Stony Brook University

The Clathrin Adaptor Protein Eps15 is Recruited to Endosomes in a Ubiquitin-dependent Manner

A Dissertation Presented

by

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to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Genetics

Stony Brook University

August 2011

Stony Brook University

The Graduate School

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Abstract of the Dissertation

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2011

Downregulation of receptor tyrosine kinases and growth factor signaling is imperative for the prevention of malignancy formation. Eps15 is a ubiquitin-binding endocytic adaptor protein essential for clathrin-dependent endocytosis and this process. Although it has been well-studied at the plasma membrane, Eps15 has also been found at low levels on early endosomes. However, the basis of this localization is currently unknown. Thus, determining why Eps15 is recruited to different membranes is important for understanding its involvement in the attenuation of downstream signaling.

Using geldanamycin (GA), we induced the internalization and ubiquitination of ErbB2, a member of the EGF family of growth receptors. We found that in geldanamycin-treated SK-BR-3 breast cancer cells, FLAG-Eps15 colocalized with ErbB2-containing vesicles remarkably well as compared to steady-state cells. This recruitment suggested Eps15 may localize to endosomes in response to ubiquitinated cargo. To further test the existence of ubiquitin-dependent binding, we used constructs containing ubiquitin fused in-frame targeted to the plasma membrane (PM-GFP-Ub) or early endosomes (GFP-FYVE(Hrs)-UbΔGG). We found that FLAG-Eps15 also colocalized with these constructs at EEA1-positive endosomes. This implied the recruitment of Eps15 to endosomes is ubiquitin-dependent. Colocalization analysis of Eps15 mutant constructs also revealed that the UIM domains (ubiquitin interacting motifs) but not the EH domains were required for endosomal recruitment.

Hrs, a highly conserved ubiquitin sorting protein localizes to early endosomes and helps traffic ubiquitinated cargo to lysosomes for degradation. One possible method of Eps15 recruitment may be via its known interaction with this key endosomal sorting protein. However, silencing of the endosomal Eps15 binding partner Hrs did not affect recruitment of Eps15 to ubiquitin-enriched endosomes, suggesting an alternate mechanism.

Taken together, our findings suggest that Eps15 can be recruited to endosomes by directly binding ubiquitinated cargo. Recruitment of Eps15 to ubiquitin-rich endosomes may act as a rheostat, sequestering the protein away from the plasma membrane, slowing endocytosis to allow time for clearance of ubiquitinated cargo from endosomes. This provides evidence for the first time that ubiquitin is sufficient in recruiting Eps15 differentially to membranes, independent of clathrin-mediated endocytosis.

Dedication Page

I dedicate this work to my son, Dylan Archer, who has completed a part of me I never knew existed. He has given my life meaning and a newfound sense of purpose. I hope I can teach him to live his own life to the fullest and without regrets; to go where there is no path and leave a trail…

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List of Abbreviations

Ack1	Cdc42-associated kinase
AF	Alexa-fluor
AP-2	Adaptor protein-2
AML	Acute myelogenous leukemia
ATP	Adenosine triphosphate
BFA	Brefeldin A
BSA	Bovine Serum Albumin
Cbl	Casitas B-lineage Lymphoma
Cdc42	Cell division control protein 42
CHIP	C-terminus of HSC70-interacting protein
CRIB	Cdc42-binding domain
DMEM	Dulbecco's modified Eagle's medium
DPF	Aspartic acid-proline-phenylalanine
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EH	Eps15 homology
ENTH	Epsin N-terminal homology
Eps15	Epidermal growth factor receptor substrate 15
EEA1	Early endosomal antigen 1
ESCRT	Endosomal sorting complex required for transport
FYVE	Fab1p-YOPB-Vps27p-EEA1 domain
GA	Geldanamycin
Grb2	Growth factor receptor-bound protein 2
GFP	Green fluorescent protein
GTP	Guanine triphosphate
HA	Hemagglutinin

Hrs	Hepatocyte growth factor regulated tyrosine kinase substrate
HRX	Histone-lysine N-methyltransferase
HRP	Horseradish peroxidase
Hsp90	Heat shock protein 90
IF	Immunofluorescence
LDL	Low-density lipoprotein
MLL	Myeloid/lymphoid or mixed-lineage leukemia
MVB	Multivesicular bodies
NPF	Asparagine-proline-phenylalanine
PI(4,5)P2	Phosphatidylinositol-4,5-biphosphate
PI(3)P	Phosphatidylinositol 3-phosphate
RTK	Receptor tyrosine kinase
SAM	Sterile α-motif
SDS-PAGE	Sodium dodecyl polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SNX9	Sorting nexin 9
STAM	Signal transducing adaptor molecule
Tfn	Transferrin
Ub	Ubiquitin
Uba	Ubiquitin-associated
UBD	Ubiquitin binding domain
Ubl	Ubiquitin-like
UIM	Ubiquitin interacting motif

Acknowledgements

First and foremost, I would like to acknowledge my thesis advisor Dr. Deborah Brown. She has proven to be an exceptional mentor, and has taught me that science requires both patience and perseverance. Debbie encouraged me to work independently and yet was always there to offer guidance and support. I am so incredibly grateful for all the time and effort she has dedicated to both my training and this project.

I would also like to give special thanks to Dr. Todd Miller and Dr. Victoria Prieto-Echagüe for the opportunity to work with them on their projects. Their enthusiasm and love for their research is truly inspiring. I look forward to our continued collaborations in the future.

I am also thankful to have had such a wonderful group of people to work with during my time in the Brown lab. I would like to express thanks to Anne-Ostermeyer-Fay for being so helpful with all of my experiments and for all that she has taught me over the years. I would like to thank Dr. Prakhar Verma for being such a great friend and always keeping things fun and interesting around the lab. I must also give thanks to Dr. Daniel Barr, for helping me out in the beginning, especially with immunofluorescence experiments and to Dr. Laura Listenberger for all of her great advice as a woman in science.

I would also like to express gratitude to the kind members of my thesis committee, Dr. Wei-Xing Zong, Dr. Michael Frohman, Dr. Robert Haltiwanger, Dr. Michael Hayman and Dr. Simon Halegoua, for all of the time and constructive criticism they have given me in regards to this project.

A major component of my work involved confocal microscopy. Therefore, I would like to thank Dr. Guowei Tian from the Central Microscopy Imaging Center at Stony Brook for all of his assistance and training.

In addition, I would like to thank the Genetics program and the members of the incoming class of 2005. In particular Nadine Ulloa, for all of the great talks we had in the cell culture room and Dr. Michael Cressy for being such a valued friend. I must also express my appreciation for the incomparable Katherine Bell for her phenomenal work as our program coordinator.

I would also like to give thanks to Dr. Tracy Callendar for the kindness and advice she has shared with me throughout graduate school. I will treasure our friendship forever.

Moreover, this would not have been possible without the love and encouragement of my family members. In particular, I would like to thank my parents Agop Kevork and Verkin Janet Demirozu. They came as immigrants to this great country with essentially nothing to their names. Despite many obstacles, they were able to give my wonderful brother Vahram and I everything we could possibly ever need. They have always been, and will continue to be my biggest inspirations.

Last but certainly not least, I would like to share this accomplishment with my husband and best friend Raymond for being so incredibly supportive throughout the years. Your love and encouragement during this process has meant more than words can express.

CHAPTER I

Introduction

INTRODUCTION

Endocytosis is a process the cell uses to communicate with its environment via the internalization of extracellular molecules. Although there are several different endocytic pathways, the clathrin-mediated pathway is most understood. Internalization by this pathway requires precise coordination of a multitude of players as it is an important component of the regulation of surface receptor expression and signal transduction.

One example of this is the attenuation of growth-factor receptors such as the epidermal growth factor receptor (EGFR). Downregulation of EGFR is essential since it is involved in vital cell functions such as survival, proliferation and differentiation. Binding of EGF to the extracellular domain of EGFR at the plasma membrane initiates autophosphorylation, ubiquitination and the activation of the receptor and subsequent signaling pathways. EGF-induced downregulation of EGFR is the result of its rapid internalization and degradation in lysosomes (Sorkin and Goh 2008). My thesis work has involved studying two different endocytic adaptor proteins involved in this process.

The bulk of my research has focused on a tyrosine phosphorylation substrate of EGFR named Eps15 (Fazioli et al. 1992). Eps15 is essential for the stimulation of clathrin-mediated internalization and the formation of clathrin-coated pits. However, it has also been found to localize to endosomes (Bache et al. 2003a; Benmerah et al. 1999). Although its role at the plasma membrane has been comprehensively studied, its presence on early endosomes is less understood. The goal of this project has been to elucidate the reason for its recruitment to endosomes.

The second part of my thesis includes work performed on the non receptor tyrosine kinase, Ack1. This work was performed in collaboration with Dr. Todd Miller and Dr. Victoria Prieto-Echagüe. Ack1 is an adaptor protein that localizes to clathrin-coated pits and has the ability to bind directly to clathrin (Teo et al. 2001). Although relatively little is known about Ack1, it has been determined to play an important role in the trafficking of receptors internalized via the clathrin pathway (Grovdal et al. 2008; Shen et al. 2007). In chapter three, I will discuss our findings on the localization of Ack1 and its effect on endocytosis.

I. Clathrin-mediated internalization

Clathrin-mediated endocytosis is used for selective internalization of specific plasma membrane proteins in functions that include nutrient uptake and down-regulation of signaling receptors (Le Roy and Wrana 2005; Polo and Di Fiore 2006; Reider and Wendland 2011). Receptor-mediated endocytosis involving clathrin is well-understood. Binding of growth factors results in kinase activation, the initiation of effector molecules and a host of downstream signaling pathways (Doherty and McMahon 2009). Clathrin-mediated endocytosis is characterized by the initiation of clathrin-coated pits on the plasma membrane. This process is highly dependent on a diverse group of adaptors and other accessory proteins that help recruit clathrin and stabilize the formation of these structures on the plasma membrane (Fig. 1-1) (Brodsky et al. 2001). Clathrin provides the structural framework for the coat surrounding internalized vesicles, while a complex array of adaptor proteins are required both for coat formation and for recruiting specific cargo proteins to vesicles (Reider and Wendland 2011).

Polymerization of clathrin alone is insufficient for inducing curvature of the membrane. Epsin, an endocytic adaptor, helps in the deformation of the membrane by interacting directly with PI(4,5)P2 via its ENTH domain (Horvath et al. 2007). Other N-BAR and BAR domain-containing proteins such as amphiphysin, endophilin and sorting nexin 9 (SNX9) have also been shown to assist with membrane curvature by the insertion of amphipathic helices into the lipid bilayer (Arkhipov et al. 2009; Shin et al. 2008). These and other cargo adaptors help to link clathrin to the plasma membrane through interactions with phosphatidylinositols and another large complex of proteins known as AP-2 (Farsad et al. 2003; Lundmark and Carlsson 2002).

The AP-2 complex is a major component of clathrin-coated pits. It helps link clathrin to the underlying membrane by directly interacting with phosphatidylinositols at the cell surface (Gaidarov and Keen 1999). The epidermal growth factor receptor pathway substrate 15 (Eps15) has also been found to help form the clathrin coat through its constitutive association with α-adaptin, a member of the AP-2 complex, and plays a key role in clathrin-mediated endocytosis (Benmerah et al. 1998; van Bergen En Henegouwen 2009). This ubiquitin-binding endocytic adaptor protein acts as a scaffold, clustering AP-2 appendages through a long, flexible C-terminal tail (Schmid et al. 2006).

The clathrin coat itself has a triskelion shape composed of three clathrin heavy and light chains that come together to form a lattice on the plasma membrane (Smith et al. 1998). The

coat then begins to invaginate forming a pit (Brodsky et al. 2001). Adaptor proteins assist in the recruitment of activated receptors, further facilitating their regulated internalization.

The internalization of clathrin-coated vesicles is an extremely dynamic process and is dependent on the GTPase dynamin. Recently, endophilin has been implicated as well and may act as a complex with dynamin at the constricted neck of clathrin coated pits (Sundborger et al. 2011). Dynamin acts by wrapping a helical polymer around the neck of the fully formed clathrin-coated pit. Upon GTP hydrolysis, the vesicle is severed from the cell surface, irreversibly releasing it and allowing it to bud off the plasma membrane into the cytosol (Loerke et al. 2009). The lipid phosphatase synaptojanin facilitates the rapid disassembly and uncoating of clathrin from the vesicle which is then later recycled (Verstreken et al. 2003). Next, the newly formed vesicle is trafficked along the endocytic pathway so that cargo can be sorted and sent to its appropriate destination (Fig. 1-2) (Doherty and McMahon 2009).

Low-density lipoprotein (LDL) and transferrin receptors are well-known examples of cargo internalized via the clathrin-mediated pathway. Although these receptors are constitutively endocytosed to facilitate the uptake of nutrients such as cholesterol and iron, they are still recruited to clathrin-coated pits with intent. Accessory proteins such as AP-2 help select and incorporate these and other receptors into the coated pit by recognizing specific cytosolic sequences, examples of which include YXXϕ, NPXY and the dileucine motif. Moreover, mutation of these critical sequences causes the inability of their recruitment to clathrin-coated pits, demonstrating the how precisely regulated clathrin-mediated internalization is (Chen et al. 1990; Jing et al. 1990; Songyang 1999).

II. EGFR Internalization

The EGF family of growth receptors is composed of 4 structurally similar receptors (ErbB1-4 or Her1-4) that have the ability to bind a number of different ligands (Sweeney and Carraway 2000). Binding of growth factors results in receptor dimerization and cross-phosphorylation of the ErbBs and the subsequent activation of signaling pathways. Members of this family are often aberrantly expressed in cancer and have been implicated in the progression of a wide variety of different human tumors, including those of the breast, ovarian, lung, prostate, colorectal, and brain. This is in part due to excessive signaling of pathways they

mediate including that of cell growth, survival, differentiation, migration, and adhesion (Citri and Yarden 2006).

Stimulation with EGF on the extracellular domain induces the tyrosine phosphorylation and subsequent ubiquitination and internalization of EGFR (Goh et al. 2010). After its rapid internalization, EGFR is efficiently sorted and downregulated. This acts as a negative-feedback loop mechanism, preventing further receptor tyrosine kinase signaling. Receptors are trafficked to endosomes and are either rapidly recycled to the cell surface or continue along the endosomal pathway to late endosomes and later lysosomes for degradation (Jovic et al. 2010).

Although ubiquitination of EGFR is important for the recruitment of receptors into coated pits, it has recently been determined this modification is not absolutely required for internalization of the receptor, particularly in cells stimulated with a low concentration of EGF (Sigismund et al. 2005). Mutation of the lysine residues found essential for ubiquitination and downregulation of EGFR did not have an effect on its tyrosine phosphorylation nor on the rate of clathrin-mediated internalization (Huang et al. 2007). Internalization of EGFR also requires the activity of its ubiquitin ligase Cbl. Expression of siRNAs targeting Cbl, the ubiquitin ligase responsible for EGFR ubiquitintation, also resulted in an inhibition of EGF internalization (Stang et al. 2004). In addition, it has been reported that siRNA-mediated suppression of ubiquitin-binding endocytic adaptors such as epsin, Eps15 and Eps15R does not specifically inhibit the clathrin-mediated internalization of EGFR (Huang et al., 2004; Sigismund et al., 2005; Vanden Broeck and De Wolf, 2006; Chen and Zhuang, 2008).

In contrast, its association with endocytic adaptors may be important for the recruitment of active EGFR to clathrin-coated pits. siRNA-mediated knockdown of the growth factor receptor binding protein 2 (Grb2) resulted in a strong inhibition of the clathrin-mediated internalization of EGFR (Stang et al., 2004). It has also been found that the constitutive interaction of AP-2 and Eps15 is required for efficient receptor-mediated endocytosis of EGFR. Expression of a dominant-negative form of Eps15 that competitively inhibits its interaction with AP-2, resulted in a decrease of both EGFR and transferrin internalization (Benmerah, 1998). Moreover, internalization of EGFR also requires the growth factor binding protein Grb2 and the ubiquitin ligase Cbl. Expression of siRNAs targeting Cbl, the ubiquitin ligase responsible for EGFR ubiquitintation, also resulted in an inhibition of EGF internalization (Stang et al. 2004).

III. Eps15

Eps15 was originally discovered in a screen to identify tyrosine phosphorylation substrates of EGFR (Fazioli et al. 1993). In addition to its interaction with activated EGFR, Eps15 is of great interest since it is encoded on chromosome 1, locus p31-p32, an area of the genome subject to a high rate of chromosomal anomalies (Wong et al. 1994). Eps15 is often rearranged with Histone-lysine N-methyltransferase (HRX also known as MLL or ALL1), a transcription factor involved in certain forms of acute myeloid leukemia (AML). The HRX-ALL1-Eps15 fusion protein is expressed exclusively to the nucleus, whereas wild-type HRX also localizes in the cytosol. This suggests fusion with Eps15 upregulates HRX activity and may induce the onset of leukemia in these cells (Rogaia et al. 1997).

Eps15 is a modular protein (Fig. 1-4). Its multiple conserved domains allow for its interaction with many different proteins in different locations throughout the cell. Three N-terminal EH (Eps15 homology) domains at the N-terminus (DI) of Eps15 preferentially bind NPF motifs on a variety of other endocytic adaptor proteins (Salcini et al. 1997). EH domains have also been found to bind phosphatidylinositols, conferring the ability to bind different membranes. It has also been suggested this ability to bind both NPF motifs as well as phosphoinositides grants specificity to the binding of Eps15 to ubiquitinated proteins on membranes versus in the cytosol (Naslavsky et al. 2007).

A central coiled-coil domain mediates Eps15 oligomerization (Tebar et al. 1997) and binding to intersectin (Sengar et al. 1999) and the Met receptor (Parachoniak and Park 2009). Eps15 contains a domain of DPF repeats that bind AP-2, an abundant adaptor protein that links cargo and clathrin (Benmerah et al. 2000; Reider and Wendland 2011). Two ubiquitin interacting motifs (UIM domains) are located near the C-terminus of Eps15 (Polo et al. 2002). These UIM domains have been found to both recognize and bind ubiquitin (Polo et al. 2002). It has been proposed that ubiquitination of the UIM domain can affect EGFR trafficking and signaling by inhibiting the internalization and degradation of the receptor, thus prolonging signaling (Fallon et al. 2006).

Ubiquitin binding by Eps15 may play another role in endocytosis as well. In addition to binding ubiquitin via UIM domains, Eps15 and other endocytic adaptor proteins (Eps15R and epsins) are themselves mono-ubiquitinated (Polo et al. 2002). These proteins can bind each other via ubiquitin-UIM interactions and through other motifs (van Bergen En Henegouwen 2009),

suggesting the existence of a UIM-ubiquitin-based protein network at endocytic sites (Polo et al. 2002). Formation of this network could enhance endocytosis by increasing the local concentration of these proteins. In yeast, formation of such a network has been proposed to be the main function of ubiquitin-binding interactions of epsins and the Eps15-like protein Ede1 (Dores et al. 2010).

Moreover, although Eps15 has been implicated in cancer, its function may also be related to several other diseases. It has been reported that Eps15 may play a role in Troyer syndrome, a hereditary spastic paraplegia (Bakowska et al. 2007). Silencing of spartin, an interaction partner of Eps15 was found to inhibit the rate of EGFR internalization and subsequent degradation. More recently, there has been some insight on the regulation of Eps15. One study showed that an E3 ubiquitin ligase implicated in the development of Parkinson's disease, parkin, interacts directly with Eps15 (Fallon et al. 2006). Upon stimulation with growth factors, Eps15 associates with the Ubl domain of parkin, resulting in the monoubiquitination of Eps15 as well as the upregulation of E3 ligase activity of parkin. In addition, internalization of EGFR was found to be enhanced in fibroblasts devoid of parkin expression and delayed in cells overexpressing the E3 ubiquitin ligase. This suggested that parkin-mediated monoubiquitination of Eps15 functionally inhibit its activity and thus help control EGFR internalization and subsequent signaling pathways.

IV. Downregulation of ErbB2

ErbB2 is unique in that it lacks a soluble ligand and is thus very stable on the cell surface. It is activated when highly overexpressed such as in breast and ovarian cancer cell lines (Barros et al. 2010). In addition, despite high levels of expression, ErbB2 is predominantly found to localize on the plasma membrane. ErbB2 is the preferred heterodimerization partner amongst the other members of the EGF family. The association with the other ErbBs has also been found to result in the activation of ErbB2 and subsequent downregulation. Interestingly, surface expression of ErbB2 was not affected by activation of EGFR in cells expressing high levels of ErbB2 (Klapper et al. 1999). Thus, it is thought in these cells lines, heterodimerization with ErbB2 increases the stability of receptors, helping to potentiate signaling in comparison to homodimers of EGFR (Citri and Yarden 2006; Klapper et al. 1999).

ErbB2 is overexpressed in approximately 20-30% of breast cancer cell lines (Harari and Yarden 2000). This prominent role in cancer has prompted the development of therapies to downregulate its expression on the plasma membrane. Humanized monoclonal antibodies such as Trastuzumab (trade name Herceptin) has been successful in inducing the internalization and degradation of ErbB2 (Baselga et al. 1998). Patients identified with ErbB2-positive tumors are often treated with a combination of Herceptin and chemotherapy (Baselga 2001).

Geldanamycin (GA), a benzoquinone ansamycin antibiotic, has also been identified as an alternate therapeutic approach. Binding of ErbB2 to the chaperone Hsp90 is required for its stability. GA works by inserting into the ATP binding pocket of Hsp90, destabilizing its constitutive interaction with ErbB2 as well as other client proteins (Grenert et al. 1997). ErbB2 is particularly sensitive to treatment with GA and undergoes several changes including internalization, caspase-dependent cleavage of the cytosolic domain and ubiquitination by the ubiquitin ligase CHIP (Tikhomirov and Carpenter 2001; Xu et al. 2002). ErbB2 is internalized by a clathrin-independent pathway upon GA-treatment. Once it has entered the cell, it later merges with the classical endocytic pathway and is targeted to MVBs independently of signaling activity (Barr et al. 2008). This presented us an opportunity to characterize the recruitment of Eps15 to early endosomes, independent of the clathrin pathway.

V. The endocytic pathway

Early endosomes are mildly acidic vesicular structures that act as sorting stations for cargo internalized from the plasma membrane (Jovic et al. 2010). The conjugation of the small polypeptide ubiquitin, a highly conserved 76-amino acid regulatory protein determines whether cargo is recycled or trafficked to the lysosomes for degradation (Barriere et al. 2007). Ubiquitination is a reversible, post-translational modification in which a ubiquitin molecule is covalently attached to lysine residues of target proteins by E1 (ubiquitin activating), E2 (conjugating) and E3 (ligase) enzymes. The final destination of internalized cargo as well as a host of other regulatory functions are dependent on the type of ubiquitin modification (Hershko and Ciechanover 1998). For example, monoubiquitin or multiple mono-ubiquitin signals for endocytosis, K48 linked polyubiquitin chains direct proteosome-dependent degradation, and K63 linked polyubiquitin chains signal for endocytosis and other cellular processes such as DNA repair (Pickart 2004).

A. Hrs and STAM

Four different complexes known as endosomal sorting complexes (ESCRT) have been identified as endosomal sorting proteins for cargo destined for degradation (Kobayashi and Nobuyuki Tanaka 2005). Ubquitinated cargo at early endosomes first interact with well-conserved members of the ESCRT-0 complex, the main components of which are the Hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) and signal-transducing adaptor molecule 1/2 (STAM). Invagination of membranes initiates endosomal maturation, creating specialized vesicles called multivesicular bodies (MVBs) (Bache et al. 2003b). Both Hrs and STAM contain ubiquitin-binding domains (UBDs) that help facilitate the sorting of endocytosed membrane proteins along the degradative pathway (Fig. 1-3).

Hrs was found to recruit and activate ESCRT-I on endosomes, helping in the trafficking of ubiquitinated cargo to MVBs, where they can be further sorted for degradation (Bache et al. 2003b). Mutation of Vps27p, the yeast homolog of Hrs, resulted in what is known as class E (endosomal) sorting defect and a buildup of ubiquitinated cargo (Bishop et al. 2002). Moreover, siRNA inhibition of Hrs resulted in decreased degradation of EGF-stimulated EGFR, suggesting Hrs is essential in the process of ligand-induced downregulation of receptors (Raiborg et al. 2008). Interestingly, overexpression of Hrs has the same affect of inhibiting trafficking of EGFR by causing an accumulation of the receptor on early endosomes (Morino et al. 2004). Drosophila embryos mutant for Hrs demonstrated increased receptor tyrosine kinase signaling, further demonstrating the importance of Hrs on early endosomes (Jekely and Rorth 2003).

