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THE UBIQUITIN LIGASE UBE4B IS REQUIRED FOR EFFICIENT EPIDERMAL GROWTH FACTOR RECEPTOR DEGRADATION

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**THE UBIQUITIN LIGASE UBE4B IS REQUIRED FOR EFFICIENT EPIDERMAL
GROWTH FACTOR RECEPTOR DEGRADATION**

by

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A

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of the Requirements
for the Degree of

MASTER OF SCIENCE

by

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Houston, Texas

May, 2010

This is dedicated to my parents.

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Many people have influenced this work through the advice, support, and encouragement they have provided.

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The ubiquitin ligase UBE4B is required for efficient Epidermal Growth Factor

Receptor degradation

Natalie Sirisaengtaksin, B.S.

Advisor: Andrew Bean, Ph.D.

The length of time that integral membrane proteins reside on the plasma membrane is regulated by endocytosis, a process that can inactivate these proteins by removing them from the membrane and may ultimately result in their degradation. Proteins are internalized and pass through multiple distinct intracellular compartments where targeting decisions determine their fate. Membrane proteins initially enter early endosomes, and subsequently late endosomes/multivesicular bodies (MVBs), before being degraded in the lysosome. The MVB is a subset of late endosomes characterized by the appearance of small vesicles in its luminal compartment. These vesicles contain cargo proteins sorted from the limiting membrane of the MVB. Proteins not sorted into luminal vesicles remain on the MVB membrane, from where they may be recycled back to the plasma membrane. In the case of receptor tyrosine kinases (RTKs), such as epidermal growth factor (EGF) receptor, this important sorting step determines whether a protein returns to the surface to participate in signaling, or whether its signaling properties are inactivated through its degradation in the lysosome. Hrs is a protein that resides on endosomes and is known to recruit sorting complexes that are vital to this sorting step. These sorting complexes are believed to recognize ubiquitin as sorting signals. However,

the link between MVB sorting machinery and the ubiquitination machinery is not known. Recently, Hrs was shown to recruit and bind an E3 ubiquitin ligase, UBE4B, to endosomes. In an assay that is able to measure cargo movement, the disruption of the Hrs-UBE4B interaction showed impaired sorting of EGF receptor into MVBs. My hypothesis is that UBE4B may be the connection between MVB sorting and ubiquitination. This study addresses the role of UBE4B in the trafficking and ubiquitination of EGF receptor. I created stable cell lines that either overexpresses UBE4B or expresses a UBE4B with no ligase activity. Levels of EGF receptor were analyzed after certain periods of ligand-induced receptor internalization. I observed that higher expression levels of UBE4B correspond to increased degradation of EGF receptor. In an *in vitro* ubiquitination assay, I also determined that UBE4B mediates the ubiquitination of EGF receptor. These data suggest that UBE4B is required for EGFR degradation specifically because it ubiquitinates the receptor allowing it to be sorted into the internal vesicles of MVBs and subsequently degraded in lysosomes.

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INTRODUCTION

1. *Endocytosis*

Endocytosis is a cellular process that allows the cell to internalize materials and molecules from its extracellular environment. This single pathway serves many functions. Endocytosis allows for nutrient uptake, and plays a critical role in maintaining cell surface homeostasis (1, 2). It can also serve as a point of entry for potentially harmful entities, such as toxins and invading pathogens (2). Endocytosis is also a key step in receptor downregulation, one of several processes that a cell utilizes to maintain homeostasis by altering its complement of cell surface localized proteins that allow the cell to interact with molecules present in the extracellular environment. (2, 3). Some cell surface proteins are internalized to inhibit further action on the plasma membrane (e.g. ion channels) and/or further signaling by temporary downregulation (e.g. AMPA receptors) or by a more permanent downregulation (e.g. receptor kinases) (4). I have focused this project on understanding the mechanisms by which a certain class of cell surface molecules, receptor tyrosine kinases, is inactivated by endocytic processing, which involves their internalization and subsequent degradation.

1.1 *Receptor-mediated endocytosis*

The theory of receptor-mediated endocytosis was first used to explain the phenomenon of cellular cholesterol metabolism regulation, which is dependent upon the binding, internalization, and degradation of low density lipoprotein receptors (5). We now know that receptor-mediated endocytosis regulates the residence time of

many molecules at the plasma membrane, such as ion channels, like the epithelial sodium channel (ENaC), and the epidermal growth factor (EGF) receptor, among many others (6, 7). In this project, I will use the EGF receptor as a model membrane protein to examine the molecular processes involved in discrete stages of endocytic processing, as there are many available reagents, the itinerary of the receptors has been well-studied, and they have been frequently used to model events in endocytosis (6).

In many cases, the binding of a ligand to its respective receptor does not trigger its internalization by endocytosis; this is to say that the binding event does not affect the expression levels of the receptor at the plasma membrane as seen in the case of ligand gated ion channels (6). For many receptors, such as G protein-coupled receptors or growth factor receptors, ligand binding stimulates their internalization and entry into the endocytic pathway (6). This leads to the immediate reduction in cell surface expression levels and, for some proteins such as EGF receptor, an eventual decrease in total receptor expressed in the cell (6).

1.2 *Internalization of EGF receptor is clathrin-dependent*

EGF receptor is frequently used to model changes in growth factor receptor endocytosis because its itinerary in the cell is well-defined (6). These receptors are degraded only by endocytosis and not by other pathways, such as the proteasome pathway (8). EGF receptors are internalized enter the endocytic pathway and meet one of two outcomes; they are either recycled up to the plasma membrane, or are degraded in the lysosome (9).

When not exposed to EGF ligand, EGF receptors are scattered about the plasma membrane (6). Upon exposure and binding of EGF ligand, the newly formed ligand-receptor complexes begin to accumulate at clathrin-coated pits, which are areas of the plasma membrane that are defined by a clathrin coat on the membrane's cytoplasmic side (6). The polyhedral clathrin lattice that delimits clathrin-coated pits is composed of two main structural molecules: clathrin and adaptor protein complexes (6, 10). The clathrin molecule is made up of three heavy chains, which are about 190 kDa, and three light chains, which are about 23 to 27 kDa (6). The assembly of the heavy and light chains forms a three-legged structure referred to as a triskelion (6). Triskelions assemble into clathrin coats through their interaction with the adaptor complex AP-2, a four-subunit complex about 267 kDa in size (6). AP-2 complexes are also tasked with ensuring that cargo proteins remain bound to the clathrin lattice (10). Rearrangements in the clathrin coat structure result in the invagination of the coated pit (6, 10). EGF ligand-receptor complexes and other surface ligand-receptor complexes are recruited to coated pits; these proteins become cargo in vesicles formed as fission of endocytic vesicles occurs (6, 10).

1.3 Overview: Endocytic Pathway

The endocytic pathway can be deconstructed into distinct phases based on cargo movement and morphology of intracellular, membrane-bound organelles called endosomal compartments (11). Following internalization, EGF ligand-receptor complexes begin their route through the cell by passing through early

endosomes, late endosomes/multivesicular bodies, and lysosomes (12, 13). This process is depicted in (figure 1).

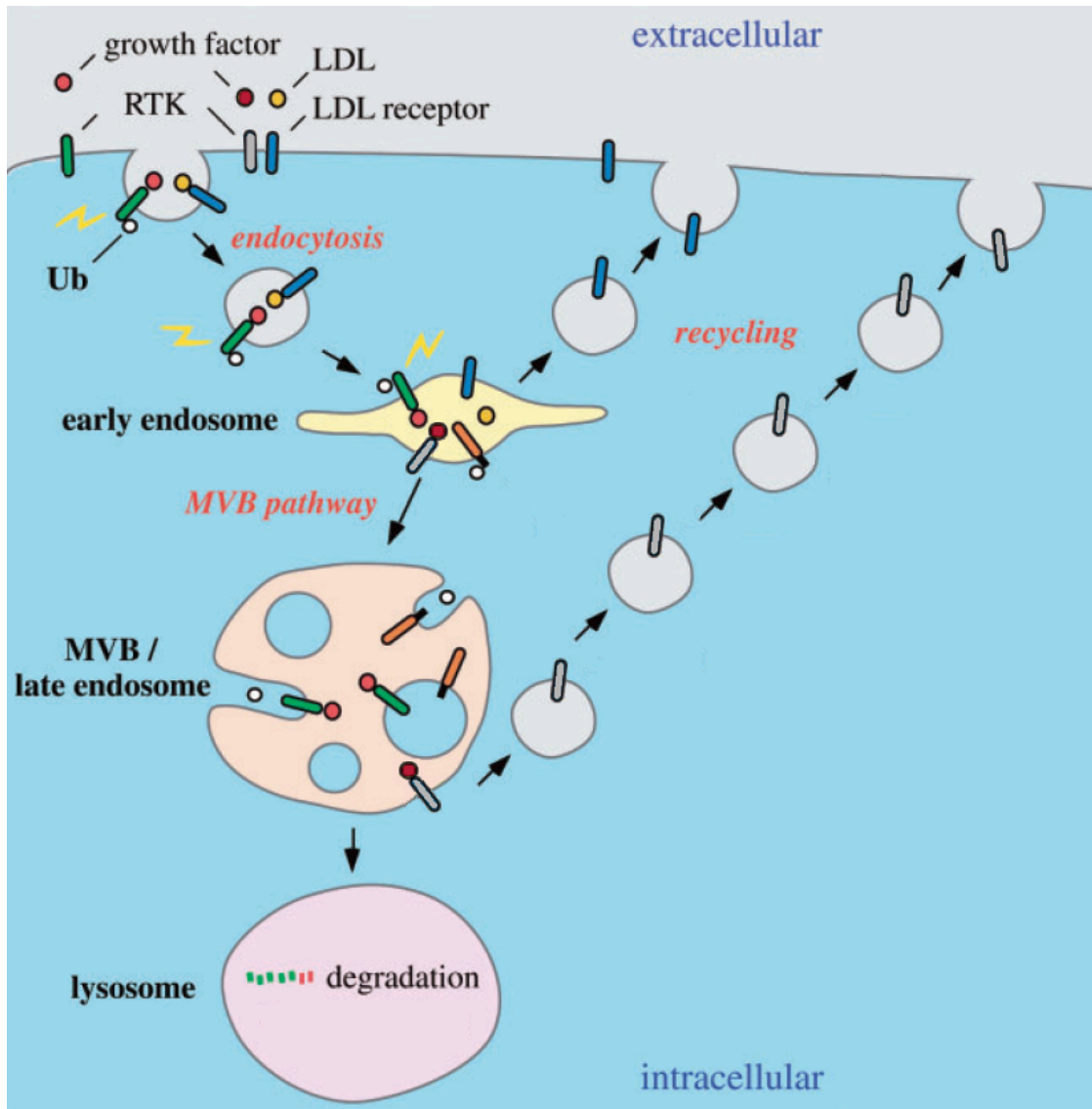


Figure 1- Endocytic pathway [adapted from original figure (14)].

After binding to its respective ligand, receptor tyrosine kinases (RTKs), such as EGF receptor, are internalized and become cargo in a clathrin-coated vesicle (6). These vesicles fuse with an early endosome, that mature or fuse with a late endosome (6). Proteins either remain on the limiting membrane and can be recycled back up the surface, or are sorted into MVBs for eventual degradation in the lysosome (14). Sorting is believed to be dependent on ubiquitin sorting signals (14).

Masayuki Komada and Naomi Kitamura, The Hrs/STAM Complex in the Downregulation of Receptor Tyrosine Kinases, The Journal of Biochemistry, 2005, Volume 137, Number 1, Pages 1-8, by permission of Oxford University Press.

First, clathrin-coated vesicles containing cargo proteins from the plasma membrane shed their coats and fuse with early endosomes, the first stop in the endocytic pathway (6). In cells incubated with EGF at 37°C, EGF ligand-receptor complexes can be seen in early endosomes as soon as two to five minutes (6). Early endosomes are tubular in shape, and tend to be located closer to the cell membrane (12).

Next, cargo proteins are delivered to late endosomes, which differ morphologically from early endosomes in that they are more spherical in shape, and are normally located near the nucleus (12). These proteins either remain on the surface of these organelles, from where they may be recycled back to the plasma membrane, or they may be sorted into multivesicular bodies (MVBs) for degradation in the lysosome (12).

