# POSTTRANSLATIONAL MODIFICATIONS AS REGULATORS OF MEMBRANE LOCALIZATION AND BIOLOGICAL ACTIVITY OF THE RHO FAMILY SMALL GTPASE, WRCH-1

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## ABSTRACT

Anastacia C. Berzat: Posttranslational modifications as regulators of membrane localization and biological activity of the Rho family small GTPase, Wrch-1
(Under the direction of Dr. Adrienne D. Cox)

Rho proteins are members of the Ras superfamily of small GTPases. They are most well known for their functions in regulating the actin cytoskeleton, but also have normal roles in nearly all aspects of cellular physiology. Aberrant regulation of Rho signaling pathways leads to transformation including uncontrolled growth, invasion and metastasis. As such, mediators of Rho protein activity are subject to intense investigation as potential targets for pharmacological inhibitors. In addition to GTP/GDP cycling, membrane localization of these GTPases is a critical determinant of their transforming ability through spatial regulation of their signaling interaction partners and downstream signaling pathways. In this dissertation, I describe my investigations into regulation of the localization and function of Wrch-1 (Wnt-regulated Cdc42 homolog-1), a novel member of the Cdc42 subfamily of Rho proteins. Nearly all Rho proteins rely on posttranslational modifications of specific residues within their carboxyl termini for proper delivery to cellular membranes. For example, a required geranylgeranyl or farnesyl isoprenoid lipid moiety is attached by the respective prenyltransferase to a conserved cysteine residue within the carboxy-terminal CAAX motif of Rho proteins. Farnesyltransferase and geranylgeranyltransferase inhibitors (FTIs,GGTIs) are under investigation as

potential anticancer drugs. I sought to determine whether Wrch-1 is a target for FTIs or GGTIs. In addition, Rho family proteins are also modified by phosphorylation and ubiquitylation that can direct protein localization and stability, but the role of these modifications in regulating Rho biological activity is much less well understood. I also investigated how posttranslational modifications might regulate the localization and transforming activity of Wrch-1. I found that Wrch-1 is an atypical Rho protein that requires the addition of palmitoyl fatty acids rather than isoprenyl groups for correct sorting to membranes and for its transforming ability. I defined three distinct membrane targeting motifs in the carboxy-terminal hypervariable domain of Wrch-1 that regulate its interaction with plasma membrane, endomembrane and nuclear locations. Finally, I uncovered a possible role for monoubiquitylation of Wrch-1 in regulating its subcellular location and trafficking. Thus, Wrch-1 biological activity is regulated by its subcellular distribution due to modification by palmitoylation, phosphorylation and ubiquitylation.

## DEDICATION

To my best friend Reggie, who has always been my number one fan. Your unwavering love and support have encouraged me during all my academic and personal endeavors. Everyone should have a friend like you.

To my brother Patrick, who has always loved and accepted me “just as I am”.

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| | |
|---|---|
| a.a. | amino acid |
| APT1 | acyl protein thioesterase 1 |
| Btn-BMCC | biotin-1-biotinamido-4-[4'-(maleimidomethyl) cyclohexanecarboxamido] butane |
| CAAX | cysteine-aliphatic-aliphatic-unconserved amino acid |
| cAMP | cyclic adenosine monophosphate |
| Cdc42 | cell division cycle 42 |
| Chp | Cdc42 homologous protein |
| CIP4 | Cdc42-interacting protein |
| CNF | cytotoxic necrotizing factor |
| CRIB | Cdc42/Rac interactive binding |
| C-terminus | Carboxyl-terminus |
| Dbl | diffuse B-cell lymphoma |
| DH | Dbl homology |
| DHHC | asparate-histidine-histidine-cysteine |
| DMEM-H | high glucose Dulbecco's modified Eagle medium |
| DUB | de-ubiquitylating enzyme |
| EGF | epidermal growth factor |
| EGFP | enhanced green fluorescent protein |
| EGFR | epidermal growth factor receptor |
| F | farnesyl group |
| FFU | focus forming units |

| | |
|---|---|
| FITC | fluorescein isothiocyanate |
| FPP | farnesyl diphosphate |
| FTase | farnesyltransferase |
| FTI | farnesyltransferase inhibitor |
| FTS | *S-trans,trans*-farnesylthiosalicylic acid |
| GAP | GTPase activating protein |
| GDP | guanine diphosphate |
| GEF | guanine nucleotide exchange factor |
| GFP | green fluorescent protein |
| GG | geranylgeranyl group |
| GGPP | geranylgeranyl diphosphate |
| GGTase | geranylgeranyltransferase |
| GGTI | geranylgeranyltransferase inhibitor |
| GGTS | geranylgeranylthiosalicylic acid |
| GST | glutathione-s-transferase |
| GTP | guanine triphosphate |
| GTS | geranylthiosalicylic acid |
| HA | hemagglutinin |
| HECT | Homlogous to E6AP Carboxyl Terminus |
| HGF/SF | hepatocyte growth factor/scatter factor |
| HGR | heregulin |
| HMG-CoA | 3-hydroxy-3-methylglutaryl coenzyme A |
| H-Ras | Harvey-rat sarcoma |

| | |
|---|---|
| HV | hypervariable |
| IBC | inflammatory breast cancer |
| ICMT | isoprenylcysteine carboxyl methyltransferase |
| JNK | c-Jun N-terminal kinase |
| K-Ras | Kirsten-rat sarcoma |
| LARG | Leukemia-associated Rho guanine exchange factor |
| MAPK | mitogen-activated protein kinase |
| MTOC | microtubule organizing center |
| NLS | nuclear localization signal |
| NRG | neregulin |
| N-terminus | amino-terminus |
| N-WASP | neural Wiskott-Aldrich syndrome protein |
| PAK | p21-activated kinase |
| Par | partitioning defective |
| PAT | protein *S*-acyltransferase |
| PDGF | platelet-derived growth factor |
| PH | Pleckstrin homology |
| PI3-K | phosphatidylinositol 3-kinase |
| PKA | protein kinase A |
| PKC | protein kinase C |
| POB1 | partner of RalBP1 |
| POSH | plenty of SH3 |
| PPT | palmitoyl protein thioesterase |

| | |
|---|---|
| P/S | penicillin/streptomycin |
| Rac | Ras related C3 |
| Ral | Ras like |
| RalBP1 | Ral-binding protein 1 |
| Ras | Rat sarcoma |
| Rce1 | CAAX-specific protease |
| Rho | Ras homologous |
| RhoBTB | Rho Broad-Complex, Tramtrack and Bric à brac |
| RhoGDI | Rho GDP dissociation inhibitor |
| RING | Really Interesting New Gene |
| Rnd | round |
| ROCK | Rho kinase |
| RTK | receptor tyrosine kinase |
| SA | soft agar |
| SAAX | serine-aliphatic-aliphatic-unconserved amino acid |
| SAPK | stress-activated protein kinase |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SENP-2 | Sumo/Sentrin/SMT3 specific isopeptidases |
| SH2 | Src homology 2 |
| SH3 | Src homology 3 |
| SMURF | Smad ubiquitylation regulatory factor |
| Spec | small protein effector of Cdc42 |
| SRF | serum response factor |

| | |
|---|---|
| STAT | Signal Transducer and Activator of Transcription |
| SUMO | small ubiquitin related modifier |
| 2-BP | 2-bromopalmitate |
| TC10 | teratocarcinoma 10 |
| TCL | TC10-like |
| TGFβ | Transforming growth factorβ |
| TGN | Trans Golgi network |
| TIAM1 | T-cell invasion and metastasis gene 1 |
| TKB | tyrosine kinase binding |
| TNF | tumor necrosis factor |
| TRITC | Texas Red isothiocyanate |
| Ub | ubiquitin |
| U-box | UFD2 homology |
| UNC-CH | University of North Carolina at Chapel Hill |
| WASP | Wiskott-Aldrich syndrome protein |
| Wnt | Wingless/Int |
| Wrch-1 | Wnt-reglated Cdc42 homolog-1 |

# CHAPTER I

# INTRODUCTION

**The Rho family of small GTPases.** Rho (Ras homologous) family small GTPases represent a major branch of the Ras superfamily of small GTPases with its members sharing 30% homology with many Ras subfamily proteins (Wennerberg and Der, 2004) (Figure 1.1A). Rho proteins have been intensively studied over 20 years for their regulation of numerous signaling pathways and biological consequences. In response to various extracellular stimuli, they receive signals from upstream molecules such as receptors and transmit the information to downstream signaling pathways for different biological activities such as actin cytoskeletal organization, cell survival, vesicular trafficking and transformation (Wennerberg and Der, 2004; Wherlock and Mellor, 2002) (Figure 1.1B). There are 20 Rho family members in humans, with conserved orthologs in many other species including yeast, worms, flies, and plants (Jaffe and Hall, 2005). Based on sequence and functional homology, the Rho subfamily is subdivided into six groups: Rho, Rac (Ras related C3), Cdc42 (cell division cycle 42), Rnd (round), and RhoBTB (Rho Broad-Complex, Tramtrack and Bric à brac) (Figure 1.1C). The Rho and Rac subgroups are best known for their roles in actin cytoskeleton rearrangements and morphological changes, whereas Cdc42-like proteins (also regulators of actin and cellular

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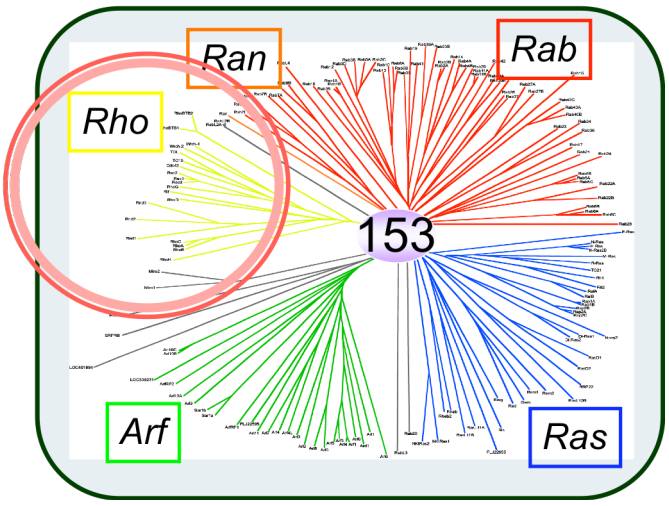


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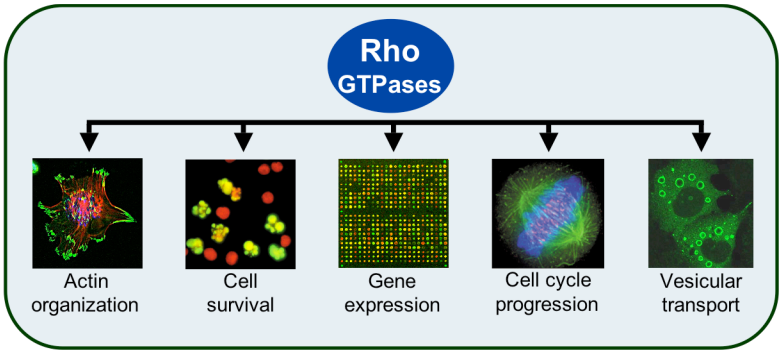


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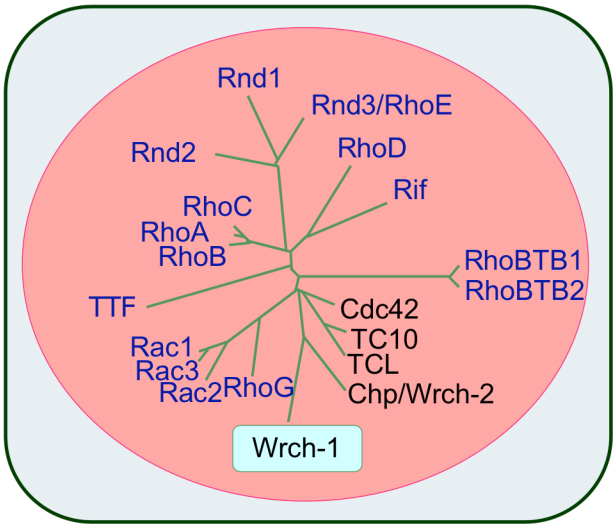


**Figure 1.1 The Rho subfamily and Cdc42 subgroup of small GTPases.** (A) The Rho subfamily (yellow font, pink circle) is one of five branches in the Ras superfamily and shares 30% homology with Ras proteins (Wennerberg and Der, 2004). (B) Rho family GTPases mediate various downstream biological functions. (C) Wrch-1 (blue bo), a new member, belongs to the Cdc42 subgroup (black font).

morphology) control cell polarity. Rnd, RhoBTB and Miro proteins are less studied and are now being investigated for their potentially distinct roles in actin rearrangements and morphology, oncogenesis and mitochondrial functions (Aspenstrom et al., 2004; Fransson et al., 2003; Siripurapu et al., 2005; Wennerberg and Der, 2004). The Cdc42 branch of Rho GTPases will be highlighted here given that Wrch-1, the focus of this dissertation, is one of the latest additions to this subgroup (Figure 1.1C).

**Cdc42-related proteins share sequence similarities and differences.** The Cdc42 group of proteins is comprised of five GTPases, Cdc42, TC10 (teratocarcinoma 10), TCL (TC10-like) and the Wrch proteins, Wrch-1 (Wnt-regulated Cdc42 homolog-1) and Chp (Cdc42 homologous protein)/Wrch-2. These proteins share high sequence identity within their GTP and effector protein binding regions and differ the most in their insert and carboxyl terminal hypervariable regions (Figure 1.2B). The GTP binding domain (Figure 1.2A) consists of two switch regions that undergo conformational changes when bound to GTP or GDP; the GTP-bound conformation is active. The residues in this domain are highly conserved throughout the Rho subfamily. The effector domain region interacts with downstream signaling partners (the effectors) resulting in activation of these effector proteins for downstream signaling cascades and biological consequences (Figure 1.2A). Because many residues in this region are conserved amongst the Cdc42 subgroup, these related proteins associate with many of the same effector molecules. This might suggest that the Cdc42-like proteins are functionally redundant and indistinct. However, sequence differences within regions like the insert (a stretch of

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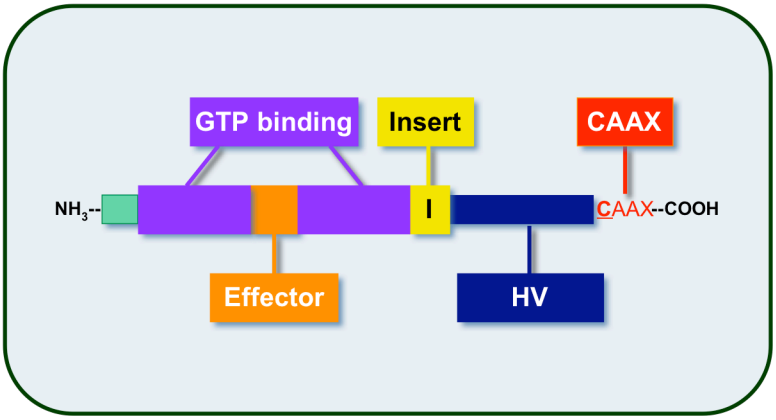


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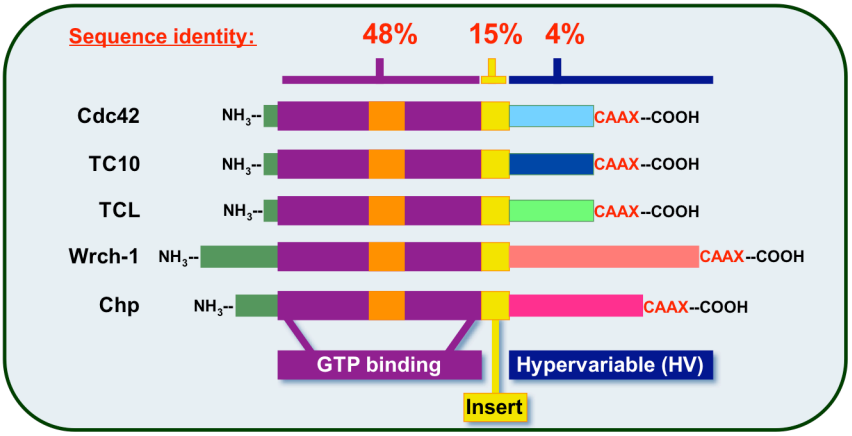


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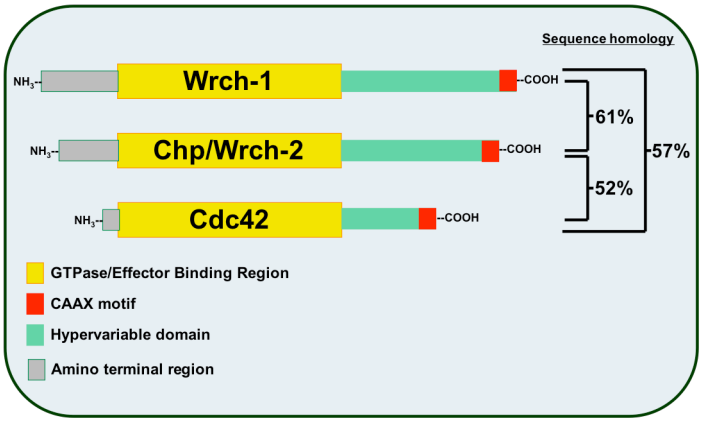



**Figure 1.2 Cdc42-related proteins share sequence similarities.** (A) Like most Rho GTPases, Cdc42 proteins have a effector binding region (orange), flanked by nucleotide binding domains (purple). The carboxyl terminus consists of the hypervariable region and a CAAX motif. (B) Cdc42-related GTPases are most similar within the GTP/effector binding region, whereas significant sequences divergence is found in the hypervariable region (each color depicts varied protein sequence among the related proteins. (C) Overall, Wrch-1 and Chp/Wrch-2 are highly homologous, but not identical to Cdc42.

residues important for effector interactions that is not present in Ras family proteins) and the hypervariable region (important for membrane localization) are divergent enough to dictate functional differences.

**Wrch proteins, novel Cdc42-related family members.** Wrch-1 (Wnt-regulated Cdc42 homolog-1) and Chp/Wrch-2 (Cdc42 homologous protein), recently identified Rho family members, share 57% and 52% sequence identity with Cdc42, respectively, and 61% sequence identity with each other (Aronheim et al., 1998; Tao et al., 2001) (Figure 1.2C). These sequence similarities classify Wrch-1 and Chp as Cdc42 subgroup members. Wrch-1 was initially discovered as a Wnt1-responsive gene that, when activated by structural mutation of the switch region to mimic GTP-bound GTPases, phenocopies Wnt1 morphological transformation of C57MG mammary epithelial cells (Tao et al., 2001). Chp was identified during a screen for proteins that interact with p21-activated kinase (PAK) (Aronheim et al., 1998). Like Cdc42, Wrch-1 and Chp contain nucleotide binding and effector domain regions, and their activity is regulated by nucleotide status, such that GDP-bound Wrch proteins are inactive and GTP-bound Wrch proteins are activated and able to interact with and signal to downstream effector targets. A leucine mutation at residue Q107 of Wrch-1 and Q89 of Chp (analogous to the 61L mutation of Cdc42 and Ras) is predicted to render them constitutively GTP-bound and activated. Both Wrch-1 and Chp messages are expressed ubiquitously, with higher levels of expression in brain, heart, skeletal muscle, liver, placental and lung tissues (Aronheim et al., 1998; Tao et al., 2001), suggesting that Wrch proteins have important signaling roles in a broad range of tissue and cell types. Plus, Wrch homologs have been found in human,

mouse, dog, chicken, *Caenorhabditis elegans* (*C. elegans,* nematodes), *Ciona intestinalis* (*C. intestinalis*, sea slug) and *Schistosoma japonicum* (a parasitic trematode), demonstrating evolutionary conservation of these GTPases. Interestingly, Wrch homologs have not been identified in other model organisms like *Drosophila*. The selectivity of Wrch homologs in other organisms is curious, and suggests that Wrch proteins may be necessary for specialized functions in higher organisms not found in flies. Taken together, this provides evidence for Wrch proteins as critical regulators of diverse biological functions, further supporting our rationale for Wrch GTPases as subjects for further exploration.

**GTP/GDP binding regulates Rho GTPases.** As indicated above, most Rho GTPases function as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound conformation (Figure 1.3A). Exceptions include the Rnd GTPases and RhoH, which are chronically associated with GTP in their wild-type forms (Li et al., 2002; Riento et al., 2005). Guanine nucleotide exchange factors (GEFs) help to promote the dissociation of a GDP molecule from GTPases in exchange for the more abundant GTP molecule. Many Rho-specific GEFs belong to the Dbl (diffuse B-cell lymphoma) family of proteins, characterized as having a Dbl homology (DH) and Pleckstrin homology (PH) domain. GTPase activating proteins (GAPs) function to stimulate the intrinsic GTPase activity of these proteins, promoting hydrolysis of GTP to GDP to deactivate Rho proteins and their signaling pathways.

A third class of negative-regulatory proteins called Rho GDP dissociation inhibitors (RhoGDIs) inhibit GDP nucleotide dissociation, preventing activation of these GTPases by GEFs, and interfere with effector interaction with GTP-bound Rho proteins (DerMardirossian and Bokoch, 2005). As will be discussed in Chapter II, RhoGDIs can also redistribute Rho proteins from cellular membranes, where their signaling partners are available, to the cytosol, where they do not induce downstream signaling cascades (DerMardirossian and Bokoch, 2005) (Figure 1.3B). There are three RhoGDI isoforms identified: ubiquitously expressed RhoGDI$\alpha$/GDI1 (associates with Cdc42, Rac1/2, RhoA and RhoC), hematopoietic-specific RhoGDI$\beta$/GDI2 (binds Cdc42, Rac1/2 and RhoA) and lung-, brain-, testis-specific RhoGDI$\gamma$/GDI3 (RhoG and RhoB) (DerMardirossian and Bokoch, 2005). Because of their inhibitory functions, RhoGDIs are sometimes used as tools to block signaling activity of Rho GTPases (as described in Chapter II).

Specific missense mutations within the switch regions of Rho GTPases can lock them in a GTP-bound conformation, thereby rendering them GTPase-deficient, constitutively activated (Figure 1.3A, lower panel) and, in many instances, oncogenic (Figure 1.3C). By analogy to Ras, laboratory-generated structural mutants of Rho family proteins are often made with oncogenic mutations at G12 and Q61, for example to valine and leucine residues, respectively (Figure 1.3A, lower panel). While mutated and constitutively activated Ras proteins, e.g., at codons 12, 13 and61, have been identified in 30% of human cancers, mutations in Rho proteins have not been found. Instead, aberrant regulation of Rho activators (GEFs, such as

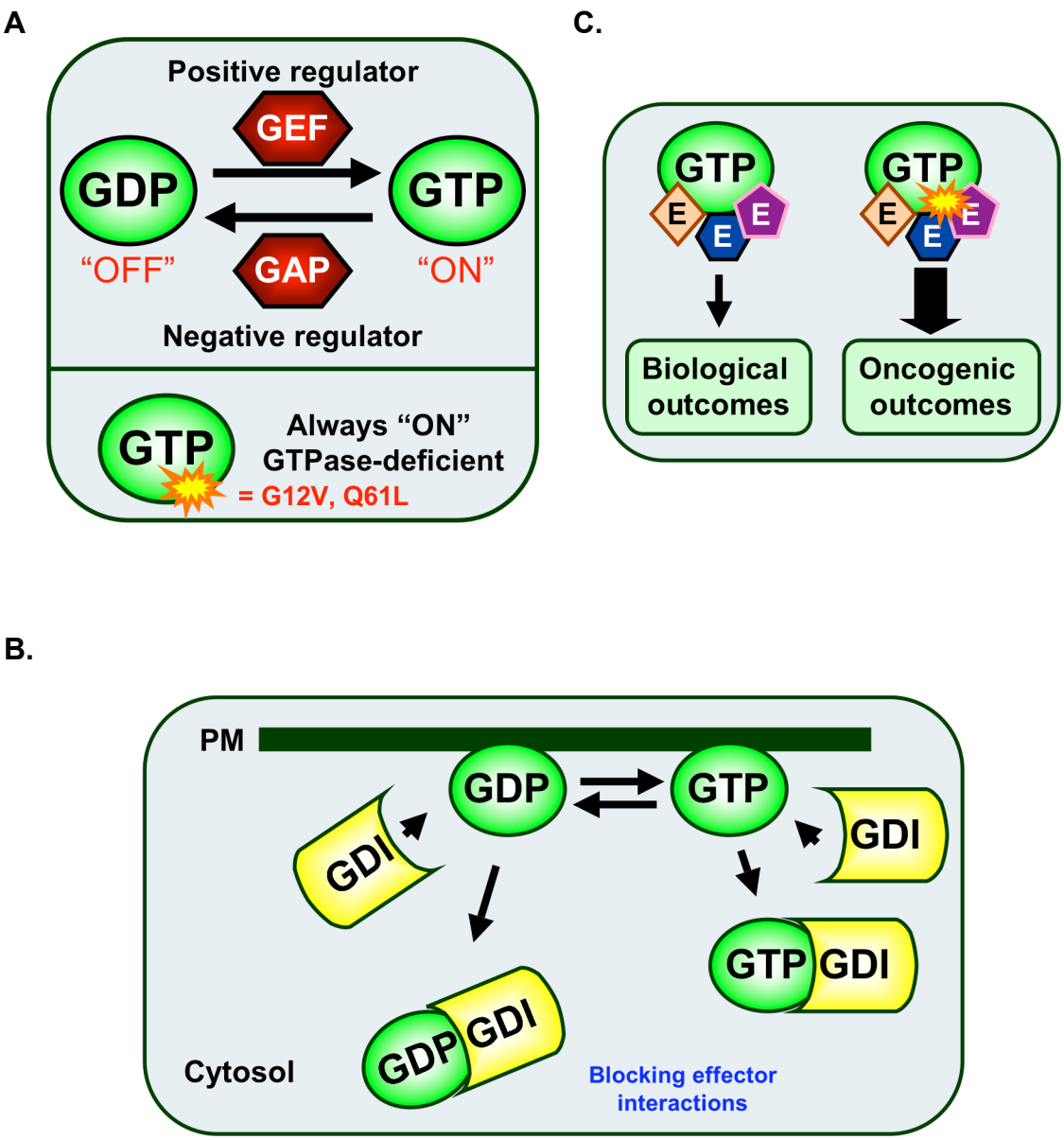



**Figure 1.3 Rho GTPases function as molecular switches.** (A) The activity of Rho proteins is tightly regulated based on their nucleotide status. GDP-bound Rho proteins are inactive, whereas GTP-bound GTPases are active. (B) GDIs negatively regulate Rho family proteins by interfering with their effector interactions or by redistributing Rho proteins from the plasma membrane to the cytosol and internal membranes. (C) Specific mutations (G12V, Q61L) within the nucleotide binding region of these proteins render them locked in a GTP-bound conformation and, therefore, constitutively activated. The orange starburst represents constitutively activated Rho GTPases.

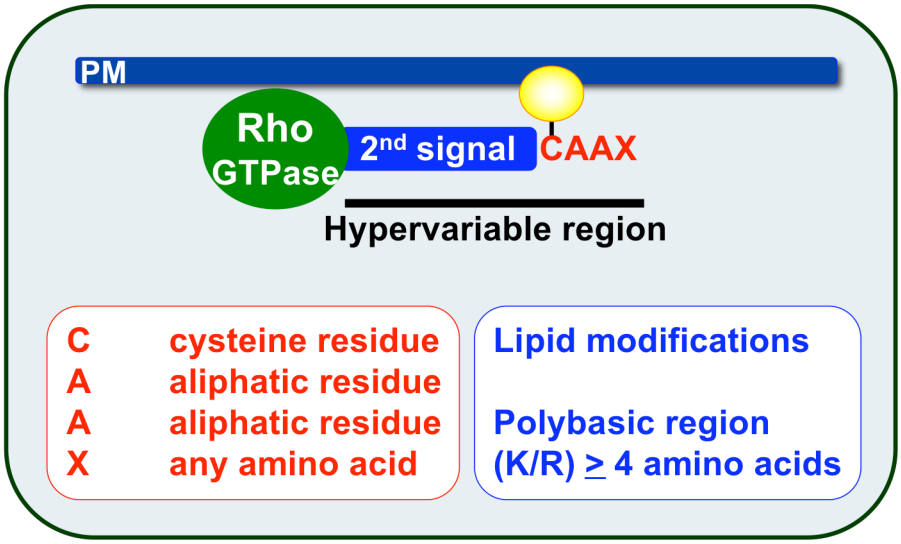
LARG and Vav2) and downregulators (GAPs, such as DLC-1) is responsible for increased Rho activity in tumors (Wennerberg and Der, 2004).

**C-terminal modifications control small GTPase localization.** Subcellular localization of small GTPases to different cellular membranes also regulates their association with downstream effector proteins and their subsequent biological activity. Posttranslational modifications at the carboxyl (C)-terminus of GTPases, which includes the hypervariable region and a conserved tetrapeptide CAAX motif, are critical for proper subcellular targeting (Adamson and Hall, 1992; Michaelson and Philips, 2001). Most Rho family members have a CAAX motif (first identified in Ras proteins) consisting of a cysteine (C) residue, two aliphatic (AA) residues and an unconserved amino acid (X) as the terminal residue. The cysteine of the CAAX motif serves as a substrate for isoprenylation by prenyltransferases enzymes such as farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase I) (Figure 1.4A), in which either a C15 farnesyl or a C20 geranylgeranyl isoprenoid lipid group (Figure 1.4B) is irreversibly attached to the cysteine residue (Casey and Seabra, 1996). If the X residue of the CAAX motif is an L or F residue (as seen in the CAAX motifs of Cdc42, Rac1/2/3 and RhoA/C), then the protein is a substrate for GGTase I; however, CAAX motifs terminating in S, M, Q or A (such as the terminating residues in Rnd proteins) are usually substrates for FTase (Cox, 1995) (Figure 1.4C). Some Rho family CAAX motifs end in unusual residues such as threonine in TC10 (FTase substrate) and an isoleucine in TCL (suspected FTase substrate) (Wennerberg and Der, 2004). Table 1.1 illustrates Rho family CAAX motifs and the terminating X residue. Interestingly, Wrch-1 also appears to have a CAAX motif, but

it terminates in another unusual residue, valine, making it difficult to predict whether Wrch-1 is a substrate for isoprenylation by FTase or by GGTase I. To complicate matters further, Chp, its closest relative, actually lacks a CAAX motif, leaving questions as to why Wrch-1 has a putative CAAX, but Chp does not. These questions will be addressed in Chapter III.

Following prenylation, the –AAX residues are proteolytically cleaved by the CAAX-specific protease Rce1, and then the newly prenylated cysteine residue is methylated by isoprenylcysteine carboxyl methyltransferase (ICMT), resulting in a fully processed and membrane-localized GTPase (Winter-Vann and Casey, 2005) (Figure 1.4A). These post-prenylation enzymatic processes are critical for proper localization of small GTPases, and failure to perform all of these reactions leads to incompletely modified proteins that do not localize to their proper membranes. For instance, in mouse cells null for either RCE1 or ICMT, CAAX-containing proteins were found mislocalized (Bergo et al., 2000; Kim et al., 1999). Interestingly, one study found that post-prenylation processing appears to be important for farnesylated GTPases, but not for geranylgeranylated GTPases, since RCE1- and ICMT-null cells resulted in mislocalization of farnesylated Ras proteins, but not geranylgeranylated Rho proteins (Michaelson et al., 2005). Substitution of farnesylated Ras CAAX motifs for geranylgeranylated CAAX sequences preserved the localization of Ras proteins in these post-prenylation enzyme-negative cells (Michaelson et al., 2005). This would suggest that localization of most Rho family proteins, the majority of which are geranylgeranylated, does not depend on proteolysis and methylation. However, farnesylated Rho proteins like TC10 and the

A.

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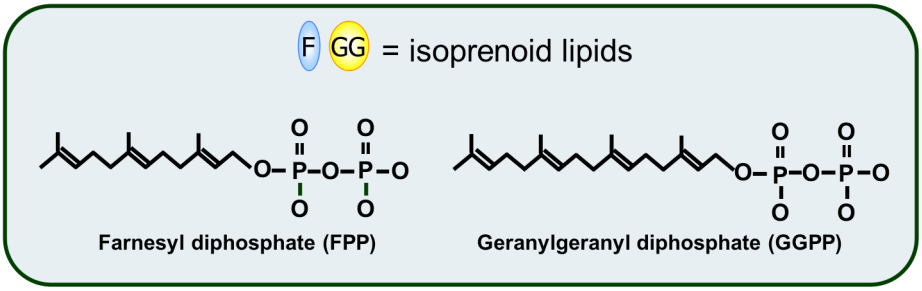


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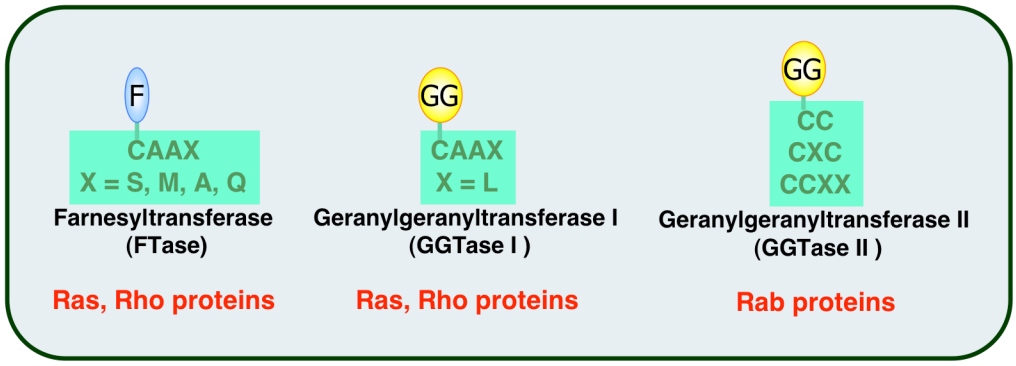



**Figure 1.4 C-terminal modifications regulate small GTPase localization.** (A) The CAAX motif and hypervariable region compose the C-terminus of Rho proteins. Lipid modification of Rho GTPases is required for proper membrane association, interaction with effector proteins and subsequent biological activity. When these proteins are not lipid modified, they are cytosolic and unable to interact with signaling partners. (B) C15 farnesyl or C20 geranylgeranyl isoprenoid moieties are irreversibly attached to a conserved cysteine residue in the CAAX motif for GTPase membrane localization. (C) Most Ras and Rho protein CAAX motifs in which the X residue terminates in S, M, Q or A are substrates for farnesylation by farnesyltransferase (FTase), whereas an L or F residue leads to geranylgeranylation by GGTase I. Rab subfamily members are substrates of GGTase II activity.

| GTPase | CAA | X | F or GG |
|---|---|---|---|
| RhoA | CLV | L | GG |
| RhoB | CKV | L | FGG |
| RhoC | CPI | L | GG |
| Cdc42 | CVL | L | GG |
| TC10 | CLI | T | F |
| TCL | CSI | I | ? |
| Wrch-1 | CCF | V | ? |
| Chp | FCF | V | X |
| Rnd3 | CTV | M | F |
| Rnd1 | CML | M | F |
| Rnd2 | CSI | M | F |

**Table 1.1 The CAAX motif and the specified isoprenoid moiety for lipid modification of Rho proteins.** Most Rho proteins terminate in a motif (X = L) that dictates geranylgeranylation of the cysteine residues of the CAAX motif. Exceptions include the Rnd subgroup, which undergo farnesylation (X = M), and RhoB, which has two populations of lipid modified protein. TC10 is also a substrate for farnesylation, despite the presence of a X = T terminating residues. Wrch-1 terminates in a valine residue that may be a substrate for geranylgeranylation (Moores et al., 1991). Interestingly, Chp does not have a CAAX motif and, instead, terminates in a FCFV sequence, making it an unlikely candidate for isoprenylation. Chapter III will evaluate how these last four amino acids contribute to Wrch-1 localization and, also, why Wrch-1 appears to have an intact CAAX, while Chp lacks the motif.

Rnd proteins likely do require full processing. If Wrch-1 (X = V) is modified by a farnesyl group, then it likely also depends on proteolysis and methylation. This question is addressed in Chapter III.

In addition to FTase and GGTase I, another prenyltransferase called geranylgeranyltransferase II (GGTase II) has been described (Seabra et al., 1992), that modifies Rab proteins terminating in CCXX, CC or CXC motifs (Khosravi-Far et al., 1992) (Figure.1.4C). Although Wrch-1 is not a Rab family GTPase, it does terminate in an apparent CCXX motif. Cysteine to serine mutations of the CAAX motif (e.g., CAAX > SAAX) prevent prenylation by prenyltransferases and result in proteins that are localized improperly and are impaired in function. For example, mutation of the RhoA CLVL > SLVL and RhoB CKVL > SKVL CAAX motifs caused mislocalization of these proteins from the cytosol to the nucleus and from cellular membranes to the cytosol and nucleus, respectively (Adamson et al., 1992b), and conversion of Rac1 CLLL to SLLL renders it insensitive to GGTase I modification and improperly distributed to the cytosol (Joyce and Cox, 2003). SAAX mutants of Wrch-1 are explored in Chapter III.

**Secondary membrane localization signals of Rho GTPases.** As mentioned previously, Rho protein sequences are very similar to each other in their GTP and effector binding regions, but differ greatly within the membrane specificity region called the hypervariable region, with sequences usually of approximately 20 amino acids long. This degree of variance is responsible in part for the distinct localizations and functions displayed by these GTPases. A prominent example is illustrated by

the Rho subgroup of GTPases. RhoA, RhoB and RhoC, which have nearly identical GTP and effector binding regions (and bind similar signaling partners), vary the most within their C-terminal membrane localization regions (Wennerberg and Der, 2004; Wheeler and Ridley, 2004). The C-terminal sequences, then, direct these GTPases to different cellular compartments, eliciting distinct effector interactions and downstream signaling outcomes (Wheeler and Ridley, 2004). For example, RhoA localizes to the plasma membrane and cytosol to mediate its effects on the actin cytoskeleton, whereas RhoB is distributed predominantly at endomembranes (Wheeler and Ridley, 2004) and regulates internalization of receptor tyrosine kinases (Gampel et al., 1999). RhoC also localizes to the cytosol and plasma membrane, but appears to direct migration and invasion phenotypes (Wheeler and Ridley, 2004).

While CAAX-signaled modifications are necessary for membrane localization, they are not sufficient for plasma membrane localization. Additional signals, sometimes called secondary signals, found upstream of the CAAX motif in the C-terminal hypervariable region, are required for proper targeting to the plasma membrane, where many small GTPases are thought to be most active (Figure 1.5A). Second signals can either be stretches of basic lysine or arginine residues that increase membrane affinity of GTPases by altering their electrostatic interactions, or cysteine residues that are substrates for acylation by C16 palmitoyl fatty acids (Hancock et al., 1989; Hancock et al., 1990) whose hydrophobicity also increases membrane affinity. Cdc42 and Rac1 have polybasic regions upstream of their prenylated CAAX motifs, whereas TC10 and RhoB, which lack polybasic regions, are palmitoylated on

upstream cysteine residues (Figure 1.5B). Inspection of the Wrch-1 hypervariable domain suggests that Wrch-1 contains a polybasic region and may also be palmitoylated on one or more upstream cysteines (Figure 1.5C). This suggests that Wrch-1 localization to plasma membranes may be regulated by palmitoylation.

Palmitoyl lipid moieties (Figure 1.5D) offer an additional level of regulation to modified proteins besides increased membrane affinity. In contrast to isoprenylation, palmitoylation is a dynamic, reversible process. The palmitoylation/depalmitoylation cycle utilizes specific protein *S*-acyltransferases (PATs) for transfer of palmitoyl fatty acids onto cysteines, and palmitoyl protein thioesterases (PPTs) for removal of the palmitates (Resh, 1999). Although there is strong evidence that Rho family GTPases are palmitoylated (Adamson et al., 1992a; Michaelson et al., 2001; Watson et al., 2003; Watson et al., 2001), the identities of small GTPase-specific human PATs have been elusive, and have been explored only for Ras. Thioesterases such as PPT1 and acyl protein thioesterase (APT1) (Duncan and Gilman, 1998) have been shown to deacylate Ras proteins in vitro. There are yeast PATs containing DHHC (aspartate-histidine-histidine-cysteine) motifs necessary for enzymatic activity that modify the yeast Ras homolog(Bartels et al., 1999; Putilina et al., 1999). Mutations within the DHHC domain of yeast PATs abolish their activity, suggesting that human DHHC-containing proteins may also palmitoylate human Ras and perhaps also Rho proteins (Bartels et al., 1999). Cycles of acylation and deacylation have been previously described biochemically for H- (Baker et al., 2003) and N-Ras (Magee et al., 1987) proteins, with palmitoylation half lives of approximately twenty minutes. A recent study visually

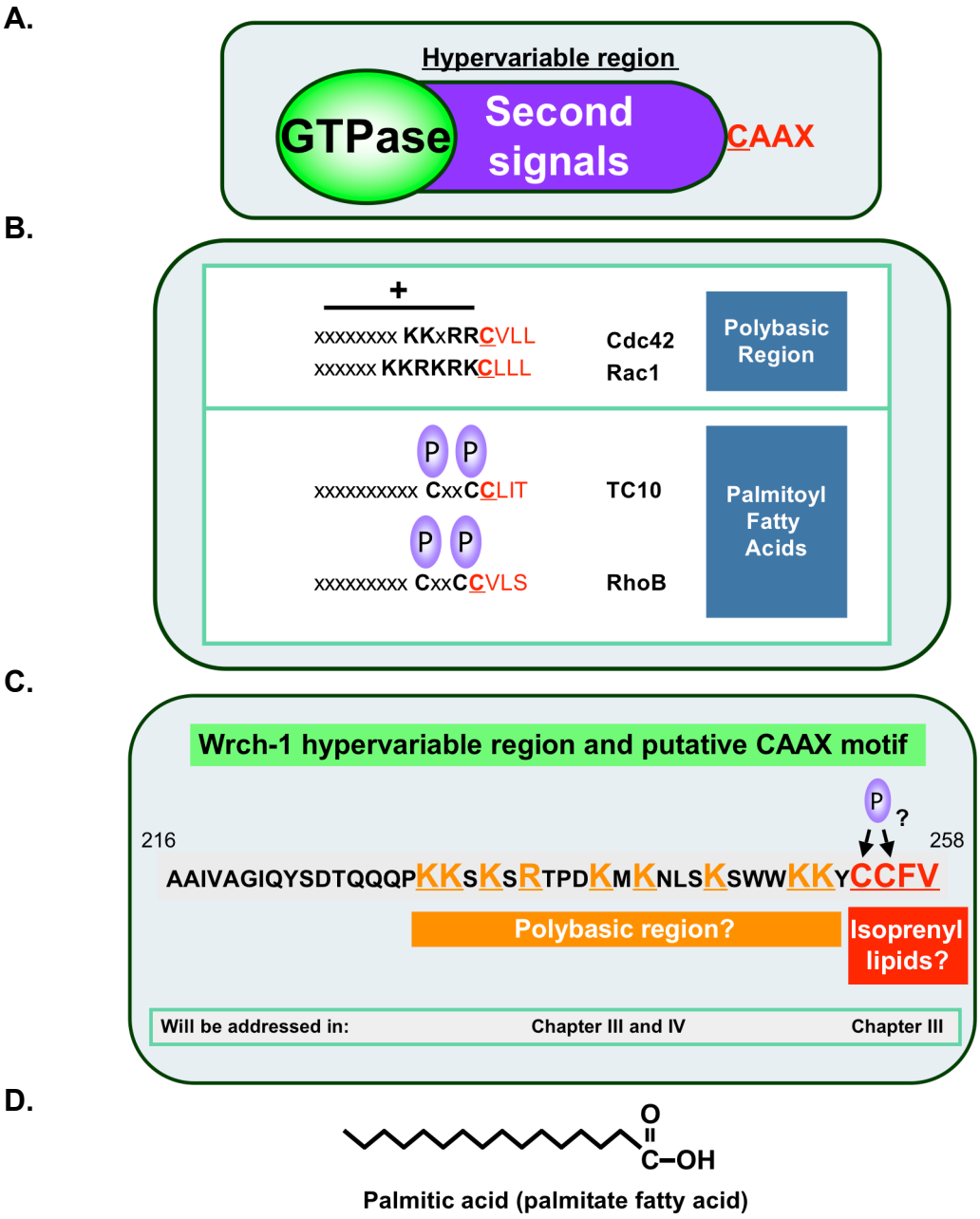



**Figure 1.5 The hypervariable region contains secondary membrane signals.** (A) Additional signals (also referred to as second signals) upstream of the CAAX motif (red) are required for cellular localization of GTPases. (B) In conjunction with isoprenylation, a stretch of basic residues such as lysines or arginines increase the membrane affinity of Cdc42 and Rac1, whereas reversible palmitoyl fatty acid modification of upstream cysteine residues occurs for TC-10 and RhoB. (C) Wrch-1 has a putative CAAX motif (CCFV) for isoprenylation and several upstream basic residues that may function as its membrane targeting signals. It is also possible that one or more cysteines in the Wrch-1 carboxyl terminus may be palmitoylated. (D) A schematic of palmitoyl fatty acid structure.

demonstrated regulation of H-Ras localization and activity through this acylation/deacylation cycle (Rocks et al., 2005).

Often, palmitoylated substrates are found associated with cholesterol-rich invaginations of the plasma membrane termed lipid rafts or caveolae (if caveolin protein is present) (Resh, 2004). Lipid rafts are specialized microdomains that feature interactions with proteins involved in activities such as endocytosis and other trafficking-related functions (Laude and Prior, 2004). Palmitoylated TC10 (Watson et al., 2001), but not unpalmitoylated Cdc42 (Gingras et al., 1998), has been found associated with these microdomains, suggesting a mechanism for their functional diversity. Signaling complexes accumulate in these microdomains, creating opportunities for protein-protein interactions and signaling consequences not available outside of these areas (Helms and Zurzolo, 2004). The association of TC10 with lipid rafts depends on the presence of palmitoylated cysteine residues, since either mutation of these cysteines to serines (Watson et al., 2003) or substitution of the TC10 hypervariable domain by the non-palmitoylatable K-Ras hypervariable domain blocks TC10 localization to lipid rafts (Watson et al., 2001).

Mutational analyses of these upstream sequences (basic residues and palmitoylated cysteine residues) have shown that loss of either second signal restricts these proteins incorrectly to endomembranes or to the cytosol, despite an intact CAAX motif. These observations suggest the possibility that Wrch-1 localization to endomembranes (Tao et al., 2001) may be dictated by dynamic regulation of palmitoylation. For example, mutation of the upstream cysteine residues of TC10 or

RhoB inhibit their palmitoylation and localization to cellular membranes (Adamson et al., 1992a; Michaelson et al., 2001; van Hennik et al., 2003; Watson et al., 2003). Additionally, mutation of the hypervariable basic residues of Rac1 to alanine residues prevents association of the Rac1 C-terminus with membrane lipids within lipid rafts (Adamson et al., 1992b; Michaelson et al., 2001; van Hennik et al., 2003; Watson et al., 2003). Thus, the CAAX motif and second signals together coordinate the localization of Rho proteins to various, distinct subcellular locations dictating diverse downstream signaling consequences. In chapters II and III, Wrch-1 C-terminal sequences are investigated to (1) determine the targeting signals used by Wrch-1 for its subcellular localization and (2) how these targeting signals contribute to Wrch-1 biological function.

