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CHARACTERIZATION OF THE UBIQUITIN LIGASE, UBE4B,

IN ENDOCYTIC TRAFFICKING

by

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CHARACTERIZATION OF THE UBIQUITIN LIGASE, UBE4B,

IN ENDOCYTIC TRAFFICKING

A

DISSERTATION

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Natalie Sirisaengtaksin, M.S.

Houston, Texas

May, 2017

This work is dedicated to my father,
who is irreplaceable.

I miss you every day.

Acknowledgements

This list is long, but not exhaustive. In truth, this particular section deserves to be longer than all the other sections combined. Everyone that I have met and known during the course of my life has in some way influenced the nature and completion of this work. This is not an exaggeration. Anyone that knows me knows that I never, ever exaggerate. Ever. However, I will respect the intent of this document (as an academic work) and keep my acknowledgements (relatively) short.

I owe everything that I have in my life to my parents, Dr. Ongard and Noemi Sirisaengtaksin. They encouraged my love for the written word, for music, and finally, for science. Growing up, my parents told my sisters and I that a good education was most important thing they could provide for us. In this regard, I hope that we have made them proud. My mom is a fearless leader, and the strongest woman that I will ever know. She exemplifies what one can achieve with hard work, determination, and confidence (above all). Most importantly, she is the glue that keeps our family together. My dad was my first mentor. He was also a professor that was loved by his students and colleagues. I grew up roaming the hallways of his university, and somewhere between all the algebra workbooks and computer science workshops I acquired an unquenchable thirst for knowledge. I will forever miss his sense of humor, sense of adventure, and his food. I will spend the rest of my life trying to leave the world better than I found it, just as he did. I am fortunate to have two younger sisters with whom I have had to keep pace with all these years. They push me to be better and constantly teach me things about life that I would never have learned without them. I am so proud of them, and look forward to celebrating their future successes.

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And now for my dear, poor friends. I’m sorry. You have seen me at my best and worst, and you still stuck around. I cannot thank you enough. Dr. Monica Gireud Goss was my foundation. Without her, I would be incredibly unsteady. She was my sounding board and confidant. I will miss sitting three feet away from her every day, but I know she’ll be slightly relieved. Sahily Reyes was my support. She was my constant cheerleader. Without her, I would be unbelievably depressed. She brought music back into my life, and I will miss her so much when she goes back to medical school. These two pushed me, both literally and figuratively, up bridges, hills, and mountains. I will never forget the time we shared together.

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My biggest regret is that I never learned the phone number of the elevators in the UTHealth Medical School Building. If anyone ever figures it out, let me know. I'll keep looking.

CHARACTERIZATION OF THE UBIQUITIN LIGASE, UBE4B, IN ENDOCYTIC TRAFFICKING

Natalie Sirisaengtaksin, M.S.

Supervisory Professor: Andrew J. Bean, Ph.D.

Endocytosis is a process by which cells internalize membrane proteins to remove them from the plasma membrane, allowing cells to regulate the cell surface expression of transmembrane proteins. In this manner, cellular responses to extracellular cues may be tuned by limiting the number of proteins available at the cell surface. One particular class of proteins, receptor tyrosine kinases (RTK), is internalized upon binding to extracellular ligands during their residence at the cell surface. The epidermal growth factor receptor (EGFR) is an RTK whose trafficking through the endocytic pathway through the cell is well-documented. Stimulation of EGFR with its cognate ligand, EGF, prompts EGFR entry into the endocytic pathway and simultaneously activates downstream signal transduction pathways that regulate physiological responses, such as survival, proliferation, and differentiation. Activated EGFRs continue to signal as they traverse the endocytic pathway until the ligand-receptor complex is included into vesicles that bud into the lumen of the multivesicular body (MVB). Inclusion into internal MVB vesicles designates EGFR for lysosomal proteolysis and extinguishes their signaling activity.

Ubiquitination is a post-translational modification that underlies some aspects of membrane protein trafficking. Ubiquitin modification allows EGFR recognition by endosomal protein complexes that mediate protein inclusion into MVB vesicles from those that remain on the MVB membrane for incorporation into other cellular structures (e.g. plasma membrane,

Golgi). The endosomal sorting machinery consists of a core group of cytosolic proteins that are recruited to the endosomal membrane, called the endosomal sorting complexes required for transport (ESCRT) machinery. A subset of ESCRT proteins bind directly to ubiquitin, allowing the sorting machinery to engage and manipulate the movement of protein cargo into inwardly budded MVB vesicles. Lack of a ubiquitin tag precludes EGFR from inclusion into vesicles that bud into the lumen of the MVB. The precise nature of coordination between the cellular machineries that govern ubiquitination and endosomal sorting are not well understood.

I have identified a protein interaction between UBE4B, an E3/E4 ubiquitin ligase, and the ESCRT-0 components, Hrs and STAM. ESCRT-0 is the first complex of the endosomal sorting machinery to bind to endosomes, and recognition by ESCRT-0 is required for EGFR sorting and degradation. Immunoprecipitation and ubiquitination assays revealed that UBE4B binds and can ubiquitinate the EGFR. Depletion of UBE4B resulted in impaired EGFR inclusion into inwardly-budded MVB vesicles. EGFR inclusion was rescued more efficiently with the addition of recombinant UBE4B versus the addition of a ubiquitination-defective mutant, UBE4B(P1140A). These data suggest that the ubiquitination of EGFR by UBE4B is required for efficient EGFR sorting. Altering UBE4B expression in neuroblastoma cells revealed a negative correlation between UBE4B expression and proliferation, as well as altered proliferative responses to EGR inhibitors. These findings suggest a mechanism by which UBE4B may regulate cell proliferation, and reveals UBE4B as a potential target in neuroblastoma therapeutic development.

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Chapter 1. Introduction

1.1 Endocytosis: An Overview

The plasma membrane is a protective barrier that sequesters the interior of a cell from its external environment. This fluid structure blocks passage of material into the cells, but for the most part is selectively permeable to certain molecules and ions. Endocytosis is a multistep process by which cells internalize molecules that cannot freely pass through the plasma membrane. Not only does this pathway allow cellular uptake of nutrients, it facilitates constant reshaping of the membrane-bound protein population residing at the cell surface. Thus, endocytosis allows cells to quickly adapt to changes in their microenviroment. Defects in endocytosis has implications in cell migration, differentiation, antigen presentation, and pathogen entry.

The first step of the endocytic process is the internalization of macromolecules from the plasma membrane. Internalization of protein “cargo” (a term used here to indicate proteins that traverse the endocytic pathway) may occur by a number of distinct mechanisms. One specific type of internalization is dependent on clathrin, a cytosolic protein that self-assembles into ball-shaped cages that encase vesicles as they bud from membranes, including those that bud into the cell from the plasma membrane (Roth and Porter, 1964; Pearse, 1976). Clathrin-dependent endocytosis was first described in the 1960s, with the advent of glutaraldehyde cell fixation (Sabatini et al., 1963; Roth and Porter, 1964). Images of clathrin acquired using electron microscopy revealed the shape and assembly of clathrin cages (Pearse, 1976).

Internalization of protein cargo may be constitutive or ligand-induced (Benmerah and Lamaze, 2007). Cargo that enter the cell constitutively are continuously internalized into clathrin-coated vesicles at the plasma membrane, as seen in the case of low density lipoprotein receptor and the transferrin receptor (Anderson et al., 1982; Watts, 1985). Conversely, receptor cargo that

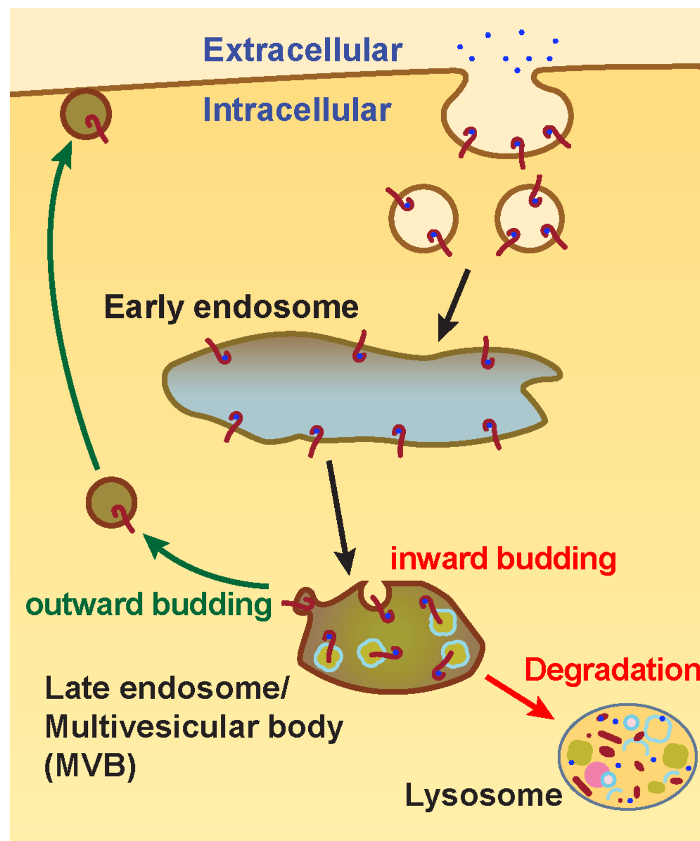

Figure 1. Membrane proteins are downregulated by the endocytic pathway.

Endocytosis is the major mechanism by which cells downregulate membrane protein expression at the plasma membrane. Proteins that reside on the cell surface of the plasma membrane are internalized into small vesicles that shuttle protein cargo from the surface to early endosomes. Proteins are incorporated into endosomal membranes upon vesicle fusion with endosomal membranes. Protein cargo remain on the limiting endosomal membrane until they are either included in membrane vesicles that bud outward from late endosomes (green arrows) or are sorted into luminal vesicles that bud into nascent multivesicular body (MVB). Protein sorting into MVB vesicles must occur in order for proteins to be degraded in the lysosome.

enter via the ligand induced pathway are internalized into clathrin-coated vesicles upon binding of cell surface receptors to extracellular ligands, which includes most growth factor receptors like

epidermal growth factor receptor (EGFR) (Sigismund et al., 2012; Beguinot et al., 1984; Benmerah and Lamaze, 2007). Both pathways converge as clathrin-coated vesicles fuse with endosomal membranes. Upon fusion, protein cargoes are integrated into endosomal membranes, where they remain until they are assigned one of three distinct metabolic fates. First, cargo may travel on membrane vesicles that bud outward from endosomes (Figure 1, green arrows). These vesicles travel from endosomes and fuse with various cellular structures, including the plasma membrane and the Golgi (Nothwehr et al., 2000; Felder et al., 1990). Second, cargo may be sorted into endosomal membrane invaginations that bud inward and are released into the lumen of endosomes (Figure 1, red arrow). These vesicles are delivered to the lysosome, resulting in protein degradation (Sigismund et al., 2012; Dunn, 1986). Third, cargo may remain on the limiting membrane of endosomes and become incorporated into lysosomal membranes upon fusion of late endosomes with lysosomes. The physical sorting of proteins into these discrete groups relies on the selective interaction of the molecular sorting machinery with protein cargo on endosomal membranes (Teo et al., 2004; Katzmann et al., 2003, 2001b; Bache et al., 2003; Babst et al., 2002b; a). This critical step occurs at a subset of late endosomes, called the multivesicular body (MVB) (Katzmann et al., 2001b; Babst et al., 2002b; Bache et al., 2003).

1.2 The ESCRT complexes sort protein cargo at the MVB

MVB formation occurs as invaginations of the late endosomal membrane bud inward and are released into the lumen of the organelle, resulting in a characteristic multivesicular morphological appearance (Teo et al., 2004; Katzmann et al., 2001b; Babst et al., 2002a; Bache et al., 2003). As a result, cargo proteins embedded within endosomal membranes are actively incorporated into luminal vesicle buds, separating them from other endocytic cargo destined for either transit to plasma membrane or lysosomal membrane (Gruenberg and Stenmark, 2004; Grant

and Donaldson, 2009; Slagsvold et al., 2006). Inclusion of protein into these endosomal vesicles require engagement with the molecular sorting machinery, called the endosomal sorting complexes required for transport (ESCRTs).

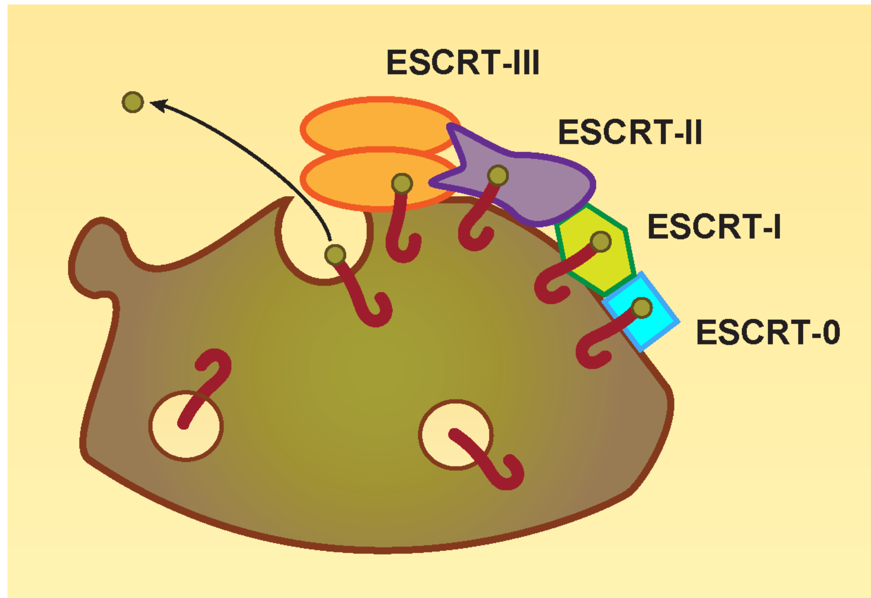

Figure 2. ESCRT complexes mediate the sorting of membrane proteins.

Cytosolic proteins are recruited to endosomal membranes to form complexes that act in sequential order. First, ESCRT-0 binds to endosomes and recognizes and concentrates ubiquitinated protein cargo. ESCRT-I also recognizes ubiquitinated cargo and complete ESCRT-I assembly initiates the formation of ESCRT-II. ESCRT-III is the least stable of the ESCRTs, and binds to endosomes long enough to mediate membrane scission of inwardly budded vesicles.

The ESCRTs comprise a multi-subunit machinery that sorts protein cargo into nascent MVBs, a highly evolutionarily conserved process (Schmidt and Teis, 2012). There are four unique ESCRT complexes: ESCRT-0, -I, -II, -III (Figure 2) (Schmidt and Teis, 2012; Katzmann et al., 2003; Babst et al., 2002b; a; Bache et al., 2003). The ESCRT complexes are comprised of

cytosolic proteins that are recruited to endosomal membranes and assemble to perform specific tasks in the protein sorting process. ESCRTs were first identified in yeast as a subset of genes called the vacuolar protein sorting (*vps*) mutants whose expression were required for cargo delivery to the yeast vacuole (Raymond et al., 1992). Systematic knockout of these *vps* genes led to defects in cargo sorting and abnormal vacuolar morphology (Raymond et al., 1992).

ESCRT-0 initiates protein sorting at endosomal membranes. It is the first complex to bind to endosomes and engage with cargo proteins (Bilodeau et al., 2002; Schmidt and Teis, 2012; Katzmann et al., 2003). The complex is comprised of a 1:1 heterodimer between HGF-regulated tyrosine kinase substrate (Hrs) and signal transducing adaptor molecule (STAM). The Hrs/STAM complex is recruited to endosomes via the FYVE (Fab1/YOTB/Vac1/EEA1) domain of Hrs, which interacts with phosphatidylinositol-3-phosphate (PI3P), a phospholipid that is highly enriched in endosomal membranes (Schmidt and Teis, 2012; Raiborg et al., 2001). Hrs is also recruited to endosomes by SNAP-25, an interaction that requires the second coiled-coils domain of Hrs (Sun et al., 2003; Pullan et al., 2006; Bean et al., 1997; Raiborg et al., 2001). The C-terminal glutamine- and proline-rich region also contributes to the endosomal localization of Hrs (Hayakawa and Kitamura, 2000). Both members of the ESCRT-0 complex contains multiple VHS domains (Vps27, Hrs and STAM domains) and ubiquitin interaction motifs (UIMs), which allows the complex to bind to ubiquitinated cargo *(please see section 1.2 for a review on ubiquitination)* (Schmidt and Teis, 2012; Bilodeau et al., 2002). Hrs binds to ubiquitin through a two-sided UIM domain and a single VHS domain (Hirano et al., 2006). The STAM protein binds ubiquitin through a single UIM domain and a single VHS domain (Bilodeau et al., 2002). In total, ESCRT-0 may bind up to five ubiquitin moieties. Ubiquitin modification of cargo is required for their recognition by ESCRTs (Henne et al., 2011; Bilodeau et al., 2002; Katzmann et al., 2001b). The multiple binding sites within the complex emphasizes the importance of ESCRT-0 in cargo recognition (Bilodeau et al., 2002; Henne et al., 2011). Knockdown of either Hrs or STAM using

RNAi resulted in impaired lysosomal degradation of receptor cargo, suggesting that ESCRT-0 function is essential for cargo inclusion into MVB vesicles (Komada, 2005). It is unclear whether ESCRT-0 contains multiple ubiquitin binding sites in order to allow binding to multiple ubiquitinated cargoes simultaneously, or to allow tighter binding to a single cargo through a single ubiquitin molecule, or to multiple ubiquitin moieties attached to the same cargo protein (Ren et al., 2009).

ESCRT-I was the first ESCRT complex that was fully characterized, and was first identified in yeast (Henne et al., 2011). The 350 kDa complex is composed of four protein subunits (Tsg101, Vps28, Vps37, and Mvb12) that come together in a 1:1:1:1 ratio (Raiborg and Stenmark, 2009; Teo et al., 2004). Unlike ESCRT-0, which binds strongly to endosomes through the Hrs-PI3P interaction, the only direct ESCRT-I association with endosomal membranes is a weak electrostatic interaction through the N-terminus of Tsg101. Instead, endosomal recruitment of ESCRT-I is dependent upon ESCRT-0 through the direct binding of Hrs to Tsg101 (Henne et al., 2011). Like ESCRT-0, ESCRT-1 also interacts with ubiquitinated cargo through the ubiquitin E2 variant (UEV) domain of Tsg101 (Pornillos et al., 2003).

ESCRT-I formation initiates the assembly of ESCRT-II (Schmidt and Teis, 2012). ESCRT-II is comprised of EAP20, EAP30, and EAP45 that bind in a 2:1:1 ratio (Hierro et al., 2004; Teo et al., 2004; Im and Hurley, 2008). EAP45 encodes a GRAM-like ubiquitin binding in EAP45 (GLUE) domain that contains a binding site for Vps28, anchoring the ESCRT-II complex to ESCRT-I (Chu et al., 2006). The GLUE domain also allows engagement of EAP45 with PI3P and ubiquitinated cargo simultaneously.

