The regulation of gene expression in response to ER stress

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by

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Abstract

Topic: An investigation into the regulation of gene expression in response to ER stress

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A disruption in endoplasmic reticulum (ER) homeostasis can lead to ER stress and the accumulation of misfolded proteins, which has been implicated with the development of diabetes and many other diseases. In reaction to this the cell mounts an adaptive response termed the unfolded protein response (UPR) to improve cell survival during ER stress through the activation of three ER stress transducers PERK, ATF6 and IRE1. However, in case of unresolved ER stress, the UPR can triggers apoptosis pathway. UPR adaptive response is intended to restore ER homeostasis through decreasing ER load, increasing ER folding capacity and increasing ER associated degradation. At the centre of the UPR is transmembrane protein PERK which upon the phosphorylation of eIF2α leads to represses of global protein synthesis coextensive with preferential translation of mRNAs, such as activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP). In this study, I investigated the molecular mechanisms of translational repression in response to ER stress in MIN6 cells and how ATF protein expression is up-regulated in response to ER stress. In conclusion, I provide evidence that the eIF2α is likely responsible for the repression of protein synthesis in the presence of ER stress and that the induction of ATF4 expression in response to ER stress is dependent on its transcriptional upregulation. PERK mediated eIF2α phosphorylation is not required for increased ATF4 expression in MIN6 cells in response to ER stress. However, in MEFs, the PERK/eIF2α pathway is required for ATF4 protein expression, IRE1-XBP1 pathway is also required for ATF4 expression which might be time dependent, and protein synthesis is essential for induction of ATF4 expression in response of ER stress. Further investigations into how ATF4 expression is up-regulated in response to ER stress may extend our understanding to develop new therapies to protect ER from stress.

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Abbreviations

ADP	Adenosine diphosphate
ASK	Apoptosis- signal regulating kinase
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor-6
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
BAD	BCL-xL/BCL-2 associated death promoter
BAK	BCL2 homologous antagonist killer
BAX	BCL2-associated X protein
BCL2	B cell leukemia/lymphoma
BID	BH3 interacting domain death agonist
BIM	BCL2 interacting mediator of cell death
BiP	Heavy chain binding protein
BSA	Bovine serum albumin
CHOP	CCAAT/enhancer binding protein (C/EBP) homologous protein
CREB	cAMP-responsive element binding
ddH_2O	double distilled water
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
eIF2α	Eukaryotic translation initiation factor 2α
ER	Endoplasmic reticulum
ERAD	ER associated degradation

EDEM	ER degradation enhancing α-mannosidase-like protein
ERO	Endoplasmic reticulum oxidoreductin
ERSE	Endoplasmic reticulum stress element
FAD	Flavin adenine dinucleotide
FFA	Free Fatty Acids
GADD	Growth arrest and DNA damage
GPR	G-protein couple receptor
GRP	Glucose regulated protein
GSIS	Glucose stimulated insulin secretion
GAP	GTPase activator protein
GCN2	General control nondepressible-2
HRI	heme-regulated inhibitor
Hsp	Heat shock protein
IKK	Inhibitor of nuclear factor-κB (IκB) kinase
INS-1	Rat Insulinoma cell line
IP3	Inositol-1, 4, 5-trisphosphate
ISR	Integrated stress response
IRE1	Inositol-requiring protein-1
JNK	Jun N-terminal kinase
MAPK	Mitogen activated kinase
MEFs	Mouse embryonic fibroblast
MIN6	Mouse Insulinoma cell line
mM	Millimolar
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NRF2	Nuclear factor (erythroid-derived 2)-like 2
ORFs	Open reading frames
PBS	Phosphate buffer saline
PDIs	Protein disulphide isomerases
PP1c	Serine/threonine protein phosphatase 1
PKA	cAMP-dependent protein kinase

PERK	Protein kinase RNA (PKR)-like ER kinase
PKR	Protein kinase R
PKC	Protein kinase C
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RIDD	Regulated IRE1-dependent decay 8
RER	Rough ER
RyR	Ryanodine receptor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SER	Smooth ER
Ser	Serine
SRP	Signal recognition particle
SERCA	Sarcoplasmic/endoplasmic reticulum Ca2+ ATPase
SREBPs	Sterol regulatory element-binding proteins
TCA	Tricarboxylic acid
Thr	Threonine
uORF	Upstream open reading frame
UPR	Unfolded protein response
UPRE	UPR element
XBP1	X-box binding protein 1

Contents

Chapter 1: Introduction

1.1 The Endoplasmic Reticulum

The endoplasmic reticulum (ER) plays an important role in the synthesis of membrane and secretory proteins, lipid biosynthesis and calcium storage (Phillips & Voeltz, 2015). It consists of several structural domains that forms an interconnected network. The largest domain of the ER is the nuclear envelope which form a double membrane barrier that envelops the cell nucleus. The peripheral ER consists of two domains: flat membrane/ sheets and tubules. The ER flat membrane is covered with ribosomes and is responsible for the biosynthesis and folding of secretory and membranes proteins. The ER tubules or smooth ER is associated with very few ribosome and spread through-out the cytosol. Its function is exclusively in lipid biosynthesis (Shibata, Shemesh, Prinz, Palazzo, Kozlov & Rapoport, 2010). Most eukaryotic cells contain both types of ER. However, the distribution of these depends on the cell function. Secretory cells such as liver or pancreatic cells tend to have large quantities of rough ER, whereas, cells producing steroid hormones such as Leydig cells of the testes, have extensive smooth ER (Rowland & Voeltz, 2012). Proteins enter the endomembrane system through two ways either inserted into the rough ER lumen co-translationally which means as the protein is synthesized on the ribosome. Or, post-translationally, proteins are imported into ER after synthesis (Hardin, 2006).

1.1.1 Protein folding and maturation

Protein folding is an important process for cellular function. In eukaryotic cells, the endoplasmic reticulum (ER) is responsible for the synthesis, folding, modification, and quality control of numerous secretory and membrane proteins. Intracellular disturbance caused by different stressors such as changes in glucose level, calcium depletion, oxidative stress, hypoxia, aging, metabolic stimulation, inflammation, as well as increases in protein synthesis can all lead to an alteration in ER homeostasis that can

lead to the accumulation of unfolded/misfolded proteins and ER stress (Chakrabarti, Chen & Varner, 2011).

Proper protein folding and maturation is considered one of the most important ER functions. Newly synthesized proteins enter the ER through the translocon or Sec61 complex in the ER membrane. These nascent proteins after entering the ER are folded and post-translationally modified (Fedorov & Baldwin, 1997). The folded proteins in ER are retained by quality control system that ensures proteins are correctly folded before exported outside of ER (Ellgaard & Helenius, 2003). A protein is considered correctly folded when accomplished its innate confirmation after modification either by co-translation or post-translation (Ellgaard, Molinari & Helenius, 1999). Proteins must attain their final fold conformation in very short time under highly controlled cellular environment (Ellgaard & Helenius, 2003).

There are two recognised chaperone systems involved in protein folding: The first system involves the two lectin chaperones, calnexin (a highly conserved 90-kDa type I ER integral membrane protein) and calreticulin (a 46-kDa ER luminal protein) (Schrag et al., 2001).This chaperone system is dependent on the presence of monoglucosylated *N*-linked glycans and unfolded protein regions. Most polypeptides entering the ER are modified by adding oligosaccharides. These oligosaccharides are modified by glucosidases I and II which form mono-glucosylated intermediates that can be recognized by the ER lectins calnexin and calreticulin (Hammond, Braakman & Helenius, 1994) and the associated oxidoreductase ERp57 (Oliver, Roderick, Llewellyn & High, 1999). Calnexin and calreticulin are localized in the ER where they bind selectively and transiently to newly synthesized glycoproteins. Calnexin is a type I membrane protein with binding carbohydrate and a hairpin, called the P-domain (Schrag et al., 2001). Calreticulin is a soluble form of calnexin. The efficiency of glycoprotein folding is increased upon binding to calnexin and calreticulin by protecting against aggregation, while the misfolded proteins are retained in the ER (Hebert, Foellmer & Helenius, 1996). ER glycoprotein chaperones interact with ERp57 to promote formation of disulphide bonds (Frickel, Riek, Jelesarov, Helenius, Wuthrich & Ellgaard, 2002). Glycoproteins cleavage by glucosidase II where are released from calnexin and calreticulin. Once they are liberated attain their native fold conformation, after that glycosylated and reutilize by the calnexin/calreticulin cycle or directed to ER-associated degradation (ERAD) (Maattanen, Gehring, Bergeron & Thomas, 2010). Continual

glycosylation and deglycosylation process give misfolded proteins enough time in the ER to be corrected (Chakrabarti, Chen & Varner, 2011).

The second major system of ER chaperones requires the presence of unfolded, hydrophobic regions that are recognized by an ER luminal chaperone. This system is similar to calnexin and calreticulin, but other proteins chaperones that recognize and associate with unfolded or misfolded proteins are involved. These increase the efficiency of correct folding by increase the residence time in the ER lumen. For instant, the Heat Shock Proteins, HSP70 family of ER-resident molecular chaperones recognize, in an ATP-dependent manner (Kaufman et al., 2002). These include Immunoglobulin Binding Protein/Glucose Regulated Protein 78 (BiP/GRP78) and oxygen-regulated protein 150kD (ORP150/HYOU1) (Gething, 1999). These chaperones occur in adenosine diphosphate (ADP) and ATP- bound states. Their cycle depend on ADP-ATP exchange and ATP hydrolysis, this process is essential for protein folding which promotes conformational changes in the unfolded proteins (Gething, 1999). The cycle of binding and release of HSP70 chaperones increase the protein folding and ensure that unfolded or misfolded proteins not exist the ER (Kaufman et al., 2002). BiP or GRP78 is the major ER-localized member of HSP70 family consists of an N-terminal ATPase domain and a C-terminal peptide binding domain, BiP is involved in polypeptide translocation, protein folding and presumably protein degradation as well, these functions are vital for cell viability (Gething, 1999).

BiP binds unfolded protein with low affinity, this binding stimulates the N-terminal ATPase activity of BiP and subsequently lead to formation of ADP-bound form that has high affinity to unfolded/misfolded proteins (Gething, 1999). HSP70 family member including BiP have high affinity for ADP is about six-fold more than ATP (Tyson & Stirling, 2000). BiP acts as an ER stress regulator through sustain the calcium level (Reddy, Mao, Baumeister, Austin, Kaufman & Lee, 2003). In addition, the three arms of unfold protein responsive transmembrane: PERK, ATF6 and IRE1 are regulated by BiP chaperons. Under normal condition BiP chaperon is bound to these ER transmembrane molecules and blocking their activity. However, under stress condition BiP disassociates which lead to activation of transmembrane molecules PERK, ATF6 and IRE1 (Bertolotti, Zhang, Hendershot, Harding & Ron, 2000).

1.1.2 Endoplasmic Reticulum Association Degradation (ERAD)

Despite the tightly controlled process of protein folding and synthesis many proteins fail to acquire their native conformations in the ER lumen. Unfolded, misfolded, or partly folded proteins are removed from the lumen or the membrane of ER through dedicated cellular pathway and target them for controlled degradation. This pathway is conserved in eukaryotes and known as ER-associated protein degradation (ERAD) (Hiller, Finger, Schweiger & Wolf, 1996). ERAD inactivation leads to ER stress by increasing the accumulation of misfolded or unfolded proteins within the lumen of the ER (Walter & Ron, 2011). Once the misfolded or unfolded proteins are recognized by ERAD, they are retortranslocated into the cytoplasm across the ER membrane (Burr, van den Boomen, Bye, Antrobus, Wiertz & Lehner, 2013). They are subjected to further modification in cytosol such as ubiquitylation and glycosylation before disposal (Ye, Shibata, Yun, Ron & Rapoport, 2004).

1.1.3 ER Calcium homeostasis

The ER also has a function as the major intracellular Ca^{2+} storage organelle in higher eukaryotic cells. Regulation of Ca^{2+} homeostasis by the ER is achieved by the existence of numerous low- affinity and large capacity Ca^{2+} binding proteins that act as buffers. These proteins chaperons such as calreticulin, calnexin, BiP/GRP78, GRP94 and protein disulphide isomerase are responsible for control of ER Ca^{2+} concentration within physiological range of ~100-200 nM (Gorlach, Klappa & Kietzmann, 2006). Any changes in ER Ca^{2+} concentration can lead to disruption of protein folding and the accumulation of unfolded or misfolded proteins in ER which activate unfolded protein response (UPR) (Michalak, Groenendyk, Szabo, Gold & Opas, 2009). Accumulation of Ca^{2+} in the ER lumen is controlled by the sarcoplasmic/ endoplasmic reticulum Ca^{2+} ATPase (SERCA). The SERCAs are encoded with three genes (SERCA1, SERCA2, and SERCA3).

SERCAs encode four domains: a nucleotide binding domain, a phosphorylation domain, an actuator domain, and transmembrane domains. SERCA2b has the highest affinity for Ca^{2+} and is primarily responsible for maintaining ER luminal Ca^{2+} concentration (Vandecaetsbeek, Vangheluwe, Raeymaekers, Wuytack & Vanoevelen, 2011). SERCA

activity is stimulated by an increase of Ca^{2+} concentration. However, SERCA also is inhibited by protein chaperons such as calnexin and calreticulin thus decreasing ER Ca^{2+} uptake (Roderick, Lechleiter & Camacho, 2000). ER calcium homeostasis is also regulated by calcium efflux via inositol 1, 4, 5-triphsphate (IP3) receptors (Smyth, Hwang, Tomita, DeHaven, Mercer & Putney, 2010), Transient Receptor Potential (TRP) channels, first identified in Drosophila (Rohacs, 2013) and ryanodine receptors (RyR). There are number of compounds that act by inhibiting of calcium uptake into ER such as thapsigargin which is a potent inhibitor of the SERCA pump even in very small concentrations (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990). Thapsigargin is a specific and irreversible inhibitor of SERCA pump in most cells types, thapsigargin inhibits endoplasmic reticulum Ca^{2+}-ATPase, to mobilize intracellular Ca^{2+} and to activate Ca^{2+} entry into cells (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990).

1.2 ER stress

The capability of cell to respond to and cope with cellular stress is vital for maintaining homeostasis. ER stress result from several factors including disruption of calcium homeostasis, glucose level, accumulation of unfolded or misfolded proteins, genetic mutation and nutrient deprivation (Rutkowski & Kaufman, 2004). Other factors such as inhibition of protein glycosylation or disulphide bond formation, hypoxia, DNA damage and virus or bacterial infection can also result in accumulation of unfolded or misfolded proteins in the ER and lead to ER stress (Pluquet, Pourtier & Abbadie, 2015). Depletion of ER Ca^{2+} provokes protein misfolding and retention of unfolded proteins in ER lumen (Schroder & Kaufman, 2005). In response to ER stress, cells have developed mechanisms to sense, rectify and avoid additional accumulation of unfolded or misfolded proteins. An important mechanism is the unfolded protein response (UPR). It is an adaptive mechanism for restoration of ER protein homeostasis by mitigating protein synthesis, increase protein folding and enhance the ERAD system, which redirects unfolded proteins from the ER to the cytosol where they are degraded (Ruggiano, Foresti & Carvalho, 2014). In case of prolonged or unresolved ER stress the UPR triggers apoptosis pathway which induce cell death (Gorman, Healy, Jager & Samali, 2012). The ER stress can occur at several levels including ER protein expression levels, ER protein

activities, or posttranslational modifications (Moenner, Pluquet, Bouchecareilh & Chevet, 2007).

1.2.1 Sensing ER stress

There are three divergent ER resident transmembrane sensors that detect ER stress and subsequently activate the UPR to maintain homeostasis of the ER; inositol-requiring protein-1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor-6 (ATF6). Both IRE1 and PERK have ER luminal domains which regulate their kinase activity through oligomerization and auto-phosphorylation (Schroder & Kaufman, 2005).Under normal condition BiP/GRP78 chaperone is bound to these ER transmembrane molecules and block their activity. However, under stress conditions BiP/GRP78 dissociates which lead to activation of the UPR transmembrane molecules PERK, ATF6 and IRE1 through three possible mechanisms as outline below.

1.2.1.1 The competition model

BiP binds to the ER luminal sensor domains of IRE1, PERK and ATF6 under normal condition to keep them inactive. In this model, the ER chaperone BiP acts as a competitor of the unfolded proteins for the binding to the luminal domains of the ER stress sensors (Shamu, Cox & Walter, 1994). However, Under ER stress, the unfolded proteins compete for BiP binding and then release from the luminal sensor domains, which lead to dimerization and activation of their cytosolic domain (Kimata et al., 2003).

1.2.1.2 The ligand-binding model or BiP independent

In this model the unfolded proteins directly bind with the ER stress sensing domains. Recent evidences suggest that ER stress sensors direct binding to unfolded proteins. For instance, the IRE1 luminal domain binds short hydrophobic peptide sequences within unfolded proteins (Pluquet *et al*., 2015).

1.2.1.3 The probing model

The probing model has been reported to play a role in the regulation of ATF6. It is found that the glycosylation in the lumenal domain of ATF6 acts as a sensor for ER homeostasis and ATF6 activation. Glycosylation of ATF6 increased the release of calreticulin

chaperone and increase translocation to the Golgi (Hong et al., 2004).Glycosylation is considered as an inducer of the UPR during ER stress, it is believed that glycosylated ATF6 acts as an alternative sensor of ER stress sensing. However, IRE1 and PERK lumenal domains also have N-glycosylation sites but they have not been linked to ER stress sensing (Carrara, Prischi & Ali, 2013). It has been reported that the bZIP domains of ATF6 is involved in the degradation of fully glycosylated ATF6, which could serve as ATF6 probing mechanism to the efficiency of the glycosylation, folding and degradation processes (Hong et al., 2004).

1.3 Unfolded Protein Response Signalling

If the unfolded/misfolded proteins accumulate inside the endoplasmic reticulum, the unfolded protein response (UPR) is induced to overcome this situation. This unfolded protein response acts by three different mechanisms to alleviate the ER stress which are: (1) increase the ER protein folding capacity, (2) reduces global protein synthesis, and (3) enhances ER-associated degradation process (ERAD) of unfolded/ misfolded proteins. These UPR mechanisms are adaptive. If the cell fails to resolve the protein-folding defect, there is another signalling pathway activated known as the cell-death pathways or the maladaptive/apoptotic response. In fact, this pathway is also considered as protective mechanism since these non-functional cells will put an extra load and harm our bodies. For instant, the elimination of β-cells in case of unresolved ER stress (Cao & Kaufman, 2012; Herbert & Laybutt, 2016). In mammalian cells, The UPR is mediated by three ER transmembrane protein sensors: (1) activating transcription factor 6 (ATF6), (2) inositol requiring kinase 1 (IRE1) and (3) double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK). Each arm or sensor of the UPR protein responds to the certain level of unfolded/misfolded protein in the ER. If the cell fails to restore or overcome the protein-folding defect, cell-death signalling pathways are activated (Lin et al., 2007).

1.3.1 Double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) pathway

1.3.1.1 The Molecular Structure and Activation of PERK

PERK is a protein kinase that belongs to eukaryotic initiation factor (eIF2α) kinase family. PERK is type I transmembrane protein or single pass molecules consisting of a lumenal sensor and cytosolic protein kinase. The cytoplasmic domain senses the accumulation of unfolded/misfolded proteins in the ER lumen. After stimulation, PERK is activated by autophosphorylation of its kinase domain which lead to phosphorylation of eIF2α (Saito et al., 2011). Similar to most typical protein kinases, the structure of the kinase domain contains a C-terminal lobe (C-lobe) and an N-terminal lobe (N-lobe) (Cui, Li, Ron & Sha, 2011). PERK is a homodimer molecule, under normal condition the cytoplasmic domain binds by the ER chaperone BiP/GRP78. The ER stress initiation the disassociation of BiP/GRP78 from the cytoplasmic domain. Then, the unfolded/misfolded proteins in the ER lumen bind to the cytoplasmic domain and after that activate the PERK homodimers and subsequently the downstream molecules of PERK are recruited and phosphorylated (Cui, Li, Ron & Sha, 2011).

1.3.1.2 PERK phosphorylation of eIF2α

A function of the PERK branch of the UPR is to control the translation in case of an accumulation of unfolded/misfolded proteins. The dissociation of BiP from PERK's sensor arm initiates phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α) (Chakrabarti, Chen & Varner, 2011).

There are four eIF2 kinases have been recognized in mammals, and each kinase has distinctive signal and different downstream response pathway to control translation under stress condition. These eIF2 kinases include general control nonderepressible-2 (GCN2) which is activated by nutritional stresses, dsRNA induced protein kinase (PKR), mediated by interferon (IFN), which is an important for an antiviral defence pathway, heme regulated inhibitor (HRI) that links protein synthesis to the availability of heme in erythroid cells, and PKR like ER kinase (PERK), which is play an essential role for overcomes of unfold/ misfold protein in the endoplasmic reticulum (ER) (Vattem et al., 2004).

PERK is highly expressed in secretory tissues such as pancreas. Activation of PERK in response to ER stress leads to PERK activation and the phosphorylation of eukaryotic translation initiation factor-2 on the α-subunit (eIF2α) at serine-51, resulting in inhibition

of total protein translation (Harding, Zhang & Ron, 1999). The phosphorylation of eIF2α leads to competitive inhibition of the guanine nucleotide exchange factor eIF2B which exchange of guanosine diphosphate (GDP) bound to eIF2 for GTP. Inhibition of eIF2B blocks eIF2 recycling and inhibit the translation (Wortham & Proud, 2015). This leads to the: (1) the global attenuation of translation initiation, thus decreasing the global protein synthesis. And (2) the relative increase in translation of mRNAs with internal ribosome entry site (IRES) and/or 5' upstream open reading frames (ORFs) (Schroder & Kaufman, 2005). e.g. activating transcription factor (ATF4).

ATF4 can ATF4 also induce the expression of pro-apoptotic transcription factors. For example, ATF4 induce the expression of the transcription factor C/EBP homologous protein (CHOP), which is linked to cell death. ER stress also activates P58IPK transcription factor. P58IPK inhibits PERK, the inhibition of PERK activity leads to dephosphorylation of eIF2α, thus removing the translational block. P58IPK expression occurs several hours after PERK activation and eIF2α phosphorylation. Thus, P58IPK induction may mark the end of UPR adaptation and the beginning of the apoptosis response (Szegezdi, Logue, Gorman & Samali, 2006). Third, CHOP induces the expression of GADD34 which in conjunction with protein phosphatase 1(PP1) leads to dephosphorylation of eIF2α. GADD34 is also induced by DNA damage and other cell stresses (Novoa, Zeng, Harding & Ron, 2001). CHOP is considered a cellular death indicator, which promotes cell death through several mechanisms such as an increase of free radicals in the ER, an increase in protein influx into the ER by increasing the expression of GADD34, and increasing the expression of the pro-apoptotic Bcl-2 family of proteins, and by increasing calcium release.

1.3.1.3 eIF2α phosphorylation induced selective translation of mRNA

In response to environmental stresses, phosphorylation of eIF2α causes a general repression of global protein translation resulting from the reduced ability of eIF2 to couple with GTP and deliver the initiator Met-tRNAi to ribosomes for start codon recognition and start the mRNA translation (Baird et al., 2014).

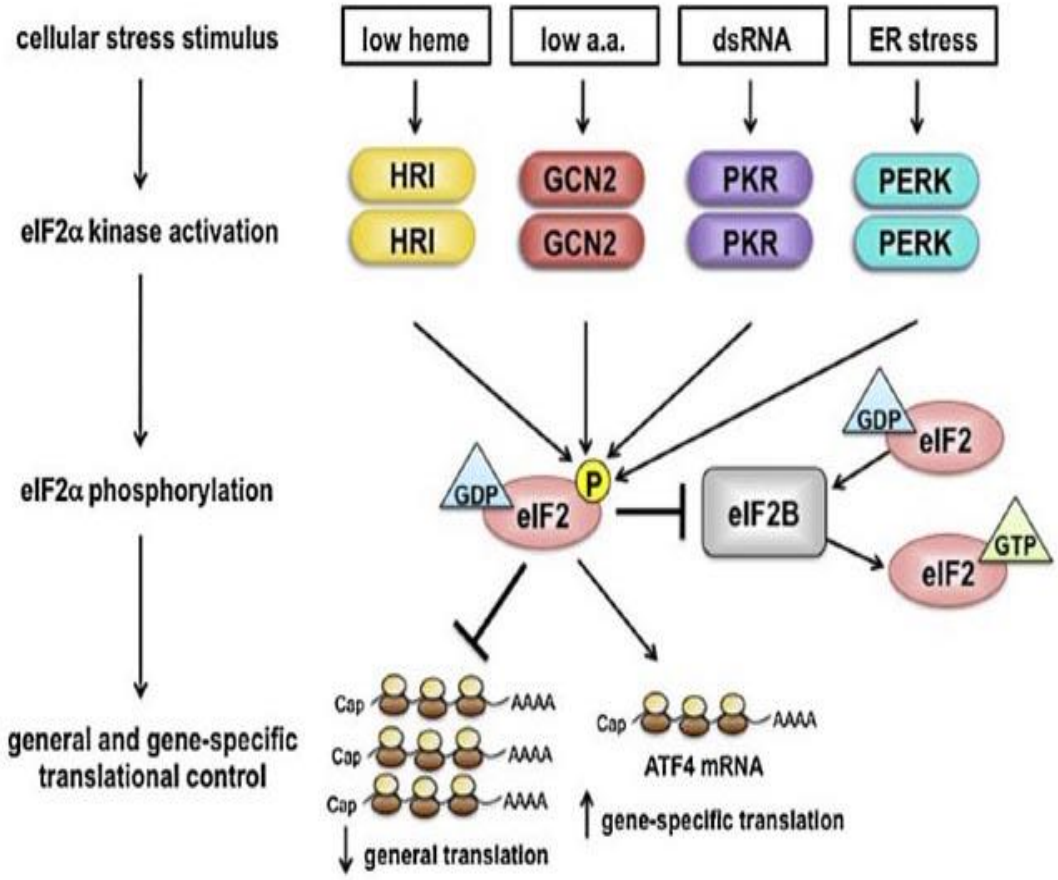

Figure 1.1 Translational control by eIF2α kinases. In response to different cellular stress stimuli, four eIF2α kinases become activated and phosphorylate the α subunit of eIF2. The guanine nucleotide exchange factor eIF2B catalyzes the exchange of inactive GDP for active GTP-bound eIF2, a process required for new rounds of translation initiation. Phosphorylation of eIF2 inhibits eIF2B activity, which blocks GDP/GTP-exchange, resulting in the reduction of general translation and the stimulation of gene-specific translation of uORF-containing such as ATF4 (Trinh & Klann, 2013).

Coincident with the inhibition of global protein translation eIF2α phosphorylation selectively enhances the translation of the activating transcription factor 4 (ATF4) mRNA, a transcriptional activator of genes involved in the UPR (Harding et al., 2000). ATF4 belongs to the activating transcription factor family and its expression is increased in response to different stress conditions (Wei, Zhu & Liu, 2015). ATF4 is a ubiquitous basic/leucine zipper domain transcription factor that belongs to the cAMP responsive element binding (CREB) protein family (Hai & Hartman, 2001). ATF4 expression is dependent on both PERK activities as well as on phosphorylation of eIF2α in response to ER stress, as examine of $PERK^{-/-}$ cells and $EIF2\alpha^{S51A/S51A}$ knock-in cells are failed to induce ATF4 activation in response to ER stress (Harding et al., 2000c; Novoa et al., 2001). The ATF4 mRNA consists of two upstream open reading frames (uORFs) in its untranslated region (UTR) which are uORF1 and uORF2. The uORF2 is out of frame and overlaps with ATF4 start codon (Vattem & Wek, 2004). During normal condition, ribosomes scanning downstream of the uORF1 and readily reinitiate at the uORF2, leading to inhibit ATF4 mRNA translation. However, stress condition delays ribosome reinitiation, allowing for the scanning ribosome to proceed through the inhibitory uORF2 result in upregulation of ATF4 expression (Hinnebusch, 2011). ATF4 expression leads to promote downstream transcriptional target genes involved in protein folding, metabolism and feedback regulation of phosphorylated eIF2α (Harding et al., 2003; Palam, Baird & Wek, 2011). In addition, under chronic ER stress, ATF4 induces the expression of transcription factor (C/EBP) homologous protein (CHOP, also known as GADD153), which directs cell to an apoptotic pathway (Harding et al., 2000).CHOP expression is abolished in $ATF4^{-/-}$ cells and overexpression of ATF4 induce CHOP expression in $PERK^{-/-}$ cells under ER stress (Harding et al., 2000).

CHOP mRNA is also subject to preferential translation ATF4 also induces the expression of growth arrest and damage inducible gene (GADD34) which acts as negative feedback to promote recovery form translational repression (Novoa, Zeng, Harding & Ron, 2001). GADD34 associates with protein phosphatase 1 (PP1) which lead to alleviate the translational repression and recovery of protein synthesis through the dephosphorylation of eIF2α (Brush, Weiser & Shenolikar, 2003; Novoa, Zeng, Harding & Ron, 2001). In presence of ER stress in $PERK^{-/-}$ and $eIF2\alpha^{S51A}$ knock-in cells, the induction of both CHOP and GADD34 mRNA were inhibited (Novoa, Zeng, Harding

& Ron, 2001), which indicates the primary role of the PERK/eIF2α phosphorylation pathway in their upregulation.

1.3.1.4 The PERK-Nrf2 signalling pathway

Nuclear factor erythroid derived 2 (Nrf2) belongs to the Cap 'n' Collar (CNC) family of basic leucine zipper (bZip) transcription factors that includes NF-E2, Nrf1-3 and Bach1-2, which are transcription regulator protein (Andrews, Kotkow, Ney, Erdjument-Bromage, Tempst & Orkin, 1993).

Nrf regulates the inducible expression of antioxidant response element (ARE), which are highly expressed in detoxifying organs such as the liver and kidneys (Moi, Chan, Asunis, Cao & Kan, 1994). Nrf2 nuclear translocation is induced under ER stress in a PERK dependent mechanism, the activation of PERK facilitates the dissociation of Nrf2/Keap1 complex and result in Nrf2 nuclear translocation (Cullinan, Zhang, Hannink, Arvisais, Kaufman & Diehl, 2003). Nrf2 activation does not required eIF2α phosphorylation or accumulation of reactive oxygen species (ROS). Nrf2 target genes, including enzymes involved in glutathione biosynthesis and chemical detoxification are induced during the UPR (Kwak, Wakabayashi, Itoh, Motohashi, Yamamoto & Kensler, 2003). As Nrf2 combats oxidative stress it is considered a cytoprotective agent (Cullinan, Zhang, Hannink, Arvisais, Kaufman & Diehl, 2003). $Nrf2^{-/-}$ cells are highly sensitive in the presence of variety ER stress inducers and Nrf2 overexpression improves cell survival in response to ER stress (Cullinan & Diehl, 2004). It is believed that PERK-dependent Nrf2 activation may contribute to protein degradation during the UPR (Cullinan & Diehl, 2006). It has been suggested that Nrf2 and ATF4 can together induce ARE-dependent gene transcription (He et al., 2001). Because of antioxidant activity of Nrf2 and its role to protect the cells apoptosis and death, the PERK/Nrf2 pathway could be beneficial for remedy of ER stress caused diseases (Zhu et al., 2015).

1.3.2 Inositol-requiring kinase 1 (IRE1) pathway

The inositol-requiring enzyme1 (IRE1) is the only identified ER stress sensor in yeast and essential for the UPR in animals and plants (Jager, Bertrand, Gorman, Vandenabeele & Samali, 2012). Thus, IRE1 is the most conserved arm of the UPR. IRE1 is type I ER transmembrane protein consisting of endoribonuclease (RNA) moiety and kinase moiety. IRE1 monitors ER homeostasis through its lumenal sensing domain and triggers

UPR through a cytoplasmic kinase domain and RNase domain (Hetz & Glimcher, 2009). IRE1 has two homologs or forms, IRE1α and IRE1β; IRE1α is present in all tissues while IRE1β is found only in the intestinal epithelia (Tirasophon, Welihinda & Kaufman, 1998). IRE1 under normal situations binds BiP. However, when there is an accumulation of unfolded / misfolded proteins BiP dissociates from IRE1. The dissociation of BiP from IRE1 leads to its activation. IRE1 activation enables both its kinase and RNA moieties to send signals at the same time. Under ER stress, IRE1 RNase is activated through conformational change, autophosphorylation, and oligomerization. The RNA moiety activity cleaves a nucleotide intron from the XBP1-mRNA which generates spliced X-box binding protein 1 (sXBP1s) that acts as a potent transcription factor (Chakrabarti, Chen & Varner, 2011). sXBP1s plays an important role in the expression of a variety of ER chaperones and protein degradation related genes. The unspliced XBP1 (uXBP1) does not have any physiological importance due to its extremely short half-life. However, it has been suggested that during the recovery phase XBP1u binds sXBP1s, stimulating degradation of sXBP1s (Yoshida, 2009).

1.3.2.1 IRE1 induced XBP1 splicing

The role of IRE1 in the UPR was established in Saccharomyces cerevisiae. Ire1p is the yeast orthologue of mammalian IRE1 (Cox, Shamu & Walter, 1993). The HAC1 encodes a basic-region leucine-zipper (bZIP) transcription factor that binds to and activates the UPR element (Mori, Kawahara, Yoshida, Yanagi & Yura, 1996), and is the substrate for Ire1p endonuclease activity. During ER stress, Ire1p cleaves the mRNA encoding Hac1 (Sidrauski & Walter, 1997). Unstressed cells show that HAC1 mRNA consists of a single 1.4-kb species. However, under stressed cells is converted to a smaller 1.2-kb species. (Cox & Walter, 1996). Hac1 mRNA translation is inhibited upon activation of Ire1p via cleaves the 5' and 3' exon-intron splice site junctions in Hac1 precursor mRNA to remove a 252 nucleotide intron (Sidrauski & Walter, 1997). The Hac1 mRNA is highly expressed but is only spliced in response to ER stress (Cox & Walter, 1996). In vivo, to accomplish HAC1 splicing, the Ire1p kinase activity is essential for HAC1 mRNA (Urano, Bertolotti & Ron, 2000). The splicing of c-terminal domain of Hac1p result in expression of a potent transcriptional factors, these transcriptional factors binds to UPR elements (UPREs), and induce the transcriptional

upregulation. Thereby, HAC1 mRNA splicing leads to an increase of protein synthesis as well as promote the protein to activate the UPR (Urano, Bertolotti & Ron, 2000).

The mammalian cells expressed two isoforms of IRE1, IRE1α and IRE1β. The IRE1α is highly expressed. IRE1α knockout mice show embryonic defects. Whereas, IRE1β knockout mice are survival (Iwawaki, Akai, Yamanaka & Kohno, 2009). IRE1α acts as a positive regulator for cell survival (Woehlbier & Hetz, 2011). In response to ER stress, IRE1 splices the transcription factorXbp1-mRNA to enhance protein folding and restore ER homeostasis. However, in case of chronic ER stress the IRE1α activates apoptosis pathway through Regulated IRE1-Dependent Decay (RIDD) (Upton et al., 2012).

Activation of IRE1 RNase eliminates a 26-nucleotide intron from unspliced X-box binding protein 1 (Xbp1u) mRNA result in cytosolic spliced form of XBP1 (XBP1s). After the newly XBP1u protein is synthesized, the 26-nucleotide intron suppress it's translation to gives enough time for hydrophobic region of the ribosome to associate with the ER membrane, subsequently the mRNA-ribosome complex is stabilized, thus enabling IRE1 to generate Xbp1s transcripts (Brewer, 2014).The XBP1s is a potent active and stable transcription factor (Yoshida, Matsui, Yamamoto, Okada & Mori, 2001). XBP1s interacts with UPR element (UPRE; TGACGTGG/A) which result in activation of ER stress responsive genes (Wang, Shen, Arenzana, Tirasophon, Kaufman & Prywes, 2000) leading to the transcription of genes encoding ER chaperones, including BiP, and proteins involved in ER associated degradation (ERAD) such as derlin-2 and ER degradation enhancing α-mannosidase-like protein (EDEM) (Oda, Okada, Yoshida, Kaufman, Nagata & Mori, 2006). XBP1s also regulates ER biogenesis (Sriburi, Jackowski, Mori & Brewer, 2004) and induces the expression of genes that enable the pass of newly synthesized protein into ER (Adachi, Yamamoto, Okada, Yoshida, Harada & Mori, 2008; Sriburi, Jackowski, Mori & Brewer, 2004; Yoshida, Matsui, Yamamoto, Okada & Mori, 2001).

Moreover, during the accumulation of misfolded proteins that induce ER stress, IRE1α is phosphorylated, resulting in the recruitment of tumour necrosis factor a (TNFα) receptor-associated factor 2 (TRAF2). The IRE1α–TRAF2 complex react with (inhibitor of kappa B) IkB kinase, which leads to the phosphorylation of IkB. The phosphorylation of IkB results in activation of its downstream inflammatory pathways. The IRE1α –

TRAF2 complex also activates c-Jun N-terminal kinase (JNK) to stimulate apoptotic pathway or cell death pathway (Szegezdi, Logue, Gorman & Samali, 2006).

1.3.2.2 Regulated IRE1-dependent decay (RIDD)

RIDD has the potential to selectively relieve the overload on the ER by reducing the subsequent influx of new proteins induced by the UPR. A subset of mRNAs are degraded during ER stress by a mechanism that is dependent on Ire1 but not Xbp1 (Coelho & Domingos, 2014). The degraded mRNAs encoded mostly proteins with signal peptide that could put an extra load on the ER folding machinery under ER stress(Brewer, 2014). These include mRNA encoding ER-translocating proteins. RIDD was first detected in Drosophila melanogaster cells, where under stress the mRNAs associated with the ER are degraded (Hollien & Weissman, 2006). RIDD also occurs in mammalian cells (Hollien, Lin, Li, Stevens, Walter & Weissman, 2009). Overexpression of IRE1 in mutant Xbp1 cells leads to hyperactivation of IRE1 and prompts cleavage of mRNAs localized ER (Benhamron, Hadar, Iwawaky, So, Lee & Tirosh, 2014). Surprisingly, IRE1 RNase products, XBP1 mRNA splicing and RIDD have an opposite action on cell fate (Han et al., 2009). The XBP1 splicing has prosurvival product, which has been observed in cancer, whereas RIDD has a proapoptotic output, which most likely happen in diseases such as diabetes (Maurel, Chevet, Tavernier & Gerlo, 2014). At normal condition, RIDD function is to sustain ER homeostasis by increase mRNA degradation which results in reducing ER protein synthesis, and inhibition of global protein synthesis by cleavage of 28S ribosomal RNA (Iwawaki & Tokuda, 2011). While, under stress status, activity of RIDD increase gradually (Hollien, Lin, Li, Stevens, Walter & Weissman, 2009). Moore *et al* has reported that under ER stress PERK mediated translational initiation attenuation plays an essential role in RIDD activity in mammalian cells (Moore & Hollien, 2015).

1.3.3 ATF6 pathways

The activating transcription factor 6 (ATF6) is an ER type II transmembrane protein belongs to the family of basic leucine zipper (bZIP) transcription factors. (Haze, Yoshida, Yanagi, Yura & Mori, 1999). In the presence of unfolded or misfolded proteins

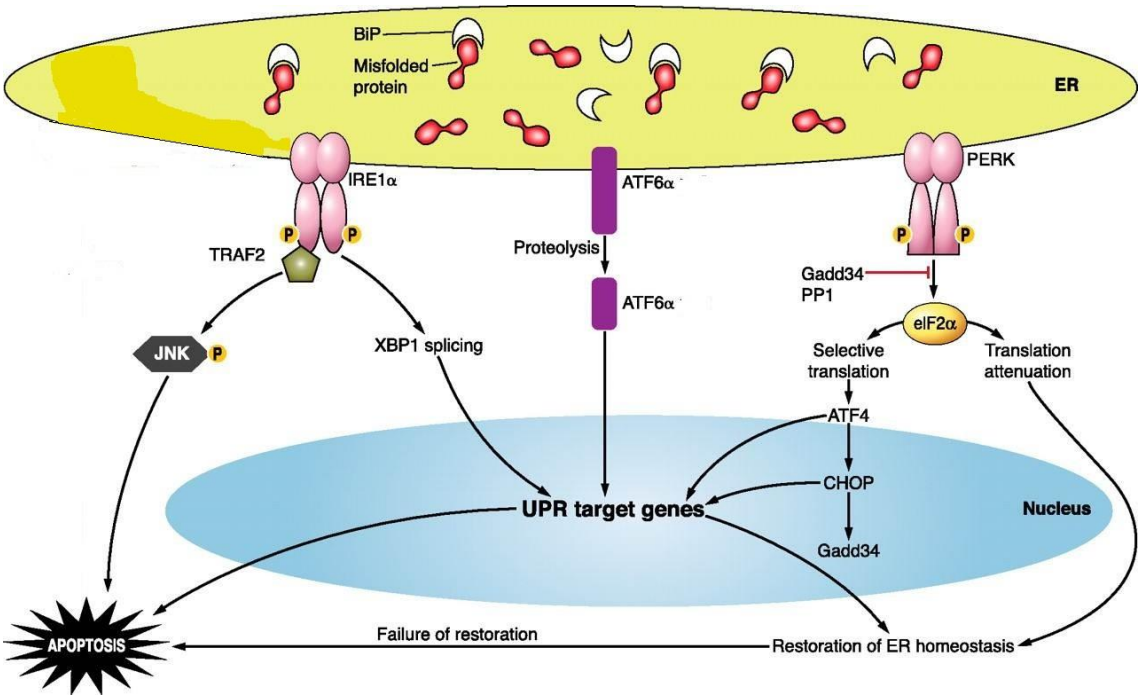

Figure 1.2 Induction of UPR by ER stress through 3 major transducers: RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring ER-to-nucleus signal kinase 1 (IRE1). Activation of PERK leads to phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which causes general inhibition of protein synthesis. In response to ER stress, ATF6 where it is cleaved by site-1 protease (S1P) and site-2 protease (S2P), yielding an active transcription factor, activated IRE1 catalyses removal of a small intron from the mRNA of the gene encoding X-box binding protein 1 (XBP1). This splicing event creates a translational frameshift in XBP1 mRNA to produce an active transcription factor. Active ATF6 and XBP1 subsequently bind to the ER stress response element and leading to expression of target genes encoding ER chaperones and ER-associated degradation (ERAD) factors involved in degradation of unfolded proteins (American Journal of Physiology).

ATF6 is activated, resulting in the up-regulation of a pro-survival transcriptional pathway. ATF6 is similar to other UPR arms, PERK and IRE1, its activation required BiP/GRP78 chaperones dissociation. However, ATF6 is the only sensor of UPR which requires proteolytic processing for activation (Chen, Shen & Prywes, 2002). In the rest state, the ER lumenal domain of ATF6 is bound to ER chaperone BiP/GRP78.Under conditions of ER stress, ATF6 dissociates from BiP and translocate to the Golgi (Shen et al., 2002). At the Golgi, ATF6 is cleaved by the site-1 (S1P) and site-2 (S2P) proteases, which lead to releases of its cytosolic domain from the ER membrane. Then, this ATF6 fragment travels to the nucleus to activate the transcription process (Brewer, 2014). Cleaved ATF6 induces a cytoprotective gene expression program, in conjunction with other bZIP transcription factors such as nuclear factor Y (NF-Y) that increases chaperone activity as well as the degradation of unfolded proteins (Yamamoto et al., 2007). It has been reported that activation of ATF6 regulates a number of ER-resident chaperones such as BiP, GRP94 , protein disulphide isomerase (PDI) and ER degradation-enhancing α-amannosidase-like protein 1 (EDEM1) expression (Okada et al., 2002). ATF6 also induces regulator of calcineurin 1 (RCAN1) expression, which inhibits calcineurin, calcium activated protein phosphatase B, that dephosphorylates Bcl2-antagonist of cell death (BAD), which result in blocking of Bcl2 by Bad, and subsequently inhibits its anti-apoptotic activity (Chakrabarti, Chen & Varner, 2011). Moreover, the X box-binding protein 1 (XBP1) is induced by ATF6 which stimulate expression of chaperones. Recently comment, it has been suggested that the unspliced form of XBP1 (XBP1u), is a negative regulator of ATF6. XBP1u may bind to ATF6 α making it more liable to proteasomal degradation (Yoshida, 2009).