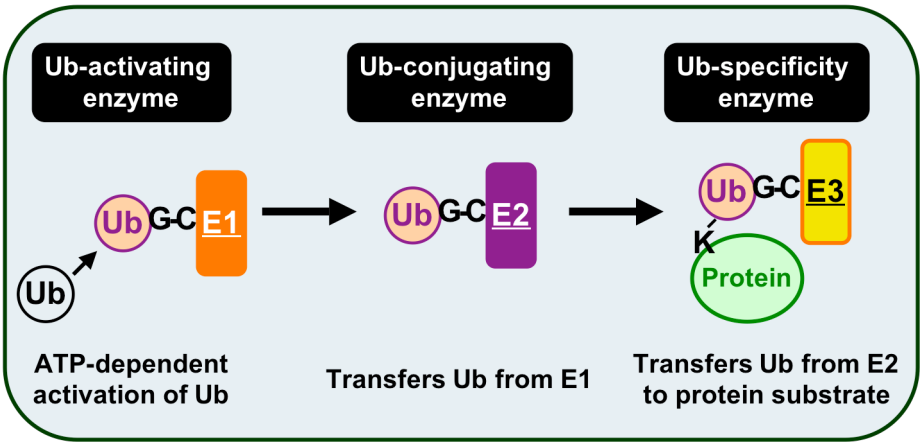
**Other localization regulatory mechanisms.** Other posttranslational modifications such as phosphorylation and ubiquitylation can also influence small GTPase localization and biology. For example, phosphorylation of serine residues within RhoA sequences can alter Rho localization. In lymphocytes, protein kinase A (PKA) activation by cAMP (a second messenger signaling molecule) leads to phosphorylation of RhoA at serine 188 of its C-terminal hypervariable region (Lang et al., 1996). Phosphorylation increased RhoA interaction with RhoGDIs, resulting in translocation of RhoA from the plasma membrane to the cytosol. Rnd3 localization is also modulated by phosphorylation events. In a recent study, platelet-derived growth factor (PDGF) stimulation of Rnd3-expressing cells caused protein kinase C(PKC)-dependent phosphorylation of Rnd3 amino-terminal serine residues S7 and S11 by Rho kinase (ROCK), translocating Rnd3 from an internal membrane and

cytosolic distribution to a mostly cytosolic localization pattern (Riento et al., 2005). Given that Rnd3 is naturally constitutively GTP-bound and active (Foster et al., 1996), its biological activities such as antagonism of RhoA-induced stress fiber formation (Riento et al., 2003) and Ras-mediated transformation (Villalonga et al., 2004) are directly affected by its localization. Properly membrane-localized, unphosphorylated Rnd3 still functions as a negative regulator of stress fibers and transformation, whereas mislocalized, unphosphorylated Rnd3 fails to perform Rnd3 functions maintaining RhoA-induced stress fibers and Ras-transformed cell phenotypes (Riento et al., 2003; Riento et al., 2005; Villalonga et al., 2004). Phosphorylation-dependent localization changes are also observed in the Ras subfamily. K-Ras phosphorylation by PKC (at an analogous C-terminal residue to the phosphorylated serine residue in RhoA) causes rapid translocation of K-Ras from plasma membranes to internal membranes including mitochondrial regions and induces apoptosis of K-Ras transformed cells (Bivona et al., 2006). The hypervariable region of Wrch-1 has several tyrosine, serine and threonine residues that may be phosphorylated (Figure 1.6C). Chapter IV investigates whether Wrch-1 is a substrate for phosphorylation.

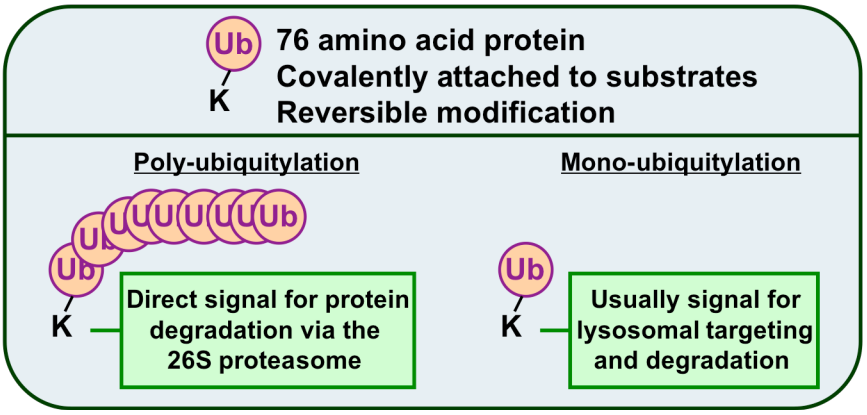
Ubiquitylation, involving the transfer of ubiquitin molecules to substrate proteins, can influence GTPase localization and activity. This process requires three sequential enzymatic reactions. The E1 activating-enzyme activates ubiquitin molecules in an ATP-dependent manner, E2 conjugating-enzymes transfer activated ubiquitin from E1 to the E3 ligases and E3 ligases deliver ubiquitin molecules to the appropriate acceptor protein (Hershko and Ciechanover, 1998; Pickart and Eddins, 2004)

(Figure 1.6A). E3 ligases, which confer specificity to ubiquitylation process through their interactions with specific substrate proteins are divided into three main categories based on sequence homology: the Homologous to E6AP Carboxyl Terminus (HECT), the Really Interesting New Gene (RING) finger and UFD2 homology (U-box) E3 ligases (Pickart and Eddins, 2004). Rho family small GTPases are modified by HECT and RING finger enzymes (see below). The small protein, ubiquitin, can attach to lysine residues either as a monomer (monoubiquitylation) or as a polymer (polyubiquitylation), chains of ubiquitin molecules linked to each other by internal lysine residues (Figure 1.6B) (Pickart and Eddins, 2004). Usually polyubiquitylation, in which chains of ubiquitin molecules associate through their K48 residue, leads to degradation of modified proteins by the26S proteasome (Figure 1.6B), a cylindrical complex of proteases dedicated to the destruction of targeted proteins (Wolf and Hilt, 2004), thus regulating protein activity through protein stability. K63-linked polyubiquitin chains can function as signals for internalization to endocytic and lysosomal membranes (Burger and Seth, 2004). Monoubiquitylation, however, sends proteins to endomembranes for trafficking functions and to lysosomes for lysosomal-mediated protein degradation (Figure 1.6B) (Pickart and Eddins, 2004). De-ubiquitylating enzymes (DUBs), also known as isopeptidases, remove ubiquitin molecules from substrate proteins making ubiquitylation a reversible process (Amerik and Hochstrasser, 2004; Soboleva and Baker, 2004). DUBs maintain a steady level of free ubiquitin molecules in the cell and are important negative regulators of protein degradation (Amerik and Hochstrasser, 2004).

A.




B.




C.

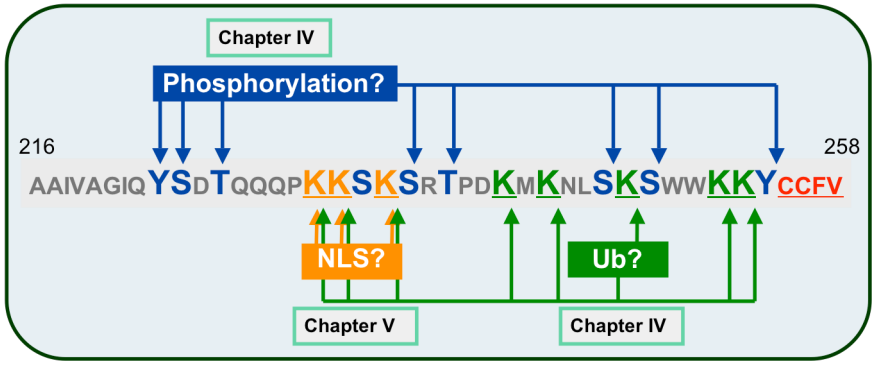



**Figure 1.6 Ubiquitylation can regulate GTPases localization and protein stability.** (A) A cascade of enzymes mediates the transfer of ubiquitin to substrate protein. E1 (activating enzyme) activates ubiquitin molecules, E2 (conjugating enzyme) transfer ubiquitin from E1 to E3 and E3 ligase (specificity enzyme) transfers ubiquitin to the appropriate substrate proteins. (B) Ubiquitin molecules attach to lysine residues for monoubiquitylation or polyubiquitylation of modified proteins. Polyubiquitylation is usually a signal for proteasomal degradation by the 26S proteasome, and monoubiquitylation functions as an internalization signal to endocytic and lysosomal membranes. (C) Wrch-1 contains possible phosphorylation (white and, ubiquitylation sites (green) and a putative NLS (orange) that may contribute to its subcellular localization.

The best characterized examples of ubiquityl-mediated regulation of protein localization come from studies of non-GTPase protein associations with E3 ligases. MDM2, a RING finger E3 ligase, regulates p53 transcription factor and tumor suppressor protein activity in part through polyubiquitylation of p53 leading to its degradation and through monoubiquitylation of p53 causing translocation of p53 from the nucleus to the cytosol (Geyer et al., 2000). Another RING finger E3 ligase, c-Cbl, regulates receptor tyrosine kinases (RTKs) like epidermal growth factor receptor (EGFR). Cbl-directed monoubiquitylation of EGFR internalizes and sorts the receptor to endocytic degradation or recycling pathways (Dikic, 2003; Levkowitz et al., 1998). Rho family members such as Cdc42 and Rac1 have contributing roles in EGFR signaling.

Thus far, no studies of Ras family ubiquitylation are published; however, ubiquitylation of Rho family small GTPases has been reported. For example, RhoA, Cdc42 and Rac1 are polyubiquitylated and degraded in response to cytotoxic necrotizing factor (CNF) bacterial toxin-mediated activation of these GTPases (Doye et al., 2006; Doye et al., 2002; Pop et al., 2004). CNF, secreted by *Escherichia coli*, deamidates the Q63 residue of RhoA and the Q61 residue of Cdc42 and Rac1 to render them constitutively GTP-bound and activated (Flatau et al., 1997; Lerm et al., 1999; Schmidt et al., 1997). Permanent activation and subsequent degradation of these Rho proteins, in response to CNF treatment, appear to function as a pro-invasive function for bacteria. In addition to CNF-mediated destruction of RhoA, polyubiquitylation by the HECT domain-containing Smad ubiquitylation regulatory factor (Smurf) family of E3 ligases negatively regulates RhoA protein stability at the

leading edge of cells. Smurfs are known for their ubiquitylation of Smad transcription factors downstream of transforming growth factor (TGF)β signaling (Zhu et al., 1999). In polarized cells, expression of Smurf1 caused increased polyubiquitylation and proteasome-mediated degradation of RhoA, but not of Cdc42 or Rac1 (Wang et al., 2003a). E3 ligase-deficient Smurf1 had no effect on RhoA protein levels and did not induce RhoA incorporation of ubiquitin molecules (Wang et al., 2003a). Lastly, the cullin3 RING finger E3 ligase complex interacts with the BTB domains in RhoBTB2 to induce polyubiquitylation and degradation of this GTPase (Wilkins et al., 2004).

There are no known consensus sequences predicting whether a particular lysine residue will be the site of ubiquitylation. Wrch-1 has eight lysines in its hypervariable region (Figure 1.6C), with several more lysine residues throughout its other protein domains. Therefore, it is not possible to guess whether Wrch-1 utilizes ubiquitin molecules for its localization based on sequence alone. Examination of Wrch-1 as a substrate for ubiquitylation is explored in Chapter IV.

Nuclear localization signals (NLSs) direct proteins to the nucleus for their degradation and other nuclear functions such as transcriptional regulation. Past reports on small GTPase contributions to nuclear functions were either indirect through MAPK signaling cascades (causing translocation of transcription factors like c-Jun and serum response factor to the nucleus) or restricted to direct effects by the Ran subfamily of small GTPases. The Ran proteins (another Ras-related GTPase subfamily) are nuclear localized when GTP-bound and cytosolic when GDP-bound

based on spatial localization of Ran-specific GEFs and GAPs (Dasso, 2002; Quimby and Dasso, 2003). Although they do not appear to have their own NLSs, Ran GTPases interact with NLS-binding proteins such as importins to regulate nuclear transport of these NLS-containing proteins, mitotic spindle formation and nuclear envelope assembly (Quimby and Dasso, 2003). The canonical NLS consensus sequence, K(K/R)X(K/R), is present in the carboxyl termini of several Rho GTPases Table 1.2. Table 1.2 illustrates putative NLS sequences identified in RhoC, RhoG, Rac1, Cdc42 (placental isoform), TCL and Rnd1 (Williams, 2003) that may control previously unknown nucleocytoplasmic shuttling and nuclear activities of these GTPases. Recently, Carol Williams' group demonstrated that Rac1 has a functional NLS that regulates its nuclear localization and degradation (Lanning et al., 2004). Fusion of Rac1 carboxyl terminal sequences containing its NLS to RhoA, which lacks a NLS and usually remains excluded from the nucleus, can lead to its localization to the nucleus (Lanning et al., 2004). Wrch-1 contains a putative non-canonical NLS within its carboxyl terminal sequences (KKSK—residues 233-236) that could contribute to Wrch-1 nuclear localization in conjunction with its other potential cellular targeting signals, adding additional layers of complexity to Rho GTPase localization signals at the carboxyl terminus (Figure 1.6C).

**Rho family members modulate the actin cytoskeleton.** RhoA, Cdc42 and Rac1, in particular, are best known for their differential regulation of the actin cytoskeleton (Jaffe and Hall, 2005). As shown in Swiss 3T3 fibroblasts, RhoA activity induces the formation of filamentous actin stress fibers and focal adhesion complexes (Figure

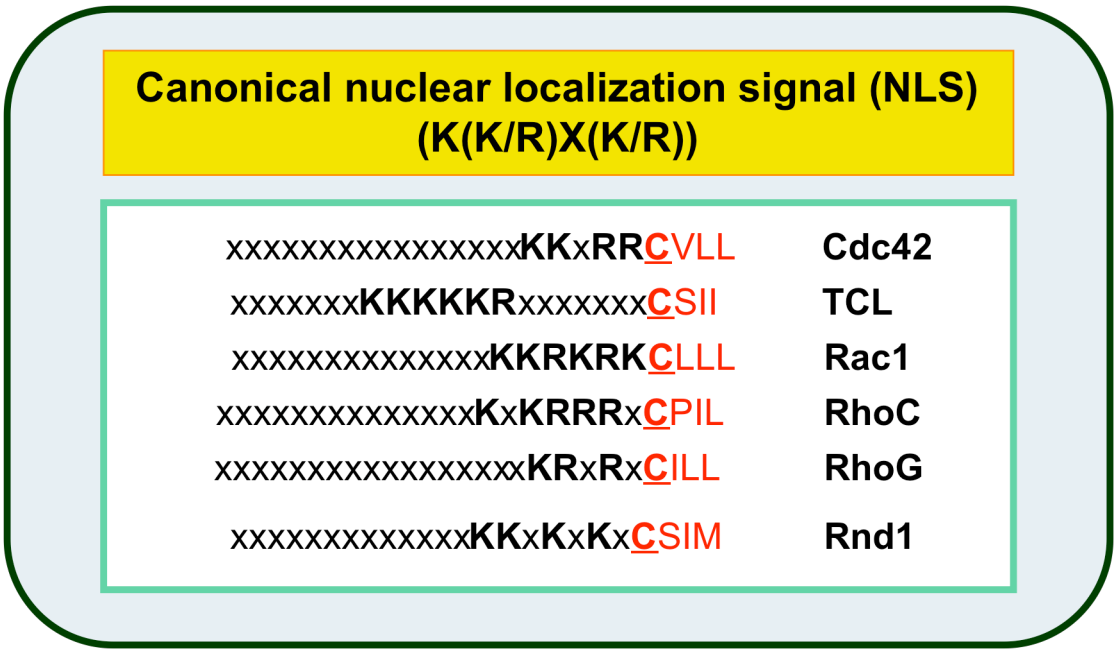



**Table 1.2 Nuclear localization signals are present in the hypervariable region of Rho GTPases.** The canonical nuclear localization signal (NLS), (K(K/R)X(K/R)) (bold), is located upstream of the CAAX motif of Rho GTPases. Most Rho proteins with a putative NLS also have polybasic regions with lysine residues that could be substrates for ubiquitylation.

1.7A), lending rigidity and structure to cells. Cdc42 and Rac1 activity antagonize RhoA-dependent stress fiber formation and, instead, cause a decrease in actin stress fibers (Jaffe and Hall, 2005; Wennerberg and Der, 2004). Additionally, Cdc42 promotes the formation of finger-like membrane protrusions called filopodia, and Rac1 stimulates lamellipodia and membrane ruffles (Figure 1.7A), extensions of the cell periphery for enhanced motility (Jaffe and Hall, 2005; Wennerberg and Der, 2004).

As mentioned previously, Wrch-1 is a Cdc42 subgroup member and, therefore, shares high sequence identity with this branch of Rho proteins. Because of this sequence similarity, Wrch-1 also has biological effects in common with Cdc42 such as dissolution of stress fibers (Tao et al., 2001). However, Wrch-1 displays differences in its modulation of these Cdc42-like events (Aspenstrom et al., 2004) that may be derived from its sequence dissimilarities. Since the specific signaling pathways utilized by Wrch-1 are largely unknown, examination of the mechanisms by which Cdc42, and its related proteins, dictate biological consequences are of special interest, and may be applicable to Wrch-1 mechanisms of function. Thus, further discussion of Rho GTPase functions will be restricted to those activities mediated by the Cdc42 and its relatives, TC10 and TCL.

**Cdc42 effector interactions and biological functions promote cellular transformation.** In addition to its effects on the actin cytoskeleton and cell morphology, Cdc42 regulates cell polarity, gene expression and cell cycle progression (Figure 1.7B). Most importantly, these biological consequences all

contribute to Cdc42 cellular transformation. It is unclear, at this point, how Wrch-1 contributes to its own transforming activities. An in-depth study of Cdc42 effector interaction, signaling pathways and biology may provide clues as to the mechanism of Wrch-1 transformation and biology. Thus, the following will be a discussion of Cdc42 and Cdc42-related protein functions in the context of transformation.

Cellular transformation is often characterized by assays measuring reduced contact inhibition, lower growth dependency on serum, anchorage-independent growth and loss of polarity and differentiation of cells. Normal cells signal for and undergo cell cycle exit when they reach confluency. Oncogenic cells bypass this signal and continue proliferation. Focus forming assays measure reductions in contact inhibition as mediated by transforming proteins, such as activated Ras proteins. Aberrant signaling from Cdc42 or TC10 is not sufficient to overcome contact inhibition; they require cooperation with other activated signaling molecules such as Raf or RhoA or Rac1 to transform cells (Khosravi-Far et al., 1995; Murphy et al., 1999; Qiu et al., 1995a; Qiu et al., 1995b; Roux et al., 1997; Whitehead et al., 1998). However, active Cdc42 is sufficient to induce anchorage-independent growth, as assessed by formation of colonies in soft agar, a phenotype that untransformed cells do not exhibit (Qiu et al., 1997). Interestingly, mutations in Cdc42 that cause elevated GDP/GTP exchange rates, in the absence of GEF activity, are more transforming than wild-type Cdc42 suggesting that cycling is important for Cdc42 function (Lin et al., 1997).

Effector proteins are signaling molecules that directly interact with the GTP-bound form of Cdc42, becoming activated for transduction of information by downstream signaling cascades to mediate Cdc42-induced biology. The specificity of these effector interactions with Cdc42 is dictated by sequences within the effector domain region of Cdc42 and within the effector protein itself. Most Cdc42 effector proteins contain the Cdc42/Rac interactive binding (CRIB) domain that is required for interaction with residues 26 - 48 (the effector domain region) of Cdc42 (Cotteret and Chernoff, 2002). Exceptions include phosphatidylinositol 3-kinase (PI3-K), CIP4 and IQGAP, which interact with the effector region of Cdc42 through other domains (Bishop and Hall, 2000; Cotteret and Chernoff, 2002). Since CRIB domains have been found in many Cdc42 effectors (Figure 1.7C), the presence of this domain in uncharacterized effector proteins can be used as an indicator for potential Cdc42 downstream effector partners (Burbelo et al., 1995). A major CRIB domain-containing effector of Cdc42 is the serine/threonine kinase, p21-activated kinase (PAK). Cdc42 binds to the CRIB domain of PAK in a GTP-dependent manner stimulating autophosphorylation of PAK (Manser et al., 1994; Martin et al., 1995). Once phosphorylated, PAK exhibits decreased affinity for Cdc42 and dissociates for interactions with additional signaling molecules (Manser et al., 1994). PAK activity downstream of Cdc42 has been implicated in many Cdc42 biological consequences including transformation, morphogenesis and cell cycle progression. For example, a mutation in the CRIB domain of PAK that prevents binding of Cdc42 inhibits Ras-induced transformation. Cdc42 is required for Ras transformation (Tang et al., 1997). Presumably the inhibitory effects of this PAK mutant are due to loss of Cdc42/PAK signaling pathways.

As mentioned earlier, activated Cdc42 reduces stress fibers and focal adhesions and induces the formation of filopodia, phenotypes characteristics of most Cdc42-related proteins (e. g. TC10, Wrch-1, Chp). Loss of cellular rigidity due to decreased stress fiber formation often correlates with reduced stress fibers and focal adhesions. For example, when activated RhoA, a promoter of stress fiber formation, is expressed in Ras-transformed cells, a promoter of stress fiber reduction, RhoA can revert Ras disruption of stress fibers (Izawa et al., 1998). Since Cdc42 functions as a negative regulator of stress fiber formation, this suggests that Cdc42-mediated transformation relies on its regulation of the cytoskeleton. Several Cdc42 effectors, including PAK, have been implicated as mediators of Cdc42 effects on the actin cytoskeleton and membrane protrusions. For example, activated Cdc42 recruits PAK1 to focal adhesion complexes and a fusion of the Cdc42 hypervariable region to PAK1 leads to the dissolution of stress fibers and focal adhesions (Manser et al., 1997). Additionally, constitutively activating structural mutations of PAK induce rapid formation of filopodia and membrane ruffles (PAK is also a Rac1 effector) upon microinjection into fibroblasts (Sells et al., 1997). Furthermore, in cells expressing both PAK4 and activated Cdc42, stress fiber reduction and filopodial formation were observed (Abo et al., 1998). These Cdc42 stimulated events are dependent on PAK4 activity since expression of a kinase dead PAK4 mutant blocks Cdc42-induced actin and morphological changes (Abo et al., 1998). There are conflicting reports concerning the contribution of PAKs to Cdc42 activities leaving it unclear whether Cdc42 activation of PAK is necessary for these functions. For example, missense mutations within the effector binding region of Cdc42, that prevent its

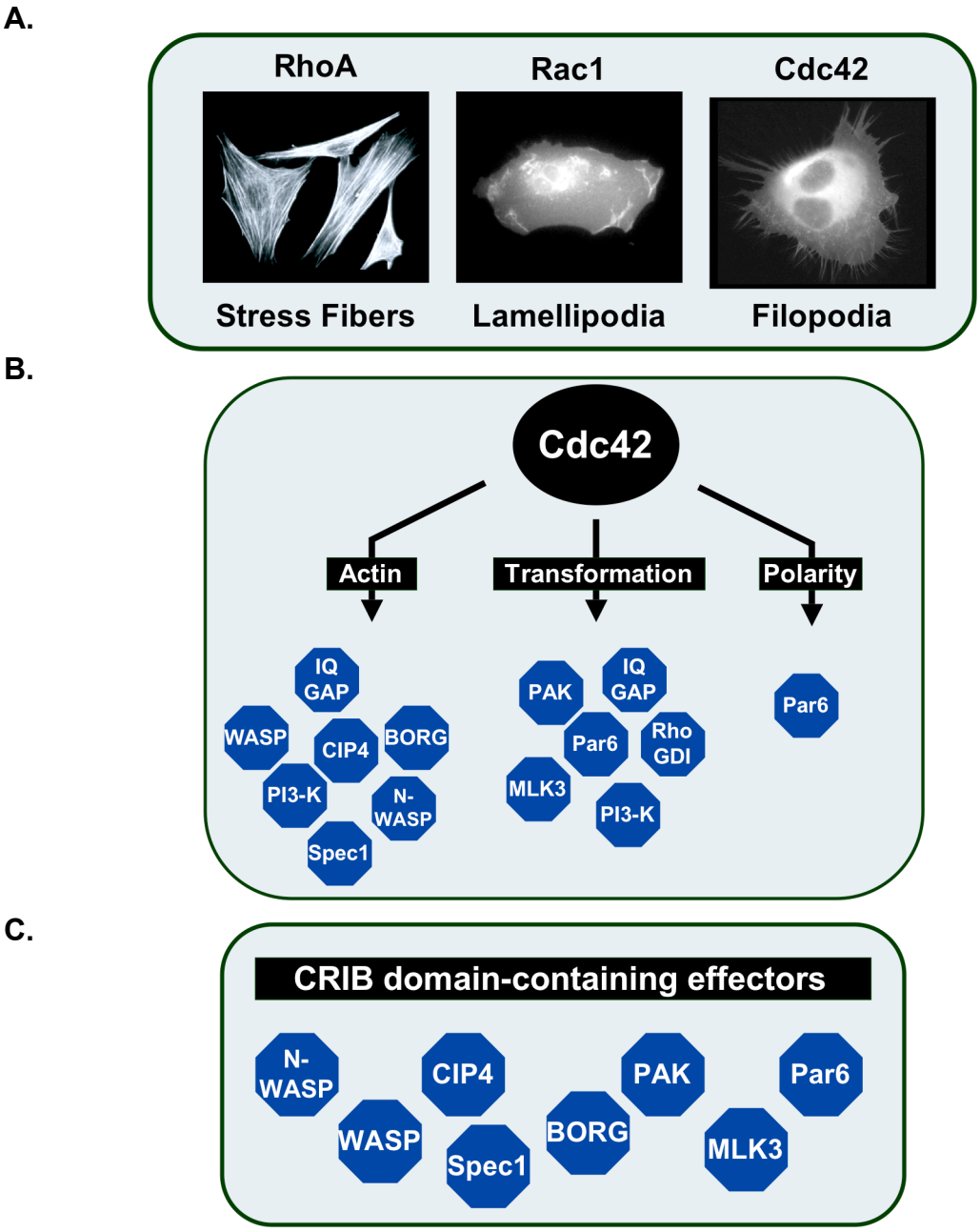



**Figure 1.7 Biological functions mediated by Cdc42 and its effectors.** (A) Rho family proteins are classically known for their effects on actin cytoskeleton organization. RhoA induces stress fibers, while Rac1 and Cdc42 induce lamellipodia (and membrane ruffles) and filopodia (tiny, finger-like protrusions of the plasma membrane). (B) Cdc42 promotes many biological functions, including actin cytoskeleton changes, transformation and polarity regulation. Cdc42 requires interactions with many effectors to perform its many functions in the cell. Most, but not all, Cdc42 effectors have a CRIB (Cdc42/Rac interactive binding) domain that interacts with the effector binding region of Cdc42 (residues 26-48).

association with PAK and, should, therefore, prevent any PAK-mediated effects on Cdc42 biology, fail to block Cdc42 filopodia induction and stress fiber reduction (Lamarche et al., 1996). Also, other studies indicate that expression of constitutively activated PAK does not induce filopodia (Manser et al., 1997), suggesting that effects on the actin cytoskeleton by Cdc42 are mediated through additional effector molecules.

PAK is also predicted to be the mediator of c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) activation induced by upstream Cdc42 signaling (Bagrodia et al., 1995), since a constitutively activating structural mutant of PAK stimulates JNK activation (Brown et al., 1996). JNK is a stress-responsive, serine/threonine kinase that phosphorylates transcription factors like c-Jun, translocating c-Jun to the nucleus for transcriptional activation of pro-proliferative and cell cycle progression genes (Johnson, 1999). It is possible that Wrch-1 activation of PAK leads to activation of JNK, but that Wrch-1 biological effects are independent of PAK activity.

The CRIB domains of Wiskott-Aldrich syndrome protein (WASP) family members bind to Cdc42 and help mediate the formation of membrane protrusions such as filopodia (Jaffe and Hall, 2005) (Figure 1.7B, lower panel). Additionally, Cdc42 interactions with the CRIB domain effectors, p21-activated kinase (PAK), Cdc42-interacting protein (CIP4) and small protein effector of Cdc42 (Spec) also contribute to actin polymerization or organization (Cotteret and Chernoff, 2002). The Wrch proteins, Wrch-1 and Chp, also cause reductions in stress fiber formation and Wrch-

1 induces a milder version of Cdc42-like filopodia (Aronheim et al., 1998; Tao et al., 2001) indicating that Wrch-1 and Chp may interact with similar Cdc42 effectors to mediate their actin organizational functions and membrane protrusions. Interestingly, a yeast 2-hybrid analysis showed that both Wrch proteins associated with PAK, but the Wrch proteins did not bind to WASP (haematopoietic-specific WASP), CIP4 or Spec1 (Aspenstrom et al., 2004) (Figure 1.8). However, Chp associated with neural (N)-WASP, a ubiquitously expressed WASP protein with higher levels of expression in neuronal tissues (Aspenstrom et al., 2004) (Figure 1.8). Taken together, this would suggest that actin cytoskeleton modulation by Wrch proteins is mediated through different signaling partners and cascades from that of Cdc42, contributing to their functional diversity. Epithelial cell polarity requires formation of distinct apical and basolateral membrane regions separated by tight junctions (Etienne-Manneville and Hall, 2003b). Additionally, establishment of polarity is necessary for directional movement of cells (Etienne-Manneville and Hall, 2003b). In transformed cells, depolarization, due to loss of tight junction formation, contributes to more motile and invasive transformed phenotypes. Cdc42 appears to be the major Rho family protein involved in regulation of cell polarity (Jaffe and Hall, 2005; Wennerberg and Der, 2004). Through interaction with partitioning defective 6 (Par6), its effector, in association with Par3 and protein kinase C (PKC), Cdc42 can control tight junction formation, Golgi apparatus and microtubule organizing center (MTOC) reorientation, and microtubule formation, all important aspects for polarization of cells. In response to Cdc42-dependent Par6 signaling, the Golgi apparatus and MTOC are repositioned in front of the nucleus towards the leading edge of a migrating cell (Etienne-Manneville and Hall, 2003b). Par6 is distinct from

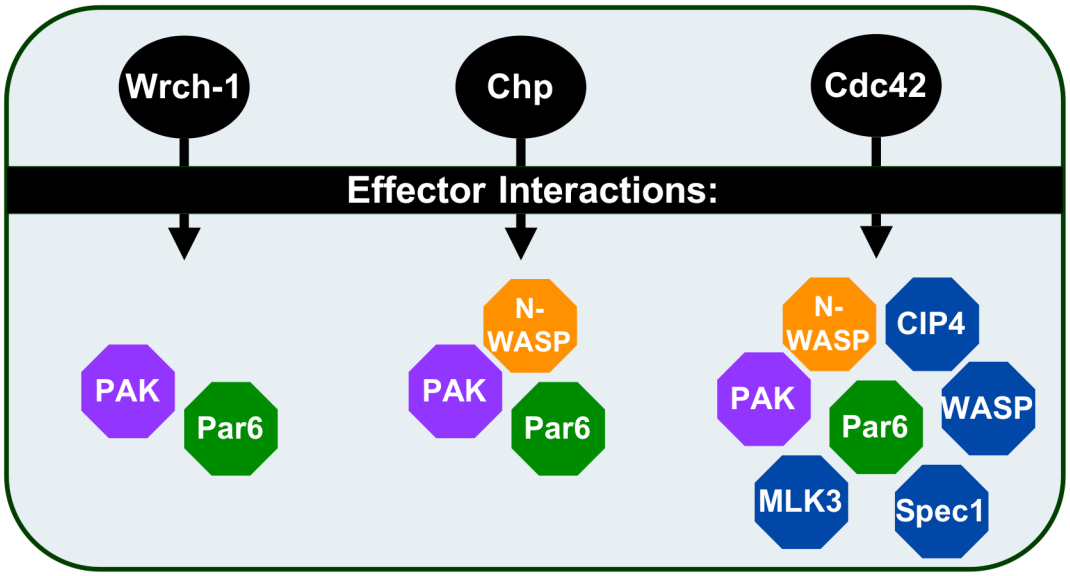



**Figure 1.8 Wrch-1 and Chp share some, but not all, effectors with Cdc42.** In a yeast 2-hybrid assay, constitutively GTP-bound Wrch-1 and Chp were analyzed for their ability to interact with different Cdc42 effector proteins. Of the effectors examined, Wrch-1 only complexed with PAK and, weakly, with Par6. However, Chp also bound to N-WASP and Par6 (with more affinity than was observed for Wrch-1). Given that Wrch-1 and Chp only interact with a subset of Cdc42 effectors and that Wrch-1 and Chp do share functional similarities with Cdc42, this would suggests that Wrc-1 and Chp have some overlapping functions and, likely signaling pathways. However, their differences in effector interactions, also suggests that Wrch-1 and Chp have functions distinctions from Cdc42 that should be explored.

other Cdc42 effectors in that it lacks an intact CRIB domain. Instead, it relies on additional sequences in the adjacent PDZ domain, together with its truncated CRIB domain sequences, to associate with Cdc42 (Etienne-Manneville and Hall, 2003b). Par6 also associates with PKC zeta to form a stable complex with Cdc42 for regulation of polarity. For example, Par6/PKC zeta negatively regulates GSK-3β, in a Cdc42-dependent manner, to polarize the centrosome for formation of membrane protrusions (Etienne-Manneville and Hall, 2003a). Additionally, Par6 and Par6/PKC zeta synergized with Cdc42 to transform cells in a focus forming assay, and a Par6 mutant lacking its CRIB domain failed to cooperate with Cdc42 (Qiu et al., 2000), implicating Par6 as a contributor to Cdc42 transformation.

In the above mentioned yeast 2-hybrid assay, Wrch proteins were also assessed for their ability to bind Par6. While both proteins associated with Par6, Wrch-1 exhibited a weak interaction suggesting that Par6 may not be a physiological effector of Wrch-1 (Figure 1.7C). The yeast 2-hybrid results do, however, indicate that the Wrch proteins may have a role in cell polarity, and they are currently being investigated in our lab for their potential contributions to cell polarity.

Cdc42 also binds to the p85 regulatory domain of PI3-K. This association stimulates activation of PI3-K, a lipid kinase, promoting cell survival pathways through activation of Akt (serine/threonine kinase) (Zheng et al., 1994). IQGAP, another Cdc42 effector, associates with the GTPase through its insert domain (residues 122-134 in Cdc42) (Li et al., 1999). Deletion of the insert region prevents IQGAP interaction with Cdc42 (Li et al., 1999). Interestingly, studies have shown that

deletion of the Cdc42 insert region blocks its transforming activity (Wu et al., 1998), suggesting that effector interactions, such as those with IQGAP, at that region may be needed for Cdc42 oncogenesis. Furthermore, IQGAP promotes enhanced motility and invasion as measured by a wound healing and matrixgel assay, respectively (Mataraza et al., 2003), consistent with a role for IQGAP in Cdc42 transformation. The insert region of Wrch-1 is not identical to Cdc42; however, it is still possible that Wrch-1 interacts with IQGAP to mediate its effects on cytoskeleton organization and transformation.

**Cdc42-related Rho GTPases and trafficking.** Cdc42 shares very high sequence identity with its closest relative, a brain-restricted splice variant of the cdc42 gene, and also with TC10 and TCL. Like Cdc42, TC10 induces formation of filopodia, activates JNK and drives SRF- and NF-κB-mediated transcription (Murphy et al., 1999). TCL also mediates formation of membrane ruffles and lamellipodia (Aspenstrom et al., 2004; Vignal et al., 2000). However, TC10 and TCL also have clear roles in cellular trafficking and, therefore, may be more functionally related to Wrch-1, than Cdc42. TC10 shares 67% sequence identity with Cdc42 and localizes mainly to the plasma membrane and intracellular membranes (Murphy et al., 2001; Murphy et al., 1999). Its localization and biological function are dependent on both palmitoyl fatty acid modification of carboxyl terminal cysteine residues and, presumably, on isoprenylation of its CAAX motif. For example, TC10 regulation of GLUT-4 translocation to the plasma membrane requires palmitoyl-assisted localization of TC10 to lipid rafts (Watson et al., 2003; Watson et al., 2001). TCL has been shown to associate with early endosomes and regulates the release of

transferrin from early/sorting endosomes to recycling endosomes (de Toledo et al., 2003). Loss of endogenous TCL protein by TCL-specific small interfering RNA or constitutive activation of TCL restricts transferrin to early endosomes and blocks its release from cells (de Toledo et al., 2003). The fusion of TCL carboxyl terminal sequences to constitutively activated Cdc42 or TC10 has a similar effect on transferrin trafficking further demonstrating importance of localization on function of closely related proteins (de Toledo et al., 2003). As mentioned previously, Wrch-1 protein is distributed to plasma and internal membranes (Tao et al., 2001). It is also possible that Wrch-1 localization to internal membranes dictates its own functions in cellular trafficking similarly to TC10 and TCL. The mechanisms by which Wrch-1 localizes to internal membranes is addressed in Chapters III and IV.

**Rho GTPases in cancer.** Although oncogenically mutated Ras family genes have been found in 30% of all human cancers (Bos, 1988), similar activating mutations in many Rho proteins, with the exception of RhoH/TTF (translocation three four)(RhoH), have yet to be identified in tumors. RhoH was initially isolated from non-Hodgkins lymphoma cells as a gene fusion with the BCL3/LAZ3 oncogene caused by a t(3;4)(q27;p11-13) chromosomal translocation (Dallery-Prudhomme et al., 1997). RhoH, a hematopoiesis-specific, GTPase-deficient and constitutively GTP-bound protein (Li et al., 2002), has also been found hypermutated in AIDS-Burkitt lymphomas (Gaidano et al., 2003). While it is currently unknown how these RhoH translocations and hypermutations contribute to lymphomas, RhoH does appear to antagonize RhoA, Cdc42 and Rac1 signaling activities in cell culture. Expression of RhoH inhibits activation of NFκB and p38 downstream of tumor

necrosis factor (TNF), RhoA, Cdc42 and Rac1 stimulation suggesting that loss of function mutations in RhoH could contribute to tumor progression (Li et al., 2002).

Overexpression of Rho family gene transcripts have been detected in numerous cancers including lung, colon, breast and pancreas (Boettner and Van Aelst, 2002). Rac1 and Rac1b, a constitutively activated splice variant of Rac1 (due to accelerated GDP/GTP exchange), have elevated mRNA and protein expression levels in colorectal and breast tumors (Jordan et al., 1999; Schnelzer et al., 2000). Additionally, upregulation of RhoC protein expression has been linked to aggressive cancers such as pancreatic adenocarcinomas (Suwa et al., 1998), gastric carcinomas with lymphatic metastases (Wang et al., 2005), non-small cell lung carcinomas (Shikada et al., 2003) and inflammatory breast cancers (IBC) (Kleer et al., 2005). Due to RhoC detection in the most aggressive breast cancers, IBC, a recent study of RhoC expression in breast tissues from various stages of tumor progression led to a proposal for RhoC expression as a marker for aggressive breast cancers (Kleer et al., 2005). Normal breast, hyperplasia, ductal carcinoma, invasive carcinoma and distant metastases tissues, showed increased RhoC expression as tumor progression increased in the tissues (Kleer et al., 2005). In addition, 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-driven downstream signaling pathways and oncogenesis (Sahai and Marshall, 2002). Leukemia-associated Rho guanine exchange factor (LARG) was isolated from acute myeloid leukemia and gain of function mutations in Dbl were detected in B-cell lymphomas, while upregulation of T-cell invasion and metastasis gene 1

(TIAM1) was found in a screen for pro-invasion genes in T-cells and mutant TIAM1 alleles were detected in renal-cell carcinoma (Boettner and Van Aelst, 2002). Detection of Rho proteins and their regulatory proteins in aggressive, metastatic cancers suggests that Rho GTPase activity and their downstream signaling pathways are also upregulated and likely have important roles in tumor progression.

While oncogenic mutations, similar to those found in Ras proteins, have not been detected for Rho proteins in human cancers, numerous labs have introduced mutations within their switch regions (e.g. codons 12 and 61) to stimulate GTPase-deficiency and constitutive activation of many Rho proteins. As mentioned previously, activation of Rho proteins leads to increased cellular proliferation, cytoskeletal reorganization and survival, indicating possible contributions of Rho proteins to metastasis and tumor progression (Sahai and Marshall, 2002). In several cell systems, cellular transformation has been evaluated using assays that measure focus forming activity and anchorage-independent growth mediated by Rho family proteins, like RhoA, Cdc42, Rac1, TC10 and Wrch-1. Ras dependency on Rho family signals for its transforming activity have been established using dominant negative mutants. Dominant negative mutants are created by single missense mutations at codons 15 and 17 in RhoA, Cdc42 and Rac1 resulting in either nucleotide-free or predominantly GDP-bound Rho proteins that bind preferentially to GEFs, sequestering them from association and activation of endogenous Rho GTPases. Ras-mediated focus forming activity is abrogated with these inhibitory mutant Rho proteins.

Interestingly, stimulation of signaling by Wnt1, a secreted glycoprotein that functions in embryonic development and oncogenesis, has been linked to activation of RhoA, Cdc42 and Rac1 proteins (Choi and Han, 2002; Habas et al., 2001) and upregulation of Wrch-1 expression (Tao et al., 2001) suggesting roles for Rho family proteins in Wnt transformation (Figure 1.9). Of the three characterized Wnt signaling pathways, the canonical Wnt/β-catenin pathway and the noncanonical Wnt/Planar cell polarity and Wnt/$Ca^{2+}$ pathways, only the latter two pathways feature Rho GTPase activity as downstream signaling events (Figure 1.9). Extensive studies in Xenopus embryos, show that activation of RhoA and Rac1 by β-catenin-independent Wnt signaling, followed by activated JNK is necessary for planar cell polarity and convergent extension during vertebrate gastrulation (Habas et al., 2003). In mammalian cells, RhoA activation is needed for Wnt3-induced cell motility (Endo et al., 2005). Additionally, the Xenopus Cdc42 homolog negatively regulates cell adhesion downstream of the Wnt/$Ca^{2+}$ pathway during convergent extension (Choi and Han, 2002). Dominant negative Cdc42 rescues this inhibition in a PKC-dependent manner (Choi and Han, 2002). Although RhoA, Cdc42 and Rac1 are required for some Wnt-driven developmental functions, direct evidence of Rho contributions to Wnt transformation have not been demonstrated. That Wrch-1 expression is upregulated in Wnt1 transformed cells and that activated Wrch-1 phenocopies Wnt1 morphological transformation suggests that Wrch-1 may regulate other Wnt1-transforming properties, possibly through its activation of JNK (like RhoA and Rac1 downstream of Wnt signals), and provides the first substantial connection for possible Rho GTPase signaling in Wnt oncogenic pathways.

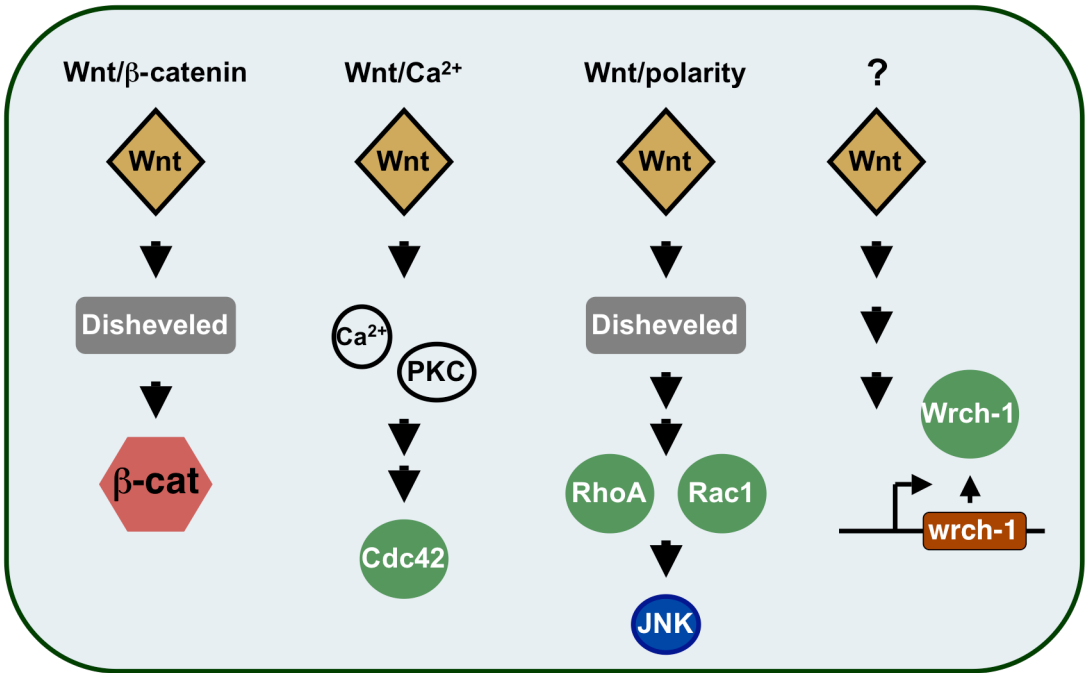



**Figure 1.9 Rho GTPases contribute to Wnt signaling pathways.** The canonical Wnt signaling pathway signals through disheveled leading to stabilization of β-catenin. Cdc42 functions downstream of Wnt signaling, in a calcium/PKC-dependent manner. Activation of RhoA and Rac1, leading to stimulation of JNK activity, occurs downstream of Wnt/polarity signaling pathways. In mammary epithelial cells, Wrch-1 message is upregulated.

**Rho GTPases as therapeutic targets.** Given that mutation and overexpression of many GTPases and their regulators are associated with late-stage and aggressive cancers, these proteins are targets for development of pharmacological inhibitors. As such, many different strategies have been employed to inhibit small GTPase signaling and function in the context of oncogenesis. Since Rho GTPases depend on their localization for protein-protein interactions and downstream signaling events, inhibitors of enzymes required for their proper subcellular targeting have been developed. As indicated above, prenyltransferases (FTase and GGTase I) regulate the transfer of isoprenoid moieties to the cysteine residues of Rho and Ras GTPase CAAX motifs for targeting those proteins to cellular membranes (Figure 1.10A). As anti-cancer therapy, small molecule inhibitors of FTase (FTIs) and GGTase I (GGTIs) were created to block these enzymes and prevent membrane association of the affected GTPases (Figure 1.10B). FTIs were initially designed to be anti-Ras drugs and had considerable effects on the reduction of H-Ras tumors in mice (Kohl et al., 1995a; Kohl et al., 1995b). However, K- and N-Ras, the predominantly mutated Ras proteins in many cancers, become alternatively prenylated by geranylgeranylation, and are therefore membrane-bound and functional in the presence of FTIs. Additionally, FTIs decreased K- and N-Ras-induced transformation, suggesting that there are other non-Ras targets of FTIs (Mangues 1998 and Sun 1995). It is possible that Rho proteins could be the physiologically relevant targets of FTI inhibition of transformation, given that RhoB, Rnd3 and TC10 are farnesylated by FTase (Cox and Der 1997, Reid 2004). Given that the large majority of Rho GTPases are substrates for geranylgeranylation by GGTase I, Rho proteins are excellent candidates for GGTI-mediated inhibition of

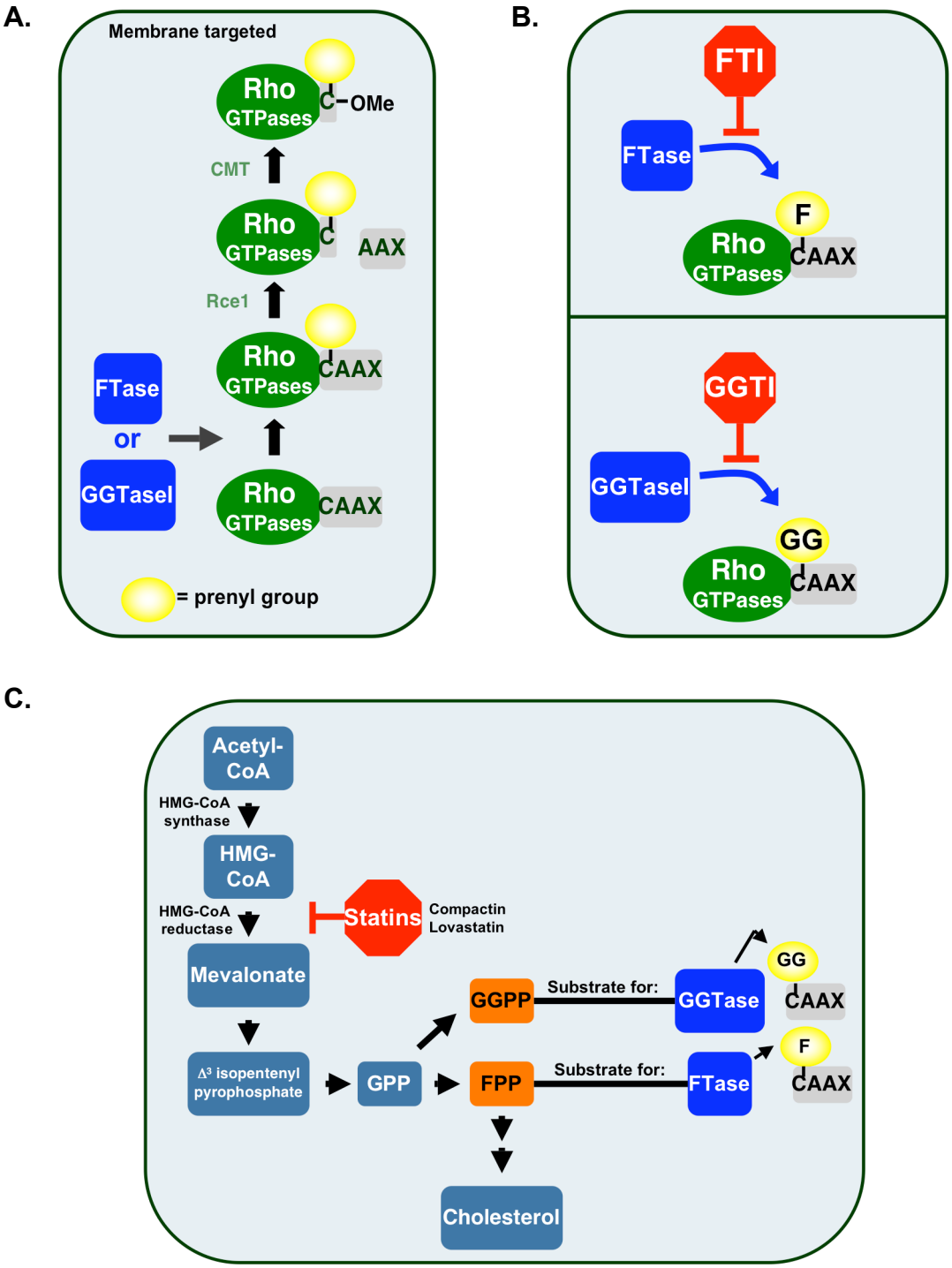



**Figure 1.10 Inhibitors block Rho family lipid modifications.** (A) The prenyltransferases, FTase and GGTase I transfer isoprenoids to the cysteine residue of Rho family CAAX motifs. (B) FTI, an inhibitor of FTase, and GGTI, a GGTase I inhibitor, blocks isoprenylation. (C) Statins, designed to block HMG-CoA reductase activity, prevent the formation of isoprenoid precursors, rendering all isoprenylation substrates unmodified and mislocalized.

their transforming activities. The localization and function of several Rho proteins including RhoA, Cdc42 and Rac1 are disrupted in the presence of GGTIs (Sebti and Hamilton, 2000b). Treatment of Rac1-expressing cells with GGTI causes mislocalization of Rac1 from cellular membranes to the cytosol and prevents Rac-stimulated membrane ruffles, focus forming activity and anchorage-independent growth (Joyce and Cox, 2003). Additionally, treatment of cells with GGTI induced $p21^{WAF1/CIP1}$ promoter activity and blocked SRF-responsive gene transcription, mimicking the results of dominant-negative RhoA expression. These data implicate inhibition of RhoA lipid modification and its membrane localization (including effector interactions), as the mechanisms for this GGTI- induced activity (Adnane et al., 1998).

