CHARACTERIZATION OF ISLET GENES IMPLICATED IN HUMAN DISEASE

By

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PRIOR PUBLICATIONS

Some of the material included in this thesis has been published. Chapter IV was published in *Biochemical Journal* (Pound LD, Sarkar SA, Benninger RK, Wang Y, Suwanichkul A, Shadoan MK, Printz RL, Oeser JK, Lee CE, Piston DW, McGuinness OP, Hutton JC, Powell DR, O'Brien RM. 2009 Jul 15; 421(3): 371-6). In addition, the analyses of the *Slc30a8* intronic enhancer and promoter referenced in Chapter VI were published in *Biochemical Journal* (Pound LD, Hang Y, Sarkar SA, Wang Y, Milam LA, Oeser JK, Printz RL, Lee CE, Stein R, Hutton JC, O'Brien RM. 2010 Dec 15; 433(1): 95-105) and *Journal of Molecular Endocrinology* (Pound LD, Sarkar SA, Cauchi S, Wang Y, Oeser JK, Lee CE, Froguel P, Hutton JC, O'Brien R. 2011 Jul 28. [Epub ahead of print]), respectively. The material described in Chapter V was recently submitted to *Biochemical Journal*.

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ABBREVIATIONS

APC	Antigen-presenting cells
CAM	Cardiovascular associated mortality
ChIP	Chromatin immunoprecipitation
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
ES	Embryonic stem
EST	Expressed sequence tag
FKHR	Forkhead transcription factor
FBG	Fasting blood glucose
FPG	Fasting plasma glucose
GCK	Glucokinase
GCKR	Glucokinase regulatory protein
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
G6PC	Glucose-6-phosphatase catalytic subunit
G6PC2	Glucose-6-phosphatase catalytic subunit, member 2
G6PC3	Glucose-6-phosphatase catalytic subunit, member 3
G6PT	G6P translocase
GSD	Glycogen storage disease
GSIS	Glucose stimulated insulin secretion
GWA	Genome wide association

HbA_{1C}	Hemoglobin A_{1C}
HNF-1	Hepatocyte nuclear factor-1
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit related protein
IRES	Internal ribosome entry site
$K_{ir}6.2$	Major subunit of the inward-rectifying ATP-sensitive K^+ channel
KO	Knockout
LD	Linkage disequilibrium
M6P	Mannose-6-phosphate
MODY	Maturity-onset diabetes of the young
NAD^+	Nicotinamide-adenine dinucleotide
$NADP^+$	NAD phosphate
Neo	Neomycin
NOD	Non-obese diabetic
PPARγ	Peroxisome proliferator-activated receptor γ
RIP	Rat insulin promoter
SNP	Single nucleotide polymorphisms
TCA	Tricarboxylic acid
TK	Thymidine kinase
WT	WT

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CHAPTER I

INTRODUCTION

The identification of genes implicated in human disease using genome wide association studies

In recent years, genome wide association (GWA) studies have replaced linkage analyses and candidate gene approaches and have been widely used to elucidate the genes involved in complex human conditions such as variations in fasting blood glucose and type 2 diabetes [1]. Previously, linkage analyses primarily had been used to identify rare familial genetic variants with large impacts on human health, such as those causing maturity-onset diabetes of the young (MODY), through the comparison of DNA segments shared among family members affected with a particular disorder [2]. *TCF7L2*, the gene encoding transcription factor 7-like 2, was identified as a type 2 diabetes-associated gene using the linkage analysis approach [3]. The candidate gene approach focused on the association of candidate genes or genes that would logically be involved in a particular disease [1]. Both *PPARG* and *KCNJ11*, which encode for peroxisome proliferator-activated receptor (PPARγ) and the major subunit of the inward-rectifying ATP-sensitive K^+ channel ($K_{ir}6.2$), respectively, were shown to be associated with type 2 diabetes using this method [4-6]. Both proteins are now targets for anti-diabetes medications [1]. While linkage analyses provide an unbiased approach to assessing a given disease, the gene must have a relatively large effect on the condition. In contrast, the candidate gene approach can be used to assess more complex diseases and genes with

relatively small effects on the given disease. The use of GWA methodologies, which assay the association of single nucleotide polymorphisms (SNP) across the human genome with altered risk for a particular disease or condition, provides both an unbiased approach and the ability to identify genes with a more modest impact on the risk of disease [1].

The GWA study approach has currently identified approximately 21 SNPs that are associated with glycemic traits (Table 1.1) and 38 SNPs that are associated with Type 2 diabetes (Table 1.2) [1]. It is important to note, however, that while the GWA study approach is useful in the identification of the genetic basis of complex human conditions and diseases, the low odds ratios associated with the variants suggest that individually, the variants do not cause changes in β-cell function. Rather, the GWA approach only indicates an increased susceptibility to the given condition. This suggests that other factors such as gene X gene or gene X environment interactions may play an important role in the manifestation of the condition.

An important caveat to the GWA study approach is that the polymorphism is typically named after the gene in with which it is in closest proximity [1]. It is important to note, however, that the variant may not be directly responsible for the observed phenotype, but rather may be in linkage disequilibrium (LD) with the causative polymorphism [1]. In addition, while the polymorphism may be causative, it may instead be modulating the activity of a long-range enhancer and thereby affecting the expression of distant genes [1]. It is, therefore, important to perform follow up studies to determine whether the gene in question is, in fact, responsible for the observed phenotype. Thus, the goal of the studies described within this thesis is to provide evidence that

Table 1.1. Genes identified in GWA studies that are associated with fasting plasma glucose (Adapted from Ref. [7]).

Locus	Marker	Location of SNP	Allele (Effect/Other)[a]	Frequency of glucose raising allele	Effect (mmol/l/allele)
G6PC2	rs560887	Intron 3	C/T	0.69	0.071
	rs13431652	Distal promoter	A/G	0.68	0.075
	rs573225	Proximal promoter	A/G	0.66	0.073
GCK	rs4607517	36 kb upstream	A/G	0.2	0.062
GCKR	rs780094	Intron 16	C/T	0.62	0.029

[a]Effect represents the allele associated with increased FPG. Other represents the allele associated with reduced FPG.

Table 1.2. Genes identified in GWA studies that are associated with Type 2 diabetes (Adapted from Ref. [7])**.**

Locus	Marker	Location of SNP	Allele (effect/other)[a]	Frequency of type 2 diabetes susceptibility allele	Odds ratio
SLC30A8	rs13266634	Arg325Trp	C/T	0.75	1.12
HHEX	rs1111875	7.7 kb downstream	C/T	0.56	1.13
TCF7L2	rs7903146	Intron 3	T/C	0.25	1.37
IGF2BP-2	rs4402960	Intron 2	T/G	0.29	1.17
PPARγ	rs1801282	Pro12Ala	C/G	0.92	1.14
KCNJ12	rs5219	Glu23Lys	G/T	0.4	1.15
FTO	rs8050136	Intron 1	A/C	0.45	1.15
CDKN2A/B	rs10811661	125 kb upstream	T/C	0.79	1.20
CDKAL1	rs7754840	Intron 5	C/G	0.31	1.12

[a]Effect represents the allele associated with increased FPG. Other represents the allele associated with reduced FPG.

polymorphisms within two genes identified in GWA studies, *G6PC2* [8] and *SLC30A8* [9], which encode an islet-specific glucose-6-phosphatase, G6PC2, and an islet-specific zinc transporter, ZnT-8, are causative for variations in fasting blood glucose and type 2 diabetes, respectively.

Hepatic glucose-6-phosphatase and glucose homeostasis

The liver plays a major role in maintaining tight regulation of whole body glucose homeostasis. In the postprandial state, the liver takes up glucose and after conversion to glucose-6-phosphate (G6P) either metabolizes it through the glycolytic or pentose phosphate pathways or stores it as glycogen. In the preprandial state when dietary glucose is not readily available, the liver is responsible for glucose production from either gluconeogenic precursors, such as glycerol, amino acids or lactate, or from glycogen breakdown. The observation that both gluconeogenesis and glycogenolysis result in a phosphorylated glucose molecule indicated that there must be an enzyme that could remove the phosphate group [10] though, because of its location within the endoplasmic reticulum (ER) membrane and its instability, isolation of the enzyme proved difficult [11, 12]. The cDNA encoding the glucose-6-phosphatase catalytic subunit (G6PC) was finally cloned in 1993, however, by Chou and colleagues who took advantage of a mouse model with radiation induced chromosomal deletions at the albino locus [13, 14]. This mouse strain displayed severe hypoglycemia and lethality shortly after birth as a result of reduced gluconeogenic enzyme activity including that of glucose-6-phosphatase [15]. Chou and colleagues screened a murine liver cDNA library with probes representing the

mRNA populations from either wild type or albino deletion mutant mice and isolated the cDNA encoding the murine glucose-6-phosphatase catalytic subunit [14, 16].

Glucose-6-phosphatase, the enzyme responsible for the terminal step of both gluconeogenesis and glycogenolysis, namely hydrolysis of glucose-6-phosphate (G6P) to produce glucose, is primarily expressed in the liver and kidney, with low expression also present in the pancreas and small intestine [14, 17, 18]. It is currently accepted that glucose-6-phosphatase is a multi-component enzyme system that consists of both the catalytic subunit as well as transporters for the substrates and products, termed the Arion substrate-transport hypothesis (Fig. 1.1) [19, 20]. In this model, the catalytic subunit, G6PC, responsible for G6P hydrolysis, has an active site that faces the ER lumen in the adult, though this orientation is reversed in the fetus [21]. Thus, the G6Pase system must also possess transporter activity to shuttle both substrate and product across the ER membrane. Subsequently, a G6P translocase (G6PT) responsible for delivering the substrate to the ER lumen was identified based on its homology to a known G6P transporter located on the inner membrane of *Escherichia coli*, UhpT [22]. Using an expressed sequence tag (EST) database, a mouse and human cDNA were identified that shared approximately 20% identity with the bacterial transporter. The G6PT is thought to lend specificity to the system as it is almost specific for G6P [20]. In intact cells, hydrolysis of mannose-6-phosphate (M6P) occurs at a rate of 5% of that of G6P [23]. When the membrane is disrupted, however, the rates of hydrolysis of G6P and M6P are similar [23]. Furthermore, UhpT is known to act as an antiporter, exchanging one molecule of a phosphate ester with one or two molecules of inorganic phosphate, depending on the pH [24]. Consistent with this observation, it is now thought that this

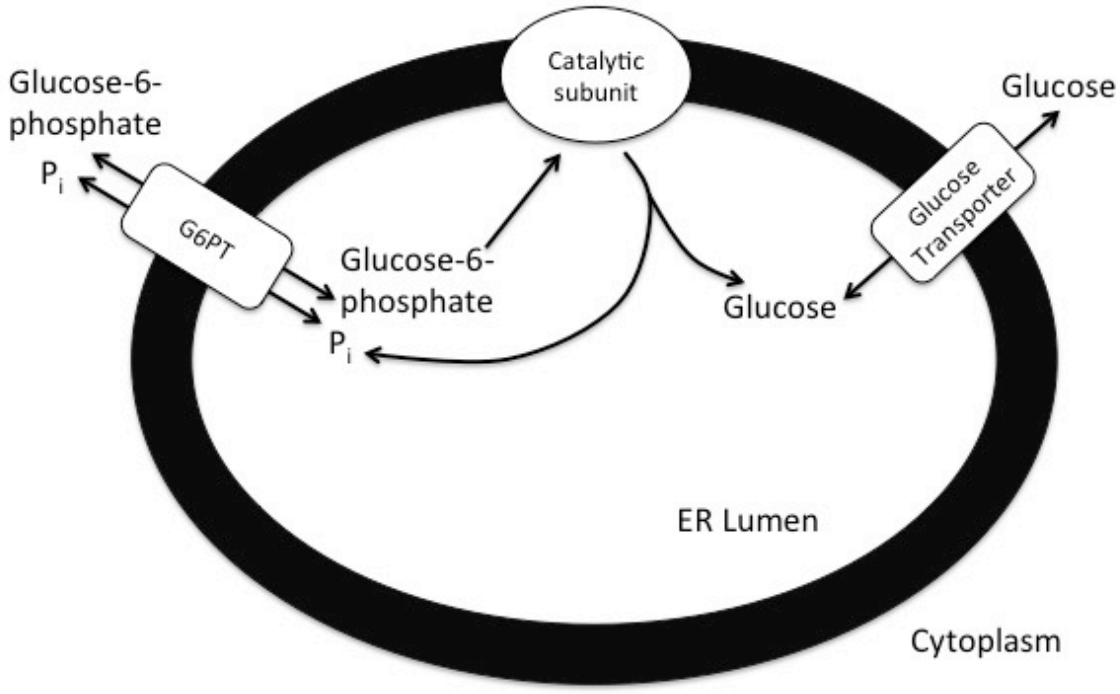


Figure 1.1. Model of the glucose-6-phosphatase multicomponent enzyme system.

same transporter shuttles inorganic phosphate back into the cytoplasm [25]. The glucose transporter in this system has yet to be identified.

Hepatic glucose-6-phosphatase and human disease

Both decreases and increases in glucose-6-phosphatase activity have been linked to human disease states. Reduced glucose-6-phosphatase activity results in glycogen storage disease (GSD), a group of autosomal recessive disorders. GSD type 1a is caused by mutations in *G6PC* and is characterized by heptomegaly and nephromegaly as a result of increased glycogen deposition, hypoglycemia in the fasted state, renal failure and growth retardation [26]. Both GSD type 1b and GSD type 1c are caused by mutations in *G6PT* and present similarly to GSD type 1a with patients also having increased susceptibility to bacterial infection due to neutrophil dysfunction [27]. Interestingly, though the G6P translocase is expressed in leukocytes, the G6PC catalytic subunit is not. This suggests an additional role of G6PT and is consistent with the presence of another isoform, G6PC3, in this cell type.

Increased glucose-6-phosphatase activity is characteristic of both type 2 diabetes and in some cases of type 1 diabetes. Typically, a 2-4-fold increase in mRNA levels and a 2-3-fold increase in G6Pase activity is observed in patients with diabetes [28-30], presumably the result of a relative (type 2) or absolute (type 1) reduction in insulin signaling. Insulin signaling has been shown to regulate *G6PC* gene transcription through an insulin response unit composed of a hepatocyte nuclear factor-1 (HNF-1) binding site located between -231 and -199 [31] and two FOXO1 binding sites located between -198 and -159 [32, 33]. HNF-1 is thought to act as an accessory factor that stabilizes the

binding of FOXO1 [34, 35]. Insulin signaling targets FOXO1 resulting in its nuclear exclusion [36-43]. Because FOXO1 acts as a transcriptional activator in this gene its removal from the nucleus results in a decrease in *G6PC* transcription. Thus, an inappropriate elevation in *G6PC* due to altered insulin signaling therefore results in inappropriate glucose production, exacerbating the hyperglycemia observed in type 2 diabetes.

The glucose-6-phosphatase gene family

The glucose-6-phosphatase gene family comprises three members: G6PC, G6PC2 and G6PC3. The identification of G6PC2 came out of the observation that though glucose-6-phosphatase activity was present in the pancreatic β-cell, the kinetics differed from that observed in the liver. Specifically, glucose-6-phosphatase activity in insulinoma microsomes is approximately one quarter of the activity observed in liver microsomes (33.1±6.4 vs. 132.7±24.9 nmol/min/mg) [44]. In addition, islet glucose-6-phosphatase displays a K_M value, pH optima, and inhibitor profile distinct from the liver, suggesting either the presence of an alternative glucose-6-phosphatase isoform in islets or a regulatory factor that alters the behavior of the same isoform that is present in liver [44, 45]. The isolation of a cDNA cloned from mouse insulinoma tissue that encodes a 355-amino acid protein and shares 50% identity with G6pc favors the former explanation. This protein was found to be restricted to the pancreatic islets and was subsequently named the islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) [44]. This protein has now been renamed G6PC2.

In addition to G6PC and G6PC2, a third isoform in the glucose-6-phosphatase gene family was identified using a BLAST search with human *G6PC2* as the query and was named the ubiquitously expressed glucose-6-phosphatase catalytic subunit related protein (UGRP) due to its expression in a wide variety of tissues, particularly heart, skeletal muscle, brain, kidney and pancreas. This protein has now been renamed G6PC3. G6PC3 shares 36% homology with G6PC [46].

Table 1.3 summarizes the characteristics of the *G6PC* family [47]. Both *G6PC* and *G6PC3* are located on chromosome 17, while *G6PC2* is located on chromosome 2. All three *G6PC* isoforms encode proteins that are located in the ER, are a similar length and are predicted to share a similar topology and 9 transmembrane domain structure.

While it has been well established that G6PC is capable of G6P hydrolysis, the glucose-6-phosphatase activity of G6PC2 and G6PC3 has been less clear. Though, based on its structure and the conservation of a number of critical residues, G6PC2 would be predicted to hydrolyze glucose-6-phosphate, initial data from the Hutton laboratory using Cos7 cells transiently transfected with G6pc2 failed to demonstrate G6P hydrolysis [44, 48, 49], as did experiments performed by the Chou laboratory [50]. Under different conditions, however, Petrolonis and colleagues were able to demonstrate that G6pc2 is capable of hydrolyzing G6P [45]. Since this report, the Hutton laboratory has re-evaluated the issue. By permeabilizing the membrane to G6P with minimal perturbation of the intracellular membrane architecture, they were able to demonstrate that G6pc2 is capable of G6P hydrolysis (Fig. 1.2) [47]. It is important to note, however, that it does so with a lower K_M and V_{MAX} than G6pc [45].

Table 1.3. The glucose-6-phosphatase catalytic subunit gene family.

Gene	G6Pase	Islet-Specific G6Pase-Related Protein (IGRP)	Ubiquitous G6Pase Related Protein (UGRP)
	G6PC	G6PC2	G6PC3
Tissue	Liver	Islet β-cell	Ubiquitous
Size	357 aa	355 aa	346 aa
% Identity	100	50	36
Chromosome	17q21	2	17q21
Location	ER	ER	ER
#Transmembrane domains	9	9	9
Substrate	G6P	G6P	G6P

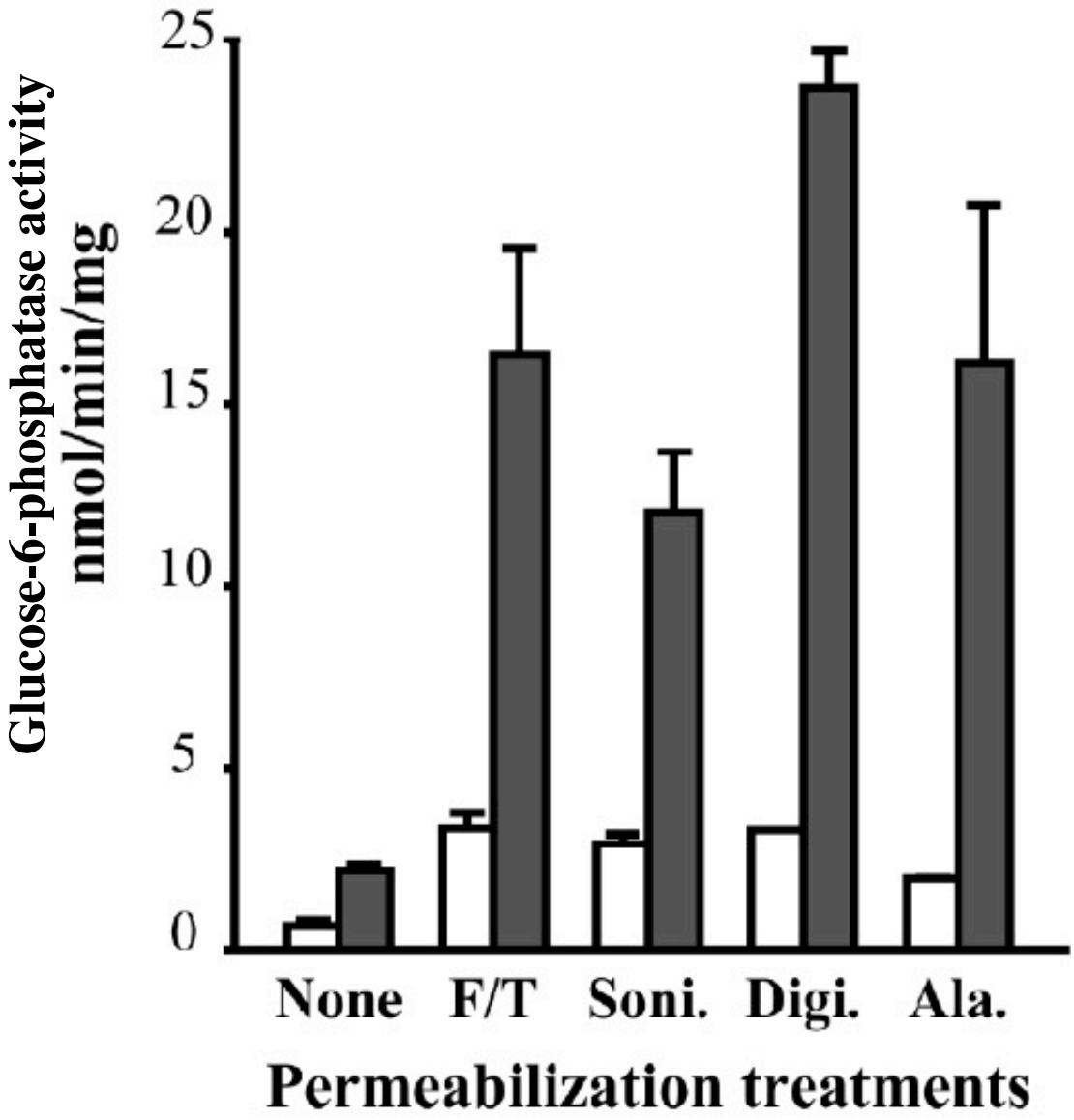


Figure 1.2. G6PC2 possesses glucose-6-phosphatase activity in permeabilized cells. The glucose-6-phosphatase activity of G6PC2 was measured in COS7 cells under several membrane permeabilization conditions. COS7 cells were transfected with either pcDNA3.1 (white bars) or pcDNA3.1 containing human G6PC2 (grey bars). 48 hours after transfection, cells were harvested, pelleted and resuspended. Cells were then permeabilized with limited disruption of the internal membranes using the indicated treatment prior to assaying for glucose-6-phosphatase activity using a radiochemical assay. F/T=freeze thawing; Soni.=sonication; Digi.=0.1% digitonin for 15 min. on ice; Ala.=0.02% alamethicin for 15 min. on ice. Results are means ± S.E.M.

Though the Hutton laboratory was also unable to demonstrate G6P hydrolysis by G6PC3 when expressed in COS cells using transient transfections at the same levels as G6PC [46], other labs have been able to show that G6PC3 can serve as a glucose-6-phosphatase when expressed at much higher levels using either stable [51] or adenoviral [52] transfections. The reason(s) for this discrepancy remain unclear. The estimated V_{Max} value for G6PC3 is approximately one sixth of G6PC while the K_M values are similar [52, 53]. Studies in *G6pc3* KO mice have confirmed that deletion of *G6pc3* impairs G6P hydrolysis in brain and testis homogenates [53]. In addition, another study found that *G6pc3* KO mice demonstrate neutropenia and defective neutrophil function [54], consistent with observations that mutations in *G6PC3* in humans result in severe congenital neutropenia syndrome [55].

Glucose-6-phosphatase activity in the ß-cell

Though the liver and to a lesser extent, the kidney, are the primary glucose producing organs, *G6PC* expression and glucose-6-phosphatase activity have been detected in other organs, including the pancreatic ß-cell [56]. This was a surprising observation because the ß-cell was not known to contribute appreciably to gluconeogenesis. Rather, it is well established that the primary function of the ß-cell is to secrete insulin in response to glucose and other nutrients. Thus, it has been hypothesized that an islet glucose-6-phosphatase would serve to modulate glucose stimulated insulin secretion (GSIS) [57].

As glucose levels rise in the blood, glucose enters the ß-cell through the high-K_M glucose transporter, GLUT2, and is then phosphorylated by glucokinase and further

metabolized by the glycolytic pathway and the tricarboxylic acid (TCA) cycle (Fig. 1.3). This results in a rise in ATP levels and consequently the ATP:ADP ratio, which causes the closure of the ATP-sensitive potassium (K_{ATP}) channels. The decrease in potassium influx leads to the depolarization of the ß-cell membrane and the opening of voltage-gated calcium channels and influx of calcium. Intracellular calcium concentrations rise, activating the exocytotic machinery, which then allows fusion of the insulin vesicles with the cellular membrane and subsequent insulin secretion.

Though this has long been the accepted model of GSIS, more recent studies suggest an important role for a K_{ATP} channel-independent mechanism of GSIS [58]. While the regulation of K_{ATP} channels appears to be particularly important for the triggering signal of GSIS [59, 60], the amplifying pathway of GSIS seems to rely on K_{ATP} channel independent mechanisms [61]. This concept is supported by the observation that mice that lack K_{ATP} channel function due to the loss of either of the channel subunits retain GSIS and maintain glucose homeostasis [62-65]. Additional studies have indicated that pyruvate metabolism, and specifically the byproducts of the pyruvate/isocitrate cycle, NADPH and α-ketoglutarate, may play a key role in the K_{ATP}-independent mechanism of GSIS [66, 67].

