REGULATION OF ENDOCYTIC RECYCLING

BY FGD4, A CDC42 GEF

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DEDICATION

Dedicated to my Family

REGULATION OF ENDOCYTIC RECYCLING

BY FGD4, A CDC42 GEF

by

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DISSERTATION

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ABSTRACT

REGULATION OF ENDOCYTIC RECYCLING

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The family of Receptor Tyrosine Kinases (RTKs) are a group of cell surface receptors with the capability of activating, through phosphorylation, multiple kinase cascades in response to activation by an extracellular ligand. This allows a cell to respond to its environment and

induce a range of cellular processes such as proliferation, differentiation, migration, and apoptosis. Unsurprisingly, these powerful transducers of extracellular signaling are often found mutated in human disease, such as cancer. Therefore, learning how these receptors are downregulated and processed once they have been activated may provide novel avenues of therapeutic intervention. How receptors are processed after internalization and fusion into the sorting endosome (also known as the early endosome) still largely remains unknown. Here, we discovered a Cdc42 GEF, FGD4, that may be important for shuttling ErbB receptors to the recycling endosome via a microtubule dependent mechanism. Through protein depletion studies we show that FGD4 is important for mitosis, microtubule stability, migration and endocytic trafficking of EGF, an ErbB1 ligand. Dynamic microtubule regulation are critical in these cell biological process, therefore we hypothesize that FGD4 may be regulating these diverse cell functions via a microtubule dependent mechanism.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Angelique W. Whitehurst, Brian O. Bodemann, **Jessica A. Cardenas**, Deborah Ferguson, Luc Girard, Michael Peyton, John D. Minna, Carolyn Michnoff, Weihua Hao, Michael G. Roth, Xian-Jin Xie, and Michael A. White. (2007) Synthetic Lethal Screen Identification of Chemosensitizer Loci in Cancer Cells. Nature, **446**, 815-9.

Birger Herzog**, Jessica A. Cardenas**, Robert K. Hall, Josep A. Villena, Philip J. Bugde, Vinvent Giguere, Daryl K. Granner, and Anastasia Kralli. (2006) Estrogen-Related Receptor Alpha is a Repressor of Phosphoenolpyruvate Carboxykinase Gene Transcription. J Biol Chem, **281**, 99-106.

Sylvia N. Schreiber, Roger Emter, M. Benjamin Hock, Darko Knutti, **Jessica A. Cardenas**, Michael Podvinec, Edward J. Oakeley, and Anastasia Kralli. (2004) The Estrogen-Related Receptor Alpha Functions in PPARgamma Coactivator 1 alpha Induced Mitochondrial Biogenesis. Proc Natl Acad Sci USA, **101**, 6472-7.

LIST OF FIGURES

LIST OF ABBREVIATIONS

RTK	Receptor Tyrosine Kinase
EGF	Epidermal Growth Factor
MAPK	Mitogen Activated Protein Kinase
JNK	c-Jun N-terminal Kinase
PI3K	Phosphoinositol-3-Kinase
TGFa	Transforming Growth Factor alpha
ERK	Extracellular signal-regulated kinase
SNARE	Soluble N-ethylmaleimide-sensitive-factor Attachement protein Receptor
TnfR	Transferrin Receptor
Tnf	Transferrin
SNX4	Sorting Nexin 4
ESCRT	Endosomal Sorting Comples Required for Transport
MVB	Multi Vesicular Body
BMP	Bone Morphogenic Protein
Gbb	Glass Bottom Boat
Nwk	Nervous Wreck
G-actin	Globular Actin
MAPs	Microtubule Associated Proteins

+TIPs	Microtubule Plus End Binding Proteins
GAP	GTPase Activating Protein
GEF	Guanylnucleotide Exchange Factor
DH	Dbl Homologous domain
PH	Pleckstrin Homology domain
PDGF	Platelet Derived Growth Factor
BCR	B-cell Receptor
siRNA	small interfering RNA
NRG1	Neuregulin 1
FGD4	Facio Genital Dysplasia – 4
FYVE	Fab1, YOTB, Vac1, EEA1
CMT4H	Charcot Marie Tooth type 4H
PHH3	phospho-Histone H3
EGTA	Ethylene Glycol Tetraacetic Acid
MHC I	Major Histocompatibility Complex Class I
QPCR	Quantitative Polymerase Chain Reaction
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate Buffered Saline
FITC	Fluorescein Isothiocyanate
FBS	Fetal Bovine Serum
RBD	Rho Binding Domain

GST	Glutathione S-transferase

CHAPTER ONE
Introduction

Surface Receptor Signaling

In order for cells to function properly and adapt to their environment they must be able to detect a multitude of extracellular signals and correctly translate this information into an appropriate behavior. This is achieved through expression and display of a variety of cell surface receptors that bind extracellular peptides to induce specific biological fates. Receptor Tyrosine Kinases are a family of 58 receptors that, once activated, can induce multiple cellular programs such as cell proliferation, cell differentiation, cell migration, and apoptosis. The subclass I of RTK receptors are comprised of ErbB1-4 which contain an extracellular ligand binding region, a transmembrane domain, and a cytoplasmic protein tyrosine kinase domain. The ErbB receptors not only play a big role in the normal development of an organism, they are also mutated in many cancer types and therefore, are a major target for therapeutic development. ErbB1, also known as EGF Receptor, is the best studied member of the ErbB family whose inactivation in mice causes such severe defects that mice who survive until birth die soon after. The development

of multiple organs such as skin, lungs, gastrointestinal tract and some parts of the brain are perturbed in the absence of ErbB1 activity. Studies have shown that perturbation of ErbB2 affects both cardiac development and Schwann cell development. The ErbB2 receptor does not have the ability to bind a ligand and instead heterodimerizes with the other ErbB receptors. ErbB2 function is indispensable for ErbB3 signaling since it does not have a functional kinase domain. Therefore, the effects of ErbB2 downregulation are likely due to its ability to heterodimerize with the other ErbB family members. ErbB2 overexpression is found in a multitude of cancer types, and also indicates a poor patient prognosis (Holbro and Hynes 2004).

Ligand binding of ErbB receptors induces heterodimerization and homodimerization, which is necessary for its kinase activity. Once activated the receptor dimers autophosphorylate each other which potentiates its kinase activity, allowing for robust activation of several downstream signaling cascades: MAPK (mitogen activated protein kinase) pathway, the PI(3)K-activated AKT pathway and the JNK pathway. The ErbB receptors also differ in their ability to activate the AKT pathway, a powerful proliferative signaling cascade. ErbB3 can directly bind and activate the PI3K subunit, p85, thereby more potently inducing the AKT

pathway. Schoeberl et al. showed that AKT phosphorylation peaked the highest when cells were stimulated with HRG1-b, an ErbB3 ligand, when compared to ErbB1 ligands such as EGF and TGFa. Interestingly, ErbB3 receptor does not contain a functional kinase domain and is obligated to heterodimerize with ErbB2 and ErbB4 to induce downstream signaling. Activation of the kinase cascades ultimately leads to the initiation of multiple transcriptional programs (e.g. fos, jun, myc) that result in changes in cell division, cell migration, cell adhesion, cell differentiation and/or cell death (Figure 1-1) (Yarden and Sliwkowski 2001).

Given that this receptor family has such robust proliferative signaling potential in response to ligands groups have found various receptor mutations and mutations of its downstream targets in several types of cancers. For example, a constitutively active mutant of ErbB1, v-ErbB, was first identified in an avian virus isolated from a spontaneous erythroleukemia in a chicken. Downward et al. characterized the viral oncogene as a truncation mutant of ErbB1 that contains the transmembrane and cytoplasmic tail, but is missing the ligand binding domain. The v-ErbB protein was further shown to dimerize with itself and available wild type ErbB1, thereby activating the kinase activity of the dimers in a ligand independent manner (Boerner, Danielsen et al. 2003).

The downstream Ras/MAPK and PI3K/AKT signaling cascades induce powerful growth and proliferation programs and members of these cascades are often found with activating mutations in many cancer types. Cancers driven by these mutation types would not be amenable to intervention at the receptor level but demonstrate the importance of the programs downstream of activated receptors. For this reason ErbB receptor trafficking and signaling downregulation has been a popular topic of research with the goal of finding new targets for intervention.

Active ErbB receptors are downregulated by endocytosis and trafficking towards the lysosome for degradation. The endocytic routes for the ErbB family of receptors differ and lead to a high degree of receptor recycling for ErbB2-4 and a high degree of receptor degradation for ErbB1. The activating phosphorylation sites on ErbB1 recruits c-Cbl, a E3 ligase that polyubiquitinates the receptor and eventually leads to endocytosis and lysosomal degradation, which will be discussed in greater detail later on. In contrast, activated ErbB2 and ErbB3 bind c-Cbl to a lesser extent, which allows these receptors to preferentially be recycled and permits multiple rounds of signaling by a single receptor. Indeed, proteins involved in various phases of receptor internalization and downregulation have been implicated in cancer cell transformation and

cancer progression (Table 1). For example, a viral oncogenic truncation mutant version of Cbl, another E3 ligase in the c-Cbl family of proteins, was discovered as a transforming gene of the Cas NS-1 virus. Expression of this mutant protein causes myeloid leukemias in mice and can transform human fibroblasts in culture (Langdon, Hyland et al. 1989). Other proteins involved in the general function of endocytic trafficking have also been implicated in cancer progression. For example, the protein cortactin is commonly found amplified in breast, head, and neck cancers and is thought to contribute to cancer cell invasion and metastasis. Cortactin coordinates actin polymerization at endocytic sites and Timpson et al. found that overexpression of this protein inhibited EGF induced ErbB1 degradation and, consequently, resulted in sustained ERK activation in response to EGF. Major players in the manifestation of key endocytic compartments have been identified and their mechanisms explored to some extent. However, the molecular players involved in sorting cargoes to different compartments are just coming to light. In the next section, I will introduce what is known about the main compartments traveled by surface receptor proteins and explore how sorting cargo might be achieved.

Endosomal sorting and trafficking

As mentioned above, receptor deactivation can be mediated by receptor internalization and degradation in the lysosomal compartment. Receptor internalization can occur through several routes: clathrin-dependent, clathrin-independent, and caveolar. However, all early endocytic carrier vesicles eventually make their way into the Rab5 positive early endosome for sorting to their final destinations. Once at the early endosome, cargo proteins can be sorted to the lysosome for degradation, the recycling endosome for a return to the plasma membrane, or the transgolgi network for further processing. As the transgolgi retrograde path is mainly traveled by acid-hydrolase receptors, transmembrane enzymes and SNAREs (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) further discussion will focus on the recycling and degradative pathways (Bonifacino and Traub 2003). The early endosome is characterized by the residency of the Rab5 GTPase, its effector protein VPS35/p150, and the phosphoinositide (PtdIns(3)P) product of their activity. This particular phosphoinositide helps manifest the identity of the endosomal compartment, which presumably aids in targeting cargo proteins to this compartment. Cargo protein sorting then occurs by grouping of proteins into discrete domains on the endosomal membrane in

preparation for endosomal scission and transport to their final destinations (Figure 2-1). For transporting proteins to the plasma membrane, transgolgi network, or recycling endosome the discrete domains begin as distinct patches of the endosomal membrane that are enriched with alternate Rab GTPases, sorting nexins, and other species of phosphoinositides. These proteins eventually deform the membrane into a tubule structure that recruit motor proteins to mediate pinching off and transport along the microtubule network. Recent work by various groups point to sorting nexins as adaptor proteins that can recruit cargo to the early endosome tubular domains destined for scission by virtue of their phospholipid binding motif (PX domain) and tendency to form protein-protein complexes (Worby and Dixon 2002). For example, the Transferrin Receptor (TnfR) is primarily recycled through the fast recycling and slow recycling routes after binding its ligand, transferrin. Traer et al. show that depletion of the Sorting Nexin 4 (SNX4) causes a decrease in TnfR protein levels and transferrin internalization. The reduction in TnfR levels was rescued by lysosomal protease inhibitors, indicating that in the absence of SNX4 the receptor is mistrafficked away from the recycling endosome to the lysosomal compartment. The group goes on to show that a SNX4-KIBRA-Dynein protein complex is necessary for transport of TnfR containing endosomes

from the cell periphery to the perinuclearly localized recycling endosome (Traer, Rutherford et al. 2007). Thus, SNX4 mediates recruitment of the Transferrin receptor into transport vesicles destined for the recycling endosome. The human genome encodes for about 36 kinesins and 32 sorting nexins, giving rise to a large number of possible combinations to link different cargoes to kinesins destined for different cellular locales.

Transport to the degradative lysosomal compartment has been studied in much more detail and is mediated by the ESCRT (endosomal sorting complex required for transport) complex. ESCRT mediated lysosomal degradation been best described for receptor tyrosine kinase (RTKs). RTKs have cytoplasmic tails that can accumulate post-translation modifications to form interactions with adaptor proteins at the plasma membrane that will have a hand in directing their endosomal trafficking. The ErbB1 receptor's fate starts with a specific auto-phosphorylation event after ligand activation at Y1045. This phosphorylation site mediates the ErbB1 interaction with the E3 ubiquitin ligase, c-Cbl, which poly-ubiquitinates the activated receptor and marks it for lysosomal degradation. Studies have shown that a mutation of this site greatly inhibits the ubiquitination of ErbB1 and it's downregulation while increasing it's recycling. This mutation can be found in the oncogenic form of ErbB1

(EGFRvII), commonly found in glioblastomas (Mosesson, Mills et al. 2008). Recruitment of c-Cbl for ubiquitination of ErbB1 as well as recruitment of endosome adaptor proteins such as CIN85, EPS15, and a component of the ESCRT complex (Hrs) are crucial to the endocytosis of the activated receptor and eventual degradation. Once the endosome containing the ErbB1/c-Cbl/endocytic proteins complex reaches the early endosome, the rest of the ESCRT complex assembles onto the existing complex to begin the invagination of the cargo proteins into a luminal vesicle. Formation of luminal vesicles along with the exchange of resident endosomal proteins marks the maturation of the early endosome into a Multi-Vesicular Body (MVB). Once the cargo proteins are contained in a luminal vesicle, they are irrevocably committed to degradation as the final step is fusion of the MVB with the lysosome where the contents of the endosomal compartment are degraded. Proteins residing on the endosomal membrane, also known as the limiting membrane, avoid degradation and can continue to signal from this locale (Lu and Hunter 2009). However, if ErbB1 can dissociate from it's ligand (EGF) and undergo deubiquitination, the receptor will no longer become associated with the ESCRT complex and instead becomes incorporated into a tubule structure that will bud off of the early endosome and merge with the

recycling endosome. The best example of this comes from a comparison of ErbB1 degradation in response to stimulation with two different ligands: TGFa (Transforming Growth Factor a) and EGF (Epidermal Growth Factor) (Zwang and Yarden 2009). Both ligands bind and activate ErbB1, but TGFa differs from EGF in its affinity for the receptor. While EGF may remain bound ErbB1 and maintain its activated state through many endocytic compartments, TGFa binding to the receptor is subject to slight changes in pH and therefore dissociates soon after internalization, allowing deactivation of the RTK. Consequently, TGFa stimulation allows for a greater percentage of ErbB1 recycling when compared to EGF simulation (100% vs. 50%, respectively).

Clearly, this fork in the road for receptor tyrosine kinases can have a profound affect on downstream signaling; depending on the sensitivity of the signaling cascade the receptor is inducing. For example, the *drosophila melanogaster* larval neuromuscular junction expands 100-fold in a four day developmental period providing a useful model for the regulation of synaptic growth signaling. During this time motor axons at the synapse establish contacts with muscles cells to form a branched synaptic terminal arbor. The Bone Morphogenetic Protein (BMP) ligand named Glass bottom boat (Gbb) is released by the muscle and stimulates

arborization on the neuronal cell membrane. Careful regulation of the signaling cascade triggered by Gbb is necessary to manage the formation of appropriate synaptic structures. The Littleton group has shown that perturbation of the presynaptic endosomal trafficking pathway that affect the Gbb receptor trafficking leads to increased arborization, presumably due to failure to attenuate the activated receptor signal (Rodal, Blunk et al.). Specifically, loss of Nervous Wreck (Nwk) an F-bar containing protein that localizes to the early endosome is thought to cause the retention of the activated Gbb receptor in the early endosome and inappropriately sustain the signaling cascade. Thus, disregulation of receptor trafficking can have profound effects in a biological system sensitive to ligand levels.

Cytoskeleton and Endosomal Trafficking

Regulating the receptor population present on the cell surface as well as downregulating activated receptor rely on the ability of the cell to transport these cargoes to the appropriate cellular compartments. In this section, I will introduce the scaffolding that provides structure to the cell and explore the role of this scaffolding in endosomal trafficking.

