TYROSINE KINASE MODULATION OF TRAFFICKING AND BIOLOGICAL FUNCTIONS OF THE ATYPICAL RHO GTPASE, WRCH-1

Jamie K. Alan

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Pharmacology

Chapel Hill
2010

Approved by

Advisor: Adrienne Cox
Reader: Channing Der
Reader: Gary Johnson
Reader: Michael Schaller
Reader: Stephen Rogers

Abstract

Jamie K. Alan
Tyrosine kinase modulation of trafficking and biological functions
of the atypical Rho GTPase, Wrch-1.

(Under the direction of Dr. Adrienne D. Cox)

Wrch-1 is an atypical Rho family small GTPase with roles in oncogenic transformation, epithelial cell morphogenesis, osteoclastogenesis, and migration. We have shown previously that Wrch-1 membrane localization and biological functions are modulated by reversible addition of a palmitate at its C-terminal membrane targeting domain. Most GTPases have at least two membrane targeting signals but no additional signals for Wrch-1 were known. We now show that the subcellular localization of Wrch-1 is responsive to growth factors contained in serum and is modulated by Src-mediated tyrosine phosphorylation. Upon stimulation with serum, Wrch-1 became phosphorylated on the evolutionarily conserved residue Y254 in its C-terminal membrane-targeting region, and relocalized from plasma membrane (PM) to endosomal compartments. Wrch-1 is known to interact with the adapter proteins Grb2 and Nck that in turn interact with growth factor receptor tyrosine kinases. We therefore next evaluated Wrch-1 modulation by RTK ligands such as EGF or PDGF. Wrch-1 was tyrosine phosphorylated in response to EGF treatment in time- and dose-dependent manner, and this was blocked by pretreatment with pharmacological inhibitors of either Src or EGFR. Wrch-1 relocalized rapidly from PM to endosomes upon EGF stimulation, similar to serum stimulation. Wrch-1 was phosphorylated at Y254 downstream of constitutively active forms of EGFR and HER2, including the deletion mutant EGFRvIII. Functionally, the

phosphodeficient Wrch-1 mutant Y254F was enhanced in Wrch-1-mediated migration, cystogenesis and transformation. Thus, EGFR, Src dependent, C-terminal tyrosine phosphorylation of Wrch-1 may represent a novel feedback mechanism to down-regulate its activity. Consistent with this hypothesis, Wrch-1-GTP and effector activation are decreased after serum stimulated tyrosine phosphorylation and subsequent endosomal relocalization. Also, we observed that phosphodeficient Wrch-1 remained GTP-bound and plasma membrane-localized in the presence or absence or serum, whereas a phosphomimetic mutant remained GDP-bound and localized at endosomes. Thus, EGFR-mediated, Src-dependent C-terminal tyrosine phosphorylation of Wrch-1 may be a negative feedback mechanism to regulate both the trafficking and biological activities of Wrch-1. C-terminal tyrosine phosphorylation represents a new paradigm in posttranslational control of small GTPase localization and biological function.

DEDICATION

To the two most important women in my life, my grandmother Mary Kathryn Farmer, and my mother Jacqueline Sue Farmer. You taught me that I can do anything, and with your love and support, I did. I am blessed to have two women like you in my life, and though one is not here on this earthly planet, she will live in my heart and in this work for all times. God blessed me when he placed both of you in my life.

To my niece Zoey Kathryn Alan. I love you with all my heart and I am glad that you are a part of the Kathryn clan. I know you will grow up to do great things.

ACKNOWLEDGMENTS

My advisor,

Dr. Adrienne Cox-thank you for you support and faith in me. I may never write anything without typos, but you helped me become a good scientist, and I am grateful.

My committee members,

Dr. Channing Der, Dr. Gary Johnson, Dr. Michael Schaller, and Dr. Steve Rogers- thank you for your insight and guidance.

Past and present members of the Cox lab,

Dr. Vanessa Gonzalez-Perez, Dr. Donita Brady, Dr. Anastacia Berzat, Dr. James Fiordalisi, James Madigan, Rhonda Wilder, Dr. Antonio Baines, Dr. Jared Snider, Dr. Trey Kidd, Lauren Parker, Dr. Heather Hodges-Loaiza, Dr. Patricia Keller, Kelly Gewain, Meredith Ward, Ethan Suttles, Brad Sanders, Sarah Grabowski, Sarah Quinn, and Alex Berger- Thank you all for a wonderful environment to work in, and for all the fun times I will never forget. I want to give special thanks to Dr. Vanessa Gonzalez-Perez, for being the best friend and lab mate a girl could ever hope for. Your love got me through the days. Also to Dr. Donita Brady, who always listened and always had sound advice for me. Finally a special thanks to Dr. Anastacia Berzat, for introducing me to Wrch-1, and to Dr. James Fiordalisi, it's been fun working on the same project with you.

My collaborators,
Dr. Lee Graves and Dr. Brian Dewer

My classmates,
Aurelie Gresset, Steve Cappell, Katy Cappell, and John Bauman. Thank you for helping me through everything. I am glad we met and went through this experience together. You made it all worthwhile.

My family members,
Mary Kathryn Farmer, Jacqueline Farmer, Jack Farmer, Jon Alan, Jared Alan, Justin Alan, Zoey Kathryn Alan, Jeff Farmer, Denise Farmer, Sadie Alan, Voltron Alan, Buffy Alan, Milo Alan, Panthro Alan, and Eric Olson. - Thank you all for you unwavering support, love and faith. Suffice it to say, none of this would be possible without you. I am blessed to have you all in my life.

TABLE OF CONTENTS

LIST OF FIGURES

LIST ABBREVIATIONS

a.a. amino acid

CAAX cysteine-aliphatic-aliphatic-unconserved amino acid

Cdc42 cell division cycle 42

Chp Cdc42 homologous protein

C-terminus Carboxyl-terminus

Dbl diffuse B-cell lymphoma

DH Dbl homology

DMEM-H high glucose Dulbecco's modified Eagle medium

EGF epidermal growth factor

EGFP enhanced green fluorescent protein

EGFR epidermal growth factor receptor

F farnesyl group

FITC fluorescein isothiocyanate

FPP farnesyl diphosphate

FTase farnesyltransferase

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

GST glutathione-s-transferase

GTP guanine triphosphate

HA hemagglutinin

H-Ras Harvey-rat sarcoma

HV hypervariable

ICMT isoprenylcysteine carboxyl methyltransferase

K-Ras Kirsten-rat sarcoma

NLS nuclear localization signal

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

2-BP 2-bromopalmitate

TC10 teratocarcinoma 10

TCL TC10-like

TKB tyrosine kinase binding

TNF tumor necrosis factor

TRITC Texas Red isothiocyanate

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 1

INTRODUCTION

1.1 The Ras superfamily of small GTPases. The Ras (Rat sarcoma) superfamily of small GTPases consists of proteins that function to transmit intracellular signals initiated from extracellular stimuli (Figure 1.1A). Ras small GTPases are involved in many divergent cellular functions including cytoskeletal reorganization, cell survival and proliferation, transformation and vesicular trafficking (Wennerberg et al., 2005) (Figure 1.1B). These small GTPases function as tightly regulated molecular switches; when they are GTP-bound they undergo a conformational change, can engage effectors, and are active. Conversely, when they are GDP-bound, they cannot engage effectors and are inactive (Figure 1.2A). Two types of proteins regulate the GDP/GTP cycle of these small GTPases. Guanine nucleotide exchange factors (GEFs) are positive modulators; they function to exchange GDP for GTP and consequently they activate small GTPases. GTPase accelerating proteins (GAPs) work by hydrolyzing the terminal phosphate of GTP, leaving the protein GDP-bound and inactive (Figure 1.2A). Oncogenically activating mutations that occur in cancer render GTPases GAP-insensitive, and therefore constitutively GTP-bound and active (Figure 1.2B). In Ras, these mutations typically occur at position 12, 13 or 61 (Figure 1.2C). These mutations can also be utilized in the laboratory to study GTPase function. The Ras superfamily is divided into 5 main subfamilies: Ras, Rho, Rab, Arf and Ran. Ras subfamily proteins are well known for their role in driving cell proliferation. The Rho subfamily of proteins are primarily known for regulating cytoskeletal dynamics. The Arf and Rab families of proteins regulate vesicular transport, whereas the Ran GTPase primarily regulates

nuclear transport (Wennerberg et al., 2005).

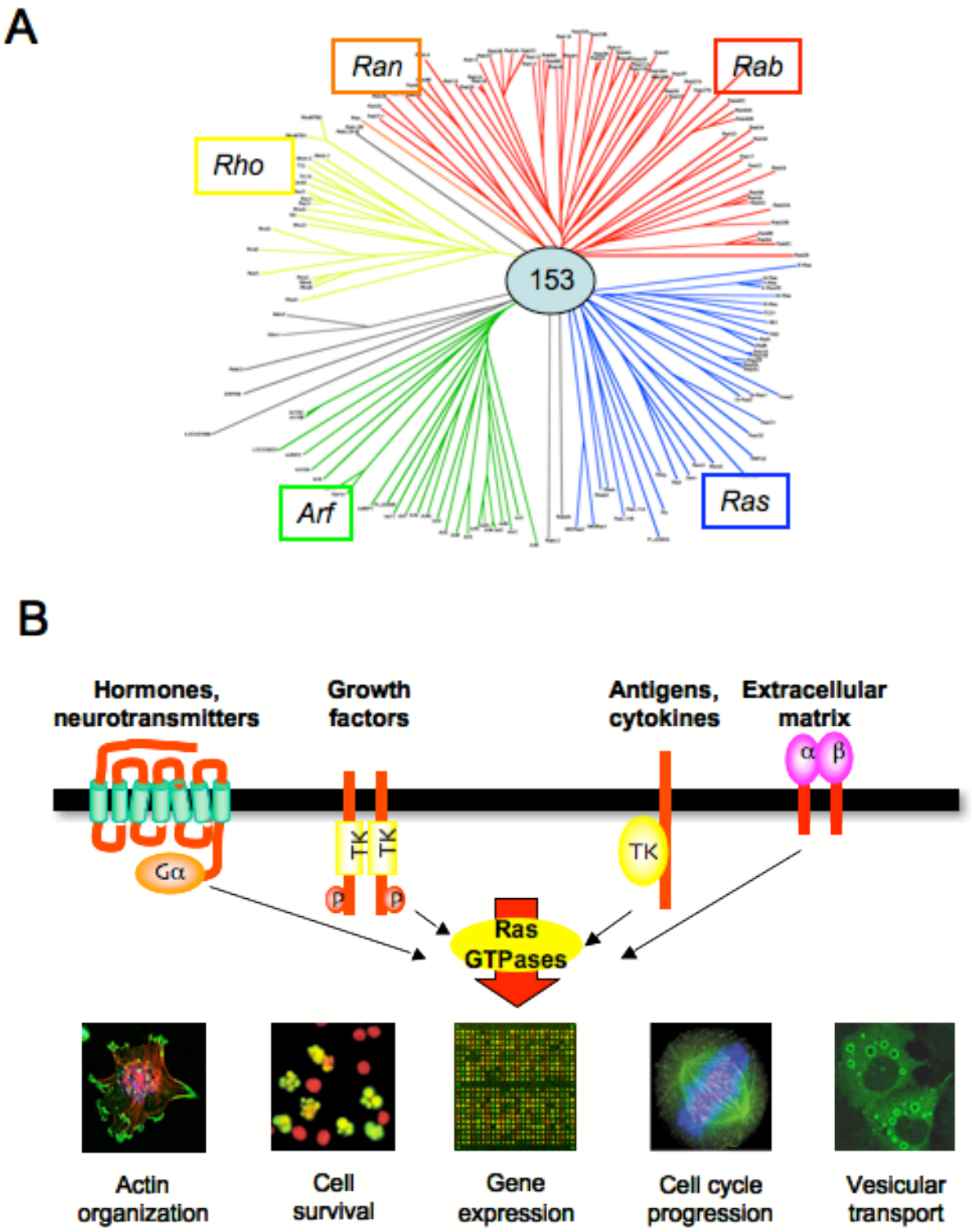


Figure 1.1 The Ras superfamily of small GTPases. A) The Ras superfamily consists of five branches (modified from Wennerberg and Der, 2004). B) Ras family GTPases mediate diverse downstream biological functions.

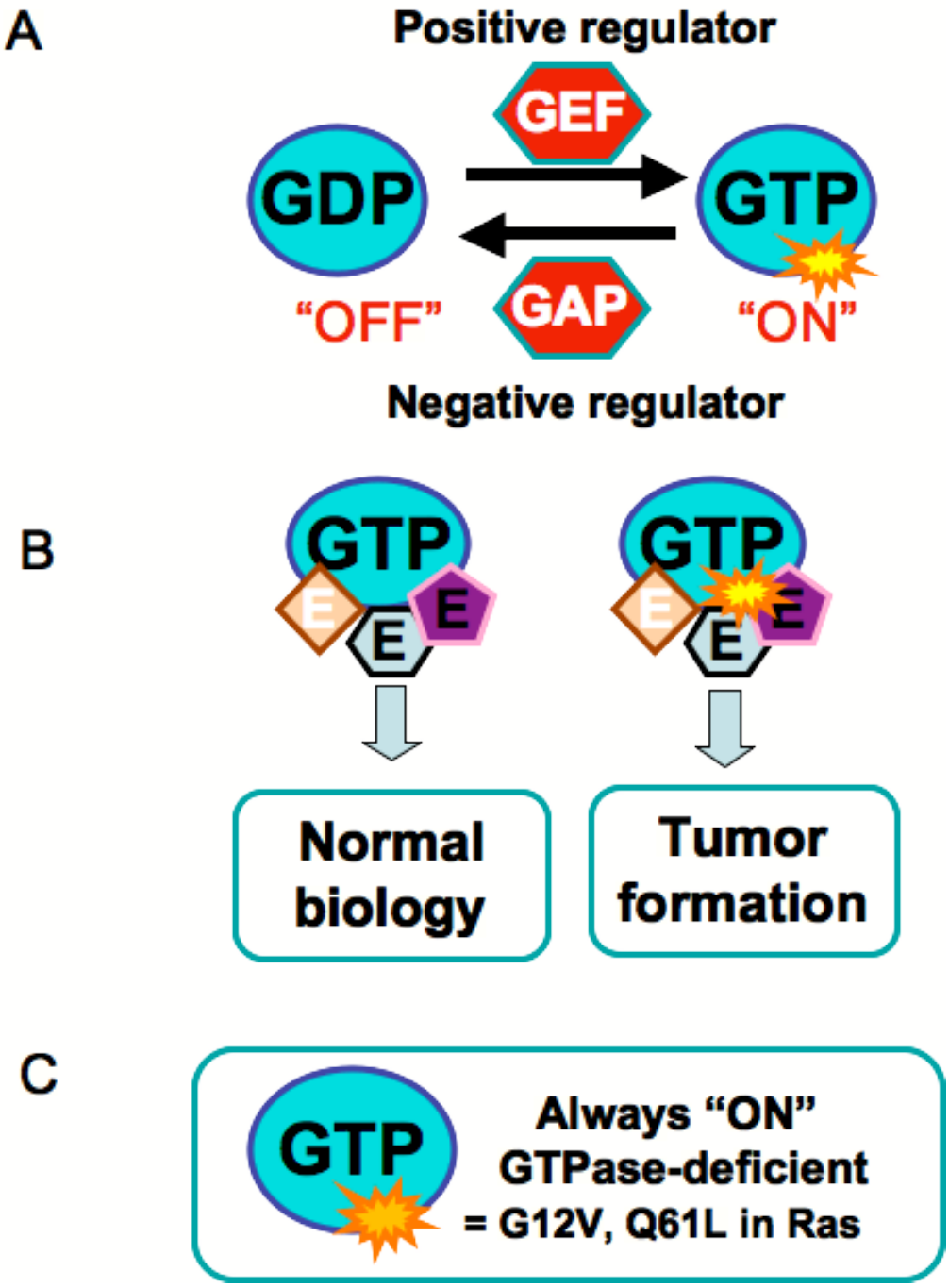


Figure 1.2 Small GTPases are regulated by GDP/GTP binding. A) GEFs promote GTP binding, resulting in an activated GTPase. GAPs hydrolyze GTP, leaving the proteins GDP-bound and inactive. B) When GTPases can cycle between the GDP and GTP state, they promote normal biological outcomes. When they are constitutively GTP-bound, they promote oncogenic outcomes. C) There are naturally occurring and synthetic mutations in GTPases that render then GTP-bound and constitutively active.

1.2 The Rho family of small GTPases. The Rho family of GTPases comprises a major subgroup of the Ras superfamily of small GTPases. Structurally, Rho GTPases are defined by a Rho insert domain between the fifth β strand and the fourth α helix in the GTPase domain. Rho family members are best known for their ability to control the actin cytoskeleton by regulating structures such as actin stress fibers, lamellipodia, and filopodia (Wennerberg and Der, 2004; Wherlock and Mellor, 2002). Within the Rho family, the core GTPase domain contains a Rho insert domain is highly conserved between family members, whereas they are divergent in their N-terminal extensions and in their C-terminal hypervariable regions. In humans there are 23 members of the Rho family, which are divided into 5 major families: Rho, Rac (Ras-related C3 substrate), Cdc42 (cell division cycle 42), Rnd (round), and RhoBTB (Rho Broad-Complex, Tramtrack and Bric à brac) (Figure 1.3). The best-studied members are RhoA, Rac1, and Cdc42. The Rho and Rac subgroups are best known for their roles in cytoskeletal rearrangement, driving formation of actin stress fibers and lamellipodia, respectively. Cdc42 family members are also involved in regulating the actin cytoskeleton by inducing filopodia formation, but additionally they play a major role in controlling cell polarity. The Rnd subgroup of Rho GTPases also plays an important role in controlling the actin cytoskeleton (Nobes et al., 1998), and Rnd3 has also been shown to be important in cell-cycle progression and transformation (Hansen et al., 2000; Villalonga et al., 2004). Although less is known about the other family members, RhoBTB and Miro proteins are being investigated for their potentially distinct roles in actin cytoskeleton control, oncogenesis, and mitochondrial functions (Aspenstrom et al., 2004; Fransson et al., 2003; Wennerberg and Der, 2004).

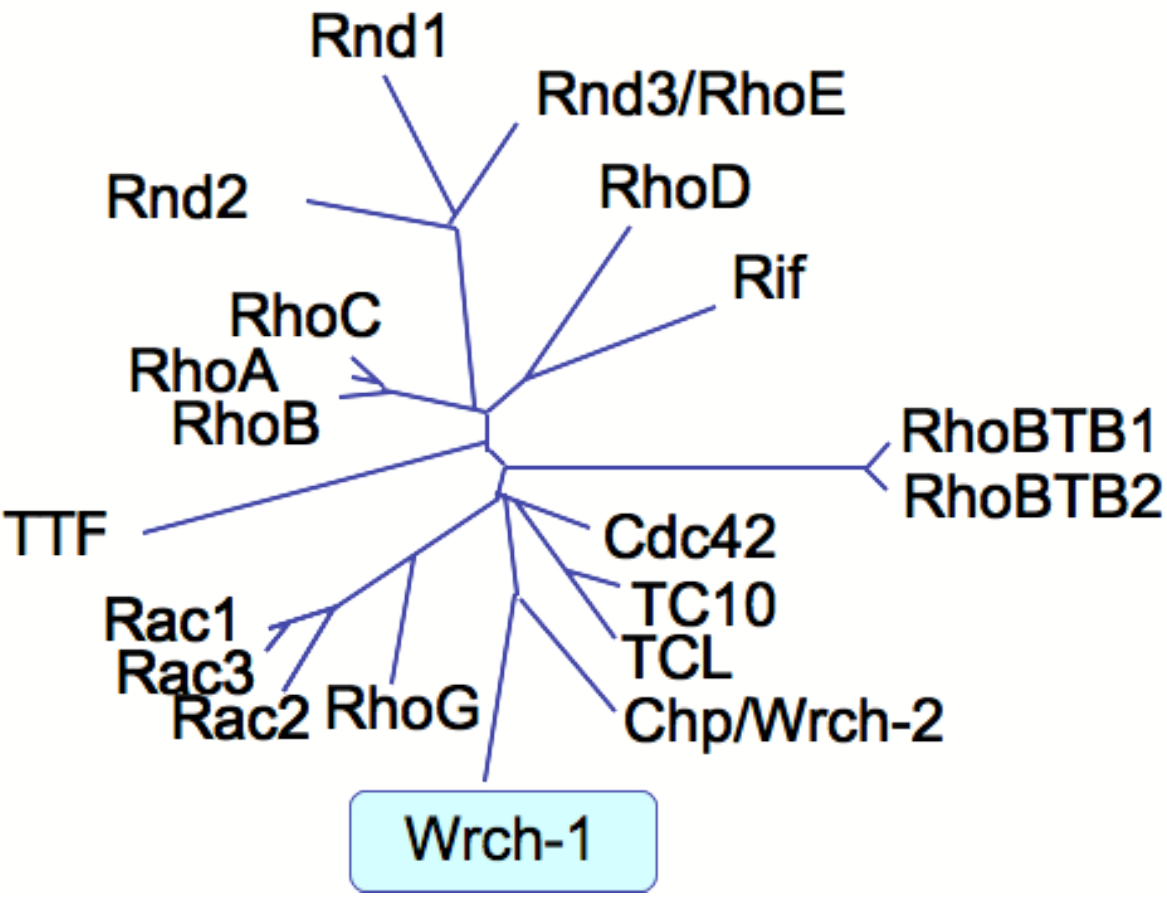


Figure 1.3 The Rho family of small GTPases. The Rho family of small GTPases contains 23 members, 20 of which are shown here. The three best studied are RhoA, Rac1, and Cdc42. Wrch-1 is a member of the Cdc42 subfamily of small GTPases.

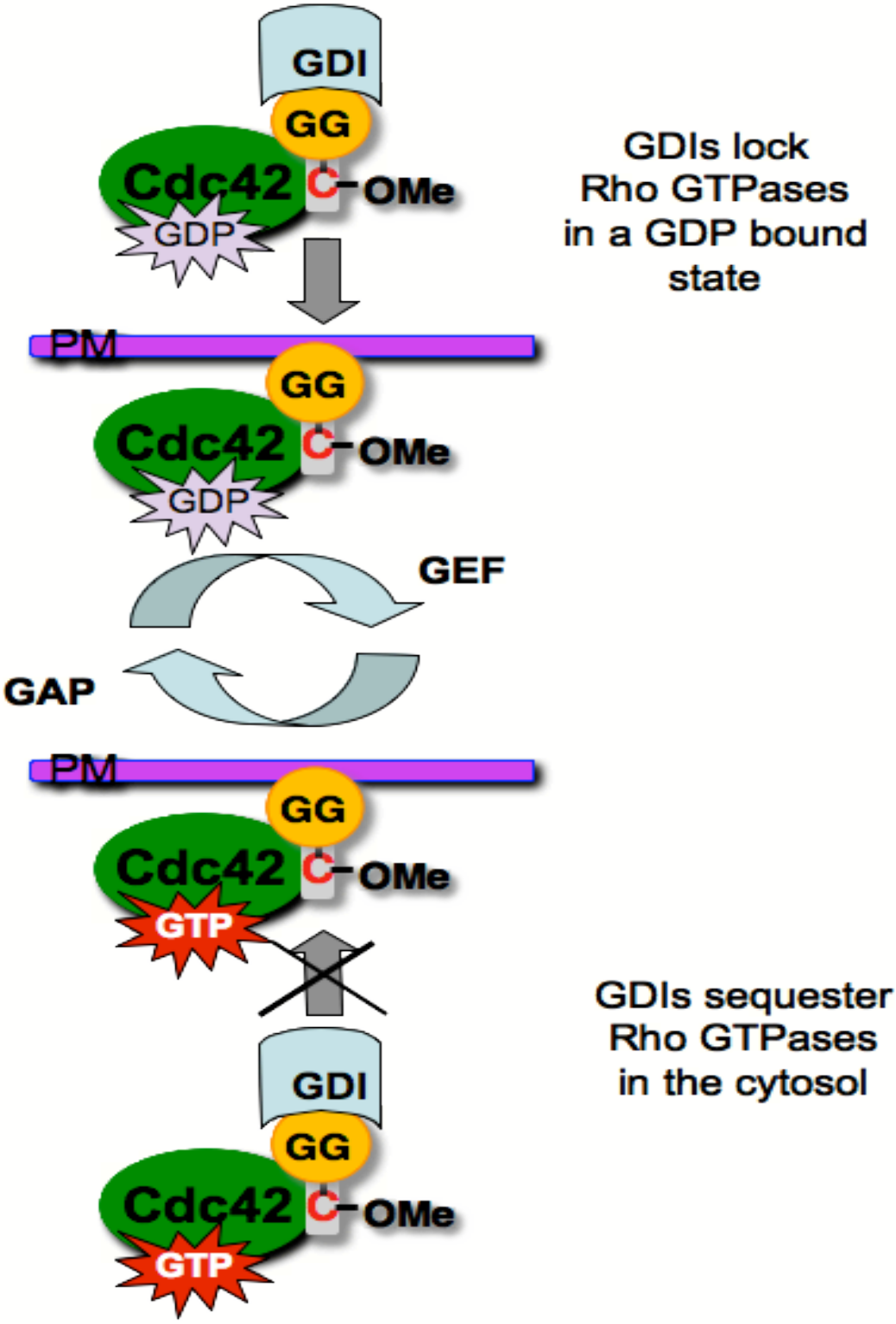


Figure 1.4 Rho GDIs regulate Rho GTPases. Rho GDIs function by inhibiting GDP dissociation and by sequestering Rho GTPases in the cytosol.

Like Ras small GTPases, Rho GTPases also function as molecular switches that cycle between a GTP-bound active state and a GDP-bound inactive state. Rho proteins are also modulated by GEFs, which exchange GDP for GTP, and GAPs which hydrolyze the terminal phosphate of GTP, rendering the protein GDP-bound. The first RhoGEF was isolated from a gene that was transforming in human diffuse B-lymphoma cells (Dbl) (Eva et al., 1988; Ron et al., 1988). Dbl is now known to be an exchange factor for Cdc42 (Hart et al., 1991). The domain in Dbl responsible for exchange was subsequently found in many other RhoGEFs and was termed the Dbl-homology (DH) domain. DH domain-containing GEFs are found in many organisms, *H. sapiens D. melanogaster*, *C. elegans*, *and S. cerevisiae* to name a few. DH domain-containing RhoGEFs also contain a pleckstrin homology (PH) domain, which is usually immediately adjacent and C-terminal to the DH domain (Rossman et al., 2005). Usually, both the DH and PH domain are minimally required to catalyze the exchange of GDP for GTP. RhoGEFs work by promoting the dissociation of GDP, the stabilizing Mg^{2+} ion, and promoting a nucleotide-free intermediate. (Cherfils and Chardin, 1999). Because of the high concentration of GTP relative to GDP in the cell, after GDP is released, GTP can replace GDP in the nucleotide-binding region of the small GTPase. Remarkably, the number of RhoGEFs outnumber target GTPases by a factor of 3, which suggests that multiple GEFs can be capable of activating the same GTPase (Rossman et al., 2005; Schmidt and Hall, 2002). For example, it is known that the RhoGEF Trio is capable of activating both Rac1 and RhoG in mammalian cells (Blangy et al., 2000). However, some GEFs are specific for a particular Rho GTPase. For example, p115RhoGEF activates RhoA exclusively (Kozasa et al., 1998). In general, RhoGEFs are regulated by their negative regulatory N-terminus; the N-terminus sterically blocks the DH domain, and prevents exchange activity. Phosphorylation of some RhoGEFs relieves the N-terminus/DH domain interaction, allowing the GEFs to become active. Vav2 is a well-characterized example of a RhoGEF that is regulated by phosphorylation. Phosphorylation of Vav2 by tyrosine kinases such as Lck

(Han et al., 1997) relieves the N-terminal inhibition of Vav2, opening up the DH domain, which can then interact with Rho GTPases (Aghazadeh et al., 2000; Crespo et al., 1997).

As previously mentioned, Rho proteins are also negatively regulated by RhoGAPs. Rho proteins contain an intrinsic ability to hydrolyze GTP to GDP, however this process is very slow, unless catalyzed by a GAP. Classically, RhoGAPs contain an "arginine finger" that is the catalytically active subunit of the GAP. This "arginine finger" functions to accelerate the hydrolysis of GTP to GDP by stabilizing the transition state of the substrate-binding site of the small GTPase (Rittinger et al., 1997). There are currently approximately 170 proteins that are predicted to possess GAP activity against various members of the Ras superfamily and about 70 of these proteins contain a predicted Rho/Rac GAP domain (Lander et al., 2001). *In vitro*, many of the GAPs that contain the Rho/Rac GAP domain interact with several members of the Rho subfamily of small GTPases; however, some GAPs are specific to a particular Rho GTPase. For example, p50RhoGAP can regulate RhoA, Rac1 (Lancaster et al., 1994), and Cdc42 (Barfod et al., 1993), whereas p190RhoGAP (Ridley et al., 1993) and RhoGAP6 (Prakash et al., 2000) are both specific for Rho. In addition to the GAP domain, RhoGAPs also contain many putative protein and lipid interaction motifs that can modify the localization, activity and/or substrate specificity of these proteins (Ligeti et al., 2004).

As a third level of regulation, in addition to GEFs and GAPs, Rho proteins are also regulated by guanine nucleotide dissociation inhibitors (GDIs). Three human RhoGDIs have been identified: RhoGDI-1/RhoGDI-α, which is ubiquitously expressed (Ueda et al., 1990), RhoGDI-2/RhoGDI-β, which is expressed in hematopoietic cells (Lelias et al., 1993; Scherle et al., 1993), and RhoGDI-3/RhoGDI-γ, which is expressed only in lung, brain, and testes (Adra et al., 1997; Zalcman et al., 1996). GDIs inhibit Rho proteins in two ways: they inhibit

GDP dissociation and also sequester Rho proteins in the cytosol, rendering them inactive (DerMardirossian and Bokoch, 2005) (Figure 1.4). When GDIs bind to Rho proteins, they interact with the effector-binding domain with high affinity (low nanomolar range (Nomanbhoy, 1996 #313)), which is comparable to or better than the affinity for the GTPase for most effectors (Nomanbhoy et al., 1999). Additionally, RhoGDIs have a hydrophobic pocket that can accommodate the geranylgeranyl or farnesyl groups attached to the CAAX boxes of most Rho family proteins (Hoffman et al., 2000).

In addition to regulation by GEFs, GAPs, and GDIs, Rho proteins are regulated by their subcellular localization. Typically Rho proteins are modified by the irreversible addition of a prenyl group to a C-terminal CAAX motif (Adamson et al., 1992; Casey and Seabra, 1996). These isoprenoid lipids are either a C15 farnesyl group or a C20 geranylgeranyl group (Figure 1.5A). It is important to note that canonical CAAX motifs are not present in Wrch-1, Chp/Wrch-2, RhoBTB1, or RhoBTB2 (Roberts et al., 2008). These lipids are added by the enzymes, farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase I), respectively. Following the addition of an isoprenoid group to the C-terminal cysteine, the AAX group is proteolytically cleaved by a CAAX-specific protease, Rce1 (Boyartchuk et al., 1997; Kim et al., 1999; Winter-Vann and Casey, 2005). Subsequently, the prenylated cysteine is then methylated by isoprenylcysteine carboxyl methyltransferase (ICMT) (Clarke et al., 1988; Winter-Vann and Casey, 2005) (Figure 1.5B). After the completion of these steps, the post-translationally processed Rho protein translocates from the cytosol to the plasma membrane (Figure 1.5B). Studies have shown that a cysteine to serine mutation in the CAAX motif (CAAX>SAAX), which render the proteins unprenylated, results in mislocalization of Rho proteins to the cytosol, resulting in their inactivity (Winter-Vann and Casey, 2005). For example, mutation of RhoA CLVL>SLVL and RhoB CKVL>SKVL causes cytosolic mislocalization of these proteins (Adamson et al., 1992). Similarly, a C>S mutation

in Rac1 (CLLL>SLLL), renders it insensitive to GGTase I modification, and results in a mislocalization of Rac1 (Joyce and Cox, 2003). Furthermore, isoprenylation alone is not sufficient to correctly target many Rho GTPases to their correct subcellular localization. Proper posttranslational processing by both Rce1 and ICMT are both required for correct localization of many Rho GTPases (Roberts et al., 2008). Rce1 activity is required for proper localization of RhoB, Rnd1, Rnd2, Rnd3, TC10, TCL, RhoD, Rif and RhoH (Roberts et al., 2008). ICMT activity is required for proper localization of RhoA, Rnd1, Rnd2, Rnd3, TC10, TCL, Rif, and RhoH (Roberts et al., 2008). In addition to the prenyl group attached to the C-terminal CAAX motif, Rho proteins are typically modified by a "second signal" upstream of the prenyl modification. This "second signal" is required for proper plasma membrane localization. There are two classic "second signals" that have been described and characterized, first for Ras proteins, and then for Rho proteins: the addition of a palmitate fatty acid, or the presence of a polybasic region upstream of the prenylation site (Hancock et al., 1990) (Figure 1.6). For example, both RhoB and TC10 require a palmitoyl modification upstream of the isoprenyl group for proper localization (Michaelson et al., 2001), whereas Cdc42 relies on an upstream polybasic region (Williams, 2003). There is data to show that "second signals" in the hypervariable domains can drive Rho proteins to distinct subcellular compartments. RhoB, which is palmitoylated, is targeted to internal membranes, Golgi, and peri-Golgi vesicles (Michaelson et al., 2001). TC10, which incorporates both a palmitoylation site and a polybasic region, localizes to both the plasma membrane and internal membranes (Michaelson et al., 2001). Rac1, which has a strong polybasic region, localizes primarily to the plasma membrane. In contrast, both Cdc42 and Rac2, which both have a weak polybasic region when compared to Rac1, localize primarily to internal membranes, with a small proportion of the protein localizing to the plasma membrane (Michaelson et al., 2001). Additionally, other additional flanking sequences in the hypervariable domain in Ras have been shown to be important for proper localization

(Willumsen et al., 1996). Additional posttranslational modifications, such as phosphorylation, can also modulate the localization of small GTPases, and this is the major focus of my dissertation.

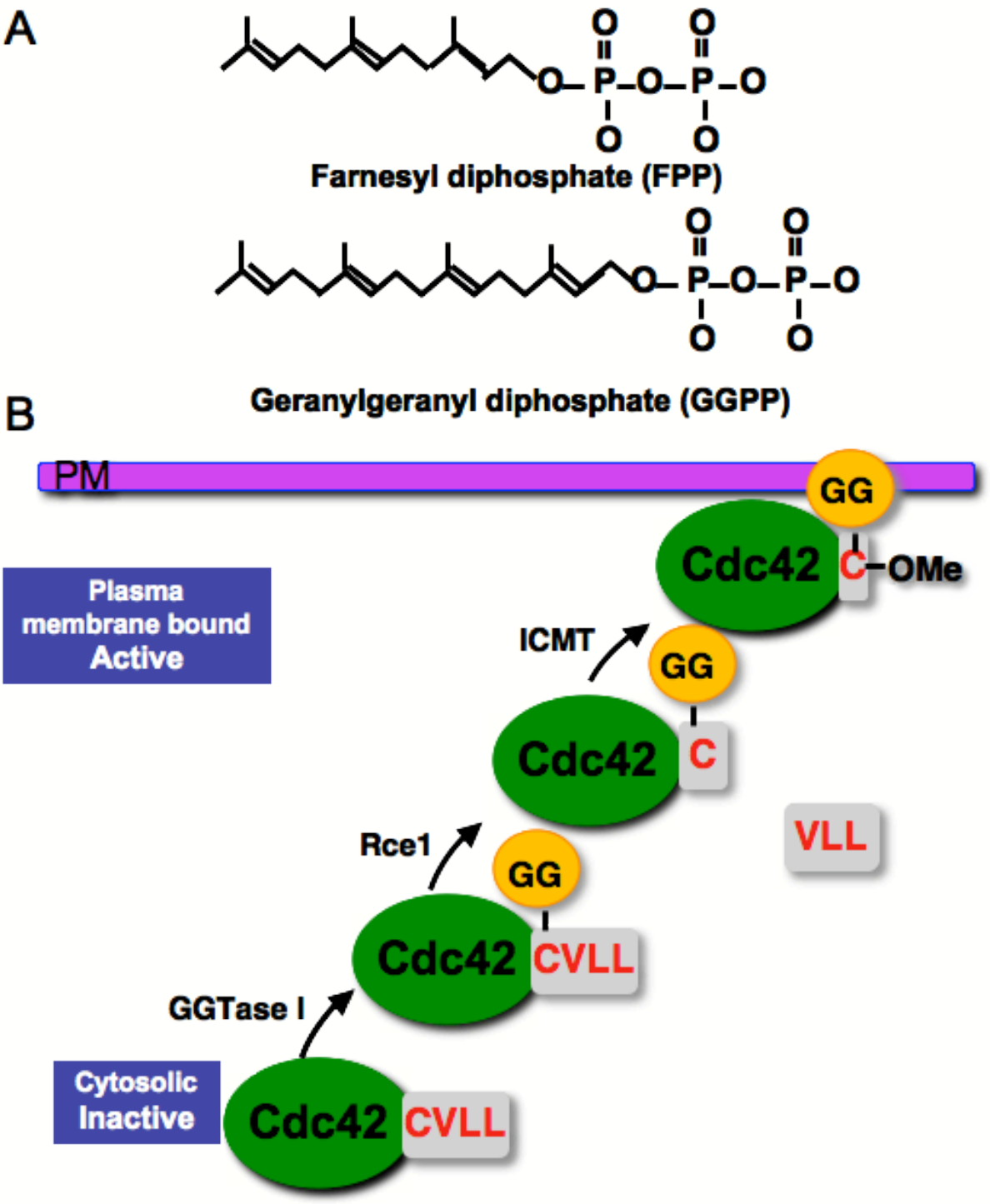


Figure 1.5 Rho proteins are modified post-translationally with isoprenoid lipids. A) Either a C15 farnesyl or a C20 geranylgeranyl group can be added to Rho proteins. B) Post-prenyl processing: after isoprenylation of the CAAX motif, Rho GTPases are further processed by Rce1 and ICMT.

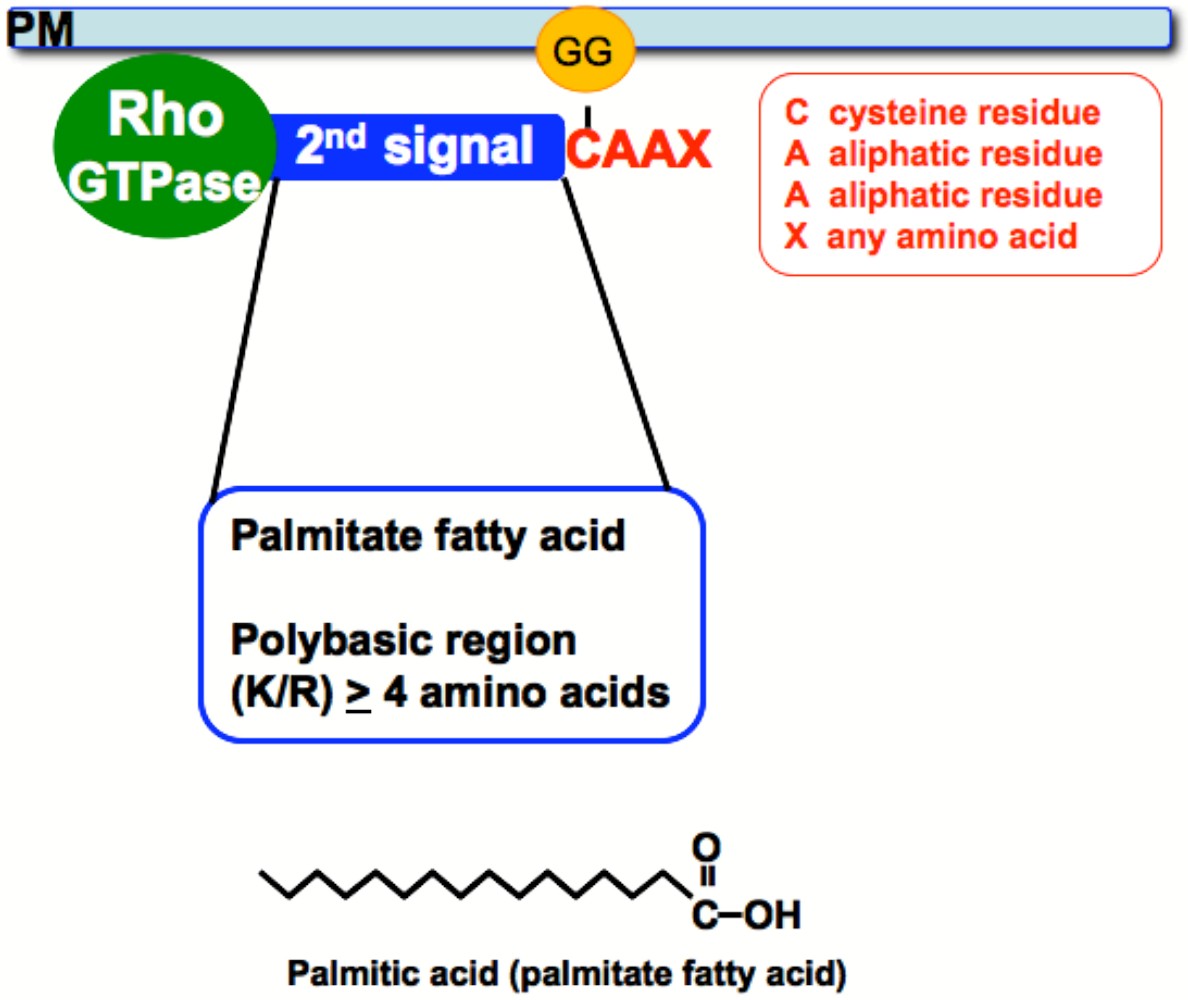


Figure 1.6 A second signal is required for proper Rho protein membrane localization. Second signals are typically comprised of either a palmitate fatty acid or a polybasic region upstream of the C-terminal CAAX motif.

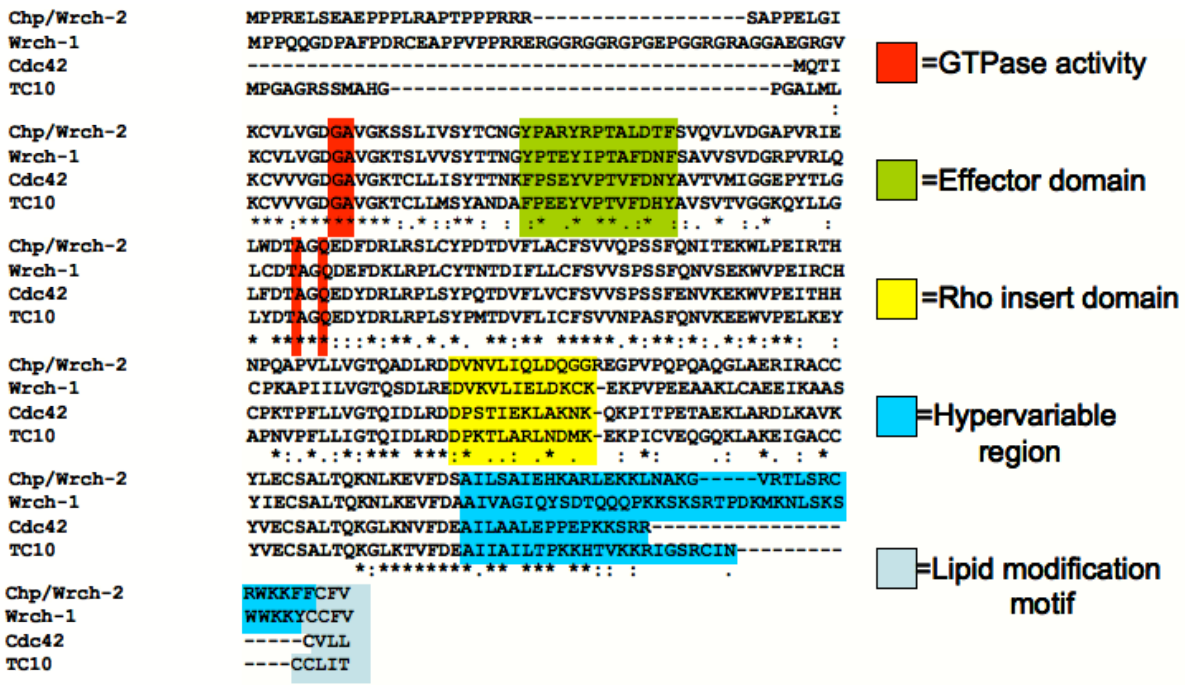


Figure 1.7 Cdc42- family members share sequence similarities.
The Cdc42 subfamily of small GTPases has 5 members that are most similar within their GTP-binding region, effector domain, and Rho insert region. Divergent sequences and modifications within their C-terminal hypervariable regions and CAAX motifs dictate different localization and function.