In addition to its existence as a complex with STAM on early endosomes, Hrs has been found to recruit and bind clathrin as well. The C-terminus of Hrs contains a clathrin box motif that interacts with the clathrin heavy chain. Hrs colocalizes with clathrin in specialized microdomains located on early endosomes. Eps15 also directly interacts with the ESCRT-0 complex in both cytosolic and membrane fractions of cells (Bache, 2003b). Moreover, it has been reported that an isoform of Eps15, Eps15b is associated with HRS on endosomes. Eps15b is a truncated isoform of Eps15, derived from an alternative start codon and may be important in the sorting function of this complex, perhaps providing an additional mode of recruiting ubiquitinated cargo to endosomes (Roxrud et al, 2008). Taken together, this suggests Eps15 may play a role in recognizing ubiquitinated cargo, independent of its role at the plasma membrane.

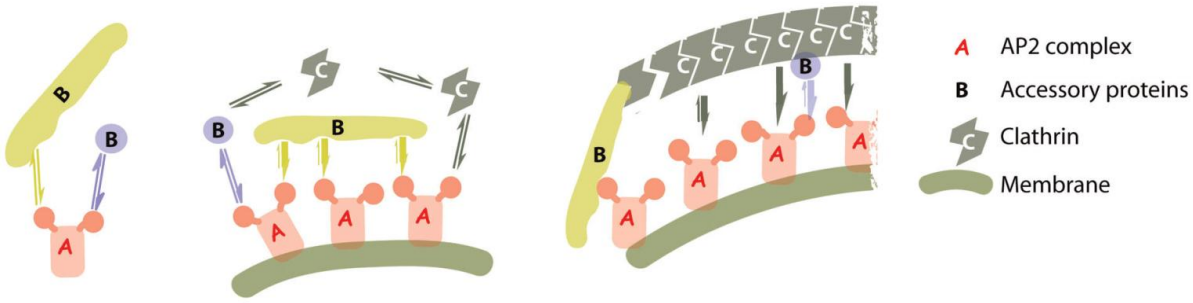

Figure 1-1. Depiction of clathrin-coated pit assembly (adapted from Schmid et al. 2006). The AP-2 complex (A) associates with accessory proteins (B), stimulating the polymerization of clathrin (C). As the pit matures and clathrin continues to polymerize and accessory proteins are displaced to the edges of the pit while it is continues to grow, forming a clathrin-coated pit.

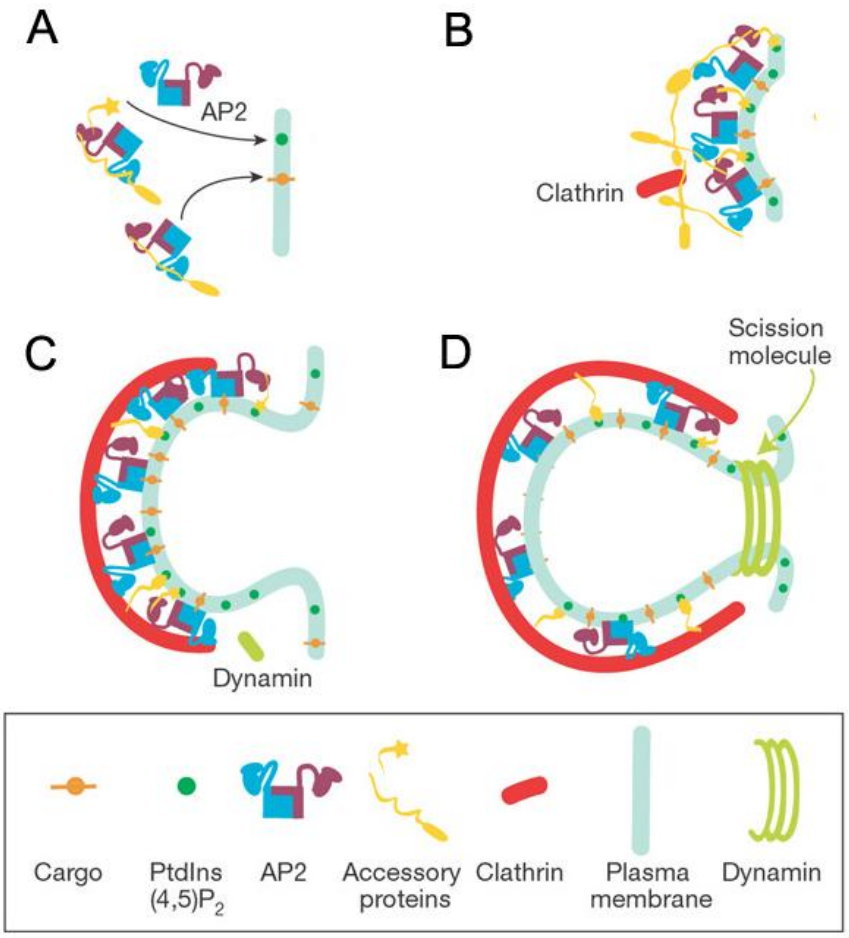

Figure 1-2. Formation and budding of clathrin-coated vesicles (adapted from Schmid and McMahon 2007). A. The clathrin coat assembles at the plasma membrane with the assistance of the AP-2 complex which links clathrin to PI(4,5)P2. B-C. Accessory proteins such as Epsin and the BAR domain-containing proteins amphiphysin and SNX9 help in the deformation and invagination of the membrane. Dynamin is then recruited (C) and the vesicle is severed from the membrane upon hydrolysis of GTP to GDP (D). The vesicle later uncoats in an ATP-dependent manner as it is trafficked along the classical endosomal pathway.

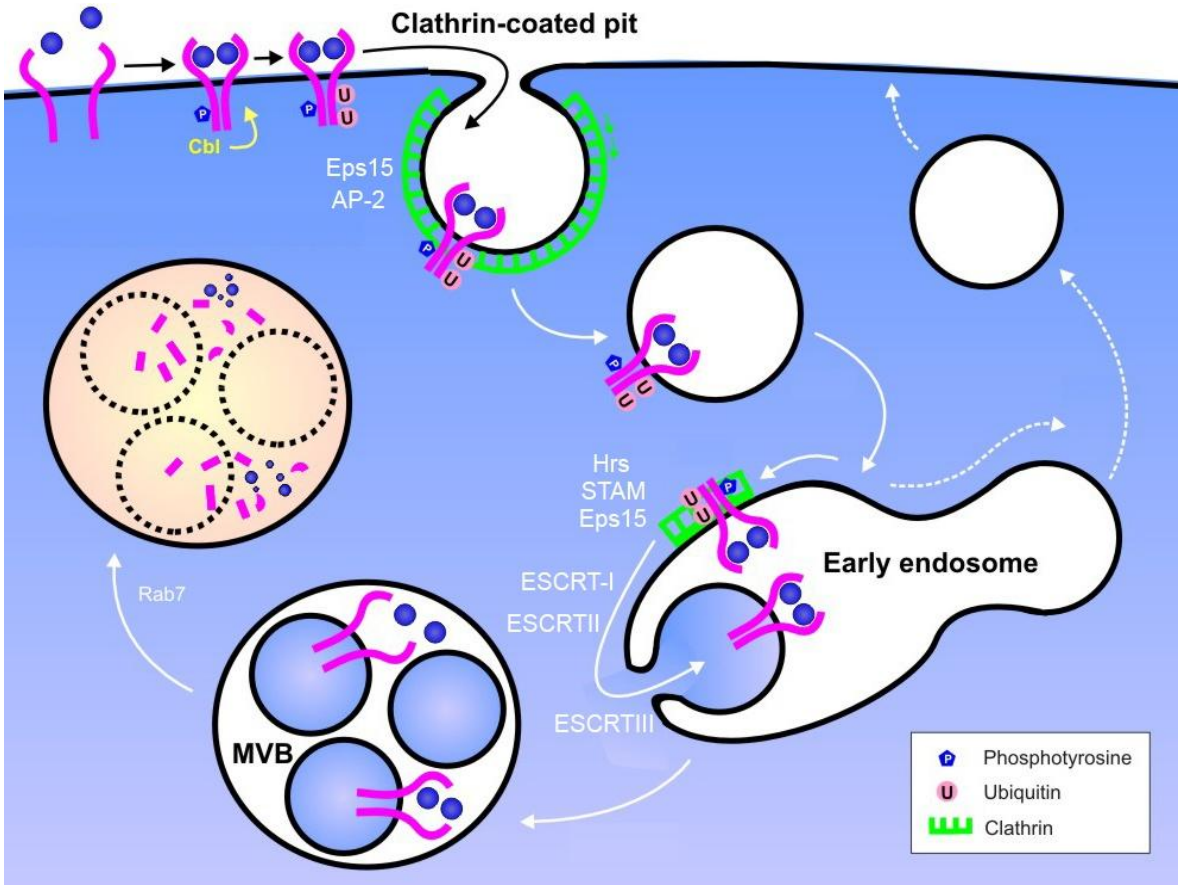

Figure 1-3. Downregulation of endocytic RTKs (adapted from Bache et al. 2004). Receptor tyrosine kinases such as EGFR are activated at the plasma membrane upon ligand binding. This triggers autophosphorylation via dimerization and ubiquitination via the E3 ubiquitin ligase Cbl. Tyrosine phosphorylation and ubiquitination of EGFR stimulates the recruitment of EGFR into clathrin-coated pits by ubiquitin-binding endocytic adaptor proteins such as Eps15. The receptor is then internalized and fuses with early endosomes where it is recognized and sorted by Hrs and STAM. Hrs and STAM pass along ubiquitinated cargo to ESCRT complexes I, II and III for additional sorting, thus facilitating transport to multivesicular bodies followed by lysosomes for degradation.

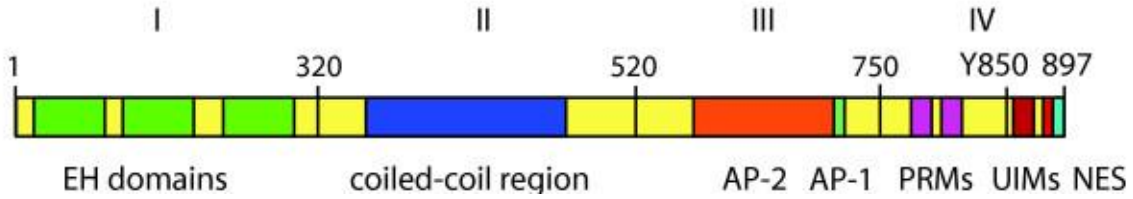

Figure 1-4. Domain structure of Eps15 (adapted from van Bergen En Henegouwe et al. 2009). Conserved domains of Eps15 are depicted in this schematic diagram. Eps15 Homology or EH domains are shown in green near the N-terminus of the protein in domain I. The coiled-coil region is centrally located and in blue in domain II. Downstream of the coiled-coiled region in domain III is the AP-2 binding regions shown in orange. Proline rich motifs are shown in purple. The UIM domains are at the C-terminus of Eps15 in domain IV.

CHAPTER II

Eps15 is recruited to endosomes in a ubiquitin-dependent manner

INTRODUCTION

Since its discovery, Eps15 has been well-studied and its role at the plasma membrane has been clearly defined. EM studies have confirmed the existence of Eps15 at the rim of clathrin-coated pits raising the possibility of its involvement in endocytosis (Cupers et al. 1998; Edeling et al. 2006; Tebar et al. 1996) and is thought to participate in the formation of the clathrin lattice. Consistent with this idea, microinjection of antibodies against Eps15 as well as dominant-negative Eps15 constructs inhibit endocytosis of diverse cargoes, including transferrin receptor and the epidermal growth factor receptor (EGFR) (Benmerah et al. 1998). Furthermore, Eps15 stimulates the rate of clathrin coat formation by the clathrin adaptor AP180 (Morgan et al. 2003).

In addition to this general role in clathrin coat assembly, Eps15 has a special relationship with EGFR and other ubiquitinated endocytic cargo. Eps15 was originally identified as an EGFR substrate (Fazioli et al. 1993), and is phosphorylated by EGFR on Tyr 850 (Confalonieri et al. 2000). Overexpression of an Eps15 mutant lacking this residue blocks endocytosis of the EGFR, but not the transferrin receptor (Confalonieri et al. 2000). Although a substantial pool of Eps15 is constitutively present in clathrin-coated pits, additional Eps15 is recruited there in response to EGFR signaling (de Melker et al. 2004; Torrisi et al. 1999). UIM domain-dependent binding of Eps15 to EGFR and other ubiquitinated proteins at the plasma membrane has been proposed to recruit ubiquitinated proteins to clathrin-coated pits and facilitate their endocytosis (Barriere et al. 2006; Catarino et al. 2011; de Melker et al. 2004; Hawryluk et al. 2006; Murakami et al. 2009).

Eps15 can localize to endosomes as well as the plasma membrane (Bache et al. 2003b; de Melker et al. 2004; Kanazawa et al. 2003; Sorkina et al. 1999; Torrisi et al. 1999). Eps15 is essentially undetectable in endosomes in resting cells (Roxrud et al. 2008; Tebar et al. 1996; van Delft et al. 1997), but is recruited there following EGFR signaling (de Melker et al. 2004; Torrisi et al. 1999). The function of endosomal Eps15 is not clear, and it is not known how the protein is targeted there by EGFR signaling. Endosomal recruitment might require tyrosine phosphorylation, either of Eps15 itself or another EGFR substrate. Alternatively, as activation of EGFR results in ubiquitination and endosomal delivery of the receptor (Hurley 2008), Eps15 might be recruited to endosomes by binding of its UIM domains to ubiquitinated EGFR. In either case, it is not known whether endosomal recruitment of Eps15 requires Hrs. Hrs, together

with its binding partner STAM, forms the ESCRT-0 complex that binds ubiquitinated cargo upon its delivery to endosomes, and then passes it on to downstream ESCRT complexes for eventual degradation in lysosomes (Hurley 2008). Eps15 can bind Hrs (Bache et al. 2003b; Bean et al. 2000), but the role of this interaction in EGF-dependent endosomal recruitment of Eps15 is not known.

We tested the role of ubiquitinated cargo in endosomal recruitment of full-length Eps15. We examined three ubiquitinated proteins that can accumulate in endosomes in the absence of EGFR or other tyrosine kinase activity. We also examined two hybrid proteins containing membrane targeting signals linked to in-frame ubiquitin (Chen and De Camilli 2005). PM-GFP-Ub is initially targeted to the plasma membrane, but accumulates in endosomes following constitutive internalization, while GFP-FYVE-UbΔGG is targeted directly to early endosomes via a PI(3)P-binding FYVE domain (Chen and De Camilli 2005). We found that Eps15 was recruited to ubiquitin-rich endosomes in all three cases. Recruitment did not require Hrs or tyrosine kinase activity, but did require the UIM domains of Eps15. We also found that Eps15 could be recruited to ubiquitin-rich plasma membrane clusters formed by PM-GFP-Ub. Together, these results suggest that Eps15 can be recruited to ubiquitin-rich endosomes via UIM-domain interactions.

MATERIALS AND METHODS

Cells and Transfection

COS-7, HeLA and SK-BR-3 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% iron-supplemented calf serum (JRH, Lenexa, KS) and penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37°C.

SK-BR-3 cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. COS-7 and HeLa cells were transfected with either Lipofectamine 2000 or polyethylenimine (PEI) reagent. For PEI transfection in 35-mm dishes; transfection mixture was prepared by adding 1 µg of DNA per 6 µL of 25mM deacylated linear PEI 22 (Sigma Aldrich, St. Louis, MO) into 100 uL of 0.15M NaCl and was immediately

vortexed. The mixtures were left at room temperature for 10 minutes before being added drop wise to cells already in regular media. The cells were then left to incubate for 24 hours at 37°C.

Antibodies, fluorescent compounds, and other reagents

Mouse monoclonal anti-ErbB2 N28 used for cell-surface detection in IF experiments was from LabVision (Fremont, CA). Lab Vision mouse monoclonal anti-ErbB2 (Ab-20 cocktail) was used for Western blotting. Anti-FLAG antibodies: for IF, rabbit polyclonal anti-DYKDDDDK was from Pierce Antibodies, Thermoscientific (Rockford, IL). For Western blotting, mouse monoclonal Anti-FLAG® M2 antibody from Sigma-Aldrich (St Louis, MO). Mouse monoclonal anti-EEA1 and anti-Eps15 (clone 17) were from BD Biosciences (San Jose, CA). Mouse monoclonal anti-EGFR mouse (Ab-12 cocktail) was Thermoscientific (Rockford, IL). Anti-Hrs (A-5) mouse monoclonal was from Alexis Biochemicals, Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). Mouse monoclonal anti-Hsp70 was from Santa Cruz (Santa Cruz, CA). Rabbit anti-HA antibodies were from Sigma Aldrich (St. Louis, MO). Horseradish peroxidase (HRP)-goat anti-mouse IgG and HRP-donkey anti-rabbit IgG was from Jackson Immunoresearch (West Grove, PA). AlexaFluor (AF)-488, 594, 647 and 680-conjugated goat anti-mouse and goat anti-rabbit IgG and AF-594-Tfn were from Invitrogen (Carlsbad, CA).

Other reagents: GA (used at 5 μM) was from 25 the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). Epidermal growth factor (EGF) was from Calbiochem, EMD Biosciences (Gibbstown, NJ).

Plasmids

pEGFP-N1 was from Clontech (Mountain View, CA). Plasmids encoding FLAG-Eps15, FLAG-Eps15-ΔUIM, FLAG-Epsin (Sigismund et al. 2005), PM-GFP, PM-GFP-Ub and pEGFP-2xFYVE-UbΔGG (Chen and De Camilli 2005) were gifts of Dr. P. DeCamilli (Yale University, New Haven, CT). pcDNA3 ErbB2-GFP (Liu et al. 2007) gift of P. Liu (Univ. North Carolina, Chapel Hill, NC). Plasmids encoding FLAG-Eps15-ΔI, FLAG-Eps15-ΔII, FLAG-Eps15-ΔIII, FLAG-Eps15-ΔIV and FLAG-Eps15-Y850F (Klapisz et al. 2002) were the gifts of E. Fon (McGill University, Montreal, Canada). HA-intersectin (Mohney et al. 2003) was a gift of Dr. John P. O'Bryan (University of Illinois at Chicago College of Medicine).

Fluorescence microscopy

Cells were seeded on acid-washed glass coverslips in 35mm dishes, transfected and examined 1 day after transfection. Cells were fixed in phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.4) containing 3% paraformaldehyde for 30 minutes, permeabilized at room temperature with PBS containing 0.5% Triton unless otherwise noted. The cells were then blocked with PBS containing 3% BSA and 10 mM glycine. Primary and secondary antibodies were diluted in 3% BSA and 10 mM glycine. Cells were incubated with primary antibodies for 1 hour at room temperature, followed by secondary antibodies for 30 minutes, also at room temperature. Cells were photographed and images were captured using a Zeiss inverted Axiovert 200 M microscope with a two-photon laser scanning confocal system. All images were acquired with 100x oil immersion objective (N.A. = 4.5) and processed with Zeiss LSM software. When necessary, images were further processed using Adobe Photoshop, adjusting contrast and/or brightness for optimal viewing. Each channel was adjusted separately and changes were applied to the whole field.

Colocalization Analysis

Images obtained from the Zeiss LSM two-photon confocal system were used for colocalization analysis. Analysis was performed with NIH Image J (http://rsb.info.nih.gov/ij/) and the JaCoP plug-in (Bolte and Cordelières 2006) as described previously (Barr et al. 2008). Manders' overlap coefficients were reported and can be interpreted as percent colocalization.

Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed then transferred to nitrocellulose and incubated with appropriate primary and HRP-conjugated antibodies. Western blotting and detection of bands on film by enhanced chemiluminescence were performed as described (Schroeder et al. 1998). Where indicated, bands were scanned and quantified using NIH ImageJ.

siRNA silencing

siRNA duplex constructs to knock down Hrs and Eps15 were made using Stealth RNAi™ siRNA technology from Invitrogen. The sequence for Eps15 was targeted to the C-terminal domain of the protein Eps15 was described previously (Huang et al. 2004; Sigismund et al. 2005). The sense sequence used was AAACGGAGCUACAGAUUAU. The targeted sequences for Hrs were GCACGGUAUCUCAACCGGAACUACU (siRNA-1), CAGAAUCUCAUGACCACCCUCCCAA (siRNA-2). The Medium GC StealthRNAi™ siRNA Duplex (Invitrogen #12935-300) was used as a control for both knockdown experiments. For Hrs knockdown, COS-7 cells grown were seeded in 35 mm dishes and transfected with 1 µg each of FLAG-Eps15 and GFP-FYVE-UbΔGG using Lipofectamine 2000 in Optimem media according to the manufacturer's specifications. Samples were left for 5 hours to incubate before 50 nM RNAi oligos along with 5 µL of Lipofectamine 2000 were added to the existing media. After 24 hours of transfection, the cells were trypsinized and divided in half. Half of the cells went into a 35mm dish with a coverslip (for immunofluorescence analysis) the other half went into a separate 35 mm dish (for Western blotting to confirm Hrs knockdown and plasmid expression). Cells were left to grow for another 24 hours in DMEM containing 10% calf serum. Samples were then processed for immunofluorescence and Western blotting as described 48 hours post-transfection. For Eps15 knockdown, SK-BR-3 cells were seeded in 35 mm dishes without coverslips. Cells were transfected using 5 µL of Lipofectamine 2000 and 100 nM RNAi oligos in Optimem media according to the manufacturer's specifications for 24 hours. After 24 hours, Optimem was replaced with DMEM containing 10% calf serum and left an additional 48 hours (72 hours total) before the ErbB2 degradation assay was performed.

ErbB2 Degradation Assay

Cells were treated with geldanamycin for 0, 2, 4 or 6 hours before performing SDS-PAGE. Triplicate samples were used in three individual experiments to control for variability. Lysates were transferred to nitrocellulose and incubated with the appropriate primary antibodies. Bands were labeled with AF-680-secondary antibodies and detected and quantified with the Odyssey-Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), using the Odyssey imaging software.

Transferrin binding and internalization

To measure cell-surface transferrin receptor levels, SK-BR-3 cells (transfected as described in the legend to Fig. 2-15) were incubated on ice for 2 hours with 50 ng/ml AF-594-transferrin in growth media, and then processed for IF as described above. To measure transferrin internalization, pre-warmed media containing 50 ng/ml transferrin was added to cells at 37˚C for 5 minutes before fixation and processing for IF. Values shown are averages from two separate experiments, analyzing at least 50 cells on each slide. All images were captured using the confocal microscope described above. The same acquisition parameters were used for all images to be compared quantitatively, and images were not further processed after acquisition. Mean intensity fluorescence (MFI) of individual cells was calculated using the histogram macro on the Zeiss LSM software.

EGFR surface expression and internalization assays

Anti-EGFR antibodies (2 μg/ml) were bound to transfected SK-BR-3 cells for 1 hour at 4˚C. To quantitate cell-surface EGFR, cells were fixed but not permeabilized before incubation with AF-594 secondary antibodies and processing for IF as described above. To measure EGFR internalization, antibody-bound cells were incubated with pre-warmed growth media containing 100 ng/ml EGF for 10 minutes at 37˚C. Residual surface-bound antibodies were then stripped from cells on ice with acid (100 mM Gly, 50 mM KCl, 20 mM MgOAc, pH 2.3), using 3 washes of 3 minutes each. Cells were then fixed, permeabilized, and incubated with AF-594 goat anti-mouse IgG antibodies. The experiment was repeated 3 times, quantitating MFI of at least 10 cells per slide as described above for transferrin experiments. Background fluorescence, determined as the average MFI in the nuclei of 5 cells, was subtracted from all values for internalized EGFR.

RESULTS

FLAG-Eps15 is recruited to endosomes in GA-treated SK-BR-3 cells

ErbB2 is expressed at high levels on the plasma membrane of SK-BR-3 breast cancer cells (Fig. 2-1). Transfected FLAG-Eps15 localized to the cytosol and small puncta in these cells, as expected (Fig. 2-1A). GA induces ErbB2 ubiquitination, internalization, and transport

to early and late endosomes and lysosomes, where it can be detected by immunofluorescence (IF) microscopy (Austin et al. 2004; Barr et al. 2008). Upon GA treatment of transfected SK-BR-3 cells, FLAG-Eps15 re-localized to endocytic organelles, where it colocalized with internalized endogenous ErbB2 (Fig. 2-1B).

