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Control of the Basal Recycling and Surface Expression of Epidermal Growth Factor Receptor by the Endocytic Recycling Regulator EHD1 Utilizing a Pathway Shared by RUSC2

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by
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Supervisory Committee:

Parmender Mehta, Ph.D. Joyce Solheim, Ph.D. Jenny Wang, Ph.D.

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Control of the Basal Recycling and Surface Expression of Epidermal Growth Factor Receptor by the Endocytic Recycling Regulator EHD1 Utilizing a Pathway Shared by RUSC2

Eric Tom, Ph.D.

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Supervisor: Hamid Band, M.D., Ph.D.

The epidermal growth factor receptor (EGFR) is a prototype receptor tyrosine kinase (RTK) and oncogene aberrantly expressed or mutated in solid tumors. Its surface expression is dynamically regulated. Display of an activation-competent pool is essential for response to ligands. Internalization and degradation of EGFR following stimulation has received the most attention, fewer studies have characterized the recycling arm of EGFR transit, basal traffic and surface display are poorly understood. Thus, we evaluated the endocytic recycling of EGFR for its therapeutic potential in EGFR driven cancers. The C-Terminal Eps15 homology (EH) domain-containing proteins have emerged as regulators of surface receptor recycling but their roles in EGFR traffic are not known. Studies presented here demonstrate EHD1 has a critical role in the positive regulation of total EGFR levels and surface display under unstimulated, basal conditions. Reduced EGFR surface expression due to EHD1 knockdown resulted in a reduction of ligand-induced signal transduction, and reduced EGF-dependent cell proliferation in two- and three-dimensional culture. Under EHD1 knockdown, EGFR accumulated in a juxta-nuclear compartment where it co-localized with markers for the Golgi apparatus. Single-cohort labeling of cell surface EGFR indicated this pool co-localizes with Rab11a, a marker of recycling endosomes, implicating EHD1 in the anterograde transport of recycling unstimulated EGFR. We have further determined that the RUN and SH3 domain containing protein (RUSC2) associates with EHD1, with both proteins co-localizing to vesicles and tubular membranes. RUSC2 knockdown led to a phenotype similar to EHD1 knockdown with reduction in total and

surface EGFR expression. Our results show EHD1 is required for the basal recycling and plasma membrane targeting of EGFR under unstimulated conditions in a pathway shared with RUSC2. This study furthers our understanding of the mechanisms by which EGFR is transported to the plasma membrane for activation, provides a new function for EHD family members in regulating the itinerary of receptor tyrosine kinases and helps validate the endocytic recycling of RTKs as an approach to augmenting existing RTK-targeted therapies.

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Abbreviations

AR	Amphiregulin
ASOR	Asialoorosomucoid
ASGPR	Asialoglycoprotein receptor
Cbl	Casitas B-lineage Lymphoma proto-oncogene
CI-M6PR	Cation-Independent Mannose-6-Phosphate Receptor
Cre	Cre recombinase
DEP-1	Density Enhanced Phosphatase-1
dEHBP1	Drosophila homolog of EH Domain Binding Protein 1
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
EEA1	Early Endosomal Antigen 1
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGFRvIII	EGFR variant Type III
EH	Eps15 homology
EPS15	Epidermal Growth Factor Receptor Substrate 15
ER	Endoplasmic Reticulum
ErbB	Erythroblastic leukemia viral oncogene homolog
ERC	Endocytic Recycling Center
ESCRT	Endosomal Sorting Complex Required for Transport
FEME	Fast Endophillin Mediated Endocytosis
GAP	GTPase activating protein
GEF	Guanine Exchange Factor
GBM	Glioblastoma Multiforme
HER1	Human Epidermal growth factor Receptor -1
HRas	V-Ha-Ras Harvey Rat Sarcoma Viral Oncogene Homolog
IGF1R	Insulin Growth Factor 1 Receptor
IF	Immunofluorescence
kDa	kiloDalton
mAbs	Monoclonal Antibodies
MAPK	Mitogen Activated Protein Kinase
MICAL-L1	Molecule Interacting with CasL-like 1
MOI	Multiplicity of Infection
MVBs	Muli-Vesicular Bodies
NGF	Nerve Growth Factor
NPF	asparagine-proline-phenylalanine
NSCLC	Non-Small Cell Lung Cancer
PBS	Phosphate-Buffered Saline
PDGFR	Platelet Derived Growth Factor Receptor
PI3K	Phosphoinositide-3-Kinase
PTB	Phosphotyrosine Binding

PTK	Protein Tyrosine Kinase
PTPs	Protein Tyrosine Phosphatase
RCP	Rab Coupling Protein
RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinase
SH2	Src Homology 2
SH3	Src Homology 3
siRNA	small interfering RNA
shRNA	small hairpin RNA
TCR	T-Cell Antigen Receptor
TGF-α	Transforming Growth Factor alpha
TKB	Tyrosine Kinase Binding
TKD	Tyrosine Kinase Domain
Trk	Tropomyosin-receptor kinase

Chapter 1
Introduction

1. Receptor Tyrosine Kinase Signaling

1.1 Role in Physiology and Disease

Patches of the cellular plasma membrane and their associated molecular components are continuously internalized and replenished as the cell mediates appropriate responses to a constant flux of nutrients, signaling molecules, and solutes comprising the extracellular milieu. Ligand receptors on the surface interpret these signals to coordinate critical cellular processes of proliferation, migration, differentiation and survival during normal tissue homeostasis and development. These processes are normally under tight control, and dysregulation can be causative of deleterious conditions including cancers, giving surface receptors deep roots in oncogenesis. One signal transducer family that has generated much interest over the last 40 years is the receptor tyrosine kinase (RTK) family and its discovery has shaped the field of molecular oncology having demonstrated efficacy as therapeutic targets.

RTKs have a conserved structural arrangement comprised of an extracellular ligand-binding region, a transmembrane span, and a cytoplasmic tyrosine kinase domain, together with regulatory sequences for recruiting downstream effectors through phospho-tyrosine binding (PTB) and Src homology-2 (SH2) domains. Functionally, upon ligand binding signals are transduced across the plasma membrane by way of receptor dimerization and phosphorylation of key residues within intracellular activation loops, in turn stimulating catalytic activity of the tyrosine kinase domain. RTKs are frequently cited as causative of hyperproliferative disorders when overexpressed or mutated, and for this are definitively referred to as proto-oncogenes. Their prototypical founding member, the epidermal growth factor receptor/erythroblastic leukemia viral oncogene homolog/ human epidermal growth factor receptor -1 (EGFR/ErbB-1/HER1) is a member of the ErbB family (ErbB1-4) and was cloned in the early 1980's from normal human placenta and A431 epidermoid carcinoma cells. Applied molecular cloning elucidated the identities of other RTKs deduced from conserved homology within the primary amino acid sequence. Today this receptor family is comprised of nearly 60 known members with

the expression of half represented as altered in various cancers (Gschwind, et al., 2004).

Genetic models have demonstrated RTKs as critical for embryonic patterning and development, as well as maintenance of adult tissue homeostasis. Whole body knock-outs are often prenatally lethal as is the case for the Platelet Derived Growth Factor Receptors (PDGFR) α and β knockout mice, which display defects in the neural crest, somites and kidney development (Soriano, 1994, 1997). EGFR-null mice succumb to multi-organ failure with impaired epithelial development and die within three weeks after birth (Miettinen,1995). Adult tissue expression of RTKs and their ligands have an established role in maintaining homeostatic conditions throughout the body. In the peripheral and central nervous systems the activity of the neurotrophin, Nerve Growth Factor (NGF) and its receptor, the tropomyosin receptor kinase (Trk) maintain differentiated neuronal phenotypes, synaptic connections and plasticity as well as neurotransmission (Chao, 2003). Causative abnormalities not being restricted to the receptors themselves, EGFR ligands and factors regulating their availability have also been demonstrated as critical contributors to organ development in the mammary gland, hair, skin and eyes among others. In the mammary gland, amphiregulin (AR) was shown to be essential for pubertal ductal outgrowth, while a triple knockout of amphiregulin, epidermal growth factor (EGF) and Transforming Growth Factor-α (TGF-α) impaired lactogenesis, alveoli organization and milk production (Luetteke, 1999).

By influencing fundamental cellular activities of growth, differentiation, migration and survival through common downstream mediators such as the mitogen activated protein kinase (MAPK) or phosphoinositide-3 kinase (PI3K) pathways, RTKs are essential for the proper development and functioning of a diverse array of tissues and cell types. This underscores the essential motive for understanding their modes of regulation, signaling and biological outcomes in human health and disease.

1.2 Phosphorylation-Dephosphorylation

The binding of ligand to the receptor extracellular domain induces conformational

activation of dimerized receptors. Ligands may be crosslinking or monomer binding and the dimerization event may be either dependent or independent on ligand binding due to the diversification of structure among various RTKs. A substantial number of permutations exist along this theme (Lemmon 2010). The modes of tyrosine kinase domain (TKD) activation exhibit extensive variation and while the *inactive* receptor conformations are very different by way of their regulatory elements it is an interesting point that the structures of *activated* receptors share similar features. All TKDs are bi-lobular, possessing an intramolecular auto-inhibition on its catalytic activation loop. This loop generally requires cross-phosphorylation at residues within the intracellular domain by its proximal dimer partner to destabilize inhibition and kinetically enhance kinase action. A notable exception is EGFR which features an allosteric mechanism mediated by contacts between two ligand bound receptor monomers (Zhang 2006).

A series of auto-phosphorylation events that follow activation are generally products of cross-phosphorylation by the dimerizing partner and are responsible for establishing docking sites for signaling adapter proteins which will bind via PTB and SH2 domains and prime the receptor for catalyzing phosphorylation of substrates (Ullrich, 1990). As a specific example, RTKs bind to the ubiquitously expressed members of the Src family of protein tyrosine kinases (PTKs) via the SH2 domain, influencing RTK activity by additional phosphorylations in a variety of contexts (Bromann, 2004).

Tyrosine phosphorylation is a reversible post-translational modification and the existence of receptor and non-receptor protein tyrosine phosphatases (PTPs) generated interest as potential tumor suppressors which counterbalance and downregulate RTK signaling (Figure 1.1). This attractive idea has been stymied through the discovery that many PTPs overexpressed in human cancers demonstrate oncogenic properties and this frequent dichotomy underpinning biological mechanisms has expanded the field. Indeed, there are phosphatases that have demonstrated roles in downregulating RTK activity. The PTP (Density Enhanced Phosphatase-1) DEP-1 was

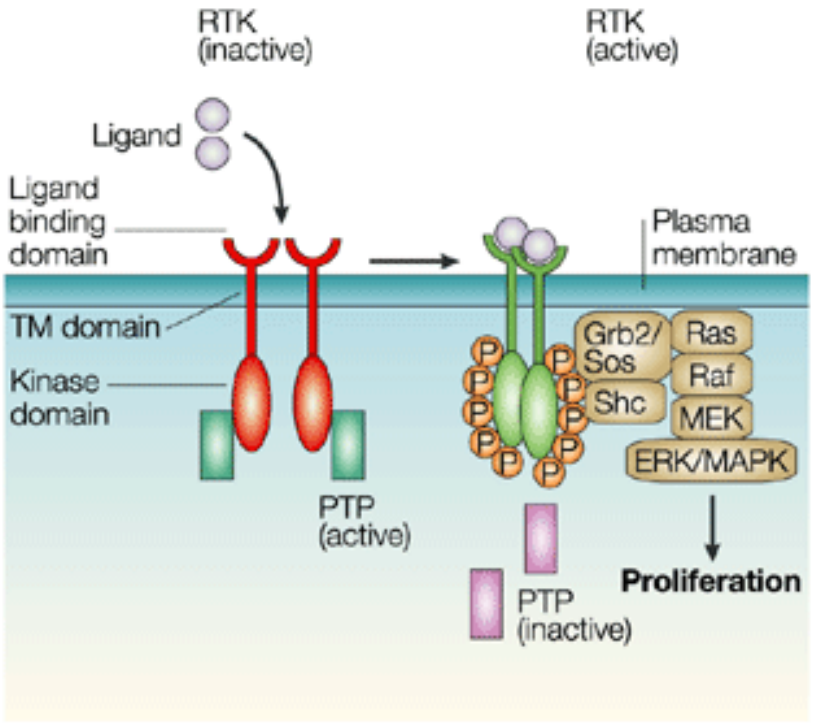


Figure 1.1 Canonical Receptor Tyrosine Kinase Signal Transduction: Ligand-induced dimerization of receptor tyrosine kinase monomers causes dimerization and intracellular autophosphorylation on tyrosine residues. Protein-tyrosine phosphatases (PTPs) regulate receptor activation. Signal adapters bind phosphotyrosine sites, which leads to the activation of a signaling cascade and biological output. Modified and used with permission from (Wetzker 2003)

recently demonstrated to serve as a negative regulator of MAPK signaling downstream of EGFR by altering receptor rate of endocytosis in Hela, MCF7, and two glioblastoma cell lines (Tarcic, 2010). Another study indicates DEP-1 knockout mice fed a high fat diet have improved insulin sensitivity and glucose tolerance, effects mediated by increasing phosphorylation events within the insulin receptor signaling cascade (Krüger, 2015). Conversely, the endoplasmic reticulum (ER)-tethered PTP1B had positive correlation with ErbB2 expression in immunohistochemistry-stained patient sections from tumors of various stages in developing breast cancer, and PTP1B inhibition was found to delay onset of ErbB2-driven mammary tumors and be protective against lung cancer metastasis (Julien, 2010). This would seem to indicate not all phosphorylation events are activating, that they are spatially resolved within the cell, or that they are context dependent. Whatever their varied modes of regulation, further experimentation will be required before any specific paradigm will clarify these seemingly contradictory roles of PTPs in signal transduction.

1.3 Ubiquitination and Degradation

The ubiquitin moiety is a small, 8.5 kDa, reversible post-translational modification with a broad role in promoting substrate degradation routed through the 26s proteasome, the lysosomal compartment, or the autophagosome, as well as influencing receptor internalization and endosomal sorting. Catalytic addition proceeds sequentially via E1, E2, and E3 enzymes to activate, transfer and bind ubiquitin to substrates. Several RTKs are determined to be targets of the Cbl (Casitas B-lineage Lymphoma proto-oncogene) -family of E3 ubiquitin ligases which tag proteins for recognition by the endosomal complex required for transport (ESCRT) system that mediates receptors first into multivesicular bodies (MVBs) and then into lysosomes for degradation. Cbl is recruited indirectly through adapters or binds directly to phosphorylated tyrosines through a tyrosine kinase binding domain (TKB), an assembly composed of a four-helical bundle, an EF hand and an SH2 domain (Levkowitz 1999, Mohapatra 2013, Waterman 2002). Ubiquitin linkages occur on lysine residues and may be single, mono-ubiquitination events or form chains of poly-ubiquitin. There is a total of seven lysines in the ubiquitin

molecule and each has potential for generating unique consequences from their 3-dimensional structure (Clague, 2010). It has not been clarified how an E3 ubiquitin-ligase such as Cbl, which displays the capacity for both mono- and poly-ubiquitination, determines which modification to catalyze (Yokouchi 2001, Ruben 2003).

PDGFR and EGFR were the first mammalian receptors identified as being tagged with ubiquitin as an outcome of ligand stimulation to the effect of modulating signal intensity and duration (Mori 1992, Galcheva-Gargova 1995). These models have in part resolved the role of this modification though not without some contention. The differences between mono- and poly-ubiquitination were probed using an EGFR chimera fused to ubiquitin mutated at lysine 48 to disrupt its ability to form poly-linked chains. This technique seemed to demonstrate that mono-ubiquitination events are sufficient for driving both receptor internalization and subsequent degradation within lysosomes (Haglund, 2003). Utilizing EGFR mutations deficient for recruiting the required machinery, several later reports suggested ubiquitination was not essential for internalization. These studies utilized EGFR mutations deficient for recruiting the required machinery. These interactions however were found to be required for degradation (Duan 2003, Huang 2007).

The roles of individual sites are not fully elucidated, but while mono-ubiquitination events regulate receptor internalization and sorting, poly-ubiquitination is primarily thought to be a mechanism tagging substrates for degradation in the proteasome with most of the published data having focused on poly-chain links of lysine 48 or 63. Recent data suggest the selectivity of sorting towards this organelle does not appear to be determined on specific linkages, as inhibition of degradation had the effect of increasing many different types of chains with the exception of the lysine-63 poly-linked species which does not appear to be involved in routing substrates towards degradation (Xu 2009); however no general consensus has been reached (Huang, 2013).

1.4 Degradative and Recycling Through Endocytic Traffic

An RTK localized at the cell surface, however internalized, is packaged into a vesicle and

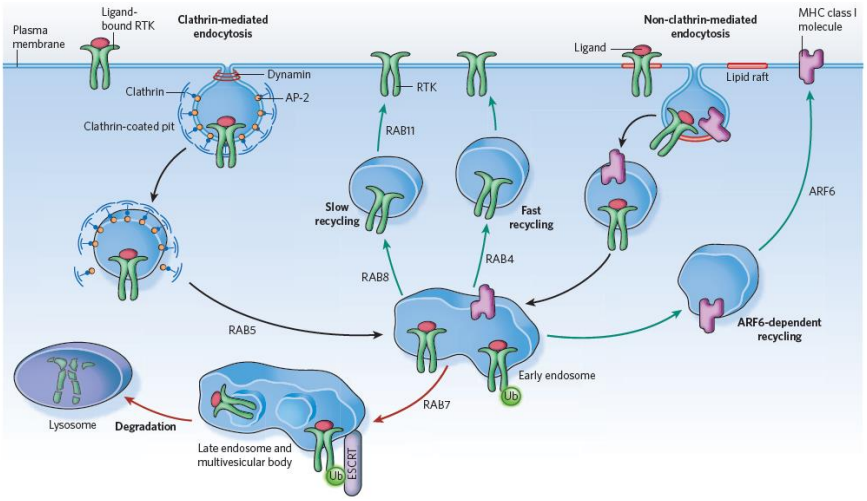


Figure 1.2 Endocytic Trafficking of Signaling Receptors: RTKs are mainly internalized through clathrin-mediated endocytosis (left). In this pathway of endocytosis, ligand binding accelerates the recruitment of receptors to clathrin (present in clathrin-coated pits) through adaptors, such as AP-2. The invaginated pit is released into the cell by dynamin. After internalization, receptors are routed to early endosomes. Trafficking in the endosomal compartment is controlled by small GTP-binding Rab proteins. From the early endosome, cargo is either recycled to the plasma membrane or degraded. Recycling can occur through a fast or a slow

enters the endocytic system, a tubular-membranous structure lying beneath the plasma membrane which receives incoming cargo and assists in determining receptor fate and ultimately its contribution to signaling. Rab proteins are a family of more than 60 small GTPases, with regulatory roles in endosomal transport. Canonically, stimulated receptors are first sent to early endosomes usually designated as positive for Rab5 or EEA-1(Early Endocytic Antigen-1) where they are sorted and from there are either rapidly recycled back to the plasma membrane for continued ligand binding, or on to late endosomes and lysosomes marked by Rab7 and Lamp-1 (Lysosomal-associated membrane protein-1) for degradation (Sigismund, 2012). New synthesis maintains a constant steady-state level. In the absence of growth factors, receptors are constitutively internalized and recycled at a much slower rate than ligand-stimulated receptors. Recycling may occur through a 'fast' pathway mediated by Rab4 and/or Rab35, as well as a 'slow' recycling pathway associated with Rab11, EHD1 and a perinuclear cell localization within the endocytic recycling compartment (ERC) (Figure 1.2). The balancing which leads receptors to degradation or recycling determines overall receptor availability, and mediates the cellular response to stimuli. Additionally, the concept of 'signaling endosomes' suggests that receptors in transit continue to signal from within vesicles as they are shuttled through compartments and buffered between organelles (Waterman, 1998).

While considerable effort has clarified the role of Cbl and ubiquitination in controlling receptor dynamics, much less is known about the alternative recycling itinerary. Recent reports indicate a truncated form of EGFR pathway substrate 15 (EPS15), a ubiquitin binding adaptor supporting clathrin-mediated internalization, which localizes to Rab11 positive endosomes and influences receptor recycling under EGF-stimulated conditions (Chi, 2011). A separate isoform, EPS15b was found to bind endosomal ESCRT protein Hrs. Both the overexpression and depletion of EPS15b, was found to inhibit EGFR ligand-induced degradation and enhance its recycling, suggesting a role in maintaining this balance (Roxrud, 2008).

2. Intracellular Trafficking

2.1 Receptor Internalization

Receptor internalization into endosomes is critical for the downregulation of available receptors from the surface, and transports them into the cell for signal propagation. In general, signaling complexes accessible to an activated receptor are different at the plasma membrane than along its transit while packaged within an endosome. While we consider the different routes available for internalization, it is an important distinction that an RTK may utilize several pathways, making explicit classification difficult (Figure 1.3). Clathrin-mediated endocytosis has been best described, and is named for the triskelion protein capable of forming lattice structures within pits around incoming vesicular cargo. Cargo selection is accomplished via receptor cytoplasmic tail association with various adapter molecules, such as AP-2, which is in part recruited by interactions with membrane lipid Phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] . The clathrin basket-like assembly promotes membrane deformations, which bud off of the plasma membrane in a dynamin GTPase-dependent scission event into intracellular clathrin-coated vesicles (CCVs). Vesicle uncoating is mediated in part by Rab5, which has an influence in regulating total PI(4,5)P2 levels and induces μ2 kinase displacement from the AP-2 complex, preventing it from continued phosphorylation of the cargo recognition subunit μ2 (Hutagalung 2011).

Clathrin-independent endocytosis is a broad term referring to vesicular cargo in order to distinguish it from phagocytosis and micropinocytosis, which are involved in the internalization of patches of plasma membrane > 1μM in size (Mayor and Pagano, 2007). The mechanisms of cargo selection are not well defined for clathrin-independent pathways. Recent published work describes a Fast Endophilin Mediated Endocytosis (FEME) pathway for several ligand-activated RTKs engaged by binding of proline-rich sequences to the SH3 domain of endophilin. FEME is dynamin, Rac, and actin polymerization dependent, and activated upon CDC42 inhibition (Boucrot 2015). Another pathway, caveolae and dynamin-dependent endocytosis is characterized by flask-shaped invaginations (caveolae) enriched in cholesterol, glycosyl phosphatidylinositol

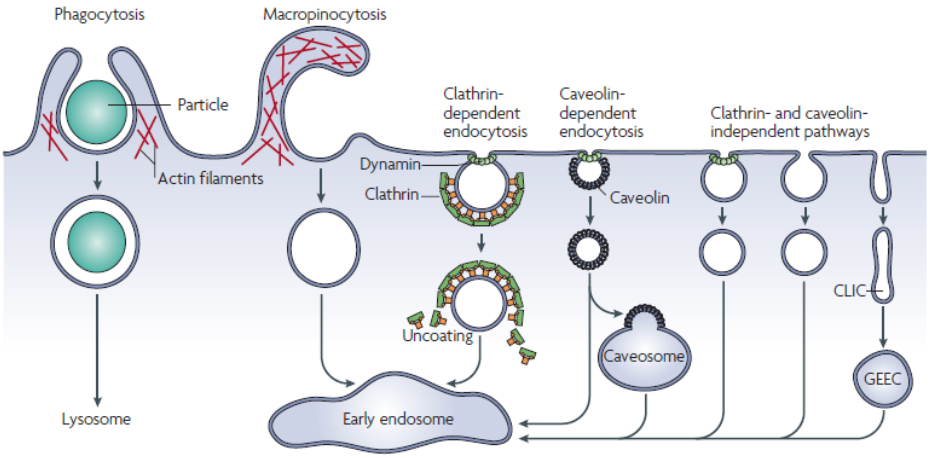


Figure 1.3 Routes of Internalization: Several mechanisms for entry into the cell have been described. Clathrin-mediated endocytosis is best characterized and internalization pathways are frequently classified upon their dependence for clathrin and dynamin. Used with permission from (Mayor 2007).

anchored proteins and the protein caveolin-1. A clathrin-independent, dynamin-independent pathway utilizing CDC42 has been described for the fluid-phase uptake of cholera toxin B. Each pathway contributes to receptor fate, signaling potential and thus conversion of stimulus into cellular output. Accordingly, it was demonstrated EGFR receptors endocytosed through the clathrin-mediated pathway are destined for recycling, and that clathrin-independent internalization commits the receptor for degradation (Sigismund 2005, 2008). The preferred pathway appears to be determinable by a concentration or gradient effect of stimulating ligand, as the clathrin-dependent pathway appears to be favored at low concentrations. This is typically cited to imply the clathrin-mediated pathway is rapidly saturated. Recent data indicate a similar system of regulation also exists for the IGF-1 and PDGF receptors (Sahat and De Donatis 2008), indicating a conserved mechanism controlling RTK propagation. The mechanism whereby cells are able to sense differences in ligand concentration and act appropriately has not been established.

