THE ROLE OF SIGMA-1 RECEPTOR IN MODULATING ENDOPLASMIC RETICULUM STRESS: PUTATIVE RELEVANCE TO ALZHEIMER DISEASE

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ABSTRACT

Alzheimer's Disease and other neurodegenerative diseases have been linked to dysfunction in proteostasis in the endoplasmic reticulum (ER). The ER provides an exclusive environment for protein synthesis and folding, which is vital to the cellular function. Under normal conditions, the synthesis and degradation of proteins remain in balance. During aging or during pathological states, disturbances of ER occur and consequently the failure of protein homeostasis. The cells rely on a system, the unfolded protein response (UPR), which regulates the homeostasis by three ER sensors: PERK, ATF6, and IRE-1. Perturbations of ER function result in UPR. In physiological condition, the cell may overcome the insult and regain homeostasis. However, prolonged or chronic UPR activates apoptotic pathways and may cause cell death. The sigma-1 receptor (Sig-1R) is a 25 kD polypeptide and a chaperone protein concentrated at the mitochondria-associated ER membrane domain (MAM). The Sig-1R plays significant roles governing calcium signalling, mitochondrial function, oxidative stress, protein chaperoning and ER stress. Results of this investigation demonstrate that immortalized mouse embryonic fibroblasts (MEFs) derived from Sig-1R$^{-/-}$(KO) mice have higher baseline activation in all three branches of the UPR in the absence of ER stress compared to MEFs derived from Wild-type mice. Despite this increase in baseline activation, the PERK and ATF6 pathways have a significantly blunted response to acute stress. Rescue experiments by expressing the Sig-1R in KO MEFs did not recover the WT MEFs phenotype. Primary Sig-1R KO MEFs did not show baseline ER stress, but did show inhibited recovery following treatment with the acute ER stressor DTT. Overall, our data suggests that Sig-1R is important for the reestablishment of proteostasis following acute stress.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AD: Alzheimer's Disease

Aβ: Amyloid-Beta

AβOs: Amyloid-Beta Oligomers (AβOs

APP: Amyloid Precursor Protein

ApoE: apolipoprotein E

ATF4: Activating transcription factor 4

ATF6: Activating Transcription Factor 6

Bcl2: Apoptosis regulator Bcl-2

BiP: Binding immunoglobulin Protein

Ca: Calcium

CHOP: C/EBP homologous protein

DTT: Dithiothreitol

eIF2 α: Eukaryotic translation initiation factor 2A

EOAD: Early-Onset Alzheimer's Disease

ER: Endoplasmic Reticulum

ERAD: Endoplasmic Reticulum Associated Degradation

ERSE: Endoplasmic Reticulum stress element

G1 phase: Gap 1 phase

GADD34: DNA damage—inducible 34

Herp: Homocysteine-induced endoplasmic reticulum protein

IKK: IκB kinase

IP3R: Inositol 1, 4, 5-trisphosphate Receptor

IRE1: Inositol-requiring enzyme 1

JNK: c-Jun N-terminal kinase

KO: Knock Out

LOAD: Late-Onset Alzheimer's Disease

MAM: Mitochondrial—endoplasmic reticulum Associated Membrane

mRNA: messenger Ribonucleic Acid

MWM: Moris Water Maze

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NF-κB: Nuclear Factor kappa B

NF—γ: Nuclear transcription Factor Gama

NFTs: NeuroFibrillary Tangles

Nrf2: Nuclear factor-erythroid 2-related Factor 2

Orai1: Calcium release-activated calcium channel protein 1

ORF: Open reading Frame

PM: Plasma Membranes

PERK: Protein kinase RNA-like Endoplasmic Reticulum Kinase

PP1C: Protein Phosphatase 1C

PS1: Presenilin 1

PS2: Presenilin 2

PTZ: Pentazocine

Rac1: Ras-related C3 botulinum toxin substrate 1

RIDD: Regulated IRE1-dependent mRNA-decay

ROS: Reactive Oxygen Species

Sig-1R: Sigma-1 receptor

SKF: 10,047: (2S,6S,11S)-1,2,3,4,5,6-Hexahydro-6,11-dimethyl-3- (2-propenyl)-2.6-methano-3-benzazocin-8-ol hydrochloride

S1P: Site 1 Protease

S2P : Site 2 Protease

SOCE: Store-Operated Ca^{2+} Entry

STIM1: stromal interaction molecule 1

Tau: Tubulin-Associated Unit

TNF-α: Tumour Necrosis Factor alpha

TRAF2: TNF receptor-associated factor 2

UPR: Unfolded Protein Response

UPRE: Unfolded Protein Response Elements

WT: Wild Type

XBP1: X-box protein

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1. INTRODUCTION

1.1 Alzheimer's Disease

1.1.1 Background

Alzheimer's disease (AD), a neurodegenerative disorder, is most common cause of dementia witnessed among aged people. AD represents 2/3 of all types of dementia. The major characteristic of AD is the decline in cognitive functions and consequently a dysfunction in social, behavioural and other daily activities. AD was first described in 1906 by the German psychiatrist Alois Alzheimer (Alzheimer et al. 1995; Maurer et al. 1997; Möller & Graeber 1998; Reitz et al. 2011; Scheltens et al. 2016). The socio-economic impact of AD amount over the billions, with considerable suffering experienced for the patients and entourage. With over 35 million people suffering from dementia worldwide, this growing social and economic burden on society is in part due to the ever-increasing aging of the worldwide population (Mota et al. 2014; Prince et al. 2013; Wimo et al. 2013). Despite decades of intense fundamental and clinical research, there are still no cures for AD and only limited therapeutic interventions are available to treat the symptoms.

AD is primarily characterized by synaptic dysfunction. This induces dysfunction of neuronal networks is manifested as episodic memory loss in early stages of the disorder which slowly transgress into dementia as the disorder progress (Jacobsen et al. 2006; Ondrejcak et al. 2010; Roy et al. 2016; Terry et al. 1991). The severity of the dementia is highly correlated with neuronal loss that succeeds to the loss of synapses in the brain (DeKosky & Scheff 1990; Shankar & Walsh 2009). The progressive loss of synapses and neurones begins in the hippocampus, a brain region essential in learning and memory

process, and vicinal region of the medial temporal lobes (Braak & Braak 1991; Braak & Braak 1997; Henderson 2014; Isik 2010; Vorhees & Williams 2014). Over the progression of the disease, synapses and neurons loss increasingly affect the association areas of the cerebral cortex, resulting in the progressive loss of short-term/working memory (also referred as visuospatial memory), cognitive flexibility, and other cognitive abilities (Carlesimo & Oscar-Berman 1992; Elcombe et al. 2014; Mucke & Selkoe 2012; Twamley et al. 2006).

There are two forms of AD, that could be determined based on the age of the onset of symptoms. The first form, a rare familial type (early onset; EOAD) is caused by autosomal dominant mutations, with an early onset that occurs before the age of 60 years. The second form and the most common one is the sporadic form (late onset; LOAD), with an onset occurring after 60 years of age (Brouwers et al. 2008; Kukull & Bowen 2002; Kukull et al. 2002; Reitz & Mayeux 2014). The EOAD is associated with mutations in the amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2) genes, all of which are linked to excessive production, accumulation, or deposition of amyloid beta (Aβ) peptides in the brain (Cummings 2004; Goate 2006; Lannfelt et al. 2014; Selkoe 1989) (Figure **1.1 Alzheimer's disease progression.**). However, despite the great amount of research on the elucidation of the complex molecular mechanisms of LOAD, the environmental and genetic components are not yet, fully understood (Balin & Hudson 2014; Zou et al. 2014).

1.1.2 Hallmarks

The pathophysiology of AD develops as a consequence of neurofibrillary tangle formation. This consists of hyperphosphorylated microtubule associated tau protein and senile plaques of amyloid-β (Aβ) peptide in specific brain regions that result in synaptic loss and neuronal death. The molecular hallmarks that characterized AD include neurofibrillary tangles (NFTs) and neuritic plaques (Ballenger 2006; Blennow et al. 2006; Dennis J Selkoe 2011a). These NFTs consist of intracellular filaments, primarily. Composed of hyperphosphorylated tubulin-associated units (tau), a protein involved in the stabilization of microtubules, which are able to self-assembly (Binder et al. 2005; Claeysen et al. 2012; Mucke & Selkoe 2012). These NFTs are basically located within cell bodies of pathological neurons of the cerebral cortex and brain stem (Braak & Braak 1991; Henderson 2014). As for the neuritic plaques, they are mainly constitute of Aβ, a peptide derived from the cleavage of APP (Mucke & Selkoe 2012; D. Puzzo et al. 2015; Zetterberg & Mattsson 2014). This peptide adopts a β-pleated sheet configuration which has the potential of self-aggregated to form soluble oligomers as well as insoluble amyloid beta sheets. This is these insoluble sheets that accumulate in the extracellular space between neurons, build up and associate with astrocytes, microglia, and dystrophic neurites (Selkoe 1989; Dennis J Selkoe 2011b; Toyn & Ahlijanian 2014). The neuritic plaques are primarily formed in the basal isocortex (layer I) and gradually spread through the entire isocortex structure as the disease progresses. In the late stages of AD, neuritic plaques are found throughout the brain, with larger density in the isocortex (Braak & Braak 1991).

However, more recently, there is increasing evidence that soluble Aβ oligomers (AβOs) are the molecules involve in the severity of the cytotoxicity and neuronal viability

as well as for the negative effects on synaptic function (Goate 2006; Lambert et al. 1998; Lannfelt et al. 2014; Mucke & Selkoe 2012; Oda et al. 1994).

1.1.3 Risk factors

Sporadic AD (LOAD), the most common form, represents more than 90% of AD case, is caused by an amalgam of environmental and genetic risk factors, for which very little are currently known. The complexity of the disease is further enhanced by a suggested sex differences in pathophysiology, which reinforcing its heterogeneity. Among the environmental risk factors, studies have identified as potential, the levels of physical activity, obesity, smoking, and alcohol consumption (Graves et al. 1991; Rovio et al. 2005) However, the predominant risk factor for AD is aging (Blennow et al. 2006; Ferreira et al. 2015; Jagust 2013; Prince et al. 2013).

As regards to genetic factors, the predominant mutation occurs in apolipoprotein E (ApoE) gene on chromosome 19, and the inheritance ApoE alleles ε4, which are associated with higher Aβ load in the brain (Andreasson et al. 2014; Selkoe 1994a; Strittmatter et al. 1993). This polymorphism correlate with an increased risk of sporadic AD that interestingly, prevail more in women than in men (Bretsky et al. n.d.; Farrer et al. 1997; Mielke et al. 2014; Reitz & Mayeux 2014). Finally, hormonal changes produced by menopause impact neuronal processes involved in cognition and has been suggested to be a risk factor in AD (Pike 2017; Vest & Pike 2013).

1.1.4 Amyloid cascade hypothesis

While most often noted for its cognitive symptoms, Alzheimer's disease (AD) is, at its core, a disease of protein misfolding/aggregation, with an intriguing inflammatory component. Defective clearance and/or abnormal production of the amyloid-β peptide (Aβ), and its ensuing accumulation and aggregation, underlie two hallmark features of AD: brain accumulation of insoluble protein deposits known as amyloid or senile plaques, and buildup of soluble Aβ oligomers (AβOs), diffusible toxins linked to synapse dysfunction and memory impairment. Although the AD heterogeneity in its complexity, the predominant theory about AD outgrowth is still amyloid cascade hypothesis. It was first been proposed in the early 1990s and it stipulates that the production and aggregation of Aβ into plaques are causing a toxic cascade responsible for neuronal dysfunctions, synaptic and neuronal loss (Glenner & Wong 1984; Hardy & Allsop 1991; Hardy & Higgins 1992; Hardy & Selkoe 2002; Karran et al. 2011; Selkoe 1991). These neuronal insult was proposed to play a role in cerebral metabolism decline, brain inflammation, cognitive impairment and brain atrophy, with stronger infringement in the hippocampus (Ball et al. 1985; Licastro et al. 2017). However, it has been more recently suggested that the primary source of toxic responsible for the major neuropathological aspect of AD is the soluble oligomeric Aβ species, rather than the insoluble plaques (Bitan et al. 2003; Frackowiak et al. 1994; Glabe & Kayed 2006; Lue et al. 1999; McLean et al. 1999; Paola et al. 2000; Walsh & Teplow 2012; Walsh et al. 2002; Wang et al. 1999). However, the Aβ is still considered as the major component in AD pathogenesis along with other pathological downstream hallmarks, including tau accumulation and neurodegeneration.