To study this behavior further, we expressed wild-type or mutant FLAG-Eps15 in COS-7 cells, and examined colocalization with the early endosome marker EEA1 under various conditions, quantitating the colocalization as described in Methods. When expressed alone (not shown) or together with GFP (Fig. 2-2A), FLAG-Eps15 had a fine punctate distribution, as in SK-BR-3 cells, both with (Fig. 2-2A, E) and without (Fig. 2-2E) GA. In agreement with earlier reports (van Delft et al. 1997), very little FLAG-Eps15 was present on early endosomes (Fig. 2-2A, E). When FLAG-Eps15 was co-expressed with GFP-tagged ErbB2 in these cells, Eps15 localization was similar to that in cells co-expressing GFP, with little endosomal localization (Fig. 2-2E). However, after GA treatment, both Eps15 and ErbB2-GFP were strongly recruited to endosomes (Fig. 2-2B, E). This required the UIM domains of Eps15, as a construct lacking these domains (Sigismund et al. 2005) did not localize to endosomes even when co-expressed with ErbB2-GFP in GA-treated cells (Fig. 2-2 D, E). Together, these results showed that Eps15 was recruited to endosomes when ubiquitinated ErbB2 accumulated there, in a UIM domain-dependent manner.

Eps15 is recruited to endosomes containing PM-GFP-Ub

As GA releases Hsp90 from many proteins (Wandinger et al. 2008), it was possible that endosomal recruitment of Eps15 depended on effects that were independent of ErbB2. To provide more direct evidence that ubiquitinated cargo could recruit Eps15 to endosomes, we examined cells expressing PM-GFP-Ub. This construct encodes ubiquitin fused to GFP and to a dually-acylated plasma membrane targeting signal derived from the Src-family kinase Lyn (Chen and De Camilli 2005; Kovarova et al. 2001). Although PM-GFP-Ub is initially targeted to the plasma membrane, it also accumulates in endosomes, probably by ubiquitin-dependent trapping by the ESCRT machinery following endocytosis (Chen and De Camilli 2005; Fig. 2-3). By contrast, PM-GFP, which lacks in-frame ubiquitin, is not enriched in endosomes. We found that Eps15 accumulated on endosomes in cells expressing PM-GFP-Ub, but not in cells expressing PM-GFP, in a UIM-dependent manner (Fig. 2-3).

PM-GFP or PM-GFP-Ub was expressed in COS-7 cells in the presence of FLAG-Eps15 or FLAG-Eps15-ΔUIM. FLAG-Eps15 but not FLAG-Eps15-ΔUIM colocalized remarkably well with PM-GFP-Ub at early endosomes (Fig 2-3B, D). Colocalization of FLAG-Eps15 and EEA1 increased significantly in the presence of PM-GFP-Ub as compared to PM-GFP (Fig. 2-3E). There was no significant difference in the colocalization of FLAG-Eps15-ΔUIM with EEA1 in cells transfected with either PM-GFP or PM-GFP-Ub, suggesting the UIM domains are responsible for this recruitment.

Eps15 is recruited to endosomes containing GFP-FYVE-UbΔGG

These results suggested that Eps15 is recruited to endosomes in a ubiquitin-dependent manner. Nevertheless, as PM-GFP-Ub is first targeted to the plasma membrane, it was possible that components required for endosomal recruitment of Eps15 – or Eps15 itself - might be transported from the plasma membrane to endosomes together with PM-GFP-Ub. For this reason, we next examined GFP-FYVE-UbΔGG, which consists of ubiquitin fused to GFP and to the Hrs FYVE domain (Chen and De Camilli 2005), targeting the protein directly from the cytosol to PI(3)P-rich endosomes. When co-expressed in COS-7 cells, Eps15 accumulated together with GFP-FYVE-UbΔGG on EEA1-positive endosomes (Fig. 2-4B). This provided further support for the idea that Eps15 was recruited to ubiquitin-rich endosomes.

Surprisingly, some Eps15 was recruited to endosomes in cells expressing GFP-FYVE itself (not shown). We speculate that overexpression of this construct disrupted endosome function by competing with endogenous FYVE domain proteins – including Hrs – for endosome binding. This could cause accumulation of endogenous ubiquitinated cargo in endosomes, which could recruit Eps15.

The UIM domains of Eps15, but not the EH or coiled-coil domains, are required for recruitment to ubiquitin-containing endosomes

We next mapped the region(s) of Eps15 required for endosomal recruitment using a series of Eps15 deletion mutants. FLAG-Eps15-ΔI lacks the N-terminal EH domains, while FLAG-Eps15-ΔII is an internal deletion lacking the central coiled-coil domain (Klapisz et al. 2002; schematized in Fig. 2-5A). FLAG-Eps15-ΔIII, FLAG-Eps15-ΔIV, and the FLAG-Eps15-ΔUIM construct used in Figs. 2-1 and 2-2 are C-terminal truncation mutants: FLAG-Eps15-ΔIII

lacks most of the DPF-repeat region required for AP-2 binding and the UIM domains, while both FLAG-Eps15-ΔIV and FLAG-Eps15-ΔUIM lack the UIM domains (Klapisz et al. 2002; Sigismund et al. 2005). Except for FLAG-Eps15-ΔIV, which for unknown reasons was poorly expressed, the constructs were expressed at similar levels in COS-7 cells (Fig. 2-5B).

We next expressed the Eps15 constructs together with GFP or GFP-FYVE-UbΔGG in COS-7 cells and measured colocalization of the Eps15 proteins with EEA1. Selected images are shown in Fig. 2-6, and quantitation of the colocalization is shown in Fig. 2-7. FLAG-Eps15-ΔI and FLAG-Eps15-ΔII were recruited quite efficiently to endosomes in cells expressing GFP-FYVE-UbΔGG, showing that the EH and coiled-coil domains were not essential for endosomal localization (Fig. 2-6). The modest decrease in endosomal recruitment of FLAG-Eps15-ΔII, compared to wild-type Eps15, might result from the inability of this construct to oligomerize (Cupers et al. 1997). As UIM domains bind ubiquitin with low affinity (Hurley et al. 2006), oligomeric Eps15 would be expected to bind more strongly to ubiquitin-rich endosomes than monomeric Eps15, even if binding occurred only through UIM-ubiquitin interactions. By contrast, constructs lacking the UIM domains (FLAG-Eps15-ΔIII, FLAG-Eps15-ΔIV, and FLAG-Eps15-ΔUIM) showed very little GFP-FYVE-UbΔGG-dependent endosomal recruitment (Fig. 2-7).

High FLAG-Eps15 expression is not required for recruitment to ubiquitin-rich endosomes

Because GFP-FYVE-UbΔGG recruited Eps15 to endosomes efficiently, without possible complications of delivery from the plasma membrane, we used it in further studies to characterize this recruitment in more detail. We could not detect endogenous Eps15 by IF with our antibodies, and we were concerned that endosomal recruitment of FLAG-Eps15 might only occur at high expression levels, after saturation of normal binding interactions. For this reason, we measured endosomal recruitment of FLAG-Eps15 in GFP-FYVE-UbΔGG-expressing COS-7 cells as a function of the level of expression of FLAG-Eps15 (Fig. 2-8). Colocalization of FLAG-Eps15 with EEA1 did not correlate with Eps15 expression: FLAG-Eps15 was recruited efficiently to endosomes even in cells with the lowest detectable FLAG-Eps15 level (Fig. 2-8).

Tyr 850 is not required for endosomal recruitment of FLAG-Eps15

The EGFR phosphorylates Eps15 on Tyr 850 (Confalonieri et al. 2000). To determine whether this modification is required for endosomal recruitment, we co-expressed a non-phosphorylatable Eps15 mutant, Eps15-Y850F, with GFP or GFP-FYVE-UbΔGG in COS-7 cells. In cells expressing GFP, Eps15-Y850F had the same punctate localization as wild-type FLAG-Eps15, and was not present on EEA1-positive endosomes (Fig. 2-9, top row). However, Eps15-Y850F was efficiently recruited to endosomes in cells expressing GFP-FYVE-UbΔGG (Fig. 2-9, bottom row). This showed that tyrosine phosphorylation on Tyr-850 is not required for endosomal localization of Eps15.

Epsin but not intersectin is also recruited to ubiquitin on endosomes.

To determine the specificity of Eps15 recruitment to ubiqutin-rich endosomes, we wanted to test for recruitment of other endocytic adaptors. Epsin, like Eps15 contains several UIM domains (Oldham et al. 2002) and interacts with clathrin adaptors at the plasma membrane. It binds directly to Eps15's EH domain (Chen et al. 1999) and is also involved in the deformation of the plasma membrane and induction of membrane curvature in clathrin-coated vesicles (Horvath et al. 2007). Although we found there to be some colocalization of epsin with endosomes in cells expressing GFP (Fig. 2-10A), there seemed to be a clear recruitment of epsin to endosomes in cells expressing GFP-FYVE-UbΔGG (Fig. 2-10B).

Intersectin is another endocytic adaptor that coordinates endocytic membrane traffic in concert with actin assembly machinery (Qualmann and Kessels 2002) and binds directly to Eps15 (Miliaras and Wendland 2004). In addition, it may also regulate the formation of clathrin-coated vesicles through its interactions with other membrane proteins (Yamabhai et al. 1998). In contrast to epsin, we did not see colocalization of HA-intersectin with endosomes in cells expressing either GFP or GFP-FYVE-UbΔGG (Fig. 2-10C, D). Taken together, this data suggests differential endosomal targeting of Eps15-binding proteins, and that recruitment is dependent on interactions with ubiquitin.

Eps15 is recruited to novel ubiquitin-rich plasma membrane clusters in a UIM-dependent manner

These results support the model that Eps15 is recruited to ubiquitin-rich endosomes through UIM domain binding to ubiquitinated cargo. If a high concentration of ubiquitin is sufficient for this recruitment, then Eps15 should also be recruited to other ubiquitin-rich sites in the cell. We took advantage of a novel feature of PM-GFP-Ub to test this prediction. In some transfected COS-7 cells, PM-FGP-Ub was present in distinctive plasma membrane clusters (Fig. 2-11A, center panel). As PM-GFP never formed these clusters, we speculated that they resulted from a ubiquitin-dependent network, analogous to that proposed to operate normally in clathrin-coated pit formation (Dores et al. 2010; Polo et al. 2002). However, these clusters probably formed aberrantly in PM-GFP-Ub-expressing cells, as a result of the high concentration of membrane-targeted ubiquitin at the plasma membrane. Consistent with this model, ErbB2 was concentrated in these clusters (Fig. 2-11D) after GA treatment of PM-GFP-UB-transfected SK-BR-3 cells (cells were not permeabilized before addition of anti-ErbB2 antibodies, showing that the clusters were on the plasma membrane). In cells not treated with GA, ErbB2 did not concentrate in the clusters, and sometimes appeared to be excluded from them (Fig. 2-11C). This suggested that ubiquitinated ErbB2 could be recruited to the clusters in a ubiquitin-dependent manner. As ubiquitinated ErbB2 is not targeted to clathrin-coated pits for internalization (Barr et al. 2008), its recruitment to the clusters is unlikely to require other proteins that participate in formation of the clathrin coat. We found that FLAG-Eps15, but not FLAG-Eps15-ΔUIM, was also strongly recruited to the PM-GFP-UB clusters (Fig. 2-11A, B). This suggested that UIM domain interactions could recruit Eps15 to these novel ubiquitin-rich sites, and that endosome-specific factors were not required for ubiquitin-dependent Eps15 recruitment.

Hrs is not required for ubiquitin-dependent recruitment of Eps15 to early endosomes

Hrs can bind Eps15 in the cytosol and on endosomes. Eps15b, which localizes primarily to endosomes even without ubiquitin enrichment, binds Hrs *in vivo* (Roxrud et al. 2008). Furthermore, Eps15b localizes to Hrs-positive endosome microdomains, suggesting that Hrs recruits Eps15b to endosomes (Roxrud et al. 2008). Eps15 can bind Hrs (Bache et al. 2003; Bean et al. 2000) though this interaction does not occur at significant levels in cells with normal,

low levels of endosomal ubiquitin (Roxrud et al. 2008). For this reason, we next suppressed Hrs expression with siRNA to determine whether Hrs were required for ubiquitin-dependent endosomal recruitment of Eps15. We first verified that Hrs expression was efficiently suppressed (by about 90%) by siRNAs targeting two different sequences in human Hrs (Hrs siRNA-1 and Hrs siRNA-2), both in human HeLa cells (not shown) and in COS-7 cells (Fig. 2-12B). We then co-expressed FLAG-Eps15 and GFP-FYVE-UbΔGG in COS-7 cells previously transfected with either Hrs siRNA-1 or a control siRNA (Fig. 2-12C). In agreement with the Western blot data (Fig. 2-12A, B), Hrs was easily detectable on endosomes in control cells, but not in cells expressing Hrs siRNA-1. (The anti-Hrs antibodies sometimes stained nuclei non-specifically, as seen in Fig. 2-12C). FLAG-Eps15 was recruited efficiently to GFP-FYVE-UbΔGG-enriched endosomes even in cells with no detectable Hrs, showing that Hrs was not required for ubiquitin-dependent endosomal recruitment of Eps15 (Fig. 2-12C). Similar results were obtained in cells expressing Hrs siRNA-2 instead of Hrs siRNA-1 (not shown).

FLAG-Eps15 is recruited to endosomes enriched in endogenous ubiquitinated cargo

If endosomal recruitment of Eps15 is physiologically relevant, it must occur at reasonable levels of endosomal ubiquitin. Our finding that FLAG-Eps15 was recruited to endosomes in GA-treated SK-BR-3 cells (Fig. 2-13) showed that artificially high, over-expressed levels of ubiquitinated cargo were not required for this recruitment. As another approach to this question, we took advantage of our finding that Hrs was not required for recruitment of FLAG-Eps15 to endosomes in GFP-FYVE-UbΔGG-expressing cells (Fig. 2-13). As Hrs is required for packaging of ubiquitinated proteins into MVBs (Hurley et al. 2008), endogenous ubiquitinated cargo should accumulate on endosome rims upon Hrs silencing. For this reason, we next examined the localization of FLAG-Eps15 in Hrs-silenced COS-7 cells. FLAG-Eps15 was recruited to endosomes in these cells, but not in control cells (Fig. 2-13A,B), showing that endogenous ubiquitinated cargo was sufficient for this recruitment.

siRNA suppression of Eps15 does not affect ErbB2 degradation

Eps15 might function in endosomes to aid the ESCRT-0 complex in collecting ubiquitinated cargo. To test this idea, we suppressed Eps15 expression to determine whether Eps15 affected the degradation of ubiquitinated ErbB2. We chose ErbB2, rather than EGFR,

because Eps15 affects EGFR internalization from the plasma membrane (Reider and Wendland 2011; van Bergen En Henegouwen 2009). This makes it hard to determine whether Eps15 also has a second role in endosomes, in facilitating EGFR degradation. By contrast, ubiquitinated ErbB2 bypasses the Eps15-dependent step at the plasma membrane, as it is internalized by a clathrin-independent pathway (Barr et al. 2008). Following internalization, however, ubiquitinated ErbB2 is packaged into vesicles in MVBs, in the same manner as ubiquitinated EGFR.

Roxrud et al. reported that siRNA directed against a sequence present in both Eps15 and Eps15b delayed EGFR degradation in Hela cells, while an siRNA that targeted a sequence present only in Eps15 had no effect (Roxrud et al. 2008). We tested an siRNA that targeted a sequence present in both Eps15 and Eps15b (Roxrud et al. 2008), used previously by others to suppress Eps15 expression (Huang, 2004; Sigismund et al. 2005). We were not able to reproducibly detect the 120 kDa Eps15b on Western blots of lysates from SKr3 or COS-7 cells, so we could not assess the efficacy of Eps15b silencing (not shown). This siRNA suppressed expression of full-length (150 kDa) Eps15 by approximately 80% in SK-BR-3 cells (Fig. 2-14A). Nevertheless, silencing Eps15 did not affect ErbB2 degradation in GA-treated cells (Fig. 2-14B, C).

Accumulation of ubiquitinated cargo in endosomes slows internalization of activated EGFR

Alternatively, Eps15 recruitment to ubiquitin-rich endosomes may function to sequester the protein away from the plasma membrane. This might provide a feed-back mechanism, slowing clathrin-mediated endocytosis when high amounts of ubiquitinated cargo were delivered to endosomes. This could provide time for the ESCRT machinery to clear the ubiquitinated proteins, preventing excessive over-accumulation in endosomes. To test this idea, we measured internalization of endogenous EGFR and transferrin receptor in SK-BR-3 cells expressing GFP-FYVE-UbΔGG. We first found that steady-state levels of both receptors at the plasma membrane were similar in GFP-FYVE-UbΔGG-expressing and control cells (Fig. 2-15A,D). However, GFP-FYVE-UbΔGG slowed internalization of both AF-594-transferrin (Fig. 2-15B) and anti-EGFR antibodies (Fig. 8E). Quantitation is shown in Figs. 2-15C and F. These findings

are consistent with the possibility that recruitment of endogenous Eps15 to endosomes slowed endocytosis by reducing its concentration at the plasma membrane.

In an attempt to determine whether this resulted from depletion of Eps15 from the plasma membrane, we repeated the experiment in cells over-expressing FLAG-Eps15, hoping to saturate endosomal ubiquitin and restore the plasma membrane pool of Eps15. However, Eps15 overexpression did not rescue EGFR internalization (data not shown). It is possible that expression levels were not high enough to saturate endosomal GFP-FYVE-UbΔGG. Alternatively, other endocytic proteins in addition to Eps15 (possibly additional components of a ubiquitin-dependent network) might also be required for efficient EGFR internalization.

DISCUSSION

It is not known how Eps15 is recruited to endosomes in response to EGFR signaling (de Melker et al. 2004; Torrisi et al. 1999). As EGF stimulates tyrosine phosphorylation of the receptor itself, of Eps15, and of other substrates, and also leads to EGFR ubiquitination, any of these events might recruit Eps15 to endosomes. It is also not known whether the endosomal Eps15 binding partner Hrs is required for this recruitment.

Our work has shown that Eps15 was recruited to endosomes enriched in ubiquitinated cargo. Recruitment did not require Eps15 Tyr850, which is phosphorylated by EGFR, or Hrs, but required the UIM domains of Eps15. Furthermore, Eps15 showed UIM-domain-dependent recruitment to novel ubiquitin-rich plasma membrane clusters. Together, these findings suggest that binding of Eps15's UIM domains to ubiquitin can determine its localization in the cell, and that EGF treatment can recruit Eps15 to endosomes by causing ubiquitinated EGFR to accumulate there.

Unlike Eps15, Eps15b is constitutively present on endosomes (Roxrud et al. 2008). Eps15b binds Hrs and colocalizes with Hrs in microdomains on endosomes. Roxrud et al. suggested that while EH domain interactions and AP-2 binding target Eps15 to the plasma membrane, Eps15b is targeted to endosomes by binding to Hrs (Roxrud et al. 2008). Thus, the UIM-dependent, Hrs-independent targeting of Eps15 to ubiquitin-rich endosomes that we

observed occurs by a different mechanism than constitutive, Hrs-dependent endosomal targeting of Eps15b.

Our findings on ubiquitin-dependent Eps15 targeting are very similar to behavior of epsin reported previously by Chen and De Camilli (Chen and De Camilli 2005). As we found for Eps15, epsin could be recruited to endosomes or other cellular sites in response to ubiquitin accumulation, in a UIM-dependent manner. However, behavior of the two proteins differed in one key way. Epsin, unlike Eps15, binds directly to clathrin (Reider and Wendland 2011). Epsin was only recruited to ubiquitin-enriched endosomes when clathrin binding was prevented, either by mutation of epsin or silencing of clathrin (Chen and De Camilli 2005). Both Eps15 and epsin have multiple binding partners at the plasma membrane, and these interactions probably counteract UIM-dependent targeting to endosomes. As an important example, binding of epsin to clathrin strongly antagonizes its recruitment to ubiquitin-rich endosomes (Chen and De Camilli 2005).

Eps15 localization is also probably determined by the balance of affinities for its various binding partners. However, our results suggest that UIM domain interactions are more likely to prevail in determining localization of Eps15 than of epsin. Although the affinity of individual UIM domains for ubiquitin is low (Hurley et al. 2006), Eps15 can form dimers and tetramers via its coiled-coil domain (Cupers et al. 1997). Thus, increasing the local concentration of ubiquitin should greatly increase the avidity of Eps15 oligomers for ubiquitin-rich sites. Our results suggest that this is enough to recruit Eps15 to ubiquitin-rich endosomes. Our results suggest that ubiquitin-dependent targeting occurs more easily for Eps15 than for epsin, and may be more likely to play an important physiological role.

In this context, an important question is whether the ubiquitin-dependent recruitment we observed occurs at physiological levels of Eps15. Alternatively, overexpressed Eps15 might saturate its normal plasma membrane binding partners, artificially creating a pool available for recruitment to endosomes. For this reason, we carefully examined endosomal recruitment in cells expressing the lowest detectable level of FLAG-Eps15 (Fig 2-7). We saw the same recruitment of FLAG-Eps15 to ubiquitin-rich endosomes at all levels of FLAG-Eps15 expression, suggesting that endosomal recruitment is not an artifact of overexpression.

Several functions for endosomal recruitment of Eps15 can be imagined. One obvious possibility is to aid the ESCRT-0 complex in processing ubiquitinated cargo for degradation.

This could occur by direct binding of Eps15 to ubiquitinated cargo, and/or by establishment of a ubiquitin-dependent protein network analogous to that at the plasma membrane (Dores et al. 2010; Polo et al. 2002). Our finding that siRNA-mediated silencing of Eps15 did not affect degradation of ubiquitinated ErbB2 in GA-treated SK-BR-3 cells (Fig. 2-12) argues against this possibility, and suggests that Eps15 is not uniquely required for degradation of ubiquitinated cargo, but does not exclude it. Eps15 function overlaps with that of the related adaptor Eps15R and of epsin at the plasma membrane (Sigismund et al. 2005). Eps15R, and possibly even epsin, might be recruited to endosomes to substitute for Eps15 after its silencing.

Roxrud et al. found that silencing of Eps15 with an siRNA that spared Eps15b did not affect EGFR degradation. By contrast, an siRNA targeting a sequence present in both Eps15 and Eps15b delayed EGFR degradation (Roxrud et al. 2008). These results suggested that Eps15b, but not Eps15, participates in EGFR degradation. The siRNA that we used was directed against a sequence present in both Eps15 and Eps15b. Thus, our results appear to contrast with those of Roxrud et al. (Roxrud et al. 2008). However, we could not reproducibly detect Eps15b on Western blots, probably because of the cell types and/or anti-Eps15 antibodies we used. Thus, we could not tell how well our siRNA suppressed Eps15b expression. Roxrud et al. reported more efficient suppression of Eps15 than Eps15b, despite using siRNA that targeting a shared sequence (Roxrud et al. 2008). If the same were true for the siRNA we used, sufficient Eps15b might have remained to aid in ErbB2 degradation.

Alternatively, or in addition, ubiquitin-dependent endosomal recruitment of Eps15 might function to sequester the protein away from the plasma membrane. Under some conditions, extensive delivery of ubiquitinated cargo in to endosomes might saturate the ESCRT machinery, causing accumulation of ubiquitinated proteins on the limiting membrane. In such cases, it might be advantageous to slow further delivery of such cargo to endosomes, to allow the ESCRT complexes to catch up, allowing them to package cargo into the MVB interior in an orderly fashion.