EGF receptors and other receptors sorted for degradation reach their final destination in the lysosome; EGF bound to EGF receptors can be detected in these compartments between 30 to 60 minutes after initial internalization (6). Cargo proteins that reach lysosomes are degraded by lysosomal proteases (15).

Still, the question remains, what determines whether a receptor is recycled to the plasma membrane or sorted for degradation?

1.4 *The multivesicular body is the site of a critical sorting event*

MVBs are a subset of late endosomes characterized by the small luminal vesicles that form as cargo proteins are sorted into the organelle (12). The mechanism by which proteins are sorted for degradation, into what some refer to as

the MVB pathway, is conserved throughout eukaryotic organisms, from yeasts to humans (14).

The distinct structure of MVBs was captured by electron microscopy as early as the 1950s (12). These early studies observed the formation of invaginations in the endosomal membrane, similar to what occurs early in endocytosis at the plasma membrane; however, while the initial event at the plasma membrane buds membrane into the cytoplasm, the infolding of the MVB membrane occurs away from the cytoplasm towards the lumen of the MVB (12). Using electron microscopy, other labs have shown that EGF and its receptor are sorted into luminal vesicles of the MVB; the MVB ultimately fuses with a lysosome, and releases its contents for exposure to the harsh, proteolytic environment of the lysosome (12). Proteins that are not included in luminal vesicles remain on the limiting membrane of the MVB, from where they are shuttled back to the plasma membrane or transported to another part of the cell (12). The implication of these studies is that EGF receptor and similar proteins must be sorted into the MVB in order to be degraded in the lysosome. Given that EGF ligand-receptor complexes are degraded only by endocytosis, MVB sorting is critical to the EGF receptor degradation process (15). While some studies have suggested that proteasomes can play a part in regulating EGF receptor degradation, proteasomes are unlikely to be involved directly in degradation of the receptor itself (15).

Studies involving the yeast species *Sacchromyces cerevisiae* have been elemental in identifying key proteins and complexes that regulate the MVB sorting event (12). One protein that plays a critical role in MVB sorting is the hepatocyte

growth factor-regulated substrate (Hrs), which is involved in the recruitment of proteins and complexes that comprise the molecular machinery thought to be required for sorting into the MVB (14).

1.5 *Hrs recruits sorting machinery to endosomal membranes*

Hrs is a 115 kDa protein found in mammals and has homologues in insects and fungi (11, 16). It is a cytosolic protein that localizes at the early endosome and directly interacts with proteins known to play a part in protein trafficking (11). Mutation or deletion of Hrs in fly, mouse, and yeast models yields an abnormal endosomal phenotype in which the organelle appears enlarged, and could be indicative of an inability to traffic its contents to other cellular destinations (11). The yeast ortholog of Hrs, Vps27, is classified as a class E Vps (vacuolar protein sorting) protein whose deletion results in impaired endocytic trafficking (17). The large, aggregated endosomal phenotype observed in yeast is also seen in mouse embryos in which the Hrs gene has been deleted (18). These data may suggest that Hrs affects cargo sorting, trafficking, or both (11).

Hrs contains many distinct domains, two of which serve to secure the protein onto the early endosome (see Figure 2). The FYVE (Fab1/YOTB/Vac1/EEA1) domain binds phosphatidylinositol(3)-phosphate [PI(3)P], which exhibits enhanced expression on endosomal membranes (14). The second coiled-coils domain (CC2) binds SNAP-25, which is also found on the membrane of endosomes, and is identified as an endosomal Hrs receptor (19).

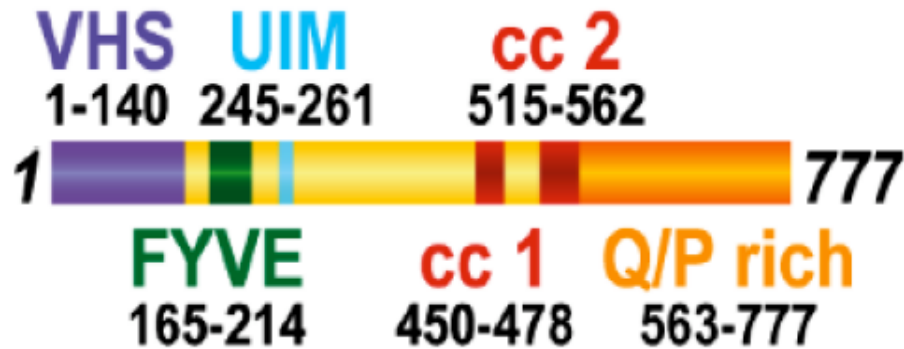


Figure 2- Domains of Hrs.

A schematic of the Hrs domain structure is shown. The VHS (Vps27/Hrs/STAM) domain is located at the N-terminal end; the FYVE (Fab1/YOTB/Vac1/EEA1) domain binds phosphatidylinositol(3)-phosphate [PI(3)P], which is abundant on endosomal membranes (14, 20). The UIM (ubiquitin-interacting motif) allows Hrs to bind to ubiquitinated protein (20). The remaining domains include two coiled-coil domains (CC1 and CC2) as well as a proline/glutamine rich domain (21). The FYVE and CC2 domains work in concert to anchor Hrs to the endosomal membrane by binding PI(3)P and SNAP-25 (syntaxin and synaptosomal associated protein of 25 kDa; serves as receptor for Hrs on endosomes) respectively (19, 21, 22).

Hrs is able to bind either STAM1 (signal-transducing adaptor molecule 1) or STAM2; both are members of a protein family collectively referred to as STAM (23). Both Hrs and STAM contain a coiled coil domain through which they bind to one another (14). Hrs recruits STAM to endosomes, and the resulting Hrs/STAM complex remains localized at endosomal membranes (20).

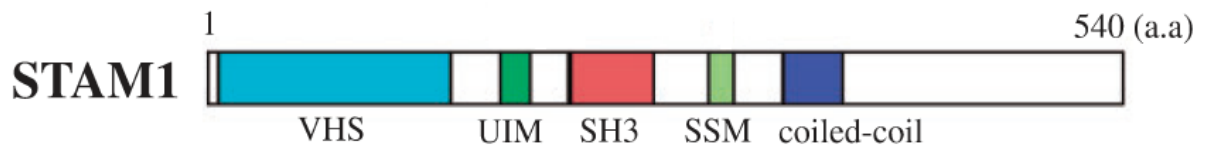


Figure 3- Domains of STAM1 [adapted from original figure (14)].

Depicted are the domains of STAM1, which shares the same domain layout as STAM2 (14). STAM is able to bind to Hrs through its coiled-coil domain (14). STAM can also recognize ubiquitinated protein through its UIM domain.

Masayuki Komada and Naomi Kitamura, The Hrs/STAM Complex in the Downregulation of Receptor Tyrosine Kinases, *The Journal of Biochemistry*, 2005, Volume 137, Number 1, Pages 1-8, by permission of Oxford University Press.

Hrs and STAM both contain a ubiquitin-interacting motif (UIM), which is capable of directly binding ubiquitin, a 76 amino acid protein covalently bound to various proteins throughout the cell (14, 24). Mutations in the yeast orthologs of Hrs and STAM, Vps27 and Hse1, result in the inability to transport ubiquitinated cargo proteins to the lumen of the vacuole; they instead remain on the vacuolar membrane (14). This would be akin to cargo proteins remaining on the membranes of MVBs instead of being sorted into luminal vesicles. Overexpression of Hrs in mammalian cells results in the accumulation of ubiquitin-tagged proteins at the endosomal membrane; this phenomenon is not observed in Hrs mutants lacking UIM domains (14). It has been suggested that by binding to Hrs, ubiquitinated proteins can be concentrated in clathrin-coated domains of the endosomal membrane where invaginations into the lumen will form (25).

Major components of the MVB sorting machinery present in mammalian cells were initially identified through the use of yeast genetics to study sorting of proteins

to the yeast vacuole and were termed vps (vacuolar protein sorting) mutants (26). Vps mutants were classified into 5 classes based on biochemical and morphological assessment of the location in the pathway affected by their mutation (27). Members of the Vps class E protein group are constituents of three ESCRT (endosomal sorting complexes required for transport) complexes: ESCRT-I, ESCRT-II, and ESCRT-III (26). Both ESCRT-I and ESCRT-II have been shown to interact with ubiquitinated cargo proteins (14). Hrs is known to bind directly to ESCRT-I through Tsg101 (tumor susceptibility gene 101), a subunit of the complex (26). Depletion of Hrs results in a decreased association of the ESCRT-I subunits with endosomes, decreased number of multivesicular bodies present in the cell, and increased late endosome size (26). Mammalian cells expressing defective Tsg101 show impaired degradation of activated EGFR (28).

Hrs serves two roles in MVB sorting. In conjunction with STAM (forming the ESCRT-0 complex), it is involved in the recognition, initial recruitment, and concentration of ubiquitinated protein cargo into clathrin-coated regions of early endosomes (26). Then through its association with Tsg101, it recruits the ESCRT-I complex to early endosomes, which furthers the sorting of cargo and formation of MVBs (14, 26).

Based on this information, the ubiquitin tag that labels certain proteins entering the endocytic pathway plays a critical role in protein sorting. Next, we will look at the process in which a protein becomes covalently bound to ubiquitin: ubiquitination.

2. *The Ubiquitin System: an overview of ubiquitination*

Ubiquitination is a basic cellular mechanism that affects many cellular processes; its effects are implicated in cell growth, proliferation, development, and apoptosis, among others (29). The ubiquitin system was initially discovered as a mechanism to mark cytosolic proteins for proteasomal degradation; post-translational ubiquitin tagging of proteins has been shown to affect the function of many proteins.

The covalent attachment of a ubiquitin molecule (7.7 KDa) to a protein substrate is a posttranslational modification utilized by the cell to regulate protein activity. Ubiquitin is attached to lysine residues of protein substrates by the ubiquitination machinery (30).

The ligation of a ubiquitin molecule onto a protein is a specific process that requires the concerted, sequential action of three enzymes. The first protein in the three enzyme cascade is the ubiquitin activating enzyme, E1 (29). In human cells, only one type of E1 is expressed; this enzyme is responsible for activating the C-terminal glycine residue of ubiquitin (29). This activation step is ATP-dependent, leads to the formation of ubiquitin adenylate, and results in the formation of a thiolester linkage between E1 and the active ubiquitin (29, 30).

Next, the activated ubiquitin is passed off to a ubiquitin-conjugating enzyme, E2, of which there are about 60 expressed in human cells (30, 31). An E2 enzyme will then pass the activated ubiquitin molecule either directly to the lysine residues of substrate proteins, or will bind to an E3 ubiquitin ligase to catalyze the attachment of ubiquitin onto the substrate protein (31).

2.1 *E3 Ubiquitin-Protein Ligases*

There are nearly 400 different E3 ubiquitin ligases expressed in human cells (30). The disproportionate number of E3 enzymes compared to E2 enzymes is due to the fastidious nature of E3s, in that one ubiquitin ligase is capable of attaching ubiquitin to a few select proteins in the cell (30). This characteristic lends precision to the ubiquitination process.

E3 ubiquitin ligases have long been categorized into two major groups. Proteins can be classified as HECT (homologous to E6AP C terminus) E3s; this family is identified by a conserved active site cysteine residue located near the C-terminal end (29). HECT E3s can accept ubiquitin from E2s and directly ligate ubiquitin onto their protein substrates (30). Proteins that promote ubiquitin ligation from the E2 to the substrate protein are RING (really interesting new gene) E3s; these proteins contain a RING finger, a domain that regulates two zinc ions (30). U-box-type ubiquitin ligases comprise a novel family of E3 ligases and contain a U-box domain that is similar to the RING finger in structure (32). The U-box domain mediates E3 ligase activity (32). These E3s also exhibit E4 polyubiquitin ligase activity, which speeds the lengthening of ubiquitin chains (30). One particular U-box-type ubiquitin ligase, UBE4B, was identified in a screen as a potential binding partner of Hrs (see Figure 4).

2.2 *The U-box-type ubiquitin ligase, UBE4B*

UBE4B (Ubiquitination factor E4B), is the mammalian homolog of yeast Ufd2 (33, 34). The gene encoding UBE4B is located on chromosome 1p and is favored

as a possible tumor suppressor gene in neuroblastoma, a cancer that affects the sympathetic nervous system of young children (33).

