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Role of WFS1 in Regulating Endoplasmic Reticulum Stress Signaling: A Dissertation

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THE ROLE OF WFS1 IN REGULATING ENDOPLASMIC RETICULUM STRESS SIGNALING

A Dissertation Presented

By

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DEDICATION

With love, I dedicate this work to my wonderful husband Carlos, and my beautiful daughters Sophia and Bianca. They fill my life with love and happiness and inspire me every day.

ACKNOWLEDGEMENTS

It is rare in life to come across people that truly inspire us. I have been blessed to encounter several, and I am not easily inspired. My husband, Carlos, is the first person that has ever really inspired me to be a better person. He has been incredibly supportive through my educational journey, encouraging me to pick myself up every time I fall. I could not have made it this far without him. He is my foundation. I thank him for being my everything and for putting his life on hold for me. Behind every strong woman, there is a strong man…

Teachers should always inspire us, but that is not always the case. I have learned a great deal from many teachers, but there is one in particular that made me very excited to learn. That teacher is Dr. William Totherow. His unconventional teaching style really inspired me – I learned so much from his classes. He is always eager to hear of my progress. His constant encouragement to pursue higher education has always stayed with me, and I hope to pass that on to my daughters. I thank him for adding such value to my life.

Although it is the 21st century, the reality is that it is still very challenging for women to have a successful career and to reach the top in science, while juggling a family. My first mentor, Dr. Susan Bonner-Weir, showed me that this was possible. She is such a strong, successful, balanced woman and I hope to follow in her footsteps. She has continued to give me support through the years, and I must thank her for this.

I will admit I was very concerned starting graduate school while having a 10 month-old daughter. I have been extremely lucky to have such a wonderful, caring mentor at UMass, Dr. Fumihiko Urano. He is truly unique. He has pushed me to do better and to think independently. I

have not only grown as a scientist under his mentorship, but I have also grown as a person. Every single day, he gives me words of wisdom. He is so excited to do research and this excitement truly rubs off on you. He really believes that every piece of data is important. This has really built my self-confidence. I have been able to achieve so much under his mentorship. I thank him from the bottom of my heart for such inspiration and encouragement. One of the most important words of wisdom that I will take with me from this experience is "*Kai Zen*": Take that which is good and make it better. I hope that I will be able to work with him in my future endeavors.

N.S.I. – *Never Stop Innovating.*

ABSTRACT

The endoplasmic reticulum (ER) is a multi-functional cellular compartment that functions in protein folding, lipid biosynthesis, and calcium homeostasis. Perturbations to ER function lead to the dysregulation of ER homeostasis, causing the accumulation of unfolded and misfolded proteins in the cell. This is a state of ER stress. ER stress elicits a cytoprotective, adaptive signaling cascade to mitigate stress, the Unfolded Protein Response (UPR). As long as the UPR can moderate stress, cells can produce the proper amount of proteins and maintain a state of homeostasis. If the UPR, however, is dysfunctional and fails to achieve this, cells will undergo apoptosis.

Diabetes mellitus is a group of metabolic disorders characterized by persistent high blood glucose levels. The pathogenesis of this disease involves pancreatic β-cell dysfunction: an abnormality in the primary function of the β-cell, insulin production and secretion. Activation of the UPR is critical to pancreatic β-cell survival, where a disruption in ER stress signaling can lead to cell death and consequently diabetes. There are several models of ER stress leading to diabetes. Wolcott-Rallison syndrome, for example, occurs when there is a mutation in the gene encoding one of the master regulators of the UPR, PKR-like ER kinase (PERK).

In this dissertation, we show that Wolfram Syndrome 1 (WFS1), an ER transmembrane protein, is a component of the UPR and is a downstream target of two of the master regulators of the UPR, Inositol Requiring 1 (IRE1) and PERK. WFS1 mutations lead to Wolfram syndrome, a non-autoimmune form of type 1 diabetes accompanied by optical atrophy and other neurological disorders. It has been shown that patients develop diabetes due to the selective loss of their

pancreatic β-cells. Here we define the underlying molecular mechanism of β-cell loss in Wolfram syndrome, and link this cell loss to ER stress and a dysfunction in a component of the UPR, WFS1. We show that WFS1 expression is localized to the β-cell of the pancreas, it is upregulated during insulin secretion and ER stress, and its inactivation leads to chronic ER stress and apoptosis.

This dissertation also reveals the previously unknown function of WFS1 in the UPR. Positive regulation of the UPR has been extensively studied, however, the precise mechanisms of negative regulation of this signaling pathway have not. Here we report that WFS1 regulates a key transcription factor of the UPR, activating transcription factor 6 (ATF6), through the ubiquitin-proteasome pathway. WFS1 expression decreases expression levels of ATF6 target genes and represses ATF6-mediated activation of the ER stress response (ERSE) promoter. WFS1 recruits and stabilizes an E3 ubiquitin ligase, HMG-CoA reductase degradation protein 1 (HRD1), on the ER membrane. The WFS1-HRD1 complex recruits ATF6 to the proteasome and enhances its ubiquitination and proteasome-mediated degradation, leading to suppression of the UPR under non-stress conditions. In response to ER stress, ATF6 is released from WFS1 and activates the UPR to mitigate ER stress.

This body of work reveals a novel role for WFS1 in the UPR, and a novel mechanism for regulating ER stress signaling. These findings also indicate that hyperactivation of the UPR can lead to cellular dysfunction and death. This supports the notion that tight regulation of ER stress signaling is crucial to cell survival. This unanticipated role of WFS1 for a feedback loop of the UPR is relevant to diseases caused by chronic hyperactivation of ER stress signaling network such as pancreatic β-cell death in diabetes and neurodegeneration.

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LIST OF ABBREVIATIONS

ASK1	Apoptosis-signaling-kinase 1
ATF3	Activating transcription factor 3
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
Bcl-2	B cell lymphoma 2
BiP	Immunoglobulin heavy chain-binding protein
b-ZIP	Basic leucine zipper
CFTR	Cystic fibrosis transmembrane conductance regulator
CHOP	C/EBP-homologous protein
CPY	Vacuolar carboxypeptidase Y
CREBH	cAMP responsive element binding protein 3-like 3
DM	Diabetes mellitus
DR5	Death receptor 5
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EDEM	ER-degradation-enhancing-α-mannidose-like protein
eIF2α	Eukaryotic initiation factor 2 α

ER	Endoplasmic Reticulum
ERAD	ER-associated protein degradation
ERSE	ER stress response element
ERO1	Endoplasmic oxidoreductin 1
FFA	Free fatty acid
GADD34	Growth arrest and DNA damage inducible gene 34
GRP94	Glucose regulated protein 94
GSIS	Glucose-stimulated insulin secretion
GSK3β	Glycogen synthase kinase 3β
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HRD1/Der3	HMG-CoA reductase degradation protein 1
IAPP	Islet amyloid polypeptide
IFN-γ	γ-interferon
IL-1β	Interleukin-1β
IPF-1	Insulin promoter factor 1
IRE1	Inositol requiring 1
IRS-1	Insulin receptor substrate-1
JNK	Jun N-terminal kinase
MafA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MEF	Mouse embryonic fibroblast

NO	Nitric oxide
NF-Y	Nuclear transcription factor-Y
OA	Optical atrophy
OASIS	cAMP responsive element-binding protein 3-like protein 1
ORP150	Oxygen-regulated protein 150
P58^{IPK}	Protein kinase inhibitor of 58 kDa
PDI	Protein disulfide isomerase
PDX-1	Pancreatic/duodenal homeobox-1
PERK	PKR-like ER kinase
RB	Retinoblastoma
RIP	Regulated intramembrane proteolysis
ROS	Reactive oxygen species
S1	Site 1 protease
S2	Site 2 protease
SEL1/HRD3	Suppressor of lin-12-like protein
SERCA2b	Sarcoendoplasmic reticulum pump Ca^{2+} ATPase2b
shRNA	Small hairpin RNA
SNAP	S-nitroso-N-acetyl-$_{D,L}$-penicillamine
SNP	Single nucleotide polymorphism
SRP	Signal-recognition particle
TCRα	α subunit of the T-cell receptor

Tg	Thapsigargin
Tm	Tunicamycin
TRAF2	TNF-receptor-associated factor 2
TRB3	Tribbles-related protein 3
uORFs	Upstream open reading frames
UPR	Unfolded Protein Response
WFS	Wolfram syndrome
WFS1	Wolfram syndrome 1
XBP1	X-box binding protein 1

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CHAPTER I

INTRODUCTION

Diabetes mellitus is a group of metabolic disorders characterized by chronic high blood glucose levels or hyperglycemia. This disease can lead to heart disease, kidney failure, and blindness because excessive amounts of glucose circulating in the blood cause micro- and macro-vascular complications[1]. The pathogenesis of this disease involves pancreatic β-cell dysfunction. A defect in endoplasmic reticulum (ER) signaling contributes to defective β-cell function and diabetes[2-5]. This thesis focuses on the discovery of a novel component of ER stress signaling, WFS1, and linking its dysfunction with ER stress-mediated diabetes.

1.1 ER Stress and the UPR

Productive folding of secretory proteins, as well as the degradation of misfolded proteins are essential processes for normal cell function. These actions occur in the ER, a compartment in eukaryotic cells that has a complement of chaperones, foldases, and oxidoreductases to perform these specialized functions of protein quality control[6]. Protein folding is an energy-demanding process and it has been estimated that even under normal conditions, approximately 30% of newly synthesized proteins are degraded[201]. Any disruption to the sensitive folding environment of the ER leads to a state of disequilibrium and an accumulation of misfolded and unfolded proteins. This state is called ER stress: the demand that a load of protein makes on the ER exceeds its folding capacity[7]. Perturbations to the ER environment include an overwhelming of chaperone capacity, alterations in redox state, reduced calcium levels, or failure in the post-

translational modifications of secretory proteins. This compromises the ability of the ER to produce properly-folded proteins. These perturbations can also be induced pharmacologically by drugs such as tunicamycin (inhibits N-linked glycosylation), thapsigargin (disrupts ER calcium levels), and dithiothreitol (disrupts ER redox state).

The Unfolded Protein Response (UPR) is an adaptive, ER-to-nucleus signal transduction system that mitigates ER stress[8]. It monitors the folding capacity of the ER and triggers signaling cascades which prevent the build-up of unproductive, potentially toxic protein products. This response is physiologically induced by protein overload, mutant protein expression, and infection[9] (Figure 1.1). The UPR has four functionally distinct responses: 1) upregulation of molecular chaperone genes to increase folding activity and reduce protein aggregation, 2) translational attenuation to reduce ER workload and prevent further accumulation of unfolded proteins, 3) ER-associated protein degradation (ERAD) to promote clearance of unfolded/misfolded proteins, and 4) apoptosis when ER function is extensively impaired[10]. This basic response is initiated by a transient inhibition of protein synthesis, followed by transcriptional induction of chaperones and ERAD activation. As long as the UPR can mitigate ER stress, cells can produce proper amounts of proteins in response to the need for them in order to perform their normal functions.

1.2 The UPR Pathways

The UPR has three ER transmembrane master regulators: inositol requiring 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6)[11] (Figure 1.2).

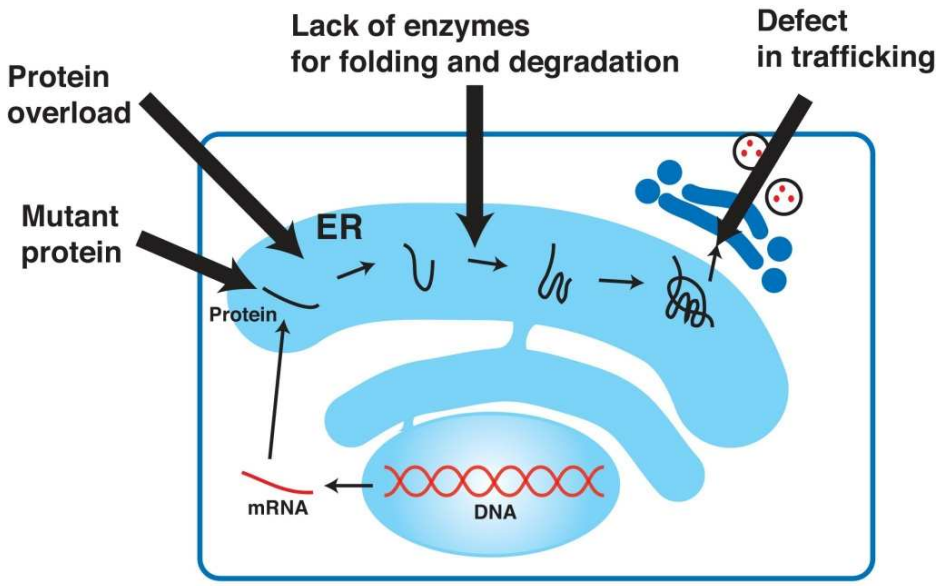

Figure 1.1 Causes of ER Stress in Cells. There are several sources of ER stress including: 1) mutant protein expression, 2) protein overload, 3) shortage of chaperones and foldases, and 4) defects in trafficking.

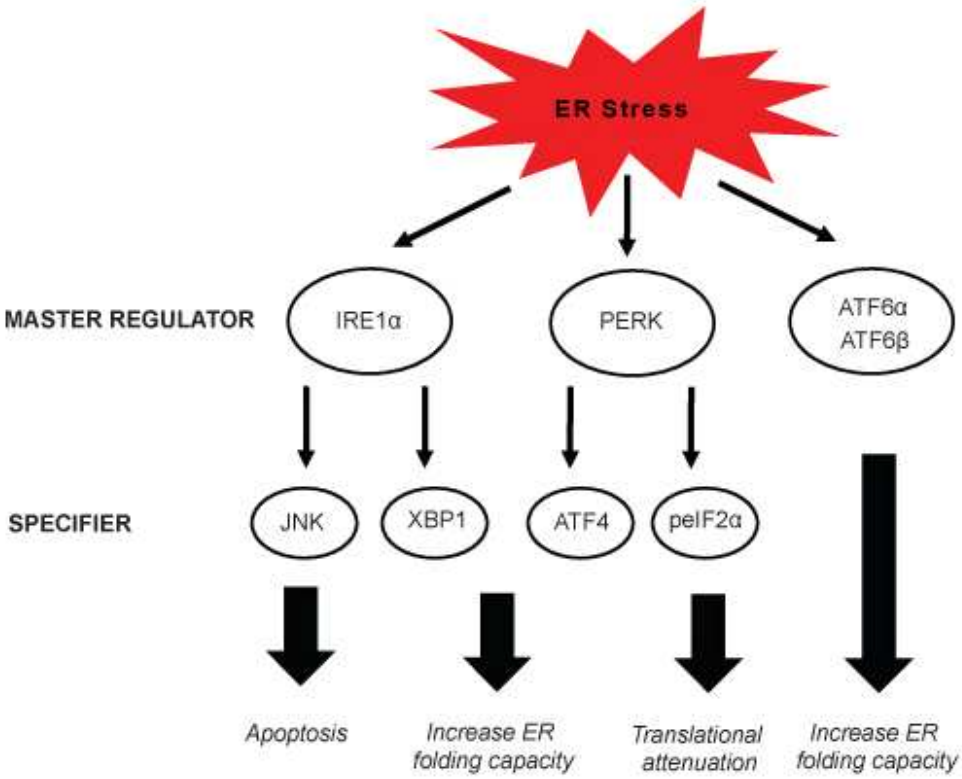

Figure 1.2 ER Stress Signaling Pathways. The UPR has (3) master regulators: IRE1, PERK, and ATF6. Each pathway serves a function in mitigating ER stress by: 1) increasing the folding capacity of the ER or 2) attenuating general protein translation. In cases in which ER stress cannot be mitigated, apoptotic pathways are activated.

Immunoglobulin heavy chain-binding protein (BiP), an ER-resident chaperone, binds to each of these regulators and is thought to keep them in an inactive state when there is no stress[12]. This stable complex is broken when stress is present in the ER lumen, as there is competition between accumulating unfolded/misfolded proteins and the UPR transducers for BiP binding. It is thought that this competitive titration leads to activation of each of these regulators[12, 13].

1.2.1 *IRE1*

IRE1, a central regulator of the UPR, is a type I ER transmembrane kinase/endoribonuclease. Its N-terminal luminal domain acts as a sensor for ER stress[14]. Mammalian cells have two IRE1 isoforms, IRE1α and IRE1β, which both have a function as sensors of the UPR. IRE1α is ubiquitiously expressed, while the β isoform is uniquely expressed in the epithelial cells of the gastrointestinal tract[199-200]. Upon dissociation from BiP, IRE1 dimerizes and undergoes trans-autophosphorylation to become active[15]. Activation of the kinase domain leads to the activation of its endoribonuclease function, whereby the mRNA of the transcription factor X-box binding protein 1 (XBP1) is spliced, resulting in the removal of a 3' 26 nucleotide intron that normally inhibits translation by binding to the 5' end of the mRNA[16-18] (Figure 1.3). Spliced XBP1, produced from this unconventional splicing reaction[19, 20], encodes a basic leucine zipper (b-ZIP) transcription factor, which translocates to the nucleus and binds the ER stress-response element reporter (ERSE) which activates a variety of UPR target genes, including genes that function in ERAD such as ER-degradation-enhancing-α-mannidose-like protein (EDEM)[21], as well as genes encoding foldases such as protein disulfide isomerase (PDI)[22]. In this manner, the IRE1-XBP1 pathway functions to increase the folding capacity of the ER. In addition to splicing XBP1, IRE1 has also been shown to mediate the cleavage of

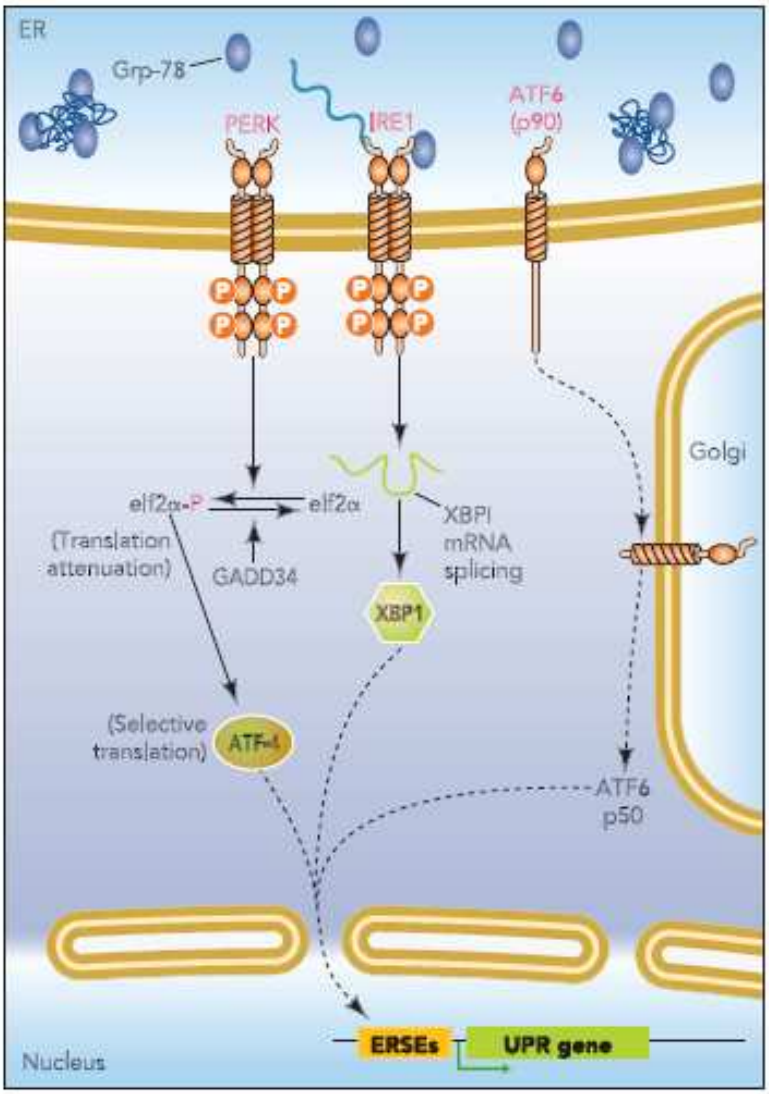

Figure 1.3 Master Regulators of the UPR. IRE1, PERK, and ATF6 are all activated by ER stress. BiP (GRP78) dissociates from these master regulators in the presence of unfolded proteins. Once activated, each regulator activates downstream targets which serve to mitigate ER stress[202].

additional mRNAs targeted to the ER[203]. There is also another known XBP1-independent pathway of IRE1, in which Jun N-terminal kinase (JNK) is the downstream effector. This pathway is activated when IRE1 activation is prolonged, or there is a state of chronic ER stress. This leads to the recruitment of TNF-receptor-associated factor 2 (TRAF2) by IRE1, activation of apoptosis-signaling-kinase 1 (ASK1), and consequently activation of JNK, leading to apoptosis[23-25].

1.2.2 *PERK*

PERK, a second transducer of the UPR, is primarily responsible for regulating protein synthesis during ER stress[26]. Like IRE1, it is a type I ER transmembrane kinase and its N-terminal luminal domain is sensitive to ER stress. When released from BiP, PERK dimerizes and undergoes trans-autophosphorylation via its cytoplasmic kinase domain[27]. Activated PERK directly phophorylates Ser51 on the α subunit of eukaryotic initiation factor 2 (eIF2α), its only identified target[28] (Figure 1.3). This in turn inhibits the formation of ribosomal initiation complexes and recognition of AUG initiation codons, hindering general protein synthesis[29]. This reduces the ER workload and protects cells from apoptosis[26]. It has been suggested that this cell survival role of PERK is mediated by the repression of cyclin D and p53 expression, which leads to cell cycle arrest[205], as well as through the activation of NF-κB as a result of inhibited IκB translation[206]. Concomitant with the inhibition of general translation is the selective translation of UPR target genes, as these polycistronic mRNAs have inhibitory upstream open reading frames (uORFs) and are thus preferentially translated by the ribosome[30]. One of these mRNAs is that of activating transcription factor 4 (ATF4), a b-ZIP transcription factor that regulates UPR targets such as C/EBP-homologous protein (CHOP) and growth arrest

and DNA damage inducible gene 34 (GADD34)[31], as well as genes involved in redox balance and amino acid synthesis[32]. Translational recovery is mediated by GADD34, a phosphatase which dephosphorylates eIF2α[204].

1.2.3 *ATF6*

ATF6 is the third transducer of the UPR and mediator of transcriptional induction. It is a type II ER transmembrane transcription factor[33]. When BiP dissociates from ATF6 upon ER stress, ATF6 senses stress in its N-terminal luminal domain and is released from the ER membrane. This dissociation also unmasks a Golgi complex localization signal[38] that allows ATF6 to transit to the Golgi where it is proteolytically cleaved by site 1 (S1) and site 2 (S2) proteases, generating an active b-ZIP factor[34] (Figure 1.3). This mechanism of activation is referred to as regulated intramembrane proteolysis (RIP). This processed form of ATF6, also referred to as p60 ATF6, translocates to the nucleus where it binds to several promoter elements in ER stress response genes and primarily upregulates UPR targets involved in protein folding such as BiP, XBP1, glucose regulated protein 94 (GRP94), PDI, and calreticulin[35, 36]. ATF6 also heterodimerizes with XBP1 to induce ERAD genes[152]. ATF6 binding to some promoters requires an additional transcription factor, nuclear transcription factor-Y (NF-Y)[148]. ATF6 transcriptional activity has also been shown to be enhanced by phosphorylation[207]. There are two isoforms of ATF6, ATF6α and ATF6β, with fairly ubiquitous tissue distribution. The α isoform has been shown to be solely responsible for transcriptional induction of ER chaperones[152]. It has been reported that unprocessed ATF6 is unstable and quickly degraded by the ubiquitin-proteasome pathway to prevent hyperactivation of the UPR[37].

1.3 Chronic ER Stress and Cell Death

There are two protein quality control outputs of the UPR: homeostatic outputs and apoptotic outputs (Figure 1.4). The goal of the UPR is to restore homeostasis, however, this may involve apoptosis to ensure that highly stressed cells do not produce damaged secretory proteins. Thus, ER stress can be classified into two groups: physiological and pathological[39]. Physiological stress occurs when there is a high demand for protein load. This is acute stress that is readily mitigated by the UPR. One example of this is acute post-prandial ER stress which occurs in the pancreatic β-cell – there is a high demand for insulin biosynthesis following a meal. This category of stress is beneficial to the cell and promotes insulin biosynthesis in these cells[40].

When ER stress is severe or prolonged such that it cannot sufficiently be resolved by the UPR, cell death occurs via apoptosis[41,42]. This prolonged stress can be attributed to an insufficient UPR response, or hyperactivation of a component of the UPR (i.e. the UPR is not regulated properly). An example of UPR hyperactivation leading to apoptosis can be seen when pancreatic β-cells are treated with an inhibitor of eIF2α dephosphorylation, salubrinal[59]. Persistent activation of the PERK-eIF2α pathway is deleterious to these cells, most likely due to the inhibition of general translation. There are at least three pathways involved in ER stress-dependent apoptosis which culminate in the activation of the effector cysteine protease, caspase-3: transcriptional activation of the transcription factor CHOP, activation of the kinase JNK, and activation of ER-associated caspase-12[43] (Figure 1.5).

1.3.1 *CHOP Pathway*

One of the main initiators of apoptosis by ER stress is CHOP activation through the PERK-ATF4 and ATF6 pathways[31, 36]. CHOP is a b-ZIP transcription factor that is a member of

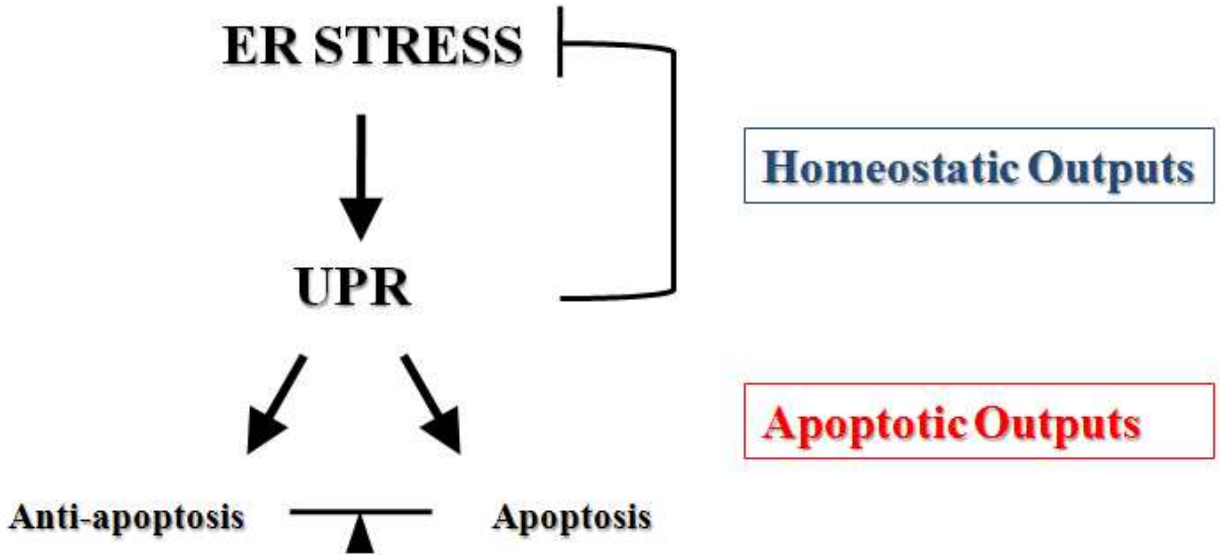

Figure 1.4 Outputs of the UPR. There are (2) main outputs of the UPR for protein quality control: 1) homeostatic outputs and 2) apoptotic outputs.

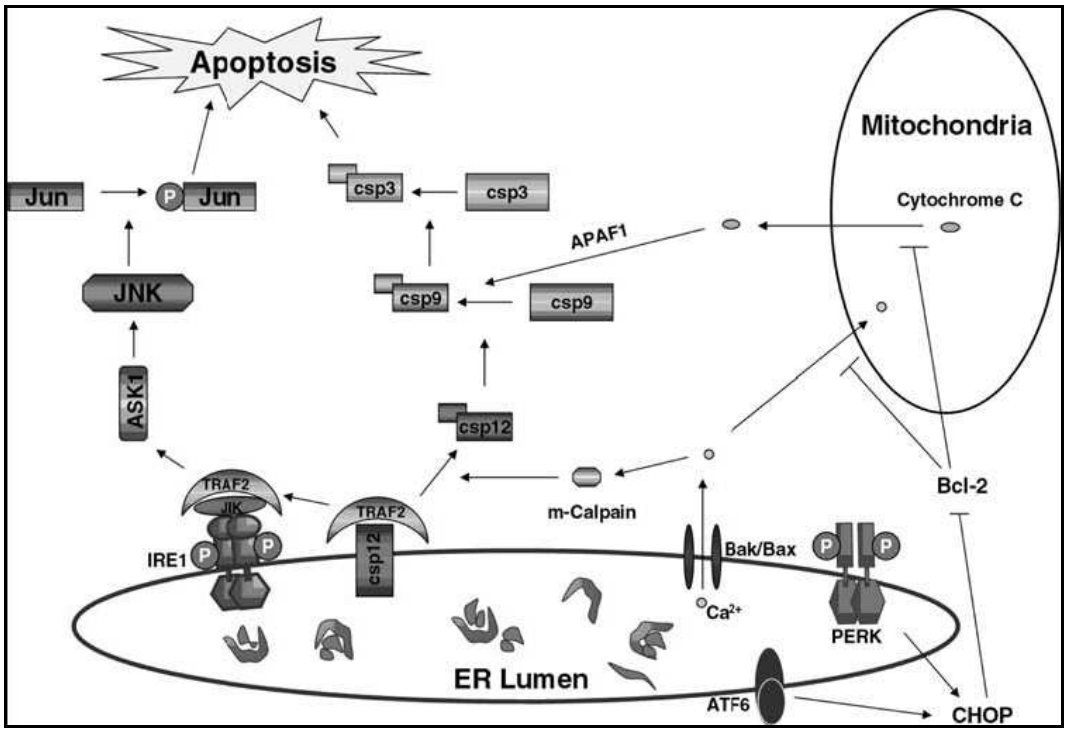

Figure 1.5 ER Stress-Mediated Apoptotic Pathways. There are (3) main apoptotic pathways which are activated when ER stress is severe and cannot be resolved: 1) IRE1-JNK, 2) Caspase-12, and 3) CHOP. Activation of these pathways results in the activation of capase-3 and induction of apoptosis[39].

the C/EBP family[208]. CHOP does not induce apoptosis directly, rather, CHOP activation leads to the repression of the B cell lymphoma 2 (Bcl-2) gene promoter[44, 45]. The Bcl-2 gene encodes an anti-apoptotic protein that inhibits cytochrome c release in the cytosol. CHOP induction of carbonic anhydrase IV[49], death receptor 5 (DR5)[50], tribbles-related protein 3 (TRB3)[51], and Bcl-X_L[44], may also be involved in ER stress-mediated apoptosis. In support of this, CHOP deletion promotes cell survival in mouse embryonic fibroblasts (MEFs) and several mouse models of diabetes[46-48]. However, CHOP activation is not the only pathway involved in ER stress-mediated apoptosis. CHOP is not upregulated in PERK -/- cells in responses to stress, however, these cells still undergo ER stress-induced cell death[64, 209].

