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ENDOCYTIC TRAFFICKING OF THE AMYLOID PRECURSOR PROTEIN IN RAT CORTICAL NEURONS

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ENDOCYTIC TRAFFICKING OF THE AMYLOID PRECURSOR PROTEIN IN RAT CORTICAL NEURONS

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ENDOCYTIC TRAFFICKING OF THE AMYLOID PRECURSOR PROTEIN IN
RAT CORTICAL NEURONS

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

Sahily Reyes-Esteves, B.S.

Houston, Texas

July 2017

Dedication

To my beloved Mami, who was my greatest cheerleader. I could write another thesis about all you taught me in life, and still continue to teach me. I hope to honor you in all I do.

“Instruye al niño en su camino, y aún cuando fuere viejo, no se apartará de él” (Prov. 22:6).

Te voy a amar para siempre.

To Papi, Ari, Nandy, Ian, Nani, and Yirán: You are my people, my tribe, my team. I can't begin to express how much you mean to me, how much I love you, and how proud I am to be your daughter, sister, and aunt.

To my husband, Francisco, because regardless of where we live, you are my rock and my home. This degree is the product of effort, sweat, tears, gray hairs, and sleepless nights for both of us, and you have remained through it all. I could never have done this without you. We are a team. I LOVE YOU!

Acknowledgments

First and foremost, I want to acknowledge the work of every member of the Bean Lab. Natalie, Monica, Kimiya, David, and Ting: Working alongside all of you, in happy times and in times of tremendous stress, has helped shaped me as a person like no other experience in recent years. I cannot thank you enough for putting up with me through these years, for providing your ideas, your creativity, your patience, and your love into this project and my life. I truly hope to remain friends with you all for many years to come.

I would also like to thank others in our department and school, including Dr. Ritika Tewari, Max Odem, Tara Fisher, Ndidi-Ese Uzor, and Felix Moruno Manchón for their help, conversations and friendship. Thank you to Sharon Gordon and Amanda Williamson for all that you do for the students in our program.

Dr. Andrew Bean, thank you for meeting with me in Fall 2013 and introducing me to the field of endocytic trafficking. Thank you for being patient, inspiring, motivational, and for challenging me to become a better scientist. Under your guidance, I have gained skills in the bench and beyond that will be invaluable for my future as a physician-scientist and a professional. After 4 years under your mentorship, I have a deeper respect and admiration for who you are as a scientist and as a person. I hope to continue collaborating with you in the future.

To the members of my committee, Dr. M. Neal Waxham, Dr. E. Terry Walters, Dr. Patrick Zweidler-McKay, Dr. Darren Boehning, and Dr. Andrey Tsvetkov, for taking their time to provide such valuable insight for the development of this project. Your thoughts and questions challenged me, but helped me gain independence in my scientific thinking. I would not have made it here without your support and teaching. For that, I am forever in your debt.

Abstract

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Amyloid-beta ($A\beta$) aggregation and deposition into extracellular plaques is a hallmark of the most common forms of dementia, including Alzheimer's disease. The $A\beta$ -containing plaques result from pathogenic cleavage of amyloid precursor protein (APP) by secretases resulting in intracellular production of $A\beta$ peptides that are secreted and accumulate extracellularly. Despite considerable progress towards understanding APP processing and $A\beta$ aggregation, the mechanisms underlying endosomal production of $A\beta$ peptides and their secretion remain unclear. Using endosomes isolated from cultured primary neurons, we determined that the trafficking of APP from the endosomal membrane into internal vesicles of late endosome/multivesicular bodies (MVB) is dependent on Endosomal Sorting Complexes Required for Transport (ESCRT) machinery. This implied that APP is ubiquitinated to allow ESCRT interactions. We then identified that the endosome-associated E3 ubiquitin ligase, UBE4B, is required for efficient endosomal APP trafficking. These results suggest that the efficiency of endosomal APP trafficking regulates $A\beta$ generation. Decreasing $A\beta$ levels in the brain may be a mechanism for disease modification in amyloid-related dementia. These experiments elucidate cellular mechanisms that are amenable to regulation and could serve as potential therapeutic targets for amyloid pathologies.

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List of Abbreviations

Alzheimer's Disease (AD)

Amyloid precursor protein (APP)

Amyloid beta (A β)

Beta-site APP Cleavage Enzyme 1 (BACE-1)

4',6-diamidino-2-phenylindole (DAPI)

Early endosomal antigen 1 (EEA1)

Endoplasmic reticulum (ER)

Epidermal growth factor receptor (EGFR)

Endosomal sorting complexes required for transport (ESCRT)

F-box and leucine rich repeat protein 2 (FBL2)

Green fluorescent protein (GFP)

Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs)

Homogenization Buffer (HB)

Multivesicular body (MVB)

Neurofibrillary tangle (NFT)

Phosphatidylinositol 3-phosphate (PI3P)

Plasma membrane (PM)

Small hairpin ribonucleic acid (shRNA)

Trans-Golgi network (TGN)

Tumor susceptibility gene 101 (Tsg101)

Ubiquitin conjugation factor E4 B (UBE4B)

Ubiquitin-interacting motif (UIM)

Vacuolar protein sorting (Vps)

Chapter 1. Introduction

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most common human neurodegenerative disease (1–4). In the United States, close to 100,000 individuals die from AD every year (5) and those affected typically die within 10 years of diagnosis (6, 7). This disease manifests as progressive loss of cognitive ability and personality alterations, a process known as dementia. Despite decades of research, there is no disease-modifying therapy or diagnostic test for this insidious disease.

AD was first described by Alois Alzheimer in publication from 1902 (8). The report described a 50-year-old female named Auguste Deter who experienced worsening paranoia and cognitive decline. Upon her death, an overall reduction in brain mass was noted, as was an accumulation of material inside and around the cells of her brain. During the 20th century, additional reports of patients experiencing similar symptoms led to the recognition that AD is the most common form of dementia.

Like other neurodegenerative diseases, brains from AD patients are characterized by marked neuronal death resulting in loss of brain mass. Unlike other similar disorders, which tend to localize to specific brain regions, the loss of neurons in Alzheimer's is robust and involves most of the cortex, as well as other key brain structures thought to be involved in memory formation such as the hippocampus (9–11). Histological examination of AD patient brains has revealed accumulation of protein aggregates *inside* and *outside* of cells, as illustrated on Figure 1A.

The classical *intracellular* pathology observed in AD is called a neurofibrillary tangle (NFT), and is mostly composed of a hyperphosphorylated form of the Tau protein. Tau is a microtubule-associated protein that becomes abnormally phosphorylated in some pathological processes, like AD and other neurological disorders (12–16).

Hyperphosphorylated Tau results in neurotoxicity due to aggregation and inability to bind to cytoskeletal microtubules, which results in microtubule disassembly and cytoskeletal dysfunction (17–19). However, phosphorylated Tau is thought to be a secondary event in the development of AD for the following reasons. First, in animal models, anomalies in Tau or its phosphorylation lead to a phenotype with marked motor dysfunction (something absent in classical AD) that more closely resembles a different disease, frontotemporal dementia (FTD) (20–23). Second, Gotz et al. (24) observed that injection of a protein called amyloid beta ($A\beta$, whose role in AD will be discussed in detail later in this chapter) into the brains of a murine strain predisposed to the deposition of NFTs accelerated the formation of tangles. A study published simultaneously (25) showed that crossing a mouse predisposed to formation of $A\beta$ with P301L mice led to accelerated formation of NFTs. Third, mice from a mutant strain predisposed to the formation of $A\beta$ have memory impairments that are dependent on the expression of Tau, although these mouse models do not form NFTs. Tau-mediated $A\beta$ toxicity has also been observed in *in vitro* studies of neurons (26). Finally, phosphorylated Tau was observed to mediate $A\beta$ toxicity in post-synaptic neurons by inducing excitotoxicity (which often leads to a seizure phenotype in AD animal models (27–29). While the controversy between these two potential etiologies for AD continues, the substantial evidence of a role for $A\beta$ in human dementia merits its investigation.

The extracellular pathology most commonly associated with AD is characterized by protein aggregates known as plaques (2–4, 18). For reasons that are as yet unclear, amyloid plaques can be diffuse or dense (30), and they can form outside of neurons or inside blood vessel walls in the brain (31–34). This latter process is called cerebral amyloid angiopathy, and while it is a distinct diagnosis from AD, the two diseases can be co-morbid (34–36) and result in dementia. The protein aggregates that form plaques are composed primarily of $A\beta$ (18, 37). $A\beta$ was first isolated and purified in 1984 (38, 39) and is a 4.2 kDa peptide most commonly found in extracellular aggregates in the brains of AD patients (3, 18,

37, 40). A β can exist in variants of 40-46 amino acids in length, with the 42-amino acid long variant, called A β 42, being the form most prone to aggregation (41).

The mechanism underlying the neuronal toxicity of A β in the central nervous system is still under investigation. In fact, some reports have linked A β to beneficial effects in the central nervous system, including enhanced neuron survival, synaptic modulation, and protection from oxidative stress (42–45). A prevailing hypothesis in the field is that the balance of A β production and clearance is disturbed in AD (41, 46). Excess A β production has been repeatedly shown to result in synaptic dysfunction, neuroinflammation, and oxidative stress, effects that are thought to ultimately produce neuronal death (41, 47–49). Although aggregation of A β is most commonly observed outside of neurons, several studies have reported that intraneuronal accumulation of A β is toxic to cells and antecedes the appearance of overt plaques in mouse models of AD (50–53).

1.2 Amyloid Precursor Protein

The amyloid precursor protein was identified in 1987 (4, 54) as the propeptide that led to A β formation. Since its discovery, APP has been strongly implicated in dementia (3, 4, 18, 41, 55). Several lines of evidence support this idea. First, familial mutations (56–61) in this protein strongly correlate with the development of many forms of early-onset AD, and many of these mutations are shown to result in the formation of A β (62, 63). Second, the APP gene is located on chromosome 21, and patients with Down syndrome (who have a trisomy of this chromosome) have a high likelihood of developing early-onset AD (64–67). Finally, APP has been shown to be the only known protein that leads to formation of A β (68–70).

Amyloid precursor protein (APP) is a Type-I transmembrane protein that exists as 8 isoforms (71–74) of 677-770 amino acids in length, of which the 695-amino acid variant is the most common in the human brain (75, 76). It is found in a variety of cell types (77–80),

and several physiological roles in neurons have been investigated. In neurons, APP contributes to cell adhesion (81), neurite outgrowth (82–84), synaptogenesis (85), and neuronal migration (86). Mice lacking APP have mild deficits compared to these described functions (87, 88) suggesting that APP's role in neuron physiology and normal function might be redundant with other related proteins, like the amyloid precursor-like proteins 1 and 2.

Cleavage of amyloid precursor protein (APP) can be amyloidogenic (leading to the formation of A β) or non-amyloidogenic (3, 89–92)(Figure 2). APP cleavage by a member of the alpha-secretase family (predominantly ADAM10)(93, 94) can occur at the plasma membrane (PM). Alpha-secretase cleavage results in the APP soluble fragment APPs α and a membrane-bound C-terminal fragment (α -stub)(3, 89, 90). Cleavage of APP by an alpha-secretase occurs in a portion of the A β sequence that renders the remaining peptides incapable of producing A β . The APPs α fragment mediates many of the described functions of full-length APP described above, such as neurite outgrowth and neuronal development (95–99). The C-terminal α -stub can be subsequently cleaved by the γ -secretase complex (composed of presenilin, nicastrin, APh-1, and PEN-2) to form the fragment P5. Interestingly, mutations in the presenilins are the most common form of inherited early-onset AD (100–102).

If APP is cleaved by the β -secretase, Beta-site APP Cleavage Enzyme 1, (BACE-1), instead of an alpha-secretase, generation of the soluble APPs β and a C-terminal fragment (β -stub, or C99) occur (103, 104). BACE-1 was first identified in 1999 by five independent groups (105–109). BACE-1 is a type-I transmembrane protein mostly expressed in the brain (3, 90, 103). BACE-1 enzyme activity has an optimal acidic pH such as is found in the Golgi and endosomes (90, 110–114). Increased expression of BACE-1 has been found in AD brains (115–117). Importantly, some familial AD-associated mutations in APP have increased susceptibility to cleavage by BACE-1(118) which then results in increased A β levels.

After BACE-1 cleavage, the β -stub can be further cleaved by the γ -secretase complex to yield the soluble A β species. The γ -secretase complex not only cleaves APP, but is essential for the cleavage of Notch, required for Notch signaling(119–121). Members of the γ -secretase complex have been localized to a variety of subcellular locations, although recent data suggests that the mature complex is mostly localized to the plasma membrane and the endosome/lysosome system(3, 122–125). For reasons that remain unclear, the γ -secretase-mediated cleavage of APP can generate A β peptides of various lengths, accounting for the variety of A β species found in AD brains(3, 126–129).

1.3 Endocytic trafficking of membrane proteins in mammalian cells

APP is synthesized and trafficked through the secretory pathway en route to the plasma membrane. In the endoplasmic reticulum (ER) and Golgi apparatus, membrane proteins can be cleaved into mature variants, post-translationally modified, or removed for degradation (130–134). Many membrane proteins are inserted into the PM after trafficking through the ER/Golgi (133–137) and from there, various mechanisms (such as binding of ligands) can promote their internalization into the cell. In neurons, these events often occur as a result of synaptic activity. Once a membrane protein is internalized from the PM, it can travel in transport vesicles to endosomes. From endosomes, membrane proteins can either recycle to the PM, or can be degraded via fusion of the endosomes with the lysosome. Because the focus of this study is on the movement of APP through endosomes, the endocytic pathway will be discussed in further detail.

The protein content of the plasma membrane is constantly renewed via insertion of proteins from the biosynthetic pathway and removal of other proteins via endocytosis. This dynamic state is a key regulatory mechanism that allows cells to adapt to their environment (138–140). This is especially important for neurons, where changes in surface protein expression (e.g. levels of glutamate receptors in the membrane are regulated by insertion

and endocytosis) can underlie functional alterations such as synaptic plasticity (141–145). The residence time of APP on the plasma membrane is approximately 10 minutes (83, 146–149). After internalization, vesicles containing membrane proteins (such as APP) can fuse with each other to form early endosomes, or fuse with existing early endosomes (150–152). Endosomes are organelles of approximately 500nm in diameter(153), that carry membrane proteins from the plasma membrane onto other cellular compartments for recycling or degradation(150–152). Endosomes mature from early into late endosomes by recruiting and discarding peripheral membrane proteins from the cytosol(154–159). Early endosomes are characterized by expression of markers such as EEA1, Rab5, Rab11, and transferrin (160–163). From early endosomes, membrane proteins can bud in vesicles that can fuse with the PM or travel to secretory compartments (163, 164). Early endosomes are thought to mature into late endosomes, characterized by markers such as Lamp1, Tsg101, CD63, and Rab7 (150, 154–159). Late endosomes are formed when the limiting membrane invaginates and buds internally producing vesicles that accumulate in the endosomal lumen. The formation of these internal vesicles can occur as a result of the involvement of cytosolic proteins that bind the endosomal limiting membrane (a process that will be discussed in Section 1.5), although some studies have demonstrated that budding into the endosomal lumen can occur in the absence of such proteins, by inward budding of lipid-containing domains present on the endosomal limiting membrane (165–168). Regardless of the mechanism of internal vesicle formation, the development of late endosomes by these inward budding events also gives them their particular structure: a larger endosomal compartment containing smaller internal vesicles between 50-200nm in size (150, 152, 169–173). For this reason, the late endosome is also known as the multivesicular body (MVB) or the multivesicular endosome (MVE). MVBs can have different itineraries in cells: (a) they can be degraded upon fusion of the late endosome with the lysosome, or (b) fuse with the PM for release of its contents. If an MVB fuses with the PM, its internal vesicles are released as

exosomes into the extracellular space (174–179). Exosomes are nanovesicles of endosomal origin (178, 180–184) secreted by a wide variety of cells (178, 185–188), including neurons (189–192). After it transits to mature endosomes, APP can be degraded upon fusion of the MVB with the lysosome (68, 69, 193). However, multiple reports suggest that APP is present on exosomes (194–196) suggesting that APP may also be present on MVBs that fuse with the plasma membrane (69, 193, 196–199).

1.4 Ubiquitination of membrane proteins for endocytic trafficking

Many membrane proteins that move from early endosomes into late endosomes (particularly into the intraluminal vesicles within the late endosome) require ubiquitination to enable association with the sorting machinery at endosomes (200–204). Ubiquitin is a 76-amino acid protein that can be conjugated to lysine residues of proteins as a post-translational modification. It is highly conserved throughout eukaryotic organisms¹, and the four genes (UBI1, UBI2, UBI3, and UBI4) that contain its code are present in both mammals and yeast. The presence of polyubiquitin chains on a protein often targets that protein for proteasomal degradation (204–208). The ubiquitination of a protein occurs in a series of steps mediated by the ubiquitin activating (E1), ubiquitin conjugating (E2), and ubiquitin ligase (E3) enzymes. There are two different E1 enzymes in mammalian cells, and these covalently bind and activate ubiquitin (207, 209, 210). From there, ubiquitin is passed to the E2 enzyme, of which there are an estimated 40 different kinds (205–208, 211–213). E2 enzymes will bind ubiquitin to the substrate, with the assistance of E3 ligases. The substrate specificity of ubiquitination is mediated by E3 ligases (214–217), of which the human genome is estimated to encode more than 600 unique proteins (214–216, 218). Ubiquitination is a reversible process and ubiquitin can be removed from proteins by the activity of deubiquitinating enzymes (219–223).

