2012-05-11

TXNIP is a Mediator of ER Stress-Induced β-Cell Inflammation and Apoptosis: A Dissertation

Christine M. Oslowski
University of Massachusetts Medical School

TXNIP IS A MEDIATOR OF ER STRESS-INDUCED β-CELL INFLAMMATION AND APOPTOSIS

A Dissertation Presented

By

Christine M. Oslowski

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MAY 11, 2012

INTERDISCIPLINARY GRADUATE PROGRAM

TXNIP IS A MEDIATOR OF ER STRESS-INDUCED β-CELL INFLAMMATION AND APOPTOSIS

A Dissertation Presented By

Christine M. Oslowski

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

Fumihiko Urano, M.D., Ph.D., Thesis Advisor

Rita Bortell, Ph.D., Member of Committee

Marcus Cooper, M.D., Member of Committee

Rohit Kulkarni, M.D., Ph.D., Member of Committee

Klaus Pechhold, M.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Reid Gilmore, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the School

Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program
May 11, 2012

ACKNOWLEDGEMENTS

“No one who achieves success does so without the help of others. The wise and confident acknowledge this help with gratitude.”- Alfred North Whitehead

Graduate school is no easy task and cannot be completed without the help of others. I am truly grateful to have received support, encouragement, guidance, and assistance from so many amazing people. I cannot thank these people enough for everything they have done to make this thesis possible.

First and foremost I wish to thank my mentor, Dr. Fumihiko Urano. He has taught me so much about the scientific process and continues to encourage me to grow as a scientist. He especially has made this experience comfortable as he has always been so understanding, supportive, responsive, and warmhearted. He truly cares about my career goals and provides me with numerous opportunities to help me achieve them. I also appreciate all of the wonderful and cultivating travel experiences he has invited me to take during my graduate career. All of these efforts and so much more has helped me complete this project and positively influence my future endeavors.

I am indebted to my committee members and colleagues who supported me during the progression of my project. I would like to express my gratitude to the members of my dissertation committee for all of their constructive feedback, recommendations, and advice. Thank you to Reid Gilmore, Rita Bortell, and Eric Baehrecke for writing recommendation letters for me. I also would like to thank current and past members of the Urano lab for their assistance and guidance. It has been an honor to work in such a collaborative environment. Thank you to Linda Leeehy of the Greiner

lab for skillfully isolating islets. Thank you to Julie Zhu for performing bioinformatics. Many thanks to Nina Bhabhalia for lab support and assistance. I wish to thank all supporting staff members of the graduate school for ensuring everything is running smoothly and assisting me in many different ways.

I would like to give a special thanks to all of my supportive friends especially one of my best friends, Dr. Emily Carino, an undergraduate colleague who recently completed graduate school in chemistry. She has provided so much inspiration, encouragement, emotional support, and advice that has helped me get through graduate life more than she will ever know. Thanks to all of my friends for their camaraderie, entertainment, help, and love.

Lastly and most importantly I would like to thank my loving family. Thank you to my mother, Fusako Oslowski for always putting herself second while raising my brother and I. She did everything she could to support us above all challenges. She is the strongest person I know and a true inspiration. One of my life goals is to provide her a comfortable life and with the completion of graduate school, I believe that will be a possibility. I also would like to convey tremendous thanks to my brother, Daniel Oslowski. Not only a brother, but also my closest friend who understands me more than anyone I know. Throughout my entire life he has provided me with love, support, and strength. I feel fortunate to have such a great brother in my life.

ABSTRACT

Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia. The pathogenesis of these diseases involves β-cell dysfunction and death. The primary function of β-cells is to tightly regulate the secretion, production, and storage of insulin in response to blood glucose levels. In order to manage insulin biosynthesis, β-cells have an elaborate endoplasmic reticulum (ER).

The ER is an essential organelle for the proper processing and folding of proteins such as proinsulin. Proteins fold properly when the ER protein load balances with the ER folding capacity that handles this load. Disruption of this ER homeostasis by genetic and environmental stimuli leads to an accumulation of misfolded and unfolded proteins, a condition known as ER stress. Upon ER stress, the unfolded protein response (UPR) is activated. The UPR is a signaling network that aims to alleviate ER stress and restore ER homeostasis promoting cell survival. Hence, the UPR allows β-cells to handle the physiological fluctuations of insulin demand.

However upon severe unresolvable ER stress conditions such as during diabetes progression, the UPR switches to pathological outputs leading to β-cell dysfunction and apoptosis. Severe ER stress may also trigger inflammation and accumulating evidence suggests that inflammation also contributes to β-cell failure, but the mechanisms remain elusive.

In this dissertation, we demonstrate that thioredoxin interacting protein (TXNIP) mediates ER stress induced β-cell inflammation and apoptosis. During a DNA microarray analysis to identify novel survival and death components of the UPR, we identified

TXNIP as an interesting proapoptotic candidate as it has been linked to glucotoxicity in β-cells. During our detailed investigation, we discovered that TXNIP is selectively expressed in β-cells of the pancreas and is strongly induced by ER stress through the IRE1α and PERK-eIF2α arms of the UPR and specifically its transcription is regulated by activating transcription factor 5 (ATF5) and carbohydrate response element binding protein (ChREBP) transcription factors.

As TXNIP has been shown to activate the Nod-like receptor protein 3 (NLRP3) inflammasome leading to the production of the inflammatory cytokine interleukin-1β (IL-1β), we hypothesized that perhaps TXNIP has a role in IL-1β production under ER stress. We show that ER stress can induce IL-1β production and that IL-1β is capable of binding to IL-1 type 1 receptor (IL-1R1) on the surface of β-cells stimulating its own expression. More importantly, we demonstrate that TXNIP does indeed play a role in ER stress mediated IL-1β production through the NLRP3 inflammasome. Furthermore, we also confirmed that TXNIP is a mediator of β-cell apoptosis under ER stress partially through IL-1β signaling.

Collectively, we provide significant novel findings that TXNIP is a component of the UPR, mediates IL-1β production and autostimulation, and induces cell death under ER stress in β-cells. It is becoming clear that TXNIP has a role in the pathogenesis of diabetes and is a link between ER stress, oxidative stress and inflammation. Understanding the molecular mechanisms involved in TXNIP expression, activity, and function as we do here will shed light on potential therapeutic strategies to tackle diabetes.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation or symbol	Term
AATF	apoptosis antagonizing transcription factor
AMPK	AMP-activated protein kinase
AP-1	activator protein 1
ASK1	apoptosis signaling kinase 1
ATF	activating transcription factor
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BiP	immunoglobulin heavy chain-binding protein
b-ZIP	basic leucine zipper
Cat1	cationic amino acids transporter 1
ChIP	chromatin immunoprecipitation
CHOP	C/EBP-homologous protein
ChoREs	carbohydrate-response elements
ChREBP	carbohydrate response element-binding protein
DNA	deoxyribonucleic acid
Dox	doxycycline
E3	ubiquitin ligase
eIF2α	eukaryotic translation initiation factor 2, α subunit
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	endoplasmic reticulum

ERAD	endoplasmic reticulum-associated degradation
ERO1α	ER oxidoreductase 1 α
ERSE	ER stress response element
FoxO1	forkhead box protein O1
GADD	growth arrest and DNA damage-inducible protein
GLP-1	glucagon-like peptide 1
GPT	N-acetylglucosamine-1-phosphate transferase
GSIS	glucose stimulated insulin secretion
HRD1	HMG-CoA reductase degradation protein 1
HSP70	heat shock protein 70
IAPP	islet amyloid polypeptide
IFN-γ	interferon γ
IKK	inhibitory κB kinase
IL-1β	interleukin 1β
IL-1R1	interleukin 1 receptor type 1
IL-1RA	interleukin 1 receptor antagonist
iNOS	inducible nitric oxide synthase
IRE1	inositol-requiring enzyme 1
IRS-1	insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
MAMs	mitochondria associated membranes
MAPK	mitogen-activated protein kinase

MEF	mouse embryonic fibroblast
Mlx	Max-like protein x
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor κB
NLRP3	Nod-like receptor protein 3
NO	nitric oxide
$p58^{ipk}$	protein kinase inhibitor of 58 kDa
PDI	protein disulfide isomerase
PDX-1	pancreatic and duodenal homeobox factor 1
PERK	PKR-like ER kinase
PGC-1α	PPARγ co-activator-1α
PI	propidium iodide
PP1	protein phosphatase 1
Q-PCR	quantitative real-time polymerase chain reaction
RACK1	receptor for activated C-kinase 1
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RPMI	media from Roswell Park Memorial Institute
SERCA	sarcoplasmic endoplasmic reticulum calcium ATPase

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	small hairpin RNA
siRNA	small interfering RNA
SP1	site 1 protease
SP2	site 2 protease
STAT3	signal transducer and activator of transcription 3
TRAF2	TNF receptor-associated factor 2
TG	thapsigargin
TM	tunicamycin
TNF-α	tumor necrosis factor α
TXNIP	thioredoxin interacting protein
UCP-1	uncoupling protein 1
UPR	unfolded protein response
UPRE	unfolded protein response element
WFS1	Wolfram syndrome 1
WT	wild-type
XBP-1	X-box binding protein 1

PREFACE

Portions of this dissertation have appeared in:

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

Oslowski, C.M. and Urano, F. A Switch From Life To Death in ER stressed β cells. *Diabetes Obes. Metab. Suppl.* **2**, 58-65, (2010).

CHAPTER I

INTRODUCTION

1.1 Diabetes and pancreatic β-cells

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia or high blood glucose levels. These diseases could lead to life threatening complications including kidney failure, blindness, amputations, heart disease, and stroke. Diabetes is one of the top ten causes of death in the United States affecting 25.8 million people or 8.3 % of the population with economic costs totaling 174 billion dollars in 2011[1, 2]. It is estimated that by 2050, 1 in 3 Americans will have diabetes. Therefore diabetes is an epidemic problem and research on the matter is vital in discovering methods to prevent and treat these diseases.

The two major forms of diabetes are type 1 and type 2. Type 1 diabetes is characterized by pancreatic β-cell loss due to autoimmunity causing an absolute deficiency of insulin. Type 2 diabetes accounts for 90 – 95% of all diabetes and develops as β-cells fail to secrete enough insulin to cope with insulin resistance. β-cells are located in the endocrine portion of the pancreas known as islets of Langerhans apart from the exocrine pancreas. These clusters of cells are largely composed of β-cells but also contain α-cells that secrete glucagon, δ-cells that secrete somatostatin and PP cells that secrete pancreatic polypeptide. All of these cells together participate in maintaining glucose homeostasis. The primary function of β-cells is to tightly regulate the production, storage, and secretion of insulin. Insulin is a hormone that functions to lower blood glucose levels. Upon a rise in blood glucose levels such as after eating a meal, β-cells secrete insulin and

stimulate insulin protein synthesis. Secreted insulin promotes blood glucose uptake by muscle and adipose tissues. In the muscle, glucose is either stored as glycogen or used for energy. In adipose tissue, glucose is stored as triglycerides and insulin prevents lipid degradation. Insulin also stimulates the liver to convert glucose as glycogen while inhibiting glucose production[3].

In the early stages of type 2 diabetes, overnutrition among other factors usually associated with obesity causes a decrease in sensitivity of peripheral tissues to insulin, a condition termed insulin resistance[4]. As a result, blood glucose levels are elevated. Pancreatic β-cells compensate by increasing insulin secretion. β-cells also increase insulin mRNA translation[5, 6] and in later phases insulin transcription[7]. However for a subset of genetically predisposed individuals, β-cells can no longer compensate for this increased insulin demand and type 2 diabetes ensues. This β-cell failure is due to a combination of β-cell dysfunction and loss. Therefore disruption of β-cell integrity is a major component during the progression of both type 1 and type 2 diabetes. Clinical, genetic, and experimental evidence suggest that endoplasmic reticulum (ER) stress has a role in β-cell dysfunction and death[8-10].

1.2 The ER, ER stress, and insulin biosynthesis

The major function of the ER is the processing and maturation of newly synthesized secreted and transmembrane proteins such as insulin. The ER lumen contains protein processing, folding, and quality control enzymes including molecular chaperones, glycosylating enzymes, and oxidoreductases supported by an oxidizing and calcium-rich

environment to ensure the proper folding of proteins. ER chaperones aid in burying hydrophobic amino acids of polypeptides through ATP hydrolytic cycles of binding and release. Glycosylating enzymes covalently attach glycans to newly synthesized proteins. Supported by an oxidizing ER, oxidoreductases promote proper disulfide formation and rearrangements between cysteine groups. Quality control enzymes ensure that only properly folded proteins are released from the ER. Misfolded proteins are recognized, retrotranslocated out of the ER, and delivered to the proteasome in a process termed endoplasmic reticulum-associated degradation (ERAD). Calcium is utilized as a cofactor by a majority of these enzymes.

Proteins fold properly when the ER chemical environment and the protein folding machinery establishes an ER folding capacity that can efficiently handle the ER protein load (Figure 1.1). This balance is termed ER homeostasis. However, an array of environmental and genetic conditions can disrupt ER homeostasis. These factors may affect the ER folding capacity such as ATP or calcium depletion, ER reduction, and dysfunctional ER folding machinery. Other factors may affect the ER protein load such as an increase in mRNA translation, expression of mutant malfolding proteins, production of radical oxygen species (ROS), and inefficient protein trafficking. Upon the loss of ER homeostasis, misfolded and unfolded proteins accumulate within the ER, a condition referred as ER stress.

Pancreatic β-cells are susceptible to physiological ER stress causing conditions. High blood glucose levels increase β-cell insulin biosynthesis in order to replenish insulin stores lost from insulin secretion. Human insulin is synthesized in the cytoplasm as

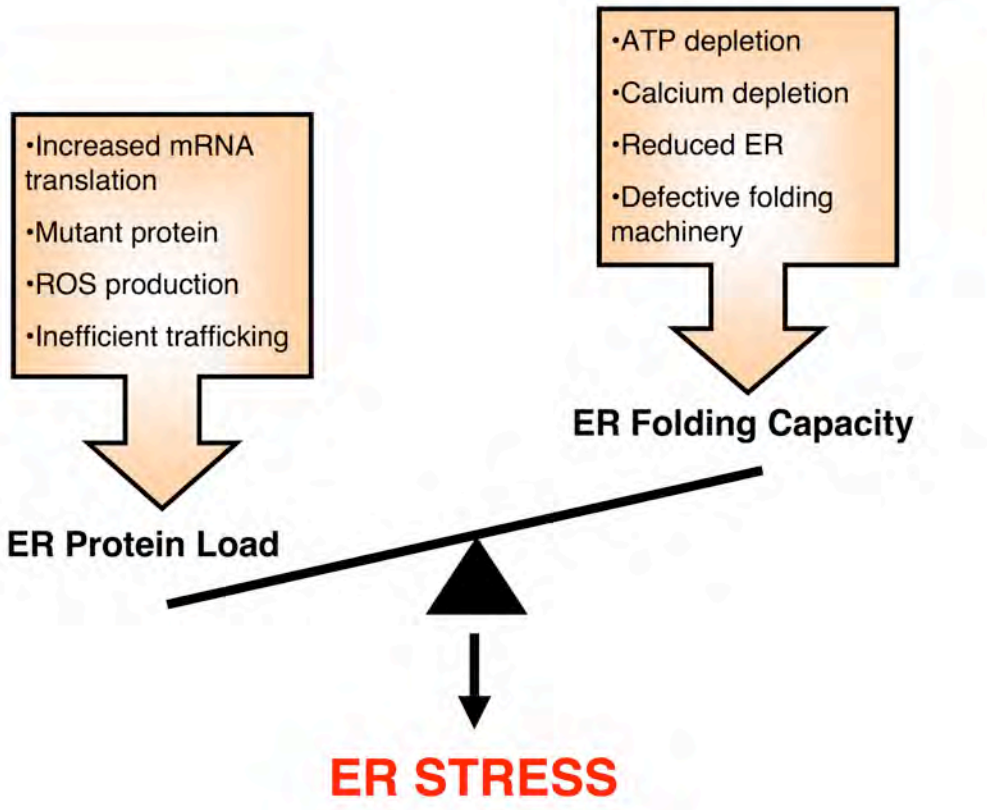


Figure 1.1 Endoplasmic reticulum (ER) stress. ER stress is caused by an imbalance between the ER protein load and the ER folding capacity that handles this load. Factors that affect the ER protein load include increased mRNA translation, expression of mutant malfolding proteins, production of radical oxygen species (ROS), and inefficient trafficking. Other factors such as ATP and calcium depletion, ER reduction, and defects in the ER folding machinery will affect the ER folding capacity. These stimuli disrupt ER homeostasis causing an accumulation of misfolded and unfolded proteins in the ER lumen.

preproinsulin. A signal peptide sequence at the N-terminal directs the protein to the ER where it is translocated into the ER lumen. In the ER, the signal sequence is cleaved producing proinsulin. Proinsulin then undergoes oxidative protein folding forming three disulfide bonds catalyzed by protein disulfide isomerase (PDI), ER oxidoreductase 1 α (ERO1α), and binding immunoglobin protein (BiP). Upon passing the ER quality control system, proinsulin is transported to the Golgi apparatus where it is packaged into secretory granules. Within these granules, proinsulin is further processed into mature insulin and is ready for release by exocytosis. β-cells ensure they maintain a readily available pool of secretory granules containing insulin in order to efficiently respond to high blood glucose levels[11, 12].

1.3 The UPR

Glucose stimulates over a 10-fold increase in preproinsulin translation creating a heavy ER protein load overwhelming the ER folding capacity and subsequently causing ER stress. Therefore β-cells are equipped with a highly developed ER and an extensive signaling network to adapt to ER stress termed the unfolded protein response (UPR) (Figure 1.2)[13, 14]. The UPR is composed of three ER transmembrane stress sensors: inositol requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6). The luminal domains are held inactive by the ER resident chaperone, BiP. Under ER stress conditions, the luminal domains sense misfolded and unfolded proteins either directly or through the titration of BiP off onto unfolded proteins[15]. Consequently, the UPR stress tranducers are activated and communicate the

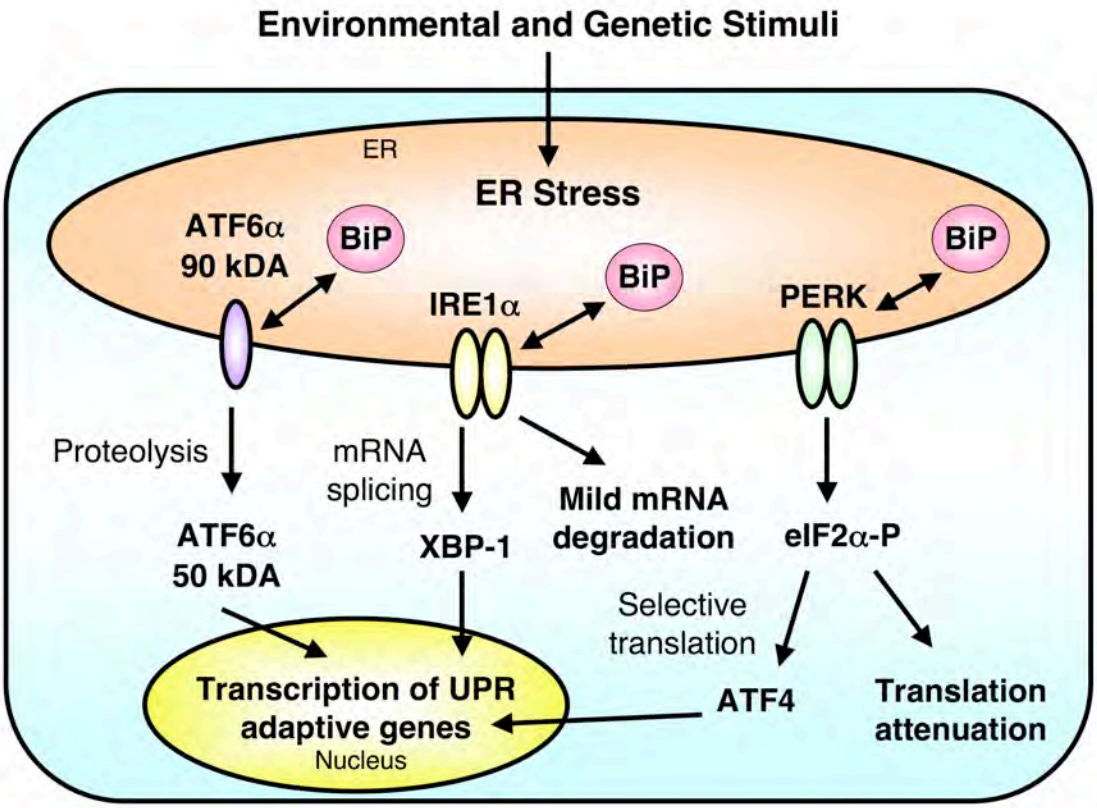


Figure 1.2 The UPR stress sensors. Upon ER stress, BiP dissociates from the luminal domains of the three UPR stress sensors, IRE1, PERK, and ATF6. As a result these UPR master regulators become activated and regulate a series of downstream effectors to mitigate ER stress and restore ER homeostasis.

ER folding status across the ER membrane to the cytosol to regulate a series of downstream effectors.

Initially the UPR triggers the adaptive response to alleviate ER stress and restore ER homeostasis (Figure 1.3). During the early phases of the UPR, global protein translation is attenuated and ER associated mRNAs are degraded to reduce ER workload and prevent further accumulation of unfolded proteins. Shortly after, the UPR also increases the expression of ERAD and autophagy components to remove malfolded proteins. To increase the ER folding and handling efficiency, the UPR upregulates the transcription of molecular chaperones, protein processing enzymes and other components of the ER protein folding machinery. The UPR also regulates the expression of genes involved in lipid biogenesis to promote the expansion of the dynamically fluid ER membrane presumably to prevent protein crowding.

IRE1 is the first stress sensor discovered and is widely conserved among species. There are two mammalian isoforms of IRE1: IRE1α and IRE1β[16, 17]. IRE1α is expressed in most cells and highly expressed in β-cells. IRE1β is primarily expressed in intestinal epithelial cells. IRE1α is a type 1 ER transmembrane serine/threonine protein kinase with endoribonuclease activity. Upon ER stress, the N-terminal luminal domain of IRE1α senses ER stress either directly by binding to misfolded proteins[18] or through the dissociation of BiP[15, 19] causing IRE1α to dimerize and transautophosphorylate its kinase domains activating its endoribonuclease activity. Activated IRE1α specifically cleaves a 26 nucleotide intron from the mRNA of the basic leucine zipper (b-ZIP) transcription factor X-box binding protein (XBP-1)[20-22]. This splicing event results in a translational

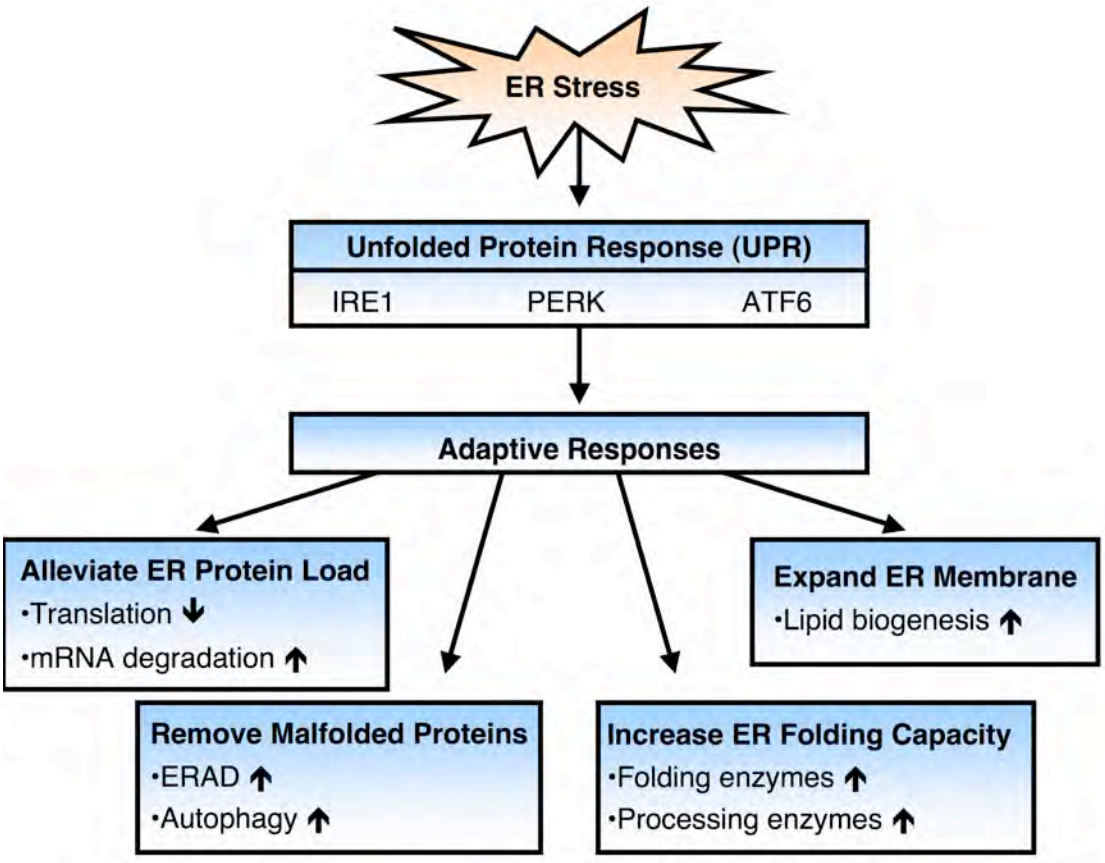


Figure 1.3 Adaptive responses. The UPR stimulates four adaptive responses: 1) alleviating the ER protein load, 2) removal of malfolded proteins, 3) increasing the ER folding capacity, and 4) expanding the ER membrane.

frameshift producing an active form of XBP-1. XBP-1 has a major role in the transcriptional regulation of several UPR homeostatic genes including those encoding chaperones, ERAD components, and phospholipid biosynthesis machinery[23-27]. IRE1α also cleaves ER associated mRNAs such as insulin to reduce ER workload[28-30].

PERK is also a type 1 ER transmembrane serine/threonine protein kinase. Similar to IRE1α, PERK senses ER stress at its N-terminal domain, dimerizes and transautophosphorylates its kinase domains. Activated PERK phosphorylates serine-51 on the α subunit of eukaryotic translation initiation factor 2 (eIF2)[31]. Phosphorylated eIF2α inhibits ribosome assembly and therefore attenuates global protein translation reducing ER protein load[32, 33]. However, translation of certain mRNAs is enhanced as the ribosome skips inhibitory upstream open reading frames. Examples of these mRNAs include activating transcription factor 5 (ATF5), cationic amino acids transporter 1 (Cat1), and the well-characterized b-ZIP transcription factor, activating transcription factor 4 (ATF4)[34-37]. ATF4 regulates UPR genes including those encoding chaperones, ERAD components, amino acid homeostasis regulators, and antioxidative stress response effectors[32, 38].

The third ER stress sensor, ATF6, has two genes, ATF6α and ATFβ expressed in all cells. Studies with mouse embryonic fibroblasts deficient in ATF6α or ATF6β demonstrate that ATF6α has a major role in the regulation of UPR genes as opposed to ATF6β[39, 40]. However, a double knockout of murine ATF6α and ATF6β causes embryonic lethality indicating that ATF6α and ATF6β may have complementary functions important for mouse development[40]. ATF6α is a 90 kDa type II ER

transmembrane transcription factor that has a unique activation mechanism. Upon ER stress, BiP dissociates from ATF6α and disulfide bonds in the luminal domain are reduced allowing ATF6α to transit to the Golgi apparatus where it is processed by site 1 (S1P) and site 2 (S2P) proteases. S1P removes the luminal domain and S2P cleaves the transmembrane anchor producing the active 50 kDa b-ZIP transcription factor[41-43]. The cytosolic fragment of ATF6α subsequently translocates to the nucleus where it functions as a transcriptional activator sometimes with XBP-1 regulating ERAD components, lipid biosynthesis, and ER chaperones such as BiP[39, 40, 44].

1.4 The UPR binary switch between life and death

In addition to the adaptive response, the UPR also determines cell fate (Figure 1.4). The current paradigm suggests that the UPR behaves as a binary switch between life and death[10, 45]. The consequence of this switch depends on the nature of the ER stress condition, the balance between UPR survival and death components, and the activation and regulation of the UPR stress sensors. The components and mechanisms involved in the UPR binary switch between life and death is cell type specific. This area remains poorly understood.

1.4.1 The UPR under tolerable ER stress

There are two types of ER stress: tolerable and unresolvable. Under tolerable ER stress, the UPR regulates adaptive and survival components mitigating ER stress, restoring ER homeostasis, and promoting cell survival (Figure 1.5). β-cells regularly

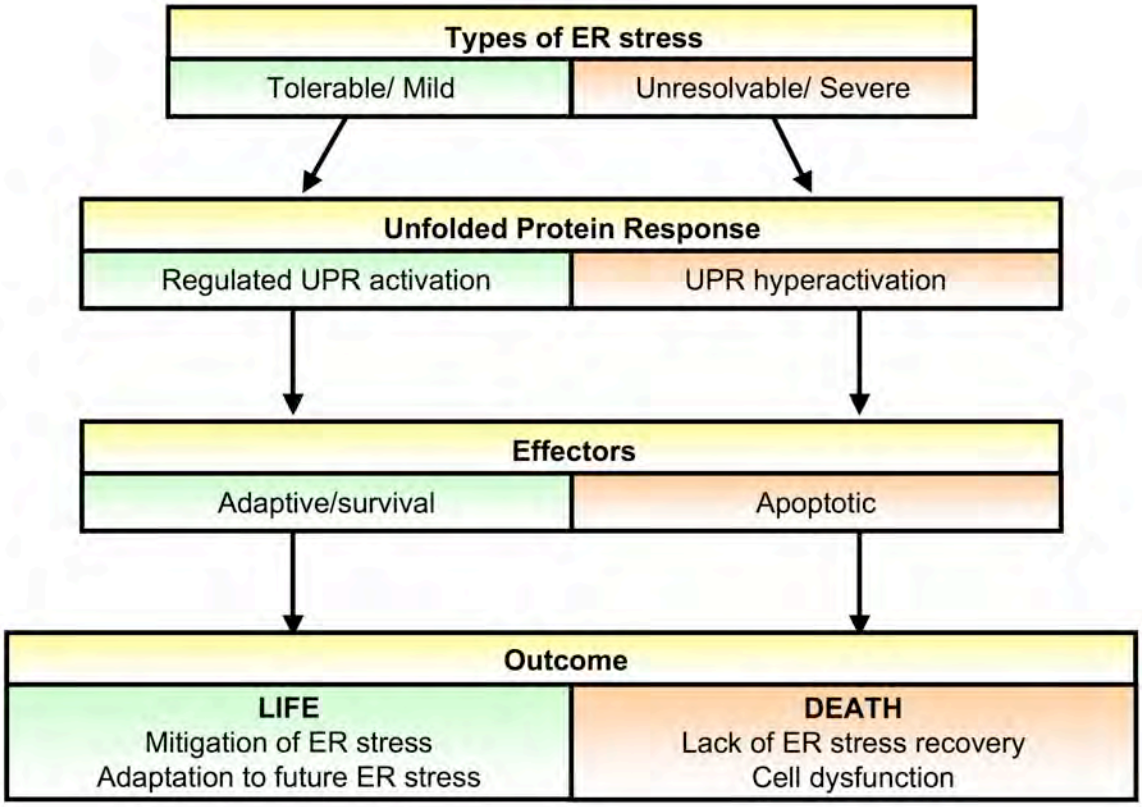


Figure 1.4 The UPR binary switch between life and death. The UPR behaves as a binary switch between life and death. The consequence of this switch depends on several factors including the type of ER stress, the degree of UPR regulation and activation, and the regulation of UPR survival and death effectors.