The cellular cytoskeleton is composed of two main matrices: the microtubule network and the actin cytoskeleton. These two systems of

cables give shape to the cell and undergo tremendous rearrangements when cells change morphology for migration, mitosis, synaptic growth, etc. The actin cytoskeleton is typically thought to provide the scaffolding and force for the formation of filopodia, lamellipodia, and microspikes. These structures are created in response to some ligand stimulus in a short time frame. The ability of actin polymers, also called microfilaments, to shrink and grow is critical for the ability of the actin cytoskeleton to reshape cell morphology. The rate limiting step for actin microfilament formation is the binding of the first three monomeric G-actin (globular actin) of the polymer. The energy required of G-actin incorporation into the nucleated polymer decreases as the polymer length increases. Therefore, actin polymerization promoting proteins are mainly involved in forming a nucleating site for microfilament growth and these proteins are regulated by Rho GTPases, a family of small GTPases that will be discussed in more detail later. The microfilaments formed by G-actin have polarity in which the “plus” end incorporates monomers at a greater rate while the actin monomers dissociate from the “minus” end. Capping proteins bind the ends of microfilaments to promote either polymerization or depolymerization of actin at the ends. In addition, a burst of actin polymerizing activity in small cell foci is often utilized to deform the cell

membrane at the leading edge of a migrating cell, and even in propelling an endosome across the cytoplasm for a short distance. These bursts of actin polymerization are typically induced via activation of actin nucleating complexes.

Microtubules provide a scaffold for maintaining cell structure and whose dynamic instability is crucial for processes that require a significant change in cell shape like mitosis, cell spreading, and cell polarization. Similar to actin microfilament polymerization, microtubule formation is also regulated by a nucleating body called the microtubule organizing center that is localized next to the nucleus. The microtubule plus ends grow away from the microtubule organizing center toward the plasma membrane, providing polarized tracks from the center of the cell to the periphery. Microtubule associated proteins (MAPs) can bind different sites on the tubulin monomers to either increase polymerization or increase depolymerization, thereby influencing the stability and longevity of microtubule networks. MAPs can also crosslink microtubules with other cytoskeletal structures, organelles, as well as the plasma membrane. Another class of proteins called microtubule plus end binding proteins (+TIPS) can also stabilize microtubules by acting as a tether between the constantly changing plus end of the microtubule and a relatively stationary

protein localized to a particular site in the cell. Signaling cascades typically influence microtubule stability by phosphorylation of certain MAPs or recruitment of +TIP proteins to sites of activity. For example, lysophophatidic acid induces stabilization of microtubules at the leading edge of migrating cells via activation of the GTPase, RhoA. Activated RhoA is thought to recruit and bind it's effector mDia at the plasma membrane, thereby relieving its autoinhibition. mDia is then able to bind the +TIP proteins EB1 and APC which then tethers the microtubule plus ends to the leading edge of the plasma membrane (Wen, Eng et al. 2004). Microtubules provide a rigid yet malleable scaffolding for cell structure.

Movement of endosomal carrier vesicles from compartment to compartment was suspected to involve the cytoskeleton when endosomes were observed to travel some distance within a cell. Indeed, perturbation of both microtubules and actin with small molecules do affect the movement of vesicles and the integrity of whole endosomal compartments. Studies of endocytic recycling and degradative pathways have revealed that endosomes utilize both cytoskeletal structures as well as their corresponding motor proteins. The actin cytoskeleton is typically involved in early endosome movements while microtubules are important for long range vesicle movements towards the perinuclear area that

occurs some time after the initial endosome internalization event. In 1996, Durrbach et al. treated mammalian cells with Cytochalasin D, a small molecule that binds monomeric G actin and prevents actin polymerization, and observed an accumulation of clathrin coated pits attached to the plasma membrane by long thin necks. This observation indicated that active actin polymerization is necessary for abscission of the vesicle from the plasma membrane. Consequently, total internalization of the Transferrin receptor was reduced as well as degradation of a2-macroglobulin was reduced by exposure to Cytochalasin D. One decade later, the Rottner group found that N-WASP, a promoter of actin polymerization, is responsible for the actin accumulation around internalizing clathrin-coated vesicles. To date, others have also shown that actin polymerizing proteins Arp2/3, N-WASP, and WASH are necessary for formation of filamentous actin comets seen juxtaposed with internalized endosomes as well. Two functions for this actin comet have been found: 1) force generation for endosomal movement; 2) interaction with microtubules and dynamin for microtubule dependent endosomal movement (Benesch, Lommel et al. 2002; Derivery, Sousa et al. 2009). Treatment of mammalian cultured cells with nocodazole, a small molecule that binds b-tubulin and prevents polymerization, causes a dramatic

disorganization of the lysosomal compartments (Durrbach, Louvard et al. 1996). Typically, the acidic lysosomes are gathered around the microtubule organizing center (MTOC) positioned next to the nucleus. Disruption of microtubules causes the dispersion of the LAMP-1 positive endosomes to the periphery of the cell. Taxol, another microtubule disrupting agent, will also disturb Transferrin trafficking to the endocytic recycling compartment and leads to its retention in peripherally located endosomes (Lin, Gundersen et al. 2002). In addition, Bananis et al. demonstrated that endocytic vesicles travel along fixed microtubules in vitro and Loubery et al. established that endocytic vesicles interacted with both kinesins and dyneins when endosomes were immunoprecipitated, further implicating microtubules and microtubule motor proteins in endocytic trafficking. Kinesins are plus end directed motor proteins while dyneins are minus end directed motor proteins, both of which can bind endosomes and whose depletion leads to perturbations of recycling and degradation of recycled proteins. For example, Kif16B is recruited to early endosomes by the PI3P production activity of the Rab5/Vps34 complex. Depletion of this kinesin inhibits Transferrin delivery to the plasma membrane and increases the degradation of EGFR after ligand stimulation. Overexpression of Kif16B, in turn, restrains the EGF induced

EGFR degradation. Kif16B is thought to be important for the delivery of recycled proteins to the plasma membrane and whose absence will cause the normally recycled proteins to be degraded, likely via delivery to the lysosome (Hoepfner, Severin et al. 2005). Several observations point toward the coordinated involvement of the actin and microtubule cytoskeletons for endosomal trafficking. For example, WASH, a promoter of actin polymerization, is important for fission of endocytic endosomes and, consequently, the recycling of Transferrin. Knockdown of WASH causes endosomal tubulation, a phenotype similar to dynamin inhibition by a small molecule inhibitor (dynasore). In addition, WASH co-immunoprecipitates with Dynamin and disruption of microtubules with nocodazole causes WASH-positive vesicles to stop moving (Derivery, Sousa et al. 2009). These observations demonstrate the interdependence of actin and microtubule roles in endosome trafficking, though how these two cytoskeletal networks interact during endocytic trafficking remains to be determined.

Rho GTPase Regulation of Cytoskeleton and Endocytosis

Rho GTPases are a subfamily of small monomeric GTPases related to the Ras superfamily of GTPases generally known to regulate the

cell cytoskeleton, cell polarization, endocytosis, and cell migration. Rho GTPases are then positioned to coordinate the function of the cytoskeleton and endocytic vesicles in regulation of receptor trafficking. In this section, I will introduce Rho GTPase function and explore their role in vesicle trafficking.

Rho GTPases are small molecular switches that toggle between the GTP bound (“on”) and GDP bound (“off”) states. The GTP bound state allows the small GTPase to engage it’s downstream effectors and execute the appropriate cellular process. These proteins have the ability to switch off their active state by hydrolyzing the bound GTP to GDP, however the basal hydrolysis activity is very low and needs to be enhanced by GTPase activating proteins (GAPs). The active state is once again engaged by exchange of the bound GDP for GTP, a process that is aided by Guanylnucleotide Exchange Factors (GEFs). GEF proteins are characterized by adjacent Dbl and PH domains, which are responsible for mediating the GDP to GTP exchange within the Rho GTPases. The Dbl homologous (DH) domain is a stretch of about 200 amino acid residues that was identified in the Dbl protein, the first GEF discovered with exchange activity for human Rho GTPases. The DH domain along with an adjacent Pleckstrin homology domain, a protein domain with the ability to

bind phosphatidylinositols, are both necessary and sufficient to exhibit exchange activity for Rho GTPases (Zheng 2001). The identified 22 human Rho GTPases can be activated by a variety of extracellular signal and they translate these stimuli into discrete cell responses. How an upstream signal activates a particular GEF is not known for all GEFs but has been well described for Vav, a Rac GEF. The Vav protein's Dbl domain is autoinhibited by an N-terminal domain when inactive. Ligand binding of several receptors such as EGFR, PDGF, and BCR lead to the phosphorylation of Vav by downstream elements, structure rearrangement, and exposure of the Dbl domain for Rac binding and activation (Bustelo 2000). As mentioned before, Rho GTPases participate in a multitude of cellular processes; therefore many laboratories have focused their research on finding how these molecular switches achieve specificity. Thus far, GTPase transduction of specific downstream signaling programs is thought to be achieved via engagement of specific combinations of the 70 GEFs, 80 GAPs, and 60 effectors encoded in the human genome. For example, the Rho GTPase, Cdc42 is embryonic lethal when knocked out in mice while the consequences of knocking out Cdc42 GEFs range from no obvious developmental defects to embryonic

lethality (Table 2), demonstrating the ability of GEFs to mediate specific phenotypes of Cdc42 activity (Rossman, Der et al. 2005).

Cdc42 in particular has been implicated as the central molecular switch that induces cell polarization. From polarized cell migration to the establishment and maintenance of epithelial cell polarity, Cdc42 and it's effectors play a pivotal role. Cdc42 was first identified as a gene product necessary for cell division (Johnson and Pringle 1990) in *Saccharomyces cerevisiae* and further studies revealed that it was critical for polarized bud growth during division. The Rho GTPase is activated at a discrete site on the plasma membrane where it coordinates the actin ring formation and endocytic delivery of raw materials necessary for bud growth (Etienne-Manneville 2004). In mammalian cells, Cdc42 activity is concentrated at the leading edge of a migrating cell where both the actin and microtubule networks undergo rearrangement during motility. Loss of Cdc42 activity does not abolish cell movement but it does result in indiscriminate lamellipodia formation at multiple sites on the cell periphery (Nobes and Hall 1999) as well as loss of polarized stabilization of microtubules at the leading edge. Active Cdc42 also recruits a key polarity complex at the migrating front of the cell: Par3/Par6/aPKC protein complex, which mediates the repositioning of the microtubule organizing center to face the

leading edge and, ultimately, direction of migration. The Par proteins may be repositioning the centrosome in a migrating cell by stabilizing microtubules at the leading edge. Par3 interacts with dynein at the cell cortex, thereby regulating the local microtubule dynamics (Schmoranzer, Fawcett et al. 2009). These studies suggest that Cdc42 may be regulating polarized migration by affecting both the actin and microtubule cytoskeleton. Cdc42 is known to stimulate actin polymerization at the migrating edge via activation of the WASP and Arp2/3 nucleation complex and now the Rho GTPase seems to have a direct role in stabilizing microtubules at the leading edge via a tethering mechanism. Fukata et al. show a Cdc42 effector, IQGAP1, is recruited to the leading edge where it binds +TIP proteins, thereby stabilizing the plus end of microtubules at the cell cortex. Thus far, several laboratories have observed that Cdc42 can regulate both the actin and microtubule cytoskeletons using multiple molecular mechanisms.

How cytoskeletal rearrangement leads to organization of proteins into apical/basal domains or migrating/trailing edges is not immediately obvious until a genome wide siRNA screen revealed that key members of the Polarity complex (Par3/Par6/PKC3 and Cdc42) were identified as potential regulators of endocytosis (Balklava, Pant et al. 2007) in a

Caenorhabditis elegans endocytosis model system. Transgenic *c. elegans* expressing green fluorescent protein (GFP) tagged yolk protein (YP170) were utilized in the screen which allows visualization of intestine secretion of YP170-GFP or oocyte uptake of the same protein. Par3, Par6, PKC3, and Cdc42 all affected endocytosis of YP170-GFP and this endocytic phenotype was also confirmed in a *c. elegans* coelomocyte endocytosis assay. To establish that the endocytic function of the polarity complex proteins are necessary in mammalian systems, the group depleted Par6 and Cdc42 in HeLa cells and saw a reduction in MHC-I protein recycling and Transferrin endocytic uptake. In addition, studies in *drosophila melanogaster* by several groups found that flystocks expressing mutant forms of Cdc42, Par6, and aPKC were characterized by unstable adherens junctions such that E-cadherin staining appears discontinuous and begins accumulating in large endocytic vesicles. These date indicate a role for Cdc42 and the polarity complex in apical endocytosis and endocytic recycling that utilize interactions with the cytoskeleton. (Etienne-Manneville, Manneville et al. 2005; Georgiou, Marinari et al. 2008).

ErbB receptor signaling is critical in both organism development and human pathologies. Appropriate trafficking to the plasma membrane and deactivation via lysosomal sorting is critical for proper induction of

downstream cellular programs. Molecular complexes involved (ESCRT complex) in sorting EGFR to the lysosomal compartment have been described, but how cargoes are shuttled to the recycling endosome is still being explored. To date, the Rho GTPase, Cdc42, has long been known to be a regulator of cell polarization and endocytosis. The recent discovery of Par polarity proteins' involvement in endocytosis leads many to speculate that Cdc42 may be a key integrator of endocytosis and polarity. Given that Cdc42 is a molecular switch utilized in various steps of cell polarization and endocytic events, mechanistic studies will be aided by probing the roles of Cdc42 GEFs, GAPs, and effectors since the specificity of Rho GTPases are often derived from their interacting proteins. The advent of siRNA screens have been revealing protein functions in cell biological processes previously unconsidered in an unbiased manner. A screen completed by our lab identifying genes involved in cancer cell survival uncovered a Cdc42 GEF, FGD4, as necessary for cancer cell viability in the presence of a microtubule drug. This functional study of FGD4 in a cell biological context implicates this GEF in microtubule stability and endocytosis. In Chapter 2, I will describe FGD4 depletion studies that demonstrate its role in a variety of cell polarizing events.

Table 1.

Protein	Function in endocytosis	Aberrations in human cancer	Oncogenic properties	Refs
CBL	Mediates multiubiquitylation of RTKs	Point mutations (e.g. R420Q in RING), insertions and deletions in AML	Inhibits ubiquitylation and downregulation of RTK FLT3	69, 167, 168
HIP1 & HIP1R	Coordinates actin remodelling during formation of clathrin-coated vesicles	*HIP1–PDGFBR* fusion in CMML	Induces cytokine-independent growth.	169
		Overexpression in primary epithelial tumours & gliomas	Transforms mouse fibroblasts to induce colonies in soft agar and tumours in nude mice	96
VPS37A	ESCRTI protein; promotes down-regulation of ubiquitylated receptors	Downregulation in HCC	Knockdown strongly stabilizes EGFR	112
ARHGEF7 or COOL1	CDC42 GEF; mediates CDC42-dependent sequestration of Cbl	Overexpression in breast cancers	Necessary for v-Src-induced transformation including tumour formation in nude mice	170
Cortactin	Coordinates actin polymerization at endocytic sites; ARP2–ARP3 activator; binds F-actin and dynamin	Amplification at gene locus (11q13) and protein overexpression in primary breast carcinomas and HNSCCs	Overexpression inhibits ligand-induced EGFR endocytosis; knockdown accelerates receptor downregulation in HNSCC cell lines	171
Clathrin heavy chain	Principal coat protein; instigates membrane invagination	*CHC–ALK* fusion in IMT and large B-cell lymphoma	Constitutive activation of ALK	172
		CHC–TFE3 fusion in paediatric renal carcinoma	Aberrant transcription factor activity?	173
SNAP91	Clathrin-binding adaptor; involved in assembly of clathrin coats	*SNAP91–AF10* fusion in ALL and AML	Fusion comprising clathrin-binding domain of SNAP91and putative transcription factor AF10	174
EPS15	Endocytic scaffold for clathrin; promotes clathrin-mediated endocytosis	*EPS15–MLL* fusion in AML	EPS15 coiled-coil domain mediates oligomerization of MLL, a DNA-binding histone methyltransferase	175
Endophilin II	Induces membrane curvature during vesicle formation.	*EEN–MLL* fusion in AML	EEN coiled coil-dependent dimerization and oncogenic activation of MLL	176
SPRY (Sprouty)	Competes with RTKs for Cbl binding	Downregulation of SPRY1 and SPRY2 in breast and prostate cancers.	Antagonist of Ras–Erk pathway. SPRY1 and SPRY2 overexpression in osteosarcoma and prostate cancer cells, respectively, inhibits cell proliferation and invasion.	177
HAX1	Regulates clathrin-mediated integrin endocytosis	Overexpression in advanced oral carcinoma	Knockdown inhibits endocytosis of integrin αvβ6 and migration of oral carcinoma cells	133
Disabled 2	Cargo-selective clathrin adaptor; recruits myosin VI to clathrin-coated structures	Downregulation in ovarian, prostate, bladder, breast, oesophageal and colorectal carcinomas	Increased expression suppresses growth of choriocarcinoma and prostate cancer cells	178
NUMB	Mediates internalization of β integrins during directional cell migration. Associates with E-cadherin in recycling endosomes	Downregulation in breast cancer	Notch antagonist. Tumour suppressor activity attributed to stabilization of p53	179
GEP100	ARF6 GEF; interacts with activated EGFR	Overexpression in invasive ductal carcinomas of the breast	Knockdown inhibits metastasis formation by breast cancer cells in nude mice	40
RAB25	Regulates receptor recycling. Promotes invasion by delivery of integrin α5β1 to the leading edge	Amplification of genomic locus (1q22) in advanced ovarian and breast cancers	Overexpression promotes increased anchorage-independent growth and tumour cell invasion.	134
NDRG1	RAB4A effector protein involved in E-cadherin recycling	Downregulation in prostate, breast and pancreatic carcinomas	Suppresses metastasis of prostate and colon cancer cells in nude mice.	180
ACK1	Binds clathrin and activated EGFR; promotes receptor degradation	Gene amplification in advanced-stage primary tumours and metastases derived from prostate and breast	Enhances tumorigenesis in nude mice. Promotes degradation of tumour suppressor protein WWOX	181
PAR3	Controls endocytosis and recycling in clathrin-dependent and independent pathways	Downregulation in HCC	Associations with tumour suppressors (VHL and PTEN) and oncogenes (e.g. *ERBB2*) impinge on regulation of cell polarity	182
Caveolin 1	Essential for caveolar biogenesis and endocytosis	Downregulation and sporadic mutation in breast cancer, correlating with oestrogen receptor expression. Upregulation in multiple cancer types; positively correlates with high tumour grade and poor clinical outcome. Amplification in aggressive breast carcinomas	Expression inversely correlates with cell cycle progression and transformation. However, ectopic expression suppresses oncogene-induced apoptosis and confers resistance to anoikis	183