1.1.5 Amyloid-beta peptide

Interestingly, APP, a highly conserved type-1 transmembrane glycoprotein located on chromosome 21 in humans, has been suggested to be essential for normal brain development as well as brain plasticity in adults; (Selkoe 1994b; Shariati & De Strooper 2013). However, the Aβ peptide is produced following a sequential proteolytic cleavage of APP (Hamley 2012; Kopan & Ilagan 2004). The proteolytic cleavage is initiated by the catalytic activity of either α- or β-secretases. The α-secretases are responsible for the cleavage of the soluble extracellular domains (αAPP) and an 83 amino acid carboxy-terminal fragments (C83) at one site. As for the β-secretase, it is responsible for the production of βAPP and C99 peptides on another cleavage site (Cummings 2004; Daniela Puzzo et al. 2015; D. J. Selkoe 2011). The maturation of the toxic peptides is then insured by the γ-secretase, a large multiprotein complex constitute, among others, of the subcomponents: PS1 and PS2. The subsequent proteolytic cleavage of βAPP by the γ-secretase then gives rise to a 40 or 42 amino acid Aβ peptide ($A\beta_{1-40}$ or $A\beta_{1-42}$) (Kopan & Ilagan 2004; Selkoe 1994a). Finally, because of the exposure of two hydrophobic alanine and isoleucine residues, the $A\beta_{1-42}$ peptide are able to self-aggregate into toxic AβOs (Mucke & Selkoe 2012; Toyn & Ahlijanian 2014; Weiner et al. 2013).

1.1.6 Clinical trials

As mentioned previously, the soluble and insoluble Aβ peptides are considered as the primary component of the AD pathogenicity in which they initiate and supply the cascade involves neuronal and cognitive dysfunction (Gharibyan et al. 2007; Hamley 2012; Lee et al. 2012; Pepys 2006; Williams & Serpell 2011). However, some clinicopathological

studies found unclear, the relationship between cognitive dysfunctions and Aβ load (Castellani & Smith 2011; Castellani et al. 2009; Gustafson et al. 2006; Näslund et al. n.d.; Vos et al. 2013). Moreover, results from therapeutic approaches targeting Aβ production or clearance has not been promising (Mangialasche et al. 2010). Several Aβ-targeting drug candidates such as secretase inhibitors and anti-Aβ antibodies have failed to improve the patient's prognostic (Barten et al. 2006; Doody et al. 2013; Doody et al. 2014; Forman et al. 2012; Hardy et al. 2014; Martenyi et al. 2012; Salloway et al. 2014). These result may suggests that they have another component that drives the pathogenicity of AD. However it is also possible that clinical trials failed because the drugs were given too late; if dementia is due to loss of cortical neurons, then drugs are unlikely to replace these lost neurons.

1.2 Sigma-1 Receptor

1.2.1 Sigma-1 Receptor background

A new player, the Sigma-1 receptor (Sig-1R), has more recently shown to be neuroprotective in models of AD. The Sigma-1 receptor is a highly conserved endoplasmic reticulum (ER) chaperone protein of 223 amino acids that contains one N-terminal transmembrane domain and that shows very little sequence homology to other mammalian proteins (Figure 1.2 **The Sig-1R homotrimer and its position in the ER membrane.** (Hanner et al. 1996; Hayashi & Su 2007; Schmidt et al. 2016). Primary studies have identified the enrichment of the Sig-1R at the mitochondrial—ER associated membrane (MAM), in neuronal and non-neuronal cells (Hayashi & Su 2003; Hayashi & Su 2007; Mavlyutov et al. 2012). Moreover, the Sig-1R has been found to be enriched in lipid rafts

where several cellular functions are regulated and possibly at the nucleus (Hayashi & Su 2003; Natsvlishvili et al. 2015; Srivats et al. 2016; Wu & Bowen 2008). In physiological conditions, the Sig-1R interacts with the ubiquitous binding immunoglobulin protein (BiP) ER chaperone (Hayashi & Su 2007). Following ER stress or ligand activation, as per an unfolded protein response (UPR) sensor, the Sig-1R dissociates from BiP and modulates ER and plasma membranes (PM) ionic channel receptors, more specifically potassium and calcium channels (Gao et al. 2012; Hayashi & Su 2007; Hayashi et al. 2012; Johannessen et al. 2009; Mavlyutov et al. 2010). The Sig-1R has been shown to be transcriptionally up regulated by the UPR, more precisely through the protein kinase RNA-like endoplasmic reticulum kinase (PERK) pathway, directly through activating transcription factor 4 (ATF4) transcriptional activation. Interestingly, the ATF4 transcription expression level has also shown to be upregulated following Sig-1R activation (Mitsuda et al. 2011; Omi et al. 2014).

One of the roles of the Sig-1R is to regulate the Ca^{2+} signalling at two distinct sites. At the cell surface, it has been shown to negatively regulate the store-operated Ca^{2+} entry (SOCE) by preventing stromal interaction molecule 1 (Stim1)- Calcium release-activated calcium channel protein 1 (Orai1) interaction (Srivats et al. 2016). In addition to SOCE modulations, at the MAM it also plays a regulatory role by promoting the calcium efflux to mitochondria through dissociation of inositol 1, 4, 5-trisphosphate receptor (IP3R) from Ankyrin and by stabilizing it in its active conformation (Wu & Bowen 2008). This same complex, in conjunction with Apoptosis regulator Bcl-2 (Bcl-2) and Ras-related C3 botulinum toxin substrate 1 (Rac1), has also been suggested to be involved in reactive

oxygen species (ROS) signalling through nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activation (Natsvlishvili et al. 2015). A more recent study has shown that the Sig-1R directly interacts with Inositol-requiring enzyme 1 (IRE1) to stabilize its active form at the ER membrane. This complex enriched at the MAM, was also suggested to monitor the ROS level generated by the mitochondria (Mori et al. 2013; Omi et al. 2014) This is further strengthened by other studies that suggested a role for Sig-1R in suppression or reduction of the accumulation/production of reactive oxygen species (ROS) neighbouring mitochondria since Sig-1R knock-down shows an increased accumulation of ROS. This accumulation is suggested to be attributed to the nuclear factor-erythroid 2-related factor 2 (Nrf2), nuclear factor kappa B (NF-κB) and tumour necrosis factor alpha (TNF-α) signalling (Allahtavakoli & Jarrott 2011; Hayashi et al. 2011; Meunier & Hayashi 2010; Mori et al. 2012; Tsai et al. 2012; Tsai et al. 2015; Zhao et al. 2014)

The activation of Sig1-R by Pentazocine (PTZ) and (2S,6S,11S)-1,2,3,4,5,6-Hexahydro-6,11-dimethyl-3-(2-propenyl)-2.6-methano-3-benzazocin-8-ol hydrochloride (SKF 10,047; SKF), two selective Sig-1R agonists, alleviated the ER stress response related UPR markers and reduced the cell lethality in a primary neuronal retinal cell lines prone to oxidative stress. This suggests a neuroprotective role for the Sig-1R (Ha et al. 2011; Omi et al. 2014). To further support this effect, overexpression of the Sig-1R decreased cell apoptotic index whereas, an increase in apoptosis is observed in Sig-1R knock out (KO) cells following ER stress activation (Ha et al. 2014; Hayashi & Su 2007). It is therefore a potential therapeutic target to overcome ER stress-related disease (Ono et al. 2013).

1.2.2 Sigma-1 receptor and Alzheimer's disease

Sig-1R had first been found to have a decreased ligand binding and lower density in ex vivo experiments and in patients suffering from AD (Jansen et al. 1993; Mishina et al. 2008). Afterward, a polymorphism found in the 5'-upstream region of Sig-1R genes (SIGMAR1), have shown to reduce its transcriptional activity, thereby reduces Sig-1R expression and have been associated with an increased risk of developing AD (Huang et al. 2011; Miyatake et al. 2004; Fehér et al. 2012; Jin et al. 2015).

Moreover, the Sig-1R role in AD has been fortified by studies which show that Sig-1R agonists attenuate memory deficits in various rodent models of amnesia (Earley et al. 1991; Matsuno et al. 1994; Matsuno et al. 1997; Maurice & Privat 1997; Maurice et al. 1994; Senda et al. 1998; Zou et al. 2000). The agonized benefit of Sig-1R as anti-amnesic agents is furfur more fortified by studies in males that shown neuroprotective and anti-amnesic effect in AD animal models (Ishikawa & Hashimoto 2010; Maurice & Su 2009; Maurice et al. 1998; Maurice et al. 2006; Meunier et al. 2009; Nguyen et al. 2015; Urani et al. 2002).

Activation of the Sig-1R with SKF has also shown to ameliorate memory deficits in an AD mouse model, and reduce oxidative stress markers induced by Aβ in-vitro (Maurice et al. 2016; Nguyen et al. 2015).

Due to its neuroprotective effects in models of AD combined with the fact it is down regulated in AD pathology, it is clear that the Sig-1R's role in AD is worth investigating further.

1.2.3 Sigma-1 receptor knockout

The absence of homology of Sig-1R with any other mammalian protein suggests a fundamental function for Sig-1Rs. The cloning of the Sig-1R was therefore a big step in the study of its structure and function but also the elaboration of Sig-1R KO mice (Langa et al. 2003). Interestingly, Sig-1R KO mice were shown to have a lower axon density as well as impaired hippocampus neurogenesis (Sha et al. 2013; Sha et al. 2015; Tsai et al. 2015). Our laboratory has revealed through electrophysiology, mild synaptic plasticity deficits in Sig-1R KO compared to the wild type (WT) male mice, without change in other aspects of basic cellular physiology. (Snyder et al. 2016).

Surprisingly, the KO mice developed normally. However it did show subtle, sex-specific, behavioural phenotypes in pain, depression, anxiety, and cognition paradigms. Behavioural studies using chemically induced and neuropathic pain models reveal an attenuated response to pain in Sig-1R KO mice from both sexes as compared to WT (Cendán et al. 2005; Entrena et al. 2009; Nieto et al. 2014; Puente et al. 2009). The depressive-like phenotype seems to predominate in male as several behavioural studies shown depressive-like phenotypes in two to eight-month-old male Sig-1R KO, but not female (Chevallier et al. 2011; Sabino et al. 2009; Sha et al. 2015; Zhang et al. 2017). Moreover, anxiety behaviours were detected only in Sig-1R KO males using behavioural tasks (Chevallier et al. 2011). However, only the Sig-1R KO female revealed learning and memory deficits in the behavioural tasks, suggesting that in the AD paradigm, the female could be hardly impacted (Chevallier et al. 2011).

1.3 Endoplasmic reticulum stress

1.3.1 Endoplasmic reticulum control of protein

Proteostasis is of prime importance for cell health and regulation. The ER, along with Golgi apparatus, is the major organelles responsible for the proteins folding, post-translational modification and, export and secretion (Ron & Walter 2007; Walter & Ron 2012). The proper folding of protein is ensured by a large variety of chaperones that also modulate degradation (Vembar & Brodsky 2008). To regulate proteostasis, the ER use highly conserved mechanism use for quality control or engage following ER stress activation. Among those mechanisms, there is the ER associated degradation (ERAD), responsible for the degradation of misfolded protein, and the UPR, trigger following the accumulation of unfolded protein (Hetz & Mollereau 2014). If the UPR fail to restore proper protein load and folding among the ER, it is known to trigger signalling cascades that induced apoptosis (Walter & Ron 2012). Defects in UPR are known to be involved in neurodegenerative disorder such as Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, frontotemporal lobar degeneration, Parkinson's disease but also other dysfunction like psychiatric disorder, diabetes and cancer (Gold et al. 2013; Hayashi et al. 2009; Kakiuchi et al. 2004; Kawada et al. 2014; Nevell et al. 2014; Prell et al. 2014; Tsai et al. 2014; Wang & Kaufman 2014). It is therefore important to get a good understanding of these mechanisms to be able to overcome these diseases.