ESCRT-III complex is the last of the ESCRTs to be recruited to endosomes. The components of the ESCRT-III complex are not well-defined, but is thought to be comprised of four core subunit proteins: CHMP2, CHMP3, CHMP4, and CHMP6 (Wollert and Hurley, 2010; Schmidt and Teis, 2012; Raiborg and Stenmark, 2009). Both CHMP2 and CHMP4 have multiple

isoforms (CHMP2A and B; CHMP4A, B, and C) (Raiborg and Stenmark, 2009). Unlike the other ESCRTs, which form stable complexes, ESCRT-III assembly on endosomes is transient. ESCRT-III assembly begins with CHMP6, which is recruited to endosomes through direct N-terminal binding to endosomal membranes as well as binding to the ESCRT-II component EAP20. Because each ESCRT-II complex contains two EAP20 subunits, the assembly of two ESCRT-III complexes may occur simultaneously for each ESCRT-II complex (Raiborg and Stenmark, 2009). In contrast to ESCRT-0, -I, and –II, ESCRT-III does not bind to ubiquitinated cargo.

The ESCRT complexes act sequentially to concentrate ubiquitinated cargo and drive the formation of membrane buds, into which cargo may be captured and sequestered into MVBs following vesicle fission (Wollert and Hurley, 2010). First, ESCRT-0 clusters ubiquitinated cargo into microdomains of the endosomal membrane (Wollert and Hurley, 2010). Then, ESCRT-I and –II induce membrane budding into the lumen of the MVB, confining the ESCRT-0-clustered protein cargo within membrane buds (Wollert and Hurley, 2010). Finally, ESCRT-III mediates membrane scission, resulting in the release of the vesicle buds into the lumen of the MVB (Hurley, 2010). Upon completion of membrane scission, ESCRT-III is disassembled by the AAA-ATPase, Vps4. It is known that ESCRT-III and Vps4 remain on the cytoplasmic face of the endosomal membrane to drive membrane budding away from the cytoplasm; however, the exact molecular mechanisms of this budding event are not completely understood (Babst et al., 2002a; Alonso Y Adell et al., 2016).

The ability of ESCRTs to recognize and bind to ubiquitinated cargo is essential to the protein sorting process, and ultimately, to lysosomal protein degradation (Bilodeau et al., 2002; Eden et al., 2012; Erpapazoglou et al., 2012; Katzmann et al., 2003; Longva et al., 2002). This suggests that ubiquitin modification is used as a signal that designates a protein for sorting into the MVB (Haglund and Dikic, 2012). Like endosomal protein sorting, ubiquitination is a highly

regulated process that is mediated by a specialized set of proteins, known as the ubiquitination machinery.

1.3 Ubiquitination: An Overview

Ubiquitination (also called ubiquitylation) is a reversible post-translational modification wherein lysine residues of target proteins are modified by small ubiquitin moieties. Ubiquitin is a small, 76 amino acid protein. Seven of these amino acids are lysines that can be modified by another ubiquitin molecule (Swatek and Komander, 2016). Proteins may be modified by ubiquitin in distinct manners: 1) monoubiquitination, in which a single ubiquitin moiety is attached to a lysine residue of the target protein; 2) multiple monoubiquitination, in which a single ubiquitin moiety is attached to multiple lysine residues of a target protein; and 3) polyubiquitination, in which multiple ubiquitin modifications result in a ubiquitin chain that is attached to a lysine residue of the target protein (Mohapatra et al., 2013; Li and Ye, 2008). Polyubiquitin chain assembly is made even more complex as linkages are formed between ubiquitin molecules via seven distinct lysine residues: K6, K11, K27, K29, K33, K48, and K63 (Li and Ye, 2008; Swatek and Komander, 2016; Nathan et al., 2013). Chains may be homogenous, and contain only one type of linkage, or chains make contain multiple linkage types (Kim et al., 2007). The two most common types of linkages are K48 and K63 (Nathan et al., 2013; Kim et al., 2007; Li and Ye, 2008).

1.4 Ubiquitination is a multi-enzyme cascade

The ubiquitination process requires the consecutive, sequential action of three types of enzymes: E1s, the ubiquitin-activating enzymes; E2s, the ubiquitin-conjugating enzymes; and E3s, the

ubiquitin ligases (Figure 3) (Komander and Rape, 2012; Swatek and Komander, 2016; Hershko and Ciechanover, 1998).

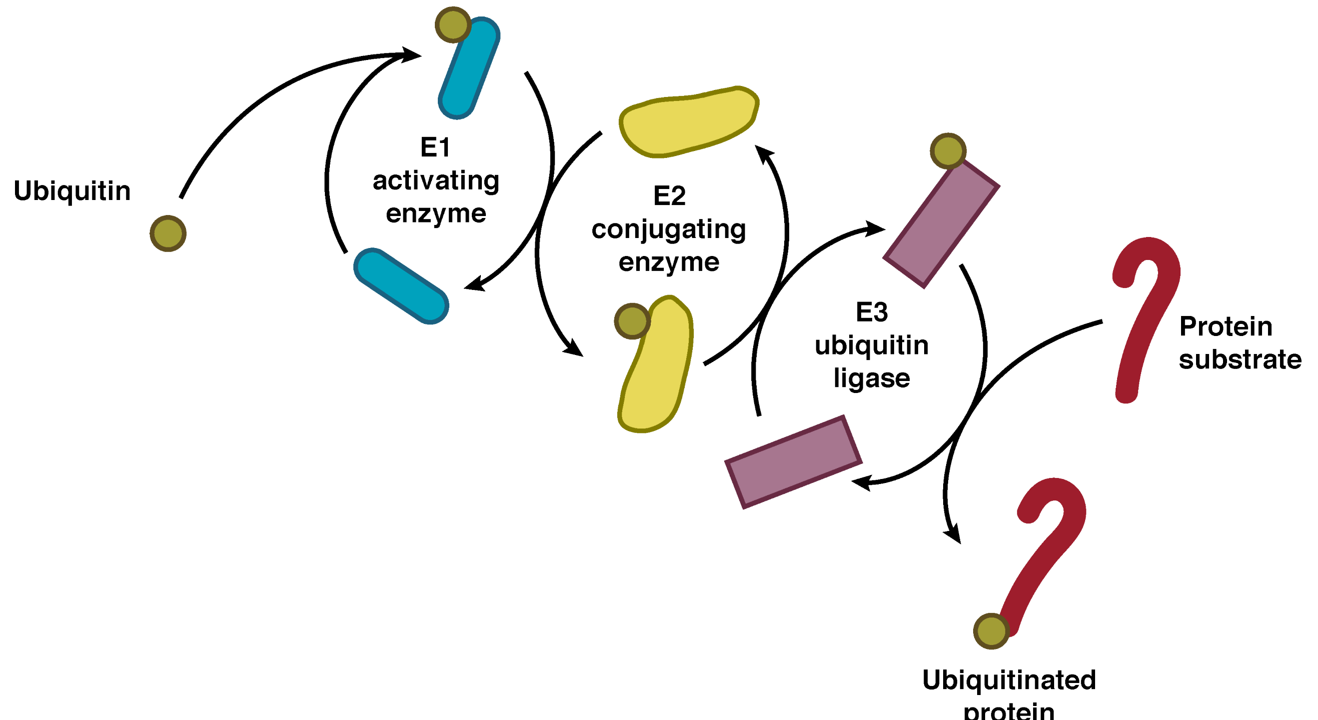

Figure 3. Ubiquitination requires the sequential action of three enzymes.

The conjugation of ubiquitin onto a target substrate is called ubiquitination. Ubiquitination is a common post-translational modification process that requires the coordinated action of E1 activating enzymes, E2 conjugating enzymes, and E3 ubiquitin ligases.

Initiation of the multienzyme ubiquitination cascade begins with the catalytic activation of ubiquitin by E1 enzymes. The human genome encodes only two ubiquitin-activating enzymes: UBA1 and UBA6 (Schulman and Wade Harper, 2009; Ye and Rape, 2009). The catalytic mechanism by which ubiquitin is activated is best characterized for UBA1. First, the E1 enzyme simultaneously binds to an ATP-Mg^{2+} complex and ubiquitin molecule. Then the E1 adds AMP to the C-terminal end of ubiquitin, resulting in the formation of a ubiquitin adenylate intermediate (Schulman and Wade Harper, 2009; Hershko and Ciechanover, 1998). The catalytic cysteine

residue of the E1 binds the ubiquitin adenylate to form a high-energy thioester linked ubiquitin-E1 complex (Hershko and Ciechanover, 1998; Schulman and Wade Harper, 2009). Binding of ubiquitin triggers a dramatic conformation change within the E1, exposing a negatively-charged groove within a ubiquitin fold domain. This region serves as an E2-binding site that is required for E1-E2 complex formation (Ye and Rape, 2009).

The ubiquitination process continues as the E1-ubiquitin complex engages an E2 ubiquitin-conjugating enzyme. The E2 family of enzymes are comprised of 38 distinct proteins that interact with UBA1 and UBA6 (Ye and Rape, 2009). E2 binding specificity for the E1 molecules depend upon lysine residues found in the α-helix 1 region of E2 proteins, which are not present in E2 enzymes that conjugate other ubiquitin-like modifiers (e.g. SUMO). Importantly, E2s also contain a ubiquitin-conjugating domain, which consists of a catalytic cysteine residue to which activated ubiquitin is transferred (Ye and Rape, 2009). In rare cases, an E2 may directly ubiquitinate a target substrate; however, the main function of an E2 is to carry activated protein to an E3 ubiquitin ligase (Cyr et al., 2002).

Finally, E3 ubiquitin ligases, either directly or indirectly, mediate the attachment of ubiquitin onto a target substrate protein. As can be inferred from the sheer number of E3s (~600 to 1,000 distinct E3s), a single E2 may interact with several E3s (Ye and Rape, 2009). The imbalance in the number of E3 enzymes compared to E2 enzymes may be attributed to the conservative interaction of E3s with select target substrates (Shenoy, 2007). The manner by which 1) E2s interact with E3s and 2) E3s transfer ubiquitin to its target substrates are dependent upon the identity of the E3, and will be detailed in the next section.

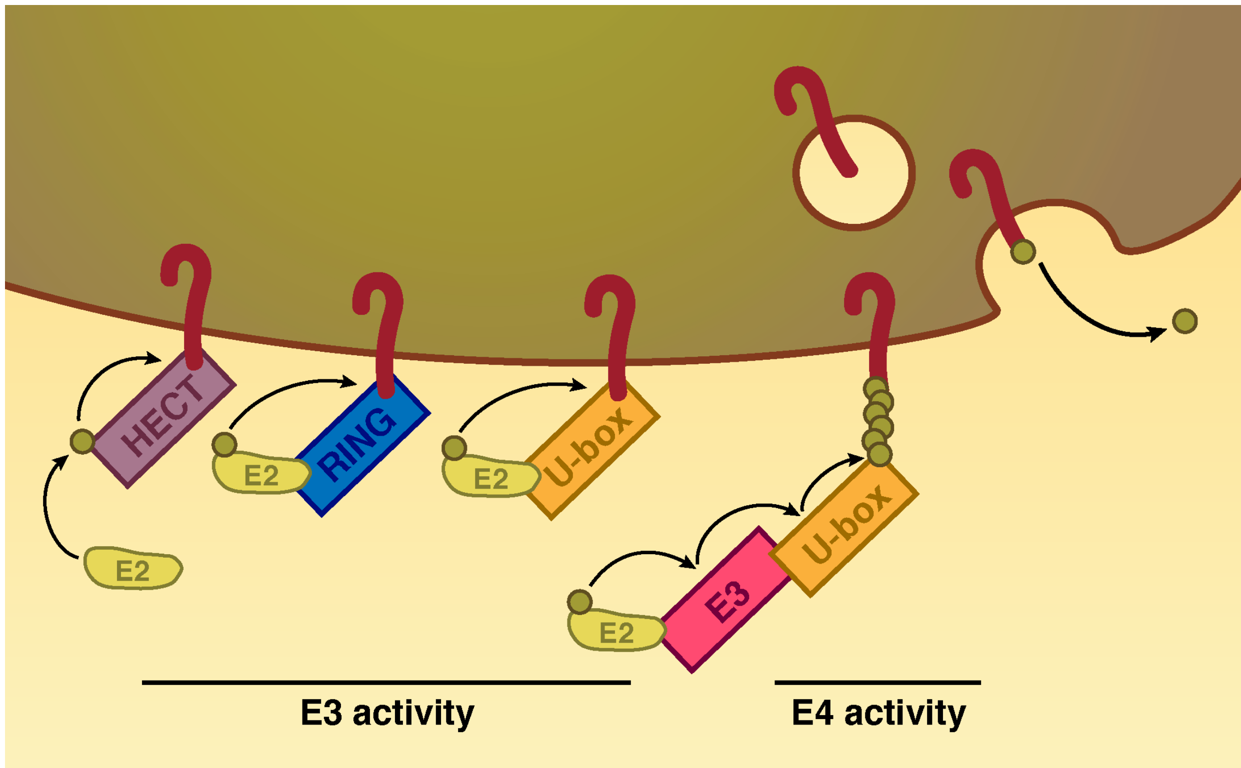

Figure 4. Ubiquitin ligases transfer ubiquitin onto target substrates in different manners.

E3 ubiquitin ligases are grouped into different classes based on their catalytic domain. Different catalytic domains transfer ubiquitin and interact with E2 conjugating enzymes in distinct ways. HECT domain ligases take ubiquitin from E2 enzymes and conjugate ubiquitin onto target substrates directly. RING domain and U-box domain ligases bind to E2s and target substrates simultaneously, to bring E2s into close proximity to target substrates so that ubiquitin may be transferred from E2s to target substrates. U-box domain ligases can also catalyze E4 activity by binding to ubiquitin and coordinating ubiquitination with E1, E2, and E3 enzymes.

1.5 E3 ubiquitin ligases

Ubiquitin protein ligases, or E3 ligases, are crucial as they confer specificity and selectivity to the ubiquitination process (Hershko and Ciechanover, 1998). An E3 may act as a

single protein with a catalytic domain, or an E3 may be a multiprotein complex composed of many proteins that act together to perform ubiquitination (d'Azzo et al., 2005). Whether acting as a single or multiprotein complex, ubiquitin ligases must possess three basic domains: 1) a substrate binding domain, 2) an E2 recognition domain, and 3) a catalytic ubiquitin ligase domain (Patterson, 2002).

E3 ligases are grouped into different classes, that are largely defined by their E2 recognition domain and their catalytic domain. The three major classes of ubiquitin ligases are the HECT domain family, the RING domain family, and the U-box domain family (Figure 4).

The human genome encodes roughly 28 HECT-domain E3s (Buetow and Huang, 2016). Ubiquitin ligases within this class contain a conserved C-terminal HECT catalytic domain and an N-terminal substrate binding domain (Buetow and Huang, 2016). HECT-domain family members are the only ubiquitin ligases that can mediate the direct transfer of ubiquitin to its target substrate (Buetow and Huang, 2016; Ardley and Robinson, 2005). In a manner similar to E1s and E2s, HECT E3s rely on a central cysteine residue within the HECT domain to receive activated ubiquitin from E2s and to form an E3-thioester intermediate. Subsequently, the E3 selects its target substrate and transfers ubiquitin directly (Buetow and Huang, 2016).

RING E3s are the largest family of ubiquitin ligases, with bioinformatics analyses predicting hundreds of putative RING E3s encoded by the human genome (Buetow and Huang, 2016; Ardley and Robinson, 2005). Members of this family contain a catalytic RING domain, which is required for recruitment of E2-ubiquitin complexes. These RING domains are enriched in cysteine and histidine, which enables this region to chelate two zinc atoms that aid in the correct folding of the RING domain (Ardley and Robinson, 2005). Unlike their HECT-domain counterparts, RING E3s are unable to catalyze protein ubiquitination directly. Instead, RING E3s promote the interaction between E2 enzymes and target proteins by binding the two proteins

simultaneously, bringing them in close proximity so that ubiquitin is transferred directly from the E2 to the substrate protein (Ardley and Robinson, 2005).

U-box E3s were the last major class of ubiquitin ligases to be identified. U-box ligases encode a 75-amino acid U-box motif that is structurally similar to the catalytic domain found in RING E3s, but lack the ability to chelate metal ions (Ardley and Robinson, 2005; Patterson, 2002). Given their strong similarities, it is not surprising that U-box ligases ubiquitinate their target substrates in a manner similar to that of RING E3s. U-box proteins bind their target substrates and E2 conjugating enzymes simultaneously, so that the E2 may transfer ubiquitin to target proteins (Ardley and Robinson, 2005). Unlike the vast number of proteins with putative RING ligase domains, there are only 19 predicted U-box domain-containing proteins encoded by the human genome (Patterson, 2002).

The prototypical U-box protein is the yeast ubiquitin ligase, Ufd2 (ubiquitin fusion degradation protein 2). Its discovery led to the introduction of a new class of ubiquitination enzymes, the E4 ubiquitin chain assembly factors (Koegl et al., 1999; Ardley and Robinson, 2005; Hatakeyama et al., 2001; Matsumoto et al., 2004; Hoppe, 2005). In conjunction with E1s, E2s, and E3s, E4s promote the efficient assembly of elongated ubiquitin chains. In the absence of an E4, construction of a ubiquitin chain is initiated, but terminates after a few ubiquitin molecules are attached (Koegl et al., 1999). E4 ligases bind to the initially attached ubiquitin moieties and coordinate the rapid elongation of the ubiquitin chains, which is required for the proteasomal degradation of certain proteins (Koegl et al., 1999; Cyr et al., 2002). Many U-box proteins can function as both an E3 or E4 ligase; whether they act as an E3 or E4 is dependent upon the target protein and E2/E3 partners that are present during ubiquitination (Cyr et al., 2002). One particular U-box protein, the mammalian homolog of Ufd2, UBE4B, has been shown to exhibit both E3 and E4 activity.

1.6 UBE4B is an E3 ubiquitin ligase and E4 ubiquitin chain assembly factor

Ubiquitination factor E4B (UBE4B), is a mammalian homolog of the yeast ubiquitin ligase, Ufd2 (Hatakeyama et al., 2001; Matsumoto et al., 2004). Like many other ubiquitin ligases, UBE4B can self-ubiquitinate (Hatakeyama et al., 2001).

Relatively little is known about interactions of UBE4B with other proteins. The E2 conjugating enzymes, UbcH5c and Ubc4 both bind and coordinate ubiquitination with UBE4B, while little enzymatic activity is detected with other E2s (Benirschke et al., 2010; Hatakeyama et al., 2001).

UBE4B has been shown to bind and polyubiquitinate ataxin-3 through its E3 ligase activity (Matsumoto et al., 2004). Ataxin-3 is a polyglutamate-containing protein that forms abnormal aggregates, and underlies neurodegeneration in Machado-Joseph disease (Matsumoto et al., 2004). UBE4B-mediated ubiquitination of ataxin-3 has been shown to promote ataxin-3 proteasomal degradation (Matsumoto et al., 2004). Thus, ubiquitination by UBE4B prevents the pathological aggregation of ataxin-3 (Matsumoto et al., 2004).