2.2 Basal Unstimulated Receptor Traffic

A newly synthesized cell receptor is translocated from the endoplasmic reticulum to the Golgi apparatus to acquire requisite glycosylations and post-translational modifications before delivery to the surface plasma membrane. The distribution may reflect the cell's commitment to functional domains such as apical or basal surfaces in polarized epithelia, or balanced between endosomal compartments and the plasma membrane (Figure 1.4). A model RTK, EGFR surface localization is not static, and when inactivated is constitutively internalized at a slow rate of 1-2% per minute, comparable to the rate of internalization of plasma membrane (Lund 1990). Under non-stimulated conditions, the receptor is primarily recycled, but a fraction is trafficked to lysosomes and degraded. The surface localization and ultimately the signaling potential is a reflection of the balance between a relatively low internalization rate, and a recycling rate that is ten-fold higher, with new synthesis keeping total levels of receptor at a consistent steady state level (Wiley 2003). Stimulation with EGF shifts this balance dramatically, clearing EGFR from

the surface with a significant proportion of receptor sent to lysosomes for degradation. It is of interest to note that much of what is known about RTK signaling and its molecular mediators comes from studying the outcomes of stimulation with EGF and other ligands, to date very few studies have sought to elucidate the pathways of unstimulated receptors and their native recycling.

2.3 EGFR Ligands, Activation and Signal Transduction

Seven EGFR ligands have been described: EGF, amphiregulin (AR), epiregulin (EPI) transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), and epigen, which upon binding induce conformational changes promoting dimerization, activation and internalization. Intracellular sorting in part is dependent on the pH sensitivity of the ligand-binding interaction, as gradual endosomal acidification accompanies progression to the late endosome/early lysosome and has an impact on the consistency of receptor ubiquitination. From a practical standpoint, this implies a ligand-receptor interaction with stable affinity at low pH favors degradation while a more sensitive binding will cause the ligand to dissociate and favor transit into recycling, accompanied with the due implications for sustained signaling as is the case exemplified by comparing the effects of EGF and TGF-α. Each ligand has a unique kinetic signature which correlates with the relative level of receptor internalization, phosphorylation, ubiquitination and degradation. In one comprehensive paper (Roepstorff 2009) that characterized the role of multiple ligands, amphiregulin stood out as an exception having a low binding affinity, very low degradation, and yet was highly acid resistant. The reasons for this are not clear, although significantly the concentration of the low affinity ligands (amphiregulin, epigen) were used at 10-fold higher concentrations than the remaining higher affinity counterparts. The authors surmise amphiregulin is released from EGFR due to its high off-rate causing rapid dissociation from the receptor in spite of pH. Interestingly, a significant difference in induced ubiquitination was observed between amphiregulin which was substantial, and the other low affinity ligand epigen which failed to induce ubiquitination at the studied post-

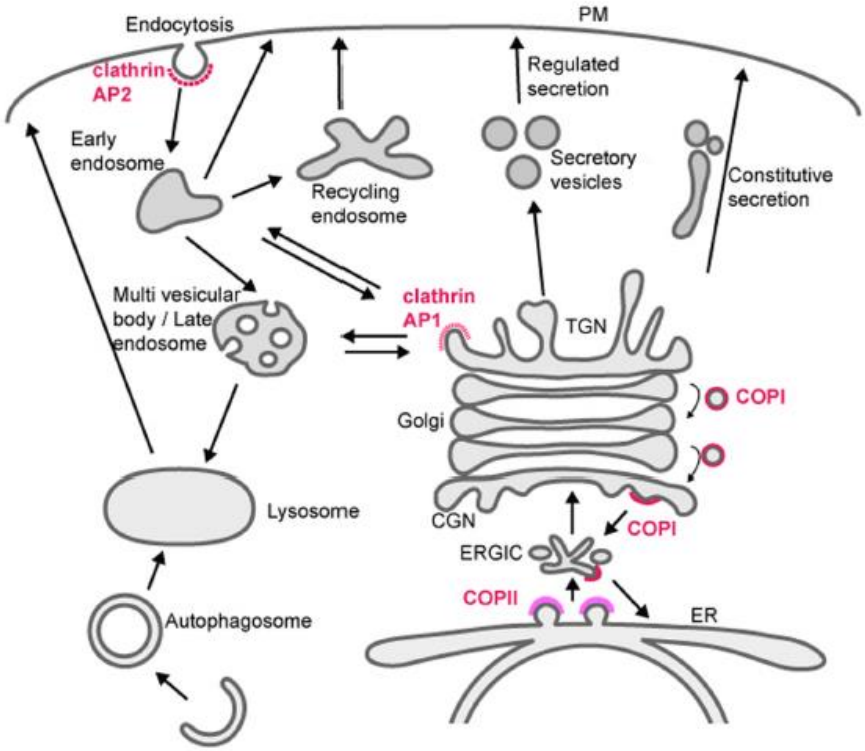


Figure 1.4: An overview of the endocytic trafficking system: transport is mediated by the scission, maturation and fusion of intracellular vesicles and tubules which are dynamically buffered between organelles. Used with permission from (Sato 2014).

stimulation intervals. In human cancers, overexpression and oncogenic mutations of EGFR lead to spontaneous dimerization and activation.

There are two types of constitutively active mutants of EGFR described, truncations and kinase domain mutations. EGFR kinase domain mutations found in non-small cell lung cancer (NSCLC) promotes receptor traffic into the ERC, and impairs receptor interaction with Cbl, resulting in defective ubiquitination-mediated degradation, which positively contributes to EGFR signaling (Padron 2007). Glioblastoma multiforme (GBM) is the most common cancer of the brain and features overexpression of wild type and mutant EGFR expression. The most commonly observed mutation, EGFRvIII, is an in-frame deletion of 267 amino acids in the extracellular domain, rendering a receptor unable to bind ligand yet constitutively active but capable of being degraded by Cbl-mediated ubiquitination, but this process is impaired (Gan 2009).

2.4 Retrograde Transport and Transcytosis

The model of endocytic traffic presented to this point has focused on the balance between recycling and degradation and has not reflected on the myriad of interconnected pathways essential for shuttling molecules between various organelles and functional domains within the cell. Mechanisms have been described for the trafficking of plasma membrane-associated molecules into an intermediary waypoint within the cell, often for its activation, before such substrates are redirected to another organelle or back to the plasma membrane where the molecules are required for a function. Transcytosis maintains apical-basolateral polarity in rat hepatic cells where newly synthesized apical membrane proteins are directed first to the basolateral membrane, followed by endocytosis and transport to the apical membrane (Bartles 1987). For sorting this mixed population of components these pathways often rely on retrograde traffic through the trans-Golgi network (TGN) along with a system of both common and polarized recycling endosomes for normal homeostatic turnover of the plasma membrane. Certain cargos may depend on the retromer complex, an evolutionarily conserved eukaryotic

machinery required for endosome-to-Golgi retrieval (Seaman 2012). Retromer is required in the maintenance of epithelial cell polarity for sorting the apical membrane protein Creb (Zhou 2011).

Recycling and re-targeting of integrins has been demonstrated as critical for cell migration with implications for metastasis. While these extracellular matrix binding proteins are constitutively recycled by Rab11a in normal cells, Rab11c (Rab25) expression is associated with aggressive cancers, and harbors a Q67L Ras-type mutation likely rendering it constitutively active. Rab25 has demonstrated a multifaceted role in driving invasion by binding to the α5β1 heterodimer to facilitate its recruitment to the forward tips of invading cells, and promotes EGFR recycling in association with the Rab coupling protein (RCP) to augment pro-invasive signaling through Akt. Further, Rab25 promotes cell detachment by redirecting an active integrin out of the lysosome to the rearward plasma membrane where it likely competes for an unidentified mediator of adhesion (Caswell 2007, 2008, Dozynkiewicz 2012).

Several activated RTKs and all four members of the EGFR family have been shown to be targeted to the nucleus where they modulate their own transcription and that of other genes relevant to the cell cycle, DNA repair, calcium signaling, and survival pathways upregulated in chemo/radio resistance. Nuclear EGFR has been observed in developing tissues and several cancers including breast, bladder, ovary, and oropharyngeal. High expression of nuclear EGFR correlates with poor patient prognosis (Lo 2006, Domingues 2011). Cell models indicate nuclear targeting is mediated by a novel tripartite cluster of arginines in the EGFR juxtamembrane domain serving as a nuclear localization signal and recruiting nuclear import factor Importin β, to facilitate docking and translocation through the nuclear pore complex through an interaction mediated by the inner nuclear membrane protein Sec61β (Hsu 2007, Wang 2010). The Fibroblast Growth Factor Receptor mediates the Importin β interaction through its ligand FGF, and several IGF receptor binding proteins are known to have nuclear localization signals binding to Importins (Reilly 2001, Iosef 2008). EGF-stimulated EGFR nuclear translocation is dependent on dynamin, which may indicate clathrin mediated internalization but this is not conclusive as dynamin-

dependent clathrin-independent endocytosis processes of FEME and Caveolin-1 pathways have not been definitively ruled out. A further study found EGF-stimulated cells promoted EGFR transport to the Golgi and to the endoplasmic reticulum (ER). Blocking of retrograde traffic using brefeldin A or an ADP ribosylation factor (ARF) dominant-negative mutant impaired EGFR transport to the ER and nuclear import. It was found EGFR physically interacts with the γ-COP subunit of the COPI coatamer retrograde trafficking complex in a time-dependent manner that occurred maximally 15 minutes after treatment with EGF and the receptor requires this interaction for COPI-mediated translocation (Wang 2010).

An exemplary system demonstrating an essential role of retrograde traffic in TrkA RTK signal transduction exists in neurons. Neuronal architecture presents a challenge in long-distance communication through axons often stretching a meter from the cell body, or soma. Stimulatory neurotrophins bind their receptors at the axonal distal tips, and this necessitates a prolonged signaling event in order to maintain neuronal survival, growth and synaptic function (Ascano 2009). The 'signaling endosome' hypothesis is frequently evoked as the mechanism whereby cells have adapted to cope with these demands (Marlin 2015). That a retrograde pathway for endosomes containing RTKs move along microtubules enabling this prolonged signaling began to be worked out in the late 1990's, the specific determinants are a source of current interest although it seems retrograde traffic in this context requires the activity of PI3 kinase (Kuruvilla 2000).

2.5 Traffic in Response to Binding by Antibodies

The advent of monoclonal antibodies (mAbs) in clinical therapeutic settings has generated a revolution in pharmacological intervention and vast amounts of information in mechanisms behind the efficacy of their use. Today there are approximately two dozen monoclonal antibodies (mAbs) approved by the FDA for a range of clinical applications in offsetting organ transplant rejection, as well as treatment of leukemia and lymphoma, solid tumors, metastatic cancers, autoimmune diseases and other conditions. The targets for these

antibodies are typically overexpressed cell surface antigens and receptors, and in certain cases, the antibodies can stimulate surface clearance or block ligand binding and signal transduction. A new generation of engineered antibodies focused on the targeted delivery of radio/cyto -toxic therapeutic payloads are in development, as are modulations of the inherent capacity to recruit effectors of the immune system, primarily through recombination of the Fc domain and manipulating its interaction with Fcγ receptors to promote stable association (Beckman 2007, Chames 2009, Lambert 2014).

While some success exemplified by Rituximab in the treatment of non-Hodgkin's lymphoma has been enjoyed, functional limitations and setbacks remain to be overcome. Delivery through a non-conducive, low-flow, and highly viscous tumor vasculature would seem to be the major obstacle, as most of the administered mAbs remain in the blood, limiting 20% of the dose to interact with the tumor in murine xenografts (Chames 2009). This challenge has on its own slowed the availability of mAbs for the treatment of solid tumors. Several antibodies have been approved by the FDA for clinical intervention against EGFR (and its most frequently overexpressed oncogenic dimerization partner ERBB2) for metastatic breast, colon and other cancers (Roskoski 2014). Cetuximab, Herceptin and Bevacizumab were the first to gain FDA approval and are long-standing models for RTK targeting in therapeutic settings. Cetuximab and Herceptin both work by targeting receptors, while Bevacizumab is directed against the VEGF ligand.

Cetuximab is a human-murine chimeric mAb currently used in the clinic to treat head and neck small cell carcinoma and colorectal cancer. It binds to the extracellular domain of EGFR. This interaction partially blocks ligand binding and sterically hinders dimerization with other HER family members. This combined action blocks phosphorylation, promotes internalization, and reduces proliferation in cancer cell line models (Sunada 1986, Goldstein 1995). Potentially contributing to its efficacy, Cetuximab recruits immune cells and induces cytotoxicity in lung cancer and other tumor cell lines *in vitro* (Kimura 2007, Kurai 2007). From a trafficking

standpoint, most receptor-antibody complexes are recycled back to the cell surface, but provoke a certain amount of degradation (Jaramillo 2006). Using the triple-negative breast cancer cell line MDA-MB-468, it was determined that Cetuximab induces some translocation of EGFR into the ER and nucleus. An interaction between EGFR and the Sec61β translocon was identified as facilitating the transport of mature transmembrane receptors between the ER and cytoplasm. Cetuximab-induced nuclear localization was counterintuitively enhanced by combinatorial treatment with AG1478, an inhibitor of EGFR tyrosine kinase activity, and in the *absence* of EGF (Liao 2009, Fig6a). The reasons for this are not immediately clear although the IC50 for AG1478 was >1 μM for 101 out of the 110 kinases tested in an *in vitro* assay, and 3nM for EGFR (Norris 2010). A similar effect of nuclear translocation was seen using the EGFR monoclonal antibody 528, which also targets the extracellular domain, blocks EGF binding to the receptor and inhibits EGF-induced proliferation (Sato 1983, Gill 1984).

Trastuzumab/Herceptin targets the extracellular domain of the EGFR dimerization partner and HER family member ERBB2. The mechanism of action for Trastuzumab is incompletely understood and there are conflicting reports on whether Trastuzumab treatment results in downregulation, recycles concurrently with the receptor or has any effect on receptor internalization (Roepstorff 2008). Cell cycle arrest is observed in some of the cell lines tested (Roskoski 2014, Austin 2004). ERBB2 has no ligand binding activity itself, is preferentially recycled, and exhibits a protective effect on EGFR either by promoting its recycling or making EGFR receptor dimers resistant to internalization, although this is not clear (Hendriks 2003, Hommelgaard 2004). An antibody directed against ERBB2 did have the effect of tumor regression, decreased EGFR tyrosine phosphorylation, and increased ubiquitination and degradation in EGFR-dependent colon cancer xenografts and cell line models (Kuwada 2004).

3. The EHD family of Endocytic Recycling Regulators

3.1 Overview

The EH domain was identified in the EGFR pathway substrate 15 (EPS15) protein as

possessing a shared homology widely conserved in eukaryotic evolution (Wong 1995). The EH domain is typically 70-100 amino acids in length and comprised of two helix-loop-helix 'EF hands' connected by an antiparallel βsheet. This motif forms a conserved binding pocket for the tripeptide asparagine-proline-phenylalanine (NPF). Many EH-domain containing proteins are shown to interact with key components of endocytosis including dynamin, the clathrin adapter AP-2, actin and ubiquitin. (Figure 1.5) Thus, a network of EH proteins are thought to act as molecular scaffolds involved in protein sorting and transport (Miliaras 2004). Eps15 is known for localizing to clathrin-associated endocytic pits after RTK stimulation but is not seen at early endosomes. Recent data indicates it has a function in regulating formation of the clathrin coat and through its ubiquitin-interacting motif, has a role in regulating internalization of surface proteins, among them: Connexin43, AMPA-type glutamate receptors and EGFR (Girão 2009, Lin 2014, Carbone 1997). EPS15 was found to be phosphorylated after stimulating cells with EGF or TGF-α, but not after PDGF or insulin, although in each case it redistributed to clathrin pits following stimulation. Its overexpression was shown to be sufficient to transform NIH 3T3 fibroblasts further suggesting a role in mediating signal transduction (van Delft 1997, Fazioli 1993). Roles in clathrin-independent internalization and the recycling of EGFR have also been identified (Sigismund 2005, Chi 2011).

The EHD proteins, are a conserved family of four highly homologous members, characterized as sharing an EH domain at their C-termini in contrast to the N-terminal domain organization of typical EH domain proteins (EPS15, Intersectin, Reps1 and 2, and others) (Miliaras 2004). Initial studies indicated EHD1 mediates the recycling of transferrin in mammalian cells and it shares nearly 70% identity with its lone *Ceanorhabditis elegans* ortholog Receptor-Mediated Endocytosis (RME-1) which presented yolk protein uptake defects into oocytes from endocytic vesicles upon knockdown or in isolated mutants, suggesting roles in regulating membrane trafficking (Grant 2001, Lin 2001). The four mammalian EHD family members share 67% or more homology with each other and the domain arrangement features an

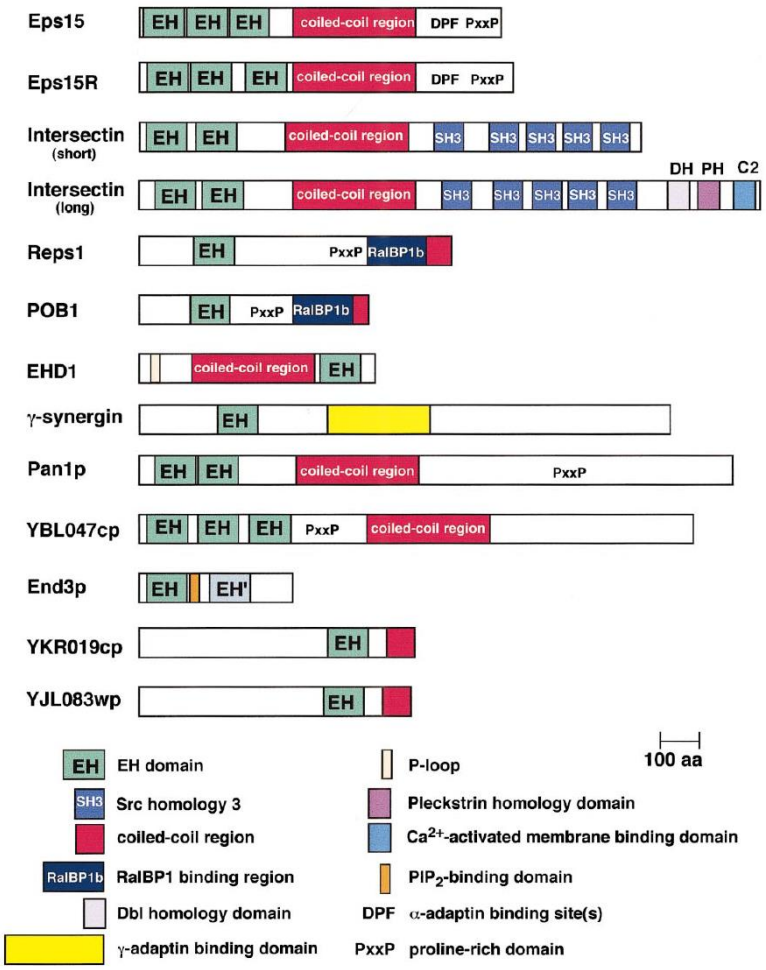


Figure 1.5 The architecture of EH domain-containing proteins: The organization of associated domains within the EH protein domain network. Modified and used with permission from (Santolini 1999).

N-terminal helical region and nucleotide binding G-domain, followed by a linker, a lipid binding coiled-coil and the C-terminal EH domain. Each EHD family member has a single module of proline-phenylalanine preceded by a variant residue (X-P-F) situated within the G-domain, with the exception of EHD2 which has two. Mutagenesis studies have indicated a critical role for this motif in regulating the hetero-dimerization, protein-protein interactions and intracellular localization of the various EHDs (Naslavsky 2011, Bahl 2015). The EHD G-domain bears a structural resemblance to the GTPase dynamin but exhibits a preference for ATP binding. It has been shown that the intrinsic ATPase activity allows EHD1 to function in a manner similar to dynamin and coordinates dynamin-dependent budding of synaptic vesicles in cooperation with amphiphysin (Daumke 2007, Jakobsson2011).

3.2 Protein-Protein Interactions

Currently of the known EHD protein interacting partners roughly half of those have been characterized as binding the EH domain via NPF motifs. Most of these partners are implicated in membrane traffic in some way and link EHDs to effectors of the cytoskeleton such as Syndapin I and II (Braun 2005), vesicle membrane targeting and fusion events exemplified by the SNARE Snap-29 (Xu 2004), effectors of Rab GTPases: Rabenosyn-5 and the Rab coupling protein RCP (Naslavsky 2004, 2006) or scaffolding hubs like MICAL-L1 (Sharma 2009). Many of these proteins bind to more than one EHD family member, as EH domains share homology on a scale comparable to that of the full length proteins and ranges between 50-80%. There are several interactions that are mediated outside of the NPF motif and most of these remain uncharacterized. Associations between clathrin heavy chain, the adapter AP-2 and the CALM protein are identified but the interacting domain has not been elucidated.

As EHD family members are shown to have both overlapping and distinct functions, a key question arises over how NPF binding specificity is achieved among the individual C-terminal EHDs and between the EH domain of other EH network members. The three-dimensional structure of the EH domain has been solved for several proteins and these have been

found to be very similar with some noticeable differences. A highly positive charged surface present in the EHDs is diametrically opposite that found in the N-terminal members EPS15 and Intersectin whose surface charge is negative (Kieken 2007). The contribution of surface charge to partner binding was in part elucidated upon examination of the residues flanking the NPF where it was found the majority of EHD interaction partners have negatively charged residues of glutamic or aspartic acid following the phenylalanine (Henry 2010).

3.3 Cell Biological Roles

Individual EHDs localize to tubular-membranes and vesicular compartments. For EHD1 this tubular distribution requires the EH domain for mediating an interaction with phosphotidylinositols (Naslavsky 2007). EHDs are demonstrated as critical regulators of endocytic recycling for a number of surface proteins. The best characterized member is EHD1, which has frequently been shown to mediate transport from the ERC back to the plasma membrane for the transferrin receptor, IGF-1 receptor, and integrins, with knockdown typically causing phenotypes of intracellular accumulation of cargo (Rotem-Yehudar 2001, Jović 2007). A role for EHD1 in mediating endosomal transit from early endosomes to the ERC has been suggested based on findings of impaired recruitment to enlarged early endosomes upon ablation of Connecdenn, a Rab35 guanine exchange factor whose knockdown interfered in the normal recycling of MHCI (Allaire 2010), and II (Walseng, 2008). Finally, similarity in phenotypic defects observed in *Caenorhabditis elegans* for yolk protein uptake in mutants of an orthologous DENN domain-containing regulator of Rab35 closely resembles loss of RME-1 (Sato 2008).

A role for EHD members has also been described in retrograde traffic. EHD1 was shown to colocalize with vacuolar protein sorting (Vps) 26 and 35, members of the retromer cargo recognition sub-complex, part of a critical evolutionarily conserved hub for sorting molecules bound for transit back to the Golgi. The retromer sorting nexin complex forms SNX-1 positive tubules during the early endosome to late endosome transition, an activity required for the diversion of substrate fate away from lysosomal degradation and into recycling (Mari 2008).