1.3.2 The Unfolded Protein Response

The UPR is mediated by three major ER receptors: Activating Transcription Factor 6 (ATF6), PERK and IRE1. The activation of these receptor result in a global decrease in gene transcription and, an up regulation of chaperones and ER stress effectors (Ron & Walter 2007). (Figure 1.3 Unfolded Protein Response (UPR)

The most conserved branch of the UPR is mediated by IRE1 receptors which remains very similar to its yeast homologue (Walter & Ron 2012). This receptor is an ER membrane resident protein consisting of, a sensor luminal domain, a single transmembrane domain and a cytoplasmic domain responsible for a double functionality kinase/endonuclease (Tirasophon et al. 1998). Following UPR activation, IRE1 dissociate from BiP, self-phosphorylate and dimerize into its active form (Carrara et al. 2015; Pincus et al. 2010). Following activation, IRE1 exerts its function through three different pathways; X-box protein 1 (XBP1), TNF receptor-associated factor 2 (TRAF2) and regulated IRE1-dependent mRNA-decay (RIDD) (Hollien & Weissman 2006; Imagawa et al. 2008; Zeng et al. 2015). The XBP1 pathway is activated following an uncanonical exon excision from XBP1 mRNA allowing the transcription of XBP1p, a transcription factor that binds to ER stress element (ERSE), sequence CCAAT-N9-CCACG, and unfolded protein response elements (UPRE), sequence TGACGTGG/A (Keisuke Yamamoto, Hiderou Yoshida, Koichi Kokame 2004; Yoshida et al. 2001) This transcription factor promote transcription of UPR target gene, cell survival, ER expansion, through lipid synthesis, ER associated degradation (ERAD) and active secretion (Reimold et al. 2001; Zhang et al. 2015; Yoshida et al. 1998; Samali et al. 2010).

The second signalling pathway consists in the recruitment of TRAF2 which activated stress kinase such as c-Jun N-terminal kinase (JNK) and IκB kinase (IKK), leading to induction of pro-inflammatory and inflammatory cytokines(Zhang et al. 2015). The last function of IRE1, RIDD, mainly occurs following IRE1 hyper-activation (Zhang et al. 2015). This function is exerted through the endonuclease activity of IRE1, on unspecific mRNA and resulted in a global decrease of the transcript (Zhang et al. 2015).

PERK, the second branch of the UPR is observed only in higher eukaryotic cells (Li et al. 2010; Walter & Ron 2012). Like the IRE1 sensor, PERK is an ER membrane resident protein that consists of a single transmembrane domain with an N-terminal luminal sensing domain but with a C-terminal domain that only exert kinase activity (Walter & Ron 2012). Following UPR activation, BiP dissociate from PERK allowing it to dimerize and self-phosphorylate in its active form (Carrara et al. 2015; Walter & Ron 2012). The active PERK complexes phosphorylate the omnipresent initiation transnational factor elF2a (Scheuner et al. 2001). This phosphorylation results in eukaryotic translation initiation factor 2A (eIF2a) inactivation which inhibits global protein translation to ultimately reduce transitory protein amount in the ER and induce the cell cycle arrest at G1 phase (Bhakta-Guha & Efferth 2015; Wek & Cavener 2007). Following the inactivation of elF2a, some small open reading frame (ORF) located in the generally 5' untranslated region of specific mRNA, are preferentially translated (Andreev et al. 2015). Among these, the activating transcription factor 4 (ATF4), induces the transcription of two key genes in the UPR: C/EBP homologous protein (CHOP), a transcription factor and growth arrest and DNA damage—inducible 34 (GADD34) a regulatory subunit (Wek & Cavener 2007). Although the signalling pathway for PERK-mediated UPR has a protective effect by promoting

chaperone synthesis, oxidative stress response, autophagy and amino acid metabolism, CHOP is controlling genes involved in apoptosis (Hetz & Mollereau 2014). This dualism is mediated in a time-dependent manner through the ratio of phospho-elf2a/elf2a that is among others, regulated by GADD34, a regulatory subunit of the PP1C phosphatase. GADD34/ PP1C phosphatase inhibits the PERK signalling through elF2a dephosphorization by feedback inhibition (Harding et al. 2009; Marciniak et al. 2004; Tsaytler et al. 2011).

The third UPR pathway, namely the ATF6 pathway, is also observed only in higher eukaryotes (Walter & Ron 2012). ATF6 is a transcription factor that is synthesized as an ER plasma membrane receptor (Haze et al. 1999; Schindler & Schekman 2009). Like IRE1 and PERK, ATF6 consists of a luminal sensing domain that is stabilized by BiP, but in addition includes a Golgi localization sequence (Shen et al. 2002; Shen et al. 2005) This domain is linked by a short transmembrane domain to a cytoplasmic portion that contains a basic leucine zipper and a transcriptional activation domain (Hetz et al. 2011). Following stress activation, BiP disassociates from ATF6 allowing ATF6 to be translocated to the Golgi apparatus by vesicular transport (Haze et al. 1999). It is then cleaved by the protease site 1/2 (S1P/S2P) at both ends of the transmembrane domain to release the N-terminal and C-terminal portions (Ye et al. 2000). The C-terminal portion, containing the transcription factor component, is then routed to the nucleus where it targets ERSE I, UPRE, ERSE II and XBP1 promoter elements (Keisuke Yamamoto, Hiderou Yoshida, Koichi Kokame 2004; Yoshida et al. 2001) ERSE is mainly responsible for activating the transcription of chaperones and can be fully activated by ATF6 in a nuclear transcription factors Gama (NF-Y) dependent manner whereas UPRE depend solely on XBP1 and is more specifically

responsible for the transcription of the ERAD component (Keisuke Yamamoto, Hiderou Yoshida, Koichi Kokame 2004). The ERSE II (ATTGG-N-CCACG), as to it, seem to be more specifically responsible for the transcription of Homocysteine-induced endoplasmic reticulum protein (Herp), an ER membrane protein. This protein is responsible for protein trafficking to proteasome through ubiquitin modulations of the ER-resident calcium release channels, aiming to maintain calcium homoeostasis, preserving mitochondria functions and suppressing caspase-3 activity (Belal et al. 2012; Keisuke Yamamoto, Hiderou Yoshida, Koichi Kokame 2004; Kim et al. 2008; Kokame et al. 2001; Slodzinski et al. 2009; Chan et al. 2004).

1.3.3 Alzheimer's Disease is an Endoplasmic Reticulum Stress disease

AD is a progressive neurodegenerative disease involving loss of synaptic function followed by neuronal loss by apoptosis. The salient features involve accumulation of aggregates of misfolded proteins occurring in conjunction with abnormalities in the redox regulation, Ca homeostasis. Accumulation of the Aβ peptide along with hyper phosphorylated Tau is seen in post-mortem samples of most AD patients. Accumulating evidence suggests that the improper regulation of proteostasis and ER stress is a major pathological hallmark for AD based on studies of several post-mortem brain tissue samples (Cornejo & Hetz 2013; Plácido et al. 2014; Kennedy et al. 2014). The AD brains show evidence for the activated UPR elements, like the phospho eIF2a has been shown in the neurons but not in the glial cells (Salminen et al. 2009). A direct correlation has been observed in the AD pathogenesis and activation of the IRE1 pathway. Further, mutations in the XBP promoter reported to be a risk factor for AD. Genetic ablation of the IRE1 or the deletion of the RNase domain

of IRE1 led to reduced amyloid accumulation indicating the activation of IRE1 pathways to be a cause for the progression of AD (Duran-Aniotz et al. 2014). The mutation in PS1linked to familial AD has been reported to down regulate the UPR response and makes cells susceptible to ER stress (Imaizumi et al. 2001; Katayama et al. 1999).

The literature suggests that ER stress is a pathological hallmark for AD (Cornejo & Hetz 2013; Kennedy et al. 2014; Plácido et al. 2014). When taken together with the reports of the Sig-1R as a protein chaperone (Hayashi & Su 2007) and its role in the regulation of ER stress (Hayashi & Su 2005; Mori et al. 2013) it makes investigation of cell biology and biochemistry of Sig-1R with respect to ER stress and cell homeostasis crucial for understanding the etiology of AD in the mouse model.

1.4 Focus of this study

Together, these studies suggest that loss of Sig-1R function may predispose an individual to AD and potentially contribute, at least partially, to the progression of AD. Therefore, to fully elucidate how loss of the Sig-1R contribute to AD and cognitive decline, apart from using animal models, it is also necessary to investigate the cell biology of Sig-1R when cells are stressed.

The overall mechanism by which Sig-1R modulates ER stress and how it exerts its protective effect remains unclear. The dissection of the UPR pathway with respect to Sig-1R will therefore allow a better understanding of the mechanism underlying neuroprotective effects of the Sig-1R.

2. HYPOTHESIS

Deletion of Sigma-1-receptor compromises the ER stress response, specifically the UPR pathways PERK, IRE1, and ATF6..

3. OBJECTIVES

1. Determine if immortalized Sig-1R-/- MEFs show baseline activation of all three branches of the UPR.
2. Characterize the UPR response during different types of ER stress for immortalized and primary Sig-1R+/+ and immortalized and primary Sig-1R-/-.
    a. Acute Stress – Tunicamycin
    b. Acute stress and recovery time - DTT

4. MATERIALS AND METHODS

4.1 MEF isolation and cell culture

Mouse embryonic fibroblasts (MEF) were isolated from WT or Sig-1R$^{-/-}$ C57/BL6 mouse embryos (E14.5) using standard procedures (Xu 2005; Wong et al. 2016). In brief, E14.5 foetuses were isolated from pregnant dames and organs removed. The tissue was trypsinized with 0.25% trypsin-EDTA (Gibco/Invitrogen, Burlington, ON Canada),

homogenized, and passed through a 70 μm cell strainer. The single cell suspension was cultured in standard MEF media consisting of DMEM (Wisent Inc., Saint-Jean-Baptiste, QC, Canada), 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine (all from Gibco/Invitrogen). MEFs were grown in a humidified 37°C, 5% CO_2 incubator, passaged every 3–4 days using trypsinization (0.05% trypsin; Gibco/Invitrogen) and plated as a monolayer at a density of ~2×10^6 cells/ml.

4.2 Semi-quantitative RT-PCR

MEFs derived from WT and Sig-1R$^{-/-}$ mice were grown to 70–90% confluence, on 35 mm dish was treated with 3 μM Tun for 8, 12, 16, or 24 h. Total RNA was isolated using 1 ml TRIzol (Invitrogen) according to the manufacturer's instructions, and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Burlington, ON, Canada). Reverse transcription was performed on 1.25 μg RNA per reaction using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. The following PCR conditions were used: an initial denaturation step at 95°C for 3 min, followed by denaturation at 94°C for 30 s, annealing at T_m (indicated in Table 1) for 30 s, extension at 72°C for 1 min repeated the indicated number of cycles (Table 1), and a final extension at 72°C for 5 min. The product was separated on 3% agarose gels and visualized using AlphaImager Mini (ProteinSimple, San Jose, CA, USA). Each data point corresponds to six separately isolated biological samples with one technical replicate.

4.3 Western blot

MEFs were plated onto 10 cm (78.54 cm^2) dishes and treated with 3 μM Tun for 4, 8, 16, or 24 h. At collection, cells were gently washed twice in ice-cold PBS (10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 137 mM NaCl, and 2.68 mM KCl; pH 7.2) and lysed on ice with 700 μl RIPA buffer (150 mM NaCl, 50 mM Tris, 0.5% sodium deoxycholate, 0.1% NP-40, 5 mM sodium pyrophosphate, 2 mM β-glycerophosphate, 1×EDTA-free protease inhibitor (Fisher Scientific), pH 7.5). MEFs were then scraped off, sonicated for 15 s at 20% effect on a FB120 sonicator (Fisher Scientific), and cleared by centrifugation. Total protein (1.5 mg per lane) was resolved on Tris-glycine SDS-PAGE and transferred onto PVDF membrane. After incubation with primary and secondary antibodies (see below), membranes were developed using Luminata Crescendo (Millipore, Darmstadt, Germany) and visualized using LI-COR Odyssey Fc (LI-COR, Lincoln, Nebraska USA). Band intensities were normalized to total protein as determined by Fast Green stain (125 μM Fast Green FCF (Sigma-Aldrich, Oakville, ON, Canada), 6.7% acetic acid, 30% methanol; [37]). Each n corresponds to a single biological replicate containing 2–4 technical replicates.

4.4 Antibodies

The following antibodies and dilutions were used: rabbit anti-Sig-1R (1:2000; Atlas Antibodies, Bromma, Sweden), rabbit and mice anti-ATF6 (1:1000; Abcam, Cambridge, MA, USA and Novus Biologicals, Oakville ON, Canada, respectively), rabbit anti-GADD34 (1:200; MyBioSource, San Diego, CA, USA), mouse anti-CHOP (1:100; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), and

mouse-Caspase 3 (1;400; Abcam, Cambridge, MA, USA). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were all purchased from Jackson ImmunoResearch (West Grove, PA, USA).

4.5 Viability assay

MEFs were plated at the density of 2×10^4 in 100 µl per well onto black 96-well clear bottom plates. After allowing 24 h for attachment, the cells were treated with 3 µM Tun as previously described. At the end of the treatment, the media was carefully aspirated from the wells, the cells washed in warm PBS, and incubated with 100 µl Calcein AM (3 µM; Invitrogen) in DPBS (PBS with 90 µM $CaCl_2$ and 50 µM $MgCl_2$; Wisent Inc.) for 30 min. Fluorescent Calcein AM hydrolyzed by live cells was measured at 535 nm (485 nm ex).

