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MERYEM TOPÇU

MASTER THESIS

DEPARTMENT OF MOLECULAR BIOLOGY

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ABSTRACT

High caloric diets, prolonged lifespan and reduced physical activity have let the humans to be subjected to the obesity that became epidemic all around the world in past two decades. In recent times obesity is accepted as major threat to the human health and also closely related to the metabolical syndrome and type-2 diabetes. Recent researches have uncovered that in obesity there are detrimental perturbations in pathways regulating energy metabolism such as gluconeogenesis and lipogenesis, SIK2 (salt inducible kinase-2) is the major kinase, which plays a central role over those vital pathways especially in the liver cells. Recent work also proposes that ER (endoplasmic reticulum) stress plays a central role in peripheral insulin resistance, obesity and type-2 diabetes at the molecular, cellular, and organismal levels. ER stress, which is related to overload of folding capacity and to misfolded proteins frequently observed in metabolically active cells at liver and adipose tissues of obese subjects. Cells make use of cellular adaptive response called as ER Associated Degradation (ERAD) in order to cope with synthetic overload in ER.

In this work, the role of SIK2 in ER stress was investigated for the first time at ER restricted proteomic level (reticulomic) levels due to the central role of SIK2 in metabolic pathways. After chemically induced ER stress by Thapsigargin (TG) in Hek293FT cells, ER isolation was performed and ER protein profiles of stressed and non-stressed conditions were compared. Next, all mammalian ERAD protein components deciphered by Christianson et al. was searched for candidate SIK2 phosphorylation sites using a modified GPS2.1 in collaboration with Xu Xue. Because VCP displayed strong SIK2 phoshorylation motif it was considered as a good candidate, however VCP level was unaffected either by SIK2 overexpression and TG treatment at protein level. On the other hand, calnexin level was decreased in SIK2 overexpressing cells similar to reduced expression level in response to ER stress induction. BiP expression level has significantly increased in TG treatment whereas its expression was inhibited in SIK2 overexpressing cells.

Key Words: Endoplasmic Reticulum (ER); SIK2; BiP; VCP; Calnexin; ERAD; ER Stress.

ÖZET

Yüksek kalorili diyetler, uzun yaşam süresi ve azalan fiziksel aktivite insanoğlunu son yirmi yılda dünya çapında yaygınlaşan obezite ile karşı karşıya getirdi. Son zamanlarda obezite insan sağlığı için büyük bir tehlike olarak görülmekle birlikte metabolik sendrom ve tip 2 diyabetle yakından ilişkilidir. Yakın zamanda yapılan araştırmalar obezite durumunda enerji metabolizmasını düzenleyen gluconegenez ve lipogenez gibi yolaklarda ciddi aksaklıklar olduğu ortaya çıkmıştır ve SIK2 (tuzla indüklenebilen kinaz 2) bu yolaklar üzerinde özellikle karaciğer hücrelerinde merkezi bir rol oynayan önemli bir kinazdır.Son araştırmalar ER (endoplazmik retikulum) stresinin çevresel insulin direnci, obesite ve tip 2 diyabetin moleküler, hücresel ve sistemsel oluşumlarında önemli bir role sahip olduğunu ileri sürmektedir. Yanlış katlanmış proteinlerin yükü ve katlama kapasitesinin aşılmasıyla ilgili olan ER stresi genellikle obez bireylerin metabolic olarak aktif olan karaciğer ve adipoz dokularında görülür. Hücreler, fazla protein birikmesiyle baş edebilmek için ER İlişkili Yıkım (ERAD) adı verilen uyarlamalı bir hücresel cevabı kullanırlar.

Bu çalışmada, SIK2'nin metabolic yolaklar üzerindeki önemli etkisi göz önüne alınarak SIK2'nin ER stresi üzerindeki etkisi, ilk defa, ER sınırlı proteomic (retikulomik) düzeyde incelenmiştir. Hek293FT hücreleri, Thapsigargin (TG) vasıtasıyla ER strese kimyasal olarak tetiklendikten sonra bu hücrelerden ER izolasyonu yapılmış ve ER protein görünümleri stress varlığında ve yokluğunda karşılaştırılmıştır. Sonrasında, Christianson ve arkadaşları tarafından deşifre edilen memelilere ait ERAD bileşenleri muhtemel bir SIK2 fosforilasyon bölgesi için Xu Xue işbirliği ile GPS2.1 programı kullanılarak taranmıştır. VCP güçlü bir fosforilasyon motifi sergilediği için iyi bir aday olarak düşünülmesine ragmen protein düzeyinde TG varlığından ya da SIK2'nin fazla anlatımından etkilenmediği görülmüştür. Öte yandan Calneksin'in protein düzeyi SIK2'nin fazla anlatıldığı durumda ve de ER stress varlığında azaldığı görülmüştür. BiP protein düzeyi TG muamelesinde ciddi ölçüde artarken SIK2'nin fazla anlatıldığı hücrelerde azalmıştır.

Anahtar Kelimeler: Endoplazmik Reticulum (ER); SIK2; Grp78/BIP; p97/VCP; Calnexin; ERAD; ER Stresi.

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LIST OF ACRONYMS AND ABBREVIATIONS

Acronyms and Abbreviations		**Definitions**
mg	:	miligram
$MgCl_2$	:	Magnesium Chloride
ml	:	Mililiter
mm	:	Milimeter
mM	:	Milimolar
$MnCl_2$	:	Manganese Chloride
NaCl	:	Sodium Chloride
Na_3VO_4	:	Sodium Orthovanadate
Nm	:	Nanometer
Ng	:	Nanogram
KCl	:	Potassium Chloride
AMPK	:	AMP-activated Kinase
APS	:	Amonium Persulfate
ATF4	:	Activating Trancription Factor 4
ATF6	:	Activating Trancription Factor
ATP	:	Adenosine Triphosphate
BiP	:	Binding Immunoglobulin Protein
BCA	:	Bicinchoninic Acid
bp	:	Base pair
BSA	:	Bovine Serum Albumine
$CaCl_2$	:	Calcium Chloride
cAMP	:	Cyclic Adenosine 5'-Monophosphate
CBP	:	CREB Binding Protein
CHOP	:	CAAT/Enhancer Binding Protein
CRE	:	cAMP-response Element
CREB	:	CRE Binding Protein
ChREBP	:	Carbohydrate-responsive Element-Binding Protein
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl Sulfoxide

DNA	:	Deoxyribonucleic Acid
DTT	:	Dithiothreitol
EDTA	:	Ethylenediaminetetraacetic Acid
EGTA	:	Ethylene Glycol Tetraacetic Acid
eIF2α	:	Eukaryotic initiation factor 2α
ERAD	:	ER-associated degradation machinery
EDEM1	:	ER degradation enhancer, mannosidase alpha-like1
ERSE	:	ER Stress Response Element
FBS	:	Fetal Bovine Serum
GFP	:	Green Fluorescent Protein
GRP78	:	Glucose Regulated Protein 78
GRP94	:	Glucose Regulated Protein 94
GTP	:	Guanosine-Triphosphate
HERP	:	Homocysteine-induced ER Protein
HRD1	:	HMG-coA reductase degradation 1
IgA	:	Immunoglobulin A
IP	:	Immunoprecipitation
IR	:	Insulin Receptor
IRE1	:	Inositol-requiring enzyme 1
IRS	:	Insulin Receptor Substrate
JNK	:	C-Jun N-Terminal Kinase
kb	:	Kilobase
KD	:	Kinase Domain
kDa	:	Kilodalton
KI	:	Kinase Inactive
LB	:	Luria Bertani Broth
LKB1	:	Liver Kinase B 1
MAPK	:	Mitogen-Activated Protein Kinase
MKP	:	Map Kinase Phosphatase
mRNA	:	Messenger Ribonucleic Acid
OD	:	Optical Density
PAGE	:	PolyAcylamide Gel Electrophoresis
pAkt	:	Phospho-Akt

PBS	:	Phosphate Buffered Saline
PCR	:	Polymerase Chain Reaction
PERK	:	PKR-like ER Kinase
PMSF	:	Phenylmethanesulfonylfluoride
PVDF	:	Polyvinylidene Fluoride
RNA	:	Ribonucleic Acid
Rpm	:	Rotations Per Minute
RTK	:	Receptor Tyrosine Kinase
SDS	:	Sodium Dedocyl Sulfate
SDS-PAGE	:	SDS- Polyacrylamide Gel Electrophoresis
Ser	:	Serine
SIK2	:	Salt Inducible Kinase 2
TBS	:	Tris Buffered Saline
TBST	:	Tris Buffered Saline Tween
TEMED	:	Tetramethylethylenediamine
Thr	:	Threonine
TG	:	Thapsigargin
TORC2	:	Transducer of Regulated CREB Activity
UBA	:	Ubiquitin-Associated
UPR	:	Unfolded Protein Response
UV	:	Ultraviolet
WB	:	Western Blot
WT	:	Wild-Type

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1. INTRODUCTION

We reported earlier that SIK2 overexpression in HEK293 cells increased ERAD whereas kinase inactive SIK2 reduced it [unpublished data] suggesting that SIK2 may be required for proper ERAD. Horike et al. reported earlier that SIK2 level and activity are low in liver cell, but high in aadipocytes of obese mouse models. Recently we reported a conver relation between ER stress and SIK2 level in Huh7 cells [Çalışkaner O., 2013].

Our data and others indicate that SIK2 might play a critical role in fine tunning of ER stress in metabolic tissues especially in adipocytes and liver cells. Here an ER restricted proteomics (Reticulomics) approach has been applied to find out putative SIK2 substrates and/or its effects constrained into ER proteins and extrapolate its possible roles in ERAD in cells chemically induced for ER stress.

A reticulomics comparison of cells overexpressing SIK2 under stress condition to control cells revealed proteins whose expression goes up and goes down respectively with respect to stress condition. These spots were collected on the basis of statistical significance at ≥ 0.005 and at least 2 fold change over detected spot intensities on the basis of assigned PI (isoelectric point) range and MW (molecular weight) standards in the reference gel and produced ER proteomics bank (see index) it would be interesting to see if the detected spots at decreased intensity upon chemical ER stress including BiP and Calnexin and VCP verify our studies.

This work will include phoshpoproteomics study to further narrow down putative candidates. Selected spots will be identified by mass spectrometry preceeding further validation of candidate proteins in functional studies.

1.1. The Aim, The Content and The Contribution of Thesis

Endoplasmic Reticulum (ER), which is present in all eukaryotic cells, serves as a protein folding factory with its special oxidative environment compared to the cytoplasm of the cell and folding of 1/3 of the proteome involving membrane proteins and secretory proteins occurs in this special ER lumen as well as the biosynthesis of steroids, cholesterol, and other lipids. Besides, ER is a dynamic Ca^{+} store and has a crucial role in sensation, integration and transmission of intracellular

signals. It is a very complex process for a protein to reach its mature conformation and it involves several steps such as biosynthesis, folding, assembly, disulphide bond formation and other posttranslational modifications [Ron and Walter, 2007]. Any delinquency in any of these steps can lead to detrimental outcomes therefore protein maturation process in ER is exposed to stringent monitoring system [Braakman et al., 2011]. ER is exquisitely sensitive to alterations that imbalance the homeostasis within ER and some physiological and pathological insults such as changes in redox balance, hypoxia, fluctuations in calcium homeostasis, nutrient deprivation, increase in demand for protein synthesis might lead to ER stress which shows up as the accumulation of misfolded and unfolded proteins within ER lumen. Eukaryotic cells have evolved a mechanism, which is called as unfolded protein response in order to handle with those stressful conditions [Schroder et al., 2005], [Ron et al., 2007].

Unfolded Protein Response (UPR) works as a double-edged sword owing to its nature, which induces both proapoptotic and prosurvival signals. In case of ER stress, if cell can cope with the situation UPR activates prosurvival signals leading to attenuation of protein translation, activation of transcription of ER chaperones and foldases and induction of endoplasmic reticulum associated degradation (ERAD), on the other hand if stress is beyond the handling capacity of the cell, UPR activates signals leading to apoptosis.

Cellular stress results in many cellular anomalies including ER stress. One third of the whole protein produced within the cell is subjected to synthesis, proper folding and maturation processes in the endoplasmic reticulum. On the other hand ER is also site of triglyceride droplet formation. In case of fatty acid accumulation within the cell, ER transforms fatty acids into triglyceride droplets with the aim of energy storage and lipid neutralizing. If there is a protein or fatty acid load over the ER it induces ER stress and in turn ER stress induces perturbations other vital pathways within the cell and this brings about an array of diseases, which are collectively known as metabolic syndrome. In normal conditions ER responds to the unfolded or misfolded protein via inducing UPR (unfolded protein response) and by means of UPR the unfolded or misfolded proteins are introduced to the folding processes over again. On the other hand if the proteins are terminally misfolded they are degraded in the cytoplasm via inducing ERAD pathway (ER associated degradation). There occurs a chronic stress over the ER stress in adipocytes and hepatocytes in a wide array of diseases such as obesity and if the cell could not

respond to the load by means of UPR the emergent ER stress leads in insulin resistance and apoptosis.

SIK, which is a member of AMPK family, is a Ser/Thr kinase and initially it was isolated from rats, which were subject to high salt diet. SIK2 isoform is especially found to be expressed in adipocytes and hepatocytes and it prevents energy loss via switching off anabolic pathways such as glycolysis and lipolysis. The most clearly known role of SIK2 is its inhibiting activity over lipogenesis by suppressing the activation of major transcription regulators such as CREB and SREBP. At the same time, in presence of cellular stress significant increase in both expression and the activity of SIK2 was observed.

In case of obesity it has shown that there is an increase in lipogenesis in hepatocytes through activation of SREBP. On the other hand an increase in both activity and expression of SIK2, in addition to the capacity of ERAD, was observed in obesity condition. When those parameters are handled together the query of whether there is a correlation between those three have come into mind. By using GPS2.1 program a canonical SIK2 phosphorylation motif was created and Human ERAD components were scanned for this putative motif.

Following experiments showed that that in case of SIK2 expression was raised also amount of ERAD increased. Exploring the elements through which SIK2 regulates or affects ERAD activity constitutes the essence and the goal of this thesis. Since SIK2 has an uba domain at the same time, it is estimated that SIK2 interacting to proteins via its uba domain. Due to its localization on ER membrane and its being increased in case of ER stress, p97/VCP is thought to be the putative ERAD target.

Results of this thesis have allowed us for the first time to obtain information about the role of SIK2 on ERAD mechanism and in turn effects of this role in the lipogenic and gluconeogenic pathways. At the same time if there is a regulation of amount of ERAD in case of ER stress by means of SIK2, this thesis also will lead up to decipher the elements through which SIK2 controls this regulation. To sum up, all those information gained throughout this work will give us the opportunity to improve better therapeutic methods or to detect new targets related with the wide range of diseases such as type 2 diabetes and obesity.

2. ENDOPLASMIC RETICULUM

The “endoplasmic reticulum” (ER) term was firstly used by Porter and his colleagues in order to refer the interconnected network of membrane vesicles around the nucleus [Palade, 1956]. The ER, emerging as a vital organelle for eukaryotic cells, comprises approximately the half of the whole cell volume. Generally it is composed of a membranous network of sac-like structures (cisterns) and greatly spanned throughout the cytoskeleton [Web 1, 2013], (Figure2.1). It has a phospholipid membrane, which encloses the space within the organelle (ER lumen), and this membrane is continuous and connected with nuclear envelope. ER is complex in both structure and function and plays essential roles for the destiny of the eukaryotic cells including Ca^{+} storage and its controlled release; synthesis, folding, posttranslational modification and transport of proteins and biosynthesis of lipids and steroids [Schröder, 2008].

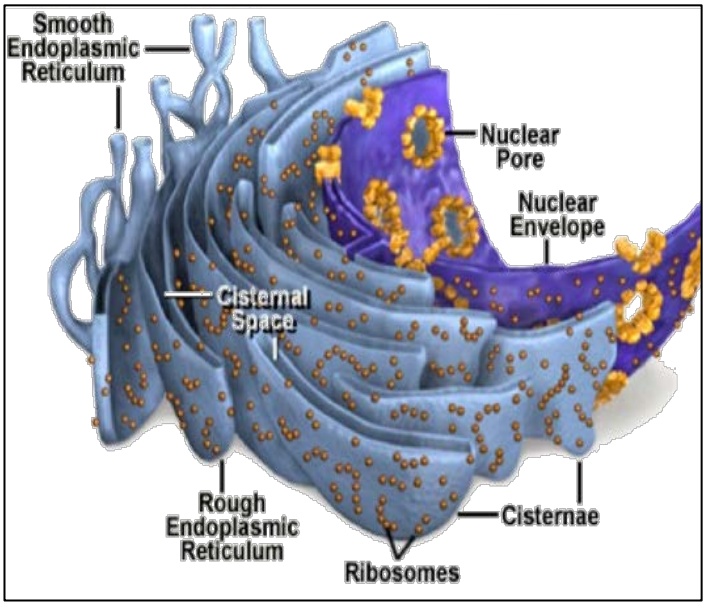


Figure 2.1: General structure of Endoplasmic Reticulum.

According to the structure, the ER can be subdivided into three well-defined domains and these are sheet-like ER, tubular ER and the nuclear envelope respectively. The sheet-like ER is rich in ribosomes so it is called as ribosome-studded rough endoplasmic reticulum (RER) on the other hand tubular ER contains fewer ribosomes and is commonly called as smooth endoplasmic reticulum (SER)

[English et al., 2009]. SER, which is more complex than RER, is found mainly in steroid synthesizing cells whereas RER is found abundantly in cells that are responsible for synthesis of large amounts of proteins. Muscle cells exceptionally possess sarcoplasmic reticulum (SR) which is a variant of SER and responsible for controlled calcium release during muscle contraction [Bravo et al., 2013], (Figure 2.2). Because of its role in protein synthesis, folding and transport RER is the major matter of concern.