EHD1 is required for the cation-independent mannose-6-phosphate receptor trafficking from the recycling endosome to the Trans Golgi Network through an interaction required for the stabilization of SNX1-positive tubule formation (Gokool 2007, Zhang 2012). A recent report from a collaborative group saw no such requirement using a chimeric CI-M6PR featuring the ectodomain of CD8 and the cytoplasmic tail of the CI-M6PR (McKenzie 2012). Like the other EHD family members, EHD2 has a role in regulating the surface presentation of proteins on the plasma membrane. Similar to EHD1, EDH2 interacts with clathrin adapter proteins. In cultured adipocytes EHD2 couples endocytosis to the actin cytoskeleton through binding the NPF motif of EHD2-binding protein 1 (EHBP1), which contains an actin-binding calponin homology domain. This interaction is required for endocytosis of transferrin and GLUT4 into EEA1-positive endosomes. Further insulin treatment significantly increased the co-immunoprecipitation of EHD2 with GLUT4 suggesting a role in the mobilization of glucose transporters to the plasma membrane (Guilherme 2004, Park 2004). EHD1 was shown to interact with IGF1 receptor and colocalized with activated IGF1R in endocytic vesicles (Rotem-Yehudar 2001).

EPS15 and CALM both undergo nuclear-cytoplasmic shuttling and all four EHD family members were identified as containing a bipartite nuclear localization signal (K/RK/R---X_{10}---K/RK/RK/R). Inhibition of nuclear exit with leptomycin B was only successful at demonstrating accumulation of EHD2, where it displayed activity as a co-repressor of the p21WAF1/Cip1 gene. EHD2 was shown to co-immunoprecipitate with Sumo1 and mutagenesis of a sumoylation consensus site (also present in all EHD members) caused nuclear accumulation. A putative nuclear export signal present in all EHD members was seen as a partial contributor (Pekar 2012).

EHD3 has been shown by the same group to undergo sumoylation and this modification is required for its localization to tubular membranes but not for its dimerization, and a sumoylation defective mutant delayed transferrin recycling from the ERC (Cabasso 2015). Interestingly EHD3 is the closest EHD1 homolog and binds similar effectors Rabenosyn-5, RCP and MICAL-L1 through their NPF motifs, however, EHD3 had not previously been implicated as

having a role in the regulating the exit of recycling cargo back to the plasma membrane. EHD3 knockdown instead displayed an impaired exit of transferrin from early endosomes, and caused a redistribution of both Rab11 and RCP from the interior to the cell periphery (Naslavsky 2006).

EHD3 was demonstrated as necessary for transport of cargo to the Golgi, and required for the maintenance and function of this organelle. The retrograde trafficking of mannose-6-phosphate receptor is essential for the export of lysosomal hydrolases. Knockdown of EHD3 or its effector Rabenosyn-5 caused mannose 6-phosphate receptor to remain in peripheral endosomes, resulting in accumulation of lysosomal hydrolase Cathepsin D. An impairment of endosome-to-Golgi movement was also seen for ShigaToxin B, SNX1, and AP-1 gamma adaptin (a mediator of mannose 6-phosphate receptor retrieval). Overall the disruption of return traffic caused Golgi morphology to appear dispersed and fragmented; however, no defects in secretion were detected upon transfection of VSV-G-GFP (Naslavsky 2009).

EHD4, like the other EHDs, requires its EH domain for localization to the tubular endosomal system. EHD4 colocalizes with markers for the early endosome, sorting nexins and newly internalized transferrin. Its knockdown caused the formation of enlarged early endosomes and prevented the transport of MHCI to the perinuclear ERC and prevented exongenous low-density-lipoprotein from reaching the lysosomal compartment. An overexpressed Rab5-binding domain from Rabaptin-5, which binds preferentially to the active conformation of GTP–Rab5, demonstrated the enlarged endosomal structures are enriched for GTP-loaded activated Rab5. This may suggest a defect in the ability to recruit the next sequential component required for GTP hydrolysis and vesicle budding or transit out of the early endosome (Sharma 2008) (Figure 1.6).

The nerve growth factor NGF binds its RTK TrkA at the nerve terminal and is unique in the sense that it avoids the typical itineraries of recycling and degradation and instead undergoes long-distance retrograde transport to the nerve cell body, and the signaling events that occur *en route* are necessary for the growth and survival of the cell. It was immediately suggested signaling events generated intracellularly within endosomes have the capacity to be qualitatively

different than those signals generated at the plasma membrane (Watson 2001).

Indeed, in the PC12 cell model, NGF acting through TrkA effectors, including Ras, Rap, and the Erk/MAP kinases mediate sustained signaling events, while the EGFR acting through the same MapK pathway generates only transient signals and may explain the inability of EGFR to mediate differentiation of neuronal phenotype and survival (Valdez 2007). The Trk-signaling endosome is formed by concentrating the receptor within membrane ruffles, a process termed 'macroendocytosis', and mediated by the Rho-GTPase, Rac, as well as EHD4 (referred to in the neuronal system literature as Pincher) (Shao2002). EHD4 is shown to be essential for the retrograde traffic of activated TrkA, and is a transcriptional target of neurotrophin signaling through this receptor (Valdez 2005). In constrast to Trk, the EGFR is not internalized by the same EHD4-dependent mechanism in this system. Further, TrkA endosomes were seen to remain Rab5 positive for longer whereas EGFR endosomes rapidly exchange Rab5 for Rab7, thereby moving into late-endosomes/lysosomes for degradation (Phillipidue 2011). Therefore, one mechanistic determinant of EHD4-mediated retrograde traffic is the ability to maintain 'immature' early endosomal character whereby the transition to Rab7 positive late endosomes/early lysosomes is prevented. This is consistent with data from Sharma et al. where EHD4 knockdown was seen to result in enlarged early endosomes and indicates EHD4 may be required to chaperone early endosomal cargos in the transition towards divergent character.

3.4 *In Vivo* Roles

Ehd1-null mice are born at sub-Mendelian ratios exhibiting embryonic lethality, in part due to defects in the closure of the neural tube (Band lab, manuscript in process). Surviving pups are smaller in size through adulthood, infertile, and display a range of spermatogenic defects resulting from disruption of spermatogenesis with abnormal acrosomal development with clumping of acrosomes, and round, misaligned spermatids missing normal elongation (Rainey 2010). Over half of surviving Ehd1-null mice display ocular abnormalities, including anophthalmia, aphakia, microphthalmia and congenital cataracts. These phenotypes appear to be

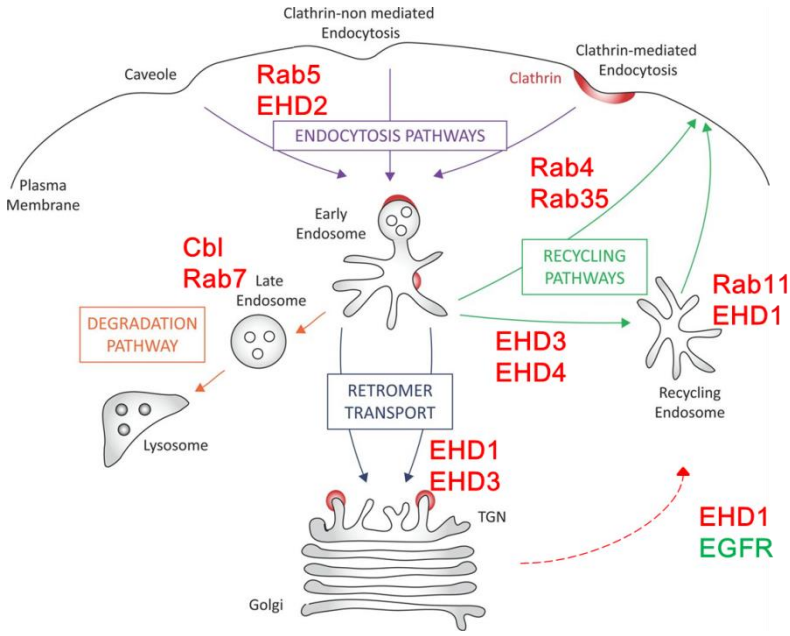


Figure 1.6 Proposed roles for EHD proteins in the regulation of endocytic transport. Modified and used with permission from (Hunt 2011).

the result of reduced lens epithelial proliferation, survival, defects in corneal endothelial differentiation and impaired maintenance of cell junctions (Arya 2015) (Figure 1.7).

At the time of writing an EHD2 knockout mouse has only recently been developed and is currently undergoing characterization in the laboratory. A previous report has indicated expression in the mouse brain, heart, lungs, mammary gland and spleen (George 2007). Future studies will focus on the developmental role of EHD2 and its characterization in these organs.

EHD3 is expressed in cardiac muscle and participates in the trafficking of the sodium-calcium ion exchanger (NCX1) to the cell membrane, and thus it has a role in regulating electrical potentiation of the heart. Both EHD3 and NCX1 have elevated expression levels in models of heart failure (Gudmundsson 2012, Curran 2014). EHD3 is also predominantly expressed in the glomerular endothelial cells of the kidney but its deletion had no obvious effect on function. Interestingly EHD3-knock out cells see an increase of the expression of EHD4, and a double EHD3/4 knock out results in death between 3-24 weeks of age. These mice display small pale kidneys, proteinuria, thrombotic lesions, thickening and duplication of glomerular basement membrane, endothelial swelling and loss of fenestrations (George 2011). Knockouts of EHD4 display a 50% reduced testis size and feature increased germ cell proliferation and apoptosis. A reduction in seminiferous tubule diameter, and dysregulation of seminiferous epithelium was observed. Like EHD1, EHD4-knockout mice display similar defects in elongated spermatids and reduced fertility. EHD4 deletion altered the expression profile of other 3 EHD members indicating a role for regulatory balance or functional redundancy (George 2010).

4. RUSC2

4.1 Overview

RUSC2 (Iporin) was identified by yeast two-hybrid screening using the constitutively active mutant of Rab1b (Rab1B Q67R) as bait (Bayer 2005). Previously known binding partners of Rab1 include GM130, Golgin-84, MICAL and p115. These components form various complexes responsible for directing vesicular traffic between the ER and the Golgi apparatus

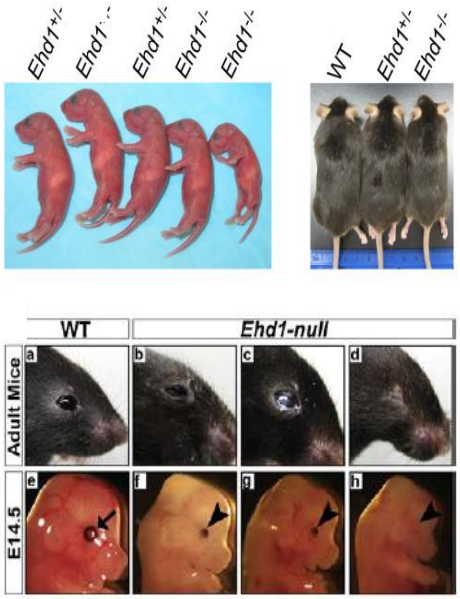


Figure 1.7 *In vivo* Roles of EHD1: Top - Newborn pups and seven month old male mice were photographed to show the size of the Ehd1-/- mice as compared to littermate controls. Bottom - Defective ocular development in Ehd1-null mice. Anatomical features of eye structures of Ehd1-null adult mice (b,c,d) and E14.5embryos (f,g, h) were compared to

(Tisdale 1992, Nuoffer, 1994). Rab1 recruits p115 to a cis-SNARE complex required for coat protein (COP) II vesicles to bud from the ER and fuse with Golgi-associated membranes in anterograde transport. Additionally, p115 coordinates the sequential tethering and docking of COP I vesicles which mediate cargo through the Golgi stack and play a role in retrograde transport back to the ER for components to be recycled. Members of the MICAL family all contain a LIM (Lin-1/Isl-1/Mec-3) domain of lesser known function and a calponin homology domain that mediates interactions with actin-binding proteins, thus linking Rab1 functions in the Golgi to transport along the cytoskeleton (Weide 2003, Fischer 2005).

RUSC (RUN and SH3 domain containing) 1 and 2 are ubiquitously expressed and well conserved. RUSC1 isoform 1(1039 aa), also called New Molecule Containing SH3 at Carboxy-terminus (NESCA) and RUSC2 (1516 aa) share 30.5% total amino-acid identity and a common domain structure with a leucine zipper between the RUN and SH3 domains. Notably, RUSC2 contains an NPF motif in its N-terminal at position 43 (NPFCPPELG) and a second NPF at position 101 (NPFLLQEGV). Both sites contain neutral residues in the +1, 2 and 3 positions followed by a negatively charged glutamic acid typical for known interaction partners of EHD family members that demonstrate binding affinity with EH-domains (Henry, 2010).

NESCA is located at chromosome 1q22 and is primarily expressed in the brain where it translocates from the cytoplasm to the nuclear membrane in response to stimulation by NGF. Its overexpression was shown to enhance neurotrophin-dependent neurite outgrowth. Knockdown significantly reduced NGF-dependent neuritogenesis. The RUN domain was determined to be required for nuclear translocation and its redistribution to be essential to promoting neurite outgrowth (MacDonald 2004).

RUSC2 is located within chromosome 9p13 (a hotspot for genomic abnormalities associated with various cancers), where sequencing revealed novel non-recurrent mutations in RUSC2 for 2 out of 35 surveyed patients with acute lymphoblastic leukemia, located within a span of ≈ 1000 base pairs (Sarhadi 2014). Its highest expression in descending order is in the

testis, brain, heart and lung. Biochemical fractionation reveals its intracellular presence to be located in the membrane fraction, with a cytosolic distribution and perinuclear concentration visualized by immunofluorescence (Bayer 2005).

4.2 Structure

The RPIP8/UNC-14/NESCA (RUN) module is found in many proteins known to interact with Rabs, and RUN domains are often in tandem with known Rab-interacting motifs such as the Rab-GTPase TBC, the phospho-inositide-3-phosphate binding FYVE, and DENN which possesses guanine exchange factor activity (Fukuda 2011). RUSC2 is comprised of a RUN, Leucine Zipper, Proline Rich domain, WW, and SH3. The SH3 and WW domain both bind proline rich-motifs, although with different consensus sequences. The WW motif which also binds to phospho-serine and phospho-threonine residues may exhibit phosphorylation dependence for SH3 domains and is frequently associated with modules typical for proteins involved in signal transduction (Chen 1995, Macias 2002). Proline-rich domains may harbor sites of regulatory phosphorylations, or in the case of Vamp2 binds an adapter (PRA1) of prenylated-Rab substrates (Martincic 1997).

The crystal structure of the NESCA RUN domain (50% homologous to the RUN of RUSC2) has been recently solved and bears considerable resemblance to the RUN of RPIPx and Rab6IP1 in the arrangement of its nine helices. A directly opposite difference was noted in the electrostatic surface distribution at the presumed GTPase interacting surface of NESCA from those of RPIPx and Rab6IP1, which are positively charged in the middle and negative on either side (Sun, 2012). Surface plasma resonance and co-immunoprecipitation experiments demonstrate the NESCA RUN binds to both TrkA and H-Ras, perhaps controversially as another group was previously unable to demonstrate the interaction with TrkA (MacDonald 2004). A crystal structure of the H-Ras-NESCA complex was not generated, but available data were algorithmically compared to the solved Rab6-Rab6IP1 structure. It was concluded the mode of interaction is likely different, resolving the actual structure might elucidate those differences.

4.3 Protein Interactions

In whole mouse brain lysates, NESCA was shown to co-immunoprecipitate with the motor protein KIF5B, its associated light chain KLC, and syntaxin-1 which is part of the membrane recruitment machinery for SNARE-mediated vesicle fusion (MacDonald 2012). Through the RUN domain, NESCA interacts with NEMO and the E3 ubiquitin ligase TRAF6, linking NGF signaling and the IKK regulatory complex of NFKB (Napolitano 2009). Other than H-Ras, no interaction with NESCA and small G-proteins have yet been successfully demonstrated (Bayer 2005, Fukuda 2011).

RUN proteins include RIPX, RUFY1, RUFY2, RUTBC1, RUTBC2, RUTBC3, RUB6IP1, and PLEKHM1 (Katoh 2004). A systematic investigation of 19 RUN domains for their ability to bind 60 different GTP-bound Rabs showed most of them did not exhibit any binding activity. However six of them; DENND5A/B, PLEKHM2, RUFY2/3, and RUSC2 interacted with hierarchical binding specificity for individual Rabs with RUSC2 exhibiting a preference for Rab35 > Rab1A > Rab1B, and Rab41 (Fukuda 2011). At the time of this writing the only other validated interaction partner is GM130, which in conjunction with its relationship with Rab1 and Rab41, strengthens a probable role in ER/Golgi transport.

Of direct interest, a split ubiquitin-based Membrane Yeast Two-Hybrid (MYTH) system and bioinformatics approach identified 87 candidate proteins potentially interacting with the ligand-unoccupied EGFR (Figure1.8). RUSC2 was identified in that screen, along with HDAC6, which the authors went on to validate. No direct links to RUSC2 function can be noted as an outcome of that screen; however, Arf6, which is frequently associated with endocytic recycling was identified, as was SNAP25, a SNARE complex protein which competes against EHD1 for binding to Snapin in PC-12 cells where EHD1 is proposed to act as a negative regulator of exocytosis (Deribe 2009, Wei 2010). Rab3a was also identified which has a role in regulation of secretory vesicles. A known Rab 3a effector is SNAP29 which interacts with EHD1 to affect clathrin-mediated endocytosis of the IGF1 receptor (Rotem-Yehudar 2001). Rabs depend on the

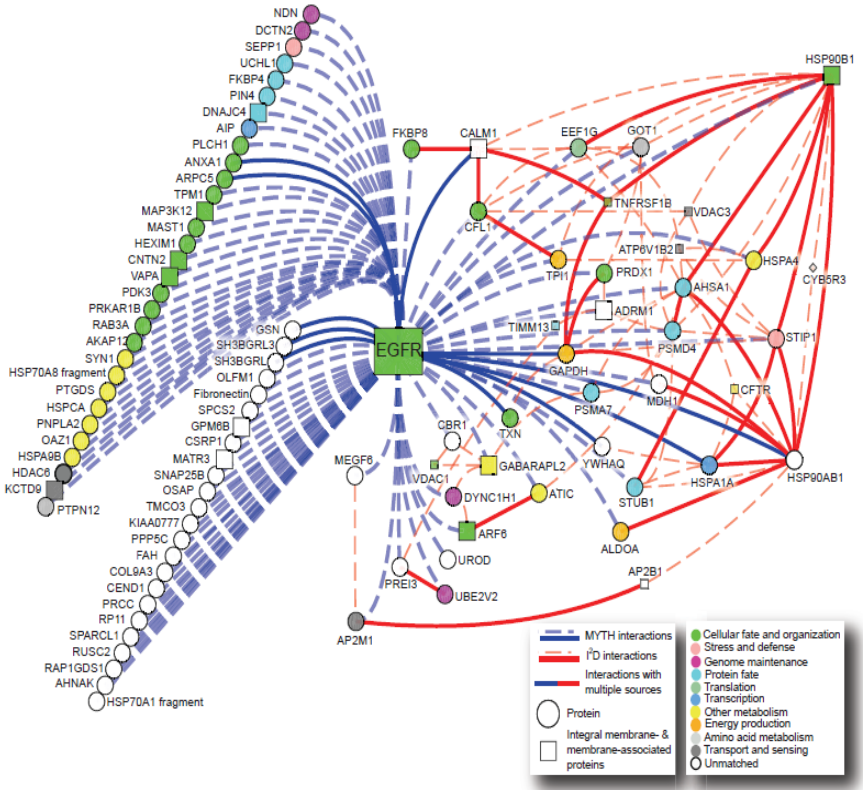


Figure 1.8 Applied Modified MYTH to the Human EGFR: An EGFR interactome was identified with a modified MYTH system. Eighty-seven EGFR interactions identified for the un-liganded condition. Modified and used with permission from (Deribe 2009).

actions of GEFs and GAPs to cycle between active GTP-bound and inactive GDP-bound states. Activated Rabs recruit effectors that control endosomal trafficking. Multiple Rabs can coexist on a single endosome, increasing the complexity of sorting decisions. Characterizing Rab effectors and their various roles is therefore quite valuable in assessing their function (Figure 1.9).

4.4 Functional Roles

Rab1a and b have both been localized to the ER and Golgi, where they interact and form complexes with p115/, GM130, giantin, golgin-84, GCC185, MICAL-1, MICAL C-terminal like, JRAB/MICAL-L2, OCRL1, INPP5B, Cog6, GBF1 and others (Hutagalung 2011). One elucidated role describes Rab1 recruitment by p115 to COPII vesicles during budding from the ER to mediate SNARE attachment to the Golgi (Allan 2000). Rab1a was also shown to regulate the sorting of early endocytic vesicles, where it is required for microtubule-based motility (Mukhopadhyay 2014). Under normal conditions, EGF accompanies its receptor to lysosomes, transferrin is recycled with its receptor back to the cell surface, and asialoorosomucoid (ASOR) dissociates from asialoglycoprotein receptor (ASGPR), which promotes degradation of the ligand, and recycling of the receptor. Significant changes were induced upon Rab1a knockdown: EGF accumulated in aggregates, failing to reach lysosomal compartments. Transferrin accumulated in a Rab11-mediated slow-recycling compartment in contrast to control cells, which instead sorted Transferrin into a fast-recycling Rab4 compartment. ASOR failed to segregate from its receptor and did not reach lysosomes for degradation. These results are in part attributed to the defective recruitment of the minus-end-directed kinesin KifC1.

An RNAi functional screen was published which identified RUSC2 and Rab1B as potential HIV-1 dependence genes but these have not been directly validated (Brass 2008). However, overexpression of a Rab1 GAP was shown to disrupt trafficking of HIV-1 envelope protein gp41 within the secretory pathway. Perturbed transit to virion assembly sites reduced gp41 association with detergent-resistant membranes, mitigating infectivity of HIV-1 virion-like particles (Nichmias 2012). Roles for Rab35 have been identified in several places

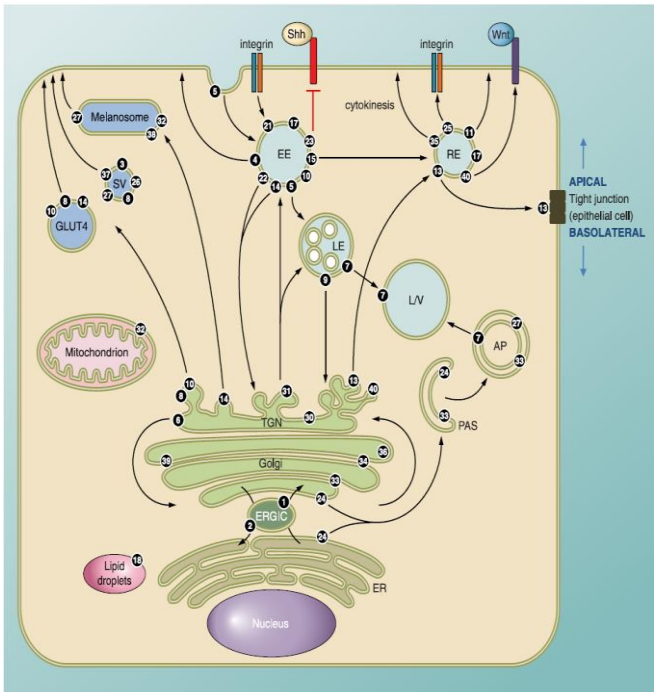


Figure 1.9 The Intracellular Distribution of Rabs: A typical cell showing the intracellular localization and associated vesicle transport pathway(s) of several Rab GTPases. Rab1 regulates ER-Golgi traffic while Rab2 is involved in recycling, or retrograde traffic, from Golgi and the ERGIC back to the ER. Rab6 regulates intra-Golgi traffic. Several Rabs including Rab8, -10, and -14 regulate biosynthetic traffic from the *trans*-Golgi network (TGN) to the plasma membrane.Used with permission from (Hutagalung 2011).

amid endocytotic routes including fast recycling, exosome fusion and cytokinesis, and its function is critical to neurite differentiation and cell shape (Chevallier 2009). NESCA and RUSC2 have both been implicated in the regulation of neurite outgrowth and differentiation (Chaineau 2013). The minimal functional binding site of the RUSC2 Rab35 binding domain has been identified as amino acids 982-1199 and is currently in use as a tool to trap activated GTP-Rab35. Its overexpression was shown to inhibit Rab35-induced neurite outgrowth in a dominant-negative manner (Fukuda 2011). NESCA appears to have a role in neuronal differentiation by regulating dendritic spine dynamics as an adaptor protein for SNARE mediated vesicle fusion events (MacDonald 2012), and as a mediator for the recruitment or dynamics of the IKK complex in response to NGF stimulation, although the significance of that event is not yet established (Napolitano 2009).

