Analysis of glucose homeostasis and beta cell mass in a mouse model of type 2 diabetes deficient in the pro-apoptotic protein BIM

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The University of Melbourne
This thesis is dedicated to
my dear husband Yuan,
our baby Max
and my parents
ABSTRACT

Loss of beta cell mass is a feature of type 2 diabetes and evidence suggests that apoptosis contributes to this loss. Previously in our lab, we identified that the pro-apoptotic BH3-only proteins BIM and PUMA are required for glucose-induced apoptosis of islet cells in vitro. High concentrations of glucose induce ER and oxidative stress that activate the downstream intrinsic apoptosis of beta cells through a BIM and PUMA dependent pathway. Islets isolated from human pancreas donors had increased expression of BIM in type 2 diabetic patients compared to non-diabetic pancreas donors. This suggests that BIM may play an important role in the loss of beta cell mass in humans as well. In this project, my aim was to investigate the role of the pro-apoptotic molecule BIM in loss of beta cell mass in vivo in type 2 diabetes. I tested the hypothesis that inhibiting BIM in type 2 diabetes will enhance islet survival and improve glucose homeostasis.

Chapter 2 describes the role of BIM in the type 2 diabetic Lepr\textsuperscript{db/db} model by studying metabolism. Global deficiency of BIM significantly protected Lepr\textsuperscript{db/db} mice by improving glucose homeostasis. Loss of BIM decreased non-fasting and fasting glucose, and improved insulin sensitivity and glucose tolerance in Lepr\textsuperscript{db/db} mice. Previous data from our lab suggest that whole body BIM deletion results in increased insulin sensitivity in muscle, liver and adipose tissue. This is due to a role for BIM in mitochondrial function, and a preference for BIM-deficient cells to utilize lipids in metabolism. Therefore, to examine the effects of BIM deficiency on beta cell death, we generated mice with beta cell specific deficiency of BIM. In contrast to global knockouts, BIM deficiency only in the beta cells of Lepr\textsuperscript{db/db} mice did not improve glucose homeostasis. These findings suggest that the improved metabolic phenotype of global BIM deficiency results from enhanced insulin sensitivity and not prevention of beta cell apoptosis.

In chapter 3, the role of BIM in beta cell survival in type 2 diabetes was studied in Lepr\textsuperscript{db/db} mice with global BIM deficiency or beta cell specific BIM deficiency. We observed a striking increase in islet size in BIM-deficient Lepr\textsuperscript{db/db} mice by histology,
while the beta-cell size was similar between Lepr$^{db/db}$BIM$^{-/-}$ and Lepr$^{db/db}$BIM$^{+/+}$ mice. This suggests that the increase in islet size was due to an increase in the number of beta cells. Quantification of islet volume with optical projection tomography revealed a significant increase in islet volume in BIM-deficient mice compared with wild-type Lepr$^{db/db}$ mice. We detected a reduced number of TUNEL positive islets cells in BIM-deficient compared with wild type Lepr$^{db/db}$ islets. We also observed about 2-fold increase in proliferating beta cells in Lepr$^{db/db}$BIM$^{-/-}$ mice compared to that in Lepr$^{db/db}$BIM$^{+/+}$ mice. However, there was no change in islet size in the Lepr$^{db/db}$ mice with beta cell specific deletion of BIM. These results indicate that BIM does not play a direct role in beta cell apoptosis in type 2 diabetic conditions, but its global inhibition generates a compensatory model by increasing beta cell mass, and this is likely due to the improved insulin sensitivity as a result of BIM inhibition.

Last, to test the hypothesis that BIM inhibition contributes to improvement of glucose homeostasis and beta cell expansion through regulating insulin sensitivity, we generated a model with severe insulin resistance using the insulin antagonist S961. Impaired glucose homeostasis and beta cell expansion was comparable in BIM-deficient and wild-type controls after S961 treatment. This indicates that BIM inhibition could not contribute to glucose homeostasis or beta cell expansion in the total absence of insulin sensitivity. This finding supports the idea that BIM’s function in regulating diabetes is related to insulin sensitivity.

Overall, my results in this thesis suggest that inhibition of BIM in beta cells only cannot prevent type 2 diabetes, however, global inhibition of BIM contributes to improvement of glucose homeostasis and beta cell expansion in type 2 diabetes through regulating insulin sensitivity.
DECLARATION

This is to certify that:

i) This thesis comprises only my original work towards the PhD except where indicated in the Preface.

ii) Due acknowledgement has been made in the text to all other material used.

iii) This thesis is less than 100,000 words in length, exclusive of tables, figures and references.

Jingjing Ge
PREFACE

My contribution to the results described in each of the chapters was as follows:

Chapter 2: 70%
Chapter 3: 80%
Chapter 4: 90%

I acknowledge the important contribution of others to the experiments in this thesis as follows:

**Chapter 2:** Jibran Wali, Christina Tan, Lorraine Elkerbout, Stacey Fynch, Lara Yachou-Wos, Megan Russell

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A1</td>
<td>BCL-2 related protein A1a</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>APAF1</td>
<td>Apoptotic protease activating factor 1</td>
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<tr>
<td>ATF6</td>
<td>Activating transcription factor-6</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<td>BAD</td>
<td>BCL-2-associated agonist of cell death</td>
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<td>BAK</td>
<td>BCL-2-antagonist/killer 1</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL-2-associated X protein</td>
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<td>BCL-2</td>
<td>B cell leukemia/lymphoma 2</td>
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<td>BCL2L11/BIM</td>
<td>BCL-2 interacting mediator of cell death</td>
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<tr>
<td>BID</td>
<td>BH3 interacting-domain death agonist</td>
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<td>BIK</td>
<td>BCL-2-interacting killer</td>
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<td>BMF</td>
<td>BCL-2 modifying factor</td>
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<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
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<tr>
<td>DAPI</td>
<td>Di-amidino-phenyl-indole</td>
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<td>DPP4</td>
<td>Dipeptidyl peptidase IV</td>
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<tr>
<td>DPX</td>
<td>Dibutylphthalate Polystyrene Xylene</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>FADD</td>
<td>Fas-associated protein with death domain</td>
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<td>FoxO</td>
<td>Forkhead box ‘Other’</td>
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<td>G-6-P</td>
<td>Glucose 6-phosphate</td>
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<td>Islet amyloid polypeptide</td>
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<td>Interleukin</td>
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<td>Inositol requiring enzyme-1</td>
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<td>IRR</td>
<td>Insulin receptor related receptor</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
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<td>JNK</td>
<td>Jun amino-terminal kinase</td>
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<td>LIRKO</td>
<td>liver-specific insulin receptor knockout</td>
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<td>Myeloid cell leukemia sequence 1</td>
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<td>MEFs</td>
<td>Murine embryonic fibroblasts</td>
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<td>OPT</td>
<td>Optical projection tomography</td>
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<td>P58</td>
<td>58-kDa protein</td>
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<td>PERK</td>
<td>PKR-like ER kinase</td>
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<td>Phorbol-12-myristate-13-acetate-induced protein</td>
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<td>PPAR</td>
<td>Peroxisome-proliferator–activated receptor</td>
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<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real-time PCR</td>
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<td>Site-1 protease</td>
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<td>Site-2 protease</td>
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<td>SAT</td>
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<tr>
<td>SGLT2</td>
<td>Sodium-glucose cotransporter 2</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
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<td>TRADD</td>
<td>TNF receptor type 1-associated death domain</td>
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<td>Treg</td>
<td>Regulatory T cell</td>
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<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end lableing</td>
</tr>
<tr>
<td>TZDs</td>
<td>Thiazolidinediones</td>
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<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<td>Visceral adipose tissue</td>
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Chapter 1.

Introduction and Literature Review
1.1 Type 2 diabetes

1.1.1 General pathogenesis and incidence of type 2 diabetes
Type 2 diabetes is a metabolic disorder of energy homeostasis. Hyperglycemia and hyperlipidemia occur as a result of beta cell failure to secrete adequate insulin to meet challenges caused by overnutrition, obesity and insulin resistance. This disease leaves a heavy burden to a nation’s healthcare system due to its rapidly growing prevalence and the severe damage it causes to many organs of the body. Thus, effective therapeutic strategies for type 2 diabetes are of great importance.

The global prevalence of diabetes has increased rapidly as a result of an ageing population, overnutrition and lack of proper exercise. The number of people with type 2 diabetes has more than doubled over the last three decades\(^1\). The latest estimate from the International Diabetes Federation is 415 million diabetic patients in 2015 and the number is estimated to rise to 642 million by 2040\(^2\). Among all diabetes, type 2 diabetes accounts for approximately 90\% of patients globally.

The heavy burden of type 2 diabetes comes from the long-term complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers and amputation. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease\(^3\).

1.1.2 Diabetes diagnosis criteria, current therapies and shortcomings
Diabetes is diagnosed by measurement of plasma glucose and glycated hemoglobin (HbA1C) levels. Diabetes is diagnosed when fasting plasma glucose (FPG) level surpasses 7.0 mmol/L or 2-hour plasma glucose level during an oral glucose tolerance test (OGTT) is above 11.1 mmol/L\(^4\). Diabetes may also be diagnosed when HbA1c is above or equal to 6.5\%. HbA1c test has some advantages compared to the FPG and OGTT such as more convenience (because fasting is not required) and less day to day variability. An important consideration when using HbA1c to diagnose diabetes, is that this is an indirect measurement of average blood glucose and other factors including age, race, and anemia might influence the test results\(^4,5\).
Management of type 2 diabetes has become increasingly complex with a wide choice of pharmacological agents available. Before therapeutic intervention, lifestyle changes are recommended to regulate glycemia and increase insulin sensitivity. Lifestyle interventions are a critical part of type 2 diabetes management. Lifestyle changes include planning meals, weight loss and exercising. When these preventative strategies fail, anti-diabetic drugs are required to gain glycemic control. The glycemia target that the American Diabetes Association (ADA) recommends to patients is lowering HbA1c to $<7\%$ in order to reduce the incidence of microvascular disease.

Metformin is the most widely used first-line drug for type 2 diabetes patients. The mechanism of action is attributed mainly to decreased hepatic glucose output and enhanced peripheral glucose uptake, although it also has lipid lowering effects in the liver due to suppression of lipogenesis. This drug has mild gastrointestinal side effects, is weight-neutral and does not increase the risk of hypoglycemia. However, metformin has side effects including nausea, vomiting, or dehydration, and its long-term use may be associated with vitamin B12 deficiency. When metformin is contraindicated or not tolerated by individuals, other classes of antidiabetic agents need be considered to replace metformin.

Another classic oral agent is sulfonylurea. These drugs stimulate insulin release by closing ATP-sensitive potassium channels on beta cells. Side effects such as modest weight gain and increased risk of hypoglycemia make this drug less popular. In addition, this drug is associated with deterioration of islet dysfunction.

Thiazolidinediones (TZDs) are peroxisome-proliferator–activated receptor (PPAR) $\gamma$ activators which have been reported to lower fasting, postprandial glucose concentrations and free fatty acid concentrations in clinical studies. The mechanism of action is to increase storage of fatty acids in adipocytes thus acting as an insulin sensitizer and also by inhibiting hepatic glucose production. TZDs do not increase the risk of hypoglycemia but have side effects including weight gain, oedema, heart failure and bone fracture.
Another class of glucose-lowering drugs is the sodium-glucose cotransporter 2 (SGLT2) inhibitors. These drugs act by inhibiting SGLT2 in the proximal nephron, hence decreasing glucose reabsorption\(^\text{15}\), which reduces the level of hyperglycemia. The mechanism is unique from other oral agents because it does not depend upon β-cell function or tissue insulin sensitivity, so SGLT2 inhibitors can be used in combination with any other oral antidiabetic drug as well as insulin\(^\text{15}\). Side effects include genital and urinary infections\(^\text{16}\).

There are also drugs that target the incretin system. Glucagon-like peptide-1 (GLP-1) receptor agonists or the oral dipeptidyl peptidase IV (DPP-4) inhibitors enhance the function of GLP-1 to stimulate insulin secretion and suppress glucagon function. They have low chance of hypoglycemia but can have mild nausea side effects. This class of antidiabetic agents has also been shown to expand β-cell mass in preclinical studies\(^\text{17}\).

When considering the application of the medications mentioned above, there are always five factors to think about: efficacy, hypoglycemia risk, weight, side effects and cost\(^\text{6}\). When monotherapy does not reduce HbA1c to the target range, proceeding to two-drug combinations will be required, and then when the target is again not reached, three-drug combinations including basal insulin will be applied. The last strategy is more complex, including multiple doses of insulin daily with one or two non-insulin antidiabetic agents\(^\text{18}\). Permanent use of insulin is inconvenient for the diabetic patient due to the requirement for multiple injections.

None of the drugs mentioned above has a direct role in regulation of beta cell mass, although loss of beta cell mass is one of the key features of type 2 diabetes. Therefore identifying ways to restore beta cell mass or inhibit the loss of beta cell mass is important for prevention and cure of type 2 diabetes. Restoration of beta cell mass will result in significantly enhanced insulin secretion, suppression of glucagon secretion and reduction of hepatic glucose production. Increased circulating insulin concentrations will also improve insulin action in peripheral tissues such as adipose tissue and muscle\(^\text{19}\).
1.2 Normal glucose homeostasis and insulin resistance

1.2.1 Islet structure

The pancreas is an organ with exocrine function for nutrient digestion and endocrine components for regulation of glucose homeostasis. The endocrine component of the pancreas consists of a group of cells called the islets of Langerhans (Figure 1.1). Islets contain at least five different endocrine cell types, including the insulin-producing β cells, which make up about 65-80% of total cells in islets\(^\text{20}\), the glucagon-producing α cells (15-20%)\(^\text{21}\), the somatostatin-producing δ cells (3-10%)\(^\text{22}\), the polypeptide-generating PP cells (1%)\(^\text{23}\) and the ghrelin-containing ε cells (1%)\(^\text{24}\).

Beta cells form the majority of the pancreatic endocrine cell mass. Beta cell secrete insulin, a 51-amino-acid peptide with strong hypoglycemic action. Insulin is vital for cellular nutrient uptake and thus essential for the survival of the organism\(^\text{25}\). Alpha cells secret glucagon as a counter-regulatory hormone with hyperglycemic action\(^\text{25}\).

![Figure 1.1 Islet structure](image)

The islets of Langerhans are small groups of cells in the pancreas that function as endocrine components. The alpha (or α) cells secrete the hormone glucagon, the beta (or β) cells secrete insulin, the delta (or δ) cells secrete somatostain, and the pancreatic polypeptide (PP) cells secrete pancreatic polypeptide. (Picture adapted from Efrat et al. 2012\(^\text{26}\))
1.2.2 Normal glucose homeostasis

In the fasted state, insulin secretion is low. Low levels of insulin lead to endogenous glucose production through increasing hepatic gluconeogenesis and promoting glycogenolysis. The brain is dependent on glucose, so in a fasted state the brain uses glucose as its source of nutrients. Adipose tissue lipolysis also increases to generate fatty acids, which provide nutrients to other tissues such as the heart and skeletal muscle. In the fed state, glucose concentrations rise because of absorption of glucose though the gut, which stimulates insulin secretion by beta cells and inhibits glucagon secretion from alpha cells. In the skeletal muscle, insulin increases glucose transport and glycogen synthesis. In the liver, insulin promotes glycogen synthesis, lipogenesis and inhibits gluconeogenesis. In the adipose tissue, insulin suppresses lipolysis and stimulates lipogenesis. Glucose concentrations become close to the fasting level within 2 hours\textsuperscript{5,27} (Figure 1.2).

![Figure 1.2 Glucose homeostasis and insulin action.](image)

In the fed state, glucose rises and stimulates secretion of insulin from beta cells. In the liver, insulin increases glycogen synthesis and lipogenesis and inhibits glucose release as well as gluconeogenesis. In the adipose tissue, insulin promotes glucose uptake and lipogenesis and inhibits lipolysis. In the muscle, insulin increases glucose uptake and promotes glycogen synthesis. In the brain, insulin regulates signals to suppress hepatic glycogenolysis, lipolysis and appetite.
1.2.3 Insulin secretion

Glucose-stimulated insulin secretion occurs by metabolizing glucose via glycolysis to pyruvate and in the mitochondria to acetyl-CoA, which is then oxidized in the TCA cycle\textsuperscript{5,28} (Figure 1.3). These actions then lead to an increase in the ATP/ADP ratio, which closes the $K^+$ channels. ATP depolarizes the plasma membrane, and opens voltage-gated $Ca^{2+}$ channels, increasing cytoplasmic calcium concentrations and triggering of insulin-granule exocytosis. In this manner, beta cells are crucial to regulate glucose concentration in a physiological range. Besides glucose, other stimuli such as fatty acids, amino acids, neurotransmitters and hormones also interact with the glucose metabolism pathway to increase insulin secretion\textsuperscript{5}. For example, the beta cell responds to other extracellular signals via GLP-1 receptor to stimulate insulin secretion. GLP-1 can also protect beta cell survival by increasing beta cell proliferation and reducing beta cell apoptosis\textsuperscript{29}. Beta cell mass can be regulated by insulin receptor signaling, where insulin receptor substrate-2 (IRS-2) activation results in enhanced beta cell survival\textsuperscript{30}.

Figure 1. 3 Insulin secretion.

Glucose stimulated insulin secretion is activated by oxidative metabolism of glucose, inducing the increased ratio of ATP/ADP. The $K_{ATP}$ channel then switches to a closed state, which causes depolarization of the plasma membrane and concentrations of cytoplasmic calcium are then increased through voltage-gated $Ca^{2+}$ channels. Exocytosis of insulin granules then occurs. The beta cells respond to other extracellular signals such as GLP-1 to regulate insulin
secretion and enhance beta cell survival. Insulin receptor signaling can also stimulate beta cell proliferation and protect beta cells from death.

1.2.4 Insulin signaling

Insulin and IGF-1 receptors act similarly because they are highly related homologous tyrosine kinase receptors. These receptors belong to a family that also includes the insulin receptor related receptor (IRR)\(^{31}\). They are tetrameric proteins that contain two \(\alpha\) subunits and two \(\beta\) subunits. Insulin binds to the \(\alpha\) subunits leading to derepression of \(\beta\) subunits, further activating the kinase activity and the downstream receptor substrates\(^{31}\) (Figure 1.4). The best-described substrates are members of the insulin receptor substrate (IRS) family including IRS-1 to IRS-4\(^{32}\). Although these substrates have similar tyrosine phosphorylation motifs, they have different roles in insulin signaling. IRS-1 knockout mice show growth retardation and impaired insulin action with impaired glucose tolerance\(^{33}\). IRS-2 knockout mice also show insulin resistance and develop early onset diabetes due to a loss of beta cell function. IRS-2 knockout mice only show defective growth in some tissues including some sites in the brain, islets and retina\(^{34,35}\). By contrast, IRS-3 and IRS-4 knockout mice have almost normal growth and metabolism, with only mild growth and insulin sensitivity defects\(^{36,37}\). IRS proteins mediate various signals that regulate cell growth, survival and metabolism. These proteins mediate insulin-related glucose homeostasis by promoting glucose uptake and regulation of genes for utilization of glucose to generate energy\(^{38,39}\).

The critical pathway downstream of IRS proteins is the PI3K-Akt pathway\(^{40,41}\). Activation of PI3K after binding the two SH2 domains to tyrosine-phosphorylated IRS proteins results in phosphorylation of phosphatidylinositol (3,4,5)-triphosphate (PIP\(_3\)), the lipid second messenger\(^{42}\). PIP\(_3\) then activates phosphorylation of Akt. The recruitment of Akt by PIP\(_3\) to the plasma membrane occurs and induces downstream signaling. Akt mediates most of insulin’s important metabolic effects, regulating glucose transport, lipid synthesis, protein synthesis, gluconeogenesis and glycogen synthesis\(^{43-45}\). Also, Akt phosphorylates many substrates to mediate a wide range of signals including some cell cycle regulating proteins, apoptosis and survival related proteins, enzymes and transcription factors\(^{46}\).
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The second insulin signaling pathway is the Grb2-SOS-Ras-MAPK pathway. After a series of activation steps, MAP kinases ERK1 and 2 are stimulated. These two kinases play a direct role in cell proliferation and regulating gene expression$^{42}$.

Figure 1. Insulin signaling.

Insulin exerts its effects by binding to insulin receptor and activating kinase activity and the downstream receptor substrates. The activated insulin receptor kinase phosphorylates the IRS. The critical pathway downstream of IRS proteins is the PI3K-Akt pathway, which transmits the major metabolic effects of insulin. The Grb2-SOS-Ras-MAPK pathway transmits signals to regulate cell proliferation. (Adapted from Kasuga et al. 2006$^{26}$)

1.2.5 Insulin resistance

In type 2 diabetes, insulin-mediated glucose uptake in the muscle is impaired. In the liver, regulation of gluconeogenesis and glycogen synthesis by insulin fails. In the adipose tissue, impaired insulin function leads to increased lipolysis. The lack of glucose clearance from the blood and the increased insulin secretion due to insulin resistance results in hyperglycemia and hyperinsulinemia. Beta cells finally fail to function properly and beta cell mass declines. This failure accelerates the sustained hyperglycemia, leading to multiple complications including kidney failure, cardiovascular disease, blindness and neuropathies. My study focuses on hyperglycemia due to loss of beta cell function.

60%-90% of cases of type 2 diabetes$^{47}$ have been shown to be associated with obesity. There is an increasing trend of obesity worldwide, with a lifestyle of higher caloric supply than what is needed and lack of adequate exercise. However, most obese and insulin resistant individuals do not develop hyperglycemia and type 2 diabetes within their lifetime. This is because the pancreatic beta cells increase insulin secretion.
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sufficiently to meet the demand caused by reduced insulin efficiency, therefore maintaining normal glucose homeostasis. Only when beta cells fail to compensate adequately for decreased insulin sensitivity does diabetes occur.

The proposed mechanisms of insulin resistance are complex, but three pathways have been considered.

1.2.5.1 Insulin resistance with ectopic lipid accumulation

Randel et al reported that fatty acids impaired insulin-mediated glucose uptake in muscle in a rodent model\textsuperscript{48}. Intramyocellular glucose and glucose 6-phosphate (G-6-P) concentrations in rodent experimental models and humans have shown that lipid-induced insulin resistance results from impaired insulin signaling and decreased insulin-stimulated glucose transport\textsuperscript{49,50}. Lipids accumulating within insulin responsive tissues, such as muscle and liver, have been shown to be a stronger predictor of insulin resistance compared to circulating fatty acids (e.g. endotoxins and prostaglandins) and can specifically cause hepatic insulin resistance\textsuperscript{27}.

1.2.5.2 Insulin resistance with endoplasmic reticulum (ER) stress

Activation of the unfolded protein response (UPR), also termed endoplasmic reticulum stress, is the second mechanism commonly accepted for the pathogenesis of insulin resistance. Activation of the three arms, inositol requiring enzyme-1 (IRE1\textsubscript{α}), PKR-like ER kinase (PERK), and activating transcription factor- 6 (ATF6) cooperates together to reduce the unfolded proteins accumulated in the ER lumen\textsuperscript{51}. Chemical inducers of UPR genes impaired insulin signaling\textsuperscript{52} and reduction of ER stress improved insulin signaling\textsuperscript{53}.

The UPR plays a role in the pathogenesis of hepatic insulin resistance through regulating lipogenesis. Overexpression of GRP78 (also termed BiP) in Lep\textsuperscript{ob/ob} mice suppressed UPR activation and reduced liver lipid content\textsuperscript{54} via decreasing expression of lipogenesis regulators SREBP1c and ChREBP\textsuperscript{27}. Mice with liver-specific reduction of IRE1\textsubscript{α} are phenotypically normal, but when challenged with a chemical activator of the UPR (tunicamycin), they developed hepatic steatosis\textsuperscript{55}. Similar observations were shown in mice lacking ATF6\textsubscript{α} challenged with tunicamycin\textsuperscript{56}. Tunicamycin treatment of these models was believed to induce increased expression of transcriptional
regulators of lipogenesis and adipose differentiation-related proteins that decrease hepatic lipid export\textsuperscript{27}.

1.2.5.3 Insulin resistance with inflammation

Obesity induces an inflammatory state that promotes the development of insulin resistance and type 2 diabetes\textsuperscript{57}. In adipose tissue, there are a large number of resident macrophages. These are normally of an anti-inflammatory M2 phenotype, but in conditions of obesity, there is a shift to the M1 or pro-inflammatory macrophages\textsuperscript{58}. TNF-$\alpha$ was the first pro-inflammatory cytokine shown to induce insulin resistance\textsuperscript{59,60}. IL-6, resistin, monocyte chemoattractant protein-1 (MCP-1), visfatin, PAI-6 and others were later shown to induce insulin resistance (reviewed in\textsuperscript{61-63}). MCP-1 and other chemokines recruit macrophages to adipose tissue. These cytokines and chemokines activate JNK and IKK$\beta$/NF-$\kappa$B pathways to promote development of insulin resistance and type 2 diabetes\textsuperscript{61}.

Recently, T cells have also been suggested to have a role in obesity and insulin resistance. Normal adipose tissue lymphocytes are mainly regulatory T cells (Treg cells) and Th2 cells, which produce cytokines that favour an anti-inflammatory environment. In obesity, Th1 type T cells are recruited to the adipose tissue and the ratio of Th1 to Treg cells increases resulting in a pro-inflammatory state, particularly in visceral adipose tissue\textsuperscript{64}. This shift correlates with an increase in M1 macrophages, insulin resistance and impaired glucose homeostasis\textsuperscript{58,64}. In humans, a high Th1/Treg cell ratio also correlates with high BMI\textsuperscript{65}.

1.3 Loss of beta cell mass in type 2 diabetes

In conditions of insulin resistance, the beta cell compensates by increasing beta cell mass and function to improve production of insulin. However, over-stressed beta cells fail to cope with the elevated concentration of glucose and fatty acids in the circulation, and are unable to secret adequate compensatory insulin to meet the body’s requirement, hence beta cell decompensation and death occur, leading to type 2 diabetes.
The traditional view of considering insulin resistance as the only main feature of type 2 diabetes has been challenged in the last few decades. Reports have shown that deficits of beta cell mass are also a feature of both T1D and T2D\textsuperscript{66}. In a normal adult human pancreas, beta cell mass is about 2\% of pancreas weight containing one million islets, which have around one billion beta cells in total\textsuperscript{67}. Beta cell mass varies widely from person to person in nondiabetic humans implying a wide range of growth of beta cell mass during infancy\textsuperscript{68}. Loss of beta cell mass has been observed in many animal models of type 2 diabetes such as Lepr\textsuperscript{db/db} and \textit{Psammomys obesus} desert gerbil\textsuperscript{70}. In human studies, a 30-70\% reduction of beta cell mass was reported in type 2 diabetes subjects\textsuperscript{66}.

1.3.1 Loss of beta cells in human studies
Deficits of beta cell mass differ in different populations and also correlate with BMI. In Korean and Japanese populations, where most diabetic patients are lean, the beta cell mass showed a correlation with BMI, with a 30\% decrease in beta cell mass. Although alpha cell density was increased by 20\%, total alpha cell mass did not change\textsuperscript{71,72}. Rahier et al. reported a slight correlation between beta cell mass and BMI. After BMI was matched, there was about 39\% lower average beta cell mass in type 2 diabetic subjects compared with matched non-diabetic controls\textsuperscript{73}. Hanley et al. also showed a similar 36\% reduction of the beta cell volume\textsuperscript{74}. Butler et al. showed a 63\% deficit in obese type 2 diabetes and a 41\% deficit in lean type 2 diabetes in relative beta cell volume compared with matched nondiabetic individuals. The reduced beta cell volume in patients with type 2 diabetes was due to a decrease in the number of beta cells rather than decreased individual beta cell size\textsuperscript{66,75}. Neither gender nor the age at diagnosis was related to beta cell mass, however duration of clinical diabetes was observed to correlate with beta cell mass\textsuperscript{73}. Interestingly, a decrease in the proportion of beta cells but not alpha cells in the islets indicates selective beta cell loss in type 2 diabetes\textsuperscript{71,75}.

