The endosomal-associated deubiquitinating enzyme USP8 regulates BACE1 ubiquitination and degradation

A thesis

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Abstract

β-site amyloid precursor protein cleaving enzyme (BACE1) is the rate-limiting enzyme in the production of amyloid beta (Aβ), the toxic peptide that accumulates in the brain of subjects affected by Alzheimer's disease. Previous studies have shown that BACE1 is degraded via the lysosomal pathway, BACE1 is ubiquitinated at lysine 501 and that blocking ubiquitination at lysine 501 results in BACE1 stabilization. Ubiquitin conjugation is a reversible process mediated by deubiquitinating enzymes (DUBs). The ubiquitin specific peptidase 8 (USP8), an endosomal-associated deubiquitinating enzyme, regulates the ubiquitination, trafficking and lysosomal degradation of several plasma membrane proteins. Thus, we hypothesized that USP8 regulates BACE1 ubiquitination, trafficking and lysosomal degradation. Here we report that USP8 directly deubiquitinates BACE1. In H4 human neuroglioma cells, RNAi-mediated depletion of USP8 increases BACE1 ubiquitination, promotes BACE1 accumulation in the early endosomes and late endosomes/lysosomes, and decreases BACE1 levels in the recycling endosomes. Furthermore, USP8 depletion reduces levels of both ectopically expressed and endogenous BACE1. Decreases in BACE1 are accompanied by the decreased formation of amyloid precursor protein C-terminal fragments (APP-CTFs) C99 and C89, products of BACE1 cleavage of APP. Moreover, Aβ levels are decreased. While USP8 depletion does not affect the levels of APP-CTFs in a manner independent of BACE1, USP8 depletion decreases the stability of the amyloid precursor protein intracellular domain (AICD) and reduces its cellular levels. Our findings demonstrate that USP8 plays a key role in the trafficking and degradation of BACE1 by deubiquitinating lysine 501. These

studies suggest that therapies able to accelerate BACE1 degradation (e.g. by increasing BACE1 ubiquitination) may represent a potential treatment for AD.

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List of Abbreviations

1,10-PNT	1,10-phenathoroline
ACTH	adrenocorticotropic hormone
AD	Alzheimer's disease
ADAM	a disintegrin and metalloproteinase domain-containing protein
AICD	amyloid precursor protein intracellular domain
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Aph1	anterior pharynx defective 1
APP	amyloid precursor protein
Aβ	amyloid beta
BACE1	β-Site amyloid precursor protein-cleaving enzyme
BRIT1	breast cancer susceptibility gene C terminus-repeat inhibitor of human telomerase repeat transcriptase expression
CDC25Mm	RAS protein-specific guanine nucleotide-releasing factor 1
CHQ	chloroquine
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
CTF	carboxyl-terminal fragment
DOR	delta (δ) -opioid receptor
DPBS	Dulbecco's phosphate-buffered saline
DUB	deubiquitinating enzyme
EEA1	early endosome antigen 1
EGFR	epidermal growth factor receptor
ESCRT	endosomal sorting complex required for transport

FAD	familial Alzheimer's disease
FLT1	FMS-Related tyrosine kinase 1
GFAP	glial fibrillary acidic protein
GGA3	Golgi-localized γ-ear-containing ARF binding protein 3
GRAIL	gene related to anergy in lymphocytes
HRS	hepatocyte growth factor regulated tyrosine kinase substrate
ILV	intraluminal vesicle
IP	Immunoprecipitation
KCA3.1	potassium intermediate/small conductance calcium activated channel, subfamily N, member 4
LAMP2	lysosomal associated membrane protein 2
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1
MCPH1	microcephalin
mEPSP	mini excitatory postsynaptic potentials
MIT	microtubule interacting and transport
MVB	multivesicular body
NEM	N-ethylmaleimide
NRDP1	neuregulin receptor degradation protein-1
NRG1	neuregulin-1
NSCLC	non-small cell lung cancer
NT	non-targeting
NTF	amino terminal fragment
PAR2	protease-activated receptor 2

Pen-2	presenilin enhancer 2
POMC	proopiomelanocortin
PS1	presenilin 1
RAB11	ras-related GTP-binding protein 11
RIPA	radioimmunoprecipitation assay
STAM	signal transducing adaptor molecule
SH3	src homology 3
STAM	signal transducing adaptor molecule
TACE	tumor necrosis factor alpha-converting enzyme
TGN	trans-Golgi network
TRKA	tropomysin–related kinase A
TDP-43	TAR DNA binding protein;
TRKA	tropomysin–related kinase A
Ub	ubiquitin
USP8	ubiquitin specific peptidase 8
UCHL1	ubiquitin carboxyl-terminal hydrolase isozyme L1
VEGFR2	vascular endothelial growth factor

Chapter 1: Introduction

1.1 Role of BACE1 in Alzheimer's disease

Alzheimer's disease (AD) is a progressive, neurological disorder of aging. Persons with AD suffer with memory, language and visuospatial impairments, and struggle with performing activates of daily living (Seeley and Miller 2015). AD is characterized by numerous pathological defects, including neuronal death, formation of intracellular hyper-phosphorylated tau aggregates, and formation of extracellular senile plaques (Seeley and Miller 2015). The extracellular senile plaques of AD are primarily composed of amyloid beta (Aβ), a 4kDa peptide, 38 to 43 amino acids in length. The physiological role of Aβ has not been well established. However, recent studies suggest that Aβ may play an antimicrobial role as part of the innate immune system (Kumar et al. 2016, Soscia et al. 2010).

AD may be divided into 2 large classes: familial (FAD) and sporadic AD (SAD). FAD, the hereditary form of AD, is characterized by an early onset and is often diagnosed in persons under the age of 60 (Seeley and Miller 2015). Both FAD and SAD are associated with an increase in total Aβ or an increase in the ratio of Aβ42 to Aβ40 (Yan and Vassar 2014). Aβ is formed by an intracellular process, whereby amyloid precursor protein (APP) is first cleaved by beta (β)-secretase, then by gamma (γ)-secretase (Yan and Vassar 2014, Kang et al. 1987).

β-site APP-cleaving enzyme (BACE1) has been identified as the β-secretase responsible for cleaving APP in the amyloidogenic pathway (Vassar et al. 1999, Sinha et al. 1999, Hussain et al. 1999, Lin et al. 2000, Yan et al. 1999). BACE1 cleavage of APP

is essential for the formation of Aβ. BACE1 knockout mice do not form Aβ, (Cai et al. 2001, Luo et al. 2001) while double transgenic rodents, which overexpress BACE1, along with wild type or mutated human APP, have increased Aβ formation (Bodendorf et al. 2002, Chiocco et al. 2004, Mohajeri, Saini, and Nitsch 2004, Ozmen et al. 2005).

BACE1 plays an integral role in both SAD and FAD (Fukumoto et al. 2002, Li et al. 2004, Tesco et al. 2007, Holsinger et al. 2002, Santosa et al. 2011, Pera et al. 2013). With regards to SAD, which accounts for 95% of AD cases (Agostinho et al. 2015), multiple investigators have demonstrated that increased BACE1 protein levels and activity are associated with increased Aβ levels (Fukumoto et al. 2002, Li et al. 2004, Tesco et al. 2007, Holsinger et al. 2002, Santosa et al. 2011, Pera et al. 2013). In FAD, several autosomal dominant mutations, predominantly in APP and γ-secretase, result in increased Aβ formation (Agostinho et al. 2015). Many of these mutations within APP, occur near the β and γ- secretase cleavage sites. For example, two mutations in APP, near the BACE1 cleavage site, are associated with FAD. This pair of mutations, known as the Swedish mutation, increases BACE1 cleavage of APP, and thus increases the formation of Aβ (Mullan et al. 1992, Yan and Vassar 2014). A mutation protective for AD at this location has also been described (Jonsson et al. 2012). The Ala673Thr mutation was found to occur more frequently in healthy controls than in AD patients. In vitro, the mutation resulted in a decrease of BACE1 cleavage of APP by 50% and resulted in a 40% decrease in both Aβ40 and Aβ42 formation, providing further support for the role of β-secretase-mediated APP processing in human cognition.

Mutations affecting BACE1-mediated APP processing are not only limited to those within APP, near the BACE1 cleavage site. 2 rare mutations in ADAM10, an alpha

(α) secretase known to cleave APP, were found to be associated with an increased risk of late-onset AD (Kim et al. 2009). These mutations resulted in decreased ADAM10 activity, and were likely due the formation of misfolded ADAM10 (Suh et al. 2013, Vassar 2013). Increased β-secretase processing of APP and increased Aβ formation were also observed *in vitro* and in transgenic mice with these ADAM10 mutations. The observed increase in BACE1 activity was likely due decreased competition from α-secretase for the substrate, APP (Suh et al. 2013).

1.2 BACE1 overview

BACE1, a 501 amino acid transmembrane protein, is an aspartyl protease with two catalytic Asp-Thr/Ser-Gly-Thr/Ser (DT/SGT/S) sites (Citron 2004). BACE1 is found in multiple brain regions including the hippocampus, where BACE1 is highly expressed (Vassar et al. 1999). BACE1 is mainly expressed in neurons (Cole and Vassar 2007), where it has been found not only in cell bodies and dendrites, but also in axons and at pre-synaptic terminals (Buggia-Prevot et al. 2014, Del Prete et al. 2014).

Although BACE1 is well known for its pathological role in AD, APP is not BACE1's only substrate. BACE1 has over 25 known substrates, suggesting that it plays an important role in maintaining normal physiological function (Hu, Fan, et al. 2015, Yan and Vassar 2014). Indeed, BACE1's role in the maintenance of normal physiology is best exemplified by the BACE1 knockout mouse, which has an abnormal phenotype due to decreased processing of multiple BACE1 substrates (Yan and Vassar 2014).

For example, Neuregulin-1 (NRG1), a well-characterized BACE1 substrate is involved in maintaining proper axonal myelination. Decreased NRG1 processing may

cause the hypomyelination observed in BACE1 knockout mice (Willem et al. 2006, Hu, Hu, et al. 2015, Hu et al. 2008, Fleck et al. 2013). Furthermore decreased NRG1 processing may be responsible for schizophrenia-like behavior seen in BACE1 knockout mice, as NRG1 is also a known schizophrenia susceptibility gene (Seshadri et al. 2010, Harrison and Law 2006, Savonenko et al. 2008). Other important substrates of BACE1 include FMS-Related Tyrosine Kinase 1 (FLT1), a tyrosine-kinase receptor important for retinal health (Cai et al. 2012), and cell adhesion molecule L1-like (CHL1), a molecule important in axon guidance and outgrowth (Rajapaksha et al. 2011, Hitt et al. 2012).

Moreover, BACE1 protein is not only elevated in AD, but is also elevated after traumatic brain injury (Blasko et al. 2004, Uryu et al. 2007, Santosa et al. 2011, Walker et al. 2012). BACE1 levels are also increased under conditions of oxidative stress, suggesting that BACE1 may be part of the cellular stress response. However, BACE1 increase, under these conditions, appears to facilitate Aβ formation and ultimately may not be protective (Bulbarelli et al. 2012, Velliquette, O'Connor, and Vassar 2005, Tamagno et al. 2012).

1.3 Amyloid precursor protein processing

BACE1 is not the only enzyme that cleaves APP. APP, a type 1 transmembrane protein, is cleaved by 3 main secretases: beta (β) secretase, gamma (γ) secretase and alpha (α) secretase (Fig. 1.1A). BACE1 is the β-secretase responsible for APP cleavage (Sinha et al. 1999, Yan et al. 1999, Hong et al. 2000, Vassar et al. 1999). BACE1 cleaves APP at one of two sites, aspartic acid 1 (Asp1, Aβ numbering) or glutamic acid 11 (Glu1, Aβ numbering), located at the beginning of the Aβ region within APP (Vassar et al.

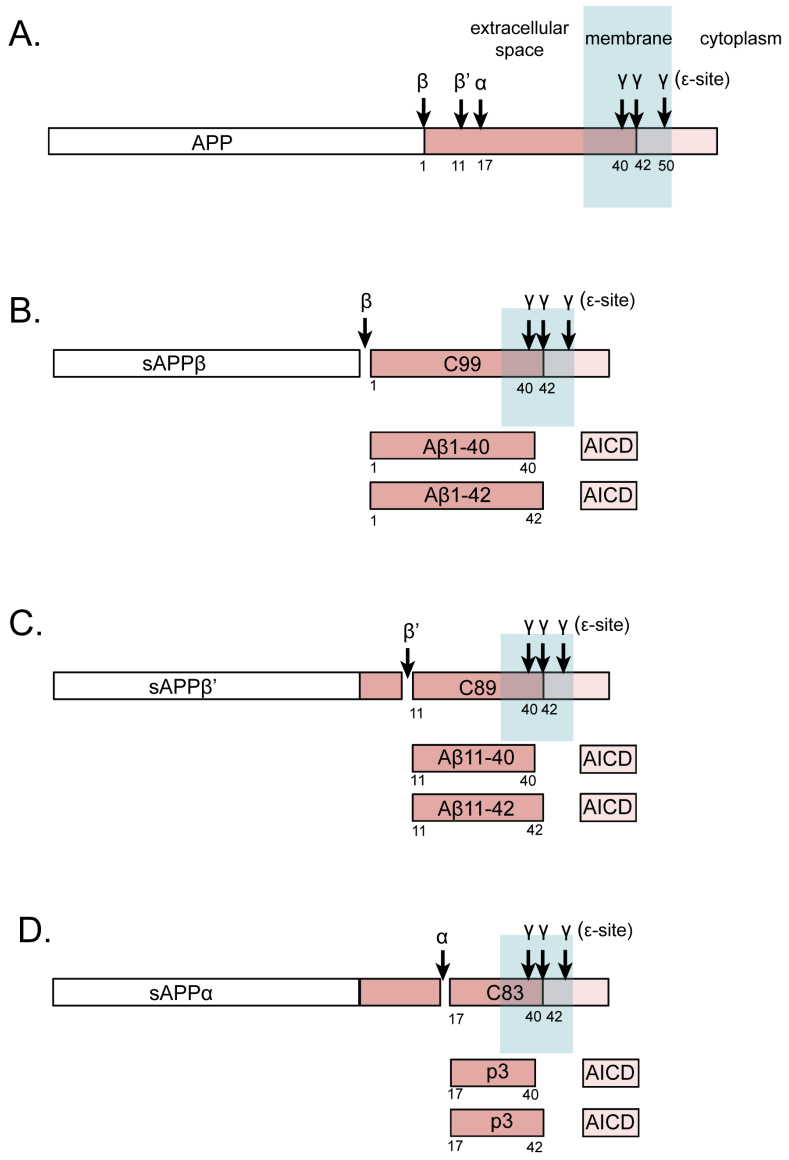

Figure 1.1

Amyloid precursor protein processing.

A) β-secretase cleaves APP at 2 locations at the start of the Aβ region. γ-secretase cleaves APP at multiple locations within the transmembrane region of APP, while α-secretase cleaves APP within the Aβ region. B) β-secretase cleaves APP at the beginning of the Aβ region (aa1) forming C99 and soluble APPβ (sAPPβ). C99 is cleaved by γ-secretase at the ε-site forming the AICD, then at aa40 or 42 forming Aβ1-40 or 1-42 C) β-secretase cleaves APP near the beginning of the Aβ region (aa11) forming C89 and soluble APPβ'. C89 is cleaved by γ-secretase forming Aβ11-40, 11-42, and the AICD. D) α-secretase cleaves APP within the Aβ region (aa17) forming C83 and sAPPα. C83 is cleaved by γ-secretase, forming p3, and an AICD.

1999). BACE1 cleavage at Asp1 results in the formation of membrane bound, APP-carboxyl terminal fragment (CTF) C99 and soluble APPβ (Fig. 1.1B). Furthermore, BACE1 cleavage of APP at Glu11, forms soluble APPβ' and APP-CTF C89 (Fig. 1.1C). Changes in BACE1 protein levels, affects the location of BACE1 cleavage. For example, overexpression of BACE1 increases the proportion of BACE1 cleavage at Glu11 (Liu, Doms, and Lee 2002).

APP-CTFs, C89 and C99, are cleaved in the intramembrane domain, by γ-secretase, resulting in the formation of Aβ, and the APP intracellular domain (AICD) (Fig1.1B-C). γ-secretase activity requires a set of four proteins including presenilin 1 and 2, nicastrin, anterior pharynx defective 1 (Aph1) and presenilin enhancer 2 (Pen-2) (Haass 2004). Presenilins are aspartyl proteases and are the catalytic subunit of the γ -secretase complex. The location of γ-secretase cleavage is variable. It can occur between amino acids 37 and 43 in the amyloid beta region of APP (Haass et al. 2012). However, within this region, γ-secretase cleavage most frequently occurs at amino acids 40 and 42. Furthermore, γ-secretase cleavage also occurs at the epsilon (ε) site, before Leu49 or Val50 (Aβ numbering), releasing the 50 or 49 amino acid long AICD (Figure 1.1A) (Dimitrov et al. 2013, Weidemann et al. 2002, Gu et al. 2001). γ-secretase cleavage at the ε-site likely occurs before cleavage at the other γ-secretase sites, ultimately forming Aβ40 and Aβ42 (Takami et al. 2009, Olsson et al. 2014).

APP may also be cleaved by α-secretase. ADAM10 (A disintegrin and metalloproteinase domain-containing protein) is believed to be the principal α-secretase (Kuhn et al. 2010). Candidate α-secretases also include ADAM9, and ADAM17, also

known as TACE (tumor necrosis factor-α converting enzyme) (Buxbaum et al. 1998, Lammich et al. 1999). α-secretase cleaves APP at Leu17 (Esch et al. 1990, Wang et al. 1991), resulting in the formation of APP-CTF C83 and sAPPα (Fig 1D). APP-CTF C83, is cleaved by γ-secretase, forming p3, a non-amyloidogenic fragment 26 or 28 amino acids in length, and an AICD.