Chapter 2
Materials and Methods

5. Materials and Methods

5.1 Generation of Doxycycline-Inducible EHD1-GFP Overexpressing Cell Lines

EHD1 overexpression and empty vector control 16A5 cells were generated by cloning EHD1 into the pDest47-GFP vector and subcloned into the pRev-Tre vector from Clonetech Laboratories Inc. using Forward Primer: 5'GATCGATCaccggtTCACCATGTTCAGCTGGGTCA-3' and Reverse Primer: 5'-GATCGATCatcgaTCATTATTTGTAGAGCTCATCCATG-3'at the AgeI/ClaI restriction sites. To create an inducible EHD1 cell line, 16A5 cells were first transfected with plasmid pRevTet-On containing the Tet-responsive transcriptional activator using Lipofectamine 2000 (Life Technologies) and selected in medium containing 0.1 μg/ml G418. The resulting G418-resistant, Tet-on clones were maintained and used for transfection with the response plasmid containing the *EHD1* gene (pRevTRE-*EHD1-GFP*) and the empty vector (pRevTRE). Tet-on cells were transfected with pRevTRE-*EHD1-GFP* and the pRevTRE empty vector using Lipofectamine 2000. Transfected cells were selected in 0.05 μg/ml hygromycin. Resistant EHD1-GFP and vector only clones were treated with 1 μg/ml doxycycline for 48 h and screened for the presence of EHD-1 GFP protein by Western blot analysis.

5.2 Generation of Stable Doxycycline-Inducible Lentiviral shRNA-Expressing Cell Lines

Doxycycline-inducible shRNA mediated Control, EHD1 and RUSC2 knockdown cell lines were generated using lentiviral constructs obtained from GE Healthcare; Control shRNA (cat. #VSC6580), EHD1 shRNA sequence #1 TGTTTCCTCGCCTCTCGAA (Source Clone ID V2IHSPGG_388688), EHD1 shRNA sequence #2 AAGGTCCATAAAGACTGAG (Source Clone ID V2IHSPGG_908952). RUSC2 shRNA sequence #1 GCCTAGACCGAAGATCACA (Source Clone ID V2IHSPGG_463376). RUSC2 shRNA sequence #2 GCTCACCAGTCATACCATG (Source Clone ID V2IHSPGG_463384). Viral transduction was performed at an MOI of 1 in the presence of 14 μg/ml of polybrene in serum-free media for 24

hours before selection with 1ug/ml puromycin for 7 days.

5.3 Antibodies and Reagents

The following antibodies were obtained from commercial sources: anti-EGFR Mouse Monoclonal 528 (cat. # Grol1) from Calbiochem, Anti- EGFR rabbit polyclonal (cat. # SC-03) anti-HSC70 (B-6) (cat. #7298) from Santa Cruz Biotechnology, anti-EHD1 (cat. #ab109311), anti-LMAN1 (cat. #125006), anti-Giantin (cat. # 37266) anti-Arf6 (cat. # 131261) from Abcam, anti-ß-Actin (cat. #A5316) from Sigma-Aldrich, anti-Turbo-GFP (cat. #PA5-22688) and anti-Calnexin (cat. #MA3-027) from Thermo Scientific, P-Erk (cat. #9101), Total-Erk (cat. #9102), P-S6 (cat. #2211), anti-P-EGF Receptor (Tyr1068) (cat. #2234), Golgin-97 (cat.# 13192) and P-AKT (cat. #9271) were from Cell Signaling Technology. Anti-GM130 (cat. #610822) was from BD Biosciences. Anti-mouse IgG2a-APC conjugate was from Biolegend (cat.# 407109). Non-specific mouse IgG2a was from BD Biosystems (cat. # 34950). Preparation of in-house rabbit anti-peptide antisera recognizing EHD1 and EHD4 were described previously (George 2007). Rabbit rabbit anti-peptide antisera against RUSC2 were generated against peptide sequences :RQRSRSYDRSLQRSPPVRLGSLER, and AGPGSPPRRVTSFAELAKGRKK.

The following reagents were obtained from commercial sources: Fugene-6 transfection reagent from Roche Applied Science (Indianapolis, IN). siRNA, smartpools and Dharmafect I transfection reagent were from Dharmacon, a division of Thermo-Fisher (Pittsburg, PA). Individual custom siRNA sequences were as follows: EHD1 2006: 5'-GACAUUGGGCAUCUCUUUCUU-3' (Naslavsky 2006). EHD4, 5'-UGGAGGACGCCGACUUGAUU (George 2007). The 3' UTR targeting oligo and smartpool against EHD1were from Thermo-Fisher (cat. #D-019022-20) and (cat. #M-019022-02) respectively as was control siRNA #5 (cat.# D-00210-05-50). Transfections were performed in Opti-Mem.

Puromycin, hygromycin, G418, doxycycline, enzyme immunoassay-grade BSA, paraformaldehyde (PFA), Monensin, Propidium Iodide, DMSO, and TritonX-100 were from

Sigma-Aldrich (St. Louis, MO). EGF and Amphiregulin were obtained from Peprotech (Rocky Hill, NJ). Halt™ Protease and Phosphatase Inhibitor Cocktail and Trypsin/EDTA was from Life Technologies. Trypsin Inhibitor was from Gibco. Normal Goat Serum (cat. #005-000-121) was from Jackson ImmunoResearch Laboratories Vectashield mounting medium was purchased from Vector Laboratories (Burlingame,CA). Polybrene was obtained from American Bioanalytical. BD Calibrite Beads and Growth Factor Reduced Matrigel were obtained from BD Biosciences. Protein A and Protein G Sepharose beads were from GE Healthcare.

Plasmids: EHD1-DsRed, EHD1-myc and EH-domain truncated EHD1-myc constructs were generated in lab and described previously (George 2007). The Iporin-HA tagged construct was a gift from Dr. Angelika Barnekow (Bayer 2005).

Media and supplements were from Life Technologies. Fetal bovine serum and tetracycline-free fetal bovine serum, were from Hyclone.

5.4 Surface EGFR Quantification by Fluorescence-Activated Cell Sorter (FACS)Analysis

16A5 cells were seeded in 35mm plates at a density of 100,000 cells and grown in the presence of doxycycline for 72 hours. Cells were then either kept at steady state or starved for 24 hours and rinsed with ice-cold PBS and released with trypsin/EDTA (Life Technologies). Trypsinization was stopped by adding trypsin inhibitor (Gibco). Cell suspensions were centrifuged at 1000 rpm for 5 minutes and transferred to Falcon 5 ml round bottom polystyrene test tubes with cell strainer caps, and washed thrice in ice-cold FACS buffer (1% bovine serum albumin in PBS). Live cells were probed with anti-EGFR (528), and stained with goat-anti-mouse Alexa Fluor 488 (Life Technologies) for transient transfection experiments or anti-mouse IgG2a-APC conjugate for stable inducible cell lines expressing Turbo-GFP. Cell suspensions were run on a BD LSRII after standardizing with BD Calibrite Beads. Data was analyzed with FlowJo cytometry data analysis software.

5.5 Immunofluorescence and Confocal Microscopy

For confocal immunofluorescence microscopy, 16A5 cells were grown on coverslips, transfected or treated as indicated, and fixed with 4% paraformaldehyde in PBS for 10 minutes. Fixed cells were permeabilized in 0.05% Triton-X-100 for 10 minutes, blocked with 10% goat serum/PBS for 25 minutes and incubated with primary antibodies in 1% goat serum/PBS overnight. After two, 5-minute washes in PBS, the cells were incubated with the indicated fluorochrome-conjugated secondary antibody for 40 minutes at room temperature. All images were acquired using a Zeiss 710 Meta Confocal Laser Scanning microscope (Carl Zeiss) by using a 40 or 63× objective with a numerical aperture of 1.0 and appropriate filters. Merged fluorescence pictures were generated and analyzed using ZEN 2012 software from Carl Zeiss ®.

5.6 Cell Proliferation and Viability Assay

Cells were plated at a density of 1500 cells/well in Nunc™ 96-Well Optical-Bottom Plates (Thermo-Fischer Scienfic) in a volume of 100µl and grown +/- doxycycline for the indicated number of days, with a media change on the 3rd day. The Cell Titer Glo® reagent (Promega) was added (50 µl) and light units read on a FLUOstar Optima plate reader from BMG Labtech.

5.7 Matrigel Colony Formation Assay

Cells (2.0^10^3) in 0.5 mL of 2% Matrigel in DFCI-1medium were plated per well of an eight-well chamber slide on top of a polymerized layer of 100% Matrigel, as described (Debnath 2003). The cultures were fed every 2 days +/- doxycycline. Phase-contrast images were obtained under 40x magnification on an EVOS FL Auto Cell Imaging System from Invitrogen.

5.8 Cell Culture

16A5 is an HPV16 E6/E7–immortalized derivative of the reduction mammoplasty-derived 76N normal MEC line grown in DFCL medium containing 2 nM EGF. Cells were used between passages 2-5 and maintained in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. When 16A5 cells were maintained in starvation conditions and for stimulation, the cells were kept in D3 media without serum or EGF for the indicated intervals (Band 1989).

EHD1 floxed mouse embryonic fibroblast cell lines were generated from C57 backcrossed to the 5th generation with Black 6 and generated according to the NIH 3T3 protocol before *in vitro* treatment with Adeno-GFP or Adeno-Cre (University of Iowa Viral Vector Core Facility) for wildtype control or $Ehd1^{-/-}$ respectively, before sorting by FACS on a BD LSRII instrument (Rainey 2010). These cells, S2013, and MDA-MB-231were maintained in DMEM containing 8.6 % FBS with 2%, 17.2 mM HEPES, .80 μM non-essential amino acids, .86 mM sodium pyruvate, 50,000 units Penicillin/Streptomycin, 1.72 mM L-Glutamine, and 47 μM β-mercapto-ethanol.

5.9 Lysate Preparation and Western Blot

Following cell culture and treatments, cells were rinsed twice with ice-cold PBS and attached cells were lysed in ice-cold Triton X-100 lysis buffer (0.5% Triton X-100, 50 mM (pH 7.5), 150 mM sodium chloride from Fisher, containing Halt ® EDTA-free protease inhibitor cocktail (Thermo Scientific)) overnight. The lysates were transferred to Eppendorf tubes, spun at 13,000 rpm for 30 minutes at 4^{o} C, and supernatants were collected and quantified for protein using the Thermo-Scientific Pierce® BCA assay. 40 μg aliquots of protein lysates were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted.

5.10 ^{35}S Pulse-Chase Labeling

16A5 cells were plated in 10 cm dishes at a density of 600,000 cells and treated with doxycycline for 3 days. Cells were pulsed for 20 mins in methionine-cysteine free DMEM with 0.1μCi of ^{35}S methionine-cysteine. Cells were placed on ice, rinsed thrice with cold PBS and chased with DMEM containing a 10-fold excess of methionine-cysteine. Lysates were taken at the indicated intervals and rocked at 4°C overnight. Lysates were spun, and protein quantified and 1 mg of total protein was incubated overnight with anti-EGFR 528 (10 μg). On recovery preparations were incubated 40 minutes with Protein-A sepharose beads and rinsed 5 times with lysis buffer before treatment adding sample buffer containing 0.5μl of β-mercaptoethanol and

boiling 5 minutes at 90°C. Samples were resolved on a 6% sodium dodecyl sulfate-polyacrylamide gel, which was fixed for 10 minutes in a solution of 50% methanol, 10% acetic acid. Fixed gels were treated with Auto-Fluor and dried on a vacuum before placement in an autoradiography cassette to be visualized.

5.11 EGF Stimulation

At 48 hours prior to stimulation cells had been grown to 80% confluence and placed in serum-free media in 10 cm plates. Cells were washed with PBS before adding serum-free media along with 2 nM EGF and incubated at 37°C for the indicated time points. Cells were washed once with PBS, lysed and rocked overnight.

5.12 Statistical Analysis

Unpaired Student's t-test was used to analyze the significance of differences between experimental groups. Data are presented as +/- standard error of the mean with $p \leq .05$ deemed significant.

CHAPTER 3

Control of the Basal Recycling and Surface Expression of Epidermal Growth Factor Receptor by the Endocytic Recycling Regulator EHD1 Utilizing a Pathway Shared by RUSC2

6. Rationale for these studies

EHD family members have been shown as linked to a variety of proteins expressed on the cell surface relevant to human disease and potentiation of tumorigenicity. EHDs have direct roles in RTK signaling events, and have been verified to be transcriptionally induced in epithelial cells as a consequence of RTK stimulation, implicating them in downstream biological outputs (Amit 2007). Roles in nutrient uptake and migration have been revealed through EHD action within the IGF1R and integrin pathways. Still, novel avenues with implications for human health remain unexplored – along this theme efflux pumps such as the ATP-binding cassette (ABC) transporters would be interesting candidates for their ability to confer multi-drug chemoresistance in prostate, lung and breast cancer (Holohan, 2013).

The ability to unbalance the endocytic recycling and eventual fate of surface proteins by promoting their shift into lysosomal degradation is an attractive hypothesis worthy of study with proof of principle well described in the literature for the activity of monoclonal antibodies and modifiers of ubiquitination (Jannot 1996, Wheeler 2008, Mizuno 2005). The Rab family of GTPases and their effectors act as molecular switches between endosomal compartments, understanding how Rabs control this sequential transition would no doubt present several strategies to explore for intervention. For instance, the adenoviral RIDα protein is capable of inducing surface downregulation of TNF receptor 1, TNF-related apoptosis-inducing ligand receptor 1, Fas and EGFR, all without the presence of ligand, in order to facilitate acute viral infectivity through prevention of apoptotic and inflammatory responses. RIDα interacts with two Rab7 effectors, Rab7-interacting lysosomal protein (RILP), and oxysterol-binding protein–related protein 1L (ORP1L) and itself seems to promote late endocytic traffic as a functional analogue of GTP-Rab7 by recruiting these components to compartments that would ordinarily be perceived as early endosomes (Shah, 2007). Rab31 overexpression has a similar effect and interacts with EGFR in a GTP-dependent manner, this association is dependent upon its effector early endosome antigen 1 (EEA1) as well as GAPex-5, a Rab GEF that modulates EGFR

ubiquitination, trafficking and degradation (Chua, 2014).

MICAL-L1 was described as a molecular scaffold and Rab GTPase effector required for the recruitment of EHD1 to tubular membranes by facilitating an EHD1 interaction with activated GTP-Rab8a (Sharma, 2009). A physical interaction was demonstrated by immunoprecipitation and GST pulldown of a GST-EH domain construct incubated with MICAL-L1 overexpressing Hela cell lysate. This interaction was dependent upon the presence of GTPγs, a non-hydrolyzable GTP analog, which indicates a dependence on the GTP-activated conformation of an enabling Rab protein. This study concluded by showing the recycling of transferrin is adversely affected in MICAL-L1 knockdown conditions.

MICAL-L1 and its isoforms are capable of binding multiple Rabs, including Rab35, which was shown as required for EHD1 recruitment and recycling of MHCI and II (Allaire, 2010). Another group showed Rab35 forms a tripartite complex with MICAL-L1 and centaurin-β2 and then sequentially recruits Rab8, Rab13, and Rab36 to perinuclear recycling endosomes in response to NGF stimulation. Functioning as scaffolds, MICAL-L1 and centaurin-β2 (an Arf6 GTPase activating protein) were shown to recruit EHD1 to positively regulate neurite outgrowth, possibly by delivering nutrients or membrane to the growing neurite tips (Kobayashi, 2013, 2014).

The cellular distribution of MICAL-L1 has been examined in MDCK cells and was found to be distributed in vesicular compartments throughout the cytoplasm. Compartmental analysis revealed it colocalized significantly with Rab7, a marker for the late endosome. Markers for early endosomes and internalization were seen as weak (Rab4, clathrin, and Eps15), or absent for EEA1. No association was observed for recycling endosomes using transferrin and Rab11, or furin (a marker for the Trans-Golgi Network) (Abou-Zeid 2011).

Given its observed localization, this study examined the effect of MICAL-L1 depletion on ligand-activated trafficking and degradation of EGFR. Treatment with a high dose of EGF (100 ng/ml) did not result in a significant difference in the ubiquitination status in knockdown

versus control. Degradation was significantly affected, with the cells markedly displaying less total EGFR 3 hours post stimulation. Notably, the initial total level of EGFR at time zero, representing a 4 hour serum deprivation in the presence of cycloheximide, was also decreased in the stably transfected shRNA-expressing cells directed against MICAL-L1 (Abou-Zeid 2011 Figure 6B), consistent with a role in regulating EGFR total levels at steady state. Immunofluorescence revealed the receptor was localized within perinuclear vesicles in control cells but were found throughout the cytoplasm in MICAL-L1-depleted cells, while MICAL-L1 overexpression caused accumulation of EGFR in the late endosomal compartment. Neither phosphorylation status nor downstream signaling to Akt or Erk was examined. Recent work demonstrated that depleting either MICAL-L1 or EHD1 impaired signals mediated downstream of EGFR through Src by restricting Src's exit from a perinuclear compartment (Reinecke 2014). It is interesting, however, that the serum-starved, pre-stimulated condition consistently exhibited a level of activated Src that was increased in the knockdown condition. Biochemical stimulation-based experiments with EGF were restricted to two time-points, 5 and 15 minutes, and displayed a decrease in Src P-Y 416. No change was seen in the amount of EGFR expressed on the cell surface in these author's study.

Based on emerging paradigms and parallels with other RTK systems, we hypothesized that EHDs play an essential role in endocytic recycling of EGFR both before and during ligand stimulation, and that EHD-dependent recycling provides an essential counterbalance to mechanisms that help sort EGFR into lysosomal degradation.

7. Results I. Biochemical and Cell Biological Consequences of EHD1 Depletion

7.1 EHD1 Knockdown Results in Depleted EGFR Total Level

The 16A5 cell line is an E6/E7 immortalized, non-malignant and EGFR dependent mammary epithelial cell line obtained from reduction mammoplasty (Band 1989). Consistent with reports in the literature, these cells display normal EGFR degradation kinetics in response to treatment with various ligands, showing substantial degradation after treatment with EGF, an apparent recycling of EGFR after stimulation with Amphiregulin, and an intermediate phenotype observed upon treatment with TGF-α (Figure 3.1).

We wanted to assess whether EHD1 had a role in regulating EGFR. To this end, we developed stable, doxycycline-inducible shRNA cell lines. Cells were treated with Doxycycline over a period of 5 days at steady state in EGF-containing media before taking lysates and resolving proteins by western blot. Our experiments indicated overall levels of EGFR were depleted in EHD1, knockdown cells (Figure 3.2).

To determine whether this was a ligand-dependent phenomenon or whether EHD1 had a role in the basal, unstimulated regulation of EGFR, we plated cells at 50% density and cultured them in the presence of doxycycline for 3 days under normal, EGF-replete, steady-state conditions to achieve sufficient knockdown. As EGF has the effect of routing stimulated EGFR to lysosomes, steady-state conditions in normal cells keep the receptor at low levels. We then deprived cells of serum and exogenous EGF by culturing cells in D3 starvation media (Band 1989), allowing newly synthesized EGFR to accumulate, which rebalances their localization favoring the plasma membrane. Upon removal of EGF we monitored EGFR total levels over a time-course of 24 hours, taking lysates at regular intervals before resolving samples by western blot (Figure 3.3A). Our observations indicated that total EGFR recovery was stunted in the two EHD1 shRNA-mediated knockdown cell lines relative to controls. We quantified this effect over 3 independent experiments, normalizing EGFR levels relative to HSC70 which was used as a loading control and shown in (Figure 3.3B). These findings were validated with siRNA-mediated

knockdown using 3 separate EHD1-targeting oligos relative to a non-targeting siRNA. Cells were transfected overnight in low-serum Opti-mem using optimized concentrations of reagent. On the following day, we changed media and allowed cells to recover for one full day under normal growth conditions, then again starved cells for 24 hours in D3 media before taking lysates (Figure 3.3C). Similarly to our initial findings, we observed total levels of EGFR were appreciably lower in the EHD1-knockdown condition relative to control.

We then sought to examine whether this phenotype represented a general and conserved phenomenon. We previously had generated $Ehd1^{-/-}$ mice using Cre/loxP-mediated genetic recombineering (Rainey 2010). Mouse embryonic fibroblasts from $Ehd1^{fl/fl}$ mice were isolated and cultured according to the NIH 3T3 protocol and treated with either Adeno-GFP or Adeno-Cre. These cells were plated and allowed to reach 80% confluence. MEFs were treated with 2 nM EGF for a period of 4.5 hours to deplete total EGFR, and starved as indicated before lysis. $Ehd1^{-/-}$ MEFs were then compared to their wild-type counterparts (Figure 3.4A). We ran similar experiments with an shRNA-inducible MDA-MB-231 cell line (Figure 3.4B), and performed a transient knockdown in S2013 pancreatic adenocarcinoma cells, consistently finding total levels of EGFR to be altered (Figure 3.4C). Interestingly, it was apparent that the process of serum starvation had the effect of inducing the expression of EHD1 and a concomitant increase in EHD4, an effect that was exacerbated when EHD1 was depleted. Occasionally, we observed an *increase* of apparent EGFR, usually in cases where EHD1 had exceptional knockdown, and where levels of EGFR are high, or when cells were routinely passaged at higher confluence. We interpreted this to mean that EGFR is first, sensitive to levels of EHD1, and some accumulative process is at work.

7.2 EHD1 Knockdown Decreases EGFR Surface Expression

Perturbations in EGFR total level consistently reflected changes in its surface expression. Flow cytometry analysis under conditions of EHD1 KD, both transient and stable, revealed a significant decrease in receptor surface localization under steady state and serum-starved

conditions. For the stable-inducible knockdowns, cells were plated at a density of 200,000 cells per 35 mM dish and cultured in the presence of doxycycline for 3 days. Cells were kept at steady state (growth factor replete) or starved accordingly and live cells were stained with anti-EGFR clone 528 and anti-mouse IgG2a APC. Cells were sorted by FACS for expression of the IRES Turbo-GFP and results were quantified for the mean fluorescent intensity of APC. Transient transfections were performed in Opti-mem overnight and allowed to recover at steady state for 24 hours before starvation and staining with anti-mouse Alexa Fluor 488 (Figure 3.5, 3.7B).

7.3 Exogenous EHD1 Compensates for Loss of Endogenous EHD1

A principal problem with RNA interference experiments is off-target effects. A rigorous demonstration of specificity is rescue of the knockdown with an RNAi-resistant target gene. We combined the expression of a doxycycline-inducible 3' UTR-targeting shRNA against EHD1 with stable doxycycline-inducible EHD1-GFP which lacks the 3' UTR regulatory sequence (Ma 2010). Control cell lines expressed non-targeting shRNA or contained an empty vector instead of EHD1-GFP. We first examined the effect of EHD1-GFP overexpression on EGFR total levels under starvation conditions. Cells were treated with the indicated concentration of doxycycline for 2 days at steady state before starving cells in D3 media also with doxycycline. We observed a concentration-dependent increase in the amount of exogenous EHD1-GFP expressed and a corresponding increase in the amount of EGFR under these conditions. In overexpressing EHD1-GFP, we also saw an increase in the endogenous expression of EHD1 and EHD4 (Figure 3.6A). The combined expression of EHD1-GFP with the shRNA targeting EHD1(Compensated) successfully rescued EGFR total levels (EHD1-GFP + EHD1 KD shRNA, (Figure 3.6B) and surface expression (Figure 3.7C) compared to empty (TET) vector plus EHD1 KD shRNA, in which EGFR levels were significantly depleted.

7.4 EGFR Signaling is Reduced During EHD1 KD

To assess the impact of EHD1 knockdown and EGFR depletion on signaling potential, a transient knockdown was prepared using the oligo directed against the EHD1 3' UTR as

described above before stimulating cells with 2 nM EGF. EGFR autophosphorylation on tyrosine residues is required for its kinase activity and serves as a marker for activation. In cells that were treated with control siRNA, stimulation with EGF caused a time-dependent increase in activation. However, in cells treated with siRNA specific to EHD1, >80% of the EHD1 was depleted, total levels of EGFR were substantially diminished and the levels of tyrosine phosphorylation (as assayed by 4G10) was correspondingly decreased relative to control cells. This indicates that EHD1 is required for EGF-induced signal propagation (Figure 3.7).