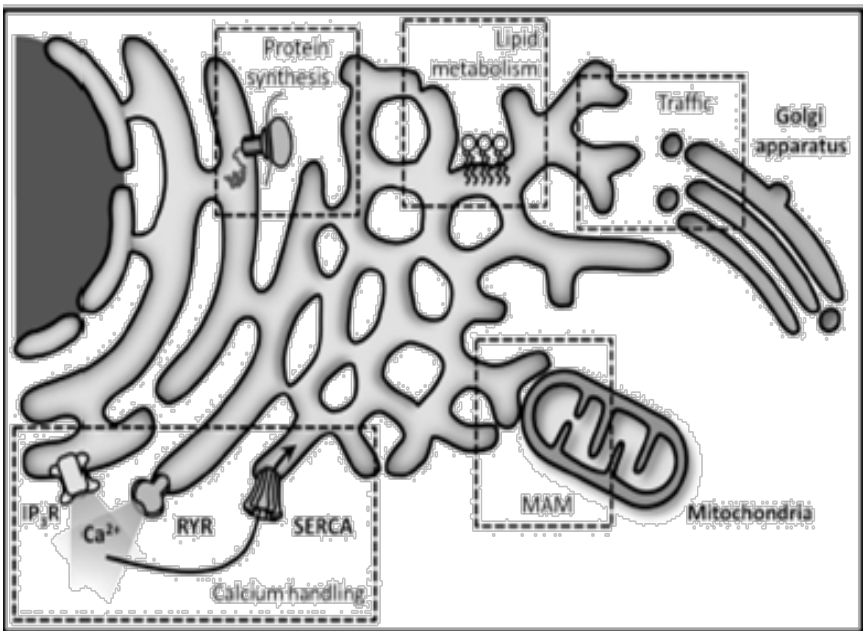


Figure 2.2: The ER fulfils diverse functions in the cell.

Rough ER, is studded with protein-manufacturing ribosomes on its outer membrane, is found throughout the cell but mainly around the nucleus and Golgi apparatus. This ribosome-bound organelle is responsible for translation and assembly of many proteins including proteins destined for secretory pathway and membrane proteins. The translocons found in RER function as binding sites for ribosomes, which must be incorporated into ER only when they initiate translation of proteins destined for secretory pathway [Görlich et al., 1992].

Nascent polypeptides have signal sequence, which is recognized via signal recognition particle (SRP) in their N-termini. Recognition of this signal sequence by SRP leads to binding of ribosome to RER and translocation of the nascent polypeptide into the RER lumen. Upon translocation the signal sequence is cleaved off by peptidases in RER lumen and from here and out folding and proper assembly

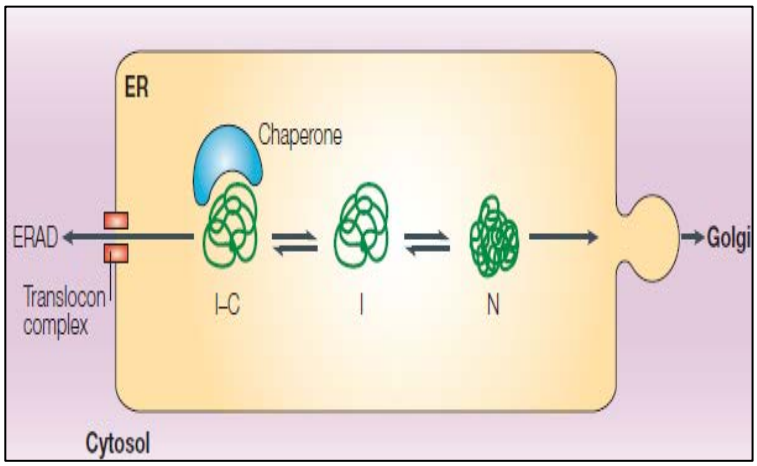
of nascent polypeptides, the major challenges of protein synthesis, are initiated [Seiser, 2000].

RER can be seen as the entry portal to the secretory pathway by being responsible for the synthesis of 1/3 of eukaryotic proteome that especially contains proteins destined to the secretory pathway and membrane proteins. Special oxidative environment of ER lumen, chaperones and folding catalysts found in ER work in harmony in a lump in order to provide the nascent polypeptides the proper folding and assembly into the native structures [Helenius and Aebi, 2004]. Due to the error-prone nature of protein synthesis endoplasmic reticulum has evolved mechanisms in order to monitor the insults and perturbations during the protein maturation process. Firstly, ER uses its protein quality control mechanisms in order to prevent accumulation of misfolded or unfolded peptides. If prevention is beyond ER capacity, aberrant protein aggregation within ER lumen induces endoplasmic reticulum stress that provoke unfolded protein response (UPR), a coordinated surveillance process, that activates well-established signaling cascades that compel the cell to make a decision between adaptation and death. In order to adapt, cell might make use of endoplasmic reticulum-associated degradation (ERAD), a complex process charged with elimination of defective proteins in ER [Vembar and Brodsky, 2008]. However if adaptation is beyond the ER capacity and if ER cannot alleviate the existing stress and restore the homeostasis, the condition becomes minaciously chronic leading to autophagy.

3. ENDOPLASMIC RETICULUM STRESS

Endoplasmic reticulum provides the favorable conditions for folding and maturation of polypeptides. During translation in ER, polypeptides undergo several post-translational modifications such as formation of disulphide bonds, removal of signaling peptides and N-glycosylation. With the help of the folding assistants found in ER such as chaperones, metal ions, lectins covalent modifications are performed, and all those modifications are required for proper folding and maturation of the polypeptides [Ellgaard L. and Helenius, A., 2003], [Schröder M. and Kaufman R.J., 2005].

After translation in ribosomes of RER, synthesized proteins enter to the ER lumen as unfolded polypeptides. This polypeptide influx into the ER lumen varies according to the environmental, physiological and differentiation conditions of the cell [Ron and Walter, 2007]. In the meantime, it's not independent from the changes in the physiological and biochemical levels such as ion balance and protein load inside the ER lumen [Zang and Kaufman, 2004]. Even worse, changes in Ca^{+} balance and redox potential, increased protein load, insufficiency of glucose, perturbations in glycosylation and overload of cholesterol may result in cellular stress in turn lead to malfunctions in protein folding process hence accumulation of those aberrant proteins in ER lumen [Zang and Kaufman, 2004], [Schröder and Kaufman, 2005].



Şekil 3.1: Translocation of mature proteins to Golgi and removal of unfolded ones via ERAD (I: unfolded protein, N: Mature protein, I-C: Unfolded protein-Chaperone complex).

In ER, the properly folded proteins are sent to Golgi in order to be transmitted to the their related destinations, however misfolded and unfolded ones are eliminated by a well-established mechanism called as ERAD and directed to cytoplasm with the intent of degradation via 26S proteasome [Elgaard and Helenius, 2003], [Zang and Kaufman, 2004], [Yoshida, 2007], (Figure 3.1).

When the amount of the proteins, synthesized in ER at a unit of time, is beyond the capacity of ER the unfolded proteins accumulate as aggregates in lumen due to their hydrophobic residues. Deposition of those unfolded proteins in ER is toxic and incline to Er stress [Yoshida, 2007], (Figure 3.2). In reply to ER stress, eukaryotic cells activate a mechanism called as UPR and by this way target to keep the ER homeostasis under the control [Zang and Kaufman, 2004], [Yoshida, 2007].

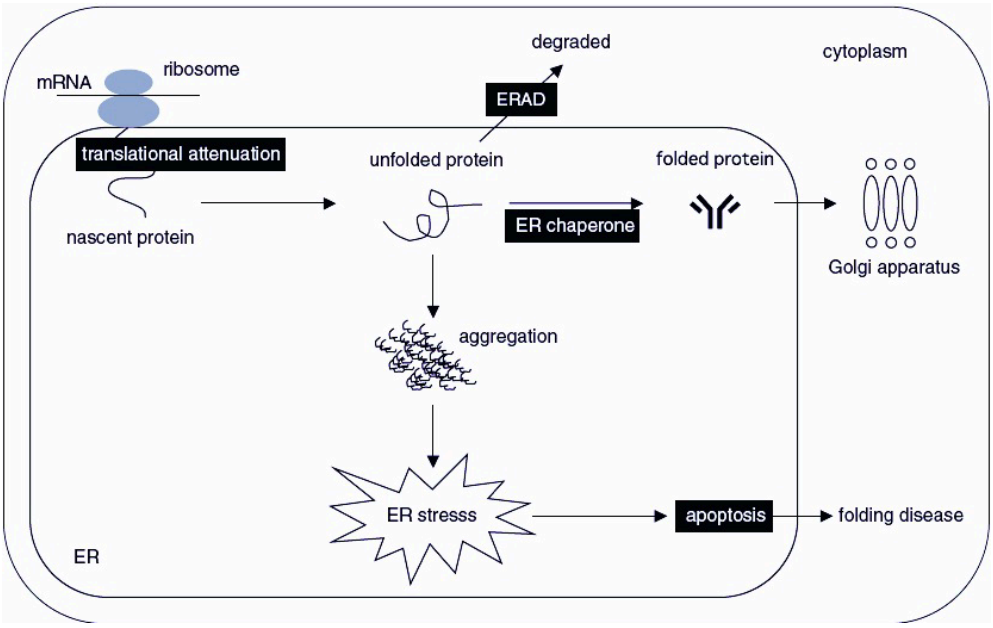


Figure 3.2: Accumulation of misfolded proteins in ER lumen and their inducing ER stress.

UPR activates several important signalling cascades through three major branches. Those branches are IRE1, PERK and ATF6 respectively. Despite the fact that they are ER stress sensors with different features activation of all is regulated by a single protein called as BiP (binding immunoglobulin protein) [Zang and Kaufman, 2004].

4. UNFOLDED PROTEIN RESPONSE

Conditions that alter ER homeostasis and proper function such as disturbances in redox balance, hypoxia, fluctuations in calcium homeostasis, nutrient deprivation and increase in demand for protein synthesis lead to ER stress by inducing accumulation of misfolded and unfolded proteins in ER lumen. Because the accumulation of aberrant proteins is toxic and imbalance in ER homeostasis is detrimental to cell integrity it is essential for a cell to restore its resting conditions for survival. Unfolded protein response, which is a well-established signaling cascade, is the cellular reply in case of ER stress.

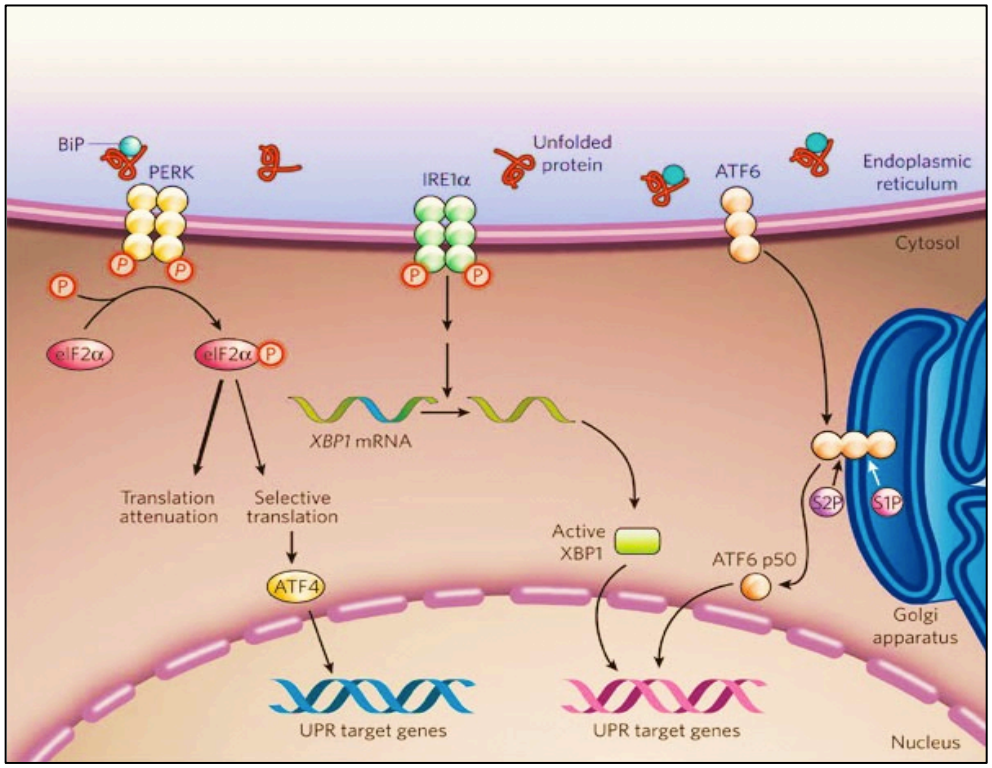


Figure 4.1: ER stress-signalling pathways.

UPR is mainly in charge of reestablishing cellular homeostasis and alleviating the existing stress. With the aim of achieving these vital goals, UPR activates several signaling cascades and force the cell to make a decision between survival and apoptosis. Thitherto, UPR make use of some strategies in order to evaluate if cell can adapt to the existing condition or not, and these strategies are enhancing expression of ER chaperones and folding assistants to prevent protein aggregation and to

promote proper folding, transitory attenuation of translation in order to reduce protein load in ER, inducing synthesis of membrane lipids in order to increase of ER volume or rather ER capacity and, least but not least, activation of ERAD in order to eliminate the aberrant proteins in ER lumen, respectively [Bravo et al., 2013].

UPR detects existing ER stress by means of three important ER stress sensors, which are protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme-1 (IRE1) and activation transcription factor 6 (ATF6), respectively [Kezhong Zhang and Randal J. Kaufman, 2008], (Figure 4.1).

4.1. IRE1 Pathway

IRE1, which is a transmembrane protein, has three domains one is luminal, one is transmembrane and the other one is cytosolic. The luminal domain is found in N-terminus and works as a sensor whereas the cytosolic domain is found in the cytosolic side and responsible for the kinase activity and the endoribonuclease activity of protein [Walter and Ron, 2011].

Much of known about IRE1 pathway is based on the studies performed in yeast. First observations have showed that IRE1 has enhanced cell viability during ER stress by inducing expression of ER chaperones [Cox et al., 1993].

In mammalian cells IRE1 has structurally similar but functionally different two paralogs; IRE1α and IRE1β. In case of ER stress IRE1α catalyzes splicing of X-box binding protein (XBP1) mRNA whereas IRE1β induces site-specific cleavage of 28S RNA and inclines a translational attenuation [Tirasophon et al., 1998; Iwawaki et al., 2001]. IRE1α not only cleaves XBP1 mRNA but also several other targets including its own mRNA [Tirasophon et al., 1998; Oikawa et al., 2010].

IRE1α activation is initiated via two-step model regulation; firstly it dissociates from BIP/Grp78, chaperones to which it is normally bound and secondly it binds to the unfolded and misfolded peptides in ER lumen. Dissociation from chaperones drives oligomerization of IRE1α, on the other hand binding of aberrant peptides induces full activation of it [Kimato et al., 2007; Pincus et al., 2010]. Upon IRE1α activation, the stress sensed in the luminal region of ER is transduced to the cytoplasm and several signaling cascades are activated. IRE1α mediated splicing of

XBP1 (X-box binding protein 1) mRNA is starting point of one of these cascades [Yoshida et al., 2001].

XBP1, which is a transcription factor that regulates the expression of genes in charge of ER protein synthesis, folding, glycosylation, ERAD, redox metabolism, autophagy, lipid biogenesis and vesicular trafficking [Walter and Ron, 2011], (Figure 4.2). At the same time, spliced XBP1 mRNA encodes a potent transcription factor that binds to the UPRE or ERSE sequence of many UPR target genes. Unspliced form of XBP1 acts as a negative feedback regulator of the protein itself, whereas the spliced form encodes a potent transcriptional activator [Yanagitani et al., 2009].

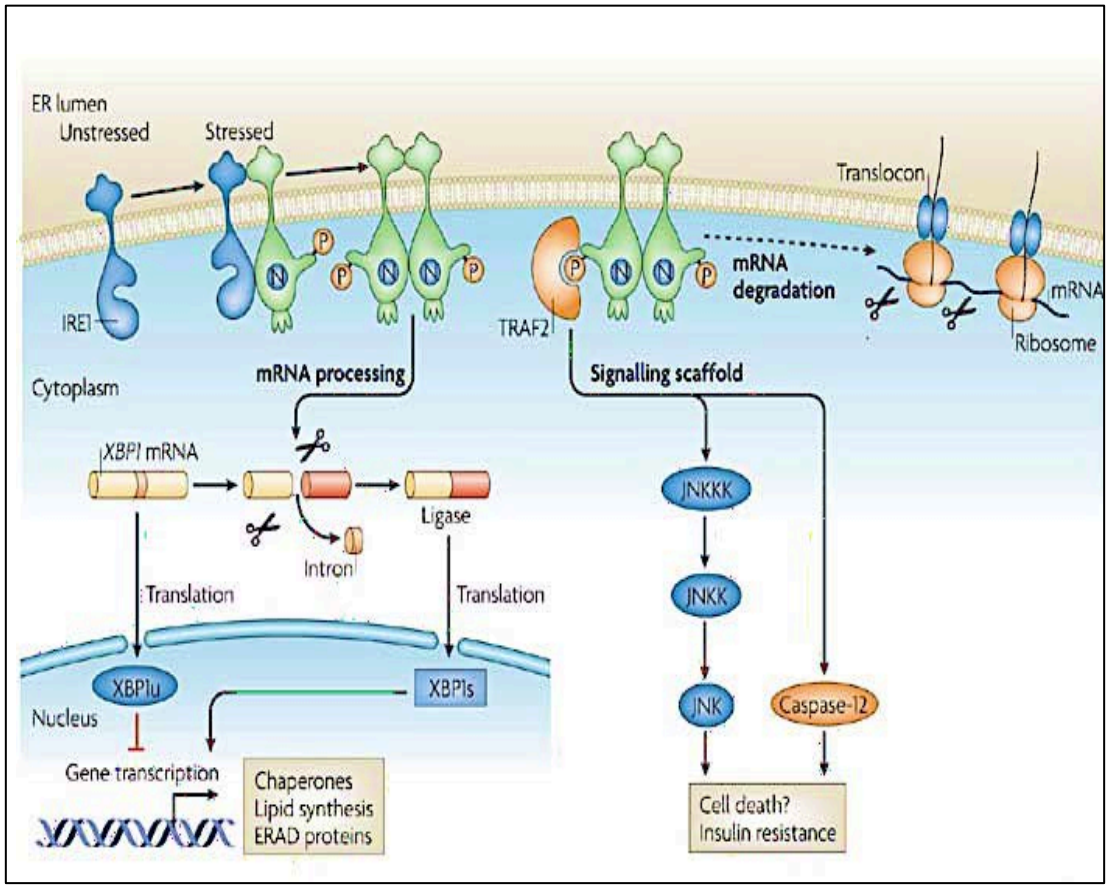


Figure 4.2: IRE1α signalling in UPR.

