EXAMINING THE ROLE OF HERP IN THE ER STRESS RESPONSE OF PANCREATIC β-CELLS

I. THE ROLE OF HERP IN MISFOLDED PROINSULIN DEGRADATION AND CELL SURVIVAL

II. ANALYSIS OF HERP EXPRESSION IN INSULINOMA CELLS AND ISLETS OF ANIMAL MODELS OF DIABETES

By

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A thesis submitted in conformity with the requirements

For the degree of Master of Science

Graduate Department of Physiology

University of Toronto

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ABSTRACT

ER stress has emerged as a potential contributor to pancreatic β-cell dysfunction and is induced by diabetic conditions such as chronically elevated glucose, free fatty acids and cytokines. The unfolded protein response, which is activated during ER stress, counteracts stress conditions by increasing folding capacity and by increasing the degradation of misfolded ER proteins by the ER-Associated Degradation (ERAD) system. Studies using an engineered insulinoma cell line with inducible expression of the Akita folding-deficient insulin (C96Y), have shown that one of the most abundantly induced genes in this cell line encodes for Herp, a protein that has been implicated in the ERAD pathway. We hypothesized that Herp is an essential protein that regulates the degradation of misfolded insulin during the ER stress response. Indeed, we found that the degradation of mutant insulin is Herp-dependent and that maintaining Herp expression is vital for maintaining cell survival. We have also observed that the expression of Herp mRNA and protein is induced in various cell culture and animal models of diabetes. These results suggest that Herp as part of the ERAD system, is an important ER stress response protein that is induced under diabetic conditions in pancreatic β-cells.
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<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATF3, ATF4, ATF6</td>
<td>activating transcription factor 3,4,6</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ASK1</td>
<td>apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>BiP</td>
<td>immunoglobulin heavy chain binding protein</td>
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<tr>
<td>b-zip</td>
<td>basic leucine zipper domain</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>Cdc48p</td>
<td>cell division control protein 48</td>
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<tr>
<td>CHIP</td>
<td>carboxyl terminus of Hsp70-interacting protein</td>
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<tr>
<td>CHOP</td>
<td>C/EBP (CCAAT/enhancer binding protein) homologous protein</td>
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<td>CHX</td>
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<td>CNX</td>
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<tr>
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<td>calreticulin</td>
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<td>Cue1p</td>
<td>coupling of ubiquitin conjugation to ER degradation protein 1</td>
</tr>
<tr>
<td>DN</td>
<td>diabetic nephropathy</td>
</tr>
<tr>
<td>Derlin-1</td>
<td>degradation in endoplasmic reticulum protein 1</td>
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<tr>
<td>Dox</td>
<td>doxycycline</td>
</tr>
<tr>
<td>Dsk</td>
<td>dual-specific kinase</td>
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<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
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<td>EDEM</td>
<td>ER degradation enhancing a-mannosidase-like protein</td>
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<td>eIF-2α</td>
<td>eukaryotic initiation factor 2α</td>
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<td>--------------</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
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<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
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<td>ERp57</td>
<td>endoplasmic reticulum resident protein 57</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activate cell sorting</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>F-box only protein 2</td>
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<td>free fatty acid</td>
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<td>G418</td>
<td>geneticin</td>
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<tr>
<td>GADD34</td>
<td>growth arrest and DNA damage 34</td>
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<tr>
<td>Gp78</td>
<td>gene 78 protein</td>
</tr>
<tr>
<td>GRP78</td>
<td>glucose-regulated protein 78</td>
</tr>
<tr>
<td>GRP94</td>
<td>glucose-regulated protein 94</td>
</tr>
<tr>
<td>GSIS</td>
<td>glucose stimulated insulin secretion</td>
</tr>
<tr>
<td>GT</td>
<td>see UGGT</td>
</tr>
<tr>
<td>HBP</td>
<td>hexose biosynthetic pathway</td>
</tr>
<tr>
<td>HERP</td>
<td>homocysteine responsive ER-resident protein</td>
</tr>
<tr>
<td>HG</td>
<td>hyperglycemic</td>
</tr>
<tr>
<td>HNF4α</td>
<td>hepatocyte nuclear factor 4 alpha</td>
</tr>
<tr>
<td>Hrd1</td>
<td>HMG-CoA reductase degradation protein 1</td>
</tr>
<tr>
<td>Hsp70-40</td>
<td>heat shock protein 70-40</td>
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<tr>
<td>HSPA5</td>
<td>heat-shock 70-kD protein 5</td>
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HUVEC  human umbilical vascular endothelial cells
I.P.   immunoprecipitation
IRE1  inositol-requiring ER-to-nucleus signal kinase 1
IRS2  insulin receptor substrate 2
JNK   c-Jun NH2-terminal kinase
MHC   major histocompatibility complex
mRNA  messenger RNA
NEFA  non-esterified free fatty acid
NO    nitric oxide
NOS   nitric oxide synthase
Npl4p nuclear protein localization protein 4
NS1 κ LC non-secreted Ig kappa light chain
OS-9  osteosarcoma-9
PBA   phenylbutyric acid
PCR   polymerase chain reaction
PDI   protein-disulfide isomerase
PDX-1 pancreatic and duodenal homeobox gene 1
PERK  double-stranded RNA-activated protein kinase (PKR)-like ER kinase
PI3K  phosphatidylinositol-3’-kinase
RMA1  protein RING membrane-anchor 1
RNA   ribonucleic acid
ROS   reactive oxygen species

xi
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Sel1</td>
<td>suppressor of Lin12-like protein</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco-endoplasmic reticulum Ca(^{2+})-ATPase pump</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>Staur</td>
<td>staurosporin</td>
</tr>
<tr>
<td>TRAF2</td>
<td>TNF receptor-associated factor 2</td>
</tr>
<tr>
<td>TRB3</td>
<td>tribbles 3</td>
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<td>UBA</td>
<td>ubiquitin-associated domain</td>
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<td>Ubc1p</td>
<td>ubiquitin conjugating enzyme E2 1</td>
</tr>
<tr>
<td>Ubc7p</td>
<td>ubiquitin conjugating enzyme E2 7</td>
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<td>UBL</td>
<td>ubiquitin-like domain</td>
</tr>
<tr>
<td>UBX</td>
<td>ubiquitin regulatory domain</td>
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<tr>
<td>Ubx2p</td>
<td>UBX domain-containing protein 2</td>
</tr>
<tr>
<td>Ufd1</td>
<td>ubiquitin fusion degradation 1</td>
</tr>
<tr>
<td>UGGT (GT)</td>
<td>uridine diphosphate (UDP)-glucose/glycoprotein glucosyltransferase</td>
</tr>
<tr>
<td>uORF</td>
<td>upstream open reading frame</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>XPB1</td>
<td>X-box binding protein 1</td>
</tr>
<tr>
<td>XTP3-β</td>
<td>xanthosine 5'-triphosphate 3-β</td>
</tr>
<tr>
<td>Yos9p</td>
<td>yeast OS-9</td>
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CHAPTER 1: INTRODUCTION
This thesis will focus primarily on examining the role that the Homocysteine responsive ER-resident Protein (Herp) plays in the ER stress response of pancreatic β-cells. The first portion of the introductory chapter will provide a summary of work that has defined ER stress-induced β-cell dysfunction and the compensatory ER stress response in the context of type 2 diabetes. We will then take a detailed look at the role that ER associated degradation (ERAD) plays in the ER stress response and an in depth description of Herp as a component of ERAD will be provided. Finally, a description of animal models in which ER stress has contributed to β-cell dysfunction and the progression of diabetes will be presented in order to obtain a broader understanding of the disease state. These animal models of diabetes are used in the thesis to examine Herp expression in islets.

1.1 Type 2 Diabetes

During an era when life expectancy has increased to such high levels, we have ironically made ourselves targets of disease states that decrease both the quality and expectancy of our lives. Some of these diseases are the result of considerable lifestyle changes including both the adaptation of western diets rich in energy and saturated fats and a lack of physical exercise (1). Type 2 Diabetes falls within this definition and is one of the major causes of morbidity and mortality today. It develops in a subset of genetically predisposed individuals and is characterized by a reduction of β-cell mass and insulin resistance in the fat, muscle and liver tissues of those affected (2; 3). However, it is ultimately pancreatic β-cell failure that causes the hyperglycemia that defines the disease. In the sections to follow, I will discuss some of the major contributing factors to the progression and exacerbation of Type 2 Diabetes in the context of the pancreatic β-cell.
1.2 Pancreatic β-cells and Type 2 Diabetes

β-cell dysfunction is a central driving factor in the progression of Type 2 Diabetes. The major defects observed in the pancreatic β-cells of subjects with Type 2 Diabetes include defects of mass, function and altered molecular features. Mass defects have been characterized by an increased amount of apoptosis, with insufficient proliferation and neogenesis. On average this loss in mass translates to approximately 30%. In addition to β-cell loss, defects in β-cell function also contribute to causing diabetes (4). Defects in function found in β-cells include a reduction in insulin secretion and altered kinetics of insulin release, especially in response to glucose. This indicates that there may be possible alterations in certain steps of glucose metabolism in diabetics, including those that take place in the mitochondria. Such alterations would result in a reduction of ATP production required for insulin biosynthesis and secretion. Finally, molecular alterations have also been noted in pancreatic islets from subjects with Type 2 Diabetes. These include changes in the expression of genes highly involved in β-cell function such as decreases in HNF4α and the insulin receptor, IRS2 and the downregulation of molecules involved in the exocytosis of insulin granules. In contrast, genes that are upregulated in diabetic islets include those involved in the differentiation and proliferation pathways such as PDX-1 and Foxo-1, as well as genes that play functional roles in the endoplasmic reticulum (ER) and are established markers of the ER stress response, namely BiP and XBP-1 (discussed in section 1.5) (4-6).

1.3 Type 2 Diabetes and lipotoxicity

High fat intake and obesity are characterized by elevated serum free fatty acid levels that can cause lipotoxicity. Lipotoxicity is associated with insulin resistance in the peripheral tissues and the β-cell, and both have been described as contributors to the progression of Type 2 Diabetes (2). The molecular mechanisms of lipotoxicity in this disease state however, remain to be fully understood.
A large body of evidence suggests that chronic exposure to high lipid concentrations contributes to β-cell death. Furthermore, non-esterified free fatty acids (NEFAs) and adipokines secreted by adipocytes have been labeled as the mediators of cross talk between adipocytes and β-cells. In fact, high levels of circulating NEFAs and low adiponectin levels are indicators for progression towards a diabetic state (7-10). Both high-fat feeding and prolonged elevation of circulating NEFAs by lipid infusion has been found to impair pancreatic β-cell function especially in individuals with a genetic predisposition to Type 2 diabetes (3; 8; 11-15). It has been suggested that the mechanisms underlying lipotoxicity most probably involve oxidative and ER stress (1; 16).

1.4 Glucotoxicity

Studies using partially pancreatectomized rats have shown that both insulin secretion and action are rendered defective by the chronic elevation of plasma glucose levels, resulting in a condition known as glucotoxicity (17; 18). The increase in generation of reactive oxygen species and of established oxidative stress markers such as nitrotyrosine in pacreata from donors with Type 2 Diabetes, demonstrate that the oxidative stress pathway plays a role in mediating the harmful effects of hyperglycemia on insulin secretion and action (19). Furthermore, treatment of pancreatic islets with intermittent high glucose levels has been found to impair glucose stimulated insulin secretion (GSIS), cause increased levels of apoptosis, changes in mitochondrial morphology and density volume and increased intracellular nitrotyrosine content (17). The changes in these markers suggests that fluctuations in glucose levels as is often the case in pre-diabetic conditions may increase the rate of loss in β-cell mass and function. An alternate and perhaps synchronous path of action of the deleterious effect of hyperglycemia on insulin action and secretion may be through the increase in flux through the hexose biosynthetic pathway (HBP). However, the relationship between the flux through the HBP pathway and insulin resistance has to-date only been correlational (20).
Most recently, ER stress has also been implicated as a mediator in the negative effects of glucotoxicity on β-cell function (21). Islets from subjects with Type 2 Diabetes show increased expression of X-box protein 1 (XBP-1) and heat shock protein 5 (HSPA5) upon high glucose treatment relative to both low glucose treated islets and islet from non-diabetic subjects (5). Rat islets cultured in high glucose (30 mM) showed a UPR activation of about 2 fold (22). In insulinoma cells exposed to high glucose, activation of the UPR was characterized by increased expression of spliced XBP-1 (XBP-1s), ER chaperones such as BiP, GRP94, EDEM and of PERK-dependent ATF3, CHOP, and GADD34 (2; 23).