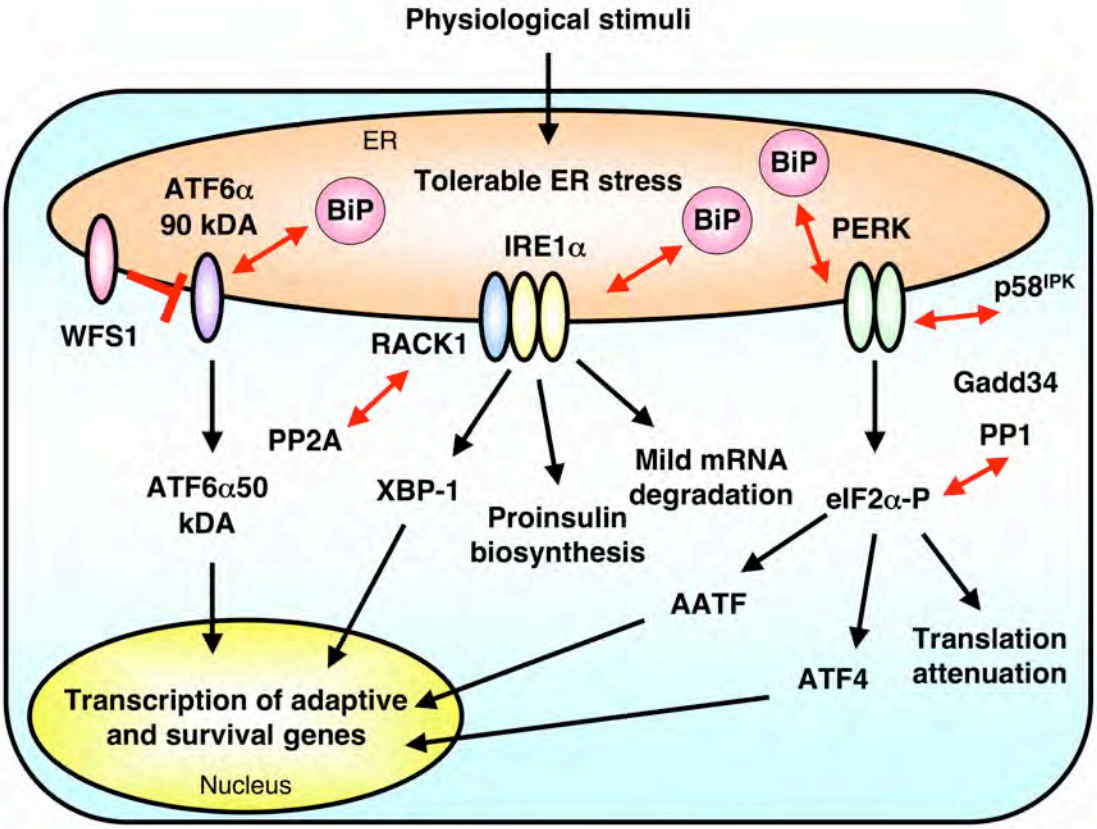


Figure 1.5 The UPR under tolerable ER stress. Under mild tolerable ER stress conditions, the UPR favors adaptive and survival pathways that alleviate ER stress and promote cell survival. As ER homeostasis is being restored, the UPR is turned off by negative feedback mechanisms.

experience physiological stress conditions that induce tolerable ER stress such as fluctuations in blood glucose levels leading to an overwhelming load of proinsulin in the ER. The PERK branch of the UPR is vital for β-cells to handle this tolerable ER stress. During basal glucose conditions, phosphorylated eIF2α levels are high[46, 47]. However, as blood glucose levels increase, levels of phosphorylated eIF2α decline allowing insulin translation[47]. As proinsulin ER load increases causing ER stress, eIF2α becomes re-phosphorylated to attenuate global translation including preproinsulin translation relieving the ER workload. Meanwhile ATF4 is translationally privileged and induces the transcription of adaptive response genes. The PERK-eIF2α pathway also induces the transcription of apoptosis antagonizing transcription factor (AATF)[48]. AATF promotes β-cell survival partly through the transcriptional upregulation of Akt1 through signal transducer and activator of transcription 3 (STAT3) leading to increased levels of activated Akt1. Akt1 is a serine/threonine protein kinase involved in multiple cellular processes including promoting cell survival.

The PERK-eIF2α pathway in particular is very important for β-cells to properly handle the dynamic increases of insulin biosynthesis as exemplified in genetic studies. Loss of function mutations in the EIF2AK3 gene encoding PERK causes the rare disorder known as Wolcott-Rallison syndrome which is characterized by infantile diabetes among other pathologies[49, 50]. Complications of this disease are most likely due to the fact that without functional PERK, eIF2α cannot be phosphorylated. Consequently, under high glucose conditions, insulin translation cannot be mitigated exacerbating ER stress leading to β-cell dysfunction and death. Indeed, PERK null mice also exhibited diabetes due to β-

cell apoptosis caused by high levels of ER stress[46, 51]. Similarly, mice with a homozygous knock in mutation at the PERK phosphorylation site in eIF2α (Ser51Ala) demonstrated extensive β-cell loss due to ER stress and die shortly after birth[52]. Further studies of heterozygous Ser51Ala mice fed a high fat diet developed glucose intolerance due to a high level of proinsulin in the ER preventing proper insulin processing and insulin secretion[53]. Collectively, these studies demonstrate the importance of the PERK-eIF2α pathway to reduce preproinsulin translation in order to reduce ER workload under high glucose. This pause in insulin biosynthesis allows the ER machinery to properly process and fold proinsulin within the ER in order to maintain a steady supply of insulin to secrete.

IRE1α is also activated under high glucose conditions. Under acute high glucose conditions, IRE1α increases the ER folding capacity through XBP-1 and promotes insulin biosynthesis and proinsulin folding in the ER[54]. Basal levels of IRE1α mediated mRNA degradation may also reduce ER workload. Therefore under physiological changes in blood glucose levels, PERK and IRE1α are activated to relieve the increased ER proinsulin load while increasing the ER folding capacity to handle this load restoring ER homeostasis and promoting β-cell survival.

1.4.2 The UPR under unresolvable ER stress

Unresolvable ER stress is severe and cannot be rescued by the UPR (Figure 1.6). Under these conditions, the adaptive and survival components of the UPR fail to achieve ER homeostasis. Instead the UPR is harmfully hyperactivated switching to pathological

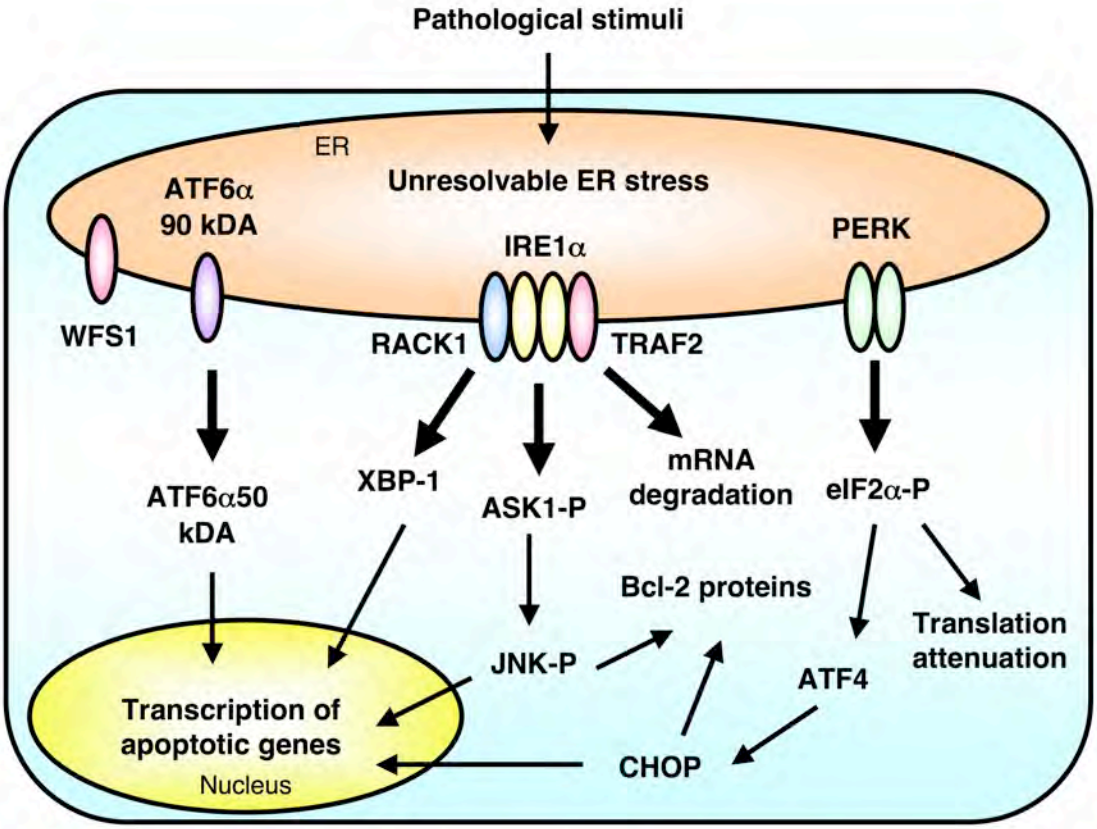


Figure 1.6 The UPR under unresolvable ER stress. Under severe unresolvable ER stress, the UPR stress sensors bypass negative feedback mechanisms and become hyperactivated favoring the expression and activation of proapoptotic effectors and hampering the ER folding capacity leading to cell death.

outputs[55]. These outputs include the expression and activation of proapoptotic effectors, deterioration of the ER folding capacity, and reduction of β-cell function. For instance under chronic high glucose conditions, the endoribonuclease domain of IRE1α becomes destructive degrading important ER-associated mRNAs including ER homeostatic proteins BiP and PDI thus reducing the ER folding capacity and promoting cell death[28]. IRE1α also recruits the adaptor protein tumor necrosis factor receptor-associated protein 2 (TRAF2) and together activates apoptosis signal regulating kinase 1(ASK1), which subsequently leads to the phosphorylation and activation of c-Jun N-terminal kinase (JNK)[56]. JNK can promote apoptosis by inactivating anti-apoptotic members of the B-cell lymphoma 2 (BCL-2) family members, Bcl-2, Bcl-XL and Mcl-1. JNK can also induce death by activating pro-apoptotic BH-3 only proteins such as Bid and Bim[57]. In addition IRE1α can trigger cell death through its association of BCL-2 family members, Bax and Bak, promoting IRE1α hyperactivation[58]. Furthermore, overproduction of spliced XBP-1 may inhibit β-cell function and promote β-cell apoptosis[59].

Under unresolvable ER stress conditions, PERK is also hyperactivated and induces β-cell dysfunction and death. Prolong translational block by phosphorylated eIF2α depletes cells of essential proteins for proper function and survival. The PERK-eIF2α pathway upregulates the translation of ATF4. ATF4 induces the transcription of activating transcription factor 3 (ATF3)[60]. ATF3 reduces transcription and activity of pancreatic and duodenal homeobox 1 (PDX-1)[61, 62]. PDX-1 is a key transcription factor in the proper function and survival of β-cells. Its expression is reduced during type 2 diabetes progression. ATF3 along with ATF4 also induces the transcription of the

proapoptotic transcription factor, CCAAT-enhancer-binding protein (C/EBP) homologous protein (CHOP)[60, 63, 64]. CHOP plays a role in apoptosis by a number of possible mechanisms including regulation of the BCL-2 family members[65-67], increasing ER radical oxygen species[66, 68], and depleting ER calcium stores[69, 70]. Accordingly, CHOP deletion reduced β-cell apoptosis and improved β-cell function in several diabetic mouse models[71, 72].

ATF6α also appears to have a role in the life and death decisions of the β-cell; however, the components and mechanisms are not clear. Basal levels of active ATF6α may be important in promoting β-cell survival[73]. Under severe ER stress conditions, ATF6α hyperactivation induces β-cell death partly through CHOP induction and plays a role in β-cell dysfunction by decreasing insulin gene expression[74]. ATF6α is also hyperactivated in Wolfram syndrome due to the lack of functional WFS1 and therefore may play a role in β-cell death in these patients[75]. Furthermore, ATF6α variants have been associated with type 2 diabetes in human genetic studies[76, 77]. ATF6α-null mice do not display any defects in β-cell function or loss in β-cell mass on a normal chow diet. However, recently it was reported that on a high fat diet, β-cells in these mice experience severe ER stress with reduced insulin content most likely due to increased β-cell apoptosis[78]. These results suggest that ATF6α has an important role in mitigating ER stress promoting β-cell survival under obese conditions.

The UPR survival and death components are also regulated at the post-transcriptional level[79]. During tolerable ER stress conditions, the stabilities of mRNAs and proteins that favor survival and adaptation such as BiP are enhanced whereas those

involved in apoptosis such as CHOP are compromised. Under unresolvable ER stress, proapoptotic components are expressed at high levels bypassing any negative regulation. The mechanisms of mRNA instabilities may involve IRE1α mediated mRNA degradation or UPR regulated miRNAs. It would be interesting to investigate the stabilities of UPR mRNAs and proteins in β-cells under tolerable and unresolvable ER stress conditions.

1.4.3 Negative feedback mechanisms of the UPR

As discussed, the UPR switches between life and death depending on the severity of the ER stress and the subsequent regulation of both survival and death effectors. The outcome of this switch also depends on the tight regulation of the UPR signal transducers themselves. Under tolerable ER stress conditions, the UPR stress sensors and downstream effectors are negatively regulated by feedback control mechanisms as ER homeostasis is restored (Figure 1.5). However during unresolvable ER stress conditions, the UPR stress sensors bypass these control mechanisms leading to harmful UPR hyperactivation promoting cell death. The effectors involved in UPR signaling regulation are the very UPR switches between life and death. Nonetheless, the components and mechanisms of this feedback regulation during mild and severe ER stress remain elusive.

One possible feedback mechanism may involve the ER resident chaperone BiP. As mentioned previously, BiP binds to the luminal domains of the UPR transducers keeping them inactive. Upon ER stress, BiP is released to participate in protein folding, which allows for activation of the UPR stress sensors. During the UPR, BiP is also

transcriptionally upregulated promoting the binding of BiP to the UPR master regulators therefore restricting their activities.

Another mechanism involves phosphatases that function to dephosphorylate certain components of the UPR. Under ER stress, ATF4 and CHOP upregulate the transcription of growth arrest and DNA damage-inducible protein 34 (GADD34)[80-83]. GADD34 interacts with protein phosphatase I (PP1) to desphosphorylate eIF2α. Consequently, protein translation is restored and the PERK branch of the UPR is turned off. A similar mechanism has been proposed for IRE1α dephosphorylation by protein phosphatase 2A (PP2A) recruited by the scaffolding protein receptor for activated C-kinase 1 (RACK1)[84].

Another regulator of the PERK pathway is the chaperone, protein kinase inhibitor of 58 kDa ($p58^{ipk}$). Under ER stress, ATF6α induces $p58^{ipk}$ transcription[85]. $p58^{ipk}$ binds to PERK and inhibits its ability to phosphorylate eIF2α therefore restoring protein translation[85, 86]. As the PERK-eIF2α pathways is important for β-cell function and survival, $p58^{ipk}$ deletion mice develop hyperglycemia with a decrease in β-cell mass due to apoptosis perhaps by severe ER stress as a result of the inability to attenuate insulin translation[87]. $p58^{ipk}$ may also function as a cochaperone with BiP in the ER lumen to assist in protein folding[88, 89] or with heat shock protein 70 (HSP70) in the cytoplasm at ER translocans to degrade newly synthesized ER-targeted proteins[90].

Upon ER stress homeostasis restoration, ATF6α is also under negative regulation by Wolfram syndrome I (WFS1)[75]. WFS1 is an ER transmembrane protein and has important roles in maintaining the ER including regulating ER calcium levels and the

UPR. Under non-stressed conditions, WFS1 recruits ATF6α to the E3 ubiquitin ligase HRD1 and the proteasome for ubiquitin-mediated degradation therefore preventing ATF6α activation. Under stressed conditions, ATF6α dissociates from WFS1 in order to upregulate adaptive response genes in the nucleus. As ER homeostasis is being restored, WFS1 expression is induced by the IRE1α and PERK pathways promoting WFS1 to negatively regulate ATF6α. Mutations in the WFS1 gene causes Wolfram syndrome, a rare autosomal recessive neurodegenerative disorder characterized by juvenile onset diabetes, optic atrophy, and deafness[91]. Analysis of pancreatic islets of Wolfram syndrome patients demonstrate β-cell loss[92]. β-cell loss may be caused by apoptosis as a result of ATF6α hyperactivation due to the lack of WFS1 negative feedback. Likewise, downregulation of WFS1 in β-cell lines exhibited high ER stress levels causing β-cell dysfunction and death. Furthermore, WFS1 knockout mice and β-cell specific WFS1 knockout mice also develop diabetes due to ER stress-induced β-cell apoptosis[93, 94]. It would be beneficial to study the genes regulated by hyperactivated ATF6α that may promote β-cell death.

1.5 β-cell specific ER stress inducers

Causes of ER stress and the activated UPR program are cell-type specific. Besides the dynamic changes in extracellular glucose, there are other environmental and genetic factors that induce ER stress specifically in β-cells (Figure 1.7). Many of these stimuli induce unresolvalable ER stress during the progression of diabetes leading to β-cell death. Elevated levels of free fatty acids due to diet and obesity have a role in β-cell

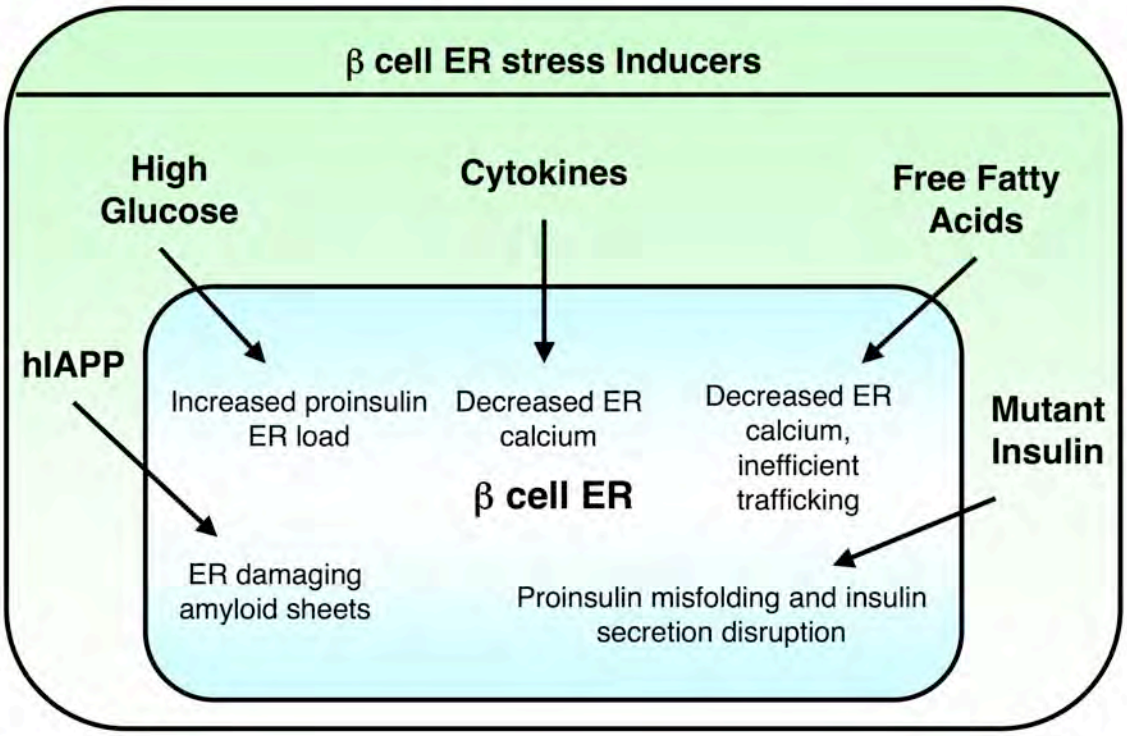


Figure 1.7 β-cell specific ER stress inducers. β-cells are susceptible to a variety of ER stress inducers including high glucose, human islet polypeptide (hIAPP), cytokines, free fatty acids and expression of mutant malfolding insulin. These conditions are capable of triggering unresolvable ER stress causing β-cell dysfunction and death.

failure during type 2 diabetes. Exposure of β-cells to the saturated free fatty acid, palmitate, causes ER stress activating all three arms of the UPR[95, 96]. Palmitate creates ER stress through inhibiting sarcoplasmic endoplasmic reticulum calcium-ATPase (SERCA) pump activity depleting ER calcium[97, 98] and disrupting ER-to-Golgi protein trafficking causing an overload of proteins in the ER[99]. Palmitate also causes carboxypeptidase E degradation leading to an accumulation of unprocessed proinsulin[100]. Palmitate has a role in lipotoxicity of β-cells by inducing the expression of CHOP and activating JNK[97, 101].

Proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) secreted from invasive immune cells are the major mediators of β-cell dysfunction and apoptosis during the progression of type 1 diabetes[102-104]. It has been proposed that cytokines induce ER stress contributing to β-cell dysfunction and demise[105-108]. However, the mechanisms remain to be clarified due to experimental differences. The general model suggests that cytokines activate the transcription factor nuclear factor-κB (NF-κB), which has a major role in regulating the transcription of proapoptotic genes including nitric oxide synthase leading to nitric oxide (NO) production. NO downregulates SERCA2b expression leading to depletion of ER calcium levels and therefore causing ER stress. Another report suggest that NO may instead stimulate the expression of UPR proapoptotic components such as CHOP, while decreasing expression of adaptive effectors[109]. Additional studies are required to clarify contradictory results.

The major function of β-cells is to produce and secrete insulin. If the insulin gene is mutated causing misfolding in the ER, severe ER stress is induced leading to β-cell

apoptosis. This idea is demonstrated in Akita mice[71, 110]. These mice express a mutated form of insulin 2 in which a cysteine is substituted for tyrosine at position 96 (C96Y). This mutation disrupts one of the three disulfide bonds between the A and B chains of mature insulin. Consequently, these mice develop diabetes due to high levels of ER stress in their β-cells leading to β-cell dysfunction and death. In the β-cells of Akita mice, the ER is distended, a hallmark of ER stress. Also ER stress markers, BiP and CHOP are upregulated. Furthermore, mRNA levels are reduced probably due to IRE1α hyperactivation. Likewise, human genetic studies revealed several mutations in the insulin gene related to proper proinsulin folding that are linked to neonatal diabetes[111, 112]. One such mutation is identical to the mutation found in the Akita mice[113].

Islet amyloid deposits are commonly found in the patients of type 2 diabetes and are linked to β-cell dysfunction and death. These amyloid formations are composed of aggregated islet amyloid polypeptide (IAPP). Even though controversial[114], IAPP may form amyloid sheets that damage the ER causing severe ER stress and β-cell death perhaps by impairing the ubiquitin-proteasome pathway. Islets of mice overexpressing the amyloidogenic human IAPP (hIAPP) demonstrated an increase in ER stress markers such as XBP-1 and CHOP, and an accumulation of polyubiquitinated proteins as opposed to islets from mice expressing rat IAPP[115, 116]. Similar findings were found in hIAPP treated mouse insulinoma MIN6 cells and isolated human pancreatic islets[117].

1.6 The UPR interconnects with oxidative stress and inflammation

The UPR is also capable of inducing other cellular stresses and crosstalking with signaling pathways. For instance in order for proinsulin to fold properly, the ER must coordinate proper formation of 3 disulfide bonds. During this oxidative protein folding, ROS is produced[118-121]. β-cells in particular are sensitive to ROS production due to their lack of antioxidant proteins such as catalase and glutathione peroxidase[122, 123]. When blood glucose rises, causing an increase in proinsulin ER load, high levels of ROS could be produced. In addition, ER stress causes calcium leakage from the ER to the mitochondria stimulating mitochondria mediated ROS production[124-126]. In turn, ROS could increase ER stress by modifying ER protein and lipids important in establishing ER homeostasis. Furthermore, ROS production has a major role in glucotoxicity of β-cells[127, 128]. Therefore ROS generated by ER stress could also have a role in glucotoxicity.

It has been suggested that the UPR could also trigger inflammation (Figure 1.8)[129-131]. For instance, all three arms of the UPR have been proposed to be involved in the activation of NF-κB, which regulates genes involved in apoptosis and the inflammatory response such as cytokines. IRE1α together with TRAF2 binds to and activates inhibitory κB kinase (IKK)[132]. IKK phosphorylates the NF-κB inhibitor IκB leading to its degradation. Subsequently, NF-κB is released and free to translocate into the nucleus to regulate transcription. The PERK-eIF2α pathway suppresses translation of IκB, which promotes NF-κB activation[133, 134]. It has also been suggested that ATF6α can activate NF-κB through phosphorylation of Akt[135]. IRE1α also activates JNK, which can also influence expression of inflammatory genes through the phosphorylation of the

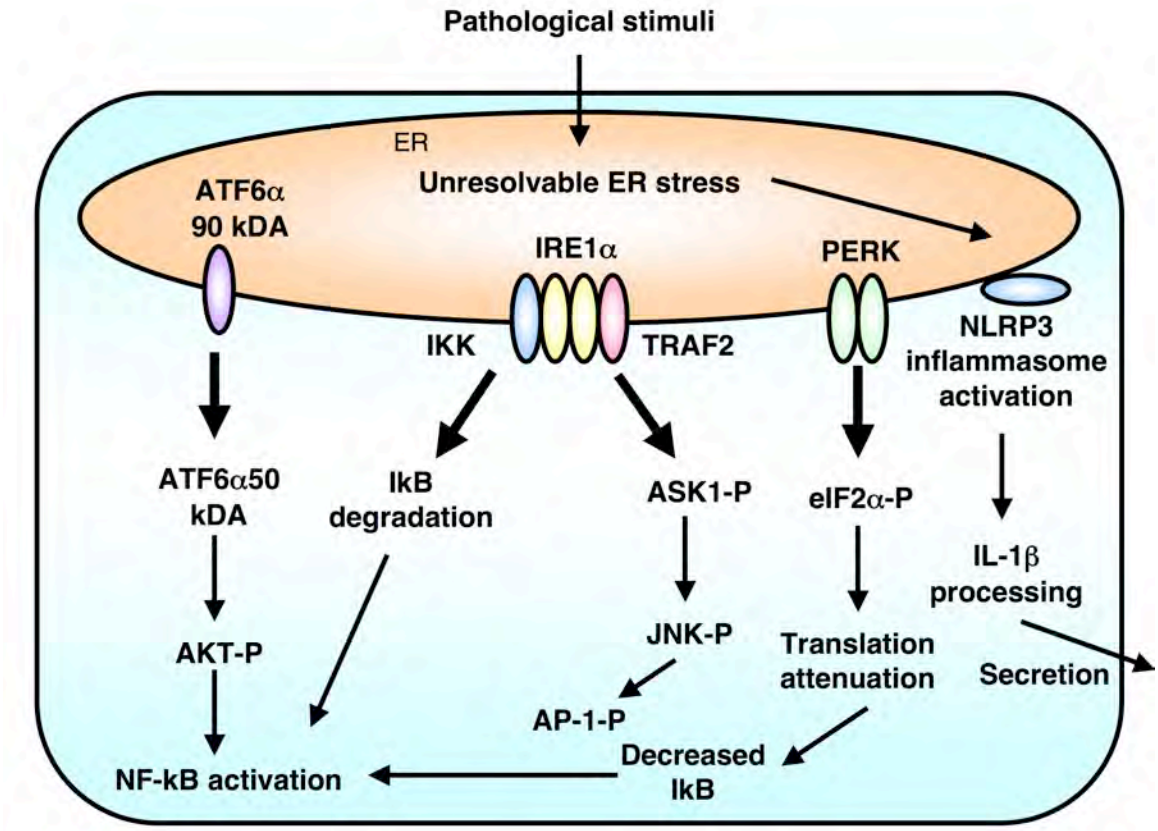


Figure 1.8 ER stress and inflammation. The UPR is capable of intersecting with inflammatory signaling. For instance, all three ER stress sensors can activate NF-κb, a key inflammatory transcription factor. IRE1α can also activate JNK, which in turn can activate inflammatory transcription factors such as AP-1. Furthermore, it has been recently reported that the NLRP3 inflammasome associates with the ER and can be activated upon ER stress conditions leading to IL-1β secretion.

transcription factor, activator protein 1 (AP-1)[56]. Recently it has been reported that inactive Nod-like receptor protein 3 (NLRP3) inflammasome associates with the ER and upon activation relocates to mitochondria-associated ER membranes (MAMs)[136], contact sites in which the ER transfers signals to the mitochondria to induce cell death[137, 138]. Furthermore, ER stress can activate the NLRP3 inflammasome[139]. The NLRP3 inflammasome is responsible for the processing and activation of the proinflammatory cytokine, IL-1β. Collectively, these observations suggest that ER stress could induce expression of inflammatory components and secretion of IL-1β potentially leading to inflammation.

1.7 Inflammation in diabetes

Inflammation is a contributing factor of β-cell destruction in type 1 diabetes and accumulating evidence links inflammation to the pathogenesis of type 2 diabetes[140, 141]. Several changes of the immune system can be detected in patients with type 2 diabetes such as elevated circulating cytokines and chemokines. The production and secretion of these cytokines and chemokines stem from several sources including β-cells as a result of metabolic overload. β-cells production and secretion of these inflammatory components, especially the cytokine IL-1β has been shown to play a key role in β-cell loss during type 2 diabetes.

