 Growth transformation assay

Single cell suspensions of MDCKII cells (3.5 x 10^3 cells per 35-mm dish) were suspended in 0.4% agar (BD Biosciences) in complete medium and layered on top of 0.6% agar as described (Shutes et al., 2004). After 14 days, colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma), and the average number of colonies on triplicate dishes was calculated.

3.6 Acknowledgements

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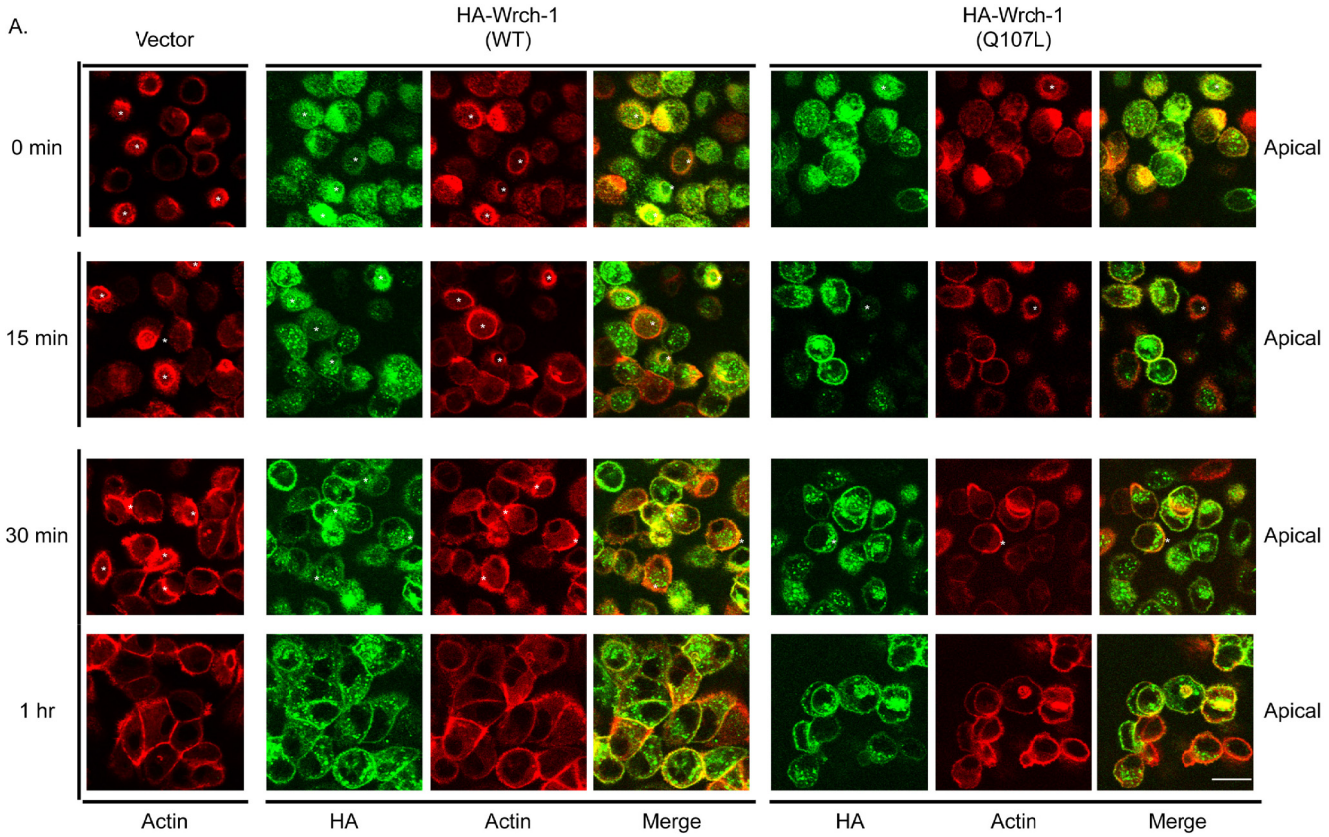

Figure 3.1A Activated Wrch-1 disrupts actin organization and cell morphology of MDCKII cells.
A) MDCKII cells stably expressing empty vector or indicated HA-tagged Wrch-1 constructs were subjected to calcium switch. Cells were fixed at indicated intervals and stained using primary antibodies against HA epitope tag (green) or with Texas-Red Phalloidin (red). Asterisks represent actin contractile rings and sites of cell-cell contact. Square panels: XY sections; rectangular panels: XZ sections (top to bottom). Scale bars = 20 μm.

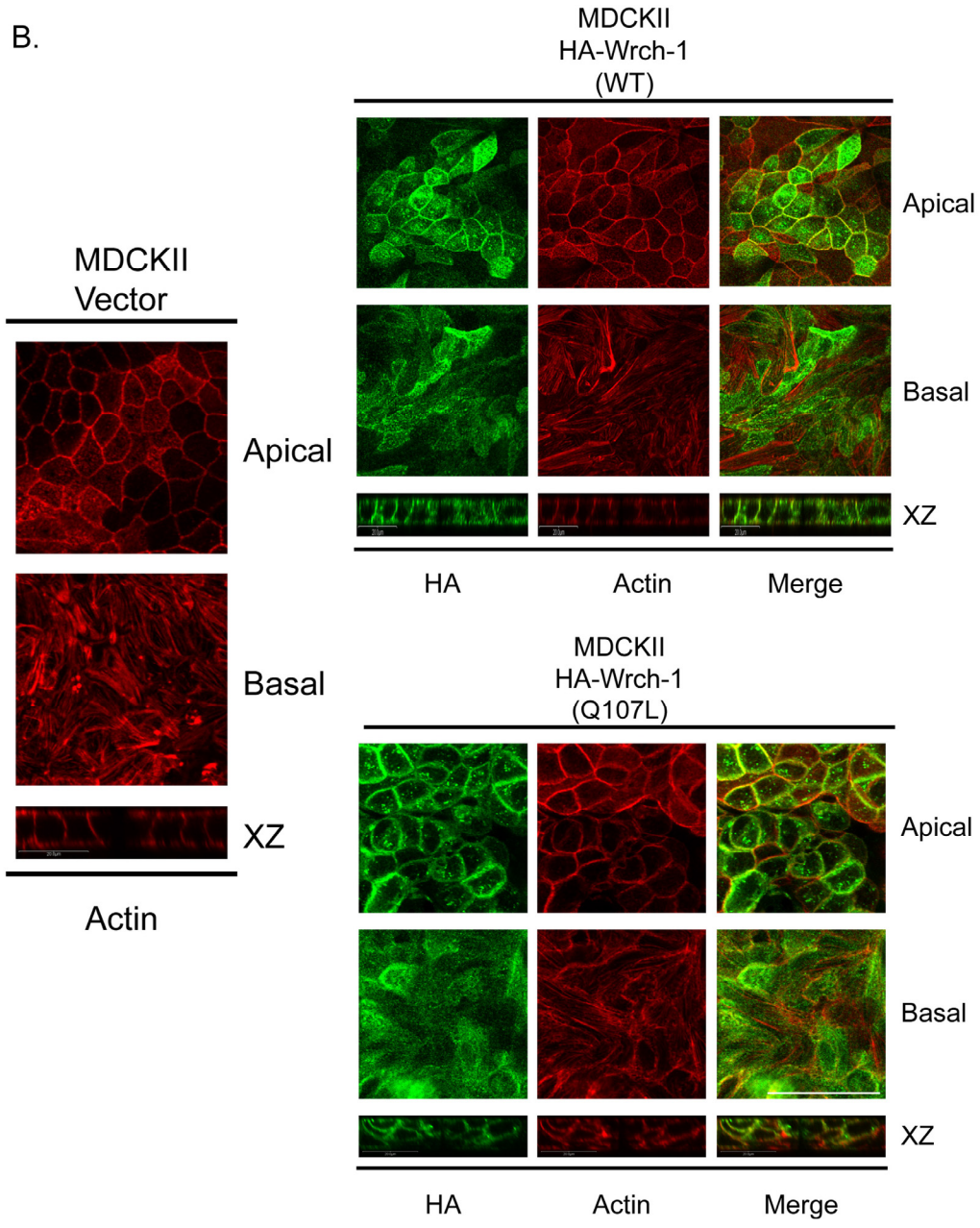

Figure 3.1B Activated Wrch-1 disrupts actin organization and cell morphology of MDCKII cells.

