Understanding mechanisms of beta cell susceptibility to type 1 diabetes

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ABSTRACT

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Type 1 diabetes mellitus (T1D) is an autoimmune disease characterized by the inflammation of the insulin-producing pancreatic beta cells, eventually leading to beta cell loss and the inability to maintain glucose homeostasis. Understanding the mechanisms of beta cell-intrinsic factors that influence the maintenance of cellular defenses and contribute to cell death when deregulated will be crucial in efforts to treat or prevent beta cell loss in individuals who are prone to autoimmunity. Through my thesis work, I have investigated beta cell-specific etiologies of T1D through both a candidate-based approach using beta cell-specific deletion of a susceptibility gene and an unbiased global exploration of beta cell factors that regulate the predisposition to insulitic injury.

Protein tyrosine phosphatase N2 (PTPN2) is a T1D candidate gene that has been shown to be critical for modulating inflammation by regulating T cell activation. PTPN2 is also highly expressed in human and murine beta cells and it has been shown to be critical for beta cell function *in vivo* and inhibit inflammatory stimuli-mediated beta cell apoptosis *in vitro*, suggesting that PTPN2-mediated defense against inflammation is two-pronged – negative regulation of inflammatory immune cells and elevation of a beta cell-intrinsic defense. To examine whether PTPN2 regulates beta cell loss upon cytotoxic stimuli by bolstering beta cell defense mechanisms *in vivo*, I deleted PTPN2 in the beta cells (Ptpn2 βKO) and subjected the mice to the diabetogenic agent streptozotocin (STZ). Animals deficient in beta cell PTPN2 are more susceptible to STZ-induced diabetes and

have poor survival due to hyperglycemia. While investigating the mechanism of PTPN2-mediated beta cell defense, I have discovered that PTPN2 interacts with pyruvate kinase M2 (PKM2), a key metabolic enzyme that normally resides in the cytosol. In response to STZ, PKM2 translocates to the nuclei of diabetic beta cells, and the lack of PTPN2 results in the hyper-accumulation of nuclear PKM2, suggesting that PTPN2 mediates nuclear export of PKM2 in stressed beta cells. In the nucleus, PKM2 mediates the transcriptional activation of key proapototic genes, which is attenuated when I modulate nuclear PKM2 *ex vivo*, in effect reconstituting the function of PTPN2. Together, deregulation of PTPN2-mediated nuclear export of PKM2 leading to excessive transcriptional activation of proapoptotic genes may be the mechanism for exacerbated diabetes in the Ptpn2 βKO mice.

To identify novel candidates that function in the beta cells to influence beta cell susceptibility to insulitic injury, I established RNA transcriptome and CpG dinucleotide methylome profiles of islets isolated from insulitis-susceptible NOD and insulitis-resistant NOR mice, prior to the onset of insulitis. Integrating these profiles with the genes nested in the human diabetic loci from the genome-wide association studies, I identified several novel candidate genes that may be involved in T1D pathogenesis in a beta cell-specific manner. Moreover, I also examined non-CpG methylation, which appears to influence gene expression independently of CpG methylation.

Collectively, my studies have expanded the understanding of beta cell-specific factors that regulate cellular defense to insulitis and may have expanded the therapeutic possibilities by implicating PKM2, inhibition of which is the focus of many cancer therapy research.

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Dedication

The years of my Ph.D. that led to this dissertation are

dedicated to my parents and my only brother,

for their unconditional love and belief that I am destined to cure diabetes …

… and to the millions of people living with diabetes.

Chapter One: Introduction

Type 1 diabetes

Diabetes mellitus is a class of endocrine disorders characterized by high levels of blood glucose due to a deficiency in or resistance to the key metabolic hormone insulin. Type 1 diabetes (T1D) develops from the autoimmune destruction of insulin producing beta cells in the pancreatic islets, whereas dysfunctional beta cells combined with insulin-resistant peripheral tissues lead to type 2 diabetes (T2D). According to the American Center for Disease Control National Diabetes Statistics Report 2014, 29.1 million people in the United States – an astounding 9.3% of the population – were living with diabetes in 2012, with 1.7 million new cases of diabetes diagnosed within the calendar year. Patients with hyperglycemia are usually diagnosed with diabetes when they present with a triad of classic clinical signs of excessive thirst (polydipsia), frequent urination (polyuria), and increased hunger (polyphagia). Left untreated, diabetes can lead to dangerous health complications such as heart disease, kidney failure, stroke, blindness, and death. Aside from being the seventh leading cause of death in the United States, diabetes poses a significant economic burden, costing the United States economy $245 billion in 2012 (CDC, 2014). As of 2014, the cure for diabetes remains elusive, and it is of great interest to prevent disease by identifying risk factors.

Although it is widely accepted that diabetes can manifest a spectrum of both T1D and T2D characteristics, islet autoantibodies can be detected in about 10% of all diabetes cases, which establishes autoimmune diagnosis. Previously known as juvenile diabetes, T1D is more commonly diagnosed in younger people, but disease initiation can occur at any age in susceptible individuals (ADA, 2014). The main treatment option is lifelong insulin replacement therapy, which requires close medical supervision, and because

peripheral tissues are sensitive to insulin action in T1D, variability in insulin absorption can lead to dangerous episodes of hypoglycemia that can substantially impair the quality of life (Frier, 2008; Pickup, 2012). Recent advances in glucose-responsive insulin (SmartInsulin) and artificial pancreas may mitigate the complications but diabetic patients still require lifelong clinical monitoring. Other treatment approaches employ immune modulators or molecules that protect beta cell function, but successful suppression of the beta cell-specific autoimmunity without rendering the patient immunocompromised remains a challenge. Furthermore, once the immune system is primed against self-antigens on beta cells, beta cell death is progressive and irreversible, and after prolonged disease progression, patients require islet transplantation. Thus, it is desirable to prevent or intervene prior to significant beta cell loss, which may be accomplished by a better understanding of disease etiology.

Current understanding of T1D etiology

The clinical manifestation of T1D is the consequence of sustained autoimmune attack on beta cells and does not occur until a significant portion of beta cell mass has been lost (van Belle et al., 2011). The most well known model of T1D timeline was proposed by Eisenbarth in 1986 (Eisenbarth, 1986). This model proposes that individuals with genetic susceptibility are exposed to variable environmental triggers. In the event that an individual possesses genetic susceptibility to T1D and is exposed to diabetogenic triggers, the combinatorial effect can initiate islet autoantibodies, leading to a linear decline in beta cell mass and progressive hyperglycemia (Fig. 1-1) It has now been established that the length of time between T1D initiation and clinical onset is highly variable. This variability may be due to the degree of genetic susceptibility including

penetrance of certain alleles and the cumulative effect of several susceptibility alleles, as well as the severity of immune response to one or more environmental triggers.

Genetic factors

Even with the same environmental triggers, only the individuals with genetic susceptibility eventually develop T1D. A significant genetic predisposition to T1D is conferred by several insulin-dependent diabetes mellitus (IDDM) loci. First, IDDM1 is the chromosomal region 6p21 commonly known as the human leukocyte antigen (HLA) complex, which contains more than 200 genes and is thought to account for most of the genetic susceptibility to T1D (Concannon et al., 2009; Nerup et al., 1974). The HLA complex is the human version of the major histocompatibility complex (MHC) molecules, which present cellular antigens to the immune cells. Class I molecules (HLA-A, B, and C) are on all nucleated cells and class II molecules (HLA-DP, DQ, DR, etc.) are only present on antigen presenting cells of the immune system. Class I alleles seem to confer a modest but consistent risk for T1D, while individuals carrying the class II DR3/4-DQ2/8 risk haplotype are at an extreme risk, as much as 55% risk of disease by the age of 12 (Aly et al., 2006).

Another well studied locus is the IDDM2 on chromosome 11p15, which contains the *insulin* (*INS*) gene (Bell et al., 1984). The promoter of the *INS* gene contains variable number of tandem repeat polymorphisms, which determines insulin gene expression in the thymus. Shorter repeats correlate with reduced gene expression and the consequent reduction in the elimination of insulin-reactive T cells in the thymus leave these individuals at a higher risk for T1D (Vafiadis et al., 1997).

Other susceptibility genes confer significant comorbidity with a number of autoimmune disorders such as rheumatoid arthritis, autoimmune thyroiditis, and inflammatory bowel disease, because they function in suppressing T cell hyperactivity. For instance, individuals with polymorphisms in the *cytotoxic T-lymphocyte protein 4* (*CTLA4*) gene, which functions in the negative regulation of T cell receptor (TCR) activation, have higher T1D risk because of the reduced expression of soluble CTLA4 (Chikuma et al., 2003; Nistico et al., 1996; Ueda et al., 2003). Additionally, a polymorphism in the *protein tyrosine phosphatase non-receptor type 22* (*PTPN22*) gene, which encodes the lymphoid protein tyrosine phosphatase (LYP), has been shown to be a gain-of-function mutation producing a more catalytically active protein phosphatase, resulting in hypophosphorylation and hypoactivation of the TCR signaling (Vang et al., 2005).

Many of the above mentioned susceptibility genes have been identified through genome-wide association studies (GWAS), which seek to identify relevant small nucleotide polymorphisms (SNPs) that occur more frequently in individuals with disease (Pearson and Manolio, 2008; Wellcome Trust Case Control, 2007). The association between genes and disease is an unbiased approach to efficiently identify novel genetic loci shared by unrelated individuals in complex polygenic diseases such as T1D. However, rigorous candidate-based inquiry into the functional consequences of disease-associated SNPs has been difficult because the SNPs may individually confer mild risk to disease without the complementary contribution of disease-triggering environment and other genetic factors. Furthermore, most of the SNPs reside in noncoding regions and the

resultant variations in gene expression are cell-type specific and mostly unknown in tissues with rare availability such as the pancreatic islets (Ward and Kellis, 2012).

Environment of the organism

Underlying genetic susceptibility leads to clinical manifestation of T1D due to a variety of environmental triggers that can be broadly classified as either infectious or dietary. As early as 1926, seasonal variation has been documented as a factor in T1D onset (Adams, 1926). Later studies have attributed this to seasonal variations in viral infections such as Coxsackie's B4 virus. Other viruses including cytomegalovirus, rubella virus and mumps virus have also been implicated (van Belle et al., 2011). Viral agents may activate autoimmunity through peptides that mimic human autoantigens (molecular mimicry), by priming the diversification of TCR during inflammation (epitope spreading), or through the expression of superantigens that dominantly activate non-specific T cells (Wucherpfennig, 2001). Bacterial imbalance in the gut microbiome may also trigger T1D by affecting gut permeability to infectious agents (Murri et al., 2013; Wen et al., 2008). However, while infection and T1D may coincide, depending on the context, infection can also confer protection from disease, and limiting the exposure to infectious agents can increase disease incidence, commonly referred to as the "hygiene hypothesis" (Bach, 2002; Okada et al., 2010). For instance, children with older siblings at home or children who attend a day-care center have a lower incidence of T1D than children with lesser degree of social mixing, due to increased exposure to infectious agents at an early age (McKinney et al., 2000).

Dietary variations among individuals may also account for variable T1D risk in susceptible individuals. First, early introduction to cow's milk possibly coupled with short duration or lack of breastfeeding increase the risk for T1D. Cow's milk has a higher albumin content, and antibodies to albumin cross react with beta cell surface protein (Karjalainen et al., 1992), and breast milk can be protective due to high content of antimicrobial lactoferrin and immunoglobin A (Patelarou et al., 2012). However, this topic has been rather controversial (Norris et al., 1996), and others have proposed that increased immunity to cow's milk may just be a reflection of impaired mucosal immune function (Harrison and Honeyman, 1999). Additional dietary components such as omega-3 fatty acids (Norris et al., 2007) and gluten (MacFarlane et al., 2003) may also increase autoimmunity to islet antigens. Furthermore, dietary vitamin D can be protective (Mathieu et al., 2005), and gene products that affect vitamin D metabolism can increase T1D risk (Bailey et al., 2007). This may be partly attributed to immunomodulatory effect of vitamin D (D'Ambrosio et al., 1998). Non-dietary sources of vitamin D such as the sunlight is also important, as the highest incidence of T1D occurs in the northernmost hemisphere, in countries like Sweden where the hours of sunshine are limited (van Belle et al., 2011).

Genetic variations may interact directly with environmental stressors, as in the case of *PTPN22* polymorphism and cow's milk exposure (Lempainen et al., 2009). However, even without genetic variations, the environment can produce variable phenotypes by modifying gene expression without directly changing the DNA sequence. One example is in the monozygotic twins. Despite the fact that they have an identical genetic makeup, identical twins do not have the same risk for T1D at a single time point

(Barnett et al., 1981). Although it has been shown that most of the discordant twins of T1D patients eventually progress to T1D with a longer follow up (Redondo et al., 2008), the fact that islet autoantibodies may appear some thirty years later in one identical twin suggests altered gene expression from identical genetics, also known as epigenetics.

Environment of the genome

Epigenetic modifications such as histone modifications and DNA methylation can change chromatin structure and limit DNA accessibility, thereby regulating gene expression. First, gene expression can be altered by the dynamic modifications of histones that change chromatin architecture. Eukaryotic chromatin is composed of nucleosome subunits, which consist of eight molecules of core histone proteins wrapped around by DNA strands. It is well established that the addition of acetyl groups to histone cores at select lysine residues can facilitate transcription factor access by unwinding the condensed chromatin. Conversely, deacetylation can lead to chromatin condensation and gene repression (Grunstein, 1997). Other posttranslational modifications of histones include methylation, phosphorylation, or ubiquitination, and different combination of these modifications, commonly known as the histone code, is linked with specific biological functions including gene expression, gene silencing, and mitosis (Strahl and Allis, 2000). Immune cells from T1D patients show differences in histone modification of T1D gene promoters such as H3K9 methylation in the promoter of *CTLA4* locus (Miao et al., 2008) and H3K9 acetylation in *HLA-DRB1* and *HLA-DQB1* promoters (Miao et al., 2012).

DNA methylation is the addition of a methyl group to cytosine residues preceding guanine, known as CpG dinucleotides. Clusters of CpG dinucleotides, known as CpG islands, can occur in regulatory regions of genes such as promoters. Hypermethylation of CpG islands correlates with transcriptional silencing events such as imprinting and X chromosome inactivation (Bird, 2002). The temporal and causative relationship between transcriptional silencing and CpG methylation is unclear. There is evidence that methylated CpG islands inhibit transcription initiation (Kass et al., 1997; Venolia and Gartler, 1983), but it has also been shown that methylation of *Hprt* gene promoter CpG sites happens after X inactivation, suggesting that DNA methylation may play a secondary role to maintain the repression of transcription (Lock et al., 1987). Nonetheless, DNA methylation is an important posttranslation modification.

Aside from its role in gene silencing, CpG methylation can be used as a biomarker of T1D. One group showed that a subset of immune cells (CD14+ monocytes) from discordant monozygotic twins showed differential methylation patterns in 132 CpG sites, including an HLA gene. Also, differences in these T1D-associated methylation variable sites seem to arise early in the disease process (Rakyan et al., 2011). B cells from discordant twin pairs also show differential CpG methylation patterns, including T1D genes such as *HLA-DOB* and *INS* (Stefan et al., 2014). Another group identified a pattern of 3 hypomethylated CpG sites in the *INS* promoter unique to the blood samples of 14% of T1D patients (Fradin et al., 2012). Hypomethylation within the *INS* gene was also seen in the sera of newly diagnosed T1D patients, and this was attributed to the presence of dead beta cell DNA (Akirav et al., 2011; Husseiny et al., 2014). Thus, DNA methylation marks are present in both the immune cells and targeted beta cells in T1D. However,

much like the relationship between methylation and transcriptional silencing, it is not known whether CpG methylation can initiate T1D pathogenesis, or whether it is a product of T1D disease process. It should be noted that DNA methylation can certainly engender human disease. For example, methylated cytosine can expedite the transition of cytosine to thymine, functioning as an endogenous mutagen, which can be carcinogenic if it occurs in tumor suppressor genes (Rideout et al., 1990). This may be subject to direct environmental influences, such as diets lacking methyl group donors like methionine, which can lead to liver cancer (Cooney et al., 2002; Poirier, 2002). Moreover, our current understanding of DNA methylation is mostly limited to CpG sites, but non-CpG methylation has also been reported in both embryonic and somatic cell types (Guo et al., 2014; Ramsahoye et al., 2000), and it is of interest to see physiological ramifications of non-CpG methylation.

Studies in murine models of T1D

Advancement in the understanding of T1D pathophysiology was set in motion with the discovery of murine models of spontaneous diabetes in the late 1970s and early 1980s. The non-obese diabetic (NOD) mouse strain was discovered during the inbreeding of the cataract-prone mice in Japan (Makino et al., 1980), and outbred colonies of the Wistar rat at the Bio-Breeding (BB) Labs in Canada displayed spontaneous diabetes (Nakhooda et al., 1977). The NOD mouse in particular, displayed many similarities with the human T1D including genetic and environmental involvement, and has become the main model of T1D (Joost et al., 2012).

The NOD mouse model has been invaluable in identifying key pathological features of the human disease, but many similarities and differences exist. Autoimmune diabetes in the NOD mouse is spontaneous and progresses in stages, beginning with immune cells being attracted to the pancreatic islets around 4-8 weeks, infiltrating and destroying beta cells starting at 12 weeks, eventually leading to overt diabetes by 16-24 weeks (Fig. 1-2) (Anderson and Bluestone, 2005). This stage-to-stage progression of worsening inflammation and heightened immune response is different from humans in which disease process is more chronic and subtle. Diabetes incidence in the NOD mouse is highly variable and depends on the pathogen levels of the environment, which may be similar to the human "hygene hypothesis" (Ohsugi and Kurosawa, 1994). It is also sexually dimorphic as most of the female mice develop diabetes by 30-40 weeks, but the incidence in males is significantly lower and it has been established that this is due to differing gut microbiome colonization that is androgen-dependent (Fox, 1992; Markle et al., 2013). This is different from humans, where incidence is not dependent on gender.

The advantage of using murine models of disease is that many therapeutic approaches may be assessed for safety and efficacy, which is required prior to initiating human trials, and that genetics can be utilized to better understand the molecular mechanisms of disease. T1D has been successfully prevented, delayed, or reversed in the NOD mice in countless ways (Ablamunits et al., 2012; Emamaullee et al., 2009; Fiorina et al., 2008; Grinberg-Bleyer et al., 2010; Jin et al., 2012; Perone et al., 2009; Tian et al., 2009; Vergani et al., 2010; Yi et al., 2012; Zhao et al., 2009). However, this has not been attainable in humans, possible due to underlying differences in humans and mice.

Aside from models of spontaneous diabetes, chemicals such as streptozotocin (STZ) or alloxan can rapidly induce beta cell death. STZ is an antibiotic from *Streptomyces achromogenes* that can selectively enter beta cells via the GLUT2 glucose transporter utilizing its glucose side chain (Fig. 1-3) (Schnedl et al., 1994; Vavra et al., 1959). Inside the beta cell, STZ can alkylate DNA using its methylnitrosourea moiety, leading to DNA fragmentation and the generation of nitric oxide (NO), and genotoxic beta cell death ensues (Lenzen, 2008; Yamamoto et al., 1981). Furthermore, STZ is an analogue of N-acetylglucosamine, which mimics and competes with the O-linked beta-N-acetylglucosamine (O-GlcNAc) modifications of cellular proteins for the beta-N-acetylglucosaminidase (O-GlcNAcase), an enzyme that cleaves O-GlcNAc (Konrad et al., 2001; Roos et al., 1998). Beta cells express unusually high levels of O-GlcNAc modifications, and transgenic mice with impaired levels of O-GlcNAc are protected from STZ-induced diabetes (Liu et al., 2000), but this has been controversial because increases in O-GlcNAc levels due to O-GlcNAcase inhibitors did not result in beta cell damage (Gao et al., 2000). In either case, STZ proves to be a potent beta cell toxin, valuable in the study of beta cell death in diabetes.

STZ can be used at different doses to induce diabetes. At low doses, multiple sequential daily injections over several days are required to achieve slow-onset modest hyperglycemia within two weeks that involves cellular immune response and the induction of apoptosis (McEvoy et al., 1984; O'Brien et al., 1996). At higher doses of STZ, a single injection can rapidly achieve significant hyperglycemia in 48 hours through the induction of cell death without involving the immune system (Deeds et al., 2011; Junod et al., 1969). The exact mode of STZ induced beta cell death without the immune

system, at least in culture, seems to be primarily via the activation of apoptosis in short-term low doses, and a combination of necrosis (22%) and apoptosis (17%) at higher doses (Morgan et al., 1994; Saini et al., 1996). Precise *in vivo* mode of STZ is most likely a combination of apoptosis and necrosis. One study used Annexin V as an early marker of apoptosis and determined that 48 hours after STZ-administration, there is significant apoptosis in both low dose (2%) and high dose (26%) models of diabetes *in vivo* (Medarova et al., 2005).

At either dose, STZ-induced diabetes is highly dependent on mouse strain background and gender (Deeds et al., 2011; Leiter, 1982). In contrast to the NOD model, where female mice are more prone to diabetes, in the STZ model, estrogens protect beta cells from apoptosis, in both low and high doses (Le May et al., 2006). Other factors such as circadian rhythm and diet, as well as methods of STZ preparation and administration, can be attributed to highly variable results in the onset and severity of diabetes (Deeds et al., 2011). Despite this, STZ is an invaluable tool to study diabetes without the need to wait for the onset of spontaneous diabetes.

A number of other mouse models of diabetes have been employed and are reviewed elsewhere (Van Belle et al., 2009). Given the complexity of T1D and the differences in humans and mice, no single mouse model can emulate the human T1D with perfection. However, the use of mouse models is efficacious and indispensable toward a better understanding of the molecular mechanisms and a cure for T1D.

Beta cell death in type 1 diabetes

In T1D, pancreatic beta cells are assaulted over the course of years by the autoimmune machinery after which beta cells start to die. Central to the rationale behind my work is the idea that T1D results from the failure of beta cells to mount sufficient defense mechanisms in response to the immune attack. Certain beta cells may be more prone to death even with the same degree of immune stimuli, in that beta cells themselves have a defense mechanism that is variably influenced by various T1D-triggering genes and environment. The fate of beta cell survival or death may be a choice implemented by the beta cells themselves, assisted by immune agents. To better understand beta cell response to the immune system, it is important to outline some of the immune events in T1D pathogenesis.

Initiation of immune events can be primed by the release of beta cell antigens upon beta cell death, which can be a result of a viral event or a sporadic physiological event. These antigens circulate and are taken up by antigen presenting cells to be presented to the T cells. Normally during immune cell development, T cells that recognize "self" beta cell antigens are effectively eliminated in the thymus by a process called negative selection. This is known as immune tolerance. However, when self-antigen recognizing T cells manage to escape thymic negative selection, abnormal recognition of self-antigens as deleterious foreign particles leads to an activation of the autoimmune machinery (Calderon and Unanue, 2012).

Decreased tolerance to beta cell antigens leads to the inflammation of the pancreatic islets, known as insulitis, first observed in 1965 (Gepts, 1965). Two of the key immune cells comprising the insulitic lesions include activated macrophages and T

lymphocytes that can produce a number of inflammatory cytokines such as interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), as well as cytotoxic free radicals (von Herrath, 2009). The inflammatory cytokines can further activate macrophages and attract T cells, becoming a positive feedback loop that further propagates the inflammatory response. Aside from cytokine production, T cells can directly participate in the extrinsic pathway of beta cell death by producing the Fas ligand (also known as CD95 or FasL) and the TNF related apoptosis inducing ligand (TRAIL), which can lead to caspase activation and cell death independently of intracellular cell death programming. Furthermore, activation of the TCR can lead to the release of toxic granules containing perforin and granzyme. Perforin perforates the target cells by opening a pore in the cell membrane, which allows for the granzyme to enter the cytosol, where it can activate cell death in synergy with the inflammatory cytokines via the intrinsic pathway, also known as mitochondrial pathway of cell death. This involves a number of other factors such as the endoplasmic reticulum (ER) stress and the intracellular balance of B-cell lymphoma 2 (BCL-2) family proteins that control the permeability of mitochondrial membrane. Imbalance in the BCL-2 proteins can lead to the release of cytochrome c from the mitochondria to the cytoplasm, which leads to the activation of programmed cell death (Delaney et al., 1997; Eizirik and Mandrup-Poulsen, 2001).

The exposure of the beta cells to a combination of pro-inflammatory cytokines leads to dramatic changes in gene expression in human and murine beta cells (Cardozo et al., 2001a; Eizirik et al., 2012; Kutlu et al., 2003). Genes that are upregulated include stress response genes that can be either protective or deleterious for beta cell survival.

Furthermore, different genes that function in the same category of stress response can be upregulated and downregulated, adding to the complexity of the beta cell response. For example, defense repair genes encoding clusters of defense enzymes including metallothionein or superoxide dismutase were induced, but glutathione peroxidase genes were downregulated in cytokine treated beta cells (Cardozo et al., 2001a; Cardozo et al., 2001b).

One of the main transcription factors that are activated in response to IL-1β is nuclear factor kappa B (NF-κB). Suppressing the activation of NF-κB through the inhibitory factor kappa B (IκB) can prevent cytokine-induced apoptosis (Giannoukakis et al., 2000; Heimberg et al., 2001). NF-κB can induce the expression of stress genes such as *CCAAT/enhancer binding protein (C/EBP) homologous protein* (*Ddit3* or *CHOP*) that can induce ER stress, and at the same time downregulate genes critical for beta cell function and the maintenance of differentiated beta cell characteristics, such as *pancreatic and duodenal homeobox 1* (*Pdx1*). The effect of NF-κB can be through a direct transcriptional activation or secondary to the expression of the *inducible nitric oxide synthase* (*iNOS*) gene, which generates cytotoxic NO in the islets (Darville and Eizirik, 1998). Cytokine-induced NO generation in the beta cells requires the activation of protein kinases including p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) (Larsen et al., 1998; Nishiki et al., 2013), and NO accumulation leads to an upregulation of the tumor suppressor protein p53, which is toxic to beta cell survival (Hoshino et al., 2014).

Gene expression changes in response to cytokines can also occur through the activation of receptor tyrosine kinase signaling. IFN-γ can bind to and activate janus

kinase 1 and 2 (JAK1/2) on the beta cell surface, and downstream of this, signal transducer and activator of transcription 1 (STAT1) becomes phosphorylated. Phosphorylated STAT1 can dimerize and migrate to the nucleus, where it can induce transcription of various genes, including *iNOS* (Eizirik and Mandrup-Poulsen, 2001). STAT1 upregulates the expression of a pro-apoptotic gene *death protein 5* (*Dp5*, also known as *activator of apoptosis harakiri* or *Hrk*), and knockdown of STAT1 protected rat beta cells from cytokine-induced apoptosis (Moore et al., 2011). This was accompanied by the retention of key beta cell gene expression including *Pdx1* and *Ins*, suggesting that STAT1 is perhaps the master regulator of beta cell apoptosis. In response to IFN-γ and TNF-α, STAT1 also upregulated *BCL-2-interacting mediator of apoptosis* (*Bim*) expression to mediate apoptosis, and silencing *Bim* protected against apoptosis (Barthson et al., 2011).

Induction of stress-activated genes in response to cytokines is followed by the ER stress response, which depletes the calcium stores in the ER (Cardozo et al., 2005; Chambers et al., 2008). Disruption of calcium homeostasis as well as the accumulation of the unfolded proteins leads to a sequence of compensatory mechanisms to restore the ER balance, which includes decreasing protein translation and degradation of unfolded proteins. Unmitigated ER stress leads to apoptosis. Cytokines can lead to ER stress via the generation of NO (Oyadomari et al., 2001) and the upregulation of p53 that can induce *p53 upregulated modulator of apoptosis* (*PUMA*) (Gurzov et al., 2010; Hoshino et al., 2014).

Key proteins that are upregulated in response to STAT1 signaling or ER stress including DP5, BIM, and PUMA, belong to a special class of the BCL-2 family

members, known as the BCL-2 homology 3 (BH3)-only proteins. Other members of the BCL-2 family contain multiple BCL-2 homology domains and can be classified as pro-survival or pro-apoptotic (Fig. 1-4). In response to apoptotic stressors, BH3-only proteins can be upregulated or activated by posttranslational modifications, and they can facilitate apoptosis by inhibiting pro-survival molecules like BCL-2, thereby freeing the pro-apoptotic molecules such as BCL-2-associated X protein (BAX) and BCL-2 antagonist killer (BAK) to form oligomers on the outer membrane of the mitochondria, leading to mitochondrial permeabilization and cytochrome c release. BH3-only molecules can also interact directly with BAX, leading to conformational changes that effect apoptosis (Czabotar et al., 2014; Lomonosova and Chinnadurai, 2008).

Together with inflammatory cytokines and cytotoxic perforin and granzymes, the complex machinery of beta cell apoptosis can be initiated, but beta cells themselves may be more prone to injury compared to other cell types, which may be a factor in lowering the threshold of apoptosis initiation. First, beta cells express unusually low levels of antioxidant enzymes such as glutathione peroxidase (~5% of liver cells), which may suggest compromised defense mechanisms (Tiedge et al., 1997). Beta cells also have a high rate of protein turnover and protein synthesis, being a secretory cell type, and may be more susceptible to ER stress (Harding et al., 2001). In fact, in the NOD mouse model, ER stress precedes significant immune attack suggesting that beta cells are innately more predisposed to stress (Tersey et al., 2012). Lastly, many candidate genes from the human T1D GWA studies have been widely studied in the context of immune signaling, but it is likely that these genes play a role in the beta cells themselves. In support of this, the majority of the candidate genes are expressed in the human islets, and the expression of

these genes are further induced with pro-inflammatory cytokines, suggesting that beta cells may contribute to their own demise (Eizirik et al., 2012).

Type 1 diabetes candidate gene *PTPN2*

Overview

One of the T1D candidate genes identified by GWAS is *protein tyrosine phosphatase non-receptor type 2* (*PTPN2,* also known as *T cell protein tyrosine phosphatase,* or *TC-PTP*), which encodes a member of the protein tyrosine phosphatase (PTP) superfamily (Todd et al., 2007). The superfamily of PTPs defines intracellular phosphotyrosine patterning in concert with protein tyrosine kinases (PTKs), and an imbalance in the PTP-ome can lead to devastating biological outcomes such as diabetes, cancer, autism, myopathy, and epilepsy (Alonso et al., 2004). In the human genome, there are 107 PTPs with the same active site motif HCX_5R but of varying substrate specificity dictated by the catalytic domain architecture, subcellular localization, and posttranlastional modifications. Of these, subcellular localization is in part determined by protein sequence: in addition to the PTP domain, receptor type PTPs contain extracellular and transmembrane domains, whereas the non-receptor type PTPs contain terminal localization domains that target the PTP to specific subcellular locations.

PTPN2 is one of the two non-transmembrane non-receptor type classical PTPs, along with PTPN1, encoded by *protein tyrosine phosphatase non-receptor type 1* (*PTPN1*, also known as *PTP1B*) (Cool et al., 1989; Tonks et al., 1988). PTPN2 has two isoforms: the major isoform TC45 (45 kDa) which has a nuclear localization signal, and the minor isoform TC48 (48 kDa) which is tethered to the endoplasmic reticulum

(Soulsby and Bennett, 2009). The substrates of nuclear TC45 include janus kinases (JAK) 1 and 3 (Simoncic et al., 2002), and signal transducer and activator of transcription (STAT) 1 and 3 (ten Hoeve et al., 2002; Yamamoto et al., 2002). Upon specific stimulation, nuclear TC45 can exit the nucleus (Fig. 1-5) (Lam et al., 2001) and dephosphorylate non-nuclear substrates such as epidermal growth factor receptor (EGFR) (Tiganis et al., 1998) and insulin receptor (IR) (Galic et al., 2003), which are also targeted by the less abundant TC48.

PTPN1 has a single isoform that is targeted to the endoplasmic reticulum (Frangioni et al., 1992). There is a partial overlap between substrates of PTPN1 and PTPN2 including STAT3 (Lund et al., 2005), EGFR (Flint et al., 1997), and IR (Kenner et al., 1996), which can be attributed to the 74% sequence identity in the catalytic domains of PTPN1 and PTPN2 (Iversen et al., 2002). However, PTPN1/PTPN2 chimera, where the PTPN2 catalytic domain was fused to the localization domain of PTPN1, does not interact with the same substrates as PTPN1, suggesting unique substrate specificity of the PTP domains despite the sequence similarities (Tiganis et al., 1998).

Lessons from the PTPN2 knockout mouse studies

Distinct substrate specificity and differential tissue expression patterns in the two closely related PTPs are highlighted by different phenotypes associated with the knockout mice. Whole body *Ptpn1* knockout mice are healthy and show increased insulin sensitivity and resistance to obesity by regulating insulin receptor signaling in muscle and liver (Elchebly et al., 1999; Klaman et al., 2000), indicating that *Ptpn1* could be an attractive metabolic target for treating obesity and T2D (Moller et al., 2000). In contrast,

whole body *Ptpn2* knockout mice die by 5 weeks after birth due to defects in hematopoiesis and significant induction of systemic inflammation (Heinonen et al., 2004; You-Ten et al., 1997). Due to this early lethality of the *Ptpn2* null mice, a number of conditional null mice have been generated to study the *in vivo* functions of PTPN2 in different tissues.