Statins also block localization of Rho family GTPases by blocking 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzymatic activity (Figure 1.10C). HMG-CoA reductase is required for the biosynthesis of cholesterol precursors farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (Figure 1.10C). FPP and GGPP are needed for isoprenylation of Rho GTPases. In human pancreatic cells, treatment with lovastatin and fluvastatin (later derivatives of the original HMG-CoA reductase inhibitor, compactin) blocked epidermal growth factor (EGF)-stimulated translocation of RhoA from the cytosol to the plasma membrane, resulting in abrogated RhoA-mediated cell invasion (Kusama et al., 2002). The effects of lovastatin and fluvastatin were reversed upon the addition of geranylgeranyl pyrophosphate, demonstrating that loss of RhoA membrane localization and invasive properties required improperly localized RhoA (Kusama et

al., 2002). In Chapter II, the use of compactin as a diagnostic tool to determine which lipid moieties affect localization of other Rho family proteins will be further discussed.

**Wrch proteins differ from the Rho GTPase norm.** As mentioned above, like other Rho GTPases, Wrch-1 and Chp bind GDP and GTP molecules. However, recent data demonstrates that Wrch-1 exchanges GDP for GTP rapidly in the absence of RhoGEF activity (i.e. Wrch-1 is a naturally fast cycling GTPase) suggesting that Wrch-1 is predominantly GTP-bound and activated independent of GEFs (Saras et al., 2004; Shutes et al., 2004). Therefore, other mechanisms to modulate Wrch-1 activity, such as subcellular targeting and protein stability may be employed for Wrch-1 regulation. These mechanisms are explored in chapters III and IV. Also, there is currently no evidence to suggest whether Wrch-1 requires GAPs for enhanced hydrolysis and downregulation; however, constitutive activation of Wrch-1 leads to higher transforming activity than wild-type Wrch-1, indicating that GAPs for Wrch-1 may exist (Shutes et al., 2004). Wrch proteins share extensive sequence identity with Cdc42; however, both Wrch-1 and Chp contain additional N-terminal and C-terminal sequences not present in Cdc42 (Figure 1.11). The N-termini of both Wrch-1 and Chp contain proline-rich regions (Figure 1.11, pink inset) that may mediate interactions with SH3 domain-containing proteins, such as the adapter proteins Nck and Grb2, which link GTPases to upstream receptors, and the Rac1 effector protein (plenty of SH3s) POSH, offering additional interaction domains for Wrch protein signaling. Interestingly, in addition to its numerous SH3 domains, POSH also has a functional RING finger domain classifying POSH as an E3 ligase

that may ubiquitylate Wrch proteins for additional protein activity and localization regulation (Alroy et al., 2005) (addressed in Chapter IV).

The carboxyl terminus of Wrch-1, on the other hand, resembles the C-terminus of Cdc42 in that it has a polybasic hypervariable region and a putative CAAX motif, CCFV (Figure 1.11, blue inset). However, the Wrch-1 C-terminus is much longer and it has tandem tryptophan residues immediately upstream of its apparent CAAX box (Figure 1.11, blue inset). In addition, the CCFV motif terminates in a valine, which is unusual for CAAX motifs (Figure 1.11, blue inset). Like Wrch-1, Chp localizes to plasma and internal membranes suggesting that its carboxyl terminal hypervariable sequences may also function as membrane targeting domains. However, Chp lacks the apparent CAAX motif present in Wrch-1 (CCFV) and other Rho proteins and, instead, terminates in a (F)CFV motif (Figure 1.11, blue inset). Because the Wrch-1 CCFV motif also includes a CFV sequence, it is possible that does not have a true prenylatable CAAX motif. Instead both Wrch-1 and Chp may utilize a novel membrane targeting signal, dictated by a CFV motif, identifying, yet another, localization signal specific for Rho proteins (Chapter III).

**Evaluating posttranslational modifications as regulators of Wrch-1 location and function.** In this dissertation, I have investigated the mechanisms by which Wrch-1 localizes to its cellular compartments and how this localization contributes to Wrch-1 signaling pathways and transforming activity. Chapter II is dedicated to the use of prenylation inhibitors as tools to evaluate posttranslational modification effects on localization and function of isoprenylated small GTPases and Wrch-1 (Figure

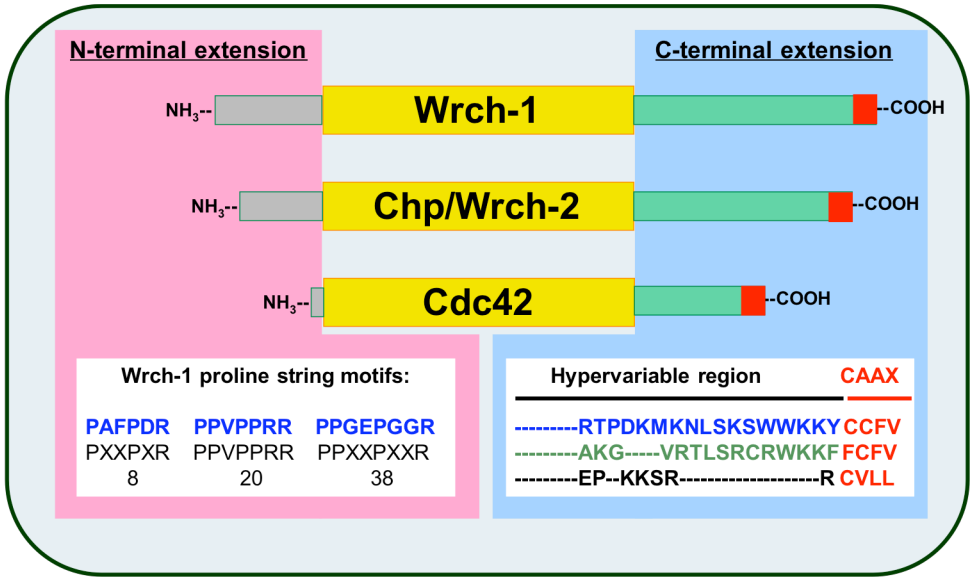



**Figure 1.11 The Wrch proteins have unusual characteristics.** Wrch-1 and Chp have longer amino and carboxyl terminal extensions than Cdc42. The amino termini of Wrch-1 (shown in blue font, pink inset, white box) and Chp (not illustrated) have several putative proline-rich regions that may interact with SH3 domain-containing proteins. Proline string motif consensus sequences and the starting residue number for each Wrch-1 proline-rich region is shown directly below the Wrch-1 protein sequences (black font, pink inset, white box). The carboxyl termini of the Wrch proteins have unique residues (partial carboxyl-terminal sequence shown in blue inset, white box). Wrch-1 has tandem tryptophan residues upstream of its putative CAAX motif (hypervariable region of Wrch-1, blue font, CAAX, red font, blue inset, white box). Chp sequences also contain a tryptophan residue, but lack a CAAX motif (hypervariable region of Chp, green font, CAAX, red font, blue inset, white box). The shorter Cdc42 carboxyl terminal sequence is illustrated below Wrch-1 and Chp sequences (black font, blue inset, white box).

1.12, purple inset). The focus of Chapter III includes examination of the putative Wrch-1 CAAX motif (CCFV) as a substrate for lipid modification by isoprenoids and palmitoyl fatty acids and how these modifications may regulate Wrch-1 localization and transformation (Figure 1.12, black inset). Work in Chapter IV investigated potential secondary membrane localization signals upstream of the Wrch-1 CCFV motif and whether these signals include posttranslational modifications such as phosphorylation and ubiquitylation (Figure 1.12, pink inset). Chapter V (not illustrated) summarizes the findings of this dissertation and suggests further studies on Wrch-1 localization and function, thus shedding light on the intricacies of Rho family function and how posttranslational modifications can play critical roles in regulation of these GTPases.

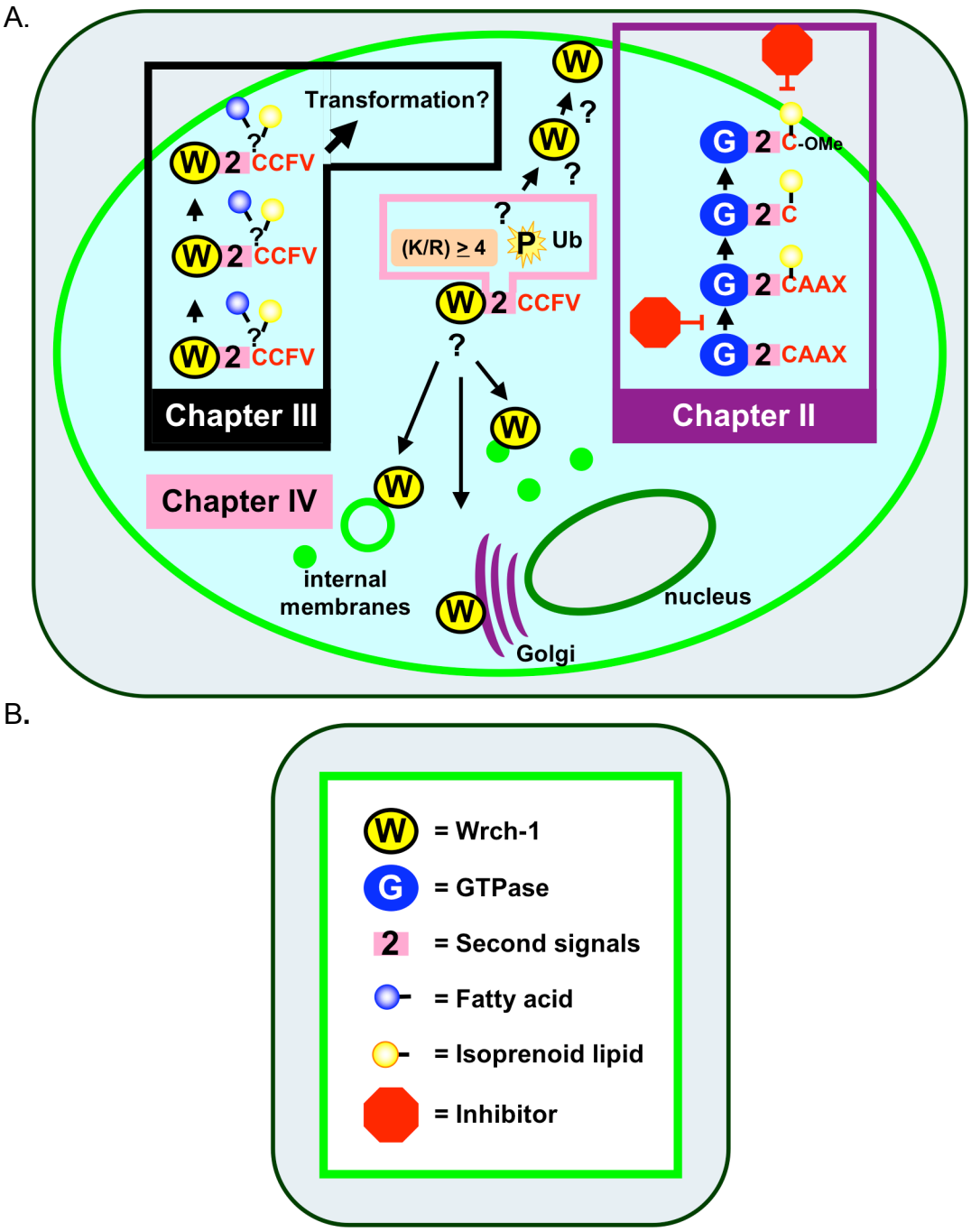



**Figure 1.12 Evaluating the regulation of Wrch-1 localization and function.** (A) schematic of each dissertation chapter. Chapter II describes the use of membrane localization inhibitors to delineate mechanisms by which small GTPases distribute to their correct subcellular compartments. Chapter III examines the functionality of the Wrch-1 putative CAAX motif and how it contributes to Wrch-1 transforming activity. Chapter IV evaluates the potential second signal modification of Wrch-1 that may help dictate Wrch-1 localization and biological function. Chapter V (not illustrated) presents future studies on Wrch-1 localization and signaling pathways. (B) Legend for panel A.

# CHAPTER II

# USING INHIBITORS OF PRENYLATION TO BLOCK LOCALIZATION AND TRANSFORMING ACTIVITY OF SMALL GTPASES

**Anastacia C. Berzat, Donita C. Brady, James J. Fiordalisi and Adrienne D. Cox**

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**ABSTRACT**

The proper subcellular localization and biological activity of most Ras and Rho family small GTPases are dependent upon their posttranslational modification by isoprenylation. Farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I) are the prenyltransferases that catalyze the irreversible attachment of C15 farnesyl (Ras, Rnd) or C20 (R-Ras, Ral, Rap, Rho, Rac, Cdc42) isoprenoid lipid moieties to these small GTPases and other proteins. Therefore, pharmacological inhibitors of FTase (FTIs) and GGTase I (GGTIs) have been developed to prevent these modifications and thereby to block the lipid-mediated association of Ras and Rho proteins with cellular membranes, and the consequent signaling and transforming activities. In addition, other small molecule inhibitors such as farnesylthiosalicylic acid (FTS) can compete with the isoprenoid moiety of small GTPases for membrane binding sites. Finally, endogenous regulatory proteins such as RhoGDIs can bind to and mask the prenyl groups of small GTPases, leading to their sequestration from membranes. We describe here methods to utilize each of these categories of prenylation inhibitors to manipulate and investigate the subcellular localization patterns and transforming potential of these Ras and Rho family GTPases.

## PREFACE

This chapter represents work published in *Methods and Enzymology*, a publication dedicated to papers describing specific experimental protocols. These types of publications have been enormously helpful for my own experimental designs, and I was thrilled contribute my own methods to this book series. I wrote all sections of this chapter, except for the "Sequestration of Prenylated Small GTPases by RhoGDIs" section. Additionally, I provided data for "Visual Analysis of FTI/GTI Inhibition of Subcellular Localization". Donita C. Brady wrote and provided data for the "Sequestration of Prenylated Small GTPases by RhoGDIs" section. James J. Fiordalisi contributed data for "Using Prenylation Inhibitors to Abrogate Ras-mediated Transformation" and "*S-trans,trans*-farnesylthiosalicylic acid (FTS)" sections. Adrienne D. Cox edited all sections of this chapter.

## INTRODUCTION

The biological functions of Ras and Rho small GTPases are critically dependent on their proper localization to specific cellular membranes. Impairment of correct membrane localization impairs the protein-protein interactions necessary for regulation of activation and effector utilization, leading to alterations in subsequent downstream biological consequences. The C-terminal region of Ras and Rho proteins, called the hypervariable domain, contains the membrane targeting motifs that dictate localization. Conserved cysteine residues in C-terminal CAAX motifs (where C = cysteine, A = aliphatic and X = "any" amino acid, but is usually S, M, A, Q or L) are sites for irreversible attachment of isoprenyl lipid moieties (farnesyl, C15 and geranylgeranyl, C20) by the prenyltransferase enzymes, farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I) (Casey and Seabra, 1996; Cox and Der, 1997). This process has been the target of many small molecule inhibitors of FTase (FTIs) and GGTase I (GGTIs) designed to block these enzymes and to prevent membrane targeting of the transforming members of the Ras and Rho families for cancer treatment (Sebti and Der, 2003; Sebti and Hamilton, 2000a). Ras and Rnd proteins are modified by FTase and are FTI targets, whereas Ral, Rap, R-Ras, Rac, Rho and Cdc42 are modified by GGTase I and are GGTI targets. Another approach to inhibiting small GTPases via interfering with their lipid-mediated interactions is represented by *S-trans,trans-farnesylthiosalicylic acid (*FTS), a synthetic, small molecule inhibitor that dislodges processed Ras family GTPases from membranes (Marom et al., 1995).

In addition to these pharmacological inhibitors, there are also endogenous proteins capable of regulating the membrane association of fully processed GTPases. Rho GDP dissociation inhibitors (RhoGDIs) are one such class of proteins. As the name implies, RhoGDIs were first described as inhibiting dissociation of GDP and subsequent loading of GTP onto Rho (Fukumoto et al., 1990). In addition, RhoGDIs also block effector molecule interactions (Chuang et al., 1993). Interestingly, these proteins have also been shown to regulate membrane association and dissociation of processed Rho family members through interactions with their isoprenyl lipid modifications. RhoGDIs contain a hydrophobic pocket that can mask the geranylgeranyl lipid moiety of many Rho GTPases, resulting in a high-affinity cytosolic complex. There are currently three known human RhoGDIs (RhoGDI$\alpha$/GDI1, Ly/D4GDI$\beta$/GDI2 and RhoGDI$\gamma$/GDI3) with distinct binding specificities for different Rho proteins. These natural inhibitors can provide selective disruption of Rho family membrane association. Since lipid modifications are critical for Rho family localization and function, RhoGDIs can function as tools to sequester Rho proteins to the cytosol, preventing membrane association and downstream biological consequences. Despite repeated attempts to identify a similar type of regulatory molecule for Ras proteins, none has been found. However, recent findings regarding the ability of galectin-1 and galectin-3 to bind H-Ras and K-Ras, respectively, and the consequences of those interactions to subcellular localization and signaling specificity of Ras proteins, suggests that galectins may actually serve a function similar to that of a "RasGDI" (Rotblat et al., 2004a). This interesting possibility awaits further investigation.

Here we describe the use of these inhibitors that either prevent lipidation of small GTPases and/or disrupt small GTPase membrane association to delineate specific localization and transforming activity of these proteins.

## PHARMACOLOGICAL INHIBITION

### *Prenyltransferase inhibitors*

***General considerations.*** Prenyltransferase inhibitors in current use were either rationally designed as peptidomimetics of specific farnesylated or geranylgeranylated CAAX motifs or identified via high throughput screens of existing libraries (Cox and Der, 1997; Sebti and Der, 2003; Sebti and Hamilton, 2000a). While most are competitive with respect to the CAAX-containing protein substrates, some are competitive with respect to the farnesylpyrophosphate (FPP) lipid moiety (e.g, manumycin, derived from *Streptomyces parvulus)*. Several FTIs have reached clinical trials, including SCH66336 (lonafarnib), R115777 (tipifarnib), L-778,123 m and BMS-214662 (Cox and Der, 1997; Sebti and Der, 2003; Sebti and Hamilton, 2000a). However, for laboratory use in cell-based assays, many more options are available. Of the commercial options, one of the best is L744,832, which was invented at Merck and has been widely licensed. Among other vendors, it is available from Calbiochem (cat. no 422720), BioMol (G-242), Alexis Labs (ALX-290-005) and Sigma-Aldrich (L7287). Other good options include the Hamilton/Sebti series that include FTI-277 / GGTI-298 and especially their newer generation relatives, e.g. GGTI-2417; these are also available from Calbiochem and others. Of note, FTI-277 and GGTI-298 are thiol-containing compounds that require the presence of DMSO, while the later generations of FTIs/GGTIs in those series do not.

Important considerations for use of FTIs and GGTIs in cell-based assays include relative potency, toxicity, and selectivity for FTase vs. GGTase. Many of the earliest generation of prenyltransferase inhibitors have relatively poor potency, while the later generations are much more effective. In general, *in vitro* $IC_{50}$s in the nanomolar range translate to practical use *in vivo* in the micromolar range. The later generations of commercially available FTIs and GGTIs can be used at 1 – 10 $\mu M$ with good efficacy and little toxicity. In general, GGTIs are more toxic on a molar basis than FTIs. In part this is because their ability to inhibit the function of Rho GTPases causes cell rounding and sloughing from the dish, followed by apoptosis. It is not recommended to use GGTIs at doses higher than 20 $\mu M$. As for any pharmacological inhibitor, it is best to use the lowest dose possible that is still effective; more is not necessarily better, and can lead to unwanted lack of specificity.

To monitor the effectiveness of FTI and GGTI treatment in blocking their respective enzymatic activities in the treated cells, a common biochemical method is to observe the shift to a slower mobility on SDS-PAGE of unprocessed forms of endogenous substrates for FTase (e.g., H-Ras, hDJ2) or GGTase I (e.g., Rap1a). Another option is the visual monitoring of ectopically expressed GFP-tagged GTPases as described elsewhere (Keller et al., 2005) and in the following.

***Visual analysis of FTI/GGTI inhibition of subcellular localization.*** To visualize the disruption of proper subcellular localization of Ras/Rho proteins by prenyltransferase inhibitors, it is convenient to monitor the localization of these small

GTPases that are tagged with enhanced green fluorescent protein (EGFP). GFP contains a putative nuclear localization signal (NLS), which results in a diffuse cytosolic and nuclear localization pattern of GFP alone (pEGFP, Clontech). When EGFP sequences are fused to Ras or Rho GTPase sequences, the lipid modification is dominant over the NLS signal, resulting in nuclear exclusion. Thus, the subcellular distribution of GFP-tagged small GTPases is indistinguishable from that of the same GTPases tagged either with smaller epitopes such as HA or of endogenous untagged GTPases (Michaelson et al., 2001). However, inhibition of their lipid modification results in subcellular localization patterns similar to those directed by EGFP alone. In addition, it is also convenient to use GFP-tagged GTPases because real-time, live cell imaging is possible. However, these localization studies can also be done by immunofluorescence using fixed cells expressing HA-, Flag- or Myc-tagged GTPases, or by evaluating endogenous proteins when suitable antibodies are available. The use of GFP-tagged small GTPases to monitor lipid modification status has been described in detail elsewhere (Keller et al., 2005). Therefore, this procedure will be discussed only briefly here, with an emphasis on conditions for FTI/GGTI treatment.

**TRANSFECTING CELLS FOR FTI AND GGTI TREATMENT**. To use this method, transient transfections of GFP-tagged small GTPases are necessary. Cells should be seeded onto glass coverslips (e.g., Corning No. 11/2, 0.16-mm thick, 18 mm) that have been sterilized in 70% ethanol overnight, rinsed briefly with 1X phosphate-buffered saline (PBS), placed in 60-mm dishes (2 coverlips per dish) and allowed to dry. Alternatively, small round coverslips in smaller dishes can also be used. For

ease of handling, we do not recommend using multiwell plates wherein each well is just large enough to contain only a single coverslip. Plate 1 x $10^5$ NIH 3T3 cells per 60-mm dish onto the dishes containing the glass coverslips, and transfect the following day by any desired method (for example, see (Fiordalisi et al., 2001)). The amount of plasmid to transfect depends on the protein to be expressed and cell type. The goal is to achieve sufficient expression to visualize the ectopic protein without gross overexpression that will lead to artefactual results. A reasonable starting point for many pEGFP-GTPase plasmids is 500 ng.

**TREATING CELLS WITH FTIs AND GGTIs.** Since prenylation is both a rapid and an irreversible post-translational process, treatment with prenyltransferase inhibitors blocks processing of newly made Ras/Rho proteins, but does not reverse the processing of endogenous proteins that have already been lipid-modified. Therefore, it is very important to treat transfected cells with the prenyltransferase inhibitors as soon as possible, before the exogenous proteins are being expressed. If using calcium phosphate transfection, the FTIs, GGTIs or vehicle should be added to the growth medium that is used immediately following the glycerol shock step. If using liposome-mediated transfection reagents under serum-free condition, add the prenyltransferase inhibitors or vehicle at the end of the serum-free incubation step when replacing with complete, serum-containing medium. In general, most of the FTIs and GGTIs are dissolved in DMSO vehicle. A reasonable starting point for most of the commercially available inhibitors mentioned above is 1 – 10 $\mu M$. Stock solutions are generally made up as 1,000X concentrations in DMSO (e.g., 10 m*M*), aliquotted into microfuge tubes and stored frozen at –80C. Make small enough

aliquots to avoid freeze/thaw as much as possible. Dilute the stock solutions 1:1,000 directly into the appropriate complete growth medium for the cells being treated. For translocation assays evaluating ectopically expressed proteins, incubate treated cells in vehicle or inhibitors for 24 hr. At this point, there should be sufficient expression of GFP-tagged small GTPases for analysis. As a reminder, FTIs and GGTIs do not remove lipids from already-processed proteins; they prevent lipidation of newly synthesized proteins. Therefore, endogenous proteins that have been synthesized prior to inhibitor treatment will remain lipid-modified. This consideration therefore does not apply to ectopic GFP-GTPases if treatment commences at the time of transfection.

**ANALYSIS OF FTI AND GGTI EFFECTS ON RAS AND RHO GTPASE LOCALIZATION.** To visually analyze the effects of prenyltransferase inhibitors on GFP-tagged GTPase localization, rinse the coverslips containing the treated cells with 1X PBS or phenol red free media to reduce cellular autofluorescence. Then add one drop of 1X PBS or phenol red free media onto a glass slide and invert the coverslips (cell-side down) on the slide. Using absorbent paper, remove any excess PBS or media and view under an epifluorescent microscope equipped with a FITC bandpass filter. Images can be captured and analyzed using MetaMorph imaging software (Universal Imaging, Corp., Downington, PA). If the EGFP-tagged Ras/Rho proteins are dependent on specific prenyltransferase activity to become lipidated and membrane localized, treatment with the appropriate prenyltransferase inhibitor will prevent prenylation and result in loss of membrane attachment and localization of the proteins to the cytosol and nucleus, similar to unprocessed EGFP protein.

Figure 2.1 demonstrates that, as expected, treatment of cells expressing GFP-H-Ras with FTI-2153 or GFP-Cdc42 with GGTI-2166 resulted in localization patterns like that of empty pEGFP vector. However, the converse was not true, demonstrating selectivity of the inhibitors and their effects on processing and localization. Consistent with the fact that K-Ras can become alternatively prenylated by GGTase I in the presence of FTIs (Whyte et al., 1997), GFP-K-Ras localization was disturbed only after treatment with both FTI and GGTI. The comparable vehicle-treated cells maintained their nuclear exclusion and proper targeting to cellular membranes.

***Using prenyltransferase inhibitors to abrogate Ras-mediated transformation.*** The function of most Ras family members is critically dependent on their specific subcellular membrane locations. Loss of this proper membrane targeting results in impairment of effector protein interactions and consequently of their downstream signaling pathways and biological outcomes. Therefore, inhibitors like FTI and GGTI that effectively block membrane localization of Ras proteins are also excellent tools for evaluating the roles of these prenylated proteins in cellular events such as transformation.

**FOCUS FORMATION ASSAYS WITH PRENYLTRANSFERASE INHIBITORS.** We commonly use two different assays to measure Ras transforming potential. The focus forming assay (FFU, focus forming units) evaluates the ability of oncoproteins to overcome contact inhibition. Normal cells proliferate only until they reach confluency and contact their neighbors; at this point, the cells cease dividing and

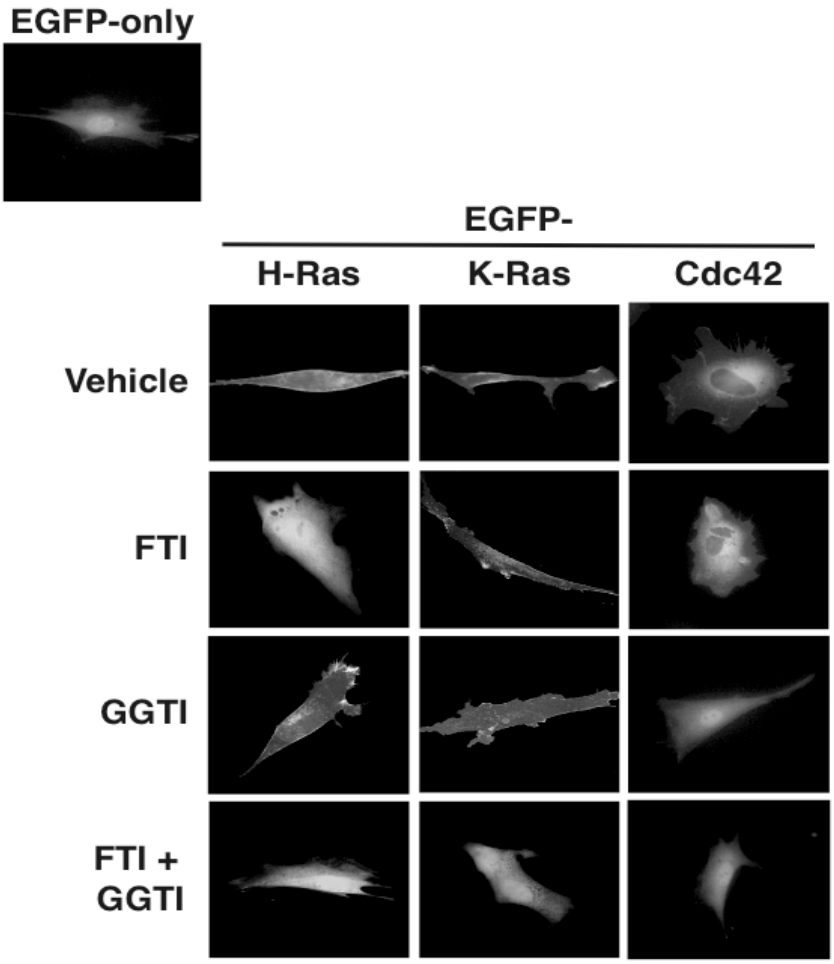


**Figure 2.1 Prenyltransferase inhibitors alter the localization patterns of Ras and Rho family members.** NIH 3T3 cells seeded on glass coverslips were transiently transfected with pEGFP expression vectors encoding H-Ras(Q61L), K-Ras(G12V), or Cdc42(Q61L) sequences and treated with either vehicle (control), 10μ*M* FTI-2153 (FTase inhibitor to block H-Ras farnesylation), 10μ*M* GGTI-2166 (GGTase I inhibitor to block Cdc42 geranylgeranylation), or 10μ*M* FTI-2153 + 10μ*M* GGTI-2166 (to block K-Ras farnesylation and subsequent geranylgeranylation). The following day, localization of each EGFP-tagged small GTPase was analyzed under epifluorescence microscopy using a FITC bandpass filter. Posttrreatment localization of EGFP-tagged proteins to the nucleus and cytosol, instead of to cellular membranes, indicates inhibition of lipid modification by the respective enzyme.

become quiescent. Cells expressing oncogenes such as Ras, however, bypass this signal and continue to proliferate and overgrow, resulting in one focus or several foci of overgrown cells derived from the initial cell (clone) that failed to arrest. In general, Ras proteins alone are capable of mediating focus formation (e.g., H-, N-, K-Ras, R-Ras, TC21, M-Ras), whereas Rho proteins (RhoA/B/C, Rac1/3, Cdc42, Wrch-1/2) require additional signals, for example from activated Raf, to abrogate contact inhibition (Khosravi-Far et al., 1995; Qiu et al., 1995a; Qiu et al., 1995b).

**USING PRENYLATION INHIBITORS TO ABROGATE FOCUS FORMING ACTIVITY.** We will describe inhibition of Ras, but not Rho, focus forming activity with prenyltransferase inhibitors. As mentioned earlier, Rho family proteins require cooperating signals from constitutively active Raf to mediate the appearance even of their nonRaf-like foci. Unfortunately, GGTIs also inhibit Raf-induced focus formation, so it is not possible to separate the effects of GGTI on Raf versus on Rho function in this assay (Joyce and Cox, 2003).

Focus assays are performed following transient transfection or viral infection of recipient cells. To perform a Ras-mediated focus assay, seed low passage NIH 3T3 cells at 2 x $10^5$ cells per 60-mm dish. Low passage cells are necessary to reduce the background levels of false positive foci. The next day, transiently transfect cells with plasmids encoding the Ras proteins of interest. We have found that the calcium phosphate DNA precipitate method yields the fewest false positives, and that GFP-tagged proteins will yield much lower FFU activity (Fiordalisi et al., 2001). A detailed

protocol for this method for use in NIH 3T3 and other cell types can be found in Fiordalisi et al., 2001.

Prenyltransferase inhibitors will be added to the growth medium after the glycerol shock step that is done to increase uptake of plasmid DNA. During the incubation period, prepare complete growth medium containing FTIs or GGTIs at final concentrations of 1 – 10 $\mu M$ as desired. When feeding the dishes during the normal course of the assay, continue to replace the spent growth medium with fresh medium containing inhibitors at least twice a week, depending on transformed phenotype and on drug half-life in serum-containing culture medium at 37C. Most prenyltransferase inhibitors require replenishment every 48 hr. Critical negative controls include both empty vector matching that from which the small GTPases are expressed, and vehicle control (generally DMSO at the same final concentration in growth medium as the inhibitors).

After 12 to 20 days, the treated cells are ready to be evaluated for the appearance of transformed foci. Ras-mediated foci can be observed by viewing the bottom of dishes with the naked eye and also by brightfield microscopy. Ras mediated-foci are characterized by large, swirling foci of highly refractile, spindle-shaped cells. Foci may be counted under the microscope or the dishes can be fixed and stained and the foci counted visually. To assist in the quantitation of foci, rinse the plates with 1X PBS, fix with 3:1 (v/v) methanol:acetic acid solution for 10 minutes and stain with 0.4% crystal violet solution in 20% ethanol for 2 minutes. Discard the crystal violet stain and rinse the plates carefully in a bucket containing running tap $H_2O$ until the

plate is cleared of crystal violet and only the foci are stained purple. Then, invert the plates and air-dry overnight. Effective treatment with prenyltransferase inhibitors may result in a reduction in numbers and/or sizes of the transformed foci. The most critical time of treatment with these inhibitors is at the time of transfection, although the best impairment of focus formation is achieved when cells are treated throughout the course of the assay.

When NIH 3T3 cells expressing oncogenic H-Ras(61L) were treated continuously with the FTI L744,832, a dramatic reduction in focus forming activity was observed in comparison to DMSO vehicle-treated cells (Figure 2.2A). Vehicle-treated cells maintained their ability to robustly induce foci. These data demonstrate that prenyltransferase inhibitors can disrupt Ras transformation.

## USING PRENYLTRANSFERASE INHIBITORS TO DISRUPT COLONY FORMING ACTIVITY IN SOFT AGAR

**General Considerations for Soft Agar Assays.** Assaying colony formation in soft agar (SA) evaluates effects on the transformed phenotype of anchorage-independent growth. Normal adherent cells require a matrix for attachment to proliferate, whereas transformed, oncogene-expressing cells secrete their own matrix proteins and can sustain growth while suspended in agar, forming colonies of proliferating cells. Unlike the focus forming assay, both Ras and Rho proteins alone are sufficient induce anchorage-independent growth, without cooperation from Raf.

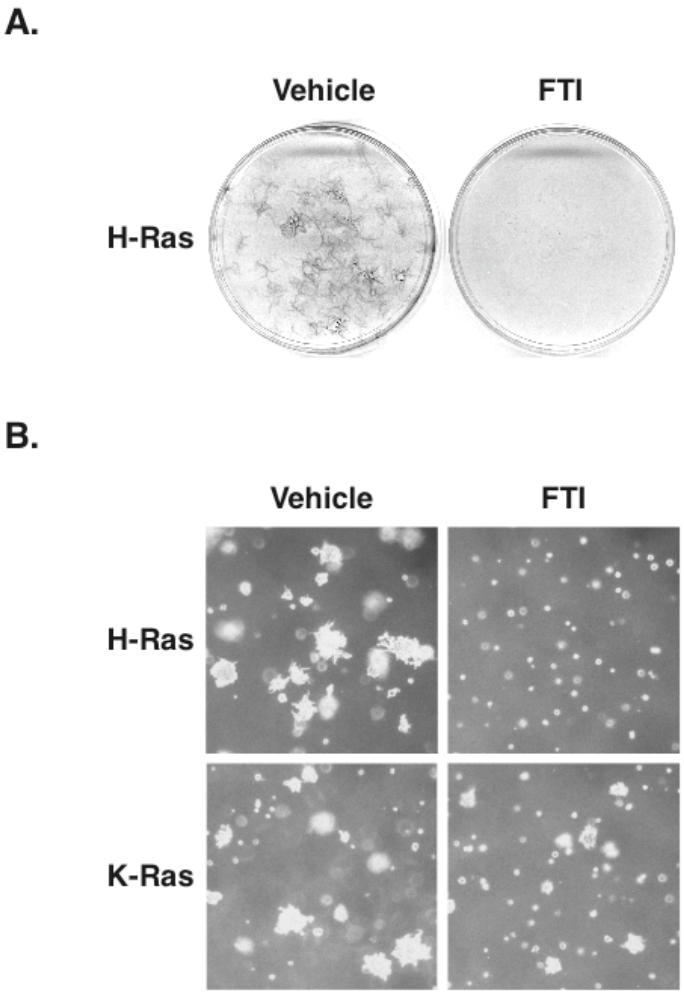


**Figure 2.2 Farnesyltransferase inhibitors selectively disrupt transforming activity of Ras.** (A) Focus formation assay: NIH 3T3 cells transiently transfected with 1 μg pCGN H-Ras(Q61L) plasmid were treated with 10 μ*M* of the FTI L744, 832 or DMSO (vehicle control) and maintained for 14 days by replacing growth media every 3 days. Cells were rinsed in 1x PBS, fixed in 3:1 methanol/acetic acid solution, and stained with 0.4% crystal violet in ethanol. Foci of transformed cells readily absorb the stain. (B) Soft agar colony formation assay: NIH 3T3 cells stably expressing H-Ras(Q61L) and K-Ras(G12V) constructs were seeded into 0.4% bacto-agar containing either DMSO vehicle control or 3μ*M* L744, 832 (FTI to block modification of H-Ras, but not K-Ras). Anchorage-independent growth was evaluated by observing colony formation after 14 days.

Also unlike the focus forming assay, in which clonal populations of transformed cells overgrow a monolayer of normal cells, soft agar assays require that essentially the entire population of cells to be tested expresses the gene of interest. To evaluate anchorage-independent growth of transforming Ras or Rho GTPases, seed stably expressing cell lines into soft agar. First, determine the number of dishes or wells necessary. We have found that 6-well plates (with 6 – 35-mm wells) are easy to handle, easy to photograph, and prevent the edge effects found on plates with smaller wells. For NIH 3T3 cells, 50,000 cells per well is sufficient, and since each cell line will be plated in triplicate (although duplicate sets can also be used), a total of $1.5 \times 10^5$ cells (50,000 cells x 3 wells) per cell line is needed. For NIH 3T3 cells, a nearly confluent (75-80%) 100-mm dish will provide a sufficient number of cells.

**Preparation of agar layers.** Each well will contain two different soft agar layers. The bottom layer (bottom agar) is devoid of cells, containing only 0.6% bacto-agar in growth medium as a solid support for the top layer. The top layer (top agar) contains the cells, seeded in a final concentration of 0.4% bacto-agar. For each plate, 2 ml bottom agar and 0.6 ml top agar will be made from 0.6% bacto-agar medium. Therefore the total volume of 0.6% bacto-agar needed depends on the number of wells needed for the experiment. Be sure to include the appropriate empty vector for each expression plasmid and the appropriate vehicle controls for each inhibitor in each assay. For a 24-well experiment, approximately 70 ml of 0.6% bacto-agar is required, so it is better to make 80 ml. Note that this solution will use both 1X and 2X versions of the growth medium appropriate to the cell type being tested. For NIH 3T3 cells, complete growth medium is DMEM-H + 10% calf serum and pen/strep.

Therefore, to make 80 ml of 0.6% bacto-agar suitable for soft agar assays of NIH 3T3 cells, add 26.7 ml 2X DMEM-H, 17.8 ml 1X DMEM-H, 8 ml calf serum ([Final] = 10%) and 0.8 ml 100X penicillin/streptomycin together in a sterile bottle. Incubate this mixture at 37°C for 10 minutes. During the incubation, melt 1.8% bacto-agar (Difco) in the microwave for about 1 minute and then cool to 55°C in a water bath. Add 26.7 ml of the melted 1.8% bacto-agar to the 37°C media mixture. It is important that the bacto-agar is not warmer than 55°C, because warmer temperatures will cause the serum proteins to precipitate. The growth medium should be at least 37°C, since cooler temperatures could cause the bacto-agar to solidify prematurely. Once the baseline 0.6% bacto-agar/medium is made, place 26 ml of it in a separate bottle to be used later for the top agar layer, and maintain at 55°C until needed. The remaining bacto-agar media will be used for the bottom agar layer. It is at this step that prenyltransferase inhibitors are first added.

For each drug and vehicle combination (i.e. FTI, GGTI or FTI + GGTI, DMSO), a separate aliquot of 0.6% bacto-agar media should be made. Then add enough drug volume to each appropriate aliquot such that the final drug concentration is 1X. Working quickly to prevent solidification of the drug-containing bacto-agar/ medium, add 2 ml of each drug condition to each appropriate well. Then allow the bacto-agar/medium to solidify for 10 to 15 minutes at room temperature. Drugs will not be added for the top agar until the cell lines are seeded, to prevent drug exposure to extended periods at 55°C.

While the bottom agar layer is forming, prepare the cells for mixing with the agar to form the top layer that will contain 0.4% bacto-agar (final concentration). Trypsinize the cells of interest and count the number of cells available. Then calculate the volume of cells needed to obtain 0.5 x $10^5$ cells per well in a volume of 0.2 ml of the appropriate growth medium (e.g., for NIH 3T3 cells, 10% calf serum-containing DMEM-H). Carefully resuspend the cells to the appropriate volume in the growth medium, and distribute the cells to polystyrene tubes. (Replicates can be pooled together.) Incubate briefly at 42°C until you are prepared to mix 0.6% bacto-agar mixture with the cells. While the cells are warming, retrieve the remaining 26 ml of 55°C, 0.6% bacto-agar that was partitioned earlier for the top agar. It is important to work as quickly as possible during the next steps to ensure that the bacto-agar does not solidify prematurely. Also, make sure that the bottom agar is completely solid in the 6-well plates before adding the top agar.

**Addition of Prenyltransferase Inhibitors to the Cells in the Top Agar Layer.** At this point, prenyltransferase inhibitors can be added to the 0.6% bacto-agar mix for the top agar. To do so, aliquot for each well 0.4 ml of 0.6% bacto-agar media to a new polystyrene tube for each stable cell line and drug treatment. (As above, replicates can be pooled together.) For each condition, add sufficient FTI or GGTI for a final concentration of 1-10 $\mu M$ as desired to the appropriate 0.6% bacto-agar containing polystyrene tube. Note that this final concentration is in reference to the entire final top agar volume, not to the volume of 0.6% bacto-agar used in this step. Leave these tubes at 42°C and remove only when immediately ready to mix bacto-agar with cells. (We find it convenient to keep a heat block in the tissue culture hood

to keep the individual tubes warm.) To avoid solidification, do not leave the 0.6% bacto-agar containing tubes for very long at 42°C.

Add the cell mixtures to their respective separate tubes containing the 0.6% bacto-agar + drugs, and mix well by pipetting up and down or by very gentle vortexing. The cells will now be in drug-containing agar/growth medium mixture at a final concentration of 0.4% agar. Immediately pour or pipet cell mixture on top of the previously solidified bottom agar. If using multiple cell lines, it is usually better to do each cell line sequentially rather than all at once. Allow the top agar to cool and solidify for 10 minutes at room temperature. Once the top agar solidifies, the seeded cells should be observed under the microscope. Only single cell suspensions should be present. However, if there are cell clumps visible, usually because cells were not thoroughly resuspended after trypsinization, then make note of their presence such that these clumps are not mistaken for bona-fide transformed colonies when the assay is scored at the end of the 14 day incubation period.

Figure 2.2B shows an example of the ability of an FTI (3 μ*M* L744, 832) to reduce SA colony formation of H-Ras-expressing cells when compared to DMSO vehicle treatment. As expected, K-Ras colony formation is uninhibited by FTI treatment since K-Ras can be geranylgeranylated in the presence of FTI, and maintain its membrane association and transforming activity.

***S-trans,trans-farnesylthiosalicylic acid (FTS)***

An alternative approach to disrupting membrane localization and biological functions of Ras/Rho family proteins involves the use of the S-farnesyl cysteine mimetic, *S-trans,trans*-farnesylthiosalicylic acid (FTS). Unlike FTIs and GGTIs, which prevent prenylation and result in an accumulation of unprocessed Ras/Rho proteins, FTS (and its counterpart GGTS, geranylgeranylthiosalicylic acid) inhibit Ras/Rho signaling by dislodging fully processed (prenylated) proteins from their respective cellular membranes (Marom et al., 1995). It is believed that FTS and GGTS (which resemble the farnesylated or geranylgeranylated cysteine, respectively) competitively interfere with membrane anchorage domains such as lipids or other membrane-bound proteins that recognize the farnesylated cysteine residue of the processed Ras/Rho C-terminus. The non-membrane bound prenylated proteins, which are no longer in a physiologically appropriate environment, are then targeted for degradation. Because FTS acts by competition with already synthesized proteins, the effects of FTS on Ras signaling occur more rapidly than those of FTIs and GGTIs (Gana-Weisz et al., 1997), which act only on newly synthesized proteins. Ras proteins have a half-life of approximately 22 hr, so it is valuable to be able to interfere with proteins already present at steady state. The use of FTS on Ras-transformed cells is described below.

***Preparation of FTS Stock and Working Solutions.*** The stock solution is 0.1 *M* FTS (Calbiochem, San Diego, CA) in chloroform (3.6 mg/100 μl). Dissolve the powdered FTS (MW = 358) in chloroform in a fume hood, and keep on ice to minimize evaporation. Distribute 10 μl aliquots into 0.7 ml microfuge tubes, closing

each tightly and wrapping in foil to keep out light and moisture. When stored at -20°C or -70°C, the FTS solution should be stable for a few weeks.

The working solution (100 X) is 2.5 m*M* FTS in 10% DMSO, made up in complete growth medium appropriate for the cells to be used. For example, the growth medium for NIH 3T3 cells is the high glucose formula of Dulbecco's modified Eagle medium (DMEM-H), supplemented with 10% calf serum (CS) and penicillin/streptomycin (P/S). Therefore, the working solutions will be prepared by diluting the stock solution in DMEM-H + 10% calf serum + P/S + 10% DMSO. Note that final concentrations of DMSO on the treated cells must not exceed 0.1% (v/v). DMSO is a free radical scavenger and can affect experimental results. This is an even more critical consideration for FTS than for FTI treatment.

Great care must be taken to avoid precipitation of the FTS. Prepare fresh working solutions of FTS immediately prior to each experiment. Evaporate the chloroform from the stock solution under a gentle stream of nitrogen. Next, add 40 μl DMSO to reconstitute the FTS and vortex. Then add 360 μl of the appropriate complete growth medium, e.g., DMEM/10% FCS. Mix well and leave the mixture at room temperature to avoid precipitation of the FTS. Do not place on ice, as this will cause freezing, and freeze/thawing causes instability of the FTS. This is now a 10 m*M* solution. For all further dilutions to make working solutions, use complete growth medium supplemented with 10% DMSO. Always thaw a new aliquot of stock solution and make a fresh FTS working solution for each experiment. Purchase fresh FTS powder every few months.

***FTS Treatment of Ras-transformed Cells for Growth Inhibition and Other Endpoints.*** Final concentrations of FTS appropriate for growth inhibition are 25 – 250 μ*M*, depending on cell sensitivity. For NIH 3T3 cells stably expressing oncogenic Ras, 25 μ*M* is sufficient to inhibit growth, whereas NIH 3T3 cells expressing only empty vector are resistant to this concentration. For growth inhibition assays whose endpoint is visual inspection, simply plate cells at 1 x $10^5$ cells per 60-mm dish. For higher throughput assays, or growth curves where cell number is measured by MTS or similar, the size of the dishes or multiwell plates and the cell number should be scaled down accordingly. Seed a sufficient number of dishes or wells to account for treatment not only with the desired conditions for FTS (e.g., different doses or time points) but also with the negative controls of vehicle (DMSO) and GTS (geranylthiosalicylic acid, also from Calbiochem). Both DMSO and GTS should be used at the same final concentration as FTS. GTS, not to be confused with GGTS, is a related thiosalicylic acid derivative but functions as a negative control because it is not recognized by the membrane anchoring domains that interact with farnesyl and geranylgeranyl moieties.