B) MDCKII cells stably expressing empty vector or indicated HA-tagged Wrch-1 constructs were grown to confluency on 12-mm Transwell filters, then fixed, stained, probed for the HA epitope tag (green) or with Texas-Red Phalloidin (red) and visualized by confocal microscopy. Square panels: XY sections; rectangular panels: XZ sections (top to bottom). Scale bars = 20 μm.

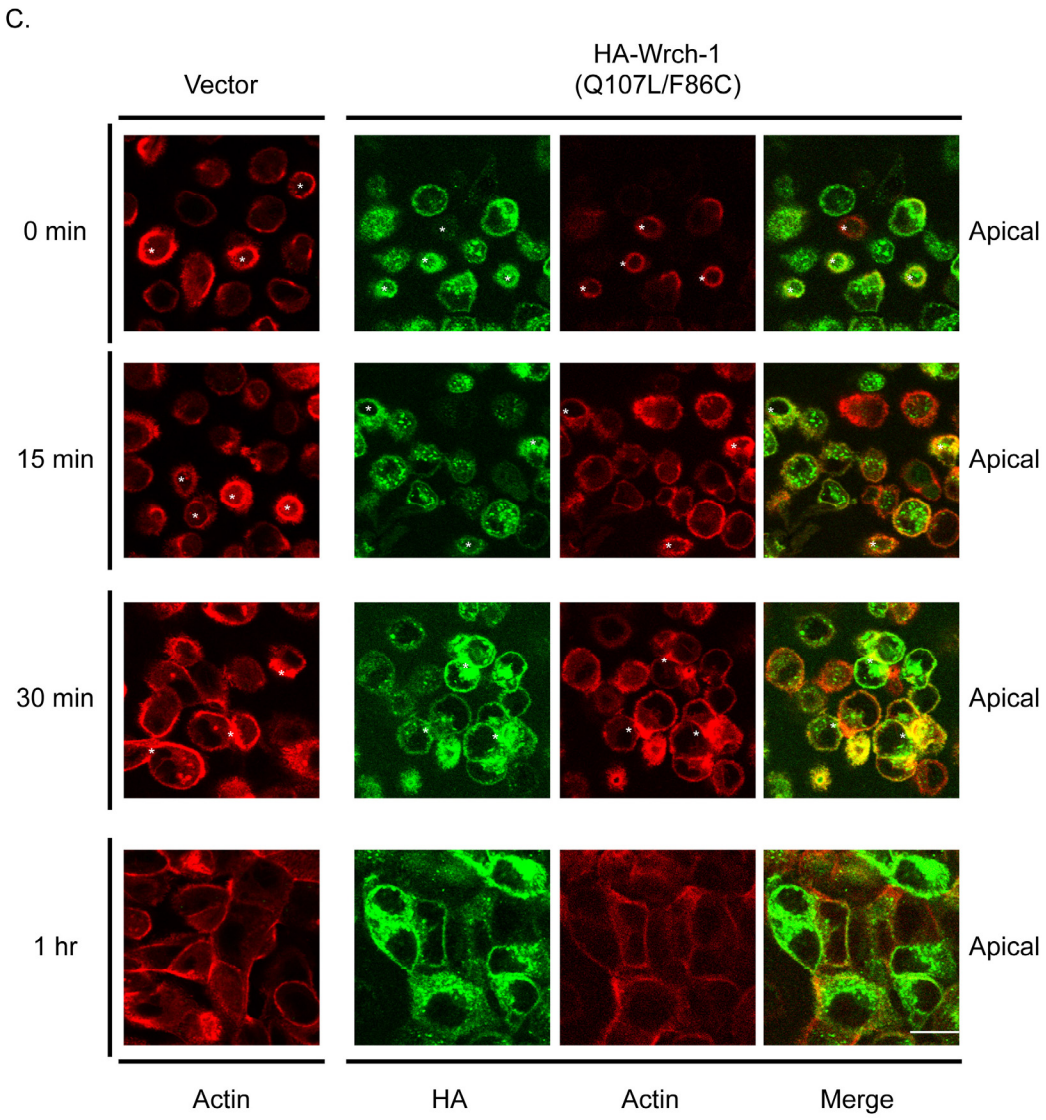

Figure 3.1C Activated Wrch-1 disrupts actin organization and cell morphology of MDCKII cells.

C) MDCK II cells stably expressing empty vector or HA-tagged Wrch-1(Q107L/F86C) were subjected to calcium switch and treated as in panel A. Square panels: XY sections; rectangular panels: XZ sections (top to bottom). Scale bars = 20 μm.