1.3 The Cdc42 subfamily of Rho GTPases. Cdc42 is the founding and most well studied member of the Cdc42 subfamily. Cdc42 was originally identified in yeast where it functions as a regulator of cell polarity and cellular division (Ziman et al., 1993). In mammalian cells, Cdc42 regulates cytoskeletal organization, especially filopodial structures. Additionally, roles for Cdc42 have been defined in normal cell growth and polarity, gene expression, cell cycle progression, and in tumorigenesis when aberrantly regulated (Aspenstrom et al., 2004; Wennerberg and Der, 2004). The Cdc42 subfamily of small GTPases consists of 5 proteins: 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 the GTP- and effector-binding regions, and differ the most in their N- and C-terminal extensions (Figure 1.7). As with all small GTPases, the GTPase domain undergoes a conformational switch when it is GTP-bound, allowing these proteins to interact with effectors through the effector-binding domain. Because the Cdc42 subfamily shares high sequence similarity within the effector-binding domain, it is easy to think that these proteins utilize similar effectors to elicit biological function and are therefore redundant. However, the presence of unique sequence elements within the effector-binding domain, and divergent hypervariable regions (important for localization) within the subfamily confer enough divergence to promote unique functions within the Cdc42 subfamily. Most Cdc42 effectors contain the Cdc42/Rac-interactive binding (CRIB) domain that is required for interactions with the effector-binding domain of Cdc42 (Cotteret and Chernoff, 2002). Exceptions to this include phosphotidylinositol 3-kinase (PI3K), CIP4 and IQGAP, which interact with a region in the effector domain that is distinct from the region that interacts with the CRIB domains (Bishop and Hall, 2000; Cotteret and Chernoff, 2002). One of the major effectors of Cdc42 is the serine/threonine kinase, p21-activated kinase (PAK), which binds through its CRIB domain to Cdc42. When Cdc42 is GTP-bound, it can then bind and activate PAK by stimulating autophosphorylation of the kinase (Manser et al., 1994; Martin

et al., 1995). PAK activity downstream of Cdc42 has been shown to be important in many Cdc42-driven biological activities, including transformation, morphogenesis and cell cycle progression.

Cdc42 and related proteins reduce stress fibers and focal adhesions, and promote the formation of filopodia. These cytoskeletal changes are thought to be induced by signaling through PAK as well as other Cdc42 effectors. Another major class of Cdc42 effectors includes the Wiskott-Aldrich syndrome protein (WASP) family members, which bind through their CRIB domains to Cdc42 (Li et al., 1999). Downstream of Cdc42, WASP proteins facilitate the formation of filopodia. Cdc42 also controls epithelial cell polarity, which is mediated by the formation of distinct apical and basolateral membrane regions, which are separated by tight junctions. Establishment of polarity is required for directional movement of cells, and if polarity is disrupted it can contribute to more motile and invasive phenotypes. Cdc42 controls tight junction formation by its interactions with two proteins; partitioning defective 6 (Par6) and atypical protein kinase Cs (aPKCs). This dissertation will focus on Wrch-1 (Wnt-regulated Cdc42 homolog-1), a more recent addition to the Cdc42 subgroup. Wrch-1 shares some effectors with Cdc42 to elicit its cellular effects, and but also utilizes other effectors, that are not associated with Cdc42.

Like Cdc42, Wrch-1 can bind to and stimulate autophosphorylation of PAK1 (Tao et al., 2001) and, as discussed later in this dissertation, activation of PAK1 by Wrch-1 is regulated by Wrch-1 phosphorylation status. Wrch-1 also binds to the Par6 and PKCζ complex (Saras et al., 2004) to control epithelial cell morphogenesis (Brady et al., 2009). Unlike Cdc42, Wrch-1 does not bind to WASP family proteins such as CIP4 or Spec1, as shown by a yeast 2-hybrid analysis (Aspenstrom et al., 2004). Taken together, this information suggests that Wrch-1 possesses some similarities to Cdc42 in terms of effector binding, but also is

divergent from Cdc42 because it can utilize different effectors.

1.4 Wrch-1, an atypical Rho family member. Wrch-1 (Wnt-regulated Cdc42 homolog-1) and Chp/Wrch-2 (Cdc42-homologous protein), the most 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). Canonical Rho GTPases include RhoA, RhoB, RhoC, Rac1, Rac2, Rac3, and Cdc42. These members of the Rho family of small GTPases are considered canonical because they are tightly regulated by GDP/GTP-binding, through regulation by GEFs, GAPs, and GDIs. Additionally, they undergo typical post-translational modifications, such as an isoprenyl group on their C-terminal CAAX motif, and an additional second signal such as a polybasic region or an upstream palmitate, which will be discussed in more detail below. Atypical Rho proteins vary in either their regulation of GTP/GDP-binding, the presence of other domains besides a Rho insert domain, and variances in their N- and C-termini and/or posttranslational modifications. For example, the Rnd subfamily of proteins are constitutively GTPase-deficient and they are not stable in either a GDP-bound or a nucleotide-free form (Nobes et al., 1998). Rnd3, also known as RhoE and Rho8, is constitutively GTP-bound *in vivo* (Foster, 1996 #4;Nobes, 1998 #231). RhoD and Rif contain additional N-terminal sequences when compared to the canonical Rho GTPases (Ellis and Mellor, 2000; Murphy et al., 1996). RhoH, also known as TTF (translocated three four), is expressed specifically in hematopoietic cells (Dallery et al., 1995), and plays a role in both non-Hodgkins lymphoma and multiple myeloma (Preudhomme et al., 2000). In RhoH, similar to the Rnd proteins, the residues analogous to G12 and Q61 in other Rho proteins are not conserved, and RhoH is likely to also be GTPase-deficient (Li et al., 2002). When compared to the other members of the Cdc42 family, Wrch-1 has elongated N-terminal and C-terminal extensions (Figure 1.8). The N-terminus has been shown to be an auto-inhibitory domain: removal of the N-terminus

increases biological activity (Berzat et al., 2005b; Shutes et al., 2006; Shutes et al., 2004). The N-termini of both Wrch-1 and Chp contain PxxP motifs, which may mediate interactions with proteins containing SH3 domains, such as the adaptor proteins Grb2 and Nck. The RhoBTB family consists of three proteins in humans: RhoBTB1, RhoBTB2, and RhoBTB3. The RhoBTB subfamily proteins are also structurally different from the canonical Rho GTPases, in that they contain a tandem repeat of BTB subdomains and lack C-terminal CAAX motifs (Ramos et al., 2002). Additionally, their GTPase domains are altered when compared to the canonical Rho GTPases. RhoBTB1 and 2 contains a Rho insert domain that is longer than usual, contains many charged residues, and also contains insertions and deletions in the GTPase domain. One of the deleted residues is analogous to Q61 in Ras, and also the G12 position is substituted by an asparagine in RhoBTB1 and 2. These proteins would be predicted to display impaired enzyme activity. Consistent with this hypothesis, *in vitro* RhoBTB2 does not bind GTP (Chang et al., 2006). Furthermore, RhoBTB3 has an extremely truncated GTPase domain, to the point where it is almost unrecognizable as a GTPase domain, and does not bind GTP (Berthold et al., 2008) but instead is an ATPase (Espinosa et al., 2009). However, like canonical Rho GTPases, RhoBTB3 does have a prenylation motif, and is farnesylated (Berthold et al., 2008). Finally, the Miro subfamily contains not only one, but two Rho GTPase domains (the N-terminal Rho GTPase domain is most similar to other Rho GTPase domains) and two EF-hand motifs (Fransson et al., 2003).

Wrch-1 is also divergent in the C-terminus, when compared to other members of the Cdc42 family. Like Chp (Chenette et al., 2006), instead of being irreversibly prenylated on a CAAX motif, Wrch-1 is reversibly palmitoylated on a CFV motif (Berzat et al., 2005b) (Figure 1.9). Although palmitoylation as a primary lipid modification has not been described for Rho GTPases other than Chp or Wrch-1, there are several proteins in *Arabidopsis* and maize,

that terminate in CXX motifs, and their localization and function depend on the addition of a palmitate fatty acid, rather than a prenyl group, on this motif (Ivanchenko et al., 2000; Lavy et al., 2002). There are eleven Rho-related proteins in *Arabidopsis*, typically referred to as Rops (Rho in plants) (Zheng and Yang, 2000) or RACs (Winge et al., 2000). Of the eleven Rho proteins in *Arabidopsis*, eight are prenylated on CAAX motifs and three rely on palmitoylation of a CXX motif for association with plasma membrane and proper elongation of the root hair (Lavy et al., 2002). Additionally, there are two ROP proteins in maize, both of which terminate in CXX motifs (Ivanchenko et al., 2000), similar to those found in *Arabidopsis*, and also similar to Wrch-1 and Chp in humans.

The palmitoylation/depalmitoylation of proteins is a rapid and reversible cycle that 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 protein palmitoylation was discovered more than 20 years ago, the enzymes responsible for this modification have only been recently described (Linder and Deschenes, 2003; Linder and Deschenes, 2004; Lobo et al., 2002; Roth et al., 2002) and their identities and modes of action are still under active exploration. Although the addition of a palmitate can occur spontaneously *in vitro* (Bharadwaj and Bizzozero, 1995; Duncan and Gilman, 1996; Quesnel and Silvius, 1994), two classes of protein palmitoylating enzymes have been discovered (Lobo et al., 2002; Roth et al., 2002). Members of the first class of PATs share a DHHC domain, which is a cysteine-rich domain with a conserved Asp-His-His-Cis motif, suggested to be the domain responsible for palmitoyl transfer (Roth et al., 2002). Examples of proteins that contain the DHHC domain are Erf2/Erf4 in yeast, which have been shown to palmitoylate Ras, and Akr1 which is the related PAT for yeast casein kinase II. Members of the second class of PATs act on proteins in the lumen of the secretory pathway (Chamoun et al., 2001; Miura et al., 2006; Zhai et al., 2004). In *D. melanogaster*, there is evidence that

skinny hedgehog (also called Rasp, Central missing, and Sightless) is required for palmitoylation of Hedgehog (Chamoun et al., 2001) and Spitz (Miura et al., 2006). Additionally, another *D. melanogaster* protein, porcupine, is required for palmitoylation of Wnt proteins (Zhai et al., 2004).

Palmitoyl protein thioesterases (PPTs) are required for the removal of palmitoyl modifications. Although no direct work has been done to demonstrate the activity of PPTs on Rho proteins, pulse-chase experiments were first done to demonstrate the turnover of palmitate on H- and N-Ras (Magee et al., 1987), the transferrin receptor (Jing and Trowbridge, 1987), and ankyrin (Staufenbiel, 1987), to name a few. Additionally, it has been shown that there is a requirement for Ras to be in the native conformation, along with a requirement for the presence of Mg^{2+} for PPT1 to remove the palmitate from H-Ras (Camp and Hofmann, 1993). Other *in vitro* work has shown that PPT1 and acyl protein thioestrase (APT1) can both remove palmitate modifications from Ras proteins (Duncan and Gilman, 1998). Importantly, the palmitoylation/depalmitoylation cycle has been shown to be dynamic and reversible (Rocks et al., 2005; Zhang and Rock, 2008), and may serve as a dynamic mechanism to regulate small GTPase localization and function. In support of this notion, palmitoylation is required for proper membrane localization and biological function of Wrch-1 (Berzat et al., 2005b). A mutant that cannot be palmitoylated (Wrch-1 SSFV) is mislocalized and causes a decrease in anchorage-independent growth (Berzat et al., 2005b). As mentioned before, many Rho proteins utilize a "second signal", such as an upstream palmitate or poly-basic region to properly regulate their localization to the plasma membrane. Currently, there is no known "second signal" for Wrch-1 plasma membrane localization; there is no identifiable polybasic region, and there is no upstream cysteine available in the C-terminus for palmitoylation.

Wrch-1 was initially discovered as a Wnt-responsive gene, whose expression (measured by mRNA) was increased in response to Wnt-1 signaling in Wnt-1-transformed cells, Wnt-1-transgene-induced mouse mammary tumors, and Wnt-1 retrovirus-infected cells (Taneyhill and Pennica, 2004; Tao et al., 2001). When Wrch-1 is mutationally activated (Q107L, analogous to a Ras Q61L mutation) it phenocopied Wnt-1-mediated morphological transformation of mammary cells (Tao et al., 2001). The Wnt family of secreted proteins are critical mediators of cell-cell signaling in development. The first Wnt gene, Wnt1, was discovered as a proto-oncogene that was activated by integration of a mouse mammary tumor virus into mammary cells. Wnt proteins have been extensively characterized in *D. melanogaster* and *C. elegans*. When cells are not exposed to Wnt, β-catenin is degraded through interactions with Axin, APC and GSK-3. When Wnt ligands interact with the transmembrane receptor Frizzled they work by inhibiting the Axin/APC/GSK-3 complex to induce elevation of cytoplasmic β-catenin, which can then interact with TCF to control transcription (Klingensmith and Nusse, 1994; Klingensmith et al., 1994; Peifer et al., 1994; Siegfried et al., 1994; Theisen et al., 1994; Zeng et al., 1997). Normally Wnt signaling is present in the developing organism, and is in place to promote normal development. However in adults, mis-regulation of the Wnt signaling pathway can lead to a variety of abnormalities and disease. Therefore, regulation of Wrch-1 in response to Wnt simulation could be hypothesized to mediate both normal development, and disease driven by aberrant Wnt signaling in adults.

Chp/Wrch-2, a protein highly related to Wrch-1, was identified in a screen that was designed to look for proteins that interact with p21-activated kinase (Pak1) (Aronheim et al., 1998). Both Wrch-1 and Chp/Wrch-2 are expressed ubiquitously at very low levels, with a higher level of expression in the brain and placental tissue (Aronheim et al., 1998; Tao et al., 2001). Interestingly, Wrch-1 and Chp are both important in chicken embryonic development,

however they are expressed in different spatial and temporal patters during chick embryogenesis, suggesting distinct roles in this process (Notarnicola, 2008 #434). Like Wrch-1, Chp also contains N- and C-terminal extensions when compared to Cdc42. The N-terminus of both Wrch-1 and Chp functions as an auto-inhibitory domain (Chenette et al., 2005; Shutes et al., 2004). When the N-terminus of either Wrch-1 or Chp is deleted, both proteins display enhanced biological functions such as increased transformation (Chenette et al., 2005; Shutes et al., 2004). Additionally, both Wrch-1 and Chp terminate in a CFV motif, and both are palmitoylated on the cysteine of this motif (Berzat et al., 2005b; Chenette et al., 2005), a modification that is necessary for their membrane association and function (Berzat et al., 2005b; Chenette et al., 2005). Because both Wrch-1 and Chp are palmitoylated instead of being prenylated, neither protein interacts with RhoGDIs (Berzat et al., 2005b; Chenette et al., 2006). Unlike Wrch-1, but similar to Cdc42, Chp requires the presence of a stretch of basic amino acids in the C-terminal membrane-targeting motif (Chenette et al., 2006). Chp also requires the presence of a conserved tryptophan in the C-terminal membrane-targeting region (Chenette et al., 2006), and although Wrch-1 possesses a tryptophan residue in the 9aa minimal targeting sequence (Berzat et al., 2005b), it has not been determined whether this residue is important for proper localization of full-length Wrch-1. Interestingly, Chp has been shown to be activated at internal membranes in response to stimulation by TNFα (Chenette et al., 2006), by a yet-undetermined mechanism; however, Wrch-1 activation in response to TNFα has not been examined.

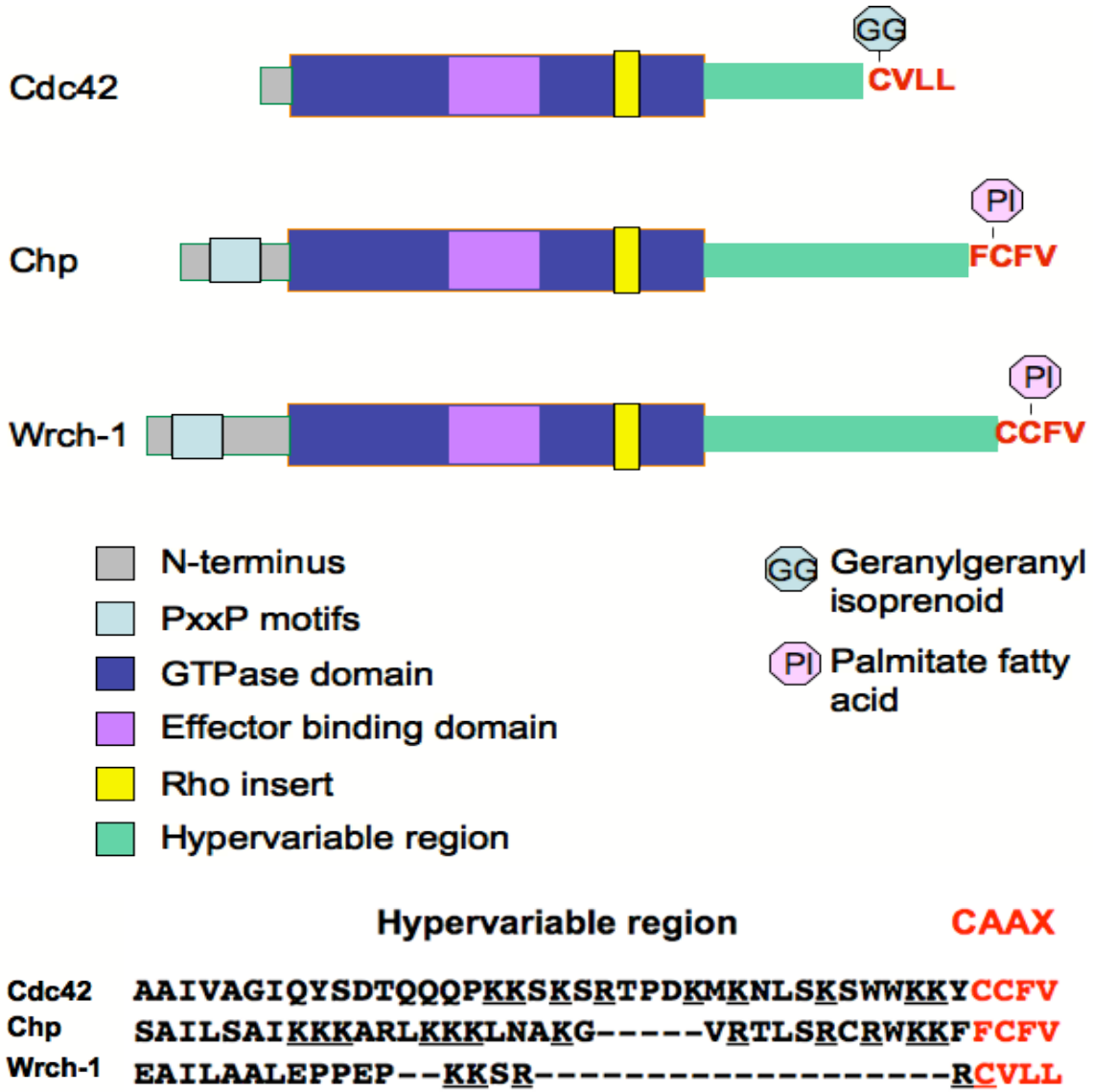


Figure 1.8 Wrch-1 contains a GTPase domain, Rho insert domain, and effector domain similar to those of Cdc42. However, unlike Cdc42, Wrch-1 also contains an N-terminal extension containing polyproline motifs and a C-terminal extension that is modified with a palmitate fatty acid instead of a geranylgeranyl isoprenoid lipid.

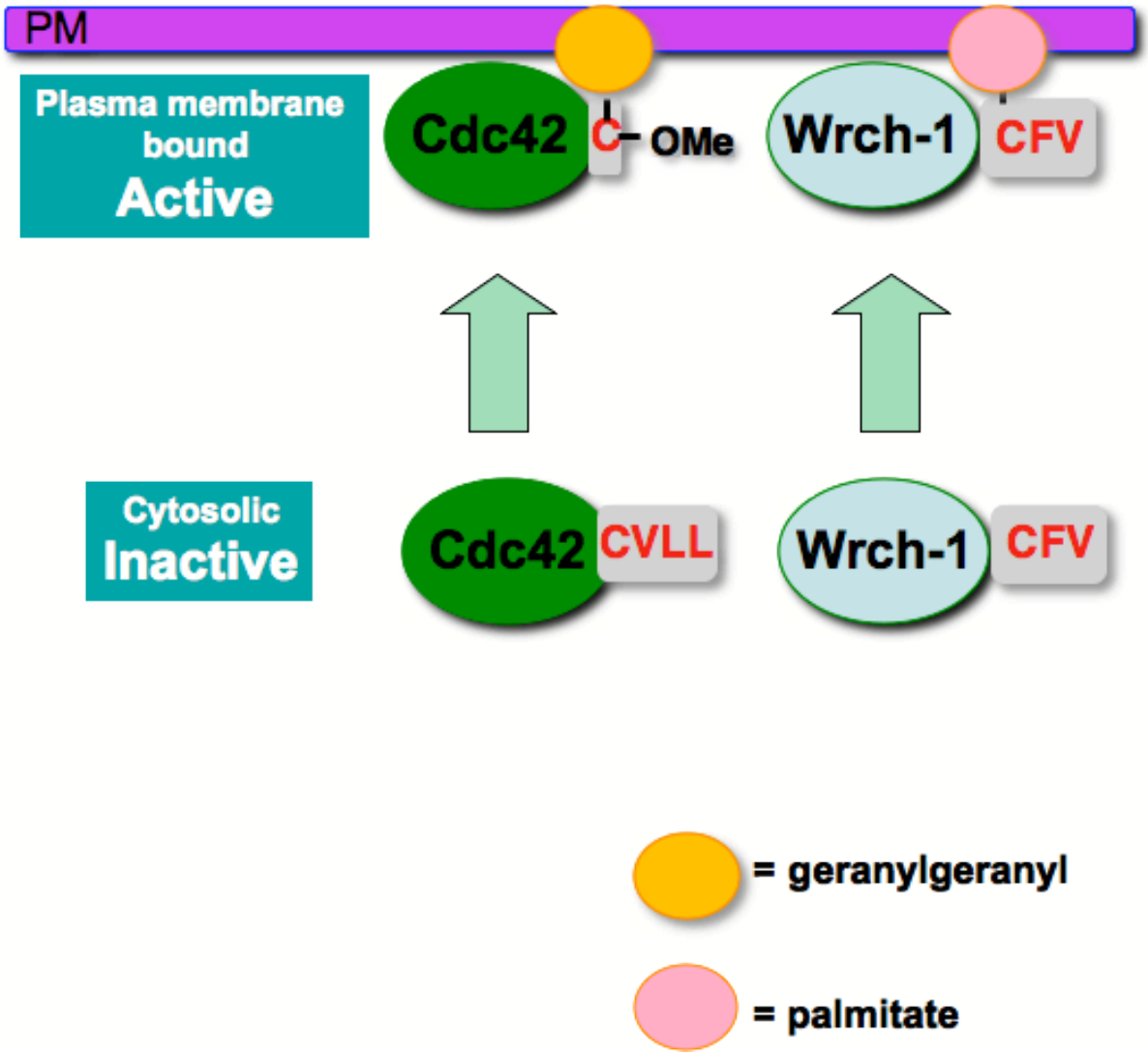
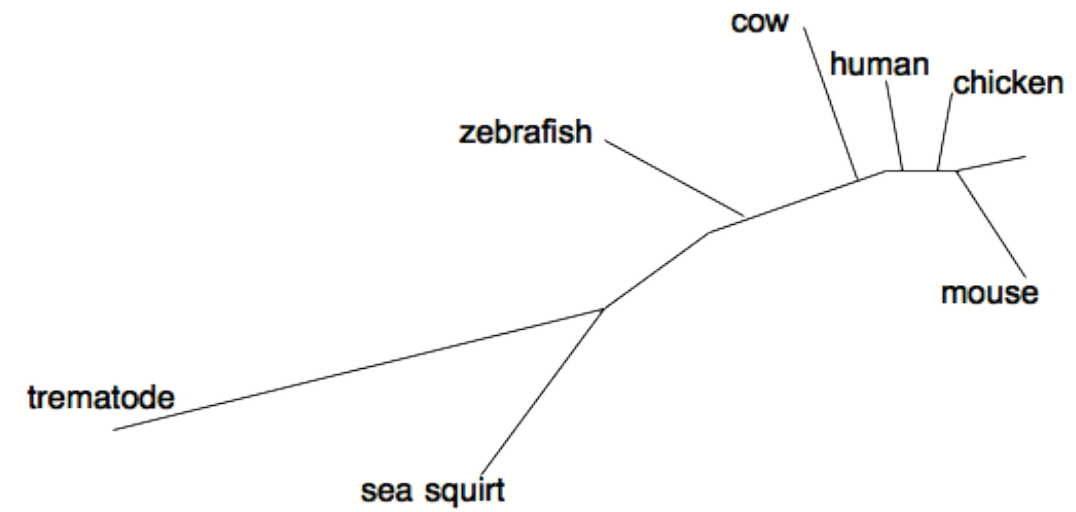


Figure 1.9 Wrch-1 utilizes a palmitate fatty acid added to its CFV motif, rather than an isoprenoid lipid on a CAAX motif, for proper membrane localization and activity



Y254

```
human          SKSR----------TPDKMKNLSKSWWKKYCCFV
mouse          SKSR----------TPDKVRDLSKSWWRKYCCL-
dog            SKSR----------TPDKMKNLSKSWWKKYCCFV
cow            SKSR----------TPDKMKTLSKSWWKKYCCFV
chicken        SKCR----------TPDKMKNLSKSWWKKYCCF-
zebrafish      LKKR----------TPDKMRKLSESWWKKYCCLA
trematode      KQRQ----------TPDKMKSLSKSWWKRYCCVA
sea squirt     RKMRSESKRSHSEKSTTPRTLKCKPRAWLRKYCCAA
```

Figure 1.10 Wrch-1 is evolutionarily conserved. Wrch-1 homologues are expressed in many organisms from sea squirts to humans. Interestingly, there is no Wrch-1 equivalent in fruit flies, a commonly used model organism. Note that the critical residue, Y254, whose regulatable modification by phosphorylation is the key subject of this dissertation, is evolutionarily conserved (red Y).

Wrch-1 homologs or orthologs have been found in many organisms, including human, mouse, dog, chicken, zebrafish, sea squirt and trematodes, demonstrating evolutionary conservation of Wrch-1 (Figure 1.9). Interestingly Wrch-1 is not found in some other important model organisms, such as fruit flies, however there is a Wrch-1/Chp hybrid found in *C. elegans*, named Chw-1. Currently there are no identified GEFs, GAPs, or GDIs known to interact with and regulate Wrch-1. *In vitro,* Wrch-1 has a very high rate of GDP/GTP exchange, suggesting that Wrch-1 may be constitutively GTP-bound in cells (Shutes et al., 2006). However, a putative dominant negative of Wrch-1, T63N, does not behave like wild type (Ruusala and Aspenstrom, 2008), supporting the idea that at least one GEF may still be important to activate Wrch-1. In addition, in biological assays, a GTPase-insensitive mutant of Wrch-1 (Q107L) shows significantly higher activity than wild-type Wrch-1 (Berzat et al., 2005b; Brady et al., 2009; Brazier et al., 2009; Chuang et al., 2007; Ory et al., 2007; Ruusala and Aspenstrom, 2008; Saras et al., 2004), suggesting that there is at least one GAP that inactivates Wrch-1 which has yet to be identified.

Although Wrch-1 is a fairly recent addition to the Cdc42 subfamily, several effectors have been identified. Wrch-1 shares some of the same effectors as Cdc42, including PAK1 and the Par6/PKCζ polarity complex (Brady et al., 2009; Saras et al., 2004; Tao et al., 2001). Wrch-1 also has effectors that it does not share with Cdc42, including the non-receptor tyrosine kinase Pyk2 (Ruusala and Aspenstrom, 2008). Several important biological functions have been ascribed to Wrch-1 including regulation of the actin cytoskeleton (Brady et al., 2009; Ruusala and Aspenstrom, 2008; Saras et al., 2004; Tao et al., 2001), anchorage-independent growth (Berzat et al., 2005b; Brady et al., 2009), focal adhesion formation (Chuang et al., 2007 Ory, 2007 #124), cell migration (Brazier et al., 2009; Chuang et al., 2007; Ory et al., 2007), osteoclastogenesis (Brazier et al., 2009; Ory et al., 2007) and epithelial cell morphogenesis (Brady, 2009 #15). Originally, Wrch-1 was discovered in a

screen designed to identify genes that were up-regulated in a murine mammary epithelial cells that stably express Wnt1 (Tao et al., 2001). In the same study, Wrch-1 was found to activate both PAK-1 and C-Jun using *in vitro* kinase assays (Tao et al., 2001). Wrch-1 also induced filopodia formation and stress fiber dissolution in Swiss 3T3 cells, and also stimulated these cells to re-enter the cell cycle (Tao et al., 2001). Expression of constitutively activated Wrch-1 caused morphological transformation of mouse mammary epithelial cells, similar to Wnt-mediated morphological transformation (Taneyhill and Pennica, 2004; Tao et al., 2001). Taken together, this data suggested that Wrch-1 may be important in Wnt-mediated transformation of cells.

Wrch-1 was also identified in another screen designed to identify mRNA that was upregulated during the differentiation of macrophages into osteoclasts (Brazier et al., 2006). Following this observation, Wrch-1 was shown to localize to the podosome belt and is associated with Src-induced podosomes in osteoclasts (Ory et al., 2007). Wrch-1 was also shown to localize to focal adhesions, where it reduces overall adherence (Brazier et al., 2009) and the number of focal adhesions in osteoclasts (Ory et al., 2007) and HeLa cells (Chuang et al., 2007). Because focal adhesions and podosomes are important structures in migration and invasion respectively, it was thought that Wrch-1 activity within these structures may regulate migration and/or invasion. Indeed, Wrch-1 overexpression increased migration in both osteoclasts and HeLa cells (Brazier et al., 2009; Chuang et al., 2007; Ory et al., 2007). Conversely, Wrch-1 depletion led to an increase in focal adhesions, by regulating myosin light chain, and a decrease in migration (Chuang et al., 2007). Wrch-1 mediated migration is thought to be accomplished through activation of Akt and JNK because Wrch-1 is necessary for wound-healing mediated by Akt and JNK activation (Chuang et al., 2007).

Wrch-1 expression reduced osteoclast adhesion onto vitronectin but not fibronectin and also increased osteoclast precursor aggregation (Brazier et al., 2009). Increased aggregation and decreased adhesion in osteoclasts was mediated through Wrch-1 binding to the cytoplasmic domain of integrin β3, and interference with adhesion induced Pyk2 and paxillin phosphorylation (Brazier et al., 2009). Wrch-1 also binds to Pyk2 in PAE/PDGFRβ cells, where activation of Wrch-1 results in increased Pyk2 phosphorylation and activation (Ruusala and Aspenstrom, 2008). In these cells, Wrch-1 requires both Pyk2 and Src expression and activity for the formation of filopodia (Ruusala and Aspenstrom, 2008).

Wrch-1 has also been shown to promote anchorage-independent growth in several cell types (Berzat et al., 2005b; Brady et al., 2009). Wrch-1-mediated anchorage-independent growth is dependent on the presence of a palmitate fatty acid on its CFV motif. A mutant of Wrch-1 that cannot be palmitoylated (Wrch-1 SSFV) decreased the ability of Wrch-1 to form colonies (Brady et al., 2009). Wrch-1-mediated anchorage-independent growth is also regulated by its N-terminus; the removal of the N-terminal auto-inhibitory domain of Wrch-1 increases anchorage-independent growth (Brady et al., 2009). In addition to FTase and GGTase I, another prenyl transferase called geranylgeranyl transferase II (GGTase II) has been described, which modifies Rab GTPases (Seabra et al., 1992). Instead of recognizing a CAAX motif, GGTase II recognizes Rab proteins which terminate in CCXX, CC, or CXC motif (Khosravi-Far et al., 1992). Although Wrch-1 ends in an apparent CCXX motif, it is not a target for GGTase II (Berzat et al., 2005b).

Most recently, we found that Wrch-1 negatively regulates the kinetics of tight junction assembly through binding to the cell polarity protein Par6 in a GTP-dependent manner (Brady et al., 2009). Polarity is important for the proper development and function of organisms (Wodarz, 2002), and loss of polarity can contribute to a transformed phenotype

(Wodarz and Nathke, 2007). Proper cell polarity is needed for the formation of apical and basolateral membranes during morphogenesis, determination of cell fate during asymmetric cell division, and proper development and migration of neuronal axons and dendrites (Nelson, 2003). Although Wrch-1 had no detectable effect on overall cell polarity in a confluent monolayer, it had a dramatic impact on the cytoskeleton and multilayering in cells grown in three-dimensional culture (Brady et al., 2009). A precisely regulated level of Wrch-1 seems to be required, because overexpression of activated Wrch-1 or knockdown of Wrch-1 disrupted epithelial cell morphogenesis (Brady et al., 2009).

Wrch-1 localizes to the plasma membrane and to internal membranes including endosomes and lysosomes, potentially suggesting a role for Wrch-1 in intracellular trafficking. As stated earlier, there is no known "second signal" for Wrch-1 plasma membrane localization; there is no identifiable polybasic region, and there is no upstream cysteine available in the C-terminus for palmitoylation. However there are many residues in the C-terminal hypervariable region that could be substrates for post-translational modification, including several lysines (ubiquitination), serines, threonines, and tyrosines (phosphorylation). This dissertation will focus on the role of C-terminal tyrosine phosphorylation in regulating the localization and function of Wrch-1. Chapters II and III will focus on the upstream kinases, and the mechanism by which C-terminal tyrosine phosphorylation regulates Wrch-1.

1.5 Cdc42-related GTPases and trafficking. There are several examples of Cdc42 family members that are involved intracellular trafficking. Cdc42 shares high sequence similarity with two other members of the Cdc42 family in addition to Wrch-1 and Chp, TC10 and TCL (Figure 1.10). Similar to Cdc42, TC10 promotes the formation of filopodia, activates JNK, and promotes SRF- and NF-κB-mediated transcription (Murphy et al., 1999). TCL promotes the formation of membrane ruffles and lamellipodia (Aspenstrom et al., 2004; Vignal et al.,

2000). Interestingly, TC10 and TCL share some overlapping localizations with Wrch-1. TC10 localizes mainly to the plasma membrane and intracellular membranes, whereas TCL localizes to endosomes. TC10 localization and biological function are dependent on both palmitoyl fatty acid modification of carboxyl terminal cysteine residues upstream of the CAAX motif, and on isoprenylation of its CAAX motif (Murphy et al., 2001; Murphy et al., 1999). Additionally, TC10 is phosphorylated by CDK5 on Thr197. Palmitoylation and phosphorylation also regulate TC10 mediated biological functions. For example, TC10 regulation of GLUT-4 translocation to the plasma membrane requires palmitoyl-assisted localization of TC10 to lipid rafts (Murphy et al., 2001; Murphy et al., 1999). Additionally, phosphorylation at Thr197 by CDK mediates the association of TC10 with lipid rafts, a requirement for its ability to modulate insulin-stimulated GLUT4 translocation (Okada et al., 2008). TCL has been previously shown to associate with early endosomes, and regulate the transfer of transferrin from early endosomes to recycling endosomes (de Toledo et al., 2003). A proper balance of TCL activity is required for this function, because both loss of TCL and constitutive activation of TCL restricts transferrin to early endosomes (de Toledo et al., 2003). Furthermore, the C-terminal tail and proper localization were crucial for TCL function, because the fusion of C-terminal sequences of TCL to either Cdc42 or TC10 had a similar effect on transferrin trafficking (de Toledo et al., 2003). This further highlights the importance of localization in regulating the function of Rho family proteins. Wrch-1 localizes to the plasma membrane and internal membranes, and as discussed in this dissertation, its localization is regulated by C-terminal terminal phosphorylation. The mechanisms of Wrch-1 relocalization and its role in trafficking are addressed in Chapters II and III.

1.6 C-terminal Ser/Thr phosphorylation of Ras and Rho proteins. There is increasing evidence that phosphorylation of small GTPases is required for both their localization and specific functions. In one of the first studies of small GTPase phosphorylation, it was found that K-Ras was phosphorylated by PKC (Ballester et al., 1987). In addition to being modified by a prenyl group on its C-terminal CAAX motif, K-Ras also possesses an upstream polybasic region that is required for proper membrane targeting (Hancock et al., 1990). Because of these modifications, K-Ras falls into a broad class of proteins that are also anchored into the plasma membrane via a lipid modification and a polybasic region, one of which is the myristoylated alanine-rich C kinase substrate (MARCKS). The lipid modifications are thought to insert into the lipid bilayer of the plasma membrane, and the basic residues are thought to interact with the positively charged head groups of the phospholipids (Leventis and Silvius, 1998). Previously, it was shown that MARCKS is a substrate for PKC phosphorylation, which causes MARCKS to dissociate from the plasma membrane by a process known as the "myristoyl-electrostatic switch" (McLaughlin and Aderem, 1995). Subsequently it was found that K-Ras also underwent a similar process, where it was phosphorylated and translocated from this plasma membrane, and this translocation was then termed a "farnesyl-electrostatic switch" (Bivona et al., 2006). In response to PKC-mediated phosphorylation of Ser181 in its C-terminal membrane-targeting domain, K-Ras4B translocates from the plasma membrane to the mitochondria, where it then promotes apoptosis instead of proliferation (Bivona et al., 2006). Similarly, RalA is a target of Aurora-A kinase-mediated phosphorylation at Ser194 and PP2A Aβ-mediated dephosphorylation; phosphorylation of this site depletes it from the plasma membrane (Lim KH, 2009; Sablina et al., 2007; Wu et al., 2005). Furthermore, AuroraA phosphorylation of RalA not only causes translocation from the plasma membrane, but also promotes activation of RalA and its effector RalBP1 (Lim KH, 2009 #172). Other members of the Ras superfamily of small GTPases are also phosphorylated: for example, Rap1 is

phosphorylated on Ser180 by protein kinase A (Quilliam et al., 1991). Rho family proteins whose localization and biological activity are regulated by phosphorylation include RhoA, Rnd3/RhoE and TC10. RhoA localization and modulation of cell spreading and migration is regulated by PKA-mediated phosphorylation on Ser188, which promotes its binding to RhoGDI (Forget et al., 2002; Lang et al., 1996). Rnd3/RhoE is phosphorylated after stimulation of PKCα, which results in translocation from the plasma membrane to internal membranes, and PKC-mediated phosphorylation is required for Rnd3 to modulate the Rho/ROCK pathway (Madigan, 2009 #435). Furthermore, Rnd3 is also phosphorylated by ROCK-1 on multiple sites in its N- and C-termini, which then increases the stability of Rnd3 and alters its subcellular localization (Riento et al., 2005). The mechanism by which ROCK phosphorylation of Rnd3 regulates its activity is thought to be mediated by promoting binding of Rnd3 to the αG helix of ROCK-1, which then positions the N-and C-termini of Rnd3 in close proximity to the kinase domain of ROCK-1 (Komander et al., 2008). TC10, which is closely related to Wrch-1, is phosphorylated by CDK5 on Thr197, which regulates its association with lipid rafts, a requirement for its ability to modulate insulin-stimulated GLUT4 translocation (Okada et al., 2008). Thus, there is significant evidence for the functional importance of phosphorylation of small GTPases.

1.7 Src family kinases V-Src, the first identified oncogene, is the transforming product of the Rous Sarcoma virus (Stehelin et al., 1977). Its cellular counterpart, c-Src, is part of a nine-member family of non-receptor tyrosine kinases. Generally the Src family is divided into 2 classes; those with a wide range of expression (Src, Yes, and Fyn) and those that have restricted expression (Lyn, Fgr, Hck, Lck, Blk, and Yrc). In general, Src family kinases are involved in cell adhesion assembly and turnover, motility, cell polarity, epithelial cell morphogenesis, cell proliferation and survival (Thomas and Brugge, 1997). Src family kinases have 6 functional domains: a myristoylation site (enables interaction with the plasma

membrane), a unique domain, SH2 and SH3 domains (for interactions with other proteins), a kinase domain (containing an autophosphorylation site), and a C-terminal regulatory domain. Src is activated by undergoing a conformational change from a "closed" state to an "open" state. Normally the SH2 domain of Src binds to a phospho group at Y527; when this happens, Src adopts a "closed" and inactive conformation. De-phosphorylation at Y527 relieves this auto-inhibition, and Src is then in an "open" conformation (Cooper et al., 1986; Cooper and King, 1986). After Src is in an "open" conformation, it can auto-phosphorylate on Y416 within the kinase domain, rendering the kinase fully active (Kmiecik et al., 1988; Kmiecik and Shalloway, 1987).

There are several well characterized effectors that Src utilizes to promote biological functions, the most well studied effector being FAK (focal adhesion kinase) (Lipfert et al., 1992). As the name suggests, FAK is a non-receptor tyrosine kinase that is localized to and involved in focal adhesion formation and turnover. In primary chicken embryo fibroblasts (CEFs), v-Src completely disrupts all focal adhesions over a 16-24 hour period, which results in marked cell detachment (Fincham et al., 1995). Subsequently, it was shown that v-Src induced tyrosine phosphorylation of FAK which mediated focal adhesion loss that accompanies transformation (Fincham and Frame, 1998; Fincham et al., 1995). Cas (Crk-and Src-associated substrate) is another Src effector that is involved in focal adhesion formation and turnover, most notably downstream of integrin signaling (Sakai et al., 1994). In addition to modulating focal adhesions, Src has also been implicated in regulating cell proliferation through the transcription factor STAT3; the levels of STAT3 are elevated in v-Src transformed cells, and dominant-negative STAT3 inhibits v-Src mediated transformation (Bromberg et al., 1998).

Src localizes to several compartments within the cell including the plasma membrane, peri-

nuclear region, endosomes, and focal adhesions (Kaplan et al., 1992). Most active Src is found either at the plasma membrane or at focal adhesions (Yamamoto et al., 2002). Src can traffick to and from endosomes within the cell after extracellular stimuli (Donepudi and Resh, 2008; Sandilands and Frame, 2008). Studies have shown that upon growth factor stimulation, inactive c-Src at the peri-nuclear region can translocate via recycling endosomes to the plasma membrane where it is active (Sandilands and Frame, 2008). Additionally, Src can traffick with RTKs such as EGFR, VEGFR, and FGFR.

1.8 EGFR family members Epidermal growth factor receptors (EGFRs) are members of the family of receptor tyrosine kinases (RTKs). The members of this family include EGFR/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4 (Hynes and Lane, 2005). All the members of this family have an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic protein-kinase domain. EGFR family members are well known for their roles in cell proliferation, angiogenesis, adhesion, migration, and invasion (Hynes and Lane, 2005). EGFR utilizes several effector pathways to promote biological effects, the most well characterized being the Ras/Raf/MEK/ERK pathway (Gupta and Davis, 1994; Hill et al., 1993; Sasaoka et al., 1994). Activation of the Ras/Raf/MEK/ERK pathway by EGFR results in activation of c-myc and c-jun transcription factors, which leads to increased transcription of genes involved in cell proliferation and survival (Hill et al., 1993). EGFR also activates PI3K (phosphoinositide 3-kinase), a lipid kinase that phosphorylates the 3' position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns) (Laffargue et al., 1999). PI3K drives several biological outputs including cell growth, proliferation, survival, differentiation, and motility (Kurosu et al., 1997; Roche et al., 1998).

Ligand binding to the EGFR family of receptors induces the formation of different homo- and heterodimers, which in turn activates the kinase domain (Ullrich and Schlessinger, 1990).

The kinase domain then autophosphorylates the receptor, creating binding sites for SH2 domains in other proteins, subsequently leading to the activation of various intracellular signaling pathways (Ullrich and Schlessinger, 1990). The duration and intensity of intracellular signaling initiated by EGFR activation is tightly regulated in cells. For example, protein phosphatases can interfere with the amplitude and duration of EGFR signaling responses (Samuels et al., 1993). Additionally, signaling initiated by there receptors is attenuated by internalization and subsequent degradation of these receptors. Following ligand binding, EGFRs are rapidly internalized via multiple different pathways (Dikic and Giordano, 2003). After internalization, these receptors are initially delivered to early endosomes, which then mature into late endosomes and multi-vesicular bodies. In the multi-vesicular bodies, the receptors are either recycled back to the plasma membrane, or directed to the lysosomes for destruction (Lai et al., 1989a; Lai et al., 1989b; Vieira et al., 1996).

There are two main pathways that EGFR utilizes for receptor internalization following ligand stimulation; ubiquitin-dependent and ubiquitin-independent pathways (Dikic, 2003; Dikic and Giordano, 2003). It widely accepted that the Cbl family of E3 ligases is a major contributor to ubiquitin-dependent EGFR endocytosis. E3 ligases are a class of proteins that, in conjunction with E1 and E2 ubiquitin conjugating ligases, allow attachment of a ubiquitin molecule onto a lysine in a target protein. These ubiquitin groups can be added as a single monomer, or in poly-ubiquitin chains. Typically mono-ubiquitination acts as a signal for intracellular trafficking, and poly-ubiquitin signals for destruction by the proteosome (Pickart and Eddins, 2004). As previously stated, after EGF stimulation, EGFR family members form dimers, and subsequently become autophosphorylated. These autophosphorylation sites serve as docking sites for the Cbl family of E3 ligases (Y1045 in EGFR) (Schmidt and Dikic, 2005). Upon binding to EGFR, Cbl ubiquitinates the receptor. After ubiquitination of

EGFR, the receptor trafficks to endosomes, where it can then either be sorted to lysosomes for destruction, or to recycling endosomes for recycling back to the plasma membrane (Dikic, 2003).

Cbl can also mediate EGFR internalization through a ubiquitin-independent pathway, through its association with CIN85 (Petrelli et al., 2002). The SH3 domain of Cbl binds to a PxxP-motif in CIN85 (where x can be any amino acid). Proline-rich motifs in CIN85 mediate its association with SH3 domains of endophilins, a family of proteins that are able to regulate curvature of the plasma membrane during the early steps of endocytosis (Schmidt et al., 1999). EGFR can utilize interaction with the Cbl-CIN85-endophilin complex, to traffick to endosomal compartments. Inhibition of the Cbl-CIN85-endophilin complex was sufficient to block EGFR endocytosis, independent of the E3 ligase activity of Cbl (Petrelli et al., 2002).