It is critical that insulin is secreted in proportion to circulating glucose levels in order to tightly maintain glycemia. The ß-cell must, therefore, have a mechanism in place for accurately coupling blood glucose concentrations to insulin secretion. There have been two primary proteins that have been considered candidates for the ß-cell glucose sensor: GLUT2 [68, 69] and glucokinase [70]. Though both proteins have K_M values within the physiological range [71, 72] a number of studies in isolated islets,

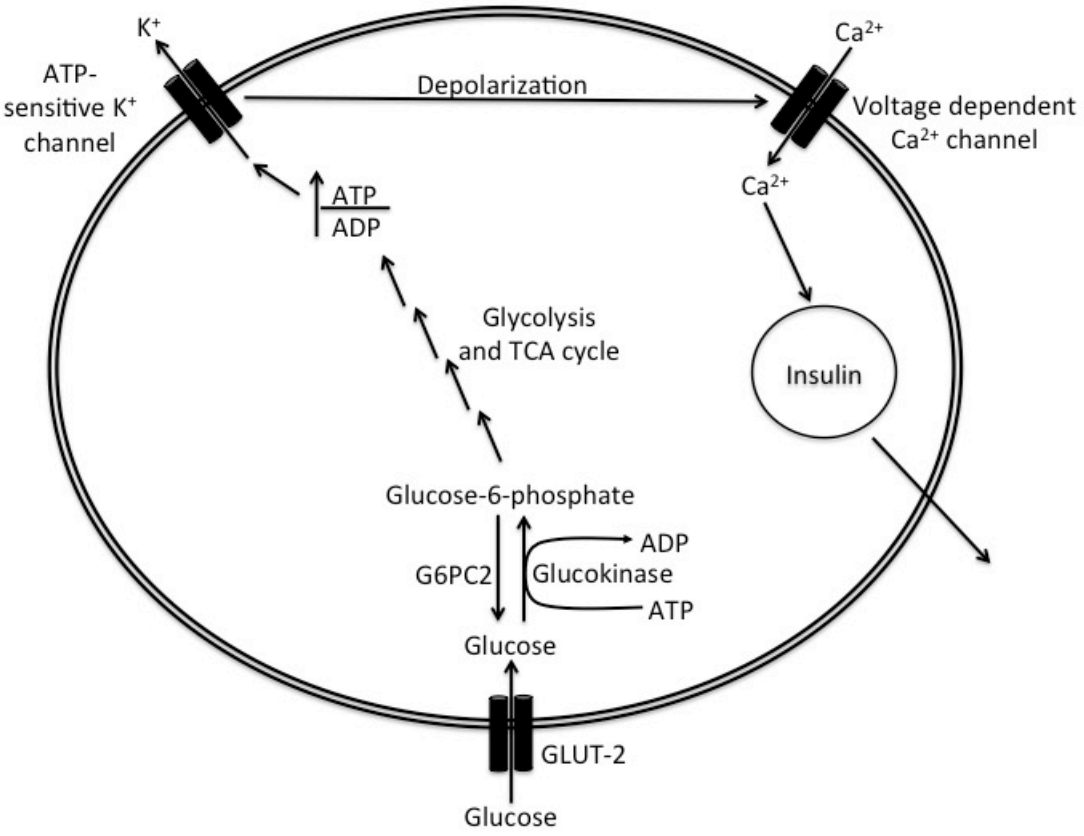


Figure 1.3. Glucose-6-phosphatase activity and the futile cycle in the pancreatic β-cell. The presence of glucose-6-phosphatase activity in the β-cell opposes glucokinase activity and results in a futile cycle. This would be predicted to result in the hydrolysis of ATP without regeneration thus lowering insulin secretion.

immortalized cell lines and transgenic mice have suggested that glucokinase, rather than GLUT2, serves as the glucose sensor and that glucose transport is not limiting [73]. For example, transgenic mice overexpressing a yeast isoform of glucokinase exhibit increased plasma insulin and reduced plasma glucose concentrations as well as enhanced GSIS from isolated islets [74]. Furthermore, transgenic mice in which glucokinase expression has been knocked down display an increased threshold for GSIS in pancreas perfusions [75]. In contrast, neither overexpression of GLUT1 [73] nor attenuation of GLUT2 expression by 90% [76] in transgenic mice affects GSIS. The prevailing dogma has, therefore, been that glucokinase serves as the ß-cell glucose sensor.

The observation that glucose-6-phosphatase activity exists in the ß-cell, however, brings this model into question. Glucokinase and an islet glucose-6-phosphatase would be predicted to form a futile cycle in which ATP is used during the phosphorylation step (Fig. 1.3). This would result in a relative decrease in the ATP:ADP ratio and attenuation of GSIS. Although this system is metabolically inefficient, it would allow multiple inputs to be integrated and the set point of GSIS to be more finely tuned than with glucokinase alone. It is, therefore, conceivable that an islet glucose-6-phosphatase could serve as a negative component of the ß-cell glucose sensor.

Consistent with this hypothesis, a number of studies have been able to detect glucose cycling in the murine islet at, albeit, low levels. Khan and colleagues, using healthy control islets and either islets isolated from ob/ob mice or streptozotocin-induced diabetic rats, estimated glucose dephosphorylation to occur at a rate of approximately 3-4.5% of glucose phosphorylated in the healthy islets and 40% and 15.7% in the ob/ob and diabetic rat islets, respectively [77, 78]. In contrast, studies using islets isolated from

healthy rats have suggested that although glucose-6-phosphatase is present, activity levels are too low to significantly affect glucose cycling and GSIS [79]. An important caveat to this study, however, is that the authors used rat islets that were freshly isolated and cultured in low glucose. It has been well established that glucose stimulates rat *G6pc* gene transcription [80-82] and, thus, in low glucose conditions, G6pc activity would be expected to be relatively low. Whether the level of glucose-6-phosphatase activity and of glucose cycling is sufficient to significantly affect GSIS *in vivo* remains unknown.

Transcriptional regulation of *G6PC2*

G6pc2 expression has been detected primarily in pancreatic ß-cells as well as a subset of α-cells [83]. Studies in our lab suggested that a short region of the promoter from -306 to +3, relative to the transcription start site, was sufficient to drive reporter gene expression in β-cell lines [84]. Surprisingly, however, while this region was sufficient to confer islet-specific transgene expression in mice beginning at embryonic day 12.5, like the endogenous *G6pc2* gene, the minimal promoter was unable to confer sustained transgene expression in the adult [83]. This observation suggests that the mechanisms by which *G6pc2* promoter activity is regulated differ in cell lines as compared to *in vivo* and that cell lines may resemble the fetal state. A number of putative enhancer elements have since been identified 5', within and 3' of the *G6pc2* gene [85], and our lab is currently in the process of determining which regions are critical for maintaining gene expression in the adult.

The transcription factors driving activity of the proximal (-306/+3) promoter have been thoroughly investigated. Originally, the goal of this work was to identify novel,

islet-enriched transcription factors that are important for islet function and/or development. Because the derivation of insulin-secreting β-cells from stem cells represents a potential cure for type 1 diabetes, the identification of novel transcription factors with a role in islet growth or differentiation could prove to be important for this process. Using *in situ* footprinting of this highly conserved proximal promoter region, key *cis*-acting sites were identified [86]. Chromatin immunoprecipitation assays and mutational analyses later indicated that Pdx-1, Pax-6, MafA, Foxa2, BETA-2 and USF bind this region in insulinoma βTC3 cells and regulate promoter activity [87-89]. Interestingly, the same group of factors has been shown to bind and regulate the insulin promoter [90-92].

Association of G6PC2 with human disease

Recent GWA studies have identified a number of SNPs within the human *G6PC2* gene that are associated with variations in fasting plasma glucose (FPG) levels in healthy, normoglycemic individuals [8, 93-95]. SNP rs560887 is located within the third intron of the *G6PC2* gene [8] and our lab has recently demonstrated that it affects splicing efficiency (Devin Baerenwald and Richard O'Brien, unpublished data). Each additional A allele, the minor allele, is associated with approximately a 1mg/dl reduction in fasting plasma glucose levels [8]. An additional SNP, rs13431652, located in the *G6PC2* promoter, was also shown to associate with a similar variation in FPG due to altered binding of the transcription factor NF-Y [96]. Specifically, the rs13431652-A allele is associated with increased FPG and increased promoter activity due to stronger NF-Y binding [96]. Interestingly, a third SNP, rs573225, was also shown to associate with FPG

and it was demonstrated that the region binds Foxa2 in insulinoma cell lines [95, 96]. The rs573225-A allele, however, is associated with elevated FPG in humans but with reduced promoter activity in cell lines [96], an observation inconsistent with expected results and the data from SNPs rs560887 and rs13431652. Our laboratory has interpreted this result as revealing the potential limitation of using insulinoma cell lines to follow up on human GWA study data.

Though these variations in FPG seem relatively minor, it is important to note that small variations in FPG can have a significant impact on human health. For example, one study demonstrated that, in a European population, an increase in fasting plasma glucose from <90 mg/dl to between 99 and 108 mg/dl is associated with a 30% increased risk of cardiovascular associated mortality (CAM) [97]. A separate study performed in an Asian population indicated that a reduction in fasting plasma glucose from 99 mg/dl to 90 mg/dl results in a 25% reduction in the risk of CAM [98]. Furthermore, it has been well established that increased fasting plasma glucose is a risk factor for type 2 diabetes [99].

More recent studies, however, have challenged these ideas. Sarwar and colleagues found that within normal FPG concentrations, between 70 and 100 mg/dl, FPG does not associate with CAM in a meta-analysis of 102 prospective studies [100]. Furthermore, it was shown that plasma glucose levels one hour after a glucose tolerance test are actually a better predictor of type 2 diabetes than FPG [101]. Finally, although SNPs within the *G6PC2* gene associate with FPG within the normal range, Heni and colleagues demonstrated that in patients with elevated FPG, SNPs within the *G6PC2* gene no longer influence glycemia [102]. While the role of G6PC2 in CAM has not yet

been directly investigated, these studies do suggest that G6PC2 may not play a significant role in disease but may rather be important for shutting off insulin secretion, as described in the next section.

In addition to *G6PC2*, a number of other genes have also been shown to be associated with variations in FPG, most notably *GCK* and *GCKR* that encode glucokinase and the glucokinase regulatory protein, respectively [103, 104] (Table 1.1). Glucokinase, a high K_M hexokinase, catalyzes the initial step in glycolysis and glycogen synthesis, the phosphorylation of glucose to G6P, in the islet as well as the liver, gut and brain [105] and thus, G6PC2 is predicted to oppose the actions of glucokinase. Glucokinase has traditionally been considered the primary glucose sensor [106]. Though mutations within the *GCK* gene have been shown to reduce glucokinase activity, leading to MODY2 [107], SNP rs4607517 is located 36 kb upstream of the transcription start site and is associated with a small but significant change in FPG levels and hemoglobin A_{1C} (HbA_{1C}) [104]. In the post-prandial state, glucokinase is present in the active form in the cytosol. In the post-absorptive state, the glucokinase regulatory protein will associate with glucokinase, resulting in nuclear localization, thus rendering glucokinase functionally inactive. A SNP within a *GCKR* intron, rs780094, is associated with a small variation in FPG [104]. Because all three genes are closely related in function, it is not surprising that all are associated with variations in FPG. This underscores the putative role of *G6PC2* as a component of the glucose sensor. These data also suggest that in this instance the SNPs identified in GWA studies are affecting the expression of the genes in which they are located rather than affecting enhancers that regulate the activity of distant genes. Finally, not only have SNPs within the *G6PC2* gene been associated with FPG, but it has also

been shown that SNP rs560887 associates with variations in hepatic glucose production as well as insulin secretion after oral and intravenous glucose load [108-110].

In addition to CAM, G6PC2 has been implicated in the pathophysiology of type 1 diabetes. G6PC2 was identified as the target of the 8.3-like T cell clone, a representative clone of the T cell population expressing a shared T cell receptor α chain (Vα17-Jα42), in the non-obese diabetic mouse (NOD) [111], a widely studied model of human type 1 diabetes [112]. It was subsequently identified as a target of cell mediated autoimmunity in humans [113, 114] as well as in "humanized mice" [113, 115, 116].

Initially, proinsulin and G6PC2 were thought to be likely candidates for the primary autoantigen as they are both β-cell specific and early targets of T-cell reactivity [111]. Subsequent studies, however, have pointed to the idea that proinsulin is the primary autoantigen, while the T cell response to G6PC2 lies downstream, suggesting that the autoimmune response to proinsulin spreads to other antigens, such as G6PC2 [117]. Specifically, using mice overexpressing either proinsulin 2 or G6PC2 in their antigen-presenting cells (APCs), it was shown that mice overexpressing proinsulin lacked G6PC2-reactive T cells and were diabetes free while those overexpressing G6PC2 still developed diabetes [117]. Furthermore, deletion of *G6pc2* from NOD mice does not prevent or delay the development of type 1 diabetes (Ken Oeser, Luc Van Kaer and Richard O'Brien, unpublished data) while NOD mice expressing a non-immunogenic form of proinsulin do not develop type 1 diabetes [118].

Though these studies point to a primary role for insulin and the existence of epitope spreading, it is not known whether this phenomenon is actually a required step in the development of diabetes. The data only suggest that autoimmunity toward G6PC2 is

not required for the development of type 1 diabetes but, given the existence of multiple autoantigens, the phenomenon of epitope spreading may still be required for disease progression.

G6pc2 function *in vivo*

The presence of glucose-6-phosphatase activity in the islet β-cell is a surprising observation given that the islet does not contribute significantly to whole body gluconeogenesis. Rather it seems more likely that an islet glucose-6-phosphatase could oppose glucokinase and modulate GSIS. In order to gain insight into the function of G6pc2, our lab obtained *G6pc2* knockout (KO) mice and examined the phenotype on a mixed C57BL/6J x 129SvEv genetic background. At 16 weeks of age, no difference in fasting plasma insulin or glucagon levels was observed but there was a small but significant reduction in fasting blood glucose (FBG) levels in both male (-14%) and female (-11%) KO mice following a 6 hour fast [119].

Hypothesis on the role of G6pc2 *in vivo*

Based on the evidence outlined here, we hypothesize that G6PC2 acts as a glucose-6-phosphatase and serves as an inhibitory component of the beta cell glucose sensor, which determines glycolytic flux and modulates the $S_{0.5}$ of GSIS. Thus, we predict that deletion of *G6pc2* in mice will result in a leftward shift in GSIS with no change in V_{MAX} (Fig. 1.4). To address this hypothesis our laboratory did perform hyperglycemic clamps on the mixed background mice to determine whether insulin secretion was elevated in the KO mice but we failed to detect a difference [119]. One

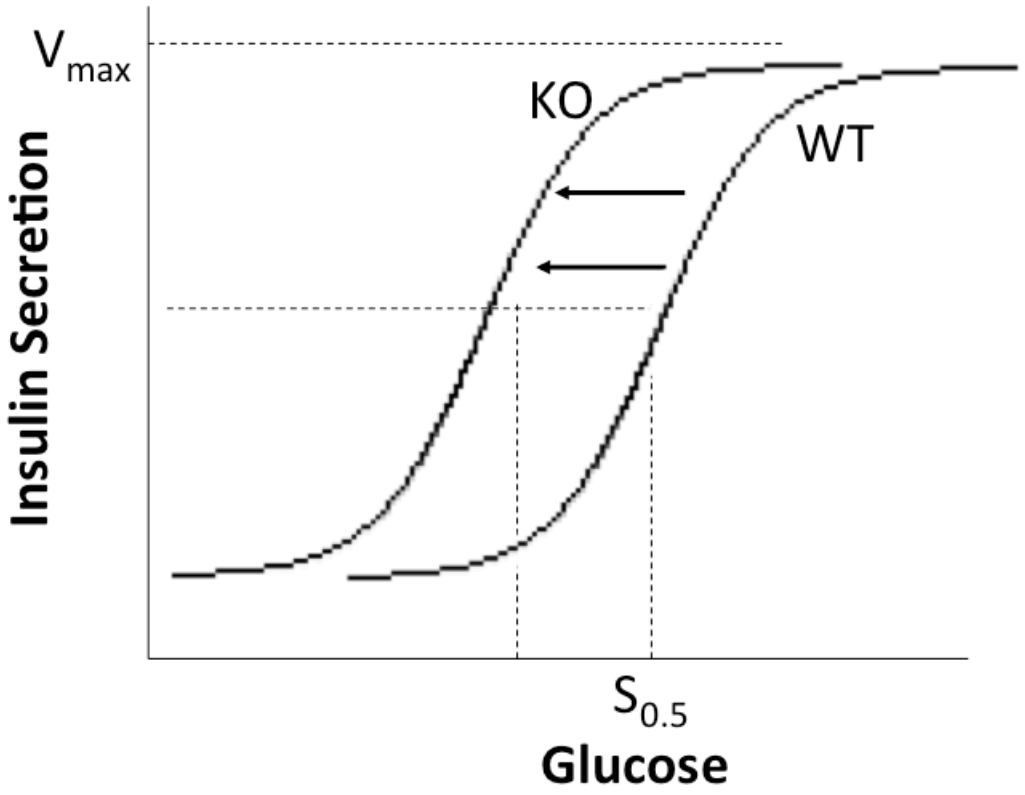


Figure 1.4. Hypothesized effect of *G6pc2* deletion in mice on GSIS dose-response curve. G6pc2 is hypothesized to serve as a negative component of the β-cell glucose sensor thus determining glycolytic flux. Deletion of *G6pc2* in mice is expected to result in a leftward shift in the dose-response curve of GSIS.

limitation to this approach is the large variation observed between mice on the mixed genetic background, which may explain why we were unable to detect a difference. Thus, more sophisticated analyses of insulin secretion have been performed on *G6pc2* KO mice on a pure C57BL/6J background and are described in Chapter III.

In addition to deletion of *G6pc2*, one study has also analyzed the effect of overexpression of *G6PC2* using a transgenic mouse model expressing human *G6PC2* under the control of the rat insulin promoter (RIP) in NOD mice. Overexpression of *G6PC2* results in increased cell death due to ER stress and consequently diabetes [120]. It was hypothesized that this is likely due to the generation of unfolded proteins in the ER [120].

Zinc homeostasis

Zinc plays a critical role in many functions *in vivo* and thus, zinc levels are tightly regulated [121]. Whole body zinc levels in adults are usually between 2 and 4 g while plasma levels are typically 12-16 μM [122]. The highest concentrations of zinc are found in the prostate, eye, brain, muscle, bones, kidney and liver [123]. Zinc acts as a cofactor and is critical for proper functioning of over 300 enzymes [124, 125]. In addition, it plays an important role in many different biological functions, including RNA and DNA metabolism, signal transduction, gene expression, metabolic enzyme activity, and protection against apoptosis and oxidative stress [121]. For example, zinc is used as a catalytic agent for both carbonic anhydrase and carboxypeptidase, enzymes responsible for carbon dioxide regulation and protein digestion, respectively [126]. Within the pancreas, some of the highest levels of zinc are present in the islet where zinc

concentrations are 10-12 mg/100 g tissue [127], approximately 4-fold higher than in the surrounding acinar tissue [128]. Zinc localizes to the insulin secretory granules [128], where it is thought to be important in the pancreatic ß-cell for proper insulin processing and secretion [129]. Alterations in zinc homeostasis are associated with a number of diseases including diabetes [122]. Thus, whole body zinc homeostasis is tightly regulated. This regulation is mediated by two classes of molecules, specifically metallothioneins and zinc transporters.

Metallothioneins

Metallothioneins are members of a superfamily of intracellular metal binding proteins and are capable of binding a number of metals including copper, cadmium and zinc [130]. Conserved cysteine residues within the proteins bind zinc and modulate zinc trafficking and storage [130]. Furthermore, metallothioneins are responsible for carrying zinc ions within a cell from one organelle to another, serving as a critical component of zinc signaling systems, and are thought to protect both α- and β-cells from oxidative stress [131, 132].

In mammals there are four genes, *MT1-4*, that encode metallothionein isoforms. In mice, the four genes are located within 50kB of one another on chromosome 8 [133]. In humans, a cluster of genes located on chromosome 16 encode the multiple isoforms of MT1, designated by the letters a, b, e, f, g, h and x, as well as MT2 while single genes on the same chromosome encode MT3 and MT4 [134-136].

Both *MT1* and *MT2* are widely expressed throughout the body [137] while *MT3* is primarily expressed in the brain, as well as liver, kidney, intestine and pancreas [138-

140], and *MT4* is detected only in squamous epithelia and maternal deciduum [141, 142]. High levels of metallothioneins are found in rapidly proliferating cells where they are not only found in the cytosol but also within the nucleus [143, 144]. Here, it is thought that metallothioneins may serve to either protect DNA from oxidative damage or to provide critical cell division enzymes and transcription factors with zinc [144-146]. In addition, the highest levels of metallothioneins are present in the liver, kidney, intestine, where they regulate both zinc absorption and excretion, and pancreas [134, 147-149].

The importance of metallothioneins in the pancreas is underscored by both rodent studies as well as human genetic data. Islets isolated from metallothionein knockout mice display reduced GSIS [150], consistent with the important role of zinc in insulin secretion. In addition, streptozotocin-treated mice and rats display increased levels of zinc in both the liver and kidney that may be explained by the observed increase in metallothionein levels in these organs [131]. Furthermore, transgenic overexpression of metallothionein in streptozotocin-treated β-cells *in vitro* and *in vivo* results in a reduction in streptozotocin-induced β-cell destruction, a delay in the onset of diabetes and a reduction in glycemia [151]. In addition, pretreatment of streptozotocin-treated rats with zinc supplementation both increases plasma and pancreatic metallothionein levels and reduces glycemia [152]. In humans, polymorphisms in both *MT1A*, rs11640851, and *MT2A*, rs1610216, have been found to be associated with type 2 diabetes, blood glucose and HbA_{1C} in a small Chinese cohort of Han descent [153]. Furthermore, polymorphisms in these same isoforms, rs11076161 and rs10636 in *MT1A* and *MT2A*, respectively, have been associated with diabetic neuropathy [153]. Finally, a polymorphism within the *MT1B* gene has been shown to be associated with obesity in humans [153]. These

observations are also consistent with data derived from the *MT1/2* knockout mouse. These mice display increased food intake, increased body weight and increased plasma leptin levels [154, 155].

Zinc transporters

In addition to the important role of metallothioneins in zinc homeostasis, two families of zinc transporters facilitate the movement of zinc into or out of cells. The SLC39 (ZIP) family is responsible for cellular uptake of zinc and/or other metal ion substrates from either the extracellular matrix or release from intracellular vesicles into the cytoplasm while the SLC30 (ZnT) family controls cellular efflux of zinc into the extracellular space or into intracellular vesicles [155]. Table 1.4 summarizes the characteristics of the ZIP (Zrt, Irt-like protein) family. Most members are predicted to share a similar structure and topology, namely an eight transmembrane domain structure with the N- and C-termini predicted to reside on the extracellular membrane [155]. In addition, a long loop region is present between domains III and IV that is highly conserved and contains a histidine-rich loop that had been predicted to serve as a metal binding region [155]. Mutational analysis using the yeast Zrt1 transporter, however, suggests that this region may instead alter subcellular localization [156]. Unlike the metallothionein or ZnT families, there is no known association of any of the Zip family members with type 2 diabetes. This is surprising given the postulated importance of zinc in islet function but it may indicate redundancy of function in this family. In contrast, a number of ZIP family members have been associated with breast cancer [157-160] and acrodermatitis enteropathica [161-163].

Table 1.4. Characteristics of the ZIP family of zinc transporters (Adapted from Ref. [155])**.**

	Dietary Zinc Response	Tissue/Cell Expression	Subcellular Localization	Knockout Phenotype	Association with Human Disease
ZIP1		Ubiquitous	In humans, membrane and ER (cell type dependent) In mice, intracellular organelles ($+Zn^{2+}$); cell membrane ($-Zn^{2+}$)	No phenotype if zinc adequate; adverse developmental effects when zinc deficient	
ZIP2		Prostate, uterus, cervical epithelium, optic nerve, monocytes			
ZIP3		Testes	Intracellular organelles ($+Zn^{2+}$); cell membrane ($-Zn^{2+}$)		
ZIP4		Small intestine, stomach, colon, kidney			
ZIP5	mRNA: unresponsive; Translation: abundance stimulated by Zn2+ supplementation	Liver, kidney, pancreas, small intestine, colon	Basolateral surface (+Zn2+); internalized (-Zn2+)		Acrodermatitis enteropathica
ZIP6		Steroid-hormone sensitive tissues: placenta, mammary gland, prostate	Plasma membrane		May play a role in breast cancer progression
ZIP7	Protein abundance repressed by Zn2+ supplementation	Ubiquitous	Golgi apparatus		May play a role in breast cancer progression

ZIP8		Lung, kidney, testis, liver, brain, small intestine, mature RBCs	Plasma membrane (-Zn2+); internalized (+Zn2+) (Exception: RBCs always membrane)	No knockout; Resistance to Cd-induced testicular toxicity observed in certain inbred mouse strains attributed to Zip8 variant	
ZIP10	Increased transcription during Zn2+ deficiency				May play a role in metastatic breast cancer progression
ZIP12					Schizophrenia
ZIP13			Golgi apparatus	Reduced osteogenesis, abnormal cartilage development, reduced dentin and alveolar bone, abnormal craniofacial features, decreased corneal stromal collagen	Spondylocheirodysplastic form of Ehlers-Danlos syndrome (SCD-EDS)
ZIP14		Liver, intestine	Plasma membrane		

*Zip 9 and Zip11 have not been characterized.