The mechanism of IL-1β production involves the interaction of thioredoxin interacting protein (TXNIP) with the NLRP3 inflammasome[142]. In β-cells, TXNIP, a member of the α-arrestin protein family, is transcriptionaly upregulated by high glucose

mediated by the transcription factors carbohydrate response element-binding protein (ChREBP) and Max-like protein X (Mlx) binding to carbohydrate-response elements (ChoREs)[143-146]. TXNIP directly binds to and inhibits the antioxidative protein thioredoxin causing an increase in intracellular ROS[147-149]. Thioredoxin also interacts and inhibits a number of apoptotic effectors such as ASK-1 and NF-κb. Therefore glucose-induced TXNIP expression causes oxidative stress leading to β-cell death. In addition, during high glucose conditions, ROS causes TXNIP to dissociate from thioredoxin. Liberated TXNIP binds to and activates the NLRP3 inflammasome leading to caspase-1 activation, which processes IL-1β into its active form. As β-cells secrete activate IL-1β, it can act upon the highly expressed IL-1 receptor type 1 (IL-1R1) on β-cells, inducing the expression of itself as well as chemokines through the transcription factor NF-κB. Meanwhile, macrophages are recruited to islets eliciting a broad inflammatory response. IL-1β and other cytokines can also induce ER stress and activate apoptotic pathways leading to β-cell death (Figure 1.9). This vicious cycle of autoinflammation may be enhanced in type 2 diabetes patients that carry a genetic variation in a gene encoding the endogenous IL-1β antagonist, IL-1 receptor antagonist (IL-1RA)[150].

1.8 Summary of Thesis Research: TXNIP mediates autoinflammation and apoptosis in β-cells under ER stress

Taken together, ER stress is triggered by an array of stimuli leading to the activation of the UPR. During mild tolerable ER stress conditions, adaptive responses are capable of mitigating ER stress and restoring ER homeostasis. Subsequently, the UPR is

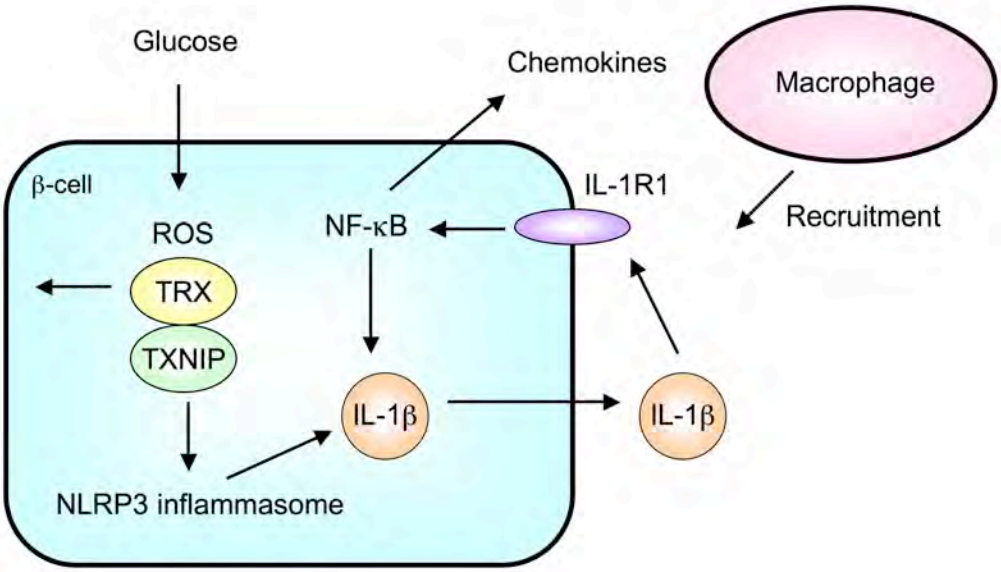


Figure 1.9 Islet inflammation. Under high glucose conditions, ROS is produced promoting the antioxidant protein thioredoxin to release TXNIP. Consequently, TXNIP binds to and activates the NLRP3 inflammasome leading to the processing and secretion of active IL-1β. IL-1β in turn can bind to the IL-1 receptor type 1 (IL-1R1) on the surface of β-cells inducing the expression of itself and chemokines through NF-κB. Meanwhile, macrophages are recruited to islets eliciting a broad inflammatory response.

properly downregulated fostering β-cell function and survival. However, under severe unresolvable ER stress conditions, the UPR becomes hyperactivated promoting the activation of apoptotic pathways, inducing ROS production, and intersecting with inflammatory signaling leading to β-cell dysfunction and death contributing to diabetes development.

We are interested in identifying the survival and death components regulated by the UPR and the mechanisms involved in the UPR binary switch between life and death in the context of the β-cell. To this end, we focused on studying genes regulated by the anti-apoptotic UPR transcription factor AATF. During a DNA microarray analysis of genes differentially expressed by the overexpression of AATF in the rat insulinoma cell line INS-1 832/13, we have identified a potential novel apoptotic UPR component, TXNIP. Interestingly during our detailed investigation of TXNIP, we discovered that TXNIP mediates ER stress induced β-cell inflammation and apoptosis.

Here we show that TXNIP is mainly expressed in β-cells of the pancreas and its expression is upregulated by both pharmacological and physiological ER stress inducers under the IRE1α and PERK-eIF2α signaling pathways of the UPR. TXNIP transcription is significantly upregulated by ER stress and while studying the potential transcription factors involved, we have identified ATF5 and ChREBP. Because it has been demonstrated that TXNIP contributes to IL-1β production, we explored if TXNIP has a role in IL-1β production by ER stress. We demonstrated that TXNIP does indeed have a role in IL-1β production through the NLRP3 inflammasome and that this IL-1β is capable of autostimulation through IL-1R1 in β-cells under ER stress. Furthermore, we

confirmed that TXNIP has a role in ER stress-mediated β-cell apoptosis partly through IL-1β signaling. Collectively, these results suggest that ER stress and the UPR induce autoinflammation and β-cell death by transcriptionally upregulating TXNIP expression leading to IL-1β secretion and autostimulation (Figure 1.10). Therefore TXNIP promises to be a potential drug target in the treatment of diabetes.

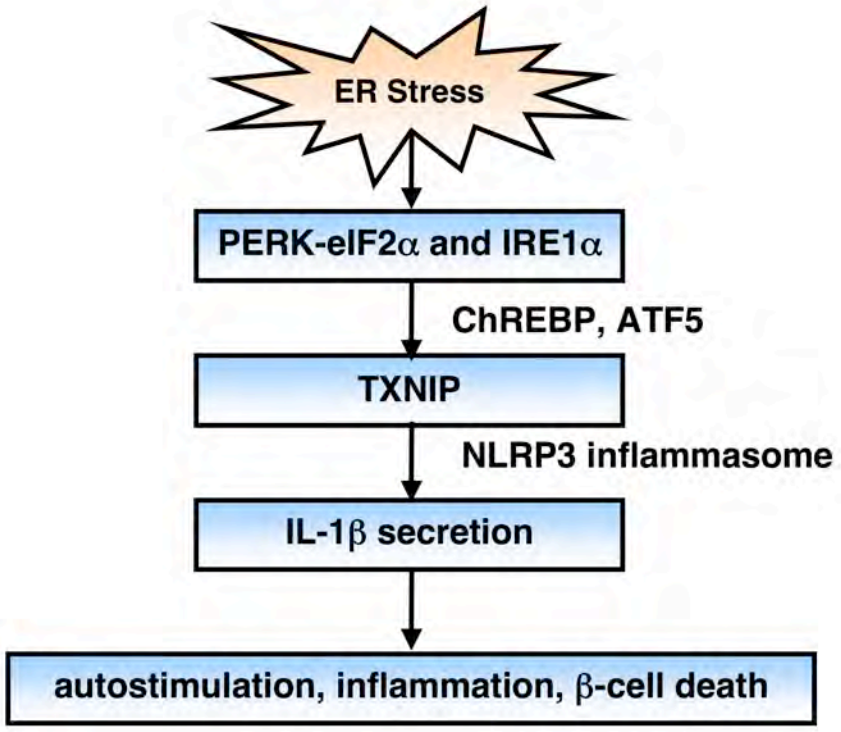


Figure 1.10 Summary of thesis research. We have discovered that TXNIP is transcriptionally upregulated under the PERK-eIF2α and IRE1α arms of the UPR through the transcription factors ChREBP and ATF5. TXNIP activates the NLRP3 inflammasome leading to the secretion of IL-1β. IL-1β is capable of autostimulation through IL-1R1, inducing a broad inflammatory response, and mediating β-cell death.

CHAPTER II

THE ROLE OF TXNIP IN ER STRESS-MEDIATED IL-1β PRODUCTION AND CELL DEATH IN β-CELLS

Summary

Pancreatic β-cells are designed to efficiently synthesize and secrete insulin in response to blood glucose levels. In order to manage insulin biosynthesis, β-cells have an especially dynamic endoplasmic reticulum (ER). The main function of the ER is to process and fold proteins such as proinsulin. Proinsulin folds properly when the ER protein folding capacity and ER protein load are in balance, a condition referred to as ER homeostasis. However, genetic and environmental stimuli can disrupt ER homeostasis leading to an accumulation of unfolded proteins. This ER stress activates the unfolded protein response (UPR), a signaling network that functions to reduce ER stress and restore ER homeostasis. Therefore the UPR allows β-cells to handle the fluctuations of insulin demand. However, if ER stress is severe such as during diabetes progression, the UPR induces β-cell dysfunction and death. Severe ER stress may also trigger inflammation and evidence suggests that inflammation also plays a role in β-cell failure, but the mechanisms remain unclear. Here we provide evidence that thioredoxin interacting protein (TXNIP) mediates ER stress-induced β-cell inflammation and death. TXNIP expression is induced by ER stress through the IRE1α and PERK-eIF2α branches of the UPR and transcriptionally regulated by activating transcription factor 5 (ATF5) and carbohydrate response element binding protein (ChREBP). TXNIP regulates the production of the inflammatory cytokine interleukin 1 β (IL-1β) through the Nod-like

receptor protein 3 (NLRP3) inflammasome and IL-1β is capable of autostimulation through IL-1 receptor type 1 (IL-1R1) in β-cells under ER stress. Furthermore, TXNIP induces β-cell apoptosis partly through IL-1β signaling. Therefore TXNIP is a potential drug target for preventing β-cell failure in diabetes.

Introduction

Type 2 diabetes is characterized by hyperglycemia as a result of insulin resistance and relative insulin deficiency[4]. In the early stages of type 2 diabetes, insulin becomes ineffective at lowering blood glucose levels. Pancreatic β-cells respond to insulin resistance by increasing insulin production and secretion. However for a subset of genetically predisposed individuals, β-cells can no longer compensate for this increased insulin demand and type 2 diabetes ensues. This β-cell failure is due to a combination of β-cell dysfunction and apoptosis. Accumulating evidence suggests that endoplasmic reticulum (ER) stress may contribute to β-cell failure[8, 9].

Pancreatic β-cells are designed to efficiently synthesize, store, and secrete insulin, a hormone that lowers blood glucose levels. When blood glucose levels rise, β-cells immediately secrete insulin from an available pool and dramatically increase insulin biosynthesis. In order to manage this insulin demand, β-cells contain a specialized ER. The ER houses an elaborate enzymatic machinery supported by a unique chemical environment to properly process and fold proinsulin. Proinsulin folds correctly when the ER protein folding capacity and ER protein load are in balance. β-cells are susceptible to conditions such as elevated levels of blood glucose that can disrupt this ER homeostasis leading to an accumulation of misfolded and unfolded proinsulin, a condition referred to as ER stress. In order for β-cells to adapt to this ER stress, the unfolded protein response (UPR) is activated[13]. This signaling network functions to mitigate ER stress and restore ER homeostasis. Therefore β-cells depend on the UPR in order to function properly.

The UPR is composed of three ER stress sensors: Inositol Requiring Enzyme 1 (IRE1), PKR-like ER kinase (PERK), and Activating Transcription Factor 6 (ATF6). Upon ER stress, these stress sensors become activated triggering the adaptive response attenuating protein translation and enhancing mRNA degradation to reduce the ER protein load, upregulating the transcription of protein folding and processing enzymes to increase the ER folding capacity, and inducing the expression of ER-associated protein degradation (ERAD) and autophagy components to remove malfolded proteins.

The UPR also determines β-cell fate behaving similar to a binary switch between life and death[10, 45]. The consequence of this switch depends on the nature of the ER stress condition, the balance between UPR death and survival components, and the activation and regulation of UPR signaling. Under healthy changes in blood glucose levels as discussed, the UPR can successfully mitigate this mild ER stress with the adaptive response and together with the increased expression of anti-apoptotic factors promote β-cell survival. As ER homeostasis is restored, negative feedback mechanisms turn off UPR signaling. However, under severe ER stress conditions such as chronic levels of glucose, free fatty acids, and cytokines, the UPR fails to restore ER homeostasis. Instead, the UPR bypasses negative regulation and becomes hyperactivated inducing the expression and activation of proapoptotic components leading to β-cell death. ER stress-mediated β-cell death has a role in the pathogenesis of type 2 diabetes.

ER stress and the UPR may also crosstalk with the inflammatory response to induce β-cell loss but a clear link has not been reported[129-131]. Several studies suggest that inflammation participates in the pathogenesis of type 2 diabetes[141]. β-cells are capable of

producing and secreting the inflammatory cytokine, interleukin-1β (IL-1β) under metabolic stress. In turn, secreted IL-1β is capable of autostimulation by binding to the highly expressed IL-1 receptor type 1 (IL-1R) on the β-cell surface. During this vicious cycle, IL-1β stimulates the production of itself and chemokines therefore recruiting macrophages to pancreatic islets leading to β-cell inflammation and death.

In this study, we provide evidence that thioredoxin interacting protein (TXNIP) induces ER stress-mediated IL-1β production and cell death in β-cells. During a DNA microarray analysis of UPR survival and death components regulated by the UPR anti-apoptotic transcription factor, apoptosis-antagonizing transcription factor (AATF), we identified TXNIP as a potential proapoptotic component. We discovered that TXNIP is strongly induced by ER stress through the IRE1α and PERK-eIF2α arms of the UPR and specifically its transcription is regulated by activating transcription factor 5 (ATF5) and carbohydrate response element binding protein (ChREBP). It has been demonstrated that TXNIP activates the Nod-like receptor protein 3 (NLRP3) inflammasome leading to the production and secretion of IL-1β. Therefore we hypothesized that ER stress induces IL-1β production through this TXNIP-NLPR3 pathway. We demonstrated that TXNIP does indeed have a role in IL-1β production through the NLRP3 inflammasome and that this IL-1β is capable of autostimulation in β-cells under ER stress. We confirmed that TXNIP is an apoptotic component of the UPR partly through IL-1β signaling. Collectively, these results suggest that the UPR can induce β-cell inflammation and apoptosis through TXNIP. Therefore TXNIP is a potential therapeutic target for the treatment of type 2 diabetes.

Materials and Methods

Cell culture and transfection of small interfering RNA

Rat insulinoma cells, INS-1 832/13, were a gift from Dr. Christopher Newgard (Duke University Medical Center). These cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% sodium pyruvate, and 0.1 % 2-mercaptoethanol. Mouse islets were cultured in RPMI 1640 supplemented with 5% FBS and 5 mM glucose. Human islets (Prodo Laboratories, Irvine, CA) were cultured in CMRL media supplemented with 5 mM glucose and grown on laminin V coated plates. Mouse embryonic fibroblasts were maintained in DMEM with 10% FBS. *Ire*$\alpha^{-/-}$ and *Perk*$^{-/-}$ fibroblasts were a gift from Dr. David Ron (University of Cambridge). *Atf6*$\alpha^{-/-}$, *eIF2*$\alpha^{S/S}$ and *eI2F2*$\alpha^{A/A}$ fibroblasts were gifts from Dr. Randal Kaufman (Sanford-Burnham Medical Research Institute).

The Cell Line Nucleofector™ kit V and the Nucleofector™ Device (Lonza, Walkersville, MD) was used to transfect small interfering RNA (siRNA) directed against IRE1, PERK, ATF6, ChREBP, ATF5 and NLRP3 into INS-1 832/13 cells. siRNAs were designed and synthesized at QIAGEN (Valencia, CA) as follows: rat IRE1 AAGCAGCAGACUUUGUCAUCA, rat PERK GGAAAUAUUUGGAACGAUC, rat ATF6 GCACAUGAGACUUACGAAA, , rat ChREBP AAAGGUUGUGGGUGCAGGAAGGGUA, rat ATF5 CAGAGUCAGUGGAACGGGA and rat NLRP3 GAGCAGCAGGCAUCGGAAA. Cells were incubated in media

overnight after siRNA transfection, and then additional treatments were performed, including ER stress induction.

Isolating islets from mouse pancreata

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital. Pancreatic islets were then isolated by pancreatic duct injection of 500 U/mL of collagenase solution followed by digestion at 37°C for 40 min with mild shaking. Islets were washed several times with Hank's buffered salt solution (HBSS), separated from acinar cells on a discontinuous Ficoll 400 gradient, viewed under a dissecting microscope, and hand-selected.

GeneChip Array Analysis

INS-1 832/13 cells were stably transduced with LV-TO/AATF, an inducible lentivirus expressing AATF. Cells were cultured with or without 2 μg/mL doxycycline to induce AATF for 48 h. Total RNA was isolated from samples of three independent experiments using RNeasy Mini Kit (Qiagen, Valencia, CA) and processed for GeneChip analysis by the Genomic core facility at University of Massachusetts Medical School (Worcester, MA). The final product was hybridized to the GeneChip® Rat Genome 230 2.0 Arrays (Affymetrix, Santa Clara, CA) and scanned with a GeneChip Scanner 3000.

RMA method[151] in Affy package from Bioconductor was used in R to summarize the probe level data and normalize the dataset to remove across array variation. Log transformed data was used in the subsequent analysis. Limma package from

Bioconductor with randomized complete block design was used to determine whether overexpressing AATF alters a gene¹s expression level significantly. Genes with p-value < 0.01 and at least one treatment has all three replicates called present from the mas5 Present/Absent call was considered significant.

Immunostaining

Frozen sections of C57BL/6 mouse pancreata were fixed in 2% paraformaldehyde for 30 min at room temperature and then permeabilized with 0.1% Triton X-100 for 2 min. The fixed cells were washed with phosphate-buffered saline, blocked with 10% bovine serum albumin for 30 min, and incubated in primary antibody overnight at 4°C. The cells were washed three times in phosphate-buffered saline/Tween 0.1% and incubated with secondary antibody for 1 h at room temperature. Images were obtained with a Leica TCS SP2 AOBS confocal microscope with LCS software. The following primary antibodies were used: anti-insulin (Dako, Carpinteria, CA), anti-glucagon (Dako, Carpinteria, CA), and anti-TXNIP (MBL, Nagoya, Japan).

Lentivirus system

For generation of cells stably suppressing TXNIP, INS-1 832/13 cells were transduced with a retrovirus expressing shRNA against rat TXNIP. The following shRNA oligos were used: GATCCCCAGCATGGCTAGCCGGACATTCAAGAGATGTCCGGCTAGCCATGCT GTTTTTGGAAA and

AGCTTTTCCAAAAACAGCATGGCTAGCCGGACATCTCTTGAATGTCCGGCTA GCCATGCTGGG. Details of this lentivirus system were described previously [152].

Plasmids

Entry vectors, destination vectors, and viral plasmids for establishing lentiviral and retroviral cell lines were provided by E. Campeau [152]. TXNIP reporter plasmids were constructed by inserting TXNIP promoter sequences into pGL4.17 (Promega, Madison, WI).

Immunoblot

Cells were lysed in M-PER buffer (Pierce, Rockford, IL) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) for 10 min on ice. The lysates were then cleared by centrifuging the cells at 14,000 g for 10 min at 4°C. Lysates were normalized for total protein (30 μg per lane), separated using a 4-20% linear gradient SDS-PAGE (BioRad, Hercules, CA) and electroblotted. Blots were probed with the following antibodies: anti-TXNIP (MBL, Nagoya, Japan), anti-CHOP (Pierce, Rockford, IL), anti-eIF2α (Santa Cruz Biotechnology, Santa Cruz, CA), anti-eIF2α-P (Cell Signaling, Danvers, MA) and anti-actin (Sigma-Aldrich, St. Louis, MO).

Real-time polymerase chain reaction

Total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen) and reverse transcribed using 1 μg of total RNA from cells with Oligo-dT primer. For the

thermal cycle reaction, the iQ5 system (BioRad, Hercules, CA) was used at 95°C for 10 min, then 40 cycles at 95°C for 10 sec and at 55°C for 30 sec.

The relative amount for each transcript was calculated by a standard curve of cycle thresholds for serial dilutions of cDNA samples and normalized to the amount of β-actin. The polymerase chain reaction (PCR) was performed in triplicate for each sample, after which all experiments were repeated twice. The following sets of primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) were used for real-time PCR: human actin, ACCATGGATGATGATATCGCC and GCCTTGCACATGCCGG; human TXNIP, CAGCCAACAGGTGAGAATGA and TTGAAGGATGTTCCCAGAGG; human IL-1β, CTCGCCAGTGAAATGATGGCT and GTCGGAGATTCGTAGCTGGAT; human IL-6, AAATTCGGTACATCCTCGACGG and GGAAGGTTCAGGTTGTTTTCTGC; mouse actin, GCAAGTGCTTCTAGGCGGAC and AAGAAAGGGTGTAAAACGCAGC; mouse TXNIP, TATGTACGCCCCTGAGTTCC and GCTCACTGCACGTTGTTGTT; rat actin, GCAAATGCTTCTAGGCGGAC and AAGAAAGGGTGTAAAACGCAGC; rat TXNIP, CAAGTTCGGCTTTGAGCTTC and ACGATCGAGAAAAGCCTTCA; rat IL-1β, CTCTGTGACTCGTGGGATGA and CGAGGCATTTTTGTTGTTCA, rat IL-6, TAGTCCTTCCTACCCCAACTTCC and TTGGTCCTTAGCCACTCCTTC.

Luciferase Assay

For reporter assays, INS-1 832/13 cells were cotransfected with a TXNIP promoter-luciferase reporter construct and various constructs as indicated by

Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 6 h the culture medium was changed to fresh medium with 2.5 mM glucose. Prior to lysis at 24 h after transfection, cells were treated with or without 0.5 μM of thapsigargin for 6 h. Luciferase activity was measured with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and transfections were normalized with the pRL-TK vector (Promega, Madison, WI) as an internal control. The light produced from the samples was read by a standard plate reading luminometer. The assays were performed independently three times.

Chromatin Immunoprecipitation

ChIP was performed using the SimpleChIP™ Enzymatic Chromatin IP Kit (Agarose Beads) (Cell Signaling, Beverly, MA) according to the manufacturer's instructions. Briefly, INS-1 832/13 cells were cultured for 6 h with or without 0.5 μM thapsigargin and crosslinked with 1% formaldehyde. DNA was isolated using lysis buffer and sonicated on ice for 20 seconds with intervals of 30 seconds and repeated for 15 cycles (Bioruptor, Denville, NJ). Chromatin immunoprecipitation was performed with the ChREBP (sc-21189), Mlx (sc-14705), ATF5 (sc-99205) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as well as rabbit IgG as a negative control. Q-PCRs were performed using human TXNIP promoter-specific primers: CCGAACAACAACCATTTTCC and CTCCCATTGGCTACTTGGTC.

Fluorescence-activated cell sorting

For flow cytometry analyses, INS-1 832/13–TetR-shTXNIP cells were plated onto 6-well plates and incubated with 2 μg/mL doxycycline for 48 h, then treated with or without 10 nM thapsigargin for 24 h. The treated cells were harvested and stained with Mitoprobe DilC1(5) kit (Invitrogen, Carlsbad, CA) and propidium iodide solution (Invitrogen) following manufacturer's protocols. After washing with PBS, the stained cells were resuspended in the 11 mM glucose-Hanks buffered salt solution. Flow cytometry analyses were performed with LSRII (BD) at the FACS core facility of University of Massachusetts Medical School (Worcester, MA). The results were analyzed by FlowJo ver.7.6.3.

Cell Viability Assay

Cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) as per manufacturer's instructions.

Caspase-Glo 3/7 Assay

Apoptosis was assessed using the Cell-Glo 3/7 assay (Promega, Madison, WI) as per manufacturer's instructions.

Reagents

Glucose, palmitate, Brefeldin A, thapsigargin, and tunicamycin were obtained from Sigma-Aldrich (St. Louis, MO) and Cell Signaling Technology (Danvers, MA). RPMI-1640, DMEM, HBSS, Mitoprobe DilC1(5) kit and propidium iodide solution were

obtained from Invitrogen (Carlsbad, CA). Human amylin (hIAPP) was ordered from Bachem (Torrance, CA). Human recombinant interleukin-1 receptor antagonist was obtained from Prospec (East Brunswick, NJ).

Animal Experiments

Txnip knockout mice were generated as previously described[153]. All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

Statistical analysis

To calculate the significance of a difference deviation between two means, we used the Mann-Whiney test, unequal variance t-test, and two-way ANOVA. A p-value of <0.05 was considered statistically significant.

Results

Gene expression profiling of β-cells overexpressing AATF reveals a novel UPR component TXNIP

Upon ER stress, the UPR transcriptionally regulates both survival and death effectors; however, the identification of these components remains incomplete. Our lab has identified a novel UPR anti-apoptotic transcription factor, AATF regulated by the PERK-eIF2α pathway[48]. AATF can promote cell survival through the transcriptional regulation of survival and death effectors[154, 155]. Indeed we revealed that AATF protects β-cells from ER stress in part by upregulating the transcription of the survival factor, Akt1 with STAT3. In order to identify other transcriptional targets of AATF, we studied genes differentially expressed in the rat insulinoma cell line, INS-1 832/13 overexpressing AATF by DNA microarray analysis. We screened the genes differentially expressed for their relation to survival or cell death and chose to focus on the apoptotic factor TXNIP, which was modestly downregulated by AATF (Figure 2.1a). TXNIP was first identified as a binding partner of thioredoxin inhibiting its protein-reducing activity[147-149]. Later it was discovered in a DNA microarray study that TXNIP expression was most strongly induced by glucose in isolated human islets[156]. Interestingly, TXNIP has a negative role in pancreatic β-cells including inhibiting glucose stimulated insulin secretion (GSIS)[157, 158] and promoting glucose-mediated β-cell death[159]. In response to the latter, we speculated that perhaps TXNIP triggers β-cell death under ER stress conditions. We further confirmed that TXNIP was downstream of AATF by Q-PCR.

A

Gene	ID	Fold Change	P-Value
AATF	1368330_at	4.81	0.00031
TXNIP	1371131_a_at	0.833	0.00547

B

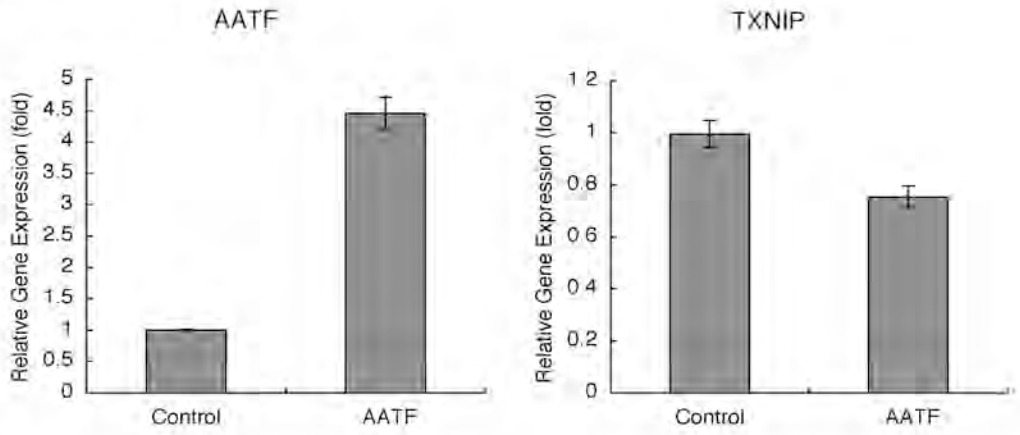


Figure 2.1 Gene expression profiling of INS-1 832/13 cells overexpressing AATF reveals a novel UPR component, TXNIP. (A) INS-1 832/13 cells were stably transduced with LV-TO/AATF, an inducible lentivirus expressing AATF. Cells were cultured with or without doxycycline (2 µg/mL) to induce AATF for 48 h. Gene expression was compared between cells overexpressing AATF and control cells using the Affymetrix GeneChip Rat Genome 230 2.0 Array. Fold change represents AATF overexpression/control. (B) INS-1 832/13 cells were treated with or without doxycycline (2 µg/ml) to induce AATF for 48 h. Expression levels of AATF and TXNIP mRNA were measured by quantitative real-time PCR (Q-PCR) and standardized to actin (n=3; results are means ± SE).

TXNIP mRNA was downregulated in AATF overexpressing INS-1 832/13 cells (Figure 2.1b).

TXNIP expression is localized in pancreatic β-cells

We primarily wanted to focus on understanding the components and mechanisms of ER stress-mediated apoptosis in β-cells as the UPR is cell-type specific. Therefore we wanted to confirm if TXNIP is preferentially expressed in pancreatic β-cells compared to other cell types in the pancreas. Immunostaining of mouse pancreas sections with anti-TXNIP antibody together with anti-insulin or anti-glucagon antibody revealed that TXNIP was mainly found in islets where it co-localizes with insulin (upper panel, Figure 2.2). TXNIP did not co-localize with glucagon suggesting that TXNIP has an important function in β-cells (lower panel, Figure 2.2).