1.9 Rho GTPases in cancer The process of carcinogenesis involves self-sufficiency in growth signal, insensitivity to anti-growth signals, the evasion of apoptosis, sustained angiogenesis, limitless replicative potential and eventually the initiation of migration and invasion into nearby and distant sites (Hanahan and Weinberg, 2000). Rho GTPases are known to regulate several of these processes, which suggests that, like Ras GTPases, Rho GTPases may be aberrantly regulated in human cancers. Ras proteins are mutated in 30% of all human cancer; however, there have been no identified naturally occurring mutations in Rho GTPases, with the notable exception of RhoH/TTF (Pasqualucci, 2001 #39; Preudhomme, 2000 #237). Although Rho GTPases are not mutationally activated in human cancers, there are documented examples of overexpression of the GTPase itself or of their positive regulators, or loss of negative regulators. Indeed, misregulation of Rho GTPases leads to increased proliferation, dedifferentiation, invasion and metastasis (Ellerbroek et al., 2003; Sahai and Marshall, 2002).

As previously mentioned, RhoH is the only Rho GTPase that is reported to be genetically altered in human cancers (Pasqualucci et al., 2001; Preudhomme et al., 2000). RhoH, also known as TTF (translocated three four), is expressed specifically in hematopoietic cells (Dallery et al., 1995), and plays a role in both non-Hodgkins lymphoma and multiple myeloma (Preudhomme et al., 2000). The rearrangement is caused by a t(3;4)(q27;p11-13) chromosomal gene translocation, which results in the expression of a BCL3/LAZ3 fusion protein (Dallery et al., 1995). Additionally, RhoH, similar to the Rnd proteins, lacks the conserved residue analogous to G12 and Q61 residues in other Rho proteins, and is likely to be GTPase-deficient (Li et al., 2002). Although there are no other known examples of genetic modifications of Rho proteins, several Rho GTPases have been reported to have altered expression in human cancers, including RhoA, RhoB, RhoC, Rac1, Rac1b, Rac2, Rac3, RhoG, Cdc42, RhoH/TTF, Rnd3/RhoE (Ellenbroek and Collard, 2007), and in some cases Wrch-1 (Kirikoshi and Katoh, 2002). Specifically RhoA is overexpressed in tumors of the colon, breast, and lung (Fritz et al., 1999), as well as testicular germ cells (Kamai et al., 2001) and head and neck squamous cell carcinoma (Abraham et al., 2001). RhoC overexpression is found in breast cancer, is involved in driving the phenotype of inflammatory breast cancer (van Golen et al., 2000) and has been shown to play a role in promoting metastasis of these tumors (Wu et al., 2004).

In addition to Rho GTPase overexpression, aberrant RhoGEF regulation, such as overexpression or constitutive activation, has been implicated in human cancers. For example, chromosomal rearrangements in leukemia can result in a MLL-LARG chimera that contributes to Rho family activation and tumorigenesis (Kourlas et al., 2000). Additionally, downregulation of negative regulators of Rho GTPase activity has also been documented. For example, genomic deletion or promoter methylation results in downregulation of

RhoGAPs such as DLC-1 and DLC-2 in hepatocellular and breast carcinoma (Ching et al., 2003; Goodison et al., 2005; Wong et al., 2003; Yuan et al., 2003a; Yuan et al., 2003b). Furthermore, down-regulation and overexpression of RhoGDIs has been reported in several types of human cancer, however the exact mechanism of how these contribute to tumor progression is still under investigation (Dovas and Couchman, 2005; Zhang and Zhang, 2006).

As previously stated, Wrch-1 was initially discovered as a Wnt responsive gene, whose expression (measured by mRNA) was increased in response to Wnt-1 signaling in Wnt-1 transformed cells, Wnt-1 transgene induced mouse mammary tumors, and Wnt-1 retrovirus infected cells (Tao et al., 2001). When Wrch-1 is mutationally activated (Q107L, analogous to a Ras Q61L mutation) it phenocopies Wnt-1 morphological transformation of mammary cells (Tao et al., 2001). Interestingly, Wrch-1 message levels are regulated by Wnt-1 in a β-catenin-independent manner (Tao et al., 2001). Wrch-1 has been shown to be involved in several mechanisms involved in tumorigenesis. Wrch-1 overexpression increases migration in both osteoclasts and HeLa cells (Brazier et al., 2009; Chuang et al., 2007; Ory et al., 2007). Conversely, Wrch-1 depletion led to an increase in focal adhesions, by regulating myosin light chain, and a decrease in migration (Chuang et al., 2007). Wrch-1-mediated migration is thought to be accomplished through activation of Akt and JNK because Wrch-1 is necessary for wound-healing mediated by Akt and JNK activation (Chuang et al., 2007). Wrch-1 expression reduced osteoclast adhesion onto vitronectin but not fibronectin and also increased osteoclast precursor aggregation (Brazier et al., 2009). Wrch-1 has also been shown to promote anchorage-independent growth in several cell types (Berzat et al., 2005b; Brady et al., 2009). Most recently, it was found that Wrch-1 negatively regulates the kinetics of tight junction assembly through binding to the cell polarity protein Par6 in a GTP dependent manner (Brady et al., 2009). Although Wrch-1 had no detectable effect on

overall cell polarity in a confluent monolayer, it had a dramatic impact on the cytoskeleton and multilayering in cells grown in three-dimensional culture (Brady et al., 2009). A proper level of Wrch-1 seems to be required, because overexpression of activated Wrch-1 or knockdown of Wrch-1 disrupted epithelial cell morphogenesis (Brady et al., 2009). Taken together, these data suggest that Wrch-1 is involved in carcinogenesis, however Wrch-1 levels have been shown to be both up- and down-regulated, depending on the type of cancer (Kirikoshi and Katoh, 2002). Further studies need to be done to address the mechanism of Wrch-1 regulation, and how this regulation contributes to Wrch-1 mediated biological functions. In this dissertation I have examined the role of C-terminal tyrosine phosphorylation of Wrch-1 in regulating both the localization and biological activity of Wrch-1.

1.10 Evaluating the role of C-terminal tyrosine phosphorylation in regulating the localization and function of Wrch-1. As stated previously, Wrch-1 expression is regulated by Wnt-1 signaling (Taneyhill and Pennica, 2004; Tao et al., 2001), and by RANKL stimulation in osteoclasts (Brazier et al., 2009; Brazier et al., 2006; Ory et al., 2007). Although Rho GTPases are regulated by GEFs and GAPs, currently there are no identified GEFs, GAPs, or GDIs known to interact with and regulate Wrch-1. *In vitro,* Wrch-1 has a very high rate of GDP/GTP exchange, suggesting that Wrch-1 may be constitutively GTP-bound in cells (Shutes et al., 2006). However, in biological assays, the GTPase-insensitive mutant of Wrch-1 (Q107L) shows higher activity than wild-type Wrch-1 (Berzat et al., 2005b; Brady et al., 2009; Brazier et al., 2009; Chuang et al., 2007; Ory et al., 2007; Ruusala and Aspenstrom, 2008; Saras et al., 2004), suggesting that there is a GAP that inactivates Wrch-1 which has yet to be identified. Additionally, Wrch-1 is palmitoylated instead of prenylated (Berzat et al., 2005b), and does not interact with RhoGDI-1 (Berzat et al., 2006). While most Rho proteins utilize a "second signal" for proper membrane localization, currently,

there is no known "second signal" for Wrch-1. However, while in search for a "second signal" for Wrch-1 membrane targeting, Anastacia Berzat, a former lab member, found that Wrch-1 was tyrosine phosphorylated in response to serum stimulation. In this dissertation, I have investigated the mechanisms controlling both tyrosine phosphorylation of Wrch-1, and how this phosphorylation event regulates the localization, trafficking and biological functions of Wrch-1. Chapter II is dedicated to defining the residue of Wrch-1 that is tyrosine phosphorylated in response to serum and the role of Src in mediating C-terminal phosphorylation of Wrch-1. Additionally Chapter II focuses on the mechanism by which this C-terminal tyrosine phosphorylation at Y254 regulates the localization and biological functions of Wrch-1 and uncovers a putative endosomally restricted GAP that downregulates Wrch-1 activity. Chapter III focuses on defining the role of EGFR in regulating C-terminal phosphorylation at Y254. Chapter III also focuses on how phosphorylation at Y254 regulates the trafficking of Wrch-1, and how it alters protein complex formation with EGFR, Src, Cbl, etc. Chapter IV summarizes the findings of this dissertation and suggests further studies on how both tyrosine phosphorylation and other posttranslational modifications play a role in regulating the localization and function of Wrch-1. These findings and potential future directions will contribute to a better understanding of how posttranslational modifications, especially tyrosine phosphorylation, can play a critical role in regulating small GTPases.

CHAPTER II

REGULATION OF THE RHO FAMILY SMALL GTPASE WRCH-1 BY SRC-MEDIATED TYROSINE PHOSPHORYLATION OF ITS C-TERMINAL MEMBRANE TARGETING REGION

2.1 Abstract

Wrch-1 is an atypical Rho family small GTPase with roles in migration, epithelial cell morphogenesis, osteoclastogenesis and oncogenic transformation. Here, we observed rapid relocalization of Wrch-1 from plasma membrane to endosomes upon serum stimulation. Exploration of the basis for this shift revealed a requirement for serum-stimulated tyrosine phosphorylation of Wrch-1 at residue Y254 within its C-terminal membrane targeting domain, mediated by the non-receptor tyrosine kinase Src. Genetic or pharmacological loss of Src kinase activity blocked both phosphorylation and relocalization of Wrch-1. Functionally, Y254 was required for proper Wrch-1 modulation of cystogenesis in three-dimensional culture, and the phosphodeficient mutant, Y254F, was enhanced in Wrch-1-mediated anchorage-independent growth. Mechanistically, C-terminal tyrosine phosphorylation and subsequent relocalization of Wrch-1 downregulated its ability to interact with and activate its effectors by decreasing active, Wrch-1-GTP, presumably by enhancing proximity to a GAP. Phosphodeficient Wrch-1(Y254F) remained trapped at the plasma membrane and GTP-bound, and continued to recruit and activate its effector PAK even upon serum stimulation. In contrast, a phosphomimetic mutant, Y254E, was constitutively endosomally localized and GDP-bound, and failed to recruit PAK unless mutated to become

GAP-insensitive. C-terminal tyrosine phosphorylation thus represents a new paradigm in posttranslational control of small GTPase localization, activation and biological function.

2.2 Introduction

Rho family proteins are Ras-related small GTPases that regulate cytoskeletal organization and dynamics, cell adhesion, motility, trafficking, proliferation and survival (Jaffe and Hall, 2005). Rho GTPases function as tightly regulated molecular switches, cycling between the active GTP-bound state and the inactive GDP-bound state with assistance from positive regulatory GEF proteins and negative regulatory GAPs. Rho GTPases are also regulated by their subcellular localization, which is directed by sequences and post-translational modifications in their C-terminal hypervariable membrane targeting regions. Most Rho GTPases are post-translationally modified by an isoprenoid lipid attached permanently to their C-terminal CAAX motifs (Adamson et al., 1992), and their correct localization requires a second signal which usually consists of a polybasic region or a palmitate fatty acid upstream of the CAAX motif (Lebowitz et al., 1997; Michaelson et al., 2001; Williams, 2003; Ziman et al., 1993). In addition, Rho GTPases typically interact with Rho-guanine nucleotide dissociation inhibitors (RhoGDIs), that bind their prenyl groups and sequester them from membranes (Isomura et al., 1991; Takai et al., 1995). Interaction of the GTP-bound proteins with their downstream effectors at specific locations then elicits their biological functions.

Wrch-1, also designated RhoU and Wrch1, is an atypical member of the Cdc42 subgroup of Rho GTPases that induce the formation of actin microspikes and filopodia. Although it shares 57% sequence identity with Cdc42 and 61% sequence identity with its closest relative, Chp/Wrch-2, Wrch-1 shares only partially overlapping localization and effector interactions with these proteins, and its activity is regulated in a distinct manner. Like Cdc42, Wrch-1 activity leads to activation of PAK1 and JNK (Chuang et al., 2007; Tao et al.,

2001), formation of filopodia (Ruusala and Aspenstrom, 2008; Saras et al., 2004) and both morphological (Brady et al., 2009) and growth transformation in multiple cell types (Berzat et al., 2005b; Brady et al., 2009). In addition, Wrch-1 also regulates focal adhesion turnover (Chuang et al., 2007; Ory et al., 2007), negatively regulates the kinetics of tight junction formation (Brady et al., 2009), plays a required role in epithelial morphogenesis (Brady et al., 2009), and modulates osteoclastogenesis (Brazier et al., 2009; Brazier et al., 2006; Ory et al., 2007). Initially discovered as a Wnt-responsive gene capable of phenocopying Wnt morphological transformation in mouse mammary epithelial cells when mutationally activated (Taneyhill and Pennica, 2004; Tao et al., 2001), Wrch-1 is transcriptionally upregulated in MEFs by Wnt in a JNK-dependent manner (Schiavone et al., 2009) and in bone marrow macrophages during osteoclastogenesis by RANKL stimulation (Brazier et al., 2006). Recently its expression has also been shown to be responsive to gp130-class cytokines, which transcriptionally regulate Wrch-1 expression via STAT3 (Schiavone et al., 2009). Finally, Wrch-1 expression has been shown to be upregulated in some cancers but downregulated in others (Kirikoshi and Katoh, 2002). Thus, modulation of Wrch-1 activity at the level of expression is a common event. However, because it is a GTP-binding protein, a more dynamic regulation of Wrch-1 activity is also required.

Wrch-1 is thought to be largely GTP-bound due to a high intrinsic exchange rate (Aspenstrom et al., 2007; Shutes et al., 2004), and no GEFs or GAPs have yet been identified. However, mutationally activated (Q107L, analogous to Q61L in Ras or Cdc42) Wrch-1 is more active than wild type Wrch-1 (Berzat et al., 2005b; Brady et al., 2009; Brazier et al., 2009; Ory et al., 2007; Tao et al., 2001), indicating that at least one or more GAPs remain to be identified. In addition, Wrch-1 contains a 46-amino acid N-terminal extension that we and others have shown confers negative regulation to Wrch-1 activity (Shutes et al., 2004). This extension contains proline-rich SH3-binding PxxP motifs that

promote interaction of Wrch-1 with Grb2, Nckβ and PLCγ1 (Saras et al., 2004; Shutes et al., 2004), and it is thought that Grb2 binding helps to relieve the inhibition of the N-terminus towards Wrch-1 activity (Shutes et al., 2004).

In addition to regulation of its activity at the level of expression as well as by at least one putative GAP and relief of its autoinhibitory domain, Wrch-1 function requires post-translational lipid modification of its C-terminal membrane targeting domain. Unlike Cdc42, Wrch-1 does not terminate in a CAAX motif that is prenylated, but instead terminates in a CFV motif that is palmitoylated (Berzat et al., 2005b). Further, lacking a prenyl group, Wrch-1 does not bind RhoGDI (Berzat et al., 2005a). We have shown previously (Berzat et al., 2005b; Brady et al., 2009) that palmitoylation, a dynamically regulated lipid modification (Mumby et al., 1994), is required for both the subcellular localization and biological activities of Wrch-1. However, whereas both prenylation and the polybasic region of Cdc42 are required for its proper localization and function (Williams, 2003), the identity of additional signals governing Wrch-1 is unknown.

There is increasing evidence that C-terminal serine/threonine phosphorylation of small GTPases near the isoprenoid moiety is required for both their localization and specific functions. In response to PKC-mediated phosphorylation of Ser181 in its C-terminal membrane targeting domain, K-Ras4B translocates from the plasma membrane to the mitochondria, where it then promotes apoptosis instead of proliferation (Bivona et al., 2006). RalA is a target of Aurora-A kinase-mediated phosphorylation at Ser194 (Wu et al., 2005) and PP2A Aβ-mediated dephosphorylation (Blake et al., 2000); phosphorylation of this site depletes it from the plasma membrane (Lim KH, 2009). Rap1 is phosphorylated on Ser180 by protein kinase A (Lerosey et al., 1991; Quilliam et al., 1991) and RhoA localization and modulation of cell spreading and migration is regulated by PKA-mediated phosphorylation

on Ser188 (Ellerbroek et al., 2003; Lang et al., 1996), which promotes its binding to RhoGDI (Ellerbroek et al., 2003). PKCα (Riento, 2005 #149; Madigan, 2009 #435) and ROCK (Riento et al., 2005) stimulate phosphorylation of Rnd3/RhoE that results in translocation from the plasma membrane to internal membranes, and PKC-mediated phosphorylation is required for Rnd3 to modulate the Rho/ROCK pathway (Madigan, 2009 #435). TC10 is phosphorylated by CDK5 on Thr197, which regulates its association with lipid rafts, a requirement for its ability to modulate insulin-stimulated GLUT4 translocation (Okada et al., 2008). Thus, there is significant evidence for the functional importance of C-terminal serine/threonine modification of small GTPases.

In the present study, we sought to determine which C-terminal elements in addition to palmitoylation contribute to the regulation of Wrch-1 subcellular localization and biological activity. Unlike the several small GTPases shown to be substrates for C-terminal Ser/Thr phosphorylation, the minimal C-terminal membrane targeting sequence of Wrch-1 does not contain a suitable serine or threonine residue. However, it does contain a potentially phosphorylatable tyrosine residue. Further, endosomal localization is often dynamically regulated by external signals such as those supplied by serum factors that stimulate engagement of growth factor receptor tyrosine kinases and their associated nonreceptor tyrosine kinase partners (Sorkin and Von Zastrow, 2002). In this report, we describe our discovery that serum stimulates Src-mediated tyrosine phosphorylation of Wrch-1 and its translocation from plasma membrane to endosomes, and that a specific tyrosine residue in the C-terminal membrane targeting domain of Wrch-1 regulates its subcellular localization, GTP/GDP-binding status, effector activation and biological activities. Thus, we have identified C-terminal tyrosine phosphorylation as a novel mechanism for regulation of small GTPase activity.

2.3 Results

2.3.1 Wrch-1 rapidly relocalizes from the plasma membrane to endosomal membranes in response to serum stimulation. We have shown previously that the atypical Rho family small GTPase, Wrch-1, localizes to both plasma membrane and internal compartments, including endosomal membranes (Berzat et al., 2005b). Because endosomal localization of other proteins, including some Rho GTPases, can be directed by external stimuli such as serum-stimulated engagement of growth factor receptors, and is therefore dynamically regulated, we sought to determine whether Wrch-1 targeting to endosomes was similarly regulated. To that end, we serum-starved overnight H1299 nonsmall cell lung cancer (NSCLC) cells transiently expressing EGFP-tagged Wrch-1 (designated GFP-Wrch-1) and then stimulated them with serum for 15 min. Whereas Wrch-1 was localized to both the plasma membrane and endosomal membranes in serum-starved cells, it underwent rapid relocalization from the plasma membrane to internal membranes upon serum stimulation (Figure 2.1). Increased endosomal localization of Wrch-1 was confirmed using transferrin, a marker for endocytic membranes (Figure 2.1). These data indicate that Wrch-1 subcellular localization to plasma membrane and endosomes is both dynamic and regulated by upstream signals.

2.3.2 Relocalization is dependent on the presence of a tyrosine at position 254 in the Wrch-1 C-terminal membrane targeting domain. We had previously determined that the carboxy-terminal nine amino acids of Wrch-1 are sufficient for its proper subcellular localization (Berzat et al., 2005b), and that mutation of the palmitoylated cysteine residues therein results in cytosolic accumulation (Berzat et al., 2005b). Within this sequence of WWKKYCCFV, the single tyrosine at residue 254 (Y254) stood out as a potential site of regulation in response to serum. Further, because subcellular localization dictates the

function of small GTPases, we hypothesized that Y254 becomes tyrosine phosphorylated in response to serum stimulation and that this phosphoryation event modulates Wrch-1 subcellular localization and activity.

To examine whether Y254 is required for the serum-stimulated re-localization of Wrch-1, we generated a Y>F mutation at position 254 [designated Wrch-1(Y254F)]. We then transfected H1299 cells with GFP-Wrch-1 or GFP-Wrch-1(Y254F) and compared the localization of the two proteins under basal conditions to its localization both in the absence of serum and after serum stimulation. We observed that the putatively phosphodeficient Wrch-1(Y254F) was preferentially localized to the plasma membrane compared to wild type Wrch-1 and was resistant to serum-stimulated relocalization: rather than being relocalized to endosomal compartments, Wrch-1(Y254F) remained trapped on the plasma membrane upon serum stimulation (Figure 2.2A). Further, cells expressing this nonphosphorylatable mutant displayed an exaggeratedly rounded phenotype under basal serum-containing conditions, which was similar to that seen in serum-starved cells expressing only WT Wrch-1 (Figure 2.2A).

These results were consistent with our hypothesis that Wrch-1 becomes tyrosine phosphorylated on Y254 in response to serum stimulation, and that this phosphorylation event is responsible for serum-stimulated relocalization of Wrch-1. In order to confirm that Wrch-1 was tyrosine phosphorylated at Y254, we first transfected HA-tagged Wrch-1 and Wrch-1(Y254F) into H1299 cells, and then immunoprecipitated Wrch-1 with an anti-HA antibody, followed by western blotting using an anti-phosphotyrosine antibody to probe for phosphotyrosine on the immunoprecipitated Wrch-1. We observed that Wrch-1 but not Wrch-1(Y254F) was detected by anti-phosphotyrosine following serum stimulation (Figure

2.2B), confirming that Wrch-1 is tyrosine phosphorylated and suggesting that Y254 is the major site of serum-stimulated phosphorylation.

Interestingly, we also determined that basal subcellular localization of Wrch-1 requires less targeting information than does either tyrosine phosphorylation or serum-stimulated relocalization. We had determined previously that the last 9 amino acids in the C-terminus of Wrch-1 are sufficient for proper basal localization of Wrch-1 (Berzat et al., 2005b). Surprisingly, we found that this short sequence [designated “9 aa tail”] (Figure 2.3A) was not sufficient to support either tyrosine phosphorylation (Figure 2.3B) or serum-stimulated relocalization (Figure 2.3C). We speculated that 9 amino acids was an insufficient length to allow binding of the kinase. Therefore we extended it, generating an additional GFP-fusion protein comprising 19 amino acids of the Wrch-1 C-terminus [designated “19 aa tail”, which still contains only a single tyrosine residue, see Figure 2.3A], and performed the same experiments as before. We found that 19 residues of the Wrch-1 C-terminus was a length sufficient both for tyrosine phosphorylation (Figure 2.3B) and for relocalization in response to serum stimulation (Figure 2.3C). A longer tail (39 aa) was indistinguishable from the 19 amino acid tail and from full length Wrch-1 (data not shown). Together, these data indicate that relocalization of Wrch-1 requires additional sequences compared to basal localization, perhaps to allow efficient kinase binding to the region encompassing Y254.

2.3.3 Src can phosphorylate Wrch-1, and Src tyrosine kinase activity is required for both tyrosine phosphorylation and serum-stimulated relocalization of Wrch-1. The nonreceptor tyrosine kinase Src transmits signaling from several serum-responsive growth factor receptor tyrosine kinases. Further, Src has many substrates, not all of which have been defined clearly. We speculated that Src could phosphorylate Wrch-1 on Y254, and indeed we found that the Src family kinase inhibitor SU6656 effectively blocked tyrosine

phosphorylation of Wrch-1 (Figure 2.4A). At the concentration that we used (5 μM), SU6656 is reported to inhibit tyrosine kinases Src, Fyn, Yes, and Lyn (Bain et al., 2007). To test whether Src alone was sufficient to phosphorylate Wrch-1, we expressed HA-Wrch-1 either in MEFs that are genetically deficient in Src, Yes, and Fyn (SYF-/-) or in MEFs that retain Src but are deficient in Yes and Fyn (YF-/-). We found that Wrch-1 was tyrosine phosphorylated in YF-/- MEFs but not in SYF-/- MEFs, indicating that Src is required for tyrosine phosphorylation of Wrch-1 (Figure 2.4B). These data indicate that Src functions upstream of Wrch-1 to mediate its tyrosine phosphorylation, but do not indicate whether Src kinase enzymatic activity is required. To address this question, we co-transfected HA-Wrch-1 or HA-Wrch-1(Y254F) along with either constitutively active or kinase-deficient Src and then measured the levels of phosphotyrosine Wrch-1 by IP/western blot. We found that Wrch-1 was tyrosine phosphorylated in the presence of kinase-active Src(Y528F) but not kinase-deficient Src(K297R), and that the Y254 residue of Wrch-1 was required for this phosphorylation, as Wrch-1(Y254F) was not phosphorylated regardless of Src kinase activity (Figure 2.4C). These data support a requirement for Src kinase activity in order for Wrch-1 to become tyrosine phosphorylated on Y254, but do not address whether the phosphorylation is direct or indirect. To answer this question, we performed an *in vitro* kinase assay with recombinant purified Src and GST-Wrch-1. We found that Wrch-1 was directly phosphorylated by Src *in vitro* (Figure 2.4D), consistent with the possibility that it may be phosphorylated directly *in vivo*.

Having established that Wrch-1 could be a substrate of Src kinase activity, and that the Y254 residue of Wrch-1 that is required for serum-stimulated relocalization is also the major site of Src-stimulated phosphorylation, we wished to test whether Src kinase activity is required for serum-stimulated relocalization of Wrch-1. To do this, we serum-starved cells expressing GFP-Wrch-1, treated them with SU6656 (5 μM) for 1 h, then serum-stimulated

them as above. We found that SU6656 prevented Wrch-1 relocalization in response to serum stimulation (Figure 2.4E). Collectively, these results indicate that Src tyrosine kinase activity is required for both the tyrosine phosphorylation and relocalization of Wrch-1.

2.3.4 Phosphorylatable residue Y254 regulates Wrch-1-mediated transformation. Correct subcellular localization is critical to regulate the biological functions of Rho GTPases (Etienne-Manneville and Hall, 2002). Therefore, we hypothesized that tyrosine phosphorylation, which alters the subcellular localization of Wrch-1, also likely alters at least some of its biological functions. We have shown previously that both wild type and constitutively active Wrch-1(107L) are capable of inducing anchorage-independent growth transformation such as colony formation in soft agar (Berzat et al., 2005b; Brady et al., 2009). In addition, we have shown that endogenous Wrch-1 is required for correct epithelial cell morphogenesis (Brady et al., 2009) and that a nonpalmitoylated mutant of Wrch-1 that fails to localize correctly to the plasma membrane is unable to modulate either of these biological functions (Berzat et al., 2005b; Brady et al., 2009). Therefore, we sought to assess the role of the tyrosine phosphorylatable residue Y254 in regulating Wrch-1-mediated anchorage-independent growth and epithelial morphogenesis. To do so, we first generated polarizable MDCKII epithelial cell lines stably expressing either HA-Wrch-1 or phosphodeficient HA-Wrch-1(Y254F) in both the WT and 107L backgrounds, and confirmed that they expressed equivalent amounts of each protein (Figure 2.5A, bottom panel). We then seeded these cells into soft agar and allowed them to grow in an anchorage-independent manner for 14 days, after which colony formation was quantitated. We predicted that the phosphodeficient Y254F mutant, which we have shown here traps Wrch-1 at the plasma membrane and prevents relocalization to endosomal compartments, would display enhanced transforming ability. In agreement with this prediction, we found that cells expressing either of the Y254F mutants formed a significantly increased number of large

colonies (>15 cells in diameter) in soft agar when compared to cells expressing parental phosphorylatable Wrch-1 proteins (Figure 2.5A). These results indicate that tyrosine phosphorylation of Y254 would represent a negative regulatory control of Wrch-1 biological activity.

2.3.5 Phosphorylatable residue Y254 regulates Wrch-1-mediated epithelial morphogenesis. Several Rho family small GTPases are required for cystogenesis in a 3-dimensional environment (Bryant and Mostov, 2008). In particular, Cdc42 is thought to be a master regulator of lumen development in tube and cyst structures, by virtue of its ability to regulate the Par6/atypical PKC polarity complex (Bryant and Mostov, 2008). Wrch-1 also binds Par6/aPKC, and we have demonstrated previously that a precise balance of endogenous Wrch-1 is also required for proper cystogenesis in a 3-dimensional environment: either too little or too much Wrch-1 causes disruption of the formation of normal, single lumen-containing, hollow cyst structures (Brady et al., 2009), and Cdc42 is unable to compensate for the loss of Wrch-1. Therefore we wished to examine the effect of Wrch-1 tyrosine phosphorylation on this crucial Wrch-1-mediated function. To this end, we performed cystogenesis assays and evaluated the formation of hollow cysts of MDCKII cells in 3-dimensional culture. MDCKII cells expressing phosphorylatable or phosphodeficient [Y254F] Wrch-1 were seeded in a matrix of collagen I. Cysts were allowed to form over a period of 10 days, after which they were stained for Texas Red-phalloidin to image the cyst structures. If phosphorylation at Y254 is a negative regulator of Wrch-1 activity, as suggested by the increased numbers of colonies formed in soft agar by cells expressing the phosphodeficient mutant Y254F, then we would expect a greater degree of disruption of cystogenesis by forms of Wrch-1 that are unable to become phosphorylated at Y254. In agreement with this hypothesis, we found that constitutively phosphodeficient Wrch-1(Y254F) disrupted epithelial cell morphogenesis, as shown by a greatly decreased

proportion of normal cysts containing a single lumen (Figure 2.5B), and this disruption was greater compared to the disruption induced by phosphorylatable Wrch-1, whether in a WT or in a constitutively GTP-bound and active (107L) background (Figure 5B). Together, these data indicate a requirement for the Y254 residue for proper function of at least two important biological activities of Wrch-1, and suggest that tyrosine phosphorylation at this site may be an important mechanism to precisely control Wrch-1 activity.

2.3.6 Wrch-1 interacts with its effector PAK at the plasma membrane, where it is GTP-bound and active, but not at endosomes, where it is GDP-bound and inactive. The lower biological activity displayed by wild type Wrch-1 compared to the phosphodeficient Wrch-1 mutant Y254F that was resistant to endosomal relocalization suggested that Wrch-1 is less able to interact with its effectors when it is endosomally localized than when it is localized to the plasma membrane. We therefore evaluated the ability of Wrch-1 to interact with its best-validated effector PAK at each of these locations, by utilizing a GFP-tagged form of the GTPase-binding domain of PAK ("GFP-PAK-PBD"). If our hypothesis was correct, then the same conditions of serum stimulation that cause Wrch-1 to become phosphorylated at Y254 and to relocalize away from the plasma membrane should also decrease its interactions with PAK-PBD. To test this, we cotransfected H1299 cells with GFP-PAK-PBD and HA-tagged Wrch-1 and observed their localization under conditions of serum starvation or stimulation. In serum-starved cells, Wrch-1 localized to both plasma membrane and endosomes, as expected (Figure 2.6A, red), and PAK-PBD (green) displayed overlapping localization with Wrch-1 (merge, yellow) at the plasma membrane. Upon serum stimulation, Wrch-1 redistributed away from the plasma membrane in a time-dependent manner, such that by 15 min it was absent from the plasma membrane and localized predominantly to endosomes. Under these conditions, PAK-PBD was distributed diffusely throughout the cell, and was recruited neither to plasma membrane nor to internal

membranes, consistent with our hypothesis that tyrosine phosphorylation and relocalization of Wrch-1 decreases its ability to interact with effectors.

Because PAK-PBD binds preferentially to the active, GTP-bound form of Wrch-1, these results indicate that Wrch-1 is active at the plasma membrane but not at internal membranes. To confirm that Wrch-1 would still be able to bind PAK-PBD at internal membranes if it were active and GTP-bound, we examined the ability of a constitutively activated, GAP-insensitive mutant of Wrch-1(Q107L) to recruit PAK-PBD. As expected, the 107L mutant still relocalized to endosomes upon serum stimulation, and still recruited PAK-PBD even to internal membranes (Figure 2.6A). Thus, the inability of WT Wrch-1 to recruit PAK-PBD following serum stimulation is not because PAK-PBD is simply unable to access the endosomal compartment or interact with tyrosine phosphorylated Wrch-1.

Our observation that Wrch-1 recruited PAK-PBD to the plasma membrane but not to internal membranes further supports our hypothesis that tyrosine phosphorylation at Y254 and subsequent internalization downregulates Wrch-1 activity. To further test this possibility, we examined recruitment of PAK-PBD by the nonphosphorylatable mutant Wrch-1(Y254F), which remains trapped on the plasma membrane and fails to relocalize in response to serum. Consistent with our hypothesis, the Y254F mutant continued to recruit PAK-PBD to the plasma membrane after serum stimulation even in the absence of a concurrent constitutively activating mutation 107L (Figure 2.6A). This result indicates that the failure of Y254F to become internalized is sufficient to abrogate C-terminal phosphorylation-mediated downregulation of Wrch-1 activity and to retain Wrch-1 in an active conformation.

Finally, if phosphorylation of Wrch-1 at Y254 is sufficient for relocalization to endosomes and subsequent downregulation of Wrch-1 activity and effector interaction, then a

phosphomimetic mutation should impair plasma membrane binding, and instead confer constitutive localization to endosomes and poor effector interaction. Consistent with this, we observed that a phosphomimetic Y254E mutant was indeed largely excluded from the plasma membrane, localized to endosomes, and failed to recruit PAK-PBD unless also constitutively GTP-bound by virtue of a concurrent 107L mutation. These results also corroborate the evidence suggesting that localization regulates Wrch-1 GTP-binding status, and that effector interaction of tyrosine phosphorylated Wrch-1 is impaired as a consequence of decreased GTP-binding rather than of decreased access to effector pools.

In order to confirm these results biochemically, we performed pulldown assays to detect active Wrch-1-GTP. Like GFP-tagged PAK-PBD, GST-tagged PAK-PBD also binds preferentially to active, GTP-bound Wrch-1, but subcellular localization plays no part in their interaction in the pulldown assay. We cotransfected H1299 cells with empty vector or vectors expressing HA-tagged Wrch-1 proteins, and then either serum starved or starved and stimulated the cells with serum for 2, 5 or 15 min, exactly as for the GFP-PAK-PBD localization assays. Cells were then lysed and incubated for 1 h with purified GST or GST-PAK-PBD coupled to glutathione-agarose beads. The resulting protein complex was pulled down by centrifugation, washed, eluted off the beads, resolved by SDS-PAGE, and immunoblotted for the active, GTP-bound HA-Wrch-1 that was pulled down by interaction with GST-PAK-PBD. GST alone pulled down no Wrch-1 (data not shown), nor did GST-PAK-PBD pull down anything detectable by anti-HA antibody in the absence of HA-Wrch-1 (Figure 6B, vector). As predicted by the recruitment of GFP-PAK-PBD, we found that prior to serum stimulation GST-PAK-PBD pulled down Wrch-1 quite strongly when Wrch-1 was able to localize to the plasma membrane (WT and Y254F) but pulled down almost none of the Wrch-1 expected to be excluded from the plasma membrane (Y254E) (Figure 2.6B). Also as predicted by GFP-PAK-PBD, the same conditions of serum stimulation that resulted

in tyrosine phosphorylation and subsequent loss of plasma membrane binding and relocalization to endosomes also conferred decreased interaction with GST-PAK-PBD. Thus, by 5 min of serum stimulation, GST-PAK-PBD still pulled down Y254F robustly but the amount of WT Wrch-1 pulled down was dramatically decreased (Figure 2.6B). Taken together, these results suggest that serum stimulation of phosphorylation of Wrch-1 at Y254 causes its translocation from plasma membrane to endosomes where it is inactivated, presumably through the activity of a yet-unidentified GAP.

2.3.7 Serum-stimulated tyrosine phosphorylation and relocalization of Wrch-1 decreases its activation of downstream effectors in a Y254-dependent manner. The decrease in Wrch-1/PAK effector interaction seen upon serum-stimulated tyrosine phosphorylation and subsequent relocalization of Wrch-1 to endosomes strongly implies that Wrch-1 activates this effector preferentially at the plasma membrane, and that internalization impairs effector activation. We next wished to determine whether relocalization to endosomes decreased not only effector interaction but also effector activation. However, PAK-PBD contains the GTPase binding domain but is not the full length PAK kinase effector of Wrch-1. Further, it was not yet clear from the previous experiments whether the decreased active Wrch-1-GTP affects PAK alone or other effectors as well. Active Wrch-1 has been shown to stimulate autophosphorylation and activation of both the serine/threonine kinase PAK1 (Tao et al., 2001) and the non-receptor tyrosine kinase Pyk2 (Ruusala and Aspenstrom, 2008). Therefore, we tested whether the same conditions that relocalized Wrch-1 and decreased active Wrch-1-GTP also altered the ability of these two kinases that are immediate downstream effectors of Wrch-1 to become activated. Immunoblot analysis shows robust phosphorylation at the autophosphorylation sites of both PAK (Figure 2.7A) and Pyk2 (Figure 2.7B) in the presence of Wrch-1 WT or Y254F but not Y254E prior to serum stimulation, consistent with their interactions with PAK-PBD. Indeed, levels of both

phospho-PAK1 and -Pyk2 were markedly decreased in the presence of phosphomimetic Wrch-1 (Y254E) and did not change regardless of serum stimulation. Similarly, serum decreased autophosphorylation of PAK and PYK2 in the presence of WT Wrch-1, whereas Y254F conferred resistance to the decrease (Figure 2.7). Taken together, our results indicate that serum stimulation induces tyrosine phosphorylation of the membrane targeting region of Wrch-1 and its subsequent relocalization to endosomes, where it is rendered GDP-bound and inactive, thereby decreasing effector interaction and activation. These results are consistent with a model in which C-terminal tyrosine phosphorylation downregulates Wrch-1 activity, presumably by bringing it into proximity with an endosomally localized GAP.

2.4 Discussion

We show here that the atypical Rho family small GTPase, Wrch-1, undergoes serum-stimulated Src-mediated tyrosine phosphorylation at residue Y254 in its C-terminal membrane targeting domain, a modification that dynamically alters its subcellular localization by promoting relocalization from the plasma membrane to endosomes. We also show that Y254 is the major site of phosphorylation, and that mutation of Y254 to a nonphosphorylatable phenylalanine residue (Y254F) prevents relocalization, remains GTP-bound, enhances recruitment of GTPase binding domain of the Wrch-1 effector PAK1 (PAK-PBD) and enhances Wrch-1-mediated effects on growth transformation and on epithelial morphogenesis in 3D culture. In contrast, both serum-stimulated WT Wrch-1 and phosphomimetic Y254E are restricted from the plasma membrane and are inactive, failing to recruit PAK-PBD or to activate the effector kinases PAK or Pyk2 unless mutationally activated to be GAP-insensitive (Q107L). These results indicate that C-terminal tyrosine phosphorylation of Wrch-1 may be important for downregulation of its biological activities, and provide evidence supporting a mechanism whereby a novel endosomally localized Wrch-1 GAP turns off previously active Wrch-1-GTP.

Our results demonstrate that Wrch-1 is GTP-bound and active at the plasma membrane, where it is capable of interacting with and activating downstream effectors such as PAK1 and Pyk2, but inactive at endosomal membranes, where it relocalizes following serum-stimulated, Src-dependent phosphorylation at Y254. That the GAP-insensitive mutant Q107L remains capable of effector interaction at endosomes even when targeted there by a C-terminal phosphomimetic mutation, Y254, indicates that phosphorylation-mediated relocalization to endosomes does not decrease Wrch-1 effector activation simply by removing Wrch-1 from its relevant effectors, at least some of which are still present at endosomes. For example, the well-validated Wrch-1 effector PAK is not only present at endosomes but is recruited and activated preferentially there by Chp/Wrch-2 in response to TNFalpha (Chenette et al., 2005). Instead, our results support a model in which endosomal relocalization of Wrch-1 promotes its deactivation by enhancing its interaction with a novel endosomally localized GAP The numbers of known GAPs capable of accelerating GTP hydrolysis on Rho family proteins already far exceeds the numbers of GTPases themselves, leading to speculation that the reason for the existence of multiple GAPs is to serve as location-specific downregulators of Rho family GTPase function. While GAPs for Wrch-1 are thought to exist, none has yet been identified. Endosomal GAPs such as p50RhoGAP (Sirokmany et al., 2006) and ARAP1 (Daniele et al., 2008; Yoon et al., 2008) have been identified for other Rho family GTPases but are not known to promote GTP hydrolysis on Wrch-1. Other mechanisms of endosomal translocation and subsequent deactivation are well-documented for receptor tyrosine kinases (RTKs). Upon autophosphorylation and activation following growth factor stimulation, many RTKs become ubiquitinated and undergo relocalization to the endosomal compartment, which attenuates their signaling (Dikic, 2003; Dikic and Giordano, 2003). Additionally, ubiquitination of the small GTPase H-Ras has been shown to promote its endosomal trafficking and thereby to attenuate its

signaling through the Raf-MEK-ERK pathway (Jura et al., 2006). We have observed that Wrch-1 is also ubiquitinated but that this modification does not appear to alter either its localization or its biological activity (data not shown). Thus, our present data best support the model of C-terminal phosphorylation of Wrch-1 leading to its downregulation by enhancing proximity to one or more GAPs.

Accumulating evidence suggests that phosphorylation of the C-terminal membrane targeting domains on small GTPases combines with other sequences and posttranslational modifications to dynamically regulate the localization and function of these proteins. To date, all the phosphorylation sites so identified, whether in Ras or Rho GTPases, have been Ser/Thr residues just upstream of C-terminal farnesyl or geranylgeranyl isoprenoid modifications (Bivona, 2006 #84; Lerosey, 1991 #165; Lim KH, 2009 #172; Lang, 1996 #41; Ellerbroek, 2003 #154; Riento, 2005 #149; Okada, 2008 #159; Madigan, 2009 #435). However, the atypical Rho family GTPase Wrch-1 is neither modified by isoprenylation nor possesses cognate serine or threonine residues. Instead, our study provides the first report of direct regulation of Rho family GTPase subcellular localization and function by tyrosine phosphorylation of its membrane targeting domain. Although Cdc42 has been reported to be tyrosine phosphorylated by Src upon EGF stimulation (Tu et al., 2003), this phosphorylation occurs at residue Y64 within its switch II region rather than in the Cdc42 C-terminus, which lacks any tyrosine residue. Whether tyrosine phosphorylation of Cdc42 alters its subcellular localization was not explored, but it was reported to promote binding of Cdc42 to RhoGDI. However, Wrch-1 is not modified by an isoprenoid, a feature required for binding of RhoGDI (Takai et al., 1995), and does not interact with RhoGDI (Berzat et al., 2005a). Thus, tyrosine phosphorylation of Wrch-1 and Cdc42 occur on distinct domains and have distinct consequences. Chp/Wrch-2, the closest relative of Wrch-1, is also palmitoylated and unprenylated (Chenette et al., 2005), but, like Cdc42, Chp lacks a tyrosine

residue near its C-terminus. This is perhaps not entirely surprising, as many closely related isoforms of small GTPases differ from each other mostly in their membrane targeting domains, possibly to provide signaling diversity.

Wrch-1 tyrosine phosphorylation at Y254 occurs only two residues away from the critical palmitoylation site C256, raising the question of whether one modification sterically hinders the other. It is clear that tyrosine phosphorylation of Wrch-1 does not require prior palmitoylation, as even a nonpalmitoylatable Cys>Ser mutant becomes tyrosine phosphorylated in the presence of Src (data not shown). Although it is not yet known whether phosphorylation can occur simultaneously with palmitoylation, there is some indirect evidence that this is the case: we have shown here that tyrosine phosphorylation results in an increased association with internal membranes, whereas an inability to be palmitoylated results in a complete lack of Wrch-1 membrane association (Berzat et al., 2005b). Taken together, these results suggest that tyrosine phosphorylation normally occurs on palmitoylated Wrch-1.

We have shown here that Wrch-1 tyrosine phosphorylation requires Src, and that Src can directly phosphorylate Wrch-1 *in vitro.* Although Y254, the major residue for tyrosine phosphorylation, does not occur in the context of a known Src kinase consensus site, and algorithms such as NetPhos or ScanSite do not predict its phosphorylation by Src, there is currently no reliable predictor of whether a given protein is in fact a substrate for Src *in vivo*. Even enolase, a commonly used positive control for Src phosphorylation, does not contain a known Src consensus site. However, many tyrosine kinases require phosphorylation themselves in order to be active, and this activating or priming phosphorylation step may be accomplished *in cis* or *in trans*. Therefore, other than direct phosphorylation of Wrch-1 by Src, another possibility is that Src activity promotes binding to Wrch-1 and/or activation of

another tyrosine kinase that can phosphorylate it. Whether Wrch-1 is a direct or an indirect downstream target of Src-mediated phosphorylation in cells remains to be determined.

Recent studies have suggested several context-dependent functional connections between Wrch-1 and Src that are likely to be pertinent regardless of whether the connection is direct or indirect. In osteoclasts, Wrch-1 colocalizes with Src in podosomes, and increased Wrch-1 activity perturbs the podosome belt (Ory et al., 2007). Thus, Src-mediated tyrosine phosphorylation of Wrch-1 could contribute to the dynamic regulation of podosome formation and assembly. In addition, Wrch-1 negatively regulates M-CSF-stimulated osteoclast migration (Brazier et al., 2009), and Src has recently been shown to be activated in osteoclasts downstream of M-CSF stimulation (Yano et al., 2008). Therefore, M-CSF may promote osteoclast migration by activating Src, to then downregulate Wrch-1 through tyrosine phosphorylation on Y254. In contrast to osteoclasts, where it decreases migration, Wrch-1 increases the migration of fibroblasts (Chuang et al., 2007), where it is localized to focal adhesions (Ory et al., 2007) and regulates their assembly. Src and several of its substrates are major components of focal adhesions. In PAE cells, Wrch-1 but not Cdc42 requires Src to induce filopodia formation (Ruusala and Aspenstrom, 2008), but it is unknown whether Src is required for an effect on Wrch-1 itself or on a downstream target not shared with Cdc42.