The ZnT family of transporters opposes the action of the ZIP family by transporting zinc out of the intracellular space. The 10 members of the ZnT family share a similar six transmembrane domain structure with both the N and C terminals located in the cytoplasm [155]. Between domains IV and V most members share a histidine-rich loop that is thought to bind zinc [155]. ZnT-6 and ZnT-10, however, have a serine-rich and a basic amino acid-rich loop, respectively, in its place [155]. Table 1.5 summarizes the characteristics of the ZnT family.

Role of zinc in the islet

Zinc levels in the pancreas are among the most abundant in the body with the highest concentrations localized to the pancreatic islets [128]. Free zinc levels are most concentrated in the insulin granules, with concentrations of approximately 20 mM [164], where zinc has been hypothesized to play a critical role in insulin processing, storage and secretion [127]. In the presence of zinc, proinsulin monomers form soluble hexamers containing at least two zinc ions coordinated to histidine residues at position B_{10} [127]. This solubility favors both transport into the Golgi apparatus, where it is thought that the zinc ions help to protect proinsulin from disulfide bond reduction, as well as the formation of evaginations that bud off into the insulin secretory vesicles [127, 165]. Proinsulin is cleaved to form insulin, which is insoluble in the presence of zinc, and the newly formed insulin hexamers form a crystalline structure within the vesicles [166]. The zinc containing crystalline structure is thought to help prevent further proteolysis of the protein [129]. Finally, when the insulin secretory granules fuse with the membrane and release insulin into the bloodstream, both the increase in pH from 5.5 to 7.4, which

Table 1.5. Characteristics of the ZnT family of zinc transporters (Adapted from Ref. [155]).

	Dietary Zinc Response	Tissue/Cell	Subcellular Localization	Knockout Phenotype	Association with Human Disease
ZnT1	Zn2+ increases mRNA abundance	Ubiquitous, specifically in tissues involved in zinc acquisition, recycling, transfer (Ex: small intestine, kidney, villous yolk sac)	Plasma membrane	Early embryonic lethality	
ZnT2	Zn2+ upregulates	Small intestine, kidney, placenta, pancreas, testis, seminal vesicles, mammary gland	Vesicles		His59Arg mutation in the mother results in neonatal zinc deficiency
ZnT3		Hippocampus and cortex of brain		Reduced zinc content in synaptic vesicles in brain	
ZnT4	Increased Zn2+ induces trafficking from trans-golgi network to cytoplasmic vesicular compartment	Mammary gland, brain intestinal epithelial cells	Vesicles, Golgi apparatus	Zinc deficient milk due to a C934T mutation; lethal	
ZnT5		Ubiquitous, high expression in β-cells	Trans-Golgi network		
ZnT6		Brain, lung	Trans-Golgi network		
ZnT7		Lung, small intestine	Golgi apparatus	Zinc deficiency; not responsive to supplementation; poor growth, reduced body fat composition	
ZnT8		Pancreatic α- and β-cell	Insulin secretory vesicles	See chapters IV and V	Type 1 and 2 diabetes

ZnT9		Embryonic lung			
ZnT10		Fetal liver and fetal brain			

results in the deprotonation of critical carboxylic acid resides, as well as the dilution of the zinc molecules results in the repulsion of the individual insulin molecules and the disintegration of the crystalline hexamer [129]. Thus, the presence of zinc within the insulin granules has long been thought to be critical for proper β-cell function.

The observation that some species, such as the guinea pig and the Atlantic hagfish, do not have a histidine residue at position B_{10} challenges this concept, however [167]. These species have much lower islet zinc levels, between 2 and 3 mg/100 g tissue, and consequently do not form insulin hexamers, suggesting that proper insulin secretion may be possible in the absence of the crystalline structure [168, 169].

Despite this, a number of studies in both mice and humans have supported the notion that the presence of zinc is, in fact, critical for β-cell function. First, zinc deficiency in rodents has been shown to result in reduced GSIS and islet insulin content [170] and well as impaired β-cell granulation [171]. In addition, in two different rodent models of type 2 diabetes, the db/db and the ob/ob mouse, zinc levels were reported to be markedly lower than in controls [172, 173]. Interestingly, zinc supplementation in both models attenuated the characteristic hyperglycemia and hyperinsulinemia [172, 173] while a zinc deficient diet in the db/db mice exacerbated the phenotype [173]. Similarly, it was observed that cadavers of individuals with type 2 diabetes had approximately a 50% reduction in pancreatic zinc content as compared to cadavers of individuals without type 2 diabetes [174]. Furthermore, patients with type 2 diabetes have reduced plasma zinc levels, increased zincuria and cellular depletion of zinc levels [175]. This presumably occurs as a result of hypersecretion of insulin. As the zinc is secreted along with the insulin hexamers, the increase in insulin secretion results in cellular depletion of

zinc and an increase in zincuria to dispose of circulating zinc. Although the β-cell can compensate, at least initially, for the increased insulin demand, it is unable to make more zinc. Thus, over time, there is a slow loss of whole body zinc. Along these same lines, zinc supplementation in humans reportedly improves glucose handling [176].

Not only is zinc thought to be important for proper β-cell function, but studies have also suggested a possible role for zinc in α-cell function. *In vitro* and *in situ* studies have suggested that the zinc that is secreted with the insulin hexamers can act as a paracrine signal by binding the SUR1 subunit of the K_{ATP} channels on the α-cell surface [177]. This results in activation of the channels and a subsequent reduction in glucagon secretion [177]. Though a separate study was unable to replicate the mechanism of these findings, they were able to confirm that zinc inhibits glucagon secretion [132]. In addition, it has been speculated that zinc molecules may also affect insulin secretion through a similar mechanism and may thereby serve as a negative feedback signal [178].

In addition to its positive effects on glucose metabolism, it has also been well-established that zinc serves an important role in the prevention of apoptosis. Zinc serves as a cofactor to a number of antioxidant enzymes including catalase, peroxidase and Cu/Zn superoxide dismutase, thus providing protection against free radicals and oxidative stress [179, 180]. Furthermore, in chemically-induced rodent models of type 1 diabetes, such as alloxan- or streptozotocin-induced diabetes, zinc supplementation was able to prevent type 1 diabetes and improve hyperglycemia [181, 182]. It is important to note, however, that though zinc deficiency is clearly harmful, increased levels, such as those seen during insulin resistance and the early stages of type 2 diabetes may also be toxic to the cells. In fact, studies performed in both the MIN6 insulinoma cell line as well as in

human islets suggested that high levels of zinc are capable of inducing apoptosis as well [183, 184].

Role of ZnT-8 in the islet

SLC30A8 is highly expressed in the pancreatic β-cell and within these cells is thought to colocalize to the insulin secretory vesicles [128]. Though it is not the only SLC30 family member that is present in the β-cell, it is the most abundantly expressed isoform, at least at the RNA level, and it is the only isoform that is primarily expressed in this tissue [185]. Thus, it has been proposed that ZnT-8 is the principal transporter responsible for supplying zinc to the insulin secretory granules [186]. It is, therefore, reasonable to expect that, in the absence of compensation from other ZnT family members, loss of ZnT-8 would result in impaired β-cell function.

In vitro studies have supported this hypothesis. Knockdown of *Slc30a8* mRNA in INS1E cells results in both a reduction in exogenous zinc uptake as well as impaired insulin secretion [187]. In contrast, overexpression of ZnT-8 in the same cell line results in increased intracellular zinc levels and enhanced GSIS at high glucose [188]. These results are consistent with the hypothesis that ZnT-8 serves as the primary zinc transporter on the insulin granules and is critical for proper insulin secretion. The role of ZnT-8 *in vivo*, however, had not been investigated. Chapter IV describes an initial characterization of the *Slc30a8* KO mice on the mixed C57BL/6J x 129SvEv genetic background. Chapter V describes a further characterization of the *Slc30a8* KO allele on the C57BL/6J genetic background as well as addressing some of the discrepancies from the different published studies on *Slc30a8* KO mice.

Association of ZnT-8 with human disease

A SNP within the human *SLC30A8* gene, rs13266634, was initially implicated in altered risk for the development of type 2 diabetes in a French cohort [9]. Since the original study, these findings have been replicated in a number of populations [103, 189, 190] and the SNP has also been found to be associated with not only type 2 diabetes, but also gestational diabetes [191], proinsulin to insulin conversion [192] and first phase insulin secretion [192]. Surprisingly, however, rs13266634 was not associated with type 2 diabetes in an African-American population [193]. It is important to note, however, that the odds ratio for rs13266634 is 1.12 (Table 1.2), suggesting that while SLC30A8 may contribute to type 2 diabetes susceptibility, this variant alone does not cause significant β-cell dysfunction.

This nonsynonymous polymorphism results in an arginine to tryptophan conversion at position 325 within the ZnT-8 protein [9] and *in vitro* studies have suggested that this SNP, which forms part of the dimer interface, mediates zinc uptake and accumulation into granules [194]. Studies performed by Nicolson *et al* have demonstrated that cells transfected with a ZnT-8 expression vector containing the tryptophan mutation have a reduction in zinc uptake and, consequently, decreased granular zinc levels as compared to cells transfected with expression vectors containing arginine at position 325 [194]. Interestingly, the same polymorphism identified in the GWA studies has also been shown to affect autoantibody epitope specificity in type 1 diabetes [195]. ZnT-8 was originally identified as an autoantigen in diabetic human

subjects [196]. It has yet to be determined whether ZnT-8 is actually recognized by human T cells and studies are currently underway to investigate this question.

Hypothesis on the role of ZnT-8 function *in vivo*

We hypothesize that ZnT-8 serves as the primary zinc transporter within the islet that provides sufficient zinc concentrations to allow for the proper maturation, storage and secretion of insulin. Thus, we predict that deletion of *Slc30a8* will result in a reduction in islet zinc content and subsequently, impaired insulin secretion. In order to test this hypothesis, we will investigate the phenotype of *Slc30a8* KO mice on both a mixed C57BL/6J x 129SvEv as well as the pure C57BL/6J genetic background.

Rationale for the use of both mixed genetic background and pure C57BL/6J genetic background mice

The studies described within this thesis have been performed on both mixed C57BL/6J x 129SvEv and pure C57BL/6J genetic backgrounds. Although the analysis of the *G6pc2* KO mice described here was performed only on the pure C57BL/6J genetic background, the analysis of the mice on the mixed genetic background has been described previously [119]. Because the mice are generated on the mixed genetic background, initial studies are performed under these conditions. The mixed genetic background is a better representation of a human population than an inbred strain is. However, there is also more mouse-to-mouse variability. Thus, more sophisticated analyses were performed on the C57BL/6J genetic background.

In contrast to the 129 strain which tends to be more glucose tolerant and insulin sensitive than most other strains, C57BL/6 mice are commonly used in the type 2 diabetes field due to their susceptibility to the development of insulin resistance and type 2 diabetes [197]. The C57BL/6 mouse bears both diabetes-promoting and diabetes-protective alleles, resulting in a strain with intermediate diabetes susceptibility [197]. Thus, C57BL/6 mice are not diabetic themselves but may become so in the context of diabetes-promoting conditions [197]. For example, islets isolated from high-fat, high-simple carbohydrate fed C57BL/6 mice displayed impaired insulin secretion as compared to the A/J strain [198, 199]. Altered susceptibility to type 2 diabetes in this strain has been mapped to the gene encoding nicotinamide nucleotide transhydrogenase (*Nnt*), an inner mitochondrial membrane enzyme that catalyzes hydride exchange between nicotinamide-adenine dinucleotide (NAD^+) and NAD phosphate ($NADP^+$) [197]. The mutant *Nnt* allele results in the increased production of reactive oxygen species and thus may impair mitochondrial ATP production [200]. Because of the susceptibility to impaired β-cell function, the use of the C57BL/6 strain may be particularly useful in our studies investigating the effect of deletion of islet-specific genes.

CHAPTER II

MATERIALS AND METHODS

Generation of the *Slc30a8*- and *G6pc2*-targeting vectors

The *G6pc2* mutant mice were generated and analyzed in collaboration with Lexicon Pharmaceuticals and the *G6pc2*-targetting vector was generated as previously described [119].

The *Slc30a8* mutant mice were generated and analyzed in collaboration with Lexicon Pharmaceuticals. An *Slc30a8*-targeting vector was derived using the Lambda KOS system [201]. The Lambda KOS phage library, arrayed into 96 superpools, was screened by PCR using exon 3-specific primers (UTT047-1, 5'-GTGAGGATAGCCAGACTCC-3' and UTT047-2, 5'-CAGCTAGTAATTCAGCACAAC-3'). The PCR-positive phage superpools were plated and screened by filter hybridization using the 516 bp amplicon derived from primers UTT047-1 and UTT047-2 as a probe. A pKOS genomic clone, pKOS-36, was isolated from the library screen and the presence of the *Slc30a8* gene was confirmed by sequence and restriction analysis. Gene-specific arms (5'-GATATTGTGCATCTCACAGGTGGACACGTTG-3' and 5'-CTATATCATTATGCATTCACTATTGCGCAATCAG-3') were appended by PCR to a yeast selection cassette containing the URA3 marker. The yeast selection cassette and pKOS-36 were co-transformed into yeast, and clones that had undergone homologous recombination were isolated. DNA sequencing confirmed that recombination had

replaced a 145 bp region, from base pair 13 of exon 3 extending to the first 10 bp of the third intron, with the yeast selection cassette. To complete the *Slc30a8*-targeting vector, the yeast cassette was subsequently replaced with a selection cassette incorporating an IRES (internal ribosome entry site), the LacZ gene, the herpes simplex virus TK (thymidine kinase) promoter and a Neo (neomycin) selectable marker (Fig.1A of the main paper). The expression of LacZ mRNA is driven by the ZnT-8 promoter with translation dependent on the IRES, whereas expression of Neo mRNA is driven by the TK promoter.

Generation of *G6pc2* and *Slc30a8* KO mice

The *G6pc2* KO mice were generated as previously described [119]. For generation of the *Slc30a8* KO mice, the NotI-linearized targeting vector was electroporated into 129SvEv^{Brd} (Lex-2) ES cells. G418/FIAU-resistant ES cell clones were isolated, and correctly targeted clones were identified and confirmed by Southern blot analysis using a 354 bp 5' external probe (9/10), generated by PCR using wild-type Lex-2 ES cell genomic DNA as the template with primers (UTT047-9, 5'-GCTGCAGACTCTTCTCATATGTAG-3' and UTT047-10, 5'-CATCTGTAGGCATATAAGTGCATGC-3'), and a 314 bp 3' internal probe (11/12), amplified by PCR using primers (UTT047-11, 5'-CACAGTCCTCTAAACCCACAGAGTG-3' and UTT047-12, 5'-GATGACTACACAAAGGTGAAGAGTG-3'). Southern blot analysis using probe 9/10 detected an 8.0 kbp wild-type band and 11.1 kbp mutant band in PstI-digested genomic DNA, whereas probe 11/12 detected a 7.6 kbp wild-type band and 12.8 kbp mutant band

in NheI-digested genomic DNA. Correctly targeted clones were also confirmed by PCR using the primers indicated below. The primers represented sequences in: exon 3, primer 1, 5'-GTGAGGATAGCCAGACTCC-3'; intron 2, primer 2, 5'-CAGCTAGTAATTCAGCACAAC-3'; intron 3, primer 4, 5'-CCCACAATAACTGCATTGACC-3'; and the Neo gene, Neo3a primer, 5'-GCAGCGCATCGCCTTCTATC-3'. Cells from the correctly targeted ES cell clone, designated 2H8 (Fig. 4.1), were microinjected into C57BL/6 (albino) blastocysts resulting in the generation of chimeric mice.

The C57BL/6J *G6pc2* and *Slc30a8* KO congenic strains were developed utilizing a speed congenic (marker-assisted) breeding strategy [202, 203] as follows. A male *G6pc2* or *Slc30a8* heterozygous mouse on the mixed 129/SvEv^{Brd} X C57BL/6 background was bred with three female C57BL/6J mice and the male offspring carrying the mutated *G6pc2* or *Slc30a8* allele and with the highest content of C57BL/6J genome, based on the analysis of microsatellite DNA, was selected for the next round of breeding with female C57BL/6J mice. Additional rounds of backcrossing were performed in the same manner. Using this approach backcrossing was complete after 6 generations, with the exception of the Y chromosome. To fix the Y chromosome an additional round of breeding was performed in which the male mouse with 100% C57BL/6J genome, based on the marker analysis, was bred with female C57BL/6J mice. Female offspring from this breeding were mated with male C57BL/6J mice such that all subsequent offspring carried the C57BL/6J Y chromosome.

Details of the speed congenic breeding strategy are as follows. A panel of 61 microsatellite markers, equally spaced throughout the genome (~30 cM intervals), were

used to differentiate the genetic background of the originating/donor (129/SvEv) and target/recipient (C57BL/6) mouse strains. Informative markers that distinguish between these strains were selected with the aid of a 'panel generator' from http://www.cidr.jhmi.edu/mouse/mmset.html. First, genomic DNA was isolated by standard proteinase K digestion protocols. Genomic DNA was then mixed with True Allele PCR Premix (Applied Biosystems, Foster City, CA) and dispensed into a panel of Mouse Mapping Primers (Applied Biosystems). The setup for the multiplexing reaction and amplification parameters were followed as per manufacturer specifications. Following PCR 4 ml of multiplexed product, 0.6 ml of GS500-ROX size standard (Applied Biosystems) and 6 ml of Hi-Di formamide (Applied Biosystems) were mixed, denatured at 94°C for 3 min and loaded onto an ABI 3100 Avant Genetic Analyzer. Chromatogram data was analyzed using GeneMapper 3.5 software (Applied Biosystems).

Once mice possessed only C57BL/6J markers, heterozygous mice were bred to generate WT, heterozygous and KO mice.

PCR genotyping of *G6pc2* and *Slc30a8* KO mice

Mouse-tail DNA was genotyped using PCR in conjunction with primers that distinguished between the WT and targeted alleles. Details of *G6pc2* genotyping can be found in ref. [119]. *Slc30a8* primers WT5' (5'-TGCGGCTCATCTCTTAATTG-3') and WT3' (5'-CCTCGATGACAACCACAAAG-3') were used to amplify a 70 bp product from the WT allele whereas primers KO5' (5'-TTTCCATATGGGGATTGGTG-3') and KO3' (5'-CTGGAATTCCGCCGATACT-3') were used to amplify a 61 bp product from the targeted allele. Tail DNA was isolated and purified by standard procedures [204].

The WT and targeted allele fragments were amplified using 2.8 ng of genomic DNA and iQ SYBR Green Supermix (Bio-Rad) under the following reaction conditions: 94◦C for 30 s; 60◦C for 30 s; and 72◦C for 30 s (for 40 cycles). Standard-curve analyses were performed for each set of samples to determine the efficiencies of the PCR reactions, which were all greater than 95%.

Animal care

The animal housing and surgical facilities used for the mice in these studies meet the American Association for the Accreditation of Laboratory Animal Care standards. The Vanderbilt University Medical Center Animal Care and Use Committee approved all protocols used. Mice were maintained on a standard rodent chow diet (LabDiet 5001; 23% protein and 4.5% fat; PMI Nutrition International) with food and water provided *ad libitum*. Where specified, mice were placed on a high fat (60% fat calories; Mouse diet F3282; BioServ) diet at 8 weeks of age and maintained on the diet for 12 weeks.

Phenotypic analysis of *G6pc2* and *Slc30a8* KO mice

Phenotypic analysis was performed on 6 h fasted mice at ~16 weeks of age. Mice were weighed after 5 h fasting and then allowed to recover for one hour prior to being anesthetized using isoflurane and bled from the retro-orbital venous plexus. Whole-blood glucose concentrations were determined using an Accu-Check Advantage monitor (Roche). EDTA (5 μl; 0.5 M) was then added to blood samples before centrifugation (16000 ***g*** for 10 min at 4◦C) to isolate plasma. Trasylol (aprotinin; 5 μl; Bayer Health Care) was added to the plasma to prevent proteolysis of glucagon. Cholesterol was

assayed using the cholesterol reagent kit (Raichem), whereas triacylglycerol and glycerol were assayed using a serum triacylglycerol determination kit (Sigma). Insulin and glucagon levels were quantified using radioimmunoassay (Millipore) by the Vanderbilt Diabetes Research and Training Center Hormone Assay Core. Proinsulin was measured using the Rat/Mouse Proinsulin ELISA kit (Mercodia) according to manufacturer's instructions.

Intraperitoneal and oral glucose tolerance tests

Intraperitoneal glucose tolerance tests (IPGTTs) and oral glucose tolerance tests (OGTTs) on fasted conscious mice were performed as previously described [205] with modifications. Duration of fast and age of mice are indicated in the respective chapters. Briefly, mice were fasted, weighed and then allowed to recover for 1 hour prior to injection or oral gavage with either 0.75 or 2.0 mg/g body weight glucose in sterile PBS, as indicated in the text. Glycemia was assessed through the analysis of tail vein blood prior to glucose injection/gavage and thereafter at 15, 30, 60, 90 and 120 min using a Freestyle glucose meter (Abbott).

In a separate study insulin secretion during IPGTTs was assessed in 13 week old male WT and KO mice. Following a 6 hour fast, mice were anesthetized using isoflurane and blood samples were isolated from the retro-orbital venous plexus to obtain basal glucose and insulin levels. Mice were allowed to recover for 15 min and were then injected with a 0.75 or 2.0 mg/kg body weight dose of glucose and the resulting glucose and insulin levels were assessed after 15 min. Blood glucose levels were measured using

a glucose monitor (Freestyle, Abbott Diabetes Care Inc., Alameda, CA, USA) and plasma insulin levels were measured by RIA (Millipore, Billerica, MA, USA).

Pancreas perfusion analysis of *G6pc2* KO mice

In situ pancreas perfusions were performed on 14 week old mice following a 3 hour fast according to the method of Bonnevie-Nielsen *et al* [206] with modifications [62, 207-209]. Briefly, mice were anesthetized and the superior mesenteric, hepatic, splenic, and right and left renal arteries were ligated and the aorta was tied off below the diaphragm so that the celiac trunk could be perfused with oxygenated Krebs-Ringer bicarbonate buffer containing 1% bovine serum albumin and 3% Dextran T70 (AmershamBiosciences) at 1 ml/min. Effluent was collected from the portal vein at 1 min intervals and insulin secretion was measured by RIA by the Vanderbilt Diabetes Center Hormone Assay Core.

Islet isolation, GSIS and arginine stimulated glucagon assays

Islets were isolated from ~13 week old male *G6pc2* mice (Chapter III), ~20 week old male *Slc30a8* mixed genetic background mice (Chapter IV) or ~18 week old *Slc30a8* C57BL/6J male and female mice (Chapter V) as described previously [209]. After isolation, islets were rinsed in three 12 ml changes of RPMI-1640 medium containing 10% (v/v) FBS (foetal bovine serum), 100 units/ml penicillin, 100 μg/ml streptomycin and 11 mM glucose, and then cultured in 10 cm non-treated plates overnight at 37˚C. The next day islets were transferred into medium with 5 mM glucose and allowed to equilibrate for 1 h at 37˚C. Following this equilibration period, 20-30 islet equivalents

(IEQs) were incubated in 5 ml of medium with 5, 11 or 16.7 mM glucose for 30 min at 37ºC. For the glucagon secretion assays, islets were cultured overnight as described above and, following the same equilibration period, were incubated in medium with 5 mM glucose or 2 mM glucose with 20 mM arginine for 30 min at 37ºC. At the end of the static incubations, islets were collected, washed and extracted in 0.2 ml of acid alcohol for 48 h at 4ºC. The medium from the static incubations was centrifuged at 600***g*** for 1 min at 4ºC. Islet extracts and static incubation media supernatants were stored at -80ºC until assayed for insulin or glucagon by radioimmunoassay (Millipore).

Calcium assays

Islets were isolated from ~18 week old male WT and *G6pc2* KO mice and cultured for two days in Krebs-Ringer buffer (2.5mM $CaCl_2$, 119mM NaCl, 4.7mM KCl, 10mM Hepes, 1.2mM $MgSO_4$, 1.2 mM KH_2PO_4, 14.4mM glucose, pH 7.35) as previously described [210]. Prior to the experimental period, islets were transferred to calcium-free Krebs-Ringer buffer (1mM EGTA, 119mM NaCl, 4.7mM KCl, 10mM Hepes, 1.2mM $MgSO_4$, 1.2 mM KH_2PO_4, 2mM glucose, pH 7.35) for 10 minutes. Thapsigargin experiments were then performed as previously described [211]. Briefly, at 145 sec., 2μM thapsigargin was added to the media and calcium levels were measured by FURA-2 fluorescence.

Immunohistochemical staining of *Slc30a8* KO mice

Pancreas tissue was fixed for 1 h in 4% (w/v) paraformaldehyde in PBS and embedded for paraffin sectioning (8 μm). Primary antisera against insulin (guinea-pig

1:100; Dako), glucagon (mouse 1:100; Sigma) and somatostatin (rat 1:100; Abcam) were combined with a rabbit polyclonal antibody raised against a 102 amino acid C-terminal human ZnT-8 peptide (amino acids 268–369; used at 1:500) and were detected with species specific secondary antibodies conjugated to Cy2 (carbocyanine), Cy3 (indocarbocyanine), Cy5 (indodicarbocyanine) and AMCA (aminomethylcoumarin) (all from Jackson Immunoresearch Laboratories).