1.3.2 Cell death
There are two major cell death processes that could result in beta cell loss: programmed cell death and passive cell death. Apoptosis, the major form of programmed cell death, is originally from the Greek word which means “leaves falling
from trees. Morphologically, apoptosis is characterized by chromatin condensation and shrinkage of the nucleus and cytoplasm, and the cell contents become bounded with ‘apoptotic bodies’, which are then quickly cleared by nearby phagocytic cells and digested in their lysosomes. With an effective clearance system by nearby macrophages, apoptosis is difficult to estimate, even in the tissues that have massive cell death such as the thymus. In contrast to apoptosis, necrosis, which is passive cell death, is characterized by swelling of the mitochondria and rupture of the plasma membrane, thereby releasing inflammatory cellular contents which might lead to release of self antigens and increase the risk of inducing autoimmunity. Although for a long time necrosis was considered an unregulated process, recent evidence reveals that necrosis can also occur in a programmed manner, defined as ‘necroptosis’ initiated by death receptors.

1.3.3 Beta cell apoptosis in type 2 diabetes
Evidence has suggested that apoptosis is an important mediator of beta cell loss in type 2 diabetes. Human pancreatic tissues from healthy and type 2 diabetic donors showed that beta cell mass is decreased and apoptosis, which is demonstrated by TUNEL staining, is increased in type 2 diabetes. Increased caspase-3 staining was shown in pancreatic sections from type 2 diabetic donors. Most studies of beta cell apoptosis have focused on animal models. Increased TUNEL or caspase 3 positive beta cells have been observed in Leprdb/db mice, as well as the desert rat, Psammomys obesus desert gerbil, domestic cat. Evidence of apoptosis was also observed in the zucker diabetic fatty rat.

1.3.4 Beta cell dedifferentiation and loss of identity in type 2 diabetes
It has been generally believed that the stress of obesity and insulin resistance lead to progressive beta cell death and reduced beta cell mass. However, recent evidence suggests that beta cells lose insulin identity, instead of proceeding to death, and de-differentiate into a progenitor state, and are able to develop into other islet cell types. FoxO1 deficient mice were examined as a model of hyperglycemia and reduced beta cell mass. Instead of islet beta cell death, beta cells de-differentiated and reverted to embryonic progenitors cells expressing markers such as Ngn3, L-Myc, Oct4 and Nanog. This finding was also observed in other type 2 diabetes mouse models.
including Lepr\textsuperscript{db/db}, Lep\textsuperscript{ob/ob}, Akita and Glut4-Cre mediated InsR knockout\textsuperscript{88-90}. In the pancreas of human type 2 diabetic subjects, beta cells co-localization with glucagon markers, suggesting loss beta cell identity\textsuperscript{91}. De-differentiated cells accounted for 32\% of beta cells in type 2 diabetic individuals vs 8.7\% in non-diabetic controls\textsuperscript{92}. The number of aldehydedehydrogenase 1A3-positive/no-hormone cells was 3-fold higher in diabetic individuals, suggesting that beta cells de-differentiated and converted to alpha cells in human type 2 diabetes\textsuperscript{92}. However, Butler et al., have challenged the above results by demonstrating only a slight increase in endocrine cells expressing no-hormone in type 2 diabetes. They suggest that these hormone negative endocrine cells in type 2 diabetes were probably newly regenerated cells\textsuperscript{93}. More intensive efforts are required in this area to uncover the potential role of de-differentiation in type 2 diabetes.

1.4 Pathways of apoptosis

1.4.1 Extrinsic pathway

There are two pathways of apoptosis: the extrinsic and intrinsic pathways (Figure 1.5). The extrinsic pathway is also called the death receptor pathway. This pathway is triggered when members of the tumor necrosis factor (TNF) receptor superfamily bind to the death receptors on the cell surface. These receptors lead to activation of caspase-8 via the adaptor Fas-associated protein with death domain (FADD) which is assisted by TNF receptor type 1-associated death domain protein (TRADD). Activation of caspase-8 then activates the effector caspases-3, -6 and -7 to induce apoptosis\textsuperscript{94}.

1.4.2 Intrinsic pathway

The intrinsic pathway or BCL-2 regulated mitochondrial pathway is regulated by activation of the pro-apoptotic members of the BCL-2 protein family. Diverse initiators, such as growth factor deprivation, oncogenic stress and cytotoxic stimuli, trigger the mitochondrial pathway\textsuperscript{94,95}. There are three groups of proteins of the BCL-2 family which share common BCL-2 homology (BH) domains: the initiators, guardians and effectors. Initiators are proteins that only have the BH3 domain, which include
BIM, PUMA, BAD, NOXA, BIK, HRK, BMF and tBID. The guardians, also known as pro-survival family members, are BCL-2, BCL-XL, BCL-W, MCL1, A1 and BCL-B. The pro-apoptotic effector proteins include BAX, BAK and BOK\(^94\) (Figure 1.6). The guardians and pro-apoptotic effectors share four BH domains\(^96\) and have similar protein structure, which is a helical bundle surrounding a central hydrophobic core helix\(^97\). This structure similarity makes it possible to generate a groove, which interacts with the BH3 domain of the pro-apoptotic members of the BCL-2 family\(^94,95\) (Figure 1.6). Diverse factors activate BH3-only proteins, which inhibit the pro-survival BCL-2 proteins, then activate the pro-apoptotic effectors BAX and BAK. BAX and BAK then form oligomers to permeabilize the mitochondrial outer membrane. Cytochrome c is released from the mitochondria, which activates caspase 9 on the scaffold protein apoptotic protease activating factor 1 (APAF1)\(^98,100\). Caspase 9 then activates effector caspases such as caspase 3 and apoptosis is initiated.

BH3-only proteins perform their pro-apoptotic function by two ways: neutralization of the pro-survival BCL-2 family proteins and direct activation of the pro-apoptotic effectors BAX and BAK. In the direct activation model, some BH3-only proteins, including tBID, BIM and PUMA (also called activators), are able to activate BAX and BAK directly\(^101-103\). The other BH3-only proteins that cannot directly activate apoptosis are called ‘sensitizers’, and these can bind with pro-survival BCL-2 members, liberating the bound BH3-only activator proteins to perform a direct activation role\(^103\). In the indirect model, all of the pro-survival proteins are required to be neutralized by BH3-only proteins, then the activated BAX and BAK become free to permeabilize the mitochondrial outer membrane\(^94,104,105\). More recently, both direct and indirect models have been integrated into a unified model. In this model, pro-survival proteins prevent apoptosis not only by neutralizing the BH3-only proteins but also controlling activated BAX and BAK\(^94\).

The extrinsic and intrinsic pathways can crosstalk between each other through BID. Activation of caspase 8 in the death receptor-mediated pathway cleaves BID to generate the active form tBID, and then the BH3 domain of tBID is exposed. By this
way BID is engaged in the mitochondrial pathway to amplify caspase activation\textsuperscript{94,95}.

Figure 1. 5 The pathways of apoptosis.

There are two major pathways of apoptosis in mammalian cells, the intrinsic and extrinsic pathways. The intrinsic pathway is activated by cellular stresses (such as ER and oxidative stress) and results in activation of the BH3-only members of the BCL-2 family (such as BIM and PUMA). The BH3-only proteins then inhibit the pro-survival BCL-2-like proteins, thereby activating BAX and BAK, which promote the loss of mitochondrial outer membrane potential. Cytochrome c is released from the mitochondria and promotes activation of caspase-9, caspase-3 and apoptosis. BH3-only proteins can also directly activate BAX and BAK. The extrinsic pathway is initiated by activation of death receptors. This results in activation of caspase-8, which promotes the activation of the caspase cascade and apoptosis. The BH3-only protein BID is essential for death receptor-mediated apoptosis and its truncated form (tBID) can engage the intrinsic pathway, thereby providing cross-talk between the two apoptotic pathways.

1.4.2.1 The role of the BCL-2 protein family in apoptosis

In this section, the general role of BCL-2 protein family in apoptosis is discussed. More specific discussion of the role of BCL-2 family proteins in beta cell apoptosis will be discussed in detail in Section 1.5 through 1.7

BIM
BCL-2-like protein 11 (BCL2L11/BIM) is expressed in many cell types including hematopoietic, epithelial, neuronal, and stem cells. There are three major forms of BIM (BIM$_S$, BIM$_L$, BIM$_{EL}$). BIM$_S$ is the most potent, and also the most difficult to detect in vivo. Activation of the forkhead transcription factor FOXO3a induces the transcription of BIM in response to cytokine withdrawal. C/EBP homologous protein (CHOP), a member of the C/EBP transcription factor family, is also reported to induce BIM transcription. An inhibitor of BIM transcription is the MiR-17-92 microRNA cluster. Activity of the other two forms of BIM (BIM$_{EL}$ and BIM$_L$) is post translationally regulated by sequestration to the microtubule-associated dynein motor complex.

BIM is required for apoptosis triggered by growth factor deprivation, regulating hematopoietic homeostasis and precluding autoimmunity. Leukocytes from blood, spleen and lymph nodes of BIM$^{-/-}$ mice were elevated due to increases in B and T cells. BIM is crucial for deletion of autoreactive T and B lymphocytes. BIM deficiency led to progressive lymphadenopathy and renal failure due to a systemic autoimmune disease similar to human systemic lupus erythematosus.

BIM also plays an important role in ER stress-induced cell death in a diverse range of cell types. ER stress activates BIM through protein phosphatase 2A mediated dephosphorylation and CHOP-C/EBPα-mediated transcriptional induction. However, there is overlap between BIM and PUMA activity to mediate apoptosis triggered by inducers such as ER stress and cytokine deprivation.

PUMA

p53 upregulated modulator of apoptosis (PUMA) encodes two proteins (PUMA-α and PUMA-β) containing BH3 domains. These two proteins function similarly either by binding to BCL-2 or directly activating BAX/BAK to induce cytochrome c release and activate programmed cell death. PUMA plays a direct role in mediating p53-induced cell death via a cytochrome c/APAF-1-dependent pathway. PUMA is a mediator of cell apoptosis in response to γ-irradiation and DNA-damage. It also mediates apoptosis induced by diverse p53-independent cytotoxic insults including cytokine deprivation, glucocorticoids, the kinase inhibitor staurosporine and phorbol ester. PUMA is also a mediator of ER stress-associated apoptosis during the early...
stages of chronic neurodegeneration in vivo\textsuperscript{120}. In neuronal cells, PUMA deficiency protects cell against oxidative stress induced apoptosis\textsuperscript{121}. CHOP interacts with FOXO3a in response to ER stress to mediate induction of PUMA\textsuperscript{122}. E2F1 up-regulates the expression of PUMA via a direct transcriptional mechanism\textsuperscript{123}.

Other BH3-only proteins

Mice deficient in other BH3-only proteins have been made and have revealed phenotypes indicating that these proteins serve unique functions in a cell type and stimulus-specific manner. Some of these are briefly summarized below.

BCL2-associated agonist of cell death (BAD) loss partially protects fibroblasts from apoptosis induced by glucose or cytokine deprivation, however BAD deficient mice appear normal\textsuperscript{124}.

BH3 interacting-domain death agonist (BID) is activated through the death receptor pathway. BID-deficient mice are saved from the lethal hepatitis mediated by activation of FAS\textsuperscript{125,126}.

Phorbol-12-myristate-13-acetate-induced protein (PMAIP1/NOXA) is direct transcriptional target of the tumor suppressor protein p53\textsuperscript{127}, which is similar to PUMA\textsuperscript{117}. It has a dominant role in the death of fibroblasts and keratinocytes triggered by ultraviolet radiation\textsuperscript{128}. NOXA and PUMA may account for all of p53 induced pro-apoptotic activity because combined loss of these two genes results in the same resistance to apoptosis as p53 loss\textsuperscript{129}, with PUMA playing the dominant role in diverse cell types\textsuperscript{129}.

BCL2 modifying factor (BMF), is sequestered to myosin V motors in healthy cells. Studies in cell lines indicated BMF-mediated apoptosis induced by loss of cell attachment (anoikis)\textsuperscript{130}. BMF-/- mice showed B cell accumulation and resistance to apoptosis induced by glucocorticoids and a range of apoptotic stimuli\textsuperscript{131} indicating that BMF regulates lymphoid homeostasis.

BCL2-interacting killer (BIK) transcription is activated by E2F1 and P53\textsuperscript{132}. BIK
deficiency leads to no obvious defects in vivo or in vitro, but male mice lacking BIK and BIM were infertile. This is due to dysfunctional spermatogenesis.

Harakiri, BCL2-interacting protein (HRK, also known as DP5) expression seems to be only in the central and peripheral nervous systems. HRK contributes to apoptosis of certain neuronal cells induced by trophic factor withdrawal but has no impact on apoptosis of haematopoietic cells.

BAX and BAK

BCL2-associated X protein (BAX) and BCL2-antagonist/killer 1 (BAK) have overlapping roles in the regulation of apoptosis. BAK-/- mice seem mostly normal and only have a modest rise in platelets due to increased platelet lifespan. BAX-/- mice also display no abnormalities except male sterility, which is because of inadequate spermatogenesis induced by excess germ cells. However, in contrast, most mice lacking both BAK and BAX die perinatally with less than 10% surviving. Those survivors all develop massive lymphadenopathy and fatal systemic lupus erythematosus-like autoimmune disease. All BAX-/-BAK-/- mice have developmental abnormalities including interdigital webs, an imperforate vaginal canal, and increased accumulation of excess cells within the central nervous and hematopoietic systems. A wide range of cells types from BAX-/-BAK-/- mice are resistant to multiple apoptotic stimuli including ER stress, gamma-irradiation, growth factor deprivation, ultraviolet radiation. Thus BAX and BAK have an essential role in mediating apoptosis, functioning downstream of BH3-only proteins.

Pro-survival proteins

Transgenic and gene knockout studies have been used to determine the functions of BCL-2 family members. When overexpressed in transgenic mice, all of the pro-survival BCL-2 members, including BCL-2, BCL-XI, MCL-1 and A1 have been shown to protect many cell types, including lymphoid and other hemopoietic cells against apoptosis induced by diverse stimuli. The different expression pattern and interaction of pro-survival proteins with other proteins may account for their varied effects in different cell types. Drugs that target pro-survival
BCL-2 proteins are in development for cancer therapy, and recently venetoclax, a drug that targets BCL-2, was approved for use in chronic lymphocytic leukemia (CLL)\textsuperscript{146,147}.

Overexpression of B cell leukemia/lymphoma 2 (BCL-2) enhanced B lymphocyte survival\textsuperscript{141} and this is the first physical evidence of a role for BCL-2 in cell survival\textsuperscript{94}. This observation has been further confirmed in T cells by the Strasser group\textsuperscript{142}. BCL-2 deletion leads to degenerative defects in several cell types\textsuperscript{148}. BCL-2-/- mice have fatal polycystic kidney disease, they develop premature greying and they suffer from immunodeficiency. Interestingly, all these defects have been reversed with concomitant loss of the BH3-only protein BIM\textsuperscript{149}. This indicates that BCL-2 and BIM are the main regulators of the life/death in these cell types\textsuperscript{95}.

BCL2-like 1 (BCL2L1/BCL-XL) ablated mice die at day 14 of embryogenesis due to severe neuronal degeneration and defective fetal erythropoiesis\textsuperscript{150}. Loss of even one BCL2L1 allele leads to spermatogenesis defects\textsuperscript{151}. BIM loss reverses the infertility and erythroid defects in BCL-XL+/- mice but does not restore neuronal defects indicating that other BH3-only proteins also regulate the death of neuronal cells\textsuperscript{95,152}.

BCL2-like 2 (BCL2L2/BCL-W) has limited roles. Loss of BCL-W only leads to defects in adult spermatogenesis\textsuperscript{153}. This is associated with loss of supporting Sertoli cells\textsuperscript{154}.

Loss of myeloid cell leukemia sequence 1 (MCL-1) has the greatest effects. MCL-1-/- embryos do not implant in utero resulting in peri-implantation embryonic lethality, although MCL-1-/- blastocysts show no evidence of increased apoptosis, so the mechanism of this remains unknown\textsuperscript{155}. Conditional MCL-1 deletion has revealed its importance in regulating the survival of many cell types including hemopoietic stem
cells$^{156}$, B and T lymphoid progenitors and mature lymphocytes$^{157}$. Cardiac-specific deletion of MCL-1 leads to fatal cardiomyopathy. Interestingly, although most of the defects have been rescued by combining with the loss of pro-apoptotic effectors BAX and BAK, there are still mitochondrial ultrastructural abnormalities suggesting that MCL-1 may also regulate mitochondrial function (see section 1.4.2.2 below)$^{158}$. Mitochondria isolated from MCL-1-deficient hearts showed impaired mitochondrial homeostasis$^{159}$. These findings are of importance because MCL-1 inhibition has been considered as a therapeutic strategy to treat cancer, but cardiac toxicity caused by impaired mitochondrial function could be an unexpected side effect.

Studies on the function of BCL-2 related protein A1a (A1) are challenging due to the difficulty of targeting three closely linked A1 genes in mice. Deletion of only one A1 gene (A1a) impaired neutrophil survival$^{160}$.

**Figure 1.** The BCL-2 family.

Pro-apoptotic BH3-only proteins have only one BCL-2 homology (BH) domain (the BH3 amphipathic helix, which mediates interaction with multi-domain BCL-2 family members), while downstream effectors (BAX, BAK and perhaps BOK) and pro-survival proteins have four BH domains (BH1-BH4). (Figure modified from Czabotar et al. 2014$^{94}$)
1.4.2.2 Association of BCL-2 proteins with mitochondrial function

Several findings have indicated that BCL-2 proteins also participate in non-apoptotic processes. Detailed evidence for their non-apoptotic role is limited and needs more work. Interfering with BCL-2 family members has been utilized in clinical therapy for cancer, raising the possibility that inhibiting these other functions might result in potential therapy side effects.

BCL-XL has been found to enhance energy metabolism. One study found BCL-XL located in the mitochondrial inner membrane in hippocampal neurons, where it enhanced the mitochondrial $F_1F_0$ATP synthase. $F_1F_0$ATP synthase directly affects mitochondrial membrane potential and BCL-XL was shown to be a binding partner for mitochondrial ATP synthase $\beta$ subunit. BCL-XL failed to protect $\beta$ subunit-deficient yeast indicating that BCL-XL contributes to mitochondrial energetic capacity.

BCL-2 has also been found localized to the mitochondrial inner membrane. A role for BCL-2 in the maintenance of mitochondrial membrane potential was demonstrated, and BCL-2 was co-localized with the $\beta$ subunit of $F_1F_0$ATP synthase in the inner mitochondrial membrane. BCL-2 has a role in maintenance of mitochondrial ROS levels, playing a unique role in adjusting cytochrome c oxidase activity.

MCL-1 is found in the mitochondrial matrix, regulating normal mitochondrial fusion, ATP production, membrane potential, respiration, cristae ultrastructure and maintenance of oligomeric ATP synthase. Cardiac-specific ablation of MCL-1 results in abnormal mitochondrial ultrastructure and defective mitochondrial respiration. This suggests that MCL-1 displays a non-apoptotic role to regulate normal mitochondrial function.

1.4.2.3 Association of BCL-2 proteins with glucose homeostasis and metabolism
Recent findings have indicated that BCL-2 family members not only control cell life and death, but also play roles in regulating glucose homeostasis and metabolism. BCL-2 and BCL-XL have been shown to dampen the beta cell response to glucose\textsuperscript{167}. BCL-2/- mice responded to glucose with increased NADPH level, cytosolic Ca\textsuperscript{2+} signals and augmented insulin secretion. Inducible deletion of BCL-XL in mouse beta cells also showed similar results. These findings indicate that BCL-2 and BCL-XL suppress glucose signaling in pancreatic beta cells.

Previously Danial et al. demonstrated that BAD plays a physiological role in beta cells that is distinct from its role in beta cell apoptosis. BAD interacts with glucokinase\textsuperscript{168}, contributing to glucose stimulated insulin secretion. Glucose and lipid metabolism has been studied in other BCL-2 family members. NOXA was shown to stimulate glucose consumption and enhance glucose turnover through the pentose phosphate pathway rather than through glycolysis\textsuperscript{169}. BID was suggested to be involved in the regulation of lipid and glucose metabolism potentially via mitochondrial carrier homolog 2 (BID receptor)\textsuperscript{170}. Wali et al. reported that BIM plays a direct role in regulating mitochondrial bioenergetics and whole body metabolism (See Appendix A). Mice lacking BIM had lower body weight and reduced adiposity as well as fasting blood glucose and improved insulin sensitivity. Lipid oxidation was increased in BIM/- mice, indicating a correlation with their metabolic phenotype. Embryonic fibroblasts from mice lacking BIM had higher mitochondrial oxygen consumption rate which was associated with higher mitochondrial complex-IV activity. This suggests BIM regulates mitochondrial oxygen consumption and lipid oxidation and therefore improved insulin sensitivity in mice (See Appendix A). In contrast, the pro-apoptotic molecule PUMA was dispensable for glucose homeostasis in lean and in high fat fed mice, but leptin levels and food intake were affected in PUMA-deficient mice\textsuperscript{171}.

All these findings indicate that in addition to their traditional role in apoptosis induction, BCL-2 family members may also play an important role in regulating mitochondrial function, glucose homeostasis and metabolism. As yet there are no unified mechanisms, and so these findings remain controversial.
1.5 ER stress activates beta cell apoptosis in type 2 diabetes

1.5.1 Unfolded Protein Response

In eukaryotic cells, protein synthesis and secretion are highly dependent on the endoplasmic reticulum (ER) to fold and process proteins. Accumulation of misfolded proteins in the ER lumen cause ER stress and activation of an important signal response called the unfolded protein response (UPR)\(^{172,173}\). The UPR aims at relieving ER stress and restoring ER homeostasis. To achieve these goals, the UPR performs several actions. First, there is a decrease in the arrival of newly-synthesized proteins into the ER by reducing protein synthesis and transfer of proteins into the ER. Second, there is an increase in capacity to deal with unfolded proteins by increasing expression of ER targeted genes including ER chaperones. When these actions fail, ER stress triggers cell apoptosis\(^{173}\).

Because the UPR works through gene transcription, signal transmission from the ER to the nucleus is important for expression of the relevant genes and proteins. This is mediated by three main arms: inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK).

In each arm, integral membrane proteins sense the protein folding status in the ER lumen and transmit the signal from the ER to the cytosol to modulate expression of the key genes and proteins\(^{174}\).

Accumulation of misfolded proteins in the ER lumen causes BiP, an important chaperone, to dissociate from IRE1\(\alpha\), ATF6, and PERK, therefore activating these three arms of the UPR. There are two forms of IRE1: IRE1\(\alpha\) is expressed in all cell types including pancreas and IRE1\(\beta\) is mainly found in epithelia of the gut\(^{175}\). Once IRE1\(\alpha\) is activated, it cleaves the mRNA encoding X-box binding protein (XBP)1 into an active, spliced form, XBP1s. XBP1s acts as a transactivator to mediate protein maturation, folding and export from the ER\(^{176,177}\). XBP1s increases transcription of various ER chaperones and co-chaperones. IRE1\(\alpha\) also degrades ER-targeted mRNAs to reduce the production of new proteins. In addition, IRE1\(\alpha\) takes part in mediating apoptosis by activating the TRAF2-JNK pathway\(^{174}\) (Figure 1.7).
Activation of ATF6 leads it to translocate to the Golgi where it is cleaved by site-1 protease (S1P) and site-2 protease (S2P). The cleaved form then translocates to the cell nucleus and binds to the ER stress relevant genes resulting in expression of ER chaperones to strengthen the folding capacity of the ER, and augment the expression of XBP1 and the pro-apoptotic gene CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP). ATF6α activates expression of ER chaperones including calnexin/calreticulin, BiP/GRP94, and co-chaperone P58IPK. PERK phosphorylates eukaryotic translation initiation factor (eIF) 2α, attenuating mRNA translation, thereby inhibiting protein synthesis. eIF2α phosphorylation reduces translation generally, but selectively increases ATF4. ATF4 regulates amino-acid import, glutathione biosynthesis, and resistance to oxidative stress. ATF4 also leads to increased expression of ATF3 and CHOP (Figure 1.7).

The integrated function of the UPR is attenuation of protein translation as well as up-regulation of ER chaperones to increase the capacity of the ER. Non-resolving ER stress-induced apoptosis is a very important pathogenic factor in many diseases including diabetes. Excessive or prolonged ER stress leads to activation of signaling through PERK, ATF6 and IRE1 to trigger apoptosis.

Phosphorylated IRE1α binds an adaptor protein called tumour necrosis factor receptor (TNFR) associated factor 2 (TRAF2) and leads to the activation of Jun amino-terminal kinase (JNK) (Figure 1.7). In vitro, dominant-negative TRAF2 inhibits activation of JNK through IRE1 activation. Thus, IRE1 is likely to mediate apoptosis through IRE1-TRAF2-JNK. JNK activation has been implicated with apoptosis. The absence of JNK in murine embryonic fibroblasts (MEFs) causes failure to release mitochondrial cytochrome c in response to apoptosis signaling. Although the mechanism of how JNK signaling leads to cell death is not clear, it has been reported that activated JNK fails to mediate death in cells lacking BAX, indicating involvement of the BCL-2 family in JNK-dependent apoptosis. JNK can also induce the expression of the pro-apoptotic BH3-only proteins DP5/HRK and BIM and inhibits pro-survival BCL-2 family members including BCL-2, BCL-XL, BCL-CL and MCL-1.
Another potential mechanism of ER stress induced apoptosis is through CHOP (Figure 1.7). CHOP was identified as an ER stress-induced transcription factor that mediates apoptosis in response to ER stress. How CHOP mediates apoptosis is still unclear. CHOP-induced apoptosis was associated with downregulation of BCL-2 and activation of BIM and PUMA\textsuperscript{109,192}. Overexpression of CHOP in cell lines resulted in downregulation of BCL-2 expression\textsuperscript{192}. But this change in BCL-2 levels was not reproduced by another group\textsuperscript{109}. CHOP can bind to a regulatory site inside the \textit{bim} gene and CHOP-dependent induction of \textit{bim} is critical for ER stress-induced apoptosis\textsuperscript{109}.

Figure 1.7 The unfolded protein response pathway.

The UPR pathway has three arms. Activation of IRE1 causes reduced protein synthesis and
increased ER chaperone expression through XBP1 splicing. Activation of the PERK arm causes reduced protein synthesis. Activity of the ATF6 arm results in increased expression of ER chaperones. When ER stress is excessive or prolonged, the apoptosis pathway will be initiated via IRE1-TRAF2-JNK or PERK-ATF4-CHOP.

1.5.2 ER stress mediates apoptosis in type 2 diabetes.

Pancreatic beta cells can produce 1 million molecules of insulin per minute and a highly developed ER is required for folding and processing of the newly synthesized insulin. Therefore beta cells are highly susceptible to ER stress\textsuperscript{193}. ER stress has been demonstrated to lead to beta cell failure and can cause diabetes in multiple animal models and rare genetic disorders.