The day after plating the cells, prepare growth medium supplemented to final concentrations of 25 μ*M* FTS + 0.1% DMSO by diluting the freshly prepared working solution [2.5 m*M* FTS + 10% DMSO in DMEM-H/10% CS] with complete medium 9:1 (v:v). Aspirate the culture medium and rinse with 1X PBS. Replace the aspirated culture medium with the FTS-containing growth medium. Three ml is sufficient for each 60-mm dish. Return to the incubator for the desired period of time. In the accompanying figure (Figure 2.3A), the effect of a 48 h treatment of FTS on NIH 3T3

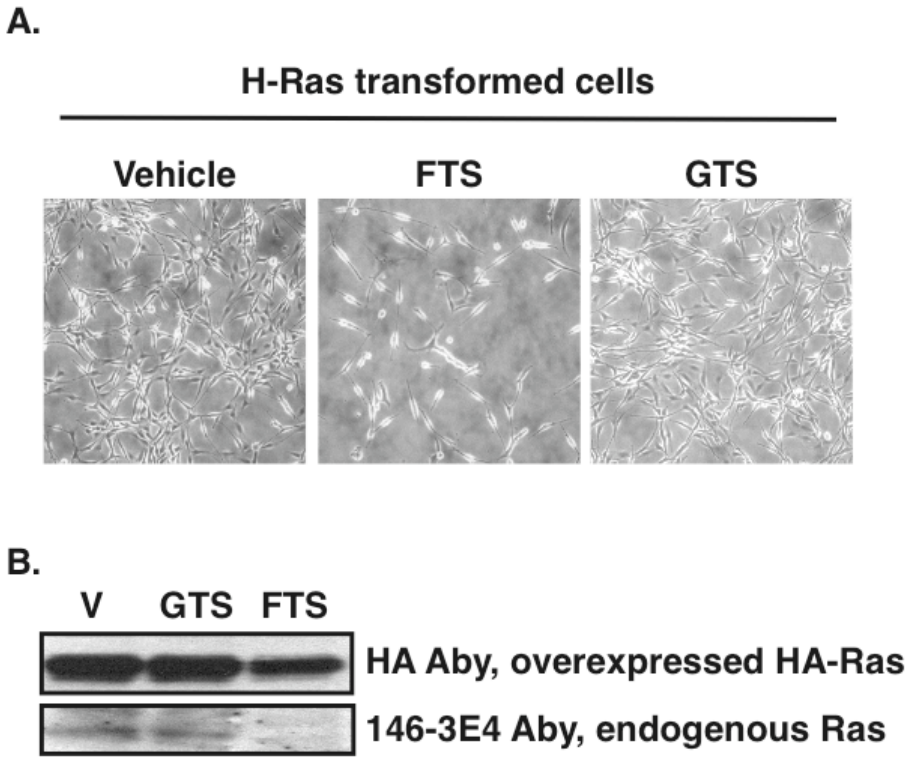


**Figure 2.3 *S-trans,trans*-farnesylthiosalicylic acid (FTS) reduces growth of H-Ras-expressing cells and H-Ras protein stability.** (A) NIH 3T3 cells stably expressing H-Ras(Q61L) were treated with either DMSO (vehicle control), 25 μ*M* FTS, or 25 μ*M* GTS (negative control for nonspecific effects of thiosalicylic acid). Cell cultures were photographed after 48h. (B) Western blot analysis of H-Ras protein levels. Ten micrograms of protein lysates prepared from NIH 3T3 cells stably expressing HA-tagged H-Ras(Q61L), treated with either DMSO (vehicle), 25 μ*M* FTS, or 25 μ*M* GTS, was resolved on 12% SDS-PAGE gels and transferred to PVDF membranes for immunoblotting with anti-HA (exogenous pCGN-H-Ras) or anti-H-Ras antibodies (endogenous H-Ras). FTS but not GTS treatment reduces the abundance of Ras proteins at steady state.

cells expressing oncogenically mutated H-Ras(61L) was evaluated by examining the cells using an inverted brightfield microscope. Whereas vehicle and GTS treatment had no effect on the growth of Ras-transformed cells, fewer cells were present in cultures treated with FTS.

The same conditions of FTS treatment can also be applied to soft agar colony formation as described above for prenyltransferase inhibitors, or to saturation density assays, apoptosis assays or other desired endpoints. The most critical aspects for successful FTS treatment are to use fresh FTS, to avoid FTS precipitation, and to limit the final concentrations of and to properly control for the effects of the DMSO vehicle. In addition, it is critical to always compare the effects of FTS to its inactive counterpart, GTS to rule out nonspecific effects of thiosalicylic acid.

***Evaluation of Ras Protein Levels on FTS Treatment.*** FTS is thought to deregulate prenylated Ras by dislodging it from the plasma membrane, thereby leading both to lower levels of active, membrane-associated Ras and also to accelerated Ras protein degradation (Marom et al., 1995). Therefore, successful FTS inhibition of Ras function will be accompanied by a decrease in levels of total Ras protein. Treatment of the same cells with DMSO vehicle or GTS controls should have no effect on Ras protein stability. To examine this, treat cells with FTS or controls as described above. Then, collect whole cell lysates by aspirating the FTS-containing culture medium, rinsing with 1X PBS and adding (for each 60 mm dish) 100 μl of magnesium lysis buffer (25 m*M* HEPES, 150 m*M* NaCl, 1% NP-40,

0.25% Na-deoxycholate, 10% glycerol, 10 m*M* $MgCl_2$ and EDTA, pH 8.0) containing a cocktail of protease inhibitors (e.g., complete protease inhibitor tablet, Roche or Sigma). Incubate the cells in lysis buffer for 5 minutes at 4°C, scrape the lysed cells off the plate and transfer into microfuge tubes. Clear the lysates by centrifugation for 5 minutes at 12,000 rpm, 4°C; save the supernatant and discard the pellet. Next, normalize protein concentrations in the cleared whole cell lysates using a DC Lowry assay (BioRad) or similar. Load 10 μg of each protein lysate, along with a protein molecular weight marker (e.g., BioRad Precision Plus dual color marker), onto each of one or more 12% SDS-PAGE gels. If it is desired to probe with different antibodies, e.g., for HA-tagged ectopic Ras protein vs. endogenous Ras protein, replicate gels can be run using multiple aliquots of the original cell lysate. It is also possible to strip and reprobe blots of a single gel. Run the gel at 25-35 mA until the 20 kD marker is well separated from the 25 kD marker to ensure good separation of HA-tagged H-Ras protein. Transfer the resolved proteins to PVDF membranes for standard western blotting analysis of Ras protein expression (Cox et al., 1995).

As seen in Figure 2.3B, Ras-transformed NIH 3T3 cells stably expressing HA-tagged H-Ras(61L) were treated with 25 μM FTS or GTS, or DMSO vehicle, and the cell lysates were resolved as indicated above. We probed for ectopic, overexpressed H-Ras (upper panel) using anti-HA antibody (Covance) as well as for endogenous H-Ras (lower panel) (anti-Ras 146-3E4 antibody (Quality Biotech, Camden, NJ); OP-40 or OP-41 clones also work well (Calbiochem or Santa Cruz)). Treatment with FTS, but not with vehicle or GTS, caused a decrease in H-Ras protein levels. Similar studies can be performed with GGTS to evaluate geranylgeranylated

members of the Ras (Ral, Rap, R-Ras) and Rho (Rho, Rac, Cdc42, etc.) family of small GTPases.

## SEQUESTRATION OF PRENYLATED SMALL GTPASES BY RHOGDIs

### *Evaluation of RhoGDI/Rho Interaction by Coimmunoprecipitation*

To determine the relevance of RhoGDIs as inhibitors of Rho family membrane association, it is important to determine whether RhoGDIs physically associate with the particular Rho protein(s) of interest. A common method of demonstrating protein:protein interaction is coimmunoprecipitation (co-IP), which can be used to evaluate RhoGDI binding to Rho proteins. This assay is based on the principle that immunoprecipitation of one protein (e.g., RhoGDI) with a specific antibody is capable of bringing down an associated protein (e.g., Rho), which can then be detected by a second antibody directed against the latter. These co-IPs can also be done in the reverse direction to assure specificity of the interaction. While it is more physiologically relevant to observe the RhoGDI/Rho interaction of endogenous proteins, the availability of antibodies specific for the particular endogenous Rho protein may be limited. To overcome this issue, it is often necessary to ectopically express epitope-tagged proteins, thereby allowing the use of generic antibodies directed against the epitope tag.

### GENERATION OF CELL LYSATES CO-EXPRESSING RHOGDI AND RHO PROTEINS.

If ectopic expression is to be used, this can be done either by use of existing stably expressing cells or following transient transfection. For transient transfections, plate NIH 3T3 fibroblasts in 60-mm dishes at a density of $2.0 \times 10^5$ cells per dish. Twenty-

four hours after plating, transfect cells with 100 ng - 1 μg of the Rho GTPase expression plasmid of interest in the presence of 100 ng - 1 μg of the RhoGDI expression plasmids. As controls for the influence of "vectorology", each expression plasmid should also be co-expressed with the corresponding empty vector for the other plasmid. The amount of plasmid should be chosen empirically, and depends on the expression level of the protein of interest, which may be cell context-dependent. It is desirable to achieve expression just sufficient for a good signal, but not so much as to introduce overexpression artifacts. This can be a delicate and difficult balancing act. We routinely begin with 500 ng of each plasmid, for example, pCGN-Rho and pCGT-RhoGDI. The Rho GTPase expressed from pCGN will be HA-tagged while the RhoDI expressed from pCGT will be T7-tagged (Fiordalisi et al., 2001). Any standard transfection protocol can be used; we routinely use calcium phosphate or liposomal reagents such as Lipofectamine Plus or FuGene. Incubate the transfected cells for 24 hr. Retroviral infection is another widely accepted alternative to transfection (for a sample protocol, see Fiordalisi et al., 2001).

To make the cell lysate, aspirate the culture medium and rinse the cells twice in 2 ml of ice-cold 1X PBS. Before addition of the lysis buffer, carefully aspirate any remaining PBS to ensure that the lysates are not diluted by residual PBS. For each 60-mm dish, lyse the cells on ice for 5 minutes in 500 μl of RIPA buffer (0.5 *M* Tris pH 7.0, 0.15 *M* NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% NP-40, 5 μg/ml aprotinin, 10 μ*M* leupeptin, 20 m*M* β-glycerophosphate, 12 m*M* p-nitrophenylphosphate, 0.5 m*M* Pefabloc, 0.1 m*M* sodium vanadate and 0.1% β-mercaptoethanol). It is important to use a stringent lysis buffer such as RIPA to

reduce the likelihood of nonspecific binding. Scrape the cells from the plate and transfer the 500 μl of cell lysate to a 1.5 ml microfuge tube.

**PERFORMING THE IMMUNOPRECIPITATION.** To clear away the cellular membranes from the lysates, centrifuge the whole cell lysates for 10 minutes at 12,000 rpm at 4$^{o}$C. Save the supernatant and discard the pellet. Determine the protein concentration using the DC Lowry protein assay (BioRad) or similar, and normalize all the samples to 1 μg/μl in 500 μl for the immunoprecipitation steps. Transfer 500 μl of each normalized lysate to a 1.5 ml microfuge tube. In addition, put aside and save on ice 10 μg of each total cell lysate (TCL) for later expression analysis using western blotting. This will be used to evaluate the input amount for each protein.

To reduce non-specific binding, it is also important to first pre-clear the lysates with the reagent that will be used to collect the immune complexes, such as Protein A/G Plus agarose beads (e.g., Santa Cruz). Add 20 μl of Protein A/G Plus agarose beads to each lysate and rotate at 4°C for 1 hour. Then pellet the beads by centrifugation of the microfuge tubes for 1 minute at ~13,000 rpm. This step can be performed at room temperature, but it is preferable to do so at 4°C for improved protein stability. Transfer the supernatant containing the precleared lysate to a new 1.5 ml microfuge tube. Save the pelleted beads and wash them 5 times with 500 μl of RIPA buffer; pellet the protein A/G beads by centrifuging for 1 minute at 13,000 rpm and decanting the supernatant in between wash steps. Resuspend the pelleted beads in 50 μl of 2.5X sample buffer and save on ice for later western blot analysis. This will be done as an important control to evaluate the degree to which the

proteins of interest bind to the beads in the absence of the specific antibodies used for precipitation.

To form the antibody-protein immune complex and immunoprecipitate HA-tagged Rho GTPases, add 5 μg (5 μl of a 1 mg/ml aliquot) of anti-HA antibody (Covance) to each precleared lysate and rotate the microfuge tubes at 4°C. After 1 h, the immune complexes should be formed. To collect them, add 20 μl of Protein A/G Plus agarose beads to each microfuge tube and rotate again at 4°C for 1 hour to allow the antibody complexes to bind to the Protein A/G. Then, pellet the beads by centrifugation for 1 minute at 13,000 rpm, and remove the supernatant carefully from the beads that now contain the bound immune complexes. It is strongly recommended to use a pipetting device rather than to aspirate at this step, to avoid the undesirable possibility of losing any beads. Wash the pelleted beads 5 times with 500 μl of RIPA buffer; pelleting by centrifugation for 1 minute at 13,000 rpm at 4°C and carefully decanting the supernatant in between wash steps. Finally, resuspend the pelleted beads directly in 50 μl of 2.5X protein sample buffer for western blot analysis of the immunoprecipitated HA-tagged Rho GTPases and their associated proteins. At this point, samples may be resolved by SDS-PAGE immediately, or frozen for later analysis. If freezing, be sure to also freeze the total cell lysate and preclear controls set aside earlier.

**DETECTING THE COIMMUNOPRECIPITATED PROTEINS.** Western blotting is used to detect which proteins are contained in the precipitated immune complexes. First elute the bound proteins from the beads by boiling each sample at 100$^{o}$C for 2

minutes. Be sure to also boil the TCL and preclear controls set aside earlier. To make it easier to load only protein and not beads onto the SDS-PAGE gel, centrifuge each boiled sample for 1 minute at 13,000 rpm at room temperature. Transfer 25 μl of the supernatant of this step (it is not necessary to transfer to a new tube) onto a 12% SDS-PAGE gel and run until the molecular weight markers indicate the desired degree of separation. Transfer the resolved proteins onto a PVDF membrane for western blotting. For detecting T7-tagged RhoGDI, a 1:1,000 dilution of anti-T7 mouse monoclonal antibody (Novagen) in TBS-T for 1 hour at room temperature is recommended. For detecting HA-tagged Rho GTPases, a 1:1000 dilution of anti-HA mouse monoclonal antibody (Covance) in TBS-T for 1 hour at room temperature is suitable.

We used the above co-immunoprecipitation method to demonstrate the specificity of RhoGDI interaction with some, but not all, Rho family proteins. Cdc42, a known geranylgeranylated substrate for RhoGDI binding and membrane targeting regulation (Hart et al., 1992; Leonard and Cerione, 1995), is used as a positive control for RhoGDI interaction. Palmitoylation of Rho family GTPases such as RhoB and TC10 has been shown to prevent interactions with RhoGDI with. We predicted that Wrch-1, a newly identified homolog of Cdc42 (Tao et al., 2001), that has both putative isoprenylation and palmitoylation signals, therefore might not interact with RhoGDI. We transiently transfected NIH 3T3 cells with pCGN-HA-Cdc42 or pCGN-HA-Wrch-1 along with pCGT-T7-RhoGDIα. We then immunoprecipitated Cdc42 and Wrch-1 using anti-HA antibody and immunoblotted the resulting precipitates with anti-HA to detect the GTPases and anti-T7 to detect RhoGDI.

As predicted, RhoGDI coimmunoprecipitated with Cdc42 but not with Wrch-1, (Figure 2.4). This is consistent with our observation that Wrch-1 entirely lacks isoprenoid modification and is, instead, palmitoylated, making it an unlikely target of RhoGDI interaction and regulation despite the fact that it is C-terminally lipidated. Thus, RhoGDI would also be predicted to sequester Cdc42 but not Wrch-1 from membrane locations.

***RhoGDI Regulation of Rho GTPase Localization***

RhoGDIs can be used as tools for membrane dissociation of the Rho GTPases with which they associate. As discussed earlier, a simple method for visually demonstrating loss of membrane association of these small GTPases utilizes EGFP-fusion proteins that are targeted to the nucleus and cytosol (the "EGFP-alone" distribution pattern) upon loss of lipid modifications or of membrane anchorage. To adapt this method to RhoGDI inhibition of Rho family membrane targeting, EGFP-tagged Rho GTPases should be expressed in cells also expressing the RhoGDI(s) at appropriate levels. If RhoGDIs bind EGFP-Rho proteins and regulate their membrane association, then a loss of membrane localization and an accumulation of nuclear and cytosolic EGFP signal will be observed. Previous studies have shown that overexpression of RhoGDIs is sufficient to cause mislocalization of Rho proteins (Michaelson et al., 2001). In addition, the molar amounts of RhoGDI in cells roughly equals the total levels of endogenous RhoA, Rac1 and Cdc42, suggesting that there is no available excess endogenous RhoGDI to bind and regulate exogenously To correlate RhoGDI interaction with Rho GTPases with its ability to regulate Rho localization, we expressed pEGFP-Cdc42 or pEGFP-Wrch-1 in the presence of

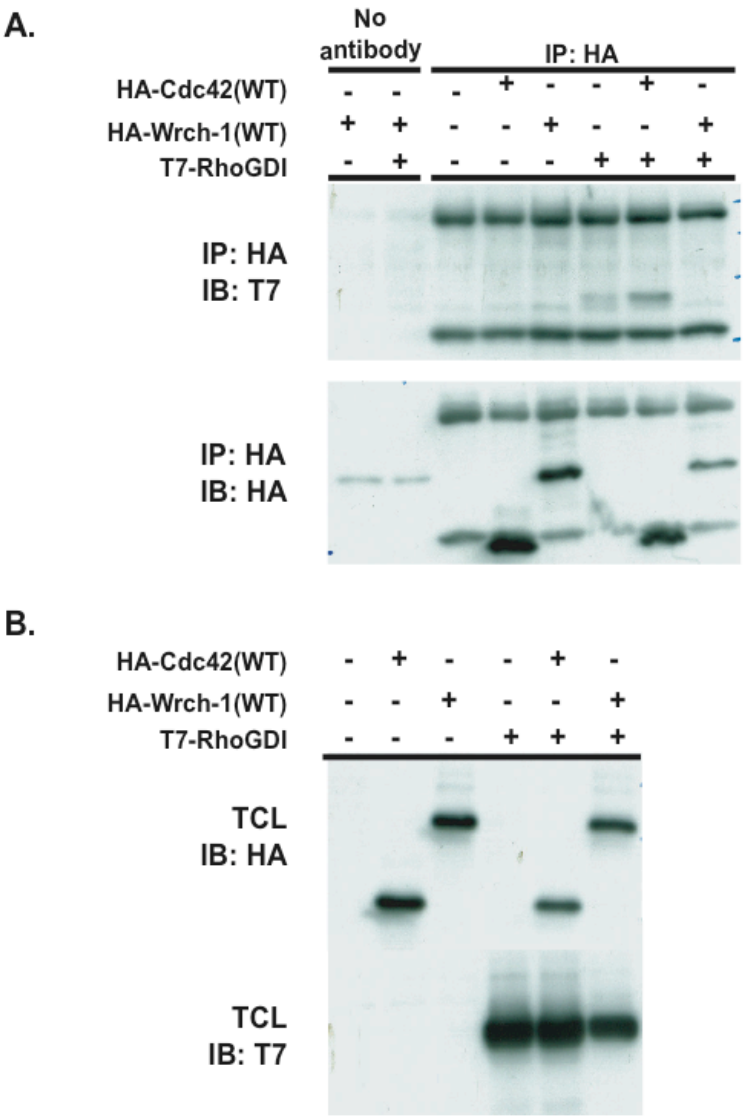


**Figure 2.4 Rho GDI interacts with geranylgeranylated Cdc42, but not with palmitoylated Wrch-1.** (A) NIH 3T3 fibroblasts were transiently transfected with HA-tagged Cdc42 or Wrch-1 in the presence or absence of T7-tagged RhoGDI. After 24 h, the HA-tagged Rho GTPases were immunoprecipitated with mouse anti-HA antibody, and RhoGDI binding was assessed by Western blot analysis using mouse anti-T7 antibody. (B) Expression levels of input HA-tagged Rho GTPases and T7-tagged RhoGDI. Ten micrograms of total cell lysates from transiently transfected NIH 3T3 fibroblasts was resolved on 12% SDS-PAGE and transferred to PVDF membrane for Western blot analysis using antibodies against the HA and T7 epitope tags.

pCGT-RhoGDI or empty pCGT vector and visualized the GFP-tagged proteins using fluorescence microscopy (Figure 2.5). As previously shown, Cdc42(WT) localization is regulated by RhoGDI (Leonard and Cerione, 1995; Michaelson et al., 2001) and showed a pronounced redistribution of GFP-Cdc42 from cellular membranes to the nucleus and cytosol, consistent with the masking of the geranylgeranyl lipid moiety by RhoGDI binding. In contrast, RhoGDI expression had no effect on the localization pattern of GFP-Wrch-1, a palmitoylated protein that is not a binding partner of RhoGDI and whose subcellular localization is, therefore, unaffected by RhoGDI expression. These data, in combination with the coimmunoprecipitation results above, illustrate how the normal activities of RhoGDIs (membrane dissociation) can be exploited as tools to investigate potential functional consequences of Rho family proteins by disrupting membrane interactions.

## CONCLUDING REMARKS

We have described here simple techniques whereby pharmacological and endogenous protein inhibitors of the lipid modifications of Ras and Rho GTPases can be used as investigative tools to understand small GTPase localization and transforming abilities. These assays can easily be adapted to different cell types. The same inhibitors can also be used in signaling assays to selectively disrupt downstream signaling pathways of Ras and Rho GTPases, providing further insight into the functions of this very diverse group of proteins.

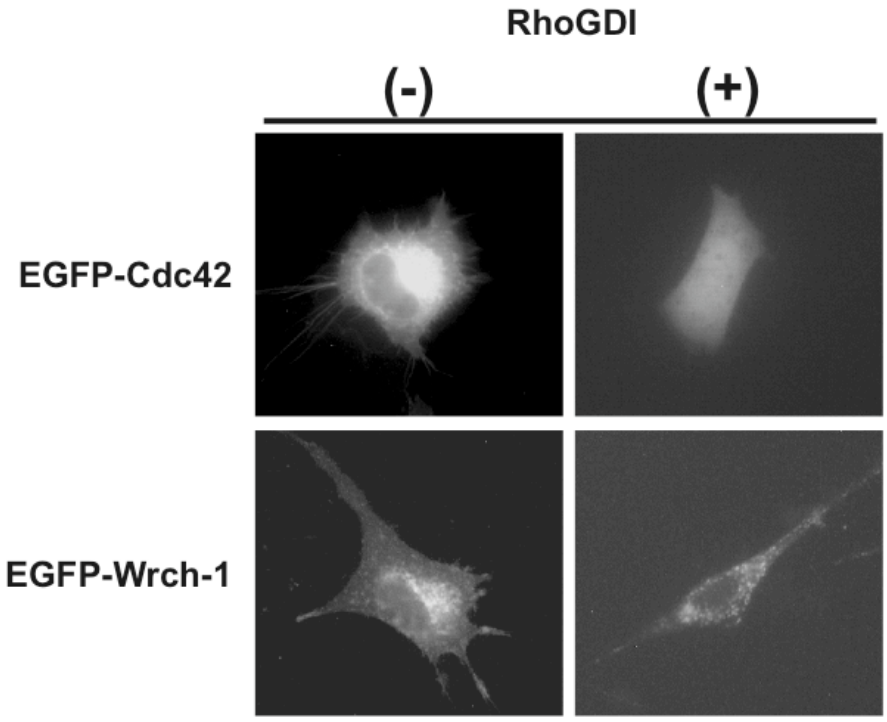


**Figure 2.5 Coexpression with RhoGDIα causes dissociation of Cdc42 but not of Wrch-1 from cellular membranes.** To assess the ability of RhoGDI to regulate Rho GTPase localization, NIH 3T3 fibroblasts seeded on glass coverslips were transiently transfected with GFP-tagged Cdc42 or Wrch-1 in the presence or absence of T7-tagged RhoGDI. After 24 h, the localization of each GFP-tagged GTPase was visualized by epifluorescence microscopy with a FITC filter. Localization of the GFP-tagged GTPase to the cytosol and nucleus in the presence of RhoGDI but not empty vector suggests that the lipid modification is masked by RhoGDI association with the GTPase.

# CHAPTER III

# TRANSFORMING ACTIVITY OF THE RHO FAMILY GTPASE, WRCH-1, A WNT-REGULATED CDC42 HOMOLOG, IS DEPENDENT ON A NOVEL CARBOXYL-TERMINAL PALMITOYLATION MOTIF

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**ABSTRACT**

Wrch-1 is a Rho family GTPase that shares strong sequence and functional similarity with Cdc42. Like Cdc42, Wrch-1 can promote anchorage-independent growth transformation. We determined that activated Wrch-1 also promoted anchorage-dependent growth transformation of NIH 3T3 fibroblasts. Wrch-1 contains a distinct carboxyl-terminal extension not found in Cdc42, suggesting potential differences in subcellular location and function. Consistent with this, we found that Wrch-1 associated extensively with plasma membrane and endosomes, rather than with cytosol and perinuclear membranes like Cdc42. Like Cdc42, Wrch-1 terminates in a CAAX tetrapeptide (C=cysteine, A=aliphatic, X=any amino acid) motif (CCFV), suggesting that Wrch-1 may be prenylated similarly to Cdc42. Surprisingly, unlike Cdc42, Wrch-1 did not incorporate isoprenoid moieties and Wrch-1 membrane localization was not altered by inhibitors of protein prenylation. Instead, we show that Wrch-1 is modified by the fatty acid palmitate, and pharmacologic inhibition of protein palmitoylation caused mislocalization of Wrch-1. Interestingly, mutation of the second cysteine of the C<u>C</u>FV motif (C<u>C</u>FV > C<u>S</u>FV), but not the first, abrogated both Wrch-1 membrane localization and transformation. These results suggest that Wrch-1 membrane association, subcellular localization and biological activity are mediated by a novel membrane-targeting mechanism distinct from that of Cdc42 and other isoprenylated Rho family GTPases.

## PREFACE

This chapter represents work published in the *Journal of Biological Chemistry.* This was my very first "first-author" publication! I wrote all sections of this chapter and contributed most of the data presented here. Janice E. Buss provided [$^3$H]palmitate metabolic labeling data in the "Wrch-1 Subcellular Localization Is Dependent on Palmitoylation" section. Emily J. Chenette performed all indirect analyses of Wrch-1 fatty acid modifications with the biotin-BMCC reagent described in the "Wrch-1 Subcellular Localization Is Dependent on Palmitoylation" section. Carolyn A. Weinbaum provided data for the incorporation of [$^3$H]farnesylpyrophosphate (FPP) and [$^3$H]geranylgeranylpyrophosphate (GGPP) by small GTPases in the "Wrch-1 Localization Is Not Dependent on Isoprenoid Modification" section. Adam Shutes contributed data demonstrating dependency of downstream activation of p21-activated kinase (PAK) on Wrch-1 localization in "The Carboxyl-terminal Cysteines Are Necessary for Wrch-1 Signaling to PAK" section. Audrey Minden provided the Wrch-1 expression vectors (originally created in the lab of Arnold Levine), which I utilized as templates to introduce missense mutations, creating constitutively activated and C-terminal mutants of Wrch-1. Channing J. Der and Adrienne D. Cox edited all sections within this chapter.

## INTRODUCTION

The Rho family of Ras-related small GTPases is a functionally diverse group of proteins that are best known for their roles in regulation of actin cytoskeleton organization, cell polarity, cell adhesion, vesicular trafficking, transcriptional regulation and cell cycle progression (Etienne-Manneville and Hall, 2002; Raftopoulou and Hall, 2004). Of the 22 known human Rho GTPases, RhoA, Rac1 and Cdc42 are the most extensively characterized family members (Wennerberg and Der, 2004).

Like Ras, Rho proteins cycle between an inactive GDP-bound state and an active GTP-bound state (Etienne-Manneville and Hall, 2002; Symons and Settleman, 2000; Van Aelst and D'Souza-Schorey, 1997). Guanine nucleotide exchange factors activate Rho proteins by promoting GDP dissociation in exchange for GTP (Schmidt and Hall, 2002; Zheng, 2001), whereas GTPase activating proteins downregulate Rho protein function by stimulating their intrinsic GTPase activity to hydrolyze GTP to GDP (Moon and Zheng, 2003). A third regulatory class of proteins includes the Rho guanine nucleotide dissociation inhibitors (RhoGDIs) that bind the carboxyl-terminus of Rho GTPases and sequester them in the cytosol (Geyer and Wittinghofer, 1997; Olofsson, 1999). Missense mutations within the switch regions of Rho proteins lock them in a GTP-bound conformation and render these proteins GTPase-deficient and constitutively activated. Activated forms of some Rho family GTPases cause growth transformation of NIH 3T3 mouse fibroblasts and aberrant activity of both regulatory proteins and effectors of the Rho signaling pathways have

been linked to human cancers (Boettner and Van Aelst, 2002; Ridley, 2004; Sahai and Marshall, 2002; Zohn et al., 1998).

Wrch-1 (Wnt-regulated Cdc42 homolog-1) is a novel member of the Rho subfamily, whose transcription is upregulated in Wnt-1 transformation of mouse mammary epithelial cells (Tao et al., 2001). Like many other Rho family members, Wrch-1 activation is regulated by its nucleotide state, and a single missense mutation at residue 107 (analogous to Q61L activating mutation in Cdc42) rendered Wrch-1 more active in signaling (Tao et al., 2001). Ectopic expression of a constitutively active form of Wrch-1(107L) caused a Wnt1-like change in the cellular morphology of mammary epithelial cells, suggesting a contribution for Wrch-1 in Wnt transformation (Tao et al., 2001). Additionally, like other Rho family proteins, Wrch-1 activation can promote growth transformation .

The correct subcellular localization and function of Ras and Rho family members is dictated by posttranslational modification of the carboxyl-terminal hypervariable domain, including the last four amino acids known as the CAAX motif (Adamson et al., 1992a; Michaelson et al., 2001). The canonical CAAX motif consists of a cysteine residue (C), two aliphatic residues (AA) and, at the last position, any amino acid (X). The conserved cysteine residue serves as the site for posttranslational modification by either farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase I), which irreversibly attaches an isoprenoid moiety (Casey and Seabra, 1996). Although CAAX-signaled prenylation is necessary (Adamson et al., 1992b; Hancock et al., 1989; Joyce and Cox, 2003; Lebowitz et al., 1997; Solski et al.,

2002; Ziman et al., 1993), a second targeting signal immediately upstream of the CAAX motif is also required for proper membrane association and subcellular localization. Some small GTPases, such as H-Ras, N-Ras and the Cdc42-related protein TC10, are covalently modified by addition of a palmitoyl fatty acid on cysteine residues, whereas others, such as K-Ras4B and Cdc42, contain several basic residues that serve as their second targeting signal (Hancock et al., 1989; Hancock et al., 1990; Michaelson et al., 2001). Finally, additional carboxyl-terminal sequences provide further specificity in targeting Ras and Rho GTPases to distinct plasma membrane microdomains or to endomembrane compartments. Thus, functionally highly related Rho GTPase isoforms (e.g., RhoA, RhoB, and RhoC), by virtue of divergent carboxyl-terminal sequences and modifications, can exhibit strikingly different biological functions dependent on distinct subcellular locations (Adamson et al., 1992b; Du et al., 1999; Wang et al., 2003b).

Although Wrch-1 has been shown to localize to plasma membrane and internal membranes (Aspenstrom et al., 2004; Tao et al., 2001), the role of the carboxyl terminus in mediating Wrch-1 biological function has not been determined. Like Cdc42, Wrch-1 induces actin reorganization and formation of filopodia, and causes activation of the PAK and JNK serine/threonine kinases (Tao et al., 2001). However, Wrch-1 contains amino-terminal sequences not found in Cdc42 and we and others recently showed that this amino-terminal extension serves as a negative modulator of Wrch-1 effector interaction (Saras et al., 2004; Shutes et al., 2004). In addition, Wrch-1 and Cdc42 differ considerably at their carboxyl termini, sharing only 25% sequence identity. Furthermore, Wrch-1 has an extended carboxyl terminus ending

in an unusual CAAX sequence (CCFV) not characteristic of known substrates for farnesyl- and geranylgeranyltransferases (Cox and Der, 2002; Fu and Casey, 1999). Therefore, in the present study we assessed the mechanism and role of the unique carboxyl terminus in regulation of Wrch-1 function. As with Cdc42, we found that Wrch-1 activation caused anchorage-dependent growth transformation of NIH 3T3 cells. However, while the carboxyl terminus was critical for Wrch-1 subcellular localization and transforming activity, Wrch-1 function was dependent not on modification by prenylation but on modification by palmitoylation. Thus, while Wrch-1 and Cdc42 share significant functional properties, they exhibit considerable divergence in lipid modifications and subcellular distribution, and consequently, may have divergent roles in cell physiology.

## MATERIALS AND METHODS

**Molecular Constructs**—pcDNA3 expression construct encoding wild type (WT) and GTPase-deficient (Q107L) human Wrch-1 were obtained from Dr. A. Levine (Tao et al., 2001). Polymerase chain reaction (PCR)-mediated DNA amplification was used to introduce 5' and 3' *Bam*HI sites flanking Wrch-1(WT) for subcloning into various epitope-tagged expression vector constructs. To create other constitutively activated mutants of Wrch-1, a glutamate to leucine mutation was generated at residue 107 using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Site-directed mutagenesis was also used to create the carboxyl-terminal CCFV motif mutants by changing cysteine residues at positions 255 and 256 to generate CCFV > SCFV (C255S), CCFV > CSFV (C256S) and CCFV > SSFV (C255S, C256S) point mutants in the

activated Wrch-1(Q107L) background. To generate glutathione-s-transferase (GST), green fluorescence protein (GFP) and hemagglutinin (HA) epitope-tagged Wrch-1 proteins for prenylation, transformation and localization assays, Wrch-1 coding regions were digested with *Bam*HI and ligated into the *Bam*HI site of pGEX-2T multiple cloning site (MCS), the 5' *Bgl*II and 3' *Bam*HI sites of the pEGFP-C1 (MCS) and the *Bam*HI site of pCGN-hygro, respectively (Fiordalisi et al., 2001). All sequences were verified by the UNC-CH Genome Analysis Facility at the University of North Carolina at Chapel Hill.

**Cell Culture and Transfections**—NIH 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Invitrogen, Carlsbad, CA) and 1% pen/strep (Invitrogen). Stable NIH cell lines were created by transfection of pCGN-wrch-1 constructs with FuGene 6 (Roche, Indianapolis, IN) per the manufacturer's instructions. After 48 h, cells were split into DMEM medium containing 200 μg/ml hygromycin B (Roche) and maintained in antibiotic selection until colonies formed. Colonies (>50) were pooled for use in soft agar assays.

**Transformation Assays**—For focus forming assays, NIH 3T3 cells were seeded at 2 x $10^5$ cells per 60-mm dish. The following day, cells were transiently co-transfected for 4 h with HA-tagged pCGN constructs encoding empty vector, activated Wrch-1(107L)-CCFV, -SCFV, -CSFV or -SSFV carboxyl-terminal mutants along with pZIP-NeoSV(x)1 empty vector or pZIP-Raf22W (encoding an amino-terminal truncated and constitutively activated variant of human Raf-1), by calcium

phosphate precipitation as previously described. After 20 to 24 days, dishes were washed with 1X phosphate-buffered saline (PBS), fixed with 3:1 (v/v) methanol:acetic acid and stained with 0.4% crystal violet solution in 20% ethanol. Non-Raf foci of transformed cells (see text) were counted and the average number of foci found on duplicate sets of dishes was then calculated.

For soft agar assays, NIH 3T3 cells stably expressing HA epitope-tagged pCGN constructs of either empty vector, activated Wrch-1(107L)-CCFV, -SCFV, -CSFV or -SSFV were suspended in DMEM medium containing 10% calf serum, 1% pen/strep, and 0.4% agar (BD Biosciences) at 5 x $10^4$ cells per 35-mm dish. The single cell suspensions were layered on top of 0.6% agar in DMEM medium. Colonies that formed after 14 – 21 days were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the average number of colonies on triplicate dishes was calculated.

**Live Cell Imaging**—To analyze subcellular localization and lipid modifications of Wrch-1 proteins, NIH 3T3 cells were seeded onto glass coverslips in 35 mm dishes. The following day, cells were placed in DMEM medium supplemented with either DMSO vehicle, 10 μ*M* FTI-2153, 10 μ*M* GGTI-2166, 10 μ*M* FTI-2153 + GGTI-2166, 20 μ*M* compactin (Sigma, St. Louis, MO), or 150 μ*M* 2-bromopalmitate (2-BP, Sigma) immediately prior to transient transfection with GFP-tagged pEGFP constructs containing Wrch-1-tail(13 a.a.), Wrch-1(Q107L)-CCFV, -SSFV, Cdc42-tail(20 a.a.), Cdc42(61L), H-Ras(61L), K-Ras4B(12V), or Rab5. FTI-2153 and GGTI-2166 were generous gifts from Andrew D. Hamilton (Yale University) and Saïd

M. Sebti (H. Lee Moffitt Cancer Center and Research Institute, University of South Florida). After 24 – 48 h, live cell images were captured on either an epifluorescent Zeiss Axioskop or Zeiss 510 LSM confocal microscope (Zeiss, Thornwood, NY) using MetaMorph imaging software (Universal Imaging Corp., Downington, PA) or LSM 5 Image browser software (Zeiss, Thornwood, NY).

To visualize localization of GFP-tagged Wrch-1(Q107L) to early endosomes, NIH 3T3 fibroblasts were transiently transfected with pEGFP-Wrch-1(Q107L). 24 h later, cells were serum-starved for 30 min with DMEM medium, rinsed in 1X PBS and then treated with DMEM medium containing 30 μg/ml Texas Red-conjugated transferrin (Molecular Probes). After 10 min incubation, cells were rinsed in 1X PBS, placed in DMEM medium, and analyzed for GFP-tagged Wrch-1(Q107L) localization using fluorescein isothiocyanate (FITC) bandpass filter and Texas Red-conjugated transferrin using a Texas Red (TRITC) bandpass filter. Co-localization of GFP-tagged Wrch-1(Q107L) and Texas Red-conjugated transferrin images were analyzed using MetaMorph imaging software (Universal Imaging Corp., Downington, PA).

**Immunofluorescence**—NIH 3T3 cells were seeded onto glass coverslips in 35-mm dishes. After 24 h, cells were transfected with HA-tagged pCGN-wrch-1(107L) constructs using FuGene 6. After 24 h, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton-X100 (TX-100) in Tris-buffered saline (TBS). Cells were then incubated with anti-HA antibody (Covance) for 1 h at room temperature. After three washes in 0.1% TX-100 in TBS, cells were incubated with Alexa-fluor 488-conjugated secondary antibody for 30 min

(Molecular Probes) and washed three times with 0.1% Tx-100 in TBS. Coverslips were mounted onto glass microslides with Vectashield Hardset mounting medium (Vector Labs, Burlingame, CA) and analyzed on the fluorescent microscope as described above.

**Metabolic Labeling**—NIH 3T3 cells were seeded at 2 x $10^5$ cells per 60-mm dish and transiently transfected with HA epitope-tagged pCGN constructs containing Wrch-1(107L) CCFV, -SCFV, -CSFV, -SSFV, H-Ras(61L), K-Ras(12V) or empty vector with FuGene 6. After 48 h, cells were labeled for 4 h with 1 mCi / ml [$^3$H]-palmitate (American Radiochemical,Inc., ARC) in DMEM medium containing 5 m*M* sodium pyruvate, 4X non-essential amino acids, 1% glutamine, 20 m*M* HEPES pH 7.2, 25 μg / ml cycloheximide and 10% calf serum. Cells were then rinsed twice with TBS and lysed in Hi-SDS RIPA buffer (1 *M* Tris pH 7.0, 5 *M* NaCl, 10% SDS, 1% Na-deoxycholate, 1% NP-40, 0.2 *M* Pefabloc, 0.05 – 0.10 TIU/ml aprotinin). For immunoprecipitation, lysates were incubated for 1 h with anti-HA antibody and then incubated for 30 min with Protein A/G beads (Santa Cruz). The immunoprecipitates were washed, resuspended in non-reducing protein sample buffer (10% SDS, 1 *M* Tris-HCl pH 6.8, 25% sucrose, 0.01% bromophenol blue), resolved on SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF; Immobilon-P, Millipore, Bedford, MA). Membranes were then sprayed with EN$^3$HANCE (PerkinElmer Life Sciences) and exposed to film at -80°C for 90 days. To detect total amount of immunoprecipitated protein available for labeling, membranes were blocked in 5% nonfat dry milk and immunoprecipitated proteins were detected with primary mouse anti-HA antibody followed by anti-mouse IgG-horseradish peroxidase

(HRP)-conjugated antibody (Amersham Biosciences, Piscataway, NJ). Membranes were incubated in SuperSignal West Dura Extended Duration substrate (Pierce, Rockford, IL) and developed on film.

**Biotin (Btn)-BMCC fatty acylthioester bond labeling**—To label Wrch-1 cysteine-palmitate thioester bonds, a modified version of a recently described method for detecting protein palmitoylation was used (Drisdel and Green, 2004). Briefly, HEK 293 human embryonic kidney cells transiently expressing GFP-tagged Wrch-1 proteins were lysed in BMCC lysis buffer (150 m*M* NaCl, 5 m*M* EDTA, 50 m*M* Tris pH 7.4, 0.02% $NaN_3$, and 2% Triton-X 100) containing Complete protease inhibitor tablet (Roche, Indianapolis, IN). Whole cell lysates were cleared and protein concentrations were normalized using DC Lowry protein assay (BioRad). For immunoprecipitation, protein lysates were pre-cleared with protein A/G-conjugated agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA), incubated with anti-GFP antibody (BD Biosciences Clontech, Palo Alto, CA) for 2 h and then incubated for 1 h with protein A/G-conjugated beads (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were washed, resuspended in 50 m*M* N-ethylmaleimide (NEM) (Sigma) in BMCC lysis buffer to block free sulfhydryls groups and incubated for 48 h. Immunoprecipitates were washed, treated for 1 h with 1 *M* hydroxylamine (Sigma) to hydrolyze any cysteine-palmitate thioester bonds, washed and then treated with 1 μ*M* Btn-BMCC (Pierce, Rockford, IL) in 50 m*M* Tris, pH 7.0 for 2 h to label cleaved thioester bonds. Immunoprecipitates were washed, resuspended in non-reducing protein sample buffer (10% SDS, 1 *M* Tris-HCl pH 6.8, 25% sucrose, 0.01% bromophenol blue), resolved on SDS-PAGE and transferred to polyvinylidene

difluoride membrane (PVDF; Immobilon-P, Millipore, Bedford, MA). Membranes were probed with streptavidin-HRP (Pierce, Rockford, IL) to detect incorporation of Btn-BMCC, incubated in SuperSignal West Dura Extended Duration substrate (Pierce, Rockford, IL) and developed on film.

**Purified protein preparation and in-vitro prenylation assay**—Recombinant GST-tagged proteins were produced from *Escherichia coli* (*E. coli*) BL21 strain as previously described (Shutes et al., 2006; Shutes et al., 2004). Protein induction was confirmed by SDS-PAGE and coomassie staining.

For isoprenylation of purified proteins, 5 μg of each purified protein was added to prenylation reaction mixture containing 30 μg bovine brain high speed supernatant (HSS) and 1 μ*M* [$^3$H]-farnesylpyrophosphate (FPP) or [$^3$H]-geranylgeranylpyrophosphate (GGPP) (8–10 Ci/mmol; American Radiochemical, Inc., ARC). Reaction mixture was incubated for 30 min at 30°C. Reaction was stopped with SDS protein sample buffer. Samples were boiled briefly, run on 4-20% SDS-PAGE, prepared for fluorography and exposed to preflashed film for 4 days.

**Western Blot Analysis**—NIH 3T3 cells transiently expressing GFP-tagged or HA-tagged Wrch-1 proteins were lysed in 1% Triton-X100 lysis buffer containing protease inhibitors (5 μg/ml aprotinin, 10 μ*M* leupeptin, 20 n*M* β-glycerophosphate, 12 m*M* *p*-nitrophenylphosphate (PNPP), Pefabloc and 0.1 m*M* sodium vanadate) or magnesium lysis buffer (25 m*M* Hepes, 150 m*M* NaCl, 1% NP40, 0.25% Na-deoxycholate, 10% glycerol, 10 m*M* $MgCl_2$ and 1 m*M* EDTA, pH 8.0 containing

Complete protease inhibitor tablet (Roche, Indianapolis, IN). Whole cell lysates were cleared and protein concentration was determined using DC Lowry protein assay (BioRad). Fifteen μg of protein lysates were prepared in sample buffer, resolved on SDS-PAGE and transferred to Immobilon-P membrane. Membranes were then blocked in 5% nonfat dry milk and probed for HA-tagged Wrch-1 proteins using mouse anti-HA antibody, for β-actin as a loading control using mouse anti-β-actin (Sigma), for endogenous phosphorylated PAK using rabbit anti-phospho-PAK1 (Ser144)/PAK2 (Ser141), for total endogenous PAK using rabbit anti-PAK1/2/3 or for GFP-tagged proteins using mouse anti-GFP antibody (BD Biosciences, Clontech), followed by anti-mouse HRP-conjugated antibody or anti-rabbit HRP-conjugated antibody and SuperSignal West Dura Extended Duration substrate as above.

## RESULTS

**The Carboxyl-Terminal Nine Residues of Wrch-1 Promote a Subcellular Distribution Distinct From That Seen For Cdc42**—As shown in Figure 3.1A, the carboxyl-termini of Wrch-1 and Cdc42 exhibit sequence differences that may result in different functional roles for Wrch-1. First, Wrch-1 terminates in an atypical CAAX motif. Second, Wrch-1 contains an additional 21 residues that have no counterpart in the Cdc42 carboxyl-terminus. To determine the role of Wrch-1 carboxyl-terminal sequences in dictating Wrch-1 membrane association and subcellular location, and to compare these properties with those of Cdc42, we expressed GFP-tagged Wrch-1 and Cdc42 in NIH 3T3 mouse fibroblasts and performed live cell imaging analyses (Figure 3.1B). Whereas GFP alone localized to the cytosol and prominently in the

nucleus, both GFP-Cdc42 and GFP-Wrch-1 were excluded from the nucleus. Consistent with previous observations (Michaelson et al., 2001), we detected Cdc42 localization strongly to the cytosol, with its predominant membrane staining in the perinuclear region. Wrch-1, however, distributed mainly to the plasma membrane and to internal membranes reminiscent of endosomes and polarized, perinuclear Golgi, demonstrating an only partially overlapping localization pattern with that of Cdc42 (Figure 3.1B) (Aspenstrom et al., 2004; Tao et al., 2001).