TXNIP expression is induced by ER stress in β-cells

We next wanted to investigate whether TXNIP expression is induced by ER stress in β-cells. We measured mRNA expression levels of TXNIP in INS-1 832/13 cells treated with the pharmacological ER stress inducers, thapsigargin and tunicamycin. INS-1 832/13 cells are commonly used in the ER stress field to study UPR signaling in β-cells due to their ability to respond to a variety of ER stress inducers, activate the UPR, and undergo cell death under severe ER stress. Thapsigargin is a strong ER stress inducer inhibiting the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) pump preventing its ability to pump calcium into the ER therefore depleting ER calcium stores. As a result, calcium dependant folding and processing enzymes cannot function properly decreasing the ER folding capacity. Tunicamycin inhibits N-acetylglucosamine-1-

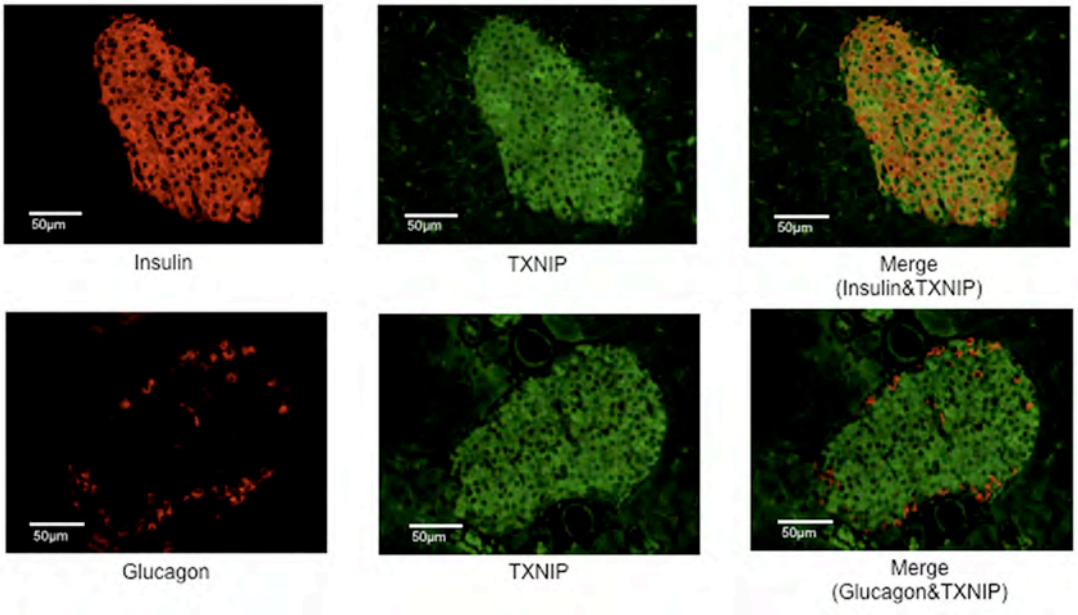


Figure 2.2 TXNIP is expressed in pancreatic β-cells. Mouse pancreata were analyzed by immunohistochemistry with anti-TXNIP, anti-insulin, and anti-glucagon antibodies. Merged images show the co-localization of TXNIP and insulin (upper panel) or TXNIP and glucagon (lower panel) (n=3).

phosphate transferase (GPT) blocking the synthesis of N-linked glycoproteins. Most proteins in the ER are N-glycosylated and N-glycosylation is important for proper protein folding therefore tunicamycin causes ER stress. This ER stress is delayed compared to thapsigargin-induced ER stress due to the dependence upon protein biosynthesis. We found that TXNIP mRNA expression levels were increased by these chemical ER stress inducers (Figure 2.3a). In addition, TXNIP protein translation is also increased by these ER stress agents (Figure 2.3b). We further confirmed these findings by treating mouse and human primary islets with thapsigargin and tunicamycin. As predicted, TXNIP mRNA expression was increased in both mouse and human primary islets (Figure 2.3c).

We also wanted to study if TXNIP expression is upregulated by pathological conditions known to cause ER stress in β-cells such as chronic high glucose and hIAPP. Chronic high glucose causes an overbearing amount of insulin biosynthesis leading to severe ER stress and a hyperactivated UPR favoring β-cell dysfunction and death[54, 160]. Human IAPP aggregation in islets is a common pathology of type 2 diabetes and has been shown to induce ER stress[115-117]. Accordingly, TXNIP mRNA was increased by chronic high glucose and hIAPP in INS-1 832/13 cells (Figure 2.3d). Furthermore, we examined pancreatic islets isolated from the Akita mouse and also found TXNIP mRNA levels were elevated as compared to islets from control mice (Figure 2.3e). These heterozygous mutant Akita mice carry an *Ins2*^{C96Y} mutation that disrupts proper disulfide bond formation within proinsulin-2[71]. This mutated protein accumulates in the ER causing severe ER stress. Lastly, the saturated free fatty acid palmitate is another pathological ER inducer that can activate the UPR in a number of β-cell models[95]. In a

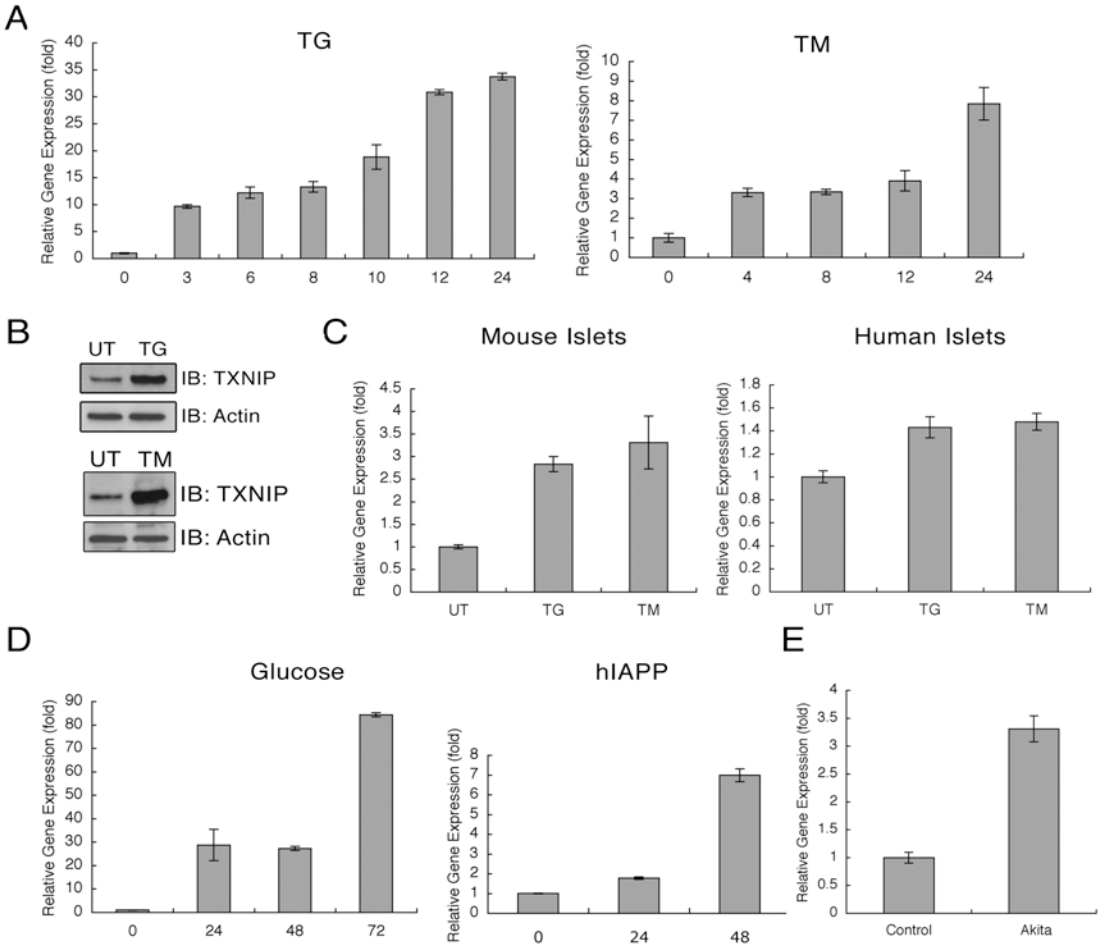


Figure 2.3 TXNIP expression is induced by ER stress. (A) INS-1 832/13 cells were treated with thapsigargin (TG, 0.25 μM) and tunicamycin (TM, 5 μg/mL) for the indicated number of hours. TXNIP mRNA levels were measured by quantitative real-time PCR (Q-PCR) and normalized to actin (n=3; values are mean ± SE). (B) INS-1 832/13 cells were treated with thapsigargin (TG, 1 μM, 8 h) and tunicamycin (TM, 5 μg/mL, 24 h). TXNIP and actin protein levels were measured by immunoblot (IB) (n=3). (C) Mouse and human primary islets were treated with thapsigargin (TG, 1 μM) and tunicamycin (TM, 5 μg/ml) for 6 h. Expression levels of TXNIP were measured by Q-PCR and normalized to actin (n=3; values are mean ± SE). (D) INS-1 832/13 cells with treated with glucose (16.7 mM) and human islet amyloid polypeptide (hIAPP, 5 μM) for the indicated number of hours. Expression levels of TXNIP were measured by Q-PCR and normalized with actin (n=3; values are mean ± SE). (E) Islets from C57BL/6 control mice and Akita mice were isolated. TXNIP mRNA levels were measured by Q-PCR and normalized to actin (n=3; values are mean ± SE).

previous study, palmitate was shown incapable of inducing TXNIP expression in isolated rat and mouse islets[161, 162]. To confirm these findings, we also examined the possibility that palmitate could induce TXNIP expression but failed to see an induction (data not shown).

TXNIP expression is regulated by the IRE1α and PERK-eIF2α UPR pathways under ER stress

Collectively, we confirmed that a variety of pharmacological and pathological ER stimuli could lead to TXINP induction at both the mRNA and protein levels. Upon ER stress, the UPR master regulators, IRE1α, PERK, and ATF6α are activated[13]. For that reason, we next wanted to identify the UPR stress sensors involved in TXNIP induction.

During our research, we have found that TXNIP expression is regulated by the IRE1α and PERK arms of the UPR. TXNIP mRNA levels were modestly reduced in IRE1α siRNA knockdown INS-1 832/13 cells as compared to control under thapsigargin treatment (Figure 2.4a). Similarly, TXNIP mRNA and protein levels were markedly attenuated in *Ire1α*$^{-/-}$ mouse embryonic fibroblasts (MEFs) as compared to wild type cells under ER stress (Figure 2.4b). TXNIP expression was also significantly reduced in both PERK siRNA knockdown INS-1 832/13 cells and *Perk*$^{-/-}$ MEFs (Figure 2.4c and 2.4d). No significant changes in TXNIP expression were observed in ATF6α siRNA knockdown INS-1 832/13 cells and *ATF6α*$^{-/-}$ MEFs (Figure 2.4e and 2.4f).

Upon ER stress, activated PERK phosphorylates eIF2α attenuating global mRNA translation and transcriptionally influences gene expression[32, 33]. To determine if eIF2α

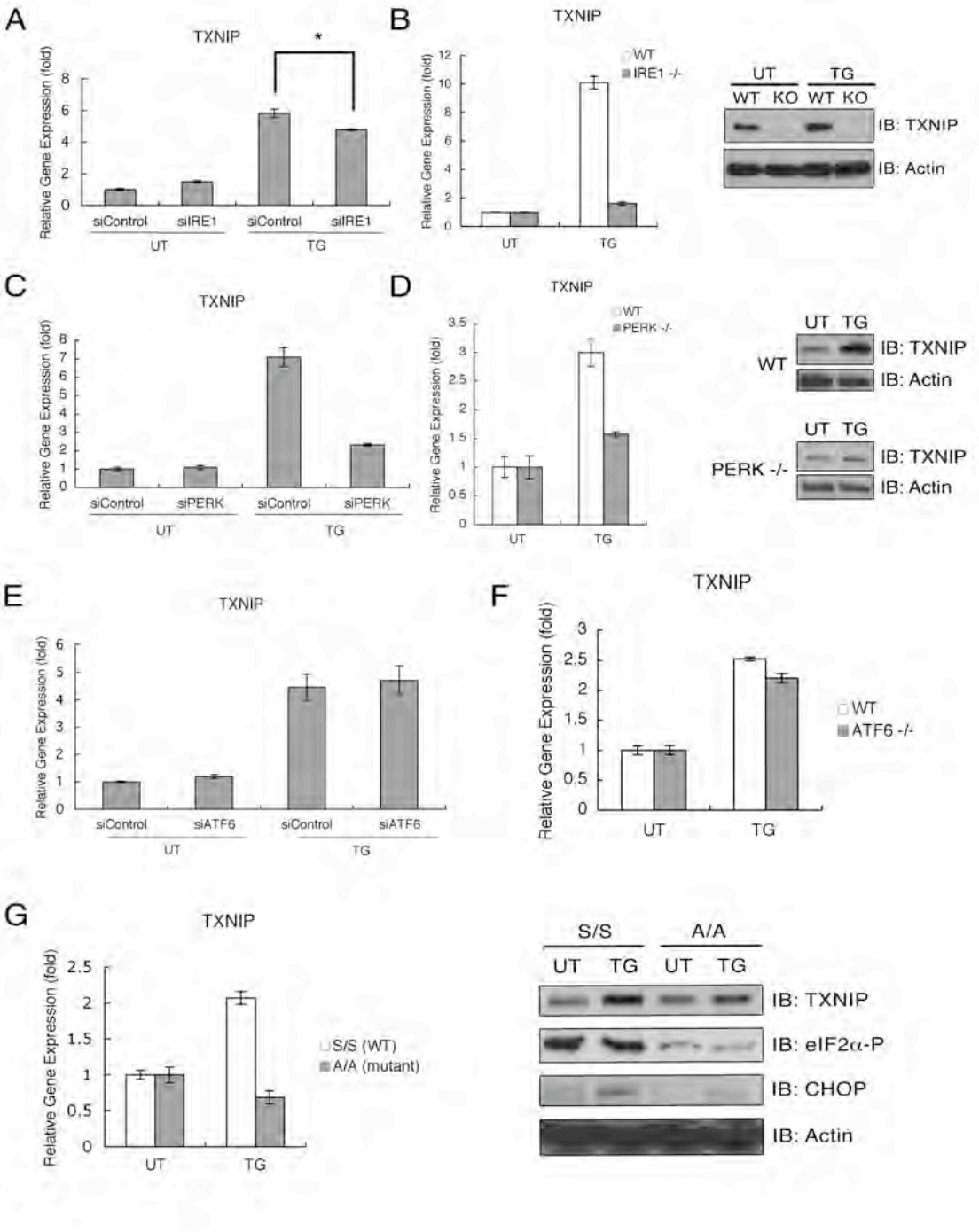


Figure 2.4 TXNIP expression is regulated under the IRE1α and PERK-eIF2α pathways of the UPR. (A) INS-1 832/13 cells were transfected with control scramble siRNA or IRE1α siRNA and then treated with thapsigargin (TG, 0.5 μM) for 6 h. TXNIP mRNA levels were measured by Q-PCR. Statistics were done by the unequal variance t-test (* $p<0.05$). (B) Wild-type (WT) and

Ire1α $^{-/-}$ (KO) MEFs were treated with thapsigargin (1.0 μM) for 3 h or untreated. Expression levels of TXNIP were measured by Q-PCR (left panel) and immunoblot (right panel). (C) INS-1 832/13 cells were transfected with control scramble siRNA or PERK siRNA and then treated with thapsigargin (TG, 0.5 μM) for 6 h. TXNIP mRNA levels were measured by Q-PCR. (D) Wild-type (WT) and *Perk*$^{-/-}$ MEFs were treated with thapsigargin (1.0 μM) for 3 h or untreated. Expression levels of TXNIP were measured by Q-PCR (left panel) and immunoblot (right panel). (E) INS-1 832/13 cells were transfected with control scramble siRNA or ATF6α siRNA and then treated with thapsigargin (TG, 0.5 μM) for 6 h. TXNIP mRNA levels were measured by Q-PCR. (F) Wild-type (WT) and *Atf6α*$^{-/-}$ MEFs were treated with thapsigargin (1.0 μM) for 3 h or untreated. TXNIP mRNA levels were measured by Q-PCR. (G) Wildtype *eIF2α*$^{S/S}$ (S/S) or mutant *eIF2α*$^{A/A}$ (A/A) MEFs were treated with thapsigargin (TG, 1.0 μm) for 3 h or untreated. TXNIP mRNA levels were measured by Q-PCR (left panel). TXNIP, phosphorylated eIF2α, CHOP, and actin protein levels were measured by immunoblot (right panel). Q-PCR results were normalized to actin and shown as means ± SE of three independent experiments. Representative immunoblots shown of three independent experiments.

phosphorylation has a role in TXNIP expression, we treated MEFS containing either a wild-type allele of eIF2α (*eIF2α*$^{S/S}$) or an allele bearing the S51A mutation (*eIF2α*$^{A/A}$) rendering eIF2α nonphosphorylatable with thapsigargin and measured TXNIP expression. We also measured expression of the PERK-eIF2α target CHOP as a positive control. Both TXNIP and CHOP expression levels were mitigated in *eIF2α*$^{A/A}$ MEFs (Figure 2.4g). Take together, theses results demonstrate that ER stress-mediated TXNIP expression is regulated by the IRE1α and PERK-eIF2α pathways.

TXNIP transcription is regulated by ATF5 and ChREBP under ER stress

According to our results, the UPR significantly induces TXNIP mRNA levels and therefore we continued our studies investigating the underlying mechanisms involved. To test if TXNIP mRNA is induced at the transcriptional level, we transfected INS-1 832/13 cells with luciferase reporter constructs containing 1.5 kb, 1.0 kb, or 0.5 kb of the TXNIP promoter. Cells were treated with or without thapsigargin 24 h post-transfection and assayed for luciferase activity. A renilla luciferase reporter vector was used as an internal control to normalize for transfection efficiency. The assay demonstrated that the 1.5 kb TXNIP luciferase reporter construct was most strongly activated by thapsigargin (Figure 2.5a).

We next investigated the influence of IRE1α and PERK on TXNIP transcription. To confirm that PERK-eIF2α signaling is involved in TXNIP transcription, we cotransfected the 1.5 kb TXNIP luciferase reporter construct with vectors expressing PERK or GADD34 in INS-1 832/13 cells followed by thapsigargin treatment. GADD34 recruits phophotases to dephosphorylate eIF2α[80-83]. We observed that PERK stimulated

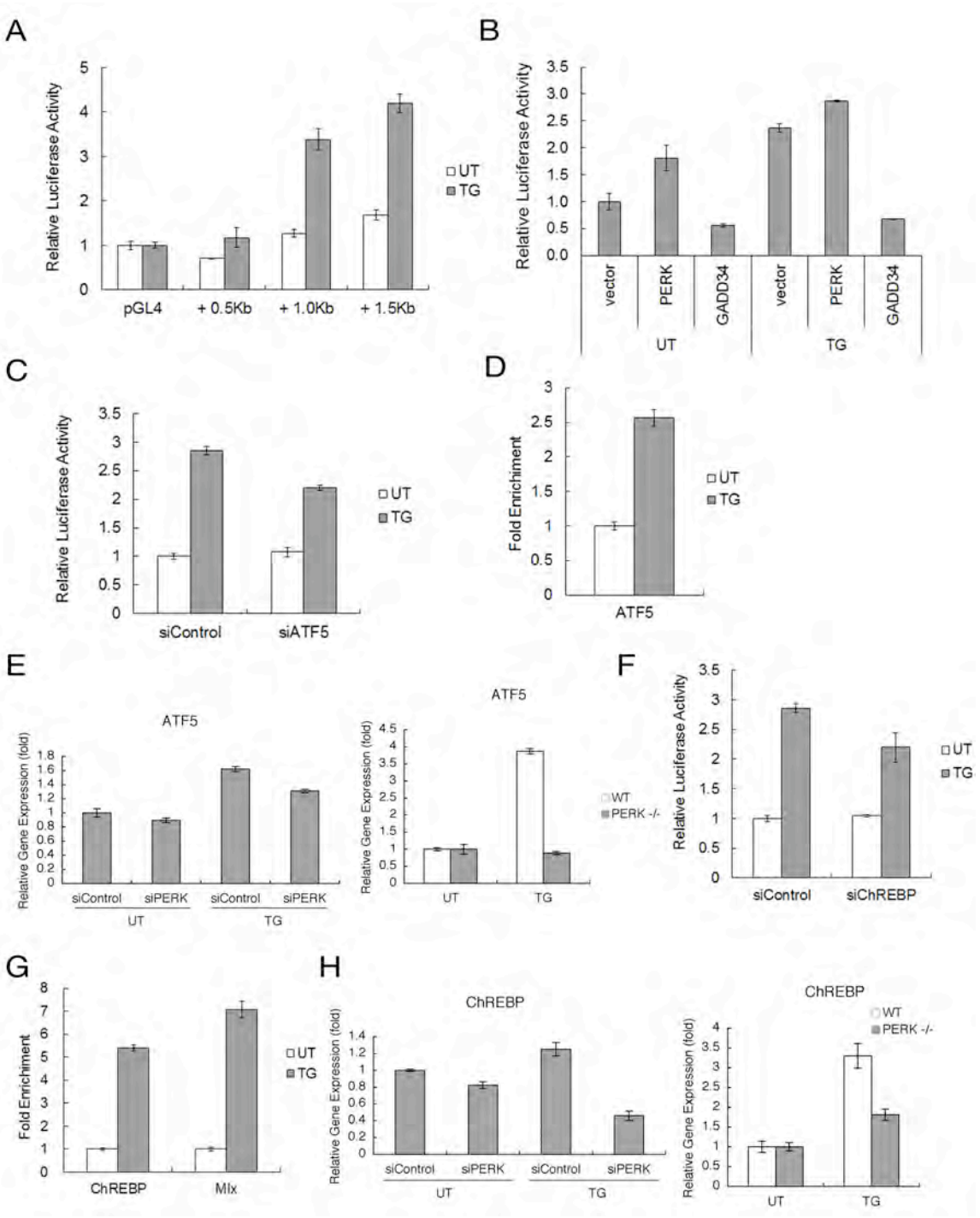


Figure 2.5 TXNIP transcription is regulated by ATF5 and ChREBP under ER stress in β-cells. (A) INS-1 832/13 cells were transfected with TXNIP luciferase reporter constructs carrying 0.5 kb, 1.0 kb, or 1.5 kb of the TXNIP promoter. After 24 h, cells were untreated (UT) or treated with thapsgargin (TG, 0.5 μM) for 6 h and then analyzed for luciferase activity. (B) INS-1 832/13 cells were co-transfected with the 1.5 kb TXNIP luciferase reporter construct together with

control, PERK expression, or GADD34 expression vectors. After 24 h, cells were untreated (UT) or treated with thapsigargin (TG, 0.5 μM) for 6 h and then analyzed for luciferase activity. (C) INS-1 832/13 cells were co-transfected with the 1.5 kb TXNIP luciferase reporter construct and control scramble siRNA or ATF5 siRNA. After 24 h, cells were untreated (UT) or treated with thapsgargin (TG, 0.5 μM) and then analyzed for luciferase activity. (D) INS-1 832/13 cells were treated with or without thapsigargin (TG, 0.5 μM) for 6 h. The association of ATF5 on the TXNIP promoter was assessed by ChIP assays and quantified by Q-PCR. (E) INS-1 832/13 cells were transfected with control scramble siRNA or PERK siRNA. Then cells were treated with or without thapsigargin (TG, 0.5 μM) for 6 h. (left panel) Wild-type and *Perk*$^{-/-}$ MEFs were treated with or without thapsigargin (TG, 1.0 μM) for 3 h (right panel). ATF5 mRNA levels were measured by Q-PCR and normalized to actin. (F) INS-1 832/13 cells were co-transfected with the 1.5 kb TXNIP luciferase reporter construct and control scramble siRNA or ChREBP siRNA. After 24 h, cells were untreated (UT) or treated with thapsgargin (TG, 0.5 μM) and then analyzed for luciferase activity. (G) INS-1 832/13 cells were treated with or without thapsigargin (TG, 0.5 μM) for 6 h. The association of ChREBP and Mlx on the TXNIP promoter was assessed by ChIP assays and quantified by Q-PCR. (H) INS-1 832/13 cells were transfected with control scramble siRNA or PERK siRNA. Then cells were treated with or without thapsigargin (TG, 0.5 μM) for 6 h (left panel). Wild-type and *Perk*$^{-/-}$ MEFs were treated with or without thapsigargin (TG, 1.0 μM) for 3 h (right panel). ChREBP mRNA levels were measured by Q-PCR and normalized to actin. Results are means ± SE of three independent experiments.

TXNIP transcriptional activity whereas GADD34 suppressed it under ER stress (Figure 2.5b). Co-transfection with IRE1α expression vector did not significantly stimulate the TXNIP reporter construct (data not shown). Therefore we concluded that the PERK-eIF2α pathway has a major role in regulating TXNIP transcription under ER stress.

Next we wanted to identify the transcription factors involved in regulating TXNIP expression under ER stress. The UPR transcription factors can bind to cis-acting response elements within UPR genes promoters[163-165]. These include ERSE (ER stress response element, 5'-CCAAT-N9-CCACG-3'), ERSE-II (ER stress response element II, 5'-ATTGG-N1- CCACG-3') and the UPRE (Unfolded Protein Response element, 5'-TGACGTGG/A-3'). To reveal potential UPR transcription factors involved in TXNIP expression, we analyzed the TXNIP promoter for these consensus sequences yet we failed to locate such sequences.

Alternatively, we screened known transcription factors of the UPR including XBP-1, AATF, ATF3, ATF4, ATF5, and CHOP for their ability to regulate TXNIP expression. As expected, we determined XBP-1 was not involved in TXNIP transcription under ER stress in XBP-1 siRNA knockdown INS-1 832/13 cells and *Xbp1*$^{-/-}$ MEFs (data not shown). The remaining transcription factors are regulated under the PERK pathway and among them, ATF5 demonstrated a role in TXNIP transcription (Figure 2.5c). Knockdown of ATF3, ATF4 and CHOP by siRNA in INS-1 832/13 cells and *Atf4*$^{-/-}$ MEFs did not disrupt TXNIP expression under ER stress (data not shown). In addition regardless of our finding that AATF overexpression suppressed TXNIP expression, its knockdown did not augment TXNIP expression under ER stress (data not shown).

ATF5 is a member of the ATF/CREB family of b-ZIP transcription factors shown to be translationally upregulated under the PERK-eIF2α pathway in a similar fashion as ATF4[37]. When we co-transfected INS-1 832/13 cells with the 1.5 kb TXNIP luciferase reporter construct with ATF5 siRNA followed by thapsigargin treatment, we measured a modest suppression of luciferase activity (Figure 2.5c) .We further investigated whether ATF5 binds to the TXNIP promoter. ChIP analysis using an ATF5 antibody demonstrated that ATF5 binding to the TXNIP promoter was enhanced by thapsigargin treatment (Figure 2.5d). We also confirmed that ATF5 expression is regulated by PERK. In PERK siRNA knockdown INS-1 832/13 cells and *Perk* $^{-/-}$ MEFs, ATF5 mRNA levels were attenuated under thapsigargin treatment (Figure 2.5e).

We also screened known transcription factors of TXNIP focusing on those that may also be induced by ER stress. One candidate was forkhead box O1 transcription factor (FoxO1). FoxO1 has been shown to participate in TXNIP transcription under high glucose conditions in endothelial cells[166]. In addition FoxO1 activation may be induced by ER stress conditions such as palmitate through JNK phosphorylation[167]. We did detect modest changes in TXNIP mRNA levels in INS-1 832/13 cells transfected with FoxO1 siRNA and FoxO1 expression vector (data not shown). However, no significant changes were observed in the activation of TXNIP luciferase reporter construct by FoxO1 knockdown or overexpression and we failed to observe FoxO1 binding to the TXNIP promoter (data not shown).

Another interesting candidate was ChREBP. TXNIP induction by glucose in β-cells is regulated by ChREBP binding to carbohydrate response elements (ChoREs) in the

TXNIP promoter[143, 145, 168]. Glucose is also known to induce ER stress and activate the UPR leading us to believe that perhaps the UPR can also regulate TXNIP transcription through ChREBP. To investigate the role of ChREBP in ER stress-induced TXNIP transcription, we transfected INS-1 832/13 cells with the TXNIP 1.5 kb luciferase reporter construct and ChREBP siRNA followed by thapsigargin treatment. Knockdown of ChREBP modestly suppressed luciferase activity under ER stress (Figure 2.5f). We confirmed that ChREBP binds to the TXNIP promoter by ChiP analysis using a ChREBP antibody (Figure 2.5g). Max-like protein (Mlx) has been shown to be a functional heteromeric partner of ChREBP in regulating the expression of ChREBP target genes raising the possibility that Mlx is also involved in TXNIP transcription. As expected, Mlx binding to the TXNIP promoter was also enhanced by thapsigargin treatment (Figure 2.5g). We also observed that ChREBP expression was induced by ER stress and regulated by PERK. ChREBP mRNA levels were significantly decreased in PERK siRNA knockdown INS-1 832/13 cells and *Perk* $^{-/-}$ MEFs (Figure 2.5h). Collectively, our results suggest that ATF5 and ChREBP regulate TXNIP transcription under ER stress in β-cells.

