PRO-APOPTOTIC BAX AND BAK CONTROL BETA-CELL DEATH AND EARLY ENDOPLASMIC RETICULUM STRESS SIGNALLING

by

Sarah White

BMSc., The University of Western Ontario, 2010

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Cell and Developmental Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

June 2013

Abstract

Functional failure and loss of pancreatic β-cells are critical events in the pathogenesis of diabetes and there is mounting evidence that suggests chronic endoplasmic reticulum (ER) stress contributes significantly to β-cell dysfunction and apoptotic death. Two core pro-apoptosis proteins, Bax and Bak, mediate the execution of mitochondrial apoptosis and have also been suggested to regulate aspects of ER physiology and stress signalling. In this study we set out to determine the relative contributions of Bax and Bak in the execution of β-cell death and examine their putative roles in β-cell ER-stress signalling under diabetogenic conditions. We generated mice in which the single or combined knockout of Bax and Bak could be induced in the pancreatic β-cell. Physiological islet function assessed both *in vivo* and *in vitro* was not affected by the knockout of Bax and/or Bak. However, Bax and Bak knockout improved β-cell survival under stress conditions. Single knockout, double knockout, and wild-type cells were assayed for ER-stress and cell death following treatment with staurosporine, thapsigargin, and culture under glucolipotoxic conditions. Time-course kinetic cell death analysis demonstrated that single and double knockout cells were protected from staurosporine, and further revealed that Bax-Bak double knockout was required for significant protection from death under glucolipotoxic conditions. ER-stress signalling was evaluated by quantitative PCR for XBP1s and CHOP. Interestingly, spliced XBP1 expression was augmented in Bax-Bak double knockout islets in the early phase of ER-stress signalling compared to wild-type controls. Stress-induced CHOP expression increased in a time-dependent manner but was not significantly different between Bax-Bak double knockout and wild-type islets. These results suggest that Bax and Bak regulate the IRE1α arm of the ER-stress response upstream of apoptosis by suppressing maximal XBP1 splicing. Under glucolipotoxic conditions, pancreatic insulin content, insulin secretion, and insulin transcription were unaffected by Bax and Bak knockout, indicating Bax and Bak do not mediate their protective effects towards β-cell death by retaining islet function. Together these data demonstrate that Bax and Bak have both individual and combined contributions to β-cell death under various stress conditions and suggest novel non-apoptotic roles regulating early ER-stress signalling in the β-cell.

Preface

The content presented in this thesis is original and unpublished work by the author, Sarah A. White.

All animal work conducted for this study was approved by the University of British Columbia Animal Care Committee. Biology and Husbandry of the Laboratory Rodent training was completed by Sarah White through the UBC Animal Care Centre (certificate #RBH-1149-10). Ethics training was completed by Sarah White and met the requirements of the Canadian Council of Animal Care (CCAC, certificate #4419-10).

Table of Contents

Abstract ii

Preface iii

Table of Contents iv

List of Tables vii

List of Figures viii

List of Abbreviations x

Acknowledgements xiii

Chapter 1: Introduction 1

1.1 Diabetes 1

1.2 β-Cell Failure and Death: The Onset of Type 2 Diabetes 2

1.3 Apoptosis and the Bcl-2 Family 4

1.3.1 Intrinsic and Extrinsic Apoptotic Pathways 4

1.3.2 Bax and Bak: Localization and Regulation 7

1.4 ER-Stress and the Unfolded Protein Response 8

1.4.1 PERK 11

1.4.2 ATF6 12

1.4.3 IRE1α 12

1.5 Potential Roles for Bax and Bak in ER Physiology and Stress Signalling 14

1.5.1 Bax and Bak Regulation of IRE1α Signalling 14

1.5.2 Bax and Bak at the ER: Cellular Physiology 15

1.5.3 Bax and Bak at the ER: A Link Between ER-Stress and Apoptosis 16

1.6 Known Roles for Bax and Bak in β-Cell Death 17

1.7 Bax-Bak Knockout Models 18

1.8 Cre-*lox* Technology and the Conditional Bax-Bak Knockout Model 19

1.9 Investigating the Roles for Bax and Bak in the Pancreatic β-Cell:

Stress and Death 23

Chapter 2: Materials and Methods 24

2.1 Reagents 24

2.2 *In Vivo* Studies 24

2.2.1 Breeding 24

2.2.2 Tamoxifen Administration for Inducible Cre Recombination 25

2.2.3 Genotyping 25

2.2.4 Intraperitoneal Glucose Tolerance Tests 26

2.3 *In Vitro* Studies 27

2.3.1 Islet Isolation and Cell Culture 27

2.3.2 Single-Cell Dispersion 28

2.3.3 *In Vitro* Stress Treatments 28

2.3.4 Cell Death Quantification 29

2.3.5 Mitochondrial Membrane Potential 30

2.3.6 Real-Time PCR Quantification of Gene Expression 30

2.3.7 Insulin Content/Insulin Secretion 32

2.4 Statistical Analysis 33

Chapter 3: Results 34

3.1 Generation of the Conditional Bax-Bak Knockout Mouse Model 34

3.2 Tamoxifen Independent Activation of Pdx1-CreER 37

3.3 Characterization of Bax and Bak Knockout on β-Cell Function 39

3.3.1 Loss of Bax and Bak Does Not Affect Glucose Homeostasis *In Vivo* 39

3.3.2 Loss of Bax and Bak Does Not Affect Islet Function *In Vitro* 41

3.4 Relative Contribution of Bax and Bak to Staurosporine-Induced Mitochondrial β-Cell Apoptosis 43

3.5 Double Knockout of Bax and Bak is Required for Significant Protection Against Glucolipotoxicity 46

3.6 Loss of Bax and Bak Augments Early UPR Signalling in Stressed β-Cells 49

3.7 Effects of UPR Signalling on Downstream Target Genes 54

3.8 Effects of Glucolipotoxicity and Thapsigargin-Induced ER-Stress on Insulin Expression in WT and BaxBak DKO β-Cells 56

3.9 Glucolipotoxic Effects on WT and BaxBak DKO β-Cell Function 59

Chapter 4: Discussion 62

4.1 Bax-Bak Knockout Model 62

4.2 Bax and Bak in β-Cell Death 64

4.3 Bax and Bak in ER-Stress 68

4.4 Potential Mechanisms for BaxBak DKO Protection Under Glucolipotoxic Stress ... 70

Chapter 5: Conclusion 72

References 73

List of Tables

Table 1. Genotyping Primer Sequences 26

Table 2. Quantitative PCR Gene Expression Primer Sequences 31

List of Figures

Figure 1. Intrinsic and Extrinsic Apoptotic Pathway 6

Figure 2. ER-Stress and the Unfolded Protein Response 10

Figure 3. Tissue-Specific Cre Recombinase Excision of the Bax Gene 21

Figure 4. Bax and Bak Genetic Modifications 22

Figure 5. Breeding Scheme for the Bax and Bak Knockout Mouse Model 35

Figure 6. Islet Specific Knockout of Bax and Bak 36

Figure 7. Ligand-Independent Pdx1-CreER Bax Recombination 38

Figure 8. Loss of Bax and Bak Does Not Affect Body Weight or *In Vivo* Glucose Tolerance 40

Figure 9. Loss of Bax and Bak Does Not Affect Islet Physiology *In Vitro* 42

Figure 10. Relative Contribution of Bax and Bak to Staurosporine-Induced Mitochondrial Islet-Cell Apoptosis 45

Figure 11. Double Knockout of Bax and Bak is Required for Significant Protection against Glucolipotoxicity 48

Figure 12. Loss of Bax and Bak Augments Early UPR Signalling in Islet Cells Under Glucolipotoxic Stress 50

Figure 13. Loss of Bax and Bak Augments Early UPR Signalling in Thapsigargin Stressed Islet-Cells 53

Figure 14. UPR Activated Gene Transcription in WT and BaxBak DKO Islets 55

Figure 15. Effects of Glucolipotoxicity and ER-Stress on Insulin Transcription in WT and BaxBak DKO Islets 58

Figure 16. Effects of Glucolipotoxicity on Glucose- and KCl-Stimulated Insulin Secretion and Insulin Content 61

List of Abbreviations

APAF-1	Apoptosis Initiating Factor-1
ASK1	Apoptosis Signal Regulating Kinase 1
ATF-6	Activating Transcription Factor-6
ATP	Adenosine Triphosphate
Bcl-2	B-Cell Lymphoma 2
BH	Bcl-2 Homology
BIP	Binding Immunoglobulin Protein (also known as GRP78)
bp	Base Pairs
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CHOP	C/EBP-Homologous Protein
Cre	Cre Recombinase
DKO	Double Knockout
DMSO	Dimethyl Sulfoxide
EDEM	ER Degradation Enhancer, Mannosidase alpha-like 1
EDTA	Ethylenediaminetetraacetic Acid
eIF2α	Eukaryotic Translation Initiation Factor 2α
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
ERDJ4	ER DnaJ Homologue 4 (also known as DNAJB9)
ERO1L	ER Oxidoreductase 1-like

EtOH	Ethanol
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid
Flox	*loxP* flanked gene
HBSS	Hanks Buffered Salt Solution
HSP90	Heat Shock Protein 90
IPGTT	Intraperitoneal Glucose Tolerance Test
IRE1α	Inositol-Requiring Protein-1α
IP3R	Inositol Triphosphate Receptor
JNK	c-Jun N-terminal Kinase
kDA	Kilodalton
KRB	Krebs Ringers Buffer
MEF	Mouse Embryonic Fibroblast
MEM	Minimum Essential Media
MOMP	Mitochondrial Outer Membrane Permeabilization
PA	Palmitate
PBS	Phosphate Buffered Saline
PDX1	Pancreatic and Duodenal Homeobox 1
PERK	PKR-like ER kinase
PI	Propidium Iodide
RIA	Radioimmunoassay
RPMI-1640	Roswell Park Memorial Institute 1640 (media acronym)

RYR	Ryanodine Receptor
SERCA	Sarco/Endoplasmic Reticulum Calcium ATPase
SKO	Single Knockout
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
Tg	Thapsigargin
TM	Tamoxifen
TMRE	Tetramethylrhodamine Ethyl Ester Perchlorate
TRAF-2	TNF Receptor Associated Factor-2
UPR	Unfolded Protein Response
XBP1s	Spliced X-box Binding Protein-1

Acknowledgements

While I sit here and stare out of my office window at an (unusual) bright and sunny Vancouver day, I still remember the phone call confirming that I would be heading to the big city of Vancouver, BC to study diabetes for my graduate degree. I would not have become the person I am today if Dr. Jim Johnson did not see potential in me to introduce me to my supervisor, Dr. Dan Luciani. I am extremely grateful for the opportunity to work for Dan, who has been an amazing supervisor over the last 3 years. I want to thank Dan for taking me on as his first graduate student, and for providing me the close supervision and guidance when needed as well as the freedom to work independently. Thanks to my committee members Dr. Bruce Verchere and Dr. Jim Johnson for providing scientific knowledge, guidance, and support to this project. I have been lucky enough to call two facilities home throughout my studies, and I'll forever be eternally grateful to Jim for allowing me to continue to work in his lab even after our move to CFRI.

The CIHR Training Program in Transplantation provided both funding support and a unique opportunity to become involved with the transplant community in Vancouver. Thanks to everyone in the Gross Anatomy and Neuroanatomy labs. I learned so much from all of you and my experience as a teaching assistant will never be forgotten.

Many thanks to everyone who taught me how to work in the lab and who provided technical assistance when needed. I owe the biggest appreciation to my co-op student and friend Lisa Zhang, the qPCR queen, as a part of this project would not have been able to progress this far without her ability to work so quickly and independently on everything I threw her way. I also thank Betty Hu for sharing her animal handling expertise and teaching me how to isolate islets, and Carol Yang, who was always there to provide assistance on anything and everything.

The people I worked with became my closest friends over the years and have made my experience in Vancouver one to remember. I love how there is always something exciting to do here year-round, from cabin trips in the summer to the Shuswap and Okanagan lakes, to skiing on the Whistler-Blackcomb mountains in the winter. I owe special thanks to Sophie Stukas for taking me in as a roommate and showing me what Vancouver had to offer. Michelle Chan and Sophie Stukas were always there for me when I just didn't understand the crazy world of graduate studies. Nicole Templeman brought RBT, essential for socializing outside of the lab and enjoying the sensational world of craft beers. Tobias Albrecht was always there for a good laugh, even if at his own expense. But most of all, I thank Blair Gage for always supporting and being there for me through the highs and lows, both in science and in life. I owe my survival through grad school to Blair, who continues to never give up on me (even when I succumb to my "I don't get it" mode).

And finally I thank Mom, Dad, and Mandi, for continuing to support my decision to move to Vancouver to achieve my goals and who are always there for me when needed; and Grandma, for always having banana bread ready when I come home to visit.

Chapter 1: Introduction

1.1 Diabetes

Diabetes is fast becoming one of the leading pandemics of the 21st century, as its incidence has steadily climbed from 2.8% of the world population in 2000 to 6.4% in 2010. Incidence rates have surpassed previously proposed estimates earlier than expected over the last decade, with newly estimated projections suggesting 7.7% of the global population will have diabetes by the year 2030, affecting 439 million people [1-3]. In Canada alone, the prevalence of diabetes has been projected to affect 3.7 million people by the year 2020, costing the Canadian health care system at least $16.9 billion per year [4].

Diabetes is a metabolic disease that affects whole body blood sugar control due to insufficiencies of the endocrine pancreas. Within the pancreas, hormone-producing clusters of endocrine cells called the islets of Langerhans contain a variety of cell types. Of the 4 major endocrine cell types (α, β, δ, and PP cells), the β-cells are responsible for producing and secreting the hormone insulin in response to elevated blood sugar levels, which allows the body to maintain euglycemia. Diabetes is characterized by impaired glucose tolerance and hyperglycemia, which leads to an increased risk for secondary complications such as diabetic nephropathy, neuropathy, and retinopathy, as well as increased mortality due to a heightened risk of cardiovascular disease and stroke [5-8].

There are multiple forms of diabetes, each with its own etiology. A small percentage of diabetic cases are comprised of gestational diabetes and genetically predisposed variants of diabetes. Type 1 diabetes (T1D) accounts for roughly 10% of diabetic cases and is caused by an autoimmune attack on the host's pancreatic β-cells, resulting in β-cell death and

virtually insulin deficiency. Type 2 diabetes (T2D) makes up roughly 90% of diabetic cases, and is a metabolic disorder characterized by insulin resistance and impaired insulin secretion due to β-cell dysfunction and increased β-cell death. The rising incidence of T2D correlates with our modern lifestyle and associated shift from moderately active to mainly sedentary and supplemented by high-calorie diets enriched with a high sugar and a high fat content [9,10]. So far genetics has been able to explain the heritability of some rare forms of diabetes within certain families and populations; however, the genetics underlying the complete etiology of diabetes still remains elusive and incomplete [11-14]. Efforts to combat diabetes are currently focused on therapeutic interventions and research on better understanding the molecular mechanisms by which β-cells become dysfunctional and die during the pathogenesis of diabetes.

1.2 β-Cell Failure and Death: The Onset of Type 2 Diabetes

The progression of T2D follows a pattern in which a pre-diabetic state of peripheral tissue insulin resistance induces a compensatory increase in β-cell function and mass in order to maintain euglycemia. This increase in β-cell function requires enhanced nutrient supply including glucose and free fatty acids (FFAs) that are usually evident in a pre-diabetic state [9]. Eventually, increasing metabolic demand overworks and exhausts the pancreatic β-cells leading to significant functional β-cell failure, increased β-cell apoptosis, and the onset of T2D [15,16]. Increased β-cell apoptosis is evident in isolated human islets from people with type 2 diabetes, and further supported the notion that it is β-cell apoptosis, not a reduction in β-cell replication or neogenesis, that is responsible for the reduction in β-cell mass seen in T2D [17-19].

Metabolic demand stems from chronic states of hyperglycemia and hyperlipidemia, both of which contribute to β-cell apoptosis [9,20]. However, the mechanisms by which apoptosis is triggered under various cell stress conditions are poorly understood. As the β-cell is responsible for a large production of insulin, it has adapted a highly resilient and functional endoplasmic reticulum (ER) that can handle the protein output required to maintain euglycemia. However, increasing cellular stress from chronic states of hyperglycemia and hyperlipidemia coupled with the sheer volume of protein processing the β-cell endures, increases the chances for improper ER function, termed ER-stress, that can lead to β-cell death [21-23]. ER-stress is one of a multitude of stress factors that contributes to β-cell dysfunction and death. Other cellular insults include oxidative stress [24-26], islet inflammation [27-29], and cytotoxicity from islet amyloid polypeptide aggregation [30,31]. The work presented here will focus on ER-stress in β-cell dysfunction and death. Evidence for increased ER-stress and apoptosis were recently found in isolated human islets from patients with T2D [32]. Similarly, human islets exposed to chronic glucotoxicity as well as lipotoxicity from elevated levels of the FFA palmitate display increased ER-stress and increased β-cell death [33-37]. While both gluco- and lipo-toxicity alone can have detrimental effects on the β-cell, the most deleterious effects of each are apparent when in combination, termed glucolipotoxicity [9,38-40]. These studies provide support that hyperglycemia and hyperlipidemia seen in the pre-diabetic state are critical and potentially causal to β-cell apoptosis and the onset of T2D.

1.3 Apoptosis and the Bcl-2 Family

Apoptosis is a highly regulated form of cell death that occurs in eukaryotic organisms to remove damaged or unwanted cells. Characteristics of apoptosis include separation of the cell from the surrounding environment, protein and organelle degradation, DNA condensation and fragmentation, and membrane blebbing, which ultimately result in phagocytic engulfment and lysosomal-mediated degradation of the apoptotic cell [41-44]. The B-cell lymphoma 2 (Bcl-2) family of proteins regulates cell survival and apoptosis. Their function is highly conserved, as orthologues of this family have been found in all metazoan species to date [45,46]. Members of this family share up to four highly conserved Bcl-2 homology (BH) regions, with BH3 being important for protein-protein interactions within the family and the control of apoptosis [46-49]. The Bcl-2 family members are categorized as either multi-domain anti-apoptotic proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl1), multi-domain pro-apoptotic proteins (Bax, Bak, Bok), or single BH3-only domain pro-apoptotic proteins (BID, BIM, BAD, BMF, NOXA, PUMA, BIK, HRK). Ultimately, the induction of apoptosis converges on the multi-domain pro-apoptotic proteins, Bax and Bak, which carry out a key step in the execution of apoptosis at the mitochondria [42,43,46,48].

1.3.1 Intrinsic and Extrinsic Apoptotic Pathways

Apoptosis can be initiated through either the intrinsic or extrinsic apoptotic pathways, both of which converge on the activation of executioner caspases that cleave an extensive array of cellular substrates to carry out apoptosis [42,46,50]. Cellular stressors and developmental cues activate apoptosis via the intrinsic apoptotic pathway, which is highly regulated by the Bcl-2 protein family. The intrinsic apoptotic pathway is irreversibly initiated when the pro-

apoptotic proteins Bax and Bak are activated and form homo-oligomeric pores in the mitochondrial outer membrane. Pore formation leads to mitochondrial outer membrane permeabilization (MOMP), which allows the release of cytochrome c into the cytosol. Cytochrome c binds to and initiates a conformation change in apoptosis initiating factor 1 (APAF-1), which establishes a heptameric apoptosome structure [43]. The apoptosome recruits and activates initiator caspase-9, which in turn activates executioner caspases-3 and -7 to carry out apoptosis (Figure 1) [42,43,51,52].

The extrinsic apoptotic pathway involves ligand activation of death receptors on the cell surface that activate initiator caspase-8. In classified type I cells, this extrinsic apoptotic signalling cascade can bypass Bcl-2 family regulation, as caspase-8 can directly cleave and activate executioner caspases-3 and -7 to carry out apoptosis [42,43,49,53,54]. In classified type II cells, the extrinsic apoptotic pathway can converge on the intrinsic apoptotic pathway via caspase-8 cleavage of the BH3-only protein BID, which can activate Bax and Bak pore formation at the mitochondria (Figure 1) [42,43,49,54-56].

Studies on the roles of Bax and Bak in apoptosis indicate their requirement for apoptotic signalling. Bax and Bak mutants that fail to oligomerize cannot initiate MOMP [57,58], and cells doubly deficient for Bax and Bak are resistant to a multitude of apoptotic stimuli (staurosporine, etoposide, ER-stress, growth factor deprivation, and UV radiation). However, single knockout of Bax or Bak leaves cells susceptible to apoptosis [59,60]. This suggests a functional redundancy in Bax and Bak function with regards to apoptosis. Despite apparent functional redundancy, these proteins still retain individuality in regards to their localization, activation, and signalling under variable cellular conditions.

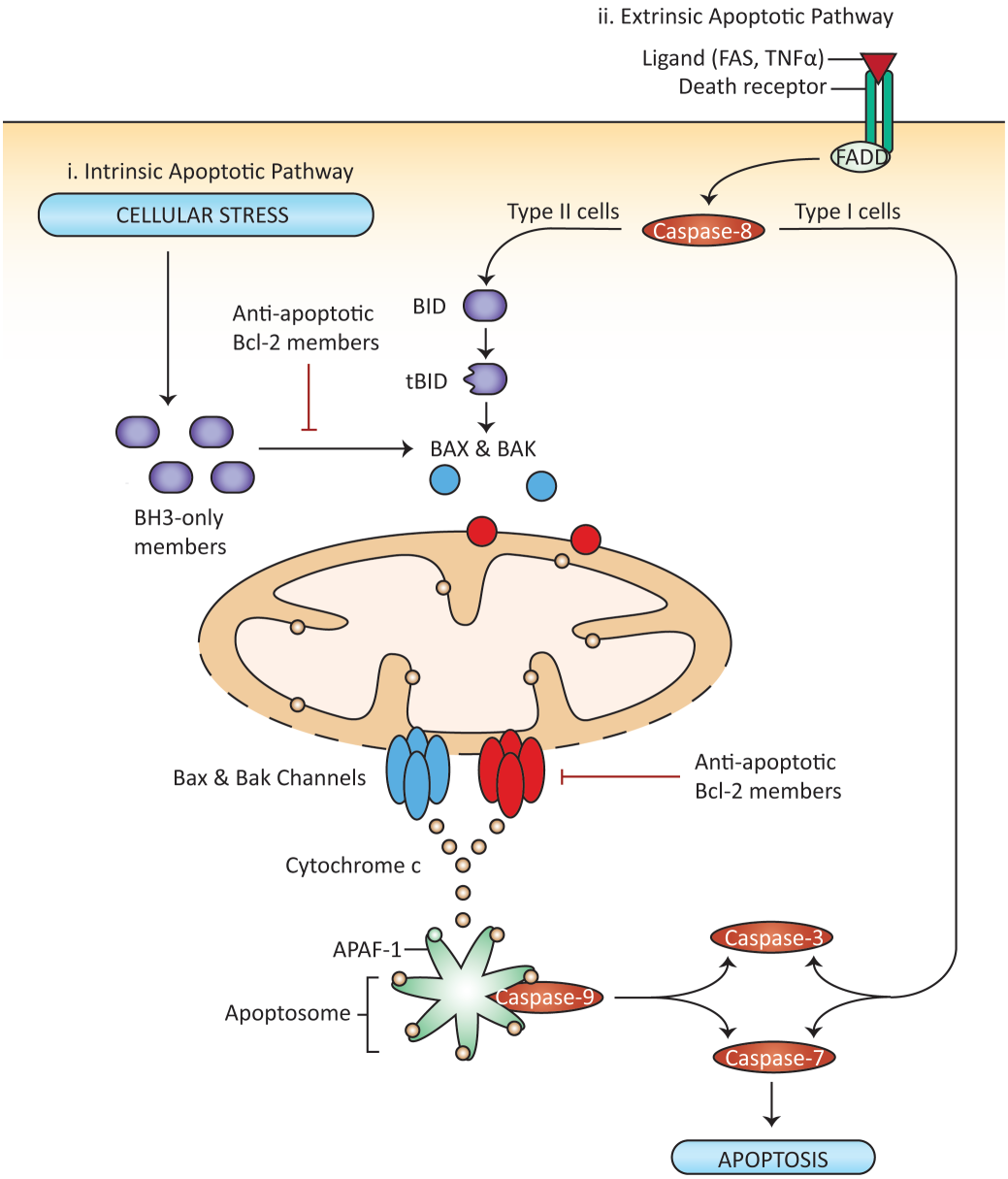

Figure 1. Intrinsic and Extrinsic Apoptotic Pathways. i. Intrinsic Apoptotic Pathway: Cellular stress leads to Bax and Bak insertion at the mitochondria after activation by pro-apoptotic BH3-only family members. Once activated, Bax and Bak form homo-oligomeric pores in the mitochondrial outer membrane leading to membrane permeabilization and the release of cytochrome c into the cytosol. Cytochrome c binds to APAF-1 to form the apoptosome that cleaves and activates initiator caspase-9, which subsequently cleaves and activates effector caspases-3 and -7 to carry out apoptosis. ii. Extrinsic Apoptotic Pathway: Death receptor activation complexes with the adaptor protein FADD to recruit caspase-8. Type I cells bypass Bcl-2 family regulation where caspase-8 directly cleaves and activates effector caspases-3 and -7. Type II cells use caspase-8 cleavage of the BH3-only protein BID to initiate the intrinsic apoptotic pathway. Anti-apoptotic Bcl-2 family members inhibit both direct and indirect pro-apoptotic activation of Bax and Bak. Black arrows, activation. Red lines, inhibition. Blue ovals, Bax. Red ovals, Bak.