1.3.2 *IRE1-JNK Pathway*

Another initiator of ER stress-mediated apoptosis is activation of JNK through the XBP1-independent pathway of IRE1, which occurs when persistent activation of IRE1 is present. Chronic activation of IRE1 leads to the IRE1-dependent recruitment of TRAF2, an adaptor protein that couples JNK activation to plasma membrane receptors through the recruitment of ASK1[24]. This has been shown to be regulated by c-Jun N-terminal inhibitory kinase (JIK). JIK overexpression promotes an interaction between IRE1 and TRAF2 and consequently JNK activation[210]. The mechanisms underlying the downstream targets of ER stress-mediated JNK activation have yet to be defined, however, proteins of the Bcl-2 family are thought to also be involved. Activation of JNK has been shown to lead to phosphorylation of Bcl-2 and Bcl-X_L and consequently inhibition of their anti-apoptotic functions[52-55].

1.3.3 *Caspase-12 Cascade*

The third pathway leading to apoptosis is activation of caspase-12 from procaspase-12. This pathway is thought to be ER stress-dependent, as procaspase-12 is cleaved in response to ER stress and not by death receptor- or mitochondria-mediated apoptotic signals[56,57]. Procaspase-9 is the substrate for caspase-12: when caspase-12 is activated, it cleaves procaspase-9 to render caspase-9 active, which in turn activates caspase-3[58]. Calpains, Ca^{2+}-dependent cysteine proteases, have also been shown to play a role in the activation of caspase-12[211].In addition to calpains, caspase-7 and TRAF2 may promote caspase-12 activation[211]. Caspase-12 -/- mice are resistant to ER stress-specific apoptosis, but not to apoptosis from other cell death stimuli[56]. Humans, however, lack functional caspase-12, but it has been suggested that human caspase-4 substitutes the function of caspase-12[212].

1.4 Pancreatic β-cells and the ER

Pancreatic β-cells, localized in specialized cell clusters called islets of Langerhans and composing approximately 2 percent of the pancreas, have the specialized function of controlling blood glucose levels by synthesizing and secreting the hormone insulin[213]. Insulin is secreted in response to acute hyperglycemia which occurs post-prandially. This secretory response stimulates the biosynthesis and translation of the precursor proinsulin in the ER[60]. This process begins as the transcription of preproinsulin is activated. Preproinsulin, which contains a signal peptide, a B chain, a connecting peptide called the C-peptide, and an A chain[213], is synthesized in the cytoplasm and co-translationally translocated into the ER. This translocation occurs

through the Sec61 translocator complex via an interaction between preproinsulin's signal peptide and a signal-recognition particle (SRP) on the ER membrane. The signal peptide is cleaved by a signal peptidase in the ER to generate proinsulin, which then undergoes precise folding in the lumen of the ER. Three disulfide bonds are formed, catalyzed by the ER-resident proteins endoplasmic oxidoreductin 1 (ERO1) and protein disulfide isomerase (PDI). Once proinsulin is properly folded, it is transported to the Golgi apparatus and packaged into secretory granules. Within these granules, proinsulin is processed to yield mature insulin which is then released from the granule in response to glucose stimulation and an elevation in blood glucose levels[61, 62] (Figure 1.6).

β-cells, with their high insulin client load, have been found to be very sensitive to disruptions to ER homeostasis[63]. These cells are exposed to frequent energy fluctuations (i.e. intermittent changes in blood glucose levels), and thus require precise and proper folding of proinsulin to respond to such changes. Any imbalance between the load of insulin translation placed on the ER and folding capacity of the ER leads to ER stress and disruption in β-cell homeostasis. A characteristic feature of these cells is a highly developed ER and high expression of the UPR transducers, IRE1 and PERK[64, 65].

The IRE1-XBP1 signaling pathway is important for ER expansion[72], and the IRE1α isoform of IRE1 is highly expressed in the pancreas[73]. β-cells also have been shown to have a baseline activation of IRE1α, as measured by its phosphorylation. IRE1 signaling additionally has a key role in insulin biosynthesis (Figure 1.7). Suppression of this signaling molecule inhibits proinsulin biosynthesis[65]. This signaling pathway is XBP1- and JNK-independent, however the

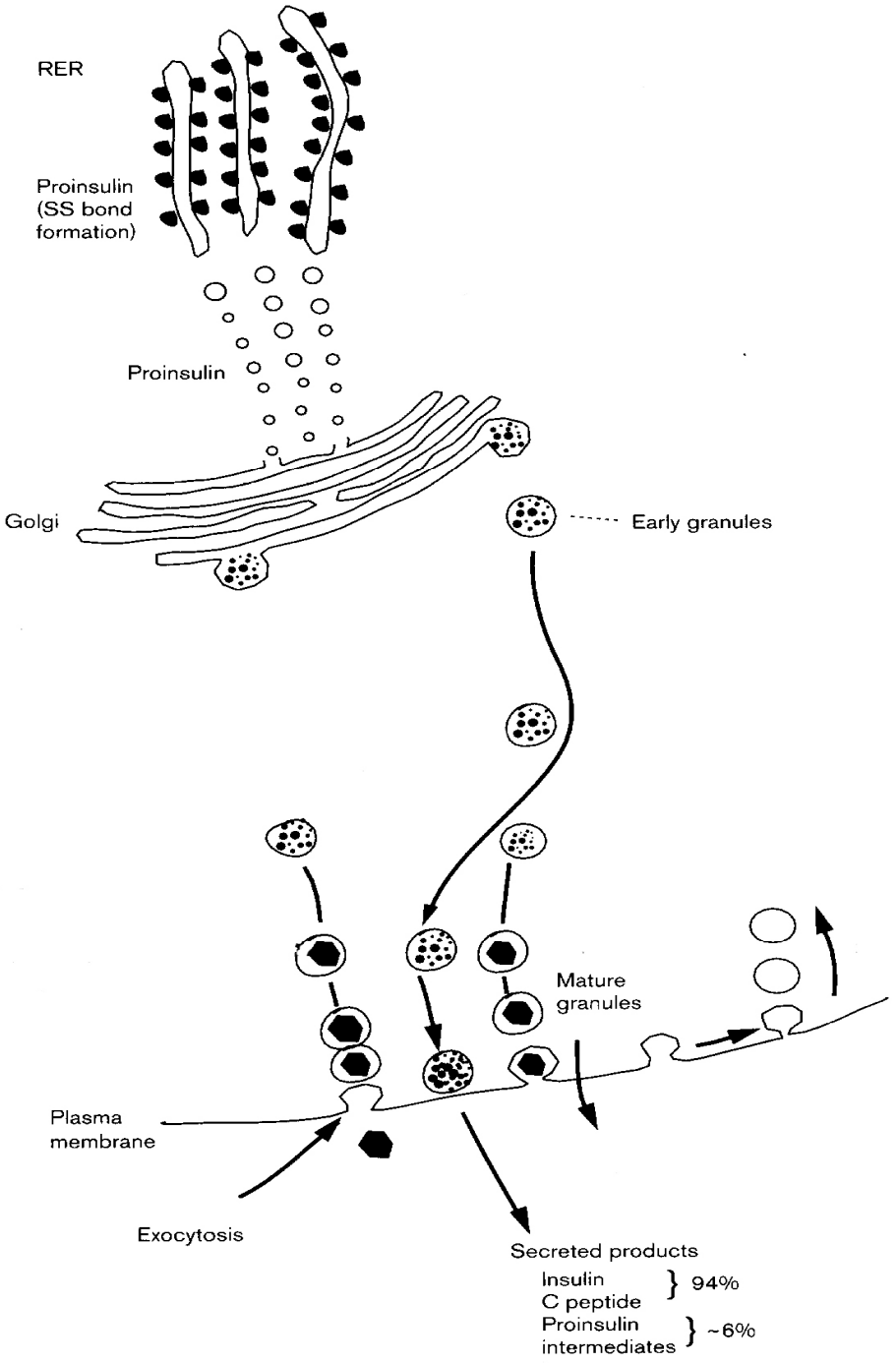

Figure 1.6 Insulin Biosynthesis. Preproinsulin is synthesized and translocated into the rough ER (RER) where its signal peptide is rapidly cleaved to form proinsulin. Here disulfide bonds are formed. Proinsulin is further processed in the Golgi and packaged into clathrin-coated vesicles. Proinsulin is cleaved to produce insulin crystals and C-peptide, which are exocytosed in equimolar proportions into the bloodstream upon stimulation by glucose or other factors. A small amount of proinsulin or other intermediates are also secreted. *Adapted from Dodson G and Steiner D, 1998.*

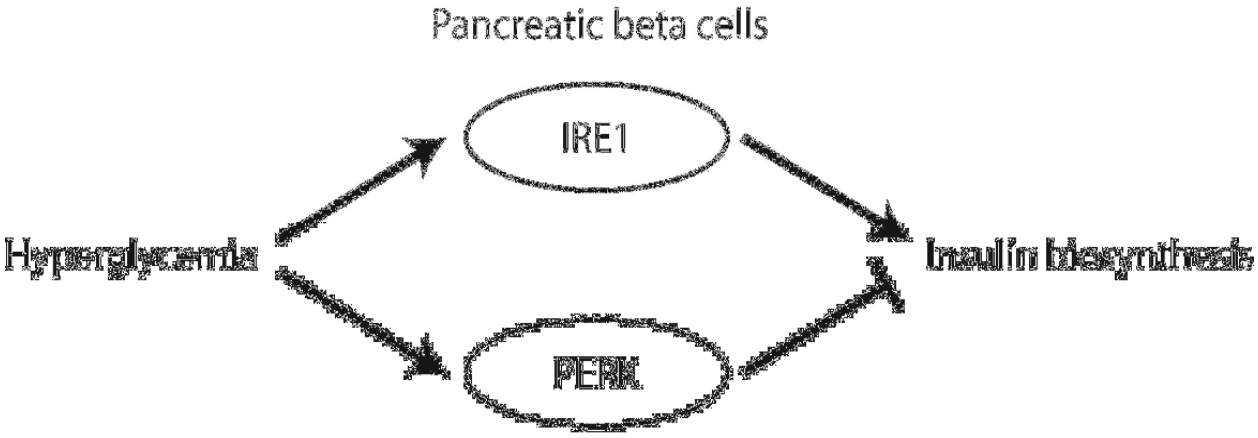

Figure 1.7 Role of IRE1 and PERK in Insulin Biosynthesis. In pancreatic β-cells, IRE1 is a positive regulator and PERK is a negative regulator of insulin biosynthesis.

downstream targets have yet to be identified. It is known, though, that the ER-resident oxidoreductase ERO1α, which functions in proinsulin folding, is upregulated. PERK is also highly expressed in pancreatic islets[74]. PERK phosphorylation (i.e. its activation) is a negative regulator of insulin biosynthesis (Figure 1.7). Insulin biosynthesis from high glucose treatment in PERK -/- mouse islets is enhanced compared to control littermate islets[75]. Recently, it has been demonstrated that ATF6 may also have a function in regulating insulin. Under ER stress, ATF6 is activated, leading to a decrease in insulin gene expression[76], suggesting that ATF6 may have dual functions: positive regulation of ER chaperones and negative regulation of insulin promoter activity.

Physiological ER stress in the β-cell is beneficial and leads to the activation of insulin biosynthesis[65]. This is attributed to acute hyperglycemia. Exposure of these cells to chronic, prolonged hyperglycemia, however, induces pathological ER stress. This leads to β-cell dysfunction and death (Figure 1.8). There are other causes of pathological ER stress in these cells: exposure to long-chain free fatty acids (e.g. palmitate)[66, 67], hyperinsulinemia which occurs in the pre-diabetic stage[68], glucose deprivation[69], islet amyloid polypeptide (IAPP) expression[70], and exposure to inflammatory cytokines such as IL-1β and IFN-γ[71] (Figure 1.9).

1.5 The Pathogenesis of Diabetes

Diabetes mellitus, defined by chronic hyperglycemia, is categorized into two groups: type 1 and type 2. The pathogenesis of both types is characterized by a reduction in the ability of the pancreatic β-cell to synthesize and secrete insulin, as well as a decrease in β-cell mass by

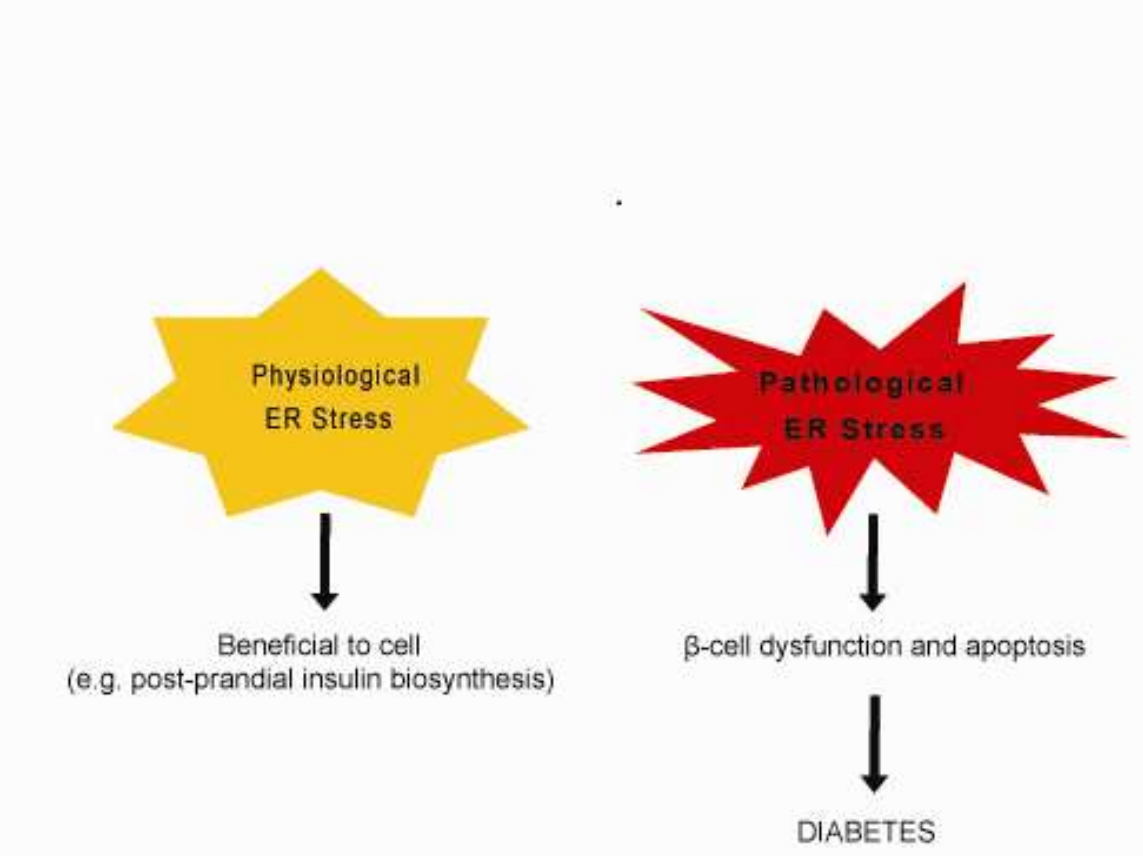

Figure 1.8 Types of ER Stress. There are two types of ER stress: physiological and pathological. Physiological ER stress is beneficial to the β-cell and activates insulin biosynthesis. Pathological ER stress, however, occurs in these cells when ER stress is chronic and not resolved by the UPR. This chronic stress can lead to β-cell dysfunction and apoptosis, which can ultimately lead to diabetes.

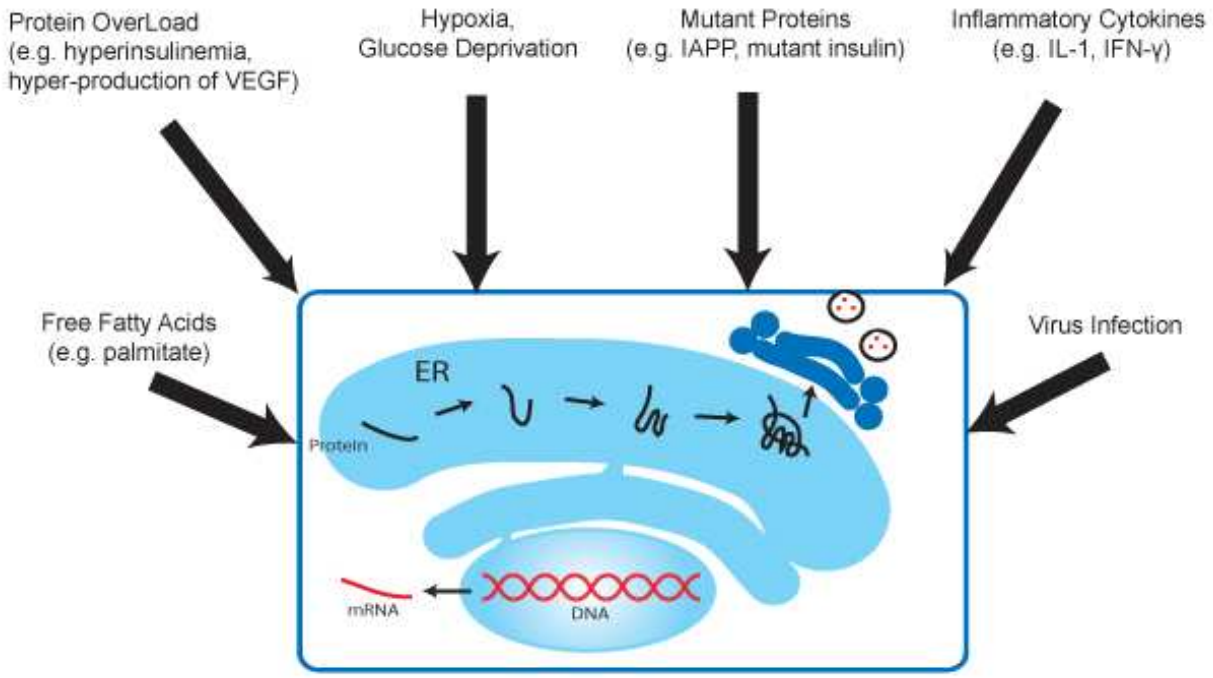

Figure 1.9 Sources of ER Stress in β-cells. There are several causes of ER stress in the β-cell including: 1) exposure to long-chain free fatty acids, 2) hyperinsulinemia, 3) glucose deprivation, 4) mutant insulin expression, 5) exposure to inflammatory cytokines, and 6) viral infection.

apoptosis[77, 78], which leads to 1) toxic glucose build-up in the blood and 2) energy depletion in peripheral tissues such as muscle, liver, and adipose. In type 1, an autoimmune response leads to the absolute deficiency of insulin, whereas type 2 is defined by a non-autoimmune relative deficiency of insulin coupled with defective insulin signaling. Of the diagnosed cases of diabetes, approximately 95% are type 2. It is predicted that the number of affected individuals with type 2 diabetes in the world will reach well over 300 million by the year 2025[79]. It is the sixth leading cause of death by disease in the United States and its estimated cost to the health care industry is over $100billion/year[80]. Diabetes in general, therefore, is a significant problem. This is a progressive disease which leads to β-cell dysfunction and ultimately β-cell failure, as these cells are no longer able to secrete enough insulin due to defects in glucose-stimulated insulin secretion (GSIS) and a reduction in β-cell mass[81]. In type 2 diabetes, β-cell loss is primarily due to a reduction in β-cell number due to an increase in β-cell apoptosis, as seen in animal models and type 2 diabetic patients[82, 83]. Chronic hyperglycemia (glucotoxicity) and chronic hyperlipidemia (lipotoxicity) are two major factors contributing to this β-cell loss[84, 85], however the underlying mechanisms of this loss are not well understood. Because β-cell dysfunction and apoptosis are main pathological components of this disease, there has been a focus in research to understand these underlying mechanisms. Recent data suggests that ER stress contributes to β-cell death, autoimmunity, and insulin resistance in patients with diabetes.

1.6 ER Stress-Mediated Diabetes

ER stress and defects in the UPR have recently been implicated in type 1 and type 2 diabetes. Pancreatic β-cells are very sensitive to perturbations in ER homeostasis which can lead

to the induction of apoptosis[63] seen in both forms of diabetes. ER stress may also lead to the production of "neo-autoantigens" caused by insulin misfolding in the ER of the β-cell[86], which may initiate the autoimmune response in type 1 diabetes.

1.6.1 *Genetic Forms of ER Stress-Mediated Diabetes*

The relationship between ER stress and diabetes was first demonstrated in a rare autosomal recessive form of juvenile diabetes, Wolcott-Rallison syndrome. In 1972, Wolcott and Rallison described two brothers and a sister with infancy-onset diabetes mellitus and multiple epiphyseal dysplasia[87]. In this syndrome, mutations have been reported in the *EIF2AK3* gene encoding PERK[88]. Because these mutations are within the catalytic domain of PERK, it is likely that they cause a loss-of-function of PERK kinase activity. This loss of PERK kinase activity leads to the reduction in the phosphorylation of eIF2α, a substrate of PERK. When a high workload is placed on the ER of the β-cell, for example when insulin demand increases post-prandially, phosphorylation of eIF2α is essential in mitigating ER stress and thereby promotes cell survival[26]. Therefore, a loss-of-function of PERK and a consequent disruption in translational attenuation during ER stress via decreased eIF2α phosphorylation, could directly attribute to β-cell apoptosis. This has been illustrated in several animal models. Indeed, PERK -/- mice develop diabetes due to excessive ER stress in their β-cells causing β-cell apoptosis[75]. Mutant mice carrying a heterozygous mutation in the phosphorylation site of eIF2α ($Eif2s1^{+/tm1Rjk}$) become obese and, due to β-cell dysfunction, diabetic when fed a high-fat diet[89]. Collectively, these observations suggest that β-cell apoptosis in Wolcott-Rallison patients is caused by excessive, unresolved ER stress and a defect in the UPR (i.e. PERK signaling).

The negative regulator of PERK signaling, $P58^{IPK}$, also functions in maintaining ER homeostasis in β-cells. $P58^{IPK}$ is an important component of a negative feedback loop used by these cells to inhibit eIF2α signaling and attenuate the UPR[90]. $P58^{IPK}$ knockout mice show a gradual onset of glucosuria and hyperglycemia associated with increased apoptosis of islet cells[91], thus this UPR component may be involved in the pathogenesis of diabetes in humans.

Permanent neonatal diabetes may also be attributed to excessive ER stress in the β-cell. Neonatal diabetes is defined as insulin-requiring hyperglycemia within the first month of life. This is typically associated with slowed intrauterine growth and is a rare disorder. Permanent neonatal diabetes, considered a genetic disorder, can be caused by several types of mutations, including mutations in insulin promoter factor 1 (IPF-1), and results in lifelong dependence on insulin injections. It has recently been shown that mutations in the human insulin gene can also cause this disorder[92]. This is an autosomal dominant disorder, with mutations primarily occurring in critical regions of preproinsulin. This presumably leads to improper folding of insulin, triggering the UPR. Severe ER stress leads to β-cell apoptosis. In the mouse models of this disease, the Munich and Akita mouse, mice have a dominant missense mutation in the Ins2 gene[93, 94]. In the Munich mouse, there is a $cysteine^{95}$-to-serine substitution, leading to a loss in an interchain disulphide bond of proinsulin. In the Akita mouse, there is a $cysteine^{96}$-to-tyrosine substitution. This mutation also leads to disruption of disulphide formation between the A and B chain of proinsulin, causing insulin to misfold and accumulate in the ER of the β-cell[94]. This accumulation of misfolded insulin leads to severe ER stress, β-cell apoptosis, and consequently diabetes[95].

1.6.2 *ER stress in Type 1 Diabetes*

Increasing evidence supports the role of ER stress-mediated β-cell death in the pathogenesis of type 1A diabetes (i.e. autoimmune diabetes). The baseline of ER stress in β-cells is higher than that of other cell types due to their exposure to frequent energy fluctuations and high client load, insulin. It is therefore possible that any additional ER stress applied to these cells by genetic or environmental factors can lead to cell death. β-cells that undergo apoptosis as a consequence of this additional, unresolved ER stress contain misfolded proteins that can act as "neo-autoantigens" – dendritic cells in the islets engulf ER stress-induced apoptotic β-cells and stimulate the maturation of β-cell-reactive T cells that mediate autoimmune destruction of remaining β-cells[96]. There are several insults to the β-cell that can lead to excessive, unresolved ER stress, triggering an apoptotic cascade and leading to the production of "neo-autoantigens:" viral infections, other environmental stresses, as well as nitric oxide (NO) (Figure 1.10).

NO plays an important role in β-cell apoptosis in type 1 diabetes[97]. Inflammatory cytokines such as γ-interferon (IFN-γ) and interleukin-1β (IL-1β) in β-cells induce the production of NO, which leads to β-cell failure and consequently cell death. There is evidence that this process is mediated by ER stress[98]. Production of NO leads to the attenuation of the sarcoendoplasmic reticulum pump Ca^{2+} ATPase2b (SERCA2b) and consequently the reduction of calcium in the ER. This calcium depletion leads to severe ER stress and the induction of the pro-apoptotic transcription factor CHOP[99, 100]. It has been shown that CHOP is induced by a NO donor, S- nitroso-N-acetyl-$_{D,L}$-penicillamine (SNAP), and pancreatic islets from CHOP -/- mice are resistant to NO-induced apoptosis[98].

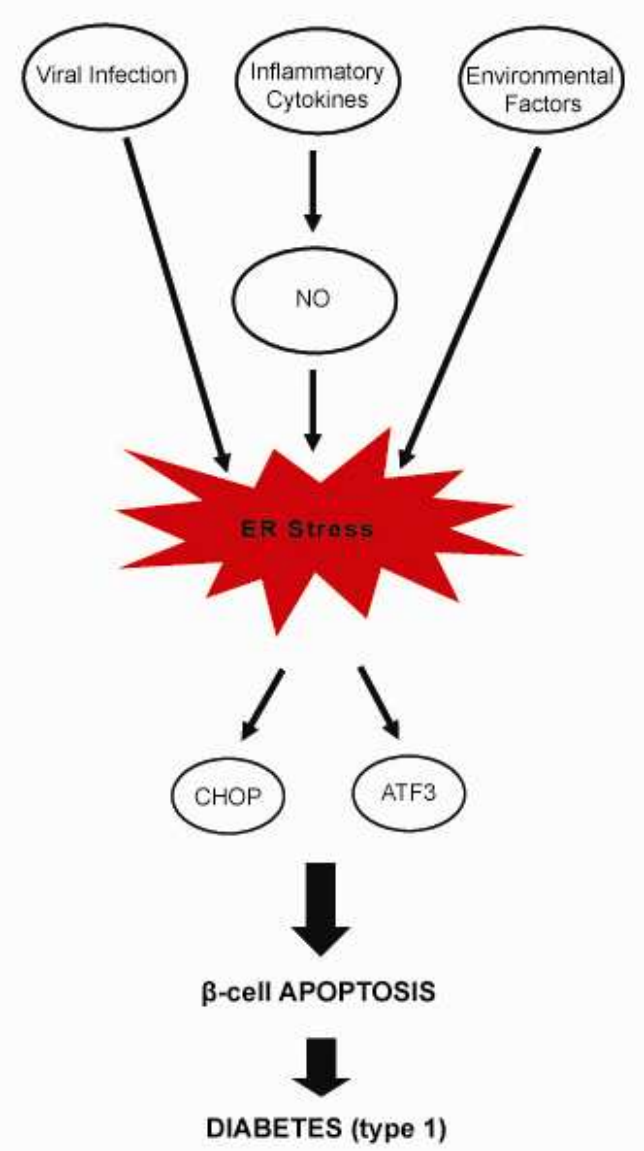

Figure 1.10 ER stress and Type 1 Diabetes. Viral infection, nitric oxide (NO), and other environmental factors cause ER stress in the β-cell leading to CHOP and ATF3 activation, and consequently apoptosis. These apoptotic β-cells are a potential source of "neo-autoantigens" which can be a potential source of an autoimmune attack, leading to type 1 diabetes.

Activating transcription factor 3 (ATF3), a pro-apoptotic transcription factor of the ATF/CREB family, may also contribute to ER stress-mediated apoptosis in type 1 diabetes. ATF3 is induced by pro-inflammatory cytokines and NO. ATF3 knockout mouse islets are partially protected from NO- and cytokine-induced β-cell apoptosis, while overexpression of this transcription factor in mouse islets leads to β-cell dysfunction[101].

1.6.3 *ER stress in Type 2 Diabetes*

As mentioned earlier, a contributing factor to the pathogenesis type 2 diabetes is the reduction of β-cell mass[77]. Resistance to insulin action in peripheral tissues (i.e. adipose, muscle, and liver) is one of the primary presenting features of this disorder. This insulin resistance leads to the hyper-production of insulin (i.e. hyperinsulinemia) in the β-cell. Hyperglycemia and type 2 diabetes develops only in patients that are unable to sustain this compensatory response of the β-cell[102]. This increase in insulin biosynthesis overwhelms the folding capacity of the ER, leading to chronic activation of the UPR. This chronic, hyperactivation of ER stress signaling can lead to β-cell dysfunction and death. There are several components of ER stress signaling that could contribute to this β-cell loss: IRE1-JNK, CHOP, and glycogen synthase kinase 3β (GSK3β). The IRE1 pathway is important in insulin biosynthesis, where transient increases in insulin production lead to IRE1 activation[40]. Chronic activation of IRE1 during prolonged increases in insulin biosynthesis, however, may lead to β-cell death through IRE1-mediated activation of JNK[24]. CHOP is also an important player of ER stress-mediated β-cell death and may promote the progression of type 2 diabetes[48]. A third signaling component, GSK3β, also plays a role in β-cell death caused by ER stress. GSK3β is a substrate of the survival kinase, Akt[103], and it has been demonstrated that attenuation of Akt

phosphorylation during ER stress mediates dephosphorylation of GSK3β, leading to ER stress-mediated apoptosis[104].

Insulin resistance, a feature of type 2 diabetes, leads to β-cell exhaustion, glucotoxicity, and hyperinsulinemia which place a massive strain on the ER of the β-cell[84]. This, however, is not the only source of stress for the ER. It has recently been shown that free fatty acids (FFAs), specifically long-chain FFAs, also induce β-cell apoptosis[59, 66-67]. Treatment of β-cell lines with the long-chain FFA, palmitate, increases levels of ER stress markers such as ATF4 and spliced XBP-1. In addition, it has been shown that circulating FFAs lead to β-cell lipotoxicity and consequently excessive ER stress[105].

Recent studies also show an involvement of ER stress in insulin resistance of liver, muscle, and adipose tissues. IRE1-JNK signaling plays an important role in the insulin-resistant liver tissue of type 2 diabetes patients. Obesity leads to hyperactivation of JNK signaling due to severe ER stress, leading to serine phosphorylation of insulin receptor substrate-1 (IRS-1), which inhibits insulin action[106]. Like β-cells, hepatocytes have a high baseline ER stress level[107], and therefore may be sensitive to additional ER stress. It has been shown that high ER stress in liver cells can be resolved via overexpression of the ER-resident chaperone oxygen-regulated protein 150 (ORP150), while suppression of this chaperone in mice inhibits insulin sensitivity[108] (Figure 1.11).