We have observed that Y254, the major site of Src-mediated tyrosine phosphorylation, negatively regulates Wrch-1-mediated anchorage-independent growth and epithelial cell morphogenesis, because mutation to the nonphosphorylatable Y254F conferred greater activity on Wrch-1 than the wild type tyrosine residue. Thus, Src-mediated tyrosine phosphorylation at Y254 may normally act as a brake for Wrch-1 function. Although Src is often thought of simply as an oncogene that leads inexorably to cellular de-differentiation, it

is clear that it can exert dual functions. For example, Src serves dual functions during epithelial cell morphogenesis in *Drosophila*, where it both antagonizes E-cadherin-mediated cell adhesion and simultaneously stimulates E-cadherin transcription (Shindo et al., 2008). Similarly, if Src modulates Wrch-1 through opposing functions, then proper cycling between the phosphorylated and the unphosphorylated state of Wrch-1 is likely to be required for the correct final outcome, regardless of whether Wrch-1 is a direct or indirect target of Src kinase activity. It will certainly also be of interest to determine which phosphatase(s) contribute to restoration of the unphosphorylated state of Wrch-1.

Our observations identify important contributors to Wrch-1 regulation and lend further credence to the emerging paradigm that C-terminal phosphorylation of small GTPases may serve as key mechanism to dynamically regulate their localization, activation and function. Thus, further investigations into such phosphorylation events will be critical for a better understanding of the regulation of Rho GTPases.

2.5 Materials and methods

2.5.1 Molecular constructs

Mammalian expression constructs for GFP-tagged and HA epitope-tagged human Wrch-1 (WT and Q107L) have been described previously (Berzat et al., 2005b). Phosphodeficient Wrch-1(Y254F) and phosphomimetic Wrch-1(Y254E) were generated by site-directed mutagenesis in both WT and Q107L backgrounds. The GFP-fusion protein of the C-terminal 9 amino acids ("9 aa tail") of Wrch-1 expressed from pEGFP has been described previously (Berzat et al., 2005b). An additional GFP-fusion containing the C-terminal 19 amino acids ("19 aa tail") was generated by site-directed mutagenesis in the same manner. All sequences were verified by the Genome Analysis Facility at UNC-CH. GFP-PAK-PBD and

GST-PAK-PBD were kind gifts from Channing Der (UNC-CH) and Keith Burridge (UNC-CH), respectively. WT Src, constitutively active Src(Y528F) and kinase-deficient Src(K297R) were expressed from the pUSE vector, all from Upstate Biotechnology. Bacterial expression constructs and purification of GST-Wrch-1 protein have been described previously (Shutes et al., 2006).

2.5.2 Cell culture, transfections, and retroviral infection

H1299 nonsmall cell lung cancer (NSCLC) cells were grown in Dulbecco's modified Eagle medium (high glucose) (DMEM-H, GIBCO/Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin-streptomycin (P/S) ("complete culture medium"), and maintained in 5% CO_2 at 37°C. H1299 cells were transfected with expression constructs encoding Wrch-1, Src, or PAK-PBD proteins by using TransIT-LT1 (Mirus) according to the manufacturer's instructions. For localization assays, cells were transfected transiently and used 24 h after transfection. For selection of stably expressing cell lines, cells were grown in complete medium supplemented with the appropriate antibiotic for 5-7 days, after which >50 colonies were pooled for use.

MDCKII cells, generously provided by Robert Nicholas (UNC-CH), were grown as above and supplemented with 1% non-essential amino acids (NEAA, Invitrogen) ("complete medium"). MDCKII cell lines stably expressing Wrch-1 were generated by retroviral infection. Retrovirus was collected following $CaCl_2$-mediated transfection of pBabe-HAII-puro, pVPack-Gag/Pol, and pVPack-Ampho (Stratagene) expression vectors into 293T cells. Cells were infected by exposure to retroviral supernant containing 8 µg/ml of Polybrene (American Bioanalytical) and maintained in puromycin for 10 days, after which the colonies were pooled for use.

SYF mouse embryo fibroblast cells (MEFs) genetically lacking Src, Yes and Fyn, or YF cells lacking Yes and Fyn but retaining Src (Klinghoffer et al., 1999), were grown in complete culture medium as above. Cells were transiently transfected with constructs encoding HA-Wrch-1 proteins by using TransIT-LT1 (Mirus) according to the manufacturer's instructions..

2.5.3 Fluorescence, immunofluorescence, confocal microscopy and localization assays

Cells were transfected transiently with pEGFP-Wrch-1 expression vectors or pEGFP-PAK-PBD as indicated above and grown in complete medium for 24 h. The cells were then either grown further overnight in complete medium ("basal conditions"), or serum-starved overnight ("serum-starved"), or serum-starved overnight then stimulated for 2, 5, or 15 min. with fresh serum-containing complete medium ("starved + stimulated"). For some experiments, cells were treated for 1 h with the Src family kinase inhibitor, SU6656 (Sigma), or DMSO vehicle prior to serum stimulation. Following incubation with Alexa 647-transferrin (Molecular Probes), cells were fixed and then visualized for GFP-Wrch-1 (green) or transferrin (red). HA-Wrch-1 was visualized by staining with a primary anti-HA antibody (Covance) and a secondary anti-mouse antibody conjugated to Alexa 647 (Invitrogen). Confocal microscopy was performed on an Olympus Fluoview 500 laser scanning confocal imaging system, configured with an IX81 fluorescence microscope fitted with a PlanApo X60 oil objective.

2.5.4 Antibodies and western blot analysis

Western blot analyses were carried out as described previously (Brady et al., 2009). Briefly, cells were lysed in Magnesium Lysis Buffer (MLB) containing 1X protease inhibitor cocktail (Roche) with or without 100 μM pervanadate, lysates were cleared by centrifugation and protein concentrations determined using the DC Lowry protein assay (BIO-RAD). Twenty μg of protein for each sample, prepared in 5X Laemmli sample buffer, were resolved by using

12% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, Millipore), blocked overnight in 3% fish gelatin, then probed for Wrch-1 by a 1 h incubation with the following primary antibodies: anti-HA epitope (HA.11, Covance), anti-phosphotyrosine (pY100, Cell Signaling Technologies (CST), and pY20, Santa Cruz), anti-GFP (JL8, Clontech), anti-PAK1/2/3 (CST), anti-phospho-PAK1(Thr423)/-PAK2(Thr402) (CST), anti-Pyk2 (Yu et al., 1996), or anti-phospho-Pyk2(Tyr402) (Biosource). Anti-β-actin (Sigma) was used to demonstrate equivalent loading. Washed membranes were incubated in anti-mouse or anti-rabbit IgG-HRP (Amersham Biosciences) or anti-mouse kappa light chain-HRP (Zymed), washed again, and developed using SuperSignal West Dura extended duration substrate (Pierce).

2.5.5 Immunoprecipitation

H1299 cells expressing GFP- or HA-tagged Wrch-1 were lysed in MLB with protease inhibitor, with or without pervanadate, as described above, at 24 h after transfection. Lysates were pre-cleared with Protein A/G beads (Santa Cruz) and then incubated overnight with anti-GFP or anti-HA antibody. After 18 h, the protein-antibody complex was recovered using Protein A/G beads. Beads were collected and washed with MLB and resuspended in Laemmli sample buffer, the precipitated proteins were resolved on SDS-PAGE, and immunoblot analysis for phosphotyrosine was performed as described above.

2.5.6 *In vitro* tyrosine kinase assay

Recombinant Wrch-1 protein was used as a substrate for purified Src kinase in a standard *in vitro* kinase assay. Bacterially expressed GST-Wrch, GST alone, or 1 mg recombinant enolase protein (Calbiochem) to serve as a standard positive control were incubated for 40 min at 30°C with or without 0.8 mg of purified recombinant Src protein (Upstate Biotechnology) in Src Kinase Reaction Buffer (100 mM Tris, pH 7.2; 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 100 mM

Na_3VO_4, and 2 mM DTT) containing [g-^{32}P]ATP (10 mCi per reaction). Reactions were terminated by the addition of 4X sample buffer and then heated at 95°C for 5 min. Protein samples were separated on 10% SDS-PAGE and visualized by Coomassie blue staining. Incorporation of radiolabel was determined by audioradiography.

2.5.7 Anchorage-independent growth transformation assay

Single cell suspensions of MDCK cells (3.5 x 10^3 cells per 35-mm dish) were suspended in 0.4% agar (BD Biosciences) in complete medium and layered on top of 0.6% agar as described previously (Brady et al., 2009). After 14 days, colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) after counting of small (6-15 cell diameters) and large (>15 cell diameters) colonies, and the average number of each type of colony on triplicate dishes was quantified. A one-way ANOVA and Tukey's *post-hoc* test were performed; *p* values of <0.01 were considered significant.

2.5.8 Epithelial morphogenesis cyst formation assay

MDCKII cells stably expressing HA-tagged Wrch-1 proteins were allowed to form cysts in 3D collagen matrices as described previously (Brady et al., 2009). Briefly, monodispersed MDCKII cells were allowed to grow and form cyst structures on collagen I gels for 10 days, when the cultures were treated with collagenase type VII (Sigma C-2399). Cyst structures were fixed and permeabilized, then incubated with fluorescent Texas-Red phalloidin (Molecular Probes) and mounted for imaging on an Olympus Fluoview confocal microscope as indicated above. Multiple XY and XZ scans were acquired for each 3D collagen gel. We have shown previously that tightly regulated endogenous Wrch-1 activity is critical for proper cystogenesis on 3D collagen I matrices in these cells (Brady et al., 2009).

2.5.9 Wrch-1 activation assay

H1299 cells were transiently transfected with either pCGN vector only, or pCGN vectors encoding HA-Wrch-1, HA-Wrch-1(Y254F), HA-Wrch-1(Y254E), HA-Wrch-1(Q107L), HA-Wrch-1(Q107L/Y254F) or HA-Wrch-1(Q107L/Y254E), by using Transit LT1 as described above. Cells were serum-starved overnight, or serum-starved, and then serum-stimulated for 2, 5 or 15 min as described above. Cells were then washed twice with ice-cold PBS (pH 7.4) and lysed in MLB as described above. Equal volumes were removed from each lysate for total protein analysis. To each lysate, glutathione-agarose beads containing 40 µg of GST-p21-activated kinase (PAK) GTPase binding domain fusion protein (GST-PAK-PBD) was added and incubated at 4°C for 60 min with rocking. Agarose-GST-PAK-PBD and associated Wrch-1 was pelleted and washed three times with 500 µL wash buffer [25 mmol/L Tris (pH 7.5), 40 mmol/L sodium chloride, and 30 mmol/L magnesium chloride]. Final pellets were resuspended in 1X protein sample buffer and resolved on SDS-PAGE. HA-Wrch-1 was detected using anti-HA antibody (Covance).

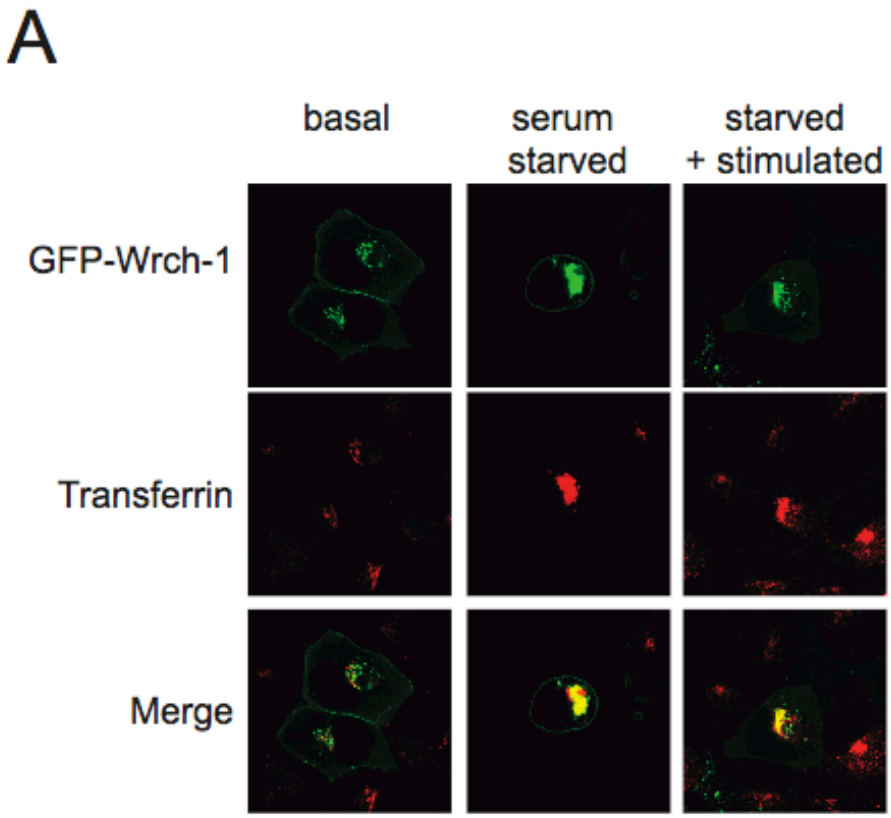


Figure 2.1 Wrch-1 rapidly relocalizes to endosomal compartments upon serum stimulation. H1299 NSCLC cells expressing GFP-Wrch-1 were grown in complete culture medium, then serum-starved overnight, or first serum-starved and then serum-stimulated. Prior to serum-stimulation, the treated cells were incubated for 1 h with Alexafluor 647-labeled transferrin to mark endosomal compartments. After 15 min of serum-stimulation, cells were fixed, then subjected to confocal microscopy for visualization of GFP-Wrch-1 (green) or transferrin (red).

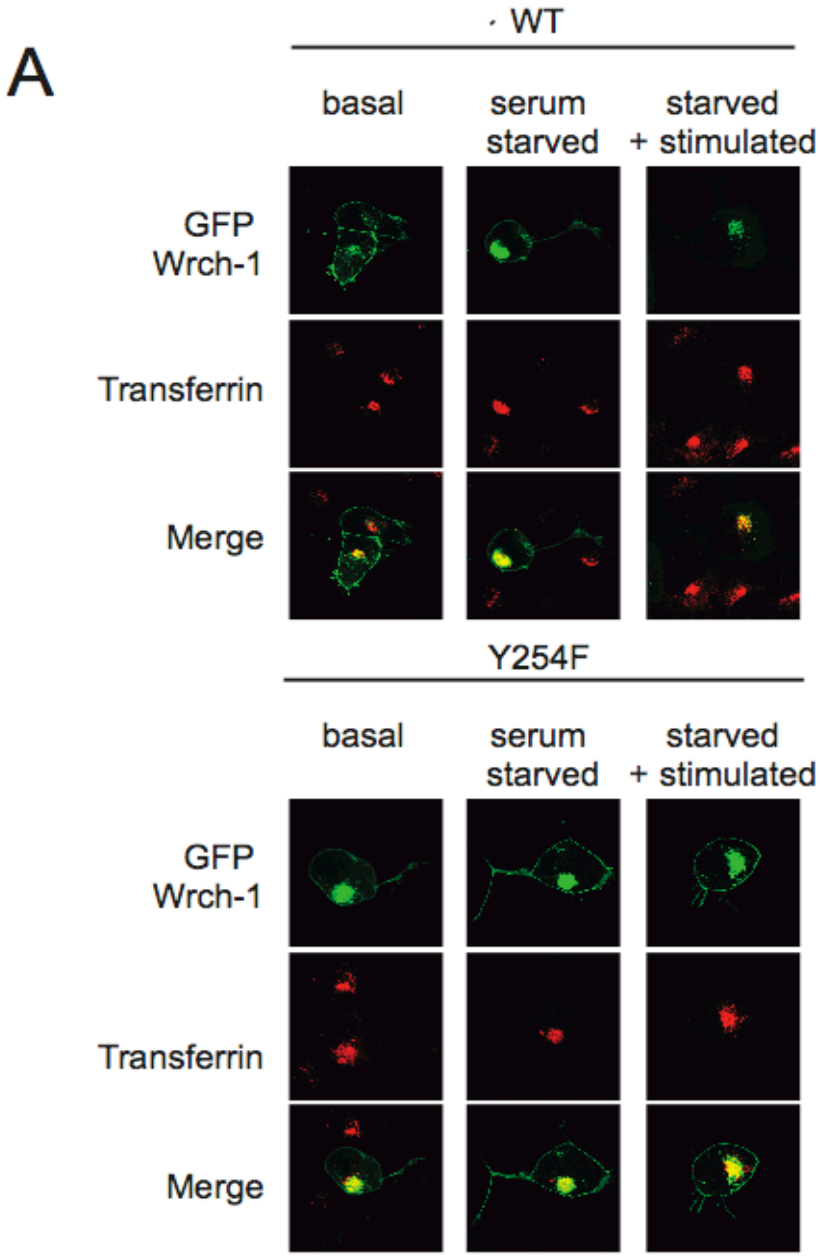


Figure 2.2A Wrch-1 is tyrosine phosphorylated on Y254 in response to serum, and this phosphorylation is required for serum-stimulated relocalization. A) *Serum-stimulated tyrosine phosphorylation of Y254.* H1299 cells expressing either GFP-Wrch-1 or GFP-Wrch-1(Y254F) were grown, treated and evaluated as in Figure 1.

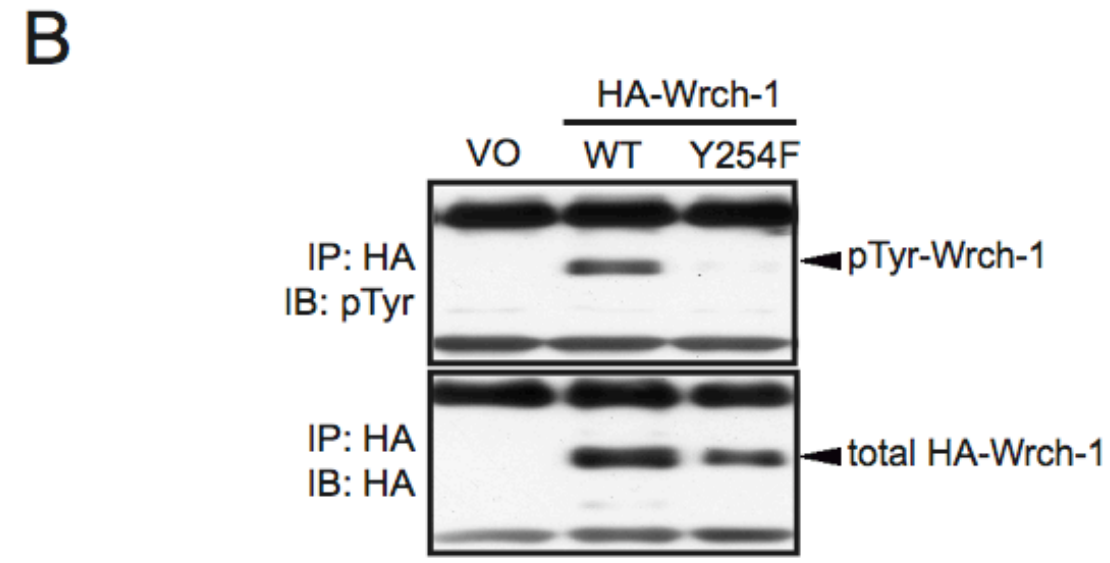


Figure 2.2B Wrch-1 is tyrosine phosphorylated on Y254 in response to serum, and this phosphorylation is required for serum-stimulated relocalization. B) *Non-phosphorylatable Wrch-1(Y254F) is resistant to serum-stimulated relocalization*. H1299 cell lysates from cells expressing empty vector, HA-Wrch-1 or HA-Wrch-1(Y254F) were incubated with anti-HA antibody. Immunoprecipitated (IP) Wrch-1 was detected by immunoblotting (IB) with anti-HA, and phosphotyrosine (p-Tyr) on Wrch-1 was detected by immunoblotting with anti-phosphotyrosine antibody. The bands above and below the Wrch-1 band represent immunoglobulin heavy chain and light chain, respectively.

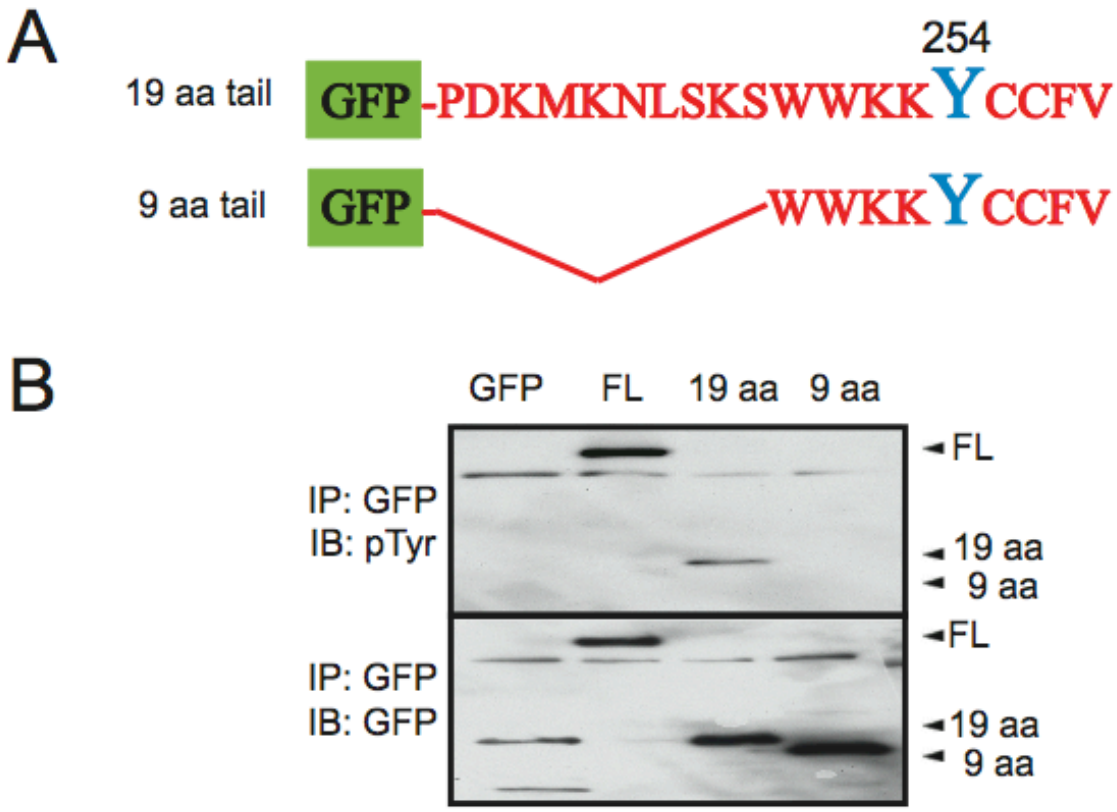


Figure 2.3A The C-terminal 19 amino acids of Wrch-1 are sufficient to become tyrosine phosphorylated and to relocalize in response to serum. A) *Schematic of 19 aa and 9 aa tails.* Shown are the sequences of GFP extended with 9 or 19 amino acids (aa) of the C-terminus of Wrch-1. Y254 is the only tyrosine residue present in each fusion protein. B) *Serum-stimulated tyrosine phosphorylation of the C-terminal 19 but not 9 amino acids of Wrch-1*. H1299 cell lysates expressing either empty vector, GFP-Wrch-1, GFP fused to the last 19 amino acids of Wrch-1 (19 aa tail) or GFP fused to the last 9 amino acids of Wrch-1 (9 aa tail) were incubated with anti-GFP antibody to immunoprecipitate Wrch-1. Wrch-1 was then detected by immunoblotting with anti-GFP antibody, and phosphotyrosine Wrch-1 was detected by immunoblotting with anti-phosphotyrosine antibody.

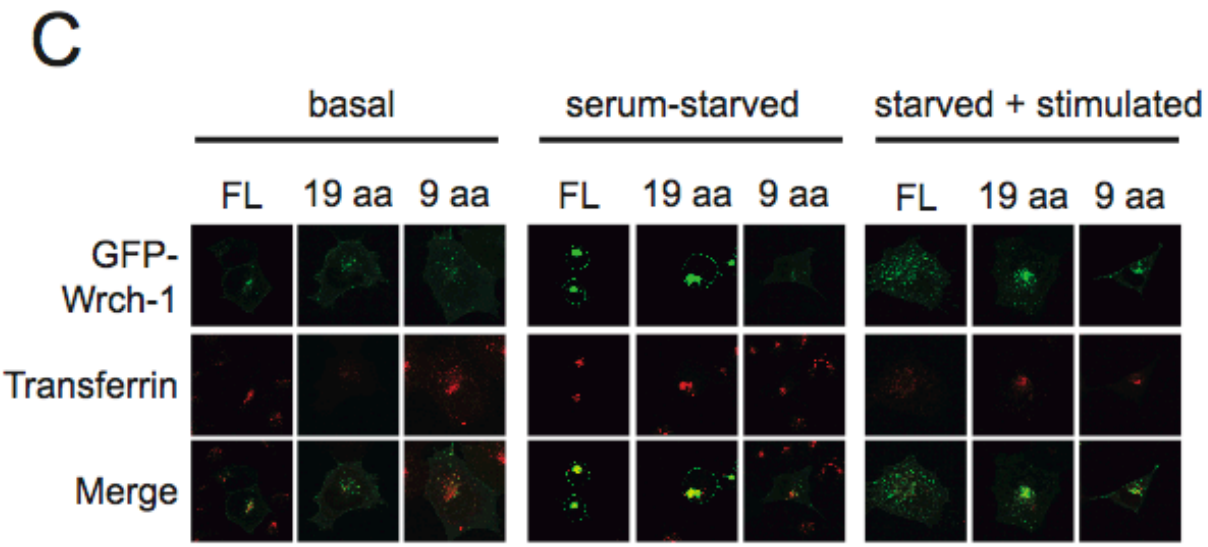


Figure 2.3B The C-terminal 19 amino acids of Wrch-1 are sufficient to become tyrosine phosphorylated and to relocalize in response to serum. C) *The C-terminal 19 amino acids of Wrch-1 are sufficient for serum-stimulated relocalization.* H1299 cells as in Panel B were grown in complete culture medium, then serum-starved overnight, or first serum-starved and then serum-stimulated for 15 min. The treated cells were incubated with Alexafluor 647-transferrin to mark endosomal compartments as in Figure 1, fixed, then visualized using confocal microscopy to detect GFP-Wrch-1 (green) or transferrin (red).

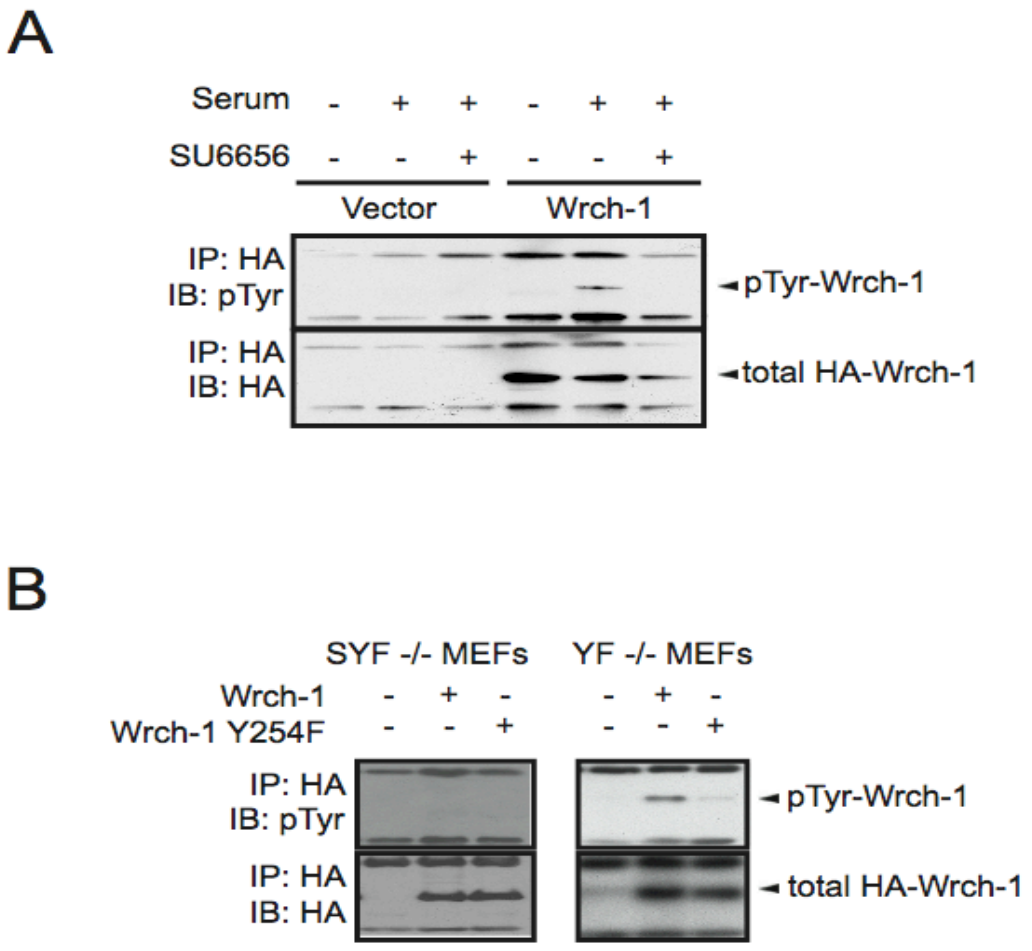


Figure 2.4A Src activity is required *in vivo* for tyrosine phosphorylation of Wrch-1.
A) *The Src family tyrosine kinase inhibitor SU6656 prevents Wrch-1 tyrosine phosphorylation in response to serum stimulation*. H1299 cells expressing HA-Wrch-1 were serum-starved overnight. Cells were then treated with 5 μM SU6656 for 1 h, and serum-stimulated for 5 min. Lysates of these cells were subjected to immunoprecipitation with anti-HA to retrieve HA-Wrch-1, following by detection of Wrch-1 (anti-HA) or phosphotyrosine (anti-p-Tyr). The bands above and below the Wrch-1 band represent immunoglobulin heavy chain and light chain, respectively. B) *Endogenous Src is required for serum-stimulated Wrch-1 tyrosine phosphorylation.* SYF -/- MEFs (MEFs lacking Src, Yes, and Fyn) and YF -/- MEFs (MEFs retaining Src but lacking Yes and Fyn) expressing either HA-Wrch-1 or non-phosphorylatable HA-Wrch-1(Y254F) were serum-starved overnight, then serum-stimulated for 5 min. The resulting cell lysates were probed for phosphotyrosine on Wrch-1 as in Panel A. The bands above and below the Wrch-1 band represent immunoglobulin heavy chain and light chain, respectively.

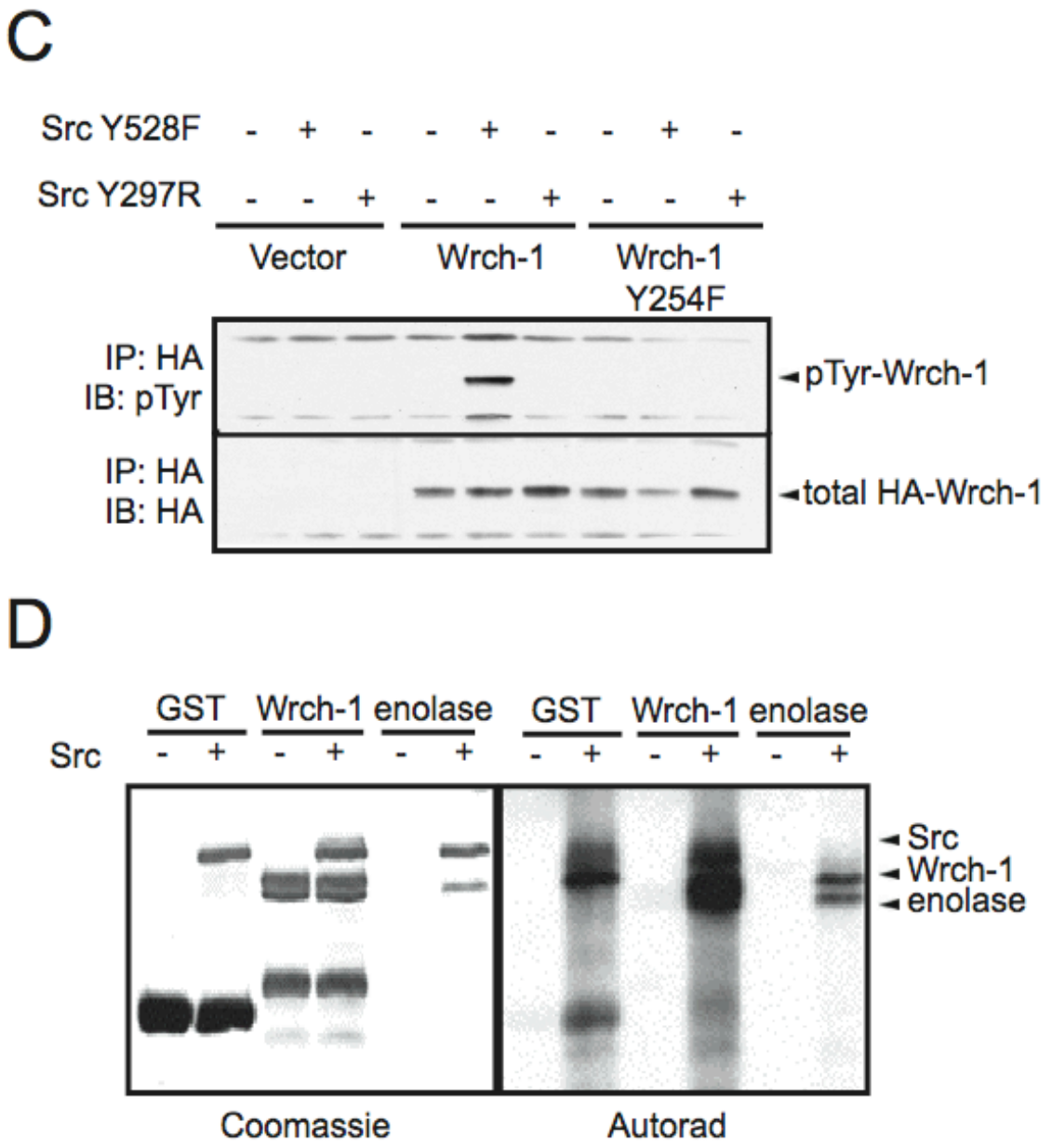


Figure 2.4A Src activity is required *in vivo* for tyrosine phosphorylation of Wrch-1. C) *Src kinase activity is required for tyrosine phosphorylation of Wrch-1.* H1299 cells were co-transfected with empty vector, HA-Wrch-1 or nonphosphorylatable HA-Wrch-1(Y254F) along with either empty vector, kinase-active Src (Src Y528F) or kinase-deficient Src (Src K297R). The resulting cell lysates were probed for phosphotyrosine on Wrch-1 as in Panel A. The bands above and below the Wrch-1 band represent immunoglobulin heavy chain and light chain, respectively. D) *Src directly phosphorylates Wrch-1* in vitro. Purified recombinant GST-Wrch-1 protein was incubated with purified recombinant Src tyrosine kinase protein and ^{32}P-ATP. Total protein was detected by Coomassie blue staining and ^{32}P-ATP incorporation was detected by autoradiography.

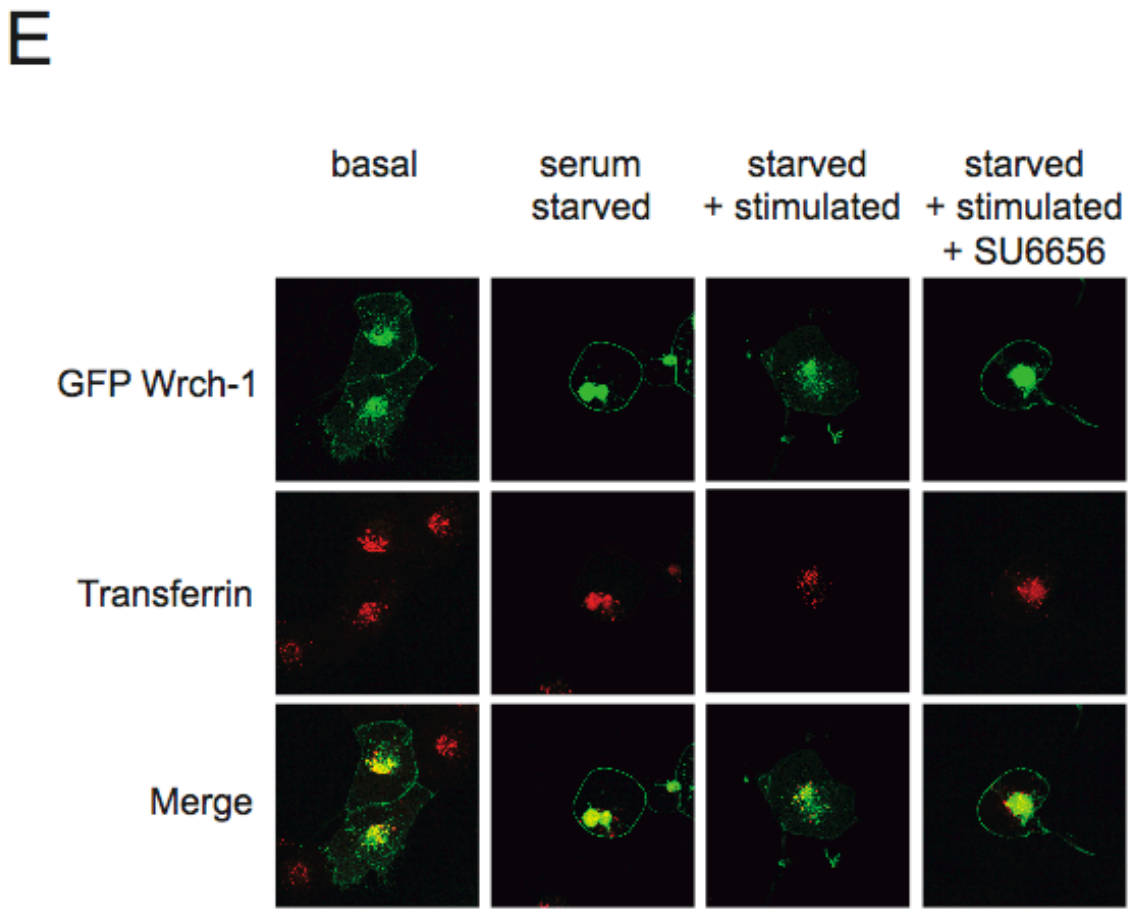


Figure 2.4C Src activity is required *in vivo* for tyrosine phosphorylation of Wrch-1.
E) *Inhibition of Src kinase with SU6656 blocks serum-stimulated relocalization of Wrch-1.* H1299 cells expressing GFP-Wrch-1 or nonphosphorylatable GFP-Wrch-1(Y254F) were grown in complete culture medium (basal), then either serum-starved overnight (serum-starved), first serum-starved and then serum-stimulated for 15 min, or starved overnight then pretreated with 5 μM SU6656 for 1 h prior to serum stimulation for 15 min. The treated cells were incubated with Alexafluor 647-transferrin as in Figure 2.1, fixed and visualized by confocal microscopy for Wrch-1 (green) or transferrin .

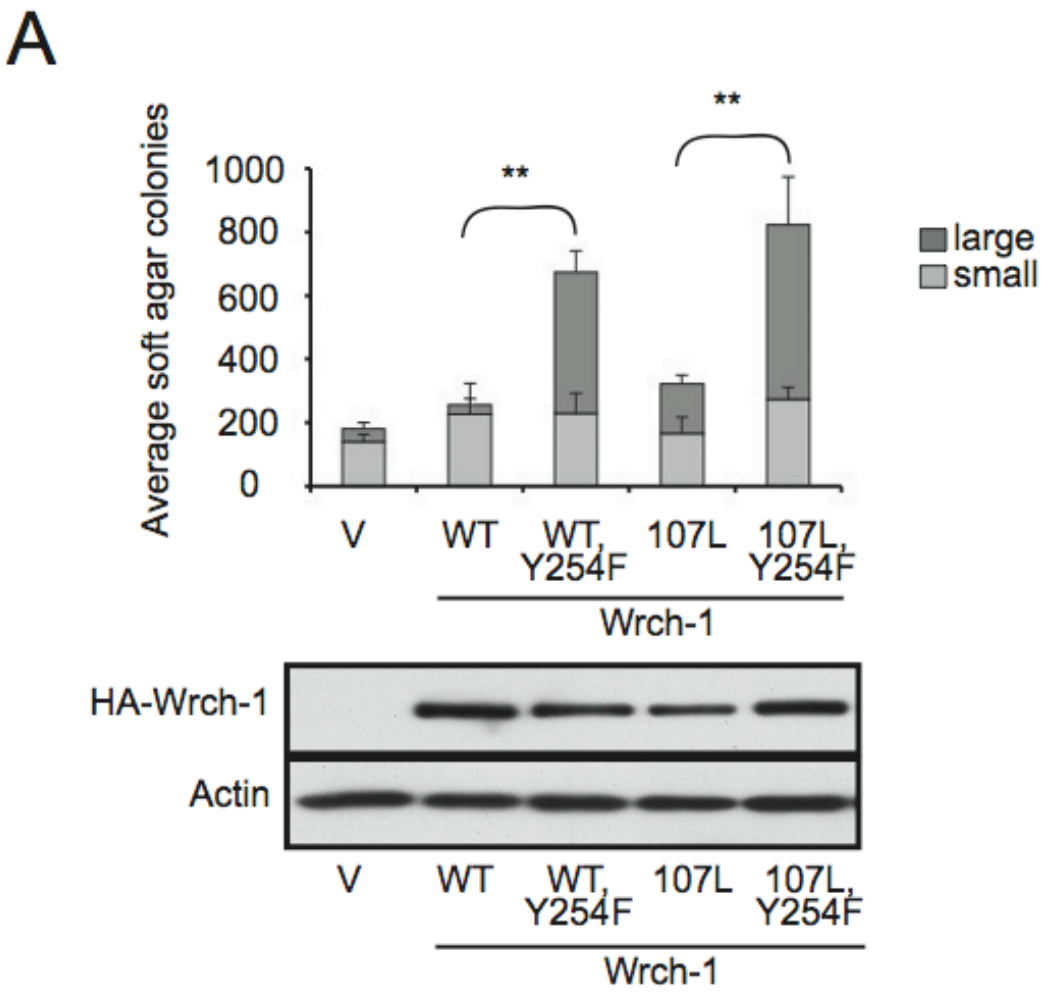


Figure 2.5A Phosphorylation at Y254 negatively regulates Wrch-1-mediated biological functions.A) *Phosphorylatable tyrosine residue Y254 negatively regulates anchorage-independent growth.* MDCKII cell lines were generated to stably express either vector only, HA-Wrch-1, HA-Wrch-1(Y254F), HA-Wrch-1(107L) or HA-Wrch-1(107L/Y254F). Equivalent expression was confirmed by immunoblotting with anti-HA. β-actin was used as a loading control. Cells were then seeded into soft agar and colonies were allowed to grow in an anchorage-independent manner for 14 days. Colonies were stained with MTT and the numbers of small (6-15 cell diameters across) and large (>15 cell diameters across) colonies were quantified. Bar graphs are representative of three independent experiments carried out in triplicate. A one-way ANOVA and Tukey's *post-hoc* test were done to determine significance of differences between numbers of colonies arising from cells expressing Wrch-1 with or without the Y254F mutation, in both the WT and constitutively activated (107L) backgrounds. Double asterisks (**) indicate a *p* value of <0.001.