1.3.2 Bax and Bak: Localization and Regulation

Pro-apoptotic proteins Bax and Bak are localized to different cellular compartments but are regulated in a similar manner by other Bcl-2 family members. Bak resides in the mitochondrial outer membrane and under apoptotic conditions Bak exposes its BH3 domain, allowing homo-dimerization with another Bak BH3 domain effectively freeing it from anti-apoptotic regulation. Alternative binding sites have been proposed to facilitate the homo-oligomerization of Bak dimers into homo-oligomeric pore formation in the mitochondrial outer membrane, but have not yet been characterized [57,61-63]. Unlike Bak, Bax is a cytosolic protein that contains a mitochondrial targeting sequence in its C-terminus. Activation of Bax requires a conformational change that exposes its C-terminal targeting sequence, freeing Bax to homo-dimerize and translocate to the mitochondria to form homo-oligomeric pores and elicit its apoptotic activity [58,64]. Bax and Bak have also been shown to localize to other membranous organelles, such as the ER and nucleus [65,66], although their roles at these organelles are less characterized.

To control their pore forming pro-apoptotic functions, Bax and Bak need to be sequestered from activation under non-apoptotic conditions and triggered to activate under severe cellular stress. This regulation is via anti-apoptotic and BH3-only Bcl-2 family members. Under non-apoptotic, physiological conditions, anti-apoptotic Bcl-2 and Bcl-xL bind to Bax, Bak, and other pro-apoptotic BH3-only family members to render them inactive [21, 22]. Under apoptotic conditions, Bax and Bak can be activated in multiple ways. When Bax and Bak outnumber Bcl-2 and Bcl-xL, they are free to oligomerize and activate MOMP at the mitochondria [42,43,67]. Pro-apoptotic BH3-only proteins can also activate Bax and Bak, with evidence supporting both a direct and indirect model. Direct activation

involves BID-like BH3-only proteins binding directly to Bax and Bak to cause a conformational change allowing them to homo-dimerize and activate. Indirect activation involves BAD-like BH3-only proteins that displace Bcl-2 and Bcl-xL from Bax and Bak, freeing them to homo-dimerize and activate [47,55,67,68].

1.4 ER-Stress and the Unfolded Protein Response

The initiation of apoptosis is well established to be irreversible once Bax and Bak are activated [21, 22]. However, the upstream signals leading to their activation and the execution of apoptosis seem to vary depending on both cell stress and cell type. In the pancreatic β-cell, the ER is highly susceptible to ER-stress caused by a variety of cellular insults. The ER is responsible for folding, modifying, and trafficking proteins to their proper cellular compartments, along with functioning as a site for the compartmentalized biosynthesis of steroids and lipids, calcium (Ca^{2+}) storage, and signalling to other cellular organelles [22,69]. Maintaining ER homeostasis is critical for the pancreatic β-cell as the ER is required for large-scale productions of the secreted hormone insulin under glucose stimulation [70]. When cellular stresses like glucotoxicity and lipotoxicity perturb ER function, misfolded proteins accumulate in the ER lumen and cause ER-stress. The unfolded protein response (UPR) is initiated as an adaptive mechanism to help the cell alleviate ER-stress. Collectively, the UPR involves coping mechanisms such as expansion of the ER, attenuation of protein translation and protein loading into the ER, upregulation of the transcription and translation of protein folding chaperones, and increasing ER-associated degradation (ERAD) to help clear the accumulated misfolded protein load within the ER lumen. When these adaptive mechanisms

fail to alleviate ER-stress, or the cell undergoes chronic ER-stress, the UPR switches from adaptive signalling to maladaptive signalling that results in apoptotic cell death [19,21,22,32,71,72].

The UPR consists of 3 transmembrane signal transducers that initiate UPR signalling and are found in all metazoan cells: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring protein 1 (IRE1) (Figure 2) [21,22]. IRE1 and PERK are type 1 transmembrane proteins that detect ER-stress via their ER luminal domains, and emit UPR signals via their cytosolic domains. In contrast, ATF6 is a b-ZIP containing type 2 transmembrane protein that detects ER-stress via its ER luminal domain, but requires transport and cleavage by site 1 and site 2 proteases in the Golgi apparatus prior to translocating to the nucleus to elicit ATF6α-mediated UPR signals [21,72]. Under physiological conditions, the 3 UPR arms are sequestered inactive via the protein chaperone BIP, which also binds misfolded proteins within the ER lumen. The accumulation of misfolded proteins during ER-stress leads to the release of BIP from the 3 UPR sensors to trigger their activation [21,22,71,72].

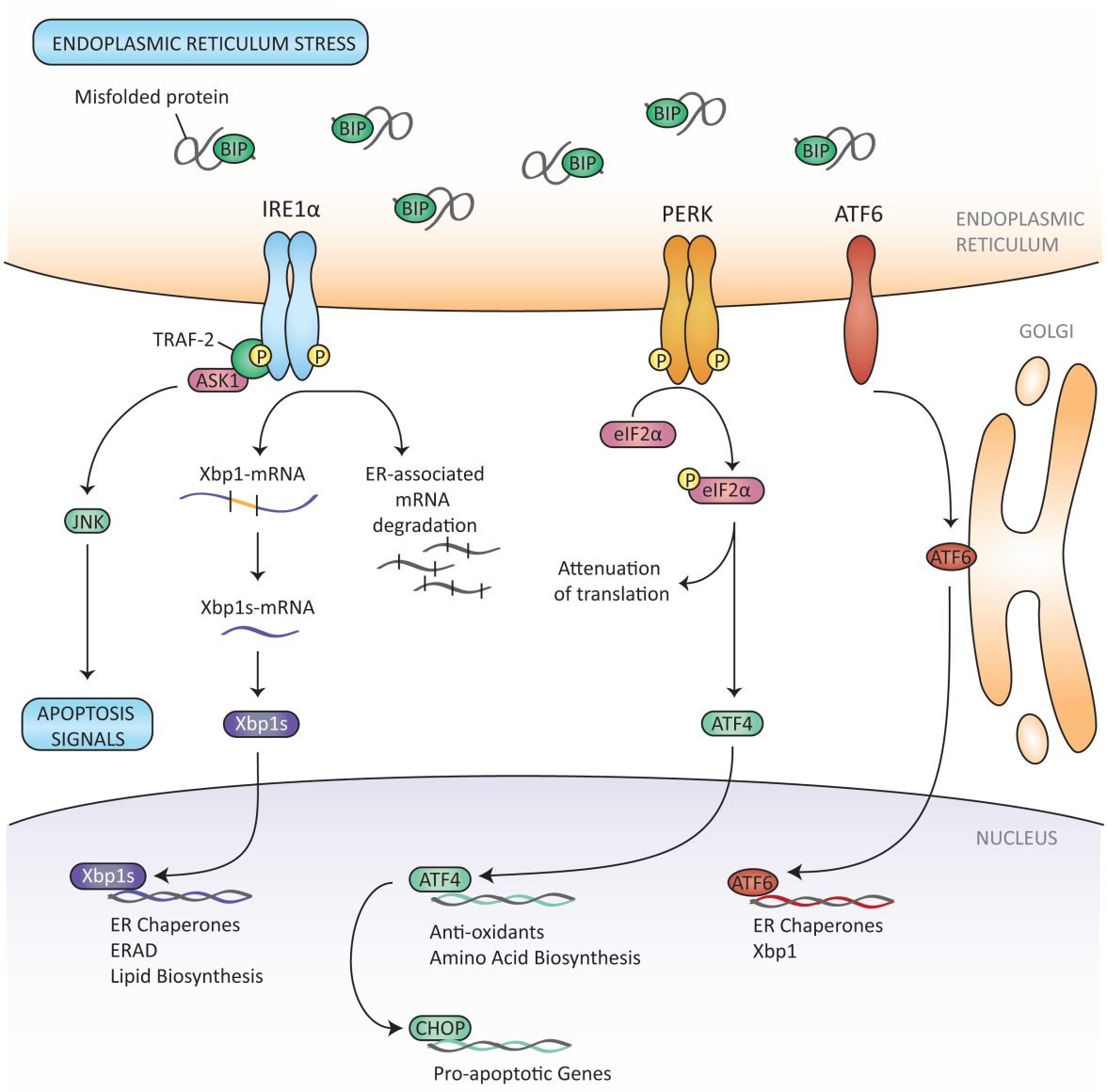

Figure 2. ER-Stress and the Unfolded Protein Response. The protein chaperone BIP preferentially binds to misfolded proteins that accumulate in the ER-lumen, releasing inhibition on the 3 UPR sensors. IRE1α and PERK activation requires dimerization and autophosphorylation. IRE1α can complex with TRAF-2 and ASK1 to initiate pro-apoptotic signaling via JNK. IRE1α uses its endoribonuclease activity to cleave ER-associated mRNAs as well as splice a 26-bp intron from XBP1 (XBP1s), which allows it to translocate to the nucleus and upregulate UPR genes. PERK activation phosphorylates eIF2α, leading to a global attenuation of translation as well as upregulation of select UPR genes via ATF4 and CHOP. ATF6 gets processed and cleaved at the golgi by site 1 and site 2 proteases, where ATF6α then translocates to the nucleus to upregulate a subset of UPR genes. Black arrows, activation. Vertical lines, mRNA cleavage. Yellow circles, phosphorylation.

1.4.1 PERK

PERK activation under ER-stress requires homo-dimerization and trans-autophosphorylation of its cytoplasmic kinase domains. PERK is a serine/threonine kinase that phosphorylates eukaryotic translation initiation factor 2 on its α subunit (eIF2α), leading to a global attenuation of mRNA translation and consequentially decreasing the protein load into the stressed ER lumen. PERK-mediated eIF2α phosphorylation is also required for selective upregulation of UPR genes. Phosphorylated eIF2α upregulates activating transcription factor 4 (ATF4), which induces expression of genes that increase amino acid biosynthesis and aid in the relief of oxidative stress [73]. ATF4 also upregulates the transcription factor C/EBP homologous protein (CHOP), a pro-apoptotic gene whose transcriptional targets include growth arrest and DNA damage-inducible protein-34 (GADD34) and ER oxidoreductase (ERO1) [22,71,74] (Figure 2). The mechanisms by which CHOP induces cell death are not fully understood; however, studies have shown that CHOP represses Bcl-2 transcription and can indirectly inhibit Bcl-2 via increasing transcription of the pro-apoptotic BH3-only protein BIM [21,71,75-77]. CHOP also acts to deplete cellular glutathione required for proper disulfide bond formation in the ER, exaggerating the production of ROS, which leads to oxidative activation of ER Ca^{2+} release onto the mitochondria, and decreases eIF2α phosphorylation thereby increasing protein loading into the ER and further exaggerating ER-stress [22,78-80]. CHOP deletion protects against ER-stress induced cell death, indicating an essential role for CHOP in ER-stress induced apoptosis [74,81]. On the other hand, evidence for the importance of PERK signalling in proper endocrine pancreas function comes from PERK knockout mice. PERK knockout mice display increased pro-insulin accumulation in the β-cell and quickly develop diabetes due to low β-cell mass.

PERK knockout mice also display chronic ER-stress with hyperactivation of the endoribonuclease IRE1α, indicating PERK is required to keep the translational load on the ER in check under normal physiological conditions in secretory cells [73,82].

1.4.2 ATF6

Unlike PERK and IRE1 that transmit UPR signals from the ER to the nucleus, ATF6α itself is a transcription factor that upregulates a subset of ER chaperones [83,84] and assists in ERAD in combination with X-box binding protein 1 (XBP1) [85]. ATF6α knockout mice show no defect in β-cell function under physiological conditions, indicating overlapping roles between ATF6 and XBP1 in regulating ER homeostasis. However, high-fat diet challenged ATF6 knockout mice display reduced glucose tolerance and an inability to cope with increasing ER-stress, indicating a requirement for ATF6α in adaptive UPR signalling [86].

1.4.3 IRE1α

IRE1 is the most evolutionarily conserved and highly studied arm of the UPR. Originally discovered in yeast, IRE1 has two mammalian isoforms. IRE1α is found in most tissues and is highly abundant in the pancreas, while IRE1β is mainly found in gastrointestinal epithelial cells [22,87]. IRE1α is a unique kinase/endoribonuclease, as the only known substrate for IRE1α phosphorylation is IRE1α itself. IRE1α activation leads to homo-dimerization and trans-autophosphorylation of its cytosolic kinase domains, along with activation of its endoribonuclease activity. Activated IRE1α splices a 26-bp intron from XBP1 mRNA, inducing a translational frameshift and production of the transcription factor

spliced XBP1 (XBP1s). XBP1s can enter the nucleus and upregulate transcription of ER chaperones required to assist in protein folding, as well as UPR genes involved with ERAD and lipid biosynthesis. The endoribonuclease activity of IRE1α has also been shown to degrade a variety of ER-associated mRNAs in response to ER-stress (Figure 2) [88,89]. In mouse islets, chronic hyperglycemia increases IRE1α activation and phosphorylation that leads to IRE1α mediated degradation of insulin 1 and insulin 2 mRNA [90]. In contrast, IRE1α activation under physiological or transient high glucose stimulation will increase pro-insulin biosynthesis without further splicing XBP1 or activating JNK signalling, indicating a beneficial role for IRE1α in pancreatic β-cell function [91]. The IRE1α-XBP1 pathway is crucial for properly regulated insulin biosynthesis and processing in pancreatic β-cells. This is illustrated by the fact that IRE1 conditional knockout mice display hypoinsulinemia and hyperglycemia [82,92], and XBP1s β-cell conditional knockout mice are glucose intolerant due to low insulin content and an impairment in insulin processing [93]. XBP1s knockout also leads to IRE1α hyperactivation, indicating a regulatory negative feedback loop of XBP1s on IRE1α activation [93]. Chronic IRE1α activation can signal towards apoptosis in combination with CHOP signalling in the PERK pathway. Along with its endoribonuclease activity, phosphorylated IRE1α can complex with TNF receptor associated factor 2 (TRAF-2) and apoptosis signal regulating kinase 1 (ASK1) to recruit and activate stress-induced c-Jun N-terminal kinase (JNK), which complexes with Bcl-2 family cell-death machinery and leads to apoptosis (Figure 2) [21,22,71,94,95]. JNK phosphorylates both Bcl-2 and BIM releasing anti-apoptotic inhibition on Bax and Bak to activate pro-apoptotic signalling [85,96,97]. Taken together, IRE1α activation leads to a multitude of effects depending on the physiological state of the cell. For the pancreatic β-cell, the IRE1α-XBP1 pathway is critical for proper

insulin production and maintenance of cellular homeostasis. However, IRE1α is also capable of signalling apoptosis once ER-stress becomes unmanageable.

1.5 Potential Roles for Bax and Bak in ER Physiology and Stress Signalling

1.5.1 Bax and Bak Regulation of IRE1α Signalling

Bcl-2 protein family members are well known as regulators of cell survival and apoptosis. It has been established that Bcl-2 proteins localize to the ER and other membranous organelles in addition to the mitochondria [66,98]. Recently, focus has shifted towards elucidating alternative roles for the Bcl-2 proteins in regulating aspects of cellular physiology and stress signalling at the ER. Bax and Bak double knockout (DKO) studies have provided evidence for Bax and Bak in regulating the IRE1α-mediated ER-stress response [65]. Conditional liver BaxBak DKO resulted in less XBP1 splicing and decreased expression of downstream XBP1s target genes in response to *in vivo* induction of ER-stress by tunicamycin injection. Decreased JNK activation and cellular apoptosis were also observed in BaxBak DKO livers, indicating Bax and Bak promote the activation of IRE1α in hepatocytes under ER-stress conditions. Co-immunoprecipitiation experiments revealed direct binding of Bax and Bak with the C-terminus of IRE1α, requiring their BH1 and BH3 functional domains, further confirming an IRE1α-Bax/Bak signalling complex within hepatocytes. Interestingly, Bax and Bak did not seem to indiscriminately regulate the UPR. PERK-mediated signalling was similar between WT and BaxBak DKO cells, suggesting targeted roles for Bax and Bak in IRE1α regulation [99,100]. This study was the first to provide

evidence for potential non-apoptotic roles of Bax and Bak in regulating adaptive UPR signalling in response to ER-stress.

1.5.2 Bax and Bak at the ER: Cellular Physiology

In addition to their reported roles in UPR regulation, there is evidence for Bax and Bak regulation of ER function under physiological conditions. In the ER lumen, optimal Ca^{2+} concentration is required for proper protein folding and chaperone function. Maintenance of steady-state ER Ca^{2+} concentration is achieved by the Ca^{2+} release channels inositol trisphosphate receptors (IP3Rs) and ryanodine receptors (RYRs), along with the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump that acts to restore luminal ER Ca^{2+} stores [101-103]. Dysregulation of ER Ca^{2+} leads to an accumulation of improperly folded proteins and activation of ER-stress. The ER is also responsible for Ca^{2+}-induced apoptosis when a heightened ER Ca^{2+} load is transferred to the mitochondria [16,72,104,105]. Multiple studies have shown that a balance between the pro- and anti-apoptotic Bcl-2 proteins plays a role in regulating a steady-state ER Ca^{2+} concentration. Pro-apoptotic Bax and Bak promote an increase in ER Ca^{2+} loading by opposing the actions of anti-apoptotic Bcl-2 and Bcl-xL [106], which increase passive IP3R-mediated ER Ca^{2+} leak [107]. Decreased ER Ca^{2+} loading in BaxBak DKO mouse embryonic fibroblasts (MEFs) resulted in decreased mitochondrial Ca^{2+} uptake from stress-induced IP3R mediated ER Ca^{2+} release, thereby decreasing cell death initiated by Ca^{2+} dependent apoptotic stimuli [108-111]. Bax and Bak were also recently shown to induce membrane permeability and cause the release of ER-luminal proteins in response to ER-stress, much like their pore-forming functions at the mitochondria [112]. These studies

suggest that Bax and Bak may have roles in sensitizing the cell to apoptotic stimuli upstream of their roles in executing apoptosis at the mitochondria.

1.5.3 Bax and Bak at the ER: A Link Between ER-Stress and Apoptosis

As increasing evidence suggests non-apoptotic functions for Bax and Bak in regulating ER physiology, Bax and Bak are still a central node of cell death regulation and provide a link between ER-stress and apoptosis. It is evident that apoptosis can be induced from unresolved ER-stress; however, the mechanisms that control ER-stress induced apoptosis seem to be dependent on both the type of stress and the cell type that experiences the stress. Currently, there is evidence for three ER-stress-induced apoptotic pathways that require Bax and Bak [74]. The first two have been discussed above; the CHOP and JNK mediated apoptosis signals. CHOP expression downregulates anti-apoptotic Bcl-2 and upregulates pro-apoptotic BIM [75]. Combined, these events ultimately lead to the activation of Bax and Bak and apoptosis. Bax and Bak have been shown to accentuate IRE1α activation leading to JNK phosphorylation and subsequent regulation of Bcl-2 proteins to converge on apoptosis signalling [99]. The third ER-stress-induced apoptotic pathway converges on the activation of caspase-12. Evidence for Bax and Bak in caspase-12 mediated apoptosis comes from stressed BaxBak DKO MEFs that cannot cleave and activate caspase-12, likely due to a reduction in ER-derived Ca^{2+} signals, which are thought to indirectly activate caspase-12 through calpains [21,74,113,114]. However, the importance of caspase-12 mediated apoptosis is still under debate as human caspase-12 is truncated and non-functional [115]. The exact signalling mechanisms leading to ER-stress-induced apoptosis still remain relatively unresolved, partly because many of the pathways and specific mechanisms appear to often be

cell-type and stress dependent. Thus, it is imperative that cell stress and cell failure should be studied with relevant cell stress in the cell-type of interest. Because of this, our work on Bax and Bak in ER-stress and apoptosis uses a primary mouse β-cell model and diabetogenic-like cell stress to overcome discrepancies we would potentially see using other cell types or highly proliferative and death-resistant β-cell-like cell lines.

1.6 Known Roles for Bax and Bak in β-Cell Death

To date, very little has been studied on the roles of Bax and Bak within the pancreatic β-cell. Loss of function studies have been limited to single Bax or Bak knockout islet experiments focusing only on the outcome of β-cell death. Islets from Bax null ($Bax^{-/-}$) and Bak null ($Bak^{-/-}$) mice each display reduced cell death in response to cytokines and stimulators of the extrinsic apoptotic pathway (FAS, TNFα). This death also depended on the presence of BID, indicating β-cells can be classified as type II cells that signal the extrinsic apoptotic pathway through cleavage of BID and induction of pro-apoptotic Bax and Bak at the mitochondria (Figure 1) [116]. Under more diabetogenic conditions, glucotoxicity was recently shown to induce cell death via the intrinsic apoptotic pathway. Interestingly $Bax^{-/-}$ islets, but not $Bak^{-/-}$ islets, displayed partial protection against glucotoxicity-induced cell death, providing evidence for potentially stress and/or cell specific roles for Bax and Bak in the adult β-cell [53]. Islets from $Bax^{-/-}$ mice have also been shown to display a slight resistance to glucolipotoxicity-induced apoptosis [117].

It is apparent that various types of cell stressors cause different signalling cascades via their own unique mechanisms. However, it is evident that a majority of cell death signals ultimately converge on Bax and Bak. Originally, these proteins were thought to have

redundant roles regulating cell death signalling, but recent evidence by Hetz *et al.* (2006) and McKenzie *et al*. (2010) suggests that Bax and Bak may also regulate ER-stress signalling upstream of apoptosis and have stress-specific roles in mediating cell death. To study the combined and individual roles of Bax and Bak within the adult β-cell, we required a mouse model in which Bax and/or Bak could be knocked out of pancreatic β-cells.

1.7 Bax-Bak Knockout Models

Programmed cell death is not exclusive to post-natal clearing of dysfunctional or dying cells, it is also a critical event for proper fetal development. Disruption or elimination of the apoptotic pathways during development can lead to severe developmental abnormalities that usually result in premature death. Bax and Bak genetic knockout studies established that these two proteins may have redundant roles in regulating apoptosis during development [118]. $Bak^{-/-}$ mice were shown to have no developmental abnormalities and normal reproductive capabilities. $Bax^{-/-}$ mice were also viable, but had a few non life-threatening abnormalities and male mice were sterile due to a defect in spermatogenesis [118]. However, most global BaxBak DKO mice died shortly after birth. BaxBak DKO mice that did survive to adulthood displayed severe developmental abnormalities such as retained interdigital webs, imperforate vaginas on the females, neurological and behavioural abnormalities, enlarged spleen and thymus tissue accompanied by increased hematopoietic components, and isolated cultured cells showed resistance to many forms of cell death stimuli [118]. Taken together it seems that at least one of the two proteins is required for proper development, and that Bax and Bak have seemingly redundant roles during the apoptosis needed for proper

fetal development. On the other hand, these studies provided insight that knocking both Bax and Bak out of a cell could be beneficial for cell survival.

The severe phenotype of BaxBak DKO mice has made study of these two proteins in an adult or tissue specific model difficult. Most global BaxBak DKO studies have been limited to MEFs [59] and Chinese hamster ovary (CHO) cells [60]. While MEFs provide a good starting point for studying these proteins within the cell, we cannot, with confidence, extrapolate evidence from MEF studies to other tissue cell types. Advances in molecular genetic technology have provided a means to investigate tissue specific and/or inducible knockout models, bypassing the side effects that accompany a global knockout model. Here we have made use of the inducible and tissue specific model of gene knockout to investigate the combined and individual roles of Bax and Bak within the pancreatic β-cell.