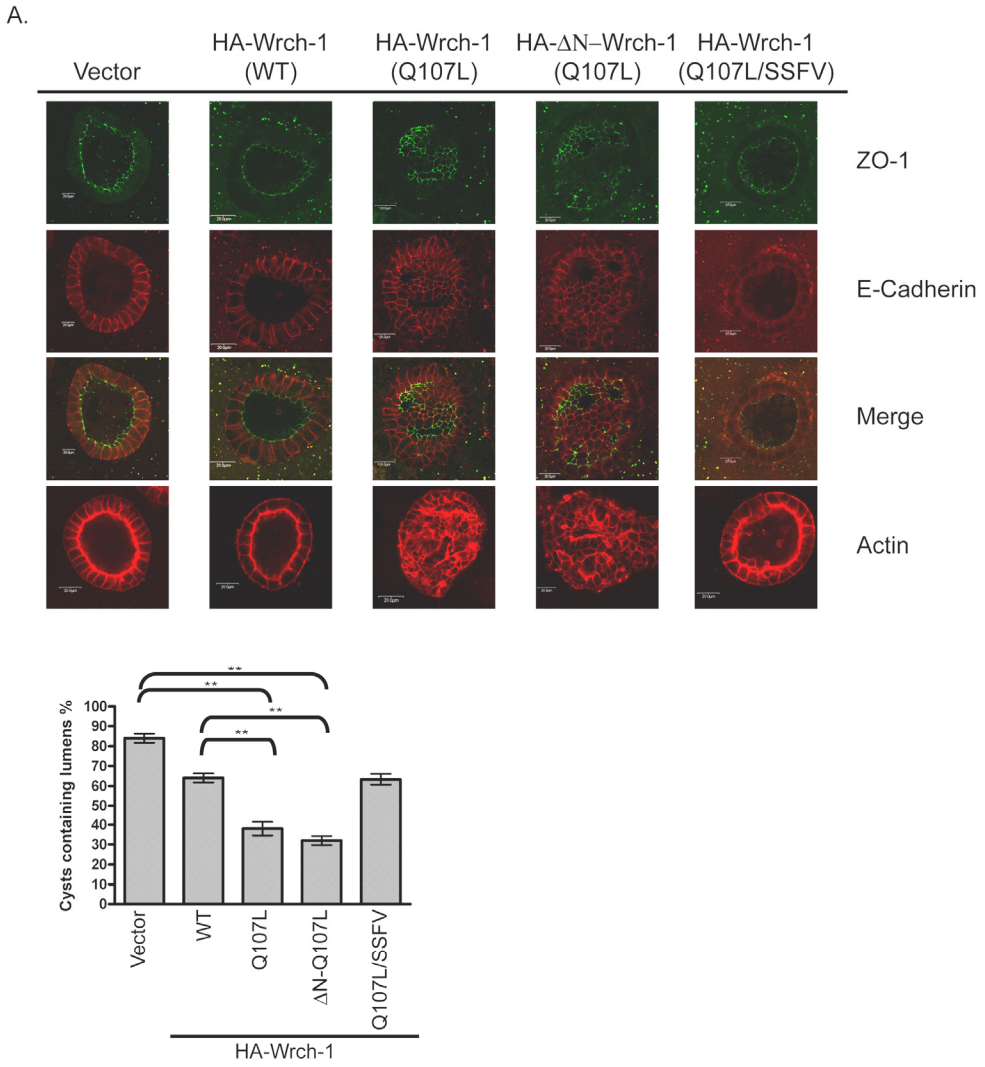

Figure 3.2A Activated Wrch-1 disrupts epithelial morphogenesis and promotes anchorage-independent growth of MDCKII cells.
A) Disruption of epithelial morphogenesis. MDCKII cells stably expressing empty vector or indicated HA-tagged Wrch-1 constructs were grown in collagen I gels. After 10 days, collagen gels were fixed and stained for ZO-1 (green) and E-cadherin (red) (top panels) to examine epithelial cell polarity, or with Texas-Red Phalloidin (red) (bottom panel) to examine epithelial morphogenesis and lumen formation. Scale bars = 20 μm. Cysts containing a single lumen were quantified by counting cysts with single luminal area as positive and cysts with no lumen as negative. Tukey's multiple comparison test was used to determine significance between cell lines. Significant p values of < 0.001 are indicated by **.

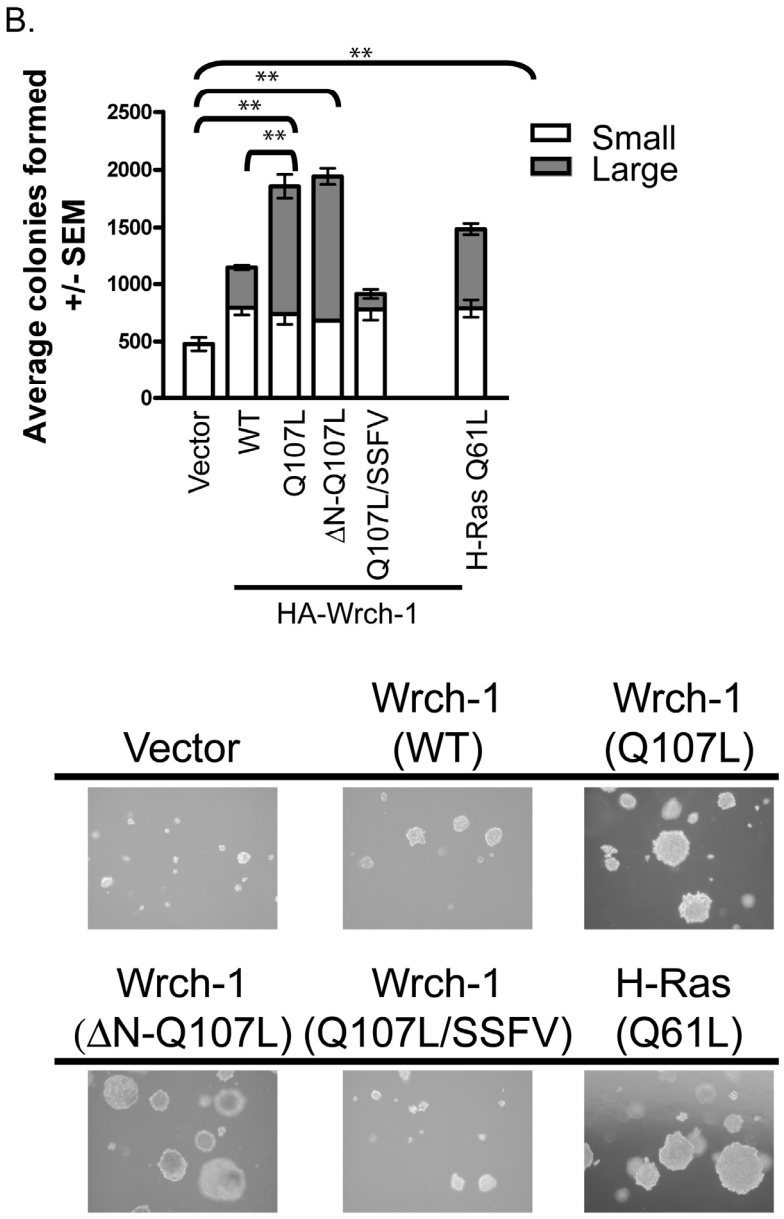

Figure 3.2B Activated Wrch-1 disrupts epithelial morphogenesis and promotes anchorage-independent growth of MDCKII cells.
B) Anchorage-independent growth. MDCKII cells were seeded into soft agar and analyzed for their ability to induce colony formation. Colonies formed after 14 days were stained, scanned, and the numbers of small (6 to 15 cells across) and large (>15 cells across) colonies were quantified. Images and bar graphs are representative of three separate experiments carried out in triplicate; shown are averages +/- SEM. Significant p values of < 0.001 obtained by using Tukey's multiple comparison test are indicated by **.

