INVESTIGATING THE ROLE OF ATF6 IN ER STRESS IN AN

IN VITRO MODEL OF ALZHEIMER'S DISEASE

by

Ondine Eken

B.S. (University of Maryland Eastern Shore) 2015

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

May 2019

Accepted:

_____	_____
Oney Smith, Ph.D.	Ann Boyd, Ph.D.
Committee Member	Director, Biomedical Science Program

_____
Ann Boyd, Ph.D.
Committee Member

_____	_____
Georgette Jones, Ph.D.	April Boulton, Ph.D.
Project Adviser	Dean of the Graduate School

STATEMENT OF USE AND COPYRIGHT WAIVER

I do not authorize Hood College to lend this mock grant proposal, or reproductions of it, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

DEDICATION

I would like to dedicate this to my family and friends who have constantly been dedicated and supportive in seeing me accomplish my goals throughout this program. Your love is gratefully appreciated.

ACKNOWLEDGEMENTS

I would like to acknowledge my committee for lending a helping hand while working on this project. I would also like to especially thank Dr. Georgette Jones who has been exceptional supportive and patient throughout the entire process.

Mock Grant Application Modeled after Department of Health and Human Services Public Health Services (based on Form PHS 398)	**LEAVE BLANK—FOR OFFICIAL USE ONLY**.		
	Type	Activity	Version – HCBMS.011712
	Review Group		Formerly
	Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*

   Investigating the role of ATF6 in ER stress in an *in vitro* model of Alzheimer's Disease

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
   (If "Yes," state number and title)

   Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR   **New Investigator** ☐ No ☒ Yes

3a. NAME (Last, first, middle) Ondine Eken	3b. DEGREE(S)	3h. eRA Commons User Name N/A

3c. POSITION TITLE Graduate Student	3d. MAILING ADDRESS *(Street, city, state, zip code)* 401 Rosemont Ave. Frederick, MD 21201
3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT Department of Biology	
3f. MAJOR SUBDIVISION Biomedical Science Program	
3g. TELEPHONE AND FAX *(Area code, number and extension)* TEL: N/A FAX: N/A	E-MAIL ADDRESS:

4. HUMAN SUBJECTS RESEARCH ☒ No ☐ Yes	4a. Research Exempt N/A	
4b. Federal-Wide Assurance No. N/A	4c. Clinical Trial ☒ No ☐ Yes	4d. NIH-defined Phase III Clinical Trial ☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes	5a. Animal Welfare Assurance No. N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)

9. APPLICANT ORGANIZATION	10. TYPE OF ORGANIZATION
Name Address Department of Biology Hood College 401 Rosemont Ave Frederick, MD 21701	Public: → ☐ Federal ☐ State ☐ Local Private: → ☒ Private Nonprofit For-profit: → ☐ General ☐ Small Business ☐ Woman-owned ☐ Socially and Economically Disadvantaged
	11. ENTITY IDENTIFICATION NUMBER
	DUNS NO. N/A Cong. District N/A

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE	13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name	Name
Title Ondine Eken	Title Ondine Eken
Address Hood College 401 Rosemont Ave Frederick, MD 21701	Address Hood College 401 Rosemont Ave Frederick, MD 21701
Tel: N/A FAX: N/A	Tel: N/A FAX: N/A
E-Mail: Ondine.Eken@gmail.com	E-Mail: Ondine.Eken@gmail.com

14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.	SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)* N/A	DATE April 15, 2019

Face Page

PROJECT SUMMARY (See instructions):

Alzheimer's disease (AD) is a neurodegenerative disease that is characterized by the accumulation of tau and amyloid β (Aβ) protein in the brain, forming neurofibrillary tangles and amyloid plaques, respectively. The endoplasmic reticulum (ER) has many critical functions such as protein processing, protein quality control, and protein folding. The ER sensors RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) are able to recognize Aβ, misfolded proteins and initiates the Unfolded Protein Response (UPR). UPR plays a role in recovering homeostasis or triggering apoptosis of irreversibly damaged cells. There have been numerous studies involving PERK and IRE1, but little is known about ATF6 and its involvement with AD. Therefore, to better understand ER stressor ATF6 in AD, suppression of ATF6 with shRNA (short hairpin RNA) in neuroblastoma cell line (SH-S5SY) treated with Aβ will be investigated. First, basal levels of ER stress will be measured to confirm the cells are an appropriate cell culture model for AD. This will be followed by knock down (KD) of ATF6 by shRNA and confirmation. Lastly, rescue of ATF6 will be performed to confirm that ATF6 is the cause of the downstream affects after silencing. Overall, these findings can contribute to the study and understanding ER stress sensor ATF6 and how delivery of shRNAs can aid in the regression of Aβ, leading closer to understanding the molecular mechanism of AD.

RELEVANCE (See instructions):

Markers of ER stress are very common in many neurodegenerative diseases as well as the accumulation of Aβ. The ability to understand the role of ATF6 in an *in vitro* model of AD would help in creating potential therapies for AD and for other neurodegenerative disease as well.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location

Organizational Name: Department of Biology, Hood College

DUNS:

Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States	Zip/Postal Code: 21702	

Project/Performance Site Congressional Districts: 6th Congressional District

Additional Project/Performance Site Location

Organizational Name:

DUNS:

Street 1:		Street 2:	
City:	County:		State:
Province:	Country:	Zip/Postal Code:	

Project/Performance Site Congressional Districts:

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Ondine Eken	POSITION TITLE Graduate student
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Maryland Eastern Shore, Princess Anne, MD	B.S.	2015	Biology
Hood College, Frederick, MD	M.S.	2019 (anticipated)	Biomedical Science

A. Positions and Honors

B. Selected peer-reviewed publications (in chronological order)

C. Research Support

Biographical Sketch Format Page

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:
N/A

Clinical:
N/A

Animal:
N/A

Computer:
N/A

Office:
N/A

Other:
N/A

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

- Centrifuge-RNA extraction, cell lysate collection
- PCR machine-qRT-PCR
- Confocal microscope-immunofluorescence
- Incubator-Cell growth
- Platform rocker- western blot
- Imaging system-western blot

The following consumables will be purchased with grant funding:

- SH-SY5Y cell line, supplements, and reagents
- Cell culture flask
- RNeasy mini prep kit
- cDNA synthesis kit
- Amyloid β 1-40 and 1-42 peptides
- Primary and secondary antibody
- Primers
- Chemiluminescent detection solution
- Phosphate Buffer Saline
- Tris Buffer Saline with 20% Tween
- Standard protein marker
- Alamar Blue Assay Kit

Resources Format Page

SPECIFIC AIMS

Alzheimer's Disease (AD) is a multifactorial neurodegenerative disease that affects the brains of 47 million people worldwide. In AD progression, misfolded Amyloid β peptides (Aβ) accumulates in the endoplasmic reticulum (ER) and induces ER stress. In response to the aggregation of these misfolded proteins the unfolded protein response (UPR) is initiated and activates the three sensors of the ER: PERK (protein kinase RNA-like ER kinase), ATF6 (activating transcription factor 6), and IRE1 (inositol requiring enzyme 1). The UPR is responsible for restoring ER homeostasis by activating various genes that remove abnormal proteins, improve protein folding, and minimize further synthesis of the mutant protein.

The main goal of this project is show silencing ATF6 in neuroblastoma cell line (SH-SY5Y) under Aβ induced ER Stress will reduce expression of endogenous Aβ. Also, essentially trying to show that ATF6 activity is associated with neuronal cell death.

1. Basal level detection of ER stress sensors in SH-SY5Y and Aβ SH-SYS5 cell to establish a working cell model for AD.

2. Demonstrating that silencing ATF6 will result in reduced ER stress and reduction of Aβ in the ER.

3. Rescue of ATF6 phenotype after KD to validate that the effects caused by KD is due to the specificity of ATF6.

Continuation Format Page

BACKGROUND AND SIGNIFICANCE

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disease that gradually slows down thinking skills and leads to loss of memory and synapse number. AD has been found to be the most common form of dementia and 60-70% of all dementia cases are contributed to this form with an estimated 24 million people affected worldwide (Mayeux 2003). Approximately 5.7 million Americans were living with Alzheimer's dementia in 2018 (Alzheimer's Association 2018). This number includes an estimated 5.5 million people age 65 and older who have late onset Alzheimer's disease (LOAD) and 200,000 individuals under age 65 who have early-onset Alzheimer's (EOAD), (Figure 1) (Herbert et al. 2013).

- 85+ years, 37%
- 75-84 years, 44%
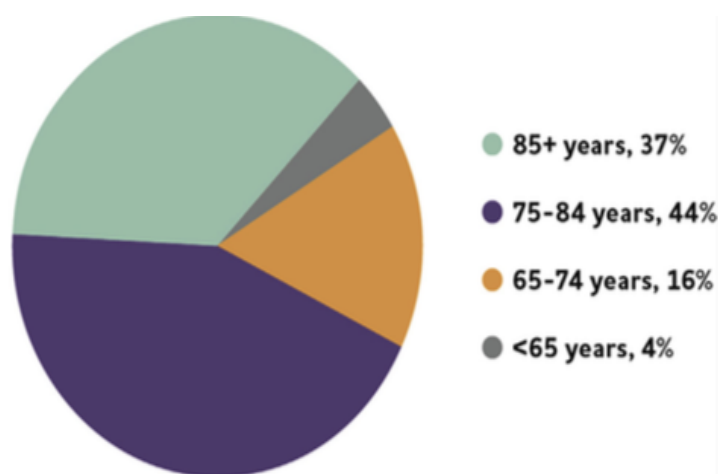- 65-74 years, 16%
- <65 years, 4%

Figure 1. Ages of people with Alzheimer's dementia in the United States, 2018. Percentages do not total 100 because of rounding. Created from data from Hebert and colleagues (Herbert et al. 2013).