1.8 Cre-*lox* Technology and the Conditional Bax-Bak Knockout Model

The advent of Cre-*lox* technology has significantly improved the temporal and spatial control of gene knockout in animal models. The 38-kDa, P1-phage-derived Cre recombinase can be used as a site-directed genetic modification tool to excise any sequence located between two floxed (34 bp *loxP* sequence-flanked) target sites [119-121]. The Cre system can be placed under the control of any tissue- or cell-specific promoter, leading to tissue-specific excision of DNA as opposed to the global knockout of DNA seen with regular genetic knockout models [119,122]. The downside to the first generation Cre system is that site-specific knockout occurs from when the accompanying promoter becomes active. It is not a suitable system if the study of a gene of interest requires controlled temporal inactivation. A gene may have different roles during various embryonic development stages and during adult cell

function. Effective study of the roles of a specific gene in the adult requires temporal- and tissue-specific knockout of the gene of interest [120,122].

To address this issue, a second generation Cre-ER™ technology was developed by multiple groups, where the gene encoding the Cre enzyme was fused to a mutant form of the estrogen steroid receptor [123-125]. The mutant estrogen receptor fails to significantly bind the naturally occurring mammalian ligand 17β-estradiol, but binds with high affinity the synthetic ligand tamoxifen [120,125]. The Cre-ER™ is sequestered inactive in the cytosol by heat shock protein 90 (HSP90) until ligand binding by tamoxifen. This induces a conformational change in the mutant estrogen receptor allowing activation and nuclear translocation of the fusion protein, which facilitates nuclear Cre enzymatic excision of the floxed DNA of interest [120,126,127]. Thus, this system allows for chemically induced tissue and temporal regulation of controlled gene knockout (Figure 3).

To study the roles of Bax and Bak in the adult β-cell, our knockout model makes use of the tissue- and temporal-specific Cre-ER™ technology in the pancreatic β-cells developed by Gu *et al.* (2002) and Zhang *et al.* (2005), where Cre-ER™ expression is under control of the Pdx1-promoter activity (Pdx1-CreER). Bax flox mice ($Bax^{flox/flox}$) were previously generated to have loxP sites flanking exons 2-4, regions containing the BH3 domain required for proper Bax function [128]. These mice were bred onto $Bak^{-/-}$ mice [118] and were available from Jackson Laboratories as double homozygous $Bax^{flox/flox}$ $Bak^{-/-}$ mice (Figure 4). We bred Pdx1-CreER mice, generously provided by Dr. Doug Melton's lab (Harvard University, Cambridge, MA, USA), onto homozygous $Bax^{flox/flox}$ $Bak^{-/-}$ mice to allow for temporal and spatial control over the Bax knockout in adult pancreatic β-cells. This model allows us to bypass the detrimental developmental defects seen in the global BaxBak DKO mouse.

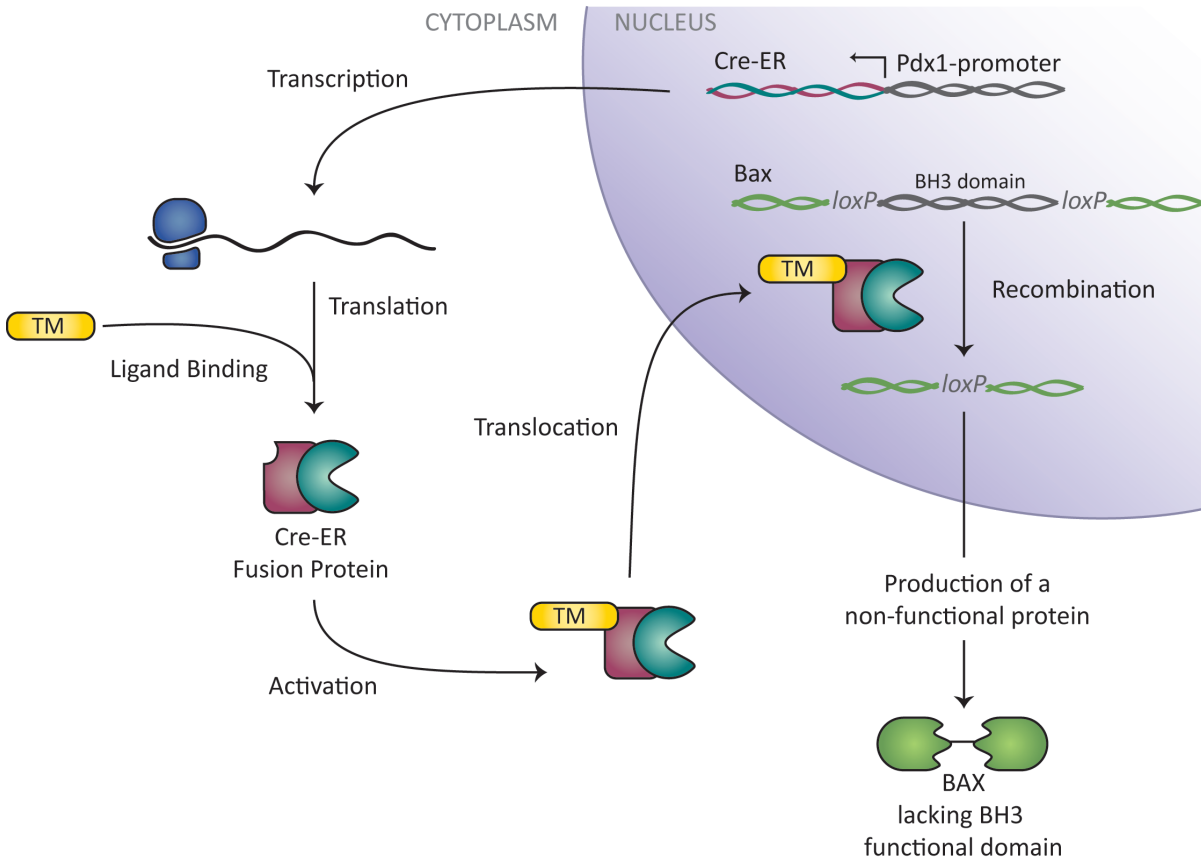

Figure 3. Tissue-Specific Cre Recombinase Excision of the Bax Gene. In the pancreatic β-cell, the Pdx1-promoter drives expression of the Cre-ER (Cre recombinase-mutant estrogen receptor) fusion protein. The mutant estrogen receptor binds the synthetic ligand tamoxifen (TM), which activates and allows translocation of Cre-ER from the cytosol into the nucleus. In the nucleus, Cre-ER recombines DNA between two *loxP* sites, excising the genetic material of interest. As an example, two *loxP* sites flank exons 2-4 of the Bax gene, the region that contains the functional BH3 domain. After Cre-ER recombination, a non-functional Bax protein is produced. TM, tamoxifen.

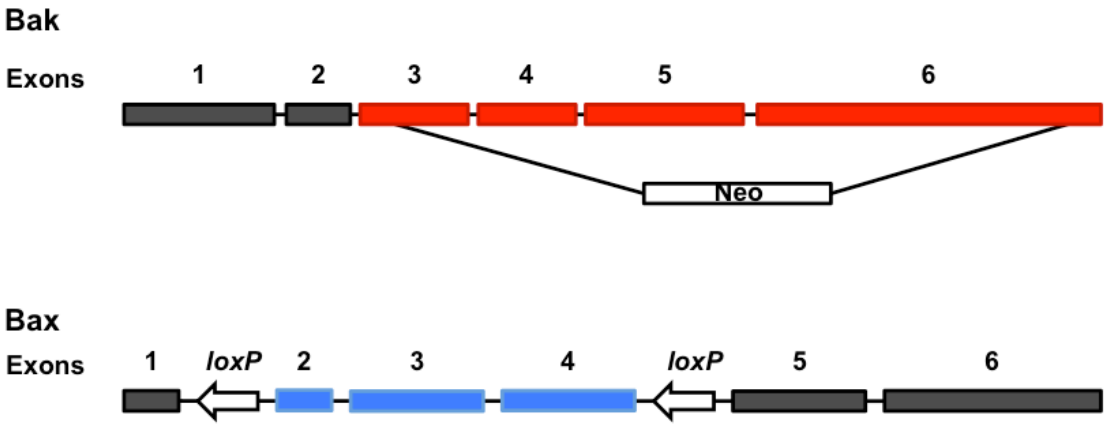

Figure 4. Bax and Bak Genetic Modifications. Schematic diagram depicting the previously generated genetic modifications to the Bak and Bax genes. Bak exons 3-6 were replaced with a neomycin resistance cassette (Neo), effectively removing the functional BH3 domain. Bax exons 2-4 were flanked with two *loxP* sites, containing the functional BH3 domain. Upon tamoxifen administration in the presence of Pdx1-CreER, exons 2-4 are excised resulting in a non-functional Bax protein.

1.9 Investigating the Roles for Bax and Bak in the Pancreatic β-Cell: Stress and Death

The present study addressed two simultaneous aims using our Bax and Bak knockout mouse model. The first aim was to investigate the combined and individual roles for Bax and Bak in β-cell death by subjecting isolated and dispersed pancreatic islet-cells to various diabetogenic stress conditions and examining the overall affect of Bax and Bak knockout on islet-cell death. Our second aim was to determine if Bax and Bak have roles in ER-stress signalling upstream of apoptosis activation by assaying UPR induction in the β-cell under ER-stress conditions. By means of our novel double knockout model, our results added to the available evidence that Bax and Bak have both individual and combined roles in mediating β-cell death under various forms of cell stress and also reveal novel roles for Bax and Bak in β-cell ER-stress regulation.

Together, with the (limited) existing work done specifically in pancreatic β-cells, our results clearly show that Bax and Bak are at the forefront of β-cell failure and cell death under a wide variety of cell stressors. Given that apoptosis-mediated loss of β-cells is a critical factor in the pathogenesis of diabetes, understanding this process may improve prevention, therapies, and even cures for diabetes. By elucidating the roles of Bax and Bak in β-cell stress signalling and death, we may better understand the mechanisms by which β-cells die in the diabetic milieu allowing future development of targeted therapeutic agents that potently promote β-cell function and survival.

Chapter 2: Materials and Methods

2.1 Reagents

InSolution™ staurosporine, *streptomyces* was from Calbiochem (EMD Millipore 569396, Billerica, MA, USA). Dimethyl sulfoxide (DMSO, D2650), thapsigargin (Tg, T9033), palmitic acid (PA, P5585), propidium iodide (PI, P4864), tetramethylrhodamine ethyl ester perchlorate (TMRE, 87917), collagenase type XI from *clostridium histolyticum* (C7657), and tamoxifen (TM, T5648) were from Sigma-Aldrich (St. Louis, MO, USA). Fura-2/AM (F-1221) and hoechst 33342 (H3570) were from Invitrogen/Life Technologies (Burlington, ON, CA).

2.2 *In Vivo* Studies

2.2.1 Breeding

Two parallel sister lines of mice were bred to create the 4 genotypes of mice used for these studies. $Bax^{flox/flox}$ $Bak^{-/-}$ mice (Jax stock number 006329, B6:129 genetic background) were bred onto Pdx1-CreER mice (provided by Dr. Doug Melton, Harvard University, Cambridge, MA, USA, CD-1 genetic background) [129] to create the Bak single knockout (Bak SKO) and BaxBak double knockout (BaxBak DKO) littermate mice. When breeding the Pdx1-CreER mice onto the $Bax^{flox/flox}$ $Bak^{-/-}$ mice, we also reintroduced the Bak wild-type allele in some offspring, which we used to establish the sister line of Bax single knockout (Bax SKO) and wild-type (WT) littermate mice (Figure 5).

2.2.2 Tamoxifen Administration for Inducible Cre Recombination

Prior to any *in vitro* or *in vivo* studies, freshly made tamoxifen was administered by intraperitoneal injection to all mice used for experiments, both Cre-positive (Bax SKO and BaxBak DKO) and Cre-negative (Bak SKO and WT). Tamoxifen was dissolved in warmed corn oil (Sigma-Aldrich, C8267) to 10 mg/mL followed by filter sterilization (Millipore Steriflip 0.22 μm polyethersulfone membrane, SCGP00525), and stored at 4°C for up to one week. Tamoxifen was administered at room temperature via intraperitoneal injection (3 mg/40 g body weight) for four consecutive days, alternating between left- and right-side injection sites daily.

2.2.3 Genotyping

Ear notches were taken from newly weaned mice (19-21 days old) to provide identification to each mouse and to use as a tissue sample for genotyping. Ear notches were individually placed in a 1.5 mL microcentrifuge tube (VWR, Mississauga, ON, CA, 87093-294) and digested at 55°C overnight in cell lysis solution (Qiagen, Toronto, ON, CA, 158906) containing the proteinase inhibitor proteinase K (New England Biolabs, Ipswich, MA, USA, P8102S). Protein precipitation solution was added to each tube of digested tissue (Qiagen, 158910), spun down for 3 minutes at 14,000 x g, and supernatant was transferred to a clean 1.5 mL microcentrifuge tube containing 100% isopropanol. The supernatant/isopropanol mix was centrifuged for 7 minutes at 14,000 x g to precipitate DNA, then washed with 70% ethanol. DNA was resuspended in DNase and RNAse free water at 65°C, and stored at -20°C. Polymerase chain reaction (PCR) was performed on each tube of isolated DNA for three genes: Bax, Bak, and Pdx1-CreER (Cre). The PCR mix contained

Crimson Taq polymerase and Crimson Taq buffer (NEB, M0324S), dNTP mix (Fermentas, Thermo Scientific, Waltham, MA, USA), primers for each individual gene (Table 1), DNAse and RNAse free water, and the isolated DNA sample. PCR reactions were run on an Applied Biosystems thermocycler (Life Technologies, Grand Island, NY, USA) for 30 cycles following the Crimson Taq polymerase thermocycling protocol. Amplified DNA was run through a 2% agarose gel (UltraPure Agarose, Invitrogen/Life Technologies, 16500500) containing SYBR Safe DNA Gel Stain (Invitrogen/Life Technologies S33102), and amplified bands were imaged on various Gel Doc Systems (Figure 6A).

Table 1. Genotyping Primer Sequences

Gene	Primer	Sequence
Bax	Forward	5'-GAATGCCAAAAGCAAACAGACC-3'
	Reverse	5'-ACTAGGCCCGGTCCAAGAAC-3'
	Vector	5'-CCACTCCCACTGTCCTTTCC-3'
Bak	Forward	5'-GGCTCTTCACCCCTTACATCAG-3'
	Reverse	5'-GTTTAGCGGGCCTGGCAACG-3'
	Vector	5'-GCAGCGCATCGCCTTCTATC-3'
Cre	Forward (Cre)	5'-AACCTGGAAGTGAAACAGGGGC-3'
	Reverse (Cre)	5'-TTCCATGGAGCGAACGACGAGACC-3'
	Forward (Control)	5'-CAAATGTTGCTTGTCTGGTG-3'
	Reverse (Control)	5'-GTCAGTCGAGTGCACAGTTT-3'

2.2.4 Intraperitoneal Glucose Tolerance Tests

Intraperitoneal glucose tolerance tests were performed after a 6 hr fast. Tail vein blood droplets were sampled 0, 15, 30, 60, 90, and 120 minutes after intraperitoneal injection of an 18% glucose in saline (0.9% NaCl) solution (2 g/kg body weight, Sigma-Aldrich, G8270). Blood glucose concentrations were read with OneTouch Ultra Blue Test Strips (Lifescan, Burnaby, BC, CA) on a glucometer (various brands).

2.3 *In Vitro* Studies

2.3.1 Islet Isolation and Cell Culture

After surgical exposure of the mouse pancreas, the bile duct was ligated at the duodenum and perfused with 3-5mL of collagenase (1000 U/mL) dissolved in 1x HBSS (with 5.5 mM D-Glucose and without Ca^{2+}, Mg^{2+}, phenol red, and sodium bicarbonate. 14185-052, Gibco, Life Technologies). The inflated pancreas was extracted, placed in a 50 mL conical centrifuge tube (BD Falcon, Franklin Lakes, NJ, USA) containing 2 mL of the collagenase solution, and then digested for 9-11 minutes in a 37°C waterbath (digestion time optimized per collagenase lot). Following digestion, the tube containing the pancreas was vigorously shaken to homogenize the tissue. The homogenized tissue was washed with 1x HBSS containing 1 mM $CaCl_2$, followed by centrifugation for 30 seconds at 1200 rpm. The supernatant was discarded and the wash and spin steps were repeated twice. Isolated islets were resuspended in 1x HBSS + $CaCl_2$, filtered through a pre-wet 70 μm nylon filter (BD Biosciences, 352350, San Jose, CA, USA), and washed with 1x HBSS + $CaCl_2$. Filtered islets were rinsed into a 100 mm petri dish with culture media, 11.1 mM glucose RPMI 1640 (Gibco, Life Technologies, 11875) containing 10% fetal bovine serum (FBS, Gibco, Life Technologies, 12483), and 2% penicillin-streptomycin (Gibco, Life Technologies, 15140). Islets were counted as they were hand-picked to remove >95% acinar cell contamination into 60 mm petri dishes containing clean culture media. Islets were cultured at 37°C under 5% CO_2 overnight before any experimental procedures commenced.

2.3.2 Single-Cell Dispersion

Isolated islets were dispersed into single cells using 0.05% trypsin-EDTA (Gibco, Life Technologies, 25300) in Ca^{2+} and Mg^{2+} free Eagle's minimum essential media (MEM) without L-glutamine (Mediatech, 15-015-CV, Manassas, VA, USA). Intact islets were collected into a 1.5 mL low retention, sterile, microcentrifuge tube (Fisher Scientific, 02-681-331, Waltham, MA, USA) and washed three times with MEM. Islets were then dispersed in a 1/5 dilution of trypsin-EDTA in MEM by pipetting up and down 30-50 times. The resulting cell suspension was spun down, and resuspended in 11.1 mM glucose RPMI 1640 complete with 10% FBS and 2% penicillin-streptomycin.

2.3.3 *In Vitro* Stress Treatments

Islets were isolated from the mouse pancreas as described above and cultured overnight prior to the addition stress treatments. For quantifications of cell death and changes in mitochondrial membrane potential, the stress and control treatments were added to dispersed islet cells, which were then studied as described below. For quantitative PCR analysis of gene expression, whole islets were placed in 35 mm or 60 mm petri dishes with an appropriate volume of treatment or control media for the time-course indicated. Thapsigargin (1 mM stock in DMSO) and staurosporine (1 mM stock in DMSO) were diluted in 11.1 mM glucose RPMI 1640, complete with 10% FBS and 2% penicillin-streptomycin, to the desired final concentrations. An equal volume of DMSO was used as a vehicle control for all thapsigargin and staurosporine treatments. For experiments involving lipotoxic treatments, palmitate media was prepared fresh before each study. A 20 mM stock solution of palmitate was created by dissolving palmitic acid in 0.03 M NaOH. In either 5

mM glucose or 25 mM glucose RPMI 1640 complete with 10% FBS and 2% penicillin-streptomycin, the 20 mM stock palmitate solution was used to prepare a 1500 μM palmitate solution in complex with 20% fatty-acid free bovine serum albumin (BSA, Sigma-Aldrich, A7030) at a 6:1 Palmitate:BSA ratio. An equal volume of 0.03 M NaOH was used in place of the 20 mM palmitate volume for vehicle control. Both control and palmitate media were sterile filtered prior to use.

2.3.4 Cell Death Quantification

Islets were dispersed into single cells as described above and seeded into 96-well flat bottom plates (Perkin Elmer ViewPlates, 6005182, Waltham, MA, USA) for 48 hours in 11.1 mM glucose RPMI 1640 complete with 10% FBS and 2% penicillin-streptomycin. Single cells from 25-30 islets were seeded into each well. Cells were stained with PI (0.5 μg/ml) and Hoechst 33342 (0.05 μg/ml) for 30 minutes before the various stress or control treatments were added, as described above. The 96-well plates were placed in an environmentally controlled (37°C, 5% CO_2) Molecular Devices ImageXpress Micro high content screening system (Molecular Devices, Sunnyvale, CA, USA) for the duration of the imaging experiment. Images of PI and Hoescht positive cells were taken at one hour intervals for up to a 72 hour time-course and the percentage of dead cells was determined as the number of PI positively stained (dead) cells relative to the number of Hoechst positive (total) cells, as analyzed by the MetaXpress software.

2.3.5 Mitochondrial Membrane Potential

Islets were dispersed into single cells as described above and seeded onto glass bottom microwell dishes (MatTek, P35G-1.5-20-C, Ashland ,MA, USA) for 48 hrs in 11.1 mM glucose RPMI 1640 complete with 10% FBS and 2% penicillin-streptomycin prior to stress treatment and imaging. Cells were then treated with new media supplemented with 1 μM staurosporine (STS) or an equal volume of DMSO as vehicle control for 24 hours. Following treatment, cells were loaded for 30 minutes with TMRE (50 nM) dissolved in DMSO, and Hoechst 33342 (0.05 ug/mL) in 11.1 mM glucose RPMI 1640 without phenol red (Gibco, Life Technologies, 11835). Mitochondrial membrane potential was assessed as the absolute TMRE fluorescence intensity by imaging a representative population of cells at 20x magnification on a Zeiss Axiovert 200 M inverted microscope using the MiCy fret-cube and coral orange emission filter. Images were analyzed using Slidebook software (3i, Denver, CO, USA).

2.3.6 Real-Time PCR Quantification of Gene Expression

Stress- and control-treated islets were collected at the indicated time-point in a 1.5 mL microcentrifuge tube (VWR, 87093-294). The media was removed and 25 μL of RNA*later* RNA Stabilization Reagent (Qiagen, 76104) was added prior to storing collected islets at -80°C. Collected islets were later homogenized using a needle and syringe technique with lysis buffer containing β-mercaptoethanol (Sigma-Aldrich, M3148). Total islet mRNA was extracted using the RNEasy Mini Kit (Qiagen, 74106) according to the manufacturer's protocol. Following mRNA isolation, cDNA was synthesized by reverse transcription using 100 ng RNA and the qScript cDNA synthesis kit (Quanta Biosciences, VWR, 95047-500),

followed by a 1:3 dilution with DNAse and RNAse free water. Target gene expression was measured using quantitative PCR (qPCR) relative to mouse β-Actin housekeeping gene on an Applied Biosystems StepOnePlus and Applied Biosystems 7500 Fast Real-Time qPCR machines (Life Technologies). All assayed genes were amplified for 35 cycles using the PerfeCTa SYBR Green SuperMix plus ROX (Quanta Biosciences, VWR, 95055) with primers synthesized from IDT (Toronto, ON, CA) (Table 2). All primers were tested for specificity to the gene of interest using NCBI blast and validating for a single amplification product using melt curve and agarose gel analyses. qPCR efficiencies were tested using a serial dilution relative standard curve method. For all primer sets examined (Table 2), efficiencies were comparable and ranged between 85-105%.

