**The Dissertation Committee for Emily Sara Stieren Certifies that this is the approved version of the following dissertation:**

# New Insights Regarding the Intracellular Effects, Trafficking, and Quality Control of the Amyloid Precursor Protein: Implications for Alzheimer's Disease Pathogenesis

**Committee:**

Darren Boehning, Ph.D., Supervisor

Henry F. Epstein, M.D.

George R. Jackson, M.D., Ph.D.

Steven A. Weinman, M.D., Ph.D.

Cam Patterson, M.D.

Dean, Graduate School

# New Insights Regarding the Intracellular Effects, Trafficking, and Quality Control of the Amyloid Precursor Protein: Implications for Alzheimer's Disease Pathogenesis

**by**

**Emily Sara Stieren, B.S.**

**Dissertation**

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

**Doctor of Philosophy**

**The University of Texas Medical Branch**

**December, 2010**

## Dedication

To my grandmother, Dr. Klara G. Janas

## Acknowledgements

I would like to thank Dr. Darren Boehning for years of support and mentorship. I gratefully acknowledge Dr. Amina El Ayadi, Dr. Megan L. Landsverk, Dr. Andres Oberhauser, Walter Werchan, Yao Xiao, Fuzhen Li, and Xinmin (Sam) Wang, who each contributed to the work presented here, in addition to Dr. Honey Herce and Alexis Galvan, who worked on earlier aspects of this project. I am indebted to Dr. José Barral for his willingness to share supplies and equipment and for valuable comments, discussions, and practical training, especially regarding protein biochemistry and chaperone biology. Throughout my graduate school career, I have benefited from sharing and discussing my research with Dr. Henry Epstein and the other members of the Boehning, Barral, and Epstein labs. This path began with Dr. Gail Ironson, who first introduced me to the thought of a career as a physician-researcher, and Dr. Qiang Tong, who taught me the basics of being a bench scientist. This work was supported by NIH grants F30-AG030878 (Emily S. Stieren) and R21-AG031948 (Darren Boehning and José M. Barral). Lastly, I am grateful to Dr. William D. Willis and those who contributed to the Jean C. and William D. William Neuroscience Research Endowment, whose generous gift made it possible for me to work in the lab of my choice.

# New Insights Regarding the Intracellular Effects, Trafficking, and Quality Control of the Amyloid Precursor Protein: Implications for Alzheimer's Disease Pathogenesis

Publication No._____

Emily Sara Stieren, Ph.D.

The University of Texas Medical Branch, 2010

Supervisor: Darren Boehning

Alzheimer's disease (AD) is the most common form of age-related dementia. Our current understanding of AD pathogenesis is largely based on data from cases of relatively rare early-onset forms of the disease, collectively known as familial AD (FAD). FAD is associated with discrete mutations in three genes: *PSEN1* and *PSEN2*, which encode the presenilin proteins, and *APP*, which encodes the amyloid precursor protein (APP). Several hypotheses exist for the pathogenesis of AD. According to the amyloid cascade hypothesis, AD pathogenesis is associated with a series of molecular events which leads to the aggregation and deposition of specific proteolytic fragments of APP. There is also evidence that disruption of intracellular calcium homeostasis contributes to AD pathogenesis, especially in cases of FAD related to *PSEN* mutations. However, important questions remain unanswered regarding the pathogenesis of AD, and thus, research has increasingly focused on identifying new genetic

associations with late-onset AD with the hope that they will shed some light on disease mechanisms. Recently, the gene encoding ubiquilin was shown to be associated with late-onset AD, but the molecular mechanism by which the ubiquilin protein contributes to disease pathogenesis is still unclear. We sought to determine: 1) whether disruption of intracellular calcium homeostasis is a common causal factor in all forms of FAD, and 2) the mechanisms by which the ubiquilin protein contributes to the pathogenesis of late-onset AD. Using calcium imaging techniques, we show that FAD-linked mutations in APP do not directly perturb intracellular calcium homeostasis, suggesting that disruption of calcium is not an initiating event in the development of all forms of AD. Using biochemical and cellular approaches, we determined that ubiquilin directly interacts with APP and regulates its trafficking and proteolytic processing. Furthermore, we show that ubiquilin possesses molecular chaperone activity and prevents the aggregation of APP. Examination of post-mortem brain tissue from AD revealed decreased ubiquilin protein levels, suggesting that the pathogenesis of late-onset AD is related to alterations in APP trafficking and quality control. Our results point to the restoration of ubiquilin protein level and/or function as therapeutic targets for AD.

# Table of Contents

# List of Tables

# List of Figures

# Abbreviations

| | |
|---|---|
| Aβ | amyloid beta |
| AICD | APP intracellular domain |
| AD | Alzheimer's disease |
| AFM | atomic force microscopy |
| APOE | apolipoprotein E |
| APLP | APP-like protein |
| APP | amyloid precursor protein |
| $APP_s$ | soluble APP ectodomain |
| BACE | beta-site APP cleaving enzyme |
| BSA | bovine serum albumin |
| CHX | cycloheximide |
| CS | citrate synthase |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | dimethyl sulfoxide |
| DSP | dithio-bis[succinimidyl propionate] |
| DTNB | dithio-1,4-nitrobenzoic acid |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol tetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| ER | endoplasmic reticulum |
| ERAD | ER-associated degradation |
| FAD | familial Alzheimer's disease |
| FRET | fluorescence resonance energy transfer |

| | |
|---|---|
| $GABA_A$ | γ-aminobutyric acid type A |
| GlcNAc-TI | N-acetylglucosaminyltransferase I |
| GFP | green fluorescent protein |
| GST | glutathione *S*-transferase |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| $IP_3R$ | 1,4,5-trisphosphate receptor |
| LTP | long-term potentiation |
| MTOC | microtubule organizing center |
| mTOR | mammalian target of rapamycin |
| MVB | multivesicular body |
| NGF | nerve growth factor |
| NMDA | *N*-methyl-D-aspartate |
| OAA | oxaloacetic acid |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PMSF | phenylmethylsulfonyl fluoride |
| RNAi | RNA interference |
| ROC | receptor-operated calcium entry |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | SDS polyacrylamide gel electrophoresis |
| SERCA | sarco/endoplasmic reticulum calcium ATPase |
| SNP | single-nucleotide polymorphism |
| SOC | store-operated calcium entry |
| Sti-1 | stress-inducible protein-1 |
| SUMO | small ubiquitin-like modifier |

| | |
|---|---|
| TPR | tetratricopeptide repeat |
| UBA | ubiquitin-associated (domain) |
| UBL | ubiquitin-like (domain) |
| UDP | ubiquitin domain protein |
| UIM | ubiquitin-interacting motif |
| UPS | ubiquitin-proteasome system |
| UTP | uridine 5’-triphosphate |
| XPCB | xeroderma pigmentosum complementation group C-binding |
| YFP | yellow fluorescent protein |

# Chapter 1: Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by deterioration of cognitive abilities. AD is the most common cause of dementia in the western world. Approximately 5.3 million people in the United States suffer from AD, which currently affects one in eight individuals over the age of 65 (Alzheimer's Association 2009). As the population ages, the prevalence of age-related dementias like AD is expected to increase. It is estimated that by 2030, there will be a 50% increase in the number of AD patients aged 65 and over (Hebert *et al.* 2003). The direct and indirect healthcare and business-related costs associated with caring for persons with dementia amounted to over 148 billion dollars in 2005, and these costs will also continue to rise as more people are diagnosed with AD. There are several FDA-approved treatments for AD, which include cholinesterase inhibitors and *N*-methyl-D-aspartate (NMDA) glutamate receptor antagonists; however, these treatments only provide temporary symptomatic relief for a fraction of AD sufferers and do not ultimately slow the progression of the disease. Therefore, it is important to elucidate the mechanisms of disease pathogenesis and to identify new therapeutic targets for the treatment of AD.

The areas of the brain most affected in patients with AD are those which contain neural networks that are critical for learning and memory. In later stages of the disease, there is often widespread degeneration throughout the cerebral cortex that may be accompanied by defects in motor function and psychiatric symptoms. The brains of AD patients contain extracellular amyloid plaques and intracellular neurofibrillary tangles, which are associated with neuronal loss

(Kumar *et al.* 2009). This neurodegeneration is evident earliest in the entorhinal cortex, and later in the hippocampus and neocortex (Kandel *et al.* 2000; Fauci *et al.* 2008; Kumar *et al.* 2009).

The diagnosis of AD remains largely one of exclusion. AD can be clinically diagnosed according to one of two major sets of criteria: 1) the *Diagnostic and Statistical Manual, 4th Ed.* (DSM-IV) criteria (First 2000), and 2) the *National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association* (NINCDS-ARDRA) criteria (McKhann *et al.* 1984) (**Table 1.1**). Both of these sets of guidelines have a high sensitivity for the diagnosis of AD at the expense of specificity (Knopman *et al.* 2001). A definitive diagnosis of AD requires histopathological evidence of AD-specific neuropathological lesions, which include extracellular neuritic plaques and intracellular neurofibrillary tangles. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria (Mirra *et al.* 1991; Mirra *et al.* 1993) involve assigning a neuritic plaque density score for each of three histological sections representing middle frontal, superior and middle temporal, and inferior parietal neocortex and correlating this score with the patient's age to obtain an age-related plaque score. Since amyloid plaque pathology increases in normal aging, a higher plaque density is required to verify the diagnosis of AD with increasing patient age. The NIA/Reagan consensus criteria (Hyman and Trojanowski 1997) combine the CERAD criteria with an assessment of the neurofibrillary tangle pathology according to the staging criteria set forth by Braak and Braak (1991).

Table 1.1: National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for the diagnosis of AD.

| |
|---|
| "Probable" AD:<br>- Dementia established by clinical exam/Mini-mental State Examination (MMSE) and confirmed with neuropsychological tests<br>- Decline in memory and *at least one non-memory intellectual function*<br>- Decline from a previous level and continuing progression<br>- Onset between 40 and 90 years of age<br>- No disturbance in consciousness<br>- Absence of other disorders or diseases that could explain deficits |
| "Possible" AD:<br>- Dementia syndrome that varies clinically from typical AD, but has no known other cause<br>- Existence of a second disorder that is sufficient to produce dementia, but is not considered the cause of the dementia<br>- Single gradually progressive cognitive deficit in the absence of any other cause |
| "Definite" AD:<br>- Clinical criteria of probable AD<br>- Histopathological evidence of AD at postmortem or biopsy |

McKhann, *et al*. (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 34(7):939-944.

The exact cause of neuronal degeneration in AD is unclear. However, according to the amyloid cascade hypothesis, AD pathogenesis is associated with a series of molecular events which leads to the aggregation and deposition of specific proteolytic fragments of the amyloid precursor protein (APP). These aggregated protein fragments constitute the core of senile neuritic plaques, a pathologic hallmark of AD.

## ALZHEIMER'S DISEASE PATHOGENESIS

### Amyloid Cascade Hypothesis

In 1907, Alois Alzheimer published a case report in which he described the symptoms and pathognomic lesions of the disease that would eventually bear his name. In his report, Alzheimer described what are now referred to as senile plaques and neurofibrillary tangles in the post-mortem brain of a patient who had suffered from progressive dementia (Alzheimer 1907; Alzheimer *et al.* 1995). Many years later, in 1984, Glenner and Wong isolated and purified amyloid β (Aβ), the core constituent of senile plaques, from the cerebral vasculature of AD brain tissue (Glenner and Wong 1984). The discovery of Aβ and the subsequent discovery of the gene encoding its precursor, APP, formed the basis of what is still the predominant theory of AD pathogenesis, the amyloid cascade hypothesis, which postulates that Aβ production and aggregation are critical initiating events in the development of AD. While there is still debate over the nature of the offending aggregate, the current body of research offers convincing evidence that APP and its proteolytic products are central to AD pathogenesis. Therefore, elucidating the molecular events involved in modulating the proteolytic

processing of APP may provide valuable information for understanding AD pathogenesis.

APP is a ubiquitously-expressed type I transmembrane protein. APP pre-mRNA is alternatively spliced within the sequence encoding the extracellular region to produce three proteins of differing lengths: $APP_{770}$, $APP_{751}$, and $APP_{695}$, which is the predominant isoform in neurons. The exact physiological function of APP is yet unknown; however, several putative roles have been suggested, including cell migration, cell adhesion, cell proliferation, and vesicle trafficking (De Strooper and Annaert 2000; Kamal *et al.* 2000; Kerr and Small 2005; Reinhard *et al.* 2005; Gralle and Ferreira 2007). There is emerging evidence for a role for APP in the modulation of gene transcription (Cao and Sudhof 2001; von Rotz *et al.* 2004; Muller *et al.* 2008).

APP matures through the secretory pathway, where it undergoes classical *N*- and *O*-linked glycosylation during transit through the endoplasmic reticulum (ER) and Golgi apparatus, respectively (Weidemann *et al.* 1989; De Strooper and Annaert 2000; Suzuki *et al.* 2006). Evidence suggests that a substantial proportion of newly-synthesized full-length APP is degraded intracellularly, most likely in lysosomes, while a significantly smaller proportion is transported to the cell surface (Caporaso *et al.* 1992; Knops *et al.* 1992; De Strooper and Annaert 2000). The half-life of the APP holoprotein at the plasma membrane is short – on the order of minutes – after which it is either proteolytically cleaved at the membrane or recycled through the endocytic pathway (Small and Gandy 2006), where it can also come into contact with proteolytic enzymes.

In normal physiology, APP is cleaved by a series of enzymes, called secretases, which generate proteolytic products of various lengths (**Fig. 1.1**). The

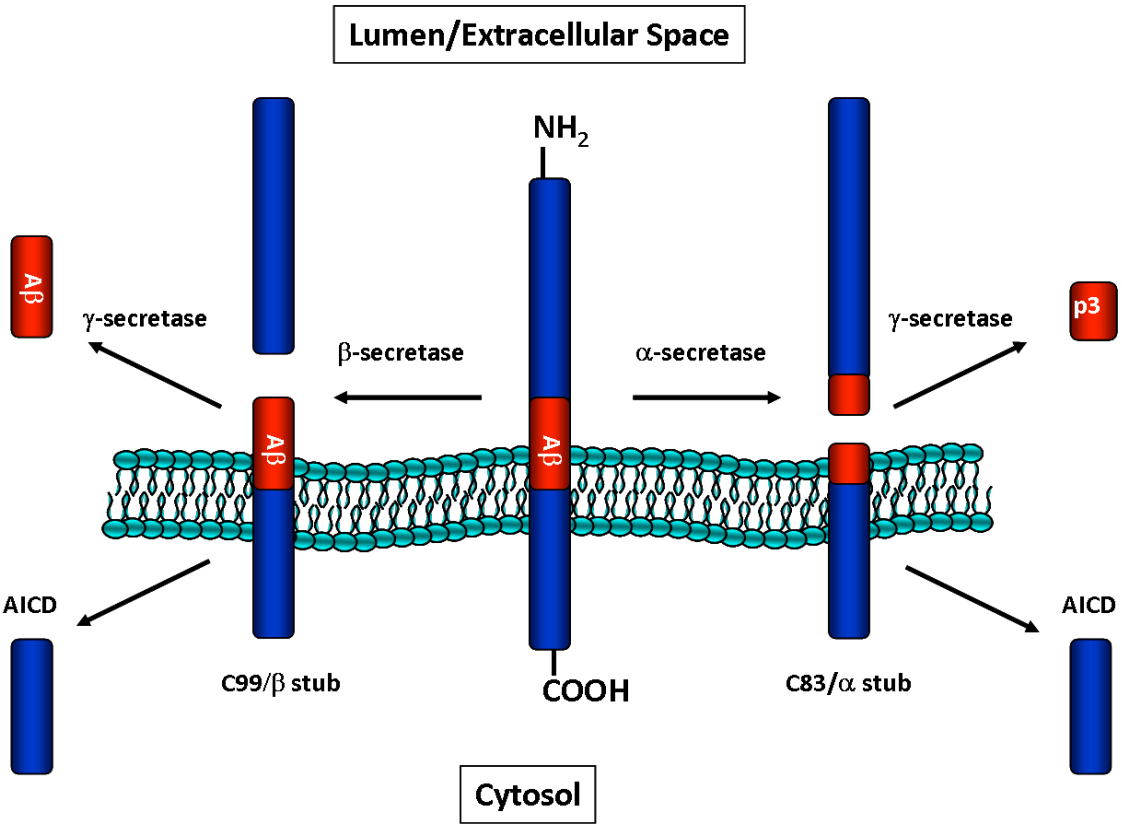
principal cleavage event is by α-secretase, which generates a large soluble ectodomain ($APP_s$) that is secreted into the extracellular space and a C-terminal fragment (C83). Several enzymes in the ADAM (a disintegrin and metalloprotease) family have been shown to function as α-secretases; however, the proportional contribution of each enzyme in the central nervous system is unknown (De Strooper *et al.* 2010). Since α-cleavage occurs within the Aβ peptide sequence, processing of APP by α-secretase precludes Aβ production and is thus, non-amyloidogenic. In an alternate processing pathway, holo-APP can be cleaved by β-secretase (β-site APP cleaving enzyme; BACE1), resulting in the production of a secreted ectodomain and the membrane-associated C99 fragment. Subsequent cleavage of C99 by γ-secretase produces the Aβ peptide and an intracellular domain (AICD) that is released from the membrane into the cytosol. The various secretase enzymes are plausible therapeutic targets for AD. However, γ-secretase activity is required for the processing and activation of numerous other critical proteins, including Notch, making it relatively unviable as a target for pharmaceutical inhibitors. Nevertheless, there are several ongoing trials involving secretase-targeting therapies for AD, some of which aim to selectively inhibit the γ-secretase cleavage of APP without affecting other target substrates (De Strooper *et al.* 2010).

Generation of the Aβ peptide has been shown to occur in many places within the cell, including the ER, trans-Golgi network, the plasma membrane, and endosomes (Cook *et al.* 1997; Daw *et al.* 2000; Small and Gandy 2006; Vetrivel and Thinakaran 2006). The γ-secretase cleaves at several different sites within APP, generating Aβ peptides of various lengths. There are two predominant forms of Aβ that differ in length by two residues, $Aβ_{40}$ and $Aβ_{42}$. $Aβ_{42}$ is more

prone to aggregation and is considered to be more cytotoxic than the shorter Aβ species, and all investigated familial AD-linked mutations that affect γ-secretase lead to an increase in the relative amount of $A\beta_{42}$ (Borchelt *et al.* 1996; Scheuner *et al.* 1996; Citron *et al.* 1997).

The highly conserved YENPTY (single amino acid code) sequence located in the cytoplasmic domain of APP functions as an internalization signal for endocytic trafficking. Deletion or mutation of this sequence leads to an increase in plasma membrane-associated APP and a significant decrease in Aβ production (Lai *et al.* 1995; Perez *et al.* 1999; De Strooper and Annaert 2000; Kerr and Small 2005), suggesting that the endocytic pathway is a major path for Aβ generation. Interestingly, one study (Perez *et al.* 1999) showed that the C-terminal tyrosine of the YENPTY sequence was important for regulating APP turnover. When this residue was mutated, cells showed a significant increase in APP half-life and a significantly greater ratio of mature to immature full-length APP. One explanation for this is that mutation of this tyrosine residue eliminates a degradation targeting signal for immature APP. Various cytoplasmic adaptor proteins have been shown to interact with the YENPTY motif, including X11 (Borg *et al.* 1996), Fe65 (Borg *et al.* 1996; Duilio *et al.* 1998), and Jip1b (Taru *et al.* 2002). These protein-protein interactions involving the AICD may ultimately affect trafficking, processing, and cellular functions of holo-APP and its C-terminal fragments, making them possible mediators of AD pathogenesis.

Figure 1.1: Proteolytic processing of APP by secretase enzymes.




In the non-amyloidogenic pathway (right side), full-length APP (center) is proteolytically cleaved by α-secretase, releasing the soluble ectodomain into the lumen/extracellular space. The membrane-associated C83 fragment is subsequently cleaved by γ-secretase to generate the AICD (residues 721-770; $APP_{770}$ numbering) and the p3 fragment (~3 kDa). In the amyloidogenic pathway (left side), full-length APP is cleaved at Asp-672 by β-secretase, and the membrane-tethered C99 fragment is subsequently cleaved by γ-secretase to generate the AICD and the Aβ peptide (~4 kDa).

## Calcium Dyshomeostasis

The idea that disruption of calcium homeostasis could be a pathogenic mechanism for AD was first introduced in 1984 by Khachaturian, who hypothesized that dysregulation of intracellular calcium storage and signaling represented a final common pathway of neural dysfunction and cell death in age-related neurodegenerative diseases (Khachaturian 1984; Khachaturian 1987). In other words, it was believed that early disease-specific biochemical events imparted a certain level of vulnerability in particular cell populations, and a subsequent age-related decline in calcium homeostasis dealt the final insult, leading to cell death and associated cognitive dysfunction. It is clear that normal aging is associated with alterations in neuronal calcium homeostasis, including impaired calcium buffering capacity and delayed post-excitatory calcium clearance (Toescu and Verkhratsky 2007). However, more recent studies using cells isolated from AD patients and animal models of AD, as well as immortalized cell lines, have suggested a more specific role for altered calcium signaling in AD pathogenesis beyond just global age-related impairments (Mattson 2004; Sperling *et al.* 2010). Indeed, the current body of evidence supports the hypothesis that calcium dysregulation plays a role in AD pathogenesis, particularly certain forms of early-onset familial AD (FAD; discussed below), and the calcium hypothesis is not mutually exclusive with the amyloid cascade hypothesis. It is still not clear, however, whether calcium dysregulation is predominantly an end result of Aβ-related toxicity or whether it represents a more proximal event in disease pathogenesis (LaFerla 2002).

Calcium is the canonical intracellular signaling molecule. Neurons rely on calcium signaling for a vast array of cellular processes, including gene

expression, neuronal excitability, neurotransmitter release, and apoptosis (Berridge 1998). In normal physiology, resting cytosolic calcium level is maintained at very low concentrations (50-300 nM) in neurons (Verkhratsky *et al.* 2004), whereas calcium concentrations in intracellular ER stores and the extracellular space are significantly higher (in the millimolar range). The calcium gradient that exists across the ER and plasma membranes is critical for proper neuronal function and is maintained by calcium ATPases and the $Na^{+}/Ca^{2+}$ exchanger on the cell surface and the sarco/endoplasmic reticulum calcium ATPase (SERCA) in the ER membrane. Calcium increases in the cytoplasm in response to various stimuli, including extracellular ligands or membrane depolarization. Calcium can enter from the extracellular space, through plasma membrane ion channels, or from intracellular stores, mainly through $IP_3$ and ryanodine receptor ion channels in the ER membrane.

Fibroblasts isolated from patients with FAD caused by a mutation in one of the presenilin genes exhibit increased calcium release from intracellular stores in response to agonist (Ito *et al.* 1994). Interestingly, these calcium disturbances were observed before the onset of cognitive symptoms in these patients, and family members who failed to develop symptoms did not exhibit abnormal calcium responses, despite the presence of the mutation. Follow-up investigations of calcium regulation in cell culture and animal models have corroborated these original findings. Expression of FAD-linked presenilin mutants in cultured cells and in animals has been shown to increase release of calcium from ER stores (Guo *et al.* 1996; Guo *et al.* 1999; Chan *et al.* 2000) and decrease store-operated calcium entry through plasma membrane channels (Leissring *et al.* 1999a; Leissring *et al.* 1999b; Leissring *et al.* 2000). Recent data

suggest that presenilins may form calcium leak channels in the ER membrane and that mutations lead to loss of function phenotypes characterized by ER calcium overload (Tu *et al.* 2006). These results are controversial, however, as others have shown that mutant presenilins alter intracellular calcium signaling *via* a direct and specific interaction with 1,4,5-trisphosphate receptors ($IP_3Rs$) in the ER membrane (Cheung *et al.* 2008). Although more research is needed to elucidate the precise mechanisms, these studies have established a role for presenilins in calcium homeostasis that is independent of their role in γ-secretase activity.

Although the presenilin effects are largely independent of any APP derivative peptides, there is evidence that exposure of cultured cells to Aβ alters calcium homeostasis. Specifically, incubation with Aβ peptides leads to increased calcium entry through the plasma membrane (Demuro *et al.* 2005; Deshpande *et al.* 2006). Furthermore, *in vivo* calcium imaging in an AD mouse model has shown increased intracellular calcium levels in neurites that are within close proximity to Aβ plaques (Kuchibhotla *et al.* 2008). Several mechanisms may be responsible for the effect of Aβ on intracellular calcium, including alteration in plasma membrane ion channel permeability, disruption of overall membrane integrity, and formation of calcium-permeable membrane pores by Aβ peptides (Demuro *et al.* 2010). These effects may also depend on whether the exposure to Aβ is acute or chronic. Short-term exposure to Aβ oligomers has been shown to increase calcium influx through NMDA receptor ion channels (Carette *et al.* 1993; Kelly and Ferreira 2006); however, chronic exposure leads to downregulation of NMDA receptors and decreased calcium entry (Snyder *et al.* 2005; Dewachter *et al.* 2009).

The consequences of ER calcium store overload and increased resting cytosolic calcium can have numerous deleterious effects in neurons. For example, long-term potentiation (LTP), which is thought to underlie certain forms of learning and memory (Kandel *et al.* 2000), relies on calcium release from ER stores (Park *et al.* 2008), and impairments in LTP may be responsible for the early decline in cognitive function in AD patients. Furthermore, sustained increases in cytosolic calcium may overwhelm the calcium buffering capacity of the cell, leading to mitochondrial dysfunction and cell death. Calcium-activated enzymes, like proteases and phosphatases, may also be activated in response to elevated calcium levels, leading to alterations in a variety of cellular processes. And finally, increased intracellular calcium can lead to increased Aβ production and/or fibrillization (Querfurth and Selkoe 1994; Querfurth *et al.* 1997; Isaacs *et al.* 2006), promoting further cellular dysfunction *via* a feed-forward cascade.

**Genetic**

***Familial Alzheimer's Disease (FAD)***

Most cases of AD are sporadic and late-onset, but rare forms of AD are early-onset and exhibit autosomal-dominant inheritance. Apparent inheritance of AD in some families was documented as early as 1981 (Heston *et al.* 1981), and this same report noted a connection between early-onset AD and Down syndrome, or trisomy 21, suggesting a possible gene dosage effect. In 1987, a genetic linkage to FAD was established within chromosome 21, where the APP gene is located (St George-Hyslop *et al.* 1987). Several years later, the first pathogenic mutation in the APP gene was described in cases of hereditary cerebral hemorrhage with amyloidosis (Van Broeckhoven *et al.* 1990). In 1995,

the first pathogenic mutations in the presenilin genes were identified (Levy-Lahad *et al.* 1995; Sherrington *et al.* 1995). Since then, 19 mutations in the APP gene and approximately 150 mutations in the genes encoding presenilin-1 and its homologue, presenilin-2, have been linked to FAD (Selkoe 2005; Tanzi and Bertram 2005).

Interestingly, FAD gene linkage studies have provided some of the most convincing evidence in support of the amyloid cascade hypothesis. Presenilins constitute the catalytic core of γ-secretase (De Strooper *et al.* 1998; Wolfe *et al.* 1999; Kimberly *et al.* 2000), and all FAD-linked presenilin mutations that have been investigated thus far lead to relative overproduction of $A\beta_{42}$ (Borchelt *et al.* 1996; Duff *et al.* 1996; Scheuner *et al.* 1996; Citron *et al.* 1997). FAD-linked mutations in APP also lead to increased amyloidogenesis, and depending on the mutation result in increased β-secretase processing, increased $A\beta_{42}/A\beta_{40}$ ratio, increased propensity of Aβ to form fibrils, or decreased clearance of Aβ peptides (Goate *et al.* 1991; Murrell *et al.* 1991; Hendriks *et al.* 1992; Kamino *et al.* 1992; Mullan *et al.* 1992; Suzuki *et al.* 1994; Tamaoka *et al.* 1994; Haass *et al.* 1995; De Jonghe *et al.* 1998; Murrell *et al.* 2000; Nilsberth *et al.* 2001). Taken together, these findings support the hypothesis that APP-derived fragments, particularly Aβ peptides, are involved in a proximal step in the pathogenesis of FAD.

***Sporadic Alzheimer's Disease***

While research focusing on FAD has contributed a great deal to our understanding of AD pathogenesis, these rare autosomal dominant forms of the disease account for less than ten percent of all AD cases (Janssen *et al.* 2003; Raux *et al.* 2005). Most AD cases are sporadic and late-onset with more complex multifactorial pathogenesis. Determining the genetic risk factors for late-onset AD

has proven challenging. There have been many genes linked to sporadic AD, and the details of these studies are listed on the publicly-available AlzGene online database (http://www.alzgene.org) (Bertram *et al.* 2007). Most studies have used candidate gene approaches to determine associations in either family-based or case-control sample populations. However, the completion of the Human Genome Project in 2003 and recent technological advancements in high-throughput methodologies have made it possible for researchers to conduct unbiased large-scale genomic screens to identify novel risk genes for common sporadic disorders like AD. Unfortunately, most of the genes identified in genome-wide association studies in AD either have not been subjected to – or have not withstood – rigorous validation measures (Bertram & Tanzi 2009). To date, only the apolipoprotein E gene (*APOE*) has been consistently and convincingly linked to sporadic AD (Strittmatter *et al.* 1993; Farrer *et al.* 1997). The lack of reproducibility between genetic screens using different samples suggests that each locus imparts a relatively small amount of risk, and indeed, the overall usefulness of genome-wide association studies for defining new therapeutic targets in late-onset AD has recently been called into question (Pedersen 2010). Nevertheless, genetic screens have provided – and continue to provide – researchers with new insights and opportunities for further investigation.

In 2005, Tanzi and colleagues (Bertram *et al.* 2005) used a candidate gene approach to identify genetic associations in family-based AD cohorts by focusing on a chromosomal region that had previously been linked to AD in a full-genome screen (Blacker *et al.* 2003). They reported that single-nucleotide polymorphisms (SNPs) in the *UBQLN1* gene, which encodes the ubiquilin-1

protein, are associated with late-onset AD. Since this original report, additional studies from other laboratories have also found associations with AD and specific SNPs in the *UBQLN1* gene (Kamboh *et al.* 2006; Golan *et al.* 2008), while some researchers have failed to find an association (Bensemain *et al.* 2006; Brouwers *et al.* 2006; Slifer *et al.* 2006; Smemo *et al.* 2006; Arias-Vasquez *et al.* 2007). Furthermore, the relatively rare incidence of the disease-related allele in certain populations may reduce its overall predictive value (Chuo *et al.* 2010).