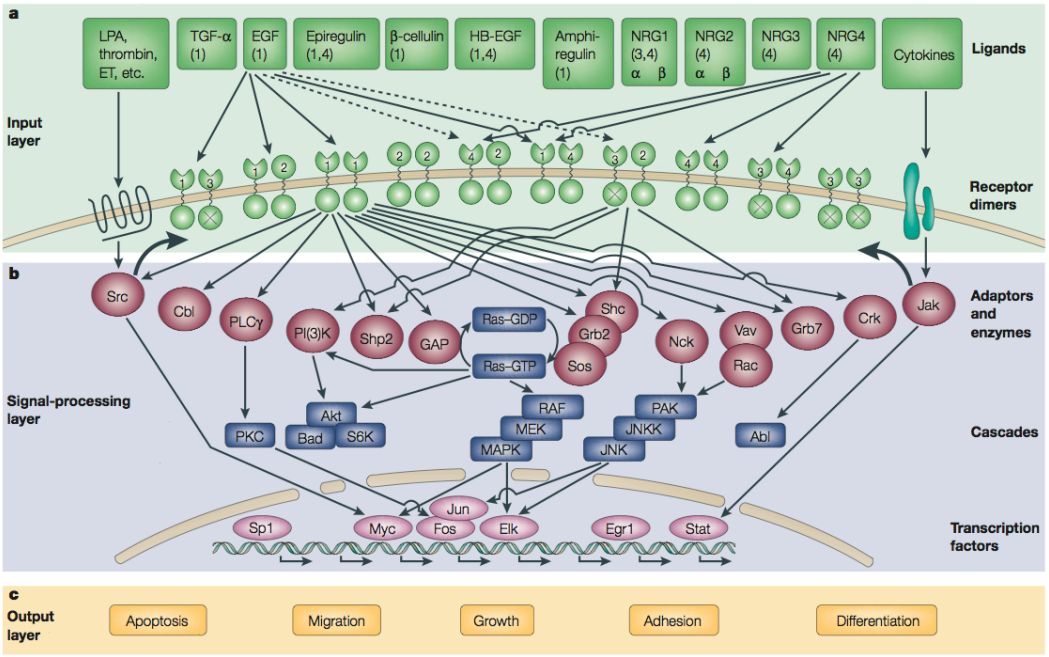
Proteins with reported roles in endocytosis also shown to be misregulated in tumor transformation, growth or progression. Figure modified from Mosesson et al., 2008.

Table 2.

Dbl Protein	Expression	Defect	References
Abr*	Widely expressed, high levels in haematopoietic and nervous tissue	Together with Bcr deficiency causes cerebellar and vestibular defects	124,125
Bcr*	Widely expressed, high levels in haematopoietic and nervous tissue	Neutrophil defect	126
Dbl	Brain, ovary and testes	Defective dendrite elongation	127
Lsc/p115-RhoGEF	Haematopoietic cells	Altered marginal zone B cell and other haematopoietic cell functions	124
RasGRF1‡	Nervous tissue, pancreatic islet cells	Long-term memory consolidation, decreased body weight, hypoinsulinaemia and glucose intolerance	128–130
RasGRF2‡	Brain, spleen and lung	None	131
Sos1‡	Widely expressed	Embryonic lethality, placental and heart defects	132,133
Sos2‡	Widely expressed	None	134
Tiam1	Widely expressed, highest levels in brain and testes	None, but impaired oncogenesis	103
Trio§	Widely expressed	Embryonic lethality, abnormal skeletal-muscle and neural-tissue development	98
Vav1	Haematopoietic cells	Partial defect in T-cell development and function, normal B-lymphocyte development and function	135–137
Vav2	Widely expressed	Normal T- and B-cell development and function, but B-cell defect in combination with Vav1 deficiency	95,96
Vav3	Widely expressed, highest levels in haematopoietic cells and brain	Normal, but increased haematopoietic cell defects when combined with loss of Vav1 and Vav2	97

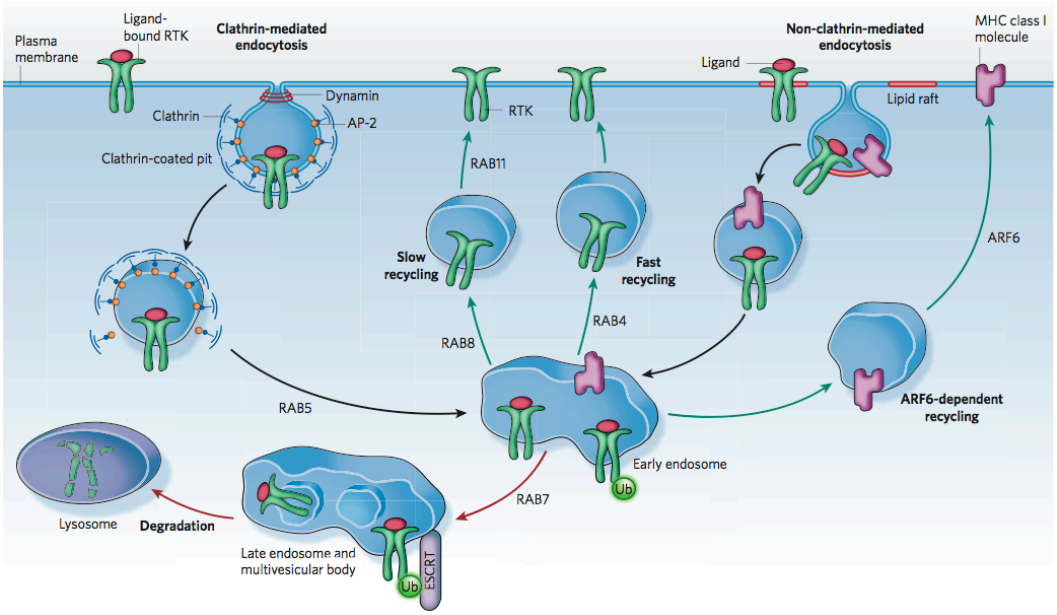
Rho GEF roles in development. The varied phenotypes caused by deficiencies in these proteins exemplify the signal specificity they provide to Rho GTPases. Table modified from Rossman et al. 2005.

Figure 1-1



The ErbB signalling network. A) Several ligands are able to bind and activate ten possible dimers formed by the four receptors of the ErbB family. ErbB2 does not bind any ligand, while ErbB3 kinase domain is inactive. B) Activate receptor induces downstream adaptors that activate three major powerful kinase pathways: PI3K/Akt, MAPK, and JNK. These pathways, in turn, induce a variety of transcription factors. C) These signaling pathways ultimately induce cell apoptosis, migration, growth, adhesion, and differentiation. Figure taken from Yarden et al., 2001.

Figure 1-2



ErbB endocytosis. Activated ErbB dimers are internalized via both the clathrin dependent, and clathrin independent pathways. Both endocytic carrier vesicles feed into the early endosome for sorting. The receptors can then be selected for recycling (both fast and slow) or degradation via ESCRT mediated internalization into a luminal vesicle in the multivesicular body. Fusion of the multivesicular body and a lysosome results in degradation of the luminal vesicles and receptors therein. Figure taken from Di Fiore et al., 2010.

CHAPTER TWO
Regulation of Endocytic Recycling by FGD4, a Cdc42 GEF

Abstract

High throughput siRNA screens are being widely used to examine the contribution of every gene to a particular biological process or disease state. Our lab has used this method to link genes and cancer cell survival. In one of our early efforts we performed a genome wide siRNA screen identifiying genes necessary for the survival of a non- small cell lung carcinoma in response to a sub-lethal dose of paclitaxel, a microtubule poison. A variety of genes were identified as necessary for cell survival in the presence of paclitaxel, but interestingly FGD4, one of more than 80 identified Rho GTPase GEFs was recognized as a high confidence hit from this screen. We are following up on our initial screen finding with a mechanistic study of FGD4 function in cell biology. FGD4 stimulates

exchange activity in Cdc42, a GTPase signaling molecule that can respond to a variety of extracellular signals and regulate various cell functions such as cell migration, cell adhesion, cell cycle progression and cytoskeletal regulation. Mutations in FGD4 also cause Charcot Marie Tooth Syndrome type 4H, a peripheral nerve demyelination neuropathy. Our studies revealed that FGD4 not only affects the actin cytoskeleton as previously published, but may also be affecting the microtubule cytoskeleton and cell processes that require acute regulation of microtubules, such as cell migration and endocytic recycling. The defects in endocytic recycling seen in FGD4 depleted cells seem to affect the signaling capabilities of ErbB receptor family members which rely on endocytic recycling to varying degrees. These finding may have implications in NRG1/ErbB3 signaling, the ligand/receptor combination that initiates myelination in peripheral nerves. We hypothesize that FGD4 may be regulating Cdc42 activity to facilitate transport of endocytic recycling vesicles.

Introduction

The advent of siRNA screening has given researchers the ability to assess the contribution of every gene in the genome to a given cellular

processes. Our lab explored the molecular pathways necessary for the survival of a non-small cell lung carcinoma (H1155) in the presence of a sublethal dose of paclitaxel using a siRNA genome screen and found a surprising range of proteins (Whitehurst, Bodemann et al. 2007). The Paclitaxil screen exposed subsets of genes involved in microtubule regulation (TubGCP2, Tuba8, DNHD1, DNAH10), which might be expected given that paclitaxel is a small compound that binds and stabilizes polymerized microtubules, abolishing their ability to reorganize. Interestingly, proteins involved in proteosome function, post-translational modifications, cell adhesion and ras GTPase signaling were also found to sensitize cells to paclitaxel, giving us another avenue to study the importance of these proteins in a cell biological context. Surprisingly, a single Rho GEF, FGD4, was identified as a high confidence paclitaxil sensitizer. FGD4 specifically has exchange activity for Cdc42, a small GTPase known to be involved in cell migration, proliferation, cell cycle progression, endocytosis, and cell polarity. Signal transduction specificity for Cdc42 is thought to be achieved through engagement of one GEF over another in response to an induction event (Rossman, Der et al. 2005). Given that the human genome encodes for ~70 Rho GEFs, we decided to follow up with a mechanistic study of FGD4 in a cell biological context.

FGD4 has been characterized as a Cdc42 GEF whose overexpression can induce microspike formation similar to those seen when constitutively active Cdc42 is overexpressed (Kim, Ikeda et al. 2002). The protein is a member of the FGD family of proteins which share similar protein domains such as the DH/PH domains necessary for Cdc42 GEF activity, a FYVE and second PH domain at the C-terminus and finally a F-actin binding domain unique to FGD4 at the N-terminus. FGD4 protein mutations has been found to cause human disease, Charcot Marie Tooth Type 4H, though its function still remains unknown (Delague, Jacquier et al. 2007). CMT4H is an inherited demyelinating neuropathy that causes progressive peripheral nerve demyelination, reduced nerve conductance velocity, distal motor weakness, muscle wasting and sensory loss. The function of FGD4 in Schwann cells, the glial cells responsible for constructing the myelin sheath around the peripheral nerve axons is still unknown. However, follow up studies of FGD4 during the Paclitaxel screen revealed that depletion of FGD4 resulted in the accumulation of mitotic figures in H1155 cells allowing us to study FGD4 in a more tractable tissue culture system as opposed to Schwann cell myelination. We found that FGD4 is important for microtubule stabilization and several cellular processes that require exquisite control of microtubule dynamics such as

mitosis, cell migration, and endocytosis. We hypothesize that FGD4 helps to stabilize microtubules at sites of dense actin meshworks such as the plasma membrane and endocytic vesicles.

Results

FGD4 depletion causes a Mad2 dependent mitotic arrest.

Our initial taxol sensitization screen revealed that a diverse group of proteins involved in such processes as proteosome function, regulation of microtubules, post-translational modifications, and cell adhesion were necessary for the survival of H1155 cells in the presence of 50 nM paclitaxel. We observed that FGD4 depletion causes an increase in the mitotic index in H1155 cells as well as sensitizing the cells to paclitaxel, indicating that FGD4 may have an important role in the completion of mitosis (Whitehurst, Bodemann et al. 2007). In order to determine if this phenotype is common to cells of diverse genetic backgrounds we transiently transfected multiple cancer cell lines with siRNA targeting FGD4 and see similar phenotypes indicating that the function of FGD4 in H1155's is not cell line specific (Fig 2-1a). Two different siRNA sequences

targeting FGD4 produce the mitotic doubling phenotype (Fig 2-1b) increasing our confidence that this phenotype is not due to an off target effect. Cells sensitized with a low dose of nocodazole (10 nM), which only mildly disrupts microtubule function, causes a 4-fold increase in mitotic cells as quantified by labeling cells with anti-PHH3 (mitotic cell marker) and FACS analysis (Fig 2-1e). Cells depleted of FGD4 and sensitized with nocodazole show an 8-fold increase in mitotic cells seen (Fig 2-1e), mimicking the original immunofluorescence phenotype.

An increase in mitotic figures can be attributed to many changes in cell proliferation such as an increase or decrease in the rate of the cell cycle. Irregularities in the structure or function of the mitotic spindle can also cause an increase in mitotic figures via activation of the Mad2 dependent mitotic checkpoint (Musacchio and Salmon 2007). This checkpoint can be bypassed by depleting key proteins of the checkpoint complex, such as Mad2 or Bubr1. To determine if the mitotic index increase seen is due to a Mad2 dependent arrest cells were co-depleted of Mad2 and FGD4, and this treatment abolishes the mitotic phenotype seen in HeLa cells (Fig 2-1d) and in HeLa cells sensitized with nocodazole (Fig 2-1e). As mentioned before, the spindle assembly checkpoint detects anomalies in the assembly of the mitotic spindle so we next took a closer look at the mitotic

spindles of the FGD4 depleted cells. Upon inspection with confocal microscopy, we see that the mitotic figures of FGD4 depleted cells seem smaller than cells transfected with control siRNA (Fig 2-2a). We quantified this by measuring the intercentrosomal distance of metaphase spindles and see a 18% (± 2%) decrease in the intercentrosomal distance between control and FGD4 depleted cells (Fig 2-2a). A search of the literature reveals that similar phenotypes were seen in drosophila S2 cells (15% decrease in intercentrosomal distance) and other mammalian cell lines, when depleted of microtubule tip binding proteins which are thought to affect stabilization of astral microtubules to the cell cortex during mitosis (Fukata, Watanabe et al. 2002; Goshima, Wollman et al. 2005). Interactions between Rho/Cdc42 effectors and MT tip binding proteins have also been reported (Tian, Nelson et al. 2000; Fukata, Watanabe et al. 2002; Yasuda, Oceguera-Yanez et al. 2004), so suspecting that FGD4 may be important for microtubule regulation we subsequently checked microtubules in other cell contexts to see if they were also affected.