Little is known about UBE4B's interaction with other proteins. It was previously shown that UBE4B interacts with VCP, an AAA-type ATPase (35). This interaction is mirrored in yeast, as the interaction between yeast orthologs Ufd2 and Cdc48 is conserved (35). It is believed that the interaction between Ufd2 and Cdc48 promotes survival of cell exposed to stressful conditions (32). UBE4B is also responsible for polyubiquitinating ataxin-3, thereby marking the abnormal protein for degradation by the ubiquitin-proteasome pathway (36). Finally, UBE4B is shown to interact, but not ubiquitinate, FEZ1 (fasciculation and elongation protein zeta 1), a protein that exerts function in neuritogenesis (37).

2.3 Ubiquitination of cargo proteins is necessary for MVB sorting

Many proteins and complexes implicated in MVB sorting contain domains that recognize and bind to ubiquitin (28). It is logical to derive that the ubiquitin moieties covalently attached to certain cellular proteins are somehow involved in the sorting process. Indeed, there is evidence that ubiquitin functions as a sorting signal that mediates the entry of cargo into the MVBs after recognition by sorting machinery (28, 38). Proteins not normally ubiquitinated or sorted into MVBs can be redirected to luminal vesicles by the addition of a ubiquitin tag (28).

Ubiquitination exerts further influence on MVB sorting early in the endocytic pathway; the ubiquitin ligase Cbl, is recruited to EGF receptors at the plasma membrane upon the formation of the EGF ligand-receptor complex, whereupon the

ligase ubiquitinates the receptor (38). A chimeric protein in which the extracellular and transmembrane domain of EGF receptor is fused to ubiquitin results in the constitutive internalization of the receptor; unlike normal EGF receptor endocytosis, this internalization is unaffected by exposure to EGF ligand (38, 39). This implies that entry of EGFR into the endocytic pathway is dependent upon ubiquitination of the receptor, which is stimulated by the complex formation of EGF and EGF receptor.

The removal of ubiquitin from a protein substrate is deubiquitination; deubiquitinating enzymes have been shown to regulate the rate of receptor downregulation (40). Overexpression of the deubiquitinating enzyme UBPY reduces overall EGF receptor ubiquitination levels and delays its degradation in cells (40). UBPY is capable of deubiquitinating EGF receptor *in vitro* and has been shown to associate with the Hrs/STAM complex (40). A similar interaction occurs between the deubiquitinating enzyme AMSH and the Hrs/STAM complex (41). A select population of EGF receptors is transported from Hrs/STAM to UBPY for deubiquitination; the removal of the ubiquitin tag prevents recognition by ESCRT-I and sorting into the MVB for degradation (40). These proteins can be reubiquitinated by c-Cbl, which facilitates their transport to lysosomes (40).

Deubiquitination is a mechanism that allows for the maintenance of a constant pool of ubiquitin (42). Deubiquitination of a cargo protein occurs before its inclusion in luminal vesicles of the MVB, and after the ESCRT complexes have performed their functions (42). Deubiquitination can also occur at the endosome prior to recognition by ESCRT-I, presumably to maintain free ubiquitin molecules

that are essential to receptor trafficking (40). Although ubiquitination of receptors occurs at the plasma membrane, reubiquitination of these receptors at the endosome must occur to ensure recognition by ESCRT-I (40). Multiple ubiquitin ligases present at membranes of endosomal compartments, including AIP4/ITCH and Nedd4 (43, 44). AIP4/ITCH has been shown to interact with Hrs (45).

In a previously performed yeast two-hybrid screen using Hrs as the bait, multiple clones encoding UBE4B were isolated indicating a potential interaction between the two proteins. This is an intriguing result for the following reasons: Hrs recruits sorting machinery to endosomes that recognize sorting signals attached to other proteins; these sorting signals are believed to be ubiquitin tags; UBE4B is a ubiquitin ligase that is likely recruited to endosomes if the Hrs/UBE4B interaction is confirmed; a solid link between the molecular components for MVB sorting and the enzymes required for ubiquitination has yet to be established.

3. *Unpublished data from the Bean Lab*

Based on the initial results of the yeast two-hybrid screen, they began to study the interaction of UBE4B and Hrs. The following is unpublished data obtained by other members of the lab regarding this interaction.

3.1 *UBE4B binds to Hrs*

The interaction between UBE4B and Hrs was shown in three ways (Figure 4).

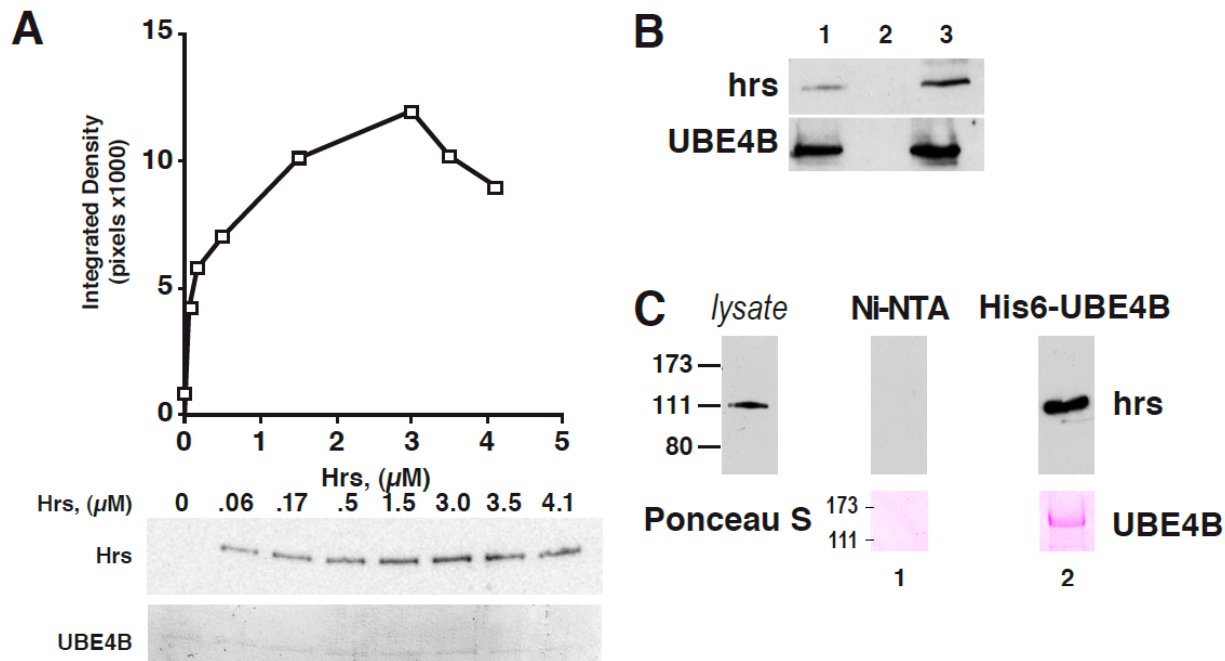


Figure 4- UBE4B binds to hrs (figure on preceding page).

(A) Recombinant hrs was incubated with a constant amount of immobilized UBE4B, washed, eluted, and run on SDS-PAGE. Increasing amounts of hrs between 0 and 3.0 μ M was bound (lanes 1-6), but when concentrations of hrs exceeded 3.0 μ M, no more could be bound to GST-UBE4B fusion protein (lanes 7 and 8). A Ponceau S stain is shown for a loading control (B) Immunoprecipitation from HeLa cell lysate (lane 1) of hrs, but not mouse IgG (lane 2) coprecipitates UBE4B (lane 3). (C) Nontransfected HeLa cell lysate (~600 μ g protein) was bound to either Ni-NTA resin (as a negative control, 1) or Ni-NTA resin with ~2 μ g of bound His₆-tagged recombinant UBE4B (2). After thorough washing, the resins were eluted with SDS-PAGE sample buffer, subjected to SDS-PAGE, and immunoblotted for Hrs and stained with Ponceau S (lysate=HeLa cell lysate ~60 μ g loaded).

They performed a yeast two-hybrid screen with hrs as the bait and isolated multiple clones encoding UBE4B. In order to confirm the hrs-UBE4B interaction, they examined whether recombinant hrs and UBE4B proteins could bind with one another. Using a constant amount of GST-UBE4B fusion protein immobilized on glutathione-agarose, increasing amounts of soluble hrs between 0 and 3.0 μ M was bound (Figure 4A, lanes 1-6). When the concentration of soluble hrs exceeded 3.0

μM , no more could be bound to the GST-UBE4B fusion protein (Figure 4A, lanes 7 and 8). This suggests that recombinant hrs and UBE4B can bind in the absence of other proteins, and that this binding is through a specific saturable site.

To see whether hrs and UBE4B can interact in situ they immunoprecipitated UBE4B from HeLa cell lysate. Hrs and UBE4B were present in the lysate (Figure 4B, lane 1) and in the immunoprecipitate (Figure 4B, lane 3). Neither hrs nor UBE4B were detectable when mouse IgG was used for the immunoprecipitation (Figure 4B, lane 2). This shows that the hrs-UBE4B interaction observed in vitro with recombinant proteins could also be detected in situ.

They used affinity chromatography to further confirm the hrs-UBE4B interaction; they lysed non-transfected HeLa cells and incubated $\sim 600\ \mu\text{g}$ of protein to either Ni-NTA resin (as a negative control) or Ni-NTA resin with $\sim 2\ \mu\text{g}$ of bound His6-tagged recombinant UBE4B (Figure 4C, lane 3). After thorough washing, the resins were eluted with SDS-PAGE sample buffer, subjected to SDS-PAGE, stained with Ponceau S and immunoblotted for hrs. They saw hrs and UBE4B present in HeLa cell lysate (Figure 4C, lane 1) and in lysate incubated with the sample containing His6 -UBE4B bound to Ni-NTS resin (Figure 4C, lane 3). However, they saw no hrs or UBE4B present in the sample of lysate incubated with only Ni-NTA resin (Figure 4C, Lane 2). This result further confirms the interaction between hrs and UBE4B.

3.2 *A region of hrs binds to UBE4B in a saturable manner*

Using various fragments of hrs, they determined the region required for the interaction with UBE4B. Hrs contains VHS, FYVE, UIM, and Q/P-rich domains as well as two coiled-coil domains (Figure 5A). Binding of UBE4B was not detectable to hrs fragments that included just the VHS, FYVE, UIM domains (Figure 5B, lane 1), both coiled-coil domains (Figure 2B, lane 3), or just the second coiled-coil domain (Figure 5B, lane 3). However, binding of UBE4B to hrs was detected using three different fragments that all contained the region of hrs between the FYVE domain and the coiled-coil region, residues 216-449 (Figure 5B lanes 2, 4, and 6). Although these fragments also encompassed the UIM domain between residues 245-261, this is most likely insufficient to account for the binding site since the N-terminal fragment, 1-258, did not bind to UBE4B (Figure 5B, lane 1) and it contains all but two residues of the UIM domain (Figure 5A, 1). The smallest region of UBE4B that was recovered in the two-hybrid interaction encompasses amino acids 63-312 of UBE4B, suggesting that this region includes the region required for Hrs binding.