To study the role of PTPN2 in insulin signaling, mice with *Ptpn2* deletion in the key organs of insulin action (muscle, liver, or bone) have been generated. The muscle-specific knockout of *Ptpn2* has no effect on insulin response and glucose homeostasis, even upon high fat diet (HFD) challenge, suggesting muscle PTPN2 is dispensable for the regulation of insulin receptor signaling (Loh et al., 2012). Liver-specific *Ptpn2* conditional deletion led to increased obesity and insulin resistance through hyperactivation of insulin-mediated STAT5 signaling (Gurzov et al., 2014). Moreover, the activation of STAT5 signaling with HFD led to the inactivation of PTPN2 by the oxidation of the PTPN2 catalytic site, creating a *de facto* liver-specific *Ptpn2* deletion in the control mice without the conditional allele, further compounding obesity and insulin resistance. It has also been shown that PTPN2 maintains whole body energy metabolism through the negative regulation of insulin receptor signaling in osteoblasts. Osteoblasts secrete the hormone osteocalcin that leads to insulin secretion and increased whole-body insulin sensitivity (Ferron et al., 2008). In turn, insulin signaling in osteoblasts leads to increased osteocalcin activity, further promoting insulin secretion and increased bone resorption by osteoclasts (Ferron et al., 2010). Therefore, PTPN2 functions as a negative regulator of insulin signaling in osteoblasts, and osteoblast-specific *Ptpn2* conditional

deletion mice exhibit increased osteocalcin activity, bone resorption, and insulin sensitivity (Zee et al., 2012).

Energy homeostasis is governed not only by insulin from the beta cells but also by leptin from the adipocytes (Halaas et al., 1995). In addition to its role in insulin signaling, PTPN2 has also been implicated as a negative regulator of leptin signaling. Hypothalamus-specific *Ptpn2* deletion hyperactivates STAT3, a signal transducer of leptin, and this leads to increased leptin signaling manifested by decreased food intake and energy expenditure, along with increased insulin sensitivity and glucose tolerance (Loh et al., 2011).

Beyond the role as a regulator of insulin signaling and leptin signaling, PTPN2 has crucial functions in T cells as a modulator of inflammatory signaling and immune proliferation. Indeed, deletion of PTPN2 can lead to a cancer of T cells known as T cell acute lymphoblastic leukemia (T-ALL) due to unchecked immunoproliferation (Kleppe et al., 2010; Kleppe et al., 2011a). To assess the molecular function of PTPN2 in T cells, T cell specific *Ptpn2* null mice were generated. In these mice, even with low-affinity antigens, PTPN2-deficient T cells show significant proliferation, and this lower threshold of activation and proliferation is mediated by enhanced TCR signaling. This leads to significant inflammation and autoimmunity in aged T cell *Ptpn2* null mice, altogether suggesting that PTPN2 is indispensable for maintaining a threshold of activation and proliferation to preclude self-antigen recognition (Wiede et al., 2014; Wiede et al., 2011). Accordingly, human *PTPN2* has been associated with a number of autoimmune disorders including T1D (Todd et al., 2007), Crohn's and celiac disease (Festen et al., 2011), and rheumatoid arthritis (Wellcome Trust Case Control, 2007).

While it is clear that PTPN2 plays a role in suppressing inflammation, the fact that whole body *Ptpn2* null mice experience much more pronounced systemic inflammation and earlier lethality than the T cell conditional null mice points to a compelling piece of evidence that PTPN2 in cell types other than T cells may contribute to suppress inflammation. It is possible that PTPN2 may function in other immune cell types such as dendritic cells or macrophages, but it is also likely that PTPN2 may modulate responses to the immune-mediated attack in cell types that are targeted for destruction, such as the pancreatic beta cells.

Beta cell PTPN2

As a T1D candidate gene, how *PTPN2* affects beta cell survival upon apoptosis signaling has received considerable attention. It has been demonstrated that levels of PTPN2 were increased in human islets, primary rat beta cells and rat INS-1E cells upon treatment with cytokines IL-1β and IFN-γ or synthetic double stranded RNA, indicating that pro-inflammatory processes upregulate PTPN2. Furthermore, cytokine stimulation induced the redistribution of exclusively nuclear PTPN2 to become both nuclear and cytoplasmic, suggesting that nuclear exit of PTPN2 may be important upon death signaling (Colli et al., 2010; Moore et al., 2009).

To assess the mechanism of PTPN2 in the beta cells, *Ptpn2* was transiently inhibited in the INS-1E cells using small interfering RNAs (siRNAs) in the presence of cytokines and this resulted in an increased percentage of apoptotic cells and hyperphosphorylated STAT1 and STAT3. Double knockdown of PTPN2 and STAT1 abolished this increase in apoptosis, supporting the notion that PTPN2 acts as a cytokine-

responsive beta cell defense mechanism by dephosphorylating and deactivating STAT1 (Moore et al., 2009). It remains unanswered whether the nucleocytoplasmic redistribution of PTPN2 works cooperatively or antagonistically with the beta cell defense mechanism.

Another avenue in which PTPN2 regulates beta cell survival has shown to be through mitigating the mitochondrial pathway of apoptosis. Because silencing PTPN2 heightened apoptosis signaling, Santin and colleagues examined the readouts of ER stress response and the mitochondrial death pathway. Genes induced by ER stress such as *CHOP*, *immunoglobulin heavy chain-binding protein* (*Bip*), and *X-box binding protein 1 spliced* (*Xbp-1s*) were not changed in cytokine treated PTPN2 knockdown beta cells. In contrast, mitochondrial death pathway was significantly activated in beta cells with cytokine stimulation and PTPN2 inhibition, as demonstrated by focal translocation of BAX to the mitochondria, cytochrome c release to the cytoplasm, and activation of caspase 3. Activation of the intrinsic death pathway seems to be a result of the hyperactivation of C-Jun N-terminal kinase 1 (JNK1) in the absence of PTPN2 that leads to the hyperphosphorylation of the BH3-only pro-apoptotic protein BIM at serine 65, and the siRNA-mediated silencing of *Jnk1* or *Bim* transcripts protected against cytokine-induced apoptosis even in the absence of PTPN2 (Santin et al., 2011).

Beta cell PTPN2 also participates in a crosstalk with another T1D candidate protein basic leucine zipper transcription factor (BACH2) to modulate apoptosis. In the study by Marroqui et al, BACH2 silencing also led to beta cell death via the hyperactivation of JNK1 and the mitochondrial pathway, and overexpressing BACH2 was protective (Marroqui et al., 2014). BACH2-inhibited cells were unable to increase PTPN2 levels in response to pro-inflammatory cytokines, and the authors concluded that

BACH2 is required to upregulate PTPN2 to ensure beta cell survival in response to apoptotic stimuli.

Until recently, PTPN2 function in beta cell survival had only been shown *in vitro*. While cultured islets and beta cells are invaluable vehicles for studying the molecular mechanisms of diabetes, cell culture conditions may not recapitulate the complex *in vivo* environment of the pancreatic islets. Crucial growth factors and hormonal signals, as well as cytokines, may be missing, insufficient, or excessive, in the culture medium supplemented with fetal bovine serum. Moreover, stress-specific signaling may not be adequately modeled with the addition of cytokines *in vitro*. Therefore, it was necessary to model PTPN2 loss in beta cells *in vivo* by utilizing a genetic approach, which was the rationale behind my project design.

As I was nearing the end of my project characterizing the *in vivo* requirement of beta cell PTPN2, two studies emerged to show that PTPN2 functions in pancreatic cell survival for both exocrine (Bettaieb et al., 2014) and endocrine cells (Xi et al., 2014) *in vivo*. These studies were conducted with whole-pancreas knockout model of PTPN2 generated by crossing the Ptpn2-floxed mice with mice carrying the *Cre* allele driven by the *Pdx1* promoter (referred to as panc-TCPTP KO in the papers). The panc-TCPTP KO mice had no gross phenotype and survived to adulthood. Upon treatment with the acinar cell toxin cerulein, in a chemical model of acute pancreatitis, PTPN2 was increased (Bettaieb et al., 2014). Cerulein treatment of panc-TCPTP KO mice led to a hyperactivation of STAT3 phosphorylation, amelioration of acinar cell apoptosis, and blunting of NF-κB inflammatory response, as well as ER stress, suggesting that PTPN2 mediates apoptosis in acinar cells. This is in stark contrast to the *in vitro* beta cell model

of PTPN2 function in decreasing the cellular inflammatory response and apoptosis (Moore et al., 2009; Santin et al., 2011).

The panc-TCPTP KO mice were also used to study the role of PTPN2 in beta cells (Xi et al., 2014). After the HFD challenge, the panc-TCPTP KO mice exhibited significant glucose intolerance and defects in glucose-stimulated insulin secretion (GSIS). This defect in GSIS was rescued *in vitro* by the reconstitution of PTPN2. The authors tested previously known substrates of PTPN2, STAT1 and STAT3, and concluded that STAT1 and STAT3 are bona fide dephosphorylation targets of beta cell PTPN2, and that panc-TCPTP KO mice have defects in beta function because of downstream effects of STAT3 hyperphosphorylation in the absence of PTPN2. In my study, I will show that beta cell PTPN2 is indeed critical for beta cell survival using an experimental model of murine beta cell loss in beta cell-specific knockout mice. Furthermore, I have discovered a novel mechanism of PTPN2-mediated beta cell survival, which may be relevant to T1D.

Mediator of cell survival and death PKM2

Overview

Cell survival is highly dependent on obtaining energy stored in various nutrients. Energy generation involves the catabolism of nutrients such as glucose into units of energy known as adenosine triophosphate (ATP). During the process of glycolysis, intracellular glucose is converted to pyruvate in the cytoplasm, producing two molecules of ATP and reduced nicotinamide adenine dinucleotide (NADH). Then, depending on the availability of the electron accepting agent oxygen, 34 more ATP molecules can be

generated from breaking down pyruvate and oxidizing electron carriers such as NADH through mitochondrial reactions of citric acid cycle and oxidative phosphorylation. In the absence of oxygen, no further energy is generated, and pyruvate is converted into lactic acid, a cellular waste product that is readily shuttled out, during which NADH is converted to the oxidized form (NAD+) for reuse in subsequent glycolysis (Vander Heiden et al., 2009). Even with a relatively low yield (only two molecules of ATP), glycolysis is an important energy producing machinery, and the final step of glycolysis generates pyruvate and ATP, catalyzed by the enzyme pyruvate kinase (PK) (Fig. 1-6).

Four isoforms of PK exist in mammalian cells encoded by two genes *Pklr* and *Pkm*. In the case of *Pklr* gene, tissue-specific promoters drive the expression of either the liver-type *Pkl* transcript (in liver, kidneys, and the intestine) or the red blood cell-type *Pkr* transcript (in red blood cells) (Noguchi et al., 1987). On the other hand, the muscle-type *Pkm* gene can produce two isoforms *M1* and *M2* by alternative splicing of exons 9 and 10 (Clower et al., 2010; David et al., 2010; Noguchi et al., 1986). The *M1* isoform is expressed in most differentiated tissues including brain and muscle, whereas the *M2* isoform is expressed in cell types with a high nutrient flux such as fetal cells and cancer cells (Christofk et al., 2008a; Netzker et al., 1992).

Understanding the functions of PKM2 through cancer

In cases of rapidly proliferating cells, high rate of nutrient uptake must be balanced by a comparable rate of utilization, and despite the lower yield of ATP, glycolysis is much more rapid than oxidative phosphorylation (Pfeiffer et al., 2001). Thus, the surplus of glucose is broken down by a high rate of glycolysis, which becomes

the primary method of energy production in cancer cells (DeBerardinis et al., 2008; Warburg, 1956). This shift toward an unusually high rate of glycolysis followed by lactic acid fermentation in cancer cells is known as the Warburg effect, and is accompanied by the preferential expression of the M2 isoform of PK. Compared to PKM1 expressing cells, PKM2 expressing cells consumed less oxygen, produced more lactate, and were not as sensitive to mitochondrial ATP synthase inhibitor oligomycin, all indicative of less oxidative phosphorylation in favor of glycolysis (Christofk et al., 2008a). Highlighted as the master regulator of cancer cell survival, PKM2 has received significant attention as the pharmacological target for cancer therapy (Mazurek, 2011).

Selective pressure for PKM2 in highly glycolytic cells seems to be stemming from the versatility of PKM2 rather than the enzymatic activity *per se*, because the PKM2 enzymatic activity is in fact much lower than that of PKM1 due to structural differences. Differing by only 22 amino acids, PKM1 exists constitutively as a high-activity tetramer, whereas PKM2 exists in equilibrium of low-activity forms of monomer, dimer, and T-state tetramer, as well as the high-activity R-state tetramer, which is dynamically regulated by structural changes caused by posttranslational modifications and allosteric binding of various intracellular metabolites (Morgan et al., 2013). In cancer cells, PKM2 exists primarily as the low-activity dimer, and the induction of high-activity tetramer configuration by the binding of upstream glycolytic intermediate fructose-1,6-bisphosphate (FBP) can inhibit tumorigenesis (Anastasiou et al., 2012; Ashizawa et al., 1991). FBP-mediated activation is inhibited by the phosphorylation of tyrosine residues in PKM2 that favors dimer conformation of PKM2 in cancer cells (Hitosugi et al., 2009; Presek et al., 1988). Furthermore, intracellular reactive oxygen species (ROS)

accumulation is characteristic of tumor growth (Ishikawa et al., 2008; Weinberg et al., 2010), and ROS inhibits the active form of PKM2 by oxidation at cysteine 358, which is critical for cancer cell survival, as replacing this cysteine with oxidation resistant serine residue resulted in increased sensitivity to oxidative stress and decreased survival (Anastasiou et al., 2011). An idea that is gaining momentum in recent years is that PKM2 may be important for cancer cell metabolism and survival because of physiological implications from its nonglycolytic functions.

PKM2 as a regulator of transcription

Many enzymes have evolved to be multifunctional, because functional plasticity could confer energy saving advantages to a cell (Jeffery, 1999). The earliest observation that PKM2 has nonglycolytic functions was in 2003, when Ignacak and colleagues discovered that nuclear PKM2 from hepatoma cells had histone H1 kinase activity (Ignacak and Stachurska, 2003). More recently, several major studies have shown that PKM2 can act as a protein kinase or a transcription factor cofactor to affect changes in gene expression.

The role of PKM2 as a transcriptional regulator is dependent on its nuclear availability. Nuclear translocation of PKM2 can be induced with specific proliferative stimuli such as interleukin 3 (Hoshino et al., 2007), as well as apoptotic stimuli such as hydrogen peroxide (Stetak et al., 2007). Transcriptional differences between cells with nuclear PKM2 induced by proliferative or apoptotic stimuli have not been defined since PKM2 as a transcriptional regulator has been characterized primarily in the context of proliferative tumor cell types.

PKM2 has been shown to directly bind phosphorylated tyrosine residues (Christofk et al., 2008b), and nuclear PKM2 can regulate gene transcription through tyrosine kinase activity. Dimeric PKM2 can phosphorylate Y705 of STAT3 in the nucleus to activate proliferative genes such as *mitogen-activated protein kinase kinase 5* (*Mek5*) (Gao et al., 2012). Inhibiting tetramer formation by mutating the positive charge of R399 residue resulted in mostly dimeric PKM2 with higher protein kinase activity, and implanting the mutant PKM2-overexpressing cells into nude mice resulted in bigger and more proliferative tumor growth than the wildtype PKM2. Another study showed that EGF transactivates β-catenin through PKM2-mediated phosphorylation of β-catenin Y333 residue, culminating in the transcription of *cyclin d1* (*Ccnd1*) and *v-myc avian myelocytomatosis viral oncogene homolog* (*Myc*) (Yang et al., 2011). Kinase activity of PKM2 may not be limited to tyrosine residues, as PKM2 can also phosphorylate threonine 11 of histone H3 to promote *Ccnd1* and *Myc* gene transcription (Yang et al., 2012a).

PKM2-mediated transcription of certain genes may contribute to PKM2 expression in a positive feedback mechanism. For instance, MYC upregulates the transcription of three heterogeneous nuclear ribonucleoproteins (hnRNP), protein products of which facilitate *Pkm2* alternative splicing from the *Pkm* locus (Clower et al., 2010; David et al., 2010). Furthermore, MYC may promote the Warburg effect by upregulating genes involved in glycolysis and lactate fermentation such as *glucose transporter 1* (*Glut1* or *Slc2a1*) and *lactate dehydrogenase A* (*Ldha*) (Osthus et al., 2000; Shim et al., 1997). Both *Glut1* and *Ldha* belong to a class of genes whose transcription is controlled by the transcription factor hypoxia-inducible factor 1 alpha (HIF-1α) (Denko,

2008). In fact, PKM2 can directly activate HIF-1α target gene transcription by acting as a cofactor (Luo et al., 2011). Furthermore, *Pkm* gene bears an intronic hypoxia response element (HRE), which leads to HIF-1α-mediated upregulation of *Pkm* expression, which may compound the positive feedback loop of HIF-1α/PKM2 induced gene transcription (summarized in Fig. 1-7).

PKM2 in beta cells

In addition to proliferating cancer or fetal tissues, PKM2 is also expressed in pancreatic islets (Chatterton et al., 1982; MacDonald and Chang, 1985). Adult islet cells are not rapidly proliferating and it is unknown why PKM2 is favored over PKM1 in the islets. It is likely that rapid uptake of glucose is important for the function of glucose sensing and insulin release from the beta cells, which could result in nutrient excess, not unlike in cancer cells. However, whereas cancer metabolism has found a way to thrive with through PKM2, it is unknown whether ROS inhibits or activates PKM2 in islets. Furthermore, beta cells are much more susceptible to injury from increases in ROS compared to other cell types, and thus, in the context of islet beta cells, PKM2 may act to promote cell death as opposed to cell survival, with ROS acting as a metabolic switch.

In my thesis work, I have addressed the function of PKM2 in beta cells that have been exposed to pro-apoptotic stimuli. I show that PKM2 interacts with T1D candidate PTPN2 in the beta cells, and that this interaction is critical for effective defense mechanism of the beta cells, as the beta cell-specific loss of PTPN2 results in exacerbated diabetes and beta cell loss. PKM2-mediated cell death mechanism appears to be through the transcriptional activation of pro-apoptotic transcripts that are regulated by HIF-

1α/PKM2 complex, and facilitating the nuclear export of PKM2 using a small molecule activator attenuates the transcription of pro-apoptotic transcripts. Furthermore, I have discovered that in the NOD genetic background, beta cell *Pkm2* levels may predispose the islets to insulitis-sensitivity or resistance. Thus, while investigating mechanisms of beta cell-intrinsic susceptibility to T1D, my studies have broadened the understanding of beta cell defense mechanisms and the therapeutic possibilities for T1D by implicating a key cancer molecule PKM2.

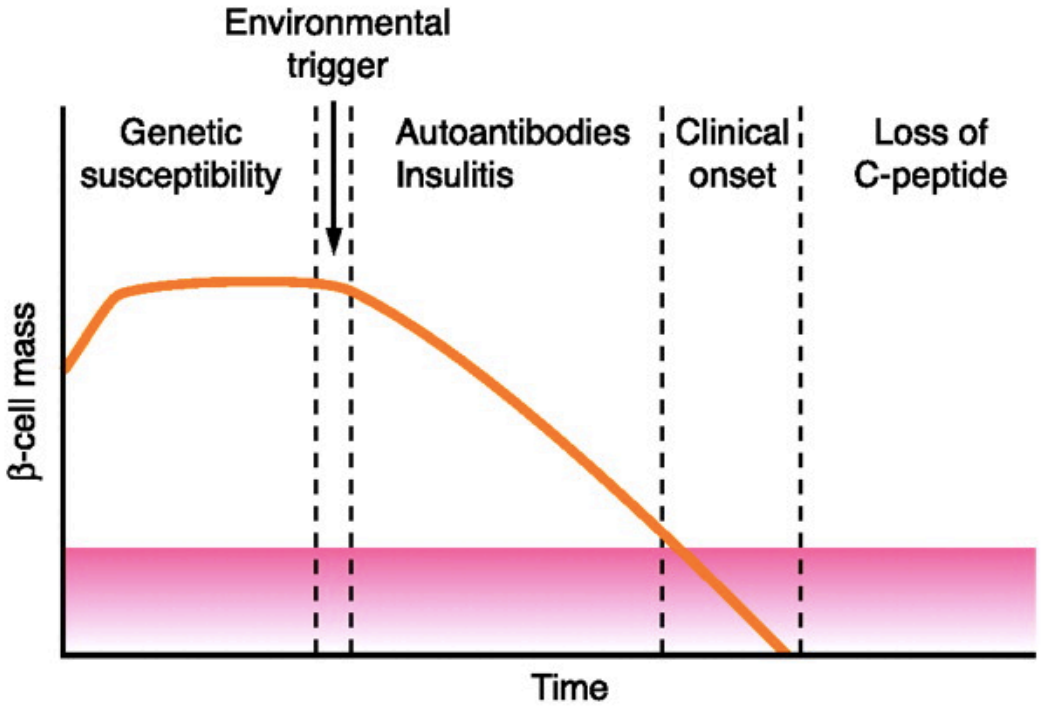


Figure 1-1. Natural history of T1D.

A model of linear beta cell mass decay postulated by Eisenbarth in 1986. Figure adapted from (van Belle et al., 2011).

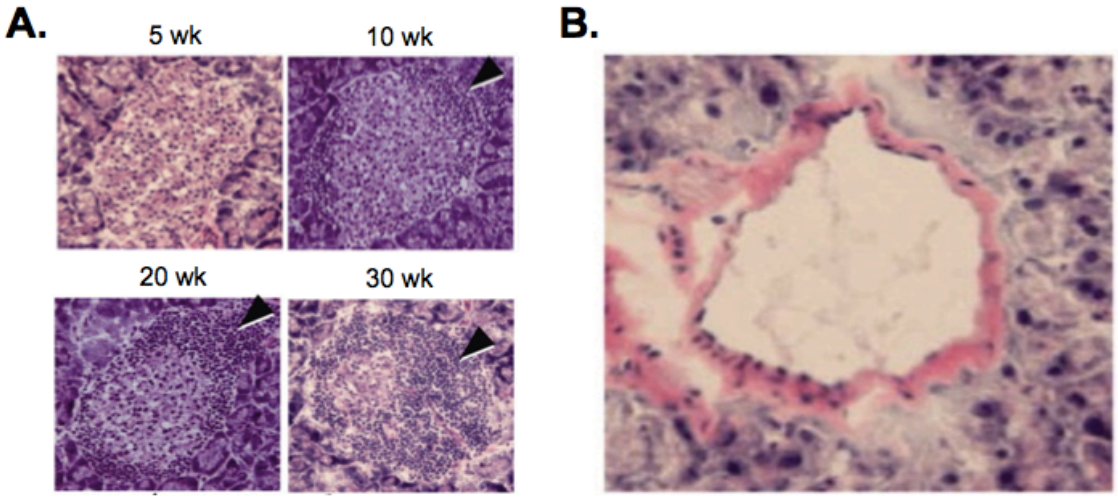


Figure 1-2. Histology of NOD islets at various stages.

(A) Prediabetic islets at various stages: 5 wk (no infiltration), 10 wk (peri-insular insulitis), 20 wk (intra-insular insulitis up to 25% of islet mass), and 30 wk (intra-insular insulitis up to 75% of islet mass). Wk, age in weeks. Arrowheads indicate immune infiltrates. **(B)** End-stage insulitis with less than 20% of islet mass remaining. Figure adapted from (Inoue et al., 2007).

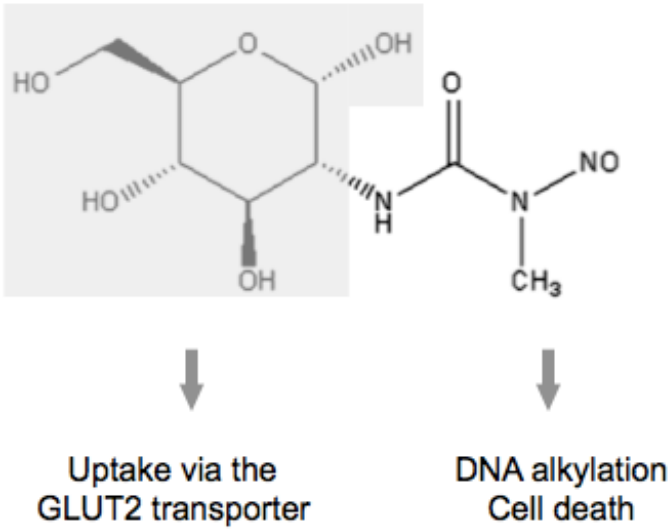


Figure 1-3. Beta cell toxin streptozotocin.

STZ is composed of glucose moiety (shaded in grey) attached to methylnitrosourea. Glucose residue facilitates beta cell specificity via uptake through GLUT2 glucose transporter. In the cell, methylnitrosourea residue alkylates and fragments DNA, leading to beta cell death.

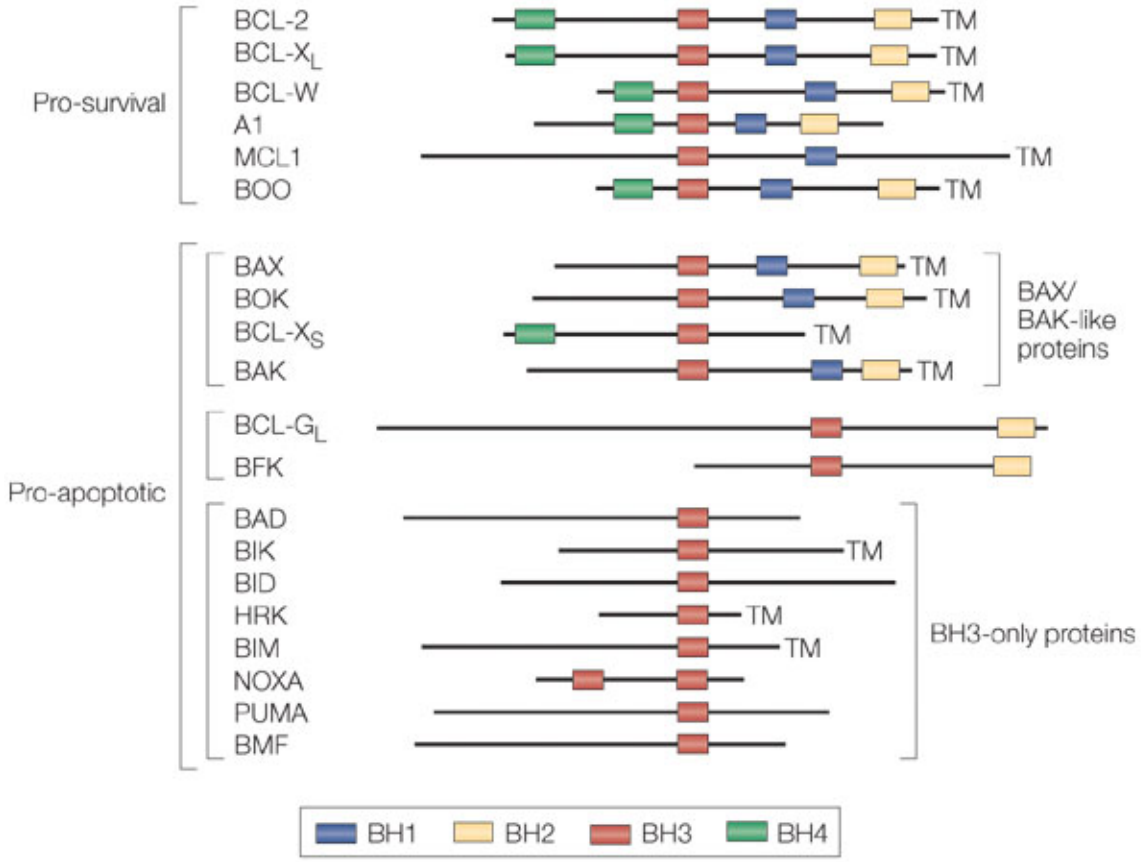


Figure 1-4. Mammalian BCL-2 family proteins.

BCL-2 family proteins with different BCL-2 homology (BH) domains are shown, categorized as pro-survival or pro-apoptotic. BH3-only proteins are pro-apoptotic BCL-2 family proteins. Figure from (Strasser, 2005).

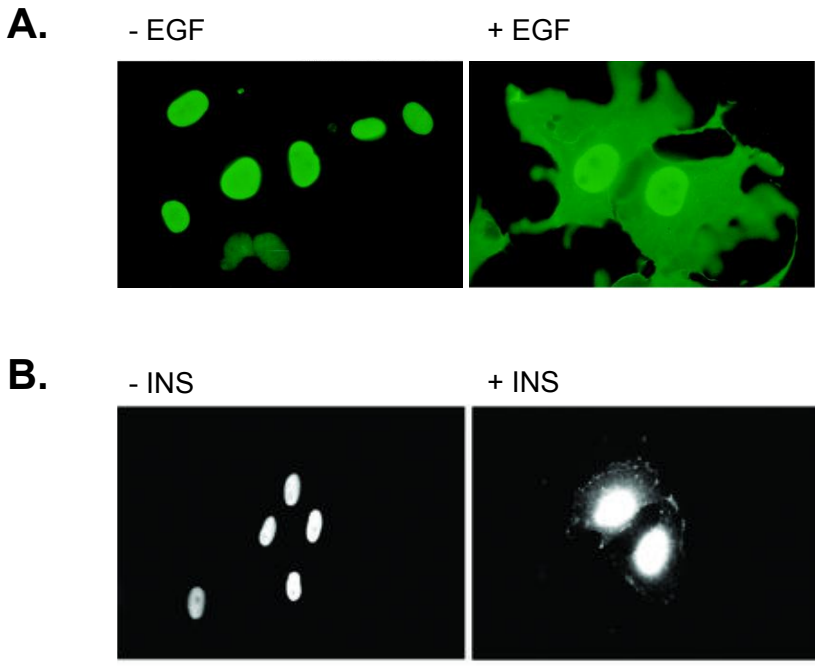


Figure 1-5. PTPN2 can exit the nucleus upon stimulation.

(A) TC45 isoform of PTPN2 is mostly nuclear (green) in unstimulated COS1 cells (-EGF). Upon epidermal growth factor stimulation for 15 minutes (+EGF), cytoplasmic and nuclear TC45 can be seen. Figure adapted from (Tiganis et al., 1998). **(B)** Fluorescence microscopy shows nuclear TC45 in unstimulated CHO/IR cells (-INS). After 30 minutes of insulin stimulation (+INS), non-nuclear TC45 can be seen. Figure adapted from (Galic et al., 2003).

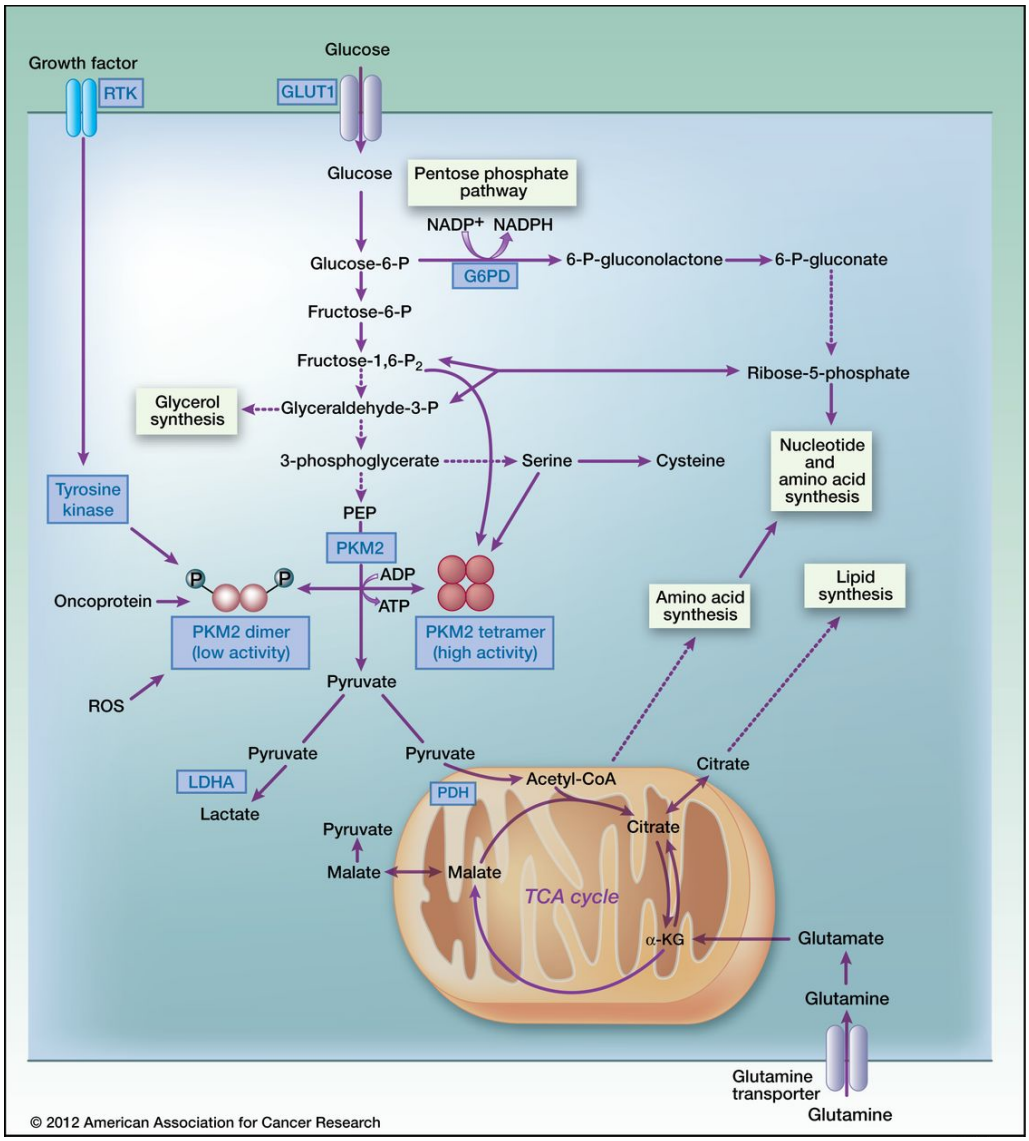


Figure 1-6. PKM2 generates pyruvate at the final step of glycolysis.