Proteins can be monoubiquitinated, multiubiquitinated (one ubiquitin is placed on

various lysine residues), or polyubiquitinated (various ubiquitin molecules form a chain anchored at one residue) (224–230). In polyubiquitination, two kinds of chains are commonly described: K48-linked chains and K63-linked chains. K48-linked chains are ubiquitin chains that bind to each other at lysine 48 of the ubiquitin sequence, forming branches that can be as long as 20 molecules. K63-linked chains occur after binding of additional ubiquitin molecules to lysine 63 of ubiquitin.

The ubiquitination of a membrane protein can signal its degradation or can be a signal important for trafficking events. However, the correlation between the type of ubiquitination present on a target protein and the role of that ubiquitin for protein disposition has not been conclusively established (217, 231), although K48-linked chains have been suggested to constitute a specific signal for proteasomal degradation (202, 208, 232). Since the proteasome functions in the breaking down of cytosolic proteins, the observation that ubiquitination could occur on membrane proteins represented a challenge to the understanding of the field (206, 207, 233–236). Studies performed in the yeast *Saccharomyces cerevisiae* were the first to discover that ubiquitination of some membrane proteins was a signal for their further movement down endocytic compartments (237, 238). In mammalian systems, the first evidence of the role of ubiquitin in the trafficking of membrane proteins through the endocytic pathway came from the study of tyrosine kinase receptors, that were observed to become ubiquitinated after binding of their ligands (239–242). For example, the epidermal growth factor receptor (EGFR) is monoubiquitinated prior to internalization from the PM (243, 244), although ubiquitination is not required for EGFR internalization (245–248). In these studies, cellular levels of EGFRs were unaffected by pharmacological proteasome inhibitors, suggesting their ubiquitination was not a signal for proteasomal degradation. Besides EGFR, a wide variety of membrane proteins can be ubiquitinated (249, 250). and both polyubiquitin and monoubiquitination can induce membrane protein internalization into endosomes (200, 251).

1.5 Recognition and trafficking of cargo by the ESCRT complexes:

Once a membrane protein is ubiquitinated *en route* to or at endosomes, it can be recognized by a sorting machinery that assembles at the endosomal limiting membrane, known as the Endosomal Sorting Complexes Required for Transport (ESCRTs) (170, 172, 248, 252–258). These proteins were discovered in a series of separate studies conducted between 1989 and 1992 by Rothman and Raymond (259–264). These investigations led to the discovery of a series of genes denominated the vacuolar protein sorting (Vps) genes. Some of the proteins encoded by these genes, denominated the ESCRT complexes, were discovered to be required for the degradation of many membrane proteins in the yeast vacuole (an organelle homologous to the mammalian endosome/lysosome) and subsequently, in mammalian endosomes (165, 203, 255, 256, 265–271).

ESCRT complexes are well conserved across eukaryotes, likely because their role in protein homeostasis at endosomes is essential for normal cell function. This is highlighted by the fact that embryonic lethality is observed in mice that contain deletions or loss-of-function mutations of various ESCRT components, such as hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and the tumor susceptibility gene 101 (Tsg101) (252, 262, 272–274). As would be expected, ESCRT deletion mutants often exhibit aberrant morphology such as enlargement and decreased internal vesicle formation (173, 256, 265, 275).

There are 4 ESCRT complexes: ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III (summarized in Figure 3). ESCRT complexes assembly on the endosomal membrane by recruitment from the cytosol. First, a member of the ESCRT-0 family, Hrs, is recruited to endosomal membranes through its membrane-lipid targeting domain FYVE(272, 276, 277), although its P/Q domain has been observed to play a role in this process (278). At endosomes, Hrs first binds membranes through interaction with phosphatidylinositol 3-

phosphate (PI3P) (256, 272, 276, 277). It can then bind to membrane proteins (often referred to as cargo) by recognizing ubiquitin with its ubiquitin-interacting motif (UIM)(251, 265). After cargo recognition, Hrs recruits other ESCRT-0 components (which help cluster targeted cargo) as well as ESCRT-I components such as Tsg101. Tsg101 can also recognize ubiquitinated cargo (166, 255, 256, 279), and also aids in the recruitment of other ESCRT-I components. ESCRT-I formation leads to recruitment of the ESCRT-II complex components (165, 203, 268). The binding of these ESCRT complexes leads to invagination of the endosomal limiting membrane toward the endosomal lumen(280) in a process that is opposite to the invaginations observed from assembly of coat proteins (257, 281). ESCRT-II is thought to recruit ESCRT-III (165, 255, 256, 268), which is believed to aid the fission of invaginated endosomal membranes (255, 264, 268). Although there are several models about how this binding of ESCRT complexes leads to invagination of membranes (255, 282), it is clear that ESCRT-0 proteins bind the ubiquitinated cargo and aid the assembly of the other ESCRT complexes.

1.6 The ubiquitin factor E4B (UBE4B) in endocytic trafficking:

The E3 ligase, UBE4B, is a ubiquitinating enzyme first described as UFD2 in yeast (283). UBE4B is part of a family of E3 ligases known as 'U-box' ligases (284–287), because they possess a 'U-box' domain that confers their ubiquitinating capacity (287–291). The 'U-Box' contains the binding site for the two E2 ligases known to bind UBE4B, UbcH5c and Ubc4 (287–292). UBE4B has at times been catalogued as a separate class of E3 ligase (also known as E4 enzymes) that, aside from aiding the ubiquitination of specific substrates as an E3 ligase, can also aid in the placement of additional ubiquitin molecules to specific substrates (289, 293).

UBE4B has been implicated in yeast survival after cell stress (284). In the adult mouse, UBE4B is predominantly expressed in neurons (285). In the brain, it has been

suggested to have a role in degeneration following axonal injury(294). UBE4B is involved in the ubiquitination and subsequent degradation of abnormal accumulations of ataxin-3, an aggregated protein that underlies spinocerebellar ataxia (292). UBE4B can also bind and decrease levels of p53, a known tumor suppressor (295). Homozygous UBE4B deficiency results in embryonic lethality in mice due to anomalies in cardiac development (289), and heterozygous UBE4B deficiency results in severe motor defects and Purkinje cell degeneration. These data suggest that UBE4B is essential for normal development of the heart, as well as maintenance of neuronal integrity in the brain.

Previous work from the Bean laboratory(296) has shown UBE4B is an endosomally-active E3 ligase. In that study, UBE4B was recruited to endosomes by binding to the early ESCRT, Hrs, that mediates its interaction with EGFR. This study also observed that UBE4B can ubiquitinate EGFR (296). The UBE4B-mediated EGFR ubiquitination is an obligatory step in efficient movement of EGFR from the endosomal limiting membrane into internal vesicles within the mature endosome. These data highlight UBE4B as an E3 ligase with an active role at the endosome, and open the doors for the discovery of new substrates for UBE4B-mediated ubiquitination at this cellular site.

1.7 APP movement and A β production through the endocytic pathway:

While BACE-1 is expressed in many subcellular locations, only the secretory pathway and the endocytic pathway contain sufficient expression(105) and adequately low pH for optimal activity of APP by BACE-1 (103, 297). Moreover, BACE-1 and APP colocalize at both of these locations (298–306). For this reason, both of these sites have been the focus of a variety of studies seeking to identify the key location of APP cleavage into A β (3, 299, 300, 305–311). Endosomes are of particular interest as a potential site of BACE-1 mediated APP cleavage for several reasons. First, blockade of APP internalization by clathrin-mediated endocytosis from the PM (312) or by removal of the YENP endocytosis

signal (146, 313, 314) decreased A β levels in conditioned media in several studies, including studies performed in neurons (315, 316). Synaptic activity mediates APP internalization from the PM and results in increased A β secretion (315–318). Moreover, synaptic activity results in colocalization of BACE-1 and APP in endosomal compartments (299, 300). These data suggest that the endocytic itinerary of APP is a crucial location for amyloidogenic cleavage.

There is conflicting evidence for a role of ESCRTs in the sorting of APP at endosomes and the secretion of A β . Depletion of early ESCRT components (Hrs and Tsg101) resulted in decreased A β secretion in some experimental models (305, 307), whereas others (306) have found that the same manipulation results in greater A β secretion. If APP trafficking is ESCRT dependent, then APP must be ubiquitinated to enable ESCRT interaction, suggesting that there may be a specific E3 enzyme that ubiquitinates APP. Interestingly, the E3 ligase FBL2 ubiquitinates APP resulting in proteosomal degradation (319), although the differences in ubiquitination requirements likely mean that a different E3 enzyme is required for endosomal trafficking. In this regard, our preliminary data suggest a role for the endosome-associated E3 ubiquitin ligase, UBE4B, in the degradation of APP.

1.8 Hypothesis statement:

This study investigated my **hypothesis (Figure 1D) that under non-pathogenic conditions, trafficking of full-length APP into the MVB is dependent on a specific E3 ligase, UBE4B, and the ESCRT machinery. Disruptions in MVB trafficking that delay APP sorting may result in amyloidogenic processing of APP.**

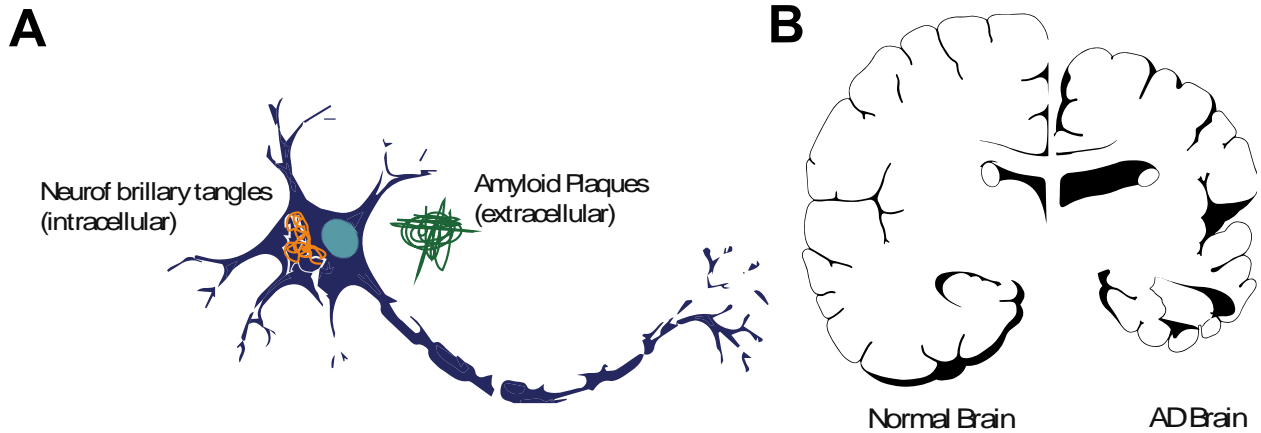


Figure 1A. Pathological findings in Alzheimer's disease (AD). (A) Accumulation of intracellular neurofibrillary tangles and extracellular amyloid plaques leads to neuronal dysfunction and death in AD. (B) Current understanding of the disease's processes behind AD suggests that these toxic accumulations lead to neuronal degeneration and death, as shown by decreased mass in affected brains.

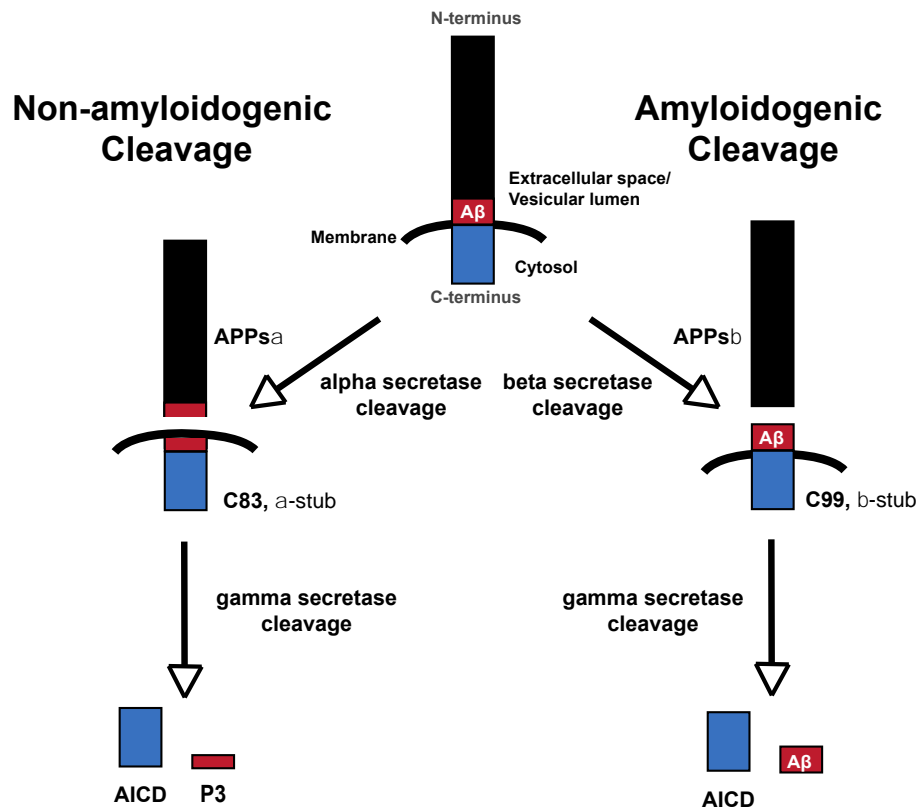


Figure 1B. Processing of the amyloid precursor protein (APP). Amyloid precursor protein can be cleaved in two main pathways. In the non-amyloidogenic cleavage, APP is cleaved into APPs α , secreted into the extracellular space, and the α -stub. This cleavage occurs through the A β sequence, precluding amyloid production after alpha-secretase cleavage. The stub is further cleaved by the gamma secretase complex into the amyloid precursor protein intracellular domain (AICD) and P3. In amyloidogenic cleavage, APP cleavage by a beta-secretase (BACE-1 in the brain), leading to generation of APPs β (released into the cytosol) and the β -stub, C99 (which faces vesicular lumens). Further cleavage by the gamma secretase complex generates AICD and A β . The subcellular site where this occurs is a matter of controversy and is the subject of this dissertation.

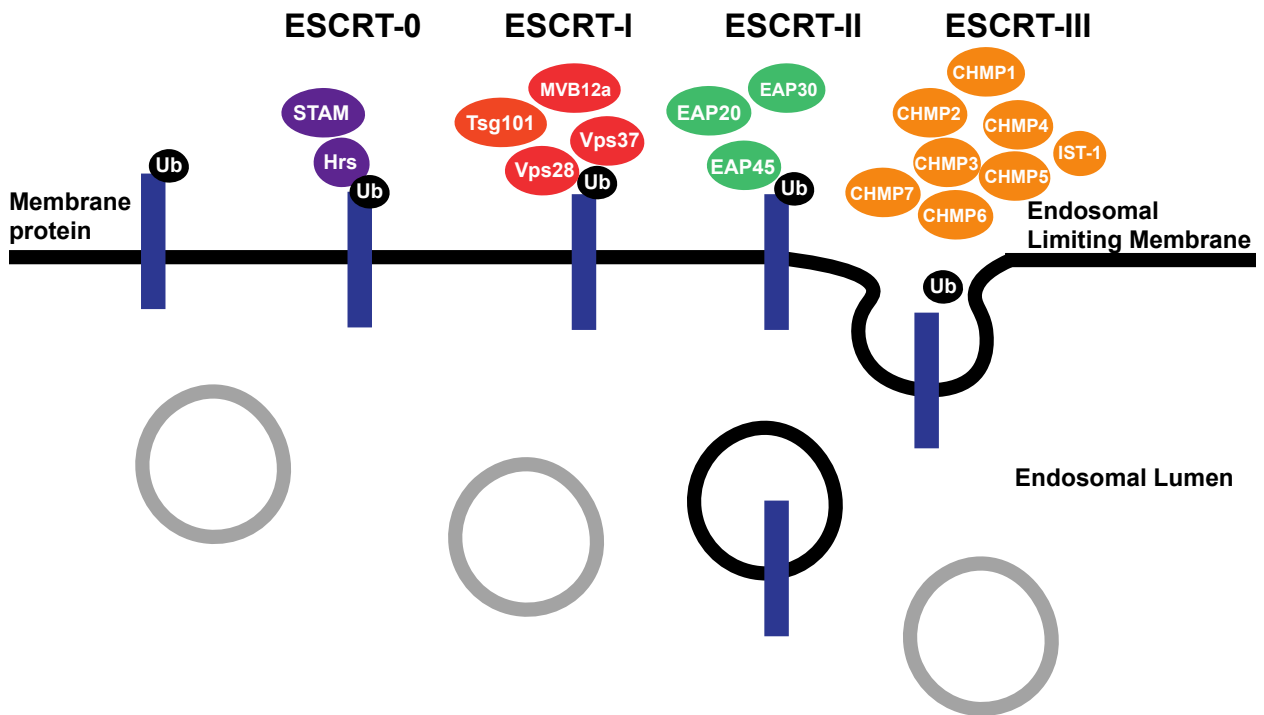


Figure 1C. Summary of ESCRT proteins involved in the endocytic trafficking of mammalian membrane proteins. ESCRTs are classified as 4 complexes in both mammals and yeast, and their metazoan names are illustrated in this schematic. ESCRT complexes sequentially bind ubiquitinated membrane proteins at the endosomal limiting membrane. These interactions are thought to mediate the necessary changes in membrane curvature that allow budding of vesicles containing membrane proteins into the endosomal lumen. Adapted from (255).