1.5 The endoplasmic reticulum and the ER stress response

The Endoplasmic reticulum (ER) is an interconnected network of membranes within the cell that has several functions including secretory and membrane protein biosynthesis, Ca^{2+} storage and lipid biosynthesis. Nascent proteins targeted for the secretory pathway are translocated from the cytosol to the ER where they undergo posttranslational modifications, processing and maturation (24). Due to the heavy involvement of the ER in protein synthesis and transport, it is no surprise that it is overwhelmed by increasing demand and made vulnerable by several types of insults. A quality control mechanism within the ER is present to monitor and assist in proper folding of these proteins. In addition to the usual protein synthesis load, insults such as misfolded and mutant proteins (i.e. mutant insulin in the Akita mouse), an imbalance in calcium levels, nutrient deprivation, and gluco-lipotoxicity present as major hurdles in the proper functioning of the ER, resulting in a condition known as ER stress. Recently, ER stress has been implicated in β-cell death in the context of diabetes due to excessive insulin production by the β-cell over a prolonged period of time to overcome a state of insulin resistance, and due to conditions associated with diabetes mentioned earlier that also cause ER stress. These conditions include chronic hyperglycemia, FFAs and cytokines.
The ER stress response or the Unfolded Protein Response (UPR) is a cellular defense in place within the ER to counteract a state of stress, resulting from conditions that perturb ER function and lead to the build-up of unfolded or misfolded proteins. Such conditions include the inhibition of protein glycosylation, altered ER redox state, calcium depletion from the ER lumen, or acute upregulation of secretory protein synthesis. The UPR senses the protein folding status of the ER and maintains folding capacity during times of ER stress (Fig. 1.1). This protective response consists of four functionally distinct pathways (25; 26). These include translational attenuation to reduce the overwhelming protein synthesis load on the ER, transient transcriptional induction to assist with the folding of misfolded proteins, ER-associated degradation (ERAD) to degrade misfolded proteins and finally apoptosis as a last resort. Apoptosis is activated if ER stress persists despite the cell protective effects (24). The three key signaling transducing arms of the UPR pathway include: 1) inositol requiring 1 (IRE1), 2) PKR-like kinase (PERK) and 3) activating transcription factor 6 (ATF6). Many downstream effectors to these signal transducers eventually converge in the nucleus to regulate the transcription of UPR target genes.

BiP or glucose-regulated protein (GRP78) (Kar2p in yeast), a luminal member of the Hsp70 family of chaperones plays various key roles in the ER, including the gating of the translocon and hence the post-translational translocation of proteins into the ER, protein folding, and calcium homeostasis (27-31). Most pertinently, BiP has been found to be involved in ER stress sensing either directly or indirectly, in the event of misfolded protein accumulation and in targeting substrates for ERAD (32-35). The former engagement of BiP involves its binding to the luminal domains of the IRE1, PERK and ATF6 signal transducers during non-stressed conditions. However upon accumulation of misfolded proteins, BiP is titrated away from the transducers, consequently activating the signal transducers.

As a member of the type I ER transmembrane kinase family, IRE1 plays a major role in the regulation of the UPR. This includes the regulation of insulin biosynthesis during conditions of ER stress associated with diabetes such as
exposure to high glucose (36). The N-terminal luminal domain IRE1 acts as an ER stress sensor of misfolded and unfolded proteins. Activation of IRE1 is achieved with its dimerization and consequent autophosphorylation. This then activates the IRE1 endoribonuclease domain which splices X-box binding protein (XBP1-s) mRNA. XBP is a basic leucine zipper transcription factor that upregulates UPR target genes such as protein disulfide isomerase (PDI), ER degradation-enhancing α-mannose-like protein (EDEM) and Homocysteine responsive ER-resident Protein (Herp) (37-39). When there is chronic stress however, IRE1 engages its pro-apoptotic pathways involving the recruitment of TNF-receptor-associated factor 2 (TRAF2) and activation of apoptosis-signaling-kinase 1 (ASK1) (24). The activation of c-Jun N-terminal protein kinase (JNK) then leads to apoptosis. Excessive IRE1 activation also results in relaxed specificity and cleavage of mRNAs for secretory proteins (40).

The PERK ER stress sensor plays a crucial role in regulating protein synthesis load during conditions of ER stress (28; 41; 42). Similar to IRE1, PERK is a type 1 ER transmembrane kinase and consists of an N-terminal luminal domain that is involved in ER stress sensing. Upon activation, PERK oligomerizes, autophosphorylates and also phosphorylates Ser51 on the alpha-subunit of eukaryotic initiation factor 2 (eIF2α). The major role of eIF2α-p entails inhibiting protein translation by blocking both the formation of ribosomal initiation complexes and the recognition of AUG start codons (24). In this manner, protein synthesis workload on the ER is reduced and ER-stress mediated apoptosis is prevented. In addition to a general arrest in protein synthesis, activation of PERK results in an increased translation of select UPR target genes such as activating transcription factor 4 (ATF4) that regulates other UPR targets such as C/EBP-homologous protein (CHOP) as well as the growth arrest and DNA damage inducible gene 34 (GADD34). The inhibitory upstream open reading frames (uORFs) of the mRNAs of these genes render them preferential to translation by the ribosome. For example, during conditions of ER stress although general translation is reduced, ATF4 is preferentially translated due to an increased eIF2α-p to eIF2–GTP ratio.
The accompanying reduction in eIF2-GTP delays the reinitiation of translation by scanning ribosomes. This in turn, allows the ribosomes to scan through the inhibitory uORF2 and reinitiate at the ATF4 coding region, thereby increasing ATF4 expression (43; 44).

Finally, the third arm of the UPR involves the activation of the ATF6 transducer, a type II ER transmembrane transcription factor that is key in mediating transcriptional induction (45). After sensing ER stress through its N-terminal luminal domain, ATF6 is trafficked to the Golgi where it is cleaved by the Site 1 and Site 2 proteases (S1 and S2), generating an activated b-ZIP component. The cleaved ATF6 component is then translocated to the nucleus where it acts a transcription factor in inducing genes involved in both protein folding and ERAD (24). Of the two isoforms of the ATF6 signaling transducer: ATF6α and ATF6β, ATF6α has been found to play a major role in the transcriptional induction of ER chaperones. ATF6β may have a similar role, as ATF6α knockout mice have been shown to be viable (46; 47). However the deletion of both ATF6α and ATF6β is embryonic lethal (46).
Figure 1.1. The ER stress response. The accumulation of misfolded proteins in the ER activates the ER stress response, which consists of four distinct pathways. These are: (1) translational attenuation, (2) transcriptional induction of various genes such as chaperones, (3) increased ERAD capacity to degrade misfolded proteins. If despite the activation of the above-mentioned pathways ER stress persists, apoptosis is initiated (4). Signaling transducers involved in this response include PERK, IRE1 and ATF6. The release of the BiP chaperone from these signaling transducers and its subsequent association with the misfolded substrates allows for their activation. Both PERK and IRE1 dimerize and are autophosphorylated. Upon activation of its kinase activity, PERK phosphorylates eIF-2α resulting in translational attenuation to reduce protein synthesis load on the ER. XBP1-s, the cleaved p50 ATF6 fragment, and ATF4, which are downstream of the signal transducers, bind to target genes in an effort to properly fold misfolded proteins. Much remains to be determined with regards to the precise mechanisms and components of ERAD. Figure adapted from Oyadamari et al. (2002)
1.6 ER associated degradation (ERAD)

When a protein synthesized in the ER is unable to achieve its proper conformation during synthesis, it is redirected from the folding cycle to be degraded by the ERAD system. ERAD consists of several functional steps in the degradation process, from recognition of misfolded proteins and retrotranslocation to the cytosol, to degradation by the ubiquitin-proteasome system (48; 49)(Fig. 1.2). Although the exact mechanisms of the recognition, engagement and retrotranslocation from the ER to the cytosol remain to be determined, research in yeast has pushed forward the understanding of ERAD dynamics and functioning in both yeast and in higher eukaryotes.

More specifically, it has become well established that the yeast ERAD system is generally divided into three components. Substrates with misfolded cytoplasmic domains are targeted to the ERAD-C pathway, whereas those with misfolded domains in the lumen are targeted to the ERAD-L pathway, and finally those with misfolded membrane domains are targeted to the ERAD-M pathway (48; 50).

1.6.1 Targeting proteins for ERAD

Various properties of misfolded substrates determine which ERAD components are involved in their degradation. The site of lesion of the misfolded protein for example is a determining factor in the mechanism of its degradation. BiP is involved in the degradation of luminal ER proteins, while Hsp70 chaperones mark proteins with cytosolic domains for degradation. Misfolded glycoproteins may be specifically recognized by the putative luminal ER lectin, EDEM.

As a natural extension of its role in the recruitment from UPR signal transducers to misfolded proteins, the BiP chaperone may be involved in the early portion of ERAD substrate recognition. Normally, BiP binds to exposed hydrophobic portions of new proteins and in the process, prevents the aggregation of misfolded proteins (35). It then releases the protein in an ATP-dependent manner, when it is
ready to fold. The Protein disulfide isomerase (PDI) chaperone is also involved in protein folding through the oxidation, reduction and isomerization of disulfide bonds. PDI is similar to BiP in its protein unfolding capabilities as well. However, one major diverging feature is its mechanism of protein release, which does not depend on ATP, but rather on its own redox state (51).

As mentioned previously, during ER stress BiP and PDI may play crucial roles in preventing the aggregation of misfolded proteins and in directing misfolded proteins to the ERAD pathway. Yeast Kar2p (BiP) together with Yos9p and Hrd3p act as a luminal surveillance complex that ensures the proper identification of misfolded substrates independent of glycosylation status and guides the substrates to degradation machinery (52) (Fig. 1.3B). PDI may also be involved in guiding misfolded substrates to retrotranslocation machinery or in reducing disulfide bonds prior to this process (24; 53). It has been specifically suggested that PDI recognizes misfolded substrates possibly by the exposure of their hydrophobic residues and targets the substrates for export to and degradation in the cytosol (53).

The lectin-like chaperones, calnexin (CNX) and calreticulin (CRT) are highly involved in glycoprotein folding in higher eukaryotes (24; 54). These chaperones interact together in a mechanism termed, the calnexin/calreticulin cycle. Although these two substrates are homologous and are both capable of binding to glycoproteins, they bind to separate and different proteins. CNX/CRT act by binding to the trimmed oligosaccharide chain: Glc$_1$Man$_{9-6}$GlcNAc$_2$, attached to Asn residues during the N-linked glycosylation of glycoproteins in the ER. The glycoprotein is then folded by an oxidoreductase such as ERp57 that is specifically involved in disulfide bond isomerization (55; 56). Subsequent to folding, glucosidase II initiates the removal of the terminal glucose at which point the glycoprotein is released from the CNX/CRT cycle. Uridine diphosphate (UDP)-glucosyl/glycoprotein glucosyl transferase (GT) then plays the role of gatekeeper by monitoring the folding status of the released protein (57; 58). GT works to reglucosylate the misfolded intermediate if has not achieved its correct conformation, after which the glycoprotein is recommitted to the CNX/CRT cycle. The correctly folded protein is
then released from the cycle and transported from the ER. Glycoproteins that are unable to achieve proper conformation however are extracted from the cycle and directed to ERAD processing.

It is believed that misfolded proteins may be specifically tagged for ERAD-mediated degradation by ER mannosidase-mediated trimming of mannose residues, resulting in the Man$_6$GlcNac$_2$ and Man$_5$GlcNAc$_2$ residues. The resulting reduced efficiency of substrate-to-CNX/CRT binding then directs the misfolded substrate to EDEM. Recently, it has been suggested that EDEM may be an actual mannosidase and that it may exhibit activity characteristic of chaperones (48).

Little is known with regards to the recognition of integral membrane substrates lacking soluble domains (i.e. those targeted to the yeast ERAD-M pathway). It has been suggested that E3 ubiquitin ligases may recognize these substrates via their multiple transmembrane-spanning domains (48). Furthermore, the mechanisms involved in the degradation of misfolded integral membrane substrates are also unclear. Hsp70-40 have however been reported as key chaperones that not only maintain the solubility of the cytoplasmic loops of the misfolded substrates but may also be involved in the interaction of integral membrane ERAD substrates with E3 ubiquitin ligases (49). In this regard, ERAD has been divided into three pathways specific to the misfolded domain of the integral membrane substrate (48).

1.6.2 Retrotranslocation of misfolded substrates (in yeast)

Subsequent to targeting and in order for misfolded proteins to be degraded in the cytosol, they must first be retrotranslocated from the ER. Two working theories presently exist in the field regarding the mechanism of retrotranslocation and may even co-exist based on the notion that the composition of the retrotranslocation pore may be substrate dependent (24; 48; 49). The first theory involves the yeast Sec61p translocation pore through which nascent polypeptides
initially enter the ER. According to this theory, Sec61p serves a dual role as both the initial translocation pore and the retrotranslocation channel. An alternative model that is gaining increasing acceptance in the field suggests that other candidates might make up the pore. These include the multimembrane-spanning ubiquitin ligases, Hrd1p, having six membrane-spanning domains, Doa10p with its 10-14 membrane spanning domains and Der1p, with its four membrane-spanning domain (24).