In the same study that examined UBE4B-mediated degradation of ataxin-3, an interaction between UBE4B and valosin-containing protein (VCP) was also identified. VCP is the mammalian homolog of yeast Cdc48. In yeast, Ufd2 (the yeast homolog of UBE4B) binding to Cdc48 has been implicated in promoting cell survival upon induction of stress (Matsumoto et al., 2004). In mammalian cells, the UBE4B-VCP interaction is hypothesized to mediate ataxin-3 delivery to the proteasome (Matsumoto et al., 2004).

UBE4B also binds and ubiquitinates FEZ1 (Fasciculation and elongation protein zeta 1) (Okumura et al., 2004). Unlike ataxin-3, the ubiquitination of FEZ1 by UBE4B does not affect its stability or lead to its degradation. Instead, the interaction promotes differentiation/neurite outgrowth in tumor cells (Okumura et al., 2004).

Most recently, UBE4B was found to act as an E3 ligase with E4 chain-modifying activity (Wu et al., 2011). UBE4B binds to MDM2 and p53 to promote the ubiquitination and degradation of p53 in medulloblastoma tumor cells (Wu et al., 2011). Because p53 is an established tumor suppressor in medullablastoma, negative regulation of p53 expression by UBE4B may be tumorigenic (Wu et al., 2011; Zeinab et al., 2012).

Although there is evidence that UBE4B may play an oncogenic role in medulloblastoma, genetic evidence suggests that UBE4B expression may be protective in some cancers. The *UBE4B* gene resides on the short arm of chromosome 1 (1p36), a region that is frequently deleted in neuroblastoma (Krona et al., 2003; Caron et al., 1996). Survival analyses of neuroblastoma patient populations found a significant correlation between 1p36 deletion and poor patient outcome (Attiyeh et al., 2005). As such, *UBE4B* has emerged as a candidate tumor suppressor gene in neuroblastoma (Krona et al., 2003; Caron et al., 1996). These 1p36 deletions are also common in hepatocellular carcinoma and glioblastoma, although studies examining the role of UBE4B in these diseases are not as extensive (Zhang et al., 2010a; Ichimura et al., 2008).

1.7 UBE4B may interact with the ESCRT-0 component, Hrs

No mechanistic studies have been performed to examine whether UBE4B may play a role in neuroblastoma. However, in a yeast two-hybrid screen using Hrs as bait, Dr. Andrew Bean identified multiple clones encoding UBE4B (Sirisaengtaksin et al., 2014). The ESCRT-0 component, Hrs, acts as a scaffolding protein to recruit proteins that play a role in endosomal sorting (see section 1.2). In addition to initiating the recruitment of the ESCRT sorting machinery components, Hrs recruits non-ESCRT components to endosomal membranes. Some of these proteins contribute to the MVB sorting of membrane proteins, suggesting that UBE4B may also bind to Hrs to contribute to endocytic sorting. Hrs binds to Eps15 in a calcium-dependent manner,

forming a stable complex at endosomes (Bean, 2000; Roxrud et al., 2008). The Hrs-Eps15 complex has been shown to mediate the endosomal sorting of epidermal growth factor receptor (EGFR) (Roxrud et al., 2008). Additionally, Hrs has been shown to bind to sorting nexin 1 (SNX1) (Chin et al., 2001). SNX1 has been shown to bind to the intracellular domain of EGFR, and SNX1 overexpression promotes the degradation of EGFR (Chin et al., 2001).

A major role of the endocytic pathway is to downregulate the expression and activity of proteins that reside at the cell surface, including EGFR, whose lysosomal degradation is dependent upon ubiquitination at endosomes (Alwan et al., 2003; Eden et al., 2012; Goh and Sorkin, 2013; Grandal et al., 2007). A potential role for UBE4B is supported by evidence that 1) the genomic region encoding UBE4B is commonly deleted in neuroblastoma; 2) UBE4B may bind to Hrs, a member of the endosomal sorting machinery; 3) the endosomal sorting of EGFR requires receptor ubiquitination; and 4) EGFR is overexpressed in neuroblastoma, suggesting a possible defect in the endocytic trafficking and degradation of EGFR.

1.8 Ubiquitination specifies proteins for degradation

Ubiquitination plays an essential role in the degradation of intracellular proteins, both by the proteasome and the lysosome (Erpapazoglou et al., 2012). The addition and removal of ubiquitin from protein substrates specifies the fate of a protein for degradation in either the proteasome or lysosome (Komander and Rape, 2012). 26S proteasomes are multi-subunit complexes that are found in either the cytosol or the nucleus (Coux et al., 1996). The 26S proteasome is comprised of two core subcomplexes: the cylindrical-shaped core particle (or 20S proteasome) and the regulatory particle (or 19S particle), which attaches to either end of the core particle (Dikic, 2017; Coux et al., 1996). Portions of the regulatory particle serve to recognize ubiquitinated substrate protein and prepare them for degradation in the core particle (Lander et

al., 2012). Both the core particle and the regulatory particle possess protease activity, and denature and degrade ubiquitinated proteins (Coux et al., 1996; Lander et al., 2012; Dikic, 2017) Lysosomes are acidic, membrane-bound organelles that contain a number of hydrolases that drive the proteolytic cleavage of proteins delivered to the lysosomal lumen (Hershko and Ciechanover, 1998).

There are two simplified conventions of the relationship between ubiquitin and protein degradation: 1) proteins are subject to proteasomal degradation or lysosomal degradation based on their cellular localization, and that 2) K48-linked ubiquitin chains specify proteasomal degradation and K63-linked specify lysosomal degradation. For example, because proteasomes are located in the cytosol and nucleus, ubiquitination of cytosolic and nuclear proteins should designate these proteins for proteasomal degradation (Dikic, 2017). Further, because lysosomes receive proteins that are sorted into the lumen of MVBs and other membrane-bound proteins, ubiquitination of luminal and membrane-bound proteins should designate lysosomal degradation (Dikic, 2017). CHIP, the U-box E3 ubiquitin ligase, is known to modify membrane-bound proteins with K63-linked polyubiquitin chains, which promote lysosomal degradation (Apaja et al., 2010). However, CHIP-mediated ubiquitination of soluble, cytosolic protein results in K48-linked ubiquitin chains, leading to proteasomal ubiquitination (Nathan et al., 2013). Interestingly, K63-linked ubiquitin chains may designate substrate proteins for lysosomal degradation because the endosomal sorting complex, ESCRT-0, binds to K63 ubiquitin chains with a higher affinity than the proteasome subunits (Nathan et al., 2013). In binding reactions, purified proteasomes were found to be capable of binding to both K48- and K63-linked chains; the addition of Hrs and STAM-containing fractions to binding assays prevented proteasomal binding of K63-linked ubiquitin chains (Nathan et al., 2013). Hrs and STAM did not bind to K48-linked ubiquitin chains (Nathan et al., 2013).

In most cases this convention is true; however, there are exceptions. For example, c-Met, a membrane-bound protein that undergoes ligand-induced endocytosis (Jeffers et al., 1997). The degradation of c-Met is blocked by a proteasome inhibitor called lactacystin, suggesting that the proteasome may contribute to c-Met proteolysis (Jeffers et al., 1997). In another example, the degradation of neuronal nicotinic acetylcholine receptor (nAChR) subunits was shown to be proteasome-dependent (Rezvani et al., 2007). Lysates isolated from cells treated with the proteasome inhibitor, PS-341, were enriched in ubiquitinated nAChR subunits compared to lysates isolated from untreated cells (Rezvani et al., 2007). However, lysates isolated from cells that were treated with a lysosome inhibitor, E-64, showed similar levels nAChR subunit levels compared to control lysates, suggesting that the proteasome is required for the degradation of ubiquitinated nAChRs (Rezvani et al., 2007).

Interestingly, a proteasome-associated protein, Ecm29, has been shown to link the 26S proteasome to specific compartments within eukaryotic cells, including endosomes, suggesting that the proteasomal and lysosomal degradation pathways may converge (Gorbea et al., 2010). Endosomal localization of proteasomes may provide a mechanism by which transmembrane proteins may be degraded, however, the purpose and mechanism of this pathway are not understood.

1.9 Deubiquitination is mediated by deubiquitinating enzymes

Ubiquitin conjugated to target substrates can be removed by ubiquitin-specific proteases called deubiquitinating enzymes (DUBs). DUBs act by cleaving the isopeptide bond formed between substrate targets and the C-terminal end of ubiquitin (Komander and Rape, 2012). The human genome encodes ~ 55 DUBs, which largely mediate ubiquitin removal in a non-specific manner (Komander and Rape, 2012). There are five major families of DUBs. The cysteine

protease DUBs are separated into four subclasses: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), Otubain domain proteases (OTUs), and Machado-Joseph disease proteases (MJDs) (Hanpude et al., 2015). An additional DUB class, contains proteins with a JAMM (JAB1/MPN/Mov34) catalytic domain, and belongs to the metalloprotease family (Hanpude et al., 2015).

The importance of the deubiquitination activity of DUBs has been established in the endocytic pathway (Raiborg and Stenmark, 2009; Alwan and van Leeuwen, 2007; McCullough et al., 2004; Hanpude et al., 2015). Deubiquitination is an opposing process to ubiquitination, and as such can reverse the degradative fate of a ubiquitinated membrane protein at endosomes (McCullough et al., 2004). A receptor that is not ubiquitinated at MVBs cannot be recognized by ESCRTs, and remain on the limiting membrane for budding from the endosomal membrane or incorporation into lysosomal membranes (McCullough et al., 2004) Although ubiquitination of protein cargo is required for their recognition by ESCRTs, the removal of ubiquitin by DUBs is also required for endosomal protein sorting (Raiborg and Stenmark, 2009). In fact, two DUBs have been demonstrated to bind the ESCRT-0 component, STAM: USP8 and AMSH (McCullough et al., 2004; Berlin et al., 2010). The sequential, temporal requirement of ubiquitination and deubiquitination makes it difficult to establish whether a DUB positively or negatively regulates the degradation of an endosomally-associated membrane protein. The lack of clarity on this issue is exemplified by the conflicting evidence that USP8 overexpression may promote or inhibit EGFR degradation (Berlin et al., 2010; Alwan and van Leeuwen, 2007; Row et al., 2006). In USP8 knockdown experiments, Row and colleagues found that decreased expression of USP8 was associated with the accumulation of ubiquitinated cargo at MVBs (Row et al., 2006). These findings were corroborated in experiments by Alwan, et al., in which the overexpression of USP8 was positively correlated with receptor cargo degradation (Alwan and van Leeuwen, 2007). Conversely, Berlin and colleagues found that the USP8 depletion was

correlated with increased receptor cargo degradation (Berlin et al., 2010) These findings were repeated by Mizuno, et al., in which depletion of endogenous USP8 resulted in increased receptor ubiquitination and degradation (Mizuno et al., 2005).

1.10 Epidermal Growth Factor Receptor: An Overview

The epidermal growth factor receptor (EGFR, also called HER1 or ErbB1) is the prototypical member of the ErbB/HER family of receptor tyrosine kinases (RTKs), which includes ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4) (Herbst, 2004). This family of transmembrane proteins binds to extracellular growth factors that activate signal transduction pathways. In this manner, EGFR influences cell differentiation, migration, survival, adhesion, and proliferation (Normanno et al., 2006; Goh et al., 2010; Ferguson, 2008; Huang et al., 2009). Impaired EGFR degradation and prolonged EGFR signaling is associated with multiple disease states, particularly in cancers (Masui et al., 1984; Di Fiore et al., 1987; Shtiegman et al., 2007; Bache et al., 2004) This suggests that defects in EGFR downregulation may favor tumorigenesis (Ferguson, 2008; Grandal et al., 2007; Bache et al., 2004; Masui et al., 1984; Prewett et al., 1996).

EGFR and its ErbB family members share the same basic domain organization, with an extensive extracellular receptor region, a single-pass, hydrophobic transmembrane domain, and an intracellular catalytic tyrosine kinase domain, and several tyrosine residues that can be phosphorylated (Herbst, 2004; Yewale et al., 2013). Through its extracellular domain, EGFR can be activated by binding to seven different types of ligand growth factors: epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin, β-cellulin, epigen, epiregulain, and heparin-binding EGF-like growth factor (Cappuzzo, 2014; Herbst, 2004; Salomon et al., 1995; Guo et al., 2003) Each ligand induces the phosphorylation of a distinct set of tyrosine residues, and therefore differentially affects the fate of EGFR (Ferguson, 2008; Roepstorff et al.,

2009). EGF and TGF-α are the two major agonists of EGFR, and bind to EGFR exclusively (Guo et al., 2003).

EGF and TGF-α bind the receptor with the highest affinity among the EGFR ligands (Roepstorff et al., 2009). Treatment with either 10 nm EGF ligand or TGF-α ligand caused similar amounts of EGFR to be removed from the cell surface (Roepstorff et al., 2009) Nearly 100% of EGFR recycled to the surface following TGF-α stimulation, only 50% of EGFR was recycled following EGF stimulation (Roepstorff et al., 2009). Stimulation by both ligands allowed receptor transport to early endosomes; however, 60 minutes after EGF stimulation, there is increased colocalization of EGFR with late endosomes, compared to cell surface localization of EGFR following TGF-α stimulation (Roepstorff et al., 2009). Ligand dissociation from EGFR underlies the difference in EGF- and TGF-α-induced EGFR trafficking. TGF-α dissociates from EGFR at much higher pH levels than EGF, leading to TGF-α dissociation shortly after plasma membrane internalization. (Roepstorff et al., 2009; Ebner and Derynck, 1991). EGF remains bound to EGFR until dissociation at lysosomes (Ebner and Derynck, 1991). These data suggest that EGFR stimulation by EGF is a better model for examining receptor sorting at the late endosomal membranes.

1.11 EGF binding enables EGFR dimerization and receptor activation

EGF-EGFR binding sets off a chain of molecular events: receptor dimerization, kinase activation, and phosphorylation of tyrosine residues (Krall et al., 2011; Chung et al., 2010). Receptor dimerization among EGFR and its ErbB family members is vastly different from dimerization events observed between other RTKs (Ferguson, 2008). For example, binding of Kit receptors (of the platelet-derived growth factor receptor family) to its cognate ligand stem cell factor (SCF) induces indirect receptor dimerization. SCF ligands form dimeric complexes that

link Kit receptors, facilitating transphosphorylation between the receptor pairs (Lev et al., 1992). In contrast, EGF binding prompts a critical conformational change in EGFR, exposing a dimerization arm and triggering structural rearrangement of the extracellular domain of EGFR (Dawson et al., 2005). Ligand-bound receptors are stabilized in this "extended" conformation of EGFR, allowing the exposed dimerization arm to mediate direct interactions with nearby receptors (Ferguson, 2008; Klein et al., 2004; Jura et al., 2009; Dawson et al., 2005). Although the exposure of the dimerization arm is required for receptor dimerization, it is not sufficient to drive dimerization alone (Dawson et al., 2005). Mutations within the intracellular domain of EGFR inhibits receptor activation (Dawson et al., 2005). Further, regions outside the dimerization arm specify homodimerization (oligomerization between EGFRs) or heterodimerization (oligomerization between EGFR and another ErbB) (Dawson et al., 2005; Ferguson, 2008).

Receptor dimerization is required for the activation of the EGFR catalytic domain (Jura et al., 2009; Mattoon et al., 2004). EGFR activation is regulated by the interaction between the tyrosine kinase domains of the dimerized receptors, wherein the kinase domain of one receptor activates the kinase domain of the second receptor (Jura et al., 2009). Receptor activation leads to EGFR autophosphorylation on cytosolic tyrosine residues (Wilson et al., 2009).

1.12 EGFR activation stimulates EGFR autophosphorylation

In all, EGFR contains 10 tyrosine residues that are phosphorylated to act as binding sites for a number of cytosolic proteins (Schulze et al., 2005; Wilson et al., 2009). These phosphorylation events play a key role in the initiation and propagation of downstream signal transduction pathways, as well as EGFR downregulation.

EGFR autophosphorylation at specific tyrosine residues enables selective binding to proteins that possess either Src homology 2 (Sh2) or phosphotyrosine binding (PTB) domains

(Ferguson, 2008; Krall et al., 2011; Normanno et al., 2006). Extensive work has resulted in the determination of the tyrosine residues of EGFR that are capable of binding signaling proteins, as well as the specific proteins that bind to each of the phosphotyrosine sites (Schulze et al., 2005; Steen et al., 2002). Phosphorylation that enables Cbl and Grb2 binding are the most relevant to EGFR sorting at the endosome.

Cbl is a RING E3 that is required for the sorting and degradation of receptor tyrosine kinases (RTKs) like epidermal growth factor receptor (EGFR) (Ardley and Robinson, 2005; Mohapatra et al., 2013; Visser Smit et al., 2009). Cbl recognizes EGFR through the direct binding of phosphotyrosine 1045 and catalyzes EGFR ubiquitination at the plasma membrane (Grandal et al., 2007). Ubiquitination is not required for the internalization of EGFR at the plasma membrane, but is required for inclusion into MVB vesicles (Smith et al., 2013; Huang et al., 2007). Cbl expression, but not its ubiquitination activity, was required for the endosomal sorting of EGFR (Smith et al., 2013). Instead, ubiquitination that is mediated by the RING E3 ligases RNF126 and Rabring7 promotes EGFR degradation (Smith et al., 2013). Cbl may also bind to EGFR indirectly through its association with Grb2, at phophotyrosines 1068 and 1173, although Cbl interaction with EGFR in this indirect manner is less effective at targeting EGFR for lysosomal degradation (Batzer et al., 1994; Grandal et al., 2007).

1.13 EGFR has been targeted to inhibit tumor growth

EGFR mediates cellular processes such as cell migration, differentiation, and proliferation. However, aberrant signaling by EGFR underlies many cancers (Ferguson, 2008). Many pharmacological approaches have been developed to limit the pathological signaling activity of EGFR, including monoclonal antibodies and tyrosine kinase inhibitors.

Monoclonal antibodies bind to the extracellular domain of EGFR and compete with EGFR agonists like EGF and TGF-α. By blocking ligand-receptor complex formation, monoclonal antibodies prevent activation of tyrosine kinase inhibitors and mediate receptor entry into the endocytic pathway (Herbst, 2004; Zage et al., 2013; Prewett et al., 1996; Shin et al., 2001; Masui et al., 1984). Compared to some small-molecule inhibitors, they achieve higher receptor specificity; therefore, EGFRs may be inhibited at lower concentrations (Herbst, 2004). One type of monoclonal antibody therapeutic, cetuximab, has undergone extensive clinical trials. It was the first EGFR-targeted antibody that received FDA-approval for used in metastatic colorectal cancer (Lenz, 2007).