IRE1α can also act by alternative means. In mammals, phosphorylated IRE1α is involved in recruitment of TRAF2 (tumour necrosis factor receptor (TNFR)-associated factor-2) thus permits it signal to JNK (Jun N-terminal kinase) [Urano et al., 2000]. JNK activation is associated on one hand with cell death [Kim et al.,

2006], on the other part with cell survival through activation of c-Jun [Zhao et al., 2008].

4.2. PERK Pathway

The second transducer PERK (RNA PKR-like ER kinase), which has a PEK-like catalytic domain, is a type 1 transmembrane protein and similar to IRE1α it oligomerizes in the plane of ER membrane in response to ER stress. Its activation requires trans-autophoshorylation of its active loop found in C terminal lobe of the protein [Cui et al., 2011]. When there is no ER stress, PERK is bound to BiP via its luminal domain. However upon activation of UPR, BiP releases PERK and permits dimerization and activation of the transducer. Kinase activity of PERK increases concomitant with its activation [Dever, 2002].

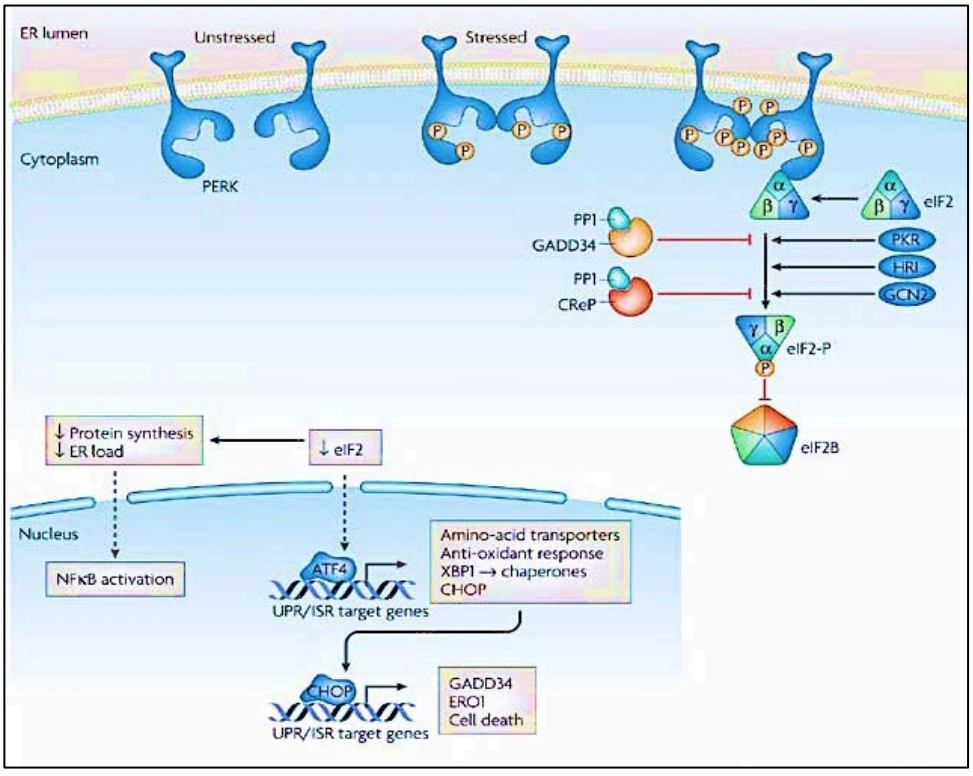


Figure 4.3: PERK signalling in UPR.

The first known substrate of PERK is eukaryotic translation initiation factor-2 (eIF2). PERK phosphorylates the protein from its α subunit on Ser^{51} and reults in eIF2α-GDP to eIF2α-GTP exchange inhibition [Dever, 2002]. This inhibition

accounts for all of the consequences of PERK activity [Ron and Walter, 2007], (Figure 4.3). Because eIF2α-GTP is a key component in the formation of active translation initiation complex, PERK activity lowers the global protein synthesis and reduces ER load [Harding et al., 2003]. eIF2 limiting conditions in turn results in increase in translation of ATF4 (translation of the activating transcription factor-4), post-transcriptional activation of NFκB (nuclear factor κB) and transcriptional activation of XBP1.

On the other hand PERK initiates apoptosis by inducing activation of CHOP (C/EBP-homologous protein) transcription by means of ATF4 phosphorylation [Fawcett et al., 1999]. In addition to this, PERK activates autophagy in case of nutrient deprivation, hypoxia and radiation [Rouschop et al., 2010]. To sum up, PERK can be seen as a key regulator between growth and survival due to its important roles in relieving ER stress, inducing apoptosis and autophagy [Blais et al., 2006].

4.3. ATF6 Pathway

Activating transcription factor-6 (ATF6) constitutes the third major branch of UPR. ATF6, which has a bZIP domain, is a single-pass type transmembrane protein and associated with regulation of genes with CRE sequences [Hai et al., 1989]. In unstressed conditions, ATF6 is reside in ER membrane and signaling thorough ATF6 is inhibited by means of interaction with BiP and Calreticulin [Shen et al., 2005]. Upon ER stress BiP binding is disrupted and in turn ATF6 is translocated into Golgi apparatus. In Golgi, ATF6 is subject to consecutive cleavage by Site-1 and Site-2 Proteases (S1P and S2P, respectively). After sequential cleavage full length ATF6 protein (90 kDa) turns into a small cytosolic fragment (50kDa) and activated ATF6 is secreted into cytosol from Golgi. Finally ATF6 is translocated into nucleus nucleus in order to activate several UPR target genes such as BiP, GRP94, CRT, Derl3, MANF and CHOP. These genes are involved in protein folding, assembly and degredation [Ron and Walter, 2007], [Web3], (Figure 4.4).

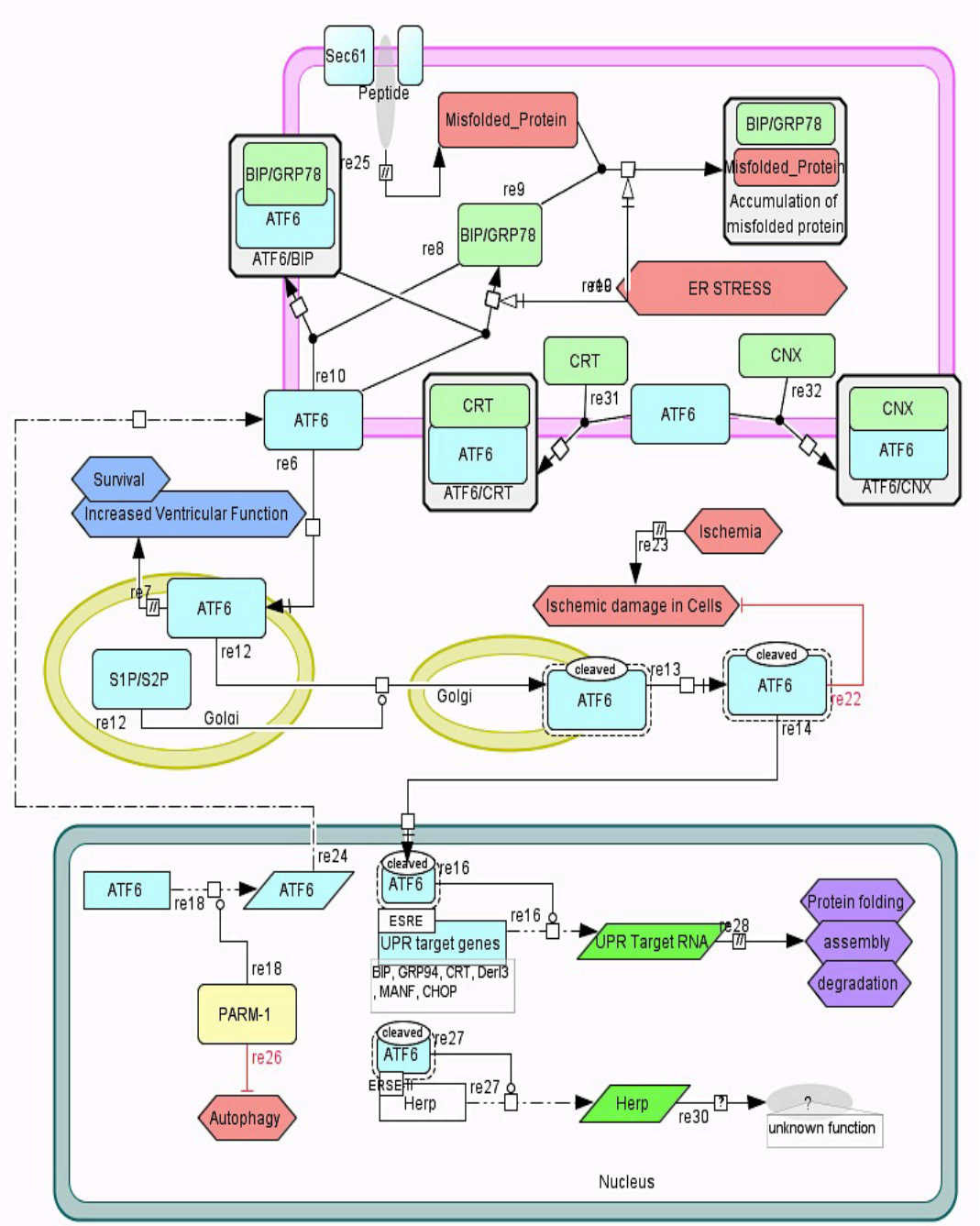


Figure 4.4: ATF6 signalling in UPR.

5. ENDOPLASMIC RETICULUM-ASSOCIATED DEGRADATION

ERAD, which is a vectorial process taking place across the ER lumen,lipid bilayer and cytoplasm, is the temporally and spatially coordinated surveillance process of the cell. It is mainly charged with the selection and the elimination of the folding-defective proteins. Much of known about this well-established process relies on the studies in yeast [Vembar and Brodsky, 2008]. ERAD comprises four distinct coupled steps, which are substrate selection, dislocation across the lipid bilayer, polyubiqitination and degradation by proteasome, respectively [Olzman et al., 2012].

5.1. Substrate Selection

Proteins that failed to achieve native structure due to mutation, translational misincorporations or folding inefficiency are the potential ERAD substrates. As nascent proteins enter the ER lumen they are cotranslationally incorporated into N-glycans ($Glc_3Man_9GlcNAc_2$) [Helenius and Aebi, 2004]. N-glycans function as the sensor of protein folding and monitor maturation of the process.

After addition of the nascent polypeptide, this high mannose core glycan is exposed to sequential deglycosylation via GlucosidaseI (GI) and GlucosideII (GII). This consecutive deglycosylation permits association of the nascent polypeptide with folding assistants Calnexin (CNX) and Calreticulin (CRT). If protein is folded properly a final deglycosylation by GII results in dissociation of CNX and CRT from the protein and the mature protein progress into ER exit sites. However if protein cannot acquire the native structure, it is reglucosylated by UGGT (UDP-glucose: glycoprotein glucosyl transferase) and reenters the CNX/CRT cycle [Aebi et al., 2010]. Further rounds of oxidative folding may rescue slightly misfolded proteins. The entry of proteins into CNX/CRT cycle is regulated by removal and readdition of glucose by GII and UGGT, respectively [Maurizio, 2007], (Figure 5.1).

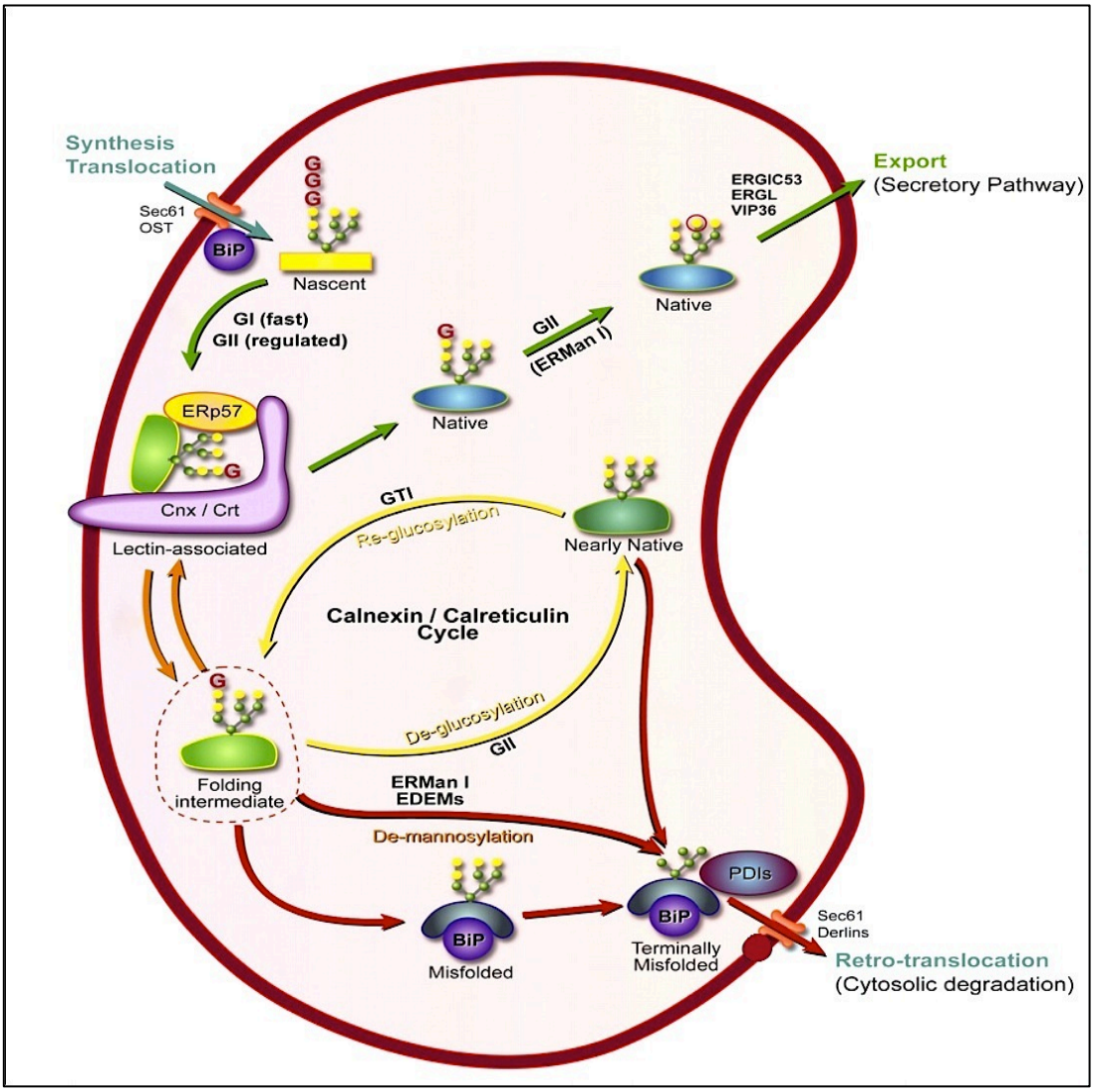


Figure 5.1: The fate of newly synthesized proteins in ER lumen.

However after rounds of folding cycles some protein retain their aberrant conformation. Those proteins are accepted as terminally misfolded and retrotranslocated from the ER to the cytosol through the dislocation channel by a process known as ER-associated degradation (ERAD) [Meusser et al. 2005], [Nakatsukasa and Brodsky 2008].

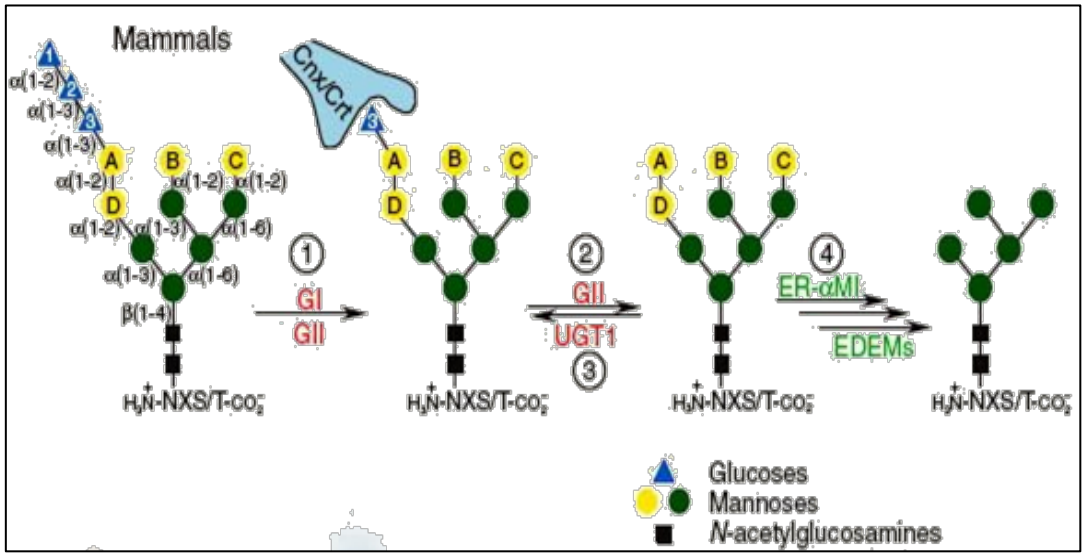


Figure 5.2: N-glycan processing in mammalian ER.

Although recognition of terminally misfolded proteins is not yet fully understood, removal of terminal mannose by mannosidases is sensed as a signal for the ERAD [Lederkremer, 2009]. ERManI [Garolez et al., 1999], EDEM1 [Olivari et al., 2006], EDEM3 [Hirou et al., 2006] and Man1C1 [Hosokowa et al., 2007] are the mannosidases in charge of progressive trimming of the terminal mannoses [Modified from Maurizio, 2007], (Figure 5.2). The resulted deglycosylated and demannosylated substrates (Man_5Man_7) are not compatible with UGGT-mediated reglucosylation. The trimmed oligosaccharides has a terminal $\alpha_{1,6}$ mannosyl linkage which is preferentially bound by soluble ER-resident lectins OS9 and XTP3-B. Upon association with lectins, ERAD substrates are directed to dislocation [Bernasconi et al., 2010].

5.2. Dislocation

Dislocation process of ERAD differs from normal protein dislocation. During normal protein dislocation the substrates to be dislocated comprise a signal sequence, whereas in ERAD substrate recognition can be performed via diverse features such as exposed hydrophobicity and glycan status. In addition to this, in normal case the substrates to be translocated must be in unfolded state, whereas in ERAD proteins of all folding states, even the tightly folded ones, can be translocated [Fiebiger et al., 2002], [Rapaport, 2007].