Normally, PKM2 can exist as the high-activity tetramer or the low-activity dimer. During oncogenic processes, PKM2 can be modified by tyrosine kinase-mediated phosphorylation events that can inhibit the tetrameric form. Generally, the high-activity tetrameric form is associated with normal metabolic pathways including the tricarboxylic acid cycle and oxidative phosphorylation. The presence of low-activity dimeric PKM2 is associated with the Warburg effect, with a high rate of conversion of pyruvate to lactate. Figure from (Tamada et al., 2012).

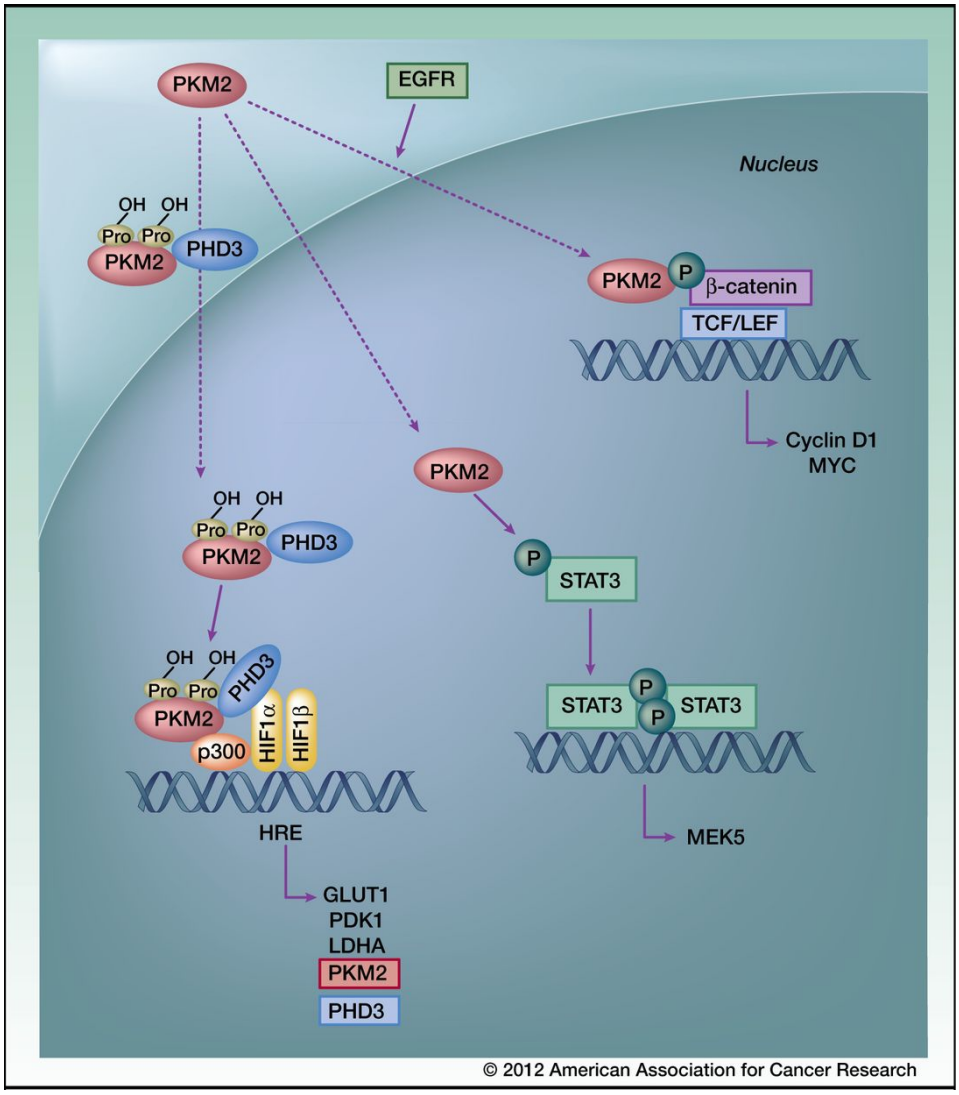


Figure 1-7. Nonglycolytic functions of PKM2 in the nuclei of cancer cells.

In the nucleus, PKM2 can transactivate HIF-1α, phosphorylate STAT3, and interact with β-catenin to regulate target gene transcription. Figure from (Tamada et al., 2012).

Chapter Two: PTPN2 promotes beta cell survival by modulating PKM2-mediated transcription of pro-apoptotic genes in diabetes

Preface

Dr. Lori Sussel and I conceived the study. *Ptpn2 fl/fl* mice were generated and provided by Dr. Gerard Karsenty and Dr. Tiffany Zee. Dr. Emily Chen performed tandem mass spectrometry. Dr. Xiaoyun Sun and I performed bioinformatics analyses for the RNA-Seq. With guidance of Dr. Lori Sussel, I generated all other data. This work was supported by the Columbia University MD/PhD Program and the National Institutes of Health DP3 Type 1 Diabetes Impact Award.

Abstract

Protein tyrosine phosphatase N2 (*PTPN2*) is a type 1 diabetes candidate gene identified from human genome-wide association studies. PTPN2 is highly expressed in human and murine islets and is elevated upon inflammation, suggesting that beta cell PTPN2 may be important for cell survival. To test this *in vivo*, we generated beta cell-specific deletion in mice (Ptpn2 βKO). While unstressed animals exhibit normal metabolic profiles, streptozotocin (STZ) treated Ptpn2 βKO mice display marked increase in hyperglycemia and death due to exacerbated beta cell loss. Global transcriptome analysis revealed that beta cell function may also be impaired in the unstressed Ptpn2 βKO islets, which confirms the role of beta cell PTPN2 in the context of high fat diet-stressed animals. However, the known substrates of PTPN2 that are critical for beta cell function could not fully explain the mechanism of beta cell defense. Here we show that PTPN2 interacts with a novel substrate, pyruvate kinase M2 (PKM2), a key metabolic enzyme that normally resides in the cytosol. Adaptation to stress stimuli such as hypoxia leads to the nuclear translocation of PKM2 in many cell types, where it can act as a transcription factor. In beta cells, PKM2 migrates to the nucleus in response to STZ, where it activates the transcription of a subset of pro-apoptotic genes. In the absence of PTPN2, there is increased accumulation of PKM2 in the nucleus, which may account for exacerbated diabetes in Ptpn2 βKO mice. We have reconstituted the proposed function of PTPN2 by modulating nuclear PKM2 using TEPP46, a small molecule activator of PKM2 that stabilizes the cytosolic conformation of PKM2. Addition of TEPP46 to STZ-treated Ptpn2 βKO islets attenuated PKM2-mediated transcription of pro-apoptotic genes. These

findings suggest that PTPN2 is an important regulator of nuclear PKM2 in beta cells facing diabetic stress.

Introduction

Autoimmune destruction of pancreatic beta cells by cytotoxic lymphocytes is the hallmark of type 1 diabetes (T1D). The initiation of insulitis leading to diabetes is multifactorial and has been attributed to both genetic and environmental factors (Todd, 2010). Many of the genetic factors of T1D have been identified in the genome-wide association studies (GWAS) (Wellcome Trust Case Control, 2007), but the molecular mechanisms by which most risk alleles increase susceptibility to T1D remain unclear. It should be noted that over 60% of these GWAS candidate genes are strongly expressed in human islets (Eizirik et al., 2012), suggesting there are beta cell features that contribute to their destruction. In addition, compared to other cell types, beta cells express lower basal levels of antioxidant protective enzymes and therefore it has been hypothesized that they more sensitive to oxidative stressors (Lenzen et al., 1996). Together, this suggests that beta cell apoptosis in T1D susceptible individuals may be, at least in part, due to dysfunctional beta cell-intrinsic defense mechanisms to inflammatory stimuli.

Protein tyrosine phosphatase N2 (*PTPN2*), also known as *T-cell protein tyrosine phosphatase* (*TC-PTP*), is a novel candidate gene for T1D (Todd et al., 2007). The association of *PTPN2* with T1D risk remains incomplete, especially since *PTPN2* is expressed in many tissue types, including T cells and pancreatic beta cells (Eizirik et al., 2012). Risk variants of *PTPN2* are noncoding (rs2542151, rs1893217, and rs478582), and one reported study correlates the risk variant rs1893217 to decreased expression of *PTPN2* transcripts in T cells of T1D patients (Long et al., 2011). Disease modeling in mice through the conditional deletion of *Ptpn2* in T cells has revealed that PTPN2 negatively regulates immune signaling to temper inflammation (Wiede et al., 2011). Mice

lacking PTPN2 in T cells die of systemic inflammation, and the whole body *Ptpn2* knockout mice have more severe phenotypes and earlier lethality due to impairment in the bone marrow microenvironment critical for hematopoiesis (You-Ten et al., 1997). Unfortunately, due to the early lethality of the whole body *Ptpn2* knockout mice, the function of PTPN2 in other cell types such as the pancreatic beta cells has been difficult. However, there is mounting evidence that PTPN2 is critical for beta cell survival, as *in vitro* PTPN2 knockdown in rodent and human primary beta cells exacerbates interferon-induced apoptosis through signal transducer and activator of transcription 1 (STAT1) signaling (Moore et al., 2009; Santin et al., 2011). On the other hand, mice lacking PTPN2 in the pancreas (panc-TCPTP KO) are euglycemic and exhibit exacerbated glucose intolerance only after prolonged high fat feeding, which has been attributed to the hyperactivation of STAT3 (Xi et al., 2014).

In this study, we report that conditional deletion of *Ptpn2* in beta cells leads to exacerbated streptozotocin (STZ) induced diabetes due to the deregulation of pyruvate kinase M2 (PKM2)-mediated pro-apoptotic gene expression. Tetrameric pyruvate kinase plays a pivotal role in energy metabolism by catalyzing the final step of glycolysis, generating pyruvate and ATP. Non-tetrameric, low-activity PKM2 has been shown translocate to the nucleus to affect gene transcription by transactivating key transcription factors such as hypoxia-inducible factor 1 alpha (HIF-1α) and β-catenin or by acting as a kinase of transcription factors like STAT3 in cancer cells (Gao et al., 2012; Luo et al., 2011; Yang et al., 2011). PKM2 is also highly expressed in islet beta cells (Chatterton et al., 1982; Martens et al., 2010), and we demonstrate that upon treatment with STZ, beta cell PKM2 translocates to the nucleus where it alters the transcription of HIF-1α-

responsive genes. We also show that overexpressing a functional mutant of PTPN2 leads to an accumulation of nuclear PKM2 with STZ stress in beta cells, which suggests that the function of wildtype PTPN2 promotes the non-nuclear, and transcriptionally inactive, PKM2 tetramer. In the absence of such regulation in the conditional *Ptpn2* beta cell knockout mice (Ptpn2 βKO), we observed a significant upregulation of HIF-1α/PKM2-responsive genes, including pro-apoptotic transcripts *Bnip3*, *Bnip3l*, and *Ddit4*. Differences in the beta cell transcriptional landscape combined with poor tolerance of STZ treatment by the Ptpn2 βKO mice reveals a novel mechanism of PTPN2-mediated beta cell defense upon apoptotic stimuli. We demonstrate that PTPN2 modulates nuclear PKM2-mediated pro-apoptotic gene transcription, and exogenous modulation of nuclear PKM2 levels attenuates pro-apoptotic gene transcription. We conclude that PTPN2 is an essential beta cell factor that modulates cell survival upon injurious stimuli and illustrate a novel mechanism of beta cell loss in diabetes through nuclear accumulation of PKM2.

Results

Generation of the beta cell-specific PTPN2 knockout mice

Given that PTPN2 knockdown exacerbates cytokine-induced apoptosis *in vitro*, we sought to test the beta cell requirement of PTPN2 *in vivo* by generating mice lacking PTPN2 in beta cells (*Ptpn2*$^{fl/fl}$; *RIP-Cre*, henceforth, Ptpn2 βKO) (Herrera, 2000; Zee et al., 2012). In the Ptpn2 βKO mice, overall levels or *Ptpn2* transcripts were unchanged, however ~81% of *Ptpn2* transcripts had a deletion of the floxed exons in the Ptpn2 βKO islets. RNA-Seq analysis of the Ptpn2 βKO islets confirmed this finding (Fig. 2-1).

Deletion of the floxed exons correlated with decreased PTPN2 protein expression in the Ptpn2 βKO islets, where PTPN2 was significantly decreased by 64%. PTPN2 expression was not changed in the hypothalamus, where ectopic *RIP-Cre* allele expression has been reported (Wicksteed et al., 2010) (Fig. 2-2).

Characterization of the Ptpn2 βKO mice

We hypothesized that PTPN2 would be required for beta cell survival and Ptpn2 βKO mice would be diabetic. However, Ptpn2 βKO animals were born at the expected Mendelian ratios and were viable, fertile, and appeared healthy overall (Fig. 2-3A). In 10-week-old animals, plasma glucose and insulin, glucose tolerance, insulin sensitivity, total insulin content, and islet morphology were comparable between Ptpn2 βKO and control littermates (Fig. 2-3B-E, 2-4). Ptpn2 βKO mice did not become spontaneously diabetic, even after aging for over 300 days (data not shown).

To investigate molecular changes in the absence of diabetes, we isolated islets from adult Ptpn2 βKO and control mice and compared the global gene expression profiles through RNA-Seq. The analysis identified 1212 genes that are differentially expressed (Fig. 2-5A). Transcripts encoding insulin, *Ins1* and *Ins2*, were comparably expressed but we observed decreased expression of a subset of beta cell transcription factors including *Nkx2-2*, *Mafa*, and *FoxO1* (Fig. 2-5B).

We then conducted pathway analysis and discovered that key canonical pathways such as toll-like receptor (TLR) and mammalian target of rapamycin (mTOR) signaling were affected (Fig. 2-5C). These signaling pathways have previously been implicated in beta cell survival or function (Fraenkel et al., 2008; Schulthess et al., 2009). Interestingly,

one of the upstream regulators contributing to the gene expression changes of the 1212 genes was nuclear factor kappa B (NF-κB) (activation Z-score = 3.11), activation of which has been implicated in both the activation and prevention of beta cell apoptosis (Chang et al., 2003; Eldor et al., 2006). Furthermore, STAT3 was predicted to be highly activated upstream of the transcriptional changes in the Ptpn2 βKO islets (activation Z-score = 3.12), and we have confirmed this finding (Fig. 2-6), which is consistent with reported hyperactivation of STAT3 in the panc-TCPTP KO islets (Xi et al., 2014). However, in the absence of overt phenotypic alterations in the adult animals, we decided to deliver exogenous metabolic stress to the Ptpn2 βKO and control mice. Unlike the panc-TCPTP KO mice, even after prolonged high fat feeding, Ptpn2 βKO mice did not display significant metabolic impairment or alterations in islet morphology compared to the control animals (Fig. 2-7), with the exception of one cohort of animals that exhibited differential weight gain due to unknown variables.

Ptpn2 βKO mice develop exacerbated experimental diabetes

In the absence of spontaneous or high fat-induced phenotypes, and considering the published *in vitro* function of PTPN2 in preventing beta cell apoptosis specifically in response to pro-inflammatory stimuli, we hypothesized that PTPN2 may become critical to beta cells facing apoptotic stimuli. Therefore, we administered multiple low-doses of streptozotocin (STZ) to chemically induce diabetes (Fig. 2-8). Differential *ad lib* hyperglycemia between Ptpn2 βKO and control animals were observed after two weeks (Fig. 2-8A), at which point glucose tolerance in Ptpn2 βKO and control animals were comparable. However, at week 3, we observed significantly exacerbated glucose

intolerance in addition to exacerbated hyperglycemia in the Ptpn2 βKO mice (Fig. 2-8C). To determine whether the hyperglycemia was due to differential beta cell loss, we examined the remaining islet cell populations using immunohistochemistry 4 months after STZ administration and determined that insulin-positive cells were less abundant in the Ptpn2 βKO islets (Fig. 2-9).

Ptpn2 βKO mice develop exacerbated hyperglycemia and glucose intolerance from low-dose STZ stress, but due to the delayed onset of differences (several weeks) we could not eliminate the possibility of glucotoxic secondary effects caused by chronic hyperglycemia. To minimize any secondary effects, we wanted to analyze differential diabetes within a week of STZ administration. Since the one-week phenotype is very mild with low-dose STZ, we employed high-dose STZ treatment. A single high-dose of STZ was sufficient to induce significant diabetes in both mutant and control mice within two days of injection, but we were unable to assess any differences in hyperglycemia due to the upper limit of the glucometer measurements (<600 mg/dl) (Fig. 2-10A). However, underlying differences were indirectly revealed through significantly impaired survival of the Ptpn2 βKO mice compared to the controls, likely from the failure to maintain glucose homeostasis due to severe beta cell loss from STZ (Fig. 2-10B).

We could not conclusively determine differential beta cell mass by immunohistochemistry because the high-dose STZ treatment obliterates most of the beta cells in the islets of both Ptpn2 βKO and control mice. Therefore, we assessed global transcriptomic changes in Ptpn2 βKO and control islets, which may be a more sensitive readout of differential beta cell survival or apoptosis. We identified 819 transcripts that were differentially expressed 5 days after high-dose STZ administration (Fig. 2-11, top

genes listed in Table 2-1). There was an overall trend of downregulation of beta cell transcripts in the Ptpn2 βKO islets, suggesting that perhaps the Ptpn2 βKO islets have fewer beta cells after STZ. Additionally, two genes involved in insulin secretion, *piccolo* (*Pclo*) and *ATP-binding cassette transporter sub-family C member 8* (*Abcc8*) were significantly decreased, suggesting that there may be functional defects in the Ptpn2 βKO beta cells (Fig. 2-11B).

Pathway analysis of differentially expressed transcripts yielded pathways of glucose metabolism, including tricarboxylic acid (TCA) cycle and oxidative phosphorylation, suggesting glucose metabolism may be altered in the Ptpn2 βKO islets (Fig. 2-11C). Genes affecting mitochondrial function made up the number one most affected pathway of the RNA-Seq, which also affect glucose metabolism. In addition, mitochondrial function is key for cell survival and death (Green and Reed, 1998). We have successfully validated many of the gene expression changes involved in the mitochondrial dysfunction category using islets harvested at different time-points after STZ, and concluded that mitochondrial dysfunction is a persistent disease process after STZ (Fig. 2-11D). Interestingly, mTOR signaling was changed in both the unstressed and post-STZ RNA-Seq, which may indicate functional alterations in the Ptpn2 βKO islets. Furthermore, consolidation of the unstressed and post-STZ islet transcriptome data revealed that genes differentially expressed between the Ptpn2 βKO and control islets were involved in increased mitochondrial membrane permeability, as well as cell death and survival, suggesting that Ptpn2 βKO islets have basal functional alterations that are amplified and may lead to increased cell death after apoptotic stimuli (Fig. 2-12).

Beta cell PTPN2 interacts with PKM2

Although it has been shown that PTPN2 inhibits the activation of transcription factor STAT3 in beta cells (Xi et al., 2014), we believe that STAT3 cannot account for all the transcriptional changes. For this reason, we sought to identify novel substrates of PTPN2. We transiently overexpressed epitope-tagged versions of wildtype and substrate-trapping form of PTPN2 in murine beta cells. After subjecting the cells to stressful stimuli, we pulled down PTPN2 using the epitope tag and examined which proteins were associated with PTPN2 using mass spectrometry. We discovered that 48 proteins consistently interacted with PTPN2 (Table 2-2). The top pathways affected by these proteins included glycolysis, TCA cycle, and gluconeogenesis, which all affect glucose metabolism or *de novo* generation, drawing parallel with the pathways affected in the RNA-Seq (Fig. 2-13A). Additionally, many of the proteins in these pathways were positioned in the mitochondria, functioning in TCA cycle and oxidative phosphorylation (Fig. 2-13B), which also correlates with mitochondrial dysfunction in the Ptpn2 βKO islets identified from the RNA-Seq. Additionally, some of the most strongly identified proteins were endoplasmic reticulum (ER) resident proteins, such as 78 kDa glucose-regulated protein (HSPA5, also known is BiP), transitional ER ATPase (VCP), and protein disulfide-isomerase A3 (PDIA3). Thus, mitochondrial and ER function could be affected by PTPN2 binding to these target proteins in the beta cells.

One of the top five PTPN2-interacting proteins was pyruvate kinase M (PKM), an important glycolytic enzyme with posttranslational modifications including tyrosine phosphorylation. A recent study reported protein tyrosine phosphatase 1B (PTP1B) as a tyrosine phosphatase that regulates the M2 isoform of PKM (Bettaieb et al., 2013). Since

PTPN2 bears close resemblance to PTP1B (74% sequence identity in the catalytic domains) (Iversen et al., 2002), we hypothesized that PTPN2 might be a regulator of PKM2. The M1 and M2 isoforms are splice variants of the *Pkm* locus and differ by one exon encoding 55 amino acids, of which 22 are unique to either isoform (Fig. 2-14A). Mass spectrometry identified the M2 isoform-specific peptides with higher frequency (Fig. 2-14B) and Ptpn2 βKO and control islets express more *Pkm2* transcripts than *Pkm1* (Fig. 2-14C). We concluded that *Pkm2* is the major isoform in beta cells and focused further analysis on the relationship between PTPN2 and PKM2.

We confirmed that PTPN2 interacts with PKM2 *in vitro* in Min6 cells and *in vivo* in mouse islets (Fig. 2-15). Unfortunately, our efforts to identify specific phosphotyrosine residue of PKM2 that is the substrate of PTPN2 through mutagenesis of known phosphotyrosine residues of PKM2 was inconclusive, possibly due to competition for PTPN2 interaction by high levels of endogenous PKM2. However, we have established that PTPN2 interacts with PKM2 in beta cells.

PTPN2 modulates nuclear localization of PKM2

Tyrosine 105 phosphorylation can inhibit the glycolytically active, cytoplasmic tetramer form of PKM2 (Hitosugi et al., 2009), and the dimeric form of PKM2 has been shown to be nuclear where it participates in transcription (Gao et al., 2012; Luo et al., 2011). Therefore, we investigated the subcellular localization of PKM2 with confocal microscopy. In both Ptpn2 βKO and control islets from healthy animals, PKM2 was excluded from the nuclei, however, after STZ treatment, strong nuclear PKM2 signal could be observed (Fig. 2-16). It was difficult to determine differential nuclear

localization of PKM2 in Ptpn2 βKO and control islets using immunohistochemistry, however *in vitro*, there was decreased nuclear localization of PKM2 in STZ-treated Min6 cells that overexpressed PTPN2 versus a mutant form of PTPN2 (Fig. 2-17). This suggests that the function of PTPN2 is critical for mitigating the accumulation of nuclear PKM2 in stressed beta cells, which is consistent with the hypothesis that diabetic Ptpn2 βKO islets accumulate more PKM2 in the nuclei.

PTPN2 regulates stress gene expression by modulating nuclear PKM2

Nuclear PKM2 functions as a transcription factor and has been identified as a coactivator of hypoxia-inducible factor 1 alpha (HIF-1α)-mediated transcriptional changes that promote cancer cell metabolism (Luo et al., 2011). Because HIF-1α is activated in response to reduced oxygen tension (Semenza, 2013) and because mitochondrial function is highly dependent on the availability of oxygen, we hypothesized that the transcriptional differences relating to increased mitochondrial dysfunction in the Ptpn2 βKO islets may be related to the cellular oxygen sensing machinery regulated by the PTPN2-PKM2 interaction. In fact, a HIF-1α target, *Egl-9 family hypoxia-inducible factor 3* (*Egln3*), was upregulated by 65-fold in the Ptpn2 βKO islets. Furthermore, pathway analysis identified HIF-1α as one of the most activated upstream regulators of differentially expressed genes (activation Z-score = 3.21), which comprised of the 19 transcripts listed in Table 2-3. Direct expression analysis of known HIF-1α target genes (Benita et al., 2009; Liu et al., 2012) in the Ptpn2 βKO and control islets identified the upregulation of additional HIF-1α/PKM2-regulated genes (Fig. 2-18). Three of the upregulated HIF-1α/PKM2 target genes included key stress-response genes

such as *BCL-2/adenovirus E1B 19kDa-interacting protein 3* (*Bnip3*), *Bnip3-like* (*Bnip3l*), and *DNA damage-inducible transcript 4* (*Ddit4*). *Bnip3* and *Bnip3l* encode pro-apoptotic proteins of the BNIP3 subfamily of the BH3-only proteins that localize to the mitochondria and induce cell death by interacting with pro-apoptotic proteins like BCL-2-antagonist/killer 1 (BAK) and BCL-2-associated X protein (BAX) and by inhibiting pro-survival proteins such as B-cell CLL/lymphoma 2 (BCL-2) (Chinnadurai et al., 2008; Czabotar et al., 2014; Lomonosova and Chinnadurai, 2008). Aside from *Bnip3* and *Bnip3l*, the transcript levels of *Bak*, *Bax*, *Bcl2*, and other BCL-2 family genes involved in apoptosis were expressed at comparable levels between Ptpn2 βKO and control islets (Table 2-4). Collectively, T1D-conducive pro-apoptotic beta cells upregulate HIF-1α/PKM2 target genes, and the degree of upregulation may be modulated by the presence of PTPN2.

Exogenous reconstitution of PTPN2 function rescues transcriptional alterations

To directly assess whether PTPN2 modulates nuclear PKM2-mediated transcription of target genes, we sought to reconstitute the nuclear exclusion of PKM2 in PTPN2-deficient beta cells. Small molecule activators of PKM2 such as TEPP46 activate the tetrameric form of PKM2 that is cytosolic (Anastasiou et al., 2012). If PTPN2 functions to lessen nuclear PKM2 in stress conditions, adding TEPP46 could compensate for the loss of PTPN2-mediated nuclear export of PKM2 by activating the cytosolic form of PKM2. We therefore stressed the Ptpn2 βKO islets with STZ in the presence or absence of TEPP46 (Fig. 2-19A). Remarkably, upregulation of HIF-1α/PKM2-responsive genes with STZ was effectively diminished by the depletion of non-tetrameric nuclear

PKM2 by TEPP46 (Fig. 2-19B). Although HIF-1α/PKM2 target genes were still significantly upregulated compared to islets not subjected to STZ stress, the degree of upregulation was milder than islets treated with STZ alone. This suggests that PTPN2 lowers nuclear levels of PKM2 in the beta cells to attenuate the transcription of pro-apoptotic genes, which ultimately serves as a survival mechanism upon diabetic stimuli (Fig. 2-20).

Discussion

Summary of the findings

In the present study, we demonstrated that PTPN2 is required in beta cells; Ptpn2 βKO mice that lack functional beta cell PTPN2 experience impaired survival in the face of the diabetic stressor STZ. We have identified PKM2 as a PTPN2-interacting protein. PKM2 translocates to the nucleus following STZ injection, where it mediates HIF-1α/PKM2-responsive gene transcription, including the key pro-apoptotic genes *Bnip3* and *Bnip3l*. PTPN2 is required to modulate the expression of *Bnip3* and *Bnip3l*, as well as other HIF-1α/PKM2-responsive genes by modulating the accumulation of PKM2 in the nuclei of stressed beta cells. Reconstituting this function of PTPN2 in Ptpn2 βKO islets by activating non-nuclear PKM2 decreases the transcriptional activation of HIF-1α/PKM2-responsive genes. This study is the first to identify transcriptional changes induced by PKM2 in a non-cancerous cell type and may have broadened therapeutic possibilities by bridging PKM2 to diabetogenesis.

Comparison with the panc-TCPTP KO phenotype

A recent study of PTPN2 requirement in pancreatic endocrine cell function (Xi et al., 2014) was conducted with a whole-pancreas knockout model of PTPN2 (referred to as panc-TCPTP KO). Consistent with the panc-TCPTP KO mice, the Ptpn2 βKO animals also had no gross metabolic phenotype and survived to adulthood. After HFD challenge, however, the panc-TCPTP KO mice exhibited significant glucose intolerance and defects in beta cell function, whereas the Ptpn2 βKO animals did not. Several differences in the study designs could account for this discrepancy. First, the panc-TCPTP KO mice were maintained in the C57BL/6J background, and the authors note the possibility that their phenotype of reduced insulin secretion may have been due to the deletion of *nicotinamide nucleotide transhydrogenase* (*Nnt*) in the genetic background (Xi et al., 2014). In our study, Ptpn2 βKO animals were maintained in a mixed background. Second, the panc-TCPTP KO mice were switched to HFD soon after weaning, whereas the Ptpn2 βKO mice were given HFD after reaching adulthood (10 weeks). It is possible that there may be an early time window during which adaptation to diet-induced obesity may be critically influenced by PTPN2. Furthermore, the panc-TCPTP KO study mentions that even in the control mice, PTPN2 expression in the pancreas significantly decreased after HFD, suggesting that differences in panc-TCPTP KO and control mice after HFD may be due to subtle differences in PTPN2 levels.

The panc-TCPTP KO study demonstrated that knocking down PTPN2 in beta cells *in vitro* led to a significant reduction of important factors influencing beta cell function, including *Glut2*, *Pdx1*, *Ins1*, *Ins2*, and *Kir6.2* (Xi et al., 2014). Consistent with this finding, we show that deletion of PTPN2 leads to an overall trend of downregulation

of key transcription factors in beta cells, including *Pdx1*, *Nkx2-2*, *Nkx6-1*, *Foxo1*, *Mafa*, and *Isl1*, which supports the idea that PTPN2 is required for the proper function of beta cells (Fig. 2-5). However, unlike the *in vitro* analysis, we did not detect a substantial decrease in *Ins1*, *Ins2*, and *Kir6.2* also known as *Kcnj11*, which encodes a pancreatic ATP-sensitive potassium channel subunit critical for insulin secretion. This may be due to differences between acute and chronic loss of PTPN2, and our *in vivo* results may be more consistent with the observed normal metabolic phenotypes in our Ptpn2 βKO and their panc-TCPTP KO mice without exogenous stress.

The mechanism of PTPN2-mediated STAT3 regulation in beta cells

The panc-TCPTP KO study identified previously known substrates of PTPN2, namely STAT1 and STAT3, as bona fide dephosphorylation targets of PTPN2 in beta cells, and defective beta cell function in the panc-TCPTP KO mice was attributed to the hyperactivation of STAT3. Beta cell function was restored by the inhibition of STAT3 in PTPN2-deficient beta cells (Xi et al., 2014). However, because STAT3 hyperactivation has been shown to be protective against NF-κB inflammatory response and apoptosis in other cell types (Bettaieb et al., 2014), it could not fully account for the changes we observed in STZ-induced beta cell loss. Furthermore, knockout mouse studies aimed at identifying the role of STAT3 in the beta cells have yielded conflicting results.

Beta cell-specific STAT3 knockout mice have defects in insulin secretion and islet architecture, accumulating alpha cells in the center of islets instead of in the periphery (Cui et al., 2004; Gorogawa et al., 2004). In addition, beta cell STAT3 knockout mice display increased obesity, partly because of STAT3 deletion in the

hypothalamic neurons due to the ectopic expression of *RIP-Cre*. To differentiate beta cell-specific requirement of STAT3 in the absence of STAT3 deletion in the hypothalamus, two groups generated pancreas-specific STAT3 knockout mice. In one study, mice with pancreas knockout of STAT3 did not develop obesity or glucose intolerance, and the islet architecture was surprisingly normal, leading to the conclusion that STAT3 is dispensable for beta cell development and function (Lee and Hennighausen, 2005). In another study, the knockout mice exhibited impairment in beta cell function and significant glucose intolerance, which was attributed to the disorganization of the islet vascular development (Kostromina et al., 2010). Thus, because the lack of STAT3 was associated with impaired beta cell function in several studies, STAT3 hyperactivation cannot fully explain the observed beta cell dysfunction in panc-TCPTP KO islets after high fat diet or exacerbated beta cell loss in the Ptpn2 βKO islets after STZ treatment. It is possible that this is due to differences in complete loss of STAT3 as opposed to the chronic activation of STAT3.