By mid-century, the number of people living with Alzheimer's dementia in the United States is projected to grow to 13.8 million, fueled in large part by the aging "baby boomer" generation. Alzheimer's disease is the sixth leading cause of death in the United States and the fifth leading cause of death for those age 65 and older (Kochanek el. al 2017). The rate of death caused from Alzheimer disease has increased since 2000 and shows a correlation with older age individuals (Figure 2). Those living with serve AD regularly experience complications such as immobility, swallowing disorders and malnutrition that significantly increase the risk of serious acute conditions that can cause death (Alzheimer's Association 2018).
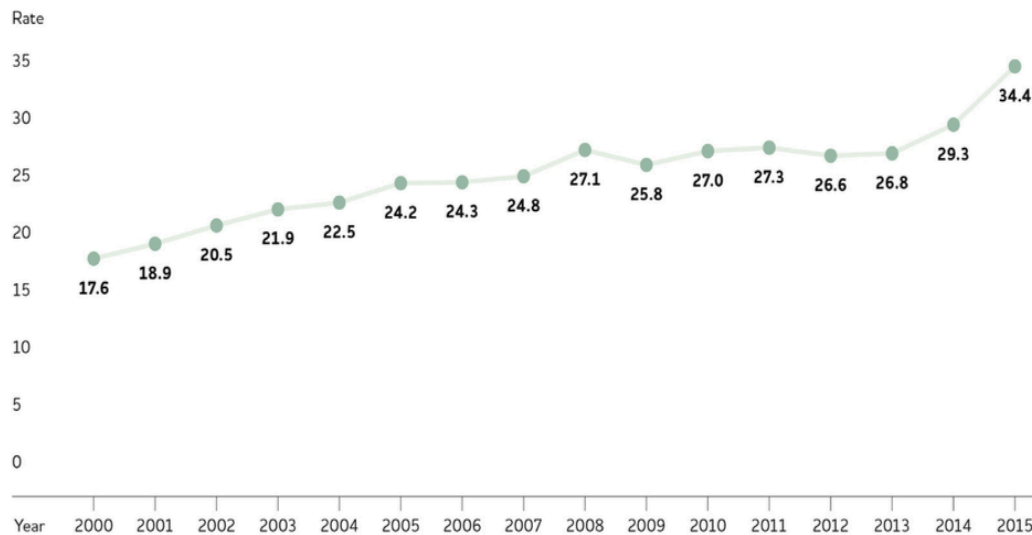

Figure 2. Percentage changes in AD death rate (per 100,000 people) between 2000 and 2015. Created from data from the National Center for Health Statistics (Murphy et al 2017)

Aβ and Tau – Plaques and NFT formation

In 1906, German psychiatrist and neuroanatomist Dr. Alois Alzheimer discovered a neurodegenerative disease in the brain tissue of his patient, Auguste Deter, who died from progressive behavioral and cognitive disorder (Hippius and Neundörfer 2003). After performing a brain autopsy he discovered abnormal shrinkage and two different types of lesions. These two lesions are now considered to be the hallmark of AD and are characterized as amyloid beta peptides (Aβ) and tau protein which get hyper-phosphorylated.
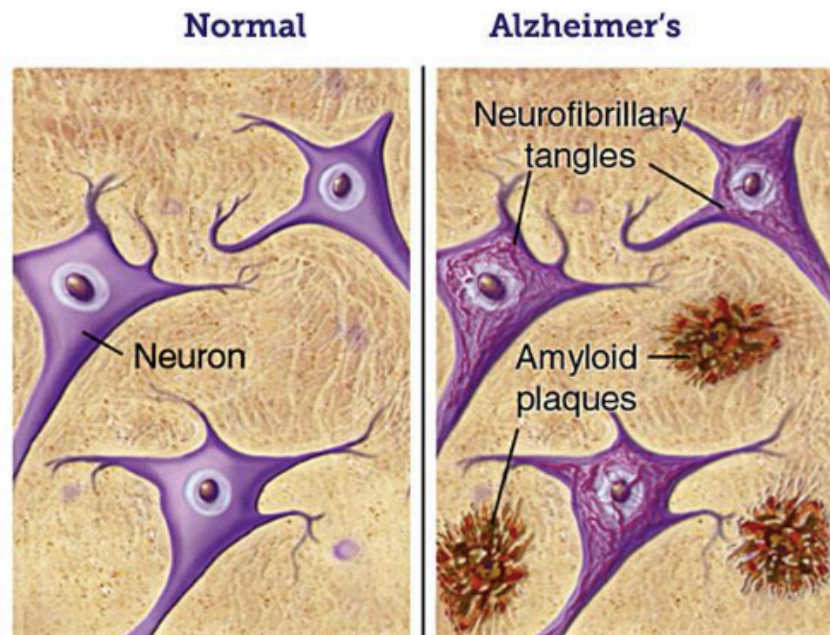

Figure 3. Normal Vs. Alzheimer's disease brain. On the left a normal functional brain contains working neurons that are able to transmit signals throughout the brain. Within the neuron, the microtubules are intact and keeps the cytoskeleton of the cells. The Alzheimer's disease brain contains non-functional neurofibrillary tangles that are found within the degenerate neurons. Amyloid beta peptides are exported from the neurons and from aggregate to from amyloid plaques that are deposited throughout the brain (Bright Focus Foundation).

Aggregation of amyloid beta and tau lead to the formation of amyloid plaques and neurofibrillary tangles (NFT), respectively (Figure 3). The brain is composed of neurons that form vast networks and synapses to transmit information from one neuron to another. The neuronal cytoskeleton is comprised of microtubules, which contain tau protein that aid in its stabilization. In AD, defective tau protein dissociates from the microtubules causing them to no longer function. Without the cytoskeleton, the neuron degenerates and connections between the neuron are lost. Tau protein then accumulates within the cell, causing the neuron to die leaving behind NFT. These specific lesions are mostly seen to affect regions such as the frontal cortex, hippocampus, and amygdala (Perl 2010).

Amyloid Precursor Protein Processing

Amyloid precursor protein (APP), is an integral type 1 membrane glycoprotein that plays a role in numerous biological activities such as neuronal development, signaling, the regulation of synapse formation, neural plasticity, and iron export. Pathogenic mutations found in presenilin-1 (PS1), presenilin-2 (PS2), and APP are known to account for Early on-set Alzheimer's Disease (EOAD), also known as Familial Alzheimer's Disease (FAD). Mutations in the gene encoding PS1 are responsible for many cases of FAD. Though APP ranges in length of 639 to 770 amino acids there are several isoforms that are preferentially expressed in neurons. The most dominant are APP751, APP770, and APP695, the latter being found the most abundant isoform expressed in neurons (Chen et al. 2017). APP can undergo processing via alternative pathways: nonamyloidgenic and amyloidgenic pathway. The amyloidgenic pathway cleaves APP by enzymes BACE 1, while non-amyloidgenic processing uses α-secretase. Both processes leave behind membrane-tethered α- or β-C terminal fragments (CTFs)

(O'Brien and Wong 2011) (Figure 4). Cleavage of APP by α-secretase releases soluble

ectodomain sAPPα from the surface and leaves behind an intracellular 83 amino acid C-

terminal fragment (C38). Processing of APP by β- secretase generates soluble

ectodomain sAAPβ and leaves a 99-amino acid CTF (C99) tethered to the membrane.

Further processing of both α- and β-CTF involves γ-secretase, which in the

nonamyloidgenic pathway releases both p3 peptide and APP intracellular domain (AICD)

and the amyloidgenic pathway releases Aβ and AICD. As APP mutations can occur in

the Aβ domain, APP proteolysis by both β- and γ-secretases can lead to the formation of

mutated Aβ peptides.

The cleavage of APP during amyloidgenic processing by γ-secretase produces

fragments of 43, 45, 46, 48, 49 and 51 amino acids that are further cleaved to form toxic

species form of Aβ monomers: the 40-amino-acid peptide (Aβ40) and the 42-amino-acid

peptide (Aβ42), (Olsson et al. 2014). Aβ42 is the most common peptide that is highly

fibrillogenic and more prone to neurotoxic assembly compared to other common variant

Aβ40. Aβ monomers are able to aggregate into toxic oligomers, protofibrils and amyloid

fibrils that are able to accumulate both intracellular and extracellular, but the main

toxicity mechanism is intracellular (Carrillo-Mora et al. 2014). The Aβ42: Aβ40 ratio is

more heightened in familial Alzheimer diseases due to increased toxicity from Aβ42.

Non-toxic, protective Aβ are dependent upon length, conformation of the structure, and

the number of peptides.

The Extracellular amyloid fibrils are larger and insoluble, and they can further

assemble into amyloid plaques, while amyloid oligomers are soluble and may spread

throughout the brain (Chen et al. 2017). Numerous studies indicate that early aggregation of Aβ40 and Aβ42 plays a key role in AD pathogenesis (Bateman et al. 2012; Jack et al. 2013). Aβ42 is known to be more prone to fibril formation and aggregation due to its hydrophobicity. Data associated with FAD studies suggest that mutation in the APP gene increases in the ratio of Aβ42/40, indicating that elevated levels of Aβ42 relative to Aβ40 play a critical role in AD pathogenesis (Borchelt et al. 1996; Chen et al. 2017) . The overproduction of amyloid β results in the accumulation of these peptides and causes senile plaque formation (Zhang et al. 2011).
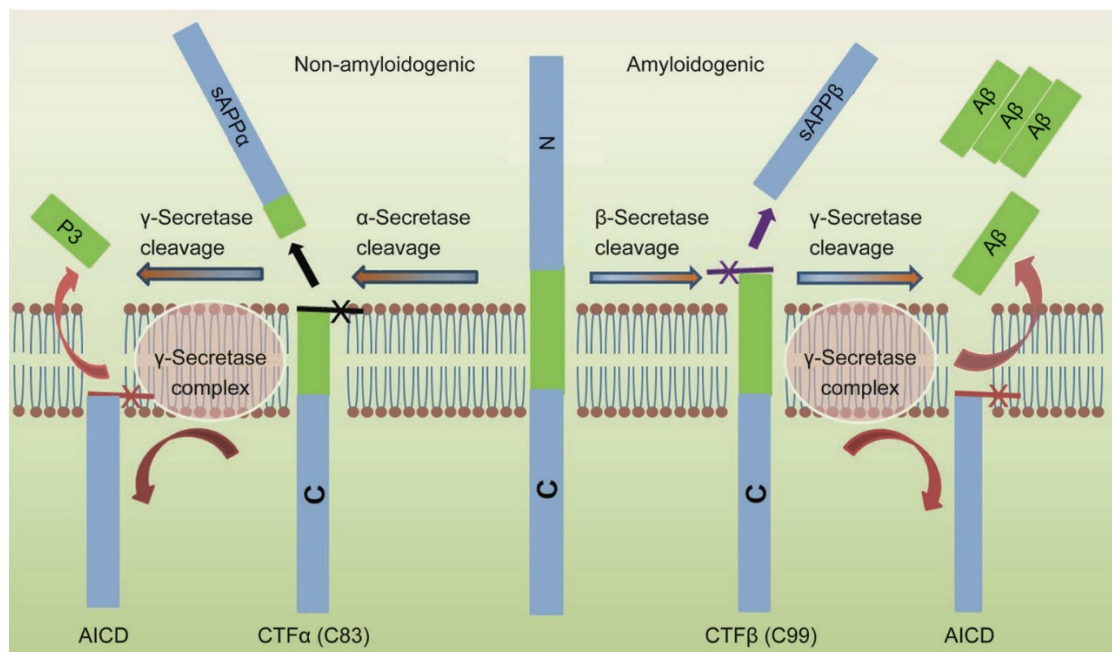

Figure 4. Cleavage of Human amyloid precursor protein. Non-amyloidogenic pathway processing of APP consist of cleavage by α-secreatase within the Aβ domain and generates the tethered C-terminal fragment CTFα (C83) and the N-terminal fragment sAPPα. γ-Secretase then cleaves CTFα releasing extracellular P3 and the APP intracellular domain (AICD). Amyloidgenic processing of APP involves cleavage by β-Secretase that generates the N-terminal fragment sAPPβ and membrane tethered C terminal fragments β (CTFβ, C99). CTFβ is then cleaved by γ-Secretase generating extracellular Aβ and the APP intracellular domain (AICD) (Chen et al. 2017).