ATF6 is ubiquitously expressed in mammals with two isoforms: ATF6α and ATF6β, both isoforms are functional and responsive to ER stress. It was reported the development of mice were not affected by deletion of either ATF6α or ATF6β. Whereas, deletion of both ATF6 isoforms leads to embryonic defect (Yamamoto et al., 2007). This indicate that ATF6α and ATF6β can compensate each other. ATF6α regulates lipid synthesis, ER biogenesis and has a physiological role in hepatocytes cells, dopaminergic neurons cells, skeletal muscle cells, pancreatic β cells, and dormant tumour cells (Brewer, 2014). There are several other tissues specific ER transmembrane ATF6-like proteins have been identified which undergo similar translocation to the Golgi and proteolysis process under ER stress, including OASIS which is expressed in astrocytes

(Kondo et al., 2005), and CREBH which is activated to induce an inflammatory response (Brewer, 2014).

1.4 ER stress and apoptosis

Despite the intensive program of the UPR to restore ER homeostasis, if the UPR fails to restore ER homeostasis or alleviates ER stress, the UPR triggers several apoptosis pathways to facilitate the execution of stressed cells (Chakrabarti, Chen & Varner, 2011).

1.4.1 ER stress and the BCL-2 family of proteins

Central to ER stress-induced cell death is the B cell lymphoma-2 (BCL-2) protein family includes both anti-apoptotic and pro-apoptotic members that carried one or more homologous domains, named the BCl-2 homology (BH) domains. BCL-2 proteins are classified based on the occurrence of one or more of BCL-2 homology (BH) domains (Wang, Olberding, White & Li, 2011). The BCL-2 family proteins are predominantly localized to the mitochondria, endoplasmic reticulum (ER) and perinuclear membrane (Rashmi, Pillai, Vijayalingam, Ryerse & Chinnadurai, 2008). BCL-2 family proteins are involved in the intrinsic death pathway, in which they regulate mitochondrial outer membrane permeability (MOMP). MOMP results in the release of apoptosis activating factors, such as cytochrome c from the mitochondrial intermembrane space into the cytoplasm in which they activate a cascade of caspases that leading to cellular death (Danial & Korsmeyer, 2004). The pro-apoptotic BCL-2 protein family is divided in to three classes, the multi-domain class such as BCL-2 associated X protein (BAX) and BCL-2-antagonist or killer (BAK), single BCL-2 homology 3 (BH3) domain-only proteins such as BIM, BID, BIK, PUMA, NOXA and BAD (Chakrabarti, Chen & Varner, 2011; Karst & Li, 2007), and less conserved BH-3 domain proteins such as BNIP1, BNIP2 and BNIP3 (Rashmi, Pillai, Vijayalingam, Ryerse & Chinnadurai, 2008). The anti-apoptotic members include BCL-2, BCLXL, BCL-W and MCL-1 which share all four BH domains (Schroder, 2008). Activation of the pro-apoptotic BCL-2 family members BAX and BAK result in changes of mitochondrial membrane permeability, which lead to release of mitochondrial membrane proteins such as cytochrome c, and subsequently initiate caspase activation and apoptosis (Tait & Green, 2010). Moreover, oligomerzation of BAX and BAK at the ER membrane initiate calcium release from the

ER to cytosol and sensitize cells to ER stress induced apoptosis (Mathai, Germain & Shore, 2005). Anti-apoptotic proteins inhibit activator BH3 proteins as well as activated BAX/BAK at the membrane (Willis et al., 2005).

IRE1 inhibits BAX and BAK by disturbing the interaction between IRE-1 BAX, and increase release of calcium from ER membrane through direct activation of BAX (Mathai, Germain & Shore, 2005). It has been shown IRE1α directly interact with BAX and BAK by forming a complex with cytosolic domain of IRE1α and modulate its signalling (Hetz et al., 2006). Knockout mice of BAX and BAK have failed to respond to IRE1α after ER stress inducing using tunicamycin; whereas, PERK arm is responded normally through phosphorylation of eIF2α (Hetz et al., 2006). The interaction of ER-localized BCL-2 with 1, 4, 5-trisphosphate receptor (IP3R) leads to inhibit calcium release and cell death; this interaction is regulated by the BH4 domain of BCL-2 binding to the regulatory coupling domain (RCD) of IP3R (Rong et al., 2009). This domain has dual function, close the inactivated IP3 receptor channel and translocate the binding ligand from the N-terminal IP3-binding domain to the C-terminal channel domain, therefore causing the channel to open (Bezprozvanny, 2011).

Disruption of ER calcium homeostasis enhances ER stress induced apoptosis via calpain-mediated caspase-12 and caspase-4 activation (Matsuzaki, Hiratsuka, Kuwahara, Katayama & Tohyama, 2010). Calpains play an important role in human endothelial cells during apoptosis. Calpain is activated in response to an increase of the intracellular Ca^{2+}, which lead to cleavage of BH3-interacting domain death agonist (Bid), cytochrome c release and activation of caspase 3. Subsequently, Calpain cleaved Bid mediates Bak and Bax oligomerization and changing the permeability of inner mitochondrial membrane (Smith & Schnellmann, 2012). Caspase-12 protein is not expressed in human, even though the transcribed of caspase-12 gene into mRNA because the translation process is interrupted. Therefore, ER stress-induced apoptosis is not regulated through human caspase-12. However, caspase-4 can function as an ER stress-induced caspase in humans which could compensate caspase-12 function in response to ER stress (Matsuzaki, Hiratsuka, Kuwahara, Katayama & Tohyama, 2010).

1.4.2 PERK signalling and apoptosis

Under unresolved condition of ER stress, and when the adaptive mechanisms of the UPR fail to alleviate ER stress, pro-apoptotic pathway is activated. Central to this is the chronic activation of PERK which triggers cell death mainly through the induction of the pro-apoptotic factor CHOP, downstream of ATF4. CHOP is belong to C/EBP family of bZIP transcription factors that is induced by ER stress, also known as GADD153 (Oyadomari & Mori, 2004). CHOP gene promoter has binding sites for the most of UPR inducers transcription factors such as ATF4, ATF6 and XBP1. Under ER stress, the inducing of CHOP is failed in $PERK^{-/-}$ and $AFT4^{-/-}$ cells and EIF2α $^{(Ser51Ala)}$ knock-in cells (Scheuner et al., 2001).

There are two main mechanisms for CHOP mediated cell death: (1) CHOP induces transcriptional regulation of apoptosis and oxidative stress genes, which involved BCL-2 family members, endoplasmic reticulum oxidoreductin 1 (ERO1α), tribbles-related protein 3 (TRB3) and death receptor 5 (DR5). CHOP can induce expression of genes encoding ER proteins, which exacerbate ER stress by increase protein loading, and promotes production of oxidative stress genes (Kim, Xu & Reed, 2008). (2) By induce the expression of GADD34 which restore the protein translation through eIF2α dephosphorylation. This in turn results in cell death (Gorman, Healy, Jager & Samali, 2012).

1.4.2.1 Role of CHOP in ER stress induced cell death

Induction of CHOP leads to up-regulation of BH3-only proteins, which belong to pro-apoptotic members of the BCl-2 family such as BIM, PUMA and NOXA (Marciniak et al., 2004). CHOP binds an element in the promoter of the gene encoding BIM proteins (McCullough, Martindale, Klotz, Aw & Holbrook, 2001) . It has been reported that under ER stress, BIM-deficient cells resist cell death in comparison with wild type cells (Kim, Xu & Reed, 2008). During ER stress, BIM is activated via transcriptional induction of CHOP-C/EBPα and phosphatase 2A-mediated dephosphorylation by preventing its degradation (Strasser et al., 2000). PUMA mRNA expression is decreased in the absence of CHOP activation, the activator protein (AP-1) is essential for PUMA regulation alongside with CHOP in response to palmitate induced ER stress (Cazanave, Elmi, Akazawa, Bronk, Mott & Gores, 2010).

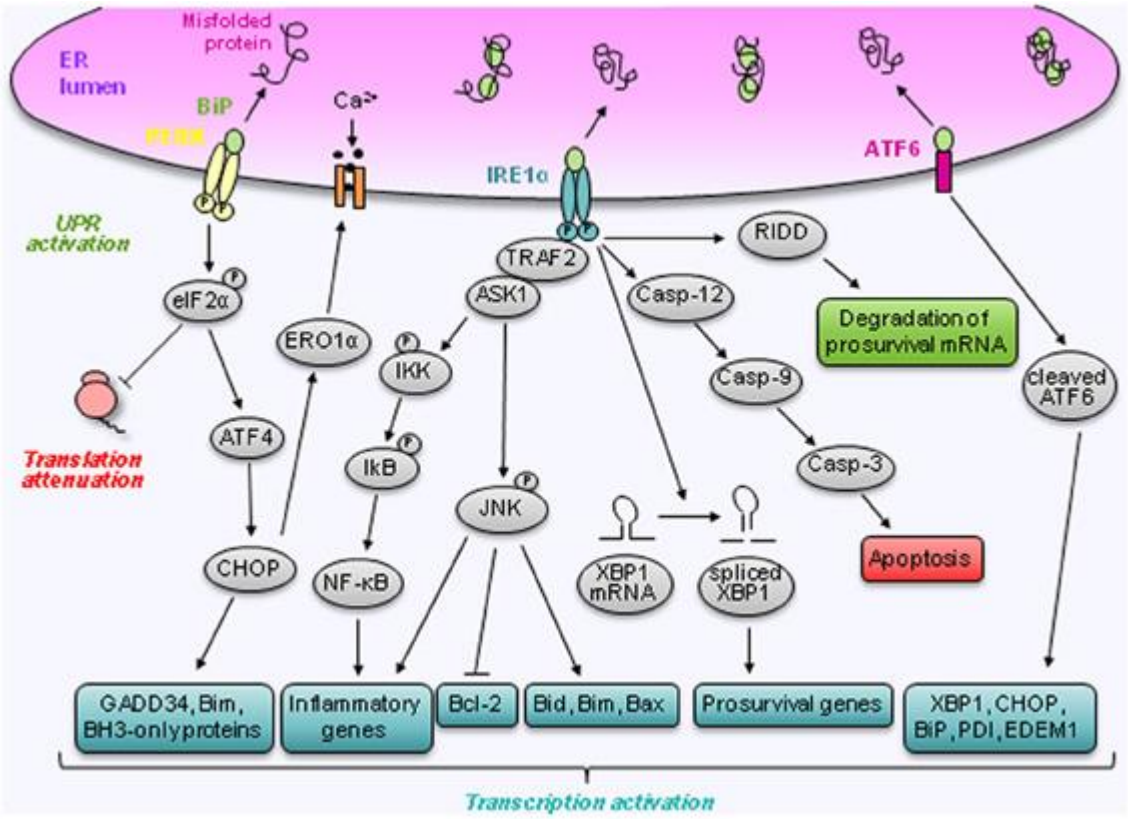

Figure 1.3 Regulation of prosurvival and apoptotic pathways by ER stress. In response to ER stress UPR is activated. This response is mediated via three ER transmembrane proteins IRE1α, PERK, and ATF6. Following dissociation of BiP, IRE1α becomes activated and induces splicing of XBP1 mRNA to XBP1s. IRE1α also activates JNK via TRAF2 and ASK1. Moreover, activation of IRE1α has been linked to downstream NF-κB activation and RIDD, which can lead to the degradation of prosurvival mRNA. Finally, IRE1α controls the activation of the caspases signalling pathway. PERK is activated following BiP dissociation. Active PERK mediates its response via phosphorylation of eIF2α leading to a translational block and cap-independent translation of ATF4. ATF4 induces CHOP which stimulates apoptosis and cell death. Following BiP dissociation, ATF6 is transported to the Golgi where it is cleaved into an active transcription factor. ATF6 regulates the expression of several genes involved in the unfolded protein response such as XBP1, CHOP, BiP, PDI, and EDEM1 (Deldicque, 2013).

Additional, CHOP can induce apoptosis by prompting the expression of the ERO1α, which keeping ER under oxidative stress. ER oxidoreductin 1-α (ERO1α), a target of CHOP, is an important oxidizing enzyme that regulates reactive oxygen species (ROS), which involved in cell death (Kim, Xu & Reed, 2008). ERO1 is a membrane-associated protein, which transfers electrons from protein disulphide isomerases (PDI) to molecular oxygen and generates hydrogen peroxide as a by-product (Malhotra & Kaufman, 2007). ERO1α expression is decreased upon disruption of CHOP leading to reduction of ROS that induce cell death in *vivo* and in *vitro*. Moreover, the decrease in the expression of ERO1α leads to an increase of ROS production through oxidizing protein disulphide isomerase (PDI) (Rao et al., 2015). ERO1α catalyse the creation of disulphide bonds of proteins in the ER, and rendering several protein disulphide isomerases (PDIs) to active form as well as ensure their correct folding of disulphide bonds (Frand, Cuozzo & Kaiser, 2000). CHOP increases the ERO1 expression leads to depletion of endogenous antioxidant, glutathione which result in an increase of ROS, subsequently leads to ER stress and cell death (Malhotra & Kaufman, 2007). Cell death is induced by oxidative stress. However, depletion of CHOP can diminish oxidative stress, decrease the production of ROS and ERO1α (Song, Scheuner, Ron, Pennathur & Kaufman, 2008). CHOP increase the leaking of ER calcium by activation of the inositol-1, 4, 5-trisphosphate receptor (IP3R). This leaking stimulates tricarboxylic acid (TCA) cycle which increase oxygen demand and hydrogen peroxide production. In addition, Ca^{2+} causes depolarization of mitochondrial membrane, resulting an increases of porosity and permeability which lead to ER stress and cell death (Landau, Kodali, Malhotra & Kaufman, 2013).CHOP is an important transcription factor that regulates death receptor 5 (DR5) expressions. CHOP directly regulates DR5 expression through a CHOP binding site in the 5-flanking region of the DR5 gene (Zou, Yue, Khuri & Sun, 2008). DR5 upregulation and apoptosis is inhibited during ER stress induced by thapsigargin; however the overexpression of DR5 in CHOP knock-out human cancer cells restore thapsigargin induced apoptosis sensitivity (Yamaguchi & Wang, 2004). ER stress-induced cell death involves sequential steps of PERK-mediated eukaryotic initiation factor α (eIF2α) phosphorylation, the preferential translation of activating transcription factor 4 (ATF4), induction of CHOP and GADD34, downstream target of CHOP. The growth arrest and DNA damage gene GADD34 is a downstream target of CHOP which

relieves the translation repression by facilitating protein phosphatase-1 (PP1) mediated dephosphorylation of eIF2α (Novoa, Zeng, Harding & Ron, 2001). GADD34 is a protein phosphatase 1 (PP1) targeting protein that directs PP1 to dephosphorylate eIF2α during the late stage of stress response (Brush, Weiser & Shenolikar, 2003). It has been reported that the localization of GADD34 to the ER by its N terminus enhancing the rate of protein turnover, and induce the apoptosis in mammalian cells (Rojas, Vasconcelos & Dever, 2015).

1.4.3 IRE1 signalling and apoptosis

In response to ER stress, IRE1α activates multiple signals through its endonuclease and kinase domains. The endonuclease domain of IRE1α promotes splicing of the mRNA encoding X-box binding protein 1 (XBP1) and regulates IRE1α dependent decay of mRNAs, including that encoding DR5. The kinase domain of IRE1α promotes the activation of c-Jun N-terminal kinase (JNK) by recruiting tumour necrosis factor α receptor-associated factor-2 (TRAF2). Under chronic ER stress, IRE1 binds to TRAF2 to form complex which interact with procaspase-12 leading to its cleavage to an active form (Yoneda et al., 2001). Moreover, the TRAF2-dependent JNK is activated by an apoptosis signal-regulating kinase (ASK1) during TNF-induced apoptosis. ASK1-deficient cells show resistant to JNK activation and ER stress- induced apoptosis, which showing that during the IRE1/TRAF2/ASK1- pathway is important for ER stress-induced apoptosis (Zheng et al., 2015). JNK becomes active after translocated to the nucleus, and subjected to phosphorylation which lead to activation of c-Jun (Chang & Karin, 2001). JNKs regulate gene expression through the phosphorylation and activation of c-Jun transcription factor. The phosphorylation of c-Jun leads to formation of activator protein 1 (AP-1), it is reported that the transcription of pro-apoptotic genes such as TNF-α and BAK is regulated by AP-1 (Dhanasekaran & Johnson, 2007; Zheng et al., 2015). Mitochondrial translocation of JNK has been implicated in cell death. JNK can interact with and phosphorylate a scaffold protein at the mitochondrial outer membrane, which leads to its translocation to mitochondria and promote signalling events, including the activation of pro-apoptotic BH3-only subgroup of BCL-2 family proteins and lead to cell death, such as BIM and BAD (Kaufman et al., 2002; Wiltshire, Gillespie & May, 2004).

Activated JNK can induce apoptosis via phosphorylation of BIM, which is pro-apoptotic BH3-only protein family member, leading to release and protect of BIM against proteasomal degradation (Morishima, Nakanishi, Tsuchiya, Shibata & Seiwa, 2004). The liberated BIM can associate with pro-apoptotic factors such as BAK and BAX thereby activates caspase to stimulate apoptosis (Putcha et al., 2003). Moreover, IRE1 can associate with TRAF2/IKK, result in activation of NF-kB signalling and lead to apoptosis (Hu, Han, Couvillon, Kaufman & Exton, 2006). It has been suggested that IRE1-deficient cells are more sensitive to changes in calcium homeostasis, oxidative stress and ER stress. These alterations leading to an accumulation of calcium in mitochondria, result in release of cytochrome C, and subsequently induce an apoptotic signalling, and eventually cell death, which might be due to ER membrane permeabilization (Kanekura, Ma, Murphy, Zhu, Diwan & Urano, 2015).

1.4.4 Caspases

Caspases are a family of cysteine dependent aspartate that directed proteases and involved in apoptosis. Eleven caspases existing in human out of fourteen caspases have been identified (MacKenzie & Clark, 2012). Generally, caspases classified into inflammatory caspases which include cytokine activators such as caspases-1, -4 and -5, and apoptotic caspases which divided into two sub-groups, the initiators such as caspases-2, -8, -9 and -10 and the effectors such as effector caspases -3, -6, or -7, this classification according to their time of entry into the apoptotic pathway (Thornberry et al., 1992). Caspases are formed in cells in inactive zymogens (inactive precursor of a protease) and subjected to proteolytic activation during apoptosis (Shi, 2002). Caspases are activated by extrinsic or intrinsic mechanisms. The extrinsic mechanism for activation of initiator caspase is responsible for removal of defective cells. However, the intrinsic mechanism is activated in response to cytotoxic stress (Boatright et al., 2003).
In response to cellular stress, mitochondria release cytochrome c into cytoplasm which is recognized by apoptosis activating factor 1(Apaf-1) protein, leading to recruitment of initiator caspase, caspase-9 to the apoptosome which leads to activation of effector caspases-3, -6 and -7 that eventually are cleavage of proteins and remove the cell (MacKenzie & Clark, 2012).

1.5 ER and role in diabetes

The disruption in the ER homeostasis, ER function or a failure to direct ER secretory proteins to their precise destinations can lead to the accumulation of misfolded/unfolded proteins and cause ER stress that leads to a number of disease such as metabolic disorders, neurodegenerative, diabetes and tumours (Schroder & Kaufman, 2005). UPR signalling is an important process for the maintenance of β-cell during severe and chronic ER stress. Mutations in the insulin gene that lead to its misfolding in the ER causes neonatal diabetes (Stoy et al., 2007). UPR not only regulates gene expression and promotes adaptation but can also activates cell death pathways (Oslowski & Urano, 2011). A high glucose level is considered one of the ER stressors in β-cells. The exposure of β-cell islets of a mouse and rat to high glucose (≥16.7mM) activates IRE1α and enhance proinsulin synthesis through XPB-1 splicing. However, prolonged IRE1α activation leads to insulin mRNA degradation and induces proapoptotic transcription factors such as CHOP and JNK which activates β-cell dysfunction and death (Jonas, Bensellam, Duprez, Elouil, Guiot & Pascal, 2009). An increase of BiP and CHOP levels was observed in pancreatic sections from Type 2 diabetic patients (Laybutt et al., 2007). The chronic high glucose level or glucose toxicity is also accompanied with release of reactive oxygen species (ROS) that induce ER stress which leads to β-cell damage, resulting in diabetes (Fonseca, Gromada & Urano, 2011a). Knockout of the CHOP recovers glycaemic control and increase the β-cell mass. Chop deletion improves ER function and protects against oxidative stress in response to ER stress in β cells. (Song, Scheuner, Ron, Pennathur & Kaufman, 2008).

PERK has a key role in the β-cell, Knockout PERK in β-cells leads to diabetes through decrease the proliferation of β-cell and insulin secretion. A mouse with a mutation at phosphorylation site of eIF2α at Ser51Ala develops β- cell deficiency and diabetes (Scheuner et al., 2005). The major cause of diabetes is β- cells death (Karaskov, Scott, Zhang, Teodoro, Ravazzola & Volchuk, 2006). Keeping of ER stress to acceptable level or promoting the UPR to triggers the survival pathway over death pathway could lead to protect the β-cells and treatment of diabetes (Laybutt et al., 2007).

1.5.1 Diabetes Mellitus

Diabetes Mellitus (DM) is a complex and chronic disease associated with multiple complications leading to an increase of morbidity and mortality. DM is characterized by chronic increase of blood glucose level with disorders of carbohydrate, lipid and protein metabolism (American Diabetes, 2014). It results from impaired insulin secretion, insulin action, or both. The main symptoms of type 1 diabetes include polyuria, polydipsia, polyphagia and weight loss (American Diabetes, 2014). Chronic diabetes can lead to development of macrovascular complications such as cardiovascular diseases and microvascular complications include retinopathy, nephropathy and neuropathy (Taubes, 2009). Patients are diagnosed as diabetic if there fasting plasma glucose is greater than 7 mmol/L (126mg/ml) , or their plasma glucose greater than 11.1 mmol/L (2000mg/dL) two hours after a 75g oral glucose load in a glucose tolerance test (WHO, 2006) or glycosylated haemoglobin (HbA1c) is greater than 6.5% (Goh, Rusli & Khalid, 2015). Diabetes Mellitus is affecting a greater proportion of the world's population each year such that the World Health Organization (WHO) predicts that DM will be the seventh leading cause of death by the year 2030. As of the year 2016, almost 422 million individuals adults living with diabetes (WHO, 2016).

1.5.1.1 Type 1 diabetes

In general diabetes mellitus can be classified into Type 1 diabetes, Type 2 diabetes, gestational diabetes mellitus and other specific types. Type 1 diabetes, formerly known as insulin-dependent diabetes or juvenile-onset diabetes, affects 5–10% of those with diabetes, it results from a cellular-mediated autoimmune destruction of β-cells leading to absolute insulin deficiency. Type 1 diabetes commonly occurs in childhood and adolescence, but it can occur at any age. Injection of insulin or synthetic insulin analogues is absolutely essential for the treatment of type1 diabetes. There are two forms of type 1 diabetes: Immune-mediated diabetes is characterised by autoimmune destruction of pancreatic β-cells. Another form is idiopathic diabetes which has no known aetiologies; it's strongly inherited, and individuals with this form of diabetes suffer from episodic ketoacidosis and show variable degrees of insulin deficiency between episodes (American Diabetes, 2010).

1.5.1.2 Type 2 diabetes

Type 2 diabetes is the most common form of diabetes, which accounts for 90 –95% of diabetes patients, previously known as non–insulin dependent diabetes, or adult-onset diabetes. Type 2 diabetes is typically a gradually progressive disease that characterized by insulin resistance in peripheral tissues with relative insulin deficiency (Hasnain, Prins & McGuckin, 2016). Insulin is the main regulator of the body nutrients that can control the use and storage of nutrients. Insulin resistance is considered the major defect in type 2 diabetes in which the insulin produces insufficient biological effect or the tissues have a lowered level of response to insulin. Insulin resistance leads to decrease the up-take of glucose by muscle and adipose tissue and increased hepatic glucose production (Cnop, Foufelle & Velloso, 2012). Type 2 diabetes is caused by a combination of lifestyle and genetic factors (Ripsin, Kang & Urban, 2009). Obesity can cause insulin resistance, which may leads to development of type 2 diabetes (Burke, Williams, Gaskill, Hazuda, Haffner & Stern, 1999). Obesity develops insulin resistance; however, small number of peoples develops type 2 diabetes. On the other hand, several reports were suggested that the β-cell dysfunction and insulin resistance are critical causes of hyperglycaemia in type 2 diabetes (Hasnain, Prins & McGuckin, 2016). It has been reported that the progressive decline in insulin secretion in type 2 diabetes is associated with decrease in β-cell mass which occur as a result of an increase in β-cell apoptosis (Butler, Janson, Soeller & Butler, 2003). ER stress is thought to lead to insulin resistance and a decline in pancreatic β-cell function and mass (Eizirik, Cardozo & Cnop, 2008).

1.5.1.3 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) which has its onset during pregnancy, it is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. GDM occurs in about 2-10% of pregnancies and most cases resolve with delivery. However, after pregnancy about 5-10% women with gestational diabetes are found to develop Type 2 diabetes (Sibartie & Quinlivan, 2015).

There are other specific forms of diabetes include genetic defects of β-cells function such as maturity-onset diabetes of the young (MODY), genetic defects in insulin action, diseases of the exocrine pancreas, endocrinopathies, drug or chemical induced diabetes and infections (American Diabetes, 2014).

1.5.1.4 Glucotoxicity and ER stress

In type 2 diabetes, insulin resistance usually leads to β-cell dysfunction. β-cell mass is increased because of increase insulin demand due to insulin resistance. However, the failure to compensate for increased insulin demand leads to a decrease of insulin levels, increase glucose level and development of type 2 diabetes (Zheng, Wu, Jin & Yan, 2016). Hyperglycaemic toxicity is the main cause of diabetes complications. Glucotoxicity results from persistent exposure to hyperglycaemia which leads to a decrease of glucose-stimulated insulin secretion (GSIS) and insulin gene expression (Robertson, Harmon, Tran & Poitout, 2004). Glucotoxicity is mediated by accumulation of excess ROS generated by several metabolic pathways (Robertson *et al*., 2004).

Oxidative stress is another factor that has damage effect on β-cell function, including suppression of insulin transcription (Kaneto & Matsuoka, 2015). Oxidative stress has an important role in β-cell dysfunction in type 2 diabetes because its highly sensitive to the overproduction of reactive oxygen species (ROS) (Zheng, Wu, Jin & Yan, 2016). Moreover, proinsulin synthesis is decreased during oxidative stress by inhibiting its mRNA expression via reduced β-cell specific transcription factors (Robertson, 2006). It has been reported that both oxidative stress and ER stress are correlated to each other because oxidative stress caused protein misfolded through the ER redox disruption, and the production of misfolded protein leads to increase ROS levels (Cadavez et al., 2014). ROS consist of molecules includes free radicals, such as nitric oxide, superoxide and hydroxyl radical, non-radicals such as hydrogen peroxide and peroxynitrite. These ROS produce during mitochondrial metabolism process or in cytoplasm such as NADPH oxidase and induced nitrogen oxide synthase (Cao & Kaufman, 2014). Oxidative stress is induced through elevation of ROS, leading to inhibition of insulin signalling via activation of protein kinase JNK (Kaneto et al., 2008).

Insulin secretory demand is increased during hyperglycaemia to regulate blood glucose level, which lead to increase insulin synthesis and disulphide bond formation that cause increase ROS production (Robertson, 2006; Zheng, Wu, Jin & Yan, 2016). It has been suggested that both oxidative and ER stress lead to mitochondrial dysfunction, cytochrome-C release, and caspase activation resulting in the death of the β-cell (Jonas, Bensellam, Duprez, Elouil, Guiot & Pascal, 2009). The β-cell does not have a strong

defence mechanism against ROS effect because the ROS detoxification mechanism is weak in the β-cell (Robertson, Harmon, Tran & Poitout, 2004). Moreover, diabetic hyperglycaemia activates many metabolic pathways such as glycation pathway, hexosamine pathway and PKC activation pathway. All these pathways induce ROS production and oxidative stress (Yan, 2014). It was reported that the chronic glucose exposure lead to activation of IRE1 and XBP1 splicing, however, acute glucose exposure from 1 to 3 h result in IRE1 activation, there is no XBP1 mRNA splicing detected (Zheng, Wu, Jin & Yan, 2016).

1.5.1.5 Lipotoxicity and ER stress

When adipose tissue cannot store excess fat, fat accumulates inappropriately in muscle and liver cells leading to what is referred to as lipotoxicity. Obesity is associated with low grade of inflammation which is implicated in insulin resistance. Accumulating evidences have been considered the obesity is the major predisposing factor for Type 2 diabetes. Obesity can lead to an increase in free fatty acids (FFAs), leading to insulin resistance, therefore compensatory mechanism becomes active to maintain normal glucose level through increase pancreatic β-cells secretion. It was reported that lipid electrophiles play an important role in ER stress and can be directly activate all UPR branches. (Hauck & Bernlohr, 2016). Palmitate can activate the UPR, resulting in PERK activation and the selective up-regulation of ATF4 and CHOP expression in β-cells and primary islets (Igoillo-Esteve et al., 2010). Obesity induced ER stress which triggers inflammatory signalling pathway via activation of JNK, leading to transcriptional upregulation of inflammatory genes (Urano et al., 2000). Under ER stress, fatty acids enhance ER load through protein modification, Ca^{2+} regulation and oxidative stress explain (Back & Kaufman, 2012; Mirmira, 2012). ER stress is induced by saturated fatty acids such as palmitate, while unsaturated fatty acids show protective effects in INS1 cells. Moreover, UPR is activated by palmitate through PERK and IRE1 pathway, and oleate abolishes this activity (Back & Kaufman, 2012). Activated JNK impairs insulin signalling through the inhibitory phosphorylation of the serine residue on insulin receptor substrate 1 (IRS-1) and thus decrease tyrosine phosphorylation of the insulin receptor and the IRS proteins, which leading to insulin resistance. IRE1 mediated JNK activation also has been shown in primary human islets and INS1E cells (Cunha et al., 2008). Obesity can induce both Toll-like receptor (TLR) and ER stress signals. TLR

activates JNK, IkB (IKK) and inflammatory gene expression such as TNFα, which all participate in insulin resistance (Milanski et al., 2009). It was suggested that in both children and adults, elevation of free fatty acids was associated with reduced β-cell function (Salgin, Ong, Thankamony, Emmett, Wareham & Dunger, 2012).

1.5.1.6 Glucolipotoxicity and ER stress

Together, hyperglycaemia and hyperlipidaemia are typical features of type 2 diabetes and obesity, prolonged exposure to high level of glucose and lipid lead to progressive β-cell dysfunction and apoptosis in type 2 diabetes. The combination of the adverse complication of glucose and lipid is termed as glucolipotoxicity. Both high glucose and high non-essential fatty acids induce oxidative stress in β-cell, leading to increase protein misfolding and ER stress (Hasnain, Prins & McGuckin, 2016). It was reported that palmitate induced ER stress in the presence of glucose by mTORC1 via increasing IRE1 protein levels and activating the JNK pathway, which results in increased β-cell apoptosis (Bachar, Ariav, Cerasi, Kaiser & Leibowitz, 2010). Another clinical study supported the glucolipotoxicity theory, by acute loading of glucose and/or lipid leading to decrease β-cell function in human. On the other hand, energy restriction or intensive insulin treatment restore β-cell function (Hasnain, Prins & McGuckin, 2016).

1.5.1.7 Islet amyloid and ER stress

Islet amyloid polypeptide (IAPP, or amylin) is a hormone that's synthesised as a 89 amino acid prohormone which is proteolytically processed to the mature hormone amylin. IAPP is stored with insulin within secretory granules in the β-cell and is released in response to an elevation in glucose (Akter et al., 2016). IAPP has a role in metabolism and glucose homeostasis as it controls gastric emptying and suppresses glucagon release (Westermark, Andersson & Westermark, 2011). Aggregated IAPP is characteristic of islets found in pancreatic sections from type 2 diabetics and these have been implicated in β-cell death (Westermark & Westermark, 2011). IAPP is one of the factors that attributed to β-cell dysfunction and death, beside to glucolipotoxicity, inflammation and lipotoxicity (Ashcroft & Rorsman, 2012). IAPP is one of the factors that leads to failure of islet cell transplants, it has been detected in transplanted human islets after islet graft failure (Westermark, Andersson & Westermark, 2011). Amyloidosis can activate

different cellular and downstream mechanisms and pathways such as receptor-mediated and non-receptor-mediated mechanisms (Bram et al., 2014). It have been reported that human IAPP (hIAPP) aggregates can cause ER stress . The role of ER stress in hIAPP mediated toxicity in vivo still unclear. Overexpression of hIAPP in transgenic mouse has shown ER stress induced β-cell dysfunction and addition of exogenous hIAPP also induced ER stress (Morita et al., 2011). However, IAPP from cultured islets does not induced ER stress (Hull, Zraika, Udayasankar, Aston-Mourney, Subramanian & Kahn, 2009). Moreover, another group suggested that IAPP accumulation is not associated with ER stress, because there is no increase ER stress makers such as BiP, ATF4, CHOP, XBPls in hIAPP transgenic mice and human islets (Hull, Zraika, Udayasankar, Aston-Mourney, Subramanian & Kahn, 2009). In case of chronic inflammation, hIAPP plays a role in β-cell dysfunction by activating an inflammatory response, which subsequently induces active caspase-1 leading to enhanced cytokines IL-1 and IL-18 activity, which play a direct role in hIAPP induced β-cell death (Hasnain, Prins & McGuckin, 2016).

1.5.1.8 Gene defective and ER stress contribute to diabetes

1.5.1.8.1 Wolcott-Rallison syndrome

Wolcott-Rallison syndrome (WRS), it is also known as multiple epiphyseal dysplasia and early-onset diabetes mellitus. WRS is characterized by insulin-dependent diabetes, which usually develops during the neonatal period (Julier & Nicolino, 2010). WRS patients develop pancreatic hypoplasia and β-cell loss (Thornton, Carson & Stewart, 1997).WRS is a rare autosomal recessive disease, it is caused by mutations in the gene encoding eukaryotic translation initiation factor 2a kinase3 (EIF2AK3) (Amos, McCarty & Zimmet, 1997). The mutation occurs in the catalytic domain of PERK leading to a failure of its kinase activity and a reduction of eIF2α phosphorylation (Senee et al., 2004). It was reported that mice deficient for PERK show a similar phenotype to human WRS such as neonatal onset diabetes, multiple epiphyseal dysplasia, hepatic dysfunction and exocrine pancreas deficiency (Julier & Nicolino, 2010). PERK-deficient mice show severe defects in neonatal β-cell proliferation and differentiation, resulting in reduction in β-cell mass, failure in proinsulin trafficking and insulin secretion, leading to development of neonatal diabetes (Zhang, Feng, Li, Iida, McGrath & Cavener, 2006). Additional studies made in β-cell lines and in PERK KO mice show reduction in cell

proliferation and insulin secretion as result of PERK ablation (Feng, Wei, Gupta, McGrath & Cavener, 2009; Gupta, McGrath & Cavener, 2010).

1.5.1.8.2 Wolfram syndrome

Wolfram syndrome is a rare genetic disorder characterized by juvenile-onset diabetes mellitus, diabetes insipidus, optic nerve atrophy, hearing loss, and neurodegeneration (Urano, 2016). Diabetes mellitus is the main and first feature of Wolfram syndrome, usually diagnosed around age of 6 months (Lou Frances, Soto de Ruiz, Lopez-Madrazo Hernandez, Macipe Costa & Rodriguez Rigual, 2008). Wolfram syndrome is developed as result of mutation in Wolfram syndrome gene 1 (WFS1) (Bespalova et al., 2001). It is established that Wolfram syndrome is example of endoplasmic reticulum disease with genetic link (Ishihara et al., 2004). The mutations in the WFS1gene can cause destruction in pancreatic β-cells and neuronal cells (Fonseca et al., 2010). ER dysfunction is considered the main pathogenic factor because WFS1 gene encodes a transmembrane protein localized to the ER. WFS1 mutation is involved in increase ER stress, β-cell dysfunction and cell death (Ajlouni, Jarrah, El-Khateeb, El-Zaheri, El Shanti & Lidral, 2002; Fonseca et al., 2005). In response to ER stress, WFS1 mRNA and protein are induced through the activation of IRE1 and PERK. However, WFS1 inactivation leads to ER stress and β-cell dysfunction (Fonseca et al., 2005) . Thus, it is believed that the WFS1 has a protective function against ER stress and the loss of its activity leads to chronic ER stress, β-cell death and the development of Wolfram syndrome (Fonseca et al., 2010). It was reported recently that β-cell death in Wolfram syndrome can be developed from depletion of ER calcium and activation of calpain (Lu et al., 2014). A large body of reviews suggested that WFS1 protein is involved in ER calcium control, which proposed that the regulation of ER calcium by small molecules can protect cell death in Wolfram syndrome (Urano, 2016).

1.5.1.9 Management of Type 2 Diabetes

Control of hyperglycaemia is considered the main target for treatment of type 2 diabetics in order to prevent the development and progression of complications. The target of the treatment is to control $HbA1_C$ level <6.5% or a fasting glucose <6.7mmol/L (120mg/dL). The first line of treatment by modifications of life style including diet, exercise, smoking cessation, reducing alcohol intake and loss weight, which are expected to improve insulin

sensitivity and improve glycaemic control (Hills et al., 2010; Stone et al., 2013). However, in case of lifestyle modification has not improved the blood glucose level within 2 months, treatment with medicine is recommended. There are some consideration should be taken for choice of pharmacological agents including, efficacy, potential side effects, obesity, hypoglycaemia risk, cost, and patient preferences. Because of progressive nature of type 2 diabetes, in many cases, monotherapy fails to improve blood glucose control and combination therapy is required. Insulin usually recommended in long term of diabetes in case of oral hypoglycaemic treatment is failed to control blood glucose level (American Diabetes, 2015).

MEDICATIONS FOR TYPE 2 DIABETES		
Classification	**Medication**	**Mechanism**
Sulfonylureas	Chlorpropamide, Glibenclamide, Glimepiride, Glipizide, Gliclazide	Increases the release of insulin from β-cells in response to glucose stimulation by inhibiting K_{ATP} channel in cell membrane
Biguanides	Metformin	Facilitation of glucose uptake, reduce of glucose absorption from small intestine
Glucosidase Inhibitors	Acarbose, Miglitol, Voglibose	Slow digestion and glucose productionby inhibiting carbohydrate degradation in gut
Thiazolidinediones	Pioglitazone, rosiglitazone	Enhance glucose utilization in peripheral tissue, improve insulin sensitivity and lowers glucose production
Meglitinides	Nateglinide, Repaglinide	Increase insulin release by closing K_{ATP} channel
Dipeptidyl peptidase 4 inhibitors	Sitagliptin, Saxagliptin	Lower glucose by inhibit the degradation of the incretins, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP).

Table 1.1 Oral hypoglycaemic medicines (American Diabetes, 2014)

1.5.2 Atherosclerosis

Atherosclerosis is a complex process that associated with increase in the arterial wall thickness as result of accumulation of lipid, cholesterol, cellular waste, Ca^{2+}, and other substances in the arterial inner lining, the arteries become hard and narrow which subsequently lead to heart attack or stroke. The main component of lipid deposit in the arterial wall is low-density lipoprotein (LDL) (Rafieian-Kopaei, Setorki, Doudi, Baradaran & Nasri, 2014). It has been reported ER stress is considered an important component that lead to development of atherosclerosis. An investigation of human and animal atherosclerotic plaques showed that ER stress is involved in at herogenesis. For example, ER stress is induced by free cholesterol and 7-ketocholesterol that facilitate macrophage apoptosis via CHOP activation (Ivanova & Orekhov, 2016; Nakamura et al., 2010; Tsukano et al., 2010). Furthermore, the induction of CHOP and cholesterol synthesis can promote macrophage apoptosis that lead to an accumulation of macrophage fragments in blood vessels, which induce atherosclerosis formation (Feng et al., 2003). Moreover, activation of the IRE1α/ASK1/JNK pathway induces CHOP leading to macrophage death. ER stress is activated during myocardial infarction (Tabas, 2009). It was reported, in heart failure model, β-adrenergic receptor blockers alleviate ER stress, suggesting that could be due to their effect on ER calcium mechanism (Dalal, Foster, Das, Singh & Singh, 2012).

1.5.3 Cancer

Microenvironment of tumour is usually characterized by poor blood stream, reduced oxygen supply and nutrient deficiency which promoting ER stress activation. In cancers, UPR has shown a cytoprotective mechanism through enhance newly synthesized proteins folding that increase the tumour growth. Under hypoxia, PERK and IRE1α are essential for survival and growth of tumour cell (Sano & Reed, 2013). Cell death is increased and angiogenesis is inhibited in tumour derived from PERK-deficient transformed mouse. Moreover, The IRE1α/XBP-1 pathway is important for angiogenesis in the primary phases of tumour development, the inhibition of XBP-1 splicing by RNAi interfered the human tumour growth (Romero-Ramirez et al., 2009). Recent studies suggested that the inhibition of the IRE1α/XBP-1 pathway could provide potential path for cancer treatment. For example, inhibition of XBP-1 splicing downstream of IRE1α

leading to suppress of myeloma cell growth in an animal model (Ri et al., 2012). It was found that inhibition of XBP1 was accompanied with an increase of apoptosis levels, proposing that XBP1 transcription factor has a role in malignant tumour prolongation (Grzmil et al., 2006; Grzmil et al., 2003). In cancer cell lines and tumour biopsies, ER chaperone BiP levels is elevated in response to different malignancies especially during the pro-survival mechanisms (Cnop, Foufelle & Velloso, 2012). It is believed that the increase of UPR activation leads to survival of carcinomas, leukaemia, lymphomas, and gliomas in the tumour microenvironment (Wang & Kaufman, 2014). Inhibiting of UPR could increase the sensitivity of cancer cells and decrease resistance to chemotherapy agents.

1.5.4 Neurodegenerative diseases

Accumulation of misfolded or unfolded proteins is involved in many neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), prion disease, Huntington's disease (HD), frontotemporal dementia (FTD), and amyotrophic lateral sclerosis (ALS). Like rest of body cells, ER in neuron cells is the main site for protein synthesis, modification and folding (Scheper & Hoozemans, 2015). Removal of misfolded proteins is essential for neurons homeostasis. However, the aggregation of misfolded proteins is considered a typical sign for many neurodegenerative diseases (Friedman et al., 2012). There is large body of studies suggested that there is a strong link between protein misfolded, ER stress and neuronal cell death in the majority of neuro-degenerative diseases.

1.5.4.1 Alzheimer's disease

Amyloidosis refers to a group of diseases such as Alzheimer's disease (AD) or the cardiac amyloidosis which characterized with abnormal protein deposition within the extracellular space (Endres & Reinhardt, 2013). In Alzheimer's disease, there is assumption suggested that the accumulation of extracellular amyloid-β and intracellular aggregates of phosphorylated tau proteins, leading to neuronal death and result in failure in cognitive processes, leading to dementia (Cuello, Allard & Ferretti, 2012; Querfurth & LaFerla, 2010). The amyloid precursor protein (APP) maturation includes translocation and cleavage. APP matures via trafficking to the ER, while cleavage takes

place in the Golgi, which is considered key player in Alzheimer's disease (Dyrks, Dyrks, Monning, Urmoneit, Turner & Beyreuther, 1993; Sambamurti, Shioi, Anderson, Pappolla & Robakis, 1992). It was reported a massive increase expression of CHOP and decrease expression of anti-apoptotic protein BCL-2 were detected in familial Alzheimer's disease (FAD) linked PS1 mutation knock-in mice (Milhavet et al., 2002). Accumulating evidences are suggested that the ER-stress acts as sensor in AD pathogenesis. At early stage of AD the XBP-1 signalling is induced, whereas PERK pathway is induced during late stage of disease (Endres & Reinhardt, 2013).

1.5.4.2 Parkinson's disease

Parkinson's disease is a neurodegenerative disease characterized by reduction in the number of dopaminergic neurons in the substantia nigra, accompanied with an increase of acetylcholine activity caused by dopamine deficiency results in dyskinesia (Tsujii, Ishisaka & Hara, 2015). Several reports suggest that ER stress is involved in the pathology of Parkinson's disease. For example, α-synuclein, which is aggregated during the Parkinson's disease condition, inhibits the activity of ATF6 (Credle et al., 2015). α-synuclein accumulation by nutrient deprivation and enhances in response to ER stress (Jiang, Gan, Lin & Yen, 2014). It was reported there is co-localization of phosphorylated PERK and phosphorylated eIF2α with α-synucleinin dopaminergic neurons of Parkinson's disease patients (Valdes et al., 2014).