Many ERAD substrates are highly hydrophobic. Association with lectins such as OS9 and XTP3-B stabilizes them. Because those hydrophobic substrates are likely to aggregate in aqueous environment all ERAD steps are tightly coupled to each other as the links in the chain. Selection of substrates is coupled to dislocation by means of variety of adaptors. The adaptors are privatized to select a diverse set of features thrugh which substrates are committed to degradation. SEL1L, erlin, insigs and F-box proteins rank as the major adaptors [Olzman et al., 2012].

Adaptors function as scaffols between recognition factors and components of dislocon. SEL1L predominantly selects soluble glycoproteins and links them to Hrd1 (ERAD-associated E_3 ubiquitin ligase), a structural component of dislocon [Carvalho et al., 2010]. SEL1L nucleates a complex involving Derlin1-2, AVP1, UBXD8, VIMP, Herp and this complex recruits VCP/p97, an ATPase that drives substrate dislocation [Christianson et al., 2012], (Figure 5.3). As SEL1L, rest of the adaptors serves as an important nexus orchestrating ERAD substrate recruitment, dislocation and ubiquitination. Erlins select for intramembrane substrates, insigs are client-specific and F-box proteins are privatized for chitobiose core of high-mannose N-linked oligosaccharides respectively.

ERdj5, a disulfide reductase required for ERAD, interacts with EDEM and BiP and promotes dislocation of misfolded proteins into the cytosol by cleaving their incorrect disulfide bonds and preventing their aggregate formation.

5.3. Ubiquitination And Degradation

ERAD is a vectorial process occurring across the ER lumen, lipid bilayer and cytoplasm. For efficient clearance of aberrant proteins all steps of ERAD are strictly coupled. For example HRD1/SEL1L complex, formed on the ER membrane, function as a scaffold and is connected to the recognition factors at the luminal side and to the degredation actors to the cytoplasmic side. The misfolded proteins that are recognized via their N-glycans are diverted to SEL1L. Later on, they are retrotanslocated from ER to cytosol with the help of VCP/p97 (AAA^+ ATPase). Finally, they are polyubiquitinated by E3 ligase HRD1 and degraded by 26S proteasome.

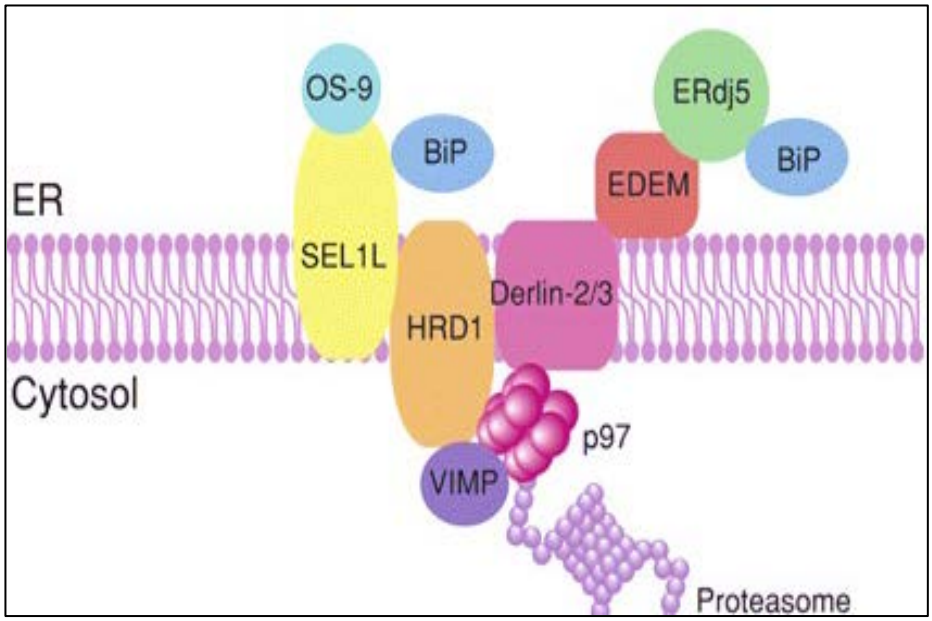


Figure 5.3. Mammalian ERAD components.

Protesomal degradation involves three important actors. The first player, E_1 (ubiquitin activating enzyme) hydrolyzes ATP and adenylates an ubiquitin molecule. Adenylated ubiquitin is transferred into a second player, E_2 (ubiquitin conjugating enzyme). Finally the third player E_3 (ubiquitin ligase) selects proteins that are destined to degradation and induces transfer of adenylated ubiquitin from E_2 to the targeted protein. Ubiquitin E_3 ligases play central functional and organizational roles in ERAD. They recruit distinct ubiquitin-conjugating enzymes (E_2) and functions as a scaffold between these enzymes and the protein to be degraded. Major ubiquitin ligases implicated in ERAD are Hrd1 [Nadav et al., 2003] and gp78 [Fong et al., 2001]. Mammalian ERAD system may harmonise with broad range of potential substrates by means of combinatorial interactions of these two central E_3s.

6. SALT-INDUCIBLE KINASE FAMILY

Salt-inducible kinase (SIK), a member of AMP-dependent protein kinases (AMPK)-related family, is a Ser/Thr kinase and initially was isolated from adrenocortical tissue of high salt diet-treated rats [Wang et al., 1999]. In comparison to AMPK family members, SIK family that comprises three members (SIK1-3) lack the AMP and glycogen sensitivity [Hardie et al., 2004], [Ruiz et al., 1994]. SIK1, first known member of SIK family, was initially found in the myocardium and later on isolated from adrenal glands of rats fed with high salt diet. [Ruiz et al., 1994], [Wang et al., 1999]. Other isoforms SIK2 and SIK3 were identified based on the sequence homology researches performed in mouse and human genome databases [Okamoto et al., 2004], (Figure 6.1).

In mammals, SIK1 is found to be expressed high amounts in brain, testes, adrenal glands and skeletal muscles and in small amounts in liver, heart and adipose tissue [Horike et al., 2003]. While SIK2 isoform is mainly expressed in liver, brain and adipose tissue, SIK3 isoform is ubiquitously expressed [Okamoto et al., 2004]. Salt inducible kinase 1 gene is located on chromosome 21; on the other hand genes of other isoforms are located in chromosome 11 [Katoh et al., 2004].

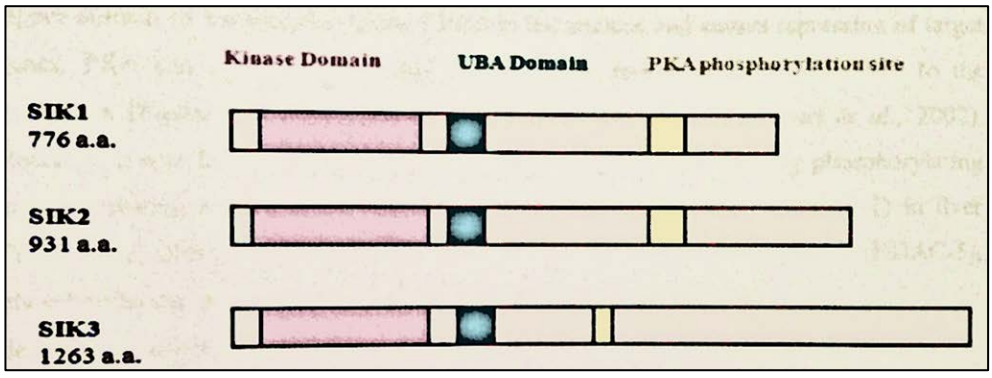


Figure 6.1. Salt inducible kinase family; SIK family comprises three members with a conserved N-terminus kinase domain, a SNF homology domain and a C-terminus PKA phosphorylation site.

All members of SIK family have an N-terminal kinase domain, an ubiquitin-associated (UBA) domain and a C-terminal PKA phosphorylation site. Upon phosphorylation by LKB1 there occurs a structural change in the N-terminal kinase domain which leading to increased kinase activity. UBA domain is thought to be

associated with kinase domain and important for phoshorylation by LKB1 whereas C-terminal PKA phoshorylation sites are adjacent to arginine-lysine rich region and thought to be associated with nuclear export of SIKs [Katoh et al., 2004], [Jaleel et al., 2006].

6.1. SIK2

SIK2, firstly identified as an adipose specific isoform of the SIK family, was suggested to be part of the early phase of adipocyte differentiation due to its induced expression in 3T3-L1 preadipocytes at the onset of differentiation [Yeh et al., 1995], [Horike et al., 2003]. SIK2 in Homo sapiens is a 926 aa long protein with a kinase domain, residues 20-271, and an uba domain, residues 295-553 (UNIPROT, Q9H0K1). SIK2 shares high degree of amino acid similarity with other isoforms os the SIK family. The researches have showed that the amino acid similarity between SIK2 and SIK1, SIK2 and SIK3 in phosphorylation domains are 73% and 33%; in kinase domains 70% and 73%; in UBA domains 70% and 40% respectively [Katoh et al., 2004].

LKB1 (Liver Kinase B 1) is the first identified kinase that phosphorylates SIK2 at Thr^{172}, and phosphorylation by LKB1 makes SIK2 approximately 30 folds more active. On the other hand PKA (Protein Kinase A) was also found to phosphorylate SIK2 at Ser^{587}, and this phosphorylation does not contribute to activation of kinase but leads to translocation of SIK2 to the cytoplasm [Horike et al., 2003].

IRS1 (insulin receptor substrate-1), important regulator of insulin pathway, is the first identified substrate of SIK2 and in human adipocytes it is phoshorylated at Ser^{794} (rat Ser^{789}) by the enzyme (Figure 6.2), [Horike et al., 2003], [Katoh et al., 2004]. Insulin binds to insulin receptor and leads to phosphorylation of IRS1 by the receptor. This phoshorylation induces activation of downstream signaling cascades. However Ser^{794} phoshorylation was found to be associated attenutation of insulin signalling [Gual et al., 2005]. It was found that Ser^{794} phosphorylation of IRS-1 by SIK2 was highly elevated in insulin-resistant rats [Qiao et al., 2002].

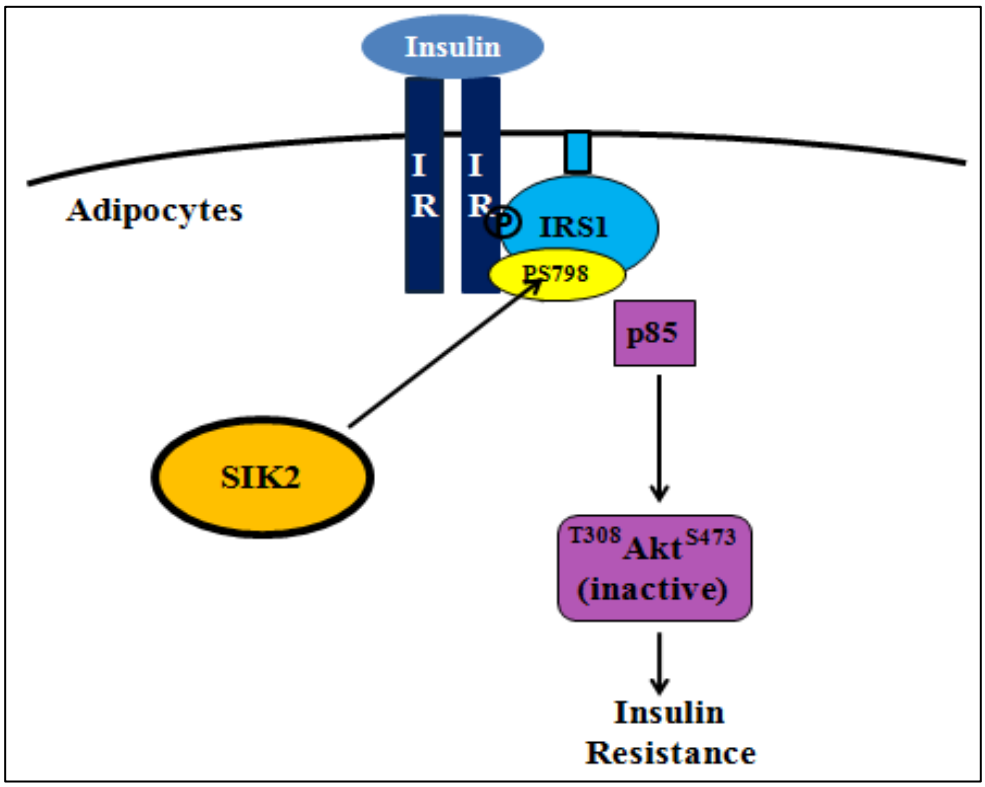


Figure 6.2: The proposed role of SIK2 in adipose insulin resistance.

Later on, another research has showed that SIK2 expression and activity were found to increased in white adipose tissue of diabetic mice [Horike et al., 2003]. All those findings suggest that SIK2 would play an important role in modulation of insulin signalling cascade and might lead to development of type-2 diabetes [Horike et al., 2003]. Furthermore, a recent work has revealed that nutrient deprivation and concomitant blockage of ATP synthesis in adipocytes have resulted in upregulation of SIK2 activity and in turn inhibition of lipogenesis by means of supressing major transcription regulators in white adipose tissue [Du et al., 2008].

TORC2 (transducer of regulated CREB activity 2), CREB co-activator, was also reported as SIK2 substrate and shown to be phosphorylated at Ser^{171} residue in pancreatic islet cells. After feeding, more precisely in presence of insulin, elevation of glucose level and gut hormone levels induce activation of PKA that normally inhibits SIK2 activity and concomitant with SIK2 inhibition TORC2 becomes dephoshorylated and translocates to nucleus in order to switch on the expression of CREB-mediated gene transcription. However during fasting, in other words in no insulin stimulation case, phoshorylation by SIK2 induces TORC2 to interact with 14-3-3 proteins and in turn this association results in cytoplasmic sequestration of

TORC2 hence inhibition of CREB dependent thermogenic gene transcription [Screaton et al., 2004], (Figure 6.3).

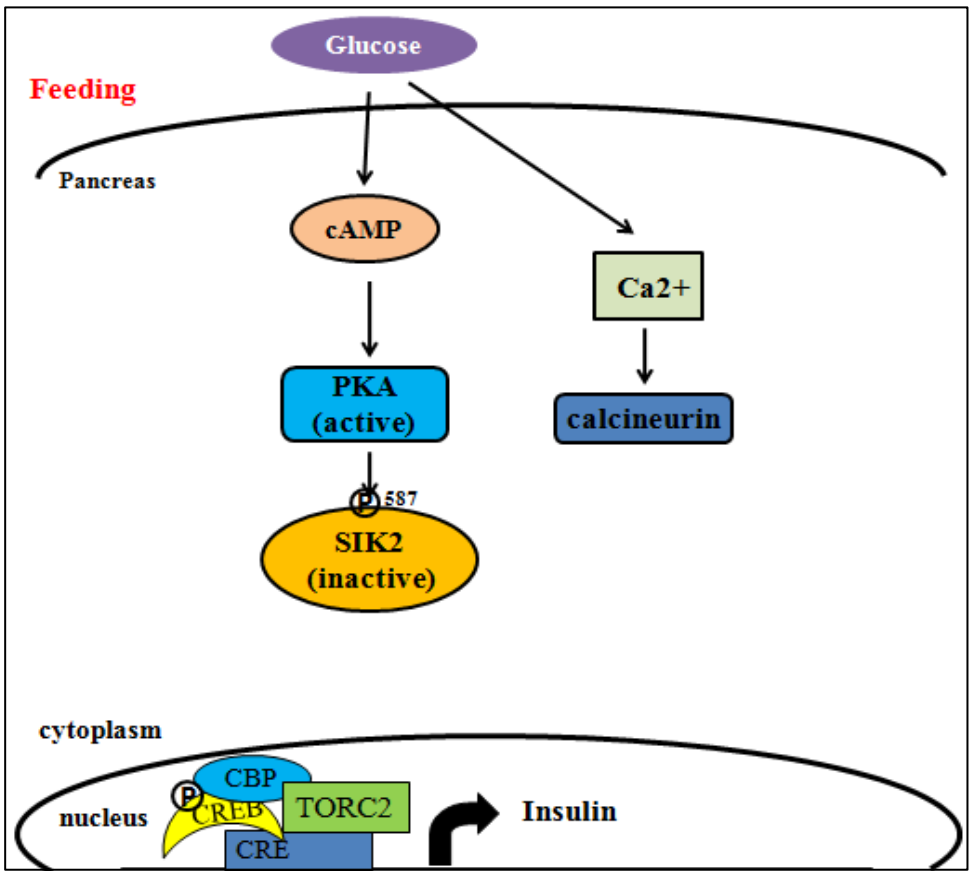


Figure 6.3: The role of SIK2 in CREB dependent thermogenic gene transcription.

SIK2 has also role in liver gluconeogenesis, generation of glucose from non-carbohydrate carbon substrates such as pyruvate, lactate and glycerol. Gluconeogenesis is the mechanism that keeps blood glucose levels from droping. During fasting, glucagon (released as blood glucose is low) triggers accumulation of cyclic AMP and in turn activation of PKA, which is a cAMP regulated kinase. PKA activation results in phoshorylation of SIK2 at Ser^{587} thereby inhibition of the enzyme. Consequently phosphorylated and inhibited SIK2 cannot phosphorylate TORC2 and dephosphorylated TORC2 translocates into nucleus in order to turn on expression of gluconeogenesis genes. However during re-feeding glucose level increases and insulin activates Akt2 in liver. Activated Akt2 in turn promotes SIK2 activation by phoshorylating the enzyme at Ser^{358} [Berdeaux, 2011], (Figure 6.4). Upon SIK2 activation, TORC2 is phosphorylated at Ser^{171} and translocates into

cytoplasm where it is associated with 14-3-3 proteins and gluconeogenic gene expression is inhibited in liver. [Dentin et al., 2007].