4.6 Generation of stable Sig-1R–eYFP-expressing Sig- 1R$^{-/-}$ MEFs

A Geneticin/G418 resistant Sig-1R-eYFP construct driven by a CAG promoter was linearized with *VspI* before transfection into Sig-1R$^{-/-}$ MEFs using *Trans*IT 2020 according to the manufacturer's instructions (Mirus Bio). After 48 h, the cells were split, diluted and selected on medium containing 1 mg/ml G418 (Sigma Aldrich). Media was changed every third day for 2 weeks until single colonies were visible. Single colonies were picked and expanded before being screened for the presence of Sig-1R-YFP using epifluorescence imaging on Zeiss Observer.A1 inverted microscope (Zeiss Instruments, Oberkirchen, Germany) after each passage. Colonies that showed less than 100% expression of Sig-1R-YFP was discarded.

4.7 Live cell Imaging

Stable Sig-1R-YFP expressing MEFs was plated on to 35 mm imaging dishes (ibidi GmbH, Martinsreid, Germany) and live-cell imaged using Airyscan confocal imaging at 488, 515, and 559 nm on an inverted Zeiss LSM 880 63× (NA 1.4) oil immersion objective (Zeiss). The ER was stained with ER-Tracker Red (Life Technologies/Thermo Fisher Scientific) and the plasma membrane was stained with CellMask Deep Red plasma membrane stain (Life Technologies/Thermo Fisher Scientific) as a morphological marker 20 min prior to imaging. Cells were imaged in Phenol Red free MEM (Wisent) containing 10% FBS on a pre-warmed 37 °C stage with 5% CO_2. For co-localization analysis, cells were imaged at a resolution of 1024×1024 pixels, dwell time of 4 μs/pixel, and Z-stacks were obtained with 0.75–1 μm step size (6–10 sections per stack). Surface-surface co-localization analysis was performed using IMARIS Imaging Software (Bitplane, Concord, MA, USA).

4.8 Generation of stable Sig-1R Knock down MEFs

WT Sig-1R MEFs were transiently transfected with Puromycin resistant shRNAs TRCN0000194512 (D10) targeting sequence (5'-TTCACCAGAGATTACTACAGG-3'), TRCN0000194052 (D11) targeting sequence (5'-TAATATCTGCATGGTATACGC-3'), and TRCN0000193914 (E1) targeting sequence (5'-ATAATAGTCAGAATCAGGGTG-3'), all purchased from GE Dharmacon. Cells were split and replated 48 h post transfection ensuring a threefold dilution of cells and selected on puromycin (3 μg/ml) selective medium. The resultant colonies were picked and expanded in puromycin-containing media before knockdown efficiency were determined *via* Western blotting. Cells stably transfected with D10 shRNA showed the largest knockdown of the 3 shRNAs tested.

4.9 Analysis and statistics

Densiometric analysis of RT-PCR results were performed using ImageJ, while Western blots were analyzed using Image Studio (LI-COR). OriginPro 8.5 (OriginLab Corporation, Northampton, MA, USA) was used to plot graphs. Data are expressed as mean ± SEM. Statistical significance was determined with one-way ANOVA and Dunnett's T3 post-hoc tests with a critical value of 0.05 using IBM SPSS Statistics 23 software (IBM, Armonk, NY, USA).

5. RESULTS

5.1 Immortalized Sig-1R KO MEFs is baseline stressed

RT-PCR analysis showed baseline activation in the effectors of the IRE1 and PERK pathways. The increased ratio of spliced XBP XBPsp over total XBP XBPtot showed that the IRE pathway is activated at baseline in the immortalized KO MEFs (**Error! Reference source not found.**). The increased transcript levels of GADD34, indicates activation of the PERK pathway (Fig. 1B). Interestingly, however, the transcript level of CHOP, another component of the PERK pathway is not up regulated in immortalized KO cells. ATF6 pathway is not activated at baseline, however, as ATF6 transcripts level remain unchanged (**Error! Reference source not found.**).

5.2 Induced, acute ER stress elicits a robust response in Sig-1R wild-type and an attenuated response in knockout

Tunicamycin induces the Unfolded Protein Response in cells. We therefore wanted to examine the effect of this acute stress on our models. In order to do so, we added tunicamycin (3µM) to cells and examined the response at various time points ranging from 8 hours to 24 hours. Following acute stress, all the effectors of the UPR pathway show a robust up regulation in the WT; however, the response is attenuated in the KO as reflected in the modest up regulation especially of the PERK, ATF6, and IRE1 (Figure 2.2: Response to **acute stress (tunicamycin).**). The observation that the immortalized KO showed a baseline stress and the attenuated response to a acute stress induced by tunicamycin suggest an adaptation to ER stress in the immortalized KO MEF line.

5.3 Recovery from an acute ER stress is similar in WT and KO

Seeing the differences in response to acute stress between immortalized WT and KO cells, we wanted to determine whether acute stress would lead to the same observation. We therefore used DTT for this experiment. DTT induces an acute stress in cells by inhibiting the formation of sulphydryl bridges in proteins and induces an unfolded protein response within minutes. Cells were stressed with 5µM DTT for 30 minutes. Cells were then washed off and allowed recover in regular media (with FBS and Pen/Strep). Response to stress was monitored for 16 hours. Cells were collected at indicated time points. Controls were not treated with DTT. The immortalized WT and KO both respond to the addition of DTT almost identically; although two of the three pathways show a modest attenuation of the response in Sig1R KO MEFs as compared with WT (CHOP and ATF6; Fig3C and 3D). However, there is no change in the recovery kinetics from the acute stress in the KO when

compared to the WT (Figure 2.3: Response to acute **stress and recovery.** . This further suggests that there is an adaptation to ER stress in our current model, immortalized cells.

5.4 Primary KO MEFs and Primary WT MEFs are not baseline stressed

We then asked the question: is the primary KO MEFs stressed at baseline as well. The primary KO MEFs are not stressed at baseline when compared with the immortalized KO MEFs (Fig 4B). This finding further argues for an adaptation to stress in the immortalized KO cells. Furthermore, the morphology of immortalized KO MEFs is very different from the immortalized WT (Fig 4A), which indicates that the mutations acquired during immortalization are different in the WT and KO cell lines. Differences in immortalization adaptation between the WT and KO MEFs may be the cause of the morphological differences we observe (Figure 2.4: **The primary Sig-1R KO MEFs are not stressed at baseline.** .

5.5 Primary Sig-1R WT and KO MEFs show a robust difference in the recovery from an acute ER stressor DTT

Preliminary experiments showed that the primary KOs respond to the acute stress tunicamycin identically to the primary WT. However, the primary KO displays a robust phenotype during the recovery from an acute stress induced by DTT (Figure 2.5. The Sig-1R **is required for the reestablishment of cell homeostasis after acute ER stress using DTT.**). WT cells were efficient at recovering from acute stress. The level of stress, indicated by the expression level of CHOP and GADD34, remained high in primary KO cells after 16 hours of recovery. This data leads us to the question of specificity. We wanted

to determine whether the response seen in Primary KO cells was caused by the absence of the Sig-1R. We therefore generated a knockdown (KD) cell line using Sig-1R shRNA. Recovery from the acute stress induced by DTT observed in the primary KO is phenocopied by the Sig-1R knockdown (Fig 5). Both Sig-1R KD and primary KO cells show a persistence of the stress phenotype for 16 hours following DTT wash off. The KD cells show the same phenotype as KO primary cells in the expression levels of effectors of the PERK pathway (CHOP and GADD34), (Figure 2.5. The Sig-1R **is required for the reestablishment of cell homeostasis after acute ER stress using DTT.**). Taken together, our data from primary cells suggests that Sig-1R is required for the re-establishment of cell homeostasis following acute ER stress.

6. DISCUSSION

6.1 Baseline ER stress in Immortalized KO not rescued by WT Sig-1R

RT-PCR analysis showed baseline activation in the effectors of the IRE1 and PERK pathways. The increased ratio of spliced XBP1 XBP1sp over total XBP1 XBP1t showed that the IRE pathway is activated at baseline in the immortalized KO MEFs (**Error! Reference source not found.**). The increased transcript levels of GADD34, indicates activation of the PERK pathway (**Error! Reference source not found.**). Interestingly, however, the transcript level of CHOP, another component of the PERK pathway is not up regulated in immortalized KO cells. This discrepancy might suggest a crosstalk between the PERK pathway and another pathway, which might regulate either GADD34 directly or might feed into CHOP (Takayanagi et al. 2013; Yao et al. 2013; Li et al. 2014; Yeganeh et

al. 2015; Chikka et al. 2013; Song et al. 2009). ATF6 pathway is not activated at baseline, however, as ATF6 transcripts level remain unchanged (Figure 2.2**: Response to acute stress (tunicamycin).**).

Further we have tried to rescue the baseline stress observed in the immortalized KO by stably over-expressing WT Sig1R and found that the baseline stress as shown by the up-regulation of the UPR elements remained intact (Appendix C: Generation of stable Sig-1R overexpressing cell lines using KO cells for rescue experiments. . This observation strongly suggests an adaptation to ER stress.

6.2 Response to ER stress is attenuated in the immortalized KO.

Tunicamycin induces the Unfolded Protein Response in cells. We therefore wanted to examine the effect of this acute stress on our models. In order to do so, we added tunicamycin (3µM) to cells and examined the response at various time points ranging from 8 hours to 24 hours. Following acute stress, all the effectors of the UPR pathway show a robust up regulation in the WT; however, the response is attenuated in the KO as reflected in the modest up regulation especially of the PERK, ATF6, and IRE1 (Figure 2.2: Response to acute **stress (tunicamycin).** . The observation that the immortalized KO showed a baseline stress and the attenuated response to a acute stress induced by tunicamycin suggest an adaptation to ER stress in the immortalized KO MEF line.

6.3 Primary Knockout and knockdown better model

Primary MEFs isolated from the KO mice strain did not show any baseline ER stress as against the immortalized KO MEFs. The process of spontaneous immortalization of Sig-

1R KO MEFs probably induced other mutations and thus the immortalized WT and KO are not isogenic. The attenuated response to ER stress and the inability of WT Sig-1R to rescue the baseline ER stress in immortalized KO, indicates an adaptation to ER stress in the immortalized KO MEFs. The immortalized cell lines of UPR mutants are known to show an attenuated response to the ER stressors and this is not uncommon in the field, although not evident from the literature (vom Brocke et al. 2006). Regardless, it is well established that immortalization process induces mutagenesis (vom Brocke et al. 2006; Busuttil et al. 2003; Fridman & Tainsky 2008) and thus the likelihood that the immortalized WT and KO are isogenic is remote.

However, the knockdown cells showed no baseline stress levels. Furthermore, the KD cells phenocopy the primary knockout MEFs in the recovery from DTT acute stress (Figure 2.5. The **Sig-1R is required for the reestablishment of cell homeostasis after acute ER stress using DTT.** . This suggests that the KD cells are a better model to study the effect of the Sig-1R in ER stress.

6.4 Cell homeostasis following acute DTT stress or a response to oxidative stress?

The primary knockout and knockdown MEFs both have a compromised recovery from DTT induced acute stress, which suggests that the Sig-1R is responsible returning the cell to homeostasis following an acute stress. This might also suggest that Sig-1R plays a role in responding to oxidative stress. Prolonged stress induced by reducing conditions has been reported to generate free radicals, which initiates an oxidative stress response (Maity

et al. 2016). Although, here we have induced ER stress by DTT (5mM) just for 30 minutes, it is possible that the Sig-1R knockout strains are susceptible to oxidative stress and are responding to oxidative stress effects. Sig-1R knockout strains have been reported to display higher levels of oxidative stress compared to the WT (Pal et al. 2012), and that activation of Sig-1R protects retinal cells from oxidative stress damage (Ha et al. 2011; Wang et al. 2016; Zhao et al. 2014). The Sig-1R KO and KD strains were not significantly different in their responses when compared to the WT MEFs when treated with either tunicamycin or thapsigargin (Appendix A: Ectopic over expression of WT Sig-R in immortalized KO MEFs does not rescue the baseline stress.). However, the Sig-1R KO and KD MEFs both displayed a robust phenotype when acutely stressed with DTT (Figure 2.5. The **Sig-1R is required for the reestablishment of cell homeostasis after acute ER stress using DTT.** . While the WT MEFs showed recovery of ER stress markers CHOP and GADD34 to baseline levels, the Sig-1R KO and KD MEFs remained elevated in these transcripts; suggesting that the Sig-1R may be important for returning the cell to homeostasis following acute ER stress. It will be important to show if Sig-1R plays a role in responding to oxidative stress and if the ER stress response observed is downstream of oxidative stress.