Carvalho and colleagues, have outlined two independent complexes that are committed to the degradation of luminally or cystosolically misfolded proteins (59). Work by this group has found that a complex consisting of Doa10p in association Ubc7p, Cue1p, Ubx2p, Cdc48p and Npl4p, degrades proteins with cytosolic mutations (via the ERAD-C pathway). Substrates with luminal mutations are degraded by a complex involving Hrd1p in association with Der1p, Yos9p, Hrd3p, Ubx2p, Cdc48p and Usa1p (via the ERAD-L pathway). These findings are suggestive of the notion that retrotranslocation and substrate modification are coupled (48).

Although, it is highly upregulated by the UPR, the function of the membrane-spanning yeast ERAD-L component, Usa1p remains unclear. It is known however that it consists of two membrane-spanning domains, an N-terminal ubiquitin-like (UBL) domain as well as a C-terminal domain on the cystosolic face of the ER (24). It is also needed to direct Der1p to Hrd1p and for the degradation of misfolded proteins found in the ER lumen. It has been suggested that mammalian Herp may be homologous in function to Usa1p as it has been able to replace Usa1p in yeast cells (59).
1.6.3 Mammalian ERAD

In contrast to Der1p which is required for the degradation of the luminal proteins alone, Derlin-1, its mammalian homologue has been found to play an essential role in the linking of luminal and cytoplasmic components such as Herp, PNGase, and US11 during ERAD (48; 60-63). Additionally, Derlin has been found to exist in complex with the cytosolic ATPase, p97 and a protein that interacts with p97 known as VIMP (Fig. 1.2).

Figure 1.2. Mammalian ERAD machinery. The mammalian ERAD consists of several proteins that function in this dynamic process. Both OS-9 and EDEM play roles in the recognition of N-glycans on misfolded substrates. Subsequent to recognition, the latter directs the substrate to Derlin-2/3 and p97. ERdj5 interacts with EDEM and BiP. It plays a functional role in the removal of substrates from the ER. With exposure to the cytosol following retrotranslocation, potentially through the Derlin channel, the substrate is then ubiquitinated by HRD1 and degraded by the 26S proteasome. Figure adapted from Hoseki et al. (2010)
1.6.4 Dislocation of ERAD substrate proteins

The location of the catalytic domains of the E1-E3s, ubiquitin-associated enzymes on the cytoplasmic surface of the ER lends support to the idea that misfolded luminal substrates must gain access to the cytoplasm via dislocation before they are degraded. Prior to degradation, complete dislocation and the unfolding of misfolded substrates is carried out with the assistance of Cdc48p (p97 in mammals), a cytosolic AAA Adenosine triphosphatase (ATPase) along with the two co-factors, Ufd1 and Npl4p that bind ubiquitinated proteins.

The Cdc48-Ufd1-Npl4p complex, a key player in what is termed the ‘escort pathway’ (64; 65), associates with the ER membrane during the removal of misfolded proteins. Studies have shown that the Cdc48/p97 complex extract ubiquitinated substrates to the cytosol by a mechanism that involves pulling a polypeptide through the retrotranslocon or by segregating a polypeptide that has been released from the ER membrane (66-70). Cdc48p is an effector of Ubx2p, which works to recruit it to the ER membrane. In addition to mediating the interaction between the Cdc48 complex and the ubiquitin ligases: Hrd1p and Doa10p, Ubx2p, regulates the interaction between Cdc48 and ERAD substrates. In this sense Ubx2p, an ER transmembrane protein that contains both a cytosolic ubiquitin associated domain (UBA) and a cytosolic ubiquitin regulatory domain (UBX), is believed to be a key link between misfolded substrates, the E3 enzymes that polyubiquitinate them, and the Cdc48 complex. Finally, the Cdc48/p97 complex may deliver the misfolded substrates to the proteasome via interaction with molecules that contain ubiquitin-binding and ubiquitin-like domains. These include Ufd2, which has been termed an E4 and Ufd3, a molecule having unknown function. Additionally Otu1, a deubiquitinating enzyme that modifies the length of the polyubiquitin chain, has also been found to interact with the Cdc48/p97 complex. Deubiquitination is necessary in the maintenance of a critical, minimal length of the polyubiquitin chain, required for the retrotranslocation of misfolded substrates (48; 50; 70). Finally, the Rad23/Dsk accessory factors, also associate with the Cdc49/p97 complex in efforts to deliver their misfolded cargo to the
proteasome for degradation (64). Studies on the above mentioned key components of ERAD machinery and their elucidated roles and interactions have outlined a working model for misfolded substrate dislocation.

Integral membrane substrates that have regions exposed to the cytoplasm on the other hand, may be ubiquitinated before dislocation. Degradation of these misfolded substrates may commence at either of its ends after dislocation through the retrotranslocon or through the direct extraction of these substrates from the membrane. An alternate model, suggests that after an endoproteolytic snip by the proteasome, degradation could commence at an internal site on a cytoplasmically exposed loop.

1.6.5 Ubiquitination of ERAD substrate proteins

Both during and following retrotranslocation from the ER, misfolded substrates are polyubiquitinated by a series of enzymatic reactions (24; 50; 71). Ubiquitin is first activated by a ubiquitin-activating enzyme known as E1. The activated ubiquitin is then directed to the ubiquitin-conjugating enzyme, E2. The ubiquitin protein ligase, E3 then attaches ubiquitin to lysine residues on the substrate.

Hrd1p is one such E3 enzyme, which as a transmembrane protein with a cytosolic RING-H2 face is stabilized by its interaction with Hrd3, another transmembrane protein with a large luminal domain (71). In addition, Hrd1p can interact with either Ubc1p or Ubc7p, both of which are E2 enzymes. Ubc7p in particular, is a soluble enzyme that associates with the ER via its interaction with Cue1p. Doa10p is another E3 protein that functions in a Ubc6p and Ubc7p-dependent manner.
Mammalian E3s include, gp78, the Fbs1/Fbs2 complex, the membrane-associated RMA1 and cytosolic CHIP (24). After repeated ubiquitination of the misfolded substrate by these E3s, the resulting polyubiquitinated substrate in both the yeast and mammalian systems, is eventually degraded by the 26S proteasome.

A point to note is that the ERAD-L, ERAD-M, and ERAD-C pathways have only been described in yeast. Distinctions among these pathways are less clear in mammalian systems as they have evolved more complex ERAD-mediated mechanisms (50). Moreover, the utilization of the Doa10 and Hrd1 ubiquitin ligases in both the yeast ERAD-L and ERAD-C pathways is indicative of an overlap between the pathways that may serve to enhance the efficiency of degradation (49; 72; 73).

That said, misfolded substrate recognition, dislocation and ubiquitination do not make up the entire degradation picture. Additionally, it is important to note that many of the ERAD components discussed above associate into specific complexes based on the nature of the misfolded substrate targeted for degradation (Fig. 1.3A,B) (24). One group has found Yos9p, which is an ER lectin that has some homology to the mannose-6-phosphate receptor, to associate with Kar2p (BiP), Hrd3p, Hrd1p, Ubx2p and USA1p to form a dislocation/degradation complex. The luminal, transmembrane and cytosolic portions of this complex allow for communication between the ER lumen and cytosol. In this complex, Yos9p and Kar2p associate with each other into a luminal subcomplex, independent of other components. Furthermore Hrd3p, which interacts with Yos9p by bridging the interaction between Yos9p and Hrd1p also recognizes misfolded substrates independent of Yos9p. Interestingly, it has been suggested that the Yos9p/Hrd3 complex may function as a surveying checkpoint before the degradation of substrates (52). This mechanism would work to prevent the inappropriate and untimely degradation of substrates.
Figure 1.3. Schematic diagram of the ERAD-L (A) and ERAD-C (B) pathways in yeast. ERAD-L involves the Hrd1p/Hrd3p complex, whereas ERAD-C involves the Doa10p complex. As part of ERAD-L, Yos9p senses N-glycans on misfolded substrates, which are subsequently ubiquitinated in the cytosol by Hrd1p before being finally degraded by the proteasome. Figure adapted from Hoseki et al. (2010)

### 1.6.6 Mammalian complexes

Two mammalian homologues of Yos9: OS-9 and XTP3-β act in a similar manner by binding to misfolded proteins and to SEL1L, which is a homologue of the yeast Hrd3 (48). This is indicative of the possible roles that OS-9 and XTP3-β may play in linking ERAD substrates to the membrane-associated ubiquitination machinery. Moreover, OS-9 has been found to interact with Grp94, a key protein involved in misfolded substrate degradation (74). Grp94 may therefore play a dual role in both the substrate recognition and/or the assembly of the OS-9-SEL1L-Hrd complex.
1.6.7  **ERAD genes induced during ER stress**

Studies in yeast looking at UPR-induced transcriptional upregulation of genes have found that only a select few of the ERAD genes are affected. Microarray analysis conducted by two groups has found that DER1, HRD1 and UBC7 are upregulated, while YOS9 and HTM1/MNL1 are not (24). This suggests one of two possibilities that either the genes that are upregulated are essential ERAD factors, or that ERAD genes that were not upregulated are indeed affected by pathways and complexes that diverge from the UPR. However, Microarray analysis studies in mammalian cells subjected to ER stress indicates that EDEM, which is the mammalian homologue of HTM1/MNL1 is actually upregulated by XPB1, a well-established component of the UPR.

1.6.8  **ERAD of non-glycosylated proteins**

Thus far I have discussed the degradation of misfolded *glycosylated* proteins. Less is known of the steps involved in the degradation of *non-glycosylated* misfolded proteins. Recent work has suggested that there is some distinction between the degradation pathways of glycosylated and non-glycosylated misfolded proteins (63). For example, Herp, a mammalian membrane-bound cytosolic protein binds to both a nonglycosylated BiP substrate known as nonsecreted Ig κ light chain (NS1 κ LC) and the 26S proteasome (Fig. 1.4), without involvement in the degradation of substrates requiring calnexin engagement (48; 63). Furthermore, reduction of Herp levels has been found to block the degradation of non-glycosylated BiP substrates, while having no effect on calnexin substrates.
Figure 1.4. ERAD pathway for misfolded non-glycosylated BiP proteins. Non-glycosylated substrates are recognized by BiP, which subsequently directs them towards ERAD machinery consisting of Herp, Hrd1 and Derlin-1. Figure adapted from Hoseki et al. (2010)

1.7 HERP

Kokame and colleagues (2000) previously identified many transcribed fragments of novel genes that were upregulated after a 4-hour treatment of human umbilical vein endothelial cells (HUVECs) with homocysteine, a sulfhydryl group containing amino acid. Among these genes, was a novel protein, which they termed homocysteine-responsive ER-resident protein (Herp).

Initial work on Herp revealed a 2.2 kilobase (kb) mRNA fragment using northern blot analysis. The resulting band increased in intensity, translating to a 50-fold increase with time dependent homocysteine treatment, which has been suggested to specifically turn on the cellular ER stress response (75). Furthermore,
both Herp mRNA and protein have been found to be induced by additional ER stress inducing compounds such as β-mercaptoethanol; a reducing agent, tunicamycin (Tm); an N-linked glycosylation inhibitor, A23187; a calcium ionophore, and thapsigargin (Tg); an ER-resident inhibitor of the Ca$^{2+}$-ATPase, indicative of its regulation by the UPR.

Herp, with its 1,176 base pair open reading frame, encodes for a 391 amino acid length protein (75). Human and mouse Herp amino acid sequences share 88.7% identity. Additional data has shown that Herp has a calculated mass of 43,719 Da and is a membrane protein with one transmembrane domain (Ser$^{285}$-Trp$^{307}$). Furthermore, a PROFILE search revealed that Herp is significantly matched to ubiquitin. In fact, Herp is a short-lived protein with a ubiquitin-like domain (ULD) (76). Although the ULD of Herp is involved in its intracellular regulation during ER stress, the same ULD is required for cell survival (77).

Studies have shown that a modular protein complex consisting of Herp, Hrd1, p97, Derlin-1 and VIMP carry out the ubiquitylation and retro-translocation of proteins from the ER (78). It has been further suggested that p97 associates directly with membrane-resident components such as Derlin-1 and Hrd1, whereas Herp binds directly to Hrd1. The coordination of ERAD is hence facilitated by the close proximity of these ERAD components within the complex.