Another class of EGFR therapy targets the tyrosine kinase activity of EGFR. Unlike cetuximab, this class of inhibitors do not interfere with cognate EGF ligand binding. Instead, this class of drugs prevent autophosphorylation events by competing for EGFR binding with ATP. Inhibition of EGFR tyrosine kinase activation by erlotinib has been showed to reduce cell proliferation and induce apoptosis (Herbst, 2004).

1.14 Rationale and summary

The major mechanism by which cells regulate EGFR expression and activity is endocytosis. EGFRs that traverse the endocytic pathway may be 1) sorted into vesicles that bud into the MVB for lysosomal degradation, 2) included into vesicles that bud out from the MVB for delivery to other organelles in the cell, or 3) remain on the endosomal membrane for incorporation into lysosomal membranes. Ubiquitination of EGFRs is a critical modification that enables receptor recognition by the endosomal ESCRT machinery, and therefore, ensures their MVB sorting, and therefore lysosomal degradation. Hrs, a critical component of ESCRT-0, recruits other ESCRTs and non-ESCRT proteins that play significant roles receptor trafficking. Hrs is

predicted to bind to a ubiquitin ligase, UBE4B, which suggests that UBE4B may play a role in receptor trafficking.

The *UBE4B* gene is located in the chromosomal region 1p36, which is commonly deleted in neuroblastoma and suggests that *UBE4B* may be a candidate tumor suppressor gene. Additionally, EGFR is shown to be overexpressed in neuroblastoma. The first aim of this work is to determine whether UBE4B plays a role in the endocytic trafficking of EGFR. The second aim of this work is to determine whether altered expression of UBE4B may affect the growth of neuroblastoma cells and their response to EGFR inhibitors.

Chapter 2. Methodology

2.1 Cell culture

The HeLa human cervical cancer cell line was obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and passaged using a 0.25% Trypsin/0.1% EDTA solution at 37°C with 5% CO_2/95% O_2.

The SK-N-AS human neuroblastoma cell line was obtained from the ATCC. The UBE4B- and UBE4B(P1140A)-expressing cell lines were generated by Drs. Wei Sun and Qing Yan in Dr. Andrew Bean's lab prior to my arrival. All cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 1% L-glutamine at 37°C with 5% CO_2/95% O_2 and passaged using 0.5 mM EDTA.

The MCF-10A human mammary epithelial cell line and the Cbl/Cbl-b double-depleted MCF-10A cell line were kindly gifted by Dr. Hamid Band from the Eppley Institute for Research in Cancer and Allied Diseases at the University of Nebraska Medical Center. Cells were maintained in mammary epithelial cell growth medium (MEGM) supplemented with the components of an MEGM Bulletkit (Lonza) and 100 ng/ml cholera toxin at 37°C with 5% CO_2/95% O_2. Cells were subcultured using a solution of 0.05% trypsin and 0.53 mM EDTA.

2.2 Antibodies

Antibodies were purchased from the following sources: EGFR, for immunoblotting (Pierce/Invitrogen, PA1-1110); EGFR, for immunoprecipitation (Santa Cruz Biotechnologies, sc-120); EEA1 (Invitrogen, PIPA517228); LAMP1 (Developmental Studies Hybridoma Bank, H4A3); UBE4B (Cocalico).

2.3 *Buffers, materials, and reagents*

Binding buffer- This buffer is a component of the recombinant protein binding assay. Binding buffer consists of 20 mM HEPES (pH 7.4), 150mM KCl, and 0.05% TWEEN® 20.

Brickey buffer- This buffer is used to resuspend cells infected with baculovirus for recombinant protein extraction, as described in (Brickey et al., 1990). Brickey buffer consists of 10 mM Tris-HCl, pH 7.5 containing 5% betaine, 1 mM EGTA, 1 mM EDTA and 0.5 mM DTT.

Homogenization buffer- This buffer is a component of the sorting assay. Homogenization buffer consists of 20 mM HEPES (pH 7.4), 0.25 M sucrose, 2mM EGTA, 2mM EDTA, and 0.1 mM DTT.

Mammalian Protein Extraction Reagent (M-PER)- This is a lysis buffer that is manufactured by Thermo Scientific (product #78501).

Protease inhibitor cocktail- A protease inhibitor cocktail was prepared using 112 μM PMSF, 3 μM aprotinin, 112 μM leupeptin, and 17 μM pepstatin.

2.4 *Separation of proteins by gel electrophoresis and specific detection of proteins by immunoblotting*

To detect specific proteins, samples were separated by SDS-PAGE (voltage was not permitted to exceed 120 V). Protein from gels were transferred to nitrocellulose membranes

(100V, 1.5 hours, 4°C). Blots were incubated with a blocking buffer consisting of 5% nonfat dry milk in 1x PBS for one hour at room temperature. Blots were then probed with antibodies directed towards the protein of interest by incubation with antibodies overnight at 4°C. Membranes were washed three times with 1x PBS, then incubated with secondary antibody diluted in blocking buffer (either anti-mouse, Invitrogen, #31430 or anti-rabbit, Invitrogen, #31460) at room temperature for one hour. Membranes were washed three times with 1x PBS. Proteins were visualized using ECL on autoradiography film and quantified using ImageJ (version 1.46r).

2.5 Production of recombinant proteins

Recombinant proteins were expressed in insect cells as described previously (Tsujimoto et al., 1999). Briefly, full-length Hrs, UBE4B, and UBE4B(P1140A) were subcloned into an Hta baculovirus vector and Hrs, UBE4B, or UBE4B(P1140A) virus was produced according to the manufacturer's protocol (Gibco). Proteins were produced by infecting a 500 mL of SF21 cells (multiplicity of infection = 0.1). Seventy-two hours after initial infection, cells were split into ten volumes and harvested by centrifugation. Pellets were stored at -80°C. Viral infections of SF21 cells were performed by the Baculovirus/mAb Shared Resource of the Proteomics Shared Resource at Baylor College of Medicine.

Cell pellets were thawed and resuspended in 800μL of Brickey buffer containing a protease inhibitor cocktail (section 2.3). Cell suspensions were rotated end-over-end for 1 hour at 4°C, followed by centrifugation at 15,000 *x g* for 30 minutes at 4°C. Resultant lysates were incubated with Ni-NTA agarose for affinity-isolation of His-tagged recombinant Hrs, STAM, UBE4B, or UBE4B(P1140A). For the recombinant protein binding assay (section 2.5), Hrs was not eluted from Ni-NTA agarose. For the ubiquitination assay (section 2.7), UBE4B was not

eluted from Ni-NTA agarose. For assays that required soluble recombinant protein, proteins were eluted using 100 mM imidazole.

2.6 Recombinant protein binding assay

To determine whether Hrs and UBE4B bound to each other directly, recombinant proteins were expressed and harvested from insect cells. First, 23.08 nM of His_6-Hrs bound to Ni-NTA agarose was incubated with increasing concentrations of purified soluble UBE4B (0 to 369.24 nM) in binding buffer and a protease inhibitor cocktail. Suspensions of recombinant protein and agarose were rotated end-over-end for 1 hour at 4°C. Suspensions were subject to centrifugation on a tabletop centrifuge for 3 seconds to pellet agarose beads and supernatants containing unbound protein were removed. Agarose beads were washed three times with 1x PBS, boiled in sample buffer, and separated by SDS-PAGE. Coomassie Blue staining was used to detect bound UBE4B and Hrs. Bands were quantified using ImageJ software (version 1.46r).

To determine whether UBE4B and STAM are capable of binding to Hrs simultaneously, 23.06 nM of His_6-Hrs bound to Ni-NTA agarose and 200 nM of soluble, free-floating STAM was incubated with increasing amounts of UBE4B (0 to 369.24 nM). Incubation and quantitation were performed as detailed above.

2.7 Immunoprecipitation of EGFR

To determine whether UBE4B and EGFR may interact *in situ*, EGFR was immunoprecipitated from cell lysates. HeLa cells were cultured in 10 cm dishes to 80% confluence. Cells were washed with 1x PBS and scraped into a 1.5 mL volume of 1x PBS. Cells were centrifuged at 1,500 *x g* for 10 minutes at 4°C. Supernatants were discarded and cell pellets were resuspended in a 50 μL M-PER buffer/protease inhibitor cocktail solution and rotated end-

over-end for one hour at 4°C. Samples were subject to centrifugation at 15,000 *x g* for 15 minutes at 4°C. Supernatants were collected and protein content was determined using a bicinchoninic acid assay (BCA assay).

Cell lysate (100 μg of protein) was incubated with antibodies specific to EGFR (overnight at 4°C). 20 μL of a 50% Protein A agarose slurry was added to samples and incubated for 4 hours while rotating end-over-end at 4°C. Beads were washed three times with 1x PBS and boiled in sample buffer. EGFR and UBE4B were detected by immunoblotting (section 2.4).

To determine whether UBE4B and EGFR can interact in the absence of Cbl, I immunoprecipitated EGFR from parental MCF-10A cells and Cbl/Cbl-b double-depleted MCF-10A cells as described above, and EGFR and UBE4B were detected by immunoblotting (section 2.4).

2.8 Ubiquitination assay

To determine whether UBE4B is capable of ubiquitinating EGFR, *in vitro* ubiquitination was reconstituted using recombinant enzymes and HeLa cell lysate as a source of EGFR. HeLa cells were cultured to 80% confluence in 10 cm dishes. The contents of one dish was scraped into PBS, pelleted, and resuspended in 30 μL of M-PER lysis buffer. Lysates were then incubated with 1 μg of anti-EGFR. This particular antibody recognizes an epitope in the N-terminal domain (extracellular) of EGFR. Samples were incubated while rotating end-over-end overnight at 4°C.

The next day, lysates were mixed with components required for ubiquitination as previously described (Hatakeyama et al., 2001). Reactions were assembled in a total volume of 20 μL containing 0.1 μg of E1, 1 μg of UbcH5c, 1 μg of ubiquitin, 2 mM ATP, 1 mM $MgCl_2$, 0.3 M DTT, 1 mM creatine phosphate, 0.5 units of phosphocreatine kinase, 20mM Tris-HCl (pH 7.5), 120 mM NaCl, and 5 μL of HeLa lysate. Some ubiquitination reactions contained recombinant

His_6-UBE4B or His_6-UBE4B(P1140A) (1ug) bound to Ni-NTA agarose. Samples were incubated in a water bath (2 hrs at 30°C). After incubation, cells were centrifuged and supernatants [containing reaction components without UBE4B or UBE4B(P1140A)] were collected. For samples that required deubiquitination, supernatants were added to a deubiquitination assay (detailed in section 2.9).

Then, supernatants were incubated with Protein A agarose, to complete the immunoprecipitation of EGFR (end-over-end for 4 hours, 4°C). Beads were washed to remove unbound protein, and proteins were eluted by boiling beads in sample buffer. Proteins were subject to immunoblotting (section 2.4).

2.9 Deubiquitination assay

Deubiquitination was performed as described by (Rezvani et al., 2007) immediately following ubiquitination assays, removal of UBE4B or UBE4B(P1140A), and immunoprecipitation of EGFR. After washing, protein A beads were resuspended in 25 mM HEPES (pH 7.4) containing 10 mM DTT and incubated with 5 μg of isopeptidase-T (Calbiochem) and 5 μg of UCH-L3 (Biomol) rotating end-over-end for 60 minutes at room temperature. Protein A agarose beads were washed and bound protein was eluted as described above. Immunoblotting was performed was performed as described above, with membranes probed using ubiquitin-specific antibodies (section 2.4).

To determine whether USP8 can deubiquitinate EGFR following UBE4B-mediated ubiquitination, 5 μg of USP8 was added to reactions in place of isopeptidase-T and UCH-L3. Then, deubiquitination was performed as described above.

2.10 Reconstitution of inward budding at MVB membranes

Generation of cytosol- HeLa ells were washed twice with 1x PBS, scraped from the plate, and collected by centrifugation (2000 *x g* for 15 minutes at 4°C). Following centrifugation, supernatant was removed and cells were resuspended in HB (100 µL per plate). Cell suspensions were lysed by sonication (Branson Sonifier 250, output 3.0, duty cycle 55%) for 10 seconds, three times. Samples were cooled for at least 30 seconds between each sonication period. Lysates were centrifuged at 2000 *x g* for 10 min at 4°C. Supernatants were collected (post-nuclear supernatant) and centrifuged at 100,000 *x g* for 1 hour at 4°C. The resulting supernatant contained the cytosolic fractions. Cytosols were collected and protein assays were performed to determine protein concentrations. Cytosols were aliquoted into volumes containing 25 µg of total protein and stored at -80°C.

Reconstitution of inward budding- The cell-free reconstitution of multivesicular body formation was performed as detailed in (Sun et al., 2010; Gireud et al., 2015) with modifications. Experiments required 1 plate for every 3 reactions (10 cm plate, 70 to 80% confluent). Plates were washed with 1 x PBS, then serum starved by adding DMEM lacking serum and incubating (2 hours at 37°C, 5% CO_2). Following serum starvation, cells were stimulated with DMEM supplemented with EGF (100 ng/mL) for 10 minutes (37°C, 5% CO_2). Cells were then placed on ice, media was removed, and cells were washed three times with cold 1x PBS. Cells were scraped into 1.5 mL 1x PBS and cell suspensions were centrifuged (1,500 *x g* for 10 minutes at 4°C). Supernatants were removed and pellets were resuspended in 100 µL homogenization buffer. Resuspended cells were drawn though a 30-gauge 1-inch needle into a 1 mL syringe 20 times while incubated on ice to burst outer cell membranes without disrupting organelle membranes. Homogenized samples were centrifuged at 800 *x g* for 5 minutes at 4°C. Without disturbing the

pellets, supernatants were transferred to a new tube and centrifuged at 1,500 *x g* for 15 minutes. Again without disturbing pellets, supernatants were transferred into a new tube and centrifuged at 9,000 *x g* for 30 minutes. Supernatants were removed and resulting pellets (which contains a crude endosomal fraction of mixed early and late endosomes) were resuspended in 80 μL homogenization buffer/protease inhibitor cocktail solution.

A standard 50 μL reaction consisted of the following:

1) 15 μL of resuspended membranes
2) volume of cytosol containing 25 μg of protein
3) 6 μL ATP regeneration system (final concentrations within 50 μL reactions listed below)
    a. 2 mM MgATP
    b. 50 μg/mL creatine kinase
    c. 8 mM phosphocreatine
    d. 1 mM DTT
4) volume of homogenization buffer to complete reaction volumes to 50 μL

For reactions requiring addition of recombinant UBE4B or UBE4B(P1140A), 180 nM of purified protein were added. Reactions were incubated for 3 hours at 37°C. Following incubation, reactions were placed on ice and reactions were incubated with trypsin (10 μL, 0.27 μg/μL) for 30 minutes. Reactions were centrifuged at 15,000 *x g* for 30 minutes at 4°C. Pellets were resuspended in sample buffer and boiled. Proteins were subject to immunoblotting as described above (section 2.4).

2.11 EGFR degradation

To examine cellular EGFR content following cell starvation and ligand treatment, cells were cultured to 80% confluence in 10 cm dishes. Each trial required three dishes per condition. Cells were washed three times with 1x PBS and incubated in serum-free media (DMEM, RPMI 1640, or MEM, where appropriate) at 37°C and 5% CO_2 for two hours. Existing media was replaced with fresh serum-free media supplemented with EGF (100 ng/mL). Plates were incubated at 4°C on ice for 1 hour to allow EGF-EGFR binding.

Following incubation, cells were rinsed three times with cold 1x PBS. One plate per condition was kept on ice. In the remaining plates, warm serum-free media was added and plates were incubated at 37°C and 5% CO_2 for either 30 or 60 minutes. Following incubations, cells were washed with 1x PBS and scraped into a 1.5 mL volume of 1x PBS. Cells were centrifuged at 1,500 *x g* for 10 minutes at 4°C and supernatant was discarded. Cell pellets were resuspended in a 50 μL M-PER buffer/protease inhibitor cocktail solution. Samples were incubated at 4°C and rotated end-over-end for one hour. After one hour, samples were centrifuged at 15,000 *x g* for 15 minutes at 4°C. Protein concentrations of supernatants were determined using a BCA assay. Fifty μg of protein from each sample was subject to immunoblotting (section 2.4).

2.12 Immunofluorescence microscopy

To determine subcellular localization of USP8 while EGFR is moving through the endocytic pathway, immunofluorescent labeling of USP8 and LAMP1 was performed. HeLa cells were plated onto sterile glass coverslips. Cells were cultured to 70% confluence. Prior to immunolabeling, cells were serum-starved for 2 hours to bring EGFR to the plasma membrane (Sirisaengtaksin et al., 2014). Cells were then incubated with media supplemented with 100 ng EGF/mL for 30 minutes on ice 0°C. Plates remained either at 0°C or were transferred to 37°C for

15 minutes to allow EGF-EGFR internalization. Cells were then fixed by incubation with 4% paraformaldehyde diluted in 1x PBS for 20 minutes. Antibodies directed towards UBE4B (1:250) and Hrs (1:500) were diluted in blocking buffer [2% normal goat serum, 0.25% saponin, 0.1 M phosphate buffer (pH 7.4)]. Coverslips were incubated in the antibody/blocking buffer solution overnight at 37°C. Cells were then washed three times with 1x PBS, then incubated with secondary antibodies at 37°C for 30 min. Coverslips were washed and mounted with *para*-phenylenediamine in 50% glycerol/0.1 M phosphate buffer (pH 7.4). Images were acquired using an LSM 510 confocal laser scanning microscope (Carl Zeiss).

2.13 Proliferation assay

SK-N-AS cell lines were plated into wells of 96-well plates (4,000 cells diluted in 100 µL of culture media). Due to the nature of measurement, each time point required a separate plate cells. At each time point (0, 24, 48, and 72 hours), 10 µL of WST-1 reagent (Roche) was added to each well and incubated for 4 hours at 37°C and 5% CO_2. WST-1 is a stable tetrazolium salt that is cleaved in response to glycolytic production of NAD(P)H by actively proliferating cells. WST-1 is cleaved to formazan dye. The amount of formazan produced directly corresponds to the number of metabolically active cells in the culture well. The amount of formazan may be measured using absorbance values obtained at 450 nm, according to the manufacturer's protocol.

To examine whether cetuximab, a monoclonal EGFR antibody therapeutic, affects the proliferation of SK-N-AS cells, proliferation following cetuximab treatment was measured over time. After the first measurement with WST-1 (0 hours), media in plates for other time points (24, 48, and 72 hour plates) was discarded and 100 µL of culture media containing cetuximab (0 nM, 400 nM, 1 µM, or 4 µM) was added to each well. Cetuximab was generously provided by the pharmacy at MD Anderson Cancer Center. Cells were subject to a WST-1 proliferation assay, and

proliferation was calculated at each time point as the percentage compared to absorbance measure at the 0 hour time point.