Table 2. Quantitative PCR Gene Expression Primer Sequences

Gene	Primer	Sequence
ACTIN	Forward	5'-GATCTGGCACCACACCTTCT-3'
	Reverse	5'-GGGGTGTTGAAGGTCTCAAA-3'
BAK	Forward	5'-GGAGCAGCTTGGGAGCG-3'
	Reverse	5'-AAAAGGCCCCTGTCTTCATGA-3'
BAX	Forward	5'-CTTTGGCTACCGTCTGGC-3'
	Reverse	5'-GGAGGAAGGAGAGGACTGTTG-3'
CHOP	Forward	5'-CTGCCTTTCACCTTGGAGAC-3'
	Reverse	5'-CGTTTCCTGGGGATGAGATA-3'
XBP1s	Forward	5'-GAGTCCGCAGCAGGTG-3'
	Reverse	5'-GTGTCAGAGTCCATGGGA-3'
BIP	Forward	5'-TCATCGGACGCACTTGGAA-3'
	Reverse	5'-CAACCACCTTGAATGGCAAGA-3'
ERO1L	Forward	5'-TCAGTGGACCAAGCATGATGA-3'
	Reverse	5'-TCCACATACTCAGCATCGGG-3'
EDEM	Forward	5'-AAGCCCTCTGGAACTTGCG-3'
	Reverse	5'-AACCCAATGGCCTGTCTGG-3'
ERDJ4	Forward	5'-TAAAAGCCCTGATGCTGAAGC-3'
	Reverse	5'-TCCGACTATTGGCATCCGA-3'
INSULIN 1	Forward	5'-GAAGTGGAGGACCCACAAGTG-3'
	Reverse	5'-ATCCACAATGCCACGCTTCT-3'
INSULIN 2	Forward	5'-GAAGTGGAGGACCCACAAGTG-3'
	Reverse	5'-GATCTACAATGCCACGCTTCT-3'

2.3.7 Insulin Content/Insulin Secretion

Prior to insulin content and insulin secretion assays, isolated islets were treated for 24 or 48 hours in 11.1 mM glucose RPMI 1640 complete with 10% FBS and 2% penicillin-streptomycin, or in 25 mM glucose RPMI 1640 with or without the addition of palmitate treatment (see above). For insulin content, 5 sized matched islets from each culture condition were washed twice with 1x PBS (Gibco, Life Technologies, 10010-023), collected in a 250 µl acid/ethanol solution (0.1 M HCl/70% EtOH), and left overnight at -20°C. Islets were then sonicated and stored at -20°C prior to assaying for insulin using either a Mouse Ultrasensitive Insulin ELISA kit (Alpco, 80-INSMSU-E10, Salem, NH, USA) or radio immunoassay (RIA, Cedarlane, RI-13K, Burlington, ON, CA) following manufacturer's protocols. According to the available manufacture data sheets, the limit of detection for the RIA was 0.1 ng/mL and relevant cross-reactivity includes 100% mouse insulin 1 and 2, 100% human insulin, 100% rat insulin, not detectable rat c-peptide, and unknown reactivity to mouse proinsulin although human proinsulin was reported to have 69% cross-reactivity. According to the available manufacture data sheets, the limit of detection for the ELISA was 0.025 ng/mL and relevant cross-reactivity includes 147% human insulin, <0.01% mouse c-peptide 1 and 2, and unknown reactivity to mouse proinsulin although intact human proinsulin was reported to have 0.27% cross-reactivity. For quantification of insulin secretion, 12 sized matched islets were first pre-incubated for one hour prior to the start of the assay in Krebs Ringers Buffer (KRB, 129 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES-Free Acid, 0.5% BSA) containing 3 mM glucose. This was followed by sequential incubation of the islets in 3 mM glucose and then 20 mM glucose for one hour each, followed by a 30 minute incubation with

KRB containing 3 mM glucose + 30 mM KCl (NaCl content adjusted for increased KCl content). Supernatant was collected after each stimulation, spun down at 200 x g to remove any cell debris, and assayed for insulin using either ELISA or RIA.

2.4 Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed in GraphPad Prism. Data were analyzed by Student's t-test, 1-way ANOVA (Analysis of Variance), or 2-way ANOVA where appropriate with the Bonferroni post-hoc test. Comparisons were made both within treatments (comparing genotypes) and within individual genotypes (comparing treatment effect). For quantitative PCR gene expression data, statistical analysis using a 2-way ANOVA with Bonferroni post-hoc test was performed on non-normalized data but often presented as the fold-change relative to the respective control condition for visual clarity. Differences were considered significant when $p<0.05$.

Chapter 3: Results

3.1 Generation of the Conditional Bax-Bak Knockout Mouse Model

To study the roles of the pro-apoptotic proteins Bax and Bak within the adult β-cell, we established a line of mice with global Bak knockout combined with a conditional and inducible Bax knockout. The most efficient breeding strategy was determined prior to the onset of breeding. As described above, $Bax^{flox/flox}$ $Bak^{-/-}$ mice were crossed with Pdx1-CreER expressing mice (Figure 5-1). The Cre positive heterozygous $Bax^{+/flox}$ $Bak^{+/-}$ progeny from breeding step 1 were backcrossed onto the parent homozygous $Bax^{flox/flox}$ $Bak^{-/-}$ strain to generate mice that could be used to create two parallel lines of knockout mice (Figure 5-2). Homozygous $Bax^{flox/flox}$ $Bak^{-/-}$ mice with and without Cre were bred together to create a line of Bak single knockout (Bak SKO; $Bax^{flox/flox}$ $Bak^{-/-}$) and BaxBak double knockout (BaxBak DKO; $Bax^{flox/flox}$ $Bak^{-/-}$ Cre^{+}) littermates (Figure 5-3). Simultaneously, $Bax^{flox/flox}$ $Bak^{+/-}$ progeny with and without Cre from breeding step 2 were bred together to reintroduce the wild-type Bak allele. Homozygous $Bax^{flox/flox}$ $Bak^{+/+}$ mice with and without Cre were bred together to create a line with Bax single knockout (Bax SKO; $Bax^{flox/flox}$ $Bak^{+/+}$ Cre^{+}) and littermate wild-type (WT; $Bax^{flox/flox}$ $Bak^{+/+}$) mice (Figure 5-3). All mice were genotyped for the Bax, Bak, and Cre genes during all breeding stages to ensure proper genetic makeup (Figure 6A). Once the two parallel sister lines were established, mice were only genotyped for the Cre transgene.

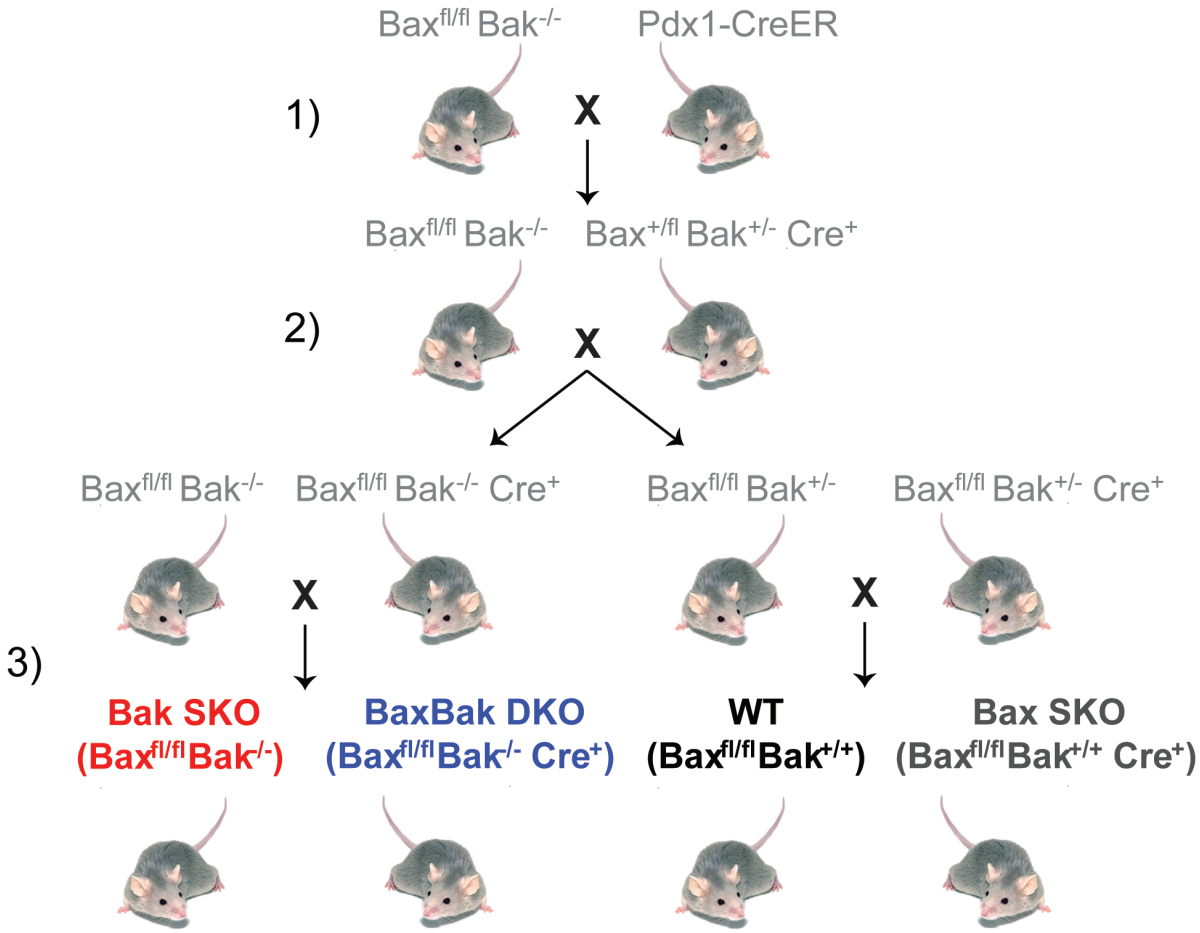

Figure 5: Breeding Scheme for the Bax and Bak Knockout Mouse Model. Pdx1-CreER mice were bred onto $Bax^{flox/flox}$ $Bak^{-/-}$ mice for the generation of WT, Bax SKO, Bak SKO, and BaxBak DKO mice. fl, *loxP* sequence flanked gene. -, global allele knockout. +, wild-type allele. Cre^{+}, Cre recombinase. x, mating pair. Arrows, subsequent generations. Numbers, breeding stages. SKO, single knockout. DKO, double knockout.

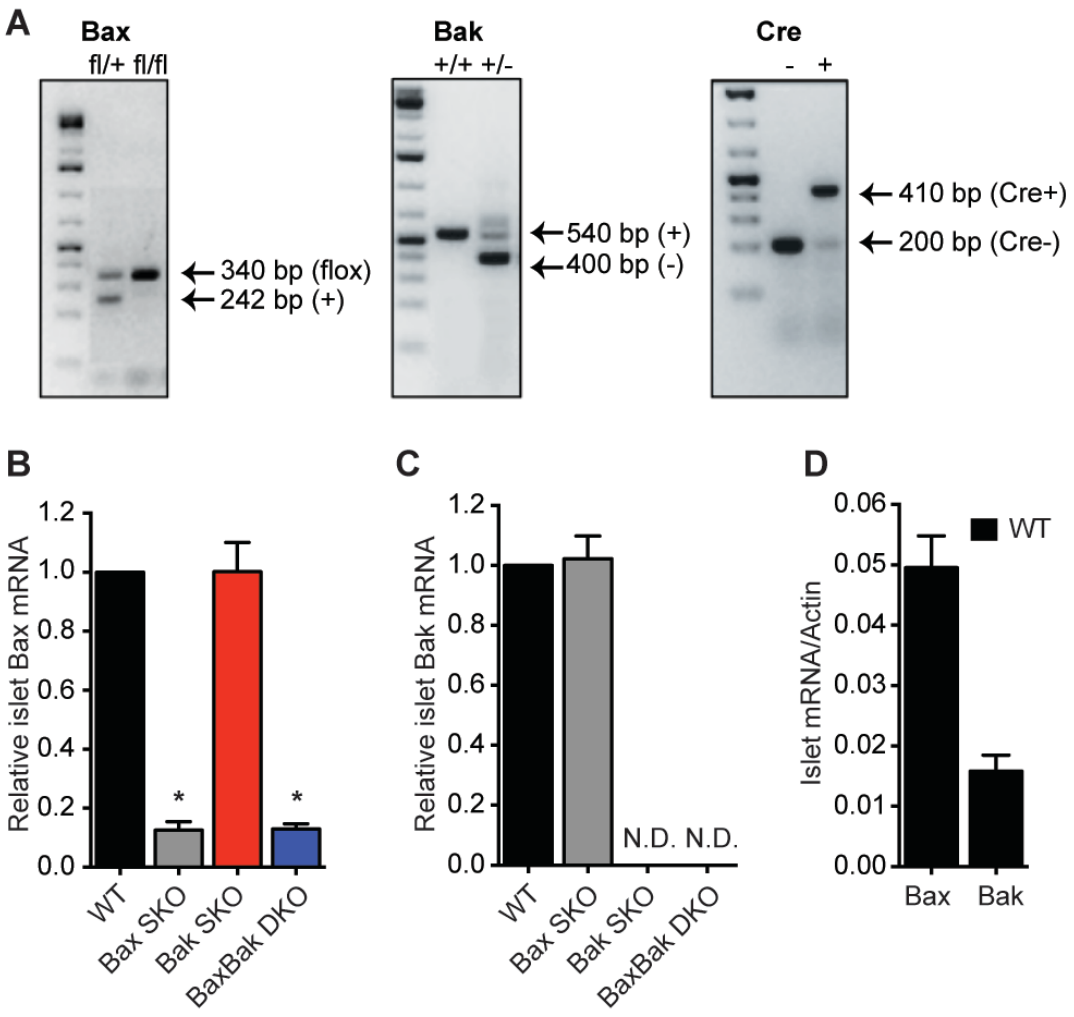

Figure 6: Islet Specific Knockout of Bax and Bak. (A) PCR products assayed for Bax, Bak, and Cre alleles genotyped post weaning. Quantification of (B) Bax (n=6) and (C) Bak (n=4) mRNA levels by quantitative PCR in islets from tamoxifen injected Bax SKO, Bak SKO, and BaxBak DKO mice relative to islets from tamoxifen injected WT mice. (D) Quantification of Bax and Bak expression levels in WT islets (n=5-9). N.D., not detected. (Data are mean ± SEM *p<0.05 vs WT).

The Pdx1-CreER mediated knockout of Bax requires intraperitoneal injection of tamoxifen [120,127,129]. Both Cre negative and Cre positive mice were injected for 4 consecutive days with tamoxifen to knock out the Bax gene in Cre positive mice and to control for any effects of tamoxifen in the Cre negative mice. Our injection protocol achieves greater than 80% knockdown of the Bax gene at the mRNA level in pancreatic islets of both Bax SKO

and BaxBak DKO mice compared to WT controls. There is no reduction of the Bax gene seen in Bak SKO or WT mice, as they are not positive for the Cre transgene (Figure 6B). The Bak gene is not detectable at the mRNA level in pancreatic islets of both Bak SKO and BaxBak DKO mice, and Bak levels are comparable between WT and Bax SKO mice (Figure 6C). At the protein level, Bax expression is greater than 80% reduced only in the islets of Bax knockout mice, and Bak protein is not detected across various tissues in Bak knockout mice [130]. Comparing the expression levels of Bax and Bak, we show that Bax expression is 3-fold higher than Bak expression in WT islets (Figure 6D). These data show that we have successfully created a mouse model to study the combined and individual roles of Bax and Bak within the adult β-cell.

3.2 Tamoxifen Independent Activation of Pdx1-CreER

The Cre-ER fusion protein is theoretically only supposed to translocate to the nucleus and perform genetic excision upon the administration of tamoxifen ligand [120]. We investigated the extent to which Pdx1-CreER could elicit genetic recombination in the absence of tamoxifen ligand. Islets were isolated from non-injected and tamoxifen-injected Bak SKO ($Bax^{flox/flox}$ $Bak^{-/-}$) and BaxBak DKO ($Bax^{flox/flox}$ $Bak^{-/-}$ Cre^{+}) mice at 3, 6, and 9 months of age (Figure 7). At 3 months of age, Bax mRNA expression is very slightly, but not significantly reduced in non-injected Cre positive (BaxBak DKO) mice (Figure 7A). However, by 6 months of age Cre positive mice have ~50% knockdown of the Bax gene in the absence of tamoxifen ligand. This tamoxifen-independent knockdown of Bax does not fully reach the level of Bax knockdown in mice injected with tamoxifen (~80%), but these results suggest the temporal control of Bax knockout may be compromised (Figure 7B). By 9

months of age tamoxifen-independent Bax knockdown is even more evident (Figure 7C). These results suggest that the tamoxifen-activated Pdx1-CreER used in our mouse model has the tendency to spontaneously activate in the absence of tamoxifen ligand over time, through an unknown mechanism. Based on our observations, all experimental mice and isolated islets were used around 3 months of age, where tamoxifen-independent activation of Cre was least evident.

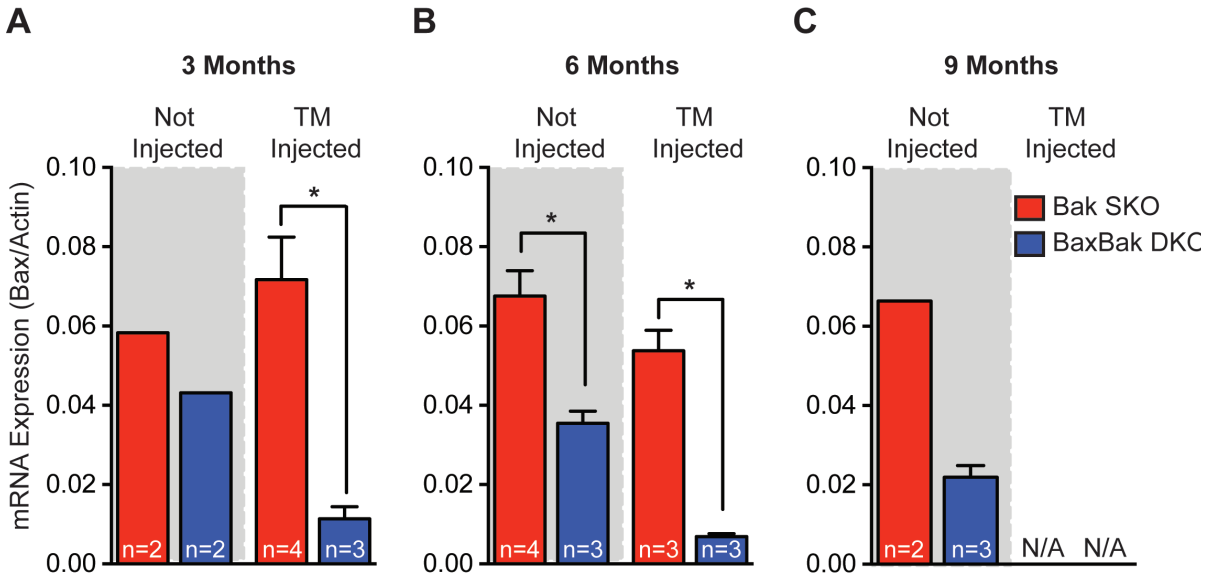

Figure 7: Ligand-Independent Pdx1-CreER Bax Recombination. Quantification of Bax mRNA expression compared to Actin housekeeping gene from Bak SKO and BaxBak DKO islets at (A) 3 months, (B) 6 months, and (C) 9 months of age. Islets isolated were from mice either not injected with tamoxifen (gray shade), or injected with tamoxifen (white shade). TM, tamoxifen. N/A, no samples available. (Data are mean ± SEM *p<0.05).

3.3 Characterization of Bax and Bak Knockout on β-Cell Function

Proper function of the mitochondria and ER are critical for β-cell function. Bax has been reported to affect mitochondrial function under non-apoptotic conditions [131] and both Bax and Bak have been shown to localize and function at the ER [108,109]. Therefore, we evaluated whether the inducible knockout of Bax and/or ablation of Bak in the adult β-cell had any effect on islet function both *in vivo* and *in vitro*.

3.3.1 Loss of Bax and Bak Does Not Affect Glucose Homeostasis *In Vivo*

We examined the effect of knocking out Bax and/or Bak on glucose tolerance and body weight. Intraperitoneal glucose tolerance tests established that single or double knockout of Bax and Bak in the adult β-cell had no effect on glucose tolerance of both male (Figure 8A,C) and female (Figure 8B,D) mice. Fasting blood glucose was comparable between genders and not affected by Bax and/or Bak knockout (Figure 8A,B). Body weight was also not affected by the knockout of Bax and/or Bak in the adult β-cell, and males tended to be ~10g heavier than females at 3 months of age (Figure 8E,F). These data suggest that knockout of Bax and/or Bak in the adult β-cell has no affect on *in vivo* glucose responsiveness in the islet, and also suggests that global Bak deletion does not affect peripheral tissue glucose homeostasis.

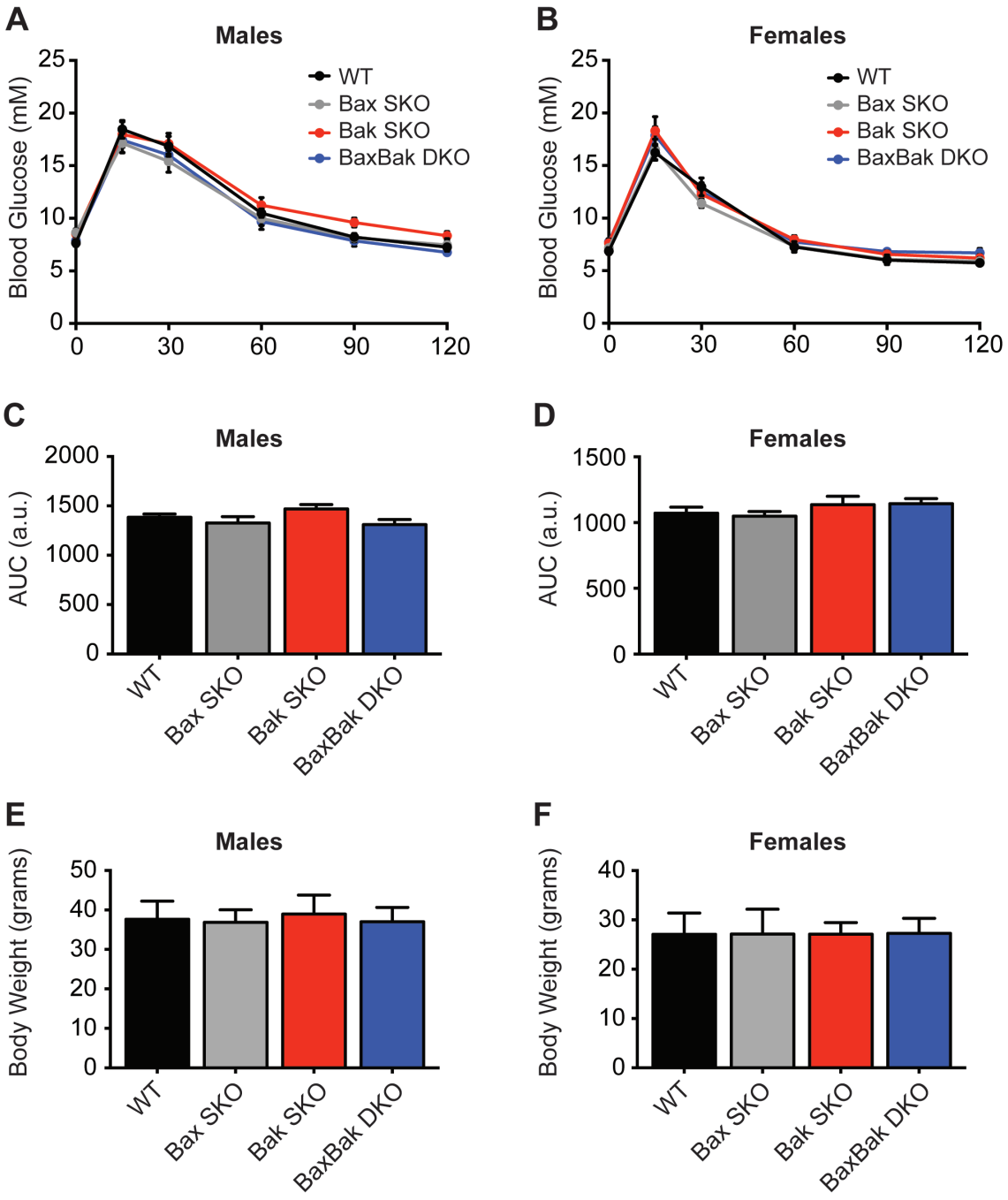

Figure 8: Loss of Bax and Bak Does Not Affect Body Weight or *In Vivo* Glucose Tolerance. (A,B) Intraperitoneal glucose tolerance tests using a 2 g/kg glucose in saline injection in 12-16 week old (A) male (n=7-12) and (B) female (n=7-11) WT, Bax SKO, Bak SKO, and BaxBak DKO mice. (C,D) Area under the curve analysis of glucose tolerance profiles in panels A and B. (E,F) Random-fed body weight data of (E) males (n=16-22) and (F) females (n=13-30) taken prior to islet isolation (age range 12-20 weeks). a.u., arbitrary units. (Data are mean ± SEM).

3.3.2 Loss of Bax and Bak Does Not Affect Islet Function *In Vitro*

The possibility that the *in vivo* environment masked any functional effect that Bax and/or Bak knockout had on adult β-cells lead us to investigate islet function *in vitro.* Islets were isolated from WT, Bax SKO, Bak SKO, and BaxBak DKO mice within 2 weeks after the tamoxifen-injections were administered. Static glucose-stimulated insulin secretion on isolated whole islets showed comparable baseline (3 mM glucose) and stimulated (20 mM glucose) insulin secretion between Bax SKO, Bak SKO, BaxBak DKO, and WT islets (Figure 9A). Whole islet insulin content was also similar between Bax SKO, Bak SKO, BaxBak DKO, and WT islets (Figure 9B), and islet insulin mRNA expression was comparable between WT and BaxBak DKO islets (Figure 9C). To further examine islet function, isolated whole islets were subjected to a stepwise glucose ramp to measure cytosolic Ca^{2+} changes. Baseline (3mM glucose), stimulated (6, 10, 15mM glucose) and KCl conditions elicited a cytosolic Ca^{2+} response that did not differ between WT and BaxBak DKO islets (Figure 9D,E). Taken together, these data demonstrate that the knockout of Bax and/or Bak in the adult β-cell has no effect on islet function, both *in vivo* and *in vitro* under physiological conditions.