We next wanted to determine which of the unique carboxyl-terminal sequences of Wrch-1 were sufficient to dictate its unique localization. Philips and colleagues (Michaelson et al., 2001) demonstrated previously that the carboxyl-terminal, CAAX- and hypervariable domain-containing 20 amino acids of Cdc42 and other Rho family GTPases were sufficient to determine their membrane targeting. As described previously, a GFP fusion protein terminating in the 20 carboxyl-terminal residues of Cdc42 exhibited the same subcellular distribution as authentic Cdc42 (data not shown) (Adamson et al., 1992b; Michaelson et al., 2001). Despite the extended length of the Wrch-1 carboxyl-terminus, we found that even fewer residues were sufficient for authentic Wrch-1 localization, as a GFP fusion protein terminating in the nine most carboxyl-terminal residues of Wrch-1 displayed the same plasma membrane and endosomal localization as authentic Wrch-1 (Figure 3.1B). To confirm that the internal membrane structures were indeed endosomes, we compared directly the localization of GFP-Wrch-1 and Texas Red-transferrin, which marks early endosomes. As shown in Figure 3.1C, merging these images demonstrated that Wrch-1 on internal membranes colocalizes with transferrin. Thus,

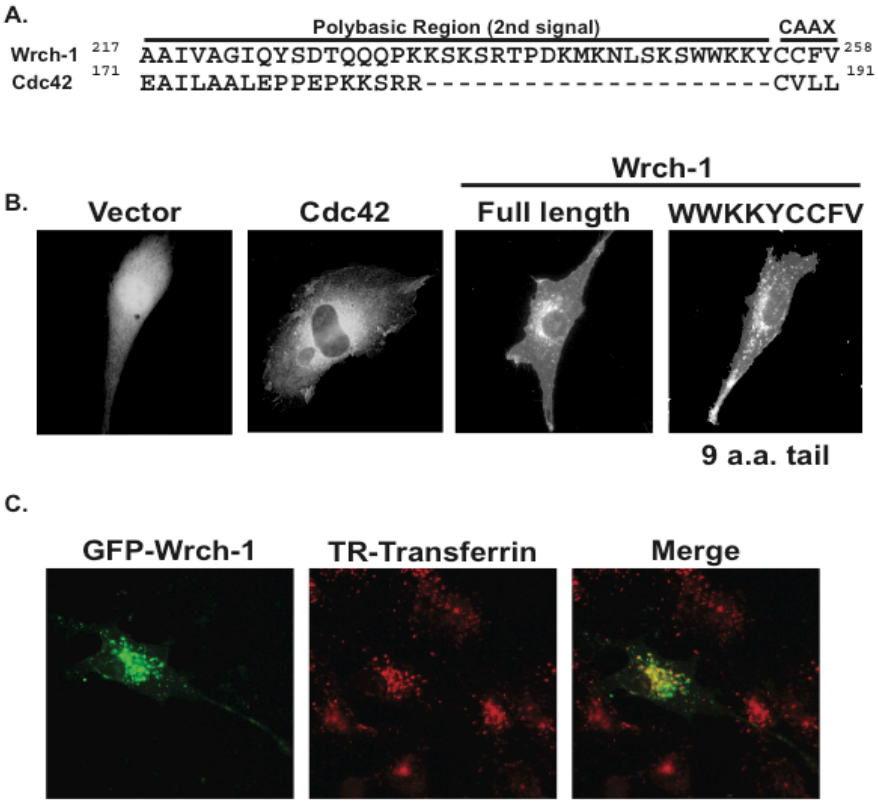



**Figure 3.1 The carboxyl-terminal hypervariable domain contributes to differences in subcellular localization of Cdc42 and Wrch-1.** (A) Sequence alignment of the hypervariable domains of Cdc42 and Wrch-1 demonstrates differences in length and composition. (B) Wrch-1 and Cdc42 exhibit distinct subcellular locations. NIH 3T3 fibroblasts were transiently transfected with pEGFP empty vector or pEGFP constructs encoding GFP-tagged full-length Cdc42 or Wrch-1, or GFP fused to the carboxyl-terminal nine residues of Wrch-1, which are sufficient to promote Wrch-1 membrane association. a.a., amino acids. (C) Endosomal localization of Wrch-1. Colocalization of GFP-Wrch-1 (green, left panel) and the early endosomal marker Texas Red-conjugated transferrin (red, middle panel) is indicated by the yellow areas in the merged image (right panel). Live cells were imaged after 24 h. Images are representative of at least three independent experiments.

although Wrch-1 and Cdc42 regulate similar downstream effector functions (Aspenstrom et al., 2004; Tao et al., 2001), they exhibit significant differences in their subcellular distribution.

**Wrch-1 Membrane Localization is Not Dependent Upon a CAAX Motif**—By analogy to Cdc42 and other CAAX-terminating small GTPases, the existence of a putative CAAX motif (CCFV) in Wrch-1 suggested that the cysteine at residue 255 (CCFV) is likely to be a target for prenylation and critical for membrane localization. To evaluate this possibility, we generated HA-tagged Wrch-1 proteins with missense mutations in the CAAX motif. Cysteine to serine substitutions of the cysteine residue of the CAAX motif abolishes the prenylation of Ras and Rho GTPases, resulting in cytosolic and inactive proteins (Adamson et al., 1992b; Hancock et al., 1989; Joyce and Cox, 2003; Lebowitz et al., 1997; Solski et al., 2002). Therefore, we generated the analogous mutant of Wrch-1 (C255S, SCFV). Most surprisingly, this mutant did not localize exclusively to the cytosol as seen in similarly mutated Ras and Rho GTPases (Adamson et al., 1992b; Hancock et al., 1989; Lebowitz et al., 1997; Solski et al., 2002; Ziman et al., 1993). Instead, it exhibited similar subcellular localization as wild type Wrch-1 (Figure 3.2). However, it did show reduced asymmetrical "Golgi-like" localization near the nucleus suggesting that the first cysteine may function as a Golgi targeting or retention signal (Figure 3.2). Thus, in contrast to Cdc42 and other Rho GTPases, an intact CAAX motif is not required for membrane localization.

The Wrch-1 CAAX motif contains a second cysteine residue at position 255 and is similar to the carboxyl-terminal CCXX motif present on proteins modified by

geranylgeranyltransferase II (GGTase II). Presently, all known substrates of GGTase II are members of the Rab small GTPase family. Thus, we speculated that Wrch-1 may possess an atypical prenylation signal sequence that is dependent instead on this second cysteine residue, alone or in combination with the first cysteine. Therefore, we generated a mutant of this cysteine residue, either alone or together with mutation of C255. Surprisingly, both Wrch-1 (C256S, CSFV) and Wrch-1 (C255S/C256S, SSFV) were mislocalized to the cytosol in a pattern similar to that of unprocessed small GTPases (Figure 3.2). However, the CSFV mutant did retain minimal plasma membrane localization. Of particular note, mutation of the second or both cysteines resulted in nucleoplasm, but not nucleosome, accumulation of the HA-tagged Wrch-1 protein implicating a potential nuclear localization signal with the carboxyl-terminus of Wrch-1. These results suggested that Wrch-1 may be prenylated at C256 rather than at C255 and that, like other Ras and Rho family proteins, additional sequences within the carboxyl-terminus may function as a secondary membrane targeting signal and/or nuclear localization signal in conjugation with modification of these cysteine residues.

**Wrch-1 Localization is Not Dependent on Isoprenoid Modification**—The importance of C256 suggested that perhaps Wrch-1 terminates in an atypical prenylation signal sequence. To address this possibility, we first determined whether Wrch-1 is a substrate for GGTase I or for FTase. We treated cells expressing GFP-tagged Wrch-1 with pharmacological inhibitors of GGTase I (GGTI) and of FTase (FTI). We have shown previously that inhibition of prenylation of GFP-tagged small GTPases results in nuclear accumulation due to the loss of the

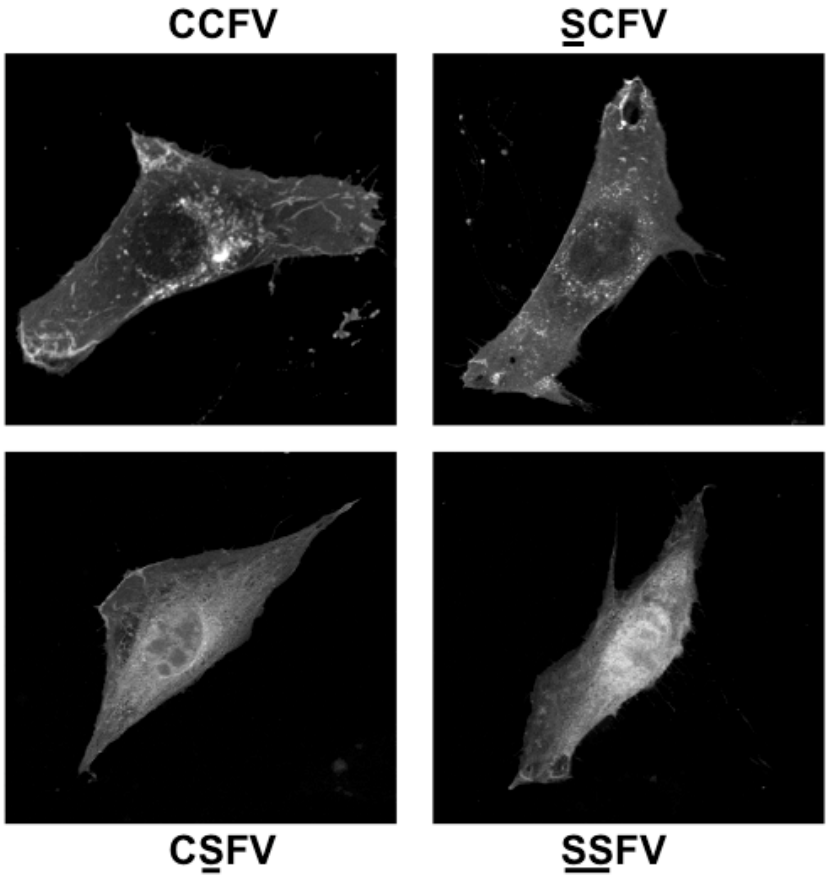



**Figure 3.2 The second cysteine of the Wrch-1 (C)CFV motif is required for Wrch-1 membrane localization.** NIH 3T3 cells were transiently transfected with the following expression constructs: HA-tagged Wrch-1(Q107L)(CCFV) or Wrch-1 bearing carboxyl-terminal mutations at cysteine residues 255 (CCFV > SCFV), 256 (CCFV > CSFV), or both (CCFV > SSFV). Cells were fixed and stained with anti-HA primary and FITC-conjugated anti-mouse secondary antibody. Wrch-1 proteins lacking cysteine 256 (CSFV, SSFV) accumulated in the cytosol. Images are representative of three independent experiments.

membrane targeting lipid and to the nuclear localization signal present within the GFP protein (Joyce and Cox, 2003; Solski et al., 2002). Therefore, sensitivity of the GFP-tagged GTPase to drug treatment was measured by redistribution of nuclear-excluded, membrane-bound proteins to the cytosol and nucleus. Efficacy and specificity of drug treatment was confirmed by their ability to cause relocalization of GFP-tagged H-Ras (farnesylated), Cdc42 (geranylgeranylated) and K-Ras (alternatively prenylated) proteins to the nucleus and cytosol upon treatment with FTI, GGTI, and FTI plus GGTI, respectively (Figure 3.3). As expected, FTI treatment caused mislocalization of H-Ras but not K-Ras4B or Cdc42, whereas GGTI treatment caused mislocalization of Cdc42 but not H-Ras or K-Ras4B. Surprisingly, Wrch-1 localization was completely unaffected by treatment with either GGTI or FTI (Figure 3.3). To eliminate the possibility that Wrch-1, like K-Ras, is alternatively prenylated by geranylgeranylation when farnesylation is blocked, cells were also treated with a combination of both FTI and GGTI. Although H-Ras, Cdc42 and K-Ras were all sensitive to the combination treatment, Wrch-1 still did not mislocalize (Figure 3.3). These results suggested that, unlike most Rho proteins, Wrch-1 is not posttranslationally modified by either FTase or GGTase I and may, therefore, be a novel substrate for prenylation by the Rab GGTase, GGTase II.

We next evaluated whether Wrch-1 is a substrate for GGTase II or for an unknown prenyltransferase. Since there are currently no pharmacological inhibitors available that specifically target GGTase II, we used compactin to treat NIH 3T3 cells transiently transfected with pEGFP-Wrch-1, -Rab5, -Cdc42 or empty vector. Compactin, an inhibitor of HMG CoA reductase, prevents the formation of all

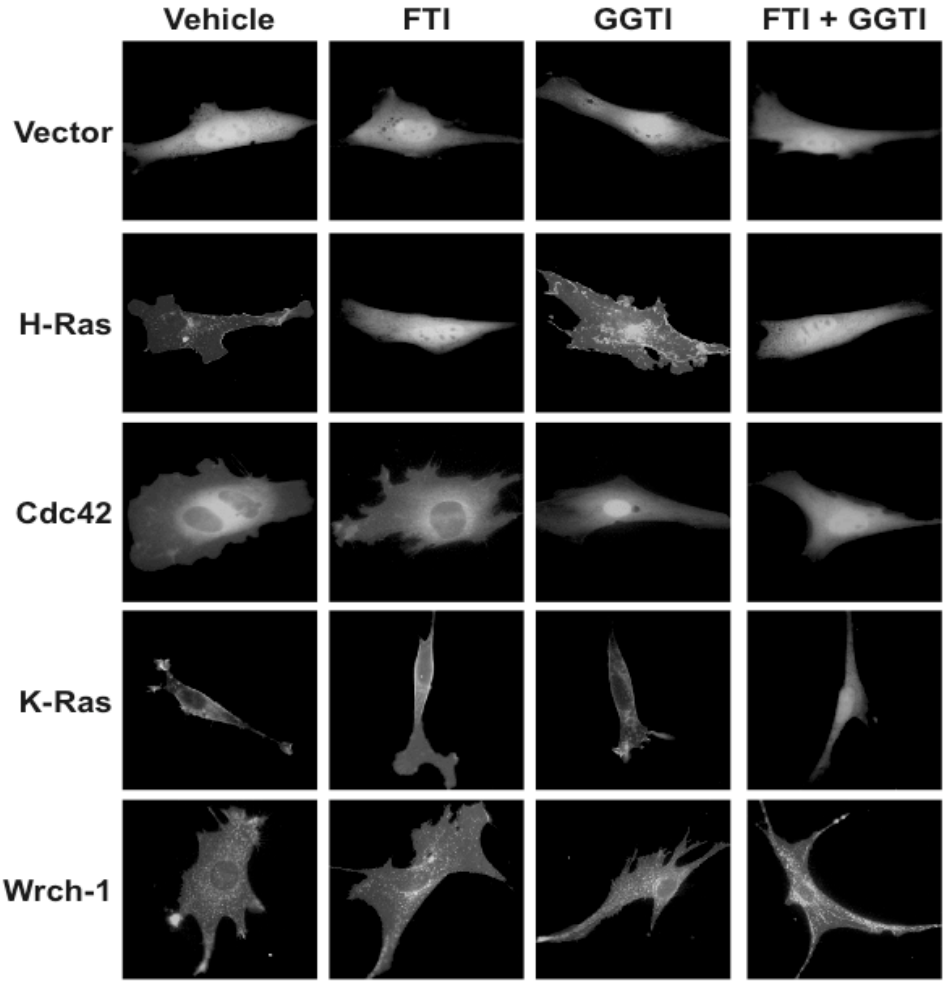



**Figure 3.3 Wrch-1 is not prenylated by either FTase or GGTase I.** NIH 3T3 cells were transiently transfected with either empty pEGFP vector or pEGFP constructs encoding GFP-tagged H-Ras (farnesylated, FTI-sensitive), Cdc42 (geranylgeranylated, GGTI-sensitive), or Wrch-1 in the presence of 10 μ*M* vehicle, FTI-2153, GGTI-2166 or a combination of both, and live cells were imaged 24 h post-transfection. Fully processed GTPases are excluded from the nucleus, whereas accumulation of GFP-tagged proteins in the nucleus following treatment with prenyltransferase inhibitors is indicative of unprocessed protein. Images are representative of at least three independent experiments.

isoprenoid precursors, thereby preventing the formation of the farnesyl and geranylgeranyl isoprenoid moieties used by all prenyltransferases, including GGTase II. Vehicle-treated cells showed nuclear exclusion, membrane and cytosolic localization of each GFP-tagged GTPase, as expected (Figure 3.4). Compactin treatment caused both Cdc42 and the GGTase II substrate Rab5 (CAAX = CCSN) to mislocalize to the nucleus and cytosol (Figure 3.4). In contrast, Wrch-1 was entirely resistant to the treatment, and remained excluded from the nucleus and targeted to the membranes and cytosol (Figure 3.4). Given that both Rab5 and Cdc42 were susceptible to compactin treatment, these results clearly demonstrate that Wrch-1 membrane association is not dependent on modification with any isoprenoid moiety.

To confirm that Wrch-1 does not utilize isoprenoid lipid groups for its membrane targeting, we performed in-vitro prenylation assays on purified, un-lipidated Wrch-1 protein to directly label Wrch-1 protein with either [$^3$H]-farnesylpyrophosphate (FPP) or [$^3$H]-geranylgeranylpyrophosphate (GGPP). Purified bacterially expressed GST-tagged Ras and Rho family proteins are unprenylated due to absence of FTase, GGTase I or II enzymes in *E. coli*. Purified H-Ras, H-Ras (FTase substrate) CVLL mutant (contains serine to leucine mutation of CAAX motif, GGTase I substrate) and Rab5 (GGTase II substrate) proteins served as standard controls for correct incorporation of [$^3$H]-FPP and [$^3$H]-GGPP by prenyltransferases. Incubation of purified, bacterially expressed Wrch-1 protein with bovine brain lysate, containing endogenous FTase, GGTase I and GGTase II enzymes, and [$^3$H]-prenylpyrophosphates followed by autoradiography demonstrated that, unlike H-Ras

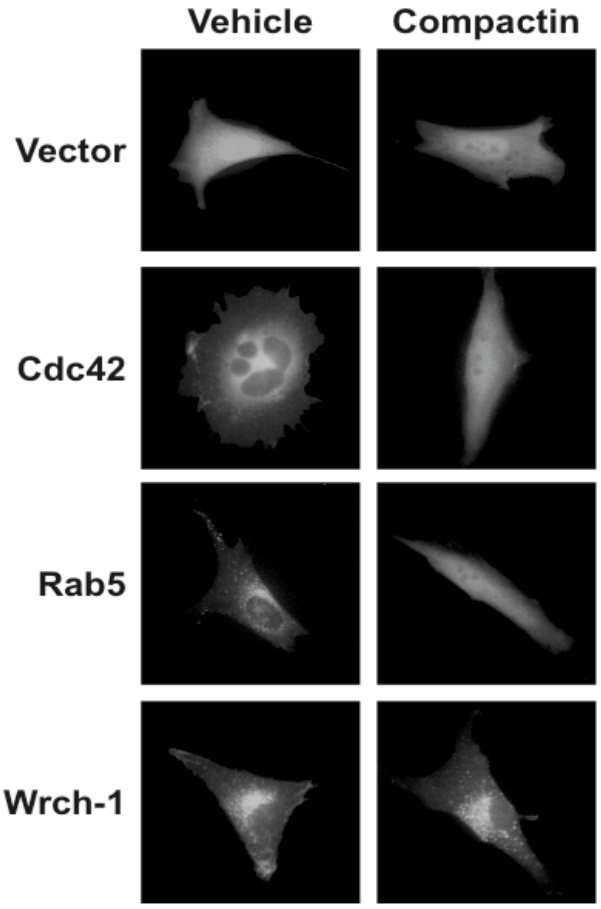



**Figure 3.4 Wrch-1 localization is not dependent on any isoprenoid modification.** NIH 3T3 cells transiently expressing either empty pEGFP vector or EGFP constructs encoding GFP-tagged Cdc42 (geranylgeranylated by GGTase I; compactin-sensitive), Rab5 (geranylgeranylated by GGTase II; compactin-sensitive), or Wrch-1 fusion proteins were treated overnight with either $Me_2SO$ vehicle or 20 $\mu M$ compactin (an inhibitor of all isoprenoid precursors). As above, mislocalization of GFP-tagged proteins to cytosol and nucleus after compactin treatment is indicative of disruption of isoprenoid-dependent localization. Images are representative of at least three independent experiments.

and Rab5, Wrch-1 was unable to incorporate [$^3$H]-FPP or [$^3$H]-GGPP (Figure 3.5). To further confirm the absence of isoprenyl modification of purified Wrch-1 protein, radioactivity of each [$^3$H]-FPP- or [$^3$H]-GGPP-bound protein reaction was determined by counting in a scintillation counter. As expected, no radioactivity was detected with Wrch-1, whereas [$^3$H]-FPP radioactivity counts were present for H-Ras WT and [$^3$H]-GGPP counts for H-Ras CVLL and Rab5 WT (data not shown). Taken together with the above prenyltransferase inhibitor data, these data indicate that, unlike most Ras and Rho family proteins, Wrch-1 is not a substrate of FTase, GGTase I or GGTase II and its membrane localization is independent of isoprenylation.

**Wrch-1 Subcellular Localization is Dependent on Palmitoylation**—The data above indicate that, unlike those of Cdc42, the last four amino acids of Wrch-1 do not function as a canonical “CAAX” motif to specify prenylation. However, since a carboxyl-terminal cysteine is clearly important for Wrch-1 localization, we then investigated other potential posttranslational modifications that might occur at cysteine residues. Palmitoylation is the reversible attachment of a palmitoyl fatty acid to cysteines via a thioester bond (Linder and Deschenes, 2003; Resh, 1999). Although no consensus signal sequence exists to aid in the prediction of which cysteine residues are likely to be palmitoylated (Linder and Deschenes, 2003; Resh, 1999), palmitoylation of cysteines in the hypervariable domains of prenylated small GTPases is common, although CAAX-signaled prenylation is a critical prerequisite for this fatty acid modification (Hancock et al., 1990; Michaelson et al., 2001; Webb et al., 2000; Willumsen et al., 1996). To determine whether the C-terminal cysteine residues of Wrch-1 are susceptible to thioester linkage to acyl groups like palmitates,

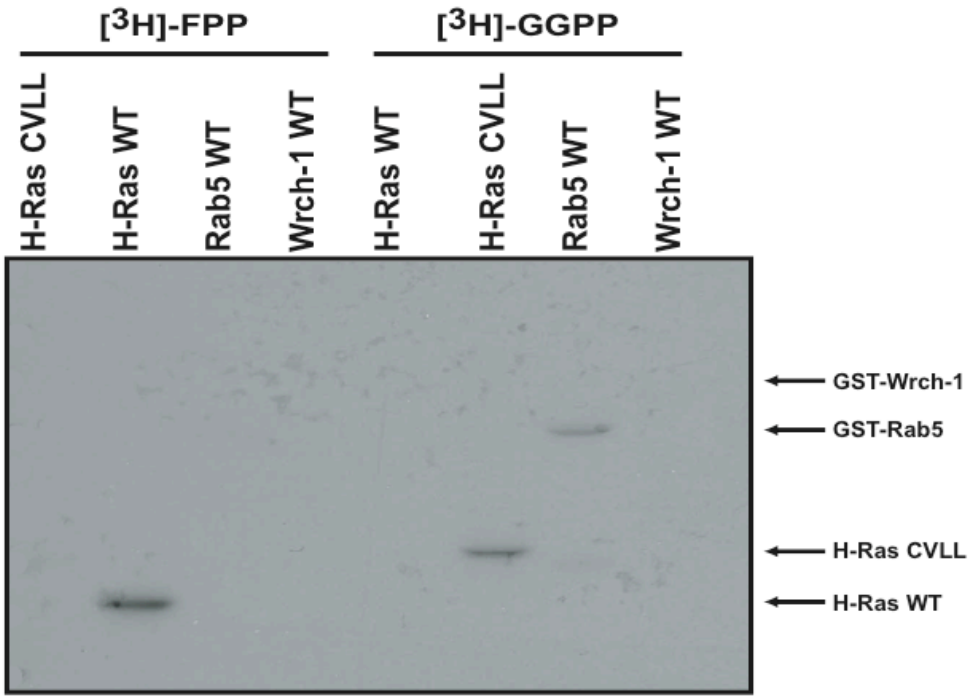



**Figure 3.5 Wrch-1 does not incorporate isoprenoid moieties.** Recombinant, purified H-Ras (FTase substrate), H-Ras CVLL mutant (GGTase I substrate), rab5 WT (GGTase II substrate), and Wrch-1 proteins were incubated in reaction mixture with bovine brain lysate containing endogenous prenyltransferase activity and [$^3$H]farnesylpyrophosphate (FPP) or [$^3$H]geranylgeranylpyrophosphate (GGPP). To detect incorporation of radioactive prenylpyrophosphates, reactions were then analyzed by SDS-PAGE and autoradiography. The presence of a band demonstrates incorporation of indicated isoprenyl groups.

we performed a recently described non-radioactive method for determining protein acylation (Drisdel and Green, 2004). This method utilizes the ability of hydroxylamine to cleave thioester bonds resulting in free sulfhydryl groups that can, then, interact with biotin-conjugated 1-biotinamido-4-[4'-(maleimidomethyl)cyclohexanecarboxamido] butane (Btn-BMCC) sulfhydryl-specific reagent, effectively labeling acylated cysteine residues. Transiently expressed GFP-tagged Wrch-1 protein immunoprecipitated from 293 HEK, human embryonic kidney cells, was suspended in N-ethyl-maleimide (NEM) to block any free, non-acylated cysteine residues, treated with hydroxylamine to cleave thioester bonds, incubated with Btn-BMCC reagent to label newly exposed sulfhydryl groups, followed by western blot analysis with streptavidin to detect any fatty acid modifications. Like H-Ras (palmitoylated on Cys181 and Cys184), Wrch-1 was labeled with the Btn-BMCC reagent suggesting that Wrch-1 contains one or more cysteine residues that are acylated (Figure 3.6A, Wrch-1 CCFV lane, top band). The non-specific lower band seen in the Wrch-1 lanes is also observed at the same molecular weight in the H-Ras lane. As expected, Btn-BMCC signal for K-Ras4B (no palmitoylated cysteines) was notably absence (Figure 3.6A).

Because Wrch-1 contains two cysteine residues at its carboxyl-terminus that may be palmitoylated, we wanted to determine whether one or both cysteine residues were susceptible to acylation. We anticipated that the second cysteine, C256, would be the preferred site for acylation due to its cytosolic and nuclear subcellular distribution when mutated to a serine residue (CSFV). However, the CSFV mutant did retain some membrane localization, it is possible that the first cysteine (C255) may also be lipid modified. As seen with parental Wrch-1 (CCFV), Wrch-1 (C255S,

SCFV) labeled with Btn-BMCC, albeit at a lower efficiency suggesting that the first cysteine residue (Cys255) may regulate acylation of the second cysteine (Cys256) (Figure 3.6A, Wrch-1 SCFV lane, top band). As expected, neither the Wrch-1 (C256S, CSFV or –(C255S/C256S, SSFV) mutants were labeled with Btn-BMCC (Figure 3.6A, Wrch-1 CSFV, -SSFV lanes, top band). Additionally, treatment of Wrch-1 with the palmitate analog 2-bromopalmitate (2-BP), a potent inhibitor of small GTPase palmitoylation (Webb et al., 2000), caused a dramatic decrease in Wrch-1 fatty acid modification suggesting that palmitate lipids are the fatty acid moieties utilized by Wrch-1 (Figure 3.6B, top panel). Expression of each protein was confirmed by western blot analysis (Figure 3.6A, bottom panel). These results indicate that the second cysteine residue of Wrch-1 (Cys256) is the primary cysteine residue for palmitate fatty acid modification.

To investigate directly whether the fatty acid modification of Wrch-1 detected by the above Btn-BMCC method is due to incorporation of palmitoyl groups at its carboxyl-terminal cysteines palmitoylation, similar to H-Ras, we used [$^3$H]-palmitate to metabolically label NIH 3T3 cells transiently expressing Wrch-1 (Willumsen et al., 1996). Correct incorporation of [$^3$H]-palmitate was confirmed using cells expressing H-Ras (palmitoylated on Cys181 and Cys184) and K-Ras (no palmitoylatable cysteines) as positive and negative controls, respectively. Immunoprecipitation of the exogenous HA-tagged Wrch-1 protein from labeled cells, followed by autoradiography, showed that Wrch-1, like H-Ras, incorporated [$^3$H]-palmitate (Figure 3.6C, top panel). The reduced level of incorporation seen in Wrch-1 compared to H-Ras may be due, in part, (potential differences in palmitoyl turnover

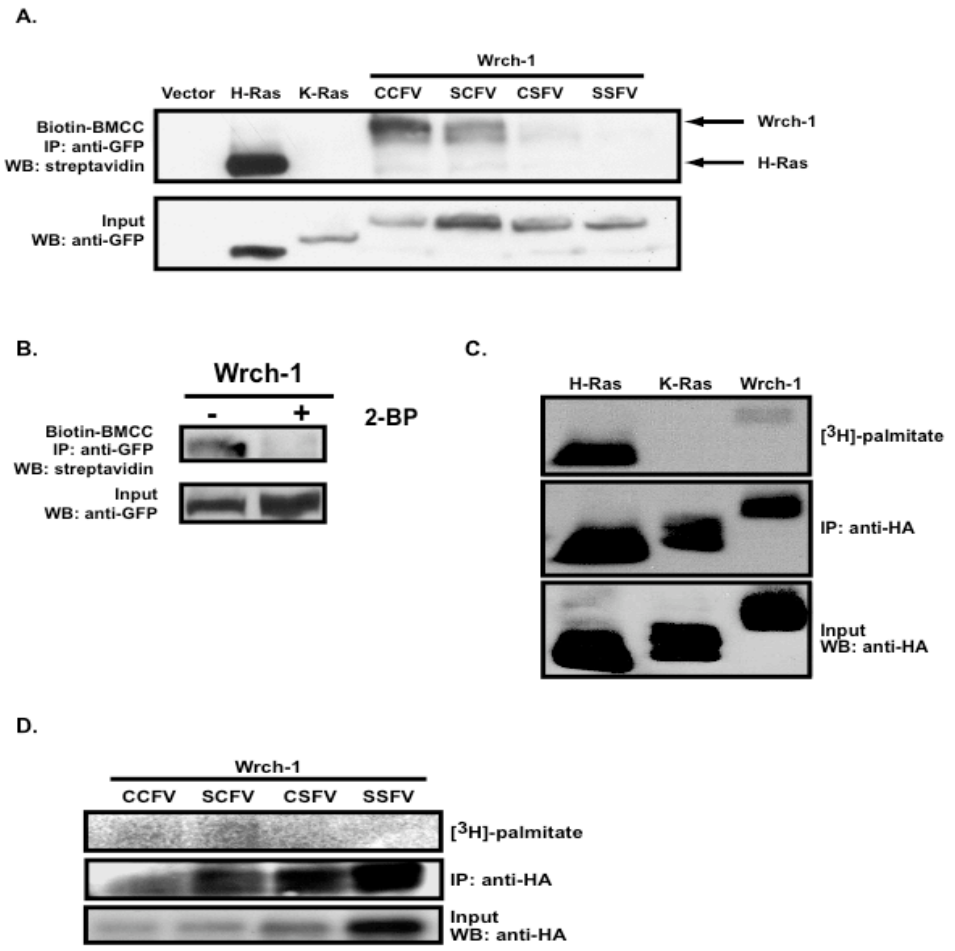



**Figure 3.6 Wrch-1 is palmitoylated.** NIH 3T3 cells were transiently transfected with either pEGFP constructs encoding GFP-tagged (A and B) or pCGN constructs encoding HA-tagged (C and D) H-Ras (palmitoylated positive control), K-Ras (nonpalmitoylated negative control), or Wrch-1 (CCFV, SCFV, CSFV, and SSFV). Two days post-transfection, proteins were immunoprecipitated with anti-GFP antibody, treated with hydroxylamine, and labeled with biotin-BMCC to detect acylated cysteine residues (A). A band indicates fatty acid modification (*top panel*). *Bottom panel* detects amount of protein available for immunoprecipitation (*IP*). *WB*, Western blot. (B) Wrch-1-expressing cells were treated with either $Me_2SO$ vehicle or 150 $\mu M$ 2-BP 12 h prior to immunoprecipitation for Btn-BMCC labeling. (C and D) Cells were labeled metabolically with a 4-h pulse of [$^3$H]palmitate, and the GTPases were immunoprecipitated with anti-HA antibody. Immunoprecipitates were subjected to SDS-PAGE and exposed to film for 4 – 6 weeks. *Top panel*, [$^3$H]palmitate autoradiogram shows that Wrch-1- and H-Ras-positive control, but not K-Ras4B-negative control, incorporated [$^3$H]palmitate (C). Mutation of carboxyl-terminal cysteines affects Wrch-1 incorporation of [$^3$H]palmitate (D). *Middle panel*, to detect total amount of GTPases retrieved for autoradiography, immunoprecipitates were probed for GTPases by Western blot using anti-HA antibody (*IP*). *Bottom panel*, to detect amount of starting protein available for immunoprecipitation (*input*), total cell lysates were probed by Western blot using anti-HA antibody.

and, therefore, steady-state levels of the palmitoylated GTPases) to the lower level of Wrch-1 protein expression, and in part to the single palmitoylated cysteine residue in Wrch-1 compared to the two palmitoylatable cysteines in H-Ras. Western blot analysis of the total input lysate input and immunoprecipitated K-Ras4B protein confirm that the absence of a [$^3$H]-palmitate signal in the K-Ras-expressing cells was not due simply to a lack of protein expression or immunoprecipitation (Figure 3.6C, middle and bottom panel). These results directly demonstrate that Wrch-1 is modified by palmitoylation.

Next, we investigated whether one or both Wrch-1 C-terminal cysteines residues are substrates for palmitoylation. Given that the Wrch-1 (C255S, SCFV) mutant, but not the Wrch-1 (C256S, CSFV or –(C255S/C256S, SSFV) mutants, retained an acylated cysteine residue, we anticipated the second cysteine would also prove to be the important cysteine residue for palmitoylation. Like the Wrch-1 (CCFV) parent, Wrch-1 (C255S, SCFV) mutant incorporated [$^3$H]-palmitate signal, whereas Wrch-1 (C255S, CSFV)- and –(C255S/C256S, SSFV)-expressing cells lacked a [$^3$H]-palmitate and, therefore, failed to incorporate [$^3$H]-palmitate (Figure 3.6D, top panel). Expression of each protein is confirmed by western blot analysis of total lysate input and immunoprecipitated CSFV and SSFV protein (Figure 3.6D, middle and bottom panels). These data show that the second cysteine residue is the required cysteine for palmitoylation. Thus, as with other palmitoylated small GTPases, this posttranslational modification may be important for Wrch-1 subcellular localization.

To determine whether palmitoylation influences Wrch-1 localization, we treated NIH 3T3 cells transiently expressing GFP-tagged Wrch-1, H-Ras, K-Ras or empty vector with 2-BP. 2-BP has been shown previously to cause the redistribution of palmitoylated GTPases, most recently the Cdc42 homologous protein Chp/Wrch-2, and other proteins within the cell (Chenette et al., 2005; Michaelson et al., 2001; Webb et al., 2000). As expected, the lack of palmitoylatable sites within the K-Ras carboxyl-terminus rendered its localization insensitive to the effects of 2-BP, whereas H-Ras was mislocalized from plasma membrane to endomembranes, thereby demonstrating substrate specificity of 2-BP for carboxyl-terminal cysteine-containing proteins (Figure 3.7). Upon treatment with 2-BP, GFP-Wrch-1 relocalized dramatically to the cytosol and accumulated in the nucleus (Figure 3.7). That Wrch-1 localization is sensitive to 2-BP treatment is consistent with our Btn-BMCC and metabolic labeling studies showing that Wrch-1 is palmitoylated. These results suggest that palmitate(s) could be the sole lipid modification normally causing retention of Wrch-1 in the cytosol and membranes.

**The Carboxyl-Terminal Cysteines are Necessary for Wrch-1 Signaling to PAK**—Since inhibition of palmitoylation and mutation of the carboxyl-terminal cysteines to serines rendered Wrch-1 improperly distributed, we predicted that loss of the palmitate modification would also affect Wrch-1 downstream signaling pathways. Previous studies have shown that activated, constitutively GTP-bound Wrch-1 results in increased auto-phosphorylation of the serine/threonine kinase p21 activated kinase (PAK) (Shutes et al., 2004; Tao et al., 2001). PAK1 is a well known

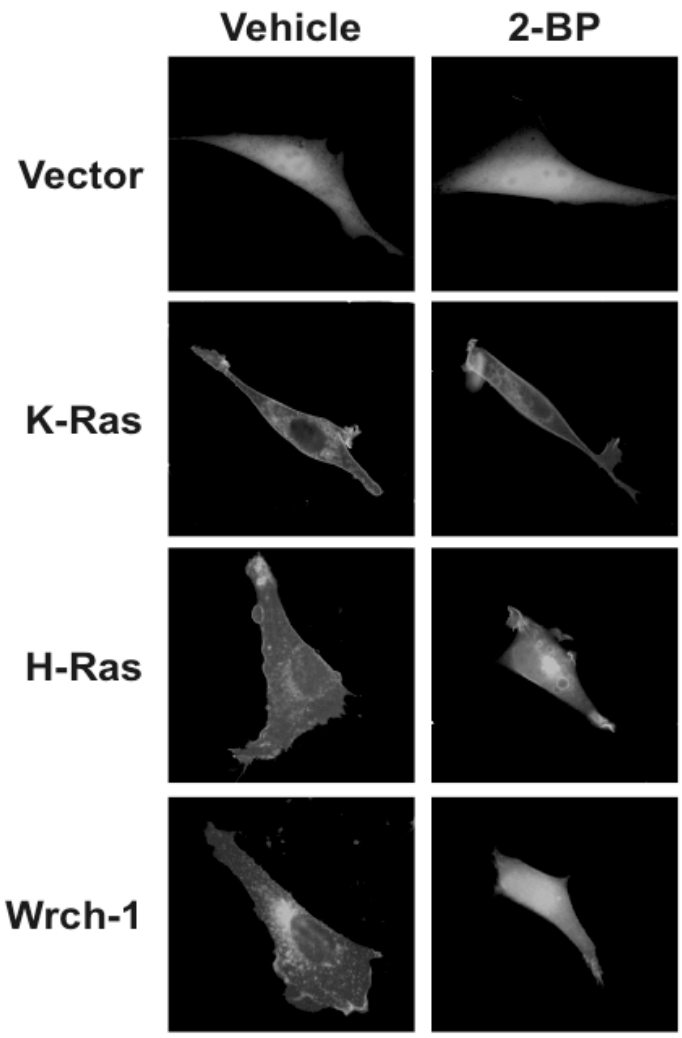



**Figure 3.7 Palmitate analog 2-BP causes mislocalization and cytosolic accumulation of Wrch-1.** NIH 3T3 cells were transiently transfected with pEGFP empty vector or pEGFP constructs encoding GFP-tagged K-Ras4B (nonpalmitoylated negative control), H-Ras (palmitoylated positive control), or Wrch-1. Cells were treated overnight with either $Me_2SO$ vehicle or 150 $\mu M$ 2-BP. The following day, 2-BP-treated cells were assessed for cytosolic and/or nuclear accumulation by live cell imaging. Images are representative of at least three independent experiments.

effector of several Rho family proteins including Cdc42 and Rac (Wennerberg and Der, 2004). To determine whether mutation of the carboxyl-terminal cysteines of Wrch-1 would affect its ability to promote auto-phosphorylation of PAK in NIH 3T3 fibroblasts, we transiently expressed the cysteine to serine mutants in an activated Wrch-1(Q107L) background and looked for differences in the PAK phosphorylation status. As shown previously by us and others (Shutes et al., 2004; Tao et al., 2001), the Wrch-1(Q107L) CCFV parent resulted in an increase in phosphorylated PAK levels when compared to vector-expressing cells (Figure 3.8). Consistent with the loss of membrane association, the ability of the Wrch-1 SCFV, -CSFV and SSFV mutants to induce phosphorylation of PAK was considerably reduced when compared to the CCFV parent. This suggests that correct localization of Wrch-1 via its palmitate modification is necessary for downstream signaling molecules.

**The Carboxyl-Terminal Cysteine 256 (CCFV), but Not 255 (CCFV), Is Required for Wrch-1 Transformation**—Given that loss of palmitate modification affected Wrch-1 downstream signaling, we predicted that loss of the palmitate modification would also affect Wrch-1 biological activity. The transforming activity of prenylated Rho family proteins is impaired when palmitate modification is blocked (Joyce and Cox, 2003). We sought to determine whether loss of palmitoylation would interfere with Wrch-1 transformation.

Previous studies showed that activated Raf can cooperate with constitutively activated Rho family members such as Cdc42 to cause synergistic transformation of NIH 3T3 cells (Khosravi-Far et al., 1995; Qiu et al., 1995a; Qiu et al., 1995b;

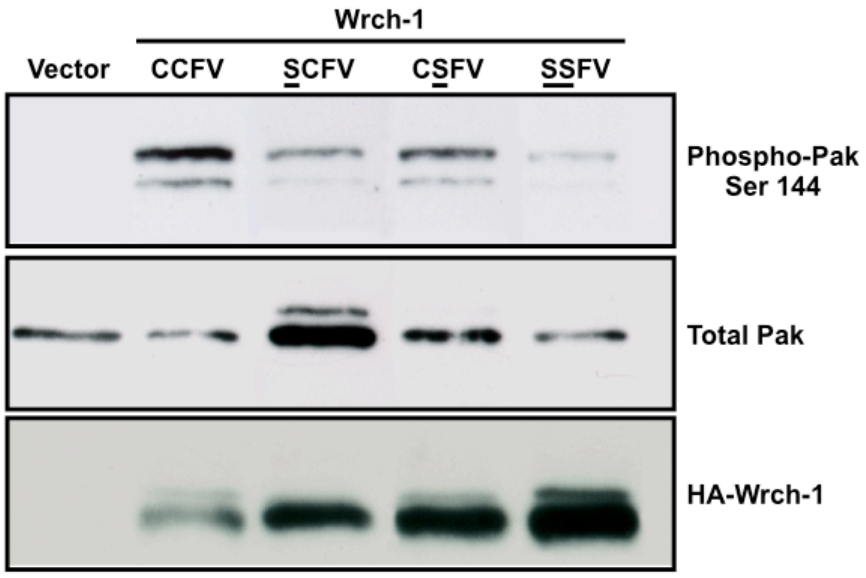



**Figure 3.8 Carboxyl-terminal cysteines regulate Wrch-1 downstream signaling to PAK.** NIH 3T3 cells were transiently transfected with pCGN constructs encoding activated Wrch-1(Q107L) and Wrch-1(Q107L) carboxyl-terminal mutants (SCFV, CSFV, and SSFV). Whole cell lysates were collected 24 h post-transfection and analyzed by Western blot for changes in endogenous phosphorylated PAK status (*top panel*). For controls, total PAK and HA-Wrch-1 protein levels were analyzed (*middle, bottom panels*). Blots are representative of three separate experiments.

Whitehead et al., 1998). To determine whether Wrch-1, like Cdc42, could also cooperate with Raf to cause focus formation, we transiently co-transfected into NIH 3T3 cells pCGN-hygro constructs expressing activated Wrch-1(Q107L), Cdc42(Q61L) or Rac1(Q61L), together with either pZIP-Raf22W or the empty pZIP-NeoSV(x)1 vector. We then evaluated the dishes for the appearance of foci of transformed cells. Raf-induced foci of transformed cells are indistinguishable in appearance from those caused by activated Ras, and are characterized by large, spreading foci of highly refractile, spindle-shaped, morphologically-transformed cells. In contrast, activated Rho GTPases cause foci of transformed cells that are very distinct from those of Raf, and are characterized by tight clusters of rounded, refractile cells that are frequently multinucleated. We anticipated that the appearance of Wrch-1 foci might resemble those of Cdc42 more than those of Raf-1. As expected, activated Wrch-1 alone was unable to induce focus formation (Figure 3.9A). In cooperation with Raf, however, activated Wrch-1 formed foci that were similar to those of Cdc42 and Rac1 (Figure 3.9A).

Next, we evaluated whether loss of the carboxyl-terminal cysteines, individually or together, impaired Wrch-1 focus formation in cooperation with Raf. The ability of activated Wrch-1-C<u>S</u>FV and -<u>SS</u>FV to form foci of transformed cells was greatly reduced compared to that of the parental Wrch-1 (Figure 3.9B). These results are consistent with a critical role for the second cysteine (C<u>C</u>FV), rather than the canonical CAAX cysteine (<u>C</u>CFV), in mediating Wrch-1 membrane association. Unexpectedly, mutation of the first cysteine residue, <u>S</u>CFV, did not impair Wrch-1 focus forming ability but rather enhanced it. This result suggests that the roles of the

two cysteines are distinct, and that the upstream cysteine plays a negative regulatory role in Wrch-1 biological activity.

To determine whether these results also applied to other aspects of the transformed phenotype, we evaluated the ability of activated Wrch-1 C-terminal mutants to promote anchorage-independent growth in soft agar. In contrast to their activity in focus formation assays, Rho proteins alone, including Wrch-1 (Shutes et al., 2004), are sufficient to confer anchorage-independent growth and do not require cooperation with Raf in soft agar assays. Therefore, NIH 3T3 cells stably expressing activated, HA-tagged Wrch-1(Q107L) were seeded into agar and analyzed for colony forming activity. Comparable expression of each Wrch-1 protein was seen (data not shown). Activated Wrch-1 potently induced colony formation in soft agar (Figure 3.9C, D). Consistent with the focus formation data, mutation of either the second cysteine (C256S, CSFV) or both cysteines (C255S/C256S, SSFV) resulted in a strong reduction of colony formation, whereas the cysteine to serine mutation at residue 255 (SCFV) led to a significant increase in Wrch-1-induced colony formation (Figure 3.9C, D). Taken together, our focus formation and soft agar analyses suggest that the second cysteine residue of the CCFV motif is required for Wrch-1 transforming activity, whereas the first cysteine may function instead as a negative regulator.

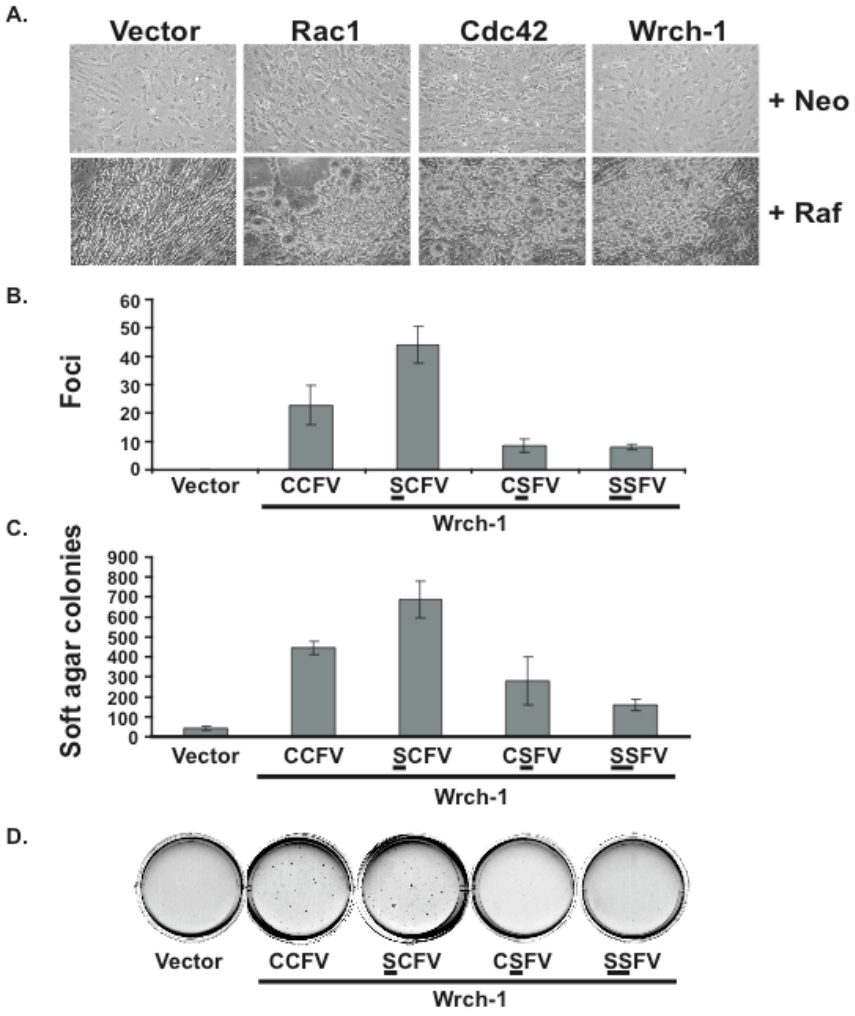



**Figure 3.9 Carboxyl-terminal cysteines of the hypervariable domain differentially modulate Wrch-1 transforming activity.** NIH 3T3 cells were transiently cotransfected with either empty vector pZIPneo or activated pZIP-Raf22W, along with pCGN constructs encoding activated Rac1(Q61L), Cdc42(Q61L), or Wrch-1(Q107L). After 14 days, dishes were fixed and stained with crystal violet, and foci of transformed cells were counted. Images are representative of at least three separate experiments carried out in duplicate. (A) Activated Wrch-1 cooperates with activated Raf to form Cdc42-like foci of transformed cells. (B) cooperation with Raf by Wrch-1 containing carboxyl-terminal mutations at cysteine residues 255 (CCFV > SCFV), 256 (CCFV > CSFV), or both (CCFV > SSFV). (C) NIH 3T3 cells stably expressing the above-mentioned pCGN Wrch-1 constructs were seeded into soft agar and analyzed for their ability to induce anchorage-independent growth. Colonies formed after 14-21 days were stained and scanned (D) and quantified (C). Images and bar graphs are representative of at least two separate experiments carried out in triplicate.

## DISCUSSION

Wrch-1 gene expression is upregulated in Wnt-1-transformed cells, and Wrch-1 activation can phenocopy the changes in cellular morphology caused by Wnt-1 (Tao et al., 2001). Wrch-1 shares significant amino acid sequence and functional identity with Cdc42, but exhibits significant divergence in carboxyl-terminal sequences. Wrch-1 terminates in an atypical CAAX tetrapeptide motif and its hypervariable domain possesses an additional 21 amino acid residues not found in Cdc42. In the present study, we evaluated the role of these unique carboxyl-terminal features in Wrch-1 membrane association and biological activity. While we found that the carboxyl-terminal nine residues of Wrch-1 alone were sufficient to promote Wrch-1 membrane association, Wrch-1 and Cdc42 exhibited very distinct patterns of subcellular localization, with significant amounts of Wrch-1 found at the plasma membrane and early endosomes. Surprisingly, we found that an intact CAAX motif was not required for Wrch-1 membrane association, but instead, mutation of a second carboxyl-terminal cysteine significantly reduced Wrch-1 membrane association. Furthermore, Wrch-1 membrane association was not dependent on isoprenoid modification, but was instead dependent on palmitoylation of the second cysteine residue. Finally, we found that Wrch-1, like Cdc42, can also promote growth transformation of NIH 3T3 cells, and that the palmitoylated cysteine was critical for this activity.