Of the four disease-related SNPs that have been identified in *UBLQN1* (**Fig. 1.2A**), three are intronic and one is located in the promoter region, suggesting that they affect alternative splicing or expression level. In fact, evidence from the original report suggested that an intronic SNP that led to production of a shortened transcript variant due to alternative splicing was correlated with late-onset AD. As discussed in the following section, the protein product of the *UBQLN1* gene displays a number of characteristics that make it a plausible AD risk modifier.

## UBIQUILIN

### Superfamily and Domain Structure

The protein product of the *UBQLN1* gene is the ubiquilin-1 protein. In humans, the ubiquilin family of proteins consists of four members: ubiquilin-1 (OMIM ID 605046) (Wu *et al.* 1999), ubquilin-2 (OMIM ID 300264) (Kleijnen *et al.* 2000), ubiquilin-3 (OMIM ID 605473) (Conklin *et al.* 2000), and ubiquilin-4 (OMIM ID 605440) (Davidson *et al.* 2000). The ubiquilin proteins belong to the UBL-UBA superfamily of proteins, which contain a ubiquitin-like (UBL) domain at their N-terminus and a C-terminal ubiquitin-associated (UBA) domain (**Fig. 1.2B**). Both

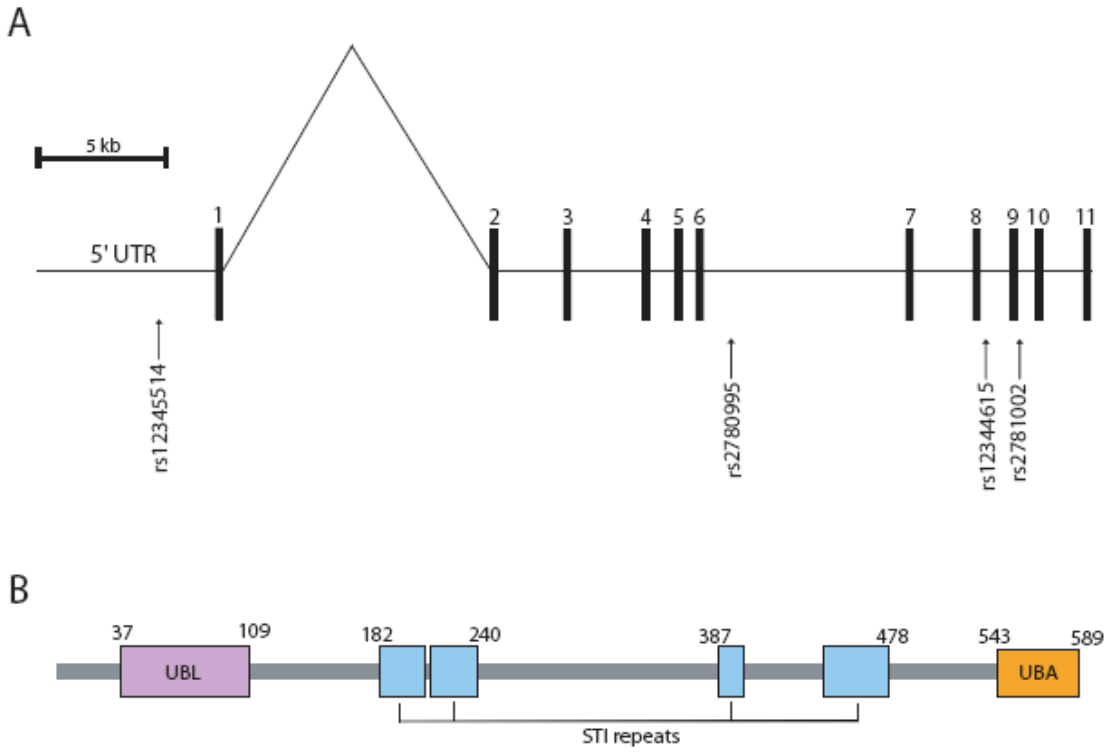
of these domains have been implicated in the targeting and degradation of proteins by the ubiquitin-proteasome system. The UBL domain is defined by a series of residues with both sequence and structural homology to ubiquitin, and it has been shown to interact with the S5a proteasomal subunit (Buchberger 2002; Walters *et al.* 2002). Type 1 UBL proteins, such as small ubiquitin-like modifier (SUMO) and NEDD8, act as ubiquitin-like protein modifiers and are covalently linked to target proteins. Type 2 UBL proteins (also referred to as ubiquitin domain proteins, or UDPs), which include ubiquilin and its homologues, contain one or more UBL domains in the context of a larger multi-domain protein. Therefore, a wider array of putative functions exists for type 2 UBL proteins. In addition to interacting directly with the proteasome, some UBL domains can mediate interactions with ubiquitin-interacting motifs (UIMs) of other proteins. For example, the UBL domain of ubiquilin has been shown to interact with the UIM of Eps15, a protein involved in endocytic trafficking (Regan-Klapisz *et al.* 2005).

The UBA domain was first identified in enzymes that are involved in the ubiquitination pathway and was also noted to be present in UBL-containing proteins (Hofmann and Bucher 1996). UBA domains form compact 3-helix bundles that directly bind to ubiquitin (Wilkinson *et al.* 2001) *via* a hydrophobic interaction involving the region surrounding $Ile^{44}$ on the ubiquitin molecule (Mueller and Feigon 2003; Ohno *et al.* 2005; Hurley *et al.* 2006). UBA domains have been shown to bind both monoubiquitin and polyubiquitin chains (Raasi *et al.* 2005; Zhang *et al.* 2008); however, they seem to prefer polyubiquitin *in vivo* (Hicke *et al.* 2005). One group defined four separate classes of UBA domains based on their ubiquitin interaction properties (Raasi *et al.* 2005): 1) those that preferentially bind $Lys^{48}$-linked polyubiquitin chains, 2) those that preferentially

bind $Lys^{63}$-linked polyubiquitin chains, 3) those that do not bind ubiquitin at all, and 4) those that bind polyubiquitin without any linkage preference, such as ubiquilin and its yeast homologue, Dsk2. The UBA domain of ubiquilin has been shown to mediate numerous protein-protein interactions, including those with the nuclear RNA-binding protein TDP-43 (Hanson *et al.* 2010), and mammalian target of rapamycin (mTOR), which functions in cell growth and survival (Wu *et al.* 2002). The UBA domain of ubiquilin is also necessary and sufficient to bind presenilin-1 and presenilin-2 (Mah *et al.* 2000). Furthermore, dimerization of Dsk2 and Rad23 occurs *via* the UBA domains (Bertolaet *et al.* 2001; Sasaki *et al.* 2005), and intramolecular UBL-UBA interactions may facilitate heterodimerization. Intra- and intermolecular UBL-UBA interactions, as have been shown for hHR23A and ubiquilin-2 (Kang *et al.* 2007), may serve a regulatory role by maintaining one or both proteins in a quiescent state until other binding partners (e.g., ubiquitinated substrates) are present in sufficient concentrations.

The central region of ubiquilin contains two STI motifs (**Fig. 1.2B**), also referred to as XPCB (*x*eroderma *p*igmentosum complementation group *C* *b*inding) domains. STI motifs are homologous to stress-inducible protein-1 (Sti-1), the yeast orthologue of the mammalian Hop protein, a co-chaperone molecule. Sti-1/Hop is a tetratricopeptide repeat (TPR)-containing protein, which functions as a co-chaperone for Hsp70 and Hsp90 (Blatch *et al.* 1997; Frydman and Hohfeld 1997; Odunuga *et al.* 2004). STI domains are found in numerous proteins and mediate protein-protein interactions and may also possess intrinsic chaperone activity (Davidson *et al.* 2000; Kamionka and Feigon 2004; Zhao *et al.* 2006).

Figure 1.2: Schematic representations of the *UBQLN1* gene and its protein product.




(A) Location of the four AD-linked SNPs within the *UBQLN1* gene sequence with intron/exon boundaries indicated. The *UBQLN1* gene contains 11 exons. The scale bar at the left is shown to approximate intron lengths (exons are not drawn to scale). The rs12345514 SNP is located in the promoter region, 1832 bp upstream of the start codon. The rs2780995 SNP is located 1473 bp from the 3' end of exon 6. The rs12344615 SNP is 70 bp from the 3' end of exon 8. Presence of the rs12344615 risk allele (G) leads to alternative splicing and removal of exon 8 (corresponding to residues 416-443 of the full-length polypeptide) without any frameshift (Bertram *et al.* 2005). The rs2781002 SNP is 67 bp from the 3' end of exon 9. (B) Domain structure of the ubiquilin-1 protein. Amino- and carboxy-terminal residue numbers are shown for each of the domains. The central region contains two STI motifs, which each consist of two repeating regions of Sti-1 homology (STI repeats).

## Established and Putative Functions

### *Proteasomal Degradation*

One of the most well-characterized UBL-UBA proteins is the *Saccharomyces cerevisae* protein Rad23, which is involved in nucleotide excision repair (Watkins *et al.* 1993) and spindle pole body (centrosome) duplication (Biggins *et al.* 1996). Following ubiquitination of a Rad23 substrate, Rad23 binds *via* one of its UBA domains. The ubiquitinated substrate is then recruited to the proteasome, where the UBL domain of Rad23 binds to the S5a proteasomal subunit (Schauber *et al.* 1998; Chen and Madura 2002). Ubiquilin and its homologues have also been shown to enhance degradation of ubiquitinated partners (Gao *et al.* 2003; Kim *et al.* 2008b) and have been shown to be involved in the proteasome-dependent ER-associated degradation (ERAD) pathway (Medicherla *et al.* 2004; Kim *et al.* 2008b; Lim *et al.* 2009). Thus, it has been suggested that UBL-UBA proteins promote degradation by serving as "shuttles" that deliver ubiquitinated substrates to the proteasome (Buchberger 2002). In contrast to this model, however, Rad23 has also been shown to stabilize ubiquitinated substrates (Hwang *et al.* 2005). This effect may be due to the ability of the UBA domain to "cap" ubiquitin chains and prevent chain elongation or de-ubiquitination (Ortolan *et al.* 2000; Su and Lau 2009). Inhibition of degradation has also been shown for numerous other UBL-UBA proteins, including ubiquilin (Kleijnen *et al.* 2000; Mah *et al.* 2000; Feng *et al.* 2004; Massey *et al.* 2004) and its *Xenopus* homolog, XDRP1 (Funakoshi *et al.* 1999). It is unclear what determines whether a UBL-UBA protein will promote or inhibit the degradation of a particular substrate, but it may depend on the substrate's

ubiquitination status, the abundance of free ubiquitin or ubiquitinated proteins in the cell, concentration of the UBL-UBA protein, or the substrate's reliance upon de-ubiquitination for degradation (Su and Lau 2009).

***Regulation of Trafficking***

In addition to regulating proteasomal degradation of substrates, ubiquitin has been shown to regulate protein trafficking and signaling events (Umebayashi 2003; d'Azzo *et al.* 2005). Monoubiquitination is a sufficient sorting signal at the plasma membrane and at the late endosome in mammalian cells, and there is evidence that UIMs can play a role in the trafficking of some proteins in both the secretory and endocytic pathways (Hicke and Dunn 2003). In fact, ubiquilin has been shown to regulate the trafficking and stability of several membrane-associated proteins, including γ-aminobutyric acid type A ($GABA_A$) receptor subunits (Bedford *et al.* 2001) and nicotinic acetylcholine receptors (Ficklin *et al.* 2005). Interestingly, ubiquilin stabilizes $GABA_A$ subunits in early secretory compartments where they are poised for rapid membrane insertion following neuronal stimulation (Saliba *et al.* 2008). Ubiquilin-2 has been shown to negatively regulate stimulus-dependent G protein-coupled receptor endocytosis (N'Diaye *et al.* 2008). This effect is mediated by the UBL domain binding to UIM-containing endocytic adaptors, Epsin and Eps15. Ubiquitin is a particularly important sorting signal at the multi-vesicular body (MVB), which is where the secretory and endocytic pathways converge. At the MVB, ubiquitin-binding proteins may determine whether a ubiquitinated substrate is recycled to the plasma membrane or degraded by the lysosome. Thus, UBL-UBA proteins may regulate non-proteasomal degradation in addition to proteasomal degradation.

***Protein Quality Control***

Mammalian cells possess three major lines of defense against the accumulation of misfolded proteins. The chaperone system comprises the first line of defense and primarily prevents protein aggregation by shielding hydrophobic regions that may participate in inappropriate intra- or intermolecular interactions. Thus, chaperones help maintain unfolded or partially folded proteins in folding-competent states, thus reducing the formation of off-pathway aggregation-prone intermediates. The second line of defense is the ubiquitin-proteasome system (UPS), which functions to degrade misfolded or folding-incompetent proteins. One important protein quality control checkpoint is in the secretory pathway, where proteins transition from the ER to the Golgi apparatus. Here, misfolded secretory proteins are translocated across the ER membrane and degraded by the UPS in a process known as ERAD (Vembar and Brodsky 2008). ERAD can be upregulated as part of the adaptive ER stress response, which is triggered when various potentially injurious stimuli, such as hypoxia or calcium depletion, lead to an increase the accumulation unfolded or misfolded proteins in the ER (Kim *et al.* 2008a). The third line of defense against the accumulation of toxic protein aggregates is the aggresome-autophagy pathway, which involves the active transport of protein aggregates to the microtubule organizing center (MTOC), where they coalesce to form large structurally-defined inclusions which can then be degraded in a lysosome-dependent process.

Ubiquilin has been shown to have a protective role in the cellular stress response. The results of one study showed that ubiquilin was upregulated in response to hypoxia and that overexpression of ubiquilin protected cells from hypoxia-induced apoptosis (Ko *et al.* 2002). Emerging evidence suggests that

ubiquilin plays a role in the cellular response to protein misfolding and aggregation (Haapasalo *et al.* 2010). Ubiquilin-1 and ubiquilin-2 were shown to be associated with polyglutamine-containing aggregates in a cellular model of Huntington's disease (Doi *et al.* 2004), which is not unexpected, given their well-established ubiquitin-binding properties. However, the Monteiro group has expanded on these findings and shown that ubiquilin protects against polyglutamine-induced cell death and toxicity in cellular and invertebrate models of Huntington's disease (Wang *et al.* 2006). This protection appears to be due, at least in part, to the ability of ubiquilin to promote the degradation of aggregation-prone potentially toxic expanded polypeptides (Wang and Monteiro 2007a). They observed similar results with proteins containing polyalanine expansions (Wang and Monteiro 2007b).

As outlined previously, ubiquilin is a well-established modulator of protein degradation *via* the UPS and is associated with the ERAD machinery (Medicherla *et al.* 2004; Kim *et al.* 2008b; Lim *et al.* 2009). In addition, recent research indicates that ubiquilin is an integral part of the aggresome-autophagy pathway as well. One group showed that endogenous ubiquilin-1 and Eps15, a UIM-containing protein, colocalized to aggresomes and that RNAi-mediated knockdown of either ubiquilin or Eps15 inhibited aggresome formation to the same extent (Heir *et al.* 2006). Furthermore, they showed that a mutation in the UBL domain of ubiquilin prevented aggresome formation without affecting the formation of peripheral aggregates, suggesting that ubiquilin plays a role in transporting aggregates along microtubules and that a UBL-UIM interaction with Eps15 is important for this function.

Brown and colleagues have recently reported that some of the cytoprotective effects associated with ubiquilin are mediated by its ability to promote maturation of the autophagosome and subsequent lysosomal fusion (N'Diaye *et al.* 2009a; N'Diaye *et al.* 2009b). Specifically, the cytoprotective effect of ubiquilin required the expression of autophagy-related genes and was dependent upon the UBA domain of ubiquilin, which interacts directly with microtubule-associated integral autophagosome proteins and does not require the presence of a ubiquitinated substrate. Interestingly, the same group had previously shown that overexpression of ubiquilin led to reorganization of cytoskeletal intermediate filaments (Wu *et al.* 1999), a process that is known to occur in aggresome formation (Kopito 2000). One well-established method of inducing autophagy in mammalian cells is with the drug rapamycin, which inhibits the kinase activity of mTOR. Ubiquilin has been shown to interact with mTOR (Wu *et al.* 2002), and therefore, may be involved in the earlier phase of autophagy induction by sequestering mTOR and preventing it from performing its cellular functions.

Ubiquilin may be important for the autophagic clearance of at least one type of protein aggregate linked to neurodegenerative disease, TDP-43. Ubiquilin has been shown to interact with polyubiquitinated TDP-43 that is mislocalized to the cytoplasm, and overexpression of ubiquilin increased the association of TDP-43 with autophagosome markers (Kim *et al.* 2009b). Also, overexpression of TDP-43 was sufficient to induce the formation of, and recruitment of endogenous ubiquilin to, large cytoplasmic aggregates. In a later study, the same group showed that, unexpectedly, ubiquilin overexpression exacerbated TDP-43 overexpression phenotypes in *Drosophila* despite an overall reduction in TDP-43

protein levels (Hanson *et al.* 2010). This may be due to sequestration of ubiquilin and/or competition for the protein degradation machinery by the TDP-43 aggregates, leading to a loss-of-function phenotype or increased accumulation of other degradation targets, respectively. There are still many unanswered questions regarding the role of ubiquilin in aggregate clearance, especially with respect to autophagy, the detailed mechanisms of which are still largely unclear.

***Potential Role in Alzheimer's Disease***

A number of studies have suggested a role for the ubiquitin-proteasome system in AD, indicating a possible role for ubiquilin. Proteasome activity is decreased (Lopez Salon *et al.* 2000) and ubiquitinated proteins accumulate in AD brains (de Vrij *et al.* 2004; Song and Jung 2004). Nunan and colleagues (Nunan *et al.* 2001) reported that the C99 fragment of APP is degraded in a proteasome-dependent fashion that is independent from γ-secretase activity. Inhibition of the proteasome results in increased processing by γ-secretase. It is possible that ubiquilin alters the processing of APP in a proteasome-dependent fashion.

Initially, ubiquilin was shown to interact with the presenilin proteins, which are thought to constitute the catalytic core of γ-secretase (De Strooper *et al.* 1998; Wolfe *et al.* 1999). Specifically, ubiquilin was shown to decrease the turnover of high molecular weight presenilin isoforms (Mah *et al.* 2000; Massey *et al.* 2004). This effect on turnover was shown to be due in part to a decrease in the ubiquitination and degradation of full length presenilin. More recently, it has been shown that knockdown of ubiquilin by RNAi accelerates APP maturation through the secretory pathway, resulting in increased secretion of the soluble APP ectodomain and the Aβ peptide in H4 neuroglioma cells and HEK293 cells (Hiltunen *et al.* 2006). These alterations in APP maturation and processing

occurred without changes in β- or γ-secretase levels, suggesting that a relationship exists between ubiquilin and APP that is independent of presenilins. In this same study, researchers used fluorescence resonance energy transfer (FRET) to establish that APP and ubiquilin came into close proximity; however, they could not confirm direct binding of the two proteins. The effect of ubiquilin knockdown on APP maturation suggests that ubiquilin may regulate trafficking from the ER to the Golgi. There is some evidence that ubiquilin associates with the ER (Ko *et al.* 2002).

Important questions remain regarding the molecular mechanisms of AD pathogenesis. The following set of studies was designed to further elucidate the mechanisms by which APP contributes to the cellular dysfunction and cell death that occurs in AD. The first set of studies (**Chapter 2**) examined the effect of FAD-linked mutations in APP on intracellular calcium homeostasis. The second set of studies (**Chapter 3**) investigated the role of the ubiquilin protein in the regulation of intracellular trafficking and degradation of APP. The third set of studies (**Chapter 4**) focused on elucidating the nature of the ubiquilin-APP interaction and established a role for ubiquilin in the quality control of the APP protein. Our studies focus specifically on the ubiquilin-1 protein, since genetic association studies have indicated a link between this particular ubiquilin family member and late-onset AD.

# Chapter 2: FAD Mutations in Amyloid Precursor Protein Do Not Directly Perturb Intracellular Calcium Homeostasis[1]


## ABSTRACT

Disturbances in intracellular calcium homeostasis are likely prominent and causative factors leading to neuronal cell death in Alzheimer's disease (AD). Familial AD (FAD) is early-onset and exhibits autosomal dominant inheritance. FAD-linked mutations have been found in the genes encoding the presenilins and amyloid precursor protein (APP). Several studies have shown that mutated presenilin proteins can directly affect calcium release from intracellular stores independently of Aβ production. Although less well established, there is also evidence that APP may directly modulate intracellular calcium homeostasis. Here, we directly examined whether overexpression of FAD-linked APP mutants alters intracellular calcium dynamics. In contrast to previous studies, we found that overexpression of mutant APP has no effects on basal cytosolic calcium, ER calcium store size or agonist-induced calcium release and subsequent entry. Thus, we conclude that mutated APP associated with FAD has no direct effect on intracellular calcium homeostasis independently of Aβ production.


## INTRODUCTION

AD is a progressive neurological disorder characterized by deterioration of cognitive abilities. AD is the most common cause of dementia in the western world, affecting one in ten individuals over 65 and nearly 50% of all persons over the age of 85 (Evans *et al.* 1989; Hebert *et al.* 2003). According to the

[1] Stieren E, Werchan WP, El Ayadi A, Li F, Boehning D. (2010) FAD mutations in amyloid precursor protein do not directly perturb intracellular calcium homeostasis. *PLoS One* 5(8):e11992. *No permissions required due to open access policy.*

predominant amyloid cascade hypothesis, AD pathogenesis is associated with a series of molecular events which leads to the extracellular deposition and aggregation of specific proteolytic fragments of APP. These aggregated protein fragments constitute the core of extracellular senile amyloid plaques, a pathologic hallmark of AD.

In normal physiology, APP is cleaved by a series of enzymes, called secretases, generating proteolytic fragments of various lengths. The principal cleavage event is by α-secretase, which generates a large soluble ectodomain ($APP_s$) that is secreted into the extracellular space and a C-terminal fragment (C83) that remains in the membrane (De Strooper and Annaert 2000). In an alternate processing pathway, holo-APP can be cleaved by β-secretase, resulting in the production of a secreted ectodomain and the membrane-associated C99 fragment (De Strooper and Annaert 2000). Subsequent cleavage of C99 by γ-secretase produces the neurotoxic Aβ peptide and an intracellular domain (AICD) that is released from the membrane into the cytosol. There are two major forms of the Aβ peptide that differ in length by two residues, $Aβ_{40}$ and $Aβ_{42}$. The $Aβ_{42}$ peptide is more prone to aggregation and is considered to be more cytotoxic than the shorter Aβ species (Finder and Glockshuber 2007).

Most cases of AD are sporadic and late-onset, but rare forms of familial AD (FAD) are early-onset and exhibit autosomal-dominant inheritance. The majority of FAD cases are linked to mutations in the presenilin genes 1 and 2 (Selkoe 2005). Presenilins constitute the catalytic core of γ-secretase, and presenilin mutations lead to relative overproduction of $Aβ_{42}$ (Borchelt *et al.* 1996; Scheuner *et al.* 1996; Citron *et al.* 1997; Wolfe *et al.* 1999). FAD-linked mutations have also been found in APP, and depending on the mutation result in

increased β-secretase processing, increased $A\beta_{42}/A\beta_{40}$ ratio, increased propensity of Aβ to form fibrils, or decreased proteolytic clearance of Aβ peptides (Goate *et al.* 1991; Murrell *et al.* 1991; Hendriks *et al.* 1992; Kamino *et al.* 1992; Mullan *et al.* 1992; Suzuki *et al.* 1994; Tamaoka *et al.* 1994; Haass *et al.* 1995; De Jonghe *et al.* 1998; Murrell *et al.* 2000; Nilsberth *et al.* 2001).

While it is clear that APP-derived fragments are involved in a proximal step in the pathogenesis of AD, the exact mechanism of neuronal loss in not known. Also, clinical symptoms do not correlate well with amyloid plaque load, suggesting that a certain level of neuronal dysfunction precedes gross architectural changes in AD brain (Braak and Braak 1991; Duyckaerts and Hauw 1997). Calcium dyshomeostasis has been implicated as a major contributor to neuronal cell death in AD (LaFerla 2002). Calcium dynamics regulate Aβ production, and Aβ peptides/fibrils directly affect multiple aspects of calcium homeostasis (Green and LaFerla 2008). There is strong evidence that mutated presenilin proteins can directly modulate calcium release from intracellular stores independently of Aβ production (Leissring *et al.* 1999a; Leissring *et al.* 1999b; Cheung *et al.* 2008), and may also form calcium permeable channels in the ER (Nelson *et al.* 2007). Similarly, several studies have suggested that APP may directly modulate calcium homeostasis independently of Aβ production (Leissring *et al.* 2002; Lopez *et al.* 2008; Rojas *et al.* 2008). However, a systematic study of the effects of FAD-associated APP mutants on intracellular calcium homeostasis has not been reported.

Here, we show that overexpression of FAD-linked APP mutants has no effect on basal cytosolic calcium concentration, ER calcium store size, or agonist-induced calcium release and subsequent entry. These results indicate

that mutant APP likely does not contribute mechanistically to alterations in calcium homeostasis in AD independently of Aβ production.

## MATERIALS AND METHODS

### Cell Lines

PC12 rat pheochromocytoma cells were purchased from ATCC. They were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin.

### Generation of APP Mutants

Point mutations in human $APP_{695}$ (kindly provided by Dr. Hui Zheng, Baylor College of Medicine) were accomplished with the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to manufacturer's instructions. Forward primers were as follows:

Swedish 5'-GGAGATCTCTGAAGTGAACCTGGATGCAGAATTCC-3'

Flemish 5'-CAAAAATTGGTGTTCTTTGGAGAAGATGTGGG-3'

Arctic 5'-GGTGTTCTTTGCAGGAGATGTGGGTTCAAAC-3'

London 5'-CATAGCGACAGTGATCATCATCACCTTGGTGATGC-3'

Indiana 5'-CATAGCGACAGTGATCTTCATCACCTTGGTGATGC-3'

V717L 5'-CATAGCGACAGTGATCCTCATCACCTTGGTGATGC-3'

The reverse primers were the exact reverse complement.

### Calcium Imaging

Calcium measurements were performed as previously described (Boehning *et al.* 2005). PC12 cells were cultured on 25 mm coverslips overnight, and were co-transfected with APP and yellow fluorescent protein (YFP) at a 4:1

ratio. Under these conditions, we have found that all YFP-positive cells express both proteins (Wozniak *et al.* 2006). For all experiments, transfection efficiency as determined by fluorescence microscopy was greater than 50%. For measurement of cytosolic calcium, cells were loaded with fura-2 as described elsewhere (Boehning *et al.* 2005) for 30 minutes at 25°C. For ER calcium measurements, cells were loaded with 5 μM mag-fura-2 for 20 minutes at 37°C followed by a 60-minute incubation at 37°C in dye-free extracellular medium (107 mM NaCl, 7.2 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$,11.5 mM glucose, 0.1% bovine serum albumin (BSA), and 20 mM HEPES pH 7.2). Coverslips were transferred to the microscope and the plasma membrane was subsequently permeabilized by a brief exposure to 0.01% saponin to release cytoplasmic dye before exchanging the solution with intracellular solution (135 mM KCl, 3 mM MgATP, 2 mM MgCl, 0.4 mM $CaCl_2$, 1 mM EGTA, and 20 mM HEPES pH 7.1) as described (Solovyova *et al.* 2002). All fields were imaged randomly, and all YFP-positive cells in a given field were imaged and quantified in the data analysis. The number of cells analyzed is indicated above each data point. Fura-2/mag-fura-2 and YFP images were acquired every 3 seconds during acquisition on a Nikon TE2000 inverted microscope using a Nikon 60X oil immersion SuperFluor objective with a 1.3 numerical aperture. All imaging was performed at 25°C. Images were captured with a Roper Scientific CoolSNAP HQ monochrome camera. Rapid filter changes for ratiometric imaging were computer controlled *via* a Ludl MAC6000 rapid filter wheel and changer and MetaFluor data acquisition and analysis software. Raw data was acquired with MetaFluor, analyzed in Excel, and graphed in Sigma Plot.

### Statistical Analysis

All experiments were performed a minimum of three times and presented as the mean ± standard error. Single cell traces from each coverslip were pooled and averaged for each data point. Total number of single cell traces is indicated over each bar. Statistical comparisons from the pooled data were performed between groups using the student's *t* test. Statistical significance was considered at *p* values <0.05.

## RESULTS

### Expression of FAD-linked APP Mutants in PC12 Cells

For our studies, we focused on six different well-characterized FAD-linked APP mutants that affect β-secretase cleavage, fibrillization, and γ-secretase cleavage (**Fig. 2.1A**). The Swedish double mutant makes APP a more favored substrate for β-secretase, shunting full-length APP down the amyloidogenic processing pathway (Mullan *et al.* 1992; Haass *et al.* 1995). The London, Indiana, and V717L mutations favor production of $A\beta_{42}$ by the γ-secretase (Goate *et al.* 1991; Murrell *et al.* 1991; Suzuki *et al.* 1994; Tamaoka *et al.* 1994; Murrell *et al.* 2000). The Flemish and Arctic APP mutations increase the propensity for fibrillization and decrease proteolytic clearance of Aβ peptides (Hendriks *et al.* 1992; Kamino *et al.* 1992; De Jonghe *et al.* 1998; Nilsberth *et al.* 2001).

Immunoblot analysis of PC12 cells overexpressing wild-type and mutant APP revealed two bands with approximate molecular weight of 110-120 kDa corresponding to immature and mature forms of the holoprotein (**Fig. 2.1B**). As APP is trafficked through the secretory pathway, a series of glycosylation events occur leading to a mature, or fully glycosylated, protein with slower mobility on

SDS-PAGE. An immunoblot for α/β-tubulin is shown as a loading control (**Fig. 2.1C**).