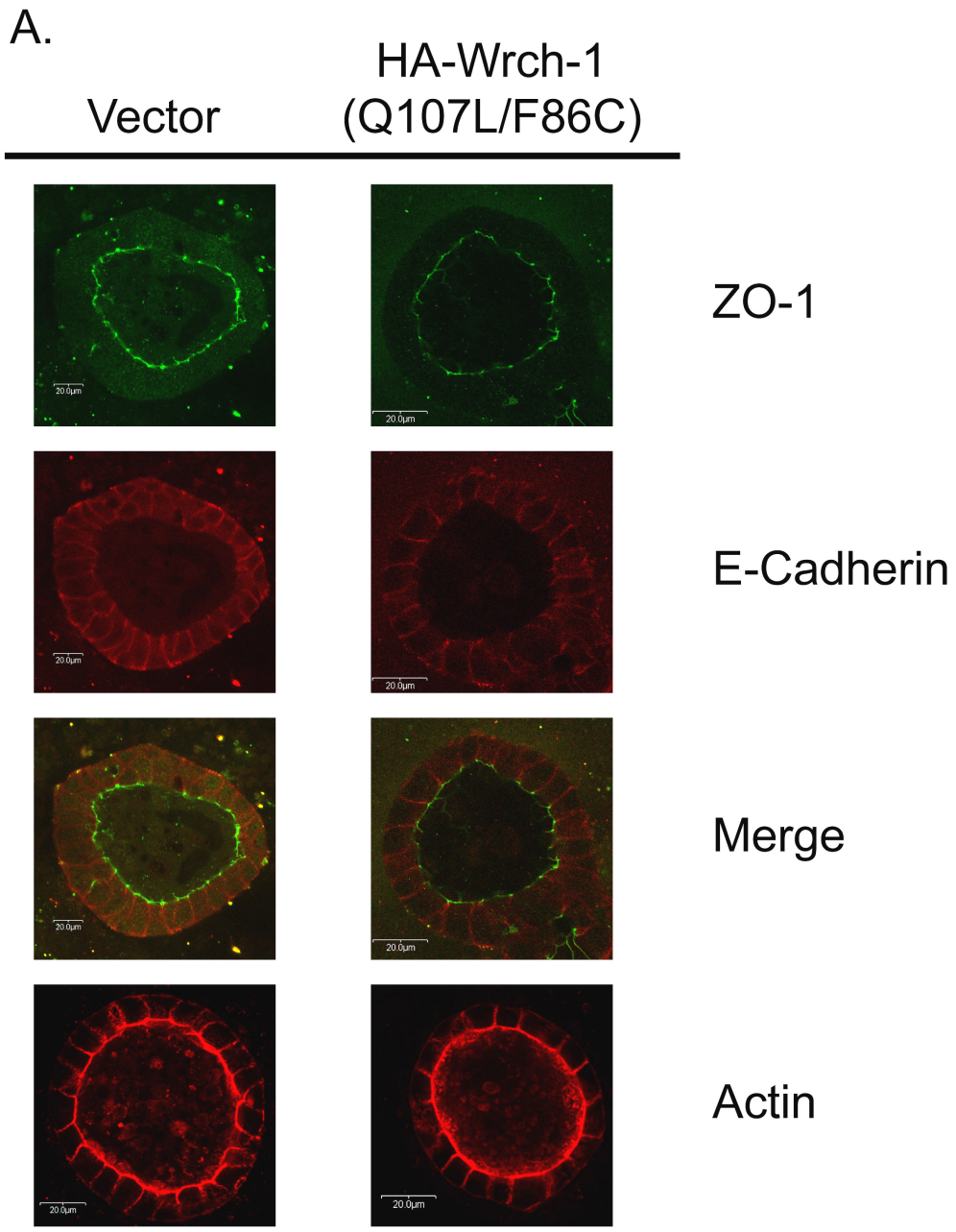

Figure 3.3A An effector domain mutation in Wrch-1 abrogates Wrch-1-mediated disruption of epithelial morphogenesis and promotion of anchorage-independent growth of MDCKII cells.
A) MDCKII cells stably expressing empty vector or HA-tagged Wrch-1(Q107L/F86C) were treated and evaluated as in Figure 3.2A.

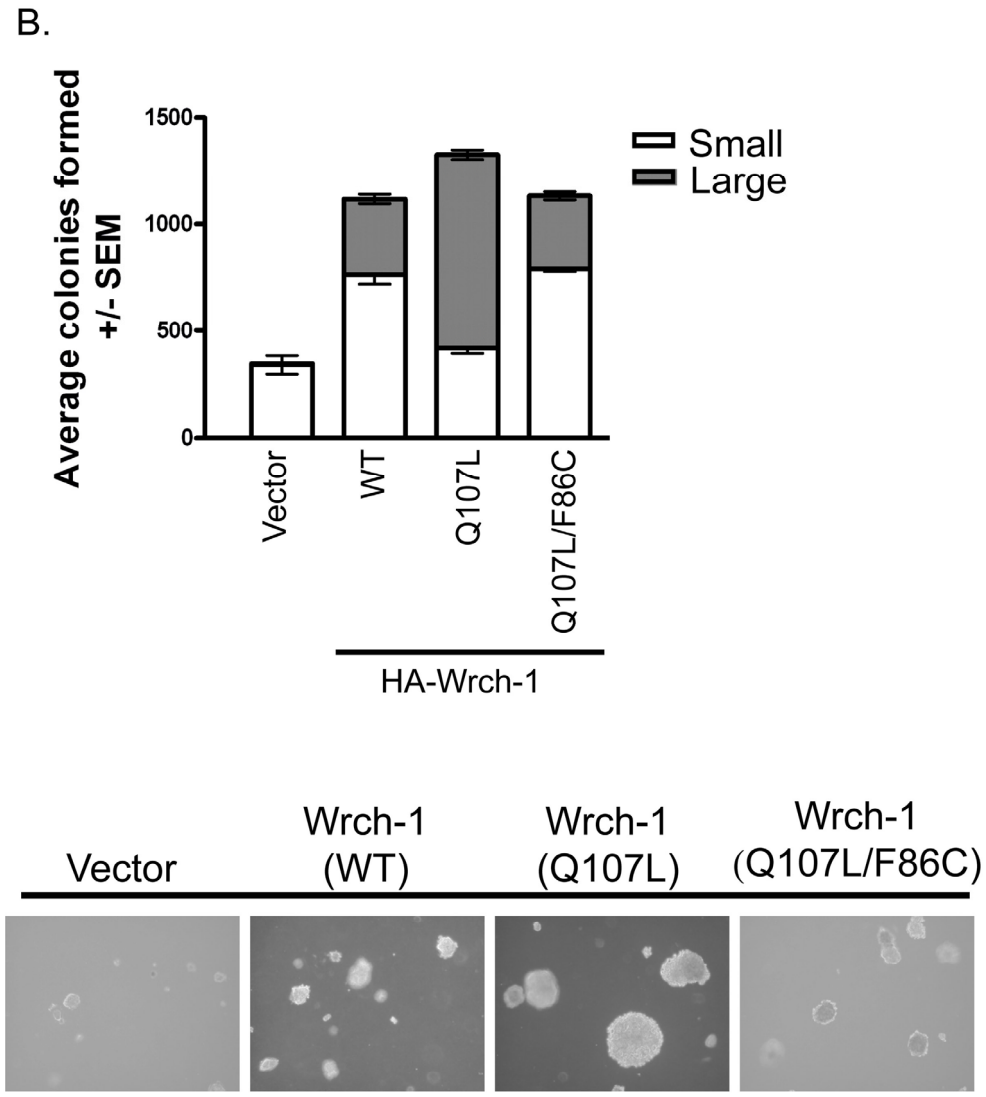

Figure 3.3B An effector domain mutation in Wrch-1 abrogates Wrch-1-mediated disruption of epithelial morphogenesis and promotion of anchorage-independent growth of MDCKII cells.

B) MDCKII cells stably expressing empty vector, Wrch-1(WT), Wrch-1(Q107L) or Wrch-1(Q107L/F86C) were seeded into soft agar and analyzed for their ability to induce anchorage-independent growth as indicated in Figure 3.2B.