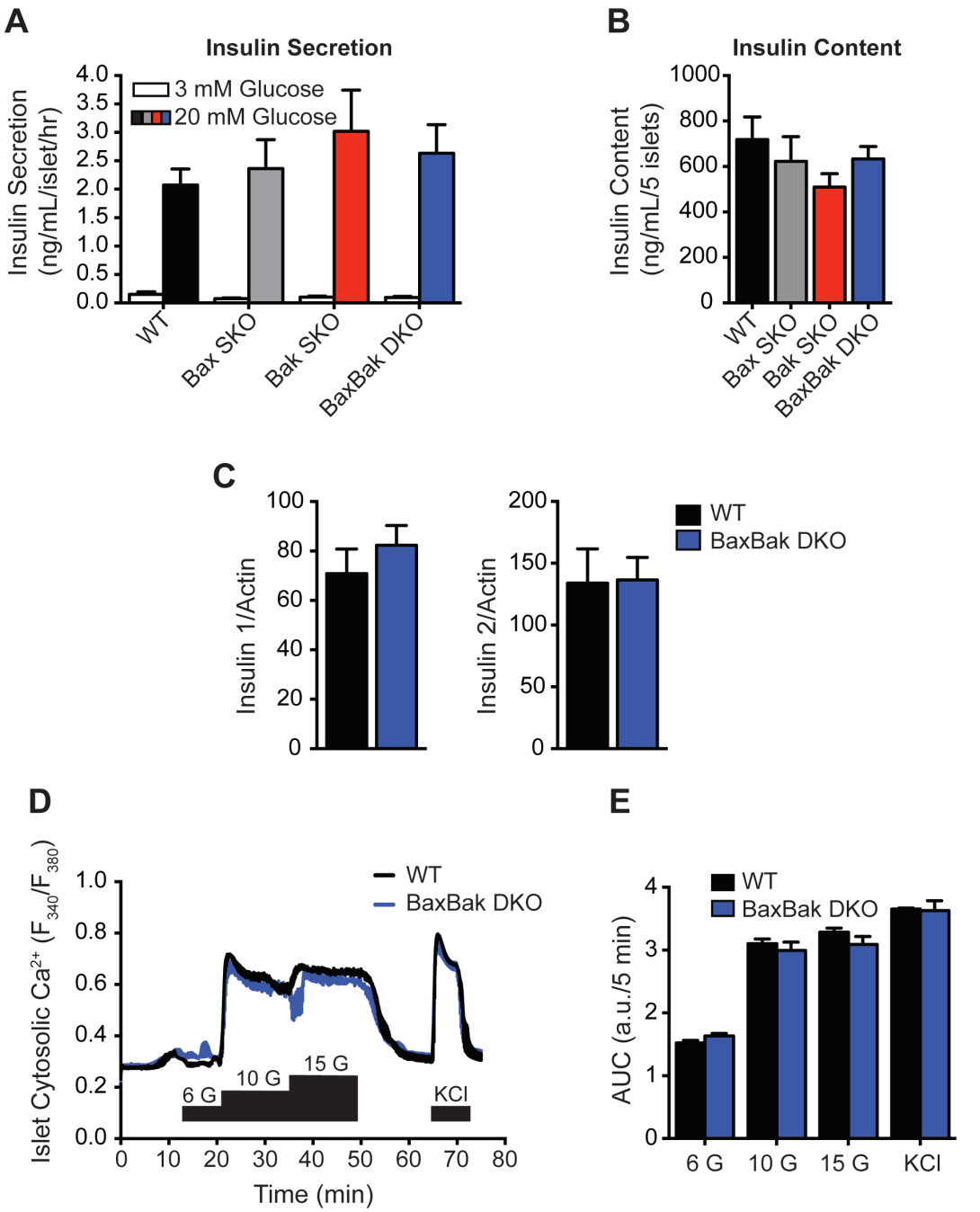

Figure 9: Loss of Bax and Bak Does Not Affect Islet Physiology *In Vitro*. (A) Glucose-stimulated insulin secretion from WT, Bax SKO, Bak SKO, and BaxBak DKO islets. One hour sequential stimulation with 3 mM then 20 mM glucose (n=5-9). (B) Insulin content of 5 size-matched islets from WT, Bax SKO, Bak SKO, and BaxBak DKO mice (n=4-8). (C) Insulin 1 and 2 mRNA expression of untreated WT and BaxBak DKO islets normalized to Actin housekeeping gene (n=4). (D) Average cytosolic Ca^{2+} responses of whole islets from WT and BaxBak DKO mice stimulated sequentially with increasing glucose concentrations and KCl (n=3-4). (E) Area under the curve (AUC) analysis per 5 min during each stimulus in panel D. a.u., arbitrary units. G, glucose. (Data are mean ± SEM).

3.4 Relative Contribution of Bax and Bak to Staurosporine-Induced Mitochondrial β-Cell Apoptosis

After establishing that Bax and Bak have no effect on physiological islet function, we next assessed whether knockout of Bax and/or Bak affected β-cell survival in response to apoptotic stimuli. As pro-apoptotic Bax and Bak are critical regulators of apoptosis at the mitochondria, we assessed overall islet-cell death in response to staurosporine (STS), a potent chemical inhibitor of protein kinases that initiates apoptosis via a Bcl-2 family and caspase-dependent manner [132,133]. Isolated and dispersed islet-cells from WT, Bax SKO, Bak SKO, and BaxBak DKO mice were treated with 1 μM STS for 54 hours. A kinetic time-course analysis revealed that knockout of either Bax or Bak provided significant resistance to STS-induced cell death compared to WT islet-cells (Figure 10A-C). WT islet-cell death was rapid in response to STS treatment, with significant cell death achieved by 16 hours. Significant induction of cell death was delayed to 25, 31, and 34 hours after STS treatment in Bax SKO, Bak SKO, and BaxBak DKO islet-cells, respectively (Figure 10A). Interestingly, the knockout of Bak provided greater protection against STS-induced cell death compared to the knockout of Bax, and the combined knockout of both Bax and Bak provided the most protection towards cell survival. However, the increased protection observed in BaxBak DKO islet-cells did not reach statistical significance over the Bak-SKO islet-cells, suggesting that Bak plays a major role in staurosporine-induced apoptosis (Figure 10A-C). All Bax and/or Bak knockout islet-cells reached a plateau of cell death after 48 hours of apoptotic stimuli, matching the total amount of WT islet-cell death (Figure 10A). These data show that removal of one pro-apoptotic protein will provide significant protection against STS-induced islet-cell death. Interestingly, Bak knockout provided the most protection against STS-

induced islet-cell death, indicating that STS induces apoptosis mostly through the induction of Bak. Taken together, these data indicate that Bax and Bak have individual roles in apoptosis induction under STS-like stress conditions in islet-cells.

We next investigated a mechanism by which BaxBak DKO islet-cells were protected from STS-induced cell death. During the initiation of apoptosis Bax and Bak form pores in the mitochondrial outer membrane, which ultimately leads to a loss of mitochondrial integrity that can be measured by a loss of mitochondrial membrane potential [57,134]. At 24 hours of 1 μM STS treatment, a significant reduction in mitochondrial membrane potential was observed in WT islet-cells compared to WT DMSO controls. This reduction in mitochondrial membrane potential was not observed in BaxBak DKO islet-cells (Figure 10D). Interestingly, baseline mitochondrial membrane potential was slightly, albeit not significantly, lower in DMSO treated BaxBak DKO islet-cells compared to DMSO treated WT islet-cells. This observation is consistent with reports that a small amount of mitochondria-associated Bax may be required for proper mitochondrial function under physiological conditions, as previous evidence showed that residual levels of mitochondrial-associated Bax were required for the proper maintenance of aerobic energy production [131]. However, the slight reduction in mitochondrial membrane potential observed in the BaxBak DKO islet-cells is not strong enough to affect islet Ca^{2+}, insulin secretion, or glucose tolerance. Overall, these results show that Bax and Bak are important mediators of islet-cell apoptosis. Knockout of Bax and/or Bak individually contributes to islet-cell survival, partly by preserving mitochondrial integrity.

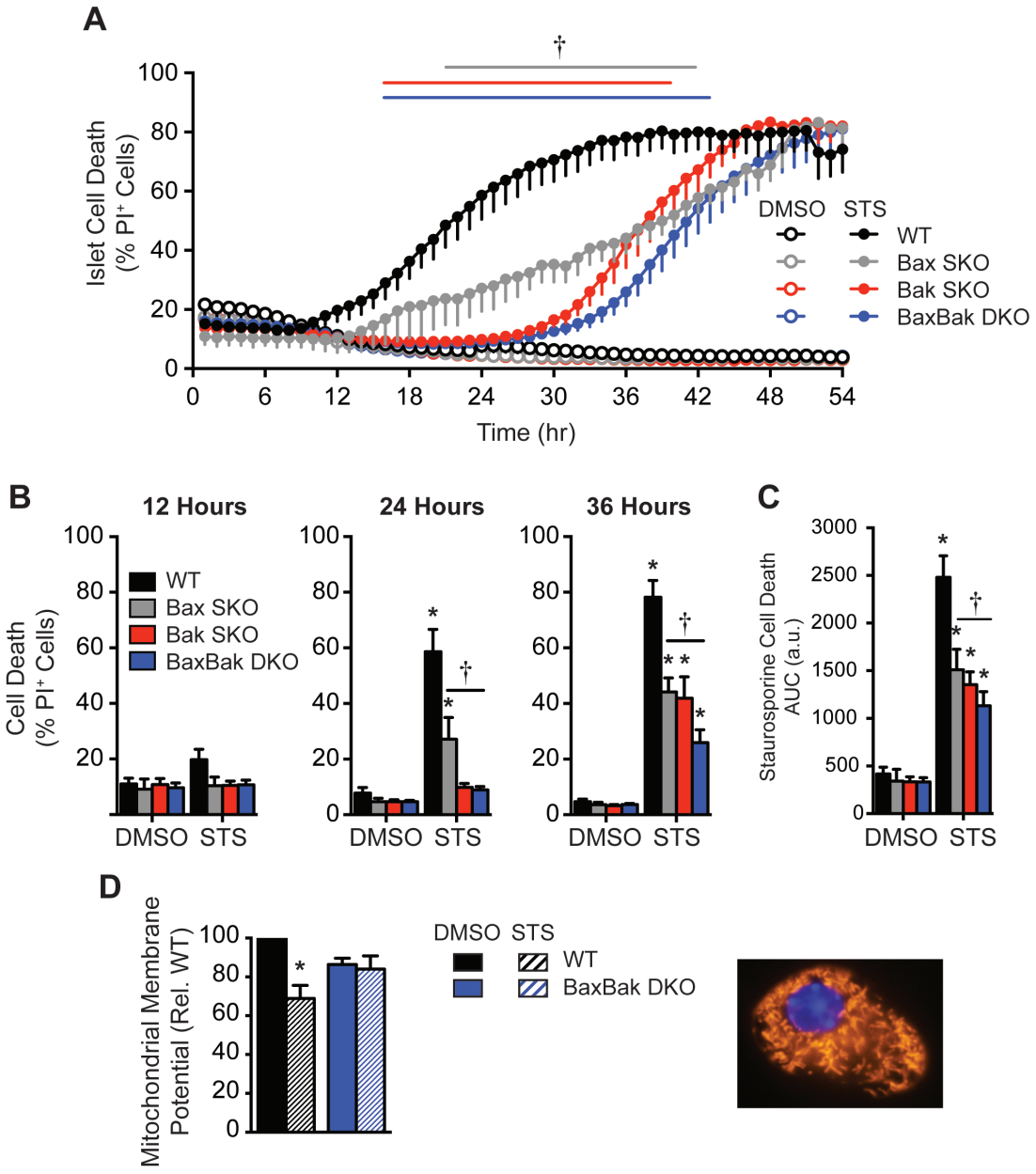

Figure 10: Relative Contribution of Bax and Bak to Staurosporine-Induced Mitochondrial Islet Cell Apoptosis. (A) Time-course cell death analysis of WT, Bax SKO, Bak SKO, and BaxBak DKO single islet-cells treated with 1 μM staurosporine (STS) or DMSO vehicle control (cells from n=4-10 mice). (B) Cell death quantified at 12-hour intervals from the time-course analysis shown in panel A. (C) Area under the curve (AUC) analysis of cell death profiles in panel A. (D) Mitochondrial membrane potential (TMRE fluorescence intensity) of WT and BaxBak DKO cells treated for 24 hours with 1 μM STS or DMSO vehicle control. Mean intensity of mitochondrial staining shown as a percentage of WT DMSO control (n=4). STS, staurosporine. a.u., arbitrary units. (Data are mean ± SEM *p<0.05 vs DMSO control, †p<0.05 vs WT).

3.5 Double Knockout of Bax and Bak is Required for Significant Protection Against Glucolipotoxicity

We next determined if knockout of Bax and/or Bak could protect the β-cell from death in the presence of diabetogenic stress. Islets in a type 2 diabetic environment are chronically exposed to high glucose (glucotoxicity) and often high FFA (lipotoxicity) conditions [16]. Dispersed islet-cells from WT, Bax SKO, Bak SKO, and BaxBak DKO mice were exposed to various gluco- and lipo-toxic conditions for 58 hours during which cell death was monitored and quantified. Low glucose (5 mM) conditions did not elicit islet-cell death (Figure 11A). Addition of 1.5 mM of the FFA palmitate (6:1 molar ratio with BSA) was used to induce lipotoxicity on the isolated islet-cells. However, in the presence of 5 mM glucose the lipotoxic conditions alone were not sufficient to induce islet-cell death over 58 hours of stress treatment (Figure 11A). High glucose (25 mM) did not elicit islet-cell death over 58 hours of culture, which is not unexpected as glucotoxicity alone seems to perturb islet function rather than induce islet-cell death [135]. When glucotoxicity does induce cell death it seems to require longer culture in much higher glucose conditions [53]. Therefore, we used 25 mM glucose as a control for glucolipotoxic stress for further ER-stress studies. While gluco- and lipo-toxicity alone are usually not sufficient to induce β-cell death, the combination is highly detrimental to β-cells [9,38]. In agreement with this, glucolipotoxicity induced by high glucose with the addition of palmitate caused significant cell death in WT, Bax SKO, Bak SKO, and BaxBak DKO islet-cells, with maximal cell death of WT islet-cells ranging between 15-25% after 58 hours (Figure 11B,C, data not shown). In all experiments, decreased levels of cell death were observed in BaxBak DKO islet-cells compared to WT, Bax SKO, and Bak SKO islet-cells under glucolipotoxic conditions (Figure 11B,C). These

findings, unlike STS-induced apoptosis where the single knockout of either Bax or Bak was sufficient to provide protection towards β-cell survival, indicate a requirement for the removal of both pro-apoptotic proteins to significantly provide protection against islet-cell death under glucolipotoxic conditions.

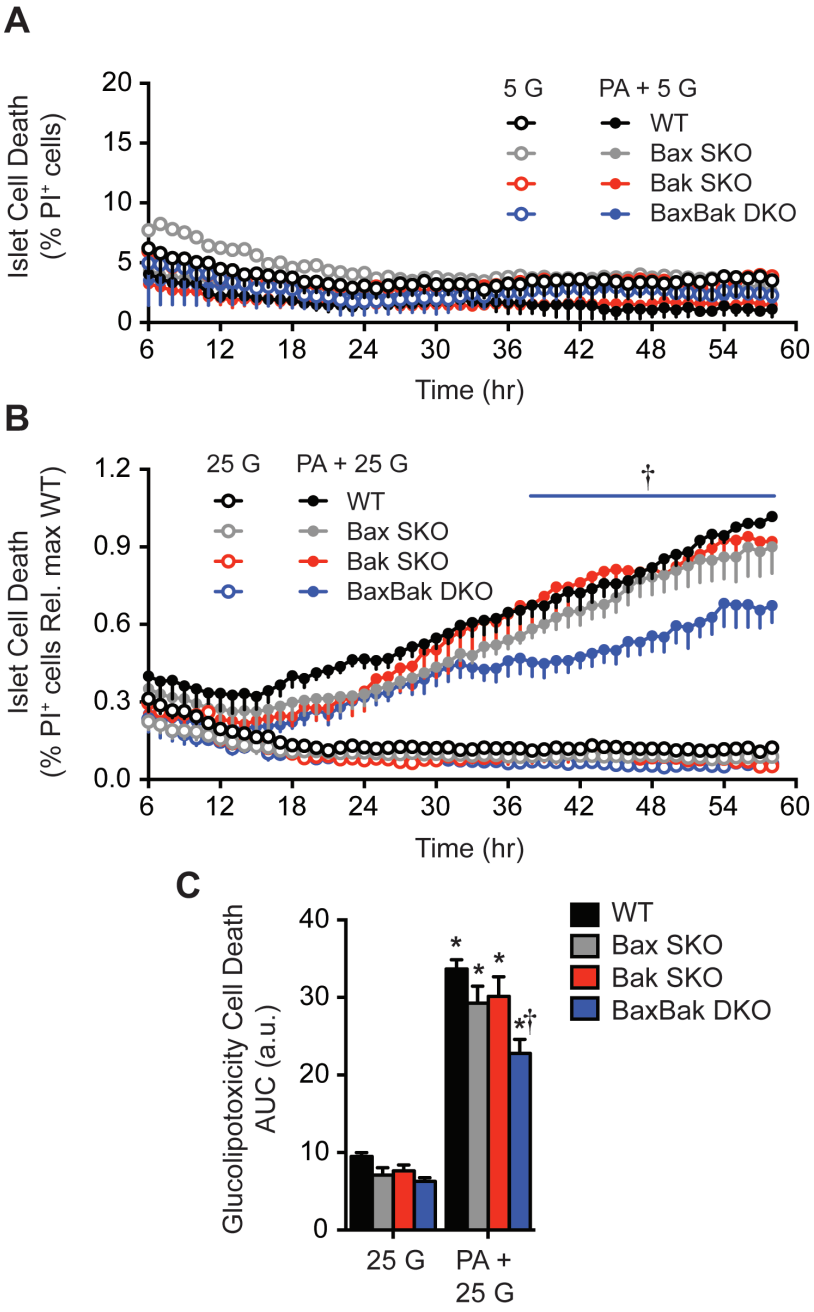

Figure 11: Double Knockout of Bax and Bak is Required for Significant Protection against Glucolipotoxicity: Time-course cell death analysis of WT, Bax SKO, Bak SKO, and BaxBak DKO islet-cells treated with (A) 5 mM low glucose control (5 G) or 5 mM glucose plus 1.5 mM palmitate (PA + 5 G) to induce lipotoxicity (n=2-4), and (B) 25 mM high glucose control (25 G) or 25 mM glucose plus 1.5 mM palmitate (PA + 25 G) to induce glucolipotoxicity (n=4-5). (C) Area under the curve (AUC) analysis of cell death profiles in panel B. a.u., arbitrary units. (Data are mean ± SEM *p<0.05 vs glucose-only control, †p<0.05 vs WT, Bax SKO and Bak SKO).

3.6 Loss of Bax and Bak Augments Early UPR Signalling in Stressed β-Cells

The emerging evidence for Bcl-2 family proteins functioning at the ER lead us to investigate whether Bax and Bak had functional roles in the ER-stress response in pancreatic β-cells. We examined UPR signalling under glucolipotoxic stress in isolated islets from WT, Bax SKO, Bak SKO, and BaxBak DKO mice. Untreated WT and BaxBak DKO islets showed similar levels of CHOP and XBP1s expression prior to ER-stress induction (Figure 12A). Under glucolipotoxic conditions there was a trend towards an increase in Bax mRNA levels after 48 hours in WT and Bak SKO islets, with no induction of residual Bax seen in islets from either of the two Bax knockout mice (Figure 12B). Bak levels did not appear to change after 48 hours of glucolipotoxic treatment. However, 48 hours of culture in 25 mM glucose trended towards a compensatory increase in Bak expression in Bax SKO islets compared to WT controls (Figure 12C). Under culture in glucolipotoxic conditions, CHOP expression increased in a time-dependent manner, with significant increases observed in islets of all four genotypes relative to islets in 25 mM glucose control conditions. However, no significant differences were observed between the four genotypes and WT controls. Bax SKO islets did not show significant CHOP induction, presumably due to a small sample size (Figure 12D). Interestingly, XBP1s mRNA levels were significantly increased early on in BaxBak DKO islets compared to Bax SKO, Bak SKO, and WT islets. After 48 hours of glucolipotoxicity, XBP1s was still significantly elevated in BaxBak DKO islets and significant induction of XBP1s was also observed in Bak SKO islets (Figure 12E). However, at this later time-point, no differences were observed between the BaxBak DKO islets and WT controls, indicating that XBP1s signalling is increased early on and sustained in BaxBak

DKO islets, where WT, Bax SKO, and Bak SKO islets show a slower time-dependent increase in XBP1s expression.

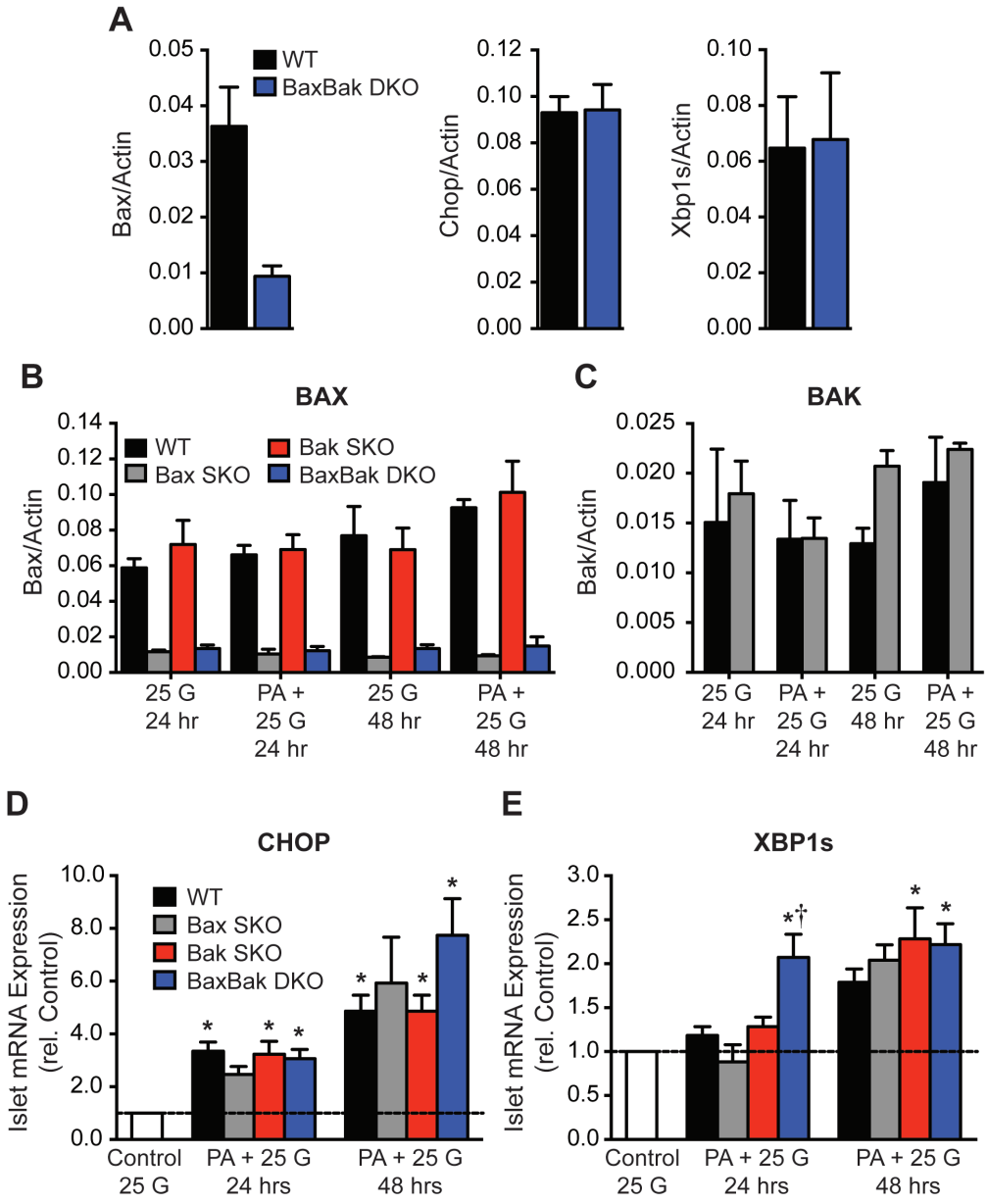

Figure 12: Loss of Bax and Bak Augments Early UPR Signalling in Islet Cells Under Glucolipotoxic Stress. (A) Quantification of BAX, CHOP, and XBP1s mRNA expression in untreated WT and BaxBak DKO islets prior to ER-stress induction (n=4). (B-E) WT, Bax SKO, Bak SKO, and BaxBak DKO islets were cultured in 25 mM glucose (25 G) or 25 mM glucose plus 1.5 mM palmitate (PA + 25 G) for 24 and 48 hours before examining mRNA expression of (B) BAX, (C) BAK (WT and Bax SKO only), (D) CHOP, and (E) XBP1s (n=3-8). (Data are mean ± SEM *p<0.05 vs 25 mM glucose control, †p<0.05 vs WT).