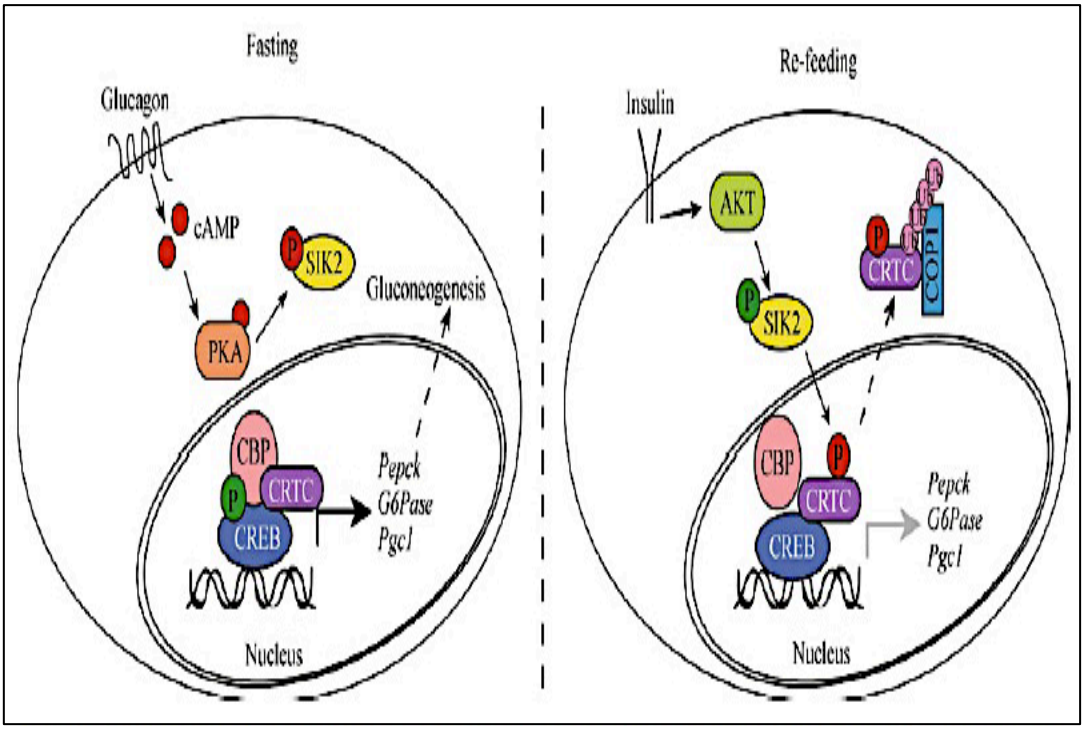


Figure 6.4: The role of SIK2 in CREB-TORC2-dependent hepatic gluconeogenesis under fasting and feeding conditions.

SIK2 has also several other roles in metabolism. A recent work has revealed that SIK2 controls liver lipogenesis. Normally in presence of glucose, histone acetyl transferase protein p300 acetylates and activates ChREBP (carbohydrate-responsive element-binding protein) thus initiates lipogenic gene expression. However SIK2 opposes this patway by phosphorylating p300 at Ser^{89} therefore reduces lipogenic gene expression by inhibiting acetylation of ChREBP [Bricambert et al., 2010], [Berdeaux, 2011], (Figure 6.5).

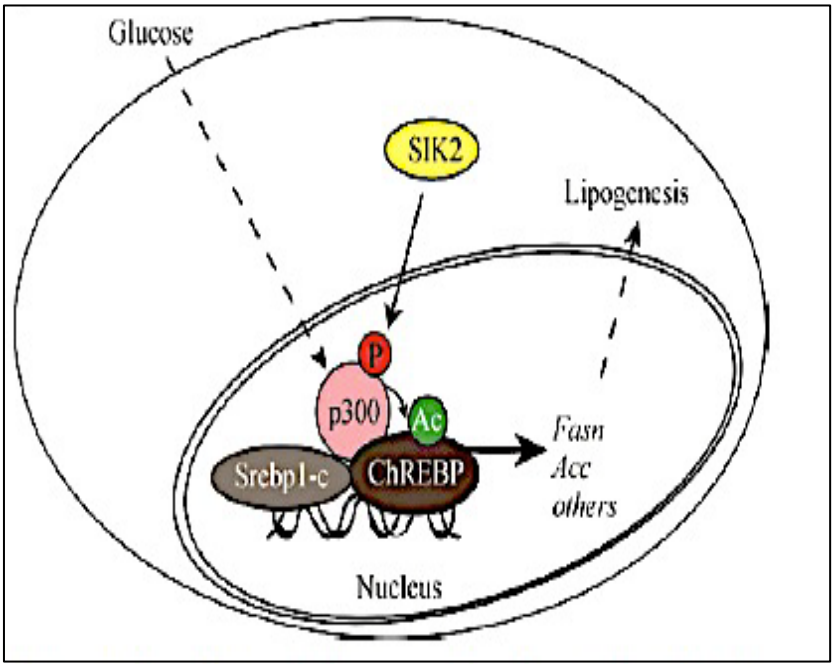


Figure 6.5: The role of SIK2 in p300-Srebp1-c-regulated hepatic lipogenesis.

Table 6.1: Known substrates of SIK2 and their phosphorylation motifs, phosphorylated residues are indicated with red.

SIK2 SUBSTRATES	SIK2 PHOSHORYLATION MOTIF
CNAP1	LXH(S/T)XSXXXL
	LHHSLSHSLL
IRS1-1	LRLSTSSGRL
	HyXBXX(S/T)XXXHy
P300 HAT	LLRSGSSPNL
CRTC2	LNRTSSDSAL
SREBP	DLVVGTEAKL
CRTC1	QYHGGSLPNVS
CRTC2	SNPSIQATL
CRTC3	SLSNPSLSTTN
HDAC4 & 5	LRKTASEPNLK

Table 6.2. Genes, which displayed SIK2 phosphorylation motifs via GPS2.1 software.

Gene Name	UNIPROT ID	Position	AA	Sequence	Motif-BHXXS/TXXH
VCPIP1	Q96JH7	204	S	RANKSQECL	Yes
EDEM1	Q92611	233	S	RVLGSLLSA	Yes
EDEM2	Q9BV94	228	S	RLWESRSDI	Yes
EDEM3	Q9BZQ6	598	S	KMGVSLIHL	Yes
EDEM3	Q9BZQ6	664	T	RVVLTAGPA	Yes
OS-9	Q13438	361	S	KVIRSPADL	Yes
OS-9	Q13438	546	T	RVRVTKLRL	Yes
MAN1B1	Q9UKM7	375	S	KIPYSDVNI	Yes
SREBF1	B0I4X4	88	S	KMYPSMPAF	Yes
CRTC2	Q53ET0	128	S	HIDSSPYSP	Yes
CRTC2	Q53ET0	393	S	HPSLSAPAL	Yes
CRTC2	Q53ET0	433	S	RVPLSPLSL	Yes
IRS1	P35568	188	T	RLCLTSKTI	Yes
IRS1	P35568	763	S	KPVLSYYSL	Yes
IRS1	P35568	828	S	RLEPSLPHP	Yes
GAB1	Q13480	130	S	KPPGSSLQA	Yes
GAB1	Q13480	197	T	KPEPTRTHA	Yes
GAB1	Q13480	276	S	KVSPSSTEA	Yes
GAB1	Q13480	306	S	HVSISYDIP	Yes
CRTC2	Q53ET0	238	S	KLSSSSSRP	Yes
IRS1	P35568	794	S	RLSTSSGRL	Yes

7. MATERIALS

7.1. Cell Lines

Hek293ft cells were kindly provided by Bosphorus University.

7.2. Chemicals, Plastic and Glassware

All chemicals used in this study were purchased from Sigma Aldrich (USA) or Merck (Germany) unless otherwise stated in the text. All solutions, plastic and glassware were sterilized by autoclaving at 121^0C for 20 minutes when possible.

7.3. Plasmids

The following plasmid was used throughout this study.

Table 7.1. Plasmid used.

pBabe-puro	Clontech, USA

7.4. Kits

The following kits were used throughout this study.

Table 7.2. Kits used.

Kit	Supplier
QIAprep Spin Miniprep kit	Qiagen, Germany
Bicinchoninic acid (BCA) assay kit	Thermo, USA

7.5. Equipment

The following equipments were used throughout this study.

Table 7.3. Equipment used.

Fluorescent Microscope	Nikon, Eclipse Ti-S)
Electrophoretic and Transfer Equipments	BioRad Mini Protean
PVDF Western Blotting Membrane	Amersham Biosciences, Hybond-P
Microcentrifuge	Beckman Coulter 22R
Microcentrifuge	Eppendorf Mini Spin
Centrifuge	Beckman Coulter Allegra X-22, Eppendorf Centrifuge 5810R
Shaker	BioSan, ES-20
Thermal Cycler	BioRad
Real Time Thermal Cycler	Roche, LC480II
Microbalance	Precisa XB320M
0.2 ml, 15 ml and 50 ml centrifuge tubes	Isolab
1.5 ml centrifuge tubes	Axygen
0.2 μm filter tips	TPP
0.2 μm vacuum filter	TPP
-80^{o}C Freezer	Symphany
Cell culture flasks and cryotubes	TPP
Glass Materials	Isolab
pH Meter	Mettler Toledo
Gel Imaging System	BioRad, ChemiDoc XRS+
Vortex	Yellowline TTS2
Liquid Nitrogen Tank	Arpege 140
Pipettor	Gilson
Micropipettor	Gilson
Filtered Micropipette Tips	Thermo
Serologic Pipette	Isolab, Precicolor, Lamtek
Pasteur Pipette	Isolab
Orbital Shaker	IKA, Yellow line
Heat blocks	VWR
Magnetic Stirrer	Heidolph
Isolecetric Focusing	Protean IEF Cell, BioRAD
IPG Strips	Ready Strip, IPG strips, BioRAD

8. METHODS

8.1. Hek293FT Culture Maintenance

Spontaneously immortalized Hek293FT kidney cells were maintained in Dulbecco's modified eagle medium (DMEM) with L-glutamine supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA) and 0.1% penicillin/streptomycin (Invitrogen, USA). Cells near confluence were washed with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), treated with 0.05% trypsin solution (0.05% trypsin in PBS) for 5 minutes and scraped. The cells were pelleted by centrifugation at 1500 rpm for 5 minutes and after resuspension in complete medium they were divided into four plates twice a week.

8.2. Agarose Gel Electrophoresis

DNA samples were mixed with 1/6 volume of 6x loading buffer (250 mg bromophenol blue, 550 mg xylene cyanol in 33 ml 150 M Tris, pH 7.6, 60 ml glycerol and 7 ml H_2O) and loaded onto 0.7 % agarose gels prepared in Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 1 mM EDTA, 20 mM acetic acid) and addition of 0.5 μg/ml ethidium bromide. The gels were run in the same buffer at 100 V. DNA size marker, Gene Ruler 100 bp DNA ladder, was purchased from Fermentas (Lithuania). The DNA bands were visualized under UV light and the images were documented with ChemiDoc imaging system (BioRad, USA) and analyzed by ImageLab software (BioRad, USA).

8.3. Preparation of the Vectors

All the used vectors, pBabepuro and pBabePuro SIK2 inserted one, were kindly gifted from Aklab, Bosphorus University, İstanbul. Those vectors were reproduced via transformation in DH5α bacterial cells.

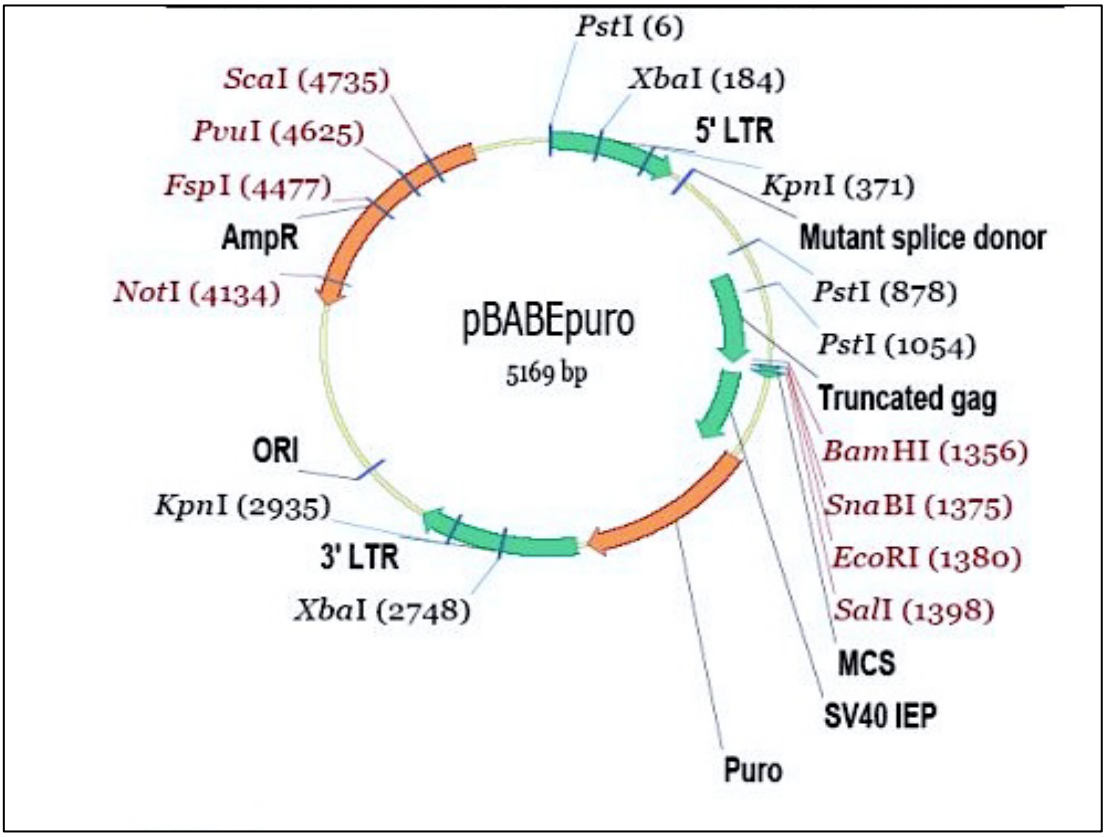


Figure 8.1. Map of pBABE-Puro vector.

8.4. Competent Cell Preparation

Stock of frozen DH5α bacterial cells were streaked on Luria Bertani broth (LB) agar plates (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl and 18 g/l agar) and incubated overnight at 37^0C. A well-isolated individual colony was transferred and grown in 5 ml of liquid LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) overnight at 37^0C. 25 ml LB medium was inoculated with 1:100 ratio of overnight culture and left incubation at 37^0C at 250 rpm shaking until the OD_{600} of the grown culture reached 0.3-0.4 (roughly two hours). Later on, the cells were spinned at 3000 rpm for 10 minutes at $+4^0$C. The bacterial pellet was resuspended gently, without vortexing, in 5 ml of ice-cold sterile 100 mM $CaCl_2$ and incubated on ice for 30 minutes. The cells were centrifuged this time at 8000 rpm for 10 minutes at $+4^0$C. The pellet was resuspended in 2.5 ml of ice-cold strerile 100 mM $CaCl_2$ containing 15% glycerol. The samples were aliquoted, 100 µl each, stored at -80^0C until used.

8.5. Transformation

For efficient DH5α transformation, 100 µl of frozen DH5α competent cells were mixed with 50 ng of appropriate vector. They were kept on ice for 30 minutes, heat-shocked at 42^0C for 90 seconds and cold-shocked on ice for 2 minutes. Subsequently 900 µl of LB medium (2 g tryptone, 0.5 g yeast extract, 1 ml 1 M NaCl in 1 l dH_2O) was added and cells were allowed to grow at 37^0C for two hours with shaking at 250 rpm. Subsequently, 100 µl and also the residual bacterial suspensions were plated on LB plates containing 100 µg/ml ampicillin (for pBabe-puro vector) and grown overnight at 37^0C.

8.6. Plasmid DNA Isolation

Selected colonies were grown overnight in LB medium containing appropriate antibiotic at 37^0C with shaking at 225 rpm and plasmids were isolated with MiniPrep kit (Qiagen, USA). Briefly bacterial cells were lysed and chromosomal DNA was denatured under strong alkaline conditions (pH 13.0), cell debris and chromosomal DNA were removed by centrifugation at 10000 xg for 10 minutes. Supernatant was applied to the QIAprep spin columns where DNA binds to the silica gel membrane in the presence of high salt. Impurities were removed by washing with wash buffer containing ethanol and plasmid DNA was eluted with TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). Plasmids were analyzed in 0.7 % agarose gels.

Stocks of bacteria carrying plasmids with the desired fragments were prepared by mixing overnight bacterial cultures with 15% glycerol and stored -80^0C until used.

8.7. Transfection of pBabe-Puro Vector to HEK293FT Cells

One day before the transfection Hek293FT cell density was measured with haemocytometer. 5 x 10^5 cells were seeded on 60 mm plates, and grown in DMEM containing 10% FBS and 0.1% streptomycin/penicillin. At the day of transfection, pBabePuro plasmids were diluted in 500 µl of OPTIMEM as 10 µg/ml concentration. The Turbofect transfection reagent (Thermo Scientific, USA): plasmid DNA ratio

was used at 3:1 and the complex in OPTIMEM was incubated for 15 minutes at room temperature. Then, all of it was added to the wells in a drop-wise manner. The plate contents were swirled on a rotating platform shaker for 30 seconds at room temperature. 24 hour after transfection culture medium was decanted and cells were washed three times with PBS and lysed.

8.8. Bicinchoninic acid (BCA) Assay

For determination of the concentration of proteins, BCA Assay kit was used. Unknowns and bovine serum albumin (BSA) dilutions ranging from 0.025 to 2 mg/ml were mixed with 200 µl of 50:1 diluted BCA Working Solution. After 30 minutes incubation at 37^0C and 5 minutes incubation at room temperature (18-25^oC), absorbance measured at 592 nm. Unknown sample concentrations were extrapolated from the standard curve.

8.9. SDS-PAGE and Western Blot

Immunoprecipitated proteins or cell lysates fractionated on 10 % polyacrylamide gels (10% acrylamide:bisacrylamide (29:1), 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 0.1% N,N,N',N'-tetramethylethylenediamine) with 6% stacking gel (6% acrylamide:bisacrylamide (29:1), 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate, 0.1% N,N,N',N'-tetramethylethylenediamine). Samples were boiled in 4X sample buffer (240 mM Tris-Cl, pH 6.8, 5 % beta-mercaptoethanol, 8% SDS, 0.04% bromophenol blue, 40% glycerol) at 95^0C for 10 minutes before loading. The gels were run in 25 mM Tris-HCl, 250 mM glycine and 0.2% SDS buffer at 90-120 V.

For some immunoprocipitation samples Sypro Ruby staining was done after incubation of the gel in fixing solution (50% methanol, 10% acetic acid in water) for 1 hour at 37°C. Subsequently, gel was gently shaken in Sypro Ruby solution (1X stain 1:2 ratio in distelled H_2O) for overnight at 37°C. Gels were rinsed with destaining solution (10% methanol, 7% acetic acid in water) for 30 minutes, then visualised under the UV via ChemiDoc.