ER Stress Response and the UPR

The ER is an essential organelle that aids in protein folding, protein secretion and plays a role in lipid synthesis and is the site of calcium (Ca^{2+}) storage. In many neurodegenerative diseases, the presence of misfolded proteins elicits cellular the response mechanism of ER stress. The ER stress response protects cells from misfolded /unfolded proteins and can be triggered by a variety of conditions such as high demand for protein synthesis and secretion, viral infection, deprivation of nutrients/oxygen, mutations affecting both client protein folding, ER folding machinery, and accumulation of unfolded or misfolded proteins (Francisco et al. 2010; Sano and Reed 2013). ER stress activates the unfolded protein response (UPR), which, promotes cell survival and restores ER homeostasis (Xiang et al. 2017). This response orchestrates intracellular signal transduction to initiate cell turnover to return to its normal function. Activation of the UPR affects the expression of different proteins with functions in almost every aspect of the secretory pathway, protein folding, quality control, protein entry into the ER, ER-associated degradation (ERAD), and autophagy-mediated degradation (Matus et al. 2011). The ERAD pathway leads to synthesis of chaperones, protein transporters, and ubiquitin-related enzymes that detect, deliver, and translocate misfolded proteins to the cytoplasm for proteasome mediated degradation (Vembar and Brodsky 2008). Overexpression of ERAD protein results in accumulation of UPR genes which can further promote ER stress if homeostasis is not restored. When UPR cannot restore normal homeostasis to the ER this can result in apoptosis of neuronal cells (Sano and Reed 2013). UPR dysfunction plays an important role in the pathogenesis of Alzheimer's disease and other neurodegenerative diseases including Parkinson's disease, amyotrophic

lateral sclerosis, and Huntington's disease, which are characterized by the accumulation and aggregation of misfolded proteins (Xiang et al. 2017).

UPR: Three pathways

UPR is signaled by three transmembrane protein receptors in the ER membrane: the protein kinase RNA (PKR)- like ER kinase (PERK), activating transcription factor-6 (ATF6), and the evolutionary conserved kinase/endoribonuclease inositol-requiring enzyme-1 (IRE1α). In the lumen of the ER, these ER stress sensors are bound to the ER resident chaperone binding immunoglobulin protein (BIP), which keeps them inactive. When unfolded/misfolded proteins accumulate in the ER, BIP is released from these complexes to assist with the folding of accumulated proteins and activates the three ER stress transducers IRE1, PERK, and ATF6 (Figure 5) (Gardner and Walter 2011).

IRE1α is a Serine/Threonine protein kinase and endoribonuclease and is activated via autophosphorylation. The active form of IRE1 undergoes unconventional splicing of the mRNA encoding the transcription factor X- Box Binding protein-1 (XBP1) (Shoulders et al. 2013; Acosta-Alvear et al. 2007). XBP1 gets translocated to the nucleus to control the induction of a subset of UPR-related genes that function in protein quality control, folding, ERAD, and ER biogenesis (Ron and Walter 2007). In addition to XBP1, activated IRE1α is able to mediate and process the cleavage of cytoprotective mRNAs through a process called IRE1α-dependent decay (RIDD) (Maurel et al. 2014; Tam et al. 2014; Hetz and Saxena 2017). IRE1 α pathway also initiates signaling mediated by Apoptosis Signal-regulating Kinase 1 (ASK1) and c-Jun-N terminal kinase (JNK) pathway further leading to apoptosis (Urano et al. 2000). The IRE1 pathway has dual pro-survival and pro-apoptotic roles (Chen and Brandizzi 2013).

PERK is responsible for controlling protein translation in the cell. Just like IRE1, PERK is activated via autophosphorylation and then phosphorylates eukaryotic initiation factor 2 (EIF2), which then leads to the attenuation of translation or markedly enhanced the expression of activating transcription factor 4 (ATF4) (Harding et al. 2000). ATF4 then binds to CHOP promoter (CCAAT/enhancer binding protein homology protein) inducing apoptotic gene expression. IRE1 and PERK are critical for cell survival after ER-stress. However functional links between ATF6 activation and cellular outcome are less well defined.
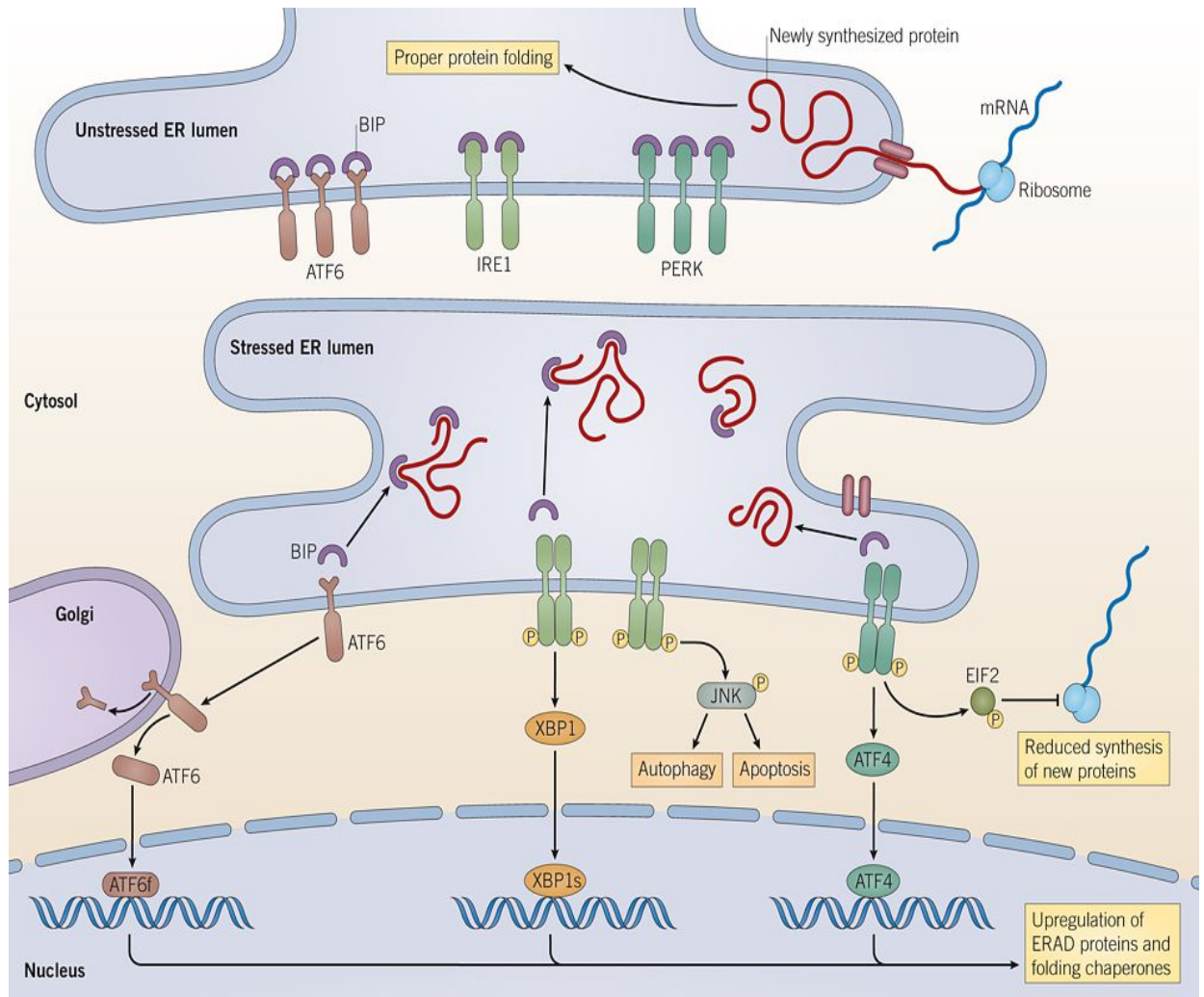
Figure 5. UPR signaling pathways initiated during ER stress. The top region represents unstressed conditions of the ER. Represented in red newly synthesized proteins will fold properly as ER homeostasis is regulated. BIP (purple) associates with the three ER transducers PERK, IRE1, and ATF6 under normal physiological conditions. Stressed ER lumen will accumulate unfolded proteins, BIP disassociate with the transducer and binds to unfolded protein to assist with folding. IRE1 and PERK will become activated and the downstream product will translocate to the nucleus and bind to UPR target genes. ATF6 upon activation will translocate to the Golgi and under cleave to release cleaved product ATF6 50. The transcription factor translocates to the nucleus and bind to UPR target gens. PERK, IRE1, and ATF6 target gene involved in protein folding, protein degradation and, attenuation of protein translation (Todd et al. 2008).

While ATF6 functions as a transcription factor, this protein is peculiar being that it is synthesized as a transmembrane protein that is embedded in the ER. The 90-kDa protein is activated after translocation to the Golgi apparatus where it is processed by site-1 protease (S1P) and site-2 protease (S2P) released as a functional 50-kDa soluble cytoplasmic fragment. The N-terminal fragment of ATF6 to enters the nucleus and activates the transcription of a specific subset of genes including ER chaperones and ERAD proteins (Jin et al. 2016)(Figure 5). There are several lines of evidence showing that IRE1α-XBP1 and ATF6 are inter-connected and both express ERAD and protein folding gene. Montibeller *et al.* (2018) found that ATF6 pathways are strongly activated in AD, and the most upregulated target gene of ATF6 was Suppressor/Enhancer of Lin-12-like (SEL1L). SEL1L is an adaptor protein for the E3 ligase and is associated with the ERAD system which serves to remove unfolded proteins by retrograde degradation using the ubiquitin-proteasome system (Saltini et al. 2006). Upregulation of ATF6 expression upregulates SEL1L expression, but its role in AD is not clearly understood specifically the correlation between SEL1L expression and Aβ production.