### ER Calcium Release, Receptor-operated Calcium Entry, and ER Calcium Store Size Are Not Affected by FAD-linked Mutant APP

Calcium release from ER-resident $IP_3Rs$ has been proposed to be a critical mediator of calcium dyshomeostasis in AD (Leissring *et al.* 1999a; Leissring *et al.* 1999b; Cheung *et al.* 2008). To determine whether mutant APP expression affects $IP_3R$-mediated calcium release, we measured the response of PC12 cells to the purinergic agonist uridine 5'-triphosphate (UTP), which selectively activates phospholipase C-coupled P2Y receptors (von Kugelgen 2006). PC12 cells were co-transfected with YFP and one of the following: empty vector control, wild-type APP, or one of six FAD-linked APP mutants. To selectively examine $IP_3R$ activity and exclude the contribution of calcium entry from the plasma membrane, UTP stimulation was done in calcium free media. As indicated in the representative fura-2 calcium traces in **Fig. 2.2A**, addition of UTP induces a robust calcium release from the ER into the cytosol in calcium-free media. Peak calcium release in response to UTP did not differ between control cells expressing empty vector, cells expressing wild-type APP, and cells expressing APP mutants (**Fig. 2.2B**).

Depletion of ER calcium stores by agonist stimulation triggers store/receptor-operated calcium entry (SOC/ROC) through channels in the plasma membrane (Putney 2007). Entry through these channels has been proposed to be compromised in fibroblasts expressing mutant presenilins (Leissring *et al.* 2000). To determine the effect of FAD-linked APP mutants on receptor-operated calcium entry, we waited until cytosolic calcium levels reached

baseline following UTP stimulation in calcium-free medium and replaced the bath solution with calcium-replete medium. As shown in **Fig. 2.2C**, receptor-operated calcium entry did not differ significantly between control cells expressing YFP alone, cells expressing wild-type APP, and cells expressing any of the six APP mutants tested.

Next, we wanted to determine the effect of FAD-linked mutant APP on ER calcium store size. To directly measure ER calcium content, we utilized ER compartmentalized mag-fura-2 (Hofer and Machen 1993; Mogami *et al.* 1998; Solovyova *et al.* 2002). As shown in **Fig. 2.2D**, ER calcium store size did not differ between control cells, wild-type APP-expressing cells, and any of the six APP mutants tested.

### Agonist-induced Calcium Release Is Not Affected By FAD-linked Mutant APP

Mutant presenilins directly modulate calcium release from ER stores (Leissring *et al.* 1999a; Leissring *et al.* 1999b; Cheung *et al.* 2008). To address whether mutant APP has similar effects, we monitored the response of PC12 cells to two different doses of UTP. As shown in the representative traces in **Fig. 2.3A**, addition of subsaturating (10 μM) and saturating (100 μM) doses of UTP resulted in transient increases in cytosolic calcium. There was no difference in the percent of cells that responded to the subsaturating dose (**Fig. 2.3B**), indicating that there were no significant differences in sensitivity to agonist-induced calcium release. There were also no differences observed in peak calcium release induced by either dose between control, wild-type APP, and the six mutant APP-expressing cells (**Fig. 2.3C**). By analyzing baseline calcium levels in each condition, we were also able to determine that resting cytosolic

calcium was not altered in cells expressing wild-type APP or FAD-linked mutant APP (**Fig. 2.3D**).

## DISCUSSION

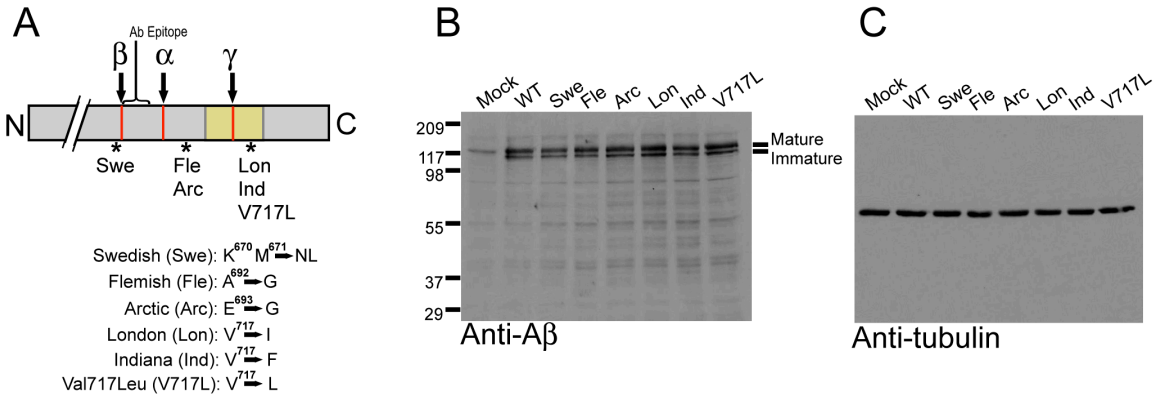
The results of these studies indicate that FAD-linked APP mutants do not directly affect calcium homeostasis independently of Aβ production, at least in this model system. Several studies have shown that overexpression of wild-type APP or FAD-linked mutant APP leads to an increase in basal cytosolic calcium concentration and an increase in ER store size. Rojas and colleagues demonstrated that neurons derived from mice possessing three copies of the APP gene have increased resting cytosolic calcium concentration and altered responses to glutamatergic and nicotinic agonists and that these effects could be partially restored by APP knockdown (Rojas *et al.* 2008). Cortical neurons from the triple-transgenic (3xTg-AD) mouse model of AD were also shown to have elevated resting cytosolic calcium (Lopez *et al.* 2008). Overexpression of the Swedish APP mutant alone was sufficient to cause the same effect. In these studies, cells were taken from young animals prior to plaque development, suggesting that the changes observed represent proximal events in the disease model. In cells from the 3xTg-AD mice, calcium entry as well as release from intracellular stores both contributed to elevated cytosolic calcium levels (Lopez *et al.* 2008).

It has been shown that agonist-induced calcium release from intracellular stores is disrupted in fibroblasts from APP null mice (Leissring *et al.* 2002). This effect is related to a decrease in ER store size and can be rescued by APP constructs containing the APP intracellular domain (AICD), suggesting that this peptide fragment is involved in regulating intracellular calcium stores (Leissring *et*

*al.* 2002). Subsequent studies showed that the loss of AICD was associated with decreased levels of ATP, perhaps impairing ER calcium uptake *via* the sarco-endoplasmic reticulum calcium ATPase (Hamid *et al.* 2007). Other studies have shown that wild-type APP is not directly involved in modulating calcium stores but does mediate the increase in store size that is seen with certain presenilin mutants and presenilin deficiency (Herms *et al.* 2003). The effect of presenilin knockdown on store size was exacerbated in cells expressing the FAD-linked APP mutant V717I, and this effect correlated with increased levels of C99 in these cells (Herms *et al.* 2003).

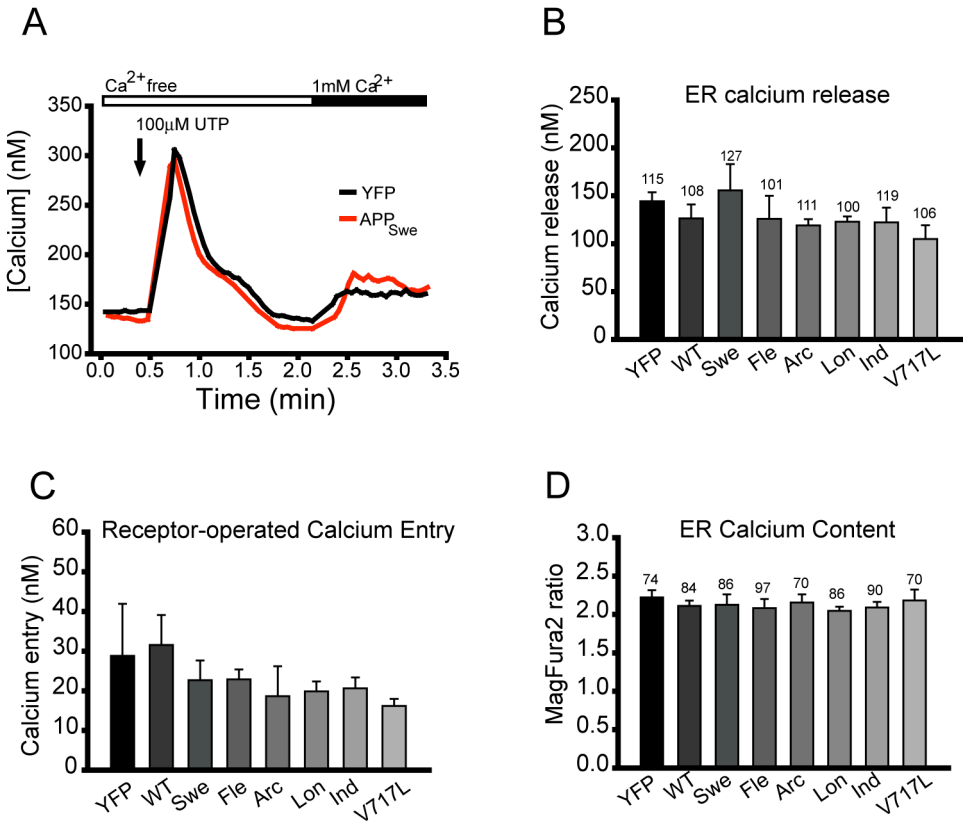
Here, we demonstrated that FAD-linked APP mutations do not directly alter calcium signaling when overexpressed in PC12 cells. These results suggest that a different mechanism must exist for the perturbations in intracellular calcium that are observed in forms of FAD that are linked to mutations in APP. Most likely, these effects are mediated directly by Aβ and not the APP holoprotein. Furthermore, our results promote the hypothesis that the disruption in calcium signaling seen with presenilin mutants is independent of the role for presenilins in APP cleavage, and may instead reflect a general role for presenilin proteins in the maintenance of intracellular calcium homeostasis.

Figure 2.1: Expression of wild-type APP and different FAD-linked APP mutants in PC12 cells.



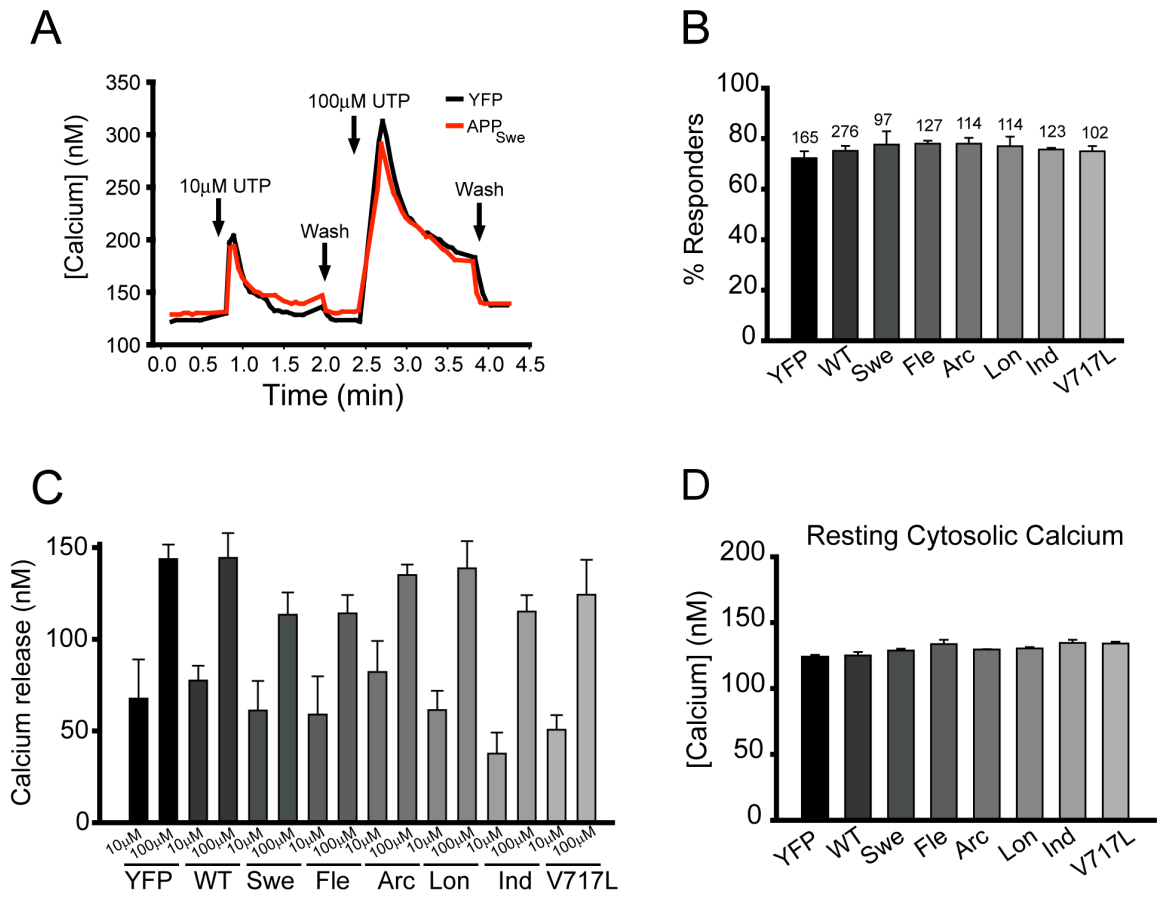

(A) Schematic diagram depicting the C-terminal portion of APP with secretase cleavage sites indicated in red and locations of FAD-linked mutations marked with asterisks. The transmembrane region is shaded in yellow. The epitope for the anti-Aβ 1-10 antibody (Millipore Cat. No. 07-592) is indicated. Specific amino acid substitutions for each mutation are shown with residue numbering corresponding to $APP_{770}$. (B) Overexpression of APP constructs in PC12 cells. Mock cells were co-transfected with YFP and empty vector. (C) Immunoblot for α/β-tubulin to demonstrate equal loading.

Figure 2.2: ER calcium release, store-operated entry, and store size are not affected by overexpression of APP mutants.




(A) Representative single cell traces of cytosolic [$Ca^{2+}$] in control cells (black) and cells expressing APP with the Swedish mutation (red). (B) Peak calcium release in response to 100 μM UTP in calcium-free medium. The total number of single cells imaged for each condition is indicated above the error bar. All cells were co-transfected with either empty vector or APP and yellow fluorescent protein (YFP) at a 4:1 ratio. WT, wild-type; Swe, Swedish; Fle, Flemish; Arc, Arctic; Lon, London; Ind, Indiana. (C) Store-operated calcium entry following addition of calcium-replete medium in control and APP-transfected cells for each of the APP constructs tested. These results are from continuous imaging of the same coverslips used for the calcium release experiments shown in (A). (D) ER store size in control and APP-transfected cells for each of the APP constructs tested. For all experiments, error bars represent standard error of the mean. There was no statistical significance between control and APP expressing cells ($p>0.05$ for all conditions).

Figure 2.3: Agonist-induced calcium entry is not affected by overexpression of FAD-linked APP mutants.




(A) Representative single cell traces of cytosolic [$Ca^{2+}$] in cells expressing YFP alone (control) or APP with the Swedish mutation (red) upon addition of 10 μM and 100 μM UTP. (B) Percentage of cells that responded to 10 μM UTP for each condition. (C) Quantification of peak calcium release induced by UTP. (D) Resting (basal) cytosolic calcium concentration in control and APP-transfected cells for each of the APP constructs tested. Error bars represent standard error of the mean. There was no statistical significance between control and APP expressing cells ($p>0.05$ for all conditions).

# Chapter 3: Ubiquilin Regulates Trafficking and Degradation of the Amyloid Precursor Protein


## ABSTRACT

Alzheimer's disease (AD) is associated with extracellular deposition of proteolytic fragments of amyloid precursor protein (APP). Although mutations in APP and the secretase enzymes that mediate its processing are known to result in familial forms of AD, the mechanisms underlying the more common sporadic forms of the disease are still unclear. Evidence suggests the susceptibility of APP to amyloidogenic processing is related to its intracelluar localization and that secretase-independent degradation may prevent the formation of cytotoxic peptide fragments. Recently, the *UBQLN1* gene has been linked to late-onset AD, and its protein product, ubiquilin, may regulate the maturation of full-length APP. Here, we show that ubiquilin inhibits the maturation of APP through the secretory pathway by sequestering it in the Golgi apparatus. Our results demonstrate that this sequestration delays the proteolytic processing of APP. Furthermore, we show that ubiquilin inhibits the proteasomal degradation of APP and increases the accumulation of ubiquitinated APP species, suggesting that ubiquitin may serve as a sorting signal in this context. Our findings support the notion that the regulation of APP trafficking by ubiquilin is an important mechanism for maintaining the proper cellular localization, and preventing excessive proteolytic processing, of APP.


## INTRODUCTION

Alzheimer's disease (AD) is associated with extracellular deposition and aggregation of proteolytic fragments of amyloid precursor protein (APP). In

normal physiology, full-length APP is cleaved by a series of enzymes, called secretases. In a non-amyloidogenic processing pathway, full-length APP is cleaved within the Aβ sequence by α-secretase, generating a large soluble ectodomain ($APP_s$) that is secreted into the extracellular space and a membrane-tethered C-terminal fragment (C83). In an alternate processing pathway, full-length APP is cleaved by β-secretase, resulting in the production of a secreted ectodomain and the membrane-tethered C99 fragment. Subsequent cleavage of C99 by γ-secretase produces the Aβ peptide and an intracellular domain (AICD) that is released from the membrane into the cytosol (De Strooper and Annaert 2000).

APP matures through the secretory pathway, where it undergoes classical *N*- and *O*-linked glycosylation during transit through the ER and Golgi, respectively (Weidemann *et al.* 1989; De Strooper and Annaert 2000; Suzuki *et al.* 2006). A small fraction of the cellular APP pool is trafficked to the cell surface, where it is only briefly associated with the plasma membrane before being recycled through the endocytic pathway. This rapid internalization occurs due to the presence of the highly-conserved YENPTY (single amino acid code) sequence in the APP cytoplasmic domain, which contains a canonical NPxY internalization signal for clathrin-mediated endocytosis. Proteolytic processing of APP has been shown to occur in various sites throughout the secretory and endocytic pathways; however, amyloidogenic processing primarily occurs after transition through the Golgi apparatus (Tomita *et al.* 1998) and in endosomal compartments, where acidic conditions promote optimal activity of the beta-site APP cleaving enzyme (BACE), or β-secretase (Small and Gandy 2006). Deletion or mutation of the YENPTY internalization signal leads to an increase in plasma

membrane-associated APP and a significant decrease in Aβ production, underscoring the importance of endocytic recycling for Aβ generation (Lai *et al.* 1995; Perez *et al.* 1999; De Strooper and Annaert 2000; Kerr and Small 2005).

Various cytoplasmic adaptor proteins have been shown to interact with the YENPTY motif, including members of the X11 family (Borg *et al.* 1996; Miller *et al.* 2006), Fe65 (Borg *et al.* 1996), Dab-1 (Homayouni *et al.* 1999), and Jip1b (Taru *et al.* 2002). Some of these interactions have been shown to affect the intracellular localization and proteolytic processing of APP (Sabo *et al.* 1999; Saito *et al.* 2008). Sorting proteins, such as sorLA (LR11), also regulate APP processing by sequestering it from endosomal compartments (Schmidt *et al.* 2007) and preventing association with secretase enzymes (Spoelgen *et al.* 2006), leading to decreased Aβ production. Production of Aβ may also be suppressed by factors that target full-length APP or its C-terminal fragments to the proteasome or lysosome for γ-secretase-independent degradation (Caporaso *et al.* 1992; Knops *et al.* 1992; Nunan *et al.* 2003). Recent studies have suggested a role for ubiquilin in the regulation of APP trafficking and processing (Hiltunen *et al.* 2006). Specifically, reduction in proteins levels of ubiquilin accelerated the maturation of APP through the secretory pathway, which was associated with increased secretion of $APP_s$ and increased production of Aβ.

Here, we show that ubiquilin can sequester APP in early secretory compartments and delay its proteolytic processing. Because ubiquilin has previously been shown to regulate the proteolysis of numerous other substrates, we also investigated the effect of ubiquilin overexpression on proteasomal and lysosomal degradation. We found that ubiquilin is a potent inhibitor of proteasomal degradation of APP. Surprisingly, however, overexpression of

ubiquilin was also associated with increased ubiquitination of APP. We hypothesize that ubiquitination in this context serves as a sorting signal that regulates the intracellular trafficking of APP and subsequent secretase processing.

## MATERIALS AND METHODS

### Cell Lines

Human HeLa cells were purchased from ATCC and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin. Rat PC12 pheochromocytoma cells were purchased from ATCC. They were cultured in propagation media, which consisted of DMEM supplemented with 10% FBS, 5% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin. For experiments in which the PC12 cells were differentiated, cells were passed such that they were approximately 30% confluent at the time of differentiation. The media was changed to differentiation media, which consisted of DMEM supplemented with 1% FBS and 2% horse serum. Nerve growth factor (NGF) was added to the differentiation media at a final concentration of 50 ng/ml. Neuronal-like processes were visible by light microscopy within 24 hours of NGF addition.

Primary cortical neurons were obtained from embryonic rat pups. Briefly, adult (~300 g) Sprague-Dawley timed pregnant (embryonic day 17-19) rats were anesthetized by i.p. injection with 150 mg/kg ketamine and 10 mg/kg xylazine. The uterus was removed and dissected to reveal the embryonic rats, and the cortex was dissected from the embryonic pups and enzymatically dissociated for *in vitro* neuronal cell culture. Primary cortical neurons were cultured on poly-D-

lysine-coated glass coverslips in Neurobasal medium (Invitrogen) supplemented with 2% B-27 supplement, 250 μM L-glutamine, 25 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin. L-glutamate at a final concentration of 25 μM was added to the medium for the initial plating. Subsequent medium changes were done by removing half the medium and replacing it with fresh medium without L-glutamate.

**Expression Constructs**

To generate an APP-GFP fusion construct, $APP_{695}$ was amplified using polymerase chain reaction (PCR), incorporating a 5' *Hind*III restriction site and a 3' *Sac*II restriction site, and was cloned in frame to the *Hind*III-*Sac*II sites of the pmaxFP$^{TM}$-Green-N mammalian expression vector (Amaxa). This vector has been described elsewhere, and the particular green fluorescent protein (GFP) variant has been shown to have no effect on proteasomal degradation (Baens *et al.* 2006). PCR primers were 5'-TTC AAG CTT CCA TGC TGC CCG GTT TGG CAC TG-3' and 5'-TCC CCG CGG GTT CTG CAT CTG CTC AAA GAA CTT GTA G-3'.

A GlcNAc-TI-mCherry fusion construct was kindly provided by Dr. Joachim Seemann. This construct encodes the cytoplasmic and transmembrane domains of N-acetylglucosaminyltransferase I, a Golgi-resident enzyme, fused to the mCherry red fluorescent protein, which replaces the C-terminal luminal catalytic domain of the enzyme.

**GlcNAc-TI Stable Cell Line**

The GlcNAc-TI-mCherry construct was linearized by digesting with *Eco*O109I/*Dra*II, which cuts once within the pmCherry-N1 vector sequence. The

linearized DNA was gel-purified and transfected into PC12 cells. One day post-transfection, the culture media was changed to selection media containing 1.5 mg/ml G418. Selection efficiency was determined by monitoring cell death in a control plate of non-transfected cells in selection media. When there were no live cells remaining in the control plate, the transfected cells were split such that the density was low enough to detect individual colonies. Individual clonal colonies were removed and expanded separately and maintained in PC12 culture media containing 1 mg/ml G418.

**Cycloheximide (CHX) Chase Experiments**

CHX (100 mg/ml in DMSO) was purchased from Sigma (Catalog No. C4859). A 1 mg/ml working stock was made by diluting the solution in deionized water immediately prior to use. The working stock was diluted in cell culture media at a final concentration of 50 µg/ml prior to adding it to the cells. All of the media for the entire experiment was prepared at the same time and added to each group simultaneously.

PC12 cells were plated on 60 mm plates (a separate plate was used for each time point). Cells in the treatment group were transfected with myc-tagged ubiquilin, and control cells were transfected with an empty vector (pcDNA3.1). Two days after transfection, cells were treated with CHX. At the specified time point, one plate from each group was snap-frozen in liquid nitrogen, and plates were stored at -80°C until processing. After completion of the time course, cells were lysed in a Triton-based lysis buffer, and lysates were run on a 4-20% gradient gel and analyzed by Western blot analysis with a polyclonal anti-Aβ antibody (Millipore). Blots were stripped and re-probed with an anti-c-myc antibody (Roche) to detect overexpressed ubiquilin.

To isolate lysosomal degradation, cells were pre-treated for 15 minutes with the proteasome inhibitor MG132 (EMD Biosciences, Cat No. 474790) at a final concentration of 10 μM. To isolate proteasomal degradation, cells were pre-treated for 15 minutes with the lysosomal inhibitor ammonium chloride ($NH_4Cl$) at a final concentration of 25 mM. Both inhibitor treatment groups were compared to controls cells pre-treated with vehicle alone (dimethyl sulfoxide; DMSO). For all CHX chase experiments, control and treatment samples were handled under identical conditions, and blots were exposed simultaneously on the same film.

**Subcellular Fractionation**

PC12 cells were transfected with myc-tagged ubiquilin or a control empty vector. The next day, cells were treated for two hours with either 50 μg/ml CHX or vehicle alone (DMSO). Cells were then collected, snap-frozen in liquid nitrogen, and lysed with a glass-on-glass dounce homogenizer in Buffer A (10 mM Tris-HCl pH 7.5, 250 mM sucrose, 1 mM EGTA, and protease inhibitors). Lysis and all subsequent fractionation steps were performed at 4°C. First, nuclei and unbroken cells were removed by centrifugation at 1000 x*g* for 15 minutes. The cleared supernatants were then centrifuged at 10,000 x*g* for 15 minutes. The 10,000 x*g* pellets, which contain rough ER, high-density Golgi, plasma membrane, and lysosomal fractions, were resuspended in Buffer A, and the supernatant was centrifuged at 15,000 x*g* for 15 minutes to remove residual mitochondria. The 15,000 x*g* supernatant was centrifuged at 100,000 x*g* in a benchtop ultracentrifuge. The 100,000 x*g* pellets, which contain smooth ER, low-density Golgi, and small vesicles, were resuspended in Buffer A. The 100,000 x*g* supernatant consisted of the cytosolic fraction.

**Cell Surface Biotinylation**

HeLa cells were plated on 10 cm plates and transfected with myc-tagged ubiquilin or a control empty vector. The next day, the media was removed, and cells were washed three times with 10 ml sterile ice-cold phosphate-buffered saline (PBS) pH 8.0 (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$), 2 mM $MgCl_2$, 1 mM $CaCl_2$. Sulfo-NHS-SS-Biotin (Pierce) was dissolved in ice-cold PBS pH 8.0, 2 mM $MgCl_2$, 1 mM $CaCl_2$ at a final concentration of 1 mM, and 6 ml of this biotin labeling solution was added to each plate. Plates were gently agitated on an orbital shaker for 3 hours at 4°C to ensure even coverage with the labeling solution. Following incubation, the labeling solution was removed, and cells were incubated in 8.5 ml quenching solution (PBS pH 8.0, 10 mM Tris, 100 mM glycine) for 10 minutes at 4°C. Cells were then lysed in 250 µl RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) containing protease inhibitors. Lysates were cleared at 10,000 x*g* for 20 minutes at 4°C, and protein content of the supernatant was determined by the Bradford method. A total of 500 µg protein (~1 mg/ml in RIPA buffer) was added to 25 µl bed volume NeutrAvidin™ agarose resin (Pierce), and mixtures were incubated overnight at 4°C with rotation. The resin was washed 10 times with RIPA buffer containing protease inhibitors and boiled at 95°C in 25 µl 2X SDS sample buffer, and eluted proteins were analyzed by SDS-PAGE and Western blot analysis.

## RESULTS

### Ubiquilin Inhibits APP Maturation and Sequesters APP in the Golgi Apparatus

To investigate the effects of ubiquilin overexpression on APP maturation and trafficking through the Golgi apparatus, we monitored the fate of APP and APP fragments in PC12 cells treated with cycloheximide (CHX), an inhibitor of protein synthesis. Trafficking through the Golgi is monitored by the transition of APP from an immature form, which migrates faster on SDS-PAGE, to a mature more slowly migrating form that has undergone *O*-linked glycosylation (Weidemann *et al.* 1989; Tomita *et al.* 1998). Addition of CHX resulted in a time-dependent loss of immature APP levels followed by a subsequent loss of mature levels at later time points, consistent with trafficking of newly synthesized APP through the Golgi, where it is glycosylated, to post-Golgi compartments where it is eventually degraded (**Fig. 3.1A**). When PC12 cells are transfected with ubiquilin, the mature form of APP is degraded much more rapidly, and the immature form of APP persists throughout the time course (**Fig. 3.1B**). These results suggest that ubiquilin may modulate APP maturation by sequestering immature APP.

To further explore the possibility that ubiquilin traps APP in early secretory compartments, subcellular fractionation experiments were performed to examine the vesicular compartmentalization of mature and immature APP in the presence and absence of ubiquilin. PC12 cells were treated with CHX or a vehicle-only control for two hours, homogenized, and subjected to differential centrifugation. Lysates were separated into 10,000 x*g* pellets (heavy membranes such as rough ER, plasma membrane, high density Golgi, and mitochondria) and 100,000 x*g*

pellets (light membranes such as smooth ER, low density Golgi, and small vesicles). In mock (GFP)-transfected cells, the 10,000 x*g* pellets contained immature and mature forms of endogenous APP when probed with an anti-Aβ antibody (**Fig. 3.2A**). An additional band with slightly faster mobility was observed (marked by an asterisk) in fractionation experiments but not observed in whole cell lysates, which may correspond to APP prior to *N*-linked glycosylation. In control cells, addition of CHX for two hours resulted in the loss of mature and immature APP in both fractions. In contrast, in PC12 cells overexpressing ubiquilin, the immature form of APP persists in the 10,000 x*g* pellet after CHX treatment, suggesting that ubiquilin stabilizes APP in high-density secretory compartments.

Sequestration of APP in the secretory pathway would prevent vesicular trafficking of APP to the cell surface. To explore this hypothesis, cell surface biotinylation experiments were performed to determine if ubiquilin overexpression was associated with decreased plasma membrane expression of APP. We observed a decrease in plasma membrane levels, and a corresponding increase in intracellular levels, of endogenous APP in cells overexpressing ubiquilin (**Fig. 3.2B**).

