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:

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Georgette Jones, Ph.D.
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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.
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**
   (If “Yes,” state number and title)
   Number:  
   Title:  

3. **PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR**
   
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   3h. eRA Commons User Name  
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   4b. Federal-Wide Assurance No.  
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   From  
   Through  

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   7b. Total Costs ($)  

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   8a. Direct Costs ($)  
   
   8b. Total Costs ($)  

9. **APPLICANT ORGANIZATION**
   
   Name  
   Department of Biology  
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10. **TYPE OF ORGANIZATION**
    
    Public:  
    Federal  
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    Local  
    
    Private:  
    Private Nonprofit  
    
    For-profit:  
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    Woman-owned  
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11. **ENTITY IDENTIFICATION NUMBER**
    
    DUNS NO.  
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12. **ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE**
    
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   SIGNATURE OF OFFICIAL NAMED IN 13.  
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   DATE  
   April 15, 2019
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.

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.
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Graduate student

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

<table>
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<th>INSTITUTION AND LOCATION</th>
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<td>M.S.</td>
<td>2019 (anticipated)</td>
<td>Biomedical Science</td>
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A. Positions and Honors

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

C. Research Support
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
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.
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).

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).](image-url)
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-secretase 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). Amyloidogenic 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). IRE1α 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.
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<td>NM_004911.4</td>
<td>ERSE</td>
<td>−68</td>
<td>ATF6</td>
<td>XBP1</td>
</tr>
<tr>
<td>os9</td>
<td>NM_006812.3</td>
<td>ERSE</td>
<td>−107</td>
<td>XBP1</td>
<td></td>
</tr>
<tr>
<td>SEL1</td>
<td>NM_005065.5</td>
<td>ERSE</td>
<td>−12331</td>
<td>ATF6</td>
<td>XBP1</td>
</tr>
<tr>
<td>SIL1</td>
<td>NM_001037633.1</td>
<td>ERSE</td>
<td>+6829</td>
<td>XBP1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Selected target genes of XBP1 and ATF6 characterized based on the transcriptional response elements (ERSE) 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 β-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]. β-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 β-actin levels and expressed relative to DMSO treated scram ctrl cells (Walter et al. 2018).
**RESEARCH 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-SYS5. 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% CO₂ 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 $2 \times 10^5$ 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% CO₂ until 70% confluency is reached. Media will be renewed every 4 to 7 days.
### Table 2

<table>
<thead>
<tr>
<th>Human Protein Sequence</th>
<th>Human Protein Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β Amyloid [1-40] PTD</td>
<td>β Amyloid [1-42] PTD</td>
</tr>
<tr>
<td>H2N-Asp-Ala-Glu-Phe-Arg-</td>
<td>H2N-Asp-Ala-Glu-Phe-Arg-</td>
</tr>
<tr>
<td>His-Asp-Ser-Gly-Tyr-Glu-</td>
<td>His-Asp-Ser-Gly-Tyr-Glu-</td>
</tr>
<tr>
<td>Val-His-His-Gln-Lys-Leu-</td>
<td>Val-His-His-Gln-Lys-Leu-</td>
</tr>
<tr>
<td>Val-Phe-Phe-Ala-Glu-Asp-</td>
<td>Val-Phe-Phe-Ala-Glu-Asp-</td>
</tr>
<tr>
<td>Val-Gly-Ser-Asn-Lys-Gly-</td>
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</tr>
<tr>
<td>Ala-Ile-Ile-Gly-Leu-Met-Val-</td>
<td>Ala-Ile-Ile-Gly-Leu-Met-Val-</td>
</tr>
<tr>
<td>Gly-Gly-Val-Val-OH</td>
<td>Gly-Gly-Val-Val-Ile-Ala-OH</td>
</tr>
</tbody>
</table>

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\(_{\alpha\beta}\): 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-A\(\beta\)42 (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-\(\beta\)-Actin (A2228, Sigma) will be used as the loading control.

**Immunofluorescence**

Immunofluorescence will be performed to detect co-localization of A\(\beta\)(1-42) and A\(\beta\)(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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stress</th>
<th>Aβ 42/40 (WB)</th>
<th>pPerk (WB)</th>
<th>pIRE1 (WB)</th>
<th>ATF6 (WB)</th>
<th>Caspase-3 (WB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Aβ</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+Aβ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stress</th>
<th>Co-localization</th>
<th>sAPPβ</th>
<th>Aβ 42/40</th>
<th>P3</th>
<th>Calnexin</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Aβ</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+Aβ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

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.1-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 CO₂ 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 CO₂ 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 CO₂ 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

<table>
<thead>
<tr>
<th></th>
<th>Human shRNAs ATF6 (TRCN0000017853)</th>
<th>Human shRNAs ATF6 (TRCN0000017855)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequence</strong></td>
<td>5’CCGGCCCAGAAGTTATCAAGACTTTTCGAGAAAATCTTGGATAACTTCTGGGTTTT-3’</td>
<td>5’-CCGGGCAGCAACCAATTATCAGTTTCTCGAGAAATGATAATTGGTTGCTGCTTTT-3’</td>
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</table>

Table 5. pLKO.1-puro shRNA AFT6 sequence
Table 6. Aim 2 experimental set up

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Positive control vector</th>
<th>Negative control vector</th>
<th>Human ATF6 shRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Aβ</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-Aβ</td>
<td>-</td>
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<td>+</td>
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</tr>
<tr>
<td>+Aβ</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

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.
<table>
<thead>
<tr>
<th></th>
<th>ATF6 Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Primer</td>
<td>5’TCCTCGGTCAGTGGACTCTTA3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5’CTTGGGTTGAATTGAAGGTTTTG3’</td>
</tr>
</tbody>
</table>

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 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 anti-Aβ42 (AB5070, Millipore), β-Amyloid (1-40) Antibody (44-136, ThermoFisher), anti-sAPPβ (SIG-39138, Biolegend), anti-P3 (APP345, Aveslab), anti-Clnexin 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.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Treatment</th>
<th>Stress</th>
<th>Viability</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-Aβ</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>+Aβ</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>KD</td>
<td>-Aβ</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>KD</td>
<td>+Aβ</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

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 CO2 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Human ATF6 shRNAs</th>
<th>Mouse cDNA exp’n vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Aβ</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+Aβ</td>
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</tr>
<tr>
<td>+Aβ</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Treatment</th>
<th>Stress</th>
<th>Viability</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-Aβ</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>+Aβ</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rescue</td>
<td>-Aβ</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rescue</td>
<td>+Aβ</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

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.
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