In the Akita mouse, mutation of one of the alleles of the insulin 2 gene disrupts the disulfide bond between the A and B chains and results in misfolded proinsulin accumulation in the ER lumen. The Akita mouse develops diabetes with beta cell loss mediated by ER stress\textsuperscript{194}.\textit{Hspa5} (the gene for BiP) and \textit{Ddit3} (the gene for CHOP) mRNA expression is induced in the pancreas. Targeted deletion of the \textit{Ddit3} gene delayed the onset of diabetes in the Akita mouse, suggesting that ER stress leads to apoptosis via CHOP\textsuperscript{195}. Wolfram syndrome is caused by mutations of the \textit{Wfs1} gene resulting in diabetes. Intracellular Ca\textsuperscript{2+} homeostasis is disrupted, leading to increased ER stress and apoptosis\textsuperscript{196,197}. Walcott Rallison Syndrome results in infancy-onset diabetes due to mutations in the eIF2\textalpha{} kinase PERK (EIF2AK3)\textsuperscript{198}. These examples in mice and humans indicate that disruption on UPR signaling is sufficient to cause diabetes.

To further support a role of ER stress in type 2 diabetes, markers of ER stress genes were shown to be increased in diabetic subjects. Islets isolated from the Lepr\textsuperscript{db/db} mouse, a type 2 diabetic mouse model with obesity and insulin resistance, had increased ER stress markers such as \textit{Atf4}, \textit{Ddit3}, \textit{Hspa5}, \textit{Dnajc3}\textsuperscript{199}. Enhanced staining intensity of CHOP, BiP and P58 was also detected in beta cells from pancreas sections of human type 2 diabetic patients compared to nondiabetic pancreas\textsuperscript{199}. Glucose, free fatty acid, and islet amyloid polypeptide have been implicated as triggers for beta cell ER stress (described in more detail under section 1.6). In addition, palmitate activates an ER stress response in beta cells\textsuperscript{200,201} and MIN-6 cells\textsuperscript{201}. High glucose treatment of rat
islets also induced a modest activation of UPR gene expression\textsuperscript{202}. These data indicate that ER stress could contribute to the development of type 2 diabetes.

1.6 Triggering factors for beta cell ER stress and apoptosis.

1.6.1 Lipotoxicity

Increased serum lipids are a feature of type 2 diabetes and a consequence of increased hepatic de novo lipogenesis. Western diet contributes to the development of obesity and insulin resistance, resulting in increased burden on the beta cells\textsuperscript{203}. Beta cells suffer from chronic exposure to high levels of free fatty acids (FFA)\textsuperscript{204}, resulting in ER stress and beta cell apoptosis\textsuperscript{205,206}. Palmitate is the most abundant free fatty acid in plasma. Chronic exposure to high levels of palmitate (0.5mmol/l) has been shown to induce increased apoptosis of rat INS-1E cells or FACS-purified primary rat beta cells\textsuperscript{201,203}. The saturated FFA palmitate caused more apoptosis than the unsaturated FFA oleate\textsuperscript{203}. This experiment has also been performed in human islets, demonstrating palmitate is toxic to human islets\textsuperscript{203}. These experiments suggest that hyperlipidemia induces apoptosis in islet cells.

It has been shown that palmitate induces apoptosis through the intrinsic apoptosis pathway\textsuperscript{207}. How FFAs cause beta cell apoptosis is not well understood, several studies have indicated this is mediated by ER stress\textsuperscript{199,203}.

In vitro treatment of INS-1E cells with 0.4 mmol/l palmitate for 24 hours induced increase of PERK, IRE1, BIP, ATF3 and CHOP expression. In human islets cells, there was also increased ATF3, CHOP, XBP1 splicing, and BIP expression after 48 hours of palmitate treatment. Inhibiting the function of CHOP or JNK reduced palmitate induced death of INS-1E cells\textsuperscript{203}. Treatment of MIN6 cells with palmitate (0.4 mmol/l) for 48 hours induced expression of CHOP, P58 and ATF4 as well as increased apoptosis. These experiments suggest that palmitate induces ER stress in pancreatic beta cells. In these studies the FFA-induced ER stress response was independent of glucose concentration, however, other studies have indicated that hyperlipidemia is deleterious only in the context of hyperglycemia\textsuperscript{208}. 

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Using microarray analysis of RNA from INS-1E cells which were treated with palmitate for 14h, there was increased expression of the BCL-2 family proteins DP5 and PUMA. The increased level of these two mitochondrial cell death genes indicates that palmitate induces apoptosis through the intrinsic pathway. This was further confirmed using RNA interference to knockdown DP5 or PUMA, which protected beta cells against palmitate. Unlike DP5 and PUMA, BIM was not increased by palmitate, indicating BIM is not a major mediator for palmitate induced beta cell death\textsuperscript{207}.

RNA sequencing was performed to map transcripts expressed in palmitate-treated human islets. Upregulation of a large number of UPR genes, including the three arms of the UPR, was observed in palmitate-treated human islets. In addition, Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis showed binding sites for the transcription factors ATF6 and IRE1-dependent XBP1 and further revealed a role for ER stress in beta cell dysfunction and death\textsuperscript{209}.

**1.6.2 Glucotoxicity**

High concentrations of glucose are an important feature of type 2 diabetes. Hyperglycemia can trigger beta cell apoptosis in type 2 diabetes and could be a mechanism for beta cell dysfunction in type 2 diabetes\textsuperscript{210}. When treating mouse or rat islets with high concentrations of glucose, there is an increased level of apoptosis detected\textsuperscript{211,212}. Islets isolated from C57BL/6 mice were treated with high glucose (33.3 mmol/l) in vitro and DNA fragmentation, which is a way of measuring apoptosis, was significantly increased compared with the control group treated with 5.6 mmol/l glucose\textsuperscript{211,212}. One caveat is that the glucose concentration in these experiments is 33.3 mmol/l, which is very high compared to the physiological environment. Another group used 16.7 mmol/l glucose to investigate its effect on apoptosis in rat islets. Increased cytoplasmic DNA fragments, caspase 3 activity, and TUNEL positive cells were observed\textsuperscript{213}. Beta cell apoptosis in response to glucose toxicity has also been observed in several animal models of type 2 diabetes such as Psammomys obesus desert gerbil\textsuperscript{70}, the Zucker diabetic fatty rat\textsuperscript{86}, and the domestic cat\textsuperscript{85}.
Similar experiments have also been performed\textsuperscript{214} in human islets. Exposure of human islets to high glucose concentrations of 11.1 and 33.3 mmol/l induced increased TUNEL positive cells compared to islets at 5.5 mmol/l glucose\textsuperscript{215,216}. Glucose-induced apoptosis of human islets was also assessed by electron microscopy, accompanied by altered mitochondrial morphology and density volume\textsuperscript{217}. These experiments suggest that high glucose concentrations induce increased apoptosis of beta cells.

Evidence suggests that high glucose concentrations induce ER stress in beta cells. Treatment of rat islets with 30 mmol/l glucose triggered XBP1 splicing and activated the ER chaperones BIP, EDEM, ATF3 and CHOP\textsuperscript{202}. Treatment of INS-1 cells with 16.7 mmol/l glucose for 72 hours increased IRE1\(\alpha\) phosphorylation\textsuperscript{218}. Exposure of INS-1 cells to 25 mmol/l glucose significantly increased the phosphorylation of PERK and eIF2\(\alpha\). There was also increased gene expression of ATF4 and CHOP after treatment of INS-1 cells or rat islets\textsuperscript{219} with high glucose. Exposure of mouse islets to the reducing sugar ribose (50 mmol/l) for 48 hours in vitro induced increased expression of \textit{Ddit3}. IRE1\(\alpha\)-mediated XBP1 splicing also gradually increased after 24 hours treatment with ribose\textsuperscript{220}. These findings indicate that glucotoxicity induces ER stress of beta cells.

Our lab and others have previously\textsuperscript{212,214} shown that glucose induces apoptosis of beta cells through the intrinsic apoptotic pathway. Islets from mice lacking apoptosis related molecules have been exposed to high concentrations of glucose\textsuperscript{212}. Deficiency of the apoptosis initiating BH3-only proteins BIM or PUMA, or the downstream apoptosis effector BAX reduced glucose-induced apoptosis. However, islets lacking IL-1 receptors or FAS did not have reduced apoptosis, which indicates inflammatory receptors or death receptors (extrinsic pathway) are not taking part in glucose induced beta cell apoptosis. Exposure of human islets to high levels of glucose in vitro lead to upregulated expression of the pro-apoptotic genes \textit{BAD} and \textit{BID} and downregulation of the pro-survival gene \textit{BCL2L1}\textsuperscript{214}, indicating that the intrinsic pathway might play an important role in mechanism of glucose induced beta cell apoptosis in human islets also.

\textbf{1.6.3 Islet amyloid polypeptide (IAPP)}
Type 2 diabetes is attributed to many factors that induce beta cell stress. Islet amyloid polypeptide (IAPP) is identified as one important factor. IAPP is the main component of amyloid. IAPP is a 37-amino acid peptide secreted by beta cells. This peptide is co-secreted with insulin when the beta cell is stimulated, such as with glucose. Over production of IAPP results in IAPP aggregation. IAPP aggregates are toxic to many cell types including neurons and beta cells. In type 2 diabetes, IAPP induced loss of cell mass appears to be beta cell specific, and glucagon-producing alpha cells are spared. Increased islet amyloid was also shown to be associated with increased beta cell apoptosis and decreased beta cell area in human pancreas.

The exact mechanisms by which IAPP aggregation causes beta cell apoptosis still remain unclear. Rodent transgenic models expressing human IAPP (hIAPP) in beta cells have been used to study the role of IAPP in beta cell failure. IAPP may induce apoptosis through both intrinsic and extrinsic pathways in a JNK-dependent manner. Islets from hIAPP transgenic mice cultured in 16.7 mmol/l glucose had JNK activation, and markers of both apoptotic pathways were increased: the extrinsic pathway molecule FAS, the intrinsic pathway molecule BIM and the pro-survival molecule BCL-XL.

hIAPP transgenic mice showed increased expression of BIP, XBP1, ATF4 and CHOP. Expression of CHOP was increased in hIAPP-expressing INS-1 cells. These findings suggest that IAPP aggregation might activate the UPR in beta cells. However, this view has been challenged. Exposure of hIAPP transgenic mouse islets to 16.7 or 33.3 mmol/l glucose, conditions considered associated with increased amyloid deposition in hIAPP transgenic islets, did not induce any changes of UPR markers including Hspa5, Atf4 or Ddit3 mRNA expression. Pancreatic sections of hIAPP transgenic mice fed with a high-fat diet for one year or human type 2 diabetic donors showed similar levels of BiP and CHOP when compared to respective controls (non-IAPP transgenic mice or non-diabetic subjects). Thus IAPP’s role in mediating ER stress in beta cells remains controversial.
Chapter 1.

1.7 Leptin receptor mutant mouse model

The leptin receptor deficient Lepr^{db/db} mouse is a model of diabetes that resembles the common form of human type 2 diabetes displaying obesity, insulin resistance and beta cell failure\(^{69,201}\). The adipocyte-specific hormone leptin is the product of the obese (ob) gene. It regulates body weight through hypothalamic effects on satiety and energy expenditure\(^{231}\). Leptin acts through the leptin receptor, which is located on mouse chromosome 4 as diabetes (db) gene\(^{232}\). Leptin receptor is expressed at a high level in the hypothalamus and is abnormally spliced in C57BL/Ks db/db mice, inducing defects in the weight-reducing effects of leptin\(^{233}\). In rodents, homozygous mutations in the leptin receptor cause obesity, hyperphagia and reduced energy expenditure. Alterations in glucose homeostasis, dyslipidemia and infertility due to hypogonadism have also been observed\(^{233}\). In human studies, a loss of function mutation in the leptin receptor is associated with early-onset obesity, no pubertal development and reduced growth hormone and thyrotropin\(^{234}\).

Lepr^{db/db} mice become obese, develop hyperglycemia at 4-8 weeks of age and have defects in insulin secretion as well as deterioration in islet structure and beta cell apoptosis\(^{69,235,236}\). This mouse model develops type 2 diabetes due to failure of beta cells to compensate for increasing obesity and insulin resistance\(^{201,236}\). Expression of multiple UPR genes was enhanced in islets of 10-12 week old Lepr^{db/db} mice, including \textit{Atf4}, \textit{Atf6}, \textit{Crebl1}, \textit{Ddit3}, \textit{Hsp90b1}, \textit{Hspa5}\(^{201}\). More recent data showed that the adaptive UPR genes progressively decline in a time-dependent manner in Lepr^{db/db} mice\(^{235}\). Treatment of islets from Lepr^{db/db} mice in vitro with a chemical chaperone to inhibit the decline in UPR partially restores changes in beta cell function genes and transcription factors. This indicates that maintenance of the adaptive UPR is related to beta cell compensation, and failure of adequate adaptive UPR expression is associated with beta cell apoptosis in Lepr^{db/db} mice. Islets from Lepr^{db/db} mice also showed increased oxidative stress. Treatment of mice with the antioxidant drug N-acetyl-L-cysteine (NAC) improved islet function and increased beta cell mass in this model\(^{69,237}\). Therefore, Lepr^{db/db} mice is a useful model to study beta cell apoptosis, ER and oxidative stress, and the role of the pro-apoptotic protein BIM in type 2 diabetes.
1.8 Aims and hypothesis

ER stress has been shown to induce insulin resistance in insulin sensitive organs and apoptosis of beta cells. Apoptosis of beta cells may contribute to loss of beta cell mass in type 2 diabetes. BIM, an important pro-apoptotic molecule, is upregulated in human islets isolated from type 2 diabetic organ donors. High glucose concentrations, also known as glucotoxicity, are believed to contribute to defective insulin secretion, beta cell function and beta cell apoptosis. After exposure to high concentrations of glucose, islets from mice lacking BIM showed less glucotoxicity, indicating that BIM may play an important role in beta cell apoptosis in type 2 diabetes. Although beta cell apoptosis has been recognized in type 2 diabetes, inhibition of apoptosis has not been fully studied in vivo in type 2 diabetic models. Therefore, this study aims to investigate the metabolic effects and islet biology of BIM inhibition in the Lepr<sup>db/db</sup> mouse model of type 2 diabetes. Inhibition of beta cell apoptosis has been suggested as a therapeutic strategy for type 2 diabetes. In this study, I hope to shed light on the potential efficacy of such a strategy.

**Aim 1. Investigate the role of BIM in regulating glucose homeostasis in type 2 diabetes.**

We hypothesize that BIM is essential for beta cell apoptosis in type 2 diabetes and thus its inhibition will lead to protection of beta cells from ER and oxidative stress. This will result in increased ability of beta cells to secrete insulin, better regulation of glucose uptake and storage in peripheral insulin sensitive tissues, and improved glucose homeostasis in type 2 diabetes.

Because BIM is ubiquitously expressed, results from whole body BIM-deficient mice may be due to a role for BIM in many cells types, including beta cells, liver, muscle and adipose tissue. Therefore, we generated mice with beta cell specific deletion of BIM to study the role of BIM directly in beta cells. We hypothesized that mice lacking BIM specifically in beta cells would have reduced beta cell death, leading to improved glucose homeostasis in type 2 diabetes.

**Aim 2. Define the role of BIM in regulating beta cell mass in type 2 diabetes.**
Beta cell mass is maintained by a balance between cell replication and death. When type 2 diabetes occurs, it is generally believed that the rate of death is greater than the rate of cell replication. Therefore, we hypothesize that blocking beta cell apoptosis will increase its mass. To study beta cell mass, we generated Lepr$^{db/db}$ mice with global knockout of BIM, and with beta cell specific deletion of BIM.

**Aim 3. Study glucose homeostasis and beta cell mass by inhibition of BIM in response to severe insulin resistance induced by the insulin receptor antagonist S961.**

Previous data from BIM deficiency in C57BL/6 mice showed a role of BIM in regulating mitochondrial bioenergetics and thereby improving insulin sensitivity (See Appendix A). We hypothesized BIM’s role of improving insulin sensitivity could contribute to the regulation of glucose homeostasis and beta cell mass. By generating a model using insulin receptor antagonist S961, we aim at studying the effect of BIM deficiency in the absence of its role in insulin sensitivity.
Chapter 2.

Preventing apoptosis in beta cells with BIM deficiency does not impact on glucose homeostasis in Lepr$^{db/db}$ mice
2.1 Summary

Loss of beta cell mass due to apoptosis is a key feature of type 2 diabetes. Our lab previously reported that ER and oxidative stress result in apoptosis of beta cells mediated by the pro-apoptotic BCL-2 family molecules BIM and PUMA. We hypothesized that preventing loss of beta cell mass by inhibiting apoptosis would improve glucose homeostasis in type 2 diabetes. To test this, we made Lepr\textsuperscript{db/db} mice, a model of type 2 diabetes, deficient in BIM globally and only in β cells.

Global BIM deficiency in Lepr\textsuperscript{db/db} mice significantly improved glucose homeostasis. Loss of BIM decreased non-fasting glucose, fasting glucose and improved insulin sensitivity and glucose tolerance in Lepr\textsuperscript{db/db} mice. In contrast, BIM deficiency only in the beta cells of Lepr\textsuperscript{db/db} mice did not improve glucose homeostasis. Body weight regulation, fasting and non-fasting glucose, glucose tolerance and insulin tolerance were all the same as in wild-type Lepr\textsuperscript{db/db} mice. This indicates that BIM deficiency in the beta cells does not contribute to the metabolic phenotype of global knockout mice, and that partial prevention of beta cell apoptosis by BIM inhibition does not prevent type 2 diabetes. The role of BIM in metabolic sensitive tissues and its possible non-apoptotic role require further investigation.

2.2 Introduction

Beta cells compensate in response to chronic insulin resistance by initially increasing insulin output. However, prolonged beta cell overwork results in reduced beta cell function and turnover\textsuperscript{57}. Eventually the beta cells fail to produce enough insulin to meet demand and diabetes occurs. High glucose concentrations (glucotoxicity) impair beta cells’ function and lead to loss of beta cell mass. A reduction of 30%-70% of the beta cell mass has been observed in type 2 diabetic individuals compared to non-diabetic controls\textsuperscript{66,71,73,75}. Studies in animal models and human individuals have shown that the increased beta cell death is due to apoptosis\textsuperscript{69,70,86}.

ER and oxidative stress are recognized mediators of beta cell glucotoxicity in vitro and in animal models and human type 2 diabetes\textsuperscript{174,219,220,229,235,238}. Previously our
laboratory has shown that glucose-induced ER and oxidative stress activate the intrinsic apoptotic pathway in islets, specifically the pro-apoptotic molecule BIM. Loss of BIM reduced ER and mitochondrial oxidative stress-induced apoptosis in islets. Increased BIM expression was observed in isolated islets from type 2 diabetes individuals. These findings suggest that BIM could play an important role in mediating beta cell apoptosis in type 2 diabetes.

Although our laboratory have established a clear role for BIM in islet cell apoptosis in vitro, we also identified a role for BIM in regulating oxidative metabolism in vivo (See Appendix A). BIM-/- mice on a C57BL/6 background fed a normal chow diet had reduced adiposity and improved insulin sensitivity. We showed that this is due to increased mitochondrial oxygen consumption and energy expenditure, with increased activity of mitochondrial complex IV (See Appendix A). However whether these phenotypes are secondary to apoptosis inhibition or an unidentified non-apoptotic role of BIM is not clear yet. Islet size and function were comparable between BIM-/- and wild type mice indicating that the improved glucose tolerance was more likely due to improved insulin sensitivity in peripheral tissues instead of increased islet function in BIM deficient mice.

Leptin receptor deficient Lepr<sup>db/db</sup> mice become obese, develop hyperglycemia at 4-8 weeks of age and have defects in insulin secretion as well as deterioration in islet structure and beta cell apoptosis. This mouse model develops type 2 diabetes due to failure of beta cells to compensate for increasing obesity and insulin resistance. Expression of multiple unfolded protein response (UPR) genes was enhanced in islets of 10-12 weeks old Lepr<sup>db/db</sup> mice, including Atf4, Atf6, Crebl1, Ddit3, Hsp90b1, Hspa5, and the adaptive UPR genes progressively decline in a time-dependent manner in Lepr<sup>db/db</sup> mice. Islets from Lepr<sup>db/db</sup> mice also showed increased oxidative stress, and treatment with the antioxidant drug N-acetyl-L-cysteine improved islet function and increased beta cell mass. Therefore, Lepr<sup>db/db</sup> mice is a useful model to study the role of pro-apoptotic BIM, which is activated by ER and oxidative stress factors in islets.
Currently there are no therapies for type 2 diabetes that directly target beta cell apoptosis, although apoptosis is thought to be an important feature of this disease. BIM is a potent pro-apoptotic molecule that can bind to all the pro-survival molecules of the BCL-2 family and therefore represents a possible therapeutic target for apoptosis inhibition. This study aims to examine the effects of apoptosis inhibition on glucose homeostasis in type 2 diabetes using BIM deficient Lepr\textsuperscript{db/db} mice. To further investigate the role of apoptosis specifically in beta cells, and to isolate the effects of β cell apoptosis from the effects of BIM deficiency on whole body metabolism, we generated Lepr\textsuperscript{db/db} mice with beta cell-specific deletion of BIM.

2.3 Research methods

2.3.1 Mice
Mice deficient for BIM on a C57BL/6 background were obtained from Dr Philippe Bouillet (WEHI, Melbourne, Australia). Mice with a spontaneous leptin receptor mutation (Lepr\textsuperscript{db}) were obtained from Dr Ross Laybutt (Garvan Institute, Sydney, Australia), originally from The Jackson Laboratories. Mice homozygous for the leptin receptor mutation (Lepr\textsuperscript{db}) become identifiably obese around 3 to 4 weeks of age with elevations of plasma insulin beginning at 10 to 14 days and of blood sugar at four to eight weeks. Affected mice are polyphagic, polydipsic, and polyuric. Homozygous mice cannot breed due to hormone dysregulation\textsuperscript{233}. Thus heterozygous (Lepr\textsuperscript{db/+}) mice, which are non-obese and non-diabetic, were used for breeding.

The beta cell defects in the Lepr\textsuperscript{db/db} mouse model depend on the C57BL/KsJ background. We were able to study the BIM contribution to beta cell apoptosis by investigating all generations from crosses between double heterozygous BIM\textsuperscript{+/−} and Lepr\textsuperscript{db/+} mice in a mixed C57BL/KsJ and C57BL/6J background. First generation Lepr\textsuperscript{db/+}BIM\textsuperscript{+/−} mice were crossed with each other to generate these six groups: Lepr\textsuperscript{db/+} BIM\textsuperscript{+/+}, Lepr\textsuperscript{db/+}BIM\textsuperscript{+/−}, Lepr\textsuperscript{db/+}BIM\textsuperscript{−/−}, Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+}, Lepr\textsuperscript{db/db}BIM\textsuperscript{+/−}, Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−}. All homozygous Lepr\textsuperscript{db/db} litters showed hyperglycemia in the presence of the wild-type BIM alleles, indicating there was sufficient C57BL/KsJ genetic background to support the diabetic phenotype.
For generating BIM deficiency specifically in beta cells, Lepr\textsuperscript{db/db} mice harboring a \textit{Bim} gene flanked by loxP sites\textsuperscript{239,240} (BIM\textsuperscript{fl/fl}, obtained from Dr Philippe Bouillet, WEHI) were crossed with mice expressing Cre under the rat insulin promoter (RIP-Cre)\textsuperscript{241} on a C57BL/6 background. BIM\textsuperscript{fl/Cre/+} mice were intercrossed to generate BIM\textsuperscript{fl/Cre/+} mice. BIM\textsuperscript{fl/Cre/+} mice were then crossed with Lepr\textsuperscript{db/+} mice to generate Lepr\textsuperscript{db/+BIM\textsuperscript{fl/Cre/+}} mice. These mice were crossed with each other to generate these following groups: Lepr\textsuperscript{db/+BIM\textsuperscript{fl/Cre/+}} (Lepr\textsuperscript{db/+BIM\textsuperscript{-/-}}), Lepr\textsuperscript{db/+BIM\textsuperscript{fl/+}} (Lepr\textsuperscript{db/+BIM\textsuperscript{+/-}}), Lepr\textsuperscript{db/+BIM\textsuperscript{fl/+}} (Lepr\textsuperscript{db/+BIM\textsuperscript{+/-}}), Lepr\textsuperscript{db/+BIM\textsuperscript{fl/+}} (Lepr\textsuperscript{db/+BIM\textsuperscript{+/-}}), Lepr\textsuperscript{db/+BIM\textsuperscript{fl/+}} (Lepr\textsuperscript{db/+BIM\textsuperscript{+/-}}). To control for any metabolic effects of overexpression of Cre in beta cells\textsuperscript{242}, we only used mice that were Cre/+. Lepr\textsuperscript{db/db} mice maintained their obese and diabetic phenotype on the mixed background.

All animal experiments were conducted with approval from the institutional animal ethics committee.

\subsection*{2.3.2 Animal housekeeping}

Mice were maintained at St Vincent’s Institute animal house on a 12 h light-dark cycle with access to normal chow food and water. Cages were regularly changed for obese and diabetic mice. Body weights and non-fasting blood glucose concentrations were measured weekly from 5 to 20 weeks of age. Fasting blood glucose was measured every four weeks from 5 weeks of age after 8 hours of fasting. At 3 months and 9 months of age, mice were fasted for 8 hours, weighed and blood glucose measured before culling for collection of serum and organs including pancreas, liver, inguinal fat pad, epididymal fat pad in male mice and peri-renal fat pad in female mice. Serum was collected from mouse heart.

\subsection*{2.3.3 Body fat quantification}

To quantify body fat, 9-month-old female Lepr\textsuperscript{db/db} mice were analysed under anaesthetic by micro-CT (SkyScan 1076 micro-CT scanner, Bruker, Brussels, Belgium). The settings for scanning were optimized for adipose tissue by following the guidelines from the manufacturer. Serial micro-CT images of the mouse abdomen
obtained from the scanner were reconstructed using NRecon software (Bruker) and abdominal and fat volumes were quantified using CT-analyser (CTAn) software (Bruker). Representative micro-CT images were generated from reconstructed scans using CTvox software (Bruker).

2.3.4 Islet isolation, culture and DNA fragmentation assay
Islets of Langerhans were isolated by collagenase P (dissolved in Hanks’ balanced salt solution containing 2 mmol/l Ca2 and 20 mmol/l HEPES) digestion and density gradient centrifugation as described previously\(^\text{212}\). Islets were washed and handpicked into 3.5 cm petri dishes containing 1.1 ml of complete CMRL. Islets were then cultured with 50mmol/l ribose or complete CMRL medium. At the end of the culture, nonattached cells and islets were washed in PBS. Islets were then dispersed with trypsin (0.1 mg/ml bovine trypsin and 2 mmol/l EDTA in PBS) for 5 min at 37°C. Islets were then gently mechanically dispersed using a pipette, washed in PBS, and left to recover in complete CMRL for 1 h at 37°C in 5% CO\(_2\). To stain the nuclear DNA, cells were then washed in PBS and resuspended in 250 µl of hypotonic buffer containing 50 µg/ml propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. Cells were then analyzed using a FACS machine in the FL3 channel as previously described\(^\text{212,220}\).

2.3.5 Insulin tolerance test
Fourteen week old mice were fasted for 4 hours before performing intra-peritoneal insulin tolerance test (IP-ITT). Insulin (Actrapid, Novo Nordisk, Denmark) was injected into the peritoneal cavity and blood glucose was measured at 0, 15, 30, 45, 60, 90 and 120 minutes. 0.75 U/kg of insulin was injected to Lepr\(^{db/c}\) mice. As Lepr\(^{db/db}\) mice have severe insulin resistance, the dose of insulin was adjusted to 2.5 U/kg.