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

4.1 Summary

When the studies addressed in my dissertation began, there were only a few publications highlighting the regulatory mechanisms of Wrch-1, while full identification and characterization of Wrch-1 downstream effectors, regulators, biological functions, and potential contributions to cancer remained elusive. Therefore, my dissertation focused on identifying downstream effectors necessary for the normal biological functions elicited by Wrch-1 that, when misregulated, may contribute to Wrch-1-mediated cellular transformation. Specifically, I set out to characterize the association between Wrch-1 and Par6, a cell polarity protein that was shown via yeast two-hybrid screen to be a potential interacting partner for Wrch-1 (Aspenstrom et al., 2004). Based on the known ability of Par6 to form an evolutionarily conserved protein complex with Par3 and aPKC that is instrumental in establishing epithelial cell polarity and in regulating tight junction formation, I hypothesized that a normal biological function for Wrch-1 may be regulating cell junctional dynamics in mammalian epithelial cells, in part through a GTP-dependent interaction between Wrch-1 and Par6 (Shin et al., 2006). Importantly, it was previously shown that aberrant activation of a GTPase-Par6-Par3-aPKC complex leads to cellular transformation (Qiu et al., 2000). These data suggest that, under normal conditions, intact cellular junctions control cell proliferation in such a way that constitutive activation of this complex disrupts cell adhesion contacts and weakens contact inhibition, resulting in excessive cell proliferation. Together,

these data suggest a potential effector pathway downstream of Wrch-1 activation that may play a role in normal epithelial cell dynamics, and that when aberrantly activated may contribute to the ability of Wrch-1 to promote cellular transformation.

Recently our lab and others established the importance and necessity of the Cdc42-related protein, Wrch-1, in regulating cellular processes in a manner that is distinct from other members of the Rho GTPase family. The studies outlined in my dissertation have added a new level of understanding of the potential normal biological functions of Wrch-1 and a potential mechanism by which Wrch-1 mediates cellular transformation. I was able to determine that Wrch-1 interacts with Par6 in a GTP-dependent manner, leading to an association with a Par6-PKCζ cassette in epithelial cells. Since the Par6-PKCζ cassette localizes to tight junctions and regulates early tight junction dynamics, I used the polarized mammalian epithelial cell line MDCKII as a powerful tool to investigate the localization and function of Wrch-1 with regards to tight junctions. To this end, I determined that Wrch-1 distributes predominantly to the basolateral membrane domain, while associating with both tight junctions and adherens junctions at sites of cell-cell contact. Activated Wrch-1 negatively regulates the kinetics of tight junction assembly during epithelial cell polarization, without a detectable effect on overall cell polarity in confluent monolayers. This is in contrast to expression of constitutively activated Cdc42, which perturbs tight junction dynamics along with cell polarity formation (Cohen et al., 2001; Kroschewski et al., 1999; Rojas et al., 2001), suggesting that in MDCK cells Cdc42 and Wrch-1 have divergent functions. Importantly, I determined that Wrch-1-mediated disruption of early tight junction dynamics may require association with Par6, since an effector domain mutant of Wrch-

1(Q107L/F86C) incapable of interacting with Par6 did not perturb tight junction formation or function.

Although I identified the cell polarity protein Par6 as a relevant effector downstream of activated Wrch-1 that is involved in Wrch-1-mediated disruption of tight junction formation and function, the cellular mechanisms that lead to delayed localization of tight junction-associated proteins and increased cellular permeability were unclear. Several hints within the literature pointed to Rho GTPase reorganization of the actin cytoskeletal network as a mechanism for Rho proteins to regulate tight junction dynamics. While evaluating the ability of Wrch-1 to regulate tight junctions, I observed that ZO-1 localization to an actin-rich perinuclear ring structure was disrupted upon expression of activated Wrch-1, suggesting that the actin organization was altered. I was able to determine that activated Wrch-1 expression in MDCKII cells causes dramatic cytoskeletal reorganization and multilayering in cells grown in two-dimensional (2D) culture. In addition, constitutive activation of Wrch-1 disrupted cystogenesis of cells grown in three-dimensional (3D) culture, resulting in aberrant multilumenal structures. I was able to correlate the loss of epithelial morphogenesis with Wrch-1-mediated transformation of MDCKII cells. Importantly, an effector domain mutation in activated Wrch-1 that prevents Par6 binding repressed transforming activity and abrogated the ability of Wrch-1 to disrupt tight junction formation, actin organization, and epithelial morphogenesis. Together these data suggest that Wrch-1 reorganization of the actin cytoskeleton perturbs tight junction dynamics, thereby causing a subsequent disruption of epithelial morphogenesis to promote a transformed phenotype, and that Wrch-1 does this in part through its association with Par6. It has been shown previously that Wrch-1 promotes proliferation and growth transformation. The data that I generated during the

course of my dissertation research, as documented in my manuscript, entitled "The transforming Rho family GTPase, Wrch-1, disrupts epithelial cell tight junctions and epithelial morphogenesis", highlight another potential mechanism by which Wrch-1 induces morphological transformation and may contribute to human cancers.

Recent publications along with our manuscript on Wrch-1 suggest that further investigation into Wrch-1 normal biological functions and contributions to human disease will be useful research endeavors. One future direction sparked in particular by the results outlined in my dissertation is determining the physiological relevance of Wrch-1 expression and activity to tight junction dynamics, cytoskeletal organization, and morphogenesis. In addition, whether Wrch-1 plays a role in Wnt-driven human breast and colon cancer remains to be determined. If so, it will be important to further investigate how the mechanisms that I have hypothesized by which Wrch-1 mediates morphological and cellular transformation contribute to the ability of Wnt family proteins to induce cellular transformation. On a different note, the use of experimental biosensor techniques to study the spatiotemporal activation of Rho GTPases during cell migration has proven to be a powerful tool to delineate the necessity of tight regulation of Rho GTPase localization and activation to dictate different cellular processes during cell migration. Therefore, I would like to employ the use of Rho GTPase biosensors during epithelial morphogenesis in 3D culture to study the localization of Rho GTPase activation during epithelial morphogenesis. This will allow me to ascertain the spatiotemporal involvement of the classic Rho GTPases, RhoA, Rac1 and Cdc42 in cystogenesis.

4.2 Future Directions

4.2.1 Determining the physiological relevance of Wrch-1 expression in mammalian epithelial cells to tight junctions, cytoskeletal organization, and morphogenesis

In this dissertation I have shown that exogenous expression of a constitutively active mutant of Wrch-1(Q107L) in MDCKII cells disrupts epithelial cell tight junctions, cytoskeletal organization, and morphogenesis, and that this is a potential mechanism by which Wrch-1 mediates cellular transformation. However, under these experimental conditions little inference as to the physiological function of Wrch-1 in mammalian epithelial cells can be made. A recent publication by Chuang et al. used small interfering RNA (siRNA) oligonucleotide duplexes to knock down Wrch-1 mRNA levels in HeLa cells, in order to address whether the loss of Wrch-1 expression affected cytoskeletal dynamics and cell migration. This group was able to determine that siRNA-mediated knockdown of Wrch-1 expression decreases focal adhesion turnover and subsequently decreases cell migration (Chuang et al., 2007). These data suggest that the use of siRNA against Wrch-1 is a useful experimental tool to help elucidate Wrch-1 normal biological functions.