To determine whether UBE4B expression alters neuroblastoma tumor cell sensitivity to cetuximab (4 μM), stable SK-N-AS cell lines expressing GFP, UBE4B, or UBE4B(P1140A) were treated with cetuximab as described above. Proliferation measurements were performed 0, 24, and 72 hours following initial drug incubation. Proliferation was calculated as described in cetuximab and erlotinib experiments.

To examine whether erlotinib, a tyrosine kinase inhibitor, affects the proliferation of SK-N-AS cells, erlotinib (4 μM, 12 μM, and 40 μM) was incubated with cells in place of cetuximab. Proliferation was calculated as in cetuximab experiments.

Chapter 3. UBE4B affects the endocytic trafficking of EGFR

The multivesicular body (MVB) is a highly specialized membrane-bound compartment found within eukaryotic cells. It is the site of the final sorting event that determines the fate of transmembrane proteins within the endocytic pathway. Protein cargo on the surface of the limiting endosomal membrane may exit the endocytic pathway by traveling on vesicles that bud outward from the endosomal membrane towards the cytosol of the cell (Sirisaengtaksin et al., 2014). Alternatively, cargo may bud inward away from the cytosol of the cell and into the lumen of the MVB (Haglund and Dikic, 2012). A third alternative for protein cargo on the endosomal membrane is that it may remain on the membrane and become part of the lysosomal membrane upon MVB-lysosomal fusion. The sorting event that determines the fate of membrane proteins on the MVB membrane uses cargo ubiquitination as a signal that designates a protein for budding inward into the MVB (Haglund and Dikic, 2012).

Ubiquitination is a process that results in the attachment of single ubiquitin moieties to target substrate proteins by E3 ubiquitin ligases. The human genome encodes over 600 ubiquitin ligases, each of which is able to ubiquitinate a select, relatively small, group of protein substrates (Ardley and Robinson, 2005). The spatial and temporal regulation of ubiquitination depends on multiple factors, including the subcellular localization of the ubiquitin ligase (Ardley and Robinson, 2005). Interestingly, only a subset of ubiquitin ligases have been reported to associate with endosomes (Marchese et al., 2003; Nakamura, 2005; Fukuda, 2006; Hassink et al., 2012; Bocock et al., 2010). These endosome-associated ubiquitin ligases (e.g. AIP4, MARCH-II, MARCH-III, Triad1, and RNF13) have been reported to play a role in protein trafficking and lysosomal degradation (Marchese et al., 2003; Nakamura, 2005; Fukuda, 2006; Hassink et al., 2012; Bocock et al., 2010). This suggests that ubiquitin ligases may be recruited to endosomes to ensure the ubiquitination of cargo to allow their recognition by the endosomal sorting machinery.

Ubiquitination underlies the mechanism by which ESCRTs affect membrane protein fate at the MVB. Cargo proteins must be conjugated to ubiquitin in order to allow recognition by the sorting machinery that resides on endosomal membranes. Select substituents of these multiprotein complexes contain ubiquitin-interacting motifs that allow binding of ESCRT complexes to ubiquitinated endosomal cargo. ESCRTs are then able to facilitate sorting of cargo into membranes that bud off into the lumen of the multivesicular body.

A yeast two-hybrid screen identified an E3 ligase, UBE4B, as a binding partner of Hrs, a constituent of the ESCRT-0 complex (Sirisaengtaksin et al., 2014). Using full-length Hrs as the bait, multiple clones encoding the ubiquitin ligase, UBE4B, were isolated (Sirisaengtaksin et al., 2014). Hrs acts as a scaffolding protein that recruits components of the sorting machinery to endosomes, endosomal trafficking while bound to endosomal membranes, this suggests that cytosolic UBE4B may be recruited endosomal membranes. Because other endosomally-localized ubiquitin ligases have been shown to promote the degradation of other transmembrane proteins (Marchese et al., 2003; Nakamura, 2005; Fukuda, 2006; Hassink et al., 2012; Bocock et al., 2010), I hypothesized that UBE4B may act at the endosome to promote the ubiquitination and degradation of a membrane protein.

The *UBE4B* gene is encoded on the short arm of chromosome 1 (1p36). Deletion of the 1p36 region is common in many types of malignancies, especially in neuroblastoma, in which these deletions are found in 30% of tumors (Caron et al., 1996; Attiyeh et al., 2005; Maris et al., 2001). This genomic region has been hypothesized to harbor a tumor suppressor in neuroblastoma, as loss of 1p36 is predictive of poor outcomes in patients diagnosed with neuroblastoma (Caron et al., 1996). Further, UBE4B expression is a prognostic indicator of survival in neuroblastoma patients such that high UBE4B expression is associated with increased survival probability, while low UBE4B expression is associated with decreased survival (Zage et al., 2013). Loss of 1p36 is not the only alteration that may underlie neuroblastoma tumor

formation, EGFR is also overexpressed in neuroblastoma (Zheng et al., 2016). The endocytic trafficking of EGFR is critical to its downregulation. I have focused on the endocytic trafficking of EGFR, not only because it plays a role in neuroblastoma tumorigenesis, but also because it is a membrane protein whose transit through the cell has been well-studied (Longva et al., 2002; Sorkin et al., 1991; Schlessinger, 2000; Haglund and Dikic, 2012). As with other membrane proteins that traverse the endocytic pathway, EGFR must be ubiquitinated in order to be recognized by sorting complexes at endosomes (Huotari and Helenius, 2011). So, I hypothesized that UBE4B acts at endosomes to ubiquitinate EGFR, and ultimately, promote its sorting into multivesicular bodies and lysosomal degradation.

3.1 UBE4B binds to Hrs

In order to confirm the UBE4B-Hrs interaction predicted by the yeast two-hybrid assay (Sirisaengtaksin et al., 2014), I performed a binding assay using purified, recombinant protein. His-tagged Hrs was immobilized by binding onto Ni-NTA agarose. Increasing amounts of soluble

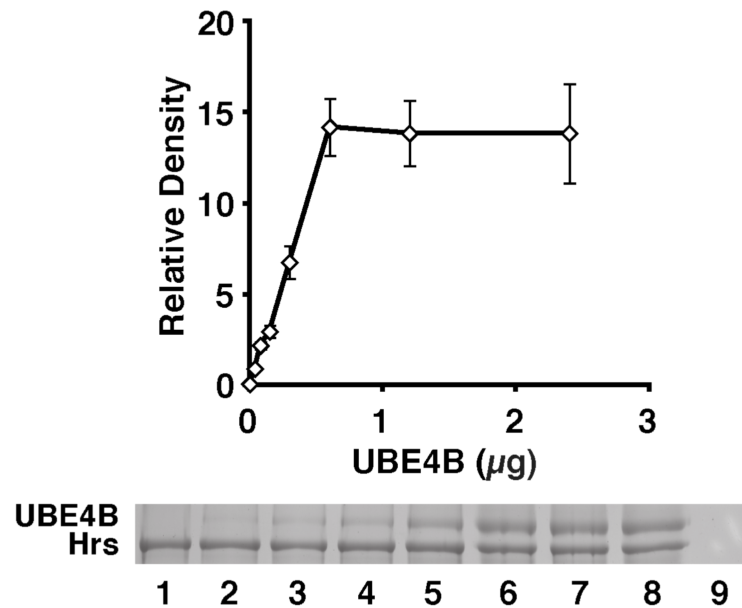

Figure 5. UBE4B binds to Hrs.

Increasing amounts of recombinant UBE4B were incubated with a constant amount of recombinant Hrs that was immobilized on Ni-NTA agarose. Following incubation, supernatants were removed and beads were washed to remove unbound protein. Addition of increasing amount of UBE4B resulted in increased binding of UBE4B to Hrs (lanes 1 to 6). Addition of an amount of UBE4B in excess of 0.6 μg resulted in no additional UBE4B binding (lanes 6 to 8).

UBE4B (0 to 2.4 μg) was added to a fixed amount of insoluble, immobilized Hrs (0.15 μg). Addition of increasing amounts of UBE4B resulted in increased binding of UBE4B to Hrs (Figure 5, lanes 1 to 6) that saturated at amounts of UBE4B in excess of 0.6 μg (Figure 5, lanes 6 to 8). The binding of UBE4B and Hrs suggest that formation of the UBE4B-Hrs complex is direct, and does not require the presence of accessory proteins to mediate the binding event.

3.2 UBE4B binds to ESCRT-0

Hrs binds to endosomal membranes and serves as a scaffold to recruit a variety of proteins (Bache et al., 2003; Pullan et al., 2006; Raiborg et al., 2001). Hrs recruits STAM, and together the two molecules form the ESCRT-0 complex (Komada, 2005; Pullan et al., 2006; Bache et al., 2003). Recognition of endosomal cargo, as well as subsequent recruitment of ESCRT-I is dependent upon the formation of ESCRT-0, so it is vital that Hrs remains bound to STAM to fulfill its role as a member of the endosomal machinery (Henne et al., 2011).

I next examined whether UBE4B binding to Hrs affects ESCRT-0 formation. Hrs (0.15 μg) immobilized on Ni-NTA agarose was incubated with a fixed, saturating amount of soluble STAM (0.7 μg) and increasing amounts of UBE4B (0 to 2.4 μg). Addition of increasing amounts of UBE4B resulted in increased binding of UBE4B to Hrs (Figure 6, lanes 1 to 6). Importantly, saturating amounts of UBE4B did not disrupt binding of STAM to Hrs (Figure 6, lanes 6 to 8). These data suggest that Hrs is capable of binding to both UBE4B and STAM simultaneously, and that UBE4B does not alter the formation of the vital ESCRT-0 complex.

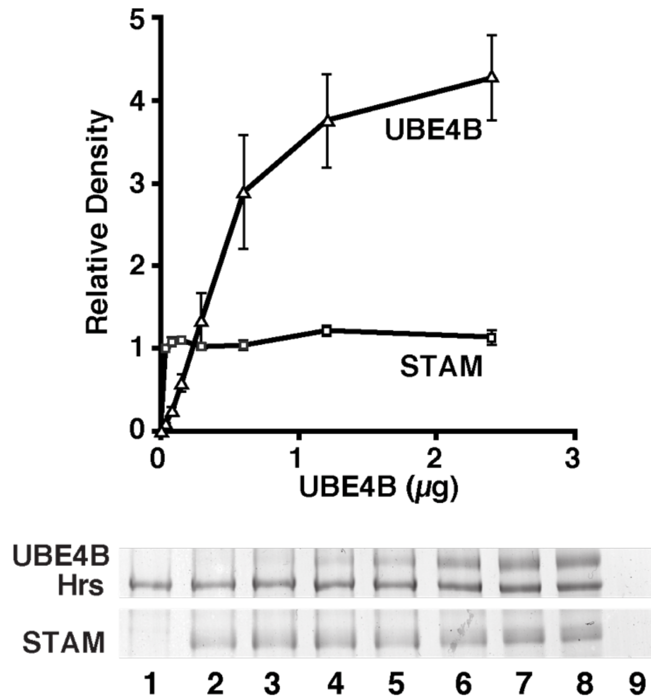

Figure 6. UBE4B binds to ESCRT-0.

Increasing amounts of soluble UBE4B was incubated with a fixed amount of soluble STAM and a fixed amount of insoluble Hrs bound to Ni-NTA agarose. Beads were washed to remove excess, unbound protein. Then, bound protein was eluted and resolved by SDS-PAGE. Concentrations of UBE4B exceeding 0.6 μg resulted in no additional binding to Hrs/STAM.

3.3 UBE4B binds to and ubiquitinates EGFR

UBE4B binds to endosomes, an interaction that is dependent upon binding of UBE4B to Hrs (Sirisaengtaksin et al., 2014). Other endosome-associated ubiquitin ligases have been shown to promote the ubiquitination, and subsequent degradation, of their target transmembrane protein substrates (Marchese et al., 2003; Nakamura, 2005; Fukuda, 2006; Hassink et al., 2012; Bocock

et al., 2010). To determine whether UBE4B interacts with EGFR, cell lysates were incubated with either rabbit IgG or an antibody directed towards EGFR. UBE4B co-precipitated with EGFR in

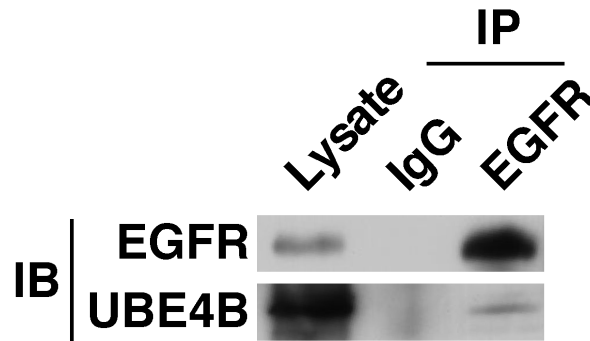

Figure 7. UBE4B binds to EGFR.

HeLa cell lysate (left lane) contains UBE4B and EGFR. Immunoprecipitation using EGFR antibodies, but not rabbit IgG (center lane), coprecipitated UBE4B (right lane).

samples incubated with α-EGFR (Figure 7, right lane), but not rabbit IgG (Figure 7, center lane).

To determine whether UBE4B can mediate EGFR ubiquitination, we reconstituted ubiquitination reactions by incubating cell lysate (as a source of EGFR substrate), recombinant UBE4B, E1, UbcH5c (E2), and an ATP regeneration system, which required for ubiquitination *in vitro*. EGFR was immunoprecipitated from reactions. Ubiquitin was co-immunoprecipitated from reactions that included UBE4B (Figure 8, first lane). Ubiquitin was not detected in samples that either did not include UBE4B (Figure 8, second lane), included deubiquitinating enzymes (Figure 8, third lane), or included a recombinant UBE4B protein containing a point mutation, UBE4B(P1140A), that is catalytically inactive and therefore incapable of ubiquitin ligase activity (Figure 8, fourth lane). These data suggest that UBE4B and EGFR interact in cells, and that

UBE4B is capable of mediating the ubiquitination of EGFR. In contrast, a point mutant of UBE4B, UBE4B(P1140A), is unable to ubiquitinate the EGFR.

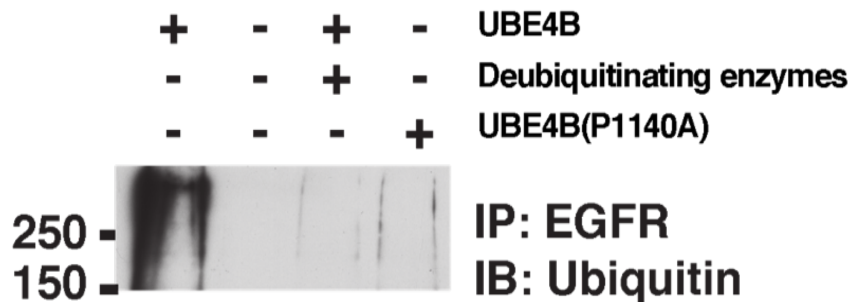

Figure 8. UBE4B can ubiquitinate EGFR.

HeLa cell lysate was incubated with recombinant UBE4B and other components required for EGFR ubiquitination. Reactions that included UBE4B contained ubiquitinated EGFR (first lane). Reactions that either did not include UBE4B (second lane), included deubiquitinating enzymes (third lane), or included a point mutant of UBE4B incapable of ubiquitination (fourth lane) did not contain ubiquitinated EGFR.

3.4 Cbl may mediate the EGFR-UBE4B interaction

Activated EGFRs signal as they traverse the endocytic pathway until the ligand-receptor complex is sorted into internal MVB vesicles and is degraded by proteolysis following MVB-lysosome fusion. Lack of a ubiquitin tag precludes the EGFR from being sorted into MVB internal vesicles, and therefore from lysosomal targeting and subsequent degradation (Goh and Sorkin,

2013; Smith et al., 2013; Eden et al., 2012). Thus, ubiquitination may play a key role in the degradation of EGFR.

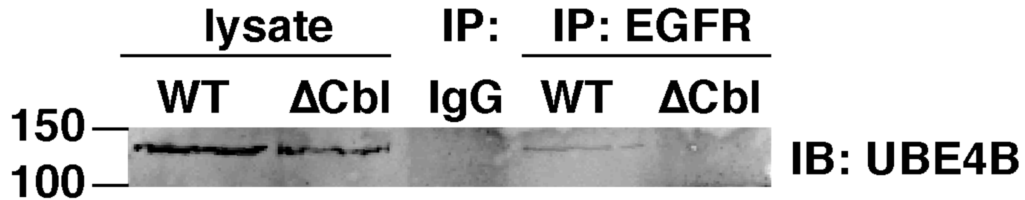

Figure 9. Cbl may mediate UBE4B-EGFR binding.

MCF-10A cell lysates were incubated with either rabbit IgG or an antibody specific for UBE4B. Both types of lysate contained UBE4B (first and second lanes). Antibodies were precipitated from samples with protein A agarose, beads were boiled in sample buffer to elute bound protein. Samples were subject to Western blotting and probed for the presence of UBE4B. Samples prepared from lysate incubated with rabbit IgG did not contain UBE4B (third lane). Samples prepared from wild-type MCF-10A cell lysate (containing Cbl) and incubated with EGFR antibody co-precipitated UBE4B (fourth lane). Samples prepared from Cbl-depleted MCF-10A cell lysate and incubated with EGFR antibody did not contain UBE4B (fifth lane).

Ubiquitination of EGFR by the ubiquitin ligase Cbl can occur at an early stage in the endocytic pathway, perhaps when the EGFR is present on the plasma membrane (Mohapatra et al., 2013; Smith et al., 2013). Interestingly, while Cbl expression is required for EGFR degradation, ubiquitination by Cbl alone is not sufficient to promote inward budding of the EGFR at the MVB membrane (Smith et al., 2013; Baldys and Raymond, 2009). Rabring7 and RNF126

are two ubiquitin ligases that are known to associate with EGFR downstream of Cbl, and their depletion results in impaired EGFR inclusion into MVB vesicles, such that EGFR is retained on late endosomal membranes (Smith et al., 2013). Association of EGFR with both Rabring7 and RNF126 required Cbl expression, suggesting that Cbl mediates the interaction between these ubiquitin ligases and EGFR (Smith et al., 2013).

I hypothesized that Cbl may mediate a similar relationship between UBE4B and EGFR. c-Cbl and Cbl-b are homologs that have a redundant function in cells (Visser Smit et al., 2009). Therefore, both c-Cbl and Cbl-b were depleted from MCF-10A cells (Mohapatra et al., 2013). Wild-type and Cbl-depleted cells were starved and stimulated with EGF to trigger EGFR entry to the endocytic pathway and UBE4B colocalization with endosomal compartments (Sirisaengtaksin et al., 2014). Cells were lysed, and immunoprecipitations were performed. Lysates containing 100 μg of total protein were incubated with either non-specific IgG antibodies or an EGFR antibody. Then, EGFR was immunoprecipitated using protein A agarose beads. Lysates prepared from both wild-type cells and Cbl-depleted cells contained UBE4B (Figure 9, first and second lanes). UBE4B did not bind to beads that were incubated with rabbit IgG (Figure 9, third lane). UBE4B was co-precipitated with EGFR from lysates isolated from cells that expressed endogenous Cbl (Figure 9, fourth lane), but not from lysates prepared from Cbl-depleted cells (Figure 9, fifth lane). These data suggest that Cbl expression is required for UBE4B-EGFR binding.