Analysis of islet number, size and cellular composition in *Slc30a8* KO mice

At least ten islets from a single or 20th consecutive pancreatic section were examined and scored from all groups of mice (n=6). Images of individual islets co-immunostained for insulin and glucagon were recorded with an Olympus BX51 microscope using a Pixera 600 digital color camera and analyzed with Image-Pro Plus software (Media Cybernetics). Briefly, the islet perimeter was marked with a pen tracer tool and Cy3 (indocarbocyanine)-stained glucagon-positive cells and Cy2 (carbocyanine)-stained insulin-positive cells with associated nuclei (stained by Hoechst 33258) were counted manually in a double-blind manner by two independent observers. Nuclei within the islet area that were not associated with either insulin- or glucagon-positive cells were designated non-α/β-cells.

Islet numbers and pancreatic area estimations were performed by scanning pancreatic sections immunostained for either insulin or glucagon using immunoperoxidase staining with diaminobenzidine as the pigment chromogen. The slides were counterstained with haematoxylin and scanned into ScanScope GL (Aperio). Using Imagescope viewing software (Aperio), total pancreatic and all individual islet

areas (endocrine) visualized within the sections were quantified using a pen tracer tool. The percentage area was calculated as: 100 x (the sum of all individual islet areas)/(total area of the pancreatic section) and averaged (*n*=6 each group).

Timm's staining analyses in *Slc30a8* KO mice

The determination of zinc content in wild-type and *Slc30a8* KO mouse pancreas was based on further modification of the revised Timm's protocol described by Danscher et al. [212]. Briefly, pancreatic tissue sections (8 μm; frozen and paraffin) fixed in 4% (w/v) paraformaldehyde were placed on glass slides and immersed in 0.1% sodium sulfide in 0.15 M sodium phosphate buffer (pH 7.4) for 1 h in glass jars inside a chemical fume hood. The slides were briefly rinsed in PBS and immersed in AMG (autometallography) developer [pH 3.8; 60 ml of gum arabic, 10 ml of sodium citrate (25.5 g of citric acid monohydrate+23.5 g of sodium citrate dihydrate in 100 ml of de-ionized water), 15 ml of reducing agent (0.056% hydroquinone in de-ionized water at 40˚C) and 15 ml of solution containing silver ions (0.008%silver lactate in de-ionized water at 40˚C) added just before use]. All glassware used for AMG development was rinsed in Farmer's solution (10% sodium thiosulfate/10% potassium ferricyanide; 9:1) and warm water. AMG development was carried out at room temperature (22˚C), in the dark and with gentle shaking. The reaction was stopped after 45 min with 5% sodium thiosulfate solution for 10 min. The slides were rinsed in warm water several times, counterstained with haematoxylin and eosin and permanently mounted.

Measurement of *Slc30a8* islet zinc content

Freshly isolated islets from WT and *Slc30a8* KO mice were washed in Ca^{2+}-free Hank's balanced salt solution and frozen down at –80˚C in 20 islet aliquots. Islet pellets were lysed by re-suspension in 1 ml of lysis buffer [1% Triton X-100 in 10 mM Tris/HCl (pH 7.4)]. The Zn^{2+} concentration in the lysate was measured using the Zn^{2+}-sensitive fluorescent dye FluoZin-3 (Invitrogen). In the presence of 1.181 μM FluoZin-3 the fluorescent signal at the emission peak (516 nm) was measured in the total sample lysate using a fluorometer (PTI Instruments). The fluorescent signal was compared with a standard curve generated from serial dilutions of $ZnSO_4$ in lysis buffer to obtain the lysate Zn^{2+} concentration and thus the Zn^{2+} content per islet. As a normalization factor, the protein content per islet was measured in the total sample lysate using the BCA (bicinchoninic acid) protein assay (Pierce). To minimize contaminating Zn^{2+}, all solutions were made in double-distilled water (18.2 MΩ), avoiding the use of any glassware. Blank samples were also prepared during the islet isolation to quantify any additional Zn^{2+} contamination.

Insulin tolerance tests

Insulin tolerance tests were performed on ~18 week old male and female *Slc30a8* mice (Chapter V) as described previously [213, 214]. Briefly, mice were weighed following a 4 hour fast and then allowed to recover for 1 hour prior to injection with a 0.75 U/kg body weight dose of insulin. Glycemia was assessed through the analysis of tail vein blood prior to insulin injection and thereafter at 7.5, 15, 22.5, 30, 37.5, 45 and 60 min using a Freestyle glucose meter (Abbott).

Electron microscopy

Primary fixation was performed by pancreas perfusion [207] with Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer with 1% calcium chloride, pH 7.4). Post fixation was performed with 1% osmium tetroxide, followed by dehydration with a graded series of ethanols, and embedding of tissue with Epon resin. After islets were located on 500 nm sections, 60-70 nm sections were collected on grids, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy with a Philips CM-12 typically operated at 80 kV. Images were obtained with a 2k x 2k CCD AMT 542 digital camera.

Analysis of *SLC30* gene expression by quantitative RT-PCR in 9-23 week old human fetal pancreas and adult human islets

SLC30 gene expression was analyzed using quantitative Real time PCR (Q-RT-PCR). Briefly, RNA was isolated from 9-23 week old fetal pancreas and adult human islets as previously described [215]. cDNA was then prepared from total RNA (1 mg) using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). cDNA samples equivalent to 0.1 μg of the original RNA sample were used as templates for amplification in a 5' nuclease assay based system using FAM ® dye labeled Taqman MGB probes for selected *SLC30* genes (Applied Biosystems, Foster City, CA, USA) and a 96-well ABI 7000 PCR system instrument. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was selected for sample normalization based on preliminary experiments with the ABI control plate (part number 430 9199). The Cycle Threshold values (CT) were measured in triplicate and the samples were re-normalized to

the CT values from 9 week old pancreas samples (control group/calibrator) and data were quantified using the $2^{-\Delta\Delta CT}$ method (ABI User Bulletin #2).

Statistical analyses

Data were analyzed using a Student's *t* test: two sample assuming equal variance. The level of significance was as indicated (two-sided test).

CHAPTER III

ANALYSIS OF THE PHENOTYPE OF *G6pc2* KNOCKOUT MICE ON A C57BL/6J GENETIC BACKGROUND

Introduction

Numerous studies have investigated the biological importance of controlling fasting blood glucose (FBG) levels. It has been well established that FPG is associated with increased risk for the development of type 2 diabetes [99]. In addition, a number of studies have indicated that even mild variations can have significant consequences on the risk of cardiovascular associated mortality. For example, a study performed on a European male population demonstrated that a small increase in fasting blood glucose levels from just 90 mg/dl to between 99 and 108 mg/dl was associated with a 30% increase in the risk of cardiovascular associated mortality [97]. Conversely, a study performed in an Asian population indicated that a reduction in fasting blood glucose levels from 99 mg/dl to 90 mg/dl was associated with a 25% reduction in cardiovascular associated mortality [98]. Because of the apparent biological importance of tightly regulating FBG levels there has been tremendous interest in understanding how this parameter is controlled.

Recent genome wide association (GWA) studies have shed light on this question by demonstrating that single nucleotide polymorphisms (SNP) within the *G6PC2* gene were associated with variations in fasting glycemia [8, 216, 217]. These GWA study data are consistent with our previous study showing that global deletion of *G6pc2* in mice on a

mixed, 129SvEv x C57BL/6J, genetic background results in a ~15% reduction in fasting blood glucose levels compared to wild type littermates [119]. These observations raise the key question as to how G6PC2 modulates FBG.

The biological role of G6PC2 has remained unclear. While G6PC2 does hydrolyze G6P *in vitro* it does so with different kinetics than G6PC [45]. Specifically, G6PC2 exhibits both a lower K_M (0.45 vs. 2.5 mM) and V_{MAX} (32 vs. 2400 nmol/mg/min) than G6PC, raising the question as to whether G6P is a physiologically important substrate for this protein. Because the pancreas does not significantly contribute to whole body glucose production, the biological purpose of a ß-cell specific glucose-6-phosphatase is unknown.

Indeed, even the question as to whether glucose-6-phosphatase activity is present in islets has been controversial, though the majority of studies have found that activity is detectable, but at a lower level than that found in liver [12, 44, 79, 218-220]. While there is now general agreement that glucose-6-phosphatase activity exists in pancreatic islets, the issue as to whether the level of activity is enough to result in significant glucose cycling and hence affect GSIS and therefore be of physiological significance, has been investigated by several groups [77, 78]. The use of *G6pc2* KO mice represents an innovative approach to address the function of G6pc2 *in vivo* and definitively answer the question as to whether it accounts for the glucose-6-phosphatase activity and hence glucose cycling present in mouse islets.

Despite this controversy over the importance of glucose-6-phosphatase activity in pancreatic islets we provide evidence here that G6PC2 opposes glucokinase and acts as an inhibitory component of the ß-cell glucose sensor thereby modulating the $S_{0.5}$ of

glucose-stimulated insulin secretion (GSIS). The data suggest that, in combination with glucokinase, G6PC2 creates a futile substrate cycle in which ATP is utilized thereby reducing the ATP:ADP ratio and hence insulin secretion. A reduction in G6PC2 expression therefore results in a leftward shift in the $S_{0.5}$ of GSIS such that under fasting conditions FBG is reduced.

While this model can explain the association between *G6PC2* and FBG it also predicts that the leftward shift the $S_{0.5}$ of GSIS resulting from a reduction in G6PC2 expression would not be associated with a change in V_{MAX}. However, we show here that this model is incomplete because G6pc2 deletion is also associated with reduced maximal insulin secretion, an observation that is consistent with the GWA study data that also revealed that SNPs within the *G6PC2* gene are not only associated with variations in fasting glycemia but also a reduction in insulin secretion during glucose tolerance tests [108]. We show here that sequestration of calcium into the ER, an important event in GSIS [221, 222], is impaired in the absence of G6PC2. This observation explains the paradoxical GWA data and indicates that G6PC2 plays dual roles in the regulation of GSIS.

Results

Phenotypic characterization of fasted G6pc2 *KO mice*

G6pc2 KO mice were generated on a mixed 129/SvEv^{BRD} x C57BL/6J genetic background as previously described [119] and subsequently backcrossed onto a pure C57BL/6J genetic background using a speed congenic breeding strategy. Cross-breeding of heterozygous *G6pc2* +/- mice resulted in a total of 325 mice where 85 were WT, 160

were +/- and 80 for KO, similar to the expected distribution for Mendelian inheritance. The ratio of males to females was 178:147. Cross-breeding of KO mice indicated that deletion of *G6pc2* does not affect fertility.

Table 3.1 summarizes the 6 hour fasted metabolic phenotype of the mice at 17 weeks of age. No significant changes were observed in body length or plasma insulin, triglycerides or glycerol in *G6pc2* KO mice compared to WT littermates. Fasting blood glucose was reduced in both male and female mice (16.4 and 14.4% respectively), consistent with our previous data obtained with mixed genetic background mice [119] and the findings of GWA studies [8, 93-95]. Fasting plasma cholesterol levels were also slightly but significantly reduced in both male and female KO mice (Table 3.1). This is consistent with the previously reported association between fasting glucose and cholesterol levels [223].

Analysis of GSIS in vivo *during intraperitoneal glucose tolerance tests*

Based on the reduction in FBG in *G6pc2* KO mice we hypothesized that G6pc2 acts as an inhibitory component of the beta-cell glucose sensor thereby modulating the $S_{0.5}$ of glucose-stimulated insulin secretion (GSIS). In this model G6pc2 would act in combination with glucokinase to create a futile substrate cycle in which ATP is utilized thereby reducing the ATP:ADP ratio and hence insulin secretion. A reduction in G6pc2 expression would therefore result in a leftward shift in the $S_{0.5}$ of GSIS with no change in V_{MAX} such that under fasting conditions, where insulin levels are identical regardless of the level of *G6pc2* expression (Table 3.1), FBG would be reduced (Fig. 1.4).

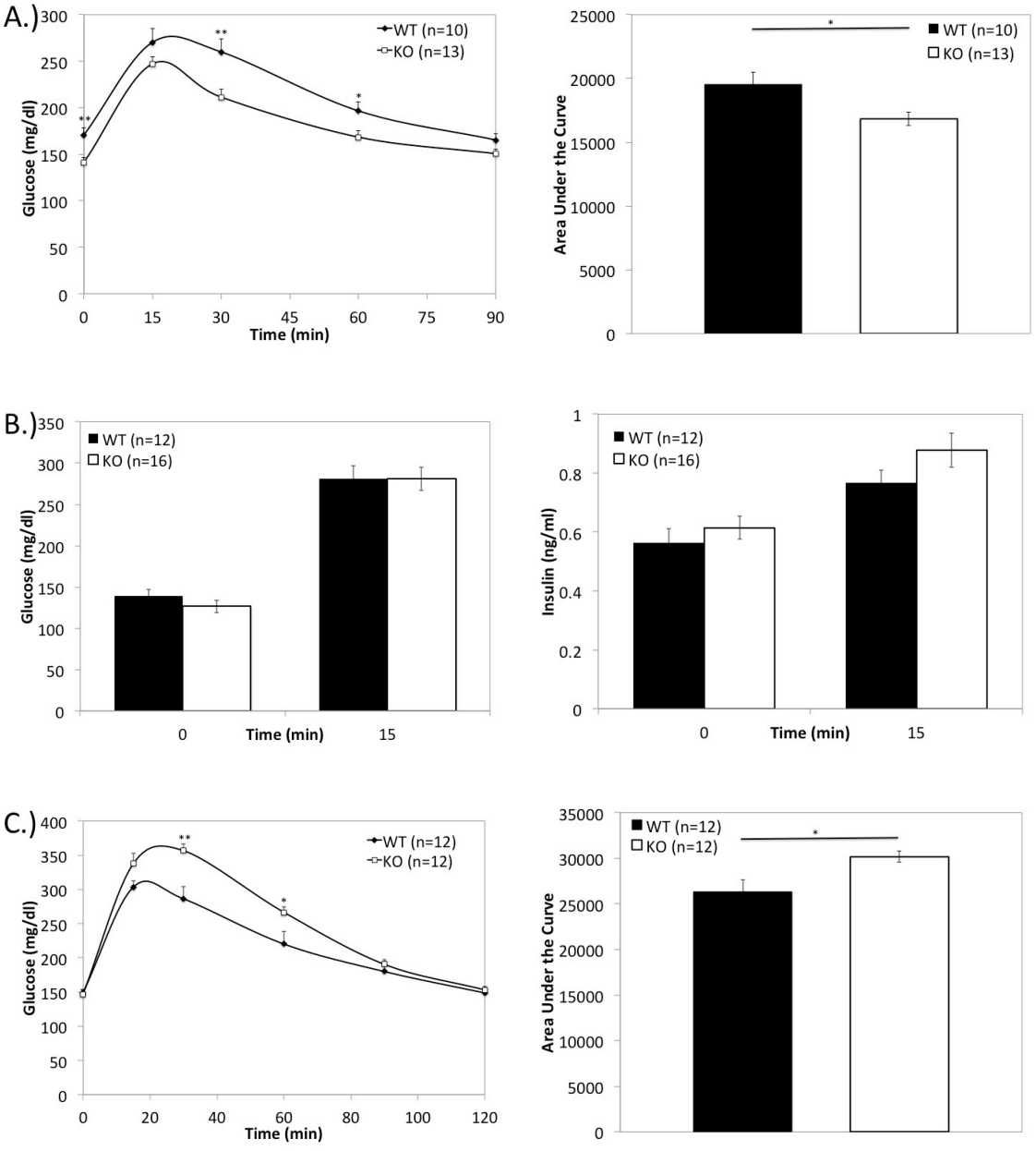
Table 3.1. Phenotypic characterization of *G6pc2* KO mice on a C57BL/6J genetic background. At 16 weeks of age mice were fasted for 5 hours and then weighed. Mice were anesthetized 1 hour later, their length was measured and blood isolated. Blood glucose and plasma cholesterol, triacylglycerol, glycerol, insulin and glucagon levels were determined as described in Chapter II. Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. WT=wild type; -/+=heterozygous; KO=knockout.

Gender & Genotype	Weight (g)	Length (mm)	Glucose (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	Glycerol (mg/dl)	Insulin (ng/ml)
Female WT	20.4 ± 0.2 (12)	96.0 ± 0.4 (12)	121.9 ± 4.3 (12)	63.8 ± 2.7 (11)	46.4 ± 1.7 (11)	2.8 ± 0.2 (10)	0.27 ± 0.03 (10)
Female -/+	20.4 ± 0.1 (37)	96.5 ± 0.3 (37)	111.1 ± 3.3 (37)	61.5 ± 1.5 (36)	47.9 ± 1.6 (34)	2.8 ± 0.1 (35)	0.28 ± 0.01 (35)
Female K/O	19.8 ± 0.2 (11) *,**	96.1 ± 0.4 (11)	101.5 ± 3.6 (11) *	57.5 ± 2.1 (11)	45.1 ± 1.1 (11)	2.8 ± 0.1 (9)	0.25 ± 0.02 (11)
Male WT	27.2 ± 0.3 (31)	101.5 ± 0.2 (29)	130.7 ± 3.6 (31)	71.6 ± 1.6 (23)	52.4 ± 1.9 (24)	2.5 ± 0.1 (24)	0.59 ± 0.07 (29)
Male -/+	27.2 ± 0.2 (51)	101.0 ± 0.2 (51)	129.4 ± 2.9 (52)	74.3 ± 1.7 (37)	53.6 ± 1.7 (38)	2.7 ± 0.1 (41) *	0.54 ± 0.04 (46)
Male K/O	27.4 ± 0.5 (17)	102.1 ± 0.4 (16) *	109.6 ± 3.6 (17) *,**	62.3 ± 3.3 (10) *, **	53.4 ± 3.0 (12)	2.8 ± 0.2 (12)	0.49 ± 0.06 (14)

1. Weight: *F WT vs. F KO, $p<0.05$; **F Het vs. KO, $p<0.05$
2. Length: *M Het vs. KO, $p<0.05$
3. Glucose: *M WT vs. M KO, $p<0.001$; **M Het vs. KO, $p<0.001$; *F WT v KO, $p<0.001$
4. Cholesterol: *M WT v KO $p<0.01$, **M Het vs. M KO $p<0.01$

To directly assess the effect of G6pc2 on the $S_{0.5}$ and V_{MAX} of GSIS, we measured insulin secretion in response to varying glucose concentrations during insulin tolerance tests *in vivo*. Following a 6-hour fast male WT and KO mice were injected with either a 0.75 or 2.0 mg/kg body weight dose of glucose and the resulting glycemia was measured over a 90 or 120 min. period, respectively. IPGTT experiments using the lower submaximal glucose dose were designed to detect the leftward shift in $S_{0.5}$ while the higher dose was designed to detect changes in V_{MAX}. Injection with the 0.75 mg/kg dose of glucose resulted in a reduction in blood glucose at all time points in the KO mice with no change in glucose tolerance, indicating that KO mice live at a lower glucose threshold (Fig. 3.1A). We next directly measured insulin secretion following injection of 0.75 mg/kg glucose (Fig. 3.1B). Although not quite statistically significant (p=0.16), *G6pc2* KO mice display a trend towards enhanced insulin secretion. These data are consistent with the concept that deletion of *G6pc2* enhances insulin secretion at a given glucose load relative to WT mice and blood glucose is more efficiently lowered. In contrast to the results from low dose injection of glucose, KO mice displayed impaired glucose tolerance following injection of 2mg/kg glucose (Fig. 3.1C). We next directly measured insulin secretion following injection of 2.0 mg/kg glucose (Fig. 3.1D). While insulin secretion did not differ significantly between WT and *G6pc2* KO mice, insulin secretion in the KO mice was clearly insufficient to achieve normal glucose tolerance.

These observations indicate that our original model of G6PC2 function, which was based solely on FBG data (Table 3.1), and which predicted that a reduction in G6PC2 expression would therefore result in a leftward shift in the $S_{0.5}$ of GSIS with no change in V_{MAX} is not correct (Fig. 1.4). However, the G6pc2 KO mouse data are


A.)
Glucose (mg/dl)
Time (min)
WT (n=10)
KO (n=13)
**
**
*
Area Under the Curve
WT (n=10)
KO (n=13)
*
B.)
Glucose (mg/dl)
WT (n=12)
KO (n=16)
Time (min)
Insulin (ng/ml)
WT (n=12)
KO (n=16)
Time (min)
C.)
Glucose (mg/dl)
WT (n=12)
KO (n=12)
**
*
Time (min)
Area Under the Curve
WT (n=12)
KO (n=12)
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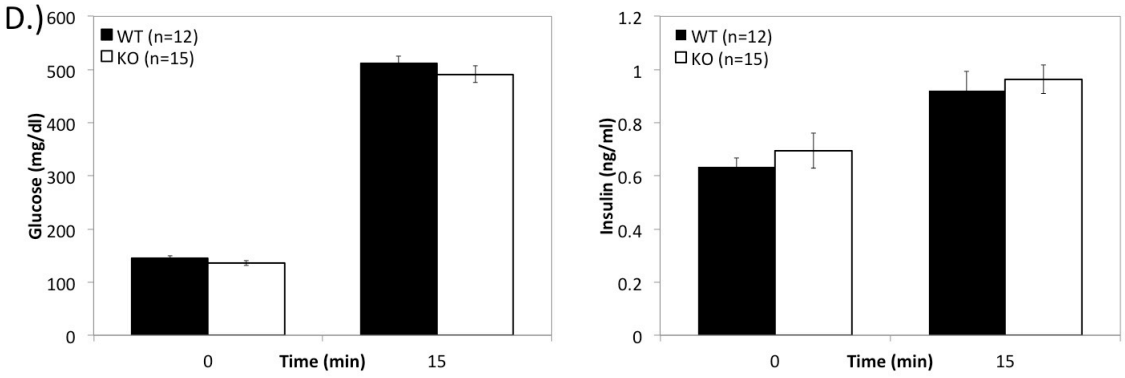


Figure 3.1. Analysis of glucose tolerance in *G6pc2* KO mice *in vivo*. Intraperitoneal glucose tolerance tests were performed on 6 hour fasted conscious wild type (closed symbols) and *Slc30a8* KO (open symbols) male mice as described in Chapter II. Following the 6 hour fast, mice were injected with either 0.75 (Panels A and B) or 2.0 (Panels C and D) mg/g body weight glucose in sterile PBS. (A, C) Results show the mean glucose concentrations ± S.E.M. in wild type (mean age 13 weeks) and *G6pc2* KO (mean age 13 weeks) animals. (B,D) Insulin secretion was assessed as described in Chapter II. Resulting mean glycemia and plasma insulin concentrations ± S.E.M. in wild type (mean age 13 weeks) and *G6pc2* KO (mean age 13 weeks) animals. WT=wild type; KO=knockout.

consistent with equivalent experiments in humans showing that *G6PC2* SNPs that are associated with elevated FBG (equivalent to WT mice) are associated with normal glucose tolerance but increased insulin secretion [108-110]. The mouse and human data suggest a new model (Fig. 3.2) in which, at low glucose concentrations, GSIS is enhanced in the absence of G6PC2 but at high glucose concentrations, GSIS is blunted in the absence of G6PC2.

Analysis of GSIS from perfused pancreata of G6pc2 *KO mice*

To address the new model the effect of G6pc2 on the $S_{0.5}$ and V_{MAX} of GSIS was directly assessed by measuring insulin secretion in response to varying glucose concentrations from perfused pancreata *in situ*. In response to submaximal concentrations of glucose, 6.5mM and 10mM, the pancreata of *G6pc2* KO mice secreted ~252% and ~90% more insulin, respectively (Fig. 3.3). In addition, in *G6pc2* KO mice, unlike WT mice, the maximal insulin response at 10 mM glucose was similar to that at 16.7mM (Fig. 3.3). In contrast, the pancreata of *G6pc2* KO mice secreted about 33% less insulin in response to 16.7mM glucose. These data suggest that there is a leftward shift in the $S_{0.5}$ of GSIS but that there may be a reduction in V_{MAX}, consistent with model 2 (Fig. 3.2).