For Western blotting, the samples fractionated on polyacrylamide gels were electroblotted to PVDF membranes (GE, USA) in transfer buffer (200 mM glycine, 25 mM Tris.HCl, 15% methanol) at 100 V for 1-1.5 hours. To equilibrate the membrane was washed in tris buffered saline-tween (TBS-T) solution (150 mM NaCl, 20 mM Tris.HCl, pH 8.0, 0.1 % Tween 20) three times for total of 30 minutes. The membrane was incubated in blocking solution (1% BSA or 1% - 5% skimmed milk powder in TBS-T) for 1 hour at room temperature with gentle shaking. The membrane was left overnight in the blocking solution containing appropriate antibodies at 4^0C, where the antibodies were diluted as given in Table 4.4. Subsequently, the membrane was washed with TBS-T three times for 5 minutes each to remove unbound antibody and incubated in Lumi-light Western blotting substrate (Cell signalling, USA) for 1 minute and images were captured using ChemiDoc visualizing system with 1 minute intervals.

Table 8.1. Antibodies, their Brands, applications, dilutions and band sizes used.

Antibody	Source	Application	Antibody dilution	MW (kDa)
SIK2	Cell Signalling	IP, WB	1/100, 1/500	130
VCP	Cell Signalling	WB	1/5000	170
Calnexin	Cell Signalling	WB	1/500	90
BIP	Cell Signalling	WB	1/500	78

8.10. Immunoprecipitation of SIK2 Protein

To be used in western blot SIK2 was immunoprecipitated. Anti-SIK2 antibody was pre-incubated 4 hours with cell lysates transfected with SIK2 and pBabePuro vector, respectively. Later on, samples were incubated with protein A-agarose beads at 4^0C overnight. Following day, incubated cell-lysate, antibody and protein A-agarose beads were centrifuged several times in order to collect the precipitates. Centrifugation steps were performed 10000 xg for 4 minutes and repeated for four times. After the final spin, the precipitates were resuspendes in cold cell lysis buffer containing protease inhibitor cocktail (50 mM HEPES, 150 mM NaCl, 1mM EDTA, 1mM EGTA, 10% Glycerol, 1% TritonX-100, 25 mM NaF, 10 μM $ZnCl_2$ with freshly added 10 μg/mol Aprotinin, 5 μg/mol Leupeptin, 1mM Na_3VO_4, 1 mM

PMSF). Half the samples were analyzed by western blotting; the remaining was used 1D visualising with Sypro Ruby.

8.11. Transfection and Endoplasmic Reticulum Isolation of 2D Samples

Firstly 5 x 10^6 cells were seeded on 100 mm plates, and grown in DMEM containing 10% FBS and 0.1% streptomycin/penicillin. At the day of transfection, pBabePuro plasmids were diluted in 500 µl of OPTIMEM as 10 µg/ml concentration. The Turbofect transfection reagent (Thermo Scientific, USA): plasmid DNA ratio was used at 3:1 and the complex in OPTIMEM was incubated for 15 minutes at room temperature. Then, all of it was added to the wells in a drop-wise manner. The plate contents were swirled on a rotating platform shaker for 30 seconds at room temperature. The day after the transfection one group is treated with 9µl Thapsigargin (300nM) and after 4-hour treatment transfection culture medium was decanted and endoplasmic reticulums of the cells were isolated.

Endoplasmic reticulum isolation process involves serial centrifugation steps. Firstly medium was removed from plates and 9 ml sterile PBS was added. After washing with PBS, PBS was removed and cells were treated 4,5 ml trypsin and incubated at 37^0C 2 minutes. 15 ml Dulbecco's modified eagle medium (DMEM) was added and cells were harvested. After 4 minutes centrifugation at 1000 rpm pellets were washed with PBS and centrigugation was repeated 2 times. After final centrifugation 1,2 ml PBS was added on pelltes and samples were transferred into 1,5 ml eppendorfs. After another 4-minute centrifuge step at 1000 rpm, the supernatants were discarded and pellets were taken on ice. The pellets were added precooled 1,2 ml 1X endoplasmic reticulum isolation buffer (10 mM HEPES pH: 7,8; 250 mM Sucrose; 25 mM Potassium Chloride; 1mM EGTA) and homogenized with dounce homogenizator at 300 rpm. After 20 strokes with homogenizator homogenized cells were transferred into precooled 1,5 ml eppendorf and centrifuged for 10 minutes at $+4^0$C at 2500 rpm. Later on, supernatants were collected and centrifuged at 14000 rpm for 15 minutes at $+4^0$C. Supernatants were collected again and transferred into ultracentrifuge tubes and centrifuged at 43000 rpm for 1 hour at 4^0C. After this final centrifugation step, supernants were transferred into different eppendorfs in order to analyse efficiency of the isolation process and the colorless pellets were dissolved in

2D lysis buffer (7 M Urea; 2M Thiourea; 4% CHAPS; 0.2% Biolytes 3-10; 0.5% Triton-X-100).

8.12. 2D Gel Electrophoresis

Protein separation by 2D gel electrophoresis consists of two steps, the first dimension is isoelectric focusing (IEF) and the second one is SDS-PAGE. First, IEF was performed in order to separate proteins according to their isoelectric points. IEF was carried out as follows:

Firstly, solubilized proteins are brought up to room temperature (18-25^{o}C). Sample volumes are adjusted to 150µg total protein in 125µL 2D-rehydration sample (IEF) buffer. Sample and IEF mixture is incubated for 30 minutes at room temperature. Mixtures are added into wells of a tray. Immobilised pH gradient (IPG) gel strips are added onto the mixtures. Tray is incubated for 30 minutes at room temperature. 1-2mL of mineral oil is overlaid into the wells in order to prevent evaporation. Rehydration is performed overnight at 25^{o}C. The next day, strips are washed with distilled water and transferred into wells of a proper tray. Wires of the tray are coated with whatmann papers in order to prevent high voltage burn out the strips. 1-2mL of mineral oil is added into the wells. IEF is run for 4 hours. The strips are washed with distilled water then incubated with Equilibrium Buffer II and I for 30 minutes at room temperature, respectively. Strips are ready for SDS-PAGE method.

SDS-PAGE was performed in order to separate proteins according to their molecular weights as described below:

10% separating gel in miniprotean (Biorad Tetracell) is casted and poured. Isopropanol is added in order to remove rough surface. Isopropanol is removed when the gel became solid. The strips in Equilibration Buffer II are put into the glass plate. Separation is performed for 1-2 hours at 50mA. Gels are incubated with fixation buffer for 2 hours at 37^{o}C. Gels are incubated with Sypro Ruby stain overnight at 37^{o}C with gentle shaking at 50 rpm. The stain is removed and gels are washed with washing buffer for 30 minutes at 37^{o}C. Gels are visualised under Ultra Violet (UV) and images are captured in gray scale using ChemiDoc XRS (BioRad).

8.13. Statistical Analysis

Spots on gels obtained by 2D gel electrophoresis were analysed by Progenesis Samespot Software. In this study, reticulum from cells under ER stress and SIK2 overexpression is compared to control cells with no stress induction and SIK2 overexpression and triplicate gels represent each experimental set up. Control gells are designated as Cnt1, Cnt2, Cnt3 and ER stressed samples as Tg1, Tg2, Tg3. Samespot automatically identified Tg2 as a reference gel due to the number of clear spots represented. All the other is normalized to Tg2 for detection of f-differences between two experimental conditions. Quality control at individual gels revealed two drawbacks in the gels: oversaturation, which can be fixed by adjusting exposure time and image acquisition in gray scale, which allows 16 bit data collection. Unfortunately in our case the data quality was 8 bit.

After all statistical analysis of different spots detected at $p < 0.05$ and fold change >2 fold adjustment produced approximately 56.9% of the 42 spots are reliable and has a power of 72% (which is slightly below 80% ideal for triplicate setup). This percentage can be increased to 95% reliability by running four replicates as suggested by the program.

One-Way Analysis of Variance (One-Way ANOVA) test was used in order to measure reliability of the differences between spots. One-Way ANOVA was used to measure the data difference between several groups. Statistical assumptions are shown below:

$p < 0.05$: statistically significant

$p < 0.01$: statistically very significant

$p < 0.001$: statistically highly significant

9. RESULTS

9.1. Endoplasmic Reticulum Isolation

In order to evaluate the efficiency of the endoplasmic reticulum isolation from HEK293FT cells, protein lysates from ERs is compared to that of cytoplasmic portion by checking presence of Er resident proteins in both samples by western blot. Four samples were prepared; SIK2 overexpressed, SIK2 overexpressed with 4-hour TG treatment, vehicle overexpressed and vehicle overexpressed with 4-hour TG treatment. In the last step of the endoplasmic reticulum isolation process supernants are taken and the pellet containing ER were dissolved in 2D lysis buffer. 60 µ samples of the lysates were used for western blot analysis. ER-resident Calnexin was selected for the isolation control. Calnexin presence was detected in endoplasmic reticulum isolation lysates, however it was absent in the supernatants of the corresponded samples. In the samples treated with TG it was seen that calnexin expression was reduced.

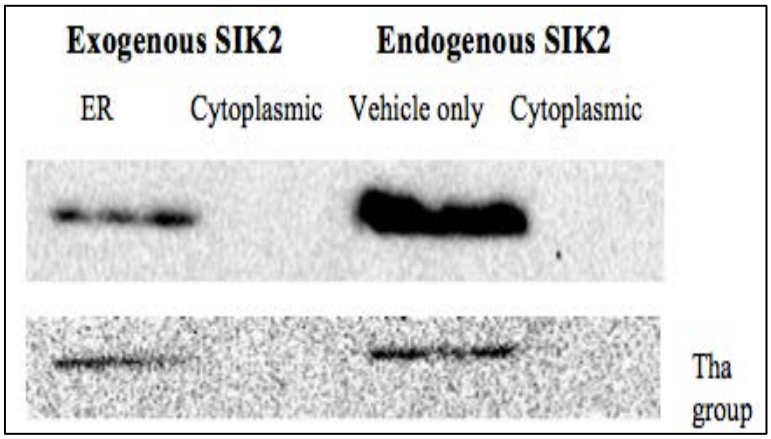


Figure9.1: Calnexin expression in endoplasmic reticulum isolation lysates and their supernatants.

Calnexin is suppressed by SIK2 overexpression whereas prevented by ER stress. We showed earlier that ER stress leads to SIK2 degredation (Fig. 9.5).

9.2. Transfection of Hek293FT cells

HEK293FT cell lysates were analysed for ERAD component p97/VCP by western blot. Four different samples were prepared; SIK2 overexpressing cells either treated or non-treated with chemical stress inducer Thapsigargin (TG) and control group included cells transfected by vehicle (pBabePuro) only and treated and nontreated with TG. Samples prepared protein lysates (ER and cytoplasmic) were resolved 10% SDS-PAGE gels. VCP, BiP and Calnexin antibodies were used in order to evaluate effect of SIK2 on expression of selected UPR and ERAD components while SIK2 antibody was used to evaluate efficiency of the overexpression. On the other hand VCP/p97 is an ATPase and this study does not address if enzymatic at this protein is affected by SIK2 activity or stress conditions.

Western blotting using VCP antibody showed that VCP expression is independent from SIK2 overexpression and TG treatment. VCP expression differences between different lysates when normalized to actin expression no significant difference detected between two biological samples (Figure 9.2).

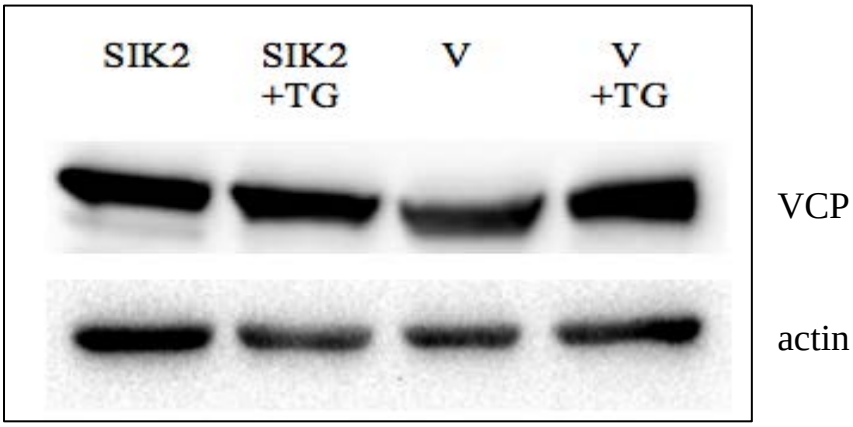


Figure 9.2: The effects of ER stress on VCP expression.

On the other hand, a well-known ER chaperone BiP (GRP78) expression is affected by both SIK2 overexpression and TG treatment. The latter increased the expression of BiP, whereas SIK2 expression markedly reduced its level similar to Calnexin. Moreover in absence of Tg treatment SIK2 overexpression was seemed to result in BiP degradation (Figure 9.3). SIK2 mediated downregulation of these two chaperones (BiP/ Calnexin) suggests that SIK2 may be a global regulators of ER

chaperones and it would be interesting to find out if this is a direct or indirect effect of SIK2. First of all, one needs to check whether overexpression causes this at the transcriptional or protein level. If the latter is true SIK2 effector proteins especially transcriptional factors including TORC/CRESB and SREB1 can be evaluated for this effect.

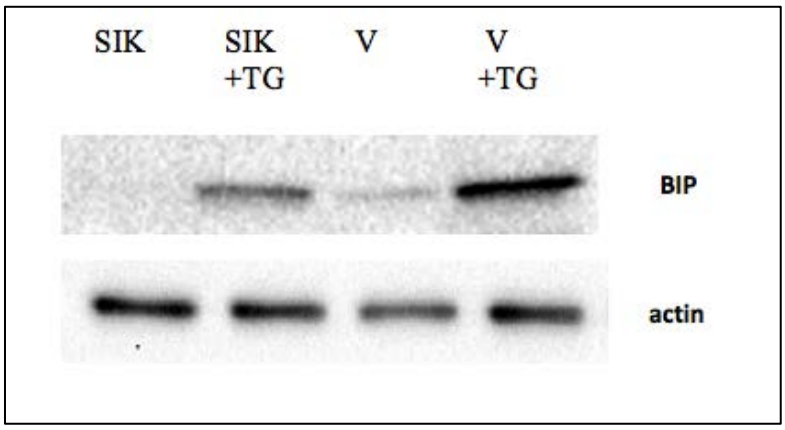


Figure 9.3: The effects of ER stress on BiP expression.

Western blotting using Calnexin antibody showed that Calnexin expression is effected by both SIK2 overexpression and TG treatment. TG treatment reduced the expression of BiP, whereas SIK2 expression resulted in degradation of Calnexin. TG interferes with N glycosylation.

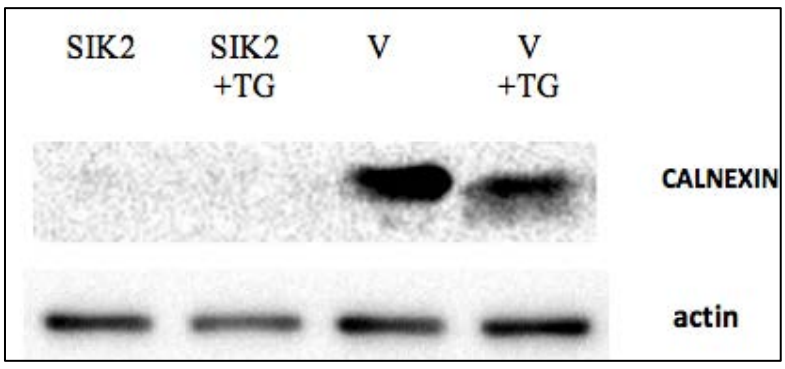


Figure 9.4: The effects of ER stress on Calnexin expression.

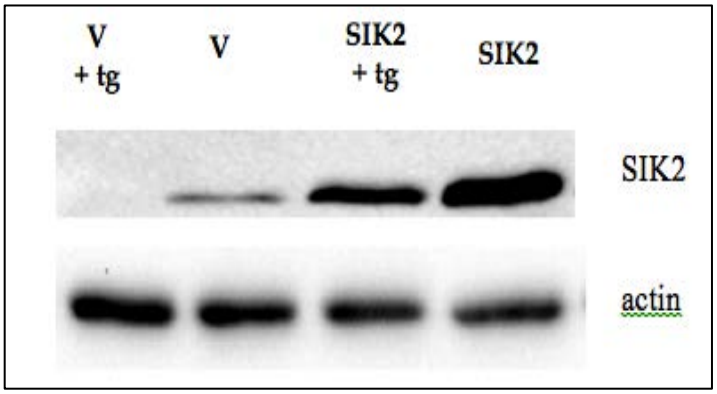


Figure 9.5: SIK2 expression in case of SIK2 overexpression and Thapsigargin treatment.

Western blotting using SIK2 antibody showed that SIK2 expression is drastically reduced by TG treatment even in the presence of SIK2 overexpression. There is approximately 2,5 fold change in SIK2 protein levels between cells overexpressing SIK2 and having vehicle only.

9.3. 2D Gel Electrophoresis of Endoplasmic Reticulum Isolation Lysates

Lysates prepared by endoplasmic reticulum isolation process were loaded into proper nonlinear IPG strips with the pH range 3-10. The IPG strips, which were focused at high voltage, were resolved in 10% SDS-PAGE gels. After treatment with Sypro Ruby, they were visualized under the UV via ChemiDoc-XRS^{+} (BioRad, UK). Following figures show the corresponding gel images.