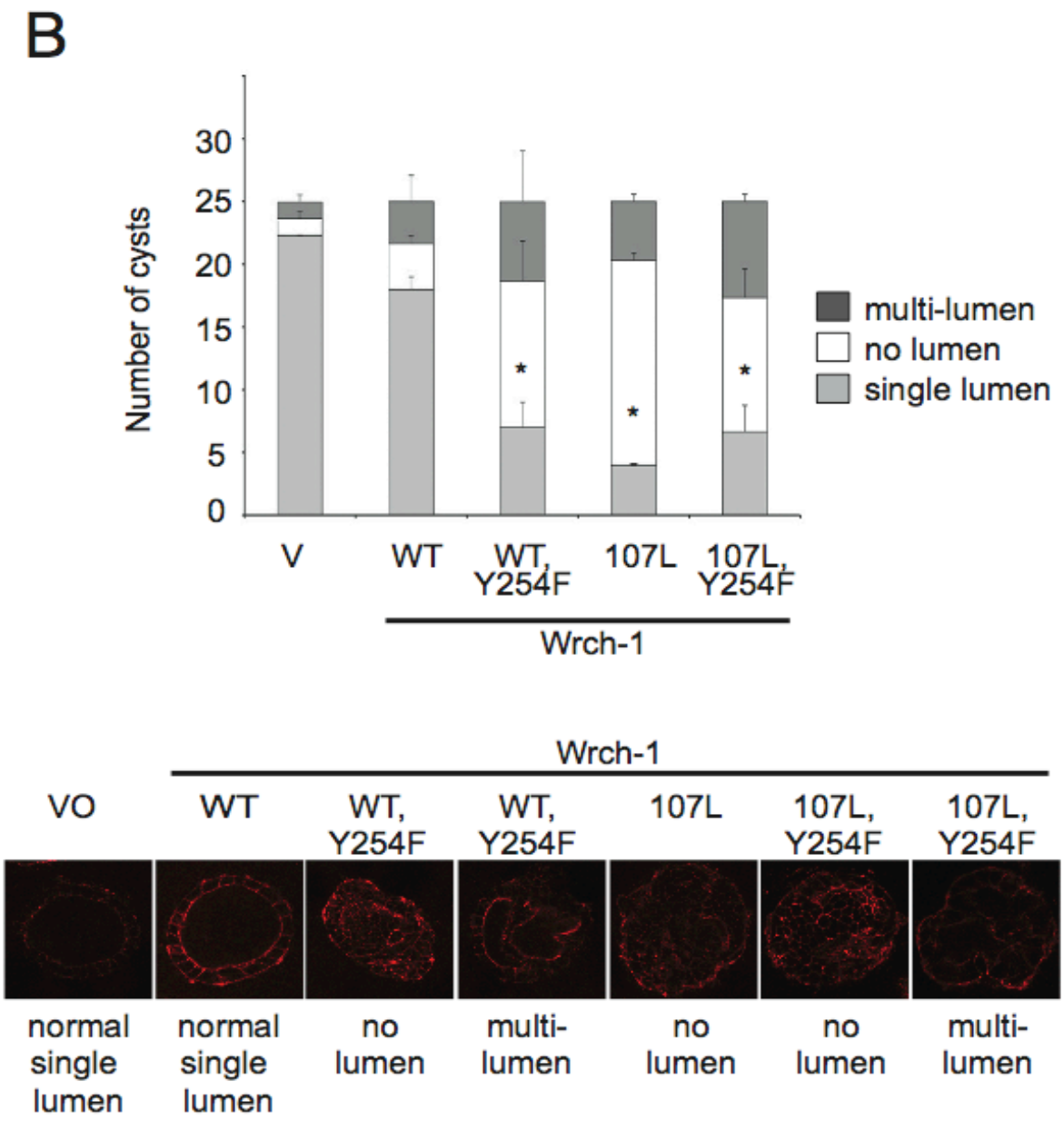


Figure 2.5B Phosphorylation at Y254 negatively regulates Wrch-1-mediated biological functions. B) *Phosphorylatable tyrosine residue Y254 negatively regulates epithelial cell morphogenesis.* MDCKII cells expressing Wrch-1 as in Panel A were seeded into 3D collagen matrices and allowed to form cysts for 10 days. Cyst structures were evaluated and quantified according to whether they contained one lumen ("normal", "single lumen"), more than one lumenal area ("multi-lumen"), or no lumen at all ("no lumen"). Bars represent the average of three independent experiments for each cell line, +/- SEM. A one-way ANOVA and Tukey's *post-hoc* test were done to determine significance of differences between numbers of normal cysts with single lumens in cells expressing Wrch-1 with or without the Y254F mutation, in both the WT and constitutively activated (107L) backgrounds. Asterisks (*) denote a *p* value of <0.01.

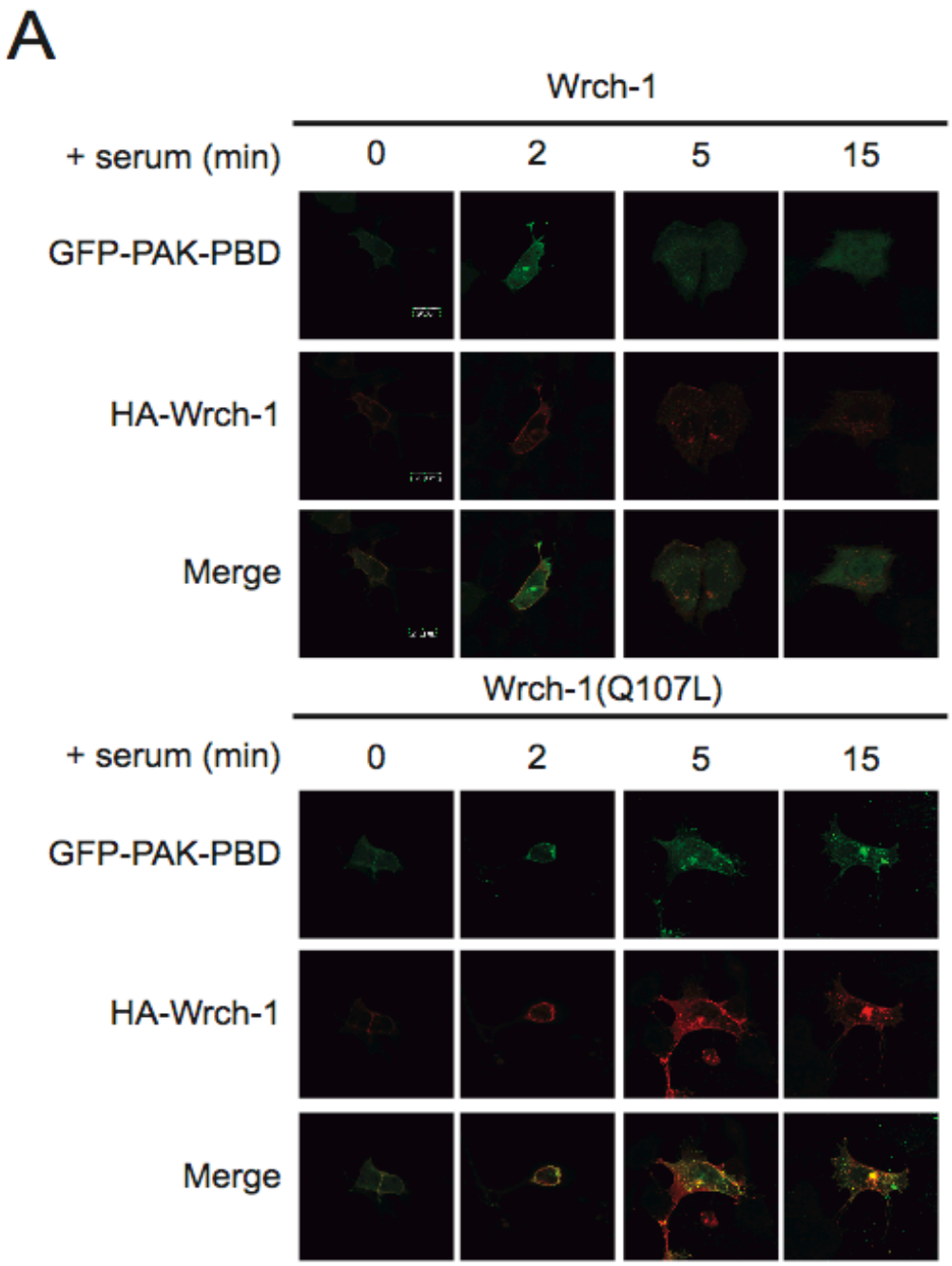


Figure 2.6A Wrch-1 is GTP-bound and active, and recruits its effector PAK1, at plasma membrane but not at endosomes. *A) Serum stimulation decreases Wrch-1 recruitment of GFP-PAK-PBD to the plasma membrane.* H1299 cells co-expressing GFP-PAK-PBD along with HA-tagged WT, phosphodeficient Y254F or phosphomimetic Y254E Wrch-1 mutants in either a WT or a GAP-insensitive (107L) background were grown in complete culture medium, then serum-starved overnight, or first serum-starved and then serum-stimulated for either 2, 5 or 15 m. After serum stimulation, cells were fixed and then stained for HA-Wrch-1 with a primary anti-HA antibody, and a secondary anti-mouse antibody conjugated to Alexa-647. The cells were then subjected to confocal microscopy for visualization of GFP-PAK-PBD (green) or HA-Wrch-1 (red). Overlapping localization (merge, yellow) demonstrates recruitment of GFP-PAK-PBD by Wrch-1.

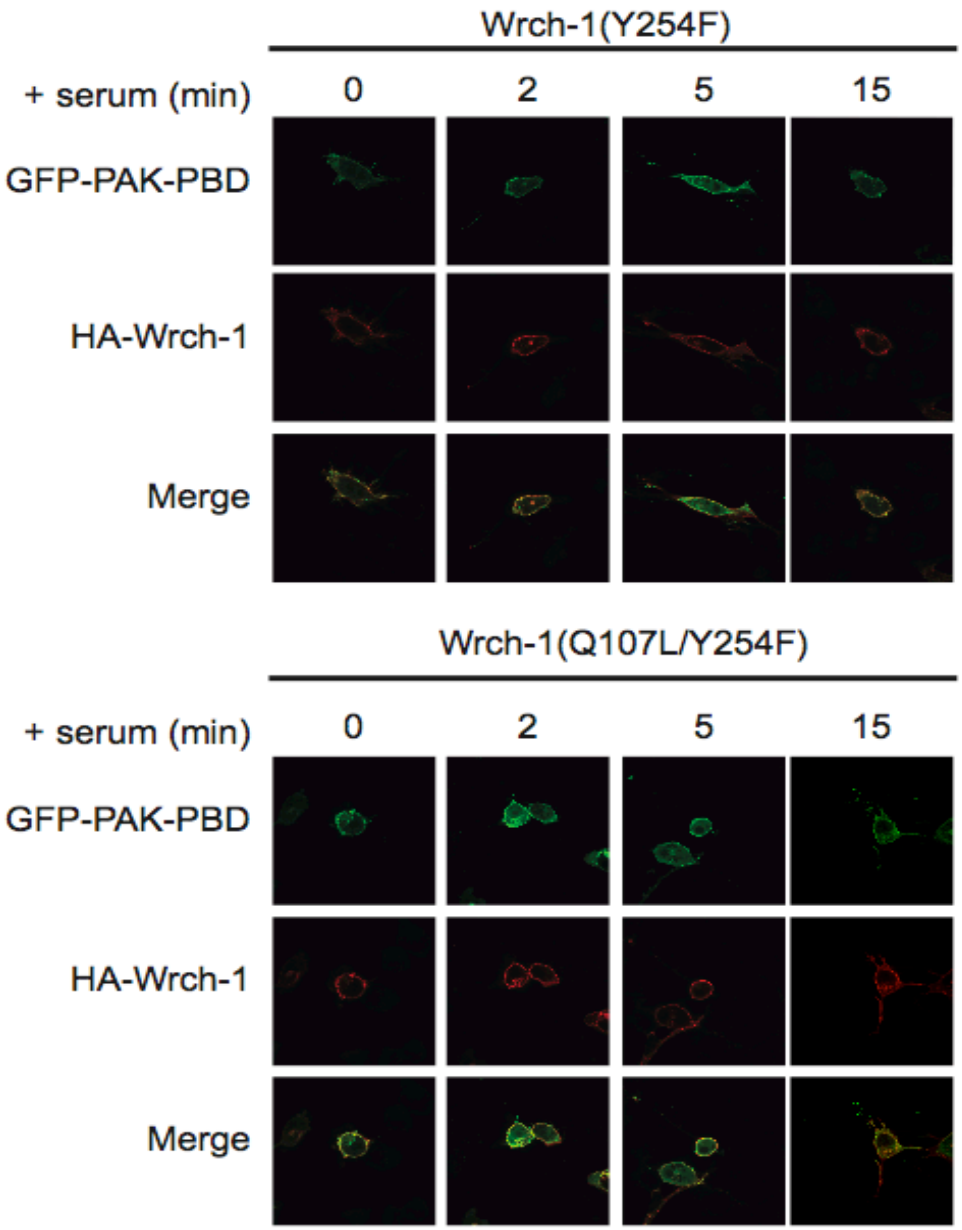


Figure 2.6A Wrch-1 is GTP-bound and active, and recruits its effector PAK1, at plasma membrane but not at endosomes. *A) Serum stimulation decreases Wrch-1 recruitment of GFP-PAK-PBD to the plasma membrane.* H1299 cells co-expressing GFP-PAK-PBD along with HA-tagged WT, phosphodeficient Y254F or phosphomimetic Y254E Wrch-1 mutants in either a WT or a GAP-insensitive (107L) background were grown in complete culture medium, then serum-starved overnight, or first serum-starved and then serum-stimulated for either 2, 5 or 15 m. After serum stimulation, cells were fixed and then stained for HA-Wrch-1 with a primary anti-HA antibody, and a secondary anti-mouse antibody conjugated to Alexa-647. The cells were then subjected to confocal microscopy for visualization of GFP-PAK-PBD (green) or HA-Wrch (red). Overlapping localization (merge, yellow) demonstrates recruitment of GFP-PAK-PBD by Wrch-1.

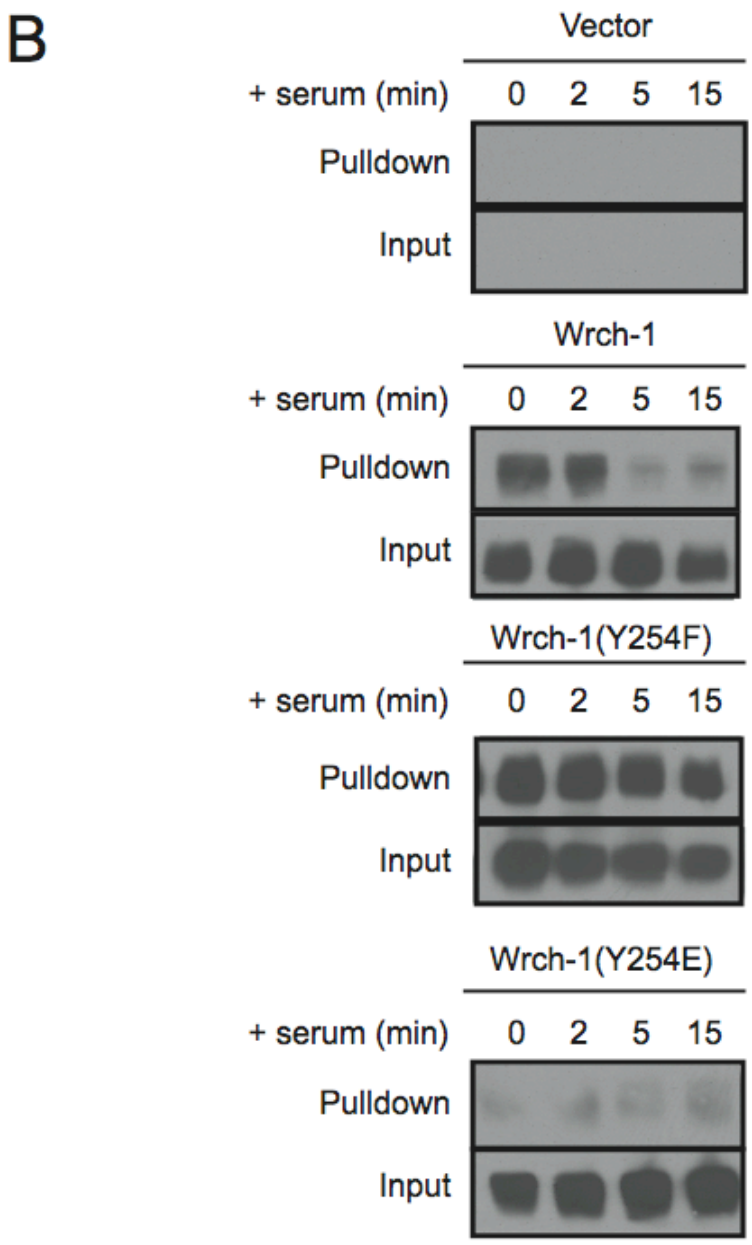


Figure 2.6B Wrch-1 is GTP-bound and active, and recruits its effector PAK1, at plasma membrane but not at endosomes. *B) Serum stimulation decreases active Wrch-1-GTP as shown by pulldown assay with GST-PAK-PBD.* To pull down active Wrch-1-GTP with GST-PAK-PBD, H1299 cells were transiently transfected with either pCGN-HA empty vector, or pCGN vector expressing HA-tagged Wrch-1 proteins, and treated as in Panel A. Resulting cell lysates were incubated with GST-p21-activated kinase (PAK) fusion protein attached to glutathione-agarose beads. The resulting Wrch-1/GST-PAK-PBD complex was collected by centrifugation, washed, eluted from the beads and resolved by SDS-PAGE. Wrch-1 was detected by immunoblot analysis using anti-HA antibody. Upper panels: pulldown followed by immunoblot, representing Wrch-1-GTP; lower panels: immunoblot of input, representing total Wrch-1 available for pulldown in that sample.

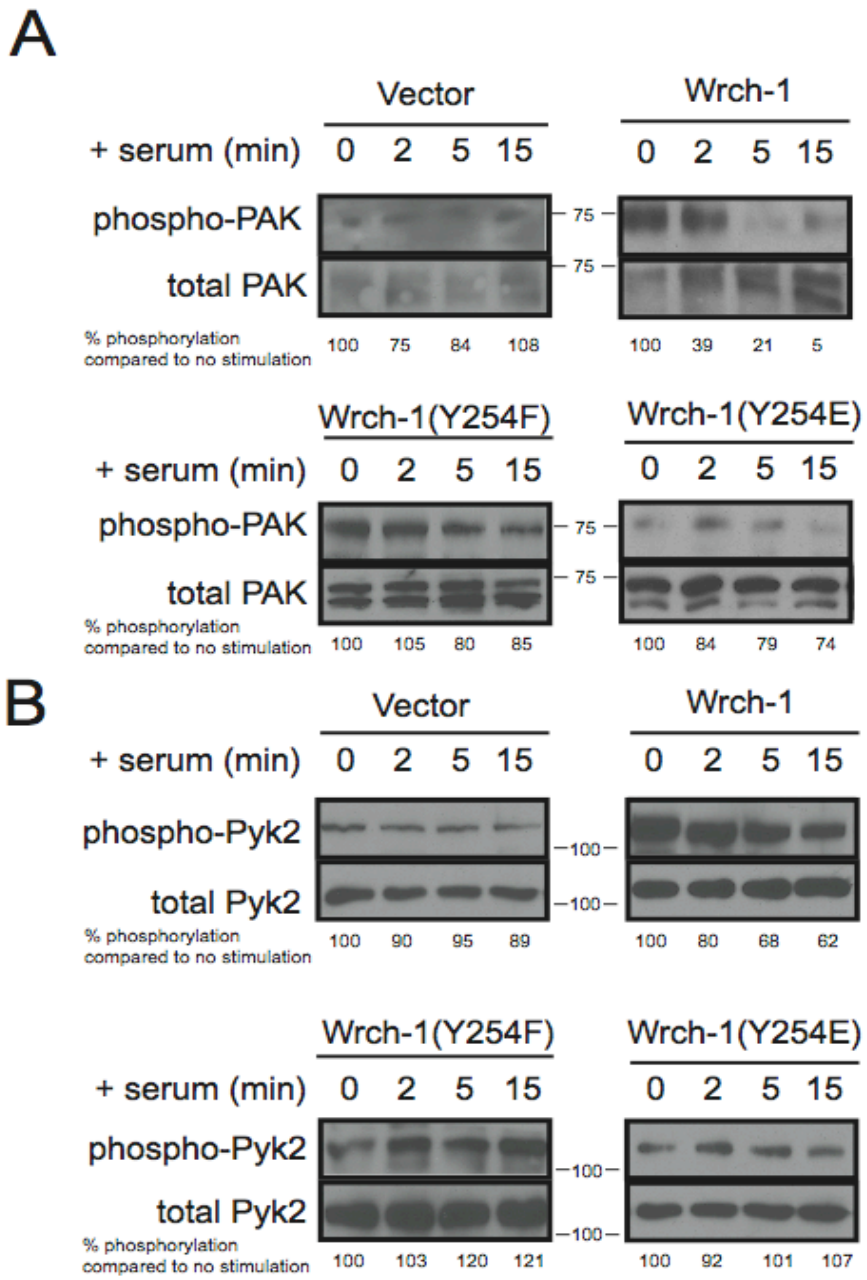


Figure 2.7 Serum-stimulated Wrch-1 tyrosine phosphorylation and endosomal relocalization results in decreased Wrch-1 effector activation. *A) Serum stimulation results in decreased autophosphorylation of PAK1.* H1299 cells expressing HA-tagged forms of the indicated Wrch-1 proteins were treated as described in the legend to Figure 6. Total PAK was detected by immunoblotting with an anti-PAK1/2/3 antibody and active, phospho-PAK was detected using an anti-phospho(Thr423)-PAK1/phospho(Thr402)-Pak2 antibody. Signal was quantitated by densitometry, phospho-protein was normalized to normal protein, then compared to no serum conditions, which was set to 100%. *B) Serum stimulation results in decreased Pyk2 autophosphorylation.* H1299 cells expressing HA-tagged forms of the indicated Wrch-1 proteins were treated and analyzed as in Panel A, except that total Pyk2 was detected by immunoblotting with anti-Pyk2 antibody and phospho-Pyk2 was detected with anti-phospho(Tyr402)-Pyk2 antibody. Quantitation was done as indicated in Panel A.

CHAPTER III

REGULATION OF MEMBRANE TRAFFICKING AND BIOLOGICAL ACTIVITIES OF THE RHO FAMILY SMALL GTPASE WRCH-1, BY THE TYROSINE KINASES EGFR AND SRC

3.1 Abstract

Wrch-1 is an atypical Rho family small GTPase with roles in oncogenic transformation, epithelial cell morphogenesis, osteoclastogensis, and migration. Wrch-1 membrane localization and biological functions are modulated by reversible addition of the fatty acid palmitate at its C-terminal membrane targeting domain. Additionally, have shown that localization of Wrch-1 is modulated by Src-mediated tyrosine phosphorylation in the C-terminal membrane-targeting region; after serum stimulation, Wrch-1 becomes tyrosine phosphorylated at Y254, and then relocalizes to endosomal compartments. Although we have shown that Wrch-1 tyrosine phosphorylation at Y254 is Src dependent, other upstream signals and kinases involved were previously unknown. We observed that Wrch-1 was tyrosine phosphorylated in response to EGF treatment in a dose and time dependent manner, and this phosphorylation can be blocked by treatment with EGFR inhibitors. We also observed rapid relocalization of Wrch-1 from the plasma membrane to endosomes upon EGF stimulation, similar to the relocalization that occurs after serum stimulation. Additionally Wrch-1 is tyrosine phosphorylated at Y254 downstream of constitutively active EGFR and HER2 receptors, including EGFRvIII, a deletion mutant found in many glioblastomas, breast cancers, etc (Kuan, 2001 #133; Huang, 2007 #136). We speculated that Wrch-1 tyrosine phosphorylation downstream of EGFR may be Src dependent. Indeed, EGF stimulated Wrch-1 tyrosine phosphorylation was blocked in the presence of the Src

family kinase inhibitor SU6656. Wrch-1 tyrosine phosphorylation downstream of EGFR maybe be facilitated by Cbl binding, and subsequently acting as an adaptor, as we found that Wrch-1 binding to Cbl increased after serum stimulation. Functionally, the phosphodeficient mutant Y254F was enhanced in Wrch-1-mediated migration. Thus, EGFR-mediated, Src dependent C-terminal tyrosine phosphorylation of Wrch-1 may represent a mechanism to regulate trafficking and biological outcomes of Wrch-1. C-terminal tyrosine phosphorylation represents a new paradigm in posttranslational control of small GTPase localization and biological function.

3.2 Introduction

Rho family proteins are Ras-related small GTPases that are known for their role in regulating cytoskeleton organization and dynamics, cell adhesion, motility, trafficking, proliferation and survival (Jaffe and Hall, 2005). Rho GTPases are tightly regulated molecular switches that cycle between the active GTP-bound state and the inactive GDP-bound state, with additional positive regulation by GEFs (guanine nucleotide exchange factors) and negative regulation by GAPs (GTPase accelerating proteins). In addition to nucleotide status, Rho GTPases are also regulated by their subcellular localization, which is directed by sequences and post-translational modifications within their C-terminal membrane targeting domains. Most Rho GTPases are post-translationally modified by an isoprenoid lipid irreversibly attached to the C-terminal CAAX motif (Adamson et al., 1992), and an additional second signal, which usually consists of a polybasic region or a palmitate fatty acid upstream of the CAAX motif (Michaelson et al., 2001; Williams, 2003). As an additional level of control, Rho GTPases can also be regulated by their interactions with RhoGDIs (Rho-guanine nucleotide dissociation inhibitors) that bind their prenyl groups and sequester the GTPases in the cytosol, rendering them inactive (Isomura et al., 1991; Takai et al., 1995).

Wrch-1, also designated RhoU or Wrch1 (Brazier et al., 2006; Kirikoshi and Katoh, 2002; Tao et al., 2001), is an atypical member of the Cdc42 subgroup of Rho GTPases. Like other members of the Cdc42 subgroup, Wrch-1 also induces the formation of actin microspikes and filopodia. Wrch-1 shares 57% sequence identity with Cdc42 and 61% sequence identity with its closest relative, Chp/Wrch-2, as well as partially overlapping localization and effector interactions. Activation of Wrch-1 leads to activation of PAK1 (Tao, 2001 #79), JNK (Chuang, 2007 #125), and Pyk2 (Ruusala, 2008 #134), to formation of filopodia (Ruusala and Aspenstrom, 2008; Saras et al., 2004), and to both morphological (Brady et al., 2009) and growth transformation (Berzat et al., 2005a; Brady et al., 2009). Wrch-1 also regulates focal adhesion turnover (Chuang et al., 2007; Ory et al., 2007), cell migration (Chuang et al., 2007), negatively regulates the kinetics of tight junction formation (Brady et al., 2009), plays a required role in epithelial cell morphogenesis (Brady et al., 2009), and modulates osteoclastogenesis (Brazier et al., 2009; Ory et al., 2007).

Wrch-1 was initially discovered as a Wnt-responsive gene that was capable of phenocopying Wnt morphological transformation in mouse mammary epithelial cells when mutationally activated (Taneyhill and Pennica, 2004; Tao et al., 2001). In addition to transcriptional upregulation by Wnt signaling, Wrch-1 is also transcriptionally upregulated in in MEFs a JNK-dependent manner (Schiavone et al., 2009) and in bone marrow macrophages during osteoclastogenesis upon RANKL stimulation (Brazier et al., 2006). Recently Wrch-1 expression has also been shown to be responsive to gp130-class cytokines, which transcriptionally regulate Wrch-1 expression via STAT3 (Schiavone et al., 2009). Furthermore, it has been shown that Wrch-1 expression varies dramatically in different cancer types. The expression of Wrch-1 is upregulated in some tumor types but downregulated in others (Kirikoshi and Katoh, 2002). Taken together, these data imply that

modulation of Wrch-1 expression is a common regulatory event, and that a proper level of Wrch-1 expression and perhaps activity is required. Because Wrch-1 is a GTP-binding protein, more dynamic regulation of its activity is also required, in addition to transcriptional regulation.

We have shown previously that phosphorylation of Wrch-1 at Y254 regulates its subcellular localization. In response to serum stimulation, this phosphorylation results in Wrch-1 relocalization from the plasma membrane to endomembranes. When it is located at the plasma membrane, Wrch-1 is GTP-bound and capable of inducing activation of at least two of its effectors, PAK1 and Pyk2. However, once it relocalizes to endomembranes, it becomes GDP-bound, and its activation of PAK1 and Pyk2 decreases. Additionally, a phosphomimetic mutant of Wrch-1 is localized constitutively to endomembranes (Y254E) and exhibits decreased anchorage-independent growth, whereas a phosphodeficient mutant is chronically localized to the plasma membrane (Y254F) and is enhanced in the same biological readout.

The pattern of growth factor-stimulated Wrch-1 relocalization suggests that there may be a specific trafficking mechanism regulating Wrch-1, similar to that regulating RTKs such as EGFR. There are several examples of Cdc42 family members that are involved intracellular trafficking. Cdc42 shares high sequence similarity with two other members of the Cdc42 family, TC10 and TCL. Like Cdc42, TC10 promotes the formation of filopodia, activates JNK, and promotes SRF- and NF-κB-mediated transcription (Murphy et al., 1999). TCL promotes the formation of membrane ruffles and lamellipodia (Aspenstrom et al., 2004; Vignal et al., 2000). Interestingly, somewhat similarly to Wrch-1 TC10 localizes mainly to the plasma membrane and intracellular membranes, whereas TCL localizes to endosomes. TC10 localization and biological function are dependent on posttranslational modifications in

its C-terminal hypervariable membrane targeting region. These modifications include isoprenylation of its CAAX motif and palmitoylation of an upstream cysteine. TC10 is phosphorylated by CDK5 on Thr197 in its C-terminal hypervariable region. Phosphorylation at Thr197 by CDK mediates the association of TC10 with lipid rafts, which modulates its ability to mediate insulin-stimulated GLUT4 translocation (Okada et al., 2008). TCL localizes mostly to endosomes, has been previously shown to associate with early endosomes, and is also able to regulate the transfer of transferrin from early endosomes to recycling endosomes (de Toledo et al., 2003). A proper balance of TCL activity is required for this function, because both loss of TCL and constitutive activation of TCL restrict transferrin to early endosomes (de Toledo et al., 2003). Like most Rho GTPases, both the C-terminal membrane targeting domain and proper localization are crucial for TCL function, because the fusion of C-terminal sequences of TCL to either Cdc42 or TC10 had a similar effect on transferrin trafficking (de Toledo et al., 2003). These studies further highlight the importance of localization in regulating the function of family proteins. Wrch-1 localizes to the plasma membrane and internal membranes, and its localization and function is regulated by C-terminal phosphorylation.

In the present study, we sought to determine the role of EGF-stimulated tyrosine phosphorylation of Wrch-1, and the role that Wrch-1 plays in intracellular trafficking. Our previous study determined that serum stimulation resulted in Wrch-1 phosphorylation on Y254, which then regulated Wrch-1 subcellular localization, activation and function. Further research was needed to elucidate the component(s) in serum responsible for tyrosine phosphorylation and subsequent regulation of Wrch-1 function. Additionally, further research was needed to understand the mechanisms controlling Wrch-1 trafficking, and the subsequent biological consequences. In this report we describe our discovery that EGF stimulates tyrosine phosphorylation of Wrch-1 in a dose- and time-dependent manner, and

that Src is required for this event. We also report that Wrch-1 tyrosine phosphorylation is important in regulating some EGFR-driven biological functions, including migration and invasion.

3.3 Results

3.3.1 Wrch-1 is tyrosine phosphorylated in response to stimulation with EGF and PDGF but not bradykinin or LPA. We have shown previously that Wrch-1 is tyrosine phosphorylated in response to cell stimulation with serum, which contains a myriad of proteins and growth factors. In order to determine which component of serum was responsible for Wrch-1 tyrosine phosphorylation, we screened a panel of ligands known to be present in serum. We serum-starved overnight H1299 NSCLC cells transiently transfected with HA-Wrch-1, and then stimulated them with a panel of ligands. We then immunoprecipitated the Wrch-1 with an anti-HA antibody, followed by western blotting using an anti-phosphotyrosine antibody to probe for phosphotyrosine Wrch-1. We observed that Wrch-1 was tyrosine phosphorylated in response to RTK ligands such as EGF and PDGF, but not ligands for GPCRs such as bradykinin, a key activator of Cdc42 (Kozma et al., 1995) or LPA, a major stimulatory component of serum (Figure 3.1A and data not shown). We found that tyrosine phosphorylation of Wrch-1 required at least 30 ng of EGF (Figure 3.1B) suggesting that it may occur under conditions of aberrant EGFR signaling. In order to determine the kinetics of the response, we stimulated cells with 30 ng of EGF for 0, 5, 15, 30, or 60 min. We found that Wrch-1 tyrosine phosphorylation in response to EGF occurred as early as 10 min, and was sustained for up to 60 min (Figure 3.1C), suggesting that this phosphorylation is induced by continuous and sustained EGFR stimulation. EGF stimulation can activate both EGFR homodimers and EGFR/HER2 heterodimers. In order to determine the specificity of EGF stimulation in our system, we serum-starved cells expressing HA-

Wrch-1 and then treated with 30 ng EGF in the presence or absence of an EGFR-selective inhibitor (erlotinib) or a dual EGFR/HER2 inhibitor (lapatinib). We found that EGF-stimulated tyrosine phosphorylation of Wrch-1 was abolished in the presence of either erlotinib or lapatinib (Figure 3.1D). Wrch-1 may thus be phosphorylated in response to EGF through either EGFR homodimers or EGFR/HER2 heterodimers. Finally, in order to determine if Wrch-1 could be tyrosine phosphorylated in the presence of mutant EGFR or HER2 receptors, we examined Wrch-1 tyrosine phosphorylation in the presence of constitutively activated EGFR (EGFRvIII) and HER2 (NeuT) receptors. We co-transfected H1299 cells with HA-Wrch-1 and either EGFRvIII or NeuT, immunoprecipitated HA-Wrch-1 and blotted as described above. We found that, in the presence of these constitutively activated EGFR family members, Wrch-1 was tyrosine phosphorylated in the absence of outside stimulus (Figure 3.1E), indicating that Wrch-1 can be tyrosine phosphorylated in response to the expression of constitutively active EGFR and HER2 receptors.

3.3.2 Wrch-1 tyrosine phosphorylation in response to EGF is Src-dependent. Previously we had determined that both Src expression and kinase activity were required for Wrch-1 tyrosine phosphorylation in response to serum stimulation. In order to determine whether Src was also required for Wrch-1 phosphorylation in response to EGF stimulation, we serum-starved H1299 cells expressing HA-Wrch-1 then stimulated the cells with EGF in the presence or absence of the Src family kinase inhibitor SU6656. We observed that in the presence of SU6656, EGF stimulation was not sufficient to induce tyrosine phosphorylation of Wrch-1 (Figure 3.2), suggesting that Src family kinase activity is required downstream of EGF stimulation to phosphorylate Wrch-1.

3.3.3 EGF stimulation causes Wrch-1 to traffic to endosomal compartments. We had previously determined that, upon serum stimulation, Wrch-1 trafficks from the plasma

membrane to internal membranes. Since EGF stimulation, like serum stimulation, results in tyrosine phosphorylation of Wrch-1, we sought to determine whether it also caused subsequent trafficking of Wrch-1 from the plasma membrane to internal membranes. In order to do this, we transiently transfected H1299 cells with GFP-Wrch-1, then serum-starved the cells overnight before stimulating with EGF. We then labeled the cells with Alexa 647-transferrin, fixed them with paraformaldehyde, and imaged them using an Olympus IX-81 confocal microscope. We observed that, like serum, EGF stimulation resulted in the relocalization of Wrch-1 from the plasma to internal membranes (Figure 3.3). These data suggest that Wrch-1 may undergo trafficking in response to EGF-stimulated phosphorylation, in a similar way to trafficking of EGFR.

3.3.4 EGF stimulation causes Wrch-1 to traffick to early endosomes, recycling endosomes and lysosomes. When we treated cells expressing Wrch-1 with EGF, we saw a relocalization similar to that of EGFR, suggesting that Wrch-1 may utilize a similar trafficking pathway. When EGFR undergoes internalization in response to EGF stimulation, it trafficks to early endosomes, and then both to recycling endosomes, which recycle EGFR back to the plasma membrane, and also to lysosomes, which degrade EGFR. In order to determine whether Wrch-1 trafficked in a similar fashion to EGFR, we sought to determine the endosomal compartment(s) to which Wrch-1 localizes after EGF stimulation. To do this, we stimulated H1299 cells expressing GFP-Wrch-1 with EGF as described above. We then either pre-incubated the cells with Lysotracker-red to label lysosomes, or co-stained with markers for early endosomes (EEA1) or recycling endosomes (Rab 11). We found that, after EGF stimulation, Wrch-1 localized to early endosomes to a small extent, along with recycling endosomes and lysosomes. These data indicate that, like EGFR, a portion of internalized Wrch-1 may be recycled back to the plasma membrane via recycling endosomes, and another portion of Wrch-1 may be degraded by the lysosomes (Figure 3.4).

3.3.5 EGF stimulation causes Wrch-1 to traffick to endosomal compartments with Src. We observed that Wrch-1 trafficking to endomembranes was similar to the pattern of EGFR trafficking after ligand stimulation. We hypothesized that Wrch-1 may also co-traffick with proteins known to traffick with EGFR, such as Src. To test this hypothesis, we transfected H1299 cells with GFP-Wrch-1, then serum-starved the cells and stimulated with EGF. We then fixed the cells and co-stained for Src, and visualized the cells as described above. We found that Wrch-1 did co-localize with Src at internal membranes after EGF stimulation, suggesting that Wrch-1 may traffick with components of the EGFR complex (Figure 3.5).

3.3.6 Tyrosine phosphorylation of Wrch-1 on Y254 negatively regulates migration and invasion. It is well accepted that Rho proteins are regulated in part by their subcellular localization. In line with this, we found that phosphorylation of Y254 that induces relocalization of Wrch-1 to the endosomes subsequently resulted in a decrease in active Wrch-1-GTP. We have shown previously that this relocalization and change in nucleotide status regulated specific biological events driven by Wrch-1, such as growth in soft agar and epithelial morphogenesis. Here we sought to determine whether C-terminal tyrosine phosphorylation of Wrch-1 also regulates EGF-driven properties also known to be modulated by Wrch-1, such as cell migration and/or invasion. To determine whether tyrosine phosphorylation of Wrch-1 regulated cell migration, we performed a scratch assay using H1299 cells stably expressing Wrch-1, and a nonphosphorylatable version of Wrch-1 (Y254F). We found that cells expressing Wrch-1(Y254F) more completely closed the scratch compared to cells expressing parental Wrch-1 (Figure 3.6A). Finally, we sought to determine whether this phosphorylation regulates Wrch-1-mediated invasion. To do this we analyzed the same cell lines in a Matrigel invasion assay. We found that nonphosphorylatable Wrch-1 [Wrch-1(Y254F)] increased Matrigel invasion, compared to parental Wrch-1 (Figure 3.6B).

Taken together with our previous studies, these data suggest that phosphorylation of Wrch-1 at Y254 may be a general mechanism for negative regulation of Wrch-1-mediated biological activities.

3.4 Discussion

We show here that the atypical Rho family small GTPase Wrch-1 undergoes tyrosine phosphorylation at a key residue in its membrane targeting domain in response to EGF stimulation and downstream of constitutively active EGFR family tyrosine kinases. Our previous work on phosphorylation of Wrch-1 showed that Src expression and kinase activity was required for Wrch-1 tyrosine phosphorylation at Y254 in the C-terminal hypervariable region. In this follow-up study, we show that phosphorylation of this residue downstream of EGFR family members regulates trafficking of Wrch-1 from the plasma membrane to internal membranes. Additionally we show that Src is required for EGFR-mediated tyrosine phosphorylation of Wrch-1 at this key residue that is important for both localization and biological activity.

Our previous study showed that Wrch-1 is GTP-bound and active at the plasma membrane where it is capable of interacting with and activating downstream effectors such as PAK1 and Pyk2, but inactive at endosomal membranes, where it localizes following both serum and EGF stimulation. Consistent with this hypothesis, plasma membrane-localized Wrch-1(Y254F) is enhanced in both migration and Matrigel invasion, whereas endosomally localized Wrch-1(Y254E) is impaired in the same biological outputs.

Our data suggest that Wrch-1 may undergo a trafficking mechanism for downregulation somewhat similar to RTKs. Upon persistent growth factor stimulation, RTKs such as EGFR under go ubiquitination and internalization to internal compartments where they are either

degraded or recycled to the plasma membrane. We have shown that when Wrch-1 becomes re-localized to endosomes, it becomes GDP-bound and inactive. In this study we show that upon growth factor stimulation, Wrch-1 relocalizes from the plasma membrane to internal membranes including early endosomes, recycling endosomes, and lysosomes. This suggests that in addition to being turned off at the endosomes, Wrch-1 may recycle back to the plasma membrane to become activated, and some portion of Wrch-1 may be degraded.. Further studies are needed to determine whether Wrch-1 expression is altered in response to growth factor stimulation.

This is the first report that receptor tyrosine kinase stimulation results in phosphorylation and translocation of a Rho small GTPase. Although Cdc42 has been reported to be tyrosine phosphorylated by Src upon EGF stimulation, this phosphorylation occurs at residue Y64 within its switch II region rather than in the C-terminus of Cdc42, which lacks any tyrosine residue (Tu et al., 2003). Whether tyrosine phosphorylation of Cdc42 alters its subcellular localization was not explored, but it was reported to promote binding of Cdc42 to RhoGDI (Wu et al., 2003). However, Wrch-1 is not modified by an isoprenoid lipid, a feature required for binding of RhoGDI, and does not interact with RhoGDI (Berzat et al., 2005a). Thus, tyrosine phosphorylation of Wrch-1 and Cdc42 occur on distinct domains and have distinct consequences.

EGFR is known to activate small GTPases, such as Ras and RhoA. Classically, after EGFR activation, the Grb2/Sos complex promotes Ras GTP-binding and activation of the Raf/MEK/ERK kinase cascade to promote biological outcomes such as cellular proliferation (Roskoski, 2004). Furthermore, it has been shown that EGFR activation can promote increased RhoA activity, leading to an increase in RhoA-driven cell motility (Mateus et al., 2007). Additionally, in NSCLC cells, EGFR has been shown to upregulate Rac1 activity via

Vav2, resulting in an increased invasive phenotype (Marcoux and Vuori, 2003; Patel et al., 2007). Currently there are no known examples of negative regulation of a small GTPase by EGFR. However, there is some evidence that tyrosine kinases can negatively regulate Rho GTPase activity. Loss of FAK in epithelial cells promoted loss of epithelial cell morphology and inhibited cell-cell contacts, which was blocked by expression of dominant negative versions of Rho and Rac (Playford et al., 2008). Inhibition of FAK decreased tyrosine phosphorylation of p190RhoGAP and increased Rho and Rac GTP levels (Playford et al., 2008).

In this work, we have shown that EGFR is an upstream mediator of Wrch-1 tyrosine phosphorylation, and that this phosphorylation regulates Wrch-1-mediated migration. Although beyond the scope of this paper, there is also the possibility that tyrosine phosphorylation of Wrch-1 may also regulate EGFR expression or signaling. In this paper, we have shown that Wrch-1 binds to Cbl in a tyrosine phosphorylation-dependent manner. Cdcc42 also binds to Cbl, and been shown to sequester Cbl and increase abundance of EGFR. Cdc42 binds to p85Cool-1 (Cloned-out-of-library)/β-Pix (Pak interacting exchange factor), which is a protein that directly associates directly with c-Cbl (Wu et al., 2003). The association of p85Cool-1/β-Pix with c-Cbl prevents EGFR ubiquitination by Cbl, therefore reducing destruction of EGFR, resulting in higher levels of EGFR protein. Activated Cdc42 was shown to block Cbl from binding to EGFR, which increased EGFR expression, sustained ERK activation and increased transformation (Wu et al., 2003). Currently, it is not known whether Wrch-1 interaction with Cbl results in the same phenomenon, and whether an intermediary protein such as p85Cool-1/β-Pix is required. Further studies aimed at addressing the role of Wrch-1 in modulating RTK signaling pathways will give us further insight into overall Wrch-1 effector pathways and biological function, and may shed light on general Rho GTPase regulation with respect to RTK signaling.

3.5 Materials and methods

3.5.1 Molecular constructs

Mammalian expression constructs for GFP-tagged and HA epitope-tagged human Wrch-(WT and Q107L) have been described previously (Berzat et al., 2005b). Phosphodeficient Wrch-1(Y254F) was generated by site-directed mutagenesis in both WT and Q107L backgrounds. All sequences were verified by the UNC-CH Genome Analysis Facility at the University of North Carolina at Chapel Hill. EGFRvIII and NeuT constructs were generous gifts from H. Shelton Earp (UNC-CH).

3.5.2 Cell culture and transfections

H1299 nonsmall cell lung cancer (NSCLC) cells were grown in Dulbecco's modified Eagle medium (high glucose) (DMEM-H, GIBCO/Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin-streptomycin (P/S) ("complete culture medium"), and maintained in 5% CO2 at 37°C. H1299 cells were transfected with expression constructs encoding Wrch-1, EGFRvIII, or NeuT proteins by using TransIT-LT1 (Mirus) according to the manufacturer's instructions. For localization assays, cells were transfected transiently and used 24 h after transfection. For selection of stably expressing cell lines, cells were grown in complete medium supplemented with the appropriate antibiotic for 5-7 days, after which >50 colonies were pooled for use.

3.5.3 Fluorescence, immunofluorescence, confocal microscopy and localization assays

Cells were transfected transiently with pEGFP-Wrch-1 expression vectors as indicated above and grown in complete medium for 24 h. The cells were then either grown overnight in complete medium ("basal conditions"), or serum-starved overnight ("serum- starved"), or

serum-starved overnight then stimulated with complete medium containing varying concentrations of EGF ("starved + stimulated"). For some experiments, cells were treated for 1 h with the EGFR-selective inhibitor erlotinib, the dual EGFR/HER2 inhibitor lapatinib, or DMSO vehicle prior to serum stimulation. Following incubation with Alexa 647-transferrin (Molecular Probes) or Lysotracker-red (Invitrogen), cells were fixed and then visualized for GFP-Wrch-1 (green), transferrin (red), or Lysotracker (red). HA-Wrch-1 was visualized by staining with a primary anti-HA antibody (Covance) and a secondary anti-mouse antibody conjugated to Alexa 647 (Invitrogen). Src was visualized using an anti-Src antibody (Cell Signaling Technologies (CST)), and a secondary anti-rabbit antibody conjugated to Alexa 594 (Invitrogen). Early endosomes were visualized using an anti-EEA1 antibody (Abcam) and Rab 11 was visualized using an anti-Rab 11 antibody (BD Biosciences). Both were followed by incubation with anti-mouse antibody conjugated to Alexa 647 (Invitrogen). Confocal microscopy was performed on an Olympus Fluoview 500 laser scanning confocal imaging system, configured with an IX81 fluorescence microscope fitted with a PlanApo X60 oil objective.