STAT3 activation in other cell types is controlled by various factors such as the suppressor of cytokine signaling 3 (SOCS3) (Yoshimura et al., 2007). It should be noted that *Socs3* was one of the most significantly upregulated transcripts in the unstressed Ptpn2 βKO islets (DESEQ P = 1.03E-07), even though STAT3 was hyperphosphorylated by immunoblotting in the Ptpn2 βKO islets, suggesting that without the chronic stress of high fat diet, the mice are able to maintain sub-threshold levels of STAT3 activation through regulatory mechanisms such as *Socs3*. Therefore, we sought to identify other substrates of PTPN2 in in Ptpn2 βKO islets that may be responsible for compromised beta cell defense.

mTOR hyperactivation in PTPN2-deficient beta cells

Our transcriptome analysis from both unstressed and STZ-stressed islets identified mammalian target of rapamycin (mTOR) signaling as being upregulated in the Ptpn2 βKO islets. Because mTOR is a serine/threonine kinase, mTOR signaling is more often assessed by the changes in phosphorylation cascades, but the transcriptional upregulation of key mTOR activators such as *ras homolog enriched in brain* (*Rheb*) suggest that mTOR signaling is affected in the Ptpn2 βKO islets, especially after STZ.

mTOR is a nutrient sensor that promotes protein translation and cellular growth (Wullschleger et al., 2006). mTOR exists as two complexes: rapamycin-sensitive complex mTORC1 with the regulatory-associated protein of mTOR (RAPTOR) and rapamycin-resistant mTORC2 with rapamycin-insensitive companion of mTOR (RICTOR) (Loewith et al., 2002). mTORC1 has been shown to induce the phosphorylation of ribosomal S6 kinase 1 (S6K1) to regulate IRS2 turnover (Shah et al., 2004). IRS2 is critical for beta cell survival, and its depletion by mTOR-mediated phosphorylation can lead to beta cell apoptosis, which can be prevented by mTOR-inhibition with rapamycin (Briaud et al., 2005; Hennige et al., 2003; Lingohr et al., 2002). Rapamycin-mediated inhibition of mTOR can also inhibit beta cell proliferation, suggesting that mTOR signaling functions in both apoptosis and proliferation in the beta cells (Liu et al., 2009). Hyperactivating mTOR by overexpressing the upstream activator of mTOR, RHEB, in the beta cells, leads to increased beta cell function and mass, as well as resistance to STZ-induced diabetes (Hamada et al., 2009). However, in the diabetes-prone NOD strain, beta cell-specific hyperactivation of mTOR through RHEB overexpression results in accelerated diabetes, and the authors conclude that the

enlargement of beta cells may in fact enhance the autoimmunity (Sasaki et al., 2011). This could also be due to increased apoptosis signaling by mTOR. The upregulation of *Rheb* transcript in the Ptpn2 βKO islets after STZ could also indicate similar increases in apoptosis, as we did not observe a significant increase in beta cell size in the Ptpn2 βKO islets. To date, the relationship between PTPN2 and mTOR is unstudied, and further investigation into whether and how PTPN2 regulates mTOR signaling may be illuminating for the understanding of beta cell loss in diabetes.

The role of PTPN2 in preventing the nuclear accumulation of PKM2

The novel finding in this study is that beta cells deficient in functional PTPN2 have a higher level of nuclear PKM2, positively regulating HIF-1α/PKM2-mediated transcription. Our model is that PTPN2 mediates nuclear-to-cytoplasmic translocation of PKM2, counteracting stressful stimuli that drive the nuclear localization of PKM2 (Fig. 2-20B). The most likely scenario is that PTPN2 dephosphorylates phosphotyrosine residues of PKM2 that inhibit the cytosolic tetramer conformation, such as the Y105 residue (Hitosugi et al., 2009). It should be noted that phosphotyrosine binding to the catalytic domain of PTP is transient, and classical studies of substrate identification of protein tyrosine phosphatases (PTPs) are conducted with substrate trapping mutants (Blanchetot et al., 2005). For instance, substrate trapping mutant of PTP1B, the only known phosphatase of PKM2 and a closely related tyrosine phosphatase family protein of PTPN2, is unable to bind Y105F mutant of PKM2 because PTP1B directly dephosphorylates the Y105 residue (Bettaieb et al., 2013). Curiously, in our study, we did not observe any significant differences in PKM2 binding affinity in wildtype and

substrate trapping mutant PTPN2 in both mass spectrometry and validation immunoblotting (Fig. 2-15, Table 2-2). Further study will be needed to determine whether PKM2 is a direct dephosphorylation target of PTPN2.

Our study has shown that loss of PTPN2 accumulates PKM2 in the beta cell nuclei, but we cannot rule out the possibility that PTPN2 mediates the nuclear export of PKM2 in a method other than direct dephosphorylation of PKM2. If PTPN2 binding to PKM2 is not specific to the catalytic domain, PTPN2 could mediate nuclear export of PKM2 by the physical translocation of the PTPN2-PKM2 complex upon death-inducing stimuli. Another uncertainty is whether the interaction of PTPN2 with PKM2 is specific to PKM2 conformation. It is possible that PTPN2 binds more strongly to the tetrameric PKM2 in the cytoplasm, in effect stabilizing the cytosolic PKM2, favoring the equilibrial shift of dimer to tetramer PKM2. Structural analysis of PTPN2-PKM2 complex is needed to determine whether PTPN2 binds more strongly to a particular PKM2 conformation. Although we cannot rule out other mechanisms of PTPN2-mediated PKM2 nuclear export, we observe significant transcriptional deregulation of HIF-1α/PKM2-responsive pro-apoptotic genes in the Ptpn2 βKO islets, suggesting that PTPN2 is critical for preventing PKM2 accumulation in the nucleus.

The ramifications of nuclear accumulation of PKM2 in diabetic islets, and evidence for increased apoptosis signaling in the Ptpn2 βKO islets

Most of the studies of nontraditional role of PKM2 as a transcription factor focus on PKM2 as a mediator of cell proliferation or survival in Warburg effect of cancer cells (Gao et al., 2012; Luo et al., 2011; Wang et al., 2014; Yang et al., 2011; Yang et al.,

2012b). Warburg effect is a phenomenon of increased ratio of glycolysis to oxidative phosphorylation, shuttling pyruvate away from oxidative phosphorylation in favor of lactate production (DeBerardinis et al., 2008; Warburg, 1956), which occurs in rapidly proliferating fetal tissues or tumors that must adapt to decreased oxygen tension and surplus of growth factors such as epidermal growth factor (EGF). Hypoxia within the tumor tissue and the activation of HIF-1α is well documented in cancers, and it has been posited that HIF-1α/PKM2-mediated transcriptional regulation in tumor cells allows for metabolic reprogramming required for cell proliferation and cancer progression (Luo et al., 2011; Yang et al., 2011). HIF-1α-induced genes facilitate the Warburg effect, but even in the context of hypoxic tumor cells, HIF-1α activation is highly cell type- and context-dependent, as it can also promote apoptosis, and HIF-1α-null tumors show accelerated growth owing to decreased HIF-1α-mediated apoptosis (Carmeliet et al., 1998). This dual role as a survival and apoptotic agent may also be at the heart of beta cell survival and apoptosis, with PKM2 acting as a regulatory switch.

In somatic cell types, only one report demonstrates HIF-1α/PKM2-mediated transcriptional changes. Inflammation and sepsis induced by lipopolysaccharides can change macrophage metabolism to that similar to Warburg effect, which further activates macrophages and stimulates the release of pro-inflammatory cytokines (Everts et al., 2012; Tannahill et al., 2013). A recent study in macrophages has shown that HIF-1α/PKM2-mediated transcription is at the heart of macrophage activation and inhibiting PKM2 protected mice from endotoxin-induced septic death by modulating macrophage activation (Yang et al., 2014). However, inflammation and immune activation involves

immune cell proliferation. Hence, there is scant evidence of PKM2-induced transcriptional changes in nonproliferating cell types.

With mounting evidence that PKM2 is a pro-survival protein in proliferating cells, the only instance of nuclear PKM2 inducing cell death was in 2007, when Stetak et al reported nuclear translocation of PKM2 in response to several pro-apoptotic stimuli. They reported cell death activation after PKM2 nuclear translocation, but did not investigate the function of PKM2 as a transcription factor in the nuclei of apoptotic cells (Stetak et al., 2007). This is what we have addressed in this study, by looking globally at the transcriptional landscape of beta cells after apoptosis-inducing stimuli, with or without PTPN2 which regulates nuclear PKM2.

Evidence of PKM2 in cell proliferation and apoptosis suggests a dual function of nuclear PKM2 – pro-survival adaptive response in cells exposed to low oxygen tension or aberrantly increased growth factors and pro-apoptotic response in cells that are by default more prone to damage, such as the pancreatic beta cells as evidenced by inherently low levels of antioxidant defense mechanism (Carlsson et al., 1998). Dual function of nuclear PKM2 may be indirectly observed from the physiological consequences of HIF-1α/PKM2 target genes. For example, *Ddit4* was significantly upregulated in an animal model of ischemic injury, and the overexpression of *Ddit4* protected breast cancer cells from hypoxia and hydrogen peroxide-induced cell death, but in non-dividing cell types, the overexpression of *Ddit4* led to increased sensitivity to cell death (Shoshani et al., 2002). Two other HIF-1α/PKM2 target genes are pro-apoptotic transcripts *Bnip3* and *Bnip3l* that are upregulated in the Ptpn2 βKO islets, and these genes may coordinately generate a pro-apoptotic environment by interacting with BCL-2 family proteins, which

were not transcriptionally activated. There may be posttranslational modifications of BCL-2 family proteins that are undetected by the RNA-Seq, but the fact that only these pro-apoptotic BCL-2 family proteins were upregulated at the transcriptional level highlights the shift towards apoptosis. Furthermore, *in vitro* studies of PTPN2 knockdown have established that another pro-apoptotic BCL-2 family protein, BIM, is activated by phosphorylation (Moore et al., 2009; Santin et al., 2011) supports our finding that the balance of cell survival to cell death is shifted to be more pro-apoptotic in the absence of PTPN2. Additional differentially expressed transcripts support the pro-apoptotic environment of Ptpn2 βKO islets. In Table 2-1, top six of the most upregulated transcripts – *Cyp2b10*, *Cidea*, *Egln3*, *Hamp*, *Ppbp*, and *Il22ra2* – and two of the transcripts only expressed in the Ptpn2 βKO islets after STZ – *Cxcl13* and *Il31ra* – have all been reported to be upregulated in inflammation or apoptosis (Feld et al., 2010; Gnana-Prakasam et al., 2008; Gonzalez-Cortes et al., 2012; Inohara et al., 1998; Lomonosova and Chinnadurai, 2008; Saez de Guinoa et al., 2011; Wei et al., 2000).

Another highly expressed transcript in the STZ-treated Ptpn2 βKO islets was *p53-induced protein 1* (*Trp53inp1*), induction of which is associated with increased apoptosis in beta cells (Okamura et al., 2001; Zhou et al., 2012). It has been shown that expression of *Trp53inp1* is controlled by a microRNA cluster known as *maternally expressed 3* (*Meg3*), which encodes several alternatively spliced non-coding RNAs (Kameswaran et al., 2014). Furthermore, in islets from T2D patients showed a decrease in *Meg3* expression and an increase in the target *Trp53inp1* mRNA levels, which the authors attribute to increased apoptosis signaling in the T2D islets. We also observed a significant decrease in *Meg3* transcript in the STZ-diabetic Ptpn2 βKO islets (DESEQ P = 0.00017),

as well as decreases in several other transcripts in the cluster (*Rian*, *Mirg*, *Rtl1*, and *Begain*), and together with the induction of *Trp53inp1* and HIF-1α/PKM2-activated stress genes may be a reliable indication of increased apoptosis signaling in the Ptpn2 βKO islets after STZ.

HIF-1α in beta cells

In addition to lower antioxidant enzyme expression, pancreatic islets may be more susceptible to damage because of lower native oxygen tension (Carlsson et al., 1998), which can activate or stabilize HIF-1α protein in the islets. Indeed, genetic stabilization of HIF-1α in the beta cells through the deletion of HIF-1α degradation protein von Hippel-Lindau (VHL) leads to impaired beta cell function, which can be rescued by the concomitant deletion of HIF-1α in the beta cells (Cantley et al., 2009; Puri et al., 2009; Zehetner et al., 2008). Mice with beta cell-specific HIF-1α deletion showed normal glucose homeostasis and slightly improved beta cell function in mixed genetic background (Cantley et al., 2009), but after 12 rounds of backcrossing into the C57BL/6J genetic background, beta cell HIF-1α-null mice exhibited significant impairment in glucose tolerance coupled with beta cell dysfunction (Cheng et al., 2010). The authors hint that subtle changes in HIF-1α dosage in different genetic backgrounds may account for such conflicting results. However, it is possible that such variable results may be linked to the dual function of HIF-1α in cell proliferation and apoptosis in response to altered oxygenation in the islets.

Normal beta cell function is dependent on mitochondrial oxidative phosphorylation, which may be why islets normally suppress glycolytic genes such as

Ldha (Sekine et al., 1994). Recently, Warburg-like effect of increased lactate production was observed in the islets of non-obese model of rat T2D (Sasaki et al., 2013). This was attributed to increased ROS, and treatment with an antioxidant mimetic to reduce ROS countered this effect by decreasing HIF-1α protein. However, despite the fact that HIF-1α is a well-studied transcription factor, this study does not characterize any transcriptional changes due to HIF-1α in diabetic islets. Furthermore, beta cell ROS also plays a significant role in T1D, and it is unknown whether this Warburg-like effect accompanies or even contributes directly to beta cell loss in T1D. It remains to be seen whether STZ-stressed beta cells also have this Warburg-like effect, but the upregulation of *Ldha* and other HIF-1α/PKM2-activated genes suggest that it is possible.

PTPN2 and cellular defense

PTPN2 has been previously implicated in other autoimmune diseases such as Crohn's disease, ulcerative colitis, and celiac disease (Festen et al., 2011; Glas et al., 2012). This is understandable since PTPN2 is required for the immunomodulation in T cells (Wiede et al., 2011). However, PTPN2 is also important in cells targeted by the immune system; Penrose et al used an activator of PTPN2, spermidine, on intestinal cells and found that they were protected from interferon γ (IFN-γ) induced mucosal barrier damage (Penrose et al., 2013). Together with our study, this suggests that PTPN2 may play a role in self-defense machinery in cell types that are targeted for destruction. It will be informative to obtain T1D patient tissues to conduct tandem DNA array and RNA-Seq, to examine the effect of any diabetes-associated *PTPN2* SNPs on the expression of *PTPN2* in diabetic beta cells. This approach will also establish whether *PTPN2* SNPs

correlate with the expression of PKM2-induced pro-apoptotic genes, and further, with disease onset or progression.

Our study demonstrates that PTPN2 controls beta cell survival by modulating PKM2-mediated transcription of apoptosis-inducing genes such as *Bnip3* and *Bnip3l*. This finding could be studied to prevent T1D and serve to understand other PTPN2-mediated disease processes such as ulcerative colitis. Lastly, this work could further differentiate pro-apoptotic or pro-survival effect of HIF-1α/PKM2-mediated transcriptional activation, which will be useful for optimizing cancer therapeutics and open a potential for preventing diabetes in susceptible individuals.

Materials and Methods

Mouse studies. All mice were maintained on a mixed strain background of C57BL/6J and 129/Sv (Jackson). *Ptpn2*-floxed mice harboring loxP sites flanking exons 5 and 7 of the *Ptpn2* gene (Zee et al., 2012) were crossed with mice expressing *Cre* under the control of rat insulin-2 promoter (*RIP-Cre*) (Herrera, 2000) to generate beta cell-specific PTPN2 knockout mice (Ptpn2 βKO). Littermates heterozygous for the *Ptpn2*-floxed allele with the *Cre* and littermates homozygous for the *Ptpn2*-floxed allele without the *Cre* were used as controls. Genotyping was performed by PCR using tail DNA as described previously (Herrera, 2000; Zee et al., 2012). For high fat feeding, mice were switched to a high fat diet at 10 weeks of age (60% kcal fat, D12492i, Research Diets). For high-dose STZ treatment, Ptpn2 βKO and littermate controls were fasted for 4 hours (h) and injected intraperitoneally (i.p.) with 150 mg STZ (Sigma) per kg body weight,

freshly dissolved in 0.1 M sodium citrate buffer (pH 4.5). All injections were done within 10 minutes of STZ solution preparation. Hyperglycemia and body weight were monitored every 2- to 3-days, and mice were monitored for survival for two weeks or euthanized at days 5 or 8 post STZ injection to harvest tissues. For multiple low-dose STZ treatment, mice were fasted for 4 h and injected i.p. with 50 mg STZ per kg body weight on five consecutive days. Only male mice 4- to 6-week-old were used for the STZ experiments. All mice were maintained on a 12-hour light/dark cycle in a barrier facility with standard chow. All animal studies were approved by the Institutional Animal Care and Use Committee at the Columbia University Medical Center.

Islet isolation and culture. Pancreatic islets were isolated from mice following a modified version of published protocol (Gotoh et al., 1985). Briefly, after the digestion of the exocrine tissue with Collagenase P (Roche), remaining tissues were separated by density gradient centrifugation using Histopaque-1077 (Sigma). After handpicking under a microscope, islets were flash-frozen and stored at -80 °C until processing, or cultured in RPMI supplemented with 10% FBS (Gemini) and 1x antibiotic-antimycotic (Fisher). Prior to the *ex vivo* STZ treatment, islets were allowed to recover from stressful islet isolation procedure. After 24 h recovery, islets were seeded 50 islets per well to 6-well plates, and islets were pretreated for 24 h with 26 μM per well of TEPP46 (Millipore) dissolved in DMSO, well beyond 300-fold over the AC50 of 92 nM, or equal volumes of DMSO for the controls. Islets were then subjected to 24 h of 1 mM STZ or buffer with or without TEPP46. STZ was used at 1 mM per well from 200 mM STZ solution, prepared immediately before treatment.

Metabolic assays. *Ad lib* or fasting (overnight) blood glucose and serum insulin were measured from tail vein blood using a glucometer (Accu-Chek) and a mouse insulin ELISA kit (Mercodia). For the glucose tolerance test (GTT), mice were fasted for 12-18 h overnight and a single i.p. glucose bolus of 2 g D-glucose (Sigma) per kg body weight was injected at time zero. Blood glucose was measured before and at times 15, 30, 60, 90, 120 min after glucose administration. For the insulin tolerance test (ITT), mice were fasted 4-6 h during the day and injected with 1 U recombinant human insulin (Humulin N, Eli Lilly) per kg body weight. Blood glucose was measured and was analyzed by calculating percent initial blood glucose of each post insulin administration time points.

Cell culture and plasmids. Mouse insulinoma Min6 cells were cultured in complete DMEM 4.5 g/L glucose (Fisher), 15% FBS (Gemini), 1x antibiotic-antimycotic (Fisher). Cells were transiently transfected using DMEM and Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. For mass spectrometry, Min6 cells were transfected with pCMV5-huPTPN2-Flag (WT or DA) or control pCMV5-Flag plasmids for 48 h, and cells were exposed to low glucose and serum-starvation (1 g/L glucose, <2% FBS) conditions for 2 h to optimize phosphorylated tyrosine substrates for the PTPN2 bait protein. Tyrosine phosphorylation after different stress conditions were optimized prior to the mass spectrometry through immunoblotting with anti-phosphotyrosine antibody (4G10 Platinum, Millipore). Flag-tagged *Pkm2* construct was generated by molecular cloning into pCMV5-Flag, and tyrosine residues of PKM2-FLAG were mutated using QuikChange II Site-Directed Mutagenesis Kit (Agilent).

RNA analysis. Total RNA was extracted using either the RNeasy Micro or Mini Kit (Qiagen) along with RNase-free DNase set (Qiagen), and cDNA was prepared using the SuperScript III First Strand Synthesis System (Invitrogen). Real time quantitative PCR (RT-qPCR) was carried out on CFX96 Real-Time PCR System (Bio-Rad) with SYBR Green Master Mix (Bio-Rad) with intron-spanning exonic primers listed in Table 2-5. Duplicate wells of each biological replicate with three or more biological replicates per genotype were used to calculate relative threshold cycle (Ct) values, which were normalized to reference genes *cyclophilin A* or *cyclophilin B*, which is indicated for each figure. To account for any differences in primer efficiency, all RT-qPCR results are shown as relative expression fold change, with the average of control samples set to 1. For the RNA-Seq, islets of all sizes from one mouse were used as one biological sample. Islet RNA samples from the STZ-treated animals were amplified for the RNA-Seq application using the Ovation RNA-Seq System V2 (Nugen). Illumina library preparation, sequencing on Illumina 2000/2500 instrument, and standard bioinformatics were performed at the JP Sulzberger Columbia Genome Center (New York, NY). Differential expression was determined by statistical testing based on negative binomial distribution using 'DESeq' package of the R software. Heatmaps were generated by normalizing FPKM values to the row mean and standard deviation values using the R software. Transcript reads were visualized with Integrative Genome Viewer (Broad Institute), and pathway analyses were performed with Ingenuity Pathway Analysis (Ingenuity Systems).

Immunoprecipitation, immunoblotting, and mass spectrometry. Protein extracts were prepared using the whole cell lysis protocol of the Active Motif co-IP Kit (Active Motif). For co-immunoprecipitation, ~100 μg (~1/2 lysate of ~1000 pooled islets) or ~500 μg (Min6 lysates) of protein were incubated with rabbit anti-PKM2 (Cell Signaling), rabbit anti-IgG (Millipore), mouse anti-FLAG M2 magnetic beads (Sigma), or mouse anti-IgG (Millipore) overnight at 4 °C with rotation. Antibody-conjugated proteins were pulled down using protein G DynaBeads (Invitrogen) unless using the antibody-conjugated beads (FLAG). Immunoprecipitated proteins were washed, eluted, and analyzed by immunoblotting. Immunoblotting was performed by running denatured protein samples on a NuPAGE 10% or 4-12% Bis-Tris gel (Invitrogen), transferring onto a nitrocellulose membrane (GE Healthcare) and probing with mouse anti-FLAG (1:1000, Sigma), mouse anti-TUBULIN (1:2000, Santa Cruz), mouse anti-TBP (1:1000, Abcam), rabbit anti-PKM2 (1:1000, Cell Signaling), mouse anti-PTPN2 (1:500, R&D Systems), rabbit anti-PTPN2 (1:1000, Santa Cruz), or mouse anti-PCAF (1:500, Santa Cruz). Immunoblots were developed by the standard ECL method (Millipore) after probing with HRP-conjugated secondary antibodies (1:10000, Jackson Immunoresearch). Western blot signals were quantified using the inverse density of light intensity measurements from the ImageJ software. FLAG-tagged Ptpn2 transfected cell lysates were co-immunoprecipitated and sent for mass spectrometry at Herbert Irving Cancer Center Proteomics Facility (New York, NY). Proteomics Facility predicted proteins with the following discovery criteria: minimum two peptides per predicted protein and <1% false discovery rate. Results were visualized with Scaffold (Proteome Software). Two independent rounds of mass spectrometry data were consolidated prior to validation.

Immunohistochemistry and microscopy. Pancreata were dissected out, fixed in 10% formalin for 24 h at 4 °C, washed, transferred to ethanol, embedded in paraffin, and sectioned 5 μm-thick. After deparaffinization, antigen retrieval was performed with 10 mM sodium citrate buffer (pH 6.0) or 10 mM Citrate-EDTA buffer (pH 6.2) for 20 min at 95 °C prior to immunofluorescence. Sections were incubated overnight at 4 °C with guinea pig anti-INSULIN (1:1000, Dako), rabbit anti-GLUCAGON (1:100, Dako), goat anti-CPA1 (1:1000, R&D Systems), and rabbit anti-PKM2 (1:50, Cell Signaling), followed by 488-, Cy3-, and Cy5-conjugated secondary antibodies (1:500, Jackson Immunoresearch) and DAPI (1:1000, Invitrogen). Slides were imaged with Confocal Laser Scanning Microscope 710 and processed with the Zen Software (Zeiss). For histology, paraffin sections were deparaffinized and stained by the standard H&E protocol. Multiple slides from at least 3 different animals per genotype were imaged.

Statistical analysis. Graphs were generated with the GraphPad Prism software. Results are expressed as mean ± SEM and significance testing was performed using a two-tailed Student's t-test. Survival analysis was done using the Kaplan-Meier method and differential survival was determined by Mantel-Cox test ($P < 0.05$).

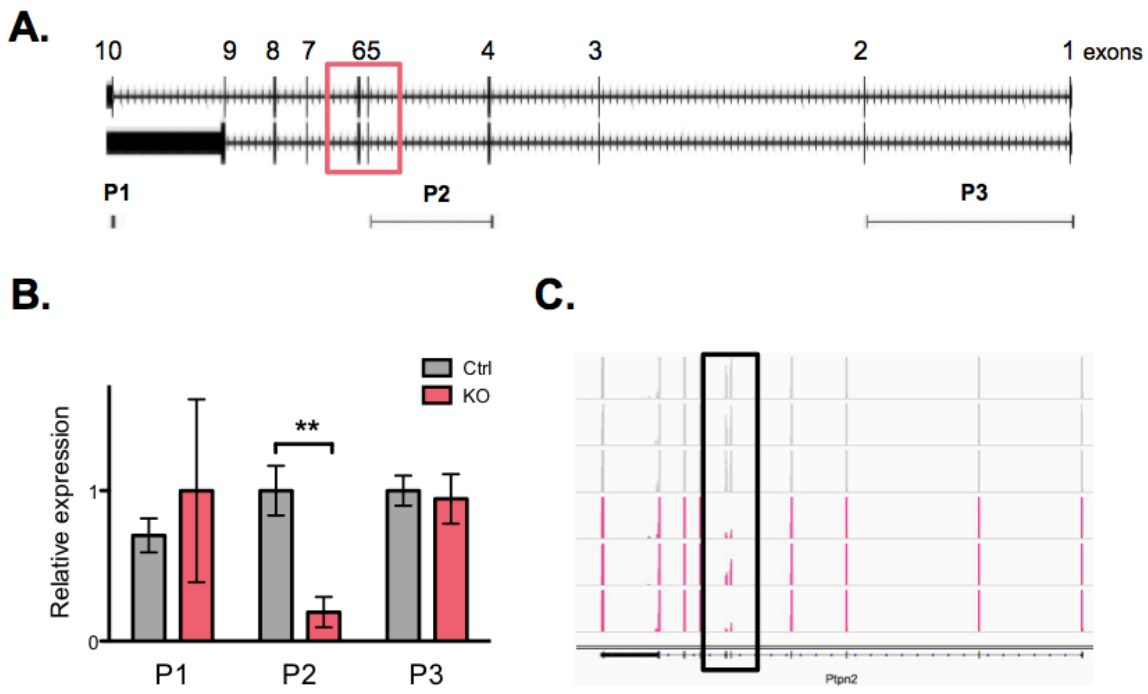


Figure 2-1. Ptpn2 βKO islets express *Ptpn2* mRNA lacking the excised exons.

(A) Schematic of the *Ptpn2* transcripts with the number of exons indicated above. Exons 5 and 6 are flanked by the loxP sites, indicated by the coral box. Below the schematic are primer sets P1, P2, and P3 aligned to the target sequences. **(B)** qPCR analysis of *Ptpn2* expression in control (Ctrl) or Ptpn2 βKO (KO) islets with floxed exon-specific (P2) or nonspecific (P1 and P3) primer sets, normalized to *cyclophilin B* reference gene (Ctrl N = 6, KO N = 4). $P < 0.01$ (**). **(C)** RNA-Seq analysis of *Ptpn2* aligned fragments in Ctrl (grey) and KO (coral histograms) islets, visualized by the IGV software, track range 10-50. Floxed exons are indicated by the black box.

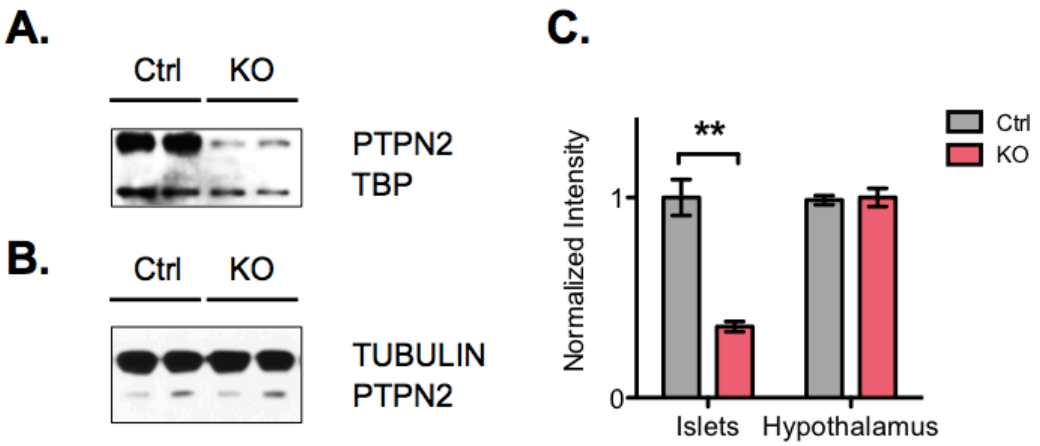


Figure 2-2. PTPN2 protein is deleted in Ptpn2 βKO islets.

Expression analysis by Western blot of whole cell extracts from control (Ctrl) and Ptpn2 βKO (KO) **(A)** islets and **(B)** hypothalamus, of PTPN2 (45 kDa) and loading controls TBP (37 kDa) or TUBULIN (55 kDa). **(C)** Quantification of PTPN2 signal intensity normalized to that of loading controls (N = 3 for each, although not shown in A and B). $P < 0.01$ (**).

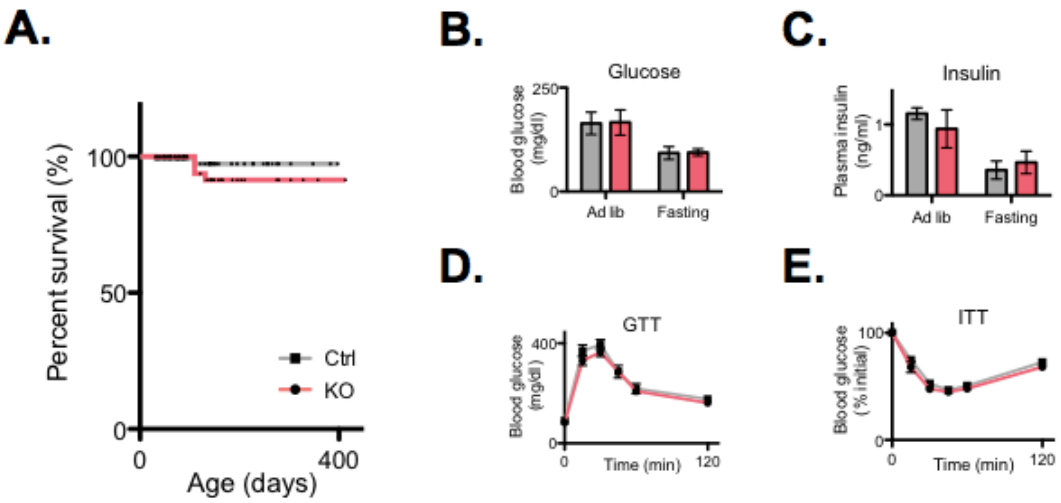


Figure 2-3. Ptpn2 βKO mice are euglycemic.

(A) Kaplan-Meier survival curve of control (Ctrl) and Ptpn2 βKO (KO) mice (Ctrl N = 176, KO N = 96). **(B-E)** Metabolic measurements in 10-week-old mice. *Ad lib* and fasting blood glucose **(B)** (Ctrl N = 16, KO N = 14), and insulin **(C)** (N = 3 each). **(D)** Glucose tolerance test (GTT) and **(E)** insulin tolerance test (ITT) (Ctrl N = 8, KO N = 10). For each panel, color key as in **(A)**, and statistical tests were not significant.