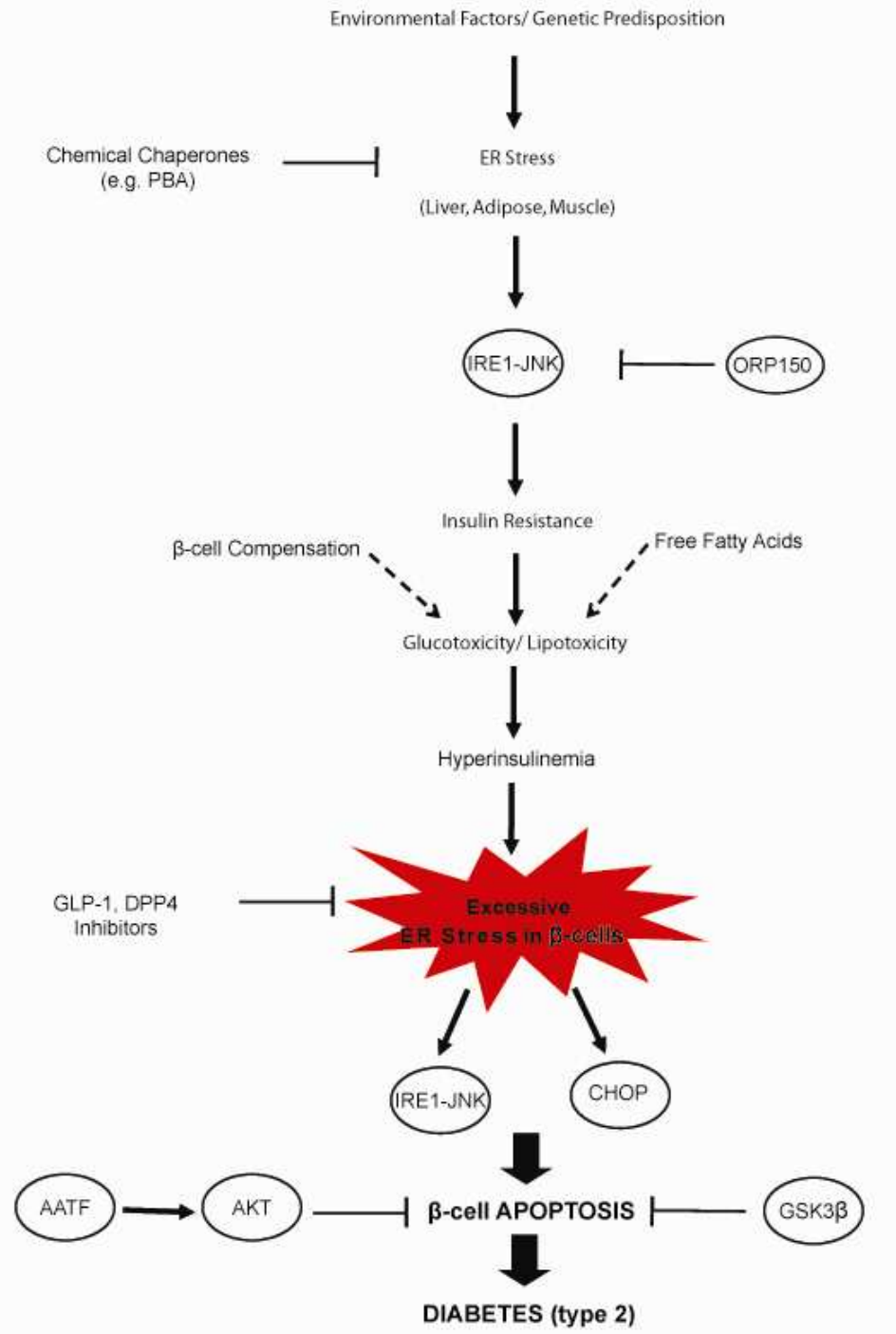

Figure 1.11 ER Stress and Type 2 Diabetes. ER stress can lead to insulin resistance which leads to β-cell compensation (i.e. hyperinsulinemia). Hyperinsulinemia (glucotoxicity) and exposure of β-cells to circulating free fatty acids (lipotoxicity) leads to excessive ER stress in these cells, leading to apoptosis and type 2 diabetes.

1.7 Wolfram Syndrome: A Link Between Diabetes and a Defective UPR

1.7.1 *WFS1: The Causative Gene for Wolfram Syndrome (WFS)*

The focus of this thesis is the role of a gene, Wolfram syndrome 1 (WFS1), in ER stress signaling and the pathogenesis of diabetes. When we began this thesis research, there was preliminary evidence that suggested that WFS1 was localized to the membrane of the ER – most proteins localized to the ER membrane are in some way involved in the UPR. WFS1 was of particular interest, as in 1998, mutations in this gene were linked to a form of diabetes called Wolfram syndrome[112] in which selective β-cell loss occurs. The question that we wanted to answer was what was the mechanism of this cell death and why was it specific to the β-cell.

Wolfram syndrome (WFS), a rare autosomal recessive disorder characterized by diabetes mellitus (DM) and optical atrophy (OA), was first described by Wolfram and Wagener in 1938[109]. This syndrome is also described as DIDMOAD (Diabetes Insipidus, Diabetes Mellitus, Optical Atrophy, and Deafness), as patients also present with secondary symptoms in addition to DM and OA, however DM and OA are the only necessary symptoms to make a diagnosis. DM presents in the first decade of life, while OA follows in the second decade[110, 111]. Postmortem studies reveal a non-autoimmune-linked selective loss of pancreatic β-cells[113]. The nuclear gene responsible for this syndrome was identified by two separate groups in 1998 and named WFS1[112, 114].

WFS1 has been shown to be mutated in 90% of patients with WFS[115]. More than 100 mutations of the WFS1 gene have been identified, most of which are inactivating mutations and located in exon 8 which encodes the protein's transmembrane and C-terminal domains[116-19].

The WFS1 protein has been shown to be localized to the ER, and while ubiquitously expressed, it is highly expressed in the pancreas[120-22]. This thesis work has identified WFS1 as being specifically localized to the β-cell of the pancreas.

1.7.2 *Significance of Studying the Pathogenesis of WFS*

WFS is a rare disease, affecting approximately 1/100,000 in North America with higher frequency in the Middle East and East Asia[123]. Despite the rarity of this disease, the carrier frequency is estimated at 1/100[123]. This thesis work illustrates WFS is an ER stress-mediated form of diabetes. β-cells in which the function of WFS1 is suppressed, exhibit high levels of ER stress. These high levels of ER stress and β-cell death attributed to WFS, may also be related to more common forms of diabetes. It has been demonstrated by various studies that first degree relatives of WFS patients, as well as carriers, have a higher prevalence of type 2 diabetes than expected by chance[115, 124-25]. This is supported by recent genome-wide studies which have identified WFS1 single nucleotide polymorphisms (SNPs) are associated with an increased risk for type 2 diabetes[126-31]. Additionally, preliminary studies show a link between WFS1 mutations and type 1A diabetes (i.e. autoimmune juvenile diabetes)[132-33]. This thesis focuses on determining the function of WFS1 in order to better understand its role in the pathogenesis of Wolfram syndrome, as well as common forms of diabetes. By determining its function, WFS1 could become a target for diabetes prevention and/or therapy.

1.7.3 *Summary of Thesis Research: Linking WFS, a Defective UPR, and Diabetes*

WFS1 has no homology with any known proteins and until this work, its function was unknown. Because WFS1 is an ER transmembrane protein, we hypothesized that it is a

component of ER stress signaling. Indeed, this thesis illustrates that WFS1 is a novel component of the UPR and is localized specifically to the β-cell of the pancreas. This data was supported by studies which showed that mice lacking WFS1 develop diabetes due to excessive ER stress in their β-cells, leading to β-cell apoptosis[134-35]. We demonstrate that WFS1 is a downstream target of IRE1 and PERK signaling, and is upregulated during ER stress. It is a vital component of the β-cell, as a lack of functional WFS1 is detrimental to these cells. Here we show that WFS1 also controls a regulatory feedback loop of the ER stress signaling network, by regulating the protein levels of the ER transcription factor, ATF6. This body of work demonstrates that WFS1 has an important function in the negative regulation of the UPR, and our data suggests that WFS1 prevents secretory cells (i.e. β-cells) from premature death caused by hyperactivation of this signaling pathway. Figure 1.12 summarizes these novel findings of WFS1 function.

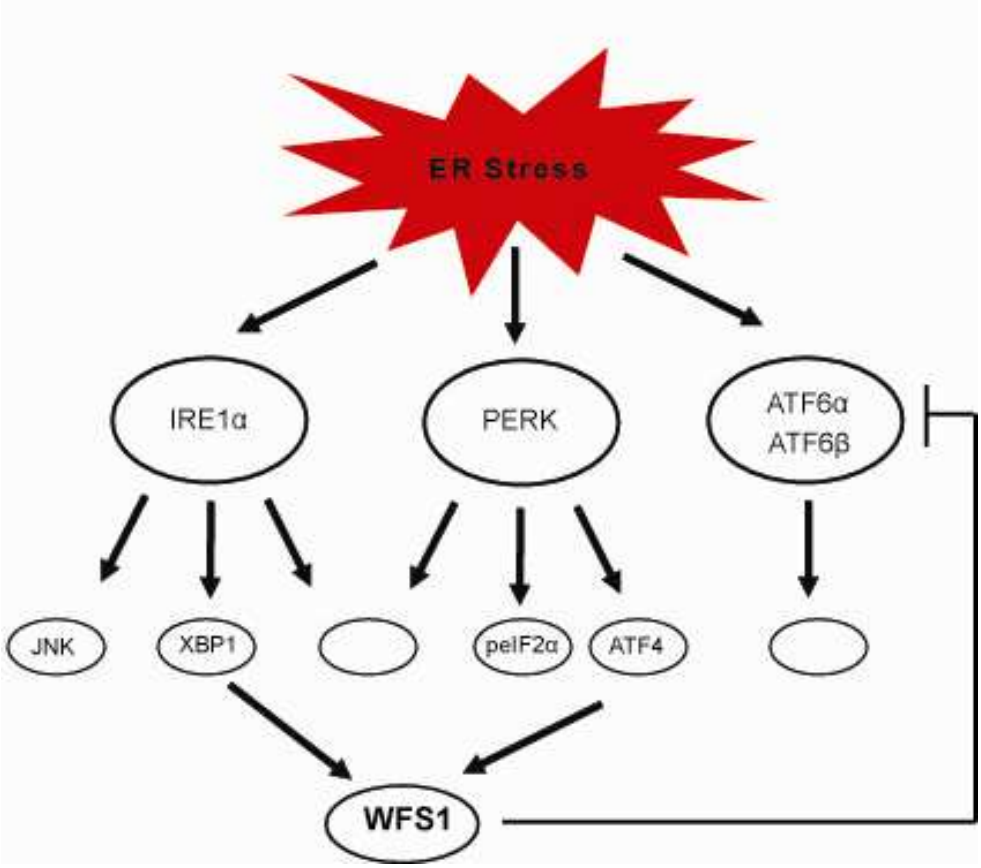

Figure 1.12 WFS1 is a Novel Component of the UPR and Regulates this Signaling Cascade. WFS1 is a downstream target of IRE1 and PERK signaling and is upregulated during ER stress. WFS1 regulates ATF6 protein through the ubiquitin-proteasone pathway and acts as a negative regulator of this pathway.

CHAPTER II

WFS1 IS A NOVEL COMPONENT OF ER STRESS SIGNALING AND MAINTAINS ER HOMEOSTASIS IN PANCREATIC β-CELLS

SUMMARY

In Wolfram syndrome, a rare form of juvenile diabetes accompanied by optical atrophy, cell death of insulin-producing pancreatic β-cells occurs, but in the absence of an autoimmune response. It had been reported that mutations in the WFS1 gene are responsible for the development of this disease, however, the underlying mechanisms behind this selective destruction of the β-cell caused by these mutations were not clear. The data in this dissertation chapter reveal that WFS1 is a novel component of the unfolded protein response (UPR) and has an important function in the maintenance of endoplasmic reticulum (ER) homeostasis in the pancreatic β-cell. An imbalance between the protein folding capacity of the ER and demand placed on this organelle, disrupts ER homeostasis and elicits ER stress. The UPR is a cytoprotective, adaptive response to mitigate this stress, however, if the UPR is dysfunctional and cannot properly abate stress, the cell will undergo apoptosis. WFS1 encodes an ER transmembrane glycoprotein and its mRNA and protein are induced by ER stress. Its expression is regulated by two master regulators of the UPR, inositol requiring 1 (IRE1) and PKR-like ER kinase (PERK), both ER transmembrane kinases. Suppression of WFS1 in β-cells causes ER stress and β-cell dysfunction. Thus, this indicates that the pathogenesis of Wolfram syndrome involves chronic ER stress and apoptosis in pancreatic β-cells due to a loss-of-function of WFS1.

INTRODUCTION

In 1938, Wolfram and Wagener analyzed eight siblings, four of which had a combination of juvenile diabetes and optical atrophy, providing the first report of Wolfram syndrome (WFS)[109]. A significant portion of patients with this disease also develop diabetes insipidus and auditory nerve deafness, thus this syndrome is also described as DIDMOAD syndrome (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, and Deafness)[110,136], however, diabetes mellitus (DM) and optic atrophy (OA) are the only minimal criteria to make a diagnosis: DM presents in the first decade of life, while OA typically follows in the second decade of life[111]. Of the other symptoms, 73% of patients develop diabetes insipidus and 62 % develop sensoneural deafness in the second decade, 58% develop renal tract complications in the third decade, and 62% develop neurological defects in the fourth decade. The median age of death is thirty years, primarily due to complications of DM, infection, respiratory failure, and suicide (Figure 2.1).

Although WFS patients are generally not obese, nor do they have insulitis, postmortem studies reveal a selective loss of β-cells in their pancreatic islets[113]. Before this thesis research was started, the mechanism underlying this cell death was unknown. The nuclear gene responsible for this syndrome was identified by two separate groups in 1998 and named WFS1[112, 114]. Families that exhibit WFS share mutations in a gene encoding WFS1 protein – WFS1 is mutated in 90% of WFS patients[112]. More than 100 mutations of WFS1 have been identified, most of which are inactivating and located in the region which encodes the transmembrane and C-terminal domain of the protein, exon 8[116-19]. In a survey of patient mutations, 35% are missense, 25% are nonsense, 21% are frameshift, 13% are inframe deletions/insertions, and 3% are splice-site[120]. A majority of WFS patients are compound

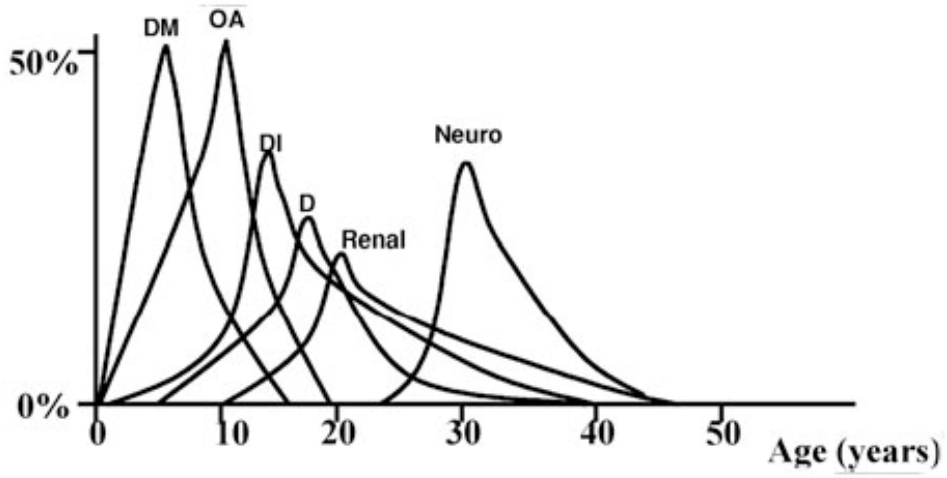

Figure 2.1 Symptom Progression in Wolfram Synndrome. The peak of each curve represents the median age of onset of the complication and the intersections with the x-axis represents the ranges. DM: diabetes mellitus; OA: optic atrophy; DI: diabetes insipidus; D: deafness; Renal: renal tract complications; Neuro: neurological complications[137].

heterozygotes for two mutations, most of which have one mutation that alters the C-terminal tail[119]. Even mutation of the last seven amino acids of the C-terminal leads to a full disease phenotype, suggesting that this region may be important to the function of WFS1 (Figure 2.2).

WFS1 is a 100 kDa glycosylated protein that is localized to the endoplasmic reticulum (ER) membrane[121]. Its N-terminal is cytoplasmic, while its C-terminal is located in the ER lumen. The C-terminal of WFS1 is highly conserved in the mouse, rat, and human. N-glycosylation is its only predicated post-translational modification and it is projected to have nine to eleven transmembrane domains (Figure 2.3). Northern blots of WFS1 mRNA in adult tissues show that highest expression is found in the pancreas and brain[112]. WFS1 has been shown to be a calcium channel; ectopic expression of WFS1 produces an increase in calcium concentration in the cytosol and exhibits novel cation-selective channel activities in the ER membrane[139]. This suggests that WFS1 may function in ER homeostasis. Thus, inactivation or suppression of WFS1 may cause an imbalance in ER homeostasis.

Imbalances in ER homeostasis elicit stress in this organelle. This organelle is critical for the folding of newly synthesized secretory proteins, such as the hormone insulin which regulates blood glucose levels. ER stress is defined as an imbalance between the folding capacity of the ER and the demand placed on the ER for folding proteins. The Unfolded Protein Response (UPR) is a cytoprotective, adaptive response to counteract this stress and return the ER to a state of homeostasis. There are three main components to this response: 1) upregulation of genes encoding folding enzymes, 2) general translational attenuation, and 3) ER-associated protein degradation (ERAD)[8, 10]. Accumulating evidence suggests that chronic and severe ER stress

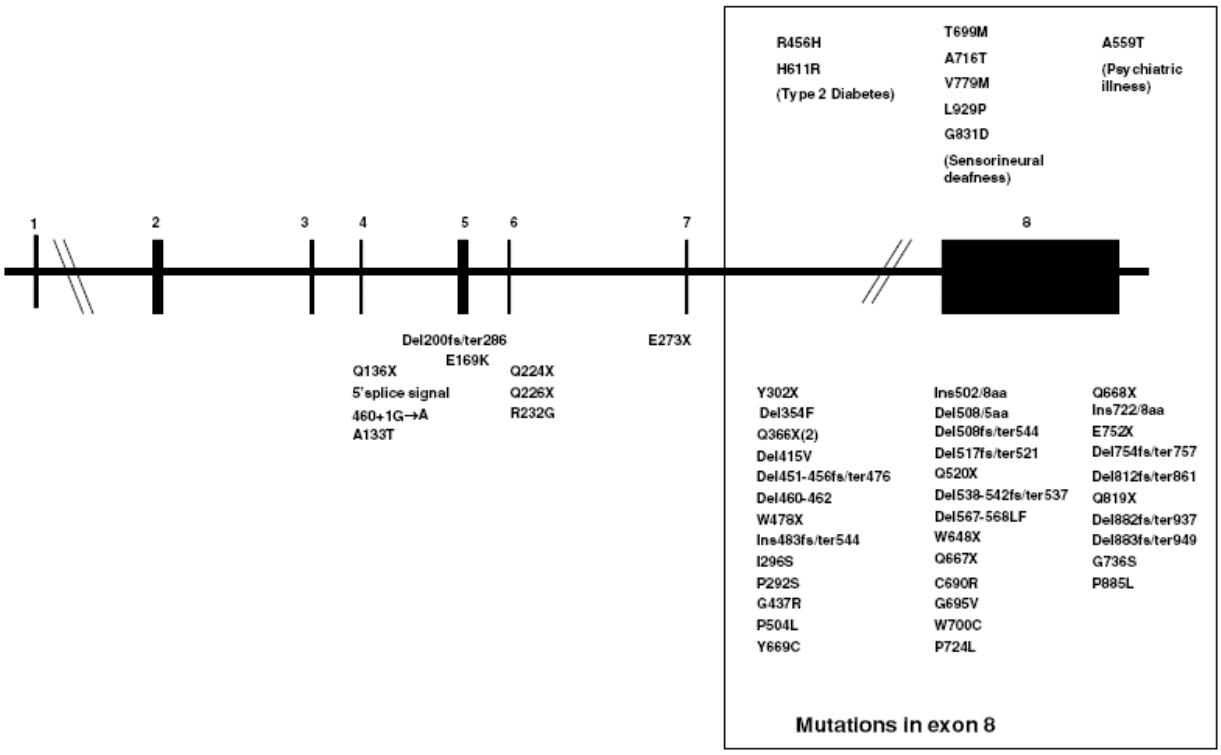

Figure 2.2 Mutations of WFS1 are Clustered in Exon 8. The WFS1 gene consists of 8 exons, of which exon 1 is noncoding. Missense, nonsense, insertions, and deletions reported in Wolfram syndrome patients are shown below the exons, while mutations reported to be associated with psychiatric illness, deafness, and diabetes are shown above the exons[137].

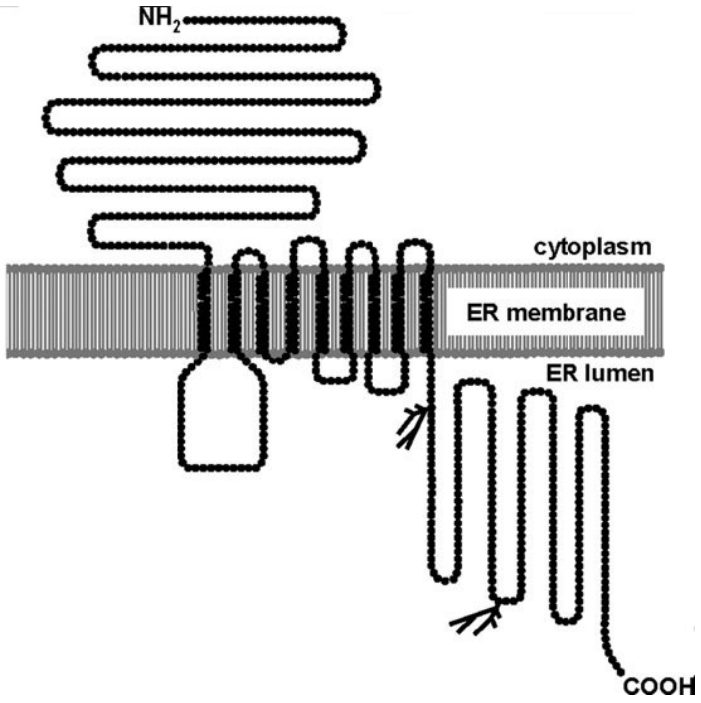

Figure 2.3 WFS1 is an ER Transmembrane Protein. WFS1 is a glycosylated ER membrane protein with its N-terminal located in the cytoplasm and its C-terminal located in the ER lumen. It is predicted to have 9-11 transmembrane domains[138].

levels and/or defects in the UPR cause β-cell death and contribute to the pathogenesis of diabetes[140].

Inositol requiring 1 (IRE1), a sensor for unfolded and misfolded proteins in the ER, is a master regulator of the UPR. IRE1α, which is ubiquitously expressed, has a high level of expression in the pancreas[73, 141]. The accumulation of unfolded proteins in the ER causes dimerization, trans-autophosphorylation, and consequent activation of IRE1. Activated IRE1 splices X-box binding protein 1 (XBP1) mRNA, leading to the synthesis of the active transcription factor XBP1 and upregulation of UPR target genes such as chaperones[18, 21]. Prolonged ER stress activates an alternative IRE1 pathway: the IRE1-JNK apoptotic pathway. Here, IRE1 recruits TNF-receptor-associated factor 2 (TRAF2)[24] which activates apoptosis signaling kinase 1 (ASK1)[25], leading to the activation of c-Jun N-terminal protein kinase (JNK)[23]. JNK activation leads to apoptosis. TRAF2 recruitment also causes clustering and activation of caspase-12 at the ER membrane, also leading to apoptosis[142]. The IRE1-JNK pathway is implicated in insulin resistance in patients with type 2 diabetes[106]. Obesity leads to ER stress in the liver, causing hyperactivation of JNK signaling and serine phosphorylation of insulin receptor substrate-1 (IRS-1). Serine phosphorylation of IRS-1 inhibits insulin action in liver cells.

PKR-like ER kinase (PERK), like IRE1, is a master regulator of the UPR and is activated by the accumulation of unfolded/misfolded proteins in the ER. PERK is also highly expressed in the pancreas[28, 74]. Activated PERK phosphorylates the α-subunit of eukaryotic translation initiation factor 2 (eIF2α), which leads to general translational attenuation and protects cells from ER stress-mediated apoptosis[26]. Mutations in PERK lead to the development of diabetes. In

Wolcott-Rallison syndrome, a rare form of juvenile diabetes, there is a mutation in the EIF2AK3 gene encoding PERK[2]. PERK knockout mice develop diabetes due to a high level of ER stress in the pancreas[75, 143]. This suggests that Wolcott-Rallison syndrome is an ER stress-mediated form of diabetes: β-cell death is caused by ER stress.

Increasing evidence supports the notion that high levels of ER stress and defective UPR signaling contribute to the pathogenesis of diabetes. It is likely that downstream components of ER stress signaling maintain ER homeostasis in pancreatic β-cells. It was therefore possible that Wolfram syndrome may fall into this category of an ER stress-mediated disease: the mutated protein involved in this disease, WFS1, is an ER transmembrane protein and most of these were somehow involved in ER stress signaling. Before this thesis work was started, the function of WFS1 was unknown. In addition to this, when we did a homology search, the results were dismal: WFS1 had no distinct homology to any known proteins. Because WFS1 is localized to the ER membrane, this led us to hypothesize that it was involved in the UPR. This chapter will outline our results from posing the question: *Is WFS1 involved in ER stress signaling?* Here we focused on investigating whether WFS1 was a component of the UPR and if it had a function in maintaining ER homeostasis in the pancreatic β-cell, since these are the primary cells that are affected in WFS. These studies revealed that, indeed, the pathogenesis of Wolfram syndrome is ER stress-mediated; WFS1 is a downstream target of IRE1 and PERK signaling, is upregulated in response to ER stress, and when suppressed, it leads to high levels of ER stress in pancreatic β-cells.

MATERIALS AND METHODS

Plasmids, Cell Culture, and Transfection

INS1 832/13 cells were a gift from Dr. Christopher Newgard (Duke University Medical Center). These cells were maintained in RPMI with 10% fetal bovine serum (FBS). They were transfected with siRNA directed against WFS1 using Cell Line Nucleofector™ Kit V with the Amaxa nucleofector device (Amaxa Biosystems, Gaithersburg, MD). siRNAs for rat WFS1 were designed and synthesized at Qiagen (Valencia, CA) as follows: for rat WFS1-1, AAGGCATGAAGGTCTACAATT; for rat WFS1-2, AAGGCCATCAGCTGCCTCAAT. COS7 cells were maintained in DMEM with 10% FBS and transfected with WFS1 expression vectors using FuGene (Roche, Basel, Switzerland). Full-length human WFS1 cDNA, as well as P724L and G695V mutant WFS1 cDNA, was tagged with a Flag epitope and each pcDNA3 plasmid was subcloned under the control of the cytomegalovirus promoter. The P724L and G695V mutations were introduced using the GeneTailor Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA). Mouse embryonic fibroblasts (MEFs) were maintained in DMEM with 10% FBS. Human fibroblasts from a patient with Wolfram syndrome and a control individual were obtained from Coriell Institute (Camden, NJ) and Dr. Alan Permutt (Washington University School of Medicine). Human fibroblasts were maintained in EMEM with 10% FBS.

Immunostaining

COS7 cells and frozen sections of mouse pancreata were fixed in 2% paraformaldehyde for 30 minutes at room temperature and then permeabilized with 0.1% Triton X-100 for 2

minutes. The fixed slides were then washed with PBS, blocked with 10% BSA for 30 minutes, and incubated in primary antibody overnight at 4°C. The slides were then washed 3 times in 0.1% Tween in PBS and incubated with secondary antibody for 1 hour at room temperature. Images were obtained using a Leica TCS SP2 AOBS confocal microscope with LCS software. FLAG M2 antibody was purchased from Sigma (St. Louis, MO) and anti-WFS1 antibody was generated as previously described[121].

Immunoblotting

Fibroblasts and INS1 832/13 cells were lysed with M-PER (Pierce, Rockford, IL) containing protease inhibitors. COS-7 cells were lysed on ice for 15 minutes with ice-cold buffer (20 mM Hepes, pH 7.5, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM EDTA) containing protease inhibitors. Insoluble material was recovered by centrifugation at 13,000 g for 15 minutes and solubilized in 10 mM Tris-HCl with 1% SDS for 10 minutes at room temperature. After addition of 4 volumes of lysis buffer, the samples were sonicated for 10 seconds. The lysates were normalized for total protein (20 µg per lane) and separated using a 4%-20% linear gradient SDS-PAGE (BioRad, Hercules, CA) and electroblotted.

Isolating Islets from Mouse Pancreata

Male C57/Bl6J and ob/ob 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 minutes with mild shaking. Islets were

then washed several times with HBSS, separated from acinar cells on a discontinuous Ficoll 400 gradient, viewed under a dissecting microscope and hand-selected.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from cells by the guanidine-thiocyanate-acid-phenol extraction method. Total RNA (1 µg) was then reverse transcribed with Oligo-dT primer. For the thermal cycle reaction, the ABI Prism 7000 sequencer detection system (Applied Biosystems, Foster, CA) was used with the following protocol: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 15 seconds each, and 60°C for 1 minute. Using mouse actin for MEFs and mouse islets, human GAPDH for human fibroblasts, and rat actin for INS1 832/13 cells as a control, the polymerase chain reaction (PCR) was performed in triplicate for each sample. All experiments were repeated 3 times. Cyber Green (BioRad, Hercules, CA) and the following primer sets were used for real-time PCR: for mouse actin, GCAAGTGCTTCTAGGCGGAC and AAGAAAGGGTGTAAAACGCAGC; for mouse WFS1, CCATCAACATGCTCCCGTTC and GGGTAGGCCTCGCCATACA; for rat actin, GCAAATGCTTCTAGGCGGAC and AAGAAAGGGTGTAAAACGCAGC; and for rat WFS1, CATCACCAAGGACATCGTCCT and AGCACGTCCTTGAACTCGCT.

RESULTS

WFS1 is a component of the IRE1 signaling pathway

The pathogenesis of Wolfram syndrome has been attributed to mutations in the WFS1 gene, a glycoprotein localized to the ER[121, 122]. The WFS1 gene encodes a 100-kDa protein containing nine to eleven transmembrane domains[112, 114]. Membrane proteins in the ER are often involved in the UPR[8, 144].

Measuring the expression levels of WFS1 by real-time PCR, revealed that WFS1 mRNA is induced by ER stress and under the control of IRE1. In wildtype mouse embryonic fibroblasts (MEFs), induction of WFS1 mRNA was 3-5 fold by two ER stress inducers, tunicamycin and thapsigargin (Figure 2.4A). Although there was a marked induction of WFS1 mRNA in wildtype cells by both ER stress inducers, induction of WFS1 at the protein level in tunicamycin-treated cells, in which N-glycosylation was inhibited, was modest (Figure 2.4B, lane 7), suggesting that WFS1 protein is unstable when N-glycosylation is inhibited. In Ire1α-/- cells, WFS1 mRNA induction under stress was attenuated (Figure 2.4A). By measuring WFS1 protein expression levels by immunoblot using anti-WFS1 antibody, this attenuation of WFS1 induction in Ire1α-/- cells was confirmed (Figure 2.4B, lanes 5 and 8) These results indicate that WFS1 is a component of the UPR and that its expression is regulated by the IRE1 signaling pathway.