1.6 Summary and thesis aims

Different molecular mechanisms participate in ER stress-induced apoptosis; however, the molecular mechanisms of switching from adaptive to pro-apoptotic responses are not fully understood. So far, the most accepted mechanisms mediating cell death under ER stress are downstream of PERK. Despite the regulation of cell death by ER stress is highly complex and may depend in an additional crosstalk with other signalling pathways. Therefore, the aims of this thesis are to:

- Investigate the molecular mechanisms of translational repression in response to ER stress and to identify which translation initiation factor/s is/are responsible for translational repression in response to ER stress.
- Demonstrate the role of PERK mediated eIF2α phosphorylation in the upregulation of ATF4 in response to ER stress and to address the underlying mechanism for ATF4 expression in response to eIF2α phosphorylation in response to ER stress.
- Gain a better understanding of how the UPR is regulated in response to cellular stresses and determine key temporal steps in the switch from an adaptive to a maladaptive UPR.

Chapter 2: Materials and Methods

2.1 Materials and Reagents

Unless stated, all chemicals were of analytical grade and were routinely purchased from Sigma-Aldrich, New England Biolabs, Melford, and Fisher Scientific. Tissue culture pipettes, plates and flasks were purchased from Nunc, TPP or VWR. Antibodies were purchased from Santa Cruz, Cell Signaling Technology, Sigma-Aldrich, Promega or Invitrogen. Anti-mouse Ig HRP-linked and anti-rabbit Ig HRP-linked antibodies were obtained from Cell Signaling Technology. Universal bottles 30ml were bought from Thermo Scientific. Centrifuge tubes 15ml and 50ml were purchased from VWR. 1.5ml Micro tubes were purchased from Sarstedt. Non-filtered and filtered tips were obtained from Axygen Scientific. RNA extraction kits were purchased from Peqlab and Ambion. Primers were purchased from Eurofins Genomic and Primer Design Ltd. Plasmids that we have used for transfection in Chapter one, Encephalomyocarditis virus (pEMCV), Cricket paralysis virus (pCrpv), Renilla/Firefly plasmid (pRF), and Hepatitis C virus (pHCV) were provided by Professor Martin Bushell-MRC, Leicester. Plasmid of 5'UTR-ATF4-Luc and adenovirus of ATF4-Luc were generated in house.

Product name	Company
Gene Jet plasmid	Thermo
4μ8C	Tocris Bioscience
Thapsigargin	Merck Millipore
PERK Inhibitor I, GSK2656157	Merck Millipore
Tran^{35}S-label No-thaw Metabolic Labeling Reagent	MP Biomedicals
Filter Immobilon-P Transfer Membranes Roll	HA
Medical X-Ray Film Blue Sensitive	Wolflab
Rec.Protein G sepharose	Generon
Transcripter First Strand cDNA Synthesis Kit	Roche
Express SYBR® GreenER^{TM}qPCR SuperMix Universal	Invitrogen
Dual-luciferase® Reporter Assay System	Promega
Lipofectamine® 2000 Reagent	Invitrogen
Viability/Cytotoxicity Kit for mammalian cells	Molecular Probes
RNAqueous-4PCR Kit	Ambion
Amplify Fluorographic Reagent	GE Healthcare

Table 2.1 General reagent list

2.2 Mammalian Cell Culture

Cell lines used in this study were Mouse Insulinoma 6 cells (obtained from Astra Zeneca (MIN6 AZ) which reveals the ability of glucose-stimulated insulin secretion and used as β-cell specific cell line, Human Embryonic Kidney 293 (HEK293) and Mouse Embryonic Fibroblast (MEFs).

2.2.1 Maintenance of Cell lines

Mouse insulinoma 6 (MIN6), human embryonic kidney 293 (HEK293) and mouse embryonic fibroblast (MEFs) cells were maintained at 37ºC and 5% CO_2 in the incubator. MIN6 cells were cultured in DMEM media containing 25mM glucose, 15% heat-inactivated FBS, 100µg/ml streptomycin, 100units/ml penicillin, 100units/ml neomycin (PSN), 40mM $NaHCO_3$ and 75µM β-mercaptoethanol. HEK293 cells were cultured in DMEM media containing 25mM glucose, 10% heat-inactivated FBS, 100units/ml PSN. MEFs cells were cultured in DMEM media containing 25mM glucose, 10% heat-inactivated FBS, 100units/ml PSN, 1× non-essential amino acids. Media was changed every 2 or 3 days.

2.2.2 Cell splitting

Cells were split at approximately 80% confluence. Media was removed and washed cells once with 1× phosphate buffered saline (PBS). Then cells were incubated with 1× trypsin/EDTA for 1-5 minutes at 37ºC dependent on cell type. Cells were re-suspended in appropriate media and split 1:3 for MIN6 cells or 1:10 for HEK293 and MEFs for maintenance or for experimental use.

2.2.3 Transfection and Infection of cell lines with plasmids and recombinant adenoviruses

2.2.3.1 Transfection with Lipofectamine

Lipofectamine mediated transfections were carried out using Lipofectamine 2000 (Invitrogen). Prior to transfection, cells were split into 24 sterile multi-well plates. 0.2µg

of plasmid was used to transfect one well of 24 wells plate. An appropriate amount of plasmid was diluted in 50µl of Opti-MEM (reduced serum media without serum from Gibco Company were placed in one tube. 1µl of lipofectamine was diluted in 50µl of Opti-MEM were placed in second tube, mix gently. Both tubes were incubated for 5 minutes at room temperature. The plasmid mixture was added to the lipofectamine mixture then incubated at room temperature for 20 min to allow plasmid-lipofectamine complex to form. The MIN6 cells media was replaced with 400µl of Opti-MEM media for each well. 100µl of the plasmid-Lipofectamine complexes were then added drop wise to the cells, and the cells then incubate at 37℃, 5% CO_2 for 4-6 h. The Opti-MEM media was then replaced with full MIN6 media and incubate at 37℃, 5% CO_2 for 48 h.

2.2.3.2 Infection of cell lines with recombinant adenoviruses

For MIN6 or MEFs cell lines: growth media was removed and replaced with 400µl DMEM (for 4cm diameter plate) containing 1% PSN. High titer or purified virus was then added into the cell and incubate at 37ºC for 1 h. Then, added 1.2ml DMEM and left the cells in the 37ºC incubator for 24 h. After 24 h, the media were changed by complete MIN6 growth media and continued incubating for another 24 h prior to experimentation.

Adenoviruses infection efficiency was determined by the presence of cells expressing GFP using a Nikon fluorescence microscope fitted with a mercury lamp. The infection efficiency was typically around 75-85% prior to experimentation.

2.2.4 Transformation of Competent cells.

50-100µl of DH5α competent cells were thawed on ice for 5 minutes, and then 1.0µg/µl of DNA was added gently to competent cells. The cells and DNA were mixed by tapping many times. Cells were incubated on ice for 30 minutes. Cells were heat shocked at 42ºC for 45 seconds, then put straight back onto ice for another 15 minutes. Cells which had been transformed with plasmid were recovered in 500µl of warm LB for 40 minutes at

37ºC with shaking. Cells were then centrifuged for 1 minute at 3,000rpm in an Eppendorf bench top microfuge and 400µl of the LB was discarded. The cells were resuspended and plated onto LB-agar plates containing an antibiotic. Plates were incubated overnight at 37ºC.

2.2.5 Plasmid DNA purification

To prepare the bacterial culture, a single colony was picked from a plate and then placed to 2ml of LB medium with the appropriate antibiotic. The culture was then incubated for approximately 8 hours at 37°C while shaking. This 'starter culture' was then added to 250ml of media containing antibiotics in a 1L conical flask and incubate overnight at 37°C with shaking. Cells were harvested by centrifugation at 5,000 × g for 10min. The bacterial pellet was then further processed or stored at -20°C. Plasmid was purified by using Gene Jet kit which based on subjected of the pelleted bacterial cells to SDS/alkaline lysis to liberate plasmid DNA. The resulting lysate was neutralized to precipitate proteins and chromosomal DNA. Cell debris and SDS precipitate were then pelleted by centrifugation. The supernatant containing plasmid DNA was applied onto the purification column. The adsorbed DNA on membrane was washed to remove contaminants and eluted with the Elution Buffer.

2.3 Experimentation

2.3.1 Cell treatment and lysis

Detailed descriptions of treatments are provided in the figure legends. After experimentation, growth medium was aspirated off the cells and the cells washed twice with 1x PBS. For measuring luciferase activity cells were scraped into 100µl of passive lysis buffer (Promega) on ice. Lysates were subject to two freeze-thaw cycles and then centrifuged at 14,000rpm at 4℃ for 10 min. The supernatant was kept for further analysis. For western blotting, all samples were harvested using ice-cold MIN6 lysis buffer or RIPA buffer. Lysates were centrifuged at 14000rpm for 10 min at 4ºC. The supernatant was kept for further analysis.

2.3.1 2.3.2 Determination of cells viability

Cells viability was determined by using the live/dead viability/cytotoxicity kit (Invitrogen). After experimentation in a 24 well plate, 20µl of 2mM EthD-1 stock solution was added to 10 ml of sterile, tissue culture PBS, vortexed 5µl of 4 mM calcein AM stock solution was the added to make the 10 ml EthD-1 working solution. 300µlof this solution was then added to each well and the cells incubated the cells for 30–45 minutes at room temperature. Following incubation, the labelled cells were viewed under the fluorescence microscope. The dye calcein is well retained within live cells, producing an intense uniform green fluorescence from ~495 nm to ~515 nm. EthD-1 enters cells with damaged membranes and binding to nucleic acids, thereby producing a bright red fluorescence in dead cells between ~495 nm and~635 nm.

2.3.2 Dual-luciferase reporter assay system

Samples were harvested and prepared to measure luciferase activity using dual-luciferase reporter assay system from Promega. The Luciferase Assay Reagent II (LARII) and Stop&Glo reagent were prepared according to manufactory instruction. After treatment, cells were harvested using ice cold 50µl passive lysis buffer (24 well plates). After that subjected to freeze-thaw cycle twice and centrifuged at 14000rpm for 10min at 4^{o}C. The supernatant was kept and the pellet discarded. Before the measurement, LARII and Stop&Glo reagent should warm up to room temperature .10µl of sample lysate was added to 96 well-read plate then 50µl LARII added to measure firefly luciferase activity using Novastar plate reader to determine luminescence. After that 50µl Stop&Glo was added to measure renilla luciferase activity using Novastar plate reader to determine luminescence.

2.3.3 Immunoprecipitation

30µl of Protein-G Sepharose beads per sample were washed twice with lysis buffer, centrifuged at 3000 rpm for 30 second and the supernatant discarded. The total volume of Protein-G Sepharose beads was resuspended in lysis buffer and incubated with the relevant antibody for one hour at room temperature on a rotator, to allow binding of the

antibody to the Protein-G Sepharose beads, the beads were then washed with 1x lysis buffer and the supernatant discarded. Protein-G Sepharose beads were resuspended in lysis buffer and then added to a lysate, and the lysate rotated for 2 hours at 4 ˚C. After incubation, the lysate was centrifuged at 3000rpm for 30 second. The supernatants were removed without disturbing the beads and stored at 80 ˚C for further analysis. The Protein-G Sepharose beads were then washed 3 times with RIPA buffer, centrifuged at 3000rpm for 30 second and resuspended in 20µl of 2X Laemmli's sample buffer. Samples were boiled for 5 minutes, then loaded straight onto SDS-PAGE, using a Hamilton syringe or were stored at -80 ˚C until required.

2.3.3.1 Autoradiography of radiolabelled proteins

12.5% SDS-PAGE gels were soaked twice in fixing solution (50% methanol, 10% acetic acid) for 15 minutes. The gels were then soaked in Amplify solution (GE Healthcare) with agitation for 20 minute. The [^{35}S]-Methionine labelled gels were then dried on 3MM paper under vacuum at 60-80 ˚C and exposed to X-ray film at either -80 ˚C or room temperature.

2.3.4 TCA Precipitation of protein and protein synthesis measurements

5 µl of cell lysate was spotted onto a 1cm^2 of 3MM Whatman filter papers in triplicate. Filter papers were boiled for 1 minute in 100 ml of 5% Trichloroacetic acid (TCA) with a pinch of L-methionine. The 5% TCA was discarded and replaced with 100ml of 5% TCA and boiled again for 1 minute. The 5% TCA was discarded again and the papers were rinsed in 5% TCA followed by washing with absolute ethanol. The papers were dried at 80℃ for one hour. The filter papers were immersed in 3ml of scintillant (Emulsifier-safe, PerkinElmer) and DPM determined by Scintillation counting using a Beckman-Coulter liquid scintillation counter .All protein synthesis measurement were expressed as a percentage of control.

2.4 Molecular Biology

2.4.1 Total RNA isolation by using The Ambion RNAqueous-4PCR Kit.

Extraction of total RNA was performed using the Ambion RNAqueous-4PCR Kit as described in the instructions. RNA concentration was determined using Nanodrop, ND1000 spectrophotometer. Extracted RNA was either directly used or kept in -80^{o}C until required.

2.4.2 Primers

ATF4 forward primer: 5'-GAA TGG ATG ACC TGG AAA C-3' (20)
ATF4 reverse primer: 5'-ACT CTC TTC TTC CCC CTT GC- 3' (22)
HKG18sRNA. 891. Forward primer: 5'GTTGGTTTTCGGAACTGAGG-3' (20)
HKG18sRNA.1090. Reverse primer: 5'GCATCGTTTATGGTCGGAAC-3' (20)

2.4.3 cDNA synthesis

To prepare cDNA from RNA, 1µg RNA was added to 2µl of oligo $(dT)_{12-18}$ ((0.5 µg/µl), (Invitrogen), 4µl dNTP mix (12.5mM) and made up to a 16µl total volume with DEPC H_2O on ice. The solution was heated at 65-80 $^{\circ}$C for 5 minutes and placed back on ice. The tubes were then spun down and then 2µl of 5X RT buffer, RNase inhibitor and 1µl MLV reverse transcriptase (Promega) were then added followed by gentle mixing and a quick centrifugation. The solution was incubated at 42 $^{\circ}$C for one hour then heat inactivated at 90 $^{\circ}$C for 5 minutes. Synthesized cDNA was stored at -20^{o}C until required for use.

2.4.4 Quantitative polymerase chain reaction (qRT-PCR)

cDNA was diluted to 1ng/2µl. Make up each 20µl master mix as follow:

Name	For ATF4	For 18s
Sybrgreen mix	10µl	10µl
Primer	1µl	Forward 0.4µl Reverse 0.4µl
DEPC H_2O	7µl	7.2µl
cDNA	2µl	2µl

Table 2.2 content of master mix

The master then was amplified using the following programme:

50°C	2min
95°C	5min
95°C	20s
59°C	30s
72°C	30s
72°C	10min
15°C	hold

Repeat 36 times

The obtained data was analysed by using Relative expression software to calculate ΔΔCt as fold change of target gene expression (Livak & Schmittgen, 2001).

2.5 Recombinant Adenovirus techniques

2.5.1 Amplification of virus

HEK 293 cells were infected with recombinant adenovirus, after 3-4 days virus were ready for harvesting. Cells were washed off from the flasks, transferred to 50ml sterile tubes and centrifuge at 1800×g for 5min at 4^{o}C. The pellets were resuspended in 1ml PBS, frozen in a dry ice/ethanol bath and then thawed at 37^{o}C in water bath and then vortex. This freeze/thaw/vortex cycle was repeated three times to fully lyse the cells and release the adenoviruses. The samples were centrifuge at 3200×g for 10min at 4^{o}C to pellet cell debris. The pellet was discarded and the viral supernatant was stored at -80^{o}C for future use.

2.5.2 Generation of High Titer Adenovirus stocks

90% confluent HEK 293 cells in two T-25 flasks were re-infected with 70% of the adenovirus supernatant. After infection, GFP expression was measured by using fluorescence microscope to determine viral production and infection efficiency. After 3-5 days post infection, when the infected cells consist of cell rounding, swelling, loss of cell-cell contact the cells were harvested and subjected to four cycles of freeze/thaw/vortex as described above. The virus supernatant from T-25 flasks was then used to infect four T-75 flasks. Finally, high titer virus was produced and only 10µl of the supernatant should able to infect one T-75 flasks, above 90% GFP expression and 80-90% floating cells should observed one day after infection.

2.6 Protein Techniques

2.6.1 Buffers and Reagents

2.6.1.1 Lysis Buffer

1% v/v Triton X-100

10mM β-glycerophosphate pH7.4

50mM Tris-HCl pH7.5

1mM EDTA pH8

1mM EGTA

1mM Sodium Orthovanadate

1mM Benzamidine

0.2mM Phenylmethylsulphonyl fluoride (PMSF)

1µg/ml Leupeptin

1µg/ml Pepstatin A

0.1% v/v β-mercaptoethanol

50mM Sodium fluoride (NaF)

2.6.1.2 RIPA buffer

0.5mM NaCl

50mM (Sodium fluoride) NaF

50mM Tris-HCl pH8

0.5% Deoxycholate (Doc)

0.1% NP40

0.5% sodium dodecyl sulfate (SDS)

1mM Sodium Orthovanadate (Na_3VO_4)

10mM β-glycerophosphate pH7.4

0.5mM Benzamidine

0.1mM

Phenylmethylsulphonyl

fluoride PMSF

1μg/ml Leupeptin

1μg/ml Pepstatin A

0.1% β-mercaptoethanol

2.6.1.3 10×Tris-glycine buffer (for 1L)

30g Tris base (Melford)

144g Glycine (Melford)

2.6.1.4 SDS-PAGE running buffer

1×Tris-glycine buffer

0.1% SDS

2.6.1.5 Wet transfer buffer

1×Tris-glycine buffer

0.01% SDS

20% Methanol

2.6.1.6 10×PBS (Phosphate buffered saline, 1L)

3g KCl

100g NaCl

14g Na_2HPO_4

3g KH_2PO_4

pH to 7.4 and make up to 1litre with

ddH_2O

2.6.1.7 PBST (PBS-Tween)

1×PBS

0.1% Tween-20 (Sigma)

2.6.1.8 Laemmli sample buffer (4×)

0.25M Tris pH 6.8

4% SDS

40% Glycerol

10% β-mercaptoethanol

20µg/ml Bromophenol blue

2.6.2 Antibodies

Name	Company	Catalog no.	Application	Dilution
p-eIF2α	NEB	9721	WB	1:1000
p-eIF2 α	GeneTex	GTX 38625	WB	1:500
ATF-4	NEB	11815S	WB	1:1000
Ribosomal ProteinS6C-8)	Santa Cruz	Sc-74459	WB	1:2000
XBP-1 (M-186)	Santa Cruz	Sc-7160	WB	1:500
eIF2α (FL-315)	Santa Cruz	Sc-11386	WB	1:1000
Anti-luc	Promega	G745A	IP	1:1000
Anti-GFP	Sigma	9E10	IP	1:5000

2.6.3 Sample preparation

2.6.3.1 Protein extraction from cells

After experimentation, cells were scraped into ice cold lysis buffer and transferred to a clean microfuge tube. The lysate was vortexed for 10sec and centrifuged at 14000rpm at 4^{o}C for 10minute. The supernatant was removed and transferred to a fresh microfuge tube. Protein concentration was determined by Bradford assay.

2.6.4 Bradford Assay

The Bradford assay was used to determine the protein content of cell lysates. Bradford reagent (Bio-Rad) was diluted with ddH_2O in a 1:5 ratio. 2µl of lysate was mixed with 8µl ddH_2O into cuvette, 1ml diluted Bradford reagent was added and then incubated for 5min at RT. The absorbance of mix was measured using the UV 1101 Biotech, WPA spectrophotometer at a wavelength of 595nm. The protein content was determined by linear regression against a standard curve of BSA protein standards. Protein contents of each sample were adjusted to the sample with the lowest protein content using triton lysis buffer. Samples were diluted with 4× laemmli sample buffer and boiled for 3min at 100^{o}C before running on SDS-PAGE gel.

2.6.5 SDS-PAGE gels preparation

Polyacrylamide gels were prepared in an ATTO system (Tokyo, Japan) mini PAGE (polyacrylamide gel electrophoresis) system by using solutions listed in table 2-3. Solutions used in the preparation of SDS-PAGE gels, for two running and three or four stacking gels (Bio-Rad, ATTO).

Solution	7.5%	10%	12.5%	15%	17.5%	20%	Stacking
40% Acrylamide	2.925ml	3.825ml	4.725ml	5.625ml	6.525ml	7.275ml	1.24ml
2% Bis-acrylamide[1]	1.56ml	2.04ml	2.52ml	3ml	3.48ml	3.75ml	0.65ml
1.5M Tris-HCl pH8.8	3.75ml	3.75ml	3.75ml	3.75ml	3.75ml	3.75ml	-
1M Tris-HCl pH6.8	-	-	-	-	-	-	1.25ml
ddH_2O	6.54ml	5.165ml	3.78ml	2.4ml	1.02ml	0ml	6.7ml
10% SDS	0.15ml	0.15ml	0.15ml	0.15ml	0.15ml	0.15ml	0.1ml
TEMED[2]	7.5µl	7.5µl	7.5µl	7.5µl	7.5µl	7.5µl	10µl
10% APS[3]	75µl	75µl	75µl	75µl	75µl	75µl	100µl

Table 2-3 Solutions of gel formation. Acrylamide: Bis acrylamide ratio 37.5:1

1: N,N'-methylene bis-acrylamide.

2: NNN' tetramethylethylendediamine

3:APS: Ammonium persulphate.

2.6.5.1 Running

SDS-PAGE gels were immersed in SDS-PAGE running buffer in ATTO system gel tanks.

Protein samples were boiled for 3min at 100^{o}C and centrifuge briefly at highest speed. After that samples are loaded onto SDS-PAGE gels alongside prestained protein markers (broad range, NEB). SDS-PAGE gels were run at 180 volts for 80min or until the bromophenol blue run off the gel.

2.6.6 Western Blotting

After run of protein samples on SDS-PAGE gels. Proteins were transferred on to immobilon-P PVDF membrane (Millipore) using wet transfer tank. Membranes were

soaked in 100% methanol for 1min. Two Whattmann 3MM papers per gel, the membrane and the gel were equilibrated in transfer buffer for at least 5 min. One Whatmann paper was placed on a foam based cell and the air bubbles removed. The gel was placed on the top of the paper, followed by the membrane and another two papers placed on the top. Foam was placed on top of the stack and the stack held firmly together in a case. The gel was transferred at 100 volts for 1hour at 4^{o}C. Transfer efficiency was determined by transfer of the pre-stained protein markers. After transfer, the membrane was blocked in 5% milk PBST for 1h at RT. Followed by three quick wash with PBST, then the membrane was incubated in specific antibody overnight at 4^{o}C. Next day, the membrane was washed three times for 10min each wash with PBST. Then the membrane was incubated in the appropriate secondary antibody for 1h at RT. Detection of proteins was performed by enhanced chemiluminescence (ECL, Amersham Biosciences) of membranes to X-ray film (Wilford). The exposure time depending on protein signal. Proteins of interest were identified by comparison of their size to the protein markers.

2.6.7 Quantification and Statistical Analysis

Statistical differences between different groups were analysed via single factor analysis of variance (ANOVA) followed by a Bonferroni or Dunnett' stests. Comparison between two sets of data was analysed using Paired t test or Mann-Whitney non-parametric test. A statistical test was only carried out when the experiments had at least n=3. Statistical significance was only presented when $p \leq 0.05$. Statistical analyses were performed using the Graphpad prism software.

Chapter 3: Investigation into the molecular mechanisms leading to a decrease in protein translation in response to ER stress.

3.1 Introduction

Translation, the first and most important phase of protein synthesis, involves a process by which mRNA is translated into proteins. The translation of mRNA into proteins involves five major components: ribosomes that perform the process of polypeptide synthesis, tRNA molecules that arrange amino acids in specific sequence within the mRNA template, aminoacyl-tRNA synthetases that attach amino acids to their tRNA molecules, mRNA that encode the amino acids sequence information for protein synthesis, and protein initiation, elongation and termination factors that facilitate the translation mechanism (Merrick, 2010). mRNA is exported from the nucleus and enters the cytosol as a messenger ribonucleoprotein (mRNP), which is an mRNA molecule coated with RNA binding proteins (Robertson & Branch, 1987). The mRNA can then be translated into protein and there are three main stages to this process: initiation, elongation and termination (Kapp & Lorsch, 2004).

3.1.1 Eukaryotic Translation initiation

The initiation of translation in eukaryotes is considered the most important stage in the regulation of protein translation. It is also a highly complex step which involves the recruitment of the 80S ribosome and the initiator methionyl-tRNA (Met-tRNAi) on to the start codon (AUG) of the mRNA. This process is facilitated by at least 12 protein initiation translation factors (eIFs) (Hinnebusch, 2011). The initiation phase is completed when the Met-tRNAi base pairs to the start codon (AUG) in the P site of the ribosome and is ready to begin the elongation phase of protein synthesis (Hinnebusch & Lorsch, 2012). Initiation of translation is considered the rate-limiting step of translation stage. In eukaryotes, the initiation step needs a number of eukaryotic initiation factors (eIFs), guanosine triphosphate (GTP), adenosine triphosphate (ATP), Met-tRNA. mRNA and two ribosomal subunits 40S and 60S, which bind to form the 80S ribosome (Figure 3.1).

3.1.1.1 Formation of the ternary complex

One of the critical steps during the initiation of translation is the formation of the translational ternary complex (TC) consisting of initiator methionyl-tRNA (Met-tRNAi) and the GTP-bound form of eukaryotic initiation factor 2 (eIF2). The TC binds to the (40S) ribosomal subunit to form 43S pre-initiation complex (PIC) (Kapp & Lorsch, 2004). Binding of the TC to the 40S subunit requires initiation factors such as eIF1, 1A, 5 and the eIF3 complex. The 43S PIC binds to the messenger RNA (mRNA) near the 7-methylguanosine cap in a process facilitated by eIF3, the poly(A)-binding protein (PABP), and eIFs 4B, 4H (in mammals), and 4F, a complex consisting of the cap-binding protein eIF4E, eIF4G, and the RNA helicase eIF4A. The eIF4F complex main role is to recruit other initiation factors to facilitate binding of PIC to mRNA. The eIF4G is a scaffold protein. It binds poly (A)-binding protein (PABP), eIF4E, eIF4A, and eIF3. The eIF4G function is to direct the ribosome to the mRNA toward 5' end of mRNA (Dmitriev et al., 2010). Once the pre-initiation complex (PIC) has bound near the cap; PIC start to scan the mRNA sequence for an AUG codon. Once AUG recognition is completed, PIC stops the scanning and eIF2. GTP is hydrolysed to eIF2.GDP helped by the GTPase activating protein eIF5. After that eIF2.GDP is released other eIFs found in the PIC dissociate. The large ribosomal subunit (60S) then joins to the small ribosomal subunit (40S) aided by of eIF5B to produce ribosomal subunit (80S) initiation complex (IC) which is ready to start the elongation stage in protein synthesis (Hinnebusch, 2011).

3.1.1.2 Formation of eIF4F complex

The formation of eIF4F complex is controlled by many stimuli (Gingras, Raught & Sonenberg, 1999). eIF4E is the critical factor for formation of eIF4F complex (Duncan & Hershey, 1989) and the binding of eIF4E to eIF4G is inhibited by eIF4E-binding proteins (4E-BPs). eIF4G and 4E-BPs compete for eIF4E binding and as result of binding eIF4E to 4E-BPs inhibits the formation of eIF4F complex (Haghighat, Mader, Pause & Sonenberg, 1995). Phosphorylation of 4E-BPs is considered to play a key role for its binding to eIF4E.

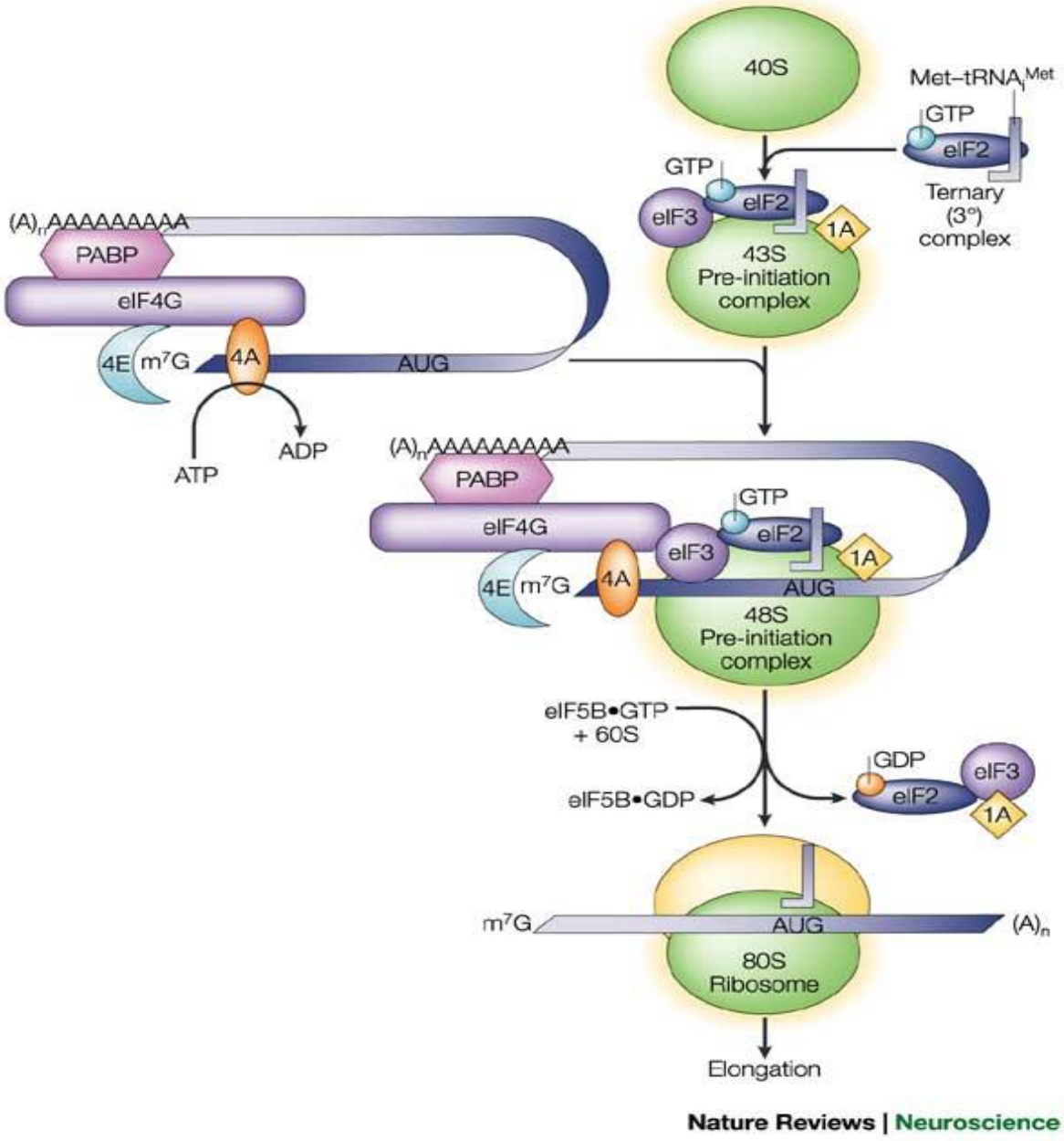

Figure 3.1 A binary complex of eukaryotic translation initiation factor 2 (eIF2) and GTP binds to methionyl–transfer RNA (Met–tRNAiMet), and the ternary complex associates with the 40S ribosomal subunit. The association of additional factors, such as eIF3 and eIF1A (1A), with the 40S subunit promotes ternary complex binding and generates a 43S pre-initiation complex. The cap-binding complex, which consists of eIF4E (4E), eIF4G and eIF4A (4A), binds to the 7-methyl-GTP (m7GTP) cap structure at the 5' end of a messenger RNA (mRNA). eIF4G also binds to the poly(A)-binding protein (PABP), thereby bridging the 5' and 3' ends of the mRNA. Following scanning of the ribosome to the AUG start codon, GTP is hydrolysed by eIF2, which triggers the dissociation of factors from the 48S complex and allows the eIF5B- and GTP-dependent binding of the large, 60S ribosomal subunit.

The affinity of eIF4E for 4EBPs is decreased by 4E-BPs phosphorylation which leads to eIF4E free to bind eIF4G (Proud, 2007). The binding of eIF4E to eIF4G is regulated by interactions with the translational repressor proteins, 4E-BPs, which prevents eIF4E for release under selected physiological conditions, thus allowing the 4E and 4G interaction and subsequent initiation of translation (Friedland, Wooten, LaVoy, Hagedorn & Goss, 2005).

The phosphorylation of eIF4E may also influence translation as it decreases its affinity for the cap structure, this may be due to the repulsion force generated between the two negative charge of phosphate group and mRNA (Scheper, van Kollenburg, Hu, Luo, Goss & Proud, 2002).

3.1.2 The untranslated regions of mRNA

In eukaryotes, the untranslated regions of mRNA have been shown to be essential in the regulation of protein synthesis , mRNA contains proximal 5' leader coding structures that are required for recruiting the translation initiation machinery as well as regulation of translation, and also has distal 3' noncoding portion that can play a role in regulation of polyadenylation, translocation, localization of mRNA and translation efficiency (Jackson, Hellen & Pestova, 2010). The 5' leaders can regulate downstream expression through upstream open reading frames (uORF), these 5' leaders structures act as codes so that ribosomes can recognize which transcripts are to be repressed or preferentially translated (Dever et al., 1993). Under stress condition certain mRNAs are preferentially translated when eIF2 is phosphorylated such as the mRNAs encoding general control non-depressible 4 (GCN4) in yeast, which is translated in response to deprivation of amino acids and other stress conditions (Hinnebusch & Lorsch, 2012).

3.1.3 Internal ribosome entry site (IRES)

Eukaryotic cells apply different mechanisms to initiate translation of their mRNAs. The cap-dependent initiation mode is responsible for 95–97% of all translation initiation in eukaryotic cells (Komar & Hatzoglou, 2005) .

However, some viral and eukaryotic cellular mRNA are translated using a cap-independent mechanism such as an IRES mediated mechanism by which the 40S ribosomal subunit is directed to a site 3' of the 5'end often by specific mRNA tertiary structures termed internal ribosome entry sites (IRESs) (Jackson, Hellen & Pestova, 2010). Poliovirus and encephalomyocarditis virus (EMCV) were the first biological systems found to translate their mRNA by the internal ribosome entry mechanism (Pelletier & Sonenberg, 1988). Later on many other virus families were also found to use this mechanism for their mRNA translation (Vagner, Galy & Pyronnet, 2001). IRES translation initiation mechanism is believed to be applied only under condition when cellular cap-dependent initiation mechanism is unavailable such as in case of cell stress (Jackson, Hellen & Pestova, 2010) .

It was found that IRES-dependent translation requires a variable number of translation initiation factors depending on the particular IRES (Hellen, 2009). For instant, the hepatitis C virus (HCV) IRES does not require any of the initiation factors of the eIF4 family (Pestova, Shatsky, Fletcher, Jackson & Hellen, 1998) and the cricket paralysis virus (CrPV) IRES is translated without the requirement for any of the canonical initiation factors including eIF2 (Jackson, Hellen & Pestova, 2010).

3.1.4 Translation control under ER stress

ER plays a central role in protein synthesis. Translation of new proteins take place on ribosomes associated with the ER. Newly synthesised membrane or secretory proteins are then folded and modified in the ER lumen (Harding & Ron, 2002). Under ER stress condition protein folding is disrupted which lead to the accumulation of unfolded proteins resulting in activation of unfolded protein response (UPR). In reaction to ER stress, UPR is intended to restore ER homeostasis through decreasing ER load, increasing ER folding capacity and increasing ER associated degradation. This decrease in ER load is classically initiated by the activation of the ER-transmembrane protein PERK, which phosphorylates the translation initiation factor eIF2α, resulting in a decrease in global protein synthesis (Back & Kaufman, 2012).

It is believed that the inhibition of protein synthesis in response to ER stress gives ER more time to qualifying the loading proteins and correctly folded. Moreover, the suppression of translation preserves energy since the translation of mRNA at ribosome highly consuming energy process (Evans-Molina, Hatanaka & Mirmira, 2013). During

the ER stress, the kinase PERK phosphorylates eIF2α which leads to translation attenuation to overcome ER stress. However, a specific mRNAs escape this translational inhibitory mechanism such as ATF4 mRNA and CHOP mRNA. Phosphorylation of eIF2α has dual action during ER stress which inhibits the general mRNA translation and promoting selective translation of specific stress responsive mRNA. (Harding et al., 2000).

We speculated that additional mechanisms/factors may be involved in repression of protein synthesis in response to ER stress. To investigate this, we exploit the differences in the dependency of viral IRES for translation initiation factors to identify which initiation factors are affected by ER stress and thus may be important in the ER stress response.

3.1.5 Aim

To investigate the molecular mechanisms of translational repression in response to ER stress in MIN6 cells.

3.2 Results

3.2.1 Bicistronic constructs initiation factors requirement for translation

In order to identify which initiation factors are responsible for the inhibition of protein synthesis in response to ER stress, we used a series of bicistronic constructs in which the translation of the upstream cistron is controlled by a cap dependent mechanism whereas the downstream cistron (cap-independent) is regulated by specific viral IRES. These bicistronic constructs have different requirements to initiate translation via their IRES. The Encephalomyocarditis (EMCV) IRES can direct translation independently of eIE4E, whereas the cricket paralysis virurs (CrPV) IRES requires no initiation factors, and directly recruits ribosomal subunits. The hepatitis C virus (HCV) IRES requires all the initiation factors except the eIF4E/4B and 4A (Meijer et al., 2013). The Renilla/firefly plasmid (pRF) which translated through cap-dependent only and has no IRES and was used as control.

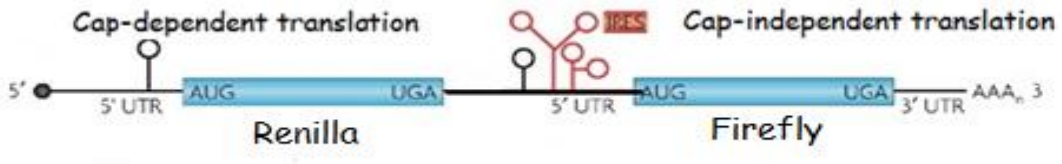

Figure3.2 A simplified diagram of bicistronic construct with internal ribosomal entry (IRES).

Bicistronic construct name	Initiation factors not required for translation of firefly (downstream cistron)
Encephalomyocarditis virus (pEMCV)	eIF4E
Hepatitis C virus (pHCV)	eIF4E, eIF4A and eIF4B
Cricket paralysis virus (pCrPV)	Does not require any initiation factors
Renilla/Firefly plasmid (pRF)	Requires all conical initiation translation factors

Table 3.1 List of bicistronic constructs with corresponding initiation factors

3.2.2 Determination of efficacy of the constructs

Initially, we set out to determine the efficacy of the constructs in investigating how protein synthesis is repressed under conditions of ER stress (Figure 3.3) by assessing: 1. the effect of ER stress on cap-dependent renilla expression (i.e. translation from the upstream cistron) and 2. The dependency of the expression of firefly (translation from the downstream cistron) on the presence of an inter-cistronic IRES. To investigate this, MIN6 cells were transfected with pRF and pEMCV. 48 h post-transfection the cells were incubated for 4 h in the presence or absence of thapsigargin and the expression renilla and firefly luciferase was determined by their activity using luminometry.

Under control conditions the activity of renilla from cells transfected with either pRF or pEMCV, were similar. However, treatment with thapsigargin caused a 20-25% decrease in renilla activity compared to untreated cells. Thus, changes in renilla activity in response to ER stress can be used as a readout of ER stress induced repression of protein synthesis (Figure 3.3a) and (Figure 3.3b). The expression of firefly in cells transfected with pRF, in either the presence or absence of thapsigargin, was negligible compared to the expression of firefly driven by the EMCV IRES in cells transfected with pEMCV (Figure 3.3c and 3.3d). Thus, the expression of the downstream cistron encoding firefly is highly dependent upon on the presence of IRES. Thus, a measure of firefly expression from different IRES with different initiation factor requirements can be used to determine the role of specific initiation factors in ER stress induced repression of protein synthesis.

3.2.3 Evidence that the repression of protein synthesis in response to ER stress is mediated by the inhibition of the initiation but not the elongation phase of protein synthesis

MIN6 cells were transfected with Cricket paralysis virus plasmid (pCrPV). 48h post transfection the cells were incubated for 4h in the presence or absence of thapsigargin and the activity of renilla and firefly determined by luminometry (Figure 3.4). The activity of renilla from cells transfected with pCrPV, under control conditions, was

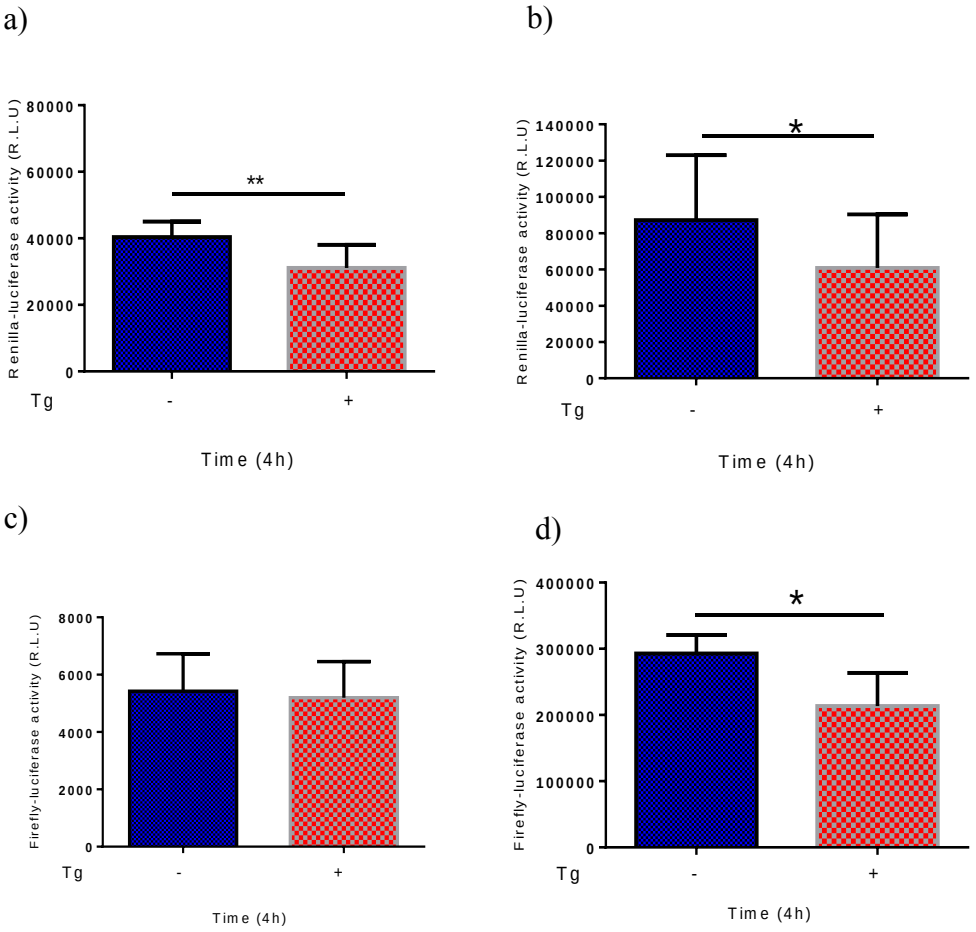

Figure 3.3 Characterisation of the reporter constructs. MIN6 cells were transfected with pRF (Renilla/Firefly plasmid) (a,c). Another set of cells were transfected with pEMCV (Encephalomyocarditis virus plasmid) (b,d) by using Lipofectamine mediated transfection method for 48h, all the cells except the control were incubated with thapsigargin (Tg,1µM) for 4 h under standard condition (37 ℃ and 5% CO_2). The luciferase activity of firefly and renilla was measured by using Dual-luciferase reporter assay system as Relative Light Unit (R.L.U).The results are ± s.e.m of n≥3 experiments, data were analysed by using Paired t test ,* $P < 0.05$.**$P<0.01$.

with pCrPV, under control conditions, was (approx.). 15,000 RLU. The addition of thapsigargin caused a 30% decrease in renilla activity, readout of ER stress induced repression of protein synthesis (Figure 3.4a). The expression of firefly in cells transfected with pCrPV, in the presence or absence of thapsigargin was unaffected and thus is able to overcome or bypass the effect of ER stress on protein synthesis repression (Figure 3.4b). In addition, the firefly/renilla ratio significantly increased in the presence of thapsigargin (Figure 3.4 c). As a control the effect of thapsigargin inhibition of protein synthesis was also determined by measuring ^{35}S-Methionine incorporation into protein (Figure 3.4 d). As the translation of CrPV IRES bicistronic construct is independent of all initiation factors, this provides evidence that the ER stress induced repression of protein synthesis is likely caused by the repression of initiation through modulation of one or more initiation factors but not through the inhibition of elongation phase of protein synthesis.