3.5.4 Antibodies and western blot analysis

Western blot analyses were carried out as described previously (Brady et al., 2009). Briefly, cells were lysed in Magnesium Lysis Buffer (MLB) containing 1X protease inhibitor cocktail (Roche) with 100 μM pervanadate, lysates were cleared by centrifugation and protein concentrations determined using the DC Lowry protein assay (BIO-RAD). Twenty μg of protein for each sample, prepared in 5X Laemmli sample buffer, were resolved by using 12% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, Millipore), blocked overnight in 3% fish gelatin, then probed for Wrch-1 by a 1 h incubation with the following primary antibodies: anti-HA epitope (HA.11, Covance), anti-phosphotyrosine (pY100, (CST), and pY20, Santa Cruz), anti-EGFR (CST), or anti-Src

(CST). Anti-actin (Sigma) was used to demonstrate equivalent loading. Washed membranes were incubated in anti-mouse or anti-rabbit IgG-HRP (Amersham Biosciences) or anti-mouse kappa light chain-HRP (Zymed), washed again, and developed using SuperSignal West Dura extended duration substrate (Pierce).

3.5.5 Immunoprecipitation

H1299 cells expressing HA-tagged Wrch-1 were lysed in MLB with protease inhibitor with pervanadate, as described above, at 24 h after transfection. Lysates were pre-cleared with Protein A/G beads (Santa Cruz) and then incubated overnight with an anti- HA antibody. After 18 h, the protein-antibody complex was recovered using Protein A/G beads. Beads were collected and washed with MLB and resuspended in Laemmli sample buffer, the precipitated proteins were resolved on SDS-PAGE, and immunoblot analysis for phosphotyrosine was performed as described above.

3.5.6 Scratch assays for cell motility

H1299 cells stably expressing HA-tagged Wrch-1 constructs were allowed to grow to confluency in a standard 35 mm tissue culture dish. A scratch wound was then made in the confluent monolayer by using a disposable P20-200 pipet tip, and any debris was washed away twice with PBS and once with media. The wound was imaged immediately after scratching and 24 h later.

3.5.7 Matrigel invasion assays

H1299 cells stably expressing HA-tagged Wrch-1 were plated on Matrigel in serum-free culture medium in the top chamber of a Matrigel multiwell assay plate (BD Biosciences) (50,000 cells per well). Cells were allowed to invade through the Matrigel and migrate to the bottom chamber, which contained 10% FBS. After 24 h, cells in the bottom chamber were

then fixed in 100% methanol, stained with 1% bromphenol blue, and quantified using a light microscope fitted with a 20x objective.

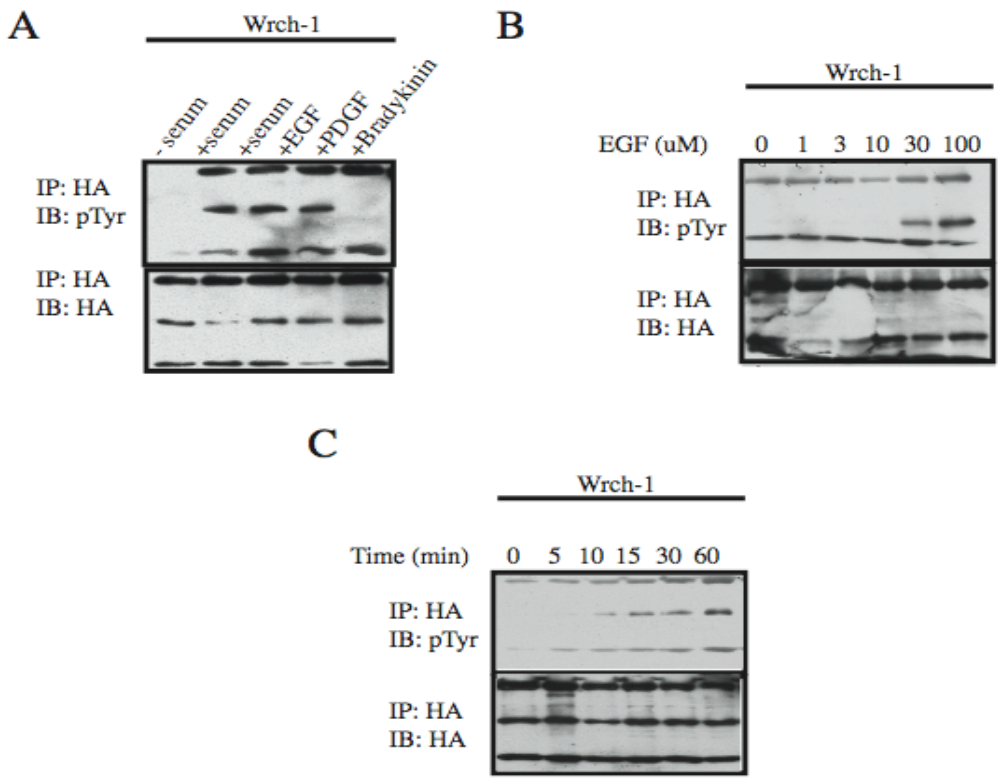


Figure 3.1A Wrch-1 is tyrosine phosphorylated in response to EGF or PDGF stimulation but not bradykinin. A) *Wrch-1 is tyrosine phosphorylated upon EGF or PDGF stimulation.* H1299 NSCLC cells expressing HA-Wrch-1 were serum-starved overnight and treated with either 10% FBS, 30 ng EGF, 100 ng PDGF, or 30 ng bradykinin for 30 min. The resulting cell lysates were incubated with anti-HA antibody. Immunoprecipitated (IP) Wrch-1 was detected by immunoblotting (IB) with anti-HA, and phosphotyrosine (p-Tyr) Wrch-1 was detected by immunoblotting with anti-phosphotyrosine antibody. The bands above and below the Wrch-1 band represent immunoglobulin heavy chain and light chain, respectively. *B) Wrch-1 is tyrosine phosphorylated in response to EGF stimulation in a dose-dependent manner.* H1299 cells expressing HA-Wrch-1 were serum starved overnight and treated with increasing concentrations of EGF, up to 100 ng for 30 min. The resulting cell lysates were incubated with anti-HA antibody. Immunoprecipitated (IP) Wrch-1 was detected by immunoblotting (IB) with anti-HA, and phosphotyrosine (p-Tyr) Wrch-1 was detected by immunoblotting with anti-phosphotyrosine antibody. The bands above and below the Wrch-1 band represent immunoglobulin heavy chain and light chain, respectively. *C) Wrch-1 is tyrosine phosphorylated in response to EGF stimulation in a time dependent manner.* H1299 cells expressing HA-Wrch-1 were serum-starved overnight and treated with 30 ng EGF for increasing amounts of time, up to 60 min. The resulting cell lysates were incubated with anti-HA antibody. Immunoprecipitated (IP) Wrch-1 was detected by immunoblotting (IB) with anti-HA, and phosphotyrosine (p-Tyr) Wrch-1 was detected by immunoblotting with anti-phosphotyrosine antibody. The bands above and below the Wrch-1 band represent immunoglobulin heavy chain and light chain, respectively.

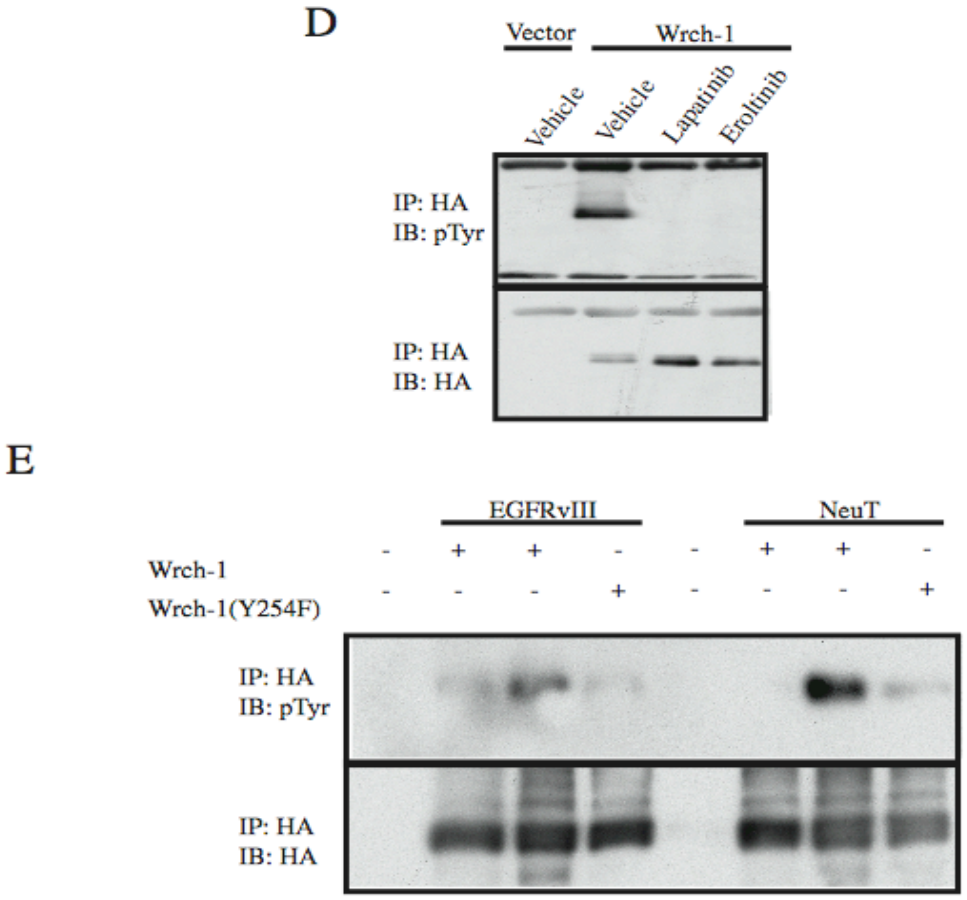


Figure 3.1 Wrch-1 is tyrosine phosphorylated in response to EGF or PDGF stimulation but not bradykinin. *D) EGF stimulated tyrosine phosphorylation of Wrch-1 can be blocked by both and EGFR and a dual EGFR/HER2 inhibitor.* H1299 cells expressing HA-Wrch-1 were serum starved overnight and treated with 30 ng EGF in the presence of either 10 μM lapatinib or 1.5 μM erlotinib. The resulting cell lysates were incubated with anti-HA antibody. Immunoprecipitated (IP) Wrch-1 was detected by immunoblotting (IB) with anti-HA, and phosphotyrosine (p-Tyr) Wrch-1 was detected by immunoblotting with anti-phosphotyrosine antibody. The bands above and below the Wrch-1 band represent immunoglobulin heavy chain and light chain, respectively. *E) Wrch-1 is tyrosine phosphorylated downstream of constitutively active EGFR family members.* H1299 cells expressing HA-Wrch-1 plus either empty vector, EGFRvIII or NeuT were lysed. The resulting cell lysates were incubated with anti-HA antibody. Immunoprecipitated (IP) Wrch-1 was detected by immunoblotting (IB) with anti-HA, and phosphotyrosine (p-Tyr) Wrch-1 was detected by immunoblotting with anti-phosphotyrosine antibody. The bands above and below the Wrch-1 band represent immunoglobulin heavy chain and light chain, respectively.

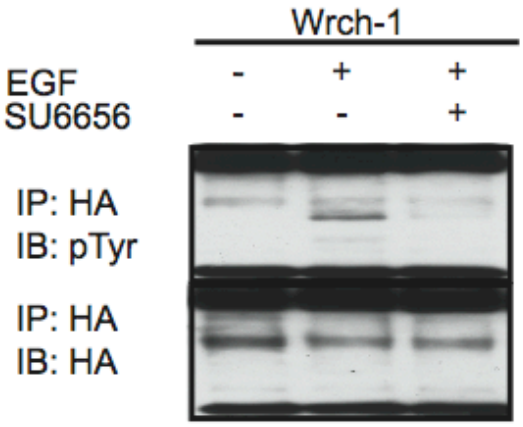


Figure 3.2 Wrch-1 tyrosine phosphorylation in response to EGF is Src dependent. H1299 cells expressing HA-Wrch-1 were serum-starved overnight and treated with 30 ng EGF for 30 min in the presence or absence of 5 μM SU6656. The resulting cell lysates were incubated with anti-HA antibody. Immunoprecipitated (IP) Wrch-1 was detected by immunoblotting (IB) with anti-HA, and phosphotyrosine (p-Tyr) Wrch-1 was detected by immunoblotting with anti-phosphotyrosine antibody. The bands above and below the Wrch-1 band represent immunoglobulin heavy chain and light chain, respectively.

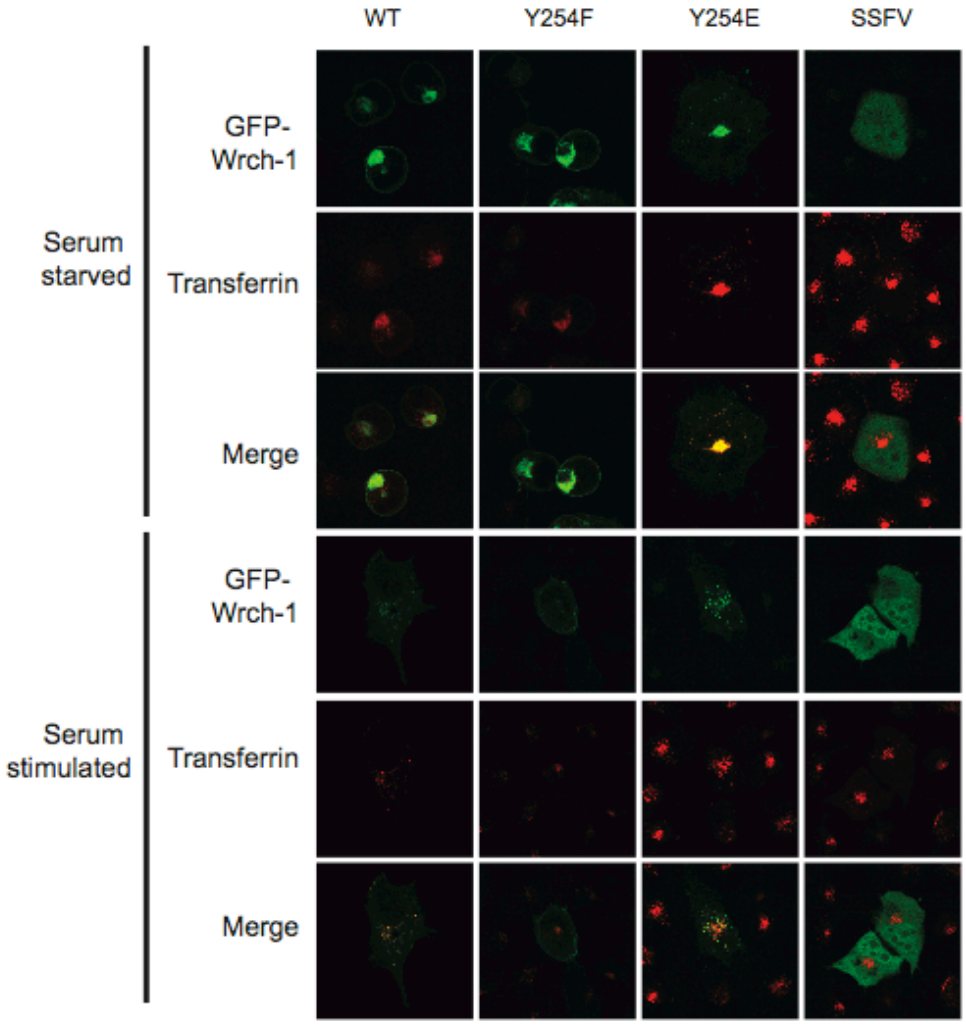


Figure 3.3 EGF stimulation causes Wrch-1 to traffick to endosomal compartments. H1299 cells expressing GFP-Wrch-1 were grown in complete culture medium, then serum-starved overnight, or first serum-starved overnight and stimulated with 30 ng EGF. Prior to EGF stimulation, the treated cells were incubated for 1 h with Alexafluor 647-labeled transferrin to mark endosomal compartments. After 30 min of EGF stimulation, cells were fixed, then subjected to confocal microscopy for visualization of GFP-Wrch-1 (green) or transferrin (red).

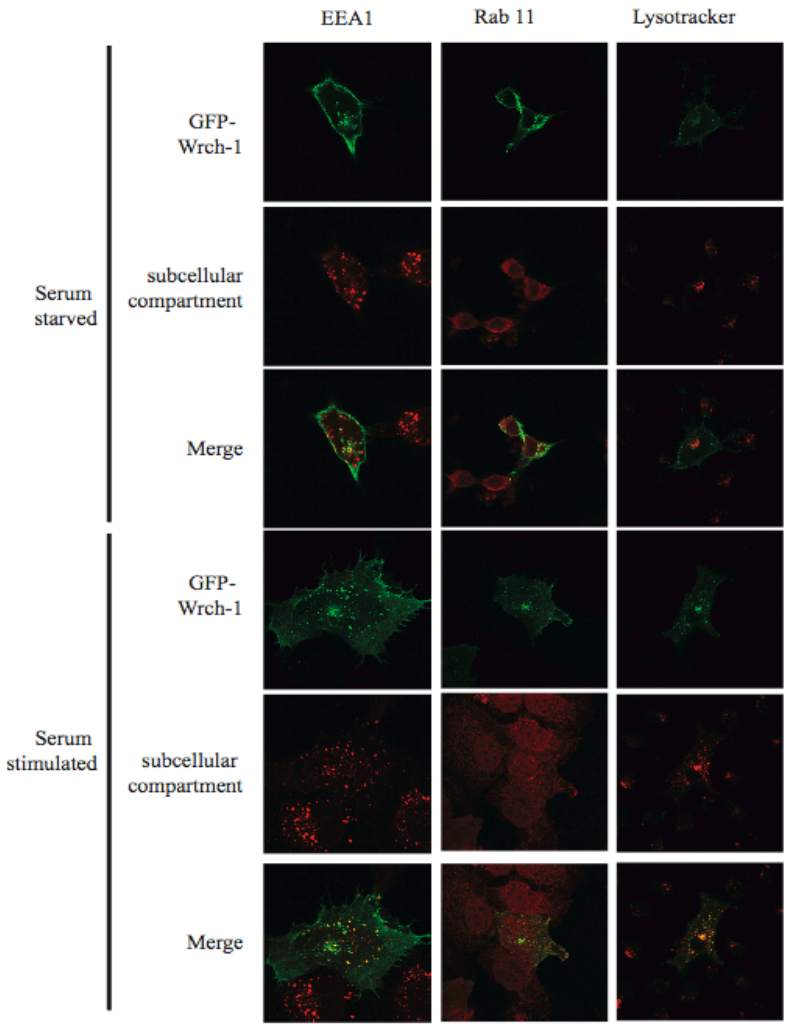


Figure 3.4 EGF stimulation causes Wrch-1 to traffick to early endosomes, recycling endosomes and lysosomes. H1299 cells expressing GFP-Wrch-1 were grown in complete culture medium, then serum-starved overnight, or first serum-starved overnight and stimulated with 30 ng EGF. Prior to EGF stimulation, the treated cells were incubated for 1 h with Lysotracker-red to mark lysosomes. After 30 min of EGF stimulation, cells were fixed, stained with EEA1 or Rab 11 then subjected to confocal microscopy for visualization of GFP-Wrch-1 (green) or EEA1 (red), Rab 11 (red), or Lysotracker (red) .

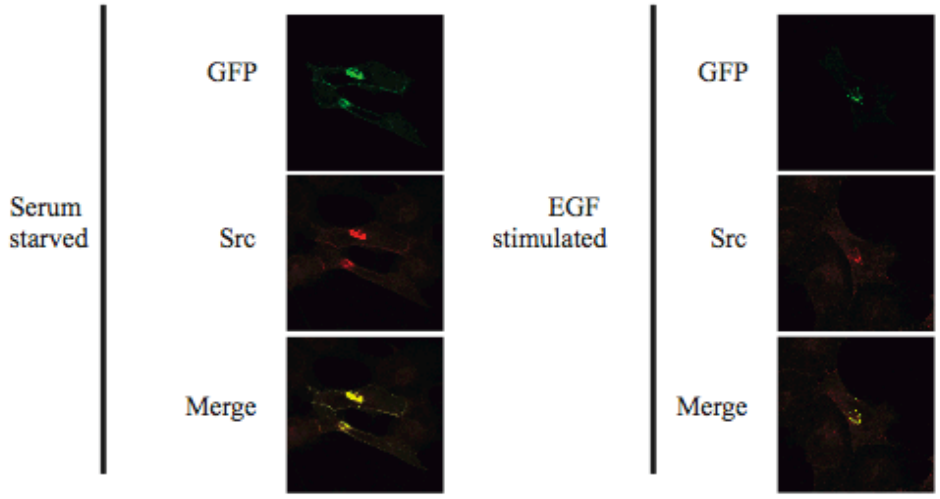


Figure 3.5 EGF stimulation causes Wrch-1 to traffic to endosomal compartments with Src. H1299 cells expressing GFP-Wrch-1 were grown in complete culture medium, then serum-starved overnight, or first serum-starved overnight and stimulated with 30 ng EGF. After 30 min of EGF stimulation, cells were fixed, stained with anti-Src, then subjected to confocal microscopy for visualization of GFP-Wrch-1 (green) or Src (red).

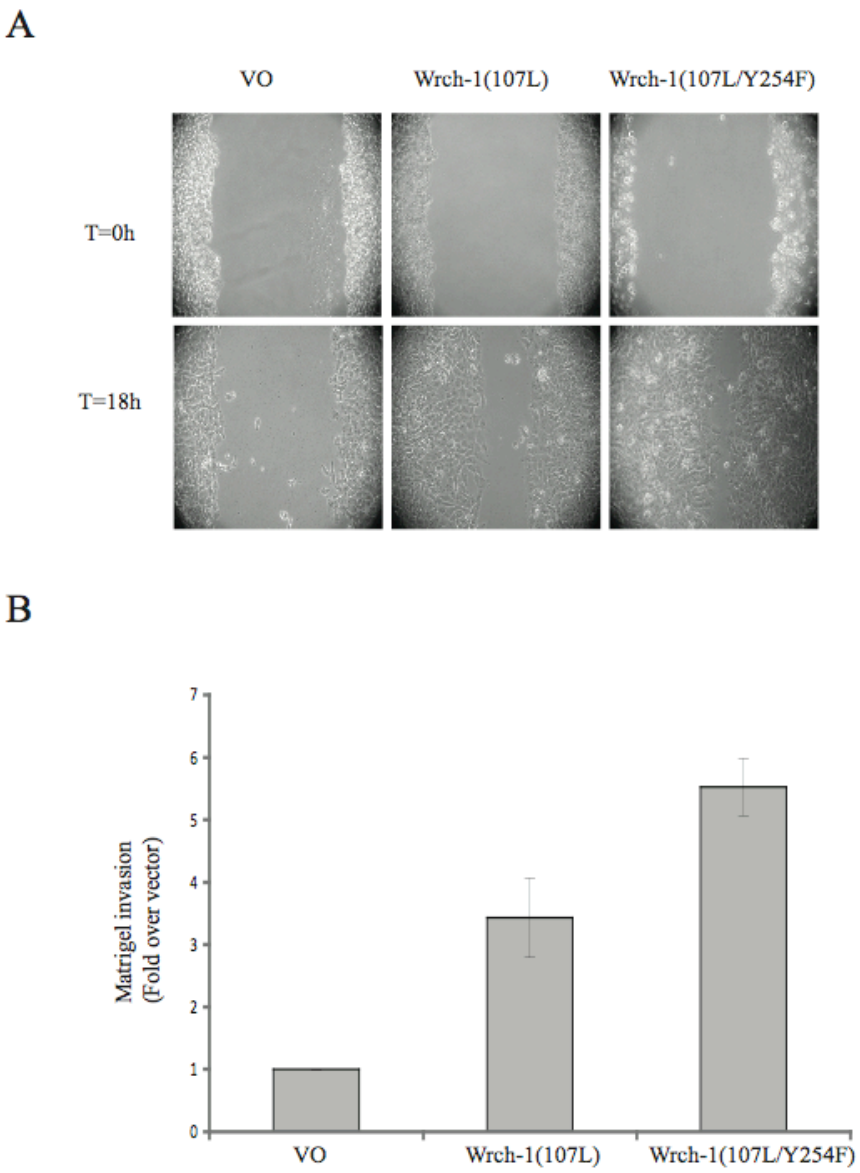


Figure 3.6 Tyrosine phosphorylation of Wrch-1 on Y254 negatively regulates migration and invasion. *A) Phosphorylatable tyrosine residue Y254 negatively regulates migration.* H1299 cell lines were generated to stably express either vector only, HA-Wrch-1(107L) or HA-Wrch-1(107L/Y254F). Cells were allowed to grow to confluency, and were then scratched with a P200 disposable pipet tip. Cells were visualized at time 0 and also 24 h later. *B) Phosphorylatable tyrosine residue Y254 negatively regulates Matrigel invasion.* H1299 cell lines were generated to stably express either vector only, HA-Wrch-1(107L), or HA-Wrch-1(107L/Y254F). Fifty thousand cells were plated into the top chamber in 0% serum, and allowed to migrate for 24 h toward the bottom chamber containing 10% serum. Cells were then fixed in 100% methanol and stained with 1% bromophenol blue. The number of cells that migrated through the Matrigel was quantified using a light microscope with a 20x objective, and represented as fold over vector. A two-tailed *t*-test was done to determine significance of differences between the cell lines.

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

4.1 Summary

My research on Wrch-1 has uncovered a novel mechanism of post-translational control of Wrch-1 localization and function. As discussed previously, phosphorylation of the membrane targeting domains of Rho proteins is emerging as a general mechanism to control their localization and function. Currently, most of the research regarding this type of phosphorylation of Rho proteins has focused on Ser/Thr phosphorylation (Okada, 2008 #159; Lang, 1996 #41; Forget, 2002 #140; Riento, 2005 #149; Madigan, 2009 #435). To date there is one report of tyrosine phosphorylation of a Rho family protein. Cdc42 was shown to be tyrosine phosphorylated, but in the switch II region, and this phosphorylation resulted in decreased Cdc42 activation by altering RhoGDI binding (Tu et al., 2003). My dissertation research has provided the first report of C-terminal tyrosine phosphorylation as a mechanism for controlling proper localization and function of Rho family small GTPases.

I have shown here that serum and growth factor stimulation results in an increase in C-terminal phosphorylation on Y254 of Wrch-1, which then results in relocalization of Wrch-1 from the plasma membrane to endosomes. Upstream receptor tyrosine kinases (RTKs) involved in this phosphorylation event include EGFR, PDGFR, and Src. Currently it is not known whether other growth factors in serum such as HGF, IGF, NGF, etc., can also induce tyrosine phosphorylation of Wrch-1. After relocalization to endosomes, Wrch-1 becomes GDP-bound, and activation of two of its effectors, PAK1 and Pyk2, is decreased. It would

be interesting to determine whether activation of other Wrch-1 effectors, such as Par6, PLCγ, and PI3K, is also altered. I have shown here that Wrch-1-mediated biological endpoints such as migration, Matrigel invasion, anchorage-independent growth, and epithelial cell morphogenesis are all negatively regulated by phosphorylation of Wrch-1 at Y254. It would also be interesting to examine other biological activities in which has been shown to play a role, such as osteoclastogenesis, cell:cell and cell: substrate adhesion, and cell cycle control.

In this dissertation, I have shown that Wrch-1 can be tyrosine phosphorylated in response to EGF and PDGF stimulation. However, I have neither characterized a role for Wrch-1 phosphorylation in regulating signaling from EGFR or PDGFR, nor determined whether Wrch-1 is necessary for any of the biological activities driven by these receptors. There is still more to discover about how Wrch-1 contributes to RTK signaling pathways. This line of inquiry may uncover novel signaling pathways and will contribute to our overall knowledge of these pathways.

Although I have concentrated here on characterizing the role of C-terminal tyrosine phosphorylation in Wrch-1 localization, activation and function, there are additional C-terminal post-translational modifications that can also contribute to Wrch-1 regulation. For example, Wrch-1 is di-mono-ubiquitinated (as discussed below), and at least one of these ubiquitin molecules is incorporated into the C-terminal membrane-targeting region. Further characterization of this modification is needed, and may help lend insight into both Wrch-1 and general Rho GTPase regulation. In this chapter, I discuss four specific research directions that are logical extensions of my dissertation work and that may prove fruitful in the near future to enhance our understanding of Wrch-1 regulation and biological activity.

4.2 Future Direction 1: Determine how PDGFR-mediated phosphorylation alters Wrch-1 localization and function. Over 20 years ago, it was shown that the transforming retroviral oncogene *v-sis* is derived from the β-chain of PDGFR (platelet-derived growth factor receptor), which highlighted the importance of PDGF signaling in carcinogenesis (Doolittle et al., 1983; Waterfield et al., 1983). PDGFR is a receptor tyrosine kinase that is an important upstream regulator of cell proliferation, cellular differentiation, and cell growth and development (Andrae et al., 2008). Although PDGFR can elicit many of the same biological functions as EGFR, it has additionally been implicated in regulating tumor stromal fibroblasts and tumor angiogenesis. The PDGF family of ligands consists of PDGF-A, -B, -C, and -D which form either homo- or heterodimers (PDGF-AA, -AB, -BB, -CC, -DD) (Bergsten et al., 2001; LaRochelle et al., 2001; Li et al., 2000). The best-studied PDGF ligands are PDGF-A and -B. The four PDGFs are inactive in their monomeric forms, and are required to dimerize to form an active ligand. PDGF receptors consist of two isoforms, α and β (Figure 4.1) (Johnsson et al., 1982). Upon ligand binding, PDGFR subunits also dimerize in αα, ββ, or αβ combinations. Once ligand-induced dimerization occurs, transphosphorylation and activation of the receptor ensues to elicit downstream signaling (Andrae et al., 2008; Kazlauskas and Cooper, 1989). The best characterized effectors of PDGFR include the Ras/mitogen-activated protein kinase (MAPK) (Zubiaur et al., 1996), PI-3 kinase (Kazlauskas and Cooper, 1990), STATs (Darnell, 1997), PLCγ (Kundra et al., 1994), and Src (Ralston and Bishop, 1985), along with the adaptor proteins Grb2 (Matuoka et al., 1993), Nck (Nishimura et al., 1993), Grb7 (Yokote et al., 1996), and Crk (Nishimura et al., 1993) (Figure 4.2). Although much remains to be learned about the regulation of PDGFR trafficking and endosomal signaling, PDGFR does undergo endosomal trafficking in a pattern similar to that of EGFR, and can elicit signaling from both the plasma membrane and endosomes (Wang et al., 2004). In this dissertation I have shown that Wrch-1 is tyrosine

phosphorylated downstream of PDGF-ββ stimulation. Previously it was shown that Wrch-1 binds the adaptor proteins Grb2 and Nckβ (Saras et al., 2004; Shutes et al., 2004)), both of which also bind PDGFR (Andrae et al., 2008; Bazenet et al., 1996). Interestingly, PDGF-ββ stimulation of cells reversed Wrch-1-induced cell rounding (Saras et al., 2004). The authors hypothesized that PDGF signals to Rac1 to overcome the rounded phenotype, because activated Rac conferred a similar phenotype. However, reversion of Wrch-1-induced rounding after PDGF stimulation is also consistent with our model in which PDGF stimulates phosphorylation of Wrch-1 at Y254, which then re-localizes Wrch-1 to endosomes, where it becomes GDP-bound and inactive. At present I have shown only that PDGF, like EGF, stimulates Wrch-1 tyrosine phosphorylation, but not whether this phosphorylation, like that mediated by EGF, also regulates trafficking of Wrch-1. Additionally it is not known whether PDGFR can phosphorylate Wrch-1 directly, or whether an intermediary kinase such as Src is required for this phosphorylation. Further investigation is needed to determine how phosphorylation of Wrch-1 downstream of PDGFR alters Wrch-1 localization and function. To test this, it will be important to first establish appropriate conditions for these assays. I would first determine a dose response curve for PDGF-stimulated phosphorylation of Wrch-1. The specificity of the stimulation could also be examined by blocking with a PDGFR inhibitor, such as CP-673451, which has been shown to be an inhibitor of PDGFRβ (Roberts et al., 2005). Once the EC_{50} is determined for PDGF in our system, this dose can be used to stimulate cells for varying amounts of time, to determine the kinetics of Wrch-1 phosphorylation by PDGFR. Additionally, it would be useful to determine which PDGFR dimer(s) are contributing to Wrch-1 tyrosine phosphorylation. This could be accomplished by either using specific PDGF ligand dimers, or by overexpressing, or knocking down specific PDGFR subunits in our system.

It would be of interest to determine whether Wrch-1 can modulate PDGFR-driven activities such as angiogenesis or fibrogenesis. Anginogenesis could be evaluated *in vitro* using a tube formation assay, or *in vivo* using a zebrafish or mouse model system. Fibrinogenesis could be evaluated *in vitro* using a hydroxyproline assay,which measures collagen content, or *in vivo* in a mouse model system by tissue harvesting and staining for fibrotic tissue, or by the hydroxyproline assay. If Wrch-1 does regulate angiogenesis or fibrogenesis, further investigation would be needed to determine whether phosphorylation of Wrch-1 by PDGFR is important for its contribution to these events. In order to investigate this possibility, Wrch-1 phosphodeficient (Y254F) or phosphomimetic (Y254E) mutants that are RNAi-resistant could be used to try to rescue knockdown of Wrch-1.

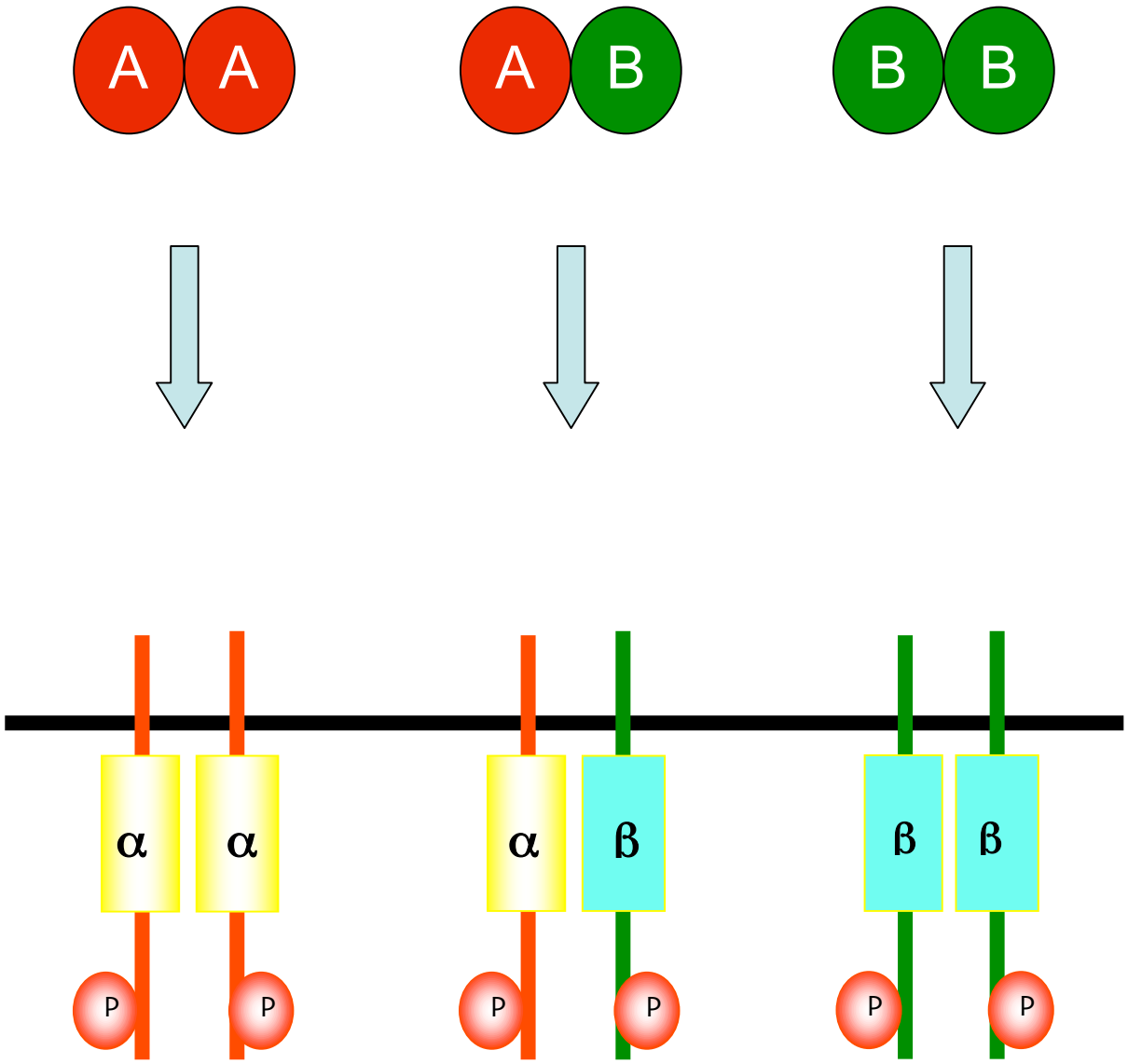


Figure 4.1 Schematic of dimerization of PDGF ligands and receptors (PDGFR). The four PDGF ligands (A,B,C,D) are inactive in their monomeric forms, and are required to dimerize to form an active ligand. PDGF receptors consist of two isoforms, α and β (upon ligand binding, PDGFR subunits also dimerize in either $\alpha\alpha$, $\beta\beta$, or $\alpha\beta$ combinations. Once ligand-induced dimerization occurs, transphosphorylation and activation of the receptor elicits downstream signaling.

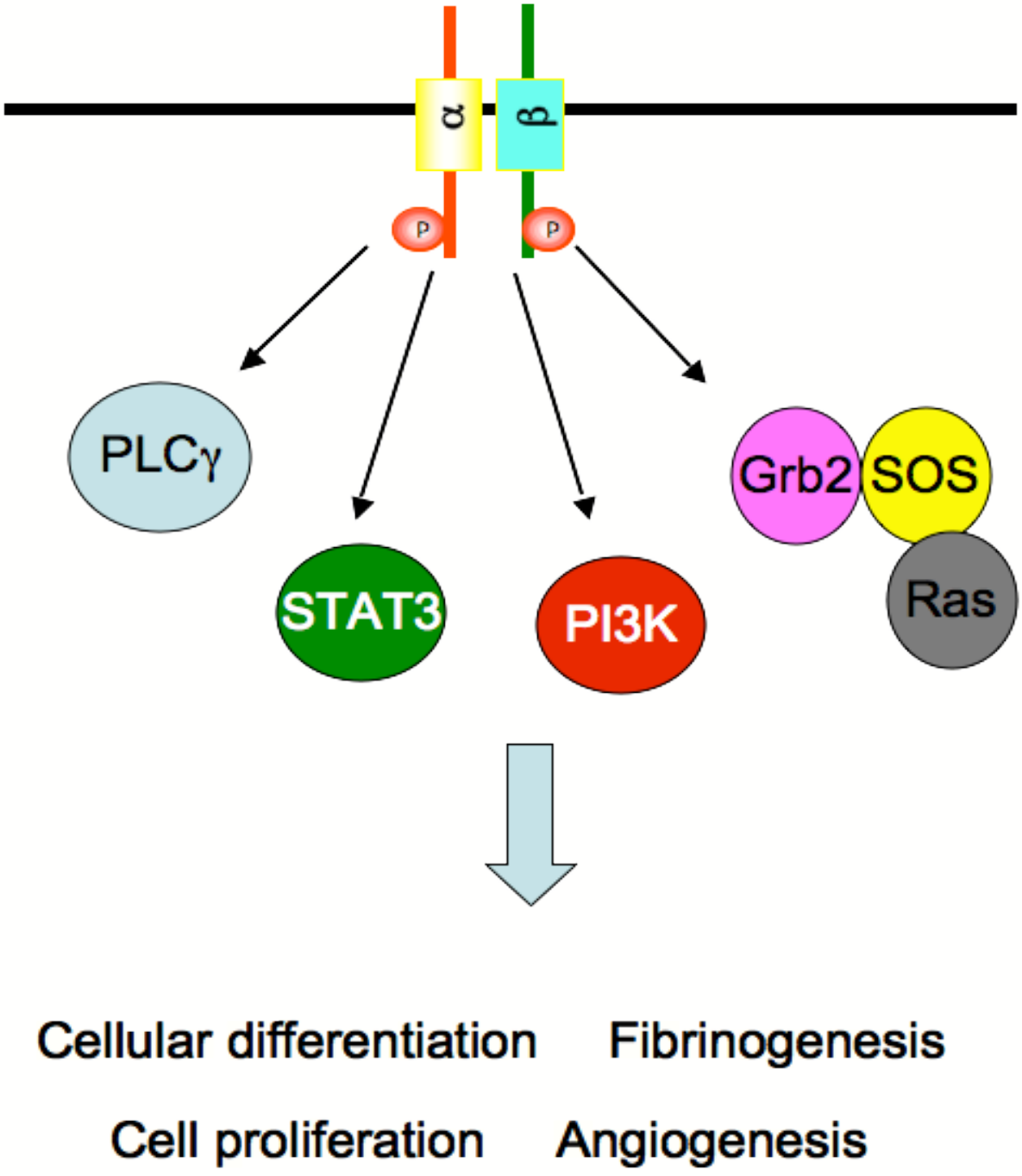


Figure 4.2 Schematic of PDGR signaling pathways. The major signaling pathways downstream of PDGFR include PLCγ, STAT3, PI3K, and the Grb2-SOS-Ras-MAPK pathway. PDGFR uses these effectors to elicit biological activities such as differentiation, proliferation, fibrinogenesis and angiogenesis.

4.3 Future Direction 2: Determine if Wrch-1 is necessary for EGFR- and PDGFR-mediated biological functions. As stated previously, EGFR is involved in cell proliferation, angiogenesis, and adhesion, migration, and invasion (Roskoski, 2004). PDGFR is involved in cell proliferation, cellular differentiation, cell growth and development, angiogenesis, and fibrogenesis (Andrae et al., 2008). Although much is known about EGFR and PDGFR functions, the role of Wrch-1 downstream of either of these receptors is not well characterized. In this dissertation, I have shown that both EGFR and PDGFR are upstream of Wrch-1 phosphorylation at Y254, and that this modification of Wrch-1 regulates its ability to mediate anchorage-independent growth, single cell migration, Matrigel invasion, and epithelial cell morphogenesis. However, it would be useful to determine whether Wrch-1 is necessary downstream of EGFR and PDGFR, by knocking down Wrch-1 and determining whether cellular responses driven by these receptors are impaired. For example, Wrch-1 could be knocked down in a cell line that utilizes EGFR signaling for functions such as cell proliferation or migration. Cell proliferation could be measured in control scrambled siRNA cells and Wrch-1 knockdown cells using BrdU incorporation or a luminescent cell viability assay, whereas migration could be measured using single cell migration assays, scratch assays and/or Matrigel invasion assays. It would also be of interest to determine whether Wrch-1 can modulate PDGFR-driven functions such as angiogenesis or fibrogenesis, as described above, and if so, whether the phosphodeficient or phosphomimetic mutants can rescue the Wrch-1 knockdown. Based on the data presented in this dissertation, we would predict that rescue with phosphodeficient Wrch-1 would restore Wrch-1-driven phenotypes such as transformation and migration, whereas phosphomimetic Wrch-1 would not.

If Wrch-1 is necessary for EGFR or PDGFR signaling, it would be useful to try to determine where Wrch-1 lies in the EGFR and/or PDGFR signaling pathway. EGFR, PDGFR and Wrch-1 interact with many of the same proteins including Grb2, Cbl, PLCγ, PI3K and Src

(Andrae et al., 2008; Chuang et al., 2007; Hynes and Lane, 2005; Shutes et al., 2006). However, the exact interplay of these RTKs and Wrch-1 signaling has not been well studied. In this dissertation I have shown that EGFR family members and PDGFR can mediate tyrosine phosphorylation of Wrch-1. Additionally I have shown that EGFR regulates tyrosine phosphorylation of Wrch-1 in a Src-dependent manner. However, I have not examined how Wrch-1 signaling downstream of EGFR and PDGFR phosphorylation interplays with other signaling pathways driven by these receptors. Wrch-1 may act in an uncharacterized linear pathway downstream of EGFR and/or PDGFR phosphorylation, or it may function in a parallel pathway. The current evidence presented in this dissertation suggests that, upon tyrosine phosphorylation by upstream kinases, Wrch-1 relocalizes to endosomes, where it becomes GDP-bound and inactive. Since Wrch-1 expression has been shown to be regulated by Wnt signaling and STAT3 activation (Schiavone et al., 2009; Taneyhill and Pennica, 2004; Tao et al., 2001), it is easy to speculate that in certain tissue types, or at certain points in development, Wrch-1 expression is increased or decreased to fine-tune RTK signaling. In support of this notion, Wrch-1 expression has been shown to be altered in cancers, but whether it is upregulated or downregulated depends on tumor type (Kirikoshi and Katoh, 2002). Further, either constitutive activation or knockdown of Wrch-1 disrupts proper epithelial cell morphogenesis (Brady et al., 2009), suggesting that a proper amount of Wrch-1 is required for cell homeostasis. In order to study precisely what signals control Wrch-1 expression, Wrch-1 levels could be measured at the transcript level by RT-PCR after stimulation by various Wnts and by increasing STAT3 activity by activating the upstream regulator Jak (Janus kinase) (Shuai et al., 1993). NSC74859, a small molecule inhibitor of STAT3 (Siddiquee et al., 2007a; Siddiquee et al., 2007b), could also be used to block the induction of Wrch-1 expression that occurs upon increased STAT3 activity. Currently, there is no commercially available antibody that can detect endogenous Wrch-1, although several attempts have been made by multiple labs and one group has claimed to have such a

reagent (Brazier et al., 2009). Further efforts should be made to generate such an antibody, as it would be a valuable tool to explore upstream factors that control Wrch-1 expression.