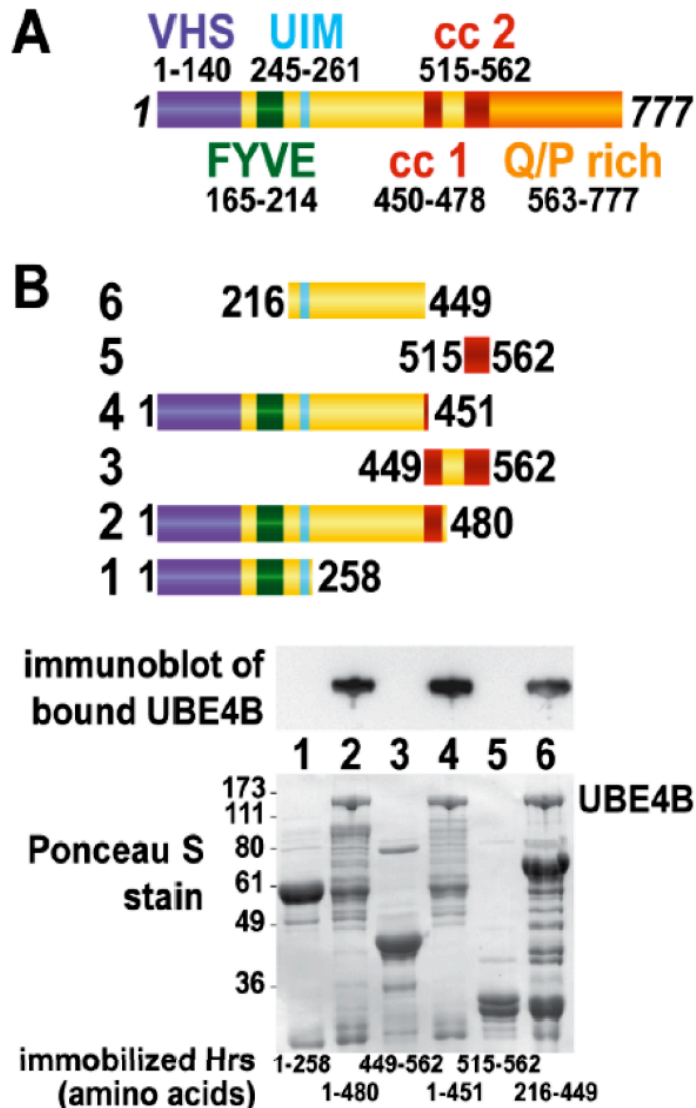


Figure 5- UBE4B binds to a region of hrs between the FYVE and helical domains.

(A) Domain structure of hrs and protein domains tested for UBE4B binding activity. (B) Binding of UBE4B to hrs fragments. Recombinant UBE4B was incubated with fragments of hrs immobilized on glutathione-agarose, washed, and remaining bound proteins were separated by SDS-PAGE. The resulting blot was probed for UBE4B. UBE4B bound to fragments 2, 4 and 6 suggesting that the minimal fragment of hrs required for UBE4B binding includes amino acids 216-449.

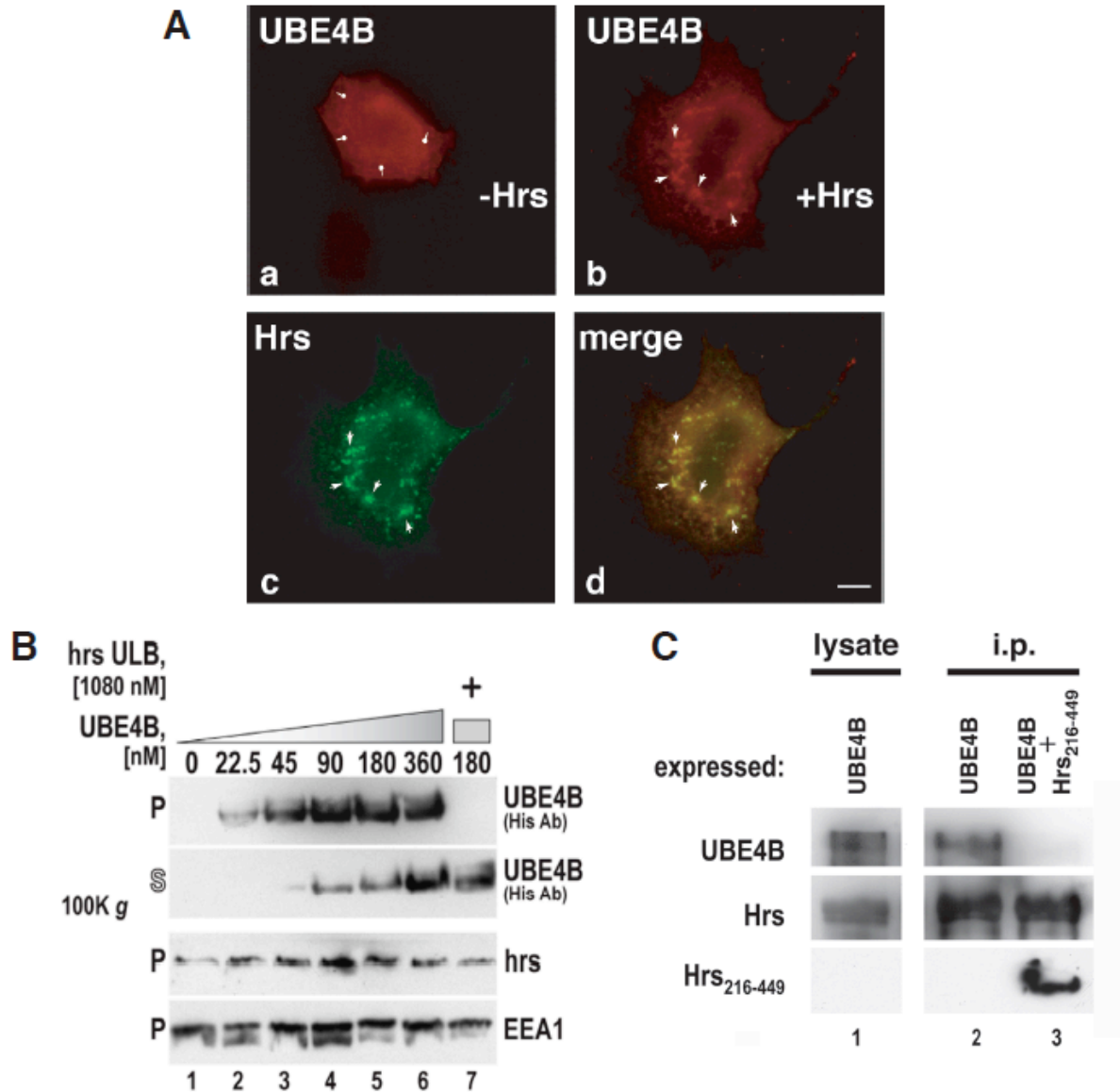


Figure 6- Hrs recruits UBE4B to endosomes.

(A) HeLa cells expressing UBE4B (a) showed a diffuse distribution, however, when UBE4B was expressed along with hrs (b) the distribution of UBE4B became punctate and the puncta also expressed hrs (c -d). The scale bar represents 2 μ m **(B)** Purified endosomes were incubated with recombinant UBE4B. UBE4B displayed saturable binding to endosomal membranes that was inhibited by addition of the region of hrs required for UBE4B binding (lane 7). Endogenous hrs is present on the purified endosomes. **(C)** Hrs₂₁₆₋₄₄₉, inhibits the binding of hrs with UBE4B *in situ*. Immunoprecipitation of hrs resulted in co-precipitation of UBE4B (lane 2), but in the presence of hrs₂₁₆₋₄₄₉ (lane 3) UBE4B was not co-precipitated.

3.3 UBE4B associates with endosomal membranes via interaction with hrs

Since hrs is thought to act in endosomal trafficking while residing on endosomal membranes they examined the localization of UBE4B to determine whether it too can associate with endosomal membranes. Immunolabeling revealed that UBE4B localized diffusely in the cytosol of HeLa cells (Figure 6A, panel A). Interestingly, expression of hrs, which results in enlarged and aggregated endosomal compartments (Figure 6A, panel C) resulted in an accumulation of UBE4B on these exaggerated endosomal membranes.

To determine whether UBE4B might bind to endosomal membranes, they incubated purified endosomes with increasing concentrations of recombinant UBE4B (Figure 6B). They observed saturable binding of UBE4B to endosomal membranes that possessed hrs and EEA-1, suggesting that a finite number of binding sites were present on this membrane. Moreover, a fragment of hrs required for its interaction with UBE4B, hrs₍₂₁₆₋₄₄₉₎, inhibited the binding of UBE4B to endosomal membranes (Figure 6B, lane 7) and inhibited the *in situ* interaction between hrs and UBE4B (Figure 6C, lane 4). This suggested that the interaction with hrs is responsible for endosomal binding of UBE4B and that hrs might be an endosomal UBE4B receptor.

They immunoprecipitated hrs from the lysate of normal HeLa cells and in HeLa cells that overexpress hrs₍₂₁₆₋₄₄₉₎. They found that while they could co-immunoprecipitate UBE4B from normal HeLa lysate, they did not detect UBE4B from lysate of cells that overexpress the hrs fragment.

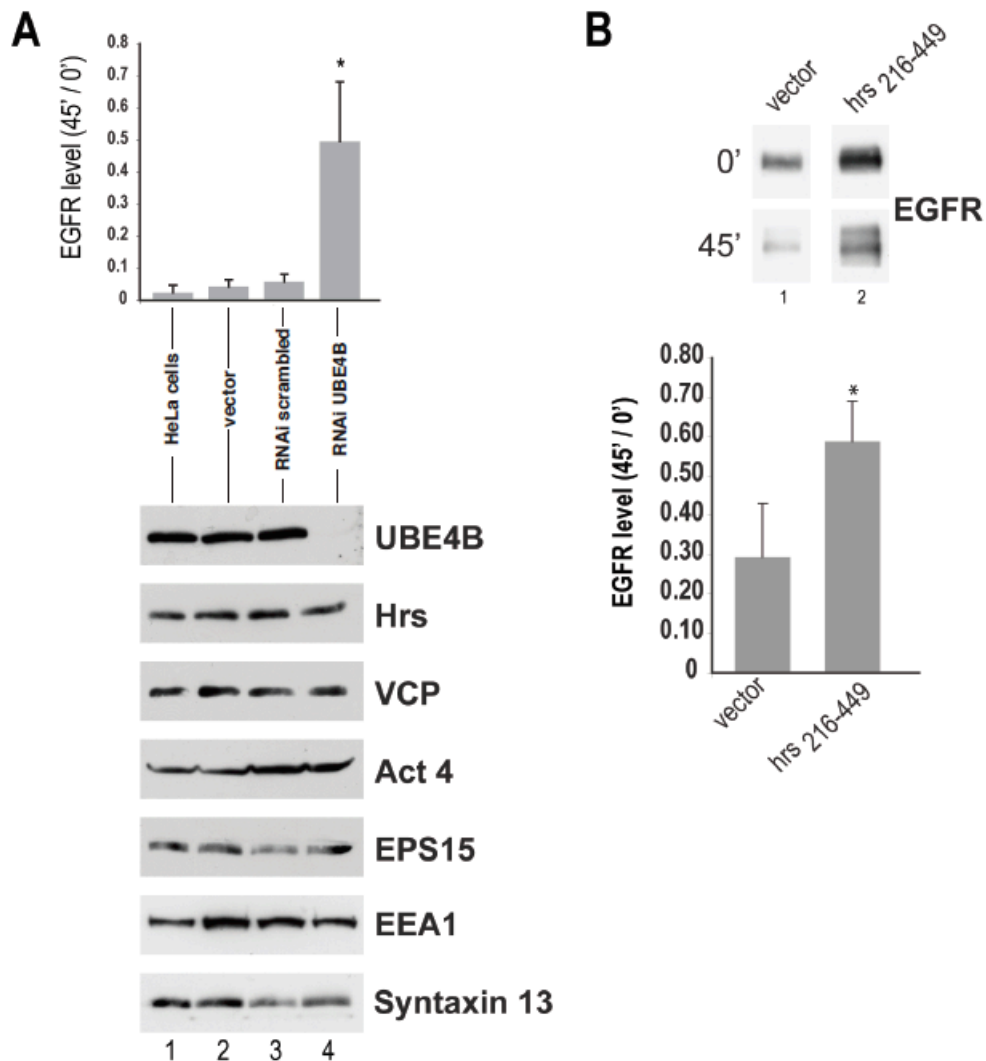


Figure 7- The effect of hrs and UBE4B on ligand-induced EGFR degradation.

(A) Depletion of UBE4B decreases EGFR degradation. UBE4B was depleted from HeLa cells using specific RNA duplexes (compare lanes 1-3 with lane 4, *bottom*) while control proteins were unaltered by UBE4B depletion. UBE4B depletion decreased ligand-induced EGFR degradation. Quantitation is presented (*top*) as EGFR remaining in the cells at 45 min/EGFR present at 0 min. * = $p \leq 0.05$. **(B)** A fragment of hrs containing the binding site for UBE4B, hrs₂₁₆₋₄₄₉, inhibited the hrs-UBE4B interaction (see Fig 6).