WFS1 is co-regulated by the PERK signaling pathway

It has been demonstrated that the three arms of the UPR, the IRE1, ATF6, and PERK pathways, communicate with each other extensively[145]. In *C.elegans,* it has been demonstrated that there is redundancy in the IRE1 and ATF6 pathways: mutations in either pathway are

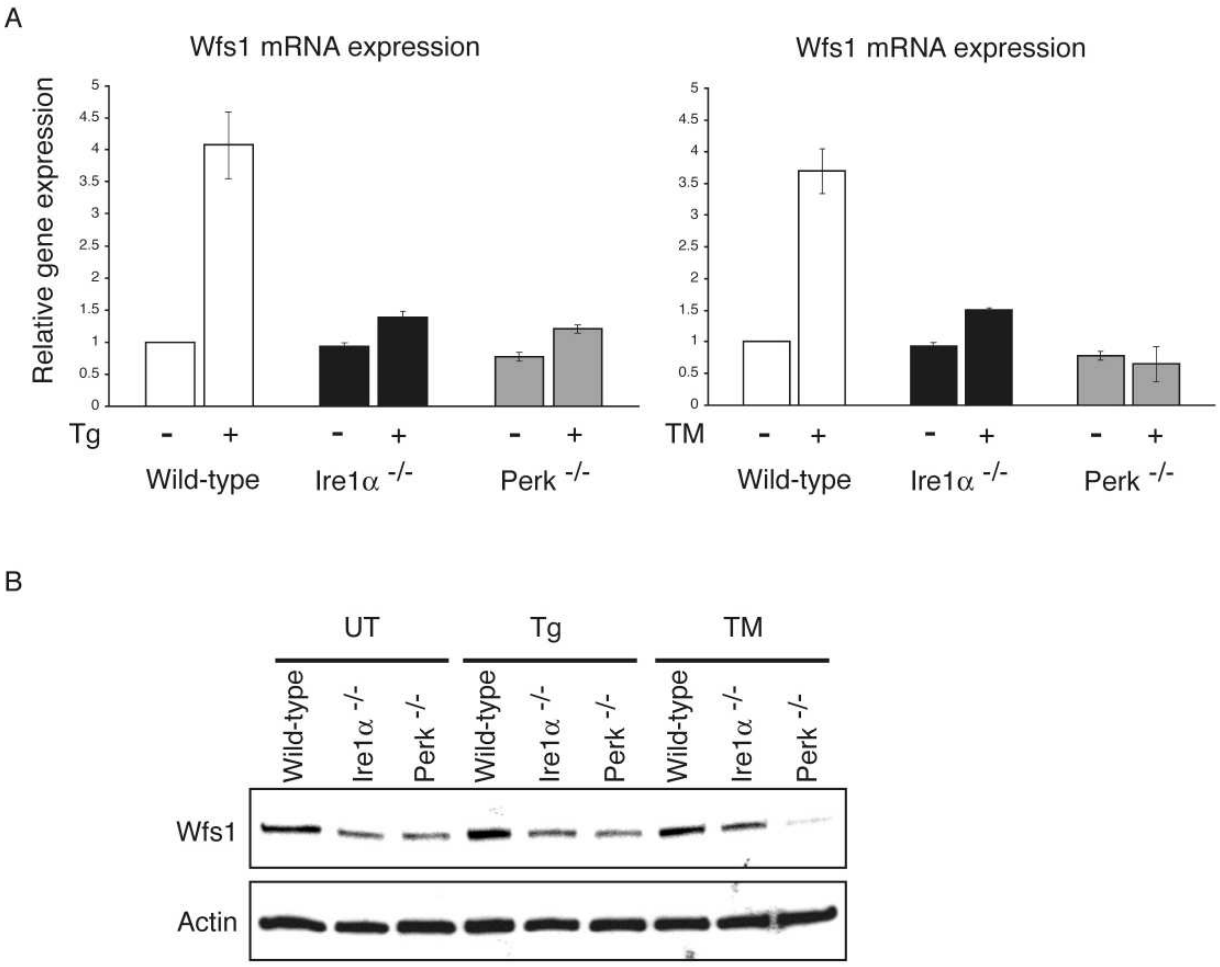

Figure 2.4 WFS1 is a Component of the UPR and Co-regulated by IRE1 and PERK Signaling. (A) Quantitative real-time PCR of WFS1 using reverse-transcribed RNA from wildtype ($Ire1\alpha^{+/+}$, $Perk^{+/+}$), $Ire1\alpha^{-/-}$, and $Perk^{-/-}$ mouse embryonic fibroblasts (MEFs). Cells were untreated or treated with 2.5 mg/ml thapsigargin (Tg) or 1 mM tunicamycin (TM) for 3 h. The amount of mouse WFS1 mRNA was normalized to the amount of actin mRNA in each sample (n=3; values are mean $\pm$ s.e.m.). (B) Immunoblot analysis of WFS1 protein using lysates from wildtype ($Ire1\alpha^{+/+}$, $Perk^{+/+}$), $Ire1\alpha^{-/-}$, and $Perk^{-/-}$ MEFs. Cells were untreated or treated with thapsigargin (Tg) or tunicamycin (TM) for 3 h (n=4). The amount of actin is shown in the lower panel.

relatively tolerated, however mutations in both arms block worm development[146]. There is also overlap in the ATF6 and PERK pathways. For example, XBP1 is transcriptionally activated by both[19, 147]. This would suggest that while WFS1 is regulated by IRE1 signaling, it is possible that it could be co-regulated by another UPR pathway based on the extensive crosstalk between the UPR pathways.

WFS1 induction under stress was also measured in PERK -/- MEFs. In PERK -/- MEFs, WFS1 mRNA induction was attenuated when the cells were treated with the ER stress inducers tunicamycin and thapsigargin as compared to control MEFs (Figure 2.4A). WFS1 protein induction was also attenuated in these cells (Figure 2.4B, lanes 6 and 9). These results suggest that WFS1 is co-regulated by IRE1 and PERK signaling.

While there was no significant difference in baseline WFS1 mRNA content between wildtype and both IRE1-/- and PERK -/- cells, at the protein level, both knockout cell types exhibited a profound decrease in WFS1 protein expression. This further suggests that WFS1 protein becomes unstable by chronic high levels of ER stress, because there exists a higher baseline stress level in IRE1-/- and PERK-/- cells which are deficient in ER stress signaling.

Mutant WFS1 does not accumulate on the ER membrane

It has been reported that WFS1 gene mutations lead to a loss-of-function of WFS1 protein. Nonsense or frameshift mutations of the WFS1 gene lead to a complete absence of WFS1 protein due to instability of the mutant protein[122]. To extend this observation, the cellular localization of mutant WFS1 was examined. Most of the WFS1 gene mutations in patients with Wolfram syndrome occur in exon 8, which encodes the protein's transmembrane and C-terminal

luminal domains[112, 114]. The full-length human WFS1 gene was cloned using human EST clones and then the P724L and G695V mutations, which occur in Wolfram syndrome, were introduced by means of PCR-based mutagenesis. Like the majority of WFS patients, these mutations are localized to exon 8.

The cellular localization of wildtype and mutant WFS1 was then determined by immunostaining cells transfected with Flag-tagged wildtype, P724L, or G695V WFS1 expression vectors. Immunostaining of cells expressing wildtype WFS1 showed a diffuse, reticular staining pattern characteristic of the ER and co-localized with ER marker ribophorin I (Figure 2.5A-C). Part of $WFS1^{P724L}$ showed a similar staining pattern and was also co-localized with ribophorin I, suggesting that mutant WFS1 is also localized to the ER membrane (Figure 2.5D-F). The signal intensity of $WFS1^{P724L}$, however, was much lower than that of wildtype WFS1 (Figure 2.5D). In addition, the ER of cells expressing mutant WFS1 showed a punctuate staining pattern, indicating that mutant WFS1 tends to aggregate (Figure 2.5D-F). These staining patterns and staining intensity suggest that newly synthesized mutant WFS1 protein, in contrast to wildtype WFS1, is unstable and not properly expressed on the ER membrane.

When the aggregation of $WFS1^{P724L}$ was assessed by SDS-PAGE immunoblot analysis of detergent-soluble and detergent-insoluble lysates from COS7 cells transiently expressing these proteins, the formation of insoluble, high-molecular-weight complexes was much more prominent in cells expressing $WFS1^{P724L}$ than in cells expressing wildtype WFS1 (Figure 2.5G, lower panel). WFS1 protein expression levels were also measured in fibroblasts from a patient with Wolfram syndrome and a control individual. WFS1 did not accumulate in the patient sample (Figure 2.5H). Again, these results indicate that most of the newly synthesized mutant

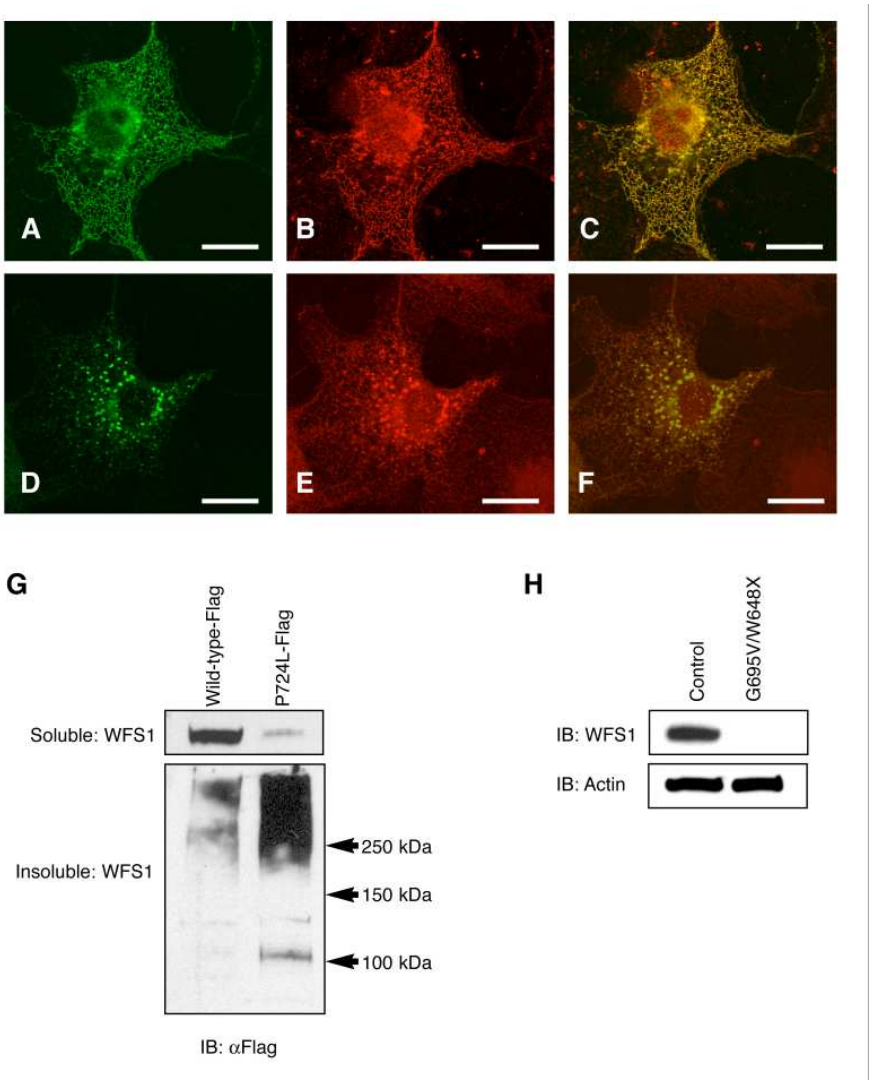

Figure 2.5 Loss-of-function of WFS1 on the ER Membrane Causes Wolfram Syndrome. (A-F) Immunocytochemical staining of COS7 cells expressing Flag-tagged human wildtype (A-C) or P724L WFS1 (D-F). Staining with anti-Flag monoclonal antibody shows the distribution of wildtype or P724L WFS1 protein (A and D). Staining of the same cells with anti-ribophorin I antibody shows the structure of the ER (B and E). Merged images show the co-localization of WFS1 and the ER marker ribophorin I (C and F) (n=3). Bars: 10 µM. (G) High-molecular-weight complexes of $WFS1^{P724L}$ in detergent-insoluble fractions. Lysates from COS7 cells transfected with Flag-tagged wildtype or P724L WFS1 expression vectors were separated into detergent-soluble (upper panel) or detergent-insoluble (lower panel) fractions and immunoblotted with anti-Flag antibody (n=3). (H) Immunoblot analysis of WFS1 protein from fibroblast lysates of a control individual (Control) and a Wolfram syndrome patient (G695V/W648X) (n=4).

WFS1 protein is unstable and not expressed on the ER membrane. It is likely, therefore, that WFS1 is caused by a loss-of-function of WFS1.

WFS1 is important in sustaining ER homeostasis in pancreatic β-cells

Immunohistochemistry experiments on mouse pancreata using anti-WFS1, anti-insulin, and anti-glucagon antibodies revealed that WFS1 was expressed in the islets of the pancreas, where it co-localized with insulin, which is specific to β-cells (Figure 2.6A). WFS1, however, did not co-localize with glucagon, which is unique to α-cells, nor was it expressed in the duct or pancreatic exocrine cells. This suggests that WFS1 is particularly important in the function of the pancreatic β-cell.

It has been shown that WFS1 may have an important role in stimulus-secretion coupling in insulin secretion[134]. To determine WFS1 gene expression levels during insulin secretion, mouse islets were pre-treated for 1 hour with 2.5 mM glucose, then stimulated for insulin secretion with the insulin secretagogues, 16.7 mM glucose and 30 mM KCl, for 1 hour. WFS1 gene expression increased after treatment with both secretagogues, as compared to an unstimulated control (Figure 2.6B). WFS1 protein expression induction was also confirmed (Figure 2.6C). These data suggest that WFS1 upregulation is important for insulin secretion.

ER homeostasis is important for insulin secretion because proinsulin, the insulin precursor, must be folded into its proper three-dimensional structure in the ER in order to become mature insulin. As a direct means of examining the relationship between the loss-of-function of WFS1 and ER homeostasis, WFS1 expression was suppressed in the β-cell line, INS1 832/13, using siRNA directed against WFS1 (Figure 2.7F). The suppression of WFS1

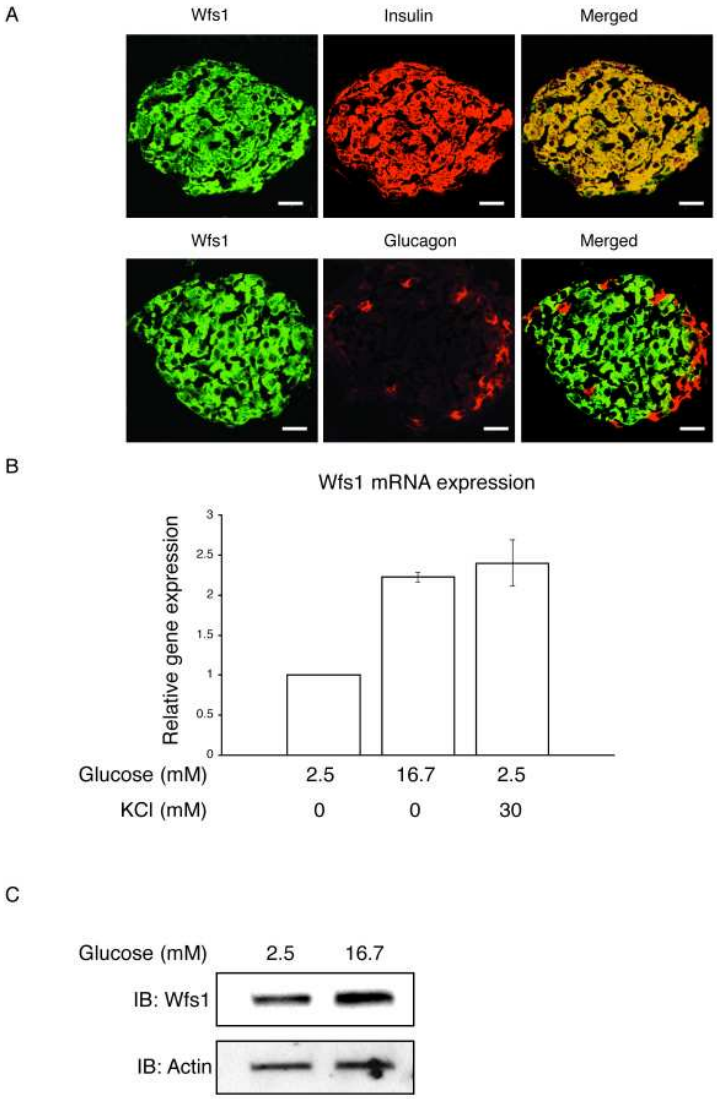

Figure 2.6 WFS1 is Localized to the Pancreatic β-cell and Plays a Role in Insulin Secretion. (A) Distribution of WFS1 in mouse pancreata analyzed by immunohistochemistry using anti-WFS1, anti-insulin, and anti-glucagon antibodies. Merged images show the co-localization of WFS1 and insulin (upper panel) and not WFS1 and glucagon (lower panel) (n=3). Scale bars: 50 µM. (B) WFS1 is upregulated by insulin secretagogues. Mouse islets were pre-treated with 2.5 mM glucose for 1 h and then stimulated with 16.7 mM glucose or 30 mM KCl for 1 h. WFS1 mRNA expression was then measured by real-time PCR and standardized to actin (n = 3; values are mean $\pm$ s.e.m.). (C) WFS1 protein is upregulated by high glucose. Mouse islets were pre-treated with 2.5 mM glucose for 1 h and then stimulated with 16.7 mM glucose. WFS1 and actin protein levels were then measured by immunoblot (n=3).

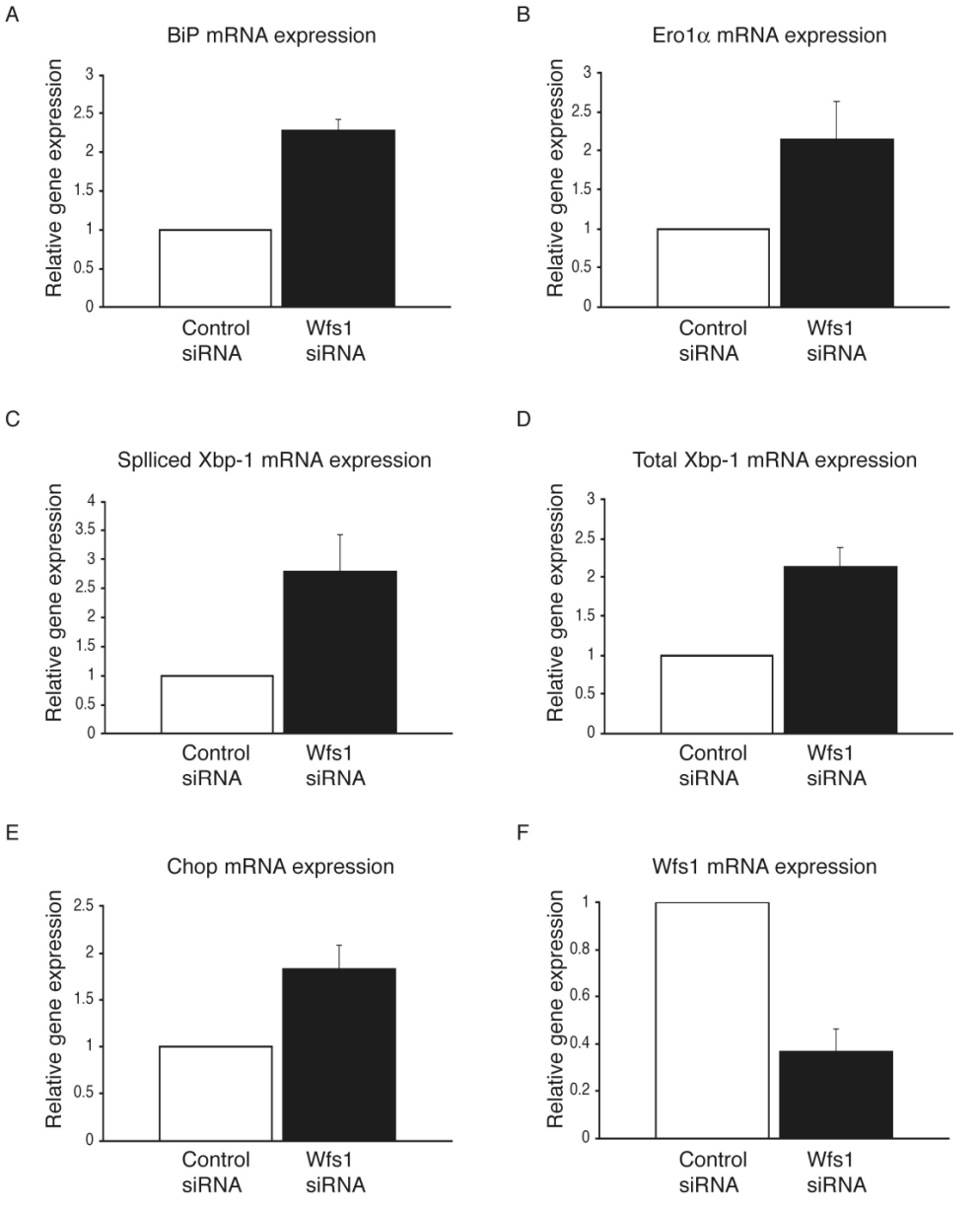

Figure 2.7 Inhibition of WFS1 Expression Causes a High Level of ER Stress in Pancreatic β-cells. INS1 832/13 cells were pre-treated with 5 mM glucose and transfected with siRNA for WFS1 or scramble siRNA. Expression levels of the ER stress markers BiP (A), Ero1α (B), spliced Xbp-1 (C), total Xbp-1 (D), Chop (E), and Wfs1 (F) were measured by real-time PCR and standardized to actin (n = 3; values are mean ± s.e.m.).

caused an increase in expression of the ER chaperone BiP (Figure 2.7A), a marker for ER stress, as well as the other stress markers ERO1α, XBP1, and spliced XBP1 (Figure 2.7B-D). This suppression also increased the expression of another ER stress marker, CHOP (Figure 2.7E). However, the induction of CHOP mRNA was modest as compared to its usual upregulation under ER stress. These results indicate that WFS1 has an important function in mitigating ER stress and maintaining ER homeostasis in pancreatic β-cells. Therefore, the suppression of WFS1 in β-cells could cause chronic ER stress and apoptosis in these cells.

In order to analyze WFS1 expression levels under pathological conditions, WFS1 mRNA expression induction was measured in islets from the ob/ob diabetes mouse model. Islets were isolated from diabetic ob/ob and control C57/Bl6 mice, and WFS1 mRNA induction measured by treating the cells with 16.7 mM glucose. Induction of WFS1 mRNA was significantly attenuated in ob/ob mice as compared to control mice (Figure 2.8). This suggests that β-cells in ob/ob mice are in a state of chronic ER stress and WFS1 induction is saturated.

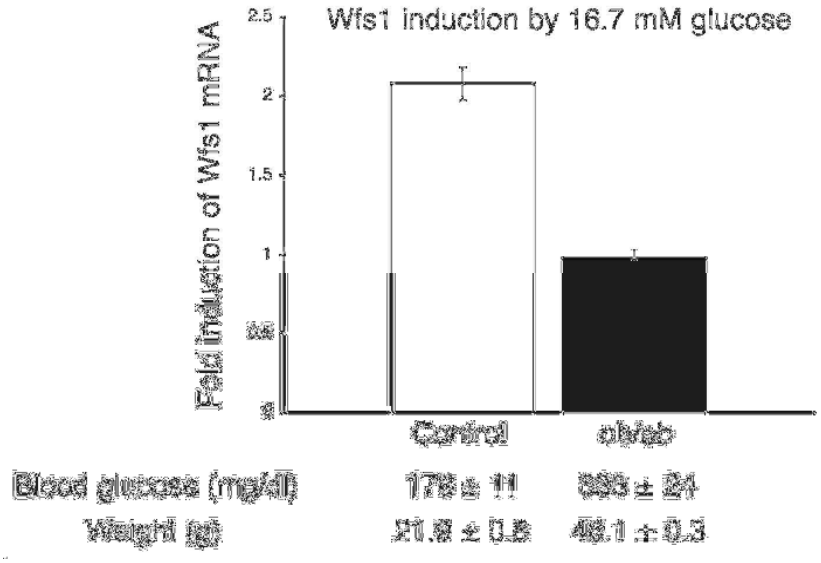

Figure 2.8 WFS1 Induction is Attenuated in the Islets of Ob/Ob Mice. Islets from control C57/Bl6 and ob/ob mice were isolated and pre-treated with 2.5 mM glucose for 1 h, then stimulated with 2.5 mM or 16.7 mM glucose for 1 h. The expression levels of WFS1 and actin were measured by real-time PCR and the induction of WFS1 by 16.7 mM glucose was calculated (n = 3; values are mean $\pm$ s.e.m.).

DISCUSSION

This study has shown that WFS1 is a component of IRE1 and PERK signaling (i.e. the UPR) and is important in the maintenance of ER homeostasis, specifically in pancreatic β-cells (Figure 2.9). WFS1 mutations in patients with Wolfram syndrome lead to a loss-of-function of WFS1. In addition, using siRNA, it was illustrated that the suppression of WFS1 leads to a high level of ER stress in β-cells. These findings suggest that WFS1 protects β-cells against ER stress and, conversely, chronic ER stress is caused by a loss-of-function of WFS1 protein. Previous studies have shown that WFS1 protein serves as a regulator of the ER ion channel, most likely acting as a calcium channel[122, 139]. It has also been reported that the increase in production of cytosolic calcium in response to glucose is lower in the islets of WFS1 knockout mice as compared to control mice[134]. These findings suggest that loss-of-function of WFS1 causes abnormal calcium homeostasis in the ER, elicits ER stress, and triggers apoptosis in pancreatic β-cells.

These findings also suggest that the pathogenesis of Wolfram syndrome can be attributed to a very high level of chronic ER stress due to the lack of functional WFS1 protein in pancreatic β-cells. WFS1 protein is localized to the β-cell of islets, but not in α-cells, duct cells, or exocrine acinar cells. Although these other cells are also active in protein secretion, WFS1 expression levels in these cells is not detectable, as compared to β-cells which are specialized in insulin biosynthesis and secretion. Therefore, our findings of WFS1 expression only in β-cells and of WFS1 upregulation during insulin secretion, suggest that WFS1 is an important component of proinsulin folding and processing in the ER of pancreatic β-cells. These results also show that pathogenic WFS1 mutants do not accumulate in the soluble fractions of cells and make insoluble

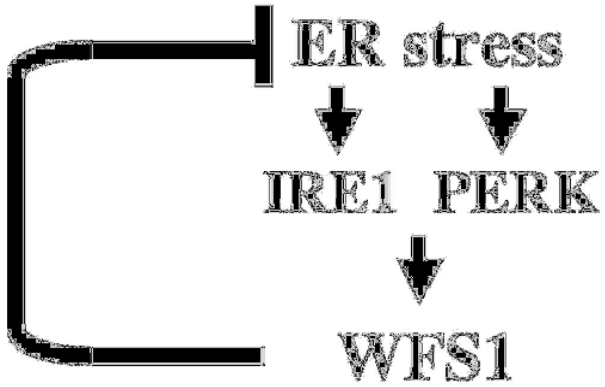

Figure 2.9 Model of the Role of WFS1 in Mitigating ER Stress. WFS1 is a component of IRE1 and PERK signaling (i.e. the UPR) and is important in the maintenance of ER homeostasis, specifically in pancreatic β-cells.

aggregates. It is possible that the accumulation of pathogenic WFS1 mutants is toxic to pancreatic β-cells, causing them to malfunction in patients with Wolfram syndrome. This would account for the selective β-cell loss which occurs in this disease.

The high levels of ER stress and pancreatic β-cell death in patients with Wolfram syndrome may be related to the β-cell dysfunction in patients with type 2 diabetes. The pathogenesis of type 2 diabetes is a result of the peripheral resistance to the action of insulin, which leads to the prolonged increase in insulin biosynthesis. Because the folding capacity of the ER is then overwhelmed, this peripheral resistance to insulin activates ER stress signaling pathways[84]. For this reason, chronic ER stress in β-cells may lead to β-cell apoptosis in patients with type 2 diabetes who are genetically susceptible to ER stress. Indeed, recent genome studies show a link between WFS1 single nucleotide polymorphisms (SNPs) and an increased risk for type 2 diabetes[126-31].

While this chapter focused on determining if WFS1 plays a role in ER stress signaling in pancreatic β-cells, the next will focus on determining the actual function of WFS1 in this pathway. This chapter demonstrated that Wolfram syndrome is an ER-stress mediated disease, however, the actual mechanisms behind WFS1 function were not defined. By defining the function of WFS1, the pathogenesis of Wolfram syndrome can be more clearly understood, as well as type 2 diabetes. The next chapter will reveal the function of WFS1 in the UPR, making it a real target for diabetes prevention and/or therapy.

CHAPTER III

NEGATIVE REGULATION OF ER STRESS SIGNALING THROUGH WFS1-MEDIATED ATF6 PROTEOLYSIS

SUMMARY

WFS1 is a transmembrane protein localized to the endoplasmic reticulum (ER). The previous chapter demonstrated that WFS1 is a component of the unfolded protein response (UPR) and mitigates ER stress in cells. Mutations in the WFS1 gene cause Wolfram syndrome, a genetic form of diabetes, optic atrophy, neurodegeneration, and psychiatric illness. Accumulating evidence indicates that pancreatic β-cell death and neural cell dysfunction in Wolfram syndrome are attributed to high levels of ER stress in affected cells. However, the function of WFS1 in the UPR, until now, has been unclear. Activation levels of the UPR are tightly regulated to maintain ER homeostasis – hyperactivation can lead to cell death. Positive regulation of the UPR has been extensively studied, however the precise mechanisms of negative regulation of this signaling pathway are not well documented. The data in this dissertation chapter reveal that WFS1 controls a regulatory feedback loop of the ER stress signaling network. WFS1 regulates a key transcription factor of the UPR, activating transcription factor 6 (ATF6), through the ubiquitin-proteasome pathway. Activation of the ER stress response element (ERSE) by ATF6 is attenuated by WFS1 expression. WFS1 recruits ATF6 to an E3 ligase, HRD1, and the proteasome, and enhances its degradation thereby suppressing the UPR. Suppression of WFS1 increases the expression of ATF6 target genes such as BiP and $P58^{IPK}$. Collectively, these data

indicate that WFS1 has an important function in the negative regulation of the UPR, protecting secretory cells from premature death caused by hyperactivation of this signaling pathway.

INTRODUCTION

Productive folding of secretory proteins and degradation of misfolded proteins are essential to ensure normal cell function. Both these processes occur in the endoplasmic reticulum (ER). Perturbations in ER function cause an imbalance between these processes, leading to the accumulation of misfolded and unfolded proteins in the organelle: a state called ER stress. Cells cope with ER stress by activating an ER stress signaling network, also called the Unfolded Protein Response (UPR). Activation of the UPR not only results in the upregulation of gene expression for molecular chaperones, but expands the size of the ER, decreases general protein translation to reduce the ER workload, and degrades abnormal proteins accumulated in the ER[9,145]. As long as the UPR can mitigate ER stress, cells can produce proper amounts of proteins in response to the need for them and perform their normal functions.

Secretory proteins are co-translationally translocated into the ER, where they undergo folding and post-translational modifications. A significant proportion of these proteins which are synthesized do not reach their final destination as functional proteins and are degraded by the endoplasmic reticulum-associated degradation pathway (ERAD)[154]. ERAD has several functions: 1) degradation of mutant proteins which cannot be appropriately folded, for example, degradation of the ΔF508 mutant cystic fibrosis transmembrane conductance regulator (CFTR)[155], 2) degradation of proteins that lack their oligomerization partners, for example, subunits of the T-cell receptor such as TCR-α[156], and 3) degradation of proteins whose activities need to be attenuated to maintain homeostatic regulation of metabolic pathways, for example, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), a rate-limiting enzyme in the mevalonate pathway in which sterols are synthesized[157].