3.2.4 Evidence that the repression of protein synthesis in response to ER stress occurs independently of the cap binding complex

To identify which translation initiation factors are required for translational repression in response to ER stress we determined: 1. the effect of ER stress on cap-dependent renilla expression compared to the effect of ER stress on EMCV IRES driven firefly expression which is known to occur independetly of eIF4E 2. the dependency of the expression of the downstream cistron encoding firefly on the presence of an inter-cistronic IRES. MIN6 cells were transfected with pEMCV. 48 h post transfection the cells were incubated for 4 h in the presence or absence of thapsigargin and the activity of renilla and firefly determined by luminometry (Figure 3.5). Under control conditions the activity of renilla from cells transfected with pEMCV, under control conditions, was (approx. 90,000RLU). Upon the addition of thapsigargin there was a 20-25% decrease in renilla activity compared to untreated cells , and thus readout of ER stress induced repression of protein synthesis (Figure 3.5a). Similarly, thapsigargin caused a 30% decrease in firefly luciferase activity compared to unteated cells (Figure 3.5b). Thus there was no significant change in the firefly/renilla ratio demonstrating that both cap-dependent and EMCV IRES dependent translation are equally represssed in response to

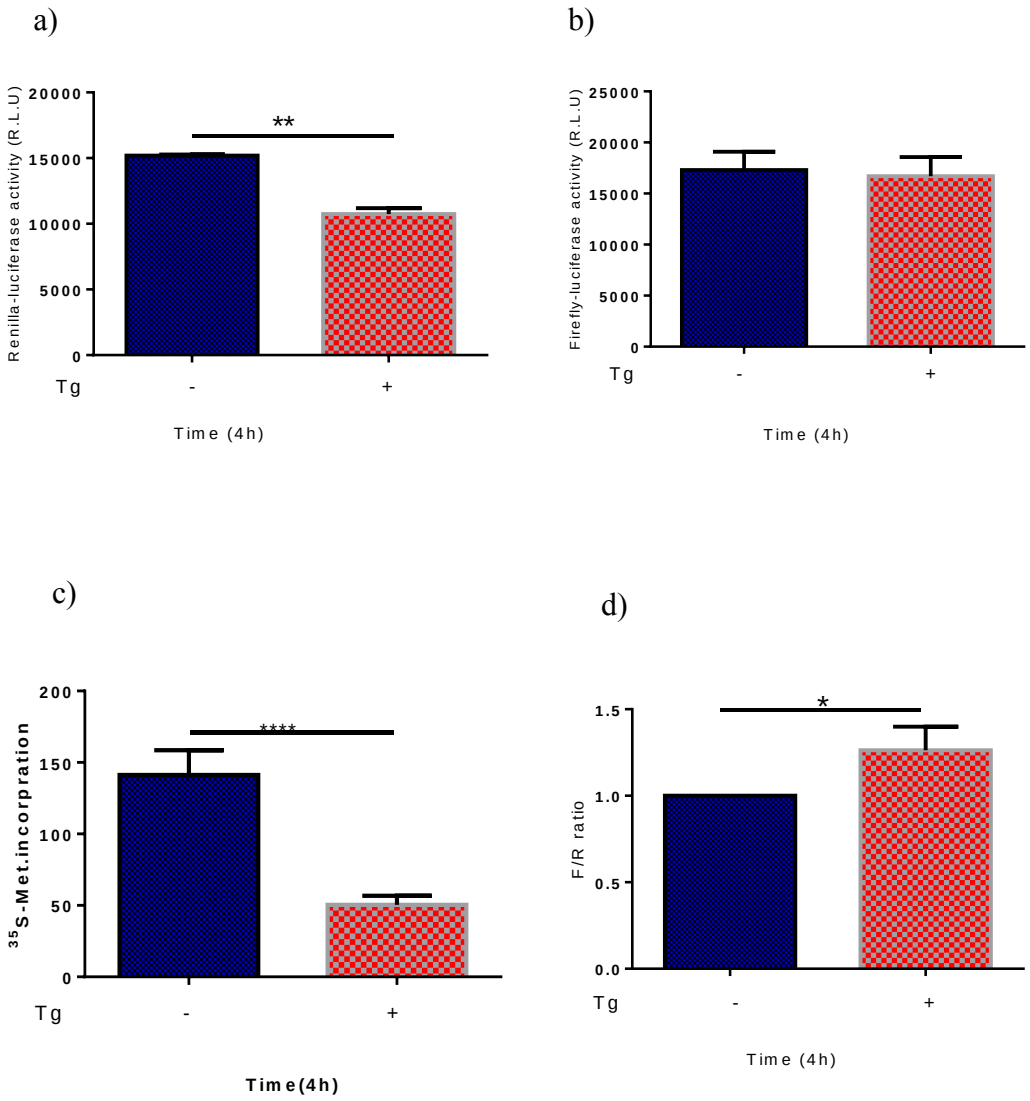

Figure 3.4 ER stress induced inhibition of protein synthesis is independent on initiation factors. MIN6 cells were transfected with pCrPV (Cricket paralysis virus plasmid) by using Lipofectamine mediated transfection for 48 h, all cells treated with thapsigargin (Tg,1µM) except control, [^{35}S]-Methionine was added to all cells include control and then incubated for 4 h under standard condition (37℃ and 5% CO_2). The luciferase activity of firefly and renilla was measured by using Dual-luciferase reporter assay system as Relative Light Unit (R.L.U) (a,b).The F/R ratio was checked (c). Total protein synthesis was determined by measuring TCA perceptible count (d). The results are ± s.e.m of n≥3 experiments, data were analysed by using Paired t test, * $P < 0.05$, **$P<0.01$. For F/R ratio data was analysed by using Mann-Whitney non-parametric test.

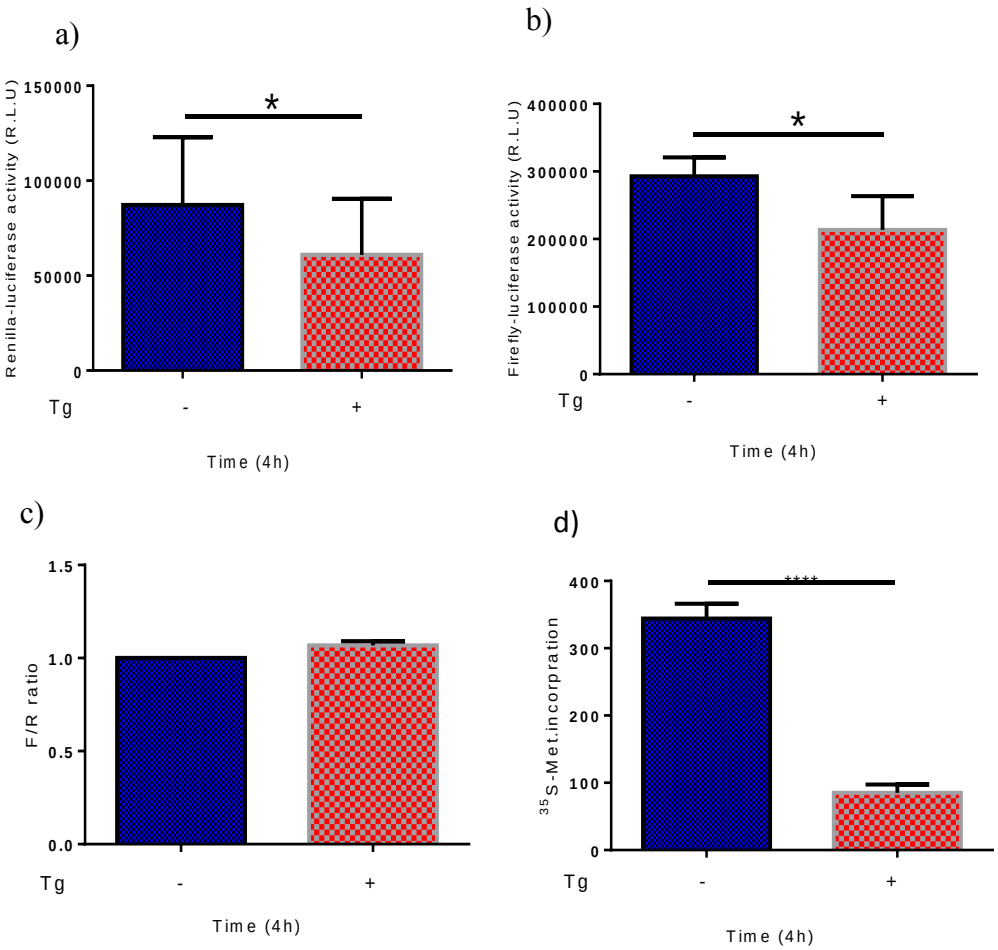

Figure 3.5 ER stress induced inhibition of protein synthesis is not dependent on eIF4E. MIN6 cells were transfected with pEMCV (Encephalomyocarditis virus palsmid) by using Lipofectamine mediated transfection method for 48 h, all cells treated with thapsigargin (Tg,1µM) except the control, and [^{35}S]-Methionine was added to all cells include control cells and then incubated for 4 h under standard condition (37 ℃ and 5% CO_2). The luciferase activity of firefly and renilla was measured by using Dual-luciferase reporter assay system as Relative Light Unit (R.L.U) (a,b). The F/R ratio was checked (c). Total protein synthesis was determined by measuring TCA perceptible count (d). The results are ± s.e.m of n≥3 experiments, data were analysed by using Paired t test, * $P < 0.05$, ****$P<0.0001$. For F/R ratio data was analysed by using Mann-Whitney non-parametric test.

ER stress (Figure 3.5c). Therefore, ER stress induced repression of protein synthesis must occurs independetly of eIF4E. As a control the inhibition of protein synthesis in cells treated with thapsigargin was also determined by measuring ^{35}S-Methionine incorporation into protein (Figure 3.5d).

3.2.5 Evidence that the repression of the initaion off protein synthesis in response to ER stress occurs independently of the cap binding complex or the RNA helicase eIF4A

MIN6 cells were transfected with pHCV and 48 h post transfection, the cells were incubated for 4 h in the presence or absence of thapsigargin and the activity of renilla and firefly determined by luminometry (Figure 3.6). The activity of renilla from cells transfected with HCV, under control conditions, was (approx. 27,000RLU).The addition of thapsigargin caused a 25-30% decrease in renilla activity, a readout of ER stress induced repression of protein synthesis (Figure 3.6a) and a 35-40 % decrease in the expression of firefly driven by the HCV IRES (Figure 3.6b). The firefly/renilla ratio showed no significant changes which demonstrates that both upstream and downstream translation are similarly inhibited by thapsigargin (Figure 3.6c). As the translation from the HCV IRES is independent of eIF4E/4B/4A, this provides evidence that ER stress induced repression of protein synthesis is independent of eIF4E, eIF4B and eIF4A. As control the rate of protein synthesis was determined in cells treated with thapsigargin was measuring ^{35}S-Methionine incorporation into protein (Figure 3.6d). Together these results demonstrate that the repression of protein synthesis in response to ER stress is independent of rates of elongation but dependent on changes in the rate of initiation and more specifically changes in an initiation factors required for EMCV and HCV IRES mediated translation. One likely initiation factor is eIF2α which is known to be phosphorylated by PERK in response to ER stress resulting in the repression of protein synthesis.

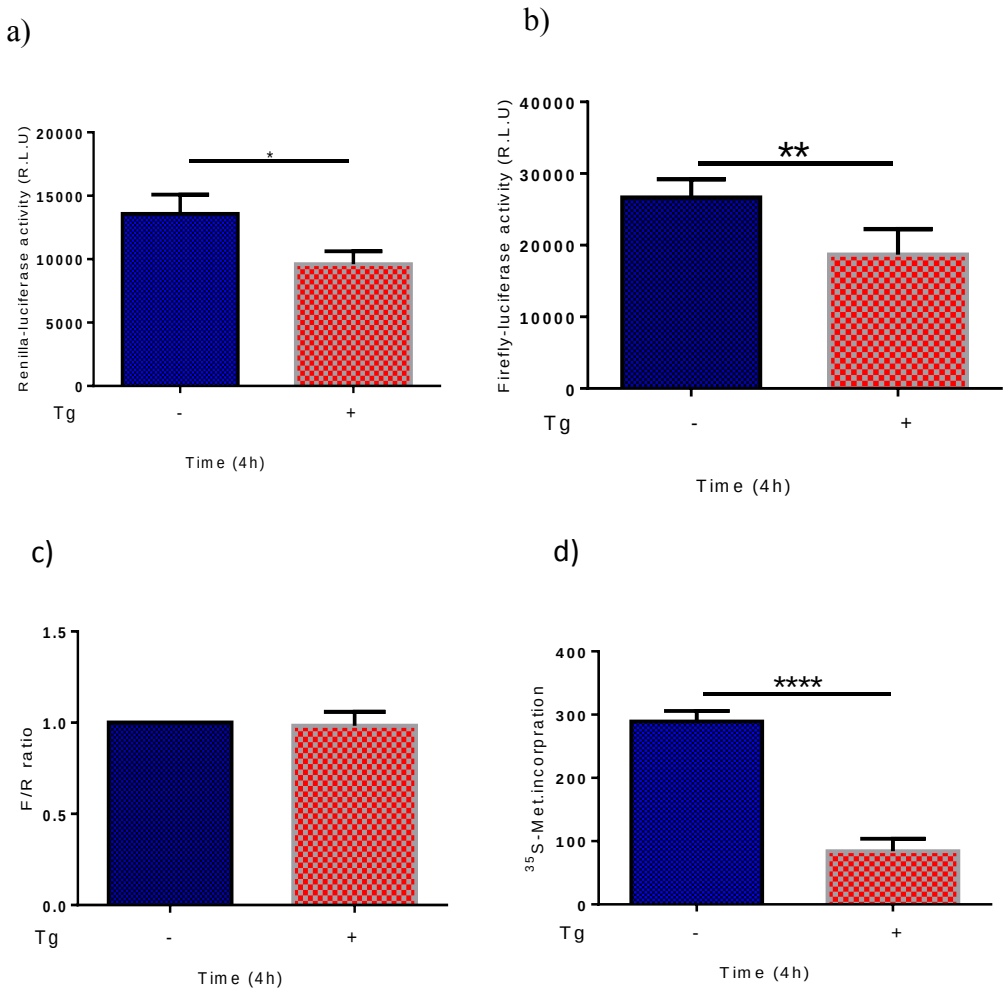

Figure 3.6 ER **stress induced inhibition of protein synthesis is independent of eIF4E/4B/4A.**MIN6 cells were transfected with HCV (Hepatitis C virus plasmid) by using Lipofectamine mediated transfection method for 48 h, all the cells treated with thapsigargin (Tg,1µM) except control, [^{35}S]-Methionine was added to all cells include the control and then incubated for 4 h under standard condition (37 ℃ and 5% CO_2). The luciferase activity of firefly and renilla was measured by using Dual-luciferase reporter assay system as Relative Light Unit (R.L. U) (a,b).The F/R ratio was checked (c). Total protein synthesis was determined by measuring TCA perceptible count (d). The results are ± S.E.M of n≥3 experiments, data were analysed by using Paired t test, * P < 0.05, **P<0.01, ****P<0.0001. For F/R ratio data was analysed by using Mann-Whitney non-parametric test.

3.2.6 Inhibition of PERK leads to restore of protein synthesis in response to ER stress in MIN6 cells.

To investigate the role of PERK dependent eIF2α phosphorylation in the repression of protein synthesis in response to ER stress. MIN6 cells were either treated or untreated with thapsigargin in the presence or absence of PERK inhibitor (GSK2656157) and the rate of total protein synthesis determined by measuring ^{35}S-Methionine incorporation into protein. The results show that upon the addition of thapsigargin for 4 h protein synthesis is inhibited by about 70%. However, in the presence of the PERK inhibitor recovery of protein synthesis was around 100%. We can therefore conclude that the PERK is responsible for the repression of global protein synthesis via its phosphorylation of the α subunit of eIF2 (Figure 3.8). After showing that the PERK/eIF2α pathway might be the main pathway that is responsible for protein repression in response to ER stress at 4 h (Figure 3.8). We investigated the role of PERK/eIF2α pathway in the repression of protein synthesis in response to ER stress after 12 h and 24 h of thapsigargin treatment. MIN6 cells were treated with thapsigargin in the presence or absence of the PERK inhibitor (GSK2656157). Total protein synthesis was determined by measuring ^{35}S-Methaninone incorporation into protein. In addition, the viability of cells was measured by monitoring the live to dead cells at 12 h and 24 h (Figure 3.9c). In the presence of thapsigargin protein synthesis was inhibited about 70% at both 12 h and 24 h. However, in the presence of the PERK inhibitor protein synthesis was restored to near control levels (i.e. 96% recovery at 12 h (Figure 3.9a), and around 84% recovery at 24 h (Figure 3.9b). At 12 h in the presence of thapsigargin the cells viability reduced around 20%, in the presence of PERK inhibitor reduced about 18%,whereas at 24 h of incubating with thapsigargin or PERK inhibitor the cells viability were inhibited about 40% (Figure 3.9c). These decreases in number of live cells due to long exposure to thapsigargin. These results indicated that PERK is likely responsible for repression of global protein synthesis via the phosphorylation of the α subunit of eIF2 at 12 h and 24 h (Figure 3.9).

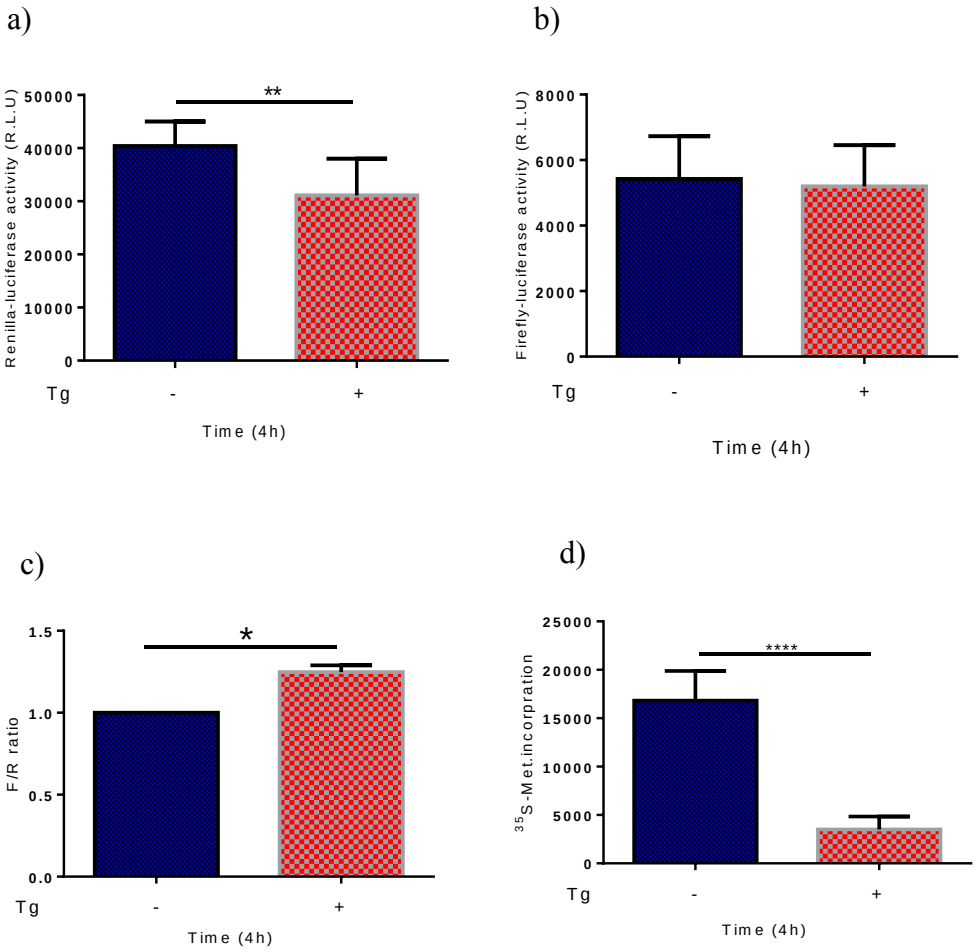

Figure 3.7 ER stress induced inhibition of protein synthesis is cap-dependent only and is not mediated through IRES. MIN6 cells were transfected with pRF (Renilla/Firefly plasmid) by using Lipofectamine mediated transfection for 48 h. The cells either treated or untreated with thapsigargin (Tg,1µM), [^{35}S]-Methionine was added to all cells include control cells and then incubated for 4 h under standard condition (37 ℃ and 5% CO_2). The luciferase activity of firefly and renilla was measured by using Dual-luciferase reporter assay system as Relative Light Unit (R.L.U) (a,b).The F/R ratio was checked (c). Total protein synthesis was determined by measuring TCA perceptible count (d). The results are ± s.e.m of n=3 experiments, data were analysed by using Paired t test, * P < 0.05, **P<0.01, ****P<0.001. For F/R ratio data was analysed by using Mann-Whitney non-parametric test.

3.2.7 ER stress induced inhibition of protein synthesis is cap-dependent only but not mediated through IRES

Next, as control, MIN6 AZ cells were transfected with renilla/firefly (pRF) bicistronic. 48 h post transfection the cells were incubated for 4h in the presence or absence of thapsigargin (a classical pharmacological inducer of ER stress) and the activity of renilla and firefly determined by luminometry (Figure 3.7). The activity of renilla from cells transfected with pRF, under control conditions, was (approx. 40,000 RLU). The addition of thapsigargin caused a 25-30% decrease in renilla activity. This is a readout of ER stress induced repression of protein synthesis (Figure 3.7a). The expression of firefly in cells transfected with pRF, was as expected very low but unaffected by the addition of thapsigargin (approx. 4,000 to 5,000 RLU) (Figure 3.7b). The firefly/renilla ratio showed a significant increase in the presence of thapsigargin (Figure 3.7c). The inhibition of protein synthesis in cells treated with thapsigargin was detected by 35S-Methionine incorporation into protein (Figure 3.7d).As expected, that cap dependent translation is significantly inhibited by thapsigargin.

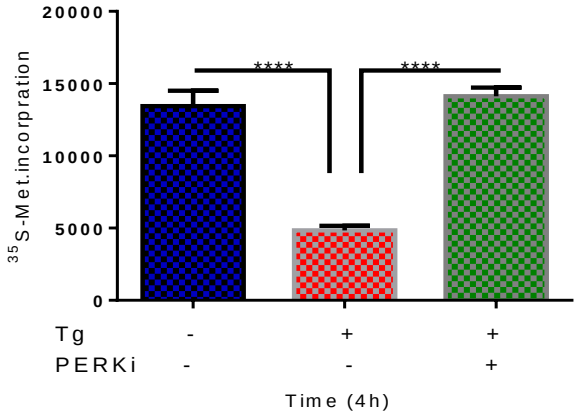

Figure 3.8 Inhibition **of PERK can restore protein synthesis in response to ER stress**. MIN6 cells were either treated or treated with thapsigargin (Tg,1µM) with or without PERK inhibitor, GSK2656157 (0.5µM added 30 min prior to thapsigargin). [^{35}S]-Methionine was posted to all cells include control and then incubated for 4 h under standard condition (37℃ and 5% CO_2). Total protein synthesis was then determined by measuring TCA perciptible count. The results are ± s.e.m of n=3, data were analysed by using one-way ANOVA and subsequently with Bonferron's test, **** $P < 0.0001$

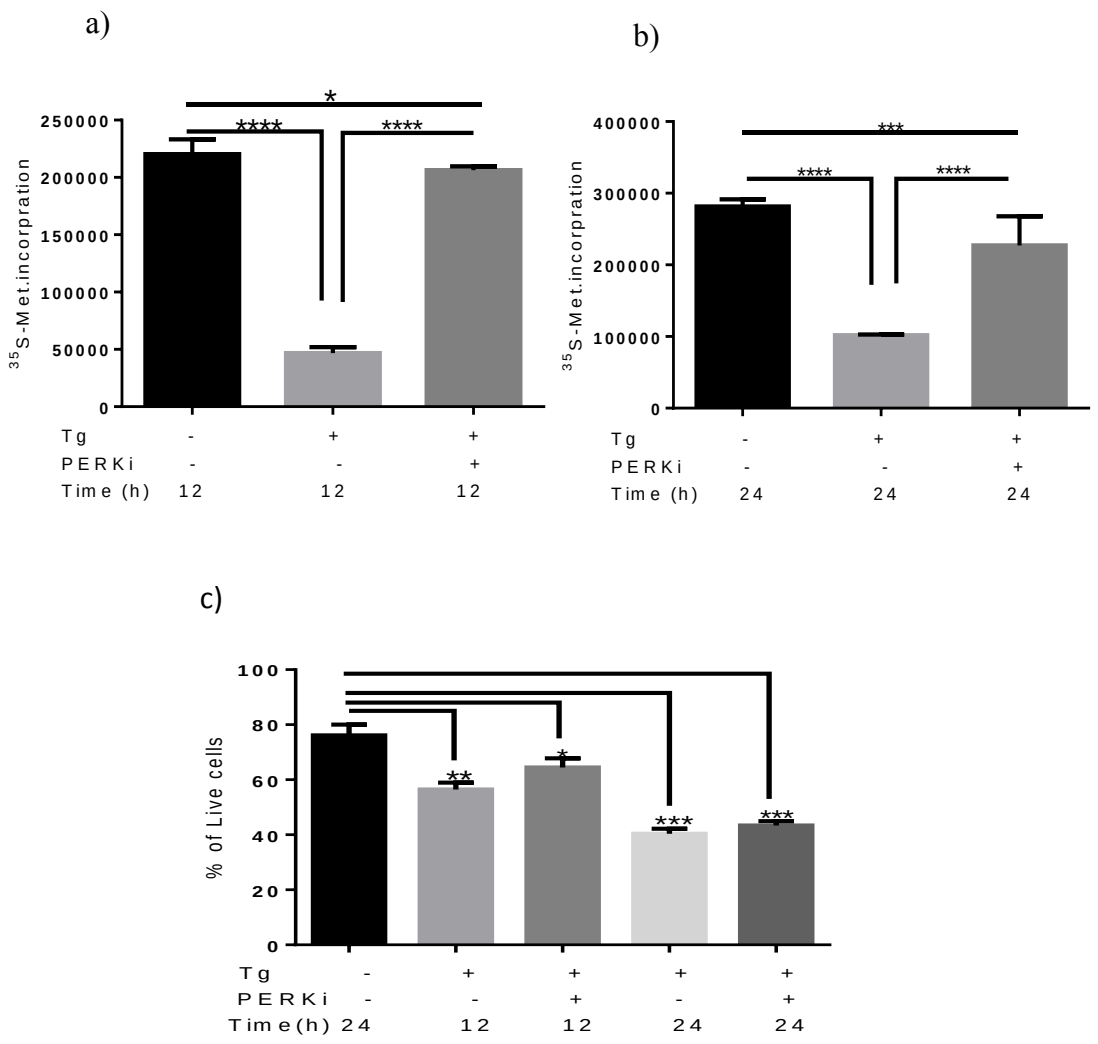

Figure 3.9 Inhibition of PERK can restore protein synthesis in thapsigargin treated MIN6 cells. MIN6 cells were either untreated or treated with thapsigargin (Tg,1µM) with or without PERK inhibitor, GSK2656157 (0.5 µM added 30 min prior to thapsigargin). Cells were then incubated for either 12 h or 24 h under standard culture conditions (37 ℃ and 5% CO_2),in the presence of [^{35}S]-Methionine ,which posted for the last 2h . Total of protein synthesis was then determined by measuring TCA perceptible counts (a, b). Cells viability was measured using a live/dead cell fluorescence assay as described in the methods (c). Data presented are the ±s.e.m, n=3. Data were analysed by one way ANOVA and subsequently with either Bonferroni's (a,b) or Dunnett's range tests (c). For * $P < 0.05$,** $P < 0.01$,*** $P < 0.001$,**** $P < 0.0001$.

3.3 Discussion

We showed that the repression in protein synthesis is independent of a group of initiation factors such as eIF4E/4A and 4B and investigated the key role of PERK arm in regulation of protein synthesis in ER stress. According to these finding we can assume that the decreased in protein synthesis in response to ER stress is likely mediated solely by the phosphorylation at eIF2α.

Ribosome binding to picornavirus IRESes such as EMCV IRES requires the complete set of initiation factors necessary for 5' end cap-dependent translation, except eIF4E. (Meijer et al., 2013; Pestova, Hellen & Shatsky, 1996). eIF4E has an important role in translation process since protein synthesis and cellular transformation in human and mouse cells are increased upon overexpression of eIF4E (Herbert et al., 2000). Under stress condition, the phosphorylation of eIF4E and increased rate of translation are not always correlated. For instance, when cells stressed by arsenite or anisomysin show an increase in eIF4E phosphorylation, however, translation rate inhibited. Indeed, the inhibition of protein synthesis could be caused by other components of the translational machinery such as eIF2α phosphorylation (Gingras, Raught & Sonenberg, 1999). In agreement with previous studies, my results show that both cap-dependent and EMCV IRES dependent translation are equally repressed in response to ER stress and there was no significant change in the firefly/renilla ratio (Figure 3.5c), which indicated that the ER stress induced repression of protein synthesis must occurs independently of eIF4E.

Regulation of eIF4E activity is through its interaction with a repressor proteins termed the eIF4E-binding proteins (4E-BPs) (Altmann, Schmitz, Berset & Trachsel, 1997). 4E-BPs inhibit cap-dependent translation, both in cell-free translation assays and when overexpressed in cells, whereas cap-independent translation is not affected (Poulin, Gingras, Olsen, Chevalier & Sonenberg, 1998). 4E-BP1 in its hypophosphorylation status inhibits the initiation of translation through the interaction with eIF4E, it prevents its binding to other partners of other initiation factor complex including eIF4G.Thus block the assembly of eIF4F complex.

I provided evidence that ER stress induced repression of protein synthesis is independent of eIF4E, eIF4B and eIF4A in HCV IRES. Since the firefly/renilla ratio showed no significant changes which demonstrates that both upstream and downstream translation are similarly inhibited by thapsigargin (Figure 3.6). It is believed that the effects of ER

stress on translation from HCV IRES is cell-type dependent. In HeLa cells, translation from the HCV IRES is sensitive to the inhibitory effect of ER stress whereas in Huh-7 cells and HEK293T it is fairly resistant. HeLa cells are equally sensitive to the inhibitory effects in response to ER stress, which are likely mediated via phosphorylation of eIF2α (MacCallum, Jack, Egan, McDermott, Elliott & Chan, 2006).

My finding are consistent with other studies that observed the degree of repression was similar in cap dependent and cap-independent translation in response to ER stress, resulting in similar IRES/Cap ratio. Moreover, using of thapsigargin shows similar suppressive effects on translational activities (Chan & Egan, 2009). It has been shown that expression of a phosphorylated eIF2α mimetic in HeLa cells repressed cap-dependent and IRES mediated translation to a similar extent (MacCallum, Jack, Egan, McDermott, Elliott & Chan, 2006) . Furthermore, HeLa cells are equally sensitive to the inhibitory effects of the envelope glycoproteins and ER stress, which are likely mediated via phosphorylation of eIF2α (Chan & Egan, 2009).

In addition, I showed that the translation of CrPV IRES was not affected by the addition of thapsigargin and able to bypass the effects of ER stress on protein synthesis. As the translation from CrPV IRES is independent of all initiation factors the repression of cap dependent/total protein synthesis is likely mediated by repression of the activity of an initiation factor (Figure 3.4). Moreover, as the efficiency of translation from the CrPV IRES is unaffected by ER stress, it is unlikely that ER stress inhibits translation elongation. Therefore, the repression of translation could be through inhibition of a number of initiation factors including eIF2α.

There is a large body of reviews suggested that in response to stresses such as accumulation of misfolded proteins in the endoplasmic reticulum leads to phosphorylation of eIF2α and resulting in the repression of global protein synthesis. It was reported that the inhibition of cellular translation is mainly caused by phosphorylation of eIF2α (Wek, Jiang & Anthony, 2006). In addition to the phosphorylation of eIF2α-dependent mechanisms for translational repression during ER stress, mTORC1 suppression activity was in parallel to the eIF2α-P mechanism. mTORC1 is involved in regulation of eIF4G, eIF4B and 4EBP1, of which 4EBP1 regulates the function of eIF4E that binds to the 5′ mRNA cap structure. Cap-binding protein eIF4E is mediated directly by mTORC1, which phosphorylates the eIF4E

inhibitors the 4EBPs. Under normal condition, hypophosphorylated 4EBP1 binds tightly to eIF4E. As 4EBP1 competes with eIF4G for binding site on eIF4E, 4EBP1 prevents eIF4G from interacting with eIF4E. However, on mTORC1 activation, hyperphosphorylated 4EBP1 dissociates from eIF4E, allowing for the recruitment of eIF4G and eIF4A to the 5′ end of an mRNA (Ma & Blenis, 2009). Then, eIF3, the small ribosomal subunit and the ternary complex are recruited to the cap, resulting in the assembly of the 48S translation preinitiation complex, ribosome scanning and translation initiation. Another mechanism of regulating protein synthesis involves eukaryotic elongation factor 2 (eEF2). Phosphorylation of eEF2 at Thr56 by eEF2 kinase (eEF2K), interferes with the binding of eEF2 to the ribosome and the translocation step during elongation (Browne & Proud, 2002).

As the repression of protein synthesis in this work was independent of eIF4E/4A and 4B initiation factors as well as elongation factors. Thus, we can conclude that the repression of protein synthesis most likely is independent of mTORC1 activation and elongation translation.

Accumulating evidence has shown the role of PERK dependent eIF2α phosphorylation in the repression of protein synthesis in response to ER stress. My data showed that there was a complete recovery of protein synthesis in the presence of the PERK inhibitor which gives us a strong evidence for the essential role of PERK in protein synthesis. This result is consistent with others finding that prove PERK is required for both the phosphorylation of eIF2α and the attenuation of translation in response to ER stress (Harding, Zhang, Bertolotti, Zeng & Ron, 2000a). There are a number of mechanisms that are involved in the repression of protein synthesis during stress conditions. It was suggested that the regulating of ternary complex formation and subsequently, global translation and protein synthesis is through the competitive inhibition of eIF2B by the phosphorylation of eIF2α (Ramaiah, Davies, Chen & Kaufman, 1994). Since, eIF2 is highly abundant with respect to eIF2B, phosphorylation of only fraction of eIF2 inhibits eIF2B and leads to block protein synthesis (Rowlands, Panniers & Henshaw, 1988). Phosphorylation of eIF2α under cellular stress leads to inhibition of eIF2B activity (Wek, Jiang & Anthony, 2006). Phosphorylated eIF2 binds tightly to the regulatory subunit of eIF2Bα, β and δ which lead to inhibit its activity. Moreover, the inhibition of phosphorylated eIF2 is prevented by deletion of eIF2Bα subunit from the complex (Wortham & Proud, 2015). Another mechanism includes the untranslated regions of mRNA, which have been shown an importance in the regulation of protein synthesis.

Indeed, specific mRNAs are selectively translated in response to stress conditions when eIF2 is phosphorylated such as mRNA for general control non-depressible 4 (GCN4) in yeast, which is translated in response to amino acid deprivation (Hinnebusch, 2014), and in mammals ATF4 mRNA is preferentially translated via eIF2α phosphorylation in response to ER stress which lead to upregulate GADD34, subsequently dephosphorylates eIF2α with aid of protein phosphatase I , result in recovery of protein synthesis.

Our data suggest that the assay system was able to determine the role of specific initiation factors in investigating how protein synthesis is repressed under conditions of ER stress in real-time based on the luciferase activity. The system has previously been used as a reporter assay in mammalian cells to monitor processing of proteins through the secretory pathway and endoplasmic reticulum monitor after treatment with the ER stress inducer thapsigargin (Browne & Proud, 2002). Indeed, the measurement of total protein synthesis using ^{35}S-Methionine incorporation as control indicated that the system we used was efficient. Since we showed about 80% decreased in total protein synthesis whereas about 30% inhibition in luciferase (Figure 3.5 and 3.6), which indicated that the inhibition was reflected the target protein that meant to be measured.

Although the firefly and renilla luciferase are evolutionarily unrelated and have different substrate specificities, it is expected that these two enzymes will have dissimilar inhibition profiles. Thus, the changes in the firefly/renilla luminescence ratio intended to be reflective of target modulation could result of the direct changes of luciferase reporter enzyme. However, the using of immunoprecipitation technique and pulldown of target protein in the presence of firefly antibody and radioactive could be used to increase the sensitivity of the test.

3.3.1 Conclusion

The data presented in this chapter is an attempt to identify which initiation factor was responsible for repression of protein synthesis in response to ER stress. We found that the eIF2α is likely responsible for the repression of protein synthesis in the presence of ER stress. Also, we showed the importance of PERK activation in repression of protein synthesis in response to ER stress.

Chapter 4: Investigation in to how ATF4 is up-regulated in response to ER stress.

4.1. Introduction

Disruption of protein folding in the endoplasmic reticulum (ER) activates the unfolded protein response (UPR) to restore ER homeostasis through activating transcriptional and translational network. At the centre of the UPR is the ER transmembrane protein PERK (PKR-like ER kinase). The activation of PERK leads to the phosphorylation of eIF2α, resulting in the repression of global protein translation by diminishing the levels of eIF2-GTP complex. However this also increases the translation of a subset of mRNAs, such as activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP) (Harding et al., 2000).

Activating transcription factor 4 (ATF4), also known as cAMP response element binding protein 2 (CREB-2), belongs to the ATF/CREB transcription factor family of basic leucine zipper domain proteins (Hai & Hartman, 2001). ATF4 can act as a transcriptional activator as well as a repressor (Harding et al., 2000). ATF4 protein consists of 351 amino acids. The organization of protein structured is essential for ATF4 dimerization, DNA binding, and stability of ATF4 in response to stress (Ameri & Harris, 2008). ATF4 belongs to the activating transcription factor family and its expression is increased in response to different stress conditions (Wei, Zhu & Liu, 2015) such as ER stress, oxidative stress and amino acid deprivation (Harding et al., 2003). Moreover, it serves as a protective gene that regulates the adaptation of cells under ER stress and oxidative stress (Ameri & Harris, 2008).

The protein expression of ATF4 is regulated by the presence of two upstream open reading frames (uORFs) located in the 5'-leader of the ATF4 mRNA that facilitates translation in response to eIF2α phosphorylation. Each one of uORFs has different role in ATF4 translation, uORF1 enhances translation of the ATF4-coding region in response to stress which acts as a positive element for translation, whereas uORF2 acts as an inhibitor element that blocks ATF4 expression in non-stressed cells (Vattem & Wek, 2004). The timing is considered an important factor for the ATF4 regulation during the

translation reinitiation, in the case of non-stressed cells the eIF2-GTP complex is copious when there is a low level of eIF2α phosphorylation, therefore ribosomes rapidly recruit next ternary complex (TC), after scanning downstream of uORF1 will start reinitiation at the next coding region which is uORF2 and then ribosomes undergo dissociate from the ATF4 mRNA which inhibits ATF4 translation. However, under stress situations the level of eIF2.GTP. Met-tRNAi complex (TC) is reduced due to phosphorylation of eIF2α which gives enough time for ribosomes to contribute to reinitiate translation and overcome the inhibitory effect of uORF2, as a result the ribosome will continue scanning and reinitiating at ATF4 start codon which leads to an increase of ATF4 translation and expression (Figure 4.1) (Lu, Harding & Ron, 2004). It is believed that the releasing factors (eRF) are essential in translation termination stage of ATF4. eRF3a plays an important role in translation termination process as the depletion of eRF3a leads to upregulation of ATF4 (Ait Ghezala et al., 2012).

The p-eIF2α/ATF4 pathway is a common downstream target of eIF2α kinases which known as integrated stress response (ISR). The ISR signalling plays an important role to elevate stress situations, such as ER stress. Genetic mutations of the eIF2α kinases lead to significant medical disorders, such as, Wolcott-Rallison syndrome, which is characterized by neonatal diabetes, degenerate of the exocrine pancreas, skeletal dysplasia, growth retardation (Harding, Zhang, Bertolotti, Zeng & Ron, 2000a). ER stress condition leads to phosphorylation of eIF2α, accumulation of misfolded proteins and inhibition of protein synthesis. Accordingly, $PERK^{-/-}$ cells are highly sensitive to agents that cause protein misfolding in ER (Harding et al., 2003). Destruction of secretory cells due to ER stress were noticed in mice and humans with PERK deficiency (Delepine, Nicolino, Barrett, Golamaully, Lathrop & Julier, 2000). In response to ER stress that induced by thapsigargin or tunicamycin there is an increase in level of caspase accumulation in $PERK^{-/-}$MEF cells wild-type MEFs (Harding et al., 2000a).

ATF4 expression is dependent on both PERK activity as well as on the phosphorylation of eIF2α in response to ER stress. ER stress failed to induce ATF4 expression in both $PERK^{-/-}$ cells and $EIF2\alpha S51A^{/S51A}$ knock-in MEFs (Harding et al., 2000; Novoa, Zeng, Harding & Ron, 2001). ATF4 knockout mice show substantial fluctuations in glucose and insulin homeostasis, pancreatic, skeletal and ocular defects (Masuoka & Townes, 2002). Elevated level of ATF4 promotes the transcription of many genes including DNA

damage-inducible gene 153 (GADD153), DNA damage-inducible gene 34 (GADD34), ATF3, and several genes important in amino acids metabolism and mitochondrial function (Ma et al., 2003). ATF4 induces GADD34 transcription which in combination with protein phosphatase 1 leads to the dephosphorylation of eIF2α and the recovery of protein synthesis (Ameri *et al*., 2008 Han; *et al*., 2013).

One of the transcriptional targets of ATF4 is CHOP, which has linked with pro-apoptotic signalling. ATF4 and CHOP can interact and activate their target genes which are involved in protein synthesis (Song, Scheuner, Ron, Pennathur & Kaufman, 2008). CHOP translation is induced by the phosphorylation of eIF2α which is driven by an uORF located in 5' leader of CHOP mRNA. CHOP has only one conserved uORF which inhibits the CHOP downstream translation in the absence of stress situation (Palam, Baird & Wek, 2011). CHOP can be upregulated through both IRE1 and ATF6 pathway but its upregulation through the PERK pathway needs selective upregulation of translation of ATF4, which consequently induce CHOP transcription (Chen, Shen & Prywes, 2002). It has been reported that CHOP over expression lead to cell apoptosis, whereas cells deficient of CHOP are resistant to ER stress induced apoptosis (Bruhat, Jousse, Carraro, Reimold, Ferrara & Fafournoux, 2000).

Recently, it has been reported that internal ribosome entry site (IRES) is involved in translational regulation of a splice variant of ATF4 in response to ER stress. It has been suggested that this splice variant could have physiological important and has been found in different tissues (Chan, Kok, Tang, Wong & Jin, 2013). Under conditions of stress such as ER stress, ATF4 expression is subject to transcriptional regulation in addition to translational regulation which both induce ATF4 expression in response to eIF2α phosphorylation (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010). It was reported that the ATF4 expression in response to different stress conditions is subjected to changes in ATF4 mRNA levels. For instance, in response to UV stress the levels of ATF4 mRNA are decreased to three fold, however, in response to ER stress ATF4 transcript levels are significantly increased (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010). In wild-type MEFs treated with thapsigargin, the ATF4 mRNA level was increased in response to ER stress, however in $eIF2^{S51A}$ knock-in MEFs this increase of ATF4 mRNA was blocked (Palam, Baird & Wek, 2011). In MEFs, ATF4 promoter activity is upregulated in

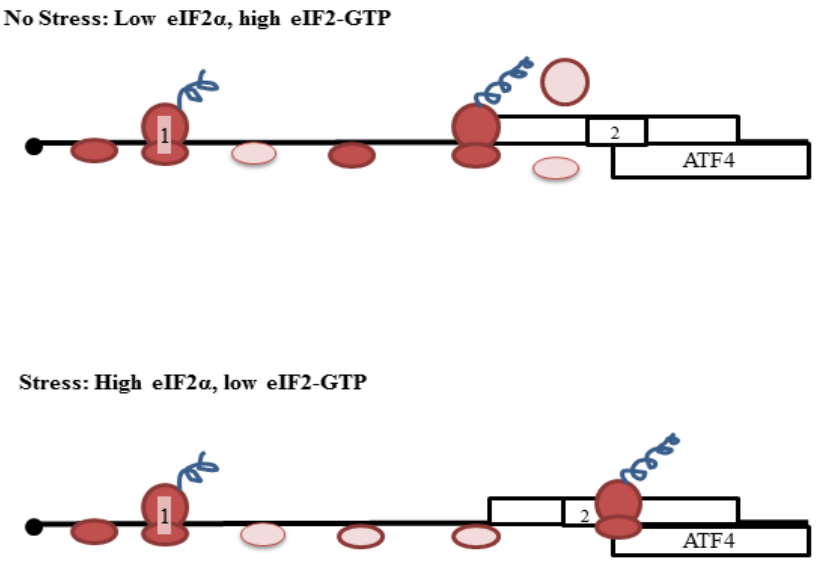

Figure 4.1 Model for ATF4 translational control by its leader sequences. The ATF4 mRNA is shown as a line with uORFs 1 and 2 and the ATF4-coding regions are illustrated as boxes. The degree of shading of the small ribosomal subunits indicates increase of being associated with the translational ternary complex (TC) (eIF2–GTP–Met-tRNA^{Meti}). In cases of no stress, the scanning ribosome readily acquired the eIF-TC and reinitiates translation at the next available uORF, i.e uORF2. The uORF2 overlaps and is out-of-frame with the coding sequence and when translated, prevents ATF4 synthesis, as shown by the dissociation of the small and large subunits after termination of uORF2 translation. During ER stress, there is an increase in p-eIF2α and low level of eIF2-GTP; ribosomes continue scanning after translation of uORF1, needs additional time to recruit another eIF2-TC. This delay in reinitiation of translation allows for ribosome to scan through uORF2 initiation codon which lead to ATF4 translation (Baird & Wek, 2012).

response to ER stress in an eIF2α phosphorylation dependent mechanism (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010).