TXNIP has a role in ER stress-mediated IL-1β production

It has been proposed that ER stress may induce inflammation but the mechanisms remain poorly characterized[129]. Remarkably we have found that tunicamycin and thapsigargin can upregulate the mRNA levels of the proinflammatory cytokine IL-1β in human islets (Figure 2.6a). In these experiments, as IL-1β is known to regulate

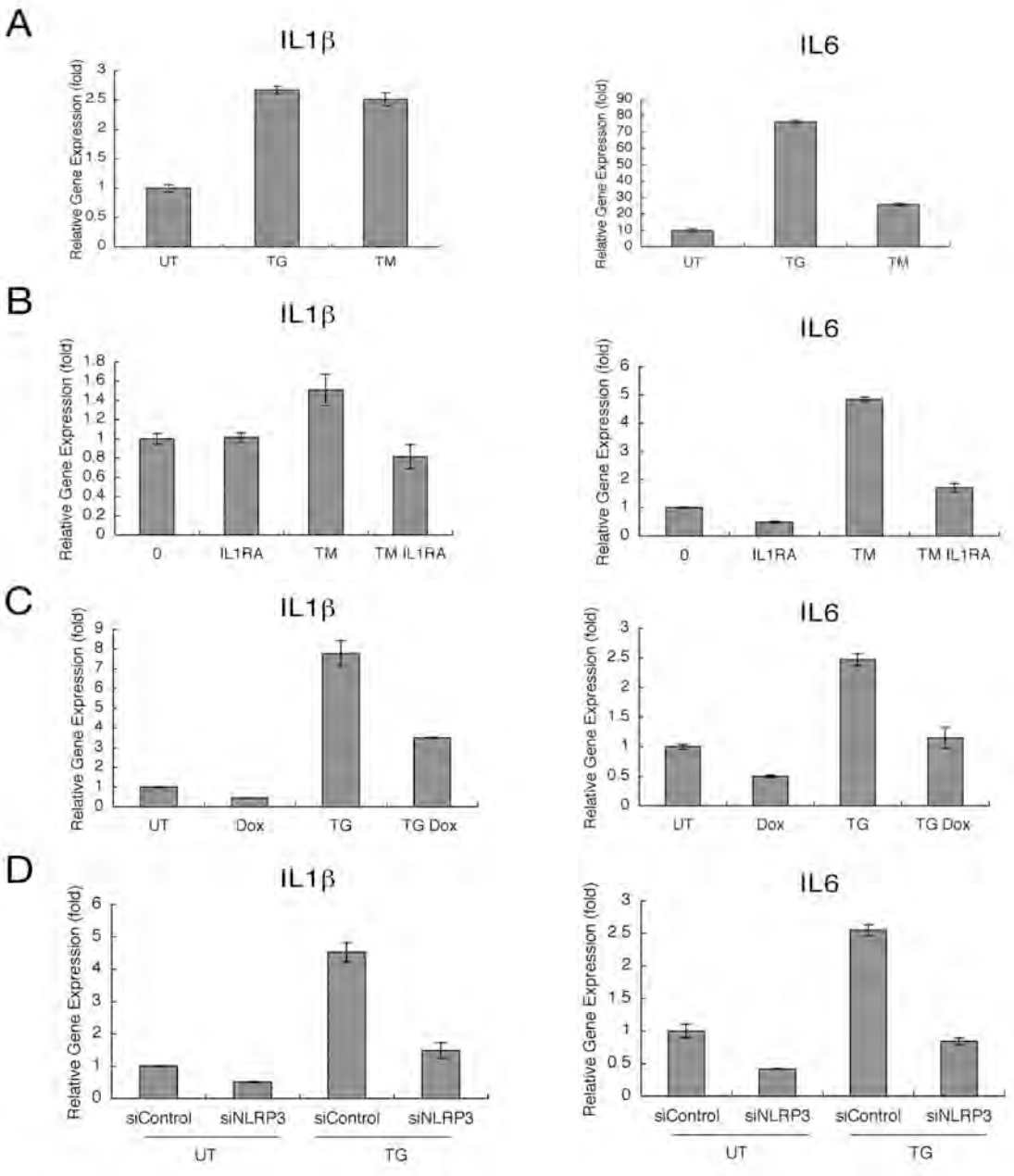


Figure 2.6 TXNIP mediates IL-1β production through the NLRP3 inflammasome under ER stress in β-cells. (A) Human islets were treated with thapsigargin (TG, 1 μM) and tunicamycin (TM, 5 μg/ml) for 6 h or untreated. IL-1β and IL-6 mRNA expression levels were measured by Q-PCR. (B) Human islets were pretreated with anakinra (IL1RA, 1 μg/mL) for 24 h followed by tunicamycin treatment (TM, 5 μg/ml) for 8 h. IL-1β and IL-6 mRNA expression levels were measured by Q-PCR. (C) INS-1 832/13 cells were stably transduced with pLenti-TO/shTXNIP, inducible lentivirus expressing shTXNIP. Cells were cultured with doxycycline (2 μg/ml) to induce shTXNIP or without doxycycline for 48 h, then challenged with thapsigargin (TG, 0.5

μM) for 6 h. IL-1β and IL-6 mRNA expression levels were measured by Q-PCR. (D) INS-1 832/13 cells were transfected with control scramble siRNA or siRNA directed against NLRP3, then treated with or without thapsigargin (TG, 0.5 μM) for 6 h. IL-1β and IL-6 mRNA expression levels were measured by Q-PCR. Q-PCR results were normalized to actin and shown as means ± SE of three independent experiments.

expression of inflammatory factors, we measured IL-6 mRNA expression levels as a control.

β-cells have been shown to secrete IL-1β and secreted IL-1β is capable of inducing its own expression through the binding of the highly expressed IL-1 receptor on the surface of β-cells[140]. Due to the low levels of IL-1β secretion and the capability of secreted IL-1β to associate with binding proteins, it is challenging to measure secreted IL-1β directly[169]. Instead we investigated if ER stress could stimulate the release of biologically active IL-1β by blocking IL-1 receptor activation with anakinra, the recombinant form of human IL-1 receptor antagonist. Human islets were preteated with anakinra for 24 h followed by tunicamycin treatment. Anakinra inhibited tunicamycin-induced IL-1β and IL-6 mRNA expression (Figure 2.6b). These results indicate that active IL-1β is secreted and capable of autostimulation under ER stress.

Under oxidative stress, TXNIP participates in the activation of the NLRP3 inflammasome leading to IL-1β production and secretion in β-cells[142]. Additionally, it has been recently reported that ER stress can activate the NLRP3 inflammasome leading to IL-1β secretion[139]. Furthermore, the NLRP3 inflammasome may be associated with the ER and upon activation relocalize to mitochondria-associated membranes (MAMs)[136], contact sites in which the ER transfers signals to the mitochondria to initiate apoptosis[137]. Together these results raise the possibility that TXNIP may be involved in IL-1β production through the NLRP3 inflammasome under ER stress in β-cells.

Significant knockdown of TXNIP by siRNA could not be established and therefore we developed a doxycycline-inducible shRNA system that allowed efficient and

regulated knockdown of TXNIP in INS-1 832/13 cells. Using this system, we discovered that TXNIP downregulation lead to the attenuation of IL-1β and IL-6 mRNA expression levels under thapsigargin (Figure 2.6c).

To confirm the involvement of the NLRP3 inflammasome, we measured IL-1β and IL-6 expression in NLRP3 siRNA knockdown INS-1 832/13 cells treated with thapsigargin. NLRP3 directly binds to TXNIP and is a key component of the NLRP3 inflammasome. We found that IL-1β and IL-6 expression was suppressed in cells deficient of NLRP3 (Figure 2.6d). Collectively, these results suggest that TXNIP has a role in ER-stress mediated IL-1β production through the NLRP3 inflammasome.

TXNIP induces β-cell death partly through IL-1β signaling under ER stress

We next wanted to explore the possibility that TXNIP is involved in ER stress mediated β-cell death. TXNIP has been shown to be a mediator of β-cell death under elevated glucose levels such as in diabetes[159]. Loss of TXNIP protected β-cells from apoptosis in several diabetic mouse models and isolated islets treated with high glucose[159, 170] and therefore may also protect β-cells from ER stress. We challenged shRNA mediated TXNIP knockdown INS-1 832/13 cells with apoptotic thapsigargin conditions and observed reduced caspase-3 cleavage levels, a key component of the mitochondria apoptotic pathway (Figure 2.7a). We further confirmed these results by staining these cells with propidium iodide (PI) to identify cells that have lost cell membrane integrity and also with mitoprobe DilC1(5), which detects active mitochondrial membrane potentials. Accordingly, TXNIP suppression decreased the

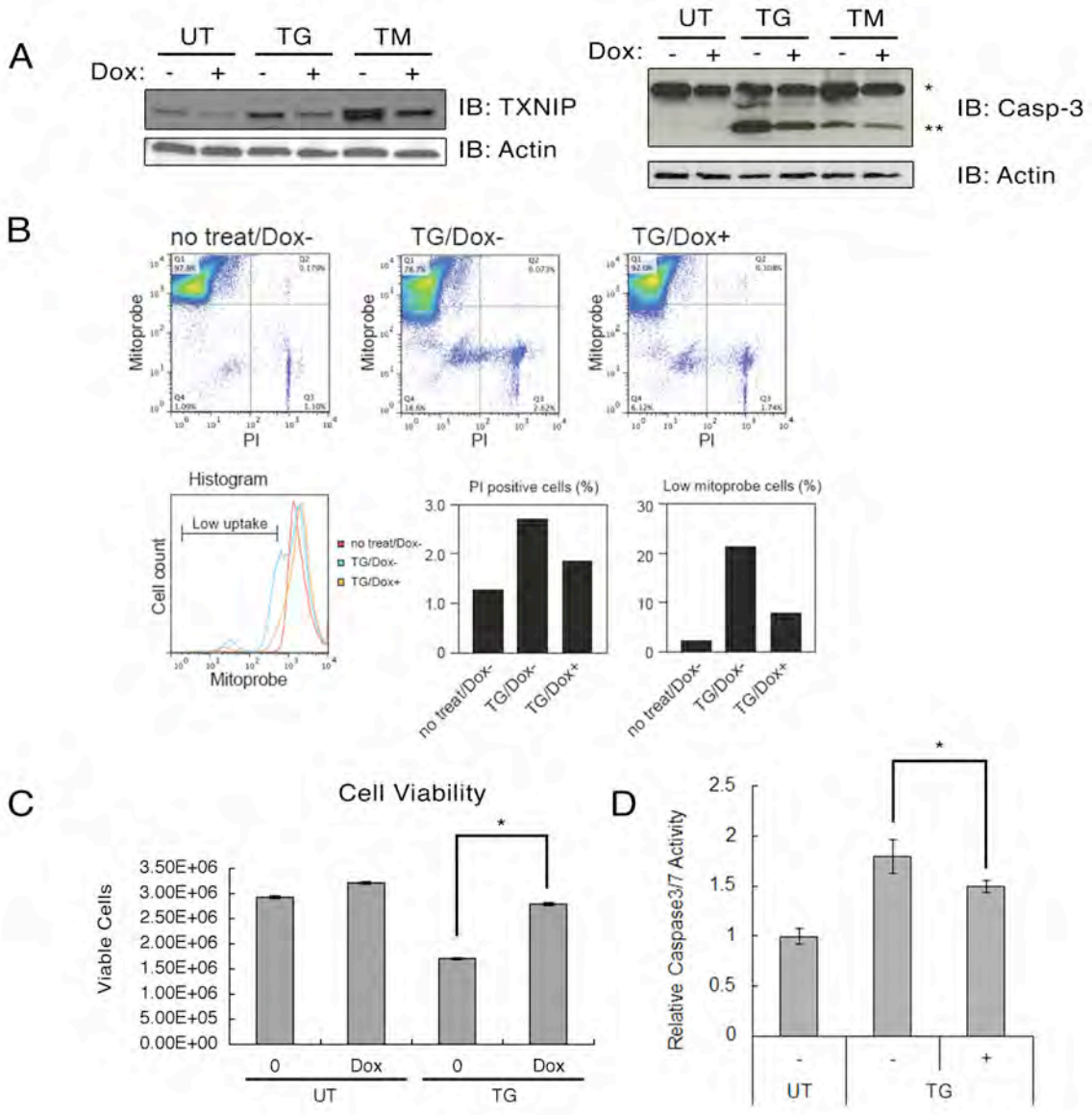


Figure 2.7 TXNIP contributes to ER stress-mediated β-cell death through IL-1β signaling. (A) INS-1 832/13 cells were stably transduced with pLenti-TO/shTXNIP, inducible lentivirus expressing shTXNIP. Cells were cultured with or without doxycycline (Dox, 2 μg/ml) to induce shTXNIP 48 h, then challenged with thapsigargin (TG, 50 nM) and tunicamycin (TM, 5 μg/ml) for 24 h. Expression levels of TXNIP, caspase-3, and actin were measured by immunoblot (IB). Single and double asterisks indicate uncleaved and cleaved caspase-3, respectively. (B) INS-1 832/13–TetR-shTXNIP cells were incubated with doxycycline (Dox, 2 μg/ml) for 48 h, then treated with thapsigargin (TG, 10 nM) for 24 h or untreated. Cells were stained with Mitoprobe DilC1(5) kit (Mitoprobe) and propidium iodide solution (PI) followed by flow cytometry analysis. (C) INS-1 832/13–TetR-shTXNIP cells were incubated with doxycycline (Dox, 2 μg/ml) for 48 h and then treated with or without thapsigargin (TG, 50 nM) for 24 h. Viable cells were determined by measuring ATP levels using CellTiter-Glo luminescent cell viability assay.

Statistics were done by the Mann-Whitney test, * $p<0.05$. (D) Human primary islets (28 year old male, BMI 21, HbA1C 5.4) were treated with thapsigargin (TG, 1 μM) in the presence of BSA (-, 1 μg/ml) or anakinra (+, 1 μg/ml) for 24 h. Caspase-3/7 activity was measured by Caspase-Glo 3/7 assay. Results are means ± SE for three independent experiments. Statistics was done by two-way ANOVA, * $p<0.05$.

amount of PI positive cells while increasing DilC1(5) stain intensities indicating protection of β-cells from ER stress-induced apoptosis (Figure 2.7b). Furthermore, TXNIP knockdown increased the number of viable cells in reflection of ATP intracellular levels (Figure 2.7c). Therefore these observations confirm that TXNIP is an integral component of ER-stress mediated β-cell death.

As demonstrated, TXNIP has a role in ER stress induced IL-1β production and autostimulation. IL-1β signaling has been shown to induce apoptosis in β-cells. To test if IL-1β mediates apoptosis under ER stress, human islets were treated with apoptotic thapsigargin in the presence of anakinra and as a result caspase-3 activity was reduced (Figure 2.7d). Collectively these findings suggest that TXNIP induced IL-1β production contributes to β-cell apoptosis under ER stress.

Discussion

Through gene expression profiling of β-cells overexpressing AATF, we have uncovered a novel component of the UPR, thioredoxin interacting protein (TXNIP). TXNIP is transcriptionally upregulated by pharmacological and physiological ER stress inducers under the IRE1α and PERK-eIF2α arms of the UPR through transcription factors ATF5 and ChREBP in β-cells. Subsequently, TXNIP activates the NLRP3 inflammasome leading to IL-1β secretion. IL-1β is capable of autostimulation through IL-1R1, eliciting a broad inflammatory response, and mediating β-cell death. Therefore, TXNIP is a mechanistic link between ER stress, inflammation, and apoptosis (Figure 2.8).

During our study we chose to focus on understanding the components and mechanisms involved in the UPR binary switch between life and death of the β-cell. Specifically, we chose to investigate TXNIP as a potential apoptotic component of the UPR in β-cells. Even though TXNIP is commonly described as a ubiquitously expressed gene, we observed that TXNIP is preferentially expressed in β-cells of pancreatic islets. We also found that a variety of ER stress stimuli can induce TXNIP expression in β-cell lines and isolated islets under IRE1α and PERK-eIF2α signaling pathways suggesting that TXNIP is an important component of the UPR in β-cells.

As of note we confirmed that TXNIP could not be induced by palmitate in β-cells. Palmitate has been shown to induce ER stress leading to β-cell death[95]. However, past studies demonstrate that TXNIP is actually repressed by palmitate in β-cells[161]. More recently a group discovered that palmitate affects TXNIP transcription by inhibiting

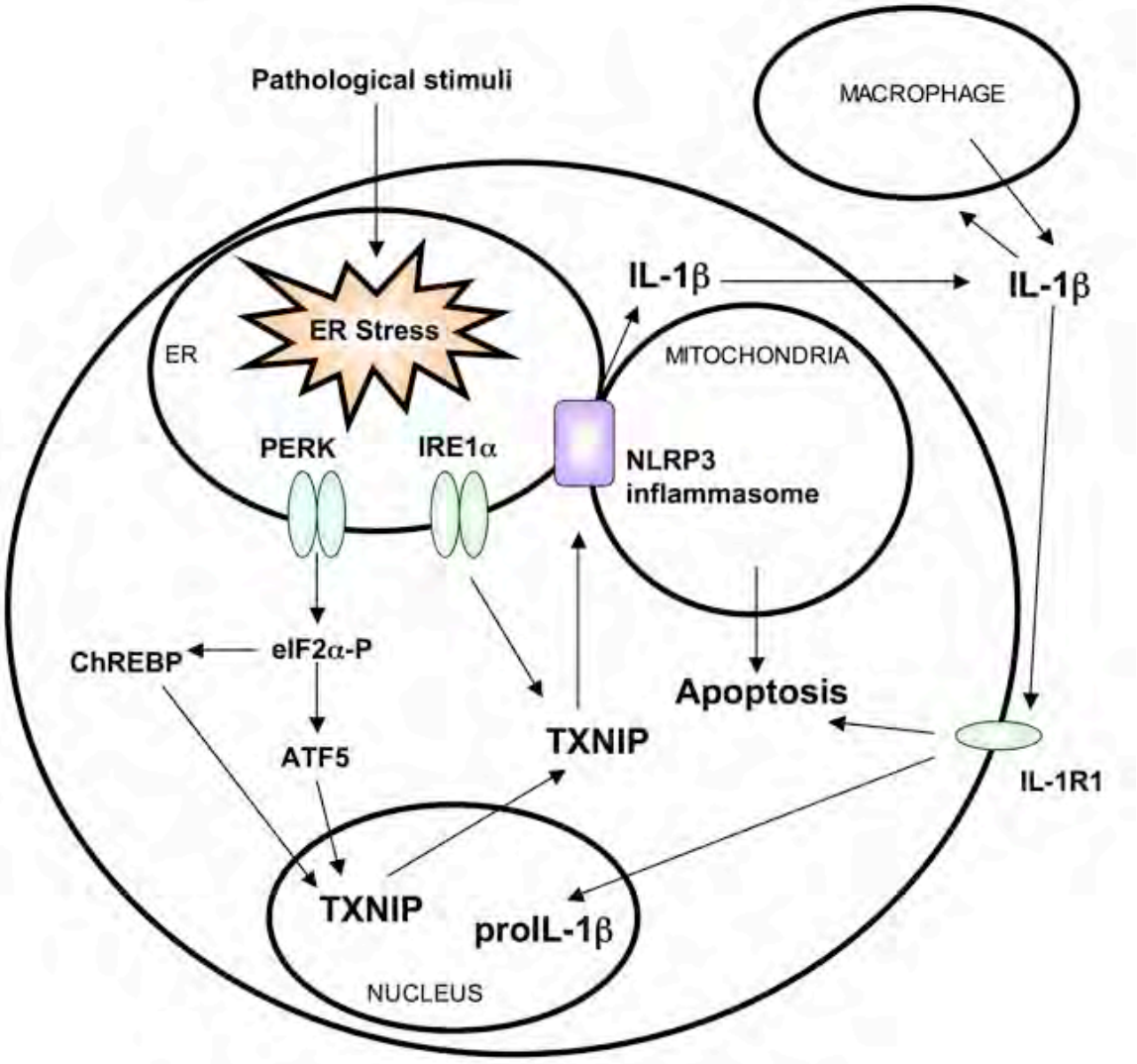


Figure 2.8 TXNIP induces ER stress-mediated β-cell inflammation and apoptosis. ER stress upregulates TXNIP expression under PERK-eIF2α and IRE1α signaling pathways and transcriptionally through ChREBP and ATF5. In turn, TXNIP activates the NLRP3 inflammasome leading to the processing and secretion of active IL-1β. IL-1β is capable of autostimulation through IL-1R1 inducing its own transcription. IL-1β also recruits macrophages to islets leading to a broad inflammatory response. In addition, TXNIP contributes to ER stress-induced β-cell death partly through IL-1β signaling.

ChREBP nuclear translocation possibly through AMP-activated protein kinase (AMPK)[162]. AMPK is an energy sensor and has several functions including regulating fatty acid metabolism. AMPK activity is enhanced by palmitate but reduced by glucose. Knockdown of AMPK partially increased TXNIP expression levels under glucose and palmitate conditions. AMPK may possibly modulate ChREBP activity by phosphorylation.

We also investigated the possible mechanisms of ER stress-mediated TXNIP transcription and revealed the involvement of transcription factors ATF5 and ChREBP. ATF5 has been demonstrated to be translationally upregulated under the PERK-eIF2α pathway[37]. Its target genes under ER stress have not been identified but a potential candidate is CHOP[171] and here we demonstrate TXNIP. Hence, ATF5 may play a role in inducing apoptotic genes in β-cells under severe ER stress. Further studies are needed to study the effect of overexpressing ATF5 and subsequently the genes it modulates in β-cells.

ChREBP is a basic helix-loop-helix leucine zipper transcription factor that partners with Mlx to bind to ChoRE motifs in the promoters of glucose-responsive genes[172, 173]. ChREBP has been well characterized for its ability to regulate genes involved in lipogenesis and glycolysis in response to glucose in liver[174]. Recently it has also been shown that ChREBP can regulate glucose-sensitive genes including TXNIP in β-cells promoting dysfunction and apoptosis[143, 175, 176]. Here we reveal that ChREBP is a novel transcription factor of the UPR. Under ER stress, ChREBP mRNA levels increase and is regulated under the PERK pathway; however, the exact mechanisms need to be

elucidated. A possible mechanism for ChREBP activation may involve ER calcium release. A new report has found that ChREBP binds to a binding protein sorcin that senses cytosolic calcium levels[177]. Upon an increase in calcium levels, sorcin releases ChREBP to allow for its translocation into the nucleus to regulate transcription. This mechanism should be explored in the context of ER stress as ER stress has been proposed to trigger ER calcium release. In addition like ATF5, it would be interesting to analyze gene targets of ChREBP that may reveal novel components of the UPR.

On the other hand, we could not confirm a role for FoxO1 in TXNIP transcription. FoxO1 belongs to the family of forkhead transcription factors. The TXNIP promoter contains a conserved FoxO consensus site. A study has demonstrated that FoxO1 can induce TXNIP transcription in neurons[178] and in another study FoxO1 upregulates TXNIP transcription under high glucose in human aortic epithelial cells[166]. In addition FoxO1 has been shown to be activated by JNK mediating ER stress and fatty acid-induced β-cell death in part through CHOP induction[167]. Therefore FoxO1 was an attractive candidate potentially involved in TXNIP transcription under ER stress in β-cells. However, even though we could see some attenuation of TXNIP expression upon FoxO1 knockdown, we did not observe FoxO1 binding to the TXNIP promoter or FoxO1 activation of the TXNIP luciferase reporter construct under ER stress. A possible explanation may be related to the recent finding that XBP-1 can bind to FoxO1 promoting its degradation by the proteosome[179]. Moreover, the UPR can also activate Akt[48, 180-182], which is an established inhibitor of FoxO1 nuclear translocation.

We also could not confirm a role for AATF in TXNIP transcriptional regulation. We originally identified TXNIP as a gene downregulated by AATF overexpression. However, upon AATF knockdown, we did not detect modulation of TXNIP expression. There are several possible explanations including overexpressed AATF acting as a negative inhibitor of TXNIP positive regulating transcription factors. Regardless, we conclude that AATF is not a major regulator of TXNIP transcription.

Despite our findings, the detailed mechanisms of ER stress-induced TXNIP expression remain incomplete. TXNIP transcription regulation under ER stress is most likely governed by additional unidentified transcription factors. A yeast one-hybrid screen in the future would reveal other key players involved. Furthermore, we revealed that IRE1α is not involved in the transcriptional regulation of TXNIP, which suggests that other mechanism are involved in TXNIP expression. Indeed Feroz Papa and his colleagues discovered that IRE1α upregulates TXNIP mRNA levels by degrading its destabilizing micro-RNA, miR-17 (unpublished). They have also revealed that TXNIP is translationally regulated by unknown mechanisms. Combining multiple means of regulation may ensure cells to efficiently increase TXNIP expression under ER stress conditions.

Accumulating evidence suggests that ER stress is a mediator of β-cell dysfunction and death during the development of diabetes[8-10]. β-cells experience ER stress on a regular basis as they increase insulin biosynthesis in response to fluctuations in blood glucose levels. As a consequence, β-cells are armed with a specialized ER and UPR to handle these physiological demands. Ironically when ER stress is unresolvable, this very

UPR system can switch over to pathological outputs including disruption in proinsulin folding and production, reduction in insulin secretion, oxidative stress, apoptosis, and inflammation. The molecular mechanisms underlying these consequences remain to be clarified. In particular, little progress has been made in understanding how ER stress can invoke inflammation. In this study, we have demonstrated that TXNIP is a key link between ER stress and inflammation.

Inflammation is a pathological component in type 1 diabetes and more recently it has been suggested to play a role in type 2 diabetes[140, 141]. Type 1 diabetes develops as a result of an autoimmune destruction of β-cells mediated by proinflammatory cytokines such as IL-1β. Interestingly, overnutrition associated with type 2 diabetes development can induce β-cells to secrete IL-1β[183]. Consequently, macrophages are recruited to islets and increase IL-1β levels[184]. Meanwhile, IL-1β can bind to IL-1R1 receptors expressed on the surface of β-cells triggering autostimulation leading to a vicious cycle of β-cell destruction. Type 2 diabetic patients who suffer from this mechanism of β-cell loss most likely have lower levels of the endogenous antagonist of IL-1 signaling, IL-1RA[185]. Accordingly, these patients respond quite well to anakinra, the recombinant form of IL-RA or neutralizing anti-IL-1β antibodies improving glycemic control and insulin production while reducing inflammatory markers[150, 186].

The mechanisms of IL-1β production in β-cells have recently been identified. Under high glucose, ROS production causes thioredoxin to release TXNIP and in turn binds to and activates the NLRP3 inflammasome[142]. The NLRP3 inflammasome processes IL-1β leading to secretion of active IL-1β. Here we demonstrate that TXNIP

regulates IL-1β production through the NLRP3 inflammasome under ER stress. Therefore excessive nutrients such as high glucose induce ER stress and activate the UPR leading to β-cell secretion of IL-1β through TXNIP. As ROS and potassium efflux are required for inflammasome activation in islets, studies should confirm their requirement specifically in β-cells.

Contradictory to our results, Menu *et al.* concluded that the classical UPR is not involved in activating the inflammasome in macrophages[139]. However, they did observe NLRP3 inflammasome activation and IL-1β secretion triggered by ER stress. Remarkably, the same group also demonstrated that upon activation, the NLRP3 inflammasome travels from the ER to mitochondria ER associated membranes (MAMs)[136]. At these contact sites, the ER transfers metabolites, lipids and calcium to the mitochondria to initiate signaling pathways including apoptosis[137]. Calcium uptake by the mitochondria can also increase mitochondrial ROS production[124], which may influence NLRP3 inflammasome activation. It would be interesting to study the localization of the NLRP3 inflammasome in the β-cell under ER stress.

We also conclude that secreted IL-1β is capable of autostimulation through its receptor, IL-1R1 on β-cells under ER stress. Presumably as a consequence, IL-1β induces expression of itself and chemokines through activation of NF-κB. Studies have demonstrated that NF-κB also regulates the expression of inducible nitric oxide synthase (iNOS) leading to nitric oxide (NO) production[187]. Even though controversial, NO suppresses SERCA2B expression contributing to the depletion of ER calcium levels and consequently ER stress[105]. In line with these observations, markers of the UPR including

spliced XBP-1, phosphorylated PERK and eIF2α, ATF4, and CHOP are detected in β-cell lines treated with IL-1β. Interestingly, BiP is actually downregulated by IL-1β therefore hampering ER folding capacity[188]. It has also been recently reported that NF-κB can directly bind to the CHOP promoter and upregulate its transcription[189]. CHOP has been implicated to play a role in ER stress mediated β-cell death. CHOP depletion partially protects β-cells from IL-1β[108]. IL-1β is also capable of activating JNK[190]. JNK is a mitogen-activated protein kinase (MAPK) that mediates β-cell cell death. Inhibiting JNK activation protects β-cells from IL-lβ[191, 192]. The JNK pathway also has a role in regulating CHOP transcription through AP-1 under cytokine treatment[64]. Collectively, IL-1β signaling is complex and needs to be studied carefully in the context of ER stress in β-cells. It may be challenging to determine if ER stress-mediated IL-1β autostimulation induces NF-κB and JNK activation as ER stress itself can induce the activation of these molecules. Nevertheless, we have found that IL-1β signaling may partially be involved in ER stress induced β-cell death.

Besides autostimulation, the UPR and TXNIP may influence IL-1β transcription directly. Our data demonstrates increased IL-1β mRNA levels under ER stress conditions and these levels are reduced by PERK and TXNIP knockdown or GADD34 overexpression. One possible mechanism involves NF-κB. NF-κB is a known transcriptional regulator of IL-1β[193]. The PERK-eIF2α pathway reduces translation of the IκB promoting NF-κB activation and perhaps leading to IL-1β transcription[133, 134]. Another possible mechanism includes TXNIP. TXNIP has been shown to localize within the nucleus possibly by importin-α and TXNIP overexpression modulates β-cell

transcription possibly as a transcriptional repressor[194-196]. Thus TXNIP may influence the activities of IL-1β transcription factors or histone modifiers. These possible mechanisms should be explored.

We also explored the possibility that TXNIP mediates β-cell death under ER stress. Studies have demonstrated that glucose-mediated TXNIP expression has an apoptotic role in β-cells[159]. Overexpressing TXNIP in β-cell lines induces apoptosis. Isolated islets from TXNIP mutant mice are protected from glucose-induced cell death. TXNIP mutant or β-cell specific knockout mice crossed with diabetic mouse models demonstrated increased β-cell mass and reduced β-cell apoptosis[170]. Likewise, we have found that TXNIP knockdown protected β-cell lines from ER stress-mediated cell death. Furthermore, Feroz Papa and his colleagues revealed that crossing TXNIP knockout mice with the heterozygous Akita mice protected these mice from β-cell apoptosis and ER stress induced diabetes (unpublished). Clearly these results confirm that TXNIP has a role in ER stress mediated β-cell death. On the contrary, Anath Shalev's group demonstrated that isolated islets from β-cell specific TXNIP knockout mice were not protected from ER stress induced-apoptosis[161]. This discrepancy is most likely due to experimental differences in ER stress induction. Moreover, the UPR is composed of other apoptotic components that are likely involved in β-cell apoptosis. Therefore, knocking down or knocking out TXNIP will partially protect β-cells from ER stress.

Even though we show a partial contribution of IL-1β signaling, the mechanisms of TXNIP induced β-cell death under ER stress remain elusive. TXNIP directly binds to the active site of thioredoxin inhibiting its protein reducing and ROS scavenging

abilities[147-149]. Hence TXNIP may increase intracellular ROS levels triggering β-cell apoptosis. Thioredoxin also binds and inhibits a variety of apoptotic factors such as ASK-1[197]. It has been proposed that under oxidative stress TXNIP shuttles from the nucleus to the mitochondria to inhibit the mitochondria resident thioredoxin 2 thereby promoting ASK-1 activation and β-cell apoptosis[194]. This mechanism may be possible in our studies as ER stress has been shown to induce oxidative stress. ER stress also activates cytoplasmic ASK-1 through IRE1α-TRAF2[56]. Thus TXNIP may contribute to ASK-1 activation by this mechanism.

TXNIP also has a role in negatively regulating glucose stimulated insulin secretion (GSIS). Mutant or TXNIP knockout mice demonstrated enhanced GSIS[198, 199]. Silencing TXNIP in INS-1 and MIN6 cells demonstrated enhanced GSIS whereas overexpressiong TXNIP suppressed GSIS[157, 158]. A potential mechanism may involve TXNIP suppression of mitochondria ATP levels by upregulating mitochondria uncoupling protein 1 (UCP-1) transcription through recruitment of PPARγ co-activator-1α (PGC-1α) to the UCP-1 promoter[158]. In addition, TXNIP may bind and inhibit Mybbp1a, a negative regulator of PGC-1α thus promoting UCP-1 transcription. UCP-1 is known to suppress mitochondria ATP levels and GSIS. Hence TXNIP may also be a link between the UPR and impaired GSIS.