Therefore, I propose the use of RNAi against Wrch-1 to determine the normal physiological role of Wrch-1 in mammalian epithelial cells. In contrast to Marc Symons and colleagues who used siRNA to transiently knockdown human Wrch-1 in HeLa cells, I propose to stably knock down canine Wrch-1 in MDCKII cells using shRNA cloned into the pRETROSUPER retroviral mammalian expression vector. As a first option to decrease Wrch-1 mRNA levels in MDCKII cells, it would be best to obtain canine shRNA targeting sequences that are complementary to the siRNA oligonucleotide duplex sequences used in Chuang et al. to successfully knock down human Wrch-1 (Chuang et al., 2007). Once reduction in Wrch-1 message was validated by RT-PCR (antibodies capable of detecting endogenous Wrch-1 protein are not available), the requirement of Wrch-1 expression to

regulate normal morphological and biological functions of model epithelial cells could be addressed. A much longer term option would be the generation of a knockout mouse.

Although there are many aspects of epithelial cell biology to which Rho GTPases contribute, I would focus first on tight junction dynamics, actin organization, and morphogenesis, based on the fact that gain-of-function experiments with Wrch-1 show dramatic alterations in these three aspects of polarized MDCKII cells. Specifically, I would perform the same experiments in MDCKII cells stably expressing shRNA against canine Wrch-1 as those described in Chapters 2 and 3, to determine if endogenous Wrch-1 expression is required for any of these three biological endpoints. In addition, by using MDCKII cells stably knocked down for Wrch-1, I could also investigate the importance of Wrch-1 in other cellular processes of epithelial cell biology, such as cell-substratum adhesion, adherens junction dynamics, microtubule network organization, vesicular trafficking, and apical basolateral polarity.

As stated earlier in my dissertation, epithelia are essential for normal physiological processes in the human body such as excretion, digestion, and leukocyte migration. During development, the movements of individual epithelial cells are coordinated in order to form epithelial cysts and tubules found in complex tissues, such as lung, pancreas, and mammary gland. Wrch-1 has been shown to be ubiquitously expressed in human tissues including lung, kidney, and liver, that mainly consist of epithelial cells. Therefore, RNAi-mediated knockdown of Wrch-1 will be a powerful tool to delineate the requirement for Wrch-1 expression in a model epithelial cell line such as MDCKII. This experimental technique could potentially help widen the scope of known biological functions of Wrch-1 in epithelial cell architecture and function that cannot be gained from overexpression systems. In

addition, if successful, the use of RNAi against Wrch-1 could be extended to other cell systems and tissues such as the brain where Wrch-1 is highly expressed. Importantly, identification of normal biological functions of endogenous Wrch-1 would help us and others determine how constitutive activation of Wrch-1 may contribute to human cancer.

4.2.2 Determining whether Wrch-1 expression plays a role in Wnt-driven human breast and colon cancer

The Wnt family of secreted, cysteine-rich glycoproteins exerts various cellular effects through autocrine and paracrine signaling. To date there are at least 19 known human Wnt genes that are developmentally regulated in a precise temporal and spatial manner (Miller, 2002). Wnt signaling mediates changes in cell morphology, cytoskeletal organization and cell proliferation, and these functions are implicated in Wnt-mediated carcinogenesis (Peifer and Polakis, 2000; Polakis, 2000). The canonical Wnt signaling pathway is initiated upon Wnt ligand binding to a seven-transmembrane-domain Frizzled receptor, and leads to the stabilization of β-catenin by inhibiting glycogen-synthase kinase-3β (Gsk-3β). Stabilized β-catenin translocates to the nucleus and activates target gene expression through an interaction with transcription factors of the T cell factor (TCF) family. In the absence of Wnt signaling, β-catenin is phosphorylated and targeted for degradation in a complex with Gsk-3β, Axin, and adenomatous polyposis colon protein (APC) (Polakis, 2000). β-catenin-independent Wnt signaling pathways include the Wnt/Ca^{2+} signaling pathway and the Wnt/planar cell polarity pathway, and these are also important for Wnt-mediated biological functions (Giles et al., 2003).

The Wnt-1 oncogene was first identified as the site of integration by the mouse mammary tumor virus (MMTV) and its expression can promote the formation of mammary

tumors in transgenic mice (Bradley and Brown, 1995; Jue et al., 1992; van Ooyen and Nusse, 1984). When misregulated, some Wnt proteins and their contributing signaling pathways can promote cellular transformation of various cell and tissue types, such as mammary and colon cells, usually through unchecked stabilization of β-catenin activity (Peifer and Polakis, 2000). Even though the canonical Wnt signaling pathways use β-catenin stabilization as a means for various cellular functions, β-catenin activation is not always sufficient (Shimizu et al., 1997). Therefore, other signaling pathways regulated by Wnt signaling may contribute to Wnt-mediated cellular transformation. Wrch-1 was first identified as a Rho GTPase whose mRNA levels increased in transformed mammary epithelial cells in response to Wnt-1 signaling, but not in response to stabilization of β-catenin (Tao et al., 2001). These data suggest that Wrch-1 may play a role in Wnt-1 β-catenin-independent signaling. Interestingly, expression of GTPase-deficient Wrch-1(Q107L) mutant induces morphological transformation of mouse mammary epithelial cells in a manner similar to expression of Wnt-1. Since Wnt-1 signaling mediates changes in cell morphology, cytoskeletal organization and cell proliferation, which are some of the cellular processes regulated by conventional Rho GTPases, Wrch-1 may mediate Wnt biological effects that contribute to its ability to induce transformation. Activation of the Wnt signaling pathway through alterations in the expression of Wnt genes and their regulators has been implicated in contributing to human breast and colon cancer (Bienz and Clevers, 2000; Turashvili et al., 2006). Therefore, it would be interesting to determine whether Wrch-1 expression is regulated by Wnt signaling in human mammary and colon epithelial cells and if it contributes to the ability of these Wnt family proteins to cause cellular transformation of these cells.

The function of the Wnt gene family in mouse mammary epithelial cell (MMEC) tumorigenesis has been investigated with ectopic expression of the Wnt gene family in the C57MG mammary epithelial cell line. In C57MG cells, expression of Wnt-1, Wnt-2, Wnt-3A, Wnt-5B, Wnt-7A and Wnt-7B induced morphological transformation with varying efficiency, while Wnt-4, Wnt-5A, and Wnt-6 were non-transforming (Shimizu et al., 1997). Although this has proved to be a useful system to determine whether Wnt genes cause cellular transformation of MMECs, C57MG cells are very difficult to work with in the lab. More importantly, many investigators have determined that those genes frequently activated in mouse mammary carcinomas, such as Wnt-1, are distinct from the most common targets of mutation in human breast cancers. However, recent studies have identified changes in Wnt gene expression, along with alterations in the positive and negative regulators of the Wnt signaling pathway in human breast cancer, which may play a role in cancer development. Specifically, upregulation of Wnt genes themselves and downregulation of negative regulators of Wnt signaling, such as Wnt inhibitory factor (WIF) and secreted frizzled-related protein 1 (SFRP1), have been identified in human breast cancer cell lines and tissue. Wnt-1, Wnt-2, Wnt-3 and Wnt-4 have been shown to be upregulated in human breast carcinomas, suggesting a possible role for Wnt signaling in human breast cancers (Ayyanan et al., 2006; Benhaj et al., 2006; Lee et al., 2004; Watanabe et al., 2004; Wissmann et al., 2003). However, whether the Wnt genes that are upregulated are also required for any aspect of breast cancer has not yet been elucidated. Nor is it known whether Wrch-1 is regulated by any Wnt gene other than Wnt-1.