FGD4 depletion destabilizes microtubules

HeLa cells were transiently transfected with control siRNA or siRNA targeting FGD4 then fixed and immunofluorescently labeled with an anti-

beta tubulin antibody. We observed a general sparseness of microtubules in the cells depleted of FGD4 (Fig 2-2b) suggesting that FGD4 may be involved in microtubule regulation. Using a more quantitative approach to observe a difference in the ratio of polymerized vs. unpolymerized tubulin, cells were partially lysed in a microtubule stabilizing buffer (Ng, Lin et al. 2006) and centrifuged to separate polymerized microtubules and soluble tubulin monomers. Cdc42 and FGD4 depleted cells had more soluble tubulin when compared to a control siRNA and FGD1, a protein in the same family as FGD4 (Fig 2-2d). One possibility that might explain the decrease in microtubule density is a decrease in microtubule stability. In order to determine whether FGD4 could be involved in microtubule stability, transfected cells were treated with 2 uM Nocodazole, a microtubule destabilizing agent, for 30 min at 37C. Cells transfected with a control siRNA had noticeably more microtubules able to withstand destabilization than in FGD4 depleted cells (Fig 2-2c). This suggests that FGD4 is impacting microtubule stability or dynamics. Long lived, stabilized microtubules are known to accumulate post-translational modifications such as acetylation and detyrosination thereby providing good indicators for levels of stabilized microtubules (Gundersen, Khawaja et al. 1987). FGD4 depleted cells have far less detyrosinated tubulin in comparison to

control cells (Fig 2-2c), also indicating that FGD4 is impacting microtubule stability.

Cdc42 has been shown to affect microtubule regulation through phosphorylation of Cofilin and Stathmin (Sumi, Matsumoto et al. 1999; Daub, Gevaert et al. 2001), proteins involved in capping and destabilizing microtubules, respectively. These Cdc42 dependent phosphorylation events are upregulated during mitosis and stimulation with EGF. In order to determine if FGD4 could be regulating Cdc42 in cofilin phosphorylation HeLa cells were transiently transfected with the indicated siRNAs then treated with 10uM Nocodazole overnight to induce mitotic arrest. While Cdc42 depletion inhibited the phosphorylation of cofilin in response to mitotic arrest, FGD4 depletion did not (Fig 2-2e). To determine if FGD4 was affecting stathmin phosphorylation, cells depleted of FGD4 were starved overnight then stimulated with EGF. FGD4 depletion did not affect the EGF stimulated phosphorylation of stathmin either. These results highlight the specificity that Cdc42 GEFs can supply to Cdc42 signaling but the downstream effectors of FGD4 function are still unknown. Cdc42 and some of its effectors (eg. Dlg1, Diaph3, IQGAP1) are known to stabilize microtubules by capturing the microtubule plus ends at the migrating front (Fukata, Watanabe et al. 2002; Etienne-Manneville,

Manneville et al. 2005). It is likely that FGD4 may be impacting microtubule regulation via the end capture mechanism and therefore important for cell migration. Thus we next wanted to determine if FGD4 depletion would affect cell migration.

FGD4 depletion inhibits cell migration and is necessary for migration induced Cdc42 activation

In a wounding assay, FGD4 depleted cells are impaired in their ability to close the scratch introduced to the monolayer when compared to cells transfected with a control siRNA (Fig 2-3a). An inspection of cell morphology at the six hour time point shows that FGD4 depleted cells are not migrating as neatly as control cells and they also seem to be unable to extend a lamellipodia (Fig 2-3a), indicating a defect in polarized migration. To quantify this we counted the number of cells at the migrating front that have relocated their centrosomes to the quadrant of the cell facing the wound, an event that is thought to indicate direction of cell migration. Within the first couple of hours of migration FGD4 depleted cells are hindered in their ability to correctly orient themselves (Fig 2-3b). Cell migration induces Cdc42 activity and an increase of GTP loaded Cdc42 can be seen with a Cdc42 activation assay. In order to test whether FGD4

is needed for this activation, stable shRNA cell lines were created targeting FGD4 that depleted mRNA levels to less than 25% of control and protein levels to less than 10% of control (Fig 2-3c). The stable shRNA cells lines expressing shGFP and shFGD4 were grown and multiple wounds were introduced to the monolayer to induce Cdc42 activation. Cdc42 activation assays were performed and an increase of GTP Cdc42 is seen after introducing wounds into the monolayer in the control shRNA stable cell line, but not in the shFGD4 stable cell line (Fig 2-3d). The observations that FGD4 is important for microtubule stabilization, mitosis and cell migration suggest that FGD4 may be integral in regulating microtubules at the plasma membrane for these cell processes. Three major protein complexes are involved in establishment and maintenance of cell polarity (Crumbs, Par, and Scribble) all of which are associated with sites on the plasma membrane where apical/basal polarity is demarcated. Several members of the Par and Scribble complexes are known Cdc42 effector proteins implicated in migrating cell polarity and thought to aid migration via their effects on microtubule stability at the leading edge. (Etienne-Manneville and Hall, 2005; Leibfried and Bellaiche, 2008; Georgiou and Baum, 2008(Joberty, Petersen et al. 2000). Specifically, the Par6-Cdc42-aPKC complex is necessary for the maintenance of apical

proteins such as E-cadherin, a-catenin, and armadillo in drosophila pupae but not maintenance of basolaterally localized proteins. Interestingly, Par6-Cdc42 complex can regulate the localization of Dlg1 and APC, proteins implicated in microtubule plus-end stabilization. It is tempting to speculate that the Par6-Cdc42 complex may maintain E-cadherin at the adherens junction since destabilization of microtubules with nocodazole inhibits the accumulation of E-cadherin at sites of cell-cell contacts (Stehbens, Paterson et al. 2006). We suspect that FGD4 may also have a role in adherens junction formation or maintenance given its affect on both cell polarity and microtubule stabilization. To test this hypothesis, we next examined the significance of FGD4 in epithelial cell adherens junctions.

FGD4 depletion destabilizes adherens junctions and compromises epithelial barrier integrity

Endogenous FGD4 staining localizes at cell junctions and only this staining is depleted upon siRNA transient depletion while non-specific cystosolic and nuclear staining is largely unchanged (Fig 2-4a). Depletion of FGD4 does not affect the formation of adherens junctions, as shown by E-cadherin staining, but interestingly E-cadherin localization is slightly irregular in FGD4 depleted cells as noted by vesicle-like structures

incorporated in the cell junctions (Fig 2-4a). To assess the integrity of the epithelial barrier function caco-2 cells were grown on transwell filters and exposed to FITC-labelled dextran (a 3kDa polysaccharide) on the apical side of the cells and samples of media on the basal side of the cells were taken at the indicated timepoints. In the control shRNA Caco-2 cell line, little FITC-Dextran signal is detected in the media of the basal side of the transwell filter. In contrast, Caco-2 cells depleted of FGD4 are more permeable to FITC-Dextran, suggesting that the epithelial barrier is compromised (Fig 2-4b).

Cdc42 has been shown to be involved in the maintenance of E-cadherin at cell cell junctions through regulation of E-cadherin endocytosis (Izumi, Sakisaka et al. 2004; Leibfried, Fricke et al. 2008). These groups report that depletion of Cdc42 and its effector, Cip4, result in the accumulation of E-cadherin in Rab5 positive vesicles culminating in the loss of cell junction integrity. Mostov's group has also reported that Cdc42 is necessary for transport of vesicles between the recycling endosome (Rab11 positive) and the plasma membrane, such that depletion of Cdc42 causes an accumulation of apical plasma membrane proteins in enlarged Rab11 positive structures during luminogenesis (Martin-Belmonte, Gassama et al. 2007; Bryant, Datta et al. 2010). These studies show that Cdc42 is

involved in various endocytic trafficking events though how and when it is activated is likely regulated by various GEFs. Similarly, FGD4 depleted cells have a loss of epithelial barrier integrity and induction of endocytosis with EGTA results in the accumulation of E-cadherin positive vesicles at the periphery of the cells (Fig 2-4c). The incorporation of newly internalized endocytic vesicles into the early endosome is likely dependent on the cytoskeleton for travel further into the perinuclear space. Indeed, microtubule motor proteins such as Kif16B and dynein, as well as the microtubule network itself are known to be important for various steps in the endocytic pathway (Bananis, Murray et al. 2000; Lin, Gundersen et al. 2002; Hoepfner, Severin et al. 2005; Driskell, Mironov et al. 2007). For example, exposure of cells to taxol, a microtubule stabilizing drug, causes a redistribution of transferring from the perinuclear endocytic compartments to the cell periphery. FGD4 is necessary for the stability of E-cadherin at cell junctions, a process likely regulated by microtubule dependent endocytic trafficking. In order to study the possible role of FGD4 in endocytosis, we next examined ligand induced EGFR endocytosis, a well studied endocytic event.

FGD4 is necessary for efficient EGFR signaling and trafficking.

HeLa cells expressing shRNAs targeting FGD4 show reduced levels of EGFR protein, but do not have decreased levels of EGFR mRNA (Fig 2-5a). When HeLa cells are stimulated with high levels of EGF (100 ng/mL), EGFR and ERK phosphorylation events are induced in FGD4 depleted cells, but at lower amplitudes when compared to control cells (Fig 2-5b). EGFR protein levels, as well as the maximum stimulated ERK phosphorylation are restored by FGD4 overexpression in HeLa cells (Fig 2-5c). In order to determine the localization of the EGFR population, surface EGFR was biotinylated and pulled down with streptavidin beads. We see that FGD4 depleted cells have an elevated percentage of EGFR at the cell surface (Fig 2-5d), indicating that the decreased p-ERK signal seen in FGD4 depleted cells is likely not due to a deficit of available receptors. Groups have shown that Dynamin, a microtubule motor protein involved in endocytosis, is essential for full ERK activation (Vieira, Lamaze et al. 1996). A growing body of work by several groups have shown that receptor signaling continues in endosomal locales and that indeed, are necessary for full activation of downstream signaling programs (Vieira, Lamaze et al. 1996; Murphy, Padilla et al. 2009). These data indicate that EGFR signaling continues in endocytic locales once removed from the plasma membrane. Cdc42 impacts the endocytic process at several points

such as the initial endocytic event at the plasma membrane and the delivery of cargo from the early endosome to the multivesicular body as shown by tracking of resident plasma membrane proteins such as E-Cadherin and MHC class I molecules (Balklava, Pant et al. 2007; Georgiou, Marinari et al. 2008). We suspect that FGD4 may regulate Cdc42 activity at one point along the EGFR endocytic track. In addition, EGF stimulation has also been shown to stimulate Cdc42 activity. To test whether FGD4 is necessary for Cdc42 activation in response to EGF stable shRNA expressing cell lines were used in a Cdc42 activation assay. We see a two-threefold increase in Cdc42 activity in response to EGF stimulation in control cells, but not in the FGD4 depleted cells (Fig 2-5d). Downregulation of EGF downstream signaling in response to FGD4 depletion can also be seen in immortalized Schwann cells (Fig 2-7b), as well as other cancer cell lines (data not shown). FGD4 may play a role in receptor trafficking away from the degradative lysosomal compartment, therefore allowing maximal EGF response in a control cells. In order to track EGFR endocytic residency after EGF induced endocytosis, we exposed HeLa cells transiently transfected with siRNA targeting FGD4 to EGF-FITC and examined co-localization with Rab5 and Rab11 vesicles (Fig 2-6a). FGD4 depleted cells with reduced amounts of (18%) EGF-FITC

colocalizing with Rab5 and an increase in colocalizing with Rab11 by 11% at 30 minutes after initial EGF-FITC exposure (Fig 2-6b,c). These observations indicate that although the EGF-FITC ligand is internalized is accumulating in the Rab11 positive recycling compartment, they do likely do not make it out to the plasma membrane and eventually get shuttled to the lysosome. Given that EGFR protein levels are reduced in response to FGD4 depletion, it is likely that the receptor is shunted to the lysosome for degradation by virtue of its c-Cbl binding site from the recycling endosome. In contrast, ErbB3 a family member of the ErbB receptor group, has a modified and weak c-Cbl binding site which allows the receptor to be preferentially recycled instead of marked for degradation as is EGFR (Baulida, Kraus et al. 1996; Levkowitz, Klapper et al. 1996). If FGD4 is indeed important for the completion of the endocytic recycling route we may expect an accumulation of ErbB3 receptor in vesicles and an increase of its signal as a consequence of its weak affinity for c-cbl binding (Waterman, Alroy et al. 1999).

FGD4 is necessary for proper ErbB3 signaling and trafficking.

ErbB3 is the receptor for NRG-1b, a small signaling protein that initiates proliferation and myelination in Schwann cells (Michailov, Sereda et al.

2004; Lemke 2006; Birchmeier and Nave 2008). Although the details of ErbB3 trafficking after ligand stimulation are poorly described, groups have shown that the majority of ErbB3 receptor escape bulk lysosomal degradation as is the fate of EGFR (Sorkin and Goh 2009). In order to determine the consequence of FGD4 depletion on ErbB3 signaling we stably infected immortalized Schwann cells with shRNAs targeting FGD4 (Fig 2-7a). Upon stimulation with lower NRG1b concentrations we see an increase in pAKT and pERK signaling in FGD4 depleted cells (Fig 2-7b,c). This phenotype was also seen in Peo1 cells (data not shown), which have been reported to express ErbB2/3 receptors and proliferate in response to NRG1b stimulation (Gilmour, Macleod et al. 2002). FGD4 also proves necessary for Cdc42 activation in response to NRG1 stimulation (Fig 2-7d). Groups have shown that NRG-1b concentration has a bimodal affect on myelination of axons. Studies in cultured primary Schwann cells demonstrate that the balance of activated AKT and activated ERK stimulated by NRG-1b dictates whether or not the Schwann cell will myelinate an axon (Syed, Reddy et al.; Ogata, Iijima et al. 2004; Sorkin and Goh 2009). Phospho-AKT signal stimulates myelination while phospho-ERK inhibits myelination, which results in lower NRG-1b more potently initiating myelin sheath formation while higher concentrations

actually inhibiting myelination. In order to see whether the shift in NRG-1b signaling FGD4 depletion causes will affect Schwann cell myelination, we carried out Schwann cell and dorsal root ganglia co-culture myelination assays. Primary Schwann cells were first infected with viruses expressing shRNAs targeting FGD4 or a control virus then co-cultured for up to four weeks with dorsal root ganglia, allowing the Schwann cells to begin myelination. In this preliminary study, we see that two of three shRNAs successfully depleted Schwann cells of FGD4 and also exhibited a decrease in myelination by about half (Fig 2-7e).

Discussion

FGD4 important for mitosis

FGD4 has previously been characterized as a Cdc42 GEF that regulates actin and microspike formation. In this study we show that FGD4 can also regulate microtubule stability and cellular processes that others have shown to heavily rely on microtubule dynamics such as cell migration, cell junction maintenance and endocytosis. We hypothesize that FGD4 is necessary for Cdc42 mediated microtubule stabilization important for the cellular process mentioned before. Groups have shown that Cdc42

effector proteins Dlg1, IQGAP1, mDia3 (Ligon, Karki et al. 2001; Fukata, Watanabe et al. 2002; Yasuda, Oceguera-Yanez et al. 2004; Etienne-Manneville, Manneville et al. 2005) stabilize microtubule plus ends at the cortical actin meshwork for anchorage of astral microtubules of the mitotic spindle whose disruption results in improper chromosomal segregation; the main function of the mitotic spindle. FGD4 depletion results in a Mad2 dependent increase in mitotic cells and confocal analysis of the mitotic spindles show a decrease in the intercentrosomal distance by 18% (±2%), a phenotype seen when astral microtubules are destabilized with depletion of microtubule plus end binding proteins (Goshima, Wollman et al. 2005). It is thought that the astral microtubules are important for anchoring the mitotic spindle and maintaining its position during chromosomal segregation when pushing/pulling forces are at play. The mechanism of microtubule end capture utilizes plus end microtubule binding proteins such as EB1, Clasp2 and APC which also stabilize microtubules in other cell contexts (Mimori-Kiyosue and Tsukita 2003). We next examined the effects of FGD4 depletion on microtubules in interphase cells.