Cleavage of APP occurs at different cellular locations. The majority of α-secretase cleavage of APP occurs on the plasma membrane (Sisodia 1992). Under physiological conditions, α-secretase cleavage of APP, is more common than β-secretase cleavage (De Strooper 2000). Meanwhile, BACE1 cleavage of APP is believed to occur in the endosomes, possibly in early or recycling endosomes (Das et al. 2013, Sannerud et al. 2011). APP, a transmembrane protein, is endocytosed from the plasma membrane and trafficked to early endosomes. A fraction of internalized APP is degradation in lysosomes (Haass et al. 1992), while the remainder is recycled to the cell surface in recycling endosomes (Haass et al. 2012) (Figure 1.2). Aβ, the product of BACE1 and γ-secretase cleavage of APP, is primarily made in intracellular compartments after APP endocytosis (Koo and Squazzo 1994). BACE1, like other aspartyl proteases, is most active under acidic conditions (Haass et al. 1993). Both early endosomes (pH 6) and recycling endosomes (pH 6.5) are acidic compartments (Maxfield and McGraw 2004), making both compartments possible locations of BACE1 cleavage of APP.

Evidence found using HeLa cells initially suggested that the early endosome was the more likely compartment for BACE1 processing. For example, sAPPβ, a product of BACE1 APP-cleavage was found in RAB5 and EEA1-positive early endosomes (Rajendran et al. 2006). Furthermore, APP-CTFs, the other product, was found

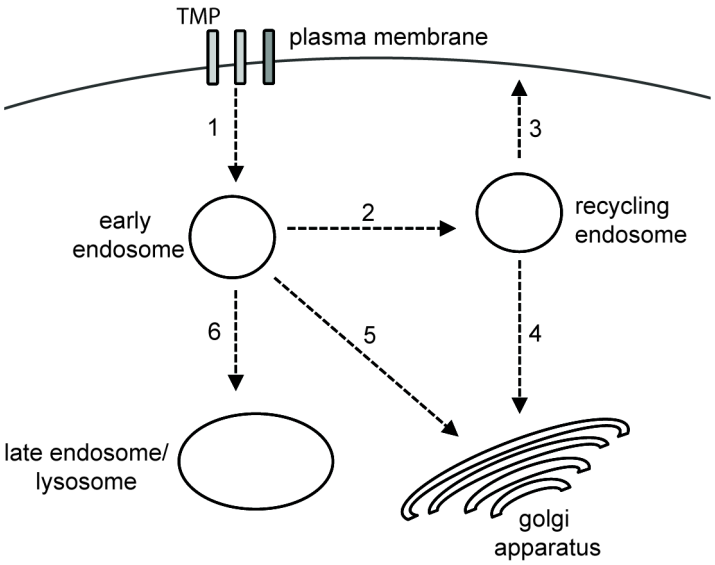

Figure 1.2

Transmembrane protein trafficking

Transmembrane proteins (TMP), including APP and BACE1, are endocytosed from the plasma membrane into vesicles that fuse with early endosomes (1). From early endosomes, TMPs may be transferred to recycling endosomes (2), and returned to the plasma membrane (3). TMPs may also be transferred to the trans-Golgi network from the recycling endosomes (4) or from the early endosomes (5). TMPs may also remain in the early endosomes, as they mature into late endosomes (6). During this process, the membrane of the endosome buds off into the endosome interior forming intraluminal vesicles. Late endosomes fuse to lysosomes, and internalized TMPs are degraded. TMP: Transmembrane proteins.

extensively in early endosomes, but sparingly in recycling endosome (Sannerud et al. 2011). More recently, investigations conducted using live imaging in neurons, showed that BACE1 processing of APP more likely occurs in the recycling endosome. In these experiments, BACE1 colocalized with stationary RAB5-positive early endosomes and mobile transferrin (TfR)-positive recycling endosomes. APP and BACE1 were primary localized in separate vesicles, however, APP and BACE1 were brought together in recycling endosomes after neuronal activation (Das et al. 2013). Neuronal activation has been associated with increased Aβ production *in vitro* (Lesne et al. 2005, Li et al. 2013, Kamenetz et al. 2003). Neuronal activation increased the localization of APP in TfR positive recycling endosomes, and also increased the co-localization of APP with BACE1. As APP and BACE1 interaction primarily occurred in the recycling endosomes, this cellular location was concluded to be the likely site of BACE1-mediated APP cleavage (Das et al. 2013).

1.4 BACE1 localization, trafficking and degradation

BACE1 is an aspartyl protease with 2 DT/SGT/S catalytic sites. It is a type 1 transmembrane protein that is synthesized in the endoplasmic reticulum (ER) and undergoes amino (N)-terminal processing in the Golgi apparatus (Huse et al. 2000). BACE1 is transported to the plasma membrane, where it may undergo endocytosis and trafficking to early endosomes (Huse et al. 2000). BACE1 internalization and localization in the endosome is facilitated by a dileucine motif (DDISLL) on the BACE1 carboxyl terminus (Huse et al. 2000).

From the early endosome, BACE1, like other transmembrane proteins, can be recycled back to the plasma membrane in recycling endosomes or transported back to the trans-Golgi network. BACE1 may also remain in the early endosomes. Early endosomes mature into late endosomes and fuse with lysosomes (Huotari and Helenius 2011), where BACE1 is degraded (Koh et al. 2005). Previous studies have shown that lysosomal inhibition by application of chloroquine and ammonium chloride results in an increase in cellular BACE1 protein levels, and an accumulation of BACE1 in the lysosome. In contrast, proteasomal inhibition with lactacystin, a specific proteasome 20S inhibitor does not result in BACE1 accumulation.

GGAs (Golgi-localized-ear-containing ARF-binding proteins) are monomeric clathrin adaptors that bind to BACE1 via a VPS27-Hrs-STAM (VHS) domain (He et al. 2003). GGA3 facilitates BACE1 lysosomal degradation, in a manner independent from BACE1 internalization. Previous studies have shown that GGA3 depletion results in an accumulation of BACE1 in the early endosomes, and a decrease of BACE1 in the lysosomes (Kang et al. 2010). GGA3 is able to negatively regulate BACE1 protein levels (Tesco et al. 2007) via an interaction with ubiquitin covalently attached to BACE1, as GGA3 binds ubiquitin via a GGA and Tom1 (GAT) domain (Bilodeau et al. 2004). BACE1 has one lysine residue (Lys 501) on its cytoplasmic tail, which is the main site of BACE1 ubiquitination. BACE1 is monoubiquitinated and lysine-63 (K63) poly-ubiquitinated at this site (Kang et al. 2010). Lys 501 is important for BACE1 degradation. Blocking ubiquitination at Lys 501 increases BACE1's half-life and causes BACE1 to accumulate in early endosomes and lysosomes (Kang et al. 2012). However, neither BACE1's rate of endocytosis nor retrograde transport to the Golgi is affected (Kang et al.

2012).

1.5 Overview of protein ubiquitination

Ubiquitination is a form of post-translational modification that serves as a signal for tagged proteins to be trafficked to different cellular compartments (Piper and Luzio 2007). The outcome of ubiquitin signaling depends on the number of ubiquitin molecules (mono- or poly-ubiquitination) attached to the target protein. While monoubiquitination serves as a signal for protein trafficking or endocytosis, polyubiquitination often serves as a signal for protein degradation (Bingol and Sheng 2011, Piper and Luzio 2007). Polyubiquitin chains can be formed by any of the 7 lysine residues on ubiquitin (K63, K48, K6, K11, K26, K29, K33), thus, the outcome of ubiquitination also depends on the type of poly-ubiquitin chain attached to the substrate (Kulathu and Komander 2012). For example, K48-linked ubiquitination mainly targets proteins for proteasomal degradation (Nijman et al. 2005). In contrast, K63-linked ubiquitin chains traffic proteins to lysosomes for degradation (Clague, Coulson, and Urbe 2012).

The proteasome is a protease complex composed of multiple subunits. Proteasomes mainly degrade intracellular, cytoplasmic ubiquitinated proteins. Whereas, lysosomes are membrane-bound acidic organelles, that contain multiple hydrolyses, including proteases and lipases. Lysosomes degrade both intracellular and extracellular ubiquitinated components, which are delivered to the lysosome via autophagy and endocytosis. Lysosomes also degrade many membrane-bound proteins (Appelqvist et al. 2013, Lecker, Goldberg, and Mitch 2006).

Ubiquitination is a reversible process. The ubiquitination of a protein is facilitated

by the serial activity of 3 enzymes: ubiquitin-activating (E1) enzymes, ubiquitin-conjugating (E2) enzymes, and ubiquitin ligase (E3) enzymes (Bingol and Sheng 2011). While ubiquitin (E3) ligases aid in the attachment of ubiquitin to proteins, and the formation of poly-ubiquitin chains, deubiquitinating enzymes (DUBs), in contrast, hydrolyze bonds between ubiquitin molecules and between ubiquitin and the tagged protein. As ubiquitination is a signal for protein degradation, DUBs, which work in opposition to ubiquitin ligases, may rescue proteins from degradation, restoring proteins to the recycling pool in the cell (Bingol and Sheng 2011).

There are approximately 100 DUBs in the human genome. These DUBS are divided into 2 classes: cysteine proteases and metalloproteases (Nijman et al. 2005). These 2 classes are further divided into 5 families. The ubiquitin C-terminal hydrolases (UCH), the ubiquitin specific proteases (USP), the ovarian tumor proteases (OTU) and the Machado Joseph disease domain proteases are cysteine proteases. The JAB1/ and MPN/ Mov34 metalloenzyme (JAMM) proteases, in contrast, are metalloproteases.

1.6 Trafficking of ubiquitinated proteins at the endosome

Ubiquitin acts like a tag, determining whether proteins are endocytosed, recycled or degraded. Monoubiquitination serves as a signal for transmembrane proteins (TMP) to be endocytosed from the plasma membrane and trafficked to early endosomes (Nijman et al. 2005). As the early endosome matures, the limiting membrane of the endosome, which contains TMPs destined for degradation, invaginates and buds off into the interior of the endosome, forming highly acidic intraluminal vesicles (ILV). As a result, mature endosomes are called multivesicular bodies (MVB) or late endosomes. Late endosomes

ultimately fuse with lysosomes and the proteins contained in ILV are degraded (Hu, Dammer, et al. 2015)

Ubiquitination serves as a signal for proteins to be sorted into ILVs (Piper and Katzmann 2007). TMPs are taken into the ILV when the limiting membrane buds inward. Endosomal sorting complexes required for transport (ESCRT) are ubiquitin-binding complexes that assist in the invagination of the endosome limiting membrane. There are 4 ESCRT complexes, ESCRT 0-III. ESCRT-0 first interacts with membrane-bound ubiquitinated proteins. ESCRT-0 is composed of 2 proteins, signal transducing adaptor molecule (STAM) and hepatocyte growth factor regulated tyrosine kinase substrate (HRS), which have multiple ubiquitin binding domains (Bache et al. 2003). ESCRT-0 recruits ESCRT-I, which in turn recruits ESCRT-II. Together ESCRT-I and ESCRT-II promote the invagination of the limiting membrane away from the cytosol. ESCRT-II recruits and activates ESCRT-III, which promotes the scission of the neck of the infolding, forming a new vesicle which buds off into the endosome (Henne, Buchkovich, and Emr 2011).

In addition to ESCRT complexes, other proteins are associated with transporting ubiquitinated TMPs at the endosome. These proteins include Golgi-localized-ear-containing ARF-binding (GGA) proteins. Like HRS and STAM, GGAs contain VHS domains and ubiquitin-binding domains. Not only can GGA3 bind ubiquitinated proteins as ESCRT-0 does, but it can also transfer ubiquitinated TMPs to ESCRT-I (Bilodeau et al. 2004, Puertollano and Bonifacino 2004).

Polyubiquitination appears to be a more effective signal than monoubiquitination, for ensuring that proteins enter ILVs, and are eventually degraded (Piper and Luzio 2007).

This is possibly due to the presence of more ubiquitin sites for ESCRT complexes to interact with. ESCRT-0, ESCRT-I and ESCRT-II bind ubiquitinated proteins (Henne, Buchkovich, and Emr 2011). ESCRT‑0 has a 50 times higher affinity to lysine (K) 63 tetra-ubiquitin chains than to monoubiquitin (Ren and Hurley 2010). Additionally, ESCRT-0 complex binds K63 chains twice as tightly as it binds K48 chains (Ren and Hurley 2010).

Since K63 polyubiquinated chains effectively tag proteins for lysosomal degradation, E3 ligases working at the endosomes, responsible for extending ubiquitin chains, promote protein degradation. For example, K63 chains are necessary for epidermal growth factor receptor (EGFR) degradation (Huang et al. 2013). In addition, Cbl proto-oncogene, an E3 ligase, polyubiquitinates EGFR, promoting EGFR degradation (Thien and Langdon 2001). Endosome-associated deubiquitinating enzymes counteract the action of E3 ligases and remove ubiquitin chains (Nijman et al. 2005), thus may act to promote protein recycling, hindering lysosomal degradation (Mizuno et al. 2005). There are 3 endosome-associated deubiquitinating enzymes, that have been associated with this function: Ubiquitin Specific Peptidase 8 (USP8), Associated molecule with the SH3 domain of STAM (AMSH) and AMSH-like protein (AMSH-LP) (McCullough, Clague, and Urbe 2004, Mizuno et al. 2005, Urbe et al. 2012, Nakamura et al. 2006)

1.7 USP8 overview

Summary:

USP8 is an endosomal associated deubiquitining enzyme.

USP8 deubiquitinates K48, K63 and K6 polyubiquitin chains.

USP8 is expressed prenatally and during adulthood.

USP8 knockout mice are embryonically lethal.

USP8 activity is regulated by phosphorylation.

USP8 activity is regulated by its cleavage.

USP8 contains SH3, MIT and 14-3-3 protein binding domains.

USP8 is a member of the Ubiquitin Specific Protease (USP) family of deubiquitinating enzymes (DUBs), the largest family of DUBs, comprising over 50 members. USP8 is one of three DUBs that have been found to be localized at the endosome (McCullough, Clague, and Urbe 2004, Mizuno et al. 2005, Urbe et al. 2012, Nakamura et al. 2006). USP8 has been found to hydrolize 3 of the 7 poly-ubiquitin chains formed by ubiquitin molecules: lysine-48 (K48) chains, lysine-63 (K63) chains (Mizuno et al. 2005), and lysine-6 (K6) chains (Durcan et al. 2014).

USP8 plays a vital role in development. USP8 knockout mice are embryonically lethal. At embryonic day (E) 9.5, these embryos are smaller than litter mates and display gross growth abnormalities, notably a lack of ventral embryonic folding (Niendorf et al. 2007), a process important in the correct formation of the foregut and the heart tube (Gavrilov and Lacy 2013). Furthermore, USP8 is vital for the survival of adult mice. USP8 conditional knockout mice die 4 to 6 days after induction of USP8 deletion (Niendorf et al. 2007). These mice show signs of liver failure, including jaundice, elevated blood plasma bilirubin levels, elevated serum aspartate transaminase and

elevated alanine transaminase. Apoptotic hepatocytes are also detected 4 days after induction of USP8 deletion, suggesting that liver failure is the cause of death for these mice (Niendorf et al. 2007).

USP8 is expressed in various organs throughout the body. Its highest expression is in the brain and the testes (Gnesutta et al. 2001). In the brain of the adult mouse, USP8 is highly expressed in the hippocampus, especially the CA1 and dentate gyrus (d'Amora et al. 2010, Bruzzone et al. 2008). In postnatal mice, USP8 is also highly expressed in the cortex (d'Amora et al. 2010). USP8 expression is first apparent in the brain at E11. At E17, USP8 expression declines and is no longer detectable between E19 and postnatal day (P) 0. However, levels of USP8 expression begin to rise again at P1 (d'Amora et al. 2010).

USP8 is mainly expressed in neurons. In adult mice, USP8 is not expressed in astrocytes. However, it is present in some CNPase-positive oligodendrocytes in the periventricular hypothalamic nucleus (Bruzzone et al. 2008). In embryos, however, USP8 labeling colocalizes with glial fibrillary acidic protein (GFAP), suggesting that USP8 may be present in astrocytes, in early development (d'Amora et al. 2010). In neurons, USP8 is found in the cell body, dendrites and axons. Moreover, it co-localizes with VAMP (vesicle associated membrane protein) a marker for presynaptic regions (d'Amora et al. 2010).

USP8 is post-translationally modified by phosphorylation. USP8 is phosphorylated after epidermal growth factor receptor (EGFR) activation, most likely by Scr-family tyrosine kinases, activated by EGFR activation (Alwan and van Leeuwen 2007). While USP8 is phosphorylated at both serine and tyrosine residues (Meijer et al.

2013), serine 680 (S680) is the major phosphorylation site in USP8 (Mizuno, Kitamura, and Komada 2007). S680 is part of the RSYS680SP sequence, which is a canonical RSXXP 14-3-3 protein-binding motif. There are seven 14-3-3 protein family members in mammals, which bind to many substrates (Fu, Subramanian, and Masters 2000). 3 of these 7 family members bind to USP8 (Mizuno, Kitamura, and Komada 2007). Notably, 14-3-3 binding is inhibited when USP8 is dephosphorylated (Mizuno, Kitamura, and Komada 2007). 14-3-3 binding does not affect USP8 localization to endosomes or USP8 binding to substrates. However, USP8 enzymatic activity is inhibited when 14-3-3 proteins bind to USP8 (Mizuno, Kitamura, and Komada 2007). This process is physiologically relevant as 14-3-3 proteins dissociate from USP8 during the mitotic (M) phase of the cell cycle. In accordance, USP8 in this phase has been reported to be more catalytically active (Mizuno, Kitamura, and Komada 2007).

USP8 is also regulated by caspase-dependent cleavage (Dufner and Knobeloch 2015, Dufner et al. 2015). USP8 is cleaved near the 14-3-3 binding site. Truncated USP8 was first observed in mutated variants of USP8 found in pituitary adenomas (Reincke et al. 2015). The mutations in USP8 were near the 14-3-3 binding site. These mutations disrupted 14-3-3 binding and facilitated cleavage of USP8. Truncated USP8 was also observed in mouse thymocytes (T cells) after T cell antigen receptor (TCR) activation, showing that wild type USP8 may also be cleaved (Dufner et al. 2015). Additionally, USP8 cleavage was prevented with pan-caspase inhibitors, demonstrating that the process was likely caspase dependent (Dufner and Knobeloch 2015, Dufner et al. 2015). The cleaved form of USP8 appears to be the more active than the full-length form. For example, truncated USP8 is more effective at deubiquitinating EGFR than full length

USP8 (Reincke et al. 2015).