7.5 EHD1 Colocalizes with EGFR in the Golgi

Given its extensively described role in endocytic traffic, we hypothesized that EHD1 might be responsible for routing the EGFR itinerary within the cell. To determine whether EHD1 colocalizes with EGFR, we starved 16A5 cells for a period of 6 hours in the presence of either DMSO or 10 μM monensin, and co-stained for endogenous EGFR and EHD1. Monensin treatment increased the accumulation of EGFR in perinuclear endocytic vesicles, indicating an effective inhibition of cargo exit from the cell interior. We observed sparse pinpoints of colocalized vesicles in the DMSO-treated cells (Figure 3.8A); however, upon monensin treatment a clear perinuclear co-localization was revealed between EGFR and endogenous EHD1 (Figure 3.8B left). This effect was markedly enhanced upon exogenous overexpression of EHD1-DsRed and structures became visible even in the absence of monensin treatment (Figure 3.8 B right). We used a combination of endogenous staining with EHD1-DsRed overexpression, and by 3-color analysis, we determined the compartment in which EHD1 and EGFR were colocalized was the Golgi, using GM130 as an established marker. Serial z-sections were taken, and an orthogonal view is presented (Figure 3.8 C, D).

7.6 EGFR is Retained within the Golgi under Conditions of EHD1 Knockdown

We next set out to examine how EHD1 affects EGFR traffic. Using the inducible knockdown and compensated cell lines, we starved cells over an interval of 24 hours and monitored EGFR localization. At time-zero, steady-state conditions, EGFR (green) displayed a

predominant intracellular distribution and was partly localized to the perinuclear area of the cell for all cell lines tested. Perinuclear localization was seen as modestly accentuated in the compensated cell line, with some visual co-localization with EHD1 (red). During starvation, EGFR distribution in the control doxycycline-negative and compensated doxycycline-positive conditions progressively localized to the cell surface membrane as expected. During uncompensated EHD1-knockdown however, we saw a significant phenotypic difference as EGFR accumulated and remained in the perinuclear region of the cell (Figure 3.9A). This compartment was once again identified as the Golgi, staining positive for GM130 (Figure 3.9B). Under knockdown conditions we were able to show EGFR localization throughout the ER-Golgi compartment, having stained positive for Calnexin, LMAN-1, GM130, Giantin, and Golgin-97, (markers of the ER, ER-Golgi intermediate compartment, cis-, medial- and trans-Golgi respectively). As EHD family members have often been cited as having both shared and distinct roles, it is notable that, we are able to show these structures as double-positive for EHD4 (Figure 3.9C).

7.7 Retrograde Traffic in EGFR Retention During EHD1 knockdown

We next were interested in whether EGFR accumulation in the Golgi was the result of a defect in either, anterograde transport or due to a defect in the retrograde pathway. To examine this, we first pre-starved cells treated either with or without doxycycline for a period of 6 hours. Cells were chilled on ice at 4°C for 20 minutes, and pulse labeled with a biotinylated anti-EGFR antibody in ice-cold D3. After the pulse media was removed, cells were rinsed with ice-cold PBS and returned to incubate at 37°C for 18 hours before fixation. Concurrently, we ran a non-surface EGFR-labeled assay in parallel, and at the end of the time course the cells were fixed, permeabilized and stained as before for GM130 and Rab11, a marker of the recycling endosome. EGFR was stained in cells that weren't previously biotinylated. Under these conditions we were able to identify an accumulation of EGFR in the EHD1 KD cells that stained double-positive for both GM130 and Rab11. This indicates that a proportion of EGFR that made it out to the cell

surface was blockaded as it attempted to recycle out of the cell interior (Figure 3.10). Radiolabel incorporation during short pulses generally reflects rates of protein synthesis, and ^{35}S metabolic labeling indicated an apparent defect in EGFR maturation. The increase in radiolabel incorporation at 4 hours is in accordance with other reports that have shown posttranslational modification of the receptor (Figure 3.11) (Franovic 2007, Gamou 1987).

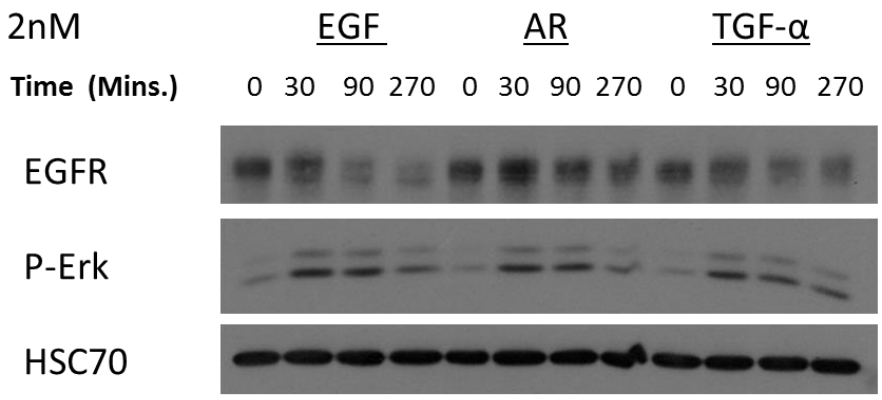


Figure 3.1: 16A5 Cells Display Expected Degradation Kinetics for EGF Stimulation versus Amphiregulin, and TGF-α.

The immortalized, non-tumorigenic and EGF-dependent, human mammary epithelial cell line 16A5 was deprived of serum and EGF in the culture medium for a period of 48 hours. Cells were then treated with 2 nM concentration for each ligand (EGF, Amphiregulin, or TGF-α) over a period of 4.5 hours.

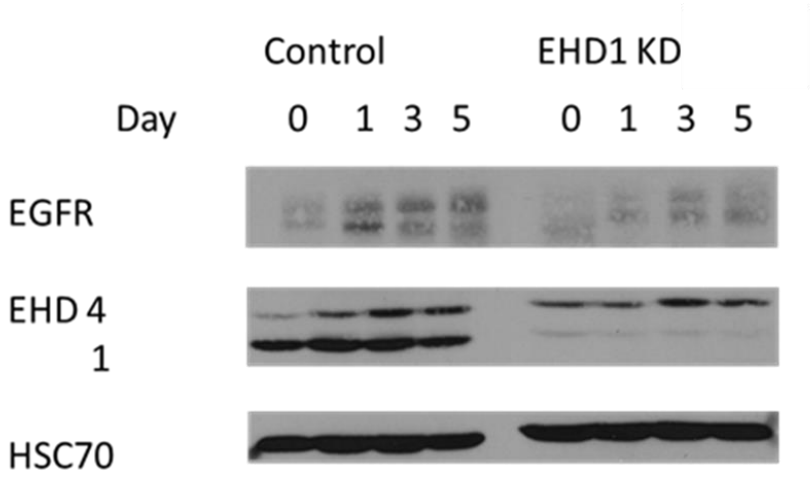


Figure 3.2: EHD1 Knockdown Depletes Total EGFR at Steady-State

Stably transduced cells lines expressing a non-targeting or, doxycycline-inducible shRNA against EHD1 were cultured in the presence of doxycycline over a period of 5 days. Cell lysates were taken on each respective day.

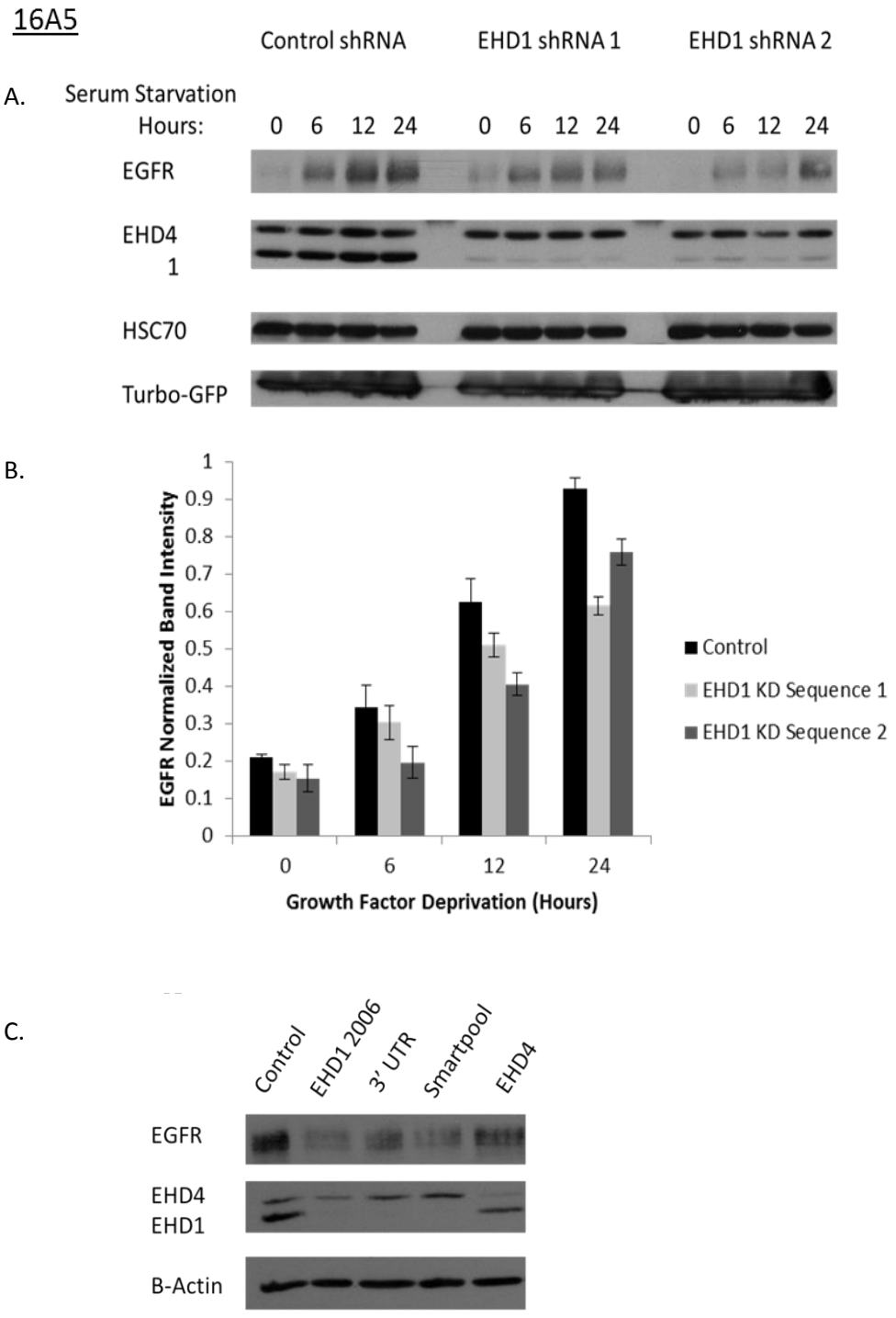


Figure 3.3: EHD1 Knockdown Depletes Total EGFR During Starvation.

A. Stably transduced 16A5 cells expressing doxycycline-inducible non-targeting shRNA or an shRNA directed against EHD1 were cultured in the presence of doxycycline for 3 days before starving for the indicated intervals. B. Densitometry quantification of EGFR normalized to HSC70, which was used as a loading control, and shown for 3 independent experiments. C. Transient siRNA transfection using various oligos against EHD1. Lysates were taken 24 hours following starvation.

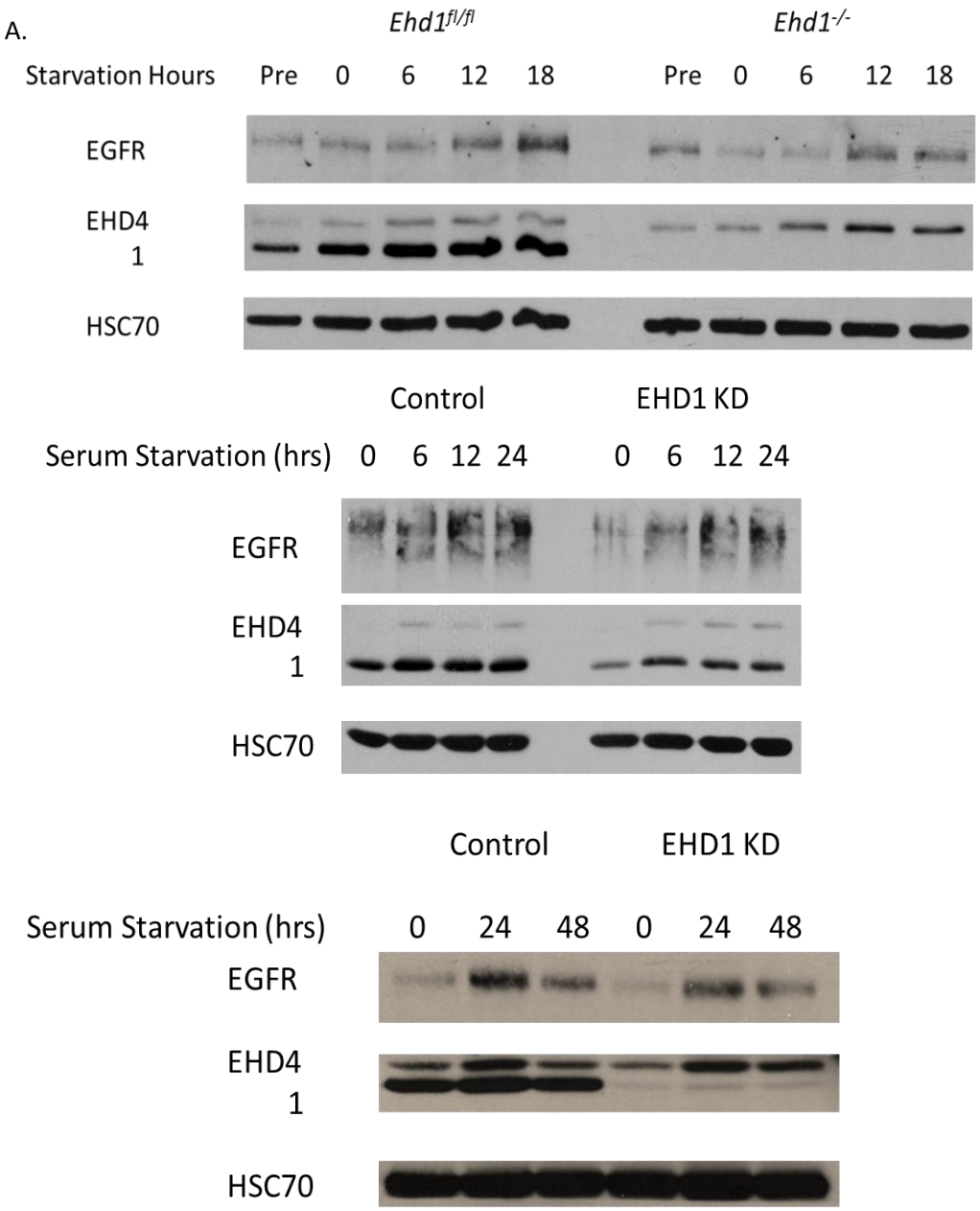


Figure 3.4: EHD1 Knockdown Depletes Total EGFR Under Starvation in MEFs and Cancer Cell Lines

A. Wild type and EHD1$^{-/-}$ MEFs were pre-stimulated with 2nM EGF before beginning serum starvation. B. Stable-inducible shRNA-mediated EHD1 knockdown in triple-negative breast cancer MDA-MB-231 cells were stimulated with 2 nM EGF before beginning starvation. C. Transient siRNA transfection in pancreatic adenocarcinoma S2013 cells.

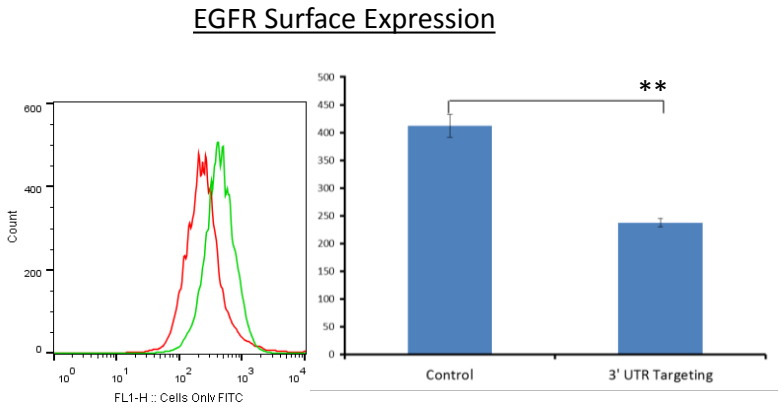


Figure 3.5: EHD1 Knockdown Decreases EGFR Surface Expression. 16A5 cells were transfected with an oligo targeting the 3' UTR of EHD1 and allowed to recover for a full day in steady state media before 24 hours of starvation. Live cells were analyzed by FACS for surface expression of EGFR. Error bars indicate the standard error of the mean for 3 replicates.

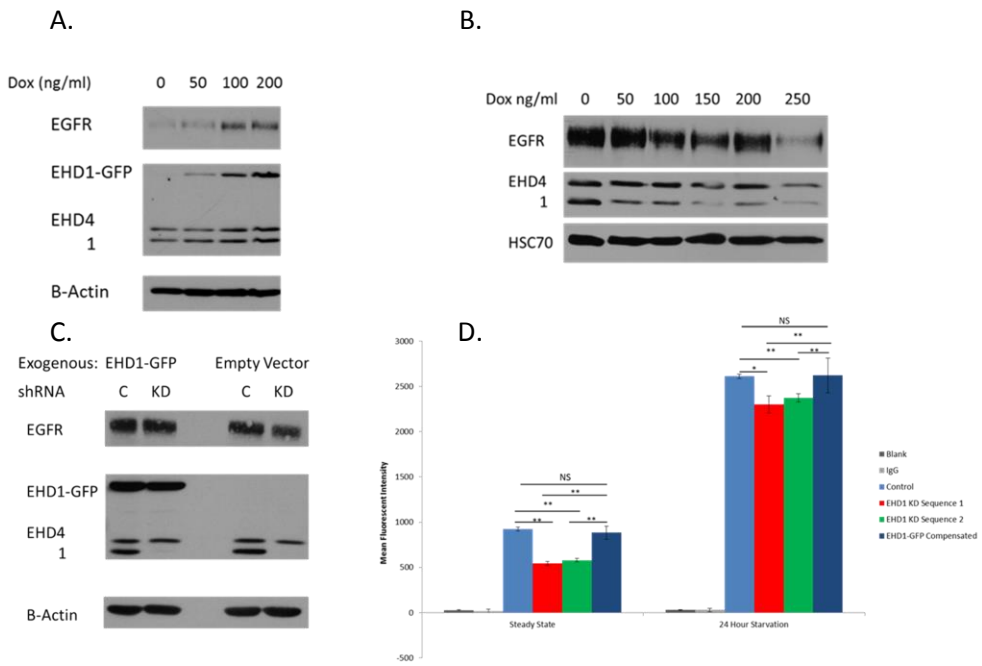


Figure 3.6 Exogenous EHD1 Compensates for Loss of Endogenous EHD1

(A) Doxycycline-inducible EHD1-GFP-overexpressing 16A5 cells were induced at the indicated concentrations and starved for 24 hours before assaying EGFR total levels. (B). Doxycycline-inducible shRNA targeting the 3' UTR of EHD1 was performed as in A. (C). Rescue of EHD1 shRNA mediated depletion by induced exogenous overexpression of shRNA resistant EHD1-GFP. (D) EGFR surface levels assayed by FACS in non-targeting control and 2 EHD1-targeting shRNA constructs with rescue by EHD1-GFP. Error bars indicate standard error of the mean. Results shown are representative of 3 independent experiments.

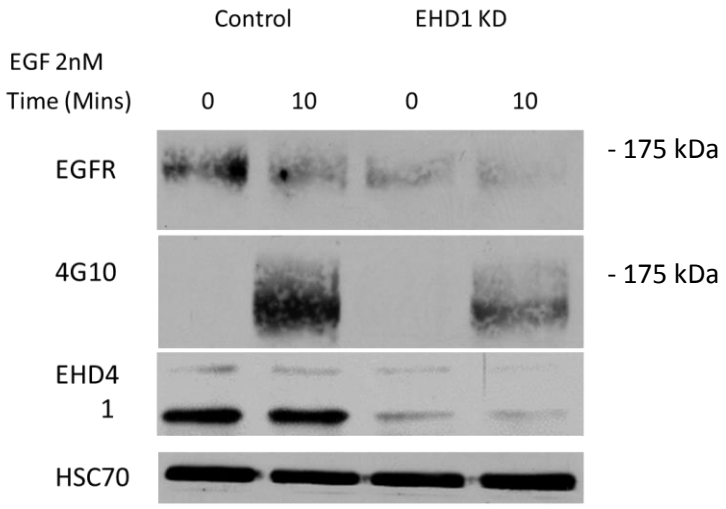


Figure 3.7 Depletion of Endocytic Recycling Regulator EHD1 impairs EGF-induced EGFR Signaling: Immortal, non-tumorigenic mammary epithelial cell line 16A5 was transfectd with a 3' UTR targeting EHD1 specific or control siRNA. These cells were growth deprived from 48 hours followed by EGF (2 nM) stimulation for 10 minutes. EHD1 knockdown (indicated byloss of the lower EHD1 band recognized by an EHD1/4 reactive antibody) promotes total level depletion of EGFR while controls show no loss of EGFR. Anti-phosphotyrosine antibody 4G10 was used to assay EGFR activation state which was comparatively reduced in the EHD1 knockdown condition (stripped and re-probed). The approximately 175 kDa phosphorylated EGFR band is shown.

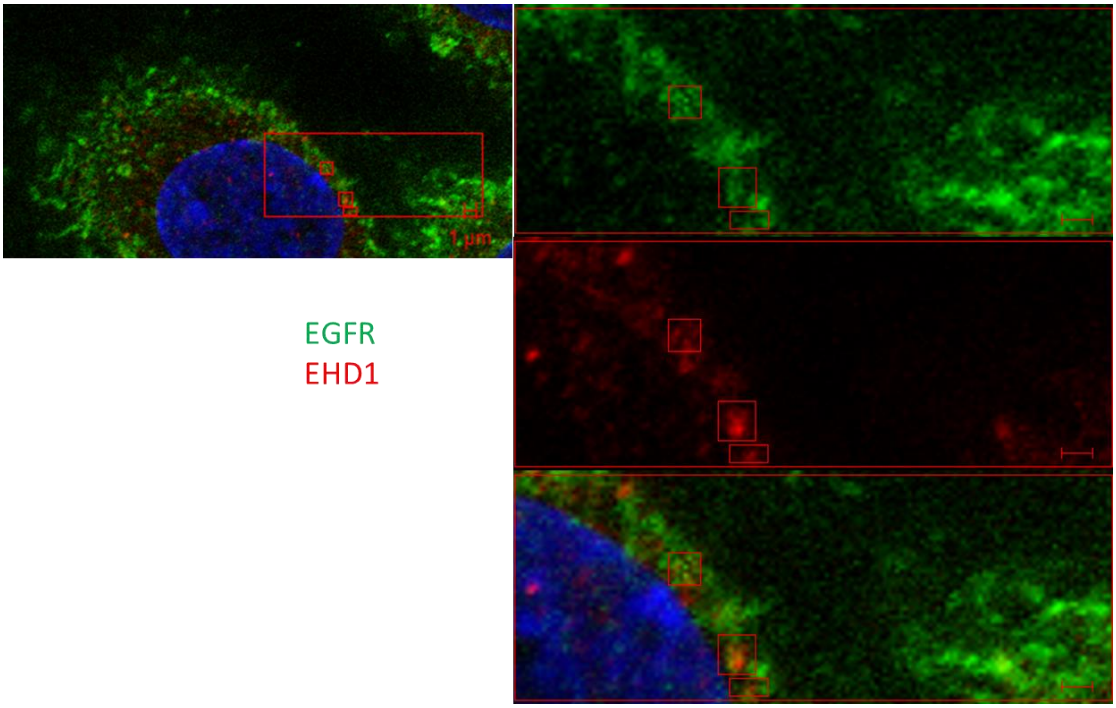


Figure 3.8 EHD1 Colocalizes with EGFR in Vesicles and in the Golgi: (A) Serum-starved 16A5 cells were immunostained after 24 hours with antibodies against EHD1 (red) and EGFR (green).