**Ubiquilin Delays the Proteolytic Processing of APP**

Real-time trafficking of APP was also investigated using live cell imaging of NGF-differentiated PC12 cells expressing an APP-GFP fusion construct consisting of a GFP moiety fused to the C-terminus of full-length APP (**Fig. 3.3A**). To visualize APP localization within the secretory pathway, we generated a stable cell line expressing the Golgi-resident enzyme N-acetylglucosaminyltransferase I (GlcNAc-TI) (Kumar *et al.* 1990) fused to the

mCherry red fluorescent protein and transiently expressed an APP-GFP fusion protein in this cell line (**Fig. 3.3B**). We found that ubiquilin expression greatly increased co-localization of APP-GFP with GlcNAc-TI, supporting the hypothesis that ubiquilin regulates exit of APP from the Golgi apparatus (**Fig. 3.3C**).

Live imaging of PC12 cells expressing APP-GFP was also used to simultaneously monitor APP trafficking and processing in real time. Proteolytic processing of the APP-GFP construct is predicted to liberate GFP (along with the AICD) from the membrane, resulting in diffuse cytosolic fluorescence (**Fig. 3.3A**). Prior to addition of CHX, a punctate pattern of GFP fluorescence was observed in cells expressing APP-GFP (**Fig. 3.4A**, upper left panel), corresponding to a predominantly vesicular localization of APP-GFP, as expected. After addition of CHX, this punctate fluorescence was gradually replaced by diffuse cytosolic fluorescence over time (**Fig. 3.4A**, upper right panel). In contrast, in cells co-expressing ubiquilin, the punctate pattern of fluorescence persisted for much longer after the addition of CHX (**Fig. 3.4A**, lower panels), suggesting that ubiquilin is able to delay the proteolytic processing of APP. The transition from punctate to diffuse fluorescence can be quantified as the punctate/diffuse index (Goldstein *et al.* 2000). As shown in **Fig. 3.4B**, APP-GFP has a reduction in the punctate/diffuse index over time after CHX addition, indicating APP processing and liberation of GFP-AICD, as expected. In contrast, co-expression of ubiquilin almost completely abrogates APP-GFP processing over this time course. Identical results were obtained with primary cortical neurons (**Fig. 3.4C**).

**Ubiquilin Inhibits the Proteasomal Degradation of APP**

Ubiquilin and other members of the UBL-UBA family of proteins have been shown to regulate the degradation of various substrates (Chen and Madura

2002; Gao *et al.* 2003; Kim *et al.* 2008b). Our results indicate that ubiquilin sequesters APP and delays secretase-dependent processing. To investigate whether ubiquilin regulates secretase-independent proteolysis of APP, the ability of overexpressed ubiquilin to modulate the half-life of endogenous APP was examined. To specifically examine lysosomal degradation of APP, cells were pre-treated with the proteasome inhibitor MG132 for 15 minutes prior to adding CHX at time zero. As shown in **Fig. 3.5A**, APP was rapidly degraded in the absence of MG132, and this degradation was partially attenuated by proteasome inhibition. Ubiquilin overexpression had no effect on lysosomal degradation. To examine proteasomal degradation, cells were pre-incubated with the lysosome inhibitor ammonium chloride ($NH_4Cl$). $NH_4Cl$ inhibited APP degradation to a greater extent than MG132, which is consistent with previous studies which show that a majority of APP is degraded in acidic compartments, including lysosomes (Caporaso *et al.* 1992; Knops *et al.* 1992). Expression of ubiquilin almost completely inhibited APP degradation in the presence of lysosome inhibitors (~80% remaining after two hours), indicating that ubiquilin is a potent inhibitor of proteasomal degradation of APP (**Fig. 3.5B**).

**Ubiquilin Increases the Ubiquitination of APP**

Ubiquitin is a small (~8.5 kDa) protein which is covalently attached to the ε-amine group of lysine residues on target proteins *via* an isopeptide bond. Polyubiquitin chains conjugated to lysine 29 ($Lys^{29}$) or $Lys^{48}$ of ubiquitin itself target a substrate protein for proteasomal degradation. We hypothesized that ubiquilin suppressed the proteasomal degradation of APP by inhibiting its ubiquitination. To investigate whether ubiquilin regulates the ubiquitination of APP, APP was immunoprecipitated from cells co-expressing hemagglutinin (HA)-

tagged ubiquitin and ubiquilin. Unexpectedly, our results indicate that expression of ubiquilin markedly increased ubiquitination of endogenous APP (**Fig. 3.5C**). This effect was much more pronounced in cells treated with MG132. Thus, ubiquilin is a potent inhibitor of APP proteasomal degradation (**Fig. 3.5B**) and also promotes ubiquitination of APP (**Fig. 3.5C**). These results suggest that ubiquitination of APP in this context does not serve as a degradation signal, but rather may function as a sorting signal.

## DISCUSSION

In the present study, we show that ubiquilin delays the maturation of APP through the secretory pathway by sequestering it in the Golgi apparatus and prevents its transport to the cell surface. We have also shown that ubiquilin is able to delay the proteolytic processing of APP and subsequent release of the AICD into the cytosol. Furthermore, we show that ubiquilin inhibits the proteasomal degradation of APP while increasing the ubiquitination of APP, suggesting that ubiquitin serves as a sorting signal, rather than a degradation signal, in this context.

Our results are in agreement with previous studies, which showed that reduced ubiquilin protein levels correlated with accelerated maturation of APP (Hiltunen *et al.* 2006). Our findings regarding the stabilization of APP in the presence of overexpressed ubiquilin are also in agreement with these previous studies, which showed the converse – ubiquilin knockdown was associated with decreased protein levels of full-length APP (Hiltunen *et al.* 2006). Ubiquilin and its homologues have also been shown to stabilize other proteasomal substrates, including the presenilins (Mah *et al.* 2000). However, in the case of the

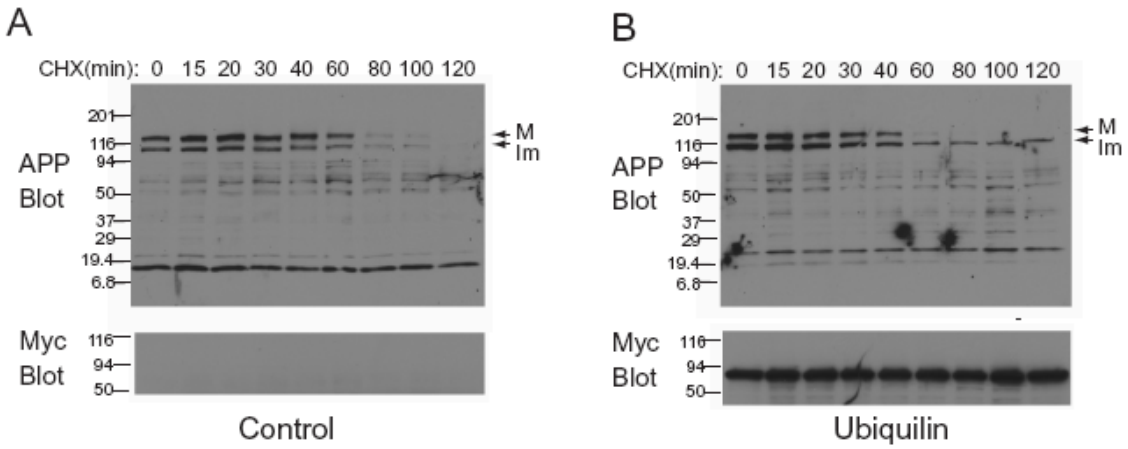
presenilins, ubiquilin leads to decreased ubiquitination (Massey *et al.* 2004). Our studies reveal that ubiquilin promotes ubiquitination of APP (**Fig. 3.5C**).

Ubiquitin can be conjugated as a single molecule, or additional ubiquitin molecules can be further conjugated to lysine residues on ubiquitin itself, forming a polyubiquitin chain. Polyubiquitin chains conjugated to $Lys^{29}$ or $Lys^{48}$ of ubiquitin serve as degradation signals, whereas monoubiquitin and $Lys^{63}$-linked polyubiquitin chains function in a signaling capacity (Hicke and Dunn 2003). Ubiquitination of proteins, particularly monoubiquitination, is a sufficient sorting signal in both the secretory and endocytic pathways. Ubiquilin and other UBA-containing proteins have been shown to bind both monoubiquitin and polyubiquitin chains (Zhang *et al.* 2008) and are known to control the cellular trafficking of ubiquitinated substrates (Bedford *et al.* 2001; N'Diaye *et al.* 2008; Saliba *et al.* 2008). It is possible that ubiquilin sequesters APP in the Golgi apparatus by binding to ubiquitinated APP and preventing recruitment of APP into vesicular trafficking complexes. The increase in APP ubiquitination that is observed in the presence of ubiquilin may be due to the promotion of monoubiquitination or K63-linked polyubiquitination of APP. It is also possible that ubiquilin binds to monoubiquitin or short K48-linked chains and prevents chain elongation, deubiquitination, and proteasomal degradation. This ubiquitin "capping" mechanism has been described for other UBL-UBA proteins (Ortolan *et al.* 2000; Hwang *et al.* 2005) and would explain how ubiquilin leads to decreased proteasomal targeting while simultaneously increasing the overall accumulation of ubiquitinated APP in the cell.

The regulatory role of ubiquilin in the coordination of APP trafficking and degradation may constitute a critical mechanism for ensuring proper protein

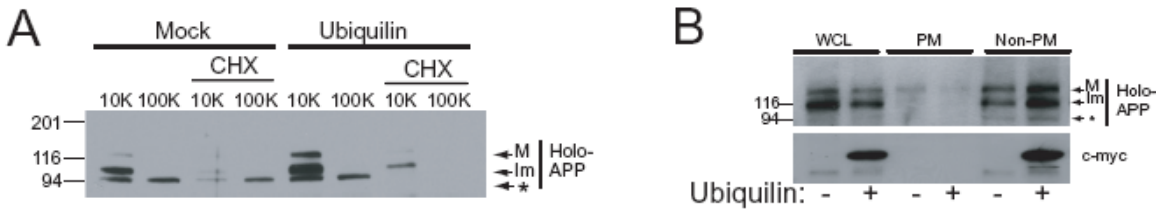
sorting and limiting contact with specific intracellular compartments. Our results suggest that ubiquilin is a component of a trafficking “checkpoint” within the secretory pathway that prevents excessive amyloidogenic processing of APP by sequestering it from secretase enzymes. More research is needed to elucidate the mechanisms by which ubiquilin regulates APP trafficking and ubiquitination and how these processes are coordinated with one another.

Figure 3.1: Ubiquilin inhibits APP maturation.



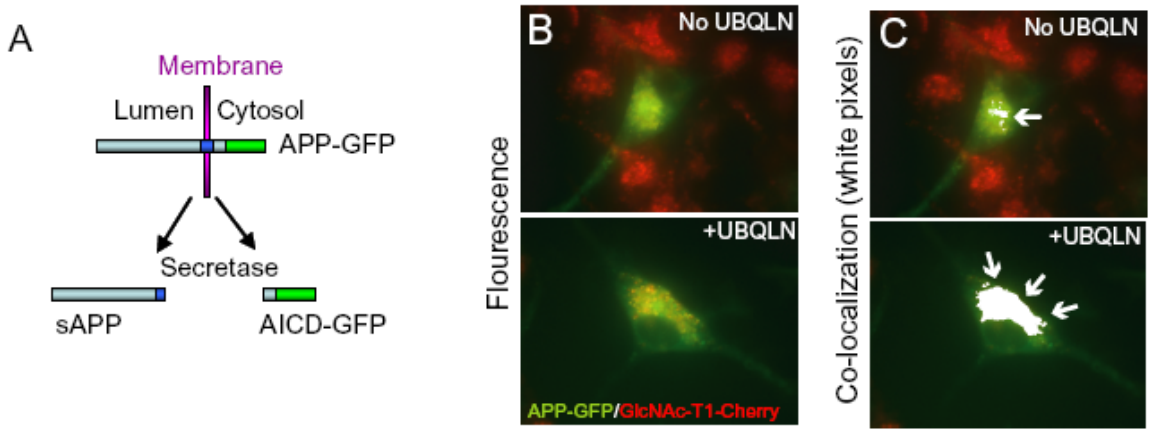

(A) Fate of mature (M) and immature (Im) levels of endogenous APP in PC12 cells after cycloheximide (CHX) addition. Time is indicated in minutes. (B) Fate of APP in PC12 cells transfected with ubiquilin as in (A). Ubiquilin expression was probed by anti-myc staining.

Figure 3.2: Ubiquilin sequesters APP in the Golgi apparatus and inhibits transport to the cell surface.



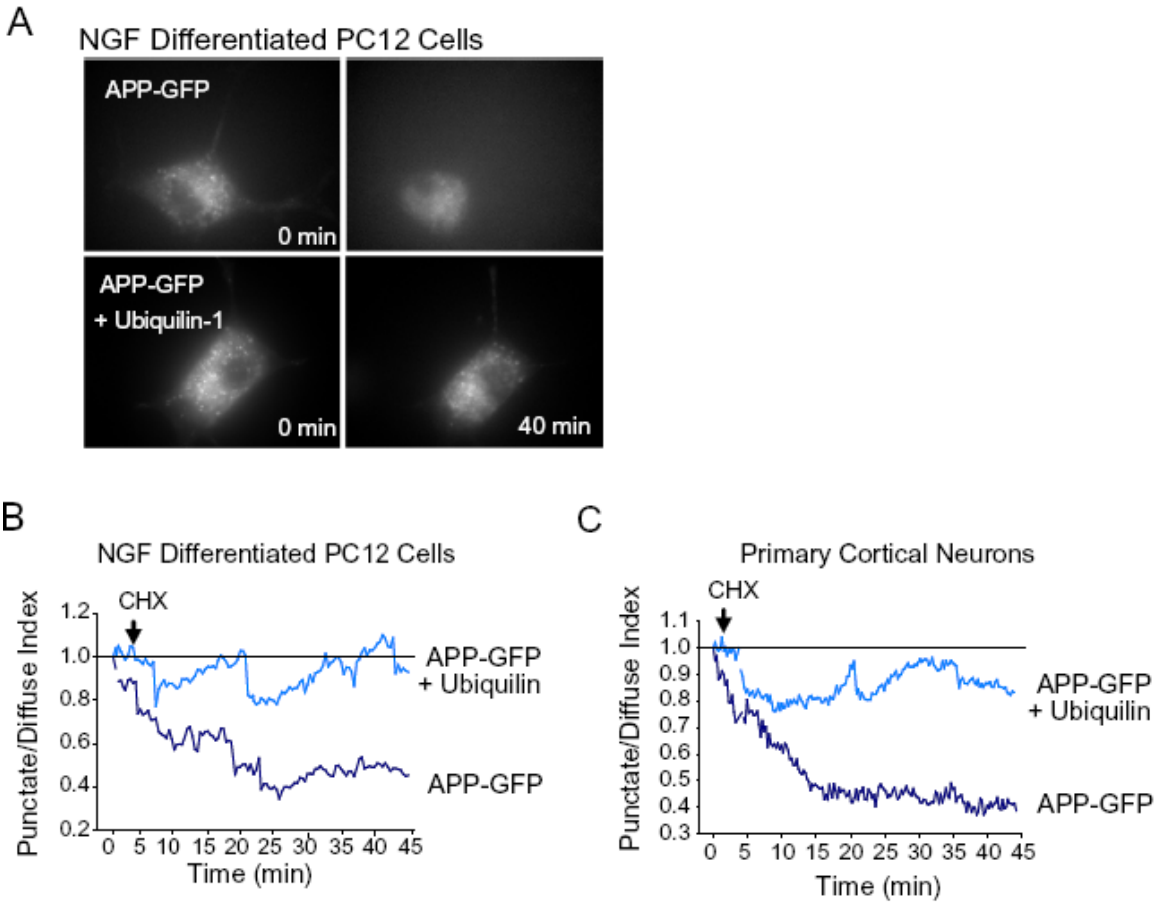

(A) Subcellular fractionation and endogenous APP distribution in GFP expressing PC12 cells (mock) or ubiquilin expressing cells before and after 2 h of CHX treatment. APP fragments are indicated. (*) indicates an unidentified reactive band which has faster mobility than Im, possibly full-length APP which has not undergone *N*-linked glycosylation in the ER. 10K, 10,000 x*g* pellet; 100K, 100,000 x*g* pellet. (B) Cell surface biotinylation of endogenous APP with or without ubiquilin expression. PM, plasma membrane; WCL, whole cell lysate.

Figure 3.3: APP-GFP co-localizes with a Golgi marker in PC12 cells overexpressing ubiquilin.



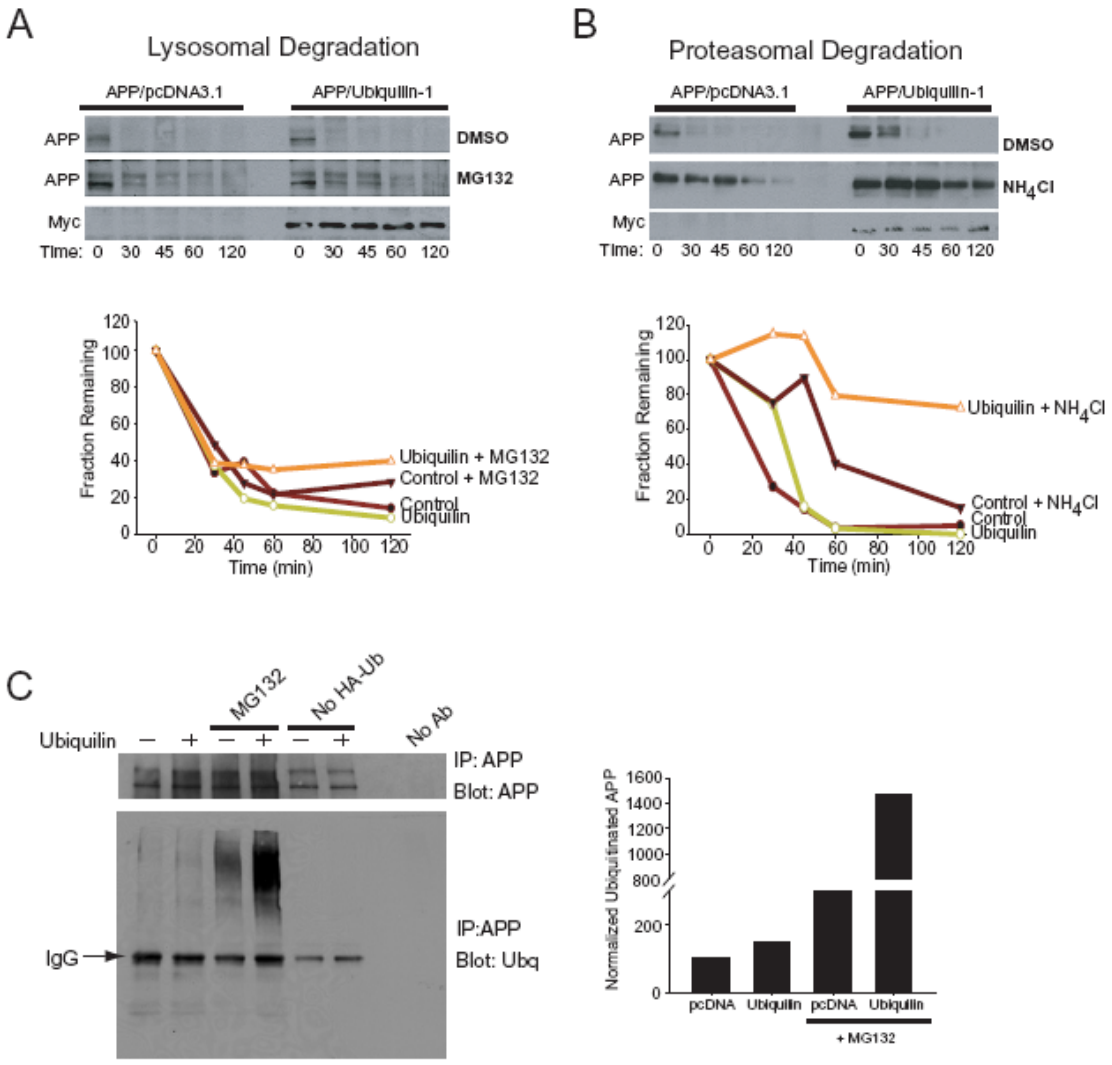

(A) Schematic representation of the membrane topology of APP-GFP. Secretase processing is predicted to release AICD-GFP from the membrane into the bulk cytosol (resulting in diffuse fluorescence). (B) APP-GFP (green) and GlcNAc-TI (red) expression in PC12 cells with or without ubiquilin. (C) Colocalized pixels (white). Note the greatly increased co-localization of APP-GFP with GlcNAc-TI in ubiquilin expressing cells. Co-localization was quantified using the ImageJ suite, with a white pixel being inserted in pixels containing fluorescence in both channels.

Figure 3.4: APP-GFP processing in PC12 cells and primary cortical neurons.




(A) Raw images of nerve-growth factor (NGF) differentiated PC12 cells expressing APP-GFP or APP-GFP plus ubiquilin. Images were taken prior to CHX addition (0 min) and 40 min after addition. (B) Quantification of the change in fluorescence from punctate to diffuse over time in single PC12 cells. The punctate/diffuse index is essentially the normalized standard deviation of GFP fluorescence in an entire cell. A reduction in this index indicates more homogeneous (i.e., diffuse) distribution of fluorescence. Solid line indicates no change. (C) Punctate/diffuse index of APP-GFP in primary rat cortical neurons as in (B).

Figure 3.5: Ubiquilin inhibits APP degradation and increases APP ubiquitination.




(A) Time course of degradation of APP after cycloheximide addition in the absence or presence of proteasomal inhibitor MG132 (thus indicative of lysosomal degradation). Quantification of band intensities is below the blots. (B) Time course of degradation of APP after cycloheximide addition in the absence or presence of lysosomal inhibitor $NH_4Cl$ (thus indicative of proteasomal degradation). Quantification of band intensities is below the blots. Chloroquine gave similar results. (C) Ubiquitination of endogenous APP in the presence or absence of MG132. APP was immunoprecipitated and the blots were probed with

an anti-ubiquitin (Ubq) antibody. In the first four lanes the cells were expressing HA-tagged ubiquitin (HA-Ub). Quantification of ubiquitinated APP levels in the presence of HA-Ub is given below the blots.

# Chapter 4: Ubiquilin is a Molecular Chaperone for the Amyloid Precursor Protein


## ABSTRACT

Alzheimer's disease (AD) is associated with extracellular deposition of proteolytic fragments of amyloid precursor protein (APP). Although mutations in APP and proteases that mediate its processing are known to result in familial, early-onset forms of AD, the mechanisms underlying the more common sporadic, yet genetically complex, forms of the disease are still unclear. Four single nucleotide polymorphisms (SNPs) within the *UBQLN1* gene have been shown to be genetically associated with AD, implicating its gene product in the pathogenesis of late-onset AD. However, genetic linkage between ubiquilin and AD has not been confirmed in studies examining different populations. Here we show that regardless of genotype, ubiquilin protein levels are significantly decreased in late-onset AD patient brains, suggesting that diminished ubiquilin function may be a common denominator in AD progression. Our interrogation of putative ubiquilin activities based on sequence similarities to proteins involved in cellular quality control showed that ubiquilin can be biochemically defined as a *bona fide* molecular chaperone and that this activity is capable of preventing the aggregation of APP both *in vitro* and in live neurons. Furthermore, we show that reduced activity of ubiquilin results in augmented production of pathogenic APP fragments as well as increased neuronal death. Our results support the notion that ubiquilin chaperone activity is necessary to regulate the production of APP and its fragments, and that diminished ubiquilin levels may contribute to AD pathogenesis.

## INTRODUCTION

Alzheimer's disease (AD) is associated with extracellular deposition and aggregation of proteolytic fragments of amyloid precursor protein (APP). In normal physiology, full-length APP is cleaved by a series of enzymes, called secretases. Sequential cleavage of APP by β-secretase followed by γ-secretase generates a soluble extracellular fragment, the amyloidogenic Aβ peptide, and the APP intracellular domain (AICD) (De Strooper and Annaert 2000). There are multiple γ-secretase cleavage sites within APP, leading to the production of various Aβ fragments of different lengths (Sisodia and St George-Hyslop 2002). The two major Aβ peptides differ in length by two residues, and the longer $A\beta_{42}$ peptide is more amyloidogenic than the $A\beta_{40}$ species (Findeis 2007). Distinct mutations in the genes for APP and the enzymes responsible for its cleavage are associated with increased amyloidogenesis and have been linked to early-onset familial forms of the disease (Tanzi and Bertram 2005). The pathogenesis of the more common sporadic late-onset form of the disease, however, is still unclear.

Recently, genetic studies have linked the *UBQLN1* gene (which encodes the ubiquilin-1 protein) to late-onset AD (Bertram *et al.* 2005; Kamboh *et al.* 2006). These studies have suggested that alterations in the levels of full length or splice variants of ubiquilin-1, due to the presence of particular SNPs in its promoter or intron regions, respectively, may be related to the development of late-onset AD (Bertram *et al.* 2005; Slifer *et al.* 2005; Kamboh *et al.* 2006). However, similar analyses with different populations have found weak (Golan *et al.* 2008) or no (Bensemain *et al.* 2006; Brouwers *et al.* 2006; Slifer *et al.* 2006; Smemo *et al.* 2006; Arias-Vasquez *et al.* 2007; Bertram *et al.* 2007; Chuo *et al.* 2010) associations. These discrepancies may be explained, at least partially, by

the presence of as yet uncharacterized SNPs throughout and beyond the ubiquilin-1 locus that modulate its production (and that of putative functional variants).

Ubiquilin-1 is a ~63 kDa multi-domain protein that is a member of the UBL-UBA family of proteins, which contain a ubiquitin-like (UBL) domain at the N-terminus and a ubiquitin-associated (UBA) domain at the C-terminus. The UBL domain has structural homology to ubiquitin and has been shown to bind the S5a subunit of the 19S proteasome cap (Buchberger 2002; Walters *et al.* 2002). The UBA domain has been shown to bind directly to ubiquitin (Wilkinson *et al.* 2001; Raasi *et al.* 2005; Zhang *et al.* 2008). One functional model suggests that UBL-UBA proteins promote protein degradation by serving as shuttles that link ubiquitinated substrates to the proteasome (Buchberger 2002). However, ubiquilin and its homologues have also been shown to stabilize a number of proteasome substrates (Kleijnen *et al.* 2000; Mah *et al.* 2000; Feng *et al.* 2004; Massey *et al.* 2004; Hwang *et al.* 2005), suggesting that the functions of UBL-UBA proteins may be subject to regulatory control. The central region of ubiquilin contains two regions of similarity to the co-chaperone Sti-1 (also known as Hop). STI domains mediate hydrophobic protein-protein interactions and may possess intrinsic chaperone activity (Kamionka and Feigon 2004; Zhao *et al.* 2006).

The molecular mechanism by which the ubiquilin protein contributes to the pathogenesis of late-onset AD is still unclear. Ubiquilin has previously been shown to interact with presenilin proteins (Mah *et al.* 2000), which constitute the catalytic core of the γ-secretase (Wolfe *et al.* 1999; De Strooper 2003). One group showed that knockdown of ubiquilin by RNA interference (RNAi) accelerated APP maturation and increased production of Aβ in cultured cells,

independently of any changes in β- or γ-secretase levels (Hiltunen *et al.* 2006). This suggests that a relationship exists between ubiquilin and APP that is independent of PS proteins. In this same study, researchers used FRET to show that APP and ubiquilin come within close proximity in live cells; however, direct binding of the two proteins could not be confirmed (Hiltunen *et al.* 2006).

Here we show that ubiquilin protein levels in patients are negatively correlated with Braak stage, an index of neurofibrillary tangle pathology. Ubiquilin was found to possess intrinsic molecular chaperone activity and protect against the aggregation of AICD *in vitro* and APP *in vivo*. Furthermore, decreased ubiquilin levels resulted in increased pathogenic processing of APP and subsequent cell death. Our results are consistent with the conclusion that ubiquilin chaperone activity regulates APP biosynthesis and processing, and that diminished ubiquilin levels may contribute to late-onset AD pathogenesis.

## MATERIALS AND METHODS

### AD Brain Samples

Human frontal cortex samples were obtained from the Joseph and Kathleen Bryan Alzheimer's Disease Research Center (Duke University Medical Center) (Hulette *et al.* 1997), genotyped by the Molecular Genomics Core at UTMB, and analyzed by SDS-PAGE and Western blotting. Ubiquilin protein level was normalized to α/β-tubulin.

### Cell Lines

Human HeLa cells, rat PC12 cells, and rat primary cortical neurons were cultured and prepared as previously described (Chapter 3).

**Antibodies**

For Western blots, immunoprecipitation experiments, filter trap experiments, and APP immunofluorescence, a polyclonal antibody directed against amino acids 1-10 of the Aβ peptide was used to detect APP (Millipore). All experiments were confirmed with anti-APP 6E10 monoclonal antibody. For dot blot experiments, a polyclonal antibody directed against the C-terminal of human $APP_{695}$ (a peptide fragment generated by ε-cleavage of C83 or C99) was used to detect purified AICD (Covance).

A monoclonal anti-ubiquilin antibody (Invitrogen) directed against a recombinant protein derived from an internal region of the human ubiquilin protein was used to detect endogenous and overexpressed ubiquilin. A monoclonal anti-c-myc antibody (Roche) was also used to verify overexpression of myc-tagged ubiquilin in cellular aggregation, Aβ detection, and cell death experiments.

**Yeast Two-hybrid**

The yeast two-hybrid screen of a rat cDNA library with human ubiquilin-1 as bait was performed with the Matchmaker GAL4 Two-Hybrid system (Clontech). Human ubiquilin cDNA was cloned into the pGBKT7 yeast expression vector to express a GAL4 DNA-binding domain fusion protein. Prey proteins from a rat brain cDNA library were expressed as GAL4 activation domain fusions. Expression was verified by Western blotting with antibodies corresponding to epitope tags encoded in the vectors. The screening methodology and library have been described elsewhere (Boehning *et al.* 2003). Positive clones grew on minimal SD agar lacking adenine, histidine, leucine, and tryptophan, and had β-gal activity.