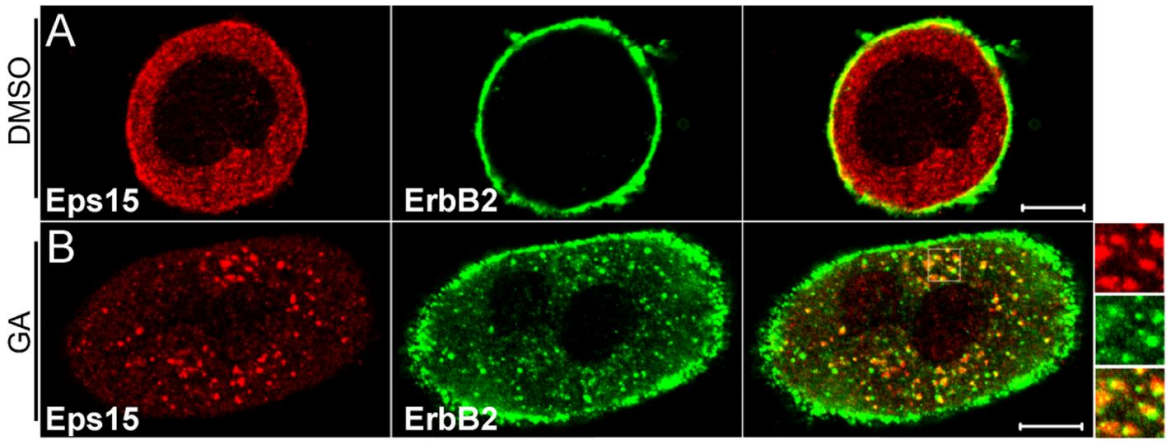

Figure 2-1. FLAG-Eps15 colocalizes with ErbB2 in geldanamycin treated cells. SK-BR-3 cells were treated with DMSO (A) or GA (B) for 4 hours before fixation and processing for immunofluorescence as described in Materials and Methods. FLAG-Eps15 (left panels; red) and detected using anti-FLAG antibodies and endogenous ErbB2 (green; center panel) is as indicated. Merged images are shown to the right of ErbB2. Enlarged views of the boxed region in the merged image demonstrating colocalization in GA-treated cells is shown in the far right panel. Scale bar; 10 μm.

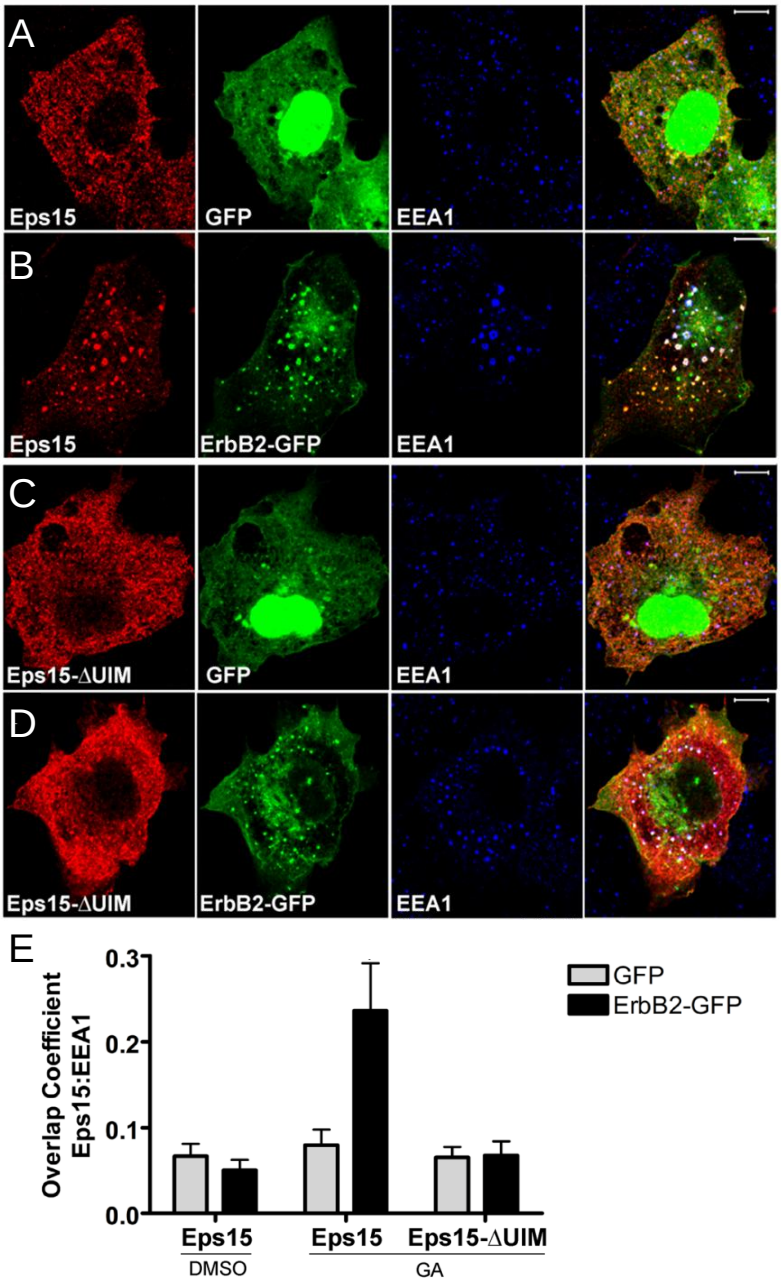

Figure 2-2. Accumulation of ubiquitinated ErbB2 in endosomes recruits FLAG-Eps15 in a UIM domain-dependent manner. A-D. COS-7 cells transfected with FLAG-Eps15 or FLAG-Eps15-ΔUIM, together with GFP or ErbB2-GFP as indicated, were treated with 5 μM GA for 4 hours and then processed for IF, detecting FLAG-Eps15 constructs with anti-FLAG antibodies. E. Colocalization of FLAG-Eps15 or FLAG-Eps15-ΔUIM with EEA1 in COS-7 cells co-expressing either GFP or GFP-ErbB2 and treated with DMSO or 5 μM GA for 4 hours was quantified using NIH ImageJ. The Manders' overlap coefficient is shown. Values shown are the average of at least 10 cells in each of 3 experiments, +/- standard error of the mean (SEM). Scale bars; 10 μm.

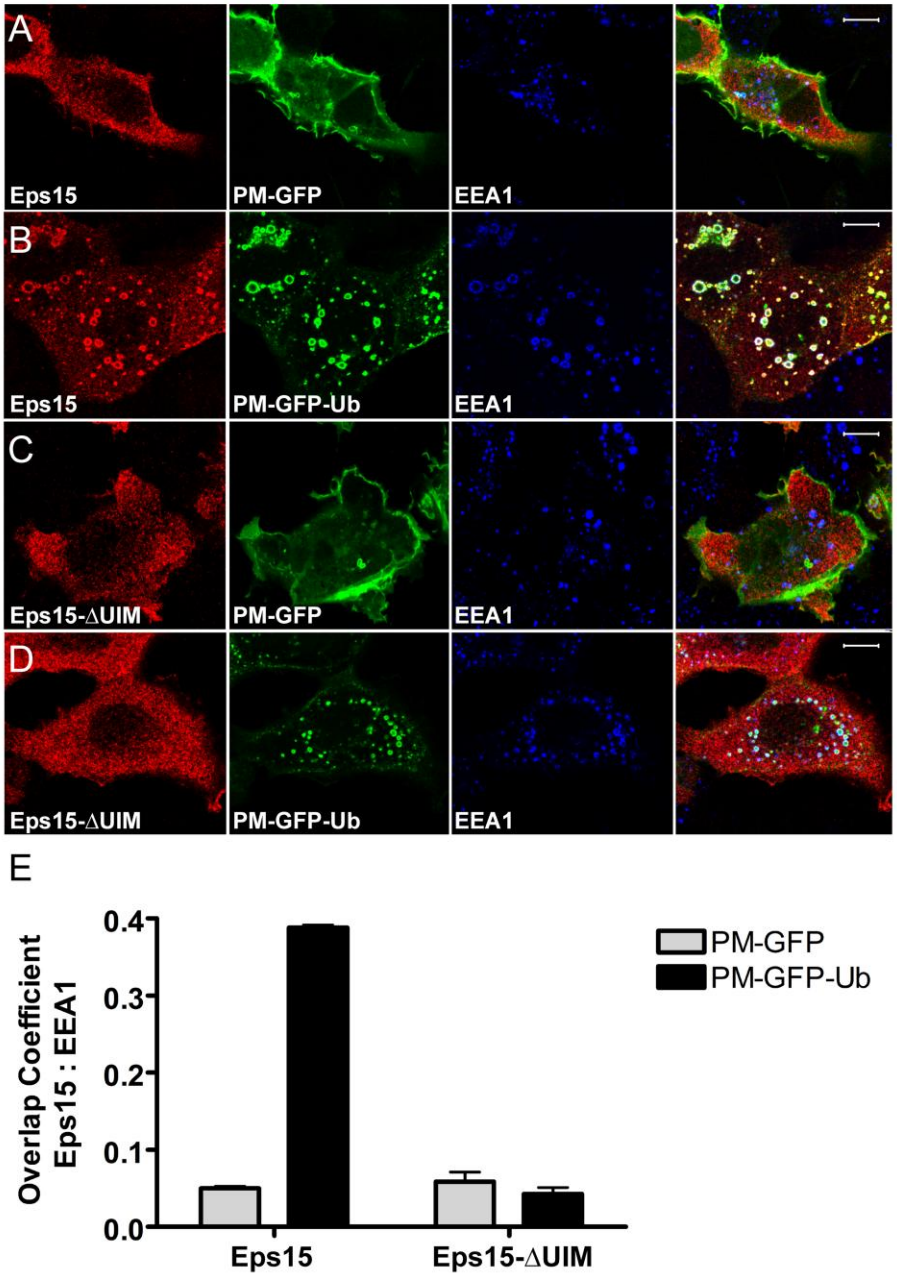

Figure 2-3. Eps15 but not Eps15-ΔUIM is recruited to endosomes containing PM-GFP-Ub. A-D. COS-7 cells transfected with FLAG-Eps15 or FLAG-Eps15-ΔUIM, together with PM-GFP or PM-GFP-Ub as indicated, were processed for IF, detecting FLAG-Eps15 constructs with anti-FLAG antibodies. E. Colocalization of FLAG-Eps15 or FLAG-Eps15-ΔUIM with EEA1 in COS-7 cells co-expressing either PM-GFP or PM-GFP-Ub was determined and is presented as in Fig. 2-2, averaging values for at least 10 cells in each of 3 experiments. Scale bars; 10 μm.

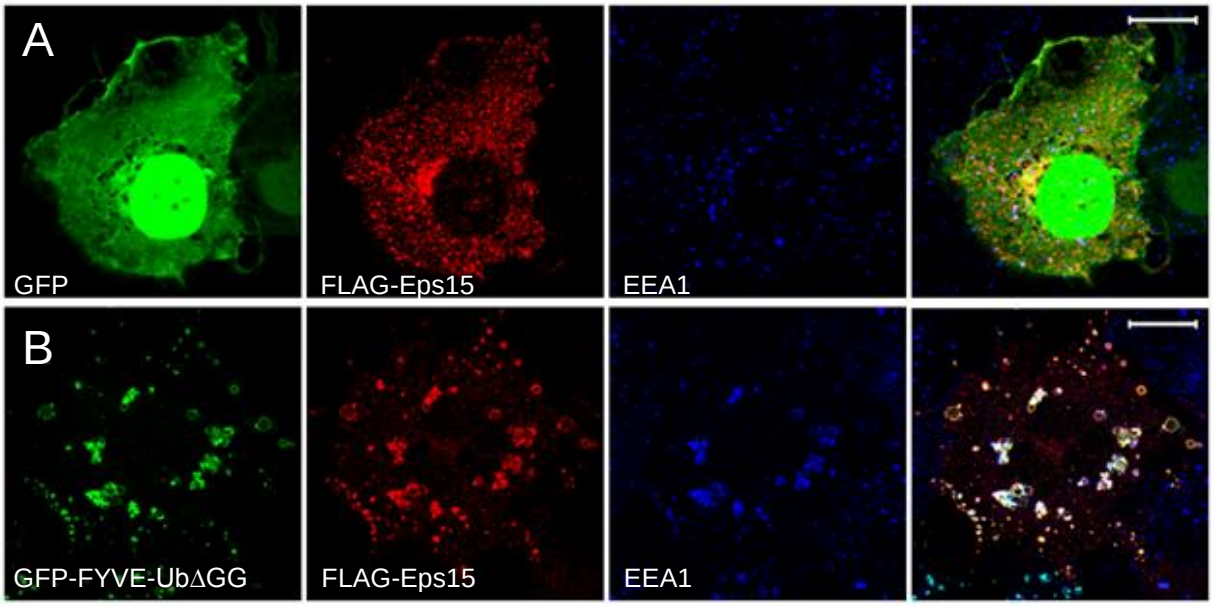

Figure 2-4. FLAG-Eps15 is recruited to GFP-FYVE-UbΔGG-enriched endosomes. COS-7 cells transfected with FLAG-Eps15 together with GFP (A) or GFP-FYVE-UbΔGG (B) as indicated, were processed for IF, detecting FLAG-Eps15 constructs with anti-FLAG antibodies and mouse anti-EEA1 antibodies and appropriate secondary antibodies (AF-594 goat anti-rabbit IgG and AF-647 goat anti-mouse IgG) as indicated. Scale bars; 10 μm.

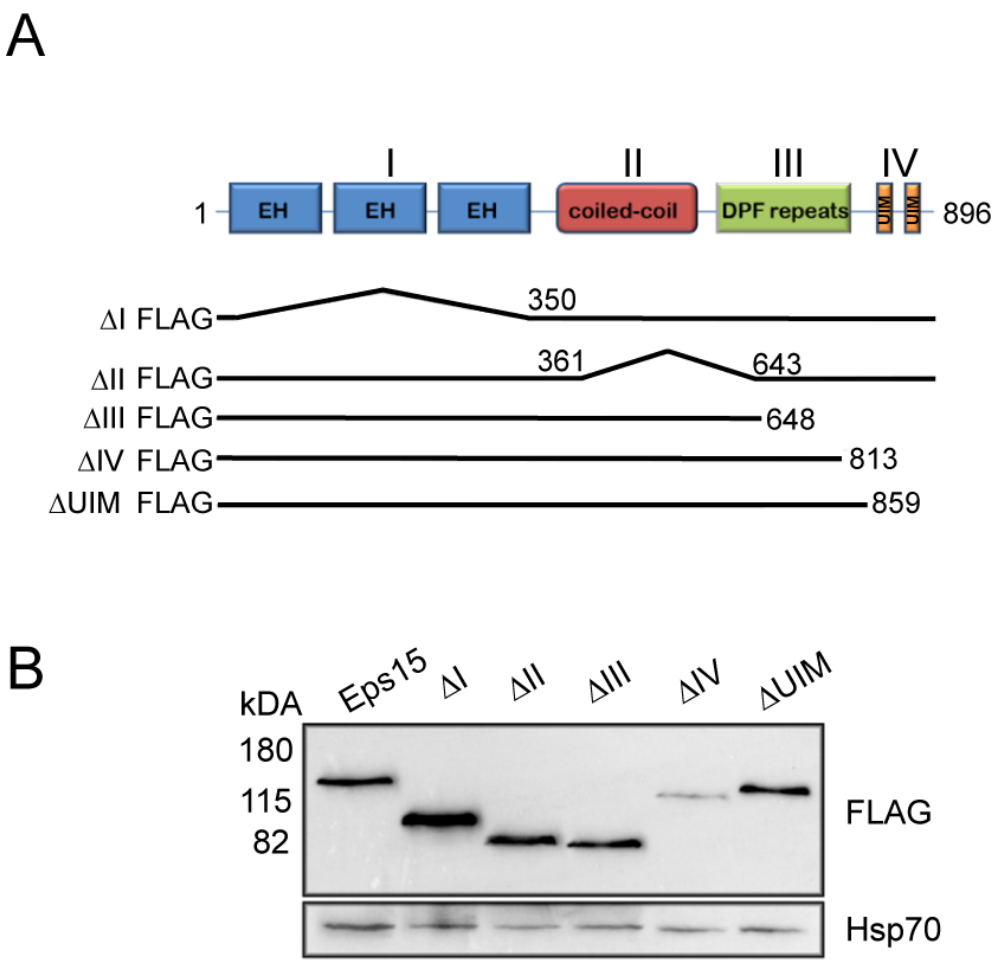

Figure 2-5. Domain architecture and Western immunoblot of wild-type and mutant Eps15. A. Schematic diagram of the Eps15 mutants used. B. FLAG-Eps15 (Eps15) or the indicated FLAG-Eps15 mutant was expressed in COS-7 cells. Proteins in cell lysates (equal volumes) were separated by SDS-PAGE and analyzed by Western blotting, probing with anti-FLAG antibodies. The blot was then probed with anti-Hsp70 antibodies to verify equal loading.

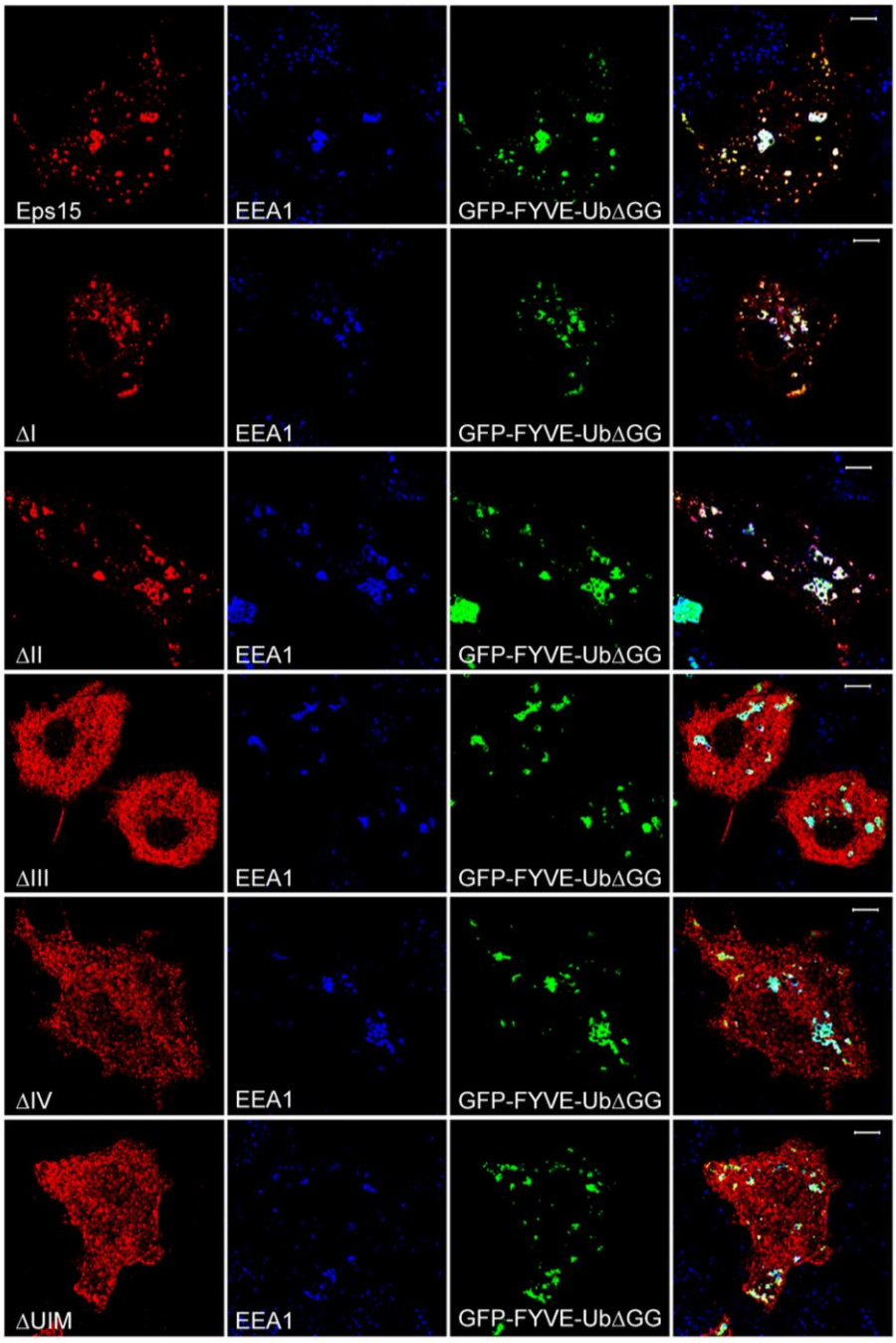

Figure 2-6. **The UIM domain(s) of Eps15 are required for recruitment to ubiquitin-containing endosomes.** Wild-type FLAG-Eps15 (Eps15) or the indicated FLAG-Eps15 mutant was expressed in COS-7 cells together with GFP-FYVE-UbΔGG. Cells were processed for IF, detecting the FLAG-Eps15 proteins with anti-FLAG and then AF-594 goat anti-rabbit antibodies, and EEA1 with anti-EEA1 and then AF-647 goat anti-mouse antibodies. From left to right in each row, anti-FLAG staining, anti-EEA1-staining (pseudo-colored blue), GFP fluorescence, and a merged image are shown. Scale bars; 10 μm.

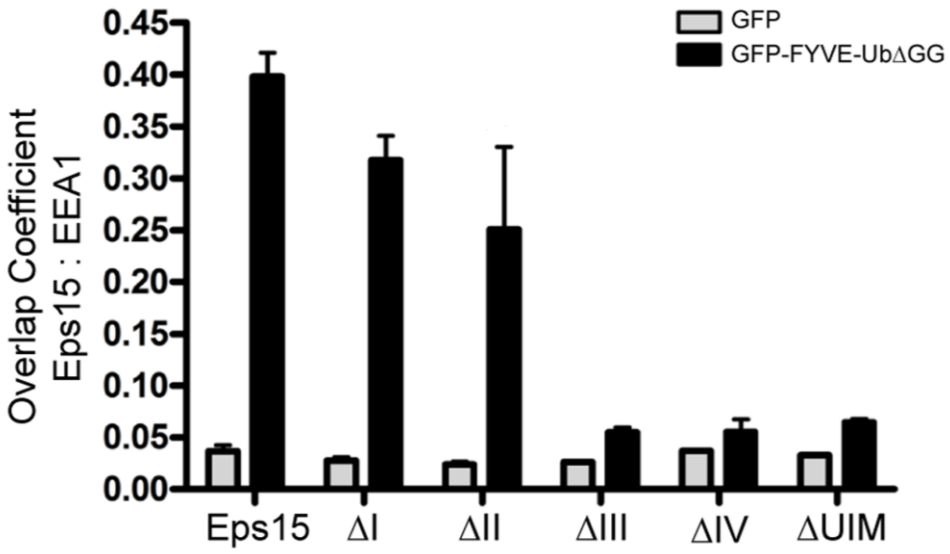

Figure 2-7. Increased colocalization of UIM-containing Eps15 constructs and endosomes in cells expressing GFP-FYVE-UbΔGG. Colocalization of wild-type and mutant Eps15 with EEA1 was performed and quantified as described in the Materials and Methods. Colocalization analysis of the FLAG-Eps15 proteins with EEA1 was performed as in Figure 2-2. Values shown are the average of 2 experiments; error bars, SEM.

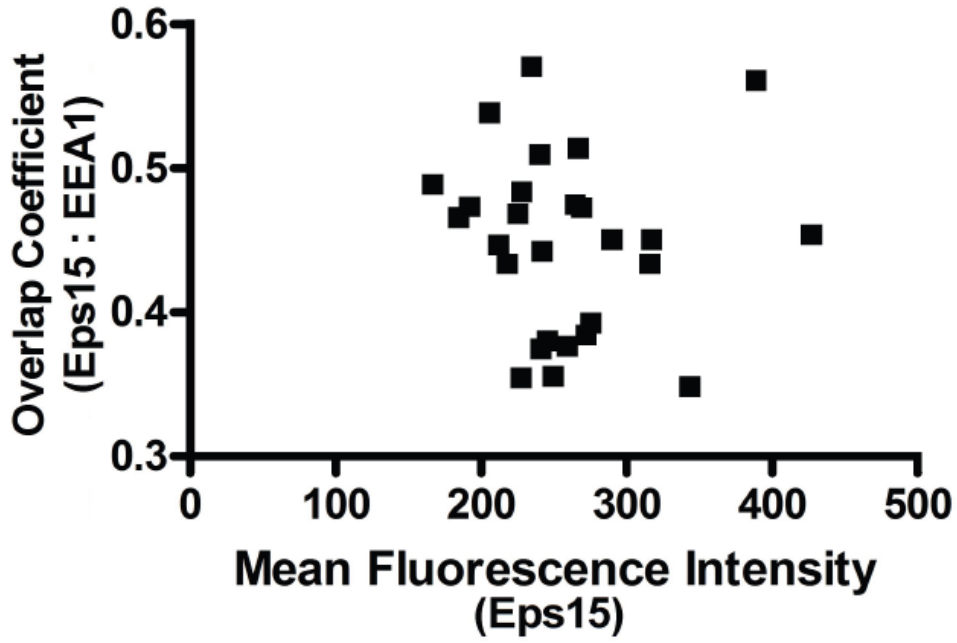

Figure 2-8. Endosomal recruitment of FLAG-Eps15 does not depend on FLAG-Eps15 expression level. FLAG-Eps15 and EEA1 were detected in FLAG-Eps15-transfected COS-7 cells with rabbit anti-FLAG and mouse anti-EEA1 antibodies and appropriate secondary antibodies (AF-594 goat anti-rabbit IgG and AF-647 goat anti-mouse IgG). The Manders' overlap coefficient for colocalization of Eps15 with EEA1 in each of 27 cells is plotted as function of the mean AF-594 fluorescence intensity in the same cell.