2.3.6 Glucose tolerance test
Sixteen week old mice were fasted for 6 hours before performing intra-peritoneal glucose tolerance test (IP-GTT). Glucose (1g/kg) dissolved in sterile water (Deerfield, IL) was injected into the peritoneal cavity and blood glucose was measured at 0, 15, 30, 45, 60 and 120 minutes. A glucometer with 50 mmol/l range was used for Lepr\(^{db/db}\) mice (GlucoDr Auto AGM4000, Allmedicus, South Korea).
2.3.7 Insulin ELISA
A rat/mouse insulin enzyme-linked immunoassay (ELISA) kit (Mercodia, Uppsala, Sweden) was used to measure the concentration of insulin in fasting serum samples as per manufacturer’s instructions. Briefly, samples and controls in duplicates were added to the ELISA plate followed by enzyme conjugate solution and incubated for 1 hour at room temperature. ELISA plates were washed 6 times. Bound conjugate was detected by adding substrate trimethylbenzidine (TMB) for 15 minutes and the reaction was stopped by adding a strong acid. The colorimetric endpoint was determined spectrophotometrically by measuring absorbance at 450nm.

2.3.8 Statistical analysis
Statistical analysis was done using GraphPad Prism Software. Comparison of multiple groups was analyzed by Student’s t test or by ANOVA with Bonferroni’s or Turkey’s post test. A p value < 0.05 was considered statistically significant and showed as *p<0.05, **p<0.01 , ***p<0.001 and ****p<0.0001.
2.4 Results

2.4.1 BIM-deficiency protects beta cells from glucose toxicity-induced apoptosis

BIM has been demonstrated to mediate pancreatic islet cell apoptosis in response to high glucose concentrations. We isolated islets from 3 groups of Lepr\textsuperscript{db/+} mice (wild-type=BIM\textsuperscript{+/+}, global knockout=BIM\textsuperscript{-/-} or beta cell specific knockout=\(\beta\text{BIM}^{-/-}\)). Ribose mimics the effects of glucose exposure over a shorter time frame and was used to examine the sensitivity to glucose toxicity\textsuperscript{243}. Islets were exposed to 50mmol/l ribose for 4 days or control medium (complete CMRL medium) and DNA fragmentation, representing apoptosis, was measured by fluorescence-activated cell sorter (FACS) (Figure 2.1). Islets from Lepr\textsuperscript{db/+}/BIM\textsuperscript{+/+} (wild-type islets) cultured in 50 mmol/l ribose showed a significant increase in DNA fragmentation. Deficiency of BIM reduced DNA fragmentation, and similarly, beta cell-specific deficiency of BIM also reduced DNA fragmentation. This confirms that BIM deficiency partially protects islets from ribose-induced apoptosis, and BIM deficiency specifically in beta cells similarly protects islets from ribose-induced apoptosis.

Figure 2.1 BIM-deficiency protects beta cells from glucose toxicity-induced apoptosis.
Islets (100) from Lepr<sup>db+</sup> mice with BIM<sup>+/+</sup>, BIM<sup>−/−</sup> or βBIM<sup>−/−</sup> were cultured in 50mmol/l ribose or control medium. DNA fragmentation was measured by FACS after 4 days of culture. Islets were isolated from a minimum of 3 mice per genotype. ****p<0.0001 compared with islets from Lepr<sup>db+</sup>BIM<sup>+/+</sup> in 50 mmol/l ribose (one-way ANOVA). UT: untreated.

### 2.4.2 Lepr<sup>db/db</sup> mice develop obesity and type 2 diabetes

We generated Lepr<sup>db</sup> mice that had BIM deficiency in all tissues (Lepr<sup>db+/</sup>BIM<sup>+/+</sup>, Lepr<sup>db+/</sup>BIM<sup>−/−</sup>, Lepr<sup>db/db</sup>BIM<sup>+/+</sup>, Lepr<sup>db/db</sup>BIM<sup>−/−</sup>, Lepr<sup>db/db</sup>BIM<sup>−−/−</sup>) or that had BIM deficiency only in beta cells (Lepr<sup>db+/</sup>βBIM<sup>+/+</sup>, Lepr<sup>db+/</sup>βBIM<sup>−/−</sup>, Lepr<sup>db/db</sup>βBIM<sup>−/−</sup>, Lepr<sup>db/db</sup>βBIM<sup>−−/−</sup>, Lepr<sup>db/db</sup>βBIM<sup>−−/−</sup>). We investigated the impact of the Lepr mutation and BIM deficiency on body weight and type 2 diabetes. Body weights were measured weekly from 5 weeks of age to 18 weeks (Figure 2.2 A-D). Both male and female Lepr<sup>db/db</sup> mice gained significantly more body weight than Lepr<sup>db+/</sup> controls as expected (Figure 2.2 A-D). Lepr<sup>db+</sup>βBIM<sup>−/−</sup> mice did not show any difference in body weight compared with Lepr<sup>db+/</sup>βBIM<sup>+/+</sup> controls (Figure 2.2 B&D), however the total body weight of whole body BIM-deficient Lepr<sup>db/db</sup>BIM<sup>/−</sup> mice was significantly higher than wild-type Lepr<sup>db/db</sup>BIM<sup>+/+</sup> mice (Figure 2.2 A&C).

Next we measured the weights of metabolic related tissues (Figure 2.2 E-H). Male BIM deficient Lepr<sup>db/db</sup> mice had a trend towards increased visceral adiposity compared to controls (Figure 2.2 G). Female Lepr<sup>db/db</sup>BIM<sup>−</sup> mice also had greater abdominal volume and total fat volume than Lepr<sup>db/db</sup>BIM<sup>+/+</sup> mice measured by microCT (Figure 2.2 E). The increased adiposity in Lepr<sup>db/db</sup>BIM<sup>/−</sup> mice could contribute to the increased body weight in these mice, although the mechanism is not clear yet. In Lepr<sup>db/db</sup> mice with BIM deficiency in beta cells only, there was no difference in tissue mass of pancreas, liver or adipose tissues in the different groups (Figure 2.2 F&H). This indicates that BIM deficiency in beta cells has no impact on body weight regulation. Thus, the increased adiposity caused by whole body BIM deficiency might result from the enhanced insulin sensitivity shown previously in BIM knockout mice on a C57BL/6 background (See Appendix A).
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Figure 2. BIM deficiency in beta cells does not affect body weight gain in Lepr<sup>db/db</sup> mice.

(A-D) Body weights in grams of females and males as indicated. Weekly body weights from
Lepr\textsuperscript{db/db} BIM mice (global knockout of BIM) were tested from 5 weeks to 20 weeks of age (A,C). Weekly body weights from Lepr\textsuperscript{db/db} BIM mice (beta cell specific knockout of BIM) were measured from 5 weeks to 18 weeks of age (B, D). n=6-20/genotype (E-H) Metabolic related tissues pancreas, liver, inguinal fat pad and epididymal fat pad (only males) weights were measured at the age of 18 weeks for female and male mice, and normalized to total body weight (mg/g). n = 5-12 mice/genotype. Abdominal fat volume was quantified by microCT in female Lepr\textsuperscript{db/db} BIM mice (E). Fat pad of male Lepr\textsuperscript{db/db} BIM mice is shown in G. Lepr\textsuperscript{db/db} BIM mice are shown in F, H.

2.4.3 Improved glucose homeostasis in BIM deficient Lepr\textsuperscript{db/db} mice is not due to protection of beta cells from apoptosis

2.4.3.1 Non-fasting glucose levels in BIM deficient Lepr\textsuperscript{db/db} mice

BIM regulates apoptosis of beta cells after exposure to high concentration of glucose in vitro. A reduction in beta cell apoptosis in vivo would result in increased capacity for the beta cell to produce insulin, resulting in lower glucose levels in Lepr\textsuperscript{db/db} mice. Both male and female Lepr\textsuperscript{db/db} mice had higher non-fasting glucose levels than Lepr\textsuperscript{db/+} controls (Figure 2.3 A-D). Whole body BIM deficient Lepr\textsuperscript{db/db} BIM\textsuperscript{-/-} mice had significantly lower blood glucose levels than Lepr\textsuperscript{db/db} BIM\textsuperscript{+/+} mice (Figure 2.3 A&C), however, this difference was not observed in the Lepr\textsuperscript{db/db} BIM\textsuperscript{-/-} mice (Figure 2.3 B&D). This indicates that while BIM deficiency improves non-fasting blood glucose levels and this is not mediated by loss of BIM in the beta cell.
Figure 2. Non-fasting glucose level in Lepr\textsuperscript{db/db} mice is not affected by BIM deficiency in beta cell.
Non-fasting blood glucose concentration (mmol/l) was measured weekly from 14 weeks to 20 weeks of age for female Lepr<sup>db/db</sup>BIM strain (A), 5-20 weeks old male mice (C) or 5 weeks to 17 weeks of age for Lepr<sup>db/db</sup>βBIM strain for both sexes (B, D) with genotypes as indicated. n=6-20/genotype. (*p<0.05, **p<0.001 Lepr<sup>db/+</sup>BIM<sup>−/−</sup> vs Lepr<sup>db/+</sup>BIM<sup>+/+</sup> mice or Lepr<sup>db/db</sup>BIM<sup>−/−</sup> vs Lepr<sup>db/db</sup>BIM<sup>+/+</sup> mice of the same sex).

2.4.3.2 Fasting glucose level in BIM deficient Lepr<sup>db/db</sup> mice

Fasting blood glucose level reflects glucose output from the liver. Fasting blood glucose levels were decreased in female and male Lepr<sup>db/db</sup>BIM<sup>−/−</sup> mice compared to Lepr<sup>db/db</sup>BIM<sup>+/+</sup>, and this was more significant in females (Figure 2.4 A&C). This could be the result of improved beta cell function generating adequate insulin to meet the demand of hyperglycemia in Lepr<sup>db/db</sup> mice. Alternatively, it may be due to the increased liver insulin sensitivity, thereby reducing glucose output. To test beta cell function, we measured fasting glucose at 5 weeks, 10 weeks, 14 weeks, and 18 weeks of age from Lepr<sup>db/db</sup>βBIM mice (Figure 2.4 B&D). The Lepr<sup>db/db</sup> mice had higher fasting blood glucose levels compared to Lepr<sup>db/+</sup> controls. However, there was no difference in fasting glucose levels between Lepr<sup>db/db</sup>βBIM<sup>−/−</sup> and Lepr<sup>db/db</sup>βBIM<sup>+/+</sup> mice (Figure 2.4 B&D). This indicates that low fasting glucose in Lepr<sup>db/db</sup>BIM<sup>−/−</sup> mice is due to improved liver insulin sensitivity.
Figure 2.4 Fasting glucose level in Lepr$^{db/db}$ mice was not affected by BIM deficiency in beta cell

Blood glucose concentration (mmol/l) was measured after 8 hours fasting from 5-6 weeks, 10 week, 14 weeks and 18 weeks of age for both sexes for Lepr$^{db/db}$BIM mice (A, C) and
Lepr\textsuperscript{db/db}βBIM (B, D). n=6-12 mice/genotype. (***)Lepr\textsuperscript{db/db}BIM\textsuperscript{-/-} vs Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+} female mice

2.4.3.3 BIM deficiency in beta cells does not improve glucose tolerance
We next tested the ability of BIM-deficient beta cells to secrete insulin and clear glucose. We performed a glucose tolerance test at 16 weeks of age (Figure 2.5 A-H). Lepr\textsuperscript{db/db} mice showed a delayed glucose clearance as compared to Lepr\textsuperscript{db/+} controls (Figure 2.5 A-H). Glucose tolerance was improved in Lepr\textsuperscript{db/db}BIM\textsuperscript{-/-} mice compared to Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+} (Figure 2.5 A&C, E&G). Serum insulin concentration of Lepr\textsuperscript{db/db}BIM\textsuperscript{-/-} mice was increased about two-fold compared to Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+} (Figure 2.5 I). However, Lepr\textsuperscript{db/db}βBIM\textsuperscript{-/-} mice showed a similar response to glucose as Lepr\textsuperscript{db/db}βBIM\textsuperscript{+/+} mice (Figure 2.5 B&F), and there was no difference in AUC for glucose (Figure 2.5 D&H). This indicates that in a mouse with type 2 diabetes and obesity, beta cell specific deletion of BIM does not change beta cell function or glucose clearance.
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Figure 2. 5 BIM deficiency in beta cells does not improve glucose tolerance.

(A, B, E, F) IP-GTT was performed on 16-17 week old female or male mice after 6 hours fasting by injecting 1g/kg of glucose for Lepr<sup>db/db</sup>BIM (A, E) and Lepr<sup>db/db</sup>βBIM (B, F). n=6-14/genotype. (*p<0.05, **p<0.01, ***p<0.001 Lepr<sup>db/+</sup>BIM<sup>−/−</sup> vs Lepr<sup>db/+</sup>BIM<sup>+/+</sup> or Lepr<sup>db/db</sup>BIM<sup>−/−</sup> vs Lepr<sup>db/db</sup>BIM<sup>+/+</sup> mice of the same sex).

(C, D, G, H) Area under the curve was measured based on the IP-GTT results above for Lepr<sup>db/db</sup>BIM (C, D) and Lepr<sup>db/db</sup>βBIM (G, H) (**p<0.01, ***p<0.001 Lepr<sup>db/+</sup>BIM<sup>−/−</sup> vs Lepr<sup>db/+</sup>BIM<sup>+/+</sup> or Lepr<sup>db/db</sup>BIM<sup>−/−</sup> vs Lepr<sup>db/db</sup>BIM<sup>+/+</sup> mice of the same sex).

I. Serum insulin concentration of 9 month old female mice measured by ELISA for Lepr<sup>db/db</sup>BIM strain. (n = 4-6 mice/genotype). (**p<0.01 Lepr<sup>db/db</sup>BIM<sup>−/−</sup> vs Lepr<sup>db/db</sup>BIM<sup>+/+</sup> mice).

2.4.3.4 Insulin sensitivity in BIM deficient Lepr<sup>db/db</sup> mice

BIM<sup>−/−</sup> mice on a C57BL/6 background were more insulin sensitive than wild type mice (See Appendix A). We tested insulin sensitivity in Lepr<sup>db/db</sup> mice using insulin tolerance tests at 14-15 weeks of age (Figure 2.6 A-L). Both lepr<sup>db/db</sup> and Lepr<sup>db/+</sup> groups were responsive to insulin. As expected, BIM-deficient Lepr<sup>db/+</sup>Bim<sup>−/−</sup> mice were more insulin sensitive than wild-type Lepr<sup>db/+</sup> mice (Figure 2.6 C&E, I&K). Whole body BIM deficient Lepr<sup>db/db</sup>BIM<sup>−/−</sup> females, but not males, were also more insulin sensitive than Lepr<sup>db/db</sup>BIM<sup>+/+</sup> females (Figure 2.6 A&E, G&K), but there was no difference detected when BIM was deficient only in beta cells (Figure 2.6 B,D,F&H,G,L). This indicates that the difference in insulin sensitivity is likely the result of whole body deficiency of BIM, and not due to improved beta cell function.
Figure 2. 6 BIM deficiency in beta cells of Lepr<sub>db/db</sub> mice had similar sensitivity to insulin compared to controls.

(A-D, G-J) IP-ITT of 14 week old mice after 4 hours fasting by injecting 0.75 U/kg of insulin
BIM has been implicated in apoptosis of beta cells in conditions that may be experienced in obesity and diabetes, such as high concentrations of glucose\textsuperscript{212,220}. We therefore tested the hypothesis that its deficiency might result in increased survival and insulin production by beta cells and thus improved glucose homeostasis in obesity and type 2 diabetes in the Lepr\textsuperscript{db/db} mouse model. Our results show that whole body BIM deficiency results in improved glucose homeostasis. However, Lepr\textsuperscript{db/db} mice with beta cell-specific BIM deletion did not show the same improved glucose homeostasis. Glucose homeostasis is regulated as a whole system, with all tissues including liver, adipose tissue, muscle as well as beta cells contributing to the metabolic phenotype.

Although the exact molecular mechanism of improved glucose homeostasis in BIM deficient Lepr\textsuperscript{db/db} mice is not clear, the metabolic phenotype is quite similar to BIM deficient mice on a non-diabetic C57BL/6 background. Previously Wali et al. showed that BIM deficient mice had lower fasting blood glucose levels that were accompanied by improved glucose tolerance as well as insulin sensitivity (See Appendix A). BIM deficient mice had reduced adiposity due to increased lipid oxidation. Further investigation showed that BIM regulates mitochondrial OXPHOS and its deficiency results in increased mitochondrial oxygen consumption and activity of complex IV. These effects of BIM deficiency have most likely contributed to the phenotype of BIM-deficient Lepr\textsuperscript{db/db} mice.

There are several studies focusing on protecting beta cells from apoptosis. For example, one group studied deficiency of the UPR-induced transcription factor C/EBP homologous protein (CHOP) in Lepr\textsuperscript{db/db} mice\textsuperscript{235}. CHOP is an ER stress factor upstream of BIM. Global deletion of CHOP in Lepr\textsuperscript{db/db} mice resulted in improved glucose homeostasis, with reduced fasting blood glucose and improved glucose
tolerance compared to Lepr\textsuperscript{db/db} controls. Islets from these CHOP\textsuperscript{+/−} mice showed increased expression of UPR genes and oxidative stress genes as well as beta cell survival, suggesting CHOP mediates the connection between ER stress, oxidative stress and apoptosis in beta cells under type 2 diabetic conditions. BIM deletion in high fat fed Pdx1\textsuperscript{+/−} mice also showed improved glucose homeostasis\textsuperscript{244}, similar to that observed in our study. Similar improvements were also shown in BIM-deficient IRS2-deficient mice fed a chow diet\textsuperscript{245}. Together with our global BIM deficiency data, these findings suggest that whole body inhibition of ER stress and apoptosis could contribute to prevent or reduce type 2 diabetes.

To our knowledge, the current study is the first where apoptosis has been inhibited specifically in the beta cells in a type 2 diabetes mouse model. In contrast to whole body knockouts, Lepr\textsuperscript{db/db} βBIM\textsuperscript{−/−} mice show that the metabolic effects of global knockout of BIM do not result from direct effect of BIM deficiency in beta cells. Possible explanations for this result include: 1) a non-apoptotic role for BIM (as discussed above); and 2) the apoptotic role of BIM in metabolic organs and/or the immune system contributing to the overall metabolic phenotype. Because BIM is ubiquitously expressed, it may be important in many cell types, including pancreatic beta cells, insulin-sensitive peripheral tissues involved in type 2 diabetes pathogenesis and also the immune system. We have ruled out the direct effect of BIM deficiency in beta cells, but further work needs to be done to rule out its role in other tissues.

In Lepr\textsuperscript{db/db} BIM\textsuperscript{+/−} mice, fasting blood glucose levels were lower than Lepr\textsuperscript{db/db} BIM\textsuperscript{+/+} mice. Fasting blood glucose represents both the ability of beta cells to secrete insulin and glucose production from the liver, which is in turn determined by hepatic insulin sensitivity. As shown in mice with beta cell specific deletion of BIM, there is no difference in the fasting blood glucose levels between groups, indicating that beta cell function has not been improved by BIM inhibition. Therefore, the liver, as a source of glucose production from glycogen, might take part in regulating fasting blood glucose levels in BIM deficient mice. It has been reported that BIM is functionally active and restrained by anti-apoptotic proteins in healthy liver\textsuperscript{246}. Hepatocyte apoptosis through the activation of BH3-only proteins is involved in the pathophysiology of various liver diseases. For example, saturated free fatty acids induce expression of BIM through
activation of FoxO3a\cite{247}. Under sustained exposure to reactive oxygen species (ROS), hepatocyte apoptosis is activated through increased expression of BIM_{EL} transcripts and decreased proteasomal degradation of BIM_{EL}\cite{248}. Inhibition of BIM in our model may result in better liver function and regulation of fasting blood glucose. The improved insulin sensitivity in global BIM knockout mice indicates that BIM in peripheral insulin-sensitive tissues has a role in regulating glucose homeostasis, confirmed by the lack of change in insulin sensitivity in beta cell specific BIM knockout Lepr\textsuperscript{db/db} mice. Our preliminary data from liver specific deletion of BIM in high fat fed mice shows that there is an improvement of glucose homeostasis (E Gurzov et al, unpublished). These findings, together with our data, suggest that hepatic insulin sensitivity is improved in the absence of BIM. Glucose levels depend on beta cell insulin secretion, hepatic glucose output and glucose extraction in muscle and adipose tissues. The glucose clearance in glucose tolerance test is faster in Lepr\textsuperscript{db/db}BIM\textsuperscript{+/−} mice and not affected in beta cell specific BIM deficient mice. Thus, although BIM deficiency does not change beta cell insulin secretion directly, it improves insulin sensitivity. In addition to hepatic insulin sensitivity as we mentioned, glucose extraction in muscle and adipose tissues could be also enhanced. Whether these findings are due to apoptosis inhibition or some undiscovered role of BIM requires further investigation.

We hypothesize that the increased adiposity and body weight in Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} mice is the result of increased insulin sensitivity. An overload of nutrients results in their uptake and storage into insulin sensitive tissues such as adipose tissue. Tissues in BIM-deficient mice are more sensitive to insulin, therefore high glucose puts less stress on beta cells, thus beta cell function or beta cell mass is preserved for longer. Therefore there is more insulin secretion from beta cells and occurrence of diabetes is delayed. In the Lepr\textsuperscript{db/db}BIM\textsuperscript{+/−} mice, severe insulin resistance causes high glucose level, mice are then in a catabolic state and thus lose body weight. However, in Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} mice, improved insulin sensitivity generates less glucotoxicity and lipotoxicity. Thus insulin concentration is increased and can store excess glucose into fat. Thus the weight threshold for development of diabetes is higher.
Immune cells have been shown to be present in the abdominal fat in diet-induced obesity models, and there is a reduction in Treg cells and an increase in inflammatory phenotype cells such as Th1 CD4$^+$ T cells and M1 macrophages$^{58,64,65}$. The role of BIM in the immune system has been studied. During development in the thymus, T lymphocytes, in association with a T-cell receptor-CD complex that engages self antigens, undergo apoptosis (negative selection) mediated essentially by BIM$^{113}$. Therefore autoreactive T cells escape this deletion in the thymus in BIM-deficient mice. However autoimmunity does not occur, because there is a preferential increase of FoxP3+CD4+ Treg cells$^{249}$. These changes could contribute to the improved insulin sensitivity in BIM deficient mice through impact on adipose tissue inflammation. However, we did not observe any changes in immune cell composition in the fat of non-diabetic BIM-deficient mice, nor did we observe increased insulin sensitivity when BIM-deficient bone marrow was transferred to wild type C57BL/6 mice (J Wali, unpublished), suggesting that BIM deficiency in the immune system alone is unlikely to contribute to the metabolic phenotype in these mice.

Although BIM has been implicated in apoptosis of beta cells induced by high concentrations of glucose, mediated through ER and oxidative stress in vitro, the more complex in vivo environment in Lepr$^{db/db}$ mice may induce apoptotic effects on beta cells that are not mediated by BIM. There are 8 pro-apoptotic BH3-only proteins that are activated in a cell type and stimulus-specific manner. PUMA was activated in beta cells after exposure to saturated free fatty acids, high glucose concentrations and ER stressors resulting in beta cell death in vitro$^{207,220,250}$. PUMA was also activated in beta cells in high fat diet treated Pdx1-deficient mice$^{244}$. However, a recent study showed that PUMA deficiency does not affect glucose homeostasis in high fat diet-induced obesity$^{171}$, indicating that PUMA does not directly regulate metabolism. The cytokine interleukin-1β (IL-1β) has also been implicated in beta cell death in type 2 diabetes. IL-1β, a proinflammatory cytokine, has been shown to inhibit beta cell function and promote beta cell apoptosis. The human IL-1-receptor antagonist improved glycemia and beta cell function and reduced systemic inflammation in type 2 diabetic patients$^{251}$. However, deficiency of IL-1 receptor did not prevent glucose-induced islet cell apoptosis, and BIM-deficiency did not prevent IL-1β-mediated beta cell death in vitro$^{212}$. Necrosis is another mechanism by which beta cells may die. Although
necrosis was believed to be an uncontrolled process which releases inflammatory cellular contents, there is evidence that this form of cell death is regulated by a sequence of signaling processes including oxidative stress, loss of Ca\textsuperscript{2+} homeostasis or ischemia\textsuperscript{252}. Recently necrosis was shown to be the main cell death pathway in CD4+ T cell-mediated autoimmune diabetes, and in that model, the programmed apoptotic cell death pathway was dispensable\textsuperscript{253}. We have yet to develop an inhibitor for necrosis to test the possibility that it may be involved in type 2 diabetes.
Chapter 3.

Deficiency of BIM results in increased islet size and islet beta cell volume in Lepr\textsuperscript{db/db} mice
3.1 Summary

Diabetes in Lepr\textsuperscript{db/db} mice is characterized by loss of functional beta cell mass and beta cell apoptosis. We, therefore, used this mouse model to study the role of the apoptosis regulator BIM in beta cell compensation and survival in type 2 diabetes. We observed a striking increase in islet size in BIM-deficient Lepr\textsuperscript{db/db} mice by histology. Beta-cell size was similar between Lepr\textsuperscript{db/db} BIM\textsuperscript{+/+} and Lepr\textsuperscript{db/db} BIM\textsuperscript{−/−} mice, suggesting that the increase in islet size was due to an increase in the number of beta cells. Quantification of islet volume with 3-dimensional optical projection tomography revealed a significant increase in islet volume in BIM-deficient compared with wild-type Lepr\textsuperscript{db/db} mice (4.2\times10^6±0.7 x10^6 vs 2.1x10^6±0.4 x10^6 µm\textsuperscript{3}). The increased islet volume was accompanied by a two-fold increase in serum insulin concentration (19.3±2.1 vs 9.7±2.3 µg/L). We detected a reduced number of TUNEL positive islets cells in BIM-deficient compared with wild-type Lepr\textsuperscript{db/db} islets, indicating that the islet phenotype may be secondary to apoptosis inhibition. We also observed approximately 2-fold increase in the number of BrdU-positive beta cells in BIM-deficient compared with wild-type Lepr\textsuperscript{db/db} mice, possibly a consequence of beta cell compensation for insulin resistance. Isolated islets from Lepr\textsuperscript{db/db} BIM\textsuperscript{−/−} mice displayed increased expression of the cell cycle gene \textit{Ccnd1}, reduced expression of inflammation genes \textit{Il6} and \textit{Cxcl1} and a trend of reduced level of UPR genes \textit{Hspa5} and \textit{Ddit3}, indicating that BIM inhibition provides a model of mild ER stress, reduced inflammation, and compensation to insulin resistance. However, we did not observe any changes in islet size in Lepr\textsuperscript{db/db} mice with beta cell-specific deletion of BIM. Taken together with the observation that glucose homeostasis was not altered in Lepr\textsuperscript{db/db} with beta cell-specific deletion of BIM, we propose that BIM does not play a direct role in beta cell apoptosis in type 2 diabetic conditions, but its global inhibition generates a compensatory model by improving glucose homeostasis and increasing beta cell mass.
3.2 Introduction

Over the past 20 years, the idea that beta cell inadequacy is an important feature of type 2 diabetes has been generally agreed. Most people with insulin resistance will never develop diabetes, type 2 diabetes only occurs when beta cell mass fails in the battle to generate adequate insulin to meet demand. Loss of beta cell mass in patients with type 2 diabetes has been observed consistently in human studies. Rodent studies also show similar observations. Evidence suggests that apoptosis is an important mediator for the loss of beta cell mass in type 2 diabetes. TUNEL and caspase 3, markers for apoptosis, have been detected in human beta cell studies. Rodent studies also reveal apoptosis as the main mechanism of beta cell loss.