Analysis of GSIS in islets isolated from G6pc2 *KO mice*

To directly assess the role of G6pc2 in islet function, GSIS was compared during static incubation in both freshly isolated islets and in isolated islets cultured overnight from male WT and KO mice. To address our hypothesis that deletion of G6pc2 results in

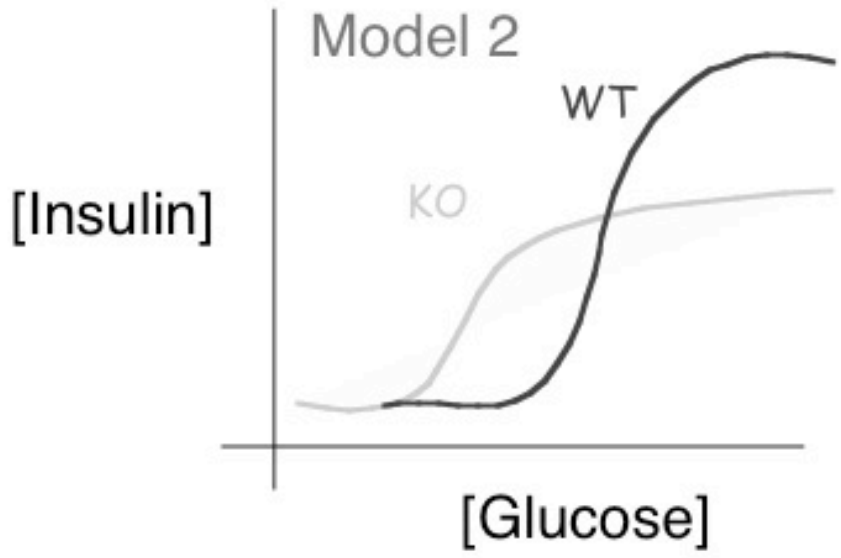


Figure 3.2. Modified hypothesis of the effect of *G6pc2* deletion in mice on GSIS dose-response curve. G6pc2 is hypothesized to serve as a negative component of the β-cell glucose sensor thus determining glycolytic flux. Deletion of *G6pc2* in mice is expected to result in a leftward shift in the dose-response curve of GSIS. Data in Chapter III suggest that, in addition to the shift in $S_{0.5}$, deletion of *G6pc2* results in a reduction in the V_{MAX} of GSIS.

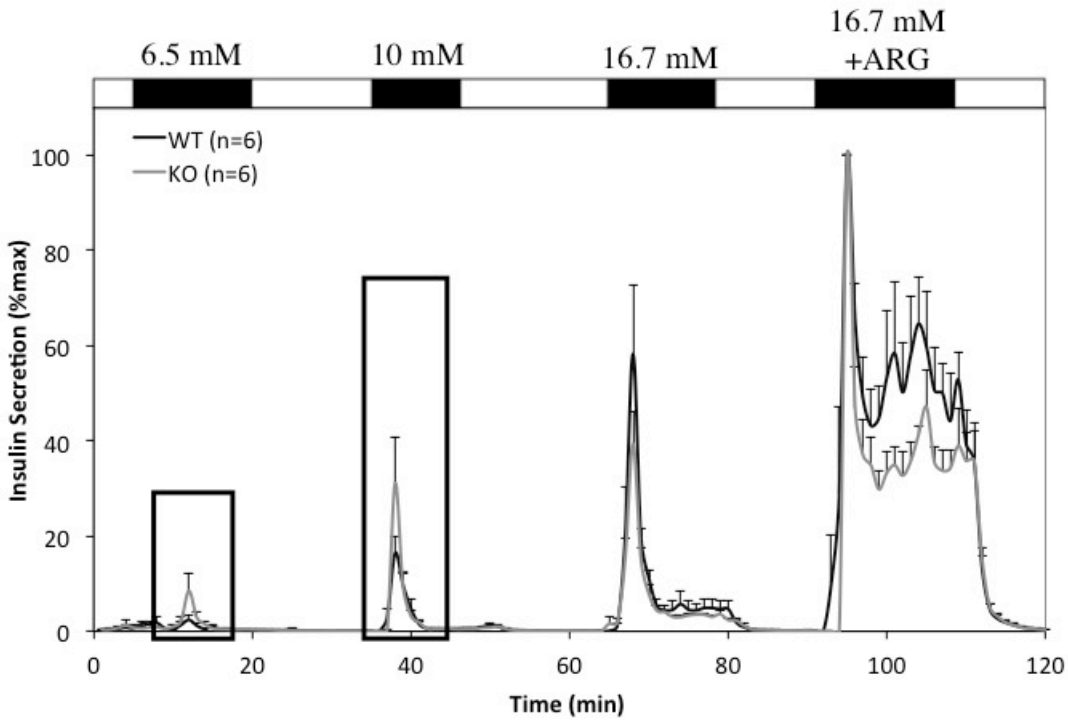


Figure 3.3. Analysis of GSIS from perfused pancreas experiments *in situ* in *G6pc2* KO mice. Pancreas perfusion studies were performed on 3 hour fasted wild type (black line) and *G6pc2* KO (gray line) animals at 14 weeks of age as described in Chapter II. Results show the mean glucose concentrations ± S.E.M. WT=wild type; KO=knockout.

a leftward shift in the dose response curve of GSIS islets were stimulated with 11mM glucose, a submaximal dose. GSIS from freshly isolated islets from KO mice did not differ from WT islets (Fig. 3.4A). In contrast, however, KO islets that had been allowed to recover from the isolation process overnight displayed a ~2.4 fold enhanced GSIS at 11mM glucose (Fig. 3.4B), consistent with a leftward shift in insulin secretion (Fig. 3.2). No differences in total insulin content were observed in these islets (Fig. 3.4C). Interestingly, a microarray analysis of WT islets indicated that freshly isolated islets lack the robust G6pc2 expression seen in islets following overnight culture (L. Pound and R. O'Brien, unpublished observations). This may explain why we are unable to see a difference in GSIS in freshly isolated KO islets relative to WT islets.

To assess the V_{MAX} of GSIS, islets were isolated, cultured overnight and then stimulated with 16.7mM glucose. In contrast to submaximal glucose concentrations, GSIS did not differ at 16.7mM (Fig. 3.4D). Again, there was no change in insulin content (Fig. 3.4E). Interestingly, however, in islets isolated from *G6pc2* KO mice, GSIS at 16.7mM glucose was actually blunted in comparison to the insulin response at 11mM. This result is consistent with model 2 in which the V_{MAX} of GSIS is lowered in the absence of *G6pc2* (Fig. 3.2).

Analysis of ER calcium uptake in isolated islets from G6pc2 *KO mice*

While the *G6pc2* KO mouse are consistent with the human GWA study data and a model (Fig. 3.2) in which reduced G6pc2 has both positive and negative effect on GSIS, the key remaining question is why the absence of G6pc2 would impair GSIS at high glucose concentrations. Because SNPs in *G6PC2* are not associated with altered insulin

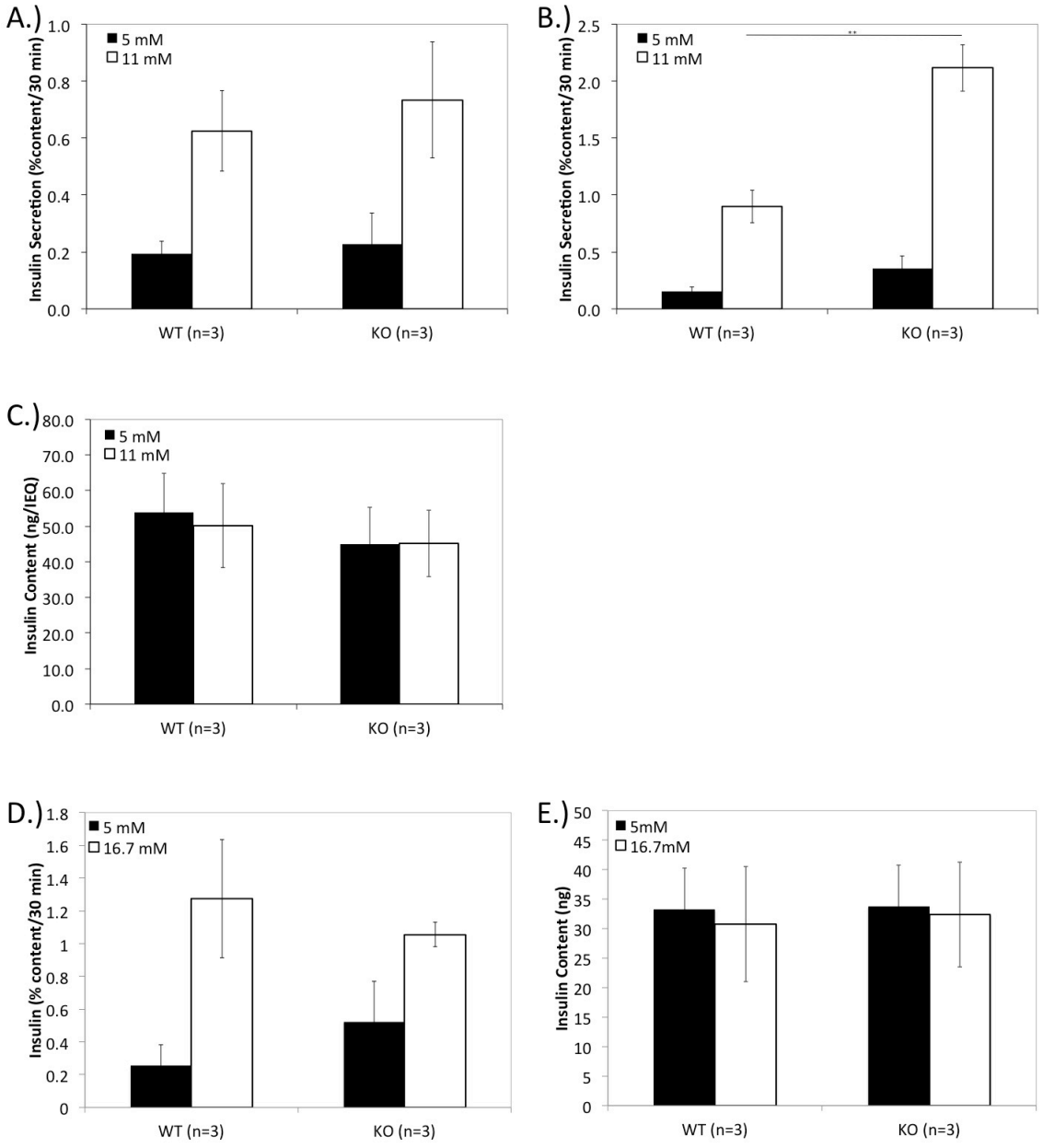


Figure 3.4. Analysis of GSIS and insulin content in islets isolated from *G6pc2* KO mice. Islets were isolated from wild type (WT) and *G6pc2* knockout (KO) mice and then GSIS following stimulation with 11mM (Panels A, B) or 16.7 mM (Panel C) and insulin content (Panels C, E) were assayed as described in Chapter II. Analysis was performed on either freshly isolated (Panel A) or overnight cultured (Panels B-E) islets. Results are the mean insulin secretion ± S.E.M. from three to four islet preparations. **p<0.01 compared with wild type 11mM glucose.

sensitivity, altered pulsatility of insulin secretion has been invoked as a possible explanation for this paradoxical observation [110]. While altered pulsatility remains a possibility, we addressed an alternate explanation, namely that sequestration of calcium into the ER, an important event in GSIS [221, 222], may be impaired in the absence of G6pc2. Thus, it has been suggested that generation of inorganic phosphate in the ER lumen, through the hydrolysis of G6P, may be important for the sequestration of calcium through the generation of calcium phosphate [224].

To address this hypothesis, islets isolated from male WT and *G6pc2* KO mice were treated with thapsigargin, a non-competitive inhibitor of sarco/endoplasmic reticulum calcium ATPases that is known to inhibit the uptake of calcium into intracellular stores, thus resulting in the emptying of calcium from these stores. Islets isolated from *G6pc2* KO mice showed an increased emptying of intracellular stores following treatment with thapsigargin (Fig. 3.5). We interpret this to indicate that *G6pc2* KO mice have impaired retention of calcium in the ER.

Discussion

In the present study, we have investigated the effect of a global deletion of G6pc2 *in vivo*, a mouse model that is directly relevant to variations in FBG in humans. We show that G6pc2 serves as a negative component of the ß-cell glucose sensor by modulating the $S_{0.5}$ of GSIS. Our data demonstrate that deletion of *G6pc2* in C57BL/6J mice results in a leftward shift in the dose response curve for GSIS. FBG levels are mildly reduced (Table 3.1) and both *in situ* pancreas perfusion studies (Fig. 3.3) as well as static islet cultures (Fig. 3.4B) indicate enhanced insulin secretion at submaximal

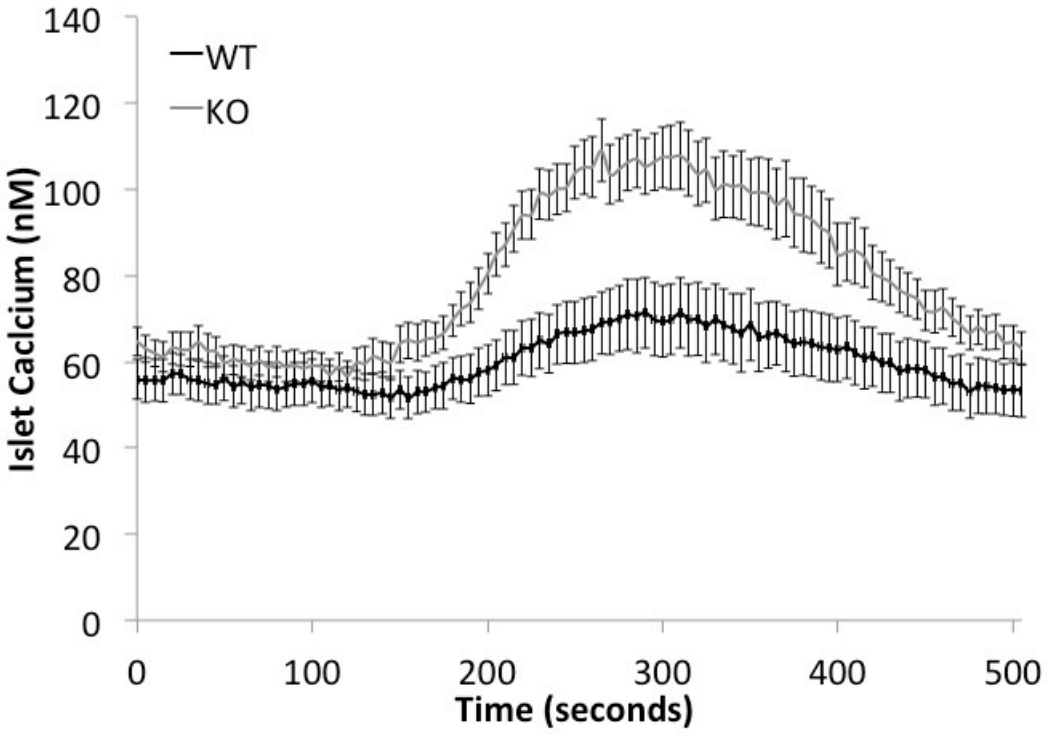


Figure 3.5. Effect of the sarco/endoplasmic reticulum calcium ATPase inhibitor, thapsigargin, on ER retention of calcium. Islets were isolated from wild type (WT) and *G6pc2* knockout (KO) male mice at ~18 weeks of age and cultured for two days as described in Chapter II. At 145 sec., 2μM thapsigargin was added to calcium-free media and calcium was assessed using the FURA-2 fluorescence method. Results are the mean calcium concentration in the media ± S.E.M. from 10-20 islets from two mice per group. All time points from t=255 sec. to t=505 sec. are statistically significant.

glucose concentrations. Furthermore, IPGTTs using a sub-maximal 0.75 mg/g glucose concentration demonstrate that the *G6pc2* KO mice live at a lower glucose set point (Fig. 3.1A). Surprisingly IPGTTs using a maximal 2.0 mg/g glucose concentration demonstrate impaired glucose tolerance (Fig. 3.1C), with inappropriately low insulin secretion (Fig. 3.1D). Importantly, these observations are consistent with human GWA studies in which SNPs within the *G6PC2* gene were found to associate with variations in fasting blood glucose levels [8, 93, 94] but paradoxically low insulin secretion during glucose tolerance tests [108-110]. We show here that the latter observation can be explained by an impairment in ER calcium sequestration in the *G6pc2* KO mice (Fig. 3.5).

A rise in intracellular calcium ions, resulting from the opening of voltage-gated calcium channels and the influx of calcium from the extracellular space and from the ER, is a key event in GSIS [221, 222]. The resulting calcium oscillations that occur contribute to pulses of insulin secretion and rely on rapid uptake and slow release of calcium by the ER [225]. Interestingly, it has been shown in liver microsomes that glucose-6-phosphatase activity is important for calcium uptake into the ER perhaps due to the production and buffering capacity of the inorganic phosphate ions [224]. Chen and colleagues, however, challenge this hypothesis because enhanced uptake of calcium into the ER is observed at phosphate concentrations below those at which significant buffering would be predicted to occur [226]. Rather, studies performed by Korge and Campbell suggest that phosphate ions may be capable of affecting calcium ATPase efficiency [227]. Our studies indicate that islets isolated from *G6pc2* KO mice and treated with thapsigargin, a noncompetitive inhibitor of the sarco/endoplasmic reticulum

calcium ATPase, display an increased level of calcium released (Fig. 3.5). Because phosphate production is important for retaining calcium in the ER and/or pumping calcium back into the ER, we interpret these data to mean that the *G6pc2* KO mice have an impaired ability to maintain ER calcium levels and thus, higher levels of calcium are leaked from the cell following thapsigargin treatment. These results may likely explain the paradoxical data obtained. At submaximal glucose concentrations, extracellular calcium levels may be sufficient for proper GSIS and the loss of G6pc2 may then serve to increase relative ATP levels. In contrast, at higher glucose concentrations, ER calcium may also be required for proper insulin secretion. Thus, loss of ER calcium may result in a reduction in GSIS.

Though the results from GWA studies [8, 93, 94, 108-110] would predict that complete loss of *G6pc2* would have a more drastic effect on FBG and GSIS, we observe a relatively modest phenotype in the *G6pc2* KO mice. One possible explanation for this is that loss of *G6pc2* during development resulted in compensatory responses such that the impact was blunted. Future studies will assess the phenotype of *G6pc2* KO mice using a conditional mouse model. However, even if the severity of beta cell failure differed in the populations studied [93, 104, 228, 229], if variations in the *G6PC2* gene universally contributed to type 2 diabetes susceptibility then this should have been apparent from the genetic analyses. Interestingly, a recent study has challenged the relative importance of FPG to type 2 diabetes risk [101]. Abdul-Ghani and colleagues found that 1 hour plasma glucose following a GTT was actually a better predictor of type 2 diabetes risk than FPG [101]. In fact, after controlling for 1 hour glycemia, FPG was no longer a significant predictor [101]. The authors suggest that the previous correlation

between FPG and type 2 diabetes risk is not due to the increase in FPG per se but is due instead to the correlation between FPG and 1 hour glycemia [101]. These data may explain why SNPs in the *G6PC2* gene are associated with FPG and not type 2 diabetes in most populations. In contrast, many other genes are in fact associated with both FPG and type 2 diabetes. It seems more likely, however, that variations in these genes not only affect FPG but also affect 1 hour glycemia. Their ability to influence the latter would then explain the association with type 2 diabetes.

It seems likely that the conflicting genetic data indicate the importance of modifier genes such that *G6PC2* is only linked with altered susceptibility to the development of type 2 diabetes in some populations and not others and that in the latter populations variations in *G6PC2* expression and/or G6PC2 activity just alter the set point for GSIS rather than affecting the health of beta cells per se. This may be explained in part by differences in the relative importance of *G6PC2* for ER calcium uptake. However, it is important to note that the SNPs examined in genetic studies to date are predicted to have only small effects on *G6PC2* expression [96]. Therefore, even though small elevation in *G6PC2* expression may not be associated with risk of type 2 diabetes in some populations [104], this would not exclude the possibility that a larger elevation in *G6PC2* expression may be associated with the risk of type 2 diabetes in all populations. Moreover, even if elevated *G6PC2* expression was not a cause of type 2 diabetes in some populations per se, compounds that inhibited G6PC2 would not only be useful for lowering FBG and the risk of CAM, but, by reducing glucotoxicity, would also protect against beta cell failure and the onset of type 2 diabetes. Importantly, a common problem with current glucose lowering therapies is that they fail to work over time [230].

Therefore, additional therapies are likely to be helpful. Moreover, therapies that control FBG are likely to be especially helpful since treating elevated FBG, which obviously occurs at night, is particularly problematic in patients with diabetes [231].

The findings of this study challenge the dogma that glucokinase is the ß-cell glucose sensor [73, 105, 232, 233]. It has been established that glucose phosphorylation rather than transport is the rate-limiting step in GSIS [106]. This conclusion is supported by studies demonstrating that overexpression of glucokinase increases GSIS [234] while overexpression of GLUT1 had no effect on GSIS [73] and a reduction in GLUT2 by >90% was required to affect GSIS [76]. Our findings demonstrate that G6pc2 can oppose glucokinase in the pancreatic ß-cell, which would be predicted to create a futile cycle in which ATP is utilized thereby reducing the ATP:ADP ratio. Thus, we propose that G6pc2 is a fundamental inhibitory component of the ß-cell glucose sensor. Future studies will examine the effect of *G6pc2* deletion on islet metabolism to confirm this conclusion.

CHAPTER IV

ANALYSIS OF THE PHENOTYPE OF *Slc30a8* KNOCKOUT MICE ON A MIXED C57BL/6J x 129SvEv GENETIC BACKGROUND

Introduction

This chapter describes an examination of the effect of a global *Slc30a8*-null mutation *in vivo*, a mouse model that is directly relevant to type 2 diabetes susceptibility in humans. The principal objectives were to identify the role of ZnT-8 in normal islet function. The results are consistent with a role for ZnT-8 in islet function, although surprisingly not in whole-body glucose metabolism.

As described in Chapter I, zinc is thought to complex with insulin hexamers and aid in proper insulin processing, storage and secretion. Because the ZnT family is responsible for movement of zinc from the cytoplasm into intracellular vesicles and ZnT-8 is highly expressed in the islet and, more specifically, the β-cell, we hypothesized that ZnT-8 serves as the primary provider of zinc to the insulin containing vesicles. Consistent with this hypothesis, overexpression of ZnT-8 in INS-1E insulinoma cells results in enhanced GSIS. In addition, recent GWAS studies have shown that a nonsynonymous SNP within the *SLC30A8* gene is associated with type 2 diabetes, gestational diabetes, proinsulin to insulin conversion and altered first phase insulin secretion, further supporting a role for ZnT-8 in islet function.

The role of Znt-8 *in vivo*, however, had not been investigated. Thus, we utilized the *Slc30a8* KO mouse model in order to elucidate the role of ZnT-8 in *in vivo* insulin

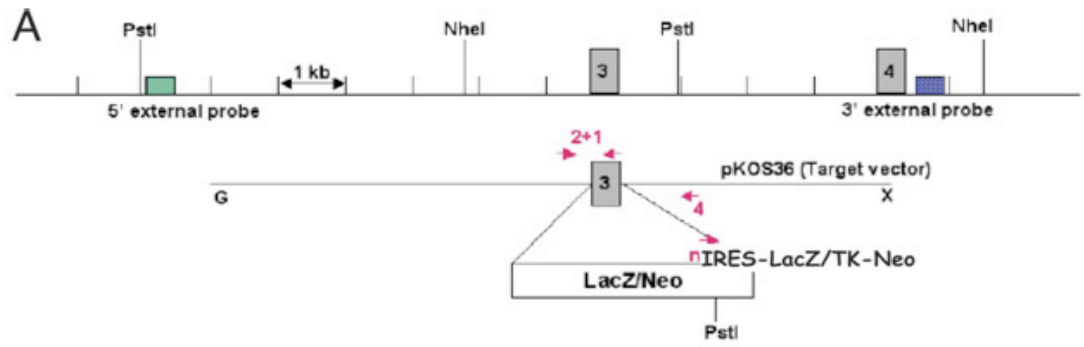
secretion. The studies described in this Chapter demonstrate that fasting insulin levels are reduced in both male and female mice and that GSIS is impaired in isolated KO mouse islets with no change in either fasting blood glucose or in glucose tolerance. These studies form the basis of the studies described in Chapter V where we further investigate the phenotype of *Slc30a8* KO but on a pure C57BL/6J genetic background.

Results

Biochemical characterization of Slc30a8 *KO mice*

The human *SLC30A8* and mouse *Slc30a8* genes contain eight exons ([235] and results not shown). A modified mouse *Slc30a8* allele, in which 135/147 bp of exon 3 and the first 10 bp of intron 3 were replaced by a LacZ/Neo cassette, was generated by homologous recombination in 129/SvEv^{Brd} (Lex-2) ES (embryonic stem) cells (Fig. 4.1A). Deletion of exon 3 disrupts two putative ZnT-8 transmembrane domains [235]. Correct gene targeting was confirmed by Southern blot (Fig. 4.1B) and PCR (results not shown) analysis prior to injection of ES cells into C57BL/6 (albino) blastocysts and subsequent generation of *Slc30a8* heterozygous mice on a mixed 129/SvEv^{Brd} x C57BL/6 background. Gene targeting and the generation of *Slc30a8* heterozygous mice were performed by Lexicon Pharmaceuticals from whom we obtained the mice.

To confirm that the targeting strategy had abolished *Slc30a8* expression, immunohistochemical staining was performed on pancreas sections prepared from a *Slc30a8* KO mouse and a WT littermate. Fig. 4.1C shows that ZnT-8 was detected in both α- and β-cells in wild-type, but not *Slc30a8* KO, mouse islets.