PRELIMINARY REPORT / PROGRESS REPORT

Recent studies have demonstrated an involvement of the IRE1α-XBP1 and ATF6 pathways in human neurodegenerative diseases. Montibeller *et al.* (2018) recently performed a study to characterize 44 target genes and selected 19 that are intensely influenced by XBP1 and ATF6 and measured the expression of a subset of genes in human post mortem AD cases and controls (Table1). This study was conducted using both frontal and temporal cortex regions from 20 healthy controls and 20 AD cases. ATF6 pathways were found to be strongly activated in AD (Montibeller and de Belleroche 2018). The most upregulated gene was Suppressor/ Enhancer of Lin-12 like (SEL1L) which is associated with ER associated degradation, and is a target of ATF6 transcription factor. Though there have been numerous studies involving PERK and IRE1 little is known about ATF6 and its involvement with AD.

In a recent study, Walter *et al.* (2018) used neuroblastoma SH-SY5Y cell lines to study the role of ATF6. This cell line is derived from a metastatic bone tumor biopsy and is a subline of the parental line SK-N-SH cells. SK-N-SH were subcloned three times; first to SH-SY, then to SH-SY5, and lastly to SH-SY5Y. SK-N-SH cells can either model a neuroblast-like or epithelial-like morphology. In the undifferentiated form, SH-SY5Y cells are characterized as having a by neuroblast-like morphologically which displays as non-polarized cell bodies with few, truncated processes. Both undifferentiated and differentiated SH-SY5Y cells can be utilized for *in vitro* experiments requiring neuronal-like cells, but differentiation involves a number of specific events, including formation and extension of neuritic processes, increased electrical excitability of the

plasma membrane, formation of synapses, and neurotransmitters exhibiting features a

mature neuron (Kovalevich and Langford 2013). Though it was not specified weather

Walter *et al.* (2018) differentiated the cells in their research, they transfected short hairpin

RNA (shRNA) vectors in SH-SY5Y to suppress expression of ATF6 and showed that

loss of ATF6 expression resulted in an increase of X box–XBP splicing, which allows

the translation of transcription factor XBP1 (Figure 8) (Walter et al. 2018). A transient

increase of IRE1 mRNA and IRE1 protein levels were detected in response to ER stress,

and expression was further increased in cells that had reduced expression of ATF6 by

silencing through shRNA constructs (Figure 6) (Figure 7) (Walter et al. 2018).

Furthermore, decreased expression of ATF6 can be compensated for by the upregulation

of IRE1 from the effects of silencing. Both IRE1 and ATF6 are inter-connected and both

lead to expression ERAD and protein folding genes. Since there is evidence to support

activation of ATF6 is linked to AD, in this study the aim is to suppress ATF6 in SH-

SYS5 cells using shRNA to demonstrate the role of ATF6 in the accumulation of Aβ in

the ER.

902   L. Montibeller, J. de Belleroche

Table 1   Selection of UPR target genes based on the response elements and transcriptional regulation by XBP1 and ATF6

Name	RefSeq	Response elements	Position to TSS	Main UPR TF	Secondary UPR TF
CANX	NM_001024649.1	ERSE	−109	XBP1	
DNAJB9	NM_012328.2	CRE-like	−1955	XBP1	
		ACGT core	−59		
DNAJC10	NM_001271581.1	UPRE	−132	XBP1	
ERLEC1	NM_015701.4	ERSE-26	−255	XBP1	
P4HB	NM_000918.3	ACGT core	−490	XBP1	
SYVN1	NM_032431.2	UPRE	−557	XBP1	
		ERSE	−521		
VCP	NM_007126.3	URPE	−452	XBP1	
		ACGT	−18,167		
		core ERSE-26	+1342		
DNAJB11	NM_016306.5	3× ERSE	−357	XBP1, ATF6	
		ACGT core	−2238		
DNAJC3	NM_006260.4	ERSE	−233	XBP1, ATF6	
EDEM1	NM_014674.2	ERSE	−411	XBP1, ATF6	
HYOU1	NM_006389.4	UPRE	−242	XBP1, ATF6	
PDIA3	NM_005313.4	ERSE	−159	XBP1, ATF6	
		UPRE	−4056		
PDIA6	NM_001282704.1	ERSE	−157	XBP1, ATF6	
XBP1	NM_005080.3	ERSE	−78	XBP1, ATF6	
HERP-UD1	NM_014685.3	ERSE-II	−167	ATF6	XBP1
		ERSE	−209		
		ACGT core	−563		
HSPA5	NM_005347.4	3× ERSE	−68	ATF6	XBP1
		ERSE-II	−107		
PDIA4	NM_004911.4	ERSE	−12,331	ATF6	XBP1
		UPRE	+6829		
OS9	NM_006812.3	ACGT core	−601	AFT6	XBP1
		ERSE	+328		
SEL1L	NM_005065.5	−	−	AFT6	
SIL1	NM_001037633.1	−	−	−	

Table 1. Selected target genes of XBP1 and ATF6 characterized based on the of ER stress response elements (ERSE) elements in their promoter regions, Position of the transcriptional start site, and the main and secondary UPR TF that is associated with the target gene. Seven genes are regulated exclusively by XBP1, one exclusively by ATF6 and 10 genes are regulated by both TFs. SIL1 was selected because it is not regulated by neither XBP1 nor ATF6 (Montibeller and de Belleroche 2018).
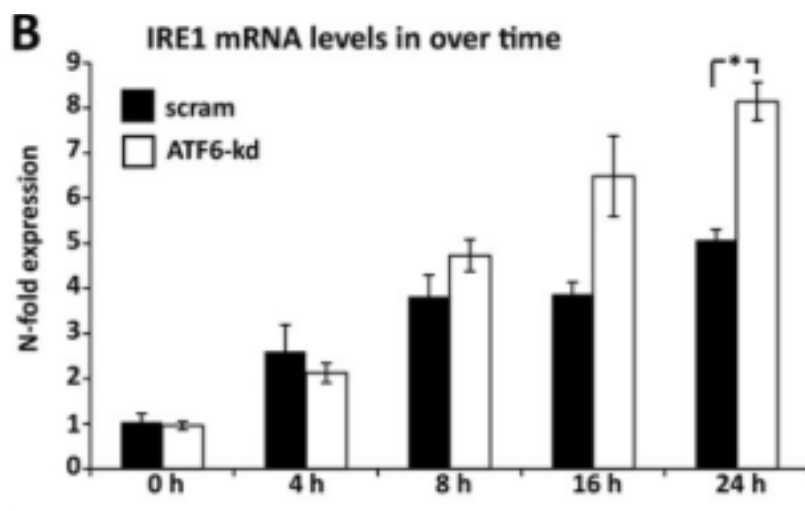
Figure 6. Real time qPCR analysis of IRE1 mRNA levels in cells silenced for ATF6 or scram control treated with tunicamycin over time in. Results were normalized to ß-actin levels and expressed relative to DMSO treated scram ctrl cells (Walter et al. 2018).

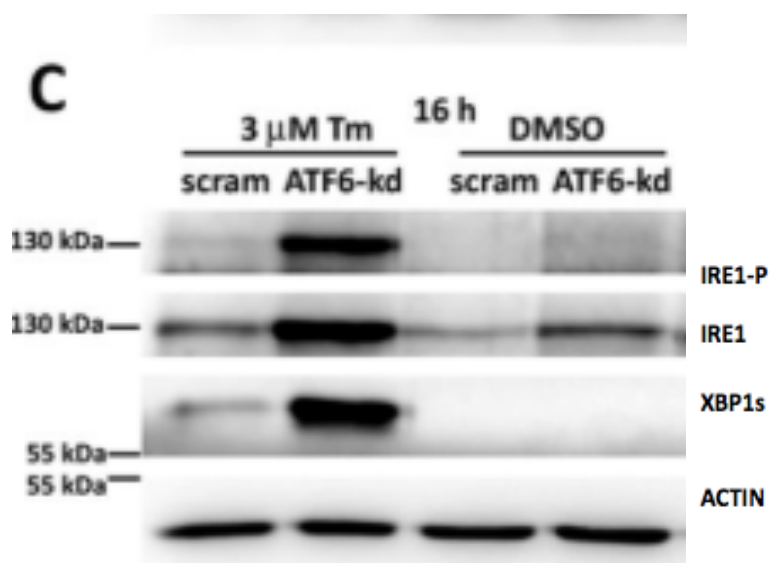Figure 7. 16 hr XBP1s- and IRE1-protein levels were analyzed by Western blot using antibodies against spliced XBP1, IRE1 and IRE1-p [Ser714]. ß-actin served as loading control. In SH-SY5Y cells induced with tunicamycin, KD of AFT6 increases protein expression of XBP1s, IRE1, and IRE1-P (Walter et al. 2018).
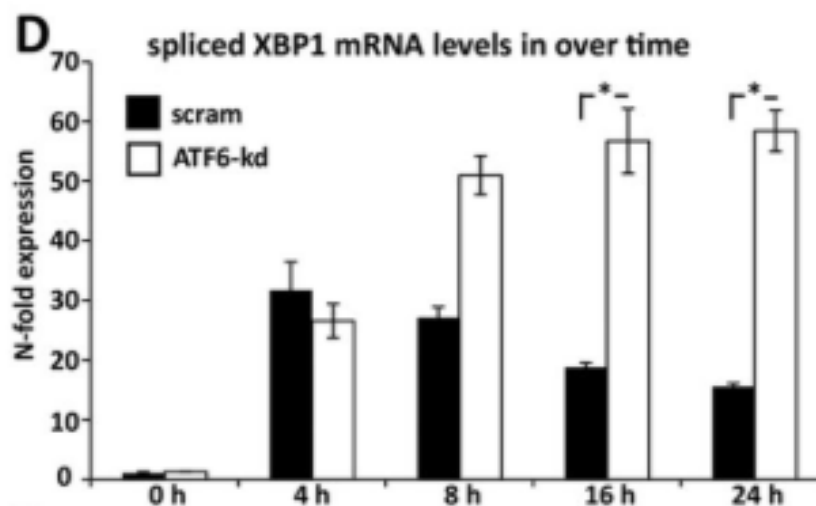
Figure 8. Real time qPCR analysis of spliced Xbp1 l mRNA levels in cells silenced for ATF6 or scram control treated with tunicamycin over time in Results were normalized to ß-actin levels and expressed relative to DMSO treated scram ctrl cells (Walter et al. 2018).