In addition to the 14-3-3 protein-binding domain, USP8 contains a Src homology (SH3)-binding domain, which allows it to interact with proteins containing SH3 domains. SH3 domains are approximately 50 amino acids in length, and usually bind to proline rich sequences (usually PXXP) (Alexandropoulos, Cheng, and Baltimore 1995). The 2 PX(V/I)(D/N)RXXKP sequences in USP8 allows it to bind to STAM, an ESCRT0 component, which it stabilizes (Kato, Miyazawa, and Kitamura 2000, Row et al. 2006). The formation of the USP8-STAM complex facilitates the binding of USP8 to EGFR, a protein which USP8 deubiquitinates (Mizuno et al. 2005). USP8's SH3-binding sequence also allows USP8 to bind to HRS binding protein (HBP). HBP's interaction with HRS, an ESCRT0 component, is integral for HRS to perform its function sorting ubiquitinated proteins for degradation (Kato, Miyazawa, and Kitamura 2000). RXXK consensus sequence also binds to SH3-domains (Liu et al. 2003). USP8 contains three RXXK regions that bind to the SH3-domains. Mutations in these regions ameliorates the ability of USP8 to deubiquitinate EGFR (Berlin, Schwartz, and Nash 2010).

Another important protein binding domain in USP8, is the Rhodanese-like domain. This sequence has been show to allow USP8 to bind to neuregulin receptor degradation protein-1 (Nrdp1), a E3 ligase, that USP8 directly deubiquitinates (Avvakumov et al. 2006). USP8 also contains a Microtubule Interacting and Transport (MIT) domain. This domain facilitates the interaction of USP8 with 2 ESCRT-III proteins, charged multivesicular body proteins, CHMP1A and CHMP1B (Row et al. 2007). Moreover USP8's MIT domain is important for its localization to the endosome. USP8, in the cytoplasm of starved cells, redistributes to endosome after EGF stimulation.

However, mutant-USP8, lacking the MIT domain, fails to relocate to the endosome, after EGF stimulation (Row et al. 2007). This is in contrast to catalytically inactive USP8, which maintains its endosomal localization (Mizuno et al. 2006, Row et al. 2007).

Finally, USP8 may also be regulated by ubiquitination. While ubiquitination of wild-type USP8 is undetectable, catalytically inactive USP8 is monoubiquitinated. This suggests that USP8 may deubiquitinate itself (Mizuno et al. 2005)

1.8 Substrates of USP8

In 1998, Naviglio et al. purified and characterized USP8. These investigations demonstrated that USP8 had deubiquitinating activity whereby the depletion of USP8 or overexpression of a catalytically inactive mutant lead to an increase of total cellular ubiquitinated proteins. Furthermore, overexpression of wild-type USP8 lead to a decrease in protein ubiquitination (Naviglio et al. 1998). Since then, the role of USP8 in regulating numerous proteins has been investigated.

USP8 has many potential substrates (outlined in Table 1). USP8 has been shown *in vitro* to directly deubiquitinate epidermal growth factor receptor (EGFR) (Mizuno et al. 2005, Alwan and van Leeuwen 2007, Sirisaengtaksin et al. 2014); tropomysin–related kinase A (TRKA) (Ceriani et al. 2015), a neurotropin receptor; gene related to anergy in lymphocytes (GRAIL) (Soares et al. 2004), an E3 ligase; and Parkin (Durcan et al. 2014), an E3 ligase, important in Parkinson disease. While USP8 has been shown in cell-free *in vitro* assays to directly deubiquitinate these substrates, changes in USP8 protein levels and activity, have been show to affect the protein levels and degradation of other proteins (outlined in Table 1). It is however unknown whether USP8 directly deubiquitinates these

potential substrates or has an indirect effect. These proteins include important neuronal proteins. USP8 has been found in immunocomplexes with TAR DNA binding protein (TDP-43) (Hans et al. 2014), a protein associated with amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration; and microcephalin (MCPH1), a protein associated with microcephaly. Furthermore, USP8 activity negatively regulates AMPA receptor ubiquitination (Scudder et al. 2014).

Many of the proteins, which USP8 regulates, are degraded in the lysosomes. This suggests a role for USP8 in regulating lysosomal degradation. These proteins include: epidermal growth factor receptor (EGFR) (Mizuno et al. 2005, Alwan and van Leeuwen 2007, Sirisaengtaksin et al. 2014), tropomysin–related kinase A, (TRKA) (Ceriani et al. 2015), AMPA receptor (Scudder et al. 2014); vascular endothelial growth factor receptor 2 (VEGFR2) (Smith et al. 2016); neuregulin receptor degradation protein-1 (NRDP1) (Wu et al. 2004); leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) (Oh et al. 2014), potassium intermediate/small conductance calcium activated channel, subfamily N, member 4 (KCA3.1) (Balut, Loch, and Devor 2011); protease-activated receptor 2 (PAR2) (Hasdemir et al. 2009); and δ-opioid receptor (DOR) (Hislop et al. 2009).

Table 1: USP8 substrates

Substrate	**Function**	**Effect on ubiquitination**	**Effect on protein levels/ degradation**	**Sources**
AMPAR†	Ionotropic glutamate receptor	USP8 depletion increases AMPAR ubiquitination and endocytosis	-	Scudder (2014)
BRIT1 (MCPH1)	DNA damage response protein and tumor inhibitor	USP8 and BRIT coIP with BRUCE. Overexpression of catalytically inactive USP8 prevents BRIT1 deubiquitination	-	Ge (2015)
CDC25Mm	Nucleotide exchange factor	USP8 WT decreases CDC25Mm ubiquitination. USP8 coIP with CDC25Mm	USP8 WT increases CDC25Mm half-life	Gnesutta (2001)
CLOCK	Transcription factor (Circadian rhythm)	USP8 knockdown decreases CLOCK ubiquitination. CLOCK coIP with USP8	USP8 depletion does not affect CLOCK protein levels	Luo (2012)
DOR †	G-coupled protein receptor	-	Knockdown of USP8 inhibited DOR degradation after opioid agonist application	Hislop (2009)
EGFR †	Protein kinase	Recombinant USP8 directly deubiquitinates BACE1 *in vitro*	USP8 depletion increases EGFR degradation. [USP8 depletion decreases EGFR degradation]	Mizuno (2005), Mizuno (2006), Alwan (2007), Sirisaengtaksin (2014), [Row (2006)]

Substrate	Function	Effect on ubiquitination	Effect on protein levels/ degradation	Sources
GRAIL	E3 ubiquitin ligase	USP8 directly deubiquitinates GRAIL *in vitro*. USP8 coIP in GRAIL complex. Catalytically inactive USP8 increases GRAIL ubiquitination	-	Soares (2004)
KCA3.1†	Voltage-independent calcium channel	USP8 overexpression increases KCA3.1 ubiquitination	USP8 overexpression decreases KCA3.1 degradation. Catalytically inactive mutant decreases rate of degradation	Balut (2011)
LRIG1 †	Negative regulator of receptor tyrosine kinases	USP8 knockdown increases LRG1 ubiquitination USP8 coIP with LRIG1	USP8 overexpression reduces LRIG1 degradation	Oh (2014)
NRDP1	E3 ubiquitin ligase	USP8 coIP with NRDP1	USP8 overexpression increases NRDP1 protein levels	Wu (2004)
PAR2†	Receptor for proteases activated by injury	Catalytically inactive USP8 and USP8 siRNA increase ubiquitination of PAR2	USP8 knockdown prevents PAR2 degradation.	Hasdemir (2009)

Substrate	Function	Effect on ubiquitination	Effect on protein levels/ degradation	Sources
Parkin	E3 ubiquitin ligase	USP8 was found to directly deubiquitinate Parkin *in vitro*.	USP8 depletion increases Parkin levels. USP8 WT overexpression decreases Parkin levels.	Durcan (2014)
STAM	Adaptor protein	USP8 depletion increases STAM ubiquitination	Knockdown of USP8 decreases STAM protein levels	Kato (2000), Row (2006)
TDP-43	Transcriptional repressor	USP8 overexpression decreases TDP-43 ubiquitination. USP8 coIP with TDP-43	USP8 depletion (in Drosophila) increased TDP protein	Hans (2014)
TrkA †	Protein kinase	USP8 directly deubiquitinates TrKA. USP8 co-IP with TrkA	USP8 depletion increases TRKA half-life	Ceriani (2015)
VEGFR2 †	Receptor tyrosine kinase	USP8 depletion increases VEGFR2 ubiquitination	-	Smith (2015)

Footnotes:

Full names: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BRIT1 (MCPH1), breast cancer susceptibility gene C terminus-repeat inhibitor of human telomerase repeat transcriptase expression (Microcephalin); CDC25Mm, RAS protein-specific guanine nucleotide-releasing factor 1; DOR, δ-opioid receptor; EGFR, epidermal growth factor receptor; GRAIL, gene related to anergy in lymphocytes; KCA3.1, potassium intermediate/small conductance calcium activated channel, subfamily N, member 4; LRIG1, leucine-rich repeats and immunoglobulin-like domains 1; NRDP1, neuregulin receptor degradation protein-1; PAR2, protease-activated receptor 2; STAM, signal transducing adaptor molecule; TDP-43, TAR DNA binding protein; TRKA, tropomysin–related kinase A; VEGFR2, vascular endothelial growth factor 2

†, Degraded in lysosome;

co-IP, Co-immunoprecipitates

1.9 Cellular Roles of USP8

Summary:

Current literature describes the involvement of USP8 in numerous processes including:

I Endocytosis of membrane bound receptors

II Promotion of protein recycling by counteracting E3 ligases and removing ubiquitin

III Promotion of protein degradation by stabilizing ESCRT-0

IV Maintenance of endosome organization

V Promotion of cell proliferation in germ cells and thymocytes (T lymocytes)

I Endocytosis of cell surface receptors

Ubiquitination regulates the movement of proteins between the plasma membrane and the cytoplasm. USP8 has been shown to affect the endocytosis of receptors, both directly through protein-protein interaction and indirectly. In 2014, Schudder et al. demonstrated that USP8 regulates AMPAR on the cell surface membranes of rat hippocampal neurons in culture (Scudder et al. 2014). In these experiments, USP8 overexpression resulted in an increase in the amount of AMPA receptors, on the surface of neurons, along with an increase in miniature excitatory postsynaptic current (mEPSC) amplitude. In contrast, USP8 knockdown resulted in decreased mEPSC amplitude, showing that USP8 can affect synaptic strength in the neuron by altering receptor localization.

Furthermore, USP8 depletion was shown to result in the accumulation of chemokine C-X-X Motif receptor 5 (CXCR5), a 7 transmembrane G protein-coupled receptor, on the cell surface membrane (Berlin et al. 2010). However, knockdown of

USP8 did not alter the ubiquitination of the receptor, indicating that USP8 was indirectly affecting the endocytosis of these cell surface receptors.

II Promotion of protein recycling

USP8 may promote protein recycling by deubiquitinating proteins, rescuing them from degradation. The role of USP8 in protein degradation has most often been investigated with respect to EGFR levels. In 2005, Mizuno et al. showed that overexpressing USP8, not only decreased ubiquitination of EGFR, but also delayed the degradation of EGFR. Furthermore, depletion of USP8, increased EGFR degradation (Mizuno et al. 2005). This finding was also replicated by Berlin et al. (Berlin, Schwartz, and Nash 2010). Moreover, USP8 conditional knockout mice, created by Niendorf et al., provided further evidence for the role of USP8 in counteracting protein degradation. Conditional knockout USP8 mice had reduced protein levels of EGFR without a change in mRNA levels. Histological examination of liver tissue, showed a decrease in EGFR labeling in hepatocytes. In addition, there was also a change in distribution of EGFR from the surface to the intracellular region of cells. Altogether, these data suggested that EGFR was internalized on USP8 depletion, and degraded (Niendorf et al. 2007).

III Promotion of protein degradation

While some investigators showed that depleting USP8 resulted in an increase of EGFR degradation, others acquired data that suggested the opposite (Row et al. 2006, Alwan and van Leeuwen 2007). Row et al. demonstrated that USP8 depletion resulted in a decrease of EGFR degradation (Row et al. 2006). The decrease in EGFR degradation

was accompanied by the accumulation of ubiquitinated EGFR in dysmorphic, enlarged late-endosomes. These data led investigators to conclude that USP8 played a role in facilitating the transport of ubiquitinated cargo from the endosome to the lysosome, and therefore facilitated protein degradation (Row et al. 2006).

Further evidence for the role of USP8 in promoting protein degradation can be found by examining some of its other substrates. While USP8 depletion increases the degradation of some substrates, USP8 depletion hinders the degradation of others, suggesting that the presence of USP8 is needed for effective protein degradation. For example, USP8 depletion increases the half-life of TrKA, a substrate, which it has been shown to directly deubiquitinate (Ceriani et al. 2015). USP8 depletion also increases the protein levels of Parkin, a known substrate (Durcan et al. 2014). Furthermore, USP8 depletion has been shown to increase the protein levels of TDP-43 (Hans et al. 2014) and prevent PAR2 degradation (Hasdemir et al. 2009). However, it is currently unknown whether USP8 directly deubiquitinates TDP-43 and PAR2.

Moreover, USP8 likely facilitates protein degradation by stabilizing HRS and STAM protein levels. The protein levels of both HRS and STAM (ESCRT0 components) are decreased when USP8 is depleted (Mizuno et al. 2005, Niendorf et al. 2007) suggesting that USP8 maintains their stability. STAM and HRS are needed to maintain lysosomal degradation. For example, depletion of HRS leads to an accumulation of APP, a lysosomal protein, within cells. Immunohistochemical labeling demonstrated that APP accumulated on the limiting membrane of the MVB (Edgar et al. 2015). Moreover, HRS depletion disrupts EGFR downregulation (Berlin, Schwartz, and Nash 2010), showing that HRS is needed for effective protein degradation.

Deubiquitinating enzymes associated with the proteasome have been show to release ubiquitin from proteins destined for proteasomal degradation (Nijman et al. 2005). This process rescues the ubiquitin itself from degradation and maintains the cellular ubiquitin pool. Similarly, it was hypothesized that deubiquitinating enzymes, such as USP8, may play a similar role at the late-endosome or multivesicular body (MVB), removing ubiquitin from the proteins before they are degraded (Balut, Loch, and Devor 2011), so that the cellular ubiquitin pool is not depleted (Clague, Coulson, and Urbe 2012). USP8 depletion, however, does not decrease cellular free ubiquitin (Row et al. 2006, Niendorf et al. 2007, Amerik and Hochstrasser 2004)

IV Maintenance of endosome and lysosome organization

Depletion of USP8 or overexpression of a dominant negative USP8 catalytically-inactive mutant has been shown to cause the accumulation of proteins within early endosomes and increase early endosome and MVB diameter (Niendorf et al. 2007, Row et al. 2006, Mizuno et al. 2006, Hasdemir et al. 2009, Bowers et al. 2006, Smith et al. 2016). Furthermore, clustering of endosomes, facilitated by the formation of electron dense regions between endosomes has been noted by electron microscopy (Row et al. 2006, Mizuno et al. 2006). Enlarged endosomes have been observed in both experiments where there is increased protein degradation and experiments where there has been decreased protein degradation. For example, this phenomenon is present in USP8 conditional knockout mice, which are noted to have decreased EGFR levels (Niendorf et al. 2007).

Abnormalities in endosomal structure have been attributed to numerous causes. First, it has been attributed to the accumulation of ubiquitinated proteins within endosomes (Niendorf et al. 2007, Row et al. 2006, Mizuno et al. 2006, Hasdemir et al. 2009). It is possible that ubiquitinated proteins form cross-linkages between endosomes and between MVBs (Row et al. 2006, Mizuno et al. 2006). Second, it has been attributed to the dysfunction of the endosomes due to loss of endosomal proteins like STAM and HRS, components of the ESCRT0 complex that bind ubiquitinated proteins. The protein levels of both HRS and STAM (ESCRT0 components) are decreased when USP8 is depleted (Mizuno et al. 2005, Niendorf et al. 2007) suggesting that USP8 maintains their stability. STAM and HRS depleted cells display similar aberrant endosomes as USP8 depleted cells (Sun et al. 2010, Komada and Soriano 1999). Thus it is possible that it is the loss of these proteins that results in the endosomal dysfunction observed with USP8 depletion.

USP8 not only plays a role in maintaining the integrity of the endosome, but also aids in maintaining functional lysosomes. Depletion of USP8 results in the secretion of Cathepsin D, a lysosomal enzyme, from cultured cells into media. Thus, in the absence of USP8, cathepsin is not transported and maintained in the correct compartment (MacDonald, Urbe, and Clague 2014).

V Cell proliferation

USP8 plays a role in cellular proliferation. Experiments done on a cell line of fibroblasts created from USP8 conditional knockout mice, revealed that 6 to 8 days after USP8 deletion, cells show impaired proliferation, unaccompanied by cell death (Niendorf

et al. 2007). USP8 depleted cells also fail to enter the synthesis (S) phase of the cell cycle (Naviglio et al. 1998). Under physiological conditions, the cellular level of USP8 oscillates between growth and nascent stages. In non-immortalized fibroblasts, USP8 is undetectable in serum-starved cells, which have entered a quiescent state (G_0 phase) (Naviglio et al. 1998). However, USP8 protein levels increase when cell re-enter G_1 phase, after cell growth is promoted by providing fetal bovine serum. Furthermore, USP8 levels decrease when cells enter growth arrest caused by increased cell-cell contact (Naviglio et al. 1998).

USP8 is highly expressed in the testes, where it is important for spermatogenesis. USP8 expression levels in frog testes fluctuate seasonally (Meccariello et al. 2007). USP8 protein levels are low in November and December, but increase from January to May when spermatogenesis occurs. Moreover, USP8 is important for thymocyte (T cell) development and proliferation. When USP8 was depleted in CD4+ expressing T cells, these cells displayed impairments in both proliferation and maturation (Dufner et al. 2015). The function of USP8 in T cells appears to be distinct from its function in other cell types, for example, HEK cells. For example, USP8 depletion does not result in a decrease in STAM in T cells. Furthermore, there was not an apparent increase in overall protein ubiquitination in T cell lysates after USP8 depletion (Dufner and Knobeloch 2015, Dufner et al. 2015).