While glucolipotoxicity is a diabetes-relevant stress factor, it induces a relatively complex β-cell stress response that includes, but is not limited to, ER stress [20,38,39,136]. To obtain a more specific picture of the roles played by Bax and Bak in UPR signalling we next examined the impact of treating islets with thapsigargin, a specific inhibitor of the SERCA pump that induces a more specific ER-stress response. Similar to glucolipotoxic treatment, Bak expression did not differ after 48 hours of 100 nM thapsigargin treatment (Figure 13A). Bax expression significantly increased after 48 hours of chronic thapsigargin treatment in both WT and Bak SKO islets, in agreement with the increasing trend seen in Bax expression under glucolipotoxic stress. No induction of residual Bax was observed in islets from both of the Bax knockout mice (Figure 13B,C). CHOP expression increased in a time-dependent manner over the 48 hour time-course, with acute and significant CHOP induction seen in all 4 genotypes after just 3 hours of thapsigargin treatment. Similar to glucolipotoxicity-induced CHOP expression, no statistically significant differences were observed between the four genotypes, indicating that Bax and Bak do not regulate PERK mediated UPR signalling (Figure 13D,E). Acute thapsigargin treatment (3 hours) induced XBP1s expression in WT, Bax SKO, and Bak SKO islets in a time-dependent manner. Interestingly, BaxBak DKO islets showed elevated and sustained XBP1s expression under acute thapsigargin treatment (3-12 hours) compared to WT controls, similar to the early elevated XBP1s expression that was observed in BaxBak DKO islets under glucolipotoxic treatment (Figure 13F). However, XBP1s expression was similar among all four genotypes after more chronic thapsigargin treatment (24-48 hours) (Figure 13G). These data suggest that chronic ER-stress upregulates Bax expression, providing a link between ER-stress and pro-apoptotic Bax induction; however, the mechanisms behind this event still remain unclear. Prior to Bax upregulation,

physiological levels of Bax and Bak collectively regulate the early ER-stress response in pancreatic β-cells by repressing IRE1α splicing of XPB1, while having no significant effect on stress-induced CHOP induction. Together these data suggest non-apoptotic roles for Bax and Bak in regulating the IRE1α ER-stress response upstream of apoptosis execution in the β-cell.

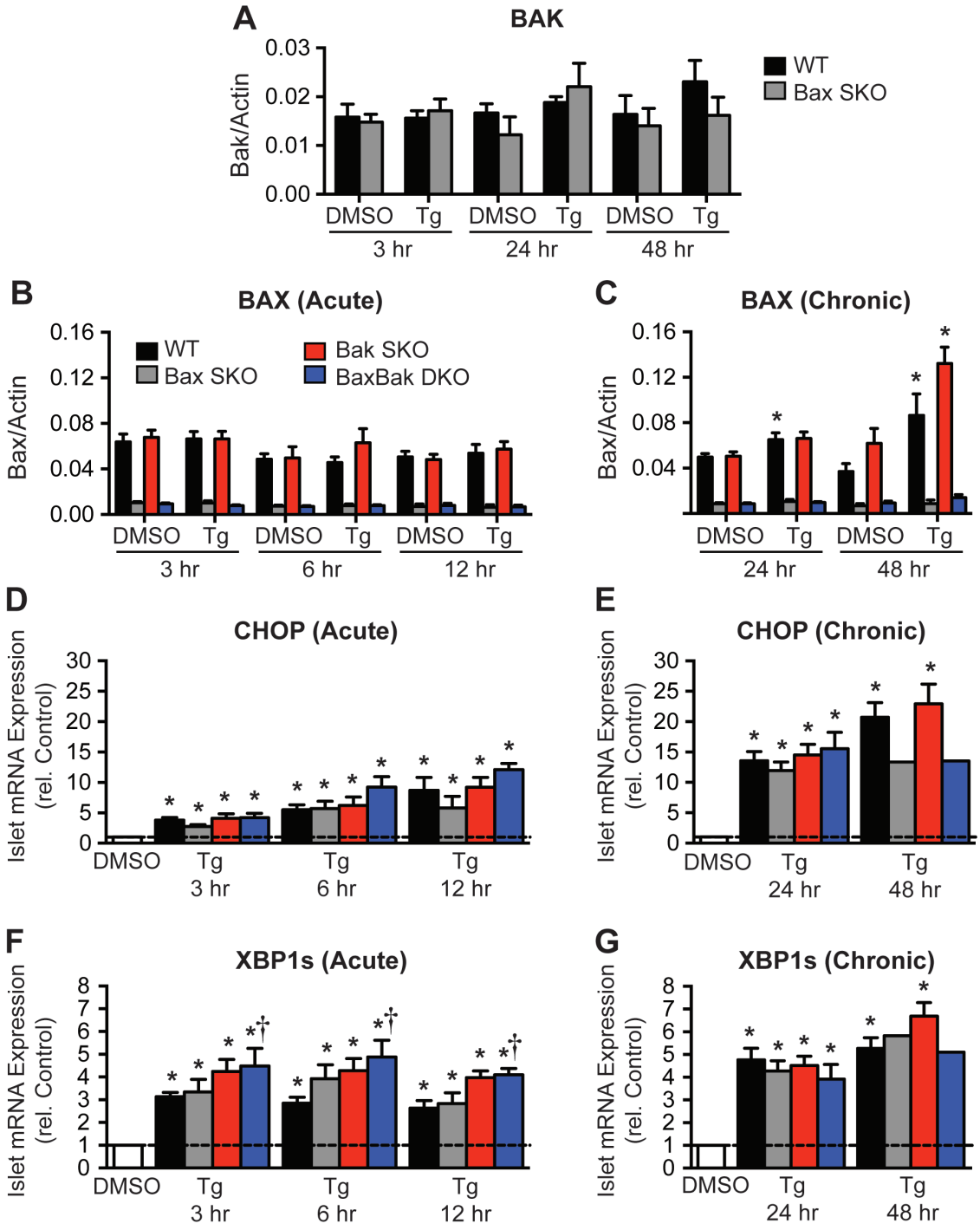

Figure 13: Loss of Bax and Bak Augments Early UPR Signalling in Thapsigargin Stressed Islet-Cells. WT, Bax SKO, Bak SKO, and BaxBak DKO islets exposed to 100 nM thapsigargin (Tg) or DMSO vehicle control for 3, 6, 12, 24, and 48 hours before examining mRNA expression levels of (A) BAK (WT and Bax SKO only), (B-C) BAX, (D-E) CHOP, and (F-G) XBP1s (n=3-10). Acute 3-12 hrs. Chronic 24-48 hrs. (Data are mean ± SEM *p<0.05 vs DMSO control, †p<0.05 vs WT).

3.7 Effects of UPR Signalling on Downstream Target Genes

As combined Bax and Bak deletion was required for islets to show significantly upregulated XBP1s compared to WT controls, islets from these two genotypes were examined further for differences in β-cell stress signalling and function under ER-stress conditions. The augmented XBP1s expression we saw under acute ER-stress in BaxBak DKO islets compared to WT controls prompted us to determine whether UPR-specific and XBP1s target genes were also differently expressed in BaxBak DKO islets under similar stress conditions. Expression of the ER-chaperone BIP trended towards a significant increase in WT islets and BIP mRNA levels were significantly upregulated in BaxBak DKO islets after 24 hours of glucolipotoxic stress (Figure 14A). BIP expression also showed a time-dependent upregulation in both WT and BaxBak DKO islets during acute thapsigargin-induced ER-stress (Figure 14B). There appeared to be a slight trend of increased BIP expression in BaxBak DKO islets compared to WT controls after short-term (6 hours) thapsigargin stress; however, due to small sample numbers this effect warrants further study. We examined the expression of the downstream UPR target genes ERO1L, ERDJ4 (DNAJB9), and EDEM, of which the latter two have been shown as distinct XBP1s target genes [137]. ERO1L expression was significantly induced over time in both WT and BaxBak DKO islets under thapsigargin-induced ER-stress (Figure 14C). Similar to BIP expression, there was a trend towards increased ERO1L expression in the BaxBak DKO islets compared to WT controls after 6 hours of thapsigargin treatment, suggesting this also warrants further study. EDEM expression similarly increased in both WT and BaxBak DKO islets under thapsigargin-induced ER-stress (Figure 14D). Interestingly, expression of the XBP1s target gene ERDJ4 was significantly upregulated in BaxBak DKO islets after 6 hours of

thapsigargin-induced ER-stress, an effect not seen in WT islets. After 12 hours of ER-stress, ERDJ4 expression was also significantly induced in WT islets to similar levels seen in BaxBak DKO islets (Figure 14E). Collectively, these data show that UPR target genes are upregulated in response to glucolipotoxic and chemical-induced ER-stress in the β-cell, and hint at augmented UPR gene expression in BaxBak DKO islets compared to WT islets in the earlier phases of the ER-stress response.

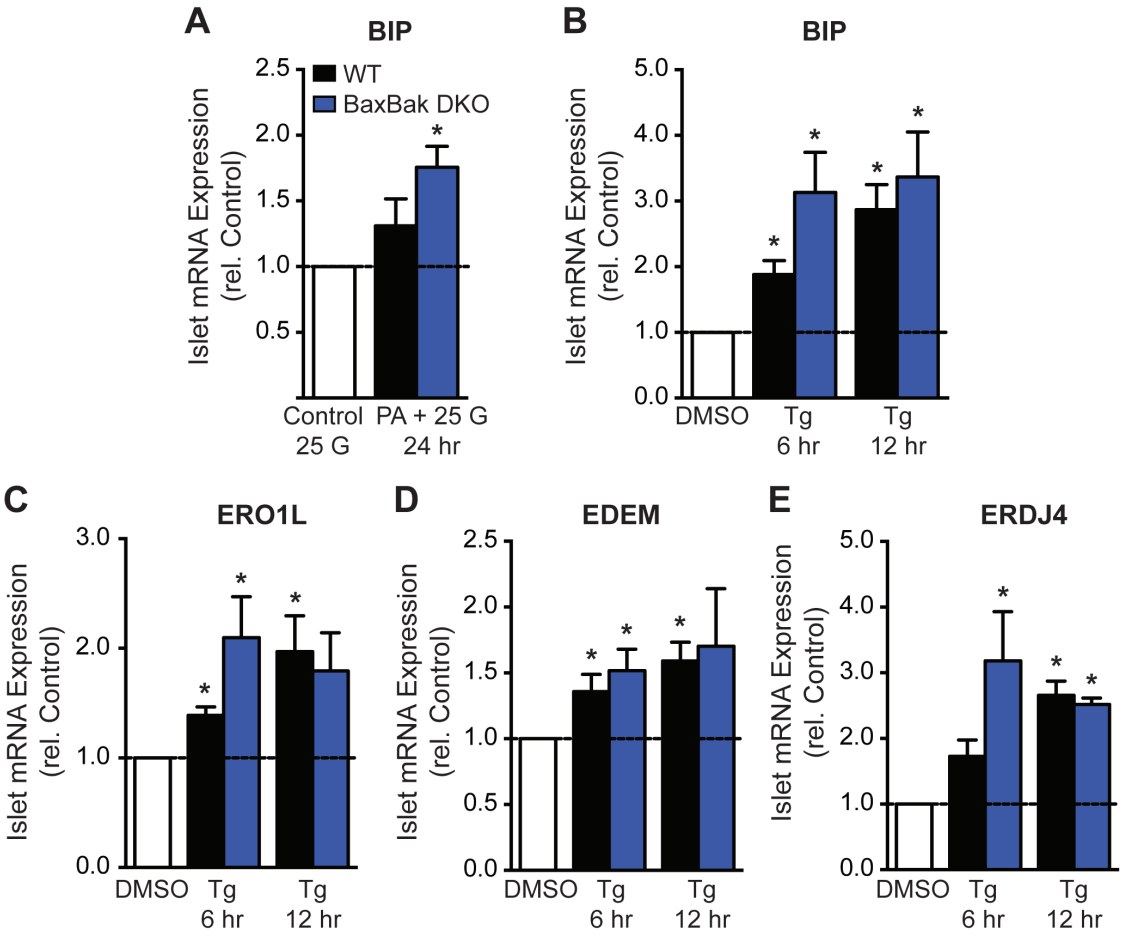

Figure 14: UPR Activated Gene Transcription in WT and BaxBak DKO Islets. (A) BIP mRNA expression in islets exposed to 25 mM glucose (25 G) control conditions or 25 mM glucose plus 1.5 mM palmitate (PA + 25 G) for 24 hours. (B-D) mRNA expression of (B) BIP, (C) ERO1L, (D) EDEM, and (E) ERDJ4 in islets exposed to 100 nM thapsigargin (Tg) or DMSO vehicle control treatment for 6 and 12 hours (n=3-6). (Data are mean ± SEM *p<0.05 vs respective control).

3.8 Effects of Glucolipotoxicity and Thapsigargin-Induced ER-Stress on Insulin Expression in WT and BaxBak DKO β-Cells

The ER-stress and β-cell death results thus far have demonstrated that deletion of Bax and Bak has protective effects on β-cell survival and also regulate the initial phases of UPR signalling in response to glucolipotoxic conditions and chemical induction of ER-stress. We next examined β-cell function by investigating the effect of Bax and Bak knockout on insulin expression under normal culture conditions, and following culture in high glucose (25 mM), glucolipotoxic, or thapsigargin conditions. To alleviate ER-stress, the β-cell will reduce insulin biosynthesis to provide relief on the ER lumen. This is partly mediated by IRE1α under both transient and chronic glucose stimulation [90,91]. Our findings that BaxBak DKO islets display increased XBP1s after 24 hours of glucolipotoxic stress is indicative of increased IRE1α signalling, and thus suggests that Bax and Bak deletion might have effects on insulin mRNA degradation and biosynthesis. After 24 and 48 hours of glucolipotoxic treatment, insulin mRNA expression was significantly decreased in both WT and BaxBak DKO islets compared to their high glucose (25mM) controls (Figure 15A,B,D,E). There were no observable differences between WT and BaxBak DKO islets (Figure 15C,F), indicating that Bax and Bak do not regulate insulin transcription or insulin mRNA degradation under glucolipotoxic stress. To test this under conditions of specific ER-stress, the effects of thapsigargin on insulin expression were investigated. In contrast to glucolipotoxicity, 6 or 24 hours of islet culture with thapsigargin induced no significant decrease in insulin mRNA levels. However, in WT islets acute (6 hours) thapsigargin treatment caused a trend toward reduced mRNA levels compared to their DMSO control, a trend that was not observed in BaxBak DKO islets (Figure 15G,H). This data suggests that stress mediated reductions in

insulin mRNA are associated with metabolism of glucose and/or FFA, rather than perturbations in ER luminal Ca^{2+} levels.

As expected from previous studies [91], high glucose treatment dramatically increased insulin mRNA levels. In WT islets we found this increase to peak after 24 hours and then drop to a lower, but still significantly elevated level after 48 hours (Figure 15A,B,D,E). Interestingly, high glucose also significantly increased insulin mRNA in BaxBak DKO islets but did so with different kinetics than in WT islets. At 24 hours the glucose-induced increase in insulin mRNA was significantly smaller than that observed in WT islets, and insulin mRNA levels remained similar following 48 hours of high glucose treatment. Essentially, BaxBak DKO islets did not show the glucose-induced peak in insulin mRNA levels seen in WT islets at 24 hours (Figure 15A,B,D,E). These data suggest that Bax and Bak may have roles in the physiological control of insulin expression, as well as potential roles in mediating specific glucotoxic effects on the adult β-cell.

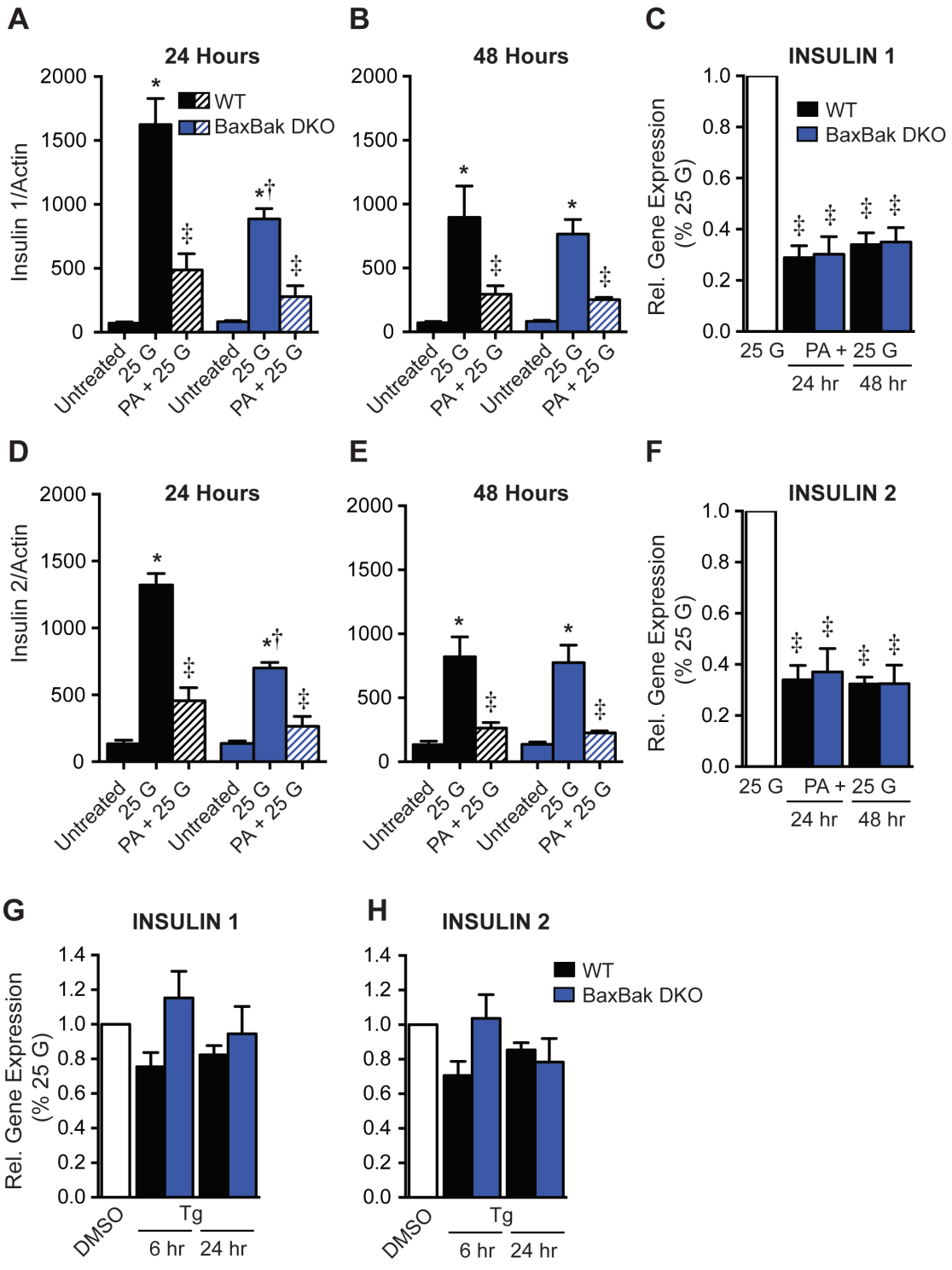

Figure 15: Effects of Glucolipotoxicity and ER-Stress on Insulin Transcription in WT and BaxBak DKO Islets. Whole islets from WT and BaxBak DKO mice were cultured in the presence of no treatment (Untreated), 25 mM glucose (25 G), or 25 mM glucose plus 1.5 mM palmitate (PA + 25 G) for 24 and 48 hours prior to quantifying (A-C) INSULIN 1 and (D-F) INSULIN 2 mRNA expression (n=3-4). (G,H) Islets from WT and BaxBak DKO mice

subjected to 100 nM thapsigargin (Tg) or DMSO vehicle control for 6 and 24 hours prior to examining (G) INSULIN 1 and (H) INSULIN 2 mRNA expression (n=3-4). (Data are mean ± SEM *p<0.05 vs untreated islets, †p<0.05 BaxBak DKO vs similarly treated WT, ‡p<0.05 vs 25 G).

3.9 Glucolipotoxic Effects on WT and BaxBak DKO β-Cell Function

Our data so far suggests that Bax and Bak have effects on IRE1α UPR signalling and potentially IRE1α-mediated insulin mRNA degradation and hyperglycemia-stimulated insulin biosynthesis. This, in turn, could have potential effects on islet insulin content and insulin secretion following hyperglycemia and/or ER-stress. Therefore, we next investigated β-cell function under glucolipotoxic conditions. Isolated islets from WT and BaxBak DKO mice were subjected to 24 or 48 hours of high glucose (25 mM) or glucolipotoxic (25 mM glucose + 1.5 mM palmitate) conditions prior to assaying insulin secretion and islet insulin content. Previously published studies have demonstrated that glucolipotoxic conditions significantly affect both glucose-stimulated insulin secretion and insulin content in whole islets[33,37,138-140]. In both WT and BaxBak DKO islets, glucolipotoxic conditions significantly increased basal (3 mM glucose) insulin secretion (Figure 16A) and decreased insulin secretion in response to glucose stimulation (20 mM glucose) and under direct depolarization with KCl (3 mM glucose + 30 mM KCl) (Figure 16A-C). However, there were no observable differences in insulin secretion from WT and BaxBak DKO islets under any of the conditions tested.

Islet insulin content was assayed after 24 and 48 hours of high glucose and glucolipotoxic conditions. Islet insulin content was similar between WT and BaxBak DKO islets after 24 and 48 hours of high glucose (25 mM) culture (Figure 16D,E). However, 24 hours of glucolipotoxic treatment significantly reduced islet insulin content in both WT and

BaxBak DKO islets. An even greater reduction in islet insulin content was seen after 48 hours. These glucolipotoxic effects on islet insulin content did not differ between WT and BaxBak DKO islets (Figure 16D,E). Taken together these data confirm that glucolipotoxicity significantly reduces β-cell insulin content and impairs insulin secretion, and furthermore establish that these effects are not mediated by Bax and Bak or are affected by their combined deletion. Although Bax and Bak may regulate insulin mRNA levels under high glucose conditions, they do not seem to regulate the detrimental effects of glucolipotoxicity on β-cell secretory function to any detectable degree.