RESEACH DESIGN / METHODS

Aim 1: Basal Level detection of ER stress sensors in SH-SY5Y and +Aβ SH-SYS5 to establish a working cell model for AD.

Cell Culture of SH-SY5Y and +Aβ SH-SY5Y

In order to establish an appropriate model for this study the basal level of ER stress will be detected in SH-SY5Y and Aβ SH-SY5Y. Human neuroblastoma cell line, SH-SY5Y (ATCC CRL-2266) will be provided by ATCC and cultured in ATCC-formulated Eagle's Minimum Essential Medium and F12 Medium. All media is supplemented with 10% fetal bovine serum (FBS), and 1X Penicillin/Streptomycin solution. Cells will be cultivated in T175 flasks at 37 °C with 5% CO_2 at saturated humidity and kept below ATCC passage + 15 to avoid cell senescence. Cells will grow until 70% confluent. β Amyloid [1-40] Pure PTD Human Protein (03138, ThermoFisher) and β Amyloid [1-42] PTD Human Protein (03112, ThermoFisher) (Table 1) will be reconstituted and prepared to induce peptide aggregation according to the manufacture instructions. SH-SY5Y cells will be removed and rinsed with 0.25% trypsin, 0.53 mM EDTA solution, additional 1 to 2 mL of trypsin solution will sit at room temperature until the cells are fully separate from the flask. Fresh medium will then be added, aspirated and combined with the floating cells recovered above and dispensed into new flasks, plated at a density of 2×10^5 cells/well in 6-well plate. β Amyloid [1 -40] and β Amyloid [1- 42] (Table 2) will be added to the cultured cells and they will incubate overnight at 37 °C, 5% CO_2 until 70% confluency is reached. Media will be renewed every 4 to 7 days.

Amyloid β Sequence	
β Amyloid [1-40] PTD Human Protein Sequence	β Amyloid [1-42] PTD Human Protein Sequence
H2N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val- Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly- Val-Val-OH	H2N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe- Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile- Ala-OH

Table. 2 Human protein sequences for β Amyloid [1-40] and 1-42

Western Blot

Protein expression levels of Aβ (1-42), (1-40), and ER markers in will be detected by western blot to determine the basal level of ER stress. SH-SY5Y and +Aβ SH-SY5Y will be collected from culture and centrifuged in PBS. Washing will be done 2 times in PBS and the supernatant will be removed. The isolates will be suspended in Protease inhibitor cocktail (P-2714, Sigma-Aldrich) and Cell Extraction Buffer (FNN0011, Invitrogen) for 30 min on ice, vortexing at 10 min intervals. The extracts will be centrifuged at 13,000 rpm for 10 min at 4°C. Lysates will be separated by SDS-PAGE

gels and transferred to polyvinylidene difluoride membranes (ThermoFisher).

Membranes will incubate with blocking solution consisting of 4% milk in TBST-

Tween20 buffer for 1 hr at room temperature with shaking, followed by incubation at 4°C

on a shaker overnight with primary antibodies. The next day, primary antibodies should

be removed and the membranes will be washed 3 times with TBST-Tween20 buffer on a

shaker for 10 min at room temperature. Secondary antibody will be added for 1 hr at

room temperature on a shaker followed by washing 3 times for 10 min with TBST-

Tween20 buffer. For chemiluminescent detection of the membranes, ECL Plus Western

Blotting Substrate (32209, ThermoFisher) will be analyzed by ImageJ. The following

primary antibodies will be used to detect the basal level expression in SH-SY5Y and SH-

SY5Y$^{A\beta}$: ATF6 Antibody (PA5-20215, Thermofisher), Phosphorylated PERK antibody

(sc-32577 Santa Cruz), Anti-phospho-IRE1 (Ser724) NB100-2323, Novus Biologicals),

beta Amyloid (1-40) Antibody (44-136, ThermoFisher), Anti-Aβ42 (AB5070, Millipore),

followed by incubation with polyclonal anti-rabbit in goat (ab6721, Abcam) or anti-

mouse in goat (ab6789, Abcam) horseradish peroxidase conjugated secondary antibodies.

Anti-β-Actin (A2228, Sigma) will be used as the loading control.

Immunofluorescence

Immunofluorescence will be performed to detect co-localization of Aβ(1-42) and

Aβ(1-40) and Calnexin in the ER to observe ER stress. Twenty-four hr post transfection,

cells will be washed twice with PBS and will be immediately fixed for 30 min at room

temperature in 4% paraformaldehyde in PBS at pH 7.4. After 1 min washing in PBS, the

cells were permeabilized for 5 min in 0.5% Triton X-100 in PBS and again washed 3

times for 10 min in PBS. Next, the samples will be blocked for 1 hr at room temperature

with 5% goat serum in PBS. All cells will then be incubated with primary antibodies

Aβ42 (AB5070, Millipore) and beta Amyloid (1-40) antibody (44-136, ThermoFisher),

anti-sAPPβ (SIG-39138, Biolegend), anti-P3 (APP345, Aveslab) anti-Calnexin antibody

(ab22595, Abcam) for 1 hr at room temperature. The primary antibodies will be washed

with PBS 3 times for 10 min. The secondary antibodies Alexa Fluor 488-labeled anti-

mouse IgG (A28175, ThermoFisher) or Alexa Fluor 568-labeled anti-rabbit IgG (A-1101,

ThermoFisher) will incubated for 1 hr at room temperature followed by washing with

PBS 3 times for 10 min. Cells will be observed using a Leica TCS SP5 confocal

microscope.

Expected results

The results of the western blot in the cells line should not show evidence of ER

stress when probing for ATF6, pPERK, and pIRE1 (Table 3). After induction of ER

stress by Aβ(1-42) and Aβ(1-40) the experimental groups is expected to show evidence

of active ER stress markers. There should be expression of Aβ(1-42), Aβ(1-40), ATF6,

pPERK, and pIRE1 in the experimental group compared to the control group, where

expression of Aβ(1-42), Aβ(1-40), ATF6, pPERK, and pIRE1 should not observed. The

loading control, Actin in both cells should have the same pattern of expression being that

actin is highly abundant. The results of immunofluorescence of the control and

experimental group should correspond to western blot analysis. In order to visualize the

co-localization of ER stress anti-sAPPβ, Anti P3, and Anti-Calnexin can be used to

confirm that cleaved Aβ is being expressed endogenously and artificial Aβ peptides are

not being detected (Table 4). Untreated cells are expected to show no evidence of ER

stress response in the ER. After activation of ER stress, by Aβ(1-42) and Aβ(1-40) the

experimental cell should display evidence of stress.

Treatment	Stress	Aβ 42/40 (WB)	pPerk (WB)	pIRE1 (WB)	ATF6 (WB)	Caspase-3 (WB)
-Aβ	-	-	-	-	-	-
+Aβ	+	+	+	+	+	+

Table 3. Aim 2 western blot results. Untreated cells will show no evidence of ER Stress. pPERk, IRE1, and ATF6 will not be expressed nor will Caspase-3 be activated. Samples treated Aβ 42 and 40 will activate ER stress and pPERk, pIRE1, and ATF6 will be expressed. Apoptosis will be seen by expression of Caspase-3.

Treatment	Stress	Co-localization	sAPPβ	Aβ 42/40	P3	Calnexin
-Aβ	-	-	-	-	+	+
+Aβ	+	+	+	+	-	+

Table 4. Aim 2 Immunofluorescence results. Untreated cells will show no evidence of ER Stress. Caspase-3 be not be activated. Samples treated Aβ 42/40 will activate ER stress and Calnexin will be visualized in the ER.

Aim 2: Demonstrating that silencing ATF6 will result in cell death and reduced ER stress.

To suppress the function of ATF6 the stable silencing vector pLKO.l-puro will be used in this experiment. The pre-cloned shRNA construct will contain Human shRNAs ATF6 (TRCN0000017853, Sigma) or Human shRNAs ATF6 (TRCN0000017855, sigma) (Table 5). Two different human shRNA construct will be used to rule out any off targets effects. The controls that will be used for this shRNA experiment will be a TRC1.5 –pLKO.1 vector (Figure 9). This vector will contain pre-cloned positive control eGFP (SHC005, Sigma) (Figure 10) targeting eGFP or pLKO.1-puro negative control (SHC016, Sigma) (Figure 11), an empty vector that does not contain an shRNA sequence. All vectors will be obtained as plasmid DNA from Sigma. SH-SY5Y cultured cells will be plated on a 12 well plate in triplicates and incubated at 37°C in a CO_2 incubator until 70% confluency is reached. Each construct will then be transfected with Lipofectamine 2000 Transfection Reagent (11668019, Invitrogen) at various concentrations (Table 6). Incubation will occur for 18-20 hr at 37°C in a CO_2 incubator. ATCC-formulated Eagle's Minimum Essential Medium, F12 Medium, and puromycin will be added to each the well. Incubation will occur for 18-20 hr at 37°C in a CO_2 incubator. The media will then be renewed with fresh puromycin. Reconstituted β Amyloid [1-40] and β Amyloid [1-42] will be added to the media. Cells will then be assay for target gene knockdown.
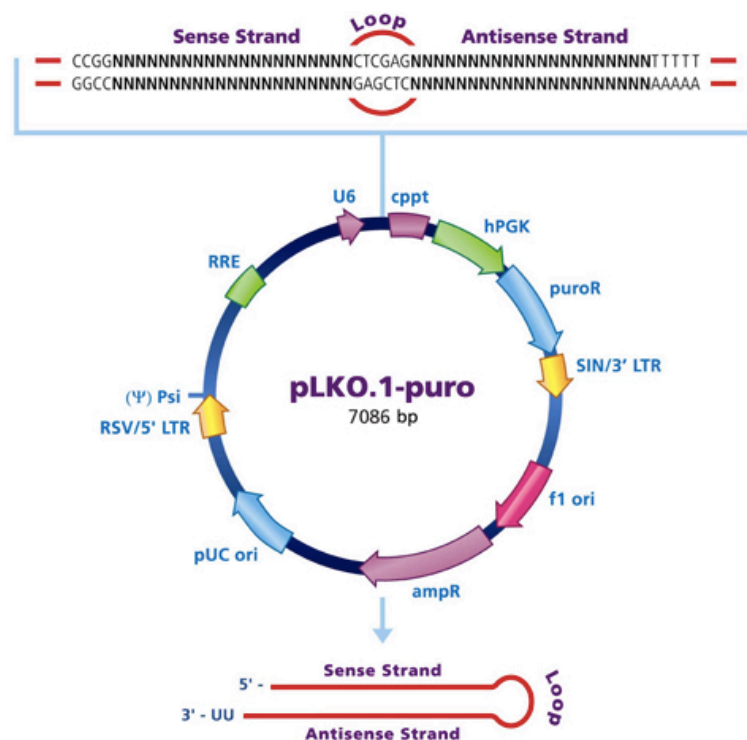
Figure 9. pLKO.1-puro mammalian shRNA vector