Northern blot analysis, examining the distribution of Herp in human organs has revealed that it is ubiquitously expressed (i.e. heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas). Moreover, Herp expression was most pronounced in pancreatic tissue (75). Within these organs, Herp has been characterized as a protein with transmembrane nature, lending support to idea that it is an integral membrane protein. Studies have indicated that Herp is specifically localized in the ER, and that both its N and C termini are exposed to the cytoplasm (75).
More recently, Okuda-Shimizu and Hendershot, who have been studying the disposal of the non-glycosylated BiP substrate, NS1 κ LC, have discovered that Herp may play a key role in ERAD-mediated degradation that is specific to non-glycosylated substrates. Herp was found in a complex with ubiquitinated proteins (79) and with the 26S proteasome, suggesting that is may serve as a linker between the substrates and the proteasome, and in this sense may function in a pro-survival manner during conditions of stress. Overexpressed Herp has also been found to associate with other BiP substrates such as the nonsecreted λ LC mutant, RE61 and the truncated, HA-tagged γ HC, HA-γ V-C11, while showing no interaction with specific calnexin substrates. Finally, results indicating either a reduction in Herp expression, or mutant p97 or Hrd1 expression inhibited the degradation of BiP substrates, while having no effect on the degradation of calnexin substrates. This is indicative of the idea that Herp may not be involved in the ERAD-mediated degradation of all misfolded substrates, but rather in the degradation of substrates exhibiting specific features such as the absence of glycosylation processing. In addition to its role in ERAD, Herp has been found to promote cell survival in neurons by stabilizing ER Ca\(^{2+}\) homeostasis and mitochondrial functions during conditions of ER stress (80).

1.8 ER stress, ERAD and the pancreatic β-cell in diabetes

As mentioned previously, it is when β-cells fail to compensate for the increased demand for insulin secretion through β-cell death and dysfunction that Type 2 Diabetes develops. Chronic activation of the UPR as an attempt to manage a state of compensatory insulin hyperproduction has been hypothesized to contribute to causing β-cell death. Moreover, as mentioned in the introduction, conditions associated with diabetes such as elevated free fatty acids, cytokines and high glucose can induce ER stress. In fact, many studies have shown an induction of UPR components as a result of these diabetic conditions (16; 81-87). It has hence
become increasingly accepted that ER stress is highly involved in the progression of the diabetic disease state through its contribution to \( \beta \)-cell dysfunction.

The role of ERAD-mediated degradation in the context of ER stress in pancreatic \( \beta \)-cells has not been studied. Several in vitro and in vivo studies have suggested its involvement by identifying potential ERAD components such as Sel1, BiP, Herp, Grp94, Erp72 and PDI, that are induced as a result of ER stress caused by diabetic conditions (i.e. FFAs, high glucose and insulin mutations) (23; 28; 85; 88-91).

Various murine models of diabetes are in line with in vitro studies. Islets from the MKR mouse model of diabetes have revealed an upregulation of components of the ER stress response including but not limited to BiP, some of which are ERAD genes (28; 85). Streptozotocin-induced Diabetic Nephropathy (DN) rat models show an increased expression of pIRE1\( \alpha \) in comparison to control rats (87). In addition to its role in sensing ER stress, IRE1\( \alpha \) induces many ERAD components in its role as a crucial regulator of the ERAD system (82). Finally, the diabetic Akita mouse model is heterozygous for a mutation to the Insulin-2, resulting in a cysteine 96 to tyrosine amino acid substitution. Studies on islets from these mice have shown an induction of the ERAD components: Hrd1, SelL and BiP (82).

Recent in vitro studies have lent support to this last study. In order to examine the ER stress response at a global level, microarray analysis studies were performed on a dox-inducible INS-1 Ins2(C96Y)-EGFP mutant protein expressing cell line that is based on the Akita mouse mutation (91). With time dependent misfolded insulin expression, many gene expression changes were noted. Interestingly, the ERAD components: Sel1 and Herp were among the many ER-stress inducible genes that were induced >2-fold (92).
1.9 Rationale and Hypothesis

Several biochemical insults (i.e. chronic glucose, free fatty acids) and genetic mutations (i.e. insulin mutations) that contribute to the development of diabetes have been found to induce the ER stress response. Knowledge in the field has grown rapidly. However, much remains to be discovered especially with regards to ERAD, a concomitant branch of the UPR. Even less is known about ERAD-mediated degradation of non-glycosylated substrates. Recently, studies (63) have found a role for Herp in the degradation of non-glycosylated BiP substrates. Interestingly enough, ER stress induced by mutant proinsulin expression has been shown to consequently induce Herp expression and thereby activate ERAD (92).

This project will attempt to enhance the understanding of Herp functionality in ERAD-mediated degradation in pancreatic β-cells by examining its expression and function under various diabetic conditions. We hypothesize that Herp is essential for the degradation of misfolded proinsulin during ER stress. More specifically, the depletion of Herp will reduce mutant insulin degradation and cell survival, while its overexpression may potentially enhance mutant insulin degradation. We further hypothesize that Herp is an essential gene in the ERAD pathway that is upregulated in ER stress conditions associated with diabetes in pancreatic β-cells.
CHAPTER 2: MATERIALS AND METHODS
2.1 Cell Culture

Rat INS-1 832/13 pancreatic β-cells were obtained from Dr. Chris Newgard (Duke University) and INS-1Ins2(C96Y)-EGFP clone #4S2 was generated previously in the Volchuk lab (92). Both cell lines were grown in RPMI 1640 (11.1mM glucose, 1mM sodium pyruvate, 10 mM HEPES) supplemented with 10% Fetal Bovine Serum (FBS), 2mM L-glutamine, and 55 µM β-mercaptoethanol containing antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). Cells were incubated at 37°C in 5% CO₂. Media, supplemented with selection drugs (200 µg/ml G418 and 50 µg/ml hygromycin) was changed for INS-1 Ins2(C96Y)-EGFP cells every 4-5 days.

AD-293 (Stratagene) cells used in the preparation of Herp adenovirus, were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (25mM glucose, 2mM L-glutamine, and 1 mM sodium pyruvate), supplemented with 10% FBS and 55 µM β-mercaptoethanol at 37°C in 5% CO₂.

2.2 Cell Treatments and Lysis

INS-1 832/13 cells were cultured in 6 well or 12 well plates and INS-1 Ins2(C96Y)-EGFP cells were cultured in 12-well plates as per experimental requirements. Mutant insulin expression was induced in INS-1 Ins2(C96Y)-EGFP cells with 2 µg/ml doxycycline (dox) treatment. Based on experimental criteria, cells were treated with 2µg/ml tunicamycin (16-18h), 5 µM lactacystin, or 0.3 µM staurosporin (24h). Treatment with thapsigargin and tunicamycin were used as positive controls for ER stress induction, while staurosporin treatment served as a positive control for apoptosis. Furthermore, INS-1 832/13 cells were treated with either 5 mM or 25 mM for 24 or 48h for high versus low glucose experiments. Filtered 500 mM stocks of D-Glucose (Sigma) were diluted in 5 mM glucose RPMI 1640 media to obtain the final concentrations.

Protein lysates were obtained by washing cells with phosphate-buffered saline (PBS) and lysing in lysis buffer (1% Triton X-100, 20 mM HEPES, pH 7.4, 100 mM
KCL, 2 mM EDTA, 1 mM PMSF, 10µg/ml leupepetin, and 10 µg/ml aprotinin). The cells were lysed on ice for 30-60 min and centrifuged or not (based on experimental requirement) at 13,000 rpm for 10 min at 4°C. The supernatants of the protein lysates were transferred to new tubes and a protein assay was performed using bicinchoninic acid (BCA) reagent (Pierce) in order to determine concentrations.

2.3 Rat Islet Isolation and Islet Lysis

Rat islets were isolated from Wistar rats as described previously (23). Rat islets were hand picked into 1.5 ml eppendorf tubes and spun down briefly. Media was then removed and the pellet was subsequently washed with PBS. Following removal of the supernatant 20-50 µl of ice cold RIPA buffer, supplemented with Aprotinin, Leupeptin, and PMSF was added to the pellets. Samples were incubated on ice for 1h with flicking every 20 min. The samples were then spun at 13,000 rpm for 10 min at 4°C and supernatants were transferred to new tubes on ice. A protein assay was performed using bicinchoninic acid (BCA) reagent (Pierce) in order to determine concentrations.

2.4 Cloning of HERP and Recombinant Adenovirus Production

Full-length Herp cDNA, which is 1200 base pairs in length, was reverse transcribed from total RNA of INS-1 832/13 cells treated with tunicamycin for 16 h based on methods provided by the QIAGEN OneStep RT-PCR. As a brief overview of the procedure, 500 ng of RNA was mixed with Qiagen RT-PCR buffer, One Step Enzyme mix, dNTPs, and the Herp primers below (ACGT Corporation; Toronto, Canada):

Herp_forward  5'-ATGGAGCCCGAGCCACAGCCCG-3'
Herp_reverse  5'-TCAGTTTGTACGCTGGTGCC-3'
PCR conditions: 30 min at 50°C, 15 min at 95°C, and 30 cycles of denaturation at 95°C for 1 min, followed by annealing at 60°C for 1 min, and extension at 72°C for 1 min. The final extension was achieved with 10 min at 72°C. The amplified RT-PCR products were subsequently resolved on 1% agarose gel. The 1200 band was then excised and purified from the agarose gel using the Qiagen Gel Extraction Kit. Following extraction, the Herp cDNA was cloned into the PCR II-TOPO vector (Invitrogen, Carlsbad, USA). The mTOPO-Herp vector was then transformed into OneShot®TOP10 chemically competent cells (Invitrogen, Carlsbad, USA) and purified using the QIAamp® DNA Mini Kit (Qiagen, Maryland, USA). Verification of the presence of the Herp cDNA insert was performed by cutting the 1200 band using the EcoRI enzyme (New England BioLabs) and running out the digested product on a 1% agarose gel. For adenovirus production, mTOPO-Herp was subsequently cloned into the pShuttleCMV vector (Stratagene) using the KpnI/EcoRV restriction enzymes (New England BioLabs). The resulting Herp-ShuttleCMV construct was transformed in the DH5α cells and plated. Kanamycin resistant clones were picked and amplified overnight. Plasmid DNA was then isolated using the QIampDNA Mini Kit (Qiagen, Maryland, USA) and proper ligation of the Herp cDNA insert into the pShuttleCMV vector was confirmed as described above.

A Herp-expressing adenovirus was produced using the Andenoviral Vector System (Stratagene). As a brief summary of the procedure, the pShuttle vector containing the insert along with a supercoiled viral DNA plasmid (pAdEasy-1) were used to produce a recombinant adenovirus plasmid via homologous recombination. The plasmid was subsequently purified and transfected into AD-293 cells (adenovirus-packaging cell line 293) using the modified MBS Mammalian Transfection Kit protocol (Stratagene). The primary viral stocks were then prepared by subjecting the infected cells to freeze-thaw cycles, followed by subjecting the resulting viral particles to one round of amplification.
A purified virus was obtained by first infecting AD-293 cells, seeded in four 10 cm dishes with the amplified virus and then purifying the virus via the Vivapure AdenoPACK 100 kit (Vivascience). The purified virus was resuspended in buffer (2 mM Tris/HCl, 25 mM NaCl, 2.5% Glycerol, pH 8.0) and aliquots of the virus were kept in storage at -80°C. The absorbance of the virus preparation at 260 nm (OD 260) was measured and the viral titer in plaque-forming units (pfu)/ml was calculated using a standard curve (pfu/ml versus OD) (Dr. Christopher Rhodes; University of Chicago).

2.5 INS-1 Ins2(C96Y)-EGFP Cell Infection

INS-1Ins2(C96Y)-EGFP cells (500,000 cells/well in a 12-well plate) were infected with 1x10^8 pfu/ml of Ad-Herp or Ad-GFP and incubated at 37°C/5% CO₂ for 2h with gentle intermittent shaking every 30 min. After the incubation period, fresh RPMI 1640 media was added to the cells and dox (2 µg/ml) was added or not as indicated in the figure legends. In the negative control condition, cells were cultured similarly without the addition of Ad-Herp or Ad-GFP. Subsequent to treatment, the cells were washed once with PBS and lysed for western blot analysis or apoptosis measurement. Samples prepared for apoptosis measurement were lysed directly in 50 µl of hot NU-PAGE lysis buffer.

2.6 Transient Transfection

In order to test the overexpression of Herp in plasmids, AD-293 cells were transiently transfected using Lipofectamine reagent. 500,000 cells/well were seeded into 12-well plates and incubates at 37°C in 5% CO₂ overnight. 50 µl of OPTI-MEM medium (Invitrogen) was combined with 2µl of Lipofectamine Reagent and incubated for 5 min the next day. Opti-MEM/Lipofectamine mix was subsequently added to pCMV-Herpmyc plasmid DNA or the pcDNA3.1 (control) and
incubated at room temperature for 20 min. Warm RPMI 1640 (500 µl) media without antibiotics was added to the DNA mixture following incubation. The plates were then removed from the incubator and the media was replaced with fresh media containing the DNA mixture. Upon addition of fresh media, the plates were returned for incubation at 37°C in 5% CO₂ for 24 or 48h. Transfection was confirmed by resolving the lysates using SDS-PAGE and western blot analysis.