FGD4 is important for microtubule stabilization

The depletion of FGD4 causes a decrease in the microtubule network density indicating a perturbance in microtubule dynamics. In order to assess polymerized microtubules in a population, we isolated soluble tubulin from polymerized microtubules using a centrifugal method (Ng, Lin et al. 2006) and see that siRNA transfection with two different sequences targeting FGD4 and a pool of four sequences targeting Cdc42 causes an increase in soluble tubulin in comparison to cells transfected with control siRNA and a pool targeting FGD1. A decrease in detyrosinated microtubules is seen in cells depleted with FGD4, which indicates a reduction in stabilized microtubules (Gundersen, Khawaja et al. 1987). Additionally, when cells are challenged with nocodazole, a microtubule destabilizing agent, less microtubules in FGD4 depleted cells remain when compared to cells transfected with a control siRNA. As mentioned before, Cdc42 regulates cofilin and stathmin phosphorylation, two proteins regulating microtubule dynamics. FGD4 depletion did not affect the stimulated phosphorylation of these proteins, suggesting that FGD4 may be instead utilizing the microtubule end capturing method of microtubule stabilization. We next wanted to investigate whether FGD4 is involved in cell migration, a cellular process that Cdc42 microtubule stabilizing effectors have been shown to be necessary.

FGD4 is important for cell migration and cell junction maintenance

FGD4 depleted cells are impaired in their ability to migrate in a directional manner, a function well known to be regulated by Cdc42 activity. Consequently, FGD4 is also necessary for induction of Cdc42 activity in response to cell migration in a wound healing assay. The Par6-Cdc42 complex involved in cell polarization during directional cell migration are also shown to be involved in maintenance of adherens junction stability (Etienne-Manneville, Manneville et al. 2005; Georgiou, Marinari et al. 2008). Caco2 cells, a human epithelial colorectal adenocarcinoma cells that establishes cell junctions once they have reached confluency do form junctions when depleted of FGD4, however E-cadherin staining reveals irregularities. Confocal images of the junctions show vesicle like E-cadherin outcroppings along the junctions in FGD4 depleted cells. Studies in drosophila have shown that Cdc42 is important for maintenance of E-cadherin at cell junctions via the use of endocytic recycling. Indeed, Cdc42 has been thought to have a role at multiple points of vesicle trafficking with different GEFs and effector combinations providing specificity. In order to determine if FGD4 is affecting cell junction maintenance through regulation

of endocytic recycling we next examined it's role in EGFR ligand stimulated internalization, a well characterized endocytic event.

FGD4 has a role in endosome recycling

Endosomal trafficking uses both actin and microtubule cytoskeletons to function. Disruption of the microtubule network itself with disruptive small molecules such as nocodazole and taxol have been shown to inhibit transport of peripherally located endosome to the perinuclear region of the cell where the endocytic recycling and lysosomal compartments are typically located (Lin, Gundersen et al. 2002; Baravalle, Schober et al. 2005). Various motor proteins have been shown to mediate endosomal traffick on the microtubule network such that selective disruption of dynein or kinesin Kif16B can stall movement of transferring or EGFR in different endocytic compartments (Bananis, Murray et al. 2000; Lin, Gundersen et al. 2002; Hoepfner, Severin et al. 2005; Driskell, Mironov et al. 2007). Endocytic trafficking is important for receptor signaling because it dictates how much receptor is available for signaling at the surface. In addition, receptors are known to continue signaling within endosomes so appropriate completion of endocytic events is necessary for proper downstream signaling. For example, some cancers exploit receptor

trafficking by selecting for mutations in the EGFR receptor Cbl domain. The Cbl domain marks EGFR for ubiquitination and lysosomal degradation, and mutations in this domain allows the receptor to escape degradation and continue providing a proliferative signal (Shtiegman, Kochupurakkal et al. 2007). Interference with EGFR recycling such as depletion of clathrin, an adaptor protein necessary for receptor internalization, causes a reduction in sustained downstream EGF signaling (Sigismund, Argenzio et al. 2008). These studies show that proper endocytic processing of internalized receptor can have repercussion on the final signaling output of the receptor. Our studies also show that FGD4 depletion causes a down regulation of EGFR protein and a decrease in downstream ERK signaling, mimicking the phenotype of a loss of receptor recycling. Interestingly, another member of the EGF receptor family, ErbB3, lacks a high affinity Cbl binding site, allowing the protein to be primarily recycled as opposed to degraded by the lysosome as is the fate of EGFR. Indeed, if the Cbl sites are switched between EGFR and ErbB3, the receptors switch fates: EGFR is primarily found in Rab11 structures, while ErbB3 is now speedily shunted to the lysosome for degradation (Waterman, Alroy et al. 1999). A decrease in proliferative signal provided by such a robust signaling molecule as EGF may not have dire

consequences for survival of cancer cells but a disturbance in receptors that regulate processes sensitive to ligand concentration would likely be greatly affected. For example, studies of Schwann cell myelination have shown that these cells are exquisitely sensitive to NRG-1b, an ErbB3 ligand that stimulates myelination. Neuronal axons display this ligand and only axons large enough, and thus display enough NRG-1b, stimulate surrounding Schwann cells to wrap around and myelinate the axon. Interestingly, Kim's group has shown that NRG1b ligand has a bimodal effect on myelination such that myelination linearly increases with NRG1b concentration until an upper threshold is reached, at which point myelination is inhibited. They go on to show that pAKT signaling drives myelination while pERK signaling inhibits myelination, thus causing the bimodal action of NRG1b concentration. In this instance, an uncoupling of the ligand concentration to downstream signaling would have a profound effect on Schwann cell action. We see that FGD4 depletion does in fact perturb pAKT and pERK response to NRG1b stimulation, resulting in a shift to the left in the NRG-1b response curve. We hypothesize that ErbB3 is retained in the Rab11 vesicle, and in the absence of a strong c-Cbl binding site, has no alternate exit. This presumably allows ErbB3 to

continue signaling; thereby causing an increase in signaling output in response to receptor activation and eventually results in hypomyelination.

In summary, we show that FGD4 has a role in stabilizing microtubules and depletion of this protein affects various cellular processes that rely on microtubule dynamics: mitosis, migration, adherens junction maintenance, and receptor recycling. However, the mechanism by which FGD4 contributes to microtubule stability remains to be determined. The FGD4 protein has many of it's domains in common with its family members, which include the DH/PH domains necessary for its Cdc42 GEF activity, a FYVE domain, and another PH domain. Its unique N-terminal F-actin binding domain (FAB) is necessary and sufficient to localize it to actin microfilaments and has been shown to carry a LIxxFE domain which demonstrates a dimerization dependent actin bundling activity independent of its Cdc42 activation activity (Banerjee et al). None of the aforementioned domains have been reported to bind tubulin, making it unlikely that FGD4 might directly bind and stabilize polymerized microtubules. As mentioned previously, several Cdc42 effector proteins (mDia2, Dlg1, IQGAP1) have been described to stabilize microtubules via a microtubule end capture mechanism (Mimori-Kiyosue et al.), offering a

possible mechanism for FGD4's effect on microtubules via it's ability to activate Cdc42.

Materials and Methods

Cell Lines and Reagents

HeLa cells were grown in DMEM with 10% FBS and Caco-2 cells were grown in DMEM with 20% FBS. Immortalized rat Schwann cells (S16) were obtained from ScienCell Research Laboratories. Protease inhibitor cocktail (Roche Diagnostics). B-tubulin monoclonal antibody (Sigma), g-tubulin monoclonal antibody (Sigma), FGD4 polyclonal antibody (SDIX), Cdc42 monoclonal antibody (BD Transduction), Actin monoclonal antibody (Sigma), Myc (9E10) monoclonal antibody (Santa Cruz Biotechnology), g-tubulin monoclonal antibody (sigma), ERK 1/2 (Cell Signaling Technology), phospho ERK 1/2 (Cell Signaling Technology), panAKT (Cell Signaling Technology), phospho AKT (Cell Signaling Technology), ErbB3 monoclonal antibody, Erbb2 monoclonal antibody(Cell Signaling Technology), EGFR monoclonal antibody (Santa Cruz Biotechnology), phospho EGFR monoclonal antibody (Santa Cruz Biotechnology).

siRNA and shRNA Sequences

All siRNA duplexes were from Dharmacon Inc siGenome Library and pGIPZ shRNA expressing plasmids were ordered from Thermo Scientific Openbiosystems.

Transfections

HeLa cells were reverse transfected with either Dharmafect 1 (Thermo Fisher Scientific) or Lipofectamine RNAiMax (Invitrogen) as per manufacturer's protocols with 50nM siRNA. For plasmid transfections Fugene 6 or Lipofectamine 2000 were used as per manufacturer's protocol. All siRNA's used were from the Dharmacon (Thermo Fisher Scientific) siGenome Pool Library. The control siRNAs used were also from the siGenome Library targeting DLNB14 or using Dharmacon's siControl. Knockdown efficiency was assessed with either western blotting or QPCR.

Immunofluorescence

After siRNA transfections, cells were incubated for 72-96 hrs then fixed with 3.7% Formaldehyde and permeabilized with 0.1% Triton X-100 for 5 min. The cells were blocked with 5% BSA, PBS, and 0.1% Triton X-100,

then incubated with the indicated primary antibodies and finally with appropriate secondary antibodies coupled to Alexa Fluorophores (Invitrogen). Epifluorescent images were taken on the Zeiss Axioplan 2E upright microscope. Confocal images were taken on Leica TSC SP5 microscope using a 63x/1.618 NA (oil) objective.

In Vivo Polymerized Tubulin Assay

The assay was performed as described in JCB 172(2):245 (2006). Briefly, after siRNA transfection, cells were incubated for 72-96 hrs, lysed in microtuble stabilizing buffer (100 mM Pipes, pH 6.9, 2 M Glycerol, 5 mM MgCl2, 2 mM EGTA, 0.5 % Triton-X100, 4 uM Taxol, 1x protease inhibitor cocktail), and centrifuged at 12000 rfc for 10 min at 4°C. The supernatant containing solubilized tubulin was isolated from the pellet containing polymerized tubulin. The pellet was washed once with microtubule stabilizing buffer before being resuspended in laemmli sample buffer.

Wound Healing Assay

Forty-eight hours post siRNA transfection, HeLa cells were starved with DMEM overnight before introducing a scratch in the cell monolayer and replacing starvation media with 10% FBS DMEM. Cells were then allowed

to incubate for the indicated times and fixed or lysates were collected for the Cdc42 activation assay.

Fluorescence Activated Cell Sorting Assay

After transfection, cells were trypsinized and resuspended in media. Cells were then washed with PBS and fixed by incubating with ice cold 75% ethanol for one hour at 4C. Cells were blocked with PBTA (PBS, 5% BSA, 0.1% Triton X-100) then incubated with a-PHH3 antibody (Cell Signaling Technology) for 1 hour at room temperature, washed and incubated with anti-rabbit FITC conjugated secondary antibody for 1 hour at room temperature. Samples were then washed and incubated with Propidium Iodide (BD Pharmingen) for 30 min at 37 C before being analyzed with a FACSCalibur Fluorescence Cell Sorter (Becton Dickinson).

Dextran Permeability Assay

Cells were plated on transwell permeable supports (Corning) and incubated until the cells formed a monolayer. FITC conjugated 3KDa dextran was then diluted to 50 ug/mL in full Caco-2 media (20% FBS in DMEM) and added to the top reservoir. The bottom reservoir was replaced with 200 uL of fresh media. Ten microliter samples were taken from the

bottom reservoir at the indicated time points and the fluorescent signal of all samples were read on the BioTek Synergy HT microplate reader.

Cell Surface Protein Biotinylation Assay

Up to 96 hrs post-transfection, cells were washed with PBS before incubating cells with 1x EZ-Link Sulfo-NHS-SS-Biotin stock solution for 15 min at room temperature. Biotinylation reaction was stopped with 1 mg/mL Lysine Solution. Cells were then washed with PBS and lysed in RIPA Buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1 % SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100, 1x Roche Protease Inhibitor Cocktail). Equivalent amounts of protein were then incubated for 1 hr, at 4C with Streptavidin beads to pull down biotinylated proteins and analyzed with western blotting.

Cdc42 Activation Assay

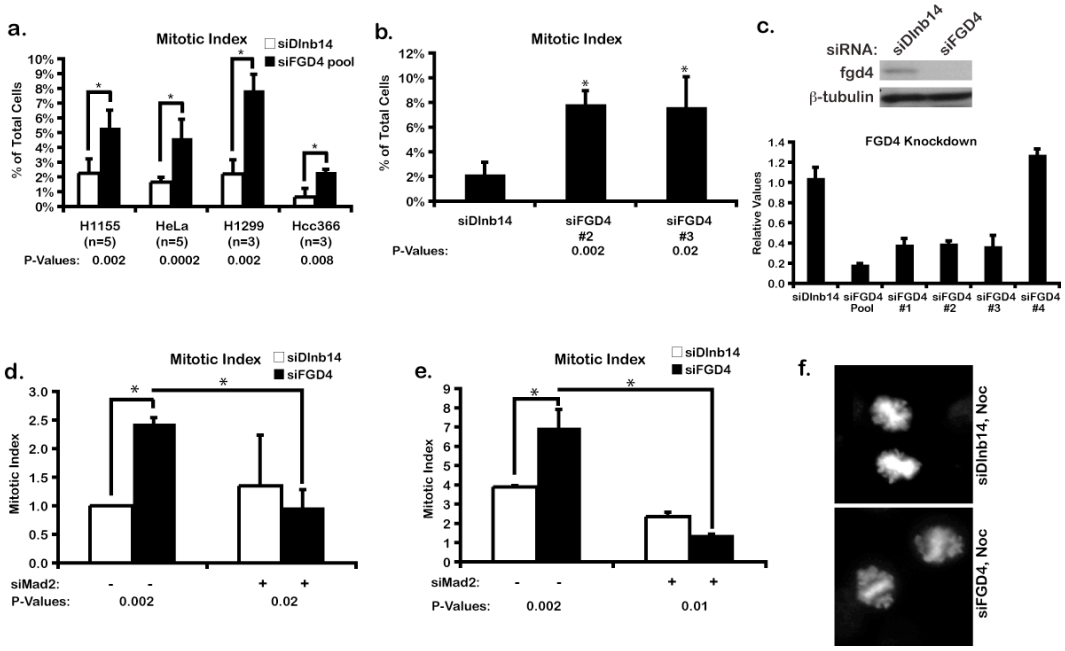
Stable cell lines were grown in 10 cm plates until confluent. Cells were then starved overnight and stimulated with ligand for the indicated time point. Lysate was made using the following cell lysis buffer: 50 mM Tris-HCl pH 7.4, 200 mM NaCl, 2.5 mM MgCl2, 1% NP-40, 10% glycerol, 1x Roche Protease Inhibitor Cocktail. Equal protein amounts for each sample

was incubated with PAK3 RBD GST fusion protein bound to GST-agarose beads for 1 hr at 4C with gentle rotation. The beads were then washed with lysis buffer and finally resuspended in 2x Lamelli sample buffer.