4.1.1 Aim

The aim of this study is to determine how ATF4 protein expression is up-regulated in response to ER stress in mouse insulinoma cells (MIN6) a pancreatic β-cell line.

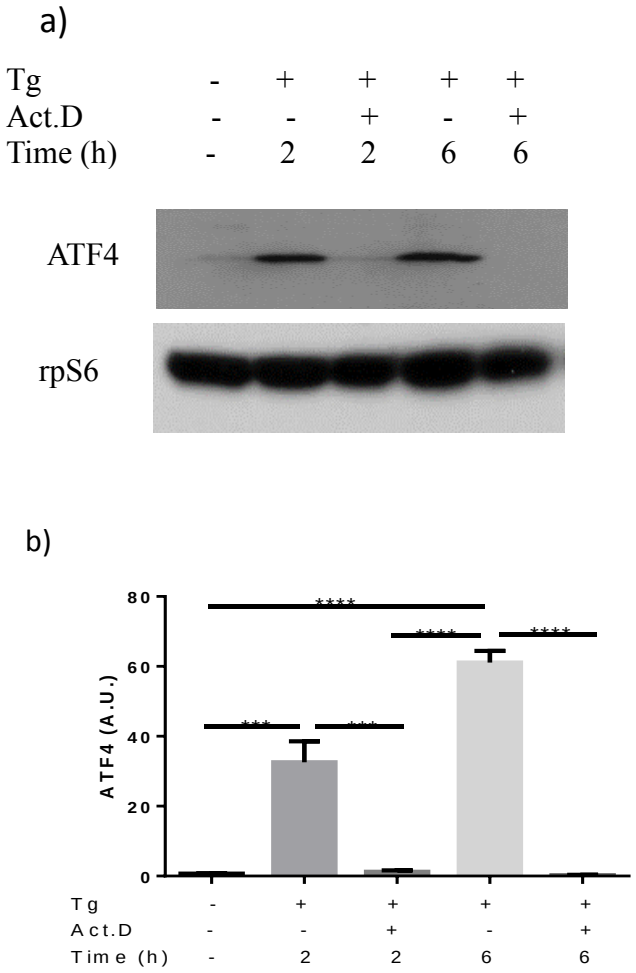

Figure 4.2 Inhibition of transcription down-regulates expression of ATF4 in MIN6 cells. Min6 cells either untreated or treated with thapsigargin (Tg,1µM) with or without actinomycin D (Act.D,1µM added 30 min prior to thapsigargin). Cells were then incubated for either 2 h or 6 h under standard culture conditions (37℃ and 5% CO_2). Changes in expression of ATF4 was detected by western blotting with rpS6 used as a loading control. a) Representative western blot. b) Densitometric analysis of ATF4 is shown in arbitrary units (A.U.). Data are representative of at least three experiments. The results are mean ± s.e.m of n=3, data were analysed by one-way ANOVA and subsequently with Bonferroni's multiple comparison tests, For *** $P < 0.001$, For **** $P < 0.0001$.

4.2 Results

4.2.1 Inhibition of transcription down-regulates expression of ATF4 in MIN6 cells.

In order to test whether ATF4 expression is dependent on induction of transcriptional regulation, MIN6 cells either untreated or treated with thapsigargin with or without actinomycin D were incubated for 2 h or 6 h (Figure 4.2). The level of expression changes of downstream of PERK pathway was determined by immunoblotting analysis. Thapsigargin led to an increase of ATF4 expression at both 2 h and 6 h (Figure 4.2a). However, ATF4 upregulation induced by thapsigargin was blocked in the presence of transcriptional inhibitor actinomycin D at both 2 h and 6 h (Figure 4.2a). The data suggest that the induction of ATF4 expression is dependent on its transcriptional regulation under ER stress.

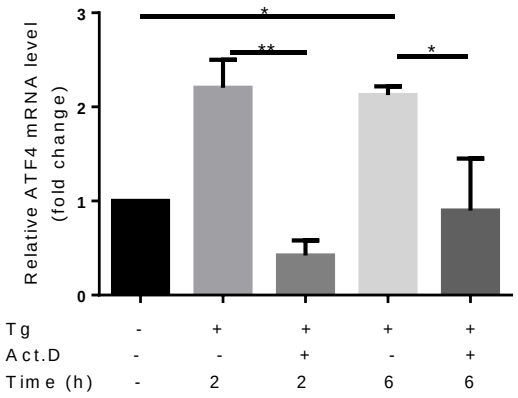

Figure 4.3 ATF4 mRNA level is inhibited by transcriptional inhibition. Min6 cells in full medium either untreated, treated with thapsigargin (Tg, 1µM) with or without actinomycin D (Act.D, 1µM added 30min prior to thapsigargin). Cells were then incubated for either 2 h or 6 h under standard culture conditions (37℃ and 5% CO_2). ATF4 mRNA was analysed using qRT-PCR and expressed as a fold change compared to untreated cells. The results are mean ± s.e.m of n=3. Data were analysed by one-way ANOVA and subsequently with Bonferroni's multiple comparisons test, For * $P < 0.05$, ** $P < 0.01$.

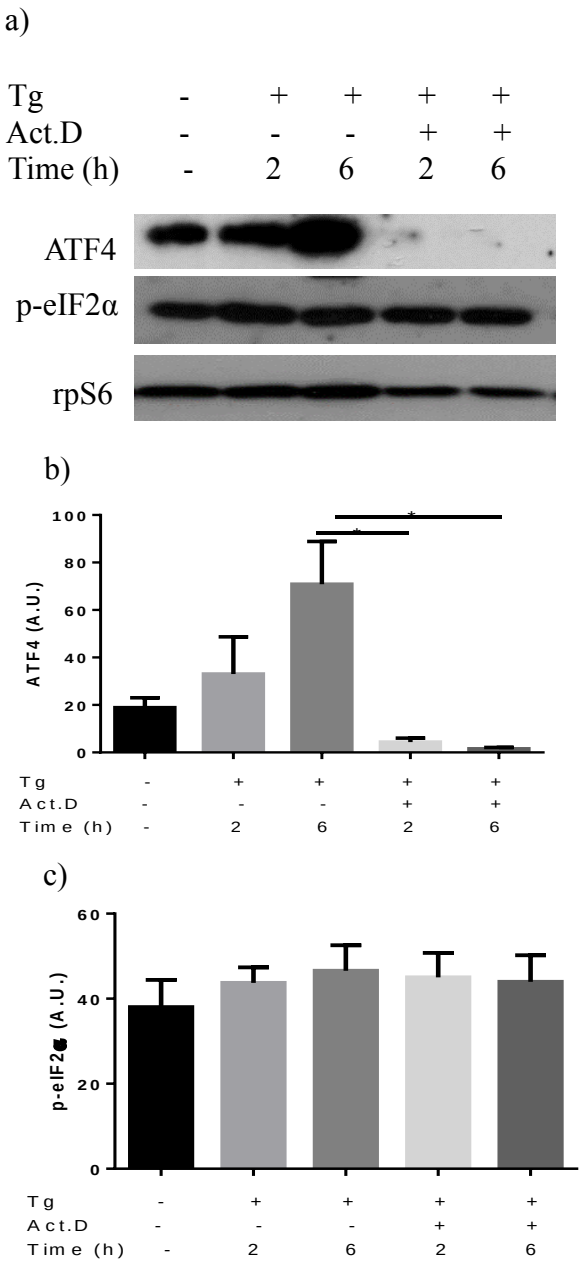

Figure 4.4 ATF4 expression is inhibited by transcriptional regulation in response to ER stress in HEK293 cells. HEK293 cells either untreated, treated with thapsigargin (Tg, 1µM) with or without actinomycin D (Act.D, 1µM added 30min prior to thapsigargin). Cells were then incubated for either 2 h or 6 h under standard culture conditions (37℃ and 5% CO_2). Changes in expression of ATF4 and phosphorylation of eIF2α (p-eIF2α) were detected by Western blotting with rpS6 was used as a loading control. a) Representative Western blot. b, c) Densitometric analysis of ATF4 and p-eIF2α are shown in arbitrary units(A.U.). Blots are representative of at least three experiments. The results are mean ± s.e.m of n=3 Data were analysed by one-way ANOVA and subsequently with either Bonferroni's (b,c) or Dunnett's multiple comparison tests, For ** $P < 0.01$.

4.2.2 Transcriptional upregulation is essential for ATF4 expression in response to ER stress in HEK293 cells

To investigate if the same was true in other cell lines, HEK293 cells either untreated or treated with thapsigargin with or without actinomycin D for either 2 h or 6 h. ATF4 expression and eIF2α phosphorylation were determined by immunoblotting. The treatment with thapsigargin led to an increase of ATF4 expression at 2 h and 6 h. However, ATF4 expression was blocked when cells were incubated with actinomycin D in the presence of thapsigargin at both 2 h and 6 h. surprisingly, the phosphorylation of eIF2α was not significantly increased upon the addition of thapsigargin (Figure 4.4). The reason for this unexpected result is unclear. These results indicate that the transcriptional upregulation of ATF4 mRNA is required for increased ATF4 expression in response to ER stress.

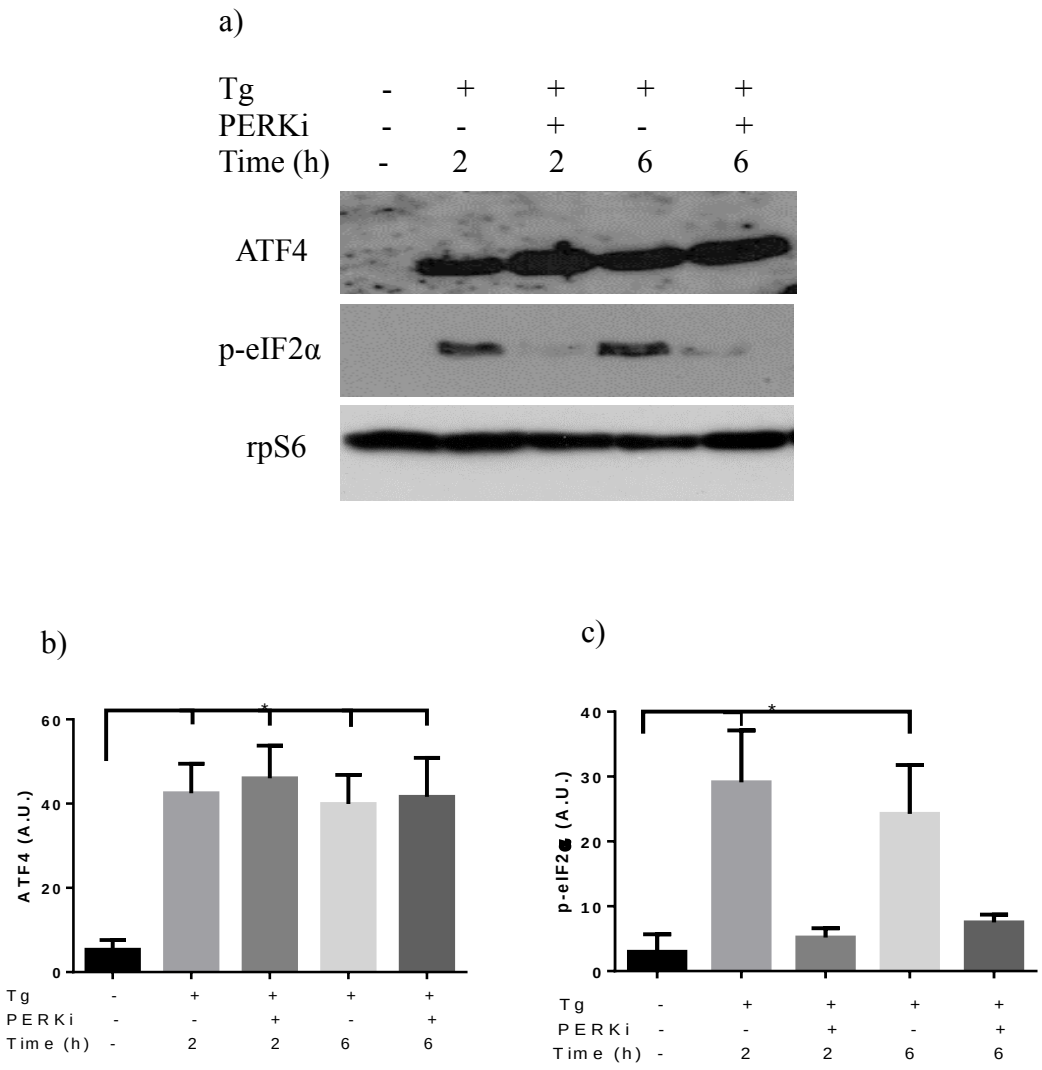

Figure 4.5 ATF4 expression is independent of PERK/eIF2α pathway in response to ER stress in MIN6 cells. Min6 cells either untreated, treated with thapsigargin (Tg,1µM) with or without PERK inhibitor, GSK2656157 (0.5µM added 30min prior to thapsigargin). Cells were then incubated for either 2 h or 6 h under standard culture conditions (37℃ and 5% CO_2). Changes in expression of ATF4 and phosphorylation p-eIF2α were detected by Western blotting with rpS6 was used as loading control. a) Representative western blot. b, c) Densitometric analysis of ATF4 and p-eIF2α are shown in arbitrary units (A.U.). Blots are representative of at least three experiments. The results are mean ± s.e.m of n=3, data were analysed by one-way ANOVA and subsequently with Bonferroni'sor Dunnett's multiple comparison test, For * $P < 0.05$.

4.2.3 ATF4 expression is independent of PERK/eIF2α pathway in response to ER stress in MIN6 cells.

To determine whether increased expression of ATF4 is dependent upon the activation of PERK, MIN6 cells were either untreated or treated with thapsigargin with or without PERK inhibitor (GSK2656157) and incubated for 2 h or 6 h (Figure 4.5). Changes in the expression of ATF4 and eIF2α phosphorylation were monitored. In these MIN6 cells thapsigargin induced expression of ATF4 was not inhibited by the PERK inhibitor at both 2 h and 6 h. However, thapsigargin induced phosphorylation of eIF2α was inhibited in the presence of PERK inhibitor. These results show that increased ATF4 expression in response to ER stress is independent of the PERK/eIF2α in these MIN6 cells.

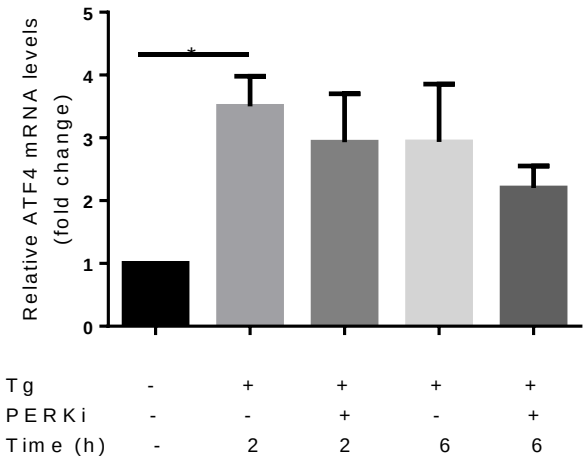

Figure 4.6 Role of the PERK/eIF2α pathway in the transcriptional upregulation of ATF4 mRNA in response to ER stress. Min6 cells were either untreated, treated with thapsigargin (Tg, 1µM) with or without PERK inhibitor, GSK2656157 (0.5 µM added 30 min prior to thapsigargin). Cells were then incubated for either 2 h or 6h under standard culture conditions (37℃ and 5% CO_2). ATF4 mRNA was analysed using qRT-PCR and expressed as a fold change compared to untreated cells. The results are mean ± s.e.m of n=3. Data were analysed by using one-way ANOVA and followed with Bonferroni's multiple comparisons test, For * $P < 0.05$.

4.2.4 Induction of ATF4 mRNA is independent PERK-eIf2α pathway in MIN6 in response to ER stress

To investigate the effect of inhibition of PERK on ATF4 mRNA level expression under ER stress, MIN6 cells either untreated or treated with thapsigargin with or without PERK inhibitor (GSK2656157) were incubated for either 2 h or 6 h. ATF4 mRNA level were measured by using qRT-PCR. Treatment with thapsigargin led to increase in ATF4 mRNA levels at 2 h and 6 h, whereas the induction of ATF4 mRNA was not significantly inhibited in the presence of PERK inhibitor (Figure 4.6). The results indicate that the induction of ATF4 mRNA in response to ER stress is not dependent upon PERK in these MIN6 cells.

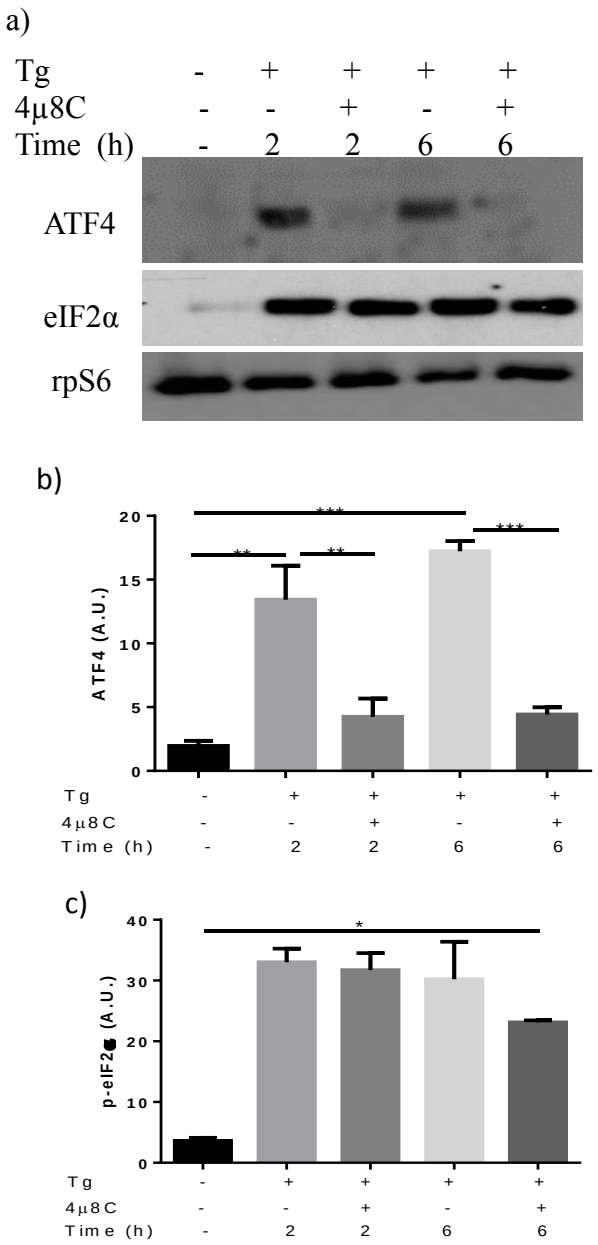

Figure 4.7 ATF4 protein expression is dependent on IRE1-XBP1 pathway in response to ER stress. MIN6 cells were either untreated, treated with thapsigargin (Tg,1µM) with or without a IRE1 inhibitor (4µ8C, 30µM added 30min prior to thapsigargin). Cells were then incubated for either 2 h or 6 h under standard culture conditions (37℃ and 5% CO_2). Changes in expression of ATF4 and phosphorylation of eIF2α (p-eIF2α) were detected by western blotting with rpS6 used as a loading. a) Representative western blot. b, c) Densitometric analysis of ATF4 and p-eIF2α are shown in arbitrary units (A.U.). Blots are representative of at least three experiments. The results are mean ± s.e.m of n=3, data were analysed by one-way ANOVA and subsequently with Bonferroni's multiple comparison tests, For * $P < 0.05$, For ** $P < 0.01$, For *** $P < 0.001$.

4.2.5 ATF4 induction is dependent on IRE1-XBP1 pathway in response to ER stress

To assess the influence of IRE1-XBP1 pathway inhibition using 4μ8C on ATF4 expression in response to ER stress, MIN6 cells were treated or untreated with thapsigargin in the presence or absence of IRE1 inhibitor, 4μ8C at 2 h and 6 h. As expected thapsigargin led to significant increase of ATF4 expression at both 2 h and 6 h. whereas, ATF4 expression is completely inhibited in the presence of IRE1 inhibitor, 4μ8C at both 2 h and 6 h (Figure 4.7a). Thapsigargin induced the phosphorylation of eIF2α and there are no significant changes in the presence of IRE1 inhibitor, 4μ8C (Figure 4.7b). This data indicate that IRE1-XBP1 pathway has an essential role in the regulation of ATF4 during ER stress in these cells (Figure 4.7).

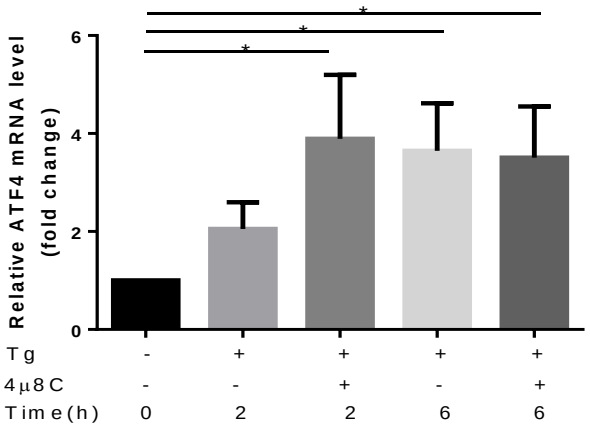

Figure 4.8 ATF4 mRNA expression is independent of IRE1-XBP1 pathway in response to ER stress of MIN6 cells. MIN6 cells in full medium either untreated, treated with thapsigargin (Tg, 1μM) with or without 4μ8C (30 μM added 30 min prior to thapsigargin). Cells were then incubated for either 2 h or 6 h under standard culture conditions (37℃ and 5% CO_2). ATF4 mRNA was analysed using qRT-PCR and expressed as a fold change compared to untreated cells. The results are mean ± s.e.m of n=3. Data were analysed by one-way ANOVA with subsequently Bonferroni's multiple comparisons test, For * $P < 0.05$.

4.2.6 Effect of IRE1-XBP1 pathway inhibition on induction of ATF4 mRNA expression

To investigate the role of IRE1-XBP1 pathway in the induction of ATF4 mRNA in response to ER stress, MIN6 cells were incubated with thapsigargin in the presence or absence of IRE1 inhibitor, 4μ8C for 2 h or 6 h. After extraction of total mRNA and cDNA synthesis, the ATF4 mRNA was measured by using qRT-PCR (Figure 4.7). Treatment with thapsigargin led to an increase of ATF4 mRNA expression around two-fold at 2 h and was significantly increase at 6 h. surprisingly, this increase in the ATF4 mRNA was not inhibited in the presence of IRE1 inhibitor, 4μ8C at 2 h or 6 h (figure 4.8). These results suggest that ER stressed induced increase in ATF4 mRNA transcription is independent of the IRE1-XBP1 pathway.

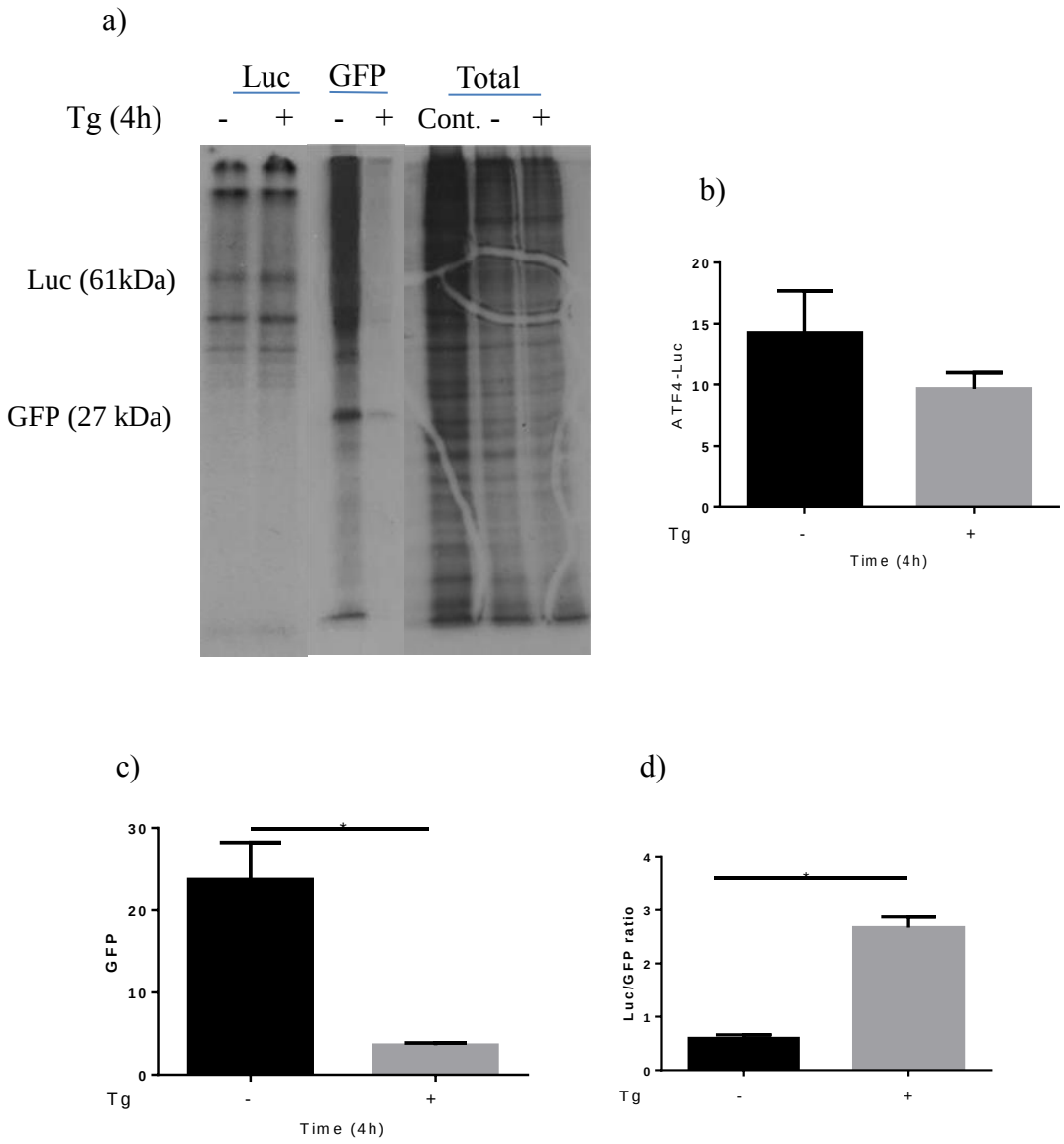

Figure 4.9 Determination of ATF4 translation regulation in response to ER stress in MIN6 cells. Min6 cells were transfected with 5'UTR-ATF4-Luc for 48h. Cells were incubated in medium without methionine or cysteine in the presence or absence of thapsigargin (Tg, 1µM). [^{35}S]-methionine was posted for last hour. Cells were incubated for 4 h under standard culture conditions (37℃ and 5% CO_2). a) Autoradiogram of SDS-PAGE of radiolabelled proteins, following immunoprecipitation of luciferase (Luc) or GFP from untreated and thapsigargin treated cells. Total lysate (Total) was included non-transfected control (Cont.) cells. b, c and d) Densitometric analysis of luciferase, GFP and Luc/GFP ratio is shown in arbitrary units. Blots shown are representative of at least three experiments. The results are mean ± s.e.m. of n=3. Data were analysed by Students paired t test, For * $P < 0.05$.

4.2.7 ATF4 translation regulation in response to ER stress in MIN6 cells

Given the lack of effect of the PERK inhibitor on ATF4 transcription in MIN6 cells I investigated whether the classical PERK/eIF2α dependent increase in ATF4 translation in response to stress is operational in MIN6 cells. MIN6 cells were transfected with 5'UTR-ATF4-Luc plasmid which encodes firefly luciferase downstream of the 5'UTR of ATF4 driven by a CMV promoter. A separate promoter drives GFP expression which can be used as a marker of transfection and translational efficiency. After 48 h of transfection, cells were labelled with radioactive isotope [^{35}S]-methionine for 4h in the presence or absence of thapsigargin. [^{35}S]-methionine labelled luciferase or GFP was immuno-precipitated using antibodies against either luciferase or GFP and the proteins separated by SDS PAGE (Figure 4.9a). After 4 h of thapsigargin treatment the expression of GFP was significantly inhibited as was total protein synthesis (Figure 4.9c). However, luciferase expression was unaffected (Figure 4.9b). The ratio of Luc/GFP indicates that in the presence of thapsigargin there was a significant increase in luciferase expression relative to GFP expression (Figure 4.9d). Therefore, the 5'UTR of ATF4 confers resistance to translational repression by ER stress in MIN6 cells. Unfortunately, the effect of PERK inhibition on ATF4 translation was not investigated in this study.

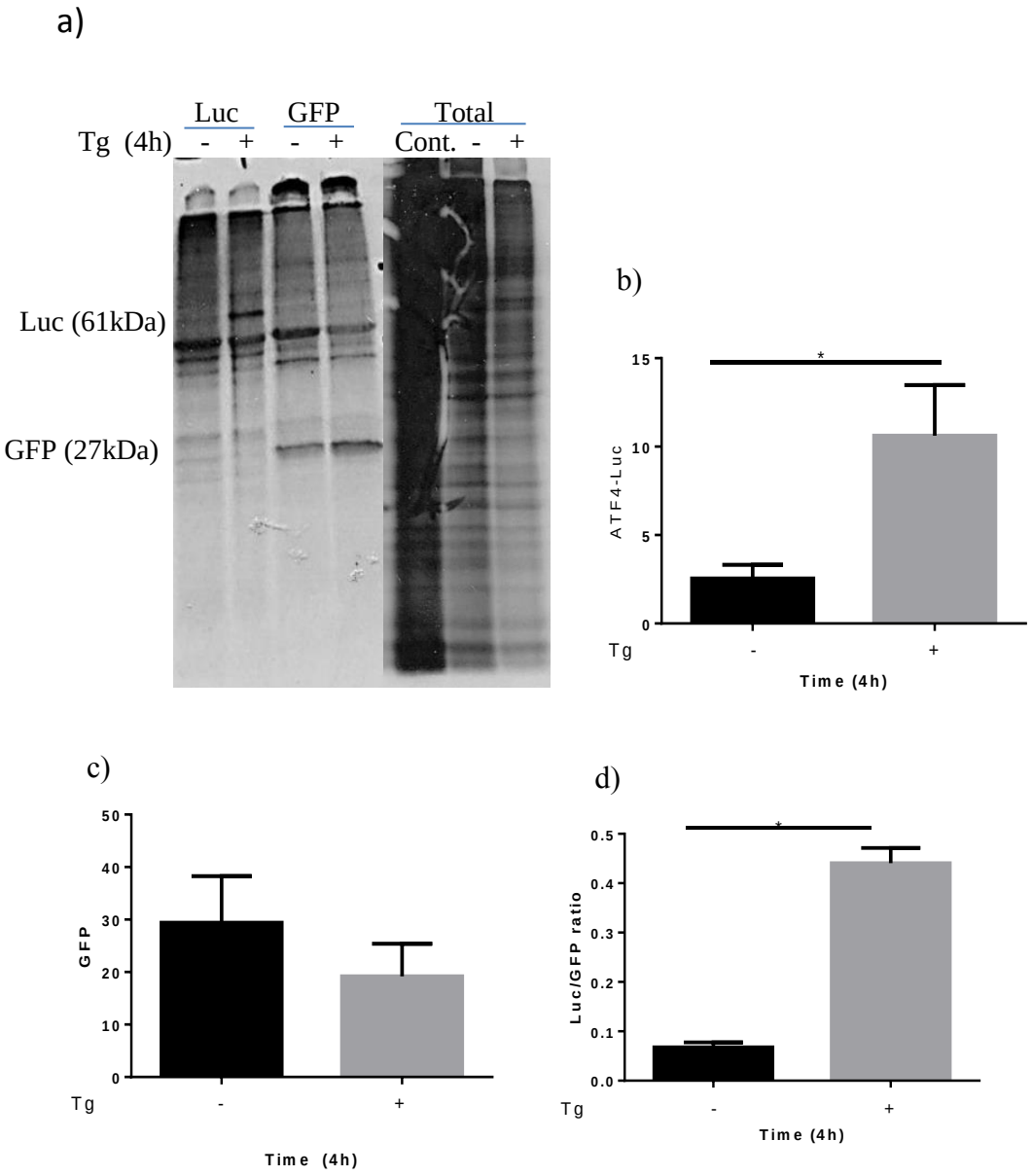

Figure 4.10 ATF4 protein translation regulations in response to ER stress in HEK293A cells: HEK293A cells were transfected with 5'UTR-ATF4-Luc for 48h. Cells were incubated in medium without methionine or cysteine in presence or absence of thapsigargin (Tg,1µM). [^{35}S]-methionine was posted for last hour. Cells were incubated for 4 h under standard culture conditions (37℃ and 5% CO_2). a) Autoradiography of SDS–PAGE of radiolabelled proteins, following immuno-precipitation of luciferase (Luc) or GFP from untreated and thapsigargin treated cells. Total lysate (Total) was included non-transfected control (Cont.) cells. b, c and d Densitometric analysis of luciferase, GFP and Luc/GFP ratio is shown in arbitrary units. Blots shown are representative of at least three experiments. The results are mean ± s.e.m of n=3. Data were analysed by Students paired t test, For * $P < 0.05$.

4.2.8 Translation upregulation is required for ATF4 expression in response to ER stress in HEK293A cells.

To test whether the ATF4-luc construct worked HEK293 cells were transfected with 5'UTR-ATF4-Luc plasmid which expresses both GFP and firefly-luciferase from separate CMV promoters. After 48 h of transfection, cells were labelled with [^{35}S]-methionine in the presence or absence of thapsigargin. [^{35}S]-methionine labelled luciferase or GFP were then immunoprecipitated using antibodies either against luciferase or GFP. Labelling proteins with [^{35}S]-methionine was detected by autoradiography of SDS–PAGE (Figure 4.10a). The presence of thapsigargin led to a significant increase of luciferase activity after 4 h (Figure 4.10b). A protein that was immunoprecipitated by luciferase antibody was detected at 61kDa (Figure 4.10a) and most likely represented luciferase protein which measuring the activity of the 5' untranslated region (5'UTR) of ATF4. Another protein which was immuno-precipitated against anti-GFP, the band was observed at 27kDa which is represented GFP expression was detected in both transfected and non-transfected cells. However, the presence of thapsigargin caused inhibition of protein synthesis by one third after 4 h (Figure 4.10c). The ratio of luciferase relative to GFP was shown significant increase in luciferase activity (Figure 4.10d). These data together indicate that ATF4 is translationally upregulated during ER stress in HEK293A cells.

I next addressed whether ATF4 translation in response to ER stress is dependent upon PERK. HEK293A cells were transfected with 5'UTR-ATF4-Luc plasmid After 48 h of transfection, cells were treated with thapsigargin in the presence or absence of PERK inhibitor and changes in GFP and firefly luciferase determined. In response to thapsigargin treatment, there was a significant increase in luciferase firefly activity compared with non-treated cells at both 12 h and 24 h (Figure 4.11b). Also, the presence of thapsigargin caused significant increase of firefly/GFP ratio at both 12 h and 24 h (Figure 4.11c). However, the incubation of the cells with PERK inhibitor led to a significant decrease in the luciferase activity at 12 h and 24 h compared to thapsigargin treated cells (Figure 4.11b). These data together indicate that PERK/eIF2α pathway play an important role in translational regulation of ATF4 in response to ER stress (Figure 4.11).

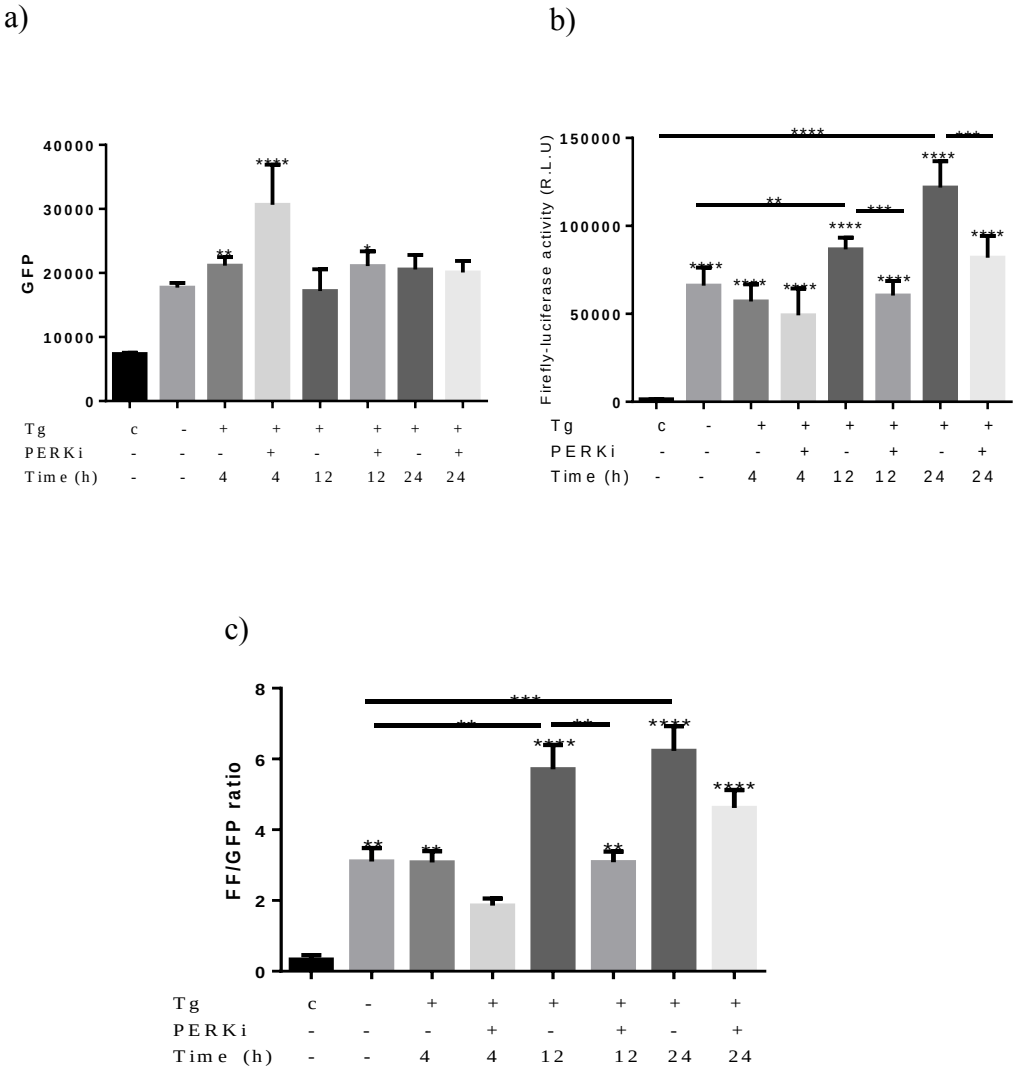

Figure 4.11 ER stress induced ATF4 translational regulation: HEK293A cells were non-transfected, control, c or transfected with 5'UTR-ATF4-Luc for 48h.Cells were either untreated or treated with thapsigargin (Tg, 1µM) with or without PERK inhibitor (PERKi), GSK2656157 (0.5 µM added 30 min prior to thapsigargin). Cells were then incubated for either 4 h,12 h or 24 h under standard culture conditions (37℃ and 5% CO_2). a) GFP was measured by CLARIO star microplate reader. b) Activity of Firefly luciferase was measured by using Dual-luciferase reporter assay system as Relative Light Unit (R.L.U). c) Firefly/GFP ratio was calculated for each condition. The results are mean ± s.e.m of n=3. Data were analysed by one-way ANOVA followed with Bonferroni's multiple comparisons test. The asterisks above the bars indicate difference from control. For * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$, **** $P < 0.0001$.

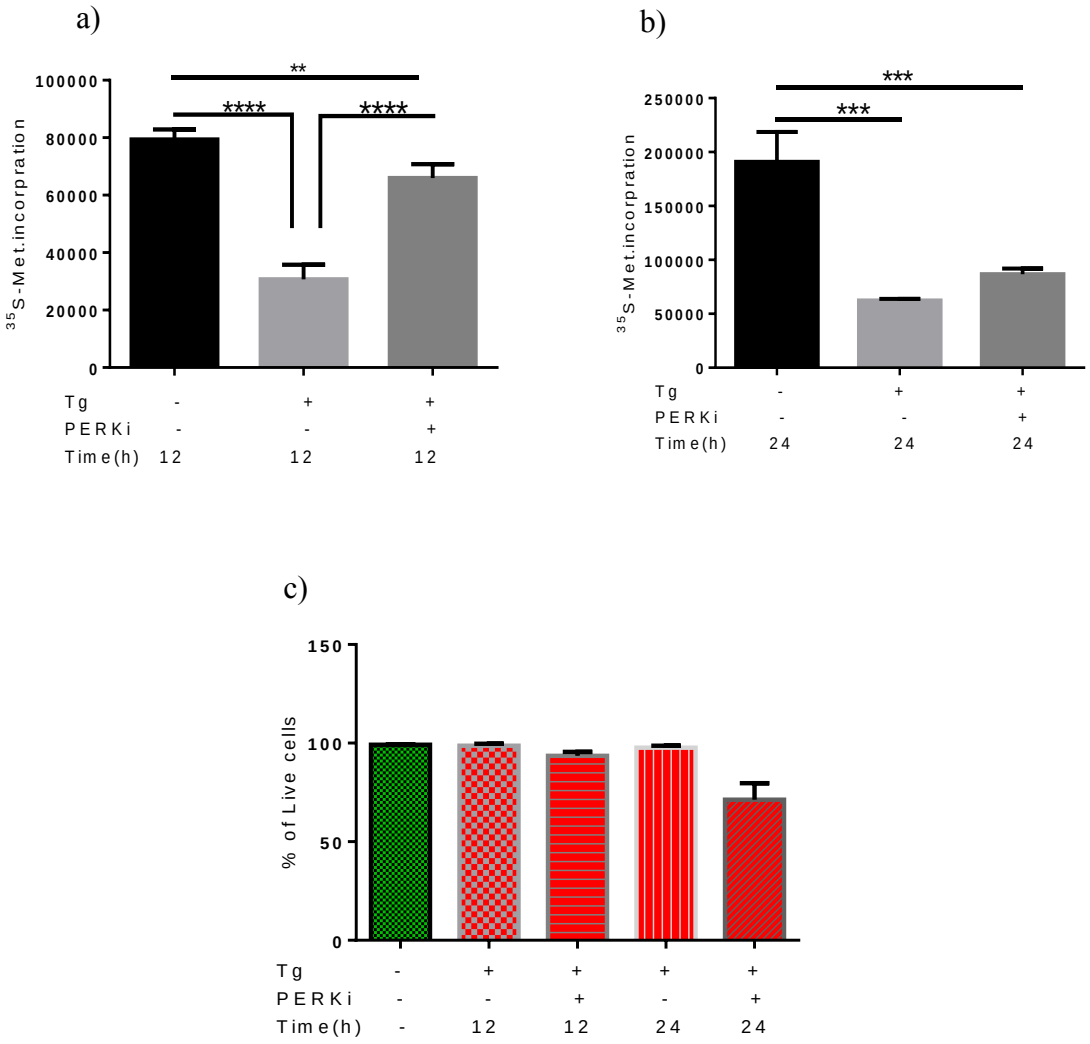

Figure 4.12 Protein syntheses can be restored with PERK inhibitor in response to ER stress in HEK293 cells. HEK293 cells were either untreated or treated with thapsigargin (Tg, 1µM) with or without PERK inhibitor, GSK2656157 (0.5 µM added 30 min prior to thapsigargin).Cells were then incubated for either 12 h or 24 h under standard culture conditions (37 ℃ and 5% CO_2), In the presence of [^{35}S]-Methionine, which posted for the last 2 h. Total of protein synthesis was then determined by measuring TCA perceptible count (a, b). Cells viability was measured using a live/dead cell fluorescence assay as described in the methods(c). Data presented are the ±s.e.m, n=3.Data were analysed by one way ANOVA and subsequently with either Bonferroni's (a,b) or Dunnett's range tests. For ** P < 0.01,*** P < 0.001,**** P < 0.0001.