2.7 Transfection of short interfering RNA (siRNA)

The knockdown of Herp mRNA was accomplished by reverse transfecting cells using RNAiMAX reagent. More specifically, 6 pmol of Herp siRNA (No. 7184602, Invitrogen) or a control siRNA directed to bacterial β-galactosidase (obtained from Dr. O. Ouerrfelli, Sloan-Kettering Institute, New York) were added and gently mixed in 200 µl of OPTI-MEM I medium (Invitrogen). The mixture was then allowed to incubate at room temperature for 5 min. After the 5 min period, 2 µl of RNAiMAX Lipofectamine (Invitrogen) was added to the siRNA mixtures and incubated at room temperature for 30 min. During the incubation period, cultured INS-1Ins2(C96Y)-EGFP cells were trypsinized and resuspended in RPMI 1640 media without antibiotics to obtain a final concentration of 250,000 cells/well or 300,000 cells/well. Subsequent to the 30 min incubation period, 1 ml of diluted cells was added to each well containing a siRNA-mixture to render a final concentration of 10 nM siRNA/Lipofectamine RNAiMAX complexes. INS-1Ins2(C96Y)-EGFP cells were then allowed to recover from siRNA treatment overnight at 37°C in 5% CO₂. The cells were treated as described in figure legends, where knockdown of Herp protein was confirmed using western blot analysis and apoptosis was quantified using the cell death detection ELISA (Roche Diagnostics).
2.8 RNA Isolation and Real-time Quantitative Polymerase Chain Reaction (PCR)

Real-time PCR analysis was performed by first isolating total RNA from cells or rat islets treated as stated in the figure legends, using TRIzol Reagent (Invitrogen) and the RNeasy Mini Kit (Qiagen). 1.95 µg of the total RNA was then reverse transcribed to single-stranded cDNA using a High-capacity cDNA reverse transcription kit (Applied Biosystems). The TaqMan Gene Expression system (Applied Biosystems) was then used to perform real-time PCR analysis on the single stranded cDNA. Rat HERP (Rn 00585371_ml) and rat β-actin (4352931E) primers were obtained from Applied Biosystems. A standard curve was generated using serial dilutions of the control condition: 5mM glucose-treated INS-1 832/13 cells. The reactions were subsequently run on and ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) using the following program: 10 min at 95°C, 40 cycles of 15sec at 95°C and 1min at 60°C. The SDS 2.1 software generated the standard curve and corresponding values for each of the samples, which were run in either duplicate or triplicate. The averaged values for each condition were then normalized to the expression of β-actin mRNA. Final data was presented as a mean ± SE of three to five independent experiments.

2.9 Immunofluorescence microscopy

INS-1 832/13 cells were seeded on glass coverslips in a 24-well dish (250,000 cells/well) and incubated at 37°C in 5% CO2 overnight. The cells were then treated as described in the figure legends. Subsequent to treatments, the INS-1 832/13 cells were washed twice with PBS and fixed for 20 min in 4% paraformaldehyde (PFA) in PBS at room temperature. Subsequent to the 20 min incubation, two washes with PBS were performed, after which the cells were incubated with 100 mM glycine/PBS for 15 min at room temperature and then washed once again with PBS. The cells were then permeabilized in 0.1% TX-100/0.5% BSA/PBS for 15 min at room temperature, washed with an additional three PBS washes and incubated in
blocking solution (2% non-fat milk/2% BSA in PBS) for 1h. The primary antibody: rabbit polyclonal anti-Herp (1:200) (Biomol, PW9705) was added to the blocking solution for 1h at room temperature. Subsequent to the 1h incubation period, the secondary antibody: Oregon green goat anti-rabbit IgG (1:1000) in blocking solution was added to the cells, and the dish was then incubated in the dark for 1h at room temperature. The cells were then subjected to an additional three 5min washes and mounted on glass slides using Fluoromount G Mounting Medium (Electron Microscopy Sciences, Inc.). Fluorescence from the samples was visualized afterwards using a fluorescence microscope (Olympus, IX71).

In some cases nuclei were stained with Hoescht 33342 dye. Briefly, INS-1 Ins2(C96Y)-EGFP cells were reverse transfected with either control siRNA or Herp siRNA on glass coverslips in a 12-well plate (300,000 cells/well) in RPMI 1640 media without antibiotics. The cells were then treated or not with dox (2µg/ml) (Clontech, 631311) and incubated at 37°C in 5% CO₂ for 24h. Following incubation, the cells were washed with PBS once, fixed in paraformaldehyde (PFA)/PBS and incubated at room temperature for 20 min. The clone cells were washed again with PBS prior to staining with 5 µg/ml Hoechst 33342 (Component A from the Vybrant Apoptosis Assay Kit #5 (Invitrogen, V13244), plus 10 µl of propidiumiodide (Component B from the Vybrant Apoptosis Assay Kit #5) in PBS and incubating for 5 min at room temperature, in the dark. Subsequent to the 5 min incubation period, cells were washed an additional two times in PBS and mounted onto glass slides using Fluoromount-G Aqueous Mounting Medium (Electron Microscopy Sciences Inc., 17984-25). The blue-fluorescent Hoeschst 33342 dye in the stained samples was then visualized with a fluorescence microscope (Olympus, IX71) the following day.
2.10 Western Blot Analysis

Subsequent to the determination of protein concentrations from cell lysates, equivalent amounts of protein were boiled in 2 or 3X Laemmli sample buffer (LSB) supplemented with 10-15% β-mercaptoethanol. The samples were then electrophoresed on SDS-PAGE gels and transferred to Hybond-ECL nitrocellulose membranes (GE Healthcare). Cleaved caspase 3 expression, was measured using 4-12% NuPage gel electrophoresis (Invitrogen) prior to the transferring process. The membrane was subsequently blocked using a blocking solution consisting of 1% dry skim milk in western wash buffer (WWB)(0.05% Tween-20 (Fisher Scientific) and 0.05% Nonidet P-40 substitute (Fluka) in PBS) and incubated in primary antibody overnight at 4°C. The following primary antibodies were used: polyclonal anti-Herp (obtained from Dr. Linda Hendershot, St. Jude Children's Hospital, Memphis, TN; 1:1000), monoclonal anti-GFP (Clontech, 632381; 1:2000), anti-cleaved caspase 3 (cell signaling, #9661S; 1:1000), polyclonal anti-GFP (obtained from Dr. James E. Rothman, Yale University; 1:1000), γ-tubulin (Sigma, T6557, 1:1000), GM130 (Transduction Laboratories, G65120, 1:500), and GOS28 (Transduction Laboratories, #G83820). Following antibody exposure, the blots were then washed three times for fifteen minutes each time with WWB, after which they were incubated in secondary antibody conjugated to horseradish peroxidase (HRP)(1:1000 in 0.1% BSA/WWB) for 1h. Protein bands were detected using the enhanced chemiluminescence system (GE Healthcare) after another three washes for fifteen minutes each in WWB. Scion Image software was used to perform densitometry analysis. The software measures the relative intensity of the protein bands that appear on photographic films of western blots. The relative intensity of the bands of loading controls was also quantified and used to normalize the signals of the proteins of interest.
INS-1Ins2(C96Y)-EGFP clone #4S2 cells were reverse transfected with Herp siRNA in two 12-well plates (300,000 cells/well) and allowed to recover overnight at 37°C in 5% CO₂. The cells were treated with dox (2 µg/ml) the following day. 24h later, existing RPMI 1640 media without antibiotics was aspirated, and fresh L-Methionine-and-L-Cystine-free 21013 media (GIBCO) was used to wash the cells. 200µCi/ml ³⁵S-Label (EasyTag Express Protein labeling Mix, PerkinElmer; NEG772002MC) in Methionine-and-L-Cystine-free 21013 media was subsequently used to pulse the cells for 10 minutes. Following the incubation, the labeling media was removed. One plate was washed with PBS, the other was replaced with RMPI 1640 media and incubated at 37°C for 30 minutes. After the incubation period, the latter plate was also washed with PBS. The samples in both plates were subsequently lysed in 1% TX-100 lysis buffer (1% Triton X-100, 20 mM HEPES, pH 7.4, 100 mM KCL, 2mM EDTA, 1 mM PMSF, 10µg/ml leupeptin, and 10 µg/ml aprotinin) on ice. The concentrations of the protein lysates were determined via bicinchoninic acid (BCA) reagent (Pierce), and an equal amount of protein per condition was used for immunoprecipitation (I.P.) (typically 80-100 µg). The I.P. was performed with either mouse IgG serum (Sigma I-5381) or monoclonal anti-GFP (Clontech, 632381). The following day, protein G-agarose beads (Roche 1 243 233) were added (50 µl per I.P.). After subsequently rotating the I.P. mixtures for two and half hours at 4°C, the unbound fractions were removed and the pellets were washed three times with 0.5 ml 1% TX-100 lysis buffer and once with 0.5 ml 0.1% TX-100 lysis buffer for 5 min each at 4°C. The pellet was then resuspended directly in Nu-PAGE sample buffer and boiled for 5 min. The samples were resolved by 4-12% Nu-PAGE. The dried gel was subsequently exposed to imaging film (Kodak BioMax Light Film) overnight and the film was then developed using a medical film processor (Konic Minolta Medical and Graphic, Inc., SRX-101A).
2.12 Apoptosis assay

Apoptosis was measured according to instructions provided by the manufacturer (Roche Diagnostics), and subsequent to treatments specified in figure legends (i.e. Herp knockdown). In order to carry out measurement of degree of apoptosis in the cell populations, the cells were lysed and oligonucleosomes in the cytoplasm (reflecting apoptosis-related DNA degradation) were measured based on Roche Diagnostics instructions. Parallel experiments were performed simultaneously to measure total protein loss. The measured protein concentrations were then used to normalize quantified apoptosis against total protein. As control samples (i.e. treated with control siRNA and no Dox) showed near background levels of cytoplasmic oligonucleosomes, this condition was used to normalize all other conditions.

2.13 Data Analysis

Results are shown as mean ± SE. The student’s two-sample t-test was used to measure statistical significance between two experimental conditions, assuming equal variance. ANOVA followed by Tukey post hoc test was used for the analysis of data obtained from several groups. A p value of <0.05 was considered statistically significant.
CHAPTER 3: EXAMINING THE ROLE OF HERP IN THE ER STRESS RESPONSE OF PANCREATIC β-CELLS

I. THE ROLE OF HERP IN MISFOLDED PROINSULIN DEGRADATION AND CELL SURVIVAL

II. ANALYSIS OF HERP EXPRESSION IN INSULINOMA CELLS AND ISLETS OF ANIMAL MODELS OF DIABETES

Some of the results presented in this chapter have been published in *BMC Cell Biology*.

I. THE ROLE OF HERP IN MISFOLDED PROINSULIN DEGRADATION AND CELL SURVIVAL

3.1 Introduction

It comes as no surprise that the protein folding machinery within the highly secretory pancreatic β-cell is complex and well developed. The endoplasmic reticulum (ER), which lies at the heart of this machinery, is constantly engaged in insulin biosynthesis and processing. Moreover, due to high protein folding demands and various biochemical insults and genetic mutations, the ER is often made vulnerable and a target of ER stress. Recently, ER stress has become associated with β-cell dysfunction in the context of diabetes due to excess secretion of insulin over a prolonged period of time to overcome a state of insulin resistance. Part of the functional architecture of the ER is a defense known as the ER stress response that allows the ER to counteract conditions of stress. These conditions include the upregulation of secretory protein synthesis and the accumulation of misfolded or mutant proteins. ER-associate degradation (ERAD) of misfolded proteins is a crucial feature of the ER stress response that is involved in relieving the stress induced by the accumulation of these proteins via degradation.

The Akita mouse model is particularly relevant to the study of the ER stress response induced by the accumulation of mutant proteins and specifically of mutant insulin. It is characterized by a heterozygous mutation for Insulin-2 gene that manifests as a cysteine 96 to tyrosine amino acid substitution. Interestingly, Akita mouse islets have exhibited an induction in ERAD components such as Hrd1, Sel1L and BiP. The induction of these components may partially explain its viability. In order to better understand the ER stress response in the pancreatic β-cell in a cell culture model, a former graduate student from the Volchuk lab, Elida Lai engineered an insulinoma cell line that mimics the Akita mouse mutation. Microarray analysis conducted, on the doxycycline-induced mutant insulin expressing cell line by another graduate student, Taila Hartley, showed a time-dependent increase of several genes including those for ERAD components such as Sel1 and Herp.
Recently, Herp, which is potentially involved in the ubiquitination and retrotranslocation of misfolded substrates, has been implicated in the degradation of non-glycosylated BiP substrates such as the NS1 κ LC. As insulin is also a non-glycosylated substrate, we aim to examine whether Herp is essential for the degradation of misfolded/mutant insulin. As an extension of this aim we will further examine whether Herp is required to maintain cell viability.

3.2 Results

3.2.1. Herp is an ER stress-inducible protein.

We first examined if Herp is induced by ER stress in pancreatic β-cells. INS-1 832/13 cells were treated with a chemical ER stress-inducing compound that inhibits N-linked glycosylation, known as Tunicamycin (Tm). Immunofluorescence studies showed that there is in fact an increase in the expression of Herp with Tm treatment (Fig. 3.1A). Herp further shows an ER-like staining pattern indicative of its localization in the ER. It is interesting, that although there is a significant induction in Herp expression with Tm treatment in comparison to the non-treated control, this induction is absent when treating with another chemical ER stress inducer known as Thapsigargin (Figure 3.1B).
3.2.2 Herp is induced in a pancreatic β-cell line that expresses a folding-deficient proinsulin-EGFP fusion protein.