Degradation from the ER is initiated by dislocation of the proteins from the ER to the cytosol through the Sec61 translocon channel, followed by ubiquitin-proteasome dependent proteolysis[158]. Ubiquitination of proteins is fundamental to this dislocation process, as blocking this pathway inhibits transport[159]. Ubiquitination of proteins requires three enzymes: 1) ubiquitin-activating enzymes (E1), which activate ubiquitin in an ATP-dependent manner, 2) ubiquitin-conjugating enzymes (E2), which conjugate ubiquitin to its essential cysteine residue, and 3) ubiquitin ligases (E3), which transfer ubiquitin to the target protein[160] (Figure 3.1). E3 ligases and the combinations of E2/E3 enzymes provide specificity for the ubiquitination of target proteins.

HMG-CoA reductase degradation protein 1 (HRD1/Der3), is one of the best characterized RING finger E3 ligases involved in ERAD, and was first identified by Hampton *et al.* in 1996[161]. It has been shown to be involved in the metabolically regulated degradation of HMGR[162], as well as other proteins such as CPY, Sec61-2p[163], and TCR-α[164]. It is a multi-spanning ER membrane protein, with its C-terminal RING-H2 finger located in the cytoplasm, and is induced by ER stress[164-65]. Induction of HRD1 is regulated by the IRE1-XBP1 pathway[166]. Suppressor of lin-12-like protein (SEL1/HRD3), a lumen-oriented ER membrane glycoprotein that mediates protein-protein interactions[167], has been shown to physically interact with and stabilize HRD1, to modulate its ligase activity[168]. SEL1 and HRD1 form a *HRD* complex and interact via the NH_2-terminal transmembrane region of HRD1. The regions of SEL1 that control HRD1 stability reside solely in the ER lumen, and in fact most of the SEL1 sequence resides in the ER lumen[215]. SEL1 has been shown to prevent the RING-H2 domain of HRD1 from programming auto-ubiquitination, thus stabilizing HRD1 protein. This chapter will illustrate that Wolfram syndrome 1 (WFS1) protein has a similar function as SEL1, and HRD1 is

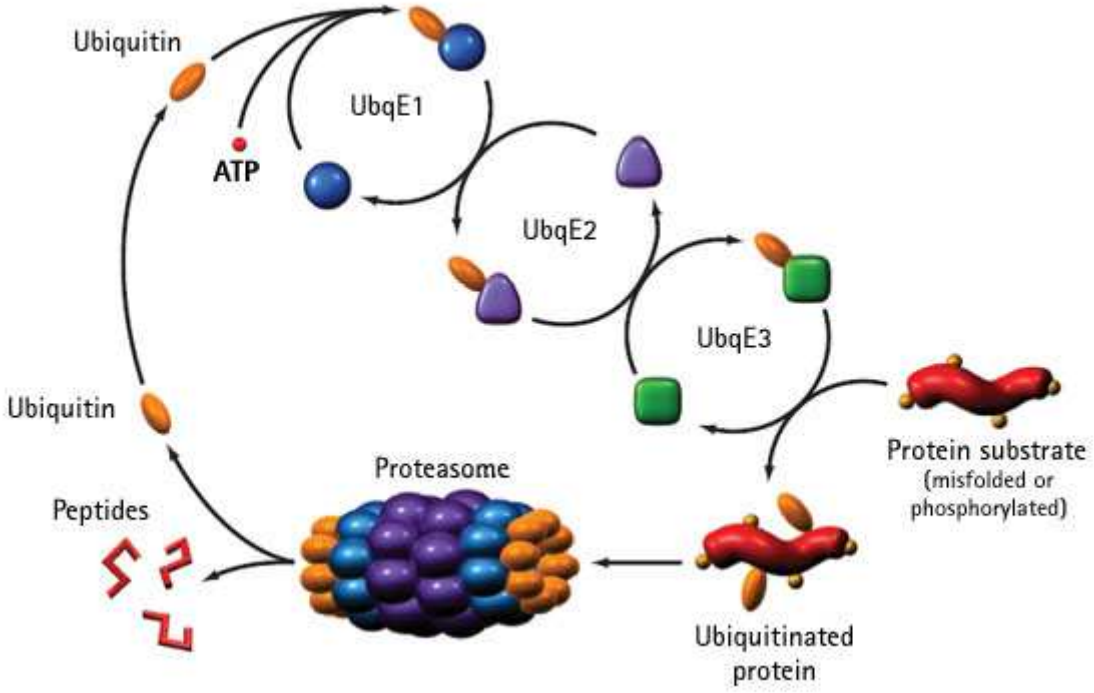

Figure 3.1 The Ubiquitin Pathway. Free ubiquitin is activated by ubiquitin-activating enzyme E1 (UbqE1), which uses ATP to form a complex with ubiquitin. Subsequently, UbqE1 is transferred to a ubiquitin-conjugating enzyme E2 (UbqE2). UbqE2 then joins to the ubiquitin protein ligase E3 (UbqE3), which facilitates the polymerization of ubiquitin molecules on the target protein. Multi-ubiquitin chains serve to mark the target protein for degradation by the 26S proteasome. *(Adapted from http://www.emdbiosciences.com/popup/cbc/proteasome_ubiquitin_path.html)*

indeed the E3 ligase for activating transcription factor 6 (ATF6), thus linking WFS1 with regulation of upstream UPR signaling.

ATF6 is one of the three master regulators of the UPR[145]. ATF6 encodes a b-ZIP-containing transcription factor localized to the ER membrane[148]. Under ER stress, the N-terminal DNA binding domain of ATF6 is cleaved and released from the ER[34]. The b-ZIP domain of ATF6 then translocates into the nucleus and upregulates downstream target genes, such as BiP and XBP1, which function in protein folding and processing[38,149-50] (Figure 3.2). Therefore, deletion of ATF6 compromises the secretory pathway during ER stress[151-52]. It has been reported that the non-cleaved form of ATF6 is unstable and quickly degraded by the ubiquitin-proteasome pathway to prevent the hyperactivation of the UPR[153]. However, the mechanism underlying this phenomenon has yet to be elucidated. Evidence in this chapter reveals that WFS1 has a novel function in regulating ATF6 by stabilizing and enhancing the function of its E3 ligase, HRD1.

WFS1 is a transmembrane protein localized to the ER[121]. It has previously been shown that WFS1 protein is a component of the UPR and mitigates ER stress in cells[169]. Deficiency of functional WFS1 causes high levels of ER stress and leads to Wolfram syndrome, a disease characterized by juvenile onset diabetes and optical atrophy[112,114]. WFS1 polymorphisms are also implicated in common type 2 diabetes[125-31]. Accumulating evidence indicates that β-cell death and neuronal cell dysfunction in Wolfram syndrome are attributed to high levels of ER stress in affected cells[134-35,169]. However, until now, the function of WFS1 in the UPR has been unclear. This chapter outlines a novel function for WFS1 in a regulatory loop of the UPR through proteolysis of ATF6. WFS1 interacts with and stabilizes the ER E3 ligase, HRD1. It then

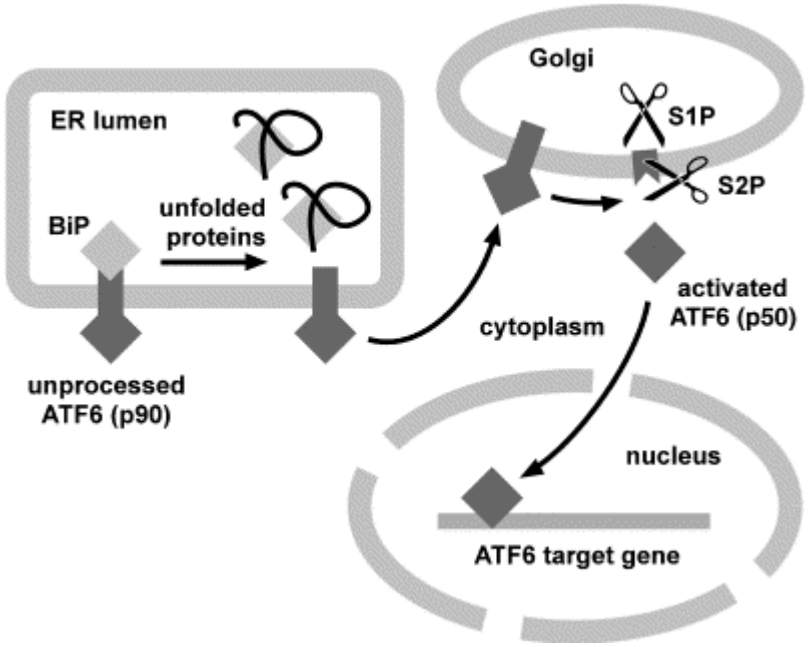

Figure 3.2 Cleavage and Processing of ATF6. Model of ATF6 activation upon accumulation of unfolded proteins in the ER. BiP dissociates from immature ATF6 (p90). As a consequence, Golgi localization signals in the lumenal domain of ATF6 are exposed and mediate exit from the ER. In the Golgi, two subsequent processing steps by the S1P and S2P proteases liberate the active ATF6 transcription factor (p50) from the membrane. The processed ATF6 is transported into the nucleus and upregulates the transcription of target genes[214].

recruits ATF6 to HRD1 which enhances ATF6 ubiquitination. Ubiquitin-tagged ATF6 is then targeted to the proteasome and undergoes proteolysis, thereby attenuating the UPR signaling pathway.

MATERIALS AND METHODS

Cell Culture

Rat insulinoma cells, INS-1 832/13, were a gift from Dr. Christopher Newgard (Duke University Medical Center) and cultured in RPMI 1640 supplemented with 10% FBS. Mouse insulinoma cells, MIN6, were maintained in DMEM with 15% FBS and 1% sodium pyruvate. COS7 and Neuro2A cells were cultured in DMEM supplemented with 10% FBS. For generation of cells inducibly overexpressing WFS1 and GFP, INS-1 832/13 stably expressing pTetR were transduced with a lentivirus expressing human WFS1-FLAG or GFP, and cultured in 2 μM doxycycline for 24 hr prior to protein/RNA isolation. For generation of cells stably suppressing WFS1 or GFP, MIN6 cells were transduced with a retrovirus expressing shRNA against mouse WFS1 or GFP. For overexpression of ATF6, HRD1, and WFS1, COS7 cells were transfected with ATF6-HA, HRD1-myc, and WFS1-FLAG expression plasmids using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN). As a control for co-expression, an equivalent amount of pcDNA3 plasmid was used. DTT, cyclohexamide, and MG132 were purchased from Sigma (Saint Louis, MO).

Plasmids

ATF6 plasmids were provided by Dr. Ron Prywes (Columbia University). GRP78 reporter plasmid was provided by Dr. Kazutoshi Mori (Kyoto University). HRD1-Myc plasmid was a gift from Dr. Masayuki Kaneko and Dr. Masayuki Nomura (Hokkaido University). TCRα plasmids were provided by Ron Kopito (Stanford University) and NHK3 plasmids were a gift

from Kazuhiro Nagata (Kyoto University). Entry vectors, destination vectors, and viral plasmids for establishing lentiviral and retroviral cell lines were provided by Dr. Eric Campeau (University of Massachusetts Medical School). shRNA against WFS1 and GFP were purchased from the shRNA Library Core Facility at the University of Massachusetts Medical School.

Immunoblotting

Cells were lysed in ice-cold TNE buffer (50mM Tris HCl [pH 7.5], 150mM NaCl, 1mM EDTA, 1% Nonidet® P 40) containing a protease inhibitor cocktail (Sigma, Saint Louis, MO) for 15 min on ice then the lysates were cleared by centrifuging the cells at 12,000 g for 20 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. Anti-WFS1 antibody was a gift from Dr. Yoshitomo Oka (Tohoku University). Anti-actin and anti-FLAG antibodies were purchased from Sigma (Saint Louis, MO). Anti-HA antibody was purchased from Stressgen (Victoria BC, Canada) and anti-ATF6 and anti-GFP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antigen retrieval was used for the anti-ATF6 antibody (membranes were incubated at 70°C for 30 min in unmasking buffer (2% SDS, 6.25 mM Tris-HCl, 100 mM β-Me)). Anti-ubiquitin and anti-IRE1 antibodies were purchased from Cell Signaling (Danvers, MA). Anti-alpha 5 20S proteasome antibody was purchased from Biomol (Plymouth Meeting, PA), and anti-PERK antibody was purchased from Rockland, Inc. (Gilbertsville, PA). Anti-c-myc antibody was purchased from Roche (Indianapolis, IN). Anti-Hrd1 antibody was generated in rabbits using a KLH-conjugated synthetic peptide,

TCRMDVLRASLPAQS. Anti-alpha-1-antitrypsin antibody was purchased from DakoCytomation (Denmark).

DTT Chase

Rat insulinoma cells, INS-1 832/13, were treated with 1mM dithiothreitol (DTT) for 2 hr. The DTT was washed out with normal media (RPMI 1640 supplemented with 10% FBS) for 0, 1, or 2 hrs. Cells were lysed and immunoprecipitated with anti-WFS1 antibodies, as previously described.

Fractionation

The endoplasmic reticulum was isolated from INS-1 832/13 cells using an Endoplasmic Reticulum Isolation kit (Sigma, St. Louis, MO). The ER pellet was then lysed in ice-cold TNE buffer containing 1% Nonidet® P 40 (NP40) and protease inhibitors, and the lysates were cleared and normalized as described above. The ER lysates (1.0 ml) were loaded on top of a glycerol gradient (10-40%) prepared in PBS containing 1 mM DTT and 2 mM ATP, and centrifuged at 4°C and 80,000 x g for 20 hr. Thirty-two fractions were collected from the top of the tubes. Two hundred µl of each fraction was precipitated with acetone and the remaining pellet was lysed with 50 µl of sample buffer. Precipitated proteins were then separated using a 4%-20% linear gradient SDS-PAGE and electroblotted.

Immunoprecipitation

Cells were lysed in ice-cold TNE buffer with 1% NP40 and protease inhibitors for 15 min on ice then the lysates were cleared by centrifuging the cells at 12,000 x g for 20 min at 4°C. For immunoprecipitation of endogenous WFS1, 500 µg of whole cell extract from each sample was pre-cleared and then incubated with Protein G Sepharose™ 4 Fast Flow beads (GE Healthcare, Upsala, Sweden) and 4 µg of anti-WFS1 antibody O/N at 4°C with rotation. After incubation, the beads were washed three times with TNE buffer followed by a final wash in 1x PBS. The immunopreciptates were resolved by SDS-PAGE and then subject to immunoblot. For immunoprecipitation of ATF6, 6 µg of anti-ATF6 antibody was used, for HA 2 µg of anti-HA antibody was used, and for Hrd1 4 µg of anti-Hrd1 antibody was used. As a control, lysates were immunoprecipitated as above using rabbit IgG.

Real-time polymerase chain reaction

Total RNA was isolated from the cells by using RNeasy Mini Kit (Qiagen, Valencia, CA) 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 sample and normalized to the amount of actin. The polymerase chain reaction (PCR) was performed in triplicate for each sample, then all experiments were repeated three times. The following sets of primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) were

used for real-time PCR: for rat actin, GCAAATGCTTCTAGGCGGAC and AAGAAAGGGTGTAAAACGCAGC; for rat BiP, TGGGTACATTTGATCTGACTGGA and CTCAAAGGTGACTTCAATCTGGG; for rat Chop, AGAGTGGTCAGTGCGCAGC and CTCATTCTCCTGCTCCTTCTCC; for rat total XBP1, TGGCCGGGTCTGCTGAGTCCG and ATCCATGGGAAGATGTTCTGG; for rat ERO1-α, GAGAAGCTGTAATAGCCACGAGG and GAGCCTTTCAATAAGCGGACTG; for rat GLUT2, GTGTGAGGATGAGCTGCCTAAA and TTCGAGTTAAGAGGGAGCGC; for rat INS2, ATCCTCTGGGAGCCCCGC and AGAGAGCTTCCACCAAG.

Luciferase Assay

COS-7 cells were mock transfected or transfected with full-length or cleaved ATF6 with pcDNA3.0 or ATF6 with WFS1 expression plasmids along with rat GRP78 (ERSE) promoter luciferase reporter gene, wildtype ATF6 binding site luciferase reporter gene (ATF6GL3), or mutant ATF6 binding site luciferase reporter gene (ATF6m1GL3) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). 48 hrs post-transfection, lysates were prepared using a Luciferase Assay System kit (Promega, Madison, WI). The light produced from the samples was read by a standard plate reading luminometer. Each sample was read in triplicate and normalized against the signal produced from mock wells. All experiments were repeated three times and normalized using β-gal.

WFS1 -/- Mice

WFS1 -/- mouse pancreata were generously provided by Dr. Alan Permutt (Washington University in St. Louis).

Apoptosis Assay

To monitor apoptosis in INS-1 832/13 cells, $5x10^5$ cells were treated with palmitate (50 μg/ml) for 24 hr and stained with Annexin V-PE followed by FACS analysis.

RESULTS

WFS1 suppresses ATF6 transcriptional activity

In order to further define the role of WFS1 in the UPR, we assessed whether WFS1 expression could impact the function of components of the UPR. We discovered that transcriptional activity of a transmembrane transcription factor and master regulator of the UPR, ATF6, is attenuated by WFS1 expression. Under ER stress, the N-terminal DNA binding domain of ATF6 is cleaved and released from the ER to upregulate UPR target genes in the nucleus[149-50]. When full-length ATF6 was transfected with the ATF6 binding site reporter gene, ATF6GL3, this reporter was induced 12-fold by ATF6 as expected[170]. In contrast, this induction was reduced to 3-fold by co-transfection with WFS1 (Figure 3.3, top panel). As a control, the ATF6 mutant site reporter gene, ATF6m1GL3, was transfected with the ATF6 and WFS1 expression plasmids. As expected, there was no activity seen with this reporter (Figure 3.3, middle panel). It has been shown that ATF6 strongly activates the BiP/GRP78 promoter[148]. To confirm that WFS1 regulates ATF6 transcriptional activity on the BiP/GRP78 promoter, full-length ATF6 or cleaved ATF6 (Δ ATF6), an N-terminal deletion mutant in which the b-ZIP domain is unmasked and simulates processed ATF6, were co-transfected with WFS1 and a rat GRP78 promoter reporter gene containing the ER stress response element (ERSE). This reporter was induced by both full-length and Δ ATF6, however, only full-length ATF6 activity was suppressed by WFS1 expression (Figure 3.3, bottom panel). Collectively, these results indicate that WFS1 suppresses ATF6 transcriptional activity before its translocation to the nucleus.

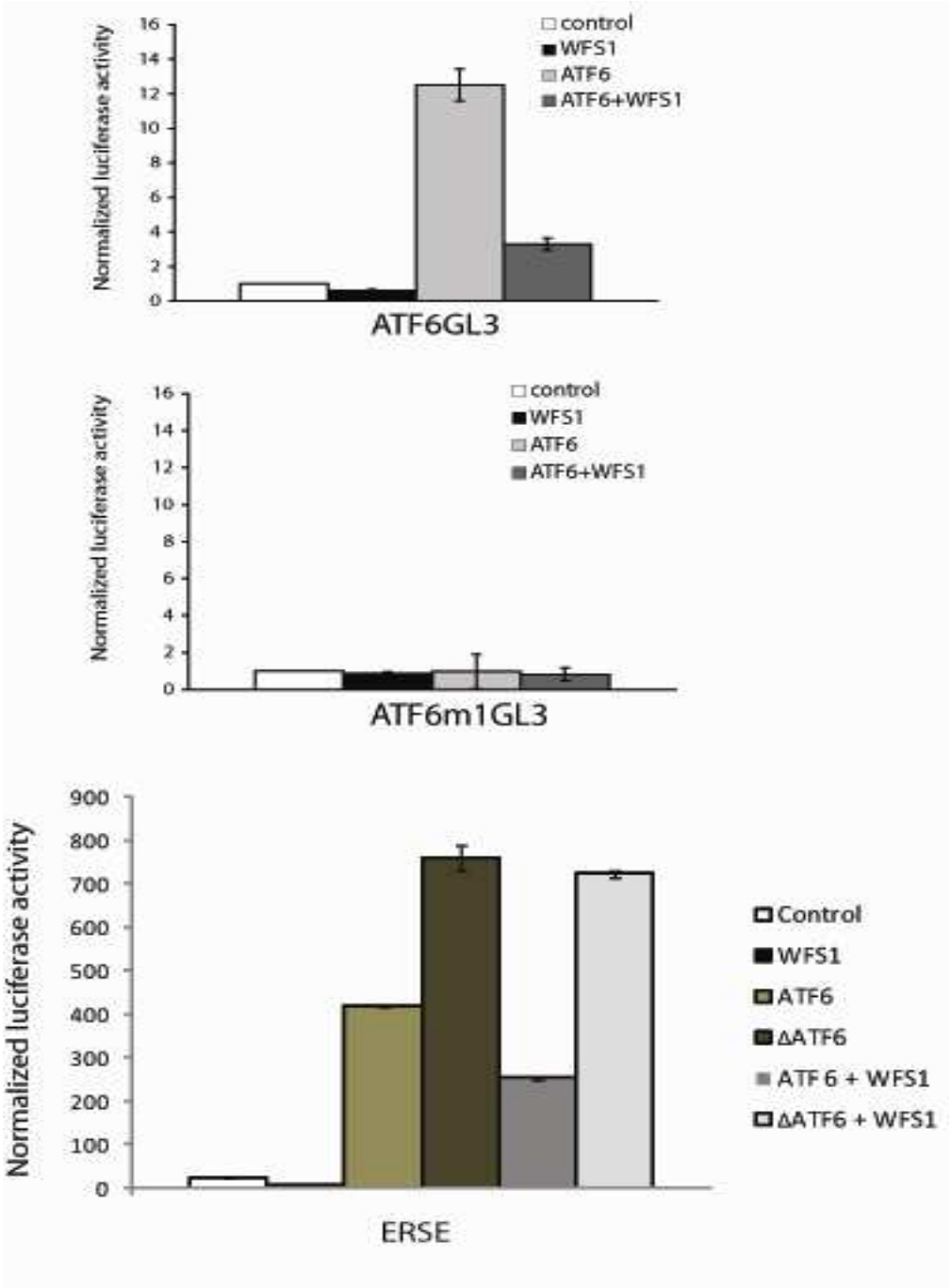

Figure 3.3 WFS1 Suppresses ATF6 Transcriptional Activity. COS-7 cells were transfected with a full-length ATF6 expression plasmid or cleaved ATF6 (ΔATF6) with a WFS1 plasmid together with the following luciferase reporter genes: ATF6 binding site reporter (ATF6GL3), ATF6 mutant site reporter (ATF6m1GL3), or rat GRP78 promoter (ERSE) (n=3).

WFS1 and ATF6 form an ER stress-mediated complex

Both WFS1 and ATF6 are transmembrane proteins localized to the ER[(121,148)], raising the possibility that the suppression of the ATF6 reporter by WFS1 might be mediated by direct interaction between the WFS1 and ATF6 proteins. To confirm this idea, the association of WFS1 with ATF6 was examined in the pancreatic β-cell line INS1 832/13 cells. Figure 3.4A (right panel, lane 2) shows that WFS1 associated with ATF6 under non-stress conditions. To examine whether this interaction was maintained during ER stress conditions, the cells were treated with the ER stress inducer dithiothreitol (DTT). Figure 3.4A (right panel, lanes 3-5) shows that DTT treatment of cells caused a dissociation of ATF6 from WFS1 in a time-dependent manner, with almost complete dissociation 3 hours post-treatment. This ER stress-dependent interaction could also be seen in cells treated with another ER stress inducer thapsigargin (Figure 3.5). To confirm that this interaction is recovered post-stress, cells were treated for 2 hours with DTT and then chased in normal media. As expected, the interaction of ATF6 and WFS1 began to recover after a 1 hour chase (Figure 3.4C, right panel). This interaction could also be seen in a neuronal cell line, Neuro2A (Figure 3.6).

WFS1 functions in the degradation of ATF6 through the ubiquitin-proteasome pathway

Suppression of ATF6 transcriptional activity by WFS1 and the formation of an ATF6-WFS1 complex led to the prediction that WFS1 regulates ATF6 function at the post-translational level. To test this prediction, we derived a pancreatic β-cell line, MIN6 cells, stably expressing a small hairpin RNA (shRNA) directed against WFS1. Figure 3.7A (left panel) shows that ATF6 protein levels were increased approximately 2-fold compared to control cells. ATF6 mRNA was unchanged in the WFS1-knockdown cells, but ATF6 target genes, such as P58^{IPK} and BiP[(151-52)] ,

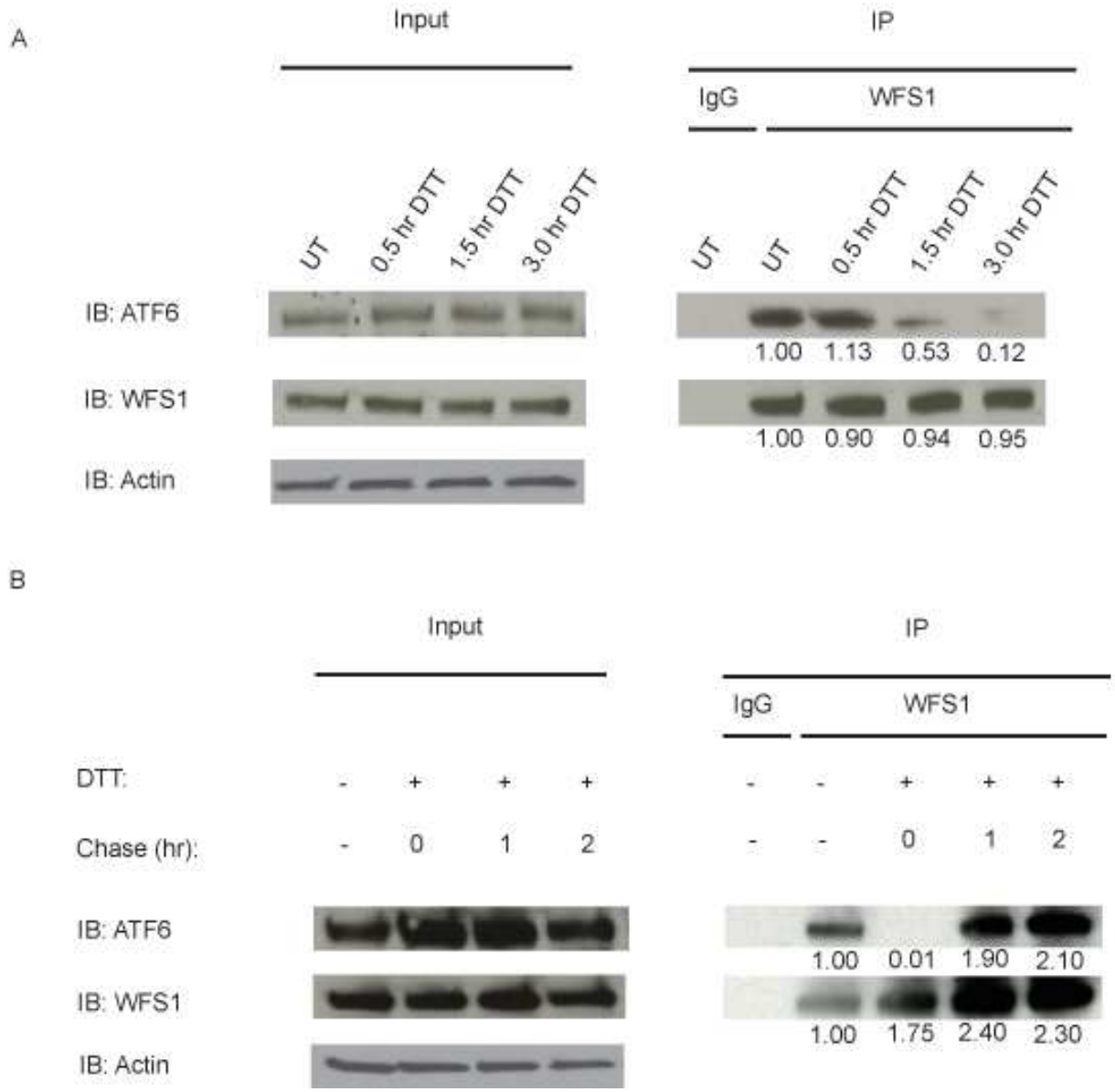

Figure 3.4 WFS1 Interacts with ATF6 in an ER Stress-Dependent Manner. (A) An anti-WFS1 antibody was used to immunoprecipitate (IP) WFS1 protein from INS1 832/13 cells untreated (UT) or treated with the ER stress inducer DTT (1 mM) for 0.5, 1.5, or 3 hr. Immunoprecipitates were then subject to immunoblot (IB) analysis using anti-ATF6 and anti-WFS1 antibodies (n=3). (B) INS1 832/13 cells were treated with DTT (1mM) for 2 hr and then chased in normal media for 0, 1, or 2 hr. WFS1 was immunoprecipitated from cell lysates and immunoprecipitates were analyzed by IB using anti-ATF6 and anti-WFS1 antibodies (n=3). The relative amount of ATF6 and WFS1 proteins were quantified using ImageJ software.

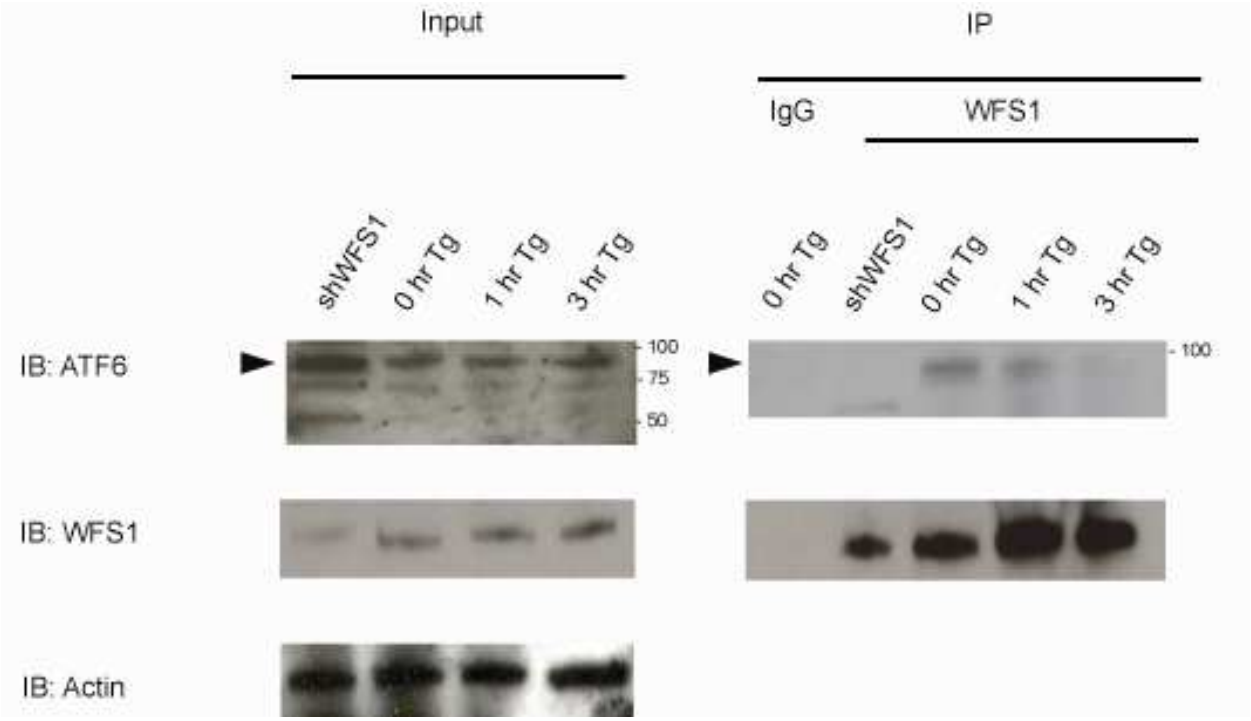

Figure 3.5 WFS1 and ATF6 Form an ER Stress-Mediated Complex. An anti-WFS1 antibody was used to immunoprecipitate (IP) WFS1 protein from INS1 832/13 cells untreated (UT) or treated with 1 µM of the ER stress inducer thapsigargin (Tg) for 0, 1, or 3 hr. Immunoprecipitates were then subject to immunoblot (IB) analysis using anti-ATF6 and anti-WFS1 antibodies (n=3).