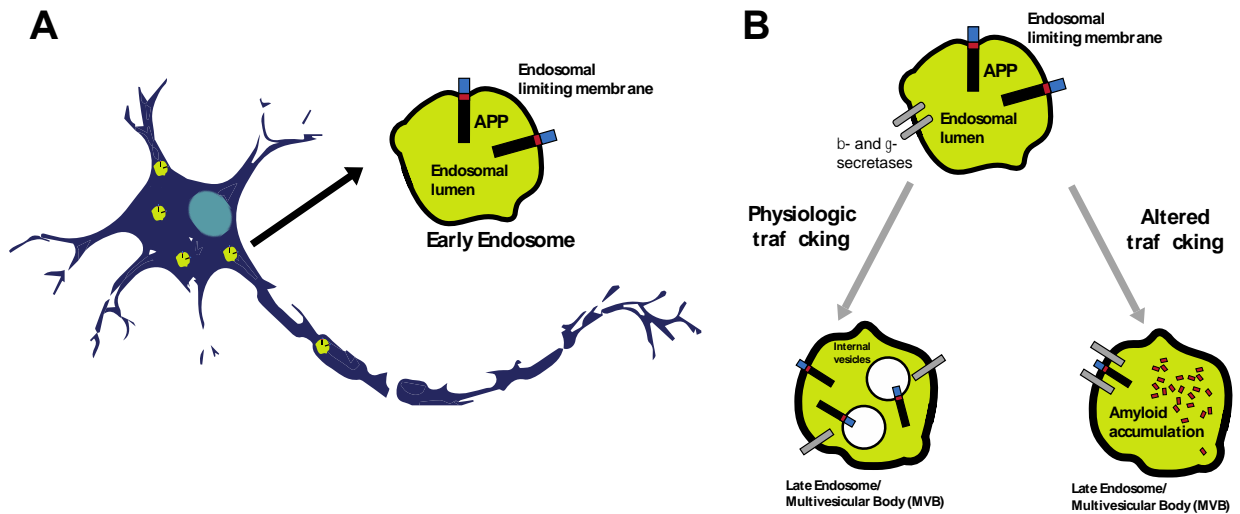


Figure 1D. Illustration of endocytic APP trafficking in neurons. (A) APP traffics through endosomes in neurons, and this is an important site of amyloidogenic cleavage (Das 2016, Morel 2013). (B) In physiological trafficking of APP through endosomes, the full-length protein is moved into internal vesicles of the maturing endosome through engagement of cytosolic proteins. When this trafficking is altered, APP can get cleaved by the secretases at the endosomal limiting membrane into A β , which will accumulate in the endosomal lumen.

Chapter 2. METHODS

2.1 Materials—Antibodies were purchased from the following commercial sources: C-terminal APP (Sigma, A8717; 5ug/mL for immunoblotting, IB; 50ug/mL for immunocytochemistry, ICC), N-terminal APP (Clone 22C11, Millipore; 10ug/mL for ICC), mouse and rabbit MAP2 (MAB3418 and AB5622, Millipore; 5ug/mL); Rab7 (D95F2 for ICC, for IB; Cell Signaling), EEA1 (2.5 ug/uL for ICC, 0.25ug/ml for IB; Thermo Fisher), BACE-1 (1ug/mL; Cell Signaling), Flotillin (Abcam), Alix (0.29 ug/ml; Abcam), Actin (0.3 ug/ml; Millipore), UBE4B (0.3 ug/ml; Abcam), Ubiquitin (1 ug/ul; Enzo), Hrs (1 ug/ul; Axxora). Chemical reagents were purchased from various sources: GW4869 (Cayman Chemical), β -Secretase Inhibitor IV – CAS 797035-11-1 (also known as C3; Millipore Sigma), and anti-fade DAPI mounting media (Life Technologies).

2.2 DNA/RNA Constructs—Short hairpin RNAs directed against UBE4B (CCGGTGGACCAACTGACGGATATTTCTCGAGAAATATCCGTCAGTTGGTCCATTTTTG; TRCN0000338294) and a scrambled shRNA (CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTT; SHC002) were purchased from Mission® shRNA (Sigma).

2.3 Mammalian Cell Culture—TLA-HEK 293T cells (ATCC) were maintained at 37°C in 5% CO₂, with DMEM (Life Technologies) and 10% FBS (Sigma), and passaged using 0.05 % trypsin/EDTA.

2.4 Primary Neuron Culture—Primary cortical neurons were obtained from E17-18 rat embryos as described (321, 322). Briefly, cortical brain tissue was extracted from rat embryonic brains, dissected on ice, enzymatically dissociated, and seeded (8 million cells)

on 100 mm² plates coated with 50ug/mL Poly-D-Lysine (Millipore). Neurons were maintained with Neurobasal Media (Life Technologies), 2% B27 (Life Technologies), 1% Glutamax (Life Technologies), and 1% Penicillin-Streptomycin (Sigma) under 5% CO₂ at 37°C. Media was replenished every 4 days. Experiments were carried out using neurons that had been cultured for 5-7 days *in vitro* unless otherwise specified.

2.5 Immunocytochemistry—Neurons (1x10⁶) were plated on coverslips coated with Poly-D-Lysine. After 5 days in culture, neurons were fixed with 4% paraformaldehyde (15 min at room temperature) and treated with 100mM glycine in PBS (10 min). Fixed neurons were permeabilized with 0.1% Triton X-100 (10 min), and blocked with 10% normal goat serum in PBS (60 min). Fixed neurons were incubated with primary antibodies (overnight at 4°C), washed 3x with PBS, and incubated with secondary antibodies (60 min at room temperature). Slides were washed 3x with PBS and mounted using anti-fade DAPI mounting media (Life Technologies). Neurons were examined using a TCS SPE microscope-camera (Leica).

2.6 Discontinuous sucrose gradient for endosome enrichment— Endosomally-enriched fractions were obtained from neuron lysates by fractionation using a discontinuous sucrose gradient, as described (323, 324). Briefly, after 5-7 days *in vitro* (DIV) neurons were washed with PBS, and gently scraped from the plate using a rubber cell scraper. Neurons were collected by centrifugation (1500 x g for 5 min) and resuspended in homogenization buffer (HB; 20 mM HEPES pH 7.4, 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, and 0.1 mM DTT) containing a protease inhibitor cocktail (112 µM leupeptin, 3 µM aproptinin, 112 µM PMSF, and 17 µM pepstatin). Neurons were lysed by passage through a 22-g needle 20 times, or until 80% of cells were lysed as demonstrated by assessing trypan blue exclusion. Post-nuclear supernatant (PNS) was obtained by centrifugation of lysed cells (2000 x g for 10

min). The concentration of sucrose in PNS was adjusted to 40.6% and PNS (440 μ L) was loaded at the bottom of a 2ml ultracentrifugation tube and overlaid with three layers of sucrose: 35% sucrose (660 μ L), 25% sucrose (440 μ L), and 8% sucrose (500 μ L). All sucrose-containing solutions also contained imidazole (3mM) and EDTA (1mM, pH 7.4). The gradient was centrifuged (150,000 x g for 60 min, model TLS55; Beckman Coulter). After centrifugation, 200uL fractions were collected from the top of the gradient (10 steps per gradient). Fractions were diluted using at least 1:1 in HB and membranes were pelleted by centrifugation (150,000xg for 30 min). A summary of the steps of this gradient, and a sample gradient after centrifugation is shown in Figure 2A.

2.7 Cytosol preparation— Mammalian cytosol: Rat brain cytosol was produced as described (Sun, JCB 2003). For cytosol prepared from rat cortical neurons, neurons were scraped and centrifuged (2000 x g for 5 min at 4 °C). This neuron pellet was resuspended in homogenization buffer (HB; described above) with protease inhibitors (112 μ M PMSF, 3 μ M aprotinin, 112 μ M leupeptin, 17 μ M pepstatin), and sonicated 3 times (8 pulses of 1 second at output control 2; Branson Sonifier 250, VWR Scientific). The resulting lysate was centrifuged (2000 x g for 10 min at 4°C) and the supernatant was further centrifuged (100,000 x g for 60 min at 4°C). The supernatant was collected as cytosol and protein concentrations were calculated using a BCA assay (Pierce).

Yeast cytosol: *Saccharomyces cerevisiae* deletion strains (Dharmacon) were plated on YPD plates (20 g/L bactopectone, 10g/L yeast extract, 16 g/L agar, 2% dextrose) with G418 (500 μ g/mL), and incubated overnight at 30°C. The parental strain was cultured without antibiotic. Colonies were inoculated in YPD media (5 mL) and incubated overnight on a shaker at 30°C. A secondary culture of YPD media (50 mL) was inoculated with the overnight culture and grown until OD₆₀₀ reached 0.8-1.0. Cells were collected (3000 x g for 10 min) and washed with ddH₂O, followed by TP buffer (20 mM Tris, pH 7.9; 0.5 mM EDTA;

10% glycerol; 50 mM NaCl, 112 μ M leupeptin, 3 μ M aprotinin, 112 μ M PMSF, and 17 μ M pepstatin). The final pellet was resuspended in 100 μ L of TP buffer. Cells were lysed using glass beads. Lysate was centrifuged (3000 x g for 10 min), and the supernatant was collected and further centrifuged (100,000 x g for 60 min) to collect cytosol. Protein concentrations were calculated using a BCA assay (Pierce). The supernatant was divided into 160 μ g aliquots and stored at -80 °C.

2.8 Immunoprecipitation and Immunodepletion— For co-immunoprecipitation experiments, neurons were collected using a rubber scraper, followed by centrifugation (1500 x g for 5 mins). Neurons were lysed using RIPA Buffer (10 mM Tris-Cl pH 8, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-1000, 0.1% sodium deoxycholate, 140 mM NaCl) containing protease inhibitors (60 min at 4°C with end-over-end rotation) followed by centrifugation (20,000 x g for 10min). For immunodepletion experiments, rat brain cytosol was isolated from lysate that was centrifuged (100,000xg for 60min). Cytosol was then incubated with 1 μ g of antibody per 100 μ g of cytosol with the addition of a protease inhibitor cocktail (overnight with end-over-end rotation). Samples were subsequently incubated with washed protein A agarose beads (15 μ L) for 4h at 4°C. Following brief centrifugation (1000 x g for 3 seconds) to separate beads from supernatant, immunodepleted supernatant was collected for use in cell-free endosomal reconstitution assays (described below). For immunoprecipitation, beads were washed (3x with 1X PBS) prior to resuspension in sample buffer and immunoblotting.

2.9 Cell-free endosomal reconstitution assay—A summary of this assay is presented in Figure 2B. Reconstitution of endosomal maturation (and formation of inwardly-budded vesicles) was performed as previously described (173, 296, 325), with some modifications. Briefly, early endosomes from primary neurons were obtained from a discontinuous sucrose

gradient (described above), and resuspended in HB. Each reaction (50 μ L) contained endosomal membranes, mammalian cytosol (75 μ g) or yeast cytosol (160 μ g), and an ATP regeneration system (2 mM MgATP, 50 μ g/mL creatine kinase, 8 mM phosphocreatine and 1mM). Reactions were incubated (3 hours at 37°C) prior to analysis (as below).

For experiments examining the movement of full-length APP into the endosomal lumen, reactions (as above) were followed by trypsin-treatment (10 μ L of 0.25 μ g/ μ L trypsin; 30 min at 4°C) and centrifugation (20,000 x *g*; 30 min at 4°C). Supernatant was aspirated and pellet was resuspended in sample buffer for immunoblotting. Resultant blots were probed with an antibody that recognizes the C-terminal epitope APP (Sigma). For experiments examining the generation of A β 42, reactions were centrifuged (20,000xg for 30 min at 4°C) and pellets were resuspended in HB for analysis by ELISA.

Western blot densitometry was quantitated using Image J (NIH). For analysis of experiments using yeast cytosol, quantitation of immunoblot bands from deletion strains were normalized to bands obtained in reactions containing parental control. For analysis of experiments using mammalian cytosol, quantitation of immunoblot bands from reactions using depleted or transfected cytosol were normalized to immunoblot bands obtained in reactions containing IgG-treated cytosol.

In experiments involving C3 (also known as β -Secretase Inhibitor IV – CAS 797035-11-1), endosomal membranes were treated with drug (20 μ M) or vehicle for 15 minutes at 37°C, prior to use in reactions containing cytosol and ATP regeneration system.

2.10 Cell transfection and lentivirus production—TLA-HEK 293T cells were transfected with third-generation lentiviral packaging plasmids (pMLg/pRRE, pRSV-Rev, pMD2.g; Addgene) and target DNA using Lipofectamine 3000 (Life Technologies), according to manufacturer's instructions. After 48 hours, media containing virus was passed through a 0.22 μ m PVDF filter (VWR) and applied to neurons. After 24 hours, virus was removed and

conditioned media (media that has been previously exposed to neurons, and as such, contains secreted factors that might aid neuronal survival in culture) added to neurons. For experiments in which exosome production was measured after lentiviral transduction, conditioned media was centrifuged (150,000 x g for 2 hours), passed through a 0.22 μ m filter, and warmed to 37°C prior to addition to neuronal cultures.

2.11 Immunoblotting— Samples were separated using SDS-PAGE and transferred onto nitrocellulose membranes (Genesee). All primary antibodies were incubated overnight at 4°C, and all secondary antibodies were incubated for 60 min at room temperature. Membranes were incubated with ECL 2 (Pierce) and developed using a Typhoon FLA 7000 (GE).

2.12 ELISA— The murine A β 42 enzyme-linked immunosorbent assay (ELISA) kit (Life Technologies) was used according to manufacturer's instructions. For analysis of endosomal A β 42, endosomal membranes generated from each reaction of the cell-free assay were sonicated (2 x 10 pulses) and loaded onto the bottom of an ELISA well. Each experiment was performed at least 3 times. For experiments measuring A β 42 secretion, 100 μ L of conditioned media was loaded onto wells and each sample was measured in duplicate. Each experiment was performed at least 3 times.

2.13 Statistical Analysis—For the comparison of two means, a paired t-test was performed for the determination of significance. For the comparison of three or more means, a one-way ANOVA with post-hoc Tukey test was used to determine significance. A *p*-value of < 0.05 was considered statistically significant with an *n* >= 3.

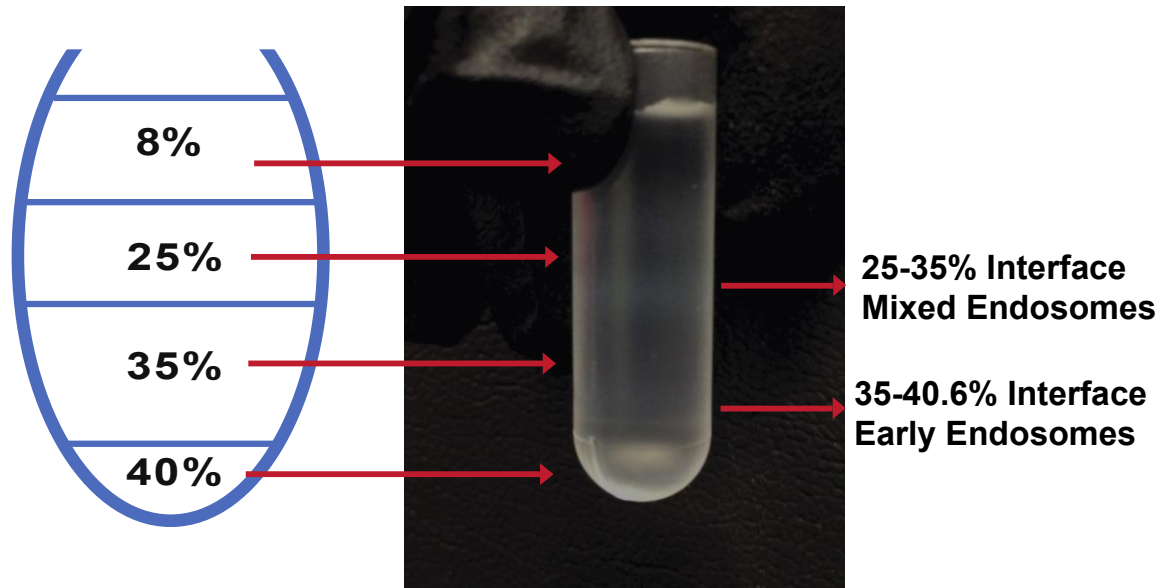


Figure 2A. Discontinuous sucrose gradient for separation of endosomal subpopulations in primary cortical neurons. Post-nuclear supernatant was loaded at the bottom of a discontinuous sucrose gradient. After ultracentrifugation ($150,000 \times g$ for 60 min) interfaces accumulate material that corresponds to endosomal subpopulations.