Highly related Rho and Ras GTPases exhibit distinct cellular functions that can be attributed in part to subcellular localizations dictated by their distinct hypervariable domains (Adamson et al., 1992b; Du et al., 1999; Wang et al.,

2003b). For example, RhoA shares 90% identity with RhoB and RhoC, and these three proteins share common regulators and effectors (Wennerberg and Der, 2004). However, despite these strong similarities, whereas RhoA can promote growth transformation, there is evidence that RhoB may function in an opposite fashion and exhibit tumor suppressor function (Chen et al., 2000; Solski et al., 2002). RhoC but not RhoA has been associated with tumor cell invasion (Clark et al., 2000; van Golen et al., 2000). These three related Rho GTPases show the greatest sequence divergence in their carboxyl-terminal sequences, and this divergence results in differences in subcellular localization that in turn promote different cellular functions (Adamson et al., 1992b; Du et al., 1999; Wang et al., 2003b). Philips and coworkers (Michaelson et al., 2001) eloquently demonstrated that the last 20 amino acids of several Rho and Ras proteins, including Cdc42, mimic the subcellular localization of the full length proteins. We have shown here that at least the last 9 amino acids of Wrch-1 are sufficient to confer proper subcellular distribution. This finding is consistent with other studies that illustrate that all of the membrane targeting information is located in the carboxyl-terminus.

Thus, while Wrch-1 and Cdc42 share significant sequence identity and functional overlap, their divergent carboxyl-terminal sequences may also impart different biological roles to these biochemically related proteins. Mutation of the cysteine residue of the CAAX motifs of Cdc42 and other Rho GTPases to prevent prenylation results in loss of membrane association and biological activity (Adamson et al., 1992b; Hancock et al., 1989; Lebowitz et al., 1997; Solski et al.,

2002; Ziman et al., 1993). Therefore, we were surprised that the analogous mutation of the Wrch-1 CAAX motif (C255S, SCFV) did not cause complete mislocalization or loss of transforming activity. Analogously to Ras family GTPases, the membrane localization of conventional Rho family proteins generally requires either a geranylgeranyl (e.g., Cdc42) or a farnesyl lipid group (e.g., Rnd3/RhoE) attached to the cysteine residue of the CAAX motif and a "second signal" consisting of either several basic residues or palmitoylated cysteine residues in the upstream hypervariable domain (Wennerberg and Der, 2004). In direct contrast, we show here that the Cdc42 homolog Wrch-1 does not utilize either a geranylgeranyl or farnesyl isoprenoid moiety for membrane targeting. Instead, its localization is regulated by a palmitoyl fatty acid, demonstrating that the Wrch-1 CAAX-like motif, CCFV, is not a canonical, prenylated CAAX.

It is unclear at this juncture whether Wrch-1 also requires a "second signal" for proper localization to plasma membranes. However, the carboxyl-terminus of Wrch-1 contains several basic residues that could form a polybasic second signal to complement the palmitate modification (Booden et al., 1999; Mitchell et al., 1994), and are included in the short stretch of nine amino acids that constitutes a minimal targeting sequence. Alternatively, by analogy to CAAX-containing palmitoylatable small GTPases, Wrch-1 may require other poorly defined but essential motifs surrounding palmitoylatable cysteines (Rotblat et al., 2004b; Willumsen et al., 1996). The Wrch-1 carboxyl-terminus also contains uncommon but conserved residues such as tandem tryptophans and a tyrosine.

The tandem tryptophan residues may represent a di-aromatic motif of the kind frequently associated with endosomal sorting (Schweizer et al., 2000) and may help to direct Wrch-1 to endosomes. The contribution of these residues to Wrch-1 membrane targeting and function is currently under investigation.

More distantly related Rho and Ras proteins also target to membranes but do not depend on carboxyl-terminal lipid modification. For example, the Rho-related proteins RhoBTB-1/2 and Miro-1/2, as well as the Ras-related proteins Rit, Rin, Gem and Rem2, are not known to undergo lipid modified, yet display distinct membrane associations (Aspenstrom et al., 2004; Finlin et al., 2000; Lee et al., 1996; Maguire et al., 1994). Conversely, although Rab proteins are prenylated, they lack a conventional CAAX motif and, instead, terminate in CCXX, CXC and XXCC sequences that, in combination with upstream residues, serve as targeting motifs for GGTase II modification (Gomes et al., 2003; Khosravi-Far et al., 1992). We have demonstrated here that the CCXX motif of Wrch-1 is not a target for GGTase II, but rather for palmitoylation. Interestingly, Chp/Wrch-2, the closest relative of Wrch-1, lacks a CAAX motif and should, therefore, not be modified by prenyltransferases (Aronheim et al., 1998). However, it shares with Wrch-1 a CFV (CXX) motif, incorporates a fatty acid modification at its carboxyl-terminal cysteine residue, and Chp membrane association is also disrupted by 2-BP treatment (Chenette et al., 2005). It is interesting to speculate that the CFV (CXX) motif may be a novel recognition site for posttranslational modification by palmitoyl transferases. While other mammalian Rho GTPases are also palmitoylated, their palmitate modification is dependent on prior modification by

prenylation. Therefore, Wrch-1 and Chp undergo unique lipid modification-dependent membrane targeting not seen with other known mammalian Ras family GTPases. Interestingly, atypical Rho-like proteins have been described in the plant *Arabidopsis* that also undergo a prenyl-independent, palmitoyl modification; however, these small GTPases terminate neither in conventional CAAX nor in CXX motifs (Ivanchenko et al., 2000; Lavy et al., 2002). In addition, their carboxyl-terminal sequences lack the basic residues found with Wrch-1 and Chp, and multiple palmitoylated cysteines appear to be required for full membrane association.

Our finding that the Wrch-1 carboxyl-terminal Cys to Ser mutants have differential effects on Wrch-1 localization and function is unusual for small GTPases and suggests that each cysteine has a distinct contribution to Wrch-1 function. Consistent with this possibility, we have shown that mutation of Cys255 (SCFV) resulted in increased transforming activity of Wrch-1, suggesting that this residue has a negative regulatory effect on Wrch-1 localization and function, whereas mutation of Cys256 (CSFV) abrogated membrane localization and transformation. Interestingly, we have made a similar observation with Chp, where mutation of the cysteine residue of the CFV motif caused mislocalization and loss of transforming activity, whereas mutation of an upstream cysteine did not alter membrane association, yet causes a significant enhancement of transforming activity (Chenette et al., 2005). It is formally possible that one cysteine regulates acylation of the second cysteine; more sensitive methods for

detection of such modifications will be necessary to determine whether this is the case.

Palmitoylation of cysteines in the hypervariable domain of Wrch-1 suggests that Wrch-1 may traffic and signal similarly to other palmitate-containing small GTPases such as H-Ras and TC10 rather than Cdc42. For example, palmitoylated H-Ras, but not polybasic-domain-containing K-Ras, transports to the plasma membrane via a Golgi-mediated process leading to the association of H-Ras with cholesterol rich lipid rafts (Apolloni et al., 2000; Choi and Han, 2002; Roy et al., 1999). Lipid rafts are specialized microdomains that contain distinct composition of lipids and signaling proteins that may organize signals impinging on the cell surface into distinct cascades (Simons and Ikonen, 1997). For some palmitoylated small Rho GTPases, such as TC10, lipid raft localization is critical to their downstream activity. For example, one study showed that TC10 could control Glut 4 activity only if specifically targeted to lipid rafts (Watson et al., 2001). TC10 has two non-tandem upstream cysteines that are substrates for palmitoylation. Mutation of the cysteine immediately upstream of the CAAX motif prevented endomembrane localization of TC10, whereas the other cysteine had no effect on TC10 subcellular distribution (Watson et al., 2003). Since palmitoylation favors association of proteins to lipid rafts, it is possible that Wrch-1 may also traffic through the exocytic pathway to interact with these lipid-rich microdomains, thereby introducing different Wrch-1 protein-protein interactions that Cdc42, lacking a palmitoylation site, may not encounter. These differences in subcellular localization suggest a potential mechanism for functional diversity.

Palmitoylation of Wrch-1 may also provide another level of regulation for Wrch-1 protein interactions and biological function. Rho and Ras protein activities are regulated by both nucleotide binding and subcellular location. For example, RhoGDIs negatively regulate Cdc42 and other Rho family proteins by binding their prenoid moieties and sequestering the proteins to the cytosol (Geyer and Wittinghofer, 1997; Olofsson, 1999). Since Wrch-1 lacks a prenyl group and does not bind RhoGDIs (see Chaper II, ((Berzat et al., 2006)), the dynamic, reversible nature of palmitoylation could serve instead as a "RhoGDI-like" regulatory entity for Wrch-1 localization. The turnover rate for H-Ras palmitoylation is a rapid ($t_{1/2}$ ~20 min) (Baker et al., 2000; Baker et al., 2003), and H-Ras lacking palmitoylatable cysteines fails to target the plasma membrane and is functionally deficient (Baker et al., 2003; Hancock et al., 1989; Willumsen et al., 1996). Most recently, a de/reacylation cycle on H-Ras has been shown to regulate its localization and activation subcellularly (Rocks et al., 2005). Palmitoylation targets H-Ras not only to the plasma membrane, but specifically to lipid rafts where dynamic GTP-dependent shifts of H-Ras in and out of rafts occur (Prior et al., 2003). Similar palmitoylation and de-palmitoylation kinetics for Wrch-1 palmitoylation could similarly cause Wrch-1 to quickly enter and exit lipid rafts and regulate Wrch-1 downstream activity in a dynamic manner. Our recent data demonstrate that Wrch-1 exchanges GDP unusually rapidly (Shutes et al., 2004). Thus, rapid movement of Wrch-1 in and out of lipid rafts may combine with the fast-cycling nature of Wrch-1, leading to regulatory control of Wrch-1 based in part on its localization.

To date, there are no published human S-acyl-transferases (PATs) shown to modify Wrch-1 or other palmitoylated small GTPases. However, recent yeast genetic screens for PAT components have identified PAT genes that are necessary for palmitoylation of yeast Ras. These yeast PAT genes contain a cysteine-rich domain (CRD and a DHHC motif required for PAT activity and this DHHC-CRD motif has been found in several human proteins that are involved in the S-acylation of specific neuronal proteins (Fukata et al., 2004; Huang et al., 2004; Linder and Deschenes, 2003; Magee and Seabra, 2005). Given that there are several DHHC-CRD-containing genes, characterization of these genes as PATs could reveal potential regulatory proteins for Wrch-1 localization and, ultimately, its downstream activity and serve as potential targets for pharmacological inhibitors.

Since Cdc42 and other Rho GTPases have been implicated in human oncogenesis (Boettner and Van Aelst, 2002; Ridley, 2004; Sahai and Marshall, 2002; Zohn et al., 1998), inhibitors of GGTase I have been considered for cancer therapy (Cox and Der, 2002; Sebti and Hamilton, 2000a). However, since Cdc42 function is important for normal cell proliferation, one concern is that GGTIs may exhibit significant normal cell toxicity. Since Wrch-1 and Chp exhibit functional overlap with Cdc42, and their functions are not dependent on GGTase I activity, perhaps these atypical Rho GTPases will provide some protection against GGTase I suppression of Cdc42 function in normal cells.

In summary, our recent delineation of a unique regulatory function of the amino-terminus of Wrch-1 (Shutes et al., 2004), together with the unusual nature of the Wrch-1 carboxyl-terminus in mediating subcellular localization identified in this study, make Wrch-1 highly distinct from the classical Rho family GTPases. Wrch-1 together with Chp represents a new class of mammalian Rho GTPases whose membrane targeting and biological activity is dependent on lipidation by palmitoyl fatty acids but not by isoprenoids. Our studies with structural mutants suggest distinct functional contributions of palmitoylation at different carboxyl-terminal cysteines. Further studies are needed to determine how the other residues of the hypervariable domain affect Wrch-1 localization and function. Additional studies of Wrch-1 and other palmitoylation-only lipidated small GTPases like Chp/Wrch-2 and *Arabidopsis* Rac proteins will also be necessary to clarify how this modification affects their ability to localize and to target downstream signaling pathways. The novel mechanism by which Wrch-1 and Chp function is regulated by carboxyl-terminal sequences and lipid modifications adds further to the complexity by which carboxyl-terminal variation may diversify the biological roles of proteins that otherwise exhibit strong biochemical similarity.

# CHAPTER IV

# UBIQUITYLATION AND SRC-DEPENDENT PHOSPHORYLATION OF WRCH-1 MAY BE REQUIRED FOR WRCH-1 LOCALIZATION TO INTERNAL MEMBRANES DOWNSTREAM OF ERBB FAMILY SIGNALING

**Anastacia C. Berzat and Adrienne D. Cox**

**ABSTRACT**

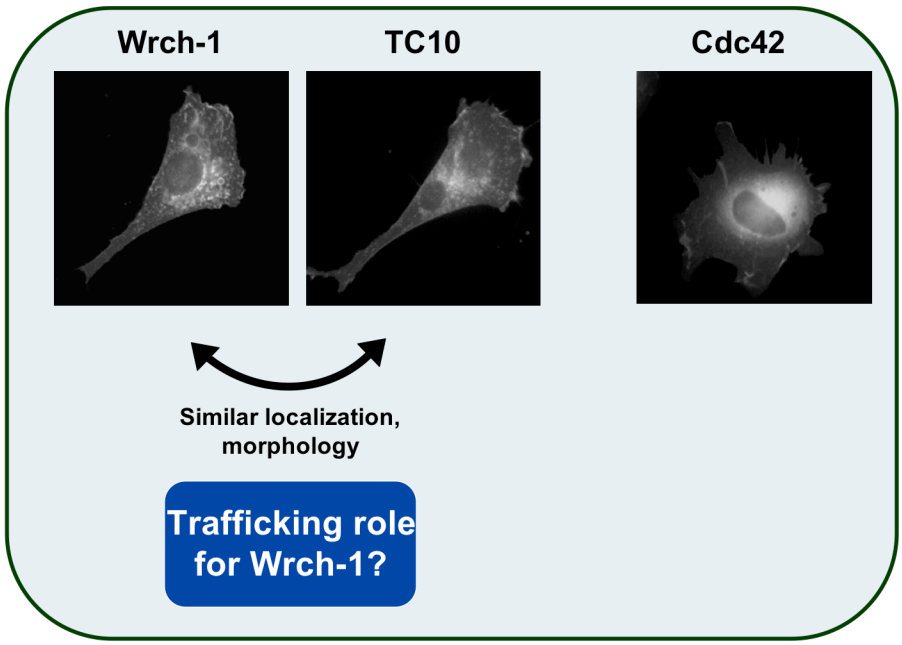
Most small GTPases require posttranslational lipid modification by farnesyl (F) or geranylgeranyl (GG) isoprenoids to correctly target to their appropriate subcellular localizations for their normal biological functions. In contrast, we have previously demonstrated that the Rho family GTPase, Wrch-1 (Wnt-regulated Cdc42 homolog-1) is not modified by either F or GG isoprenoids. Instead, Wrch-1 incorporates palmitate fatty acids for its primary lipid-mediated localization to cellular membranes and subsequent transforming activity. In addition to isoprenoid lipids, membrane targeting of small GTPases also requires a "second signal", which is usually either a stretch of basic residues or a palmitate, and, in some cases, phosphorylation. The minimal membrane targeting sequence for Wrch-1 lacks both a polybasic region and additional palmitoylated residues, suggesting that there are other, uncharacterized, membrane signals contributing to its localization. Here, evaluation of the minimal sequences necessary to target Wrch-1 to cellular membranes reveals the presence of three distinct targeting motifs, including potential ubiquitylation and phosphorylation sites. We also demonstrate that Wrch-1 is a substrate for mono-ubiquitylation and Src-dependent tyrosine phosphorylation. Additionally, Wrch-1 localizes to both endosomes and lysosomes in a pattern reminiscent of mono-ubiquitylated receptor tyrosine kinases (RTKs). Interestingly, the epidermal growth factor receptor (EGFR/ErbB) family regulates the Src-dependent Wrch-1 tyrosine phosphorylation, implicating Wrch-1 activity downstream of ErbB family signaling. Taken together, these data suggest a possible role for ubiquitylation and phosphorylation of Wrch-1 as regulators of its localization and trafficking.

## PREFACE

The basis for the work in this chapter began with an observation that endosomal localization of Wrch-1 (shown previously in Chapter III) did not account for all the internal compartments in which Wrch-1 is found. Only a subset of Wrch-1 protein overlapped with endosomal markers (Chapter III), suggesting that Wrch-1 localizes to other internal membranes. Additionally, expression of GFP-tagged Wrch-1 and TC10 in live cells revealed an internal membrane subcellular localization pattern of Wrch-1 more closely resembling that of TC10 (Figure 4.1A) rather than Cdc42. So, perhaps Wrch biological functions are more similar to TC10-like chaperone functions in cellular trafficking.

These ideas begged the question of what other internal membranes are playing host to Wrch-1 localization and function. Since activated Wrch-1 increased apoptosis (Tao et al., 2001), I examined whether Wrch-1 apoptotic activities were dependent on its localization to mitochondrial membranes. In a recently published paper, K-Ras internalization to the mitochondria during PKC-driven electrostatic switches leads to its promotion of apoptosis (Bivona et al., 2006). Perhaps like K-Ras, Wrch-1 effects on apoptosis are mediated directly by its association with mitochondrial membranes rather than by signaling cascades originating from the plasma membrane. Co-staining of Wrch-1 with the mitochondrial marker Mitotracker clearly demonstrated no overlap of Wrch-1 with those membranes (Figure 4.1B) indicating that Wrch-1 induction of apoptosis is not dependent on mitochondrial localization.

A.




B.

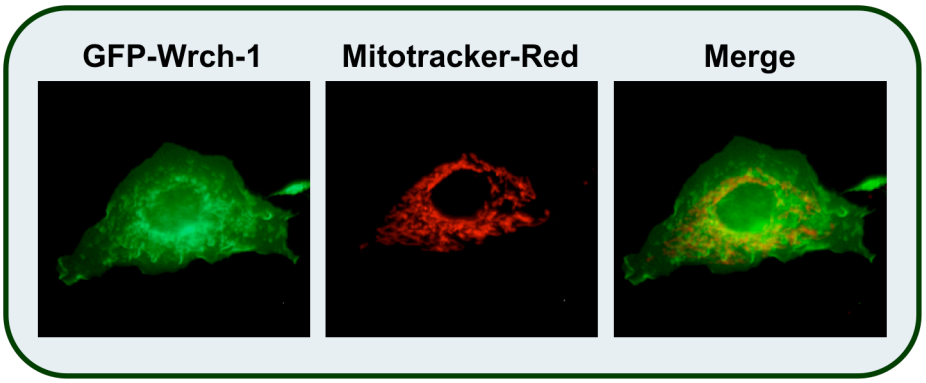



**Figure 4.1 Wrch-1 localization to unknown subcellular compartments may contribute to its biological functions.** (A) Wrch-1 cellular distribution to internal membranes (punctate structures), morphological changes (leading edge membrane ruffles, white arrow) resembles that of TC10 rather than Cdc42. NIH 3T3 fibroblasts are transiently expressing GFP-tagged Wrch-1, -TC10 and –Cdc42 and live cell images were captured 24 hours post-transfection. (B) NIH 3T3 cells expressing GFP-tagged Wrch-1(Q107L) were stained live with Mitotracker (Molecular Probes) to detect mitochondria. GFP-tagged Wrch-1 localization patterns do not overlap with those of mitochondria.

In early 2005, I attended a seminar given by Dr. Yoko Itahana, a postdoctoral fellow in Dr. Yanping Zhang's lab at UNC-CH, in which she described the mechanisms by which ubiquitin and ubiquitin-like protein small ubiquitin related modifier (SUMO) modifications control their substrate proteins. I left her seminar very energized and excited about the prospect of Wrch-1 utilizing either of these modifications for its localization and signaling activities. Many ubiquitylated proteins are directed to internal membranes including endosomes, where Wrch-1 also localizes (Chapter III), and to lysosomes. There are several types of endosomal bodies including early endosomes (marked by transferrin), late endosomes (marked by Rab 9) and recycling endosomes (marked by Rab 11) (Mohrmann and van der Sluijs, 1999) each of which have distinct functions and biological consequences. Thus far, I have only tested and confirmed Wrch-1 localization to early endosomes (Berzat et al., 2005). However, it is likely that Wrch-1 could also target to these other endocytic membranes, since Wrch-1 localization to early endosomes accounted for some, but not all, of its internal membrane localizations.

Because I am interested in carboxyl terminal modifications as regulatory mechanisms of Wrch-1 localization, I checked for potential ubiquitin and SUMO attachment sites in the hypervariable region of Wrch-1. Wrch-1 lacked a sumoylation consensus site (ΨKXE, where Ψ is a large, hydrophobic residue and K is the site of sumoylation (Gill, 2004)), but it has several lysine residues that could be substrates for ubiquitylation. Since there are no consensus sequences for ubiquitin modification, all the carboxyl terminal lysine residues are candidates for ubiquitylation. In addition, there are other lysine residues that could also be

ubiquitylated. Dr. Itahana kindly provided HA-tagged ubiquitin, lysis buffer recipes for optimal ubiquitylation observations and protocols for determining whether proteins incorporate ubiquitin modifications. These were invaluable resources that helped me obtain the preliminary data on which this chapter and project are based.

This project, although preliminary and a “work-in-progress”, is an outgrowth of my own independent thoughts and has reinforced my passion for wanting to learn more. The work in this chapter describes my efforts to date to learn whether posttranslational modifications like ubiquitylation and, also phosphorylation, can function as second signals to regulate Wrch-1 localization and biological function. At the very least, this chapter describes data that are, in my opinion, very tantalizing.

## INTRODUCTION

Wrch-1 (Wnt-regulated Cdc42 homolog-1) is a member of the Rho family of small GTPases, whose activity is regulated by continuous cycling between an inactive GDP-bound state and an active GTP-bound state (Saras et al., 2004; Shutes et al., 2004). Similar to the related Rho proteins, a single missense mutation in its switch region renders Wrch-1 constitutively activated (Tao et al., 2001). Wrch-1 activity modulates many Rho-related biological functions such as actin cytoskeleton organization, activation of the serine/threonine kinases PAK and c-Jun, and growth transformation (Aspenstrom et al., 2004; Berzat et al., 2005; Shutes et al., 2004; Tao et al., 2001). In Wnt-transformed mouse mammary epithelial cells, transcriptional upregulation of Wrch-1, but not of RhoA, Rac or Cdc42, occurs (Tao et al., 2001). Constitutively activated Wrch-1 mimics Wnt-induced morphological changes in mammary epithelial cells (Tao et al., 2001), implicating a role for Wrch-1 in Wnt transforming pathways. Additionally, Wrch-1 has an extended amino terminal region not found in other closely related Rho GTPases that we have shown recently to negatively regulate Wrch-1 effector interaction and transforming activity (Shutes et al., 2004).

Correct subcellular localization is a critical component of Rho protein biology, regulating protein-protein interactions and downstream signaling activities. Membrane targeting of most Rho proteins requires posttranslational modifications dictated by specific sequences within their carboxyl-terminal hypervariable region (Adamson et al., 1992; Michaelson et al., 2001). Of significant importance are the last four amino acids known as the CAAX motif. Conserved in many Rho proteins,

the cysteine residue is the site for irreversible attachment of isoprenoid lipid moieties by farnesyltransferase or geranylgeranyltransferase I enzymes, followed by two aliphatic residues (AA) and any amino acid (X) in the last position (Casey and Seabra, 1996). Additional targeting signals are located upstream of the isoprenylated cysteine residue and are required for correct localization to cellular membranes. For H-, N-Ras and TC10, palmitoyl fatty acids modify upstream cysteine residues whereas stretches of basic residues help recruit K-Ras and Cdc42 to membranes (Hancock et al., 1989; Hancock et al., 1990; Michaelson et al., 2001). Finally, there are also less characterized signals in the hypervariable domain directing GTPase membrane localization that can confer specificity to their membrane targeting and contribute to the functional diversity of these proteins.

Ubiquitylation of Rho family small GTPases has been previously described (Doye et al., 2006; Doye et al., 2002; Pop et al., 2004; Wang et al., 2003; Wilkins et al., 2004). Ubiquitylation involves covalent attachment of ubiquitin, a small 8 kDa protein, to other cellular proteins, thereby modulating their localization, stability and biological functions (Pickart and Eddins, 2004). Through a three-step enzymatic process involving E1 activation and E2 conjugation enzymes, E3 ligases transfer ubiquitin molecules to lysine residues of their cognate substrates (Hershko and Ciechanover, 1998; Pickart and Eddins, 2004). Generally, ubiquitin can associate with proteins either as a single monomer (monoubiquitylation) or as a long polymer chain (polyubiquitylation) by isopeptide linkages at residues K48, K63 or K24 within ubiquitin itself (Pickart and Eddins, 2004). Polyubiquitylated proteins with polymers linked through K48 of ubiquitin are usually targeted for degradation by the 26S

proteasome (Wolf and Hilt, 2004), whereas mono-ubiquitylated proteins utilize this posttranslational modification as a membrane targeting signal for localization to internal membranes of endosomal and lysosomal origin (Pickart and Eddins, 2004).

Regulation of cellular membrane localization by mono-ubiquitylation of small GTPases proteins has not yet been described. However, extensive studies of several receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) (Levkowitz et al., 1998) and hepatocyte growth factor receptor (Met) (Peschard et al., 2001) revealed that they undergo mono-ubiquitylation as a mechanism for their internalization to endocytic membranes. Additionally, it has been shown that tyrosine phosphorylation of these receptors is necessary for recruitment of E3 ligases for ubiquitylation (Levkowitz et al., 1998; Peschard et al., 2001). The carboxyl-terminus of RhoA (Lang et al., 1996) and K-Ras (Bivona et al., 2006), and the amino terminus of Rnd3 (Riento et al., 2005), contain several serine residues that when phosphorylated by protein kinase A (PKA) or PKC relocalized these GTPases from the plasma membrane to internal membranes and to the cytosol for alternate signaling activity. These results provide support for the idea that localization can be controlled at the level of phosphorylation. While translocation of small GTPases by tyrosine phosphorylation has not yet been described, phosphorylation of tyrosine residues within transcription factors like STATs can direct these proteins to the nucleus for transcriptional upregulation.

A typical CAAX motif is not present in Wrch-1 carboxyl terminal sequences. Instead, Wrch-1 terminates in a CCFV motif that undergoes palmitoylation rather than the traditional isoprenylation (Berzat et al., 2005). Our recent study of Wrch-1 carboxyl

terminus sequence contributions to its localization revealed that the minimal membrane targeting sequence of Wrch-1 lacks any upstream cysteine residues for additional palmitoylation, and also lacks a polybasic region of at least four lysine residues for increased membrane affinity (Berzat et al., 2005). However, tandem lysine residues and a tyrosine residue do reside within the minimal membrane localization sequences; these may be ubiquitylated and phosphorylated. Additionally, Wrch-1 contains a putative carboxyl terminal nuclear localization signal that may serve as a substrate for ubiquitylation and translocation to the nucleus. Furthermore, there are other interesting residues not typically found in the carboxyl termini of Rho GTPases that may serve as secondary signals for Wrch-1 cellular distributions. In this study, we assess whether Wrch-1 undergoes further posttranslational modifications and whether these modifications are dictated by the carboxyl terminal sequences of Wrch-1. We found that Wrch-1 is a substrate for additional modifications by ubiquitin and phosphate groups and that its carboxyl terminal sequences can direct proteins to different membrane compartments. Thus, although, like other Rho proteins, Wrch-1 likely contains secondary and tertiary membrane targeting signals necessary for its proper cellular distribution, these signals are distinct from those previously described for Rho proteins and may represent novel mechanisms by which Rho protein membrane localization, and ultimately function, can be regulated.

## MATERIALS AND METHODS

**Molecular Cloning**–Wrch-1 gene sequences flanked by 5' and 3' BamHI end were generated as previously described (Berzat et al., 2005) for subcloning into BamHI

sites of myc-tagged pCMV 3B expression vector for ubiquitylation assays. PCR-mediated DNA amplification was used to fuse different lengths of Wrch-1 carboxyl terminal tail sequences to enhanced green fluorescence protein (EGFP) creating amino terminally EGFP-tagged. short Wrch-1 tail gene sequences. Then, the PCR-amplified EGFP-tagged Wrch-1 short tails with flanking 5' Nhe I and 3' BamHI sites were digested by Nhe I and BamHI restriction enzymes and subcloned into Nhe I and BamHI restriction sites of pEGFP-C1 expression vector (Clontech) for localization assays. For tyrosine phosphorylation and localization assays, previously generated pCGN expression vectors coding hemagglutinin (HA)-tagged Wrch-1 sequences were used (Berzat et al., 2005; Shutes et al., 2004). pCMV expression plasmids encoding HA-tagged ubiquitin (HA-Ub) was obtained from Dr. Yanping Zhang to assess ubiquitylation status of Wrch-1. DNA coding sequences were verified by the Genome Analysis Facility at the University of North Carolina, Chapel Hill.

**Cell Culture and Transfections**–NIH 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemente with 10% calf serum (Invitogen) and 1% penicillin/streptomycin (Invitrogen). H1299 human non small cell lung carcinoma cells (ATCC) were maintained in DMEM containing 10% fetal calf serum (Sigma) and 1% penicillin/streptomycin. For transient expression of various Wrch-1 constructs, cells were seeded in 60 mm dishes one day prior to transfection with Lipofectamine Plus (Invitrogen) per manufacturer's instructions. 24 h later, cells were either treated with appropriate inhibitors and lysed for cellular protein analysis or visually analyzed for localization of membrane proteins.

**Live Cell Imaging**–NIH 3T3 cells were seeded onto glass coverslips in 60-mm dishes. 24 h later, cells were transfected with pEGFP expression vectors as described previously with Lipofectamine Plus. The following day, live cell images were captured on an epifluorescent Zeiss Axioskop microscope (Zeiss, Thornwood, NY) using MetaMorph imaging software (Universal Imaging Corp., Downington, PA).

**Immunofluorescence**–NIH 3T3 and H1299 cells were seeded onto glass coverslips in 60-mm dishes and transfected the following day with appropriate expression vectors. After 24 h, cells were washed twice with Tris-buffered saline (TBS), fixed with 3.7% formaldehyde in phosphate-buffered saline and permeabilized with 0.5% Triton-x-100 in TBS. After 5 washes with TBS, cells were mounted onto glass microslides with Vectashield Hardset mounting medium (Vector Laboratories, Burlingame, CA). The localization patterns of EGFP-tagged Wrch-1 were analyzed using either epifluorescence microscopy as previously described or an Olympus Confocal FV300 microscope with an Argon laser for FITC/Alexa-488. Images were analyzed with Fluoview imaging software. To visualize EGFP-tagged Wrch-1 at lysosomes, cells were treated for 30 min with DMEM supplemented with 100 n*M* Lysotracker Red DND-99 (Molecular Probes, Invitrogen) prior to TBS washes and fixation with 3.7% formaldehyde in PBS.

**Ubiquitylation Assay**–H1299 cells were seeded at 2 x $10^5$ cells per 60-mm dish and transiently co-transfected with Myc epitope-tagged pCMV 3b constructs expressing Wrch-1(Q107L), $\Delta NH_3$ Wrch-1(Q107L) (1-46 amino acids deleted),

SENP-2 (Itahana et al., 2006), or empty vector along with either pCMV HA-tagged ubiquitin or empty vector with Lipofectamine Plus. After 24 h, cells were rinsed twice with PBS and lysed in 1% sodium dodecyl sulfate (SDS) and 1% Nonidet P-40 (NP-40) in PBS. To determine whether proteasomal inhibition enhances the abundance of the putatively ubiquitylated Wrch-1 proteins, cells were treated with DMEM supplemented with either 20 μM MG132 (Calbiochem) or $Me_2SO$ vehicle for 6 h prior to cell lysis as described above. Lysates were sheared using 27 1/2 gauge needle and protein concentrations was determined using DC Lowry protein assay. Approximately 150 μg of protein was diluted in ten times 0.1% NP-40 in PBS containing 10 μ*M* Leupeptin to decrease SDS concentration. For immunoprecipitation, lysates were pre-cleared for 1 h with protein A/G protein beads (Santa Cruz Biotechnology), incubated for 1 h with anti-Myc antibody (9B11) (Cell Signaling Technology) and then incubated for 30 min with protein A/G beads. Immunoprecipitates were washed four times in 0.1% NP-40 lysis buffer (0.1% NP-40, 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 50 m*M* NaF) including 10 μ*M* Leupeptin and resuspended in reducing protein sample buffer (10% SDS, 1 M Tris-HCl, pH 6.8, 25% sucrose, 0.01% bromophenol blue), resolved on SDS-PAGE, and transferred to polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore, Bedford, MA). Membranes were blocked in 5% non-fat dry milk and probed for HA-tagged ubiquitin associated with Myc-tagged Wrch-1 and SENP-2 proteins using mouse anti-HA antibody (Covance), anti-mouse HRP-conjugated antibody, SuperSignal West Dura extended duration substrate (Pierce), and film development. Membranes were then stripped with 62.5 n*M* Tris-HCl, pH 6.8, 2% SDS and 100 m*M* β-mercaptoethanol (BME) and re-probed for total immunoprecipitated Myc-tagged Wrch-1 and SENP-2

proteins using mouse anti-Myc antibody (Cell Signaling Technology) followed by anti-mouse HRP-conjugated antibody and SuperSignal West Dura extended duration substrate as described above.

**Tyrosine Phosphorylation Assay**–H1299 cells were seeded at 2 x $10^5$ cells per 60-mm dish and transiently transfected with HA epitope-tagged pCGN construct containing Wrch-1(WT) using Lipofectamine Plus. After 24 h, cells were treated with freshly prepared 0.1 m*M* sodium pervanadate (a phosphatase inhibitor to preserve phosphorylated state of proteins) for 30 min prior to cell lysis in 1% SDS and 1% NP-40 in PBS as described above. For physiological stimulation of Wrch-1 phosphorylation, transfected cells were starved overnight in DMEM medium without serum and treated for 30 min with 0.1 m*M* sodium pervanadate prior to addition of DMEM medium supplemented with either 10% fetal calf serum or 50 ng/ml EGF (Sigma) plus 1% penicillin/streptomycin and 0.1 m*M* sodium pervanadate for 5 min and cell lysis. For inhibition of EGFR/HER2 and Src family kinases, after overnight serum starvation, cells were treated for 1 h with either $Me_2SO$ vehicle, 5 μ*M* non-specified, unnamed dual specificity EGFR/ErbB2 inhibitor (from Dr. Carolyn Sartor), or 5 μ*M* SU6656 (Calbiochem). During 1 h incubation period, 0.1 m*M* sodium pervanadate was added to the appropriate cells 30 min before serum stimulation and cell lysis. Lysates were sheared and protein concentrations normalized as described above. For immunoprecipitation, approximately 500 μg of protein were pre-cleared for 1 h with protein A/G protein beads (Santa Cruz Biotechnology), incubated for 1 h with anti-HA antibody (Covance) and then incubated for 30 min with protein A/G beads. Immunoprecipitates were washed four times in 0.1% NP-40

lysis buffer including 10 μ*M* Leupeptin and resuspended in reducing protein sample buffer, resolved on SDS-PAGE, and transferred to polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore, Bedford, MA). Membranes were blocked in 5% non-fat dry milk and probed for tyrosine phosphorylated Wrch-1 using mouse anti-P-Tyr-100 antibody (Cell Signaling Technology), anti-mouse HRP-conjugated antibody, SuperSignal West Dura extended duration substrate (Pierce), and film development. Membranes were then stripped with 62.5 n*M* Tris-HCl, pH 6.8, 2% SDS and 100 m*M* β-mercaptoethanol (BME) and re-probed for total immunoprecipitated HA-tagged Wrch-1 protein using mouse anti-HA antibody (Covance) followed by anti-mouse HRP-conjugated antibody and SuperSignal West Dura extended duration substrate as described above.

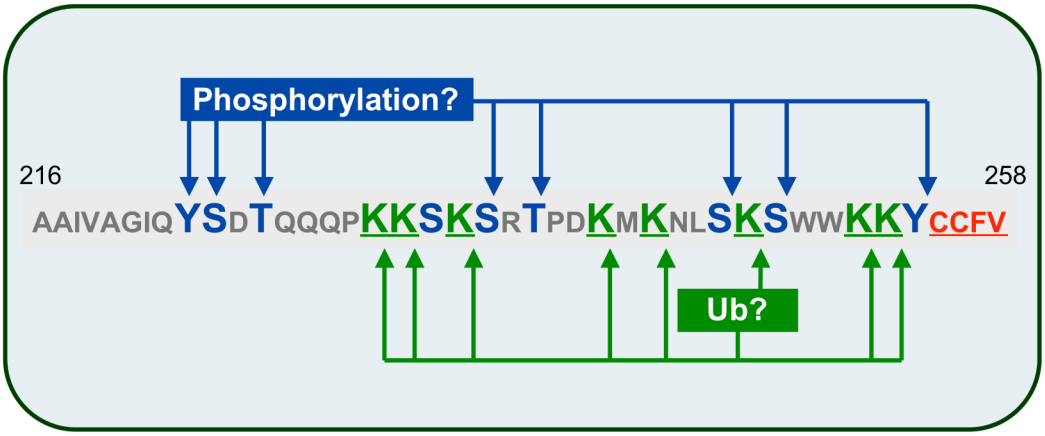
## RESULTS

**Putative Wrch-1 carboxyl-terminal membrane-targeting motifs are sufficient to regulate the subcellular distribution of GFP.** In addition to palmitoylated cysteine residues, the Wrch-1 carboxyl-terminal hypervariable domain has several putative phosphorylation sites and basic residues that may function as secondary and tertiary membrane targeting motifs as seen with other Ras and Rho family members (Figure 4.2A). We have recently shown that fusion of the last nine carboxyl-terminal amino acids of Wrch-1 to EGFP is sufficient to mimic the localization patterns for full-length EGFP-tagged Wrch-1 (Berzat et al., 2005). To determine whether there are additional signals for localization within this region, we fused short stretches of these 9 amino acid sequences to EGFP (EGFP-short tails), which contains no membrane targeting information. Then, we expressed the EGFP-short tails in cells and

examined their effect on EGFP localization. As shown previously, empty EGFP vector distributed predominantly to the nucleus and cytosol, whereas the EGFP fusion containing the last nine amino acids (EGFP-WWKKYCCFV) localized similarly to full-length Wrch-1 at plasma and internal membranes (Figure 4.2B). Surprisingly, deletion of a single tryptophan (residue 250-EGFP-WKKYCCFV) directed EGFP mainly to internal membranes and to the nucleus, suggesting that both tryptophan residues may be required to exclude EGFP from the nucleus or to anchor Wrch-1 to other cellular membranes (Figure 4.2B). Deletion of both tryptophan residues (EGFP-KKYCCFV) leads to localization more closely resembling the cellular distribution of the EGFP-WKKYCCFV fusion protein. Most interestingly, the absence of the tandem lysine residues (EGFP-YCCFV) resulted in nuclear localization, a dramatic accumulation of EGFP in trans-Golgi network (TGN)-like structures and loss of EGFP at endosomal-like internal membranes (Figure 4.2B). The EGFP fusion protein containing only the last 4 amino acids of Wrch-1 (EGFP-CCFV) localized to structures similar to that of the EGFP-YCCFV protein. These data indicate that the carboxyl-terminus of Wrch-1 possesses three different membrane targeting signals, including a possible YCCFV motif, basic lysine residues and tandem tryptophan residues directing Wrch-1 to plasma membrane and internal membranes.

To confirm that the minimal membrane targeting sequence of Wrch-1 is located within its last nine amino acids, a direct comparison of the EGFP short tail fusion protein localizations with that of full-length, endogenous Wrch-1 would be optimal. However, an antibody that recognizes endogenous Wrch-1 protein is not yet

A.




B.

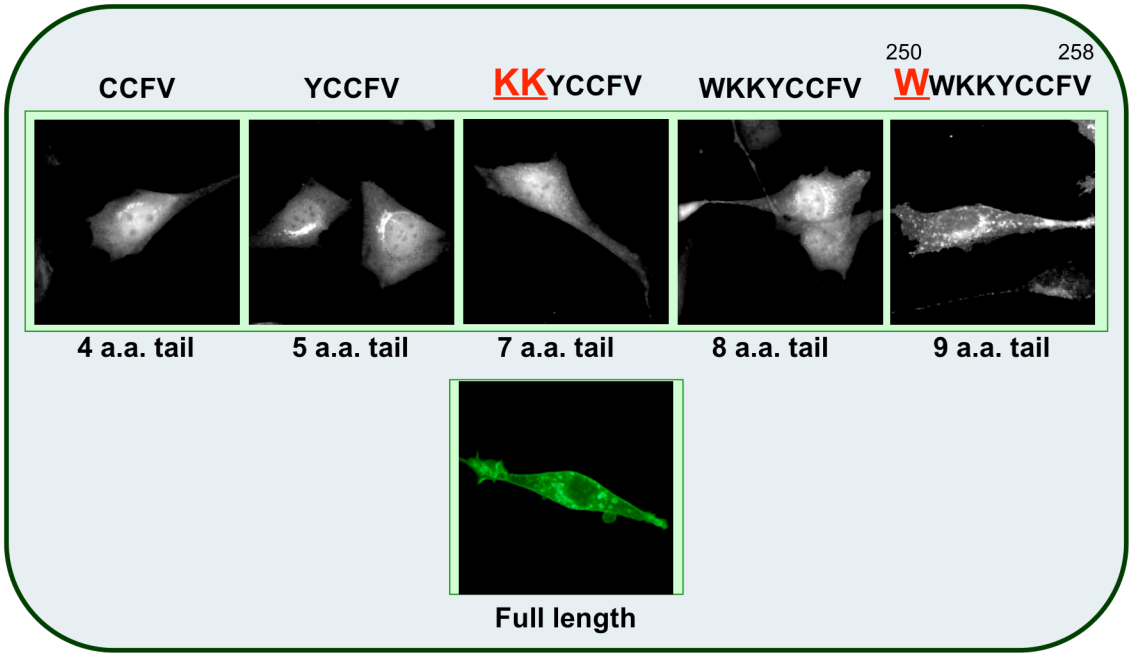



**Figure 4.2 The carboxyl-terminus of Wrch-1 has at least three potential membrane targeting motifs.** (A) The hypervariable domain sequence of Wrch-1 has several residues of interest for secondary membrane targeting signals. (B) EGFP fusion of short Wrch-1 carboxyl-terminal sequences alters EGFP localization. NIH 3T3 fibroblasts (or H1299) transiently expressing EGFP fused to the last nine amino acids (EGFP-WWKKYCCFV), eight (EGFP-WKKYCCFV), seven (EGFP-KKYCCFV), five (EGFP-YCCFV), and four (EGFP-CCFV) were analyzed for EGFP localization changes.

available. Therefore, I plan to co-express EGFP-tagged short tails and HA-tagged full-length Wrch-1 and probe for HA-tagged Wrch-1 cellular distributions with an antibody against HA. To detect any overlapping membrane localizations of the EGFP short tails and HA-tagged full-length Wrch-1, HA-tagged and EGFP-tagged images obtained using appropriate bandpass filters will be merged and analyzed for positive colocalization staining. If full-length Wrch-1 cellular distributions precisely match with the EGFP-tagged minimal membrane targeting sequence (WWKKYCCFV), but not the other shorter EGFP-tagged Wrch-1 hypervariable sequences, this would further support the last nine amino acids of Wrch-1 as the minimal sequence needed for Wrch-1-like membrane targeting.

**Wrch-1 is a substrate for di-monoubiquitylation.** The dramatic shift of GFP from Golgi-like structures to smaller internal membranes by the addition of two lysine residues led us to question how lysine residues, in the absence of a typical polybasic stretch, could contribute to Wrch-1 localization patterns. As mentioned previously, lysine residues can also serve as substrates for modification by ubiquitin molecules (Pickart and Eddins, 2004) and ubiquitylation has been shown to modulate localization of proteins. Since Wrch-1 contains several lysine residues, including the two critical lysines within the last 9 amino acids, it is possible that Wrch-1 may incorporate ubiquitin molecules that may function as membrane targeting signals. To determine whether Wrch-1 is a substrate for ubiquitylation, we co-transfected H1299 non-small cell lung carcinoma cells with Myc-tagged Wrch-1 and Sumo/Sentrin/SMT3 specific isopeptidase (SENP-2), a positive ubiquitylation control (Itahana et al., 2006), in the presence or absence of HA-tagged ubiquitin. SENP2, a

mediator of sumoylation and de-sumoylation (Nishida et al., 2001), is a substrate for polyubiquitylation by E3 ligases. Because ubiquitylation can also lead to degradation of proteins by the 26S proteasome, all samples were treated with MG132 proteasome inhibitor to prevent any potential degradation of ubiquitin-modified Wrch-1 protein. Immunoprecipitated Myc-tagged Wrch-1 was then analyzed for the presence of HA-ubiquitin using western blot analysis. In the presence of exogenous ubiquitin, two discrete bands were observed at molecular weights consistent with the 10 kDa molecular weight shift expected of Myc-tagged Wrch-1 protein modified by one or two, independent mono-ubiquitylation events as compared to the molecular weight of unmodified Wrch-1 (Figure 4.3). Unlike the control protein, a known target for 26S proteasome degradation after polyubiquitylation, in which a smear resulted due to the attachment of many chains of ubiquitin molecules to the control protein, the two single bands observed in the Wrch-1 lane suggests the addition of two single ubiquitin molecules at two separate lysine residues (di-monoubiquitylation) (Figure 4.3). Since monoubiquitylation can direct its target proteins to distinct subcellular locations, it is possible that this modification also functions as a membrane targeting modification for Wrch-1.

**Di-monoubiquitylation of Wrch-1 is not a degradation signal.** Given that ubiquitylation can send marked proteins to the 26S proteasome for processing into its constituent amino acids (Wolf and Hilt, 2004), it is possible, though unlikely, that the mono-ubiquitylation events observed with Wrch-1 are also signals for protein degradation by the 26S proteasome. To test whether ubiquitin-modified Wrch-1 does not cause proteasomal breakdown of Wrch-1 protein, we examined H1299

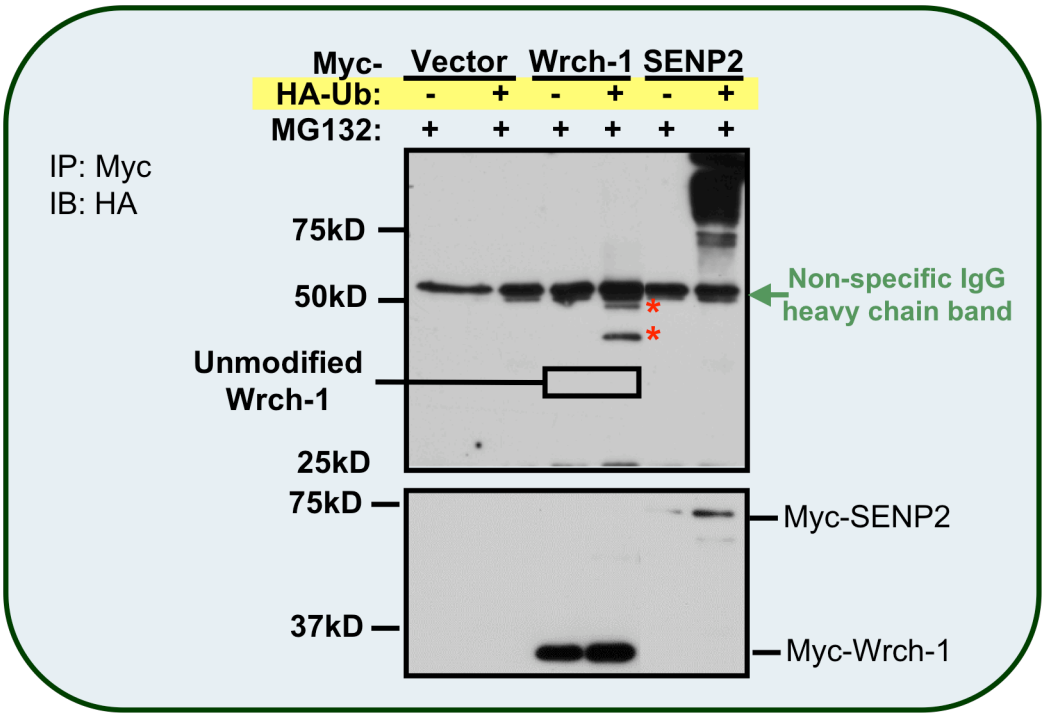



**Figure 4.3 Wrch-1 incorporates ubiquitin molecules.** Myc-tagged Wrch-1 and SENP2 (ubiquitylation positive control) were expressed in H1299 lung carcinoma cells in the presence or absence of HA-tagged ubiquitin. All cells were treated with MG132 (proteasome inhibitor) for 6 h to increase presence of ubiquitylated proteins. Myc-tagged proteins were immunoprecipitated with anti-myc antibody. Immunoprecipitates were subjected to SDS-PAGE and Western blot analysis for incorporation of ubiquitin molecules using anti-HA antibody. Non-specific bands at 50 kDa and 25 kDa are Ig heavy chain and light chain, respectively. Astericks identify suspected mono-ubiquitylated Wrch-1 bands.

cells expressing Myc-tagged Wrch-1 and HA-ubiquitin in the presence or absence of MG132, a potent proteasomal inhibitor. As a control, we also analyzed MG132 effects on SENP2, a 26S proteasome substrate (Itahana et al., 2006). If ubiquitylation does mark Wrch-1 for proteasomal degradation, then treatment with MG132 should block proteasomal degradation, resulting in increased detection of ubiquitylated Wrch-1 protein, whereas no treatment will show little or no ubiquitylated Wrch-1 protein. As expected, poly-ubiquitylation of the control protein is greatly enhanced in the presence of MG132, demonstrating that MG132 is properly inhibiting proteasomal degradation of ubiquitylated proteins (Figure 4.4). In the case of Wrch-1, however, the intensity of the di-monoubiquitin bands are unaffected by MG132 treatment (Figure 4.4). This suggests that ubiquitylation of Wrch-1 is not a direct signal for 26S proteasomal degradation. Instead, the data support the idea that mono-ubiquitylation of Wrch-1 is more likely a signal for targeting Wrch-1 to specific cellular membranes.