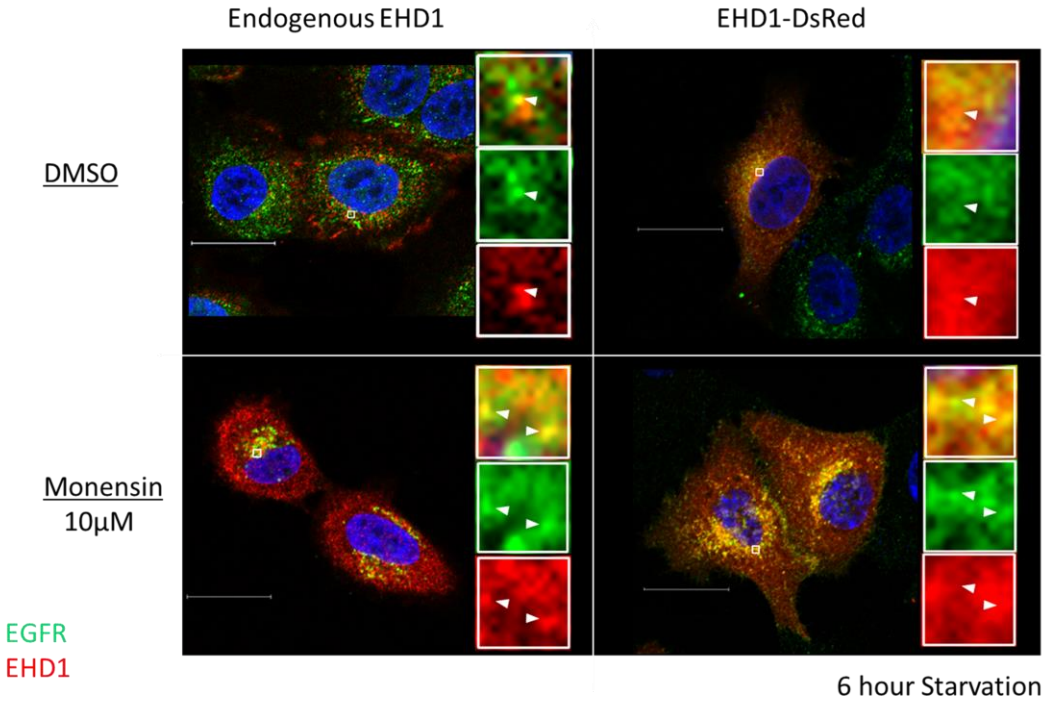
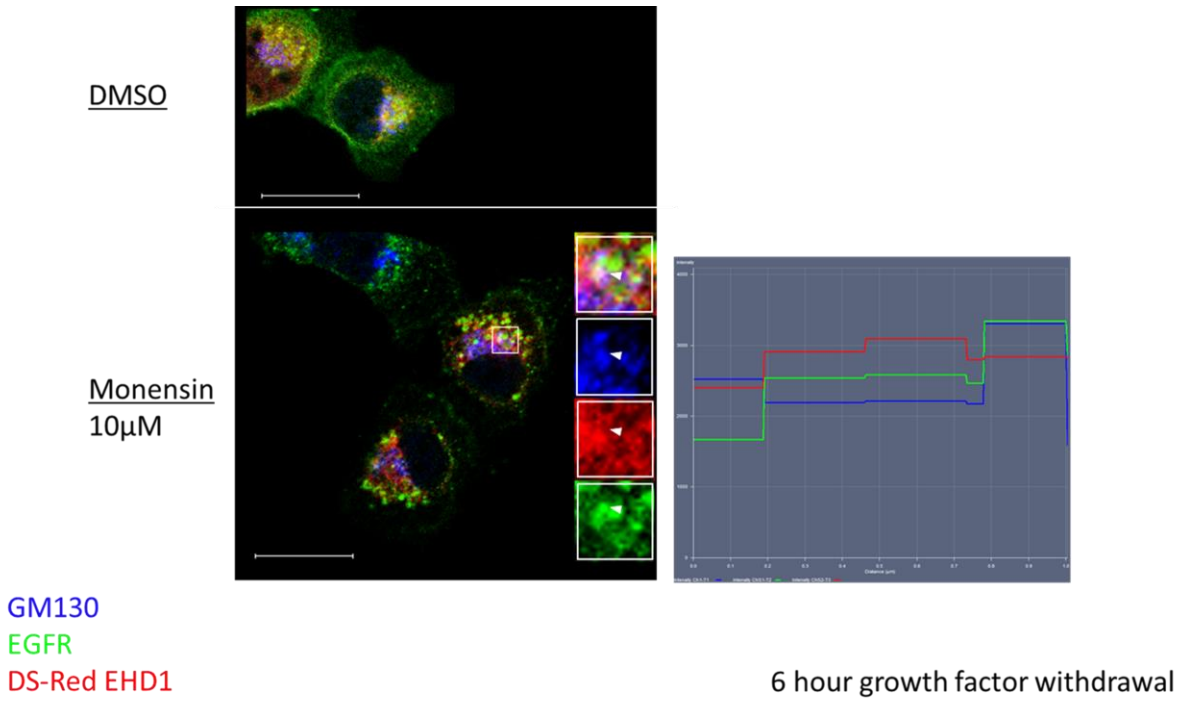


Figure 3.8 EHD1 Colocalizes with EGFR in Vesicles and in the Golgi: (B) (Left) 16A5 cells were stained for endogenous EGFR and EHD1in the (top) absence or (bottom) presence of 10 µM monensin. (Right) Overexpressed EHD1-DsRed. Serum starvation interval 6 hours.

Scale bar 20 µm



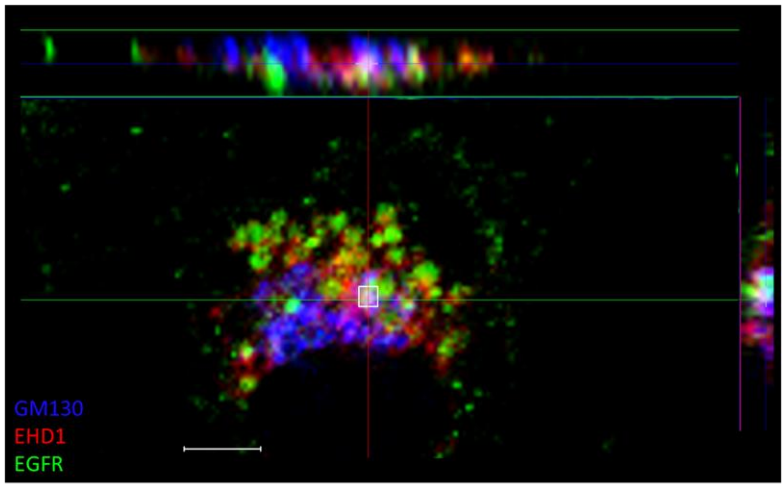


Figure 3.8 EHD1 Colocalizes with EGFR in Vesicles and in the Golgi: (C) Three-color colocalization analysis of EHD1-Dsred, EGFR (green) and GM130 (blue), with and without monensin concurrent with serum starvation. (D) Orthogonal view of serial Z-section interval of 0.4µM.

Scale bar 20µm (Upper)

Scale bar 5µm (Lower)

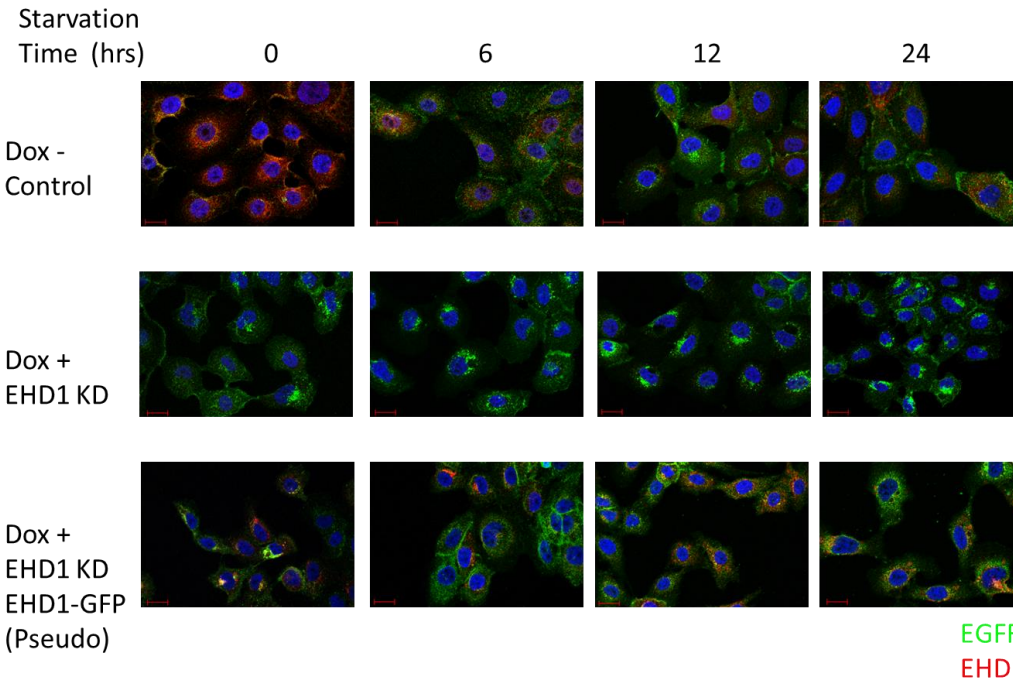


Figure 3.9 EGFR is Retained within the Golgi under Conditions of EHD1 Knockdown (A) (Top) Stable, doxycycline inducible control (Dox -) and (Middle) EHD1-depleted cells (Dox +) demonstrating accumulation of EGFR in an intracellular compartment upon induction of growth factor and serum deprivation. (Bottom) Doxycycline inducible EHD-1 GFP expression compensates for KD of endogenous EHD1, and rescues observed intracellular accumulation.

Scale bar 20μm

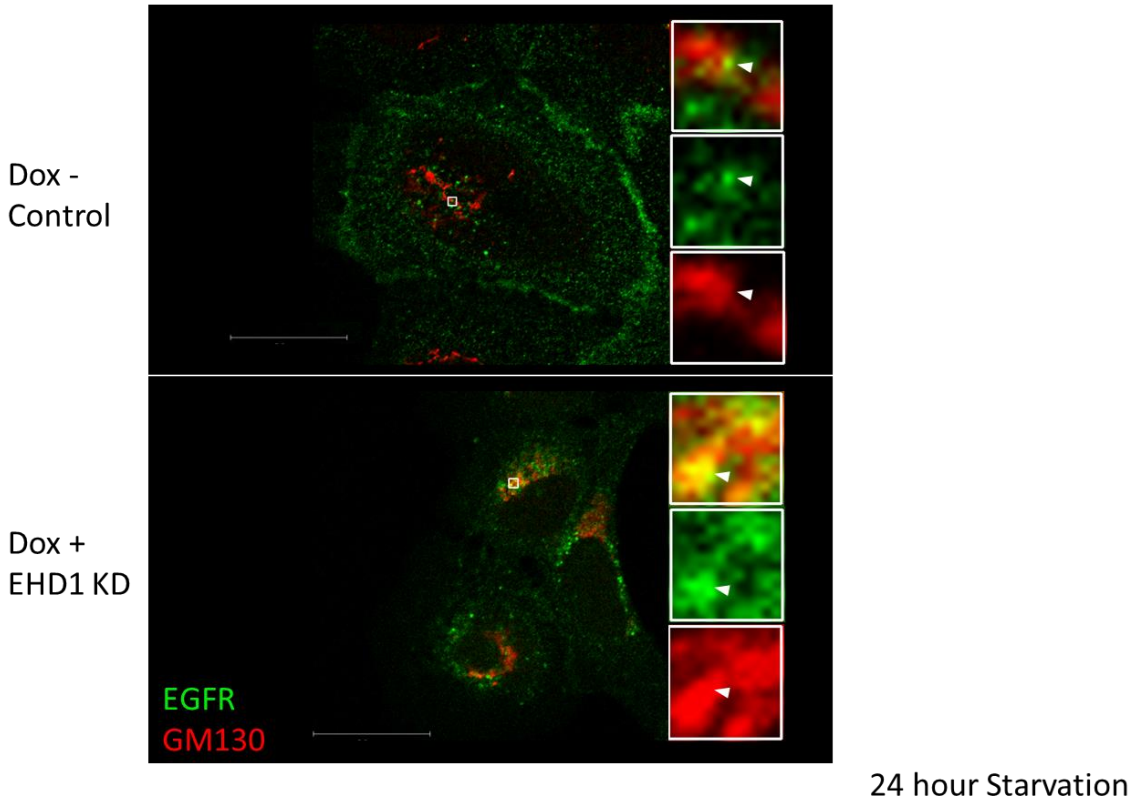


Figure 3.9 EGFR is Retained within the Golgi under Conditions of EHD1 Knockdown (B) Stable, doxycycline-inducible control (Dox -) and EHD1-depleted cells (Dox +) demonstrating accumulation of EGFR within the Golgi upon induction of growth factor and serum deprivation.

Scale bar 20μm

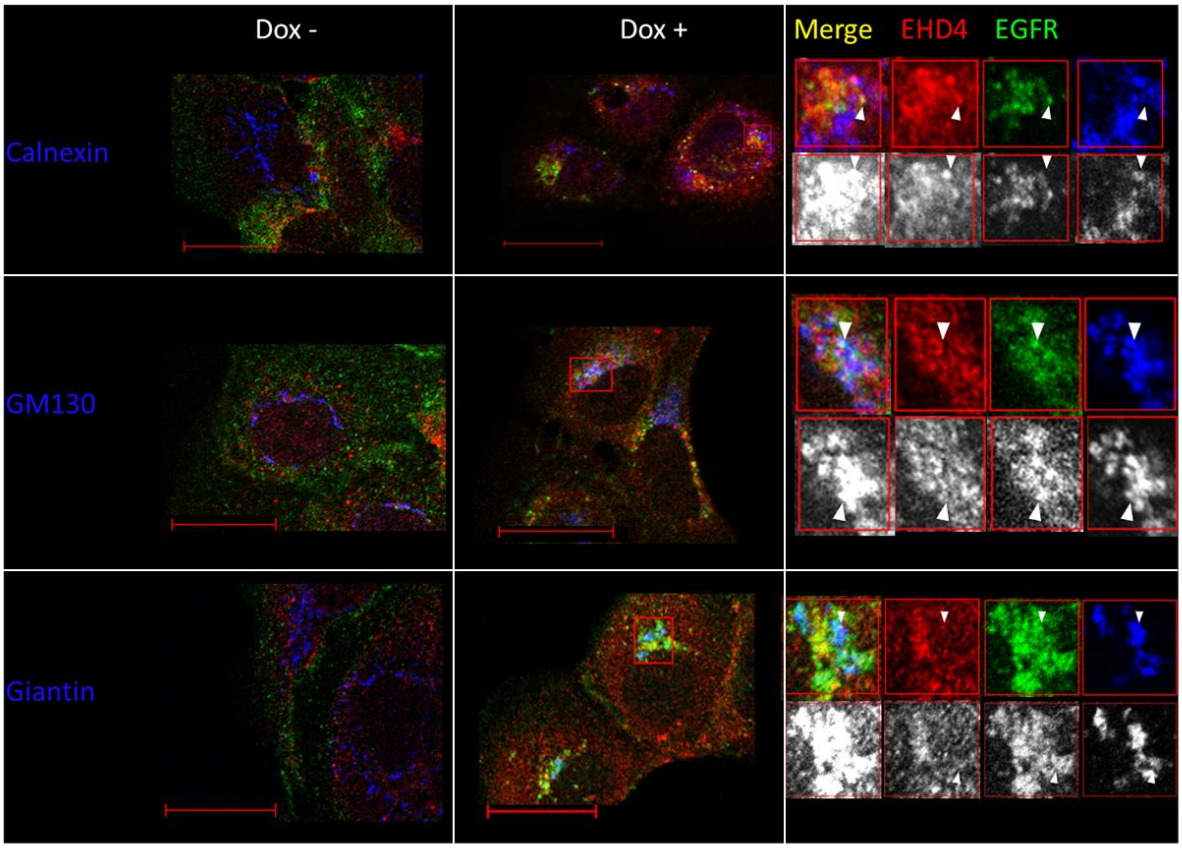


Figure 3.9 EGFR is Retained within the Golgi under Conditions of EHD1 Knockdown (C) Stable, doxycycline-inducible control (Dox -) and EHD1-depleted cells (Dox +) compartmental analysis. EGFR accumulates throughout the ER and Golgi in compartments also containing EHD4 upon induction of growth factor and serum deprivation.

Scale bar 20 μm

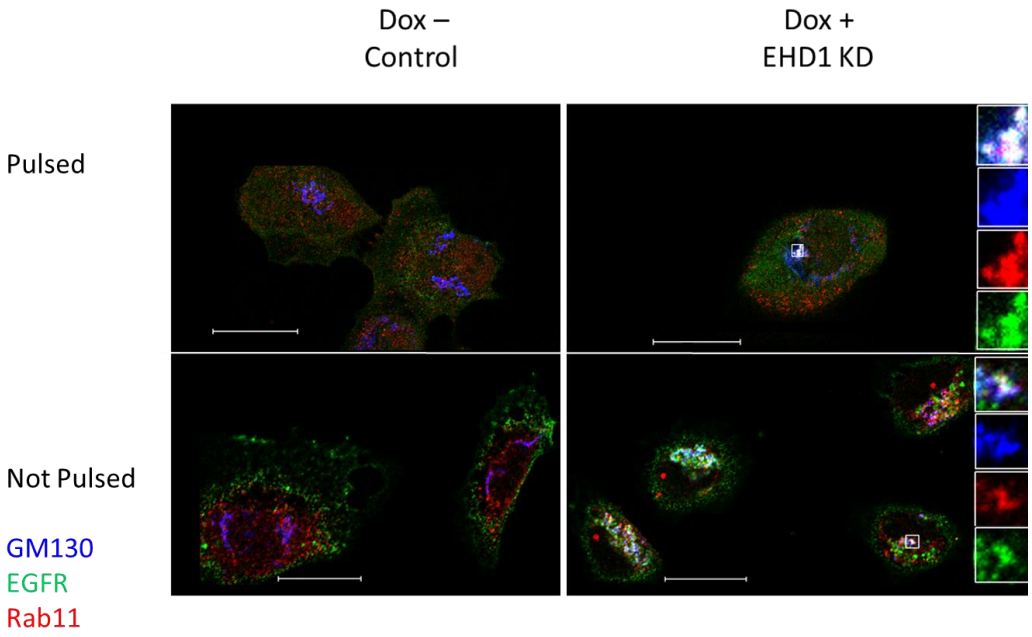


Figure 3.10 Retrograde Traffic is Involved in EGFR Retention During EHD1 Knockdown: Cells were pre-starved for 6 hours before being chilled on an ice water bath and labeled (Pulsed) with a biotinylated antibody against EGFR (green) in Control (Dox -) or EHD1 KD (Dox +). Media and unbound antibody were removed, cells were washed and chased in media without antibody for 18 hours. Cells were then fixed, permeabilized and stained for a marker of recycling endosomes, Rab11 (red), and the Golgi, GM130 (Blue). Cells were also stained for EGFR using the 528 clone (green) (not pulsed).

Scale bar 20 μm

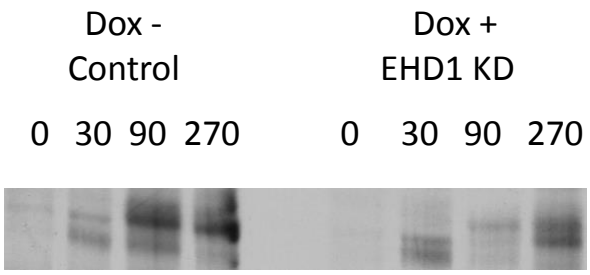


Figure 3.11 EHD1 Knockdown Slows EGFR Maturation: ^{35}S Metabolic labeling was performed in Dox -/Dox+ conditions to induce EHD1 KD. Cells were washed and kept in methionine/cysteine-depleted media for 30 minutes and pulse labeled with .1mCi of hot ^{35}S methinone/cysteine for 20 minutes. Cells were washed and chased with a 100-fold excess of unlabeled methionine/cysteine for the indicated intervals before lysis and resolving by SDS-PAGE. Gel was fixed and incubated with Auto-Fluor before drying and visualization by auto-radiography. Representative of 3 independent experiments.

7. Results II. Functional Impact of EHD1 Depletion

7.8 EHD1 KD Reduces Cell Proliferation in 2-D Culture

To determine the functional consequences of EHD1 depletion on signaling downstream of EGFR, we assayed the proliferative capacity of 16A5 cells in the presence of doxycycline over several days. Both EHD1 knockdown sequences displayed significant reductions in their proliferation as assayed by Cell Titer Glo assay relative to control shRNA cells. Similar results were observed in an EHD1 knockdown line when cultured +/- doxycycline (Figure 3.12 Top, Bottom).

7.9 EHD1 KD Attenuates Matrigel Colony Formation

We examined the ability of 16A5 cells to form organoid colonies in Matrigel under EHD1 Knockdown. 5,000 cells were mixed in 5% matrigel and plated in 8-well chambers and cultured +/- doxycycline for a period of 9 days. Colonies >/= 100μM in size were counted and measured. We observed a significant difference in the number of colonies in the control versus the knockdown condition. Overall differences in colony size were found not to be significant (Figure 3.13).

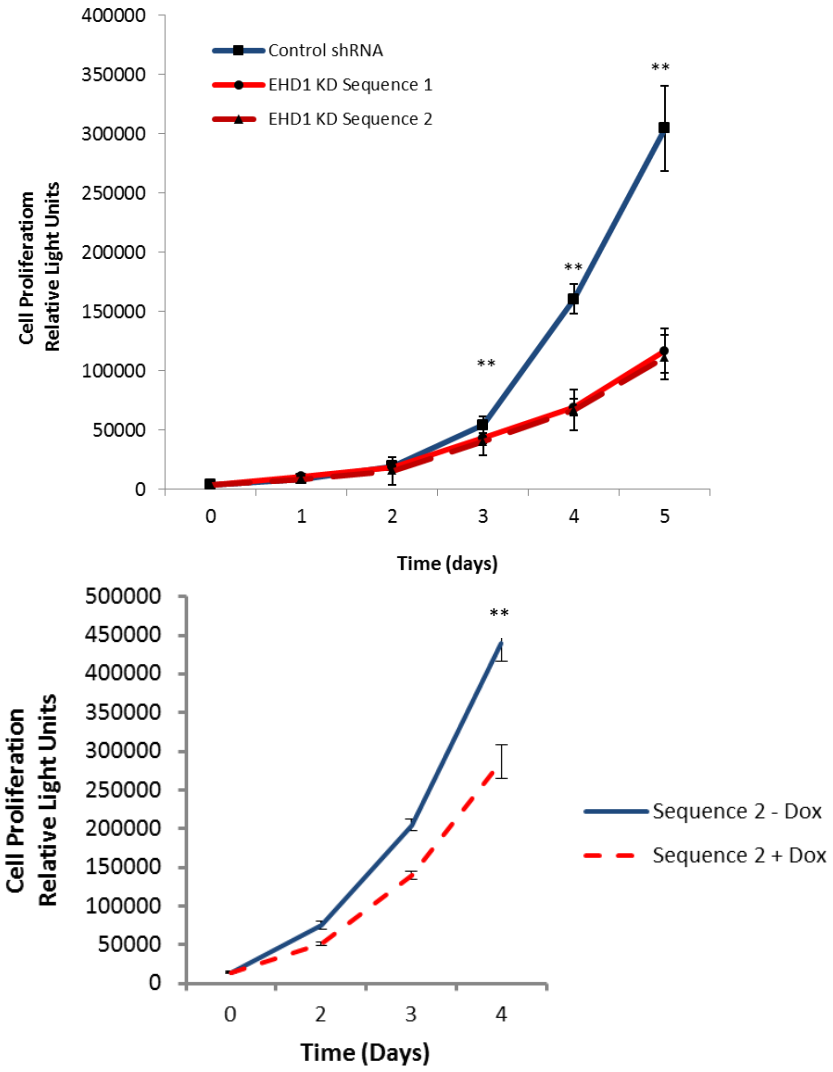


Figure 3.12 EHD1 KD Reduces 16A5 Cell Proliferation in 2-D Culture: (Top) 1500 cells per well were plated in 20 wells in a 96-well format and cultured for 5 days in the presence of doxycycline for Control shRNA and 2 different sequences targeting EHD1. Readouts were taken on each day with Cell Titer glo reagent and read on a FLUOstar Optima plate reader for luminescence. (Bottom) As above, +/- dox for the EHD1 KD sequence #2.