In order to explore the role of Wrch-1 in modulating RTK signaling, it would be beneficial to further examine overlapping effector pathways utilized by EGFR, PDGFR and Wrch-1. In this dissertation, I have shown that growth factor stimulation results in the relocalization of Wrch-1 from the plasma membrane to internal membranes, and this relocalization results in deactivation of Wrch-1. I have examined activation of two downstream effectors, Pak1 and Pyk2. However, activation of other downstream effectors such as PI3K and PLCγ has not been examined. PI3K activity downstream of wild-type, constitutively active, and dominant negative Wrch-1 can be measured using a FRET based sensor (Ananthanarayanan et al., 2008) or by measuring phosphorylation states of PI3K substrates. PLCγ activity downstream of Wrch-1 can be assessed using radioactive assays measuring [^{3}H] PIP_2.

Furthermore, long-term effects of growth factor stimulation on Wrch-1 phosphorylation, localization, activation, and biological outcomes have yet to be examined. My preliminary evidence suggests that Wrch-1 recycles back to the plasma membrane after phosphorylation-stimulated relocalization to internal membranes (data not shown), suggesting that cycling between a phosphorylated and unphosphorylated state occurs. Further studies aimed at addressing the spatiotemporal aspects of Wrch-1 tyrosine phosphorylation need to be done. Here, I have studied Wrch-1 activation status by pulldowns using GST-PAK-PBD and by imaging its ability to recruit GFP-PAK-PBD. Wrch-1-GTP could also be assessed by using a split GFP complementation assay (Chun et al., 2007), where GFP is split into two parts, one half that would be associated with either Wrch-1 and another half associated with an effector such as Pak, which come together to form a functional GFP molecule only when Wrch-1 is GTP-bound and therefore capable of effector

interaction. Alternatively, the two halves of GFP could be fused to Wrch-1 itself, and engineered so that when the switch I and switch II regions underwent a conformational change after GTP binding, the two halves would come together to produce a measurable fluorescent signal, which would allow monitoring of Wrch-1 independent of an exogenous effector. These probes could provide a dynamically reversible system to study spatiotemporal control of Wrch-1 activation in response to growth factor stimulation and subsequent tyrosine phosphorylation.

An additional possibility is that Wrch-1 may modulate RTK signaling by altering receptor abundance. For example, Cdc42 has been shown to sequester Cbl and increase expression of EGFR. Cdc42 binds to p85Cool-1 (Cloned-out-of-library)/β-Pix (Pak interacting exchange factor), which is a protein that directly associates directly with c-Cbl (Wu et al., 2003). The association of p85Cool-1/β-Pix with c-Cbl prevents EGFR ubiquitination by Cbl, therefore reducing destruction of EGFR, resulting in higher EGFR expression levels. Activated Cdc42 was shown to block Cbl from binding to EGFR, which increased EGFR expression, sustained ERK activation and increased transformation (Wu et al., 2003) (Figure 4.3). Currently, it is not known whether Wrch-1 interaction with Cbl results in the same phenomenon. First, it would be useful to determine whether Wrch-1 binds to p85Cool-1/β-Pix. On the other hand, Wrch-1 has been shown to bind Cbl in a phosphorylation-dependent manner; therefore Wrch-1 could act by directly sequestering Cbl, even if Wrch-1 does not bind to p85Cool-1/β-Pix. Next it would be useful to determine whether Wrch-1 activation increases EGFR expression levels, EGF-stimulated ERK activation, and/or EGFR-dependent transformation. Further studies aimed at addressing the role of Wrch-1 in modulating RTK signaling pathways will give us further insight into overall Wrch-1 effector pathways and biological function, and may provide insight on general Rho

GTPase regulation with respect to RTK signaling.

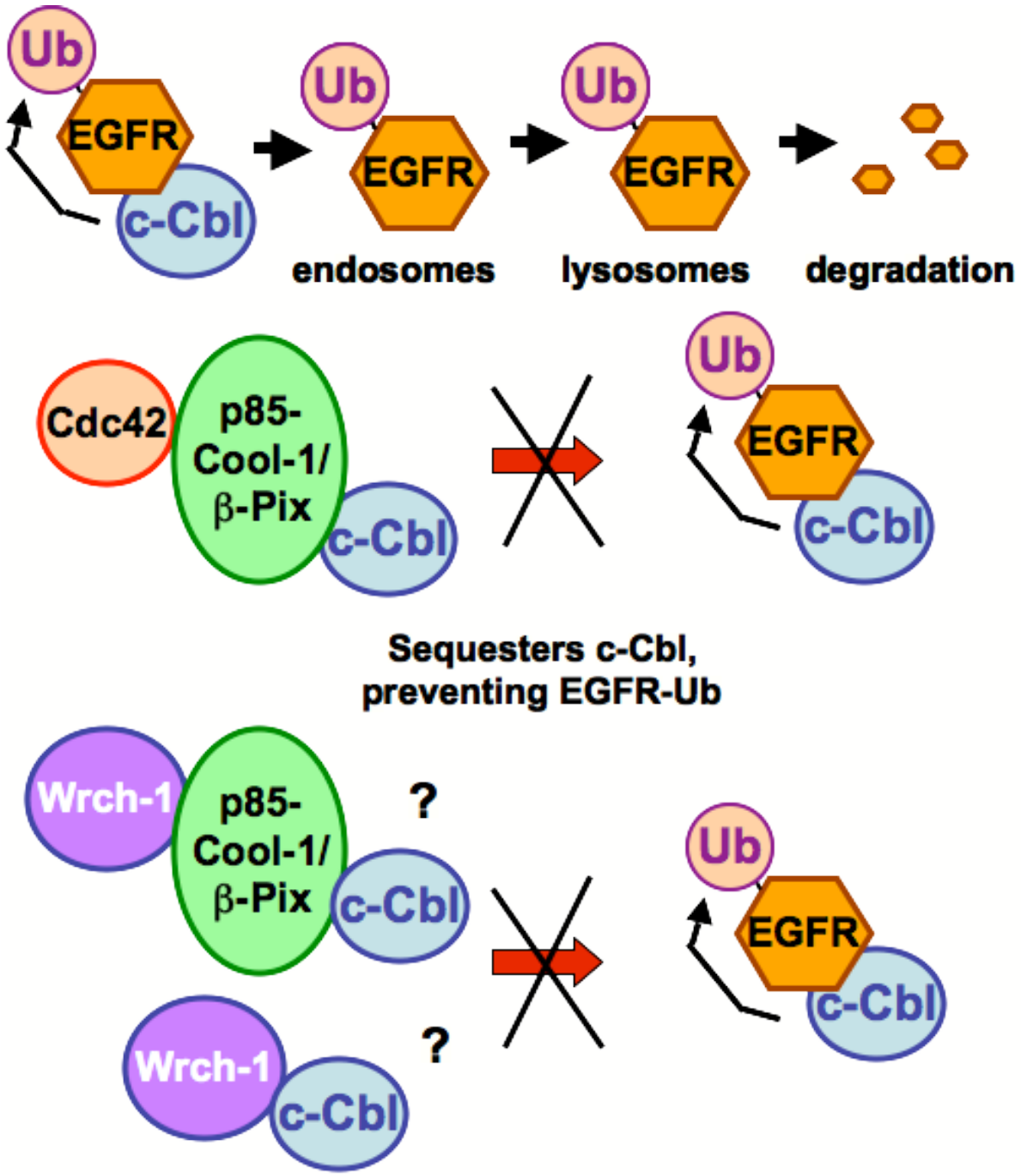


Figure 4.3 Cdc42 sequesters c-Cbl through binding to p85-Cool-1/β-Pix, resulting in decreased degradation and increased abundance of EGFR. Wrch-1 binds to Cbl in a serum-dependent manner, and may also prevent degradation of EGFR either by binding to p85-Cool-1/β-Pix or by directly sequestering c-Cbl.

4.4 Future Direction 3: Determine how Wrch-1 C-terminal ubiquitination contributes to biological function. Wrch-1 has eight lysines in its C-terminal tail that may be subject to mono-ubiquitination (Figure 4.4A). As previously determined by Anastacia Berzat, a former graduate student in our group, Wrch-1 is di-mono-ubiquitinated (Figure 4.4B). Ubiquitination involves the covalent attachment of ubiquitin (an 8 kDa protein) to cellular proteins (Hershko and Ciechanover, 1998; Pickart and Eddins, 2004). Ubiquitin is activated in an ATP-dependent manner by a ubiquitin-activating enzyme known as an enzyme-1 (E1) (Walden et al., 2003). The E1 along with a ubiquitin-conjugating enzyme, or E2, and a ubiquitin-protein ligase (E3) covalently attaches ubiquitin to a lysine in the substrate protein (Huang et al., 1999; Pickart and Eddins, 2004). Ubiquitin is covalently attached to proteins as either a single monomer (mono-ubiquitination) or a long chain (polyubiquitination). Polyubiquitination usually serves as a signal for degradation by the 26S proteasome (Finley et al., 1994; Pickart and Eddins, 2004), whereas mono-ubiquitination regulates protein trafficking, often to internal cellular membranes such as endosomes and lysosomes (Figure 4.5) (Finley et al., 1994; Haglund and Dikic, 2005). Some Rho family proteins, such as RhoA, Cdc42, and Rac1, are polyubiquitinated and degraded in response to the bacterial toxin CHF (Doye et al., 2006). Ubiquitination of H-Ras regulates its localization and decreases its signaling to the MAPK pathway (Jura et al., 2006). Wrch-1 is mono-ubiquitinated at one of three lysines, K242, K244, or K248, in its C-terminal tail (Figure 4.6A), and this ubiquitination does not result in an increase in Wrch-1 degradation (Figure 4.6B). The exact lysine that is ubiquitinated cannot be determined because Wrch-1 is still a substrate for ubiquitination even after a single or double mutation of each of these lysines (Figure 4.7C), presumably due to nonspecific ubiquitination upon loss of the preferred residue. We hypothesized that ubiquitination of Wrch-1 may regulate its biological function. Consistent with this, we found that mutation of the ubiquitinated lysines of Wrch-1 to arginines negatively regulated Wrch-1-mediated anchorage-independent growth (Figure 4.8). We hypothesized that, like tyrosine

phosphorylation, mono-ubiquitination of Wrch-1 may regulate its subcellular localization and subsequent biological function. However, we were unable to detect an alteration in Wrch-1 localization (Figure 4.9).

Alternatively, Wrch-1 ubiquitination may alter effector binding. In a recent study, it was found that ubiquitin could bind to a subset of SH3 domains (Stamenova et al., 2007). Classically, SH3 domains promote protein-protein interactions by binding to PxxP motifs. Certain proteins, such as the yeast protein Sla1 and the mammalian protein CIN85, contain an SH3 domain capable of binding ubiquitin molecules (He et al., 2007; Stamenova et al., 2007). PLCγ, a known effector of Wrch-1, contains an SH3 motif that is highly homologous to the SH3 domain of CIN85. Therefore it is possible that ubiquitination of Wrch-1 could modulate binding to effectors, such as PLCγ. In order to test this hypothesis, co-immunoprecipitations could be performed with parental Wrch-1 or mutants of Wrch-1 that cannot be ubiquitinated, along with known effectors of Wrch-1 that contain an SH3 domain, such as PLCγ, Grb2, or Nckβ. In order to further explore this possibility, activation of effectors could be examined in the presence of Wrch-1 that can and cannot be ubiquitinated.

It was also discovered that ubiquitin molecules could compete with PxxP motifs for interaction with SH3 domains (Stamenova et al., 2007). Wrch-1 contains several PxxP motifs in its N-terminal extension that are capable of binding to adaptor proteins such as Grb2 and Nckβ. Binding of the N-terminal PxxP motifs in Wrch-1 to SH3-containing proteins is thought to relieve Wrch-1 auto-inhibition by its N-terminal domain. Therefore, ubiquitination of Wrch-1 could compete for SH3 binding, thus promoting increased auto-inhibition of Wrch-1, and negatively regulating Wrch-1 function. This hypothesis could be

addressed in part by using GST-PAK-PBD pulldown assays and GFP-PAK-PBD recruitment assays to examine GTP levels in Wrch-1 proteins that can or cannot be ubiquitinated.

Additionally, further investigation into how ubiquitination alters Wrch-1 biological function is needed. To date, I have examined only ubiquitin regulation of Wrch-1-mediated anchorage-independent growth. However, Wrch-1 is known to contribute to a myriad of other biological functions such as adhesion, motility, migration, invasion, osteoclastogenesis, and epithelial cell morphogenesis. To further test the hypothesis that ubiquitination regulates Wrch-1 biological function, I propose to examine the contribution of mutants of Wrch-1 that can or cannot be ubiquitinated to single cell migration, Matrigel invasion, and epithelial cell morphogenesis.

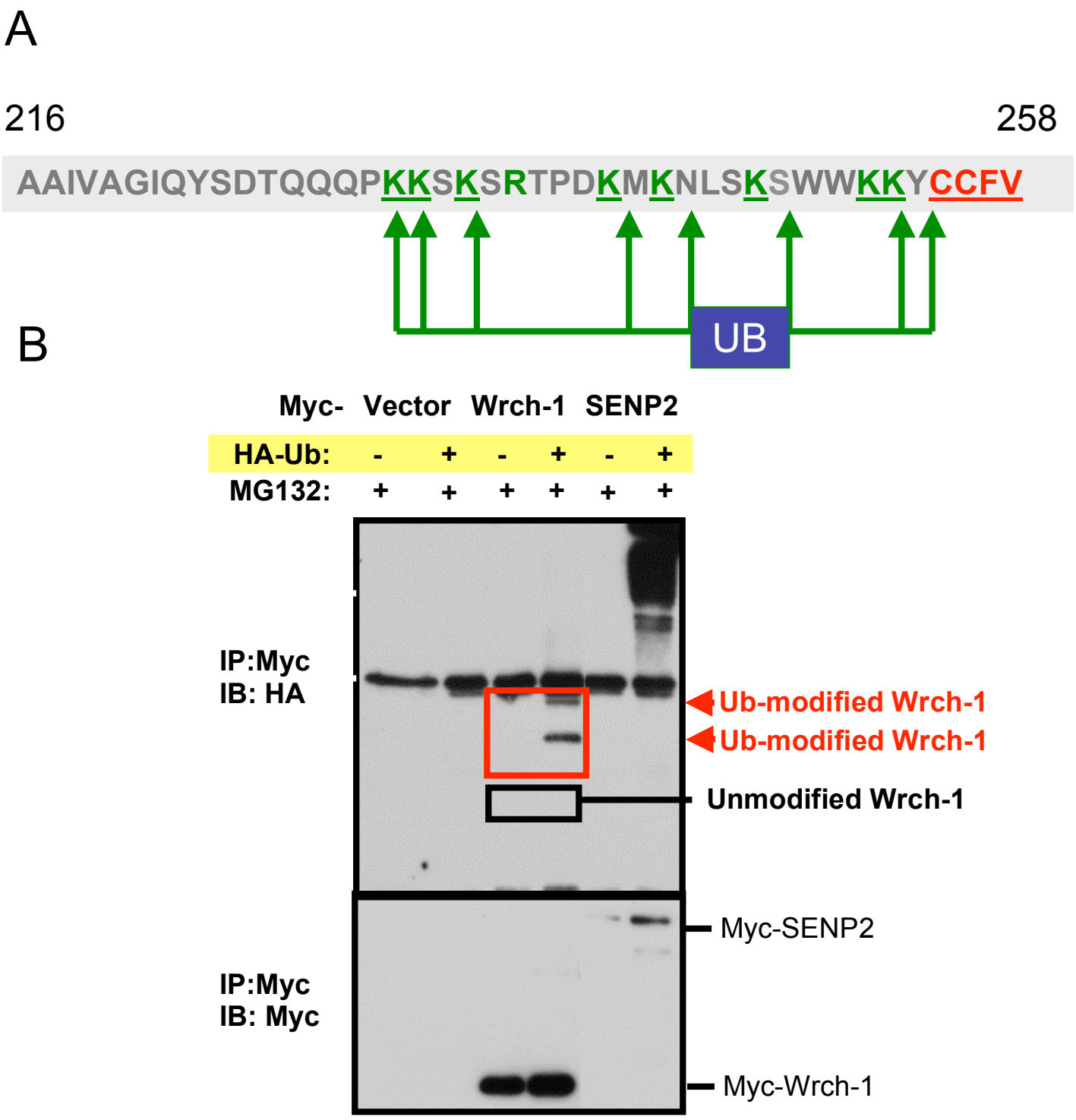


Figure 4.4 Wrch-1 incorporates ubiquitin molecules. A) The Wrch-1 C-terminus contains several potentially ubiquitinated lysines. B) Myc-tagged Wrch-1 or the positive control protein SENP2 were expressed in H1299 cells in the presence or absence of HA-tagged ubiquitin. All cells were treated with MG132 (proteasome inhibitor) for 6 h. 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. Data courtesy of Anastacia Berzat.

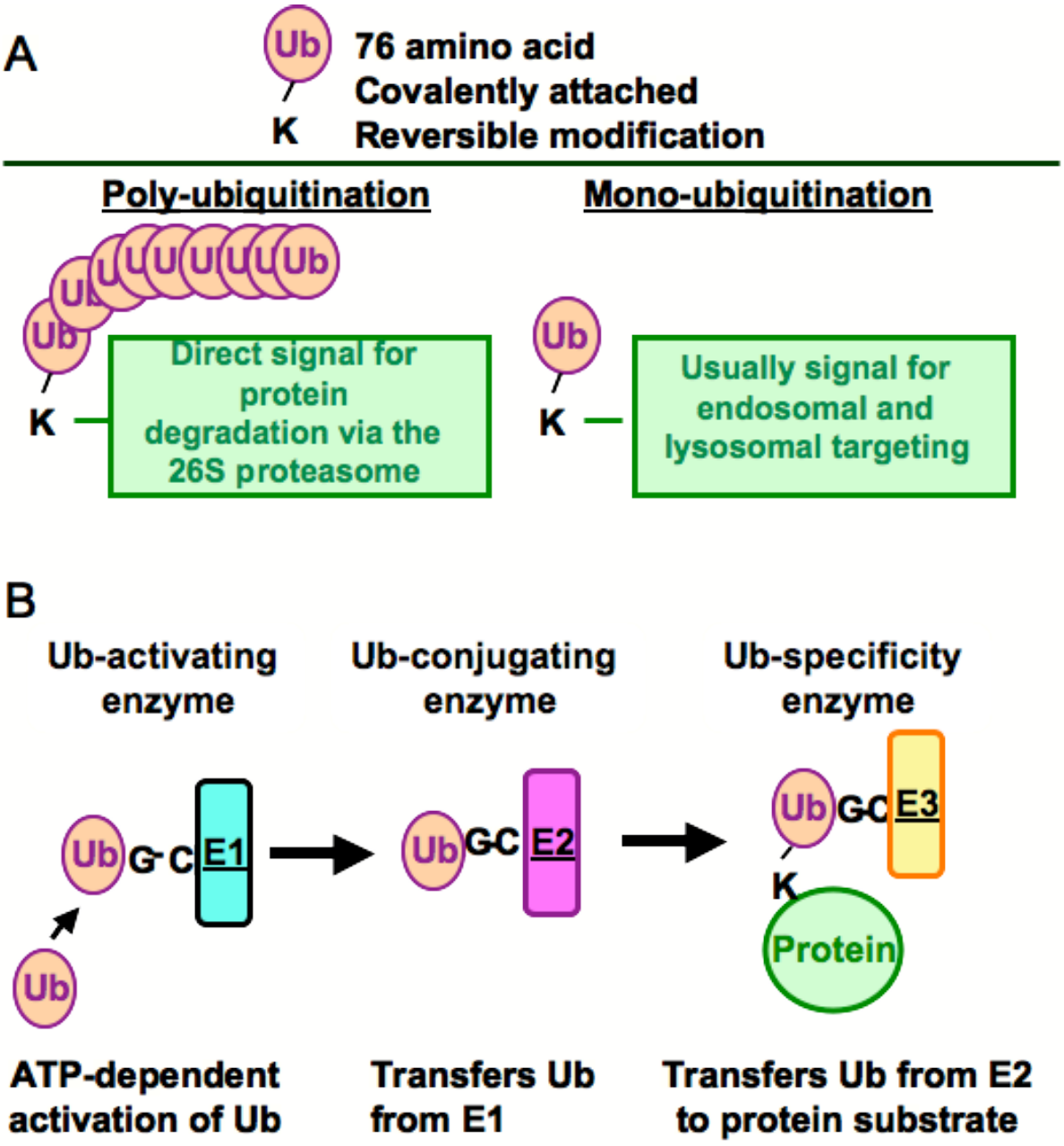


Figure 4.5 Process of mono- versus poly-ubiquitination. A. Ubiquitin is covalently attached to proteins as either a single monomer (mono-ubiquitination) or a long chain (polyubiquitination). Polyubiquitination usually serves as a signal for degradation of the protein by the 26S proteasome. Mono-ubiquitination of proteins regulates protein trafficking to cellular membranes. B. Ubiquitin is activated in an ATP-dependent manner by a ubiquitin-activating enzyme known as an enzyme-1 (E1). The E1 along with a ubiquitin-conjugating enzyme, or E2, and a ubiquitin-protein ligase (E3) covalently attaches ubiquitin to a lysine in the substrate. protein.

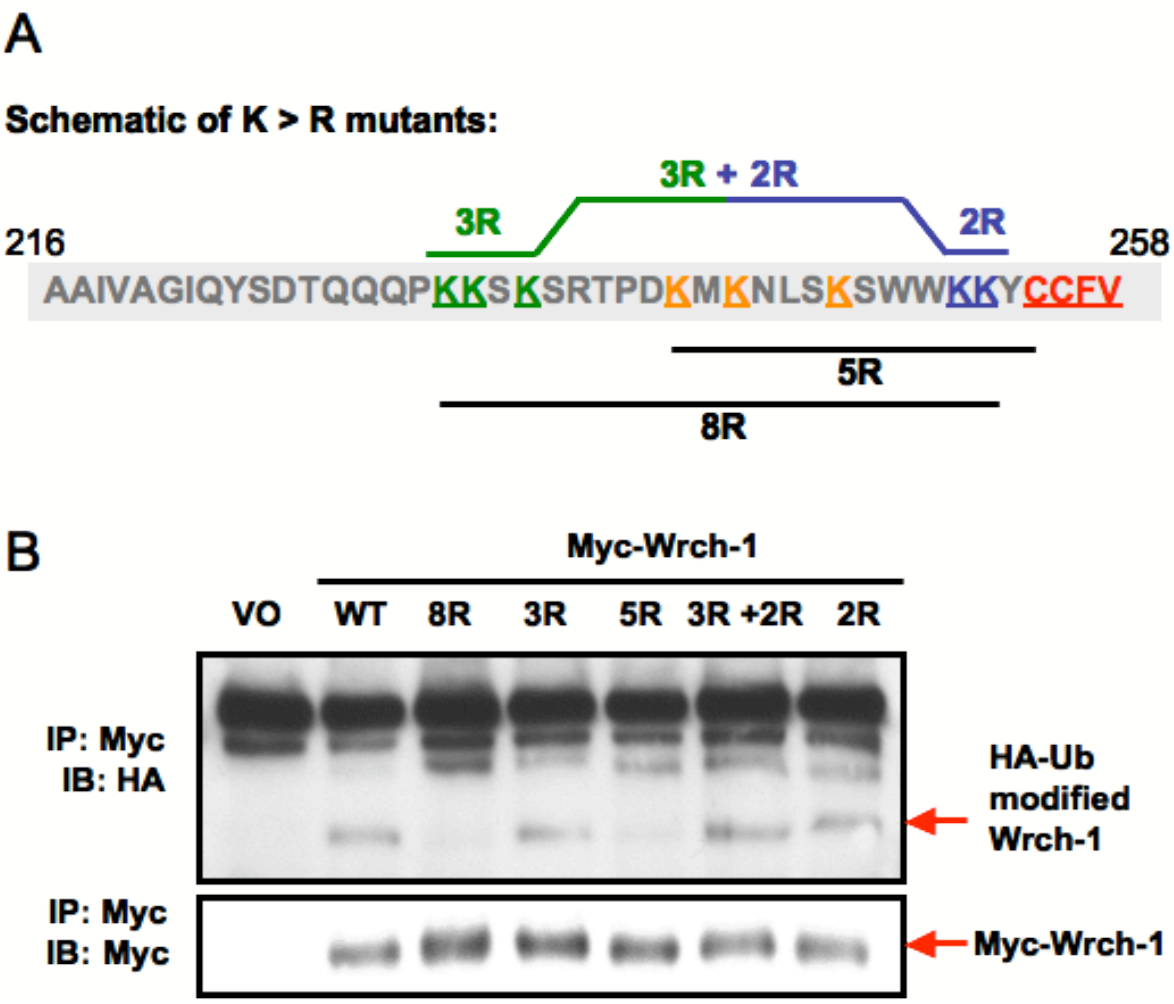


Figure 4.6 A) Schematic of multiple K>R mutations made in the C-terminal membrane targeting region of Wrch-1. B) Myc-tagged Wrch-1 and Wrch-1 K>R mutants were expressed in H1299 cells in the presence of HA-tagged ubiquitin. Myc-tagged proteins were immunoprecipitated with anti-myc antibody. Immunoprecipitates were subjected to SDS-PAGE and western blot analysis for incorporation of ubiquitin molecules using anti-HA antibody.

Figure 4.7 A) Schematic of single K>R mutations made in the C-terminal membrane targeting domain of Wrch-1. B) Myc-tagged Wrch-1 and Wrch-1 K>R mutants were expressed in H1299 cells in the presence 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.

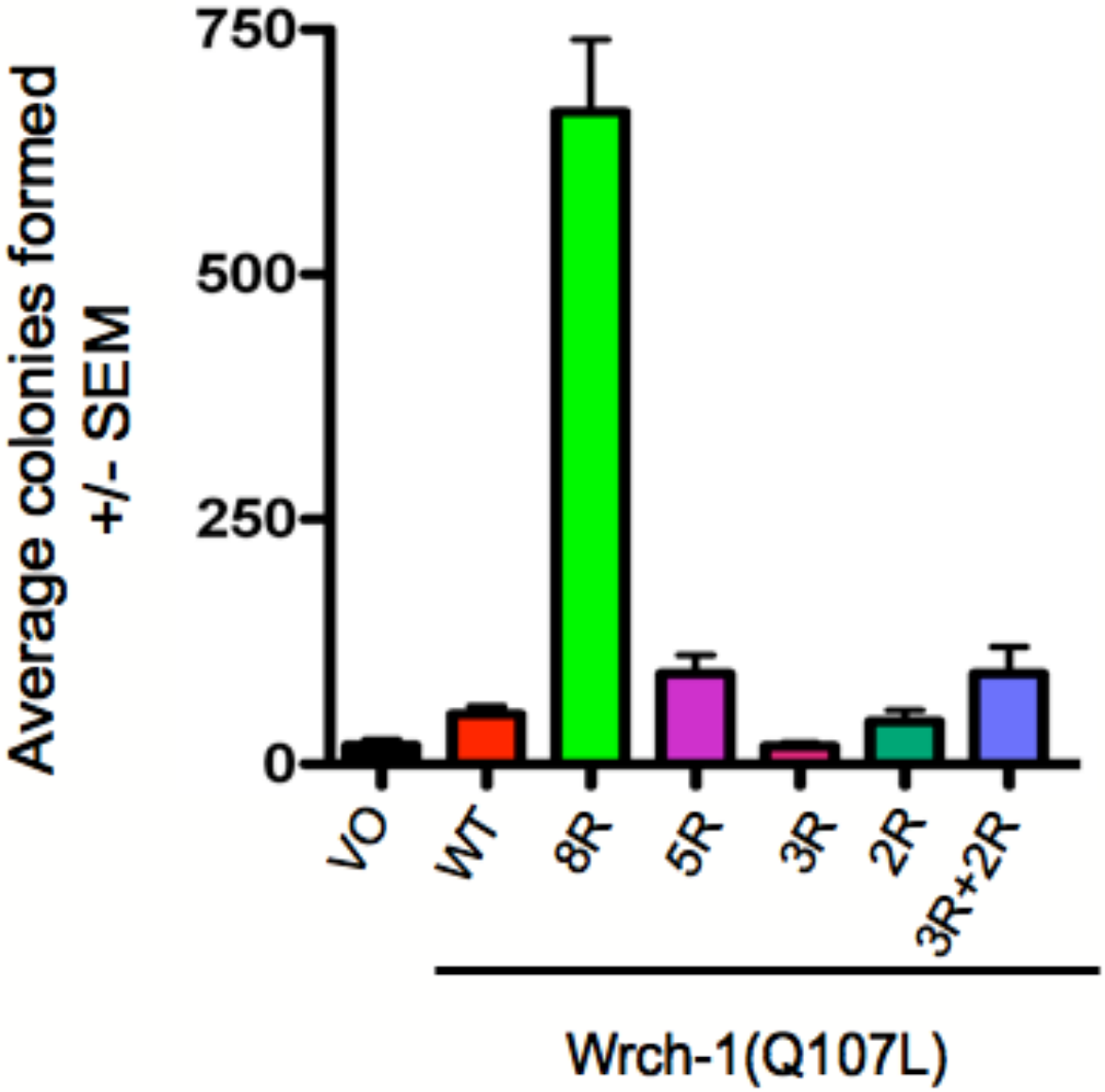


Figure 4.8. Potentially ubiquitinated lysines cannot be completely substituted with arginines to support Wrch-1-mediated anchorage-independent growth. H1299 cells stably expressing HA-tagged Wrch-1 were seeded in soft agar and colonies were allowed to grow for 21 days. Viable colonies were stained with MTT and quantified.

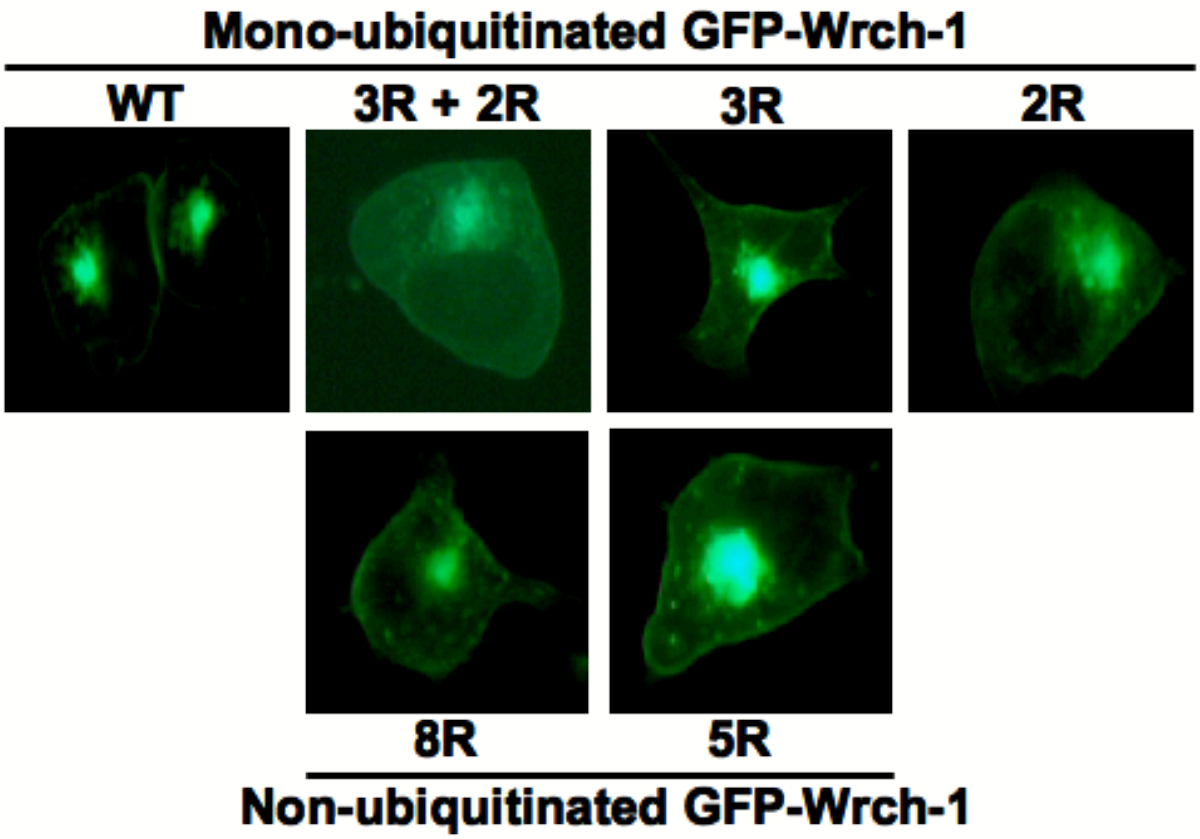


Figure 4.9. Wrch-1 localization is not altered, despite K>R mutations. GFP-tagged Wrch-1 and Wrch-1 K>R mutants were expressed in H1299 cells. Cells were visualized live using a Zeiss Axioscope.

4.5 Future Direction 4: Determine how Wrch-1 is negatively regulated at endosomes.

In this dissertation, I have shown that Wrch-1 becomes tyrosine phosphorylated on Y254 in response to growth factor stimulation, and that this phosphorylation results in a relocalization of Wrch-1 from the plasma membrane to internal membranes. I have also shown that after Wrch-1 relocalizes to internal membranes, it becomes GDP-bound and inactive, and that Wrch-1-mediated PAK1 and Pyk2 activation is decreased. These data indicate that Wrch-1 is turned off when relocalized to endosomes, but do not tell us whether this is due to increased interaction with an endosomally localized GAP, decreased interaction with a plasma membrane-associated GEF, or both. To date, no GEFs, GAPs or GDIs have been shown to regulate Wrch-1, but on the other hand no published studies have been specifically designed to probe GEF or GAP regulation of Wrch-1. It has been shown that Wrch-1 does not interact with RhoGDI-1 (Berzat et al., 2006), which is not surprising given that RhoGDIs recognize isoprenoid lipids, whereas Wrch-1 is not isoprenylated (Berzat et al., 2005b).

Since Wrch-1 is GFP-bound at the plasma membrane but GDP-bound at endosomes, Wrch-1 may be regulated by an endosomally localized GAP. There are examples of endosomally localized RhoGAPs, including p50RhoGAP, and ARAP1 (Simova et al., 2008; Sirokmany et al., 2006). p50RhoGAP, the first RhoGAP identified, has been shown to localize to endosomes, a subcellular localization that is mediated by its Sec-14-like domain (Sirokmany et al., 2006). ARAP1 has both Arf and Rho GAP domains, and has been shown to regulate trafficking and degradation of EGFR (Daniele et al., 2008; Yoon et al., 2008). Based on the localization of these RhoGAPs, p50RhoGAP and ARAP1 are good candidates for down-regulating Wrch-1 activity at endosomes. To test whether these GAPs are capable of regulating Wrch-1 nucleotide binding, I could perform *in vitro* GTPase assays as described in Shutes et al (Shutes et al., 2006). Additionally, co-immunoprecipitations using Wrch-1

and Wrch-1 phosphorylation mutants could be performed to determine whether Wrch-1 interacts with these GAPs in a localization-dependent manner. Finally, I could knock them down and then determine if endosomally localized Wrch-1 remains GTP-bound in their absence.

Although p50RhoGAP and ARAP1 are good candidates for regulation of Wrch-1 activity at endosomes, there could be other unidentified endosomally localized GAPs that could be regulating Wrch-1 instead of or in addition to p50RhoGAP and/or ARAP1. To identify such candidate GAPs, mass spec analysis of proteins that co-immunoprecipitate with mutationally activated Wrch-1(Q107L) but not with chronically GDP-bound Wrch-1(T63N) could be utilized. To determine which GAPs interact with Wrch-1 at the endosomes versus the plasma membrane, Wrch-1 phosphorylation mutants (Y254F and Y254E) could be used to identify proteins that bind Wrch-1 at the plasma membrane and internal membranes, respectively.

Many other interesting questions remain. For example, it will also be interesting to determine the identity of the phosphatase(s) that restore Wrch-1 to the plasma membrane, to more precisely characterize the stoichiometry of the pools of Wrch-1 that are localized to different sites in the cell, and to define the roles of these different pools in specific Wrch-1-mediated biological functions.

In this dissertation, I have tried to emphasize how Wrch-1 C-terminal tyrosine phosphorylation downstream of growth factor stimulation regulates Wrch-1 localization and function. More work is needed to further define the role of Wrch-1 phosphorylation and its interplay with other RTK signaling pathways. Additionally, more work is required to characterize how other post-translational modifications, such as ubiquitin, regulate Wrch-1

function. Further investigation into how post-translational modifications regulate Wrch-1 will provide insight on how Wrch-1 is regulated in normal and cancer cells, and may shed light on novel modes of Rho protein regulation.

REFERENCES

Abraham, M.T., M.A. Kuriakose, P.G. Sacks, H. Yee, L. Chiriboga, E.L. Bearer, and M.D. Delacure. 2001. Motility-related proteins as markers for head and neck squamous cell cancer. *Laryngoscope*. 111:1285-9.

Adamson, P., C.J. Marshall, A. Hall, and P.A. Tilbrook. 1992. Post-translational modifications of p21rho proteins. *J Biol Chem*. 267:20033-8.

Adra, C.N., D. Manor, J.L. Ko, S. Zhu, T. Horiuchi, L. Van Aelst, R.A. Cerione, and B. Lim. 1997. RhoGDIgamma: a GDP-dissociation inhibitor for Rho proteins with preferential expression in brain and pancreas. *Proc Natl Acad Sci U S A*. 94:4279-84.

Aghazadeh, B., W.E. Lowry, X.Y. Huang, and M.K. Rosen. 2000. Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation. *Cell*. 102:625-33.

Ananthanarayanan, B., Q. Ni, and J. Zhang. 2008. Chapter 2: Molecular sensors based on fluorescence resonance energy transfer to visualize cellular dynamics. *Methods Cell Biol*. 89:37-57.

Andrae, J., R. Gallini, and C. Betsholtz. 2008. Role of platelet-derived growth factors in physiology and medicine. *Genes Dev*. 22:1276-312.

Aronheim, A., Y.C. Broder, A. Cohen, A. Fritsch, B. Belisle, and A. Abo. 1998. Chp, a homologue of the GTPase Cdc42Hs, activates the JNK pathway and is implicated in reorganizing the actin cytoskeleton. *Curr Biol*. 8:1125-8.

Aspenstrom, P., A. Fransson, and J. Saras. 2004. Rho GTPases have diverse effects on the organization of the actin filament system. *Biochem J*. 377:327-37.

Aspenstrom, P., A. Ruusala, and D. Pacholsky. 2007. Taking Rho GTPases to the next level: the cellular functions of atypical Rho GTPases. *Exp Cell Res*. 313:3673-9.

Bain, J., L. Plater, M. Elliott, N. Shpiro, C.J. Hastie, H. McLauchlan, I. Klevernic, J.S. Arthur, D.R. Alessi, and P. Cohen. 2007. The selectivity of protein kinase inhibitors: a further update. *Biochem J*. 408:297-315.

Ballester, R., M.E. Furth, and O.M. Rosen. 1987. Phorbol ester- and protein kinase C-mediated phosphorylation of the cellular Kirsten ras gene product. *J Biol Chem*. 262:2688-95.

Barfod, E.T., Y. Zheng, W.J. Kuang, M.J. Hart, T. Evans, R.A. Cerione, and A. Ashkenazi. 1993. Cloning and expression of a human CDC42 GTPase-activating protein reveals a functional SH3-binding domain. *J Biol Chem*. 268:26059-62.

Bazenet, C.E., J.A. Gelderloos, and A. Kazlauskas. 1996. Phosphorylation of tyrosine 720 in the platelet-derived growth factor alpha receptor is required for binding of Grb2 and SHP-2 but not for activation of Ras or cell proliferation. *Mol Cell Biol*. 16:6926-36.

Bergsten, E., M. Uutela, X. Li, K. Pietras, A. Ostman, C.H. Heldin, K. Alitalo, and U. Eriksson. 2001. PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor. *Nat Cell Biol*. 3:512-6.

Berthold, J., K. Schenkova, S. Ramos, Y. Miura, M. Furukawa, P. Aspenstrom, and F. Rivero. 2008. Characterization of RhoBTB-dependent Cul3 ubiquitin ligase complexes--evidence for an autoregulatory mechanism. *Exp Cell Res*. 314:3453-65.

Berzat, A.C., D.C. Brady, J.J. Fiordalisi, and A.D. Cox. 2005a. Using inhibitors of prenylation to block localization and transforming activity. *Methods Enzymol*. 407:575-97.

Berzat, A.C., D.C. Brady, J.J. Fiordalisi, and A.D. Cox. 2006. Using inhibitors of prenylation to block localization and transforming activity. *Methods Enzymol*. 407:575-97.

Berzat, A.C., J.E. Buss, E.J. Chenette, C.A. Weinbaum, A. Shutes, C.J. Der, A. Minden, and A.D. Cox. 2005b. Transforming activity of the Rho family GTPase, Wrch-1, a Wnt-regulated Cdc42 homolog, is dependent on a novel carboxyl-terminal palmitoylation motif. *J Biol Chem*. 280:33055-65.

Bharadwaj, M., and O.A. Bizzozero. 1995. Myelin P0 glycoprotein and a synthetic peptide containing the palmitoylation site are both autoacylated. *J Neurochem*. 65:1805-15.

Bishop, A.L., and A. Hall. 2000. Rho GTPases and their effector proteins. *Biochem J.* 348 Pt 2:241-55.

Bivona, T.G., S.E. Quatela, B.O. Bodemann, I.M. Ahearn, M.J. Soskis, A. Mor, J. Miura, H.H. Wiener, L. Wright, S.G. Saba, D. Yim, A. Fein, I. Perez de Castro, C. Li, C.B. Thompson, A.D. Cox, and M.R. Philips. 2006. PKC regulates a farnesyl-electrostatic switch on K-Ras that promotes its association with Bcl-XL on mitochondria and induces apoptosis. *Mol Cell*. 21:481-93.

Blake, R.A., M.A. Broome, X. Liu, J. Wu, M. Gishizky, L. Sun, and S.A. Courtneidge. 2000. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol*. 20:9018-27.

Blangy, A., E. Vignal, S. Schmidt, A. Debant, C. Gauthier-Rouviere, and P. Fort. 2000. TrioGEF1 controls Rac- and Cdc42-dependent cell structures through the direct activation of rhoG. *J Cell Sci*. 113 (Pt 4):729-39.

Boyartchuk, V.L., M.N. Ashby, and J. Rine. 1997. Modulation of Ras and a-factor function by carboxyl-terminal proteolysis. *Science*. 275:1796-800.

Brady, D.C., J.K. Alan, J.P. Madigan, A.S. Fanning, and A.D. Cox. 2009. The transforming Rho family GTPase Wrch-1 disrupts epithelial cell tight junctions and epithelial morphogenesis. *Mol Cell Biol*. 29:1035-49.

Brazier, H., G. Pawlak, V. Vives, and A. Blangy. 2009. The Rho GTPase Wrch1 regulates osteoclast precursor adhesion and migration. *Int J Biochem Cell Biol*. 41:1391-401.

Brazier, H., S. Stephens, S. Ory, P. Fort, N. Morrison, and A. Blangy. 2006. Expression profile of RhoGTPases and RhoGEFs during RANKL-stimulated osteoclastogenesis: identification of essential genes in osteoclasts. *J Bone Miner Res*. 21:1387-98.

Bromberg, J.F., C.M. Horvath, D. Besser, W.W. Lathem, and J.E. Darnell, Jr. 1998. Stat3 activation is required for cellular transformation by v-src. *Mol Cell Biol*. 18:2553-8.

Bryant, D.M., and K.E. Mostov. 2008. From cells to organs: building polarized tissue. *Nat Rev Mol Cell Biol*. 9:887-901.

Camp, L.A., and S.L. Hofmann. 1993. Purification and properties of a palmitoyl-protein thioesterase that cleaves palmitate from H-Ras. *J Biol Chem*. 268:22566-74.

Casey, P.J., and M.C. Seabra. 1996. Protein prenyltransferases. *J Biol Chem*. 271:5289-92.

Chamoun, Z., R.K. Mann, D. Nellen, D.P. von Kessler, M. Bellotto, P.A. Beachy, and K. Basler. 2001. Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal. *Science*. 293:2080-4.

Chang, F.K., N. Sato, N. Kobayashi-Simorowski, T. Yoshihara, J.L. Meth, and M. Hamaguchi. 2006. DBC2 is essential for transporting vesicular stomatitis virus glycoprotein. *J Mol Biol*. 364:302-8.

Chenette, E.J., A. Abo, and C.J. Der. 2005. Critical and distinct roles of amino- and carboxyl-terminal sequences in regulation of the biological activity of the Chp atypical Rho GTPase. *J Biol Chem*. 280:13784-92.

Chenette, E.J., N.Y. Mitin, and C.J. Der. 2006. Multiple sequence elements facilitate Chp Rho GTPase subcellular location, membrane association, and transforming activity. *Mol Biol Cell*. 17:3108-21.

Cherfils, J., and P. Chardin. 1999. GEFs: structural basis for their activation of small GTP-binding proteins. *Trends Biochem Sci*. 24:306-11.

Ching, Y.P., C.M. Wong, S.F. Chan, T.H. Leung, D.C. Ng, D.Y. Jin, and I.O. Ng. 2003. Deleted in liver cancer (DLC) 2 encodes a RhoGAP protein with growth suppressor function and is underexpressed in hepatocellular carcinoma. *J Biol Chem*. 278:10824-30.