HEK293 cells were also treated with thapsigargin in the presence or absence of PERK inhibitor (GSK2656157) for 12 h or 24 the rate of total protein synthesis was determined by measuring ^{35}S-Methionine incorporation into protein. The results show that upon the addition of thapsigargin for 12 h, protein synthesis is inhibited by about 65%. In cells treated with the PERK inhibitor, prior to the addition of thapsigargin, protein synthesis was recovered by around 90% (Figure 12a). 24 h of treatment with thapsigargin inhibited protein synthesis by about 75%, while in the presence of PERK inhibitor recovery of protein synthesis 45%. This could be due to increase in cells death (Figure 4.12b). To validate the integrity of tested cells we were measured the viability of cells by monitoring the live to dead cells during the experiment (Figure 4.12c). After 12 h of treatment with thapsigargin or PERK inhibitor cells viability was 100%. Treatment with thapsigargin for 24 h had no effect on cells viability whereas in presence of PERK inhibitor decreased by 20%. The incubation of HEK293 cells in the presence of thapsigargin or PERK inhibitor did not have that effect on cells viability (Figure 4.12c). These data provides evidence that PERK activation in response to thapsigargin is unlikely to be the main cause for the repression of global protein synthesis in HEK293 cells at 24 h (Figure 4.12).

4.3 Discussion

In response to ER stress, PERK phosphorylation of eIF2α leads to a reduction in global protein synthesis. This repression in translation is accompanied with preferential translation of ATF4 mRNA, encoding a transcription activator of genes that play an essential role of adaptive mechanism. ATF4 as stress responsive gene is also involved in activation of downstream transcription factors, such as CHOP/GADD153, that can induce apoptosis. In this chapter, I investigated how ATF4 expression is regulated in response to ER stress in these MIN6 cells and HEK293 cells. From my experimental results, I provide evidence that: (1) The transcriptional regulation of ATF4 is essential for its expression in response to ER stress (this is also the case in HEK293 cells) ;(2) ATF4 induced expression is not dependent on PERK/eIF2α pathway in response to ER stress in MIN6 cells unlike HEK293 cells, (3) IRE1-XBP1 pathway has an essential role in MIN6 cells in the up-regulating ATF4 expression during ER stress but not through increasing ATF4 mRNA.

4.3.1 The transcriptional upregulation of ATF4 in response to ER stress

Induction of ATF4 expression is dependent on its transcriptional upregulation under ER stress. The up-regulation of the expression of ATF4 in response to ER stress in both MIN6 cells and HEK293 cells was blocked with actinomycin D indicating that the transcriptional upregulation of ATF4 mRNA is essential for increased ATF4 protein expression (Figure 4.2, 4.3 and 4.8). It is known that ATF4 is transcriptionally up-regulated in response to ER stress (Pirot et al., 2007). However, it was not been reported that the upregulation of ATF4 mRNA is essential for the rapid increase in protein expression in response to ER stress. The elevation ATF4 transcription increases the availability of ATF4 transcript for preferential translation through eIF2α phosphorylation (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010). It has been reported that ATF4mRNA and its downstream gene target CHOP mRNA levels were blocked in response to thapsigargin induced ER stress in $eIF2^{S51A}$ knock in or $PERK^{-/-}$ MEF cells (Harding et al., 2000; Palam, Baird & Wek, 2011; Scheuner et al., 2001). It is

conceivable that, the activation of UPR leads to mediated of JNK/AP-1 pathway, and activated JNK can bind and phosphorylate c-Jun (Lizundia, Chaussepied, Huerre, Werling, Di Santo & Langsley, 2006), which increase the ATF4 protein level. It has been suggested that c-JUN is essential either to increase translation or decrease turnover of ATF4 protein in response to amino acids deprivation (Fu, Balasubramanian, Shan, Dudenhausen & Kilberg, 2011).

4.3.1.1 Role of PERK

I found that in response to ER stress the induction of ATF4 protein expression is independent of eIF2α phosphorylation in MIN6 cells (Figure 4.4). This results are supported by earlier studies from the Herbert laboratory (Zhao XC, 2013). In addition, I showed that in these MIN6 cells the induction of ATF4 mRNA is not dependent on PERK in response to ER stress (Figure 4.5). However, these results contrast with what is found in other cells / cell types or indeed primary islets. For example, it has been reported that under ER stress the expression of ATF4 and CHOP proteins were blocked in $PERK^{-/-}$ and $MEFs^{eIF2\alpha S51A}$ knock-in cells (Harding et al., 2000; Scheuner et al., 2001). It is not unexpected that different cell lines respond to ER stress differently. Indeed, I found that in wild-type MEFs, (shown in chapter 5) and in HEK 293 cells (this chapter) that thapsigargin increases the expression of ATF4 via a PERK dependent mechanism. Moreover, it has been reported that the inhibition of PERK by GSK2656157 does not reiterate the effects of genetic inactivation of PERK on eIF2α phosphorylation in stressed cells, which suggested that the PERK inhibitors induce different pathways that may interfere with eIF2α kinases activity (Krishnamoorthy, Rajesh, Mirzajani, Kesoglidou, Papadakis & Koromilas, 2014). However, subsequent experiments by members of the Herbert lab found that PERK inhibits ATF4 mRNA transcription and ATF4 expression. These conflicting results may be due to different clones of MIN6 cells that respond differently to ER stress.

During ER stress, the transcription of ATF4 is increased resulting in additional ATF4 transcript accessible for preferential translation, thus augmenting expression of ATF4. This combination of transcriptional regulation with translational control could govern which genes allow for preferential translation by phosphorylation of eIF2α to be selectively induced in response environmental stresses (Dey*et al*., 2010). It was reported

that the ATF4 mRNA levels are decreased 3-fold in response to UV stress; however, ATF4 transcript levels are significantly increased during ER stress (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010). Furthermore, it has been reported that in response to amino acid starvation the level of ATF4 mRNA is elevated (Siu, Bain, LeBlanc-Chaffin, Chen & Kilberg, 2002). Therefore, ATF4 regulation in response to ER stress could vary according to the type of cell line or even clones of the same cell line used as well as nature of the stress.

Certainly, the expression of ATF4 in the absence of eIF2α phosphorylation in Min6 cells (Figure 4.4) suggests that there is another signalling pathway that could be involved in the upregulation of ATF4 protein translation in response to ER stress. Besides elF2α, PERK can also phosphorylate nuclear erythroid factor 2 (NRF2), preventing oxidative stress by induction of antioxidant genes (Zhu et al., 2015). It has been suggested that Nrf2 and ATF4 can together induce ARE-dependent gene transcription (He et al., 2001). Moreover, ATF4 mRNA is inhibited through down-regulation of Nrf2 in response to ER stress induced via thapsigargin even without phosphorylation of eIF2α, which indicates Nrf2 could play a role in the regulation of ATF4 (Miyamoto et al., 2011). Therefore, investigation into the role of the PERK/Nrf2 signalling pathway in the regulation of ATF4 in MIN6 cells would be prudent.

Recent study has been suggested that the eIF2-P/ATF4/mTORC1 pathway has a role in the translational control during prolonged ER. The mTORC1 activity during chronic ER stress leads to an increase of protein synthesis through the up-regulation of the translational mechanism involved ATF4. The translational inhibition in the absence of eIF2α phosphorylation in response to ER stress involved inhibition translation of mRNAs encoding anabolic proteins such as proteins controlling the translational process and ribosomal proteins. So, mTORC1 is considered as a downstream target of the metabolic function of p-eIF2α/ATF4 pathway. Thus, this pathway could have beneficial to study the survival or death decisions during ER stress (Guan et al., 2014).

4.3.1.2 Role of IRE1

In response to ER stress, IRE1 splices the transcription factor Xbp1-mRNA to enhance protein folding and restore ER homeostasis. However, in case of chronic ER stress the

IRE1α activates apoptosis pathway (Upton et al., 2012).The expression of IRE1α and its substrate XBP1mRNA was regulated separately by ATF4 and ATF6 that enable cells to cope with various types stress (Moore & Hollien, 2015). Inhibition of IRE1 activation by over-expressing of a dominant-negative mutant resulted in enhanced CHOP mRNA in response to ER stress induced by tunicamycin, which suggested IRE1 may has a role in the transcriptional regulation of ATF4 (Wang, Harding, Zhang, Jolicoeur, Kuroda & Ron, 1998). In my results, I observed that IRE1 has an essential role in the regulation of ATF4 in response to ER stress, since the ATF4 protein expression inhibited completely in the presence of pharmacological inhibition of IRE1. However, the levels of ATF4 mRNA were not changed in the presences of IRE1 pharmacological inhibitor, which suggested that ATF4 mRNA induction is not dependent on IRE1-XBP1 pathway (Figure 4.7). The results show that inhibition of IRE1 activation did not influence ATF4 mRNA level, whereas ATF4 protein was completely inhibited under ER stress. Indeed, the expectation to see inhibition in ATF4 mRNA level in presence of IRE1 inhibitor but we have to take in our consideration that mRNA level does not always predict its protein level. For example, Shebl *et al*. who did study for measuring of cellular mRNA levels of 20 cytokines produced by peripheral blood mononuclear cells, they are concluded that mRNA expression changes should not be necessarily reflect changes in corresponding protein levels (Shebl et al., 2010). In another study, also it is reported that BAX mRNA and protein levels were obviously different, mRNA levels were low whereas protein levels were higher (Stark et al., 2006). The role of IRE1 in regulating ATF4 protein synthesis still unclear and need more extensive study.

4.3.2 The translational up-regulation of ATF4 in response to ER stress

Increased ATF4 translation in response to ER stress in ES cells is dependent on both PERK activities as well as on phosphorylation of eIF2α in response to ER stress. Also, ATF4 translation is mediated by its 5’ UTR in response to ER stress (Harding et al., 2000). Therefore, I investigated ATF4 translation upregulation in response to ER stress, using luciferase as a reporter to measure the activity of 5’UTR of ATF4 and immunoprecipitation followed by autoradiography. I showed that there is no increase in luciferase activity in response to ER stress within 4 h. However, luciferase expression

was maintained despite a significant decrease in total protein synthesis (Figure 4.9). In contrast, I observed a significant increase of luciferase activity with other cell lines such as HEK293 (Later in this chapter) and MEFs (shown in Chapter 5). One scenario is that ATF4 protein expression could be regulated independent of eIF2α phosphorylation in MIN6 cells, which is in line with my previous finding. It has been reported that the translational upregulation of ATF4 can be mediated by its 5'UTR but this is also dependent on eIF2α phosphorylation in response to ER stress (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010; Harding et al., 2000).

4.3.2 Conclusion

Although the p-eIF2α /ATF4 pathway has been extensively described in regulating of gene expression programs in response to ER stress, in this chapter I demonstrate that PERK mediated eIF2α phosphorylation is not required for ATF4 activation in these MIN6 cells in response to thapsigargin induced ER stress. In addition, I show that the transcriptional upregulation of ATF4 in response to ER stress is essential for the upregulation of ATF4 protein and that this increase occurs independently of p-eIF2α /ATF4 pathway. Further studies into the alternative mechanisms for how ATF4 expression is upregulated in response to ER stress such as PERK/Nrf2 signalling pathway oreIF2 -P/ATF4/mTORC1 pathway may extend our understanding to develop new therapies to protect ER from stress.

Chapter 5: Investigation to the transcriptional and translational up-regulation of ATF4 expression in response to ER stress using MEFs.

5.1 Introduction

ER stress develops as result of accumulation of unfolded/misfolded proteins in the endoplasmic reticulum leading to the activation of UPR. During the UPR, there is a transcriptional and translational reprogramming of the cell which ensures selective gene expression to promote cell survival. Central to the reprogramming is PERK/eIF2/ATF4 pathway. The activation of this pathway through the PERK-dependent phosphorylation of eIF2 leads to a shutdown of global translation. However, it also selectively increases the translation from a subset of mRNAs including that encoding ATF4 (Baird et al., 2014).

To further explore ATF4 expression is regulated in response to ER stress. I investigated the effects of thapsigargin in a mouse embryonic fibroblasts (MEFs). It has been reported that in MEFs under ER stress ATF4 expression is subjected to transcriptional regulation in addition to the translational regulation via eIF2 phosphorylation (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010). It was reported that ATF4 expression is blocked in $PERK^{-/-}$ MEFs in response to ER stress, whereas its expression is abolished in $GCN2^{-/-}$ MEFs in response to leucine deprivation (Harding et al., 2000). In addition, thapsigargin induces ER stress in MEFs wild-type cells shows an increase of eIF2α phosphorylation accompanied with increased ATF4 protein expression. (Palam, Baird & Wek, 2011; Scheuner et al., 2001).

Besides to translational regulation, transcriptional regulation has been reported play a key role in the regulation of both ATF4 and CHOP expression. Thapsigargin induced ER stress in wild-type MEFs, which increased both ATF4 and CHOP mRNA levels but this increased of mRNA level is blocked in $PERK^{-/-}$ MEFs cells or eIF2αS51A knock in MEFs cells (Harding, Zhang, Bertolotti, Zeng & Ron, 2000b; Palam, Baird & Wek, 2011).

The PERK pathway is not only responsible for translational control but also plays a role in transcriptional control in mammals via the induction of translation of transcription factor ATF4 in response to ER stress (Harding *et al*., 2000a). Accordingly, transcription

of UPR target genes is affected in MEFs lacking PERK (Harding *et al*., 2003). Induction of ER chaperones is less extensive in PERK-knockout MEFs than in wild-type cells (Harding *et al*., 2000a), or in knock-in MEFs in which the phosphorylation site of the α subunit of eukaryotic initiation factor 2 (eIF2α), serine51, has been replaced by alanine (Scheuner *et al*., 2001). MEFs lacking IRE1α or XBP1 can induce transcription of major ER chaperones such as BiP and GRP94 in response to ER stress (Yamamoto et al., 2007). The phosphorylation of eIF2α at Ser51 induced by thapsigargin was prolonged in GADD34$^{-/-}$MEF. ER stress stimuli induced expressions of Bip and CHOP in wild-type MEFs. These expressions were reduced in GADD34$^{-/-}$MEF, which suggests that GADD34 has a role in up-regulation of Bip and CHOP, and recovery of protein synthesis (Brown et al., 2016).

5.1.1 The aim of this study is to:

- Determine the role of PERK/eIF2α pathway in the regulation of ATF4 expression in response to ER stress in MEFs cells.
- Determine the role of IRE1-XBP1 pathways in the regulation of ATF4 expression in response to ER stress in MEFs cells.
- Determine the role of protein synthesis in the regulation of ATF4 expression in response to ER stress MEFs

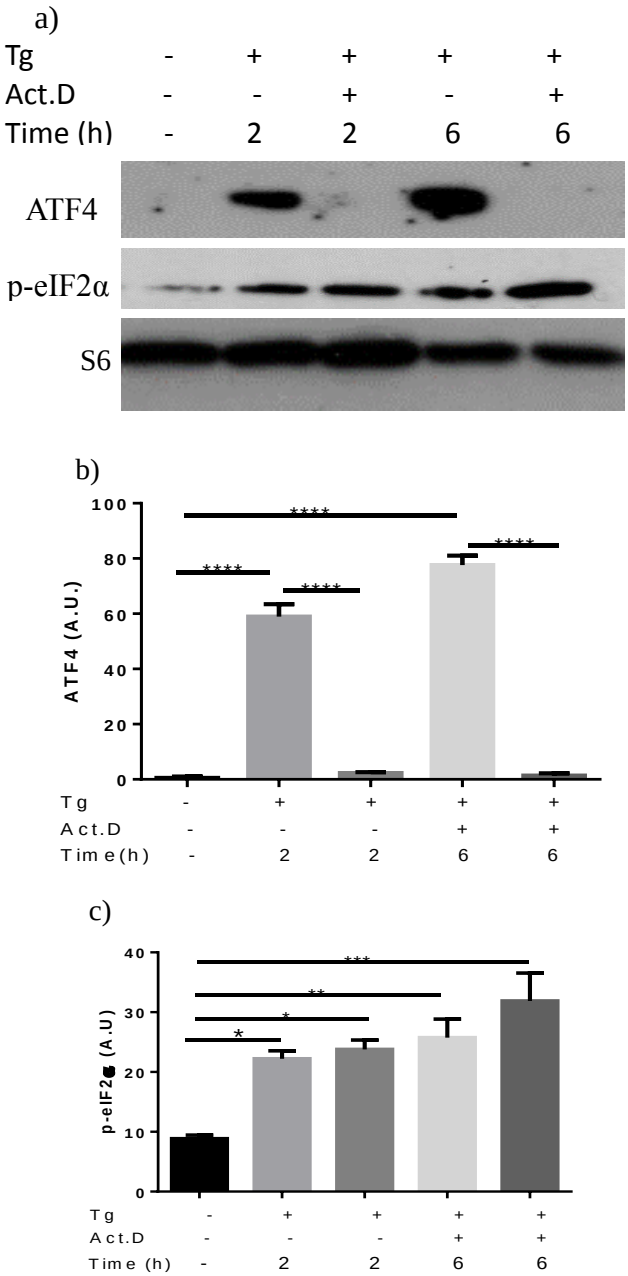

Figure 5.1 Induction of ATF4 protein expression in response to ER stress is dependent on transcription. MEFs in full medium were either untreated or treated with thapsigargin (Tg, 1µM) with or without actinomycin D (Act.D, 1µM added 30 min prior to thapsigargin). Cells were then incubated for either 2 h or 6 h under standard culture conditions (37℃ and 5% CO_2). Changes in the expression of ATF4 and the phosphorylation of eIF2 α (p-eIF2α) were detected by Western blotting with rpS6 used as a loading control. a) Representative Western blot. b, c) Densitometric analysis of ATF4 and p-eIF2α are shown in arbitrary units (A.U.). Blots are representative of at least three experiments. The results are mean ± s.e.m., n=3. Data were analysed by one-way ANOVA test and subsequently with Bonferroni's range tests. For * $P < 0.05$,**** $P < 0.0001$.

5.2 Results

5.2.1 ER stress induced ATF4 expression is dependent on transcriptional regulation.

To investigate whether the induction of ATF4 expression in response to ER stress is dependent on transcriptional regulation, MEFs either untreated or treated with thapsigargin in the presence or absence of the transcriptional inhibitor, actinomycin D for either 2 h or 6 h (Figure 5.1). Changes in the expression of ATF4 and phosphorylation of eIF2α were determined by Western blotting. The Phosphorylation of eIF2α and the expression of ATF4 protein were increased after thapsigargin treatment. However, thapsigargin induced ATF4 expression was blocked when cells were incubated with actinomycin D in the presence of thapsigargin (Figure 5.1a). eIF2α phosphorylation was not effected in the presence of actinomycin (Figure 5.1c). The data suggested that transcriptional upregulation of ATF4 plays an important role in its induction upon ER stress.

As a control, I then measured changes in ATF4 mRNA by qRT-PCR. ATF4 mRNA expression was increased upon the addition of thapsigargin at 2 h and 6 h. As anticipated in the presence of actinomycin D the ATF4 mRNA level was inhibited at both 2 h and 6 h (Figure 5.2). The results indicate that transcription upregulation of ATF4 mRNA in response to ER stress plays an essential role for the upregulation of ATF4 protein expression.

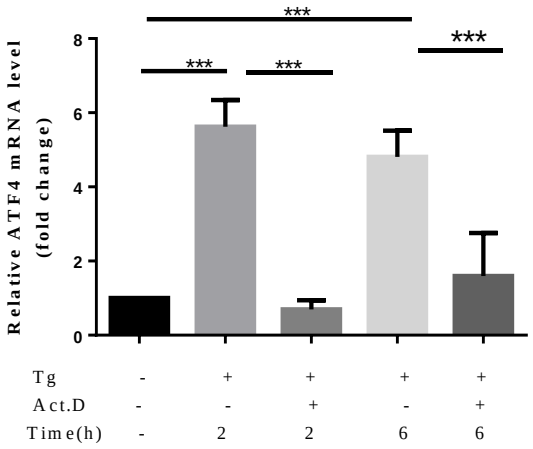

Figure 5.2 ER stress induced ATF4 mRNA expression is inhibited by actinomycin D in MEFs. MEFs were either untreated, treated with thapsigargin (Tg, 1µM) with or without actinomycin D (Act.D, 1µM added 30min prior to thapsigargin). Cells were then incubated for either 2 h or 6 h under standard culture conditions (37℃ and 5% CO_2). ATF4 mRNA was analysed using qRT-PCR. Data were normalized to 18S ribosomal RNA levels and expressed as fold change to the value of untreated sample. The results are mean ± s.e.m. of n=3. Data were analysed by one-way ANOVA with Bonferroni's multiple comparisons test, For *** $P < 0.001$.

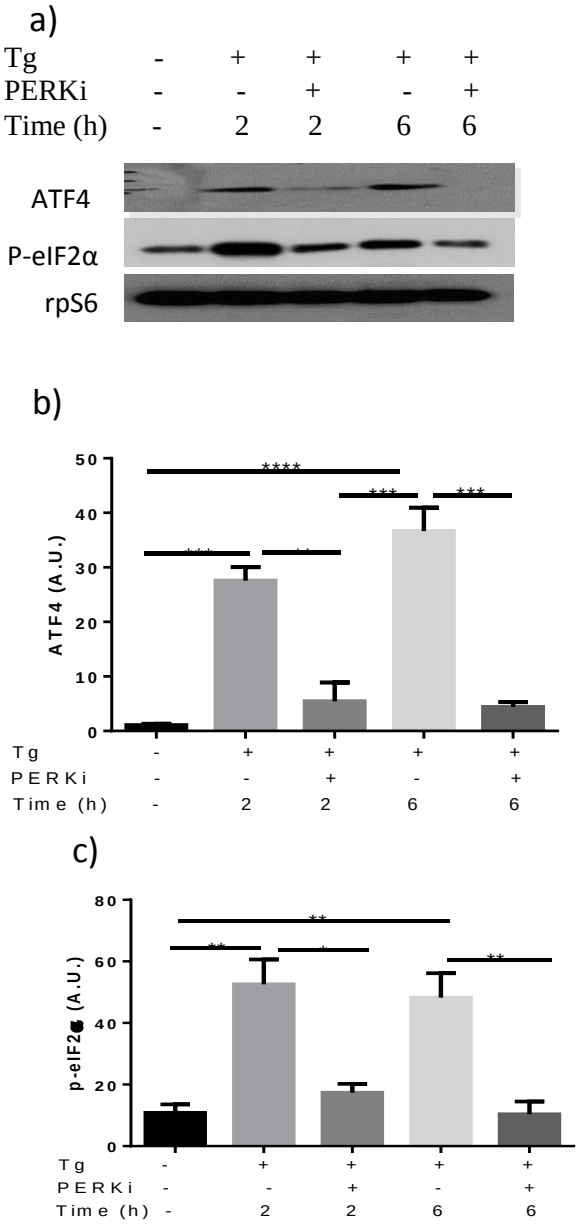

Figure 5.3 PERK is required for ER stress-induced ATF4 expression in MEFs. MEFs were either untreated, treated with thapsigargin (Tg, 1μM) with or without the PERK inhibitor, GSK2656157, (PERKi, 0.5 μM added 30 min prior to thapsigargin). Cells were then incubated for either 2 h or 6 h under standard culture conditions (37℃ and 5% CO_2). Changes in expression of ATF4 and phosphorylation of eIF2α (p-eIF2α) were detected by Western blotting with total rpS6 as a loading control. a) Representative Western blot**.** b, c) Densitometric analysis of ATF4 and p-eIF2α are shown in arbitrary units (A.U.). Blots are representative of n=3. The results are mean ±s.e.m., n=3. Data were analysed by one-way ANOVA followed by Bonferroni's multiple comparison test, For * P< 0.05 ** P< 0.01, *** P < 0.001, **** P < 0.0001.

5.2.2 PERK activation is required for ER stress induced ATF4 expression in MEFs.

To determine the whether the activation of PERK was required for increased expression of ATF4 in response to ER stress. MEFs were incubated with thapsigargin for 2 h or 6 h in the presence or absence of PERK inhibitor (GSK2656157). Treatment of MEFs with thapsigargin for either 2 h or 6 h resulted in a significant increase in the expression of ATF4 as well as the phosphorylation of eIF2α. However, in the presence of the PERK inhibitor (Figure 5.3b). Thapsigargin induced phosphorylation of eIF2α and the expression of ATF4 were significantly inhibited (Figure 5.3c). Therefore, the activation of PERK is required for ER stress-induced ATF4 expression in MEFs.

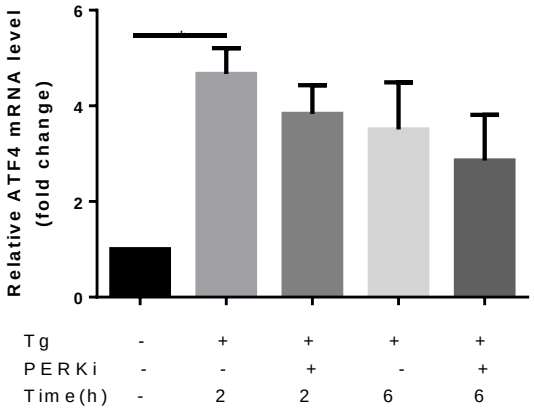

Figure 5.4 Investigating the role of PERK in the upregulation of ATF4 mRNA expression in response to ER stress. MEFs in full medium were either left untreated (control) or treated with thapsigargin (Tg, 1µM) for 2 or 6h with or without the PERK inhibitor, GSK2656157 (0.5 µM added 30 min prior to thapsigargin). Changes in ATF4 mRNA expression was determined using qRT-PCR. Data were normalized to 18S ribosomal RNA levels and expressed as fold change to the value of untreated sample. The results are ± s.e.m., of n=3, data were analysed by using one-way ANOVA test, Bonferroni's multiple comparisons test. , For * $P < 0.05$, ** $P < 0.01$.

5.2.3 An increase of ATF4 mRNA is independent of PERK in response to ER stress.

To investigate the effect of PERK inhibition on ATF4 mRNA expression under ER stress, MEFs were incubated with thapsigargin in the presence or absence of PERK inhibitor for 2 h and 6 h. changes in ATF4 mRNA expression was measured by using qRT-PCR. As anticipated the addition of thapsigargin caused an increase in ATF4 mRNA level at 2 h and 6 h, yet in the presence of PERK inhibitor the induction of ATF4 mRNA was not inhibited at either 2 h or 6 h (Figure 5.4). This data shows that ATF4 mRNA induction is not dependent on PERK in MEFs.

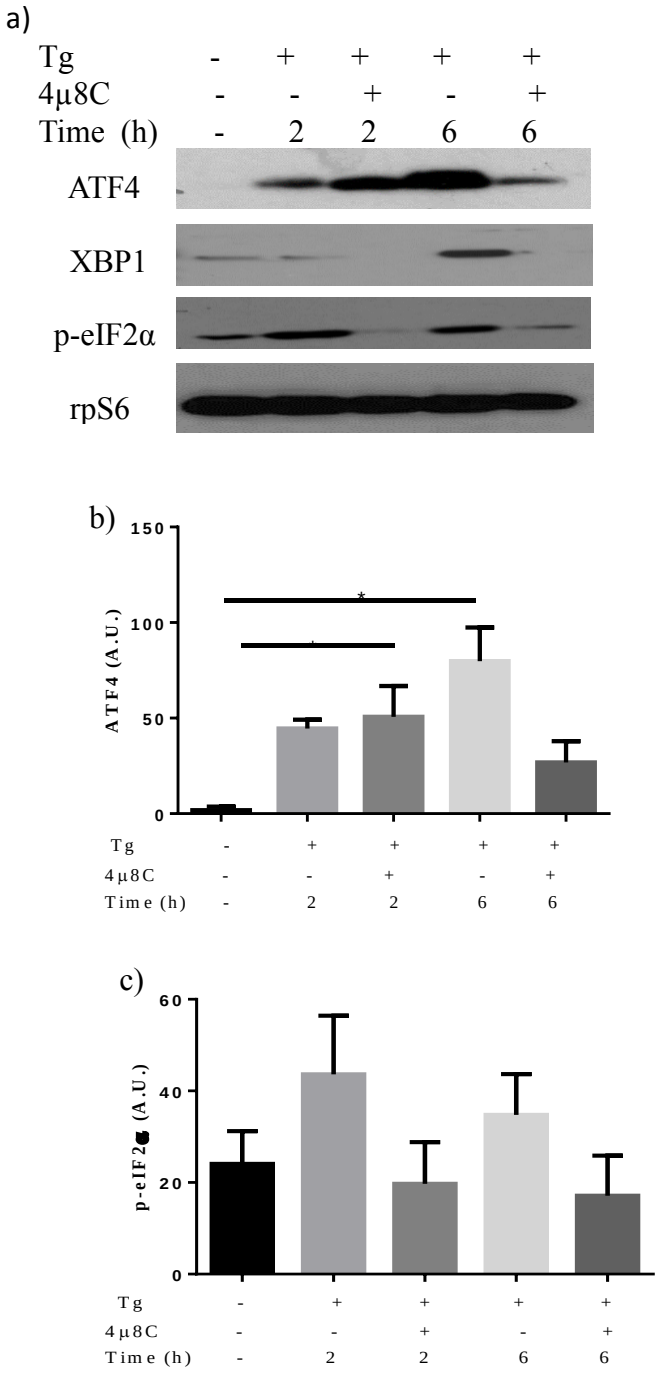

Figure 5.5: Role of IRE1 in ER stress induced expression of ATF4 in MEFs. a) MEFs were either untreated (control) or treated with thapsigargin (Tg, 1μM) for 2 or 6h with or without a IRE1 inhibitor (4μ8C,30μM added 30min prior to thapsigargin). Changes in the expression of ATF4 protein expression and the phosphorylation of eIF2α (p-eIF2α) were determined by Western blot analysis. The expression of rpS6 was used for loading control. b,c) Densitometric analysis of ATF4 and p-eIF2α expression are shown in arbitrary units (A.U.). Blots are representative of at least three experiments; n=3. The results are mean ± s.e.m. of n=3, data were analysed by one-way ANOVA test and subsequently with Bonferroni's multiple comparison tests, For * $P < 0.05$.

5.2.4 Inhibition of IRE1-XBP1 pathway using the IRE1 inhibitor 4μ8C inhibits ATF4 expression at 6 h in MEFs.

In order to investigate the role of IRE1 on expression of ATF4 in response to ER stress, MEFs incubated with or without thapsigargin in the presence or absence of IRE1 inhibitor, 4μ8C. Changes in the expression of ATF4, phosphorylation of eIF2α and XBP1 were determined by western blotting (Figure 5.5a). There was a significant increase of ATF4 protein expression upon addition of thapsigargin at both 2 and 6 h. In the presence of the IRE1 inhibitor there was inhibition in the expression of ATF4 at 6 h but there was no complete inhibition at 2 h (Figure 5.5b). Thapsigargin treatment induced phosphorylation of eIF2α. However, the presence of IRE1 inhibitor led to decrease of eIF2α phosphorylation at 2 h and 6 h. XBP1 expression was decreased at 2 h and 6 h upon the addition of IRE1 inhibitor, 4μ8C (Figure 5.5). These data indicate that the IRE1-XBP1 pathway is required for ATF4 protein expression at 6 but not at 2 h.

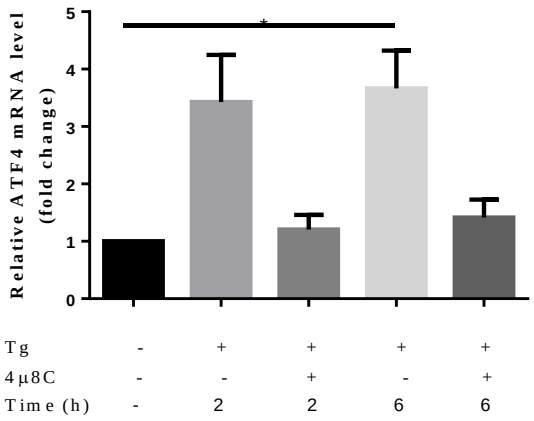

Figure 5.6 Investigate the effect of inhibition of IRE1-XBP1 pathway on ATF4 mRNA level in response of ER stress in MEFs. MEFs in full medium either untreated (control), treated with thapsigargin (Tg, 1μM) with or without 4μ8C (30 μM added 30 min prior to thapsigargin). Cell were then incubated for either 2 h or 6 h under standard culture conditions (37℃ and 5% CO_2). cDNA preparation and ATF4 mRNA was analysed using qRT-PCR. Data were normalized to 18S ribosomal RNA levels and expressed as fold change to the value of untreated sample. The results are ± s.e.m. of n=3, data were analysed by using one-way ANOVA test, Bonferroni's multiple comparisons test.

5.2.5 IRE1-XBP1 pathway is required for the ATF4 mRNA regulation in response to ER stress

To assess the effect of IRE1 inhibitor on the expression level of ATF4 mRNA in response to ER stress, MEFs either untreated or treated with thapsigargin with or without IRE1 inhibitor were incubated for either 2 h or 6 h. Changes in the expression of ATF4 mRNA level were measured by qRT-PCR. ATF4 mRNA level was increased in response to thapsigargin at 2 h and 6 h, nevertheless this increase is not significantly inhibited in the presence of IRE1 inhibitor (Figure 5.6). These data shows that IRE1-XBP1 pathway might be has a role in expression of ATF4 mRNA.

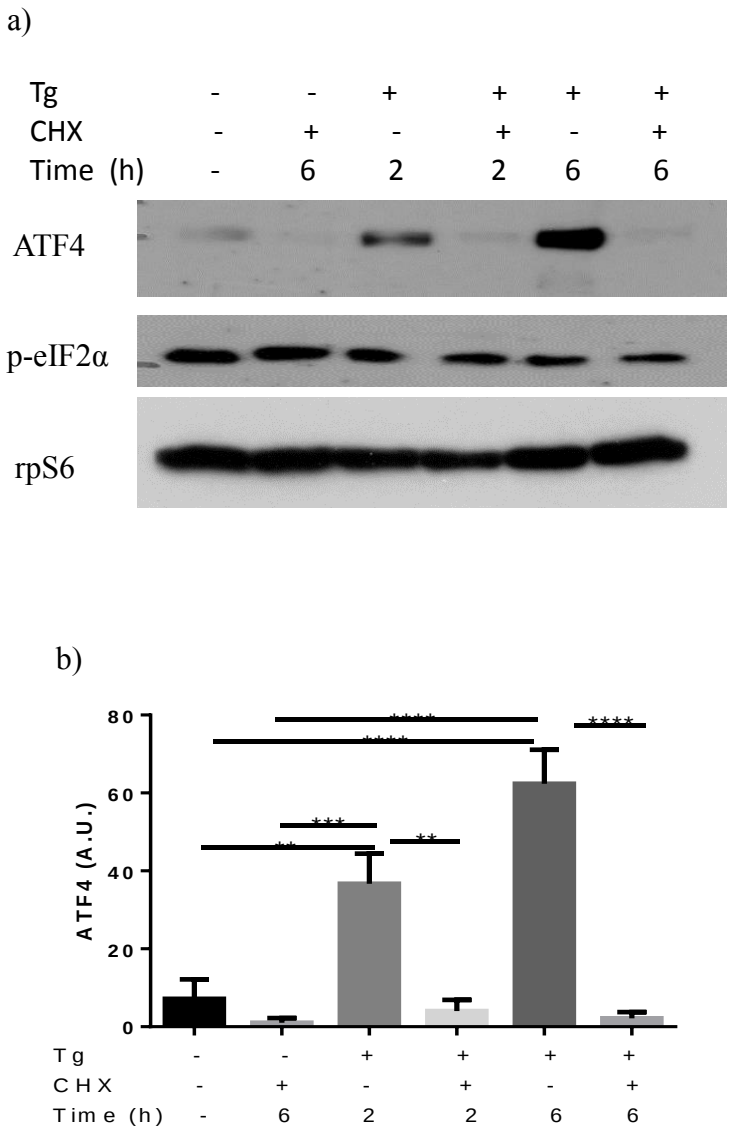

Figure 5.7 Effect of inhibition of protein synthesis on the expression of ATF4 in response to ER stress in MEFs. a) MEFs were either untreated (control) or treated with thapsigargin (Tg,1µM) for either 2 h or 6 h with or without a cycloheximide, CHX (CHX, 3.5 µM was added 15 min prior to thapsigargin). Changes in the expression of ATF4 and the phosphorylation of eIF2α were detected using Western blotting; rpS6 was used for loading control. b) Densitometric analysis of ATF4 expression is shown in arbitrary units (A.U.). Blots are representative of at least three experiments; n=3. The results are mean ± s.e.m. of n=3, data were analysed by one-way ANOVA test and subsequently with Bonferroni's multiple comparison tests, For ** $P < 0.01$, *** $P < 0.001$.**** $P < 0.0001$.

5.2.6 Protein synthesis is required for induced expression of ATF4 in response to ER stress.

Our results indicate that ATF4 is transcriptionally regulated in response to ER stress. To investigate the nature of these transcriptional regulators and effect of protein synthesis during ER stress on expression of ATF4, MEFs were stressed with thapsigargin in the presence or absence of protein synthesis inhibitor, cycloheximide for 2 h or 6 h. Change in the expression of ATF4 protein and mRNA was determined by western blotting and qPCR respectively. As expected thapsigargin caused an increase in both ATF4 protein and mRNA expression. Cycloheximide inhibited protein synthesis as determined by ^{35}S-met incorporation into protein and inhibited ATF4 protein expression. Interestingly ATF4 mRNA expression was also blocked. Therefore, ongoing protein synthesis is required for the transcriptional upregulation in ATF4 mRNA expression (Figure 5.8).

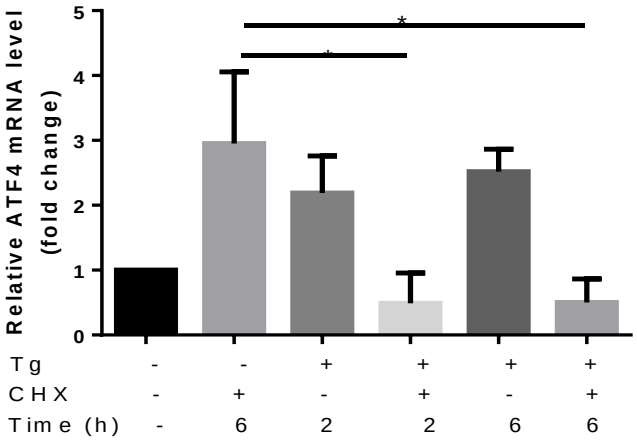

Figure 5.8 induction of ATF4 mRNA is dependent on de novo protein synthesis in response of ER stress. MEFs were either untreated (control) or treated with thapsigargin (Tg, 1µM) with or without cycloheximide, CHX (CHX, 3.5 µM was added 15 min prior to thapsigargin). Cell were then incubated for either 2 h or 6 h under standard culture conditions (37℃ and 5% CO_2). cDNA preparation and ATF4 mRNA was analysed using qRT-PCR. Data were normalized to 18S ribosomal RNA levels and expressed as fold change to the value of untreated sample. The results are ± S.E.M of n=3, data were analysed by using one-way ANOVA test, Bonferroni's multiple comparisons test. For * $P < 0.05$.

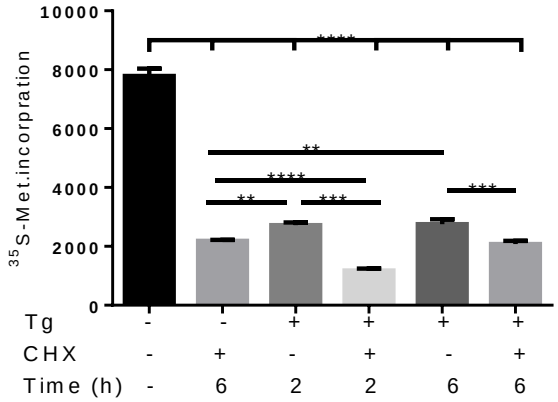

Figure 5.9 Determination of total protein synthesis. In the presence of [^{35}S]-Methionine MEFs were either untreated (control), treated with thapsigargin (Tg,1µM) for 2 or 6 h with or without cycloheximide, CHX (CHX, 3.5 µM was added 15 min prior to thapsigargin). Total protein synthesis was then determined by measuring ^{35}S met. incorporation into protein. Data presented are the ±SEM, n=3. Data were analysed by one way ANOVA and subsequently with Bonferroni's test. For * $P < 0.05$, ** $P < 0.01$,*** $P < 0.001$,**** $P < 0.0001$.

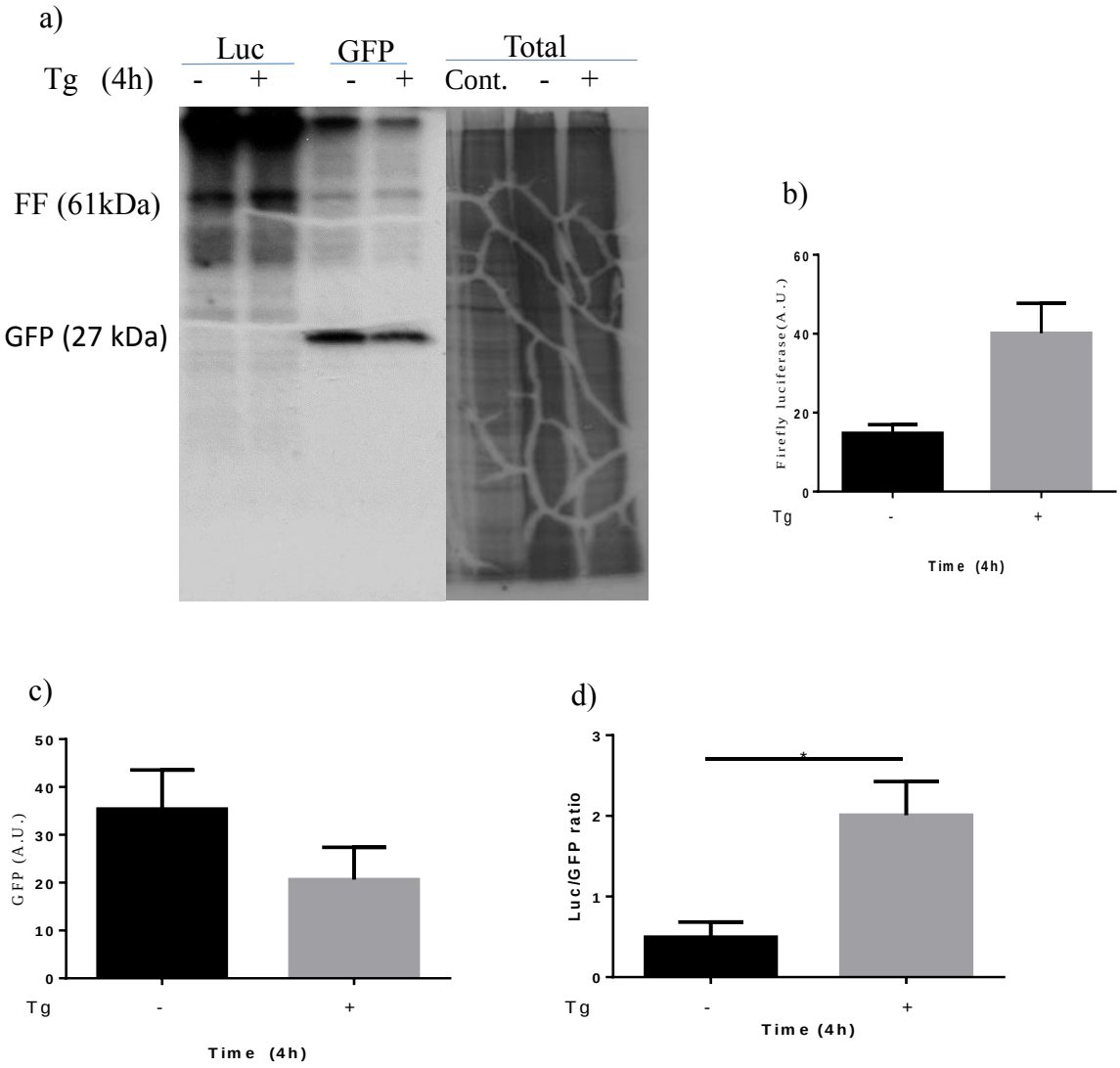

Figure 5.10 Pull-down of luciferase and GFP associated ATF4 after incorporation with [^{35}S]-methionine isotope: MEFs were infected with Ad ATF4-Luc for 48 h. Cells were incubated in medium without methionine, cysteine and in presence or absence of thapsigargin (Tg, 1µM) for 4 h. Cells were labelled with [^{35}S] - methionine which posted at last hour and incubated under standard culture conditions (37℃ and 5% CO_2). a) Autoradiograph of SDS-PAGE of radiolabelled proteins, the labelling followed by immunoprecipitation of luciferase (Luc) and GFP from untreated and thapsigargin treated cells. Total lysate (Total) was measured which included non-infected cells control (Cont.). b, c and d) Densitometry analysis of luciferase, GFP and Luc/GFP ratio is shown in arbitrary units, blots shown are representative of at least three experiments; n=3. The results are ± s.e.m., of n=3, data were analysed by paired t test. For * $P < 0.05$.