3.5 UBE4B is required for EGFR sorting

Following EGF binding, EGFR is internalized from the plasma membrane and traverses a well-defined pathway from the early endosome to the late endosome. On endosomal membranes, EGFRs may: 1) remain on the limiting membrane of the multivesicular body, from which receptors may be directed to other parts of the cell (e.g. the plasma membrane), or 2) bud inward

into endosomes, and action that ultimately targets EGFRs to lysosomes for degradation (Huotari and Helenius, 2011; Smith et al., 2013; Chin et al., 2001; Felder et al., 1990; Roepstorff et al., 2009) or 3) remain on the limiting membrane of the endosome for incorporation into the lysosomal membrane. Formation of intraluminal vesicles occur from endosomal membranes as ESCRTs and other proteins drive the formation of membranes buds that are released into the lumen (Bache et al., 2003; Katzmann et al., 2001a; Babst et al., 2002b; a). The choice between recycling and degrading is determined by the ubiquitination state of proteins (Longva et al., 2002; Alwan et al., 2003; Eden et al., 2012). ESCRTs recognize and capture ubiquitinated endosomal cargo, while leaving nonubiquitinated receptors for recycling back to the plasma membrane (Sorkin et al., 1991; Eden et al., 2012; Goh and Sorkin, 2013).

To determine whether UBE4B is required for the endosomal movement of the EGFR, we used a cell-free assay that allows reconstitution of multivesicular body (MVB) formation and measurement of the movement of membrane spanning receptors from the limiting membrane of endosomal membranes into luminal vesicles of MVBs (Sun et al., 2010; Sirisaengtaksin et al., 2014; Gireud et al., 2015). During these reactions, MVB formation is reconstituted by incubating endosomal membranes with cytosol and an ATP regeneration system. The movement of EGFR from the limiting membrane of endosomes into inwardly budding vesicles can be examined by assessing the protection of an intracellular epitope. Thus, samples are treated with trypsin protease, that digests the exposed intracellular domain of EGFR that remains outside of the endosomal membrane. Digestion of EGFR by trypsin renders the protein undetectable by Western blotting, when using antibodies specific for an intracellular epitope of EGFR. EGFR that has been recognized by the sorting machinery is sorted into vesicles that bud into the endosomal lumen. Internalized EGFR is protected from protease cleavage as trypsin cannot pass through endosomal membranes and is recognized by EGFR antibodies that recognize an intracellular epitope on the EGFR.

To reconstitute endosomal sorting and membrane budding, endosomal membranes were incubated with an ATP regenerating system and cytosol. Cytosol was isolated from transfected HeLa cells (Fig 6A). Cytosol isolated from untreated cells (Fig 6, first lane) and cells transfected with non-specific siRNA (Figure 10, fourth lane) were able to support the sorting of EGFR. Addition of recombinant UBE4B (Figure 10, second and fifth lanes) or recombinant UBE4B(P1140A) (Figure 10, third and sixth lanes) had no effect on EGFR sorting. Compared to controls, cytosol isolated from UBE4B-depleted cells was unable to support the endosomal sorting of EGFR (Figure 10, seventh lane). Addition of recombinant UBE4B rescued EGFR sorting (Figure 10, eighth lane). Addition of recombinant UBE4B(P1140A) also partially rescued EGFR sorting, but the amount of EGFR sorted was less than the rescue with wild-type UBE4B (Figure 10, ninth lane). This apparent rescue with the addition of UBE4B(P1140A) is unexpected, as the point mutation of the proline lies within the catalytic U-box domain, and should abolish the E3 ligase activity of UBE4B (Hatakeyama et al., 2001). However, the complete inhibition of EGFR sorting into endosomes was also unexpected, as UBE4B depletion has been shown to only partially inhibit EGFR sorting (Sirisaengtaksin et al., 2014). These data suggest that UBE4B-mediated ubiquitination of EGFR facilitates the sorting of EGFR into endosomes.

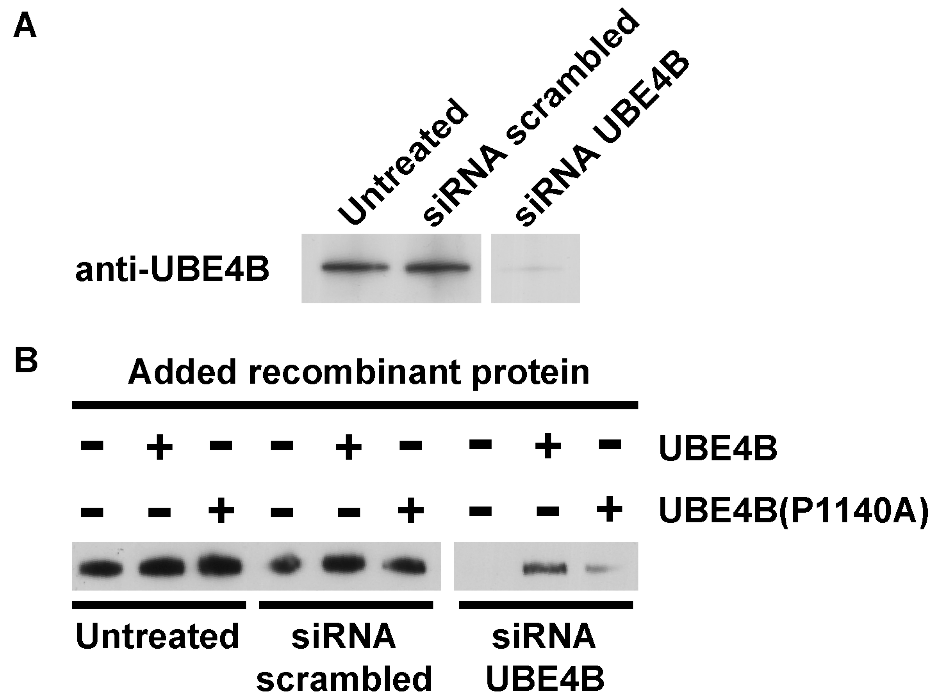

Figure 10. Depletion of endogenous UBE4B inhibits EGFR sorting; impaired EGFR sorting can be rescued with the addition of exogenous UBE4B.

A) HeLa cells were transfected using either a scrambled siRNA or with siRNA specific for UBE4B. Transfection with scrambled RNA did not result in altered UBE4B expression (middle lane) versus control (left lane). Transfection with siRNAs specific for UBE4B resulted in UBE4B depletion (right lane) compared to control. B) Cell-free sorting assays were performed to determine the requirement of UBE4B in EGFR sorting. Reactions including cytosols prepared from cells containing endogenous UBE4B (lane 1 and lane 4) contained similar amounts of EGFR sorted, and EGFR sorting in these reactions was not affected with the addition of exogenous UBE4B (lane 3 and lane 5) or UBE4B(P1140A) (lane 3 and lane 6). Compared to these control reactions, reactions that included cytosol prepared from UBE4B-depleted cells showed a dramatic decrease in EGFR sorting (lane 7). EGFR sorting in these reactions were rescued with the addition of exogenous UBE4B (lane 8), more than with the addition of UBE4B(P1140A) (lane 9).

3.5 Increased UBE4B expression increases EGFR degradation following ligand stimulation

Because I observed that UBE4B is required for EGFR sorting into endosomes, a step that is required for EGFR delivery to lysosomes for degradation, I next examined whether UBE4B may regulate EGFR degradation.

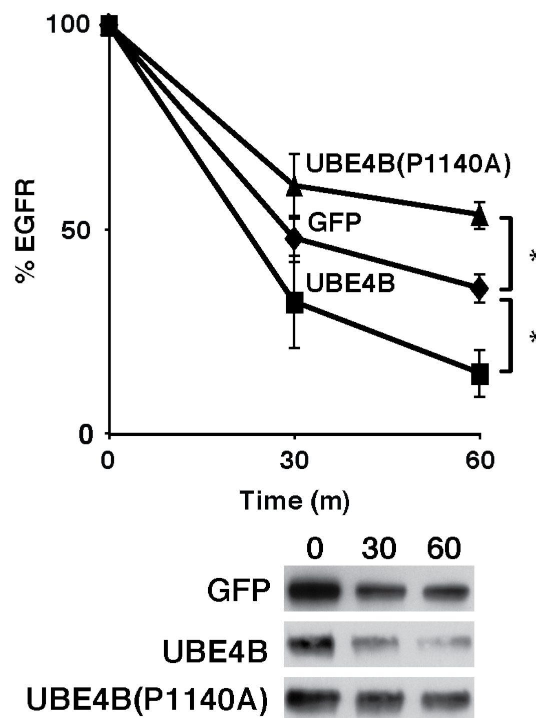

Figure 11. Overexpression of UBE4B affects EGFR degradation.

*SK-N-AS cell lines were starved and stimulated with EGF. Cells were collected after 0, 30, and 60 minutes, and cellular EGFR content was analyzed. Cells that overexpressed UBE4B degraded significantly more EGFR than control cells (GFP). Cells that overexpressed the ubiquitination-deficient mutant, UBE4B(P1140A), degraded significantly less EGFR than control cells. Data represent the mean ± standard error. * denotes $p < 0.05$, $n = 3$.*

The SK-N-AS human neuroblastoma cell line was chosen for these experiments because deletions of the 1p36 chromosomal region are common in neuroblastoma, suggesting that this region may harbor a tumor suppressor gene (Caron et al., 1996). The location of the *UBE4B* gene within 1p36, coupled with the low expression of UBE4B in high-stage neuroblastoma cases suggest that *UBE4B* is a promising candidate for a neuroblastoma tumor suppressor (Caron et al., 1996).

I determined whether increasing UBE4B expression affects EGFR degradation in neuroblastoma cells. SK-N-AS cells were infected with lentiviruses to create three stable cell lines: overexpressing UBE4B, overexpressing UBE4B(P1140A), and a control overexpressing green fluorescent protein (GFP) (Zage et al., 2013). The overexpression of the proteins were driven by the ubiquitin promoter resulting in a overexpression of UBE4B to approximately five times endogenous levels (Sirisaengtaksin et al., 2014).

To examine EGFR degradation, cell lines were starved for two hours and stimulated with EGF (50ng/ml for 0, 30, and 60 min), to synchronize entry of EGFR into the endocytic pathway. Cells were collected and EGFR content was analyzed by Western blotting. After 60 minutes of incubation with EGF, cells that overexpressed UBE4B had less than 20% of total EGFR remaining, compared to control cells expressing GFP, that had about 40% EGFR remaining (Figure 11). The amount of EGFR degraded at 60 minutes in cells that overexpress UBE4B was significantly different ($p < 0.05$). Additionally, expression of UBE4B(P1140A), resulted in significantly impaired degradation compared to control cells expressing GFP (Figure 11), with about 55% of total EGFR remaining in cells. The difference in EGFR degradation between control cells and cells that express UBE4B(P1140A) was also significant ($p < 0.05$). These data suggest a correlation between UBE4B expression and EGFR degradation. Moreover, the enzyme activity of UBE4B appeared to be required for efficient degradation.

3.6 The deubiquitinating enzyme, USP8, can remove ubiquitin from EGFR

EGFR must be ubiquitinated at endosomal membranes to be recognized by the UIM domains of ESCRT proteins to enable concentration of proteins into domains of the endosomal membrane and internalization into luminal vesicles of multivesicular bodies. However, prior to vesicle scission and release to complete the inward budding event, ubiquitinated cargo proteins, presumably including EGFRs, are hypothesized to be deubiquitinated (Katzmann et al., 2001a; Alwan et al., 2003).

USP8 is a deubiquitinating enzyme that is recruited to endosomes and is predicted to play a role in EGFR degradation (Alwan et al., 2003; Row et al., 2006; Mizuno et al., 2005; Alwan and van Leeuwen, 2007). However, the exact nature of USP8 regulation of EGFR degradation is controversial, as multiple studies have reported either inhibition or promotion of EGFR degradation following USP8 overexpression (Alwan et al., 2003; Berlin et al., 2010; Alwan and van Leeuwen, 2007; Row et al., 2006). Interestingly, USP8 binds to STAM, the ESCRT-0 component that is recruited to endosomes by Hrs (Alwan and van Leeuwen, 2007; Berlin et al., 2010; Mizuno et al., 2005). Based on this interaction I hypothesized that a protein complex containing UBE4B, Hrs, STAM, and USP8 may form to allow concerted ubiquitination/deubiquitination events to be confined to a microdomain of the endosomal membrane and provide efficient tagging and untagging of membrane protein cargo. Thus, EGFRs could be ubiquitinated by UBE4B, recognized by the ESCRT-0 complex to initiate protein sorting into endosomes, then deubiquitinated by USP8 before inclusion into intraluminal vesicles.

To determine whether USP8 is capable of deubiquitinating EGFR that was previously ubiquitinated by UBE4B, EGFR ubiquitination and deubiquitination assays were performed in succession. HeLa cell lysate (as a source of EGFR target substrate) was incubated with recombinant UBE4B, as well as E1, UbcH5c (E2), and an ATP regeneration system, components

that required for ubiquitination *in vitro.* EGFR was then immunoprecipitated from the initial reactions and ubiquitination assay components were removed by centrifugation. Reactions were then incubated with either recombinant non-specific deubiquitinating enzymes (UCH-L3 and isopeptidase-T) or USP8. Ubiquitinated EGFR could only be detected in reactions that included UBE4B during incubation (Figure 12, lane 1). Decreased levels of ubiquitinated EGFR were detected in reactions in which UBE4B, UBE4B(P1140A), or non-specific deubiquitinating enzymes were added (Figure 12, lanes 2, 3, and 4). Incubation with USP8 alone resulted in no detectable ubiquitinated EGFR (Figure 12, lane 5). These data suggest that USP8 is capable of deubiquitinating EGFR following UBE4B-mediated ubiquitination.

Figure 12. USP8 can deubiquitinate EGFR following UBE4B-mediated ubiquitination.

HeLa cell lysate was incubated with recombinant UBE4B and other components required for EGFR ubiquitination. Reactions that included UBE4B contained ubiquitinated EGFR (first lane). Reactions that either did not include UBE4B (second lane) or included a point mutant of UBE4B incapable of ubiquitination (third lane) did not contain ubiquitinated EGFR. Samples that included UBE4B in ubiquitination reactions were subsequently subject to deubiquitination. The control deubiquitination reaction that included the non-specific deubiquitinating enzymes, UCH-L3 and isopeptidase T, showed decreased co-precipitated ubiquitination. Additionally, the deubiquitination reaction that included the STAM-associated deubiquitinating enzyme, USP8, contained no detectable ubiquitin.

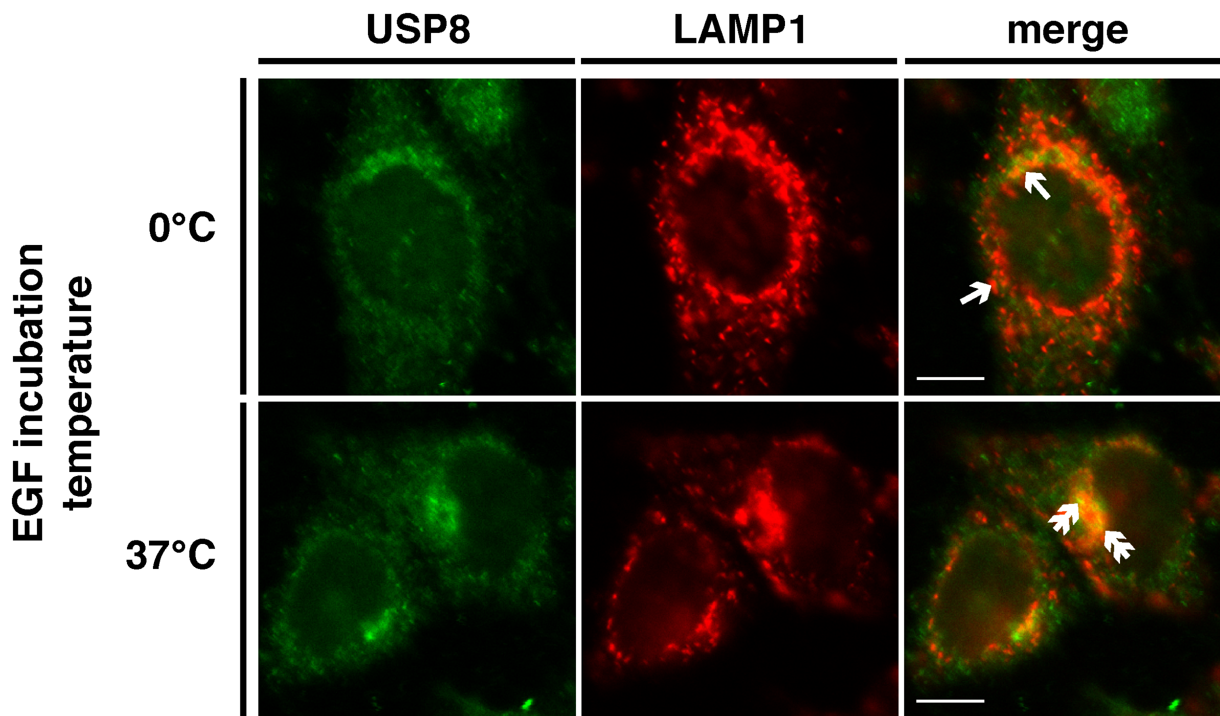

Figure 13. USP8 colocalizes with the late endosome-associated protein, LAMP1, following cellular exposure to EGF.

HeLa cells were plated on coverslips, starved, and incubated with EGF (100ng/ml). Cells were fixed and immunolabeled with antibodies directed against LAMP1 (red, Alexa Fluor 568) and USP8 (green, Alexa Fluor 488). Distinct punctate localization of LAMP1 and USP8 was observed in cells incubated at 0°C (single arrow), while colocalization of LAMP1 and USP8 was observed in cells that were incubated at 37°C (double arrow) Scale bars = 10 µm.

To determine whether USP8 can be recruited to endosomal membranes with a time course similar to that of UBE4B, HeLa cells were serum-starved, and stimulated with EGF for 15 minutes at either 0°C or 37°C. Cells were then fixed and immunolabeling was performed with antibody directed against USP8 and LAMP1. Localization of USP8 and LAMP1 in cells incubated with EGF at 0°C was distinct (Figure 13, top). However, cells that were incubated with EGF (15 min

at 37°C) had increased incidence of overlapping localization of USP8 and LAMP1, suggesting that EGFR movement through the endocytic pathway is correlated in time with endosomal recruitment of USP8. UBE4B is recruited to endosomes in a similar manner under the same conditions (Sirisaengtaksin et al., 2014).