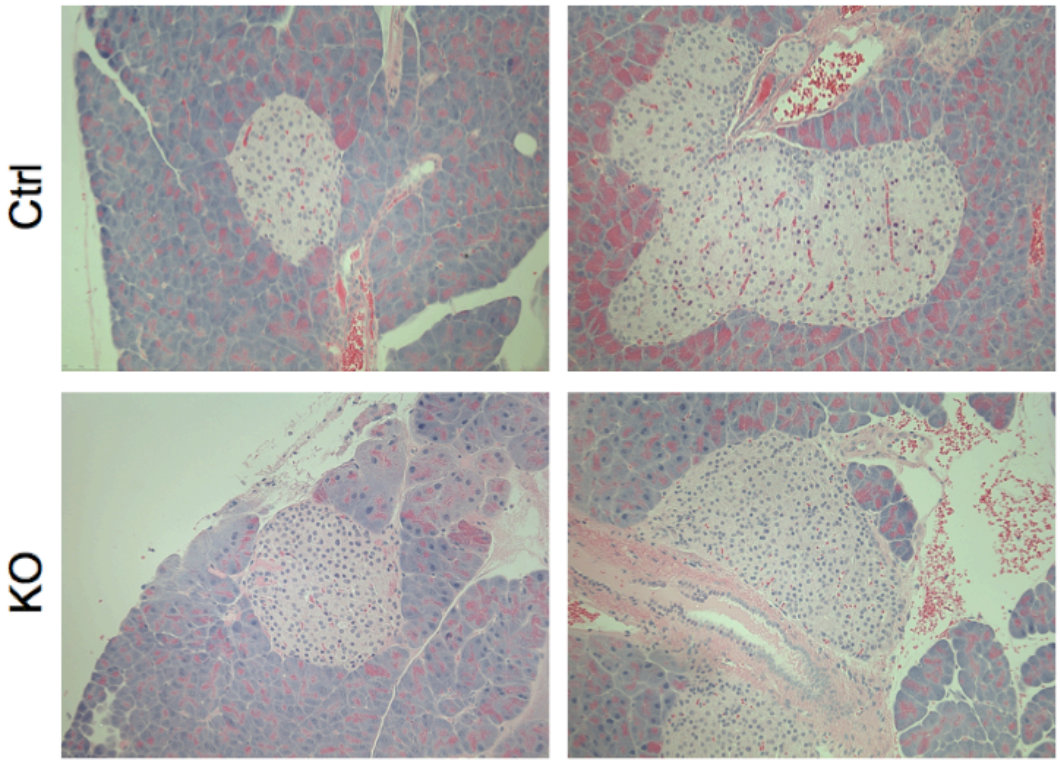
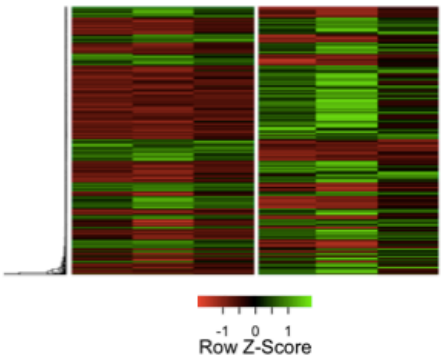


Figure 2-4. Islet morphology is not altered in Ptpn2 βKO versus control islets.

H&E-stained paraffin sections showing representative of islet morphology in Ptpn2 βKO (KO) and control (Ctrl) mice. Multiple sections from at least 3 animals of each genotype were stained.

A.



B.

Gene	Log ratio	P
Islet hormone transcripts		
Ins1	0.069	0.513
Ins2	-0.029	0.883
Gcg	**-0.201**	**0.033**
Sst	0.221	0.344
Ppy	0.359	0.190
Key transcription factors		
Pdx1	-0.233	0.307
Nkx2-2	**-0.414**	**0.003**
Nkx6-1	-0.192	0.098
Foxo1	-0.210	0.058
Mafa	**-0.419**	**0.034**
Isl1	-0.101	0.342
Insulin secretion related		
Chga	**-0.341**	**0.003**
Slc2a2	-0.299	0.394
Pclo	-0.428	0.080
Abcc8	-0.037	0.701
Kcnj11	-0.111	0.291

C.

Canonical pathways	Score
Integrin signaling	5.2
ILK signaling	5.1
Toll-like receptor signaling	4.9
Sertoli cell-Sertoli cell junction signaling	4.7
mTOR signaling	4.0

Figure 2-5. RNA-Seq analysis of Ptpn2 βKO and control islets.

(A) RNA-Seq identified 1212 transcripts that are differentially expressed in control (Ctrl) and Ptpn2 βKO (KO) islets isolated from 8-week-old mice (N = 3 each). DESEQ P < 0.05. **(B)** Table of key islet hormone transcripts, transcription factors expressed highly but not necessarily exclusively in beta cells, and genes associated with secretory granules or insulin secretory function. Statistically significant genes are in bold (DESEQ P < 0.05). Log ratio is the log fold change of Ctrl to KO baseMean values from DESEQ analysis, where negative sign indicates downregulation in the KO samples. P, DESEQ P values. **(C)** Top 5 canonical pathways from IPA analysis of differentially expressed transcripts. Score, pathway enrichment score calculated from -log(Fisher P), with threshold set to P < 0.05 or -log(0.05) > 1.3.

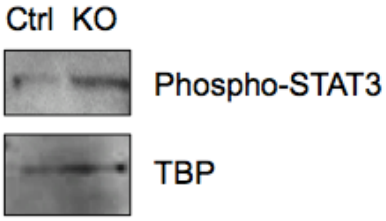


Figure 2-6. STAT3 is hyperphosphorylated in the Ptpn2 βKO islets.

Western blot analysis of phosphorylated STAT3 at tyrosine 705 (86 kDa) in Ptpn2 βKO (KO) and control (Ctrl) islets and loading control TBP (37 kDa).

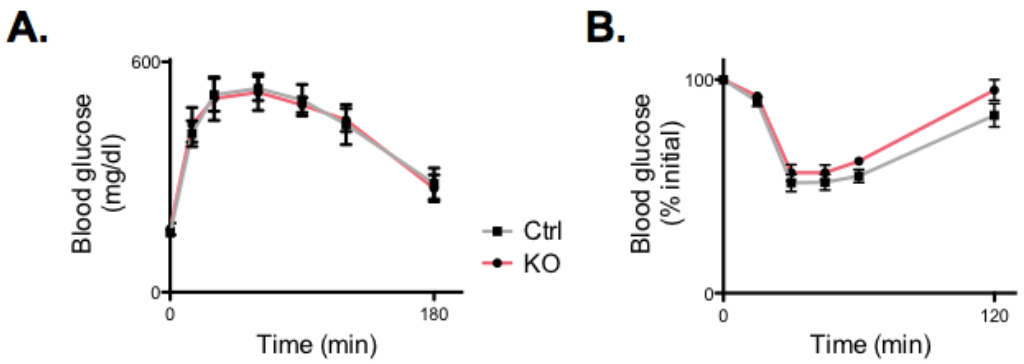


Figure 2-7. Ptpn2 βKO mice develop glucose intolerance upon high fat feeding comparable to that of the control mice.

Three cohorts of animals were given high fat diet (HFD) at 10 weeks of age, and they were monitored for weight gain, *ad lib* and fasting blood glucose, as well as glucose and insulin tolerance. In the initial cohort, in which Ptpn2 βKO animals gained ~8 g less weight than the control, they exhibited improved glucose tolerance, but in the latter two cohorts, where weight gain was comparable, metabolic differences were not detected. Representative **(A)** GTT and **(B)** ITT from one of the two latter cohorts of animals with comparable weight gain after 10 weeks of HFD is shown (N = 5 each).

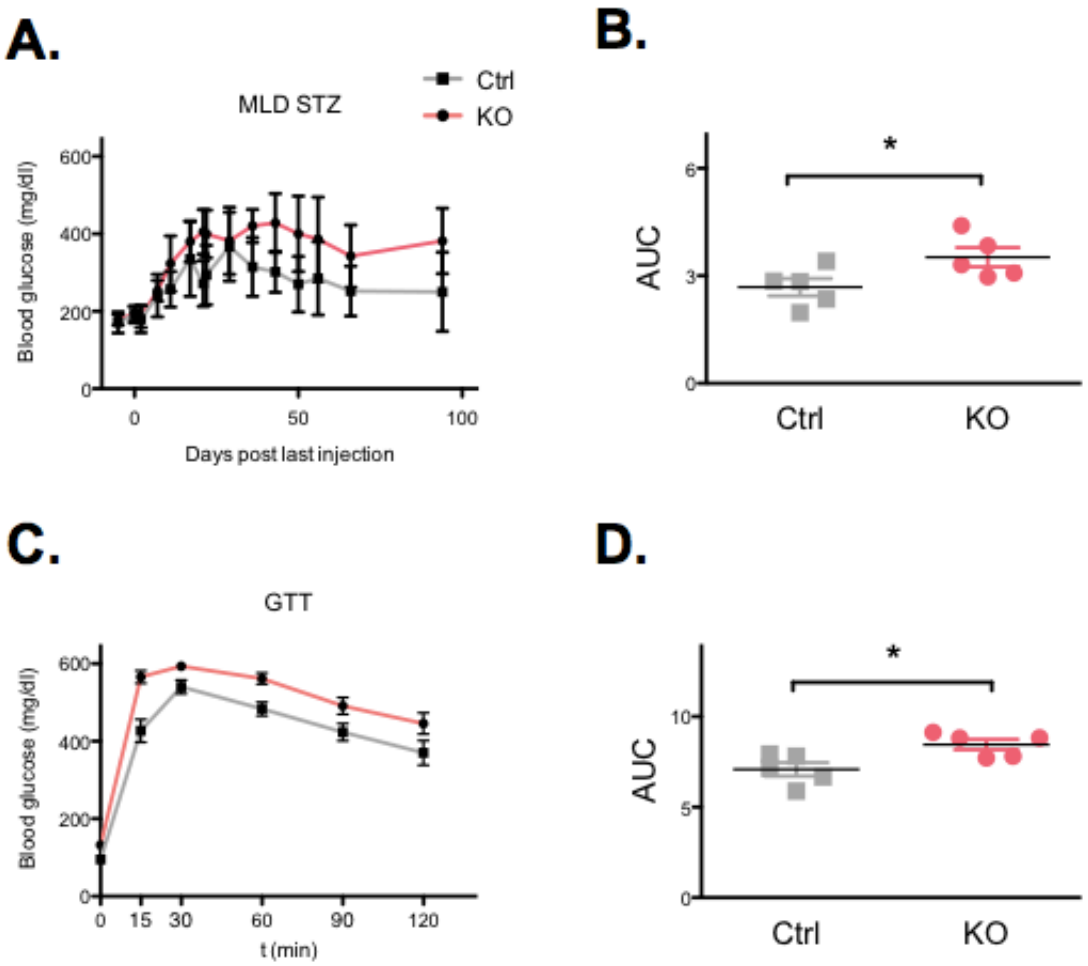


Figure 2-8. Ptpn2 βKO mice develop heightened diabetes with low-dose STZ.

(A) *Ad libitum* blood glucose levels during (day -5 to day 0) and after five consecutive STZ administrations in multiple low-dose (MLD) STZ-induced diabetes in control (Ctrl) and Ptpn2 βKO (KO) mice (N = 5 each). **(B)** Area under the curve (AUC) of **(A)**. **(C)** Glucose tolerance test (GTT) of MLD STZ mice three weeks after the final STZ administration. **(D)** AUC of **(C)**. For each panel, color key as in **(A)**. $P < 0.05$ (*).

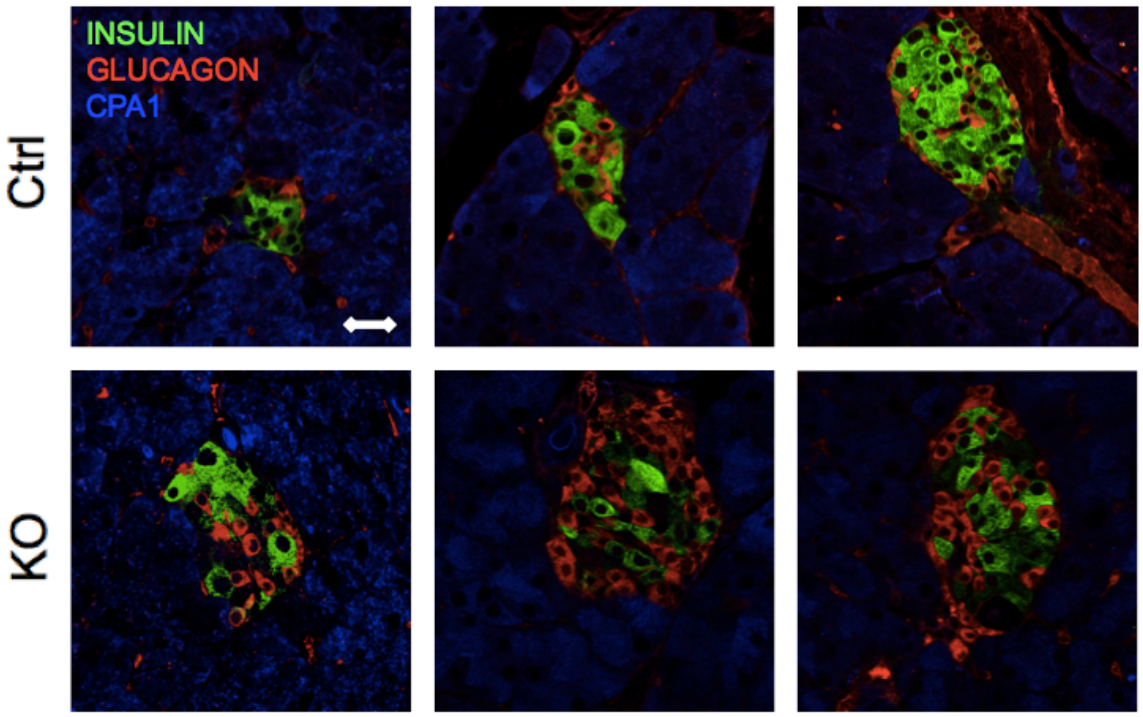


Figure 2-9. Ptpn2 βKO islets display increased beta cell loss in STZ-diabetes.

Islets from control (Ctrl) and Ptpn2 βKO (KO) animals 4 months after multiple low-dose injections of STZ were stained with anti-INSULIN (green), anti-GLUCAGON (red), and exocrine marker anti-CPA1 (blue). Multiple sections from at least 3 animals from different cohorts of STZ were stained. Merged images are shown. Scale bar, 40 μm.

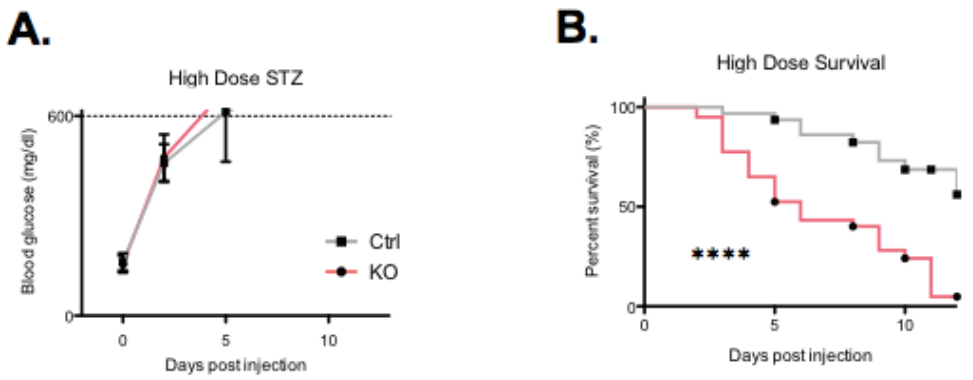
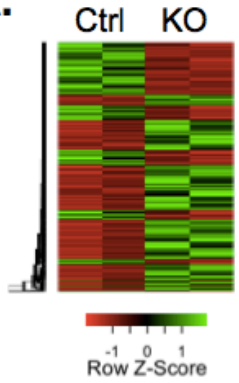


Figure 2-10. Ptpn2 βKO mice show survival impairment with high-dose STZ.

(A) *Ad libitum* blood glucose levels of surviving control (Ctrl) and Ptpn2 βKO (KO) animals after single high-dose STZ induced diabetes (Ctrl N = 16, KO N = 17). The limit of our glucometer (600 mg/dl) is indicated by the dotted line. In order to plot the measurements beyond our limit, unreadable high measurements were assigned the same nonspecific value above the limit (800 mg/dl). **(B)** Kaplan-Meier survival curve of high-dose STZ mice (Ctrl N = 31, KO N = 40). Color key as in **(A)**. $P < 0.0001$ (****).

A.



B.

Gene	Log ratio	P
Islet hormone transcripts		
Ins1	-0.608	0.814
Ins2	-0.895	0.605
Gcg	1.057	0.216
Sst	-0.819	0.077
Ppy	**-2.023**	**<0.001**
Key transcription factors		
Pdx1	-1.113	0.103
Nkx2-2	-0.832	0.087
Nkx6-1	-1.249	0.094
Foxo1	0.725	0.138
Mafa	-0.549	1.000
Isl1	-0.655	0.157
Insulin secretion related		
Chga	-0.296	0.752
Slc2a2	-0.797	0.628
Pclo	**-1.508**	**0.001**
Abcc8	**-1.307**	**0.003**
Kcnj11	-0.595	0.233

C.

Canonical pathways	Score
Mitochondrial dysfunction	14.3
Oxidative phosphorylation	11.4
TCA cycle II (Eukaryotic)	6.2
EIF2 signaling	6.1
mTOR signaling	4.8

D.

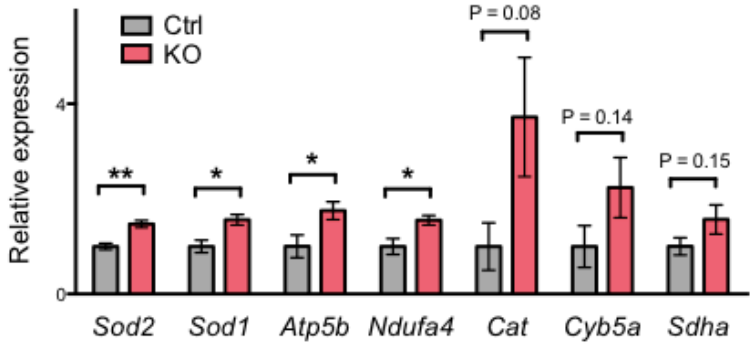
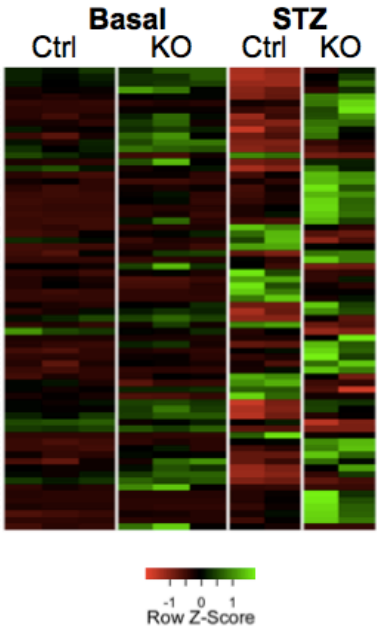


Figure 2-11. Transcriptome analysis of STZ-Ptpn2 βKO islets.

(A) RNA-Seq identified 819 transcripts that are differentially expressed in control (Ctrl) and Ptpn2 βKO (KO) islets isolated 5 days after a single high-dose STZ administration, shown as a heatmap (N = 2 each). DESEQ $P < 0.05$. **(B)** Table of key islet hormone transcripts, transcription factors expressed highly but not necessarily exclusively in beta

cells, and genes associated with secretory granules or insulin secretory function. Statistically significant genes are in bold (DESEQ $P < 0.05$). Log ratio and P, as in Figure 2-5. **(C)** Top 5 canonical pathways from IPA analysis of differentially expressed transcripts. Score, as in Figure 2-5. **(D)** Validation qPCR analysis of genes in the top category, mitochondrial dysfunction, normalized to *cyclophilin A* reference gene (Ctrl N = 5, KO N = 4). $P < 0.05$ (*), $P < 0.01$ (**).

A.



B.

Pathway analysis	Score
Network	
Cell death and survival, free radical scavenging	51.1
Cancer, organismal injury and abnormalities	27.0
Gene expression, protein synthesis	26.0
Toxic response	
Increases transmembrane potential of mitochondria and mitochondrial membrane	3.3
Oxidative stress	3.2
NRF2-mediated oxidative stress response	3.1
Canonical pathways	
mTOR signaling	4.6
EIF2 signaling	3.6
Phospholipase C signaling	3.0

Figure 2-12. Integration of unstressed and post-STZ gene expression profiles.

(A) Integration of the 1212 differentially expressed genes from unstressed (basal) islets and 819 differentially expressed genes in islets after STZ yielded 71 common genes, shown as a heatmap. DESEQ $P < 0.05$. **(B)** Top 3 network, toxic response, and canonical pathway analyses from IPA. Score, as in Figure 2-5.

A.

Canonical pathways	Score
TCA cycle II (Eukaryotic)	11.7
tRNA charging	10.4
Glycolysis I	8.2
Gluconeogenesis I	8.2
Protein ubiquitination pathway	7.6

B.

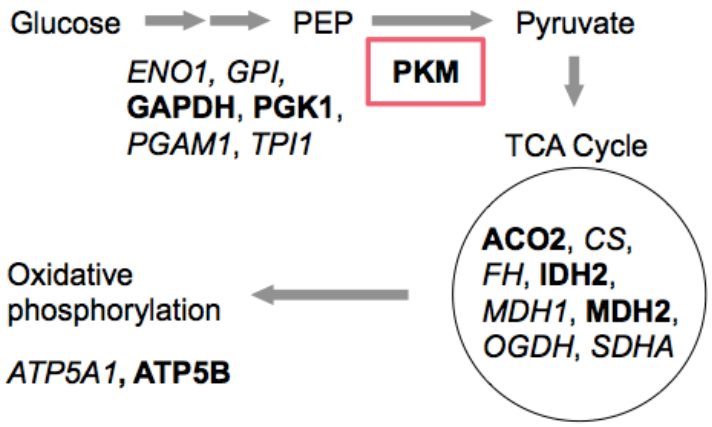


Figure 2-13. PTPN2 interacting proteins are involved in glucose metabolism.

Tandem mass spectrometry (MS) analysis of eluates from FLAG immunoprecipitation of Min6 cells transfected with FLAG-tagged human PTPN2. **(A)** Top 5 canonical pathways from IPA analysis of MS-identified PTPN2-interacting proteins (N = 2). Score, as in Figure 2-5. **(B)** Schematic of the glucose metabolic flux. PTPN2 interacting proteins identified in both rounds of MS are indicated in bold, and those identified in only one round of MS are in italics. Coral box highlights pyruvate kinase M. PEP, phosphoenolpyruvate. TCA Cycle, tricarboxylic acid or Krebs cycle. Names of MS identified proteins are listed in Table 2-2.

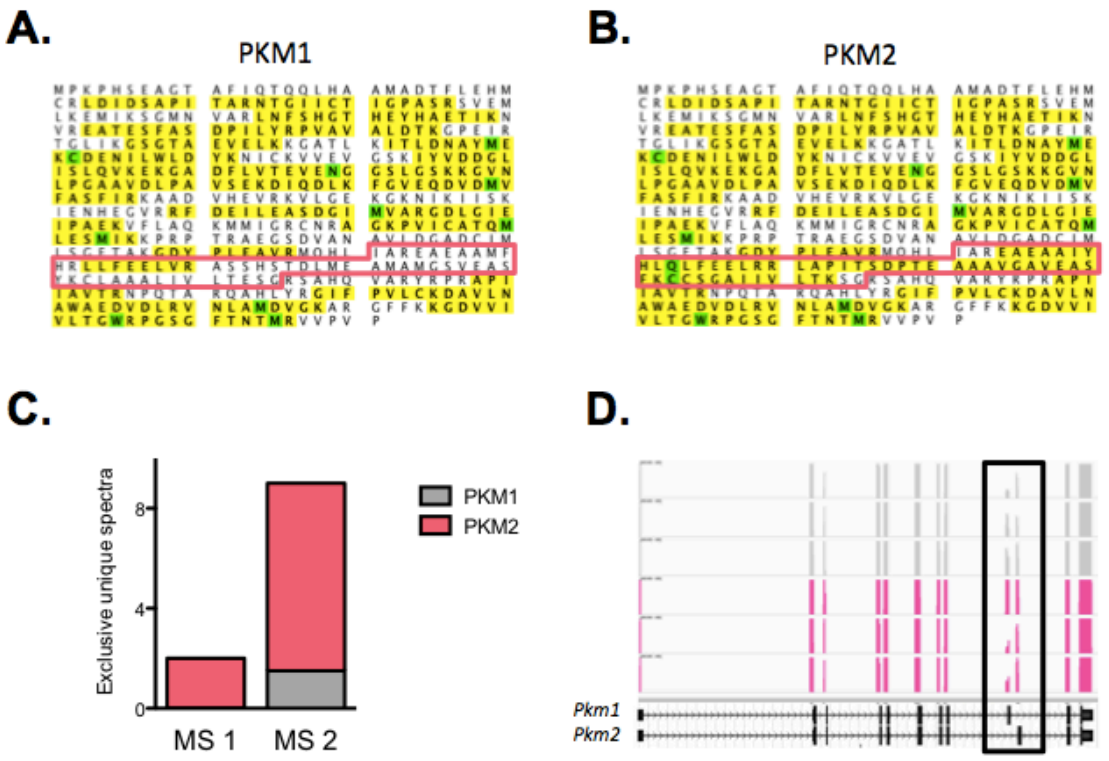


Figure 2-14. PTPN2 interacts with PKM1 and PKM2.

Peptides mapping to PKM1 **(A)** or PKM2 **(B)** proteins are highlighted in yellow. Representative coverage of one of the hPTPN2-WT-Flag-transfected sample is shown. Most peptides map to both isoforms. Region of 55 amino acids of either exon are indicated by the coral outline. Amino acids in green are tags used in the MS protocol. **(C)** Spectral counts of peptides mapping exclusively to PKM1 or PKM2 in both rounds of mass spectrometry (MS1 and MS2). **(D)** RNA-Seq analysis of *Pkm*-aligned fragments in control (grey) and Ptpn2 βKO (coral) islets (IGV track range 200-300). Alternatively spliced exons indicated by the black box (exon 9 excised out in *Pkm2*, exon 10 excised out in *Pkm1*).

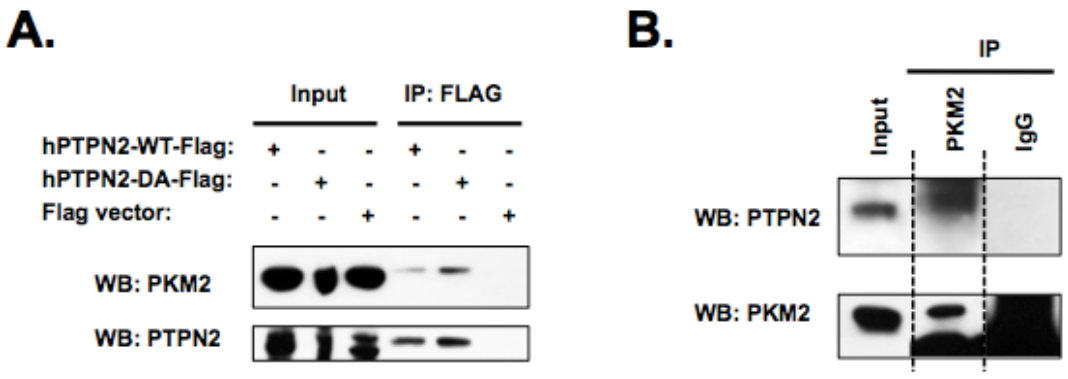


Figure 2-15. PTPN2 interacts with PKM2 *in vitro* and *in vivo*.

(A) Validation immunoprecipitation (IP) and Western blot using identical IP conditions as mass spectrometry sample preparation. Western blot representative of three separate transfections for each plasmid. **(B)** Reciprocal IP with PKM2 antibody using whole cell extracts from pooled mouse islets. IgG, non-specific antibody control for IP. Dashed line shows cropped boundaries.

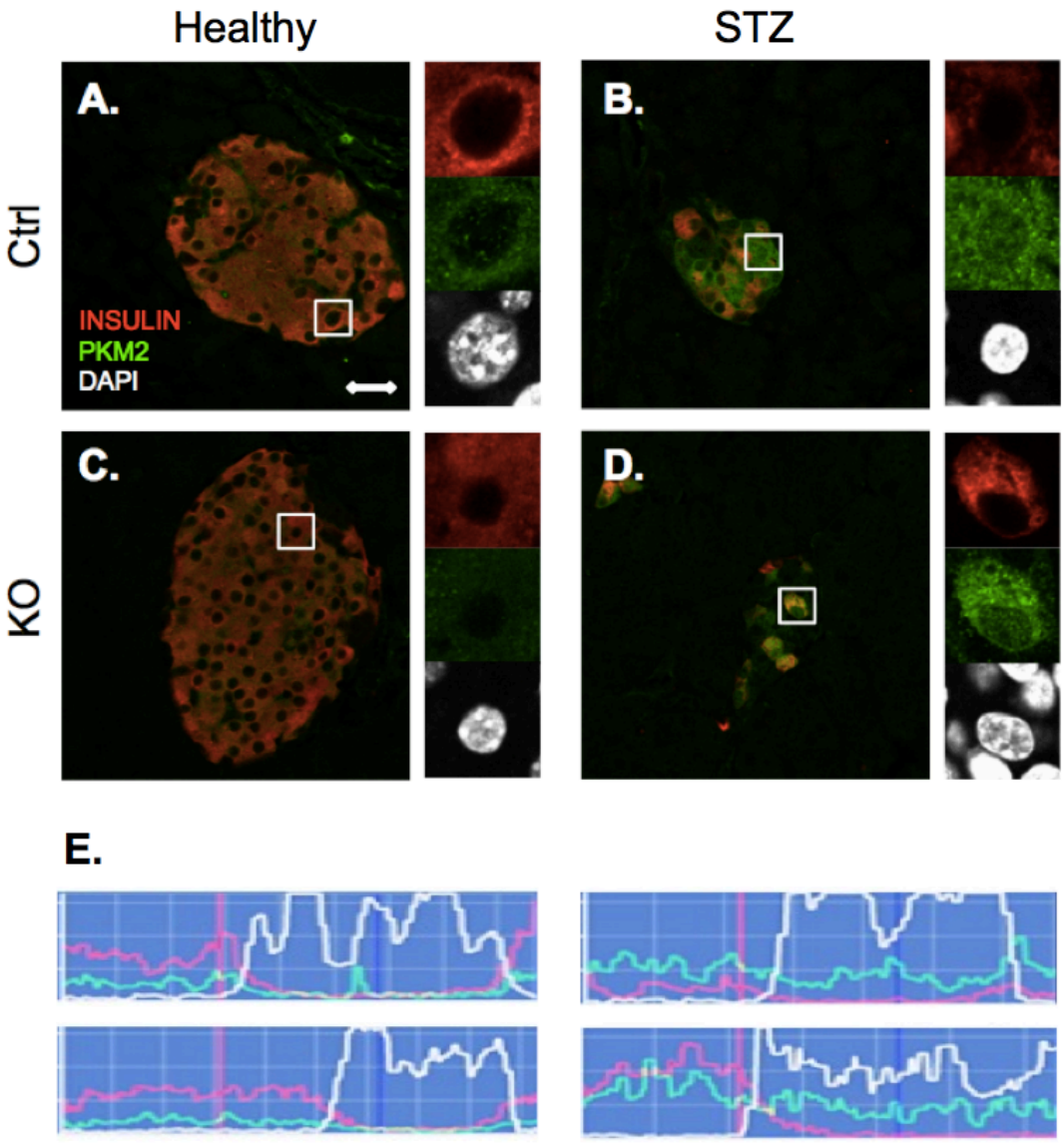


Figure 2-16. PKM2 translocates to the nuclei in diabetic islets.

Subcellular localization of PKM2 in healthy and STZ-diabetic islets. Diabetic condition is within one week after a single high-dose injection of STZ. Control islets from healthy **(A)** and STZ **(B)** mice, and Ptpn2 βKO islets from healthy **(C)** and STZ **(D)** mice were stained with anti-INSULIN (red), anti-PKM2 (green), and nuclear stain DAPI (white). Multiple sections from at least 3 animals of each condition were stained. Merged images

are shown. Framed regions are zoomed and separated by channel on the right side. Scale bar, 30 μm. **(E)** Line profiles of relative fluorescence intensities of INSULIN (red), PKM2 (green), and DAPI (white) signals from the framed cells of corresponding panels in **(A)-(D)**.

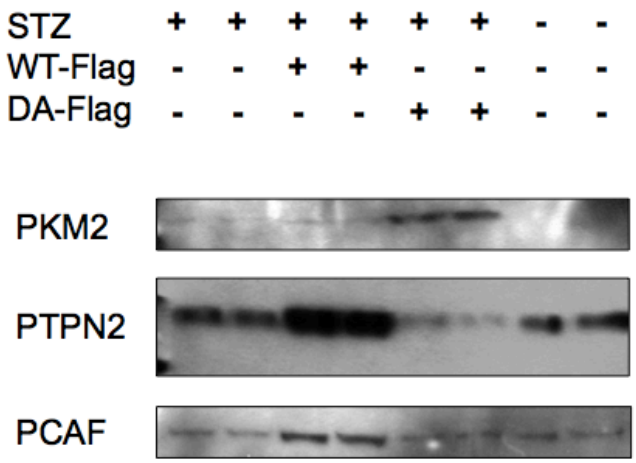


Figure 2-17. PKM2 nuclear translocation is modulated by PTPN2.

Min6 cells were transfected with constructs encoding wildtype (WT-Flag) and functional mutant (DA-Flag) human PTPN2. After exposing the cells to 1 mM STZ for 24 hours, nuclear fractions were extracted. Cells transfected with mutant PTPN2 displayed decreased PTPN2 and increased nuclear PKM2. Cells not subjected to STZ stress did not accumulate nuclear PKM2. PCAF was used as a loading control.