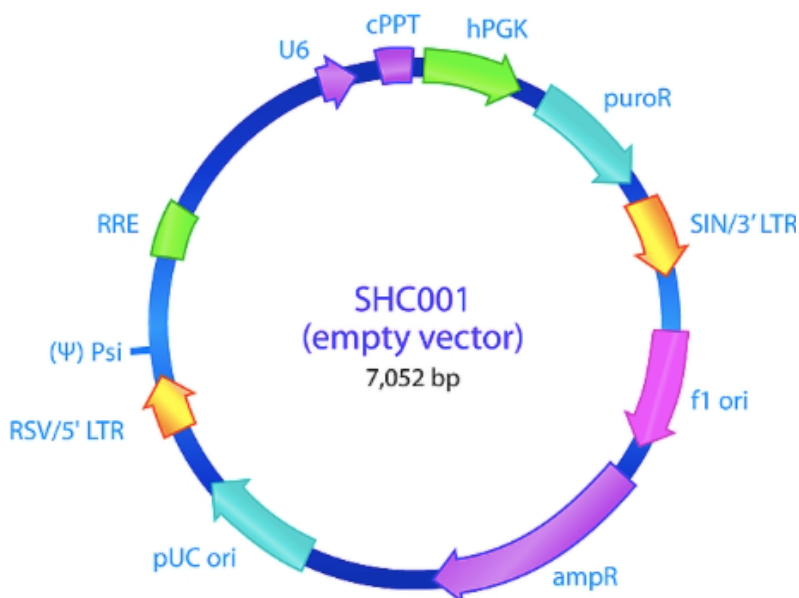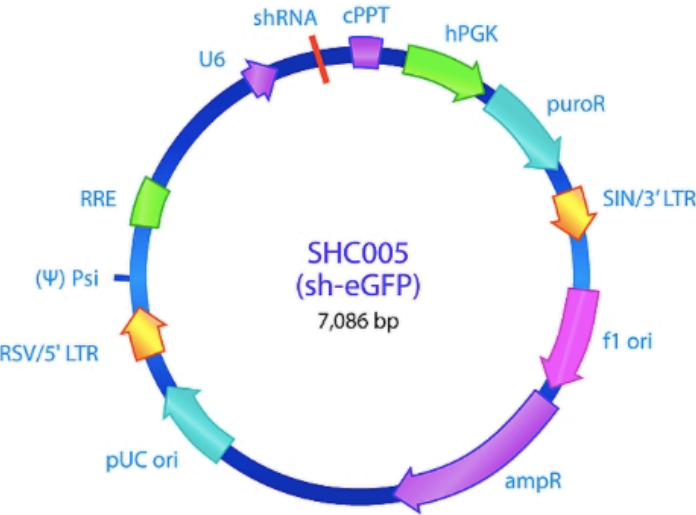Figure 10. pLKO.1 empty vector that contain no shRNA sequence

Figure 11. pLKO.1 positive control sh-eGFP

plLKO.1-puro Human ATF6 shRNA		
	Human shRNAs ATF6 (TRCN0000017853)	Human shRNAs ATF6 (TRCN0000017855)
Sequence	5' - CCGGCCCAGAAGTTA TCAAGACTTTCTCGAG AAAGTCTTGATAACTT CTGGGTTTT-3'	5'- CCGGGCAGCAACCAATT ATCAGTTTCTCGAGAAA CTGATAATTGGTTGCTG CTTTTT-3'

Table 5. pLKO.1-puro shRNA AFT6 sequence

Treatment	Positive control vector	Negative control vector	Human ATF6 shRNAs
-Aβ	+	-	-
-Aβ	-	+	-
-Aβ	-	-	+
+Aβ	+	-	-
+Aβ	-	+	-
+Aβ	-	-	+

Table 6. Aim 2 experimental set up

RNA extraction and quantitative-PCR

To validate specific knockdown of ATF6 q-PCR will be performed 48h after transfection. RNA will be extracted with TRIZOL reagent (15596026, ThermoFisher) and isolated with RNeasy Mini Kit (74104, Qiagen,). Cells from Extracted RNA will be treated with DNase I for 10 min and purified using the RNAqueous-Micro Kit (AM1931, ThermoFisher). To produce cDNA from RNA, High Capacity cDNA Reverse Transcriptase Kit (4368813, ThermoFisher) will be used described by the manufacturer. Primer sequences will be obtained from the Primer Bank and used in q-PCR with a QuantStudio 6 Flex Real-Time PCR System. ATF6 Forward and Reverse Primer will be added to cDNA samples and plated in triplicates (Table 7). A non-template control will be applied to monitor contamination and will not include cDNA samples. β-Actin will be used as the internal loading control to normalize expression. For quantitation, the delta-delta Ct method of relative gene expression will be used to determine the mean expression of each target gene normalized to the mean of actin.

ATF6 Primer	
Forward Primer	5'TCCTCGGTCAGTGGACTCTTA3'
Reverse primer	5'CTTGGGTGAATTGAAGGTTTTG3'

Table 7. Aim 2 ATF6 Forward and reverse primer

Western Blot

Protein expression levels of Aβ(1-42) and Aβ(1-40) and ER markers in will be

detected by western blot to determine levels after silencing. All cells lines will be

collected from culture and centrifuged in PBS. Washing will be done 2 times in PBS and

the supernatant will be removed. The isolates will be suspended in Protease inhibitor

cocktail (P-2714, Sigma-Aldrich) and Cell Extraction Buffer (FNN0011, Invitrogen) for

30 min on ice, vortexing at 10 min intervals. The extracts will be centrifuged at 13,000

rpm for 10 min at 4°C. Cell lysates will be separated on SDS-PAGE gels and transferred

to polyvinylidene difluoride membranes (ThermoFisher). Membranes will incubate with

blocking solution consisting of 4% milk in TBST-Tween20 buffer for 1 hr at room

temperature with shaking, followed by incubation at 4°C on a shaker overnight with

primary antibodies. The next day, primary antibodies should be removed and the

membranes will be washed 3 times with TBST-Tween20 buffer on a shaker at room

temperature for 10 min. Secondary antibody will be added for 1 hr at room temperature

on a shaker followed by washing 3 times for 10 min with TBST-Tween20 buffer. ECL

Plus Western Blotting (32209, ThermoFisher) will be used for chemiluminescent

detection. The following primary antibodies were used: ATF6 Antibody (PA5-20215,

Thermofisher), Anti-phospho-IRE1 (Ser724) NB100-2323, Novus Biologicals), beta

Amyloid (1-40) Antibody (44-136, ThermoFisher), Anti-Aβ42 (AB5070, Millipore), followed by incubation with polyclonal anti-rabbit in goat (ab6721, Abcam) or anti-mouse in goat (ab6789, Abcam) horseradish peroxidase conjugated secondary antibodies (Sigma-Aldrich). Anti-β-Actin (A2228, Sigma) will be used as the loading control. Following incubation, the membranes will be imaged and analyzed using ImageJ.

Immunofluorescence

Immunofluorescence will be performed to detect co-localization of Aβ 42/40, sAPPbeta, P3, and calnexin in the ER to observe ER stress after KD of ATF6. Using cell from 24 hr post transfection, cells will be washed twice with PBS and will be immediately fixed for 30 min at room temperature in 4% paraformaldehyde in PBS at pH 7.4. After 1 min washing in PBS, the cells will be permeabilized for 5 min in 0.5% Triton X-100 in PBS and again washed 3 time for 10 min in PBS. Next, samples will be blocked for 1 hr at room temperature with 5% goat serum in PBS. All cells will then be incubated with primary antibodies anti-Aβ42 (AB5070, Millipore), β-Amyloid (1-40) Antibody (44-136, ThermoFisher), anti-sAPPβ (SIG-39138, Biolegend), anti-P3 (APP345, Aveslab), anti-Calnexin antibody (ab22595, Abcam), and anti-β-Actin (A2228, Sigma) for 1 hr at room temperature. The primary antibodies will be washed with PBS 3 times for 10 min. The secondary antibodies Alexa Fluor 488-labeled anti-mouse IgG (A28175, ThermoFisher) or Alexa Fluor 568-labeled anti-rabbit IgG (A-1101, ThermoFisher) will incubate for 1 hr at room temperature before being washed with PBS 3 times for 10 min. Slides will be observed using a Leica TCS SP5 confocal microscope.

Cell Survival

Alamar Blue assay will be used to assess cells viability for *in vitro* cytotoxicity of all

samples. Sample will be treated with reagents to promote a color formation and will

incubate over time. absorbance will then be read by fluorescence spectrophotometer

(DAL102, Sigma).

Expected results

Based on aim 1 expression pPERk, IRE1, and ATF6 and co-localization of

sAPPbeta, P3, Aβ 42, Aβ 40, and calnexin indicates ER stress. q-PCR, western blot, and

immunofluorescence samples that will contain −Aβ and control should not activate ER

stress response (Table 8). Those samples that will contain +Aβ and control are expected

to show evidence of ER stress. Cells that will contain pLKO.1 ATF6 shRNA and -Aβ

are expected to not show evidence of ER stress. Cells that will contain pLKO.1 ATF6

shRNA and +Aβ are expected to not illustrate evidence of ER stress. Accuracy and

reduce assay variability should be expected due to triplicate transfection. Actin level in

both Aβ treated cells and untreated cells should have same pattern of expression. Cell

death should only occur in samples that contain +Aβ and pLKO.1 shRNA vectors.