Human mammary epithelial cells (HMECs) and the human mammary epithelial cell line, MCF-10A, have been used to investigate the role of the Wnt-1 pathway in human breast

cancer (Benhaj et al., 2006). Therefore, I propose to use MCF-10A cells to investigate the role of Wnt genes and Wrch-1 in the morphological and growth transformation of human mammary epithelial cells. These experiments will aid in the development of a system to determine whether Wnt-mediated cellular transformation is linked to upregulation of Wrch-1. The ability of a panel of Wnt genes to induce growth transformation of MCF-10A cells could be investigated with a soft agar assay, which measures the ability of cells to grow independent of anchorage. I would expect that in soft agar assays, Wnt-1, Wnt-2, Wnt-3 and Wnt-4 will induce colony formation of MCF-10A, cells, while Wnt-5a will not. However, these predictions are different from what has been elucidated for Wnt ligand-induced transformation of C57MG, which are mouse mammary epithelial cells. The reasons for these predictions are as follows: Wnt-1, Wnt-2, Wnt-3, and Wnt-4 have been shown to be overexpressed in human breast cancer, while Wnt-5a has been shown to be downregulated in human breast cancer (Ayyanan et al., 2006; Tao et al., 2001; Watanabe et al., 2004). I speculate that Wnts that are upregulated may be those that have gain-of-function phenotypes and are positively associated with transformation. Although a requirement for the Wnt ligands in the transformed phenotype has not been elucidated in human breast cancer, these data would help support further investigation and help set up a simple system to address which Wnt ligands may be involved in transformation of human mammary epithelial cells.

As stated previously, Wrch-1 was first identified as being upregulated in mouse mammary tumor induced by Wnt-1 (Tao et al., 2001). In addition, expression of activated Wrch-1(Q107L) mimics morphological transformation observed in the Wnt-1-expressing mouse mammary epithelial cells, C57MG. These data suggest that Wrch-1 expression may contribute to Wnt-mediated transformation. Therefore, it would be relevant to investigate

whether Wrch-1 upregulation also occurs in MCF-10A human mammary epithelial cells in response to Wnt signaling. To this end I propose measuring Wrch-1 mRNA levels in MCF-10A cells expressing a panel of Wnt genes in order to correlate upregulation of Wrch-1 expression and the ability of those Wnt genes to induce cellular transformation. If, as predicted, a function of the upregulation of Wrch-1 expression by Wnt signaling is to promote the tumor program associated with aberrant Wnt signaling, then those Wnt genes able to transform human mammary epithelial cells should express increased Wrch-1 mRNA. Specifically, I predict that Wrch-1 mRNA as determined by RT-PCR will be increased in MCF-10A cells expressing Wnt-1, Wnt-2, Wnt-3, and Wnt-4, while Wnt-5A expressing cells will not show an increase in Wrch-1 mRNA. Further experiments involving RNAi-mediated knockdown of Wrch-1 expression could be used to determine whether Wrch-1 expression is required for Wnt-mediated cellular transformation of MCF-10A cells and predict wto hether Wrch-1 expression will play a role in human breast cancers that have aberrant Wnt signaling.

Another advantage to using MCF-10A cells is that, much like MDCKII cells, under 3D culture conditions MCF-10A cells proliferate and organize into spheroids, commonly called 'acini', which are notable for the presence of a centrally-localized, hollow lumen, and for the polarization of cells surrounding this lumen (Debnath et al., 2003). Such 3D culture of MCF-10A cells in extracellular matrix has been used to study the early stages of carcinogenesis in culture in response to oncogenes like Erb2 (Muthuswamy et al., 2001). Therefore, I propose to use this 3D culture system to investigate whether stable expression of a panel of Wnt genes in MCF-10A perturbs normal epithelial morphogenesis of mammary epithelial cells and resembles tumorigenesis. More importantly, this same MCF-10A culture system could be used to determine if Wrch-1 expression is required for the transformed

phenotype that will potentially be induced by several Wnt genes (Wnt-1, Wnt-2, Wnt-3 and Wnt-4) in a similar manner to those experiments described above. These studies would be the first investigation into the functional involvement of Wrch-1 in human cancer.

In colon cancer, loss of function mutations in APC and activating mutations in β-catenin lead to β-catenin stabilization and gene transcription associated with tumorigenesis. Specifically, loss-of-function mutations in the β-catenin regulator APC and gain-of-function mutations within β-catenin lead to β-catenin stabilization and to gene transcription events that contribute to tumorigenesis (Polakis, 2000). An investigation into the expression levels of Wnt signaling components in both normal and colon cancer cell lines (Caco-2, HT29, and Colo205) suggests that changes in the expression of some Wnt ligands and Frizzled receptors can be detected in colon cancer when compared to normal colon tissue. The data from this study indicated that altered expression of Wnt signaling components, distinct from APC and ß-catenin, is associated with the progression of colon carcinogenesis (Holcombe et al., 2002).

Since most colon cancers harbor mutations in APC or β-catenin, I propose to use the colorectal adenocarcinoma cell line Caco-2 for my studies. Caco-2 (APC mutation) is an adherent cell line that forms moderately well-differentiated adenocarcinomas in nude mice. In order to investigate the involvement of Wrch-1 in tumorigenesis of these cells, I would use shRNA targeted against human Wrch-1 to knock it down in Caco-2 cells and then assess the ability of exogenously expressed Wnt genes to promote the growth of these cells in soft agar. Recently Alan Hall and colleagues began using Caco-2 cells in 3D culture studies because they are capable of forming cysts like those described for MCF-10A cells and for the MDCKII cells that I have used in my dissertation studies (A. Hall, personal communication). Therefore, it would be interesting to exogenously express various Wnt genes or constitutively

activated Wrch-1 in Caco-2 cells and determine whether normal epithelial morphogenesis is lost. Once it is clear which Wnts are transforming in this background, I could also knock down Wrch-1 to determine if there is a requirement for Wrch-1 expression for Caco-2 morphogenesis and transformation. The studies postulated here would be the first investigation of the physiological relevance of the small GTPase Wrch-1 to human cancer and would begin to validate whether Wrch-1 is a potential drug target for the treatment of human breast and colon cancer. In addition, they would begin to address whether the potential mechanisms by which Wrch-1 promotes cellular transformation described in Chapter 2 and 3 using a model cell system are also valid in human cancer cell lines.

4.2.3 Use of Rho GTPase biosensors during epithelial morphogenesis in 3D culture

Until the recent development of fluorescent probes capable of deciphering the spatiotemporal activation of Rho GTPases, the presumed local activity of a given GTPase could be inferred only from the appearance of specific cytoskeletal and adhesive structures. Klaus Hahn and colleagues have been at the forefront of the development of fluorescence-based probes capable of detecting the spatiotemporal activation of the Rho GTPases RhoA, Rac1, and Cdc42 (Pertz and Hahn, 2004). The structures of these biosensors are all unique, and their readout has allowed researchers to determine the spatiotemporal activation of these Rho GTPases. Most of these biosensors are advantageous because they allow for non-destructive measurement of the nucleotide state of Rho GTPases in individual living cells (Gardiner et al., 2002; Kraynov et al., 2000; Nalbant et al., 2004; Pertz et al., 2006).