Since they knew that UBE4B directly binds to hrs, a protein known to play a role in receptor trafficking, they wanted to see if the expression of UBE4B would affect the degradation of membrane proteins. They looked at the degradation rates of a prototypical membrane protein, EGF receptor, in normal HeLa cells and HeLa cells depleted of UBE4B by siRNA transfection, as well as control samples of HeLa cells transfected with either an empty vector or a nonspecific oligonucleotide. The depletion of UBE4B left levels of hrs, as well as levels of other key proteins, unaffected. Levels of EGFR were compared before and after stimulation with the EGF ligand. HeLa cells depleted of UBE4B had about 50% of EGF receptor remaining after 45 minutes of EGF ligand stimulation (**Figure 7A, lane 4**), while normal HeLa cells and the other two control cell samples degraded over at least 95% of EGF receptor after ligand stimulation (**Figure 7A, lanes 1-3**).

They looked at the importance of the hrs-UBE4B interaction in EGFR degradation by analyzing EGFR levels before and after 45 minutes of EGF stimulation in HeLa cells with the hrs-UBE4B interaction intact, and in cells in which the fragment hrs₍₂₁₆₋₄₄₉₎ disrupts the interaction. Cells which overexpress the hrs fragment had nearly twice the amount of EGF receptor remaining after EGF ligand stimulation as compared to cells without (**Figure 7B**). This shows that the hrs-UBE4B interaction is at least partly required for the efficient degradation of EGF receptor.

They saw that the depletion of UBE4B from HeLa cells slows the degradation rate of EGF receptor as compared to normal HeLa cells. They showed that SKNAS, a human neuroblastoma cell line, endogenously expresses UBE4B at low levels as

compared to HeLa cells (**Figure 8A**). They compared the degradation rates of HeLa and SKNAS cells and found that the neuroblastoma cells degrade EGF receptor at a rate much slower than HeLa cells (**Figure 8B**).

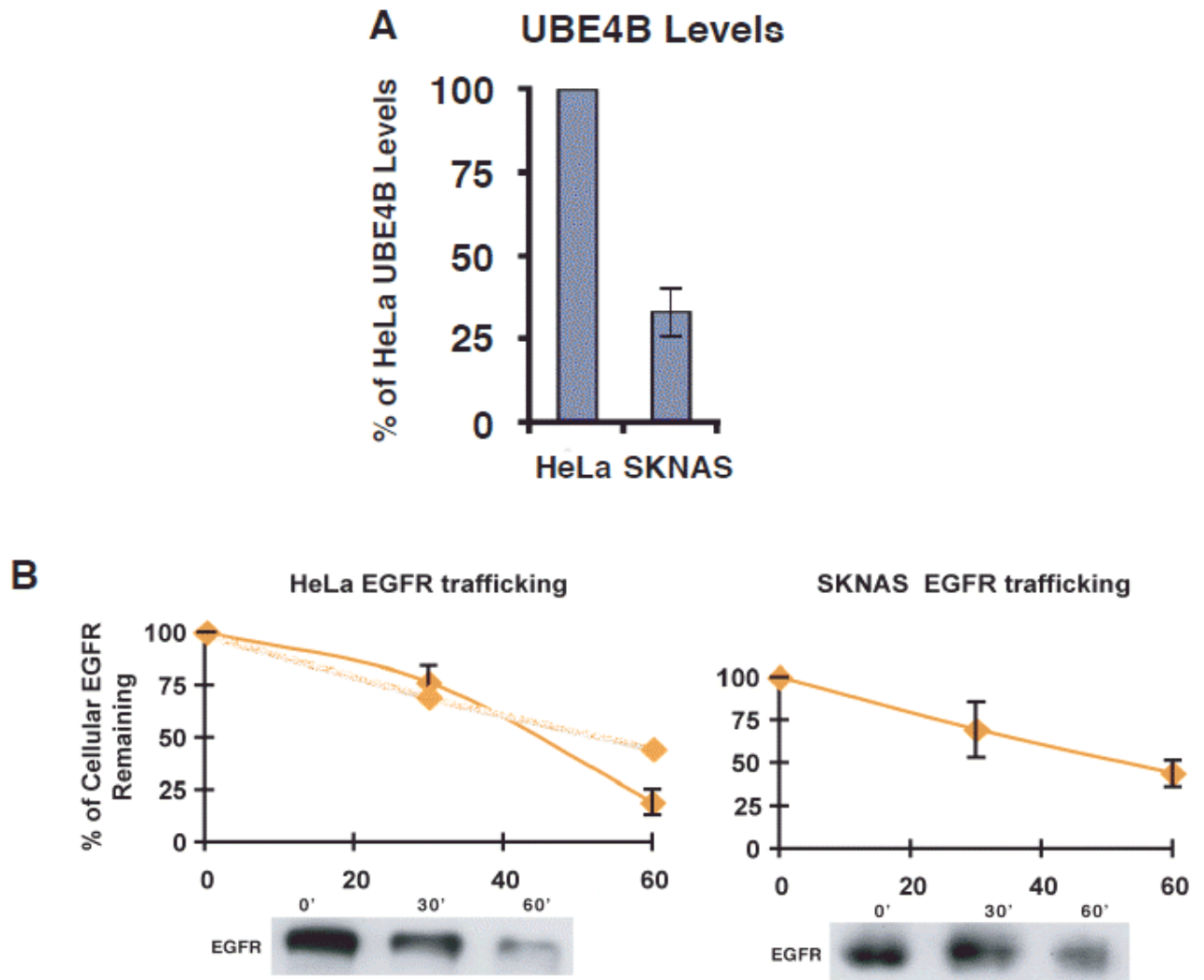
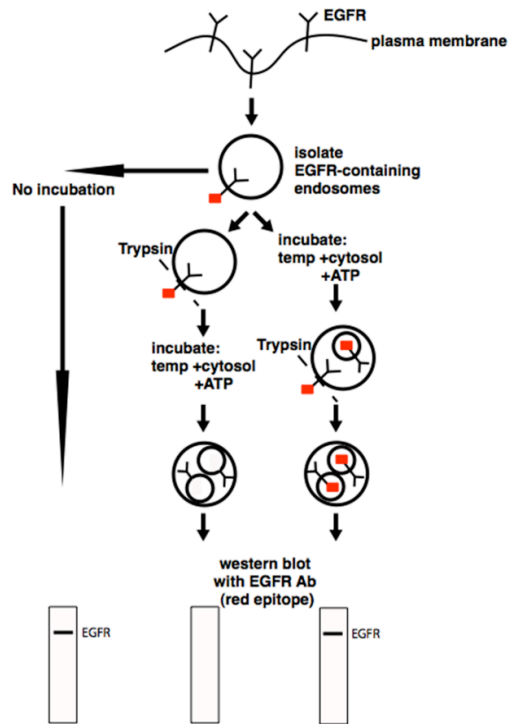


Figure 8- SKNAS cells degrade EGFR slowly.

(A) Endogenous levels of UBE4B in SKNAS cells were compared to levels in HeLa cells. SKNAS cells express about 1/3 the amount of UBE4B than HeLa cells. **(B)** HeLa cells degrade EGFR faster than SKNAS cells. HeLa and SKNAS cells were serum-starved for 2 hours, then stimulated with EGF ligand for either 0, 30, or 60 minutes. Cell lysates were collected and 100 μ g of protein from each sample was loaded, resolved by SDS-PAGE, then analyzed for EGFR content. SKNAS cells degrade EGFR more slowly, and have greater amounts of EGFR remaining in the cell (right and left, speckled line) than HeLa cells (left, solid line).

A Cell-free Assay for Endosomal Sorting



B

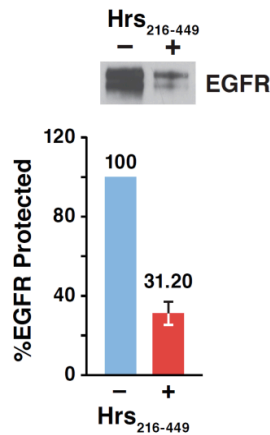


Figure 9- The blockade of hrs-UBE4B binding inhibits sorting of EGFR sorting.

(A) Schematic for a cell-free assay that reconstitutes receptor sorting (46). (B) Amounts of EGFR protected in a cell-free assay were analyzed. Incubation with the hrs₂₁₆₋₄₄₉ fragment were compared to normal assay conditions. Only about 1/3 of EGFR is protected in samples that include the hrs fragment (lane 2) as compared to the sample without the fragment (lane 1).

3.4 *Protection of EGFR into multivesicular bodies*

Because they saw that hrs recruits UBE4B to endosomal membranes, they examined whether the interaction between hrs and UBE4B affects the sorting of receptors. When cell surface receptors are internalized by endocytosis they can be recycled to the surface or degraded in the lysosome. This sorting decision is made at the multivesicular body (MVB), an intermediate organelle that is found between the endosome and lysosome. Receptors that remain on the limiting membrane of the MVB can be recycled back to the surface of the cell, while receptors that are internalized into the MVB are sent for degradation into the lysosome. They used a cell-free assay to reconstitute the sorting of EGF receptors into the MVB (46). Under normal assay conditions, membranes are collected from HeLa cells and incubated with rat brain cytosol and an ATP regeneration system at 37°C for 30 minutes. Inclusion of the fragment of hrs₍₂₁₆₋₄₄₉₎ in these reactions prevent protection of nearly 70% of EGF receptor as compared to normal reaction conditions (Figure 9B). This suggests that UBE4B affects the degradation of receptors by promoting the sorting of proteins into MVBs for degradation into the lysosome and that disrupting the interaction between UBE4B and Hrs results in inhibition of MVB sorting.

MATERIALS AND METHODS

4.1 *Materials*

Reagents for cell culturing were obtained from the following sources: Dulbecco's modified essential medium (DMEM, Mediatech, Manassas, VA); fetal bovine serum (FBS, Sigma); 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO); RPMI-1640 (Mediatech); 1% L-Glutamine (Sigma-Aldrich); and Trypsin EDTA 1x (0.25% Trypsin/2.21 mM EDTA in HBSS, Mediatech).

Reagents for lentivirus production, harvesting, and infection were obtained from the following sources: Opti-MEM I (Invitrogen, Carlsbad, CA); Lipofectamine 2000 (Invitrogen); and fetal calf serum (Invitrogen).

Reagents for the degradation assay were obtained from the following sources: Bovine serum albumin (BSA, Fisher, Pittsburgh, PA); leupeptin (Sigma); pepstatin (Sigma); PMSF (Sigma), aprotinin (Sigma), Mammalian Protein Extraction Reagent (M-PER, Pierce); rabbit polyclonal anti-EGFR (Affinity BioReagents, Golden, CO), enhanced chemiluminescence (ECL, Pierce) reagent.

Reagents for the ubiquitination assay were obtained from the following sources: mouse monoclonal anti-EGFR (for IP, Santa Cruz, Santa Cruz, CA); recombinant rabbit E1 (Boston Biochem, Cambridge, MA); UbCH5c (Boston Biochem); ubiquitin (Sigma); mouse monoclonal anti-EGFR (for Western Blot,

Millipore, Billerica, MA); anti-ubiquitin (Sigma).

Reagents for the deubiquitination assay were obtained from the following sources: His6-tagged UCH-L3 (Biomol, Farmingdale, NY); Isopeptidase T, Rabbit (Calbiochem, Gibbstown, NJ).

4.2 Cell culture

Cells were cultured as a monolayer in 10 cm plastic dishes at 37°C under 5% CO₂. HeLa cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. SKNAS cell lines were cultured in RPMI-1640 containing 10% FBS, 1% penicillin/streptomycin, and 1% L-Glutamine. Before each experiment, HeLa cells were split with trypsin/EDTA and seeded in 10 cm plastic dishes.

4.3 Lentivirus Production, harvesting, and infection

(This procedure was performed by another member of the Bean lab, but is included in this section for clearer understanding of results that will be presented).