Samples	Treatment	Stress	Viability	Apoptosis
Control	-Aβ	-	+	-
Control	+Aβ	+	+	-
KD	-Aβ	-	+	-
KD	+Aβ	-	-	+

Table 8. Aim 2 western blot, immunofluorescence, and cell viability results. After
knockdown (KD) ER stress should not be expressed. Control cells that will contain +Aβ
should be the only cells that should portray ER stress. Samples that include +Aβ and KD

vector is expected to undergo apoptosis, while the all other cells should survive.

Aim 3: Rescue of ATF6 after Knockdown

Once the effect of ATF6 KD is observed a rescue experiment will be performed to recover the phenotype of human ATF6 and validate that the cause of all the downstream affects after silencing is due to specifically human AFT6. In order for ATF6 to be recused a cDNA expression plasmid from a different species will be used. Mus musculus activating transcription factor 6 (Atf6), (NM_001081304.1, NP_0010747731) shares 86% sequence similarity with human ATF6 (NM_007348). Them being from different species makes it more likely that the shRNAs that won't target the cDNA. cDNA expression vector, pCMV-SPORT6 containing Mouse Atf6 cDNA (MMM1013-202707547) (Figure 12) will be transiently transfecting into SH-SY5Y cells containing pLKO.1 ATF6 shRNAs or control vector using Lipofectamine 2000 Transfection Reagent (11668019, Invitrogen) (Table 9). A range of volume will be used to determine the optimal transfection efficiency. Incubations will occur at least 48 (up to 72) hr to allow time for the vector components to be expressed. Incubation will occur for 18-20 hr at 37°C in a CO_2 incubator. The media will then be renewed with fresh media and reconstituted β Amyloid (1-40) and β Amyloid (1-42) will be added.

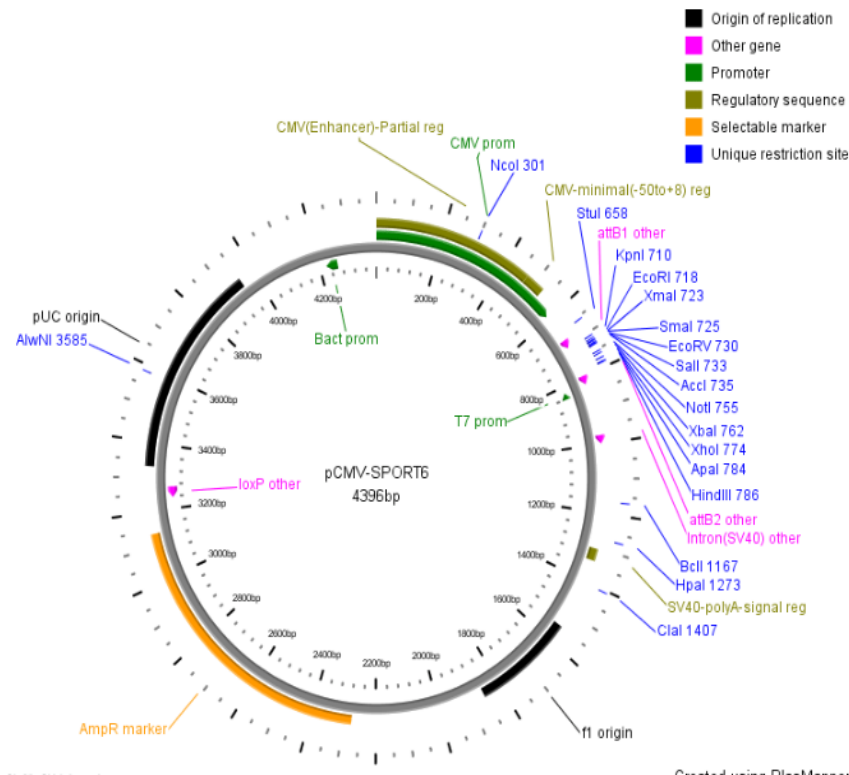Figure 12. pCMV cDNA expression vector

Treatment	Control	Human ATF6 shRNAs	Mouse cDNA exp'n vector
-Aβ	+	+	+
+Aβ	+	+	+
-Aβ	-	+	+
+Aβ	-	+	+

Table 9. Aim 3 experimental set up

RNA extraction and q-PCR

To confirm expression of mouse ATF6 cDNA q-PCR will be performed. Gene expression will be confirmed 48h after transfection by q-PCR. RNA will be extracted with TRIZOL reagent (15596026, ThermoFisher) and isolated with RNeasy Mini Kit (74104, Qiagen). Extracted RNA will be treated with DNase I for 10 min and purified using the RNAqueous-Micro Kit (AM1931, ThermoFisher). To produce cDNA from RNA, High Capacity cDNA Reverse Transcriptase Kit (4368813, ThermoFisher) will be used provide by the manufacturer. Primer sequences will be obtained from the Primer Bank and used in q-PCR with a QuantStudio 6 Flex Real-Time PCR System. ATF6 Forward and Reverse Primer will be added to cDNA samples and plated in triplicates. ATF6 primer will be designed from NCBI Primer-Blast against the pairwise sequence alignment of human ATF6 NM_007348 and mouse ATF6 NM_001081304.1 to target only mouse ATF6. A non-template control will be applied to monitor contamination and will not include cDNA samples. β-Actin will be used to normalize expression of ATF6. For quantitation, the delta-delta Ct method of relative gene expression will be used to determine the mean expression of each target gene normalized to the mean of actin.

Western Blot

Protein expression of Aβ (1-42), (1-40) and ER markers will be detected by western blot to determine if mouse ATF6 will be reinstated back to normal levels in –Aβ and +Aβ cells. Samples will be collected from culture and centrifuged in PBS. Washing will be done 2 times in PBS and the supernatant will be removed. The isolates will be suspended in Protease inhibitor cocktail (P-2714, Sigma-Aldrich) and Cell Extraction Buffer (FNN0011, Invitrogen) for 30 min on ice, vortexing at 10 min intervals. The

extracts will be centrifuged at 13,000 rpm for 10 min at 4°C. Cell lysates will be

separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes

(ThermoFisher). Membranes will incubate with blocking solution consisting of 4% milk

in TBST-Tween20 buffer for 1 hr at room temperature with shaking, followed by

incubation at 4°C on a shaker overnight with primary antibodies. After 24 hr primary

antibodies should be removed and the membranes will be washed 3 times with TBST-

Tween20 buffer on a shaker at room temperature for 10 min. Secondary antibody will be

added for 1 hr at room temperature on a shaker followed by washing 3 times for 10 min

with TBST-Tween20 buffer. ECL Plus Western Blotting (32209, ThermoFisher) will be

used for chemiluminescent detection. The following primary antibodies were used to

detect the protein expression in all samples ATF6 Antibody (PA5-20215, Thermofisher),

Anti-phospho-IRE1 (Ser724) NB100-2323, Novus Biologicals), β-Amyloid (1-40)

Antibody (44-136, ThermoFisher), β42 (AB5070, Millipore), followed by incubation

with polyclonal anti-rabbit in goat (ab6721, Abcam) or anti-mouse in goat (ab6789,

Abcam) horseradish peroxidase conjugated secondary antibodies (Sigma-Aldrich). Anti-

β-Actin (A2228, Sigma) will be used as the loading control. Following incubation, the

membranes will be imaged and analyzed using ImageJ.

Immunofluorescence

Immunofluorescence will be performed to detect co-localization of Aβ 42/40,

sAPPbeta, P3, and calnexin in the ER to observe ER stress after rescue of ATF6.

Twenty-four hr post transfection, cells will be washed twice with PBS and will be

immediately fixed for 30 min at room temperature in 4% paraformaldehyde in PBS at pH

7.4. After 1 min washing in PBS, the cells will be permeabilized for 5 min in 0.5% Triton X-100 in PBS and again washed 3 times for 10 min in PBS. Next, samples will be blocked for 1 hr at room temperature with 5% goat serum in PBS. All cells will then be incubated with primary antibodies ATF6 Antibody (PA5-20215, Thermofisher), Anti-Aβ42 (AB5070, Millipore) and beta Amyloid (1-40) Antibody (44-136, ThermoFisher), Anti-Calnexin antibody (ab22595, Abcam) Anti-β-Actin (A2228, Sigma) for 1hr at room temperature. The primary antibodies will be washed with PBS 3 times for 10 min. The secondary antibodies Alexa Fluor 488-labeled anti-mouse IgG (A28175, ThermoFisher) or Alexa Fluor 568-labeled anti-rabbit IgG (A-1101, ThermoFisher) will incubate for 1 hr at room temperature before being washed with PBS 3 times for ten min. Slides were observed using a Leica TCS SP5 confocal microscope.

Cell Survival

Alamar Blue assay will be used to assess cells viability for *in vitro* cytotoxicity of all samples that will be KD. All sample will be treated with regents to promote a color formation and will incubate over time. absorbance will then be read by fluorescence spectrophotometer (DAL102, Sigma).

Expected results

Based on aim 1 expression of pPERk, IRE1, and ATF6 and co-localization of sAPPbeta, Anti P3, Aβ 42, Aβ 40, and Anti-Calnexin will indicate ER stress. After q-PCR, western blot and immunofluorescence analysis samples that will contain –Aβ, mouse cDNA ATF6, control should not activate ER stress response (Table 10). Those

that will contain + Aβ, mouse cDNA ATF6, and control are expected to show evidence of ER stress. Cells that will contain −Aβ, mouse cDNA ATF6, and pLKO.1 ATF6 shRNAs are expected to not show evidence of ER stress. Samples that contain +Aβ, mouse cDNA ATF6, pLKO.1 ATF6 shRNAs are expected to show evidence of ER stress meaning normal levels of ATF6 will be reinstated. Actin level in both Aβ treated cells and untreated cells should have same pattern of expression. All cells should be viable and cell death should not occur.

Samples	Treatment	Stress	Viability	Apoptosis
Control	-Aβ	-	+	-
Control	+Aβ	+	+	-
Rescue	-Aβ	-	+	-
Rescue	+Aβ	+	+	-

Table 10. Aim 3 western blot, immunofluorescence, and cell viability results. Samples that do not contain Aβ should not display ER stress. Samples that contain +Aβ should show proof of ER stress. All samples should be viable and should not undergo apoptosis.

Future directions

Suppressor/Enhancer of Lin-12-like (SEL1L) is a highly activated gene in AD by ATF6 pathway. It is associated with the ERAD system which serves to remove unfolded proteins by retrograde degradation. Though this gene is highly active in AD cases there seems to be no clearance of unfolded proteins. A future proposal would be to observe the role of SEL1L after KD of ATF6.