**Wrch-1 localizes to lysosomal compartments.** We have previously shown that Wrch-1 localization to internal membrane structures includes those of endocytic origin (Berzat et al 2005). However, complete overlap of Wrch-1 localization with an endosomal marker was not seen, suggesting that Wrch-1 is present at other internal organelles. Mono-ubiquitylation has been shown to direct membrane proteins like receptor tyrosine kinases to endosomes and lysosomes (Dikic, 2003). Given that Wrch-1 distributes to endomembranes and incorporates ubiquitin molecules, we predicted that Wrch-1 would also localize to lysosomal membranes. To determine whether Wrch-1 localization to internal membranes includes lysosomes, we treated

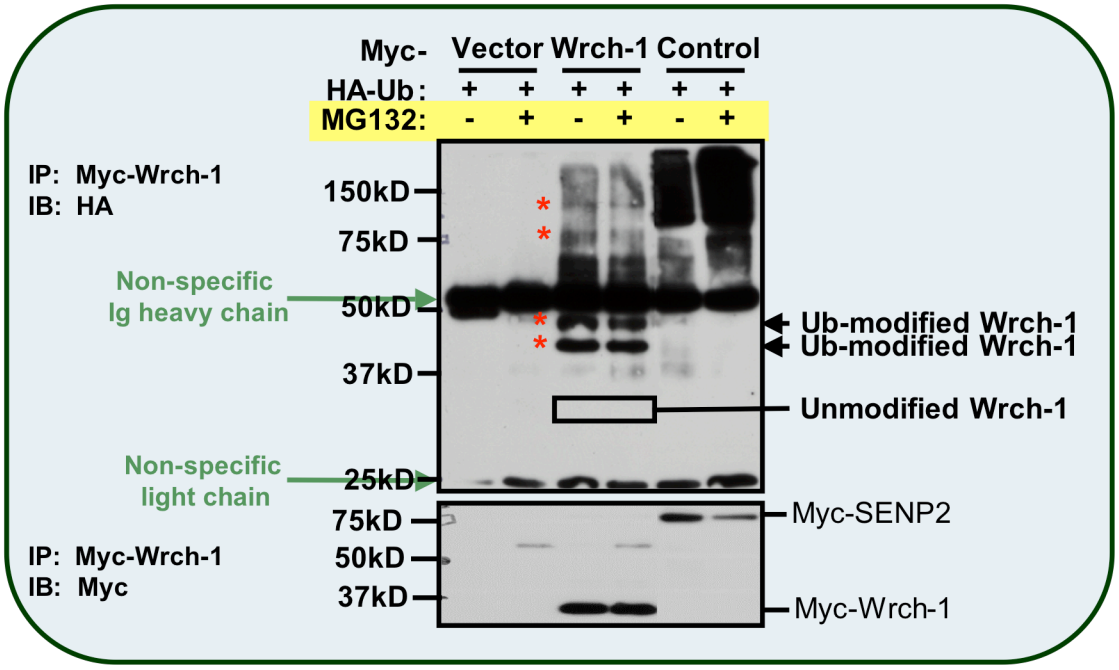



**Figure 4.4 Wrch-1 ubiquitylation is not enhanced by proteasomal degradation inhibition.** HA-tagged ubiquitin and either Myc-tagged Wrch-1 or SENP2 (ubiquitylation positive control) and HA-tagged ubiquitin were co-expressed in H1299 lung carcinoma cells in the presence or absence of MG132 proteasomal inhibitor. 6 h posttreatment, Myc-tagged proteins were immunoprecipitated with anti-myc antibody. Immunoprecipitates were subjected to SDS-PAGE and Western blot analysis for changes in levels of ubiquitylated Wrch-1 and Ub control protein using anti-HA antibody. Non-specific bands at 50 kDa and 25 kDa are Ig heavy chain and light chain, respectively.

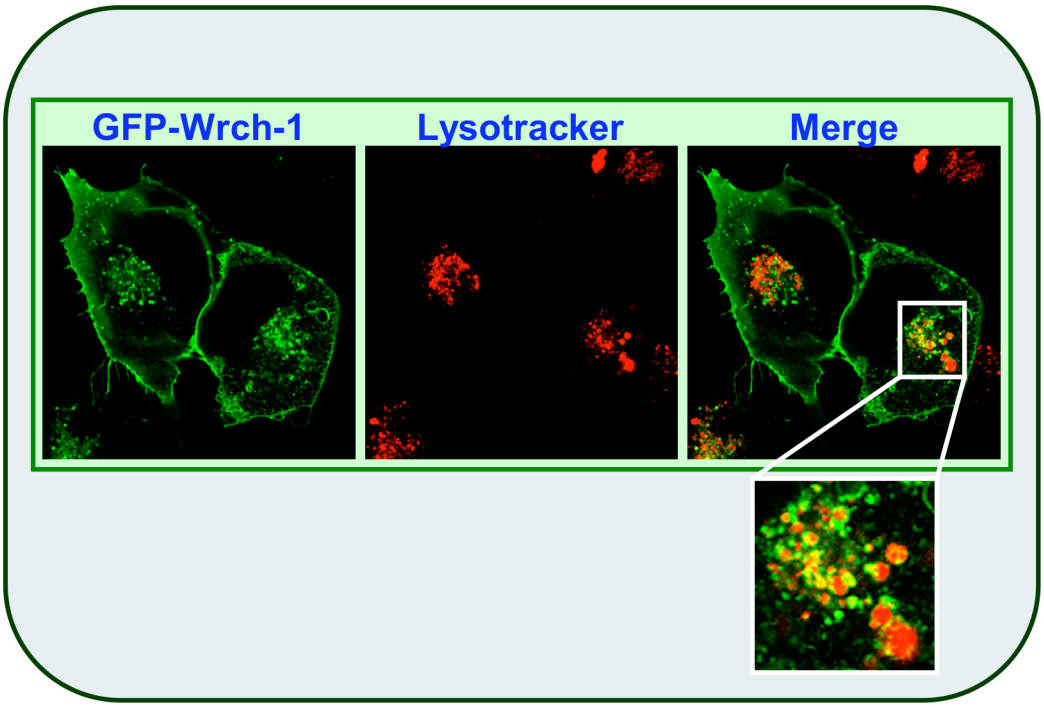



**Figure 4.5 Wrch-1 localizes to lysosomal membranes.** H1299 cells transiently expressing EGFP-tagged full-length or K > R mutant Wrch-1 proteins were stained with the lysosomal marker Lysotracker Red DND-99. Localization of EGFP-Wrch-1 (green) to lysosomal membranes (red) is indicated by the merge image.

H1299 cells transiently expressing EGFP-Wrch-1 with Lysotracker Red DND-99, a known marker for lysosomal compartments. A merge of the EGFP-Wrch-1 and Lysotracker Red DND-99 images revealed localization of Wrch-1 to the surrounding membranes of lysosomes (Figure 4.5). These data are consistent with the possibility of ubiquitylation as a regulator of Wrch-1 distribution to endosomes and lysosomes. It will be interesting to determine whether mutation of lysines critical for ubiquitylation results in loss of Wrch-1 at lysosomal and endosomal membranes. Such experiments are underway. If so, it will provide support for the notion that ubiquitylation targets Wrch-1 away from the plasma membrane and onto internal membranes. Such relocalization is likely to lead to interaction with a distinct pool of regulators and effectors, resulting in different biological outcomes. This could explain the differential transforming activity of the SCFV and CSFV mutants of Wrch-1 described in Chapter III. If the SCFV mutant directs Wrch-1 away from cellular compartments where Wrch-1 would become ubiquitylated and, therefore, targeted to internal membranes, then non-ubiquitylated Wrch-1 protein would remain localized to the plasma membrane, promoting further transforming signaling pathways (See further discussion in Chapter V).

**Di-monoubiquitylation is not dependent on N-terminal extension.** A cascade of three enzymatic reactions catalyzed by E1 activating enzyme, E2 conjugating enzyme and E3 ligase enzyme is required for ubiquitylation of target substrates (Hershko and Ciechanover, 1998; Pickart and Eddins, 2004). Of these enzymes, E3 ligases are substrate specific and are, therefore, responsible for conferring specificity to the ubiquitylation process (Pickart and Eddins, 2004). Many Rho family

small GTPases, but not all, have been shown to either be modified by or to interact with Homologous to E6AP Carboxy Terminus (HECT) domain-containing such as the Smurf family (Wang et al., 2003) and Really Interesting New Gene (RING) domain-containing E3 ligases (Wu et al., 2003). Therefore, it is likely that Wrch-1 is modified by a similar type E3 ligase. Plenty of SH3s (POSH) is an E3 ligase that contains a RING domain and several SH3 domains that can interact with proline-rich regions (Alroy et al., 2005). Interestingly, Wrch-1 contains three proline- rich regions within its amino-terminal extensions that have been shown to bind SH3-containing proteins *in vitro* and *in vivo* (Saras et al., 2004; Shutes et al., 2004). Since Wrch-1 is a substrate for mono-ubiquitylation, interactions between Wrch-1 proline strings and POSH SH3 domains could lead to ubiquitin modification of Wrch-1 through the E3 ligase activity of POSH. To determine whether the proline string motifs within the amino-terminal extension of Wrch-1 are necessary for Wrch-1 ubiquitylation and potential POSH binding sites, we used an N-terminally truncated Wrch-1 mutant previously described (Shutes et al., 2004) that lacks the first 46 amino acids of Wrch-1 protein sequence containing those three proline-rich regions. Surprisingly, deletion of the proline string motifs had no effect on the incorporation of ubiquitin molecules by Wrch-1 (Figure 4.6). This would suggest that association of Wrch-1 with POSH through these N-terminal proline strings is not the mechanism by which Wrch-1 becomes ubiquitylated. These data do not eliminate, however, the possibility that POSH may interact with Wrch-1 through other domains to mediate Wrch-1 ubiquitylation.

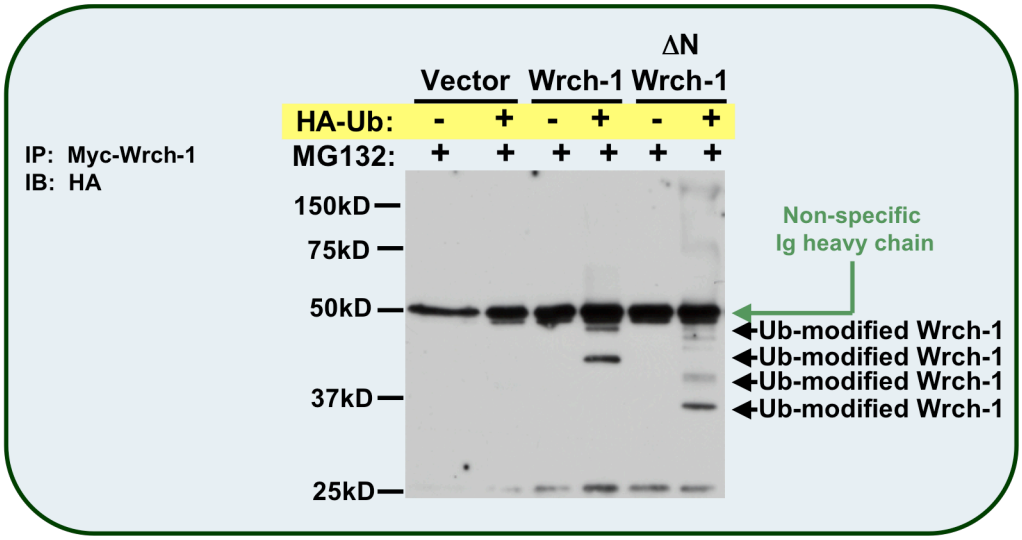



**Figure 4.6 The amino-terminal extension of Wrch-1 is not required for Wrch-1 incorporation of ubiquitin molecules.** Myc-tagged Wrch-1 (parental) and amino-terminally truncated Wrch-1 (first 46 amino acids are deleted) were expressed in H1299 lung carcinoma cells in the presence or absence of HA-tagged ubiquitin. Myc-tagged proteins were immunoprecipitated with anti-myc antibody. Immunoprecipitates were subjected to SDS-PAGE and Western blot analysis for incorporation of ubiquitin molecules using anti-HA antibody. Non-specific bands at 50 kDa and 25 kDa are Ig heavy chain and light chain, respectively.

**Wrch-1 is tyrosine phosphorylated.** Tyrosine phosphorylation is a prerequisite for recruitment of some E3 ligases to their substrates for mono-ubiquitylation (Dikic et al., 2003). It is possible that phosphorylation of Wrch-1 at one of its carboxyl-terminal tyrosine residues may serve as a signal for its ubiquitylation. To determine whether Wrch-1 is tyrosine phosphorylated, we expressed HA-tagged Wrch-1 in H1299 cells, immunoprecipitated Wrch-1 with HA-specific antibody and then performed western blot analysis to detect phosphorylated tyrosine residues in Wrch-1. Cells were also pre-treated with sodium per-vanadate for 30 minutes to inhibit cleavage of phosphate groups by tyrosine phosphatases. In the presence of pervanadate, a phosphotyrosine band is detected at the same molecular weight as HA-Wrch-1, whereas the band is either absent or noticeably diminished without pervanadate (Figure 4.7). A re-probe for the total amount of HA-Wrch-1 immunoprecipitated reveals that Wrch-1 protein is present in all lanes (Figure 4.7). These data show that Wrch-1 is tyrosine phosphorylated and that this modification is sensitive to de-phosphorylation by tyrosine phosphatases.

**Serum stimulation, but not EGF, enhances tyrosine phosphorylation of Wrch-1**. Since deregulation of receptors by tyrosine phosphorylation and mono-ubiquitylation occurs in response to external stimuli (Dikic, 2003), we wanted to determine whether the phospho-tyrosine modification of Wrch-1 is also similarly regulated. For receptor tyrosine kinases such as EGFR, auto-tyrosine phosphorylation occurs within 5 minutes of stimulation, leading to recruitment of its cognate E3 ligase (c-Cbl) for ubiquitylation (Amann et al., 2005). Since Wrch-1 is similarly localized to the plasma membrane, and likely in close proximity to the initial

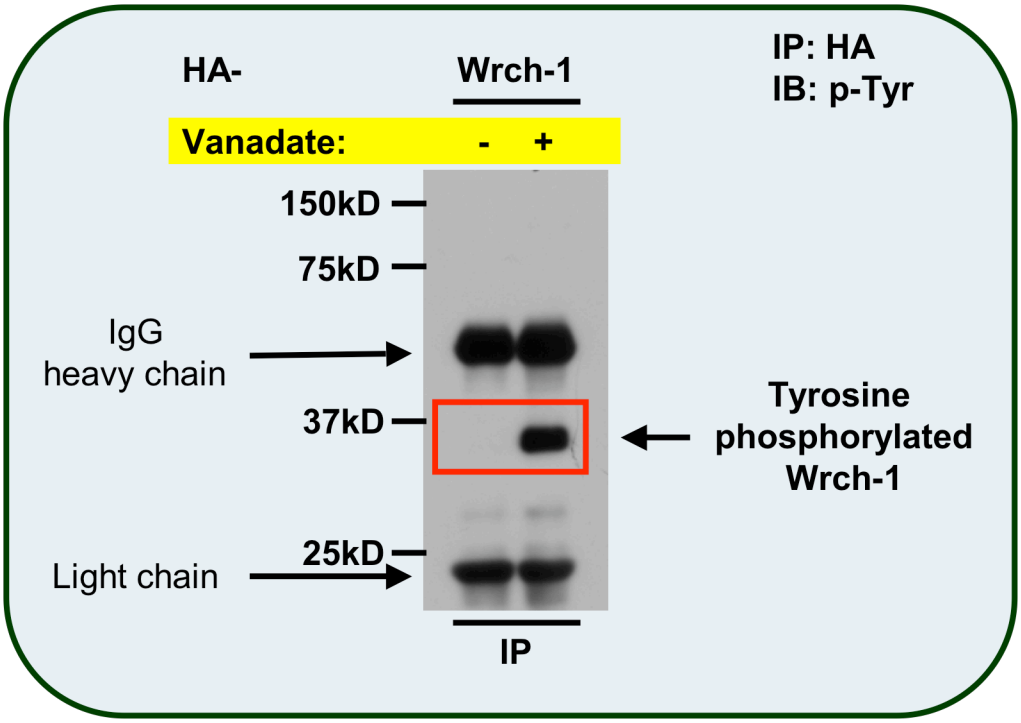



**Figure 4.7 Wrch-1 is tyrosine phosphorylated.** H1299 cells transiently expressing HA-tagged Wrch-1 were treated for thirty minutes with either freshly prepared per-vandate (tyrosine phosphatase inhibitor) or vehicle. HA-tagged Wrch-1 was immunoprecipitated with anti-HA antibody and the immunoprecipitates were SDS-PAGE and Western blot analyzed to detect presence of tyrosine phosphorylated Wrch-1 using anti-phospho-tyrosine antibody. Nonspecific bands at 50 kDa and 25 kDa are Ig heavy and light chains, respectively.

signaling event, we hypothesized that Wrch-1 would undergo the tyrosine phosphorylation with kinetics similar to those of EGFR. To that end, we starved HA-Wrch-1 expressing cells overnight and treated these cells with serum for 5 minutes. While serum starved cells had very little detectable amounts of phosphorylation, HA-Wrch-1 cells treated with serum showed robust tyrosine phosphorylation, suggesting that this modification is physiologically relevant, and rapidly turned over by tyrosine phosphatases (Figure 4.8).

If serum-stimulated phosphorylation of Wrch-1 is a downstream consequence of EGFR receptor stimulation and subsequent phosphorylation, then recruitment of c-Cbl to EGFR would also place c-Cbl in close proximity to Wrch-1, allowing for possible interactions of c-Cbl with Wrch-1 in the context of EGFR signaling. Since there are numerous agonists in serum that could be responsible for Wrch-1 tyrosine phosphorylation, we wanted to determine whether EGF stimulation would lead to tyrosine phosphorylation of Wrch-1. We treated HA-Wrch-1 expressing cells with EGF and serum to compare whether similar or different levels of tyrosine phosphorylation were stimulated. While serum stimulation resulted in robust phosphorylation of Wrch-1, a clear induction of Wrch-1 phosphotyrosine levels by EGF was not observed (Figure 4.9). Instead, EGF treatment resulted in barely detectable increases in phospho-Wrch-1 levels compared to starved, vanadate treated cells (Figure 4.9), suggesting that the stimulus in serum is not EGF. However, there are other ligands such as the neregulin (NRG) family that bind to the EGFR family of kinases that could lead to phosphorylation of Wrch-1. Interestingly, EGF treatment appears to slightly decrease levels of Wrch-1 phosphorylation,

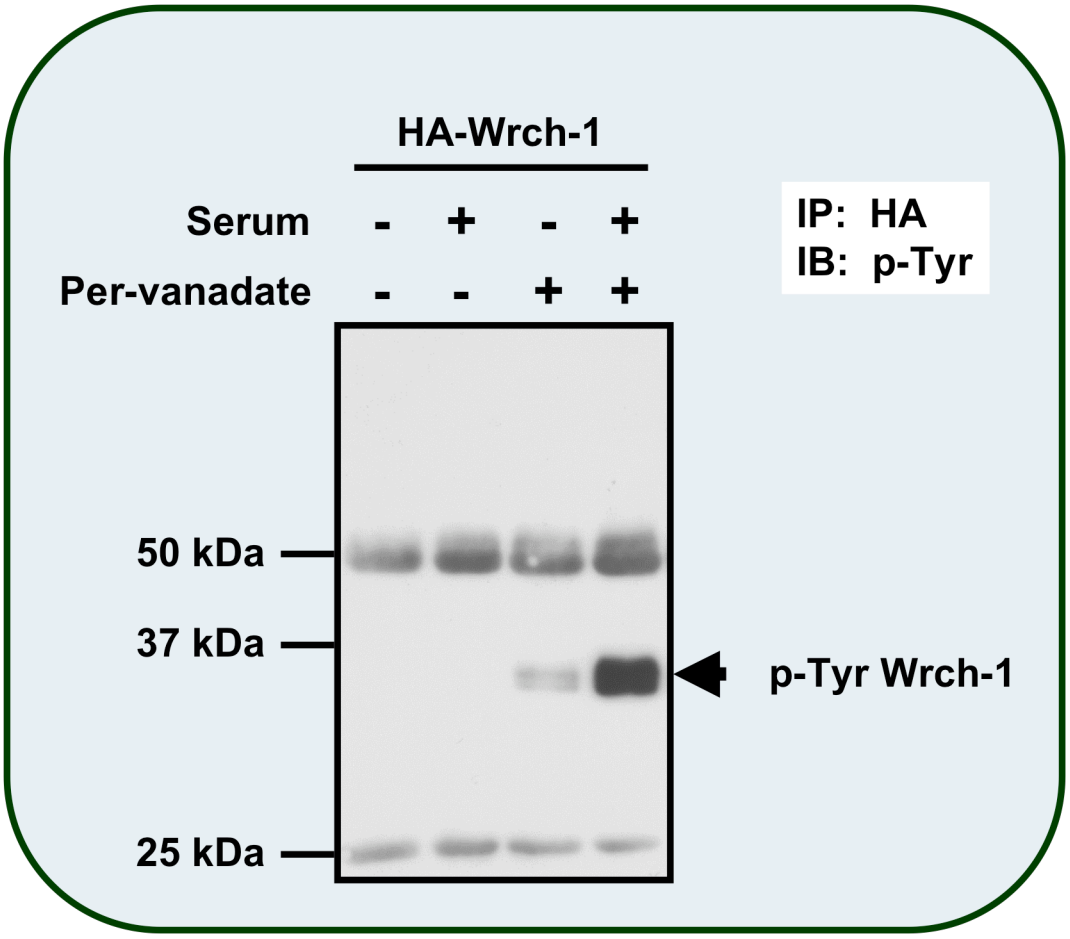



**Figure 4.8 Serum stimulation induces Wrch-1 tyrosine phosphorylation.** H1299 cells transiently expressing HA-tagged Wrch-1 were serum starved overnight and treated for thirty minutes with either freshly prepared per-vandate (tyrosine phosphatase inhibitor) or vehicle. Then cells were incubated for five minutes in media with or without 10% serum prior to cell lysis and immunoprecipitation of HA-tagged Wrch-1 with anti-HA antibody. Immunoprecipitates were SDS-PAGE and Western blot analyzed to detect presence of tyrosine phosphorylated Wrch-1 using anti-phosphotyrosine antibody. Nonspecific bands at 50 kDa and 25 kDa are Ig heavy and light chains, respectively.

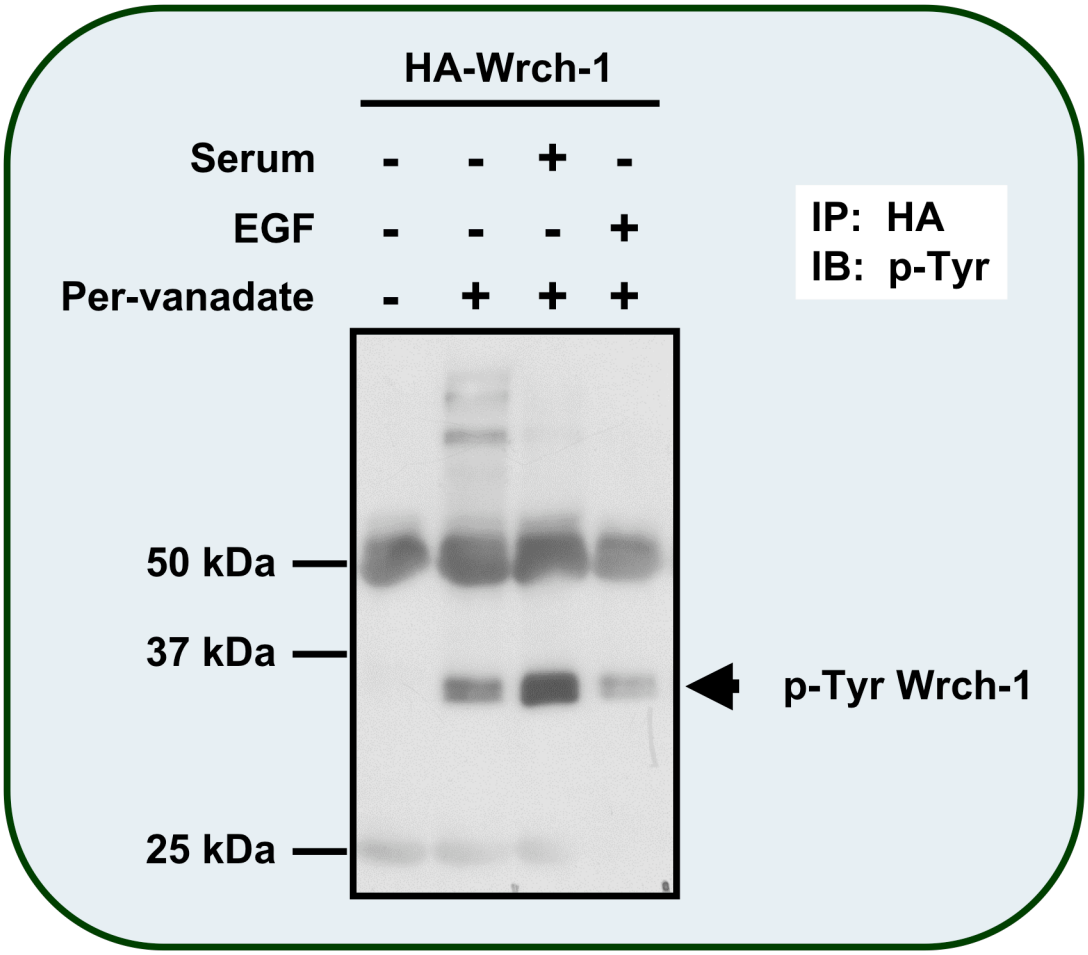



**Figure 4.9 EGF does not induce phosphorylation of Wrch-1.** H1299 cells transiently expressing HA-tagged Wrch-1 were serum starved overnight and treated for thirty minutes with either freshly prepared per-vandate (tyrosine phosphatase inhibitor) or vehicle. Then cells were incubated for five minutes in media with or without 10 μg/μl EGF prior to cell lysis and immunoprecipitation of HA-tagged Wrch-1 with anti-HA antibody. Immunoprecipitates were SDS-PAGE and Western blot analyzed to detect presence of tyrosine phosphorylated Wrch-1 using anti-phosphotyrosine antibody. Nonspecific bands at 50 kDa and 25 kDa are Ig heavy and light chains, respectively.

suggesting a possible negative regulatory role for ErbbB family signaling on Wrch-1 modifications.

**EGFR family receptors negatively regulate Src-dependent Wrch-1 tyrosine phosphorylation.** To determine whether ErbB family signaling has any effect on Wrch-1 phosphorylation levels, we treated HA-Wrch-1 expressing cells with non-specified dual specificity inhibitor of EGFR and ErbB2 signaling. The H1299 cell line has multiple copies of EGFR and expresses high levels of EGFR protein, whereas ErbB2 protein levels are not detectable by western blot and ErbB3 protein is minimally detected (Amann et al., 2005). Surprisingly, treatment with the EGFR/ErbB2 inhibitor resulted in an increase, not decrease, in phospho-Wrch-1 levels (Figure 4.10). This would suggest that signaling through EGFR/ErbB2 negatively regulates phosphorylation of Wrch-1, correlating well with our data that shows very little, if any increases in Wrch-1 phosphorylation after EGF stimulation.

Interestingly, a recent paper described a Src-dependent compensatory function of ErbB3 in which inhibition of EGFR and ErbB2 resulted in increased activity by ErbB3, lending protection against loss of EGFR/ErbB2 signaling (Contessa et al., 2006). It is possible that the increase in Wrch-1 phosphorylation observed in the presence of the EGFR/ErbB2 inhibitor is due to this compensatory ErbB3 kinase activity. Since the ErbB3 kinase activity requires Src activity, perhaps the Wrch-1 tyrosine phosphorylation event also depends on Src function. We treated HA-Wrch-1 expressing cells with the Src family inhibitor SU6656, which has been

shown to block the kinase activity of Src, Fyn and Yes (Blake et al., 2000), and analyzed its effect on serum-stimulated Wrch-1 tyrosine phosphorylation. Treatment with SU6656 almost completely abrogated the increase in phospho-Wrch-1 levels compared to the phosphorylated Wrch-1 levels in vehicle-treated cells (Figure 4.10). Since endogenous Src-dependent ErbB3 kinase activity towards Wrch-1 may occur in the presence of EGFR/ErbB2 inhibition, we treated cells with EGFR/ErbB2 and SU6656 inhibitors. Inhibition of Src by SU6656 decreased, but did not abrogate, phosphorylated Wrch-1 levels (Figure 4.10), suggesting that Src is partially necessary for EGFR/ErbB2-independent modification of Wrch-1.

These results also further support the idea of EGFR/ErbB2 signaling as a negative regulator of Wrch-1 phosphorylation. Inhibition of Src, in the presence of uninhibited EGFR/ErbB2, almost completely blocks Wrch-1 phosphorylation, suggesting that negative regulation of Wrch-1 phosphorylation by EGFR/ErbB2 and inhibition of Src synergize to ablate this modification. However, when the negative effects of EGFR/ErbB2 are lifted (through EGFR/ErbB2 inhibition), then complete inhibition of Wrch-1 phosphorylation by the Src inhibitor SU6656 is not possible. Taken together, our data on the effects of serum, EGF, and inhibitors of EGFR family and Src on Wrch-1 tyrosine phosphorylation suggest that Wrch-1 may function downstream of the EGFR family of receptors, and that this modification may regulate subcellular localization and trafficking of Wrch-1.

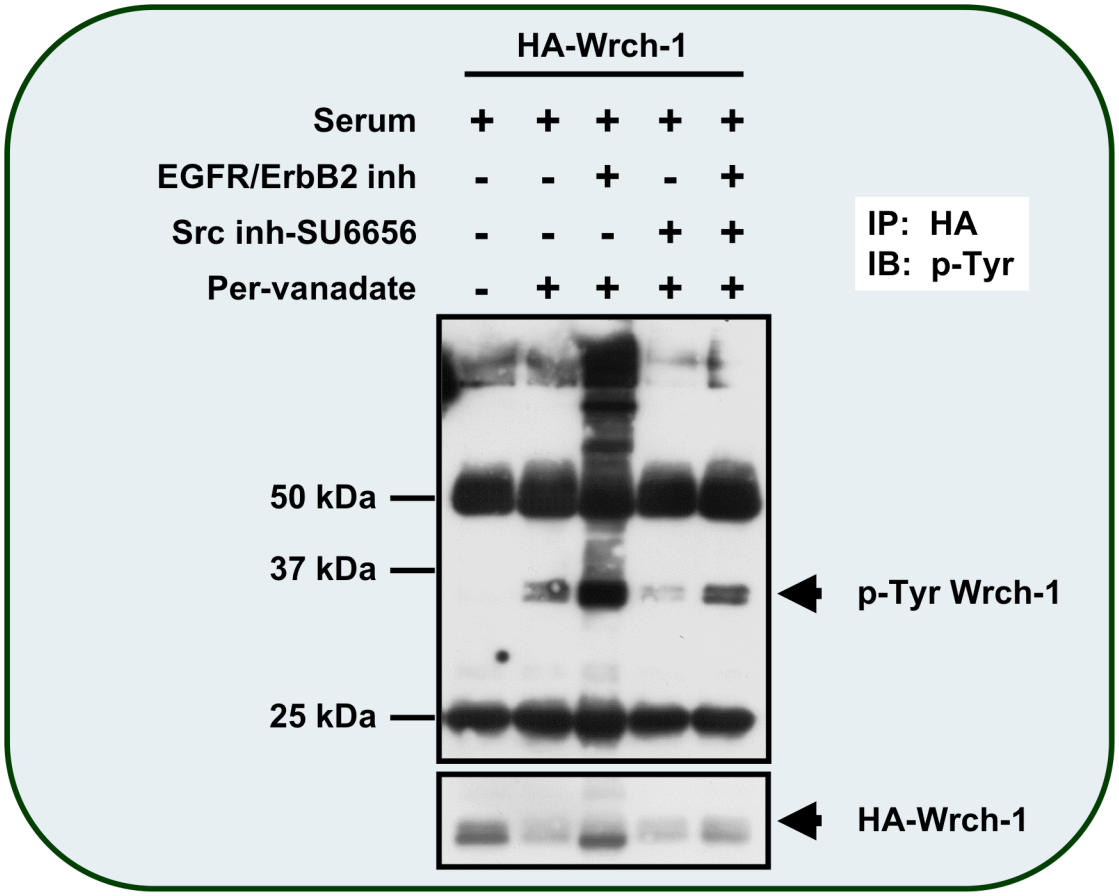



**Figure 4.10 Inhibitors of EGFR/ErbB2 enhance Src-dependent Wrch-1 phosphorylation.** H1299 cells transiently expressing HA-tagged Wrch-1 were serum starved overnight and treated 1 h with either $Me_2SO$ vehicle, EGFR/ErbB2 inhibitor, or SU6656 (Src family kinase inhibitor). During 1 h incubation, cells were also treated for thirty minutes with either freshly prepared per-vanadate (tyrosine phosphatase inhibitor) or vehicle prior to five minute incubation in media with or without 10% serum. Cell lysis and immunoprecipitation of HA-tagged Wrch-1 with anti-HA antibody followed. Immunoprecipitate were SDS-PAGE and Western blot analyzed to detect presence of tyrosine phosphorylated Wrch-1 using anti-phosphotyrosine antibody. Nonspecific bands at 50 kDa and 25 kDa are Ig heavy and light chains, respectively. family kinases suggest that Wrch-1 may utilize phosphorylation as a mechanism to recruit E3 ligases like c-Cbl for its mono-ubiquitylation.

## DISCUSSION

In the present study, we have investigated the possibility of additional membrane targeting signals for Wrch-1 localization. We have found that the carboxyl terminal hypervariable region of Wrch-1 contains at least three potential membrane targeting motifs that may help coordinate Wrch-1 subcellular distributions. We have also shown that, in addition to endocytic membrane localization (Berzat et al., 2005), Wrch-1 protein distributes to lysosomal compartments, suggesting a potential role for Wrch-1 in cellular trafficking. Additionally, the pattern of Wrch-1 incorporation of ubiquitin molecules implicates that Wrch-1 is a substrate for monoubiquitylation, not polyubiquitylation. Treatment with a proteasomal inhibitor indicates that Wrch-1 ubiquitylation is not a signal for its protein degradation. The amino terminus of Wrch-1 has several proline string motifs that interact with SH3 domains and negatively regulate Wrch-1 function (Saras et al., 2004; Shutes et al., 2004). Interestingly, we found that these motifs are not necessary for di-monoubiquitylation of Wrch-1. We have also shown here that Wrch-1 is tyrosine phosphorylated in a serum-dependent manner. Src family kinase activity is required for phosphorylation of Wrch-1, and EGFR family members appear to negatively regulate this event.

Most Ras and Rho proteins depend on a secondary signal, in addition to a primary lipidation event, for proper localization to cellular membranes (Hancock et al., 1989; Hancock et al., 1990; Michaelson et al., 2001). For example, K-Ras and Rac 1 both utilize a polybasic region containing a stretch of at least 4 basic residues that are critical for their membrane targeting (Hancock et al., 1989; Hancock et al., 1990; Michaelson et al., 2001). Mutation of these residues leads to mislocalization of fully

lipidated K-Ras and Rac. Additionally, palmitoylation of cysteine residues upstream of the isoprenylated CAAX motifs of H-, N-Ras and TC10 are necessary second signals for these proteins in the absence of a polybasic region (Hancock et al., 1989; Hancock et al., 1990; Michaelson et al., 2001). While we have previously shown that Wrch-1 does utilize palmitoyl fatty acids, instead of isoprenoid lipids (Berzat et al., 2005), as its main lipid moiety for localization, other potential C-terminal membrane targeting signals have not been characterized. A functional polybasic region usually requires at least 4 basic residues. Given that the last nine amino acids of Wrch-1, which contain only two basic residues, are sufficient to mimic the localization patterns of full-length Wrch-1, it is unlikely that Wrch-1 uses a polybasic "region" for its localization. Additionally, there are no cysteines available for palmitoylation upstream of the cysteine residues at position 255 and 256.

Ubiquitylation is another posttranslational modification that has been previously shown to modulate subcellular localizations of membrane-bound signaling proteins. The most commonly recognized ubiquitylated substrates include receptor tyrosine kinases (RTKs) (Dikic, 2003) and tumor suppressor proteins like p53 (Geyer et al., 2000). To date, there are a few small GTPases reported as substrates for ubiquitylation. Of those identified, no evidence of Ras subfamily ubiquitylation has been published; however, many groups have indicated that Rho family proteins are regulated by this modification. For example, RhoA and Rac1 are polyubiquitylated and proteasomally degraded in response to CNF1 (Doye et al., 2006; Doye et al., 2002; Pop et al., 2004), a bacterial toxin that constitutively activates RhoA, Rac1 and Cdc42 through deamidation of glutamine 63 (RhoA) and glutamine 61 (Rac1/Cdc42)

to glutamate (Flatau et al., 1997; Lerm et al., 1999; Schmidt et al., 1997). Additionally, RhoA, but not Rac1 or Cdc42, has been identified as a substrate for ubiquitylation by the Smurf family of HECT-containing E3 ligases in migrating cells (Wang et al., 2003). RhoBTB, an unusual Rho family protein in that it contains tandem BTB (Broad-Complex, Tramtrack, and Bric a brac) domains required for binding to cullin E3 ligase subunits, forms a complex with the E3 ligase cullin 3 and other molecules as part of a multi-subunit complex necessary for polyubiquitylation of RhoBTB (Wilkins et al., 2004). Wrch-1 is unique in that it appears to mainly undergo mono-ubiquitylation. Thus far, only polyubiquitylation in conjunction with proteasomal degradation has been demonstrated for the other Rho proteins (Doye et al., 2006; Doye et al., 2002; Pop et al., 2004; Wang et al., 2003). That Wrch-1 incorporates single ubiquitin molecules, suggests that Wrch-1 may use mono-ubiquitylation as a second signal for more specialized regulation of its localization and function.

Given that there are no consensus sequences for the attachment of ubiquitin to lysine residues, it is possible that, under certain circumstances, other Ras and Rho family proteins are mono-ubiquitylated and that this modification could control their localization to specific internal membranes. H- and N-Ras are both localized to internal membranes through palmitoylation (Baker et al., 2003; Magee et al., 1987) and other unknown mechanisms, and have several potentially ubiquitylated lysines residues within their C-termini. While Rac1 is mostly found at the plasma membrane and cytosol, Rac2 and Rac3 are more endomembrane localized (Joyce and Cox, 2003; Keller et al., 2005; Wennerberg and Der, 2004), and previously unappreciated

mono-ubiquitylation could also contribute to their localizations. A survey of many Ras and Rho proteins is currently underway to determine their mono-ubiquitylation status and its possible contribution to their subcellular localization.

It is interesting to speculate which E3 ligases may act upon Wrch-1. POSH, a RING finger-containing E3 ligase, was an obvious candidate because it contains several SH3 domains (Alroy et al., 2005) that could potentially interact with the proline string motifs located in the amino-terminal region of Wrch-1 leading to its mono-ubiquitylation. However, we have shown that deletion of these proline string motifs has no effect on ubiquitylation of Wrch-1, suggesting that modification of Wrch-1 by POSH is not mediated by interaction with the amino-terminus of Wrch-1. That said, it is still possible for POSH to associate with Wrch-1 through other domains. For example, POSH, previously described as a scaffolding protein that activates JNK and causes translocation of NFκB to the nucleus, interacts in a GTP-dependent manner with Rac1 (Tapon et al., 1998). Mutations within the effector binding region of Rac1 (Y40C) disrupt this interaction (Pop et al., 2004). Since Wrch-1 shares sequence homology with Rac1 in this region, Wrch-1 could also associate with POSH in a similar manner. Additionally, beyond the effector domain, the insert region of Rac and Cdc42 contain important sequences required for binding to effector proteins and their transforming activity (Karnoub et al., 2001; Li et al., 1999; Tapon et al., 1998). Thus, POSH is still a viable candidate as an E3 ligase activity for Wrch-1. Preliminary data suggests that Wrch-1 may be ubiquitylated by POSH (data not shown) and more conclusive studies are currently being pursued.

In addition to ubiquitylation, there are other less characterized signals that have been reported to contribute to the localization of small GTPases and, may also function as secondary membrane targeting signals for Wrch-1. Phosphorylation of specific serine, threonine or tyrosine residues, for example, can dictate changes in cellular localization of several small GTPases. For example, a recent study showed that atypical PKC-induced phosphorylation at serine 181 of K-Ras promotes a farnesyl electrostatic switch, resulting in K-Ras translocation from the plasma membrane to the mitochondria for pro-apoptotic functions (Bivona et al., 2006). A similar mechanism was described for Rnd3/RhoE in which serine phosphorylation at N-terminal serine residues by serine/threonine kinase ROCK caused cytosol distribution of Rnd3 in a membrane flotation assay, whereas phosphodeficient Rnd3 mutants were distributed between membrane and cytosolic fractions (Riento et al., 2005). An earlier study of the phosphorylation status of RhoA revealed that cAMP-mediated PKA phosphorylation of RhoA could alter its localization from the plasma membrane to the cytosol (Lang et al., 1996).

Evidence of tyrosine phosphorylation as a regulator of small GTPase localization is less clear. However, there are many examples in other proteins that utilize tyrosine phosphorylation as a mechanism for translocation. The signal transducers and activators of transcription (STATs) are transcription factors that upon tyrosine phosphorylation by Janus kinases (JAKs) or Src relocalize to the nucleus where they promote transcriptional activation (Silva, 2004). Also, tyrosine phosphorylation of paxillin, a focal adhesion protein, is important for its co-localization with actin at focal adhesions (Nakamura et al., 2000). Mutation of tyrosine residues prevents

phosphorylation of paxillin and alters paxillin targeting to focal adhesions (Nakamura et al., 2000). In addition, phosphorylation can also be a precursor to mono-ubiquitylation of certain membrane and cytosolic proteins such as RTKs. As previously mentioned, ubiquitylation of the ErbB family (Levkowitz et al., 1998) and c-Met (Peschard et al., 2001) RTKs is dependent on phosphorylation of tyrosine residues for recruitment of E3 ligases to the receptors. Our data demonstrating that Wrch-1 is tyrosine phosphorylated supports the idea of this posttranslational modification as a possible mechanism for both localization alterations and for priming of Wrch-1 ubiquitylation. We are currently determining whether ubiquitylation of Wrch-1 is dependent on its phosphorylation status and also whether phosphorylation of Wrch-1 affects its subcellular distributions as a secondary membrane targeting signal.

In addition to the tyrosine residue, the last nine amino acids within the C-terminus of Wrch-1 contains at least two other potential membrane-targeting signals that could function as secondary and tertiary signals for Wrch-1 membrane localization. The first of these is a pair of tryptophan residues at residues 250 and 251 that could be a di-aromatic motif of the type that has been implicated in endocytic membrane targeting of several transmembrane proteins (Bonifacino and Traub, 2003; Nair et al., 2003; Schweizer et al., 2000). Di-aromatic motifs are thought to sequester proteins away from lysosomal destinations and direct proteins instead to recycling endosomal compartments, preventing their degradation (Bonifacino and Traub, 2003). Interestingly, this signal appears to work in conjunction with palmitoylation such that di-aromatic-containing palmitoylated proteins are localized to endosomes,

whereas unpalmitoylated proteins are directed to lysosomes (Nair et al., 2003). Given that Wrch-1 is also palmitoylated, its putative di-aromatic motif may have a role in Wrch-1 localization to these internal compartments. In support of this, the GFP fusion proteins containing the di-aromatic tryptophan motif localizes to Wrch-1-containing internal membranes, whereas the GFP fusion protein containing only one tryptophan residue is unable to recapitulate Wrch-1 localization to endocytic membranes. Chp/Wrch-2, a close relative of Wrch-1, is also palmitoylated, but lacks the tandem tryptophan residues present in Wrch-1 (Chenette et al., 2005). Instead, Chp contains two phenylalanine residues at residues 232 and 233 very near its C-terminal end. It is unclear at this juncture whether these tandem phenylalanines of Chp constitute a functional di-aromatic motif. However, Chp does have a single tryptophan residue, analogous in position to the Wrch-1 tryptophans, that when mutated results in mislocalization of palmitoylated Chp (Chenette EJ, personal communication). Taken together, it is interesting to speculate that Wrch-1 and Chp localization to endosomes or to lysosomes requires both aromatic and palmitoyl fatty acid contributions, adding an additional layer of complexity to Wrch-1 and Chp membrane localization and suggesting potential roles for these proteins in cellular trafficking.

The minimal membrane targeting sequence of Wrch-1 also contains another possible internal membrane localization signal, the YXX∅ motif. The YXX∅ motif consists of a conserved tyrosine residue, any two hydrophilic residues (XX) and a hydrophobic residue (∅) and has been described as an endosomal/lysosomal-localization motif (Bonifacino and Traub, 2003). Usually endocytic motifs are

situated within the protein, whereas lysosomal motifs occur within 6-7 residues from the C-termini of proteins (Bonifacino and Traub, 2003). Previous studies have shown that this motif is also important for trafficking of proteins to the trans-Golgi network (TGN) (Honing et al., 1996). Interestingly, not only does Wrch-1 have a putative YXX∅ motif (YCCF) very near its C-terminal end, suggesting a potential alternative mechanism for its lysosomal targeting, but the GFP fusion protein containing only the YXX∅ motif (YCCFV) localized to TGN-like structures. While the hypervariable regions of other Ras and Rho family proteins do not reveal evidence of these exact motifs, the possibility of these motifs as functional secondary and tertiary membrane signals correlates well with the fact that additional signals, in conjunction with lipid modifications, are necessary for correct membrane targeting of small GTPases. Furthermore, if only Wrch-1 proteins contain such functional motifs, these may represent Wrch-specific mechanisms of regulation.

Localization of Wrch-1 to endocytic and lysosomal membranes suggests that Wrch-1 could play a role in cellular trafficking. If so, it is possible that Wrch-1 localization to these internal membranes represents sorting of the Wrch-1 protein itself or Wrch-1-assisted delivery of other proteins to endocytic vesicles, similar to the function of Rab small GTPases. Rab proteins have well-defined roles in endocytosis, trafficking, vesicular fusion and exocytosis (Seachrist et al., 2000; Seachrist and Ferguson, 2003; Seachrist et al., 2002). For example, Rab5 is a prominent modulator of early endosomal functions, whereas Rab4 and Rab11 are also localized to recycling endosomes and mediate recycling of proteins back to the plasma membrane (Seachrist and Ferguson, 2003). Rab7, on the other hand,

controls distribution of some receptors to lysosomes for degradation (Seachrist and Ferguson, 2003). Clearly, attachment of geranylgeranyl isoprenoid moieties contributes greatly to localization of Rab proteins to their intracellular compartments (Seabra et al., 1992). However, some Rab proteins also contain other putative membrane motifs similar to those that have been described previously for Wrch-1. For example, both Rab8 and Rab24, which localize to ER, Golgi and late endosomes, contain possible di-aromatic motifs within their C-termini (Seabra et al., 1992). While Rab10 does not have tandem aromatic residues, it does have a single tryptophan residue upstream of its geranylgeranylated cysteine residues (Seabra et al., 1992), which may help to control its cellular distribution.

To date, there are no known Rab family proteins modified by ubiquitin molecules. However, there are several lysines within the C-termini of many Rab proteins that might be ubiquitylated. In support of this possibility, a recent paper demonstrated that dominant-negative Rab24 resulted in accumulation of the protein in nuclear inclusions where ubiquitin and Hsp70 were also colocalized (Maltese et al., 2002). Hsp70 has been previously shown to interact with Rab GEFs containing E3 ligase domains (Rosa and Barbacid, 1997; Rosa et al., 1996). It would be interesting to determine whether Wrch-1 mimics any Rab-mediated functions at internal membranes and also whether these functions require ubiquitin inputs.