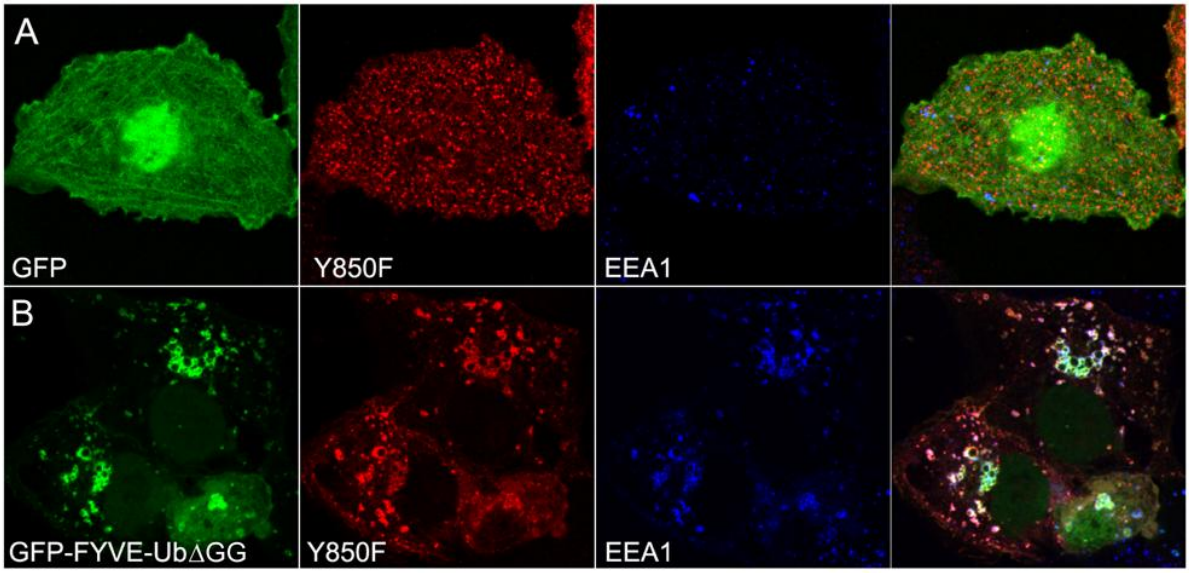

Figure 2-9. Tyr 850 is not required for endosomal recruitment of FLAG-Eps15. FLAG-Eps15 Y850F was co-expressed in COS-7 cells with either GFP (top) or GFP-FYVE-UbΔGG (bottom), and cells were processed for IF microscopy. FLAG-Eps15 Y850F was detected with anti-FLAG antibodies and AF-594 goat anti-rabbit antibodies, while EEA1 was detected with anti-EEA1 antibodies and AF-647 goat anti-mouse antibodies (pseudo-colored blue).

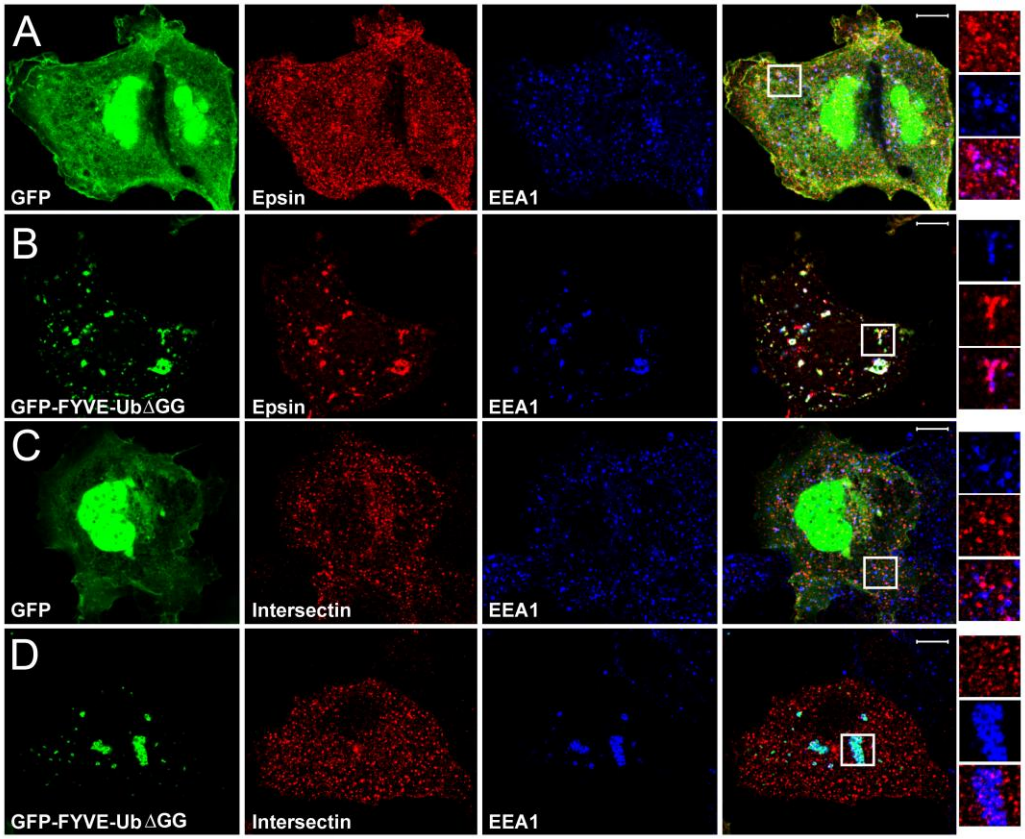

Figure 2-10. Epsin but not intersectin is recruited to ubiquitin on endosomes. COS-7 cells expressing either GFP (A,C) or GFP-FYVE-UbΔGG (B,D) and FLAG-epsin (A,B) or HA-intersectin (C,D) are shown as indicated. Merged images of all three channels are shown in the panel to the right of EEA1. Enlarged views of boxed regions in the merged image of only FLAG-epsin or HA-intersectin and EEA1 are shown on the far right. Scale bar; 10 μm.

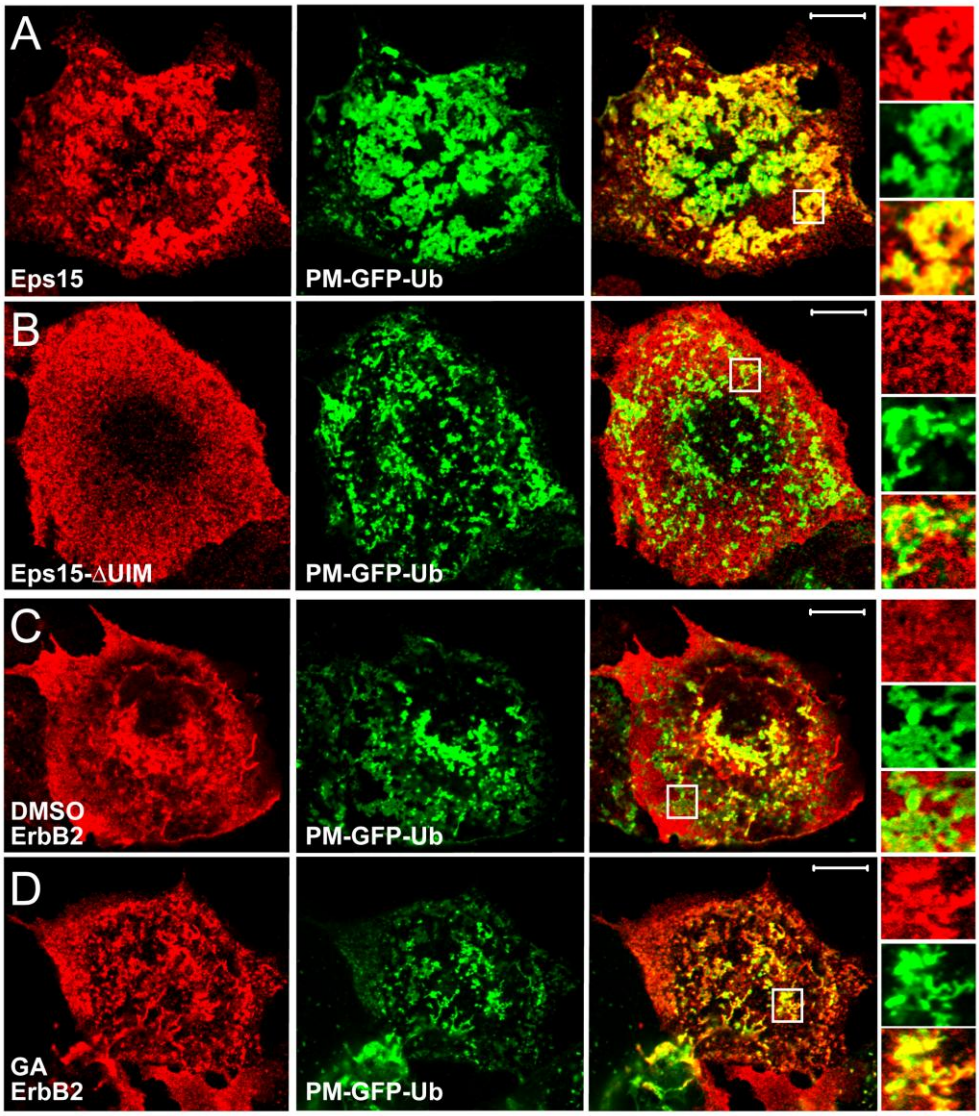

Figure 2-11. FLAG-Eps15 is recruited to ubiquitin-rich PM-GFP-Ub clusters on the plasma membrane. PM-GFP-Ub was expressed in COS-7 cells together with FLAG-Eps15 (A), FLAG-Eps15-ΔUIM (B), or ErbB2 (C, D). Cells in Panel D were treated with 5 μm GA for 1 hour before fixation. All cells were fixed and processed for IF as described in Experimental Procedures, except that cells shown in C and D were not permeabilized. Images of cells exhibiting characteristic PM-GFP-Ub plasma membrane clusters (about 20% of PM-GFP-Ub-expressing cells) are shown. Left panels; FLAG-Eps15 constructs were detected with anti-FLAG antibodies, and ErbB2 was detected with anti-ErbB2 antibodies; AF-594 secondary antibodies were used in both cases. Middle panels; GFP fluorescence. Right panels; merged images. Enlargements of the boxed regions in the merged images are shown at the far right. Scale bars; 10 μm.

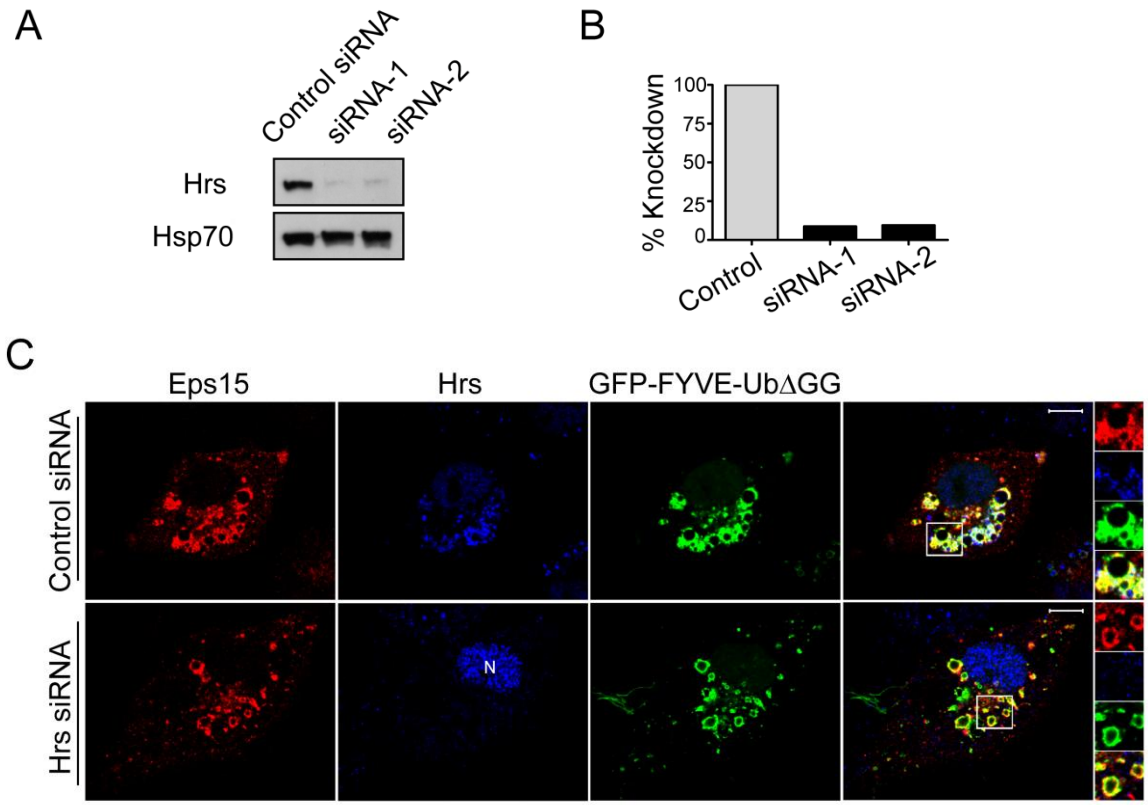

Figure 2-12. Hrs is not required for recruitment of Eps15 to GFP-FYVE-UbΔGG-enriched endosomes. COS-7 cells were transfected with siRNA targeting Hrs or control siRNA, and also with plasmids encoding FLAG-Eps15 and GFP-FYVE-UbΔGG. A. Proteins in lysates (equal volumes from each sample) were separated by SDS-PAGE and then analyzed by Western blotting, probing with anti-Hrs antibodies (top) or anti-Hsp70 antibodies as a loading control (bottom). B. Bands on the blot shown in Panel A were quantitated by densitometry, normalizing to the signal in the control sample. C. Cells transfected with the indicated constructs were processed for IF microscopy, staining with anti-FLAG and AF-594 goat anti-rabbit IgG to detect FLAG-Eps15 and with anti-Hrs and AF-647 goat anti-mouse IgG to detect Hrs (pseudo-colored blue). Merged images are shown at the right. Scale bars; 10 μm.

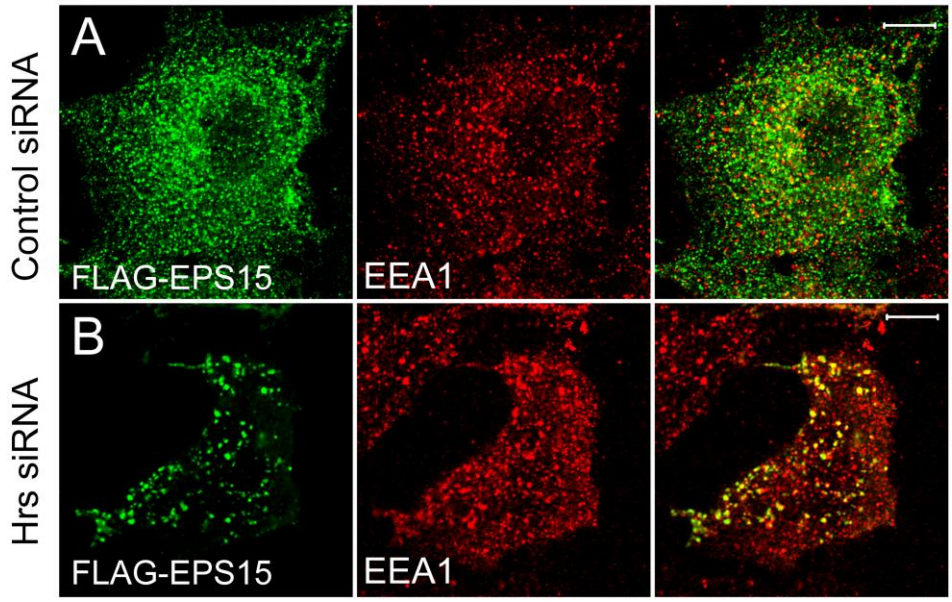

Figure 2-13. FLAG-Eps15 is recruited to endosomes enriched in endogenous ubiquitinated cargo. A,B. COS-7 cells transfected with control siRNA (A) or Hrs siRNA-2 (B) and with FLAG-Eps15 were processed for IF microscopy, detecting FLAG-Eps15 with anti-FLAG antibodies and AF-488 goat anti-rabbit IgG, and EEA1 with anti-EEA1 antibodies and AF-594 goat anti-mouse IgG. C,D. COS-7 cells co-transfected with FLAG-Eps15 together with either GFP or GFP-Rab5Q79L were processed for IF microscopy, detecting FLAG-Eps15 with anti-FLAG antibodies and AF-594 goat anti-rabbit IgG, and EEA1 with anti-EEA1 antibodies and AF-647 goat anti-mouse IgG (pseudo-colored blue). Scale bars; 10 μm.

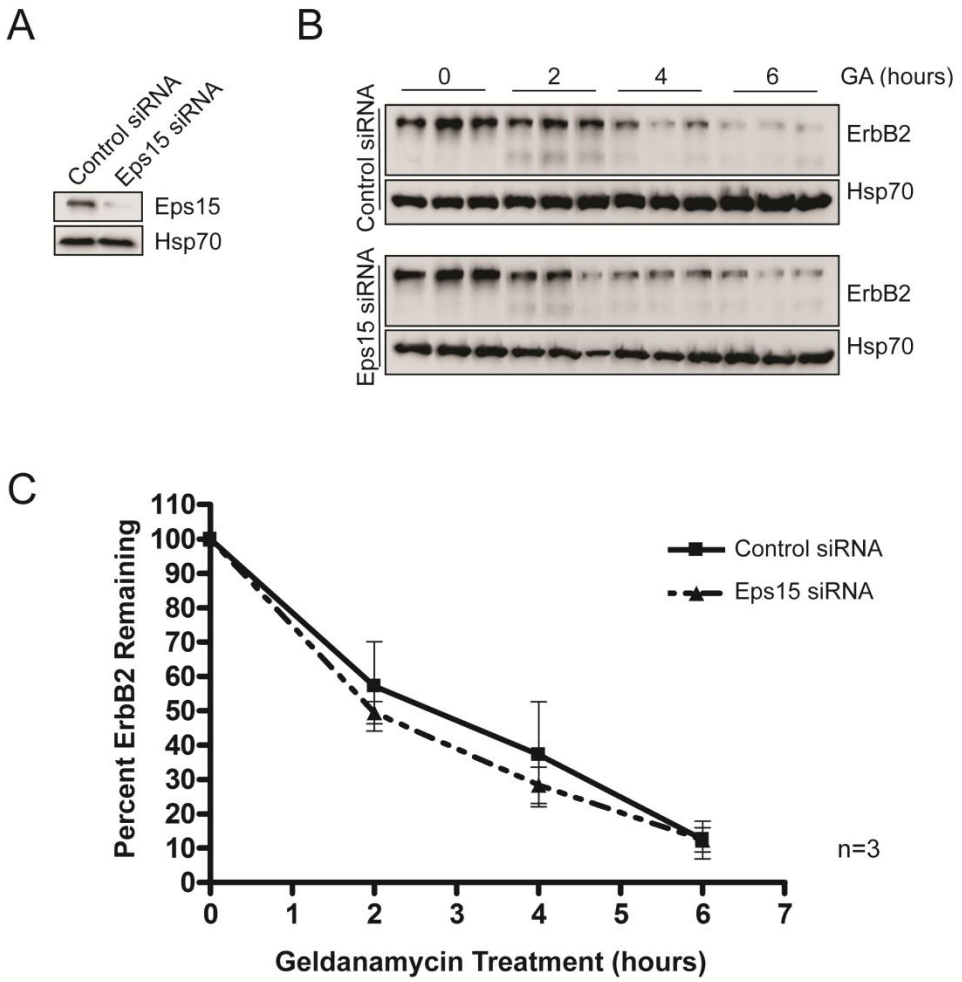

Figure 2-14. Eps15 silencing does not affect ErbB2 degradation in GA-treated SK-BR-3 cells. A. SK-BR-3 cells were transfected with siRNA targeting Eps15 or a control siRNA as indicated. Proteins in equal volumes of cell lysate were separated by SDS-PAGE and analyzed by Western blotting, probing with anti-Eps15 or anti-Hsp70 antibodies as indicated. B. Triplicate dishes of SK-BR-3 cells transfected with siRNA targeting Eps15, or a control siRNA, were incubated with 5 μM GA for the indicated times, lysed, and subjected to SDS-PAGE and Western blotting. Equal volumes of each lysate were loaded on the gel. Blots were probed with anti-ErbB2 or anti-Hsp70 antibodies as indicated, and HRP-conjugated secondary antibodies for detection by chemiluminescence. C. SK-BR-3 cells were transfected and processed as in B, except that blots were probed with AF-680-conjugated secondary antibodies for quantitation of bands using the Odyssey infrared imaging system and the associated software. Results shown are the average of 3 separate experiments, +/- SEM.

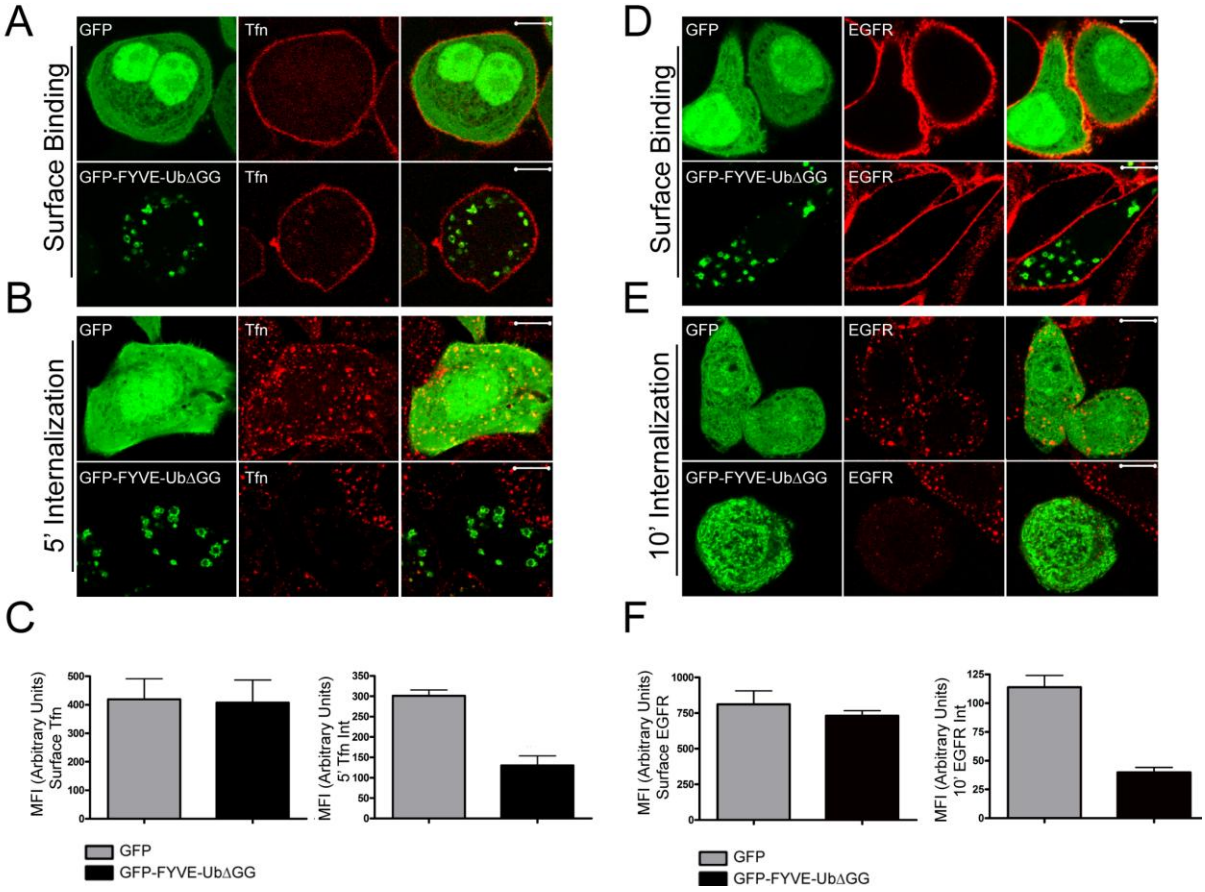

Figure 2-15. GFP-FYVE-UbΔGG expression inhibits endocytosis of EGFR and transferrin receptor in SK-BR-3 cells. GFP or GFP-FYVE-UbΔGG as indicated was expressed in SK-BR-3 cells. A,D. AF-594 transferrin (A) or anti-EGFR antibodies (D) were bound to cells at 4°C, as detailed in Experimental Procedures, before fixation. B, E. AF-594 transferrin (B) or anti-EGFR antibodies (E) were allowed to internalize at 37°C as detailed in Experimental Procedures. A,B,D,E; Cells were processed for fluorescence microscopy as in Fig. 1, except that cells shown in A, B, and D were not permeabilized. Tfn, transferrin. Scale bars; 10 μm. D, E. Anti-EGFR antibodies were detected with AF-594-goat anti-mouse IgG. C, F. AF-594-transferrin (C) or AF-594 goat anti-mouse IgG (F) fluorescence was quantitated as described in Experimental Procedures. In each panel, MFI of cells with surface-bound probes is shown on the left; MFI of cells containing internalized fluorophores is on the right. Values for GFP-expressing cells are shown with gray bars; values for GFP-FYVE-UbΔGG-expressing cells are shown with black bars. Results shown are the average of 2 separate experiments, +/- SEM.