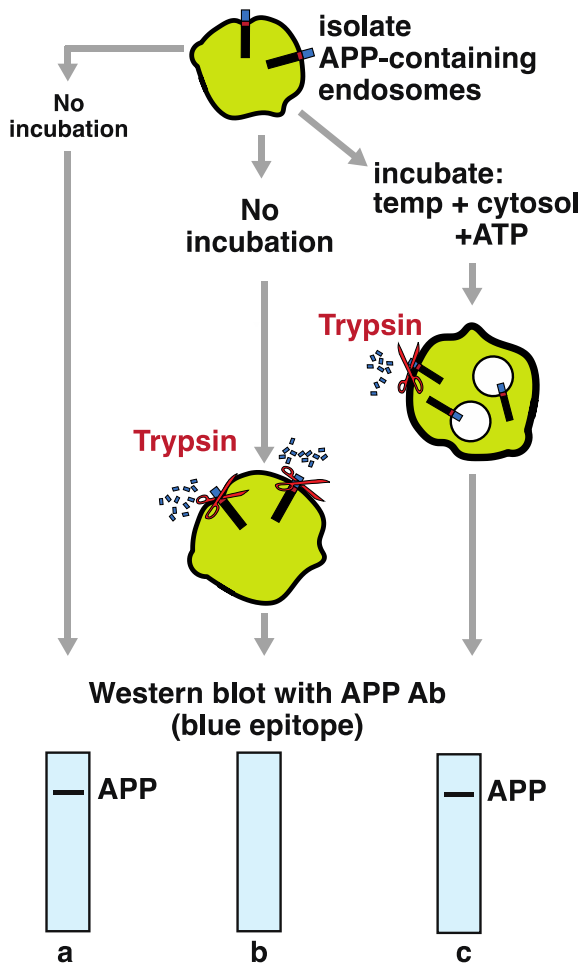


Figure 2B. Schematic of the cell-free endosomal maturation assay. After obtaining an enriched population of early endosomes from a discontinuous sucrose gradient, the location of APP on these endosomes can be determined via susceptibility to trypsin cleavage. (a) Early endosomes from primary cortical neurons contain APP, as shown by immunoblot. (b) Treatment of early endosomes with trypsin results in a loss of APP immunoreactivity, suggesting APP is localized on the limiting membrane of these endosomes. (c) In our cell-free assay, early endosomes are incubated with cytosol and an ATP regeneration system, that supports the formation of internal vesicles within endosomes. If APP is transported into internal vesicles it will be protected from protease treatment and APP will be visible on immunoblot.

Chapter 3. Endogenously Expressed Amyloid Precursor Protein (APP) is localized on neuronal endosomes

Rationale: Pathological cleavage of APP into A β fragments underlies the plaque pathology that is a hallmark of Alzheimer's Disease. While we understand much about the enzymes involved in APP cleavage(3, 4, 89), the cellular location of these cleavage events is unclear. There is considerable evidence suggesting that endosomes may be the location of pathologic APP cleavage. However, APP trafficking through endosomes is the subject of significant controversy in the scientific literature. In particular, the cytosolic requirements for APP movement from early to late endosomes and the implications of this movement for pathogenic processing are unclear. To address this scientific problem, I chose to use a cellular model, cultured primary cortical neurons, that is both physiologically relevant for Alzheimer's Disease and is tractable for mechanistic examination. In this chapter, I address the characterization of this model.

APP trafficking through endosomes has been observed in a variety of cell types, (194, 300, 305–307, 315). However, many of the published experiments examining APP trafficking have employed approaches including overexpression of exogenous APP or examination of trafficking events in heterologous non-neuronal cells. These experiments have greatly contributed to our understanding of APP movement through subcellular compartments, but it is unclear whether the results obtained are comparable to the itinerary of endogenous APP in neurons. To begin my approach of this scientific question, I examined whether endogenous APP is expressed in neurons in my preparation (cultured primary cortical neurons from E17 rat pup brains) at the time in culture when I would be investigating endosomal trafficking. Using immunocytochemistry and two different APP antibodies (Figure 3A), I observed that APP is expressed primarily in neurons (as shown by colocalization of APP with the neuronal marker, MAP2).

After establishing that APP was expressed in cultured neurons, I investigated whether APP is colocalized with endosomal markers in the absence of exogenous stimuli. I immunolabeled neurons using APP antibodies, as well as antibodies against endosomal marker proteins early endosomal antigen 1 (EEA1, a marker of early endosomes) and Rab7 (a small GTPase that identifies late endosomal compartments). I observed that APP could be localized to distinct puncta that also contained one or another of these markers (Figure 3B).

Because I was interested in examining the movement of APP through the endosomal pathway, I next determined whether early and late endosomal compartments from cultured neurons could be separated using velocity gradients, and whether APP would fractionate with endosomal markers in these preparations. Discontinuous sucrose gradients have been used extensively in the literature to separate fractions enriched in early and late endosomes (323, 324, 326). Using this technique, separation of post-nuclear supernatant derived from primary cortical neurons resulted in two major peaks (Figure 3C). The density of both endosomal peaks corresponds to reported values for the density of early and late endosomes (327). The peak observed in fractions 4 and 5 corresponds to lighter fractions in which both early and late endosomal markers have increased immunoreactivity. The peak observed in fractions 8 and 9 corresponds to more dense fractions where there is a peak in immunoreactivity for the EEA1 early endosomal marker. APP is enriched in both early and mixed endosomal peaks, although the APP-cleaving secretases BACE-1 and PS1 (a component of the gamma-secretase complex) are only enriched in the mixed endosomal fractions.

The Bean Laboratory has developed and characterized a cell-free endosomal reconstitution assay to investigate the movement of transmembrane proteins through endosomes (173, 296, 325). This approach examines the movement of a membrane protein from the limiting endosomal membrane into the internal endosomal vesicles that

corresponds to a critical step in which the cell determines whether a protein will be: (1) degraded in the lysosome, (2) become embedded in the lysosomal membrane, or (3) bud from the endosomal membrane for transport to other cellular compartments. In this approach, protease protection is used to follow the fate of an intracellular epitope of membrane proteins. Thus, when a transmembrane protein invaginates from the endosomal limiting membrane into internal vesicles within the endosomal lumen, it becomes inaccessible to the trypsin protease therefore assessing whether a membrane protein has entered the endosome lumen (refer to Figure 2B in Chapter 2). The movement of APP into endosomes renders its C-terminal tail inaccessible to trypsin, while APP residing on the endosomal membrane is susceptible to trypsin cleavage. Protection of the APP C-terminal tail would happen as APP moves from the endosomal limiting membrane into internal vesicles.

We examined how accessible an APP C-terminal epitope was to trypsin treatment on endosomes that had been separated using a discontinuous sucrose gradient. We hypothesized that in the early endosome, APP would be localized on the endosomal limiting membrane, suggesting that its C-terminal tail would be facing the exterior of the vesicle and would be cleaved by trypsin treatment. However, on the late endosome, we hypothesized that APP would have moved from the endosomal limiting membrane into internal vesicles within the mature endosome, and the APP C-terminal epitope would be inaccessible to trypsin.

We observed that APP was present on fractions enriched with early endosomes, and upon treatment by trypsin, immunoreactivity to APP using a C-terminal antibody was eliminated (Figure 3D). This suggested that the C-termini of most of the APP molecules present on early endosomes were exposed to the protease and that APP was therefore mostly residing on the limiting membrane of early endosomes. Interestingly, APP was also present on mixed endosomal fractions (that contain both early and late endosomes).

However, C-terminal APP immunoreactivity was not reduced by trypsin treatment on mixed endosomal fractions suggesting that the C-terminal of APP molecules on late endosomes was protected from proteolysis, perhaps because a large fraction of these APP molecules were already within the internal vesicles of late endosomes.

These results suggest that endogenous APP is present in cultured primary cortical neurons and that endosomal fractions from these neurons can be separated and used in cell-free reconstitution reactions to investigate the mechanisms by which APP travels from the limiting endosomal membrane into internal vesicles, a critical step in determining the fate of the APP that transits this pathway.

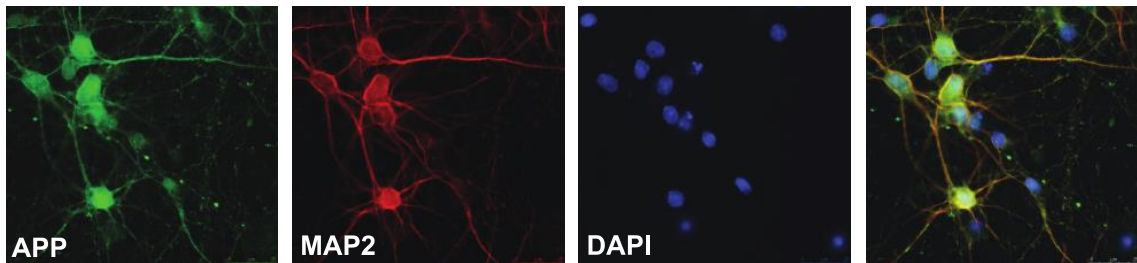
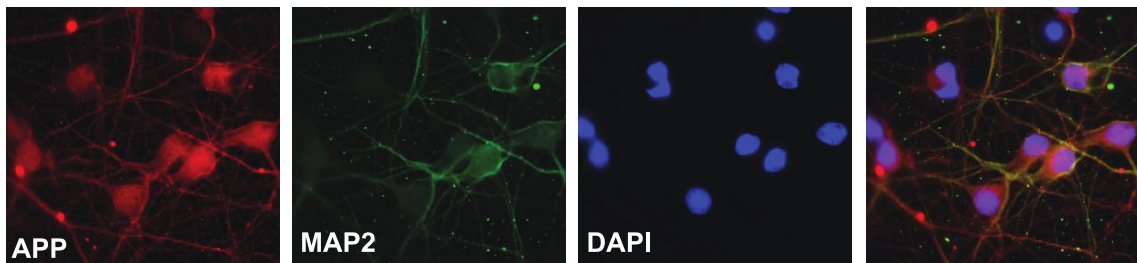
A**B**

Figure 3A. Endogenous APP is expressed in cultured rat cortical neurons.

Cultured neurons were stained with fluorescent antibodies against APP, MAP2 (a neuronal marker), and DAPI (a nuclear stain), as shown by the first three panels A and B. The last panel is a merged image of all fluorescent channels. (A) Cells present in the preparation were stained for both DAPI and MAP2 (rabbit antibody, Millipore). Most of the staining for APP (mouse antibody, Millipore) colocalizes with MAP2, suggesting the APP present in our culture is primarily expressed in neurons. (B) A different set of antibodies (rabbit anti-APP, Sigma; mouse anti-MAP2, Millipore) against the same target proteins reveal a similar result as in Panel A. Scale bar = 25 μm . *The data shown in this figure was performed in collaboration with Francisco Rivera-Milián.*

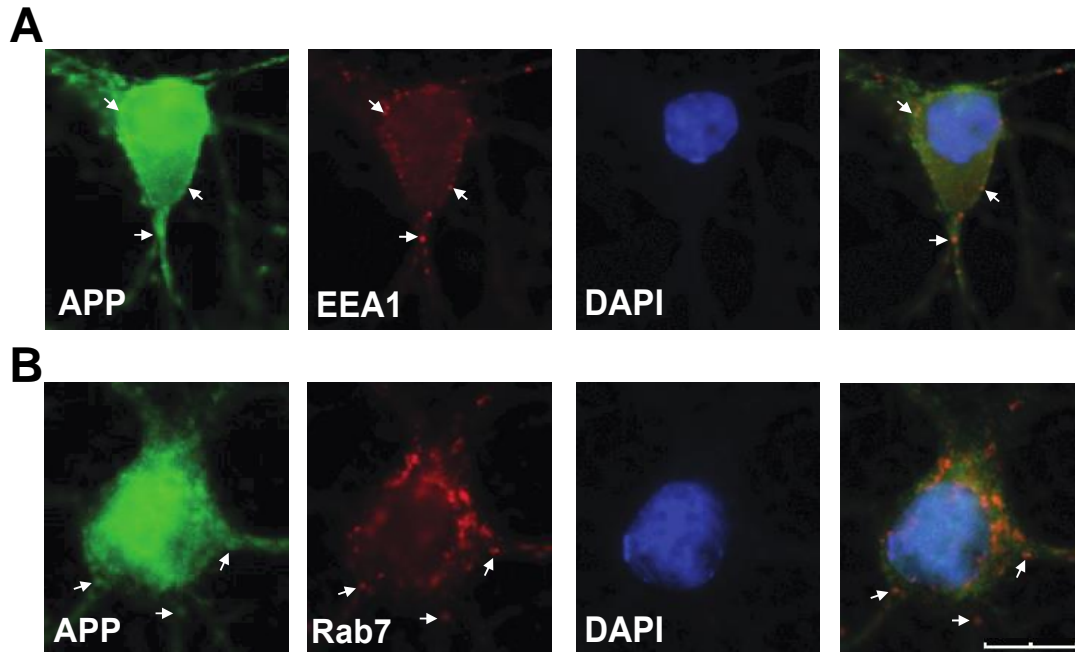


Figure 3B. Endogenous APP colocalizes with endosomal markers in primary cortical neurons at steady state. Neurons were fixed and stained with APP (first panel), endosomal markers (second panel), and the nuclear stain DAPI (third panel). The fourth panel represents a merged image of all fluorescent channels. Arrows mark distinct punctae where endosomal markers colocalize with APP. (A) EEA1 is an early endosomal marker that colocalizes with endogenous APP at steady state. (B) Rab7 is a late endosomal marker that colocalizes with endogenous APP at steady state. Scale bar = 10um. *The data shown in this figure was performed in collaboration with Francisco Rivera-Milián.*

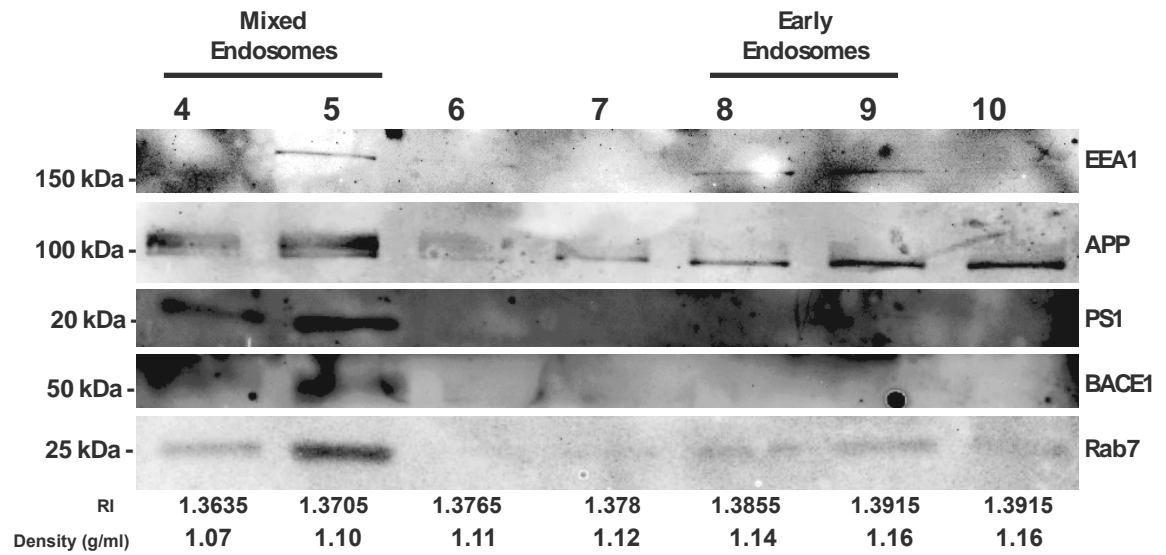


Figure 3C. Enrichment of endosomal subpopulations from cortical neurons through discontinuous sucrose gradient. Post-nuclear supernatant was loaded onto a discontinuous 8-40.6% sucrose gradient and centrifuged. Fractions were immunoblotted for endosomal markers (EEA1 for early endosomes and Rab7 for late endosomes), APP, and APP-modifying enzymes BACE1 and presenilin 1 (PS1, a component of the gamma-secretase complex). Refractive index (RI) and calculated density of each fraction is reported at the bottom. A mixed endosomal peak was observed at fractions 4 and 5 (as shown by immunoreactivity to both EEA1 and Rab7), and an early endosomal peak was observed at fractions 8 and 9 (as shown by immunoreactivity to EEA1).

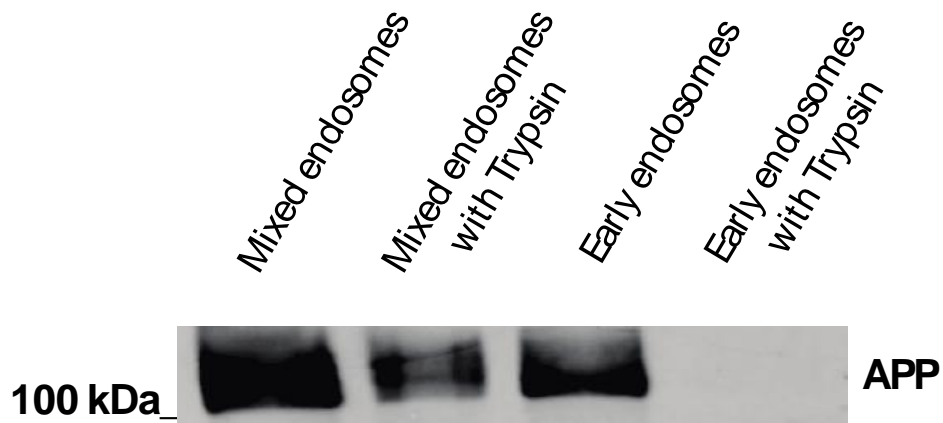


Figure 3D. APP localization on endosomes can be assessed by protease protection. Endosomal subpopulations were obtained from post-nuclear supernatant extracted from primary neurons, as described observed in Figure 3C. Untreated mixed endosomal fractions were analyzed via immunoblotting for presence of APP (lane 1). Upon treatment with trypsin, a protease, APP immunoreactivity persisted in this fraction (lane 2), suggesting APP is localized in a compartment inaccessible to trypsin (such as internal vesicles within the late endosome). Early endosome fractions were also analyzed for presence of APP (lane 3). Upon protease treatment, APP immunoreactivity was abolished (lane 4), suggesting APP in these endosomes is localized to a site accessible to trypsin, such as the endosomal limiting membrane. This experiment is representative of an experiment performed at least 3 times.