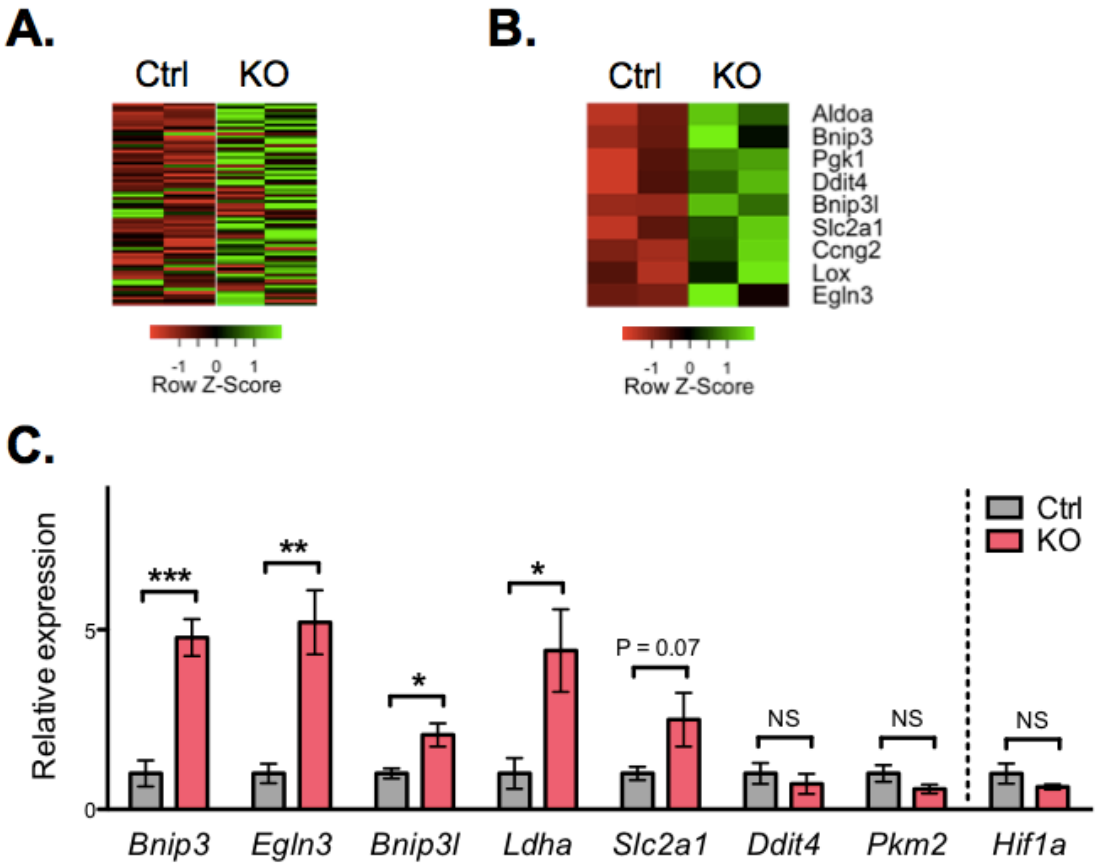


Figure 2-18. A subset of upregulated transcripts are HIF-1α targets.

(A) 70 validated and predicted HIF-1α-induced genes were compiled from literature. Regardless of the statistical significance from DESEQ analysis, visual inspection via heatmap representation of the FPKM values shows an overall upregulation of HIF-1α-induced genes in the Ptpn2 βKO (KO) versus control (Ctrl) islets from STZ-diabetic mice. **(B)** Statistically significant HIF-1α target transcripts are shown. DESEQ $P < 0.05$. **(C)** Validation qPCR analysis of HIF-1α target genes, as well as expression of *Hif1a* and *Pkm2*, normalized to *cyclophilin A* reference gene (Ctrl N = 5, KO N = 4). $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). NS, not significant.

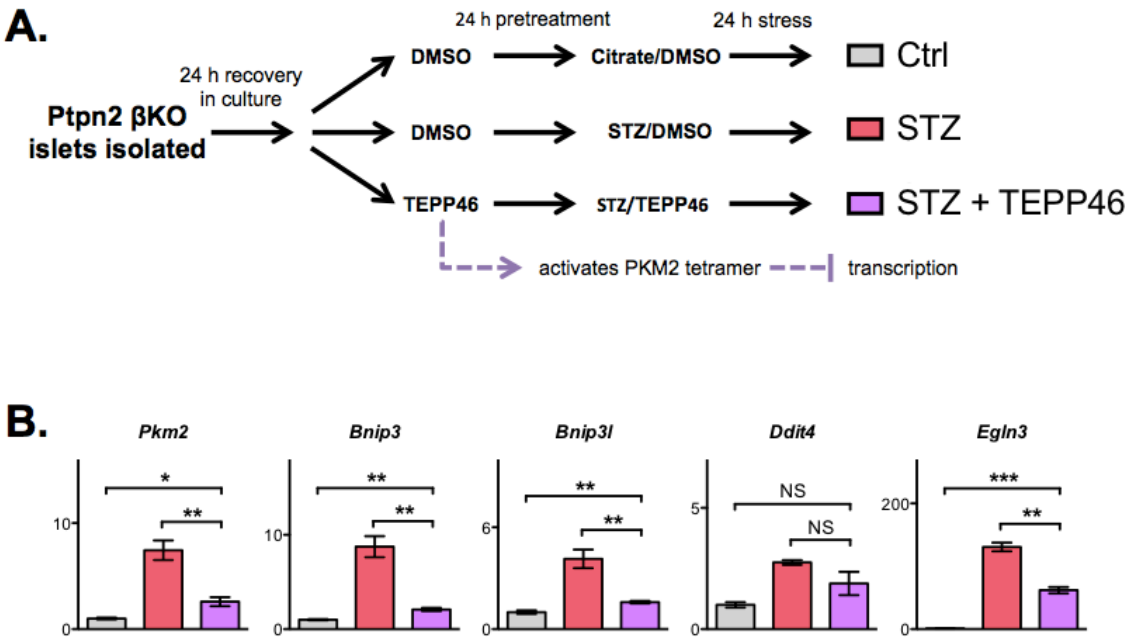


Figure 2-19. *Ex vivo* reconstitution of proposed PTPN2 function in Ptpn2 βKO islets rescues the transcriptional alterations.

(A) Schematic of the reconstitution experiment. Islets were isolated from Ptpn2 βKO mice, and islets in culture were stressed with STZ with or without the PKM2 tetramer activator, TEPP46, after a period of pretreatment conditions. Equal amounts of DMSO were used as a vehicle control for TEPP46, and sodium citrate buffer was used as a vehicle control for STZ. Function of TEPP46 is indicated below. **(B)** Gene expression changes in isolated Ptpn2 βKO islets treated with STZ and TEPP46 compared to no treatment islets or islets treated with STZ alone, normalized to *cyclophilin A* reference gene (N = 3 each). *Ptpn2* transcripts were assayed with primer sets in Figure 2-1 and the majority of the transcripts were floxed transcripts in all samples compared to islets from control mice. Y-axis labels (relative expression) left out for visual simplicity. Color scheme as in **(A)**. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). NS, not significant.

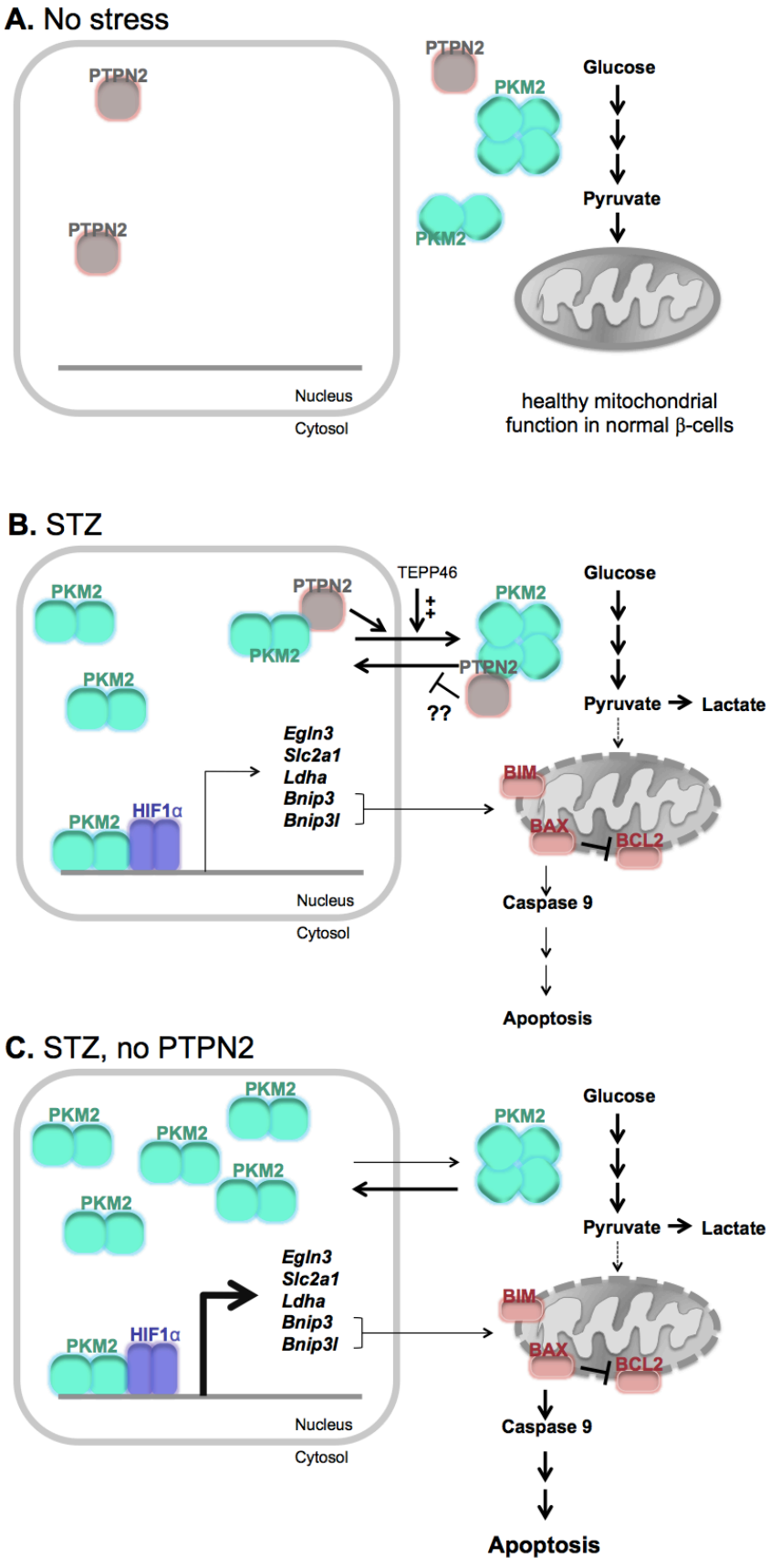

A. No stress
PTPN2
PTPN2
PTPN2
PKM2
PKM2
Glucose
Pyruvate
Nucleus
Cytosol
healthy mitochondrial
function in normal β-cells
B. STZ
PKM2
PKM2
PTPN2
PKM2
TEPP46
++
PKM2
PTPN2
??
Glucose
Pyruvate → Lactate
Egln3
Slc2a1
Ldha
Bnip3
Bnip3l
PKM2
HIF1α
BIM
BAX
BCL2
Caspase 9
Nucleus
Cytosol
Apoptosis
C. STZ, no PTPN2
PKM2
PKM2
PKM2
PKM2
PKM2
Glucose
Pyruvate → Lactate
Egln3
Slc2a1
Ldha
Bnip3
Bnip3l
PKM2
HIF1α
BIM
BAX
BCL2
Caspase 9
Nucleus
Cytosol
Apoptosis

Figure 2-20. Proposed model depicts PTPN2 as the regulator of nuclear PKM2 in pro-apoptotic beta cells.

(A) In healthy beta cells with normal mitochondrial function, PKM2 is cytosolic. **(B)** In pro-apoptotic beta cells, as in STZ stress, PKM2 translocates to the nucleus and mediates HIF-1α-responsive transcription. PTPN2 balances the levels of nuclear PKM2, as does tetramer activator TEPP46. It is also possible that PTPN2 binds to the cytosolic PKM2, stabilizing its tetramer conformation. **(C)** In the absence of PTPN2, nuclear PKM2 accumulates, and activates HIF-1α/PKM2-mediated transcription with vigor, leading to increased beta cell death and poor survival upon STZ stress.

Table 2-1. RNA-Seq analysis of differentially expressed genes in diabetic islets

A. Top 10 upregulated genes

Gene	Ctrl	KO	Fold change	P	Description	Other names
Cyp2b10	0.7	172.6	258.55	0.0328	cytochrome P450, family 2, subfamily b, polypeptide 10	Cyp2b1
Cidea	1.2	81.4	66.08	< 0.0001	cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	Cide-A
Egln3	0.6	36.7	65.14	0.0299	egl-9 family hypoxia-inducible factor 3	Hif-p4h-3
Hamp	7.1	401.9	56.31	0.0455	hepcidin antimicrobial peptide	Hepc1
Ppbp	2.6	62.5	24.35	< 0.0001	pro-platelet basic protein	Cxcl7
Il22ra2	1.3	25.4	19.04	0.0070	interleukin 22 receptor, alpha 2	Il-22bp
Gys2	7.9	138.4	17.50	< 0.0001	glycogen synthase 2	Lgs
Lhpp	1.1	19.5	17.29	0.0236	phospholysine phosphohistidine inorganic pyrophosphate phosphatase	Hdhd2b
Lhx8	1.1	18.7	16.61	0.0297	LIM homeobox protein 8	Lhx7
Ehhadh	91.1	1429.3	15.69	< 0.0001	enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	Lpb

B. Transcripts only detected in KO islets

Gene	Ctrl	KO	Fold change	P	Description	Other names
Cxcl13	0	52.0	-	< 0.0001	chemokine (C-X-C motif) ligand 13	Angie2
Hoxd9	0	19.3	-	0.0041	homeobox D9	Hox-5.2
Slc17a1	0	17.9	-	0.0055	solute carrier family 17, member 1	Npt1
Hoxc6	0	15.8	-	0.0115	homeobox C6	Hox-3.3
Coro6	0	15.1	-	0.0153	coronin 6	-
Aox3	0	16.3	-	0.0229	aldehyde oxidase 3	Aoh2
H2-M9	0	30.2	-	0.0248	histocompatibility 2, M region locus 9	M9
Hgd	0	16.8	-	0.0252	homogentisate 1,2-dioxygenase	Aku
Il31ra	0	11.6	-	0.0381	interleukin 31 receptor A	Gpl
Tectb	0	11.3	-	0.0420	tectorin beta	Tctnb

C. Top 10 downregulated genes

Gene	Ctrl	KO	Fold change	P	Description	Other names
Fam171a2	13.0	0.3	0.023	0.0359	family with sequence similarity 171, member A2	RGD1307966
Gm872	14.7	0.5	0.036	0.0282	chromosome 12 open reading frame 55	C12orf55
Tas1r1	13.0	0.5	0.040	0.0350	taste receptor, type 1, member 1	Tas1r1
Gm10532	14.7	0.6	0.041	0.0498	predicted gene 10532	-
Ipw	14.6	0.6	0.042	0.0427	imprinted gene in the Prader-Willi syndrome region	ncRNA00002
Rs1	12.2	0.5	0.043	0.0480	retinoschisin 1	Xlrs1
Clic3	19.9	1.1	0.057	0.0153	chloride intracellular channel 3	-
4930544O15Rik	14.3	0.8	0.058	0.0431	testis expressed 38	Tex38
Grm7	18.6	1.1	0.061	0.0229	glutamate receptor, metabotropic 7	mGlu7
1700019N12Rik	28.7	2.1	0.074	0.0468	testis expressed 40	Tex40

Table 2-1. RNA-Seq analysis of differentially expressed genes in diabetic islets.

DESEQ analysis of differentially expressed transcripts from control (Ctrl) and Ptpn2 βKO (KO) islets isolated 5 days after STZ treatment. After filtering for statistical significance (DESEQ $P < 0.05$), transcripts were sorted by the magnitude of fold change (KO/Ctrl baseMean values). Listed are the top 10 upregulated genes (A) and top 10 downregulated genes (C) in the order of fold change magnitude. In (B), transcripts only detected in KO islets are listed. No differentially expressed transcripts were only detected in Ctrl islets. Ctrl and KO columns list baseMean values from the DESEQ analysis (N = 2 each).

Table 2-2. Mass spectrometry-identified PTPN2-interacting proteins

Gene	WT	DA	EV	D:W	Spec%	Description	Accession
Hspa5	281	240	105	85	60	78 kDa glucose-regulated protein	P20029
Tubb5	209	188	79	90	60	cluster of Tubulin beta-5 chain	P99024
Acly	192	157	67	82	62	ATP-citrate synthase	Q3V117
Pkm	134	108	46	81	62	cluster of Pyruvate kinase M	P52480
Vcp	118	103	40	87	64	transitional ER ATPase	Q01853
Pdia3	78	63	16	81	77	protein disulfide-isomerase A3	P27773
Atp2a2	66	47	11	71	81	sarcoplasmic/endoplasmic reticulum calcium ATPase 2	O55143
P4hb	81	56	25	69	64	protein disulfide-isomerase	P09103
Hnrnpk	78	57	26	73	61	heterogeneous nuclear ribonucleoprotein K	P61979-2
Atp5b	61	55	26	90	55	ATP synthase subunit beta, mitochondrial	P56480
Ppm1b	49	51	19	104	62	protein phosphatase 1B, Mg dependent	Q99NF7
Uba1	42	25	6	60	82	ubiquitin-like activating enzyme 1	Q02053
Cct3	30	20	0	67	100	T-complex protein 1 subunit gamma	P80318
Gapdh	40	35	14	88	63	glyceraldehyde-3-phosphate dehydrogenase	P16858
Dpysl2	34	23	7	68	75	dihydropyrimidinase-related protein 2	O08553
Ganab	29	20	3	69	88	isoform 2 of Neutral alpha-glucosidase AB	Q8BHN3
Pdia6	27	24	5	89	80	protein disulfide-isomerase A6	Q3TML0
Eif4b	39	39	19	100	51	eukaryotic translation initiation factor 4B	Q8BGD9
Ddx3x	29	20	5	69	80	cluster of ATP-dependent RNA helicase	Q62167
Actn4	28	11	0	39	100	cluster of Alpha-actinin-4	P57780
Alb	39	25	13	64	59	serum albumin	P07724
Cltc	24	12	0	50	100	clathrin heavy chain	Q5SXR6
Hspa9	28	17	6	61	73	stress-70 protein, mitochondrial	P38647
Atp1a1	28	15	5	54	77	cluster of Sodium/potassium-transporting ATPase subunit alpha-1	Q8VDN2
Aco2	20	13	0	65	100	aconitate hydratase, mitochondrial	Q99KI0
Ero1lb	17	21	3	124	84	ERO1-like protein beta	Q8R2E9
Hyou1	23	8	0	35	100	hypoxia up-regulated protein 1	Q9JKR6
Ddx5	29	19	9	66	63	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	Q61656
Cct4	18	12	0	67	100	T-complex protein 1 subunit delta	P80315
Mcm2	21	12	2	57	88	DNA replication licensing factor	P97310
Hnrnpu	21	7	0	33	100	heterogeneous nuclear ribonucleoprotein U	Q8VEK3
Fasn	16	9	0	56	100	fatty acid synthase	P19096
Mcm3	18	7	0	39	100	DNA replication licensing factor	P25206
Mdh2	21	15	6	71	67	malate dehydrogenase, mitochondrial	P08249
Mthfd1	18	5	0	28	100	C-1-tetrahydrofolate synthase, cytoplasmic	Q922D8
Hadha	22	14	7	64	61	trifunctional enzyme subunit alpha	Q8BMS1
Mcm5	14	8	0	57	100	DNA replication licensing factor	P49718
Cct2	12	13	2	108	84	T-complex protein 1 subunit beta	P80314
Nsf	12	8	0	67	100	N-ethylmaleimide sensitive fusion protein	P46460
Ap1b1	14	6	0	43	100	cluster of AP-1 complex subunit beta-1	O35643
Ap3b2	9	10	0	111	100	cluster of AP-3 complex subunit beta-2	Q9JME5
Khsrp	10	6	0	60	100	far upstream element-binding protein 2	Q3U0V1
Idh2	13	9	4	69	64	isocitrate dehydrogenase, mitochondrial	P54071
Oat	5	9	0	180	100	ornithine aminotransferase, mitochondrial	P29758
Sfpq	14	7	4	50	62	splicing factor, proline- and glutamine-rich	Q8VIJ6
Thrap3	17	7	6	41	50	thyroid hormone receptor-associated protein 3	Q569Z6
Pgk1	8	9	4	113	53	cluster of Phosphoglycerate kinase 1	P09411
Srrm2	5	0	0	0	100	serine/arginine repetitive matrix protein 2	Q8BTI8

Table 2-2. Mass spectrometry-identified PTPN2-interacting proteins.

Tandem mass spectrometry (MS) analysis of eluates from FLAG-immunoprecipitation of Min6 cells transfected with Flag-tagged wildtype (WT) or substrate-trapping mutant (DA) forms of human PTPN2, or empty vector control (EV). Two rounds of MS were consolidated for proteins identified in both rounds. Numbers in WT, DA, and EV columns are spectral intensities from the second round of MS, which had much higher peptide coverage. D:W is the ratio of DA to WT spectral intensities, represented as a percentage. Spec% is the specificity, calculated by subtracting the background EV intensity from the average of WT and DA, represented as a percentage of the average of WT and DA. The list shows proteins of very specific interactions with PTPN2 (filtered by Spec% > 50%) in the order of specificity weighted by spectral intensity. Accession column corresponds to UniProt accession numbers.

Table 2-3. IPA-identified transcripts downstream of HIF-1α

Gene	Log ratio	Description	HIF-1α effect	HIF-1α state
Slc2a1	1.610	solute carrier family 2 (facilitated glucose transporter), member 1	Upregulates (22)	Activated
Sdc4	1.406	syndecan 4	Upregulates (1)	Activated
Ppara	2.012	peroxisome proliferator activated receptor alpha	Upregulates (4)	Activated
Pgk1	1.253	phosphoglycerate kinase 1	Upregulates (10)	Activated
Maff	1.927	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog F	Upregulates (1)	Activated
Lox	2.458	lysyl oxidase	Upregulates (9)	Activated
Igf1	2.165	insulin-like growth factor 1	Upregulates (2)	Activated
Hist1h1c	2.149	histone cluster 1, H1c	Upregulates (1)	Activated
Gbe1	1.457	glucan (1,4-alpha-), branching enzyme 1	Upregulates (1)	Activated
Fhl3	3.839	four and a half LIM domains 3	Upregulates (1)	Activated
Egln3	6.025	egl-9 family hypoxia-inducible factor 3	Upregulates (21)	Activated
Bnip3l	1.188	BCL-2/adenovirus E1B 19kDa interacting protein 3-like	Upregulates (7)	Activated
Bnip3	2.625	BCL-2/adenovirus E1B 19 kDa interacting protein 3	Upregulates (10)	Activated
Apoe	1.833	apolipoprotein E	Upregulates (2)	Activated
Aldoa	1.468	aldolase A, fructose-bisphosphate	Upregulates (6)	Activated
Kifc2	-1.654	kinesin family member C2	Upregulates (1)	Inhibited
Cyb5	3.072	cytochrome b5 type A (microsomal)	Downregulates (1)	Inhibited
Cyp2s1	-2.709	cytochrome P450, family 2, subfamily S, polypeptide 1	Regulates (2)	Affected
Hamp	5.815	hepcidin antimicrobial peptide	Regulates (2)	Affected

Table lists 19 differentially expressed transcripts from RNA-Seq that were categorized as being downstream of HIF-1α by the IPA software's Upstream Analysis function. Log ratio is the log fold change of control (Ctrl) to Ptpn2 βKO (KO) baseMean values, where negative sign indicates downregulation in KO samples. HIF-1α effect column lists known HIF-1α effect the transcript expression, with number of published articles to this effect in parenthesis. HIF-1α state column lists prediction of HIF-1α activation or inhibition upstream of these transcripts, based on the consolidation of log ratio direction with known HIF-1α effect.

Table 2-4. RNA-Seq analysis of genes encoding members of the BCL-2 family proteins

Gene	Ctrl	KO	Log ratio	P	Description	Other names
Pro-survival						
Bcl2	233.7	262.1	0.165	0.8427	B cell leukemia/lymphoma 2	
Bcl2l2	352.9	289.4	-0.286	0.6801	BCL-2-like 2	BCL-W
Bcl2a1a	9.3	0.9	-3.356	0.4803	BCL-2-related protein A1a	A1
Bcl2l10	3.3	1.6	-1.082	0.7798	BCL-2-like 10	Boo
Pro-apoptotic						
Bax	183.5	154.8	-0.246	0.8070	BCL-2-associated X protein	
Bok	2.4	16.2	2.778	0.1055	BCL-2-related ovarian killer	
Bak1	75.8	69.4	-0.127	0.8966	BCL-2-antagonist/killer 1	BAK
Bcl2l14	0.0	0.9	-	0.8899	BCL-2-like 14	BCL-GL
Bcl2l15	7.0	1.7	-2.013	0.4853	BCL-2-like 15	BFK
Pro-apoptotic, BH3-only						
Bad	34.8	48.0	0.464	0.4859	BCL-2-associated agonist of death	
Bik	11.0	8.0	-0.458	0.7431	BCL-2-interacting killer	
Bid	18.0	31.4	0.809	0.4239	BH3 interacting domain death agonist	
Hrk	2.8	5.1	0.844	0.8264	activator of apoptosis harakiri	DP5
Bcl2l11	143.4	299.2	1.061	0.1005	BCL-2-like 11	BIM
Pmaip1	10.9	14.3	0.384	0.6952	phorbol-12-myristate-13-acetate-induced protein 1	NOXA
Bbc3	44.5	19.4	-1.196	0.2427	BCL-2-binding component 3	PUMA
Bmf	77.3	123.0	0.670	0.3250	BCL-2-modifying factor	
Bnip3l	**350.0**	**797.2**	**1.188**	**0.0145 ***	**BCL-2/adenovirus E1B 19kDa interacting protein 3-like**	
Bnip3	**384.3**	**2371.3**	**2.625**	**0.0268 ***	**BCL-2/adenovirus E1B 19kDa interacting protein 3**	
Both pro-survival and pro-apoptotic						
Bcl2l1	354.3	337.1	-0.072	0.8607	BCL-2-like 1	BCL-X
Mcl1	900.0	1694.1	0.913	0.0621	myeloid cell leukemia 1	

Table lists transcripts encoding BCL-2 family proteins listed in Figure 1-4 and additional known BCL-2 family members, with expression baseMean values from control (Ctrl) and Ptpn2 βKO (KO) islets 5 days after STZ treatment. P, DESEQ P values. Log ratio is the log fold change of baseMean values. Statistical significance indicated by *. *Bcl2l1* and *Mcl1* both encode long and short forms that function in pro-survival and pro-apoptotic pathways, respectively. Pro-apoptotic *Bok* and *Bcl2l11*, as well as dual-functioning *Mcl1* showed increased expression in the KO samples, with P values just above 0.05.

Table 2-5. Primer sequences

Gene	Forward	Reverse	cDNA	gDNA
Ptpn2_P1	GATGTGCAAAGCAAGACCTGAAG	TGCAAGTCTTCTGCTGGTGG	141	141
Ptpn2_P2	AACACATGCTGCCATTTCTG	CACACTGAATCCCGTTTCCT	156	7469
Ptpn2_P3	ATCGAGCGGGAGTTCGA	TCTGGAAACTTGGCCACTC	110	12624
CycA	CAGTGCTCAGAGCTCGAAAGT	GTGTTCTTCGACATCACGGC	109	2324
CycB	GCAAAGTTCTAGAGGGCATGGA	CCCGGCTGTCTGTCTTGGT	63	686
Sod2	GCTTGATAGCCTCCAGCAAC	ACTGAAGTTCAATGGTGGGG	107	2903
Sod1	TACTGATGGACGTGGAACCC	GAACCATCCACTTCGAGCA	103	1953
Atp5b	GGGTCAGTCAGGTCATCAGC	CACAATGCAGGAAAGGATCA	90	1990
Ndufa4	TCTGGGCCTTCTTTCTTCAG	CCAGAGCCATGGAACAAACT	92	4703
Cat	CCCGCGGTCATGATATTAAGT	GATGAAGCAGTGGAAGGAGC	102	8154
Cyb5a	AGGACTTCTTCTCCACCAGGA	GCACAAAGACAGCAAGAGCA	96	19993
Sdha	AACAGAGAAGTGAAAGCCGC	CGCAGTTTCGAGGCTTCTT	97	5305
Bnip3	TGAAGTGCAGTTCTACCCAGG	CCTGTCGCAGTTGGGTTC	94	10628
Egln3	CTGGATAGCAAGCCACCATT	CATCAACTTCCTCCTGTCCC	110	17654
Bnip3l	CCATTTCCATTCTCATTGCC	AACAACAACTGCGAGGAAGG	110	9093
Ldha	GTGCCCAGTTCTGGGTTAAG	CTGGGTCCTGGGAGAACAT	99	917
Slc2a1	GAGTGTGGTGGATGGGATG	AACACTGGTGTCATCAACGC	99	11735
Ddit4	ACAGGTGCTCATCCTCGG	CTGGACAGCAGCAACAGTG	109	396
Pkm2	CTCTGGAGGCTGTTCGCATG	GGCGGAGTTCCTCGAATAGC	87	3581

Sequences of qRT-PCR primers designed using Primer-BLAST (NCBI). cDNA, base pair amplicon size of the transcript. gDNA, genomic DNA amplicon size, maximized whenever possible to avoid amplifying any contaminant genomic DNA.

Chapter Three: Identification of novel type 1 diabetes candidate genes by integrating non-obese diabetic mouse islet gene expression and CpG methylation data with human genome-wide association studies

Preface

Dr. Lori Sussel conceived the study. Dr. Peter Nagy and colleagues performed Illumina Genome Analyzer II. Dr. Xiaoyun Sun and I performed bioinformatics analyses for the RNA-Seq. Dr. Angad P. Singh, Dr. Wenji Ma, and Dr. Yufeng Shen performed DMR analysis for the methylation data. With guidance of Dr. Lori Sussel, I generated all other data. This work was supported by the Columbia University MD/PhD Program and the National Institutes of Health DP3 Type 1 Diabetes Impact Award.

Abstract

Genome-wide association studies (GWAS) in humans have linked over 40 loci to type 1 diabetes (T1D) susceptibility. Although many of the genes nested in these loci have not been functionally linked to disease pathogenesis, many are expressed in the human islets, suggesting that the beta cells of certain individuals may be predisposed to autoimmunity. To identify candidate genes that could influence beta cell susceptibility to insulitis, we integrated the human T1D GWAS data with differential RNA transcriptome and DNA methylome of insulitis-susceptible (NOD) versus resistant (NOR) murine pancreatic islets, prior to the autoimmune assault. Aligning the 312 positional candidate genes from 52 GWAS-orthologous regions to the 1745 genes that were differentially expressed in NOD versus NOR islets identified 30 known and novel diabetes candidate genes. Further incorporation of the DNA methylation data identified three potential islet-specific T1D candidates that may be involved in disease pathogenesis. Together, our study describes a useful approach in identifying T1D susceptibility genes through integrating mouse gene expression and DNA methylation data.

Introduction

The pathogenesis of type 1 diabetes (T1D) involves the interplay of injurious immune cells and the targeted pancreatic beta cells that result in autoimmune insulitis and gradual beta cell destruction over the course of months to years (Todd, 2010). Human genome-wide association studies (GWAS) have linked over 40 loci to T1D susceptibility (Barrett et al., 2009). However, many of these loci span large genomic regions containing multiple transcripts, hindering efforts to identify a specific causal gene in a given susceptibility locus. Identification of functional candidate genes through transcriptome analysis is further complicated by the multidimensional nature of T1D etiology. Twin studies have shown that T1D is highly concordant over a long-term follow up (Redondo et al., 2008), but even with identical genetics, monozygotic twins do not necessarily have the same risk for T1D at a single time point (Barnett et al., 1981), suggesting environmentally-induced non-genetic factors may also be involved. Consistent with this idea, epigenetic modifications such as DNA methylation and histone modifications have been associated with altered gene expression in T1D (Dang et al., 2013).

The non-obese diabetic (NOD) mouse strain develops spontaneous autoimmune diabetes similar to human T1D (Atkinson and Leiter, 1999; Makino et al., 1980). In NOD mice, immune cells are targeted to the pancreatic islets around 4-8 weeks, where they infiltrate and destroy the beta cells beginning around 12 weeks, and eventually cause overt diabetes by 16-24 weeks (Anderson and Bluestone, 2005). Gene expression profiling of NOD islets during the various stages of insulitis has been instrumental in identifying a conserved sequence of immunological activation and inflammatory responses during the progression to T1D (Carrero et al., 2013). Such an unbiased

approach to identify pivotal changes throughout T1D offers insights into the events leading up to disease initiation; however, a major challenge associated with these global expression datasets is prioritizing functional candidate genes and identifying those that are associated with human T1D initiation and progression. Similarly, development of diabetes in the NOD mice is not fully penetrant, suggesting DNA methylation may also play a role in predisposing NOD islets to insulitis.