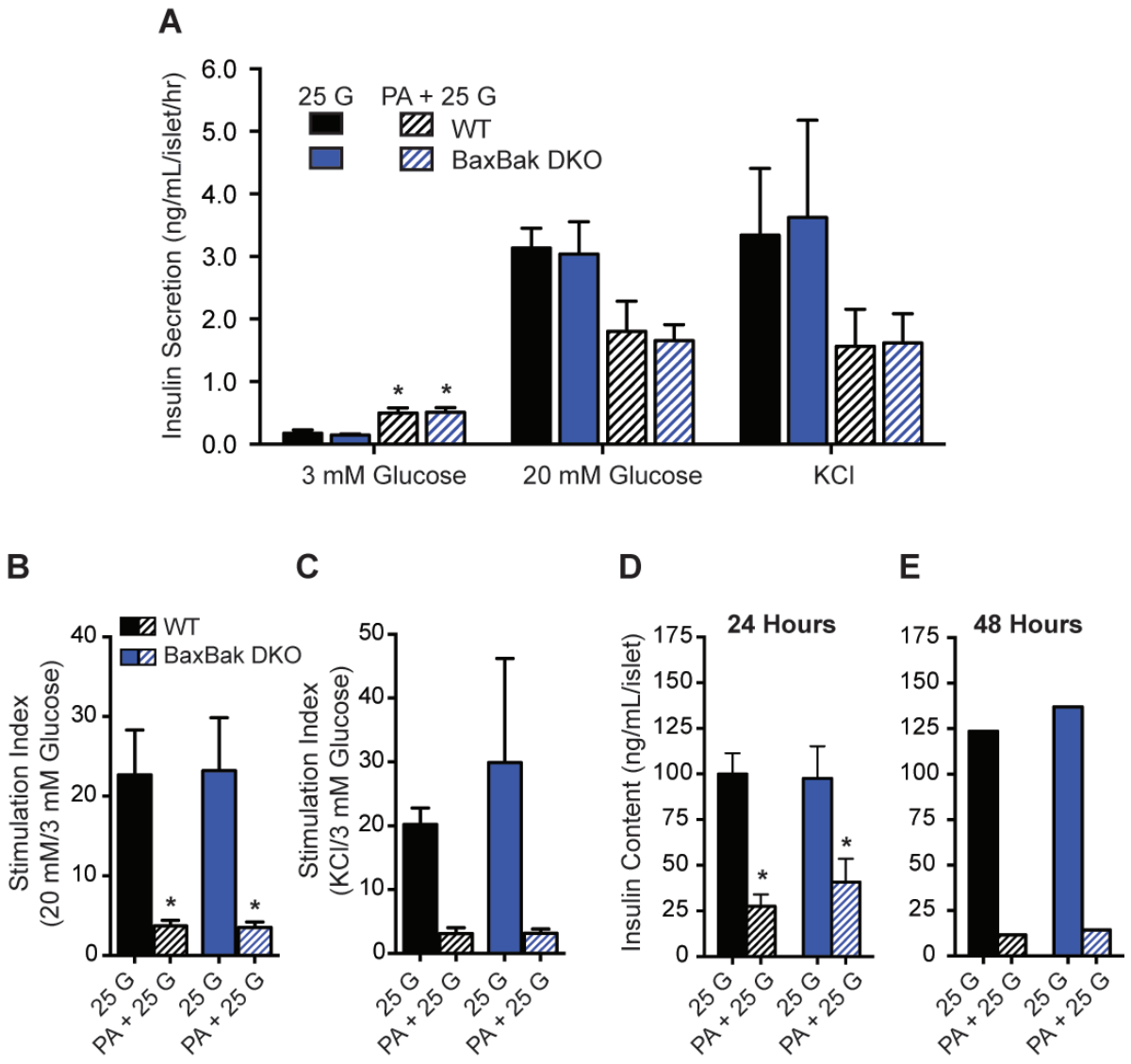

Figure 16: Effects of Glucolipotoxicity on Glucose- and KCl-Stimulated Insulin Secretion and Insulin Content. Whole islets from WT and BaxBak DKO mice subjected to 25 mM glucose (25 G) control conditions or 25 mM glucose plus 1.5 mM palmitate (PA + 25 G) for 24 or 48 hours. (A) Glucose-stimulated insulin secretion after 24 hours of treatment (n=4-5). 3 mM and 20 mM glucose stimulation were for one hr, KCl (3 mM glucose + 30 mM KCl) stimulation for 30 minutes (KCl secretion values shown x2). Fold increase of (B) 20 mM glucose-stimulated insulin secretion and (C) KCl-stimulated insulin secretion over 3 mM glucose basal insulin secretion. (D,E) Islet insulin content after 24 hours (n=4) and 48 hours (n=2) of treatment. (Data are mean ± SEM *$p<0.05$ vs respective control).

Chapter 4: Discussion

4.1 Bax-Bak Knockout Model

To better understand the mechanisms by which β-cells undergo functional failure and death in the context of diabetes we investigated the roles of the pro-apoptotic Bcl-2 family proteins Bax and Bak in β-cell death and ER-stress signalling. We have established a mouse model in which the combined and individual roles of Bax and Bak can be studied in the adult β-cell. By combining a β-cell-specific and inducible Bax knockout with the global Bak knockout we were able to bypass the detrimental developmental effects encountered with a global double knockout mouse model [118]. These are the first studies to use an *in vivo* loss of function model to examine the effects of double Bax and Bak knockout on the pancreatic β-cell. We have shown that Bax and Bak have both combined and individual roles in regulating β-cell apoptosis depending on the cell stress encountered, indicating these pro-apoptotic proteins work in concert with each other to mediate cellular apoptosis. We have also provided evidence to show that Bax and Bak have roles in the β-cell UPR by regulating IRE1α splicing of XBP1 upstream of apoptosis activation. Collectively, we have provided insight into stress-specific mechanisms of β-cell death and show that the pro-apoptotic proteins Bax and Bak have non-apoptotic roles in the UPR that may be unique to the β-cell.

To investigate the roles of Bax and Bak in the adult β-cell, we generated two background-matched sister lines of mice in which the individual or combined knockout of Bax and Bak could be achieved. With a daily tamoxifen injection regime, we achieve over 80% knockdown of Bax mRNA and protein in the islets of mice carrying the floxed Bax alleles and expressing Cre recombinase. The residual 20% of Bax expression observed is

presumed to be associated with other islet cell types that have not undergone recombination, mainly α-cells and to some extent δ- and PP-cells [141,142]. Therefore we can conclude that we achieve near maximal Bax and Bak ablation in mouse β-cells after tamoxifen injection. Due to evidence showing that another β-cell specific inducible recombination system, the RIP-CreER mouse, undergoes high genetic recombination in the absence of tamoxifen administration [143], we investigated whether the Pdx1-CreER used in our mouse model [129] could elicit Bax recombination in the absence of tamoxifen ligand. The Pdx1-CreER used in our mouse model was previously investigated to show only very slight (<5%) "spontaneous" recombination in 4 month old mice [143], and a report on a similar Pdx1-CreER mouse model suggested a slight "leakiness" (~1% of total cells) in small islet cell clusters [127]. Our data shows that by 3 months of age there is statistically non-significant evidence of tamoxifen-independent activation of Pdx1-CreER; however, significantly higher recombination rates are observed as animals age. Further investigation is required behind the limitations of ligand-dependent CreER systems, as our data suggest these temporal specific models could be compromised depending on genetic background of the Cre-target lines and the age at which animals are being studied.

Bax and/or Bak knockout mice are phenotypically normal compared to WT littermates and islet function does not appear to be affected by either the global Bak knockout or inducible Bax knockout. Intraperitoneal glucose tolerance tests showed no marked differences between genders or genotypes, indicating there is no baseline effect of Bak knockout on target tissues and that Bak is not required for proper islet development and function, and when combined with the induced ablation of Bax does not affect islet function under physiological conditions. These results strongly suggest Bax and/or Bak deletion also

does not induce differences in insulin tolerance or insulin secretion *in vivo*. In isolated islets *in vitro,* we did not observe any functional abnormalities in insulin message, content, or secretion, further confirming that Bax and Bak are not required for basal islet physiology under non-stressed conditions. However, BaxBak DKO β-cells displayed slightly decreased mitochondrial membrane potential compared to WT β-cells, suggesting that one or both of these proteins may be involved in the regulation mitochondrial integrity under physiological conditions. This observation is in agreement with a recent study demonstrating that a small portion of mitochondria-associated Bax is required to maintain proper mitochondrial bioenergetics under non-apoptotic conditions in HCT-116 cells and primary hepatocytes [131]. However, our data suggest such roles of Bax and/or Bak in β-cells do not reach an extent that changes overall Ca^{2+} and insulin secretion responses to a glucose stimulus.

4.2 Bax and Bak in β-Cell Death

Bax and Bak have been extensively studied as key regulators of apoptosis. As BaxBak DKO mice rarely survive past birth, combined global Bax and Bak knockout has been difficult to study in adult cell types and has been limited to MEF and CHO cells [59,60]. The temporal control of Bax knockout makes it possible to study the combined double knockout of Bax and Bak in adult tissue types, which so far has been studied in hepatocytes, neurons, T-cells, and B-cells [99,128,144,145]. So far, there have been only few investigations of Bax and Bak knockout in the β-cell, and while these have only focused on single gene deletion they have provided some indications of stress specific roles for these proteins in the β-cell [53,117]. Originally thought to have redundant roles, we have shown that Bax and Bak have both individual and combined roles in regulating apoptosis depending on the cell stress

encountered. Here we have shown that Bax and Bak have individual roles regulating apoptosis in the β-cell under staurosporine treatment, but have combined roles when regulating cell death via glucolipotoxicity. Time-course analysis of β-cell death by staurosporine treatment shows that Bax and Bak have different susceptibilities to staurosporine-induced stress. Although the ablation of Bax and/or Bak promotes β-cell survival, Bak SKO β-cells are much more resistant to staurosporine than Bax SKO β-cells, suggesting that Bak plays a major role in initiating cell death under staurosporine treatment. Previously it was demonstrated that overexpressing Bak sensitized cells to staurosporine [146], further supporting that Bax and Bak may have individual susceptibilities to various stress conditions.

We did not observe detectable β-cell death after 3-days in either high glucose (25 mM) or lipotoxic (5 mM glucose + 1.5 mM palmitate) culture. One of the prevailing views on gluco- and lipo-toxicity is that the β-cell can cope with either one alone through substrate detoxification, but the combination of both is detrimental to the β-cell [9,38]. High glucose can accentuate the toxicity of lipids by impairing the FFA detoxification process. Complex, unoxidized FFAs accumulate within the cytoplasm leading to cellular toxicity and eventual death [147]. The glucose-dependent toxicity of FFA has been supported by studies showing that palmitate only impairs insulin expression, content, and elicits β-cell death when in complex with stimulatory glucose levels [139,148-150]. These results support why we detect no β-cell death in lipotoxic conditions, as our lipotoxic culture was only in basal glucose (5 mM glucose) conditions. Under glucolipotoxic conditions (25 mM glucose + 1.5 mM palmitate) we detect β-cell death that was comparable between WT, Bax SKO, and Bak SKO β-cells, whereas BaxBak DKO β-cells were less susceptible to glucolipotoxic stimuli. This suggests that Bax

and Bak play similar roles in glucolipotoxic cell death, further indicating that variable forms of cell stress can initiate differential induction of Bax and Bak. While the deleterious effects of high glucose and high FFA alone are potentiated when in combination, other mechanisms of glucolipotoxic-induced cell death include FFA production of ceramides that lead to a decrease in Bcl-2 expression and an increase in apoptosis [151], as well as increases in both oxidative and ER-stress [32,152,153]. The decrease, but still detectible amounts of β-cell death observed only in BaxBak DKO islets could be the result of multiple stress pathways, such as the ones listed above, activated in the presence of glucolipotoxicity. Since the mechanisms by which cell death is activated under various types of stress remains unclear, there is a possibility that the cell death observed in BaxBak DKO islets could be other forms of non-apoptotic cell death. Also, the balance between pro- and anti-apoptotic Bcl-2 family members under each stress pathway activated may contribute to the overall amount of cell death seen in our WT, SKO, and DKO β-cells under glucolipotoxic conditions. Further work should focus on the mechanistic activation of Bax and Bak and cell death under the multiple signalling pathways that could contribute to glucolipotoxic cell death.

It was previously shown that $Bax^{-/-}$ islets displayed increased resistance to 24 hours of glucolipotoxicity compared to WT islets, whereas we showed that Bax SKO islets were not different from WT controls [117]. However, the glucolipotoxic conditions in that study used a 15 mM glucose concentration and a 2:1 ratio of the unsaturated fatty acid oleate with the saturated FFA palmitate. Oleate is shown to be less cytotoxic than palmitate, and some evidence suggests oleate may have protective effects against palmitate-induced cytotoxicity [153-155]. Our studies used a higher glucose concentration (25 mM) in combination with only the saturated FFA palmitate. Despite the glucolipotoxic preparation differences, there was a

trend in our studies towards decreased cell death in Bax SKO, Bak SKO, and BaxBak DKO islets compared to WT controls during early (18-24 hours) culture in glucolipotoxic stress. As glucolipotoxic stress continued over the 58 hour time-course, the overall protective effect continued only in BaxBak DKO islets. This observation highlights the advantage of performing kinetic cell death analyses, as small trends in cell-death are more easily observed that could be hidden in end-point analysis studies.

Previous studies have reported that BaxBak DKO cells are resistant to multiple forms of apoptotic stimuli [59,60]. Most reports provide a single snapshot of BaxBak DKO resistance to cell death compared to their WT controls at a select time, leaving the notion that BaxBak DKO cells are indefinitely protected. In our time-course analyses of islet-cell death, we showed that BaxBak DKO islet-cells eventually succumb to cell death in the case of staurosporine treatment, or are only partially protected from cell death under glucolipotoxic treatment. This indicates that inhibition of Bax and Bak can indeed provide β-cell protection, though chronic stress in the absence of Bax and Bak may ultimately lead to the activation of alternate death mechanisms, such as necrosis, necroptosis, or autophagy.

Work from Mckenzie *et al.* (2010) has previously demonstrated that severe glucotoxicity induces cell death preferentially via Bax. In combination with our work, showing that staurosporine induces cell death preferentially via Bak and glucolipotoxicity induces cell death through both Bax and Bak, this suggests that Bax and Bak have both combined and individual roles in the execution of β-cell death under various stress conditions. Further work should investigate the means by which Bax and Bak are differentially activated, focusing both on the mechanisms by which apoptosis is signaled

under various stress conditions as well as the activation of Bax and Bak by other specific Bcl-2 family members.

4.3 Bax and Bak in ER-Stress

Recent evidence has pointed to roles for the Bcl-2 family proteins in the ER-stress response [65,99]. We investigated the time-course expression of CHOP and XBP1s as indicators of UPR activation. UPR signalling preceded the upregulation of Bax after chronic ER-stress, providing another link between ER-stress and apoptosis. However, how Bax becomes upregulated after chronic ER-stress still remains unknown. Here we show that XBP1s expression is augmented under early stages of ER-stress in BaxBak DKO islets compared to WT controls. However, after chronic ER-stress XBP1s expression is comparable between WT and BaxBak DKO islets. The augmentation of XPB1s expression in BaxBak DKO islets is observed under both acute thapsigargin and in the early stages of glucolipotoxic stress. On the other hand, time-dependent CHOP expression increased similarly in all knockout and WT islets during stress, indicating that Bax and Bak do not significantly regulate the PERK arm of the UPR.

Using co-immunoprecipitation, Hetz *et al.* (2006) previously demonstrated that Bax and Bak can bind to the C-terminus of IRE1α in MEFs, suggesting direct roles in IRE1α regulation. Similar to our findings, they reported that Bax and Bak deletion in both liver cells and MEFs had no effect on CHOP and PERK signalling. However, BaxBak DKO livers and MEFs displayed reduced or ablated XBP1 protein expression, along with attenuated phosphorylation of JNK, indicative of *decreased* IRE1α activity. The conclusions of the Hetz *et al.* (2006) study suggest that Bax and Bak stabilize IRE1α, thereby enhancing stress-

reducing effects of XBP1s and promoting cell survival during early stages of ER-stress [156]. Such protective roles of Bax and Bak at the ER would seem to counteract their apoptotic roles at the mitochondria. In contrast to what was observed in hepatocytes and MEFs, our finding that BaxBak DKO islets have augmented XBP1s mRNA expression is indicative of *enhanced* IRE1α signalling. This suggests that in β-cells, endogenous Bax and Bak may normally suppress IRE1α-mediated splicing of XBP1, and thereby impede stress-adaptation, during the early UPR [157]. While these apparent differences suggests cell-specific roles of Bax and Bak in UPR signalling, it remains possible that the discrepancy between our results and those of Hetz *et al.* (2006) lie in the separation between message and protein expression. XBP1s message was not investigated in the Hetz study, and we have yet to examine protein expression for XBP1s, activated IRE1α, and phosphorylated JNK in our BaxBak DKO and WT islets.

To further explore the possibility that Bax and Bak regulate adaptive signals in β-cells under ER stress, we also investigated the expression of downstream UPR target genes. The UPR chaperone BIP and the oxidoreductase ERO1L both increased upon ER-stress induction and trended towards increased expression in BaxBak DKO islets relative to WT controls. The specific XBP1s target ERDJ4 was significantly upregulated in BaxBak DKO islets under early ER-stress compared to WT controls. Small sample size and low mRNA content were a limitation to this study and it requires further examination, but taken together these data suggest that the early augmented XBP1s expression seen in BaxBak DKO islets under acute ER-stress may cause earlier induction of downstream UPR target genes to better help with adaptation to ER-stress.

Taken together, these data suggest that the pancreatic β-cell may have differences in UPR signalling compared to other cell types. The β-cell is a fairly quiescent and long-living cell and is prone to ER-stress from the vast amount of protein processing it must execute throughout its lifespan. This inherent functional difference between β-cells and the MEFs and liver cells that have been examined to date, suggests that the mechanisms regulating ER-stress and apoptosis may be unique to the β-cell, although this has yet to be fully explored.

4.4 Potential Mechanisms for BaxBak DKO Protection Under Glucolipotoxic Stress

Our data demonstrated that BaxBak DKO islets are resistant to glucolipotoxic cell death, and showed indications of increased adaptive UPR signalling under early stages of ER-stress. To investigate the potential protective effects of the altered UPR signalling, we examined if Bax and Bak deletion affected the impact of glucolipotoxic conditions on β-cell function. It has been well studied that FFA increase basal insulin secretion and decrease glucose-stimulated insulin secretion, insulin content, and insulin biosynthesis [33,37,138-140]. In agreement with this we found that WT and BaxBak DKO islets exposed to 24 hours of glucolipotoxic conditions showed perturbations in insulin content and insulin secretion. Both WT and BaxBak DKO islets had increased basal insulin secretion and decreased glucose-stimulated insulin secretion. Insulin content and insulin transcription were also decreased under glucolipotoxic conditions in both WT and BaxBak DKO islets. However, no differences were observed between WT and BaxBak DKO islets suggesting that the augmentation of early UPR signalling does not preserve insulin processing or secretion under the stress conditions tested. Consequently, the improved survival of BaxBak DKO β-cells is not a result of retaining crucial aspects of β-cell function.

Although knockout of Bax and Bak did not preserve islet function under glucolipotoxic conditions, other unexplored mechanisms may contribute to the pro-survival effects of Bax and Bak knockout in β-cells. It has been previously shown that Bcl-2 family proteins regulate ER Ca^{2+} homeostasis, with Bax and Bak promoting increased ER Ca^{2+} levels and subsequently increased Ca^{2+} loading onto the mitochondria under apoptotic conditions [98,106,108,111]. Inferring data from these studies, our BaxBak DKO β-cells might also display reduced ER Ca^{2+} levels, which could ultimately affect ER homeostasis and UPR activation. Exploring stress-induced ER Ca^{2+} dynamics could elucidate whether this plays a role in the observed differences in UPR signalling between WT and BaxBak DKO islets. The evidence that Bax and Bak modulate IRE1α signalling further indicates that JNK signalling may also vary between BaxBak DKO and WT islets. JNK can regulate Bcl-2 family proteins to signal apoptosis [21,22,94,95], and might contribute to the differences observed in β-cell death between BaxBak DKO and WT islets, making it an important topic for further study.

Overall, the reduction in cell death observed in BaxBak DKO islets may be explained by reduced sensitivity of BaxBak DKO islets to apoptotic stimuli. Taken together, our results suggest that a major component of this protection results from the loss of the mitochondrial apoptosis 'executioners'. In addition, our findings provide novel evidence that Bax and Bak may regulate the ER-derived stress signals that ultimately trigger their own apoptotic functions at the mitochondria.

Chapter 5: Conclusion

In this study, we established a line of conditional and inducible Bax and Bak knockout mice to study the individual and combined roles of the pro-apoptotic Bax and Bak proteins in the pancreatic β-cell. Global ablation of Bak and/or induced knockout of Bax does not affect physiological islet function both *in vivo* and *in vitro*. However, under apoptotic conditions Bax and Bak have both individual and combined contributions to β-cell death in response to various apoptotic stimuli. We also provided evidence for non-apoptotic roles for Bax and Bak in the ER-stress response, as BaxBak DKO islets display augmented XBP1s expression under early ER-stress signalling. While these data suggest that Bax and Bak suppress maximal IRE1α splicing of XBP1 under early UPR signalling, it remains to be established if this aspect of Bax and Bak function significantly affects β-cell function or survival in response to stress. Overall, the knockout of both Bax and Bak is beneficial for β-cell stress signalling and survival, highlighting that these proteins may be important targets for diabetic therapies where maintaining islet survival is crucial.

References

1. Zimmet, P., Alberti, K. & Shaw, J. Global and societal implications of the diabetes epidemic. *Nature* **414**, 782–787 (2001).
2. Shaw, J. E., Sicree, R. A. & Zimmet, P. Z. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice* **87**, 4–14 (2010).
3. Wild, S., Roglic, G., Green, A., Sicree, R. & King, H. Global prevalence of diabetes. *Diabetes Care* **27**, 1047–1053 (2004).
4. Doucet, G. & Beatty, M. The cost of diabetes in Canada: The economic tsunami. *Canadian Diabetes Association* **34**, 27–29 (2009).
5. Forbes, J. M. & Cooper, M. E. Mechanisms of diabetic complications. *Physiological Reviews* **93**, 137–188 (2013).
6. Tominaga, M. *et al.* Impaired glucose tolerance is a risk factor for cardiovascular disease, but not impaired fasting glucose. *Diabetes Care* **22**, 920–924 (1999).
7. Ryden, L. & Mellbin, L. Glucose perturbations and cardiovascular risk: Challenges and opportunities. *Diabetes and Vascular Disease Research* **9**, 170–176 (2012).
8. Ohinmaa, A., Jacobs, P., Simpson, S. & Johnson, J. A. The projection of prevalence and cost of diabetes in Canada: 2000 to 2016. *Canadian Journal of Diabetes* **28**, 1–8 (2004).
9. Prentki, M. Islet beta-cell failure in type 2 diabetes. *J. Clin. Invest.* **116**, 1802–1812 (2006).
10. Kahn, B. B. Type 2 diabetes: when insulin secretion fails to compensate for insulin resistance. *Cell* **92**, 593–596 (1998).
11. Leahy, J. L. Pathogenesis of type 2 diabetes mellitus. *Archives of Medical Research* **36**, 197–209 (2005).
12. Barnett, A. H., Eff, C., Leslie, R. D. G. & Pyke, D. A. Diabetes in identical twins. *Diabetelogia* **20**, 87–93 (1981).
13. Boerwinkle, E. *et al.* A genome-wide search for human non-insulin-dependent (type 2) diabetes genes reveals a major susceptibility locus on chromosome 2. *Nature* **13**, 161–166 (1996).
14. Ho, M. M. *et al.* Diabetes genes identified by genome-wide association studies are regulated in mice by nutritional factors in metabolically relevant tissues and glucose concentrations in islets. *BMC Genet* **14**, 10 (2013).
15. Rhodes, C. J. Type 2 diabetes: A matter of beta-cell life and death? *Science* **307**, 380–384 (2005).
16. Cnop, M. *et al.* Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes. *Diabetes* **54**, S97–S107 (2005).
17. Butler, A. E. *et al.* Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* **52**, 102–110 (2003).
18. Deng, S. *et al.* Structural and functional abnormalities in the islets from type 2 diabetic subjects. *Diabetes* **53**, 624–632 (2004).
19. Eizirik, D. L., Cardozo, A. K. & Cnop, M. The role for endoplasmic reticulum stress in diabetes mellitus. *Endocrine Reviews* **29**, 42–61 (2008).