Lentivirus production and harvesting was performed to prepare for virus infection into the SKNAS cell line. The overexpression of wild-type UBE4B, UBE4BP1140A, and GFP within each respective SKNAS cell line is driven by the ubiquitin promotor of the FUGW plasmid. First, 3 µg each of pFUGW, p8.9-1, and pVSV-1 DNA was diluted in 1.5 mL of serum-free Opti-MEM I. A 35 µL volume of Lipofectamine 2000 was diluted into a separate 1.5 mL of Opti-MEM I, and

incubated for 5 minutes at room temperature. The DNA and Lipofectamine 2000 solutions were combined and incubated for 20 minutes at room temperature, then mixed in a new flask with 5 mL DMEM (10% fetal calf serum). The resultant volume is sufficient for one T75 flask of 90% confluent cells. Cells were lifted from flasks with trypsin EDTA 1x and resuspended in 5 mL of DMEM supplemented with 10% FCS. The cell suspension was then added to the flask containing the DMEM solution containing DNA and Lipofectamine 2000 and placed in an incubator (37°C at 5% CO₂) overnight. The next day, the media was changed to DMEM (10% FCS, 1% L-Glutamine) and incubated for another 48 to 72 hours.

After the growth period, media was collected in a 15 mL tubes, and 1% SDS was added to the remaining cells before disposal. The supernatant was put through a 0.45 µm filter. The collected supernatant can be used directly, or stored at -80°C. For a more concentrated virus dilution, spin with a SW41 rotor at 25000 rpm for 90 minutes at 4°C. Resuspend pellet in 50 µL PBS and store at -80°C.

SKNAS cells were plated in six-well dishes for 15 to 20% confluence, and placed into an incubator (5% CO₂ at 37°C) for 4 to 5 hr. Media was aspirated from wells, and 2 µL of lentivirus solution was diluted in 3 mL of growth media and added to each well. Cells were placed back into the incubator allowed to grow for 5 days. The lentivirus/media solution was aspirated from the wells, and cells were dissociated from wells with EDTA (0.5 mM in PBS). Cells were transferred to 10 cm dishes, and maintained as normal SKNAS cells. Expression of virus was confirmed by immunohistochemistry, using either a mouse monoclonal anti-GFP or anti-his as primary antibodies.

4.4 EGF receptor Degradation Assay

For each trial, three 10 cm plates of cells of one cell line (either HeLa, SKNAS, GFP, UBE4B, or P1140A cell lines) were cultured to 80% confluency to examine cellular EGFR content after stimulating receptor internalization with EGF. Cells were washed three times with medium A (DMEM containing 1% BSA), and starved for 2 hours at 37°C under 5% CO₂. Media was replaced with ice-cold medium A supplemented with EGF (50 ng/mL). Plates were placed on ice, set on an orbital shaker at low speed, and incubated at 4°C.

Cells were rinsed three times with cold medium A and either kept on ice (0 mins), or incubated with warm medium A and placed in 5% CO₂ at 37°C for either 30 or 60 minutes. After incubations, cells were rinsed three times with cold phosphate-buffered saline (PBS), and scraped into a 1.5 mL solution of PBS containing 5 µL each of 10 mM leupeptin, 1 µg/µL pepstatin, 0.3 mM aprotinin, and 1.74 µg/µL PMSF per sample. Samples were centrifuged at 2,500 x *g* for 10 minutes at 4°C. The supernatant was discarded, and cell pellets were resuspended in 30 µL lysis buffer. The lysis buffer consisted of 100 µL of M-PER containing 1 µL each of leupeptin, pepstatin, aprotinin, and PMSF. Samples were rotated end over end at 4°C for 1 hour, then centrifuged at 15,000 x *g* for 15 minutes at 4°C. Supernatants were collected, protein concentrations were determined, and 50 µg of protein from each sample was resolved by SDS-PAGE. After transfer (100 V for 2 hours) to nitrocellulose membranes, blots were blocked with 5% nonfat dry milk in PBS. EGFR content within samples was analyzed by quantitative Western blotting by probing blots with a rabbit polyclonal anti-EGFR (ABR, 1:1000 dilution, incubated

overnight at 4°C). Proteins were visualized with ECL and exposed to autoradiography film; signals were quantified using ImageJ (ver 1.42).

4.5 Ubiquitination Assay

Two samples of HeLa lysate were collected as above, with each sample containing the cells of one 10 cm plate of 80% confluent cells resuspended in 30 µL of lysis buffer. The lysates were incubated overnight at 4°C with either 1 µg of mouse monoclonal anti-EGFR or 1 µg of mouse IgG control.

E. coli lysate was used as a control for the ubiquitination assay. An overnight culture of *E. coli* cells was pelleted and resuspended in 300 µL of homogenization buffer (20 mM HEPES 7.4, 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 0.1 mM DTT and a protease inhibitor cocktail (PMSF 112 µM, aprotinin 3 µM, leupeptin 112 µM, pepstatin 17µM). The cells were then lysed by sonication, then centrifuged at 15,000 x *g* for 15 minutes at 4°C. Supernatants were collected.

The ubiquitination of EGFR was performed as detailed in (34). Four reaction conditions were assembled to a total volume of 20 µL. For the positive *E. coli* and HeLa conditions, 0.1 µg of E1, 1 µg of Ubch5c, 1 µg of His₆-UBE4B bound to nickel beads, 1 µg of ubiquitin, 2 mM ATP, 1 mM MgCl₂, 0.3 mM DTT, 1 mM creatine phosphate, 0.5 units of phosphocreatine kinase, 25 mM Tris-HCl (pH 7.5), 120 mM NaCl, and either 2 µL of *E. coli* lysate or 5 µL of HeLa lysate. The negative *E. coli* condition included all above components with the exception of the E1. The negative HeLa condition included all above components with the exception of UBE4B. Samples were incubated at 30°C for 2 hours. After the incubation time, samples

were centrifuged and the supernatant containing all but the His₆-UBE4B bound to nickel beads was transferred to another tube. Samples containing HeLa lysate were incubated with 20 µL of 50% packed protein A resin. Samples were boiled then resolved by SDS-PAGE. Transfer and Western blotting were carried out as above. EGFR and ubiquitin content within samples were analyzed by probing the blot with either a mouse monoclonal anti-EGFR or anti-ubiquitin at manufacturer recommended concentrations.

4.6 Deubiquitination Assay

The deubiquitination of EGFR was performed as detailed in (47). Deubiquitinating enzymes were added to positive reactions immediately following removal of UBE4B and incubation with protein A beads. After washing, protein A beads were resuspended in 25 mM HEPES, pH 7.4, and 10 mM DTT. Then, 5 µg each of isopeptidase-T and UCH-L3 were added. Samples were incubated for 60 minutes at room temperature. Protein A beads were then centrifuged and eluted by the addition of SDS-PAGE sample buffer. Samples were boiled for 5 minutes, then subject to SDS-PAGE. Transfer and Western blotting were performed as detailed above. Membranes were then probed with anti-ubiquitin.

RESULTS

5.1 *Lentivirus infection does not affect EGFR degradation in SKNAS cells*

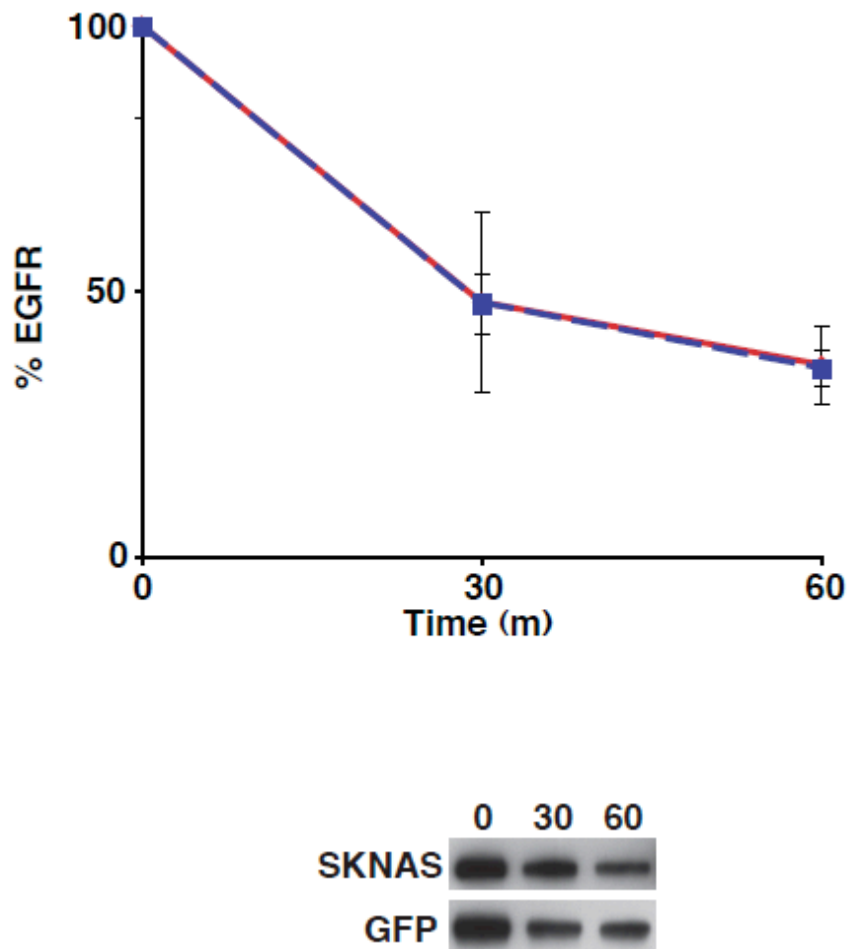


Figure 10- EGF receptor degradation in SKNAS and GFP cell lines.

SKNAS and GFP cell lines were incubated EGF ligand and analyzed for EGFR content after 0, 30, or 60 minutes of EGF internalization. Lysates of each sample were collected, and 50 μ g of protein were resolved by SDS-PAGE. Western blotting was performed, and resultant membranes were probed for EGFR. Bands were quantitated using ImageJ, and average results of trials are depicted (top). A representative blot for each cell line is shown (bottom).

The SKNAS parental cell line and SKNAS-GFP cell line (referred to as GFP cell line) were stimulated with EGF for 0, 30, or 60 minutes. Lysate from cell were collected, protein concentrations were determined; 50 µg of protein were resolved by SDS-PAGE, and EGFR content analyzed through Western blotting. The results of individual trial were averaged together (SKNAS, n=3; GFP, n=5). Degradation of EGFR in the SKNAS and GFP cell lines are very similar. Average % EGFR remaining in SKNAS is 47.7% after 30 minutes and 35.9% after 60 minutes. Average % EGFR remaining in GFP is 47.6% after 30 minutes and 35.3% after 60 minutes. These results are depicted on the graph above (Figure 10, top), and a representative Western blot is shown (Figure 10, bottom).

5.2 *Expression levels of UBE4B affect degradation rates of EGFR*

SKNAS cells were infected with a lentivirus that led to stable expression of GFP or UBE4B (referred to as UBE4B cell line). A stable cell line that expressed UBE4B_{P1140A} (referred to as P1140A cell line), a UBE4B point mutant lacking E3 ubiquitin ligase activity, was also created. Levels of UBE4B in SKNAS, UBE4B, and P1140A cell lines were determined (Figure 11).