The present study was designed to identify novel candidate genes that may contribute to T1D by integrating the gene expression and DNA methylation profiles of non-obese insulitis-resistant NOR and insulitis-sensitive NOD mouse islets with the human T1D GWAS susceptibility loci. Alignment to the mouse positional candidate genes identified in the human GWAS identified 30 differentially expressed candidate genes, some of which have been studied in the context of diabetes while others are novel candidates. We did not find any differentially methylated genes that were identified in the human GWAS, but three hypomethylated genes had correlated increases in gene expression, and are potential novel T1D candidates.

Results

Gene expression changes in NOD islets are enriched for human diabetes candidate genes.

To identify transcriptional changes associated with insulitis-prone islets, we isolated islets from NOD and NOR mice at 4 weeks of age, and identified their respective RNA profiles by high throughput sequencing. The four-week time point was chosen to

optimize the degree of islet maturity, while avoiding any secondary changes induced by insulitis (Carrero et al., 2013). To control for beta cell-specific changes, we used the genetically similar NOR strain that has the NOD congenic adaptive immune system, but does not initiate an autoimmune response (Prochazka et al., 1992). Transcriptome analyses identified 1745 transcripts that were differentially expressed (DESEQ $P < 0.05$) in the insulitis-prone islets. Biological pathway analysis of these genes revealed enrichment in immune response signatures (Fig. 3-1), in agreement with a previous NOD islet study that identified immune response signatures as early as 4 weeks, despite the islets having less than 0.1% CD4 and CD8 immune cells by flow cytometry (Carrero et al., 2013).

To discover novel gene expression changes that were potentially relevant for human T1D, we evaluated the overlap of the 1745 differentially expressed transcripts with the genes residing in regions of the mouse genome that were orthologous to the human T1D susceptibility loci. Because many of these positional candidate genes identified in the human loci have not been previously studied in the context of diabetes, the comparative data represents a source of potential T1D candidate genes. The overlap revealed that 30 protein-coding positional candidate genes from the human loci are differentially expressed in the NOD islets (Fig. 3-2). This list contained some well-known T1D candidate genes such as *cathepsin H* (*Ctsh*), but also revealed many candidate genes not previously implicated in T1D etiology, including *osteosarcoma amplified 9* (*Os9*). Expanding the analysis to encompass non-protein-coding transcripts identified additional genes, including the known candidate gene *maternally expressed 3* (*Meg3*), which has

been linked to both type 1 (Wallace et al., 2010) and type 2 diabetes (Kameswaran et al., 2014) (data not shown).

Islet CpG methylation changes in insulitis-prone islets.

To investigate whether there was a correlation of CpG methylation and gene expression changes in the NOD islets, we prepared CpG site-enriched reduced representation bisulfite sequencing (RRBS) DNA libraries (Gu et al., 2011) from 4-week-old NOD and NOR islets to perform methylation sequencing. Differentially methylated region (DMR) analysis with stringent discovery criteria compiled 46 unique genomic regions that were differentially methylated in the NOD islet genome. Of these regions, 41 mapped within or in close proximity (< 60 kb from the transcriptional start site) to protein-coding genes; 38 were hypomethylated in NOD islets and 3 were hypermethylated. Eight of the CpG regions were not located within 60 kb of the transcriptional start site of any single gene. Of the 38 hypomethylated regions, three genes were identified by duplicate DMRs: *erbin* (*Erbb2ip*), *forkhead box A2* (*Foxa2*), and *UPF1 regulator of nonsense transcripts homolog* (*Upf1*). All other genes were mapped by single DMR regions, with a total of 38 differentially methylated genes (Table 3-1). Interestingly, many of these genes have not been previously implicated in beta cell pathophysiology and T1D. We aligned the 38 differentially methylated genes with the T1D positional candidate genes and did not identify any overlap. However, three of the hypomethylated genes were identified in the RNA-Seq to be significantly upregulated in the NOD islets: *eukaryotic translation initiation factor 4a1* (*Eif4a1*), *collagen type 1 alpha 1* (*Col1a1*), and *FAT atypical cadherin 1* (*Fat1*). Collectively, integration of the

NOD versus NOR transcriptome results with human GWAS and islet DNA methylation data led to the identification of potential T1D candidate genes in NOD islets which may be beta cell modulators of diabetogenesis.

Discussion

Using the NOD mouse model of T1D, we identified gene expression and methylation changes in insulitis-prone and insulitis-resistant mouse islets. By combining the islet datasets with positional candidate genes derived from the human GWAS loci, we found 30 differentially expressed candidate genes. By combining the dataset further with CpG methylation data, we found three additional candidate genes that may be regulated by promoter methylation.

Some of the candidate genes identified by RNA-Seq have been previously implicated in diabetes or beta cell function, which allowed us to use them as internal positive controls to validate our data mining approach. One such gene, *Ctsh*, has recently been reported to affect disease progression in newly diagnosed pediatric patients (Floyel et al., 2014). The authors propose increased *Ctsh* expression in beta cells as a potential mechanism of protection against immune damage. Our data shows that *Ctsh* expression is downregulated in NOD islets (Fig. 3-2), perhaps suggesting that even at this early pre-insulitic time point, NOD islets have compromised protection against immune damage.

Among the novel candidate genes, *Os9* is upregulated in NOD islets. *Os9* is upregulated in response to endoplasmic reticulum (ER) stress in mammalian cell lines (Alcock and Swanton, 2009), and it has been established that in 6-, 8-, and 10-week-old islets, beta cell ER stress precedes clinical diabetes in the NOD mouse model (Tersey et

al., 2012). At the studied time points, there is progressive insulitis, but the fact that *Os9* is upregulated in our 4-week-old NOD islets suggests that ER stress may in fact be present much earlier than reported and possibly before significant insulitis. This suggests that there are beta cell intrinsic differences in insulitis-prone and insulitis-resistant islets.

Another novel candidate was *ribosomal protein S6 kinase, polypeptide 2* (*Rps6ka2*), which encodes a ribosomal S6 kinase family member of serine/threonine kinases that act as downstream effectors of the mitogen-activated kinase (MAPK) signaling pathway to modulate cell survival and proliferation (Anjum and Blenis, 2008). This gene has been associated with diabetic cataracts in type 2 diabetics (Lin et al., 2013), as well as pancreatic (Milosevic et al., 2013), colorectal (Slattery et al., 2011), and ovarian (Bignone et al., 2007) cancer models. In fact, several of our novel candidates from the RNA-Seq/GWAS overlap had been identified in the context of cancer, including *teratocarcinoma-derived growth factor 1* (*Tdgf1*), *breast cancer anti-estrogen resistance 1* (*Bcar1*), and *Os9*. Furthermore, DMR candidate genes also included a number of genes that are oncogenic, including *erbin* (*Erbb2ip*), also known as *ERBB2 interacting protein*. Much like the aberrant cancer cell environment, it is possible that these gene products produce a dysfunctional beta cell milieu leading to immune targeting and diabetes. How this leads to beta cell death as opposed to tumor-like proliferation is not well understood, and it may be worthwhile to study these genes in primary beta cells and tumor tissues.

Of the three hypomethylated genes with correlating increases in gene expression, none had been previously implicated in T1D pathogenesis or beta cell function. *Col1a1* had been studied as a genotypic marker to identify T1D patients with lower bone mineral density and higher risk for osteopenia, a common complication of T1D (Hampson et al.,

1998). *Fat1* is an oncogenic gene that is highly upregulated in glioblastoma, and mediates inflammation and neoplasia (Dikshit et al., 2013). Because the expression of these genes appears to be influenced by promoter methylation at an early age, it would be interesting to study the functions of EIF4A1, COL1A1, and FAT1 in the context of beta cell development, function, and inflammation.

Most of the genes identified by islet CpG methylation differences were not significantly changed in RNA expression. However, it is possible that the methylation signatures are maintained over time, and islet gene expression at the time of immune attack may be significantly different. It is worthwhile to mention that *nitric oxide synthase interacting protein* (*Nosip*) is hypomethylated in the NOD islets. In neuronal cell types, neuronal nitric oxide synthase (nNOS) interacted with NOSIP and the protein complex colocalized with synapses in rat brains (Dreyer et al., 2004). Overexpressing NOSIP decreased nNOS in tertiary dendrites, and authors suggest that NOSIP sequesters nNOS to prevent nNOS trafficking to distal dendrites. Beta cells also express nNOS, which localizes to the membrane of insulin secretory granules where it is critical for insulin secretion (Lajoix et al., 2001). It is unknown whether NOSIP is also expressed on the membrane of the insulin granules, but drawing a parallel from the neurons, NOSIP interaction may also be the mechanism in beta cells that spatially restricts nNOS to the insulin granules. Hypomethylation of the CpG region in the promoter of *Nosip* suggests less steric hindrance for *Nosip* gene expression. It is thus possible that NOD islets may overexpress *Nosip* at a later time point, which may decrease nNOS on insulin granules and play a role in beta cell dysfunction. Additionally, pro-inflammatory cytokines promote nitric oxide production by inducing the expression of *inducible nitric oxide*

synthase (*iNOS*) to trigger beta cell apoptosis (Kaneto et al., 1995) and ER stress (Oyadomari et al., 2001). It is not known whether NOSIP interacts with iNOS, but if it does, it could shed new light on regulatory mechanisms of beta cell apoptosis.

Our study is somewhat limited by the variable diabetes phenotype in the NOD mice. Although we limited our study to the female mice, which have higher (80%) T1D penetrance than their male counterparts (30%), not knowing which mouse would have become diabetic in the future could have diluted our dataset if our sample mice were to be diabetes-free by chance. We tried to address this by increasing the number of replicates (N = 6 for the NOD), and by visualizing the sample-to-sample heterogeneity within genotypes with heatmaps and hierarchical clustering analysis, it was clear that most of the NOD samples were distinct from the NOR islets. For example, in Figure 3-2, the NOD sample in column 5 mirrors the NOR samples for many of the genes, and perhaps this replicate contained islets from mice that would have been more diabetes-resistant. This could have diluted the significance statistic in differential expression and methylation analysis and perhaps this led to the exclusion of some critical candidate genes. Another caveat is that the NOD mouse model, although it is a well-validated model of T1D, has some type 2 diabetes (T2D) characteristics (Chaparro et al., 2006). This may in fact be an advantage in identifying diabetes candidate genes in our datasets, because in humans, clinical similarities have blurred the distinction between different classifications of diabetes (Cervin et al., 2008). We kept this in mind when categorizing candidate genes as novel. One of the hypomethylated genes, *Foxa2*, is not a reported T1D candidate but is a well confirmed T2D gene (Banasik et al., 2012; Tabassum et al., 2008) that controls the expression of important beta cell transcription factors such as

neurogenin 3 (*Ngn3*), *forkhead box protein O1* (*FoxO1*), and *pancreatic and duodenal homeobox 1* (*Pdx1*) (Chen et al., 2011; Ejarque et al., 2013; Lee et al., 2002). Our identification of T2D-associated *Foxa2* hints at the usefulness of the NOD mouse model in studying T2D as well as T1D, and further analysis of beta cell *Foxa2* promoter methylation in diabetic islets and diabetes-susceptible islets may help us to understand the epigenetic mechanisms of beta cell dysfunction.

Other limitations of the study include the RRBS method of detecting differential methylation, which covers genomic regions with relatively high CpG density, overlooking regions that are not CpG-rich. Other methods with higher genomic coverage that may be used to detect DMRs include affiny-based methods such as methylated DNA immunoprecipitation deep sequencing (MeDIP-Seq) and methylated DNA capture by affinity purification (MethylCap-Seq). However, comparative analysis of the DMR detection methods concluded that despite lower genomic coverage, the RRBS method yields accurate DNA methylation levels, because CpG-rich sequences are most consistently methylated overall and CpG-poor sequences have variable methylation levels within biological replicates (Bock et al., 2010). Moreover, we annotated each DMR as regulating the gene that is most proximal to the DMR. Methylated DNA can impose steric hindrance to the chromatin, but we cannot rule out the possibility that differential methylation of a regulatory region that is far from the promoter of a gene may also regulate the chromatin structure through tertiary interactions. And lastly, interplay of DNA methylation, histone modifications, and non-coding RNAs may coordinately regulate differential gene expression in the NOD versus NOR islets, and limiting our study to just DMRs may overlook important epigenetic mechanisms.

In conclusion, even with some limitations, we have successfully identified novel candidate genes for T1D, including three genes with correlating RNA expression and DNA methylation changes. Integrating positional candidate genes from GWAS with disease-susceptible mouse data is a powerful method to analyze high throughput datasets. Such global integrative screening prior to molecular characterization and functional confirmation of the candidate genes has the potential for a more efficient and possibly more clinically relevant candidate gene selection that could lead to a better understanding of T1D.

Materials and Methods

Mice. Female NOD/ShiLtJ (NOD) and NOR/LtJ (NOR) mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and their islets were harvested within a week of arrival at our facility to limit environmental effects. All animal studies were approved by the Institutional Animal Care and Use Committee at the Columbia University Medical Center.

Islet isolation. Pancreatic islets were isolated from 4-week-old NOD and NOR mice, following a modified version of published protocol (Gotoh et al., 1985). After handpicking, islets were flash-frozen and stored at -80 °C until processing.

RNA-Seq analysis. For each sample, islets from two mice were pooled to make one biological replicate. Total RNA was extracted using either the RNeasy Micro Kit or Mini

Kit (Qiagen). Illumina library preparation, sequencing on Illumina 2000/2500 instrument, and standard bioinformatics were performed at the Columbia Genome Center (New York, NY). Differential expression was determined by statistical testing based on negative binomial distribution using 'DESeq' package of R software.

Methylation analysis. For each sample, islets from three mice were pooled to make one biological replicate. Genomic DNA was extracted from isolated islets and purified with two to three rounds of the standard phenol-chloroform extraction. Reduced representation bisulfite sequencing libraries were prepared as described (Gu et al., 2011), and libraries were sequenced with 36-bp single-end sequencing with 10 million aligned reads per library on the Illumina Genome Analyzer II. Raw reads were aligned using Genomic Short-read Nucleotide Alignment Program (GSNAP) (Wu and Nacu, 2010) along with Goby API support. The methylation rates for the two groups (NOD vs. NOR) were determined using the Goby package and differentially methylated regions (DMRs) across the groups were determined using the BSmooth, a replicate-aware program that accounts for biological variation by performing local smoothing of the data (Hansen et al., 2012), followed by a two-tailed t-test for DMR detection with $P = 0.025$. We focused our DMR analysis only on the CpG sites, and only sites with a minimum 10-fold coverage in at least 3 of the 4 samples in each group were considered for the analysis.

Integration with human data. A total of 478 genes within 52 mouse orthologous regions associated with 49 human T1D loci were obtained from the T1DBase (Burren et al., 2011). This gene set was parsed to extract 312 protein-coding genes. These positional

candidate genes were used as input into the RNA-Seq and methylation datasets and evaluated for any significant differences with threshold of DESEQ $P < 0.05$ using the R software.

Pathway analyses and heat maps. Pathway analyses were performed using the Ingenuity Pathway Analysis (Ingenuity Systems) and DAVID (NIH). Heatmaps were generated by normalizing FPKM values to the row mean and standard deviation values using the R software.

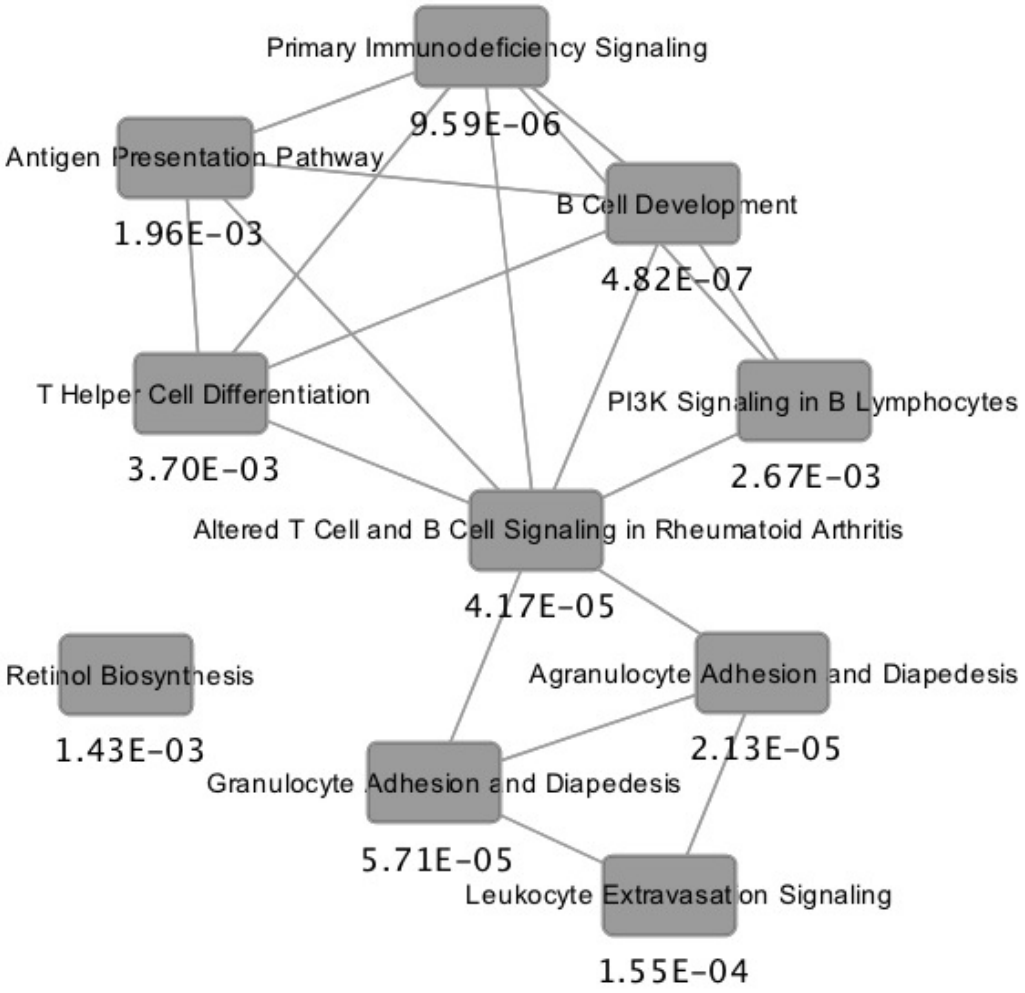
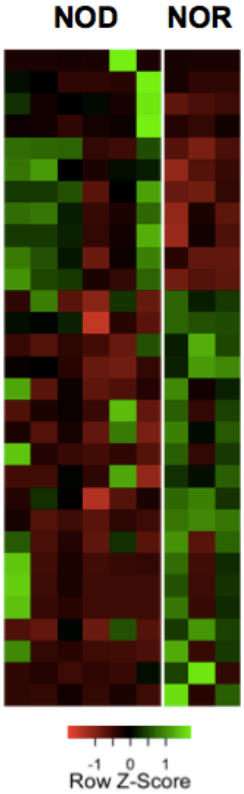


Figure 3-1. Gene expression changes in the 4-week-old NOD islets are involved in immune response signaling.

Significant IPA canonical pathways enriched in 1745 genes differentially expressed in the NOD islets (DESEQ $P < 0.05$). Numbers under each pathway represents Fisher's Exact P value to score enrichment density of the gene sets in a pathway. N = 6 for NOD and N = 3 for NOR islets.



Gene	Fold change	P	Description	GWAS locus
Clec2h	**6.02**	**1.09E-07**	**C-type lectin domain family 2, member h**	**12p13.31**
Tdgf1	**4.05**	**2.87E-04**	**teratocarcinoma-derived growth factor 1**	**3p21.31**
Gsdma	**2.15**	**3.10E-07**	**gasdermin A**	**17q12**
Igf2	1.16	5.94E-05	insulin-like growth factor 2	11p15.5
Olfml3	**0.59**	**3.15E-02**	**olfactomedin-like 3**	**1p13.2**
Rps6ka2	**0.45**	**1.04E-03**	**ribosomal protein S6 kinase, polypeptide 2**	**6q25.3**
Ddc	**0.40**	**4.95E-04**	**dopa decarboxylase**	**7p12.2**
Suox	0.34	2.03E-02	sulfite oxidase	12q13.2
Bcar1	0.30	2.38E-02	breast cancer anti-estrogen resistance 1	16q23.1
Atp6ap1	0.27	2.18E-02	ATPase, H+ transporting, accessory protein 1	Xq28
Os9	**0.24**	**2.58E-02**	**amplified in osteosarcoma**	**12q14.1**
Cabp7	**-0.34**	**4.50E-02**	**calcium binding protein 7**	**22q12.2**
Sstr3	-0.36	5.64E-03	somatostatin receptor 3	22q12.3
Pan2	**-0.38**	**7.53E-03**	**pancreas protein 2**	**12q13.2**
Ascc2	**-0.44**	**3.32E-03**	**activating signal cointegrator 1 complex subunit 2**	**22q12.2**
Ctsh	-0.46	4.26E-02	cathepsin H	15q25.1
Nupr1	-0.50	2.12E-02	nuclear protein 1	16p11.2
Slc1a5	**-0.53**	**3.56E-02**	**solute carrier family 1, member 5**	**19q13.32**
Ikzf3	**-0.61**	**5.65E-03**	**IKAROS family zinc finger 3**	**17q12**
Slc39a5	-0.63	5.67E-03	solute carrier family 39, member 5	12q13.2
Th	-0.64	9.05E-05	tyrosine hydroxylase	11p15.5
Slx1b	**-0.66**	**2.51E-03**	**slx1 structure-specific endonuclease subunit homolog B.**	**16p11.2**
B4galnt1	-0.79	1.17E-02	beta-1,4-N-acetyl-galactosaminyl transferase 1	12q14.1
Rac2	**-0.91**	**1.49E-05**	**RAS-related C3 botulinum substrate 2**	**22q12.3**
Cd19	-0.96	4.02E-05	CD19 antigen	16p11.2
Ptpn22	-1.27	5.03E-04	protein tyrosine phosphatase, non-receptor type 22	1p13.2
Dbp	-1.37	2.66E-09	D site albumin promoter binding protein	19q13.33
Ikzf1	-1.46	4.82E-08	IKAROS family zinc finger 1	7p12.2
Ltf	-2.01	8.72E-04	lactotransferrin	3p21.31
Fgf21	-3.35	1.67E-04	fibroblast growth factor 21	19q13.33

Figure 3-2. Human positional candidate genes differentially expressed in NOD islets. Heatmap depicts the expression profiles of 30 positional candidate genes in NOD and NOR islets. Each column is one biological replicate (NOD, N=6; NOR, N=3). Colors represent transcripts upregulated (green) or downregulated (red), scaled to row Z-scores. The table catalogs gene symbol, full name, log-2 ratio of NOD baseMean to NOR baseMean fold change from DESEQ analysis, P value from DESEQ analysis, and the corresponding human T1D susceptibility locus. Genes in bold have not been previously reported in diabetes literature or implicated in beta cell function and/or survival.

Table 3-1. Differentially methylated genes in NOD islets

Gene	Change	NOD	NOR	Description	DM
Hypermethylated					
Arrb2	0.17	0.34	0.17	arrestin, beta 2	*
Masp2	0.14	0.53	0.40	mannan-binding lectin serine peptidase 2	
Cux1	0.10	0.19	0.08	cut-like homeobox 1	
Hypomethylated					
Gm7120	-0.18	0.68	0.86	predicted gene 7120	
Eif5a	-0.13	0.30	0.43	eukaryotic translation initiation factor 5A	*
Plscr3	-0.38	0.35	0.72	phospholipid scramblase 3	
Erbb2ip	-0.20	0.47	0.67	Erbin	
Dusp10	-0.24	0.70	0.94	dual specificity phosphatase 10	
Mrpl14	-0.18	0.53	0.71	mitochondrial ribosomal protein L14	
Tmem63b	-0.18	0.53	0.71	transmembrane protein 63b	
Asgr2	-0.43	0.42	0.85	asialoglycoprotein receptor 2	
Zfp664	-0.12	0.47	0.59	zinc finger protein 664	
Neurl4	-0.18	0.30	0.48	neuralized E3 ubiquitin protein ligase 4	
Plekha2	-0.25	0.52	0.78	pleckstrin homology domain containing, A2	
Pex14	-0.27	0.47	0.74	peroxisomal biogenesis factor 14	
Lbr	-0.25	0.55	0.81	lamin B receptor	
Mbnl2	-0.17	0.70	0.87	muscleblind-like 2	
Mgl1	-0.27	0.53	0.80	macrophage N-acetyl-galactosamine specific lectin	
BC025816	-0.17	0.60	0.78	kelch-like 36	
Galnt13	-0.16	0.35	0.51	N-acetylgalactosaminyltransferase 13	
Eif4a1	**-0.14**	**0.32**	**0.45**	**eukaryotic translation initiation factor 4A1**	
Upf1	-0.13	0.71	0.84	UPF1 regulator of nonsense transcripts homolog	
Col1a1	**-0.17**	**0.60**	**0.77**	**collagen, type I, alpha 1**	
Mylc2pl	-0.13	0.38	0.52	myosin, light chain 10	
Foxa2	-0.14	0.38	0.51	forkhead box A2	*
Gdf11	-0.17	0.34	0.50	growth differentiation factor 11	
Cd37	-0.10	0.50	0.61	CD37 antigen	
Osmr	-0.15	0.66	0.80	oncostatin M receptor	
Git2	-0.26	0.54	0.80	G protein-coupled receptor kinase-interactor 2	
Stpg1	-0.25	0.45	0.70	sperm tail PG rich repeat containing 1	
Fat1	**-0.12**	**0.79**	**0.90**	**FAT atypical cadherin 1**	
Pcdh1	-0.32	0.52	0.85	protocadherin 1	
Ryr1	-0.25	0.54	0.79	ryanodine receptor 1	*
Trpc2	-0.28	0.47	0.75	transient receptor potential cation channel, C2	
Nosip	-0.18	0.45	0.63	nitric oxide synthase interacting protein	
Cacng4	-0.17	0.66	0.82	calcium channel, voltage-dependent, g4	
Atp6v0a2	-0.19	0.49	0.68	ATPase, H+ transporting, lysosomal V0 subunit a2	
Lyl1	-0.20	0.20	0.40	lymphoblastic leukemia associated 1	

Table categorizes genes that are hypo- and hypermethylated in the NOD islets compared to the NOR islets (N=4 each). NOD and NOR columns list the ratio of CpG methylation per region, and change is the mean difference in the ratios. Asterisk (*) in DM category indicates that the gene has been previously linked to diabetes or beta cell function. Rows in bold are the genes with correlating changes in RNA expression.

Chapter Four: PKM2-mediated insulitis in the NOD islets

Preface

With guidance of Dr. Lori Sussel, I generated all data. NOD islet microarray data obtained from Gene Expression Omnibus dataset GSE41203, associated with Carrero et al., 2013.

Results and Discussion

Protein tyrosine phosphatase N2 (PTPN2) modulates hypoxia-inducible factor 1 alpha/pyruvate kinase M2 (HIF-1α/PKM2) regulated gene expression in the beta cells after the onset of diabetes (Chapter 2). To determine whether this is a phenomenon specific to the streptozotocin (STZ) treated mice, we performed *in silico* analysis of the HIF-1α/PKM2 target genes using the islet gene expression data from NOD mice of various ages from Carrero et al (Carrero et al., 2013). Although almost all of the genes were not significantly changed by statistical methods, analysis of relative expression revealed subtle but progressive increases in many of the HIF-1α/PKM2 target genes with increasing age, reaching maximum expression in diabetic islets (Fig. 4-1). We compared this NOD islet gene expression data to our RNA-Seq data from STZ-diabetic Ptpn2 βKO and control islets in the context of HIF-1α/PKM2 target genes. We confirmed that the Ptpn2 βKO islets clustered more tightly with the diabetic NOD islets and that the control islets from our study clustered tightly with younger NOD islets (Fig. 4-2), perhaps reflecting the fact that Ptpn2 βKO animals are more severely diabetic. This indicates that the upregulation of HIF-1α/PKM2 target genes is a conserved feature of diabetic islets and suggests that greater increases in HIF-1α/PKM2 target genes may be an indication of exacerbated disease progression in islets.

We also analyzed the HIF-1α/PKM2 target gene expression in the prediabetic 4-week-old NOD islets (RNA-Seq data from Chapter 3). There was no obvious trend or differences in the NOD compared to the NOR islets at this early time point, but surprisingly, *Pkm2* expression was significantly upregulated in the 4-week-old NOD islets (Fig. 4-3). Re-examining the time-course NOD islet expression data from Carrero et

al, we found that *Pkm2* expression was comparable among the NOD and age-matched control islets at 6 weeks. However, the control strains in this study were NOD.*Rag1*-null, B6.g7, and C57BL/6J strains (Carrero et al., 2013). None of these strains develop insulitis similar to the NOD mice, as the NOD.*Rag1*-null mice are NOD congenic strain without the adaptive immune system, B6.g7 strain is the C57BL/6J strain carrying the NOD-matched H2g7 histocompatibility locus, and the C57BL/6J strain is the normal wildtype strain. In our study, we used the NOR strain, which is the NOD congenic strain that accumulates pancreatic leukocytic aggregates (peri-insulitis) without becoming diabetic. Furthermore, the NOR strain is completely resistant to cyclophosphamide-induced diabetes (Prochazka et al., 1992; Serreze et al., 1994), suggesting that even in the face of heightened immune or chemical attack, the islets in this strain resist cell death. Our finding that *Pkm2* expression was significantly upregulated in the NOD islets compared to the NOR islets suggests that NOR islets resist diabetes possibly because they express less PKM2 and thus may have blunted expression of HIF-1α/PKM2 target genes critical for beta cell death.

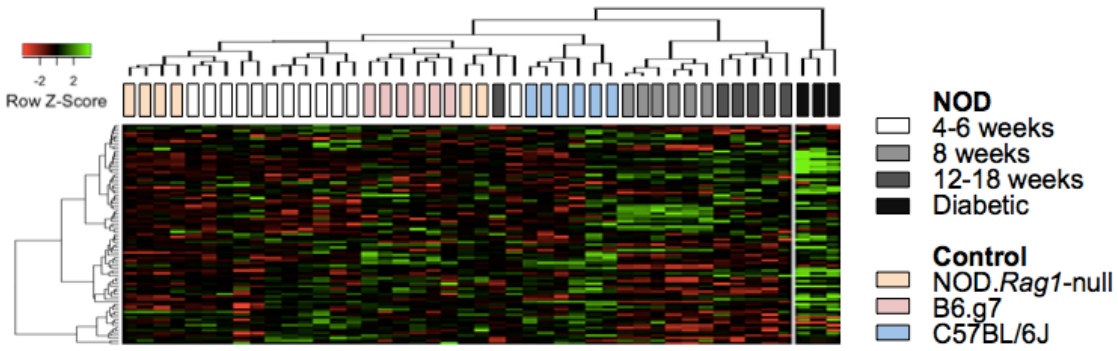


Figure 4-1. *In silico* analysis of HIF-1α/PKM2 gene expression pattern in NOD islets of different ages.

Analysis of time-course NOD islet microarray for HIF-1α/PKM2-responsive genes, represented as a heatmap of relative microarray intensities. Diabetic islets (3 lanes at the right) cluster tightly together and have increased HIF-1α/PKM2-responsive transcription. Raw data obtained from (Carrero et al., 2013).

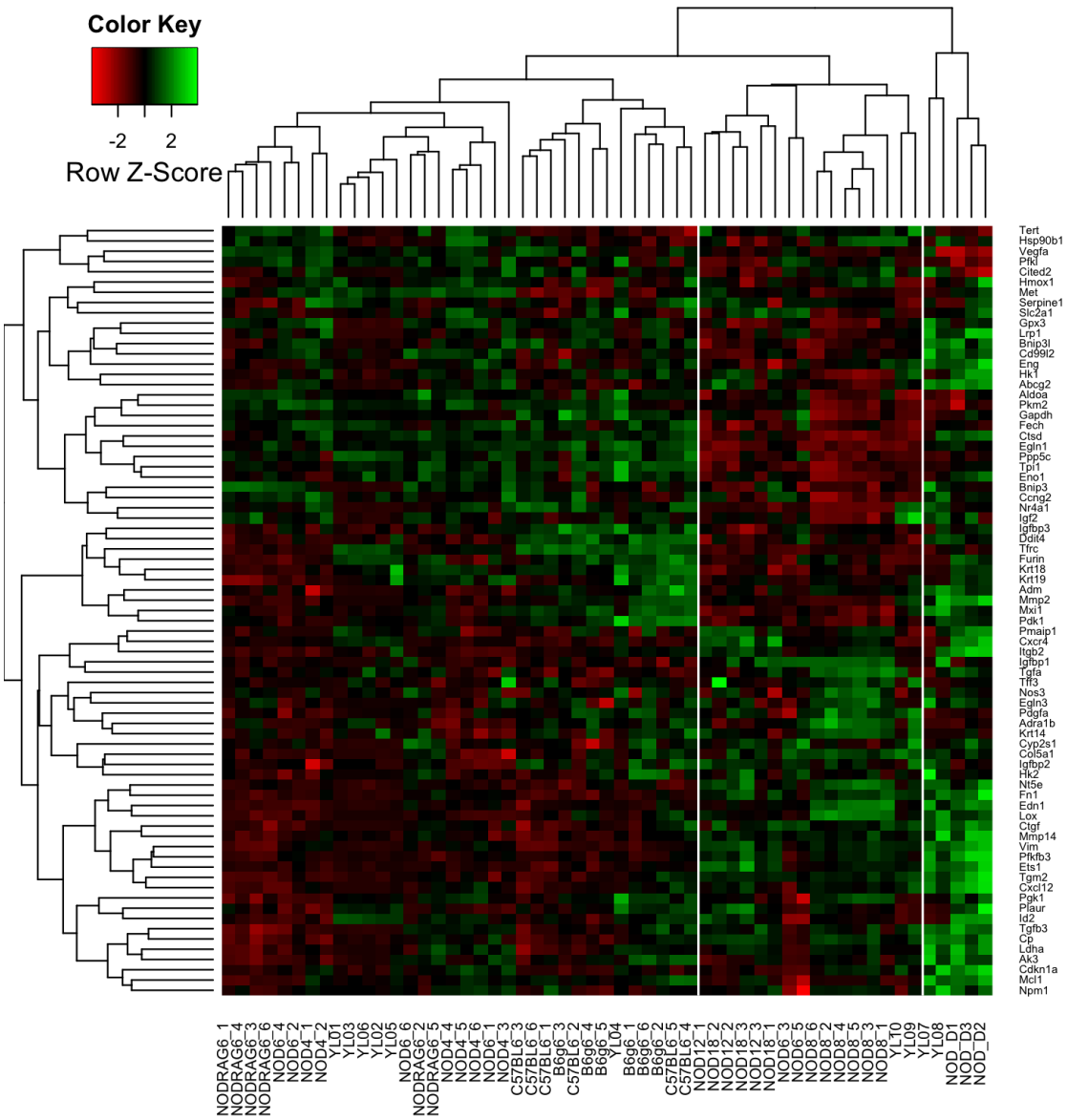


Figure 4-2. Integration of Ptpn2 βKO islet RNA-Seq data with the NOD islet microarray *in silico* analysis.