6.5 Exogenous Aβ peptide induces autophagic and apoptotic response

Alzheimer's disease (AD) is a neurodegenerative disease that impairs memory and cognitive function. The clinical manifestation of AD, and many other neurodegenerative diseases, is initiated by alterations in protein functionalities of distinctive neuronal populations. This disruption of functionality has been shown to be correlated to the

accumulation of misfolded proteins, which may be caused by the disturbances in the Endoplasmic Reticulum (ER) function. Studies have shown that ER stress markers have been detected in post-mortem samples from early AD patients, specifically in the temporal cortex and hippocampus (Hoozemans et al. 2005). Interestingly, recent studies have shown that GRP78 (also known as BiP), an ER chaperone that interacts with Sig-1R, directly binds to APP and inhibits the generation of Aβ peptide (Hoshino et al. 2007; Yang et al. 2017).

We therefore wanted to examine the effect of losing Sig-1R; and whether this loss of Sig-1R would result in an increase in expression levels of ER stress markers. In line with this prediction, preliminary data from our lab shows that ER stress markers, CHOP, GADD34 and XBP1, remain unchanged in the presence of Sig-1R (Appendix C: **Generation of stable Sig-1R overexpressing cell lines using KO cells for rescue experiments.** . We predict that in the absence of Sig-1R, the expression level of these markers would go up. Interestingly, toxicity induced by Aβ peptides caused an increase in relative expressions of autophagy markers as well as apoptotic markers, but not of ER-stress markers in wild-type cells (Appendix D**: $A\beta_{25-35}$ activates autophagy and apoptosis in WT cells and in ex-vivo organotypics. Appendix D: $A\beta_{25-35}$ activates autophagy and apoptosis in WT cells and in ex-vivo organotypics.**). Furthermore, an increase in caspase activity, which is indicative of apoptosis, was seen in WT mouse organotypic slices (ex-vivo). Taken altogether, this data suggests that Sig-1R may be involved in the clearance of Aβ peptides. Future studies should, therefore, look at the effect of Aβ on our KO and KD models to unravel the significance of Sig-1R in ER stress; and ultimately its role in AD.

6.6 Future Directions

While the results obtained from this study indicate that the Sig-1R may play a role in the ER stress response and recovery in immortalized and primary MEF cell lines, the molecular mechanism underlying its effect it is still unclear. Is the Sig-1R interacting with the three branches of the UPR at their initiation (PERK, IRE1, and ATF6), or is there a process upstream of ER stress that the Sig-1R is a part of, such as the oxidative stress response? To elucidate the proteins and pathway(s) for which the Sig-1R is a direct interactor we propose to use a BioID assay to map the Sig-1R interactome at baseline and under cellular stress.

The BioID assay was first developed by the Roux lab (Roux et al. 2012) to successfully identify the proteins that interact with and are proximal to the lamin-A protein, a well-characterizes structural component of the nuclear envelope. The BioID assay relies on the expression of a biotin ligase enzyme fused to a protein of interest. Upon expression of the fusion protein, the biotin ligase enzyme will biotinylate proteins that interact with, or are proximal to, the protein of interest. Biotinylated proteins can then be pulled down through affinity purification and subsequently identified via mass spectrometry. Roux lab followed up their work with an improved BioID tag that was much smaller in size (Kim et al., 2016). We used this tag and generated a tetracycline inducible Sig-1R-BioID (Appendix D: Aβ25-35 activates autophagy and apoptosis in WT cells **and in ex-vivo organotypics.** using the vector deposited in addgene (addgene #24418) by the Hsaio lab (Hsiao et al. 2011). A pulse-chase experiment will be conducted to determine the optimal induction time for the Sig-1R-BioID to reach endogenous Sig-1R protein levels. Optimal induction time along with biotin supplementation to the culture media results in the Sig-1R-BioID construct being expressed and able to biotinylate any proteins that interact with or are proximal to the Sig-1R-BioID. Knowing the Sig-1R interactome under baseline and

under cellular stress is a crucial step towards understanding its role in cellular function, the molecular mechanism underlying its ability to modulate ER stress response and recovery, and finally enables the scientific community to make more informed decisions when using the Sig-1R as a pharmacological target for treating AD.

7 CONCLUSION

Taken together, the data shows that immortalized Sig-1R KO MEFs are baseline stressed whereas primary Sig-1R KO MEFs are not. I showed that using immortalized KO MEFs may not be a reliable model especially when examining ER stress pathways. Using primary WT and Sig-1R KO MEFs, however, I have shown that there is a hindered recovery from the acute ER stressor DTT in the KO MEFs; suggesting a role for the Sig-1R in returning the cell to homeostasis following ER stress. Future studies will investigate the effect of a Sig-1R KO on Aβ toxicity and focus on elucidating the molecular pathway for which the Sig-1R is a part of using Bio-ID.

8 FIGURE

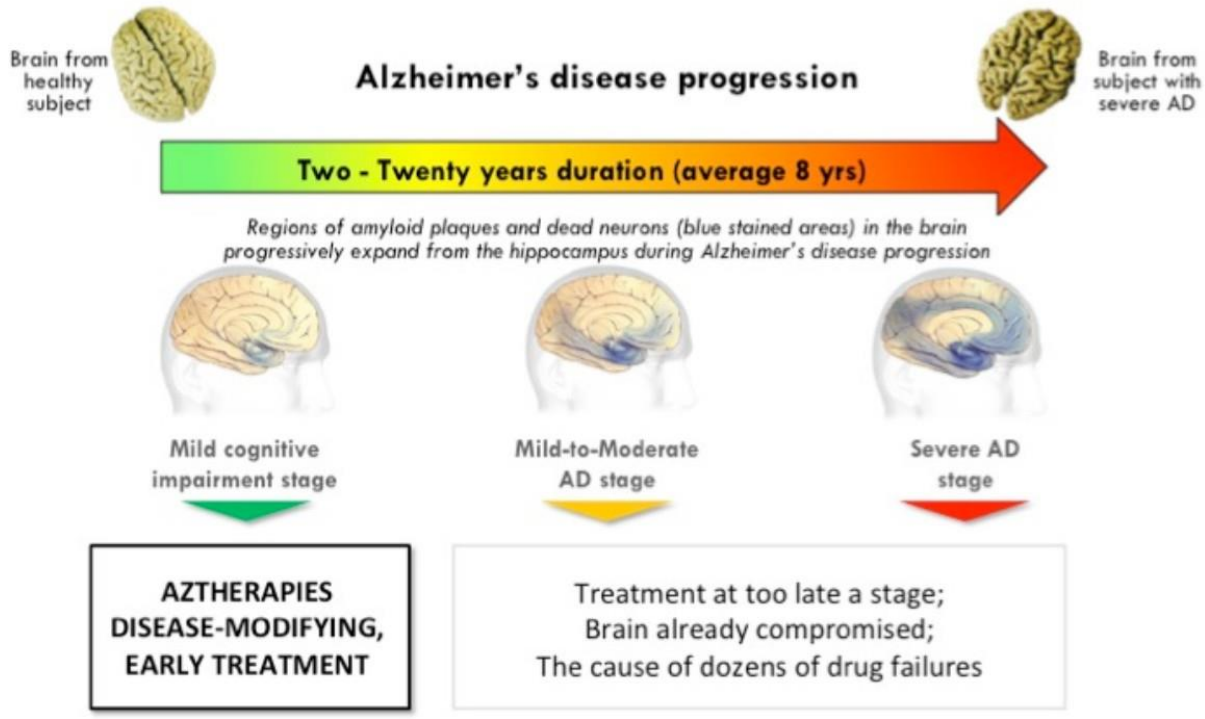


Figure 1.1 Alzheimer's disease progression. Image shows the progression of Alzheimer's disease starting from mild impairment caused by deposition of amyloid plagues and dead neurons to the severe stage. Many clinical trials have tried and failed to help improve the conditions of patients with severe AD. At the severe stage of AD, the brain is comprised which leads to the failures of medications.

Reference: (AZTherapies n.d.)

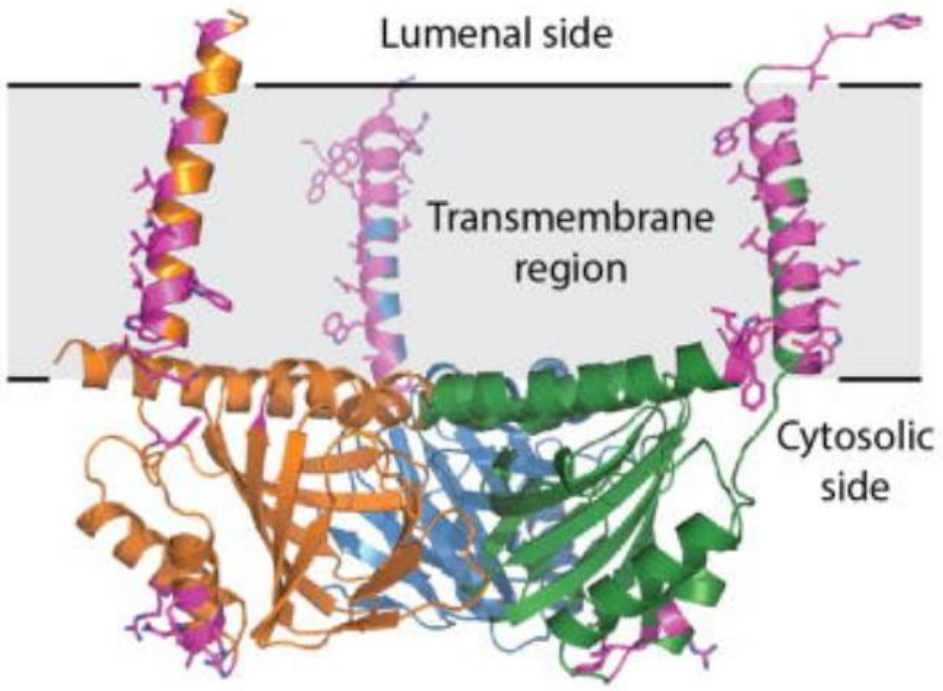


Figure 1.2 The Sig-1R homotrimer and its position in the ER membrane.

Reference: (Schmidt et al. 2016)

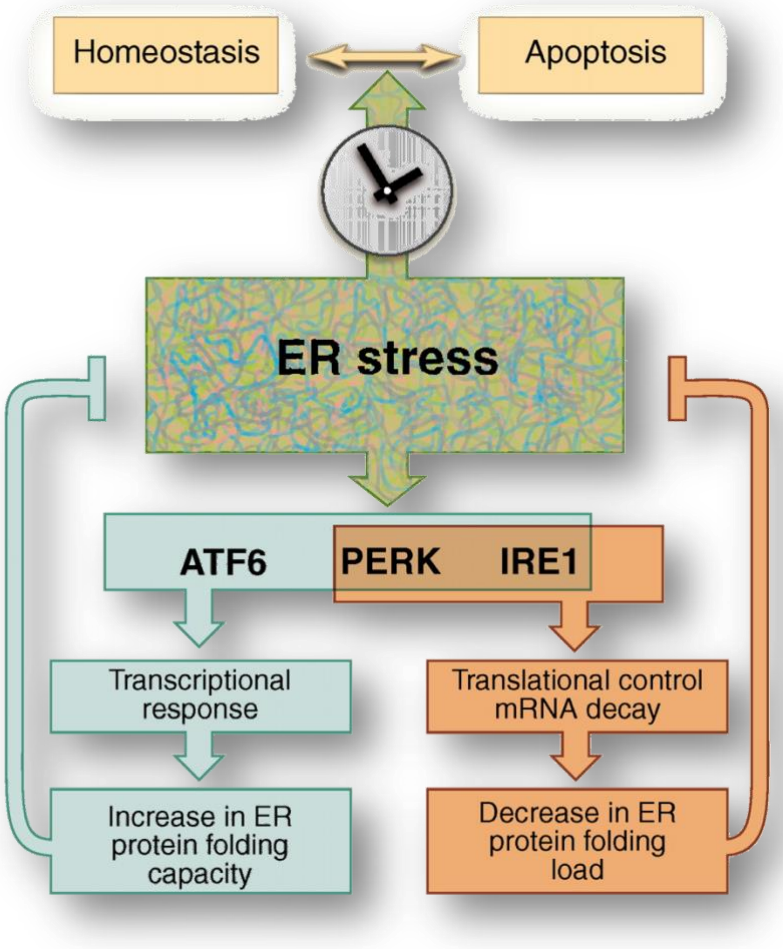


Figure 1.3 Unfolded Protein Response (UPR), a network of intracellular signalling pathways that maintain the proper protein folding within the ER. This stress response has been shown to be conserved between all mammalian species, as well as yeast. Cells utilize this response in the effort to maintain homeostasis.