Chapter 4. Inward budding of endogenous APP into the mature endosome lumen

Rationale: From the endosomal limiting membrane, transmembrane proteins can travel to different cellular destinations. The canonical route taken by membrane proteins involves movement from the endosomal limiting membrane into internal vesicles within the maturing endosome. Following transit into internal vesicles, membrane proteins can be degraded upon endosome-lysosome fusion or can be exported outside of the cell upon endosome-plasma membrane fusion. For membrane proteins to move from the endosomal limiting membrane into internal vesicles, cytosolic machinery is often required to sort proteins destined for internal vesicles from those that will remain on the limiting membrane. This movement of APP from the endosomal limiting membrane into internal vesicles has been suggested by others (305–307) to be a key location for amyloidogenic cleavage of APP. My previous results (Chapter 3) showed that APP is present on endosomes and that it is susceptible to protease cleavage on early, but not late endosomes suggesting that I could examine the requirements for endosomal APP trafficking, as well as how alterations in this trafficking might affect the fate of APP.

To investigate the mechanisms of endogenous APP trafficking from the endosomal limiting membrane into endosome lumen, I used a cell-free reconstitution assay previously characterized by the Bean laboratory (173, 296, 325). This assay relies on protease protection of cargo to examine movement of proteins (173, 328, 329), and it recapitulates endosomal maturation including accumulation of internal vesicles, the movement of membrane proteins from the limiting endosomal membrane into internal vesicles, and the dependence of membrane protein movement into internal vesicles on the cytosolic

machinery known as Endosomal Sorting Complexes Required for Transport (ESCRTs) (173, 296).

To confirm APP movement from the limiting endosomal membrane to internal vesicles, I separated early endosomes on a velocity gradient and incubated the early endosomes with rat brain cytosol or cytosol isolated from a parental *S. cerevisiae* (yeast) strain and an ATP regenerating system (Figure 4A). Inclusion of either mammalian or yeast cytosol in the reconstitution reactions was required to protect the APP C-terminal epitope from trypsin cleavage, suggesting that cytosolic components are necessary for the movement of APP from the endosomal membrane into internal vesicles.

As summarized in the Introduction, the requirement of ESCRT machinery for the endosomal trafficking of APP is unclear. In particular, there is conflicting data on the role of early (ESCRT 0 and I) and late (ESCRT II and III) ESCRT complexes on APP trafficking (305, 307). In particular, deletion of early ESCRT components have been observed to both increase (306) and decrease (305, 307) amyloid production and secretion. I employed the cell-free reconstitution approach (as described in Figure 2B of Chapter 2) to directly determine whether ESCRT proteins are involved in APP movement from the endosomal membrane into its lumen.

The capability of yeast cytosol to support APP movement into internal vesicles within endosomes allowed me to screen a yeast deletion library for proteins that might be required for the movement of APP from the limiting endosomal membrane into internal vesicles. I observed that components of all 4 ESCRT complexes are necessary for efficient APP movement into internal vesicles (Figures 4B, 4C). Thus, absence of ESCRT components from each of the four ESCRT complexes resulted in significant decreases in the protease protection of the C-terminal APP epitope, suggesting that these components are required for efficient movement of APP from the limiting endosomal membrane into the endosome lumen (Figure 4C).

The production of A β fragments is affected after deletion of ESCRT components, although previous studies did not address whether A β production in the absence of ESCRT components was endosomal (305–307). I investigated whether absence of ESCRT components affects endosomal production of A β 42 within endosomes isolated from cultured neurons that had been used in the cell-free assay. Because of the limited endosomes obtained from each neuron, and the sensitivity limit of the ELISA, our assay does not allow for assessment of multiple A β species from one sample. I focused on the A β 42 species of APP cleavage product because of its reported increased pathogenicity and aggregation potential (41), compared to A β 40, the other most common form of amyloid found in the human brain.

Using the cell-free approach, with cytosol isolated from yeast strains lacking individual ESCRT components, I measured levels of endosomal A β 42 following incubation of endosomal membranes with yeast cytosol lacking individual ESCRT components. I observed that A β 42 levels were significantly increased in the absence of both early and late ESCRT components (Figure 4D), conditions that also resulted in decreased movement of APP into internal vesicles (see Figure 4C).

I next examined whether immunodepletion of ESCRT components from mammalian cytosol would also affect endosomal A β 42 levels. In particular, I wanted to understand whether deletion of an early ESCRT component affected APP trafficking, as the role of early ESCRT components in APP trafficking have been observed to both increase and decrease A β production and secretion (305–307). To this end, I immune-depleted rat brain cytosol of the mammalian early ESCRT, Hrs, and employed this cytosol in our cell-free endosomal reconstitution assay. I confirmed the depletion of Hrs using immunoblotting (Figure 4E). I incubated Hrs-depleted cytosol with APP-containing early endosomes in the cell-free endosome maturation assay and observed that the protease protection of APP was significantly reduced in Hrs-depleted conditions compared to IgG-treated cytosol (Figure

4F). Using a similar experimental design, I used endosomes in reactions with Hrs-depleted and control (IgG treated) cytosols and examined A β 42 production. Interestingly, A β 42 production was dramatically increased in the absence of Hrs (Figure 4G).

The results from these experiments suggest that ESCRT complexes may assist in the trafficking of APP through endosomes and confirm that absence of early and late ESCRT components result in increased A β 42 accumulation in the endosomal lumen.

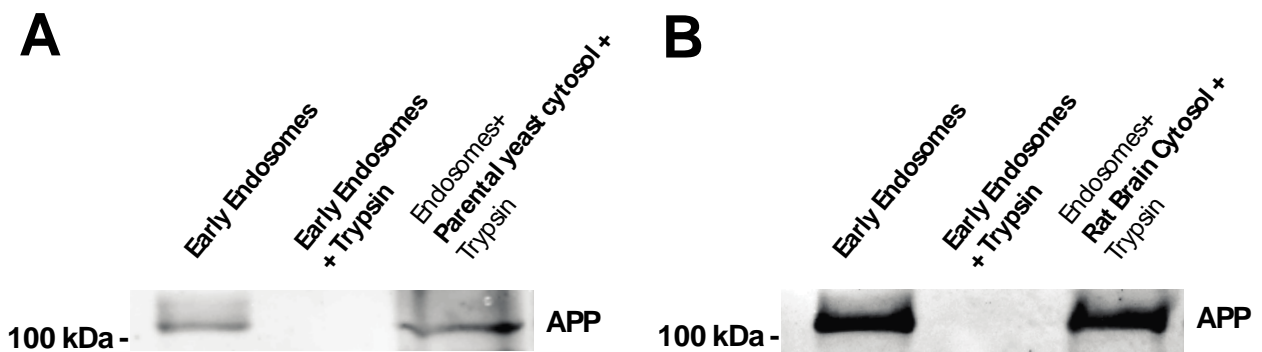


Figure 4A. APP movement from the endosomal limiting membrane into internal vesicles is mediated by cytosolic components. Early endosomes were isolated from rat cortical neurons using velocity gradients, as described (see Chapter 2), and incubated with cytosol isolated from yeast or rat brain. (A) Immunoblotting of early endosome fractions demonstrates that this fraction contains APP protein (lane 1). Treatment with trypsin results in the loss of APP immunoreactivity (lane 2), suggesting APP is present on the endosomal limiting membrane. Incubation of early endosomes with parental yeast cytosol results in persistent APP immunoreactivity despite trypsin treatment (lane 3). This suggests that cytosolic components can induce the movement of APP into trypsin-resistant compartments, such as the endosomal lumen. (B) As in A, APP is present on early endosomal membranes (lane 1), and is susceptible to trypsinization (lane 2). Incubation with rat brain cytosol results in persistent APP immunoreactivity despite trypsin treatment (lane 3), suggesting that rat brain cytosol also supports movement of APP into internal vesicles.

Name	ESCRT Mammalian Homolog	ESCRT Complex	Genotype	Source
BY4741	<i>*parental strain</i>	---	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0</i>	Dharmacon
Δhse1	<i>STAM1,2</i>	<i>ESCRT-0</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hse::KANMX6</i>	Dharmacon
Δvps23	<i>Tsg101</i>	<i>ESCRT-I</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 vps23::KANMX6</i>	Dharmacon
Δsnf8	<i>Vps22/EAP30</i>	<i>ESCRT-II</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 snf8::KANMX6</i>	Dharmacon
Δvps24	<i>CHMP3</i>	<i>ESCRT-III</i>	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 vps24::KANMX6</i>	Dharmacon

Figure 4B. Yeast deletion strains used to examine the role of ESCRT machinery in APP endosomal trafficking. This table summarizes the *Saccharomyces cerevisiae* deletion strains for ESCRT components used in this study.

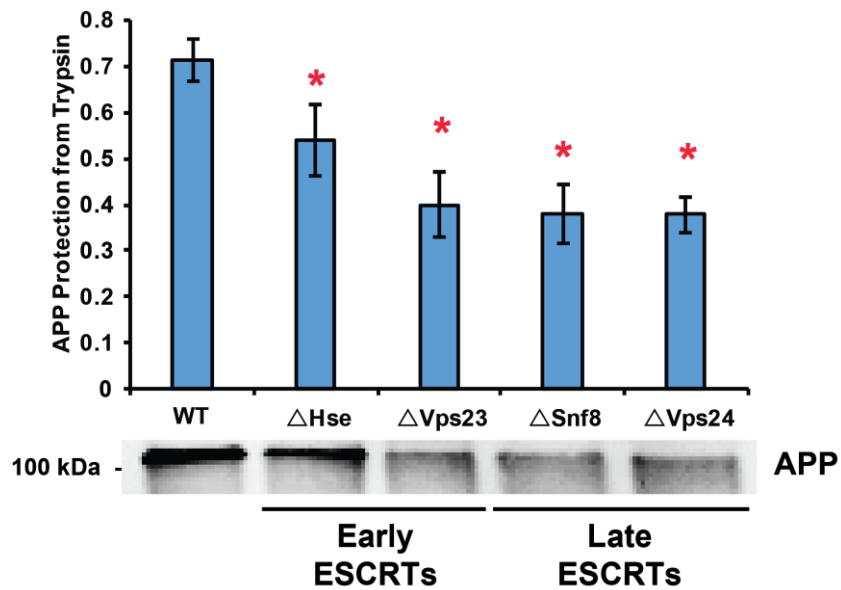


Figure 4C. Efficient trafficking of full-length APP depends on components of all 4 ESCRT complexes. Early endosomal membranes containing APP were obtained using velocity gradients (see Figure 2C of Chapter 2). Endosomal membranes were incubated with cytosol isolated from yeast strains lacking ESCRT components (Δ Hse for ESCRT-0, Δ Vps23 for ESCRT-I, Δ Snf8 for ESCRT-II, and Δ Vps24 for ESCRT-III). Cytosol isolated from a parental yeast strain served as a reaction control. For these experiments, $n=4$ and (*) denotes $p < 0.05$.

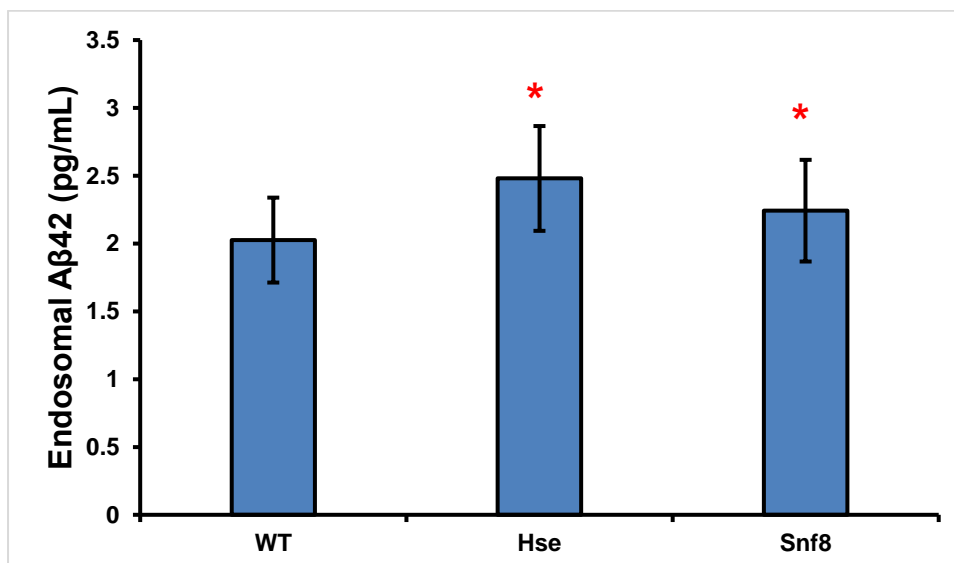


Figure 4D. Absence of early and late ESCRT components leads to increased A β 42 production. Early endosomes containing APP were isolated with from velocity gradients and incubated with yeast cytosol isolated from yeast strains lacking an early ESCRT component (Δ Hse) or a late ESCRT component (Δ Snf8). After incubation, endosomes were collected and analyzed via ELISA for A β 42 levels. The amount of A β 42 present in endosomes following reactions containing cytosol from each deletion condition was normalized to the A β 42 level obtained in a reaction containing cytosol isolated from a parental yeast control. For these experiments, n=4 and (*) denotes p<0.05.



Figure 4E. Immunodepletion of Hrs from rat brain cytosol. Rat brain cytosol was prepared as previously described (173). Hrs was immunoprecipitated from cytosol, and equal amounts of cytosol were separated using SDS-PAGE and immunoblotted using anti-Hrs antibody. .

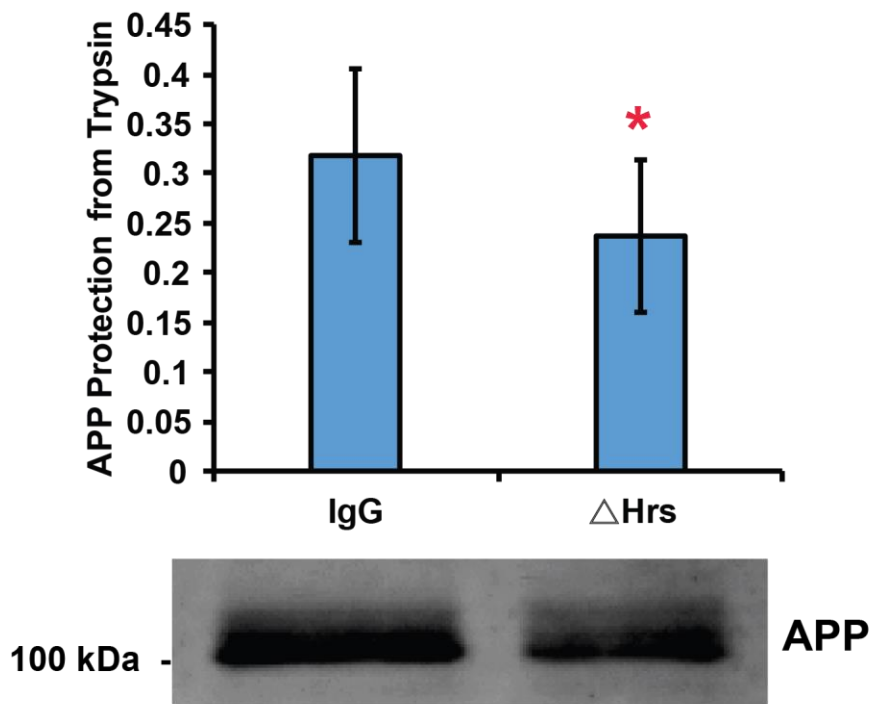


Figure 4F. Depletion of Hrs significantly reduces APP movement into endosomal internal vesicles. Rat brain cytosol was immunodepleted of Hrs (see Figure 4E) and incubated with APP-containing early endosomes obtained from velocity gradients. After incubation and trypsin treatment, APP levels are significantly decreased in the Δ Hrs condition, suggesting that Hrs is required for efficient movement of APP into internal vesicles within the endosomal lumen. For these experiments, $n=3$ and $p=0.025$.

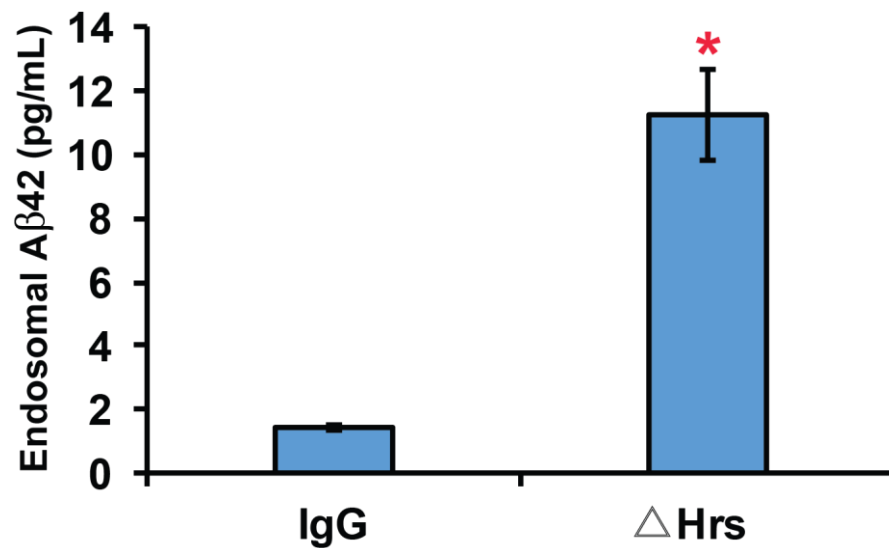


Figure 4G. Absence of Hrs results in significantly increased Aβ42 in endosomes.