**Recombinant Proteins**

Recombinant polyhistidine (His)-tagged ubiquilin proteins were generated by amplifying fragments of human ubiquilin cDNA (kindly provided by Dr. Mervyn J. Monteiro), incorporating a 5' *Sal*I restriction site and a 3' *Xba*I restriction site, and cloning in frame to the *Sal*I-*Xba*I sites of the pProEx-HT bacterial expression vector (Invitrogen). PCR primers were as follows: 5'-AAA GTC GAC AAT GGC CGA GAG TGG TGA AAG C-3' (forward); 5'-CGC TCC TGC TCT AGA CTA TGA TGG CTG GGA-3' (full-length, reverse); and 5'-GCT CTA GAC TAA GAC AAA AGT TGT CGC TGC ATC TGA CT-3' (UBL, reverse). The UBL construct consists of residues 1-181 of human ubiquilin. Proteins were purified using TALON cobalt-based metal affinity resin (Clontech). Purified protein was stored in PBS with 10% glycerol at -80°C, unless otherwise noted.

Recombinant glutathione *S*-transferase (GST)-tagged AICD was generated by amplifying the AICD sequence (amino acid residues 649-695; $APP_{695}$ numbering) from full-length $APP_{695}$ (kindly provided by Dr. Hui Zheng), incorporating a 5' *Bam*HI restriction site and a 3' *Eco*RI restriction site, and cloning into the *Bam*HI-*Eco*RI sites of the pGEX-4T-1 vector (GE Life Sciences), which encodes a thrombin recognition site for removal of the tag from the protein product. PCR primers were 5'-CGC GGA TCC AAG AAA CAG TAC ACA TCC ATT-3' and 5'-CGG AAT TCC CTA GTT CTG CAT CTG CTC AAA GAA-3'. Pure AICD for *in vitro* aggregation experiments was generated by cleavage of GST with thrombin protease. Thrombin was added to GST-AICD at an approximate final concentration of 1 NIH unit per 70 µg protein, and the reaction was incubated overnight at 37°C to induce cleavage. GST was removed by sequential extractions with glutathione sepharose beads. Complete removal of GST was

verified by SDS-PAGE followed by Coomassie staining. A dot blot with an AICD-specific antibody was used to verify presence of pure AICD in the final eluate. For every 70 µg of GST-AICD in the reaction, approximately 3 µg of pure AICD was recovered. Pure AICD was stored in PBS with 10% glycerol at -80°C in single-use aliquots until use, unless otherwise noted. Although the AICD aliquots used for experiments contained low levels of thrombin, we determined with control experiments that the thrombin did not contribute any spectral artifacts or have any effects on aggregation or protection of AICD.

**Expression Constructs**

The APP-GFP construct was described previously (Chapter 3). For the UBL domain expression constructs, the UBL domain sequence was cut from the previously generated pProEx-HT-UBL bacterial expression construct with *Sal*I and *Xho*I, which generated a fragment containing the UBL sequence with a short C-terminal stretch of the pProEx-HT multiple cloning site. This fragment was subsequently cloned into the *Sal*I-*Xho*I sites of the pCMV-Myc mammalian expression vector (Clontech).

**RNA Interference**

Stealth-modified double-stranded RNA against the human ubiquilin-1 gene (sense sequences, 5'-ACA AAC GUU GGA ACU UGC CAG GAA U-3'; 5'-GGA ACC AAU GCU GAG UGC UGC ACA A-3'; 5'-CCU UGU UAC AGA UUC AGC AGG GUU U-3') and the rat ubiquilin-1 gene (sense sequences, 5'-GCC GCA AGA UAA UUC AGC UCA GCA A-3'; 5'-CCC UUU GUG CAG AGC AUG CUC UCA A-3'; 5'-GAG CCU UGA GCA ACC UAG AAA GUA U-3') was obtained from Invitrogen. Transfection with Lipofectamine 2000 (Invitrogen) of various doses

and combinations of the RNA duplexes was used to determine that a combination all three duplexes at a final concentration of 100 pmol per milliliter of cell culture media gave maximal knockdown. Universal control oligomers (medium GC content) were also obtained from Invitrogen.

**Citrate Synthase Inactivation**

Citrate synthase (CS) inactivation experiments were carried out by plotting initial velocity measurements of mixtures of the enzyme exposed to 43°C in the presence and absence of ubiquilin, essentially as described (Buchner *et al.* 1998). Purified CS from porcine heart was purchased from Sigma. The original ammonium sulfate storage solution was exchanged for Tris-EDTA (TE) buffer (50 mM Tris, 2 mM EDTA, pH 8.0) *via* dialysis, and protein concentration was determined using absorbance at 280 nm. Single-use aliquots were snap-frozen in liquid nitrogen and stored at -80°C until use. The CS activity assay is based on the reaction in which CS catalyzes the condensation of oxaloacetic acid (OAA) and acetyl-CoA to citrate and coenzyme A. When Ellman's reagent (dithio-1,4-nitrobenzoic acid, or DTNB) is included in the reaction mixture, an observable increase in absorption at 412 nm occurs as DTNB is reduced by the reaction product coenzyme A. The linear slope of the initial increase in absorption represents the CS activity. For each activity assay sample, the reaction mixture consists of 186 µl of TE buffer, 2 µl of 10 mM OAA in 50 mM Tris, 6 µl of 5 mM acetyl-CoA in TE buffer, and 4 µl of the inactivation reaction (enzyme ± putative chaperone or control protein). Immediately prior to measurement, 2 µl of 10 mM DTNB in TE buffer is added. The inactivation reaction is made up in 40 mM HEPES-KOH, pH 7.5 and contains 0.15 µM CS in a total volume of 50 µl (final concentration of CS in each sample is 3 nM). Full-length ubiquilin was purified

immediately prior to inactivation experiments to avoid freeze-thaw and cryopreservation artifacts. Before determining inactivation kinetics, the background slope was determined by measuring the increase in absorption in the absence of enzyme. This background slope was then subtracted from subsequent measurements to get the true enzymatic activity. Initial maximal enzymatic activity (zero time point) for each reaction was determined at 25°C, then the reaction was transferred to a 43°C shaking thermomixer to induce inactivation. Absorption measurements were taken at 0, 5, 10, 15, 20, 30, and 60 minutes. The slopes for each time point were then plotted to determine the inactivation kinetics.

### *In vitro* Aggregation of Purified AICD

For light scattering and sedimentation experiments, AICD was freshly prepared to avoid freeze-thaw and cryopreservation artifacts. For light scattering experiments, AICD was diluted to a final concentration of 1 μM in PBS, and the final concentration of ubiquilin was 0.5 μM. Samples were maintained at a constant temperature of 43°C to induce thermal aggregation. Absorbance readings were taken every 10 minutes.

For sedimentation experiments, freshly cleaved and purified AICD and purified ubiquilin (full-length and UBL) were incubated in at a final concentration of 145 nM in 20 mM Tris (pH 8.0), 150 mM NaCl at 37°C to induce aggregation. Aliquots of each aggregation reaction were removed and snap-frozen in liquid nitrogen at 0, 24, and 48 hour time points for later processing. Each frozen sample was thawed and half of each sample was centrifuged at 21,000 x*g* to sediment aggregated material. Pellets were then resuspended in 20 mM Tris (pH 8.0), 150 mM NaCl, and both total and pellet fractions were spotted onto a

nitrocellulose membrane. Membranes were blotted with an AICD-specific antibody.

**Atomic Force Microscopy (AFM)**

For AFM sample preparation, frozen single-use aliquots of AICD were thawed and incubated at a final concentration of 17.3 μM in a total volume of 15 μl with equimolar concentrations of ubiquilin proteins where applicable. Samples were incubated at 43°C with shaking for 2 days to induce thermal aggregation. Each sample was diluted 1:20 in deionized $H_2O$ prior to imaging. For imaging, 2 μl of the diluted reaction was placed onto a freshly cleaved mica surface and allowed to dry overnight. Samples were imaged in air with a 'home-built' AFM using a Nanotec Scanning Probe Microscope Control System (Dulcinea, Nanotec Electronica) operating in tapping mode, using MSNL cantilevers (0.1N/m, Veeco). Typical tapping amplitudes at imaging were 10-20 nm at the resonance frequency of the cantilevers in air (30-50 kHz). Images were acquired at rates of 60 seconds per image (256 pixels, 2000 nm). Image processing was performed with WSxM software (www.nanotec.es) (Horcas *et al.* 2007). Standard image processing consisted of plane subtraction and flattening.

**Cellular Aggregation Assays**

For filter trap assays, PC12 cell pellets were resuspended in a Triton-based lysis buffer, and total protein concentration was determined *via* the Bradford method. Samples were briefly sonicated to shear DNA and centrifuged at 16,000 x*g* for 30 minutes to remove cellular debris. Supernatants were spotted onto a cellulose acetate membrane (0.22 μm pore size) in a dot blot apparatus attached to a vacuum source. Gentle vacuum was applied to pull samples

through the membrane, trapping protein aggregates larger than 0.22 μm in the cellulose acetate. The membrane was then blotted with an APP antibody to detect the presence of Aβ-containing aggregates.

For quantification of aggregates in cells, APP-GFP was overexpressed in differentiated PC12 cells. Cells were fixed on glass coverslips in 4% paraformaldehyde for 20 minutes. APP-GFP could be visualized directly in fixed cells. For immunofluorescence of $APP_{695}$, cells were treated with formic acid prior to addition of the primary antibody to recover the epitope. An Alexa Fluor 350-conjugated secondary antibody (Invitrogen) was used. Quantification of aggregates was performed in a double-blind manner, where slides were prepared and cells were counted by two separate experimenters. Fluorescence expression patterns were determined manually by an experimenter who was blinded to the experimental condition.

**Enzyme-linked Immunosorbent Assay (ELISA)**

Secreted $A\beta_{40}$ and $A\beta_{42}$ levels were determined using colorimetric peptide-specific ELISA kits (Invitrogen, CA, USA; Cat. Nos. KHB3481 and KHB3544). Conditioned media from HeLa cells over-expressing the construct of interest was collected 48 hours after transfection and supplemented with phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 1 mM to prevent proteolysis of the Aβ peptide. The conditioned media was cleared at 1000 x*g* for 5 minutes at 4°C, and the supernatant was used for ELISA without dilution and according to the manufacturer's instructions.

**Cell Death**

Cell death was determined as described (Wozniak *et al.* 2006).

### Statistical Analyses

Cell counts of APP-GFP were statistically analyzed using Fisher's Exact test using a 2x2 contingency table comparing multiple aggregates *versus* aggregate free cells or single aggregates *versus* aggregate free cells. Statistical significance in all other figures was determined by using unpaired two-tailed *t* tests. All comparisons were considered significant if $p<0.05$ with the exception of APP-GFP cell counts, in which *p* values less than 0.01 were determined to be significant. Actual *p* values are provided in the figures.

## RESULTS

### Ubiquilin protein levels are reduced in AD cortex

In order to determine whether ubiquilin protein levels are altered in AD patients, we genotyped and examined post-mortem frontal cortex samples from 20 late onset AD patients (Braak stages III-VI) and 20 gender- and age-matched individuals with no cognitive impairment (Braak stages I-II) (**Fig. 4.1A**; **Table 4.1**). We found that the accumulation of both ubiquilin splice variants detected by Western blotting correlated inversely with the Braak stage classification of the sample (**Fig. 4.1A,B**). Brain samples classified as Braak stages II-VI show marked reduction in total ubiquilin protein levels compared with stage I (**Fig. 4.1B**). Remarkably, we found no correlation between any of the genotyped ubiquilin-1 SNPs with ubiquilin protein levels or with Braak stage. However, consistent with previous findings (Strittmatter and Roses 1996), the ε4 allele of the apolipoprotein E (*APOE*) gene displayed strong association with occurrence of AD in our patient cohort (**Fig. 4.1A**; **Table 4.1**). The marked reduction in ubiquilin protein in these samples is not likely to simply result from brain atrophy

characteristic of late Braak stages, as the levels of the cytoskeletal protein tubulin are essentially unaltered in all samples examined. Moreover, since there is no significant neuropathology in Braak stage II (Braak and Braak 1991), yet ubiquilin levels are already drastically diminished by that stage, our results suggest that reduction in ubiquilin protein levels precede the neuropathological changes and associated neuronal death in the cortices of AD patients, regardless of the particular constellation of SNPs present throughout the ubiquilin-1 locus (Braak and Braak 1991).

**Ubiquilin interacts with APP**

In order to explore a possible mechanism for the role of ubiquilin in AD, we performed a yeast two-hybrid screen of a rat cDNA library with the human full length ubiquilin-1 protein to identify putative interaction partners. Ten percent of the clones isolated in the screen corresponded to the AICD or the intracellular domain of its homolog APP-like protein (APLP) (**Fig. 4.2A**). To determine if this interaction was relevant in a mammalian system, we transfected HeLa cells with ubiquilin singly and in combination with APP and performed co-precipitation experiments. Ubiquilin could be co-precipitated with both endogenous and overexpressed APP (**Fig. 4.2B**), but only when cells were treated prior to lysis with Lomant's reagent (Dithio-bis[succinimidyl propionate]; DSP), a cell-permeant crosslinking compound. This finding suggests that complex formation between ubiquilin and APP is transient. To determine whether purified recombinant ubiquilin was capable of interacting with native, endogenous APP, we performed immunoprecipitations from rat brain lysates. Marginal binding of ubiquilin to immobilized APP was detected at 4°C and this association was found to be considerably strengthened by incubation at 37°C, which suggests that it is

mediated by hydrophobic interactions (**Fig. 4.2C**). Both the transient nature and the hydrophobic character of the interaction between ubiquilin and APP are compatible with a chaperone-client relationship. This notion is supported by the presence of the two STI domains within the ubiquilin sequence, which are known to possess chaperone-like properties (Zhao *et al.* 2006).

**Ubiquilin displays properties of a molecular chaperone**

To investigate whether ubiquilin possesses chaperone activity, we tested its capacity to protect the model client protein citrate synthase (CS) against thermal inactivation, a biochemical hallmark of molecular chaperones (Buchner *et al.* 1998). Upon exposure to elevated temperatures (43°C), CS is rapidly inactivated *via* the production of reversible early unfolding intermediates that subsequently aggregate. Productive binding and release of these intermediates by a molecular chaperone increases their kinetic partition towards productive refolding, thereby effectively stabilizing the native enzyme (Jakob *et al.* 1995). We found that mixtures of CS containing ubiquilin were significantly stabilized upon exposure to elevated temperature compared to those without it: the $t_{1/2}$ of inactivation increased from ~3 min to ~19 min (**Fig. 4.3A**). Moreover, ubiquilin provided considerably greater protection against thermal denaturation than the well-characterized molecular chaperone Hsp90 at equivalent stoichiometries ($t_{1/2}$ ~6 min). Thus, ubiquilin can function as a *bona fide* molecular chaperone for this model client.

**Ubiquilin is a chaperone for the AICD *in vitro***

We next investigated whether ubiquilin exerts chaperone activity on APP, a biologically relevant client. Having found that ubiquilin and the AICD are

capable of forming complexes (**Fig. 4.2**), we tested whether ubiquilin was capable of preventing the aggregation of AICD. We found that aggregation of purified AICD could be induced by elevated temperature, a process that can be followed kinetically by monitoring light scattering at 420 nm (**Fig. 4.3B**). In contrast to AICD alone, mixtures containing ubiquilin and AICD displayed no increase in light scattering over the entire duration of the experiment, indicating that ubiquilin is capable of significantly suppressing AICD aggregation under these conditions. In order to ascertain that AICD formed *bona fide* aggregates, we examined its solubility properties by dot-blot analyses and its structural characteristics by atomic force microscopy (AFM), which can image aggregates with nanometer resolution and does not require staining or fixation (Gosal *et al.* 2006). Mixtures containing AICD and ubiquilin were found to be virtually free of insoluble AICD, in contrast to mixtures containing only its UBL domain (**Fig. 4.3C**). When imaged by atomic force microscopy, total reaction mixtures containing AICD and ubiquilin were found to contain no detectable aggregates, whereas large (~50 nm) amorphous aggregates were prominently found in reactions incubated without ubiquilin (**Fig. 4.3D**). Thus, it appears that ubiquilin is capable of exerting chaperone activity on the AICD, preventing its aggregation under various *in vitro* conditions, and that this activity does not reside on its UBL domain.

**Ubiquilin is a chaperone for APP in living cells**

We next wished to determine whether ubiquilin functions as a molecular chaperone for APP in living cells. We began by studying the behavior of the neuronal splice variant of APP ($APP_{695}$) upon overexpression in nerve-growth factor differentiated PC12 cells. We observed three distinct distribution patterns:

(1) a diffuse or vesicular pattern, consistent with the localization of APP to secretory compartments (~65% of the cells); (2) a single large aggregate, which may correspond to an aggresome due to its size and perinuclear localization (Johnston *et al.* 1998) (~20% of the cells); or (3) multiple aggregates present throughout the soma and neurites (~15% of the cells) (**Fig. 4.4A**). Overexpression of a fusion protein of APP with a C-terminal GFP moiety resulted essentially in the same proportions and patterns of distribution (**Fig. 4.4A**), and thus was utilized for subsequent experiments. When ubiquilin was co-overexpressed in these cells, a significant reduction in the proportion of cells containing single (~8%) and multiple (~12%) aggregates was consistently observed, with a corresponding increase in the proportion of cells with no aggregates (~80%) (**Fig. 4.4B**). We next wished to ascertain whether the ability of ubiquilin to prevent APP aggregation was preserved in intact neurons and thus performed similar overexpression experiments in rat primary cortical neurons (**Fig. 4.4C**). We observed essentially the same trend: the number of cells without any aggregates increased significantly when ubiquilin was co-overexpressed compared to cells overexpressing APP-GFP alone (~69% *vs.* ~39%, respectively), with the corresponding reduction in the number of cells containing single (11% *vs.* 16%) and multiple (20% *vs.* 45%) aggregates. If the endogenous steady-state levels of ubiquilin play a role in modulating the aggregation properties of APP, then RNAi-mediated reduction of ubiquilin protein levels should result in different patterns of APP localization. When we performed such an experiment in PC12 cells, we observed that indeed, the fraction of cells with multiple aggregates was increased when compared to cells treated with control RNA (15.7% *vs.* 8.3%, respectively) (**Fig. 4.4D**). Interestingly, a reduction of ~5%

in the fraction of cells containing single aggregates was noted upon ubiquilin knockdown, which may be related to an impairment of aggresome formation under these conditions (Regan-Klapisz *et al.* 2005). In order to confirm that the material described as aggregated in the above experiments actually corresponded to insoluble APP, we performed filter-trap assays (Bailey *et al.* 2002) (**Fig. 4.4E**). Overexpressed APP was preferentially retained on the filter compared to endogenous APP and, consistent with our microscopy results, overexpression of ubiquilin resulted in a decrease in the accumulation of insoluble overexpressed and endogenous APP (**Fig. 4.4E**). Taken together, our results suggest that ubiquilin functions as a molecular chaperone to prevent the aggregation of APP in neuronal cells.

**Ubiquilin protects against Aβ toxicity**

Since proteolytic products of APP, particularly Aβ peptides, play a critical role in AD pathogenesis (Hardy and Selkoe 2002), and ubiquilin appears to function in its cellular quality control, we sought to determine whether alterations in ubiquilin levels led to effects on the amyloidogenic processing of APP. Levels of secreted $A\beta_{42}$ and $A\beta_{40}$ were measured in the media of HeLa cells overexpressing $APP_{695}$. Since elevated production of $A\beta_{42}$, which is more aggregation prone than the $A\beta_{40}$ species, has been shown to correlate with disease states (Findeis 2007), we determined the fraction of $A\beta_{42}$ to $A\beta_{40}$ in our experiments. Overexpression of $APP_{695}$ in HeLa cells markedly elevated the ratio of secreted $A\beta_{42}/A\beta_{40}$ compared to non-expressing cells (**Fig. 4.5A**). In contrast, co-overexpression of APP with ubiquilin resulted in a significant decrease in the ratio of secreted $A\beta_{42}/A\beta_{40}$, while co-overexpression of APP with the UBL domain had no effect (**Fig. 4.5A**). This capacity of ubiquilin to reduce the production of

pathogenic $A\beta_{42}$ peptides prompted us to investigate whether ubiquilin may protect against APP-induced toxicity and death (**Fig. 4.5B**). We found that ~25% of HeLa cells die within 24 hours upon overexpression of APP. Significantly, cells co-overexpressing APP and ubiquilin showed considerably reduced cell death (~15%). If our notion that ubiquilin is able to protect cells against APP-induced toxicity, then RNAi-mediated knockdown of endogenous ubiquilin should render cells more susceptible to APP-induced cell death. When we performed such an experiment, we observed that indeed the cell death rates were markedly increased (~60%) (**Fig. 4.5B**). Thus, the strongly reduced ubiquilin protein levels detected in the brains of AD patients (**Fig. 4.1A,B**) may render them particularly susceptible to APP-related toxicity.

## DISCUSSION

In the present study, we have shown that ubiquilin directly interacts with the intracellular domain of APP. We have also shown that ubiquilin is able to delay the thermal denaturation of CS and prevent the aggregation of AICD *in vitro* and that overexpression of ubiquilin can prevent the aggregation of APP in living cells. Furthermore, in agreement with previous studies (Hiltunen *et al.* 2006), we show that overexpression of ubiquilin reduces amyloidogenic processing of APP. Ubiquilin overexpression also protects cells against APP-induced toxicity, while ubiquilin knockdown exacerbates APP-induced cell death. Coupled with the evidence that ubiquilin protein levels are decreased in brain tissue from patients with tangle pathology, our results suggest that ubiquilin is a *bona fide* molecular chaperone for APP and that decreased quality control of APP may be an important mechanism for the pathogenesis of late-onset AD.

Ubiquilin has previously been shown to interact with presenilin proteins (Mah *et al.* 2000), which constitute the catalytic core of the γ-secretase. Specifically, ubiquilin decreases endoproteolysis of high molecular weight presenilin species (Massey *et al.* 2004). Endoproteolysis of full-length presenilin proteins, which may rely on the proteasome (Honda *et al.* 1999), is required to generate the C- and N-terminal fragments that participate in γ-secretase assembly. Overexpression of ubiquilin decreased the levels of presenilin fragments and two other γ-secretase components (Massey *et al.* 2005). This suggests that decreased γ-secretase activity may accompany ubiquilin overexpression, although this has not been directly tested. The results of the present study indicate that a relationship exists between ubiquilin and APP that is independent of the presenilin proteins. These findings are in agreement with a previous study which showed that knockdown of ubiquilin by RNAi accelerates the maturation of APP through the secretory pathway, independently of any changes in β- or γ-secretase levels (Hiltunen *et al.* 2006).

In the present study, we provide evidence of a direct interaction between ubiquilin and APP. The UBL domain is insufficient to prevent the aggregation of APP, suggesting that the interaction involves the STI domains, the UBA domain, or both. Furthermore, we show that ubiquilin is able to prevent the aggregation of purified recombinant AICD, indicating that ubiquitination of APP is not required for this interaction. It is plausible that the interaction with AICD involves the central region of ubiquilin, which contains two tandem STI domains. STI domains resemble co-chaperone molecules and have been shown to mediate hydrophobic interactions (Kamionka and Feigon 2004). The AICD contains a hydrophobic stretch of amino acids N-terminal to the conserved YENPTY

sequence and encompassing the caspase cleavage site. Interestingly, the hydrophobicity profile of the AICD is remarkably similar to the intracellular domain of APLP, which was among the verified clones from our yeast two-hybrid with ubiquilin.

According to our results, the ubiquilin-APP interaction displays characteristics of a chaperone-client interaction. The interaction between ubiquilin and the AICD is transient in nature, as evidenced by the crosslinking requirement for co-precipitation experiments (**Fig. 4.2B**). The association is also enhanced with elevated temperature (**Fig. 4.2C**), suggesting that hydrophobic surfaces are involved, since higher temperatures are known to strengthen hydrophobic interactions in macromolecules (Lesk 2003). Elevated temperatures may also promote the partial unfolding of polypeptide chains, which exposes hydrophobic surfaces that are normally buried in the native three-dimensional structure. Molecular chaperones transiently associate with exposed hydrophobic regions of client proteins, preventing inappropriate hydrophobic interactions that may lead to aberrant folding or aggregation (Hartl and Hayer-Hartl 2009). Interestingly, other groups have reported that ubiquilin plays a role in the cellular response to protein misfolding and aggregation (Haapasalo *et al.* 2010). Specifically, ubiquilin has been shown to protect against polyglutamine-induced toxicity in cellular and invertebrate models of Huntington's disease (Wang *et al.* 2006; Wang and Monteiro 2007a). Ubiquilin is also involved in aggresome formation (Heir *et al.* 2006) and may promote removal of cellular aggregates *via* autophagy (N'Diaye *et al.* 2009a; N'Diaye *et al.* 2009b). Taken together, our results and the results from previous studies suggest that ubiquilin plays an important role in quality control of aggregation-prone cellular proteins.

The AICD is thought to be natively unstructured in solution and has a very short half-life following cleavage by the γ-secretase, unless it is associated with a cytoplasmic binding partner (Kroenke *et al.* 1997; Ramelot *et al.* 2000; Kimberly *et al.* 2001; Edbauer *et al.* 2002). For example, association of the AICD with Fe65 promotes nuclear translocation of the complex and subsequent transcriptional regulation of various genes (Muller *et al.* 2007; Muller *et al.* 2008). Other physiological functions for AICD have also recently been indentified, including regulation of cytosolic calcium (Leissring *et al.* 2002; Hamid *et al.* 2007) and apoptosis (Ozaki *et al.* 2006; Nakayama *et al.* 2008). Quality control of free AICD may be important for ensuring that it retains the ability to interact with other binding partners, undergo transport between cellular compartments, and perform its normal cellular functions. Interestingly, overexpression of AICD and Fe65 in a transgenic mouse model is sufficient to produce Alzheimer's-like neuropathology and cognitive deficits (Ghosal *et al.* 2009). It is possible that the overexpressed proteins overwhelm the quality control machinery, leading to aggregation and loss of function. Further studies are needed to address the mechanism by which the AICD-Fe65 contributes to the neurodegenerative phenotype and whether ubiquilin can ameliorate these effects.

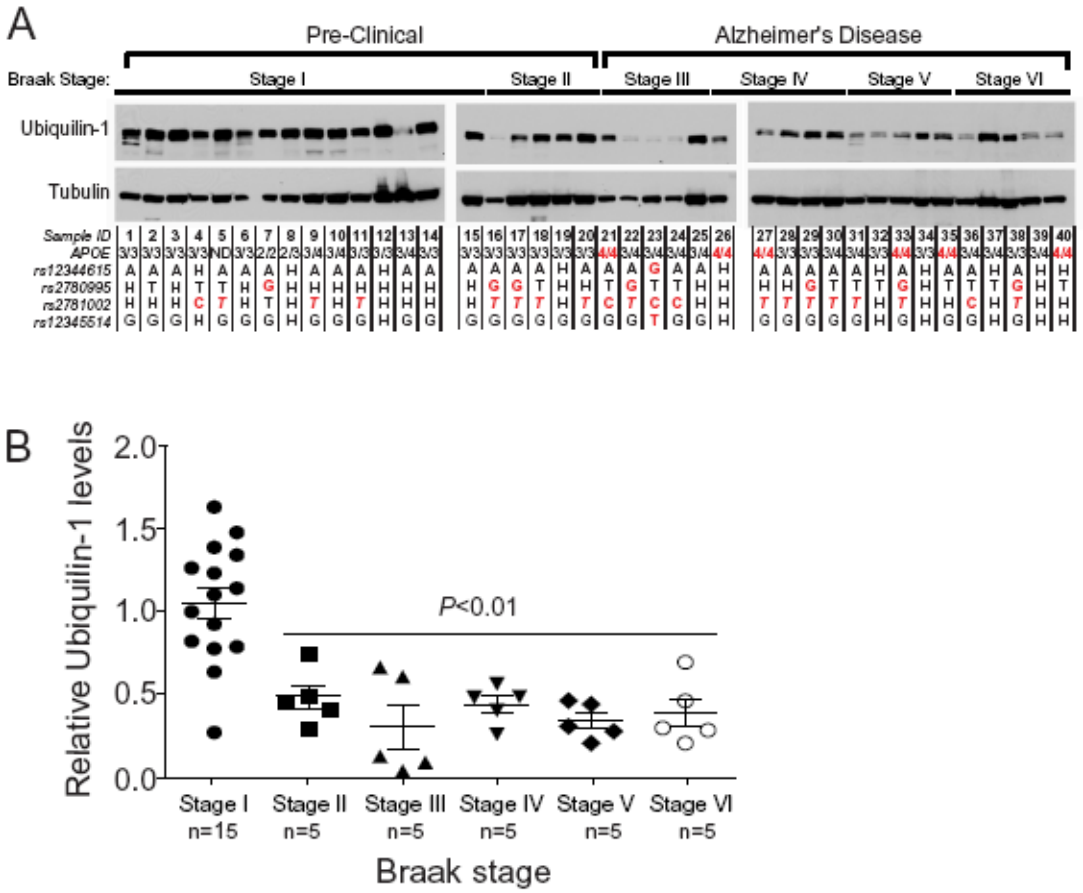
The chaperone function of ubiquilin described here may constitute a critical component of a protein quality control network necessary to prevent irreversible aggregation and amyloidogenic processing of APP. Our results underscore the importance of protein-protein interactions within the AICD in the cellular homeostasis of APP and point towards the maintenance and restoration of adequate levels of ubiquilin and other cellular quality control molecules as therapeutic targets to prevent APP-related cognitive decline in late onset AD.