Reference: (Walter & Ron 2012)

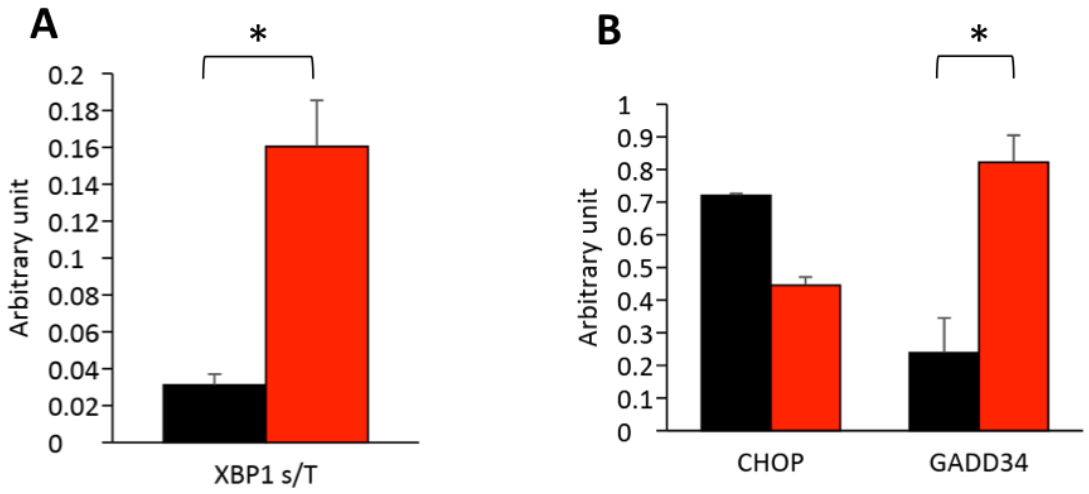


Figure 2.1 Baseline activation of UPR pathways. (A) Baseline activation of the IRE1 pathway. Transcript levels of spliced XBP1 over the total XBP reflect activation of the IRE1 pathway. Black bar= Immortalized WT; Red Bar= Immortalized KO; n=18; asterisk represents 5% significance level. (B) Baseline activation of the PERK pathway. CHOP and GADD34 are both effectors of the PERK pathway. GADD34 is clearly up regulated in the immortalized KO indicating a baseline activation of the PERK pathway. Black bar= Immortalized WT; Red Bar= Immortalized KO; n=18.

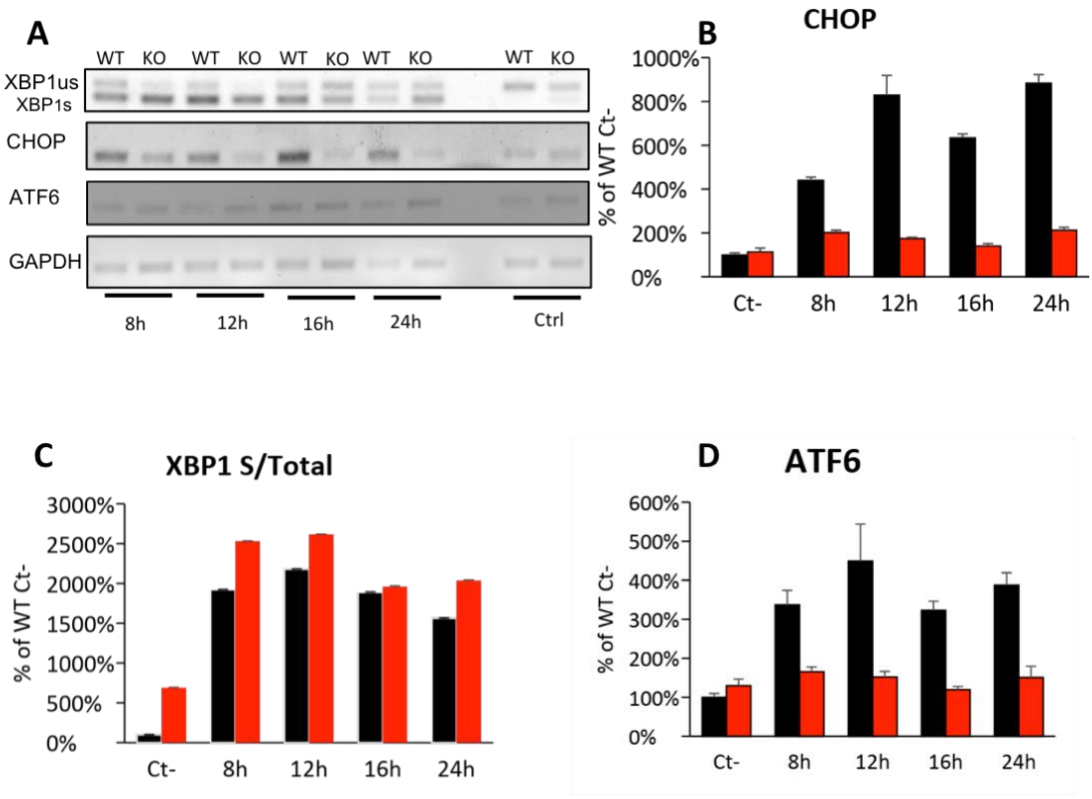


Figure 2.2: Response to acute stress (tunicamycin). Tunicamycin (3µM) was added to the growth medium for the times indicated. Controls have no tunicamycin added, n=6. (A) Graphical representation of transcript levels. (B) PERK pathway. CHOP transcript levels expressed as a percentage over the WT control. (C) IRE1 pathway. Response to acute stress. Tunicamycin (3µM) was added to the growth medium for the times indicated. Controls have no tunicamycin added, n=6. Spliced XBP (XBP1s) transcript expressed as a ratio over total (spliced and un-spliced XBP1s +XBP1-us). The transcript levels are expressed as a percentage over the WT control; n=18. (D) ATF6 pathway. Response to acute stress (tunicamycin). Tunicamycin (3µM) was added to the growth medium for the times indicated. Controls have no tunicamycin added, n=6. ATF6 transcript levels expressed as a percentage over the WT control; asterisk represents 5% significance level.

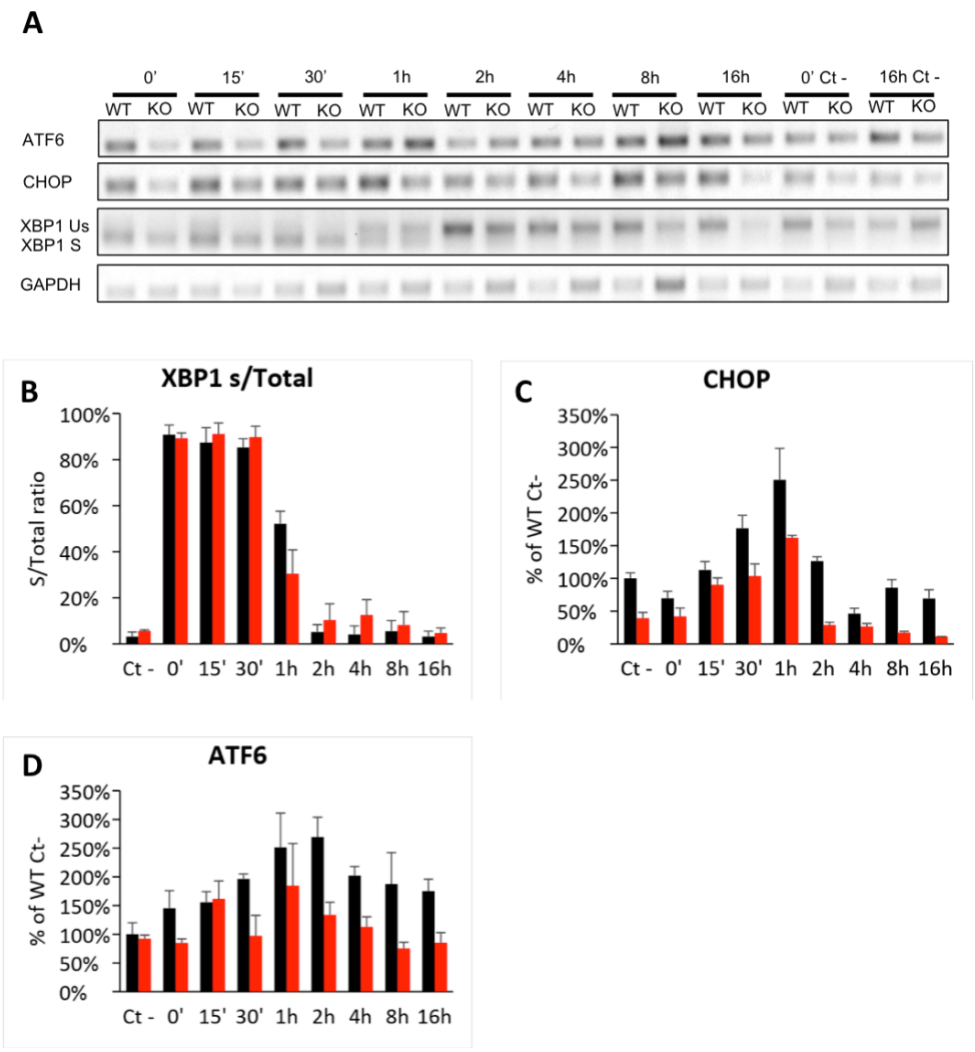


Figure 2.3: Response to acute stress and recovery. Cells were treated with DTT 5µM for 30 minutes, and then washed off. Cells were collected at wash off (0 min), 15 min, 30 min, 1h, 2h, 4h, 8h and 16 h after wash off. (A) Graphical depiction of transcript levels. **(B)** The spliced XBP transcripts over total is shown which reflects the activation and recovery of the IRE1 pathway, n=3. (C) The levels of CHOP transcripts in the WT and KO are expressed as percent of the WT control, n=3; asterisk represents 5% significance level. (D) The levels of ATF6 transcripts in the WT and KO are expressed as percent of the WT control, n=3.

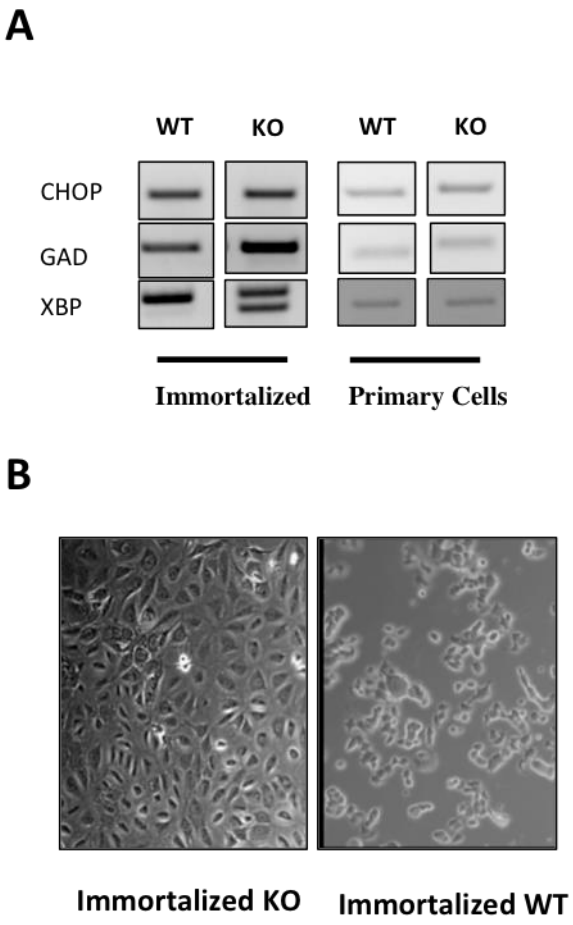


Figure 2.4: The primary Sig-1R KO MEFs are not stressed at baseline. (A) Expression levels of CHOP, GADD34 and XBP1 in the immortalized WT, immortalized KO, primary WT and primary KO. (B) Images of immortalized WT and KO MEFs showing the differences in morphology.