APP-containing early endosomal membranes were incubated with cytosol that had been immunodepleted using anti-Hrs antibodies (see Figure 4E). After cell-free endosomal reconstitution reactions resulting endosomal membranes were recollected and analyzed by ELISA for Aβ42 levels. For these experiments, n=3, p=0.02.

Chapter 5. UBE4B is an E3 ubiquitin ligase that binds to, ubiquitinates APP and mediates efficient APP trafficking on endosomes

Rationale: My data, along with the work of other groups, suggests a role for ESCRT proteins in the endosomal trafficking of APP. However, it is thought that ESCRT recognizes ubiquitinated cargo at the endosomal membrane. If so, APP must be ubiquitinated at this cellular location by a specific E3 ligase. I examined a candidate enzyme, UBE4B, and show that it affects APP trafficking at endosomes. My work suggests that UBE4B is a specific ligase that ubiquitinates APP at endosomes.

To determine whether APP can be ubiquitinated at steady state in cultured cortical neurons I immunoprecipitated APP and observed that it is ubiquitinated (Figure 5A). I hypothesized that endosomally localized E3 ubiquitin ligases might be potential APP modifying enzymes that could affect APP trafficking at endosomes. In collaboration with Drs. Ritika Tewari, Monica Goss and Ting Wang we observed that one of these endosomally localized E3 ubiquitin ligases, UBE4B, co-immunoprecipitates with APP (Figure 5B) from neurons suggesting that APP and UBE4B can bind to each other.

To further elucidate the relationship between APP and UBE4B, I obtained SK-N-AS (a neuroblastoma cell line) that overexpressed UBE4B from another member of the lab, David Savage. I observed that SK-N-AS cells expressing increased UBE4B possessed significantly decreased levels of APP (Figure 5C) suggesting that there may be a relationship between UBE4B expression and APP stability.

I next used an shRNA against UBE4B in neurons to determine whether depletion of UBE4B would alter neuronal APP levels. The efficiency of lentiviral delivery was confirmed by infection of neurons with a virus containing GFP (Figure 5D). I then used this strategy to deliver an shRNA targeted toward UBE4B and a control shRNA containing a scrambled sequence. I observed that the shRNA containing a UBE4B target sequence resulted in

UBE4B protein depletion and decreased levels of APP in neuron lysate (Figure 5E), compared to the scrambled shRNA.

To assess whether UBE4B levels affected the state of APP ubiquitination in cortical neurons, I immunoprecipitated APP from neurons that had been treated with scrambled shRNA or shRNA against UBE4B. I observed that absence of UBE4B led to significantly decreased levels of APP ubiquitination (Figure 5F).

To determine whether UBE4B affects endosomal APP trafficking, I immunodepleted UBE4B from rat brain cytosol (Figure 5G) and used this cytosol in reactions containing early endosomes. Interestingly, immunodepletion of UBE4B did not significantly affect other endosomally associated cytosolic proteins that are known to bind UBE4B, such as Hrs (Figure 5H). Incubation of endosomes with UBE4B-depleted cytosol resulted in significantly decreased protease protection of the C-terminal epitope of APP compared to IgG-treated control cytosol (Figure 5I). These data suggest that UBE4B can ubiquitinate APP and that movement of APP into internal vesicles within the maturing endosome cannot occur efficiently in the absence of UBE4B.

I next examined whether incubation of endosomes with UBE4B-depleted cytosol resulted in increased endosomal A β 42 levels (Figure 5J). The increase observed was comparable to the A β 42 increase observed when the early ESCRT Hrs was depleted in a similar experiment (See Chapter 4). To confirm that the A β 42 was produced at endosomes during our cell-free assay, I performed cell-free reactions that included endosomes and the UBE4B-depleted cytosol, in the presence and absence of the BACE inhibitor, C3. I observed that reactions performed in the presence of the BACE1 inhibitor resulted in significantly decreased levels of A β 42 compared to the amount of A β 42 generated in the absence of this drug (Figure 5K). These data suggest that absence of UBE4B leaves APP more susceptible to cleavage by BACE-1, potentially due to increased time spent on the endosomal limiting membrane.

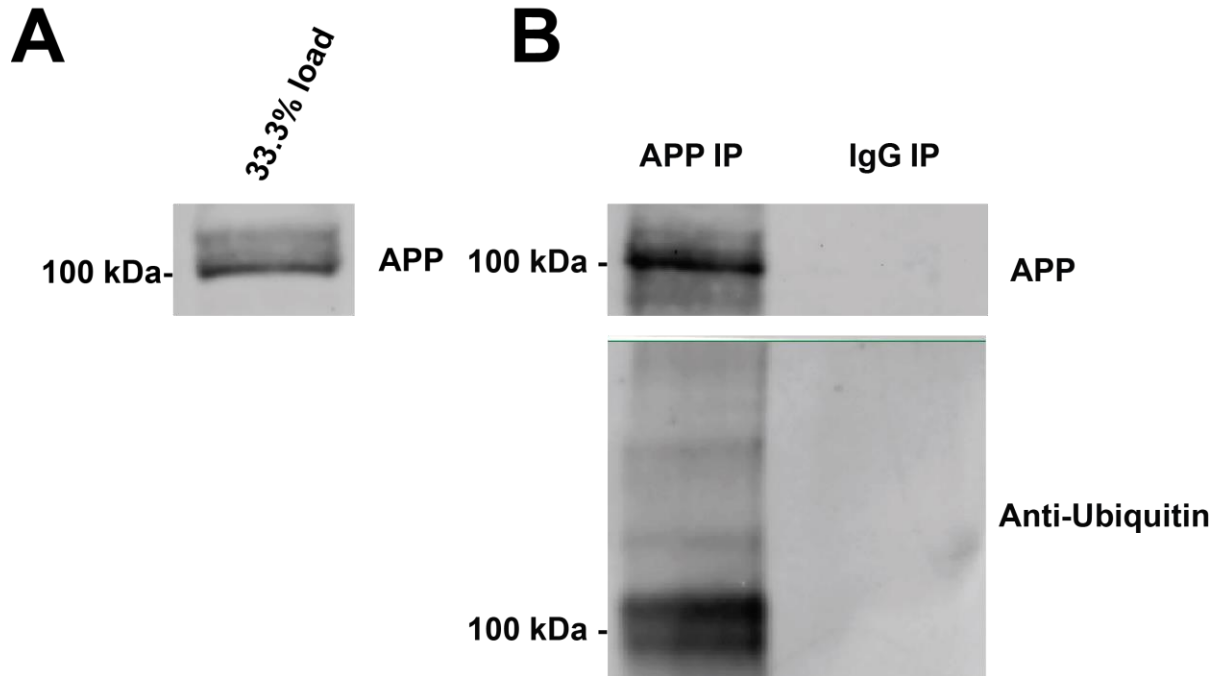


Figure 5A. APP is ubiquitinated at steady-state in primary cortical neurons. (A) APP was immunoprecipitated from neuron lysate (Lane 1). As a control, neuron lysate was incubated with a non-specific IgG control (Lane 2). (B) Ubiquitin co-immunoprecipitated with APP (Lane 1) but not with a nonspecific IgG (Lane 2). Together, these data suggest APP is ubiquitinated at steady state in primary cortical neurons.

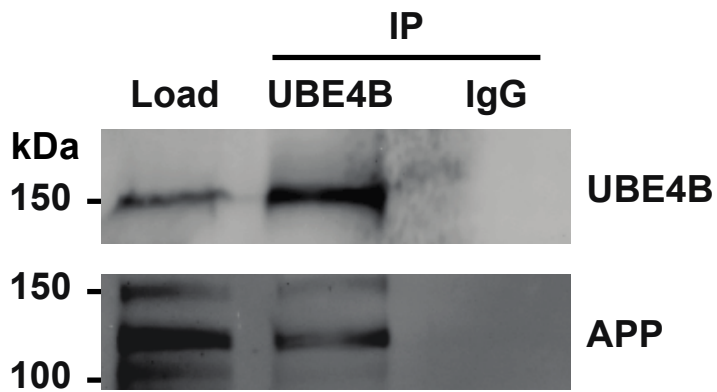


Figure 5B. UBE4B co-immunoprecipitates with APP. Using lysate from the neuron-like cell line SK-N-AS, UBE4B was immunoprecipitated. Loaded material is shown in Lane 1. On immunoblotting, APP is shown to co-immunoprecipitate with UBE4B (Lane 2), but not with an IgG-treated control (Lane 3). *Experiments performed in conjunction with Dr. Ritika Tewari.*

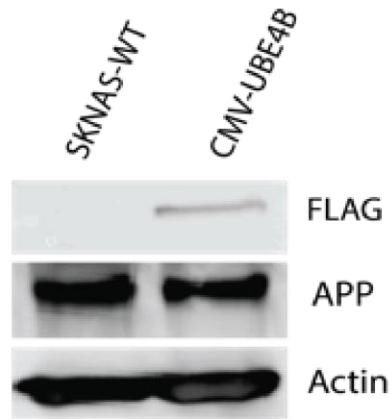
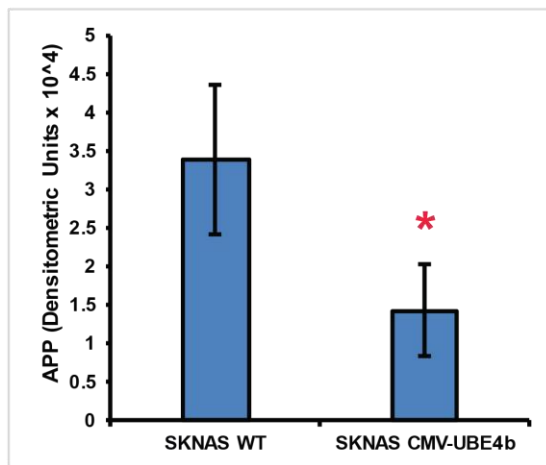
A**B**

Figure 5C. Overexpression of UBE4B leads to decreased APP levels in lysate. (A)

UBE4B was overexpressed under a CMV promoter in SK-N-AS, a neuron-like human cell line (Lane 2). As a control, untransfected SK-N-AS cells are shown in Lane 1. (B) Increased UBE4B led to significantly reduced levels of APP in cell lysate, as demonstrated by quantitation. For these experiments, n=3 and p=0.03.

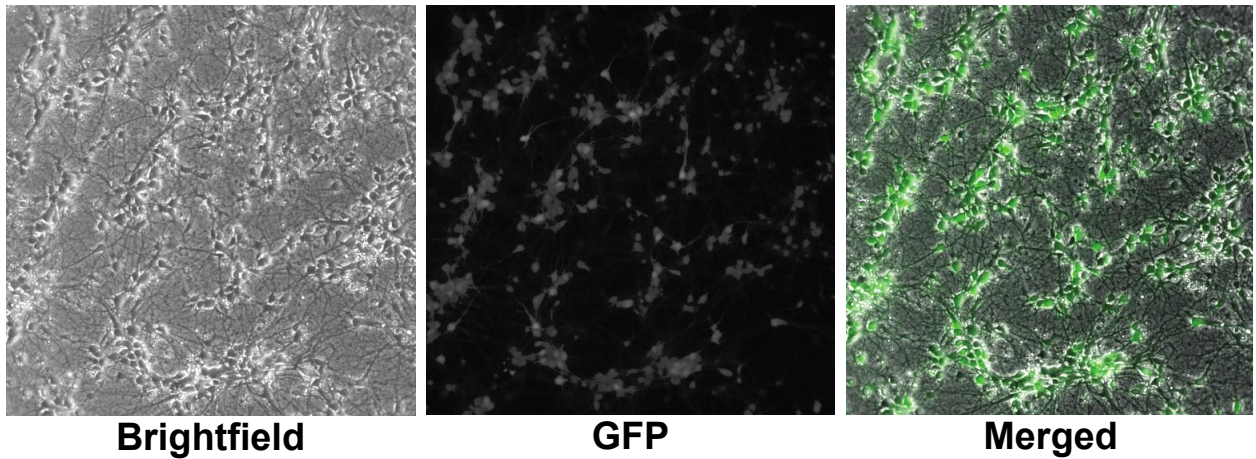


Figure 5D. Efficiency of lentiviral transfection in primary cortical neurons. Primary cortical neurons (as shown in the first panel) were successfully transduced with lentiviral particles packaged with a pFUGW plasmid (which encodes the GFP gene), as shown by the abundant expression of GFP in culture (second panel). The merged (third) panel demonstrates the high efficiency of transduction.

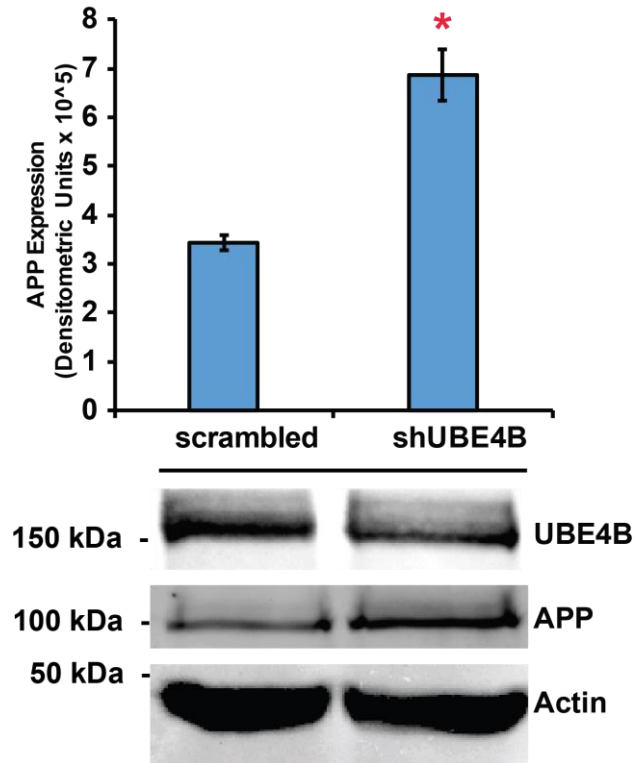


Figure 5E. UBE4B depletion in primary cortical neurons leads to increased APP levels in lysate. UBE4B was depleted from primary cortical neurons using lentivirally-delivered shRNA (as detailed in Chapter 2). As a control, neurons were also transduced with virus containing a non-specific shRNA (scrambled, Lane 1). Depletion of UBE4B was confirmed by immunoblotting (Lane 2). Depletion of UBE4B leads to significant increases in APP levels in lysate, as shown by quantitation. For these experiments, n=3 and p=0.04.

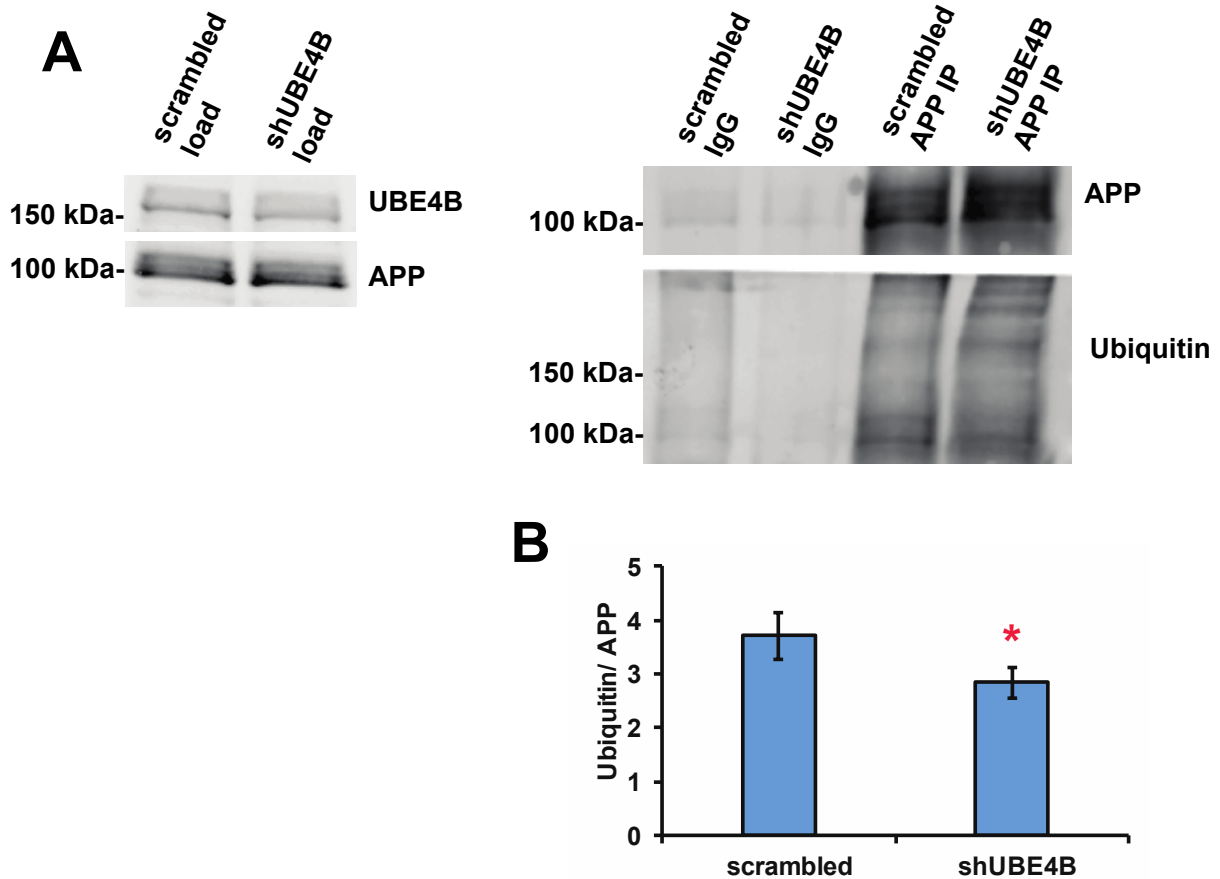


Figure 5F. Absence of UBE4B leads to decreased APP ubiquitination. Lysates for immunoprecipitation were obtained from primary cortical neurons treated with lentivirus packaged with either scrambled or scrambled shRNA against UBE4B. (A) Left, a third of the material used for immunoprecipitation was used as a loading control. Right, lysates were immunoprecipitated with anti-APP or a nonspecific IgG control antibody. Immunoprecipitated materials were immunoblotted with antibodies against APP or ubiquitin. Immunoblot against ubiquitin was quantitated and normalized to immunoprecipitated APP in the scrambled and shUBE4B conditions. For these experiments, $n=5$ and $p=0.041$.