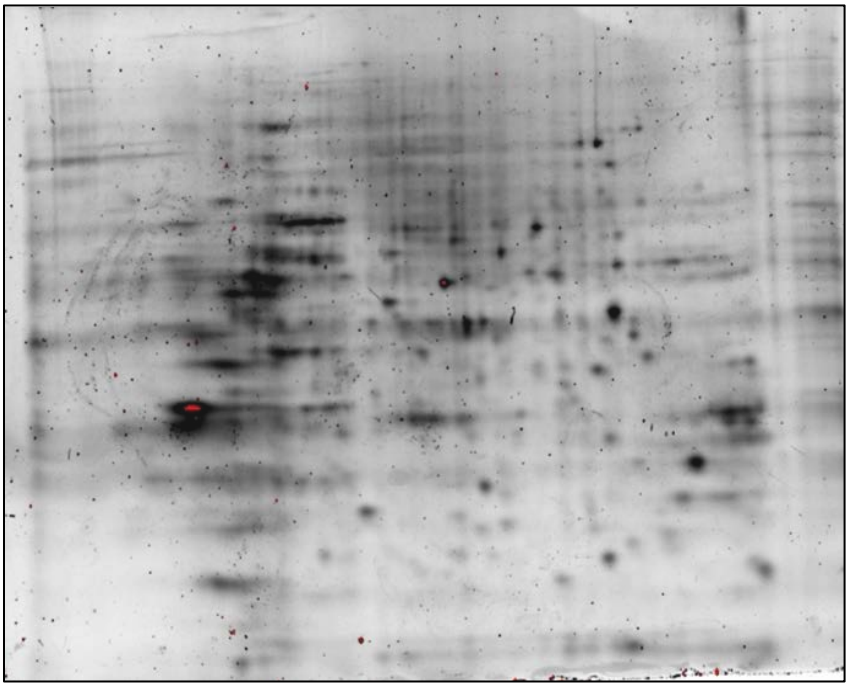


Figure 9.6: Control Group; ER lysate prepared from Hek293FT cells overexpressing SIK2 (0.5 sec exposure time under UV).

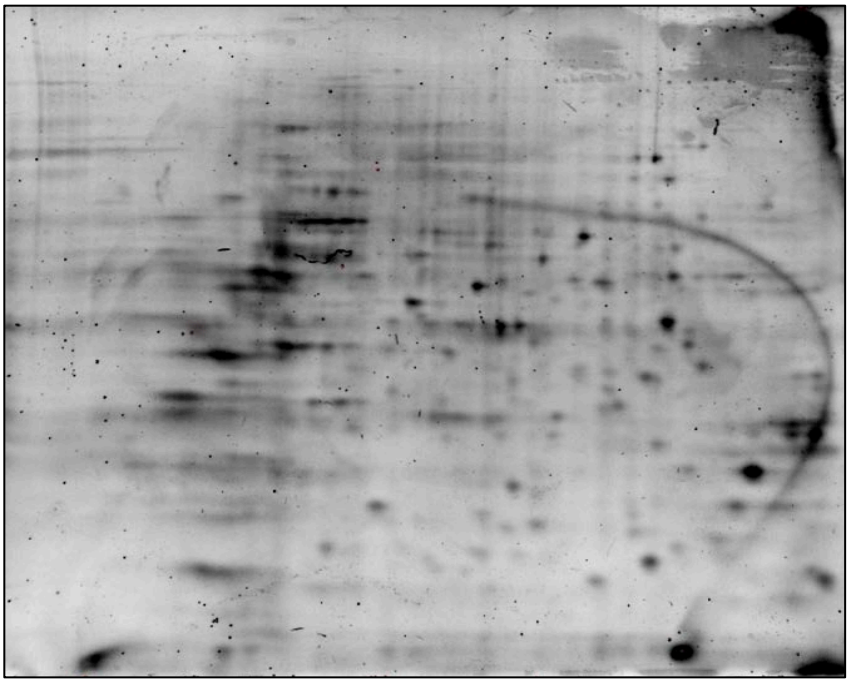


Figure 9.7: Second replicate of gel number 1.

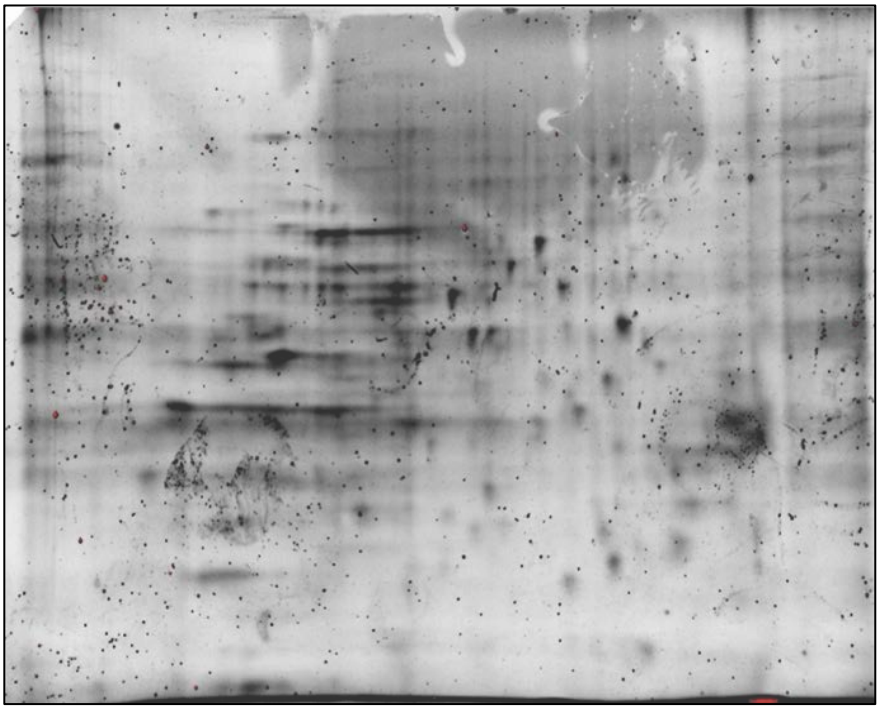


Figure 9.8: Third replicate of gel number 1.

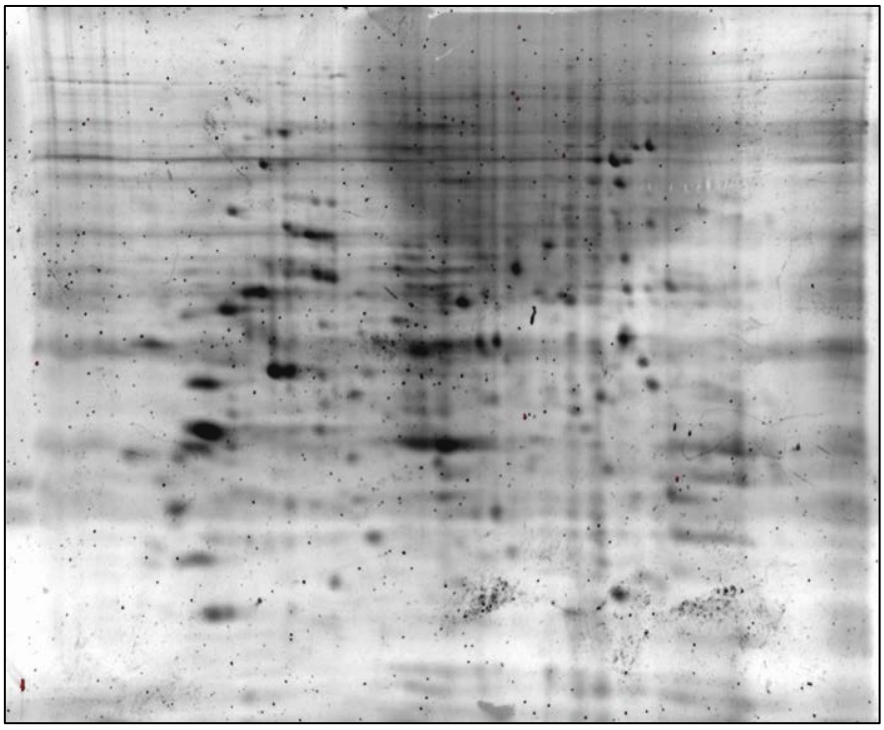


Figure 9.9: TG1; ER lysate from cells overexpressing SIK2 and treated with TG for 4h.

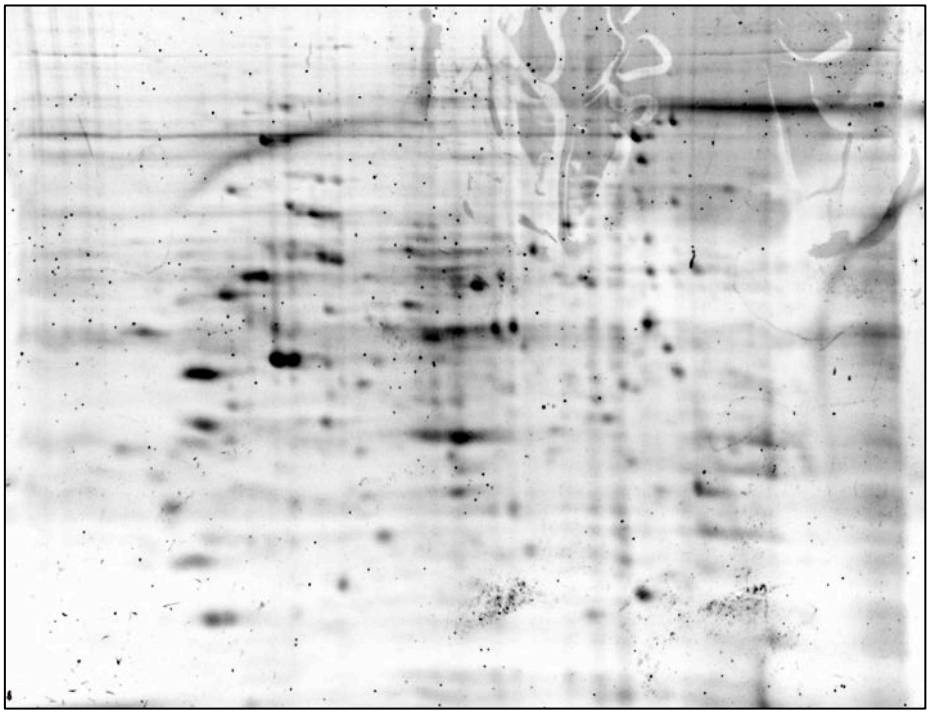


Figure 9.10: Second replicate of TG1.

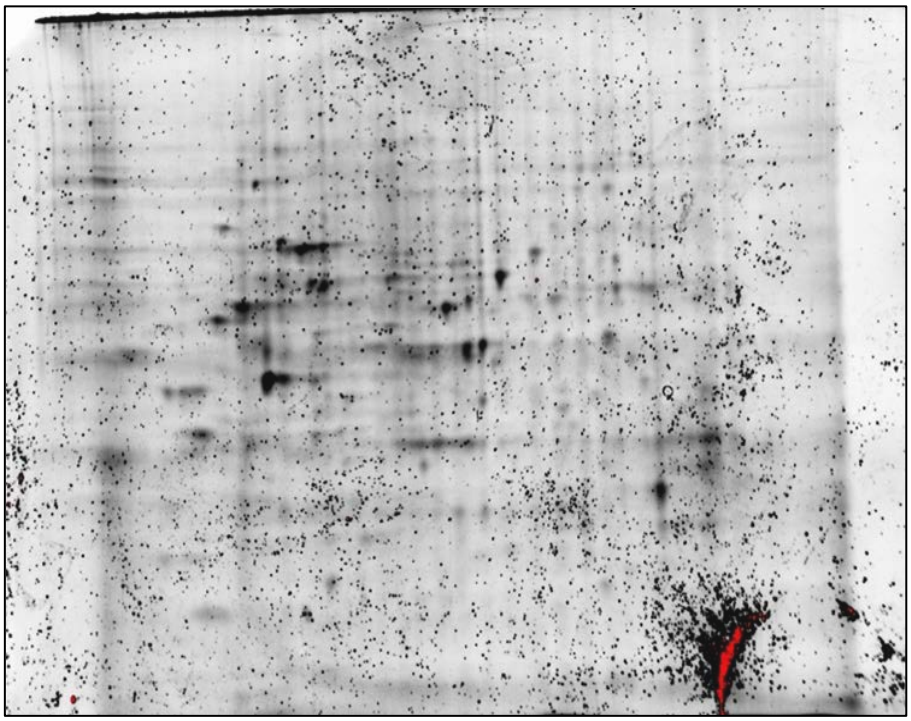


Figure 9.11: Third replicate of TG1.

9.4. Analysis of 2D Gels by Progenesis SameSpot Software

Gel images visualized under the UV light were analysed by using Progenesis Samespot Software (Nonlinear Dynamics, UK). According to the program a new experiment design and two different groups for individual staining each included triplicate gels for reliable statistical analysis were selected. Both group used reticulum from cells overexpressing SIK2 while only the later was inducted with TG treatment. Each biological sample was loaded into three different IPG (pH 3-10 NL) strips and three different gel images were obtained at UV Channel corresponding to one sample.

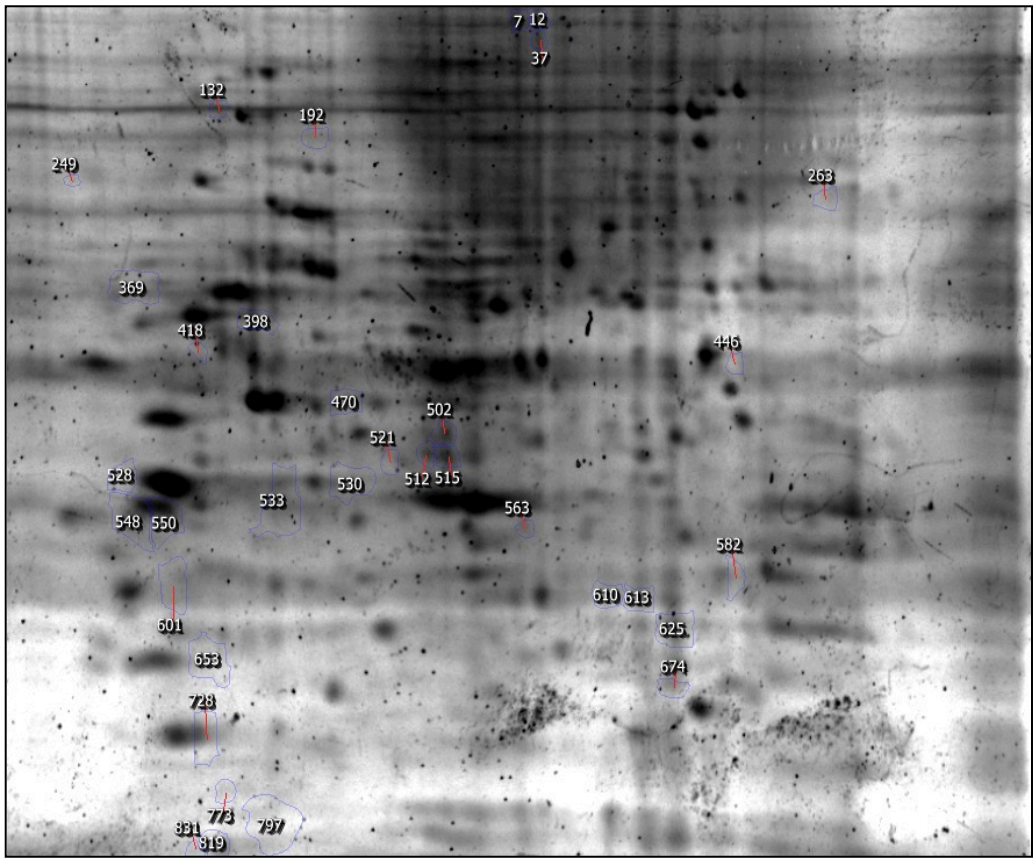


Figure 9.12: Aligment of whole gel images by using Progenesis Samespot Software

After bading triplicate gels for each sample, the quality control check by the software exhibited few drawbaks. Due to the better resolution and the quality of TG2 gel picture the program assigned it as a reference gel to align with others for the

master gel production representing all the spots found commonly in al six gels. For the detection of distinct spots between two experimental setup, program merged master gel (Figure 9.12). On the basis of user defined statistical parameters $p > 0.05$ and >2 fold change difference the program detected both up and down regulated proteins with confidence at 56.9 % power. Table 9.1 lists the number of spots either up or down regulated upon TG treatment.

Table 9.1: Spots found using Progenesis Samespot Software.

#	Anova (p)	Fold	Tags	pI	MW	Average Normalised Volumes	
						Normal Untreated	Thapsigargin Treatment
502	4,969e-004	2,3		6,03	55	3856,223	8967,989
249	9,005e-004	2,1		3,42	105	2030,730	956,460
192	0,001	2,4		5,16	123	1,490e+004	6107,055
533	0,001	2,6		4,84	49	1,096e+005	4,299e+004
515	0,001	2,8		6,03	53	4797,432	1,328e+004
625	0,002	2,1		7,61	37	2,183e+004	4,494e+004
37	0,004	3,7		6,66		1355,078	5053,872
521	0,004	2,2		5,67	52	1,010e+004	4543,330
653	0,004	3,2		4,43	36	7,189e+004	2,263e+004
470	0,004	2,1		5,26	61	2,740e+004	1,334e+004
263	0,004	2,1		8,7	99	9503,723	4482,201
512	0,004	2,1		5,92	53	3930,342	8090,681
831	0,004	8,4		4,33		8645,191	1030,021
773	0,006	2,6		4,51		6306,469	2457,538
369	0,007	2,6		3,94	81	5,612e+004	2,169e+004
12	0,007	2,5		6,66		4013,077	9938,527
528	0,008	5,8		3,88	50	2,942e+004	5076,477
601	0,009	3,0		4,2	39	6,005e+004	1,977e+004
563	0,009	2,0		6,57	45	6212,776	3103,913
728	0,009	2,3		4,29		5,503e+004	2,386e+004
446	0,010	2,9		8,02	66	7820,528	2703,204
530	0,013	2,8		5,3	50	5,980e+004	2,167e+004
7	0,014	2,1		6,52		4278,530	8823,120
550	0,016	2,5		4,04	47	9,550e+004	3,761e+004
674	0,017	2,1		7,6		4236,165	8862,848
797	0,023	2,3		4,76		6,284e+004	2,753e+004
398	0,023	2,4		4,61	78	4,924e+004	2,039e+004
610	0,025	2,1		7,14	39	6538,819	1,390e+004

Table 9.1: Spots found using Progenesis Samespot Software (continue).

#	Anova (p)	Fold	Tags	pI	MW	Average Normalised Volumes	
						Normal Untreated	Thapsigargin Treatment
418	0,027	2,5		4,32	71	1,087e+004	4419,695
582	0,029	2,2		7,98	41	1,913e+004	8539,767
548	0,046	2,3		3,95	47	1,015e+005	4,504e+004
819	0,046	3,2		4,37		3,755e+004	1,184e+004
613	0,047	2,7		7,37	39	8909,005	2,437e+004
132	0,047	2,2		4,43	139	4357,204	9620,161

Table 9.2: Tags used to define conditions in Progenesis Samespot Software.