Table 4.1: Demographic, genotypic, and pathological characteristics of AD subjects and tissue samples.

| Sample ID | Braak Stage | Age | Sex | *APOE* genotype | rs12344615 (intron 8) | rs2780995 (intron 6) | rs2781002 (intron 9) | rs12345514 (promoter) | Amyloid Score | Notes |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | I | 82 | M | ε3/ε3 | A/A | G/T | C/T | G/G | Mild | |
| 2 | I | 86 | M | ε3/ε3 | A/A | T/T | C/T | G/G | | |
| 3 | I | 80 | F | ε3/ε3 | A/A | G/T | C/T | G/G | Mild | |
| 4 | I | 78 | F | ε3/ε3 | A/G | T/T | C/C | G/T | | |
| 5 | I | 78 | M | | A/A | T/T | T/T | G/G | | |
| 6 | I | 85 | F | ε3/ε3 | A/A | G/T | C/T | G/G | | |
| 7 | I | 75 | M | ε2/ε2 | A/A | G/G | T/T | G/G | | Lacunes |
| 8 | I | 88 | M | ε2/ε3 | A/G | G/T | C/T | G/T | | |
| 9 | I | 72 | F | ε3/ε4 | A/A | G/T | T/T | G/G | | |
| 10 | I | 79 | F | ε3/ε4 | A/A | G/T | C/T | G/G | | |
| 11 | I | 80 | M | ε3/ε3 | A/A | G/T | T/T | G/G | | |
| 12 | I | 83 | M | ε3/ε3 | A/G | G/T | C/T | G/T | | |
| 13 | I | 80 | M | ε3/ε4 | A/A | G/T | C/T | G/G | | |
| 14 | I | 82 | F | ε3/ε3 | A/A | G/T | C/T | G/G | Mild | |
| 15 | I | 75 | M | ε3/ε3 | A/A | G/T | C/T | G/G | | |
| 16 | II | 82 | F | ε3/ε3 | A/A | G/G | T/T | G/G | | Infarcts |
| 17 | II | 72 | F | ε3/ε3 | A/A | G/G | T/T | G/G | | |
| 18 | II | 85 | M | ε3/ε3 | A/A | T/T | T/T | G/G | Mild | |
| 19 | II | 85 | F | ε3/ε3 | A/G | G/T | C/T | G/G | Mild | |
| 20 | II | 85 | M | ε3/ε3 | A/A | G/T | T/T | G/G | Severe | |
| 21 | III | 80 | F | ε4/ε4 | A/A | T/T | C/C | G/G | | |
| 22 | III | 80 | F | ε3/ε4 | A/A | G/G | T/T | G/G | | Moderate amyloid angiopathy; lacunes |
| 23 | III | 79 | F | ε3/ε4 | G/G | T/T | C/C | T/T | | |
| 24 | III | 79 | M | ε3/ε4 | A/A | T/T | C/C | G/G | Moderate | Mild amyloid angiopathy; lacunes |
| 25 | III | 80 | F | ε3/ε4 | A/A | G/T | C/T | G/G | Moderate | Severe amyloid angiopathy |
| 26 | IV | 80 | F | ε4/ε4 | A/G | G/T | C/T | G/T | Mild | Severe amyloid angiopathy |
| 27 | IV | 79 | F | ε4/ε4 | A/A | G/T | T/T | G/G | | Moderate amyloid angiopathy |
| 28 | IV | 80 | F | ε3/ε3 | A/A | G/T | T/T | G/G | | |
| 29 | IV | 80 | M | ε3/ε3 | A/A | G/G | T/T | G/G | | |
| 30 | IV | 80 | F | ε3/ε4 | A/A | T/T | T/T | G/G | | Mild amyloid angiopathy |
| 31 | V | 80 | F | ε3/ε4 | A/A | T/T | T/T | G/G | | Mild amyloid angiopathy; infarcts |
| 32 | V | 80 | F | ε3/ε3 | A/G | T/T | C/T | G/T | Mild | |
| 33 | V | 80 | F | ε4/ε4 | A/A | G/G | T/T | G/G | Moderate | Moderate amyloid angiopathy |
| 34 | V | 80 | F | ε3/ε3 | A/G | G/T | C/T | G/T | | |
| 35 | V | 80 | M | ε4/ε4 | A/A | T/T | C/T | G/G | | Mild amyloid angiopathy |
| 36 | VI | 75 | F | ε3/ε4 | A/A | T/T | C/C | G/G | Severe | |
| 37 | VI | 77 | F | ε3/ε4 | A/G | T/T | C/T | G/T | | Moderate amyloid angiopathy |
| 38 | VI | 75 | F | ε3/ε3 | A/A | G/G | T/T | G/G | Mild | |
| 39 | VI | 75 | M | ε3/ε4 | A/G | G/T | C/T | G/T | Moderate | |
| 40 | VI | 77 | M | ε4/ε4 | A/G | T/T | C/T | G/T | Mild | Mild amyloid angiopathy |

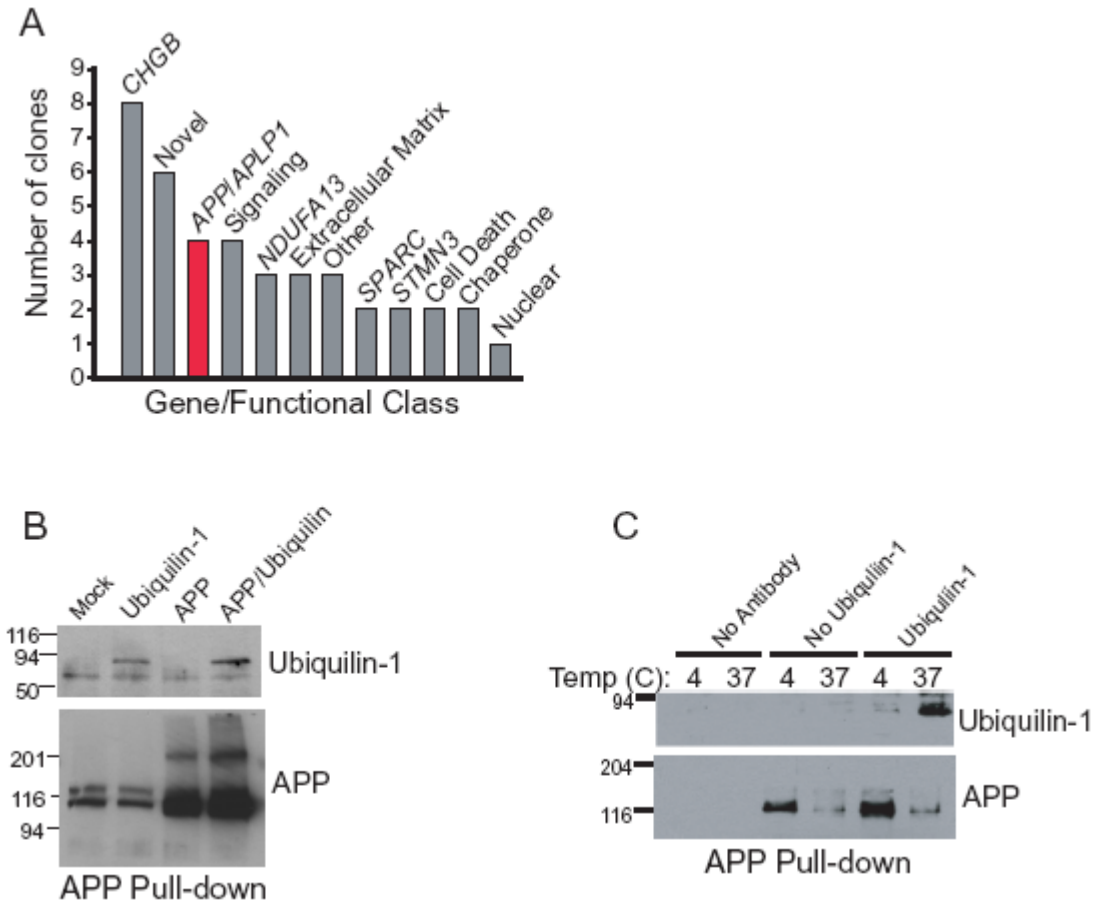
Risk allele for rs12344615 is G (Bertram et al., N Engl J Med 2005, 352:884-94)
Risk allele for rs2780995 is G (Bertram et al., N Engl J Med 2005, 352:884-94)
Risk allele for rs2781002 is either C (Bertram et al., N Engl J Med 2005, 352:884-94) or T (Golan et al., Dement Geriatr Cogn Disord 2008, 25:366-71)
Risk allele for rs12345514 is T (Bertram and Tanzi, N Engl J Med 2005, 352:2752-3)

Figure 4.1: Ubiquilin protein levels are decreased in late-onset AD patient brains.



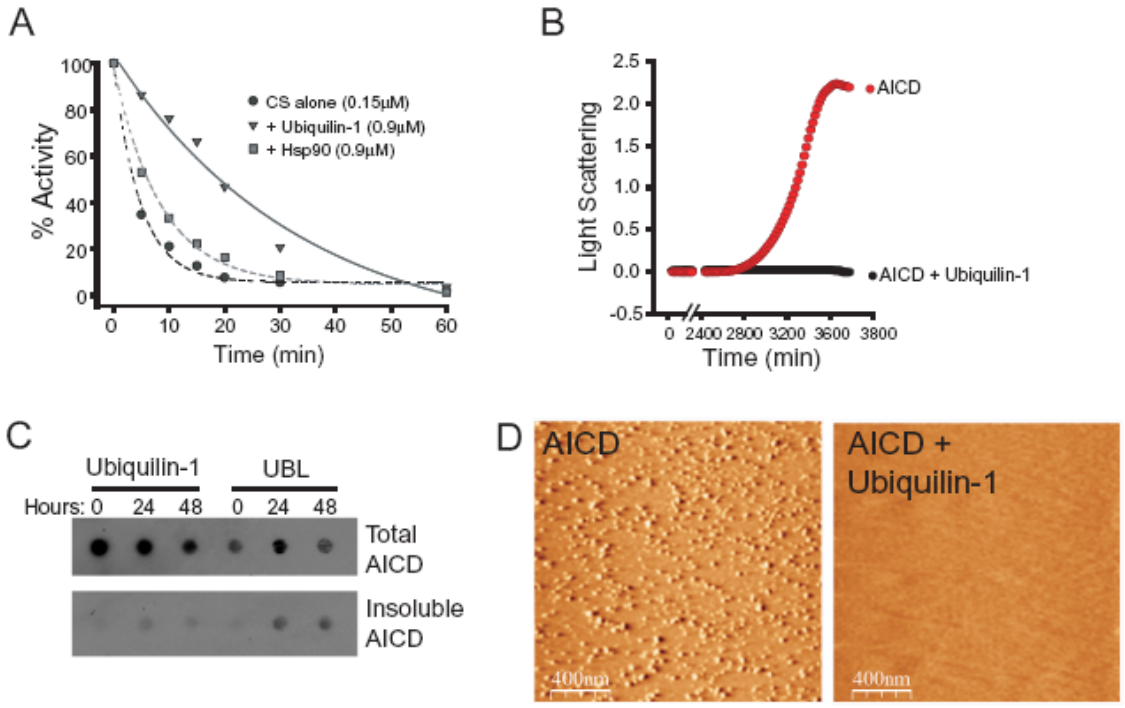

(A) Western blot of ubiquilin and α/β-tubulin levels in post-mortem cortical brain samples (50 µg of total protein per lane) of human patients (ages 72 – 85 years) arranged by Braak staging, as indicated. All samples were handled under identical conditions and blots were exposed simultaneously on the same film. The genotypes of each patient for common ubiquilin-1 and APOE SNPs are indicated below each lane. H indicates heterozygosity for that SNP. SNPs highlighted in red indicate risk alleles. The rs2781002 risk allele has been proposed to be either C (Bertram *et al.* 2005) or T (Golan *et al.* 2008) (in italics). (B) Quantification of ubiquilin protein levels as a function of Braak stage, displayed as ratios of the intensities of the bands corresponding to ubiquilin over α/β-tubulin, determined densitometrically for each sample (symbols) and their means ± s.e.m. (bars). *p* values were determined by *t* test comparison to Stage I.

Figure 4.2: A transient and temperature-dependent interaction exists between ubiquilin and APP.



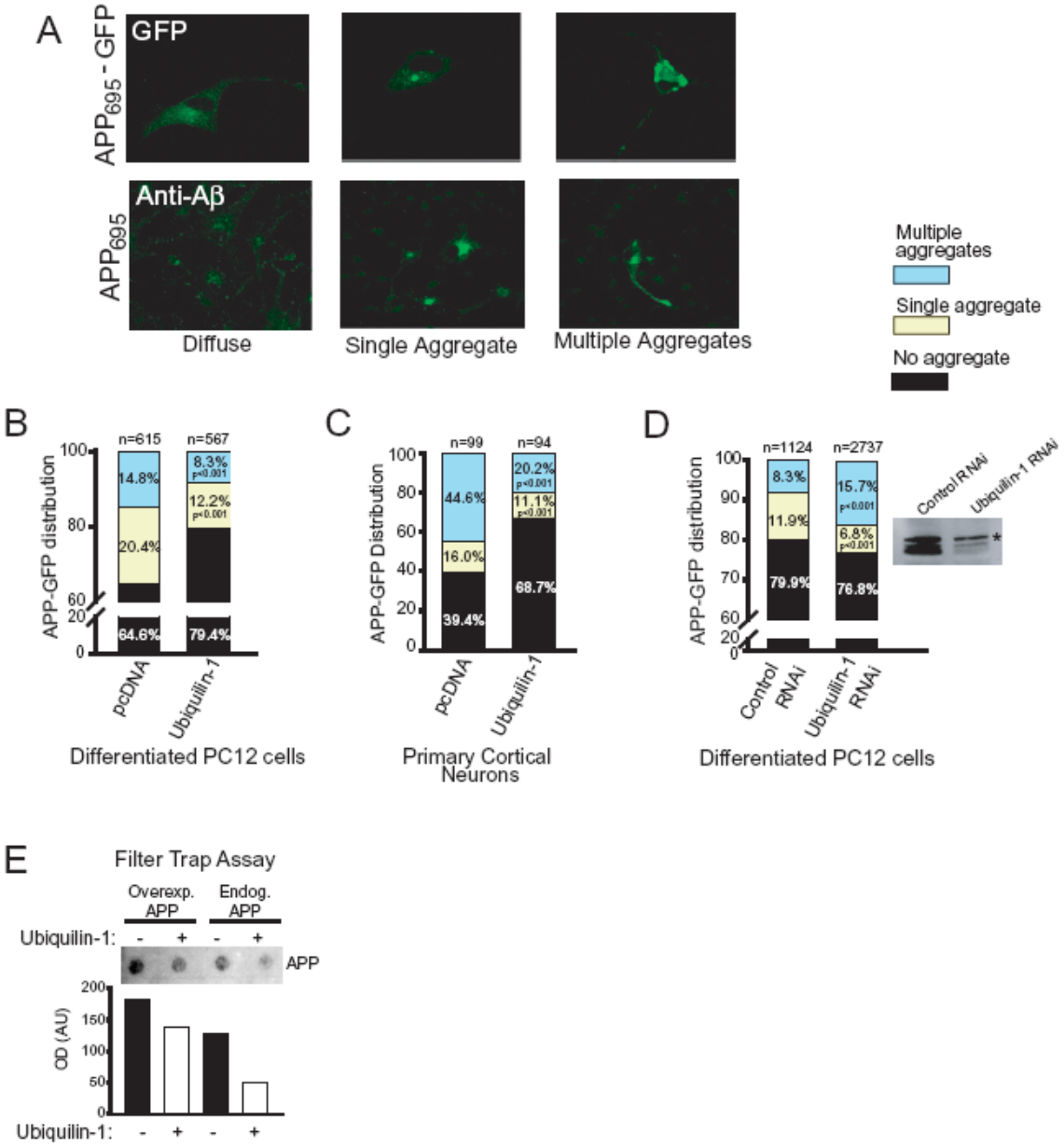

(A) Histogram depicting interaction partners of ubiquilin-1 derived from a yeast two-hybrid screen, displayed as individual genes (italicized) or organized into functional classes. (B) Metal affinity pull-down of APP from HeLa cells overexpressing c-myc tagged ubiquilin and/or APP, as indicated. Cells were pre-treated with Lomant's reagent prior to lysis. Co-precipitating ubiquilin was detected with an anti-c-myc antibody. Mock cells were transfected with an empty vector. (C) Purified ubiquilin binding to APP immunoprecipitated from rat brain at the indicated temperatures. "No Antibody" lanes received no anti-APP antibody, and "No Ubiquilin-1" lanes received no ubiquilin-1 supplementation.

Figure 4.3: Ubiquilin exerts chaperone activity on APP *in vitro*.




(A) Inactivation kinetics of CS alone or in mixtures supplemented with purified Hsp90 or ubiquilin, as indicated, upon exposure to 43°C. (B) Aggregation kinetics of purified AICD alone or in combination with ubiquilin, upon exposure to 43°C, monitored by light scattering. (C) Dot-blot analyses with an anti-AICD antibody of *in vitro* aggregation mixtures of AICD with ubiquilin or its UBL domain, as indicated, upon exposure to 37°C. Aliquots were removed at the indicated times and insoluble material was sedimented by centrifugation. Total and insoluble fractions were spotted onto a nitrocellulose membrane and probed with an anti-AICD antibody. (D) AFM images of aliquots of total material from mixtures set up as in b after 48 h of incubation at 43°C. Aggregates had a diameter of 46 ± 24 nm (n=611 particles).

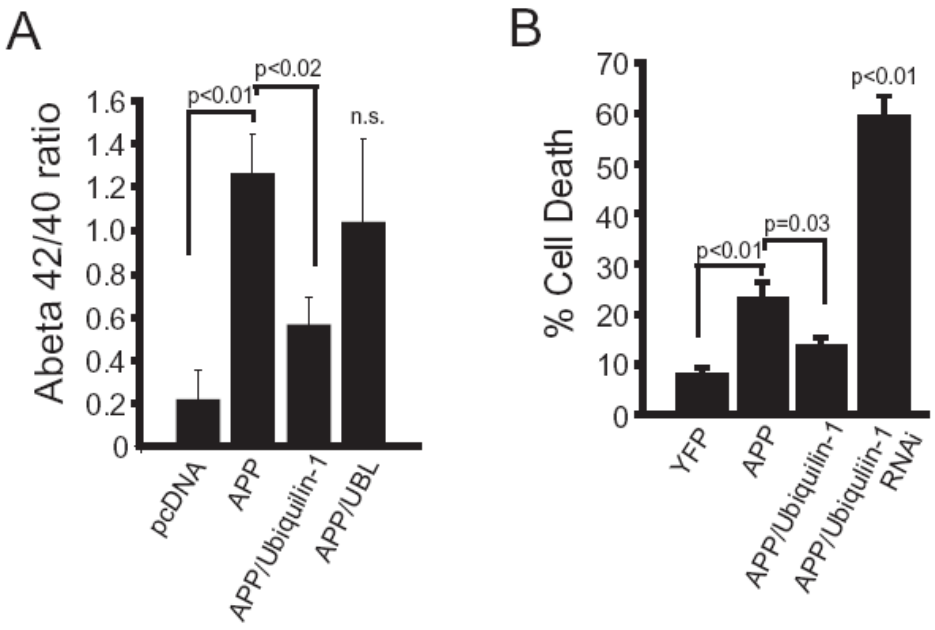
Figure 4.4: Ubiquilin exerts chaperone activity on APP *in vivo*.




(A) Representative images of the three patterns of $APP_{695}$ and APP-GFP localization upon overexpression in differentiated PC12 cells observed by immunofluorescence and fluorescence microscopies, respectively. (B)

Quantification of APP-GFP fluorescence patterns in differentiated PC12 cells co-transfected with APP-GFP and vector (pcDNA), or ubiquilin, as indicated. Total numbers of scored cells are displayed above each bar and significant *p* values are indicated. (C) Quantification of APP-GFP fluorescence patterns in rat primary cortical neurons, as in (B). (D) Quantification of APP-GFP fluorescence patterns in differentiated PC12 cells treated with RNAi oligonucleotides targeting ubiquilin-1 or control RNAi oligonucleotides (left panel) and Western blot confirming decreased levels of ubiquilin-1, but not ubiquilin-2 (asterisk), protein levels only in cells treated with ubiquilin-1 RNAi oligonucleotides (right panel). (E) Filter-trap assay of total lysates from cells as in (B), filtered through cellulose acetate membranes (0.22 μm) probed with an anti-APP antibody. *p* values in B-D were determined by Fisher's Exact test and are indicated within the bars.

Figure 4.5: Ubiquilin reduces amyloidogenesis and associated cell death.




(A) Quantification of $A\beta_{42}/A\beta_{40}$ production in HeLa cells co-transfected with vector only (pcDNA), APP alone, APP and ubiquilin, or APP and UBL domain, as indicated. n.s., not significant relative to APP. (B) Quantification of cell death determined by propidium iodide staining in HeLa cell transfected with the yellow fluorescent protein (YFP), APP, APP and ubiquilin, or APP and ubiquilin RNAi oligonucleotides, as indicated.

# Chapter 5: Conclusions

The studies in this dissertation were inspired by fundamental questions that remain regarding the pathogenic mechanisms of Alzheimer's disease. First, is disruption of intracellular calcium homeostasis a common causal factor in all forms of familial AD? Second, what are the mechanisms of disease pathogenesis in the most common form of AD, late-onset AD, for which discreet genetic associations have not been established? To answer these questions, we focused our investigations on APP for several reasons. First, although a it had previously been suggested that calcium dysregulation is a common pathogenic feature of AD (LaFerla 2002), a systematic investigation of the effects of FAD-linked APP mutants on intracellular calcium homeostasis had not been reported. Second, recent evidence from genetic studies suggested a link between the *UBQLN1* gene and late-onset AD (Bertram *et al.* 2005; Kamboh *et al.* 2006), and evidence from our lab (**Fig. 4.2A**) and others suggested that the protein product of the *UBQLN1* gene may directly interact with APP and affect its maturation (Hiltunen *et al.* 2006).

Although there is still a great deal of debate regarding the chronology of events in disease pathogenesis and the nature of the offending protein aggregates, the current body of research overwhelmingly supports a role for APP and its cleavage products in the pathogenesis of both forms of AD (Querfurth and LaFerla 2010). Mutations in three different genes have been linked to FAD: the two *PSEN* genes, which encode presenilin-1 and presenilin-2, and the gene encoding APP itself (Tanzi and Bertram 2005). Biochemical, cellular, and animal studies incorporating FAD-linked mutant proteins have provided the basis for the

amyloid cascade hypothesis of AD, which postulates that aggregation-prone proteolytic fragments of APP disrupt neuronal function and lead to cell death and cognitive decline in affected individuals. An alternate hypothesis, which is not mutually exclusive with the amyloid cascade hypothesis, is the calcium dysregulation hypothesis, which suggests that alterations in intracellular calcium signaling are proximal events in disease pathogenesis and lead to abnormalities in synaptic transmission and subsequent cell death (LaFerla 2002). Calcium signaling abnormalities were first associated with FAD when researchers discovered that cells isolated from patients expressing FAD-linked mutant presenilin exhibited abnormally elevated agonist-induced calcium responses (Ito *et al.* 1994). Subsequent analyses have supported the notion that calcium dyshomeostasis is an early alteration in FAD linked to mutant presenilins and that it may even be independent of Aβ production (Guo *et al.* 1996). In particular, there is a growing body of evidence that mutant presenilin proteins directly mediate calcium disturbances in AD by modulating calcium release from the ER (Leissring *et al.* 1999a; Leissring *et al.* 1999b). However, conclusive evidence regarding the effect of FAD-linked APP mutations on intracellular calcium has been lacking.

We were interested in determining whether the calcium dyshomeostasis hypothesis could be generalized to all forms of FAD, and thus chose to perform a systematic analysis of the intracellular calcium dynamics associated with six different FAD-linked APP mutants (Chapter 2). Notably, we found that none of the mutations tested had any effect on calcium signaling when overexpressed in PC12 cells, suggesting that perturbations in intracellular calcium associated with APP mutants are most likely mediated by Aβ or other cleavage products and not

the APP holoprotein. Since our calcium imaging experiments were performed 24 hours following transient transfection, it is possible that Aβ had not been produced at levels sufficient to disrupt calcium signaling. Our results promote the hypothesis that the presenilin proteins have a physiological role in the maintenance of calcium homeostasis that is independent of their role in APP processing. The mechanism by which mutant presenilins lead to calcium dysregulation is still unclear. It may reflect a loss-of-function phenotype as has been suggested by Bezprozvanny and colleagues, who have evidence to suggest that wild-type presenilin proteins form calcium leak channels in the ER membrane (Tu *et al.* 2006). Alternatively, it may reflect a gain-of-function phenotype due to mutant presenilins interacting with and altering the gating properties of $IP_3Rs$, as has been suggested by Foskett and colleagues (Cheung *et al.* 2008). The mutant presenilin phenotypes may also be a consequence of altered function of the γ-secretase, which is required for the cleavage of several cellular targets and may have additional, as yet undefined, substrates. A major conclusion that can be drawn from our data is that calcium dysregulation in models of AD that are not a result of presenilin mutations is a relatively late event in disease pathogenesis. Thus, treatment modalities aimed at maintaining intracellular calcium homeostasis would be ineffective in modifying disease progression, which may explain why the NMDA receptor antagonist memantine is only marginally effective in treating AD (van Marum 2009).

A fundamental question regarding the pathogenesis of AD is whether or not the mechanisms underlying FAD apply to the more common late-onset form of the disease. Discrete mutations in the *PSEN* and *APP* genes lead to early-onset FAD, and the assumption is that the results of these mutations – increased

Aβ production and/or aggregation and deposition – are the same events that initiate late-onset AD. However, this has not been validated. To complicate matters, the only gene that has been consistently linked to late-onset AD, *APOE*, contributes to disease risk by an as yet undetermined mechanism (Kim *et al.* 2009a). The need to define the molecular mechanisms of late-onset AD has led researchers to genome-wide association studies, which use an unbiased approach to identify new genes that may be associated with complex disorders like AD. Unfortunately, none of the linkages that have been discovered in genome-wide association studies have been validated using other approaches or even consistently replicated between genome scans (Bertram *et al.* 2007). However, this is not unexpected if one considers the matrix model of AD pathogenesis proposed by Hunter and colleagues (Hunter *et al.* 2010), which postulates that AD is a syndrome that results from the convergence of multiple pathways with distinct initiating factors that may differ between patients. Although a genetic association may not achieve statistical significance in all populations tested, it may still represent a viable candidate for further study into the mechanisms underlying AD. We propose that the results of our biochemical and cellular studies with ubiquilin, the protein product of the *UBQLN1* gene, elucidate molecular mechanisms that are relevant to the pathogenesis of late-onset AD in general, despite an ambiguous genetic association.

In Chapter 3, we provide evidence that ubiquilin is able to regulate the ubiquitination, trafficking, and degradation of APP. Specifically, we show that ubiquilin sequesters APP in early secretory compartments and consequently prevents or delays contact with the enzymes responsible for amyloidogenic processing (**Figs. 3.1–3.4**). Secondly, we show that ubiquilin inhibits the

proteasomal degradation of APP and increases the ubiquitination of APP (**Fig. 3.5**). We propose that the effects on APP trafficking are related to the effects of ubiquilin on the ubiquitination of APP and are mediated, at least in part, by a UBA-ubiquitin interaction. We hypothesize that the observed increase in ubiquitination corresponds to an increase in monoubiquitination or $Lys^{63}$-linked polyubiquitination, which each have been shown to modulate protein sorting (Hicke and Dunn 2003), rather than $Lys^{29}$- or $Lys^{48}$-linked polyubiquitination, which serve as degradation signals. These hypotheses can be tested by co-expressing ubiquitin with mutations in $Lys^{48}$, $Lys^{63}$, or both and determining what effect each has on the ability of ubiquilin to alter trafficking and enhance ubiquitination. Also, linkage-specific antibodies can be used to distinguish between $Lys^{48}$- and $Lys^{63}$-linked chains. It is possible that ubiquilin inhibits exit from the Golgi apparatus by competing with cargo adaptors, which also bind to $Lys^{63}$-linked polyubiquitin chains and couple cargo to vesicular transport machinery (Lauwers *et al.* 2009).

The inhibition of proteasomal degradation may also be mediated by a ubiquitin-UBA interaction. As has been shown for Rad23, UBL-UBA proteins can “cap” monoubiquitin or short polyubiquitin chains and prevent further elongation or deubiquitination, which may in turn inhibit degradation by the proteasome (Ortolan *et al.* 2000; Hwang *et al.* 2005). This may also explain the increased accumulation of ubiquitinated APP in cells overexpressing ubiquilin. Additional experiments with a ubiquilin construct lacking the UBA domain – or containing mutations in critical ubiquitin-binding residues – are needed to determine if UBA interactions are required for the trafficking and stabilization effects observed in our studies.

An alternative explanation is that ubiquilin actively recruits E2/E3 ligases to APP or stimulates their activity, leading to increased ubiquitination. E3 ligases have been shown to be associated with ubiquilin and its homologues (Hara *et al.* 2005; Kim *et al.* 2008b). Using whole-genome Affymetrix analysis followed by quantitative PCR, we have found that knockdown of ubiquilin results in significant downregulation of many zinc/RING finger E3 proteins, including cyclin B1-interacting protein 1, ring finger protein 34, and polycomb group ring finger 5. This suggests that ubiquilin may functionally interact with a large family of E3 ligases, and provides an avenue for future investigation.