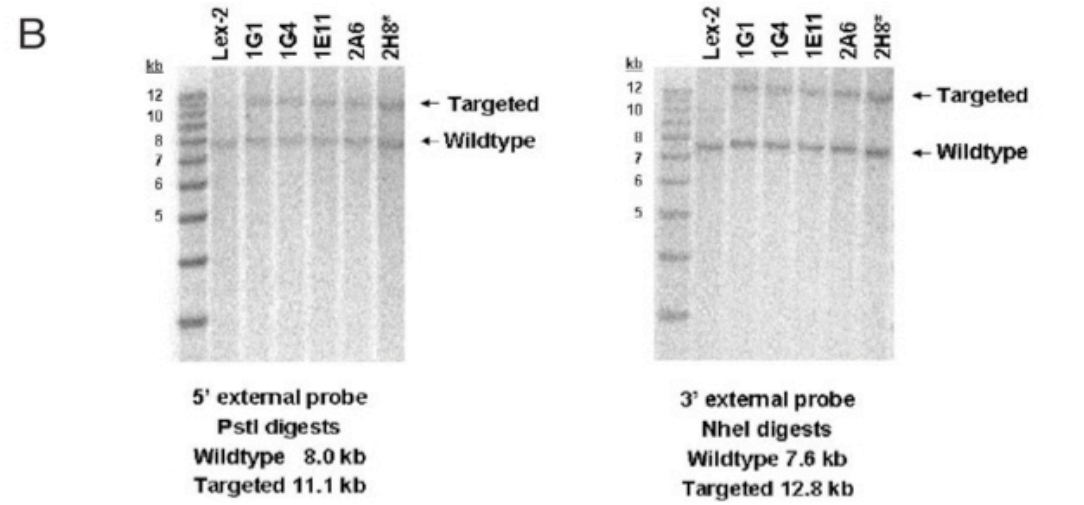


Southern Strategies

Probe	5' external	3' external
Enzyme	PstI	NheI
Wildtype	8.0 kb	7.6 kb
Targeted	11.1 kb	12.8 kb

PCR Strategies

Stratagy	WT-specific	Mutant-specific
Primers	1+2	Neo3a+4
Wildtype	516 bp	--------
Targeted	--------	673 bp



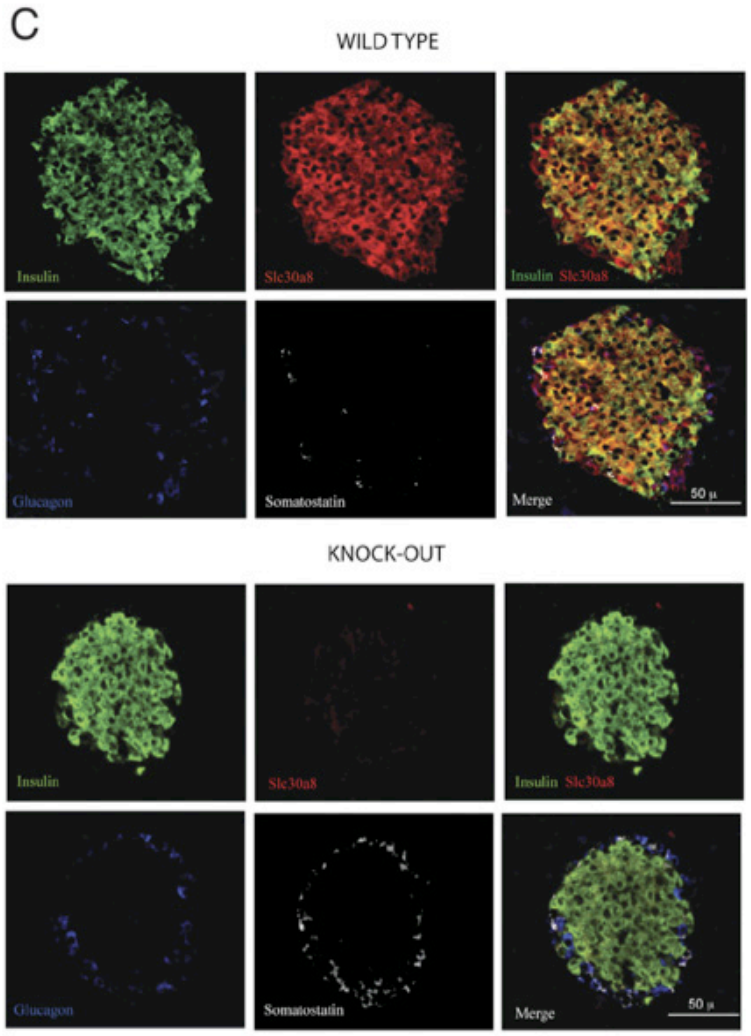


Figure 4.1. Generation and biochemical characterization of *Slc30a8* KO mice. (A) Strategy used to generate *Slc30a8* KO mice by homologous recombination in ES cells. A schematic representation of the wild type murine *Slc30a8* locus and the targeting construct are shown. Exon 3 was replaced with a cassette containing an IRES (internal ribosome entry site), the LacZ gene and a TK (thymidine kinase)-neomycin selectable marker. Correctly targeted clones were identified by Southern blot analysis using the indicated probes and were confirmed by PCR using the primers indicated. The primers represented sequences in exon 3 (primer 1), intron 2 (primer 2), intron 3 (primer 4) and the Neo gene (Neo3a primer). (B) Southern blot analysis of the *Slc30a8* locus using genomic DNA extracted from the indicated targeted ES cell lines, or wild type ES cell genomic DNA, designated Lex-2, as a control, using 5' and 3' diagnostic probes (A). The sizes of the wild type locus, targeted allele and DNA markers are indicated. Clone 2H8 was used to achieve germline transmission. (C) Immunohistochemical staining of wild type and *Slc30a8* KO mouse pancreas with antisera raised against insulin, glucagon, somatostatin and ZnT-8 was performed as described in Chapter II. Representative pictures (200x magnification) are shown.

The size and number of islets in *Slc30a8* KO animals were indistinguishable from WT littermates, as were the relative numbers of α- and β-cells (Fig. 4.2). Histological analysis of zinc content on frozen pancreatic sections using a modified Timm's staining procedure that involves silver enhancement of metal sulfide precipitation showed that, in WT mouse pancreas, islets contained abundant zinc relative to the exocrine tissue (Fig. 4.3A). This contrasted with *Slc30a8* KO mouse pancreas in which no difference was observed in Timm's staining between islets and exocrine tissue, although gross islet morphology was preserved (Fig. 4.3A). These results are consistent with analyses of zinc content in isolated islets using an assay that detects free and loosely bound zinc (Fig. 4.3B). Figure 4.3B shows that zinc content was markedly reduced in islets isolated from *Slc30a8* KO mice relative to those isolated from WT mice. The concentration of zinc detected in WT islets was similar to that previously reported in the islet derived INS1 cell line [188].

Phenotypic characterization of Slc30a8 *KO mice*

Genotype analysis of 383 3-week-old pups generated by crossbreeding heterozygous *Slc30a8* mice demonstrated that 83 mice were WT, 203 were heterozygous and 97 were *Slc30a8* KO, a distribution close to the expected pattern for Mendelian inheritance. The ratio of male to female mice was 206:177. Cross-breeding experiments revealed that both male and female homozygous *Slc30a8* mice are fertile.

The activity and behavior of *Slc30a8* KO mice were indistinguishable from their WT and heterozygous littermates at all ages, from birth up to 1 year in age. No gross anatomical changes were observed either externally or to major internal organs, and no

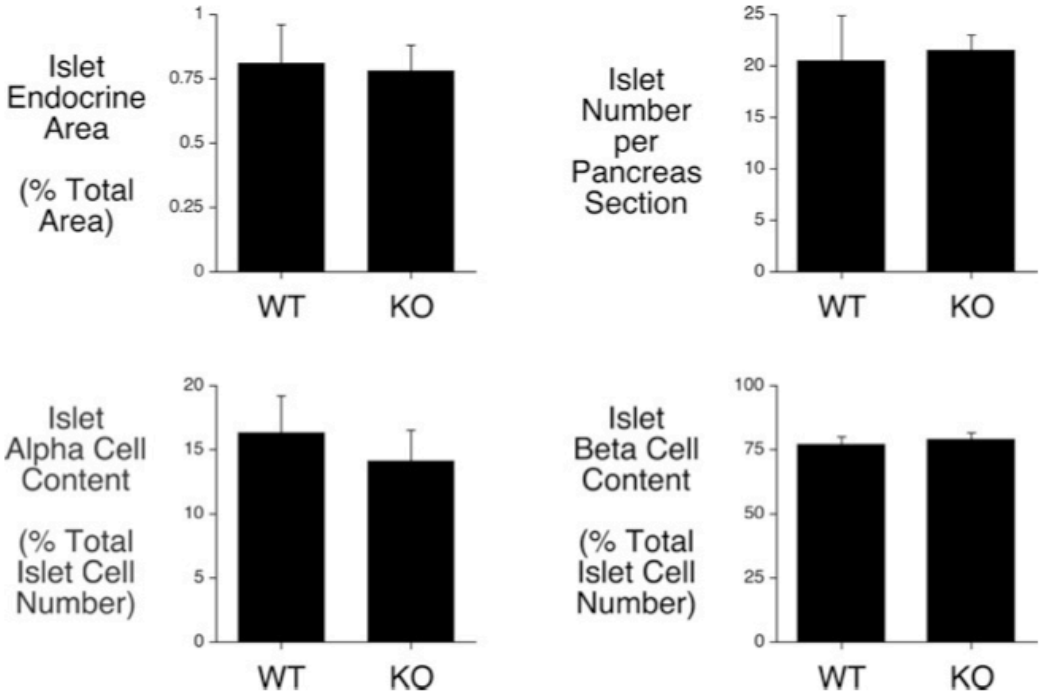


Figure 4.2. Analysis of islet number, size and composition in *Slc30a8* KO mice. Pancreas tissue was isolated from male wild type (WT) and knockout (KO) mice. Fixation, preparation of mouse pancreatic slices, immunohistochemical staining with antibodies raised to insulin and glucagon and quantitation of islet size, islet number and α- and β- cell numbers were then performed as described in Chapter II. Results are presented as means ± S.E.M.

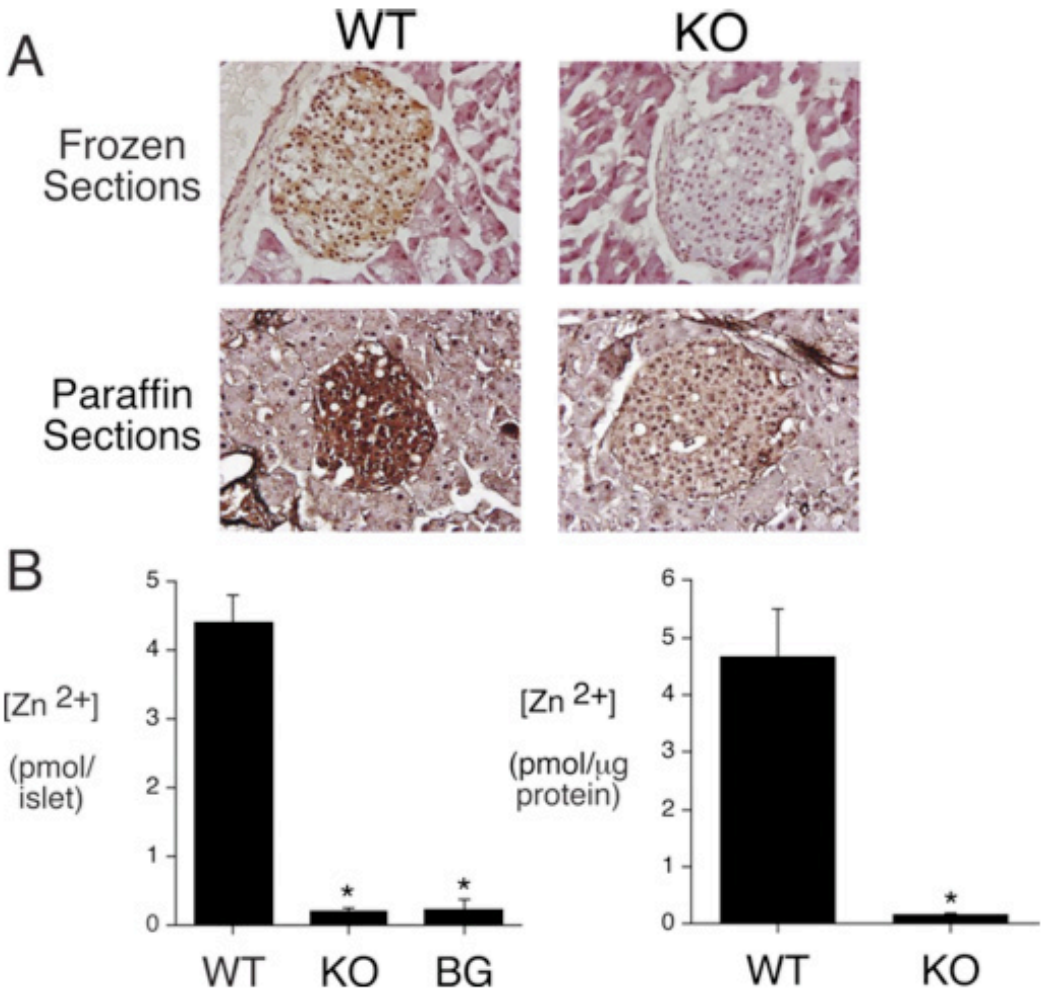


Figure 4.3. Analysis of islet zinc content in *Slc30a8* KO mice. (A) A modified Timm's staining protocol was used to assess zinc content in both frozen and paraffin pancreas sections prepared from male wild type and *Slc30a8* KO mice as described in Chapter II. Representative pictures (100x magnification) are shown. (B) The zinc content in isolated islets was determined as described in Chapter II. Results are the means ± S.E.M. (n=12). *p<0.05 compared with wild type. BG=background; WT=wild type; KO=knockout.

differences were seen in the weights or lengths of *Slc30a8* KO compared with WT mice (Table 4.1).

Table 4.1 summarizes metabolic parameters in these animals assayed at 16 weeks of age following a 6 h fast. No marked changes in plasma cholesterol, triacylglycerol or glycerol were observed in either male or female *Slc30a8* KO mice relative to WT animals (Table 4.1). Blood glucose and glucagon concentrations were also unchanged in both male and female *Slc30a8* KO mice relative to WT animals; however, a statistically significant difference in plasma insulin concentrations was observed (Table 4.1). This result suggests that, although the absence of ZnT-8 might affect islet function, it has a limited effect on whole-body glucose metabolism. In addition, since a statistically significant difference in plasma insulin concentrations was not observed between male or female *Slc30a8* heterozygous mice relative to WT animals, this suggests that loss of a single *Slc30a8* allele is insufficient to affect insulin secretion (Table 4.1). *Slc30a8* KO mice showed gender-related variation in the majority of these metabolic parameters that were in the same direction and of similar magnitude to the gender-related differences in WT mice. Thus in males compared with females, insulin, triacylglycerols, cholesterol and glucose were all higher, whereas glucagon was lower (Table 4.1).

Since some metabolic disturbances only become readily apparent under stimulatory rather than basal conditions intraperitoneal glucose tolerance tests were used to provide a measurement of dynamic islet function *in vivo*. Following glucose injection (2 g/kg of body weight) blood glucose was assessed over a 120 min period (Fig. 4.4). The data show no impairment in glucose clearance between WT and *Slc30a8* KO mice

Table 4.1 Phenotypic characterization of *Slc30a8* KO mice on a mixed C57BL/6J x 129SvEv genetic background. At 16 weeks of age mice were fasted for 5 hours and then weighed. Mice were anesthetized 1 hour later, their length was measured and blood isolated. Blood glucose and plasma cholesterol, triacylglycerol, glycerol, insulin and glucagon levels were determined as described in Chapter II. Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. WT=wild type; -/+=heterozygous; KO=knockout.

Gender	Genotype	Weight (g)	Length (mm)	Glucose (mg/dl)	Cholesterol (mg/dl)	Triacylglycerol (mg/dl)	Glycerol (mg/dl)	Insulin (ng/ml)	Glucagon (pg/ml)
Female	WT	23.8 ± 0.4 (29)	98.8 ± 0.5 (28)	110.1 ± 3.9 (29)	75.1 ± 4.0 (26)	49.2 ± 2.5 (27)	2.5 ± 0.1 (27)	0.45 ± 0.05 (19)	70.5 ± 5.6 (23)
Female	−/+	23.3 ± 0.3 (67)	98.7 ± 0.3 (65)	110.0 ± 1.8 (66)	87.8 ± 2.4 (64)*	49.5 ± 1.5 (62)	2.6 ± 0.1 (63)	0.34 ± 0.03 (38)	71.8 ± 3.4 (56)
Female	KO	23.5 ± 0.4 (32)	98.8 ± 0.4 (31)	116.9 ± 3.7 (32)	82.5 ± 2.8 (30)	46.1 ± 1.7 (30)	2.6 ± 0.1 (30)	0.31 ± 0.05 (20)†	74.2 ± 6.3 (26)
Male	WT	32.4 ± 0.5 (35)	105.5 ± 0.4 (34)	135.7 ± 3.9 (35)	100.7 ± 4.4 (34)	68.4 ± 2.7 (32)	2.5 ± 0.1 (33)	1.50 ± 0.23 (12)	63.2 ± 5.6 (29)
Male	−/+	32.4 ± 0.4 (63)	104.7 ± 0.3 (60)	135.7 ± 3.0 (63)	103.6 ± 3.2 (58)	73.2 ± 2.0 (58)	2.7 ± 0.1 (59)	1.07 ± 0.22 (15)	66.4 ± 3.5 (54)
Male	KO	31.6 ± 0.7 (34)	105.3 ± 0.6 (32)	137.2 ± 3.4 (34)	111.7 ± 3.6 (33)	70.6 ± 2.9 (32)	2.5 ± 0.1 (31)	0.79 ± 0.11 (16)‡	52.0 ± 4.7 (26)§

*$P < 0.01$, female wild-type compared with female heterozygote.
†$P = 0.05$, female wild-type compared with female knockout.
‡$P < 0.01$, male wild-type compared with male knockout.
§$P < 0.05$, male heteozygote compared with male knockout.

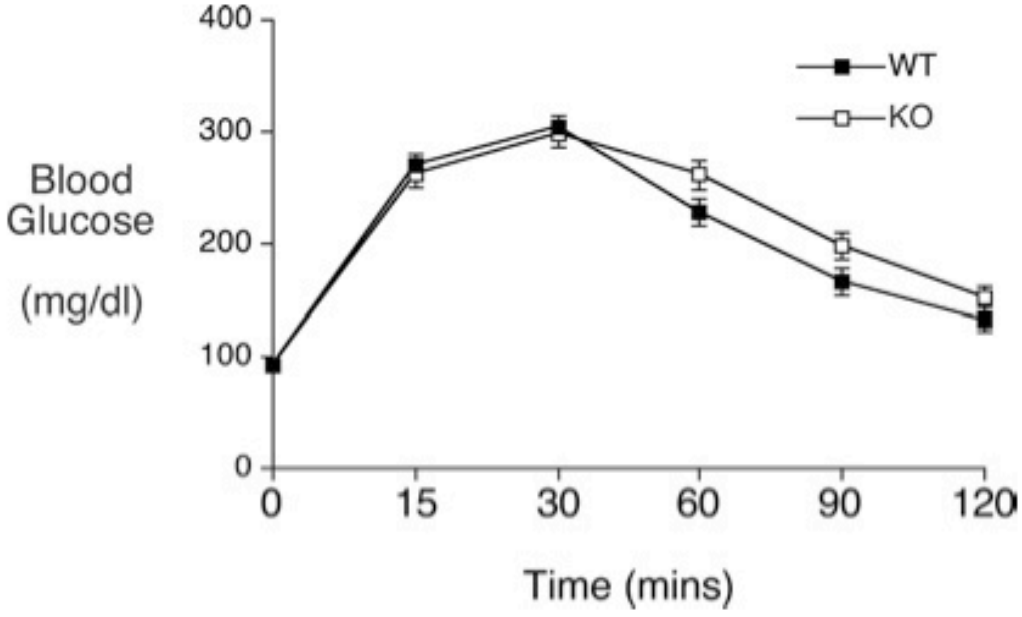


Figure 4.4. Analysis of glucose tolerance in *Slc30a8* KO mice *in vivo*. Intraperitoneal glucose tolerance tests were performed on overnight fasted conscious wild type (closed symbols) and *Slc30a8* KO (open symbols) male mice as described in Chapter II. Results show the mean glucose concentrations ± S.E.M. in wild type (n=30; mean age 20 weeks) and *Slc30a8* KO (n=36; mean age 20 weeks) animals. WT=wild type; KO=knockout.

(Fig. 4.4). This result again suggests that, although the absence of ZnT-8 might affect insulin secretion it has a limited impact on whole-body glucose metabolism, at least under the conditions examined.

Insulin secretion from Slc30a8 *KO mouse islets*

To directly assess the impact of ZnT-8 on islet function, GSIS was compared in islets isolated from male WT and *Slc30a8* KO mice in static incubations. Figure 4.5A shows that insulin content did not differ between WT and *Slc30a8* KO mouse islets, whereas Figure 4.5B shows that GSIS from *Slc30a8* KO mouse islets was reduced ~33% relative to that from WT mouse islets.

Discussion

In the present study we examine the effect of a global *Slc30a8*-null mutation *in vivo*, a mouse model that is directly relevant to type 2 diabetes susceptibility in humans. The study addresses the hypothesis that, based on GWA study data, changes in the activity or stability of the ZnT-8 protein may result in islet dysfunction, which contributes to the pathogenesis of type 2 diabetes. The results indicate that deletion of the *Slc30a8* gene results in a mild metabolic phenotype on a mixed 129SvEvBrd x C57BL/6J background. Plasma insulin is reduced in both male and female *Slc30a8* KO mice following a 6 h fast (Table 4.1). Consistent with this observation, GSIS from isolated islets is impaired (Fig. 4.5B) and islet zinc content is markedly reduced (Fig. 4.3), although islet size, number and cellular composition are unaffected (Fig. 4.2). These observations in *Slc30a8* KO mice are consistent with the demonstration that

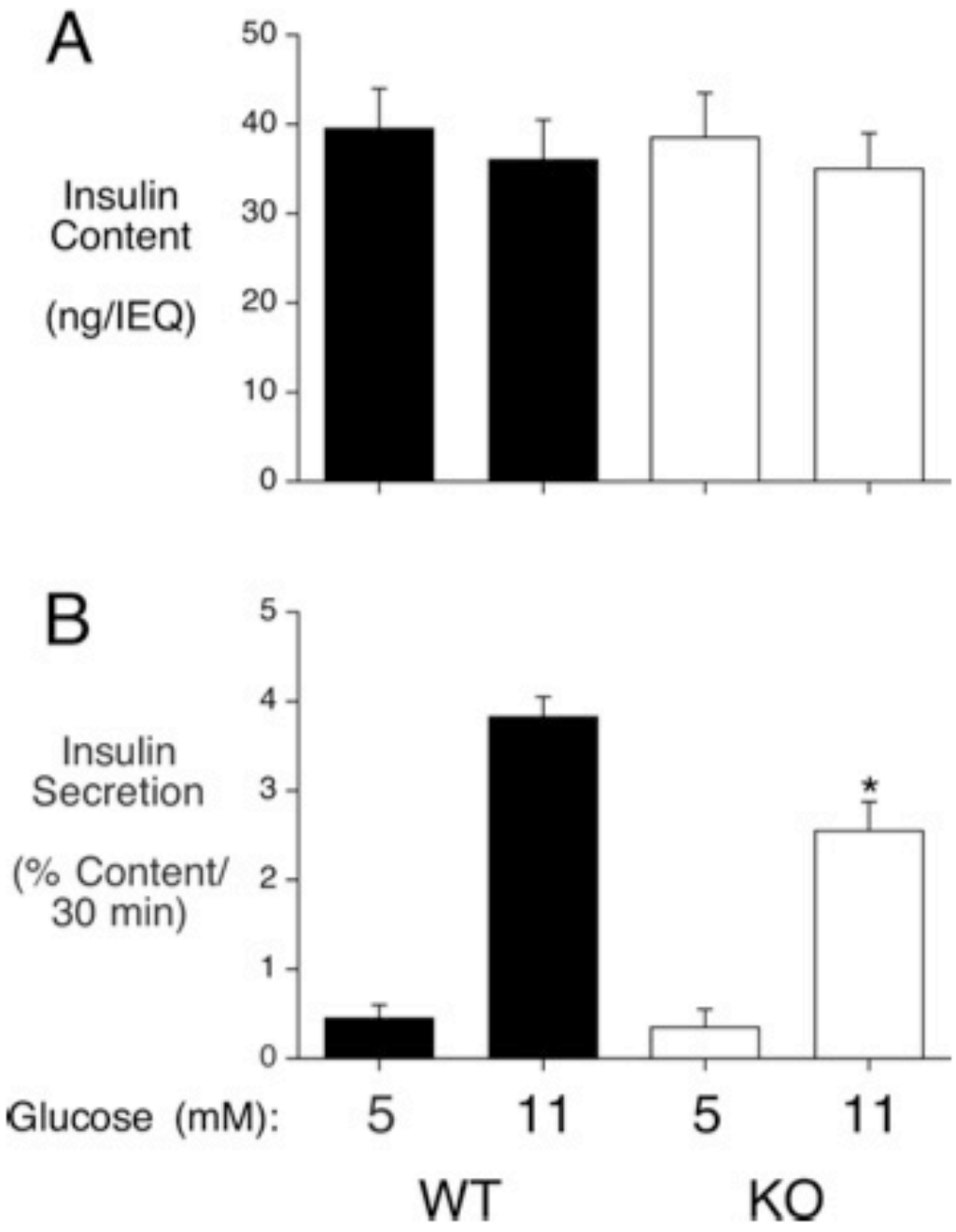


Figure 4.5. Analysis of insulin content and GSIS in isolated *Slc30a8* KO mouse islets. Islets were isolated from wild type (WT) and *Slc30a8* knockout (KO) mice and then insulin content (A) and GSIS (B) were assayed as described in Chapter II. Results are the means ± S.E.M. from three to four islet preparations. *p<0.05 compared with wild type 11mM glucose.

overexpression of ZnT-8 in INS-1 cells has the opposite effect, stimulating zinc accumulation and GSIS [188]. Although the loss of ZnT-8 function only has a mild effect it could be argued that this is nonetheless consistent with the small contribution of *Slc30a8* mutations to type 2 diabetes risk, as reflected in the odds ratio of 1.12 [9, 103, 189, 190]. However, that argument depends on a quantitative assessment of the deleterious nature of the identified *SLC30A8* SNP on ZnT-8 function. For example, if the SNP completely disables ZnT-8 then the low odds ratio would be consistent with the mild phenotype of *Slc30a8* KO mice. On the other hand, if the SNP only has a mild effect on ZnT-8 then a more dramatic phenotype would have been expected in *Slc30a8* KO mice, with the caveats that the importance of ZnT-8 may vary between mice and humans and, in addition, compensatory events may have occurred in the *Slc30a8* KO mice. The latter concept is consistent with observations *in vitro* in INS-1E insulinoma cells. Nicolson and colleagues demonstrated that while cells transfected with a ZnT-8 expression vector encoding the tryptophan variant display a significant reduction in zinc uptake, there is no significant change in GSIS [194]. We considered additional studies on these mixed genetic background mice that would have been designed to examine whether the absence of ZnT-8 affects glucose metabolism under conditions more favorable for the development of glucose intolerance, such as following high-fat feeding or in older animals. However, we decided to postpone those studies until the KO allele was backcrossed onto the C57BL/6J genetic background, thus reducing variability between animals.