3.5 Increased UBE4B expression decreases neuroblastoma tumor cell sensitivity to EGFR inhibitors

Proliferation of neuroblastoma cellular (SK-N-AS) is significantly inhibited in cells that overexpress UBE4B, compared to control cells and cells that expressed UBE4B(P1140A) (Zage et al., 2013). These proliferative responses were observed regardless of whether the cells were incubated in media that was supplemented with FBS or with EGF (Zage et al., 2013). The correlation of UBE4B expression with the ability of cells to degrade EGFR suggested that cellular expression of UBE4B and resultant changes in EGFR degradation may affect the response of neuroblastoma tumor cells to treatment with EGFR inhibitors. To explore this possibility, the SK-N-AS lentivirus-infected cell lines were treated with cetuximab, a monoclonal antibody that blocks EGFR function by binding to the extracellular domain of the EGFR and competing with EGFR ligands (e.g., EGF) for EGFR binding (Mehra et al., 2008; Lenz, 2007). In addition to blocking ligand binding, cetuximab also triggers receptor internalization and entry into the endocytic pathway (Lenz, 2007).

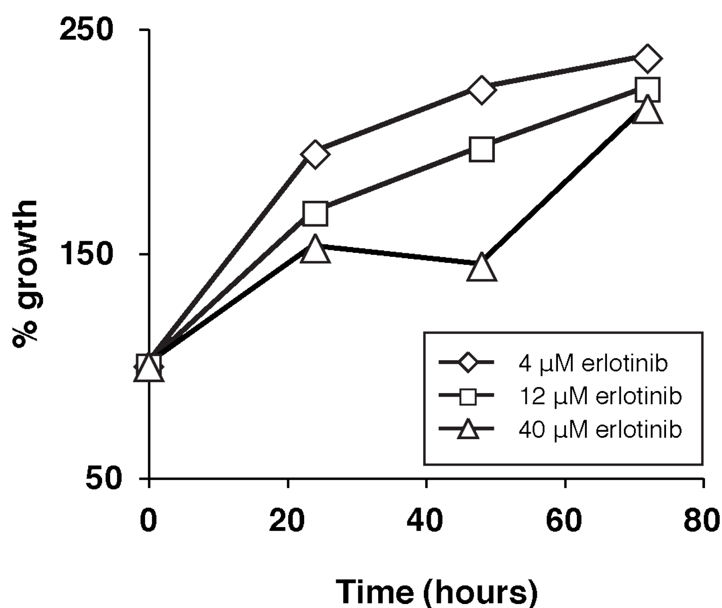

Figure 14. Erlotinib treatment does not inhibit neuroblastoma tumor cell proliferation.

Parental SK-N-AS cells were treated with increasing concentrations of erlotinib: 4 µM, 12 µM, and 40 µM. Cell proliferation was measured at 0, 24, 48, and 72 hours using the WST-1 spectrophotometric assay. Erlotinib treatment did not inhibit the proliferation of SK-N-AS cells at any concentration tested.

The proliferation of SK-N-AS cells treated with various concentrations (4 µM, 12 µM, 40 µM) of a tyrosine kinase inhibitor, erlotinib, was not different than the proliferation of untreated cells (Figure 14).

SK-N-AS cells were treated with three concentrations of cetuximab (400 nM, 1 µM, and 4 µM) and proliferation was measured over time (0, 24, 48, and 72 hours) using a WST-1-based spectrophotometric assay. The proliferation of cells treated with the highest cetuximab concentration (4 µM) was significantly inhibited compared for untreated cells (Figure 15). The

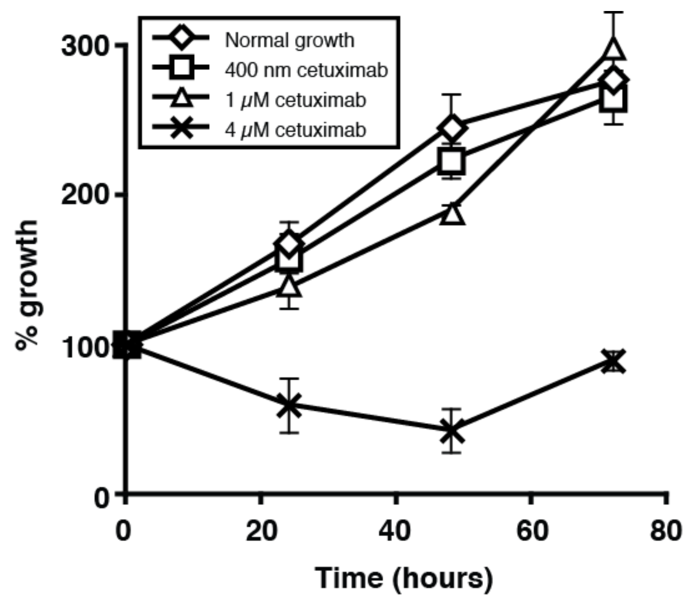

Figure 15. Treatment with 4 μM cetuximab inhibits neuroblastoma tumor cell growth.

Parental SK-N-AS cells were treated with increasing concentrations of cetuximab. Cell proliferation was measured at 0, 24, 48, and 72 hours after initial drug treatment. Proliferation was assessed using the WST-1 spectrophotometric assay. Cetuximab treatment using less than 4 μM concentrations did not inhibit proliferation of SK-N-AS cells.

proliferation of cells treated with lower concentrations (400 nM and 1 μM) of cetuximab was not significantly different than untreated cells (Figure 15).

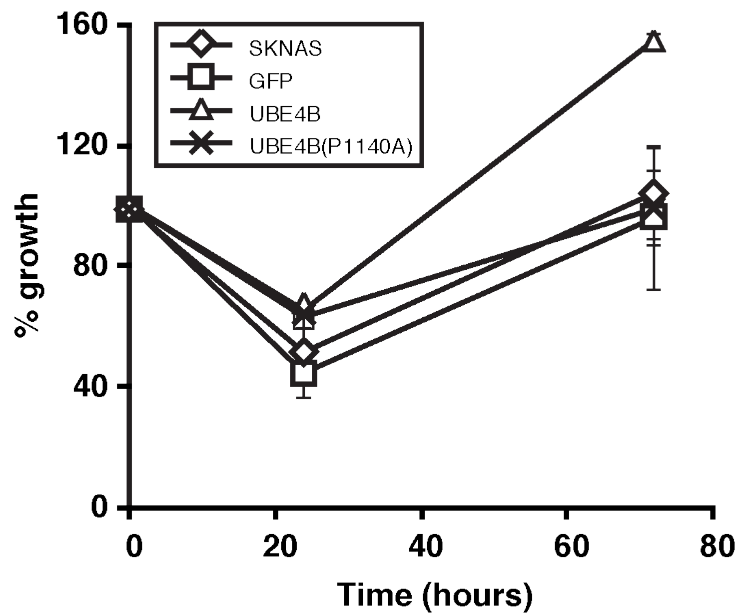

Figure 16. UBE4B expression decreases neuroblastoma cell sensitivity to cetuximab treatment.

Parental SK-N-AS cells and cell lines derived from the SK-N-AS cell line expressing either GFP, UBE4B, or UBE4B(P1140A) were grown in the presence of 4 μM cetuximab with cell viability assessed at 0, 24, and 72 hours as detailed above.

To determine whether UBE4B expression affects SK-N-AS sensitivity to cetuximab, parental SK-N-AS cells and the SK-N-AS stable cell lines expressing UBE4B, UBE4B(P1140A), or GFP were treated with 4 μM cetuximab. Cell proliferation was then evaluated at 0, 24, and 72 hours. Proliferation of parental SK-N-AS cells, as well as cells expressing GFP and UBE4B(P1140A), was inhibited (Figure 16). In contrast, expression of UBE4B resulted in an increase in cell proliferation (Figure 16). These data suggest that increases in UBE4B expression result in decreased sensitivity to cetuximab.

Chapter 4. Discussion

Introduction

The removal of membrane proteins that reside at the cell surface occurs by endocytosis, a vital mechanism that regulates the protein residence time on the surface. One particular class of proteins, receptor tyrosine kinases, is internalized upon binding to extracellular ligands. Ligand binding initiates signal transduction pathways that propagate throughout the cell. Following receptor internalization from the plasma membrane, receptors travel in transport vesicles that allow movement of membrane and proteins that travel as cargo through a well-defined pathway called endocytosis (Goh and Sorkin, 2013; Goh et al., 2010; Doherty and McMahon, 2009). This physical removal of protein from the surface allows constant remodeling of the transmembrane surface protein population. In this manner, endocytosis allows fine-tuning of signaling in response to extracellular cues by limiting the availability of signaling molecules at the surface and the time the receptor spends signaling as it moves through the endocytic pathway (Goh et al., 2010). These signaling events mediate basic cell processes like differentiation, migration, plasticity, and proliferation (Goh et al., 2010; Schlessinger, 2000).

Following internalization, protein cargo travels on transport vesicles that fuse with the early endosomal organelle (Beguinot et al., 1984; Huang et al., 2007). They remain on endosomal membranes until they are either 1) sorted into vesicles that bud inward into a late endosome/MVB for eventual delivery to lysosomes, 2) included in small vesicles that bud outward into the cytosol for eventual incorporation into other cellular structures (e.g. the plasma membrane or Golgi), or 3) remain on membranes of the MVB and become incorporated into the membranes of lysosomes upon MVB-lysosomal fusion (Nothwehr et al., 2000; Felder et al., 1990; Sigismund et al., 2012; Dunn, 1986). The sorting event requires the recruitment of the multisubunit ESCRT complexes that act sequentially to concentrate cargo into membrane buds that are released into the lumen of

the MVB (Teo et al., 2004; Katzmann et al., 2001a, 2003; Bache et al., 2003; Babst et al., 2002a). ESCRTs engage protein cargo that have been covalently conjugated to ubiquitin, a small molecule that designates endosomally-associated membrane proteins for lysosomal degradation (Katzmann et al., 2001a; Bilodeau et al., 2002). Nonubiquitinated cargo cannot be engaged by ESCRTs, which precludes them from a degradative fate. Therefore, the action of the cellular machineries that regulate ubiquitination and endosomal sorting of membrane protein cargo are required for cargo degradation; however, the mechanisms by which the machineries coordinate their functions are unknown. UBE4B is an E3 ubiquitin ligase that can catalyze the covalent attachment of ubiquitin onto its target substrates. Prior to my work, UBE4B was predicted to bind to the ESCRT-0 component, Hrs, an interaction that suggests a role for UBE4B in the endocytic pathway (Sirisaengtaksin et al., 2014). The goal of this work was to examine whether UBE4B couples the action of the ubiquitination and ESCRT machinery to promote the sorting and degradation of the membrane protein EGFR.

UBE4B binds to ESCRT-0

Hrs is the first ESCRT sorting machinery component to bind to endosomal membranes (Raiborg et al., 2001; Bache et al., 2003). It acts as a scaffolding protein that recruits ESCRT and non-ESCRT proteins that promote endocytic cargo sorting (Chin et al., 2001; Bean, 2000; Komada, 2005; Pullan et al., 2006; Roxrud et al., 2008; Yan et al., 2005). In preliminary experiments, a yeast two-hybrid assay predicted an interaction between Hrs and UBE4B (Sirisaengtaksin et al., 2014). This interaction was further confirmed when we observed that Hrs bound to UBE4B following incubation of recombinant UBE4B with cell lysate as a source of Hrs (Sirisaengtaksin et al., 2014). While these data suggest that Hrs and UBE4B may bind *in vitro*, it was unknown whether the interaction required the presence or action of an accessory protein to

mediated Hrs-UBE4B binding. I performed a binding assay between recombinant Hrs and recombinant UBE4B and found that the interaction between the two proteins can occur in the absence of other protein, and that binding is saturable (Figure 5).

Hrs recruits proteins (e.g. Eps15, SNX1) to endosomes, but its association with the ESCRT-0 component, STAM, is required to promote endocytic sorting of membrane protein cargo (Henne et al., 2011; Komada, 2005; Pullan et al., 2006; Bean, 2000; Chin et al., 2001). Therefore, it was important to establish whether UBE4B binding to Hrs disrupted the formation of ESCRT-0. I found that *in vitro* incubation of UBE4B, Hrs, and STAM resulted in the saturable binding of UBE4B to ESCRT-0 (Figure 6). Importantly, incubation with excess UBE4B did not affect ESCRT-0 complex formation or stability (Sirisaengtaksin et al., 2014). The binding of UBE4B to the complete ESCRT-0 complex suggests that UBE4B may localize to endosomes and play some role in receptor trafficking. Endosomal binding was confirmed by another member of the Bean Lab, who incubated purified endosomes with increasing concentrations of recombinant UBE4B (Sirisaengtaksin et al., 2014). He observed saturable binding of recombinant UBE4B to endosomal membranes. He also observed that this association was dependent on UBE4B binding to Hrs, suggesting that Hrs acts as an endosomal receptor for UBE4B binding. Taken together, these data show that UBE4B is recruited by Hrs to endosomes and binds to ESCRT-0, a complex that recognizes and concentrates ubiquitinated cargo. This suggests a potential role for UBE4B, in which ubiquitination of endosomal cargo by UBE4B may enable cargo recognition by the endosomal sorting machinery.

UBE4B binding to and ubiquitination of EGFR may be regulated by Cbl

While Hrs is required for cargo recognition at MVBs, its recruitment of other proteins to endosomes, such as SNX1 and Eps15, has also been shown to promote cargo protein sorting,

especially EGFR (Chin et al., 2001; Roxrud et al., 2008; Bean, 2000). Immunofluorescence experiments performed in collaboration with other members of the Bean Lab suggest the involvement of UBE4B in EGFR endocytosis. She observed the localization of Hrs and UBE4B following EGF-mediated EGFR endocytosis. She starved cells to synchronize EGFR internalization, incubated cells with ligand, and labeled cellular protein with antibodies. I quantified the colocalization of UBE4B with Hrs and EEA1, a protein marker for the early endosomal compartment. Under conditions that prevented EGFR internalization, I found that UBE4B was diffusely localized in the cytosol and did not appear to colocalize with Hrs or EEA1 (Sirisaengtaksin et al., 2014). Under conditions that permitted EGFR internalization, I found that UBE4B colocalization with Hrs and EEA1 was significantly increased (Sirisaengtaksin et al., 2014). These data strongly suggest that UBE4B is recruited to endosomes to interact with EGFR as it moves through the endocytic pathway.

To confirm the UBE4B-EGFR interaction, I immunoprecipitated EGFR and observed that UBE4B was coprecipitated from cell lysate, suggesting a physiological interaction between UBE4B and EGFR (Figure 7). I also identified EGFR as a substrate for UBE4B-mediated ubiquitination (Figure 8). Ubiquitination of EGFR is required for its recognition by the endosomal sorting machinery, and eventual inclusion into vesicles that are targeted for lysosomal degradation (Goh et al., 2010; Eden et al., 2012; Goh and Sorkin, 2013). Therefore, the ubiquitination state of EGFR at endosomal membranes plays a role in determining whether EGFR is degraded or whether it remains in the cells for continued participation in signaling.

Many ubiquitin ligases are reported to associate with endosomes (including Cbl, AIP4, MARCH-II, MARCH-III, Triad1, and RNF13) and are predicted to play some role in protein trafficking (Visser Smit et al., 2009; Marchese et al., 2003; Nakamura, 2005; Fukuda, 2006; Hassink et al., 2012; Bocock et al., 2010). The role of Cbl-mediated ubiquitination in EGFR degradation has been well-established (Visser Smit et al., 2009; Mohapatra et al., 2013; Ardley

and Robinson, 2005; Grandal et al., 2007; Smith et al., 2013). Cbl is recruited to plasma membranes upon EGFR activation, and binds directly to EGFR to catalyze ubiquitination early in the endocytic pathway (Grandal et al., 2007). EGFR ubiquitination at the plasma membrane is not required for EGFR entry into the cell (Huang et al., 2007). However, EGFR may not gain entry into MVBs by virtue of Cbl-mediated ubiquitination alone (Smith et al., 2013). Instead, ubiquitination by RING E3 ligases RNF126 and Rabring7 may promote efficient EGFR degradation, although the presence of Cbl was still required (Smith et al., 2013). While it is likely that RNF126 and Rabring7 play some role in EGFR degradation, their role in EGFR sorting remains unclear. The mechanism by which these RING E3 ligases regulate EGFR degradation is unclear because their ubiquitination activity was not demonstrated to directly enable EGFR sorting. However, the dependence of RNF126 and Rabring7 on Cbl to mediate the degradation of EGFR provides insight into similar interactions that may occur, especially between Cbl and UBE4B.

I observed that the co-immunoprecipitation of UBE4B and EGFR from cell lysates were dependent upon Cbl expression (Figure 9), suggesting that Cbl may directly or indirectly mediate the interaction between UBE4B and EGFR. While no E4 activity by RNF126 and Rabring7 have been detected, it is well known that U-box domain proteins like UBE4B are capable of E4 ubiquitin chain assembly enzymes (Hatakeyama et al., 2001; Patterson, 2002; Kaneko et al., 2003). E4 activity requires the coordinated action of E1, E2, and E3 enzymes to enable the efficient, rapid elongation of ubiquitin chains that are attached (Ardley and Robinson, 2005; Koegl et al., 1999). It is possible that EGFR ubiquitination by Cbl primes EGFR for UBE4B

binding and ubiquitin chain elongation. Alternatively, Cbl may be required for the physical mediation of the EGFR-UBE4B interaction.

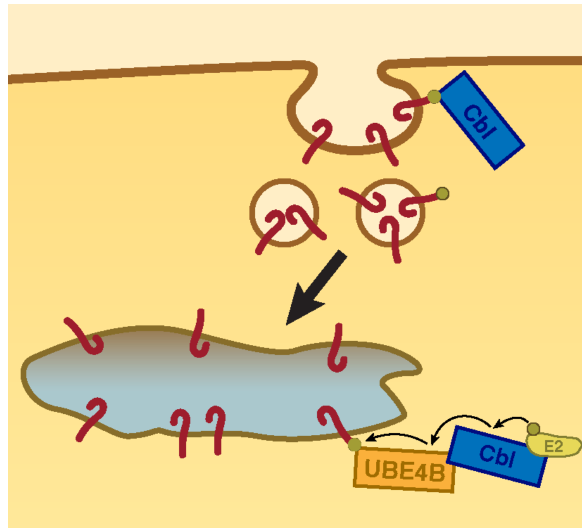

Figure 17. Cbl may mediate the UBE4B-EGFR interaction.

Cbl ubiquitination may prime EGFR for suitable binding to UBE4B. Alternatively, Cbl may be required to mediate E4 chain elongation activity with UBE4B.