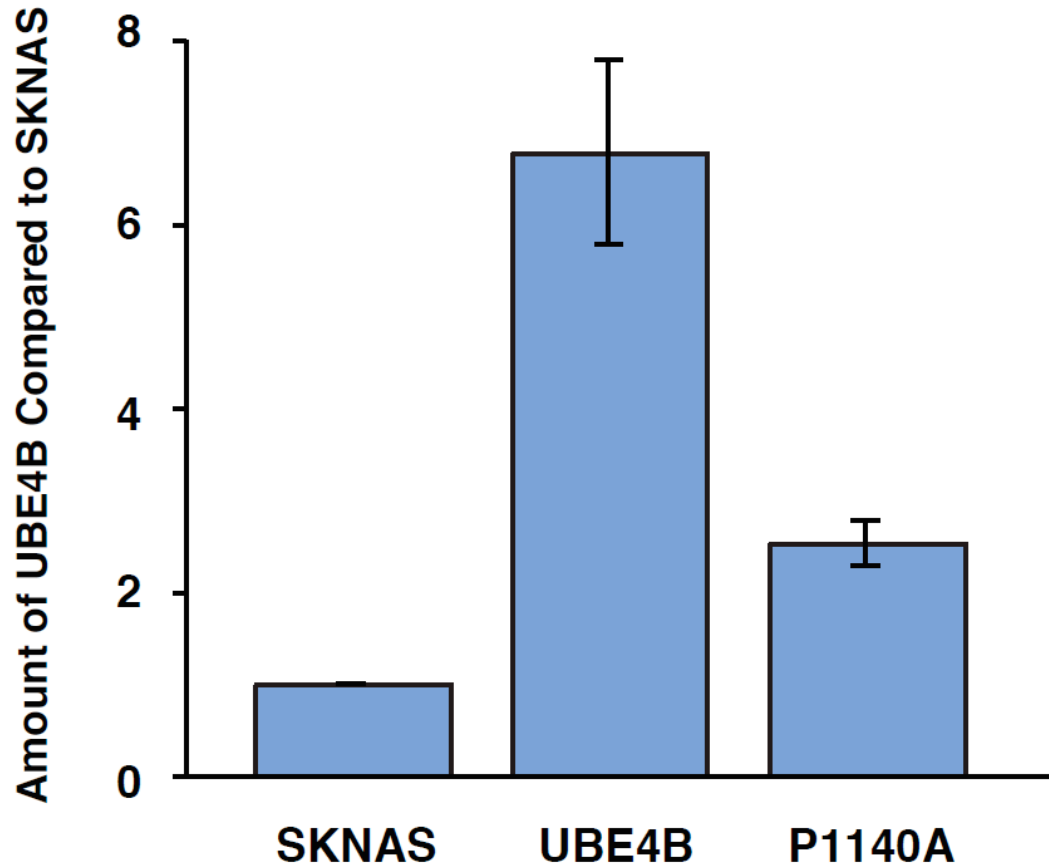


Figure 11- UBE4B expression levels in experimental cell lines.

Lysates of SKNAS, UBE4B, and P1140A cells were collected, and 50 μ g of protein from each sample was resolved by SDS-PAGE. UBE4B content was visualized through Western blotting. Results were quantitated using ImageJ, and average UBE4B levels as compared to the SKNAS cell line are depicted above (n= 4). Error bars indicate standard error.

Next, EGFR degradation of UBE4B and P1140A cell lines were performed and analyzed as above (UBE4B, n=3; P1140A, n=3). Results were compared to GFP degradation (Figure 12).

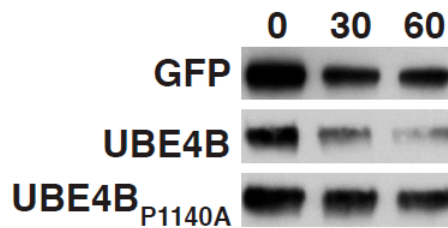
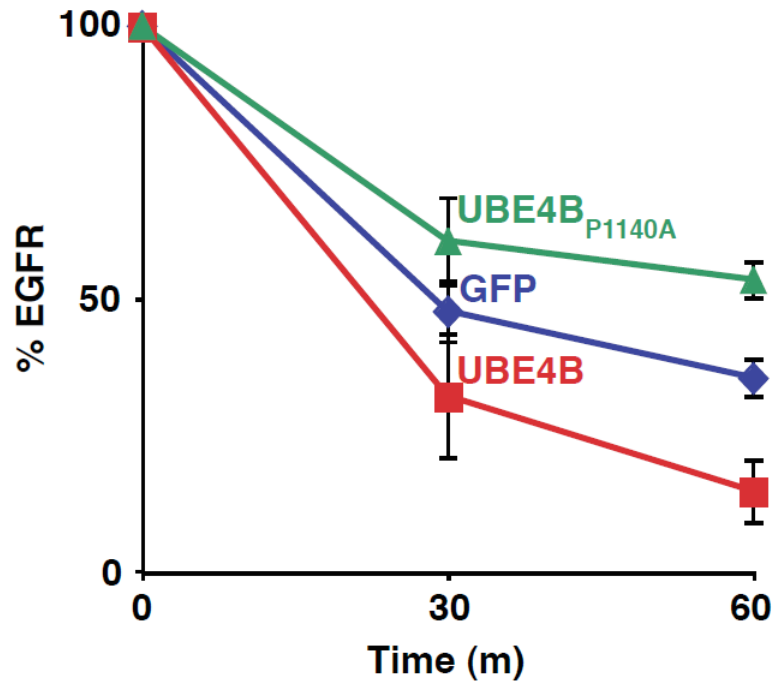


Figure 12- Degradation of EGF receptor in novel SKNAS cell lines.

UBE4B and P1140A cell lines were treated with EGF ligand. Cell lysates were collected, and EGFR content in 50 µg of protein was analyzed after samples were resolved by SDS-PAGE. Western blotting was performed, and membranes were probed for EGFR. Average % EGFR remaining in UBE4B and P1140A cell lines are depicted above, and compared to average % EGFR remaining in the GFP cell line (top). A representative blot for each cell line is shown (below).

They compared the average amount of %EGFR remaining in each cell line after 60 minutes of EGF stimulation (Figure 13).

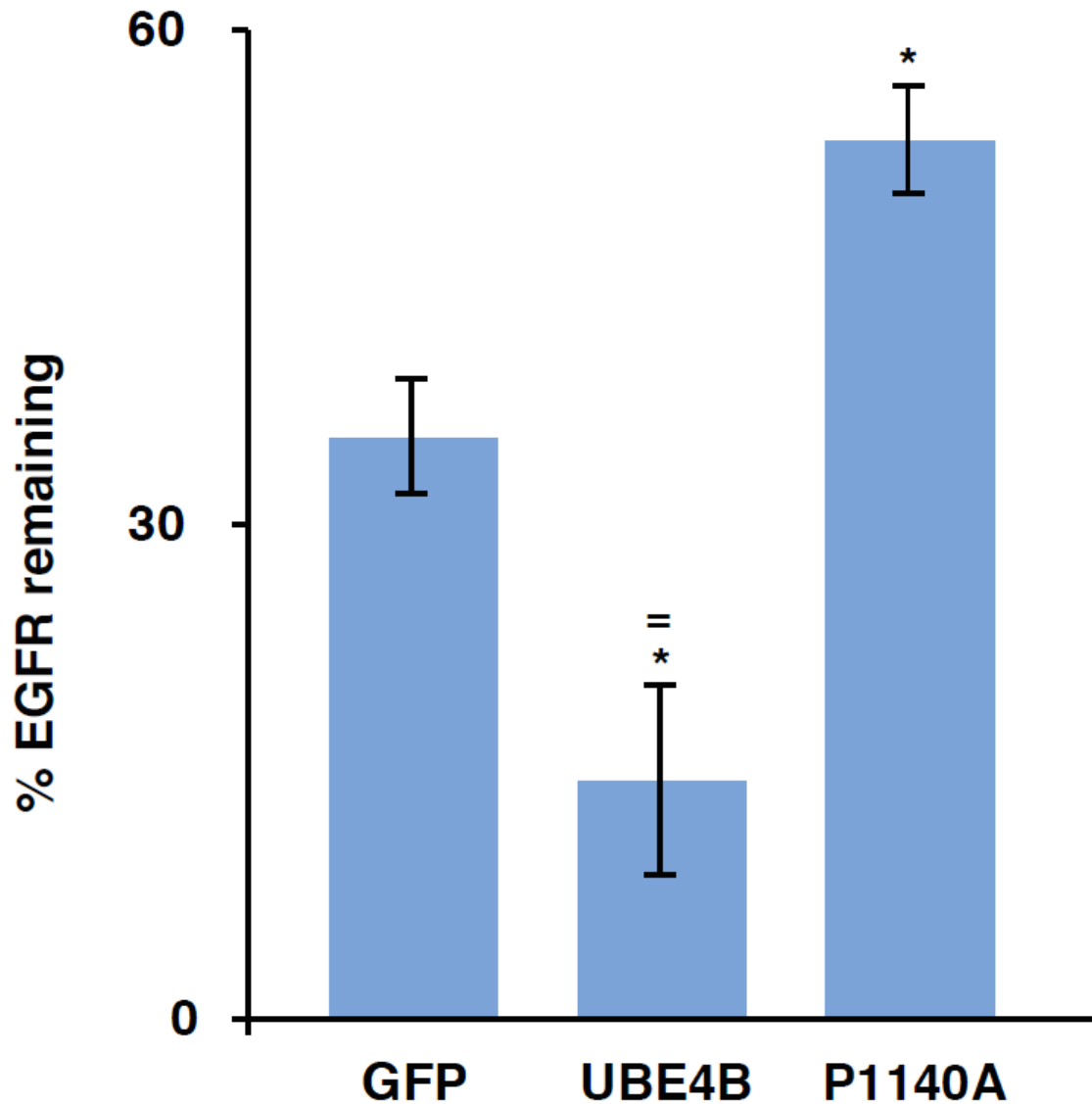


Figure 13- Percentage of EGF receptor remaining in cells after 60 minutes of EGF stimulation.

The percentage of EGF receptor remaining after 60 minutes of EGF ligand stimulation was determined. Average results were compared between the GFP, UBE4B, and P1140A cell lines. Error bars indicate standard error. (* signifies $p < .05$ between GFP; = signifies $p < .01$ between P1140A).

A one-way ANOVA and Tukey HSD test was used to analyze differences in the amounts of EGFR remaining in the cell after 60 minutes of EGF stimulation for

GFP, UBE4B, and P1140A cell lines. Results show that the differences between the GFP cell line and both the UBE4B and P1140A cell lines are significant ($P < .05$). The difference between the UBE4B and P1140A cell line is also significant ($P < .01$).

5.3 *UBE4B can ubiquitinate EGF receptor*

UBE4B is a ubiquitin ligase that resides on the endosomal membrane. It has been previously shown that the ubiquitination and subsequent deubiquitination of EGFR is required for its sorting into luminal vesicles of the multivesicular body. An *in vitro* ubiquitination assay was performed using UBE4B as the sole E3. The complete ubiquitination reaction requires an E1, E2 (UbcH5c), and an E3 (in this case, UBE4B), as well as ubiquitin, ATP regenerating system, and a potential substrate. In this assay, I use HeLa lysate as my source of potential substrate, and *E. coli* lysate as my control. *E. coli* lysate contains proteins that are able to be ubiquitinated, but the organism itself does not have the cellular machinery required for ubiquitination. The HeLa lysates were incubated with an anti-EGFR antibody that recognizes an epitope in the extracellular region of the receptor. Lysates were then added to either complete ubiquitination reactions, or reactions lacking UBE4B. In my reactions, recombinant UBE4B is bound to nickel beads, in order to allow easy removal of UBE4B from the reactions. Reactions were incubated at 30°C for 2 hours, after which UBE4B was removed from the samples. UBE4B is removed because the protein is known to self-ubiquitinate, and I only wanted to look at ubiquitination of EGFR. After the removal of UBE4B, samples were incubated with Protein A beads in order to pull down any EGFR that is associated with the EGFR

antibody previously added to the HeLa lysates. Samples were then washed to remove extraneous protein, and sample buffer was added to the remaining beads. Samples were resolved by SDS-PAGE and Western blotting was performed. Membranes were probed initially for ubiquitin content, then probed for EGFR content (Figure 14A, 14C). IP controls (Figure 14D) and the *E. coli* controls (Figure 5B) for the ubiquitination assay are also shown. I saw that in the absence of UBE4B, little to no EGFR is ubiquitinated (Figure 14A,-) while I saw a dramatic increase in EGFR ubiquitination with the inclusion of UBE4B in the assay (Figure 14A,+). After looking at ubiquitin content, membranes from figure 14A were reprobed for EGFR. Results show that although there is no ubiquitination in samples without UBE4B, there is EGFR present (Figure 14C). The *E. coli* lysate controls confirm that the ubiquitination assay is working correctly, as there is ubiquitination with E1 included, and no ubiquitination when not added to the assay (Figure 14B). The IP controls confirm immunoprecipitation of EGFR (Figure 14D, 1) versus incubation with a non-specific mouse IgG (Figure 14D, 2). An additional control was run to ensure that signal seen in Figures 14A and 14B were due to ubiquitination. Additional samples of complete ubiquitination reactions were run alongside the positive and negative reactions; following removal of UBE4B deubiquitination was performed. A significantly decreased ubiquitination signal is seen after deubiquitination (Figure 14E, + Dub).