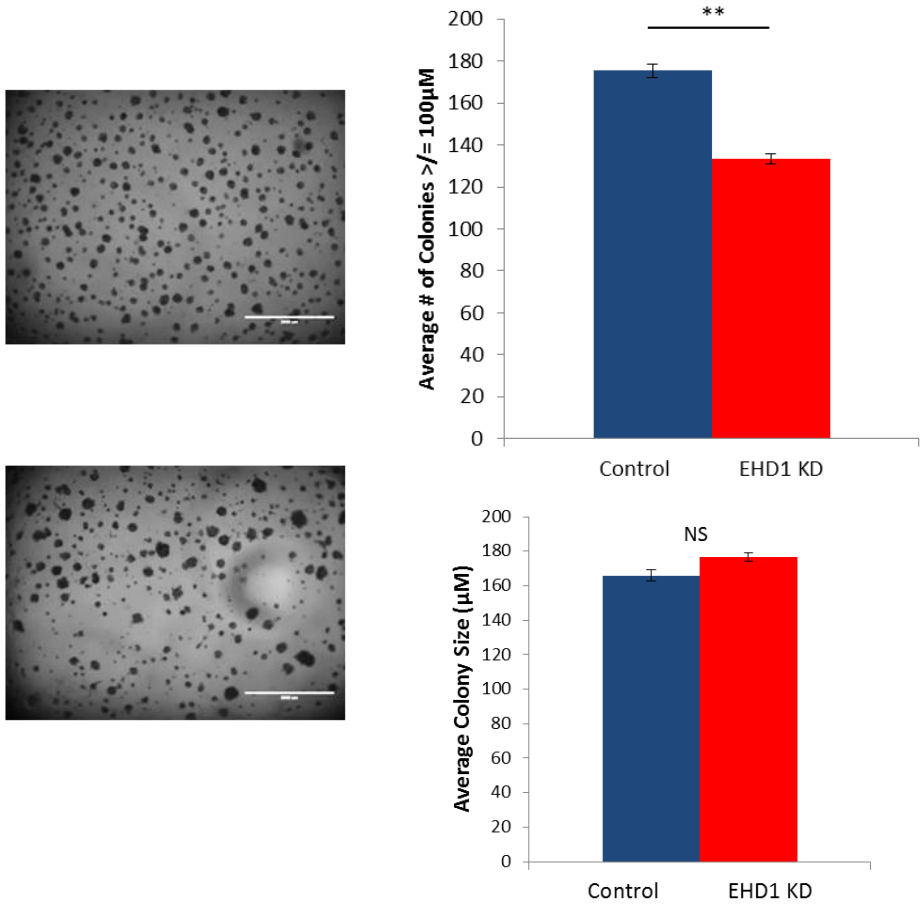


Figure 3.13 EHD1 KD Reduces 16A5 Cell Proliferation in 3-D Matrigel Culture: 5,000 cells were mixed in 5% matrigel and plated in 8-well chambers and cultured +/- doxycycline for a period of 9 days. Colonies >/= 100µM in size were counted and measured. Scale bar 200 µm

CHAPTER 4:

A Shared Mechanism Between EHD1 and RUSC2 Responsible for EGFR Basal Trafficking

8.1 Introduction

We were intrigued by results (Deribe 2009) indicating RUSC2 could have a potential role in the unstimulated basal traffic of EGFR. RUSC2 was noted to have two NPF motifs, and a RUN domain implicated as a Rab effector for Rab35 and a closely related family member, NESCA with a published role in mediated signaling downstream of the Trk receptor tyrosine kinase. The RUSC2 interaction with GM130 further strengthened our hypothesis that interactions mediated through RUSC2 were in part responsible for our findings with EHD1. A peptide-based screen performed in the lab was positive for 9-mer representing RUSC2 binding to the EH domain of EHD1 (data not shown). Parallels with the model proposed by (Sharma 2009) and (Kobayashi 2010) suggest RUSC2 might be an effector for either Rab35, which has a known role in recruitment of EHD1 mediated by MICAL-L1, or Rab1a, known to participate in traffic through the Golgi and endosomal sorting. We developed stable shRNA cell lines and conducted confocal based studies to examine our hypothesis.

8.2 RUSC2 Knockdown Results in Decreased Total Levels of EGFR

We again plated cells at 50% density and cultured them in the presence of doxycycline for 3 days under normal, EGF-replete, steady-state conditions to achieve sufficient knockdown. We then deprived cells of serum and exogenous EGF by culturing cells in D3 starvation media to cause EGFR accumulation. We monitored EGFR total levels over a time-course of 24 hours taking lysates at regular intervals before resolving samples by western blot (Figure 3.3A). Our observations indicated that total EGFR recovery was stunted in the two RUSC2 shRNA-mediated knockdown cell lines relative to controls. RUSC2 knockdown resulted in an even more dramatic depletion of EGFR than the EHD1 knockdowns.

8.3 RUSC2 KD Results in Decreased EGFR Surface Expression

We hypothesized that, along with EHD1, RUSC2 might be involved in recycling of internalized EGFR to the cell surface. To test this hypothesis, flow cytometry analyses under identical conditions were performed for RUSC2 KD; this revealed a significant decrease in receptor

surface localization under steady state and serum-starved conditions similar to that seen for EHD1 (Figure 4.1).

8.4 RUSC2 Knockdown Reduces Cell Proliferation in 2-D Culture

To determine the functional consequences of RUSC2 depletion on signaling downstream of EGFR, we assayed the proliferative capacity of 16A5 cells in the presence of doxycycline over several days. Both RUSC2 knockdown sequences displayed significant reductions in their proliferation as assayed by Cell Titer Glo assay relative to control shRNA cells (Figure 4.2).

8.5 RUSC2 Colocalizes with EHD1

On the basis of the co-localization between GM130 and EHD1, we predicted that RUSC2 might also associate with EHD1 vesicles. To determine the localization of RUSC2 in 16A5 cells and whether RUSC2 and EHD1 co-localize, EHD1-DsRed and HA-RUSC2 were co-transfected and stained with anti-HA antibody. RUSC2 displayed a tight correlation to EHD1 on an array of tubular structures and vesicles. Artifacts of overexpression were ruled out using a newly developed in-house antibody specific for RUSC2. Endogenous RUSC2 showed a very similar distribution pattern to that of the overexpressed construct, and co-localized with endogenous EHD1 upon similar structures, although to a lesser extent than when both proteins were co-overexpressed. We were also able to demonstrate endogenous EHD1 co-localization with overexpressed RUSC2-HA (Figure 4.3).

8.6 EHD1 Knockdown Places EGFR in Association with RUSC2 and GM130

Due to a published role of RUSC2 as an effector of Golgi-associated Rab1, its direct-interaction with GM130 and its prominent co-localization with EHD1, we hypothesized that an interaction mediated between EHD1 and RUSC2 may be responsible for transiting EGFR out of the Golgi during the basal recycling process. We first sought to determine whether the intracellular compartment where we see accumulation of EGFR under EHD1 knock-down conditions also contains RUSC2. This was verified, as we were able to see significant 3-color co-localization between EGFR, RUSC2 and its expected binding partner GM130 (Figure 4.4).

Notably, RUSC2 tubulation was significantly reduced and its distribution appeared mostly in vesicles, relative to conditions where either it or EHD1 was overexpressed (Figure 4.3).

8.7 RUSC2 Knockdown Causes EHD1 Accumulation in the Golgi

We suspected RUSC2 might serve as an effector to recruit EHD1 to a Golgi-compartment whereby it could mediate traffic of EGFR. Cells expressing doxycycline-inducible shRNA targeting RUSC2 were plated onto coverslips with or without doxycycline for 3 days before initiating starvation conditions. After 24 hours, the cells were fixed and stained for GM130 and EHD1. Results were quantified by measuring the average weighted co-localization coefficient for the GM130 channel. To our surprise, we observed an increase in the amount of EHD1 in association with this marker. Heterogeneity of Golgi morphology made determination of structural alterations in the RUSC2 knockdown condition uninterpretable (Figure 4.5).

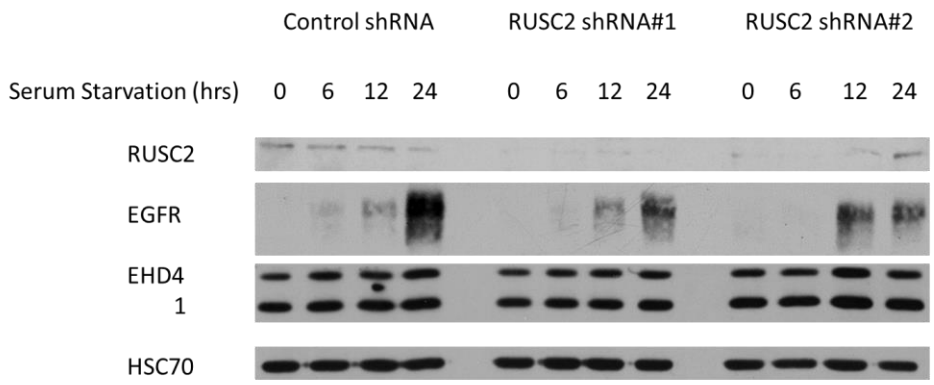
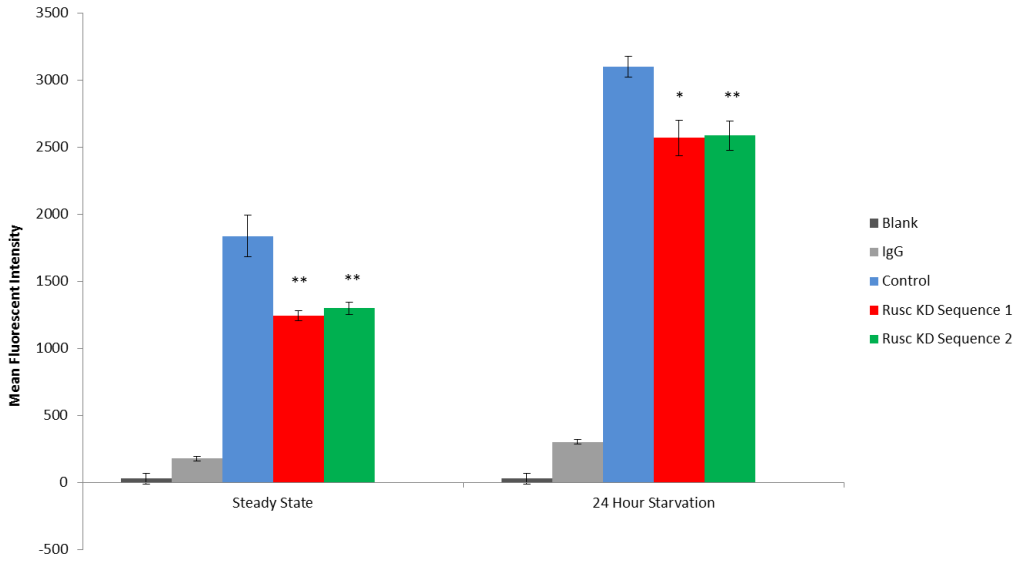
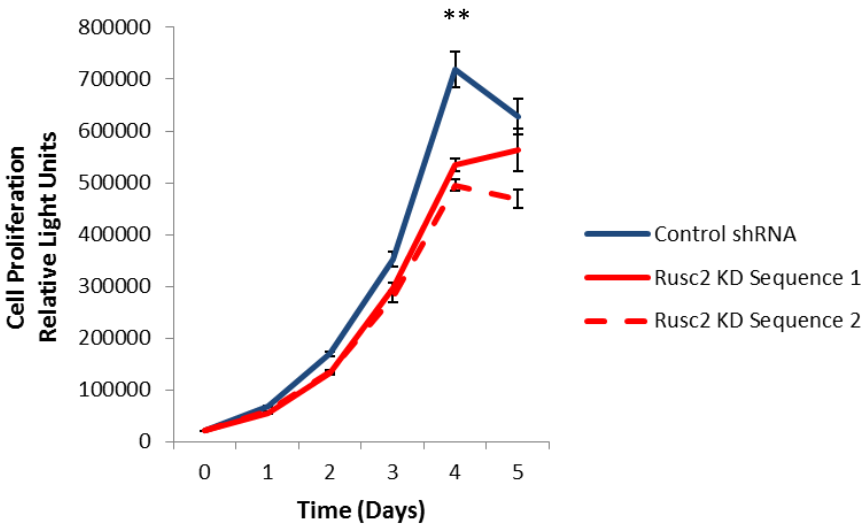


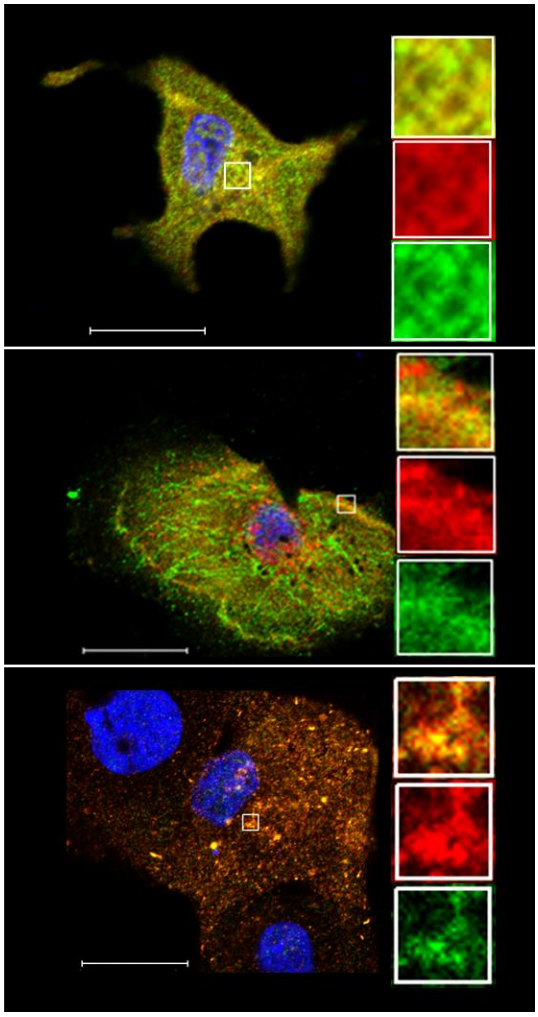
Figure 4.1: RUSC2 Knockdown Results in Decreased Total Levels of EGFR: Stably transduced 16A5 cells expressing doxycycline-inducible non-targeting shRNA or an shRNA directed against RUSC2 were cultured in the presence of doxycycline for 3 days before starving for the indicated intervals.



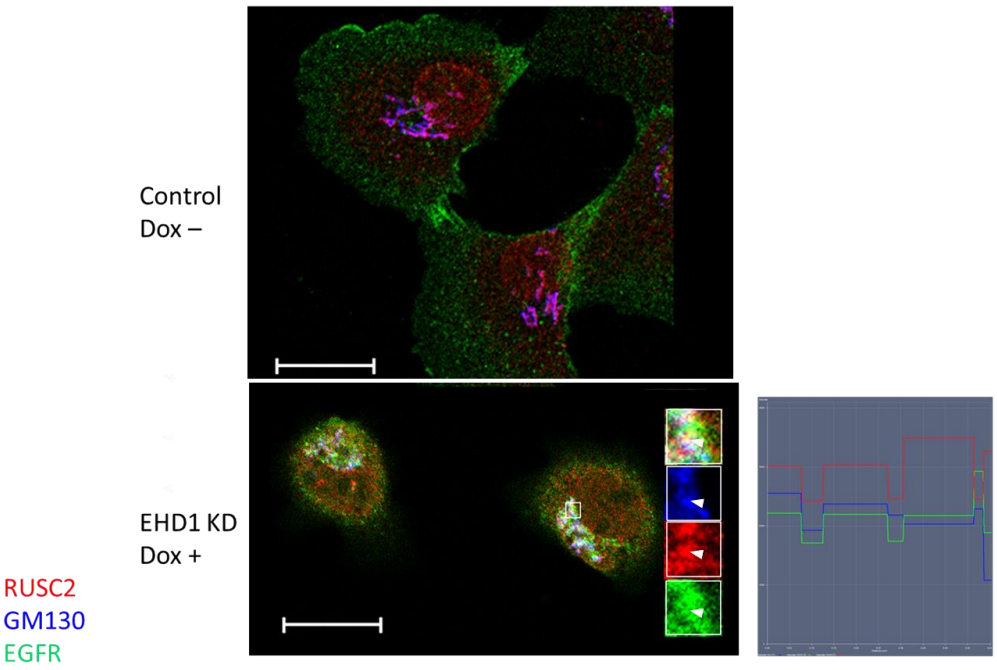
4.2 RUSC2 KD Results in Decreased EGFR Surface Expression: EGFR surface levels assayed by flow cytometry in non-targeting control and 2 different RUSC2 targeting shRNA constructs with rescue by EHD1-GFP. Error bars indicate standard error of the mean for 3 replicates of 10,000 cells.



4.3 RUSC2 Knockdown Reduces Cell Proliferation in 2-D Culture: (Top) 1500 cells per well were plated in 20 wells in a 96-well format and cultured for 5 days in the presence of doxycycline for control shRNA and 2 different sequences targeting EHD1. Readouts were taken on each day with Cell Titer Glo reagent and read on a FLUOstar Optima plate reader for luminescence.



4.4 RUSC2 Colocalizes with EHD1: (Top) EHD1-DsRed was co-overexpressed with RUSC2-HA in 16A5 cells, fixed and stained with an antibody against the HA tag. (Middle) Endogenous RUSC2. (Bottom) Endogenous EHD1. Scale Bar 20 μm.



4.5 EHD1 Knockdown places EGFR in association with RUSC2 and GM130 : 16A5 cells were treated with doxycycline to induce knockdown of EHD1 and then were growth-factor deprived for 24 hours. Three-color imaging of endogenous RUSC2, EGFR and GM130 shows specific co-localization in the perinuclear area in starved cells.

Scale Bar 20μm

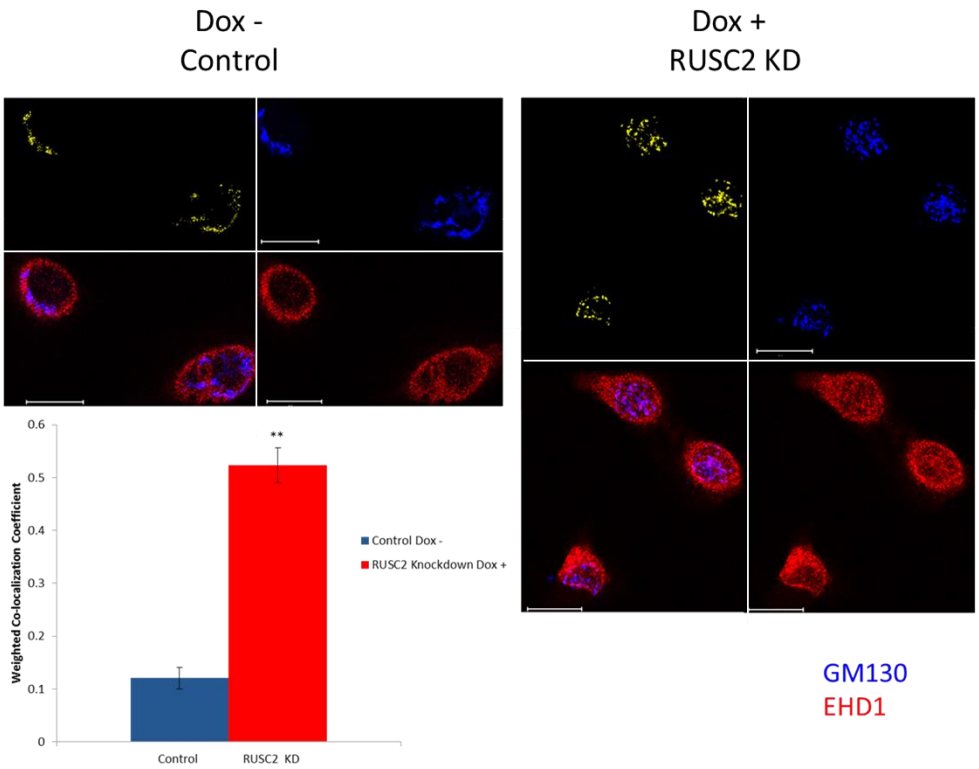


Figure 4.6 RUSC2 Is Required for EHD1 Transit Out of the Golgi:

Doxycycline-induced RUSC2 knockdown quantitatively increases the weighted correlation coefficient for GM130 and EHD1 in serum-depleted cells. Golgi morphology is visibly altered from its usual peripheral nuclear localization. Image and quantification is representative of 20 cells for each condition. Images were taken as 3-slice, serial Z-stacks from the cell apex towards the center at an interval of 0.4μm, and the average was taken for the highest observed weighted co-localization coefficient at GM130, the more static of the two markers.
Scale Bar 20 μm

9. Discussion

RTK activation is a critical mechanism regulating cell growth and survival, and has demonstrated key roles in the potentiation of oncogenesis, generating considerable interest in the mechanisms controlling RTK downstream signaling. Key studies have contributed to our understanding of RTK regulation in response to stimulation with their various ligands and the influence these have in mediating their signaling and traffic through the endosomal membrane system. A prototypical RTK, EGFR has been a model system for elucidating the molecular determinants and characteristics of transit for a great variety of cell surface receptors.

Using immunofluorescence analysis, we found that EGFR localizes to EHD1-positive membranes situated at the Golgi in human mammary epithelial cells under conditions of serum starvation and that EHD1 is required for the basal maintenance of receptor traffic out of a recycling-endosomal compartment marked by Rab11, GM130, and a novel uncharacterized protein, RUSC2. We saw an increase in EHD1-EGFR co-localization in the presence of monensin, which is known to block both anterograde traffic to distal organelles and also perturbs retrograde Golgi to ER transport which notably, occurs through the formation of Golgi-derived tubules (Barzilay 2005). While we have not exclusively ruled out a role for EHD1 in the biosynthetic transport of EGFR, we observed no anomalies in receptor maturation using ^{35}S metabolic labeling. We were able to demonstrate this compartment contains receptors internalized from the cell surface and the impact of an EHD1 knockdown-induced blockade has the effect of reducing total levels, surface expression and signal potentiation downstream of EGFR.

We observed similar phenotypic effects on EGFR upon knockdown of RUSC2. Of the RUN-domain containing proteins, RUSC2 is not well described but has several distinguishing features. Its closest homolog, NESCA, displays only ~30% identity in its amino acid sequence. NESCA has been demonstrated as binding to TrkA and has a direct role in neuronal differentiation by regulating dendritic spine dynamics as an adaptor protein for SNARE-mediated

vesicle fusion events and as a link to molecular motor complexes. NESCA has a cytoplasmic distribution that localizes to the nuclear envelope upon treatment with NGF (MacDonald 2004, 2012). Unlike NESCA, RUSC2 contains 2 NPF motifs, and displays strong co-localization with EHD1on tubules and vesicles. We suspect a physical interaction is dependent on the EH domain of EHD1 and may depend on the active conformation of a Rab-GTPase, a study currently underway in the lab.

RUSC2 has two unverified functional roles in the literature; one, in the basal unstimulated traffic of EGFR (Deribe, 2009) and another as an HIV-1 susceptibility gene (Brass 2008). We have now verified one of these, and are able to draw very apparent functional parallels to another EHD1 and Rab35 effector, MICAL-L1. Based on their described similarities (Sharma 2009), we expected EHD1 recruitment to the Golgi to be inhibited upon RUSC2 knockdown. Determination of whether such a role exists in EHD1-to-Golgi recruitment is substantially informative. Our preliminary study in RUSC2-inducible knockdown cells has attempted to answer this. Under control starvation conditions, a very limiting amount (of less than 2%) of the total intracellular EHD1 was present in the GM130 compartment, reflecting the dynamic nature of EHD1 vesicular localization. To our surprise, the RUSC2 knockdowns were seen as causing a statistically significant increase in the amount of EHD1 associated with GM130. If this result is correct, it would imply the EHD1-RUSC2 interaction is required for efficient EHD1 transit out of the Golgi, along with its associated cargo.