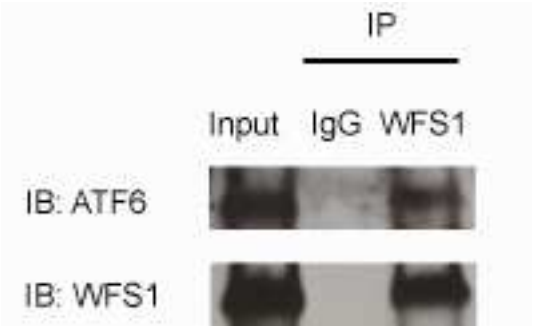

Figure 3.6 The WFS1-ATF6 Complex in Neuronal Cells. An anti-WFS1 antibody was used to immunoprecipitate (IP) WFS1 protein from Neuro2A cells. Immunoprecipitates were then analyzed using anti-ATF6 and anti-WFS1 antibodies (n=3).

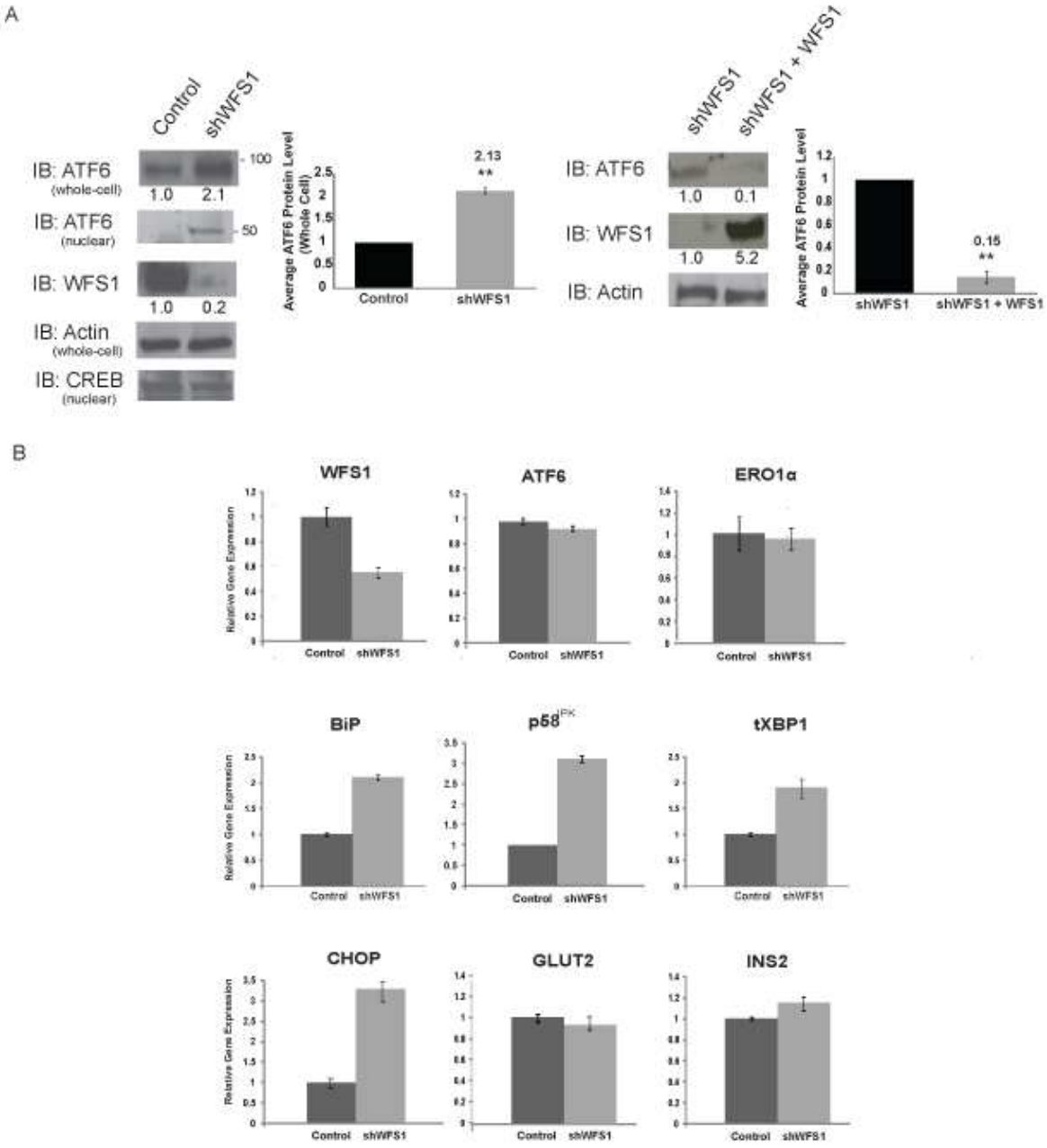

Figure 3.7 WFS1 Suppression Enhances ATF6 Protein and ATF6 Target Gene Expression. (A) Total cell lysates or nuclear extracts were prepared from mouse β-cell lines, MIN6, stably transduced with a retrovirus expressing shRNA against GFP (control) or mouse WFS1, and analyzed by immunoblot using anti-WFS1, anti-ATF6, anti-CREB, or anti-actin antibodies (left panel). MIN6 cells expressing shWFS1 or expressing shWFS1 and rescued with a WFS1 expression plasmid were immunoblotted with anti-WFS1, anti-ATF6, and anti-actin antibodies (right panel) (n=3; values are mean $\pm$ SD). (B) Total mRNA was prepared from INS1 832/13 cells inducibly expressing shRNA against WFS1 (Control = UT, shWFS1 = 2 μM doxycycline treatment for 48 hrs). Expression levels of WFS1, ATF6, BiP, total XBP1, $p58^{IPK}$, CHOP, ERO1α, GLUT2, and INS2 were measured by quantitative real-time PCR (n=3; values are mean $\pm$ SD). ** p-value < 0.01

were upregulated as predicted (Figure 3.7B). We reintroduced a lentivirus expressing WFS1 into the cells expressing shRNA directed against WFS1. Figure 3.7A (right panel) shows that ATF6 protein expression levels were again reduced when WFS1 was reintroduced. ATF6 protein levels were also measured in INS1 832/13 cells overexpressing WFS1. ATF6 protein was suppressed in these cells (Figure 3.8A), while there was no significant change in protein levels of the other two master regulators of the UPR, IRE1 and PERK. This could also be seen in a neuronal cell line (Figure 3.9). ATF6 target gene mRNA levels were also suppressed in these β-cell lines overexpressing WFS1 (Figure 3.8B). The relationship of WFS1 and ATF6 protein expression was found to be dose-dependent: increased expression of WFS1 leads to a decrease in ATF6 protein expression (Figure 3.10A). This is also a proteasome-dependent relationship. Treatment of cells overexpressing WFS1 with the proteasome inhibitor MG132 could rescue ATF6 protein levels (Figure 3.10B-C). Two mutant variants of WFS1 cloned from patient samples were found not to affect ATF6 protein levels in MIN6 cells expressing shRNA directed against WFS1 (Figure 3.11A). This was also confirmed in INS1 832/13 cells (Figure 3.11B) and neuronal cells (Figure 3.11C).

To assess the impact of WFS1 on ATF6 protein degradation, cyclohexamide experiments were performed. In MIN6 cells expressing shRNA directed against WFS1, there was a block in ATF6 protein degradation (Figure 3.12A), while in cells overexpressing WFS1, there was very little ATF6 protein expression (Figure 3.12B). WFS1 could not enhance the degradation of two other ER proteins which are susceptible to misfolding (Figure 3.13A-B), indicating that WFS1 specifically degrades ATF6 protein. WFS1 could also enhance the ubiquitination of ATF6. In cells expressing shRNA directed against WFS1, there was a decrease in ATF6 ubiquitination after blocking the proteasome (Figure 3.14A), while in cells overexpressing WFS1 there was an

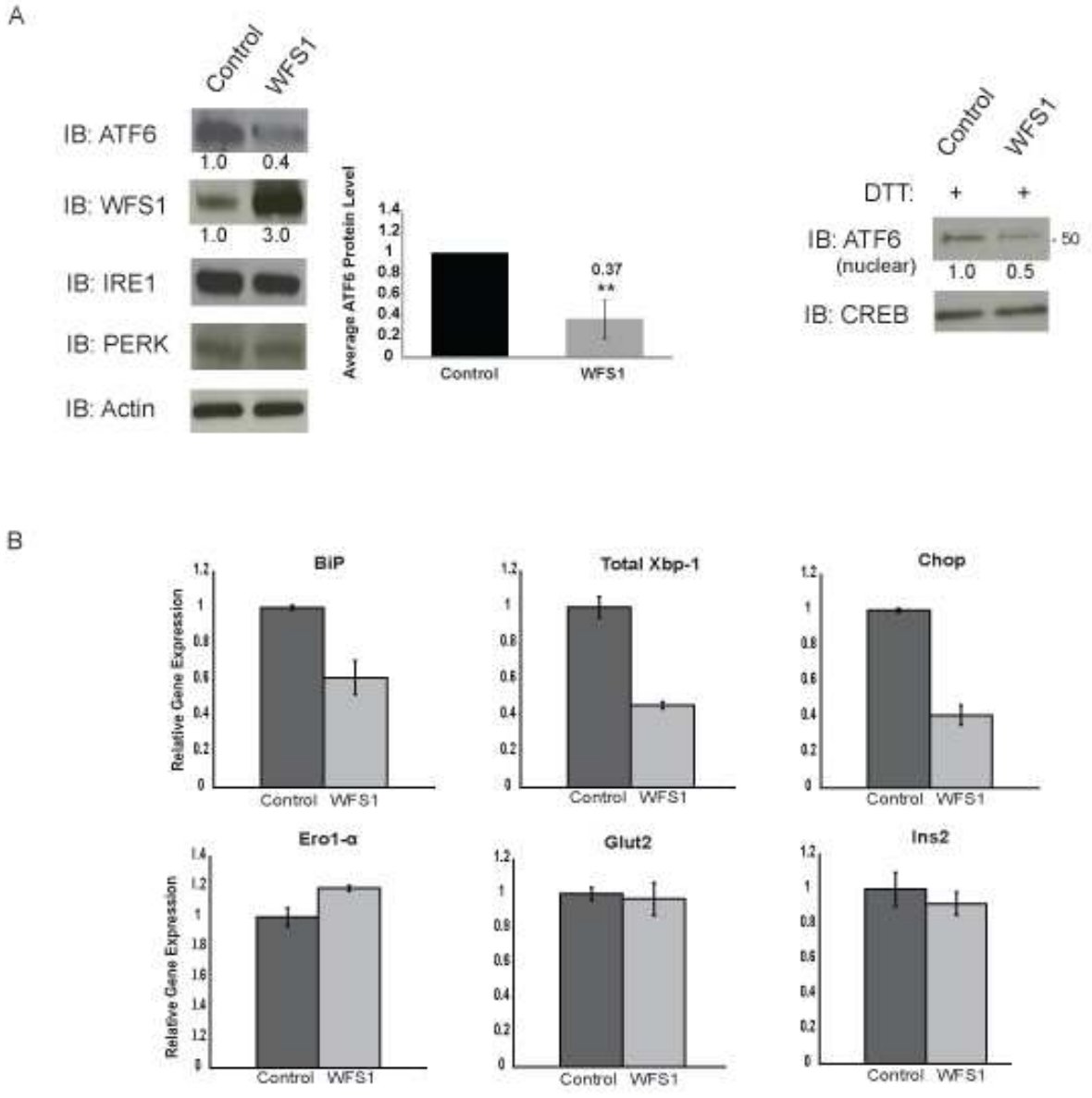

Figure 3.8 WFS1 Suppresses ATF6 Protein Expression. (A) Total cell lysates and nuclear extracts were prepared from rat β-cell lines, INS1 832/13, transduced with an inducible lentivirus expressing GFP (control) or human WFS1, treated with 2 μM doxycycline for 24 hr, and analyzed by immunoblot (IB) using anti-WFS1, anti-ATF6, anti-IRE1, anti-PERK, anti-CREB or anti-actin antibodies. For nuclear extracts, cells were treated with 1mM DTT for 3 hr (n=3; values are mean ± SD). (B) Total mRNA was prepared from INS1 832/13 cells overexpressing GFP (control) or WFS1. Expression levels of BiP, total XBP1, CHOP, ERO1α, GLUT2, and INS2 were measured by quantitative real-time PCR (n=3; values are mean ± SD). ** p-value < 0.01

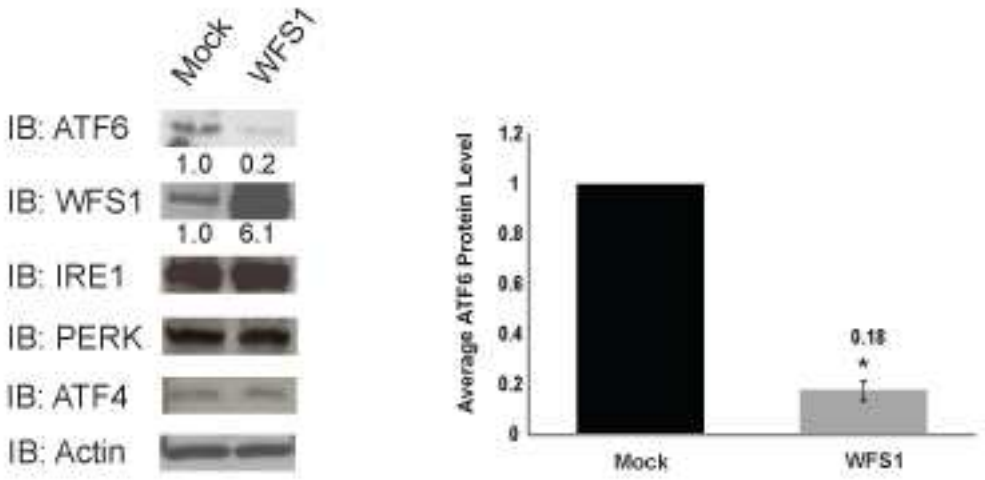

Figure 3.9 WFS1 Regulates ATF6 Protein in Neuronal Cells. Neuro2A cells were either mock transfected or transfected with a WFS1 expression plasmid. Lysates were analyzed by immunoblot (IB) using anti-ATF6, anti-WFS1, anti-IRE1, anti-PERK, anti-ATF4, and anti-actin antibodies (n=3; values are mean $\pm$ SD). The relative amounts of WFS1 and ATF6 protein were quantified using ImageJ software. * p-value < 0.05

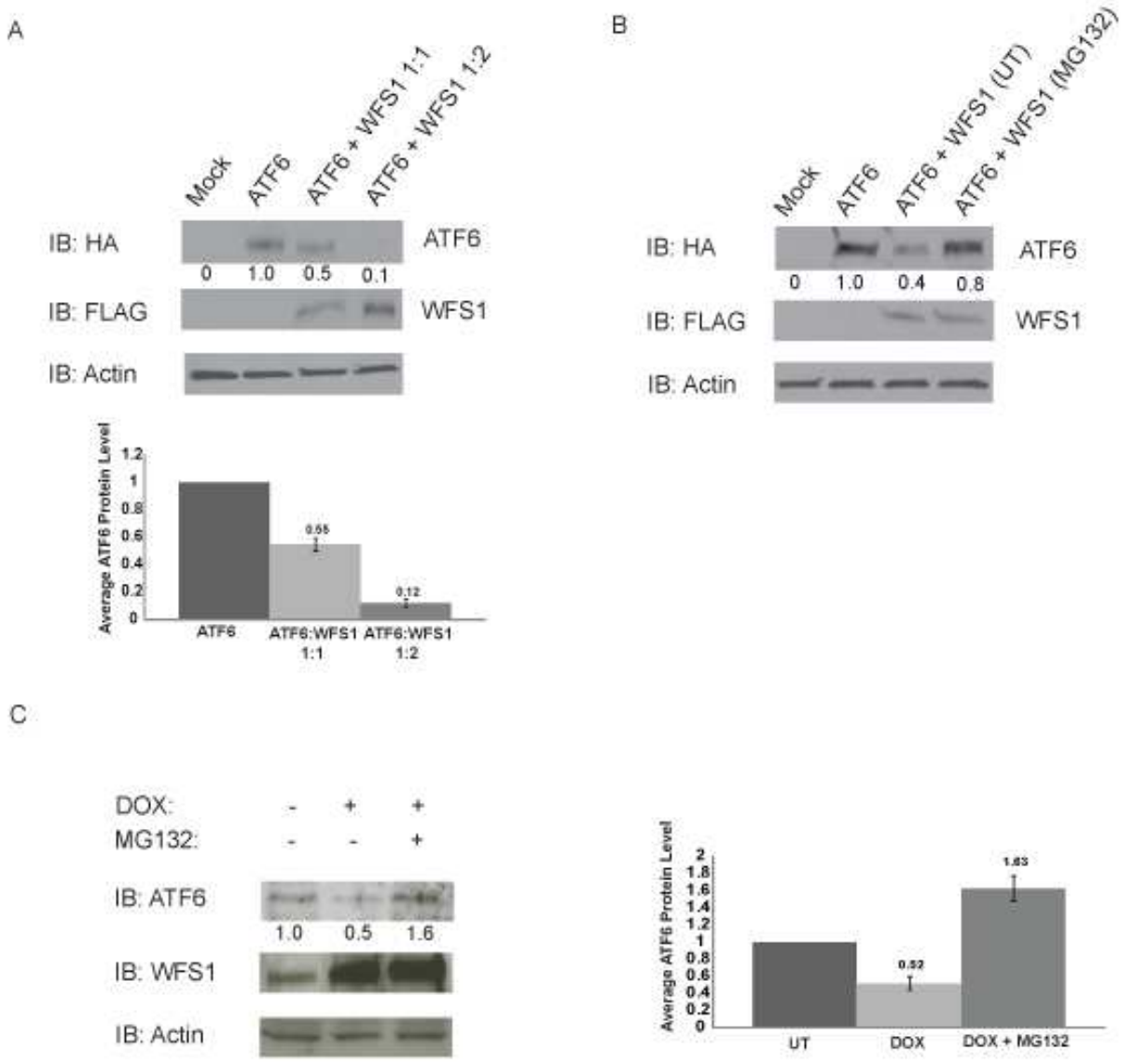

Figure 3.10 WFS1 controls ATF6 Protein in a Dose-Dependent and Proteasome-Dependent Manner. (A) COS7 cells were transfected with ATF6-HA or ATF6-HA and WFS1-FLAG at a 1:1 or 1:2 ratio of ATF6:WFS1. Whole cell extracts were then subject to immunoblot (IB) using anti-HA, anti-FLAG, and anti-actin antibodies (n=3; values are mean ± SD). (B) COS7 cells were transfected with ATF6-HA or ATF6-HA and WFS1-FLAG and then untreated (UT) or treated with the proteasome inhibitor MG132 (20 µM) for 3 hr. Whole cell extracts were then subject to immunoblot (IB) using anti-HA, anti-FLAG, and anti-actin antibodies (right panel) (n=3). (C) INS1 832/13 cells inducibly expressing WFS1 were treated with 2 µM doxycycline treatment for 24 hr followed by MG132 (20 µM) for 3 hr. Lysates were analyzed by IB using anti-WFS1, anti-ATF6, and anti-actin antibodies (n=3; values are mean ± SD). Image J software was used to quantify relative amounts of ATF6 protein.

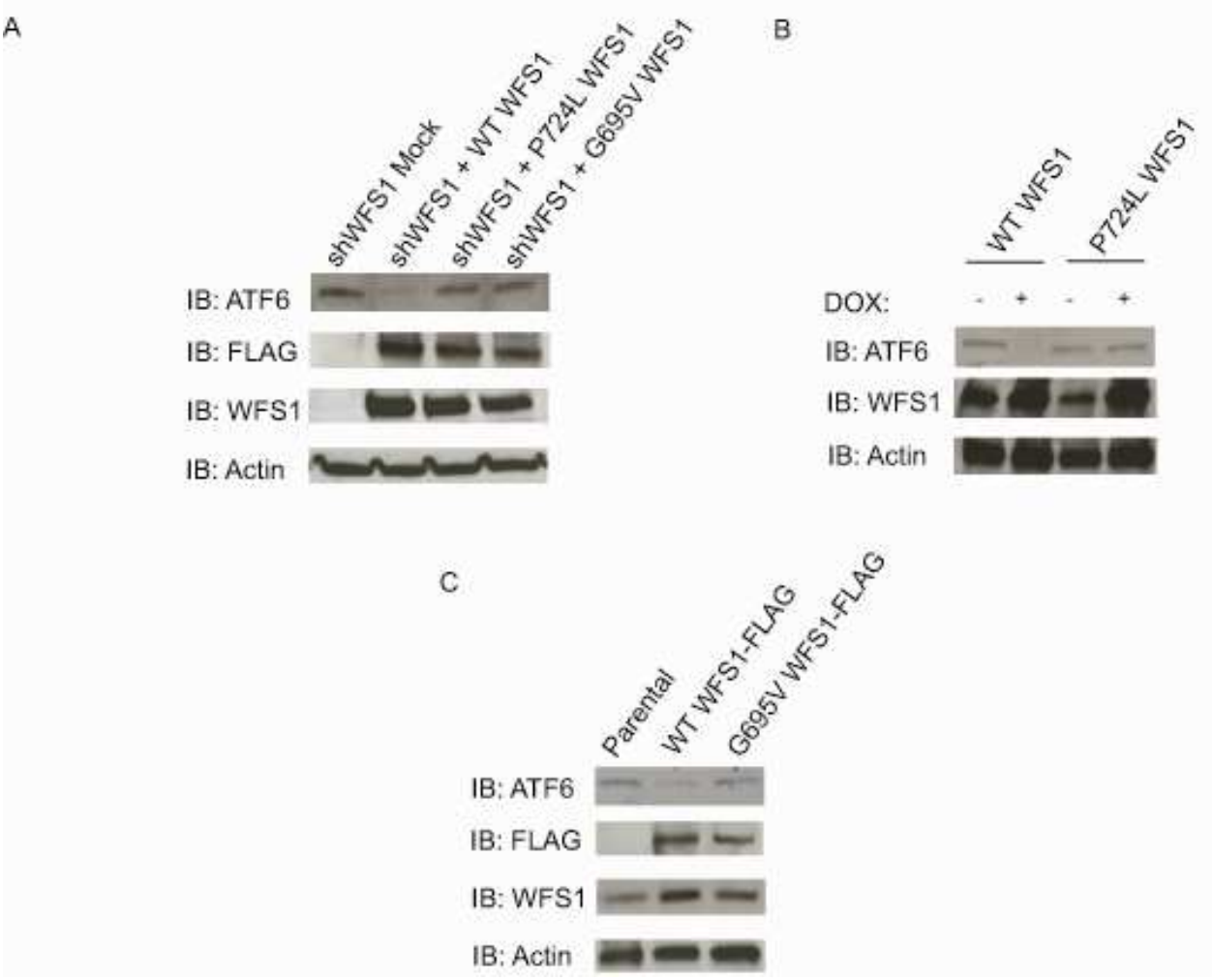

Figure 3.11 Wolfram Syndrome Disease-Causing Mutant WFS1 Variants Do Not Regulate ATF6 Protein. (A) Total cell lysates were prepared from mouse β-cell lines, MIN6, stably transduced with a retrovirus expressing shRNA against mouse WFS1 and either mock transfected, or transfected with WT WFS1-Flag or the mutant variants P724L WFS1-Flag, and G695V WFS1-Flag. Lysates were then analyzed using anti-ATF6, anti-Flag, anti-WFS1, and anti-actin antibodies (n=3). (B) WT WFS1 or a disease-causing mutant variant, P724L WFS1, expression was induced in INS1 832/13 cell lines with 2 μM doxycycline (24 hr). Whole cell lysates were prepared and immunoblotted (IB) with anti-ATF6, anti-WFS1, and anti-actin antibodies (n=3). (C) Lysates from parental Neuro2A cells and cells stably expressing WT WFS1 or the mutant variant G695V WFS1 were analyzed by IB using anti-ATF6, anti-FLAG, anti-WFS1, and anti-actin antibodies (n=3).

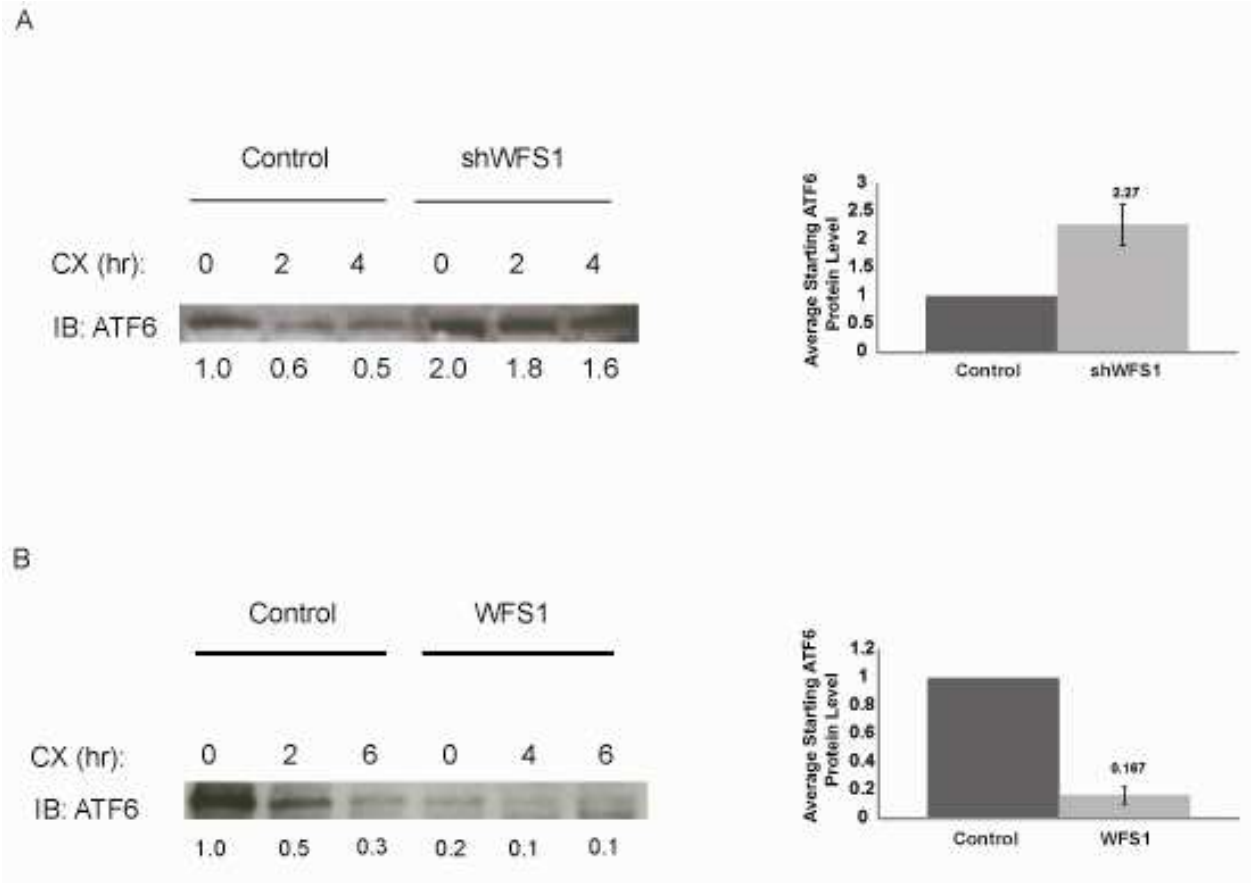

Figure 3.12 WFS1 Enhances ATF6 Degradation. (A) MIN6 cells stably expressing shRNA to GFP(control) or shRNA to WFS1 (shWFS1) were treated with 40 µM cyclohexamide (CX) for 0, 2, and 4 hr. Total cell lysates were analyzed using an anti-ATF6 antibody (n=3; values are mean $\pm$ SD). (B) INS1 832/13 cells expressing GFP (control) or WFS1 were treated with 40 µM cyclohexamide (CX) for 0, 2, and 6 hr. Total cell lysates were analyzed using an anti-ATF6 antibody (n=3; values are mean $\pm$ SD). The relative amount of ATF6 protein was quantified using ImageJ software.

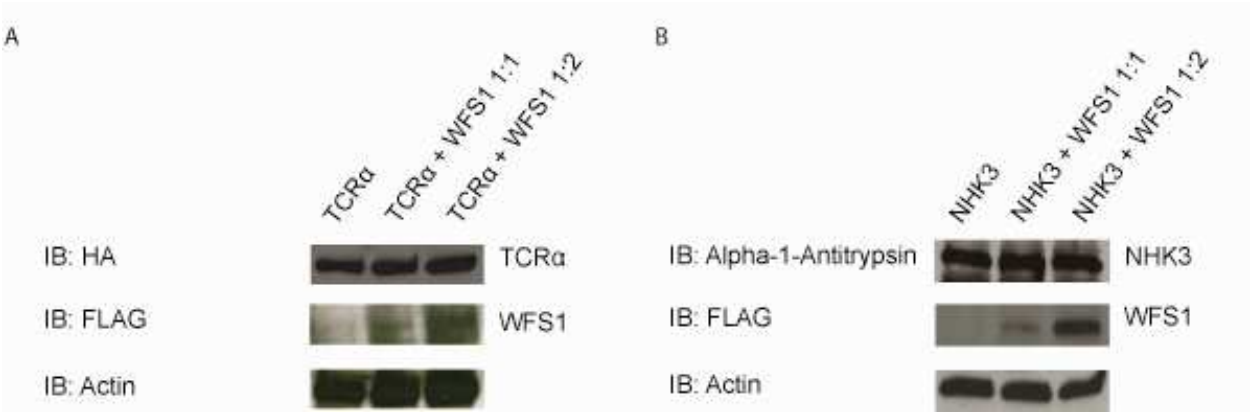

Figure 3.13 WFS1 Specifically Degrades ATF6. (A) COS7 cells were transfected with TCRα-HA or TCRα-HA and WFS1-FLAG at a 1:1 or 1:2 ratio of TCRα:WFS1. Lysates were then immunoblotted with anti-HA, anti-FLAG, and anti-actin antibodies (n=3). (B) COS7 cells were transfected with mutant alpha-1-antitrypsin (NHK3) or NHK3 and WFS1-FLAG at a 1:1 or 1:2 ratio of NHK3:WFS1. Lysates were then immunoblotted with anti-alpha-1-antitrypsin, anti-FLAG, and anti-actin antibodies (n=3).