CHAPTER THREE

Localization of Ack1 and its effect on endocytosis

The text and figures of this chapter is taken in part from the following manuscripts with some modifications:

Prieto-Echagüe VP, Gucwa A, Craddock BP, Brown DA, Miller WT. Cancer-associated Mutations Activate the Nonreceptor Tyrosine Kinase Ack1. J Biol Chem. 2010 Jan 28.

Prieto-Echagüe VP, Gucwa A, Brown DA, Miller WT. Regulation of Ack1 Localization by the Amino-terminal SAM domain. BMC Biochem. 2010 Oct 27.

INTRODUCTION

The Rho family of GTPases are best known for their regulation of cytoskeletal reorganization of actin, cell morphology and migration (Heasman and Ridley 2008). Increased activity and overexpression of the Rho family members results in increased invasiveness, a hallmark of cancer progression as well as metastasis. Ack1 is a non-receptor tyrosine kinase and a direct effector molecule of Cdc42, a small GTPase in the Rho family. It was originally identified in a screen for its ability to bind specifically GTP-bound Cdc42 but not Rac1or RhoA (Manser et al. 1993)

Ack1 itself has also been implicated in many type of cancers (Mahajan and Mahajan 2010). It is often amplified in primary tumors and correlates with poor prognosis and increased metastatic properties (van der Horst et al. 2005). In addition, overexpression of Ack1 can induce transformation of cells through the promotion of migration and non-anchored growth (Prieto-Echagüe et al. 2010a). Moreover, cancer-associated somatic missense mutations of Ack1 have demonstrated increased autophosphorylation and kinase activity. Two mutations in the N-terminus (R34L and R99Q) were identified in lung adenocarcinoma and ovarian carcinoma, respectively; a mutation in the kinase catalytic domain (E346K) was identified in ovarian endometroid carcinoma; and a mutation in the SH3 domain (M409I) was found in gastric adenocarcinoma (Prieto-Echagüe et al. 2010a). For example, E346K seems to destabilize the auntoinhibited conformation of Ack1, resulting in constitutively high Ack1 activity.

Ack1 is unique as a non receptor tyrosine kinase since it is the only member of the family with a SH3 domain C-terminal to the kinase domain. It has multiple conserved domains including an N-terminal sterile alpha motif (SAM) domain that can mediate specific protein-protein interactions (Galisteo et al. 2006), a Cdc42-binding (CRIB) domain (Teo et al. 2001), a proline-rich region, a clathrin-binding motif and a ubiquitin binding domain (Shen et al. 2007). Notably in the C-terminal part of the protein there also is a region that shares a high homology with Mig6 (Zhang et al. 2007), a domain that has been found to participate in inhibitory intramolecular interactions.

Cdc42 has been linked to EGFR function and downregulation. Binding of EGF stimulates the activation of Cdc42 and downstream effectors (Wu et al. 2003). Cdc42 also has

been found to bind the E3 ubiquitin ligase responsible for ubiquitination of EGFR, Cbl, preventing its degradation and consequently prolonging signaling. Other studies have focused on the role of Ack1 in EGF receptor trafficking and downregulation. Activation of EGFR results in the recruitment of Ack1 to EGFR and the AP-2 complex (Shen et al. 2007). Ack1 also binds directly to clathrin, the major structural component of these vesicles. This helps promote the activation of Ack1 and in turn the subsequent degradation of the receptor. In addition, Ack1 interaction between Cdc42 and the adaptor protein Grb2 has also been found essential for clathrin-dependent internalization (Kato-Stankiewicz et al. 2001).

Upon EGF stimulation, Ack1 phosphorylates and activates the Dbl, a guanine exchange factor which activates the Rho family of GTPases (Kato et al. 2000). Furthermore, cells overexpressing Ack1 have also been found to block internalization of both transferrin receptor as well as EGFR. Furthermore, siRNA-mediated suppression of Ack1 inhibited the degradation of activated EGFR and increased the rate of recycling to the plasma membrane, suggesting it may play a role in its degradation (Shen et al. 2007). Taken together, this strongly suggests Ack1 is involved in the regulation of EGFR.

Little is known about the mechanisms that regulate Ack1 activity. Four somatic missense mutations of Ack1 were identified in cancerous tissue; however the effects of Ack1 activity and function of these mutants had not been described (Greenman et al. 2007). The mutations of these four mutants occurred in the N-terminal region, the C-lobe of the kinase domain and the SH3 domain. The Miller lab demonstrated that cancer-associated mutations increased Ack1 autophosphorylation in mammalian cells and helped promote the proliferation and migration, suggesting these point mutants as a mechanism for Ack1 dysregulation (Prieto-Echagüe et al. 2010a). My contribution to this paper, detailed below in Section A of the Results and shown in Fig. 3-1 - 3-3, was to show that this effect was not due to the mislocalization of these mutants and that they all had similar affects on clathrin and clathrin-mediated internalization.

Our next objective was to clarify the mechanisms that regulate the activity of Ack1 by addressing the importance of the SAM domain. The N-terminal region of Ack1 contains the SAM domain. SAM domains have been found to share a common fold and mediate protein-protein interactions in a wide variety of proteins. The Miller lab found that Ack1 deletion mutants lacking the N-terminus displayed significantly reduced autophosphorylation in cells. A

minimal construct, comprising the N-terminus and kinase domain (NKD) was autophosphorylated while the kinase domain (KD) alone was not (Prieto-Echague et al. 2010b).

The coimmunoprecipitation experiments they performed showed stronger interactions between full length Ack1 and NKD than between full length Ack1 and KD, indicating the N-terminus was important for Ack1 dimerization (Prieto-Echague et al. 2010b). My results, described in Section B of the Results and presented in Figs. 3-4 and 3-5, showed that Ack1 deletion mutants lacking the N-terminus displayed reduced autophosphorylation in cells. We found the minimal construct comprising the N-terminus and kinase domain (NKD) was autophosphorylated, while the kinase domain (KD) was not. Moreover, I showed that the localization of these constructs differed in mammalian cells.

Lastly, included in the final portion of the Results section are experiments I performed to try and further understand how overexpression of Ack1 is affecting the cell. These unpublished results are can be found in Section C of the Results section (Figs. 3-6 - 3-9).

MATERIALS AND METHODS

Cell culture and transfection

COS-7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium with 10% iron-supplemented calf serum (JRH, Lenexa, KS) and penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37°C. Transient transfection of cells seeded on acid-washed glass coverslips was performed using fully-deacylated polyethylenimine (PEI) reagent, prepared from 200kDa poly(2-ethyl-2-oxazoline) (Sigma-Aldrich, St. Louis, MO) as described (Thomas et al. 2005). Transfection mixtures contained 2 μg DNA and 12 μl PEI reagent per 35 mm dish. Cells were examined one day after transfection.

Antibodies, fluorescent compounds, and other reagents

Primary antibodies; rabbit anti-HA antibodies were from Sigma Aldrich (St. Louis, MO). Mouse anti-EEA1and mouse gamma-adaptin antibodies were from BD Biosciences (San Jose, CA). Mouse monoclonal anti-ErbB2 (N28) antibodies were from Lab Vision (Fremont, CA). Alexa Fluor (AF)-594-transferrin and AF-594-cholera toxin B subunit (CTxB) was from

Molecular Probes, Invitrogen (Carlsbad, CA). Fluorophore-conjugated secondary antibodies; goat anti-mouse or anti-rabbit AF-488/AF-594 were from Molecular Probes, Invitrogen (Carlsbad, CA).

Plasmids

A plasmid encoding EGFP-clathrin light chain A in pEGFP-C3 (Wu et al. 2001) was the gift of Lois Greene (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD). A plasmid encoding PM-GFP (Chen and De Camilli 2005) was a gift of Dr. P. DeCamilli (Yale University, New Haven, CT). Full length Ack1 was a gift from Dr. Edward Manser (Institute of Molecular and Cell Biology, Singapore) given to us by Dr. Todd Miller (Stony Brook University, New York). Ack1 mutant constructs were also all gifts of Dr. Todd Miller (Stony Brook University, New York) and created by Dr. Victoria Prieto-Echagüe as described (Prieto-Echague et al. 2010a; Prieto-Echague et al. 2010b). CI-MPR-pEGFP was a gift of Dr. Jeffrey Pessin (Albert Einstein College of Medicine, New York). Human ErbB2 in pcDNA3 was from Len Neckers (NIH, Maryland).

Fluorescence microscopy

Cells were seeded on acid-washed glass coverslips in a 35mm dishes, transfected and examined 1 day after transfection. Cells were fixed in phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH7.4) containing 3% paraformaldehyde for 30 minutes, permeabilized with PBS containing 0.5% Triton X-100 and blocked with PBS containing 3% bovine serum albumin (BSA) and 10 mM glycine for 1 hour. Primary and secondary antibodies were diluted in 3% BSA and 10 mM glycine. Cells were incubated with primary antibodies for 1 hour at room temperature, followed by secondary antibodies for 30 minutes, also at room temperature. Cells were photographed and images were captured using a Zeiss Inverted Axiovert 200 M Microscope with a two photon laser scanning confocal system and a 100x oil immersion objective. Images were processed with Zeiss LSM software. Where indicated, cells were incubated with AF-594-Tfn (35 μg/ml) for 20 minutes at 37°C to the regular growth medium before fixation. Cells were then washed three times with PBS before processing for fluorescence microscopy as described above. For quantification, a region of interest (ROI) was drawn around individual cells, and the histogram macro on the Zeiss LSM software was used to

quantify the mean fluorescence intensity of each cell in both red and green channels, and the ratio of anti-pY284 to anti-HA staining in each cell was calculated. Acquisition parameters were the same for all cells, and images shown as well as those used for quantitation were not further processed. 30 cells expressing each construct were analyzed in each of three separate experiments.

RESULTS

A. Cancer-associated mutations activate the nonreceptor tyrosine kinase Ack1

Cell localization studies

The four missense mutations in Ack1 were identified in a screen searching for somatic mutations in protein kinase genes (Greenman et al. 2007). Two mutations in the N-terminus (R34L and R99Q) were identified in lung adenocarcinoma and ovarian carcinoma, respectively; a mutation in the kinase catalytic domain (E346K) was identified in ovarian endometroid carcinoma; and a mutation in the SH3 domain (M409I) was found in gastric adenocarcinoma (Prieto-Echagüe et al. 2010a). The aim of this paper was to show that these mutants had increased Ack1 activity. One potential explanation for the increased activity of the cancer-associated mutant forms of Ack1 would be a change in subcellular localization. To test this possibility, I examined the localization of wild-type and mutant Ack1 proteins in COS-7 cells by fluorescence microscopy. The Ack1 proteins all had similar distribution patterns: all were localized to small discrete puncta and larger amorphous perinuclear structures (Fig. 3-1). As reported earlier for wild-type HA-Ack1 (Grovdal et al. 2008; Shen et al. 2007; Teo et al. 2001), all the Ack1 proteins showed partial colocalization with EEA1 on early endosomes. In addition, both wild-type Ack1 and the cancer-associated mutants were present on larger amorphous, perinuclear structures. These did not stain for EEA1, but often appeared to surround and embed EEA1-positive endosomes (Fig. 3-1). These structures are probably the same as large reticular complexes and endosome-associated tubules seen in Ack1-overexpressing cells by electron microscopy (Grovdal et al. 2008), and may be endosome-derived tubules. These results showed that altered localization did not explain the increased activity of the cancer-associated Ack1 mutants.

Ack1 and cancer mutant affects inhibit transferrin internalization

We thought the amorphous structures seen in the localization studies (Fig. 3-1) may be similar to the reticular membrane described previously (Grovdal et al. 2008). These structures resembled recycling tubules that form to facilitate the recycling of transferrin to the plasma membrane (Harding et al. 1983). To determine if wild-type Ack1 and the four cancer-associated mutants colocalized with these structures, I performed a transferrin internalization experiment (Fig. 3-2). Interestingly, I found that transferrin internalization was efficiently blocked in both the wild-type and cancer mutant expressing cells. Neighboring cells that were untransfected exhibited apparently normal internalization of AF-594 transferrin. Thus, in addition to similar distribution patterns, the cancer-associated Ack1 mutants also possess similar functions in regulating endocytosis, further confirming the missense mutations did not alter Ack1 function.

Ack1 and cancer-associated mutants sequester clathrin light chain

I found that all the Ack1 proteins caused a dramatic accumulation of clathrin (visualized using co-expressed GFP-clathrin light chain A) on these amorphous structures (Fig.3-3) as reported earlier for wild type Ack1 (Teo et al. 2001). This suggested that Ack1 over-expression induced the accumulation of endosome-associated tubular structures that sequestered clathrin, inhibiting transferrin internalization by reducing the amount of clathrin available for coated pit formation at the plasma membrane. All the cancer-associated mutants showed the same behavior as wild-type Ack1 (Fig. 3-3B-E), suggesting that activation of the mutants did not stem from differences in cellular localization.

B. Regulation of Ack1 localization by the amino-terminal SAM domain. (Prieto-Echague et al. 2010b)

Analysis of Ack1 autophosphorylation by quantitative immunofluorescence.

Together with the Miller lab, we next investigated the role of the N-terminal SAM domain on Ack1 localization and activity (Prieto-Echague et al. 2010b) . The N-terminus of Ack1 contains a sterile alpha motif (SAM) domain (Galisteo et al. 2006). This conserved domain is known to mediate protein-protein interactions and may also play a role in the regulation of Ack1. Fusion of the Ack1 SAM domain sequence to GFP resulted in localization at the plasma membrane (Fig.

3-4). In an effort to verify NKD was indeed targeted to the plasma membrane, we expressed wild-type and mutant Ack1 along with a GFP-fusion protein that localizes to the plasma membrane (Chen and De Camilli 2005). Indeed NKD localized exclusively to the plasma membrane in contrast to the intracellular localization of wild-type Ack1 and ΔNT constructs (Fig. 3-4). Moreover, the minimal construct KD, had a diffuse cytosolic staining pattern. The Miller lab found that deletion of the SAM domain from full-length Ack1 reduced the level of autophosphorylation in HeLa cells, suggesting the involvement of the N-terminus in enzyme regulation (Prieto-Echague et al. 2010b).

Next, I tested whether the various forms of Ack1 were active in all cellular sites. I studied the subcellular localization of the autophosphorylated, activated forms of Ack1 and the mutants by immunofluorescence analysis using anti-pY284 Ack1 antibody. In the cases where it was detected, the signal for pY284 Ack1 colocalized exactly with the distribution of total Ack1 (Fig. 3-4A). To determine the relative activities of the constructs, we quantitated anti-pY284 staining (for autophosphorylated Ack1) and anti-HA staining (for total Ack1) (Fig. 3-5B, C). The ratios of fluorescence intensities (pY284/HA) were normalized to the value corresponding to wild type (WT=100). Fig. 3-5B shows results from a representative experiment, while Fig. 3-5C shows the merged data from three experiments. Consistent with the Western blotting results obtained by Victoria (Prieto-Echagüe et al. 2010b; Fig1B, C) the level of autophosphorylation was significantly decreased by the deletion of the N-terminus, both in the context of the full length and the minimal construct. The construct ΔNt showed a ~2-fold decrease in autophosphorylation compared to wild type, while the isolated kinase domain (KD) had a ~9-fold decrease in autophosphorylation compared to NKD. The autophosphorylation level of KD was not significantly different from the negative control (K158R, a kinase dead Ack1 mutant).

C. Effects of Ack1 on clathrin-independent endocytosis and TGN morphology and dynamics.

Wild-type Ack1 inhibits internalization of geldanamycin-treated ErbB2

To determine if the inhibition of internalization is specific to the clathrin-mediated pathway, I tested the internalization of several different molecules. First, I wanted to confirm the affect of Ack1 on clathrin-dependent internalization. Serum starved cells expressing Ack1 and GFP-EGFR were left untreated or stimulated with EGF (Fig. 3-6). As expected, cells expressing

Ack1 completely blocked EGFR internalization in EGF-treated cells (Fig. 3-6B), suggesting overexpression of Ack1 has an adverse affect on clathrin-mediated internalization. In agreement with my previous findings, this affect was also seen with the cancer mutants (data not shown).

ErbB2 is internalized via a non-clathrin, non-caveolar pathway in GA-treated cells (Barr et al. 2008). I next wanted to test if Ack1 would also inhibit internalization of ErbB2. COS-7 cells expressing either ErbB2 (Fig. 3-7A, C) or Ack1 and ErbB2 (Fig. 3-7 B, D) were treated with GA to induce the endocytosis of ErbB2. Although treatment with GA is efficient for the downregulation of ErbB2 (Zhou et al. 2003), overexpression results in high levels of plasma membrane staining. The residual staining of ErbB2 on the cell surface makes it difficult to distinguish internalized receptor. In order to clearly visualize only internalized ErbB2 receptors, I acid washed the cells to remove residual surface-bound antibody before fixation and processing for immunofluorescence (Fig. 3-7, bottom panel). Internalization was unaffected in control cells only expressing ErbB2 (Fig. 3-7 A, C). In these samples, ErbB2 that was not acid washed (Fig. 3-7A) displayed both surface staining of ErbB2 as well as some internalized puncta. Acid washed cells (Fig. 3-7C) displayed internalized puncta indicating the GA-treatment was effective in this experiment. In contrast, overexpression of Ack1 completely inhibited the internalization of ErbB2. ErbB2 was highly expressed on the surface of cells that were not acid washed (Fig. 3-7B), however, there was no staining seen in cells where residual surface-bound antibodies was removed (Fig 3-7D). These results suggest the overexpression of Ack1 also blocked the GA-induced internalization of ErbB2.

Overexpression of Ack1 has a global affect on internalization and trafficking

I next tested to see if there was any relationship between the main focus of my thesis research, Eps15 and the nonreceptor tyrosine kinase Ack1. In an immunofluorescence experiment (Fig. 3-8A) we surprisingly saw that overexpression also sequestered Eps15 at the plasma membrane as well (Fig. 3-8B) as compared to cells expressing Eps15 (Fig. 3-8A). Moreover, the mannose-6-phosphate receptor (MPR) binds newly synthesized lysosomal hydrolases in the trans-Golgi network (TGN) and delivers them to pre-lysosomal compartments (Duncan and Kornfeld 1988). We found in cells expressing Ack1, a GFP-tagged form of cation-independent MPR was also sequestered at the plasma membrane (Fig. 3-8D) as compared to CI-MPR-expressing cells (Fig. 3-8C). Additionally, we internalized cholera toxin subunit B

(CTxB) which is endocytosed by the caveolar pathway (Pelkmans and Helenius 2002) (Fig. 3-8E, F). These experiments revealed that the overexpression of Ack1 also inhibits the uptake of CTxB (Fig. 3-8F). Taken together, this data strongly suggests overexpression of Ack1 is having a global affect on the vesicular transport and internalization.

Ack1 overexpression induces the disruption and tubularization of the trans-Golgi network

Since my studies found that Ack1 resides in amorphous structures of unknown origin, I performed experiments with additional markers in order to characterize its localization further. In an effort to fully characterize the localization of Ack1, I tested different markers including that of the Golgi apparatus. Often, Ack1 overexpression induced tubularization of gamma adaptin-containing TGN elements (Fig. 3-9). These were similar to TGN-derived tubules previously described in brefeldin A (BFA)-treated cells (Alcade et al. 1992). This effect was specific to the trans-Golgi network and was not seen with a marker for cis-Golgi maker, GM130 (data not shown). Thus, I found in addition to its effects on internalization, overexpression of Ack1 disrupts the localization of gamma-adaptin**.**

DISCUSSION

Ack1 is a direct effector of activated Cdc42 and is amplified and overexpressed in a number of tumors (Mahajan and Mahajan 2010). Its strong implications in cancer and associations with the Rho family member makes it of great interest. However, until recently, little was known about this non receptor tyrosine kinase and its regulation.

The four non-synonymous mutations in Ack1 studied (Prieto-Echagüe et al. 2010a), were identified in a study in which a large sample of cancer tissues were screened for somatic mutations in protein kinase genes (Greenman et al. 2007). These four point mutations, R34L, R99Q, E346K and M409I were found to activate Ack1. It was not initially known if these cancer-associated mutations represented an additional mechanism for the transgenic properties of Ack1. The Miller lab found that cells expressing these cancer-associated mutants exhibited hallmark properties of a transformed phenotype. This data reinforced the previous implications

of Ack1 as an oncogene and suggested that these missense mutations may contribute to the development of cancer.

Our main objective was to determine the localization of Ack1 and the somatic missense mutations. This was to verify that the increased kinase activity of the cancer-associated mutant forms of Ack1 was not due to a mislocalization of the protein. The observed activation of Ack1 by these mutations could potentially have been explained by the mislocalization of Ack1 caused by the disruption of the membrane localization domain of the N-terminus. However, the intracellular localization of Ack1 was not affected by the cancer-associated mutations located in the N-terminus. Immunofluorescence experiments I performed revealed the localization of wild-type and mutant Ack1 proteins had a similar distribution pattern. Ack1 appeared to localize in puncta throughout the cell, but also in large amorphous perinuclear structures. Both wild-type and mutant Ack1 proteins demonstrated what we think is partial colocalization with the endosomal marker, EEA1. These structures were very similar to endosome-associated tubular structures that had previously been described (Grovdal et al. 2008).

Since the localization of Ack1 and mutant proteins was not clearly defined, I performed additional experiments to ensure the localization and activity of the mutants was one and the same. It had previously been reported that Ack1 had an effect on clathrin-dependent internalization (Grovdal et al. 2008; Kato-Stankiewicz et al. 2001). In my studies, I found that in cells expressing wild-type Ack1, transferrin internalization was completely blocked. I also found this phenotype for all of the cancer mutations. In addition, EGFR internalization was completely blocked by overexpression of wild-type Ack1. It is clear that altering the expression levels of Ack1 dysregulates clathrin-mediated internalization and the subsequent downregulation of EGFR. Thus, increased kinase activity may be the mechanism by which these cancer-associated mutants facilitate prolong signaling at the cell surface, promoting the transformation of cells.