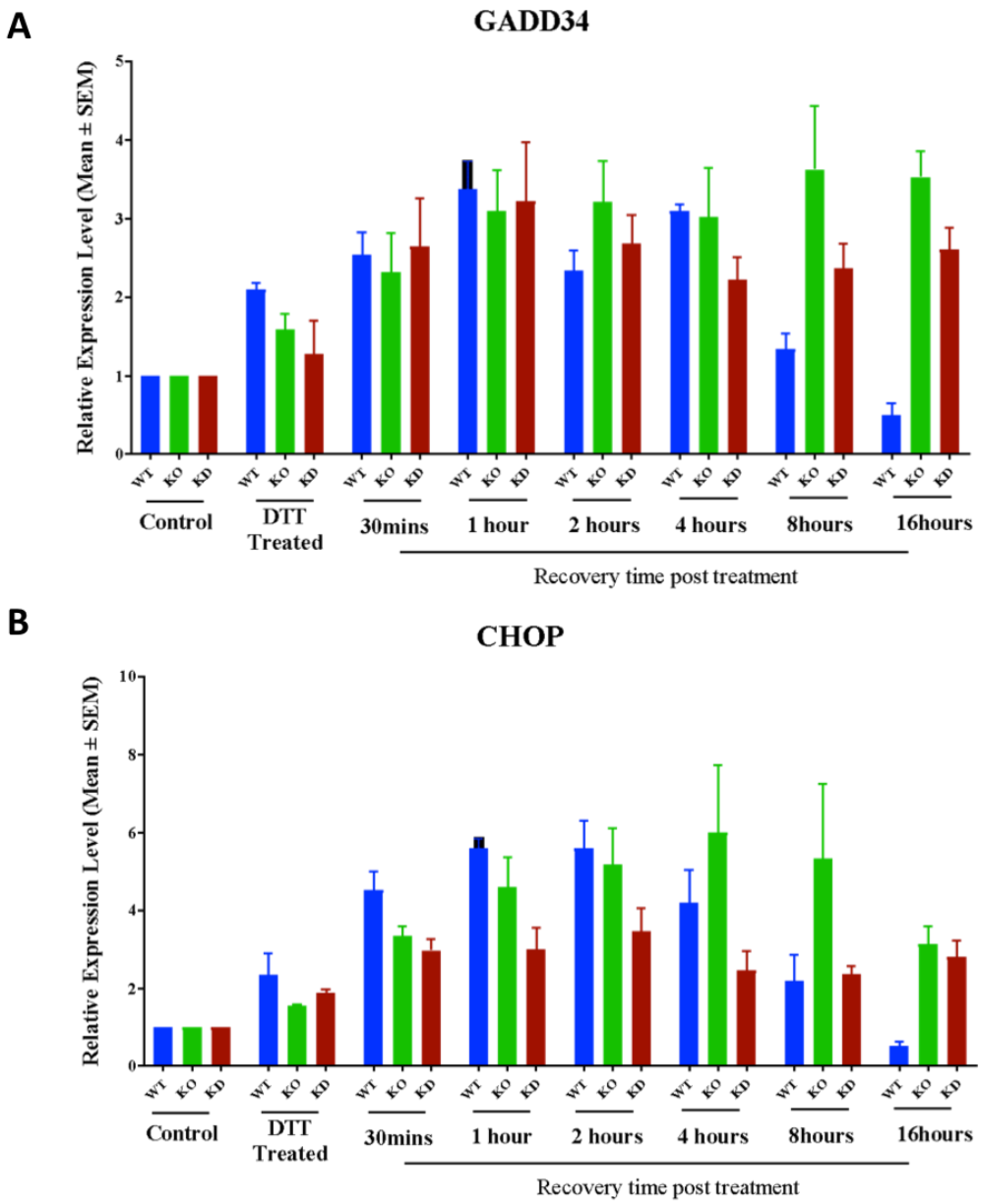
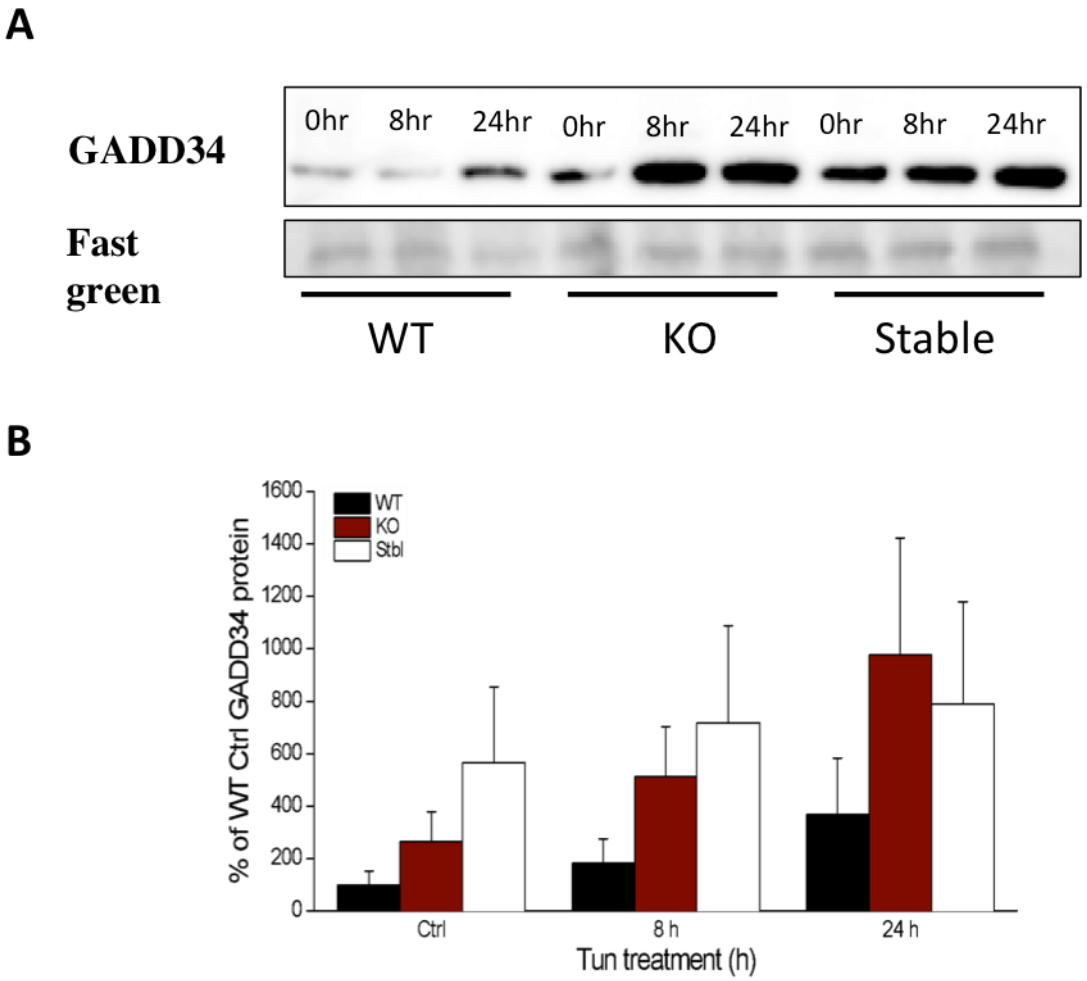
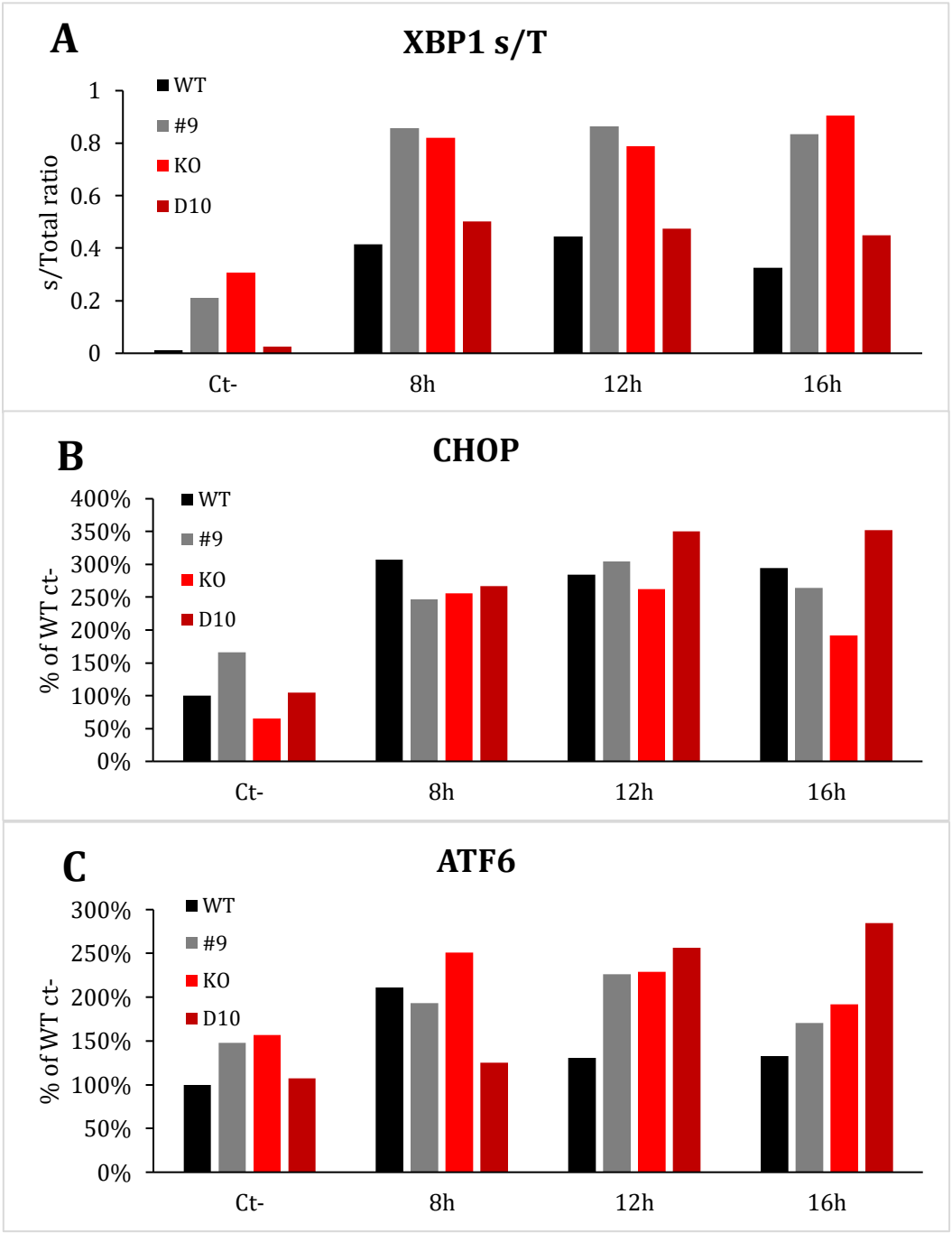


Figure 2.5. The Sig-1R is required for the reestablishment of cell homeostasis after acute ER stress using DTT. Primary WT (blue bars), Primary KO (green bars), and Knockdown line (burgundy) were treated with DTT (5uM) for 30 minutes, and then washed off. Cells were collected at wash off (DTT treated), 30 min, 1h, 2h, 4h, 8h, and 16h post wash off. (A) and (B) GADD34 and CHOP expression levels are shown here normalized to the WT control. Controls were not treated with DTT.