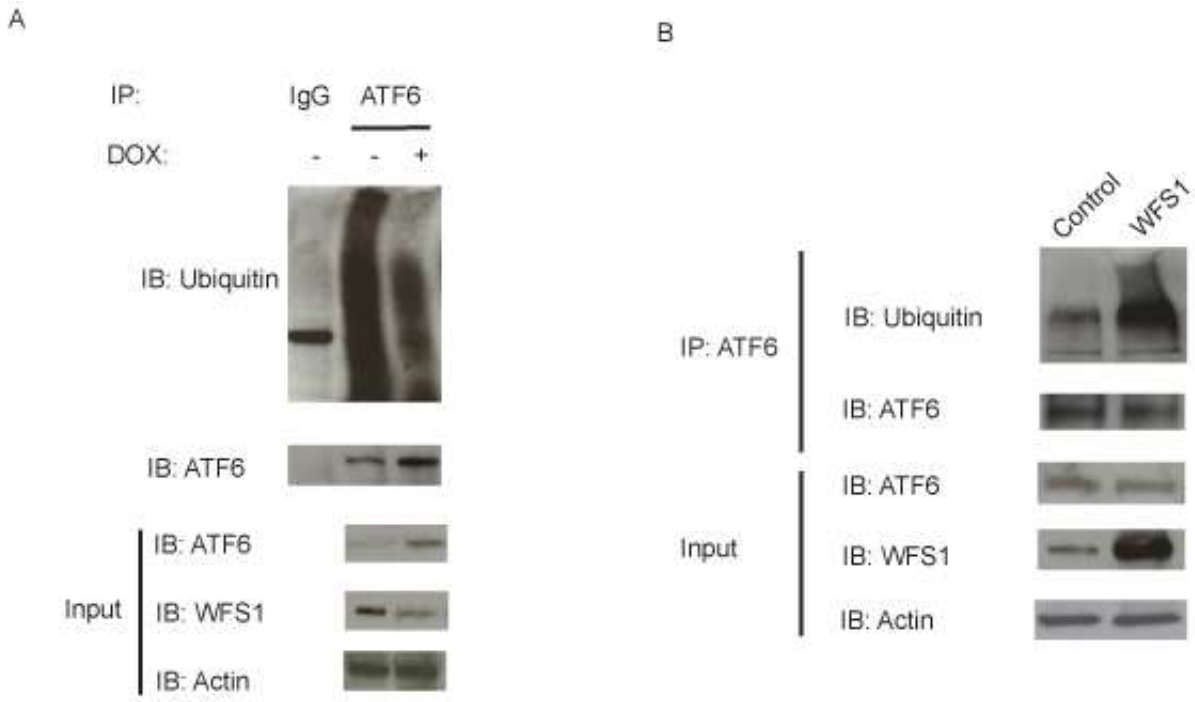

Figure 3.14 WFS1 Enhances ATF6 Ubiquitination. (A) ATF6 was immunoprecipitated (IP) using anti-ATF6 antibody from INS1 832/13 cell lines inducibly expressing shWFS1 (treated for 48 hrs with 2 µM doxycycline) and treated with MG132 (20 µM) for 3 hr. Immunoprecipitates were then immunoblotted with anti-ubiquitin and anti-ATF6 antibodies, and input lysates were blotted with anti-ATF6, anti-WFS1, and anti-actin antibodies (n=3). (B) ATF6 was immunoprecipitated, using an anti-ATF6 antibody, from INS1 832/13 cells expressing GFP (control) or WFS1, then treated with MG132 (0.1 µM) O/N. Immunoprecipitates were then immunoblotted with anti-ubiquitin and anti-ATF6 antibodies, and input lysates were blotted with anti-ATF6, anti-WFS1, and anti-actin antibodies (n=3).

enhancement of ATF6 ubiquitination (Figure 3.14B). In WFS1 -/- mouse pancreata, ATF6 protein expression was strikingly higher than in control littermate pancreata (Figure 3.15A), indicating that WFS1 functions in ATF6 protein expression *in vivo*. In samples from patients with WFS1 mutations, there was a higher expression of ATF6 protein, as compared with control samples (Figure 3.15B). Together, these results indicate that WFS1 is important for regulating ATF6 protein expression. Thus, when WFS1 is not present, there is increased expression of ATF6 protein and hyperactivation of its downstream effectors.

These data raised the possibility that WFS1 is recruiting ATF6 to the proteasome for its degradation. As we predicted, WFS1 formed a complex with the proteasome (Figure 3.16A), and when glycerol-gradient fractionation was performed on ER-isolated lysates, the proteasome, ATF6, and WFS1 co-migrated in the same high-molecular weight fractions (Figure 3.16B) and a complex between them was formed (Figure 3.16C).

WFS1 stabilizes HRD1, a novel E3 ligase for ATF6

Based on a homology search (http://www.genome.ad.jp/kegg/ssdb/), WFS1 has a very distant homology to an integral membrane protein of the ER, SEL1/HRD3, which has an important function in 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) degradation[(161)]. SEL1/HRD3 has been shown to interact with and stabilize the E3 ligase HRD1[(168)], raising the possibility that WFS1 could also interact with HRD1. Indeed, WFS1 and HRD1 form a complex (Figure 3.17A). WFS1 also plays a role in stabilizing HRD1 protein. In cells expressing shWFS1, there was suppression in HRD1 protein expression (Figure 3.17B-C). In WFS1-/- mouse pancreata, HRD1 expression was undetectable (Figure 3.18A), while in patient samples

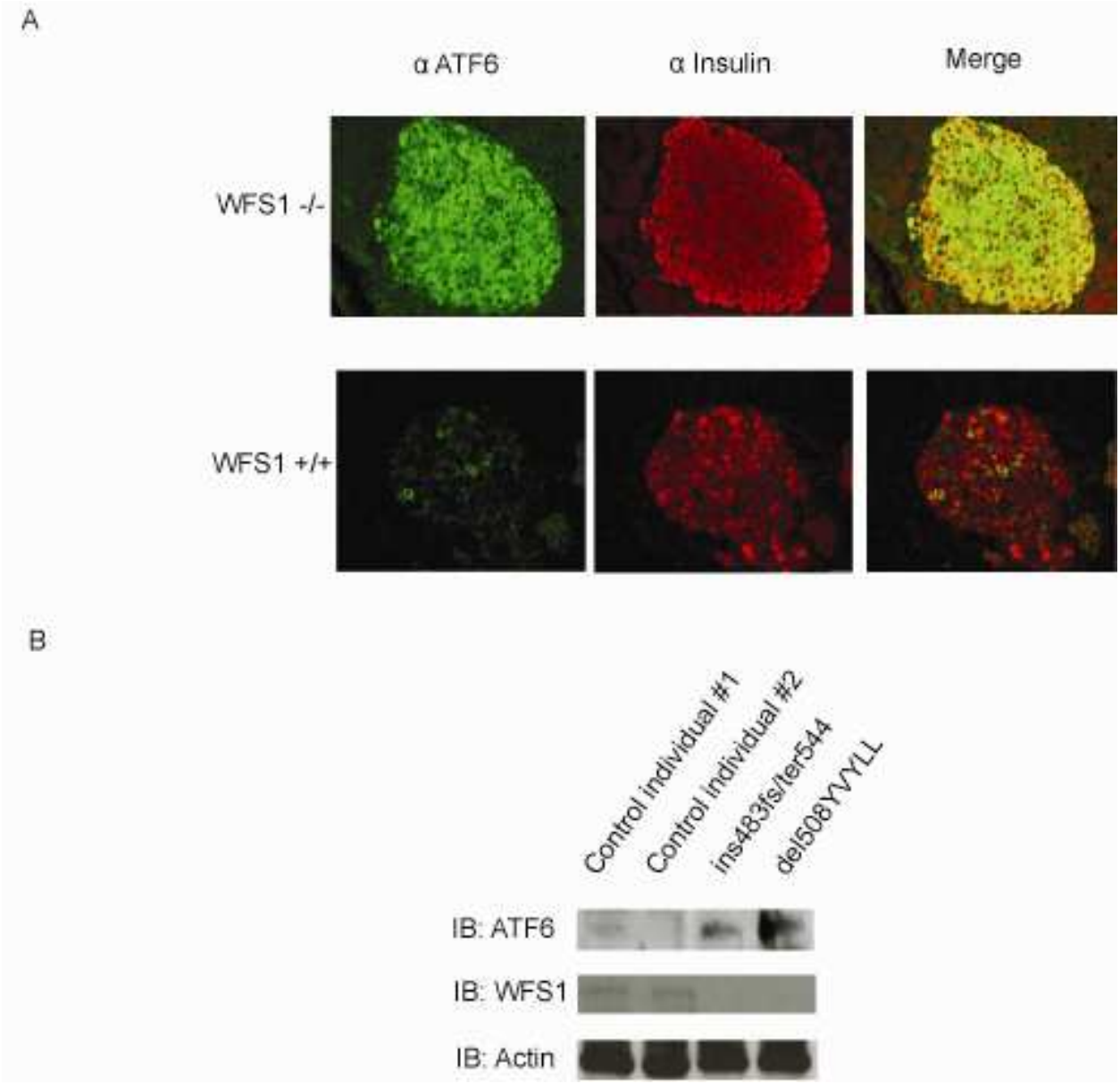

Figure 3.15 WFS1 Regulates ATF6 *In Vivo*. (A) WFS1 -/- and wildtype littermate mouse pancreata were analyzed by immunihistochemistry using anti-ATF6 and anti-insulin antibodies (n=3). (B) Lysates from Wolfram syndrome patient (ins483fs/ter544 and del508YVYLL) lymphoblasts and control individuals were immunoblotted with anti-ATF6, anti-WFS1, and anti-actin antibodies (n=3).

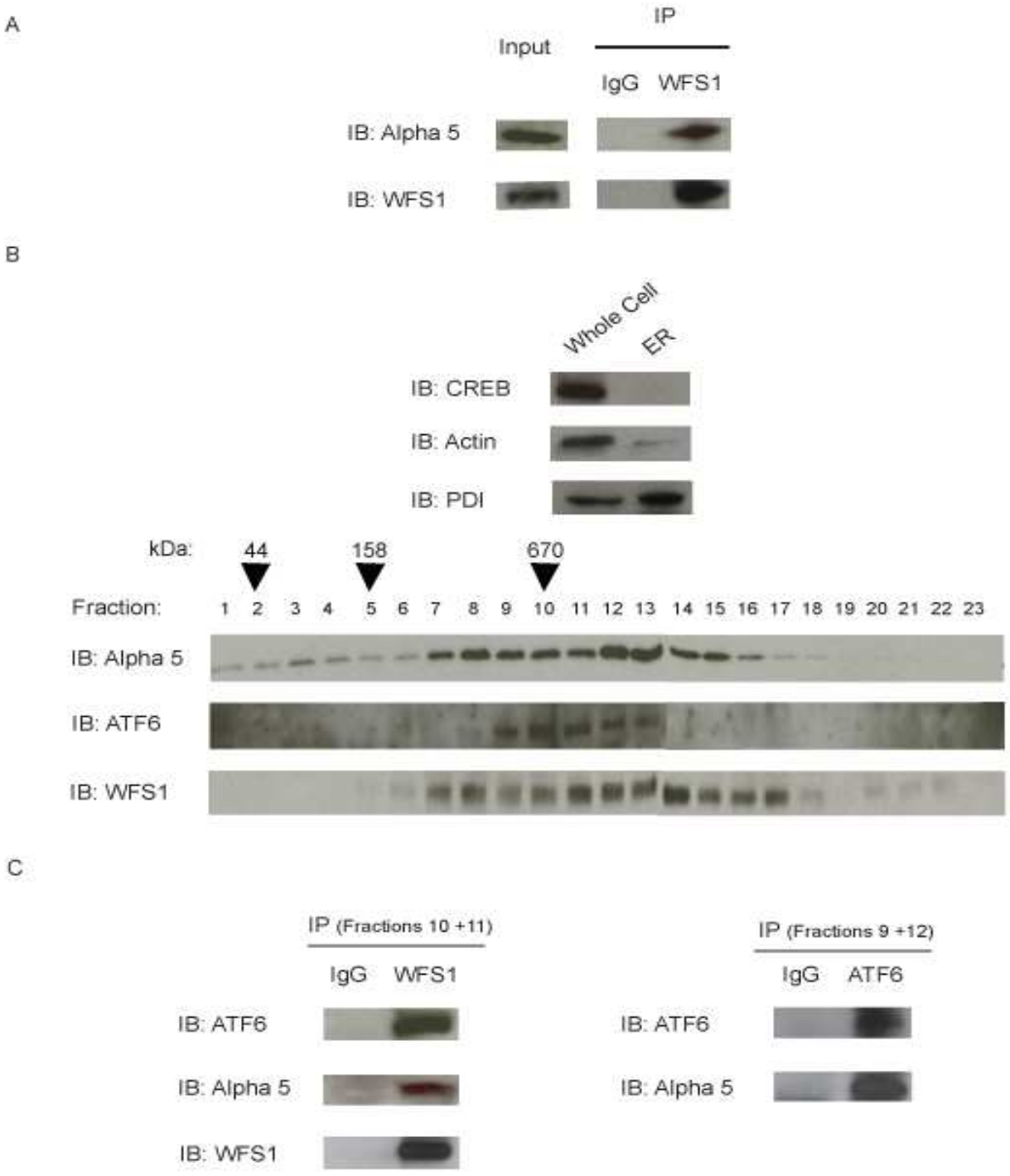

Figure 3.16 WFS1 Forms a Complex with ATF6 and the Proteasome. (A) WFS1 was immunoprecipitated from INS1 832/13 cells using an anti-WFS1 specific antibody. Immunoprecipitates were immunoblotted (IB) with anti-alpha 5 20S proteasome and anti-WFS1 antibodies (n=3). (B) Whole cell lysates or ER-isolated lysates of INS1 832/13 cells were subject to immunoblot using anti-CREB, anti-actin, and anti-PDI antibodies (upper panel). ER-isolated lysates of INS1 832/13 cells were subject to fractionation using a 10-40% glycerol gradient. Fractions were analyzed by immunoblot using anti-alpha 5 20s proteosome, anti-ATF6, and anti-WFS1 antibodies (lower panel)

(n=3). (C) WFS1 was immunoprecipitated (IP) from a mixture of fractions 10-11 using an anti-WFS1 antibody and IP products subject to immunoblot analysis using anti-alpha 5 20s proteosome, anti-ATF6, and anti-WFS1 antibodies (left panel). ATF6 was immunoprecipitated from a mixture of fractions 9 and 12, and IP products analyzed by immunoblot with anti-alpha 5 20s proteosome and anti-ATF6 (right panel) (n=3).

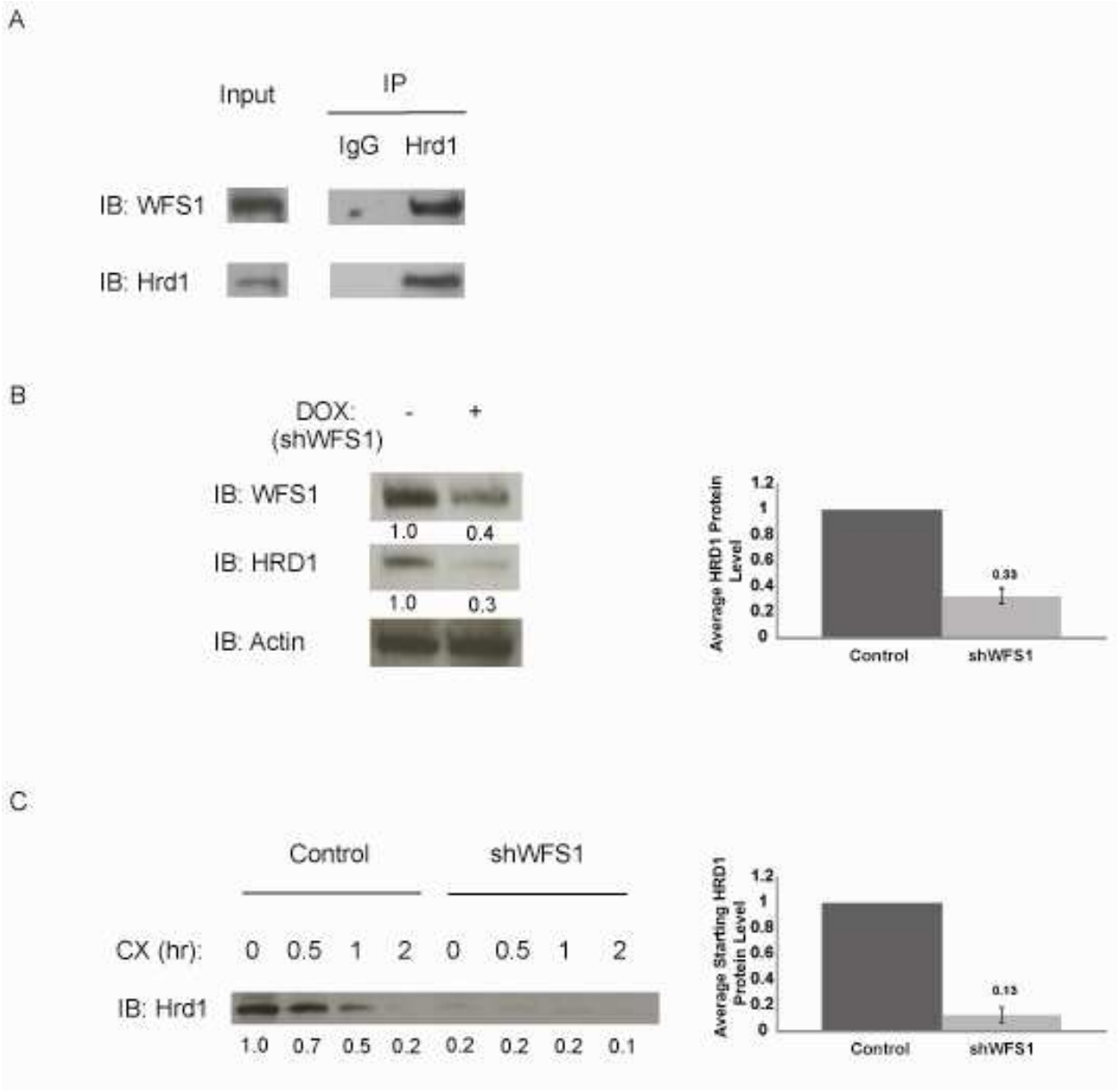

Figure 3.17 WFS1 Interacts with and Stabilizes the E3 Ligase HRD1. (A) Hrd1 was immunoprecipitated (IP) from INS1 832/13 cells and immunoprecipitates were subject to immunoblot (IB) analysis using anti-WFS1 and anti-Hrd1 antibodies (n=3). (B) Total lysates from INS1 832/13 cells inducibly expressing shWFS1 (treated with doxycycline (2 μM) for 48 hrs) were analyzed by IB using anti-WFS1, anti-Hrd1, and anti-actin antibodies (n=3; values are mean ± SD). (C) MIN6 cells stably expressing shRNA to GFP (control) or shRNA to WFS1 (shWFS1) were treated with 40 μM cyclohexamide (CX) or 0, 0.5, 1, and 2 hr. Total lysates were analyzed using an anti-Hrd1 antibody (n=3; values are mean ± SD). The relative amount of HRD1 protein was quantified using ImageJ software.

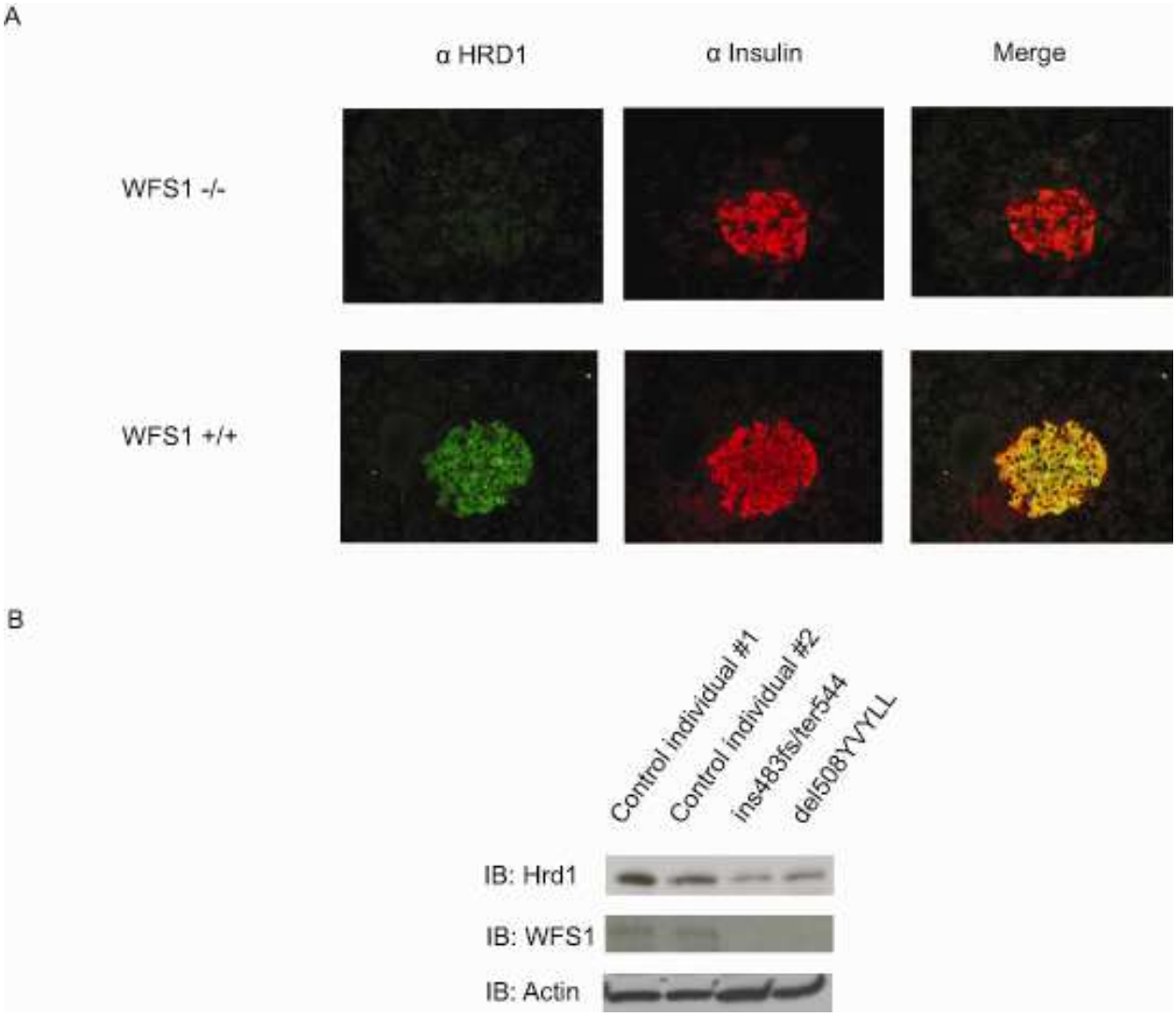

Figure 3.18 WFS1 Stabilizes HRD1 *In Vivo*. (A) WFS1 -/- and wildtype littermate mouse pancreata were analyzed by immunohistochemistry using anti-Hrd1 and anti-insulin antibodies (n=3). (B) Lymphoblast lysates from Wolfram syndrome patients (ins483fs/ter544 and del508YVYLL) and control individuals were immunoblotted (IB) with anti-Hrd1 and anti-actin antibodies (n=3).

with Wolfram syndrome, there was less HRD1 protein expression compared to control samples (Figure 3.18B). HRD1 expression did not affect WFS1 protein expression (Figure 3.19). These results demonstrate that WFS1 stabilizes and enhances the function of the E3 ligase HRD1.

Based on the ability of WFS1 to regulate ATF6 protein, as well as its function in stabilizing HRD1, it followed that WFS1 may be recruiting ATF6 to HRD1, and that ATF6 is a substrate of HRD1. In glycerol-gradient fractionation experiments of ER-isolated lysates, HRD1, ATF6, and WFS1 were found to form a complex (Figure 3.20A). HRD1 suppression in cells enhanced ATF6 protein stability (Figure 3.20B), while overexpression of HRD1 enhanced ATF6 protein degradation (Figure 3.20C). HRD1 also enhanced ATF6 ubiquitination (Figure 3.21A), while the lack of HRD1 decreased ATF6 ubiquitination (Figure 3.21B). Collectively, these results indicate that the WFS1-HRD1 complex enhances ATF6 ubiquitination and degradation.

WFS1 protects cells from hyperactivation of the UPR

Based on evidence that WFS1 regulates ATF6 in an ER stress-dependent manner, it follows that in Wolfram syndrome, in which there is a lack of functional WFS1 protein, β-cell death is due to a hyperactivation of the UPR through the ATF6 pathway. Indeed, pancreatic β-cells expressing shWFS1 are significantly more susceptible to ER-stress mediated apoptosis from palmitate treatment compared to control cells (Figure 3.22). Together, the data in this chapter indicate that WFS1 is important for regulating ATF6 protein expression. When WFS1 is not present, there is increased expression of ATF6 protein, hyperactivation of its downstream effectors, and enhancement of ER stress-mediated apoptosis.

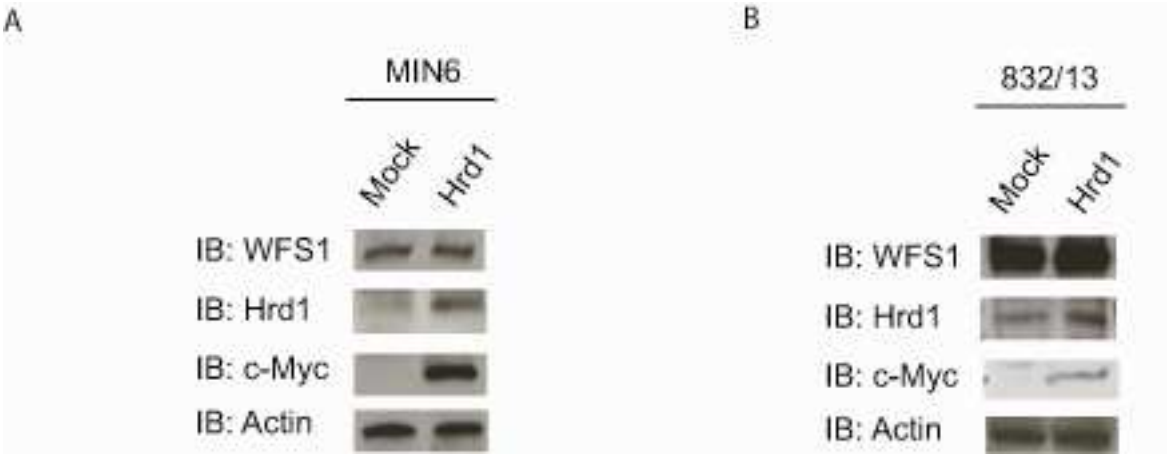

Figure 3.19 HRD1 Does Not Regulate WFS1 Protein. (A) MIN6 cells were mock transfected or transfected with a Hrd1-Myc expression plasmid and lysates were subject to IB using anti-WFS1, anti-Hrd1, anti-c-Myc, and anti-actin antibodies (n=3). (B) INS1 832/13 cells were mock transfected or transfected with a Hrd1-Myc expression plasmid and lysates were subject to IB using anti-WFS1, anti-Hrd1, anti-c-Myc, and anti-actin antibodies (n=3).

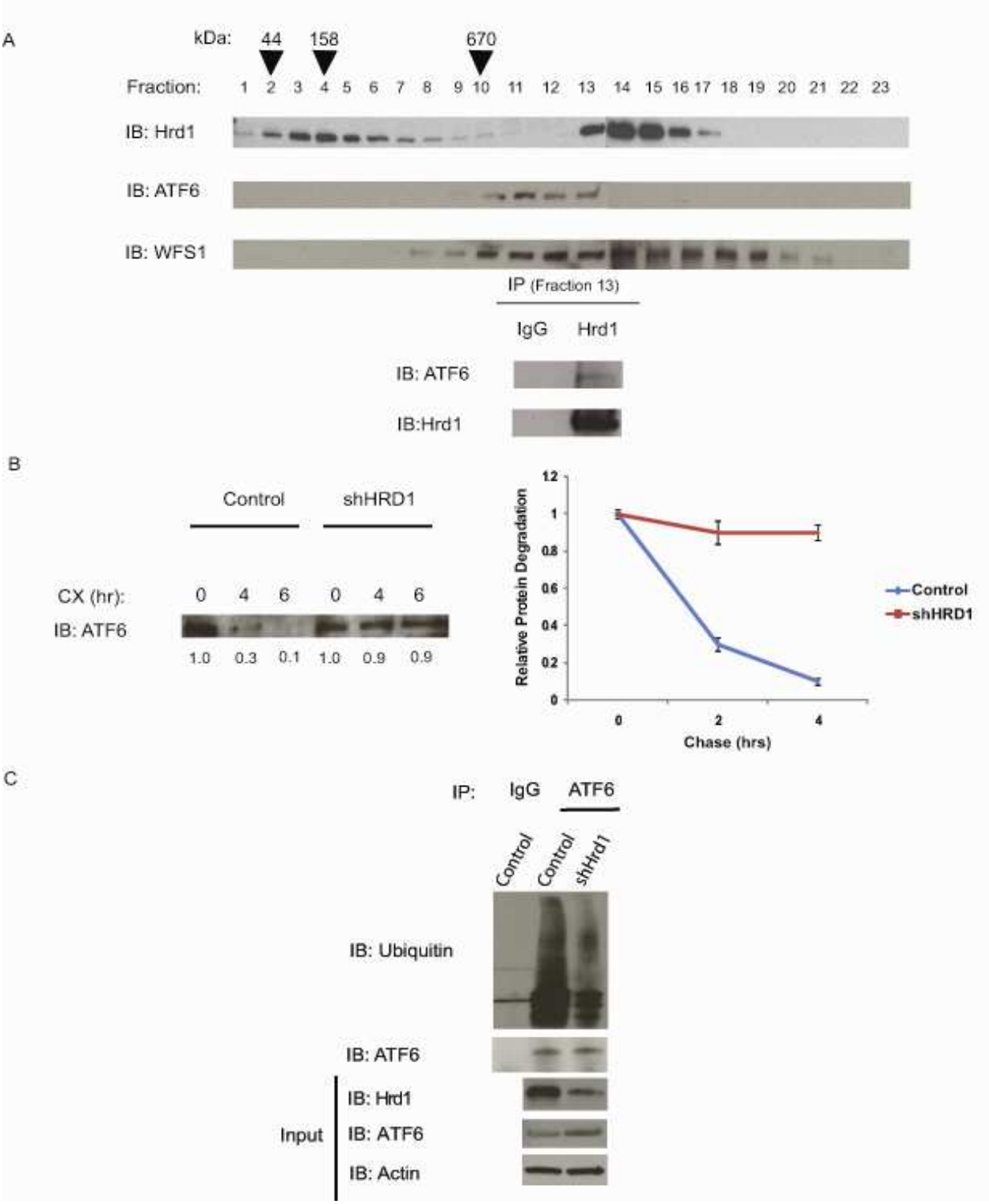

Figure 3.20 HRD1 is an E3 Ligase for ATF6. (A) ER-isolated lysates of INS1 832/13 cells were subject to fractionation using a 10-40% glycerol gradient. Fractions were analyzed by immunoblot using anti-Hrd1, anti-

ATF6, and anti-WFS1 antibodies (upper panel). Hrd1 was immunoprecipitated from fraction 13, and IP products analyzed by immunoblot with anti-Hrd1 and anti-ATF6 antibodies (lower panel) (n=3). (B) MIN6 cells stably expressing shRNA to GFP (control) or shRNA to HRD1 (shHRD1) were treated with 40 μM cyclohexamide (CX) for 0, 4, and 6 hr. Total lysates were analyzed using an anti-ATF6 antibody (n=3). The relative amount of ATF6 protein was quantified using ImageJ software. (C) ATF6 was immunoprecipitated (IP) using an anti-ATF6 antibody from MIN6 cells stably expressing shGFP (control) or shHRD1 and treated with MG132 (20 μM) for 3 hr. Immuoprecipitates were then immunoblotted with anti-ubiquitin and anti-ATF6 antibodies, and input lysates were blotted with anti-Hrd1, anti-ATF6, and anti-actin antibodies (n=3).