Figure 5G. Immunodepletion of UBE4B from rat brain cytosol. UBE4B was immunodepleted from rat brain cytosol, and confirmed via immunoblotting (Lane 2). As a control, a separate sample of rat brain cytosol was immunodepleted with a nonspecific IgG control (Lane 1). UBE4B levels are markedly decreased in the Δ UBE4B condition, compared to IgG-treated cytosol.

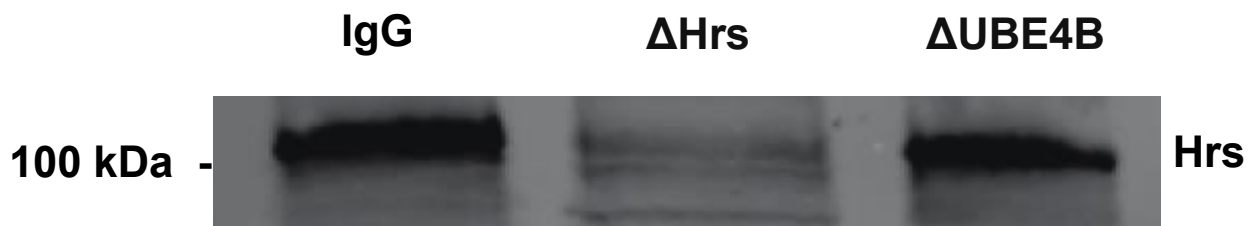


Figure 5H. Depletion of UBE4B does not affect Hrs levels in cytosol. UBE4B was immunodepleted from rat brain cytosol, and confirmed via immunoblotting (See Figure 5G). Immunodepletion of UBE4B did not affect levels of the early ESCRT component, Hrs, known to bind UBE4B and required for the efficient trafficking of many membrane proteins at endosomes, including APP (detailed in Chapter 4). Lane 1 shows IgG-treated cytosol. Lane 2 shows cytosol immunodepleted for Hrs. Lane 3 shows cytosol immunodepleted for UBE4B.

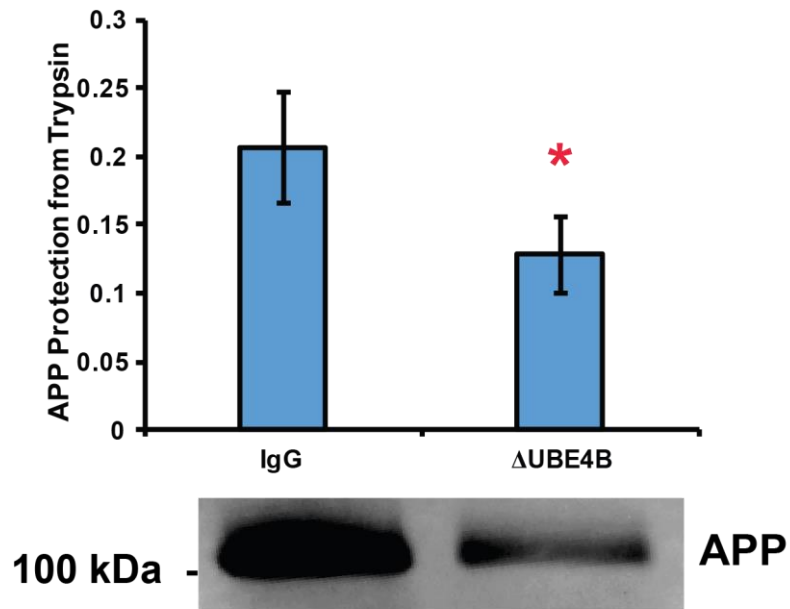


Figure 5I. Absence of UBE4B leads to decreased APP protection from trypsin. APP-containing early endosomes were incubated with IgG-treated cytosol (Lane 1) or Δ UBE4B cytosol (Lane 2). After trypsin cleavage, APP levels are significantly decreased in the Δ UBE4B condition, when compared to IgG control. In these experiments, $n=4$ and $p=0.033$.

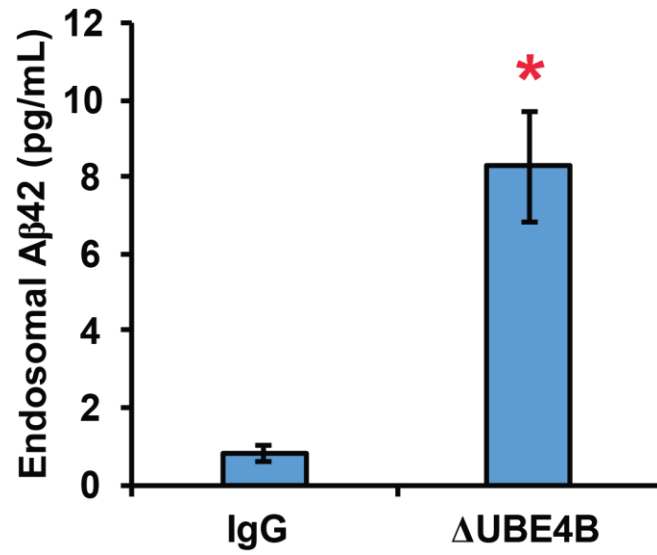


Figure 5J. Absence of UBE4B leads to significantly increased levels of Aβ42 in endosomes. APP-containing early endosomes were incubated with IgG-treated cytosol (Lane 1) or ΔUBE4B cytosol (Lane 2). After incubation, endosomes were recollected and analyzed for Aβ42 via ELISA. Aβ42 levels significantly increased in the ΔUBE4B condition, when compared to IgG control. In these experiments, n=3, p=0.04.

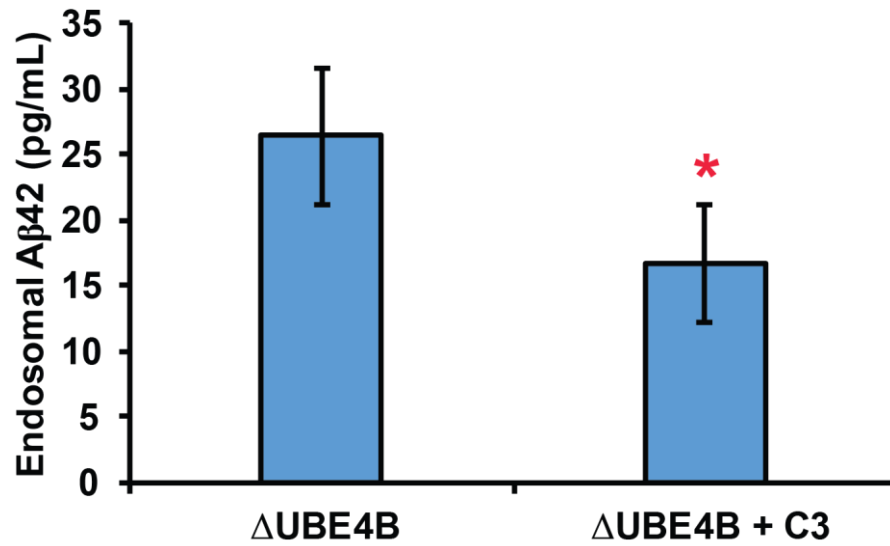


Figure 5K. Production of Aβ42, caused by absence of UBE4B, can be significantly decreased in the presence of the BACE inhibitor, C3. APP-containing early endosomes were incubated with 20uM C3 prior to incubation with ΔUBE4B cytosol. After endosomal recollection, endosomes were analyzed for Aβ42 levels via ELISA. For these experiments, n=4, p=0.019.

Discussion

The trafficking of APP through subcellular compartments has been a matter of intense investigation because of the role that these trafficking events may play in its amyloidogenic cleavage. Reports suggesting that APP may be cleaved at specific locations in the secretory and endocytic pathways have not clarified the primary sites underlying pathogenic APP fragment formation (3, 330–334). While the trans-Golgi network (TGN) and endosomes are likely important sites for production of A β , I focused my investigation on endosomal APP trafficking because of the strength of the evidence in favor of amyloid production at this cellular site. First, abnormal endosomal morphology and increased endosomal accumulation of pathogenic A β 40 and A β 42 peptides in the brain of individuals with dementia suggest that endosomal dysfunction may be key to its pathogenesis (335, 336). Second, APP and β -secretase (BACE1) co-localize on endosomes (299, 300, 337, 338). Third, both BACE1 (103, 112, 297) and the γ -secretase complex are enzymatically active at endosomes (83, 96, 123, 125). Fourth, BACE1 has optimal activity at the low pH found in endosomes (298, 339), and BACE1 inhibitors tethered to endosomes result in decreased APP processing into A β *in vitro* (340). Given the topology of APP on the endosomal membrane, amyloidogenic APP cleavage into A β would result in A β accumulation in the lumen of the endosome. Fifth, internalization of APP from the PM into endosomes is required for secretion of A β into the extracellular space (312, 315). These data point to endosomes as an important site for pathogenic APP cleavage. The findings of my dissertation contribute to this important scientific discussion as they approach the problem in a novel way, focusing on neuronal endosomes containing endogenous APP using biochemical and cell biological approaches.

6.1 Endogenously Expressed Amyloid Precursor Protein (APP) is localized on neuronal endosomes

The available literature on APP trafficking through endosomes has been confounded by the differences in cellular models (e.g. heterologous cell lines, neurons differentiated *in vitro*) used to study this process (3, 4, 89). Murine models have been used extensively in studies that inform our current understanding of A β production in the brain (341–344). I used a tractable model system that is relevant for APP processing in brain, primary cultured rat cortical neurons, to understand the cell biology of APP trafficking,

To confirm that cultured cortical neurons from my preparations expressed APP, I performed a series of experiments using two different APP antibodies, as well as antibodies against the neuronal marker, MAP2 (see Figure 3A). The results from that experiment confirmed that APP is mostly expressed in neuronal cells in my preparations. Moreover, their morphology provides evidence that the neurons within my preparation are healthy (321, 345, 346). It has been my experience that alterations in the culturing of these delicate cells greatly impacts the quality of the results obtained, and variance in neuronal health can interfere with observations. Aside from morphology, colocalization of APP with a neuronal marker (MAP2) provides evidence that in my preparation, APP is expressed in mostly in neurons, as opposed to glial cells or other brain associated cells that could contaminate cultures. In my experiments, no pharmacological agents were used to suppress the growth of supporting cells, so all experiments were performed between 5-10 days *in vitro*. It is important to note that these neurons are still considered developing neurons, which could impact my results. However, it is important to remember the conserved nature of the biological problem being examined and the fact that the mechanisms I have studied are similarly preserved in organisms across the eukaryotic taxon.

Much of the previous research examining APP trafficking through endosomes has been performed using cell lines overexpressing APP, expressing tagged APP, or with

altered compartments that facilitate examination of their endosomal trafficking. This work has provided an essential framework that informs our current understanding of the itinerary of this protein within the cell, and that has laid the foundation for the work exposed in this dissertation. However, there are abundant examples in the scientific literature in which overexpression of a protein can saturate trafficking mechanisms in the cell in both endocytic and secretory pathways (347–349), and can obscure the interpretation of data generated under such conditions. Moreover, fusion of large epitope or fluorescent tags (e.g. green fluorescent protein (GFP) can change the protein structure or cause multimerization (350–352), that can potentially alter protein function. For these reasons, examining the trafficking of endogenously expressed APP without the addition of tags is appealing and likely more accurately reflects trafficking in the human condition where APP is only overexpressed under limited circumstances, as in Down Syndrome (55, 64–66).

To examine the trafficking of a membrane protein through endosomes, many studies employ pulse-chase methods in which binding of a ligand to a surface protein, triggers its internalization into endosomal compartments (353–356). However, the absence of an established ligand for APP (4) made the possibility of a pulse-chase experiment unfeasible. Although some studies have observed that events like synaptic activity can trigger APP internalization (315–317, 357), I wanted to understand whether, in the absence of investigator-driven stimuli, APP was localized in endosomal compartments. Our results confirmed that APP colocalized with early and late endosomal markers within our cellular model (Figure 3B), suggesting that in the absence of exogenous stimuli, APP enters the endocytic pathway as part of its typical itinerary.

The use of velocity gradients to separate cellular organelles in a variety of tissues and cells (including neurons) has been extensively described (324, 327, 358, 359). I observed APP in fractions enriched in both early endosomes and fractions enriched in mixed (early and late) endosomes (Figure 3C). It is likely that the mixed endosomal peak

reflects intermediate states of the maturing endosome (150, 152, 154–156, 360).

Interestingly, I observed that APP also peaks in fractions that coincide with the endosomal peaks, suggesting that, at steady state, APP can localize to endosomes. However, BACE1 and PS1 (the enzymes that modify APP) seem to only be enriched in the mixed endosomal fraction. Other studies in the literature have observed APP-modifying enzymes to be present in endosomes (299, 300, 337), but their absence in early endosomal fractions suggests they can encounter APP at specific endosomal subpopulations. Despite this, it is still plausible that these enzymes are present (although not enriched) in early endosomal fractions, as evidenced by the accumulation of amyloid beta in *in vitro* assays, a product of the specific cleavage of APP by these enzymes.

Since the fractions obtained from this gradient are not truly pure preparations of each endosomal subpopulation, I confirmed that protease protection (173, 328) was a way to assess the location of APP with respect to the maturing endosome (Figure 3D). The differential response I obtained to protease protection with respect to the C-terminal epitope of APP in these two subpopulations allowed me to tailor a previously developed cell-free endosomal reconstitution assay (173, 296, 325) to the investigation of APP movement through endosomes.

It is important to note that this gradient isolation of endosomes only separates these organelles based on their density. It cannot distinguish between endosomes destined to target particular neuronal projections, such as axons or dendrites, versus those localized to the neuronal soma (361–363). Two recent studies(299, 300) showed that APP interacted with BACE-1 preferentially at endosomes from dendritic spines, presynaptic boutons, and axons. However, while these endosomal subpopulations might have different cellular destinations, they have been characterized using endosomal markers similar to those used in this dissertation (299, 300). It is currently not possible to identify or separate these

subpopulations for *in vitro* use. Future studies could identify the neuronal location where the endosomal proteins identified in these studies affect APP trafficking in intact neurons.

6.2 Inward budding of endogenous APP into the mature endosome lumen

I observed that both yeast and mammalian cytosol supported the movement of APP from the endosomal limiting membrane into internal vesicles, as shown by the increase in protease protection that resulted from incubating these early endosomes with each type of cytosol (Figure 4A). This result provides further evidence of the conserved nature of endosomal trafficking machinery (as discussed in the Introduction). Furthermore, the successful implementation of this *in vitro* examination of APP trafficking in neuronal endosomes will allow future studies to examine the trafficking of other membrane proteins in the brain using this approach.

The data presented supports our interpretation that trypsin protection can assess the location of APP with regard to the maturing endosome, and that cytosolic components support the development of internal vesicle formation in endosomes, even under cell-free conditions. Previous studies from the Bean laboratory have characterized this assay using other tools such as electron microscopy (173, 296, 325). However, it could be that the differential response to trypsin we observe in early vs mixed endosomal populations results from other factors (e.g. inhibitors to trypsin present in the preparation). In collaboration with Dr. Neal Waxham, I have performed preliminary experiments to address these questions and confirm that our *in vitro* assay can recapitulate endosomal maturation and internal vesicle formation in primary cortical neurons. In these experiments, we are using cryo-electron microscopy to examine the distribution of multi-vesicular endosomes (which have a distinct morphology) in endosomal fractions obtained from discontinuous sucrose gradients. This will allow us to assess the degree of endosomal subpopulation separation yielded by the gradient by using a different parameter (endosome morphology) besides endosomal

molecular markers. This assessment will also allow us to confirm whether the cell-free endosomal reconstitution assay leads to quantifiable increases in multi-vesicular endosomes within our assay reactions. Published work from the Bean laboratory has previously used electron microscopy to confirm this in heterologous cell lines (173), but not in primary neuronal cultures.

The acquisition of early endosomal membranes by using velocity gradients, and subsequent cell-free reactions yields very small amounts of material. I observed that successful immunoblot detection of APP on endosomes required at least 10 million neurons per cell free reaction. For this reason, I limited my investigation of the role of ESCRT proteins in APP trafficking to one component per ESCRT complex (described in Figure 4B). Using the described assay and yeast deletion strains for a component of all ESCRT complexes, I observed that members of the ESCRT 0, I, II, and III complexes are needed for efficient movement of APP from the endosomal limiting membrane into internal vesicles (Figure 4C). These data confirm previous observations (305–307) that ESCRT proteins are involved in APP endosomal trafficking, and strengthen the evidence supporting the endocytic pathway as an important cellular site for APP trafficking in neurons. However, there are differences in how these previous studies hypothesized endosomal production of A β occurs as a result of the absence of early (ESCRT-0, I) versus late (ESCRT-II, III) ESCRT proteins. As discussed in the introduction, Choy et al.(305) observed that deletion of early ESCRT components retained APP at endosomes and inhibited A β 40 secretion, whereas deletion of a late ESCRT component redirected APP into secretory compartments and enhanced secretion of A β 40. Morel et al.(306) observed that deletion of early ESCRT components also resulted in APP retention in endosomes (specifically, the endosomal limiting membrane), but led to increased A β 40 secretion. Finally, Edgar et al.(307) observed that deletion of early ESCRT components led to increased intracellular A β 40, but decreased A β 40 secretion.