Condition	Normal Untreated	Thapsigargin Treatment
Replicates	3	3

Table 9.3: Tags used in Progenesis Samespot Software to define spots.

TAGS	
	Anova p-value ≤ 0,05
	Max fold change ≥ 2
	Edited
	goes up w TG
	goes down w TG

9.5. SIK2 and p97/VCP Relation

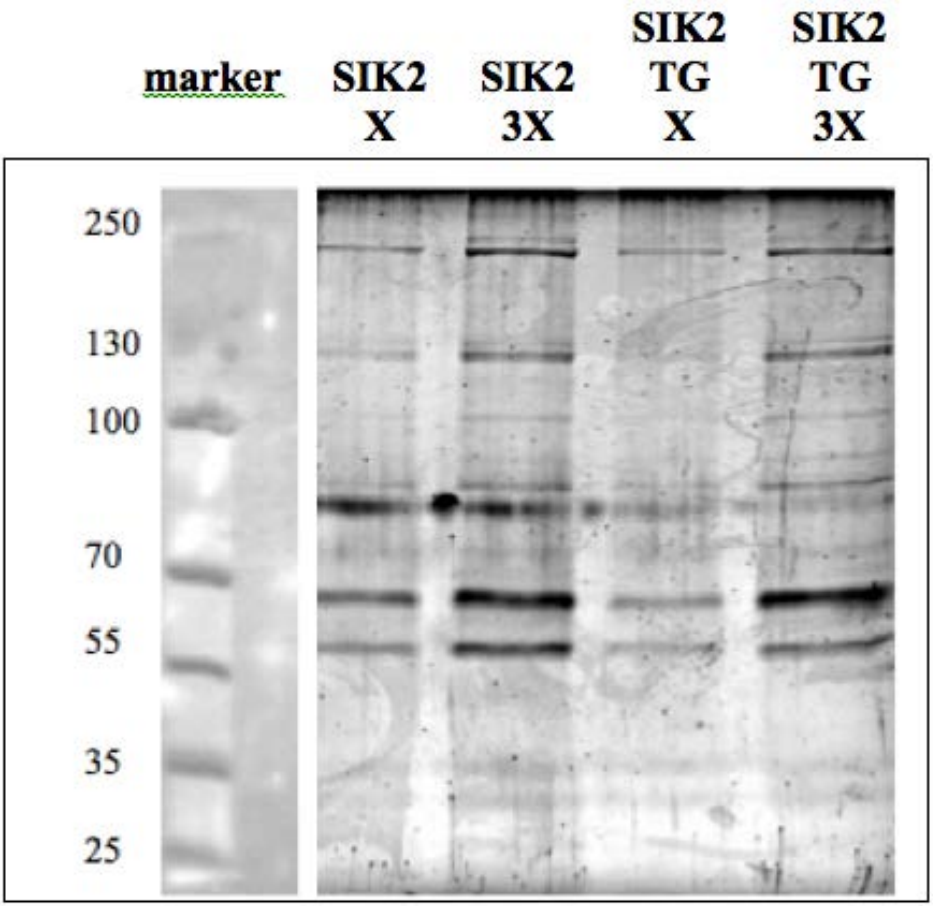


Figure 9.13: SyproRuby staining of SIK2 immunoprecipitation in 1D SDS-PAGE (10%).

Cells overexpressing SIK2, which treated or non-treated with TG were compared. Three-fold excess was loaded for each application for proper evaluation of intensities.

10. DISCUSSION

Obesity has become one of the major threats to human health in past the two decades due to high caloric diets, prolonged lifespan and reduced physical activity. Recent researches have showed that obesity predisposes individuals to various diseases collectively known as metabolic syndrome and type-2 diabetes. It was thought that under ER stress conditions aberrant protein load in ER lumen alters homeostasis of ER and intervene most of the vital functions of endoplasmic reticulum which in turn leaves the cell in the lurch. Eukaryotic cells have evolved several well-established mechanisms in order to handle with this aberrant situation namely the UPR and the ERAD. UPR functions as a switch between survival and apoptosis. Firstly it evaluates the degree of the ER stress, it tries to cope with the situation by inhibiting overall protein synthesis while inducing expression of chaperones and ERAD components. However if the situation is far beyond ER capacity, UPR induces expression of genes related to autophagy in a final attempt to escape apoptosis and favour survival.

SIK2 is a major kinase, which plays a central role over the vital metabolical processes. Recent researches have showed that there are detrimental perturbations in pathways regulating energy metabolism such as gluconeogenesis and lipogenesis in obese subjects associated with low-grade inflammation and chronic ER stress in respective tissues. In this study and ER specific proteomics (reticulomics) approach is favoured for the sake of ER stress associated complexity in whole proteome. Here we explored the effects of ER stress on reticulomics in cells over expressing a metabolically critical Ser/Thr kinase SIK2. Initially, all mammalian ERAD protein components deciphered by Christianson et al. was searched for candidate SIK2 phosphorylation sites using a modified GPS2.1 in collaboration with Xu Xue. Among others listed in Table 6.2 VCP displayed strong SIK2 phoshorylation motif and considered as a good candidate for further investigation here. However there were no changes in VCP levels between the SIK2 overexpressing and nonexpressing cells without regard to TG treatment. It was concluded that VCP/p97 expression is independent from both SIK2 overexpression and TG treatment despite we did not included a functional study for VCP roles in these circumstances.

Calnexin was selected because of its key role in protein folding. Calnexin, one of the master chaperones, is in charge of assistance of properly folded and assembled proteins to be delivered to the further destinations. At the same time Calnexin retains unfolded and unassembled ones in the ER lumen. It binds to Man_9Glc_1 N-glycans and ensure their migration out of ER. It was shown that Calnexin, an ER resident chaperone, was markedly reduced by SIK2 overexpreesion. Calnexin is one of the major assistants of protein folding process. At the same time expression level of Calnexin was reduced in ER stress presence.

BiP/GRP78, which is master regulator of all UPR sensors; IRE1α, PERK and ATF6 was also investigated in this study. BiP is the most abundant chaperone, which is functional in both UPR and ERAD pathways. It binds to nascent protein chains during retrotranslocation. BiP was shown to be markedly affected by both SIK2 overexpression and TG treatment. ER stress increased BiP level, however SIK2 overexpression acted opposite.

2D gel electrophoresis was performed in order to analyse possible differences in reticulomics of TG treated and nontreated cells in the presence of SIK2 overexpression. By using Progenesis Samespot Software it was shown that there are fourty two spots identified with over 2 fold changing in protein level upon stress condition. Among them five exhibited over three-fold change. This shows that there significant differences of protein levels in case of ER stress, it is practically impossible to say that this changes is due to SIK2 overexpression. The spots were not sent to mass analysis so their correct identities were not determined. Although empirical determination of their isoelectric points and molecular weights may be used to extrapolate about their identities when compared to computational values for respective reticulomics.

Due to low transfection efficiencies with hepatocarcinoma cell line Huh7, HEK293FT cell line with superior transfection efficiency was used in 2D reticulomics study. Recently, we are attemting to use retrovirus to create stable Huh7 cells to reproduce current work in relevant cell lines. Gel qualities will be improved to achieve at least 80% power, which means 80 percent of the spots detected. Phosphoproteomics study will be conducted along with the 2D work for better understanding of SIK2's role in ER associated processes including UPR and ERAD. Finally , mass spectra analysis will be exploited for identification of spots with low p values and high fold changes with over 95% confidence in identified spots.

REFERENCES

Bravo R., Parra V., Gatica D., Rodriguez A.E., Torrealba N., Paredes F., Wang Z. V., A. Zorzano,. Hill J.A., Jaimovich E., Quest A.F.G, Lavandero S., (2013), "Endoplasmic Reticulum and the Unfolded Protein Response: Dynamics and Metabolic Integration'', International Review of Cell and Molecular Biology, 301, 215-290.

Bricambert J., Miranda J., Benhamed F., Girard J., Postic C., Dentin R., (2010), "Salt-inducible kinase 2 links transcriptional coactivator p300 phosphorylation to the prevention of ChREBP-dependent hepatic steatosis in mice" , American Society for Clinical Investigation, 120(12), 4316-4331.

Bright, N. J., C. Thornton, and D. Carling, (2009), "The Regulation and Function of Mammalian AMPK-Related Kinases", Acta Physiologica (Oxf), 196(1), pp. 15-26.
Chemistry, 279, 25935-25938.

Christianson J.C., Olzmann J.A., Shaler T.A., Sowa M.E., Bennett E.J., Richter C.M, Tyler RE, Greenblatt EJ, Harper JW, Kopito RR., (2011), "Defining human ERAD networks through an integrative mapping", Nature Cell Biology, 14(1), 93-105.

Claessen J. P. H.L., Kundrat L., Ploegh H. L., (2012), ''Protein quality control in the ER: balancing the ubiquıtin checkbook'', Trends in Cell Biology, 22(1), 22-32.

Çalışkaner O., (2013), "Endoplasmik Retikulum Stresin SIK2 yolağı üzerindeki etkisi", Yüksek Lisans tezi, Gebze Yüksek Teknoloji Enstitüsü.

Dentin, R., Y. Liu, S. H. Koo, S. Hedrick, T. Vargas, J. Heredia, J. Yates and M. Montminy, (2007), "Insulin Modulates Gluconeogenesis by Inhibition of the Coactivator TORC2", Nature, 449 (7160), 366-369.

Du J., Chen Q., Takemori H., and Xu H., (2008), "SIK2 Can Be Activated by Deprivation of Nutrition and it Inhibits Expression of Lipogenic Genes in Adipocytes", Obesity (Silver Spring), 16(3), 531-538.

Ellgaard L. and Helenius A., (2003), "Quality Control in The Endoplasmic Reticulum", Molecular Cell Biology, 4, 181-191.

Feldman J. D., Vician L., Crispino M., Hoe W., Baudry M., and. Herschman H. R, (2000), "The Salt-Inducible Kinase, SIK, is Induced by Depolarization in Brain", Journal of Neurochemistry, 74(6), 2227-2238.

Gual P., Marchand-Brustel Y. L., and J. F. Tanti, 2005, "Positive and Negative Regulation of Insulin Signaling Through IRS-1 Phosphorylation", Biochimie, 87(1), 99-109.

Hagiwara M., Maegawa K., Suzuki M., Ushioda R., Araki K., Matsumoto Y., Hoseki J., Nagata K., Inaba K., (2011), ‘’ Structural basis of an ERAD pathway mediated by the ER-resident protein disulfide reductase ERdj5’’, Molecular Cell Biology, 41(4), 432-444.

Hebert D. N and Molinari M., (2007), ‘’In and out of the ER: protein folding, quality control, degradation, and related human diseases’’, Physiological Reviews., 87(4), 1377-408.

Horike N., Kumagai A., Shimono Y., Onishi T., Itoh Y., T. Sasaki, K. Kitagawa, Hatano O., Takagi H., Susumu T., Teraoka H., Kusano K., Nagaoka Y., H. Kawahara, and H. Takemori, 2010, “Downregulation of SIK2 Expression Promotes the Melanogenic Program in Mice”, Pigment Cell and Melanoma Research, 23(6), 809-819.

Horike N., Takemori H., Katoh Y., Doi J., Min L., Asano T., Sun X. J., Yamamoto H., Kasayama S., Muraoka M., Nonaka Y., and Okamoto M., (2003), “Adipose-Specific Expression, Phosphorylation of Ser794 in Insulin Receptor Substrate-1, and Activation in Diabetic Animals of Salt-Inducible Kinase-2”, Journal of Biological Chemistry, 278(20), 18440-18447.

Hoseki J., Ushioda R., Nagata K., (2009), “Mechanism and components of endoplasmic reticulum-associated degradation”, The Journal of Biochemistry, 147(1), 19-25.

Hoseki J., Ushioda R., Nagata K., (2010), ‘’ Mechanism and components of endoplasmic reticulum-associated degradation.’’, Journal of Biochemistry, 147(1), 19-25.

Jaleel M., Villa F., Deak M., Toth R., Prescott A. R., Van Aalten D. M. and Alessi D. R., (2006), “The Ubiquitin-Associated Domain of AMPK-Related Kinases Regulates Conformation and LKB1-Mediated Phosphorylation and Activation”, Biochemical Journal, 394(3), 545-555.

Katoh, Y., H. Takemori, N. Horike, J. Doi, M. Muraoka, L. Min, and M. Okamoto, (2004), “Salt-Inducible Kinase (SIK) Isoforms: Their Involvement in Steroidogenesis and Adipogenesis”, Molecular and Cellular Endocrinology, 217, (1-2), 109-112.

Määttänen P., Gehring K., Bergeron J.J., Thomas D.Y., (2010), ‘’Protein quality control in the ER: the recognition of misfolded proteins’’, Seminars in Cell and Developmental Biology, 21(5), 500-511.

Molinari M., (2007), ‘’ N-glycan structure dictates extension of protein folding or onset of disposal’’, Nature Chemical Biology, 3, 313 – 320.

Muraoka M., Fukushima A., Viengchareun S., Lombès M., Kishi F., Miyauchi A., Kanematsu M., Doi J., Kajimura J., Nakai R., Uebi T., Okamoto M., Takemori H., (2009), ‘’Involvement of SIK2/TORC2 signaling cascade in the regulation of insulin-induced PGC-1alpha and UCP-1 gene expression in brown adipocytes’’, American Journal of Physiology Endocrinology and Metabolism, 296(6), 1430-1439.

Nakatsukasa K., Huyer G., Michaelis S., Brodsky J.L., (2008), ‘’Dissecting the ER-associated degradation of a misfolded polytopic membrane protein’’, Cell, 132(1), 101-112.

Okamoto M., Takemori H. and Katoh Y., (2004), “Salt-Inducible Kinase in Steroidogenesis and Adipogenesis”, Trends in Endocrinology and Metabolism, 15(1), 21-26.

Olzmann J.A., Kopito R.R., Christianson J.C., (2013), “The Mammalian endoplasmic reticulum-associated degradation system”, Cold Spring Harbor Perspective in Biology, 5(9) , 1-12.

Ron D. and Walter P., (2007), “Signal integration in the endoplasmic reticulum unfolded protein response”, Nature Reviews Molecular Cell Biology, 8(7), 519-529.

Sage A. T., Holtby-Ottenhof S., Shi Y., Damjanovic S., Sharma A. M., Werstuck G.H., (2011), “Metabolic syndrome and acute hyperglycemia are associated with endoplasmic reticulum stress in human mononuclear cells”, Silver Spring, 20(4), 748-755.

Samali A., Fitzgerald U., Deegan S., Gupta S., 2009), “Methods for monitoring endoplasmic reticulum stress and the unfolded protein response”, International Journal of Cell Biology, 2010, 1-11.

Sasaki T., Takemori H., Yagita Y., Terasaki Y., Uebi T., Horike N., Takagi H., Susumu T., Teraoka H., Kusano K., Hatano O., Oyama N., Sugiyama Y., Sakoda S., and Kitagawa K., (2011), “SIK2 is a Key Regulator for Neuronal Survival after Ischemia via TORC1-CREB”, Neuron, 69(1), 106-119.

Schröder M. and Kaufman R. J., (2005), ‘’ER stress and unfolded protein response’’, Mutation Reseach, 569, 29-63

Seiser, R. M., (2000), "The Fate of Membrane-bound Ribosomes Following the Termination of Protein Synthesis", Journal of Biological Chemistry, 275 (43): 33820–33827.

Sharma N.K., Das S.K., Mondal A.K., Hackney O.G., Chu W.S., Kern P.A., Rasouli N., Spencer H.J., Yao-Borengasser A., Elbein S.C., (2008), “Endoplasmic reticulum stress markers are associated with obesity in nondiabetic subjects.” , Journal Clinical Endocrinology and Metabolism, 93(11), 4532-4541.

Sinici I., (2008), “Endoplasmic Reticulum Quality Control and Pharmacological Chaperones”, FABAD Journal Pharmaceutical Sciences, 33, 163-176.

Takemori H., Okamoto M., (2007), “Regulation of CREB-mediated gene expression by salt inducible kinase” , Journal of Steroid Biochemistry and Molecular Biology, 108(2008), 287-291.

Vembar S.S. and Brodsky J.L., (2008), ‘’One step at a time: endoplasmic reticulum-associated degradation’’, Nature Reviews Molecular Cell Biology, 9(12), 944-957.

Wang Y., Vera L., Fischer W.H., Montminy M., (2009), ‘’ The CREB coactivator CRTC2 links hepatic ER stress and fasting gluconeogenesis’’, Nature, 460 ,534-537.

Wang Y., Fischer H. W., Vera L., Montminy M., (2009), "The CREB coactivator CRTC2 links hepatic ER stress and fasting gluconeogenesis", Nature , 460(7254), 534-535.

Wang Z., Takemori H., Halder S. K., Nonaka Y., and Okamoto M., (1999), "Cloning of a Novel Kinase (SIK) of the SNF1/AMPK Family from High Salt Diet-Treated Rat Adrenal", FEBS Letters, 453(1-2), 135-139.

Web 1, (2013), http://www.nature.com/nature/journal/v454/n7203/full/nature07203.html, (Access Date: 05/09/2013).

Web 2, (2013), http://micro.magnet.fsu.edu/cells/endoplasmicreticulum/endoplasmicreticulum.html, (Access Date: 04/09/2013).

Web 3, (2013), http://cidms.org/pathways/er_stress/s2.html, (Access Date: 04/09/2013).

Ye R., Jung D.Y., Jun J.Y., Li J., Luo S., Ko H.J., Kim J.K., Lee A.S., (2010), ‘’ Grp78 heterozygosity promotes adaptive unfolded protein response and attenuates diet-induced obesity and insulin resistance’’, Diabetes, 59(1), 6-16.

Yoshida H., (2007), ‘’ER Stress and Diseases’’, The Febs Journal, 274, 630–658.

Zhang K., Kaufman R. J., (2008), "From endoplasmic-reticulum stress to the inflammatory response" , Nature, 454(7203), 455-462.

Zhang K., Kaufman R.J., (2004), ‘’Signaling the unfolded protein response from the endoplasmic reticulum’’, Journal of Biological Chemistry, 279(25), 25935-25938.

Zhou L., Zhang J., Fang Q., Liu X., Liu M., Jia, W., Dong L. Q. and Liu F. (2009), ‘’Autophagy-mediated Insulin Receptor Down-regulation Contributes to ER Stress-induced Insulin Resistance’’, Molecular Pharmacology, 76(3), 596-603.

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