Beta cell mass is maintained by the balance between cell birth and death. Beta cell birth occurs through replication from preexisting beta cells or neogenesis, the newly generated islets from pancreatic ductal cells. Human studies have shown that 0.5% beta cells are positive for the replication marker Ki67 in pancreatic samples collected from surgery. When human islets are transplanted into mice, 0.2-0.7% of beta cells are positive for Ki67. Although the rate is very low, if beta cell mass keeps a 0.5% Ki67 positivity for 12 hours, beta cell mass could more than double within one year. It is mostly believed that neogenesis occurs in the neonatal period when beta cells originate from ductal precursor cells, or in circumstances of injury. However, in a recent human study of insulin resistant and insulin sensitive individuals, islet neogenesis was detected in insulin resistant individuals. Because there is no specific marker of neogenesis, the origin of newly generated beta cells remains unclear. Rodents studies also suggest that beta cells have the capacity to expand when metabolic load and insulin demand is increased and we consider this as a compensation stage. At this stage, the balance between proliferation and cell death in beta cells has been switched in favor of proliferation. However, when prolonged metabolic load or excessive metabolic demands exceed the capacity of increasing beta cell mass, beta cell mass declines and this is a stage of decompensation. Increased glucose might trigger the beta cell mass expansion initially, but prolonged high levels lead to glucotoxicity resulting in beta cell apoptosis and loss of beta cell mass. At this stage, the apoptotic rate is more than the proliferation rate.
Beta cell death is detected by TUNEL staining. It is only possible to detect limited numbers of positive cells because of quick clearance of dead cells by surrounding cells such as macrophages. Hence the real rate for beta cell death is usually underestimated. Increased frequency of apoptosis was detected in obese type 2 diabetes compared to non-diabetic patients\(^{66}\). In lean individuals, there were 0.47 apoptotic cells per islet in type 2 diabetic subjects compare to 0.07 apoptotic cells per islet in non-diabetic subjects. In the same study, no changes in replication rate were detected between type 2 diabetic and non-diabetic subjects\(^{66}\). These findings indicate that although apoptosis and replication occur at a very low rate, a small increase in the apoptosis rate could lead to significant loss of beta cell mass in type 2 diabetes. Because of the limited techniques for measuring beta cell mass in humans, most data about beta cell mass comes from rodents. The homozygous Lepr\(^{db/db}\) mouse is extensively used as a model of type 2 diabetes. These mice become obese, insulin resistant and diabetic at around 8 weeks of age\(^{262}\). It was recently shown that beta cell mass in male Lepr\(^{db/db}\) mice increased from 2.1 ± 0.3 mg at a very young age (5 weeks) to 4.84 ± 0.26 mg at the peak of compensation (12 weeks), then reduced to 3.27 ± 0.44mg during the decompensation stage (34 week�\(^{263}\). During the compensation stage it is hypothesized that proliferation is greater than apoptosis.

Previously we have identified that the pro-apoptotic BH3-only protein BIM is required for glucose-induced apoptosis of islet cells in vitro\(^{212}\). High concentrations of glucose induce ER and oxidative stress that activate the downstream intrinsic apoptosis of beta cells through a BIM-dependent pathway. Islets isolated from human pancreas donors had increased expression of BIM in type 2 diabetic patients compared to non-diabetic pancreas donors\(^{220}\). This suggests that BIM may play a role in the loss of beta cell mass in humans as well.

In this study, we investigated the role of BIM in regulating beta cell mass in type 2 diabetes. We generated Lepr\(^{db/db}\) mice with either global or beta cell-specific BIM deficiency. We studied the role of BIM in pancreatic beta cell mass during the compensation and decompensation phases of type 2 diabetes development and investigated whether preventing BIM-dependent apoptosis resulted in protection of beta cell mass.
3.3 Research methods

3.3.1 Mice
Mice were bred and maintained at St Vincent’s Institute animal house on a 12 h light-dark cycle with access to normal chow food and water as described in chapter 2.

3.3.2 Animal housekeeping
At 3 months, 6 months and 9 months of age, mice from the diabetic groups (Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+}, Lepr\textsuperscript{db/db}BIM\textsuperscript{-/-}) and non-diabetic controls (Lepr\textsuperscript{db/+}BIM\textsuperscript{+/+}, Lepr\textsuperscript{db/+}BIM\textsuperscript{-/-}) were sacrificed for collection of pancreas tissues, a small piece of spleen was also harvested to be used as a positive control for proliferation and apoptosis staining. Mice from groups Lepr\textsuperscript{db/db}βBIM\textsuperscript{+/+}, Lepr\textsuperscript{db/db}βBIM\textsuperscript{-/-}, Lepr\textsuperscript{db/+}βBIM\textsuperscript{+/+} and Lepr\textsuperscript{db/+}βBIM\textsuperscript{-/-} were culled for the collection of the pancreas at 18 weeks of age. Duodenal lobes of the pancreas were put into 4% paraformaldehyde (PFA) for Optical Projection Tomography (OPT), the remainder of the pancreas were fixed for histology (4% PFA for TUNEL staining and formalin for H&E, insulin-fluorescence and BrdU staining). To keep sex consistent, all the analyses done in this chapter were performed in female mice only.

3.3.3 Histological studies
3.3.3.1 Pancreatic Tissue Processing
Pancreas samples were fixed and embedded in paraffin for analysis. Five-micrometer sections were cut at three levels separated by 100 µm and stained with H&E or by immunofluorescence.

3.3.3.2 Hematoxylin and Eosin (H&E)
Slides were dewaxed and stained in haematoxylin, blued in Alkaline Solution, dehydrated in 70% ethanol, 90% ethanol and 100% ethanol multiple times, cleared in Histoclear and mounted with mounting medium Dibutylphthalate Polystyrene Xylene (DPX).

3.3.3.3 Insulin and glucagon immunofluorescence
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Formalin-fixed sections were stained with insulin and glucagon, and counterstained with DAPI. Primary antibodies were guinea pig anti-insulin antibody (1:200, Catalogue number # A0564-01-2, incubated 1 hour in 10% FCS in PBS, Dako, Glostrup, Denmark) and mouse anti-glucagon antibody (1:100, #G265-4-100ul, Sigma-Aldrich, St. Louis, MO). Secondary antibodies were Alexa Fluor®555-conjugated goat anti-guinea pig IgG antibody (1:100, # A056401-2, incubated 1 hour in 10% FCS in PBS, Dako, Glostrup, Denmark) and mouse anti-glucagon antibody (1:100, #G2654-100ul, Sigma-Aldrich, St. Louis, MO). Nuclei were stained with DAPI reagent. Images were taken on a Nikon A1R-A1 confocal microscope (Nikon Corporation, Tokyo, Japan).

3.3.3.4 Insulin immunohistochemistry
Formalin-fixed sections were stained with insulin or glucagon. The primary antibody was guinea pig anti-insulin (Dako, Glostrup, Denmark), the secondary antibody was anti-guinea pig HRP (1:40, #P0141, Dako, Denmark). Sections were then reacted with 3,3’-diaminobenzidine (DAB) and counterstained with haematoxylin. Slides were then left to dry overnight and scanned on an Aperio slide scanner. Each insulin+ area was manually measured using the image software Aperio imageScope.

3.3.3.5 Proliferation and Apoptosis
To evaluate beta cell proliferation, BrdU (100µg/g body weight, BrdU powder dissolved in Baxter water) was injected to mice intraperitoneally as described previously. Due to the very low rate of proliferation in adult mice, we continuously labelled cells with BrdU by giving BrdU water (1mg/ml BrdU dissolved in drinking water) for 9 days. BrdU water bottles were wrapped with aluminum foil to prevent light exposure and made freshly every other day. Pancreas tissue was harvested and processed as described above. Sections were stained with mouse anti-BrdU antibody (Sigma-Aldrich). The percentage of BrdU-positive cells was analyzed by quantifying BrdU+Insulin+ cells divided by the total number of islets in the 9-month-old group or by total insulin+ staining area in the 3-month-old group as previously described. To determine insulin+ staining area, images were collected using a Nikon microscope and analyzed using ImageJ64.
A TUNEL assay kit (1:100 dUTP-bio in TUNEL buffer, # 1093070, Roche) was used to determine the percentage of TUNEL positive cells by quantifying TUNEL$^+$Insulin$^+$-positive cells divided by the total number of islets in the 9-month-old group or by total insulin$^+$-staining area in the 3-month-old group. Representative images were collected using a Nikon microscope. BrdU$^+$Insulin$^+$ or TUNEL$^+$Insulin$^+$ cells were blinded to the mouse genotype and manually counted using a fluorescent microscope.
3.3.4 Optical Projection Tomography (OPT)
Duodenal lobes of pancreas were fixed in fresh 4% PFA, dehydrated in methanol, incubated in MeOH:DMSO:H$_2$O$_2$ to quench the autofluorescence, put into -80°C 5 times for 1 hour each time and back to room temperature to ensure antigens in deeper parts of the tissue were accessible. Then tissues were stained with guinea pig anti-insulin antibody for 2 days and washed thoroughly in TBST buffer, then stained with Alexa Fluo®555-conjugated goat anti-guinea pig IgG antibody (Life Technologies) for 2 days and washed. Tissues were then mounted in 1% low melting agarose and trimmed into a block with pancreas tissue in the center. The four corners of the agarose were cut off. Agarose was then dehydrated in methanol for 24 hours and cleared in 1:2 Benzyle alcohol, Benzyle benzoate (BABB solution) for 24 hours. Samples were then scanned at Monash University using a Bioptonic s 3001 OPT scanner (Skyscan). Tomographic reconstruction was generated using the Nrecon software (Brucker). Iso-surfaces of insulin-positive beta cell mass were created using Imaris software (Bitplane, Switzerland). The beta cell volumes and pancreas tissue volumes of each pancreas sample were quantified using Imaris software (Bitplane, Switzerland).

3.3.5 Islet isolation and qPCR analysis
Islets of Langerhans were isolated as described previously$^{212}$. RNA was prepared using the NucleoSpin RNA XS (Macherey Nagel, Dülmen, Germany). First-strand cDNA was prepared from 200 to 300ng RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). c-DNA was diluted (1 : 100) and real-time PCR was performed using the Rotor-Gene RG-3000 machine (Corbett Research; Qiagen, Hilden, Germany) and the TaqMan PCR Master Mix (AmpliTaq Gold with GeneAmp kit; Applied Biosystems) in 20ml reaction volumes. To determine relative expression, the value obtained from each product was normalized to a control gene (GAPDH) and expressed as a fold change of the value in control extracts. Data analyses were performed using GAPDH as an internal control.

3.3.6 Statistical analysis
All analysis was performed using GraphPad Prism 7 Software. All values are shown as mean ± SEM. Data were analyzed by t-test or one-way ANOVA for comparison of
multiple columns with a p-value < 0.05 considered significant. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.4 Results

3.4.1 BIM deficiency increases beta cell area in Lepr\textsuperscript{db/db} mice

In order to examine whether BIM inhibition has an impact on islets, we studied the islet morphology in pancreatic sections. There was a gradual increase in islet size of 3 month, 6 month and 9 month old BIM deficient Lepr\textsuperscript{db/db} mice (Figure 3.1 A&C). It was previously shown that the beta cell mass in Lepr\textsuperscript{db/db} mice starts to decrease after failure of beta cell compensation in response to insulin resistance\textsuperscript{263}. While we did not see a decrease in islet size in Lepr\textsuperscript{db/db} BIM\textsuperscript{+/+} mice, there was no increase in islet size as mice aged. There was no difference in islet size between Lepr\textsuperscript{db/+} BIM\textsuperscript{+/+} and Lepr\textsuperscript{db/+} BIM\textsuperscript{-/-} mice, and these islets were smaller than those of Lepr\textsuperscript{db/db} mice (Figure 3.1 A&C). There was a significant increase in the insulin-positive area and no change was observed in glucagon-positive area (Figure 3.1 B&D). Islets of Lepr\textsuperscript{db/db} BIM\textsuperscript{-/-} mice showed an organized, oval shape, with continuous circumference, while the shape of islets from Lepr\textsuperscript{db/db} BIM\textsuperscript{+/+} was disorganized and distorted (Figure 3.1 D). In H&E staining of pancreas sections from 9 month old mice, more red blood cells (RBC) were observed inside the islets of Lepr\textsuperscript{db/db} BIM\textsuperscript{-/-} mice (Figure 3.1 C). A recent study\textsuperscript{265} made a similar observation in Lep\textsuperscript{ob/ob} mice, and demonstrated that these lesions can contribute to 15% of total beta cell volume.
Figure 3. BIM deficiency increases beta cell area in \( \text{Lepr}^{db/db} \) mice.

A. Mean islet area from 3, 6 and 9 month old female mice was compared based on insulin immunohistochemistry. \( n=4-6/\text{Lepr}^{db/db} \) genotype of each age, \( n=3-6/\text{Lepr}^{db/+} \) genotype of each age. 47-134 islets/genotype were measured. Data are shown as mean ± SEM where **\( P<0.01 \), ***\( P<0.001 \) and ****\( P<0.0001 \).

B. Percentage of glucagon positive area compared to total islet area was quantified for pancreatic sections described in (D).

C. Islet morphology by H&E staining. Analysis was performed on samples from 9 month old female mice.

D. Islet morphology by immunofluorescence staining. Formalin fixed pancreatic sections from 3 month old and 9 month old female mice were stained with insulin (green), glucagon (red) and nuclei (DAPI, blue).
3.4.2 BIM deficiency increases beta cell volume in Lepr\textsuperscript{db/db} mice

It has been shown that total islet beta cell volume, islet number and islet density can vary among the different lobes of the pancreas\textsuperscript{266}. Therefore the variation in relative distribution of islets between pancreatic lobes may affect the quantification of beta cell mass. In particular, most current data that examine beta cell mass are based on two-dimensional data. The 3D imaging technique optical projection tomography (OPT) was previously used to address this issue by studying beta cell mass distribution among three pancreas lobes from healthy, eight-week old C57BL/6 mice. It was revealed that the duodenal lobe displayed a 20% higher relative islet count and a 40% higher beta cell mass compared with the splenic lobe\textsuperscript{266}.

We applied OPT, choosing the duodenal lobes of pancreas from Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+} and Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} mice (Figure 3.2). Our data shows that islet beta cell volume was increased approximately two-fold in Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} compared with Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+} mice (Figure 3.2 B&C&D), consistent with the two-dimensional immunohistochemistry data. There was no significant difference in the islet density (islet number per cm\textsuperscript{3} pancreas) (Figure 3.2 E). We then tested if islets of different sizes have similar expansion of beta cell mass. We distributed the individual islet beta cell volumes into three different size categories, small (\(<1 \times 10^6 \mu m^3\)), medium (\(1 \times 10^6 \mu m^3 - 5 \times 10^6 \mu m^3\)) and large (\(> 5 \times 10^6 \mu m^3\)) based on a recent publication\textsuperscript{265}. There was a significant increase of the total beta cell volume in the large size category in Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} compared to Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+}. No difference was detected in the small and medium size groups (Figure 3.2 F). There was a trend towards an increase in the total number of large islets (Figure 3.2 G), although the data did not reach statistical difference. The data also indicate that the islets that are in the large size category take up more than half of the total beta cell volume, although the total number of islets in large size category only make up 10\% of the total number of islets in the whole duodenal lobe, consistent with previously published results\textsuperscript{267}. 


Figure 3. BIM deficiency increases islet volume in Lepr\textsuperscript{db/db} mice.

A. OPT images of the duodenal lobes of pancreas from 9-month Lepr\textsuperscript{db/db} mice stained with insulin. The islet volumes were reconstructed based on the insulin-staining (red) and pancreas outline (grey), which is based on the signal from tissue autofluorescence. B. Isosurface rendered OPT images of representative duodenal lobes from Lepr\textsuperscript{db/db}BIM\textsuperscript{+/-} and Lepr\textsuperscript{db/db}BIM\textsuperscript{-/-}.
pancreas. C. Average insulin volume in the duodenal lobe of pancreas from 3, 6 and 9 month old female Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+} and Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} mice. D. Graph showing the average insulin volume dynamics from 3 month old, 6 month old and 9 month old female Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+} and Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} mice. E. Graph showing islet density using total islet numbers divided by total pancreas volume. F. Histogram showing the average total insulin volume within each size category in 9 month old female Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+} and Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} mice. G. The average total number of islets within each size category in 9 month old female Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+} and Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} mice. Data are shown as mean ± SEM (n = 4-6/genotype). ****P<0.0001.

3.4.3 Increased beta cell mass in Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} mice is caused by beta cell hyperplasia

To test whether increased beta cell mass is due to an increase in beta cell numbers or beta cell size, we measured the mean individual beta cell cross-sectional area within the two groups and found no evidence of cell hypertrophy, suggesting this does not contribute to the increase in islet size (Figure 3.3 A). Therefore the increased islet size is more likely caused by beta cell hyperplasia.

To reveal how BIM deletion affects beta cell mass, we studied beta cell replication and apoptosis at 9 months and 3 months of age (Figure 3.3 B-H). At 9 months of age, TUNEL staining showed reduced beta cell apoptosis in Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} mice (Figure 3.3 D&E), suggesting that BIM-dependent apoptosis pathways may be activated in beta cells of Lepr\textsuperscript{db/db} mice. Because the rate of proliferation is extremely low in adult animals, we performed continuous 9 days’ administration of BrdU in vivo, and then analyzed pancreas sections for proliferation. At 9 months of age, there was no difference between the groups in the number of BrdU positive beta cells per islet (Figure 3.3 B&C). However, at 3 months of age, a stage when beta cells reach the peak of their compensatory expansion in response to insulin resistance, the number of BrdU positive beta cells per islet was two-fold greater in the islets of Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} mice compared to those from Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+} (Figure 3.3 F&G). At this age, apoptosis, determined by TUNEL immunostaining, was undetectable (Figure 3.3 H). This shows that apoptosis at this compensatory stage is much lower than at the later stage. Therefore the islet hyperplasia in Lepr\textsuperscript{db/db}BIM\textsuperscript{−/−} mice is due to increased proliferation of beta cells at the compensation stage and reduced apoptosis at the stage when beta cells fail to compensate.
Figure 3. Increased beta cell mass in Lepr<sup>db/db</sup>BIM<sup>−/−</sup> mice is caused by beta cell hyperplasia.

(A). Mean individual beta cell size in Lepr<sup>db/db</sup>BIM<sup>+/−</sup> and Lepr<sup>db/db</sup>BIM<sup>−/−</sup> female mice at the age of 9 months. n=3-5 mice/genotype. At least 1000 beta cells were counted for each mouse. (B, C, F, G). Proliferation in islets of 9 month old mice (B, C) and 3 month old mice (F, G) in Lepr<sup>db/db</sup>BIM<sup>+/−</sup> and Lepr<sup>db/db</sup>BIM<sup>−/−</sup> mice. BrdU staining was performed and the total number of positive cells within islet areas was quantified. n=3-9 mice/genotype. Average BrdU-positive cells were quantified as described in the methods. (D, E, H). Apoptosis from 9 month old mice (D, E) and 3 month old mice (H) in Lepr<sup>db/db</sup>BIM<sup>+/−</sup> and Lepr<sup>db/db</sup>BIM<sup>−/−</sup> mice. TUNEL staining was performed. n=2-3 mice per genotype in (D, E), n=3-4 mice/genotype in (F).
3.4.4 BIM deficiency in beta cells has a limited role in changing beta cell mass

We expected decreased apoptosis in BIM deficient mice, but increased proliferation was unexpected. Hence we considered the possibility that improved glucose homeostasis in BIM deficient mice, along with decreased apoptosis, may have accounted for increased beta cell mass. Therefore we studied islet area in Lepr^{db/db} mice with beta cell-specific deletion of BIM. No increase in islet size was observed in Lepr^{db/db}βBIM^{-/-} mice compared with Lepr^{db/db}βBIM^{+/+} (Figure 3.4). This suggests that BIM deletion in beta cells alone does not affect beta cell mass in type 2 diabetes, but that the islet phenotype might be the consequence of whole body effects of BIM deficiency.

Figure 3.4 BIM deficiency in beta cells has a limited role in changing beta cell mass.

Formalin fixed pancreatic section from 4.5 month old female Lepr^{db/db}BIM^{+/+} and Lepr^{db/db}BIM^{-/-} mice were immunostained with insulin and counterstained with DAPI. Islet area was quantified. n=6 mice/genotype. 66-134 islets/genotype were measured.

3.4.5 Changes in mRNA levels of islet-associated transcription factors, inflammation, UPR and cell cycle genes in BIM-deficient mice

Next, we explored potential mechanisms of increased beta cell mass in BIM^{-/-} mice by studying gene expression in islets. We analyzed isolated islets from Lepr^{db/db} mice with or without BIM for expression of the islet-associated transcription factor Pdx1, and genes associated with inflammation (Il6 and Cxcl1), unfolded protein response (Hspa5 and Ddit3), and cell cycle (Ccnb1 and Cdk4) (Figure 3.5). The expression level of Pdx1, a transcription factors important for islet development and maintenance of beta cell differentiation, was similar in Lepr^{db/db}BIM^{-/-} and Lepr^{db/db}BIM^{+/+} islets, although
reduced compared to Lepr\(^{db/+}\) islets (Figure 3.5 A). There is evidence that islet inflammation is associated with obesity and type 2 diabetes. We observed significantly decreased expression of \(Il6\) and \(Cxcl1\) in islets of Lepr\(^{db/db}\)BIM\(^{-/-}\) compared to wild type (Figure 3.5 B). This reduced islet inflammation is likely to be associated with successful beta cell compensation, in a similar manner to Lep\(^{ob/ob}\) mice published previously\(^{235}\). A trend of decreased expression of UPR genes was observed in Lepr\(^{db/db}\)BIM\(^{-/-}\) mice compared to Lepr\(^{db/db}\)BIM\(^{+/+}\) mice (Figure 3.5 C). The cell-cycle regulators cyclin D1/D2 and CDK4 and cell-cycle inhibitors such as P15\(^{INK4b}\) and P18\(^{INK4c}\) have been shown to control beta cell proliferation\(^{268-271}\). There was no difference of expression of the cell cycle gene \(Ccnd1\) in Lepr\(^{db/db}\)BIM\(^{-/-}\) mice compared to Lepr\(^{db/db}\)BIM\(^{+/+}\) mice (Figure 3.5 D). Analysis of a larger set of genes and more mice needs to be done to confirm these preliminary findings.

Figure 3.5 Changes in mRNA expression of the islet-associated transcription factor Pdx1 (A), inflammation genes (B), UPR genes (C) and cell cycle genes (D) at 3 months of age.
Islets were isolated from 3-4 mice/group. mRNA levels are shown as fold change relative to age-matched controls (Lepr\textsuperscript{db}/BIM\textsuperscript{+/+}). Results are mean ± SEM. **P<0.01.

3.5 Discussion

In this study, we have tested the hypothesis that inhibition of beta cell apoptosis could increase islet size in conditions of insulin resistance and type 2 diabetes. Our results show that whole body BIM deficiency results in gradually increasing beta cell mass with age in obese and diabetic mice. Used the RipCre-LoxP recombination system, we show that beta cell specific BIM deletion plays a limited role in regulating beta cell mass. BIM inhibition globally generates a compensating model for type 2 diabetes with increased beta cell proliferation, reduced apoptosis, and possible reduced systemic inflammation. It is therefore likely that BIM-deficiency in Lepr\textsuperscript{db/db} protects beta cell mass secondarily to improved insulin sensitivity.

Beta cell apoptosis is thought to be an important mediator of the loss of beta cell mass in type 2 diabetes\textsuperscript{66}. Although initially there is a stage of compensation of beta cell mass to meet the demand when hyperglycemia occurs, decompensation happens when beta cells fail to secrete enough insulin and loss of beta cell mass occurs. Apoptosis has been considered the main cause of beta cell loss, and our data shows that partial inhibition of apoptosis by BIM deficiency does not protect from diabetes. However, we cannot rule out apoptosis totally because it could occur by BIM-independent mechanisms. This could be tested on mice deficient in the apoptosis effectors BAX and BAK, but this would be a difficult experiment to do because these mice do not survive after birth and our experience is that it is extremely difficult to generate Lepr\textsuperscript{db/db} mice deficient in multiple genes. Caspases are the major components of the cell suicide machinery. Caspase 8 is a critical component of the death receptor-mediated apoptosis pathway. Mice with beta cell specific deficiency of caspase 8 had improved glucose tolerance and increased islet mass when fed a high fat diet\textsuperscript{272}. Therefore it is possible that the death receptor-mediated apoptosis pathway plays a role in regulating beta cell apoptosis, although McKenzie et al. found that deficiency of the Fas receptor did not diminish glucose-induced apoptosis in vitro\textsuperscript{212}.
CHOP deficiency in Lepr\textsuperscript{db/db} mice generated a phenotype similar to whole body BIM deficiency, including increased beta cell mass, increased insulin production and improved glucose tolerance\textsuperscript{69}. BIM was shown to be regulated by CHOP in diverse cell types, although interestingly BIM gene expression was not altered in CHOP-deficient islets or in conditions of ER stress\textsuperscript{220}. CHOP deletion improved ER function and protected against oxidative stress in response to ER stress\textsuperscript{69}. Wali et al. showed that BIM deficiency protects islets from ER stress\textsuperscript{220}. Therefore the phenotype of the CHOP deficient mice could also be a non-islet phenotype in a similar manner to the whole body BIM deletion. ER stress can induce insulin resistance in liver and fat\textsuperscript{52}. Inhibiting ER stress in a number of ways has a similar effect to whole body BIM or CHOP deletion with improved glucose homeostasis and increased beta cell mass\textsuperscript{53,273,274}. Treatment of obese and diabetic mice with chemical chaperones that alleviate the ER stress restored glucose homeostasis\textsuperscript{53}. Reducing ER stress by overexpression of oxygen-regulated protein 150, a molecular chaperone that protects cells from ER stress, in Lepr\textsuperscript{db/db} mice significantly improved insulin resistance and markedly ameliorated glucose tolerance\textsuperscript{273}. A recent report showed that mild activation UPR ex vivo increases beta cell proliferation and beta cell number, through activation of ATF6. It could be possible that BIM deletion improves ER function in a similar manner to deletion of CHOP, and our results suggest that global inhibition of ER stress is required for downstream positive effects on beta cell mass. The difference between CHOP and BIM-deficient mice is the improvement of insulin sensitivity with BIM deficiency. It remains to be studied whether improved ER stress is followed by insulin sensitivity or these two processes cooperate together with BIM playing a non-apoptotic role in regulating insulin sensitivity to generate this phenotype.

It has been suggested that insulin resistance directly impacts islet biology\textsuperscript{259,273}. Although Lepr\textsuperscript{db/db}BIM\textsuperscript{-/-} mice have improved insulin sensitivity, they are still insulin resistant compared to non-diabetic mice. Thus, a state of mild insulin resistance directly stimulates an increase in beta cell mass. This would be similar to insulin-resistant obese patients, who show increased islet size, compared to diabetic patients, where there is evidence of loss of beta cell mass\textsuperscript{259}. Treating Lepr\textsuperscript{db/db} mice with the insulin sensitivity drug pioglitazone resulted in a phenotype similar to Lepr\textsuperscript{db/db}BIM\textsuperscript{-/-} mice, including reduced insulin resistance, increased beta cell proliferation and mass,
and increased expression of antioxidant and proliferation genes\textsuperscript{275}. Another similar phenotype is from food restriction. Food restriction in Lepr\textsuperscript{db/db} mice promoted insulin secretion, improved insulin sensitivity and decreased islet inflammation\textsuperscript{276,277}. A recent report showed that pair feeding in Lepr\textsuperscript{db/db} mice results in reduction of beta cell dedifferentiation, indicating restoration of beta cell function\textsuperscript{278}. Liver-specific BIM deficient mice were more insulin sensitive than wild type mice when fed a high fat diet (E. Gurzov, unpublished). It would be interesting to test whether liver-specific deletion of BIM in Lepr\textsuperscript{db/db} mice also results in increased beta cell mass to test whether improved insulin sensitivity alone is able to drive beta cell expansion. Defining the threshold of insulin resistance/sensitivity in Lepr\textsuperscript{db/db} mice that can stimulate increased beta cell mass is an important question.