Although the results indicate that ZnT-8 is important for normal islet function, surprisingly whole-body glucose metabolism appears unaltered based on an assessment

of fasting glucose levels (Table 4.1) and glucose tolerance tests (Fig. 4.4). These observations suggest that ZnT-8 is not necessary for glucose homoeostasis, at least under the conditions examined. There appear to be two possible explanations for the decrease in plasma insulin without a concomitant increase in blood glucose (Table 4.1). There is a statistically significant decrease in plasma glucagon (~20%) between male *Slc30a8* heterozygous and *Slc30a8* KO mice, although not between *Slc30a8* WT and *Slc30a8* KO mice (Table 4.1). If the latter simply reflects a lack of power to detect a small change in plasma glucagon, the former would imply that glucagon secretion is also impaired in male *Slc30a8* KO mice. In this event an offsetting decrease in both insulin and glucagon secretion could result in normal blood glucose. Indeed, the insulin/glucagon ratios in individual animals were not statistically different between male WT and *Slc30a8* KO mice (results not shown). We realized that future experiments studying glucagon secretion from isolated islets would address this possibility but we also decided to postpone those studies until the KO allele was backcrossed onto the C57BL/6J genetic background, thus reducing variability between animals.

In contrast to the males, in female mice there are no statistically significant differences in glucagon levels between groups (Table 4.1). This suggested that there must be a different explanation for the reduced FBG in female mice without a concomitant change in insulin and glucagon levels. One possibility was that there may be differences in insulin sensitivity between female WT and *Slc30a8* KO mice. This would be consistent with the results of post-hoc QUICKI calculations [236] suggesting a statistically significant difference in insulin sensitivity between female WT and *Slc30a8* KO mice (results not shown). Such a difference could have arisen as an adaptive change

during development to compensate for low plasma insulin. Alternatively, a difference in insulin sensitivity could arise if *Slc30a8* were expressed in other tissues, specifically ones which directly or indirectly modulate insulin dependent glucose disposal. Interestingly, Murgia et al. [237] have recently demonstrated that *Slc30a8* is expressed at low levels in tissues other than islets. We considered additional experiments to directly compare insulin sensitivity in female WT and *Slc30a8* KO mice using hyperinsulinemic clamps but once again we decided to postpone those studies until the KO allele was backcrossed onto the C57BL/6J genetic background, thus reducing variability between animals. Finally, zinc is required not only for stabilizing the insulin crystal within the insulin storage granule, but may also be essential for the conversion of proinsulin to insulin [129]. Increased circulating pro-insulin is a feature of early type 2 diabetes and impaired glucose tolerance [238, 239] and in a group genetically at risk of developing type 2 diabetes, the *SLC30A8* allele was associated with reduced proinsulin into insulin conversion, although not insulin secretion [240]. Since proinsulin has only ~3% of the potency of insulin [241], if proinsulin secretion was markedly increased in the female *Slc30a8* KO mice this could also explain the reduced plasma insulin levels associated with unchanged blood glucose levels. Unfortunately, commercially available assays for murine proinsulin were unavailable when these studies were performed.

Chimienti et al. [235] had previously reported that ZnT-8 is only expressed in pancreatic islet β-cells, but using antiserum raised against a 102-amino-acid C-terminal human ZnT-8 peptide (amino acids 268–369) we found that ZnT-8 is also clearly expressed in α-cells (Fig. 4.1C). This observation is consistent with the results of Gyulkhandanyan et al. [132] who examined ZnT-8 expression in dispersed islet cells.

The specificity of our antiserum was confirmed by the absence of staining in sections prepared from *Slc30a8* KO mouse pancreas (Fig. 4.1C). The hypothesis that glucagon secretion may be impaired in male *Slc30a8* KO mice would therefore be consistent with the expression of ZnT-8 in α-cells (Fig. 4.1C). However, the possibility also exists that glucagon secretion from α-cells has been indirectly affected by the absence of ZnT-8 in β-cells. Indeed, although the mechanism(s) involved are disputed, zinc release from β-cells inhibits glucagon secretion from α-cell [132, 177, 242].

The SNP that linked the human *SLC30A8* gene to increased type 2 diabetes susceptibility [9, 103, 189, 190] is located in the C-terminus of ZnT-8 and represents a nonsynonymous polymorphism that changes the sequence of amino acid residue 325 [235]. In theory this ZnT-8 variant could represent either a gain- or loss-of-function, but because overexpression of ZnT-8 enhances GSIS [188] and because deletion of the *Slc30a8* gene in mice impairs GSIS (Fig. 4.5B) we would predict that this human sequence variant impairs ZnT-8 function. Future experiments will be needed to address this hypothesis.

CHAPTER V

ANALYSIS OF THE PHENOTYPE OF *Slc30a8* KNOCKOUT MICE ON A PURE C57BL/6J GENETIC BACKGROUND

Introduction

This chapter describes studies performed to directly follow up on those described in Chapter IV and to address the hypothesis that the absence of ZnT-8 results in impaired islet function and thereby contributes to type 2 diabetes. The studies described in Chapter IV indicate that global deletion of *Slc30a8* in mice results in a significant reduction in islet zinc content, a reduction in fasting plasma insulin levels and GSIS with, surprisingly, no change in fasting blood glucose levels or in glucose tolerance. Though alterations in insulin secretion are consistent with the proposed role of zinc in the islet, the studies performed *in vitro*, and the GWA studies, the observation that both FBG and glucose tolerance were normal was unanticipated.

Soon after the publication of the data described in Chapter IV, a number of other studies were published that also described the phenotype of *Slc30a8* KO mice. These studies all demonstrated a significant loss of zinc within the islet [194, 243, 244], supporting the proposed role of ZnT-8. In contrast, however, the reported effect of *Slc30a8* deletion on insulin secretion from isolated islets varied between these studies. Wijesekara *et al* demonstrated that GSIS was reduced in isolated *Slc30a8* KO mouse islets, consistent with the results of our study, though the authors also reported no change in insulin secretion in KO mice during an IPGTT *in vivo* despite mildly impaired glucose

tolerance [243]. In contrast, Lemaire *et al* found no impairment in GSIS [244], while Nicolson *et al* reported an increase in GSIS from isolated *Slc30a8* KO mouse islets, though this result varied with age and gender [194]. Surprisingly, however, this same study demonstrated, in young mice, a reduction in insulin secretion in *Slc30a8* KO mice during an IPGTT as well as impaired glucose tolerance [194]. Finally, only one study examined plasma insulin levels following a 6 hour fast and found no change in a β-cell specific *Slc30a8* KO mouse model [243]. The differences between these studies have been attributed to variations in the age and genetic background of the mice examined [245]; however, there were also marked differences in the numbers of mice analyzed in these different studies, which could be significant given the mild phenotypes reported [246].

Due to the variation in reported phenotypes, we sought to remove the conflicting effect of 129SvEv-specific modifier genes by examining the impact of *Slc30a8* deletion in the context of the pure C57BL/6J genetic background. Because binding of zinc to insulin is thought to be important for proper β-cell function, we predicted that a lack of granular zinc would again result in impaired insulin secretion in C57BL/6J *Slc30a8* KO mice, consistent with observations in *Slc30a8* KO mice on a mixed genetic background (Chapter IV). Surprisingly, we find that, in contrast to the observations made with *Slc30a8* KO mice on a mixed genetic background, on the C57BL/6J background only female *Slc30a8* KO mice displayed a reduction in fasting plasma insulin. Furthermore, despite this reduction in fasting plasma insulin there was no change in FBG, glucose tolerance or GSIS from isolated islets. Our data therefore suggest that, despite the

marked loss of zinc in islet insulin secretory granules, the absence of ZnT-8 does not have a substantial impact on normal mouse physiology.

Results

Generation of C57BL/6J Slc30a8 *KO mice*

Slc30a8 KO mice were initially generated on a mixed 129SvEv^{Brd} x C57BL/6J genetic background (Chapter IV, [247]). Using a speed congenic breeding strategy, mice were backcrossed onto a pure C57BL/6J genetic background.

Genotype analysis of 360 three week old pups generated by cross breeding C57BL/6J *Slc30a8* heterozygous mice demonstrated that 88 mice were *Slc30a8* WT, 184 were *Slc30a8* heterozygous, and 88 were *Slc30a8* KO, a distribution close to the expected pattern for Mendelian inheritance. The ratio of male to female mice was 201: 159. Cross breeding experiments revealed that both male and female *Slc30a8* KO mice are fertile. Furthermore, as with mice on a mixed genetic background, no gross anatomical or behavioral abnormalities were observed in *Slc30a8* KO mice compared to WT or heterozygous mice on the C57BL/6J genetic background.

Phenotypic characterization of male C57BL/6J Slc30a8 *KO mice following a glucose challenge*

On a mixed genetic background the effect of deleting the *Slc30a8* gene was more apparent in male mice (Chapter IV, [247]), thus we initially analyzed the phenotype of male *Slc30a8* KO mice on the pure C57BL/6J genetic background.

We first performed IPGTTs to gain insight into islet function *in vivo*. Following a 6 hour fast, male mice were injected with glucose (2 mg/g body weight) and glycemia was measured over a 120 min period. As in male *Slc30a8* KO mice on a mixed genetic background (Chapter IV, [247]), no defect in glucose tolerance was observed (Fig. 5.1A). Furthermore, male *Slc30a8* KO mice also displayed normal glucose tolerance when glucose was administered by gavage (Fig. 5.1B). Thus, the results of glucose tolerance tests are consistent with the phenotype observed on a mixed genetic background (Chapter IV, [247]) and, most significantly, the conclusion that global deletion of the *Slc30a8* gene has little effect on whole body glucose metabolism.

Phenotypic characterization of fasted male C57BL/6J Slc30a8 *KO mice*

We next investigated the phenotype of male C57BL/6J *Slc30a8* KO mice following a 6 hour fast. As with male *Slc30a8* KO mice on a mixed genetic background (Chapter IV, [247]), no marked differences in body weight or length, fasting blood glucose or plasma, cholesterol, glycerol, triglycerides or glucagon were observed (Table 5.1). But surprisingly, male C57BL/6J *Slc30a8* KO mice also displayed normal fasting plasma insulin levels (Table 5.1) in marked contrast to the ~50% reduction observed with male *Slc30a8* KO on the mixed genetic background (Chapter IV, [247]). This result suggests that the phenotype of male *Slc30a8* KO mice is strongly dependent upon 129SvEv-specific modifier genes.

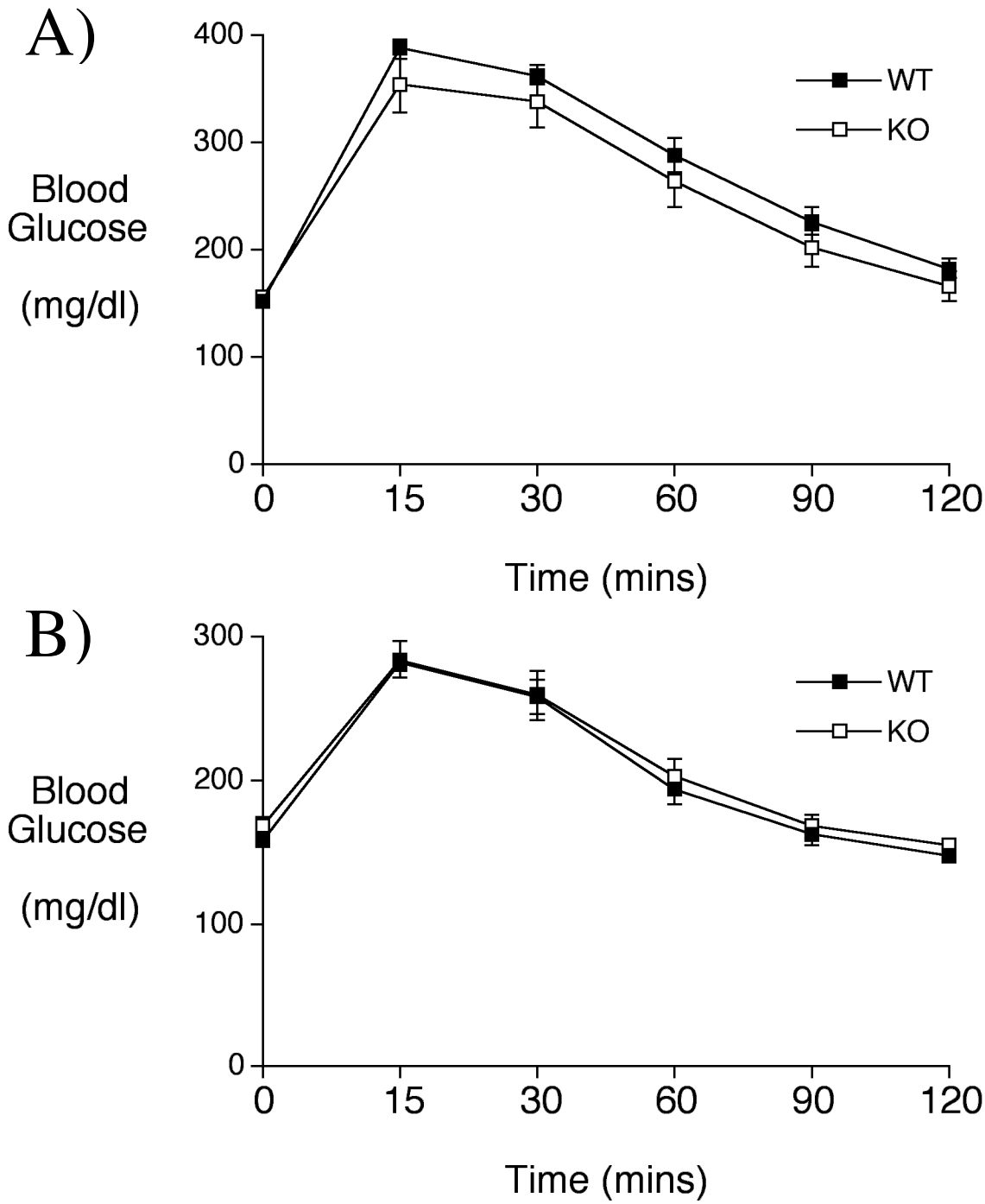


Figure 5.1. Analysis of glucose tolerance in male C57BL/6J *Slc30a8* KO mice *in vivo*. Intraperitoneal (Panel A) and oral (Panel B) glucose tolerance tests were performed on 6 hour fasted conscious C57BL/6J WT (closed symbols) and *Slc30a8* KO (open symbols) male mice as described in Chapter II. (A) Results show the mean glucose concentrations ± S.E.M. in WT (n=11; mean age ~20 weeks) and *Slc30a8* KO (n=9; mean age ~20 weeks) animals. (B) Results show the mean glucose ± S.E.M. in WT (n=16; mean age ~22 weeks) and *Slc30a8* KO (n=9; mean age ~22 weeks) animals.

Table 5.1. Phenotypic characterization of *Slc30a8* KO mice on a C57BL/6J genetic background. At 16 weeks of age mice were fasted for 5 hours and then weighed. One hour later mice were anaesthetized, their length was measured and blood isolated. Blood glucose and plasma cholesterol, triacylglycerol, glycerol, insulin and glucagon levels were determined as described in Chapter II. Results represent mean data ± S.E.M. obtained from the indicated number of animals in parentheses.

Gender & Genotype	Weight (g)	Length (mm)	Glucose (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	Glycerol (mg/dl)	Insulin (ng/ml)	Proinsulin (pg/ml)	Glucagon (pg/ml)
Female WT	20.1 ± 0.3 (15)	97.4 ± 0.3 (14)	113.7 ± 4.0 (15)	63.1 ± 2.3 (13)	46.6 ± 2.0 (12)	2.9 ± 0.2 (12)	0.57 ± 0.04 (15)	25.4 ± 3.8 (17)	48.4 ± 6.7 (10)
Female -/+	20.0 ± 0.2 (33)	97.1 ± 0.3 (33)	113.5 ± 2.1 (31)	58.8 ± 1.7 (20)	44.2 ± 1.6 (21)	3.3 ± 0.1 (22)	0.41 ± 0.02 (30) *		47.3 ± 3.5 (18)
Female KO	20.2 ± 0.2 (14)	96.1 ± 0.3 (12) *	122.1 ± 4.9 (14)	64.0 ± 2.2 (11)	46.7 ± 4.4 (11)	3.3 ± 0.3 (11)	0.45 ± 0.03 (14) **	16.0 ± 2.8 (11)	38.3 ± 5.5 (9)
Male WT	26.3 ± 0.2 (24)	101 ± 0.3 (24)	133.1 ± 4.7 (23)	74.5 ± 1.5 (13)	46.6 ± 2.1 (13)	2.8 ± 0.2 (13)	0.67 ± 0.04 (19)	60.1 ± 7.5 (17)	42.4 ± 2.0 (12)
Male -/+	26.1 ± 0.2 (49)	100.5 ± 0.2 (48)	135.7 ± 2.6 (49)	68.5 ± 1.0 (31) *	45.9 ± 1.4 (32)	2.8 ± 0.1 (31)	0.65 ± 0.03 (37)		39.4 ± 2.8 (29)
Male KO	26.0 ± 0.4 (27)	100.7 ± 0.3 (26)	135.0 ± 4.5 (27)	72.5 ± 1.4 (19)	47.5 ± 2.1 (19)	2.9 ± 0.2 (20)	0.60 ± 0.04 (24)	44.1 ± 3.8 (22) *	38.8 ± 2.9 (19)

1. Length: *F WT vs. F KO, $p<0.01$
2. Cholesterol: *M WT vs. M Het $p<0.01$
3. Insulin: *F WT vs. F Het, $p<0.001$; **F WT vs. F KO, $p<0.05$
4. Proinsulin: *M WT vs. KO, $p<0.05$

Insulin and glucagon secretion from male C57BL/6J Slc30a8 *KO mouse islets*

Even though fasting plasma insulin and glucose were unaltered in male C57BL/6J *Slc30a8* KO mice (Table 5.1), we next analyzed GSIS in islets isolated from ~18 week old male mice so as to allow a direct comparison with the equivalent data obtained with islets isolated from *Slc30a8* KO mice on a mixed genetic background. Following overnight culture in 5 mM glucose, islets were incubated in either 5 mM or 11 mM glucose for 30 min. No change in insulin content was observed in C57BL/6J *Slc30a8* KO mouse islets (Fig. 5.2A), consistent with data obtained with islets isolated from *Slc30a8* KO mice on a mixed genetic background [247]. However, in marked contrast to the reduced GSIS observed using islets isolated from *Slc30a8* KO mice on a mixed genetic background [247], islets from male C57BL/6J *Slc30a8* KO mice displayed no change in GSIS (Fig. 5.2B). These data are again consistent with the conclusion that the phenotype of male *Slc30a8* KO mice is strongly dependent upon 129SvEv-specific modifier genes.

Because *Slc30a8* is also expressed in α cells, we investigated both glucagon content and arginine-stimulated glucagon secretion in isolated male C57BL/6J *Slc30a8* KO mouse islets. Despite the extensive literature describing a role for zinc secretion from β cells in the regulation of glucagon secretion from α cells [132, 177, 242], neither glucagon content (Fig. 5.2C) nor amino acid stimulation of glucagon secretion (Fig. 5.2D) were altered.

Phenotypic characterization of fasted female C57BL/6J Slc30a8 *KO mice*

Although the phenotype of male *Slc30a8* KO mice observed on a mixed genetic background was not retained on the C57BL/6J background, female *Slc30a8* KO mice on

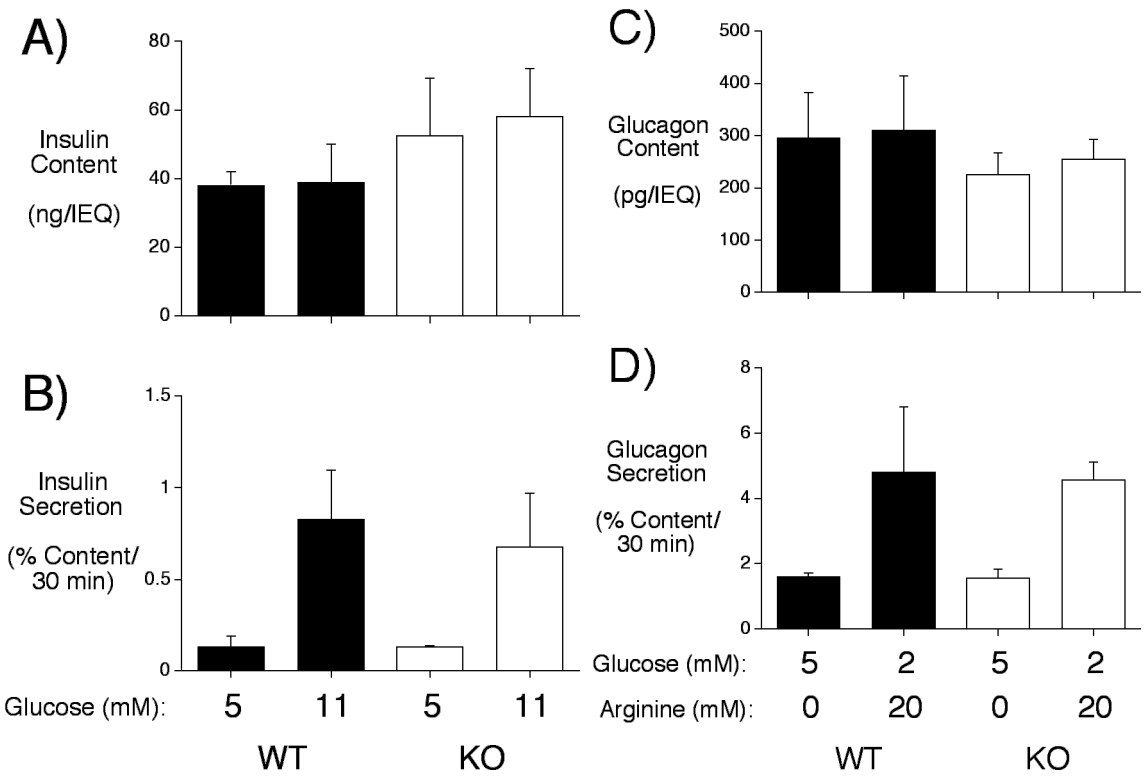


Figure 5.2. Analysis of insulin content, glucagon content, GSIS and arginine-stimulated glucagon secretion in male C57BL/6J *Slc30a8* KO mouse islets *in situ*. Islets were isolated from male C57BL/6J WT and *Slc30a8* KO mice and then insulin content (Panel A), GSIS (Panel B), glucagon content (Panel C) and arginine-stimulated glucagon secretion (Panel D) were assayed as described in Chapter II. Results show the mean data ± S.E.M. from 3 islet preparations isolated from ~18 week old male mice. *, $p<0.05$ *versus* WT 11mM glucose.

the mixed genetic background also displayed reduced fasting insulin levels [247]. We therefore assessed the phenotype of the female C57BL/6J *Slc30a8* KO mice following a 6 hour fast at ~16 weeks of age (Table 5.1). As with the males, female C57BL/6J *Slc30a8* KO mice displayed no marked differences in body weight or length or fasting plasma cholesterol, glycerol, triglycerides or glucagon. But, consistent with observations on the mixed genetic background, female C57BL/6J *Slc30a8* KO mice displayed a marked reduction (~20%) in fasting plasma insulin levels with no change in fasting blood glucose levels (Table 5.1), though the magnitude of this reduction was somewhat greater in female *Slc30a8* KO mice on the mixed genetic background (~30%) [247]. This result implies that, in contrast to male mice, the phenotype of female *Slc30a8* KO mice is less dependent upon the influence of 129SvEv-specific modifier genes.