REFERENCES

Acosta-Alvear D, Zhou Y, Blais A, Tsikitis M, Lents NH, Arias C, Lennon CJ, Kluger Y, Dynlacht BD. 2007. XBP1 Controls Diverse Cell Type- and Condition-Specific Transcriptional Regulatory Networks. Molecular Cell. 27(1):53–66. doi:10.1016/j.molcel.2007.06.011. [accessed 2018 Nov 7]. http://www.sciencedirect.com/science/article/pii/S1097276507004005.

Bateman RJ, Xiong C, Benzinger TLS, Fagan AM, Goate A, Fox NC, Marcus DS, Cairns NJ, Xie X, Blazey TM, et al. 2012. Clinical and Biomarker Changes in Dominantly Inherited Alzheimer's Disease. New England Journal of Medicine. 367(9):795–804. doi:10.1056/NEJMoa1202753. [accessed 2019 Feb 4]. https://doi.org/10.1056/NEJMoa1202753.

Borchelt DR, Thinakaran G, Eckman CB, Lee MK, Davenport F, Ratovitsky T, Prada C-M, Kim G, Seekins S, Yager D, et al. 1996. Familial Alzheimer's Disease–Linked Presenilin 1 Variants Elevate $A\beta1–42/1–40$ Ratio In Vitro and In Vivo. Neuron. 17(5):1005–1013. doi:10.1016/S0896-6273(00)80230-5. [accessed 2019 Feb 4]. https://www.cell.com/neuron/abstract/S0896-6273(00)80230-5.

Carrillo-Mora P, Luna R, Colín-Barenque L. 2014. Amyloid Beta: Multiple Mechanisms of Toxicity and Only Some Protective Effects? Oxidative Medicine and Cellular Longevity. doi:10.1155/2014/795375. [accessed 2019 Apr 15]. https://www.hindawi.com/journals/omcl/2014/795375/.

Chen G, Xu T, Yan Y, Zhou Y, Jiang Y, Melcher K, Xu HE. 2017. Amyloid beta: structure, biology and structure-based therapeutic development. Acta Pharmacologica Sinica. 38(9):1205–1235. doi:10.1038/aps.2017.28. [accessed 2019 Feb 1]. http://www.nature.com/doifinder/10.1038/aps.2017.28.

Chen Y, Brandizzi F. 2013. IRE1: ER stress sensor and cell fate executor. Trends Cell Biol. 23(11). doi:10.1016/j.tcb.2013.06.005. [accessed 2019 Mar 6]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3818365/.

Francisco AB, Singh R, Li S, Vani AK, Yang L, Munroe RJ, Diaferia G, Cardano M, Biunno I, Qi L, et al. 2010. Deficiency of Suppressor Enhancer Lin12 1 Like (SEL1L) in Mice Leads to Systemic Endoplasmic Reticulum Stress and Embryonic Lethality. Journal of Biological Chemistry. 285(18):13694–13703. doi:10.1074/jbc.M109.085340. [accessed 2019 Mar 18]. http://www.jbc.org/lookup/doi/10.1074/jbc.M109.085340.

Gardner BM, Walter P. 2011. Unfolded Proteins Are Ire1-Activating Ligands That Directly Induce the Unfolded Protein Response. Science. 333(6051):1891–1894.

doi:10.1126/science.1209126. [accessed 2018 Nov 7].
http://science.sciencemag.org/content/333/6051/1891.

Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D. 2000. Regulated
Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells.
Molecular Cell. 6(5):1099–1108. doi:10.1016/S1097-2765(00)00108-8. [accessed 2018
Dec 13]. https://www.cell.com/molecular-cell/abstract/S1097-2765(00)00108-8.

Hetz C, Saxena S. 2017. ER stress and the unfolded protein response in
neurodegeneration. Nature Reviews Neurology. 13(8):477–491.
doi:10.1038/nrneurol.2017.99. [accessed 2018 Sep 12].
https://www.nature.com/articles/nrneurol.2017.99.

Hippius H, Neundörfer G. 2003. The discovery of Alzheimer's disease. Dialogues Clin
Neurosci. 5(1):101–108. [accessed 2018 Dec 12].
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3181715/.

Jack CR, Knopman DS, Jagust WJ, Petersen RC, Weiner MW, Aisen PS, Shaw LM,
Vemuri P, Wiste HJ, Weigand SD, et al. 2013. Tracking pathophysiological processes in
Alzheimer's disease: an updated hypothetical model of dynamic biomarkers. The Lancet
Neurology. 12(2):207–216. doi:10.1016/S1474-4422(12)70291-0. [accessed 2019 Feb 4].
http://www.sciencedirect.com/science/article/pii/S1474442212702910.

Jin S, Kedia N, Illes-Toth E, Haralampiev I, Prisner S, Herrmann A, Wanker EE,
Bieschke J. 2016. Amyloid-β(1–42) Aggregation Initiates Its Cellular Uptake and
Cytotoxicity. J Biol Chem. 291(37):19590–19606. doi:10.1074/jbc.M115.691840.
[accessed 2018 Nov 7]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5016693/.

Kochanek KD. 2017. Mortality in the United States, 2016. (293):8.

Kovalevich J, Langford D. 2013. Considerations for the Use of SH-SY5Y Neuroblastoma
Cells in Neurobiology. Methods Mol Biol. 1078:9–21. doi:10.1007/978-1-62703-640-
5_2. [accessed 2019 Apr 5]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5127451/.

Matus S, Glimcher LH, Hetz C. 2011. Protein folding stress in neurodegenerative
diseases: a glimpse into the ER. Current Opinion in Cell Biology. 23(2):239–252.
doi:10.1016/j.ceb.2011.01.003. [accessed 2018 Dec 13].
http://www.sciencedirect.com/science/article/pii/S0955067411000056.

Maurel M, Chevet E, Tavernier J, Gerlo S. 2014. Getting RIDD of RNA: IRE1 in cell
fate regulation. Trends in Biochemical Sciences. 39(5):245–254.
doi:10.1016/j.tibs.2014.02.008. [accessed 2018 Nov 7].
http://www.sciencedirect.com/science/article/pii/S0968000414000334.

Mayeux R. 2003. Epidemiology of Neurodegeneration. Annual Review of Neuroscience. 26(1):81–104. doi:10.1146/annurev.neuro.26.043002.094919. [accessed 2018 Nov 7]. https://doi.org/10.1146/annurev.neuro.26.043002.094919.

Montibeller L, de Belleroche J. 2018. Amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) are characterised by differential activation of ER stress pathways: focus on UPR target genes. Cell Stress and Chaperones. 23(5):897–912. doi:10.1007/s12192-018-0897-y. [accessed 2018 Sep 12]. https://doi.org/10.1007/s12192-018-0897-y.

O'Brien RJ, Wong PC. 2011. Amyloid Precursor Protein Processing and Alzheimer's Disease. Annu Rev Neurosci. 34:185–204. doi:10.1146/annurev-neuro-061010-113613. [accessed 2018 Dec 12]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3174086/.

Olsson F, Schmidt S, Althoff V, Munter LM, Jin S, Rosqvist S, Lendahl U, Multhaup G, Lundkvist J. 2014. Characterization of Intermediate Steps in Amyloid Beta (Aβ) Production under Near-native Conditions. J Biol Chem. 289(3):1540–1550. doi:10.1074/jbc.M113.498246. [accessed 2019 Feb 4]. http://www.jbc.org/content/289/3/1540.

Ron D, Walter P. 2007. Signal integration in the endoplasmic reticulum unfolded protein response. Nature Reviews Molecular Cell Biology. 8(7):519–529. doi:10.1038/nrm2199. [accessed 2018 Dec 13]. https://www.nature.com/articles/nrm2199.

Saltini G, Dominici R, Lovati C, Cattaneo M, Michelini S, Malferrari G, Caprera A, Milanesi L, Finazzi D, Bertora P, et al. 2006. A novel polymorphism in SEL1L confers susceptibility to Alzheimer's disease. Neuroscience Letters. 398(1):53–58. doi:10.1016/j.neulet.2005.12.038. [accessed 2019 Mar 7]. http://www.sciencedirect.com/science/article/pii/S0304394005014436.

Sano R, Reed JC. 2013. ER stress-induced cell death mechanisms. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 1833(12):3460–3470. doi:10.1016/j.bbamcr.2013.06.028. [accessed 2018 Nov 7]. http://www.sciencedirect.com/science/article/pii/S0167488913002516.

Shoulders MD, Ryno LM, Genereux JC, Moresco JJ, Tu PG, Wu C, Yates JR, Su AI, Kelly JW, Wiseman RL. 2013. Stress-Independent Activation of XBP1s and/or ATF6 Reveals Three Functionally Diverse ER Proteostasis Environments. Cell Reports. 3(4):1279–1292. doi:10.1016/j.celrep.2013.03.024. [accessed 2018 Nov 7]. http://www.sciencedirect.com/science/article/pii/S2211124713001319.

Tam AB, Koong AC, Niwa M. 2014. Ire1 Has Distinct Catalytic Mechanisms for XBP1/HAC1 Splicing and RIDD. Cell Reports. 9(3):850–858.

doi:10.1016/j.celrep.2014.09.016. [accessed 2018 Nov 7].
http://www.sciencedirect.com/science/article/pii/S2211124714007840.

Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. 2000. Coupling
of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase
IRE1. Science. 287(5453):664–666.

Vembar SS, Brodsky JL. 2008. One step at a time: endoplasmic reticulum-associated
degradation. Nature Reviews Molecular Cell Biology. 9(12):944–957.
doi:10.1038/nrm2546. [accessed 2018 Dec 13].
https://www.nature.com/articles/nrm2546.

Walter F, O'Brien A, Concannon CG, Düssmann H, Prehn JHM. 2018. ER stress
signaling has an activating transcription factor 6α (ATF6)-dependent "off-switch."
Journal of Biological Chemistry. 293(47):18270–18284. doi:10.1074/jbc.RA118.002121.
[accessed 2018 Dec 5]. http://www.jbc.org/lookup/doi/10.1074/jbc.RA118.002121.

Xiang C, Wang Y, Zhang H, Han F. 2017. The role of endoplasmic reticulum stress in
neurodegenerative disease. Apoptosis. 22(1):1–26. doi:10.1007/s10495-016-1296-4.
[accessed 2018 Nov 7]. https://doi.org/10.1007/s10495-016-1296-4.

Zhang Y, Thompson R, Zhang H, Xu H. 2011. APP processing in Alzheimer's disease.
Mol Brain. 4:3. doi:10.1186/1756-6606-4-3. [accessed 2019 Feb 1].
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3022812/.