A previous graduate student, Elida Lai generated a doxycycline-inducible INS-1 Ins2(C96Y)-EGFP expressing cell line on which the first aim of my project is based. As shown previously, with increasing time dependent treatment of dox (2μg/ml) there is an increased expression of mutant insulin shown by an increased amount of fluorescence (Fig. 3.2). We then looked at whether Herp is upregulated in the mutant insulin expressing cell line. With time dependent dox (2μg/ml) treatment there is an increased expression of Herp (Fig. 3.3A,B).
Figure 3.2. Doxycycline inducible mutant insulin expression in the INS-1 Ins2(C96Y)-EGFP cell line. An inducible mutant insulin expressing cell line was generated to study the ER stress response in β-cells. A stable Insulin2(C96Y)-EGFP expressing INS1 clone was treated with 2 µg/ml doxycycline for the times indicated, fixed and visualized by confocal fluorescence microscopy. The data presented in this figure was obtained by Elida Lai.

Figure 3.3. Herp is induced by doxycycline in a time dependent manner. A stable Insulin2(C96Y)-EGFP expressing INS-1 clone was treated with 2 µg/ml doxycycline for the times indicated and lysed in TX-100 lysis buffer. Western blot analysis was performed on electrophoresed samples to examine Herp and GOS28 protein expression in the indicated conditions (A). The blot was quantified for relative Herp expression as described in the Methods (B).

3.2.3 Herp knockdown results in increased expression of mutant insulin.

To study the potential role of Herp in mutant insulin degradation and cell survival we sought to knockdown Herp expression using siRNA. To test the effectiveness of our Herp siRNA we treated INS-1 Ins2(C96Y)-EGFP with both control and Herp siRNA. The following day the mutant insulin expressing cells were treated or not with dox (2 µg/ml) for 24h. We found that Herp siRNA was in fact
effective at knocking down Herp protein levels by approximately 50% (Fig. 3.4B). Furthermore, in comparison to the control siRNA-treated condition, treatment with Herp siRNA resulted in increased mutant insulin expression (Fig. 3.4A,C). This result was most obvious in the no dox treated conditions, but was nonetheless also observed in the dox treated condition to a lesser extent.

Figure 3.4. Herp knockdown is accompanied by an increase in steady state levels of mutant insulin. INS-1 Ins2(C96Y)-EGFP cells were transfected with control or Herp siRNA and treated or not the following day with Dox (2 µg/ml) for 48h. The cells were then lysed in TX-100 lysis buffer, electrophoresed on a 10% SDS gel and immunoblotted for Herp and mutant insulin (A). The blots were quantified for relative Herp expression (B). Western blots were quantified to determine relative expression of mutant insulin (INS-1Ins2(C96Y)-EGFP) (C). Shown is the relative expression between Herp siRNA and control siRNA-treated cells normalized to control siRNA. Student’s t-test was performed on the densitometry data from N=4 experiments. *p<0.05 Herp siRNA-treated cells versus control siRNA-treated cells.
3.2.4. Examining if Herp is required for mutant insulin degradation.

To examine if Herp is involved in mutant insulin degradation we used cycloheximide to inhibit new protein synthesis, and monitored steady-state mutant insulin levels. Mutant insulin expressing cells were treated with dox (2ug/ml) for 48h and subsequently treated or not with cycloheximide (CHX), a protein synthesis inhibitor for 3 or 6h. We observed that with time dependent CHX treatment steady state levels of mutant levels remained constant, whereas Herp levels rapidly degraded (Fig. 3.5A-C). Both the constant steady levels of mutant insulin and rapid degradation of Herp in the absence of new protein synthesis suggest that mutant insulin degradation may be Herp dependent.

Figure 3.5. Mutant insulin remains at constant steady state levels in the presence of decreased Herp expression. INS-1 Ins2(C96Y)-EGFP cells were treated with Dox (2ug/ml) for 48h and treated or not with cycloheximide for 3h or 6h. The stable mutant insulin expressing cells were subsequently lysed in TX-100 lysis buffer. Western blot analysis was conducted to examine Herp and mutant insulin expression at the indicated conditions. Shown are representative western blots from two independent experiments (A). Western blots were quantified as described in the Methods, and shown is the relative expression of Herp (B) and mutant insulin (C). Data shown are the mean ± from three independent experiments.
To further examine if Herp is required for mutant insulin degradation we performed a 35-S pulse-chase degradation assays (Fig. 3.6A,B). INS-1 Ins2(C96Y)-EGFP cells were induced with dox then labeled with 35S-methionine/cysteine for 10 min in cells treated with control siRNA or Herp siRNA. The cells were subsequently chased in cold media 0 or 30 min. At time 0 min and 30 min, cells were lysed and mutant insulin was immunoprecipitated by an anti-GFP antibody. Although there tended to be more mutant insulin present in the Herp siRNA treated cells after 30 min of chase (Fig. 3.6A), overall the results were not significant (Fig. 3.6B).

**Figure 3.6. Effect of Herp knockdown on degradation of mutant insulin.** The INS-1 Ins2(C96Y)-EGFP cell line was transfected with control or Herp siRNA and treated with dox (2μg/ml) the following day. 24h later the samples were labeled with 35S-methionine/cysteine for 10 min. Following the labeling period, t=0 min samples were washed in PBS and lysed, whereas t=30 min samples were incubated in “cold” media for 30 min, then washed in PBS and lysed in TX-100 lysis buffer. Protein concentrations of the cell lysates were quantified and monoclonal anti-GFP antibody was used to immunoprecipitate the mutant insulin. The samples were then electrophoresed on a NU-PAGE gel. The gel was dried and exposed to a phosphoimager screen (A). The mutant insulin bands on the gel were quantified based on their relative intensity using ImageQuant Software (B). Band intensity data was normalized to the t=0 min time point for both control-siRNA and Herp siRNA-treated samples. Data shown are the mean ±SE from four independent experiments.
3.2.5. Herp is essential for cell survival.

Work performed by Taila Hartley, a previous graduate student in the Volchuk lab, revealed that dox-induced Ins2(C96Y)-EGFP mutant protein expression induces apoptosis after several days of expression. Using a TUNEL assay to measure apoptosis, it was found that even after several days of mutant insulin expression apoptosis was detected in only 14% of the cell population (92). We hypothesized that upregulation of ERAD was maintaining cell survival by degrading mutant insulin.

To examine the role of Herp in cell survival, we knocked-down Herp expression in INS-1 Ins2(C96Y)-EGFP cells and treated or not with dox. Apoptosis was subsequently measured using an ELISA cell death detection kit (Fig. 3.7B) or by cleaved caspase 3 levels (Fig. 3.7C,D). The ELISA kit works by measuring relative amounts of cytoplasmic mono and oligonucleosomes that are reflective of DNA degradation specific to apoptosis. Protein amount was measured in the same conditions in order to control for protein loss (Fig. 3.7A). Measurements of apoptosis obtained from the ELISA kit indicate that there are increasing apoptosis levels with Herp siRNA treatment relative to control siRNA treatment. This increasing trend is present in both the no dox and dox treated conditions. It is important to note that although protein loss is significant in the Herp siRNA plus dox treated condition (Fig. 3.7A), controlling for protein loss was insufficient to detect a statistically significant change between control siRNA and Herp siRNA in the dox-treated condition (Fig. 3.7B). However, by measuring cleaved caspase 3 levels we clearly observed that cleaved caspase 3 was markedly increased in Herp siRNA-treated cells, in both the absence and presence of dox (Fig. 3.7C,D).
**Figure 3.7. Herp knockdown induces apoptosis.** (A,B) INS-1 Ins2(C96Y)-EGFP cells were transfected with control or Herp siRNA and treated or not with dox (2µg/ml) the following day. 48h later the cells were lysed and total protein was measured or apoptosis was measured using an ELISA cell death detection kit as described in the Methods. Total protein/well in a 12-well plate from each condition (A). Data shown are the mean ±SE from four independent experiments, *p<0.05. The apoptosis signal was normalized to control siRNA-treated cells (B). INS-1 Ins2(C96Y)-EGFP cells were transfected with control siRNA or Herp siRNA for 72h. Cells were lysed in sample buffer and immunoblotted fro cleaved caspase 3 and tubulin (C). INS-1 Ins2(C96Y)-EGFP cells were treated as described in A, then lysed and immunoblotted as described in C (D).

We also examined nuclear morphology in control siRNA and Herp siRNA-treated cells by Hoescht 33342 staining ([Fig. 3.8](#)). After 24 h of dox induction, fragmented and condensed nuclei were readily detected in Herp siRNA-treated cells compared to control siRNA-treated cells.
Figure 3.8. **Nuclei become fragmented with Herp siRNA treatment.** As part of a qualitative approach, nuclei morphology was examined in mutant insulin-expressing cells that were reverse transfected with control or Herp siRNA and treated or not with dox for 24h. The cells were then fixed and stained with Hoechst 33342 and visualized using an Olympus IX71 fluorescence microscope.

### 3.2.6. Steady state levels of mutant insulin remain constant with Herp overexpression.

In addition to knock-down studies, we hypothesized that overexpression of Herp may increase ERAD efficiency and enhance mutant insulin degradation. We constructed a recombinant adenovirus expressing human Herp and a GFP control virus (generated by Liling Zhang). Western blot analysis was used to test expression in pancreatic β-cell lines (**Fig. 3.9**). Once Herp overexpression was confirmed, we examined the effect of Herp adenovirus infection on steady state mutant insulin levels in the INS-1 Ins2(C96Y)-EGFP cell line. 24h after dox-induced mutant insulin expression in the appropriate conditions, the cells were lysed and analysed using western blot analysis. Results showed that treatment with dox resulted in increased mutant insulin expression in comparison to conditions not treated with dox. However, it was also revealed that infection of the cells with Herp adenovirus made no significant difference in mutant insulin steady state levels in comparison to the untreated and GFP overexpressing cells (**Fig. 3.10A-C**). This suggests that Herp overexpression does not enhance mutant insulin degradation as was initially hypothesized.
Figure 3.9. Herp overexpression in INS-1 832/13 and AD-293 cells. Both INS-1 832/13 and AD-293 cells were infected or not with varying doses of Herp adenovirus as described in the Methods to test for Herp adenovirus efficiency (1=1x10^8 pfu/ml, 2=1x10^9 pfu/ml, 3=1x10^10 pfu/ml, 4=1x10^11 pfu/ml). The cells were subsequently lysed and equal amounts of protein were resolved by SDS-PAGE and immunoblotted for the indicated proteins.

Figure 3.10. Herp overexpression has no effect on steady state levels of mutant insulin. INS-1 In2(C96Y)-EGFP cells were infected or not with Herp adenovirus or GFP adenovirus (1x10^8 pfu/ml), and subsequently treated with dox (2µg/ml). 24h later the cells were lysed, resolved by SDS-PAGE and immunoblotted for the indicated proteins. Data shown are the mean ±SE from three independent experiments (A). Western blots were quantified and Herp expression (B) or Ins2(C96Y)-EGFP mutant insulin expression (C) relative to control uninfected cells is presented.
3.3 Discussion

It has become evident that ER stress is implicated in β-cell failure in the context of diabetes. During the development of this disease state, the ER is overwhelmed by increased and prolonged demand to produce the non-glycosylated, mature insulin molecule in order to overcome a state of insulin resistance. As a protective mechanism, the UPR responds to perturbation in ER function. It works to restore optimal ER functioning by attenuating the translation of proteins, increasing the transcription of various genes, and degrading misfolded proteins via ERAD-mediated degradation.

In the first part of this project, we have examined the role of Herp in ERAD-mediated mutant insulin degradation and in cell survival during conditions of ER stress using the INS-1 Ins2(C96Y)-EGFP insulinoma cell line generated previously in our lab. In this cell line, our lab has discovered that Herp is induced at the mRNA level in response to mutant insulin expression (92). This study is of particular interest, as Herp has been shown to be specifically involved in the degradation of non-glycosylated BiP substrates (63), and proinsulin is non-glycosylated.