In Chapter 4, we provide evidence for the chaperone function of ubiquilin. Specifically, ubiquilin is able to prevent the aggregation of APP *via* an association with the AICD (**Figs. 4.2, 4.3, and 4.4**). Furthermore, ubiquilin inhibits amyloidogenesis and associated toxicity in cells overexpressing APP (**Fig. 4.5**), which may be, in fact, associated with the trafficking and sorting effects of ubiquilin presented in Chapter 3. We also show that ubiquilin protein levels are reduced in the brain tissue of patients with neurofibrillary tangle pathology (**Fig. 4.1**), suggesting that decreased quality control of APP by ubiquilin may be an important mechanism for the pathogenesis of late-onset AD. We show that ubiquilin is able to prevent the aggregation of purified AICD *in vitro* (**Fig. 4.3B-D**), indicating that the interaction between the two proteins involves the AICD, which is expected since ubiquilin is a cytosolic protein. However, the results of our studies do not establish the nature of the aggregates observed in cells overexpressing full-length APP (**Fig. 4.4**). We do not expect the aggregates involving the AICD to be highly ordered fibrillar structures like those involving Aβ. Instead, as the results of our AFM experiments reveal, the AICD aggregates are

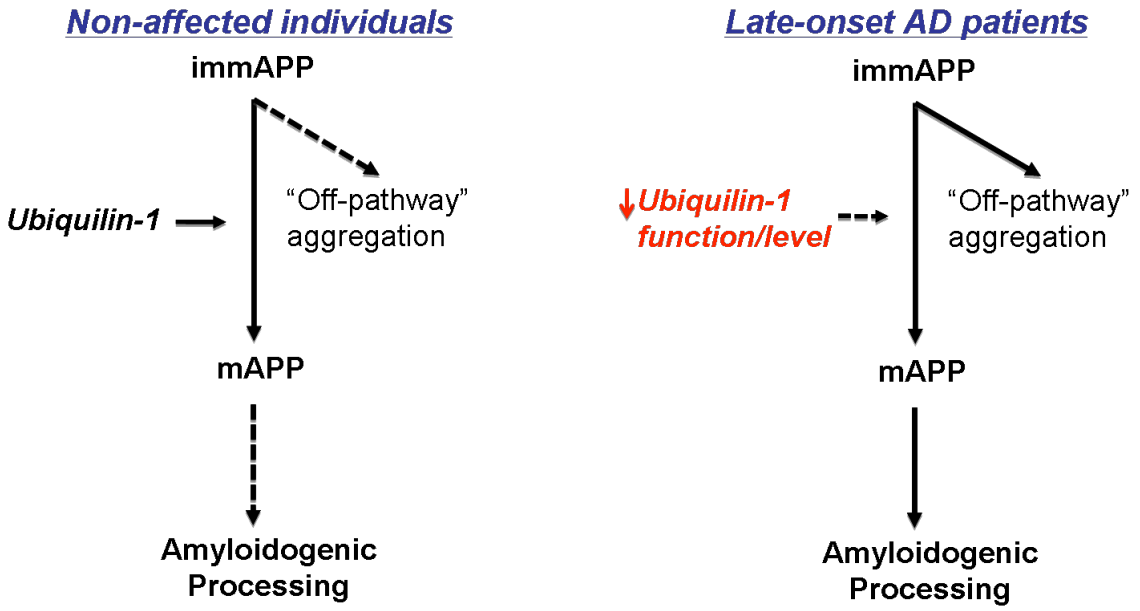
relatively amorphous (**Fig. 4.3D**). Furthermore, the results of our light scattering experiments (**Fig. 4.3B**) indicate that the kinetics of AICD aggregation are on the order of days, which is relatively slow compared to $A\beta_{42}$ and highly fibrillogenic $A\beta_{40}$ variants, which have been shown to aggregate *in vitro* within minutes (Jan *et al.* 2010). Instead, we hypothesize that the aggregation of AICD is based on its intrinsically unstructured nature, which allows for inappropriate or unproductive intra- and intermolecular contacts unless it is stabilized by a transient binding partner. While our data indicate that ubiquilin is able to perform this function, it is possible that other binding partners are able to stabilize the AICD in a similar fashion. One such candidate protein is C-terminus Hsp70-interacting protein (CHIP), a co-chaperone molecule that has been shown to interact with APP (Kumar *et al.* 2007). Cytosolic heat shock proteins, which have been shown to prevent the aggregation of Aβ (Koren *et al.* 2009), may also be able to stabilize the AICD. Based on APP immunofluorescence experiments using an antibody directed against the Aβ sequence (**Fig. 4.4A**, bottom panels), we can conclude that a significant proportion of the aggregates contain the Aβ peptide. We hypothesize that the interaction of ubiquilin with the AICD prevents the aggregation of *full-length* APP and Aβ-containing membrane-tethered C-terminal fragments. We further propose that the aggregation of full-length APP and its C-terminal fragments is a novel mechanism for the pathogenesis of late-onset AD and that the protective effect of ubiquilin is partly related to its ability to prevent this aggregation.

Intracellular APP aggregates may contribute to synaptic dysfunction and cell death by a number of mechanisms. Aggregation of secretory proteins is known to initiate the ER stress response, which can result in apoptosis if the

repair responses are insufficient to restore normal cellular function (Kim *et al.* 2008a). Also, aggregates may sequester other proteins or components of the degradation machinery and prevent their normal physiological function. Another possibility is that aggregation of APP inhibits its normal physiological function, which may be related to vesicle trafficking or transcriptional regulation (De Strooper and Annaert 2000; Gralle and Ferreira 2007; Muller *et al.* 2008), both of which are critical for the changes in synaptic plasticity that are thought to underlie learning and memory (Kandel 2001). Unfolded or misfolded APP may also impair cellular function by participating in inappropriate hydrophobic interactions with other proteins or even cell membrane lipids (Stefani and Dobson 2003). Each of these effects can render cells vulnerable to subsequent insults, which may include alterations in intracellular calcium or Aβ-mediated toxicity. Furthermore, small intracellular APP aggregates may serve as “seeds” that promote further APP aggregation, contributing to a feed-forward mechanism of cellular dysfunction.

Our data supports the notion that ubiquilin offers protection against cellular dysfunction and death at multiple levels, including APP biosynthesis, folding and maintenance of proper structure, trafficking, degradation, and amyloidogenic processing. Our hypothesis of ubiquilin function is summarized in **Fig. 5.1**, which emphasizes a decrease in ubiquilin protein level or function in the pathogenesis of late-onset AD.

Figure 5.1: Working model of late-onset AD pathogenesis.




In non-affected individuals (left panel), ubiquilin interacts with immature APP (immAPP) along the secretory pathway and inhibits the transition of mature APP (mAPP) to vesicular and membrane compartments where amyloidogenic processing occurs. This interaction also prevents the formation of off-pathway folding intermediates by shielding hydrophobic surfaces within the AICD. In patients with late-onset AD, reduced ubiquilin protein level or function leads to accelerated maturation and amyloidogenic processing of APP, as well as increased off-pathway aggregation, which renders neurons particularly vulnerable to subsequent insults. Broken lines indicate inhibited pathways.

The role of ubiquilin in the regulation of APP folding, trafficking, and proteolysis may represent a universal mechanism for cytoprotection in the nervous system. Furthermore, decreased ubiquilin levels or function may be associated with other neurodegenerative disease, many of which also involve the misfolding and aggregation of cellular proteins. The results presented here have important implications for the treatment of AD and related disorders, and point to

the restoration of chaperone function and other elements of the protein quality control machinery as potential therapeutic targets. Combination therapies that target various aspects of APP quality control are likely to beneficial in the treatment of AD and may be superior to existing therapies, which are unable to prevent the progression of the disease. Furthermore, our results favor a view that recognizes late-onset AD and early-onset familial AD as two separate and distinct pathological processes. Whereas Aβ accumulation and aggregation may be the fundamental mechanisms of early-onset inherited AD, Aβ may only be a partial contributor in the pathogenesis of late-onset AD, which is most likely initiated before substantial amounts of Aβ have accumulated. Thus, research into the mechanisms of late-onset AD should focus on these earlier aspects of the disease process, and researchers should recognize that therapies targeted at Aβ or the secretase enzymes may not be clinically efficacious in late-onset AD.

# Bibliography


Alzheimer A. (1907) Über eine eigenartige Erkrankung der Hirnrinde. *Allg Zietschr Psychiatr Psychiatr-Gerichtl Med* 109:146-148.

Alzheimer A, Stelzmann RA, Schnitzlein HN, Murtagh FR. (1995) An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde". *Clin Anat* 8(6):429-431.

Alzheimer's Association. (2009) 2009 Alzheimer's disease facts and figures. *Alzheimers Dement* 5(3):234-270.

Arias-Vasquez A, de Lau L, Pardo L, Liu F, Feng BJ, Bertoli-Avella A, Isaacs A, Aulchenko Y, Hofman A, Oostra B, Breteler M, van Duijn C. (2007) Relationship of the Ubiquilin 1 gene with Alzheimer's and Parkinson's disease and cognitive function. *Neurosci Lett* 424(1):1-5.

Baens M, Noels H, Broeckx V, Hagens S, Fevery S, Billiau AD, Vankelecom H, Marynen P. (2006) The dark side of EGFP: defective polyubiquitination. *PLoS One* 1:e54.

Bailey CK, Andriola IF, Kampinga HH, Merry DE. (2002) Molecular chaperones enhance the degradation of expanded polyglutamine repeat androgen receptor in a cellular model of spinal and bulbar muscular atrophy. *Hum Mol Genet* 11(5):515-523.

Bedford FK, Kittler JT, Muller E, Thomas P, Uren JM, Merlo D, Wisden W, Triller A, Smart TG, Moss SJ. (2001) GABA(A) receptor cell surface number and subunit stability are regulated by the ubiquitin-like protein Plic-1. *Nat Neurosci* 4(9):908-916.

Bensemain F, Chapuis J, Tian J, Shi J, Thaker U, Lendon C, Iwatsubo T, Amouyel P, Mann D, Lambert JC. (2006) Association study of the Ubiquilin gene with Alzheimer's disease. *Neurobiol Dis* 22(3):691-693.

Berridge MJ. (1998) Neuronal calcium signaling. *Neuron* 21(1):13-26.

Bertolaet BL, Clarke DJ, Wolff M, Watson MH, Henze M, Divita G, Reed SI. (2001) UBA domains mediate protein-protein interactions between two DNA damage-inducible proteins. *J Mol Biol* 313(5):955-963.

Bertram L, Hiltunen M, Parkinson M, Ingelsson M, Lange C, Ramasamy K, Mullin K, Menon R, Sampson AJ, Hsiao MY, Elliott KJ, Velicelebi G, Moscarillo T, Hyman BT, Wagner SL, Becker KD, Blacker D, Tanzi RE. (2005) Family-

based association between Alzheimer's disease and variants in UBQLN1. *N Engl J Med* 352(9):884-894.

Bertram L, McQueen MB, Mullin K, Blacker D, Tanzi RE. (2007) Systematic meta-analyses of Alzheimer disease genetic association studies: the AlzGene database. *Nat Genet* 39(1):17-23.

Biggins S, Ivanovska I, Rose MD. (1996) Yeast ubiquitin-like genes are involved in duplication of the microtubule organizing center. *J Cell Biol* 133(6):1331-1346.

Blacker D, Bertram L, Saunders AJ, Moscarillo TJ, Albert MS, Wiener H, Perry RT, Collins JS, Harrell LE, Go RC, Mahoney A, Beaty T, Fallin MD, Avramopoulos D, Chase GA, Folstein MF, McInnis MG, Bassett SS, Doheny KJ, Pugh EW, Tanzi RE. (2003) Results of a high-resolution genome screen of 437 Alzheimer's disease families. *Hum Mol Genet* 12(1):23-32.

Blatch GL, Lassle M, Zetter BR, Kundra V. (1997) Isolation of a mouse cDNA encoding mSTI1, a stress-inducible protein containing the TPR motif. *Gene* 194(2):277-282.

Boehning D, Patterson RL, Sedaghat L, Glebova NO, Kurosaki T, Snyder SH. (2003) Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nat Cell Biol* 5(12):1051-1061.

Boehning D, van Rossum DB, Patterson RL, Snyder SH. (2005) A peptide inhibitor of cytochrome c/inositol 1,4,5-trisphosphate receptor binding blocks intrinsic and extrinsic cell death pathways. *Proc Natl Acad Sci U S A* 102(5):1466-1471.

Borchelt DR, Thinakaran G, Eckman CB, Lee MK, Davenport F, Ratovitsky T, Prada CM, Kim G, Seekins S, Yager D, Slunt HH, Wang R, Seeger M, Levey AI, Gandy SE, Copeland NG, Jenkins NA, Price DL, Younkin SG, Sisodia SS. (1996) Familial Alzheimer's disease-linked presenilin 1 variants elevate Abeta1-42/1-40 ratio in vitro and in vivo. *Neuron* 17(5):1005-1013.

Borg JP, Ooi J, Levy E, Margolis B. (1996) The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. *Mol Cell Biol* 16(11):6229-6241.

Braak H, Braak E. (1991) Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol* 82(4):239-259.

Brouwers N, Sleegers K, Engelborghs S, Bogaerts V, van Duijn CM, De Deyn PP, Van Broeckhoven C, Dermaut B. (2006) The UBQLN1 polymorphism, UBQ-8i, at 9q22 is not associated with Alzheimer's disease with onset before 70 years. *Neurosci Lett* 392(1-2):72-74.

Buchberger A. (2002) From UBA to UBX: new words in the ubiquitin vocabulary. *Trends Cell Biol* 12(5):216-221.

Buchner J, Grallert H, Jakob U. (1998) Analysis of chaperone function using citrate synthase as nonnative substrate protein. *Methods Enzymol* 290:323-338.

Cao X, Sudhof TC. (2001) A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science* 293(5527):115-120.

Caporaso GL, Gandy SE, Buxbaum JD, Greengard P. (1992) Chloroquine inhibits intracellular degradation but not secretion of Alzheimer beta/A4 amyloid precursor protein. *Proc Natl Acad Sci U S A* 89(6):2252-2256.

Carette B, Poulain P, Delacourte A. (1993) Electrophysiological effects of 25-35 amyloid-beta-protein on guinea-pig lateral septal neurons. *Neurosci Lett* 151(1):111-114.

Chan SL, Mayne M, Holden CP, Geiger JD, Mattson MP. (2000) Presenilin-1 mutations increase levels of ryanodine receptors and calcium release in PC12 cells and cortical neurons. *J Biol Chem* 275(24):18195-18200.

Chen L, Madura K. (2002) Rad23 promotes the targeting of proteolytic substrates to the proteasome. *Mol Cell Biol* 22(13):4902-4913.

Cheung KH, Shineman D, Muller M, Cardenas C, Mei L, Yang J, Tomita T, Iwatsubo T, Lee VM, Foskett JK. (2008) Mechanism of Ca2+ disruption in Alzheimer's disease by presenilin regulation of InsP3 receptor channel gating. *Neuron* 58(6):871-883.

Chuo LJ, Wu ST, Chang HI, Kuo YM. (2010) Extremely rare incidence of the UBQLN1 polymorphism (UBQ-8i) in Taiwan Chinese with Alzheimer's disease. *Neurosci Lett* 475(2):108-109.

Citron M, Westaway D, Xia W, Carlson G, Diehl T, Levesque G, Johnson-Wood K, Lee M, Seubert P, Davis A, Kholodenko D, Motter R, Sherrington R, Perry B, Yao H, Strome R, Lieberburg I, Rommens J, Kim S, Schenk D, Fraser P, St George Hyslop P, Selkoe DJ. (1997) Mutant presenilins of

Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice. *Nat Med* 3(1):67-72.

Conklin D, Holderman S, Whitmore TE, Maurer M, Feldhaus AL. (2000) Molecular cloning, chromosome mapping and characterization of UBQLN3 a testis-specific gene that contains an ubiquitin-like domain. *Gene* 249(1-2):91-98.

Cook DG, Forman MS, Sung JC, Leight S, Kolson DL, Iwatsubo T, Lee VM, Doms RW. (1997) Alzheimer's A beta(1-42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Nat Med* 3(9):1021-1023.

d'Azzo A, Bongiovanni A, Nastasi T. (2005) E3 ubiquitin ligases as regulators of membrane protein trafficking and degradation. *Traffic* 6(6):429-441.

Davidson JD, Riley B, Burright EN, Duvick LA, Zoghbi HY, Orr HT. (2000) Identification and characterization of an ataxin-1-interacting protein: A1Up, a ubiquitin-like nuclear protein. *Hum Mol Genet* 9(15):2305-2312.

Daw EW, Payami H, Nemens EJ, Nochlin D, Bird TD, Schellenberg GD, Wijsman EM. (2000) The number of trait loci in late-onset Alzheimer disease. *Am J Hum Genet* 66(1):196-204.

De Jonghe C, Zehr C, Yager D, Prada CM, Younkin S, Hendriks L, Van Broeckhoven C, Eckman CB. (1998) Flemish and Dutch mutations in amyloid beta precursor protein have different effects on amyloid beta secretion. *Neurobiol Dis* 5(4):281-286.

De Strooper B. (2003) Aph-1, Pen-2, and Nicastrin with Presenilin generate an active gamma-Secretase complex. *Neuron* 38(1):9-12.

De Strooper B, Annaert W. (2000) Proteolytic processing and cell biological functions of the amyloid precursor protein. *J Cell Sci* 113(11):1857-1870.

De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F. (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* 391(6665):387-390.

De Strooper B, Vassar R, Golde T. (2010) The secretases: enzymes with therapeutic potential in Alzheimer disease. *Nat Rev Neurol* 6(2):99-107.

de Vrij FM, Fischer DF, van Leeuwen FW, Hol EM. (2004) Protein quality control in Alzheimer's disease by the ubiquitin proteasome system. *Prog Neurobiol* 74(5):249-270.

Demuro A, Mina E, Kayed R, Milton SC, Parker I, Glabe CG. (2005) Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers. *J Biol Chem* 280(17):17294-17300.

Demuro A, Parker I, Stutzmann GE. (2010) Calcium signaling and amyloid toxicity in Alzheimer disease. *J Biol Chem* 285(17):12463-12468.

Deshpande A, Mina E, Glabe C, Busciglio J. (2006) Different conformations of amyloid beta induce neurotoxicity by distinct mechanisms in human cortical neurons. *J Neurosci* 26(22):6011-6018.

Dewachter I, Filipkowski RK, Priller C, Ris L, Neyton J, Croes S, Terwel D, Gysemans M, Devijver H, Borghgraef P, Godaux E, Kaczmarek L, Herms J, Van Leuven F. (2009) Deregulation of NMDA-receptor function and down-stream signaling in APP[V717I] transgenic mice. *Neurobiol Aging* 30(2):241-256.

Doi H, Mitsui K, Kurosawa M, Machida Y, Kuroiwa Y, Nukina N. (2004) Identification of ubiquitin-interacting proteins in purified polyglutamine aggregates. *FEBS Lett* 571(1-3):171-176.

Duff K, Eckman C, Zehr C, Yu X, Prada CM, Perez-tur J, Hutton M, Buee L, Harigaya Y, Yager D, Morgan D, Gordon MN, Holcomb L, Refolo L, Zenk B, Hardy J, Younkin S. (1996) Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. *Nature* 383(6602):710-713.

Duilio A, Faraonio R, Minopoli G, Zambrano N, Russo T. (1998) Fe65L2: a new member of the Fe65 protein family interacting with the intracellular domain of the Alzheimer's beta-amyloid precursor protein. *Biochem J* 330(1):513-519.

Duyckaerts C, Hauw JJ. (1997) Diagnosis and staging of Alzheimer disease. *Neurobiol Aging* 18(4 Suppl):S33-42.

Edbauer D, Willem M, Lammich S, Steiner H, Haass C. (2002) Insulin-degrading enzyme rapidly removes the beta-amyloid precursor protein intracellular domain (AICD). *J Biol Chem* 277(16):13389-13393.

Evans DA, Funkenstein HH, Albert MS, Scherr PA, Cook NR, Chown MJ, Hebert LE, Hennekens CH, Taylor JO. (1989) Prevalence of Alzheimer's disease in a community population of older persons. Higher than previously reported. *JAMA* 262(18):2551-2556.

Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, Myers RH, Pericak-Vance MA, Risch N, van Duijn CM. (1997) Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *JAMA* 278(16):1349-1356.

Fauci AS, Braunwald E, Kasper DL, Hauser SL, Longo DL, Jameson JL, Loscalzo J, editors. 2008. Harrison's Principles of Internal Medicine. 17th ed. New York: McGraw-Hill Professional.

Feng P, Scott CW, Cho NH, Nakamura H, Chung YH, Monteiro MJ, Jung JU. (2004) Kaposi's sarcoma-associated herpesvirus K7 protein targets a ubiquitin-like/ubiquitin-associated domain-containing protein to promote protein degradation. *Mol Cell Biol* 24(9):3938-3948.

Ficklin MB, Zhao S, Feng G. (2005) Ubiquilin-1 regulates nicotine-induced up-regulation of neuronal nicotinic acetylcholine receptors. *J Biol Chem* 280(40):34088-34095.

Findeis MA. (2007) The role of amyloid beta peptide 42 in Alzheimer's disease. *Pharmacol Ther* 116(2):266-286.

Finder VH, Glockshuber R. (2007) Amyloid-beta aggregation. *Neurodegener Dis* 4(1):13-27.

First MB. 2000. Diagnostic and Statistical Manual of Mental Disorders – 4th Ed. (DSM-IV-TR™, 2000). Washington, DC: American Psychiatric Association.

Frydman J, Hohfeld J. (1997) Chaperones get in touch: the Hip-Hop connection. *Trends Biochem Sci* 22(3):87-92.

Funakoshi M, Geley S, Hunt T, Nishimoto T, Kobayashi H. (1999) Identification of XDRP1; a Xenopus protein related to yeast Dsk2p binds to the N-terminus of cyclin A and inhibits its degradation. *EMBO J* 18(18):5009-5018.

Gao L, Tu H, Shi ST, Lee KJ, Asanaka M, Hwang SB, Lai MM. (2003) Interaction with a ubiquitin-like protein enhances the ubiquitination and degradation of hepatitis C virus RNA-dependent RNA polymerase. *J Virol* 77(7):4149-4159.

Ghosal K, Vogt DL, Liang M, Shen Y, Lamb BT, Pimplikar SW. (2009) Alzheimer's disease-like pathological features in transgenic mice expressing the APP intracellular domain. *Proc Natl Acad Sci U S A* 106(43):18367-18372.

Glenner GG, Wong CW. (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun* 120(3):885-890.

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, et al. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349(6311):704-706.

Golan MP, Melquist S, Safranow K, Styczynska M, Slowik A, Kobrys M, Zekanowski C, Barcikowska M. (2008) Analysis of UBQLN1 variants in a Polish Alzheimer's disease patient: control series. *Dement Geriatr Cogn Disord* 25(4):366-371.

Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR. (2000) The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat Cell Biol* 2(3):156-162.

Gosal WS, Myers SL, Radford SE, Thomson NH. (2006) Amyloid under the atomic force microscope. *Protein Pept Lett* 13(3):261-270.

Gralle M, Ferreira ST. (2007) Structure and functions of the human amyloid precursor protein: the whole is more than the sum of its parts. *Prog Neurobiol* 82(1):11-32.

Green KN, LaFerla FM. (2008) Linking calcium to Abeta and Alzheimer's disease. *Neuron* 59(2):190-194.

Guo Q, Fu W, Sopher BL, Miller MW, Ware CB, Martin GM, Mattson MP. (1999) Increased vulnerability of hippocampal neurons to excitotoxic necrosis in presenilin-1 mutant knock-in mice. *Nat Med* 5(1):101-106.

Guo Q, Furukawa K, Sopher BL, Pham DG, Xie J, Robinson N, Martin GM, Mattson MP. (1996) Alzheimer's PS-1 mutation perturbs calcium homeostasis and sensitizes PC12 cells to death induced by amyloid beta-peptide. *Neuroreport* 8(1):379-383.

Haapasalo A, Viswanathan J, Bertram L, Soininen H, Tanzi RE, Hiltunen M. (2010) Emerging role of Alzheimer's disease-associated ubiquilin-1 in protein aggregation. *Biochem Soc Trans* 38(1):150-155.

Haass C, Lemere CA, Capell A, Citron M, Seubert P, Schenk D, Lannfelt L, Selkoe DJ. (1995) The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway. *Nat Med* 1(12):1291-1296.

Hamid R, Kilger E, Willem M, Vassallo N, Kostka M, Bornhovd C, Reichert AS, Kretzschmar HA, Haass C, Herms J. (2007) Amyloid precursor protein intracellular domain modulates cellular calcium homeostasis and ATP content. *J Neurochem* 102(4):1264-1275.

Hanson KA, Kim SH, Wassarman DA, Tibbetts RS. (2010) Ubiquilin modifies TDP-43 toxicity in a Drosophila model of amyotrophic lateral sclerosis (ALS). *J Biol Chem* 285(15):11068-11072.

Hara T, Kamura T, Kotoshiba S, Takahashi H, Fujiwara K, Onoyama I, Shirakawa M, Mizushima N, Nakayama KI. (2005) Role of the UBL-UBA protein KPC2 in degradation of p27 at G1 phase of the cell cycle. *Mol Cell Biol* 25(21):9292-9303.

Hardy J, Selkoe DJ. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297(5580):353-356.

Hartl FU, Hayer-Hartl M. (2009) Converging concepts of protein folding in vitro and in vivo. *Nat Struct Mol Biol* 16(6):574-581.

Hebert LE, Scherr PA, Bienias JL, Bennett DA, Evans DA. (2003) Alzheimer disease in the US population: prevalence estimates using the 2000 census. *Arch Neurol* 60(8):1119-1122.

Heir R, Ablasou C, Dumontier E, Elliott M, Fagotto-Kaufmann C, Bedford FK. (2006) The UBL domain of PLIC-1 regulates aggresome formation. *EMBO Rep* 7(12):1252-1258.

Hendriks L, van Duijn CM, Cras P, Cruts M, Van Hul W, van Harskamp F, Warren A, McInnis MG, Antonarakis SE, Martin JJ, et al. (1992) Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the beta-amyloid precursor protein gene. *Nat Genet* 1(3):218-221.

Herms J, Schneider I, Dewachter I, Caluwaerts N, Kretzschmar H, Van Leuven F. (2003) Capacitive calcium entry is directly attenuated by mutant presenilin-1, independent of the expression of the amyloid precursor protein. *J Biol Chem* 278(4):2484-2489.

Heston LL, Mastri AR, Anderson VE, White J. (1981) Dementia of the Alzheimer type. Clinical genetics, natural history, and associated conditions. *Arch Gen Psychiatry* 38(10):1085-1090.

Hicke L, Dunn R. (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol* 19:141-172.

Hicke L, Schubert HL, Hill CP. (2005) Ubiquitin-binding domains. *Nat Rev Mol Cell Biol* 6(8):610-621.

Hiltunen M, Lu A, Thomas AV, Romano DM, Kim M, Jones PB, Xie Z, Kounnas MZ, Wagner SL, Berezovska O, Hyman BT, Tesco G, Bertram L, Tanzi RE. (2006) Ubiquilin 1 modulates amyloid precursor protein trafficking and abeta secretion. *J Biol Chem* 281(43):32240-32253.

Hofer AM, Machen TE. (1993) Technique for in situ measurement of calcium in intracellular inositol 1,4,5-trisphosphate-sensitive stores using the fluorescent indicator mag-fura-2. *Proc Natl Acad Sci U S A* 90(7):2598-2602.

Hofmann K, Bucher P. (1996) The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway. *Trends Biochem Sci* 21(5):172-173.

Homayouni R, Rice DS, Sheldon M, Curran T. (1999) Disabled-1 binds to the cytoplasmic domain of amyloid precursor-like protein 1. *J Neurosci* 19(17):7507-7515.

Honda T, Yasutake K, Nihonmatsu N, Mercken M, Takahashi H, Murayama O, Murayama M, Sato K, Omori A, Tsubuki S, Saido TC, Takashima A. (1999) Dual roles of proteasome in the metabolism of presenilin 1. *J Neurochem* 72(1):255-261.

Horcas I, Fernandez R, Gomez-Rodriguez JM, Colchero J, Gomez-Herrero J, Baro AM. (2007) WSXM: a software for scanning probe microscopy and a tool for nanotechnology. *Rev Sci Instrum* 78(1):013705.

Hulette CM, Welsh-Bohmer KA, Crain B, Szymanski MH, Sinclaire NO, Roses AD. (1997) Rapid brain autopsy. The Joseph and Kathleen Bryan Alzheimer's Disease Research Center experience. *Arch Pathol Lab Med* 121(6):615-618.

Hunter S, Friedland RP, Brayne C. (2010) Time for a change in the research paradigm for Alzheimer's disease: the value of a chaotic matrix modeling approach. *CNS Neurosci Ther* 16(4):254-262.

Hurley JH, Lee S, Prag G. (2006) Ubiquitin-binding domains. *Biochem J* 399(3):361-372.

Hwang GW, Sasaki D, Naganuma A. (2005) Overexpression of Rad23 confers resistance to methylmercury in saccharomyces cerevisiae via inhibition of

the degradation of ubiquitinated proteins. *Mol Pharmacol* 68(4):1074-1078.

Hyman BT, Trojanowski JQ. (1997) Consensus recommendations for the postmortem diagnosis of Alzheimer disease from the National Institute on Aging and the Reagan Institute Working Group on diagnostic criteria for the neuropathological assessment of Alzheimer disease. *J Neuropathol Exp Neurol* 56(10):1095-1097.

Isaacs AM, Senn DB, Yuan M, Shine JP, Yankner BA. (2006) Acceleration of amyloid beta-peptide aggregation by physiological concentrations of calcium. *J Biol Chem* 281(38):27916-27923.

Ito E, Oka K, Etcheberrigaray R, Nelson TJ, McPhie DL, Tofel-Grehl B, Gibson GE, Alkon DL. (1994) Internal Ca2+ mobilization is altered in fibroblasts from patients with Alzheimer disease. *Proc Natl Acad Sci U S A* 91(2):534-538.

Jakob U, Lilie H, Meyer I, Buchner J. (1995) Transient interaction of Hsp90 with early unfolding intermediates of citrate synthase. Implications for heat shock in vivo. *J Biol Chem* 270(13):7288-7294.

Jan A, Hartley DM, Lashuel HA. (2010) Preparation and characterization of toxic Abeta aggregates for structural and functional studies in Alzheimer's disease research. *Nat Protoc* 5(6):1186-1209.

Janssen JC, Beck JA, Campbell TA, Dickinson A, Fox NC, Harvey RJ, Houlden H, Rossor MN, Collinge J. (2003) Early onset familial Alzheimer's disease: Mutation frequency in 31 families. *Neurology* 60(2):235-239.

Johnston JA, Ward CL, Kopito RR. (1998) Aggresomes: a cellular response to misfolded proteins. *J Cell Biol* 143(7):1883-1898.

Kamal A, Stokin GB, Yang Z, Xia CH, Goldstein LS. (2000) Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. *Neuron* 28(2):449-459.

Kamboh MI, Minster RL, Feingold E, DeKosky ST. (2006) Genetic association of ubiquilin with Alzheimer's disease and related quantitative measures. *Mol Psychiatry* 11(3):273-279.

Kamino K, Orr HT, Payami H, Wijsman EM, Alonso ME, Pulst SM, Anderson L, O'Dahl S, Nemens E, White JA, et al. (1992) Linkage and mutational analysis of familial Alzheimer disease kindreds for the APP gene region. *Am J Hum Genet* 51(5):998-1014.

Kamionka M, Feigon J. (2004) Structure of the XPC binding domain of hHR23A reveals hydrophobic patches for protein interaction. *Protein Sci* 13(9):2370-2377.