1.10 USP8 in disease

Cushing's disease

Mutations in USP8 were recently found in pituitary adenomas of patients with Cushing's disease (Ma et al. 2015, Reincke et al. 2015). Cushing's disease is caused by the presence of a non-cancerous tumor called a pituitary adenoma, secreting excess amounts of adrenocorticotropic hormone (ACTH). Excess ACTH secretion results in an excess of serum corticosteroids. This in turn cases a range of symptoms including weight gain, skin thinning, hypertension, glucose intolerance, proximal muscle weakness, depression, and cognitive defects (Arlt 2015). ACTH-secreting pituitary adenomas often have high EGFR levels. EGFR activation is believed to promote ACTH secretion by promoting the transcription of proopiomelanocortin (POMC), the ACTH precursor (Fukuoka et al. 2011).

USP8 gain-of-function mutations were found in ACTH-secreting pituitary adenomas. As USP8 directly deubiquitinates EGFR, deterring EGFR degradation (Mizuno et al. 2005, Mizuno et al. 2006), USP8 over-activity would be expected to increase EGFR protein levels. *In vitro*, these USP8 mutants promoted deubiquitination of EGFR, slowed EGFR degradation and promoted EGFR recycling (Ma et al. 2015, Reincke et al. 2015). Furthermore, mutant USP8 increased EGFR-induced POMC expression (Reincke et al. 2015). The findings support the hypothesis that USP8 promotes EGFR recycling and deters its degradation. This study provided a potential mechanism whereby USP8-overactivity could lead to increased EGFR protein levels and ultimately increase ACTH production. Furthermore, it identified USP8 as a potential therapeutic target in the treatment of pituitary adenomas (Ma et al. 2015).

Lung cancer

Non-small cell lung cancer (NSCLC) is a subclass of lung cancer. 10-35% cases of NSCLC contain EGFR mutations (Horn, Lovly, and Johnson 2015). NSCLC is treated with drugs like genfitinib, which are EGFR-tyrosine kinase inhibitors (Byun et al. 2013). However, while treatment is initially successful, a subset of the malignancies develops resistance towards the drug. Since USP8 is known to inhibit EGFR degradation, Byun et al. hypothesized that USP8 depletion could be used as an alternative therapy to treat genfitinib-resistant NSCLC. Both siRNA-mediated depletion of USP8, as well as application of a USP8 inhibitor decreased protein levels of EGFR and its family members ErbB2 and ErbB3 in NSCLC cells. These interventions decreased the proliferation and viability of NSCLC cells in vitro, but importantly did not decrease the viability of normal human bronchial epithelial cells and lung fibroblasts (Byun et al. 2013).

These experiments demonstrated that USP8 inhibitors might be important therapeutic agents. USP8 inhibitor used in these experiments, 9-Ethyloxyimino-9H-indeno[1,2-b]pyrazine- 2,3-dicarbonitrile, was synthesized in an effort to derive more potent inhibitors of USP7 and USP8. This class of inhibitor appears to inhibit USP8 100X more effectively than USP7 (Colombo et al. 2010).

1.11 DUB regulation of BACE1 protein levels

Given that BACE1 is one of the two enzymes responsible for Aβ formation, it is an important drug target for AD therapy. As shown in the case of non-small cell lung cancer, DUB inhibition may be a potential mechanism for increasing the degradation of ubiquitinated proteins, as it increases EGFR lysosomal degradation and lowers EGFR

protein levels (Byun et al. 2013). BACE1, like EGFR, is K-63 ubiquitinated and degraded in lysosomes (Koh et al. 2005, Kang et al. 2010). Thus, DUB inhibition, increasing BACE1 lysosomal degradation, may be a potential mechanism for BACE1 inhibition.

Previous to our experiments, the only DUB recognized as influencing BACE1 degradation was Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1) (Choi et al. 2004). UCHL1 is a deubiquitinating enzyme that is highly expressed in the brain, where it constitutes 1-5% of soluble brain proteins (Wilkinson et al. 1989). AD, in human patients, has been associated with decreased soluble UCHL1. In fact, the amount of soluble UCHL1 was inversely proportional to the amount of neurofibrillary tangles (Choi et al. 2004). Decreased UCHL1 protein levels have also been observed in AD cortices with increased BACE1 protein (Guglielmotto et al. 2012). In mouse models, overexpression of UCHL1 has been shown to decrease BACE1 protein levels, APP levels and amyloid beta formation. Furthermore, genetic and pharmacological inhibition of UCHL1 have been associated with an increase in BACE1 (Zhang et al. 2012).

Although UCHL1 overexpression increases BACE1 degradation, it is unknown whether BACE1 is a direct substrate of UCHL1. Moreover, UCHL1 is believed to have multiple roles in protein ubiquitination, and may act as a DUB or as a ligase (Liu et al. 2002). The effect of UCHL1 on BACE1 degradation is likely indirect. While UCHL1 depletion does not significantly affect BACE1 ubiquitination, it does decrease the activity of cathepsin D, a lysosomal protease (Guglielmotto et al. 2012). Thus, UCHL1 likely regulates BACE1 degradation by influencing the lysosomal machinery. Furthermore, the concomitant UCHL1 decrease and BACE1 increase are likely linked via Aβ-mediated

NFκB nuclear activation (Guglielmotto et al. 2012) as NFκB acts as a transcriptional activator for BACE1 and a repressor for UCHL1 (Guglielmotto et al. 2012, Wang et al. 2011).

Although UCHL1 regulates BACE1 protein levels, it is not a likely candidate for DUB inhibition, as UCHL1 must be overexpressed to observe a decrease in BACE1. DUB inhibition as a therapy, is likely more practical than DUB overexpression, since several screens to identify small molecule inhibitors for specific DUBs have recently been developed (Chauhan et al. 2012, Weinstock et al. 2012, Arnst et al. 2013).

Since USP8, an endosomal-associated DUB, deubiquitinates lysosomal degraded proteins, and inhibits their degradation, we hypothesized that likewise, USP8 may prevent the degradation of BACE1, by directly deubiquitinating BACE1. We hypothesized that depleting cellular levels of USP8, would result in increased BACE1 ubiquitination. As BACE1 ubiquitination is important for BACE1 trafficking to the lysosomes and degradation (Kang et al. 2010, Kang et al. 2012), we hypothesized that increased BACE1 ubiquitination would increase BACE1 trafficking to the lysosomes, and as a consequence increase degradation of BACE1 in the lysosome. Furthermore, we hypothesized that by decreasing recycling BACE1, there would be a resultant decrease in BACE1-mediated APP processing and a decrease in amyloid beta production. In the following chapters we show that RNAi-mediated knockdown of USP8 results in a decrease in BACE1 protein levels owing to increased BACE1 ubiquitination and degradation in human H4 neuroglioma cells. As a consequence BACE1 mediated APP-cleavage and amyloid beta formation are also decreased in USP8 depleted cells.

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Chapter 2: Material and methods

2.1 Overview

β-site amyloid precursor protein cleaving enzyme (BACE1) is the rate-limiting enzyme in the formation of Aβ from amyloid precursor protein (APP). Previous studies have shown that BACE1 is degraded via the lysosomal pathway, that BACE1 is ubiquitinated at lysine 501 and that lack of ubiquitination at lysine 501 produces BACE1 stabilization (Kang et al. 2010, Kang et al. 2012). Ubiquitination of plasma membrane proteins regulates their endocytosis and sorting to lysosomes for degradation. The ubiquitin specific peptidase 8 (USP8), an endosomal-associated deubiquitinating enzyme, regulates the ubiquitination, trafficking and lysosomal degradation of several transmembrane proteins. We hypothesized that USP8 regulates BACE1 ubiquitination, recycling and degradation.

The materials and methods described in this chapter were used to determine:

i) whether depletion of USP8 increases BACE1 ubiquitination and degradation,

ii) whether BACE1 is a direct substrate of USP8 by observing *in vitro* deubiquitination,

iii) whether USP8 depletion increases BACE1 trafficking to the lysosome, while decreasing recycling BACE1 using immunocytochemistry, and

iv) whether USP8 depletion results in decreased BACE1-mediated APP processing and amyloid beta formation.

2.2 Experimental procedures

To investigate our hypotheses, we employed the following methods and used the following materials:

Antibodies and expression vectors

Antibodies used include: anti-GFP (Molecular Probes; cat#A11122); anti-BACE1 (clone D10E5) (Cell signaling, cat#5606S); anti-GAPDH (Millipore, Cat#MAB374); anti-USP8 (Cell signaling, cat#8728); anti-FLAG (Sigma, cat#F3165); anti anti-ADAM10 (Millipore; cat#AB19026); anti-ubiquitin (clone P4D1) (Santa Cruz, cat# sc-8017); anti PS1-NTF (AB14), kindly provided by Dr. Samuel E. Gandy, Mount Sinai Hospital; anti-LAMP2 (BD biosciences; cat#555803); anti-EEA1 (BD biosciences, cat#610456); anti-RAB11 (Cell signaling; cat#5589S); anti-APP C-Terminal fragment (clone C1/6.1) (Biolegend, cat#802801) and anti-beta amyloid 1-16 (clone 6E10) (Biolegend, cat#803001). The BACE1-GFP plasmid was a gift from Dr. Tae Wan Kim, Columbia University, New York, NY, USA. The APP-C99 plasmid was a gift from Dr. Stefan Lichtentaler, TUM School of Medicine, Munich, Germany. The APP-C60 plasmid was a gift from Dr. Dennis Selkoe, Brigham and Women's Hospital.

Site-Directed Mutagenesis: BACE1-K501R-GFP Mutant

Site-directed mutagenesis was performed using the QuikChange II site-directed mutagenesis kit (Agilent, cat#200523-5) according to manufacturer's instructions. The

mutation was introduced with the following primer (Primer: 5' GACATCTCCCTGCTGAGAAAGGGCAATTCTGCAG 3)'(along with reverse complement primer). Mutagenesis was confirmed by DNA sequencing.

Generation of H4 neuroglioma stable cell lines expressing BACE1-GFP and BACE1K501R-GFP

50,000 H4 neuroglioma cells were seeded in 6-well tissue culture plates (Falcon, cat#353064, growth area: 9.6 cm^2) and transiently transfected with 1.2µg BACE1-GFP plasmid (a gift form Dr. Tae Wan Kim) or BACE1K501R-GFP plasmid. 24 hours post-transfection, cells were transferred to a 150 x 25mm Falcon tissue culture dish (cat#353025, growth area: 156.35cm^2). Cells were cultured in media containing 300µg/ml G418 sulfate, Genticin (Calbiochem, cat#345812) for selection of colonies positive for expression vector. Isolated clones were screened for levels of BACE1 by fluorescence microscopy and western blotting using rabbit anti GFP antibody (Molecular Probes; cat#A11122). Colonies were maintained using media containing 200 µg/ml G418 sulfate.

Generation of H4 neuroglioma stable cell lines expressing APP-C99

125,000 H4 neuroglioma cells were seeded in 6-well tissue culture plates (Falcon, cat#353064, growth area: 9.6 cm^2) and were transiently transfected with 2 µg C99 plasmid (a gift from Dr. Stefan Lichtentaler). 48 hours post-transfection, approximately 30,000 cells were sparsely plated in 150 x 25mm Falcon tissue culture dishes (cat#353025, growth area: 156.35cm^2). Cells were cultured in media containing

400μg/ml hygromycin B (Invitrogen, cat#10687-010) for selection of colonies positive for expression vector. Colonies formed from surviving cells were screened by western blotting for C99 expression using anti-APP C-Terminal fragment (clone C1/6.1) (Biolegend, cat#802801) and anti beta amyloid 1-16 (clone 6E10) (Biolegend, cat#803001) antibodies. Colonies were maintained using media containing 200 μg/ml Hygromycin B.

siRNA-mediated USP8 knockdown

Reverse transfections were performed in 6-well tissue culture plates (Falcon, cat#353064, growth area: 9.6cm^2) using Lipofectamine RNAimax Transfection reagent (Invitrogen, cat#13778075) and 25nM siRNA. For reverse transfections, suspended cells were added to wells in which RNAi-Lipofectamine complexes were formed. Briefly, RNAi - Lipofectamine complexes were formed in wells using 6ul Lipofectamine RNAimax Transfection reagent, 500ul Opti-MEM reduced serum media (Gibco, cat# 31985-070) and Human USP8#3 siRNA (Dharmacon; cat# J-00523-08; CUUCGUAACUUAGGAAAUA); Human USP8#2 siRNA (Dharmacon; cat# J-00523-07; CCACUAGCAUCCACAAGUA) or Non targeting (NT) siRNA (Dharmacon; cat# D-00181-10-05; UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, UGGUUUACAUGUUUUCCUA). 400,000 H4-BACE1-GFP cells in 2ml antibiotic-free media were added to wells for a final volume of 2.5ml. Cells were incubated at 37°C and harvested 96 hours post transfection. Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer [10mM Tris pH 8, 150mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 5mM EDTA, 1% Protease Inhibitor

cocktail (ThermoScientific, cat#78425)]. Cell lysates were analyzed using western blotting.

Reverse transfections of H4 Naïve cells were performed using a final concentration of 5nM siRNA. Initial trials using a concentration of 25nM siRNA with this cell line resulted in cell toxicity. Thus, the siRNA concentration was reduced to 5nM and treatment time was reduced to 48hrs. Briefly, 400,000 H4 naïve cells were reverse transfected with 5nM of Human USP8#3 (Dharmacon; cat# J-00523-08) or Non targeting siRNA (Dharmacon; cat# D-00181-10-05). Cells were harvested 48 hours post transfection and lysed using RIPA Buffer. Cell lysates were analyzed using western blotting.

Transient transfection of APP C60

2 million H4 cells were seeded in 100 x 20 mm tissue culture dishes (Falcon, cat#353803, growth area: 58.95cm^2) and were transiently transfected with 10µg C60 (a gift from Dr. Dennis Selkoe) using Lipofectamine 2000 transfection reagent (Invitrogen, cat#1168027). 24 hours post transfection, cells were trypsinized, reverse transfected with 5nM siRNA USP8 or NT siRNA in 6-well plates as described above. 48 hours post reverse transfection, samples were lysed with RIPA buffer, supplemented with 1% Protease Inhibitor cocktail (ThermoScientific) and 5mM 1,10-Phenantholine.

Western blot and densitometry analysis

Equal amounts of sample were separated by SDS-PAGE using 4-12% Bis-Tris gels (Invitrogen or Bio-Rad) and transferred to a PVDF membrane (Bio-Rad). Membranes

were probed with the following antibodies: anti-GFP (Molecular Probes, cat#A11122; 1:2000); anti-BACE1 (clone D10E5) (Cell signaling, cat#5606S, 1:1000); anti-GAPDH (Millipore, cat#MAB374, 1:2500); anti-USP8 (Cell signaling, cat#8728; 1:1000); anti-FLAG (Sigma, cat#F3165, 1:1000); anti-ADAM10 (Millipore, cat#AB19026, 1:1000); anti-ubiquitin (clone P4D1) (Santa Cruz, cat# sc-8017, 1:1000); and anti PS1-NTF (AB14), (1:3000), kindly provided by Dr. S. Gandy.

In order to detect full-length APP and APP-C-terminal fragments, equal amounts of each sample were separated by SDS-PAGE using 16.5% Tris-tricine gels (BioRad) and transferred to a PVDF membrane. Membranes were probed with anti-APP C-Terminal fragment (clone C1/6.1) antibody (Biolegend, 1:1000) or anti beta amyloid 1-16 (clone 6E10) (Biolegend, 1:1000). Densitometry analysis was performed using a LAS-4000 Fuji Imaging System and Quantity One software (BioRad). Protein levels were normalized to GAPDH levels, which served as a loading control given that no significant changes were observed in the levels of GAPDH in treated and untreated cells. Each sample was analyzed at least twice by Western blot. Each experiment included duplicate or triplicate samples for each treatment. At least 3 experiments were performed.

Lysosomal inhibition of BACE1-GFP cells

H4-BACE1-GFP cells were reverse transfected with Human USP8#3 (Dharmacon; cat# J-00523-08), or non-targeting siRNA Dharmacon (cat# D-00181-10-05). 72 hours post transfection, cells were washed with DPBS and treated with media containing 25 μM chloroquine (Sigma, cat#C6628), for approximately 16 hours. Cells were washed with

DPBS and lysed in RIPA buffer. BACE1-GPF protein levels in cell lysates were analyzed using western blotting.

Immunoprecipitation of Ubiquitinated BACE1-GFP

H4-BACE1-GFP (or H4-BACEK501R) cells were reverse transfected with Human USP8#3 (Dharmacon; cat# J-00523-08), or non-targeting siRNA. 96 hours post transfection, cells were washed with DPBS and lysed in IP lysis buffer [50mM Tris-HCl pH7.4, 0.15M NaCl, 1mM EDTA, 1% NP-40, 10% glycerol, 5mM 1,10-Phenantholine, 10mM N-ethylmaleimide (NEM), 50uM PR617 (Life sensors)], and Protease Inhibitor cocktail (ThermoScientific, cat#78425)]. Protein extracts were centrifuged at 14000 rpm for 10 minutes. 200µg of protein was precleared with 20µl of equilibrated Protein A/G Plus-agarose beads (Santa Cruz) for 30 mins at 4°C. BACE1-GFP was immunoprecipitated from lysates using 5ul anti-GFP antibody (Molecular Probes, cat#A11122) and 40ul protein A/G agarose beads in 500ul of IP lysis buffer overnight at 4°C. Beads were washed with TBST and proteins were eluted with 4x sample buffer/ 3% β-mercaptoethanol, for 10 minutes at 95°C. Samples were separated by SDS page. Western blotting with an anti-ubiquitin antibody (P4D1, Santa Cruz) was used to detect ubiquitinated BACE1.

Cell free BACE1 deubiquitination assay

Ubiquitinated BACE1-GFP was immunoprecipitated with Protein A/G plus agarose beads from USP8-depleted samples, as described above. Beads were washed twice with TBST and once with DUB reaction buffer [50mM Tris pH7.4, 1mM DTT, 0.5mM

EDTA]. Beads were split into 3 equal parts, combined with 0.5uM human recombinant Flag-USP8 (BPS Bioscience, cat# 80358) in 50ul DUB reaction buffer and incubated at 37°C for 120 minutes with shaking. 5mM NEM, a cysteine protease inhibitor that inhibits DUBs, was included in some reactions. Reaction was stopped by adding 4x sample buffer/ 3% β-mercaptoethanol, and incubation at 95°C for 10 minutes. Samples were separated by SDS-PAGE and BACE1 ubiquitination levels were evaluated using Western blotting.