Accumulating evidence indicate that TXNIP has a role in the pathogenesis of type 2 diabetes. A recent study found that TXNIP polymorphisms were associated with type 2 diabetes and high blood pressure[200]. Carriers of one of these polymorphisms exhibited higher levels of TXNIP expression in smooth muscle cells. Lymphocytes from type 2

diabetic patients and patients with impaired glucose tolerance demonstrated higher TXNIP expression levels[201]. Parikh *et al*. found that TXNIP expression in skeletal muscle was higher in prediabetic and diabetic patients[202]. They further discovered that TXNIP is a negative regulator of blood glucose uptake by fat and muscle cells. Accordingly, TXNIP deficient mice exhibit increased insulin sensitivity[153, 199]. The mechanisms may involve TXNIP downregulation of insulin signaling components such as Akt phosphorylation and insulin receptor substrate 1 (IRS-1)[158]. Thus it would be important to study TXNIP regulation and function under ER stress in muscle and fat cells.

Furthermore, TXNIP expression is induced by glucose in fat and muscle cells like β-cells and in all of these cell types, insulin suppresses TXNIP expression[202, 203]. Therefore TXNIP behaves as a homeostatic switch controlling glucose uptake by peripheral tissues and insulin secretion by β-cells. A balance between glucose and insulin levels ensures that TXNIP expression is properly regulated. However, when β-cells can no longer compensate for insulin resistance, glucose and insulin levels are dysregulated favoring TXNIP overexpression exacerbating insulin resistance and β-cell failure leading to type 2 diabetes.

Collectively, these results along with ours establish TXNIP as a potential target in diabetes therapy. Diabetes ensues only when the β-cell fails. TXNIP clearly has a role in β-cell dysfunction and death during diabetes development. Understanding the mechanisms involved in TXNIP regulation under glucose and insulin conditions may uncover strategies to modulate its expression or activity. Moreover, exploring how

TXNIP elicits its harmful affects will also shed some light on therapeutic interventions Here we demonstrate that the UPR through the PERK-eIF2α and IRE1α pathways regulate TXNIP expression. The PERK-eIF2α pathway regulates TXNIP at the transcriptional level through ChREBP and ATF5. Additionally, we discovered that TXNIP has apoptotic and inflammatory roles under ER stress through the NLPR3 inflammasome production of IL-1β. Targeting TXNIP and components of these pathways are candidates for developing diabetes treatment.

CHAPTER III

PERSPECTIVES

Diabetes mellitus is a major health problem affecting 366 million people worldwide in 2011 and will rise to 552 million by 2030[204]. About 90-95% of diabetes cases are type 2 diabetes. Type 2 diabetes is a result of genetic and environmental factors that lead to insulin resistance and β-cell failure causing hyperglycemia. Hyperglycemia gives rise to several life threatening long term complications including heart attacks, strokes, diabetic retinopathy, kidney failure, and amputations. Therefore it is clear the importance of understanding the pathogenesis of diabetes to develop novel therapies.

Metabolic overload often due to obesity impairs insulin action in the skeletal muscle, adipose tissue, and liver[4, 205]. As a result glucose uptake is reduced in the muscle and adipose tissues. Adipose tissues also release excess levels of free fatty acids and other factors that promote insulin resistance in the muscle and liver. Reduced insulin sensitivity in the liver leads to increased glucose production. As a result, insulin resistance leads to higher blood glucose levels. Pancreatic β-cells compensate by increasing insulin secretion. However, for a subset of genetically predisposed individuals, β-cells fail to compensate due to dysfunction and loss. Consequently, type 2 diabetes develops with chronic high blood glucose levels along with increased circulating free fatty acids. These elevated levels of nutrients exacerbate β-cell failure by inducing oxidative stress, inflammation, and ER stress[8, 9, 141].

The ER is the major site for proinsulin folding and processing in the β-cell. During physiological changes in glucose levels, the increased proinsulin ER load overwhelms the ER folding capacity causing an accumulation of unfolded proteins[54]. As β-cells often experience ER stress, they have an elaborate ER and UPR to activate adaptive mechanisms that mitigate ER stress, restore ER homeostasis, and promote cell survival. However, upon conditions associated with type 2 diabetes, ER stress is severe and the UPR becomes hyperactivated causing β-cell dysfunction, oxidative stress, inflammation, and death. Thus the UPR switches from physiological to pathological outcomes[10, 45]. Understanding the mechanisms and components involved in this switch will shed light on β-cell failure and potential targets for drug development.

In this study, we began our focus on identifying the survival and death components of the UPR in the β-cell. During a DNA microarray analysis, we have selected TXNIP as an interesting candidate to study. Accumulating evidence indicates that TXNIP has a central role in maintaining glucose homeostasis. At physiological conditions, TXNIP expression is reciprocally regulated by insulin and glucose and functions as a break for insulin secretion in β-cells[157, 158]. However as insulin resistance ensues, higher glucose levels lead to pathological levels of TXNIP expression inhibiting GSIS and promoting β-cell apoptosis[159]. Hence we hypothesized that TXNIP may be involved in ER stress mediated β-cell apoptosis.

Interestingly during our research, we discovered that TXNIP has a role in β-cell inflammation and apoptosis under ER stress. We demonstrated that TXNIP is mainly expressed in β-cells of the pancreas and is induced by both pharmacological and

pathological ER stress inducers under the IRE1α and PERK-eIF2α arms of the UPR. At the transcriptional level, TXNIP is regulated by ATF5 and ChREBP transcription factors. As we explored possible functions of TXNIP, we have found that TXNIP has a role in ER stress mediated IL-1β production and autostimulation. Furthermore, we confirmed that TXNIP does indeed contribute to ER stress-induced β-cell apoptosis partially through IL-1β signaling.

TXNIP not only has a role in β-cell failure as we and others have demonstrated, but TXNIP also reduces glucose uptake in peripheral tissues[202] and increases hepatic glucose production[206]. Therefore TXNIP is an aggravator of type 2 diabetes. Notably, TXNIP is a potential molecular link between ER stress, oxidative stress, and inflammation (Figure 3.1a). Such a model implicates that as blood glucose levels increase due to insulin resistance, ER stress becomes severe in the β-cell and induces pathological factors such as TXNIP. TXNIP in turn can inhibit thioredoxin[147-149] promoting activation of apoptotic pathways and ROS production and/or activate the NLRP3 inflammasome leading to IL-1β secretion, autostimulation, and inflammation[142]. In addition, studies demonstrate that increased TXNIP expression also suppresses GSIS[157, 158], thus this phenomenon is probable under ER stress. As a result, the functional β-cell mass decreases which further increases blood glucose levels and TXNIP expression levels in β-cells as well as in peripheral tissues creating a downward spiral to overt type 2 diabetes (Figure 3.1b). Collectively, these studies select TXNIP as an attractive target for type 2 diabetes therapies.

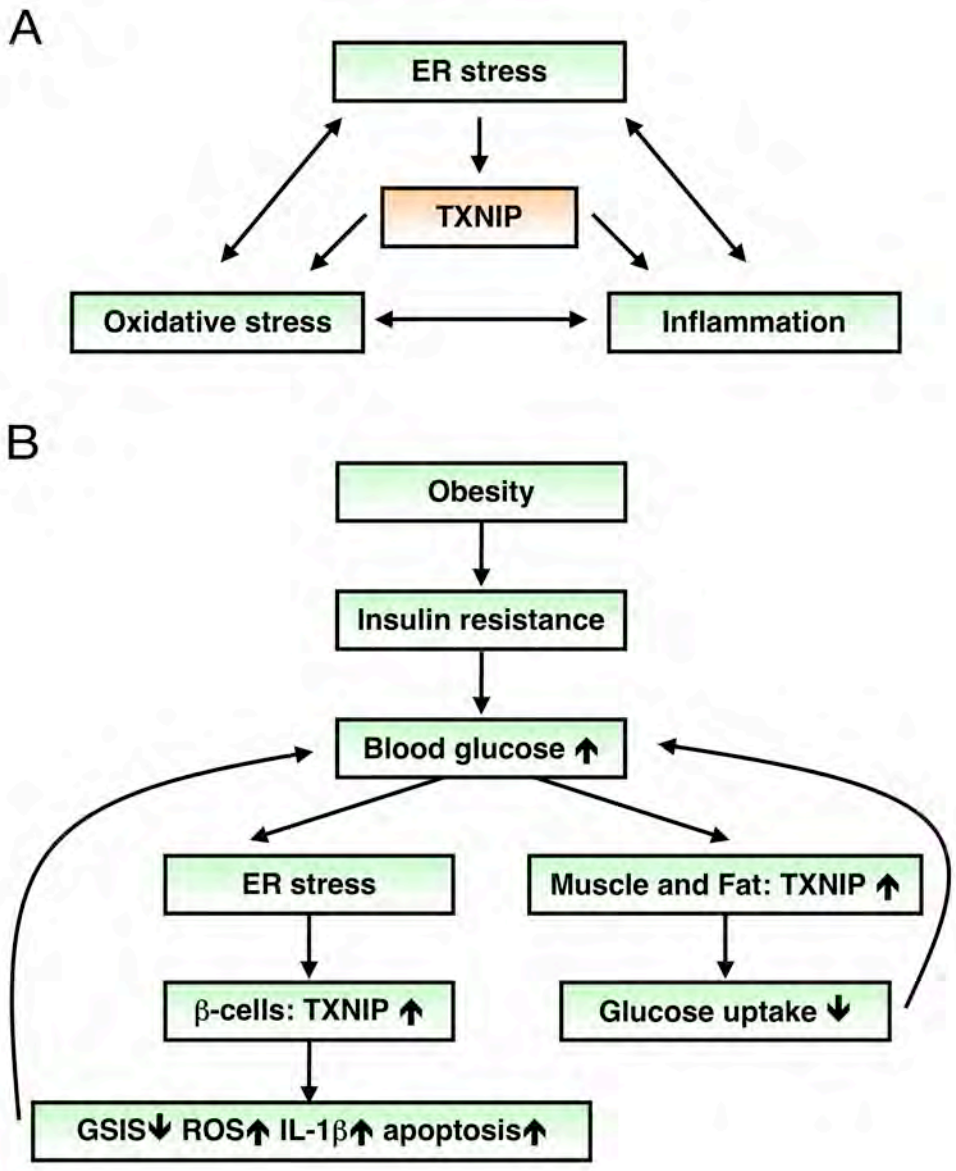


Figure 3.1 TXNIP expression contributes to type 2 diabetes. (A) TXNIP is a link between ER stress, oxidative stress, and inflammation in β-cells. These different pathological outputs can feed into each other causing a vicious cycle leading to β-cell failure. (B) During insulin resistance, as blood glucose levels increase causing ER stress in β-cells, TXNIP expression is induced leading to decreased GSIS and increased levels of ROS, IL-1β and apoptosis. High glucose also induces TXNIP expression in muscle and fat cells leading to impaired glucose uptake. Taken together, TXNIP expression worsens insulin resistance and β-cell failure leading to overt type 2 diabetes.

In particular, it is becoming more apparent that early therapeutic intervention alleviating β-cell function and promoting survival could potentially prevent the transition to diabetes[207]. Currently 79 million Americans have prediabetes[1] characterized by impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) mostly due to insulin resistance. As the functional β-cell mass declines, about 40-50% of IGT patients will progress to type 2 diabetes. Once type 2 diabetes develops, β-cells continue to deteriorate even with therapeutic interventions[208] and severe complications are likely to develop. Therefore early intervention for high-risk prediabetic individuals could prevent or delay the progression to type 2 diabetes. Even though diet and exercise reduces conversion to diabetes by 50-60%, it is difficult to establish and maintain[207]. A more effective strategy is with use of pharmacological therapies that especially focus on preserving functional β-cell mass.

As TXNIP is a mediator of β-cell failure, understanding the mechanisms of TXNIP expression, activity, and function will shed light on strategies to prevent diabetes. In this work we present significant novel findings that TXNIP is a component of the UPR, mediates IL-1β production and autostimulation, and has a role in β-cell death under ER stress. These findings among others have begun to reveal different components of ER stress, oxidative stress, and inflammation that could be therapeutic targets against β-cell dysfunction and death.

One strategy is to target ER chaperone capacity with chemical chaperones such as 4-phenylbutryic acid (PBA) and tauroursodeoxycholic acid (TUDCA). During ER stress, the ER folding capacity is overwhelmed by the ER protein load. Chemical chaperones

could potentially improve the ER folding capacity therefore alleviating ER stress. Use of these chemical chaperones demonstrated improvement of hyperglycemia and insulin resistance in ob/ob mice[209]. TUDCA demonstrated improvement of insulin secretion in isolated islets[210]. More recently, *in vivo* administration of PBA and TUDCA in 48 h high glucose treated rats reduced β-cell ER stress and restored β-cell function[211]. However, such compounds have yet to be shown effective in humans and lack specificity potentially affecting a wide range of cells. Furthermore, these chaperones are effective at high dosages, which may be toxic in humans.

A more specific drug may be BiP inducer X (BIX), which induces transcription of the ER resident chaperone BiP. Studies show that BIX is capable of protecting neurons from ER stress mediated-cell death[212, 213]. In the β-cell, BiP overexpression improved proinsulin levels and GSIS under high glucose conditions[214] and has a protective effect against free fatty acids[101]. Taken together, it would be interesting to study the effects of BIX and in general other means of inducing chaperones on β-cell failure *in vitro* and *in vivo*.

Another strategy is the use of antioxidants. Under high glucose and fatty acid conditions, excessive amounts of ROS are produced contributing to β-cell failure. Several mechanisms are responsible for ROS production including oxidative protein folding in the ER[119] and calcium leakage from the ER to the mitochondria increasing mitochondrial ROS production[124]. TXNIP binding to the antioxidant thioredoxin also results in ROS production[215]. ROS inhibits ER and β-cell function, and leads to β-cell apoptosis. In addition, β-cells lack antioxidants and are especially vulnerable to ROS[122].

Administration of antioxidants such as N-acetyl cysteine[216, 217] and probucol[218, 219] have shown improvement of diabetic symptoms *in vitro* and *in vivo*. Interestingly, probucol reduces oxidative stress in islets in part by increasing thioredoxin expression while decreasing TXNIP expression. However, evidence for antioxidants usefulness in improving β-cell failure in humans is lacking.

An additional strategy may involve maintaining an oxidized ER. Under healthy conditions, the ER houses an oxidized environment to promote disulfide bond formation during protein folding[119, 220]. Stimuli such as high glucose and hIAPP reduce the ER as a mechanism to cause ER stress and consequently apoptosis. Our lab has developed a method to monitor ER redox states in the β-cell line, INS-1 832/13. These cells express a modified redox-sensitive green fluorescent protein (GFP) named MERO-GFP (Mammalian Endoplasmic reticulum-localized RedOx-sensitive GFP)[221]. Upon ER reduction, a change of fluorescence of MERO-GFP as a result of disulfide bond disruption can be quantified. Using this redox monitoring system, we screened pharmacologically active compounds that can maintain an oxidizing ER under ER stress and discovered two compounds, apomorphine and pioglitazone (unpublished). We were able to confirm the ability of these drugs to maintain an oxidized ER under ER stress and also to protect cells from ER stress-induced apoptosis. Apomorphine is a dopamine agonist used to treat Parkinson's disease[222]. Interestingly, pioglitazone is a drug used to treat type 2 diabetes that improves insulin sensitivity by activating peroxisome proliferator-activated receptor γ (PPARγ) and also preserves β-cell function[223, 224]. A

study demonstrated a 72% reduction in conversion from IGT to type 2 diabetes using pioglitazone and therefore is a recommended drug for diabetes prevention[225].

These drugs discussed thus far are capable of relieving ER stress, but more direct targets of the UPR signaling network would be ideal for drug development. Targeting upstream components such as the UPR master regulators, IRE1α, PERK, and ATF6α and also eIF2α phosphorylation should be considered with caution. These components switch from physiological to pathological outputs. As β-cells require these upstream UPR components for adaptation and survival, inhibiting them would reduce β-cell defenses against ER stress. Likewise, activating these pathways may cause a switch to β-cell failure. Therefore techniques that focus on activating UPR homeostatic survival components and inhibiting components involved in dysfunction and death are more promising therapeutic strategies.

One proposed method is to inhibit the kinase activity of IRE1α. Under physiological ER stress conditions, IRE1α dimerizes, autophosphorylates, and splices XBP-1 mRNA to produce mature XBP-1 transcription factor. XBP-1 regulates homeostatic UPR genes. However under severe ER stress, IRE1α becomes hyperactivated triggering decay of ER associated mRNAs including preproinsulin and activating apoptotic pathways. During such conditions, it is hypothesized that IRE1α luminal domains undergo higher order oligerimization leading to increased phosphorylation, RNase activity, and promiscuous mRNA degradation[28]. Kinase activity is required for this harmful mRNA decay. Thus identifying kinase inhibitors that prevent IRE1α autophosphorylation and favor activation of IRE1α RNase domains that promote

XBP-1 mRNA splicing without destructive mRNA decay are promising tools to alleviate β-cell failure. Such compounds such as APY29 show promise in uncoupling the different RNase activities of IRE1α.

Another method is to inhibit the RNase activity of IRE1α altogether. In patients with prediabetes, glucose levels may be high enough to hyperactivate IRE1α leading to ER mRNA decay. Even though XBP-1 is still being processed, its homeostatic activity is negligible and in fact it has been reported that extensive XBP-1 splicing may trigger apoptosis. Therefore inhibiting endoribonucleolytic activity of IRE1α should promote β-cell function and survival. Feroz Papa and colleagues took this approach with the IRE1α endoribonuclease inhibitor STF083010[226]. Their group discovered that IRE1α RNase activity promotes TXNIP mRNA stability by degrading the miRNA, miR-17. Using STF083010 to prevent this pathway, TXNIP mRNA is degraded preventing IL-1β production (unpublished). Further studies on the effect of these kinase and RNase inhibitors on β-cell function and survival should be conducted *in vivo*.

Further downstream the UPR stress sensors, regulators of TXNIP transcription may be good targets. One approach in this regard is targeting ChREBP. Under basal glucose levels, sorcin binds to ChREBP restricting it to the cytoplasm[177]. Upon high glucose, calcium influx is sensed by sorcin triggering ChREBP release and subsequently translocation into the nucleus. Consequently, ChREBP upregulates pathological genes such as TXNIP and downregulates genes involved in β-cell function[143, 175]. Therefore finding drugs that inhibit ChREBP activity could potentially be used to prevent β-cell failure. Interestingly, a recent study demonstrated that the calcium channel blocker,

verapamil, reduces TXNIP expression by preventing ChREBP nuclear translocation perhaps through increased ChREBP phosphorylation and/or inhibiting calcineurin signaling[227]. The possibility of sorcin involved in ChREBP nuclear exclusion by verapamil should also be investigated. Regardless, verapamil was able to enhance β-cell function and survival improving diabetic symptoms in diabetic mice. Because verapamil is already an approved drug, it is an especially strong candidate to prevent β-cell failure in humans.

A new group of diabetic drugs, the GLP-1 analogues have been shown to rescue β-cell failure partly through reducing ER stress and TXNIP expression[228]. GLP-1 is an incretin released from the large intestines after a meal that stimulates insulin secretion and biosynthesis, reduces glucagon secretion, delays gastric emptying, and promotes satiety. One group of GLP-1 analogues are the GLP-1 receptor agonists including the approved drugs liraglutide and exenatide. Exenatide is the synthetic form of exendin-4. Exendin-4 has been shown to reduce expression of ER stress, oxidative stress, and inflammatory markers of β-cells *in vitro* and *in vivo*[229-231]. In addition exendin-4 also reduces TXNIP expression levels through cyclic AMP (cAMP) signaling leading to TXNIP proteasomal degradation[232, 233], which provides an explanation for the drug's β-cell protective effects. Because GLP-1 analogues promote β-cell function and survival, cause weight loss, lower blood pressure, have little side effects and are long lasting, they are great candidates to prevent type 2 diabetes[207].

As mentioned previously, pioglitazone was discovered for its ability to maintain an oxidized ER and prevent β-cell death under ER stress. Pioglitazone may also target

TXNIP expression through PPARγ. PPARγ, a member of the nuclear receptor superfamily, regulates several biological processes including adipogenesis and glucose metabolism. PPARγ has been shown to negatively regulate TXNIP transcription in proximal tubule kidney cells and in adipose tissues perhaps by directly binding to peroxisome proliferator response elements (PPREs) in the TXNIP promoter[234-236]. Under high glucose conditions, PPARγ expression is suppressed and TXNIP expression is induced. But treatment with pioglitazone or the similar PPARγ agonist rosiglitazone reduced TXNIP expression in kidney and fat cells. Similarly, we have found that pioglitazone was able to suppress ER stress-induced TXNIP expression in β-cells (data not shown). In addition, PPARγ has anti-inflammatory roles including decreasing the expression of cytokines perhaps through TXNIP[236, 237]. Collectively, these data further explain the beneficial effects of pioglitazone in preventing diabetes.

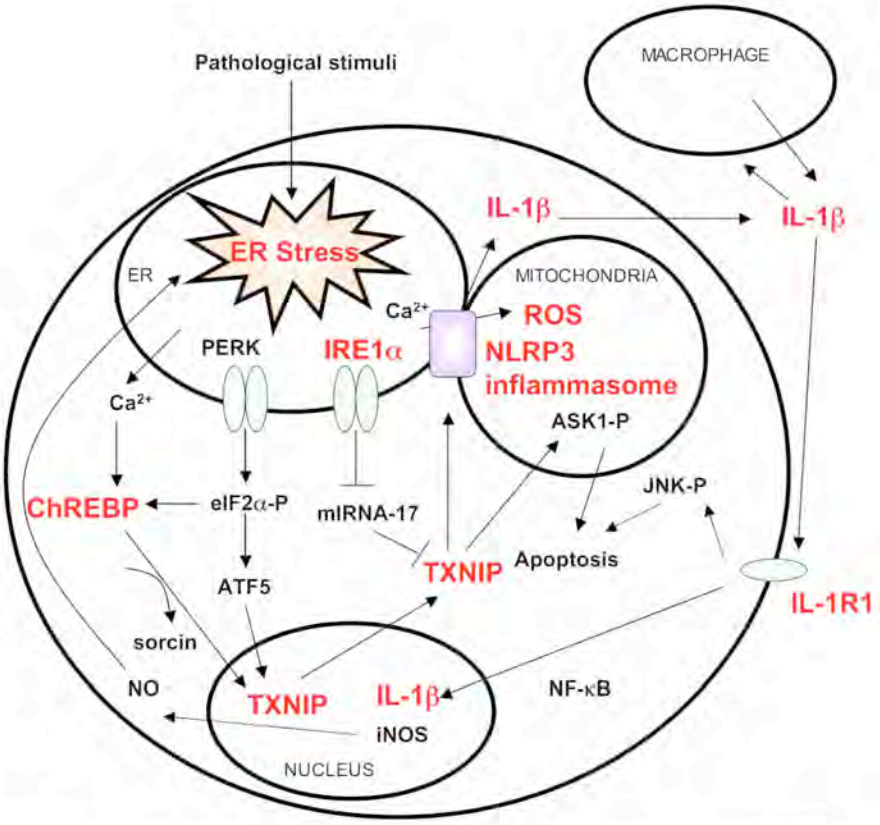
TXNIP also activates the NLRP3 inflammasome leading to the production of the proinflammatory cytokine IL-1β[142]. A number of studies propose that IL-1β signaling and inflammation contribute to the pathogenesis of type 2 diabetes[140, 141]. Several markers of inflammation are found in diabetic patients such as increased IL-1β serum levels and increased islet-localized macrophages. Inflammation in type 2 diabetic patients are most likely due to decreased expression of the endogenous IL-1β antagonist, IL-1RA. Clinical trials with anakinra, the recombinant form of IL-1RA, and IL-1β specific antibodies were able to improve glycemia and β-cell function especially for those individuals that carry IL-1RA variants[150]. Because these therapeutic strategies are safe and long lasting, they may be ideal candidates to treat prediabetic patients.

Modulating NLRP3 inflammasome activity is also an approach to examine. A study demonstrated that the type 2 diabetic drug, glibenclamide, was able to reduce TXNIP expression levels and IL-1β production in mouse islets[142]. Glibenclamide also known as glyburide is a potassium ATP channel inhibitor that leads to calcium influx and insulin release. As the NLRP3 inflammasome requires potassium efflux for its activation, glibenclamide was able to inhibit its activation. Thus glibenclamide and development of more specific NLRP3 inhibitors may be beneficial in preventing diabetes progression.

Collectively, potential therapies are beginning to emerge that target ER stress, oxidative stress, and inflammation (Figure 3.2). As these different cellular processes are connected in the context of type 2 diabetes, targeting components that link these pathways will be especially effective in preventing β-cell failure. Approved drugs that already demonstrate mechanisms targeting these pathways such as the calcium channel blocker verapamil, the GLP-1 analogue exanatide, the PPARγ agonist pioglitazone, the IL-1β antagonist anakinra, and the ATP potassium channel blocker glibenclamide could potentially be administered to prediabetic patients to prevent type 2 diabetes. As the other compounds discussed must be tested *in vivo* and in humans, research on more selective drugs targeting signaling components of ER stress, oxidative stress and inflammation will provide improved therapies for diabetes.

Moreover it is essential as we do here to decipher the signaling network involved in ER stress, oxidative stress, and inflammation to reveal novel drug targets. TXNIP seems to link these different processes and therefore is an attractive molecule to

A



B

Target	Drug Type/Function	Example
ER folding capacity	Chemical chaperones	PBA, TUDCA
BiP	Induce BiP expression	BIX
Oxidative stress	Antioxidants	Probucol
ER redox state	Maintain oxidized ER	Apomorphine, pioglitazone
IRE1α kinase activity	Kinase inhibitors	APY29
IRE1α RNase activity	RNase inhibitors	STF083010
ChREBP	Calcium channel blockers	Verapamil
TXNIP	GLP-1 analogues	Exendin-4
TXNIP	PPARγ agonists	Pioglitazone
IL-1β	IL-1RA	Anakinra
NLRP3 inflammasome	Potassium ATP channel inhibitors	Glibenclamide

Figure 3.2 Targeting components of TXNIP signaling network for diabetes intervention. (A) Progress is being made on deciphering the signaling pathways involved in TXNIP induction and function in β-cells. The components involved are potential therapeutic targets to prevent or delay the onset of diabetes. (B) Several drugs are being recognized for their ability to target different components of TXNIP signaling network and demonstrate promise in preventing type 2 diabetes.

investigate. We have found that TXNIP mediates ER stressed induced β-cell death and inflammation. But several unanswered questions remain (Figure 3.3). Further studies should elucidate how TXNIP mediates β-cell death, does TXNIP bind and inhibit thioredoxin, does this result to activation of apoptotic pathways and/or production of ROS, and where do TXNIP and the NLRP3 inflammasome localize and how does this localization change under ER stress in β-cells. These future studies in addition to others will pave the road to delay or even prevent type 2 diabetes.

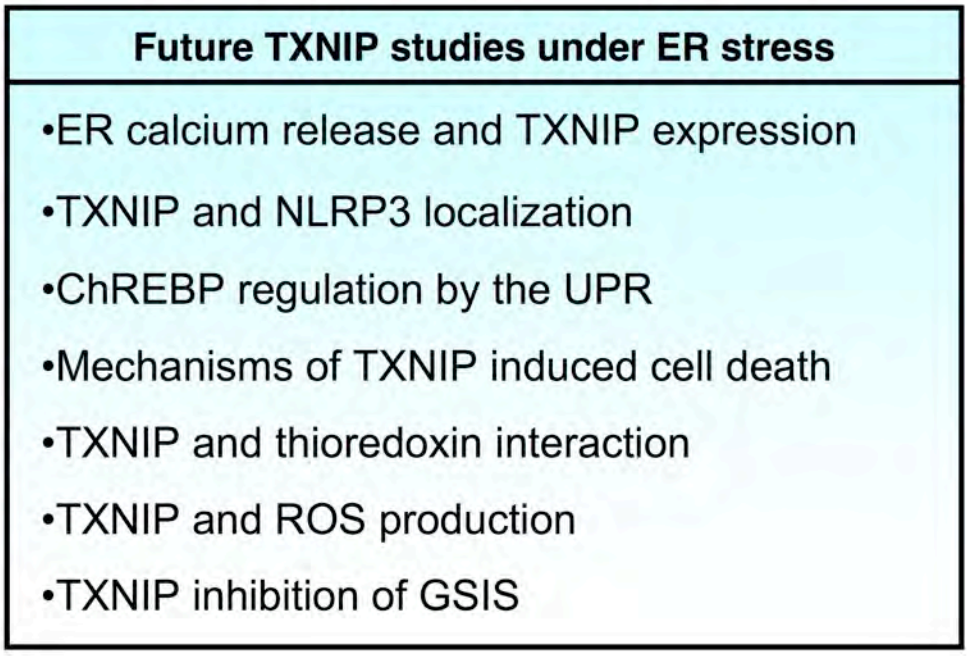


Figure 3.3 Future TXNIP studies under ER stress. Regardless of the progress being made on understanding the mechanisms involved in TXNIP induction and function several remaining questions require investigation.