Kandel ER. (2001) The molecular biology of memory storage: a dialogue between genes and synapses. *Science* 294(5544):1030-1038.

Kandel ER, Schwartz JH, Jessell TM, editors. 2000. Principles of Neural Science. 4th ed. New York: McGraw-Hill Health Professions Division.

Kang Y, Zhang N, Koepp DM, Walters KJ. (2007) Ubiquitin receptor proteins hHR23a and hPLIC2 interact. *J Mol Biol* 365(4):1093-1101.

Kelly BL, Ferreira A. (2006) beta-Amyloid-induced dynamin 1 degradation is mediated by N-methyl-D-aspartate receptors in hippocampal neurons. *J Biol Chem* 281(38):28079-28089.

Kerr ML, Small DH. (2005) Cytoplasmic domain of the beta-amyloid protein precursor of Alzheimer's disease: function, regulation of proteolysis, and implications for drug development. *J Neurosci Res* 80(2):151-159.

Khachaturian ZS. (1984) Scientific challenges and opportunities related to Alzheimer's disease. *Clin Pharm* 3(5):522-523.

Khachaturian ZS. (1987) Hypothesis on the regulation of cytosol calcium concentration and the aging brain. *Neurobiol Aging* 8(4):345-346.

Kim I, Xu W, Reed JC. (2008a) Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov* 7(12):1013-1030.

Kim J, Basak JM, Holtzman DM. (2009a) The role of apolipoprotein E in Alzheimer's disease. *Neuron* 63(3):287-303.

Kim SH, Shi Y, Hanson KA, Williams LM, Sakasai R, Bowler MJ, Tibbetts RS. (2009b) Potentiation of amyotrophic lateral sclerosis (ALS)-associated TDP-43 aggregation by the proteasome-targeting factor, ubiquilin 1. *J Biol Chem* 284(12):8083-8092.

Kim TY, Kim E, Yoon SK, Yoon JB. (2008b) Herp enhances ER-associated protein degradation by recruiting ubiquilins. *Biochem Biophys Res Commun* 369(2):741-746.

Kimberly WT, Xia W, Rahmati T, Wolfe MS, Selkoe DJ. (2000) The transmembrane aspartates in presenilin 1 and 2 are obligatory for gamma-

secretase activity and amyloid beta-protein generation. *J Biol Chem* 275(5):3173-3178.

Kimberly WT, Zheng JB, Guenette SY, Selkoe DJ. (2001) The intracellular domain of the beta-amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in a notch-like manner. *J Biol Chem* 276(43):40288-40292.

Kleijnen MF, Shih AH, Zhou P, Kumar S, Soccio RE, Kedersha NL, Gill G, Howley PM. (2000) The hPLIC proteins may provide a link between the ubiquitination machinery and the proteasome. *Mol Cell* 6(2):409-419.

Knopman DS, DeKosky ST, Cummings JL, Chui H, Corey-Bloom J, Relkin N, Small GW, Miller B, Stevens JC. (2001) Practice parameter: diagnosis of dementia (an evidence-based review). Report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* 56(9):1143-1153.

Knops J, Lieberburg I, Sinha S. (1992) Evidence for a nonsecretory, acidic degradation pathway for amyloid precursor protein in 293 cells. Identification of a novel, 22-kDa, beta-peptide-containing intermediate. *J Biol Chem* 267(23):16022-16024.

Ko HS, Uehara T, Nomura Y. (2002) Role of ubiquilin associated with protein-disulfide isomerase in the endoplasmic reticulum in stress-induced apoptotic cell death. *J Biol Chem* 277(38):35386-35392.

Kopito RR. (2000) Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol* 10(12):524-530.

Koren J, 3rd, Jinwal UK, Lee DC, Jones JR, Shults CL, Johnson AG, Anderson LJ, Dickey CA. (2009) Chaperone signalling complexes in Alzheimer's disease. *J Cell Mol Med* 13(4):619-630.

Kroenke CD, Ziemnicka-Kotula D, Xu J, Kotula L, Palmer AG, 3rd. (1997) Solution conformations of a peptide containing the cytoplasmic domain sequence of the beta amyloid precursor protein. *Biochemistry* 36(26):8145-8152.

Kuchibhotla KV, Goldman ST, Lattarulo CR, Wu HY, Hyman BT, Bacskai BJ. (2008) Abeta plaques lead to aberrant regulation of calcium homeostasis in vivo resulting in structural and functional disruption of neuronal networks. *Neuron* 59(2):214-225.

Kumar P, Ambasta RK, Veereshwarayya V, Rosen KM, Kosik KS, Band H, Mestril R, Patterson C, Querfurth HW. (2007) CHIP and HSPs interact with beta-APP in a proteasome-dependent manner and influence Abeta metabolism. *Hum Mol Genet* 16(7):848-864.

Kumar R, Yang J, Larsen RD, Stanley P. (1990) Cloning and expression of N-acetylglucosaminyltransferase I, the medial Golgi transferase that initiates complex N-linked carbohydrate formation. *Proc Natl Acad Sci U S A* 87(24):9948-9952.

Kumar V, Abbas AK, Fausto N, Aster J, editors. 2009. Robbins and Cotran Pathologic Basis of Disease. 8th ed. Philadelphia, PA: Saunders Elsevier.

LaFerla FM. (2002) Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease. *Nat Rev Neurosci* 3(11):862-872.

Lai A, Sisodia SS, Trowbridge IS. (1995) Characterization of sorting signals in the beta-amyloid precursor protein cytoplasmic domain. *J Biol Chem* 270(8):3565-3573.

Lauwers E, Jacob C, Andre B. (2009) K63-linked ubiquitin chains as a specific signal for protein sorting into the multivesicular body pathway. *J Cell Biol* 185(3):493-502.

Leissring MA, Akbari Y, Fanger CM, Cahalan MD, Mattson MP, LaFerla FM. (2000) Capacitative calcium entry deficits and elevated luminal calcium content in mutant presenilin-1 knockin mice. *J Cell Biol* 149(4):793-798.

Leissring MA, Murphy MP, Mead TR, Akbari Y, Sugarman MC, Jannatipour M, Anliker B, Muller U, Saftig P, De Strooper B, Wolfe MS, Golde TE, LaFerla FM. (2002) A physiologic signaling role for the gamma -secretase-derived intracellular fragment of APP. *Proc Natl Acad Sci U S A* 99(7):4697-4702.

Leissring MA, Parker I, LaFerla FM. (1999a) Presenilin-2 mutations modulate amplitude and kinetics of inositol 1, 4,5-trisphosphate-mediated calcium signals. *J Biol Chem* 274(46):32535-32538.

Leissring MA, Paul BA, Parker I, Cotman CW, LaFerla FM. (1999b) Alzheimer's presenilin-1 mutation potentiates inositol 1,4,5-trisphosphate-mediated calcium signaling in Xenopus oocytes. *J Neurochem* 72(3):1061-1068.

Lesk AM. (2003) Hydrophobicity--getting into hot water. *Biophys Chem* 105(2-3):179-182.

Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, Yu CE, Jondro PD, Schmidt SD, Wang K, et al. (1995) Candidate gene for the

chromosome 1 familial Alzheimer's disease locus. *Science* 269(5226):973-977.

Lim PJ, Danner R, Liang J, Doong H, Harman C, Srinivasan D, Rothenberg C, Wang H, Ye Y, Fang S, Monteiro MJ. (2009) Ubiquilin and p97/VCP bind erasin, forming a complex involved in ERAD. *J Cell Biol* 187(2):201-217.

Lopez JR, Lyckman A, Oddo S, Laferla FM, Querfurth HW, Shtifman A. (2008) Increased intraneuronal resting [Ca2+] in adult Alzheimer's disease mice. *J Neurochem* 105(1):262-271.

Lopez Salon M, Morelli L, Castano EM, Soto EF, Pasquini JM. (2000) Defective ubiquitination of cerebral proteins in Alzheimer's disease. *J Neurosci Res* 62(2):302-310.

Mah AL, Perry G, Smith MA, Monteiro MJ. (2000) Identification of ubiquilin, a novel presenilin interactor that increases presenilin protein accumulation. *J Cell Biol* 151(4):847-862.

Massey LK, Mah AL, Ford DL, Miller J, Liang J, Doong H, Monteiro MJ. (2004) Overexpression of ubiquilin decreases ubiquitination and degradation of presenilin proteins. *J Alzheimers Dis* 6(1):79-92.

Massey LK, Mah AL, Monteiro MJ. (2005) Ubiquilin regulates presenilin endoproteolysis and modulates gamma-secretase components, Pen-2 and nicastrin. *Biochem J* 391(3):513-525.

Mattson MP. (2004) Pathways towards and away from Alzheimer's disease. *Nature* 430(7000):631-639.

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 34(7):939-944.

Medicherla B, Kostova Z, Schaefer A, Wolf DH. (2004) A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. *EMBO Rep* 5(7):692-697.

Miller CC, McLoughlin DM, Lau KF, Tennant ME, Rogelj B. (2006) The X11 proteins, Abeta production and Alzheimer's disease. *Trends Neurosci* 29(5):280-285.

Mirra SS, Hart MN, Terry RD. (1993) Making the diagnosis of Alzheimer's disease. A primer for practicing pathologists. *Arch Pathol Lab Med* 117(2):132-144.

Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L. (1991) The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* 41(4):479-486.

Mogami H, Tepikin AV, Petersen OH. (1998) Termination of cytosolic Ca2+ signals: Ca2+ reuptake into intracellular stores is regulated by the free Ca2+ concentration in the store lumen. *EMBO J* 17(2):435-442.

Mueller TD, Feigon J. (2003) Structural determinants for the binding of ubiquitin-like domains to the proteasome. *EMBO J* 22(18):4634-4645.

Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L. (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. *Nat Genet* 1(5):345-347.

Muller T, Concannon CG, Ward MW, Walsh CM, Tirniceriu AL, Tribl F, Kogel D, Prehn JH, Egensperger R. (2007) Modulation of gene expression and cytoskeletal dynamics by the amyloid precursor protein intracellular domain (AICD). *Mol Biol Cell* 18(1):201-210.

Muller T, Meyer HE, Egensperger R, Marcus K. (2008) The amyloid precursor protein intracellular domain (AICD) as modulator of gene expression, apoptosis, and cytoskeletal dynamics-relevance for Alzheimer's disease. *Prog Neurobiol* 85(4):393-406.

Murrell J, Farlow M, Ghetti B, Benson MD. (1991) A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* 254(5028):97-99.

Murrell JR, Hake AM, Quaid KA, Farlow MR, Ghetti B. (2000) Early-onset Alzheimer disease caused by a new mutation (V717L) in the amyloid precursor protein gene. *Arch Neurol* 57(6):885-887.

N'Diaye EN, Debnath J, Brown EJ. (2009a) Ubiquilins accelerate autophagosome maturation and promote cell survival during nutrient starvation. *Autophagy* 5(4):573-575.

N'Diaye EN, Hanyaloglu AC, Kajihara KK, Puthenveedu MA, Wu P, von Zastrow M, Brown EJ. (2008) The ubiquitin-like protein PLIC-2 is a negative regulator of G protein-coupled receptor endocytosis. *Mol Biol Cell* 19(3):1252-1260.

N'Diaye EN, Kajihara KK, Hsieh I, Morisaki H, Debnath J, Brown EJ. (2009b) PLIC proteins or ubiquilins regulate autophagy-dependent cell survival during nutrient starvation. *EMBO Rep* 10(2):173-179.

Nakayama K, Ohkawara T, Hiratochi M, Koh CS, Nagase H. (2008) The intracellular domain of amyloid precursor protein induces neuron-specific apoptosis. *Neurosci Lett* 444(2):127-131.

Nelson O, Tu H, Lei T, Bentahir M, de Strooper B, Bezprozvanny I. (2007) Familial Alzheimer disease-linked mutations specifically disrupt Ca2+ leak function of presenilin 1. *J Clin Invest* 117(5):1230-1239.

Nilsberth C, Westlind-Danielsson A, Eckman CB, Condron MM, Axelman K, Forsell C, Stenh C, Luthman J, Teplow DB, Younkin SG, Naslund J, Lannfelt L. (2001) The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Abeta protofibril formation. *Nat Neurosci* 4(9):887-893.

Nunan J, Shearman MS, Checler F, Cappai R, Evin G, Beyreuther K, Masters CL, Small DH. (2001) The C-terminal fragment of the Alzheimer's disease amyloid protein precursor is degraded by a proteasome-dependent mechanism distinct from gamma-secretase. *Eur J Biochem* 268(20):5329-5336.

Nunan J, Williamson NA, Hill AF, Sernee MF, Masters CL, Small DH. (2003) Proteasome-mediated degradation of the C-terminus of the Alzheimer's disease beta-amyloid protein precursor: effect of C-terminal truncation on production of beta-amyloid protein. *J Neurosci Res* 74(3):378-385.

Odunuga OO, Longshaw VM, Blatch GL. (2004) Hop: more than an Hsp70/Hsp90 adaptor protein. *Bioessays* 26(10):1058-1068.

Ohno A, Jee J, Fujiwara K, Tenno T, Goda N, Tochio H, Kobayashi H, Hiroaki H, Shirakawa M. (2005) Structure of the UBA domain of Dsk2p in complex with ubiquitin molecular determinants for ubiquitin recognition. *Structure* 13(4):521-532.

Ortolan TG, Tongaonkar P, Lambertson D, Chen L, Schauber C, Madura K. (2000) The DNA repair protein rad23 is a negative regulator of multi-ubiquitin chain assembly. *Nat Cell Biol* 2(9):601-608.

Ozaki T, Li Y, Kikuchi H, Tomita T, Iwatsubo T, Nakagawara A. (2006) The intracellular domain of the amyloid precursor protein (AICD) enhances the p53-mediated apoptosis. *Biochem Biophys Res Commun* 351(1):57-63.

Park MK, Choi YM, Kang YK, Petersen OH. (2008) The endoplasmic reticulum as an integrator of multiple dendritic events. *Neuroscientist* 14(1):68-77.

Pedersen NL. (2010) Reaching the limits of genome-wide significance in Alzheimer disease: back to the environment. *JAMA* 303(18):1864-1865.

Perez RG, Soriano S, Hayes JD, Ostaszewski B, Xia W, Selkoe DJ, Chen X, Stokin GB, Koo EH. (1999) Mutagenesis identifies new signals for beta-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including Abeta42. *J Biol Chem* 274(27):18851-18856.

Putney JW, Jr. (2007) Recent breakthroughs in the molecular mechanism of capacitative calcium entry (with thoughts on how we got here). *Cell Calcium* 42(2):103-110.

Querfurth HW, Jiang J, Geiger JD, Selkoe DJ. (1997) Caffeine stimulates amyloid beta-peptide release from beta-amyloid precursor protein-transfected HEK293 cells. *J Neurochem* 69(4):1580-1591.

Querfurth HW, LaFerla FM. (2010) Alzheimer's disease. *N Engl J Med* 362(4):329-344.

Querfurth HW, Selkoe DJ. (1994) Calcium ionophore increases amyloid beta peptide production by cultured cells. *Biochemistry* 33(15):4550-4561.

Raasi S, Varadan R, Fushman D, Pickart CM. (2005) Diverse polyubiquitin interaction properties of ubiquitin-associated domains. *Nat Struct Mol Biol* 12(8):708-714.

Ramelot TA, Gentile LN, Nicholson LK. (2000) Transient structure of the amyloid precursor protein cytoplasmic tail indicates preordering of structure for binding to cytosolic factors. *Biochemistry* 39(10):2714-2725.

Raux G, Guyant-Marechal L, Martin C, Bou J, Penet C, Brice A, Hannequin D, Frebourg T, Campion D. (2005) Molecular diagnosis of autosomal dominant early onset Alzheimer's disease: an update. *J Med Genet* 42(10):793-795.

Regan-Klapisz E, Sorokina I, Voortman J, de Keizer P, Roovers RC, Verheesen P, Urbe S, Fallon L, Fon EA, Verkleij A, Benmerah A, van Bergen en Henegouwen PM. (2005) Ubiquilin recruits Eps15 into ubiquitin-rich cytoplasmic aggregates via a UIM-UBL interaction. *J Cell Sci* 118(19):4437-4450.

Reinhard C, Hebert SS, De Strooper B. (2005) The amyloid-beta precursor protein: integrating structure with biological function. *EMBO J* 24(23):3996-4006.

Rojas G, Cardenas AM, Fernandez-Olivares P, Shimahara T, Segura-Aguilar J, Caviedes R, Caviedes P. (2008) Effect of the knockdown of amyloid precursor protein on intracellular calcium increases in a neuronal cell line derived from the cerebral cortex of a trisomy 16 mouse. *Exp Neurol* 209(1):234-242.

Sabo SL, Lanier LM, Ikin AF, Khorkova O, Sahasrabudhe S, Greengard P, Buxbaum JD. (1999) Regulation of beta-amyloid secretion by FE65, an amyloid protein precursor-binding protein. *J Biol Chem* 274(12):7952-7957.

Saito Y, Sano Y, Vassar R, Gandy S, Nakaya T, Yamamoto T, Suzuki T. (2008) X11 proteins regulate the translocation of amyloid beta-protein precursor (APP) into detergent-resistant membrane and suppress the amyloidogenic cleavage of APP by beta-site-cleaving enzyme in brain. *J Biol Chem* 283(51):35763-35771.

Saliba RS, Pangalos M, Moss SJ. (2008) The ubiquitin-like protein Plic-1 enhances the membrane insertion of GABAA receptors by increasing their stability within the endoplasmic reticulum. *J Biol Chem* 283(27):18538-18544.

Sasaki T, Funakoshi M, Endicott JA, Kobayashi H. (2005) Budding yeast Dsk2 protein forms a homodimer via its C-terminal UBA domain. *Biochem Biophys Res Commun* 336(2):530-535.

Schauber C, Chen L, Tongaonkar P, Vega I, Lambertson D, Potts W, Madura K. (1998) Rad23 links DNA repair to the ubiquitin/proteasome pathway. *Nature* 391(6668):715-718.

Scheuner D, Eckman C, Jensen M, Song X, Citron M, Suzuki N, Bird TD, Hardy J, Hutton M, Kukull W, Larson E, Levy-Lahad E, Viitanen M, Peskind E, Poorkaj P, Schellenberg G, Tanzi R, Wasco W, Lannfelt L, Selkoe D, Younkin S. (1996) Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat Med* 2(8):864-870.

Schmidt V, Sporbert A, Rohe M, Reimer T, Rehm A, Andersen OM, Willnow TE. (2007) SorLA/LR11 regulates processing of amyloid precursor protein via

interaction with adaptors GGA and PACS-1. *J Biol Chem* 282(45):32956-32964.

Selkoe DJ. (2005) Defining molecular targets to prevent Alzheimer disease. *Arch Neurol* 62(2):192-195.

Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, et al. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375(6534):754-760.

Sisodia SS, St George-Hyslop PH. (2002) gamma-Secretase, Notch, Abeta and Alzheimer's disease: where do the presenilins fit in? *Nat Rev Neurosci* 3(4):281-290.

Slifer MA, Martin ER, Bronson PG, Browning-Large C, Doraiswamy PM, Welsh-Bohmer KA, Gilbert JR, Haines JL, Pericak-Vance MA. (2006) Lack of association between UBQLN1 and Alzheimer disease. *Am J Med Genet B Neuropsychiatr Genet* 141(3):208-213.

Slifer MA, Martin ER, Haines JL, Pericak-Vance MA. (2005) The ubiquilin 1 gene and Alzheimer's disease. *N Engl J Med* 352(26):2752-2753; author reply 2752-2753.

Small SA, Gandy S. (2006) Sorting through the cell biology of Alzheimer's disease: intracellular pathways to pathogenesis. *Neuron* 52(1):15-31.

Smemo S, Nowotny P, Hinrichs AL, Kauwe JS, Cherny S, Erickson K, Myers AJ, Kaleem M, Marlowe L, Gibson AM, Hollingworth P, O'Donovan MC, Morris CM, Holmans P, Lovestone S, Morris JC, Thal L, Li Y, Grupe A, Hardy J, Owen MJ, Williams J, Goate A. (2006) Ubiquilin 1 polymorphisms are not associated with late-onset Alzheimer's disease. *Ann Neurol* 59(1):21-26.

Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK, Greengard P. (2005) Regulation of NMDA receptor trafficking by amyloid-beta. *Nat Neurosci* 8(8):1051-1058.

Solovyova N, Fernyhough P, Glazner G, Verkhratsky A. (2002) Xestospongin C empties the ER calcium store but does not inhibit InsP3-induced Ca2+ release in cultured dorsal root ganglia neurones. *Cell Calcium* 32(1):49-52.

Song S, Jung YK. (2004) Alzheimer's disease meets the ubiquitin-proteasome system. *Trends Mol Med* 10(11):565-570.

Sperling RA, Dickerson BC, Pihlajamaki M, Vannini P, LaViolette PS, Vitolo OV, Hedden T, Becker JA, Rentz DM, Selkoe DJ, Johnson KA. (2010) Functional alterations in memory networks in early Alzheimer's disease. *Neuromolecular Med* 12(1):27-43.

Spoelgen R, von Arnim CA, Thomas AV, Peltan ID, Koker M, Deng A, Irizarry MC, Andersen OM, Willnow TE, Hyman BT. (2006) Interaction of the cytosolic domains of sorLA/LR11 with the amyloid precursor protein (APP) and beta-secretase beta-site APP-cleaving enzyme. *J Neurosci* 26(2):418-428.

St George-Hyslop PH, Tanzi RE, Polinsky RJ, Haines JL, Nee L, Watkins PC, Myers RH, Feldman RG, Pollen D, Drachman D, et al. (1987) The genetic defect causing familial Alzheimer's disease maps on chromosome 21. *Science* 235(4791):885-890.

Stefani M, Dobson CM. (2003) Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. *J Mol Med* 81(11):678-699.

Stieren E, Werchan WP, El Ayadi A, Li F, Boehning D. (2010) FAD mutations in amyloid precursor protein do not directly perturb intracellular calcium homeostasis. *PLoS One* 5(8):e11992.

Strittmatter WJ, Roses AD. (1996) Apolipoprotein E and Alzheimer's disease. *Annu Rev Neurosci* 19:53-77.

Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD. (1993) Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci U S A* 90(5):1977-1981.

Su V, Lau AF. (2009) Ubiquitin-like and ubiquitin-associated domain proteins: significance in proteasomal degradation. *Cell Mol Life Sci* 66(17):2819-2833.

Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L, Jr., Eckman C, Golde TE, Younkin SG. (1994) An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. *Science* 264(5163):1336-1340.

Suzuki T, Araki Y, Yamamoto T, Nakaya T. (2006) Trafficking of Alzheimer's disease-related membrane proteins and its participation in disease pathogenesis. *J Biochem (Tokyo)* 139(6):949-955.

Tamaoka A, Odaka A, Ishibashi Y, Usami M, Sahara N, Suzuki N, Nukina N, Mizusawa H, Shoji S, Kanazawa I, et al. (1994) APP717 missense mutation affects the ratio of amyloid beta protein species (A beta 1-42/43 and a beta 1-40) in familial Alzheimer's disease brain. *J Biol Chem* 269(52):32721-32724.

Tanzi RE, Bertram L. (2005) Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. *Cell* 120(4):545-555.

Taru H, Iijima K, Hase M, Kirino Y, Yagi Y, Suzuki T. (2002) Interaction of Alzheimer's beta -amyloid precursor family proteins with scaffold proteins of the JNK signaling cascade. *J Biol Chem* 277(22):20070-20078.

Toescu EC, Verkhratsky A. (2007) The importance of being subtle: small changes in calcium homeostasis control cognitive decline in normal aging. *Aging Cell* 6(3):267-273.

Tomita S, Kirino Y, Suzuki T. (1998) Cleavage of Alzheimer's amyloid precursor protein (APP) by secretases occurs after O-glycosylation of APP in the protein secretory pathway. Identification of intracellular compartments in which APP cleavage occurs without using toxic agents that interfere with protein metabolism. *J Biol Chem* 273(11):6277-6284.

Tu H, Nelson O, Bezprozvanny A, Wang Z, Lee SF, Hao YH, Serneels L, De Strooper B, Yu G, Bezprozvanny I. (2006) Presenilins form ER Ca2+ leak channels, a function disrupted by familial Alzheimer's disease-linked mutations. *Cell* 126(5):981-993.

Umebayashi K. (2003) The roles of ubiquitin and lipids in protein sorting along the endocytic pathway. *Cell Struct Funct* 28(5):443-453.

Van Broeckhoven C, Haan J, Bakker E, Hardy JA, Van Hul W, Wehnert A, Vegter-Van der Vlis M, Roos RA. (1990) Amyloid beta protein precursor gene and hereditary cerebral hemorrhage with amyloidosis (Dutch). *Science* 248(4959):1120-1122.

van Marum RJ. (2009) Update on the use of memantine in Alzheimer's disease. *Neuropsychiatr Dis Treat* 5:237-247.

Vembar SS, Brodsky JL. (2008) One step at a time: endoplasmic reticulum-associated degradation. *Nat Rev Mol Cell Biol* 9(12):944-957.

Verkhratsky A, Mattson MP, Toescu EC. (2004) Aging in the mind. *Trends Neurosci* 27(10):577-578.

Vetrivel KS, Thinakaran G. (2006) Amyloidogenic processing of beta-amyloid precursor protein in intracellular compartments. *Neurology* 66(2 Suppl 1):S69-73.

von Kugelgen I. (2006) Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. *Pharmacol Ther* 110(3):415-432.

von Rotz RC, Kohli BM, Bosset J, Meier M, Suzuki T, Nitsch RM, Konietzko U. (2004) The APP intracellular domain forms nuclear multiprotein complexes and regulates the transcription of its own precursor. *J Cell Sci* 117(19):4435-4448.

Walters KJ, Kleijnen MF, Goh AM, Wagner G, Howley PM. (2002) Structural studies of the interaction between ubiquitin family proteins and proteasome subunit S5a. *Biochemistry* 41(6):1767-1777.

Wang H, Lim PJ, Yin C, Rieckher M, Vogel BE, Monteiro MJ. (2006) Suppression of polyglutamine-induced toxicity in cell and animal models of Huntington's disease by ubiquilin. *Hum Mol Genet* 15(6):1025-1041.

Wang H, Monteiro MJ. (2007a) Ubiquilin interacts and enhances the degradation of expanded-polyglutamine proteins. *Biochem Biophys Res Commun* 360(2):423-427.

Wang H, Monteiro MJ. (2007b) Ubiquilin overexpression reduces GFP-polyalanine-induced protein aggregates and toxicity. *Exp Cell Res* 313(13):2810-2820.

Watkins JF, Sung P, Prakash L, Prakash S. (1993) The Saccharomyces cerevisiae DNA repair gene RAD23 encodes a nuclear protein containing a ubiquitin-like domain required for biological function. *Mol Cell Biol* 13(12):7757-7765.

Weidemann A, Konig G, Bunke D, Fischer P, Salbaum JM, Masters CL, Beyreuther K. (1989) Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* 57(1):115-126.

Wilkinson CR, Seeger M, Hartmann-Petersen R, Stone M, Wallace M, Semple C, Gordon C. (2001) Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. *Nat Cell Biol* 3(10):939-943.

Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ. (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. *Nature* 398(6727):513-517.

Wozniak AL, Wang X, Stieren ES, Scarbrough SG, Elferink CJ, Boehning D. (2006) Requirement of biphasic calcium release from the endoplasmic reticulum for Fas-mediated apoptosis. *J Cell Biol* 175(5):709-714.

Wu AL, Wang J, Zheleznyak A, Brown EJ. (1999) Ubiquitin-related proteins regulate interaction of vimentin intermediate filaments with the plasma membrane. *Mol Cell* 4(4):619-625.

Wu S, Mikhailov A, Kallo-Hosein H, Hara K, Yonezawa K, Avruch J. (2002) Characterization of ubiquilin 1, an mTOR-interacting protein. *Biochim Biophys Acta* 1542(1-3):41-56.

Zhang D, Raasi S, Fushman D. (2008) Affinity makes the difference: nonselective interaction of the UBA domain of Ubiquilin-1 with monomeric ubiquitin and polyubiquitin chains. *J Mol Biol* 377(1):162-180.

Zhao G, Zhou X, Wang L, Li G, Kisker C, Lennarz WJ, Schindelin H. (2006) Structure of the mouse peptide N-glycanase-HR23 complex suggests co-evolution of the endoplasmic reticulum-associated degradation and DNA repair pathways. *J Biol Chem* 281(19):13751-13761.

# VITA

Emily S. Stieren was born on March 15, 1980 in Clearwater, Florida to Angelika Stieren and Gerald W. Stieren, Jr. After graduating from John Foster Dulles High School in Sugar Land, Texas, Ms. Stieren attended the University of Miami in Coral Gables, Florida. She graduated summa cum laude in May, 2002 with a Bachelor of Science in Psychobiology. During her undergraduate studies, she conducted research in the Department of Behavioral Medicine under the mentorship of Dr. Gail Ironson, and she earned departmental honors for completing an undergraduate thesis entitled "*Cortisol- and stress-mediated effects of initiation of HAART on CD4$^+$ cell level and disease progression in HIV-positive individuals*." After graduation, she took a position as a research technician in the Department of Pediatrics at Baylor College of Medicine in the laboratory of Dr. Qiang Tong. Ms. Stieren enrolled in the M.D.-Ph.D. Combined Degree Program at UTMB in the summer of 2004. After completing the first two years of medical school, she joined the laboratory of Dr. Darren Boehning to complete her dissertation research. During her graduate school career, Ms. Stieren has earned several honors and awards. Her research has been funded by a predoctoral National Research Service Award from the National Institute on Aging and the Jean C. and William D. Willis Neuroscience Research Endowment. Ms. Stieren has been awarded the Arthur V. Simmang Academic Scholarship (2007), the Leroy Olson, Ph.D. Endowed Scholarship (2007), the Jen Chieh and Katherine Huang Scholarship (2009), and the Dr. and Mrs. Seymour Fisher Academic Excellence Award (2010) by the UTMB Graduate School of Biomedical Sciences. Ms. Stieren has been active in tutoring medical

students in neuroscience, cardiovascular, and pulmonology courses. She has also served the UTMB community as a student representative in the Neuroscience Graduate Program and the Graduate Student Organization.

Permanent address: 3400 Edloe St. #1904, Houston, Texas, 77027

This dissertation was typed by Emily S. Stieren.