Beta cell growth in humans and rodents has been shown to be due to self-replication of preexisting beta cells\textsuperscript{279,280}. Compensatory islet hyperplasia in response to insulin resistance is a known feature of diabetes. It has recently been shown that the liver is a source of circulating factors that mediate this replication. Crosstalk between liver and pancreatic islets has been verified using liver-specific insulin receptor knockout (LIRKO) mice\textsuperscript{281}. SerpinB1 is a hepatocyte factor that regulates beta cell proliferation in humans, mice and zebrafish in response to insulin resistance\textsuperscript{282}. Although there was no difference between SerpinB1 gene expression levels in Lepr\textsuperscript{db/db}BIM\textsuperscript{-/-} compared to Lepr\textsuperscript{db/db}BIM\textsuperscript{+/+} mice (data not shown), this, or other similar proteins, might contribute to the increased proliferation of beta cells in Lepr\textsuperscript{db/db}BIM\textsuperscript{-/-} mice.

Among other possible circulating regulators of beta cell mass, the incretin hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)\textsuperscript{29,283} increase insulin secretion and promote beta cell replication. Adipocyte-derived adipokines including leptin\textsuperscript{284} and adiponectin\textsuperscript{285}, muscle-derived myokines such as IL-6\textsuperscript{286} have also been implicated as regulators of beta cell mass. Insulin, placental lactogen, prolactin, and osteoblast-secreted hormone osteocalcin are also indicated to play a role\textsuperscript{287}. However, none of them regulate beta cell mass specifically, and their effect on beta cell mass is quite modest, which limits their therapeutic value.
Previous studies have shown tissue inflammation related to obesity and type 2 diabetes\textsuperscript{288,289}. We observed a significant reduction in inflammatory gene expression including $IL6$ and $Cxcl1$ in islets of Lepr\textsuperscript{db/db} BIM\textsuperscript{-/-} mice, and this reduction of inflammation in islets might contribute to the improved phenotypes in Lepr\textsuperscript{db/db} BIM\textsuperscript{-/-} mice. IL-6 is also an indicator of islet endothelial cell dysfunction and is expressed at much higher levels in endothelial cells compared to beta cells or other islet cells\textsuperscript{290}. Therefore, improved endothelial cell function may play a role in the islet phenotype of Lepr\textsuperscript{db/db} BIM\textsuperscript{-/-} mice. Endothelial cells produce hepatocyte growth factor, which can stimuli beta cell proliferation\textsuperscript{291}. Laybutt et al. reported that hypoxia is induced in Lepr\textsuperscript{db/db} mice\textsuperscript{235}, we hypothesize that islets of Lepr\textsuperscript{db/db} BIM\textsuperscript{-/-} mice are not hypoxic because we observed rich islet capillaries and many red blood cells in Figure 3.1 C, which could be due to the improved endothelial cell function. A role for endothelial cells could be tested using BIM\textsuperscript{flox/flox} mice with cre expressed under control of an endothelial cell-specific promoter.

In this study, I have shown that inhibition of apoptosis in vivo specifically in beta cells does not change glucose homeostasis or beta cell mass, suggesting that beta cell apoptosis may not play a direct role in type 2 diabetes. However, global inhibition of BIM generates a compensatory model of improved glucose homeostasis and beta cell expansion, possibly due to a non-apoptotic role of BIM.
Chapter 4.
BIM inhibition does not regulate glucose homeostasis and islet size in response to insulin receptor antagonism
4.1 Summary

BIM is a potent BH3-only protein that mediates intrinsic apoptosis in response to stresses such as ER stress in many cell types. Although we have established a role for BIM in regulating glucose homeostasis and beta cell expansion in type 2 diabetes, the mechanism remains unclear. We hypothesized that these findings are related to enhanced insulin sensitivity. Therefore we generated a model with severe insulin resistance by administration of the insulin receptor antagonist S961.

Global BIM knockout mice had increased blood glucose, glucose intolerance, and insulin resistance after treatment with S961 for 28 days. BIM deficient mice showed significant beta cell mass expansion after treatment, but this was comparable to increased beta cell mass in controls. Similar results were observed in mice with beta cell specific-BIM deletion and in mice lacking BIM specifically in the liver.

Our data indicate that under conditions where insulin sensitivity is completely lacking, BIM plays a limited role in regulating glucose homeostasis and beta cell mass expansion. BIM-deficiency improved insulin sensitivity in normal mice, but this was bypassed in mice treated with S961 by inhibiting insulin receptor signaling. This suggests that BIM’s function in regulating diabetes is associated with insulin sensitivity.

4.2 Introduction

The majority of new beta cells are generated by self-duplication\textsuperscript{292}. Beta cells replicate at a very low rate in adult rodents\textsuperscript{264} and humans\textsuperscript{280}. However, beta cells possess the capacity to replicate under circumstances where there is increased demand for insulin secretion, including pregnancy\textsuperscript{293}, high blood glucose\textsuperscript{294}, pancreatic injury\textsuperscript{295} and peripheral insulin resistance\textsuperscript{296,297}. In type 2 diabetes, beta cell function and mass progressively decline with time\textsuperscript{66,71}.

Several systemic or circulating factors can regulate beta cell mass and replication, including glucose\textsuperscript{294}, glucokinase\textsuperscript{298} and hormones such as insulin, prolactin\textsuperscript{287},
glucagon-like peptide 1 (GLP-1) and glucose-dependent peptide (GIP)\(^{20}\). These factors lack beta cell specificity and have only a mild effect on beta cell mass.

Liver-specific deletion of insulin receptors results in pancreatic beta cell proliferation\(^{206}\). Recently, the compensatory beta cell expansion in response to insulin resistance has been reported to be mediated by liver-derived circulating factors in liver-specific insulin receptor knockout (LIRKO) mice\(^{281}\). One of the relevant proteins involved in increased beta cell mass is serpinB1, a protease inhibitor generated by the liver\(^{282}\). This liver-derived secretory protein regulates beta cell proliferation in humans and mice. Small-molecule compounds that mimic the function of serpinB1 promote proliferation of mouse and human pancreatic beta cells. Adaptive beta cell proliferation fails in mice lacking serpinB1 that have been fed a high fat diet to make them insulin resistant\(^{282}\).

S961 is a single chain peptide of 43 amino acids\(^{299}\). Its affinity for the insulin receptor is comparable to that of insulin, and its specificity for the insulin receptor compared to the IGF-1 receptor is stronger than that of insulin itself. S961 antagonizes insulin signaling in vitro and in vivo in rats and mice\(^{299}\). Thus, S961 completely inhibits insulin action and generates a model of acute peripheral insulin resistance. Beta cell replication and expansion of beta cell mass can be induced within one-week of S961 administration\(^{300,301}\).

As previously mentioned, we have observed a significant expansion of beta cell mass in global BIM deficient Lepr\(^{db/db}\) mice. We concluded that this was related to enhanced insulin sensitivity. In this study, we generated BIM-deficient mice in which insulin sensitivity could not be enhanced. We created mice with peripheral insulin resistance on a C57BL/6 background using S961, and studied the effects of BIM deficiency globally, specifically in beta cells or in the liver. We hypothesized that BIM deletion would not lead to improvement of glucose homeostasis and beta cell expansion because it was unable to affect insulin sensitivity in this model. Thus, we would further confirm our hypothesis that global BIM inhibition regulates glucose homeostasis and beta cell mass expansion by enhancing insulin sensitivity.
4.3 Research methods

4.3.1 Mice
Mice deficient in BIM, were generated on a C57BL/6 genetic background and kindly given by Dr Philippe Bouillet (WEHI)\(^\text{112}\). Wild-type C57BL/6 mice were purchased from Animal Resources Centre (Canning Vale, Australia). Mouse breeding procedures were conducted at St Vincent’s Institute. BIM gene flanked by loxP sites (BIM\(^{fl/fl}\)) were obtained from Dr Philippe Bouillet (WEHI). For generating mice with BIM deficiency specifically in beta cells, BIM\(^{fl/fl}\) mice were crossed with mice expressing Cre under control of the rat insulin promoter (RIP-Cre) on a pure C57BL/6 background. BIM\(^{fl/+}\)Cre/+ mice were intercrossed to generate BIM\(^{fl/fl}\)Cre/+ mice. We generated the following groups: BIM\(^{fl/fl}\)RIPCre/+ (\(\beta\)BIMKO) and BIM\(^{fl/fl}\)RIPCre-/-(\(\beta\)BIMWT). For generating mice with BIM deficiency specifically in liver cells, BIM\(^{fl/fl}\) mice were crossed with mice expressing Cre under control of the albumin promoter (ALB-Cre) to get the following groups: BIM\(^{fl/fl}\)ALBCre/+ (LBIMKO) and BIM\(^{fl/fl}\)ALBCre/- (LBIMWT). All animals were housed at St Vincent’s Institute Bioresources Centre, and all experiments were approved by the animal ethics committee. Mice were genotyped for Bim, the floxed Bim allele and Cre as previously described\(^240,241,302\).

4.3.2 Mouse housekeeping
Eight week old male mice from each genotype: global knockout of BIM (BIMWT, BIMKO), beta cell-specific BIM deficiency (\(\beta\)BIMWT, \(\beta\)BIMKO) and liver-specific BIM deficiency (LBIMWT, LBIMKO) were maintained on a 12 hour light/12 hour dark cycle with access to normal chow food and water. Non-fasting blood glucose was measured on day 0 (immediately before S961 treatment), day 2, 3, 5, 7, second week, third week and day 28 using a glucometer (Accu-Chek). At the end of the study (day 28), mice were sacrificed and the pancreases were collected for histology.

4.3.3 S961 administration
S961 was synthesized and kindly provided by Dr. Lauge Schäffer (Novo Nordisk). S961 was continuously infused at 20 nmol/l/week using Alzet osmotic minipumps
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(2004 model, Durect) embedded under the skin of mice for 28 days. Mice were anaesthetized first and minipumps were implanted subcutaneously on the back of mice.

4.3.4 Glucose tolerance test
At the second week after beginning S961 infusion, an intra-peritoneal glucose tolerance test (IP-GTT) was performed. Mice were fasted for 6 hours then 2g/kg glucose solution (Baxter, Deerfield, 1L) was injected and blood glucose was measured at 0, 15, 30, 45, 60 and 120 minutes.

4.3.5 Insulin tolerance test
At the third week after S961 infusion, mice were fasted for 4 hours before performing an intra-peritoneal insulin tolerance test (IP-ITT). Insulin (Actrapid, Novo Nordisk, Bagsværd, Denmark), at a dose of 0.75 U/kg, was injected intra-peritoneally and blood glucose was measured at 0, 15, 30, 45, 60, 90 and 120 minutes.

4.3.6 Histological study
After mice were sacrificed, pancreas samples were fixed in formalin and sectioned. Sections were stained for insulin using polyclonal guinea pig anti-insulin antibody (DAKO dilution 1:200). Anti-guinea pig HRP (Dako, Denmark) was used as a secondary antibody. Sections were then developed with DAB and counterstained with haematoxylin. Slides were scanned on an Aperio slide scanner at 20X magnification with 0.5 micron/pixel. The insulin+ (beta cell) area was measured using the Aperio imageScope.

4.3.7 Statistical analysis
All values are shown as mean ± SEM. Data were analyzed by t-test or one-way ANOVA for comparison of multiple columns.

4.4 Results

4.4.1 Inhibition of BIM does not change glucose homeostasis or beta cell mass compared to wild type mice after infusion of the insulin receptor antagonist S961.
Previous work has shown that blocking insulin receptors in vivo leads to beta cell mass expansion. My previous results showed that BIM inhibition was associated with regulating insulin sensitivity and resulted in expansion of beta cell mass in Lepr$^{db/db}$ mice. To investigate the association between BIM inhibition and insulin sensitivity, we used S961, an insulin receptor blocker, to block insulin signaling, resulting in severe insulin resistance. We used this model to study whether BIM inhibition could expand beta cell mass in the absence of enhanced insulin sensitivity. We infused 20 nmol/l S961 into adult C57B6 mice for 28 days using osmotic minipumps. Body weights of mice ranged from 23-25 grams. No changes in body weight were observed during S961 treatment (Data not shown). Infusion of the insulin receptor antagonist S961 caused a profound increase in plasma glucose after two days in both BIMKO and BIMWT mice (Figure 4.1 A). Mice treated with S961 were severely insulin resistant, glucose intolerant and showed no response to insulin injection (Figure 4.1 B&C). This was associated with a compensatory expansion of beta cell mass in both BIMKO and BIMWT mice (Figure 4.1 D). These results show that insulin receptor antagonism causes glucose intolerance and beta cell expansion, and BIM does not play a role in regulating this compensatory response.
Figure 4.1 Administration of the insulin receptor antagonist S961 induces glucose intolerance, hyperglycemia, lack of insulin sensitivity and beta cell mass expansion in BIMKO mice and wild type mice.

A. Non-fasting blood glucose levels in global BIMKO mice and BIMWT mice continuously treated with the insulin receptor antagonist S961 (20 nmol/l/week) for 28 days. Glucose was measured every other day for the first week of treatment and then weekly for two weeks.

B. Intraperitoneal glucose tolerance test was performed after overnight fasting in male BIMKO mice and BIMWT mice treated with S961 (20 nmol/l/week) in the third week of the study.

C. Insulin tolerance test performed after fasting in male BIMKO mice and BIMWT mice treated with S961 (20 nmol/l/week) in the fourth week of the study.

D. Average beta cell area in BIMKO and BIMWT mice treated with S961 (20 nmol/l/week) for 28 days. All groups had six to eight mice. Data are not significantly different.

4.4.2 Insulin receptor antagonist promotes glucose intolerance and beta cell expansion in beta cell specific BIM deficient mice.
To investigate whether BIM inhibition protects beta cell mass from death and results in greater beta cell mass expansion during S961 treatment, we treated beta cell specific BIM deficient mice with the insulin receptor antagonist S961 for 28 days. Blood glucose levels were significantly increased after two days of treatment and sustained until the end of treatment. However, βBIMKO and βBIMWT mice showed a similar pattern (Figure 4.2 A). Both βBIMKO and βBIMWT mice showed glucose intolerance, with a compensatory increase in beta cell area in both βBIMKO and βBIMWT mice (Figure 4.2 B&D). Lack of insulin sensitivity was observed in both βBIMKO and βBIMWT mice (Figure 4.2 C). These data suggest that insulin receptor antagonism causes glucose intolerance and beta cell expansion by a mechanism that does not require functional BIM in beta cells.
Figure 4. Administration of the insulin receptor antagonist S961 induces glucose intolerance, hyperglycemia, lack of insulin sensitivity and beta cell mass expansion in βBIMKO mice and wild type mice.

A. Non-fasting blood glucose level in βBIMKO mice and βBIMWT mice continuously treated with the insulin receptor antagonist S961 (20 nmol/l/week) for 28 days. Glucose was measured every other day during the first week of treatment.

B. Intraperitoneal glucose tolerance test was performed after overnight fasting in male βBIMKO mice and βBIMWT mice treated with S961 (20 nmol/l/week) in the third week of the study.

C. Insulin tolerance test performed after fasting in male βBIMKO mice and βBIMWT mice treated with S961 (20 nmol/l/week) in the fourth week of the study.

D. Average beta cell area in βBIMKO and βBIMWT mice treated with S961 (20 nmol/l/week) for 28 days. All groups had six to eight mice. Data are not significantly different.
4.4.3 Insulin receptor antagonist promotes glucose intolerance and beta cell mass expansion in LBIMKO and LBIMWT mice

Global BIM deficient or liver-specific BIM deficient mice have improved glucose homeostasis associated with enhanced insulin sensitivity\(^{303}\). We treated liver-specific BIM deficient mice with S961 for 28 days and measured blood glucose. Plasma glucose was increased after two days of treatment and high glucose levels were sustained throughout the treatment. However, there was no difference in glucose levels between LBIMKO and LBIMWT mice (Figure 4.3 A). Mice treated with S961 showed similar glucose intolerance and lack of insulin sensitivity (Figure 4.3 B&C), with no difference between LBIMKO and LBIMWT mice. S961 treatment significantly increased beta cell growth, however this was independent of BIM inhibition (Figure 4.3 D). Our results indicate that loss of BIM has no impact on insulin secretion, glucose tolerance and insulin sensitivity in conditions where severe insulin resistance is induced by blocking insulin signaling.
Figure 4. 3 Administration of the insulin receptor antagonist S961 induces glucose intolerance, hyperglycemia, lack of insulin sensitivity and beta cell mass expansion in LBIMKO mice and wild type mice.

A. Non-fasting blood glucose level in LBIMKO mice and LBIMWT mice continuously treated with the insulin receptor antagonist S961 (20 nmol/l/week) for 28 days. Glucose was measured every other day for the first week and then weekly for two weeks.
B. Intraperitoneal glucose tolerance test was performed after overnight fasting in male LBIMKO mice and LBIMWT mice treated with S961 (20 nmol/l/week) in the third week of the study.
C. Insulin tolerance test performed after fasting in male LBIMKO mice and LBIMWT mice treated with S961 (20 nmol/l/week) in the fourth week of the study.
D. Average beta cell area in LBIMKO and LBIMWT mice treated with S961 (20 nmol/l/week) for 28 days. All groups had six to eight mice. Data are not significantly different.
4.5 Discussion

The main finding of the study is that BIM inhibition does not regulate glucose homeostasis and islet size in response to insulin receptor antagonism. We have also confirmed the findings that administration of S961 causes severe glucose intolerance and beta cell expansion in three mouse models.

Wali et al. found that BIM inhibition improved glucose homeostasis by enhancing insulin sensitivity through a non-classical role of BIM (See Appendix A). We have confirmed this finding by showing improvement of glucose homeostasis and increased beta cell mass in Lepr\textsuperscript{db/db} mice with global BIM inhibition, and no change in glucose homeostasis and beta cell size in Lepr\textsuperscript{db/db} mice with beta cell specific BIM inhibition. In this S961 model, blocking insulin receptors leads to total lack of insulin sensitivity. Therefore, in this model, BIM inhibition was unable to enhance insulin sensitivity because it is blocked at the level of the insulin receptor. Thus, the enhanced insulin sensitivity in BIM-deficient mice is important for expansion of beta cell mass. It is also possible that the maximum threshold of beta cell mass expansion was reached in the S961 model, and BIM-deficiency was unable to further expand beta cell mass.

Increased beta cell mass in the S961 model of insulin resistance is through the generation of circulating factors that trigger beta cell expansion. However, we did not observe any additional increase in beta cell mass in BIM knockout mice, no matter whether we deleted BIM globally, beta cell-specifically or liver specifically. This generates the conclusion that BIM regulates glucose homeostasis and beta cell mass independently from insulin-resistant related circulating factors such as liver-secreted serpinB1. Liver generated serpinB1 was shown to promote beta cell growth in a model of hepatic insulin resistance in mice and in human cells\textsuperscript{382}. We observed no difference in SerpinB1 expression in the livers of BIMKO and BIMWT Lepr\textsuperscript{db/db} mice (Data not shown). In this study, we can confirm that the insulin receptor antagonist S961 caused hyperglycemia. High glucose levels could also contribute to expansion of beta cell mass. Glucose infusion stimulates beta cell proliferation\textsuperscript{294}. Glucokinase has also been shown to stimulate beta cell proliferation\textsuperscript{298}. Other factors remain to be discovered. However, different rodent models might depend on different factors to regulate beta
cell proliferation. More challengingly, S961 administration does not increase proliferation of transplanted human beta cells\textsuperscript{304}, and human beta cells are perhaps less responsive to the insulin-resistant state. This is a gap worth considering between studies in mice and potential human application.
Chapter 5.

General discussion
5.1 Summary of results

The standard paradigm is that beta cell apoptosis is an important pathogenic feature of type 2 diabetes. We hypothesized that inhibition of apoptosis with BIM deficiency would protect beta cell mass and as a result would improve glucose homeostasis. My results showed that global BIM deficiency in a mouse model of type 2 diabetes significantly improved glucose homeostasis. There was a striking increase in islet size in BIM-deficient Lepr\(^{db/db}\) mice, which was accompanied by increased proliferation of beta cells and reduced apoptosis rate in older mice. Islets of BIM deficient Lepr\(^{db/db}\) mice showed reduced expression of inflammation genes and a trend to reduced unfolded protein response genes, indicating a compensation state. In contrast, mice with BIM deficiency specifically in beta cells did not show any improvement in glucose homeostasis and no increase in islet size was observed in these mice. These findings indicate that BIM plays a limited role in regulating beta cell apoptosis in the early stages of type 2 diabetes in vivo, which directly challenges the traditional view that beta cell apoptosis is important at least during this stage. Because our studies were limited to younger mice, the results leave open the possibility that apoptosis may be important in later stages of diabetes.

The improved glucose homeostasis in global BIM-deficient mice was likely a consequence of the increased sensitivity to insulin. In a state of severe insulin resistance induced by the insulin receptor antagonist S961, there was no difference in glucose homeostasis and beta cell mass in global BIM-deficient mice compared to wild type controls. These data suggest that the improved glucose homeostasis and increased beta cell mass in global BIM-deficient Lepr\(^{db/db}\) mice are associated with insulin sensitivity, because these did not occur when insulin sensitivity was prevented in mice treated with S961.

Together, my results indicate that beta cell apoptosis may not play a direct role in type 2 diabetes, but global BIM deletion generates a compensatory model by increasing insulin sensitivity. Thus, my results challenge the standard paradigm.
5.2 Evidence of beta cell apoptosis in type 2 diabetes

5.2.1 It is difficult to be sure there is any apoptosis in human type 2 diabetes

Loss of beta cell mass has been observed in many human studies in type 2 diabetic subjects, however because of the technical difficulty in measuring apoptosis due to rapid clearance of dying cells and limited numbers of available human samples, there are less reports showing beta cell apoptosis in type 2 diabetes. I review the available evidence of beta cell apoptosis in human type 2 diabetes here. The most widely cited study is from Butler et al.\(^6\), showing TUNEL staining in pancreatic sections from type 2 diabetes donors (n=57) and non-diabetic donors (n=48). The frequency of beta cell apoptosis was increased 10-fold in lean (0.47 TUNEL\(^+\)/islets Vs 0.07 TUNEL\(^+\)/islets) and 3-fold in obese (0.31 TUNEL\(^+\)/islets Vs 0.2 TUNEL\(^+\)/islets) cases of type 2 diabetes compared with their respective nondiabetic control group. Although this study includes a large number of subjects, I found it difficult to assess because the TUNEL assay is greatly dependent on technical details, including fixation conditions and pretreatment procedures that need be optimized. In addition, the authors do not show photos of their staining. In a more recent study, an approximately 4.5-fold increase of TUNEL staining in pancreatic sections from type 2 diabetes donors (n=13) compared to non-diabetic donors (n=16) was observed\(^8\). However, only half of the samples in the diabetic group had more TUNEL staining compared to controls, and the number of positive TUNEL cells overall was low. There were no TUNEL\(^+\) cells detected in another human study\(^8\). Another way of measuring apoptosis is caspase 3 staining. Islets from type 2 diabetic subjects showed a higher rate of caspase 3 positive cells at 8.7% in total islets cells compared to 4.7% in controls\(^8\). Apoptosis, measured by caspase 3 and caspase 8, using an ELISA kit on isolated islets, showed approximately 2-fold increase in optical density in type 2 diabetes subjects compared to controls\(^8\). Limitations for this study include the small number of subjects studied: 6 with type 2 diabetes and 10 controls, and the use of isolated islets. While the evidence above suggest that beta cell apoptosis is increased in type 2 diabetes, the small number of studies and the sometimes unconvincing data indicate that we need be more careful when concluding that beta cell apoptosis is an important feature in human type 2 diabetes. I believe more convincing evidence is required in future.
There are also challenges to improve the staining methods. First, the specificity of the TUNEL technique relies on optimizing technical details such as the TdT concentration, fixation conditions and pretreatment procedures\textsuperscript{305}. Variations among different studies may be because of lack of a standard protocol. Also, the TUNEL technique can also display cell necrosis making this technique not specific for apoptosis. Second, number of positive cells identified by TUNEL and caspase 3 is often different, with more positive cells detected by caspase 3. Caspase 3 staining can sometime stain morphologically healthy cells, which may indicate they are at an early stage of apoptosis, thus causing more caspase 3 positive cells compared to TUNEL positive cells in the same tissues. Third, use of adjacent sections to identify positive beta cells has limitations, as it is difficult to be sure that the TUNEL positive cells are beta cells. Therefore, staining TUNEL with insulin on the same section is preferred. The very low frequency of TUNEL or caspase 3 positive cells warrants careful interpretation of role of beta cell apoptosis in type 2 diabetes.

In conclusion, although it is clear that beta cell mass is reduced, it is difficult to be sure there is any apoptosis in human type 2 diabetes based on evidence until now.

5.2.2 Evidence of beta cell apoptosis in Lepr\textsuperscript{db/db} mice

Similarly, the frequency of detectable apoptotic beta cells was very low in Lepr\textsuperscript{db/db} mice. In 8 week old mice, there is rarely any TUNEL positive beta cells observed in Lepr\textsuperscript{db/db} mice\textsuperscript{306}. We also made a similar observation in 12 week old Lepr\textsuperscript{db/db} mice. In 16 week old mice, 4.8 TUNEL\textsuperscript{+}/100 islets were observed by another group\textsuperscript{237}. In 9-10 month old Lepr\textsuperscript{db/db} mice, although there was a 10 fold increase of TUNEL\textsuperscript{+} cells compared to that in Lepr\textsuperscript{db/+} mice\textsuperscript{69}, the total amount of positive cells was small. Puff et al. observed an increase in apoptosis related to increasing age in the Lepr\textsuperscript{db/db} mice, from 5 TUNEL\textsuperscript{+}/100 islets in 7-8 week old mice to 9 TUNEL\textsuperscript{+}/100 islets in 10-12 week old mice\textsuperscript{307}. However, this finding was challenged by Dalbøge at al. by showing that there is no difference in caspase 3 staining among 5 week old, 10 week old and 24 week old mice\textsuperscript{263}. In our study, we also detected a very low frequency of TUNEL\textsuperscript{+} cells even in 9 month old mice. Again, because the inevitable difficulty in apoptosis quantification in vivo, it requires caution to consider beta cell apoptosis as a key feature of type 2 diabetes in Lepr\textsuperscript{db/db} mouse model.
5.3 Inhibition of beta cell apoptosis in type 2 diabetes

Obesity often leads to insulin resistance, however only a subset of obese and insulin resistant individuals develop type 2 diabetes. In both animal models and humans, beta cell failure has been recognized as the triggering factor\(^{308}\). Beta cell failure is characterized by a decrease in beta cell mass and a decline in beta cell function. Chronic exposure of islets to elevated levels of nutrients such as glucose and fatty acids in vitro leads to beta cell dysfunction and beta cell death. To meet the demand of increased insulin secretion placed on the beta cells by the increased nutrient level, the cell responds by activating the UPR to increase ER chaperones, improve protein folding capacity and reduce protein translation\(^{172,173}\). Although initially the UPR protects the ER, prolonged or too much ER stress leads to failure of the UPR and apoptosis can be activated. Activation of ER stress-induced apoptosis in other cell types has been shown to occur via IRE1-TRAF2-JNK-BIM or PERK-eIF2\(\alpha\)-CHOP-BIM pathways\(^{109,174,309}\). Previously, our in vitro data showed that BIM mediates apoptosis in beta cells exposed to high concentrations of glucose, and this was reduced by blocking ER stress\(^{220}\). Therefore, we reasoned that deficiency of BIM in beta cells would protect them in vivo from ER stress induced apoptosis caused by high levels of circulating glucose and fatty acids in type 2 diabetes.