Immunocytochemistry

H4 BACE1-GFP cells were transfected with USP8 siRNA, or non-targeting siRNA. 96 hours post- transfection, cells were washed with DPBS and fixed with 4% formaldehyde in DPBS for 15 minutes at room temperature. Coverslips were washed twice with 0.1M glycine in DPBS, then cells were permeabilized with 0.1% Triton X-100 in DPBS for 5 minutes. Coverslips were blocked with 5% BSA in DPBS and incubated overnight at 4°C with primary antibody. Late endosomes/lysosomes and early endosomes were labeled with monoclonal antibodies against LAMP2 (1:800; BD biosciences) and EEA1 (1:800; BD biosciences) respectively. Recycling endosomes were labeled with antibodies against RAB11 (1:400; Cell signaling). Coverslips were incubated in secondary antibody, goat anti mouse Alexa Fluor 568, or goat anti rabbit Alexa Fluor 568 (Molecular Probes; 1:500) for 1 hour at room temperature (RT). Coverslips were incubated in DAPI dilactate (1:10,000) for 5 minutes. Coverslips were mounted onto slides using Fluoromount-G (Southern Biotech). Images were acquired with a Nikon Eclipse T*i* inverted microscope, equipped with a Nikon A1R+ confocal system, and a Nikon Plan Apo VC 60x oil/1.4

numerical aperture objective (1024x1024 resolution). Images were acquired using NIS Elements software (Nikon). Z- stack projections thorough cells were acquired taking 0.5μM steps. Images were analyzed using a custom macro on Image J. Briefly, the sum of the slices were generated. Background subtraction (Rolling ball radius=20), median filter (radius=1) and triangle threshold were applied to images with BACE1, EEA1, RAB11 and LAMP2 labeling to delineate regions positive for labeling. Percentage of BACE1 in the early-endosome was determined by finding the quotient of the total GFP intensity in EEA1-positive regions and the total GFP intensity within cell. Percentage of BACE1-GFP in lysosomes and recycling endosomes was calculated in a similar manner using LAMP2–positive and RAB11-positive regions, respectively.

Amyloid Beta ELISA

Conditioned media was collected from H4-BACE1-GFP and H4 Naïve cells treated with USP8 or NT siRNA. 72 hours post transfection (H4-BACE1-GFP) or 24 hours post transfection (H4 Naïve), media was removed and replaced with 0.8ml fresh media. 24 hours later, media was collected. Media was centrifuged immediately after collection, for 10 minutes at 10,000g, to remove cellular debris. Cells were also harvested for western blot analysis. The most abundant amyloid beta isoform (Aβ 1-40) in samples was measured using a Human Amyloid beta 1-40 ELISA kit (ThermoFisher Scientific, cat# KHB3481). Aβ concentration in media (pg/ml) was normalized to the protein concentration of cell lysates from the corresponding well (μg/μl).

Statistical analysis

Statistical analysis was performed using Prism 6 (Graph pad software). Mean of samples were compared using either One-way analysis of variance or the unpaired t-test with Welch's correction. Values were expressed as mean and standard error of the mean (S.E.M.). The alpha level was set as 0.05. ELISA data were analyzed using IBM SPSS, version 22 (IBM). Samples were compared using a linear mixed effect model, where the fixed effect was siRNA treatment (USP8 or NT) and the random effect was each independent experiment. Sample size (n) was each individual sample.

Chapter 3: Results

3.1 USP8 depletion decreases BACE1 and Aβ levels

USP8 depletion decreases BACE1-GFP but not ADAM10 and presenilin 1 protein levels.

Depletion of USP8 has been shown to decrease protein levels of USP8 substrates, as a result of increased substrate ubiquitination and degradation (Mizuno et al. 2005, Niendorf et al. 2007). To determine whether USP8 depletion resulted in a decrease in BACE1, we performed siRNA-mediated knockdown of USP8, using two different siRNA targeting USP8 in a H4 neuroglioma cell line overexpressing BACE1-GFP (H4-BACE1-GFP). Treatment of cells with USP8 siRNA#3 and USP8 siRNA#2, resulted in an 87±7% and 85±5% reduction of USP8 protein levels, respectively, compared to NT siRNA treated cells (mean±S.E.M., $p<0.0001$; One way ANOVA and Tukey's multiple comparison test, n=3 independent experiments). Knockdown with each siRNA resulted in an independent decrease in BACE1-GFP protein in H4-BACE1-GFP cells ($p=<0.05$; One way ANOVA and Tukey's multiple comparison test, n=4 independent experiments) (Fig. 3.1A-B). Furthermore, to determine whether this protein decrease was specific for BACE1, we examined the protein levels of two other proteins important in APP processing, ADAM10, the prevalent alpha secretase of APP, and presenilin 1 (PS1), the catalytic subunit of the γ-secretase complex. USP8 depletion did not result in any change in ADAM10 or PS1 protein levels (Fig. 3.1C-E) (n.s. $p>0.05$; One way ANOVA and Tukey's multiple comparison test, n=3 independent experiments). The decrease in BACE1 protein but not ADAM10 or PS1, indicated that the effect of USP8 depletion is specific to BACE1, and not to other enzymes that also cleave APP.

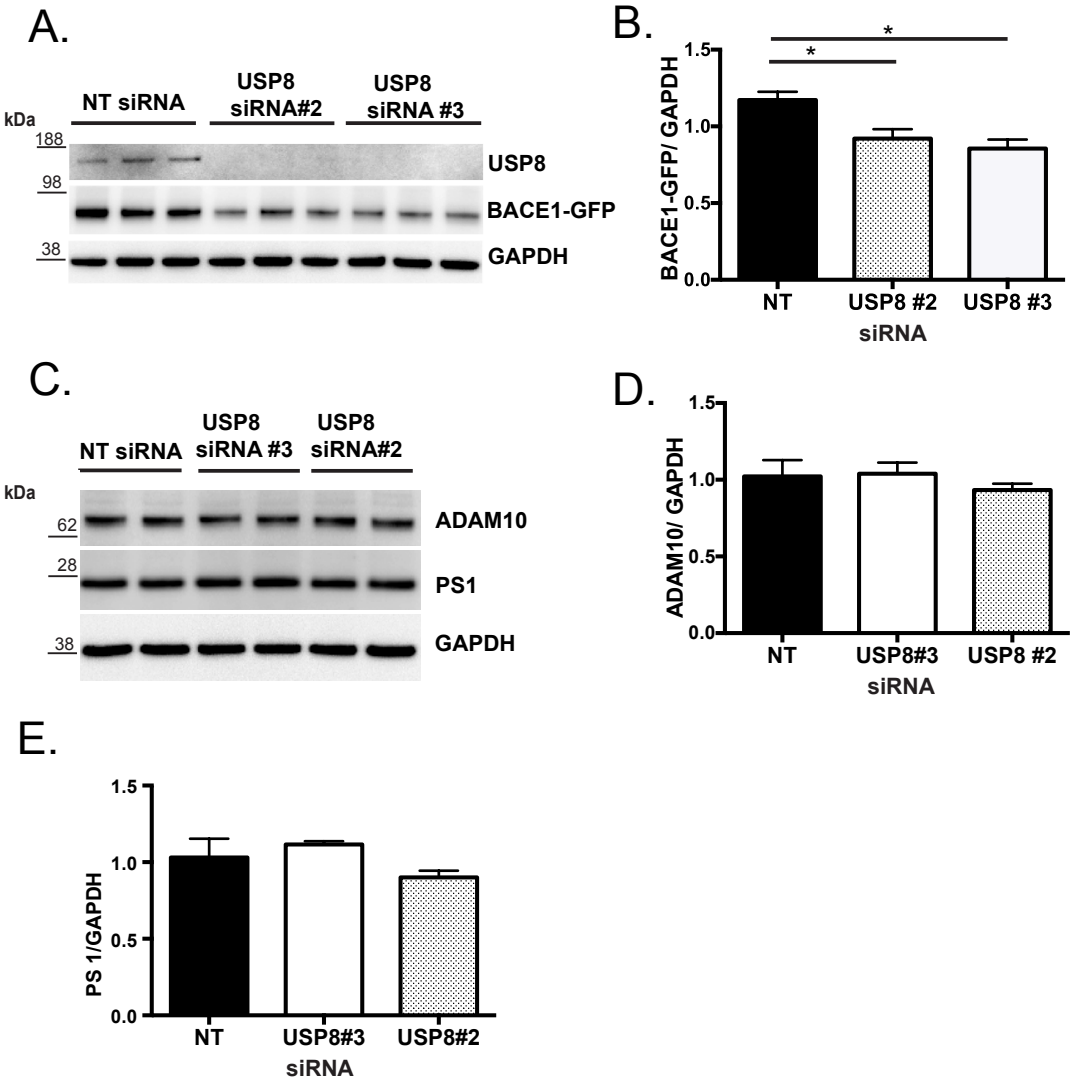


Figure 3.1

USP8 depletion decreases BACE1-GFP protein levels, but not ADAM10 and presenilin 1 protein levels.

A) USP8 knockdown using 2 separate siRNA independently decreases BACE1-GFP in BACE1-GFP overexpressing cell line. Representative Western blot with B) Quantification of BACE1-GFP normalized to GAPDH. C) USP8 knockdown does not decrease ADAM10 or Presenilin 1 protein levels. Representative Western blot. D) Quantification of ADAM10 normalized to loading control, GAPDH and E) Quantification of PS1 normalized to GAPDH. Graphs show mean and S.E.M. n=3 independent experiments; *, $p<0.05$; One-way ANOVA and Tukey's multiple comparison test. NT, Non targeting.

USP8 depletion decreases endogenous BACE1 protein levels.

Next we determined whether USP8 depletion resulted in a similar decrease in endogenous BACE1. USP8 was knocked-down in H4 cells, which express endogenous levels of BACE1. Initial trials using a concentration of 25nM siRNA with this cell line resulted in toxicity. Thus, the siRNA concentration was reduced to 5nM and treatment time was reduced to 48 hours rather than 96 hours. Western blot analysis of lysates obtained from USP8-depleted or NT siRNA treated cells revealed a decrease in USP8 accompanied by a decrease in endogenous BACE1 ($p=0.0001$; Unpaired t-test with Welch's correction; n=4 independent experiments) (Fig. 3.2A-B). This demonstrated that USP8 depletion resulted in a decrease in endogenous BACE1, similar to the observed decrease in BACE1-GFP.

Inhibition of lysosomal degradation rescues USP8-dependent BACE1 protein decrease.

As BACE1 is degraded in the lysosomes, we hypothesized that the decrease in BACE1 protein levels observed with USP8 depletion, was due to increased degradation of BACE1 in the lysosomes. To determine whether lysosomal degradation was a possible mechanism for the decrease in BACE1 protein levels in USP8 depleted cells, we treated USP8-depleted and NT siRNA transfected cells with chloroquine, a weak base, which inhibits lysosomal hydrolases. Chloroquine treatment resulted in an accumulation of BACE1-GFP in both USP8 and NT siRNA treated samples (Fig. 3.3A-B). Thus, lysosomal inhibition rescued the decrease of BACE1-GFP protein levels in USP8–depleted cells, indicating that USP8 regulates lysosomal degradation of BACE1.

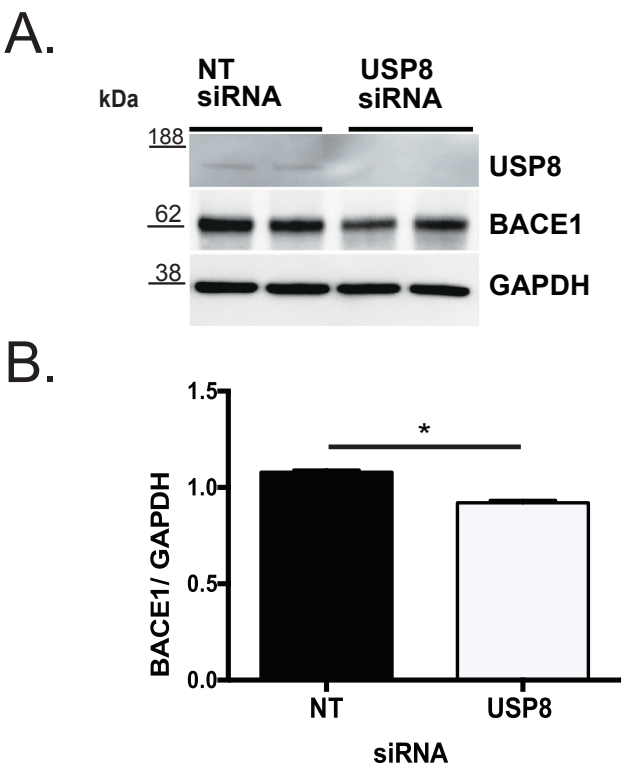


Figure 3.2

USP8 knockdown decreases endogenous BACE1 protein levels in H4 cells.

Protein levels of endogenous BACE1 were evaluated in H4 cell lysates 48 hours post-transfection. A) Representative Western blot with B) Quantification of BACE1 normalized to loading control, GAPDH. Graph shows mean and S.E.M.; n=4 independent experiments; *, p=0.0001; Unpaired t-test with Welch's correction. NT, Non targeting.

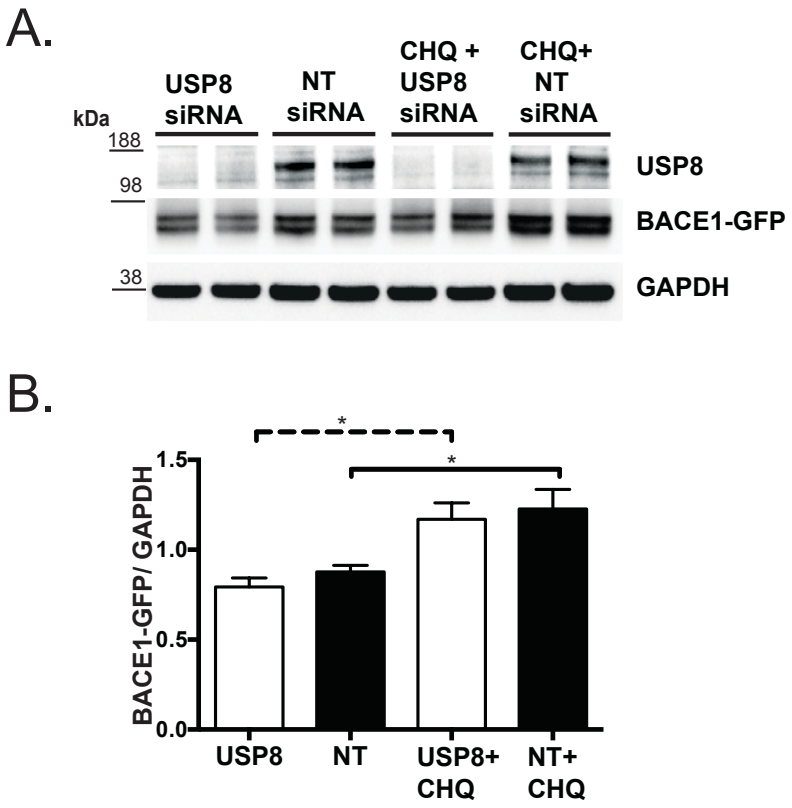


Figure 3.3

Inhibition of lysosomal degradation rescues USP8-dependent BACE1 protein decrease.

Chloroquine (25μM) treatment caused an accumulation of BACE1-GPF protein in both USP8 and NT siRNA treated cells. A) Western blot with B) Quantification of BACE1-GFP normalized to loading control, GAPDH. Graph shows mean and S.E.M.; n=5 independent experiments; *, $p<0.05$; One-way ANOVA and Tukey's multiple comparison test. CHQ, chloroquine; NT, Non targeting.

USP8 deubiquitinates BACE1 at Lysine 501

Depletion of USP8 results in an increase in the ubiquitination of its substrates (Smith et al. 2016, Niendorf et al. 2007, Row et al. 2006, Mizuno et al. 2005, Scudder et al. 2014, Oh et al. 2014). Thus, we tested whether USP8 depletion would result in increased BACE1 ubiquitination. BACE1-GFP was immunoprecipitated with anti-GFP antibody in lysates of cells treated with either USP8 or NT siRNA for 96 hours. Western blot analysis of immunoprecipitated BACE1-GFP, using anti-Ub antibody, revealed that BACE1-GFP was significantly more ubiquitinated in USP8-depleted than control samples (Fig. 3.4A-B)($p=0.0003$; unpaired t-test with Welch's correction; n= 4 independent experiments). We have previously shown that BACE1 is ubiquitinated at K501 and that the K501R mutation prevents BACE1 ubiquitination (Kang et al. 2010, Kang et al. 2012). Thus, we tested whether USP8 deubiquitinates BACE1 at K501 by depleting USP8 in H4-BACE1-K501R cell line. BACE1-GFP was immunoprecipitaed from cell lysates using anti-GFP antibody. Western blot analysis of immunoprecipitates with anti-Ub antibody revealed that BACE1-K501R GFP had negligible levels of ubiquitination. Furthermore, depletion of USP8 did not lead to the large increases in ubiquitination, observed in wild-type BACE1-GFP (Fig. 3.4A). These data indicate that USP8 is responsible for deubiquitinating BACE1 at K501. However, indirect effects of USP8 could not be ruled out as the experiment was carried out in cells.