20. Poitout, V. & Robertson, R. P. Glucolipotoxicity: fuel excess and beta-cell dysfunction. *Endocrine Reviews* **29**, 351–366 (2007).
21. Malhotra, J. D. & Kaufman, R. J. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or double edged sword? *Antioxidants and Redox Signaling* **9**, 2277–2293 (2007).
22. Back, S. H. & Kaufman, R. J. Endoplasmic reticulum stress and type 2 diabetes. *Annu. Rev. Biochem.* **81**, 767–793 (2012).
23. Johnson, J. D. & Luciani, D. S.in *The Islets of Langerhans, Advances in Experimental Medicine and Biology* **654**, 447–462 (Springer Science, 2010).
24. Lowell, B. B. Mitochondrial Dysfunction and Type 2 Diabetes. *Science* **307**, 384–387 (2005).
25. Brownlee, M. A radical explanation for glucose-induced beta-cell dysfunction. *J. Clin. Invest.* **112**, 1821–1830 (2003).
26. Lenzen, S. Oxidative stress: the vulnerable β-cell. *Biochem. Soc. Trans* **36**, 343 (2008).
27. Ehses, J. A. *et al.* Increased Number of Islet-Associated Macrophages in Type 2 Diabetes. *Diabetes* **56**, 2356–2370 (2007).
28. Ehses, J. A., Ellingsgaard, H., Böni-Schnetzler, M. & Donath, M. Y. Pancreatic islet inflammation in type 2 diabetes: From α and β cell compensation to dysfunction. *Archives of Physiology and Biochemistry* **115**, 240–247 (2009).
29. Boni-Schnetzler, M. *et al.* Increased interleukin (IL)-1beta messenger ribonucleic acid expression in beta-cells of individuals with type 2 diabetes and regulation of IL-1beta in human islets by glucose and autostimulation. *Journal of Clinical Endocrinology & Metabolism* **93**, 4065–4074 (2008).
30. Meng, F. *et al.* The Sulfated Triphenyl Methane Derivative Acid Fuchsin Is a Potent Inhibitor of Amyloid Formation by Human Islet Amyloid Polypeptide and Protects against the Toxic Effects of Amyloid Formation. *Journal of Molecular Biology* **400**, 555–566 (2010).
31. Potter, K. J. *et al.* Islet amyloid deposition limits the viability of human islet grafts but not porcine islet grafts. *PNAS* **107**, 4305–4310 (2010).
32. Laybutt, D. R. *et al.* Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. *Diabetologia* **50**, 752–763 (2007).
33. Lupi, R. *et al.* Prolonged exposure to FFA has cytostatic and pro-apoptotic effects on human pancreatic islets. *Diabetes* **51**, 1437–1442 (2002).
34. Marchetti, P. *et al.* The endoplasmic reticulum in pancreatic beta cells of type 2 diabetes patients. *Diabetologia* **50**, 2486–2494 (2007).
35. Fonseca, S. G., Burcin, M., Gromada, J. & Urano, F. Endoplasmic reticulum stress in β-cells and development of diabetes. *Current Opinion in Pharmacology* **9**, 763–770 (2009).
36. Federici, M. *et al.* High glucose causes apoptosis in cultured human pancreatic islets of langerhans. *Diabetes* **50,** 1290–1301 (2001).
37. Jeffrey, K. D. *et al.* Carboxypeptidase E mediates palmitate induced beta-cell ER stress and apoptosis. *PNAS* **105**, 8452–8457 (2008).
38. Kim, J.-W. & Yoon, K.-H. Glucolipotoxicity in Pancreatic β-Cells. *Diabetes Metab J* **35**, 444 (2011).

39. Poitout, V. *et al.* Glucolipotoxicity of the pancreatic beta cell. *BBA - Molecular and Cell Biology of Lipids* **1801**, 289–298 (2010).
40. Cnop, M. Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes. *Biochem. Soc. Trans* **36**, 348-352 (2008).
41. Kerr, J., Wyllie, A. H. & Currie, A. R. Apoptosis a basic biological phenomenon with wide ranging implications in tissue kinetics. *British Journal of Cancer* **26**, 239–257 (1972).
42. Taylor, R. C., Cullen, S. P. & Martin, S. J. Apoptosis: controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology* **9**, 231–241 (2008).
43. Tait, S. W. G. & Green, D. R. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nature Reviews Molecular Cell Biology* **11**, 621–632 (2010).
44. Savill, J. & Fadok, V. Corpse clearance defines the meaning of cell death. *Nature* **407**, 784–788 (2000).
45. Aouacheria, A. Phylogenomics of Life-Or-Death Switches in Multicellular Animals: Bcl-2, BH3-Only, and BNip Families of Apoptotic Regulators. *Molecular Biology and Evolution* **22**, 2395–2416 (2005).
46. Youle, R. J. & Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. *Nature Reviews Molecular Cell Biology* **9**, 47–59 (2008).
47. Lutz, R. J. Role of the BH3 (Bcl-2 homology 3) domain in the regulation of apoptosis and Bcl-2-related proteins. *Biochem. Soc. Trans* **28**, 51–56 (2000).
48. Hardwick, J. M. & Youle, R. J. SnapShot: BCL-2 Proteins. *Cell* **138**, 404.e1–404.e2 (2009).
49. Westphal, D., Dewson, G., Czabotar, P. E. & Kluck, R. M. Molecular biology of Bax and Bak activation and action. *BBA - Molecular Cell Research* **1813**, 521–531 (2011).
50. Lüthi, A. U. & Martin, S. J. The CASBAH: a searchable database of caspase substrates. *Cell Death Differ* **14**, 641–650 (2007).
51. Zou, H., Henzel, W., Liu, X., Lutschg, A. & Wang, X. Apaf-1 a human protein homologous to c elegans CED4 participates in cytochrome c dependent activation of caspase 3. *Cell* **90**, 405–413 (1997).
52. Li, P. *et al.* Cytochrome c and dATP dependent formation of apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479–489 (1997).
53. McKenzie, M. D. *et al.* Glucose Induces Pancreatic Islet Cell Apoptosis That Requires the BH3-Only Proteins Bim and Puma and Multi-BH Domain Protein Bax. *Diabetes* **59**, 644–652 (2010).
54. Meng, X. W. *et al.* High Cell Surface Death Receptor Expression Determines Type I Versus Type II Signaling. *Journal of Biological Chemistry* **286**, 35823–35833 (2011).
55. Wei, M. C. *et al.* tBID a membrane targeted death ligand oligomerizes bak to release cytochrome c. *Genes & Development* **14**, 2060–2071 (2000).
56. Kim, H. *et al.* Stepwise Activation of BAX and BAK by tBID, BIM, and PUMA Initiates Mitochondrial Apoptosis. *Molecular Cell* **36**, 487–499 (2009).
57. Dewson, G. & Kluck, R. M. Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. *Journal of Cell Science* **122**, 2801–2808 (2009).

58. George, N. M., Evans, J. J. D. & Luo, X. A three-helix homo-oligomerization domain containing BH3 and BH1 is responsible for the apoptotic activity of Bax. *Genes & Development* **21**, 1937–1948 (2007).
59. Wei, M. *et al.* Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292**, 727–730 (2001).
60. Cost, G. J. *et al.* BAK and BAX deletion using zinc-finger nucleases yields apoptosis-resistant CHO cells. *Biotechnol. Bioeng.* **105**, 330–340 (2009).
61. Dewson, G. *et al.* To Trigger Apoptosis, Bak Exposes Its BH3 Domain and Homodimerizes via BH3:Groove Interactions. *Molecular Cell* **30**, 369–380 (2008).
62. Griffiths, G. J. *et al.* Cell damage induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. *The Journal of Cell Biology* **144**, 903–914 (1999).
63. Cheng, E. H. Y. VDAC2 Inhibits BAK Activation and Mitochondrial Apoptosis. *Science* **301**, 513–517 (2003).
64. Gross, A., Jockel, J., Wei, M. C. & Korsmeyer, S. J. Enforced dimerization of BAX results in its translocation mitochondrial dysfunction anda poptosis. *EMBO* **17**, 3878–3885 (1998).
65. Hetz, C. & Glimcher, L. The daily job of night killers: alternative roles of the BCL-2 family in organelle physiology. *Trends in Cell Biology* **18**, 38–44 (2008).
66. Gajkowska, B., Motyl, T., Olszewska-Badarczuk, H. & Godlewski, M. M. Expression of bax in cell nucleus after experimentally induced apoptosis revealed by immunogold and embedment-free electron microscopy. *Cell Biology International* **25**, 725–733 (2001).
67. Lindsay, J., Esposti, M. D. & Gilmore, A. P. Bcl-2 proteins and mitochondria—Specificity in membrane targeting for death. *BBA - Molecular Cell Research* **1813**, 532–539 (2011).
68. Letai, A. *et al.* Distinct BH3 domains either sensitize or activate mitochondrial apoptosis serving as prototype cancer therapeutics. *Cancer Cell* **2**, 183–192 (2002).
69. Berridge, M. J. The ER: A multifunctional signaling organelle. *Cell Calcium* **32**, 235–249 (2002).
70. Schuit, F. C., In't Veld, P. A. & Pipeleers, D. G. Glucose Stimulates Proinsulin Biosynthesis by a Dose-Dependent Recruitment of Pancreatic Beta Cells. *PNAS* **85**, 3865–3869 (1988).
71. Ron, D. & Walter, P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews Molecular Cell Biology* **8**, 519–529 (2007).
72. Schröder, M. & Kaufman, R. J. ER stress and the unfolded protein response. *Mutation Research* **569**, 29–63 (2005).
73. Harding, H. P. *et al.* An Integrated Stress Response Regulates Amino Acid Metabolism and Resistance to Oxidative Stress. *Molecular Cell* **11**, 619–633 (2003).
74. Oyadomari, S., Araki, E. & Mori, M. Endoplasmic reticulum stress mediated apoptosis in pancreatic beta-cells. *Apoptosis* **7**, 335–345 (2002).
75. McCullough, K. D., Martindale, J. L., Klotz, L. O., Aw, T. Y. & Holbrook, N. J. Gadd153 Sensitizes Cells to Endoplasmic Reticulum Stress by Down-Regulating Bcl2 and Perturbing the Cellular Redox State. *Molecular and Cellular Biology* **21**, 1249–1259 (2001).

76. Puthalakath, H. *et al.* ER Stress Triggers Apoptosis by Activating BH3-Only Protein Bim. *Cell* **129**, 1337–1349 (2007).
77. Merino, D. *et al.* The role of BH3-only protein Bim extends beyond inhibiting Bcl-2-like prosurvival proteins. *The Journal of Cell Biology* **186**, 355–362 (2009).
78. Marciniak, S. J. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes & Development* **18**, 3066–3077 (2004).
79. Song, B., Scheuner, D., Ron, D., Pennathur, S. & Kaufman, R. J. Chop deletion reduces oxidative stress, improves β cell function, and promotes cell survival in multiple mouse models of diabetes. *J. Clin. Invest.* **118**, 3378–3389 (2008).
80. Han, J. *et al.* ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat Cell Biol* **15**, 481–490 (2013).
81. Zinszner, H. *et al.* CHOP is implicated in programmed cell death in response to imapired funciton of the ER. *Genes & Development* **12**, 982–995 (1998).
82. Wagner, M. & Moore, D. D. Endoplasmic Reticulum Stress and Glucose Homeostasis. *Current Opinion Clinical Nutrition and Metabolic Care* **14**, 376–373 (2011).
83. Yoshida, H. *et al.* Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6-alpha and 6-beta that activates the mammalian unfolded protein response. *Molecular and Cellular Biology* **21**, 1239–1248 (2001).
84. Iwawaki, T. & Oikawa, D. The role of the unfolded protein response in diabetes mellitus. *Semin Immunopathol* **35**, 333–350 (2013).
85. Yamamoto, K., Ichijo, H. & Korsmeyer, S. J. Bcl-2 is phosphorylated and inactivated by ASK1/JNK protein kinase pathway normally activated at G2/M. *Molecular and Cellular Biology* **19**, 8469–8478 (1999).
86. Usui, M. *et al.* Atf6α-null mice are glucose intolerant due to pancreatic β-cell failure on a high-fat diet but partially resistant to diet-induced insulin resistance. *Metabolism* **61**, 1118–1128 (2012).
87. Tirasophon, W., Welihinda, A. A. & Kaufman, R. J. A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. *Genes & Development* **12**, 1812–1824 (1998).
88. Oslowski, C. M. & Urano, F. A switch from life to death in endoplasmic reticulum stressed β-cells. *Diabetes, Obesity and Metabolism* **12**, 58–65 (2010).
89. Hollien, J. & Weissman, J. S. Decay of Endoplasmic Reticulum Localized mRNAs During the Unfolded Protein Response. *Science* **313**, 101–104 (2006).
90. Lipson, K. K., Ghosh, R. & Urano, F. The role of IRE1a in the degradation of insulin mRNA in pancreatic beta-cells. *Plos One* **3**, e1648 (2008).
91. Lipson, K. L. *et al.* Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1. *Cell Metabolism* **4**, 245–254 (2006).
92. Iwawaki, T., Akai, R. & Kohno, K. IRE1α disruption causes histological abnormality of exocrine tissues, increase of blood glucose level, and decrease of serum immunoglobulin level. *Plos One* **5**, e13052 (2010).

93. Lee, A.-H., Heidtman, K., Hotamisligil, G. S. & Glimcher, L. H. Dual and opposing roles of the unfolded protein response regulated by IRE1-alpha and XBP1 in proinsulin processing and insulin secretion. *PNAS* **108**, 8885–8890 (2011).
94. Urano, F. *et al.* Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287**, 664–666 (2000).
95. Widenmaier, S. B., Ao, Z., Kim, S. J., Warnock, G. & McIntosh, C. H. S. Suppression of p38 MAPK and JNK via Akt-mediated Inhibition of Apoptosis Signal-regulating Kinase 1 Constitutes a Core Component of the -Cell Pro-survival Effects of Glucose-dependent Insulinotropic Polypeptide. *Journal of Biological Chemistry* **284**, 30372–30382 (2009).
96. Szegezdi, E., Logue, S. E., Gorman, A. M. & Samali, A. Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* **7**, 880–885 (2006).
97. Lei, K. & Davis, R. J. JNK phosphorylation of Bim related members of the Bcl2 family induces Bax dependent apoptosis. *PNAS* **100**, 2432–2437 (2003).
98. Nutt, L. K. Bax and Bak Promote Apoptosis by Modulating Endoplasmic Reticular and Mitochondrial Ca2+ Stores. *Journal of Biological Chemistry* **277**, 9219–9225 (2002).
99. Hetz, C. *et al.* Proapoptotic bax and bak modulate the UPR by a direct interaction with IRE1a. *Science* **312**, 570–572 (2006).
100. Sha, H., He, Y., Yang, L. & Qi, L. Stressed out about obesity: IRE1a-XBP1 in metabolic disorders. *Trends in Endocrinology & Metabolism* **22**, 374–381 (2011).
101. Varadi, A., Molnar, E., Ostenson, C.-G. & Ashcroft, S. J. Isoforms of SERCA are differentially expressed in normal and diabetic islets of langerhans. *Biochemical Journal* **319**, 521–527 (1996).
102. Lee, B., Jonas, J.-C., Weir, G. C. & Laychock, S. G. Glucose regulates expression of IP3R isoforms in isolated rat pancreatic islets. *Endocrinology* **140**, 2173–2182 (1999).
103. Johnson, J. D. Ryanodine receptors in human pancreatic beta cells: localization and effects on insulin secretion. *The FASEB Journal* **18**, 878–880 (2004).
104. Oyadomari, S. *et al.* NO induced apoptosis in pancreatic beta cells is mediated by the ER stress pathway. *PNAS* **98**, 10845–10850 (2001).
105. Cardozo, A. K. *et al.* cytokines downregulate the SERCA and deplete ER calcium leading to induction of ER stress in pancreatic beta cells. *Diabetes* **54**, 452–461 (2005).
106. Oakes, S. A. *et al.* Proapoptotic Bax and Bak Regulate Type 1 IP3R and Ca2+ Leak from the ER. *PNAS* **102**, 105–110 (2005).
107. White, C. *et al.* The endoplasmic reticulum gateway to apoptosis by Bcl-XL modulation of the InsP3R. *Nat Cell Biol* **7**, 1021–1028 (2005).
108. Scorrano, L. BAX and BAK Regulation of Endoplasmic Reticulum Ca2+: A Control Point for Apoptosis. *Science* **300**, 135–139 (2003).
109. Oakes, S. A., Opferman, J. T., Pozzan, T., Korsmeyer, S. J. & Scorrano, L. Regulation of endoplasmic reticulum Ca2+ dynamics by proapoptotic BCL-2 family members. *Biochemical Pharmacology* **66**, 1335–1340 (2003).
110. Luciani, D. S. *et al.* Roles of IP3R and RyR Ca2+ Channels in Endoplasmic Reticulum Stress and -Cell Death. *Diabetes* **58**, 422–432 (2008).

111. Johnson, J. D., Bround, M. J., White, S. A. & Luciani, D. S. Nanospaces between endoplasmic reticulum and mitochondria as control centres of pancreatic β-cell metabolism and survival. *Protoplasma* **249**, 49–58 (2011).
112. Wang, X., Olberding, K. E., White, C. & Li, C. Bcl-2 proteins regulate ER membrane permeability to luminal proteins during ER stress-induced apoptosis. *Cell Death Differ* **18**, 38–47 (2010).
113. Zong, W.-X. *et al.* Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. *The Journal of Cell Biology* **162**, 59–69 (2003).
114. Nakagawa, T. *et al.* Caspase-12 mediates endoplasmic reticulum specific apoptosis and cytotoxicity by amyloid B. *Nature* **403**, 98–103 (2000).
115. Fischer, H., Koenig, U., Eckhart, L. & Tschachler, E. Human caspase 12 has acquired deleterious mutations. *Biochemical and Biophysical Research Communications* **293**, 722–726 (2002).
116. McKenzie, M. D. *et al.* Proapoptotic BH3-only protein Bid is essential for death receptor-induced apoptosis of pancreatic beta-cells. *Diabetes* **57**, 1284–1292 (2008).
117. Kim, S. J. Glucose-dependent insulinotropic polypeptide (GIP) stimulation of pancreatic beta-cell survival is dependent upon phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) signaling, inactivation of the forkhead transcription factor Foxo1, and down-regulation of *bax* expression. *Journal of Biological Chemistry* **280**, 22297–22307 (2005).
118. Lindsten, T. *et al.* The combined functions of proapoptotic Bcl2 family members Bak and Bax are essential for normal tissue development of multiple tissues. *Molecular Cell* **6**, 1389–1399 (2000).
119. Sauer, B. & Henderson, N. Site specific DNA recombination in mammalian cells by the cre recombinase of bacteriophage P1. *PNAS* **85**, 5166–5170 (1988).
120. Hayashi, S. & McMahon, A. P. Efficient Recombination in Diverse Tissues by a Tamoxifen-Inducible Form of Cre: A Tool for Temporally Regulated Gene Activation/Inactivation in the Mouse. *Developmental Biology* **244**, 305–318 (2002).
121. Nagy, A. Cre recombinase: the universal reagent for genome tailoring. *genesis* **26**, 99–109 (2000).
122. Rossant, J. & McMahon, A. 'Cre'-ating mouse mutants - a meeting review on conditional mouse genetics. *Genes & Development* **13**, 142–145 (1999).
123. Feil, R. *et al.* Ligand activated site-specific recombination in mice. *Proc Natl Acad Sci* **93**, 10887–10890 (1996).
124. Zhang, Y. *et al.* Inducible site-directed recombination in mouse embryonic stem cells. *Nucleic Acids Research* **24**, 543–548 (1996).
125. Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K. & McMahon, A. P. Modification of gene inactivity in mouse embryos *in utero* by a tamoxifen inducible form of cre recombinase. *Current Biology* **8**, 1323–1326 (1998).
126. Picard, D. Regulation of protein function through expression of chimaeric proteins. *Current Opinion in Biotechnology* **5**, 511–515 (1994).
127. Zhang, H., Fujitani, Y., Wright, C. V. E. & Gannon, M. Efficient recombination in pancreatic islets by a tamoxifen-inducible Cre-recombinase. *genesis* **42**, 210–217 (2005).
128. Takeuchi, O. *et al.* Essential role of Bax,Bak in B cell homeostasis and prevention of autoimmune disease. *PNAS* **102**, 11272–11277 (2005).

129. Gu, G., Dubauskaite, J. & Melton, D. A. Direct evidence for the pancreatic lineage NGN3 cells are islet progenitors and are distinct from duct progenitors. *Development* **129**, 2447–2457 (2002).
130. Luciani, D. S. *et al.* Bcl-2 and Bcl-xL suppress glucose signaling in pancreatic beta-cells. *Diabetes* **62**, 170–182 (2012).
131. Boohaker, R. J., Ge, Z., Carlson, A. L., Nemec, K. N. & Khaled, A. R. BAX supports the mitochondrial metwork, promoting bioenergetics in nonapoptotic cells. *American Journal of Physiology and Cellular Physiology* **300**, 1466–1478 (2011).
132. Karaman, M. W. *et al.* A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol* **26**, 127–132 (2008).
133. Posadas, I., Vellecco, V., Santos, P., Prieto-Lloret, J. & Ceña, V. Acetaminophen potentiates staurosporine-induced death in a human neuroblastoma cell line. *British Journal of Pharmacology* **150**, 577–585 (2009).
134. van Delft, M. F. & Huang, D. C. How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Res* **16**, 203–213 (2006).
135. Robertson, R. P., Harmon, J., Tran, P. O., Tanaka, Y. & Takahashi, H. Glucose toxicity in beta cells T2D good radicals gone bad and the glutathione connection. *Diabetes* **52**, 581–587 (2003).
136. Giacca, A., Xiao, C., Oprescu, A. I., Carpentier, A. C. & Lewis, G. F. Lipid-induced pancreatic beta-cell dysfunction: focus on in vivo studies. *AJP: Endocrinology and Metabolism* **300**, E255–E262 (2011).
137. Lee, A. H., Iwakoshi, N. N. & Glimcher, L. H. XBP-1 Regulates a Subset of Endoplasmic Reticulum Resident Chaperone Genes in the Unfolded Protein Response. *Molecular and Cellular Biology* **23**, 7448–7459 (2003).
138. Zhou, Y.-P. & Grill, V. E. Long term exposure of rat pancreatic islets to FFA inhibits glucose induced insulin secretion and biosynthesis through a glucose fatty acid cycle. *J. Clin. Invest.* **93**, 870–876 (1994).
139. Bollheimer, C. L., Skelly, R. H., Chester, M. W., McGarry, D. J. & Rhodes, C. J. Chronic exposure to FFA reduces beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. *J. Clin. Invest.* **101**, 1094–1101 (1998).
140. Iizuka, K. *et al.* Metabolic consequence of long-term exposure of pancreatic beta cells to FFA with special reference to glucose insensitivity. *Biochimica et biophysica* **1586**, 23–31 (2002).
141. Granot, Z. *et al.* LKB1 Regulates Pancreatic β Cell Size, Polarity, and Function. *Cell Metabolism* **10**, 296–308 (2009).
142. Fu, A. *et al.* Loss of Lkb1 in adult beta cells increases beta cell mass and enhances glucose tolerance in mice. *Cell Metabolism* **10**, 285–295 (2009).
143. Liu, Y. *et al.* Tamoxifen-Independent Recombination in the RIP-CreER Mouse. *Plos One* **5**, e13533 (2010).
144. Biswas, S. *et al.* A role for proapoptotic Bax and Bak in T-cell differentiation and transformation. *Blood* **116**, 5237–5246 (2010).
145. Reyes, N. A. *et al.* Blocking the mitochondrial apoptotic pathway preserves motor neuron viability and function in a mouse model of amyotrophic lateral sclerosis. *J. Clin. Invest.* **120**, 3673–3679 (2010).

146. Neise, D. *et al.* Activation of the mitochondrial death pathway is commonly mediated by a preferential engagement of Bak. *Oncogene* **27**, 1387–1396 (2008).
147. Prentki, M., Joly, E., El-Assaad, W. & Roduit, R. Malonyl CoA ignaling, lipid partitioning, and glucolipotoxicitiy. *Diabetes* **51**, S405–S413 (2002).
148. Jacqueminet, S., Briaud, I., Rouault, C., Reach, G. & Poitout, V. Inhibition of insulin gene expression by long-term exposure of pancreatic beta cells to palmitate is dependent on the presence of a stimulatory glucose concentration. *Metabolism* **49**, 532–536 (2000).
149. El-Assaad, W. Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology* **144**, 4154–4163 (2003).
150. Nolan, C. J. *et al.* Beta cell compensation for insulin resistance in Zucker fatty rats: increased lipolysis and fatty acid signalling. *Diabetologia* **49**, 2120–2130 (2006).
151. Maedler, K., Oberholzer, J., Bucher, P., Spinas, G. A. & Donath, M. Y. Monounsaturated FFA prevent the deleterious effects of palmitate and high glucose on human pancreatic beta cell turnover and function. **52**, 726–733 (2003).
152. Piro, S. *et al.* Chronic exposure to free fatty acids or high glucose induces apoptosis in rat pancreatic islets: Possible role of oxidative stress. *Metabolism* **51**, 1340–1347 (2002).
153. Gwiazda, K. S., Yang, T. L. B., Lin, Y. & Johnson, J. D. Effects of palmitate on ER and cytosolic Ca2+ homeostasis in beta-cells. *AJP: Endocrinology and Metabolism* **296**, E690–E701 (2009).
154. Karaskov, E. Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis. *Endocrinology* **147**, 3398–3407 (2006).
155. Maedler, K. *et al.* Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. *Diabetes* **50**, 69–76 (2001).
156. Hetz, C. & Glimcher, L. H. Fine-tuning of the unfolded protein response: assembling the IRE1-alpha interactome. *Molecular Cell* **35**, 551–561 (2009).
157. Lin, J. H. *et al.* IRE1 signaling affects cell fate during the UPR. *Science* **318**, 944–949 (2007).