In an attempt to study the role of Herp in mutant insulin degradation, we treated the insulinoma cell line with Herp siRNA and measured mutant insulin expression levels. We found that mutant insulin levels are increased with Herp knockdown, suggesting that mutant insulin degradation may be Herp dependent. We lent further support to this possibility by showing that Herp is rapidly degraded, while steady state levels of mutant insulin remain constant in the absence of new protein synthesis. These experiments were then followed by S<sup>35</sup> pulse-chase studies to measure the kinetics of mutant insulin degradation in Herp-depleted cells. Although results showed a trend toward increased mutant insulin expression with Herp knockdown in comparison to the control siRNA, there was no statistical difference in the level of mutant insulin after a 30 min chase. The variability may be partially due to the large amount of protein loss experienced with Herp knockdown.
and the difficulty in obtaining sufficient material, or perhaps the rather inefficient knockdown achieved with our siRNAs. Moreover, only one time point of chase was used. Perhaps shorter chase periods might reveal a difference. Another point to consider, is that degradation may also occur by autophagy, which is known to be involved in the degradation of some ER proteins (91). Finally, Okuda-Shimizu et al., have shown that Herp depletion resulted in a relatively small difference in degradation. Again, this may have been due to siRNA inefficiencies. Overall, however, our results support the notion that Herp as part of the ERAD process is involved in the degradation of the Insulin2 C96Y-EGFP protein.

That said, the inability to obtain sufficient amounts of protein to perform the abovementioned radioactive degradation and apoptosis assays may be partially explained by yet another phenomenon. It has been shown in neurons that the ability of Herp to prevent ER-stress induced death is associated with its ability to stabilize Ca\(^{2+}\) homeostasis within the cell (80). The accumulation of ER-released Ca\(^{2+}\) in the mitochondria during ER stress causes it to depolarize and release cytochrome c. The released cytochrome c then activates caspase 3, resulting in apoptosis. Herp however, is able to regulate the Ca\(^{2+}\)-mediated pro-apoptotic cross-talk between the ER and the mitochondria, thereby precluding apoptosis. Interestingly, once ER-stress induced apoptosis occurs, the resulting activated caspase 3 works quickly to render Herp dysfunctional by cleaving its ubitquitin-like domain (ULD) (80). In this manner, the effects of Herp knockdown may be exacerbated by the further reduction in Herp levels that occurs with ER stress-induced apoptosis and cleaved caspase 3 expression. Nonetheless, much remains to be known about the role Herp plays in apoptotic pathways, and a better understanding would especially prove to be of therapeutic benefit (80).

A point to be noted is that although ERAD may appear to be a prime mechanism in the degradation of misfolded proteins, many misfolded substrates are disposed of through alternate pathways (93-96). Subsequent secretory organelles for example have their own mechanisms for quality control. Autophagy is another degradative pathway that may be engaged when it is appropriate (48; 97).
By definition, autophagy is an alternative mechanism engaged by the cell to eliminate misfolded substrates, particularly those substrates that form aggregates in the secretory pathway or cytoplasm (48). In fact studies in which the proteasome has been inhibited, have demonstrated an activated autophagic pathway. This suggests that both the proteasomal and autophagic systems may be coupled to one another, or that the former may function in a compensatory manner for the latter. Furthermore, studies have found that the autophagic pathway may not solely be involved in the degradation of bulk aggregates, but may also function selectively (91; 98).

Studies in yeast have even shown that autophagy may play an important role during the ER stress response (99; 100). Interestingly enough, the formation of autophagosomes in such contexts includes the ER membrane and sequesters the material without really degrading the aggregates (99; 101). Autophagy is however required in the degradation of aggregates of mutant proteins such as mutant vasopressin and dysferin that have accumulated within the ER. Moreover, studies have shown data in favour of a link between UPR signaling and autophagic pathways. Both the IRE1 and PERK pathways have been implicated in autophagy. The JNK kinase for example, which is needed for the induction of autophagy, is a downstream effector of IRE1 signal transduction. That said, although autophagy is not apparently engaged in mutant insulin expressing cells, whether it is involved in mutant insulin degradation in this cell line is yet to be determined.

With regards to cell survival, we found that with Herp knockdown levels of apoptosis measured by both the ELISA cell death detection kit and cleaved caspase 3 expression, were increased. Once again, due to the significant amount of protein loss with Herp knockdown and dox-induced mutant insulin expression, apoptosis was difficult to quantify even after normalizing for protein amount present in each condition. Nonetheless, the increased expression of cleaved caspase 3, significant protein loss and fluorescence microscopy images showing fragmented and smaller nuclei with Herp knockdown strongly suggest that Herp is essential for cell survival.
Furthermore, Herp has been found to suppress the activation of pro-apoptotic factors such as caspase 12 and JNK (80).

Studies have supported the notion that by constantly degrading misfolded substrates such as mutant insulin, ERAD in particular may play a major role in relieving stress and protecting the cell against death. It has been shown that despite the increased expression of pro-apoptotic transcription factors such as CHOP and TRB3, apoptosis remains at low levels, while inhibiting the proteasome primes the cell for apoptosis. In this manner, the suggested role Herp may play in possibly linking the substrate to the proteasome for degradation or in enhancing ubiquitination may be essential for ERAD function and its protective effects. Specifically, the ULD in the N-terminus of Herp has been shown to be crucial for its function. It has been suggested that the ULD may interact with the proteasome (75). This perhaps explains why it is required in order to confer ER stress resistance in F9 embryonic carcinoma cells (76).

The viable diabetic Akita mouse model, which is heterozygous for a mutation to the Insulin-2 gene that results in a cysteine 96 to tyrosine amino acid substitution, is another example of the importance of Herp and ERAD function. Studies on islets from these mice have shown an induction of the ERAD components: Hrd1, SelL and BiP (82). Microarray studies performed in our lab on the Akita mouse mutation-based INS-1 Ins2(C96Y)-EGFP cell line have shown an induction of both Sel1 and Herp.

In line with this finding, we constructed a Herp adenovirus to examine our hypothesis that Herp overexpression may potentially enhance mutant insulin degradation. Infection of the insulinoma cell line with the Herp adenovirus however, showed no changes in steady state mutant insulin levels in comparison to control conditions. This may have been due to the idea that simply increasing the expression of one of the components of the degradation machinery, such as Herp may not optimize the function of the entire machinery as each component is not a rate-limiting reaction.
Specifically, Herp has been found to have a direct interaction with the Hrd1 ligase, but not with other members of the retrotranslocation complex involved in the degradation of misfolded substrates including Derlin-1, VIMP and the p97 hexamer (78). Thus, the increased expression of the other components of the complex may also be required to enhance the degradation of mutant insulin.

An alternate possibility for the inability of Herp overexpression to enhance mutant insulin degradation may be its suggested involvement in enhancing protein folding capacities in the ER (76). Here, although Herp has been found to bind to full-length presenilins and enhance amyloid beta peptide production, Herp overexpression does not change the steady state levels of the presenilins. This suggests that Herp is not involved in enhancing the proteolysis of these substrates. Furthermore, as an ATF6-dependent gene, Herp along with GRP78 are induced in F9 embryonic carcinoma cells within 4h of Tm treatment. This lends support to its secondary involvement in protein folding.

Although we have shown that Herp, as a component of ERAD may play an important role in mutant insulin degradation and may thereby enhance cell survival, many other factors must be taken into consideration in order to obtain a clear picture of Herp mechanism and function. Changes in experimental parameters such as the use of shorter time points as well as the use of more effective siRNAs would perhaps render a more clearly defined role of Herp function in mutant insulin degradation. This may be further enhanced by taking into consideration measures of cleaved caspase 3 function on the Herp ULD as well as autophagy as an alternative and perhaps coupled mechanism of degradation. Moreover, the consideration of Herp’s secondary roles, such as in protein folding may prove to be beneficial in the future design of experimental protocols.
II. ANALYSIS OF HERP EXPRESSION IN INSULINOMA CELLS AND ISLETS FROM ANIMAL MODELS OF DIABETES

3.3 Introduction

Cultured cell lines are effective models for primary cells, and have lent support to the notion that ER stress is involved as a mediator in the negative effects of glucotoxicity on β-cell function. Specifically, insulinoma cells treated with high glucose have exhibited UPR activation by the increased expression of XBP-1s, and the ER chaperones BiP, GRP94 and EDEM, to name a few (21). Although, cultured cells may have similarities to primary cells, there are nonetheless some notable differences. It is for this reason, that in addition to conducting experiments with insulinoma cell lines, we have extended our studies to animal models.

Animal models of diabetes have served as a valuable tool in gaining a better understanding of the disease state and fuelling research geared towards therapeutic benefit. In the sections to follow, I will briefly discuss three animal models of diabetes that were used in our studies, i.e. the Akita and MKR mice models and the hyperglycemic rat model.

The mutant Akita mouse model reveals a phenotype where it spontaneously develops hyperglycemia in combination with reduced β-cell mass, but without insulitis or obesity (81). This phenotype is the result of a Cys96Tyr mutation in the insulin 2 gene of the mouse. This mutation precludes the formation of a disulfide bond between the A and B chains of the proinsulin molecule resulting in major conformational change and preventing the proper folding and processing of the protein. The mutant, misfolded insulin-2 is then retained in the ER of the pancreatic β-cell, by ER quality control (102). Oyadomari (2002) et al have found that increasing and prolonged hyperglycemia in these mice results in CHOP induction via PERK activation and eIF2α phosphorylation (103), and subsequent β-cell apoptosis. Furthermore, the development of diabetes in these mice resulted in the increased expression of both BiP and CHOP at both the mRNA and protein levels in the
pancreas of these mice. The Akita mouse is believed to be a good model for ER stress-induced diabetes due to the fact that its phenotype reflects a gain-of-function via ER stress activation, as opposed to a loss of functional insulin.

During physiological functioning, β-cell mass increases in response to a state of insulin resistance in order to maintain normoglycemia. However, it is less certain whether insulin resistance may fuel β-cell cell dysfunction in the absence of a pre-existing state of β-cell vulnerability. In efforts to gain a better understanding in this regard, Asghar et al (2006) studied the β-cell phenotype in the MKR mouse model of diabetes (104).

This mouse model exhibits insulin resistance in the skeletal muscle and the development of diabetes due to muscle-specific dominant negative expression of human IGF-1 receptor cDNA. This expression leads to the functional inactivation of both the INSR and IGF1R pathways. The MKR mouse also exhibits hyperglycemia and defective glucose stimulated insulin secretion (GSIS). Interestingly, although demand for insulin secretion was high, increased insulin content and β-cell mass via hypertrophy and hyperplasia were evident in the pancreas of these mice. Furthermore, treatment with a high-fat-sucrose diet increased the severity of hyperglycemia in MKR mice without a corresponding increase in weight gain. These findings demonstrate that heightened insulin resistance can render GSIS defective. Such a phenotype would eventually lead to a state of hyperglycemia due to an inability of the β-cell to compensate. Furthermore, the presence of intact insulin stores lends support to a pre-existing state of β-cell susceptibility and dysfunction underlying the secretory defects of the β-cell in the MKR mouse.

Lu et al (2008) have proposed a model where prior to β-cell failure, defective metabolic coupling and cell stress may result from an increased demand for the β-cell of the MKR mouse to secrete insulin (85). Specifically, defective GSIS in this model may be the result of the inappropriate expression of insulin processing and secretory proteins, reflected by an increased expression of many UPR and ERAD proteins. Furthermore, increased levels of ER stress and the resulting increase in
UPR activation may reduce the synthesis of proteins that contribute to insulin secretion. Such conditions are believed to lower glucose sensitivity and insulin secretion, both of which are responsible for β-cell failure. The consequent state of hyperglycemia is believed to worsen the initial defect and eventually contribute the development of the Type 2 diabetes.

Chronic hyperglycemia has been found to contribute to β-cell dysfunction in vitro via an oxidative stress pathway. In efforts to better understand the involvement of oxidative stress in the glucose-induced β-cell death, Giacca et al. (2007) have conducted studies in Wistar rats infused with glucose for 48h to achieve 20 mmol/l hyperglycemia. β-cell function (i.e. GSIS, insulin secretion/sensitivity), reactive oxygen species and total superoxide levels were subsequently measured. Measurements from these hyperglycemic rat models exhibited decreased GSIS and increased levels of ROS, and of total and mitochondrial superoxide in isolated islets. Furthermore, co-infusion of these rats with tempol (TPO), a membrane-permeable and metal independent superoxide dismutase mimetic, prevented the β-cell dysfunction and the increase in total and mitochondrial superoxide levels observed with chronic and elevated glucose infusion alone.

This section will focus on our aim to examine Herp expression in β-cell lines and islets from animal models of diabetes to elucidate if Herp is induced in these models.
3.4 Results

3.4.1 Herp mRNA is induced with high glucose treatment.

To examine expression of Herp in cell culture models of diabetes, studies with high and low glucose were conducted on the rat INS-1 832/13 cell line. Real-time PCR results showed that after 24h of treatment with high glucose (25 mM), there was no significant increase in Herp expression (Fig. 3.11A). There is nonetheless, a slight increase in Herp induction with 25 mM glucose treatment indicative of an underlying trend. At the 48 h time point however, there was a significant increase in Herp expression with high glucose relative to the low glucose (5 mM) treated condition (Fig. 3.11B). This suggests that Herp mRNA is induced under ER stress-associated diabetic conditions such as high glucose.