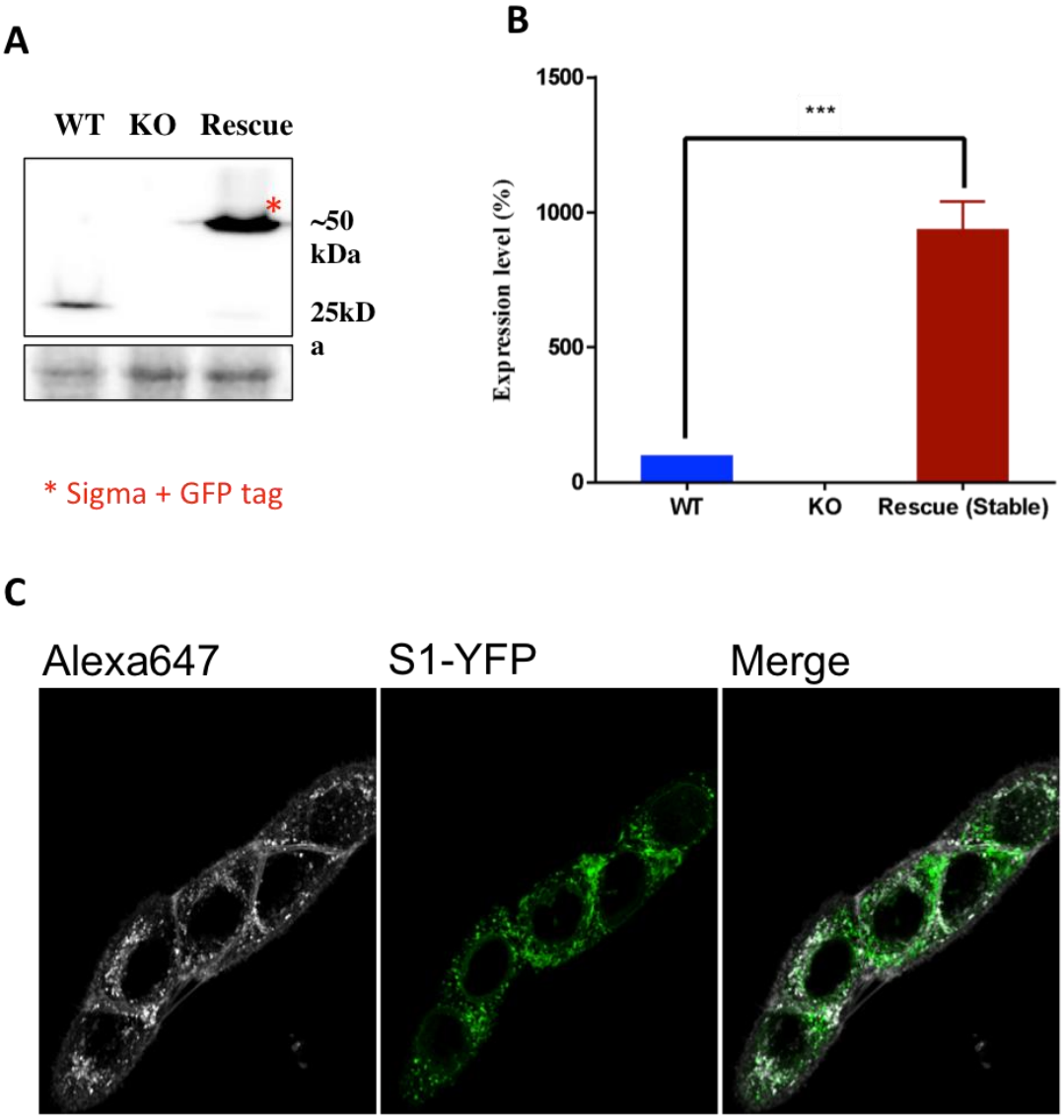
9 APPENDIX



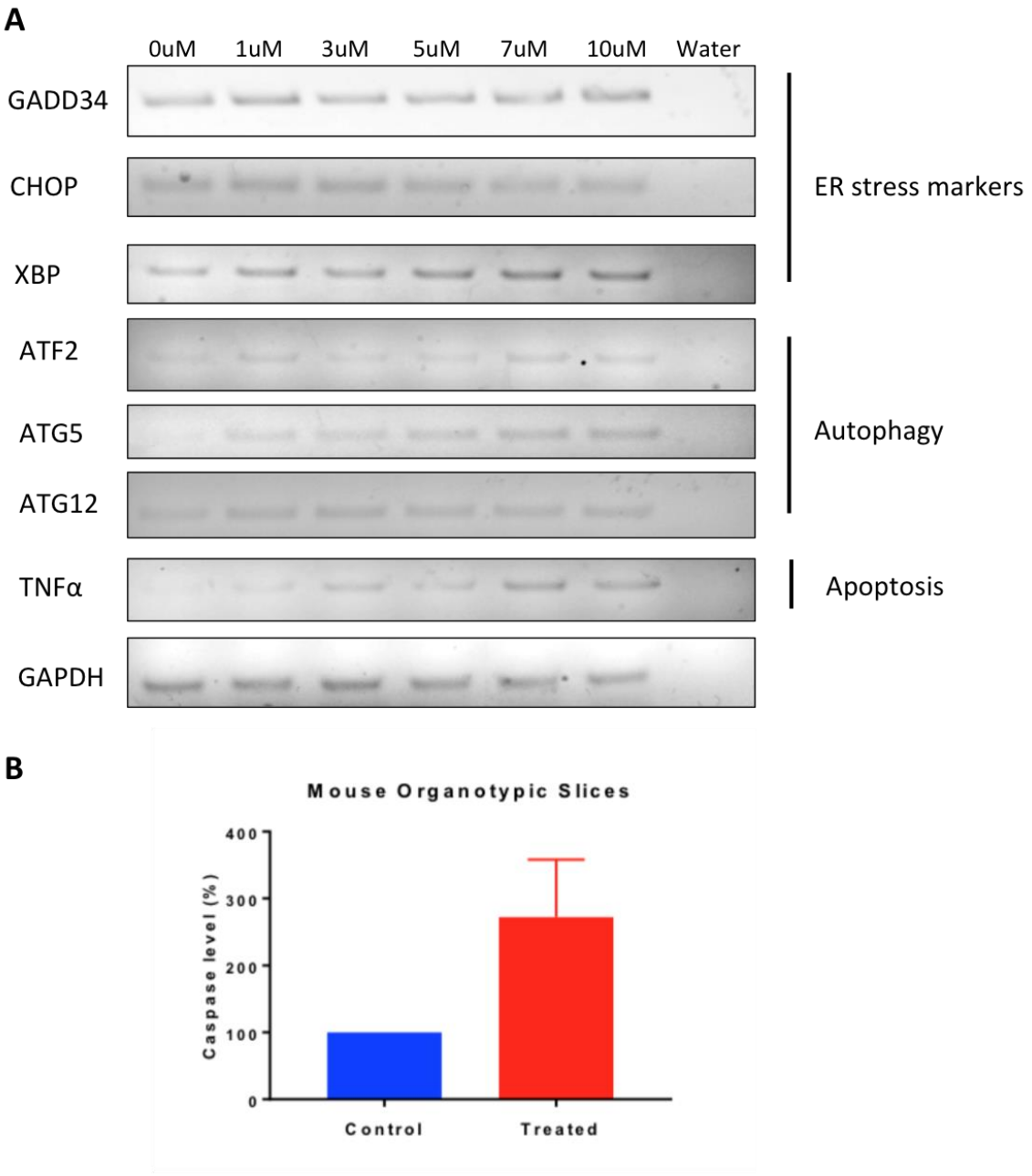
Appendix A: Ectopic over expression of WT Sig-R in immortalized KO MEFs does not rescue the baseline stress. Tunicamycin (3µM) was added to the growth medium for the times indicated. Controls have no tunicamycin added **(A)** Western blots showing the expression levels of GADD34 in WT, KO and Stable cell lines **(B)** Quantification of gel images showing the expression levels of GADD34 at baseline and post treatment in WT, KO, and Stable cell lines.



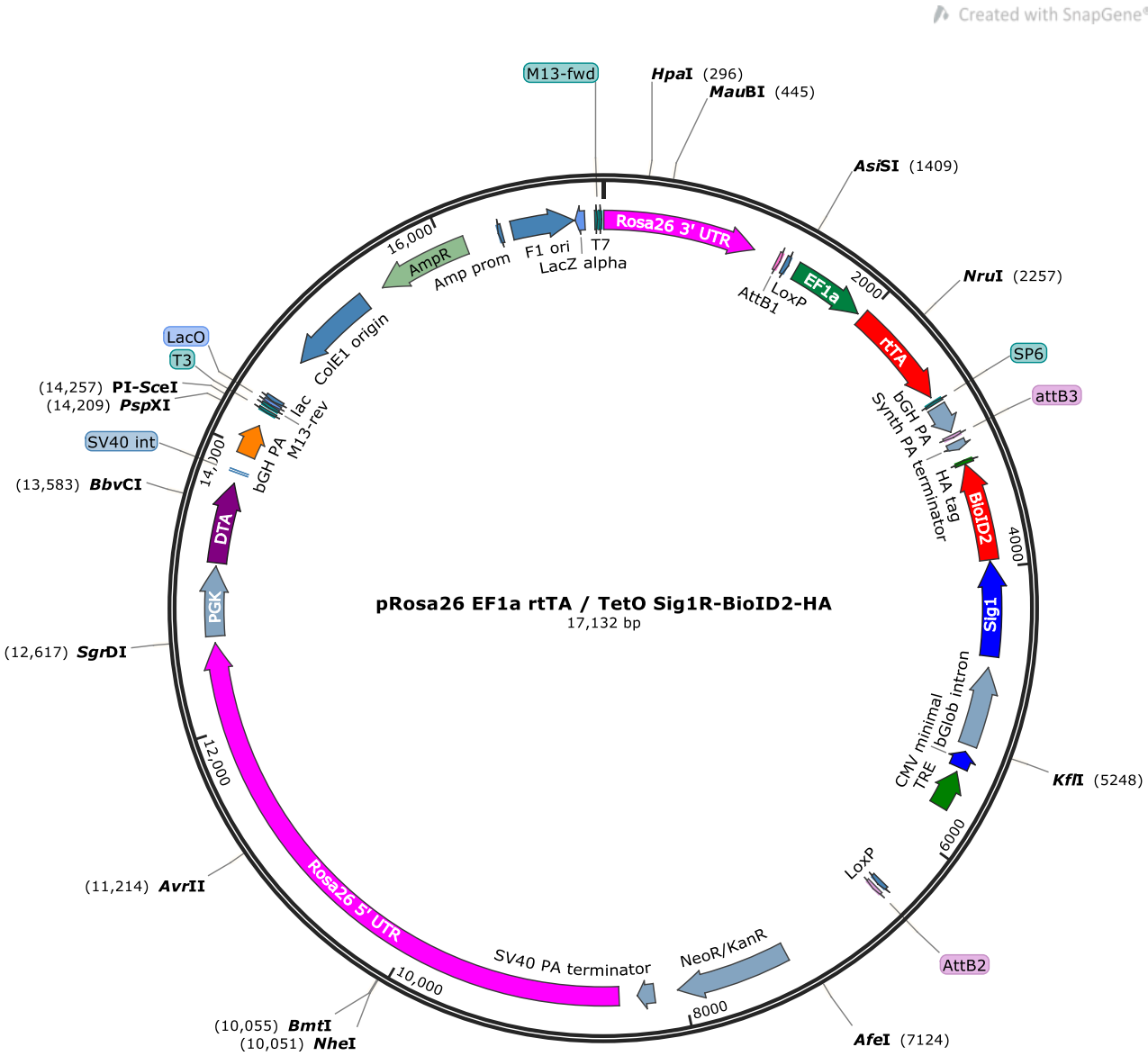
Appendix B: No significant difference between Sig-1R KO and KD following Tunicamycin treatment. (A) The spliced XBP transcripts over total is shown which reflects the activation of the IRE1 pathway, n=4. (B) The levels of CHOP transcripts in the WT and KO are expressed as percent of the WT control, n=4. (C) The levels of ATF6 transcripts in the WT and KO are expressed as percent of the WT control, n=4.



Appendix C: Generation of stable Sig-1R overexpressing cell lines using KO cells for rescue experiments. (A) and **(B)** Western blot shows the protein level of Sig-1R in Sig-1R overexpressing line (Rescue) is significantly higher compared to WT cells. **(C)** AiryScan images show that Sig-1R is expressed in KO cells after being stably transfected with Sig-1R expression vector.



Appendix D: Aβ$_{25-35}$ activates autophagy and apoptosis in WT cells and in ex-vivo organotypics. (A) Expression level of ER stress, autophagy and apoptosis markers **(B)** Caspase 3 activity in WT mouse organotypic slice (Ex-Vivo)



Appendix E: TetO Sig1R-BioID2-HA/rtTA stable expression vector. Transfection vector targeting the rosa26 locus, including double insulated cassette : TetO Sig1R-BioID2-HA/EF1a rtTA and geneticin resistance.

Gene	Direction	Sequence
ATF6	FWD	AAC AAC CAC AGA GAC GAC CC
	REV	AGC GCA CTT CCT GTT CTC TC
CHOP	FWD	CTG CCT TTC ACC TTG GAG AC
	REV	CGT TTC CTG GGG ATG AGA TA
GADD34	FWD	CAC GAT CGC TTT TGG CAA CC
	REV	GAG CCG CAG CTT CTA TCT GA
GAPDH	FWD	CCT GGA GAA ACC TGC CAA GTA T
	REV	AGA GTG GGA GTT GCT GTT GAA G
XBP1	FWD	GAA CCA GGA GTT AAG AAC AGG
	REV	AGG CAA CAG TGT CAG AGT CC

Appendix F: List of primers. Listed 3' to 5'**.**

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