The chronic and continuous progress of ER stress may explain why humans remain obese and insulin resistant for many years before eventually beta cell decompensation occurs leading to diabetes. This has been recently supported in the Lep\(^{ob/ob}\) model compared to Lepr\(^{db/db}\) model\(^{235}\). In the Lep\(^{ob/ob}\) mice, adaptive UPR genes were progressively induced in islets. In contrast, these genes declined in diabetic Lepr\(^{db/db}\) mice\(^{235}\). These findings indicate that the adaptive UPR generates a beta cell compensation model, however failure of the UPR leads to beta cell decompensation, beta cell death and diabetes (Figure 5.2).

ER stress also contributes to the pathogenesis of peripheral insulin resistance. In Lep\(^{ob/ob}\) mice\(^{53}\), chemical chaperones that alleviate ER stress can restore glucose homeostasis by normalizing hyperglycemia, improving insulin sensitivity and
enhancing insulin action in liver, muscle and adipose tissues. Similarly, in humans, hepatic and muscle insulin sensitivity increased after treatment with the chemical chaperone tauroursodeoxycholic acid (TUDCA)\textsuperscript{310}. Therefore targeting ER stress represents a new therapeutic way for treating type 2 diabetes.

Although the exact mechanism of the improved glucose homeostasis in the global BIM knockout Lepr\textsuperscript{db/db} mice is not known, we hypothesized that BIM deficiency has an impact on reducing ER stress globally and therefore reducing peripheral insulin resistance. Thus, when there is less glucotoxicity caused by insulin resistance, the beta cell mass is preserved and diabetes is delayed (Figure 5.1). Less glucotoxicity means there is less ER stress on the beta cell, therefore beta cells can maintain the UPR in the adaptive stage\textsuperscript{235}, similar to beta cells in Lep\textsuperscript{ob/ob} mice. In the compensation stage, the beta cell mass is increased to meet the demand\textsuperscript{274}. Our preliminary qPCR data show a trend of reduced ER stress gene expression in Lepr\textsuperscript{db/db}BIM\textsuperscript{-/-} mice, which indicates that the improved glucose homeostasis is accompanied by less ER stress (Figure 5.2).

A number of ways have been investigated to preserve beta cell mass by inhibiting beta cell apoptosis in type 2 diabetes, however none of them is beta cell-specific. We are the first to study the role of apoptosis specifically in beta cells in a type 2 diabetes model. Interestingly, our results challenge the traditional view on the role of the apoptotic molecule BIM in type 2 diabetes. Our study showed that the potent BH3-only molecule BIM, which can bind to all the pro-survival proteins of the BCL-2 family, plays a limited role in regulating beta cell apoptosis in type 2 diabetic conditions in vivo. My results suggest that beta cell apoptosis does not play a direct role in type 2 diabetes. However, it remains possible that another pro-apoptotic BH3-only molecule, such as PUMA, could compensate for loss of BIM in vivo, allowing apoptosis to occur in BIM-deficient mice. This could be definitively tested using mice with beta cell-specific deletion of BAX and BAK, the two downstream effector molecules.

\textbf{5.4 Non-apoptotic role of BH3-only proteins}
In addition to its possible role in ER stress, new evidence indicates that BIM may have a role in regulating glucose homeostasis and metabolism via a non-apoptotic role. Wali et al. have reported that BIM regulates mitochondrial oxygen consumption and lipid oxidation, which leads to improved insulin sensitivity (See Appendix A). We hypothesize that this mechanism is also responsible for improved insulin sensitivity in global BIM deficient Lepr<sup>db/db</sup> mice, thereby improving beta cell function by reducing pressure on the beta cell to produce insulin. The absence of a phenotype in metabolism or beta cell mass in beta cell specific BIM deficient Lepr<sup>db/db</sup> mice supports this possibility, because in these mice BIM deficiency did not affect any other cell types. S961 treated C57BL/6 BIM-/− mice were also the same as wild-type mice treated with S961. These data also support the idea that BIM’s function is associated with regulating insulin sensitivity because in this model, insulin sensitivity is unlikely to be changed due to complete blockade of the insulin receptor. Mice lacking BIM specifically in the liver showed improved glucose homeostasis when fed a high fat diet<sup>303</sup>, and this was due to increased insulin sensitivity, further supporting a role for BIM in regulating insulin sensitivity.

BCL-2 family members have been reported to function beyond their canonical role in apoptosis<sup>311</sup>. These non-apoptotic roles include regulation of mitochondria (morphology, permeability transition, metabolism), endoplasmic reticulum (UPR, calcium homeostasis), glucose and lipid metabolism, cell cycle and DNA damage response<sup>311</sup>. This new area enables us to have a wider eyesight to explore the network of BCL-2 family proteins.

BCL-2 family members were shown to regulate metabolism at the inner mitochondrial membrane. BCL-2 is localized to the inner mitochondrial membrane (IMM)<sup>164</sup>, regulates mitochondrial respiration and plays a role in adjusting cytochrome c oxidase activity<sup>165</sup>. BCL-XL, also localizing to the IMM, interacts with F<sub>1</sub>F<sub>0</sub>ATP synthase to maintain mitochondrial membrane potential<sup>161</sup>. MCL-1 has also been suggested to operate at the IMM to regulate mitochondrial function<sup>166</sup>. In addition to the distinct role of regulation of mitochondrial function, BCL-2 family proteins also possess the capacity to regulate metabolism. Danial et al. have
demonstrated a role for BAD in glucose metabolism. BAD resides in a glucokinase-containing complex that regulates glucose-driven mitochondrial respiration\textsuperscript{312} and has a physiological role in glucose-stimulated insulin secretion by beta cells. Inhibition of BAD restores functional beta cell mass in diabetes\textsuperscript{168}. Other BCL-2 proteins were also shown to regulate glucose and lipid metabolism. NOXA stimulates glucose consumption via the pentose phosphate pathway\textsuperscript{169}. In addition, BID is involved in lipid metabolism through its action on mitochondrial carrier homologue 2 (MTCH2)\textsuperscript{170,313}. Although these proteins apparently affect glucose and lipid metabolism, the exact mechanisms remain unclear and therefore require further investigation.

Future experiments to study the impact of BIM deletion on insulin sensitivity would be performed in mice with liver specific deletion of BIM in Lepr\textsuperscript{db/db} mice. Examining beta cell mass in these mice would determine whether the increased islet size is a result of increased insulin sensitivity or another mechanism. One alternative mechanism is that BIM is required for endothelial cell homeostasis. We observed an increase in the number of islet blood vessels in the islets of Lepr\textsuperscript{db/db}BIM\textsuperscript{-/-} mice, which could be due to reduced endothelial cell apoptosis. Endothelial cell-specific deletion of BIM could also be studied to test this idea.

5.5 Clinical significance of our study

Currently the therapies for management of type 2 diabetes focus on stimulating insulin secretion from beta cells, insulin injection, enhancing insulin sensitivity and reducing glucose reabsorption. There is currently no treatment to target beta cell apoptosis. We are the first to try blocking the intrinsic apoptosis pathway specifically in beta cells in a type 2 diabetic model. Our data show that blocking apoptosis in beta cells did not delay the occurrence of type 2 diabetes. However, excitingly, we observed a non-classic role of BIM in type 2 diabetes, which may lead to the development of novel treatments for type 2 diabetes. There are several drugs in clinical use that increase insulin sensitivity such as TZDs. This class of antidiabetic agents, peroxisome proliferator-activated receptor (PPAR) agonists, very effectively improve glycemic control in type 2 diabetic patients\textsuperscript{12,314} and were shown to reduce the incidence of type 2 diabetes by 55\% in women with a history of gestational diabetes. These drugs are
associated with improved insulin sensitivity, less glucotoxicity and better preservation of beta cell mass\textsuperscript{275}. Global BIM deficiency results in a similar phenotype, with improved insulin sensitivity and possible protection of beta cells from glucotoxicity. This suggests that targeting BIM could be a new strategy to treat type 2 diabetes.

BIM was shown to regulate mitochondrial oxygen consumption and loss of BIM resulted in increased lipid metabolism. We believe that BIM blockade could be a novel treatment for type 2 diabetes, targeting the mitochondrial respiratory chain to oxidize fatty acid and improve insulin sensitivity. A new class of antidiabetic drug called glimins act by increasing the mitochondrial capacity to oxidize fatty acids. In type 2 diabetic patients, glimins were as effective as metformin in regulating glucose homeostasis. Glimins showed a favourable tolerability compared to metformin and therefore could be suitable for combination with other classes of antidiabetic agents and may be available to a larger patient population\textsuperscript{315}. In a similar manner, BIM could be another potential therapeutic target for type 2 diabetes by regulating mitochondrial capacity.

5.6 Limitations of this study

We have analysed the importance of BIM on metabolism and islet biology in mice lacking this pro-apoptotic protein globally at early (3 months) and late (9 months) time points in type 2 diabetes. Beta cell specific deletion of BIM in mice was only studied in the early phase of type 2 diabetes (4.5 months). However, it remains possible that BIM may impact beta cell apoptosis and metabolic phenotypes later in the disease. At 4.5 months of age, our mice may not yet be at the expected “decompensation” stage with increased apoptosis. This could be tested in future in older Lepr\textsuperscript{db/db} \( \beta \) BIM\textsuperscript{−/−} mice, at a stage when the beta cells are decompensating with increased apoptosis.

5.7 Conclusions

In summary, in this thesis, three in vivo models were used to study the effects of BIM inhibition under insulin resistance and type 2 diabetes. The main findings are:
1. Global BIM deletion regulated glucose homeostasis and beta cell apoptosis in vivo in type 2 diabetes. This was possibly through a mechanism of improved insulin sensitivity in BIM-deficient mice and thereby protection of beta cell mass from ER stress induced by hyperglycemia.

2. β-Cell specific deficiency of BIM did not change beta cell size or metabolic status, indicating the phenotype of global BIM deletion is not related to BIM’s direct impact on the β-cell. In other words, in vivo inhibition of beta cell apoptosis does not prevent type 2 diabetes.

3. BIM-deficiency in Lepr<sup>db/db</sup> protects beta cell mass secondarily to improved insulin sensitivity. The insulin receptor antagonist model shows that inhibition of BIM could not additionally increase beta cell mass independent of its effect on insulin sensitivity. The mechanism by which inhibition of BIM improves insulin sensitivity requires further investigation, possibly through BIM’s non-apoptotic role in regulating OXPHOS and energy expenditure.

Figure 5.1 The working model of BIM deficiency in Lepr<sup>db/db</sup> mice.
Obesity and insulin resistance in Lepr\textsuperscript{db/db} mice becomes worse with time. In BIM wild-type Lepr\textsuperscript{db/db} mice, insulin sensitivity is impaired and contributes to glucose toxicity on beta cells, which leads to loss of functional beta cell mass, therefore insufficient amount of insulin is secreted from beta cells and type 2 diabetes occurs. However, in the Lepr\textsuperscript{db/db} mice with global BIM deficiency, insulin sensitivity is improved, thereby beta cell mass is preserved longer and this results in delayed diabetes. This effect was not observed in Lepr\textsuperscript{db/db} mice with beta cell specific-BIM deficiency as a result of lack of improvement in insulin sensitivity.

Figure 5. 2 Model for increased beta cell mass in BIM deficient Lepr\textsuperscript{db/db} mice.

Increased caloric intake in homozygous Lepr\textsuperscript{db/db} mice leads to obesity and insulin resistance. Insulin resistance initially induces a compensatory increase in beta cell mass. Adaptive UPR could contribute to the compensatory actions by increasing beta cell proliferation. However, chronic and progressive insulin resistance leads to beta cell stress and induces apoptosis of beta cells, therefore loss of beta cell mass occurs. This could be reversed by global BIM deficiency, and in this case the islet phenotype is maintained at the compensatory stage. This effect is likely related to the improved insulin sensitivity by global BIM deficiency because there are no changes in beta cell mass in the Lepr\textsuperscript{db/db}βBIM\textsuperscript{+/--} mice.
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Appendix A
Appendix A

Appendix A Supporting information not for examination

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Loss of BIM increases mitochondrial oxygen consumption and lipid oxidation, reduces adiposity and improves insulin sensitivity in mice

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Running title: Role for BIM in substrate metabolism
ABSTRACT

BCL-2 proteins are known to engage each other to determine the fate of a cell after a death stimulus. However, their evolutionary conservation and the many other reported binding partners suggest an additional function not directly linked to apoptosis regulation. To identify such a function, we studied mice lacking the BH3-only protein BIM. BIM<sup>−/−</sup> cells had a higher mitochondrial oxygen consumption rate that was associated with higher mitochondrial complex-IV activity. The consequences of increased oxygen consumption in BIM<sup>−/−</sup> mice were significantly lower body weights, reduced adiposity and lower hepatic lipid content. Consistent with reduced adiposity, BIM<sup>−/−</sup> mice had lower fasting blood glucose, improved insulin sensitivity and hepatic insulin signalling. Lipid oxidation was increased in BIM<sup>−/−</sup> mice, suggesting a mechanism for their metabolic phenotype. Our data suggest a role for BIM in regulating mitochondrial bioenergetics and metabolism and support the idea that regulation of metabolism and cell death are connected.
INTRODUCTION

Type 2 diabetes is characterised by insulin resistance and reduced functional mass of pancreatic beta cells. In addition, the content and oxidative capacity of mitochondria is reduced in insulin responsive tissues such as skeletal muscle, liver and white adipose tissue of subjects with insulin resistance and type 2 diabetes, and these changes are believed to contribute to diabetes pathogenesis.  
The inner mitochondrial membrane is the site of oxidative phosphorylation (OXPHOS) and energy metabolism. OXPHOS involves the transfer of electrons liberated during \( \beta \)-oxidation and the citric acid cycle across the protein complexes of the electron transport chain. This transfer of electrons, together with the pumping of protons from the mitochondrial matrix to the intermembrane space is required for generation of ATP and release of heat. 

In contrast, the outer mitochondrial membrane is important in apoptotic cell death signalling. In mammalian cells, the intrinsic apoptosis pathway, also known as the BCL-2 regulated or mitochondrial pathway, is activated by cellular stresses such as radiation exposure, DNA damage and growth factor withdrawal. Activation of the pro-apoptotic BH3-only BCL-2 family members, such as BIM or PUMA, inhibits the pro-survival BCL-2 family members, including BCL-2, BCL-XL and MCL-1, allowing activation of the pro-apoptotic multi-BH domain effectors BAX and BAK, leading to mitochondrial outer membrane permeabilisation with consequent release of cytochrome \( c \) into the cytoplasm, and activation of the caspase cascade that eventually causes cell death.

BIM is a potent BH3-only factor that can bind with high affinity to all the pro-survival members of the BCL-2 family. It mediates apoptosis in a variety of cell types in response to cellular stresses, such as growth factor deprivation, ER stress, oxidative...
stress, and corticosteroid exposure\textsuperscript{5-8}. Although BIM is expressed at very low levels in the absence of stress, it has been observed in the mitochondria of non-apoptotic cells, in association with the translocase of the outer membrane (TOM) complex that imports proteins into the mitochondria \textsuperscript{9,10}. This indicates that it may have a mitochondrial role, even in resting cells. Further, the protein sequence of BIM is conserved across species \textsuperscript{11}, and binding partners additional to pro-survival BCL-2 proteins have been described \textsuperscript{9}. We have therefore studied BIM-deficient mice to investigate whether BIM could have a physiological role in resting cells separate to its apoptotic function. BIM\textsuperscript{−/−} mice had increased lipid oxidation, associated with increased activity of mitochondrial complex IV and an increased mitochondrial oxygen consumption rate (OCR) at a cellular level. As a consequence, BIM-deficient mice had lower fasting blood glucose, improved insulin sensitivity and reduced adiposity. Our results suggest that BIM has a role in mitochondrial function that is independent of apoptosis, and its deficiency results in altered whole body metabolic homeostasis.

RESULTS

BIM-deficient cells have increased mitochondrial oxygen consumption rate and reduced mitochondrial membrane potential

We investigated whether BIM is involved in regulating cellular mitochondrial oxidative metabolism. The mitochondrial oxygen consumption rate (OCR) was measured in mouse embryonic fibroblasts (MEF) under serum-free conditions. BIM\textsuperscript{−/−} MEFs had substantially higher basal OCR than wild-type controls (Fig. 1A&B). Similar to wild-type cells, around 70% of basal OCR in BIM\textsuperscript{−/−} MEFs was coupled to ATP synthesis suggesting that there was a proportional increase in uncoupled and ATP-coupled OCR in BIM\textsuperscript{−/−} cells (Fig. 1A&B). Strikingly, we noticed an increase of
more than 3-fold in the maximal mitochondrial respiratory capacity of BIM$^{-/-}$ MEFs compared to wild-type controls (Fig. 1A&B). Similar differences were seen in basal and maximal cellular respiratory capacity (Supplementary Fig. 1A). The rate of oxygen consumption coupled to flux through complex-I and complex-III was determined after sequentially adding rotenone and antimycin A to the cells and was also higher in BIM$^{-/-}$ cells (Supplementary Fig. 1A). The mitochondrial OCR of BAX$^{-/-}$ BAK$^{-/-}$ MEFs was not different from wild-type controls, suggesting that increased OCR in BIM$^{-/-}$ cells may be separate from its pro-apoptotic function (Fig. 1A&B) These data indicate that in addition to its known role as a pro-apoptotic protein, BIM is also involved in regulating mitochondrial oxidative phosphorylation (OXPHOS). 

Mitochondrial membrane potential, measured by JC-1 staining, was lower in BIM$^{-/-}$ MEFs than wild-type MEFs (Fig. 1C), indicating changes in ionic equilibrium across the inner mitochondrial membrane. In contrast, the intensity of JC-1 staining in BAX$^{-/-}$ BAK$^{-/-}$ MEFs was similar to wild-type controls (Fig. 1C). The reduction in mitochondrial potential could be a consequence of increased mitochondrial ATP turnover in BIM$^{-/-}$ MEFs. These data show that BIM has a fundamental role in regulating mitochondrial function, cellular bioenergetics and energy expenditure.

Hepatocytes from BIM$^{-/-}$ mice had a similar amount of mitochondrial DNA compared to wild-type hepatocytes (Supplementary Fig. 1B). BIM deficiency resulted in increased mean mitochondrial cross-sectional area in hepatocytes (Supplementary Fig. 1C & D). This could be due to altered mitochondrial fusion or fission$^{12, 13}$. BIM-deficient cells also had reduced number of mitochondria per cell and mitochondria were relatively more round in shape, which is likely to be due to their bigger size (Supplementary Fig. 1C & D).
BIM-deficient hepatocytes have increased activity of complex IV

To understand how BIM could control OCR, activity of mitochondrial complexes I-IV was measured in liver mitochondrial preparations from control and BIM\(^{-/-}\) mice. These assays assess the intrinsic capacity of mitochondrial complexes independent of cellular energetics, which was assessed in the experiments using MEFs. Citrate synthase activity (a marker for mitochondrial content) was slightly reduced in BIM\(^{-/-}\) livers (Fig. 2A). We observed an increase of about two-fold in the activity of complex IV in BIM-deficient liver tissue, while no difference was observed in the activities of other complexes, indicating that the effects of BIM deficiency are specific to complex IV and not generalised to the entire OXPHOS machinery (Fig. 2B).

In blue native PAGE (BN-PAGE) analysis, a cocktail of antibodies against complexes I, II, III and IV (anti-OXPHOS) showed no difference in the expression of individual complexes (Supplementary Fig. 2). Further, no difference in the assembly of CI/CIII/CIV supercomplexes was observed between wild-type and BIM\(^{-/-}\) liver tissue samples suggesting that loss of BIM does not increase complex IV activity by facilitating improved assembly of supercomplexes (Supplementary Fig. 2).

Lipid oxidation and energy expenditure is greater in BIM-deficient mice than wild-type controls

Our data in MEFs show that BIM regulates cellular energy expenditure that is supported by mitochondrial oxidative metabolism. Mitochondria are an important site of cellular macronutrient metabolism. This includes glucose oxidation via the TCA cycle. In addition, $\beta$-oxidation of lipids and the subsequent oxidation of the resultant acetyl-CoA, also through the TCA cycle, produces reducing equivalents for the generation of ATP molecules through oxidative phosphorylation\(^{14, 15}\). To find out
whether changes in mitochondrial OXPHOS due to BIM deficiency alter whole body energy expenditure and substrate metabolism, we studied mice in metabolic cages. Consistent with data obtained in MEFs, mice lacking BIM had increased energy expenditure (Fig. 3A). These mice had a lower respiratory quotient under the basal conditions of light cycle (Fig. 3B), which was associated with increased lipid utilization by BIM−/− mice (Fig. 3C). Carbohydrate oxidation was reduced in the light cycle in BIM−/− mice (Supplementary Fig. 3A), indicating that BIM also regulates whole body substrate utilisation. No significant differences between wild type and BIM−/− mice were observed in physical activity (Supplementary Fig. 3B).

**BIM-deficient mice have altered lipid and glucose metabolism in the liver**

The liver is a key organ in regulating glucose and fat metabolism in the body. We examined metabolism of these micronutrients in the liver. Isolated hepatocytes lacking BIM had a greater capacity to oxidise palmitate than wild-type hepatocytes (Fig. 3D). Further, BIM−/− mice had lower serum triglycerides, while cholesterol concentrations were similar between wild-type and BIM−/− mice (Fig. 3E). We hypothesize that due to increased energy expenditure, BIM−/− mice metabolise the glycogen stores relatively rapidly and this is followed by greater lipid metabolism in the resting state, resulting in reduced hepatic lipid and glycogen content. Indeed, fasting liver glycogen content was reduced in BIM−/− livers (Fig. 3F). Although serum albumin was slightly reduced, deficiency of BIM did not affect concentrations of total proteins (Supplementary Fig. 3C) and liver enzymes (not shown) in the serum suggesting that synthetic liver function in BIM−/− mice is overall normal.

**BIM deficiency is associated with reduced body weight and adiposity**
Appendix A

Given the increased energy expenditure and lipid oxidation, and favourable serum lipid profile in BIM-deficient mice, we next investigated total body weight and adiposity in mice on a chow diet and those challenged by a 45% high fat diet (HFD) for a total of 18 weeks. Compared with wild-type mice on similar diet, mice lacking BIM gained less weight (Fig. 4A). Consistent with increased energy expenditure, BIM\(^{-/-}\) mice had slightly greater cumulative food intake than the wild-type controls, despite the reduced weight gain (Fig. 4B). Heterozygous BIM\(^{+/+}\) mice were intermediate between wild-type and knock-out mice showing that effects of BIM deficiency are allele dosage dependent (Fig. 4A&B). BIM deficiency did not affect length (Supplementary Fig. 4A) or histological structure of the small intestine (Supplementary Fig. 4B) suggesting that reduced weight in BIM\(^{-/-}\) mice is not associated with altered intestinal morphology that could potentially affect nutrient absorption.

Consistent with lower body weight, BIM-deficient mice also had lower visceral (VAT) and subcutaneous (SAT) white fat pad mass and inter-scapular brown fat adiposity on chow and high fat diets indicating a generalized decrease in adiposity in BIM\(^{-/-}\) mice (Fig. 4C). This was confirmed by MRI at 18 weeks of age, with reduced percentage fat mass and increased percentage lean body mass and unchanged percentage mass of water observed in BIM\(^{-/-}\) compared to wild-type mice (Fig. 4D). Adipocyte cell size was smaller in BIM-deficient mice than wild-type counterparts on both a chow diet and HFD (Fig. 4E&F). Liver weights were similar across all the mouse groups (Supplementary Fig. 4C).

**Deficiency of BIM improves glucose homeostasis in mice**
Appendix A

Increased energy expenditure and reduced body fat is commonly associated with improvements in insulin sensitivity and glucose homeostasis. Compared with mice on a chow diet, high fat feeding increased fasting blood glucose in wild-type mice (Fig. 5A&B). BIM\(^{-/-}\) and BIM\(^{+/+}\) mice had lower fasting blood glucose concentrations when compared with chow or high fat fed wild-type mice (Fig. 5A&B). No significant differences in non-fasting blood glucose were observed (Supplementary Fig. 4D). Lower fasting blood glucose could be due to enhanced release of insulin from islets or improved sensitivity to insulin in peripheral tissues.

Islet function was analysed by intra-peritoneal glucose tolerance testing in 20-week-old mice, after 14 weeks on either chow or high fat diet. BIM\(^{-/-}\) mice had improved glucose tolerance compared to wild-type mice (Fig. 5C&E). High fat feeding made the mice glucose intolerant, but BIM\(^{-/-}\) mice still had improved glucose tolerance compared to BIM\(^{+/+}\) mice (Fig. 5D&E). Glucose tolerance in heterozygous BIM\(^{-/-}\) mice was intermediate between wild-type and BIM\(^{-/-}\) mice (Fig. 5C-E).

Insulin mediated glucose disposal (measured in an intraperitoneal insulin tolerance test) was significantly increased in mice lacking BIM compared to wild-type controls (Fig. 5F&H). Although the high fat diet impaired sensitivity to insulin in all mice, BIM\(^{-/-}\) mice remained more sensitive to injected insulin than BIM\(^{+/+}\) mice (Fig. 5G&H). Sensitivity of heterozygous BIM\(^{-/-}\) mice to insulin was intermediate between wild-type and BIM\(^{-/-}\) mice (Fig. 5F-H).

Hyperinsulinaemia is a compensatory response by islets to high fat-induced insulin resistance. Deficiency of BIM was associated with lower plasma insulin concentrations after intravenous glucose injection compared to wild-type controls under both dietary conditions (Fig. 5I&J and Supplementary Fig. 4E). These data suggest that the increased insulin sensitivity in BIM-deficient mice results in reduced
Appendix A

insulin requirements than wild-type mice to maintain their basal glucose and to normalize hyperglycaemia induced by high fat feeding. Consistent with this we observed no differences in islet size or fractional insulin or glucagon positive areas (Supplementary Fig. 4F&G). Both basal and glucose-stimulated insulin secretion were the same in wild-type and BIM-deficient islets (Supplementary Fig. 4H). Overall, these data suggest that improved peripheral tissue insulin sensitivity, and not islet function, results in the metabolic phenotype of BIM−/− mice.

Phosphorylation of Akt was greater in the livers of insulin-treated BIM−/− mice suggesting that improved insulin signalling contributes to better insulin sensitivity in these mice (Fig. 5K). No difference was observed in AMPK phosphorylation between BIM+/+ and BIM−/− livers suggesting that increased insulin signalling is not secondary to AMPK mediated metabolic changes (such as AMPK driven increased lipid metabolism) in BIM-deficient hepatocytes (Supplementary Fig. 5A). We observed a similar trend of increased Akt phosphorylation in the skeletal muscle (gastrocnemius) of BIM−/− mice (Supplementary Fig. 5B).

Age-related intra-cellular lipid droplet accumulation, visualized by histological analysis and oil red O staining, was reduced in hepatocytes of BIM-deficient mice compared to wild-type controls and this was observed in both chow-fed and high fat-fed mice (Supplementary Fig. 5C-E). Reduced hepatic lipid content may be the result of reduced lipogenesis, however, we did not observe any change in lipogenic or lipid uptake enzyme expression in liver or visceral fat (Supplementary Fig. 5F). Ectopic lipid accumulation in the liver reduces hepatic insulin sensitivity. Therefore, reduced hepatocyte lipid accumulation in BIM-deficient mice could contribute to increased hepatic insulin sensitivity.
DISCUSSION

In this study, we show that BIM regulates mitochondrial OXPHOS and its deficiency leads to increased mitochondrial oxygen consumption and activity of complex IV, the terminal enzyme in the electron transport chain. Mitochondria are the major site of cellular ATP synthesis and oxidation of lipids, so changes in mitochondrial OXPHOS are likely to affect whole body macronutrient metabolism. In particular, liver and muscle are the main sites of lipid oxidation in the body, and most likely to be affected by increasing complex IV activity. Taking advantage of the fact that BIM-deficient mice survive to adulthood and do not show any significant pathology on a C57BL/6 genetic background at a young age, we found that altered bioenergetics observed with BIM deficiency led to increased use of lipids for resting energy metabolism resulting in reduced body fat, reduced glycogen stores and increased insulin sensitivity. Consistent with our data, feeding a high fat diet to mice for 12 weeks induced insulin resistance and reduced the expression of complex IV in visceral fat\textsuperscript{20}. However, mice lacking the complex IV assembly factor Surf1 have reduced complex IV activity and this is associated with increased mitochondrial biogenesis, reduced adiposity, increased lipid oxidation and improved insulin sensitivity\textsuperscript{21}. This shows that the impact of alterations in complex IV activity on insulin sensitivity and substrate metabolism is dependent on the underlying mechanism and metabolic context. Our study shows that increased mitochondrial activity caused by BIM deficiency is a mechanism for improving insulin sensitivity.