![Graph A](image1.png)  

![Graph B](image2.png)

**Figure 3.11. Chronic high glucose induces Herp mRNA expression in the INS-1 832/13 cell line.** INS-1 832/13 cells were treated with 5 mM, 25 mM or 5 mM with tumincamycin (Tm, 2µg/ml). 24h later (A) or 48h later (B) total RNA was isolated and the mRNA levels relative to cellular β-actin was quantified using real-time PCR. The data shown in A and B are the mean ±SE of five and three independent experiments respectively *p<0.05. 25 mM-treated versus 5 mM-treated (Student’s t-test).
3.4.2 Temporal Biphasic Herp response to high glucose treatment at the protein level.

Western blot analysis revealed that Herp protein expression is not changed in INS-1 832/13 cells treated for 24 h with high glucose (25 mM) relative to low glucose (5 mM) -treated cells (Fig. 3.12A,B). In contrast, 48 h high glucose treatment resulted in a significant downregulation of Herp expression in comparison to low glucose treatment (Fig. 3.12C,D). These findings indicate that unlike the mRNA, which is induced by high glucose, Herp protein levels seem to be reduced by chronic high glucose.

Figure 3.12. Chronic high glucose downregulates Herp protein expression in the INS-1 832/13 cell line. INS1 832/13 cells were treated with 5 mM, 25 mM or 30 mM glucose for 24h (A,B) or 48h (C,D). Control cells grown in 11.1 mM glucose-supplemented media were treated or not with tunicamycin (Tm, 2µg/ml) for 16h. Cells were lysed in lysis buffer and equal amounts of protein were electrophoresed on a 10% SDS PAGE and immunoblotted for the indicated proteins. Relative Herp expression based on western blots shown in A (B). Relative Herp expression based on western blots shown in C normalized to control cells (D) *p<0.05 relative to the 5 mM glucose-treated condition.
3.4.3 Effect of chronic high glucose on Herp mRNA and protein level in rat islets.

Cultured cell lines are good models for primary cells but do not always behave the same. Thus, one must test effects in primary cells to examine if they behave similarly. We therefore, examined the effects of high glucose in primary islets. Isolated Wistar rat islets were treated with high glucose at both the 24h and 48 h time points. Real-time PCR analysis did not show significant changes in Herp mRNA levels with 25 mM glucose treatment relative to control conditions (5 mM) at either the 24h or 48h time points (Fig. 3.13A). There does however appear to be a decreasing trend in Herp expression where expression is reduced with high glucose treatments.

Western blot analysis was performed on similarly treated Wistar rat islets to examine Herp expression at the protein level (Fig. 3.13B,C,D). Herp expression was somewhat variable between experiments in low and high glucose (5 mM versus 25 mM) treatments at both the 24h and 48h time points. Similar to results with the insulinoma cell line, islets exposed to high glucose for 48h tended to have reduced Herp protein expression. The effect of Tm to induce Herp expression was less than with insulinoma cells as primary rat islets are more resistant to ER stress (88).
Figure 3.13. Herp mRNA and protein levels are not affected by high glucose treatment in primary rat islets. Wistar rat islets were treated with 5 mM, 25 mM glucose or 5mM glucose with Tm (2 µg/ml) for 18h. Total RNA was isolated and the mRNA levels relative to cellular β-actin were quantified using real-time PCR (A). Similar treatment conditions were repeated with Wistar rat islets to measure Herp protein levels. Islets were lysed and equal amounts of protein were electrophoresed on a 10% SDS PAGE and immunoblotted for the indicated proteins (B). Western blots were quantified and relative Herp expression is shown (C,D). Data shown are the mean ±SE from three independent experiments.
3.4.4 Herp is induced in the hyperglycemic rat model.

Having tested the effect of high glucose in insulinoma cells and primary islets in vitro, we wanted to examine if hyperglycemia in vivo affects Herp expression in islets. As mentioned in the introduction, the laboratory of Dr. Adria Giacca has established a chronic hyperglycemia model where animals are made hyperglycemic with continuous infusion of a glucose solution (105). After 48h infusion of 20 mM glucose, the β-cells become dysfunctional, but no cell death is observed. Hyperglycemic rat islet samples and pancreas sections were obtained from Dr. Adria Giacca’s laboratory. Preliminary western blot analysis of islet samples and immunofluorescence studies on pancreas sections from these rats reveal that Herp expression is induced in comparison to samples from saline-treated rats (Fig. 3.14A,B). Insulin expression is low in high glucose-treated islets as expected, given the constant stimulation these cells are under. Thus, unlike insulinoma cells and rodent islets exposed to high glucose in vitro, in vivo high glucose treatment leads to an increase in Herp expression. It should be noted that other markers of ER stress have also been observed in islets from high glucose-treated animals (A. Volchuk; A. Giacca, unpublished observations).
Figure 3.14. Herp is induced in islets from the Hyperglycemic rat model. Equal amounts of protein from rat islets isolated from rats infused with saline or high glucose were resolved on a 10% SDS-PAGE and immunoblotted for the indicated proteins. As a positive control for Herp expression, INS-1 Ins2(C96Y)-EGFP cells treated or not with Dox is also shown (A). Pancreas tissue from saline or high glucose-treated rats was excised, fixed and embedded in paraffin. Pancreas sections were co-stained with anti-Herp and anti-insulin antibodies (B). HG rat islets and pancreas sections were generously provided by Dr. Adria Giacca.
3.4.5 *Herp is induced in islets from the MKR mouse model.*

Finally, we examined Herp expression in a more physiological diabetes model, where severe insulin resistance in the MKR mouse overworks the β-cell, causing it to become dysfunctional. Although islets from the MKR mouse model are relatively larger in size and demonstrate increased hyperplasia in comparison to islets from control mice, apoptosis remains at basal levels. This is indicative of a potential protective mechanism in place in the islets of these mice. In order to examine Herp expression in islets, we electrophoresed islet samples obtained from Dr. Wheeler's laboratory on a 10% SDS and performed western blot analysis (*Fig. 3.15A,B*). Islets from the MKR mouse show a two-fold increase in Herp expression in comparison to islets from control mice. Immunofluorescence studies performed by Liling Zhang confirmed these results (*Fig. 3.15C*). Thus it appears that in diabetic islets, Herp expression is increased, and since apoptosis is not detected it is possible that Herp and ERAD are maintaining cell survival under such conditions.
Figure 3.16. **Herp is induced in MKR mouse islets.** Equal amounts of protein from MKR mouse islets were resolved using SDS-PAGE and immunoblotted for the indicated proteins (A). Herp expression was quantified by gel densitometry (B). Pancreas tissue from control mice (WT FVB) or MKR mice was excised, fixed and embedded in paraffin. Pancreas sections were co-stained with anti-Herp and anti-insulin antibodies (C). MKR mouse islets and pancreas sections were generously provided by Dr. Michael Wheeler.
3.5 Discussion

The second part of my thesis aimed to examine Herp expression in cultured cell lines and primary islets from models of hyperglycemia and diabetes. The emphasis was placed on ER stress induced by glucotoxicity.

In efforts to examine Herp expression in vitro, we treated the rat INS-1 832/13 cell line with low or high glucose concentrations (i.e. 5 mM versus 25 mM) for either 24h or 48h. At the 24h time point, treatment with high glucose showed no significant change in Herp at both the mRNA and protein levels. With regards to chronic (i.e. 48h) high glucose treatment, Herp mRNA is significantly induced relative to control conditions, whereas Herp protein levels are reduced with high glucose treatment. This suggests that there is an increased demand for Herp function likely due to hyperglycemia-induced ER stress (21) resulting in its transcriptional induction. However, increased Herp transcription does not lead to increased Herp translation for reasons unknown. It is possible that Herp translation is dysfunctional or there is increased degradation of Herp under hyperglycemic conditions.

Similar experiments to those conducted in the INS-1 832/13 cell were performed in isolated rat islets to examine Herp expression during high glucose-induced-ER stress, and whether results would be reflective of results obtained in the cell line. Interestingly, there were no significant changes in Herp expression with high glucose treatment (i.e. 25 mM glucose) at either the mRNA or protein levels. This is indicative of the possibility that primary islets have more complex compensatory mechanisms, or are more resistant to high glucose treatments. Indeed, primary islets have been shown to be more resistant to ER stress than cultured cell lines (88). Further studies are required to determine whether high glucose for longer times induces Herp expression in rat islets.

In contrast, studies conducted with islets and pancreas sections from pathological models, namely the hyperglycemic rat model and MKR mouse, show a significant increase in Herp expression. Interestingly, in both animal models the
islets are hyperplastic and hypertrophic, without detectable apoptosis. This suggests that a protective mechanism may be in place within these islets. ERAD has been suggested to play a role in preventing ER-stress-induced apoptosis (80). Thus, as a component of ERAD, Herp may play a protective role in the islets. Additional studies would be warranted to better understand the role of Herp in both these animal models of diabetes.

As mentioned earlier, a state of glucotoxicity may cause rat islets to turn on alternative degradation pathways to ERAD, such as autophagy. In the context of diabetes, chronic hyperglycemia has resulted in the development of ubiquitinated protein aggregates both in pancreatic β-cell lines and in animal models of diabetes (i.e. Zucker diabetic fatty rat). Although it is not known why, it has been proposed that the aggregates may function as storage sites for dysfunctional proteins during stress. Autophagy would then be activated once the stress is alleviated, in order to dispose of the aggregates. Proteins targeted to these aggregates in the β-cell remain to be identified. The elevation of circulating free fatty acids is another condition associated with the development of diabetes. Evidence of autophagy in these conditions has also been observed (91; 106).

Although recent studies have found that autophagy plays a crucial role in the maintenance of pancreatic β-cell mass and function, and the protection against diabetes in mouse models (107), the involvement of autophagy during the ER stress response in pancreatic β-cells remains to be examined. This degradative pathway may be especially important during conditions of chronic stress (i.e. hyperglycemia and hyperlipidemia) where the heightened accumulation of aggregate proteins overwhelms the ERAD system (91).

Overall, the studies reported in this chapter have examined the transcript and protein levels of Herp in a cultured β-cell line and isolated rodent islets. It appears that Herp protein expression is increased in islets from hyperglycemic or diabetic models. Interestingly, in the animal models examined, islet apoptosis is not observed. This suggests that similar to the effect we observed in the mutant insulin
cell line, Herp as part of the ERAD process may protect β-cells from hyperglycemia-induced ER stress. This hypothesis requires future experimental testing.
3.6 Summary and Future Directions

The Akita mouse mutation based INS-1 Ins2(C96)-EGFP cell line generated previously in the Volchuk lab has allowed us to study the functional role of Herp in mutant insulin degradation and cell survival. We have shown that Herp knockdown in this insulinoma cell line is accompanied by increased mutant insulin expression. At the same time, we have found that the rapid degradation of Herp in the absence of new protein synthesis is accompanied by constant steady state levels of mutant insulin. Although both these results suggest that Herp is essential for mutant insulin degradation, we have been unable to conclusively confirm this by pulse-chase studies. Future experiments that optimize the pulse-chase could be performed. This includes the use of shorter chase times after the pulse. It is possible that autophagy is also degrading some of the mutant insulin, which would be more prevalent at longer chase times. The effect of autophagy could be tested using inhibitors such as 3-methyladenine.

With regards to cell survival, we have shown that Herp is indeed essential for cell viability and that its depletion results in increased levels of apoptosis in the cell population. In contrast, Herp overexpression appears to have no effect on steady state levels of mutant insulin. However, the effect of Herp overexpression on cell survival was not monitored and could be in future studies.

The study of Herp expression in animal models has increased our understanding of Herp expression and function during diabetic conditions that cause ER stress. We have shown that chronic high glucose treatment induces Herp mRNA, while reducing Herp protein expression in the INS-1 832/13 cell line. The mechanism behind this interesting result needs to be studied, i.e. is it altered translation of the Herp mRNA or increased Herp degradation that accounts for this result.

Studies in the pathological MKR mouse and HG rat models on the other hand, have shown significantly increased Herp expression, and have thereby suggested a potential protective role for Herp in the islets of these murine models. The role of
Herp in vivo could be studied in knock-out mice, although these might be neonatal lethal. For this reason, tissue-specific (i.e. β-cell specific) mice could be generated. It may also be worthwhile to examine Herp expression in additional models such as the Zucker Diabetic Fatty rat and the ob/ob mouse. These models exhibit β-cell phenotypes reflective of lipotoxicity. Furthermore, another current project in the Volchuk lab is focused on generating a BiP overexpressing mouse. It has been suggested that BiP may play a role in transporting misfolded substrates to the Herp/Derlin-1 complex for degradation (63). It would therefore be interesting to examine Herp expression in islets of these mice after chronically infusing them with high glucose.

Future studies to further the understanding of the role of Herp in the ER stress response of pancreatic β-cells would include observing the dynamics of a Herp-GFP construct during both stressed and non-stressed conditions and identifying interacting proteins to better define the biochemistry of Herp function. Also, the mechanism of Herp transcription (promoter), and how Herp is targeted to ER membranes could be studied to better understand Herp function.
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