5.2.7 ATF4 protein expression is translationally regulated in response to ER stress

In order to address whether the expression of ATF4 is translationally upregulated in response to ER stress, MEFs were infected with Ad ATF4-Luc which expresses both GFP and firefly-luciferase from separate CMV promoters. After 48 h of infection, cells were labelled with [^{35}S]- methionine in the presence or absence of thapsigargin. [^{35}S]-methionine labelled luciferase or GFP were then immunoprecipitated using antibodies either against luciferase or GFP and the labelled proteins separated and detected by SDS–PAGE followed by autoradiography (Figure 5.10a). 4h of thapsigargin treatment led to a statistically insignificant increase in luciferase expression (Figure 5.10b). (Figure 5.10a), as well as a statistically insignificant decrease in GFP (Figure 5.10c). However there was a significant increase in the ratio Luciferase expression relative to GFP (Figure 5.10d) indicating that ATF4 expression is increased relative to GFP expression in response to ER stress in MEFs.

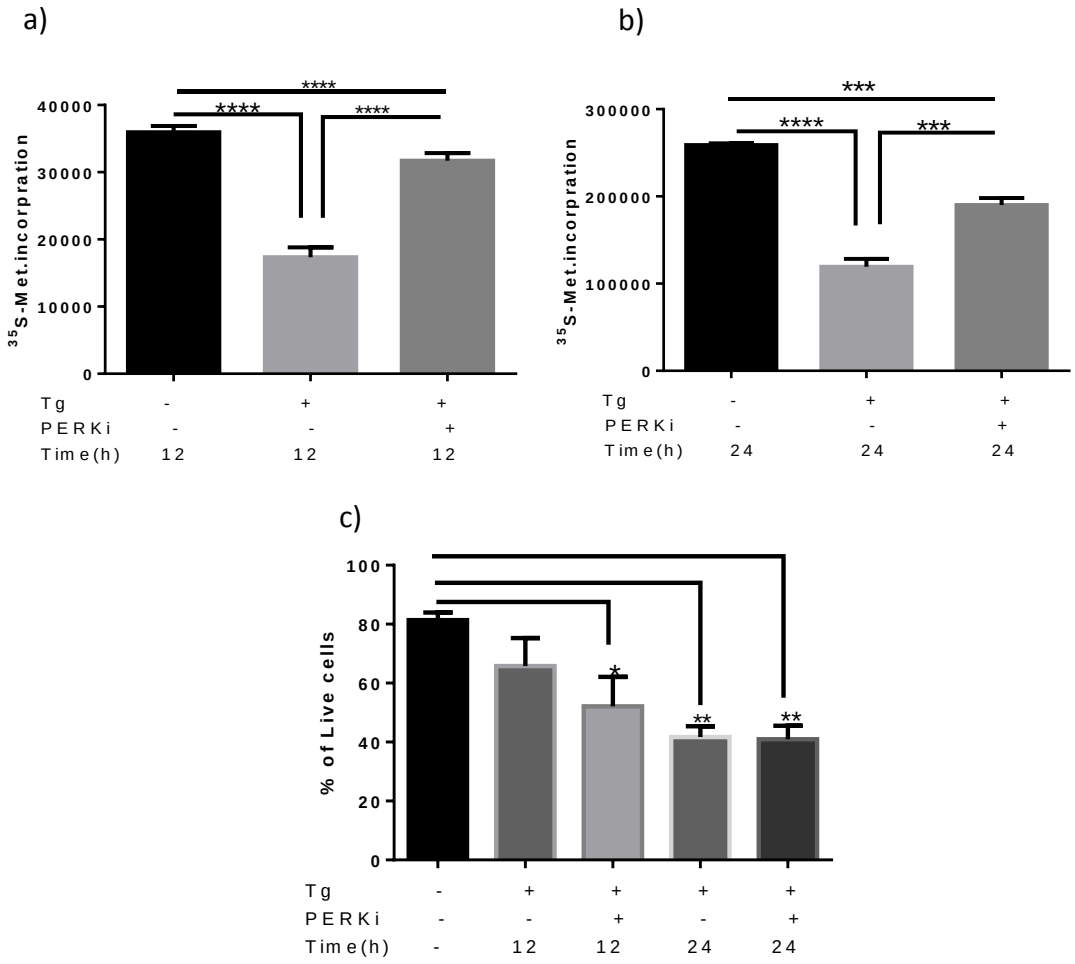

Figure 5.11 Inhibition of PERK can restore protein synthesis in thapsigargin treated MEFs: MEFs were either untreated (control), treated with thapsigargin (Tg, 1µM) with or without PERK inhibitor, GSK2656157 (0.5 µM added 30 min prior to thapsigargin).Cell were then incubated for either 12 h or 24 h under standard culture conditions (37 ℃ and 5% CO_2). [^{35}S]- Methionine was added for the last 2 h. Total protein synthesis was then determined by measuring TCA perceptible count (a, b). Cell viability was measured using a live/dead cell fluorescence assay as described in the methods (c). Data presented are the ±s.e.m., n=3. Data were analysed by one way ANOVA and subsequently with either Bonferroni's (a,b) or Dunnett's range tests. For * $P < 0.05$,** $P < 0.01$,*** $P < 0.001$,**** $P < 0.0001$.

5.2.8 Inhibition of PERK leads to repression of protein synthesis in response to ER stress in MEFs.

To address the role of PERK inhibition in repression of protein synthesis in response to ER stress, I investigated the role of PERK/eIF2α pathway in the repression of protein synthesis in response to ER stress induced by thapsigargin. MEFs were treated with thapsigargin in the presence or absence of PERK inhibitor for 12 h or 24 h. After radioactive labelling of newly synthesized protein using [^{35}S]-Methionine isotope, the incorporation into protein was measured by protein precipitation technique. The results show that upon the addition of thapsigargin for 12 h, protein synthesis is inhibited by about 65%. However, cells treated with PERK inhibitor prior the addition of thapsigargin were shown recovery of protein synthesis around 95% (Figure 5.11a). After 24 h of incubation with thapsigargin cells were shown repression in protein synthesis about 70%, while in the presence of PERK inhibitor caused recovery of protein synthesis around 70% (Figure 5.11b). The viability of cells was checked, the presence of thapsigargin caused drop of 12% after 12 h and 50% after 24 h, while the presence of PERK inhibitor caused 32% and 50% respectively (Figure 5.11c). Together these dates showed that the PERK/eIF2α pathway is likely responsible for the inhibition of protein synthesis of MEFs at 12 h and 24 h in response to ER stress (Figure 5.11).

5.3 Discussion

The PERK/eIF2α pathway of the UPR has been suggested to be central for the conservation of cellular functional integrity by mediating recovery from ER stress via the inhibition of protein translation to increase folding capacity within ER (Harding, Zhang, Bertolotti, Zeng & Ron, 2000b). During ER stress, the phosphorylation of eIF2α leading to elevation ATF4 transcription which increased the availability of ATF4 transcript for preferential translation (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010). In this chapter, I aimed to investigate the transcriptional and translational up regulation of ATF4 as well as the protein synthesis in response to ER stress using MEFs as different cell model to address the underlying mechanism responsible for regulation of ATF4.

In chapter 4, I showed that the expression of ATF4 protein was dependent on transcriptional upregulation in response to ER stress in MIN6 cell line (Figure 4.2). Also, I found that the induction of ATF4 expression was dependent on its transcriptional regulation under ER stress in MEFs. This finding was completed by using actinomycin-D to block transcription followed with inducing ER stress using thapsigargin. I observed both ATF4 protein expression and ATF4 mRNA are completely inhibited in the presence of actinomycin-D (Figure 5.2). Previous reports have also shown that the ATF4 is transcriptionally up-regulated in response to ER stress (Pirot et al., 2007). Moreover, MEFs wild-type cells showed high level of ATF4 mRNA during ER stress (Harding et al., 2000). In consistent with my observation, it has been reported that the transcriptional upregulation of ATF4 is essential for the changes in ATF4 mRNA levels in response to ER stress conditions (Lu, Harding & Ron, 2004).

I showed that the PERK/eIF2α pathway is required for ATF4 protein expression in response to ER stress (Figure 5.3). This observation was supported with previous studies that reported under ER stress the expression of ATF4 and CHOP proteins were blocked in PERK$^{-/-}$ and MEFs $^{eIF2\alpha S51A}$ knock-in cells (Harding et al., 2000; Scheuner et al., 2001). Also, I showed that the induction of ATF4 mRNA is not dependent on PERK in response to ER stress (Figure 5.4). It has been reported that ER stress leads to ATF4 activation which result in an increase of ATF4 transcript for selective translation and increase the expression of ATF4 protein (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010). In previous study has been reported that in unstressed cells the mRNA of ATF4

is inefficient to be translated. However, under ER stress mRNA is liberated in stressed cells (Harding et al., 2000). In my results, I observed an increase of ATF4 mRNA in the presence of PERK inhibitor (Figure 5.4). This is in agreement with previous study, in $PERK^{-/-}$ MEFs where the ATF4 mRNA level was increased in response to ER induced by thapsigargin, which indicated that $PERK^{-/-}$ MEFs could be compromised with ER stress through different mediator of UPR (Gupta et al., 2012). Indeed, the increase of ATF mRNA in the presence of PERK inhibitor was not accompanied with an increase of ATF4 protein expression, this could be due to the mRNA efficiency that the mRNA level does not usually reflect its protein level (Shebl et al., 2010). This result appears to differ from my findings in chapter 4, MIN6 cells line. I am currently uncertain as to the underlying reason for this difference, but it may reflect the different cell type.

I showed that the inhibition of IRE1-XBP1 pathway using 4μ8C caused an inhibition of ATF4 protein expression at 6 h but not at 2 h in response to ER stress (Figure 5.5). In same context, in the presence of ER stress I showed that the ATF4 mRNA level was reduced in the presence of IRE1 inhibitor, which indicated that the IRE1-XBP1 pathway could play a role in ATF4 mRNA expression in response to ER stress (Figure 5.6). It was reported that IRE1 activation was observed in PERK deficient cells and PERK knockout mice compared with wild-type, which believe there is a compensatory mechanism between both arms (Harding, Zhang, Bertolotti, Zeng & Ron, 2000a). Previous studies suggested that IRE1- deficient cells are more sensitive to ER stress and calcium homeostasis. Since, the calcium accumulation promotes cytochrome c release, leading to induce apoptotic pathway including ATF4 and downstream CHOP pathway (Kanekura, Ma, Murphy, Zhu, Diwan & Urano, 2015; Mattson & Chan, 2003). This could describe the increase of ATF4 protein expression at 2 h in the presence of IRE1 inhibitor in response to ER stress (Figure 5.5). The ATF4 mRNA and protein have been shown low stability and rapidly degraded (Rutkowski et al., 2006). Many studies have been reported that the level of mRNA does not reflect the protein expression. For instant, BAX mRNA and protein levels were obviously different, mRNA levels were low whereas protein levels were higher (Stark et al., 2006).

I provide an evidence that the protein synthesis is essential for induced ATF4 expression in response of ER stress. I confirmed this using protein synthesis inhibitor, cycloheximide then incubated in the presence or absence of thapsigargin. I found

complete inhibition of ATF4 protein in the presence of cycloheximide (Figure 5.7). In agreement with previous studied, my results suggested that ATF4 is transcriptionally regulated in response to ER stress and this transcriptional regulation is essential to ATF4 expression (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010). Also, I showed complete inhibition of ATF4 protein expression in the presence of cycloheximide only (Figure 5.7a), which is confirmed that ATF4 protein is expressed under stress condition and protein synthesis is required for its upregulation. My results show that under ER stress ATF4 mRNA was inhibited in the presence of cycloheximide (Figure 5.8), which suggests that transcription factors are synthesized in response to ER stress and essential for transcriptional control of ATF4 expression. Moreover, I showed a significant increase in ATF4 mRNA level in the cells treated with cycloheximide only, and this result in agreement with proposed cycloheximide mechanism of action that cycloheximide inhibits the elongation phase of eukaryotic translation; it binds the ribosome and inhibits eEF2-mediated translocation (Dai, Shi, Chen, Iqbal, Liu & Gong, 2013; Schneider-Poetsch et al., 2010).

I showed that the total of protein synthesis was repressed in the presence of protein synthesis inhibitor in response to ER stress. I confirmed this by incubated the cells with cycloheximide in the presence of [^{35}S]-methionine to allow for their incorporation into newly synthesized proteins (Figure 5.9). Cycloheximide was strongly inhibited the translation and protein synthesis. However, the presence of thapsigargin beside to cycloheximide potentiate this inhibition, which indicate that the inhibition of translation might be occurred at elongation phase by inhibits eEF2 and through eIF2α phosphorylation at initiation translation phase, which result in drastic protein synthesis repression. It was reported that cycloheximide allows one translocation process before inhabits elongation. Also, it was suggested that the accumulation of 80S could inhibit of either a late step in translation initiation or an early step in elongation(Schneider-Poetsch et al., 2010). Studies suggest that cell survival is determined by reduced protein synthesis but not increase ATF4 synthesis (Han et al., 2013).

In my results, I showed that the ATF4 protein expression is likely subjected to translation upregulation in response to ER stress. This finding was accomplished by immunoprecipitated of ATF4 luciferase binding protein after 4 h of thapsigargin induced ER stress and labelling with [^{35}S]-methionine isotope (Figure 5.10), the luciferase

activity was increased by more than 2-folds in treated thapsigargin cells which indicated that the translational upregulation is required for ATF4 protein expression in response to ER stress. This results in agreement with previous studies that reported the ATF4 translational upregulation is mediated by its 5'UTR is dependent on eIF2α phosphorylation in response to ER stress (Harding *et al*., 2000c; Dey *et al*., 2010), the phosphorylation of eIF2α inhibit the global translation with coincidence selective translation of certain mRNAs under stress conditions (Ameri *et al*., 2008; Guan *et al*., 2014). Moreover, it was reported that the luciferase activity was significantly increased in MEFs transfection with ATF4-luc plasmid were treated with thapsigargin compared with non-treated cells (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010). Overall, ATF4 is likely translationally upregulated in response to ER stress. However, protein synthesis was inhibited in the presence of thapsigargin after 4 h (Figure 5.10). It was shown that the overexpression of ATF4 in MEFs deficient eIF2α-P (S51A mutation) cells leads to increase protein synthesis (Guan *et al*., 2014). It is noteworthy that the phosphorylation of eIF2α leads to global inhibition of protein synthesis. It is proposed that the PERK/eIF2α-P/ATF4 pathway acts as suppresser of protein synthesis inhibition during chronic ER stress (Guan et al., 2014).

As expected, the presence of PERK inhibitor leads to recovery of protein synthesis which indicates that the PERK/eIF2α pathway could be the main responsible for repression of protein synthesis in response to ER stress (Figure 5.11). It was reported that during acute ER stress, the phosphorylated eIF2α leads to repression of protein synthesis, whereas during chronic ER stress, dephosphorylation of eIF2α leads to translational recovery (Back *et al*., 2009). eIF2α phosphorylation diminished the availability of ternary complexes (eIF2-GTP-Met-tRNAi), which leads to inhibition of global translation initiation rates (Baird & Wek, 2012). It was suggested that PERK is essential for eIF2α phosphorylation and translation inhibition during ER stress (Harding, Zhang, Bertolotti, Zeng & Ron, 2000b). My results showed that after 24 h of cells treatment with PERK inhibitor caused 50% cell death (Figure 5.11c). It has been reported that $PERK^{-/-}$ cells show low survival and more susceptible when exposed to ER stress (Harding, Zhang, Bertolotti, Zeng & Ron, 2000b).

5.3.1 Conclusion

In this chapter, I provide evidence that under ER stress the induction of ATF4 expression is dependent on its transcriptional regulation, PERK/eIF2α pathway is required for ATF4 protein expression, and the IRE1-XBP1 pathway plays a role in ATF4 expression which is time dependent. Also, I provide data shown that the protein synthesis is essential for induced ATF4 expression in response of ER stress. These results improve our knowledge to understanding the signalling pathways in controlling of protein synthesis and protect the cells form ER stress which could provide new strategies for treatment of many chronic diseases.

Chapter 6: Final discussion

6.1 Translation regulation by eIF2α phosphorylation

Traditionally, cap-dependent translation can be divided into three stages: initiation, elongation and termination. Initiation is considered the main stage that regulated protein synthesis rather than during elongation or termination (Dever, 2002). Translation initiation is the process of assembly of 80S ribosomes, which involves the recruitment of the 80S ribosome and the initiator methionyl-tRNA (Met-tRNAi) on to the start codon (AUG) of the mRNA. This process is facilitated by at least 12 protein initiation translation factors (eIFs) (Hinnebusch, 2011).

Phosphorylated eIF2 is able to form an initiation complex eIF2-TC, after its release, phosphorylated eIF2–GDP tightly binds to and blocks the guanine nucleotide-exchange factor eIF2B, inhibit its activity. Subsequently, eIF2-TC levels drop and for most mRNAs translation is reduced, but protein synthesis from certain mRNAs can be stimulated. For instance, the transcription factor ATF4 whose expression is increased (Zhou, Palam, Jiang, Narasimhan, Staschke & Wek, 2008). GDP/GTP exchange on eIF2 can be controlled by the guanine nucleotide exchange factor (GEF) eIF2B or GTPase activating protein (GAP) eIF5. Moreover, the inhibitory action of phosphorylated eIF2 is prevented by deletion of eIF2Bα from the eIF2B complex (Elsby, Heiber, Reid, Kimball, Pavitt & Barber, 2011). Mutation of eIF2B results in the neurological disorder leukoencephalopathy with Vanishing white matter (VWM) (Dev, Qiu, Dong, Zhang, Barthlme & Hinnebusch, 2010; Wortham & Proud, 2015).

In this thesis, I provide evidence that the repression of protein synthesis in response to ER stress is independent of eIF4E/4A and 4B initiation factors as well as elongation factors. Thus, the repression of protein synthesis most likely is independent of mTORC1 activation and elongation. Therefore, based on my work, eIF2α phosphorylation is primarily responsible for the repression of general protein synthesis in response to ER stress in MIN6 cell (Figure 3.4, 3.5, 3.6, 3.7 and 3.8). This is consistent with other reports (Wek, Jiang & Anthony, 2006). Indeed, the phosphorylation of eIF2α is one of the main mechanisms that controls translation rates *in vivo* and *in vitro* (Kaufman, 2004). Also, I showed that there was a complete recovery of protein synthesis in the presence

of the PERK inhibitor that gives us a strong evidence for the essential role of PERK in the repression of protein synthesis (Figure 3.8, 3.9, 4.12 and 5.11). This is in line with a large body of evidence that stated PERK is required for both the phosphorylation of eIF2α and the attenuation of translation in response to ER stress (Harding *et al.*, 1999, Harding *et al*., 2000b). Overall, eIF2α is pivotal for translation regulation and repression of protein synthesis in response to ER stress. Therefore, eIF2α kinases and downstream target genes offer attractive targets for develop new therapies to alleviate ER stress.

6.2 ER and disease

The ER is the organelle responsible for calcium storage, lipid synthesis, and protein folding as well as trafficking. In eukaryotic cells, newly synthesized polypeptides are co-translationally translocated to the ER as unfolded polypeptide chains, where the native conformation and maturation of these proteins are attained with the help of a group of ER-based folding factors and chaperones. The ER has a capability to accommodate increases in the demand for protein folding. However, extracellular stimuli and changes in intracellular homeostasis cause protein misfolding in the ER (Jaenicke & Seckler, 1997). Perturbations in ER function, Ca^{2+} homeostasis, redox imbalance, altered protein glycosylation, or protein folding defects lead to accumulation of unfolded or misfolded proteins in the ER lumen, result in a condition named ER stress. In response to ER stress, cells have an integrated signalling system to restore homeostasis and normal ER function called the unfolded protein response (UPR) (Senft & Ronai, 2015). This response is required to restore proper protein folding which requires regulation of gene expression such as transcription, translation, translocation into the ER lumen, and ERAD (Schroder & Kaufman, 2005).

Misfolded or unfolded proteins that are trapped within ER lumen and directed into proteasomal degradation after retro-translocation into the cytosol, a process known as ER-associated degradation (ERAD). This process is considered a first line of defence to restore ER homeostasis. ERAD is essential in cells that cannot induce the UPR (Walter & Ron, 2011). If the UPR fails to restore ER homeostasis, the UPR triggers caspase-dependent apoptosis or caspase-independent necrosis pathways, which ultimately lead to cell death (Kim, Xu & Reed, 2008). UPR can act as an apoptotic executor of non-functional cells. This may occur in type 2 diabetes when pancreatic β-cells fail to meet

the excessive demand of insulin (Fonseca, Gromada & Urano, 2011b). However, UPR can also act as cytoprotective such as in multiple myeloma (MM) which inhibits the rapid growth of tumour (Walter & Ron, 2011).

There is a huge body of data suggest that ER stress as well as UPR are responsible for a large number of diseases including metabolic disease, neurodegenerative disorders and cancer. Accordingly, it is essential to improve our understanding of the molecular mechanism of UPR and develop compounds that can selectively treat ER stress related diseases. There are three ER-localized transmembrane of the UPR have been identified. These transducers are two protein kinases, IRE1and PERK (Harding, Zhang, Bertolotti, Zeng & Ron, 2000b), and the transcription factor ATF6 (Adachi, Yamamoto, Okada, Yoshida, Harada & Mori, 2008).

In this thesis, I showed that the phosphorylation of eIF2α is responsible for the repression of protein synthesis in response to ER stress. I then investigated how ATF4 expression is up-regulated in response to ER stress in different cell lines. I provide evidence that under ER stress the transcriptional up-regulation of ATF4 expression is essential for its induction in three different cell lines. The up-regulation of the expression of ATF4 protein in response to ER stress in MIN6 cells, HEK293 cells as well as MEFs was blocked with actinomycin D (Figure 4.2, 4.8 and 5.1). It is known that ATF4 is transcriptionally up-regulated in response to ER stress (Pirot *et al.*, 2007). Also, I showed that the transcriptional upregulation of ATF4 mRNA in response to ER stress in MIN6 cells and MEFs was completely inhibited in the presence of actinomycin D (Figure 4.3 and 5.2). It has been reported that ATF4 mRNA and its downstream gene target CHOP mRNA levels were blocked in response to thapsigargin induced ER stress in $eIF2^{S51A}$ knock in or $PERK^{-/-}$ MEF cells (Harding *et al.*, 2000b; Scheuner *et al.*, 2001; Palam *et al.*, 2011). Consistent with my observation, it has been reported that the transcriptional upregulation of ATF4 is essential for the changes in ATF4 mRNA levels in response to ER stress conditions (Lu, Harding & Ron, 2004).

Classically in response to ER stress an increase in ATF4 expression requires PERK and eIF2α phosphorylation. Based on experiment conducted in $PERK^{-/-}$ and $eIF2\alpha^{S51A}$ knock-in PERK activation and eIF2α phosphorylation is required in ER stress induced expression of ATF4 (Harding et al., 2000; Palam, Baird & Wek, 2011; Scheuner et al.,

2001). Likewise, I found that PERK activation and eIF2α phosphorylation was required for ATF4 protein induction in response to ER stress in MEFs (Figure 5.3). However, I provide evidence that ER stress induced induction of ATF4 protein is independent of PERK activation or eIF2α phosphorylation in these MIN6 cells (Figure 4.5,4.6). This is in agreement with previous study from Herbert lab (Zhao XC, 2013). Furthermore, I showed that in these MIN6 cells the induction of ATF4 mRNA is not dependent on PERK in response to ER stress (Figure 4.5). These finding are in contrast to what is found in other cells/cell types or indeed primary islets. Furthermore, there are lines of MIN6 cells whose expression of ATF4 is dependent on PERK and eIF2α phosphorylation (Herbert, unpublished results). Therefore, it is possible that these conflicting results could be due to different clonal lines of MIN6 cells that respond differently to ER stress. Regardless my results demonstrate that ER stress can upregulate ATF4 expression independent of PERK/eIF2α phosphorylation. In my hands, an increase of ATF4 mRNA in MEFs was observed in the presence of a PERK inhibitor (Figure 5.4). This is in agreement with previous study, in $PERK^{-/-}$MEFs where the ATF4 mRNA level was increased in response to ER induced by thapsigargin, which indicated that $PERK^{-/-}$MEFs could be compromised with ER stress through different mediator of UPR (Gupta et al., 2012). Indeed, mRNA level does not usually predict its protein level (Shebl et al., 2010). This may be explained why the increase of ATF4 mRNA in the presence of PERK inhibitor was not accompanied with an increase of ATF4 protein expression in my results.

The expression of ATF4 protein in the absence of eIF2α phosphorylation in MIN6 cells suggests that there is an additional or alternative mechanism is involved in the up-regulation of ATF4 protein in response to ER stress (Figure 4.5). It has been suggested that Nrf2 and ATF4 can together induce ARE-dependent gene transcription (He et al., 2001). It was reported that ATF4 mRNA is inhibited through down-regulation of Nrf2 in response to ER stress induced via thapsigargin even without phosphorylation of eIF2α, which indicates that Nrf2 could play a role in the regulation of ATF4 (Miyamoto et al., 2011). These data suggest that the up-regulation of ATF4 in response to ER stress could be through the phosphorylation of Nrf2, which is independent of eIF2α phosphorylation (Miyamoto et al., 2011). Thus, PERK/Nrf2 pathway may also play a role in the regulation of ATF4 under ER stress. Therefore, investigation into the role of the

PERK/Nrf2 signalling pathway in the regulation of ATF4 in MIN6 cells would be prudent.

Under chronic ER stress the mTORC1 activity leads to an increase of protein synthesis through the up-regulation of the translational mechanism involved ATF4 (Guan et al., 2014). In response to ER stress, the inhibition of translation in the absence of eIF2α phosphorylation involved inhibition of mRNAs translation encoding anabolic proteins such as proteins controlling the translational process and ribosomal proteins (Guan et al., 2014) . Thus, the p-eIF2α/ATF4 pathways considered as an alternative pathway for up-regulation of ATF4 in the absence of eIF2α phosphorylation and could have beneficial to study the survival or death decisions during ER stress.

ATF4 expression can also be regulated by the IRE1-XBP1 pathway. IRE1α and its substrate XBP1 mRNA can be regulated separately by ATF4 and ATF6 that enable cells to cope with various types of stresses (Moore & Hollien, 2015). In these MIN6 cells ATF4 protein expression was inhibited completely in the presence of pharmacological inhibition of IRE1 (Figure 4.6). Yet my results suggest that ER stress induced an increase in ATF4 mRNA expression, which is independent of the IRE1-XBP1 pathway in these MIN6 cell line (Figure 4.7). I found that the inhibition of ATF4 protein synthesis was not accompanied with inhibition in ATF4 mRNA level in response to ER stress as I was expected. Usually, the mRNA expression changes should not be necessarily reflected changes in corresponding protein levels (Shebl et al., 2010). However, in MEFs the inhibition of IRE1 leads to inhibition of ATF4 protein expression at 6 h but not at 2 h in response to ER stress (Figure 5.5). The ATF4 mRNA level was inhibited in the presence of IRE1 inhibitor, which indicated that the IRE1-XBP1 pathway could play a role in ATF4 mRNA expression in response to ER stress (Figure 5.6).

ATF4 translation is mediated by its 5’ UTR in response to ER stress (Harding, Zhang, Bertolotti, Zeng & Ron, 2000a) and I investigated ATF4 translation upregulation in response to ER stress, using luciferase as a reporter to measure the activity of 5’UTR of ATF4. In MIN6 cells the luciferase expression was maintained in response to ER stress although there was a significant inhibition in total protein synthesis (Figure 4.9). This indicates that ATF4 protein expression bypasses the inhibitory effect of eIF2α phosphorylation in MIN6 cells and the rate of translation in stressed cells is maintained.

However, in contrast I observed a significant increase of luciferase activity in other cell lines such as HEK293 (Figure 4.1) and MEFs (Figure 5.10), which suggested that the ATF4 protein expression is up-regulated in response to ER stress. It has been reported that selectively increases the translation of certain mRNAs under stress conditions including ATF4 (Ameri & Harris, 2008; Guan et al., 2014). Dey et al found that in MEFs, thapsigargin led to significant increase of the luciferase activity compared with non-treated cells (Dey, Baird, Zhou, Palam, Spandau & Wek, 2010). My results show that under ER stress ATF4 mRNA was inhibited in the presence of cycloheximide in MEFs cells (Figure 5.8), which suggests that transcription factors are synthesized in response to ER stress and essential for transcriptional control of ATF4 mRNA expression. Surprisingly, ATF4 mRNA level in MEFs the cells treated with protein inhibitor, cycloheximide shows a significant increase.

UPR becomes a tractable drug targets to develop a pharmacological compound that have ability to modulate its outputs to regulate ER stress. For example, PERK inhibitors from GlaxoSmithKline (GSK2606414 and GSK2656157) and compounds that inhibit PERK auto-phosphorylation by inhibiting eIF2 phosphatase (e.g. salubrinal) (Atkins et al., 2013). It was shown that GSK2656157, significantly decreased the growth of human tumour xenografts in mice (Atkins et al., 2013), and GSK2606414 has been shown to prevent neurodegeneration in prion-infected mice (Moreno et al., 2013) indicating that the PERK inhibitors can be used for the treatment of diseases associated with decontrolled UPR. It is worthy to mention that inhibition of PERK by GSK2656157 does not have the same effects as genetic inactivation of PERK on eIF2α phosphorylation in stressed cells (Krishnamoorthy, Rajesh, Mirzajani, Kesoglidou, Papadakis & Koromilas, 2014). Compounds that inhibit the RNase activity of IRE1α through its RNase domain, include 3-methoxy-6-bromosalicylaldehyde, 4μ8C. Another IRE1 inhibitor that interacts with the kinase domain of IRE1 is the clinically approved drug sunitinib which is an effective treatment for metastatic renal- cell carcinoma (Ravaud et al., 2016). Recently KIRA3 have been identified, which inhibits the kinase and RNase activities of IRE1α in vitro and in cells (Wang et al., 2012). A proteasome inhibitor, such as Bortezomib (BTZ), inhibits NF-κB pathway that plays a critical role in the pathogenesis of multiple myeloma (Ri, 2016). The indirectly targeting of the UPR upstream or downstream through pharmacological interventions or genetic modifications has been suggested for treatment of ER stress diseases. For instant, CHOP is activated by ER

stress through the PERK pathway as well as IRE1α and ATF6α. CHOP has been implicated to ER stress-mediated β-cell apoptosis in diabetes (Tabas & Ron, 2011). In addition, CHOP deficiency can delay the onset and symptoms of diabetes. Therefore, compounds that inhibit the expression or activity of CHOP could improve β-cell functional, and may have therapeutic potential for diabetes treatment (Duan et al., 2016). Another target is apoptosis signal-regulating kinase 1(ASK1) which is activated via different stimuli include oxidative stress, calcium influx, ER stress and DNA damage-inducing agents. The ASK1-JNK1/2 pathways were activated in diabetes (Kozar et al., 2004). Deleting ASK1 gene significantly reduced the rate of heart defects, Moreover, ASK1 deletion diminished diabetes-induced JNK1/2 phosphorylation and its downstream transcription factors and endoplasmic reticulum (ER) stress markers (Wang, Wu, Quon, Li & Yang, 2015).

It has been suggested that inhibition of eIF2α phosphorylation has promising potential to alleviate ER stress in several diseases (Boyce et al., 2005). For instant, Salubrinal indirectly inhibits GADD34–PP1 complex, which leads to phosphorylation of eIF2α, decrease translation rate and induces ATF4 expression (Boyce et al., 2005). Salubrinal reduces neuronal death and improves degeneration of Parkinson's disease (Colla et al., 2012). Guanabenz is another compound that directly binds to GADD34, preventing GADD34–PP1 complex. Guanabenz has been clinically approved as α2‑adrenergic receptor agonist for hypertension treatment (Matus, Glimcher & Hetz, 2011). Chemical chaperones have been shown to improve ER function by attenuating protein misfolding and reducing ER stress such as 4‑phenylbutyrate (4‑PBA) which has shown improve of glucose tolerance (Lindquist & Kelly, 2011). However, inhibition of ER stress may interrupt the adaptive stage of ER stress that has function as protective mechanism during early response of stress. Using gene therapy to modulate UPR signalling by directing an active UPR component to specific tissue could be most applicable and beneficial for treatment of ER stress related diseases. (Hetz, Chevet & Harding, 2013).

In conclusion, ER dysfunction is considered an important factor in a wide range of diseases, and the ER stress sensors pathways offer interesting therapeutic targets.

References

Adachi Y, Yamamoto K, Okada T, Yoshida H, Harada A, & Mori K (2008). ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. Cell Struct Funct 33**:** 75-89.

Ajlouni K, Jarrah N, El-Khateeb M, El-Zaheri M, El Shanti H, & Lidral A (2002). Wolfram syndrome: identification of a phenotypic and genotypic variant from Jordan. Am J Med Genet 115**:** 61-65.

Akter R, Cao P, Noor H, Ridgway Z, Tu LH, Wang H*, et al.* (2016). Islet Amyloid Polypeptide: Structure, Function, and Pathophysiology. J Diabetes Res 2016**:** 2798269.

Altmann M, Schmitz N, Berset C, & Trachsel H (1997). A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E. EMBO J 16**:** 1114-1121.

Ameri K, & Harris AL (2008). Activating transcription factor 4. Int J Biochem Cell Biol 40**:** 14-21.

American Diabetes A (2010). Diagnosis and classification of diabetes mellitus. Diabetes Care 33 Suppl 1**:** S62-69.

American Diabetes A (2014). Standards of medical care in diabetes--2014. Diabetes Care 37 Suppl 1**:** S14-80.

American Diabetes A (2015). Standards of medical care in diabetes-2015 abridged for primary care providers. Clin Diabetes 33**:** 97-111.

Amos AF, McCarty DJ, & Zimmet P (1997). The rising global burden of diabetes and its complications: estimates and projections to the year 2010. Diabet Med 14 Suppl 5**:** S1-85.

Andrews NC, Kotkow KJ, Ney PA, Erdjument-Bromage H, Tempst P, & Orkin SH (1993). The ubiquitous subunit of erythroid transcription factor NF-E2 is a small basic-leucine zipper protein related to the v-maf oncogene. Proc Natl Acad Sci U S A 90**:** 11488-11492.

Ashcroft FM, & Rorsman P (2012). Diabetes mellitus and the beta cell: the last ten years. Cell 148**:** 1160-1171.

Atkins C, Liu Q, Minthorn E, Zhang SY, Figueroa DJ, Moss K*, et al.* (2013). Characterization of a novel PERK kinase inhibitor with antitumor and antiangiogenic activity. Cancer Res 73**:** 1993-2002.

Bachar E, Ariav Y, Cerasi E, Kaiser N, & Leibowitz G (2010). Neuronal nitric oxide synthase protects the pancreatic beta cell from glucolipotoxicity-induced endoplasmic reticulum stress and apoptosis. Diabetologia 53**:** 2177-2187.

Back SH, & Kaufman RJ (2012). Endoplasmic reticulum stress and type 2 diabetes. Annual review of biochemistry 81**:** 767-793.

Baird TD, Palam LR, Fusakio ME, Willy JA, Davis CM, McClintick JN*, et al.* (2014). Selective mRNA translation during eIF2 phosphorylation induces expression of IBTKalpha. Mol Biol Cell 25**:** 1686-1697.

Baird TD, & Wek RC (2012). Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. Adv Nutr 3**:** 307-321.

Benhamron S, Hadar R, Iwawaky T, So JS, Lee AH, & Tirosh B (2014). Regulated IRE1-dependent decay participates in curtailing immunoglobulin secretion from plasma cells. Eur J Immunol 44**:** 867-876.

Bertolotti A, Zhang Y, Hendershot LM, Harding HP, & Ron D (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nature cell biology 2**:** 326-332.

Bespalova IN, Van Camp G, Bom SJ, Brown DJ, Cryns K, DeWan AT*, et al.* (2001). Mutations in the Wolfram syndrome 1 gene (WFS1) are a common cause of low frequency sensorineural hearing loss. Hum Mol Genet 10**:** 2501-2508.

Bezprozvanny I (2011). Role of inositol 1,4,5-trisphosphate receptors in pathogenesis of Huntington's disease and spinocerebellar ataxias. Neurochem Res 36**:** 1186-1197.

Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM*, et al.* (2003). A unified model for apical caspase activation. Mol Cell 11**:** 529-541.

Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D*, et al.* (2005). A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. Science 307**:** 935-939.

Bram Y, Frydman-Marom A, Yanai I, Gilead S, Shaltiel-Karyo R, Amdursky N*, et al.* (2014). Apoptosis induced by islet amyloid polypeptide soluble oligomers is neutralized by diabetes-associated specific antibodies. Sci Rep 4**:** 4267.

Brewer JW (2014). Regulatory crosstalk within the mammalian unfolded protein response. Cell Mol Life Sci 71**:** 1067-1079.

Brown M, Strudwick N, Suwara M, Sutcliffe LK, Mihai AD, Ali AA*, et al.* (2016). An initial phase of JNK activation inhibits cell death early in the endoplasmic reticulum stress response. J Cell Sci 129**:** 2317-2328.

Browne GJ, & Proud CG (2002). Regulation of peptide-chain elongation in mammalian cells. Eur J Biochem 269**:** 5360-5368.

Bruhat A, Jousse C, Carraro V, Reimold AM, Ferrara M, & Fafournoux P (2000). Amino acids control mammalian gene transcription: activating transcription factor 2 is essential for the amino acid responsiveness of the CHOP promoter. Mol Cell Biol 20**:** 7192-7204.

Brush MH, Weiser DC, & Shenolikar S (2003). Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. Mol Cell Biol 23**:** 1292-1303.

Burke JP, Williams K, Gaskill SP, Hazuda HP, Haffner SM, & Stern MP (1999). Rapid rise in the incidence of type 2 diabetes from 1987 to 1996: results from the San Antonio Heart Study. Arch Intern Med 159**:** 1450-1456.

Burr ML, van den Boomen DJ, Bye H, Antrobus R, Wiertz EJ, & Lehner PJ (2013). MHC class I molecules are preferentially ubiquitinated on endoplasmic reticulum luminal residues during HRD1 ubiquitin E3 ligase-mediated dislocation. Proc Natl Acad Sci U S A 110**:** 14290-14295.

Butler AE, Janson J, Soeller WC, & Butler PC (2003). Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid. Diabetes 52**:** 2304-2314.

Cadavez L, Montane J, Alcarraz-Vizan G, Visa M, Vidal-Fabrega L, Servitja JM*, et al.* (2014). Chaperones ameliorate beta cell dysfunction associated with human islet amyloid polypeptide overexpression. PLoS One 9**:** e101797.

Cao SS, & Kaufman RJ (2012). Unfolded protein response. Current biology : CB 22**:** R622-626.

Cao SS, & Kaufman RJ (2014). Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. Antioxid Redox Signal 21**:** 396-413.

Carrara M, Prischi F, & Ali MM (2013). UPR Signal Activation by Luminal Sensor Domains. International journal of molecular sciences 14**:** 6454-6466.

Cazanave SC, Elmi NA, Akazawa Y, Bronk SF, Mott JL, & Gores GJ (2010). CHOP and AP-1 cooperatively mediate PUMA expression during lipoapoptosis. Am J Physiol Gastrointest Liver Physiol 299**:** G236-243.

Chakrabarti A, Chen AW, & Varner JD (2011). A review of the mammalian unfolded protein response. Biotechnol Bioeng 108**:** 2777-2793.

Chan CP, Kok KH, Tang HM, Wong CM, & Jin DY (2013). Internal ribosome entry site-mediated translational regulation of ATF4 splice variant in mammalian unfolded protein response. Biochim Biophys Acta 1833**:** 2165-2175.

Chan SW, & Egan PA (2009). Effects of hepatitis C virus envelope glycoprotein unfolded protein response activation on translation and transcription. Arch Virol 154**:** 1631-1640.

Chang L, & Karin M (2001). Mammalian MAP kinase signalling cascades. Nature 410**:** 37-40.

Chen X, Shen J, & Prywes R (2002). The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. J Biol Chem 277**:** 13045-13052.

Cnop M, Foufelle F, & Velloso LA (2012). Endoplasmic reticulum stress, obesity and diabetes. Trends in molecular medicine 18**:** 59-68.

Coelho DS, & Domingos PM (2014). Physiological roles of regulated Ire1 dependent decay. Front Genet 5**:** 76.

Colla E, Coune P, Liu Y, Pletnikova O, Troncoso JC, Iwatsubo T*, et al.* (2012). Endoplasmic reticulum stress is important for the manifestations of alpha-synucleinopathy in vivo. J Neurosci 32**:** 3306-3320.

Cox JS, Shamu CE, & Walter P (1993). Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. Cell 73**:** 1197-1206.

Cox JS, & Walter P (1996). A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. Cell 87**:** 391-404.

Credle JJ, Forcelli PA, Delannoy M, Oaks AW, Permaul E, Berry DL*, et al.* (2015). alpha-Synuclein-mediated inhibition of ATF6 processing into COPII vesicles disrupts UPR signaling in Parkinson's disease. Neurobiol Dis 76**:** 112-125.

Cuello AC, Allard S, & Ferretti MT (2012). Evidence for the accumulation of Abeta immunoreactive material in the human brain and in transgenic animal models. Life Sci 91**:** 1141-1147.

Cui W, Li J, Ron D, & Sha B (2011). The structure of the PERK kinase domain suggests the mechanism for its activation. Acta Crystallogr D Biol Crystallogr 67**:** 423-428.

Cullinan SB, & Diehl JA (2004). PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. J Biol Chem 279**:** 20108-20117.

Cullinan SB, & Diehl JA (2006). Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway. Int J Biochem Cell Biol 38**:** 317-332.

Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, & Diehl JA (2003). Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol Cell Biol 23**:** 7198-7209.

Cunha DA, Hekerman P, Ladriere L, Bazarra-Castro A, Ortis F, Wakeham MC*, et al.* (2008). Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. J Cell Sci 121**:** 2308-2318.

Dai CL, Shi J, Chen Y, Iqbal K, Liu F, & Gong CX (2013). Inhibition of protein synthesis alters protein degradation through activation of protein kinase B (AKT). J Biol Chem 288**:** 23875-23883.

Dalal S, Foster CR, Das BC, Singh M, & Singh K (2012). Beta-adrenergic receptor stimulation induces endoplasmic reticulum stress in adult cardiac myocytes: role in apoptosis. Mol Cell Biochem 364**:** 59-70.

Danial NN, & Korsmeyer SJ (2004). Cell death: critical control points. Cell 116**:** 205-219.

Deldicque L (2013). Endoplasmic reticulum stress in human skeletal muscle: any contribution to sarcopenia? Front Physiol 4**:** 236.

Delepine M, Nicolino M, Barrett T, Golamaully M, Lathrop GM, & Julier C (2000). EIF2AK3, encoding translation initiation factor 2-alpha kinase 3, is mutated in patients with Wolcott-Rallison syndrome. Nat Genet 25**:** 406-409.

Dev K, Qiu H, Dong J, Zhang F, Barthlme D, & Hinnebusch AG (2010). The beta/Gcd7 subunit of eukaryotic translation initiation factor 2B (eIF2B), a guanine nucleotide exchange factor, is crucial for binding eIF2 in vivo. Mol Cell Biol 30**:** 5218-5233.

Dever TE (2002). Gene-specific regulation by general translation factors. Cell 108**:** 545-556.

Dever TE, Chen JJ, Barber GN, Cigan AM, Feng L, Donahue TF*, et al.* (1993). Mammalian eukaryotic initiation factor 2 alpha kinases functionally substitute for GCN2 protein kinase in the GCN4 translational control mechanism of yeast. Proc Natl Acad Sci U S A 90**:** 4616-4620.