Polarized Migration Assay

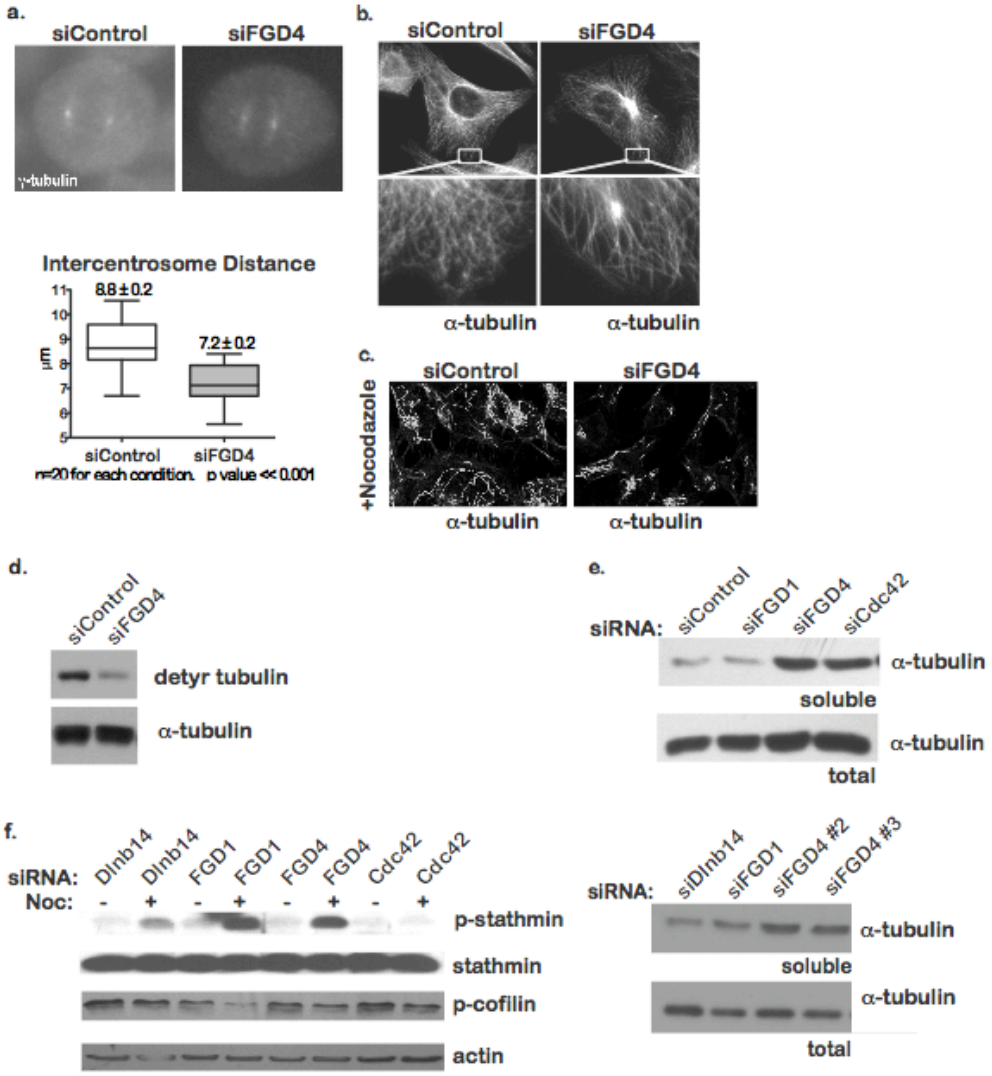
HeLa cells were siRNA transfected then 24 hrs later, transferred to glass slip containing tissue culture plates. Forty-eight hours later, when cells were confluent, a scratch was introduced to the monolayers and incubated for the indicated times at which point they were fixed with 3.7% formaldehyde. Cells were immunofluorescently labeled using the protocol mentioned above.

Figure 2-1. FGD4 depletion causes a Mad2 dependent mitotic arrest.



(a) A variety of cancer cell lines were transfected with siRNA pools targeting DNLB14 as control or FGD4 with Dharmafect Transfection reagents. After 72-96 hrs, cells were fixed with paraformaldehyde and stained with DAPI. Mitotic index was determined by counting mitotic figures per field. (b). HeLa cells were transfected with single siRNAs targeting DLNB14 or FGD4 using Dharmafect 1. (c). HeLa cells were transfected with pooled siRNAs and single siRNAs using Dharmafect 1. After 96 hours, RNA was isolated from the cells and the level of FGD4 mRNA was determined with QPCR. The protein level of FGD4 was also determined using a western blot. (d) HeLa cells were co-depleted with the indicated siRNAs using Dharmafect 1 for transfections. After 96 hrs, cells were fixed with paraformaldehyde, stained with DAPI and the mitotic index determined for each condition. (e). HeLa cells were co-depleted with the indicated siRNAs using Dharmafect 1 for transfections. After 72 hours, cells were sensitized with a low dose of nocodazole (10 ng/mL) for 16 hours. Cells were then collected, fixed with 70% ethanol, and labeled with anti-PHH3 Alexa Fluor 488-conjugated antibody before being subjected to FACS analysis.

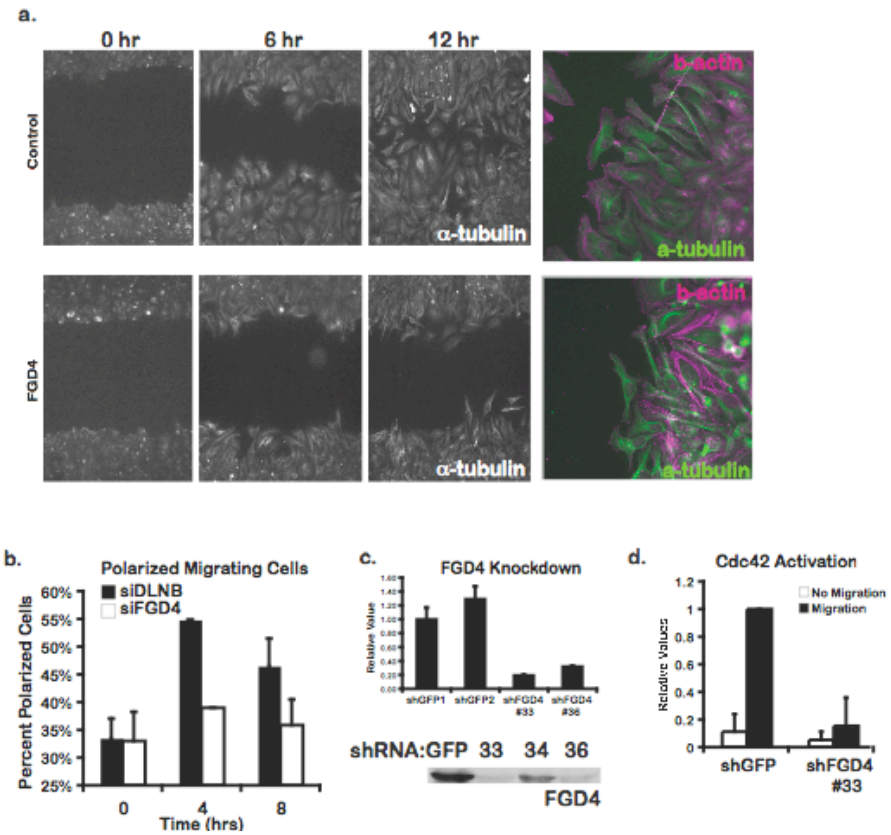
Figure 2-2. FGD4 depletion destabilizes microtubules.



(a) HeLa cells were transfected with siRNA pools targeting DLNB14 or FGD4 using Dharmafect 1. After 72 hrs cells were fixed and stained for g-Tubulin. Images of the cells were taken using a confocal microscope and the intercentrosome distance measured. (b) HeLa cells were transfected with siRNA as described above then fixed with paraformaldehyde and stained for anti-beta tubulin. Images were taken using an epifluorescence microscope. (c). HeLa cells were transfected with siRNA, and after 96 hrs treated with 20 uM nocodazole for 10 min. Cells were then permeabilzed with 0.1% Triton in PBS for 1 min before being fixed and stained for anti-beta tubulin. (d). HeLa cells were transfected with the indicated siRNA

pools and after 96 hrs collected in Microtubule stabilizing lysis buffer. Polymerized microtubules were separated by centrifugation at 14000 x g for 15 min at 4C.

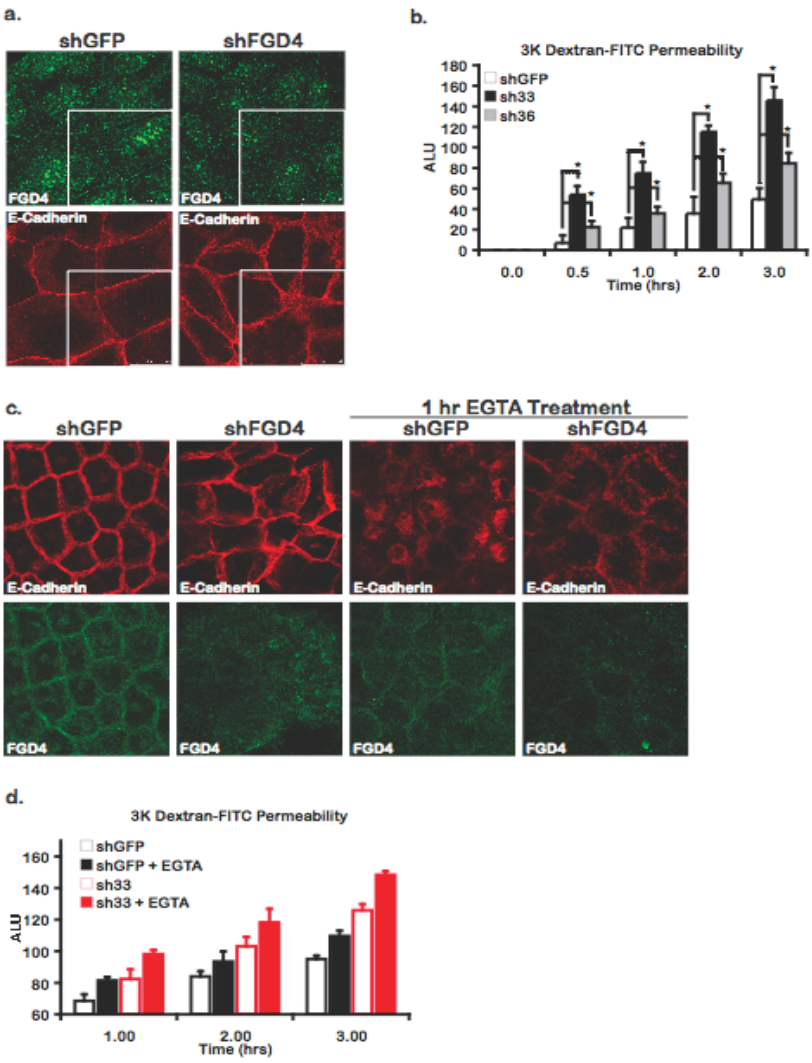
Figure 2-3. FGD4 is necessary for cell migration and cell migration induced Cdc42 activity.



(a). HeLa cells were transfected with siRNA pools targeting DLNB14 or FGD4 using Dharmafect 1. After 48 hrs, cells were starved overnight, a scratch was introduced into the monolayer and the starvation media was replaced with full media (10% FBS). Cells were then fixed with paraformaldehyde at the indicated timepoints and stained with anti -beta tubulin. (b). Cells were transfected and wounds introduced as described above. Cells were then fixed and stained with anti-pericentrin and DAPI at the time points indicated. Percent of cells polarized was then counted at the migrating front as described in Materials and Methods. (c). HeLa cells were infected with lentiviruses expressing shRNAs targeting GFP and FGD4. Cells were then selected with puromycin, RNA was isolated from the stable cells lines, and mRNA levels of FGD4 were determined using QPCR. FGD4 protein levels were also determined with immunoblotting. (d). HeLa stable cell lines were grown to 100% confluency and wounds

were introduced to the monolayer until about 50% of cells were scratched off. Cells were then collected and subjected to a Cdc42 activation assay.

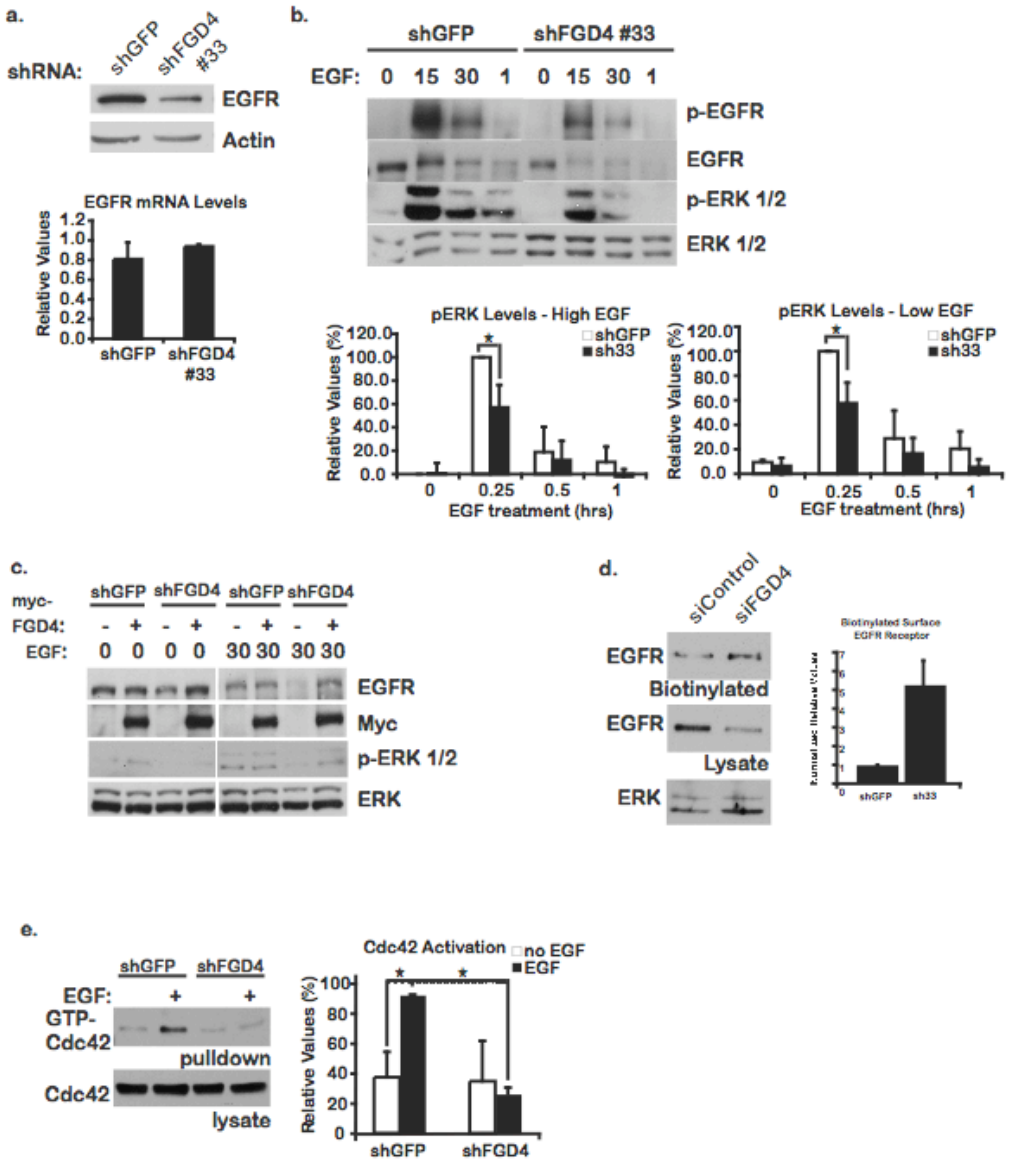
Figure 2-4. FGD4 depletion compromises adherens junction integrity.



(a). Caco-2 cells were infected with lentiviruses expressing the indicated shRNAs and selected with puromycin. Cells were grown until 100% confluent and stained with anti-E-Cadherin and FGD4. (b). Caco-2 stable cell lines were grown until 100% confluent on transwell filters. 3K Dextran conjugated to Alexa Fluor 488 was then added to the upper chamber. Samples of the lower chamber were then taken at the indicated times and the fluorescent signal measured. (c). Caco-2 stable cell lines were grown until 100% confluent on transwell filters. Cells were then treated with EGTA for 1 hr then fixed and stained with anti-E-cadherin and anti-FGD4.

(d). 3K Dextran conjugated to Alexa Fluor 488 permeability was also determined for the EGTA treated cells.

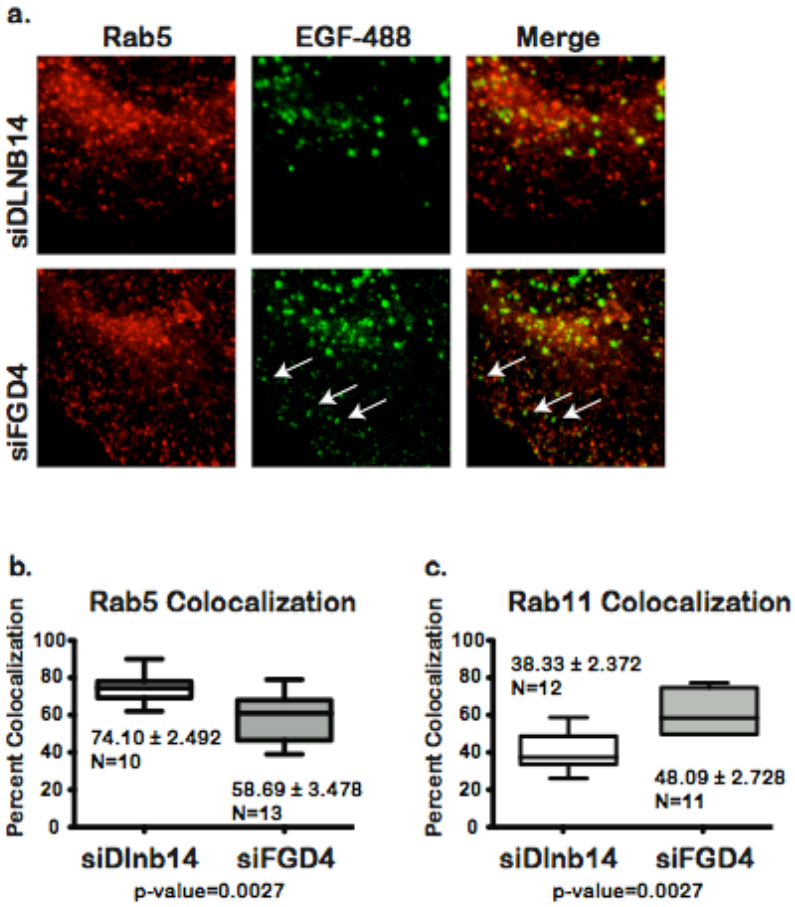
Figure 2-5. FGD4 is necessary for efficient ErbB1 signaling.