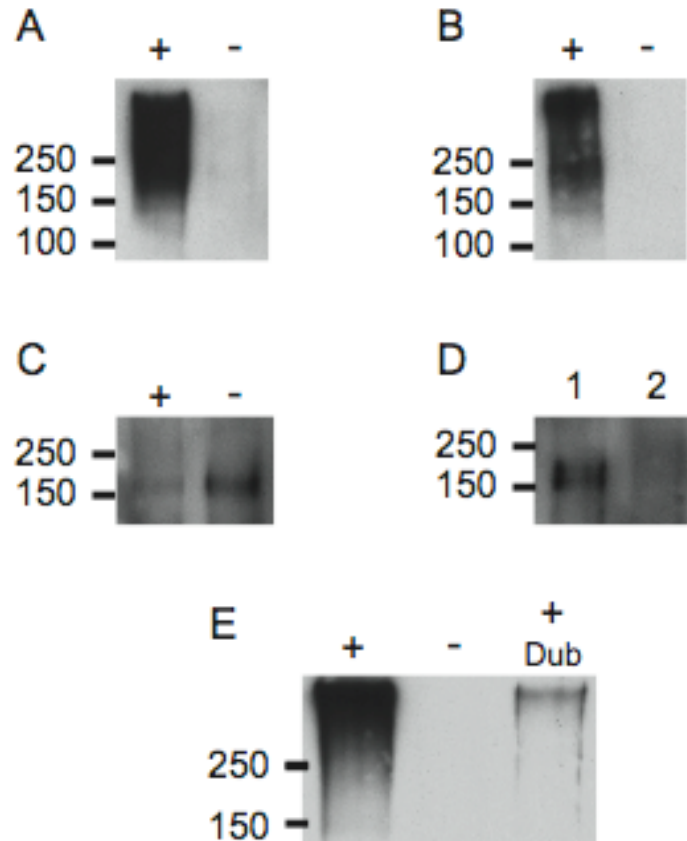


Figure 14- UBE4B mediates the ubiquitination of EGF receptor *in vitro*.

(A) HeLa lysate was added to a complete ubiquitination reaction (+) or a reaction lacking UBE4B (-). Reactions were incubated for 2 hours at 30°C. UBE4B was removed from the complete reaction, and EGFR was immunoprecipitated from samples. Proteins were resolved by SDS-PAGE. Western blotting was performed, and membranes were probed for ubiquitin content. (B) *E. coli* lysate was added to a complete ubiquitination reaction (+) or a reaction lacking E1 (-). Reactions were treated as in (A). Ubiquitin content from each sample is shown. (C) Membranes from (A) were probed for EGFR content. (D) Control for the immunoprecipitation. HeLa lysate was incubated with either an antibody for EGFR (1) or mouse IgG (2). Samples were resolved by SDS-PAGE, Western blotting was performed, and membranes were probed for EGFR. (E) Additional ubiquitination reactions were performed with the positive and negative HeLa reactions as in (A). An extra positive ubiquitination reaction was run along side the other two reactions, and following removal of UBE4B, deubiquitination was performed (+ Dub).

DISCUSSION

6.1 *UBE4B potentiates sorting of membrane proteins by mediating their ubiquitination*

The degradation of membrane proteins is a highly regulated process that requires the collaborative activity of many different proteins and complexes. Cargo proteins pass through distinct compartments as they progress along the endocytic pathway. The sorting of an internalized cargo protein at the limiting membrane of the MVB is arguably the most critical step in determining its fate; this decision seems to be dependent upon whether it is covalently bound to a ubiquitin molecule (38). While the ubiquitin ligase Cbl mediates the ubiquitination of the EGF receptor at the plasma membrane, deubiquitinating enzymes, such as UBPY, have the ability to reverse this attachment (38, 40). Deubiquitination of a protein precludes its recognition by the MVB sorting complex, ESCRT-I, thereby barring inclusion into the luminal vesicles of the MVB (40).

I have shown that UBE4B is capable of ubiquitinating the EGF receptor (figure 14) in an *in vitro* ubiquitination assay, and that the exclusion of UBE4B from the assay results in little to no ubiquitination of EGF receptors. Through unpublished data obtained by other members of our lab, UBE4B is also known to bind to endosomal membranes through Hrs, but the reason for its localization on endosomes remains unclear. I propose that UBE4B is present on endosomal membranes to ensure that proteins, like the EGF receptor, are reubiquitinated to ensure their recognition by ESCRT-I. This conclusion is supported by results of a cell-free assay that is able to measure the movement of cargo proteins from the

MVB membrane to the luminal compartment (46). As the data in Figure 9B show, the interaction between UBE4B and Hrs is necessary for most of the EGF receptors to be sorted into MVBs. This means that UBE4B must be able to bind to Hrs; in other words, UBE4B must be able to be recruited to endosomes. Once it is present on endosomes, it can mediate ubiquitination of proteins lacking a ubiquitin tag; these proteins would otherwise have remained on MVB membranes, undetected by sorting complexes, and likely would have been recycled (39).

This is not to say that UBE4B is the sole ubiquitin ligase responsible for protein reubiquitination. Figure 9B shows that even with the disruption of the Hrs-UBE4B interaction, 31.2% of EGF receptors are still sorted into the MVB. Other ubiquitin ligases are known to be present at endosomal membranes, including AIP4/ITCH and Nedd4, though it is not known whether these proteins are capable of ubiquitinating EGF receptor (43, 44). It is unclear whether proteins that are ubiquitinated at the plasma membrane before internalization can retain their ubiquitin tag for use in the sorting event at the MVB membrane. This possibility seems unlikely because of the large number and promiscuity of deubiquitinating enzymes present in the cell.

The ubiquitin ligase responsible for the initial ubiquitination of EGF receptor at the plasma membrane, c-Cbl, has been shown to remain associated with EGF receptor as it progresses through the endocytic pathway; c-Cbl can mediate the multiple monoubiquitination of EGF receptor, meaning that many, individual ubiquitin moieties are found covalently attached to multiple lysine residues of the protein (38, 39). It was once believed that EGF receptors could only be

monoubiquitinated by ubiquitin ligases like c-Cbl; however, more recent studies have shown that more than 50% of ubiquitin molecules found attached to EGF receptors were part of poly-ubiquitin chains (48). This is an interesting result since convention dictates that the degradation of monoubiquitinated proteins occurs in the lysosome, while polyubiquitinated proteins are degraded in the proteasome (38).

I have presented UBE4B as an E3 ubiquitin ligase, but UBE4B and other U-box proteins were once classed as novel ubiquitination enzymes, called E4s (32). It was believed that the original function of E4 ubiquitination enzymes was to facilitate the addition of ubiquitin onto existing single ubiquitin molecules present on monoubiquitinated proteins; this addition results in the formation of poly-ubiquitin chains (35, 49, 50). However, members of the U-box family of ligases are now described as E3 ubiquitin ligases with E4 activity (32). The ability of UBE4B to lengthen existing ubiquitin chains is intriguing, since most of the ubiquitin associated with EGF receptor are linked to other ubiquitin molecules, and may explain the existence of polyubiquitinated EGF receptor (48).

6.2 *Effects of UBE4B promoted sorting of EGF receptor*

Other members of the lab observed that disruption of the Hrs-UBE4B interaction resulted in inhibition of the sorting of EGF receptors into MVBs (Figure 9B). I have observed that HeLa cells, that express nearly 6 times the amount of UBE4B compared to normal SKNAS cells, degrade over 75% of EGF receptor present in the cell over the course of 60 minutes of ligand stimulation, while in that time SKNAS cells only degrade about 50% of EGF receptor. Stable SKNAS cell

lines were created in order to determine whether increasing the amount of UBE4B in cells would affect its EGF receptor degradation. There is evidence of this in Figure 13; the average amount of EGF receptor remaining in the UBE4B cell line is less than 15%, compared to the ~35% remaining in the GFP control cell line. The amount of UBE4B expressed in the UBE4B cell line is more than four times greater than that of endogenous (SKNAS parental) levels. The amount of EGF receptor remaining in the cell after EGF stimulation seems to negatively correlate with the amount of UBE4B expressed by the cell. This is interesting in light of unpublished data from the lab suggesting a significant negative correlation between UBE4B levels and EGF receptor levels in tumor biopsy samples.

The UBE4B overexpressed by the stable UBE4B cell line retains its ability to attach ubiquitin to its substrate proteins. By contrast, the P1140A cell line expresses an enzyme-dead version of UBE4B; this mutant is not capable of E3 ubiquitin ligase activity. According to Figure 11, P1140A cells express 1.5 times the amount of UBE4B than endogenous levels.; however, the P1140A cell line shows a much slower degradation than the GFP control, with more than 50% of EGF receptor remaining in cells after 60 minutes of EGF stimulation, compared to the ~35% remaining in GFP cells. This indicates that the ubiquitin ligase activity of UBE4B is required for normal degradation of EGF receptor.

I believe that the effect I see in degradation of EGF receptor between cell lines is due to the simple fact that there is more UBE4B available in the cell to ubiquitinate EGF receptors, and possibly other proteins in the cell. Although receptors are ubiquitinated upon plasma membrane internalization, deubiquitination

may occur before the protein reaches the membrane of the MVB. It is not known what the exact function of the deubiquitination process serves. Conflicting reports suggest that deubiquitination can either promote or inhibit sorting (51). Deubiquitination may simply be a means of sustaining a reserve pool of free ubiquitin (41). However, the fact remains that if a protein is not ubiquitinated, it cannot be recognized by the sorting complexes that sort proteins into the MVB for degradation in lysosomes (40). Overexpressing UBE4B in SKNAS cells increases the amount of UBE4B available to be recruited to endosomes and to tag unubiquitinated proteins on MVB membranes.

6.3 *Implications of EGF receptor degradation*

Growth factors mediate cell growth and division, by binding to their respective receptors and initiating intracellular signaling cascades (15). The endocytosis of growth factor receptors, such as the EGF receptor, is a means of downregulating the proteins from the plasma membrane and limiting their signaling (3). Upon ligand binding, receptors are internalized and enter the endocytic pathway; when these receptors reach the limiting membrane of the MVB they may be sorted for degradation or recycled back to the cell surface, where they may continue to participate in signaling (14). When a receptor is transferred to the lysosome and degraded, it no longer takes part in signal-transduction (3). So, the fate of a receptor is determined at the MVB by its state of ubiquitination, which will determine whether it is recognized by sorting complexes, and ultimately directs its destination (40).

Based on my hypothesis, UBE4B promotes EGF receptor degradation by ubiquitinating the receptor therefore in its absence, or when mutated, I would expect less degradation of the receptor. If this hypothesis were correct, cells that have reduced levels of UBE4B or express an enzyme-inactive mutant, would contain more EGF receptors and might grow faster than cells expressing wild-type UBE4B. Interestingly, unpublished data suggests that cells expressing lower levels of UBE4B or UBE4BP1140A grow at faster rates than cells with higher levels of UBE4B.

CONCLUSIONS

The presence or absence of a ubiquitin tag on a membrane protein at the MVB determines whether or not the protein is sorted into the luminal compartment of the MVB. The collective effect of individual sorting decisions ultimately impacts physiological processes of the whole cell.

UBE4B is a ubiquitin ligase that is recruited to endosomes, where it is in close proximity to membrane proteins that may not be ubiquitinated. Proteins on the MVB membrane that lack ubiquitin tags cannot be included in luminal vesicles (40). Other members of the lab found that when UBE4B is allowed to bind to endosomes, EGF receptor is more efficiently sorted into MVBs than when the binding is disrupted. This is likely a direct effect of ubiquitination of EGF receptors by UBE4B.

The sorting of membrane proteins is related to their degradation; proteins that enter the luminal compartment of the MVB are eventually degraded in the lysosome (14). I observed that the amount of wild-type UBE4B expressed by cells

negatively correlates with the amount of EGF receptor remaining in cells after a fixed period of ligand stimulation. Therefore, degradation of a receptor that enters the endocytic pathway following plasma membrane internalization can be altered by varying expression levels of a ubiquitin ligase that can mediate its ubiquitination on endosomal membranes.

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