Thus, to determine whether USP8 directly deubiquitinated BACE1, a cell-free reaction was prepared. BACE1-GFP was immunoprecipitated with anti-GFP antibody from USP8-depleted samples, where BACE1-GFP was highly ubiquitinated. Immunoprecipitates immobilized on Protein A/G plus agarose beads were mixed with

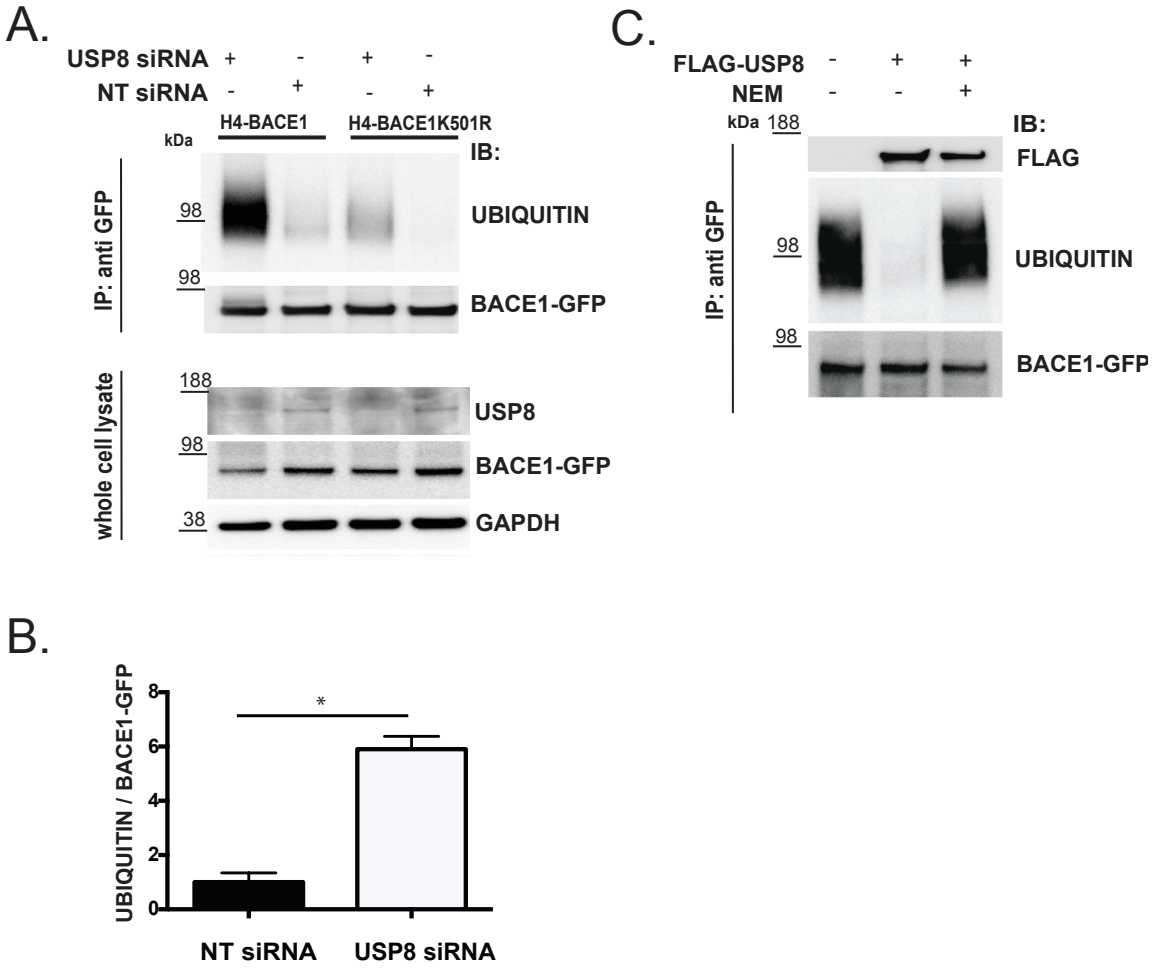


Figure 3.4

USP8 deubiquitinates BACE1 at Lysine 501.

A) USP8 depletion increases BACE1 ubiquitination. Ubiquitinated BACE1-GFP or BACE1-K501R was immunoprecipitated from cells treated with either USP8 or NT siRNA. USP8 depletion increased ubiquitination of immunoprecipitated BACE1. B) Quantification of ubiquitin normalized to immunoprecipitated BACE1-GFP. Graph shows mean and S.E.M.; n= 4 independent experiments; *, p=.0003, unpaired t-test with Welch's correction. C) USP8 deubiquitinates BACE1 in cell-free assay. Western blot showing ubiquitinated BACE1 after incubation with recombinant FLAG-USP8 in cell free assay with or without 5mM NEM for 120 minutes at 37°C. NT, Non targeting.

human recombinant Flag-USP8 and incubated at 37°C for 120 minutes. Furthermore, 5mM NEM, a cysteine protease inhibitor, known to inhibit DUBs, was included as an experimental negative control in some samples. In this cell-free reaction, samples containing recombinant Flag-USP8 had very low levels of ubiquitination after 120 minutes, compared to samples where no recombinant USP8 was included. Furthermore, addition of NEM with recombinant FLAG-USP8, inhibited the decrease in ubiquitination (Fig. 3.4C). These data indicate that USP8 directly deubiquitinates BACE1.

USP8 depletion results in BACE1 accumulation in early endosomes and lysosomes and reduction in recycling endosomes.

Lysine-63-linked polyubiquitination is a known signal for protein trafficking to the lysosomes. We have previously reported that BACE1 is degraded by the lysosomes and that it is Lysine 63-linked polyubiquitinated (Kang et al. 2010, Kang et al. 2012). As USP8 depletion resulted in an increase in BACE1 ubiquitination, we hypothesized that there would be a resultant increase in BACE1 trafficking to the endosomes and then to the lysosomes for degradation. Subcellular localization of BACE1-GFP was assessed in H4-BACE1-GFP cells, 96 hours post-transfection of USP8 or NT siRNA. To identify the different subcellular compartments, cells were immunostained with markers for early endosomes (EEA1), late endosomes/lysosomes (LAMP2) and recycling endosomes (RAB11). Confocal microscopy showed that BACE1-GFP percentage intensity was increased in EEA1-positive regions, in USP8-depleted compared to NT siRNA treated cells ($p=0.0025$, unpaired t-test with Welch's correction) (Fig. 3.5A-B). We also found an increase in the percentage intensity of BACE1-GFP in LAMP2 positive regions in

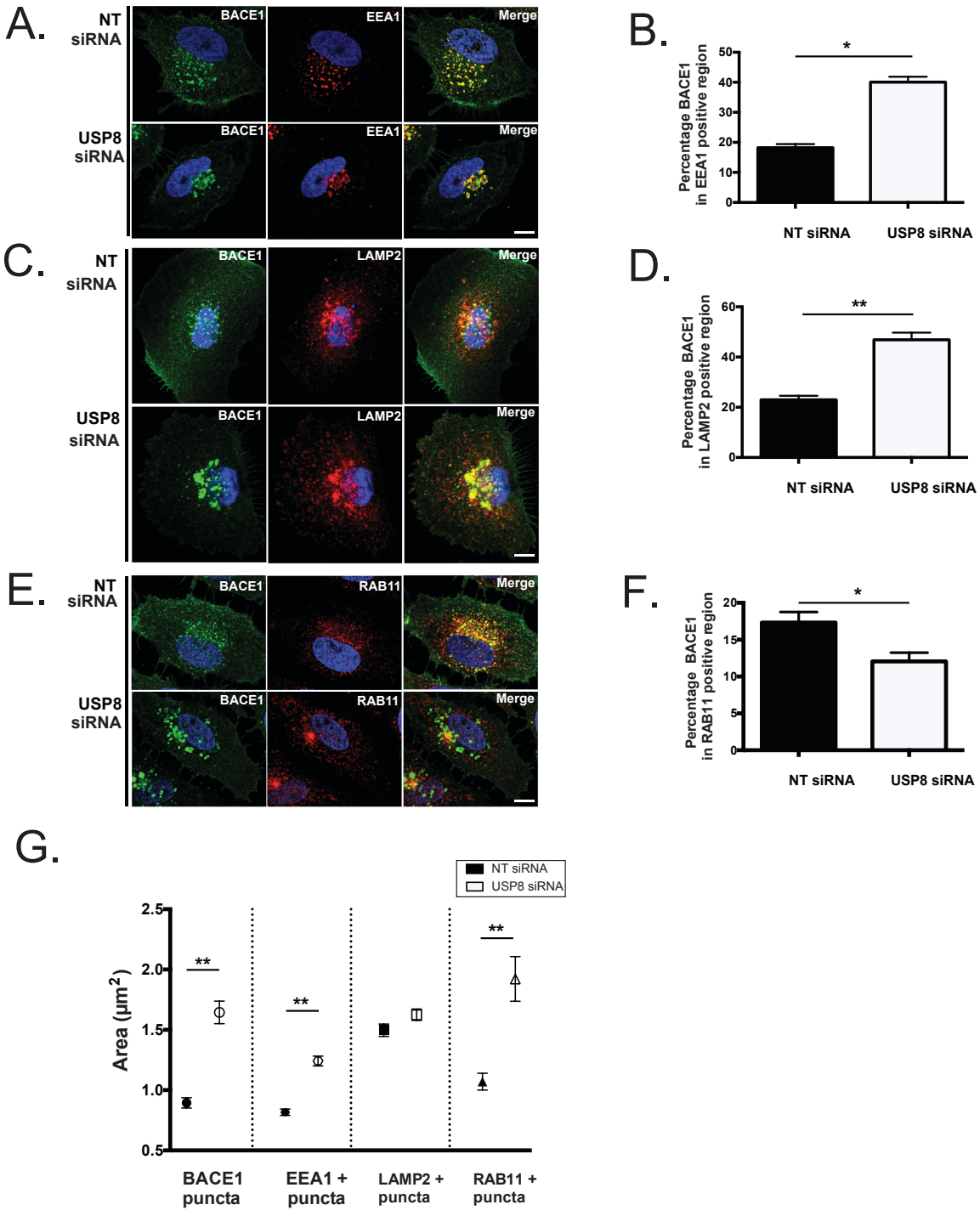


Figure 3.5

USP8 depletion results in BACE1 accumulation in early endosomes and lysosomes and reduction in recycling endosomes.

Figure 3.5

USP8 depletion results in BACE1 accumulation in early endosomes and lysosomes and reduction in recycling endosomes.

A) Representative photomicrograph of H4-BACE-GFP cell treated with NT or USP8 siRNA, labeled with early endosome marker, EEA1. B) USP8 depletion increases the percentage of BACE1 in EEA1-positive regions. Percentage was derived by dividing total intensity of BACE1-GFP in EEA1-positive regions by total GFP intensity in the cell. C) Representative photomicrograph of H4-BACE-GFP cell treated with NT or USP8 siRNA, labeled with late endosome/lysosome maker, LAMP2. D) USP8 depletion increases the percentage of BACE1 in LAMP2-positive regions. E) Representative photomicrograph of H4-BACE-GFP cell treated with NT or USP8 siRNA, labeled with recycling endosome maker, RAB11. F) USP8 depletion decreases the percentage of BACE1 in RAB11-positive regions. G) USP8 depletion results in an increase in the area of BACE1-positive puncta, EEA1-positive early endosomes and RAB11-positive recycling endosomes, but not LAMP2-positive lysosomes. Nuclei, shown in blue, are labeled with DAPI. Graphs show mean and S.E.M., n=43-55 cells per condition, from 3 independent experiments. *, $p<0.01$; **, $p<0.0001$; Unpaired t-test with Welch's correction. Scale bar= 10 μm. NT, Non targeting.

USP8-depleted cells ($p=<0.0001$, unpaired t-test with Welch's correction) (Fig. 3.5C-D). In contrast, we found a decrease in the percentage intensity of BACE1-GPF in RAB11-positive regions, when USP8 was depleted ($p=0.0057$, unpaired t-test with Welch's correction) (Fig. 3.5E-F). These data suggest that USP8 regulates the trafficking of BACE1 after its internalization by favoring the recycling of BACE1, while negatively regulating its lysosomal degradation.

We also noted a 1.8 fold increase in the size of BACE1-positive puncta ($p<0.0001$, unpaired t-test with Welch's correction). Moreover, the size of EEA1-labelled endosomes and RAB11-positive recycling endosomes was increased by 1.5 and 1.8 fold, respectively ($p<0.0001$, unpaired t-test with Welch's correction). In contrast there was no change in the size of the LAMP2-positive lysosomes ($p=0.068$, unpaired t-test with Welch's correction) (Fig. 3.5G). Our findings are in agreement with previous reports showing that

depletion of USP8 or overexpression of a dominant negative USP8 mutant causes the accumulation of ubiquitinated proteins within endosomes, and a corresponding increase in endosome size (Niendorf et al. 2007, Row et al. 2006, Mizuno et al. 2006, Hasdemir et al. 2009, Bowers et al. 2006, Smith et al. 2016).

USP8 knockdown decreases APP-C99 and C89 generation, but not APP-CTF degradation.

As USP8 depletion resulted in a decrease in BACE1 protein levels, we next determined whether there would be an accompanying decrease in BACE1-mediated APP processing. BACE1 cleaves amyloid precursor protein (APP) at 2 sites forming APP-C99 and C89, while α-secretase generates APP-C83. These C-terminal fragments (CTF) may exist in non-phosphorylated and phosphorylated forms. Accordingly 5 APP-CTFs may be resolved on Western blotting, corresponding to APP-CTFs pC99, C99, pC89, C89+pC83, and C83 in H4 cells (Fig. 3.6A). However, 3 bands corresponding to pC99, pC89, C89+pC83, were the most apparent in H4-BACE-GFP cell lysates owing to increased β-secretase processing, and were hence quantified (Fig. 3.6A).

Lysates from H4-BACE1-GFP cells treated with USP8 and NT siRNA were separated by SDS-PAGE using 16.5% Tris-Tricine gels (BioRad) and incubated with C1/6.1 antibodies (Biolegend) to detect full-length APP and APP CTFs. USP8 depletion increased the amount of full-length APP (Fig. 3.6B-C), which is consistent with decreased BACE1 processing. Furthermore, USP8 depletion decreased the ratio of pC99/APP and pC89/APP (Fig. 3.6D-E). However, the ratio of C89+pC83 to APP remained unchanged (Fig. 3.6F)

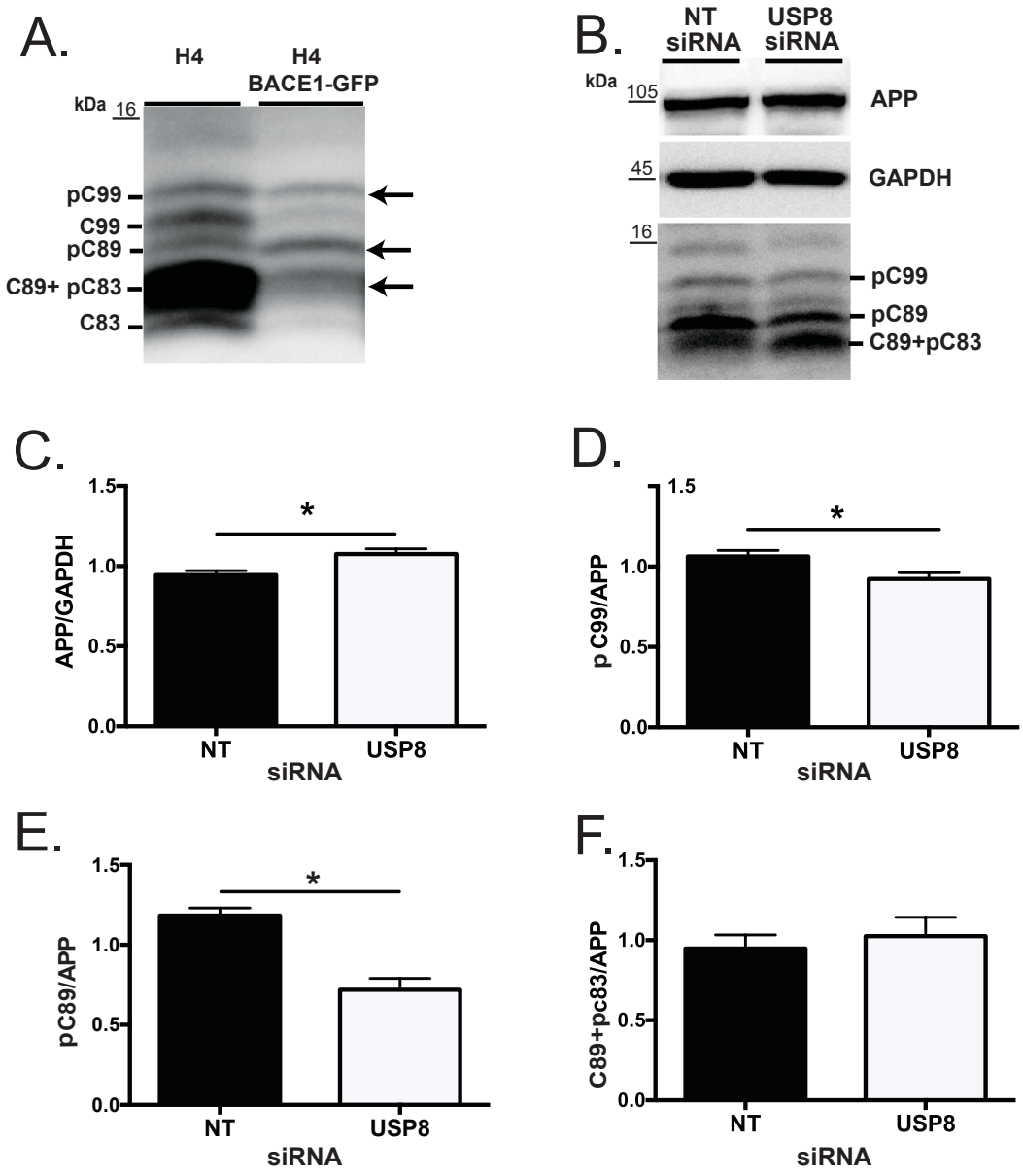


Figure 3.6

USP8 knockdown decreases APP-C99 and C89 generation in H4-BACE-GFP cells

A) USP8 knockdown decreases the ratio of pC99, pC89 but not C89+pC83 to full length APP. Representative Western blot showing prominent APP-CTF bands in untreated cell lysates. B) Lysates from H4-BACE-GFP cells were separated by SDS-PAGE using 16.5% Tris-Tricine gels and incubated with C1/6.1 antibodies to label full length APP and APP C- terminal fragments. Representative Western blot. C) Quantification of full length APP normalized to GAPDH; *, p=0.0199. D) Ratio of pC99 to APP; *, p=0.0255. E) Ratio of pC89 to APP; *, p=0.0005; F) Ratio of C89+pC83 to APP; p=0.602. Graphs show mean and S.E.M.; n=6 independent experiments; Unpaired t-test with Welch's correction. NT, Non targeting

($p<0.05$; Unpaired t-test with Welch's correction, n=6 independent experiments). Since C99 and C89 are products of BACE1-mediated APP cleavage, decreased protein levels of these APP-CTFs suggested a decrease in β-secretase processing of APP.