Using yeast deletion strains, I observed that absence of early and late ESCRT components led to a small but statistically significant increase in endosomal accumulation of A β 42 (Figure 4D). Due to limitations in the amount of neuronal material available, I chose to only examine A β 42 production in endosomes because of its increased pathogenicity and greater association with the development of AD (see Introduction), and because previous studies on the endosomal production of amyloid did not examine production of this species. Also, since there was significant controversy in the literature specifically regarding the role of early ESCRT proteins in amyloid production at endosomes (305–307), I examined how depletion of an early ESCRT, Hrs, affected APP trafficking. My results show that Hrs is required for efficient movement of full-length APP into the endosomal lumen (Figures 4E-F). Absence of Hrs dramatically increased A β 42 production at endosomes (Figure 4G).

There were differences in the levels of endosomal A β 42 generated in cell-free reactions containing yeast cytosol compared with reactions containing mammalian cytosol. Although the endosomal trafficking machinery is generally well conserved across species, it appears that the requirements for amyloid production through cleavage of APP are less efficient in reactions containing yeast cytosol in our *in vitro* reconstitution assay. BACE-1, is a transmembrane protein (104) that is not present in the yeast genome (364), so it must therefore be contributed by its presence on neuronal endosomes. BACE-1 can cleave APP in cell-free conditions (365), suggesting it does not require other cofactors for this activity. The γ -secretase complex requires the integration of 4 members of the complex (presenilins 1 and 2, APH, and nicastrin), none of which are expressed in yeast (366, 367) suggesting that they too are contributed on neuronal endosomes. The γ -secretase complex has been reconstituted by expression in yeast and shown to be sufficient for the cleavage of APP (368, 369). Thus, while both BACE-1 and the γ -secretase complex are capable of APP cleavage in the absence of co-factors, amyloidogenic cleavage of APP requires localization of APP and BACE-1 on lipid rafts (370–373). It is possible that the yeast cytosol does not

efficiently recapitulate this clustering of APP and the secretases in the appropriate endosomal microdomains.

The observation of intra-endosomal accumulation of A β 42 under conditions where both early (examined using yeast and mammalian cytosols) and late (examined using yeast cytosols) ESCRT components are deleted contrasts with the observations made by Choy et al.(305), where only absence of late ESCRTs led to increased amyloid production. Although my studies did not investigate the secretion of A β under conditions where ESCRTs were deleted from whole cells, my results are in accordance with the observations made by Morel et al. (306) and Edgar et al.(307) who showed that deletion of early ESCRTs causes generation of amyloid species in endosomes. The discrepancies in the results of these investigations might be due to differences in the cell model used, the type of APP examined (human vs. murine), and the degree of expression employed (exogenous vs. endogenous). However, my experiments do not address all aspects of the fate of APP in the absence of ESCRTs. It is likely that only a fraction of APP gets cleaved into amyloid at the endosome, and that some APP may be exported from endosomes in budded vesicles. This hypothesis is consistent with the observations made by Choy et al. (305), who showed that deletion of Hrs led to increased recycling of APP into the TGN. The cell-free assay used herein has been recently adapted to examine budding of vesicles from the endosomal limiting membrane (unpublished work from Bean laboratory). Such an approach could be used to examine whether APP may traffic from endosome-TGN in the absence of ESCRTs, and to elucidate the molecular machinery that may be required for this itinerary.

My observations are limited to the production of A β 42 at endosomes, but do not address where these endosomes may be targeted within neurons. Late endosomes may fuse with the lysosome (174, 374, 375) or with the plasma membrane (168, 376, 377) although the mechanisms distinguishing these fusion events are unclear. A β production in these two different populations of endosomes would have different outcomes:

in one case A β would remain inside neurons and in the other A β would be exported into the extracellular environment. Although the classical AD pathology involves aggregation of amyloid species outside of neurons, many studies (4, 51–53) have observed that intraneuronal accumulation of A β is toxic to neurons and might precede extracellular deposition.

Finally, it is not clear whether the degree of amyloid production caused by endosomal disturbances is sufficient to drive AD pathogenesis. As discussed in the Introduction, animal models deficient in ESCRT proteins have severely affected phenotypes, and any *in vivo* investigation of the role of ESCRTs in amyloidopathies would be obscured by the many other afflictions that these animals would develop. However, Morel et al. (306) observed decreased expression of components of the endosomal sorting machinery in the brains of people with AD, suggesting that these defects might contribute to pathogenesis in humans.

6.3 UBE4B is an E3 ubiquitin ligase that binds to, ubiquitinates APP and mediates efficient APP trafficking on endosomes

The involvement of ESCRT proteins in APP trafficking suggests that APP is ubiquitinated in its passage through the endocytic pathway, since ESCRT proteins enable endosomal sorting by binding to ubiquitin moieties on cargo proteins at the endosomal limiting membrane (203, 217, 256, 273, 378). In this regard, a previous publication(306) has demonstrated that APP is ubiquitinated at endosomes and that this ubiquitination facilitates its movement into mature endosomes. Aberrant ubiquitination has been correlated with AD (379–383), specifically the abnormal accumulation of ubiquitinated proteins inside endosomes of diseased neurons (384, 385). However, the identity of the E3 ligase (the ubiquitinating enzyme that is thought to provide specificity to the ubiquitination process, as detailed in the Introduction) that ubiquitinates APP at endosomes has been unclear.

APP ubiquitination has been correlated with its trafficking in previous studies. Ubiquitin-mediated ubiquitination of APP has been observed, although it occurs in secretory compartments (386, 387), prior to insertion in the plasma membrane. Also, the E3 ligase FBL2 was identified by microarray data as a gene downregulated in the hippocampus of AD subjects (388). A follow-up study discovered that overexpression FBL2 decreased production of A β 40 and A β 42 in murine hippocampal neurons (319). Although FBL2 expression did not significantly impact cellular APP levels, the study demonstrated that FBL2 can ubiquitinate APP at lysine 726 and increase its degradation (319). Interestingly, ubiquitination of APP by FBL2 at the cell surface inhibited APP endocytosis suggesting that FBL2 may play a role at the plasma membrane regulating APP internalization (Watanabe 2012). In this regard, the ubiquitination of membrane proteins at the plasma membrane can, but does not always, trigger the internalization of membrane proteins (389–393). In another study (306), mutations of lysine residues 724-726 of APP led to decreased ubiquitination of APP, as well as increased A β 40 production and retention of APP at the endosomal limiting membrane.

In my experiments, I observed that the E3 ligase UBE4B affects the endocytic trafficking of APP in neurons. It binds to APP, potentially affects its ubiquitination, and alters APP levels in lysate. My observation that APP levels are significantly increased in neuronal lysate in the absence of UBE4B underscores the importance of this ligase in APP homeostasis in neurons. The only other E3 ligase known to ubiquitinate APP, FBL2, does not affect cellular levels of full-length APP.

As discussed in the Introduction, UBE4B has been implicated in the endosomal trafficking of other membrane proteins (296). In this previous study from the Bean laboratory, UBE4B is recruited to endosomes by binding to the early ESCRT protein Hrs. Depletion of UBE4B in HeLa cells did not affect levels of Hrs or other endosomally associated proteins in lysate, suggesting that it does not indiscriminately affect expression of

other important trafficking proteins (296). Of note, I observed that immunodepletion of UBE4B from rat brain cytosol did not affect Hrs levels, suggesting that the effects of UBE4B on APP endosomal trafficking observed with the cell-free assay are not due to depletion of Hrs.

My results demonstrate that UBE4B aids in the efficient movement of APP through endosomes. It is important to underscore that the interaction between APP and UBE4B I examined in cell-free reconstitution reactions occurs at endosomes, where APP is located prior to incubation with UBE4B-containing cytosol. These results do not exclude upstream interactions between APP and UBE4B molecules, but highlight the consequences of this interaction at the endosomal limiting membrane. While these results emphasize the importance of UBE4B as an endosomally-associated peripheral membrane protein, it is important that future investigations identify whether other protein targets are affected by UBE4B at endosomes. If UBE4B has many endosomally localized targets whose trafficking is affected by its activity, it would complicate the potential exploitation of the UBE4B-APP interaction for therapeutic purposes.

In my studies, the absence of UBE4B led to dramatic increases in endosomal A β 42. The 7-fold increase in A β 42 production observed in the absence of UBE4B is similar to the effect that deleting an ESCRT protein, Hrs, has on A β 42 production. Further, the observation that pharmacological inhibition of BACE-1 in our cell-free assay leads to significant inhibition of the increases in A β 42 production produced by Hrs or UBE4B depletion, suggests that active cleavage of APP by BACE-1 during the reaction incubations results in amyloid production. These observations suggest that endosomal sorting of wild-type APP from the endosomal limiting membrane to internal vesicles within the endosomal lumen is a key regulatory step in the balance between APP elimination and A β production. In the context of endogenous expression of APP, depletion of Hrs and UBE4B leads to such

dramatic changes in A β 42 production that it is possible that alterations in this APP trafficking step could result in pathological levels of A β in patients.

Although the *in vitro* experiments performed in this dissertation propose a role for UBE4B in the formation of A β , the relevance of this result to *in vivo* conditions has yet to be examined. Deletion of UBE4B results in embryonic lethality in mice (289), so conditional knockouts would have to be generated to investigate whether absence of UBE4B in the brain could lead to accumulation of amyloid species, as would be predicted by my results.

E3 ligases like UBE4B have been observed to confer specificity to the ubiquitination process, and are thought to have limited protein targets. This makes them suitable as potential therapeutic targets (200, 214, 216, 394), since alteration of their activity would likely have decreased potential for off-target effects. Future experiments could investigate the therapeutic role of UBE4B in the context of A β -related diseases like AD. For example, one might envision that overexpression of UBE4B could stimulate increased trafficking of APP into the endosomal lumen and may prevent A β production. Future investigations could explore UBE4B as a drug target for AD and other amyloidopathies of the brain.

6.4 Future Directions

The results discussed in this dissertation have examined the endocytic itinerary of APP, and determined that disruptions in this trafficking result in increased amyloid production. These findings also raise new questions that could drive further research in this field.

1. To determine whether endosomally produced A β 42 is actively secreted from neurons.

A pressing question raised by the observation of endosomal accumulation of A β 42 is whether this A β 42 is secreted from cells. As discussed, intracellular A β can also be toxic to neurons, but previous work has shown that endosomal anomalies lead to increased A β

accumulation in conditioned media from cells (305–307). To address this question, I am pursuing experiments to examine how knockdown of UBE4B in neurons affects the accumulation of A β 42 extracellularly.

If it is the case that A β 42 is secreted as a result of abnormalities at endosomes, how is this A β 42 exported? As discussed in the introduction, endosomes can potentially fuse with the plasma membrane, and release its luminal contents and internal vesicles to the extracellular space. Evidence for this event is the observation of nanovesicles in the extracellular space that contain endosomal markers such as Tsg101 and the ESCRT-associated protein Apoptosis-Linked Gene 2-Interacting Protein X (Alix) (178, 181, 183, 377, 395). Upon secretion, these nanovesicles of endosomal origin are called exosomes, and are characterized as 100-200nm vesicles expressing endosomal proteins, such as Alix and Tsg101 (396), as well as lipid raft markers such as Flotillin(396, 397). Exosome release has been documented in a variety of cell types and body fluids (185, 186, 398, 399), including neurons (189–191). There is controversy regarding what physiological function exosomes have, and how they are secreted. However, in a 2008 study(168), Trajkovic and others observed that inhibition of nSMase2 (led to decreased release of exosomes. Since then, multiple studies have replicated this effect in a variety of cells (400, 401), including neuron-like cells (192, 402). This effect has also been observed in *in vivo* experiments (403–405). I am actively pursuing experiments to address whether inhibition of endosome-PM fusion can block the release of endosomally accumulated A β 42.

2. To determine whether UBE4B can inhibit A β formation in the presence of disease-inducing APP mutants.

Another interesting aspect of these studies that could be explored is the potential for UBE4B to be used therapeutically to modulate production of A β . In the 2004 study by Matsumoto et al., the authors observed that overexpression of UBE4B led to decreased

accumulation of another toxic protein, ataxin 3, that is also a target of UBE4B ubiquitination. A similar strategy could be used in our investigations, where increased UBE4B could force degradation of the full-length APP, and lead to decrease A β 42 levels induced by disease-causing mutations in APP.

The results of this dissertation suggest that UBE4B can ubiquitinate APP in living cells. However, the details of this ubiquitination were not explored. The work from Watanabe et al. and More et al. (306, 319) suggested that lysine residues 724-726 are crucial for APP ubiquitin. It would be interesting to verify whether these residues are ubiquitinated by UBE4B. This information would be beneficial not only for our mechanistic understanding of this relationship, but also because it would allow a more specific targeting of this interaction for therapeutic purposes.

3. To determine whether the itinerary of APP in endosomes leads to its degradation in lysosomes or its release via endosome-PM fusion.

The observation of APP in exosomes (194, 199) is in contrast with previous work showing that APP gets degraded at the lysosome (122, 146, 147, 197, 406). This raises questions regarding the itinerary of APP after arrival at the late endosome, and could also be the subject of future investigations. Furthermore, if the itinerary of APP is release on exosomes via endosome-PM fusion, what advantage would this give neurons? Is exosomal APP physiologically relevant? Future projects could help clarify whether APP is lysosomally degraded in neurons, or exported in exosomes upon endosome-plasma membrane fusion. Moreover, if both observations are true, it would be of interest to investigate whether different subgroups of late endosomes lead to these divergences in the itinerary of APP, and what signals this segregation of APP on endosomes.

4. To explore whether APP can traffic from endosomal compartments into outwardly budded vesicles, and how the retromer complex modulates this process.

The subcellular site of APP cleavage into A β species has been highly controversial in the scientific literature (3, 4). My results suggesting that APP is processed through the amyloidogenic pathway at endosomes do not exclude the possibility that APP also gets cleaved into A β in secretory compartments. The retromer complex is a coat complex that has been established in retrograde trafficking from endosomes into the trans-Golgi network, TGN (137, 407–410). The retromer complex has been implicated in Alzheimer's disease, although the direction of that relationship (whether increased or decreased expression of retromer components leads to A β formation) has been controversial (reviewed in (411)). Choy et al. (305) observed that ESCRT deficiencies led to retromer-dependent trafficking of APP into the TGN, where it was processed into A β . While my investigations did not examine this route of trafficking for APP, recent unpublished work by the Bean laboratory has adapted our cell-free assay to the examination of outward budding events from the endosomal limiting membrane. This assay could be adapted to evaluate whether neuronal endosomes traffic APP outwardly in a retromer-dependent matter, and whether those vesicles already contain endosomally produced A β or whether the processing truly occurs in the TGN.

6.5 Conclusions

The findings of my dissertation contribute to the ongoing conversation regarding APP trafficking through endosomal compartments, and the production of A β as a result of trafficking disturbances. They address controversies in the literature regarding the role of ESCRT proteins in APP trafficking and A β production. Although these observations stem from *in vitro* reconstitution experiments, they suggest that, in the absence of pathogenic mutations in APP or the secretases, disturbances in endocytic trafficking of APP can lead to

marked increases in A β production. According to the amyloid cascade hypothesis (2, 41), imbalances in the production and clearance of A β can create a scenario that leads to brain disease, and the dramatic increases in A β production observed as a result of endocytic deficiencies in this study suggest that such mechanisms could underlie the pathogenesis of at least some individuals with AD and other diseases where A β accumulates.

A particular weakness of this investigation is the use of cell-free conditions to elucidate APP endosomal trafficking. While this assay has yielded information that I believe helps advance the field, the use of such assays disregards any regulatory mechanisms that the cell might employ to correct disturbances in trafficking. For example, it is feasible to envision how decreased UBE4B might, over time, lead the cell to also downregulate APP expression as a compensatory mechanism. In that sense, future experiments should design strategies that confirm these findings in whole cells, particularly neurons. However, the observation of how UBE4B impacts APP levels in lysate, as well as APP ubiquitination, lead me to believe that this compensatory mechanism might not be occurring, or at least might not occur before harm is done to the cell.

Like with other complex diseases of aging, it is likely that individuals develop similar disease phenotypes of dementia due to distinct pathological mechanisms (412, 413). In that sense, the findings of this study do not disprove that A β production can happen at other cellular sites as have been described, particularly the secretory pathway (120, 147, 299, 305). However, because A β accumulation is a common observation in demented patients, and dementia is so prevalent in our society, it is likely that endosomal cleavage of APP into A β is a relevant disease mechanism for at least some individuals.

Despite the significant controversies surrounding the pathogenesis of Alzheimer's disease and the role that A β plays in that process, there is an overwhelming literature that suggests excess A β is toxic to neurons. However, multiple failures in the development of therapeutic strategies that target A β have led to decreased enthusiasm for A β as a target for

dementia (4, 48, 413). Studies during the last 5 years (including this dissertation) have allowed enhanced mechanistic understanding of A β production in cells, and have particularly highlighted the endocytic pathway. This new understanding of the cell biology of APP could aid in the development of new therapeutics for AD, especially in patients in which the disease is caused by endocytic trafficking disturbances.

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Vita –

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