# CHAPTER V

# SUMMARY AND FUTURE DIRECTIONS

Since beginning this dissertation project, there have been several recent publications providing further information about the regulatory mechanisms of the Wrch proteins. As mentioned in Chapter I, both Wrch-1 and Chp/Wrch-2 have amino-terminal extensions with several proline string motifs that could potentially be interaction sites for SH3 domain-containing proteins. In support of this idea, we and others independently demonstrated that Wrch-1 has proline string motif-dependent interactions with the SH3 domains of Nck (in vitro) and Grb2 (in vivo) (Shutes et al., 2004) and NCKβ (in vivo) (Saras et al., 2004). At this juncture, there are no published accounts examining interactions of the Chp amino terminal domain with SH3 domain-containing proteins.

It is now known that the amino termini of Wrch-1 and Chp function as negative regulators of their effector interactions and signaling and transforming activities. In collaboration with Adam Shutes and Channing Der (UNC-CH), we have recently shown that deletion of the Wrch-1 amino terminus (the first 46 amino acids, including the proline string motifs) significantly enhances Wrch-1 transforming activity as measured by anchorage-independent growth of Wrch-1 cells in soft agar, causes

higher levels of phosphorylated PAK compared to full length Wrch-1 and increases interaction of Wrch-1 with the PAK binding domain (PBD) or CRIB domain of PAK (Shutes et al., 2004). In a separate paper, similar results were observed upon deletion of the amino terminus of Chp (Chenette et al., 2005), suggesting that the amino terminal domains of Wrch-1 and Chp may either provide docking sites for proteins that negatively regulate Wrch-1 and Chp signaling activities or function as an auto-inhibitory region for their remaining protein domains.

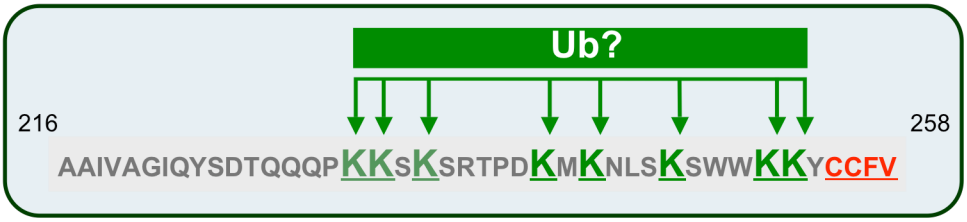
Chp has an unusual carboxyl terminus for Rho family proteins in that it lacks a CAAX motif and, instead, terminates with FCFV residues, suggesting that it may not be isoprenylated. Although Wrch-1 did appear to have a typical CAAX motif (CCFV), I demonstrated in Chapter III that Wrch-1 fails to incorporate isoprenoid lipids and utilizes palmitoyl fatty acids as its lipid modification. Interestingly, the predominant cysteine residue for Wrch-1 palmitate incorporation (Cys 256, C<u>C</u>FV) is analogous to the cysteine residue in the last four amino acids of Chp (Cys 234, F<u>C</u>FV) suggesting that the Wrch proteins may truly have a novel lipid binding motif, CFV. In support of this, a recent paper from Channing Der's lab demonstrated that, like Wrch-1, Chp incorporates fatty acids at Cys 234 and that its localization is dependent on palmitoyltransferase, not isoprenyltransferase, activity (Chenette et al., 2005).

In summary, posttranslational modifications are critical determinants in the regulation of small GTPase localization and biological function. Of the many different modifications, lipid moieties such as isoprenoid and palmitate lipids, phosphate groups and ubiquitin molecules are key players for small GTPase control. We have

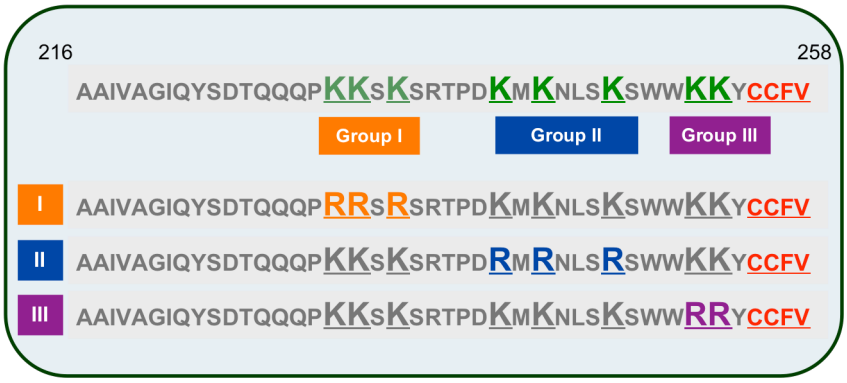
shown that palmitoylation of Wrch-1 reveals opportunities for tightly controlled regulation of this protein by mechanisms previously under-investigated in other small GTPases with more conventional membrane targeting signals. The fact that Wrch-1 also incorporates ubiquitin molecules leads to additional levels of complexity of regulation and biological activity of Wrch-1. Additionally, investigation of the specific modifications of Wrch-1 could uncover potential novel downstream signaling partners that may serve as targets for new pharmacological inhibitors. There is still more to discover in terms of Wrch-1 regulation including how palmitoylation specifically regulates the activity and signaling pathways of Wrch-1, how Wrch-1 becomes ubiquitylated and what effect this modification has on Wrch-1 biological function on a larger scale.

**Future Direction 1: Determine how ubiquitylated Wrch-1 lysine residues contribute to its subcellular localization and biological function.** The Wrch-1 protein sequence has many lysine residues that may be subjected to mono-ubiquitylation. Of particular interest are eight lysine residues located within the carboxyl terminus of Wrch-1 that could function as secondary membrane targeting signals in coordination with palmitoyl fatty acids (Figure 5.1A). As previously shown in Chapter IV, Wrch-1 is a substrate for mono-ubiquitylation. However, the exact residues targeted by this modification have yet to be determined. To determine whether the carboxyl terminal region of Wrch-1 is the site of ubiquitin modification, epitope-tagged expression vectors encoding only the carboxyl terminal sequences of mutants), which cannot link to ubiquitin, by using site-directed mutagenesis should block ubiquitylation and can be used to delineate the ubiquitin-modified lysine

A.




B.




C.

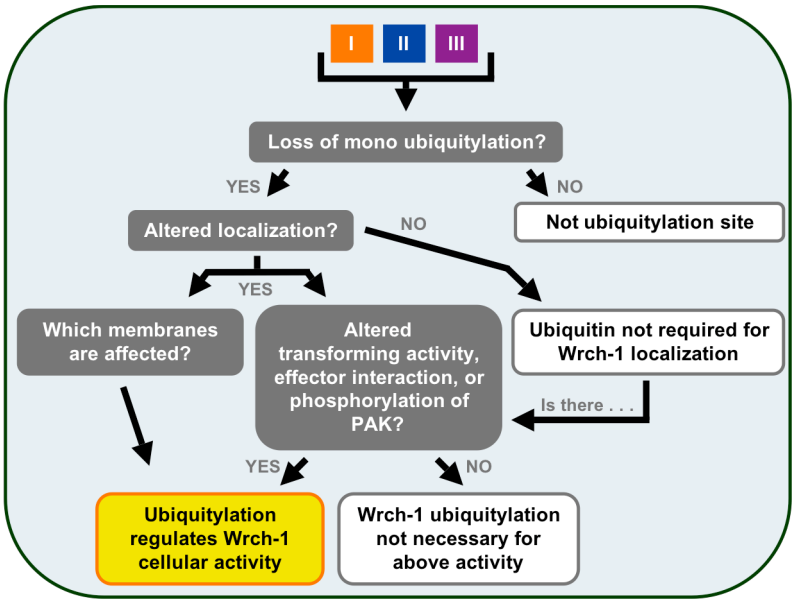



**Figure 5.1 Future Direction 1: How do ubiquitylated lysine residues regulate Wrch-1?** (A) Wrch-1 has several potential carboxyl terminal ubiquitylation sites. (B) Initially, groups of lysine residues will be mutated to arginine residues (K > R mutations, group I-orange, group II-blue, group III-purple) to determine which residues are ubiquitylated. Later, single K > R mutants can be created to determine the specific ubiquitylated residues. (C) A flowchart illustrates experimental approaches using K > R mutants and possible conclusions from anticipated results.

residues to arginine residues (K > R mutants), which cannot link to ubiquitin, by using site-directed mutagenesis should block ubiquitylation and can be used to delineate the ubiquitin-modified lysine residues of Wrch-1. Given that there are eight lysines in question, the lysine residues can be mutated in clusters (Figure 5.1B) such that groups of lysines will be evaluated versus creating eight different single K > R mutants. If mutation of a group results in loss of Wrch-1 ubiquitylation, then further individual mutations can be made within that particular group of lysine residues.

Our hypothesis that ubiquitylation of Wrch-1 functions as an internal membrane targeting signal can be evaluated using the Wrch-1 K > R mutants to examine whether Wrch-1 localization to endomembranes is lost when mono-ubiquitylation is also blocked (Figure 5.1C). Earlier, we described how modulation of Wrch-1 palmitoyl status regulates not only Wrch-1 localization, but also its transforming activity (Chapter III). If ubiquitylation of Wrch-1 directs its subcellular distribution, then these same K > R mutants can be utilized to examine how ubiquitylation-dependent localization perturbations may affect Wrch-1 transforming activity (Figure 5.1A). If ubiquitylation of Wrch-1 is blocked, then expression of K > R mutants during Wrch-1 transformation assays should abrogate Wrch-1 transforming ability if ubiquitylation of Wrch-1 is required for localization of Wrch-1 to pro-transformation protein signaling complexes.

**Future Direction 2: Determine which E3 ligase mediates mono-ubiquitylation of Wrch-1.** A cascade of enzymes requiring E3 ligases to catalyze the transfer of ubiquitin to its cognate substrate mediates the ubiquitylation process (Hershko and

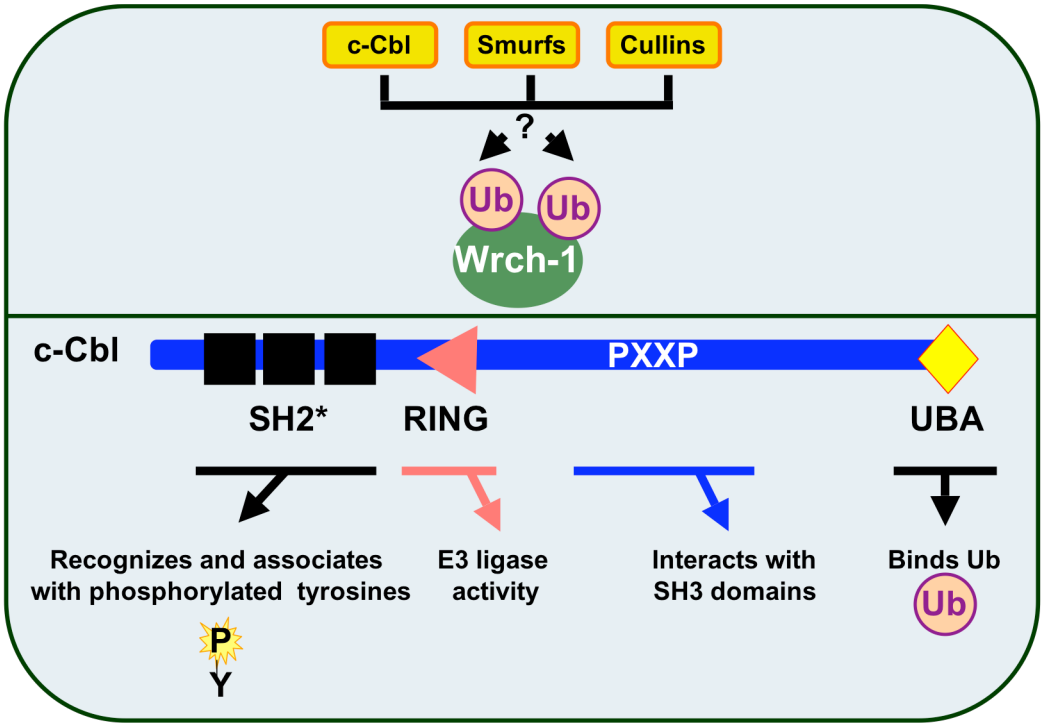
Ciechanover, 1998; Pickart and Eddins, 2004). Given that there are over 500 known human E3 ligases, the majority of which have no known substrate, it is a difficult task to determine the specific E3 ligase responsible for modification of many ubiquitylated proteins, including Wrch-1. That said, there are a few E3 ligases that have either been identified as the enzymes responsible for ubiquitylation of Rho A (i.e. Smurfs) (Wang et al., 2003a) and RhoBTB (i.e. cullins) (Wilkins et al., 2004) small GTPases or have been found in protein complexes with Cdc42 (i.e. c-Cbl, cullins) (Senadheera et al., 2001; Wu et al., 2003), Rac 1, 2 and 3 (i.e. cullins) (Senadheera et al., 2001). Although the exact mechanism by which they interact with these Rho proteins is still uncharacterized, these E3 ligases may also be candidates for ubiquitylation of Wrch-1.

Since we have demonstrated that Wrch-1 is mono-ubiquitylated (Chapter IV), E3 ligases that mono-ubiquitylate their substrates are of interest as potential E3 ligases for Wrch-1. The RhoA and RhoBTB E3 ligases Smurfs and cullins cause polyubiquitylation of their small GTPase substrates (Wang et al., 2003a; Wilkins et al., 2004), whereas c-Cbl is a known mediator of mono-ubiquitylation of receptor tyrosine kinases (RTKs) (Levkowitz et al., 1998). As mentioned earlier, c-Cbl has been found associated with Rho proteins are with their biological activities. For example, constitutively activated Rac rescues membrane ruffling and lamellae formation blocked by mutant c-Cbl lacking SH3 domains critical for its localization to these peripheral actin structures (Scaife and Langdon, 2000). Additionally, Cdc42 interaction with cool/pix (a Cdc42 GEF) and c-Cbl leads to sequestration of c-Cbl and prevention of c-Cbl-mediated mono-ubiquitylation of EGFR (Wu et al., 2003).

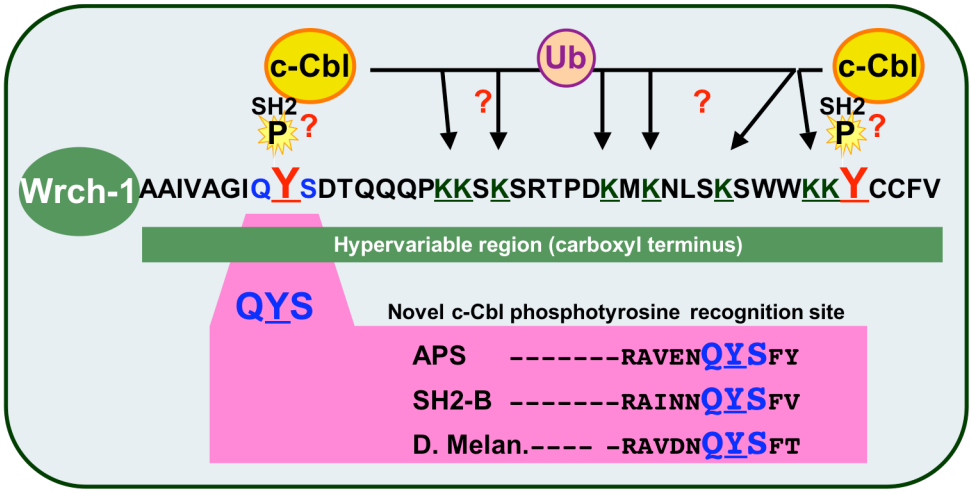
Interestingly, our data in Chapter IV demonstrating that the ErbB family of RTKs (including EGFR) regulates the tyrosine phosphorylation status of Wrch-1 suggests a possible signaling mechanism by which c-Cbl, downstream of EGFR activity, could potentially interact with Wrch-1, making c-Cbl an intriguing candidate E3 ligaswe for Wrch-1.

c-Cbl contains a variant SH2 domain that interacts with the phosphorylated tyrosine residues of EGFR family. Tyrosine phosphorylation has previously been shown to be necessary for recruitment of c-Cbl to its target RTKs, whereby interaction between phospho-tyrosine residue of the RTKs and SH2 domain of c-Cbl occur and are followed by subsequent mono-ubiquitylation of the RTKs (Dikic, 2003). Wrch-1 also contains several tyrosine residues that may be phosphorylated, and we have demonstrated that Wrch-1 can be tyrosine phosphorylated under physiologically relevant conditions. Normally, the consensus sequence for c-Cbl binding to phosphorylated residues is NXpY(S/T)XXP (Hu and Hubbard, 2005), a sequence not found in Wrch-1. However, a recent paper described a novel noncanonical phosphotyrosine recognition consensus sequence, RA(V/I)XN**Q**$_p$**Y**(**S**/T) for c-Cbl (Hu and Hubbard, 2005), in which the critical QYS residues are located in the C-terminal hypervariable region of Wrch-1 (Figure 5.2B). If Wrch-1 does, in fact, associate with c-Cbl in this manner, then a plausible model for Wrch-1 and c-Cbl binding would involve modification of Wrch-1 by c-Cbl after Wrch-1 tyrosine phosphorylation, resulting in internalization of Wrch-1 to internal membranes for either chaperone or degradation activities (Figure 5.2C).

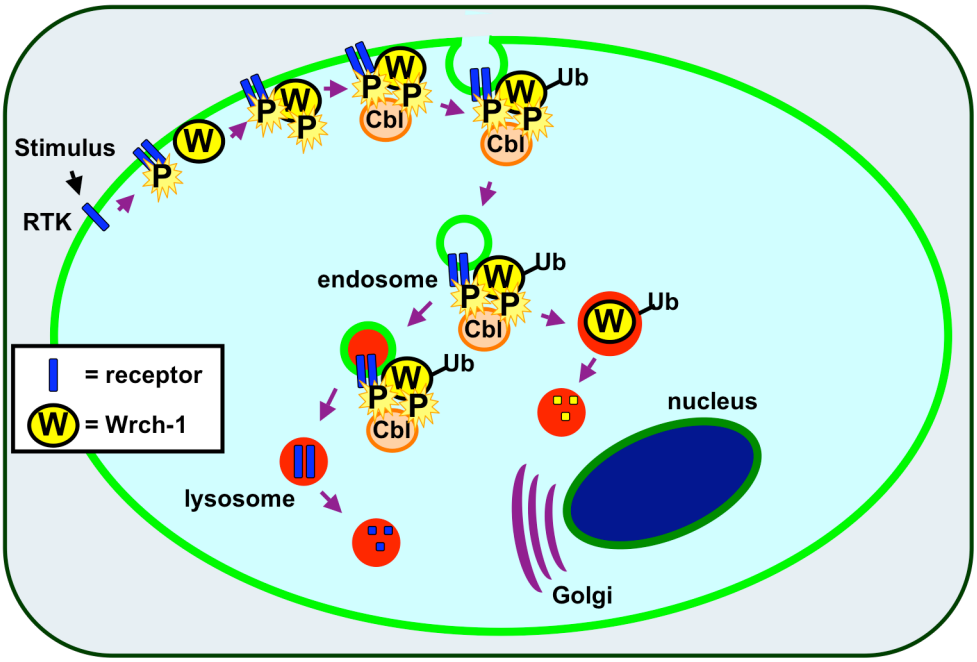
A.


c-Cbl
Smurfs
Cullins
?
Ub
Ub
Wrch-1
c-Cbl
PXXP
SH2*
RING
UBA
Recognizes and associates with phosphorylated tyrosines
E3 ligase activity
Interacts with SH3 domains
Binds Ub
Ub
P
Y

B.


c-Cbl
SH2
P
?
Ub
?
?
c-Cbl
SH2
P
?
Wrch-1
AAIVAGIQYSDTQQQPKKSKSRTPDKMKNLSKSWWKKYCCFV
Hypervariable region (carboxyl terminus)
QYS
Novel c-Cbl phosphotyrosine recognition site
APS -------RAVENQYSFY
SH2-B -------RAINNQYSFV
D. Melan.---- -RAVDNQYSFT

C.




D.

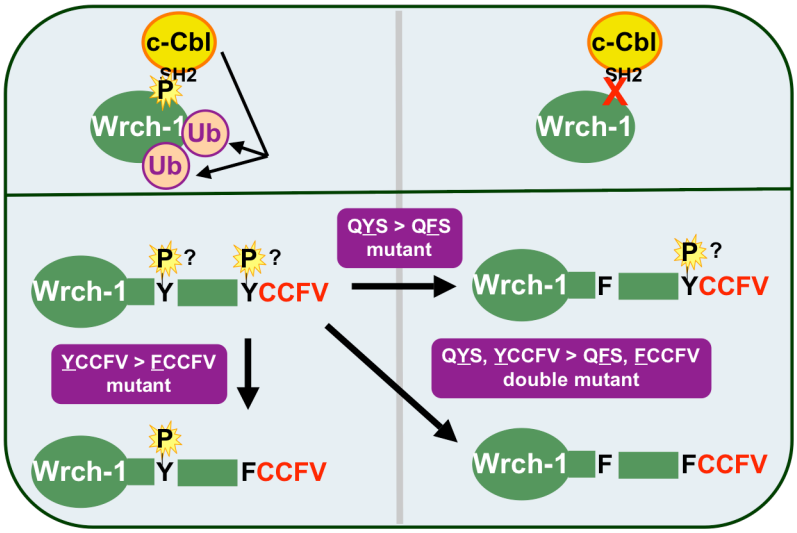



**Figure 5.2 Future Direction 2: Which E3 ligases modify Wrch-1?** (A) c-Cbl, Smurfs and cullins are candidate Wrch-1 E3 ligases (upper panel). C-Cbl has a SH2 domain that interacts with phosphorylated tyrosine residues (lower panel). (B) Wrch-1 has two carboxyl terminal tyrosines, including a putative c-Cbl phosphotyrosine recognition motif (QYS-blue, pink inset) that might host such an interaction. (C) A model for c-Cbl association and ubiquitylation of Wrch-1 downstream of RTK signaling and trafficking functions. (D) Wrch-1 phosphorylation could lead to c-Cbl binding and Wrch-1 monoubiquitylation (upper left panel), whereas inhibition of phosphorylation may block c-Cbl interactions with Wrch-1, preventing its ubiquitylation (upper right panel). Tyrosine to phenylalanine mutations could delineate the phosphorylated Wrch-1 residue and its effects on c-Cbl binding and possible ubiquitylation of Wrch-1.

To determine whether Wrch-1 actually does have a QYS docking site for c-Cbl interaction and also whether phosphorylation of Wrch-1 occurs at this residue (Y225), leading to mono-ubiquitylation of Wrch-1 by c-Cbl, several approaches could be used to address these questions. One such approach involves site directed mutagenesis to mutate residue 225 (QYS > QFS) of Wrch-1 from a tyrosine to a phenylalanine, which cannot be phosphorylated. If this is the critical tyrosine residue for Wrch-1 interaction with c-Cbl (Figure 5.2D), then a Y225F mutant of Wrch-1 will not be able to interact with c-Cbl. Alternatively, if phosphorylation of residue 225 is regulated for interactions with other E3 ligases, then the Wrch-1 Y225F mutant will also not be ubiquitylated

A second approach would be to determine whether Wrch-1 and c-Cbl are in a complex together, suggesting a physical interaction between the proteins. Immunoprecipitation of exogenously expressed epitope-tagged Wrch-1 (antibodies for endogenous detection of Wrch-1 are not yet available) in H1299 non-small lung carcinomas, followed by detection of endogenous c-Cbl using anti-c-Cbl antibody could be used to confirm an interaction. Antibodies directed against the epitope tag would then be used to evaluate the presence of Wrch-1 protein in association with c-Cbl. Should an interaction occur, then the QYS > QFS Wrch-1 mutant also could be use to show loss of c-Cbl association and mono-ubiquitylation when tyrosine phosphorylation is abrogated.

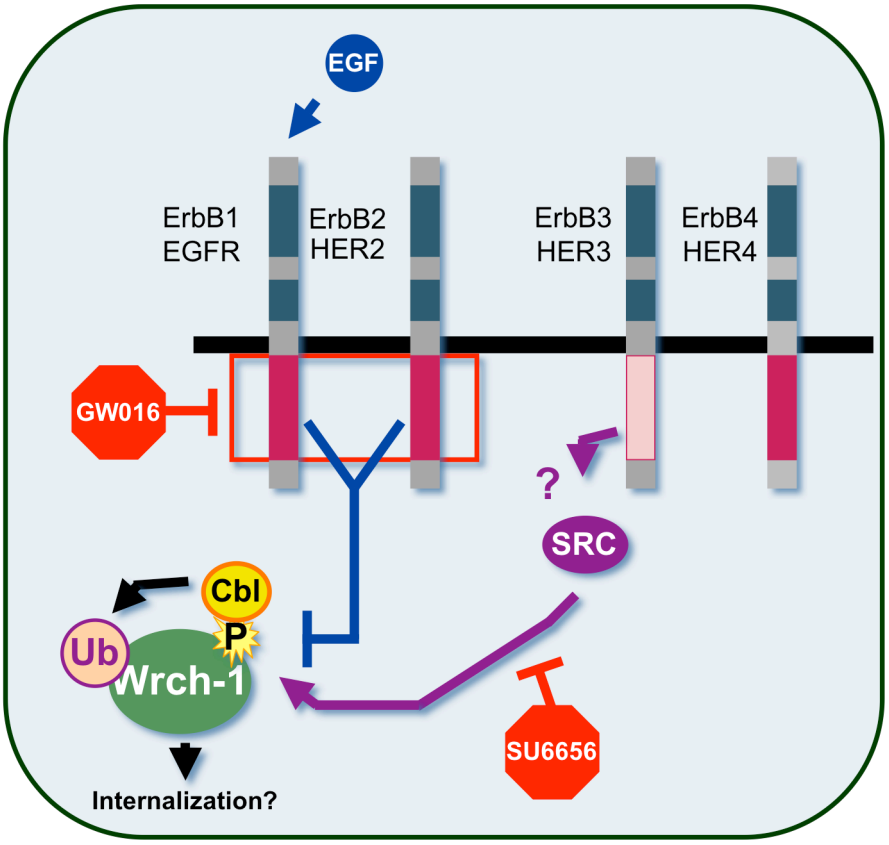
**Future Direction 3: Determine how Wrch-1 may contribute to downstream receptor tyrosine kinase signaling pathways.** Our data showing differential

effects on tyrosine phosphorylation of Wrch-1 in the presence of EGFR/ErbB2 and Src inhibitors, and a recent paper describing compensatory activity of ErbB3 and Src during EGFR/ErbB2 inhibition (Contessa et al., 2006), suggests that ErbB3 and Src kinase activity may be upstream of Wrch-1. If so, this brings up interesting questions as to how Wrch-1 could be contributing to EGFR family signaling pathways. The unspecified, dual specificity EGFR/ErbB2 inhibitor data in which GW016 treatment results in enhanced Wrch-1 tyrosine phosphorylation, while EGF stimulation causes slight increases in phosphorylation, together would suggest that EGFR and ErbB2 negatively regulate Wrch-1 phosphorylation. One reason for this negative regulation may involve a role for Wrch-1 in internalization, recycling and promotion of EGFR signaling pathways. Ubiquitylated Wrch-1 may assist in c-Cbl-induced EGFR invagination to endosomes and its downregulation through delivery to lysosomes (Figure 5.2C, 5.3A). If so, then EGFR reduction of Wrch-1 phosphorylation would block c-Cbl-mediated ubiquitylation of Wrch-1, thereby preventing downregulation of EGFR through Wrch-1 chaperone services and prolonging EGFR signals (Figure 5.3A). It would be interesting to determine whether Wrch-1 actually functions downstream of EGFR to promote either its signaling cascades or its downregulation through endocytosis.

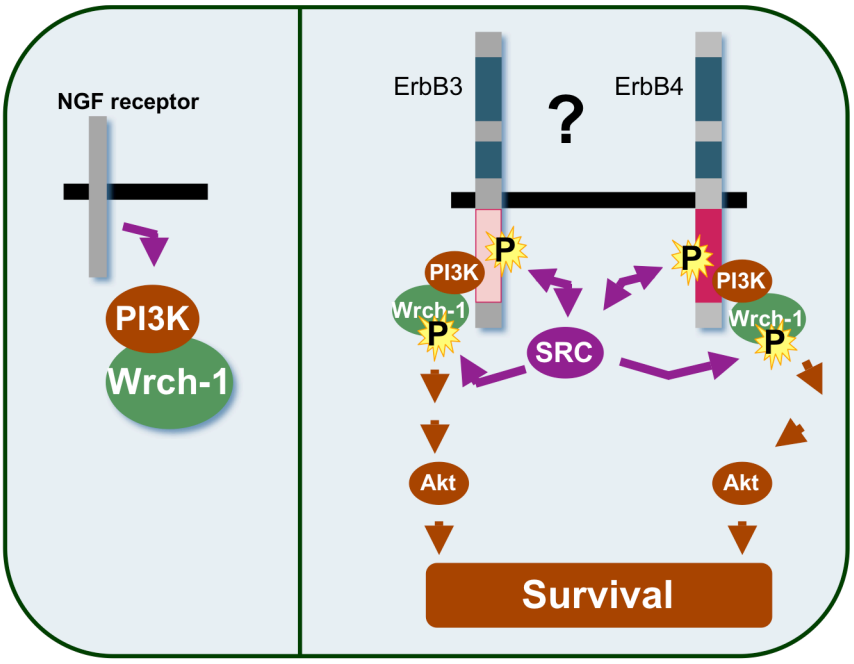
As mentioned above, ErbB3 can compensate for loss of EGFR/ErbB2 signaling in a Src-dependent manner (Contessa et al., 2006). Given that inhibition of EGFR/ErbB2 resulted in increased Wrch-1 phosphorylation and that this increased phospohorylation requires Src activity, it is possible that the higher phosphotyrosine levels of Wrch-1 are due to compensatory action by ErbB3. At first glance,

enhancement of Wrch-1 phosphorylation by ErbB3 seems to contradict the rescue of EGFR/ErbB2 activity if Wrch-1 has a role in c-Cbl-mediated downregulation of EGFR. However, ErbB3 is not regulated by c-Cbl internalization mechanisms (Levkowitz et al., 1996). Therefore, phosphorylation of Wrch-1 downstream of ErbB3 and Src could actually promote positive ErbB receptor signals, providing another possible role for Wrch-1 downstream of these receptors. In support of this idea, both ErbB3 and ErbB4 (which dimerizes with ErbB3) are overexpressed in several cancers including breast and lung (Kraus et al., 1993; Lemoine et al., 1992; Poller et al., 1992; Yi et al., 1997). Further, both wild type and constitutively activated forms of Wrch-1 promote cellular transformation (Berzat et al., 2005; Shutes et al., 2004; Tao et al., 2001), providing a potential link for these proteins in an oncogenic context. PI3-K, a pro-survival signaling molecule, associates with activated ErbB3 via several p85 subunit consensus sites (YXXM—Y is a tyrosine residue, XX are any amino acid and M is methione) found most frequently in ErbB3 and activated ErbB4 (Hellyer et al., 1998; Hynes and Lane, 2005; Kim et al., 1994). Additionally, in gefitinib (EGFR tyrosine kinase inhibitor)-sensitive cells, treatment with gefitinib inhibits ErbB3 and PI3-K interaction and decreases phospho-Akt levels (Engelman et al., 2005), suggesting a link between ErbB3 signaling and PI3-K/Akt survival pathway. Interestingly, Wrch-1 also interacts with PI3-K, through association with the GAP related domain (GRD), and partially with SH3 domains of PI3-K downstream of NGF receptor in N2A neuronal cells (Shutes, A, personal communication). It would be interesting to determine whether, upon ErbB3/B4 stimulation, Src-dependent activation of ErbB3 occurs, leading to binding of ErbB3 to PI3-K and PI3-K-dependent recruitment of Wrch-1 to ErbB3 and Src and to

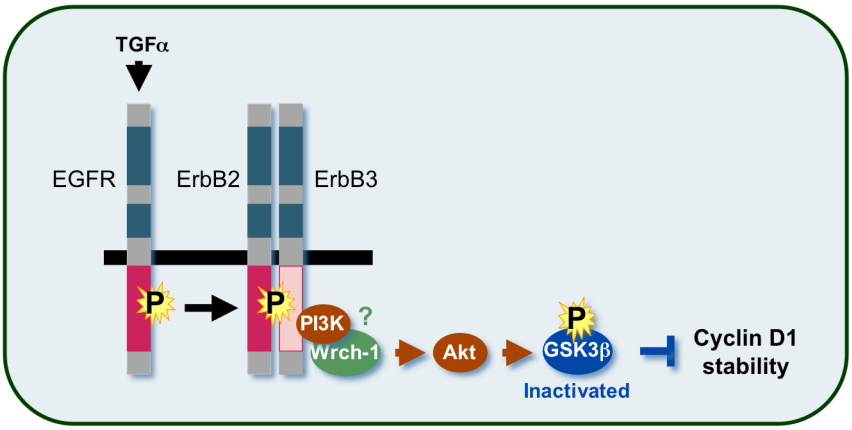
A.


EGF
ErbB1
EGFR
ErbB2
HER2
ErbB3
HER3
ErbB4
HER4
GW016
?
SRC
Cbl
P
Ub
Wrch-1
SU6656
Internalization?

B.


NGF receptor
PI3K
Wrch-1
ErbB3
?
ErbB4
P
PI3K
Wrch-1
P
SRC
Akt
Survival

C.




D.

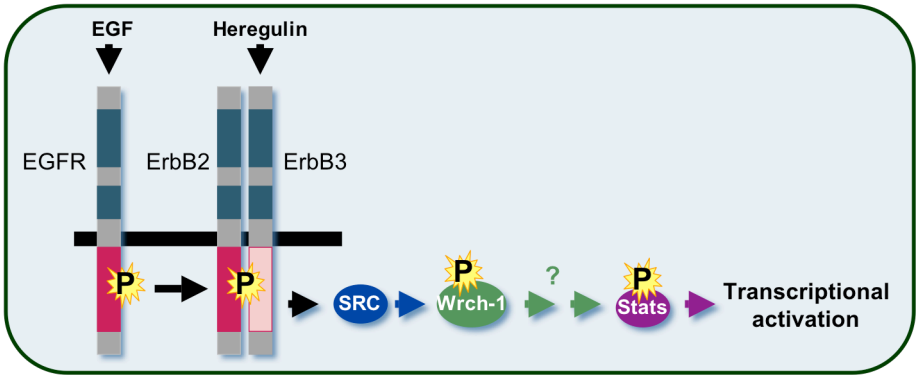



**Figure 5.3 Future Direction 3: Wrch-1 activity downstream of ErbB receptors.** (A) Enhanced Src-dependent phosphorylation of Wrch-1 in the presence of a dual specificity EGFR/ErbB2 inhibitor, suggests that EGFR signaling negatively regulates Wrch-1 tyrosine phosphorylation. If Wrch-1 participates in the downregulation of stimulated EGFR, a possible model for EGFR negative regulation of Wrch-1 phosphorylation is as follows: EGF-stimulation of EGFR leads to recruitment of Cbl to the receptor and Wrch-1. To prolong its activity, EGFR signaling decreases Src-mediated Wrch-1 tyrosine phosphorylation to prevent Cbl association and monoubiquitylation of Wrch-1 (and perhaps reducing endocytic targeting signals for Wrch-1). (B) Wrch-1 interacts with PI3-K downstream of the nerve growth factor (NGF) receptor (left panel). There are many sites for PI3-K interaction with ErbB3 and PI3-K signals downstream of ErbB3 to promote survival pathways. Since Wrch-1 phosphorylation is dependent on Src kinase activity, it is possible that Wrch-1 signals downstream of ErbB3 through interactions with PI3-K. (C) GSK3β is inactivated downstream of ErbB3/PI3-K/Akt signaling. Since Wrch-1 signaling also causes inactivation of GSK3β it would be interesting to determine whether ErbB3/PI3-K inhibition of GSK3β is mediated through Wrch-1. (D) STATS, transcription factors are regulated by Rho family activity (RhoA and Rac1) and also by ErbB family signaling and Src. Perhaps, after ErbB family stimulation, phosphorylation of Wrch-1 contributes to translocation of STATS to the nucleus for transcriptional activation.

subsequent tyrosine phosphorylation of Wrch-1 (Figure 5.3B).

It is intriguing to speculate which type of signaling pathways downstream of such interactions could be mediated by Wrch-1. For example, phosphorylation of the negative regulator GSK-3β is an inactivating event downstream of PI3-K/Akt activity to promote survival and cell cycle progression pathways (Liang and Slingerland, 2003). GSK-activity inhibits many molecules including β-catenin and cyclin D1 (Liang and Slingerland, 2003). A recent paper confirmed and extended the observation that PI3-K/Akt negative regulation of GSK-3β and increased stability of cyclin D1 is downstream of TGFα-induced ErbB2 and ErbB3 dimerization (Yakes et al., 2002) (Figure 5.3C). Since constitutively activated Wrch-1 also causes phosphorylation of GSK-3β at the same inactivating residue (Shutes A and Brady DC, personal communication), it is possible that Wrch-1 could function upstream of this PI3-K/Akt downregulation event (Figure 5.3C), thereby providing a link between ErbB receptor signaling and Wrch-1 signaling pathways.

Another pro-survival pathway downstream of EGFR family receptor signaling involves the STAT (Signal transducer and activator of transcription) family of transcription factors. STATs are cytosolic transcription factors that rapidly translocate to the nucleus upon tyrosine phosphorylation. In the nucleus, STATs upregulate transcription of cell cycle control genes like cyclin D1 and c-Myc (Kiuchi et al., 1999; Turkson et al., 1998). Activation of STATs in several cancers including breast and lung has been investigated (Bowman et al., 2000). While they are best known for their promotion of transforming and pro-proliferative gene upregulation downstream of cytokine receptors and serine/threonine JAKs (Janus kinases)

(Horvath and Darnell, 1997), several papers have described a dependency of STAT family activity on Src tyrosine kinase function downstream of ErbB receptor stimulation in lung carcinomas cells (Song et al., 2003). For example, inhibition of Src results in decreased EGF- and HGR-induced STAT3 activity and G2 cell cycle arrest (Song et al., 2003). Additionally, STAT3 activation is required for Src transforming activity (Bromberg et al., 1998) and STAT5b has been shown to enhance Src-mediated transformation (Kazansky and Rosen, 2001). Several Rho family members have been cited as regulators of STAT activity. For instance, RhoA, Cdc42 and Rac1 can induce transcriptional activation of STAT3 via tyrosine and serine phosphorylation in both epithelial and fibroblastic cells (Debidda et al., 2005). One study demonstrated that activated RhoA leads to Src- and JAK-induced tyrosine phosphorylation of STAT3 and that STAT3 could cooperate with RhoA for transformation of human epithelial cells (Aznar et al., 2001). As mentioned previously, Wrch-1 becomes tyrosine phosphorylated after cellular stimulation and this phosphorylation is dependent on Src family kinases. In addition, the ErbB receptors modulate this Src-induced phosphorylation. So, it conceivable that, similar to other Rho family small GTPases, Wrch-1 activity, in the context of ErbB and Src activation, may lead to activation of STAT family transcriptional activity (Figure 5.3D).

Lastly, Wrch-1 phosphorylation and mono-ubiquitylation may be downstream of other receptor tyrosine kinases that upon activation recruit c-Cbl in a Src-dependent manner. Hepatacyte growth factor/scatter factor (HGF/SF) receptor (better known as c-Met) is a RTK known for its role in invasion and tumorigenesis (Hammond et

al., 2004). When stimulated, c-Met interacts with many of the same signaling molecules as the EGFR family of receptors (Hammond et al., 2004). For example, one study revealed that c-Met undergoes a novel interaction with the tyrosine kinase binding (TKB) domain of c-Cbl using a previously uncharacterized, conserved DpYR motif (Peschard et al., 2004). Additionally, c-Met associates with the p85 subunit of PI3-K and modulates the function of the STAT3 transcription factor to promote pro-oncogenic phenotypes (Maulik et al., 2002). In light of the previously mentioned posttranslational modifications, protein-protein interactions and possible signaling consequences of Wrch-1, Wrch-1 may be a downstream partner of c-Met receptor activity.

**Future Direction 4: Determine the role of Wrch-1 palmitoylation in cellular trafficking.** There are many areas distributed through the plasma membrane termed lipid rafts for their unique concentration of cholesterol and other lipid moieties (Laude and Prior, 2004). Recruitment of caveolin, caveolae-specific molecules, to lipid rafts leads to a transition of lipid rafts to invaginated membrane pits called caveolae (Laude and Prior, 2004). Localization of caveolin to caveolae depends not only on its high binding affinity for cholesterol, but also on its palmitoylated cysteine residues (Laude and Prior, 2004; Waterman and Yarden, 2001). In addition to caveolin, there are also ubiquitin-binding proteins that reside in caveolae and that have been shown to interact with the ubiquitin molecules attached to receptor tyrosine kinases marked for endocytosis. Epsins and the heterotrimeric adaptor protein (AP) complexes are two such families of proteins in which association with ubiquitylated receptors is critical for internalization of these receptors during

endocytosis (Salcini et al., 1999). Several Ras and Rho family small GTPases such as H-, N-Ras and TC10 can localize to caveolae based on their palmitoyl lipid status (Watson et al., 2001; White and Anderson, 2001). Other Ras proteins require additional signaling complexes for localization to caveolae. For example, Ral, a Ras-related small GTPase, localizes to caveolae and regulates endocytosis through interactions with its effector RalBP1 (Ral-binding protein 1) in a complex with POB1 (partner of RalBP1) and Epsin and Eps 15 (Nakashima et al., 1999).

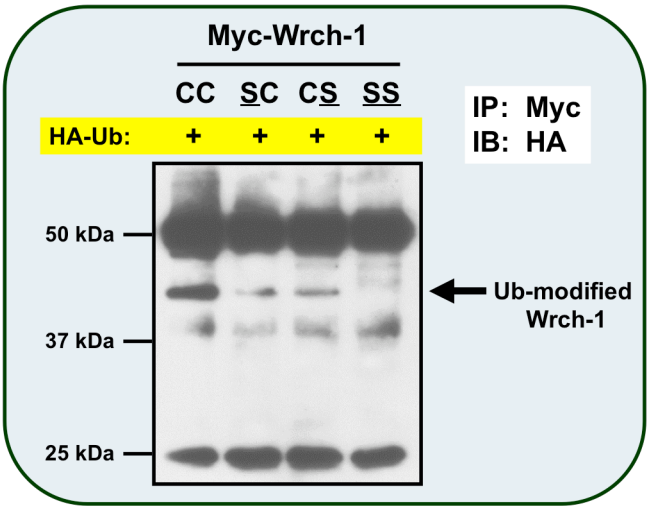
Our data demonstrate localization of Wrch-1 to internal vesicles, endosomes and lysosomes, which suggesting a possible role for Wrch-1 in trafficking similar to that seen with other small GTPases and RTKs. Given that Wrch-1 is both palmitoylated and ubiquitylated, it would be intriguing to determine whether palmitate moieties may direct Wrch-1 to lipid rafts and caveolae where the ubiquitin modified-Wrch-1 could interact with epsins leading to internalization of Wrch-1 into endocytic vesicles.

Our recent findings demonstrate that mutation of the carboxyl-terminal cysteine residues of Wrch-1 have differential effects on Wrch-1 localization and function. While second cysteine residue (Cys-256) appears to be the main target of palmitoylation based on palmitate incorporation analysis, the first cysteine residue (Cys-255) clearly has a role in Wrch-1 function. For example, mutation of Cys-255 to a serine (SCFV mutant) resulted in increased transforming activity of Wrch-1 and reduced localization to endosomes and lysosomes. Instead, the SCFV mutant localized predominantly to the plasma membrane and to unknown vesicular structures throughout the cytosol. Generally, the transition of mono-ubiquitylated RTKs from internalized vesicles to early and late endosomes for degradation

requires further ubiquitylation of the RTKs by E3 ligases (Wiley and Burke, 2001). Failure to do so sends receptors to recycling endosomes where they are sorted back to the plasma membrane (Wiley and Burke, 2001). It is possible that the altered function and localization observed with the SCFV mutant is due to a decrease in ubiquitylation of Wrch-1 and, therefore, a reduction in pools of Wrch-1 dedicated to endocytic trafficking functions. In support, preliminary data shows that mutation of either cysteine residue results in decreased incorporation of ubiquitin molecules (Figure 5.4A). Interestingly, expression of the Wrch-1 SCFV mutant in H1299 cells clearly shows loss of Wrch-1 at endosomal and lysosomal membranes (as seen in NIH 3T3 fibroblasts) and the appearance of Wrch-1 at internal vesicle structures, reminiscent of recycling endosomes. If Wrch-1 does localize to recycling endosomes when mutated at Cys-255, this could potentially explain the increased transforming activity observed and also allude to a regulatory mechanism for Wrch-1. The reduction in ubiquitylation seen with the CSFV may be due to a decrease in plasma membrane localization where Wrch-1 would potentially interact initially with E3 ligases.

Thus, when Wrch-1 is doubly palmitoylated at both cysteine residues, Wrch-1 localizes to plasma membrane where it participates in transforming signaling cascades and it is also ubiquitylated for localization to endosomes and lysosomes for trafficking functions. However, loss of palmitoylation at the first cysteine residue prevents efficient ubiquitylation of Wrch-1 for sorting to endosomes and lysosomes. Instead, Wrch-1 is shuttled to recycling endosomes for delivery back to the plasma membrane and transforming signaling complexes leading to an enhanced

A.

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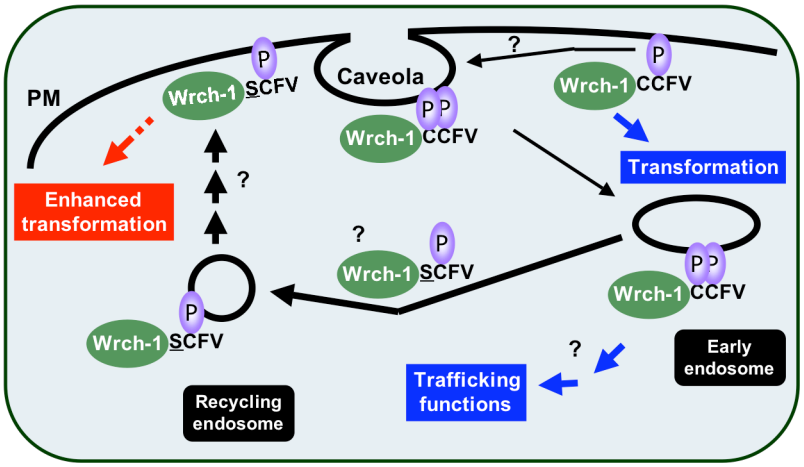


**Figure 5.4 Future Direction 4: Palmitoylation of Wrch-1 may dictate trafficking functions.** (A) Preliminary data indicate that mutation of cysteine 255 (CCFV > SCFV) or cysteine 256 (CCFV > CSFV) reduces incorporation of HA-tagged ubiquitin molecules by Wrch-1. (B) A model for palmitoylation of Wrch-1 in trafficking. When singly palmitoylated (whether physiologically due to presumed activity from PATs and PPTs or mutationally, cysteine to serine mutants), Wrch-1 is retained at the plasma membrane, promoting transformation pathways. This retention may be due to loss of internalization signals such as ubiquitin molecules and the additional palmitate moiety such that Wrch-1 never leaves the plasma membrane, or, maybe, reduced palmitoylation and ubiquitylation direct Wrch-1 to recycling endosomes for delivery back to the plasma membrane. Hence, a reduction in the population of Wrch-1 involved in early and late endosomal trafficking occurs, leaving more Wrch-1 at the plasma membrane for enhanced transformation. When Wrch-1 is doubly palmitoylated and, also properly ubiquitylated, it associates with caveolae for internalization to endocytic compartments, where it may function as a chaperone protein for shuttling of cargo to endosomes and lysosomes.

transforming activity. A model to explaining this phenomenon is shown (Figure 5.4B). It is also possible that mutation of the first cysteine residue prevents Wrch-1 from localizing to caveolae where Src is also localized. Perhaps doubly palmitoylated Wrch-1 localizes to one type of caveolae whereas singly palmitoylated Wrch-1 targets to a different type that directs proteins down the recycling endosome pathway. We have previously shown that phosphorylation of Wrch-1 is dependent on Src kinase activity. If Wrch-1 is not localized to lipid rafts because both palmitates are needed, then the Wrch-1 (SCFV) may not be as phosphorylated as the parent leading to a reduction in the number of E3 ligases recruited to Wrch-1 and a reduction of ubiquitylated Wrch-1 leading to recycling endosomes for Wrch-1.

In this dissertation, I have tried to emphasize the magnitude by which different posttranslational modifications can modulate Wrch-1 localization and, ultimately, how they can dictate specific protein-protein interactions with Wrch-1 and other signaling molecules. Clearly, more work is needed to define the specific mechanisms by Wrch-1 utilizes these modifications for its cellular functions. However, these modifications can still provide clues to the function of the biological regulation of Wrch-1 in normal and cancer cells.

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