REFERENCES

1. Centers for Disease Control and Prevention National Diabetes Fact Sheet: national estimates and general information on diabetes and prediabetes in the United States 2011. *U.S. Department of Heath and Human Services* (2011).
2. Association, A.D. Economic Costs of Diabetes in the U.S. in 2007 *Diabetes Care* **31**, 596-615 (2008).
3. Bonner-Weir, S. Islet growth and development in the adult. *J Mol Endocrinol* **24**, 297-302 (2000).
4. Muoio, D.M. & Newgard, C.B. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat Rev Mol Cell Biol* **9**, 193-205 (2008).
5. Itoh, N. & Okamoto, H. Translational control of proinsulin synthesis by glucose. *Nature* **283**, 100-102 (1980).
6. Welsh, M., Scherberg, N., Gilmore, R. & Steiner, D.F. Translational control of insulin biosynthesis. Evidence for regulation of elongation, initiation and signal-recognition-particle-mediated translational arrest by glucose. *Biochem J* **235**, 459-467 (1986).
7. Permutt, A., Chirgwin, J., Giddings, S., Kakita, K. & Rotwein, P. Insulin biosynthesis and diabetes mellitus. *Clin Biochem* **14**, 230-236 (1981).
8. Eizirik, D.L., Cardozo, A.K. & Cnop, M. The role for endoplasmic reticulum stress in diabetes mellitus. *Endocr Rev* **29**, 42-61 (2008).
9. Scheuner, D. & Kaufman, R.J. The unfolded protein response: a pathway that links insulin demand with beta-cell failure and diabetes. *Endocr Rev* **29**, 317-333 (2008).
10. Oslowski, C.M. & Urano, F. The binary switch that controls the life and death decisions of ER stressed beta cells. *Curr Opin Cell Biol* **23**, 207-215 (2011).
11. Dodson, G. & Steiner, D. The role of assembly in insulin's biosynthesis. *Curr Opin Struct Biol* **8**, 189-194 (1998).
12. Rhodes, C.J. Processing of the insulin molecule *Diabetes Mellitus* 27-50 (2004).
13. Ron, D. & Walter, P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* **8**, 519-529 (2007).
14. Kaufman, R.J., Scheuner, D., Schroder, M., Shen, X., Lee, K., Liu, C.Y. & Arnold, S.M. The unfolded protein response in nutrient sensing and differentiation. *Nat Rev Mol Cell Biol* **3**, 411-421 (2002).
15. Bertolotti, A., Zhang, Y., Hendershot, L.M., Harding, H.P. & Ron, D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* **2**, 326-332 (2000).
16. Wang, X.Z., Harding, H.P., Zhang, Y., Jolicoeur, E.M., Kuroda, M. & Ron, D. Cloning of mammalian Ire1 reveals diversity in the ER stress responses. *Embo J* **17**, 5708-5717 (1998).
17. Tirasophon, W., Welihinda, A.A. & Kaufman, R.J. A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional

protein kinase/endoribonuclease (Ire1p) in mammalian cells. *Genes Dev* **12**, 1812-1824 (1998).
18. Credle, J.J., Finer-Moore, J.S., Papa, F.R., Stroud, R.M. & Walter, P. On the mechanism of sensing unfolded protein in the endoplasmic reticulum. *Proc Natl Acad Sci U S A* **102**, 18773-18784 (2005).
19. Kimata, Y., Oikawa, D., Shimizu, Y., Ishiwata-Kimata, Y. & Kohno, K. A role for BiP as an adjustor for the endoplasmic reticulum stress-sensing protein Ire1. *J Cell Biol* **167**, 445-456 (2004).
20. Calfon, M., Zeng, H., Urano, F., Till, J.H., Hubbard, S.R., Harding, H.P., Clark, S.G. & Ron, D. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415**, 92-96 (2002).
21. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T. & Mori, K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107**, 881-891 (2001).
22. Shen, X., Ellis, R.E., Lee, K., Liu, C.Y., Yang, K., Solomon, A., Yoshida, H., Morimoto, R., Kurnit, D.M., Mori, K. & Kaufman, R.J. Complementary signaling pathways regulate the unfolded protein response and are required for C. elegans development. *Cell* **107**, 893-903 (2001).
23. Lee, A.H., Iwakoshi, N.N. & Glimcher, L.H. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol Cell Biol* **23**, 7448-7459 (2003).
24. Lee, K., Tirasophon, W., Shen, X., Michalak, M., Prywes, R., Okada, T., Yoshida, H., Mori, K. & Kaufman, R.J. IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev* **16**, 452-466 (2002).
25. Lee, T.J., Jung, E.M., Lee, J.T., Kim, S., Park, J.W., Choi, K.S. & Kwon, T.K. Mithramycin A sensitizes cancer cells to TRAIL-mediated apoptosis by down-regulation of XIAP gene promoter through Sp1 sites. *Mol Cancer Ther* **5**, 2737-2746 (2006).
26. Oda, Y., Okada, T., Yoshida, H., Kaufman, R.J., Nagata, K. & Mori, K. Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. *J Cell Biol* **172**, 383-393 (2006).
27. Yoshida, H., Matsui, T., Hosokawa, N., Kaufman, R.J., Nagata, K. & Mori, K. A time-dependent phase shift in the mammalian unfolded protein response. *Dev Cell* **4**, 265-271 (2003).
28. Han, D., Lerner, A.G., Vande Walle, L., Upton, J.P., Xu, W., Hagen, A., Backes, B.J., Oakes, S.A. & Papa, F.R. IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. *Cell* **138**, 562-575 (2009).
29. Hollien, J. & Weissman, J.S. Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science* **313**, 104-107 (2006).
30. Pirot, P., Naamane, N., Libert, F., Magnusson, N.E., Orntoft, T.F., Cardozo, A.K. & Eizirik, D.L. Global profiling of genes modified by endoplasmic reticulum

stress in pancreatic beta cells reveals the early degradation of insulin mRNAs. *Diabetologia* **50**, 1006-1014 (2007).
31. Kimball, S.R. Eukaryotic initiation factor eIF2. *Int J Biochem Cell Biol* **31**, 25-29 (1999).
32. Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M. & Ron, D. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* **6**, 1099-1108 (2000).
33. Harding, H.P., Zhang, Y. & Ron, D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* **397**, 271-274 (1999).
34. Fernandez, J., Bode, B., Koromilas, A., Diehl, J.A., Krukovets, I., Snider, M.D. & Hatzoglou, M. Translation mediated by the internal ribosome entry site of the cat-1 mRNA is regulated by glucose availability in a PERK kinase-dependent manner. *J Biol Chem* **277**, 11780-11787 (2002).
35. Lu, P.D., Harding, H.P. & Ron, D. Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. *J Cell Biol* **167**, 27-33 (2004).
36. Vattem, K.M. & Wek, R.C. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc Natl Acad Sci U S A* **101**, 11269-11274 (2004).
37. Zhou, D., Palam, L.R., Jiang, L., Narasimhan, J., Staschke, K.A. & Wek, R.C. Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. *J Biol Chem* **283**, 7064-7073 (2008).
38. Harding, H.P., Zhang, Y., Zeng, H., Novoa, I., Lu, P.D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D.F., Bell, J.C., Hettmann, T., Leiden, J.M. & Ron, D. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* **11**, 619-633 (2003).
39. Wu, J., Rutkowski, D.T., Dubois, M., Swathirajan, J., Saunders, T., Wang, J., Song, B., Yau, G.D. & Kaufman, R.J. ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress. *Dev Cell* **13**, 351-364 (2007).
40. Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., Harada, A. & Mori, K. Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. *Dev Cell* **13**, 365-376 (2007).
41. Haze, K., Yoshida, H., Yanagi, H., Yura, T. & Mori, K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell* **10**, 3787-3799 (1999).
42. Nadanaka, S., Okada, T., Yoshida, H. & Mori, K. Role of disulfide bridges formed in the luminal domain of ATF6 in sensing endoplasmic reticulum stress. *Mol Cell Biol* **27**, 1027-1043 (2007).
43. Shen, J., Chen, X., Hendershot, L. & Prywes, R. ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Dev Cell* **3**, 99-111 (2002).

44. Bommiasamy, H., Back, S.H., Fagone, P., Lee, K., Meshinchi, S., Vink, E., Sriburi, R., Frank, M., Jackowski, S., Kaufman, R.J. & Brewer, J.W. ATF6alpha induces XBP1-independent expansion of the endoplasmic reticulum. *J Cell Sci* **122**, 1626-1636 (2009).
45. Oslowski, C.M. & Urano, F. A switch from life to death in endoplasmic reticulum stressed beta-cells. *Diabetes Obes Metab* **12 Suppl 2**, 58-65 (2010).
46. Zhang, P., McGrath, B., Li, S., Frank, A., Zambito, F., Reinert, J., Gannon, M., Ma, K., McNaughton, K. & Cavener, D.R. The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. *Mol Cell Biol* **22**, 3864-3874 (2002).
47. Zhang, W., Feng, D., Li, Y., Iida, K., McGrath, B. & Cavener, D.R. PERK EIF2AK3 control of pancreatic beta cell differentiation and proliferation is required for postnatal glucose homeostasis. *Cell Metab* **4**, 491-497 (2006).
48. Ishigaki, S., Fonseca, S.G., Oslowski, C.M., Jurczyk, A., Shearstone, J.R., Zhu, L.J., Permutt, M.A., Greiner, D.L., Bortell, R. & Urano, F. AATF mediates an antiapoptotic effect of the unfolded protein response through transcriptional regulation of AKT1. *Cell Death Differ* **17**, 774-786 (2010).
49. Delepine, M., Nicolino, M., Barrett, T., Golamaully, M., Lathrop, G.M. & Julier, C. EIF2AK3, encoding translation initiation factor 2-alpha kinase 3, is mutated in patients with Wolcott-Rallison syndrome. *Nat Genet* **25**, 406-409 (2000).
50. Senee, V., Vattem, K.M., Delepine, M., Rainbow, L.A., Haton, C., Lecoq, A., Shaw, N.J., Robert, J.J., Rooman, R., Diatloff-Zito, C., Michaud, J.L., Bin-Abbas, B., Taha, D., Zabel, B., Franceschini, P., Topaloglu, A.K., Lathrop, G.M., Barrett, T.G., Nicolino, M., Wek, R.C. & Julier, C. Wolcott-Rallison Syndrome: clinical, genetic, and functional study of EIF2AK3 mutations and suggestion of genetic heterogeneity. *Diabetes* **53**, 1876-1883 (2004).
51. Harding, H.P., Zeng, H., Zhang, Y., Jungries, R., Chung, P., Plesken, H., Sabatini, D.D. & Ron, D. Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. *Mol Cell* **7**, 1153-1163 (2001).
52. Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S. & Kaufman, R.J. Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell* **7**, 1165-1176 (2001).
53. Scheuner, D., Vander Mierde, D., Song, B., Flamez, D., Creemers, J.W., Tsukamoto, K., Ribick, M., Schuit, F.C. & Kaufman, R.J. Control of mRNA translation preserves endoplasmic reticulum function in beta cells and maintains glucose homeostasis. *Nat Med* **11**, 757-764 (2005).
54. Lipson, K.L., Fonseca, S.G., Ishigaki, S., Nguyen, L.X., Foss, E., Bortell, R., Rossini, A.A. & Urano, F. Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1. *Cell Metab* **4**, 245-254 (2006).

55. Tabas, I. & Ron, D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol* **13**, 184-190 (2010).
56. Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H.P. & Ron, D. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287**, 664-666 (2000).
57. Weston, C.R. & Davis, R.J. The JNK signal transduction pathway. *Curr Opin Cell Biol* **19**, 142-149 (2007).
58. Hetz, C., Bernasconi, P., Fisher, J., Lee, A.H., Bassik, M.C., Antonsson, B., Brandt, G.S., Iwakoshi, N.N., Schinzel, A., Glimcher, L.H. & Korsmeyer, S.J. Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha. *Science* **312**, 572-576 (2006).
59. Allagnat, F., Christulia, F., Ortis, F., Pirot, P., Lortz, S., Lenzen, S., Eizirik, D.L. & Cardozo, A.K. Sustained production of spliced X-box binding protein 1 (XBP1) induces pancreatic beta cell dysfunction and apoptosis. *Diabetologia* **53**, 1120-1130 (2010).
60. Jiang, H.Y., Wek, S.A., McGrath, B.C., Lu, D., Hai, T., Harding, H.P., Wang, X., Ron, D., Cavener, D.R. & Wek, R.C. Activating transcription factor 3 is integral to the eukaryotic initiation factor 2 kinase stress response. *Mol Cell Biol* **24**, 1365-1377 (2004).
61. Jang, M.K., Park, H.J. & Jung, M.H. ATF3 represses PDX-1 expression in pancreatic beta-cells. *Biochem Biophys Res Commun* **412**, 385-390 (2011).
62. Kim, W.H., Jang, M.K., Kim, C.H., Shin, H.K. & Jung, M.H. ATF3 inhibits PDX-1-stimulated transactivation. *Biochem Biophys Res Commun* **414**, 681-687 (2011).
63. Ma, Y., Brewer, J.W., Diehl, J.A. & Hendershot, L.M. Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. *J Mol Biol* **318**, 1351-1365 (2002).
64. Pirot, P., Ortis, F., Cnop, M., Ma, Y., Hendershot, L.M., Eizirik, D.L. & Cardozo, A.K. Transcriptional regulation of the endoplasmic reticulum stress gene chop in pancreatic insulin-producing cells. *Diabetes* **56**, 1069-1077 (2007).
65. Gotoh, T., Terada, K., Oyadomari, S. & Mori, M. hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria. *Cell Death Differ* **11**, 390-402 (2004).
66. McCullough, K.D., Martindale, J.L., Klotz, L.O., Aw, T.Y. & Holbrook, N.J. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol Cell Biol* **21**, 1249-1259 (2001).
67. Puthalakath, H., O'Reilly, L.A., Gunn, P., Lee, L., Kelly, P.N., Huntington, N.D., Hughes, P.D., Michalak, E.M., McKimm-Breschkin, J., Motoyama, N., Gotoh, T., Akira, S., Bouillet, P. & Strasser, A. ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* **129**, 1337-1349 (2007).
68. Ciccaglione, A.R., Marcantonio, C., Tritarelli, E., Equestre, M., Vendittelli, F., Costantino, A., Geraci, A. & Rapicetta, M. Activation of the ER stress gene gadd153 by hepatitis C virus sensitizes cells to oxidant injury. *Virus Res* **126**, 128-138 (2007).

69. Li, G., Mongillo, M., Chin, K.T., Harding, H., Ron, D., Marks, A.R. & Tabas, I. Role of ERO1-alpha-mediated stimulation of inositol 1,4,5-triphosphate receptor activity in endoplasmic reticulum stress-induced apoptosis. *J Cell Biol* **186**, 783-792 (2009).
70. Seimon, T.A., Obstfeld, A., Moore, K.J., Golenbock, D.T. & Tabas, I. Combinatorial pattern recognition receptor signaling alters the balance of life and death in macrophages. *Proc Natl Acad Sci U S A* **103**, 19794-19799 (2006).
71. Oyadomari, S., Koizumi, A., Takeda, K., Gotoh, T., Akira, S., Araki, E. & Mori, M. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest* **109**, 525-532 (2002).
72. Song, B., Scheuner, D., Ron, D., Pennathur, S. & Kaufman, R.J. Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. *J Clin Invest* **118**, 3378-3389 (2008).
73. Teodoro, T., Odisho, T., Sidorova, E. & Volchuk, A. Pancreatic beta-cells depend on basal expression of active ATF6alpha-p50 for cell survival even under non-stress conditions *Am J Physiol Cell Physiol* **302**, 992-1003 (2012).
74. Seo, H.Y., Kim, Y.D., Lee, K.M., Min, A.K., Kim, M.K., Kim, H.S., Won, K.C., Park, J.Y., Lee, K.U., Choi, H.S., Park, K.G. & Lee, I.K. Endoplasmic reticulum stress-induced activation of activating transcription factor 6 decreases insulin gene expression via up-regulation of orphan nuclear receptor small heterodimer partner. *Endocrinology* **149**, 3832-3841 (2008).
75. Fonseca, S.G., Ishigaki, S., Oslowski, C.M., Lu, S., Lipson, K.L., Ghosh, R., Hayashi, E., Ishihara, H., Oka, Y., Permutt, M.A. & Urano, F. Wolfram syndrome 1 gene negatively regulates ER stress signaling in rodent and human cells. *J Clin Invest* **120**, 744-755 (2010).
76. Meex, S.J., van Greevenbroek, M.M., Ayoubi, T.A., Vlietinck, R., van Vliet-Ostaptchouk, J.V., Hofker, M.H., Vermeulen, V.M., Schalkwijk, C.G., Feskens, E.J., Boer, J.M., Stehouwer, C.D., van der Kallen, C.J. & de Bruin, T.W. Activating transcription factor 6 polymorphisms and haplotypes are associated with impaired glucose homeostasis and type 2 diabetes in Dutch Caucasians. *J Clin Endocrinol Metab* **92**, 2720-2725 (2007).
77. Thameem, F., Farook, V.S., Bogardus, C. & Prochazka, M. Association of amino acid variants in the activating transcription factor 6 gene (ATF6) on 1q21-q23 with type 2 diabetes in Pima Indians. *Diabetes* **55**, 839-842 (2006).
78. Usui, M., Yamaguchi, S., Tanji, Y., Tominaga, R., Ishigaki, Y., Fukumoto, M., Katagiri, H., Mori, K., Oka, Y. & Ishihara, H. Atf6alpha-null mice are glucose intolerant due to pancreatic beta-cell failure on a high-fat diet but partially resistant to diet-induced insulin resistance. *Metabolism* (2012) In press.
79. Rutkowski, D.T., Arnold, S.M., Miller, C.N., Wu, J., Li, J., Gunnison, K.M., Mori, K., Sadighi Akha, A.A., Raden, D. & Kaufman, R.J. Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. *PLoS Biol* **4**, e374 (2006).
80. Brush, M.H., Weiser, D.C. & Shenolikar, S. Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the

endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. *Mol Cell Biol* **23**, 1292-1303 (2003).

81. Connor, J.H., Weiser, D.C., Li, S., Hallenbeck, J.M. & Shenolikar, S. Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1. *Mol Cell Biol* **21**, 6841-6850 (2001).
82. Kojima, E., Takeuchi, A., Haneda, M., Yagi, A., Hasegawa, T., Yamaki, K., Takeda, K., Akira, S., Shimokata, K. & Isobe, K. The function of GADD34 is a recovery from a shutoff of protein synthesis induced by ER stress: elucidation by GADD34-deficient mice. *Faseb J* **17**, 1573-1575 (2003).
83. Novoa, I., Zeng, H., Harding, H.P. & Ron, D. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. *J Cell Biol* **153**, 1011-1022 (2001).
84. Qiu, Y., Mao, T., Zhang, Y., Shao, M., You, J., Ding, Q., Chen, Y., Wu, D., Xie, D., Lin, X., Gao, X., Kaufman, R.J., Li, W. & Liu, Y. A crucial role for RACK1 in the regulation of glucose-stimulated IRE1alpha activation in pancreatic beta cells. *Sci Signal* **3**, ra7 (2010).
85. van Huizen, R., Martindale, J.L., Gorospe, M. & Holbrook, N.J. P58IPK, a novel endoplasmic reticulum stress-inducible protein and potential negative regulator of eIF2alpha signaling. *J Biol Chem* **278**, 15558-15564 (2003).
86. Yan, W., Frank, C.L., Korth, M.J., Sopher, B.L., Novoa, I., Ron, D. & Katze, M.G. Control of PERK eIF2alpha kinase activity by the endoplasmic reticulum stress-induced molecular chaperone P58IPK. *Proc Natl Acad Sci U S A* **99**, 15920-15925 (2002).
87. Ladiges, W.C., Knoblaugh, S.E., Morton, J.F., Korth, M.J., Sopher, B.L., Baskin, C.R., MacAuley, A., Goodman, A.G., LeBoeuf, R.C. & Katze, M.G. Pancreatic beta-cell failure and diabetes in mice with a deletion mutation of the endoplasmic reticulum molecular chaperone gene P58IPK. *Diabetes* **54**, 1074-1081 (2005).
88. Petrova, K., Oyadomari, S., Hendershot, L.M. & Ron, D. Regulated association of misfolded endoplasmic reticulum lumenal proteins with P58/DNAJc3. *Embo J* **27**, 2862-2872 (2008).
89. Rutkowski, D.T., Kang, S.W., Goodman, A.G., Garrison, J.L., Taunton, J., Katze, M.G., Kaufman, R.J. & Hegde, R.S. The role of p58IPK in protecting the stressed endoplasmic reticulum. *Mol Biol Cell* **18**, 3681-3691 (2007).
90. Oyadomari, S., Yun, C., Fisher, E.A., Kreglinger, N., Kreibich, G., Oyadomari, M., Harding, H.P., Goodman, A.G., Harant, H., Garrison, J.L., Taunton, J., Katze, M.G. & Ron, D. Cotranslocational degradation protects the stressed endoplasmic reticulum from protein overload. *Cell* **126**, 727-739 (2006).
91. Inoue, H., Tanizawa, Y., Wasson, J., Behn, P., Kalidas, K., Bernal-Mizrachi, E., Mueckler, M., Marshall, H., Donis-Keller, H., Crock, P., Rogers, D., Mikuni, M., Kumashiro, H., Higashi, K., Sobue, G., Oka, Y. & Permutt, M.A. A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome). *Nat Genet* **20**, 143-148 (1998).

92. Karasik, A., O'Hara, C., Srikanta, S., Swift, M., Soeldner, J.S., Kahn, C.R. & Herskowitz, R.D. Genetically programmed selective islet beta-cell loss in diabetic subjects with Wolfram's syndrome. *Diabetes Care* **12**, 135-138 (1989).
93. Ishihara, H., Takeda, S., Tamura, A., Takahashi, R., Yamaguchi, S., Takei, D., Yamada, T., Inoue, H., Soga, H., Katagiri, H., Tanizawa, Y. & Oka, Y. Disruption of the WFS1 gene in mice causes progressive beta-cell loss and impaired stimulus-secretion coupling in insulin secretion. *Hum Mol Genet* **13**, 1159-1170 (2004).
94. Riggs, A.C., Bernal-Mizrachi, E., Ohsugi, M., Wasson, J., Fatrai, S., Welling, C., Murray, J., Schmidt, R.E., Herrera, P.L. & Permutt, M.A. Mice conditionally lacking the Wolfram gene in pancreatic islet beta cells exhibit diabetes as a result of enhanced endoplasmic reticulum stress and apoptosis. *Diabetologia* **48**, 2313-2321 (2005).
95. Cnop, M., Ladriere, L., Igoillo-Esteve, M., Moura, R.F. & Cunha, D.A. Causes and cures for endoplasmic reticulum stress in lipotoxic beta-cell dysfunction. *Diabetes Obes Metab* **12 Suppl 2**, 76-82 (2010).
96. Cnop, M., Igoillo-Esteve, M., Cunha, D.A., Ladriere, L. & Eizirik, D.L. An update on lipotoxic endoplasmic reticulum stress in pancreatic beta-cells. *Biochem Soc Trans* **36**, 909-915 (2008).
97. Cunha, D.A., Hekerman, P., Ladriere, L., Bazarra-Castro, A., Ortis, F., Wakeham, M.C., Moore, F., Rasschaert, J., Cardozo, A.K., Bellomo, E., Overbergh, L., Mathieu, C., Lupi, R., Hai, T., Herchuelz, A., Marchetti, P., Rutter, G.A., Eizirik, D.L. & Cnop, M. Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. *J Cell Sci* **121**, 2308-2318 (2008).
98. Gwiazda, K.S., Yang, T.L., Lin, Y. & Johnson, J.D. Effects of palmitate on ER and cytosolic Ca2+ homeostasis in beta-cells. *Am J Physiol Endocrinol Metab* **296**, E690-701 (2009).
99. Preston, A.M., Gurisik, E., Bartley, C., Laybutt, D.R. & Biden, T.J. Reduced endoplasmic reticulum (ER)-to-Golgi protein trafficking contributes to ER stress in lipotoxic mouse beta cells by promoting protein overload. *Diabetologia* **52**, 2369-2373 (2009).
100. Jeffrey, K.D., Alejandro, E.U., Luciani, D.S., Kalynyak, T.B., Hu, X., Li, H., Lin, Y., Townsend, R.R., Polonsky, K.S. & Johnson, J.D. Carboxypeptidase E mediates palmitate-induced beta-cell ER stress and apoptosis. *Proc Natl Acad Sci U S A* **105**, 8452-8457 (2008).
101. Cunha, D.A., Ladriere, L., Ortis, F., Igoillo-Esteve, M., Gurzov, E.N., Lupi, R., Marchetti, P., Eizirik, D.L. & Cnop, M. Glucagon-like peptide-1 agonists protect pancreatic beta-cells from lipotoxic endoplasmic reticulum stress through upregulation of BiP and JunB. *Diabetes* **58**, 2851-2862 (2009).
102. Eizirik, D.L., Colli, M.L. & Ortis, F. The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. *Nat Rev Endocrinol* **5**, 219-226 (2009).
103. Rabinovitch, A. & Suarez-Pinzon, W.L. Role of cytokines in the pathogenesis of autoimmune diabetes mellitus. *Rev Endocr Metab Disord* **4**, 291-299 (2003).

104. Thomas, H.E., McKenzie, M.D., Angstetra, E., Campbell, P.D. & Kay, T.W. Beta cell apoptosis in diabetes. *Apoptosis* **14**, 1389-1404 (2009).
105. Cardozo, A.K., Ortis, F., Storling, J., Feng, Y.M., Rasschaert, J., Tonnesen, M., Van Eylen, F., Mandrup-Poulsen, T., Herchuelz, A. & Eizirik, D.L. Cytokines downregulate the sarcoendoplasmic reticulum pump Ca2+ ATPase 2b and deplete endoplasmic reticulum Ca2+, leading to induction of endoplasmic reticulum stress in pancreatic beta-cells. *Diabetes* **54**, 452-461 (2005).
106. Chambers, K.T., Unverferth, J.A., Weber, S.M., Wek, R.C., Urano, F. & Corbett, J.A. The role of nitric oxide and the unfolded protein response in cytokine-induced beta-cell death. *Diabetes* **57**, 124-132 (2008).
107. Kharroubi, I., Ladriere, L., Cardozo, A.K., Dogusan, Z., Cnop, M. & Eizirik, D.L. Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress. *Endocrinology* **145**, 5087-5096 (2004).
108. Oyadomari, S., Takeda, K., Takiguchi, M., Gotoh, T., Matsumoto, M., Wada, I., Akira, S., Araki, E. & Mori, M. Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. *Proc Natl Acad Sci U S A* **98**, 10845-10850 (2001).
109. Chan, J.Y., Cooney, G.J., Biden, T.J. & Laybutt, D.R. Differential regulation of adaptive and apoptotic unfolded protein response signalling by cytokine-induced nitric oxide production in mouse pancreatic beta cells. *Diabetologia* **54**, 1766-1776 (2011).
110. Wang, J., Takeuchi, T., Tanaka, S., Kubo, S.K., Kayo, T., Lu, D., Takata, K., Koizumi, A. & Izumi, T. A mutation in the insulin 2 gene induces diabetes with severe pancreatic beta-cell dysfunction in the Mody mouse. *J Clin Invest* **103**, 27-37 (1999).
111. Colombo, C., Porzio, O., Liu, M., Massa, O., Vasta, M., Salardi, S., Beccaria, L., Monciotti, C., Toni, S., Pedersen, O., Hansen, T., Federici, L., Pesavento, R., Cadario, F., Federici, G., Ghirri, P., Arvan, P., Iafusco, D. & Barbetti, F. Seven mutations in the human insulin gene linked to permanent neonatal/infancy-onset diabetes mellitus. *J Clin Invest* **118**, 2148-2156 (2008).
112. Edghill, E.L., Flanagan, S.E., Patch, A.M., Boustred, C., Parrish, A., Shields, B., Shepherd, M.H., Hussain, K., Kapoor, R.R., Malecki, M., MacDonald, M.J., Stoy, J., Steiner, D.F., Philipson, L.H., Bell, G.I., Hattersley, A.T. & Ellard, S. Insulin mutation screening in 1,044 patients with diabetes: mutations in the INS gene are a common cause of neonatal diabetes but a rare cause of diabetes diagnosed in childhood or adulthood. *Diabetes* **57**, 1034-1042 (2008).
113. Stoy, J., Edghill, E.L., Flanagan, S.E., Ye, H., Paz, V.P., Pluzhnikov, A., Below, J.E., Hayes, M.G., Cox, N.J., Lipkind, G.M., Lipton, R.B., Greeley, S.A., Patch, A.M., Ellard, S., Steiner, D.F., Hattersley, A.T., Philipson, L.H. & Bell, G.I. Insulin gene mutations as a cause of permanent neonatal diabetes. *Proc Natl Acad Sci U S A* **104**, 15040-15044 (2007).
114. Hull, R.L., Zraika, S., Udayasankar, J., Aston-Mourney, K., Subramanian, S.L. & Kahn, S.E. Amyloid formation in human IAPP transgenic mouse islets and

pancreas, and human pancreas, is not associated with endoplasmic reticulum stress. *Diabetologia* **52**, 1102-1111 (2009).
115. Huang, C.J., Haataja, L., Gurlo, T., Butler, A.E., Wu, X., Soeller, W.C. & Butler, P.C. Induction of endoplasmic reticulum stress-induced beta-cell apoptosis and accumulation of polyubiquitinated proteins by human islet amyloid polypeptide. *Am J Physiol Endocrinol Metab* **293**, E1656-1662 (2007).
116. Huang, C.J., Lin, C.Y., Haataja, L., Gurlo, T., Butler, A.E., Rizza, R.A. & Butler, P.C. High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress mediated beta-cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes. *Diabetes* **56**, 2016-2027 (2007).
117. Casas, S., Gomis, R., Gribble, F.M., Altirriba, J., Knuutila, S. & Novials, A. Impairment of the ubiquitin-proteasome pathway is a downstream endoplasmic reticulum stress response induced by extracellular human islet amyloid polypeptide and contributes to pancreatic beta-cell apoptosis. *Diabetes* **56**, 2284-2294 (2007).
118. Malhotra, J.D. & Kaufman, R.J. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid Redox Signal* **9**, 2277-2293 (2007).
119. Tu, B.P. & Weissman, J.S. Oxidative protein folding in eukaryotes: mechanisms and consequences. *J Cell Biol* **164**, 341-346 (2004).
120. Santos, C.X., Tanaka, L.Y., Wosniak, J. & Laurindo, F.R. Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. *Antioxid Redox Signal* **11**, 2409-2427 (2009).
121. Shimizu, Y. & Hendershot, L.M. Oxidative folding: cellular strategies for dealing with the resultant equimolar production of reactive oxygen species. *Antioxid Redox Signal* **11**, 2317-2331 (2009).
122. Lenzen, S., Drinkgern, J. & Tiedge, M. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic Biol Med* **20**, 463-466 (1996).
123. Welsh, N., Margulis, B., Borg, L.A., Wiklund, H.J., Saldeen, J., Flodstrom, M., Mello, M.A., Andersson, A., Pipeleers, D.G., Hellerstrom, C. & et al. Differences in the expression of heat-shock proteins and antioxidant enzymes between human and rodent pancreatic islets: implications for the pathogenesis of insulin-dependent diabetes mellitus. *Mol Med* **1**, 806-820 (1995).
124. Deniaud, A., Sharaf el dein, O., Maillier, E., Poncet, D., Kroemer, G., Lemaire, C. & Brenner, C. Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. *Oncogene* **27**, 285-299 (2008).
125. Lee, G.H., Kim, H.K., Chae, S.W., Kim, D.S., Ha, K.C., Cuddy, M., Kress, C., Reed, J.C., Kim, H.R. & Chae, H.J. Bax inhibitor-1 regulates endoplasmic reticulum stress-associated reactive oxygen species and heme oxygenase-1 expression. *J Biol Chem* **282**, 21618-21628 (2007).