Hierarchical clustering analysis of the HIF-1α/PKM2-responsive genes in time-course NOD islet microarray data and the RNA-Seq of islets from both unstressed and STZ-administered Ptpn2 βKO (KO and STZ-KO) and control (Ctrl and STZ-Ctrl) mice is shown. Because microarray intensities and RNA-Seq FPKM values are not directly

comparable, relative Z-scores were calculated within each datasets, which were then combined to generate the heatmap. Three major clusters are separated. The first cluster contains mostly NOD control islets (6-week-old NOD.*Rag1*-null, NODRAG*6*; 6-week-old B6.g7, B6G*6*; and 6-week-old C57BL/6J, C57BL*6*), NOD islets from 4-6 weeks of age (NOD*4* and NOD*6*), and unstressed KO (YL04-06) and Ctrl islets (YL01-03). The second cluster contains prediabetic NOD samples (8-, 12-, and 18-week-old, NOD*8*, NOD*12*, and NOD*18*) and STZ-Ctrl islets (YL09 and 10). The last cluster contains diabetic NOD samples (NOD_*D*) and STZ-KO islets (YL07 and 08). Microarray raw data obtained from (Carrero et al., 2013).

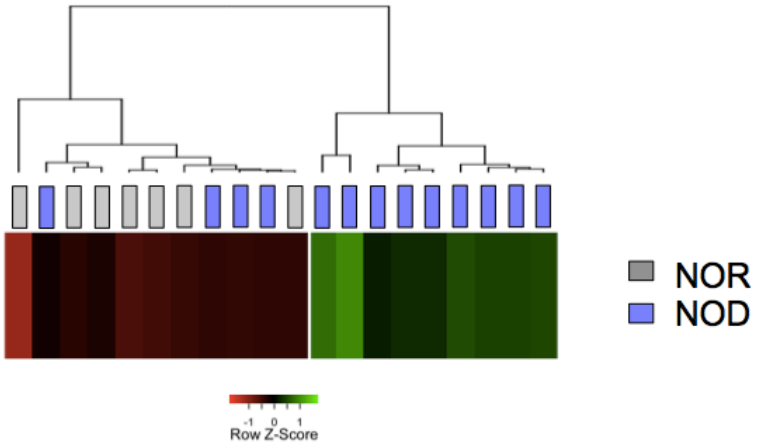


Figure 4-3. *Pkm2* expression in 4-week-old NOR and NOD islets.

The expression of *Pkm2* from RNA-Seq is shown (Chapter 3). DESEQ analysis identified *Pkm2* as being differentially expressed in the NOD islets at this time point (P = 0.002). Hierarchical clustering reveals that 4 of the NOD samples cluster with NOR samples; interestingly, 3 of these samples clustering with NOR were from male NOD mice that have a lower incidence of T1D (NOD female N = 7, NOD male N = 8, NOR female N = 3, NOR male N = 4). Males and females were grouped together based on genotype for visual simplicity.

Chapter Five: The effect of non-CpG DNA methylation on gene expression

Preface

Islet sample preparation, methylation sequencing, and RNA-Seq same as Chapter 3.

Differential CpT analysis conducted by Dr. Angad P. Singh.

Results and Discussion

Cytosine methylation in mammalian DNA is a key epigenetic modification controlling chromatin structure (Bird and Wolffe, 1999). While most of the DNA methylation is found on CpG dinucleotides, non-CpG methylation has been observed. One study reported that the majority of all DNA methylation was at non-CpG nucleotides in the human spleen (Woodcock et al., 1987). A more recent study reported non-CpG methylation in mammalian cell lines and posited that non-CpG methylation is commonly underestimated due to current methylation analysis methods (Yan et al., 2011). Another study reported *de novo* DNA methylation at both CpG and non-CpG dinucleotides in the promoter of adenovirus type 2 after integration into host genome, suggesting such propagation of *de novo* methylation in response to viral infection may hinder the expression of integrated foreign DNA (Toth et al., 1990).

To address the question of whether non-CpG methylation influences islet gene expression, we analyzed the existence of CpT methylation in our reduced representation bisulfite sequencing (RRBS) data from non-obese diabetic (NOD) and non-obese insulitis resistant (NOR) islets (Chapter 3). Surprisingly, we discovered that there were 4654 differentially methylated CpT sites in the NOD and NOR islets (DMR $P < 0.05$). To determine whether any of the CpT methylation correlated with gene expression data, we combined the list of candidate genes with promoter CpT methylation differences with the list of 658 genes with significantly different gene expression (RNA-Seq data from Chapter 3, with adjusted $P < 0.05$) and found that of 109 genes appeared in both candidate lists. Of these, 46 genes had correlating inverse relationship between gene expression and promoter methylation. All 46 genes were CpT-hypomethylated in the

NOD islets and had increased gene expression, and no candidate gene displayed hypermethylation and reduced gene expression (Table 5-1; Fig. 5-1A). Interestingly, one of the genes was *chromogranin A* (*Chga*), which is a T1D autoantigen specifically recognized by self-reactive T cells (Stadinski et al., 2010).

We then analyzed whether a majority of these candidate genes were involved in a common signaling pathway. Interestingly, the most significantly changed category was endoplasmic reticulum (ER) stress pathway, which has been shown to precede islet inflammation and the onset of hyperglycemia in the NOD mice (Ize-Ludlow et al., 2011; Tersey et al., 2012). We performed quantitative PCR analysis with NOD and NOR islet RNA and successfully validated the RNA-Seq findings (Figure 5-1B). Given that the NOD islets develop ER stress signatures as early as 6 weeks, the lack of CpT methylation at the promoters of key ER stress genes may facilitate the aberrant overexpression of these genes even earlier than 6 weeks, as our samples are from 4-week-old mice. Whether the CpT methylation is maintained over time until the onset of hyperglycemia in NOD mice has not yet been investigated, but our study highlights that non-CpG methylation events may be important for the regulation of gene expression in islet cells.

It is not known whether non-CpG methylation is found in clusters as in CpG islands. A global bisulfite sequencing strategy would detect all non-CpG methylation events. Our study was somewhat limited in that the original RRBS library for the methylation sequencing was a method to enrich for CpG islands by CpG-specific enzymatic cleavage of the genome. However, even if our study has only detected differential CpT methylation near CpG islands, the fact that we detected correlating changes in ER stress genes and *Chga* suggests that a global analysis of CpT methylation

and other non-CpG methylation events may be functionally significant in understanding differential susceptibility to autoimmune attack of the NOD and NOR beta cells.

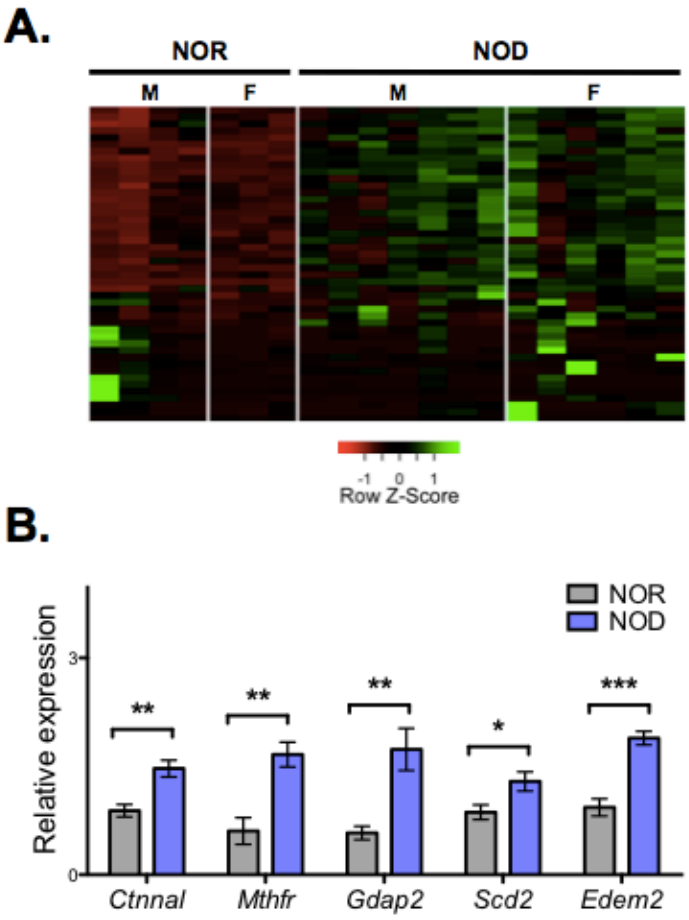


Figure 5-1. Gene expression of candidates with CpT methylated promoters.

(A) 46 candidate genes with correlating changes in RNA expression and CpT methylation (presumed inverse relationship) were all highly expressed and had hypomethylated loci in the NOD islets. Islet RNA-Seq data for the 46 genes shown as a heatmap. F and M indicate female and male gender of the mouse (NOR M, N =4; NOR F, N = 3; NOD M, N = 7; NOD F, N = 6). Full list of genes is in Table B-1. **(B)** Validation qPCR analysis using fresh islet RNA from 4-week-old female NOD and NOR mice, normalized to *Hprt* reference gene (NOR N = 4, NOD N = 7). $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

Table 5-1. Genes with differential CpT methylation and RNA expression

Gene	RNA P	Fold change	CpT P	Delta mCpT	Description
Chga	0.0001	1.43	0.0081	-2.05	chromogranin A
Pyy	<0.0001	1.43	0.0007	-1.82	peptide YY
Pdia6	0.0488	1.29	0.0202	-1.87	protein disulfide isomerase associated 6
Scd2	0.0188	1.30	<0.0001	-2.18	stearoyl-Coenzyme A desaturase 2
Mafa	<0.0001	1.69	0.0482	-1.96	v-maf avian musculoaponeurotic fibrosarcoma oncogene A
Edem2	0.0001	1.46	0.0223	-2.63	ER degradation enhancer, mannosidase alpha-like 2
Pdia4	0.0272	1.33	0.0052	-1.93	protein disulfide isomerase associated 4
Vdr	0.0333	1.33	0.0237	-1.96	vitamin D receptor
KIAA0513	0.0252	1.31	0.0125	-1.94	RIKEN cDNA 6430548M08
Creld2	0.0083	1.40	<0.0001	-2.70	cysteine-rich with EGF-like domains 2
Akp3	<0.0001	28.69	0.0215	-4.41	alkaline phosphatase 3
Peg10	<0.0001	2.04	0.0084	-1.71	paternally expressed 10
Ckb	0.0002	1.52	0.0071	-2.35	creatine kinase, brain
Coa5	0.0031	1.48	0.0006	-4.40	cytochrome C oxidase assembly factor 5
Gdap2	0.0054	1.50	0.0481	-2.32	ganglioside induced differentiation associated protein 2
Mthfr	<0.0001	1.62	0.0473	-1.95	5,10-methylenetetrahydrofolate reductase
Myo1d	<0.0001	1.66	0.0304	-1.73	myosin ID
Rps6ka2	0.0327	1.37	0.0338	-1.90	ribosomal protein S6 kinase 2
Ncald	0.0149	1.41	0.0456	-1.77	neurocalcin delta
Tuba4a	0.0173	1.43	0.0046	-1.81	tubulin, alpha 4a; tubulin, alpha 4A
Ctnnal1	0.0049	1.45	0.0347	-2.16	catenin, alpha like 1
Arhgap18	<0.0001	8.05	0.0155	-2.34	Rho GTPase activating protein 18
Mttp	0.0003	1.67	0.0167	-1.46	microsomal triglyceride transfer protein
Mgp	0.0001	1.70	0.0110	-2.67	matrix Gla protein
Ckmt1	0.0015	1.56	0.0319	-2.07	creatine kinase, mitochondrial 1
Ovol2	<0.0001	4.87	0.0087	-1.43	ovo-like 2
Cnnm1	0.0005	1.96	0.0088	-2.14	cyclin M1
Nfil3	0.0001	2.23	0.0124	-1.88	nuclear factor, interleukin 3
Cry1	0.0017	1.78	0.0471	-1.99	cryptochrome circadian clock 1
Pak1	0.0461	1.59	0.0014	-2.03	p21 protein (Cdc42/Rac)-activated kinase 1
Fam159b	0.0075	1.81	0.0125	-1.59	family with sequence similarity 159B
Cbln4	0.0027	2.35	0.0119	-1.67	cerebellin 4 precursor
Tgfb3	0.0078	1.90	0.0414	-1.98	transforming growth factor, beta 3
Myo1a	<0.0001	17.54	0.0041	-2.65	myosin IA
Prtg	<0.0001	9.35	0.0399	-1.36	protogenin
Myo7b	0.0004	3.05	0.0475	-1.89	myosin VIIB
Cybrd1	<0.0001	6.15	0.0297	-1.34	cytochrome b reductase 1
L1td1	<0.0001	12.46	0.0007	-1.92	LINE-1 transposase domain containing 1
Cxcl10	0.0011	8.28	0.0028	-1.62	chemokine (C-X-C motif) ligand 10
Sectm1b	0.0007	7.58	0.0385	-2.44	secreted and transmembrane 1B
Rmrp	0.0031	8.40	0.0001	-2.53	RNA of mitochondrial RNAase P
Ankrd1	0.0459	4.05	0.0009	-1.71	ankyrin repeat domain 1
Rpph1	0.0021	14.81	0.0001	-2.31	ribonuclease P RNA component H1
Muc6	0.0173	6.43	0.0035	-2.12	mucin 6
Utf1	0.0462	29.71	0.0012	-2.09	undifferentiated embryonic cell transcription factor 1
Nlrp6	0.0207	7.17	0.0378	-1.37	NLR family, pyrin domain containing 6

Table 5-1. Genes with differential CpT methylation and RNA expression.

Table categorizes genes that are hypo- and hypermethylated at the CpT sites in the NOD islets compared to the NOR islets (N = 4 each). RNA P and fold change are the adjusted P value and the ratio of NOD to NOR baseMean values from DESEQ analysis. CpT P and delta mCpT are the Fisher P value and the mean difference in the ratio of CpT methylation. Genes are listed in the order of highest to lowest average baseMean values.

Chapter Six: Conclusions and perspective

Dissertation summary and major findings

Type 1 diabetes (T1D) is a chronic disease characterized by the autoimmune attack on pancreatic beta cells leading to substantial beta cell loss and the inability to maintain glucose homeostasis. I have sought to identify novel mediators of beta cell loss that are specific to the beta cell as opposed to the autoreactive immune system. In Chapter 2, analysis of the Ptpn2 βKO islets compared to the control islets after streptozotocin administration revealed a novel mode of beta cell loss by a beta cell-intrinsic mechanism of nuclear accumulation of PKM2, which is modulated by the T1D candidate PTPN2. My study suggests that PKM2 hyperaccumulation in the nuclei of Ptpn2 βKO beta cells leads to increased apoptosis signaling and preventing PKM2 nuclear accumulation may be a solution to preventing insulitic injury. Furthermore, in the insulitis-prone NOD genetic background, the NOD beta cells with higher expression of *Pkm2* are sensitive to insulitis, and congenic NOR beta cells with lower expression of *Pkm2* are resistant to insulitis, suggesting a possible mechanism for the NOR beta cells to escape insulitic injury and diabetes by having a desensitized system of PKM2-mediated apoptosis signaling (Chapter 4). In Chapter 3, global gene expression profiling and promoter CpG methylation analysis of NOD and NOR islets at a time point prior to insulitis, coupled with T1D loci from human genome-wide association studies, have revealed novel candidate genes that may be involved in beta cell-mediated pathogenesis of T1D. In Chapter 5, analysis of the CpT methylation profiles suggests that non-CpG methylation may also play a role in regulating differential gene expression in the NOD and NOR islets. Together, my studies contribute to identifying beta cell-centric mechanisms of T1D through a combination of candidate-based and global inquiries.

Integration of the signaling pathways affected by the loss of PTPN2 through PKM2

During the analysis of Ptpn2 βKO and control islet transcriptomes, mammalian target of rapamycin (mTOR) was highly upregulated in the Ptpn2 βKO islets in both the unstressed and stressed conditions. It is unknown how PTPN2 regulates mTOR signaling and how this contributes to beta cell loss or dysfunction in diabetes pathogenesis. PTPN2-deficient beta cells overexpress the upstream activator of mTOR, *ras homolog enriched in brain* (*Rheb*), but how does the lack of PTPN2 translate to increased expression of *Rheb* transcripts? Are there transcription factors that are regulated by PTPN2-mediated tyrosine dephosphorylation? Does PTPN2 also inhibit mTOR directly? Or does PTPN2-catalyzed dephosphorylation of phosphotyrosine residues facilitate serine/threonine phosphorylation by mTOR, as differential phosphorylation of serine or tyrosine residues can compete for positive or negative regulation of signal transduction pathways, which is a layer of regulation of insulin receptor substrate 1 by mTOR (Ozes et al., 2001). Moreover, how does the relationship of PTPN2 and mTOR contribute to the regulation of nuclear PKM2? Further studies are needed to address these questions. For example, one could use rapamycin to inhibit mTOR in the Ptpn2 βKO and control islets, with or without prior stress with STZ, and assess global changes in the phosphoproteomes. Another approach to address these questions may be to generate beta cell-specific double knockout mice with the genetic deletion of both *Ptpn2* and an activator of mTOR such as *Rheb*.

The crossover of mTOR and PKM2 deserves our attention. It has been shown that mTOR can upregulate *Pkm2* transcription in cancer cells through a downstream effector MYC, which can activate the transcription of hnRNP proteins that play a role in

preferential alternative splicing of *Pkm2* (Sun et al., 2011). This may be a self-perpetuating feedback loop, since PKM2 can phosphorylate histone H3 to drive the transcription of *Myc* (Yang et al., 2012a). In my study, *Myc* was highly upregulated in the unstressed Ptpn2 βKO islets (DESEQ P = 0.01, fold change = 2.24), and although it did not reach statistical significance, the upregulation of *Myc* was also present in the STZ-stressed Ptpn2 βKO islets (DESEQ P = 0.23, fold change = 3.01). The expression of *Pkm2* was not different between Ptpn2 βKO and control islets, but we cannot rule out mTOR/MYC-mediated *Pkm2* expression in the Ptpn2 βKO islets at a more acute time point. Another study implicated microRNA *miR-99a* in regulating the mTOR/PKM2 axis. In cancer cells, insulin decreased *miR-99a* expression, which was accompanied by increased mTOR, HIF-1α, and PKM2; and the overexpression of *miR-99a* abrogated the insulin-mediated upregulation of PKM2 and HIF-1α, similar to rapamycin treatment in these cells (Li et al., 2013). If mTOR-mediated beta cell proliferation and apoptosis were to be compared with mTOR signaling in tumor cells, it is possible that mTOR activation-mediated increases in *Pkm2* and HIF-1α signaling after stressful stimuli in the beta cells could lead to beta cell proliferation or apoptosis. My data suggests that PKM2 nuclear translocation is accompanied by increased beta cell loss and exacerbated survival, suggesting that in our case, mTOR/PKM2/HIF-1α is involved in beta cell apoptosis, counteracted by PTPN2.

In addition mTOR signaling, I have observed hyperphosphorylation of STAT3 in the Ptpn2 βKO islets, which was supported by the recent panc-TCPTP KO study (Xi et al., 2014). mTOR positively regulates STAT3 to mediate tumorigenesis (Zhou et al., 2007), PKM2 can act as a kinase to mediate STAT3 phosphorylation (Gao et al., 2012),

and together with decreased PTPN2-mediated dephosphorylation of STAT3, mTOR and PKM2 may compound the hyperactivation of STAT3 in the Ptpn2 βKO islets. It is interesting to note that in addition to its role in beta cell function, STAT3 is activated in response to the inflammatory cytokine interleukin 22 (IL-22) and it helps mucosal wound healing upon injury in cell types targeted by other autoimmune diseases, such as the intestinal epithelial cells in inflammatory bowel disease (Pickert et al., 2009).

IL-22 is secreted by the immune cells, and IL-22 was highly upregulated in islets from recently-diagnosed T1D donors, suggesting that IL-22 signaling may be upregulated in response to islet cell injury (Arif et al., 2011). Therefore, much like its role in aiding epithelial barrier healing, STAT3 hyperactivation may be protective in the beta cells. However, exposure to interferon alpha (IFN-α) can activate STAT1, mediated by IL-22 signaling, and the increase in IL-22 in T1D islets may indicate STAT1 activation rather than STAT3 (Arif et al., 2011; Bachmann et al., 2013). IFN-α and STAT1 activation have been linked to T1D initiation, and beta cell PTPN2 has been shown to dephosphorylate both STAT1 and STAT3 (Li and McDevitt, 2011; Moore et al., 2011; Xi et al., 2014), suggesting that IL-22-mediated potentiation of STAT1 signaling by IFN-α may be another avenue of cellular defense regulated by PTPN2. Furthermore, beta cells express high levels of IL-22 receptor IL-22R1 (Shioya et al., 2008), and in my studies, I have found that *Il22ra1* expression is significantly increased in the unstressed Ptpn2 βKO islets (DESEQ P = 0.016). In the STZ-stressed islets, *Il22ra1* is not differentially expressed, but *Il22ra2*, encoding another IL-22 receptor, is highly expressed in the Ptpn2 βKO islets (DESEQ P = 0.007), again suggesting that IL-22 signaling, and perhaps also STAT1-mediated apoptosis signaling, may be increased in the Ptpn2 βKO islets, much

like the increased IL-22 levels in the islets of the T1D patient (Arif et al., 2011). Moreover, IL22RA2 has been shown to mediate both wound healing and tumorigenesis after chronic inflammation (Huber et al., 2012), highlighting the parallel between cancer cells and apoptotic cells upon inflammatory signaling.

It is unclear how the crosstalk between PTPN2, PKM2, mTOR, STAT3, and STAT1 contributes to beta cell apoptosis, and several questions remain unanswered. Does the kinase function of PKM2 extend to the phosphorylation of STAT1 in addition to STAT3? Does STAT3 hyperactivation facilitate STAT1-mediated apoptosis signaling observed in PTPN2-knockdown beta cells (Santin et al., 2011)? Does increased IL-22 in T1D islets potentiate STAT1 and STAT3, or preferentially activate one versus the other? Does PTPN2-mediated nuclear export of PKM2 ameliorate STAT1-mediated apoptosis in beta cells, or is nuclear PKM2-responsive transcription of apoptotic genes independent of STAT1-mediated apoptosis? PKM2, mTOR, STAT3, and PTPN2 have all been implicated in tumorigenesis, but how does their interaction result in beta cell loss, while mediating proliferative, pro-survival signaling in tumor cells? Further investigation into the coordinated regulation of mTOR, STATs, and PKM2 by PTPN2 will be illuminating for both diabetes and cancer.

Diabetes to cancer, and the therapeutic implications of this work

Parallels between diabetes and cancer can be seen in many facets of my study, including mTOR, STAT3, HIF-1α, and PKM2. Another parallel can be seen in the mode of diabetes induction in my studies. I used STZ to induce diabetes, but STZ has been shown to induce beta cell tumors, known as insulinomas (Kazumi et al., 1978; Rakieten

et al., 1971; Yamagami et al., 1985). A significant portion of the mice surviving a single low-dose injection of STZ over 10 months (42%) went on to develop insulinomas. It has been suggested that poly(ADP-ribose) synthase is activated in response to DNA fragmentation induced by STZ, which leads to the depletion of NAD+ that is incompatible with beta cell survival, and combination of STZ with inhibitors of poly(ADP-ribose) synthase potentiates STZ-mediated insulinoma incidence (up to 100% after 10 months). However, STZ alone can mediate tumorigenesis in the beta cells, which suggests that islet cells that survive the apoptotic stimuli eventually succeed in proliferating and surviving through poly(ADP-ribose) synthase-independent pathway, perhaps through the accumulation of nuclear PKM2, which can mediate both apoptosis and tumorigenesis.

PTPN2 is another example of convergence between oncogenic and apoptotic signaling. Although there is no evidence of *PTPN2* deletion in insulinoma patients, inactivating mutations or deletion of *PTPN2* can result in Hodgkin's lymphoma or T-cell acute lymphoblastic leukemia (T-ALL), which has been attributed to the disinhibition of JAK1 signaling (Kleppe et al., 2010; Kleppe et al., 2011b). It is possible that in addition to JAK1 regulation, PTPN2 is required to mediate the nuclear export of PKM2, which can inhibit carcinogenesis. Further study will be required to determine if this is the case.

It remains to be seen whether beta cell-specific ablation of *Pkm2* influences beta cell loss or function. I have recently begun to investigate this by obtaining the *Pkm2 fl/fl* mouse and crossing it to the *RIP-Cre* mice. If nuclear PKM2-mediated apoptotic transcriptional reprogramming is required for beta cell loss in T1D, beta cells with *Pkm2* deletion could be resistant to diabetes, as in the NOR strain, which we have shown to

have decreased *Pkm2* transcript levels and is known to be insulitis-resistant despite the accumulation of pancreatic immune infiltrates (Prochazka et al., 1992). Alternatively, *Pkm2* deletion could result in contradicting phenotypes, as in the beta cell-specific HIF-1α knockout mice, due to the duality of PKM2 function in cell proliferation and cell death. Accordingly, in cancer cells, *Pkm2* deletion resulted in a different ratio of high- to low-proliferation cells in the tumor, but cancer formation or progression was not significantly affected (Israelsen et al., 2013).

In place of tissue-specific genetic ablation of *Pkm2*, which is impossible in humans as a therapeutic approach, PKM2 can be regulated by shifting the ratio of tetramer-to-dimer conformation through small molecule activators of PKM2 such as TEPP46, which was utilized in my studies. In proliferating cell types, nuclear PKM2 mediates proliferation, and PKM2 activator treatment has resulted in increased cell death *in vitro* (Anastasiou et al., 2011) and delayed xenograft tumor growth *in vivo* in a multi-day dosing regimen without any toxicity or weight loss in mice (Anastasiou et al., 2012). In my study, in nonproliferating islets, cytotoxic stressor-mediated increase in apoptotic transcripts were downregulated upon co-treatment with TEPP46, suggesting that in cell types where PKM2 mediates apoptosis, PKM2 activators may be useful for decreasing cell death signaling. This suggests a potential avenue for testing whether the *in vivo* administration of PKM2 activators would prevent or delay diabetes in diabetes-prone mice, such as the NOD strain.

Similar to the ability of TEPP46 to stabilize cytosolic PKM2, inhibiting the nuclear import of PKM2 may be another approach to prevent insulitic injury. In one study, a small molecule inhibitor of nuclear import was shown to prevent diabetes and

attenuate insulitis in the pancreas of NOD mice, which was attributed to the inhibition of nuclear import of stress-responsive transcription factors such as NF-κB in the immune cells, resulting in decreased activation of immune cells (Moore et al., 2010). However, the study does not address the attenuation of nuclear import of stress-responsive transcription factors in the beta cells leading to apoptosis. It is my belief that the nuclear import inhibitor used in this study may have prevented beta cell loss partly by inhibiting the nuclear import of PKM2 in the beta cells. Additional studies using nuclear import inhibitors is crucial in determining whether this is a viable therapeutic strategy for preventing beta cell loss.

HIF-1α/PKM2-responsive genes in human T1D

In this study, I show that exacerbated beta cell loss in PTPN2-deficient beta cells is accompanied by a significant upregulation of HIF-1α/PKM2-driven genes, and that decreased expression of *Pkm2* in the NOR islets could be a reflection of attenuated beta cell apoptotic response. However, human and mice are inherently different, as seen in the differences between T1D pathogenesis in humans and the NOD mouse model (discussed in Chapter 1). Therefore, I investigated whether the upregulation of HIF-1α/PKM2 target genes is observed in the human diabetic islets. Unfortunately, global gene expression data of islets from human T1D patients do not exist yet. The closest approximation to T1D islet gene expression data is provided by the RNA-Seq data from human islets treated with pro-inflammatory cytokines (Eizirik et al., 2012). I performed *in silico* analysis of select HIF-1α/PKM2 target genes, and our results demonstrate that these genes are highly upregulated in the cytokine treated human islets (Fig. 6-1). Further study with primary

islets needs to be performed to address whether the HIF-1α/PKM2 target gene expression is regulated by PTPN2 in human beta cells, and whether PKM2 accumulates in the nuclei of diabetic beta cells at the initiation of T1D and whether this accumulation persists throughout disease progression. It is highly probable that PTPN2 mediated regulation of nuclear accumulation of PKM2 is also a mechanism of human beta cell loss, given that the original identification of *PTPN2* as a T1D candidate gene was from human genome-wide association studies.

Conclusions

T1D is a chronic disease characterized by progressive beta cell loss and the inability to maintain glucose homeostasis. To prevent beta cell loss and disease progression, it is critical to understand the mechanisms of beta cell defense. My thesis work provides a foundation for further understanding of beta cell-intrinsic factors that may be responsible for cell survival or death upon cytotoxic stimuli. The fact that PKM2 accumulates in the diabetic beta cells and that nuclear PKM2-mediated activation of pro-apoptotic transcripts may be a novel mechanism of beta cell loss have never been shown. Furthermore, through the global approach, I have discovered novel candidate genes that may be critical for beta cell defense or survival, functional studies of which may uncover additional mechanisms of beta cell loss that may be targeted for developing T1D therapies. Finally, my dissertation has bridged diabetes and cancer pathogenesis through a single molecule, PKM2, through the investigation of T1D candidate PTPN2, and figuring out how to prevent the nuclear accumulation of PKM2 may be the system through which to inhibit both diabetes and cancer progression.

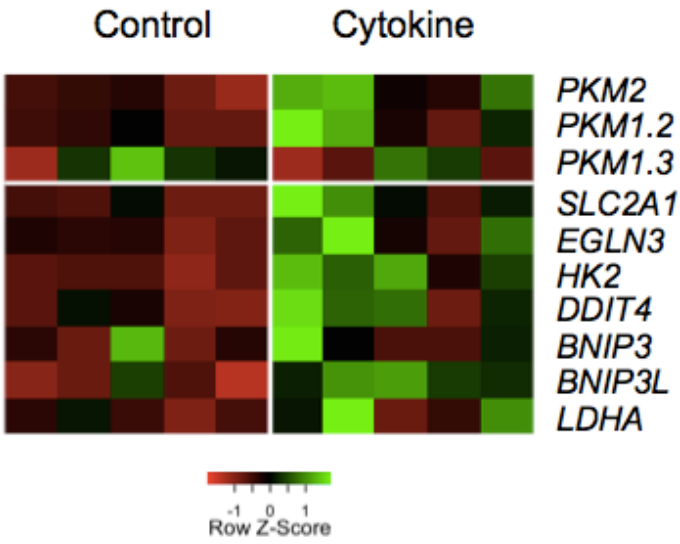


Figure 6-1. *In silico* analysis of RNA-Seq data from human islets treated with pro-inflammatory cytokines.

Analysis of human islet RNA-Seq, represented as a heatmap. Three transcripts from the *PKM* locus were detected, with transcript variant 1 encoding *PKM2* (RefSeq ID, NM_002654) and variants 2 and 3 encoding *PKM1* (RefSeq IDs, NM_182470 and NM_182471, indicated as *PKM1.2* and *PKM1.3* in the heatmap, respectively). Some of the HIF-1α/PKM2-responsive genes are shown. Four different transcript variants of *LDHA* were detected, but only the most highly expressed transcript variant (RefSeq ID, NM_005566) is shown. Raw data obtained from (Eizirik et al., 2012).

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