(a). EGFR protein levels in the HeLa stable cell lines were measured with immunoblotting. EGFR mRNA levels were also measured using QPCR. (b). HeLa stable cell lines were starved overnight and stimulated with 100 ng/mL EGF. Lysates were then collected at the indicated times and

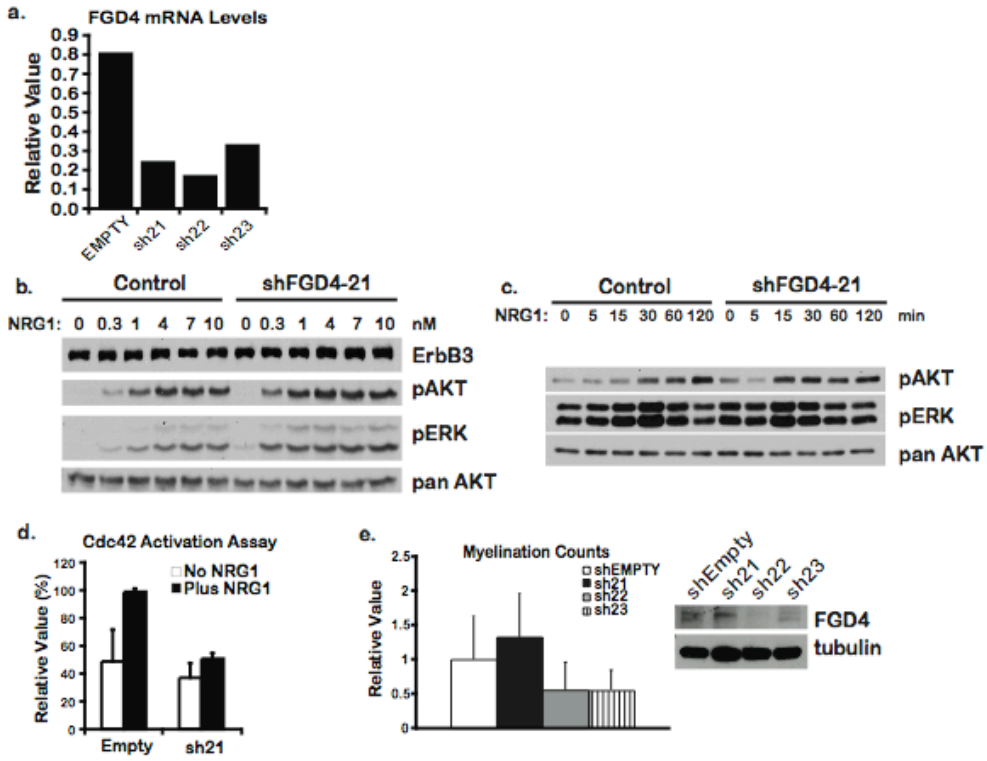
immunoblotted with the indicated antibodies. (c). HeLa shRNA stable cell lines were transfected with myc-FGD4 and stimulated with 100 ng/mL EGF. Lysates were collected at the indicated times and immunoblotted with the indicated antibodies. (d). HeLa cells were transfected with the indicated siRNAs. After 72 hours, surface receptors were biotinylated for 15 min at room temperature then biotinylated receptors were pulled down with streptavidin beads. Lysates and pulldown samples were immunoblotted for EGFR and ERK. (e). HeLa shRNA stable cell lines were stimulated with 100 ng/mL EGF for 15 min and subject to a Cdc42 activation assay.

Figure 2-6. FGD4 is necessary for efficient ErbB1 endocytic trafficking



a) Cells were transfected with the indicated siRNA then transfected with either mCherry-Rab5 or mCherry-Rab11. After 72 hours of the initial siRNA transfection, samples were incubated with EGF-488 on ice, then washed and incubated in a 37 C incubator. Cells were then fixed at 30 min and Z-stack images taken using a confocal microscope with a 60X objective and processed using Imaris software. b) Co-localization of mCherry-Rab5 and EGF-488 was calculated using Imaris software function “Coloc.” c) Co-localization of mCherry-Rab11 and EGF-488 was calculated using Imaris software function “Coloc.”

Figure 2-7. FGD4 is necessary for efficient ErbB3 signaling



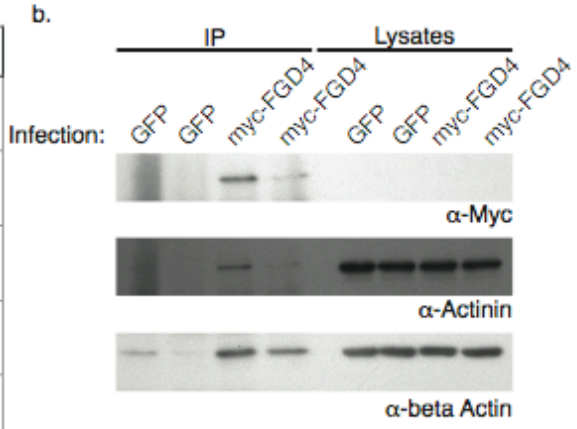
a) Immortalized Schwann cells were infected with the indicated shRNAs and selected for those that successfully integrated the shRNA cassette. Efficient knockdown of FGD4 was quantified by QPCR. b) The indicated Schwann cell stable lines were starved overnight then stimulated with increasing concentrations of NRG1 for 15 minutes. The lysates were then immunoblotted for ErbB3, pAKT, pERK, and pan AKT. c) The indicated Schwann cell stable lines were starved overnight then stimulated with with 1 nM NRG1 for the indicated times. The lysates were then immunoblotted for pAKT, pERK, and pan AKT. d) The stable Schwann cell lines were starved overnight then stimulated with 1nM NRG1 for 15 minutes. The lysates were then prepared and used for a Cdc42 activation assay. e) Primary Rat Schwann cells were infected with the indicated shRNA viruses and co-cultured with Rat Neurons. After up to 4 weeks, slides were prepared of the co-cultures and stained for myelin basic protein and nuclei. Percent of myelinated cells were calculated by counting myelin basic protein positive cells and dividing by the number of nuclei in that field.

Figure 3-1. Immunoprecipitation and Mass Spectrometry Analysis

a.

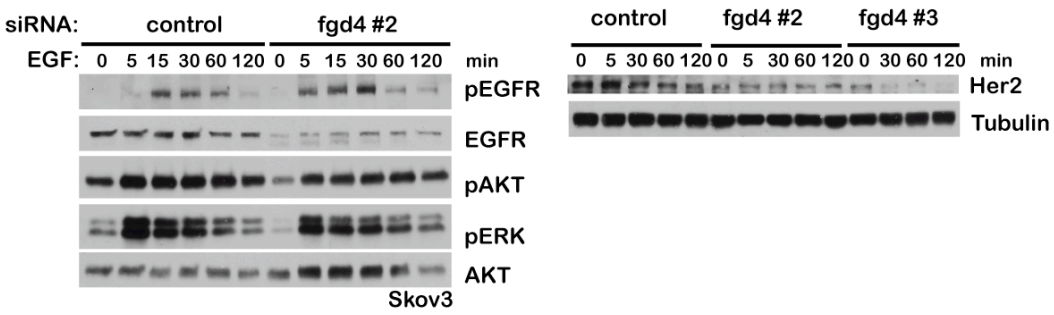
MW on Gel	Protein Identified	MW of Protein
190	filamin A, alpha	276.4
	SPTAN1 protein	282.1
115	myosin IC isoform b	119.6
	actinin, alpha 4	278
82	actinin, alpha 4	104.8
	Junction plakoglobin (Desmoplakin -3)	81.6
64	vimentin	53.6
	tubulin alpha 6	49.9
55	beta actin	41.7

b.



a) Table of identified co-immunoprecipitated proteins b) Confirmation of interaction. Cells were infected with a viral vector expressing GFP or myc-FGD4. Lysates were harvested and immunoprecipitated with anti-myc conjugated beads. The immunoprecipitate was then immunoblotted with the indicated antibodies.

Figure 3-2. EGF induced degradation of ErbB1 and ErbB2 in Skov3 cells. a.



a) Skov3 cells were transfected with siRNAs targeting FGD4 or Dlnb14 (control). Forty-eight hours after transfection, cells were starved overnight and then stimulated with EGF. Lysates were harvested at the indicated times and immunoblotted with the listed antibodies.

CHAPTER THREE
Conclusions and Future Directions

Conclusions

ErbB receptor signaling is a powerful proliferative signal that originates from an extracellular ligand, thereby activating the receptor tyrosine kinase and its various downstream signaling pathways. As discussed in the introductory chapter, downregulation of the active receptor signal requires endocytosis and trafficking toward the lysosomal compartment for degradation or toward the recycling compartment for future signaling potential. Trafficking to particular endocytic compartments likely require interaction of cargo proteins with a complex that can mediate the hand off. For example, ErbB1 lysosomal degradation requires ubiquitination for eventual ESCRT complex binding and inhibition of

ubiquitination exhibits increased downstream signaling and reduced receptor degradation (Shtiegman, Kochupurakkal et al. 2007). ErbB receptors and their ligands can be found overexpressed in a myriad of cancer yet the therapeutic potential of the endocytic route of these receptors has yet to be explored. This is likely due to the scarcity of knowledge on the key players that regulate endocytic trafficking. This data works towards elucidating the molecular players that help traffick ErbB receptors toward recycling versus degradation.

In this work, we see that FGD4, a Cdc42 GEF, is involved in microtubule stabilization, efficient polarized cell migration, appropriate downstream receptor signaling, and receptor sorting. Using siRNA and shRNA methods to deplete FGD4, we see that the microtubule network becomes sparse and the microtubules are less stable and long-lived. Also, activation of Cdc42 in response to cell migration, EGF, and NRG1 requires FGD4 suggesting that FGD4 depletion phenotypes are mediated by Cdc42 activity. These phenotypes implicate an underlying defect in signal induced microtubule stabilization as each cellular process affected relies on regulation of microtubule dynamics as discussed in Chapter one. The literature reveals that Cdc42 can regulate both the microtubule cytoskeleton as well as the actin cytoskeleton. Cdc42 utilizes a

microtubule end capture mechanism to stabilized microtubules at the leading edge in migrating cells via interaction with one of it's microtubule stabilizing effectors (e.g. mDia1, IQGAP1, Par6) (Fukata, Nakagawa et al. 2003). FGD4 likely makes use of this mechanism to affect migration. However in *Dictyostelium discoideum*, Myosin-1C, an actin motor protein, has been implicated in microtubule stabilization offering another microtubule stabilizing mechanism that exploits the actin cytoskeleton (Rump, Scholz et al. 2011).

FGD4 depletion also leads to downregulation of EGF ligand induced signaling. Tracking of EGF-488 labeled ligand demonstrated that the EGFR is being retained in the Rab11 compartment, suggesting that FGD4 may be important for the trafficking of the receptor from a Rab11 compartment back to the plasma membrane. Many groups report that microtubules are important for certain long-range vesicle trafficking events, as previously discussed in the introduction. This data suggests that FGD4 may be important in coordinating microtubule stabilization and vesicle delivery to the plasma membrane. A change in receptor trafficking in the absence of FGD4 likely depends on the affinity of the activated ErbB receptor for c-cbl. ErbB1 has a strong affinity for c-cbl, while its family members ErbB2 and ErbB3 do not. This difference results in an increase

in EGFR degradation in response to ligand stimulation, while ErbB3 levels remain the same when FGD4 is depleted. Subsequently, EGFR downstream signaling is reduced while ErbB3 signaling is upregulated. Uncoupling ligand concentration from downstream signaling can have negative consequences in biological processes sensitive to ligand amounts. For example, peripheral nerve myelination is very sensitive to Neuregulin levels and will likely be vulnerable to perturbations of ErbB3 receptor trafficking. Indeed, mutation in several proteins involved in regulating endocytosis such as Rab7, Fig4, MTMR2, MTMR13, SH3TC2 cause the human disease Charcot-Marie-Tooth neuropathy, which is characterized by progressive demyelination of the peripheral nerves. The role of these proteins in receptor regulation has not been explored but these mutations may ultimately be affecting ErbB3 receptor signaling and, therefore, Schwann cell myelination of peripheral axons. Our studies show that FGD4 depletion affects NRG1 stimulation of ErbB3 receptor signaling, but the molecular partners and exact mechanism of action are still not understood. The following section will describe some follow up experiments that will further elucidate the mechanism of FGD4 in endocytic recycling.

Future Directions

FGD4 function in microtubule stability

Our data show that FGD4 depletion causes a decrease in microtubule network density and a decrease in microtubule stability. FGD4 is likely utilizing one or both of the following mechanisms 1) microtubule end capture and 2) interactions with microtubule stabilizing motor proteins. Cdc42 and a few of its effector proteins interact with microtubule tip binding proteins (APC, EB1, Clip170) to stabilize tubules at foci at the plasma membrane (Mimori-Kiyosue and Tsukita 2003). FGD4 may be utilizing these molecular interactions during migration to stabilize microtubules at the leading edge and presumably orient cell motility. Immunoprecipitation of FGD4 with lysates of cells in an exponential growth phase and mass spectrometry of the co-immunoprecipitated proteins was carried out to find novel interacting partners. Unfortunately, mainly actin and actin binding proteins were identified in the immunoprecipitate (Figure 3-1) as has been reported in the literature (Kim, Ikeda et al. 2002; Banerjee, Fischer et al. 2009). Interestingly, one of proteins identified is Myosin 1C, an actin motor protein recently shown to bind and stabilize microtubules during mitosis in *Dictyostelium discoideum*, providing an

alternate mechanism (Rump, Scholz et al. 2011). In order to determine which mechanism is utilized by FGD4 immunoprecipitation can be done in cells that are actively migrating and in cells in which endocytosis has been induced as is described in Chapter 2. The necessity of the candidate proteins could then be assessed in ErbB1 signaling, EGF endocytosis, migration and microtubule stability.

In addition, the FGD4 protein domains necessary for microtubule stabilization can also be explored by overexpressing truncated forms of FGD4 and immunoblotting for the post-modifications characteristic of long-lived microtubules (e.g. acetyl-tubulin, glu-tubulin).

FGD4 function in polarized cell migration

We demonstrate that FGD4 depletion results in retarded cell wound closure in a wounding assay, possibly due to reduced polarization of cells at the migrating front. As mentioned previously, Cdc42 stabilizes microtubules at the leading edge of migrating cells to orient the cell. Complementation studies using truncation forms of FGD4 can be utilized to ascertain which domains are important for this phenotype. The same method may be used for complementation of Cdc42 activity induced by cell migration.

FGD4 function in ErbB1 signaling and endocytosis

FGD4 depletion causes a reduction in EGF induced ErbB1 signaling and a retention of EGF ligand in the Rab11 positive recycling compartment. Again, complementation with FGD4 truncation mutants would tell us which domains are important for its function in this instance. The same complementation method may be applied to Cdc42 activation studies in response to EGF stimulation.

Immunofluorescence studies using truncation mutants during EGF ligand endocytosis will also help pinpoint FGD4 site of action. A caveat to this assay is that high expression vectors should be avoided since any excess FGD4 binds actin stress fibers and does not allow for visualization of any fine structures.

Our data show that in FGD4 depleted cells accumulate 488 labled EGF ligand in the Rab11 positive recycling compartment after 30 minutes using confocal microscopy. Time-lapse studies using fluorescently tagged Rab proteins and fluorescently labeled ligands can also be used to track the ligand over a continuous time period. This would allow us to again pinpoint where the ligand endocytic path diverges in FGD4 depleted cells in comparison to control cells.

FGD4 function in ErbB2/3 signaling and myelination

Our initial studies of FGD4 involvement in ErbB2/3 signaling in response to NRG1 show that in the absence of FGD4, downstream ErbB2/3 signaling is upregulated. This is likely due to the receptors failing to proceed through the endocytic compartments and continuing to signal from an endosome. Immunofluorescence studies of the ErbB2 and ErbB3 receptors after ligand induced endocytosis would help identify the compartment in which the receptors remain.