Since USP8 depletion resulted in a decrease in products of BACE1-mediated APP processing in H4-BACE1-GFP cells, we sought to determine whether similar effect could be observed in H4 cells, with endogenous levels of BACE1. Lysates from H4 cells treated with USP8 and NT siRNA were separated by SDS-PAGE using 16.5% Tris-Tricine gels and incubated with C1/6.1 antibody (Biolegend) to detect full-length APP and APP CTFs. Like in H4-BACE1-GFP cells, USP8 depletion led to a slight increase in the amount of full-length APP (Fig. 3.7A-B). Furthermore, USP8 depletion decreased the ratio of pC99/APP, C99/APP, pC89/APP and C89+pC83/APP (Fig. 3.7C-F). However, the C83/APP ratio remained unchanged (Fig. 3.7G) ($p<0.05$; Unpaired t-test with Welch's correction, n=3-4 independent experiments). These results demonstrated that the decrease in endogenous BACE1 with USP8 depletion (Figure 3.2), was sufficient to decrease BACE1 mediated APP processing.

Given that C99 and C89 are products of BACE1-mediated APP cleavage, decreased protein levels of these APP-CTFs suggested a decrease in β-secretase processing of APP. However, APP-CTFs are ubiquitinated and degraded in the lysosomes (Morel et al. 2013), thus, a decrease in pC99 and pC89 may also have arisen from increased APP-CTFs degradation in a BACE1-independent manner. Thus, H4 cells overexpressing APP-C99 were transfected with either USP8 or NT siRNA. Western blot analysis revealed that USP8 depletion did not alter the levels of overexpressed C99 ($p=0.4561$; Unpaired t-test with Welch's correction; n=3 independent experiments),

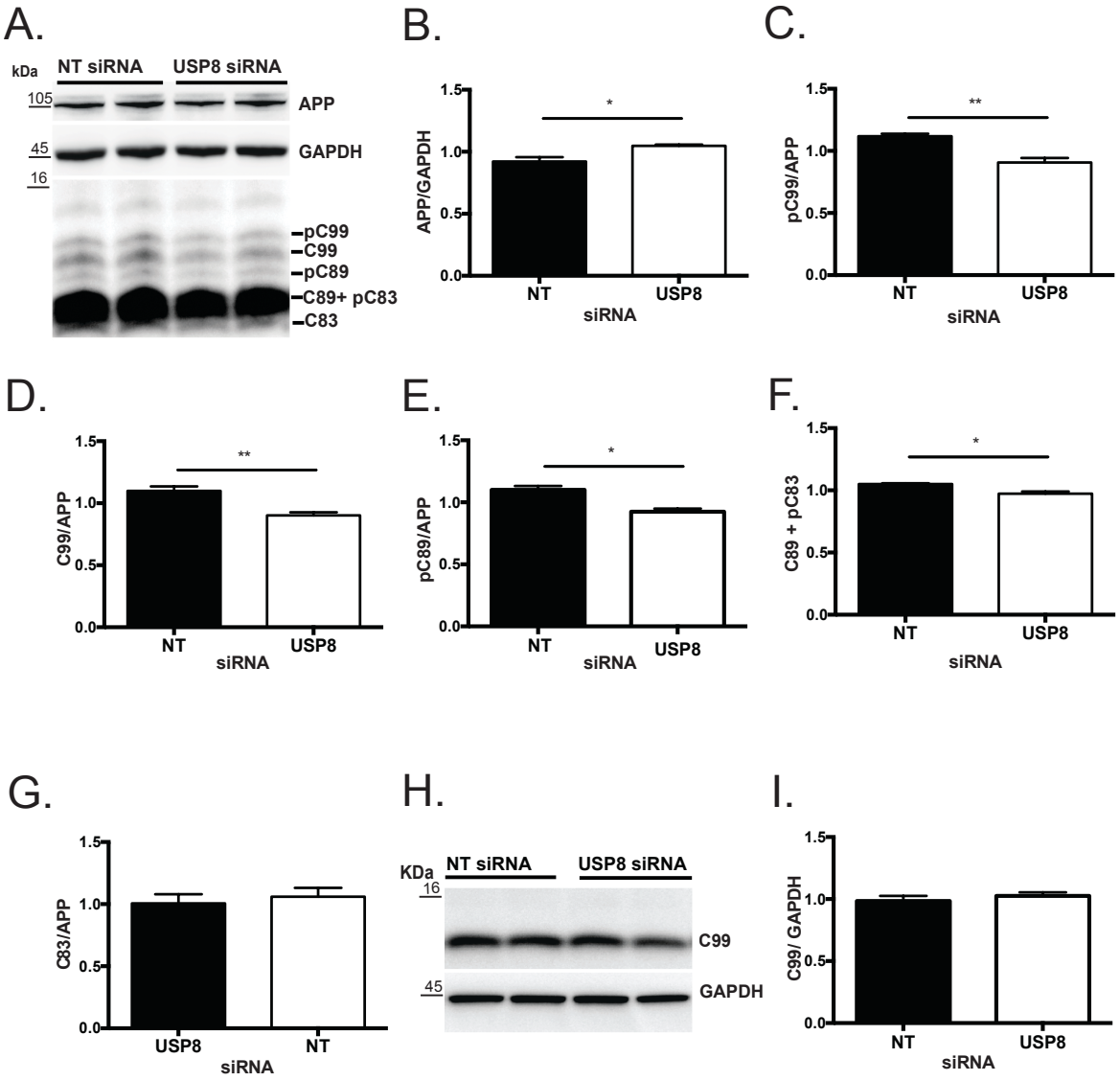


Figure 3.7

USP8 knockdown decreases APP-C99 and C89 generation in H4 cells

A) USP8 knockdown decreases the ratio of pC99, C99, pC89, and C89+pC83, to full length APP, but not C83. Representative Western blot. B) Quantification of full length APP normalized to GAPDH; *, p=0.0315. C) Ratio of pC99 to APP; *, p=0.0049. D) Ratio of C99 to APP, *, p=0.0059; E) Ratio of pC89 to APP; *, p=0.0124; F) Ratio of C89+pC83 to APP; *, p=0.0137; G) Ratio of C83 to APP; p=0.602. H) USP8 depletion does not affect overexpressed C99 levels in H4-C99 cell line. Representative Western blot and I) Quantification of C99 normalized to GAPDH. Graphs show mean and S.E.M.; n=3-4 independent experiments; Unpaired t-test with Welch's correction, NT, non targeting.

indicating that USP8 depletion did not decrease APP-CTFs degradation in a BACE1-independent manner (Fig. 3.7H-I). Altogether, these data indicate that the decrease in BACE1 protein levels observed with USP8 depletion leads to a decrease in BACE1-mediated APP processing.

USP8 knockdown decreases AICD protein levels

Moreover, we sought to determine whether USP8 depletion affected the stability of the AICD. The AICD is rapidly degraded in the cytoplasm by insulin-degrading enzyme (IDE) (Edbauer et al. 2002, Farris et al. 2003). While treatment of cells with proteasome inhibitors has no effect on AICD levels, treatment of cells with ammonium chloride (NH_4Cl) and chloroquine, known lysosomal inhibitors, increases AICD levels (Eisele et al. 2007, Vingtdeux, Hamdane, Begard, et al. 2007). To determine whether USP8 depletion affected the stability of the AICD, we overexpressed APP-CTF C60 (Kimberly et al. 2001), in H4 cells, as endogenous levels of AICD in H4 cell lysates were low and difficult to quantify.

The APP-C60 plasmid expresses a protein containing the terminal 59 amino acid residues of APP, along with an initiating methionine residue. This sequence corresponds to the portion of the APP-CTF that occurs after the γ-secretase cleavage site at position 40 within Aβ (Kimberly et al. 2001). Overexpressing C60 allowed the effect of USP8 depletion on AICD levels to be measured independently of the changes in CTF levels. Endogenous AICD is a product resulting from γ-secretase cleavage of C99, C89 and C83, thus AICD levels may be influenced by changes in CTF levels. USP8 depletion resulted

in decreased C60 protein levels (p=0.0187; Unpaired t-test with Welch's correction; n=3 independent experiments), suggesting that USP8 may play a role in stabilizing the AICD (Figure 3.8).

USP8 depletion decreases Aβ production in H4 cells.

As there was a decrease in the ratio of pC99 and pC89 to full-length APP in H4-BACE1-GFP cells, we hypothesized that there would be a decrease in the level of Aβ production with USP8 depletion. Aβ1-40 content was measured in conditioned media from H4-BACE1-GFP cells treated with USP8 or NT siRNA. However, depletion of USP8 did not result in a decrease of Aβ1-40 in this cell line (data not shown). Given that the overexpression of BACE1 has been shown to promote APP processing at the β'-site resulting in the increased production of +11 Aβ species (Vassar et al. 1999, Urbe et al. , Creemers et al. 2001), it is possible that in H4-BACE1-GFP cells the decrease in BACE1 results in a reduction of +11Aβ which is not detected by the ELISA. Accordingly, the levels of pC89 are decreased to a greater extent than pC99 in H4-BACE1-GFP cells depleted of UPS8 (Fig. 3.6B, D-E). Thus, we determined whether a decrease in BACE1 protein levels resulted in a decrease in Aβ formation in H4 cells expressing endogenous BACE1. We measured Aβ1-40 content in conditioned media from H4 cells treated with USP8 or NT siRNA and found that there was a statistically significant decrease (p=0.002) in Aβ1-40 production with USP8 depletion (Fig. 3.9). These data indicate that a decrease in BACE1 protein levels observed in USP8 depleted cells, results in a corresponding decrease in Aβ formation.

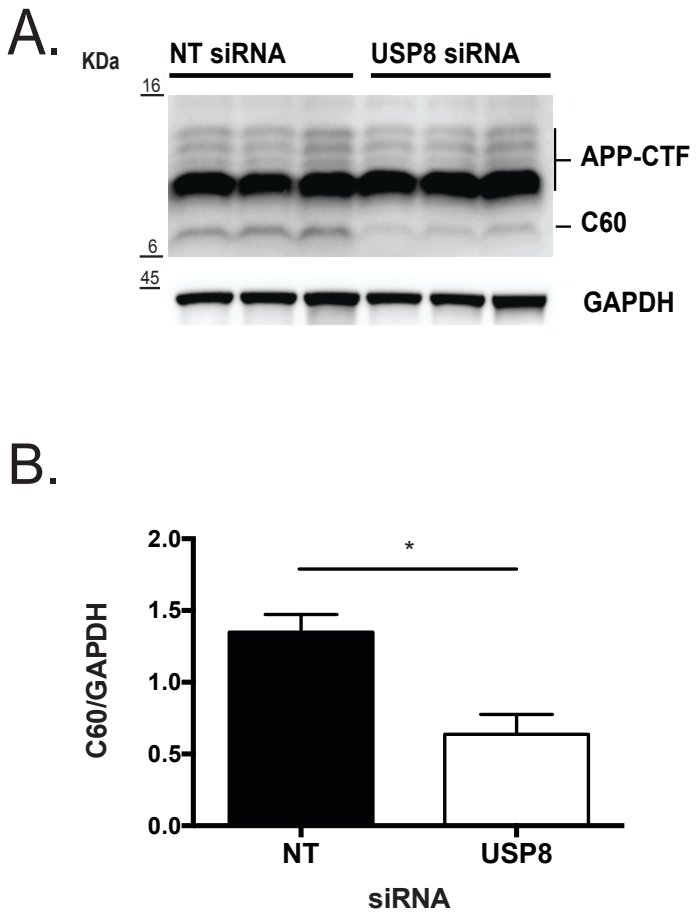


Figure 3.8

USP8 knockdown decreases AICD protein levels

USP8 depletion decreases ectopically-expressed AICD protein levels. APP-C60 was overexpressed in H4 cells by transient transfection. 24 post-transfection, cells were treated with USP8 or NT siRNA. A) Representative Western blot and B) Quantification of APP-C60 normalized to GAPDH. Graphs show mean and S.E.M; n=3 independent experiments; p=0.0187; Unpaired t-test with Welch's correction. NT, Non targeting.

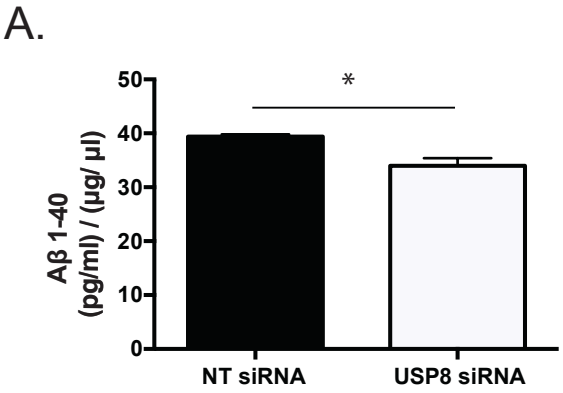


Figure 3.9

USP8 depletion decreases amyloid beta production in H4 cell line.

A) Conditioned media was collected from H4 Naïve cells treated with USP8 or NT siRNA. Aβ 1-40 in conditioned media was measured using ELISA kit. Aβ concentration (pg/ml) was normalized to the protein concentration of cell lysates from the corresponding well (µg/µl). USP8 depletion reduced the amount of Aβ 1-40 in conditioned media collected for H4 naïve cells; Graph shows mean and S.E.M, n=8-9 samples per condition from 3 independent experiments; *, p=0.002, linear mixed model. NT, Non targeting.

Chapter 4: Discussion

4.1 USP8 depletion increases BACE1 ubiquitination and degradation

Previous investigators have shown that USP8 depletion leads to increased turnover of EGFR, a lysosomal degraded protein (Berlin, Schwartz, and Nash 2010, Mizuno et al. 2005, Niendorf et al. 2007). We sought to determine whether USP8 depletion, would lead to a similar decrease in BACE1 protein levels, and a subsequent decrease in Aβ formation. In our investigations, we found that USP8 depletion decreased levels of ectopically expressed BACE1-GFP, as well as endogenous BACE1 in H4 cells. USP8 depletion, however, did not affect ADAM10 and PS1 protein levels. This showed that USP8 depletion affected BACE1 but not other APP-cleaving proteins.

Given that BACE1-GFP cDNA transcription is regulated by the CMV promoter, the observed decrease in BACE1-GFP protein levels was less likely due to changes in transcription or translation. Thus, we hypothesized that increased lysosomal degradation was likely responsible for decreased BACE1 protein levels in cells depleted of USP8. Accordingly, we found that treatment of USP8-depleted cells with chloroquine, a lysosomal inhibitor, prevented BACE1 protein decrease. Furthermore, chloroquine treatment resulted in a similar accumulation of BACE1-GFP in both USP8-treated and NT-treated samples, demonstrating that lysosomal function is preserved when USP8 is depleted.

We hypothesized that an increase in BACE1 ubiquitination followed by increased lysosomal degradation was the mechanism for the BACE1 protein decrease. Accordingly, we found that USP8 depletion increased BACE1 ubiquitination at K501. Moreover, we determined that USP8 directly deubiquitinated BACE1 in an *in vitro* cell free assay.

Together, these data showed that BACE1 is a direct substrate of USP8 deubiquitination, supporting a model of direct interaction, over the possibility that USP8 was influencing BACE1 ubiquitination and protein levels, indirectly e.g. by deubiquitinating other DUBs and/or ubiquitin ligases.

Currently, it is unknown whether USP8 deubiquitinates any other DUBs, although USP8 is known to bind to and deubiquitinate three E3 ligases, NRDP1 (Wu et al. 2004, Avvakumov et al. 2006), GRAIL (Soares et al. 2004), and Parkin (Durcan et al. 2014). Furthermore, USP8 depletion is known to result in decreased STAM and HRS protein levels (Niendorf et al. 2007, Row et al. 2006). These two components of ESCRT0, are important for functional recruitment of ubiquitinated proteins to endosomes and for lysosomal degradation. However, an indirect effect of USP8 through these proteins is less likely, as it would lead to a decrease in BACE1 degradation, and a resultant BACE1 accumulation, rather than the observed BACE1 protein decrease. Given that GGA3 is able to bind ubiquitinated cargoes and transfer them to the tumor susceptibility gene (TSG101), an ubiquitin-binding subunit of ESCRT complex I (Puertollano and Bonifacino 2004, Bilodeau et al. 2004, Prag et al. 2005, Kawasaki et al. 2005), functioning like HRS, it is possible that BACE1 is trafficked to the lysosomes by GGA3 even when levels of STAM are decreased in USP8-depleted cells.

Previous investigators have shown that USP8 depletion results in the accumulation of ubiquitinated proteins in the endosomes (Niendorf et al. 2007, Row et al. 2006, Mizuno et al. 2006). We hypothesized that the decrease in BACE1 protein levels was a result of increased trafficking of BACE1 to the lysosomes, and reduction of BACE1 in recycling endosomes, the intracellular site where BACE1 meets and processes

APP (Das et al. 2016, Das et al. 2013, Buggia-Prevot et al. 2013). We found that USP8 depletion increased BACE1 localization in the early endosomes and the lysosomes, while decreasing BACE1 contained in the recycling endosomes. Furthermore, there was an increase in early and recycling endosome puncta size, whereas there was no change in lysosome size.

Depletion of USP8 or overexpression of a dominant negative USP8 mutant has been shown to cause the accumulation of proteins within early endosomes and increase early endosome and MVB diameter. Furthermore, clustering of endosomes, facilitated by the formation of electron dense regions between endosomes, has been observed by electron microscopy (Row et al. 2006, Mizuno et al. 2006). These abnormalities have been attributed to numerous causes. First, it has been attributed to the accumulation of ubiquitinated proteins within endosomes (Niendorf et al. 2007, Row et al. 2006, Mizuno et al. 2006, Hasdemir et al. 2009). Second, it has been attributed to the dysfunction of the endosomes due to loss of endosomal proteins like STAM and HRS (Mizuno et al. 2005, Niendorf et al. 2007). Third, it has been suggested that USP8 is necessary for the promotion of lysosomal degradation, namely the need of USP8 to deubiquitinate proteins before they are finally transferred to the lysosomes (Oh et al. 2014). In our experiments, it is likely that there is an increased amount of ubiquitinated proteins within the early endosomes. However, it is unlikely that there is a complete inhibition of protein transfer to the lysosomes, as the decrease in BACE1 protein suggests that there is successful protein degradation.