Chuang, Y.Y., A. Valster, S.J. Coniglio, J.M. Backer, and M. Symons. 2007. The atypical Rho family GTPase Wrch-1 regulates focal adhesion formation and cell migration. *J Cell Sci*. 120:1927-34.

Chun, W., G.S. Waldo, and G.V. Johnson. 2007. Split GFP complementation assay: a novel approach to quantitatively measure aggregation of tau in situ: effects of GSK3beta activation and caspase 3 cleavage. *J Neurochem*. 103:2529-39.

Clarke, S., J.P. Vogel, R.J. Deschenes, and J. Stock. 1988. Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proc Natl Acad Sci U S A*. 85:4643-7.

Cooper, J.A., K.L. Gould, C.A. Cartwright, and T. Hunter. 1986. Tyr527 is phosphorylated in pp60c-src: implications for regulation. *Science*. 231:1431-4.

Cooper, J.A., and C.S. King. 1986. Dephosphorylation or antibody binding to the carboxy terminus stimulates pp60c-src. *Mol Cell Biol*. 6:4467-77.

Cotteret, S., and J. Chernoff. 2002. The evolutionary history of effectors downstream of Cdc42 and Rac. *Genome Biol*. 3:REVIEWS0002.

Crespo, P., K.E. Schuebel, A.A. Ostrom, J.S. Gutkind, and X.R. Bustelo. 1997. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature*. 385:169-72.

Dallery, E., S. Galiegue-Zouitina, M. Collyn-d'Hooghe, S. Quief, C. Denis, M.P. Hildebrand, D. Lantoine, C. Deweindt, H. Tilly, C. Bastard, and et al. 1995. TTF, a gene encoding a novel small G protein, fuses to the lymphoma-associated LAZ3 gene by t(3;4) chromosomal translocation. *Oncogene*. 10:2171-8.

Daniele, T., G. Di Tullio, M. Santoro, G. Turacchio, and M.A. De Matteis. 2008. ARAP1 regulates EGF receptor trafficking and signalling. *Traffic*. 9:2221-35.

Darnell, J.E., Jr. 1997. STATs and gene regulation. *Science*. 277:1630-5.

de Toledo, M., F. Senic-Matuglia, J. Salamero, G. Uze, F. Comunale, P. Fort, and A. Blangy. 2003. The GTP/GDP cycling of rho GTPase TCL is an essential regulator of the early endocytic pathway. *Mol Biol Cell*. 14:4846-56.

DerMardirossian, C., and G.M. Bokoch. 2005. GDIs: central regulatory molecules in Rho GTPase activation. *Trends Cell Biol*. 15:356-63.

Dikic, I. 2003. Mechanisms controlling EGF receptor endocytosis and degradation. *Biochem Soc Trans*. 31:1178-81.

Dikic, I., and S. Giordano. 2003. Negative receptor signalling. *Curr Opin Cell Biol*. 15:128-35.

Donepudi, M., and M.D. Resh. 2008. c-Src trafficking and co-localization with the EGF receptor promotes EGF ligand-independent EGF receptor activation and signaling. *Cell Signal*. 20:1359-67.

Doolittle, R.F., M.W. Hunkapiller, L.E. Hood, S.G. Devare, K.C. Robbins, S.A. Aaronson, and H.N. Antoniades. 1983. Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. *Science*. 221:275-7.

Dovas, A., and J.R. Couchman. 2005. RhoGDI: multiple functions in the regulation of Rho family GTPase activities. *Biochem J*. 390:1-9.

Doye, A., L. Boyer, A. Mettouchi, and E. Lemichez. 2006. Ubiquitin-mediated proteasomal degradation of Rho proteins by the CNF1 toxin. *Methods Enzymol*. 406:447-56.

Duncan, J.A., and A.G. Gilman. 1996. Autoacylation of G protein alpha subunits. *J Biol Chem*. 271:23594-600.

Duncan, J.A., and A.G. Gilman. 1998. A cytoplasmic acyl-protein thioesterase that removes palmitate from G protein alpha subunits and p21(RAS). *J Biol Chem*. 273:15830-7.

Ellenbroek, S.I., and J.G. Collard. 2007. Rho GTPases: functions and association with cancer. *Clin Exp Metastasis*. 24:657-72.

Ellerbroek, S.M., K. Wennerberg, and K. Burridge. 2003. Serine phosphorylation negatively regulates RhoA in vivo. *J Biol Chem*. 278:19023-31.

Ellis, S., and H. Mellor. 2000. The novel Rho-family GTPase rif regulates coordinated actin-based membrane rearrangements. *Curr Biol*. 10:1387-90.

Espinosa, E.J., M. Calero, K. Sridevi, and S.R. Pfeffer. 2009. RhoBTB3: a Rho GTPase-family ATPase required for endosome to Golgi transport. *Cell*. 137:938-48.

Etienne-Manneville, S., and A. Hall. 2002. Rho GTPases in cell biology. *Nature*. 420:629-35.

Eva, A., G. Vecchio, C.D. Rao, S.R. Tronick, and S.A. Aaronson. 1988. The predicted DBL oncogene product defines a distinct class of transforming proteins. *Proc Natl Acad Sci U S A*. 85:2061-5.

Fincham, V.J., and M.C. Frame. 1998. The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. *EMBO J*. 17:81-92.

Fincham, V.J., J.A. Wyke, and M.C. Frame. 1995. v-Src-induced degradation of focal adhesion kinase during morphological transformation of chicken embryo fibroblasts. *Oncogene*. 10:2247-52.

Finley, D., S. Sadis, B.P. Monia, P. Boucher, D.J. Ecker, S.T. Crooke, and V. Chau. 1994. Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. *Mol Cell Biol*. 14:5501-9.

Forget, M.A., R.R. Desrosiers, D. Gingras, and R. Beliveau. 2002. Phosphorylation states of Cdc42 and RhoA regulate their interactions with Rho GDP dissociation inhibitor and their extraction from biological membranes. *Biochem J*. 361:243-54.

Fransson, A., A. Ruusala, and P. Aspenstrom. 2003. Atypical Rho GTPases have roles in mitochondrial homeostasis and apoptosis. *J Biol Chem*. 278:6495-502.

Fritz, G., I. Just, and B. Kaina. 1999. Rho GTPases are over-expressed in human tumors. *Int J Cancer*. 81:682-7.

Goodison, S., J. Yuan, D. Sloan, R. Kim, C. Li, N.C. Popescu, and V. Urquidi. 2005. The RhoGAP protein DLC-1 functions as a metastasis suppressor in breast cancer cells. *Cancer Res*. 65:6042-53.

Gupta, S., and R.J. Davis. 1994. MAP kinase binds to the NH2-terminal activation domain of c-Myc. *FEBS Lett*. 353:281-5.

Haglund, K., and I. Dikic. 2005. Ubiquitylation and cell signaling. *Embo J*. 24:3353-9.

Han, J., B. Das, W. Wei, L. Van Aelst, R.D. Mosteller, R. Khosravi-Far, J.K. Westwick, C.J. Der, and D. Broek. 1997. Lck regulates Vav activation of members of the Rho family of GTPases. *Mol Cell Biol*. 17:1346-53.

Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell*. 100:57-70.

Hancock, J.F., H. Paterson, and C.J. Marshall. 1990. A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell*. 63:133-9.

Hansen, S.H., M.M. Zegers, M. Woodrow, P. Rodriguez-Viciana, P. Chardin, K.E. Mostov, and M. McMahon. 2000. Induced expression of Rnd3 is associated with transformation of polarized epithelial cells by the Raf-MEK-extracellular signal-regulated kinase pathway. *Mol Cell Biol*. 20:9364-75.

Hart, M.J., A. Eva, T. Evans, S.A. Aaronson, and R.A. Cerione. 1991. Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product. *Nature*. 354:311-4.

He, Y., L. Hicke, and I. Radhakrishnan. 2007. Structural basis for ubiquitin recognition by SH3 domains. *J Mol Biol*. 373:190-6.

Hershko, A., and A. Ciechanover. 1998. The ubiquitin system. *Annu Rev Biochem*. 67:425-79.

Hill, C.S., R. Marais, S. John, J. Wynne, S. Dalton, and R. Treisman. 1993. Functional analysis of a growth factor-responsive transcription factor complex. *Cell*. 73:395-406.

Hoffman, G.R., N. Nassar, and R.A. Cerione. 2000. Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI. *Cell*. 100:345-56.

Huang, L., E. Kinnucan, G. Wang, S. Beaudenon, P.M. Howley, J.M. Huibregtse, and N.P. Pavletich. 1999. Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. *Science*. 286:1321-6.

Hynes, N.E., and H.A. Lane. 2005. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer*. 5:341-54.

Isomura, M., A. Kikuchi, N. Ohga, and Y. Takai. 1991. Regulation of binding of rhoB p20 to membranes by its specific regulatory protein, GDP dissociation inhibitor. *Oncogene*. 6:119-24.

Ivanchenko, M., Z. Vejlupkova, R.S. Quatrano, and J.E. Fowler. 2000. Maize ROP7 GTPase contains a unique, CaaX box-independent plasma membrane targeting signal. *Plant J*. 24:79-90.

Jaffe, A.B., and A. Hall. 2005. Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol*. 21:247-69.

Jing, S.Q., and I.S. Trowbridge. 1987. Identification of the intermolecular disulfide bonds of the human transferrin receptor and its lipid-attachment site. *EMBO J*. 6:327-31.

Johnsson, A., C.H. Heldin, B. Westermark, and A. Wasteson. 1982. Platelet-derived growth factor: identification of constituent polypeptide chains. *Biochem Biophys Res Commun*. 104:66-74.

Joyce, P.L., and A.D. Cox. 2003. Rac1 and Rac3 are targets for geranylgeranyltransferase I inhibitor-mediated inhibition of signaling, transformation, and membrane ruffling. *Cancer Res*. 63:7959-67.

Jura, N., E. Scotto-Lavino, A. Sobczyk, and D. Bar-Sagi. 2006. Differential modification of Ras proteins by ubiquitination. *Mol Cell*. 21:679-87.

Kamai, T., K. Arai, T. Tsujii, M. Honda, and K. Yoshida. 2001. Overexpression of RhoA mRNA is associated with advanced stage in testicular germ cell tumour. *BJU Int*. 87:227-31.

Kaplan, K.B., J.R. Swedlow, H.E. Varmus, and D.O. Morgan. 1992. Association of p60c-src with endosomal membranes in mammalian fibroblasts. *J Cell Biol*. 118:321-33.

Kazlauskas, A., and J.A. Cooper. 1989. Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins. *Cell*. 58:1121-33.

Kazlauskas, A., and J.A. Cooper. 1990. Phosphorylation of the PDGF receptor beta subunit creates a tight binding site for phosphatidylinositol 3 kinase. *EMBO J*. 9:3279-86.

Khosravi-Far, R., G.J. Clark, K. Abe, A.D. Cox, T. McLain, R.J. Lutz, M. Sinensky, and C.J. Der. 1992. Ras (CXXX) and Rab (CC/CXC) prenylation signal sequences are unique and functionally distinct. *J Biol Chem*. 267:24363-8.

Kim, E., P. Ambroziak, J.C. Otto, B. Taylor, M. Ashby, K. Shannon, P.J. Casey, and S.G. Young. 1999. Disruption of the mouse Rce1 gene results in defective Ras processing and mislocalization of Ras within cells. *J Biol Chem*. 274:8383-90.

Kirikoshi, H., and M. Katoh. 2002. Expression of WRCH1 in human cancer and down-regulation of WRCH1 by beta-estradiol in MCF-7 cells. *Int J Oncol*. 20:777-83.

Klingensmith, J., and R. Nusse. 1994. Signaling by wingless in Drosophila. *Dev Biol*. 166:396-414.

Klingensmith, J., R. Nusse, and N. Perrimon. 1994. The Drosophila segment polarity gene dishevelled encodes a novel protein required for response to the wingless signal. *Genes Dev*. 8:118-30.

Klinghoffer, R.A., C. Sachsenmaier, J.A. Cooper, and P. Soriano. 1999. Src family kinases are required for integrin but not PDGFR signal transduction. *EMBO J*. 18:2459-71.

Kmiecik, T.E., P.J. Johnson, and D. Shalloway. 1988. Regulation by the autophosphorylation site in overexpressed pp60c-src. *Mol Cell Biol*. 8:4541-6.

Kmiecik, T.E., and D. Shalloway. 1987. Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. *Cell*. 49:65-73.

Komander, D., R. Garg, P.T. Wan, A.J. Ridley, and D. Barford. 2008. Mechanism of multi-site phosphorylation from a ROCK-I:RhoE complex structure. *EMBO J*. 27:3175-85.

Kourlas, P.J., M.P. Strout, B. Becknell, M.L. Veronese, C.M. Croce, K.S. Theil, R. Krahe, T. Ruutu, S. Knuutila, C.D. Bloomfield, and M.A. Caligiuri. 2000. Identification of a gene at 11q23 encoding a guanine nucleotide exchange factor: evidence for its fusion with MLL in acute myeloid leukemia. *Proc Natl Acad Sci U S A*. 97:2145-50.

Kozasa, T., X. Jiang, M.J. Hart, P.M. Sternweis, W.D. Singer, A.G. Gilman, G. Bollag, and P.C. Sternweis. 1998. p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. *Science*. 280:2109-11.

Kozma, R., S. Ahmed, A. Best, and L. Lim. 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol Cell Biol*. 15:1942-52.

Kundra, V., J.A. Escobedo, A. Kazlauskas, H.K. Kim, S.G. Rhee, L.T. Williams, and B.R. Zetter. 1994. Regulation of chemotaxis by the platelet-derived growth factor receptor-beta. *Nature*. 367:474-6.

Kurosu, H., T. Maehama, T. Okada, T. Yamamoto, S. Hoshino, Y. Fukui, M. Ui, O. Hazeki, and T. Katada. 1997. Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110beta is synergistically activated by the betagamma subunits of G proteins and phosphotyrosyl peptide. *J Biol Chem*. 272:24252-6.

Laffargue, M., P. Raynal, A. Yart, C. Peres, R. Wetzker, S. Roche, B. Payrastre, and H. Chap. 1999. An epidermal growth factor receptor/Gab1 signaling pathway is required for activation of phosphoinositide 3-kinase by lysophosphatidic acid. *J Biol Chem*. 274:32835-41.

Lai, W.H., P.H. Cameron, J.J. Doherty, 2nd, B.I. Posner, and J.J. Bergeron. 1989a. Ligand-mediated autophosphorylation activity of the epidermal growth factor receptor during internalization. *J Cell Biol*. 109:2751-60.

Lai, W.H., P.H. Cameron, I. Wada, J.J. Doherty, 2nd, D.G. Kay, B.I. Posner, and J.J. Bergeron. 1989b. Ligand-mediated internalization, recycling, and downregulation of the epidermal growth factor receptor in vivo. *J Cell Biol*. 109:2741-9.

Lancaster, C.A., P.M. Taylor-Harris, A.J. Self, S. Brill, H.E. van Erp, and A. Hall. 1994. Characterization of rhoGAP. A GTPase-activating protein for rho-related small GTPases. *J Biol Chem*. 269:1137-42.

Lander, E.S., L.M. Linton, B. Birren, C. Nusbaum, M.C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczky, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J.P. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, N. Stange-Thomann, N. Stojanovic, A. Subramanian, D. Wyman, J. Rogers, J. Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J.C. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R.H. Waterston, R.K. Wilson, L.W. Hillier, J.D. McPherson, M.A. Marra, E.R. Mardis, L.A. Fulton, A.T. Chinwalla, K.H. Pepin, W.R. Gish, S.L. Chissoe, M.C. Wendl, K.D. Delehaunty, T.L. Miner, A.

Delehaunty, J.B. Kramer, L.L. Cook, R.S. Fulton, D.L. Johnson, P.J. Minx, S.W. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J.F. Cheng, A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier, et al. 2001. Initial sequencing and analysis of the human genome. *Nature*. 409:860-921.

Lang, P., F. Gesbert, M. Delespine-Carmagnat, R. Stancou, M. Pouchelet, and J. Bertoglio. 1996. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *Embo J*. 15:510-9.

LaRochelle, W.J., M. Jeffers, W.F. McDonald, R.A. Chillakuru, N.A. Giese, N.A. Lokker, C. Sullivan, F.L. Boldog, M. Yang, C. Vernet, C.E. Burgess, E. Fernandes, L.L. Deegler, B. Rittman, J. Shimkets, R.A. Shimkets, J.M. Rothberg, and H.S. Lichenstein. 2001. PDGF-D, a new protease-activated growth factor. *Nat Cell Biol*. 3:517-21.

Lavy, M., K. Bracha-Drori, H. Sternberg, and S. Yalovsky. 2002. A cell-specific, prenylation-independent mechanism regulates targeting of type II RACs. *Plant Cell*. 14:2431-50.

Lebowitz, P.F., W. Du, and G.C. Prendergast. 1997. Prenylation of RhoB is required for its cell transforming function but not its ability to activate serum response element-dependent transcription. *J Biol Chem*. 272:16093-5.

Lelias, J.M., C.N. Adra, G.M. Wulf, J.C. Guillemot, M. Khagad, D. Caput, and B. Lim. 1993. cDNA cloning of a human mRNA preferentially expressed in hematopoietic cells and with homology to a GDP-dissociation inhibitor for the rho GTP-binding proteins. *Proc Natl Acad Sci U S A*. 90:1479-83.

Lerosey, I., V. Pizon, A. Tavitian, and J. de Gunzburg. 1991. The cAMP-dependent protein kinase phosphorylates the rap1 protein in vitro as well as in intact fibroblasts, but not the closely related rap2 protein. *Biochem Biophys Res Commun*. 175:430-6.

Leventis, R., and J.R. Silvius. 1998. Lipid-binding characteristics of the polybasic carboxy-terminal sequence of K-ras4B. *Biochemistry*. 37:7640-8.

Li, R., B. Debreceni, B. Jia, Y. Gao, G. Tigyi, and Y. Zheng. 1999. Localization of the PAK1-, WASP-, and IQGAP1-specifying regions of Cdc42. *J Biol Chem*. 274:29648-54.

Li, X., X. Bu, B. Lu, H. Avraham, R.A. Flavell, and B. Lim. 2002. The hematopoiesis-specific GTP-binding protein RhoH is GTPase deficient and modulates activities of other Rho GTPases by an inhibitory function. *Mol Cell Biol*. 22:1158-71.

Li, X., A. Ponten, K. Aase, L. Karlsson, A. Abramsson, M. Uutela, G. Backstrom, M. Hellstrom, H. Bostrom, H. Li, P. Soriano, C. Betsholtz, C.H. Heldin, K. Alitalo, A. Ostman, and U. Eriksson. 2000. PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. *Nat Cell Biol*. 2:302-9.

Ligeti, E., M.C. Dagher, S.E. Hernandez, A.J. Koleske, and J. Settleman. 2004. Phospholipids can switch the GTPase substrate preference of a GTPase-activating protein. *J Biol Chem*. 279:5055-8.

Lim KH, B.D., Kashatus DF, Ancrile BB, Der CJ, Cox AD, and Counter CM. 2009. Aurora-A phosphorylated, activates and relocalizes RalA. *MCB*. under revision.

Linder, M.E., and R.J. Deschenes. 2003. New insights into the mechanisms of protein palmitoylation. *Biochemistry*. 42:4311-20.

Linder, M.E., and R.J. Deschenes. 2004. Model organisms lead the way to protein palmitoyltransferases. *J Cell Sci*. 117:521-6.

Lipfert, L., B. Haimovich, M.D. Schaller, B.S. Cobb, J.T. Parsons, and J.S. Brugge. 1992. Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. *J Cell Biol*. 119:905-12.

Lobo, S., W.K. Greentree, M.E. Linder, and R.J. Deschenes. 2002. Identification of a Ras palmitoyltransferase in Saccharomyces cerevisiae. *J Biol Chem*. 277:41268-73.

Madigan JP, B.B., Brady DC, Dewer BJ, Keller PJ, Leitges M, Philips MR, Ridley AJ, Der CJ and Cox AD. 2009. Regulation of Rnd3 localization and function by PKCa-mediated phosphorylation. *Biochem J*. Sept. 1 epub ahead of print.

Magee, A.I., L. Gutierrez, I.A. McKay, C.J. Marshall, and A. Hall. 1987. Dynamic fatty acylation of p21N-ras. *EMBO J*. 6:3353-7.

Manser, E., T. Leung, H. Salihuddin, Z.S. Zhao, and L. Lim. 1994. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature*. 367:40-6.

Marcoux, N., and K. Vuori. 2003. EGF receptor mediates adhesion-dependent activation of the Rac GTPase: a role for phosphatidylinositol 3-kinase and Vav2. *Oncogene*. 22:6100-6.

Martin, G.A., G. Bollag, F. McCormick, and A. Abo. 1995. A novel serine kinase activated by rac1/CDC42Hs-dependent autophosphorylation is related to PAK65 and STE20. *Embo J*. 14:4385.

Mateus, A.R., R. Seruca, J.C. Machado, G. Keller, M.J. Oliveira, G. Suriano, and B. Luber. 2007. EGFR regulates RhoA-GTP dependent cell motility in E-cadherin mutant cells. *Hum Mol Genet*. 16:1639-47.

Matuoka, K., F. Shibasaki, M. Shibata, and T. Takenawa. 1993. Ash/Grb-2, a SH2/SH3-containing protein, couples to signaling for mitogenesis and cytoskeletal reorganization by EGF and PDGF. *EMBO J*. 12:3467-73.

McLaughlin, S., and A. Aderem. 1995. The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions. *Trends Biochem Sci*. 20:272-6.

Michaelson, D., J. Silletti, G. Murphy, P. D'Eustachio, M. Rush, and M.R. Philips. 2001. Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. *J Cell Biol*. 152:111-26.

Miura, G.I., J. Buglino, D. Alvarado, M.A. Lemmon, M.D. Resh, and J.E. Treisman. 2006. Palmitoylation of the EGFR ligand Spitz by Rasp increases Spitz activity by restricting its diffusion. *Dev Cell*. 10:167-76.

Mumby, S.M., C. Kleuss, and A.G. Gilman. 1994. Receptor regulation of G-protein palmitoylation. *Proc Natl Acad Sci U S A*. 91:2800-4.

Murphy, C., R. Saffrich, M. Grummt, H. Gournier, V. Rybin, M. Rubino, P. Auvinen, A. Lutcke, R.G. Parton, and M. Zerial. 1996. Endosome dynamics regulated by a Rho protein. *Nature*. 384:427-32.

Murphy, G.A., S.A. Jillian, D. Michaelson, M.R. Philips, P. D'Eustachio, and M.G. Rush. 2001. Signaling mediated by the closely related mammalian Rho family GTPases TC10 and Cdc42 suggests distinct functional pathways. *Cell Growth Differ*. 12:157-67.

Murphy, G.A., P.A. Solski, S.A. Jillian, P. Perez de la Ossa, P. D'Eustachio, C.J. Der, and M.G. Rush. 1999. Cellular functions of TC10, a Rho family GTPase: regulation of morphology, signal transduction and cell growth. *Oncogene*. 18:3831-45.

Nelson, W.J. 2003. Adaptation of core mechanisms to generate cell polarity. *Nature*. 422:766-74.

Nishimura, R., W. Li, A. Kashishian, A. Mondino, M. Zhou, J. Cooper, and J. Schlessinger. 1993. Two signaling molecules share a phosphotyrosine-containing binding site in the platelet-derived growth factor receptor. *Mol Cell Biol*. 13:6889-96.

Nobes, C.D., I. Lauritzen, M.G. Mattei, S. Paris, A. Hall, and P. Chardin. 1998. A new member of the Rho family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion. *J Cell Biol*. 141:187-97.

Nomanbhoy, T.K., J.W. Erickson, and R.A. Cerione. 1999. Kinetics of Cdc42 membrane extraction by Rho-GDI monitored by real-time fluorescence resonance energy transfer. *Biochemistry*. 38:1744-50.

Okada, S., E. Yamada, T. Saito, K. Ohshima, K. Hashimoto, M. Yamada, Y. Uehara, T. Tsuchiya, H. Shimizu, K. Tatei, T. Izumi, K. Yamauchi, S. Hisanaga, J.E. Pessin, and M. Mori. 2008. CDK5-dependent phosphorylation of the Rho family GTPase

TC10(alpha) regulates insulin-stimulated GLUT4 translocation. *J Biol Chem*. 283:35455-63.

Ory, S., H. Brazier, and A. Blangy. 2007. Identification of a bipartite focal adhesion localization signal in RhoU/Wrch-1, a Rho family GTPase that regulates cell adhesion and migration. *Biol Cell*. 99:701-16.

Pasqualucci, L., P. Neumeister, T. Goossens, G. Nanjangud, R.S. Chaganti, R. Kuppers, and R. Dalla-Favera. 2001. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. *Nature*. 412:341-6.

Patel, V., H.M. Rosenfeldt, R. Lyons, J.M. Servitja, X.R. Bustelo, M. Siroff, and J.S. Gutkind. 2007. Persistent activation of Rac1 in squamous carcinomas of the head and neck: evidence for an EGFR/Vav2 signaling axis involved in cell invasion. *Carcinogenesis*. 28:1145-52.

Peifer, M., D. Sweeton, M. Casey, and E. Wieschaus. 1994. wingless signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. *Development*. 120:369-80.

Petrelli, A., G.F. Gilestro, S. Lanzardo, P.M. Comoglio, N. Migone, and S. Giordano. 2002. The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. *Nature*. 416:187-90.

Pickart, C.M., and M.J. Eddins. 2004. Ubiquitin: structures, functions, mechanisms. *Biochim Biophys Acta*. 1695:55-72.

Playford, M.P., K. Vadali, X. Cai, K. Burridge, and M.D. Schaller. 2008. Focal adhesion kinase regulates cell-cell contact formation in epithelial cells via modulation of Rho. *Exp Cell Res*. 314:3187-97.

Prakash, S.K., R. Paylor, S. Jenna, N. Lamarche-Vane, D.L. Armstrong, B. Xu, M.A. Mancini, and H.Y. Zoghbi. 2000. Functional analysis of ARHGAP6, a novel GTPase-activating protein for RhoA. *Hum Mol Genet*. 9:477-88.

Preudhomme, C., C. Roumier, M.P. Hildebrand, E. Dallery-Prudhomme, D. Lantoine, J.L. Lai, A. Daudignon, C. Adenis, F. Bauters, P. Fenaux, J.P. Kerckaert, and S. Galiegue-Zouitina. 2000. Nonrandom 4p13 rearrangements of the RhoH/TTF gene, encoding a GTP-binding protein, in non-Hodgkin's lymphoma and multiple myeloma. *Oncogene*. 19:2023-32.

Quesnel, S., and J.R. Silvius. 1994. Cysteine-containing peptide sequences exhibit facile uncatalyzed transacylation and acyl-CoA-dependent acylation at the lipid bilayer interface. *Biochemistry*. 33:13340-8.

Quilliam, L.A., H. Mueller, B.P. Bohl, V. Prossnitz, L.A. Sklar, C.J. Der, and G.M. Bokoch. 1991. Rap1A is a substrate for cyclic AMP-dependent protein kinase in human neutrophils. *J Immunol*. 147:1628-35.

Ralston, R., and J.M. Bishop. 1985. The product of the protooncogene c-src is modified during the cellular response to platelet-derived growth factor. *Proc Natl Acad Sci U S A*. 82:7845-9.

Ramos, S., F. Khademi, B.P. Somesh, and F. Rivero. 2002. Genomic organization and expression profile of the small GTPases of the RhoBTB family in human and mouse. *Gene*. 298:147-57.

Resh, M.D. 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim Biophys Acta*. 1451:1-16.

Ridley, A.J., A.J. Self, F. Kasmi, H.F. Paterson, A. Hall, C.J. Marshall, and C. Ellis. 1993. rho family GTPase activating proteins p190, bcr and rhoGAP show distinct specificities in vitro and in vivo. *EMBO J*. 12:5151-60.

Riento, K., N. Totty, P. Villalonga, R. Garg, R. Guasch, and A.J. Ridley. 2005. RhoE function is regulated by ROCK I-mediated phosphorylation. *Embo J*. 24:1170-80.

Rittinger, K., P.A. Walker, J.F. Eccleston, S.J. Smerdon, and S.J. Gamblin. 1997. Structure at 1.65 A of RhoA and its GTPase-activating protein in complex with a transition-state analogue. *Nature*. 389:758-62.

Roberts, P.J., N. Mitin, P.J. Keller, E.J. Chenette, J.P. Madigan, R.O. Currin, A.D. Cox, O. Wilson, P. Kirschmeier, and C.J. Der. 2008. Rho Family GTPase modification and dependence on CAAX motif-signaled posttranslational modification. *J Biol Chem*. 283:25150-63.

Roberts, W.G., P.M. Whalen, E. Soderstrom, G. Moraski, J.P. Lyssikatos, H.F. Wang, B. Cooper, D.A. Baker, D. Savage, D. Dalvie, J.A. Atherton, S. Ralston, R. Szewc, J.C. Kath, J. Lin, C. Soderstrom, G. Tkalcevic, B.D. Cohen, V. Pollack, W. Barth, W. Hungerford, and E. Ung. 2005. Antiangiogenic and antitumor activity of a selective PDGFR tyrosine kinase inhibitor, CP-673,451. *Cancer Res*. 65:957-66.

Roche, S., J. Downward, P. Raynal, and S.A. Courtneidge. 1998. A function for phosphatidylinositol 3-kinase beta (p85alpha-p110beta) in fibroblasts during mitogenesis: requirement for insulin- and lysophosphatidic acid-mediated signal transduction. *Mol Cell Biol*. 18:7119-29.

Rocks, O., A. Peyker, M. Kahms, P.J. Verveer, C. Koerner, M. Lumbierres, J. Kuhlmann, H. Waldmann, A. Wittinghofer, and P.I. Bastiaens. 2005. An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science*. 307:1746-52.

Ron, D., S.R. Tronick, S.A. Aaronson, and A. Eva. 1988. Molecular cloning and characterization of the human dbl proto-oncogene: evidence that its overexpression is sufficient to transform NIH/3T3 cells. *EMBO J*. 7:2465-73.

Roskoski, R., Jr. 2004. The ErbB/HER receptor protein-tyrosine kinases and cancer. *Biochem Biophys Res Commun*. 319:1-11.

Rossman, K.L., C.J. Der, and J. Sondek. 2005. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol*. 6:167-80.

Roth, A.F., Y. Feng, L. Chen, and N.G. Davis. 2002. The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase. *J Cell Biol*. 159:23-8.

Ruusala, A., and P. Aspenstrom. 2008. The atypical Rho GTPase Wrch1 collaborates with the nonreceptor tyrosine kinases Pyk2 and Src in regulating cytoskeletal dynamics. *Mol Cell Biol*. 28:1802-14.

Sablina, A.A., W. Chen, J.D. Arroyo, L. Corral, M. Hector, S.E. Bulmer, J.A. DeCaprio, and W.C. Hahn. 2007. The tumor suppressor PP2A Abeta regulates the RalA GTPase. *Cell*. 129:969-82.

Sahai, E., and C.J. Marshall. 2002. RHO-GTPases and cancer. *Nat Rev Cancer*. 2:133-42.

Sakai, R., A. Iwamatsu, N. Hirano, S. Ogawa, T. Tanaka, H. Mano, Y. Yazaki, and H. Hirai. 1994. A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. *EMBO J*. 13:3748-56.

Samuels, M.L., M.J. Weber, J.M. Bishop, and M. McMahon. 1993. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. *Mol Cell Biol*. 13:6241-52.

Sandilands, E., and M.C. Frame. 2008. Endosomal trafficking of Src tyrosine kinase. *Trends Cell Biol*. 18:322-9.

Saras, J., P. Wollberg, and P. Aspenstrom. 2004. Wrch1 is a GTPase-deficient Cdc42-like protein with unusual binding characteristics and cellular effects. *Exp Cell Res*. 299:356-69.

Sasaoka, T., W.J. Langlois, J.W. Leitner, B. Draznin, and J.M. Olefsky. 1994. The signaling pathway coupling epidermal growth factor receptors to activation of p21ras. *J Biol Chem*. 269:32621-5.

Scherle, P., T. Behrens, and L.M. Staudt. 1993. Ly-GDI, a GDP-dissociation inhibitor of the RhoA GTP-binding protein, is expressed preferentially in lymphocytes. *Proc Natl Acad Sci U S A*. 90:7568-72.

Schiavone, D., S. Dewilde, F. Vallania, J. Turkson, F. Di Cunto, and V. Poli. 2009. The RhoU/Wrch1 Rho GTPase gene is a common transcriptional target of both the gp130/STAT3 and Wnt-1 pathways. *Biochem J*. 421:283-92.

Schmidt, A., and A. Hall. 2002. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev*. 16:1587-609.

Schmidt, A., M. Wolde, C. Thiele, W. Fest, H. Kratzin, A.V. Podtelejnikov, W. Witke, W.B. Huttner, and H.D. Soling. 1999. Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. *Nature*. 401:133-41.

Schmidt, M.H., and I. Dikic. 2005. The Cbl interactome and its functions. *Nat Rev Mol Cell Biol*. 6:907-19.

Seabra, M.C., J.L. Goldstein, T.C. Sudhof, and M.S. Brown. 1992. Rab geranylgeranyl transferase. A multisubunit enzyme that prenylates GTP-binding proteins terminating in Cys-X-Cys or Cys-Cys. *J Biol Chem*. 267:14497-503.

Shindo, M., H. Wada, M. Kaido, M. Tateno, T. Aigaki, L. Tsuda, and S. Hayashi. 2008. Dual function of Src in the maintenance of adherens junctions during tracheal epithelial morphogenesis. *Development*. 135:1355-64.

Shuai, K., A. Ziemiecki, A.F. Wilks, A.G. Harpur, H.B. Sadowski, M.Z. Gilman, and J.E. Darnell. 1993. Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. *Nature*. 366:580-3.

Shutes, A., A.C. Berzat, E.J. Chenette, A.D. Cox, and C.J. Der. 2006. Biochemical analyses of the Wrch atypical Rho family GTPases. *Methods Enzymol*. 406:11-26.

Shutes, A., A.C. Berzat, A.D. Cox, and C.J. Der. 2004. Atypical mechanism of regulation of the Wrch-1 Rho family small GTPase. *Curr Biol*. 14:2052-6.

Siddiquee, K., S. Zhang, W.C. Guida, M.A. Blaskovich, B. Greedy, H.R. Lawrence, M.L. Yip, R. Jove, M.M. McLaughlin, N.J. Lawrence, S.M. Sebti, and J. Turkson. 2007a. Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity. *Proc Natl Acad Sci U S A*. 104:7391-6.

Siddiquee, K.A., P.T. Gunning, M. Glenn, W.P. Katt, S. Zhang, C. Schrock, S.M. Sebti, R. Jove, A.D. Hamilton, and J. Turkson. 2007b. An oxazole-based small-molecule Stat3 inhibitor modulates Stat3 stability and processing and induces antitumor cell effects. *ACS Chem Biol*. 2:787-98.

Siegfried, E., E.L. Wilder, and N. Perrimon. 1994. Components of wingless signalling in Drosophila. *Nature*. 367:76-80.

Simova, S., M. Klima, L. Cermak, V. Sourkova, and L. Andera. 2008. Arf and Rho GAP adapter protein ARAP1 participates in the mobilization of TRAIL-R1/DR4 to the plasma membrane. *Apoptosis*. 13:423-36.

Sirokmany, G., L. Szidonya, K. Kaldi, Z. Gaborik, E. Ligeti, and M. Geiszt. 2006. Sec14 homology domain targets p50RhoGAP to endosomes and provides a link between Rab and Rho GTPases. *J Biol Chem*. 281:6096-105.

Sorkin, A., and M. Von Zastrow. 2002. Signal transduction and endocytosis: close encounters of many kinds. *Nat Rev Mol Cell Biol*. 3:600-14.

Stamenova, S.D., M.E. French, Y. He, S.A. Francis, Z.B. Kramer, and L. Hicke. 2007. Ubiquitin binds to and regulates a subset of SH3 domains. *Mol Cell*. 25:273-84.

Staufenbiel, M. 1987. Ankyrin-bound fatty acid turns over rapidly at the erythrocyte plasma membrane. *Mol Cell Biol*. 7:2981-4.

Stehelin, D., D.J. Fujita, T. Padgett, H.E. Varmus, and J.M. Bishop. 1977. Detection and enumeration of transformation-defective strains of avian sarcoma virus with molecular hybridization. *Virology*. 76:675-84.

Takai, Y., K. Kaibuchi, A. Kikuchi, and T. Sasaki. 1995. Effects of prenyl modifications on interactions of small G proteins with regulators. *Methods Enzymol*. 250:122-33.

Taneyhill, L., and D. Pennica. 2004. Identification of Wnt responsive genes using a murine mammary epithelial cell line model system. *BMC Dev Biol*. 4:6.

Tao, W., D. Pennica, L. Xu, R.F. Kalejta, and A.J. Levine. 2001. Wrch-1, a novel member of the Rho gene family that is regulated by Wnt-1. *Genes Dev*. 15:1796-807.

Theisen, H., J. Purcell, M. Bennett, D. Kansagara, A. Syed, and J.L. Marsh. 1994. dishevelled is required during wingless signaling to establish both cell polarity and cell identity. *Development*. 120:347-60.

Thomas, S.M., and J.S. Brugge. 1997. Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol*. 13:513-609.

Tu, S., W.J. Wu, J. Wang, and R.A. Cerione. 2003. Epidermal growth factor-dependent regulation of Cdc42 is mediated by the Src tyrosine kinase. *J Biol Chem*. 278:49293-300.

Ueda, T., A. Kikuchi, N. Ohga, J. Yamamoto, and Y. Takai. 1990. Purification and characterization from bovine brain cytosol of a novel regulatory protein inhibiting the dissociation of GDP from and the subsequent binding of GTP to rhoB p20, a ras p21-like GTP-binding protein. *J Biol Chem*. 265:9373-80.

Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell*. 61:203-12.

van Golen, K.L., Z.F. Wu, X.T. Qiao, L.W. Bao, and S.D. Merajver. 2000. RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. *Cancer Res*. 60:5832-8.

Vieira, A.V., C. Lamaze, and S.L. Schmid. 1996. Control of EGF receptor signaling by clathrin-mediated endocytosis. *Science*. 274:2086-9.

Vignal, E., M. De Toledo, F. Comunale, A. Ladopoulou, C. Gauthier-Rouviere, A. Blangy, and P. Fort. 2000. Characterization of TCL, a new GTPase of the rho family related to TC10 andCcdc42. *J Biol Chem*. 275:36457-64.

Villalonga, P., R.M. Guasch, K. Riento, and A.J. Ridley. 2004. RhoE inhibits cell cycle progression and Ras-induced transformation. *Mol Cell Biol*. 24:7829-40.

Walden, H., M.S. Podgorski, and B.A. Schulman. 2003. Insights into the ubiquitin transfer cascade from the structure of the activating enzyme for NEDD8. *Nature*. 422:330-4.

Wang, Y., S.D. Pennock, X. Chen, A. Kazlauskas, and Z. Wang. 2004. Platelet-derived growth factor receptor-mediated signal transduction from endosomes. *J Biol Chem*. 279:8038-46.

Waterfield, M.D., G.T. Scrace, N. Whittle, P. Stroobant, A. Johnsson, A. Wasteson, B. Westermark, C.H. Heldin, J.S. Huang, and T.F. Deuel. 1983. Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. *Nature*. 304:35-9.

Wennerberg, K., and C.J. Der. 2004. Rho-family GTPases: it's not only Rac and Rho (and I like it). *J Cell Sci*. 117:1301-12.

Wennerberg, K., K.L. Rossman, and C.J. Der. 2005. The Ras superfamily at a glance. *J Cell Sci*. 118:843-6.

Wherlock, M., and H. Mellor. 2002. The Rho GTPase family: a Racs to Wrchs story. *J Cell Sci*. 115:239-40.

Williams, C.L. 2003. The polybasic region of Ras and Rho family small GTPases: a regulator of protein interactions and membrane association and a site of nuclear localization signal sequences. *Cell Signal*. 15:1071-80.

Willumsen, B.M., A.D. Cox, P.A. Solski, C.J. Der, and J.E. Buss. 1996. Novel determinants of H-Ras plasma membrane localization and transformation. *Oncogene*. 13:1901-9.

Winge, P., T. Brembu, R. Kristensen, and A.M. Bones. 2000. Genetic structure and evolution of RAC-GTPases in Arabidopsis thaliana. *Genetics*. 156:1959-71.

Winter-Vann, A.M., and P.J. Casey. 2005. Post-prenylation-processing enzymes as new targets in oncogenesis. *Nat Rev Cancer*. 5:405-12.

Wodarz, A. 2002. Establishing cell polarity in development. *Nat Cell Biol*. 4:E39-44.

Wodarz, A., and I. Nathke. 2007. Cell polarity in development and cancer. *Nat Cell Biol*. 9:1016-24.

Wong, C.M., J.M. Lee, Y.P. Ching, D.Y. Jin, and I.O. Ng. 2003. Genetic and epigenetic alterations of DLC-1 gene in hepatocellular carcinoma. *Cancer Res*. 63:7646-51.

Wu, J.C., T.Y. Chen, C.T. Yu, S.J. Tsai, J.M. Hsu, M.J. Tang, C.K. Chou, W.J. Lin, C.J. Yuan, and C.Y. Huang. 2005. Identification of V23RalA-Ser194 as a critical mediator for Aurora-A-induced cellular motility and transformation by small pool expression screening. *J Biol Chem*. 280:9013-22.

Wu, M., Z.F. Wu, C. Kumar-Sinha, A. Chinnaiyan, and S.D. Merajver. 2004. RhoC induces differential expression of genes involved in invasion and metastasis in MCF10A breast cells. *Breast Cancer Res Treat*. 84:3-12.

Wu, W.J., S. Tu, and R.A. Cerione. 2003. Activated Cdc42 sequesters c-Cbl and prevents EGF receptor degradation. *Cell*. 114:715-25.

Yamamoto, Y., T. Maruyama, N. Sakai, R. Sakurai, A. Shimizu, T. Hamatani, H. Masuda, H. Uchida, H. Sabe, and Y. Yoshimura. 2002. Expression and subcellular distribution of the active form of c-Src tyrosine kinase in differentiating human endometrial stromal cells. *Mol Hum Reprod*. 8:1117-24.

Yano, A., S. Tsutsumi, S. Soga, M.J. Lee, J. Trepel, H. Osada, and L. Neckers. 2008. Inhibition of Hsp90 activates osteoclast c-Src signaling and promotes growth of prostate carcinoma cells in bone. *Proc Natl Acad Sci U S A*. 105:15541-6.

Yokote, K., B. Margolis, C.H. Heldin, and L. Claesson-Welsh. 1996. Grb7 is a downstream signaling component of platelet-derived growth factor alpha- and beta-receptors. *J Biol Chem*. 271:30942-9.

Yoon, H.Y., J.S. Lee, and P.A. Randazzo. 2008. ARAP1 regulates endocytosis of EGFR. *Traffic*. 9:2236-52.

Yu, H., X. Li, G.S. Marchetto, R. Dy, D. Hunter, B. Calvo, T.L. Dawson, M. Wilm, R.J. Anderegg, L.M. Graves, and H.S. Earp. 1996. Activation of a novel calcium-dependent protein-tyrosine kinase. Correlation with c-Jun N-terminal kinase but not mitogen-activated protein kinase activation. *J Biol Chem*. 271:29993-8.

Yuan, B.Z., M.E. Durkin, and N.C. Popescu. 2003a. Promoter hypermethylation of DLC-1, a candidate tumor suppressor gene, in several common human cancers. *Cancer Genet Cytogenet*. 140:113-7.

Yuan, B.Z., X. Zhou, M.E. Durkin, D.B. Zimonjic, K. Gumundsdottir, J.E. Eyfjord, S.S. Thorgeirsson, and N.C. Popescu. 2003b. DLC-1 gene inhibits human breast cancer cell growth and in vivo tumorigenicity. *Oncogene*. 22:445-50.

Zalcman, G., V. Closson, J. Camonis, N. Honore, M.F. Rousseau-Merck, A. Tavitian, and B. Olofsson. 1996. RhoGDI-3 is a new GDP dissociation inhibitor (GDI). Identification of a non-cytosolic GDI protein interacting with the small GTP-binding proteins RhoB and RhoG. *J Biol Chem*. 271:30366-74.

Zeng, L., F. Fagotto, T. Zhang, W. Hsu, T.J. Vasicek, W.L. Perry, 3rd, J.J. Lee, S.M. Tilghman, B.M. Gumbiner, and F. Costantini. 1997. The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. *Cell*. 90:181-92.

Zhai, L., D. Chaturvedi, and S. Cumberledge. 2004. Drosophila wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires porcupine. *J Biol Chem*. 279:33220-7.

Zhang, Y., and B. Zhang. 2006. D4-GDI, a Rho GTPase regulator, promotes breast cancer cell invasiveness. *Cancer Res*. 66:5592-8.

Zhang, Y.M., and C.O. Rock. 2008. Membrane lipid homeostasis in bacteria. *Nat Rev Microbiol*. 6:222-33.

Zheng, Z.L., and Z. Yang. 2000. The Rop GTPase: an emerging signaling switch in plants. *Plant Mol Biol*. 44:1-9.

Ziman, M., D. Preuss, J. Mulholland, J.M. O'Brien, D. Botstein, and D.I. Johnson. 1993. Subcellular localization of Cdc42p, a Saccharomyces cerevisiae GTP-binding protein involved in the control of cell polarity. *Mol Biol Cell*. 4:1307-16.

Zubiaur, M., L.W. Forman, L.L. Stice, and D.V. Faller. 1996. A role for activated p21 ras in inhibition/regulation of platelet-derived growth factor (PDGF) type-beta receptor activation. *Oncogene*. 12:1213-22.