One reason for the inhibition of clathrin-mediated internalization may be that overexpression of Ack1 and the cancer-associated mutants are disrupting the localization of clathrin itself. Upon EGF stimulation, it has been found that Ack1 is recruited to clathrin-coated pits (Teo et al. 2001). I therefore compared the localization of Ack1 and the cancer-associated mutants to that of clathrin. Both Ack1 and mutant-Ack1 sequestered clathrin-light chain-GFP (CLC-GFP) in large amorphous structures within the cell. In contrast, surrounding cells that did

not express Ack1 had the expected localization pattern of clathrin in small puncta throughout the cell and in the perinuclear region.

We next explored the role of the N-terminal domain in Ack1 localization and regulation. The minimal constructs NKD and KD showed very distinct intracellular localizations: NKD is clearly localized at the plasma membrane and phosphorylated, while KD is diffuse and cytosolic, and unphosphorylated. The situation is even more complex in the cases of WT and ΔNt. They both show a punctate distribution that is not identical to either NKD or KD. This suggests that the localization of full-length Ack1 results from the cumulative effect of interactions mediated by the SAM domain and other regions of the protein. It is likely that interactions between full-length Ack1 and other adaptor proteins play a role in determining subcellular localization. The SAM domain has the potential to drive Ack1 to the plasma membrane, although in the context of full-length Ack1 other protein-protein interactions make important contributions

We next wanted to continue studying alternate effects of Ack1 on endocytosis. It was clear that Ack1 had an effect on clathrin-mediated internalization, but we were curious to see if Ack1 would also inhibit the internalization through a non-clathrin endocytic pathway. Thus, I treated cells with GA to stimulate the internalization of ErbB2 through a pathway independent of clathrin (Barr et al. 2008). I found that in GA-treated cells, ErbB2 internalization was also completely blocked in cells overexpressing Ack1. Additionally, cholera toxin uptake, which occurs via caveolae (Pelkmans and Helenius 2002), was also blocked by the overexpression of Ack1. Interestingly, we also found that Ack1 overexpression also sequestered the endocytic adaptor Eps15 at the plasma membrane. Although at this time we do not know what the mechanism for these occurrences, it is clear that the overexpression and/or increased kinase activity of Ack1 has global affects on the cell and endocytosis, regardless of which pathway. This is especially intriguing because very little is known about the molecular basis of the clathrin-independent endocytic pathway used to internalize ErbB2. The fact that Ack1 overexpression inhibits ErbB2 internalization suggests that one or more proteins regulated by Ack1 may act both in clathrin-dependent and clathrin-independent endocytosis.

The most interesting and novel affect I found with the overexpression of Ack1 however was the disruption of the trans-Golgi network (TGN). I found that overexpression of Ack1 changes the localization of gamma-adaptin. It is often found in tubules that look like TGN tubules in brefeldin A (BFA)-treated cells (Wagner et al. 1994). In addition, I found another

TGN marker, the mannose-6-phosphate receptor (MPR) is sequestered to the plasma membrane. BFA causes the TGN and MPR to form tubules (Wagner et al. 1994). BFA inhibits GTP exchange on BFA causes the TGN marker and MPR to form tubules that look similar to the ones we observe here. One possibility for this occurrence is that the MPR spends time cycling between the TGN and endosomes, and occasionally to the plasma membrane, where it is then sequestered (Anitei et al. 2010). Gamma-adaptin on the other hand is involved in the transport from the TGN to lysosomes. However, additional studies must be performed to further elucidate exactly how the overexpression of Ack1 is has such global affects on these cellular functions.

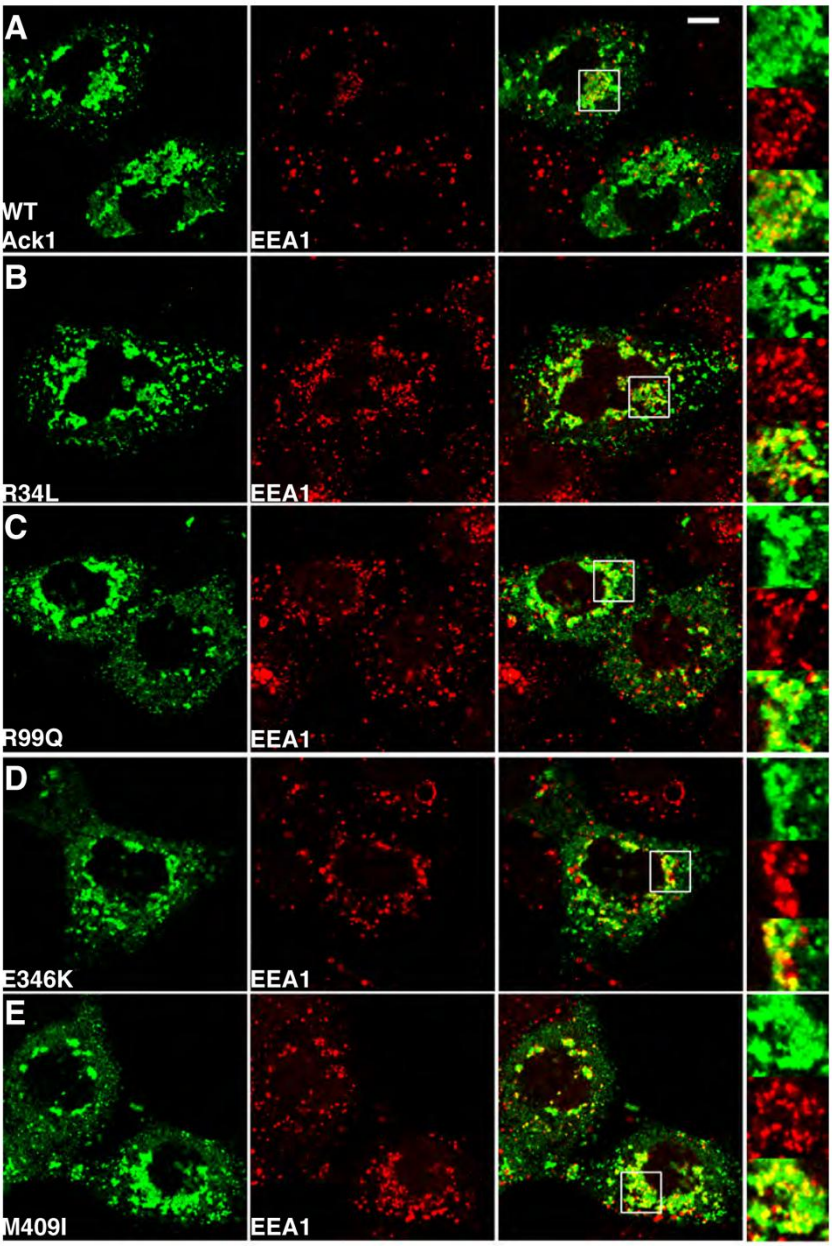

Figure 3-1. Subcellular localization of Ack1 and cancer-associated mutants. COS-7 cells expressing wild-type HA-tagged Ack1 (A) or the indicated mutant (B-E) were prepared for immunostaining and confocal microscopy as described in Materials and Methods, detecting Ack1 proteins with anti-HA antibodies (left panels; green) and endogenous EEA1 (red) as indicated. Merged images are shown to the right of the EEA1 panels. Enlarged views of the boxed regions in the merged images are shown to the right of the merged images. All Ack1 proteins partially co-localized with EEA1, but were also seen in larger, amorphous structures that often surrounded punctate endosomes. Scale bar: 10 μm.

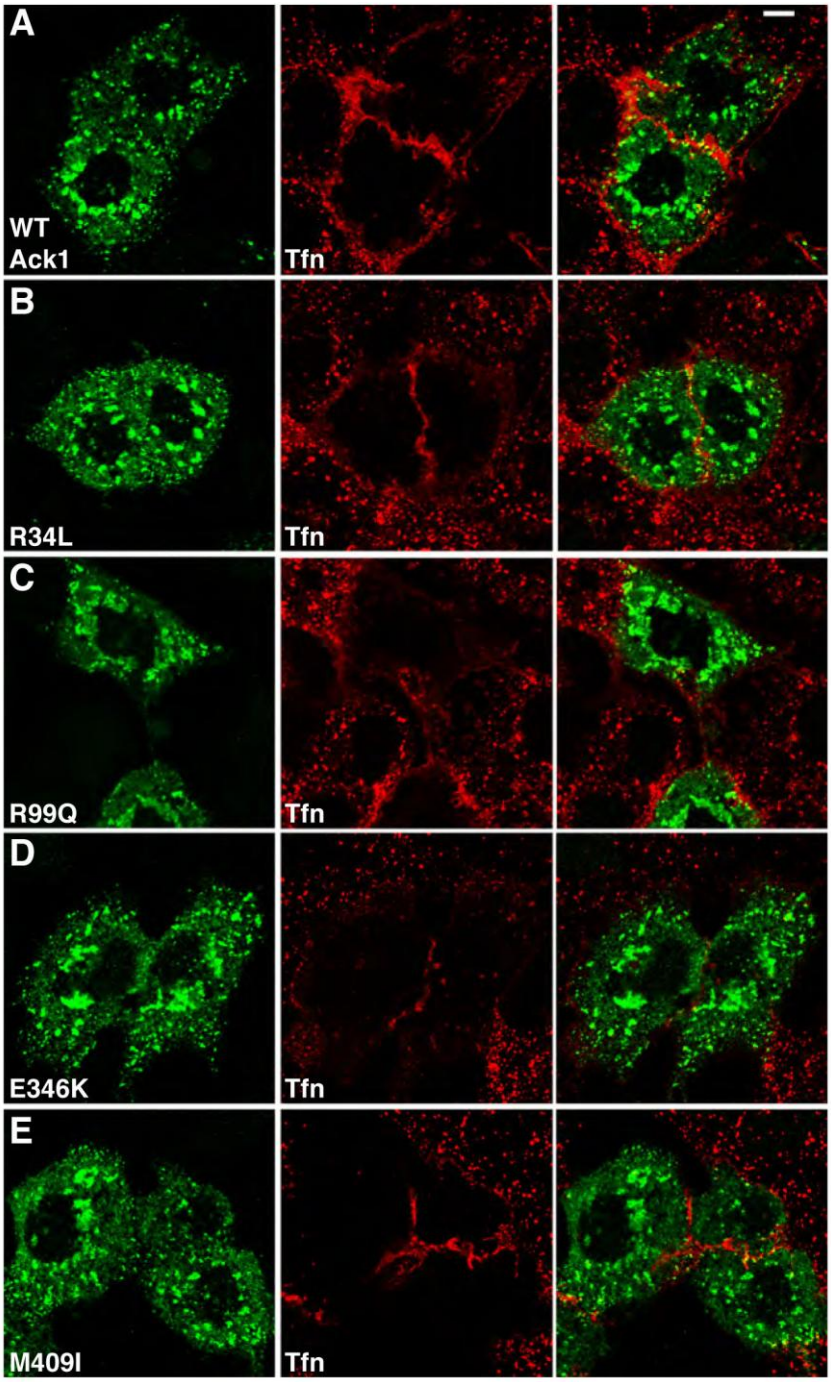

Figure 3-2. Wild-type ACK1 and cancer-associated mutants inhibit transferrin uptake. AF-594-transferrin (35 µg/ml) was added to COS-7 cells expressing wild-type HA-Ack1 (A) or the indicated Ack1 mutant (B-E) for 20 minutes at 37°C before fixation and processing for immunostaining and confocal microscopy. Ack1 proteins (left-hand panels; green) were detected with anti-HA antibodies. AF-594-transferrin (Tfn) fluorescence is shown in the center panels, and merged images in the right-hand panels. Transferrin internalization was blocked in cells expressing any of the Ack1 constructs, but occurred normally in neighboring untransfected cells. Scale bar; 10 µm.

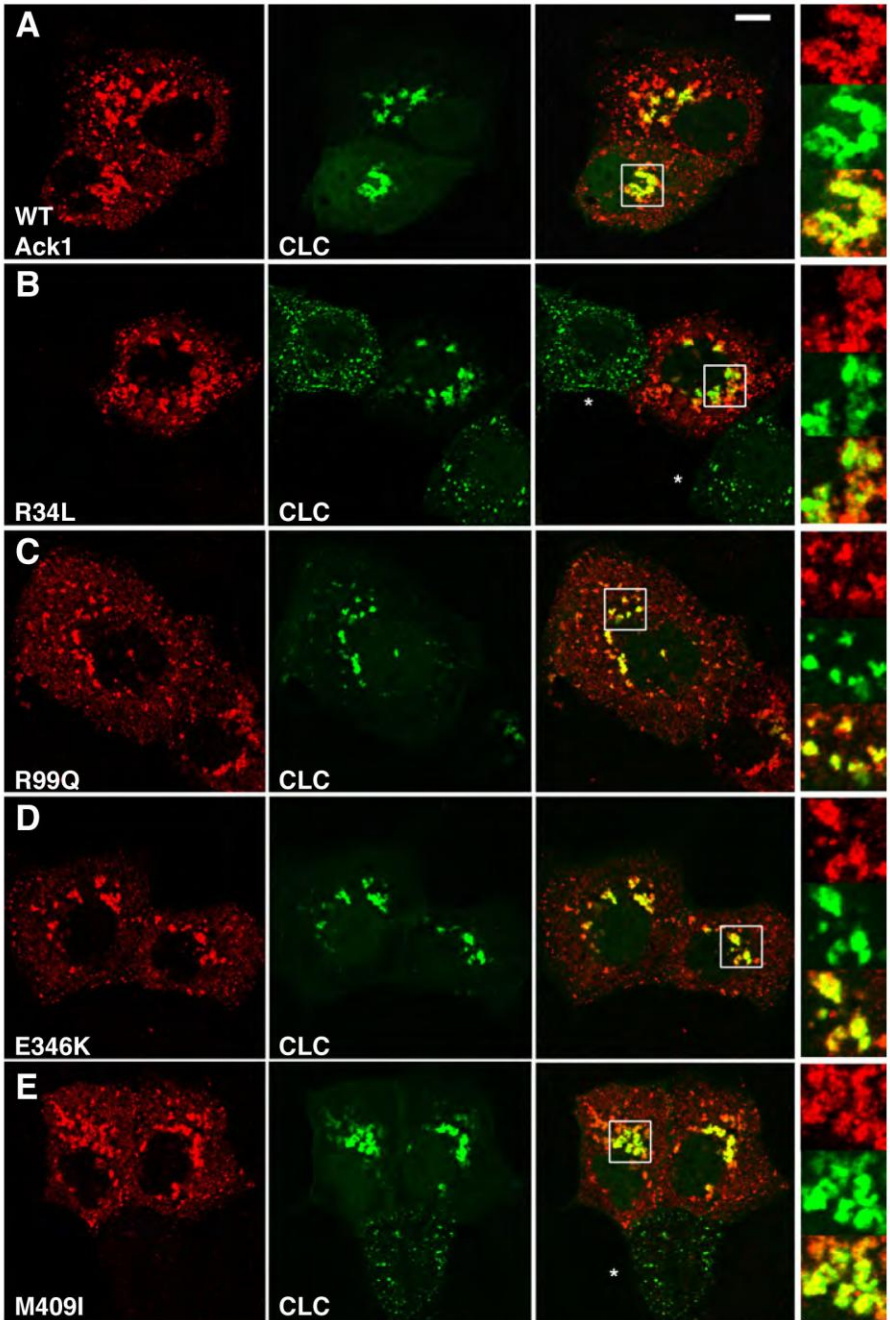

Figure 3-3. Wild-type Ack1 and cancer-associated mutants colocalize with clathrin and alter its localization. COS-7 cells transfected with GFP-clathrin light chain A together with wild-type ACK1 (A) or the indicated Ack1 mutant (B) were prepared for immunostaining and confocal microscopy. Ack1 proteins (left-hand panels; red) were detected with anti-HA antibodies. GFP-clathrin light chain A (CLC) fluorescence is shown in green. Merged images are shown to the right of the CLC panels. Enlarged views of the boxed regions in the merged images are shown to the right of the merged images. Asterisks; cells expressing GFP-clathrin light chain A only, showing the normal clathrin localization pattern. All Ack1 constructs induced redistribution of clathrin to the amorphous structures that also contained the Ack1 proteins. Scale bar; 10 μm.

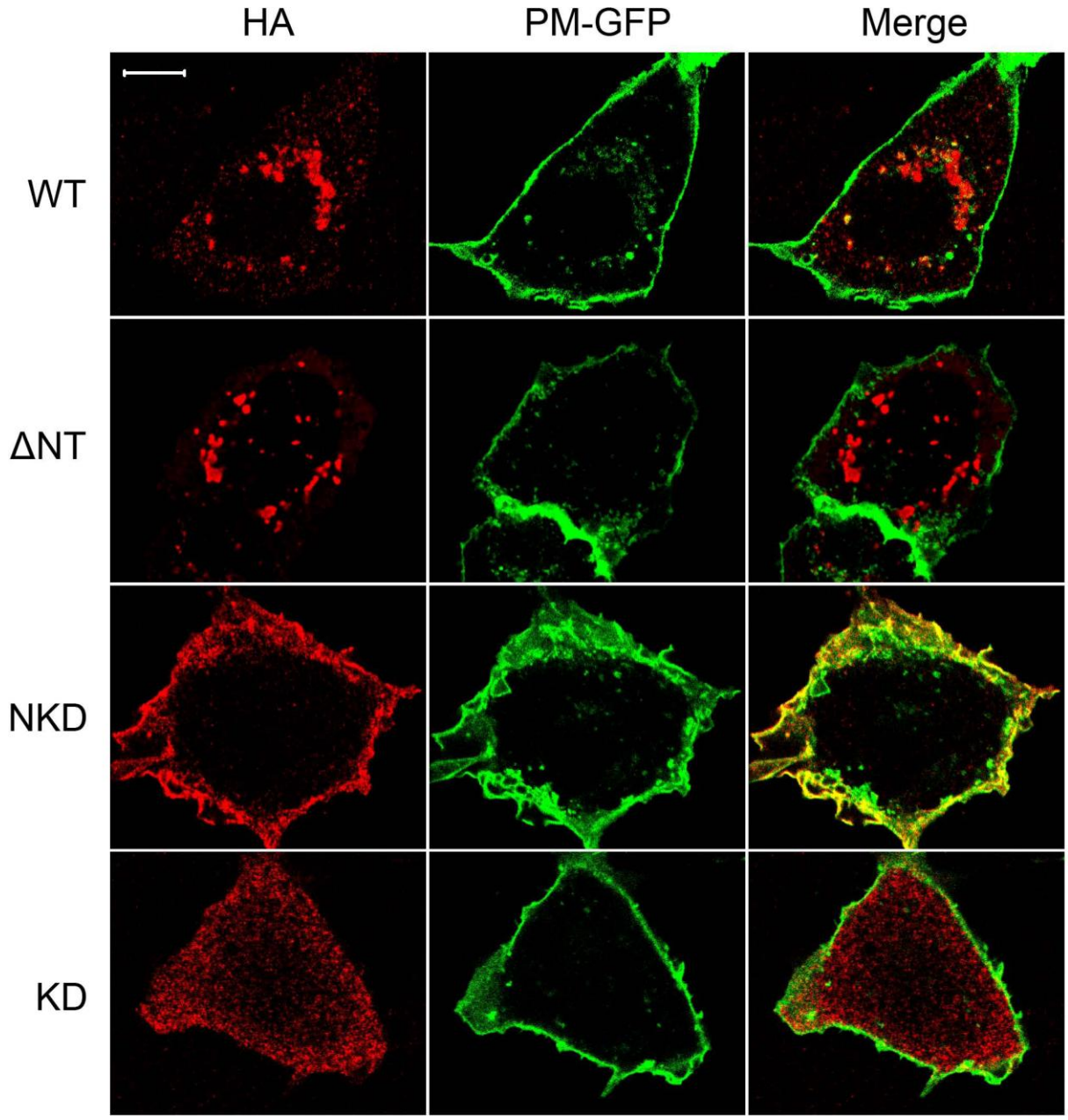

Figure 3-4. Localization of Ack1 proteins. COS-7 cells co-expressing wild-type or mutant HA-tagged Ack1 along with a membrane targeted form of GFP (PM-GFP) were prepared for immunostaining and confocal microscopy as described in Materials and Methods. Ack1 proteins were detected with anti-HA antibodies. Anti-HA; left panels; PM-GFP, middle panels; merged images, right panels. Scale bar; 10 μm.

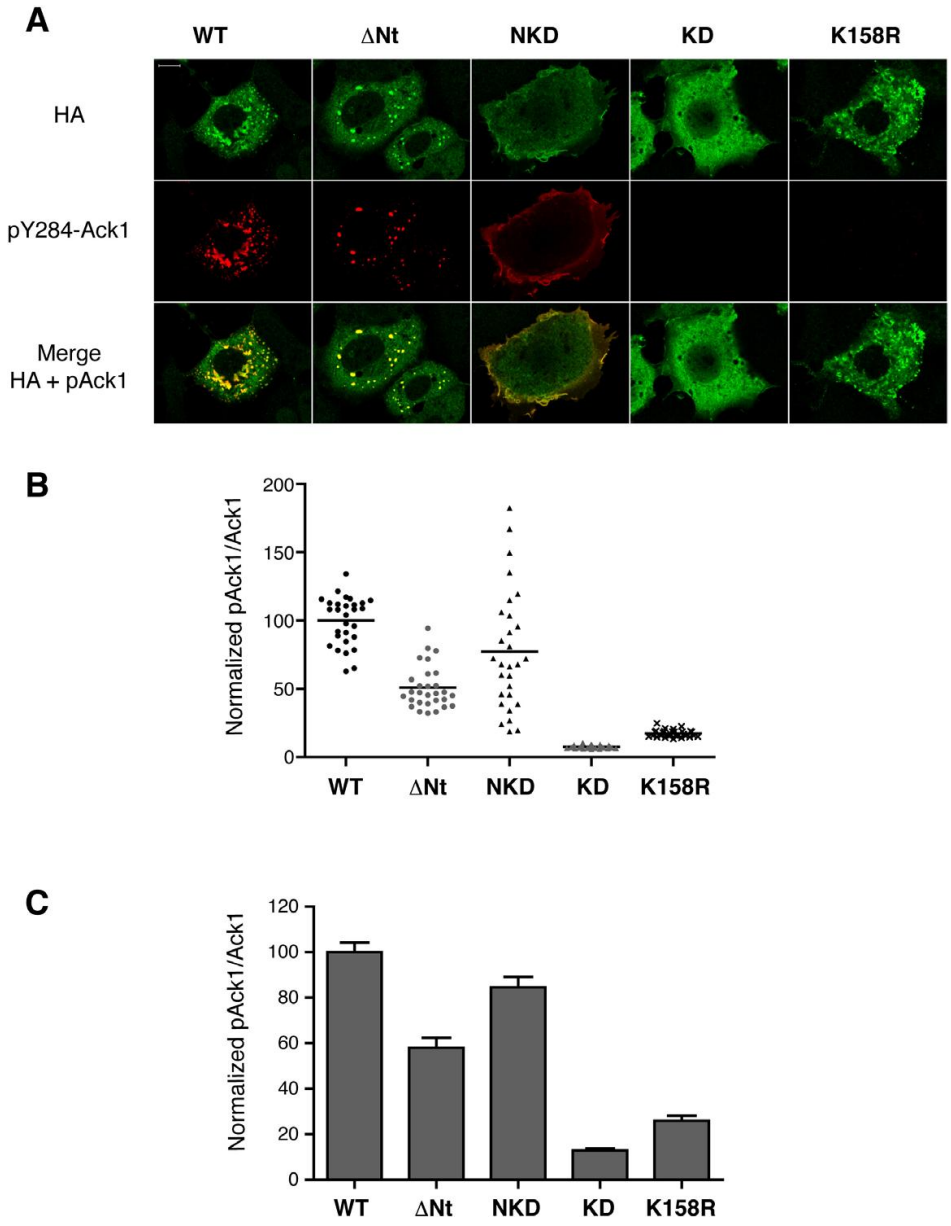

Figure 3-5. Quantitative immunofluorescence analysis. A. COS-7 cells expressing wild type or truncated forms of Ack1 were prepared for immunofluorescence using anti-phospho-Ack1 (pY284) and anti-HA antibodies. Top row: HA staining; middle row: pY284 staining; bottom row: merged images. The images in panel A were taken from the set used for quantitation and statistics in panels B and C. B, Immunofluorescence (anti-pY284 and anti-HA) was measured in three separate experiments (30 cells per experiment). The ratio of pY284Ack1/HA in each cell was calculated and normalized with respect to WT Ack1. Acquisition parameters were the same for all cells, and images shown as well as those used for quantitation were not further processed. A scatter plot for one representative experiment, with a line at the mean is shown. C, Bar graph showing the combined data of the three experiments. The data were compared using a one-way ANOVA test ($P < 0.0001$), followed by a Tukey's test of honest significant differences. All groups were significantly different from each other; $P < 0.001$ for all pairings, with the exception of KD vs. K158R ($P < 0.01$). Errors bars indicate standard error.