ErbB receptor overexpression can frequently be found in various cancers such as breast cancer, gliomas, ovarian, etc. It is also known that co-expression of two ErbB receptors can augment the proliferative effect of EGF signaling (Olayioye, Neve et al. 2000). In addition, tumors that co-express two ErbB receptors have a poor clinical outcome (Baselga and Swain. 2009). Many have speculated that heterodimerization of ErbB1 with any other ErbB family member allows it to escape lysosomal downregulation and continue signaling. FGD4's role in aiding receptor recycling to the plasma membrane may have interesting implications for degradation of ErbB2 and ErbB3 receptors, proteins that are typically recycled. In a preliminary experiment, FGD4 depletion leads to reduced

ErbB1 levels and ErbB2 levels in response to EGF stimulation when compared to control cells (Fig. 3-2). This suggests that if the recycling avenue is impaired, then ErbB1 trafficking to the lysosome then becomes dominant and carries the heterodimer toward degradation. These results should be replicated in other cancer cell lines that express more that one ErbB receptor. A study by Schoeberl et al. demonstrates that stimulation with NRG1 does not induce ErbB1 heterodimerization with ErbB3, indicating that FGD4 depletion may not push the ErbB3 receptor toward degradation as seen with EGF stimulation. This hypothesis should be tested in a cell line that expresses both ErbB1 and ErbB3.

Perturbation in ErbB3 receptor trafficking may have profound consequences on peripheral nerve myelination by uncoupling NRG1 concentration and downstream signaling. We hypothesize that FGD4 mutations are causing Charcot-Marie-Tooth syndrome through this mechanism. Further characterization of FGD4 depletion on NRG1 signaling should be explored in primary Schwann cells to confirm the observation seen in immortalized Schwann cells.

FGD4 activation by extracellular stimulus

The question still remains of how FGD4 is activated by the various extracellular signals (e.g EGF, NRG1). Other GEFs are activated by phosphorylation of key residues, and rearrangement of GEF structure to relieve autoinhibition (Rossman, Der et al. 2005), which are possible mechanisms used for FGD4 activation. Considering that FGD4 may have a role in endocytic trafficking and many of it's domains are known to bind phospholipids (two PH domain and one FYVE domain), we hypothesize that FGD4 is likely autoinhibited and activation by an upstream signal allows it to reveal its phospholipids binding domains for recruitment to particular endosomal membranes. To explore this possibility, a phospholipids binding screen might be conducting with FGD4 to narrow down which phospholipid species the protein is likely binding in vivo. Overexpression of FGD4 have been unsuccessful until this point, as the F-actin binding domain within the protein is extremely dominant. Viral expression of a mutant without the F-actin binding domain may help solve this problem.

In addition, discovering phosphorylation sites in response to EGF stimulation is also a major step in finding the mechanism of FGD4 activation. One unbiased method to employ is to immunoprecipitate FGD4

after EGF stimulation and use mass spectrometry to directly identify phosphorylation sites.

BIBLIOGRAPHY

Balklava, Z., S. Pant, et al. (2007). "Genome-wide analysis identifies a general requirement for polarity proteins in endocytic traffic." Nat Cell Biol **9**(9): 1066-73.

Bananis, E., J. W. Murray, et al. (2000). "Microtubule and motor-dependent endocytic vesicle sorting in vitro." J Cell Biol **151**(1): 179-86.

Banerjee, J., C. C. Fischer, et al. (2009). "The amino acid motif L/IIxxFE defines a novel actin-binding sequence in PDZ-RhoGEF." Biochemistry **48**(33): 8032-43.

Baravalle, G., D. Schober, et al. (2005). "Transferrin recycling and dextran transport to lysosomes is differentially affected by bafilomycin, nocodazole, and low temperature." Cell Tissue Res **320**(1): 99-113.

Baulida, J., M. H. Kraus, et al. (1996). "All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired." J Biol Chem **271**(9): 5251-7.

Benesch, S., S. Lommel, et al. (2002). "Phosphatidylinositol 4,5-biphosphate (PIP2)-induced vesicle movement depends on N-WASP and involves Nck, WIP, and Grb2." J Biol Chem **277**(40): 37771-6.

Benesch, S., S. Polo, et al. (2005). "N-WASP deficiency impairs EGF internalization and actin assembly at clathrin-coated pits." J Cell Sci **118**(Pt 14): 3103-15.

Birchmeier, C. and K. A. Nave (2008). "Neuregulin-1, a key axonal signal that drives Schwann cell growth and differentiation." Glia **56**(14): 1491-7.

Boerner, J. L., A. Danielsen, et al. (2003). "Ligand-independent oncogenic signaling by the epidermal growth factor receptor: v-ErbB as a paradigm." Exp Cell Res **284**(1): 111-21.

Bonifacino, J. S. and L. M. Traub (2003). "Signals for sorting of transmembrane proteins to endosomes and lysosomes." Annu Rev Biochem **72**: 395-447.

Bryant, D. M., A. Datta, et al. (2010). "A molecular network for de novo generation of the apical surface and lumen." Nat Cell Biol **12**(11): 1035-45.

Bustelo, X. R. (2000). "Regulatory and signaling properties of the Vav family." Mol Cell Biol **20**(5): 1461-77.

Daub, H., K. Gevaert, et al. (2001). "Rac/Cdc42 and p65PAK regulate the microtubule-destabilizing protein stathmin through phosphorylation at serine 16." J Biol Chem **276**(3): 1677-80.

Delague, V., A. Jacquier, et al. (2007). "Mutations in FGD4 encoding the Rho GDP/GTP exchange factor FRABIN cause autosomal recessive Charcot-Marie-Tooth type 4H." Am J Hum Genet **81**(1): 1-16.

Derivery, E., C. Sousa, et al. (2009). "The Arp2/3 activator WASH controls the fission of endosomes through a large multiprotein complex." Dev Cell **17**(5): 712-23.

Driskell, O. J., A. Mironov, et al. (2007). "Dynein is required for receptor sorting and the morphogenesis of early endosomes." Nat Cell Biol **9**(1): 113-20.

Durrbach, A., D. Louvard, et al. (1996). "Actin filaments facilitate two steps of endocytosis." J Cell Sci **109 (Pt 2)**: 457-65.

Etienne-Manneville, S. (2004). "Cdc42--the centre of polarity." J Cell Sci **117**(Pt 8): 1291-300.

Etienne-Manneville, S. and A. Hall (2003). "Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity." Nature **421**(6924): 753-6.

Etienne-Manneville, S., J. B. Manneville, et al. (2005). "Cdc42 and Par6-PKCzeta regulate the spatially localized association of Dlg1 and APC to control cell polarization." J Cell Biol **170**(6): 895-901.

Feng, Y., S. M. Hartig, et al. "The Cdc42-interacting protein-4 (CIP4) gene knock-out mouse reveals delayed and decreased endocytosis." J Biol Chem **285**(7): 4348-54.

Fukata, M., M. Nakagawa, et al. (2003). "Roles of Rho-family GTPases in cell polarisation and directional migration." Curr Opin Cell Biol **15**(5): 590-7.

Fukata, M., T. Watanabe, et al. (2002). "Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170." Cell **109**(7): 873-85.

Georgiou, M., E. Marinari, et al. (2008). "Cdc42, Par6, and aPKC regulate Arp2/3-mediated endocytosis to control local adherens junction stability." Curr Biol **18**(21): 1631-8.

Gilmour, L. M., K. G. Macleod, et al. (2002). "Neuregulin expression, function, and signaling in human ovarian cancer cells." Clin Cancer Res **8**(12): 3933-42.

Goshima, G., R. Wollman, et al. (2005). "Length control of the metaphase spindle." Curr Biol **15**(22): 1979-88.

Gundersen, G. G., S. Khawaja, et al. (1987). "Postpolymerization detyrosination of alpha-tubulin: a mechanism for subcellular differentiation of microtubules." J Cell Biol **105**(1): 251-64.

Hoepfner, S., F. Severin, et al. (2005). "Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B." Cell **121**(3): 437-50.

Holbro, T. and N. E. Hynes (2004). "ErbB receptors: directing key signaling networks throughout life." Annu Rev Pharmacol Toxicol **44**: 195-217.

Hu, J., F. Troglio, et al. (2009). "F-BAR-containing adaptor CIP4 localizes to early endosomes and regulates Epidermal Growth Factor Receptor trafficking and downregulation." Cell Signal **21**(11): 1686-97.

Izumi, G., T. Sakisaka, et al. (2004). "Endocytosis of E-cadherin regulated by Rac and Cdc42 small G proteins through IQGAP1 and actin filaments." J Cell Biol **166**(2): 237-48.

Joberty, G., C. Petersen, et al. (2000). "The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42." Nat Cell Biol **2**(8): 531-9.

Johnson, D. I. and J. R. Pringle (1990). "Molecular characterization of CDC42, a

Saccharomyces cerevisiae gene involved in the development of cell polarity." J Cell Biol **111**(1): 143-52.

Kim, Y., W. Ikeda, et al. (2002). "Association of frabin with specific actin and membrane structures." Genes Cells **7**(4): 413-20.

Langdon, W. Y., C. D. Hyland, et al. (1989). "The c-cbl proto-oncogene is preferentially expressed in thymus and testis tissue and encodes a nuclear protein." J Virol **63**(12): 5420-4.

Leibfried, A., R. Fricke, et al. (2008). "Drosophila Cip4 and WASp define a branch of the Cdc42-Par6-aPKC pathway regulating E-cadherin endocytosis." Curr Biol **18**(21): 1639-48.

Lemke, G. (2006). "Neuregulin-1 and myelination." Sci STKE **2006**(325): pe11.

Levkowitz, G., L. N. Klapper, et al. (1996). "Coupling of the c-Cbl protooncogene product to ErbB-1/EGF-receptor but not to other ErbB proteins." Oncogene **12**(5): 1117-25.

Ligon, L. A. and E. L. Holzbaur (2007). "Microtubules tethered at epithelial cell junctions by dynein facilitate efficient junction assembly." Traffic **8**(7): 808-19.

Ligon, L. A., S. Karki, et al. (2001). "Dynein binds to beta-catenin and may tether microtubules at adherens junctions." Nat Cell Biol **3**(10): 913-7.

Lin, S. X., G. G. Gundersen, et al. (2002). "Export from pericentriolar endocytic recycling compartment to cell surface depends on stable, detyrosinated (glu) microtubules and kinesin." Mol Biol Cell **13**(1): 96-109.

Lu, Z. and T. Hunter (2009). "Degradation of activated protein kinases by ubiquitination." Annu Rev Biochem **78**: 435-75.

Martin-Belmonte, F., A. Gassama, et al. (2007). "PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42." Cell **128**(2): 383-97.

Meng, W., Y. Mushika, et al. (2008). "Anchorage of microtubule minus ends to

adherens junctions regulates epithelial cell-cell contacts." Cell **135**(5): 948-59.

Michailov, G. V., M. W. Sereda, et al. (2004). "Axonal neuregulin-1 regulates myelin sheath thickness." Science **304**(5671): 700-3.

Mimori-Kiyosue, Y. and S. Tsukita (2003). ""Search-and-capture" of microtubules through plus-end-binding proteins (+TIPs)." J Biochem **134**(3): 321-6.

Mosesson, Y., G. B. Mills, et al. (2008). "Derailed endocytosis: an emerging feature of cancer." Nat Rev Cancer **8**(11): 835-50.

Murphy, J. E., B. E. Padilla, et al. (2009). "Endosomes: a legitimate platform for the signaling train." Proc Natl Acad Sci U S A **106**(42): 17615-22.

Musacchio, A. and E. D. Salmon (2007). "The spindle-assembly checkpoint in space and time." Nat Rev Mol Cell Biol **8**(5): 379-93.

Newbern, J. and C. Birchmeier "Nrg1/ErbB signaling networks in Schwann cell development and myelination." Semin Cell Dev Biol **21**(9): 922-8.

Ng, D. C., B. H. Lin, et al. (2006). "Stat3 regulates microtubules by antagonizing the depolymerization activity of stathmin." J Cell Biol **172**(2): 245-57.

Nobes, C. D. and A. Hall (1999). "Rho GTPases control polarity, protrusion, and adhesion during cell movement." J Cell Biol **144**(6): 1235-44.

Ogata, T., S. Iijima, et al. (2004). "Opposing extracellular signal-regulated kinase and Akt pathways control Schwann cell myelination." J Neurosci **24**(30): 6724-32.

Rodal, A. A., A. D. Blunk, et al. "A presynaptic endosomal trafficking pathway controls synaptic growth signaling." J Cell Biol **193**(1): 201-17.

Rossman, K. L., C. J. Der, et al. (2005). "GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors." Nat Rev Mol Cell Biol **6**(2): 167-80.

Rump, A., T. Scholz, et al. (2011). "Myosin-1C associates with microtubules and

stabilizes the mitotic spindle during cell division." J Cell Sci **124**(Pt 15): 2521-8.

Schmoranzer, J., J. P. Fawcett, et al. (2009). "Par3 and dynein associate to regulate local microtubule dynamics and centrosome orientation during migration." Curr Biol **19**(13): 1065-74.

Schoeberl, B., E. A. Pace, et al. (2009). "Therapeutically targeting ErbB3: a key node in ligand-induced activation of the ErbB receptor-PI3K axis." Sci Signal **2**(77): ra31.

Shtiegman, K., B. S. Kochupurakkal, et al. (2007). "Defective ubiquitinylation of EGFR mutants of lung cancer confers prolonged signaling." Oncogene **26**(49): 6968-78.

Sigismund, S., E. Argenzio, et al. (2008). "Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation." Dev Cell **15**(2): 209-19.

Sorkin, A. and L. K. Goh (2009). "Endocytosis and intracellular trafficking of ErbBs." Exp Cell Res **315**(4): 683-96.

Stehbens, S. J., A. D. Paterson, et al. (2006). "Dynamic microtubules regulate the local concentration of E-cadherin at cell-cell contacts." J Cell Sci **119**(Pt 9): 1801-11.

Sumi, T., K. Matsumoto, et al. (1999). "Cofilin phosphorylation and actin cytoskeletal dynamics regulated by rho- and Cdc42-activated LIM-kinase 2." J Cell Biol **147**(7): 1519-32.

Syed, N., K. Reddy, et al. "Soluble neuregulin-1 has bifunctional, concentration-dependent effects on Schwann cell myelination." J Neurosci **30**(17): 6122-31.

Tian, L., D. L. Nelson, et al. (2000). "Cdc42-interacting protein 4 mediates binding of the Wiskott-Aldrich syndrome protein to microtubules." J Biol Chem **275**(11): 7854-61.

Timpson, P., D. K. Lynch, et al. (2005). "Cortactin overexpression inhibits ligand-

induced down-regulation of the epidermal growth factor receptor." Cancer Res **65**(8): 3273-80.

Traer, C. J., A. C. Rutherford, et al. (2007). "SNX4 coordinates endosomal sorting of TfnR with dynein-mediated transport into the endocytic recycling compartment." Nat Cell Biol **9**(12): 1370-80.

Vieira, A. V., C. Lamaze, et al. (1996). "Control of EGF receptor signaling by clathrin-mediated endocytosis." Science **274**(5295): 2086-9.

Vieira, A. V., C. Lamaze, et al. (1996). "Control of EGF receptor signaling by clathrin-mediated endocytosis." Science **274**(5295): 2086-9.

Voronina, E., G. Seydoux, et al. "RNA Granules in Germ Cells." Cold Spring Harb Perspect Biol.

Waterman, H., I. Alroy, et al. (1999). "The C-terminus of the kinase-defective neuregulin receptor ErbB-3 confers mitogenic superiority and dictates endocytic routing." Embo J **18**(12): 3348-58.

Wen, Y., C. H. Eng, et al. (2004). "EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration." Nat Cell Biol **6**(9): 820-30.

Whitehurst, A. W., B. O. Bodemann, et al. (2007). "Synthetic lethal screen identification of chemosensitizer loci in cancer cells." Nature **446**(7137): 815-9.

Worby, C. A. and J. E. Dixon (2002). "Sorting out the cellular functions of sorting nexins." Nat Rev Mol Cell Biol **3**(12): 919-31.

Yarden, Y. and M. X. Sliwkowski (2001). "Untangling the ErbB signalling network." Nat Rev Mol Cell Biol **2**(2): 127-37.

Yasuda, S., F. Oceguera-Yanez, et al. (2004). "Cdc42 and mDia3 regulate microtubule attachment to kinetochores." Nature **428**(6984): 767-71.

Zheng, Y. (2001). "Dbl family guanine nucleotide exchange factors." Trends Biochem Sci **26**(12): 724-32.

Zwang, Y. and Y. Yarden (2009). "Systems biology of growth factor-induced receptor endocytosis." Traffic **10**(4): 349-63.