One caveat of our work is the observation that BIM-deficient mice have perturbed immune homeostasis. In BIM-deficient mice, autoreactive T cells escape deletion in the thymus. However, widespread autoimmunity is avoided because a subset of FoxP3\textsuperscript{+}CD4\textsuperscript{+} Treg cells is preferentially increased in BIM\textsuperscript{-/-} mice\textsuperscript{22}. This shift in the
balance of immune subsets could contribute to the improved insulin sensitivity in BIM<sup>−/−</sup> mice. However, we did not observe any changes in immune cell populations in the visceral adipose tissue of BIM<sup>−/−</sup> mice (not shown). Also, it is likely that all tissues contribute to the metabolic phenotype in BIM<sup>−/−</sup> mice, and tissue-specific knockout mice will display a partial phenotype. Preliminary data in hepatocyte-specific knockouts show insulin sensitivity consistent with the whole body data (ENG and HET unpublished data).

Our data suggest that BIM inhibits both ATP coupled and uncoupled respiration and this is seen in non-apoptotic conditions in the absence of apoptotic stimuli known to activate BIM. Consistent with this, increased accumulation of mitochondrial reactive oxygen species (ROS) and reduced mitochondrial respiration were observed in neuroblastoma cells conditionally expressing BIM<sup>23</sup>. BIM has tumour suppressor properties<sup>24, 25</sup> and it is possible that BIM-mediated inhibition of mitochondrial activity provides the initial trigger for tumour cell apoptosis by generating ROS in the mitochondria<sup>23</sup>. Therefore, it is possible that metabolic effects of BIM could be connected with apoptosis, and elucidating this connection will be determined in future studies.

Changes in mitochondrial OXPHOS have been observed previously <em>in vitro</em> in cells with altered expression of the pro-survival factors MCL-1, BCL-2 and BCL-XL<sup>26-29</sup>. Overexpression of these pro-survival factors increased mitochondrial activity, and although the effects on OXPHOS are not exactly the same, they are consistent with our findings that deletion of BIM increased mitochondrial function and complex IV activity. The exact mechanism by which BIM regulates these effects remains unclear. It was recently reported that a variant of <em>BCL2</em> is associated with altered insulin sensitivity in a large genome wide association study<sup>30</sup>.<em>BCL2</em> is also associated with
type 2 diabetes and body fat distribution. It is currently unknown if BIM directly binds to a subunit of complex IV or if its deletion leads to increased availability of pro-survival factors in the mitochondrial matrix. The highly unstructured nature of BIM suggests that it may have a secondary role in the mitochondria through association with another protein, such as one of the pro-survival factors. While the pro-apoptotic proteins BAD and BNip3 have demonstrated roles in mitochondrial function and liver energy metabolism, PUMA did not have a similar metabolic phenotype. Our new data showing a role of pro-apoptotic BIM in metabolism, together with previous data showing an effect of pro-survival members of the same family suggest that the family of proteins is involved in both cell death and metabolism.

AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor proteins (PPARs) are important regulators of lipid oxidation, and have been implicated in protection from apoptosis of BIM-deficient neuronal cells, hepatocytes and beta cells. Further, activation of AMPK has been shown to increase mitochondrial content and activity of complexes III and IV. This raises the possibility that by inhibiting apoptosis, BIM deficiency could permit prolonged and sustained AMPK activation resulting in increased oxidative metabolism of fatty acids. However, we did not observe evidence that BIM and AMPK mediate cross-talk between apoptosis and metabolism, and therefore we suggest that the increased lipid oxidation in BIM−/− mice is not likely to be AMPK mediated, but depends on downstream increase in mitochondrial activity.

Overall, we have shown that in addition to its known role as a potent inducer of apoptosis, BIM is involved in the regulation of mitochondrial oxygen consumption and complex IV activity and its loss results in increased lipid metabolism and reduced body fat. Future work will determine what domain(s) in BIM is important in regulating
mitochondrial activity. Targeting the mitochondrial respiratory chain to oxidize fatty acids and improve insulin sensitivity is a novel treatment option for type 2 diabetes. For example, glimins, a new class of drug that act by increasing the mitochondrial capacity to oxidize fatty acids, are as effective as metformin in preliminary human studies. The lipid lowering effects of metformin are dependent on AMPK target enzymes ACC1 and ACC2, and metformin treatment does not lead to substantial increases in mitochondrial OXPHOS. It is possible that targeting the mitochondrial role of BIM could be an option to amplify the lipid lowering effects of metformin by increasing mitochondrial activity, providing of course that this does not impact on BIM’s critical role in regulation of cell death.

MATERIALS AND METHODS

Mice

Mice deficient in Bim generated on a pure C57BL/6 genetic background were kindly provided by Dr Philippe Bouillet (Walter and Eliza Hall Institute of Medical Research, Parkville, Australia). BIM−/− and BIM+/− mice were bred at St Vincent’s Institute. Wild-type C57BL/6 mice were purchased from Animal Resources Centre (Canning Vale, Australia). All animals were housed at St Vincent’s Institute Bioresources Centre, and experiments were approved by the St Vincent’s Hospital Animal Ethics Committee.

High fat diet and metabolic analysis

Six-week-old male BIM−/−, BIM+/− or BIM+/+ mice on C57BL/6 background were randomly placed on a chow diet (Specialty Feeds, Glenn Forest, Australia) or a high fat diet (HFD, 45% kCal/fat, Specialty Feeds) for 18 weeks. Mice were maintained on a 12 h light/12 h dark cycle. Mice were weighed and their food intake was calculated
weekly. Non-fasting blood glucose was measured every two weeks while fasting blood glucose was measured after 7-8 h of fasting at 0, 6, 10, 14 and 18 weeks using a glucometer (Accu Chek Performa, Roche, Basel, Switzerland). At 18 weeks, mice were sacrificed and pancreas, liver, inguinal fat pad (subcutaneous adipose tissue: SAT), epididymal fat pad (visceral adipose tissue: VAT) and interscapular fat pad (brown adipose tissue) were weighed, and collected for further analysis.

Mice lacking BIM and wild-type controls fed a chow-diet were housed in metabolic cages (Columbus Instruments, Columbus, OH) for 48 h at 18 weeks of age. After 24 h of acclimatisation, physical activity, energy expenditure and substrate utilization were measured by indirect calorimetry. Body composition was determined by MRI scanning (EchoMRI, Houston, TX).

Blood samples were obtained from fasted mice by cardiac puncture. Serum was isolated from the blood samples by centrifugation and the measurements for lipids, proteins and liver enzymes were performed on a clinical chemistry analyser (Beckman Coulter AU5800 Clinical Chemistry System, Brea, CA).

**Insulin and glucose tolerance tests**

Mice were fasted for 4 h at 18 weeks of age before performing an intra-peritoneal insulin tolerance test (IP-ITT). Insulin (Actrapid, Novo Nordisk, Bagsværd, Denmark), at a dose of 0.75 U/kg, was injected intra-peritoneally and blood glucose was measured at 0, 15, 30, 45, 60, 90 and 120 min after tail bleeding.

At 20 weeks of age, mice were fasted for 6 h before an intra-peritoneal glucose tolerance test (IP-GTT). Glucose solution (2g/kg, Baxter, Deerfield, IL) was injected and blood glucose was measured at 0, 15, 30, 45, 60 and 120 min. An intra-venous glucose tolerance (IV-GTT) test was performed on 24 week old mice after 6 h of fasting according to previously described methods. Briefly, 1g/kg glucose was
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injected and plasma samples were obtained at 0, 2, 5, 10, 15 and 30 min to measure insulin concentration by ELISA (Mercodia, Uppsala, Sweden).

**Glucose stimulated insulin secretion**

Islets of Langerhans were isolated as described previously\(^47\). Twenty islets of similar size were pre-incubated for 30 min in Krebs-Ringer bicarbonate (KRB) buffer containing 3 mM glucose and 0.1% BSA, after which they were incubated for 30 min with KRB buffer containing either 20 mM glucose or 3 mM glucose and 0.1% BSA. The secreted insulin was measured using a rat/mouse insulin ELISA kit (Mercodia).

**Western blotting and blue native PAGE**

20-week-old male mice were fasted for 6 h and then injected intra-peritoneally with PBS or insulin (0.65 mU/g). Liver and muscle samples were collected in liquid nitrogen 10 min after insulin injection. Lysates were prepared and western blotting was performed according to previously described methods \(^48\). Anti-phospho-S473-Akt, anti-Akt, anti phosphoT172-AMPK and anti-pan-alpha AMPK antibodies from Cell Signaling Technology (Danvers, MA), and anti-BIM antibody from Alexis Biochemicals (Plymouth Meeting, PA) were used. Blotting with anti-actin or anti-tubulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used for loading control.

Blue native polyacrylamide gel electrophoresis (BN-PAGE) of liver tissue samples was performed as described \(^49\). Immunoblotting was performed using an antibody cocktail (Abcam, Cambridge, UK) against NDUFB8 (ab110242), CII-30kDa (ab14714), CIII-core protein 2 (ab14745), CIV subunit I (ab14705) and CV alpha subunit (ab14748).

**Seahorse studies and JC-1 staining**

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Wild-type, BIM<sup>−/−</sup> and BAX<sup>−/−</sup>BAK<sup>−/−</sup> mouse embryonic fibroblasts (MEFs), isolated from mouse lines made on a mixed B6/129 background and subsequently backcrossed onto B6 for more than 6 generations, were analysed on a Seahorse XF24 Flux Analyzer (Seahorse Bioscience, North Billerica, MA) in serum-free conditions according to a previously described protocol. After plating 25 000 MEFs/well and recording three basal oxygen consumption rate (OCR) measurements, readings were taken after sequentially injecting oligomycin (1 µM), FCCP (1 µM), rotenone (1 µM) and antimycin A (1 µM). Calculations of parameters of mitochondrial respiratory function included subtraction of non-mitochondrial respiration from all mitochondrial respiration parameters. The data was normalised to total protein content/well measured by BCA assay (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions.

Mitochondrial potential was measured in wild-type, BIM<sup>−/−</sup> and BAX<sup>−/−</sup>BAK<sup>−/−</sup> MEFs using JC-1 dye (10 µg/mL) according to manufacturer’s instructions. After three washes with PBS, red and green fluorescence was measured using an excitation wavelength of 488nm and emission wavelengths of 522 and 605nm respectively, using a Flexstation II microplate reader (Molecular Devices, Sunnyvale, CA). Activity of electron transport chain complexes was measured in liver tissue samples as previously described.

**Histology**

For all the histological analysis, tissue was fixed in formalin, and sections (5 µm) were stained according to standard procedures. Guinea pig anti-insulin antibody (Dako, Glostrup, Denmark) was detected with Alexa Fluor®555-conjugated goat anti-guinea pig IgG antibody (Life Technologies, Carlsbad, CA). Mouse anti-glucagon antibody (Sigma-Aldrich, St. Louis, MO) was detected with Alexa Fluor®647-conjugated goat...
anti-mouse IgG antibody (Life Technologies). Nuclei were stained with DAPI reagent (Life Technologies). Images were analysed on a Nikon A1R-A1 confocal microscope (Nikon Corporation, Tokyo, Japan). The percentage of insulin and glucagon positive staining per islet was analysed using ImageJ software (NIH, Bethesda, MD).

Hepatic lipid content was analysed on frozen sections of BIM\textsuperscript{+/+} and BIM\textsuperscript{-/-} liver by oil red O staining according to a previously published protocol \textsuperscript{52}. Slides were imaged using an Aperio digital slide scanner (Leica, Wetzlar, Germany) and oil red O positive areas were quantified using the Aperio software.

**Electron microscopy**

Transmission electron microscopy was performed as described previously \textsuperscript{53}. For isolated hepatocytes, cells were fixed in 2\% paraformaldehyde, 0.05\% glutaraldehyde in 0.08 M Sorensens’s phosphate buffer then processed using standard procedures. Ultrathin sections approximately 70 nm thickness were cut on Ultracut-S ultramicrotome (Leica, Wein, Austria) and contrasted with uranyl acetate and lead citrate. Images were analysed by ImageJ software (NIH, Bethesda, MD).

**Real-time PCR**

Total RNA was isolated using Nucleospin RNA kits (Macherey-Nagel, Düren, Germany) and reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) following the manufacturer’s protocol. Real-time PCR was performed using AmpliTaq Gold DNA Polymerase (Applied Biosystems) and a LightCycler 480 (Roche). TaqMan primers for \textit{Scd1}, \textit{Srebf1}, \textit{Pparg}, \textit{Acaca}, \textit{Fasn}, \textit{Cd36} and \textit{Actin-b} were purchased from Applied Biosystems. Data analysis was performed using the \(\Delta \Delta CT\) method: relative mRNA expression levels were calculated by normalising to the signal for \textit{Actin-b} mRNA in each sample and then comparison with wild-type samples.
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**Palmitate oxidation assay**

Measurement of palmitate oxidation capacity of hepatocytes using $[^{14}\text{C}]$ palmitate has been described previously $^{43}$. The radioactivity of the culture medium as well as the acid soluble intermediates after chemical extraction of cellular lipids was measured. Total oxidation was then calculated as a function of both $[^{14}\text{C}]$ CO$_2$ produced and incomplete oxidation products.

**Liver glycogen quantification**

Liver tissues were harvested from 24-week-old male mice after 6 h of fasting. Glycogen content was measured using a glucose oxidase reaction-based glycogen assay kit according to manufacturer’s directions (Sigma).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism Software (San Diego, CA). All data shown as bar graphs are mean±SEM. Data were analysed by t-test, one-way or two-way ANOVA with Bonferroni’s or Tukey’s post-test for comparison of multiple columns (as appropriate). A p-value of $<0.05$ was considered statistically significant and *p$<0.05$, **p$<0.01$ and ***p$<0.001$. Area under the curve (AUC) was calculated by applying the trapezoidal method.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

JAW, SG, CT, ENG, AEF, TC, JG, DS, CV and CS performed experiments, analyzed data and revised the manuscript. JAW, SG, BK, TWHK, SLM and HET designed the study and wrote the manuscript. MTR, DRT, and BEK contributed to conception, design and interpretation of this work, provided essential reagents and critically revised the manuscript.

SUPPLEMENTARY INFORMATION

Supplementary information is available at Cell Death and Differentiation’s website.
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Appendix A


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FIGURE LEGENDS

**Figure 1.** BIM-deficient cells have increased mitochondrial oxygen consumption rate (OCR) and reduced mitochondrial potential

**(A)** Mitochondrial OCR was measured in wild-type, BIM\(^{-/-}\) and BAX\(^{-/-}\)/BAK\(^{-/-}\) MEFs using a Seahorse XF24 bioanalyzer, n=3 independent MEF isolations/genotype. After recording three baseline OCR measurements, oligomycin (Oligo), FCCP, rotenone (Rot) and antimycin A (Anti) were sequentially added to the cells and OCR measurements taken thrice after each treatment. (B) Quantification of parameters of mitochondrial respiration measured by the Seahorse bioanalyzer. n=3 independent experiments. ***p<0.001 BIM\(^{-/-}\) vs wild-type MEF (one-way ANOVA). (C) Mitochondrial potential was measured in wild-type, BIM\(^{-/-}\) and BAX\(^{-/-}\)/BAK\(^{-/-}\) MEFs by JC-1 staining. n=3 independent experiments, *p<0.05 BIM\(^{-/-}\) vs wild-type MEFs (one-way ANOVA). Data show mean±SEM.
**FIGURE 2**

**Figure 2. BIM deficiency increases activity of complex IV**

(A, B) Activity of (A) citrate synthase and (B) mitochondrial electron transport chain complexes (I-IV) measured in liver samples of wild-type and BIM−/− mice and normalised to citrate synthase activity. Complex IV activity is enlarged. n=6 mice/genotype. Data show mean±SEM. *p<0.05, **p<0.01 BIM−/− vs wild-type liver tissue samples (Student’s t-test).
Figure 3. BIM-deficient mice have altered liver lipid and glucose metabolism

(A) Energy expenditure (kcal/kg), (B) mean respiratory quotient (RQ) measured hourly for 24 h (from 7am to 7am), and (C) lipid oxidation (g/kg), measured in metabolic cages for chow-fed male mice at 18 weeks of age, n=7-8 mice/genotype. *p<0.05 BIM\(^{-/-}\) vs wild-type mice (Student’s t-test). Energy expenditure and lipid oxidation normalized to mouse weight (g/kg body mass). (D) Total palmitate oxidation measured in hepatocytes isolated from 20-week-old chow-fed male wild-type and BIM\(^{-/-}\) mice. One representative experiment is shown. *p<0.05 BIM\(^{-/-}\) vs wild-type mice calculated from fold change of pooled data from n=3 independent experiments (Student’s t-test). (E) Concentrations of triglyceride and cholesterol species in blood collected from 24-week-old fasted (6 h) chow-fed male mice. n=5-8 mice/genotype. **p<0.01 BIM\(^{-/-}\) vs wild-type mice (Student’s t-test). (F) Liver glycogen content in samples harvested from 24-week-old male fasted (6h) chow-fed mice. n=8-9
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mice/genotype. *p<0.01 BIM<sup>−/−</sup> vs wild-type mice (Student’s t-test). Data show mean±SEM.

**Figure 4. BIM-deficient mice have reduced body weight and adiposity**

(A, B) Body weights in g (A) and cumulative calorie intake (B) of wild-type, BIM<sup>+/−</sup> and BIM<sup>−/−</sup> male mice on chow (left) or high fat diet (right) measured weekly from 6 weeks of age for 18 weeks. n=14-16 mice/genotype. *p<0.05, **p<0.01 BIM<sup>−/−</sup> vs wild-type mice on similar diet (two-way ANOVA). (C) Weight of inguinal fat pad (SAT), epididymal fat pad (VAT) and interscapular brown fat pad measured after 18 weeks.
weeks (24 weeks of age) and normalized to total body weight (mg/g body weight). n=9-16 mice/genotype *p<0.05, **p<0.01, ***p<0.001 BIM\textsuperscript{-/-} vs wild-type mice on similar diet (one-way ANOVA). (D) Body composition determined at 18 weeks of age in chow-fed mice by MRI scanning. % fat, lean and water mass are shown. n=7-8 mice/genotype. **p<0.001 BIM\textsuperscript{-/-} vs wild-type mice (Student’s t-test). (E) Formalin-fixed sections of inguinal fat pads (SAT; left) and epididymal fat pads (VAT; right) isolated from 24-week-old chow-fed or HFD-fed male wild-type mice or BIM\textsuperscript{-/-} mice stained with H&E. Representative images of n=4 mice/genotype. Scale bar 100 µm. (F) Quantification of adipocyte cross-sectional area of SAT and VAT from chow-fed and HFD-fed mice, n=4 mice/genotype. **p<0.01, ***p<0.001 BIM\textsuperscript{-/-} vs wild-type mice on similar diet (one-way ANOVA). Data show mean±SEM.
Figure 5. Improved glucose homeostasis in BIM-deficient mice

(A, B) Blood glucose concentration (mmol/L) measured every four weeks after 7-8 h of fasting for mice on chow (A) and high fat diet (B). n=14-16 mice/genotype. *p<0.05 BIM⁻/⁻ vs wild-type mice on similar diet (two-way ANOVA). (C, D) IP-GTT performed on 20-week-old mice after 6 h of fasting by injecting 2 g/kg of glucose. Results for the chow-fed mice (C) and high fat fed mice (D) are shown. n=14-16 mice/genotype. (E) Area under the curve (AUC) for the IP-GTT data from chow and high fat fed mice. n=14-16 mice/genotype. For (C-E) *p<0.05, **p<0.01 and
***p<0.001 BIM\(^{-/-}\) vs wild-type mice on similar diet (two-way ANOVA). (F, G) IP-ITT was performed on 18-week-old mice after 4 h of fasting by injecting 0.75 U/kg of insulin. Results are expressed as a percentage of basal blood glucose concentration and are shown for the chow-fed mice (F) and high fat fed mice (G). n=14-16 mice/genotype. (H) Area under the curve (AUC) for the IP-ITT data from chow and high fat fed mice. n=14-16 mice/genotype. For (F-H) *p<0.05, **p<0.01 and ***p<0.001 BIM\(^{-/-}\) vs wild-type mice on similar diet (two-way ANOVA). (I, J) Plasma insulin concentrations for samples obtained during IV-GTT determined by ELISA. Results for the chow-fed mice (I) and high fat fed mice (J) are shown. n=9-11 mice/genotype. *p<0.05 (Student’s t-test). (K) Western blot of phosphorylated AKT (pAKT), total AKT and actin (loading control) performed on liver lysates isolated from 20-week-old male mice after 6 h of fasting. Liver samples were isolated 10 min after intra-peritoneal injection of insulin. n=5-6 mice/genotype. Quantification of western blots for pAKT normalized to total AKT. *p<0.05 BIM\(^{-/-}\) vs wild-type mice on chow diet (Student’s t-test). Data show mean±SEM.
Supplementary Figure 1. BIM-deficient cells have increased mitochondrial respiration

(A) Quantification of parameters of cellular and mitochondrial (mt) respiration as indicated measured in wild-type, BIM\(^{-/-}\) and BAX\(^{-/-}\)/BAK\(^{-/-}\) MEFs using a Seahorse bioanalyzer. (n=3 independent experiments). Data show mean±SEM. ***p<0.001 BIM\(^{-/-}\) vs wild-type MEFs (one-way ANOVA). (B) Mitochondrial DNA (mtDNA) copy number from livers of wild-type (n=5) and BIM\(^{-/-}\) (n=5) mice, measured relative to 18s rRNA. p=0.1 (Student’s t-test). (C) Representative images of transmission electron microscopy showing mitochondria from wild-type (n=6 mice) and BIM\(^{-/-}\) (n=6 mice) hepatocytes, scale bar is 1 µm. (D) Quantification of mean mitochondrial...
(mt) cross-sectional area, number of mitochondria/cytoplasmic area, total mitochondrial area and shape factor, with a total of 32-34 hepatocytes/genotype (n=4 mice/genotype) scored. *p<0.05, **p<0.01 BIM<sup>−/−</sup> vs wild-type (Student’s t-test).

**Supplementary Figure 2. Expression of mitochondrial complexes is unchanged in BIM<sup>−/−</sup> liver**

Mouse liver mitochondria (50 µg/lane) were solubilised in either 1% (w/v) digitonin or 1% (w/v) Triton X-100 and analysed by BN-PAGE. The gel was subsequently transferred to PVDF for immunoblotting with an antibody cocktail against mitochondrial respiratory chain complexes I-V (n = 3 mice/genotype).
Supplementary Figure 3. The physical activity is not changed in BIM\textsuperscript{−/−} mice

(A) Carbohydrate oxidation by 18-week-old mice during 24 h, light cycle and dark cycle in the metabolic cages after normalizing to mouse weight (g/kg body mass). n=7-8 mice/genotype. *p<0.05 BIM\textsuperscript{−/−} vs wild-type mice (Student’s t-test). (B) Physical activity of 18-week-old mice over 24 h in metabolic cages (n=7-8 mice/genotype). Data are not statistically different. (C) Concentrations of total protein, albumin and bilirubin from 24-week-old male mice fasted for 6 h, measured on a clinical chemistry analyser. n=5-8 mice/genotype. **p<0.01 BIM\textsuperscript{−/−} vs wild-type mice on chow diet (Student’s t-test). Other data are not statistically different. Data show mean±SEM.
Supplementary Figure 4. Small intestine length and liver weight and islets are normal in BIM\textsuperscript{+/−} mice

(A) Length of small intestine (cm) of 24-week-old male wild-type and BIM\textsuperscript{+/−} mice on a normal chow diet (n=6-7 mice/genotype). Data are not statistically different. (B) Formalin-fixed sections of small intestine from 24-week old chow-fed male wild-type mice and BIM\textsuperscript{+/−} mice were stained with H&E. Representative images of n=3 mice/genotype are shown. Scale bar 200 µm. (C) Weights of liver measured at 24
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weeks of age and normalized to total body weight (mg/g body weight). n=14-16 mice/genotype. Data are not statistically different. (D) Non-fasting blood glucose concentrations (mmol/L) measured between 3-5 pm every two weeks for mice on chow (left) and high fat diet (right) for 18 weeks. N=14-16 mice/genotype. Data not statistically different. (E) Non-fasting insulin concentrations measured in wild-type (n=3) or BIM°/° (n=4) mice, p=0.07, Student’s t-test. (F) Formalin-fixed pancreatic sections from 24-week-old wild-type and BIM°/° mice on a chow-diet were stained for insulin (green), glucagon (red) and DAPI (blue). Representative sections from n=4 mice/genotype are shown. Scale bar 50 µm. (G) Total islet area, percentage insulin⁺ and percentage glucagon⁺ area was quantified for the pancreatic sections described in (F). Data are not statistically different. n=4 mice/genotype. (H) Glucose-stimulated insulin secretion of islets isolated from wild-type and BIM°/° mice. Islets were exposed to 3 mM or 20 mM glucose for 30 min, and the amount of insulin secreted into the buffer was quantified by ELISA. Results of n=4 independent experiments with 2-4 replicates in each experiment. Data are not statistically different. For (A, C, D, E, G, H) Data show mean±SEM.
Supplementary Figure 5. Muscle insulin signalling and liver morphology in wild-type and BIM\(^{-/-}\) mice

(A, B) Western blots and quantification of phosphorylated AMPK (pAMPK), total AMPK, phosphorylated AKT (pAKT), total AKT, and actin or \(\alpha\)-tubulin (loading controls) performed on liver (A) or skeletal muscle (B) lysates from 20-week-old male mice after 6 h of fasting. Samples were isolated 10 minutes after intra-peritoneal injection of insulin or PBS. n=4 mice/genotype for insulin treatment and n=2
mice/genotype for PBS treatment. Data are not statistically different. (C) Formalin-fixed sections of liver samples isolated from 24-week-old chow-fed or HFD-fed male mice stained with H&E. Representative figure of n=3 mice/genotype. Intra-hepatocyte lipid droplets (black arrows) and liver macrophages (white arrows) are visible. Scale bar 100 µm. (D) Frozen sections of liver samples isolated from 24-week-old chow-fed male mice stained with oil red O. Representative figure of n=4 mice/genotype. Scale bar 200 µm. (E) Area of oil red O staining in liver sections quantified using the Aperio digital slide scanner. *p<0.05 BIM−/− vs wild-type liver samples (Student’s t-test). n=4 mice/genotype. (F) Quantitative real-time PCR of lipogenic (Scd1, Srebf1, Pparg, Acaca, Fasn) and lipid uptake (Cd36) genes from liver (left) and visceral adipose tissue (VAT, right). Relative mRNA expression levels were calculated by normalising to the signal for Actin-b in each sample, then BIM−/− were compared with wild-type samples for each gene. N=6-8 mice/genotype Data are not significantly different. For (A, B, E, F) Data show mean±SEM.