The RhoA biosensor designed by Klaus Hahn's group is a genetically encoded single-chain biosensor that responds to RhoA activation with intramolecular fluorescence resonance energy transfer (FRET). Specifically, the RhoA biosensor consists of a portion of the Rho-

binding domain (RRB) of the Rho effector Rhotekin that binds specifically to active, GTP-bound RhoA, followed by cyan fluorescent protein (CFP), an amino acid linker, yellow fluorescent protein (YFP), and full-length RhoA. Therefore, when RhoA is in its active GTP-bound conformation, the N-terminal Rhotekin RBD binds the C-terminal active RhoA, which results in a change in the relative orientation of the central CFP and YFP fluorophores and thereby increases FRET. Thus, changes in the FRET/CFP emission ratio allow for the determination of RhoA activation at a given subcellular localization (Pertz et al., 2006).

Genetically encoded single chain biosensors for Rac1 and Cdc42 have also been engineered by others. Specifically, like the RhoA biosensor described above, these genetically encoded single chain probes contain YFP followed by a portion of the Pak1 CRIB domain, either Rac1 or Cdc42, and CFP. Therefore, when a cell is exposed to extracellular stimuli capable of activating Rac1 or Cdc42, the now GTP-bound Rac1 or Cdc42 portion of the probe binds to the CRIB domain of Pak1 within the probe, bringing the YFP and CFP molecules into close enough proximity to result in FRET (Itoh et al., 2002). These FRET-based biosensors, known as Raichu-Rac1 and Raichu-Cdc42 probes, designed by Matsuda and colleagues, lack a functional CAAX motif. Therefore they bypass Rac and Cdc42 regulation by Rho GDI and are capable of measuring the activation of Rac and Cdc42 only at the plasma membrane. However, because these GTPases are also active at other subcellular locations, the Raichu probes do not reveal the full extent of Rac and Cdc42 spatiotemporal control. The design of biosensor probes that can specifically track all of the physiologically important activities of individual small GTPases remains very challenging.

Recently, Klaus Hahn and colleagues described the design and optimization of genetically encoded single chain fluorescence-based probes for detecting Rho GTPase

activation in a spatiotemporally regulated manner. Simplistically, these probes consist of the protein to be studied, an "affinity reagent" that binds preferentially to the active conformation of the protein being studied, and mutants of GFP, such as YFP and CFP, that are capable of undergoing FRET (Hodgson et al., 2008). One advantage of these fluorescent probes is that they can be cloned into many different mammalian expression vectors, including retroviral or lentiviral vectors, and expressed in various cell types. Therefore, I propose to use these newly designed genetically encoded single chain FRET-based biosensors for Rho GTPases to investigate the spatiotemporal activation of the classical Rho GTPases, RhoA, Rac1, and Cdc42, during epithelial morphogenesis. If a similar useful probe could be constructed for Wrch-1, potentially based on its ability to bind Par6, that too could be used to compare its spatiotemporal activation to those of the classical Rho GTPases. In addition, extending this type of study to other members of the Cdc42-related family, especially the more highly related proteins Wrch-2/Chp and TCL, could be highly informative. Such studies would be longer term goals, in part due to lack of currently available biosensor probes and in part due to lack of information on the role of these proteins in epithelial morphogenesis.

Interestingly, Mostov and colleagues recently used the PH domain of phospholipase-Cδ1 or the PH domain of AKT fused to GFP as high affinity biosensors to identify the intracellular pools of the phosphoinositides PtdIns(4,5)P_2, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 during epithelial morphogenesis of MDCKII cells grown in 3D culture (Martin-Belmonte et al., 2007). In addition, these researchers used the CRIB domain of the Cdc42 effector WASP fused to GFP, known as CBD-GFP, to monitor the subcellular localization of Cdc42 activation during epithelial morphogenesis. However, the use of CBD-GFP is not specific to Cdc42 since many other Cdc42-related proteins are also known to interact with WASP in a

GTP-dependent manner. The experimental design for these studies was simple in that monodispersed MDCKII cells stably expressing the biosensors described above were plated in Matrigel. At various time points (1, 2, 3, 4 or 5 days) after MDCKII cells were plated in Matrigel, the newly forming cysts were fixed and mounted on coverslips and the localization of the fluorescence-based biosensors was visualized using confocal microscopy. If MDCKII cells stably expressing the biosensors were not obtainable, cysts were incubated for a short time in trypsin containing Ca^{2+} and Mg^{2+}. After trypsin-mediated digestion of the Matrigel, the cysts were then infected with adenovirus encoding the fluorescent probes and later fixed and visualized using confocal microscopy.

I propose to generate retroviral expression vectors encoding the single chain FRET-based biosensors capable of detecting activated RhoA, Rac1, and Cdc42 (Hodgson et al., 2008). These retroviral expression vectors can be used to create MDCKII stable cell lines expressing the fluorescent biosensors. The generation of these stable cell lines will allow for the investigation of the spatiotemporal activation of these GTPases during epithelial morphogenesis. Hopefully these experiments would help uncover previously unidentified information about the activation of Rho GTPases in 3D culture systems. In addition, as mentioned above, this experimental strategy could be used to engineer biosensors for many of the Rho GTPase family members whose function and activation in epithelial morphogenesis has not been elucidated.

4.2.4 Summary of future directions

The future directions to my dissertation outlined here would add further insight into the cellular functions of the atypical Rho GTPase Wrch-1 and members of the Rho GTPase family as a whole. The first two future directions hinge upon the use of RNAi technology as

a powerful tool to reduce Wrch-1 expression levels as a means to determine the importance of Wrch-1 in epithelial morphogenesis and in Wnt-mediated transformation, while the third direction relies on FRET-based imaging techniques to approximate the spatiotemporal activation of Rho family GTPases during epithelial morphogenesis. The rationale for wanting to use shRNA knockdown to uncover the physiological functions of Wrch-1 is two-fold. First, these studies could help address the necessity of Wrch-1 compared to other Cdc42-related proteins in normal epithelial cell functions. In addition, they could potentially lead to the discovery of cellular signaling pathways and functions that, when aberrantly activated, could contribute to Wrch-1-mediated cellular transformation. These would be the first studies aimed at elucidating the involvement of a panel of Wnt genes in the cellular transformation of human breast and colon epithelial cells. In addition, it would allow for the development of a cell system capable of determining whether Wrch-1 contributes to Wnt-mediated cellular transformation and could potentially lead to the use of Wrch-1 expression levels as a diagnostic marker for Wnt-mediated breast and colon cancer. The third future direction is dependent upon the use of genetically encoded single chain FRET-based probes capable of approximating the spatiotemporal localization of the classic Rho GTPases, RhoA, Rac1, and Cdc42 during epithelial morphogenesis in 3D culture. Similar biosensors engineered and optimized by Klaus Hahn's group here at UNC-CH have been instrumental in deciphering the subcellular localization of RhoA, Rac1, and Cdc42 activation during cell migration. Although it is well appreciated that Rho family GTPase activation and inactivation contribute to epithelial morphogenesis, these studies would allow for identification of previously unknown spatial and temporal coordinates of GTPase cycling during the multistep progression of cystogenesis in 3D culture. Together with the data in my

dissertation, these future directions could open many new avenues to understanding of the atypical Rho GTPase Wrch-1 and of Rho proteins in general.

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