The RUSC2 binding partner GM130 has a recently described role in nucleation of microtubules through sequestration of importin-α and activation of spindle assembly factor TPX2 (Wei 2015). In muscle cells, microtubules were found to array uniquely in a grid-like distribution with Golgi elements at the vertices as major sites of nucleation in addition to elements at the nuclear envelope (Rios 2015). The shRNA-mediated knockdown of RUSC2 has possibly altered the distribution and proper functioning of GM130, and so an objective examination of the morphological appearance is under further review. Concurrent with knockdown of RUSC2, we

were able to observe a clear and quantifiable increase in the amount of EHD1 in association with Golgi-membrane marker GM130. Vesicular transport depends upon motor protein movement along microtubules. MICAL-L1 was demonstrated as being a critical link between EHD1 vesicles and dynein motors mediated (through an interaction with the collapsin response mediator protein-2 (Crmp2)), and required for EHD1 export from the ERC (Rahajeng 2010). It is intriguing to think that EHD1-dependent cargo transport is reliant either on Golgi-associated membranous tubules, or on the microtubule nucleating activity of GM130 at this organelle, and that this is somehow sensitive to RUSC2 inhibition. It would be interesting to see how microtubule morphology is affected by RUSC2 knockdown. How then do we reconcile a change in GM130 configuation and the direct interaction between RUSC2-EHD1 with retention at this compartment? Stable co-localization between RUSC2 and GM130, and no apparent Golgi morphological change upon EHD1-knockdown supports the idea that RUSC2 recruits EHD1 to facilitate its association with the microtubule based-motor assemblies required for forward cargo transport out. The observed increase in localization of EHD1 at this compartment under RUSC2-knockdown conditions is consistent with and suggestive of a second, unexamined mechanism responsible for bringing in EHD1.

It is also possible a shared and compensatory role for EHD4 exerts an effect on the observed EHD1 knockdown phenotype. We saw substantial upregulation of both EHD1 and 4 in response to serum depletion, and the rise in EHD4 was further accentuated during EHD1 knockdown. Interestingly, preliminary data in double-floxed EHD1/4 MEFs suggests that there is an accumulation of EGFR total levels similar to what we occasionally observed during EHD1 single knockdown experiments (data not shown). An exacerbation of phenotype would no doubt reflect a more substantial accumulation of intracellular EGFR. This seems reasonable given we were able to co-localize EHD4 to the same Golgi compartment where EHD1 knockdown promoted EGFR retention. Given the described role of EHD4 in promoting early endosomal character critical for the lysosomal avoidance and retrograde traffic of TrkA, we might presume a

similar circumstance exists for EGFR. We also saw EHD3 localization to this same compartment, while EHD2 was the standout and had very little correlation with EGFR under these conditions. EHD3 is required for maintenance of normal Golgi morphology, its knockdown resulting in dispersal that inhibited retrograde transport of Shiga Toxin, and altered the distribution of mannose-6-phosphate receptors, but didn't impact the secretion of VSV-G (Naslavsky 2009). EHD3 has a proposed role as an endosomal tubulator (in contrast to EHD1 and 4 as tubule-vesiculators), which was demonstrated in an *in vitro* study (Cai 2013), and implies that EHD3 might have a different but essential role within these same compartments intrinsic to the basic biological function of EHD family-mediated trafficking. We saw no comparable gross anomalies of Golgi morphology upon EHD1 knockdown, and to the best of our knowledge none have been reported. A couple of recent studies indicate EHD1 depletion altered Golgi polarization relevant to cytokinesis, while a MICAL-L1 knockdown partially disrupted orientation of the Golgi towards the direction of a scratch wound (Reinecke 2014, 2015).

EHD1 has been cited as having roles in the retromer-mediated endosome-to-Golgi retrieval process for the cation-independent mannose-6-phosphate receptor, and was required for the biosynthetic transport of VSVG-GFP (Gokool 2007, Zhang 2012). While EHD1 knockdown is frequently observed to have the consequence of impaired endocytic recycling and accumulation of cargo in a juxtanuclear compartment, no reports have directly tied this activity to the Golgi specifically, and no report has tied an EHD1-mediated *recycling* cargo to impaired Golgi exit.

A role for Rab11 in delivering early endosomal cargo to the Golgi has been known for some time (Wilcke 2000), while some of these pathways serve to maintain the composition and function of the Golgi, an examination of a role in sorting for a return trip to the surface membrane has only recently come into focus. This pathway seems to be regulated in part through the Rab11/EHD1 interactor FIP1/RCP which was recently shown to mediate traffic between early endosomes/recycling endosomes and the Trans-Golgi network through binding to Golgin-97 (Jing 2010). Recently explored work on an array of recycling surface receptors have parallel to the

work presented here. Studies implicate a GM130-Rab11 mediated pathway for the surface expression of the T-cell antigen receptor (TCR), which utilizes recycling to accumulate at the immunological synapse (Onnis 2015). This new phenomenon also doesn't appear to be restricted to RTKs. LGR5 is a Wnt pathway-associated G protein-coupled receptor (GPCR) constitutively internalized and trafficked to the TGN with as yet unknown functional outcome (Snyder 2013).

The newly identified Drosophila *Tempura* gene encodes a novel protein prenyltransferase subunit required for the normal localization of the Drosophila homolog of the EH Domain Binding protein 1 (dEHBP1). It exerts this effect through the geranyl-geranylation of Rab11, but was also shown to target Rab1. *Tempura* mutants cause a 'notum balding' phenotype, a condition describing the loss of external sensory organs from the dorsal thoracic region of the adult fly, a condition typical of impaired Notch signaling and faulty differentiation of cell fate from common progenitors (Charng 2014).

Loss of *Tempura* catalytic activity was shown to cause the intracellular accumulation of Delta and Sca, two proteins required for Notch signaling. Sca was found in a compartment that was GM130-positive (a marker for the cis- and medial- Golgi), but was not localized with Syntaxin 16 (a trans-Golgi marker), suggesting a defect in secretion. Delta, for its proper function, is endocytosed and recycled through a Rab11–Sec15–dEHBP1-dependent route to be re-targeted along an apical actin rich structure, where it is proposed to activate Notch receptor at the interface between two differentiating cells of the developing external sensory organ. *Tempura* mutants completely restricted the secretion of Sca, however, Delta localized normally (albeit was reduced) at the surface membrane, and also co-localized with Sca together within the GM130 compartment as well as in vesicles positive for the lysosomal marker Lamp-1. Concurrently, dEHBP1 was found to accumulate at the basal surface, while Rab11 was found aberrantly to accumulate apically. This is significant for two reasons; first, it implies that surface-membrane associated cargo dependent on EHD family members for their recycling get stuck in the Golgi

when that system goes awry. Second, those substrates are mixed with secretory cargo where defects can promote sorting to the endo-lysosomal system for degradation.

Endosomal accumulation of phosphorylated-EGFR upon ablation of Rab7 was found to be an apoptotic stimulus in Hela and MDA-MB-468 cells (Rush 2012). It is likely the purpose of recycling EGFR back and through the Golgi has roots in maintaining the homeostatic balance between new synthesis and the amount of unstimulated receptor available at the surface. Accumulation throughout this organelle and its channels may be a signal that receptor levels are high and should be attenuated. As overexpression of EGFR is transformative, this would seem to be a critical component of the cells failsafe mechanism.

Chapter V. Future Studies

10. Significance of EGFR Regulation by EHD Family Members

10.1 Mutant Variants of EGFR and their Recycling

Aberrant EGFR signaling as a result of receptor overexpression and/or mutation occurs in many types of cancer, and is frequently observed as an oncogenic driver in glioblastoma and NSCLC. Kinase domain mutations exhibit constitutive signaling and escape from Cbl-dependent ubiquitination and degradation that may include entry into the recycling endosome system. This study therefore is presented as an initial step to delineate the relationship between EGFR mutants and how their endocytic traffic drives oncogenic processes. The work described here may provide eventual insight towards novel therapeutic avenues in cancers that frequently develop resistance to the first line of treatment, tyrosine kinase inhibitors.

EGFR gene amplification and overexpression are observed in about half of all cases of glioblastoma medulla. The EGFR Type III, (EGFRvIII) mutant is detected in about one-third of all cases and half of cases where wild type EGFR is overexpressed. EGFRvIII is generated from a deletion of exons 2 to 7, resulting in an in-frame deletion of 267 amino acids from the extracellular domain of the receptor. EGFRvIII is unable to bind ligand and yet the receptor signals constitutively (Guo 2015). Most importantly, EGFRvIII has been shown to be defective in Cbl-mediated lysosomal degradation, is internalized at a decreased rate, and that pool of internalizing EGFR is preferentially recycled, highlighting our interest in elucidating the factors that characterize the routes the EGFR transit (Grandal 2007).

Lung cancer is the leading cause of cancer deaths worldwide, responsible for one-third of all cancer-related mortalities. A subset of those cancers, the NSCLCs of epithelial origin, make up 80% of all lung cancers (Sharma 2007). Conventional chemotherapy outcomes have been modest without significant benefit to patients. EGFR over-expression occurs in 60% of NSCLC cases, and its expression is correlated with a poor prognosis (Blackhall 2006). A subset of these, the bronchio-alveolar adenocarcinomas, exhibit EGFR mutations in over half of all cases, primarily among non-smokers of East Asian descent (Haneda 2006). The most common NSCLC-

associated mutations are in-frame deletions of codons 746-750 (EGFR Δ746-750) and a leucine to arginine point mutation at codon 858 in the full-length EGFR (Chung 2009). These EGFR mutations are located in the catalytic domain near the ATP-binding pocket, which is also the binding site for the clinical tyrosine kinase inhibitors gefitinib and erlotinib. These mutants display increased kinase activity potentiating these receptors with hyperactive, sustained signaling and enabling them with oncogenic properties. Remarkably, these render a subset of cancers particularly susceptible to therapy, likely the constitutively open conformation promotes drug binding and may also result in reduced affinity for ATP, which also must now compete against the drug. Still, rapid drug resistance develops within 6-12 months of current therapeutic intervention strategies. In 50% of relapsing patients, a secondary 'gatekeeper' mutation in the neighboring exon (T790M) is present, either the result of selected tumor outgrowth or pre-existing in a smaller number of total malignant cells. These complexities clearly underscore the necessity of furthering our understanding of the molecular regulation of EGFR.

Of other possible modes of acquired resistance to therapy, alterations in endocytic traffic have been proposed due to its intrinsic function woven into the fundamental biology of surface receptors. Avoidance of Cbl-dependent ubiquitination has been suggested and reported (Yang 2006, Padrón 2007). Mutant EGFR activation/phosphorylation is ligand-independent, and shown to be constitutively internalized at an accelerated rate relative to wild type EGFR under serum starved conditions in a panel of immortalized human bronchial epithelial cell lines stably transfected with various NSCLC-associated EGFR mutants (Chung 2009). At steady-state, these mutants were shown to be localized predominantly at lysosomes, but also within compartments containing transferrin, a marker of recycling endosomes. Using similar conditions to those utilized in the course of this work (Figure 3.8), Chung et. al. starved both mutant and wild type cell lines for 48 hours before treating them for 3 hours with an identical concentration of monensin, 10μM. Under these conditions, detected mutant EGFR was detected within a transferrin containing compartment, but not wild type EGFR (seen in both +/- EGF conditions).

The difference between those results and the data presented here could stem from the marker used (transferrin), but this is unlikely as EHD1 has been shown to regulate transferrin through the ERC. Another possibility might be that the starvation interval itself is not conducive to making a direct comparison between wild type and mutant EGFR. In Figure 3.4C, the pancreatic adenocarcinoma cell line S2013 has a clear reduction of EGFR at 48 hours of starvation relative to 24 hours. We have seen a similar reduction in total EGFR at 48 hours in 16A5 cells, suggesting this is a general phenomenon. This is hypothesized to be due to autophagy, as the cell's commitment to maintaining high levels of EGFR under serum starvation conditions may be uneconomical at longer intervals. Unfortunately, a biochemical assessment of whole cell lysates over time was not performed with the NSCLC and wildtype cell lines. This might reveal whether mutant receptors are more resistant to a degradation event under these experimental conditions. Total levels were assessed at the 48-hour time point in (Chung 2009) and results were mixed for mutant lines. As EGFR was overexpressed in all cell lines tested, wild type and mutant, it seems unlikely that the duration of monensin treatment was a significant factor, which was 3 hours opposed to the 6 performed for this work. It remains possible that if mutant EGFR is more favored for recycling, that under these conditions an undetectable amount of wild type receptor has escaped observation. Co-localization between mutant EGFR and EHD1-GFP was reported, but wild type EGFR was not examined.

The phosphorylation status of the receptor is another point of interest. As these mutant EGFRs are shown to be constitutively phosphorylated, they're also resistant to ubiquitination. That the EH network contains ubiquitin-recognizing motifs is not in question, however our results imply the machinery that recruits and directs EGFR to EHD1 compartments is phosphorylation-independent. Chung et. al.(2009) also reported increased mutant EGFR interaction with Src, which was required for transformation, contributes to constitutive phosphorylation and downstream activation of STAT3, Akt, Erk and Src. Src inhibitors reduced EGFR-Src association as well as functional outputs. EHD1 displays strong co-localization with Src, and

seems to be required for its activation. It would be interesting to examine whether the observed phenotype we have described is somehow Src-dependent under non-phosphorylated conditions. As Src is shown to be required for integrity of the Golgi via a dynamin2-dependent mechanism, what role it might have in directing EGFR traffic to this compartment (Weller 2010) could be interesting to assess. Also of interest, crystal structures have revealed that auto-inhibited, inactive EGFRs adopt a 'Src-like'/ 'CDK-like' conformation, which is refractory to binding with Erlotinib, and may explain why activating mutations potentiate the effectiveness of Gefitinib which is closely related (Zhang 2006). We assume the complex mediating EGFR traffic is through receptor binding to RUSC2, and this in turn interacts with EHD1 through its NPF motif, the possibility the event is dependent on this inactive 'Src-like' structural conformation is intriguing and a critical future consideration for rational therapeutic design.

9.2 Amphiregulin

Endocytic sorting directly regulates EGFR signaling. After encountering its ligand at the surface membrane, EGFR is internalized into a vesicle and is either recycled to the cell surface, or it is transported to lysosomes to be degraded. Sorting to lysosomes is controlled by Cbl-mediated ubiquitination of the receptor. A maturing endo-lysosome is characterized by a gradual increase in its pH, and sustained ubiquitination is dependent on the pH sensitivity and binding affinity of the ligand. There are seven ligands available to EGFR, each with unique differences in their oncogenic potential. Receptor recycling after binding with amphiregulin, TGF-α, or epigen allows for continued rounds of signaling, making them potent mitogens whereas EGF promotes degradation and the receptor must be resynthesized before initiating signaling cascades can continue. The clinical importance of this is highlighted in breast, ovarian and androgen-independent prostate cancer (Révillion 2008, Lafky 2008, Tørring 2000).

Amphiregulin (AR) is expressed in a number of epithelial cells types during development and is a critical regulator of homeostasis. It has demonstrated roles in mammary gland development, promotes tissue repair by stimulating proliferation of keratinocytes, and has

recently been described as being secreted by immune cells, whereby it assists with the resolution of cell-mediated inflammation (Zaiss 2015). Its low affinity interaction with EGFR has a distinct impact on its signaling potential, and may result in reduced internalization, sustained signaling, reduced degradation and modulates the MAP-kinase signaling pathway to differentially direct gene expression and cell response (Shankaran 2010). In this way, AR is capable of inducing both proliferation and cell differentiation and this is the basis for its name (Shoyab 1988).

The uniqueness of EGFR ligand-induced degradation makes it an ideal system in which to study the impact of EHD family members on the recycling fate under stimulation with its various ligands. It is our hypothesis that EHD family members are critical regulators of the recycling fate, and that EHD1 knockdown would accentuate degradation and attenuate the surface of expression of EGFR following stimulation with the ligands associated with recycling. We performed initial experiments aimed at testing this hypothesis. We transiently knocked-down EHD1 with either a control non-targeting, or siRNA directed against the 3' UTR of EHD1. Cells were serum-starved for 48 hours in preparation for stimulation by ligand, and treated with 2 nM AR for the indicated time course (Figure 5.1). We observed overall depleted levels of total EGFR in the EHD1 knockdown condition. Stimulation with AR caused activation of phospho-Erk, which peaked at 30 minutes in the control. Levels of phospho-Erk were diminished in the EHD1 knockdown condition. EGFR total levels appeared consistent and unchanged in the control over the observed interval, in line with our expectations. However, in the EHD1-knockdown condition total levels of EGFR may be slightly diminished at 90 minutes post-stimulation. Interestingly, levels of EGFR appeared to recover at later time-points. We further examined this phenomenon using flow cytometry. Cells were treated with siRNA and starved, and stimulated as before. After stimulation for the indicated intervals, cells were trypsinized and analyzed by flow cytometry for surface EGFR (Figure 5.2). We observed a statistically significant decrease in EGFR surface expression between knockdown and control. At 30 minutes surface EGFR appears to have decreased at comparable levels, suggesting the receptor is internalized at comparable

rates. At 270 minutes, (the endpoint of the assay) EGFR surface expression in the control cells was decreased slightly, however, we observed a significant increase in EGFR total level in the EHD1 knockdown condition. Finally, we sought to determine whether EGFR and EHD1 co-localize under conditions of AR treatment. 16A5 cells were plated onto coverslips and starved for 48 hours and treated with AR for 15 minutes before fixation. These cells were stained and visualized using confocal microscopy. Consistent with our predictions, we were able to see co-localization between EGFR and EHD1 in a juxtanuclear compartment (Figure 5.3).

EHD1 appears to regulate EGFR stimulated with AR. It is both interesting and counterintuitive that at later time points both total levels and surface expression appear to exceed those in the initial, pre-stimulated condition during EHD1-knockdown. These results must reflect at least some new synthesis of receptor. Its possible EHD1 is required to permit transit through the ERC where it undergoes 'slow' recycling. EHD1-knockdown under these conditions may have the effect of shunting EGFR traffic through the 'fast' recycling pathway, which was shown to be under control of Rab35 (Chaineu 2013). This may explain why surface levels seem to recover over those seen at time 0. This implies that fast-recycling keeps EGFR in closer proximity to the plasma membrane, which may also attenuate degradation by restricting itinerary away from machinery located deeper within the cell. Further study will be required to interpret these findings.

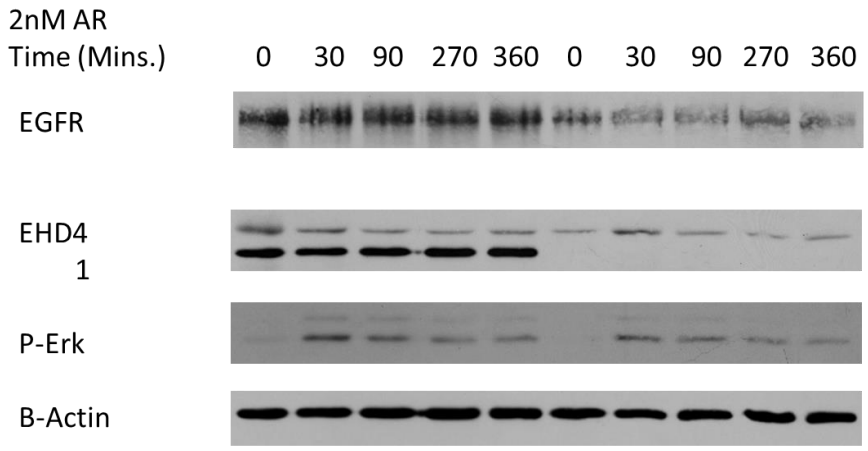


Figure 5.1: EHD1 KD Reduces Signal Potentiation Downstream of EGFR Upon Stimulation with Amphiregulin: 16A5 cells were depleted of serum and EGF for 48 hours. Cells were then stimulated with 2 nM Amphiregulin for the indicated time points. Whole cell lysates were resolved by SDS-PAGE and transferred to nylon membrane and probed with antibodies specific for the indicated proteins. The relatively unchanged EGFR levels upon stimulation are consistent with predominant recycling without ligand-induced degradation.

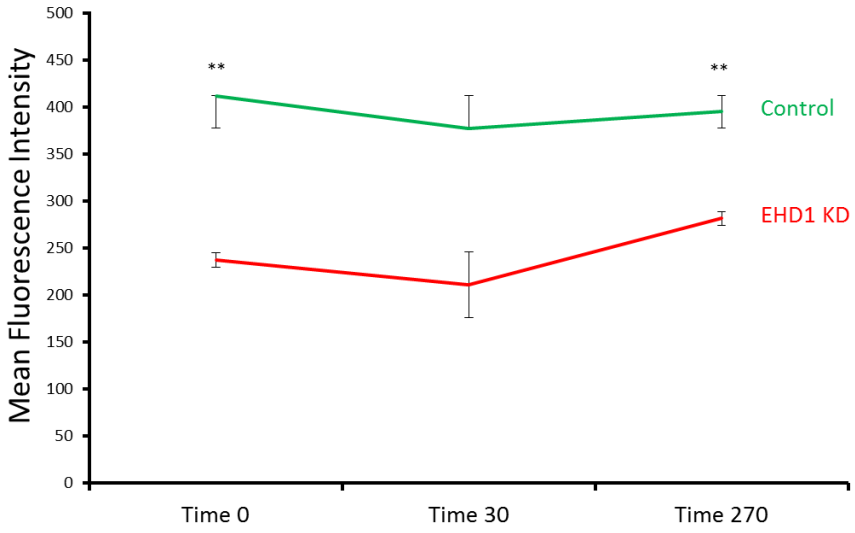


Figure 5.2 EGFR Surface Expression is Increased After Stimulation with Amphiregulin During EHD1-Knockdown: EGFR surface levels assayed by flow cytometry in non-targeting control and siRNA against EHD1. Error bars indicate standard error of the mean for 3 replicates of 50,000 cells.

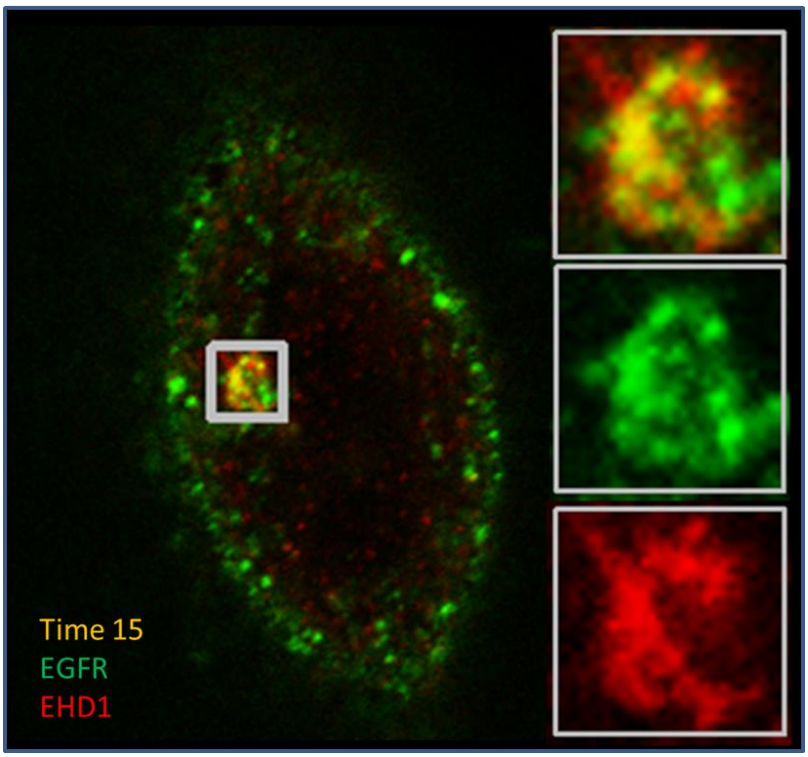


Figure 5.3 Amphiregulin Stimulation Localizes EGFR to an EHD1 Compartment: 16A5 cells were plated on coverslips, starved 48 hours, and stimulated for 15 minutes with amphiregulin before fixation and immunofluorescent staining.

Chapter 6
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