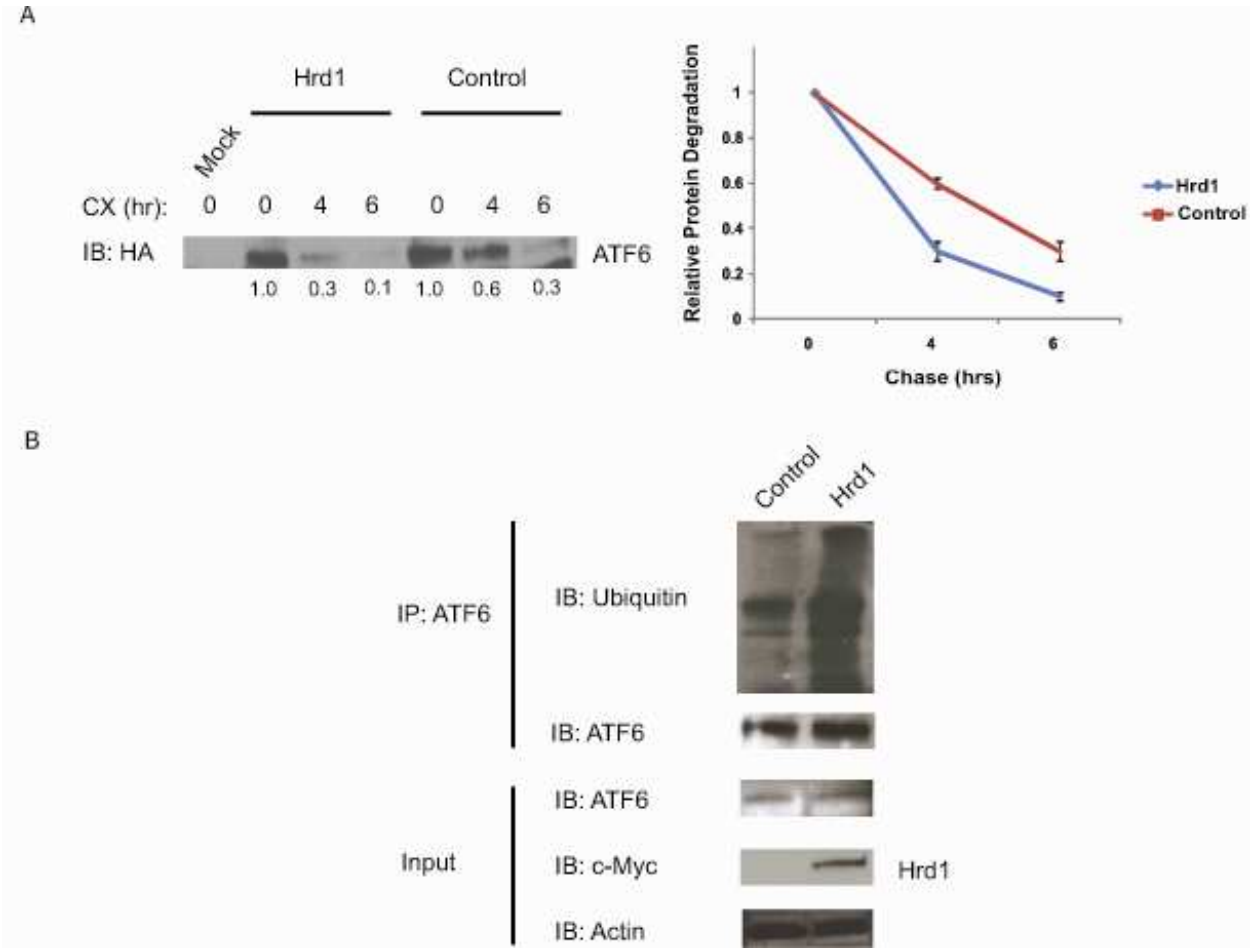

Figure 3.21 HRD1 Enhances ATF6 Degradation Through its Interaction with WFS1. (A) COS7 cells transfected with ATF6-HA expression plasmid (control) or ATF6-HA together with Hrd1-myc expression plasmids (Hrd1) were treated with 40 µM cyclohexamide for 0 hr, 4hr, and 6 hr. Whole cell lysates were subject to immunoblot (IB) with an anti-HA antibody (n=3). The relative amounts of HRD1 and ATF6 proteins were quantified using ImageJ software. (B) ATF6 was immunoprecipitated (IP) using an anti-ATF6 antibody from INS1 832/13 cells either mock transfected (control) or transfected with a Hrd1-Myc expression plasmid and treated with MG132 (20 µM) for 3 hr. Immuoprecipitates were then immunoblotted with anti-ubiquitin and anti-ATF6 antibodies, and input lysates were blotted with anti-ATF6, anti-c-Myc, and anti-actin antibodies (n=3).

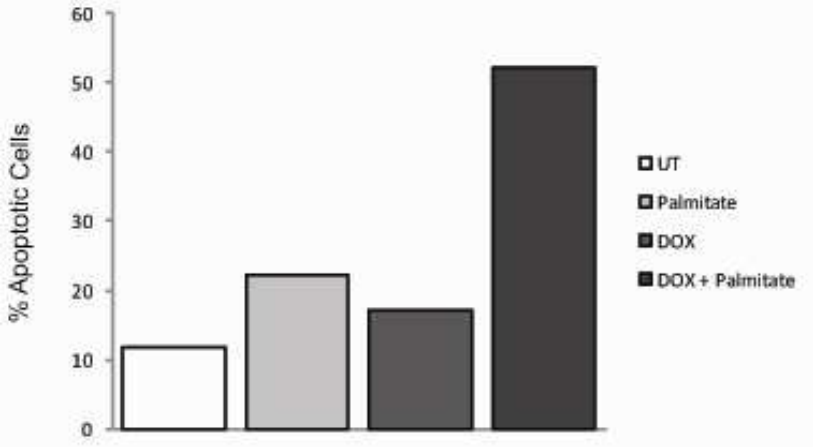

Figure 3.22 Suppression of WFS1 Enhances Free Fatty Acid-Induced Apoptosis. INS1 832/13 cells inducibly expressing shWFS1 (treated with doxycycline (2 µM) for 48 hrs) were treated with palmitate (50 µg/ml) for 24 hr. Cells were then stained Annexin V-PE followed by FACS analysis (n=3).

DISCUSSION

In this chapter, evidence was provided that WFS1 plays a crucial role in regulating ATF6 transcriptional activity through the HRD1-mediated ubiquitination and proteasome-mediated degradation of ATF6 protein. Based upon the data provided, we propose a pathway for a negative-feedback regulation of ER stress signaling network by WFS1 (Figure 3.23). Under non-ER stress conditions, WFS1 prevents premature activation of ER stress signaling through the ATF6 pathway by recruiting it to HRD1 and the proteasome for ubiquitin-mediated degradation (Figure 3.23, upper panel). When stress is applied to the ER, such as through the chemical ER stress inducer DTT, ATF6 is released from WFS1. It is then released from the ER membrane and translocates to the nucleus where it upregulates stress signaling targets (Figure 3.23, lower panel). While the concept of protein degradation-mediated control of signaling has been established, for example the activation of the famed NF-κB transcription factor through degradation of its inhibitor IκB[171], it is a novel mechanism in the ER stress signaling network.

ER stress is caused by both physiological and pathological stimuli that can lead to the accumulation of unfolded and misfolded proteins in the ER. Pathophysiological stimuli include viral infection and mutations that impair client protein folding[172]. Physiological ER stress can be caused by a large biosynthetic load placed on the ER, for example, during postprandial stimulation of proinsulin biosynthesis in pancreatic β-cells. This stimulation leads to the activation of ER stress signaling and enhancement of insulin synthesis[40]. Under physiological ER stress conditions, activation of ER stress signaling must be tightly regulated because hyperactivation or chronic activation of this signaling pathway can cause cell death. For example, when eukaryotic translation initiation factor 2α (eIF2α), a downstream component of ER stress signaling, is hyperphosphorylated by the compound salubrinal in pancreatic β-cells, the

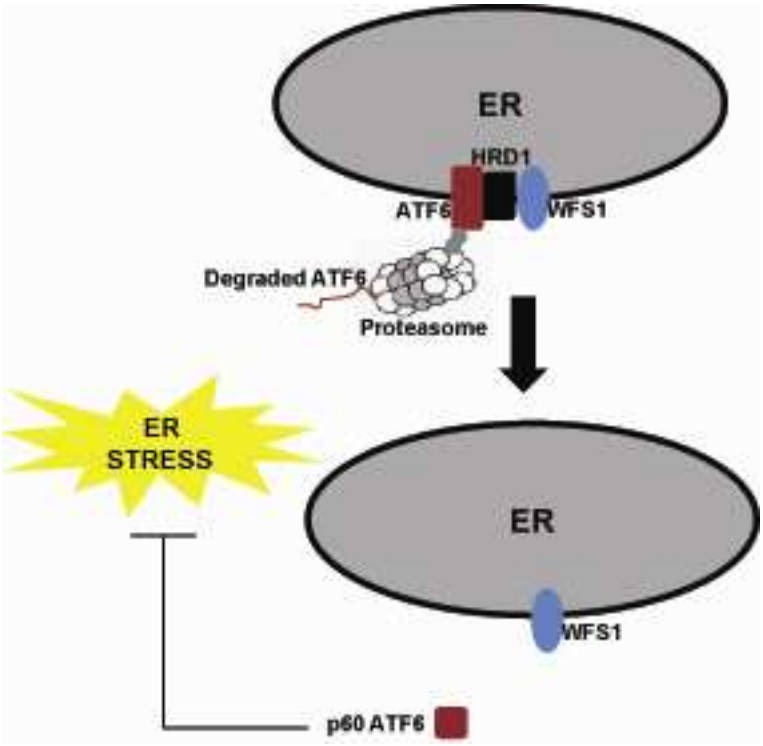

Figure 3.23 WFS1 Negatively Regulates the UPR Through ATF6 Proteolysis. WFS1 controls steady-state levels of ATF6 protein and actvation. Under non-ER stress conditions, WFS1 recruits the ER transcription factor ATF6 to the E3 ligase HRD1. HRD1 marks ATF6 with ubiquitin for proteasomal degradation (upper panel). Under ER stress conditions, ATF6 dissociates from WFS1, undergoes proteolysis, and its soluble amino-portion (p60 ATF6) translocates to the nucleus where it upregulates ER stress target genes such as BiP, CHOP, and XBP1 (lower panel).

result is the induction of apoptosis in these cells[59]. Our results show that WFS1 has an important function in the tight regulation of ER stress signaling through its interaction with a key transcription factor, ATF6, thereby protecting cells from the damaging effects of hyperactivation of this signaling pathway; hyperactivation of ATF6 can lead to CHOP-induced apoptosis, as this pro-apoptotic component of the UPR is a downstream target of ATF6.

WFS1 is highly expressed in pancreatic β-cells which are specialized for the production and regulated secretion of insulin to control blood glucose levels. In β-cells, ER stress signaling needs to be tightly regulated for adaptation to the frequent fluctuations of blood glucose levels and to produce the proper amount of insulin in response to the need for it[59,107]. Higher expression of WFS1 in β-cells, therefore, prevents hyperactivation of ER stress signaling in these cells which are particularly sensitive to disruption of ER homeostasis and dysregulation of the UPR. Therefore, WFS1 has a role in protecting β-cells from premature death by acting as an ER stress signaling suppressor.

Mutations in the WFS1 gene cause Wolfram syndrome, a genetic form of diabetes and neurodegeneration. It has been proposed that a high level of ER stress causes β-cell death and neurodegeneration in this disorder. Collectively, our results suggest that a loss-of-function of WFS1 causes the instability of an E3 ligase, HRD1, leading to the upregulation of ATF6 protein and hyperactivation of ATF6 signaling. Therefore, we predict that a loss-of-function or hypomorphic mutations of the WFS1, HRD1, or ATF6 gene can cause ER stress-related disorders, such as diabetes and neurodegeneration. Indeed, it has been shown recently that common variants in WFS1 confer risk of type 2 diabetes[126] and there is a link between WFS1

mutations and type 1 diabetes[132-33]. It has also been shown that ATF6 polymorphisms and haplotypes are associated with impaired glucose homeostasis and type 2 diabetes[173].

Excessive β-cell loss is a component of both type 1 and type 2 diabetes[77], therefore WFS1 may have a key role in the protection of these cells from apoptosis through the tight regulation of ER stress signaling, thereby suppressing the diabetes phenotype. In addition, about 60% of patients with Wolfram syndrome have some mental disturbance such as severe depression, psychosis, or organic brain syndrome, as well as impulsive verbal and physical aggression[174]. Heterozygotes who do not have Wolfram syndrome are 26-fold more likely than non-carriers to have a psychiatric hospitalization[175], and the relative risk of psychiatric hospitalization for depression is estimated to be 7.1[176]. Therefore, it is possible that dysregulation of a negative feedback loop of ER stress signaling may have a pathological role in psychiatric illness, as well.

While the experiments in this chapter convincingly demonstrate that a complex between HRD1, ATF6, and WFS1 exists, more detailed studies will be performed in the future to illustrate this more directly. For example, it will be assessed whether or not HRD1 suppression affects complex formation. In addition, experiments will be performed in which the interaction between ATF6 and HRD1 can be better visualized. In this case, treating cells with the proteasome inhibitor, MG132, would presumably enhance the interaction between this E3 ligase and its substrate. The possibility also exists that the affect that WFS1 expression has on ATF6p could be due to post-translational modifications (e.g. phosporylation of ATF6). While this seems unlikely, if this is occurring, the ATF6 antibody used may no longer bind to the modified ATF6. Simple experiments will be performed using labeled amino acids to assess the post-translational

modifications of ATF6p. To further assess the role of WFS1 on such modifications, ATF6 antibodies will be developed (e.g. a phospho-specific ATF6 antibody).

From this data, it is evident that WFS1 plays a similar role in mammals, as HRD3 in yeast, in that it promotes the stability and enhances the activity of HRD1p. The loss of WFS1, thus, may have two effects on ER stress: 1.) increasing ATF6 signaling by increasing the pool of ATF6p and 2.) decreasing HRD1p stability. While the first effect leads to hyperactivation of the ATF6 pathway, the second effect on HRD1p may independently contribute to ER stress by promoting the build-up of misfolded proteins in the ER. In addition, it must be noted that the induction of HRD1 by ER stress coupled by the stabilizing effect of WFS1 may act as a switch from the ATF6-mediated branch of the UPR to the IRE1 branch which acts later in the UPR. This is achieved through the destruction of ATF6 by a factor, WFS1, which is induced by IRE1.

In this study, we focused on determining the physiological function of WFS1 in ER stress signaling because of its implication in diabetes and neurodegeneration. WFS1 is a downstream target of IRE1 and PERK signaling in the UPR, is upregulated under ER stress, and then acts as a switch, turning off the ATF6 pathway (Figure 1.12). We propose that WFS1 has a critical function in the regulation of ER stress signaling and prevents secretory cells, such as pancreatic β-cells, from dysfunction and premature death caused by hyperactivation of ER stress signaling through its interaction with the transcription factor ATF6. WFS1 could therefore be a key target for prevention and/or therapy of ER stress-mediated diseases such as diabetes and neurodegenerative diseases. While this chapter revealed a novel role for WFS1 in a negative feedback loop of the UPR through its interaction with ATF6, the next will demonstrate other possible roles for WFS1, including maintaining the maturation status of the pancreatic β-cell.

CHAPTER IV

DISCUSSION AND PERSPECTIVES

Diabetes mellitus is a disease that is reaching epidemic proportions, with the predicted number of affected individuals with type 2 diabetes reaching over 300 million by the year 2025[79] and its estimated cost in the US reaching over $100 billion/year[80]. A major problem with current treatments for diabetes is the failure of medications in the long-term maintenance of normoglycemia – patients must change medications every 5-10 years and the issue of halting the progression of the disease has not been adequately addressed, a state called "secondary failure"[177]. It is therefore critical to understand the pathogenesis of this disease.

The progression of diabetes stems from chronic hyperglycemia which leads to β-cell dysfunction. This is a state of "glucotoxicity" – prolonged exposure of β-cells to high glucose concentrations leads to reduced insulin secretion and gene expression due to the decreased binding of the transcription factors PDX-1 and MafA to the insulin gene promoter[216]. One mechanism to account for these detrimental effects is the production of reactive oxygen species (ROS)[217], however, chronic hyperglycemia also induces ER stress[68]. Accumulating evidence suggests that there is a link between ER stress and β-cell function[65,86], suggesting that one of the mechanisms involved in β-cell dysfunction is defective ER stress signaling or an inadequate UPR response. Indeed, it has been suggested that β-cell failure is initiated by one or more of the following defects in the β-cell: mitochondrial dysfunction, dysfunction in triglyceride/FFA cycling, glucolipotoxicity, oxidative stress, and ER stress[178]. In fact, ER stress and defects in the UPR have been implicated in both type 1 and type 2 diabetes[24, 48, 98, 105]. This thesis work was undertaken to further understand the mechanisms of ER stress in β-cell death and dysfunction in

the progression of diabetes. Knowledge of these mechanisms could potentially lead to the development of novel therapies to prevent and treat this complex disease.

The goal of this work was to identify key regulators of the UPR that may contribute to β-cell death and dysfunction in the progression of disease. We took on studying a rare disease, Wolfram syndrome, because research in uncommon diseases can help lead to answers for more common diseases. Often, there is a single genetic defect which can allow one to focus on the function of a protein or gene, and often that gene/protein is involved in common diseases. An example of this is retinoblastoma (RB), a rare, juvenile disease in which tumors develop from the immature retina. Here, there is a loss of the Rb gene from chromosome 13[218]. Since this loss is linked with retinoblastoma, and Rb is found in all cell types, studying the molecular mechanisms of tumor suppression by Rb has given insight into other forms of cancer. Thus, Wolfram syndrome seemed like a great target, as this disease involves a selective loss of β-cells leading to the primary phenotype of diabetes. At the time that this thesis work was undertaken, indeed it had been shown that Wolfram syndrome was caused by a mutation in a single gene, WFS1[112,114]. It had also been determined that WFS1 is an ER transmembrane protein[121]. This is when we made the link that WFS1 may have a function in ER stress signaling, due to its subcellular localization, and that β-cell loss in Wolfram syndrome may be ER stress-mediated. Thus we attempted to take an orphaned, neglected disease and relate it to common forms of diabetes in which loss of β-cell mass via apoptosis also occurs[77,96].

In Chapter II, we presented evidence that WFS1 is indeed a component of the UPR and is localized specifically to the β-cell of the pancreas. We demonstrated that WFS1 is a downstream target of IRE1 and PERK signaling, and is upregulated under ER stress. Suppression of WFS1 in

β-cells causes ER stress and cell dysfunction. This data was supported by a study which was published concomitantly with ours, which showed that mice lacking WFS1 developed diabetes and this was attributed to excessive ER stress and ER stress-mediated apoptosis in their β-cells[135]. This suggested to us that the pathogenesis of Wolfram syndrome involves chronic ER stress and apoptosis in pancreatic β-cells due to a loss-of-function of WFS1. Indeed our data supports this hypothesis. These findings increased our understanding of the link between ER stress and diabetes, however we did not have a clear insight into the precise mechanisms involved.

In order to understand the precise mechanism of Wolfram syndrome and find ways to relate this to more common forms of diabetes, we sought to determine the function of WFS1 protein in the UPR. This was challenging, since WFS1 does not have a distinct homology to any known proteins. We therefore took the approach of looking at interacting partners of WFS1. We started out determining if WFS1 could form a complex with any of the three master regulators of the UPR: IRE1, PERK, and ATF6. In Chapter III, we presented evidence of an endogenous interaction between WFS1 and the master transcription factor of the UPR, ATF6. We hypothesized that this interaction was dependent on the ER stress status of the cell. Our findings supported this hypothesis and we found that the relationship of WFS1 and ATF6 is ER stress-mediated. We determined that WFS1, in fact, regulates ATF6 protein through the ubiquitin-proteasome pathway: WFS1 recruits ATF6 to the E3 ligase, HRD1, and the proteasome, and enhances its degradation. This was quite an unexpected role of WFS1 in the UPR as a negative regulator. These findings further supported our observations that Wolfram syndrome is an ER stress-mediated disease, as well as gave us insight into the precise molecular mechanism of β-cell loss in the presence of WFS1 dysfunction.

Collectively, these data support the previous findings that hyperactivation of the UPR is detrimental to the cell – for example, hyperactivation of IRE1, as measured by its chronic phoshorylation, leads to cell death via the JNK pathway[59,65,86]. Hyperactivation of any of the three major pathways of the UPR, IRE1, PERK, or ATF6 ultimately leads to activation of the mitochondrial-caspase-3 pathway. With a lack of functional WFS1, which occurs in Wolfram syndrome, the ATF6 pathway becomes hyperactivated. As seen in our cell lines in which WFS1 is suppressed, as well as in WFS1-/- mouse pancreata and Wolfram syndrome patient cells, ATF6 protein expression is higher, as well as ATF6 target gene expression. This hyperactivation of the ATF6 pathway may be the primary reason why β-cells are selectively destroyed in this disease. This is supported by the data that CHOP, a pro-apoptotic component of ER stress signaling, is a target of ATF6[36,46-48].

The body of this work demonstrates that there indeed is a link between ER stress and diabetes, and that WFS1 is an important component of ER stress signaling in the β-cell. We were then pleased, and not so much surprised, when several genome-wide studies were published that identified WFS1 polymorphisms are associated with an increased risk of type 2 diabetes[126-31]. It is likely that this is at least partly due to a loss of β-cell function, as these polymorphisms are associated with a reduction in insulin secretion[128]. But the question remained: *Why are β-cells particularly susceptible to hyperactivation of the UPR through the WFS1-ATF6 pathway?* There are several possibilities which exist that could provide an explanation for this. We always get asked why other secretory cells are not particularly affected by ER stress-mediated apoptosis. β-cells should technically be robust cells, as they encounter more energy fluctuations than other cells of the body and they are under constant pressure to produce and secrete insulin. Thus, through evolution these cells should have developed a defense mechanism against such stress.

However, because of the constant demands of insulin biosynthesis, this has made β-cells dependent on an efficient UPR. In fact, baseline ER stress levels are higher in these cells than other cells[65,73]. Any additional stress, therefore, applied to the cell causes there to be an imbalance between the homeostatic outputs (e.g. chaperone induction) and apoptotic outputs (e.g. JNK and CHOP activation) of the UPR. Lack of WFS1 causes such an imbalance.

Another theory is that there exists WFS1-like molecules in other secretory cells and that WFS1 confers specificity on the β-cell. While WFS1 is expressed in other cell types, it is highly expressed in the pancreatic β-cell. Therefore, mutations in WFS1 will primarily affect these cells, explaining why the first symptom to present in Wolfram syndrome is diabetes. This could be defined as a cell type-specific ER stress response. There exist other examples of this. OASIS, for example, regulates the signaling of the UPR specifically in astrocytes[179], and CREBH has been identified as a hepatocyte-specific UPR transducer[180].

While mutations in WFS1 lead to hyperactivation of the ATF6 pathway, prior to cell death, β-cell dysfunction also occurs in which there is an impairment of stimulus-coupling insulin secretion[134]. Our studies also show that WFS1 is upregulated during insulin secretion. We also demonstrated that WFS1 is a downstream target of IRE1 and PERK signaling, both of which are involved in insulin biosynthesis. Activation of IRE1 enhances insulin biosynthesis[65], while PERK is a negative regulator of this process[26]. It follows that WFS1 may have an additional function of regulating insulin biosynthesis and/or secretion in the β-cell. Interestingly, activation of ATF6 by ER stress has also been shown to decrease insulin gene expression[76]. ATF6 polymorphisms and haplotypes have recently been shown to be associated with impaired glucose homeostasis and type 2 diabetes[173]. This raises the possibility that the interaction

between WFS1 and ATF6 not only functions as a method to regulate the UPR, but also to regulate insulin: by restricting activation of ATF6 by recruiting it to the proteasome, WFS1 prevents ATF6-mediated suppression of insulin gene expression. This may also in part explain the association of WFS1 polymorphisms and type 2 diabetes: in type 2 diabetes, there is a classic decrease in insulin gene expression[181]. Thus, there is a possible dual function of the WFS1-ATF6 complex, which is quite unexpected.

WFS1 has proven to be quite an interesting protein to research. Not only is it a negative regulator of the UPR, but it is also possibly a regulator of insulin biosynthesis. WFS1 may have several other roles, as well. We have preliminary data which suggests that WFS1 may have a function in the transdifferentiation of non-β-cells of the pancreas into β-cells. Based on published findings, this is not all that far-fetched. Published data indicate that several components of the UPR play a key role in the development and differentiation of various secretory cells from precursors. IRE1 and its downstream effector, XBP1, are required for B cell lymphopoeisis[182-84]. XBP1 has also been shown to be central to the differentiation of dendritic cells[185], hepatocytes[186], and exocrine pancreatic acinar cells[187]. PERK, another master regulator of the UPR, is principal for bone and cartilage development[188], while activation of the UPR transcription factor ATF6, is important for myogenesis[189]. It has also been shown that both pancreatic acinar cells, as well as duct cells can transdifferentiate into β-cells[190-91]. The role of the UPR in β-cell differentiation and maturation has not been tested, but since the UPR has been established to play a central role in the differentiation of other secretory cells, and non-β-cells can be transdifferentiated into β-cells, it follows that components of the UPR may be involved in the maturation and differentiation of β-cells from non-β-cell components of the pancreas. We discovered that WFS1 expression in an acinar cell line, AR42J, can induce insulin gene

expression (Figure 4.1). In addition, long-term suppression of WFS1 in β-cell cell lines leads to a change in cell morphology, perhaps indicating a reversion to a more immature state (Figure 4.2). Control cells form clusters similar to islets, while shWFS1 cells grow in a monolayer and lose this ability to form three-dimensional structures. This suggests that WFS1 may be involved in maintaining the mature status of the β-cell which is defined as a glucose-responsive, insulin-producing cell. This data is supported by a study which showed altered cell morphology and growth when WFS1 antisense constructs were expressed in BRIN-BD11 β-cells[193].This is quite an interesting prospect and needs further exploration.

WFS1 may not just be important in the pancreatic β-cell. While it is highly expressed in these cells, it is also expressed in the brain[192]. Neurological dysfunction also presents as a symptom of Wolfram syndrome, however this typically occurs in the second decade of the disease. The question that needs to be addressed is *why are β-cells the primary cells to be affected when WFS1 is mutated?* Again, if we go back to the concept that β-cells are professional secretory cells with a critical dependence on the UPR, it makes sense why there would be a delay in disease phenotype in neuronal cells. However, it cannot be disputed that WFS1 does have an important function in the brain. Several studies show that WFS1 heterozygotes are more likely to have psychiatric illness than homozygotes[174-76]. Thus, because WFS1 has a function in the brain, it follows that it may also be implicated in other neurologic diseases, most of which are protein misfolding diseases, such as Alzheimer's and Parkinson's. WFS1, therefore, may be more important than what we originally thought, because according to Dr. Gregory Petsko, we are now facing an epidemic of neurologic diseases. The main reason for this is that the age pyramid is flattening because lifespan has more than doubled since 1840 – for ages 65^{+} the risk for Alzheimer's and Parkinson's increases exponentially. By the year 2050, 32 million people in

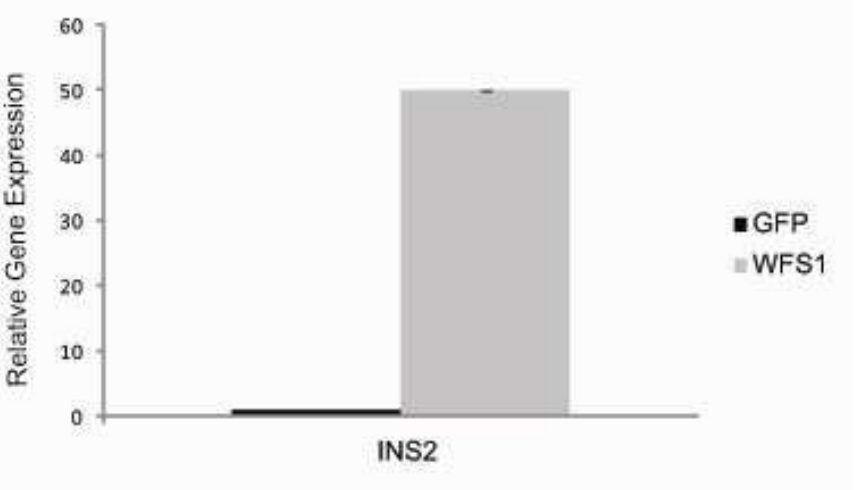

Figure 4.1 Expression of WFS1 in Acinar Cells Leads to Expression of Insulin mRNA (INS2). Rat acinar cells, AR42J, were infected with GFP or WFS1 expression lentiviral vectors. The cells were cultured for 7 days in normal media and insulin gene expression then measured by quantitative real-time PCR and standardized to actin (n=3; values are mean $\pm$ SD).

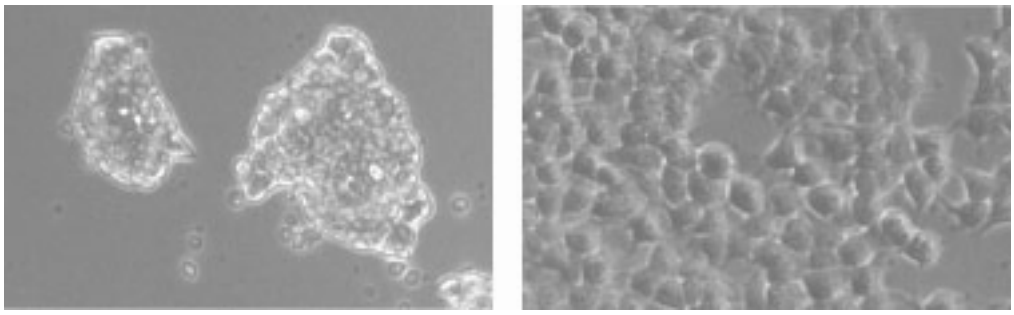

Figure 4.2 Suppression of WFS1 in β-cells Changes Cell Morphology. Mouse β-cells, MIN6, were infected with scramble shRNA (left panel) or shRNA directed against WFS1 (right panel) lentivirus and cultured for 30 days in normal media (n=5).

the US will be over the age of 80 and half of them will have Alzheimer's. The current cost of these diseases is over $330 million per year[194]. Thus, understanding components of the UPR is critical to approaching this epidemic.

Not only is understanding the mechanisms of the UPR important for studying protein misfolding diseases, such as neurodegenerative disorders, as well as diabetes, but there is increasing evidence that it may have a substantial role in the pathogenesis of cancer. Cancer cells, unlike normal cells, display elevated activity of the anti-apoptotic branch of the UPR[195]. Tumor cells have elevated levels of BiP, thought to be a defensive strategy for their survival[196-97]. WFS1 could be considered a member of the anti-apoptotic branch of the UPR, as suppression of WFS1 leads to the suppression of several anti-apoptotic molecules, such as AATF[198]. Thus, manipulation of WFS1 expression could be an attractive therapeutic modality for cancer treatment (e.g. suppress WFS1 during chemotherapy).

Collectively, this thesis work suggests that WFS1 may be an attractive therapeutic target for multiple diseases such as diabetes, cancer, and neurodegenerative disorders. Based on evidence presented in this work, WFS1 modulation may also be a solution to finding methods of enhancing pancreatic β-cell function, replication, and survival as a treatment for diabetes. These data illustrate that, indeed, it is important to not neglect researching rare diseases, such as Wolfram syndrome. Our studies have provided insight into the negative regulation of the UPR, an area of research that has not been well-studied. We have shown that WFS1 is a complex protein, and much more research needs to be undertaken to fully understand its multi-functional mechanisms. One area that would be particularly interesting to look at is how WFS1 is actually activated, an area that has elucidated us and others.

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