4.2 USP8 depletion decreases BACE1-mediated APP processing

BACE1 is the rate-limiting enzyme in Aβ production. Since USP8 depletion resulted in a decrease in BACE1 protein levels, we expected a decrease in BACE1-mediated APP cleavage and also a decrease in Aβ formation. As expected, we found that USP8 depletion was accompanied by a decrease in the ratio of pC99 and pC89 to APP in H4-BACE1-GFP cells. pC99 and pC89 are phosphorylated forms of C99 and C89, direct products of BACE1-APP cleavage. Furthermore, USP8 depletion led to the decrease in pC99/APP, C99/APP and pC89/APP in H4 cells, which did not overexpress BACE1. This demonstrated that the decrease in endogenous BACE1 after USP8 depletion was sufficient to decrease BACE1-mediated APP processing, and that the effect was not limited to cells that overexpressed BACE1. Moreover, the decrease in BACE1-generated APP-CTF levels was likely due to a decrease in APP-CTF formation rather than a result of increased APP-CTF degradation, as USP8 depletion did not increase the degradation of overexpressed C99. This was important as APP CTFs can be ubiquitinated and are degraded in the lysosomes (Morel et al. 2013, Bustamante et al. 2013).

Furthermore, we also showed that USP8 depletion decreases ectopically-expressed AICD protein levels, independent of decreases in APP-CTFs. Although AICD is known to be rapidly degraded in the cytoplasm (Edbauer et al. 2002), there is evidence to suggest that AICD may also be degraded in lysosomes, as drugs that prevent acidification of endosomes and lysosomes stabilize AICD levels (Vingtdeux, Hamdane, Begard, et al. 2007). Furthermore, AICD is present in exosomes, which are derived from the intraluminal vesicles of MVBs or late endosomes, suggesting that AICD may be

present within the endosome-lysosome system (Vingtdeux, Hamdane, Loyens, et al. 2007).

The effect of USP8 depletion on AICD may be direct or indirect. The AICD sequence contains multiple lysine groups (Slomnicki and Lesniak 2008), which may be potential sites of ubiquitination. Moreover, the stability of AICD may be affected by changes in the stability of its binding partners. For example, the AICD is stabilized by binding to Fe65, an APP-binding adaptor protein (Kimberly et al. 2001). Fe65, a proteasomal degraded protein, is K-63 ubiquitinated (Lee et al. 2009, Matz et al. 2015). Fe65 ubiquitination may be increased under conditions of USP8 depletion, as USP8 deubiquitinates K-63 chains. A resultant Fe65 decrease would account for a loss of AICD stability under conditions of USP8 depletion.

Furthermore, USP8 depletion decreased Aβ1-40 production in H4 cells. This was consistent with the decrease in pC99 and C99 observed after USP8 depletion. However, USP8 depletion, unexpectedly, did not result in a decrease of Aβ1-40 in H4-BACE1-GFP cells. Aβ1-40 is the most common species secreted in the media of H4 cells. Thus, we chose to measure this variant. It is possible that USP8 depletion in H4-BACE1 GFP cells may have resulted in a reduction of Aβ11 variants, as BACE1 overexpression shifts BACE1 cleavage of APP to the Glu11 site (Aβ numbering) (Liu, Doms, and Lee 2002). Furthermore, in this cell line, USP8 depletion resulted in a greater reduction of pC89 than pC99.

In summary, we found that depletion of USP8 decreases BACE1 protein levels by increasing BACE1 ubiquitination, decreasing the recycling of BACE1 while increasing its trafficking to the early endosome and lysosomes. As a consequence BACE1-mediated

APP processing and Aβ formation is decreased in USP8 depleted cells owing not only to the decreased levels of BACE1 but also to reduced localization of BACE1 in the recycling endosomes where BACE1 meets and processes APP.

4.3 Significance of findings

By conducting the experiments outlined in the methods, we were able to find strong evidence to support the novel hypothesis that BACE1 is a direct substrate of USP8. Furthermore, we found that USP8 depletion decreased BACE1 protein levels, and increased BACE1 accumulation in the early endosomes and lysosomes, while decreasing BACE1 trafficked in the recycling endosomes. This suggests that USP8 may be important in regulating the recycling of BACE1 back to the plasma membrane, just as it is important in promoting EGFR recycling via the recycling endosomes (Reincke et al. 2015).

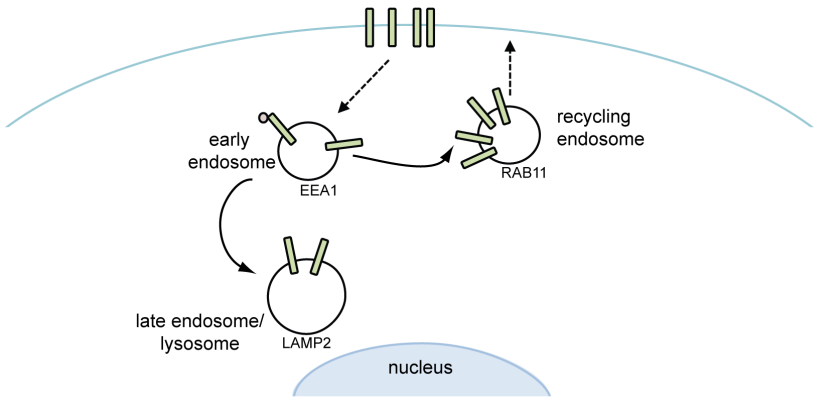
Previous experiments demonstrated that BACE1 ubiquitination at lysine 501 is important for BACE1 trafficking (Kang et al. 2012). We found additional evidence supporting the importance of ubiquitination in BACE1 trafficking by showing that USP8 depletion (which increases BACE1 ubiquitination) alters BACE1 trafficking, so that BACE1 is increased in early endosomes and late endosomes/lysosomes, and decreased in recycling endosomes. Moreover, we were able to provide additional evidence to support the finding that USP8 is important for endosome structure, as we have found enlarged aberrant endosomes on USP8 depletion, similar to what was reported by other investigators (Niendorf et al. 2007, Row et al. 2006, Mizuno et al. 2006, Hasdemir et al. 2009).

Finally, our experiments show that depletion of USP8 leads to decreased BACE1 protein levels and activity, suggesting that therapies able to accelerate BACE1 degradation (e.g. by increasing BACE1 ubiquitination) may represent a potential treatment for AD. BACE1 is an important target for drug therapy. Numerous BACE small-molecule inhibitors, targeting both BACE1 and its homologue, BACE2, have been developed and are at various phases of clinical trial (Vassar 2014). However, it remains to be determined, the extent of BACE1 inhibition needed to reach efficacy and whether such a dose would produce side effects owing to BACE1-mediated processing of multiple substrates. The indirect inhibition of BACE1 through the modulation of regulatory mechanisms that control BACE1 levels or BACE1 trafficking to acidic compartments where it is optimally active, represent an alternative approach to direct inhibition of BACE1. Such an approach, used in conjunction with direct BACE inhibition may produce additive effects and more importantly may reduce the dose of BACE inhibitor needed to be effective, reducing the possibility of non-target-related side effects. Thus, DUBs, like USP8, may in the future, be an attractive pharmacological target.

4.4 Future studies

1. We were able to demonstrate that USP8 regulates the trafficking of BACE1. We found that USP8 depletion increases BACE1 localization in the early endosomes and the lysosomes, while decreasing the proportion of BACE1 in the recycling endosomes. However, it was not determined whether USP8 affects the trafficking of BACE1 to and from the plasma membrane (Figure 4.1). Future experiments will include, determining whether USP8 depletion decreases the proportion of BACE1 on the plasma membrane.

A. Control



B. USP8 depleted

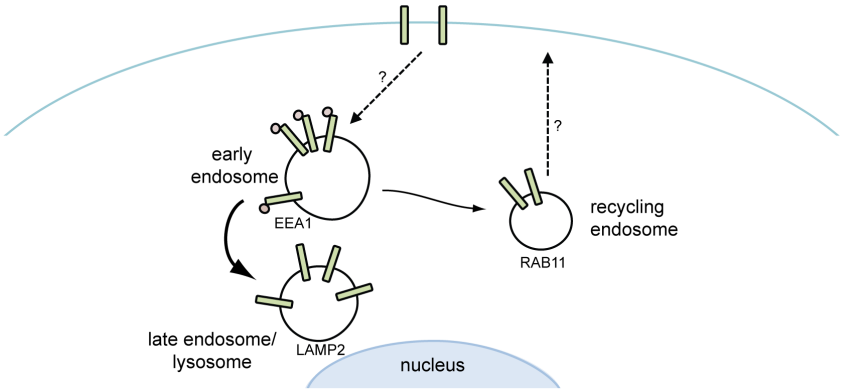


Figure 4.1

Schematic

A) BACE1 is endocytosed from the plasma membrane into vesicles that fuse with EEA1-positive early endosomes. From early endosomes, BACE1 is transferred to RAB11-positive recycling endosomes, and returned to the plasma membrane. BACE1-containing early endosomes also develop into late endosomes and fuse to LAMP-positive lysosomes, where BACE1 is degraded. B) USP8 depletion causes an accumulation of BACE1 in EEA1-positive early endosomes and increases BACE1 trafficking to LAMP2-positive late endosomes/lysosomes, while decreasing BACE1 trafficking to RAB11-positive recycling endosomes. However, it is unknown whether USP8 depletion increases the rate of BACE1 endocytosis or decreases the rate of BACE1 exocytosis at the plasma membrane.

Furthermore, we will determine whether USP8 depletion increases the rate of BACE1 endocytosis and decreases the rate of BACE1 exocytosis, using cell surface biotinylation and iodine-125 labeling (Sorkin and Duex 2010).

USP8 depletion results in decreased levels of EGFR on the plasma membrane (Niendorf et al. 2007). Similarly, USP8 depletion may decrease the percentage of BACE1 on the plasma membrane. However, USP8 depletion may also have the opposite effect. USP8 appears to act in opposition to NEDD4, an E3 ligase, preventing the internalization of the AMPAR receptors from the cell surface of neurons (Scudder et al. 2014). While USP8 depletion may affect the amount of BACE1 on the plasma membrane, it may not increase the rate of BACE1 endocytosis, as BACE1 ubiquitination at BACE1 K501 does not appear to influence the rate of BACE1 internalization (Kang et al. 2012). However, under USP8 –depleted conditions, there may be a decreased rate of return of BACE1 to the plasma membrane, as there is less BACE1 contained in recycling endosomes.

2. USP8 depletion results in an increase in endosome size. To ensure that the changes in BACE1 trafficking observed under conditions of USP8 depletion are due to increased BACE1 ubiquitination, rather than to changes in endosome morphology, we can perform various control experiments. First, we can examine the trafficking of other lysosomal degraded transmembrane proteins, such as transferrin receptor (TfR). TfR can be rapidly recycled back to the plasma membrane following ubiquitin-mediated endocytosis or degraded in the lysosome (Tachiyama et al. 2011, Fujita et al. 2013). Provided that USP8 depletion does not affect TfR ubiquitination, we would expect that TfR recycling would remain unchanged. Second, a BACE1-GFP-Ub construct can be created, where the cytoplasmic C-terminus of BACE1 is fused to human ubiquitin

(Raiborg et al. 2002). This construct would allow us to observe ubiquitinated-BACE1 trafficking, in the absence of the enlarged endosomes produced with USP8 depletion. We would expect BACE1-GFP-Ub to be trafficked in a similar manner to ubiquitinated BACE1 under USP8-depleted conditions. That is, we expect to see increased BACE1-GFP-Ub localization in the early endosomes and late endosomes/lysosomes, and less BACE1-GFP-Ub in the recycling endosomes.

3. USP8 depletion experiments in this study were conducted using H4 neuroglioma cells and stable cells lines overexpressing BACE1-GFP or APP-CTF99 derived from H4 cells. These stable cell lines provided an invaluable, reproducible system for determining how USP8 depletion affects BACE1 protein levels, ubiquitination and localization. H4 cells endogenously produce BACE1 and APP, and secrete measurable amounts of Aβ. Using these cell lines, we were able to obtain sufficient protein lysates to conduct the biochemical studies outlined in the methods, without the loss of animal life which results from dissecting murine embryos or neonates to produce primary neuronal cell cultures. Furthermore, the use of H4 cells allowed us the advantage of examining human BACE1, APP and Aβ, rather than the murine variants present in rodent primary cell cultures.

Nevertheless, since AD is primarily a disorder of the brain, future investigations will determine the effect of USP8 deletion in primary neurons. USP8 can be depleted in murine and rat primary neuronal cultures using lentiviral particles (Duncan et al. 2006, Scudder et al. 2014). H4 cells, unlike neurons are non-polarized cells, thus using primary neuronal cultures, will allow the effect of USP8 depletion on BACE1 trafficking to be examined in different regions of the neuron, since USP8 is not only found in the neuronal

cell body, but also in the dendrites and in the axons, where it colocalizes with presynaptic markers (d'Amora et al. 2010). In our experiments, we found that USP8 depletion, decreased APP-CTF (C99 and C89) formation and decreased secreted Aβ1-40 in H4 cells. Using primary neuronal cultures will allow us to determine whether USP8 depletion leads to a similar, lesser or greater effect in neurons. We expect to observe a decrease in BACE1 and Aβ1-40, when USP8 is depleted in primary neuronal cultures, similar to the effect observed in USP8-depleted H4 cells.

Moreover, using primary neuronal cultures, we can determine whether neuronal activity increases USP8 recruitment to the endosome and as a result, increases BACE1 deubiquitination by USP8. Treatment of cultured cells with EGF promotes EGFR ubiquitination, internalization (Yokouchi et al. 1999), degradation, as well as, USP8 recruitment to the endosome (Row et al. 2007). Similarly, neuronal activity has been shown to increase the internalization of cell surface receptors, by promoting the activity of E3 ligases at the plasma membrane (Lussier, Nasu-Nishimura, and Roche 2011, Widagdo et al. 2015). However, the role of neuronal stimulation on USP8 localization and activity remains unknown. Neuronal activation has been associated with increased Aβ production *in vitro* (Lesne et al. 2005, Li et al. 2013). Moreover, neuronal activation has been shown to increase the colocalization of APP with BACE1 in recycling endosomes (Das et al. 2013). Since, USP8 depletion decreases the localization of BACE1 in the recycling endosomes, it is possible that USP8 may play a role in trafficking BACE1 to the recycling endosome by decreasing BACE1 ubiquitination, under conditions of increased neuronal activity.

4. Specific small-molecule inhibitors for USP8 have been developed (Colombo et al. 2010). One of these inhibitors 9-Ethyloxyimino-9H-indeno[1,2-b]pyrazine- 2,3-dicarbonitrile has been used to decrease EGFR levels in lung cancer cells *in vitr*o (Byun et al. 2013, Jeong 2015). In order to determine whether USP8 inhibitors are efficacious in decreasing BACE1 protein levels and BACE1-mediated APP processing, we can screen USP8 inhibitors using H4-BACE1-GFP cells. Using this cell line, BACE1 protein levels can be determined by measuring GFP fluorescence. Thus, this cell line may be used in moderate or high throughput screens. Validated USP8 inhibitors can be subsequently used to treat neurons, to determine whether USP8 inhibition with small molecules has a similar effect as RNAi-mediated USP8 reduction. We expect to observe a similar decrease in BACE1 and Aβ1-40, when USP8 is depleted using USP8-specific inhibitors.

Ultimately, USP8 inhibitors can be used *in vivo* to determine whether pharmacological inhibition can be used to decrease BACE1-mediated APP processing and Aβ production in Aβ plaque-forming transgenic rodents. At present, there are no reports of USP8 inhibitors being used *in vivo*. USP8 conditional knockout mice survive only 4 to 6 days post induction of USP8 deletion due to liver failure (Niendorf et al. 2007). However, mice treated with validated USP8 inhibitors are expected to fare better, as there would only be a partial decrease in USP8, rather than a total knockout. Furthermore, with a pharmacological inhibitor, USP8 depletion could be regulated by adjusting the dosing, and thus potential side effects, can be mitigated by reducing the dose.

5. Furthermore, we would like to determine whether USP8 inhibition decreases BACE2 protein levels, as well as, BACE1. BACE2 is 64% homologous to BACE1, when peptide sequences are compared (Bennett et al. 2000). Unlike BACE1, which cleaves APP at the β-site, BACE2 cleaves APP near the α-site, acting like an α-secretase, rather than a β-secretase (Yan et al. 2001). Current BACE inhibitors, inhibit the activity of both BACE1 and BACE2. BACE2 inhibition has its own deleterious effect, as BACE2 has roles independent of BACE1. For example, BACE2 appears to play a role in pigmentation. BACE2 knockout mice have an abnormal coat color (Rochin et al. 2013). Thus it would be important to determine whether USP8 inhibition leads to a specific decrease in BACE1, over BACE2. If USP8 inhibition specifically decreases BACE1, it can serve as an invaluable tool, which may be used alongside or in lieu of BACE inhibitors.

6. Finally, in order to determine the role of USP8 in AD, future experiments will involve determining whether USP8 levels are altered in postmortem cortical or hippocampal samples of AD patients compared to healthy age-matched controls. Protein level of UCHL1, another DUB, was found to be decreased in post mortem samples of AD patients (Choi et al. 2004, Guglielmotto et al. 2012). Similarly, protein levels of USP8 and other DUBs may be altered.

4.5 Conclusion

In summary, we found that depletion of USP8 decreases BACE1 protein levels by increasing BACE1 ubiquitination, decreasing the recycling of BACE1 while increasing its trafficking to the early endosome and lysosomes. Owing to the decreased levels of BACE1, and reduced localization of BACE1 in the recycling endosomes, where BACE1 processes APP, BACE1-mediated APP processing and Aβ formation was reduced. These experiments have identified USP8 as a DUB that directly deubiquitinates BACE1 at K501. Furthermore, this study has provided further evidence for the role of USP8 in promoting protein recycling over degradation. Finally, the results of this study aid not only the understanding of BACE1 degradation, but also provide an alternate mechanism by which BACE1 and its product, Aβ, could be reduced, indicating that therapies able to accelerate BACE1 degradation (e.g. by increasing BACE1 ubiquitination) may represent a potential treatment for AD.

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