126. Walter, L. & Hajnoczky, G. Mitochondria and endoplasmic reticulum: the lethal interorganelle cross-talk. *J Bioenerg Biomembr* **37**, 191-206 (2005).
127. Kaneto, H., Katakami, N., Kawamori, D., Miyatsuka, T., Sakamoto, K., Matsuoka, T.A., Matsuhisa, M. & Yamasaki, Y. Involvement of oxidative stress in the pathogenesis of diabetes. *Antioxid Redox Signal* **9**, 355-366 (2007).
128. Newsholme, P., Haber, E.P., Hirabara, S.M., Rebelato, E.L., Procopio, J., Morgan, D., Oliveira-Emilio, H.C., Carpinelli, A.R. & Curi, R. Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. *J Physiol* **583**, 9-24 (2007).
129. Hasnain, S.Z., Lourie, R., Das, I., Chen, A.C. & McGuckin, M.A. The interplay between endoplasmic reticulum stress and inflammation. *Immunol Cell Biol* **90**, 260-270 (2012).
130. Hotamisligil, G.S. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* **140**, 900-917 (2010).
131. Zhang, K. & Kaufman, R.J. From endoplasmic-reticulum stress to the inflammatory response. *Nature* **454**, 455-462 (2008).
132. Kaneko, M., Niinuma, Y. & Nomura, Y. Activation signal of nuclear factor-kappa B in response to endoplasmic reticulum stress is transduced via IRE1 and tumor necrosis factor receptor-associated factor 2. *Biol Pharm Bull* **26**, 931-935 (2003).
133. Deng, J., Lu, P.D., Zhang, Y., Scheuner, D., Kaufman, R.J., Sonenberg, N., Harding, H.P. & Ron, D. Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. *Mol Cell Biol* **24**, 10161-10168 (2004).
134. Jiang, H.Y., Wek, S.A., McGrath, B.C., Scheuner, D., Kaufman, R.J., Cavener, D.R. & Wek, R.C. Phosphorylation of the alpha subunit of eukaryotic initiation factor 2 is required for activation of NF-kappaB in response to diverse cellular stresses. *Mol Cell Biol* **23**, 5651-5663 (2003).
135. Yamazaki, H., Hiramatsu, N., Hayakawa, K., Tagawa, Y., Okamura, M., Ogata, R., Huang, T., Nakajima, S., Yao, J., Paton, A.W., Paton, J.C. & Kitamura, M. Activation of the Akt-NF-kappaB pathway by subtilase cytotoxin through the ATF6 branch of the unfolded protein response. *J Immunol* **183**, 1480-1487 (2009).
136. Zhou, R., Yazdi, A.S., Menu, P. & Tschopp, J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* **469**, 221-225 (2011).
137. Grimm, S. The ER-mitochondria interface: The social network of cell death. *Biochim Biophys Acta* **1823**, 327-334 (2011).
138. Hayashi, T., Rizzuto, R., Hajnoczky, G. & Su, T.P. MAM: more than just a housekeeper. *Trends Cell Biol* **19**, 81-88 (2009).
139. Menu, P., Mayor, A., Zhou, R., Tardivel, A., Ichijo, H., Mori, K. & Tschopp, J. ER stress activates the NLRP3 inflammasome via an UPR-independent pathway. *Cell Death Dis* **3**, e261 (2012).
140. Dinarello, C.A., Donath, M.Y. & Mandrup-Poulsen, T. Role of IL-1beta in type 2 diabetes. *Curr Opin Endocrinol Diabetes Obes* **17**, 314-321 (2010).

141. Donath, M.Y. & Shoelson, S.E. Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol* **11**, 98-107 (2011).
142. Zhou, R., Tardivel, A., Thorens, B., Choi, I. & Tschopp, J. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* **11**, 136-140 (2009).
143. Cha-Molstad, H., Saxena, G., Chen, J. & Shalev, A. Glucose-stimulated expression of Txnip is mediated by carbohydrate response element-binding protein, p300, and histone H4 acetylation in pancreatic beta cells. *J Biol Chem* **284**, 16898-16905 (2009).
144. Peterson, C.W., Stoltzman, C.A., Sighinolfi, M.P., Han, K.S. & Ayer, D.E. Glucose controls nuclear accumulation, promoter binding, and transcriptional activity of the MondoA-Mlx heterodimer. *Mol Cell Biol* **30**, 2887-2895 (2010).
145. Stoltzman, C.A., Peterson, C.W., Breen, K.T., Muoio, D.M., Billin, A.N. & Ayer, D.E. Glucose sensing by MondoA:Mlx complexes: a role for hexokinases and direct regulation of thioredoxin-interacting protein expression. *Proc Natl Acad Sci U S A* **105**, 6912-6917 (2008).
146. Yu, F.X. & Luo, Y. Tandem ChoRE and CCAAT motifs and associated factors regulate Txnip expression in response to glucose or adenosine-containing molecules. *PLoS One* **4**, e8397 (2009).
147. Junn, E., Han, S.H., Im, J.Y., Yang, Y., Cho, E.W., Um, H.D., Kim, D.K., Lee, K.W., Han, P.L., Rhee, S.G. & Choi, I. Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. *J Immunol* **164**, 6287-6295 (2000).
148. Nishiyama, A., Matsui, M., Iwata, S., Hirota, K., Masutani, H., Nakamura, H., Takagi, Y., Sono, H., Gon, Y. & Yodoi, J. Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. *J Biol Chem* **274**, 21645-21650 (1999).
149. Yamanaka, H., Maehira, F., Oshiro, M., Asato, T., Yanagawa, Y., Takei, H. & Nakashima, Y. A possible interaction of thioredoxin with VDUP1 in HeLa cells detected in a yeast two-hybrid system. *Biochem Biophys Res Commun* **271**, 796-800 (2000).
150. Larsen, C.M., Faulenbach, M., Vaag, A., Ehses, J.A., Donath, M.Y. & Mandrup-Poulsen, T. Sustained effects of interleukin-1 receptor antagonist treatment in type 2 diabetes. *Diabetes Care* **32**, 1663-1668 (2009).
151. Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U. & Speed, T.P. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249-264 (2003).
152. Campeau, E., Ruhl, V.E., Rodier, F., Smith, C.L., Rahmberg, B.L., Fuss, J.O., Campisi, J., Yaswen, P., Cooper, P.K. & Kaufman, P.D. A versatile viral system for expression and depletion of proteins in mammalian cells. *PLoS One* **4**, e6529 (2009).
153. Hui, S.T., Andres, A.M., Miller, A.K., Spann, N.J., Potter, D.W., Post, N.M., Chen, A.Z., Sachithanantham, S., Jung, D.Y., Kim, J.K. & Davis, R.A. Txnip

balances metabolic and growth signaling via PTEN disulfide reduction. *Proc Natl Acad Sci U S A* **105**, 3921-3926 (2008).
154. Passananti, C. & Fanciulli, M. The anti-apoptotic factor Che-1/AATF links transcriptional regulation, cell cycle control, and DNA damage response. *Cell Div* **2**, 21 (2007).
155. Passananti, C., Floridi, A. & Fanciulli, M. Che-1/AATF, a multivalent adaptor connecting transcriptional regulation, checkpoint control, and apoptosis. *Biochem Cell Biol* **85**, 477-483 (2007).
156. Shalev, A., Pise-Masison, C.A., Radonovich, M., Hoffmann, S.C., Hirshberg, B., Brady, J.N. & Harlan, D.M. Oligonucleotide microarray analysis of intact human pancreatic islets: identification of glucose-responsive genes and a highly regulated TGFbeta signaling pathway. *Endocrinology* **143**, 3695-3698 (2002).
157. Rani, S., Mehta, J.P., Barron, N., Doolan, P., Jeppesen, P.B., Clynes, M. & O'Driscoll, L. Decreasing Txnip mRNA and protein levels in pancreatic MIN6 cells reduces reactive oxygen species and restores glucose regulated insulin secretion. *Cell Physiol Biochem* **25**, 667-674 (2010).
158. Yoshihara, E., Fujimoto, S., Inagaki, N., Okawa, K., Masaki, S., Yodoi, J. & Masutani, H. Disruption of TBP-2 ameliorates insulin sensitivity and secretion without affecting obesity. *Nat Commun* **1**, 127 (2010).
159. Chen, J., Saxena, G., Mungrue, I.N., Lusis, A.J. & Shalev, A. Thioredoxin-interacting protein: a critical link between glucose toxicity and beta-cell apoptosis. *Diabetes* **57**, 938-944 (2008).
160. Dowling, P., O'Driscoll, L., O'Sullivan, F., Dowd, A., Henry, M., Jeppesen, P.B., Meleady, P. & Clynes, M. Proteomic screening of glucose-responsive and glucose non-responsive MIN-6 beta cells reveals differential expression of proteins involved in protein folding, secretion and oxidative stress. *Proteomics* **6**, 6578-6587 (2006).
161. Chen, J., Fontes, G., Saxena, G., Poitout, V. & Shalev, A. Lack of TXNIP protects against mitochondria-mediated apoptosis but not against fatty acid-induced ER stress-mediated beta-cell death. *Diabetes* **59**, 440-447 (2010).
162. Shaked, M., Ketzinel-Gilad, M., Cerasi, E., Kaiser, N. & Leibowitz, G. AMP-activated protein kinase (AMPK) mediates nutrient regulation of thioredoxin-interacting protein (TXNIP) in pancreatic beta-cells. *PLoS One* **6**, e28804 (2011).
163. Mori, K., Ogawa, N., Kawahara, T., Yanagi, H. & Yura, T. Palindrome with spacer of one nucleotide is characteristic of the cis-acting unfolded protein response element in Saccharomyces cerevisiae. *J Biol Chem* **273**, 9912-9920 (1998).
164. Yoshida, H., Haze, K., Yanagi, H., Yura, T. & Mori, K. Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *J Biol Chem* **273**, 33741-33749 (1998).

165. Kokame, K., Kato, H. & Miyata, T. Identification of ERSE-II, a new cis-acting element responsible for the ATF6-dependent mammalian unfolded protein response. *J Biol Chem* **276**, 9199-9205 (2001).
166. Li, X., Rong, Y., Zhang, M., Wang, X.L., LeMaire, S.A., Coselli, J.S., Zhang, Y. & Shen, Y.H. Up-regulation of thioredoxin interacting protein (Txnip) by p38 MAPK and FOXO1 contributes to the impaired thioredoxin activity and increased ROS in glucose-treated endothelial cells. *Biochem Biophys Res Commun* **381**, 660-665 (2009).
167. Martinez, S.C., Tanabe, K., Cras-Meneur, C., Abumrad, N.A., Bernal-Mizrachi, E. & Permutt, M.A. Inhibition of Foxo1 protects pancreatic islet beta-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis. *Diabetes* **57**, 846-859 (2008).
168. Minn, A.H., Hafele, C. & Shalev, A. Thioredoxin-interacting protein is stimulated by glucose through a carbohydrate response element and induces beta-cell apoptosis. *Endocrinology* **146**, 2397-2405 (2005).
169. Arend, W.P., Malyak, M., Smith, M.F., Jr., Whisenand, T.D., Slack, J.L., Sims, J.E., Giri, J.G. & Dower, S.K. Binding of IL-1 alpha, IL-1 beta, and IL-1 receptor antagonist by soluble IL-1 receptors and levels of soluble IL-1 receptors in synovial fluids. *J Immunol* **153**, 4766-4774 (1994).
170. Chen, J., Hui, S.T., Couto, F.M., Mungrue, I.N., Davis, D.B., Attie, A.D., Lusis, A.J., Davis, R.A. & Shalev, A. Thioredoxin-interacting protein deficiency induces Akt/Bcl-xL signaling and pancreatic beta-cell mass and protects against diabetes. *Faseb J* **22**, 3581-3594 (2008).
171. Yamazaki, T., Ohmi, A., Kurumaya, H., Kato, K., Abe, T., Yamamoto, H., Nakanishi, N., Okuyama, R., Umemura, M., Kaise, T., Watanabe, R., Okawa, Y., Takahashi, S. & Takahashi, Y. Regulation of the human CHOP gene promoter by the stress response transcription factor ATF5 via the AARE1 site in human hepatoma HepG2 cells. *Life Sci* **87**, 294-301 (2010).
172. Havula, E. & Hietakangas, V. Glucose sensing by ChREBP/MondoA-Mlx transcription factors. *Semin Cell Dev Biol* (2012).
173. Stoeckman, A.K., Ma, L. & Towle, H.C. Mlx is the functional heteromeric partner of the carbohydrate response element-binding protein in glucose regulation of lipogenic enzyme genes. *J Biol Chem* **279**, 15662-15669 (2004).
174. Uyeda, K. & Repa, J.J. Carbohydrate response element binding protein, ChREBP, a transcription factor coupling hepatic glucose utilization and lipid synthesis. *Cell Metab* **4**, 107-110 (2006).
175. da Silva Xavier, G., Sun, G., Qian, Q., Rutter, G.A. & Leclerc, I. ChREBP regulates Pdx-1 and other glucose-sensitive genes in pancreatic beta-cells. *Biochem Biophys Res Commun* **402**, 252-257 (2010).
176. Poungvarin, N., Lee, J.K., Yechoor, V.K., Li, M.V., Assavapokee, T., Suksaranjit, P., Thepsongwajja, J.J., Saha, P.K., Oka, K. & Chan, L. Carbohydrate response element-binding protein (ChREBP) plays a pivotal role in beta cell glucotoxicity. *Diabetologia* (2012).

177. Leclerc, I., Rutter, G.A., Meur, G. & Noordeen, N.A. Roles of Ca2+ Ions in the Control of ChREBP Nuclear Translocation. *J Endocrinol* (2012).
178. Papadia, S., Soriano, F.X., Leveille, F., Martel, M.A., Dakin, K.A., Hansen, H.H., Kaindl, A., Sifringer, M., Fowler, J., Stefovska, V., McKenzie, G., Craigon, M., Corriveau, R., Ghazal, P., Horsburgh, K., Yankner, B.A., Wyllie, D.J., Ikonomidou, C. & Hardingham, G.E. Synaptic NMDA receptor activity boosts intrinsic antioxidant defenses. *Nat Neurosci* **11**, 476-487 (2008).
179. Zhou, Y., Lee, J., Reno, C.M., Sun, C., Park, S.W., Chung, J., Lee, J., Fisher, S.J., White, M.F., Biddinger, S.B. & Ozcan, U. Regulation of glucose homeostasis through a XBP-1-FoxO1 interaction. *Nat Med* **17**, 356-365 (2011).
180. Hosoi, T., Hyoda, K., Okuma, Y., Nomura, Y. & Ozawa, K. Akt up- and down-regulation in response to endoplasmic reticulum stress. *Brain Res* **1152**, 27-31 (2007).
181. Hyoda, K., Hosoi, T., Horie, N., Okuma, Y., Ozawa, K. & Nomura, Y. PI3K-Akt inactivation induced CHOP expression in endoplasmic reticulum-stressed cells. *Biochem Biophys Res Commun* **340**, 286-290 (2006).
182. Srinivasan, S., Ohsugi, M., Liu, Z., Fatrai, S., Bernal-Mizrachi, E. & Permutt, M.A. Endoplasmic reticulum stress-induced apoptosis is partly mediated by reduced insulin signaling through phosphatidylinositol 3-kinase/Akt and increased glycogen synthase kinase-3beta in mouse insulinoma cells. *Diabetes* **54**, 968-975 (2005).
183. Maedler, K., Sergeev, P., Ris, F., Oberholzer, J., Joller-Jemelka, H.I., Spinas, G.A., Kaiser, N., Halban, P.A. & Donath, M.Y. Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. *J Clin Invest* **110**, 851-860 (2002).
184. Ehses, J.A., Perren, A., Eppler, E., Ribaux, P., Pospisilik, J.A., Maor-Cahn, R., Gueripel, X., Ellingsgaard, H., Schneider, M.K., Biollaz, G., Fontana, A., Reinecke, M., Homo-Delarche, F. & Donath, M.Y. Increased number of islet-associated macrophages in type 2 diabetes. *Diabetes* **56**, 2356-2370 (2007).
185. Maedler, K., Sergeev, P., Ehses, J.A., Mathe, Z., Bosco, D., Berney, T., Dayer, J.M., Reinecke, M., Halban, P.A. & Donath, M.Y. Leptin modulates beta cell expression of IL-1 receptor antagonist and release of IL-1beta in human islets. *Proc Natl Acad Sci U S A* **101**, 8138-8143 (2004).
186. Geiler, J. & McDermott, M.F. Gevokizumab, an anti-IL-1beta mAb for the potential treatment of type 1 and 2 diabetes, rheumatoid arthritis and cardiovascular disease. *Curr Opin Mol Ther* **12**, 755-769 (2010).
187. Darville, M.I. & Eizirik, D.L. Regulation by cytokines of the inducible nitric oxide synthase promoter in insulin-producing cells. *Diabetologia* **41**, 1101-1108 (1998).
188. Kacheva, S., Lenzen, S. & Gurgul-Convey, E. Differential effects of proinflammatory cytokines on cell death and ER stress in insulin-secreting INS1E cells and the involvement of nitric oxide. *Cytokine* **55**, 195-201 (2011).

189. Shao, C., Lawrence, M.C. & Cobb, M.H. Regulation of CCAAT/enhancer-binding protein homologous protein (CHOP) expression by interleukin-1 beta in pancreatic beta cells. *J Biol Chem* **285**, 19710-19719 (2010).
190. Ammendrup, A., Maillard, A., Nielsen, K., Aabenhus Andersen, N., Serup, P., Dragsbaek Madsen, O., Mandrup-Poulsen, T. & Bonny, C. The c-Jun amino-terminal kinase pathway is preferentially activated by interleukin-1 and controls apoptosis in differentiating pancreatic beta-cells. *Diabetes* **49**, 1468-1476 (2000).
191. Bonny, C., Oberson, A., Negri, S., Sauser, C. & Schorderet, D.F. Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. *Diabetes* **50**, 77-82 (2001).
192. Wang, Q., Zhang, H., Zhao, B. & Fei, H. IL-1beta caused pancreatic beta-cells apoptosis is mediated in part by endoplasmic reticulum stress via the induction of endoplasmic reticulum Ca2+ release through the c-Jun N-terminal kinase pathway. *Mol Cell Biochem* **324**, 183-190 (2009).
193. Cogswell, J.P., Godlevski, M.M., Wisely, G.B., Clay, W.C., Leesnitzer, L.M., Ways, J.P. & Gray, J.G. NF-kappa B regulates IL-1 beta transcription through a consensus NF-kappa B binding site and a nonconsensus CRE-like site. *J Immunol* **153**, 712-723 (1994).
194. Saxena, G., Chen, J. & Shalev, A. Intracellular shuttling and mitochondrial function of thioredoxin-interacting protein. *J Biol Chem* **285**, 3997-4005 (2010).
195. Nishinaka, Y., Masutani, H., Oka, S., Matsuo, Y., Yamaguchi, Y., Nishio, K., Ishii, Y. & Yodoi, J. Importin alpha1 (Rch1) mediates nuclear translocation of thioredoxin-binding protein-2/vitamin D(3)-up-regulated protein 1. *J Biol Chem* **279**, 37559-37565 (2004).
196. Minn, A.H., Pise-Masison, C.A., Radonovich, M., Brady, J.N., Wang, P., Kendziorski, C. & Shalev, A. Gene expression profiling in INS-1 cells overexpressing thioredoxin-interacting protein. *Biochem Biophys Res Commun* **336**, 770-778 (2005).
197. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K. & Ichijo, H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *Embo J* **17**, 2596-2606 (1998).
198. Hui, T.Y., Sheth, S.S., Diffley, J.M., Potter, D.W., Lusis, A.J., Attie, A.D. & Davis, R.A. Mice lacking thioredoxin-interacting protein provide evidence linking cellular redox state to appropriate response to nutritional signals. *J Biol Chem* **279**, 24387-24393 (2004).
199. Oka, S., Yoshihara, E., Bizen-Abe, A., Liu, W., Watanabe, M., Yodoi, J. & Masutani, H. Thioredoxin binding protein-2/thioredoxin-interacting protein is a critical regulator of insulin secretion and peroxisome proliferator-activated receptor function. *Endocrinology* **150**, 1225-1234 (2009).
200. Ferreira, N.E., Omae, S., Pereira, A., Rodrigues, M.V., Miyakawa, A.A., Campos, L.C., Santos, P.C., Dallan, L.A., Martinez, T.L., Santos, R.D., Mill, J.G., Krieger, J.E. & Pereira, A.C. Thioredoxin interacting protein genetic variation is

associated with diabetes and hypertension in the Brazilian general population. *Atherosclerosis* **221**, 131-136 (2012).
201. Gokulakrishnan, K., Mohanavalli, K.T., Monickaraj, F., Mohan, V. & Balasubramanyam, M. Subclinical inflammation/oxidation as revealed by altered gene expression profiles in subjects with impaired glucose tolerance and Type 2 diabetes patients. *Mol Cell Biochem* **324**, 173-181 (2009).
202. Parikh, H., Carlsson, E., Chutkow, W.A., Johansson, L.E., Storgaard, H., Poulsen, P., Saxena, R., Ladd, C., Schulze, P.C., Mazzini, M.J., Jensen, C.B., Krook, A., Bjornholm, M., Tornqvist, H., Zierath, J.R., Ridderstrale, M., Altshuler, D., Lee, R.T., Vaag, A., Groop, L.C. & Mootha, V.K. TXNIP regulates peripheral glucose metabolism in humans. *PLoS Med* **4**, e158 (2007).
203. Shaked, M., Ketzinel-Gilad, M., Ariav, Y., Cerasi, E., Kaiser, N. & Leibowitz, G. Insulin counteracts glucotoxic effects by suppressing thioredoxin-interacting protein production in INS-1E beta cells and in Psammomys obesus pancreatic islets. *Diabetologia* **52**, 636-644 (2009).
204. Diabetes Atlas. *International Diabetes Federation* **4** (2011).
205. Lin, Y. & Sun, Z. Current views on type 2 diabetes. *J Endocrinol* **204**, 1-11 (2010).
206. Chutkow, W.A., Patwari, P., Yoshioka, J. & Lee, R.T. Thioredoxin-interacting protein (Txnip) is a critical regulator of hepatic glucose production. *J Biol Chem* **283**, 2397-2406 (2008).
207. DeFronzo, R.A. & Abdul-Ghani, M. Type 2 diabetes can be prevented with early pharmacological intervention. *Diabetes Care* **34 Suppl 2**, S202-209 (2011).
208. Turner, R.C., Cull, C.A., Frighi, V. & Holman, R.R. Glycemic control with diet, sulfonylurea, metformin, or insulin in patients with type 2 diabetes mellitus: progressive requirement for multiple therapies (UKPDS 49). UK Prospective Diabetes Study (UKPDS) Group. *Jama* **281**, 2005-2012 (1999).
209. Ozcan, U., Yilmaz, E., Ozcan, L., Furuhashi, M., Vaillancourt, E., Smith, R.O., Gorgun, C.Z. & Hotamisligil, G.S. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* **313**, 1137-1140 (2006).
210. Lee, Y.Y., Hong, S.H., Lee, Y.J., Chung, S.S., Jung, H.S., Park, S.G. & Park, K.S. Tauroursodeoxycholate (TUDCA), chemical chaperone, enhances function of islets by reducing ER stress. *Biochem Biophys Res Commun* **397**, 735-739 (2010).
211. Tang, C., Koulajian, K., Schuiki, I., Zhang, L., Desai, T., Ivovic, A., Wang, P., Robson-Doucette, C., Wheeler, M.B., Minassian, B., Volchuk, A. & Giacca, A. Glucose-induced beta cell dysfunction in vivo in rats: link between oxidative stress and endoplasmic reticulum stress. *Diabetologia* (2012).
212. Kudo, T., Kanemoto, S., Hara, H., Morimoto, N., Morihara, T., Kimura, R., Tabira, T., Imaizumi, K. & Takeda, M. A molecular chaperone inducer protects neurons from ER stress. *Cell Death Differ* **15**, 364-375 (2008).

213. Oida, Y., Izuta, H., Oyagi, A., Shimazawa, M., Kudo, T., Imaizumi, K. & Hara, H. Induction of BiP, an ER-resident protein, prevents the neuronal death induced by transient forebrain ischemia in gerbil. *Brain Res* **1208**, 217-224 (2008).
214. Zhang, L., Lai, E., Teodoro, T. & Volchuk, A. GRP78, but Not Protein-disulfide Isomerase, Partially Reverses Hyperglycemia-induced Inhibition of Insulin Synthesis and Secretion in Pancreatic {beta}-Cells. *J Biol Chem* **284**, 5289-5298 (2009).
215. Schulze, P.C., Yoshioka, J., Takahashi, T., He, Z., King, G.L. & Lee, R.T. Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. *J Biol Chem* **279**, 30369-30374 (2004).
216. Kaneto, H., Kajimoto, Y., Miyagawa, J., Matsuoka, T., Fujitani, Y., Umayahara, Y., Hanafusa, T., Matsuzawa, Y., Yamasaki, Y. & Hori, M. Beneficial effects of antioxidants in diabetes: possible protection of pancreatic beta-cells against glucose toxicity. *Diabetes* **48**, 2398-2406 (1999).
217. Tanaka, Y., Gleason, C.E., Tran, P.O., Harmon, J.S. & Robertson, R.P. Prevention of glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats by antioxidants. *Proc Natl Acad Sci U S A* **96**, 10857-10862 (1999).
218. Gorogawa, S., Kajimoto, Y., Umayahara, Y., Kaneto, H., Watada, H., Kuroda, A., Kawamori, D., Yasuda, T., Matsuhisa, M., Yamasaki, Y. & Hori, M. Probucol preserves pancreatic beta-cell function through reduction of oxidative stress in type 2 diabetes. *Diabetes Res Clin Pract* **57**, 1-10 (2002).
219. Liu, J.H., Liu, D.F., Wang, N.N., Lin, H.L. & Mei, X. Possible role for the thioredoxin system in the protective effects of probucol in the pancreatic islets of diabetic rats. *Clin Exp Pharmacol Physiol* **38**, 528-533 (2011).
220. Travers, K.J., Patil, C.K., Wodicka, L., Lockhart, D.J., Weissman, J.S. & Walter, P. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101**, 249-258 (2000).
221. Merksamer, P.I., Trusina, A. & Papa, F.R. Real-time redox measurements during endoplasmic reticulum stress reveal interlinked protein folding functions. *Cell* **135**, 933-947 (2008).
222. Stocchi, F. Use of apomorphine in Parkinson's disease. *Neurol Sci* **29 Suppl 5**, S383-386 (2008).
223. Waugh, J., Keating, G.M., Plosker, G.L., Easthope, S. & Robinson, D.M. Pioglitazone: a review of its use in type 2 diabetes mellitus. *Drugs* **66**, 85-109 (2006).
224. Gastaldelli, A., Ferrannini, E., Miyazaki, Y., Matsuda, M., Mari, A. & DeFronzo, R.A. Thiazolidinediones improve beta-cell function in type 2 diabetic patients. *Am J Physiol Endocrinol Metab* **292**, E871-883 (2007).
225. DeFronzo, R.A., Tripathy, D., Schwenke, D.C., Banerji, M., Bray, G.A., Buchanan, T.A., Clement, S.C., Henry, R.R., Hodis, H.N., Kitabchi, A.E., Mack, W.J., Mudaliar, S., Ratner, R.E., Williams, K., Stentz, F.B., Musi, N. & Reaven,

P.D. Pioglitazone for diabetes prevention in impaired glucose tolerance. *N Engl J Med* **364**, 1104-1115 (2011).

226. Papandreou, I., Denko, N.C., Olson, M., Van Melckebeke, H., Lust, S., Tam, A., Solow-Cordero, D.E., Bouley, D.M., Offner, F., Niwa, M. & Koong, A.C. Identification of an Ire1alpha endonuclease specific inhibitor with cytotoxic activity against human multiple myeloma. *Blood* **117**, 1311-1314 (2011).
227. Xu, G., Chen, J., Jing, G. & Shalev, A. Preventing beta-Cell Loss and Diabetes With Calcium Channel Blockers. *Diabetes* **61**, 848-856 (2012).
228. Gallwitz, B. Glucagon-like peptide-1 analogues for Type 2 diabetes mellitus: current and emerging agents. *Drugs* **71**, 1675-1688 (2011).
229. Tsunekawa, S., Yamamoto, N., Tsukamoto, K., Itoh, Y., Kaneko, Y., Kimura, T., Ariyoshi, Y., Miura, Y., Oiso, Y. & Niki, I. Protection of pancreatic beta-cells by exendin-4 may involve the reduction of endoplasmic reticulum stress; in vivo and in vitro studies. *J Endocrinol* **193**, 65-74 (2007).
230. Yusta, B., Baggio, L.L., Estall, J.L., Koehler, J.A., Holland, D.P., Li, H., Pipeleers, D., Ling, Z. & Drucker, D.J. GLP-1 receptor activation improves beta cell function and survival following induction of endoplasmic reticulum stress. *Cell Metab* **4**, 391-406 (2006).
231. Wu, J.D., Xu, X.H., Zhu, J., Ding, B., Du, T.X., Gao, G., Mao, X.M., Ye, L., Lee, K.O. & Ma, J.H. Effect of exenatide on inflammatory and oxidative stress markers in patients with type 2 diabetes mellitus. *Diabetes Technol Ther* **13**, 143-148 (2011).
232. Chen, J., Couto, F.M., Minn, A.H. & Shalev, A. Exenatide inhibits beta-cell apoptosis by decreasing thioredoxin-interacting protein. *Biochem Biophys Res Commun* **346**, 1067-1074 (2006).
233. Shao, W., Yu, Z., Fantus, I.G. & Jin, T. Cyclic AMP signaling stimulates proteasome degradation of thioredoxin interacting protein (TxNIP) in pancreatic beta-cells. *Cell Signal* **22**, 1240-1246 (2010).
234. Qi, W., Chen, X., Holian, J., Tan, C.Y., Kelly, D.J. & Pollock, C.A. Transcription factors Kruppel-like factor 6 and peroxisome proliferator-activated receptor-{gamma} mediate high glucose-induced thioredoxin-interacting protein. *Am J Pathol* **175**, 1858-1867 (2009).
235. Chutkow, W.A., Birkenfeld, A.L., Brown, J.D., Lee, H.Y., Frederick, D.W., Yoshioka, J., Patwari, P., Kursawe, R., Cushman, S.W., Plutzky, J., Shulman, G.I., Samuel, V.T. & Lee, R.T. Deletion of the alpha-arrestin protein Txnip in mice promotes adiposity and adipogenesis while preserving insulin sensitivity. *Diabetes* **59**, 1424-1434 (2010).
236. Koenen, T.B., Stienstra, R., van Tits, L.J., de Graaf, J., Stalenhoef, A.F., Joosten, L.A., Tack, C.J. & Netea, M.G. Hyperglycemia activates caspase-1 and TXNIP-mediated IL-1beta transcription in human adipose tissue. *Diabetes* **60**, 517-524 (2011).
237. Schmidt, M.V., Brune, B. & von Knethen, A. The nuclear hormone receptor PPARgamma as a therapeutic target in major diseases. *ScientificWorldJournal* **10**, 2181-2197 (2010).