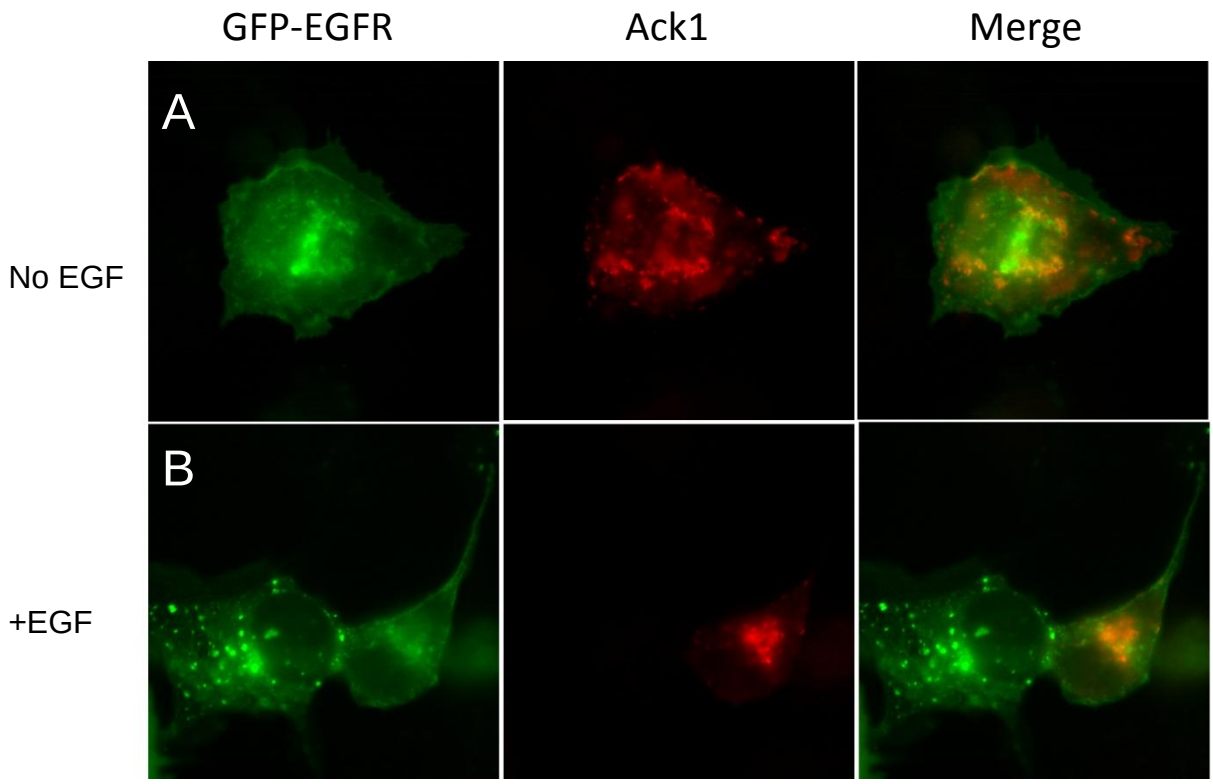

Figure 3-6. Ack1 inhibits EGFR internalization. COS-7 cells expressing wild-type HA-Ack1 and GFP-EGFR was either left untreated (top panel, A) or stimulated with 100 ng/mL EGF for 10 minutes at 37°C (bottom panel, B) before fixation and processing for immunostaining. Ack1 proteins (center panel; red) were detected with anti-HA antibodies. Merged images are shown in the right-hand panels. EGFR internalization was inhibited in cells expressing any of the Ack1 constructs, but occurred normally in neighboring untransfected cells.

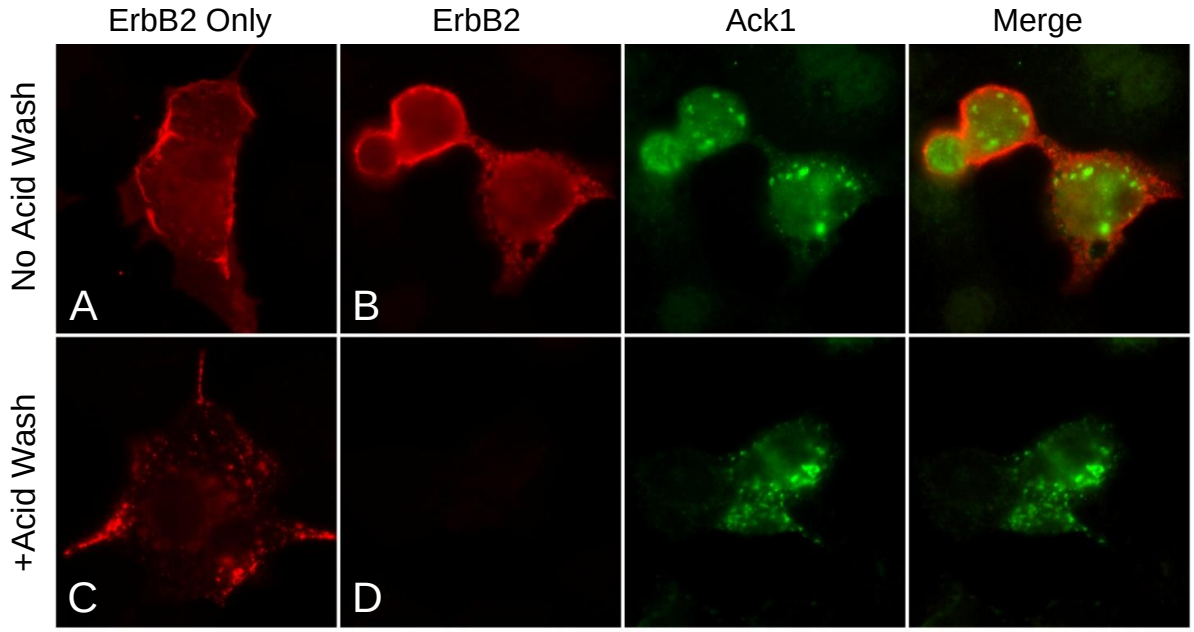

Figure 3-7. Wild-type Ack1 inhibits internalization of geldanamycin-treated ErbB2. COS-7 cells were transfected with either wild-type HA-Ack1 and ErbB2 (B,D) or ErbB2 alone (A,C). Cells were treated with GA for 1 hour. The bottom panel was acid washed to remove residual surface staining in order to distinguish internalized ErbB2 from surface staining. Cells were then fixed and processing for immunostaining. Ack1 proteins (center panels; green) were detected with anti-HA antibodies. ErbB2 was detected with anti-ErbB2 antibodies (left and center panels; red). Merged images are shown in the panels on the far right.

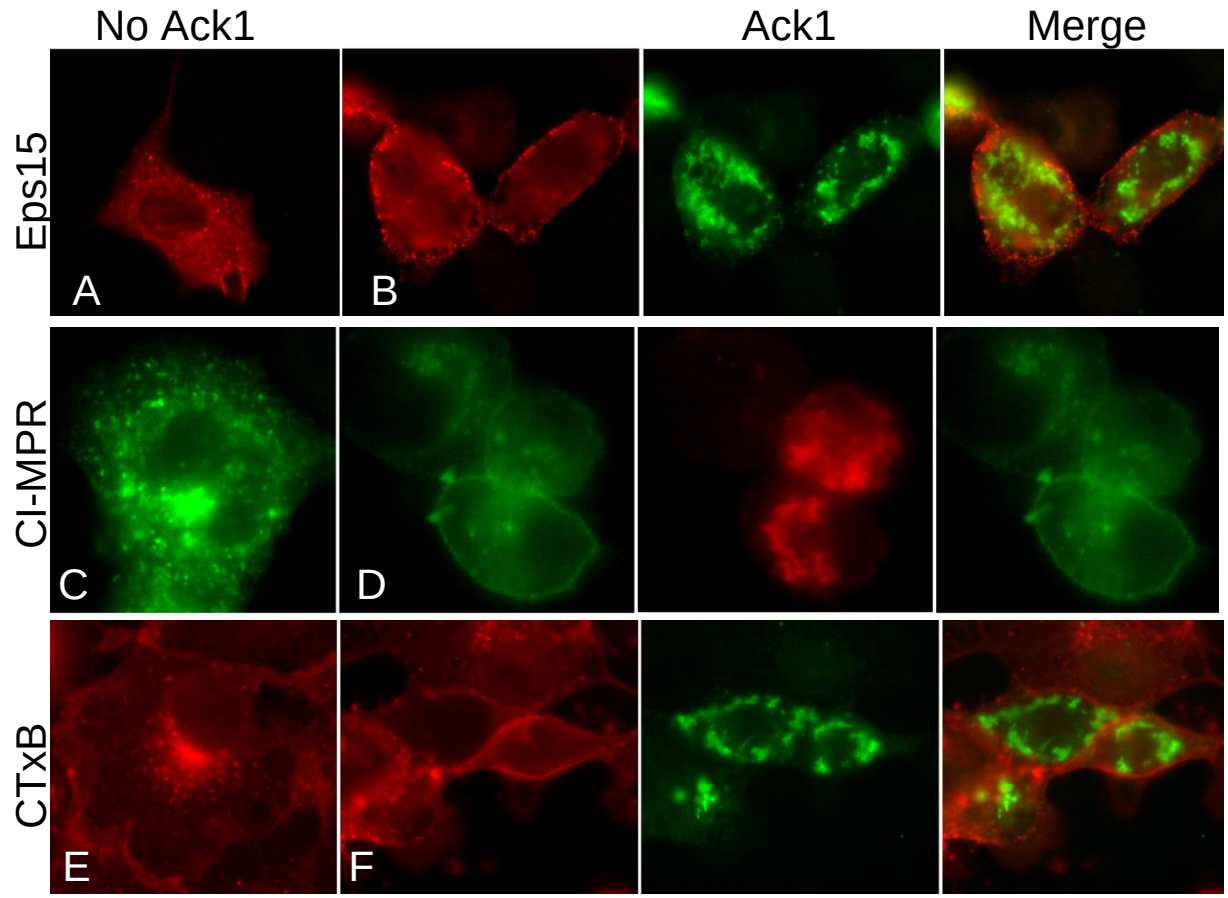

Figure 3-8. Overexpression of Ack1 has a global affect on internalization and trafficking. Immunofluorescence experiment demonstrating the affects of Ack1 overexpression on different internalization pathways. A. COS-7 cells were transiently transfected with FLAG-Ep15 only (A) or FLAG-Eps15 and HA-Ack1(B) together. FLAG antibodies were used to visualize FLAG-Eps15 and HA antibodies were used to visualize HA-Ack1 (green) as indicated. Merged panels are shown on the far right. B. COS-7 cells were transiently transfected with cation independent mannose-6-phosphate receptor (CI-MPR) (C) or CI-MPR and HA-Ack1 together (D). HA antibodies were used to visualize HA-Ack1 (red) as indicated. Merged panels are shown on the far right. C. COS-7 cells were left untransfected (E) or transiently transfected with HA-Ack1 (F). Cholera toxin subunit B (CTxB) was added to the cells at 100 µg/mL for 20 minutes at 37ºC. HA antibodies were used to visualize HA-Ack1 (green) as indicated. Merged panels are shown on the far right.

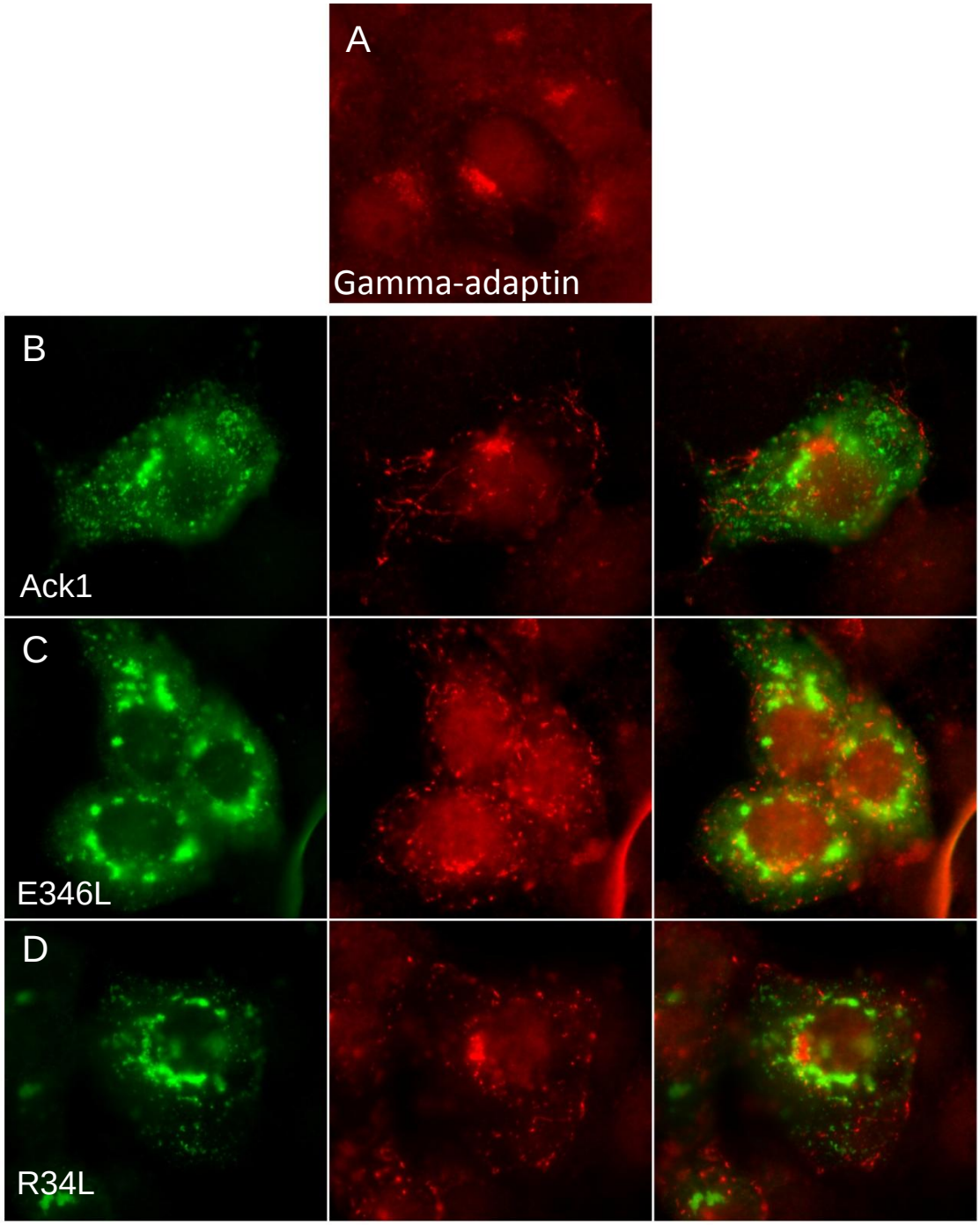

Figure 3-9. Ack1 induces the disruption and tubularization of the trans-Golgi network. COS7 cells were untransfected (A) transfected with either full-length HA-Ack1 (row B) or the cancer-associated mutants E346L (row C) or R34L (row D). HA-Ack1 and cancer mutants were visualized with anti-HA antibodies as indicated (left panel; green) along with endogenous gamma-adaptin (center panel; red). Merged images are shown in the panel on the right.

CHAPTER IV
Current and future perspectives

The main goal of my research has been further clarify the function of Eps15 at early endosomes since little is known about how it is recruited there or why. At the cell surface, Eps15 helps form the clathrin coat and is an important component for the internalization and subsequent downregulation of EGFR (Cupers et al. 1998; Edeling et al. 2006; Tebar et al. 1996) . Most of what is known about Eps15 concerns its role at the plasma membrane. Eps15 localizes to the plasma membrane in its constitutive association with AP-2, a key component of the clathrin coat (Hinrichsen et al. 2003), and also in response to stimulation with growth factors such as EGF (Torrisi et al. 1999). Our work suggests that increasing the amount of ubiquitinated cargo is sufficient in recruiting Eps15 to both early endosomes as well as the cell surface, regardless of signaling. Determining the mode of Eps15's recruitment to different membranes is fundamental in understanding how its localization may affect the downregulation of receptor tyrosine kinases.

We found that Eps15 was efficiently recruited in a UIM-dependent manner to endosomes after inducing the internalization and subsequent ubiquitination of ErbB2 in GA-treated cells. In addition, the localization of Eps15 to early endosomes increased significantly when expressing the ubiquitin constructs PM-GFP-Ub and GFP-FYVE-UbΔGG. This finding was also dependent on the UIM domains but not the EH or coiled-coiled domains of Eps15 and was not affected by the expression level of Eps15.

Low levels of Eps15 had been previously reported on endosomes (Roxrud et al. 2008; Tebar et al. 1996; van Delft et al. 1997). Moreover, Eps15 is known to interact with the ubiquitin sorting machinery at endosomes, Hrs and STAM (Bache et al. 2003; Bean et al. 2000). Roxrud et al. recently identified a short isoform of Eps15, Eps15b, that lacks the three EH domains and instead has a short 30 amino acid sequence at the N-terminus. Eps15b is otherwise structurally identical to full-length Eps15. In this study, they found full-length Eps15 preferentially associates with AP-2 at the cell surface. Thus, they surmise Eps15b is the major form of Eps15 localizing to endosomes in association with Hrs. Our findings however suggest the localization of Eps15 is dependent the amount of ubiquitinated cargo at endosomes. Under conditions where endosomes are rich in ubiquitin, Eps15 is efficiently localized there in comparison to steady-state levels.

This finding then raises the question as to whether recruitment to endosomes was dependent on its association with its interaction partner Hrs. Our studies revealed that siRNA-

mediated suppression of Hrs did not deter the recruitment of Eps15 in cells expressing GFP-FYVE-UbΔGG. This suggested Eps15 is recruited to endosomes by an alternate mechanism and that ubiquitin is sufficient for the recruitment and localization of Eps15 to endosomal membranes. This provided evidence for the first time that Eps15 localizes to endosomes independent of its association with Hrs and STAM.

In addition, overexpression of PM-GFP-Ub induced the formation of ubiquitin clusters on the plasma membrane. Although we do not believe the occurrence of these clusters to be physiologically significant, we suspect they are caused by an increase in the local concentration of ubiquitin and ubiquitin-binding adaptors forming a network on the cell surface. Consistent with this idea, these unique clusters did not form when expressing PM-GFP. Moreover, as expected, GA-treated ErbB2 also localized to these ubiquitin-rich clusters on the plasma membrane but were often excluded from them in untreated cells. Our studies revealed the colocalization of Eps15 with these clusters was dependent on the UIM domain, suggesting this recruitment was indeed ubiquitin-dependent. This finding allowed us to study the ubiquitin-dependent recruitment of Eps15 to the plasma membrane independent of clathrin-mediated endocytosis and activated EGFR.

To determine the specificity of Eps15 recruitment to ubiquitin-rich endosomes, we also expressed the endocytic adaptors epsin and intersectin. Epsin is a ubiquitin-binding endocytic adaptor that helps induce curvature of clathrin-coated pits at the plasma membrane and cooperates with a host of other endocytic adaptors to internalize clathrin-coated pits (Horvath et al. 2007). We found that epsin was also efficiently recruited to endosomes in cells expressing GFP-FYVE-UbΔGG although its comparison to GFP-expressing cells was not quantified. Binding of epsin with clathrin normally prevents the UIM domains of epsin from binding to concentrated ubiquitinated cargo on endosomes (Chen and De Camilli 2005). This is most likely because the overall affinity of epsin UIM domains for ubiquitin is lower than the affinity of the clathrin-binding domains for clathrin. Our overexpression of epsin may saturate clathrin binding and improve the availability of epsin to bind ubiquitinated proteins. In contrast, we do not see this effect with intersectin, an adaptor that is not known to bind ubiquitin. This makes recruitment of endocytic adaptors with ubiquitin-binding capabilities a reasonable explanation for the differential endosomal targeting of different UIM-domain containing or Eps15-binding proteins.

Our intention was to determine Eps15's function at early endosomes. However, this has proven to be quite challenging. First, Eps15 is a complex protein with a multitude of binding partners. It is also has been found to localize to many different areas of the cell including the plasma membrane, cytosol, early endosomes and the trans Golgi network. Therefore, it has been difficult to define a function for Eps15 on endosomes. Additionally, Eps15 is part of a group of structurally related proteins that includes Eps15R and Eps15b. These proteins may partially substitute for Eps15, making it difficult to dissect the true function of Eps15 since they may possess redundant functions under certain conditions.

Our model is that Eps15 works as molecular sensor at early endosomes. That is, under conditions where there is increased ubiquitinated cargo, Eps15 is sequestered at early endosomes and clathrin-dependent internalization is decreased. We saw this in cells transfected with GFP-FYVE-UbΔGG. Both transferrin and EGFR internalization were decreased in these cells although surface expression was similar in GFP and GFP-FYVE-UbΔGG transfected cells.

We cannot say with absolute certainty this effect is due to the decreased availability of Eps15 for clathrin-mediated internalization. This hypothesis makes sense because in cases where Eps15 is inhibited, clathrin-dependent internalization is adversely affected. We would predict that either full-length FLAG-Eps15 or FLAG-Eps15-ΔUIM would be able to rescue this phenotype. However, we were not able to achieve this with any success.

Thus, while this study has found ubiquitin to be sufficient for recruitment of Eps15, many questions remain unanswered. Several functions for endosomal recruitment of Eps15 can be imagined. One obvious possibility is to aid the ESCRT-0 complex in processing ubiquitinated cargo for degradation. This could occur by direct binding of Eps15 to ubiquitinated cargo, and/or by establishment of a ubiquitin-dependent protein network analogous to that at the plasma membrane (Dores et al. 2010; Polo et al. 2002). Our finding that siRNA-mediated silencing of Eps15 did not affect degradation of ubiquitinated ErbB2 in GA-treated SK-BR-3 cells (Fig. 2-12) argues against this possibility, and suggests that Eps15 is not uniquely required for degradation of ubiquitinated cargo, but does not exclude it. Eps15 function overlaps with that of the related adaptor Eps15R and of epsin at the plasma membrane (Sigismund et al. 2005). Eps15R, and possibly even epsin, might be recruited to endosomes to substitute for Eps15 after its silencing. Roxrud et al. found that silencing of Eps15 with an siRNA that spared Eps15b did not affect EGFR degradation. By contrast, an siRNA targeting a sequence present in both Eps15 and

Eps15b delayed EGFR degradation (Roxrud et al. 2008). These results suggested that Eps15b, but not Eps15, participates in EGFR degradation. The siRNA that we used was directed against a sequence present in both Eps15 and Eps15b. Thus, our results appear to contrast with those of Roxrud et al. (Roxrud et al. 2008). However, we could not reproducibly detect Eps15b on Western blots, probably because of the cell types and/or anti-Eps15 antibodies we used. Thus, we could not tell how well our siRNA suppressed Eps15b expression. Roxrud et al. reported more efficient suppression of Eps15 than Eps15b, despite using siRNA that targeting a shared sequence (Roxrud et al. 2008). If the same were true for the siRNA we used, sufficient Eps15b might have remained to aid in ErbB2 degradation.

Alternatively, or in addition, ubiquitin-dependent endosomal recruitment of Eps15 might function to sequester the protein away from the plasma membrane. Under some conditions, extensive delivery of ubiquitinated cargo in to endosomes might saturate the ESCRT machinery, causing accumulation of ubiquitinated proteins on the limiting membrane. In such cases, it might be advantageous to slow further delivery of such cargo to endosomes, to allow the ESCRT complexes to catch up, allowing them to package cargo into the MVB interior in an orderly fashion.

Since its initial discovery, a function of Eps15 has been determined in many different cell processes. However, much work still needs to be done to understand the intricacies of Eps15 localization, interactions and regulation. Thus, we are only at the beginning of understanding the many roles this multifunctional endocytic protein plays in cellular functions.

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