Dey S, Baird TD, Zhou D, Palam LR, Spandau DF, & Wek RC (2010). Both transcriptional regulation and translational control of ATF4 are central to the integrated stress response. J Biol Chem 285**:** 33165-33174.

Dhanasekaran DN, & Johnson GL (2007). MAPKs: function, regulation, role in cancer and therapeutic targeting. Oncogene 26**:** 3097-3099.

Dmitriev SE, Terenin IM, Andreev DE, Ivanov PA, Dunaevsky JE, Merrick WC*, et al.* (2010). GTP-independent tRNA delivery to the ribosomal P-site by a novel eukaryotic translation factor. J Biol Chem 285**:** 26779-26787.

Duan H, Arora D, Li Y, Setiadi H, Xu D, Lim HY*, et al.* (2016). Identification of 1,2,3-triazole derivatives that protect pancreatic beta cells against endoplasmic reticulum stress-mediated dysfunction and death through the inhibition of C/EBP-homologous protein expression. Bioorg Med Chem 24**:** 2621-2630.

Duncan RF, & Hershey JW (1989). Protein synthesis and protein phosphorylation during heat stress, recovery, and adaptation. J Cell Biol 109**:** 1467-1481.

Dyrks T, Dyrks E, Monning U, Urmoneit B, Turner J, & Beyreuther K (1993). Generation of beta A4 from the amyloid protein precursor and fragments thereof. FEBS Lett 335**:** 89-93.

Eizirik DL, Cardozo AK, & Cnop M (2008). The role for endoplasmic reticulum stress in diabetes mellitus. Endocr Rev 29**:** 42-61.

Ellgaard L, & Helenius A (2003). Quality control in the endoplasmic reticulum. Nat Rev Mol Cell Biol 4**:** 181-191.

Ellgaard L, Molinari M, & Helenius A (1999). Setting the standards: quality control in the secretory pathway. Science 286**:** 1882-1888.

Elsby R, Heiber JF, Reid P, Kimball SR, Pavitt GD, & Barber GN (2011). The alpha subunit of eukaryotic initiation factor 2B (eIF2B) is required for eIF2-mediated translational suppression of vesicular stomatitis virus. J Virol 85**:** 9716-9725.

Endres K, & Reinhardt S (2013). ER-stress in Alzheimer's disease: turning the scale? Am J Neurodegener Dis 2**:** 247-265.

Evans-Molina C, Hatanaka M, & Mirmira RG (2013). Lost in translation: endoplasmic reticulum stress and the decline of beta-cell health in diabetes mellitus. Diabetes Obes Metab 15 Suppl 3**:** 159-169.

Fedorov AN, & Baldwin TO (1997). Cotranslational protein folding. J Biol Chem 272**:** 32715-32718.

Feng B, Yao PM, Li Y, Devlin CM, Zhang D, Harding HP*, et al.* (2003). The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. Nature cell biology 5**:** 781-792.

Feng D, Wei J, Gupta S, McGrath BC, & Cavener DR (2009). Acute ablation of PERK results in ER dysfunctions followed by reduced insulin secretion and cell proliferation. BMC Cell Biol 10**:** 61.

Fonseca SG, Fukuma M, Lipson KL, Nguyen LX, Allen JR, Oka Y*, et al.* (2005). WFS1 is a novel component of the unfolded protein response and maintains homeostasis of the endoplasmic reticulum in pancreatic beta-cells. J Biol Chem 280**:** 39609-39615.

Fonseca SG, Gromada J, & Urano F (2011a). Endoplasmic reticulum stress and pancreatic beta-cell death. Trends Endocrin Met 22**:** 266-274.

Fonseca SG, Gromada J, & Urano F (2011b). Endoplasmic reticulum stress and pancreatic beta-cell death. Trends in endocrinology and metabolism: TEM 22**:** 266-274.

Fonseca SG, Ishigaki S, Oslowski CM, Lu S, Lipson KL, Ghosh R*, et al.* (2010). Wolfram syndrome 1 gene negatively regulates ER stress signaling in rodent and human cells. The Journal of clinical investigation 120**:** 744-755.

Frand AR, Cuozzo JW, & Kaiser CA (2000). Pathways for protein disulphide bond formation. Trends Cell Biol 10**:** 203-210.

Frickel EM, Riek R, Jelesarov I, Helenius A, Wuthrich K, & Ellgaard L (2002). TROSY-NMR reveals interaction between ERp57 and the tip of the calreticulin P-domain. Proc Natl Acad Sci U S A 99**:** 1954-1959.

Friedland DE, Wooten WN, LaVoy JE, Hagedorn CH, & Goss DJ (2005). A mutant of eukaryotic protein synthesis initiation factor eIF4E(K119A) has an increased binding affinity for both m7G cap analogues and eIF4G peptides. Biochemistry 44**:** 4546-4550.

Friedman LG, Lachenmayer ML, Wang J, He L, Poulose SM, Komatsu M*, et al.* (2012). Disrupted autophagy leads to dopaminergic axon and dendrite degeneration and promotes presynaptic accumulation of alpha-synuclein and LRRK2 in the brain. J Neurosci 32**:** 7585-7593.

Fu L, Balasubramanian M, Shan J, Dudenhausen EE, & Kilberg MS (2011). Auto-activation of c-JUN gene by amino acid deprivation of hepatocellular carcinoma cells reveals a novel c-JUN-mediated signaling pathway. J Biol Chem 286**:** 36724-36738.

Gething MJ (1999). Role and regulation of the ER chaperone BiP. Semin Cell Dev Biol 10**:** 465-472.

Gingras AC, Raught B, & Sonenberg N (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annual review of biochemistry 68**:** 913-963.

Goh SG, Rusli BN, & Khalid BA (2015). Evolution of diabetes management in the 21st century: the contribution of quality of life measurement in Asians. Asia Pac J Clin Nutr 24**:** 190-198.

Gorlach A, Klappa P, & Kietzmann T (2006). The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. Antioxid Redox Signal 8**:** 1391-1418.

Gorman AM, Healy SJ, Jager R, & Samali A (2012). Stress management at the ER: regulators of ER stress-induced apoptosis. Pharmacol Ther 134**:** 306-316.

Grzmil M, Kaulfuss S, Thelen P, Hemmerlein B, Schweyer S, Obenauer S*, et al.* (2006). Expression and functional analysis of Bax inhibitor-1 in human breast cancer cells. J Pathol 208**:** 340-349.

Grzmil M, Thelen P, Hemmerlein B, Schweyer S, Voigt S, Mury D*, et al.* (2003). Bax inhibitor-1 is overexpressed in prostate cancer and its specific down-regulation by RNA interference leads to cell death in human prostate carcinoma cells. Am J Pathol 163**:** 543-552.

Guan BJ, Krokowski D, Majumder M, Schmotzer CL, Kimball SR, Merrick WC*, et al.* (2014). Translational control during endoplasmic reticulum stress beyond phosphorylation of the translation initiation factor eIF2alpha. J Biol Chem 289**:** 12593-12611.

Gupta S, Giricz Z, Natoni A, Donnelly N, Deegan S, Szegezdi E*, et al.* (2012). NOXA contributes to the sensitivity of PERK-deficient cells to ER stress. FEBS Lett 586**:** 4023-4030.

Gupta S, McGrath B, & Cavener DR (2010). PERK (EIF2AK3) regulates proinsulin trafficking and quality control in the secretory pathway. Diabetes 59**:** 1937-1947.

Haghighat A, Mader S, Pause A, & Sonenberg N (1995). Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. EMBO J 14**:** 5701-5709.

Hai T, & Hartman MG (2001). The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. Gene 273**:** 1-11.

Hammond C, Braakman I, & Helenius A (1994). Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. Proc Natl Acad Sci U S A 91**:** 913-917.

Han D, Lerner AG, Vande Walle L, Upton JP, Xu W, Hagen A*, et al.* (2009). IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. Cell 138**:** 562-575.

Han J, Back SH, Hur J, Lin YH, Gildersleeve R, Shan J*, et al.* (2013). ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. Nature cell biology 15**:** 481-490.

Hardin BK (2006) *The world of the cell.*: USA.

Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M*, et al.* (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell 6**:** 1099-1108.

Harding HP, & Ron D (2002). Endoplasmic reticulum stress and the development of diabetes: a review. Diabetes 51 Suppl 3**:** S455-461.

Harding HP, Zhang Y, Bertolotti A, Zeng H, & Ron D (2000a). Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol Cell 5**:** 897-904.

Harding HP, Zhang Y, & Ron D (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 397**:** 271-274.

Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M*, et al.* (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell 11**:** 619-633.

Harding HP, Zhang YH, Bertolotti A, Zeng HQ, & Ron D (2000b). Perk is essential for translational regulation and cell survival during the unfolded protein response. Molecular Cell 5**:** 897-904.

Hasnain SZ, Prins JB, & McGuckin MA (2016). Oxidative and endoplasmic reticulum stress in beta-cell dysfunction in diabetes. J Mol Endocrinol 56**:** R33-54.

Hauck AK, & Bernlohr DA (2016). Oxidative stress and lipotoxicity. J Lipid Res.

Haze K, Yoshida H, Yanagi H, Yura T, & Mori K (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 10**:** 3787-3799.

He CH, Gong P, Hu B, Stewart D, Choi ME, Choi AM*, et al.* (2001). Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation. J Biol Chem 276**:** 20858-20865.

Hebert DN, Foellmer B, & Helenius A (1996). Calnexin and calreticulin promote folding, delay oligomerization and suppress degradation of influenza hemagglutinin in microsomes. EMBO J 15**:** 2961-2968.

Hellen CU (2009). IRES-induced conformational changes in the ribosome and the mechanism of translation initiation by internal ribosomal entry. Biochim Biophys Acta 1789**:** 558-570.

Herbert TP, & Laybutt DR (2016). A Reevaluation of the Role of the Unfolded Protein Response in Islet Dysfunction: Maladaptation or a Failure to Adapt? Diabetes 65**:** 1472-1480.

Hetz C, Bernasconi P, Fisher J, Lee AH, Bassik MC, Antonsson B*, et al.* (2006). Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha. Science 312**:** 572-576.

Hetz C, Chevet E, & Harding HP (2013). Targeting the unfolded protein response in disease. Nat Rev Drug Discov 12**:** 703-719.

Hetz C, & Glimcher LH (2009). Fine-tuning of the unfolded protein response: Assembling the IRE1alpha interactome. Mol Cell 35**:** 551-561.

Hiller MM, Finger A, Schweiger M, & Wolf DH (1996). ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. Science 273**:** 1725-1728.

Hills AP, Shultz SP, Soares MJ, Byrne NM, Hunter GR, King NA*, et al.* (2010). Resistance training for obese, type 2 diabetic adults: a review of the evidence. Obes Rev 11**:** 740-749.

Hinnebusch AG (2011). Molecular mechanism of scanning and start codon selection in eukaryotes. Microbiol Mol Biol Rev 75**:** 434-467, first page of table of contents.

Hinnebusch AG (2014). The scanning mechanism of eukaryotic translation initiation. Annual review of biochemistry 83**:** 779-812.

Hinnebusch AG, & Lorsch JR (2012). The mechanism of eukaryotic translation initiation: new insights and challenges. Cold Spring Harb Perspect Biol 4.

Hollien J, Lin JH, Li H, Stevens N, Walter P, & Weissman JS (2009). Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. J Cell Biol 186**:** 323-331.

Hollien J, & Weissman JS (2006). Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. Science 313**:** 104-107.

Hong M, Luo S, Baumeister P, Huang JM, Gogia RK, Li M*, et al.* (2004). Underglycosylation of ATF6 as a novel sensing mechanism for activation of the unfolded protein response. J Biol Chem 279**:** 11354-11363.

Hu P, Han Z, Couvillon AD, Kaufman RJ, & Exton JH (2006). Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. Mol Cell Biol 26**:** 3071-3084.

Hull RL, Zraika S, Udayasankar J, Aston-Mourney K, Subramanian SL, & Kahn SE (2009). Amyloid formation in human IAPP transgenic mouse islets and pancreas, and human pancreas, is not associated with endoplasmic reticulum stress. Diabetologia 52**:** 1102-1111.

Igoillo-Esteve M, Marselli L, Cunha DA, Ladriere L, Ortis F, Grieco FA*, et al.* (2010). Palmitate induces a pro-inflammatory response in human pancreatic islets that mimics CCL2 expression by beta cells in type 2 diabetes. Diabetologia 53**:** 1395-1405.

Ishihara H, Takeda S, Tamura A, Takahashi R, Yamaguchi S, Takei D*, et al.* (2004). Disruption of the WFS1 gene in mice causes progressive beta-cell loss and impaired stimulus-secretion coupling in insulin secretion. Hum Mol Genet 13**:** 1159-1170.

Ivanova EA, & Orekhov AN (2016). The Role of Endoplasmic Reticulum Stress and Unfolded Protein Response in Atherosclerosis. International journal of molecular sciences 17.

Iwawaki T, Akai R, Yamanaka S, & Kohno K (2009). Function of IRE1 alpha in the placenta is essential for placental development and embryonic viability. Proc Natl Acad Sci U S A 106**:** 16657-16662.

Iwawaki T, & Tokuda M (2011). Function of yeast and amphioxus tRNA ligase in IRE1alpha-dependent XBP1 mRNA splicing. Biochem Biophys Res Commun 413**:** 527-531.

Jackson RJ, Hellen CU, & Pestova TV (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nat Rev Mol Cell Biol 11**:** 113-127.

Jaenicke R, & Seckler R (1997). Protein misassembly in vitro. Adv Protein Chem 50**:** 1-59.

Jager R, Bertrand MJ, Gorman AM, Vandenabeele P, & Samali A (2012). The unfolded protein response at the crossroads of cellular life and death during endoplasmic reticulum stress. Biol Cell 104**:** 259-270.

Jiang P, Gan M, Lin WL, & Yen SH (2014). Nutrient deprivation induces alpha-synuclein aggregation through endoplasmic reticulum stress response and SREBP2 pathway. Front Aging Neurosci 6**:** 268.

Jonas JC, Bensellam M, Duprez J, Elouil H, Guiot Y, & Pascal SM (2009). Glucose regulation of islet stress responses and beta-cell failure in type 2 diabetes. Diabetes Obes Metab 11 Suppl 4**:** 65-81.

Julier C, & Nicolino M (2010). Wolcott-Rallison syndrome. Orphanet J Rare Dis 5**:** 29.

Kanekura K, Ma X, Murphy JT, Zhu LJ, Diwan A, & Urano F (2015). IRE1 prevents endoplasmic reticulum membrane permeabilization and cell death under pathological conditions. Sci Signal 8**:** ra62.

Kaneto H, & Matsuoka TA (2015). Role of pancreatic transcription factors in maintenance of mature beta-cell function. International journal of molecular sciences 16**:** 6281-6297.

Kaneto H, Miyatsuka T, Kawamori D, Yamamoto K, Kato K, Shiraiwa T*, et al.* (2008). PDX-1 and MafA play a crucial role in pancreatic beta-cell differentiation and maintenance of mature beta-cell function. Endocr J 55**:** 235-252.

Kapp LD, & Lorsch JR (2004). The molecular mechanics of eukaryotic translation. Annual review of biochemistry 73**:** 657-704.

Karaskov E, Scott C, Zhang L, Teodoro T, Ravazzola M, & Volchuk A (2006). Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis. Endocrinology 147**:** 3398-3407.

Karst AM, & Li G (2007). BH3-only proteins in tumorigenesis and malignant melanoma. Cell Mol Life Sci 64**:** 318-330.

Kaufman RJ (2004). Regulation of mRNA translation by protein folding in the endoplasmic reticulum. Trends Biochem Sci 29**:** 152-158.

Kaufman RJ, Scheuner D, Schroder M, Shen X, Lee K, Liu CY*, et al.* (2002). The unfolded protein response in nutrient sensing and differentiation. Nat Rev Mol Cell Biol 3**:** 411-421.

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

Kimata Y, Kimata YI, Shimizu Y, Abe H, Farcasanu IC, Takeuchi M*, et al.* (2003). Genetic evidence for a role of BiP/Kar2 that regulates Ire1 in response to accumulation of unfolded proteins. Mol Biol Cell 14**:** 2559-2569.

Komar AA, & Hatzoglou M (2005). Internal ribosome entry sites in cellular mRNAs: mystery of their existence. J Biol Chem 280**:** 23425-23428.

Kondo S, Murakami T, Tatsumi K, Ogata M, Kanemoto S, Otori K*, et al.* (2005). OASIS, a CREB/ATF-family member, modulates UPR signalling in astrocytes. Nature cell biology 7**:** 186-194.

Kozar K, Ciemerych MA, Rebel VI, Shigematsu H, Zagozdzon A, Sicinska E*, et al.* (2004). Mouse development and cell proliferation in the absence of D-cyclins. Cell 118**:** 477-491.

Krishnamoorthy J, Rajesh K, Mirzajani F, Kesoglidou P, Papadakis AI, & Koromilas AE (2014). Evidence for eIF2alpha phosphorylation-independent effects of GSK2656157, a novel catalytic inhibitor of PERK with clinical implications. Cell Cycle 13**:** 801-806.

Kwak MK, Wakabayashi N, Itoh K, Motohashi H, Yamamoto M, & Kensler TW (2003). Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway. Identification of novel gene clusters for cell survival. J Biol Chem 278**:** 8135-8145.

Landau G, Kodali VK, Malhotra JD, & Kaufman RJ (2013). Detection of oxidative damage in response to protein misfolding in the endoplasmic reticulum. Methods Enzymol 526**:** 231-250.

Laybutt DR, Preston AM, Akerfeldt MC, Kench JG, Busch AK, Biankin AV*, et al.* (2007). Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. Diabetologia 50**:** 752-763.

Lin JH, Li H, Yasumura D, Cohen HR, Zhang C, Panning B*, et al.* (2007). IRE1 signaling affects cell fate during the unfolded protein response. Science 318**:** 944-949.

Lindquist SL, & Kelly JW (2011). Chemical and biological approaches for adapting proteostasis to ameliorate protein misfolding and aggregation diseases: progress and prognosis. Cold Spring Harb Perspect Biol 3.

Livak KJ, & Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25**:** 402-408.

Lizundia R, Chaussepied M, Huerre M, Werling D, Di Santo JP, & Langsley G (2006). c-Jun NH2-terminal kinase/c-Jun signaling promotes survival and metastasis of B lymphocytes transformed by Theileria. Cancer Res 66**:** 6105-6110.

Lou Frances G, Soto de Ruiz S, Lopez-Madrazo Hernandez MJ, Macipe Costa R, & Rodriguez Rigual M (2008). [Wolfram syndrome. Clinical and genetic study in two families]. An Pediatr (Barc) 68**:** 54-57.

Lu PD, Harding HP, & Ron D (2004). Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. J Cell Biol 167**:** 27-33.

Lu S, Kanekura K, Hara T, Mahadevan J, Spears LD, Oslowski CM*, et al.* (2014). A calcium-dependent protease as a potential therapeutic target for Wolfram syndrome. Proc Natl Acad Sci U S A 111**:** E5292-5301.

Ma XM, & Blenis J (2009). Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol 10**:** 307-318.

Maattanen P, Gehring K, Bergeron JJ, & Thomas DY (2010). Protein quality control in the ER: the recognition of misfolded proteins. Semin Cell Dev Biol 21**:** 500-511.

MacCallum PR, Jack SC, Egan PA, McDermott BT, Elliott RM, & Chan SW (2006). Cap-dependent and hepatitis C virus internal ribosome entry site-mediated translation are modulated by phosphorylation of eIF2alpha under oxidative stress. J Gen Virol 87**:** 3251-3262.

MacKenzie SH, & Clark AC (2012). Death by caspase dimerization. Adv Exp Med Biol 747**:** 55-73.

Malhotra JD, & Kaufman RJ (2007). The endoplasmic reticulum and the unfolded protein response. Semin Cell Dev Biol 18**:** 716-731.

Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R*, et al.* (2004). CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes & development 18**:** 3066-3077.

Masuoka HC, & Townes TM (2002). Targeted disruption of the activating transcription factor 4 gene results in severe fetal anemia in mice. Blood 99**:** 736-745.

Mathai JP, Germain M, & Shore GC (2005). BH3-only BIK regulates BAX,BAK-dependent release of Ca2+ from endoplasmic reticulum stores and mitochondrial apoptosis during stress-induced cell death. J Biol Chem 280**:** 23829-23836.

Matsuzaki S, Hiratsuka T, Kuwahara R, Katayama T, & Tohyama M (2010). Caspase-4 is partially cleaved by calpain via the impairment of Ca2+ homeostasis under the ER stress. Neurochem Int 56**:** 352-356.

Mattson MP, & Chan SL (2003). Neuronal and glial calcium signaling in Alzheimer's disease. Cell Calcium 34**:** 385-397.

Matus S, Glimcher LH, & Hetz C (2011). Protein folding stress in neurodegenerative diseases: a glimpse into the ER. Current opinion in cell biology 23**:** 239-252.

Maurel M, Chevet E, Tavernier J, & Gerlo S (2014). Getting RIDD of RNA: IRE1 in cell fate regulation. Trends Biochem Sci 39**:** 245-254.

McCullough KD, Martindale JL, Klotz LO, Aw TY, & Holbrook NJ (2001). Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 21**:** 1249-1259.

Meijer HA, Kong YW, Lu WT, Wilczynska A, Spriggs RV, Robinson SW*, et al.* (2013). Translational repression and eIF4A2 activity are critical for microRNA-mediated gene regulation. Science 340**:** 82-85.

Merrick WC (2010). Eukaryotic protein synthesis: still a mystery. J Biol Chem 285**:** 21197-21201.

Michalak M, Groenendyk J, Szabo E, Gold LI, & Opas M (2009). Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. Biochem J 417**:** 651-666.

Milanski M, Degasperi G, Coope A, Morari J, Denis R, Cintra DE*, et al.* (2009). Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. J Neurosci 29**:** 359-370.

Milhavet O, Martindale JL, Camandola S, Chan SL, Gary DS, Cheng A*, et al.* (2002). Involvement of Gadd153 in the pathogenic action of presenilin-1 mutations. J Neurochem 83**:** 673-681.

Mirmira RG (2012). Saturated free fatty acids: islet beta cell "stressERs". Endocrine 42**:** 1-2.

Miyamoto N, Izumi H, Miyamoto R, Bin H, Kondo H, Tawara A*, et al.* (2011). Transcriptional regulation of activating transcription factor 4 under oxidative stress in retinal pigment epithelial ARPE-19/HPV-16 cells. Invest Ophthalmol Vis Sci 52**:** 1226-1234.

Moenner M, Pluquet O, Bouchecareilh M, & Chevet E (2007). Integrated endoplasmic reticulum stress responses in cancer. Cancer Res 67**:** 10631-10634.

Moi P, Chan K, Asunis I, Cao A, & Kan YW (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci U S A 91**:** 9926-9930.

Moore K, & Hollien J (2015). Ire1-mediated decay in mammalian cells relies on mRNA sequence, structure, and translational status. Mol Biol Cell 26**:** 2873-2884.

Moreno JA, Halliday M, Molloy C, Radford H, Verity N, Axten JM*, et al.* (2013). Oral treatment targeting the unfolded protein response prevents neurodegeneration and clinical disease in prion-infected mice. Sci Transl Med 5**:** 206ra138.

Mori K, Kawahara T, Yoshida H, Yanagi H, & Yura T (1996). Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. Genes Cells 1**:** 803-817.

Morishima N, Nakanishi K, Tsuchiya K, Shibata T, & Seiwa E (2004). Translocation of Bim to the endoplasmic reticulum (ER) mediates ER stress signaling for activation of caspase-12 during ER stress-induced apoptosis. J Biol Chem 279**:** 50375-50381.

Morita S, Sakagashira S, Shimajiri Y, Eberhardt NL, Kondo T, Kondo T*, et al.* (2011). Autophagy protects against human islet amyloid polypeptide-associated apoptosis. J Diabetes Investig 2**:** 48-55.

Nakamura T, Furuhashi M, Li P, Cao H, Tuncman G, Sonenberg N*, et al.* (2010). Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis. Cell 140**:** 338-348.

Novoa I, Zeng H, Harding HP, & Ron D (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. J Cell Biol 153**:** 1011-1022.

Oda Y, Okada T, Yoshida H, Kaufman RJ, Nagata K, & Mori K (2006). Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. J Cell Biol 172**:** 383-393.

Oliver JD, Roderick HL, Llewellyn DH, & High S (1999). ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin. Mol Biol Cell 10**:** 2573-2582.

Oslowski CM, & Urano F (2011). The binary switch that controls the life and death decisions of ER stressed beta cells. Current opinion in cell biology 23**:** 207-215.

Oyadomari S, & Mori M (2004). Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ 11**:** 381-389.

Palam LR, Baird TD, & Wek RC (2011). Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance CHOP translation. J Biol Chem 286**:** 10939-10949.

Pelletier J, & Sonenberg N (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature 334**:** 320-325.

Pestova TV, Hellen CU, & Shatsky IN (1996). Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. Mol Cell Biol 16**:** 6859-6869.

Pestova TV, Shatsky IN, Fletcher SP, Jackson RJ, & Hellen CU (1998). A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. Genes & development 12**:** 67-83.

Phillips MJ, & Voeltz GK (2015). Structure and function of ER membrane contact sites with other organelles. Nat Rev Mol Cell Biol.

Pirot P, Ortis F, Cnop M, Ma Y, Hendershot LM, Eizirik DL*, et al.* (2007). Transcriptional regulation of the endoplasmic reticulum stress gene chop in pancreatic insulin-producing cells. Diabetes 56**:** 1069-1077.

Pluquet O, Pourtier A, & Abbadie C (2015). The unfolded protein response and cellular senescence. A review in the theme: cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. American journal of physiology Cell physiology 308**:** C415-425.

Poulin F, Gingras AC, Olsen H, Chevalier S, & Sonenberg N (1998). 4E-BP3, a new member of the eukaryotic initiation factor 4E-binding protein family. J Biol Chem 273**:** 14002-14007.

Proud CG (2007). Signalling to translation: how signal transduction pathways control the protein synthetic machinery. Biochem J 403**:** 217-234.

Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B*, et al.* (2003). JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. Neuron 38**:** 899-914.

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

Rafieian-Kopaei M, Setorki M, Doudi M, Baradaran A, & Nasri H (2014). Atherosclerosis: process, indicators, risk factors and new hopes. Int J Prev Med 5**:** 927-946.

Ramaiah KV, Davies MV, Chen JJ, & Kaufman RJ (1994). Expression of mutant eukaryotic initiation factor 2 alpha subunit (eIF-2 alpha) reduces inhibition of guanine nucleotide exchange activity of eIF-2B mediated by eIF-2 alpha phosphorylation. Mol Cell Biol 14**:** 4546-4553.

Rao J, Zhang C, Wang P, Lu L, Qian X, Qin J*, et al.* (2015). C/EBP homologous protein (CHOP) contributes to hepatocyte death via the promotion of ERO1alpha signalling in acute liver failure. Biochem J 466**:** 369-378.

Rashmi R, Pillai SG, Vijayalingam S, Ryerse J, & Chinnadurai G (2008). BH3-only protein BIK induces caspase-independent cell death with autophagic features in Bcl-2 null cells. Oncogene 27**:** 1366-1375.

Ravaud A, Motzer RJ, Pandha HS, George DJ, Pantuck AJ, Patel A*, et al.* (2016). Adjuvant Sunitinib in High-Risk Renal-Cell Carcinoma after Nephrectomy. N Engl J Med.

Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, & Lee AS (2003). Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. J Biol Chem 278**:** 20915-20924.

Ri M (2016). Endoplasmic-reticulum stress pathway-associated mechanisms of action of proteasome inhibitors in multiple myeloma. Int J Hematol.

Ri M, Tashiro E, Oikawa D, Shinjo S, Tokuda M, Yokouchi Y*, et al.* (2012). Identification of Toyocamycin, an agent cytotoxic for multiple myeloma cells, as a potent inhibitor of ER stress-induced XBP1 mRNA splicing. Blood Cancer J 2**:** e79.

Ripsin CM, Kang H, & Urban RJ (2009). Management of blood glucose in type 2 diabetes mellitus. Am Fam Physician 79**:** 29-36.

Robertson HD, & Branch AD (1987). RNA processing--a meeting review. Genes & development 1**:** 415-417.

Robertson RP (2006). Oxidative stress and impaired insulin secretion in type 2 diabetes. Curr Opin Pharmacol 6**:** 615-619.

Robertson RP, Harmon J, Tran PO, & Poitout V (2004). Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. Diabetes 53 Suppl 1**:** S119-124.

Roderick HL, Lechleiter JD, & Camacho P (2000). Cytosolic phosphorylation of calnexin controls intracellular Ca(2+) oscillations via an interaction with SERCA2b. J Cell Biol 149**:** 1235-1248.

Rohacs T (2013). Regulation of transient receptor potential channels by the phospholipase C pathway. Adv Biol Regul 53**:** 341-355.

Rojas M, Vasconcelos G, & Dever TE (2015). An eIF2alpha-binding motif in protein phosphatase 1 subunit GADD34 and its viral orthologs is required to promote dephosphorylation of eIF2alpha. Proc Natl Acad Sci U S A 112**:** E3466-3475.

Romero-Ramirez L, Cao H, Regalado MP, Kambham N, Siemann D, Kim JJ*, et al.* (2009). X box-binding protein 1 regulates angiogenesis in human pancreatic adenocarcinomas. Transl Oncol 2**:** 31-38.

Rong YP, Bultynck G, Aromolaran AS, Zhong F, Parys JB, De Smedt H*, et al.* (2009). The BH4 domain of Bcl-2 inhibits ER calcium release and apoptosis by binding the regulatory and coupling domain of the IP3 receptor. Proc Natl Acad Sci U S A 106**:** 14397-14402.

Rowland AA, & Voeltz GK (2012). Endoplasmic reticulum-mitochondria contacts: function of the junction. Nat Rev Mol Cell Biol 13**:** 607-625.

Rowlands AG, Panniers R, & Henshaw EC (1988). The catalytic mechanism of guanine nucleotide exchange factor action and competitive inhibition by phosphorylated eukaryotic initiation factor 2. J Biol Chem 263**:** 5526-5533.

Ruggiano A, Foresti O, & Carvalho P (2014). Quality control: ER-associated degradation: protein quality control and beyond. J Cell Biol 204**:** 869-879.

Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM*, et al.* (2006). Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. PLoS Biol 4**:** e374.

Rutkowski DT, & Kaufman RJ (2004). A trip to the ER: coping with stress. Trends Cell Biol 14**:** 20-28.

Saito A, Ochiai K, Kondo S, Tsumagari K, Murakami T, Cavener DR*, et al.* (2011). Endoplasmic reticulum stress response mediated by the PERK-eIF2(alpha)-ATF4 pathway is involved in osteoblast differentiation induced by BMP2. J Biol Chem 286**:** 4809-4818.

Salgin B, Ong KK, Thankamony A, Emmett P, Wareham NJ, & Dunger DB (2012). Higher fasting plasma free fatty acid levels are associated with lower insulin secretion in children and adults and a higher incidence of type 2 diabetes. J Clin Endocrinol Metab 97**:** 3302-3309.

Sambamurti K, Shioi J, Anderson JP, Pappolla MA, & Robakis NK (1992). Evidence for intracellular cleavage of the Alzheimer's amyloid precursor in PC12 cells. J Neurosci Res 33**:** 319-329.

Sano R, & Reed JC (2013). ER stress-induced cell death mechanisms. Biochim Biophys Acta 1833**:** 3460-3470.

Scheper GC, van Kollenburg B, Hu J, Luo Y, Goss DJ, & Proud CG (2002). Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. J Biol Chem 277**:** 3303-3309.

Scheper W, & Hoozemans JJ (2015). The unfolded protein response in neurodegenerative diseases: a neuropathological perspective. Acta Neuropathol 130**:** 315-331.

Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P*, et al.* (2001). Translational control is required for the unfolded protein response and in vivo glucose homeostasis. Mol Cell 7**:** 1165-1176.

Scheuner D, Vander Mierde D, Song B, Flamez D, Creemers JW, Tsukamoto K*, et al.* (2005). Control of mRNA translation preserves endoplasmic reticulum function in beta cells and maintains glucose homeostasis. Nature medicine 11**:** 757-764.

Schneider-Poetsch T, Ju J, Eyler DE, Dang Y, Bhat S, Merrick WC*, et al.* (2010). Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. Nat Chem Biol 6**:** 209-217.

Schrag JD, Bergeron JJ, Li Y, Borisova S, Hahn M, Thomas DY*, et al.* (2001). The Structure of calnexin, an ER chaperone involved in quality control of protein folding. Mol Cell 8**:** 633-644.

Schroder M (2008). Endoplasmic reticulum stress responses. Cell Mol Life Sci 65**:** 862-894.

Schroder M, & Kaufman RJ (2005). The mammalian unfolded protein response. Annual review of biochemistry 74**:** 739-789.

Senee V, Vattem KM, Delepine M, Rainbow LA, Haton C, Lecoq A*, et al.* (2004). Wolcott-Rallison Syndrome: clinical, genetic, and functional study of EIF2AK3 mutations and suggestion of genetic heterogeneity. Diabetes 53**:** 1876-1883.

Senft D, & Ronai ZA (2015). UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. Trends Biochem Sci 40**:** 141-148.

Shamu CE, Cox JS, & Walter P (1994). The unfolded-protein-response pathway in yeast. Trends Cell Biol 4**:** 56-60.

Shebl FM, Pinto LA, Garcia-Pineres A, Lempicki R, Williams M, Harro C*, et al.* (2010). Comparison of mRNA and protein measures of cytokines following vaccination with human papillomavirus-16 L1 virus-like particles. Cancer Epidemiol Biomarkers Prev 19**:** 978-981.

Shi Y (2002). Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 9**:** 459-470.

Shibata Y, Shemesh T, Prinz WA, Palazzo AF, Kozlov MM, & Rapoport TA (2010). Mechanisms determining the morphology of the peripheral ER. Cell 143**:** 774-788.

Sibartie P, & Quinlivan J (2015). Implementation of the International Association of Diabetes and Pregnancy Study Groups Criteria: Not Always a Cause for Concern. J Pregnancy 2015**:** 754085.

Sidrauski C, & Walter P (1997). The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. Cell 90**:** 1031-1039.

Siu F, Bain PJ, LeBlanc-Chaffin R, Chen H, & Kilberg MS (2002). ATF4 is a mediator of the nutrient-sensing response pathway that activates the human asparagine synthetase gene. J Biol Chem 277**:** 24120-24127.

Smith MA, & Schnellmann RG (2012). Calpains, mitochondria, and apoptosis. Cardiovasc Res 96**:** 32-37.

Smyth JT, Hwang SY, Tomita T, DeHaven WI, Mercer JC, & Putney JW (2010). Activation and regulation of store-operated calcium entry. J Cell Mol Med 14**:** 2337-2349.

Song B, Scheuner D, Ron D, Pennathur S, & Kaufman RJ (2008). Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. The Journal of clinical investigation 118**:** 3378-3389.

Sriburi R, Jackowski S, Mori K, & Brewer JW (2004). XBP1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum. J Cell Biol 167**:** 35-41.

Stark AM, Pfannenschmidt S, Tscheslog H, Maass N, Rosel F, Mehdorn HM*, et al.* (2006). Reduced mRNA and protein expression of BCL-2 versus decreased mRNA and increased protein expression of BAX in breast cancer brain metastases: a real-time PCR and immunohistochemical evaluation. Neurol Res 28**:** 787-793.

Stone MA, Charpentier G, Doggen K, Kuss O, Lindblad U, Kellner C*, et al.* (2013). Quality of care of people with type 2 diabetes in eight European countries: findings from the Guideline Adherence to Enhance Care (GUIDANCE) study. Diabetes Care 36**:** 2628-2638.

Stoy J, Edghill EL, Flanagan SE, Ye H, Paz VP, Pluzhnikov A*, et al.* (2007). Insulin gene mutations as a cause of permanent neonatal diabetes. Proc Natl Acad Sci U S A 104**:** 15040-15044.

Strasser A, Puthalakath H, Bouillet P, Huang DC, O'Connor L, O'Reilly LA*, et al.* (2000). The role of bim, a proapoptotic BH3-only member of the Bcl-2 family in cell-death control. Ann N Y Acad Sci 917**:** 541-548.

Szegezdi E, Logue SE, Gorman AM, & Samali A (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO reports 7**:** 880-885.

Tabas I (2009). Macrophage apoptosis in atherosclerosis: consequences on plaque progression and the role of endoplasmic reticulum stress. Antioxid Redox Signal 11**:** 2333-2339.

Tabas I, & Ron D (2011). Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nature cell biology 13**:** 184-190.

Tait SW, & Green DR (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol 11**:** 621-632.

Taubes G (2009). Insulin resistance. Prosperity's plague. Science 325**:** 256-260.

Thastrup O, Cullen PJ, Drobak BK, Hanley MR, & Dawson AP (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase. Proc Natl Acad Sci U S A 87**:** 2466-2470.

Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ*, et al.* (1992). A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature 356**:** 768-774.

Thornton CM, Carson DJ, & Stewart FJ (1997). Autopsy findings in the Wolcott-Rallison syndrome. Pediatr Pathol Lab Med 17**:** 487-496.

Tirasophon W, Welihinda AA, & Kaufman RJ (1998). A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. Genes & development 12**:** 1812-1824.

Trinh MA, & Klann E (2013). Translational control by eIF2alpha kinases in long-lasting synaptic plasticity and long-term memory. Neurobiol Learn Mem 105**:** 93-99.

Tsujii S, Ishisaka M, & Hara H (2015). Modulation of endoplasmic reticulum stress in Parkinson's disease. Eur J Pharmacol 765**:** 154-156.

Tsukano H, Gotoh T, Endo M, Miyata K, Tazume H, Kadomatsu T*, et al.* (2010). The endoplasmic reticulum stress-C/EBP homologous protein pathway-mediated apoptosis in macrophages contributes to the instability of atherosclerotic plaques. Arterioscler Thromb Vasc Biol 30**:** 1925-1932.

Tyson JR, & Stirling CJ (2000). LHS1 and SIL1 provide a lumenal function that is essential for protein translocation into the endoplasmic reticulum. EMBO J 19**:** 6440-6452.

Upton JP, Wang L, Han D, Wang ES, Huskey NE, Lim L*, et al.* (2012). IRE1alpha cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. Science 338**:** 818-822.

Urano F (2016). Wolfram Syndrome: Diagnosis, Management, and Treatment. Curr Diab Rep 16**:** 6.

Urano F, Bertolotti A, & Ron D (2000). IRE1 and efferent signaling from the endoplasmic reticulum. J Cell Sci 113 Pt 21**:** 3697-3702.

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

Vagner S, Galy B, & Pyronnet S (2001). Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. EMBO reports 2**:** 893-898.

Valdes P, Mercado G, Vidal RL, Molina C, Parsons G, Court FA*, et al.* (2014). Control of dopaminergic neuron survival by the unfolded protein response transcription factor XBP1. Proc Natl Acad Sci U S A 111**:** 6804-6809.

Vandecaetsbeek I, Vangheluwe P, Raeymaekers L, Wuytack F, & Vanoevelen J (2011). The Ca2+ pumps of the endoplasmic reticulum and Golgi apparatus. Cold Spring Harb Perspect Biol 3.

Vattem KM, & Wek RC (2004). Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proc Natl Acad Sci U S A 101**:** 11269-11274.

Walter P, & Ron D (2011). The unfolded protein response: from stress pathway to homeostatic regulation. Science 334**:** 1081-1086.

Wang F, Wu Y, Quon MJ, Li X, & Yang P (2015). ASK1 mediates the teratogenicity of diabetes in the developing heart by inducing ER stress and inhibiting critical factors essential for cardiac development. Am J Physiol Endocrinol Metab 309**:** E487-499.

Wang L, Perera BG, Hari SB, Bhhatarai B, Backes BJ, Seeliger MA*, et al.* (2012). Divergent allosteric control of the IRE1alpha endoribonuclease using kinase inhibitors. Nat Chem Biol 8**:** 982-989.

Wang M, & Kaufman RJ (2014). The impact of the endoplasmic reticulum protein-folding environment on cancer development. Nat Rev Cancer 14**:** 581-597.

Wang X, Olberding KE, White C, & Li C (2011). Bcl-2 proteins regulate ER membrane permeability to luminal proteins during ER stress-induced apoptosis. Cell Death Differ 18**:** 38-47.

Wang XZ, Harding HP, Zhang Y, Jolicoeur EM, Kuroda M, & Ron D (1998). Cloning of mammalian Ire1 reveals diversity in the ER stress responses. EMBO J 17**:** 5708-5717.

Wang Y, Shen J, Arenzana N, Tirasophon W, Kaufman RJ, & Prywes R (2000). Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. J Biol Chem 275**:** 27013-27020.

Wei N, Zhu LQ, & Liu D (2015). ATF4: a Novel Potential Therapeutic Target for Alzheimer's Disease. Mol Neurobiol 52**:** 1765-1770.

Wek RC, Jiang HY, & Anthony TG (2006). Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans 34**:** 7-11.

Westermark GT, & Westermark P (2011). Localized amyloids important in diseases outside the brain--lessons from the islets of Langerhans and the thoracic aorta. FEBS J 278**:** 3918-3929.

Westermark P, Andersson A, & Westermark GT (2011). Islet amyloid polypeptide, islet amyloid, and diabetes mellitus. Physiol Rev 91**:** 795-826.

Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI*, et al.* (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes & development 19**:** 1294-1305.

Wiltshire C, Gillespie DA, & May GH (2004). Sab (SH3BP5), a novel mitochondria-localized JNK-interacting protein. Biochem Soc Trans 32**:** 1075-1077.

Woehlbier U, & Hetz C (2011). Modulating stress responses by the UPRosome: a matter of life and death. Trends Biochem Sci 36**:** 329-337.

Wortham NC, & Proud CG (2015). eIF2B: recent structural and functional insights into a key regulator of translation. Biochem Soc Trans 43**:** 1234-1240.

Yamaguchi H, & Wang HG (2004). CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. J Biol Chem 279**:** 45495-45502.

Yamamoto K, Sato T, Matsui T, Sato M, Okada T, Yoshida H*, et al.* (2007). Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. Dev Cell 13**:** 365-376.

Yan LJ (2014). Pathogenesis of chronic hyperglycemia: from reductive stress to oxidative stress. J Diabetes Res 2014**:** 137919.

Ye Y, Shibata Y, Yun C, Ron D, & Rapoport TA (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature 429**:** 841-847.

Yoneda T, Imaizumi K, Oono K, Yui D, Gomi F, Katayama T*, et al.* (2001). Activation of caspase-12, an endoplastic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. J Biol Chem 276**:** 13935-13940.

Yoshida H (2009). ER stress response, peroxisome proliferation, mitochondrial unfolded protein response and Golgi stress response. IUBMB life 61**:** 871-879.

Yoshida H, Matsui T, Yamamoto A, Okada T, & Mori K (2001). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107**:** 881-891.

Zhang W, Feng D, Li Y, Iida K, McGrath B, & Cavener DR (2006). PERK EIF2AK3 control of pancreatic beta cell differentiation and proliferation is required for postnatal glucose homeostasis. Cell Metab 4**:** 491-497.

Zhao XC OO, Smith D, Herbert TP. (2013). Endoplasmic reticulum stress can upregulate activating transcription factor 4 (ATF4) and C⁄EBP homologous protein (CHOP) expression via a PKR-like ER kinase (PERK) indepen-dent pathway in pancreatic beta cells. Diabet Med.

Zheng GF, Cai Z, Meng XK, Zhang Y, Zhu W, Pang XY*, et al.* (2015). Unfolded protein response mediated JNK/AP-1 signal transduction, a target for ovarian cancer treatment. Int J Clin Exp Pathol 8**:** 6505-6511.

Zheng H, Wu J, Jin Z, & Yan LJ (2016). Protein Modifications as Manifestations of Hyperglycemic Glucotoxicity in Diabetes and Its Complications. Biochem Insights 9**:** 1-9.

Zhou D, Palam LR, Jiang L, Narasimhan J, Staschke KA, & Wek RC (2008). Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. J Biol Chem 283**:** 7064-7073.

Zhu YF, Li XH, Yuan ZP, Li CY, Tian RB, Jia W*, et al.* (2015). Allicin improves endoplasmic reticulum stress-related cognitive deficits via PERK/Nrf2 antioxidative signaling pathway. Eur J Pharmacol 762**:** 239-246.

Zou W, Yue P, Khuri FR, & Sun SY (2008). Coupling of endoplasmic reticulum stress to CDDO-Me-induced up-regulation of death receptor 5 via a CHOP-dependent mechanism involving JNK activation. Cancer Res 68**:** 7484-7492.