MVB sorting and lysosomal degradation of EGFR requires ubiquitination by UBE4B

For lysosomal degradation EGFR, the receptor must be sorted into membrane buds that are released into the lumen of MVBs. Protein sorting is regulated by ESCRTs, which recognize ubiquitinated cargo. I observed that UBE4B was able to ubiquitinate EGFR, so I examined whether UBE4B ubiquitination would enable EGFR sorting (entry into luminal MVB vesicles). In a cell-free sorting assay, we are able to measure the movement of EGFRs from the limiting membrane of endosomes into luminal vesicles. Depletion of proteins that are required for cargo sorting, such as the ESCRT component Hrs, results in impaired sorting of EGFR (Sun et al., 2010;

Gireud et al., 2015). Likewise, depletion of UBE4B impaired the sorting of EGFR (Figure 10, lane 7), suggesting that UBE4B is required for efficient sorting of EGFR. While addition of recombinant UBE4B partially rescued the sorting of EGFR (Figure 10, lane 8), addition of recombinant UBE4B(P1140A) did not rescue EGFR sorting (Figure 10, lane 9). These data suggest that the enzymatic activity of UBE4B is required for the efficient endosomal sorting of EGFR.

Altered expression of UBE4B in neuroblastoma cells affect EGFR degradation. Significant differences in EGFR degradation were observed 60 minutes after EGF stimulation (Figure 11). Neuroblastoma cells that overexpressed UBE4B degraded significantly more EGFR than control cells that expressed GFP (Figure 11, UBE4B and GFP). Neuroblastoma cells that expressed the point mutant UBE4B(P1140A) degraded significantly less EGFR than control cells (Figure 11, UBE4B(P1140A) and GFP. These data suggest that UBE4B expression is correlated with EGFR degradation such that cells with higher levels of UBE4B degrade more EGFR than cells with lower levels of UBE4B.

The endosomal trafficking of EGFR allows cells to regulate the activity and duration of signaling in cells; aberrant regulation of receptor activity is often associated with diseases such as cancer (Katzmann et al., 2002; Baldys and Raymond, 2009; Roepstorff et al., 2009; Shtiegman et al., 2007). The sorting and degradation data I show are complementary, and suggest a correlation between UBE4B expression/ubiquitination activity and EGFR sorting and degradation. Additionally, we observed a profound increase in steady-state levels of EGFR expression when UBE4B is depleted (Sirisaengtaksin et al., 2014), suggesting that decreased UBE4B expression may underlie disease. The *UBE4B* gene resides on chromosome 1p36, a genomic region that is commonly deleted in cancers such as neuroblastoma, glioblastoma, and hepatocellular carcinoma (Maris et al., 2001; Caron et al., 1996; Attiyeh et al., 2005; Krona et al., 2003; Ichimura et al., 2008; Zhang et al., 2010b). Additionally, EGFR overexpression or deregulation of the EGFR

signaling pathway is shown to underlie the development of these cancers (Zheng et al., 2016; Keller et al., 2016; Thorne et al., 2016; Cloughesy et al., 2014; Huang et al., 2014; Lanaya et al., 2014). I observed that the addition of recombinant UBE4B partially rescued EGFR sorting and overexpression of UBE4B increased EGFR degradation. These data suggest that UBE4B may be a novel target for drug development in diseases where loss of UBE4B may underlie tumor formation. A treatment that can inhibit the proteasomal degradation of UBE4B or one that may deliver recombinant UBE4B to malignant cells to act in the endocytic pathway would provide additional UBE4B to ubiquitinate EGFR in the endocytic pathway. For example, UBE4B is a ubiquitin ligase that is capable of self-ubiquitination. The ubiquitination of cytosolic proteins designates them for proteasomal degradation (Komander and Rape, 2012; Ardley and Robinson, 2005). I have shown that recombinant UBE4B may be used to rescue the depletion of endogenous UBE4B in my EGFR sorting experiments (Figure 10). Therefore, targeted delivery of UBE4B (via a nanoparticle or liposomal vehicle) to cells with either 1) decreased UBE4B expression due to abnormal genetic alterations (e.g. 1p36 deletions) or 2) cells that overexpress EGFR, may provide a therapeutic mechanism by which EGFR degradation is increased upon UBE4B delivery.

UBE4B and USP8 coordinate ubiquitination/deubiquitination through ESCRT-0 binding

Ubiquitination of EGFRs at endosomal membranes is critical for their recognition by the ESCRT machinery (Smith et al., 2013; Katzmann et al., 2001a). However, deubiquitination of EGFRs is also important for receptor inclusion into MVB vesicles (Alwan and van Leeuwen, 2007; Berlin et al., 2010; Row et al., 2006; Mizuno et al., 2005). Katzmann, et al. proposed that deubiquitination of receptors occurs following ESCRT recognition in order to maintain the cellular pool of free ubiquitin (Katzmann et al., 2001a). Interestingly, the ESCRT-0 component, STAM, recruits a deubiquitinating enzyme, USP8, to endosomes (Alwan and van Leeuwen, 2007;

Berlin et al., 2010; Row et al., 2006; Mizuno et al., 2005). Conflicting evidence regarding the precise role of USP8 in EGFR degradation has been reported. While some have reported that USP8 activity was required for EGFR degradation (Alwan and van Leeuwen, 2007; Row et al., 2006), others have found that USP8 activity inhibited EGFR degradation (Mizuno et al., 2005; Berlin et al., 2010). The difficulty in determining the exact role of USP8 in EGFR degradation highlights the importance of the temporal regulation of ubiquitination. I have shown that USP8 is recruited to endosomal membranes under a similar time course to UBE4B [Figure 13 and (Sirisaengtaksin et al., 2014)]. Additionally, I found that USP8 was able to deubiquitinated EGFRs that were previously ubiquitinated by UBE4B. These data suggest that USP8 and UBE4B may form a complex with ESCRT-0 to coordinate efficient ubiquitination and deubiquitination. However, numerous attempts to isolate a four-part complex of UBE4B-Hrs-STAM-USP8 were unsuccessful.

Based on these observations, I propose a model in which UBE4B and USP8 are recruited to endosomes to regulate the ubiquitination of cargo at endosomal membranes (Figure 18). Hrs binds to endosomal membranes, where it recruits STAM to form the ESCRT-0 complex, to which UBE4B binds (Figure 18A). The endosomal localization of UBE4B brings it in close proximity to EGFR, which must be ubiquitinated in order to be recognized by ESCRT-0. UBE4B ubiquitinates ESCRT-0 (Figure 18B), allowing cargo engagement by ESCRT-0 (Figure 18C). UBE4B-Hrs binding is disrupted by USP8, which binds to STAM and forces UBE4B dissociation from endosomes (Figure 18C and D). Endosomal sorting continues as ESCRT-I and -II encage ubiquitinated cargo and mediate inward membrane budding (Figure 18D and E). USP8 deubiquitinates EGFR just before ESCRT-III mediates vesicle scission and ESCRT disassembly occurs (Figure 18E and F).

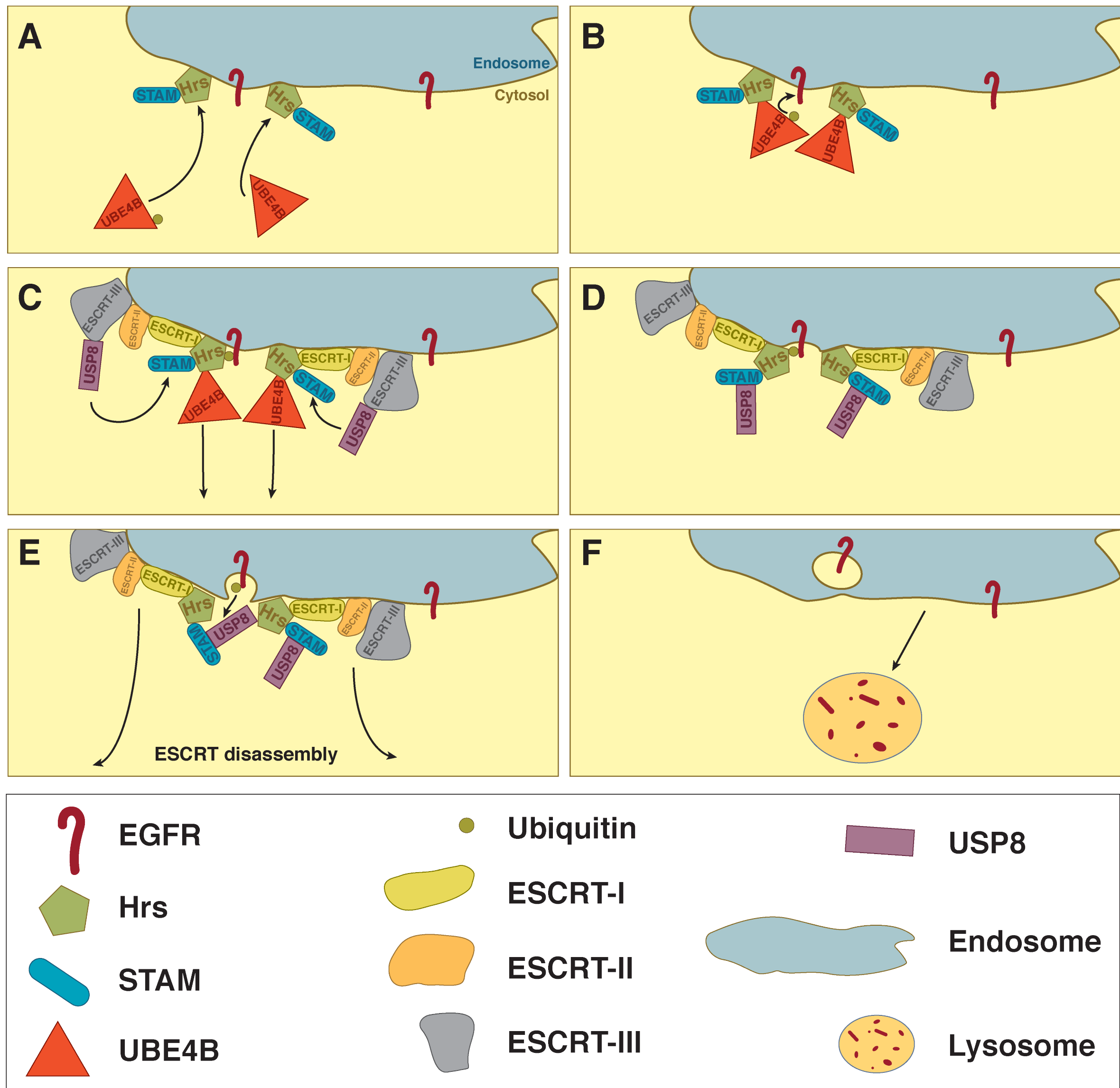

Figure 18. Proposed model of UBE4B and USP8 interaction with ESCRT-0 and role in EGFR sorting at the endosome.

I propose a model in which UBE4B is recruited to endosomes to aid in EGFR sorting into internal MVB vesicles. A) UBE4B is recruited to endosomes by binding to the ESCRT-0 complex via Hrs. B) UBE4B binds and ubiquitinates EGFR, allowing recognition of the receptor by ESCRT-0 ubiquitin-binding domains. C) USP8 displaces UBE4B from its endosomal localization by binding to STAM. D) ESCRT-I and ESCRT-II then bind to facilitate membrane budding inward towards the MVB lumen. E) USP8 then mediates the deubiquitination of EGFR while the ESCRT-III facilitates the dissociation of the ESCRTs, then F) scission of endosomal membrane to release the newly formed vesicle into the endosomal lumen.

UBE4B expression may predict neuroblastoma response to EGFR inhibitors

Neuroblastoma is the most common extracranial solid tumor in children. The overall survival rates for children that are diagnosed with advanced stage cases are ~30% with the current treatment options that are available, highlighting the need for improved treatment options. A number of studies have looked to adapt therapies that have been approved to treat adult cancers for use in pediatric cancers (Rössler et al., 2009). Because EGFR is overexpressed in neuroblastoma, I chose to evaluate the efficacy of two EGFR inhibitors that are approved therapies in other cancers (Zheng et al., 2016). While the tyrosine kinase inhibitor, erlotinib, did not inhibit the proliferation of neuroblastoma cells (Figure 14), in contrast, treatment with a monoclonal antibody, cetuximab, did inhibit neuroblastoma cell proliferation at a 4 μM concentration (Figure 15).

We previously showed that UBE4B expression affected neuroblastoma cell growth, such that increased UBE4B expression was correlated with decreased cell proliferation (Zage et al., 2013). Additionally, we observed that steady-state expression of EGFR was negatively regulated by UBE4B (Sirisaengtaksin et al., 2014). So, it was not surprising that increased expression of UBE4B resulted in decreased sensitivity to 4 μM treatments of cetuximab (Figure 16). Compared to control cell lines that expressed endogenous amounts of UBE4B and cells that expressed UBE4B(P1140A), proliferation was not inhibited in cells that overexpressed UBE4B (Figure 16). These data link UBE4B and its role in the EGFR trafficking pathway in the sensitivity of neuroblastoma tumor cells to EGFR inhibitors. However, further investigation into the mechanisms by which altered UBE4B expression regulates cell sensitivity to cetuximab, and another monoclonal antibody that targets EGFR, panitumumab, may lead to expanded treatment options for high-risk neuroblastoma patients (Martinelli et al., 2009).

Future directions

1. To characterize the ubiquitination activity of UBE4B at endosomes.

Ubiquitin ligases mediate the attachment of ubiquitin onto target substrates. Attachment of additional ubiquitin moieties onto one of seven lysine residues may occur to form a polyubiquitin chain (Li and Ye, 2008; Ardley and Robinson, 2005; Hatakeyama et al., 2001). It is thought that the linkages that compose polyubiquitin chains encode structural and functional information that specify the fate of the tagged protein (Li and Ye, 2008; Erpapazoglou et al., 2012). It would be beneficial to fully characterize the interaction between UBE4B and EGFR, as a model for other ubiquitin ligase/protein substrate pairings. First, it should be determined whether UBE4B mediates the monoubiquitination or polyubiquitination of EGFR. If UBE4B mediates polyubiquitination, the types of linkages mediated by UBE4B may provide insight into the importance of ubiquitin ligase/protein substrate relationship. It may also speak to why ubiquitination by Cbl is not sufficient for EGFR sorting (Smith et al., 2013).

Another interesting aspect of ubiquitination in endosomal sorting is that MVB vesicles can also be released as exocytic cargo when MVBs fuse with the plasma membrane. While this may not be common for EGFRs that enter the endocytic pathway, it has been shown that other protein cargo escapes deubiquitination and become incorporated into MVB vesicles destined for exocytosis (Buschow et al., 2005). It is possible that UBE4B and other EGFR-associated ubiquitin ligases mediate the ubiquitination of EGFR using certain linkages that are susceptible to deubiquitination, while other types of linkages are difficult to remove. Discerning the differences between these two types of ubiquitination events may clarify why receptors are degraded and others are released into the extracellular space.

2. **To determine whether UBE4B-mediated ubiquitination of endosomal cargo occurs through E3 or E4 activity.**

Like other U-box ubiquitin ligases, UBE4B can ubiquitinate target substrates independently of other E3 ligases (through its E3 activity) or in conjunction with another E3 ligase (through its E4 activity). The yeast homolog of UBE4B, Ufd2, was the first E4 ubiquitination enzyme that was discovered. Without an E4 ligase, polyubiquitin chains may be initiated, but terminate after a few ubiquitin molecules are attached to a substrate protein (Koegl et al., 1999). It is unclear whether monoubiquitination or polyubiquitination destines EGFR for lysosomal degradation, as both types of ubiquitination have been identified as sufficient for EGFR sorting (Haglund et al., 2003; Huang et al., 2013). Elucidating the mechanism by which UBE4B ubiquitinates EGFR may provide information about a protein-protein interaction that can be exploited in to develop novel therapies for diseases, such as neuroblastoma.

3. **To examine the nature of Cbl's contribution to the sorting of endosomal cargo**

If it is determined that UBE4B mediates EGFR ubiquitination through its E4 activity, Cbl is a promising candidate for an E3 ligase partner. Cbl expression is required for EGFR sorting and degradation, but ubiquitination by Cbl cannot mediate entry of EGFR into MVBs (Smith et al., 2013). Additionally, Cbl has been shown to localize to endosomes (Visser Smit et al., 2009; de Melker et al., 2001; Levkowitz et al., 1999). There are many different ways in which Cbl may mediate the EGFR-UBE4B interaction. 1) Cbl ubiquitination may enable UBE4B binding to EGFR. 2) Cbl may bind directly to EGFR, and may remain bound to EGFR in order to mediate UBE4B binding. 3) UBE4B may bind to EGFR and elongate existing ubiquitin polypeptides with the aid of the E3 activity of Cbl.

In a broader sense, the mediation of the UBE4B-EGFR relationship by Cbl may speak to an organization of control that one class of ubiquitin ligases (to which Cbl belongs) exerts upon

another class of ubiquitin ligases (to which UBE4B belongs). Plasma membrane proteins may require interaction with two ubiquitin ligases (or one ubiquitin ligase and one E4 enzyme) to gain entry to endosomes.

4. To determine whether delivery of recombinant UBE4B may overcome loss of endogenous UBE4B expression

Recombinant UBE4B was able to rescue the sorting of EGFR (Figure 10) in my experiments. Moreover, overexpression of UBE4B in neuroblastoma cells can accelerate the degradation of EGFR and affect cell proliferation [Figure 11 and (Zage et al., 2013)]. Delivery of recombinant UBE4B to tumor cells may compensate for genetic defects that underlie tumor formation, such as deletions in 1p36 (as seen in neuroblastoma, glioblastoma, and hepatocellular carcinomas), overexpression of EGFR, or mutations in EGFR that lead to defective receptor degradation (Caron et al., 1996; Ichimura et al., 2008; Zhang et al., 2010b; Keller et al., 2016; Grandal et al., 2007). Therefore, targeted delivery of UBE4B (via a nanoparticle or liposomal vehicle) to cells with either 1) decreased UBE4B expression due to abnormal genetic alterations (e.g. 1p36 deletions) or 2) cells that overexpress EGFR, may provide a therapeutic mechanism by which EGFR degradation is increased upon UBE4B delivery.

5. To examine whether UBE4B mediates ubiquitination of other endosomal cargo

UBE4B has been shown to interact with and ubiquitinate other target substrates (Wu et al., 2011; Zeinab et al., 2012; Matsumoto et al., 2004; Okumura et al., 2004). However, other than EGFR, protein targets of UBE4B ubiquitination are cytosolic. Out of more than 600 ubiquitin ligases, a smaller subset have been E3 ligases have been shown to localize at endosomes, including AIP4, MARCH-II, MARCH-III, Triad1, and RNF13 (Marchese et al., 2003; Nakamura, 2005; Fukuda, 2006; Hassink et al., 2012; Bocock et al., 2010). Over 6,700 membrane proteins

are predicted to be encoded by the human genome (Almén et al., 2009). For each membrane protein to be ubiquitinated, each ligase would have to associate with at least ten different membrane proteins. It is probable that UBE4B can mediate the ubiquitination of membrane proteins other than EGFR. One strategy would be to examine whether UBE4B can ubiquitinate proteins that are similar to EGFR. Another strategy would be to identify cancers in which UBE4B expression may be lost (cancers with 1p36 deletions) and identify proteins that overexpressed in these cancers.

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Vita

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