Phenotypic characterization of female C57BL/6J Slc30a8 *KO mice following a glucose challenge*

We next determined whether the reduction in fasting plasma insulin in female C57BL/6J *Slc30a8* KO mice resulted in impaired glucose tolerance. Both intraperitoneal (Fig. 5.3A) and oral (Fig. 5.3B) glucose tolerance tests indicated that glucose tolerance is normal in female C57BL/6J *Slc30a8* KO mice. A similar observation was previously made with male *Slc30a8* KO mice on a mixed genetic background in which a marked reduction in fasting plasma insulin was not associated with a change in glucose tolerance [247].

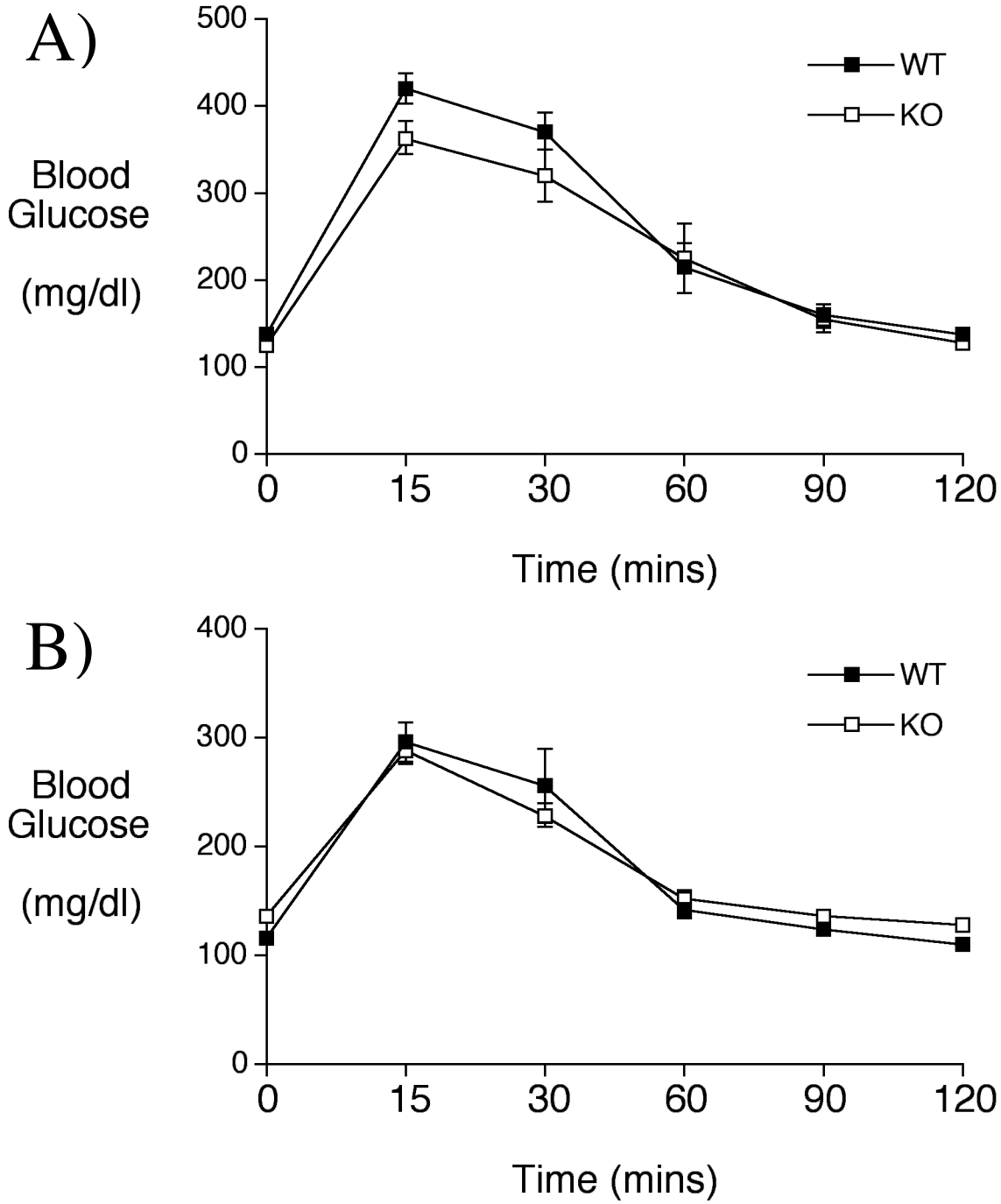


Figure 5.3. Analysis of glucose tolerance in female C57BL/6J *Slc30a8* KO mice *in vivo*. Intraperitoneal (Panel A) and oral (Panel B) glucose tolerance tests were performed on 6 hour fasted conscious C57BL/6J WT (closed symbols) and *Slc30a8* KO (open symbols) female mice as described in Chapter II. (A) Results show the mean glucose concentrations ± S.E.M. in WT (n=6; mean age ~20 weeks) and *Slc30a8* KO (n=8; mean age ~20 weeks) animals. (B) Results show the mean glucose concentrations ± S.E.M. in WT (n=9; mean age ~22 weeks) and *Slc30a8* KO (n=12; mean age ~22 weeks) animals.

Insulin secretion from female C57BL/6J Slc30a8 *mouse islets*

In male *Slc30a8* KO mice on a mixed genetic background, although glucose tolerance was not altered, the marked reduction in fasting plasma insulin was associated with impaired GSIS [247]. We therefore investigated whether the reduction in fasting plasma insulin in female *Slc30a8* KO mice was also associated with impaired GSIS. Islets were isolated from ~18 week old female C57BL/6J WT and *Slc30a8* KO mice and insulin content and secretion were measured following static incubations in either 5 mM or 11 mM glucose. No differences in insulin content between WT and KO islets were observed (Fig. 5.4A). And surprisingly, despite the reduction in fasting insulin levels, GSIS in female C57BL/6J *Slc30a8* KO mouse islets did not differ from WT islets (Fig. 5.4B). Thus, in contrast to male *Slc30a8* KO mice on the mixed genetic background, reduced fasting insulin levels were not associated with reduced GSIS from isolated female C57BL/6J *Slc30a8* KO mouse islets.

Analysis of the mechanism by which fasting plasma insulin is reduced in female C57BL/6J Slc30a8 *KO mice with no change in glucose tolerance*

To address the issue as to how fasting plasma insulin levels could be altered with no change in fasting blood glucose, we considered three possibilities. First, it was possible that female C57BL/6J *Slc30a8* KO mice would also manifest a corresponding reduction in fasting plasma glucagon levels, such that the KO mice are simply living at a different metabolic equilibrium. However, no significant difference in fasting plasma glucagon levels was observed (Table 5.1). This is consistent with the female *Slc30a8* KO

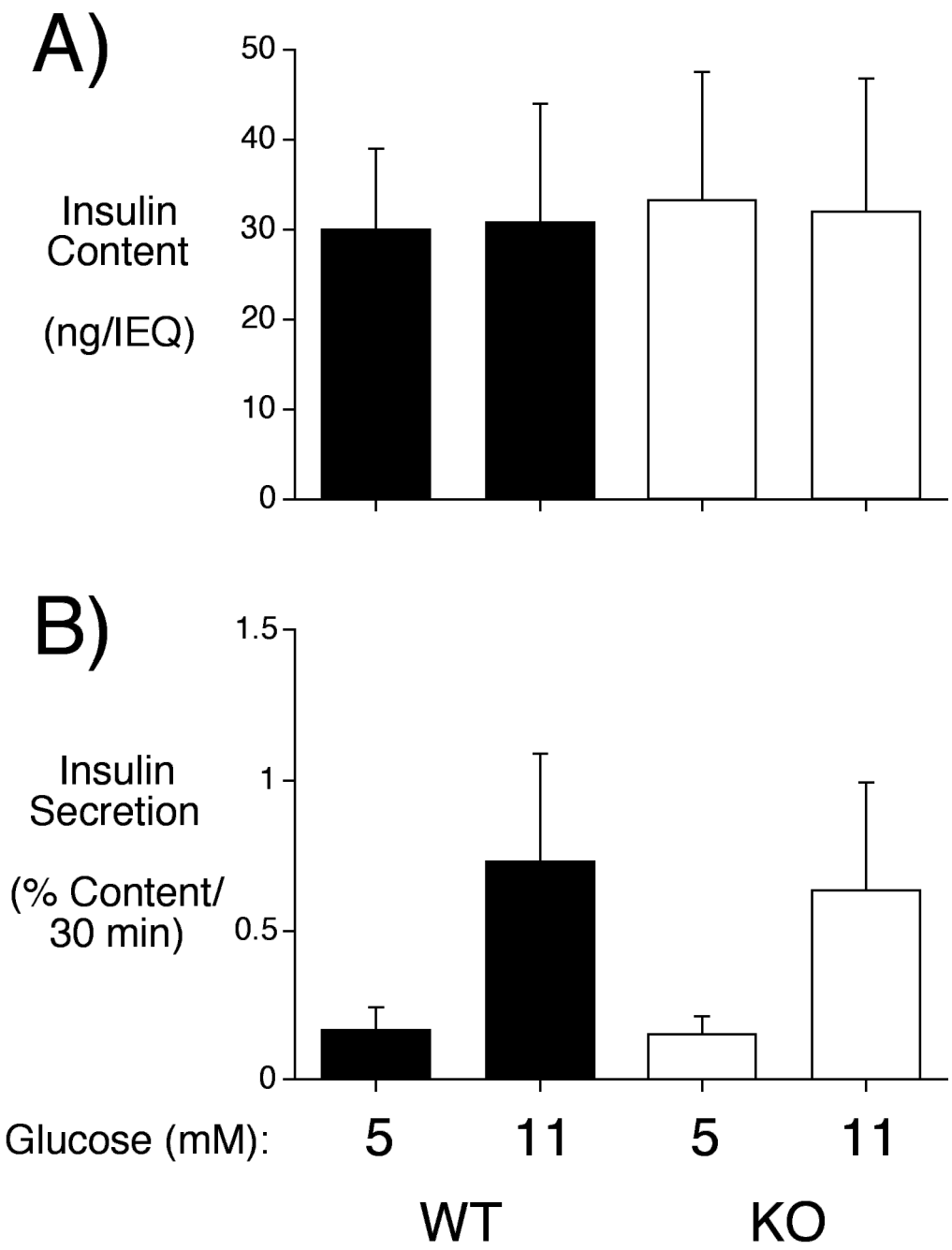


Figure 5.4. Analysis of insulin content and GSIS in female C57BL/6J *Slc30a8* KO mouse islets *in situ*. Islets were isolated from female WT and *Slc30a8* KO mice and then insulin content (Panel A) and GSIS (Panel B) were assayed as described in Chapter II. Results show the mean data ± S.E.M. from 6 islet preparations isolated from ~18 week old female mice.

mice on a mixed genetic background in which a reduction in fasting insulin was not associated with a change in fasting glucagon [247].

Second, because it has been proposed that zinc is important for proinsulin to insulin conversion [129] and, in humans, variations in the *SLC30A8* gene are associated with impaired proinsulin conversion [240], it was conceivable that increased proinsulin levels may have compensated for the reduction in insulin secretion. Proinsulin can bind, albeit with low affinity relative to insulin, to the insulin receptor and thus activate insulin signaling pathways [241]. However, fasting proinsulin levels were unchanged in female *Slc30a8* KO mice following a 6 hour fast (Table 5.1), though the difference between WT and KO was close to significance ($p<0.06$). Interestingly, male C57BL/6J *Slc30a8* KO mice displayed a significant reduction in proinsulin levels following a 6 hour fast (Table 5.1), suggesting that insulin processing is altered, though not sufficiently to lead to a reduction in fasting glucose or insulin levels. However, even this effect appears subject to the influence of modifier genes since it is not observed in male *Slc30a8* KO mice on a mixed genetic background (Fig. 5.5).

Finally, we considered the possibility that insulin sensitivity was enhanced in the female C57BL/6J *Slc30a8* KO mice to offset the reduction in fasting insulin secretion. Such a change could have arisen either indirectly, as an adaptive compensation to offset the reduction in fasting insulin secretion, or directly as a consequence of the absence of ZnT-8 in several other tissues where it is expressed at low levels [237]. Insulin tolerance tests, however, indicated that neither female (Fig. 5.6A) nor male (Fig. 5.6B) C57BL/6J *Slc30a8* KO mice display altered insulin sensitivity.

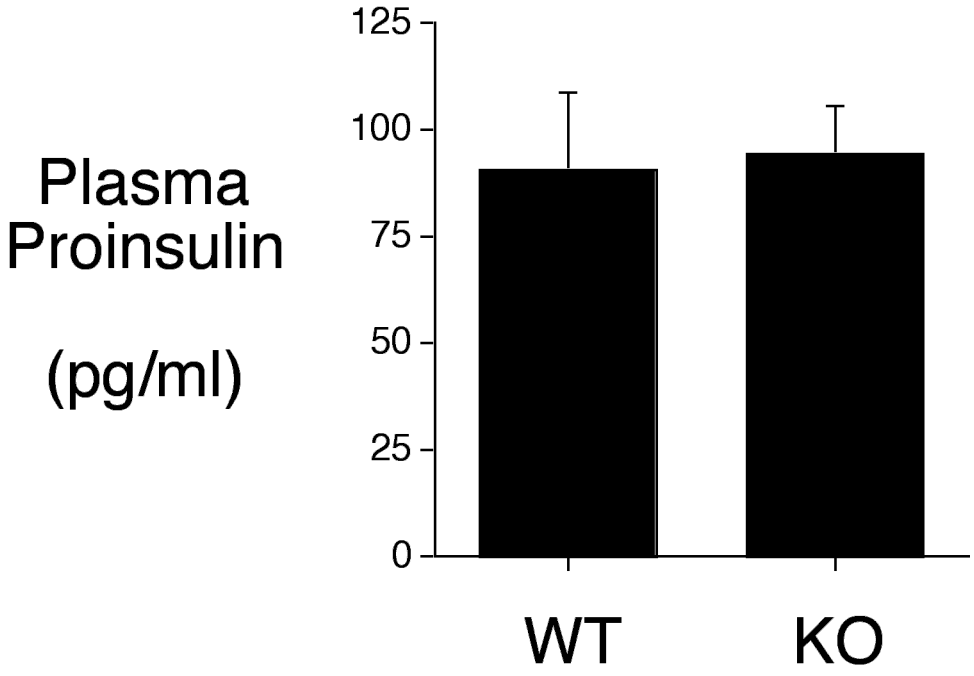


Figure 5.5. Analysis of plasma proinsulin in mixed genetic background *Slc30a8* KO mice *in vivo*. At 16 weeks of age mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized, their length was measured and blood isolated. Plasma proinsulin levels were determined as described in Chapter II. Results represent mean data ± S.E.M. obtained from the indicated number of animals in parentheses.

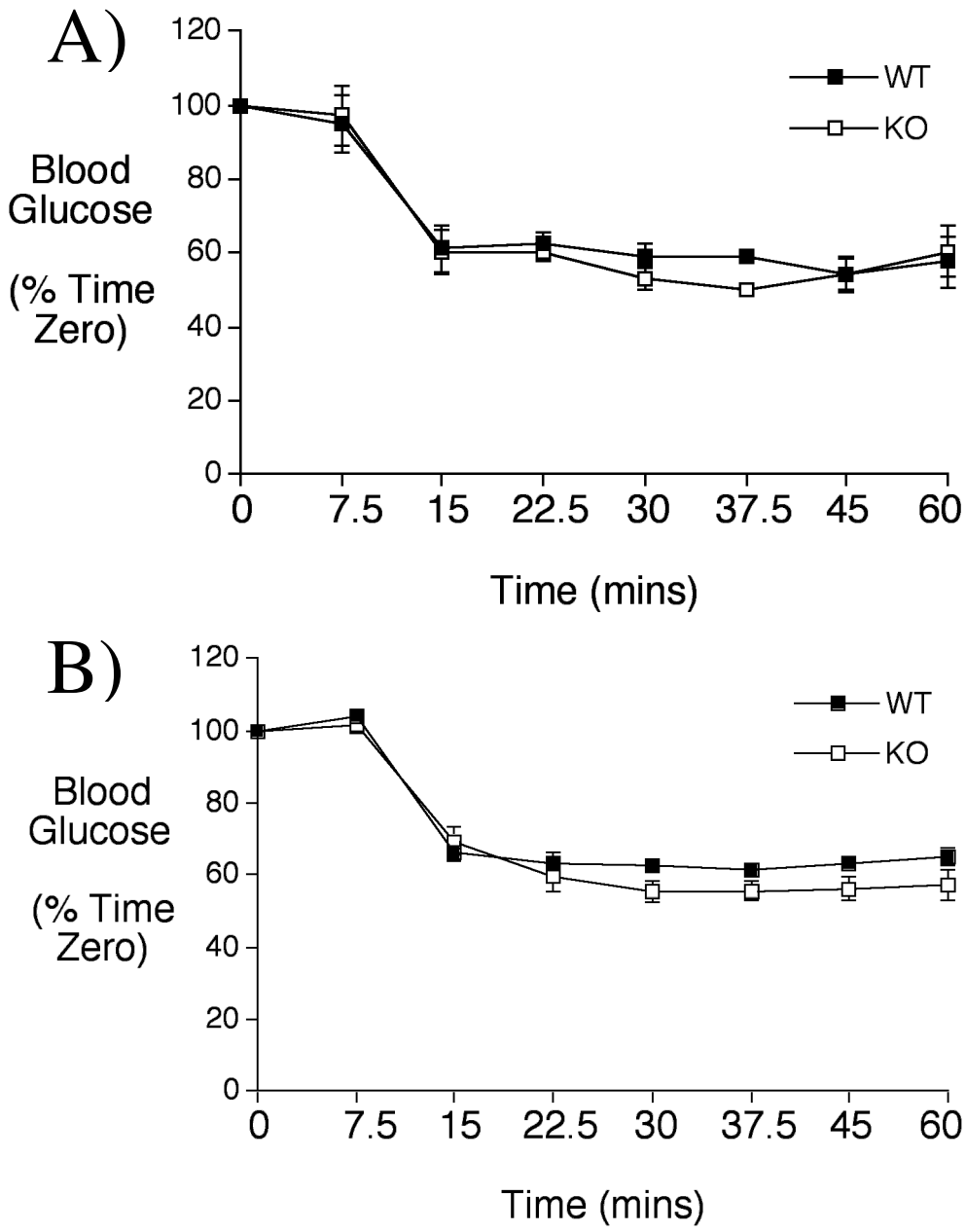


Figure 5.6. Analysis of insulin sensitivity in C57BL/6J *Slc30a8* KO mice *in vivo*. Insulin tolerance tests were performed on 5 hour fasted conscious C57BL/6J WT (closed symbols) and *Slc30a8* KO (open symbols) female (Panel A) and male (Panel B) mice as described in Chapter II. (A) Results show the mean glucose concentrations ± S.E.M. in WT (n=8; mean age ~18 weeks) and *Slc30a8* KO (n=8; mean age ~18 weeks) animals. In these groups of mice the initial glucose concentration at time zero was higher in KO mice (142.6 ± 6.1 vs 120.8 ± 4.9 mg/dl; $p<0.02$). (B) Results show the mean glucose concentrations ± S.E.M. in WT (n=11; mean age ~18 weeks) and *Slc30a8* KO (n=6; mean age ~18 weeks) animals. In these groups of mice the initial glucose concentration at time zero was not different between WT (153.0 ± 4.8 mg/dl) and KO (165.0 ± 2.3 mg/dl) mice.

Analysis of C57BL/6J Slc30a8 *KO mouse islets using electron microscopy*

Previous studies [194, 243, 244] have reported markedly altered islet secretory granule morphology in *Slc30a8* KO mice, which seems at odds with the mild metabolic phenotype. We therefore re-examined islet beta cell secretory granule morphology in the C57BL/6J *Slc30a8* KO mice. The results demonstrate that female C57BL/6J *Slc30a8* KO granules are indistinguishable from control mice (Fig. 5.7). The size and shape of both the granules and the electron dense cores did not appear to differ from control islets (Fig. 5.7). This suggests that female C57BL/6J *Slc30a8* KO mice have normal insulin crystallization and packaging. In the published studies Nicholson et al. [194] and Wijesekara et al. [243] both fixed isolated islets whereas Lemaire et al. [244] fixed intact pancreas by immersion. In contrast, our fixation method involved the more challenging fixation of the pancreas by vascular perfusion *in situ*. This method has the advantage of minimizing perturbations to cell structure by rapidly and uniformly delivering the fixative to the tissue using the animals' own vasculature. We speculate that perfusion of the fixative results in the maintenance of true islet architecture, which may explain the differences between our results and published observations.

Analysis of pancreatic SLC30 developmental expression

The association between *SLC30A8* and susceptibility to type 2 diabetes appears at odds with the very mild phenotype of *Slc30a8* KO mice. While there are multiple possible explanations for this apparent inconsistency we considered the possibility that ZnT-8 may play a more important role during islet development in humans than mice. Indeed, islet development appears significantly different between humans and mice with

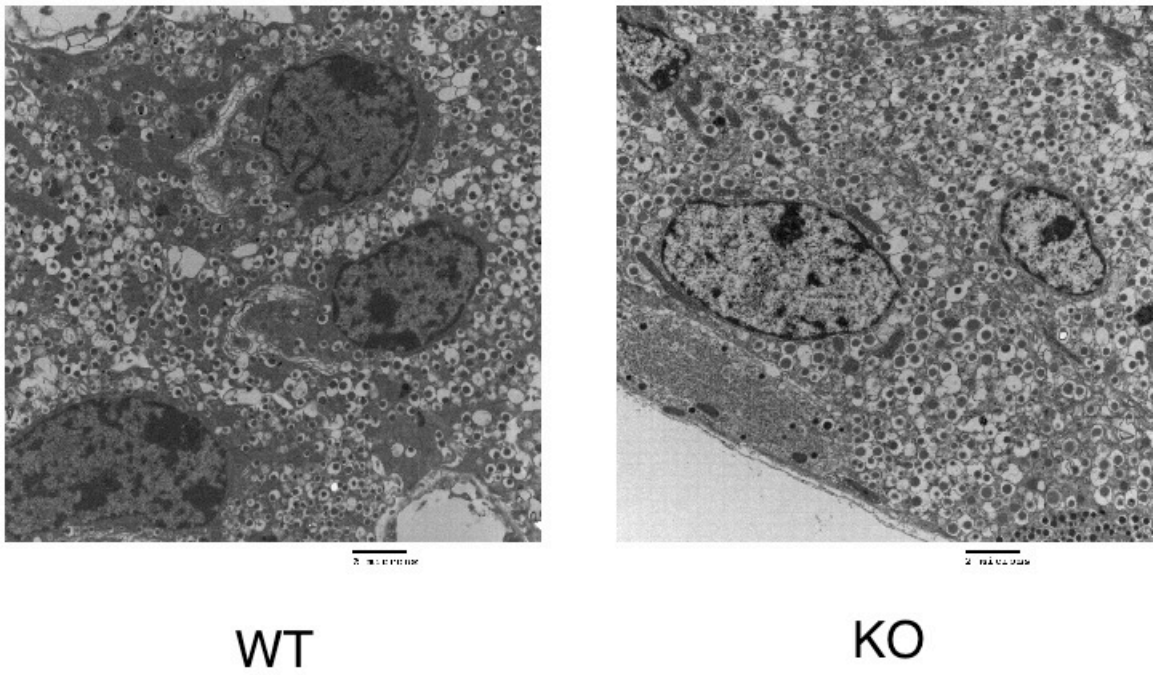


Figure 5.7. Analysis of insulin secretory granule structure in C57BL/6J *Slc30a8* KO mice. Islets were fixed in situ using pancreas perfusion and electron microscopy was then performed on pancreas sections as described in Chapter II.

the human equivalent of a mouse endocrine secondary transition not evident, either in terms of morphology or in dramatic changes in endocrine-specific transcriptional regulators [215]. We have previously shown that a marked increase in islet *Slc30a8* gene expression is observed between e15.5 and e17.5 in mice whereas *insulin* expression is already clearly evident at e15.5 [248]. In humans *insulin* transcripts are already present at 9-10 weeks and only increase ~50% further by 23 weeks, commensurate with the expansion of endocrine cell volume [215]. Figure 6 shows that *SLC30A8* expression increases markedly between 9 weeks and 23 weeks, indicating that in humans as in mice, high *insulin* expression precedes that of *SLC30A8*. Figure 5.8 also shows that *SLC30A8* is the major *SLC30* isoform in human islets, as it is in mice [194]. These observations do not support the concept of a dramatic difference between mice and humans with respect to a role for ZnT-8 during development.

Discussion

Our data demonstrate that the consequences of *Slc30a8* gene deletion in mice are both gender- and genetic background-specific. A decrease in fasting insulin was observed in female *Slc30a8* KO mice on both the pure C57BL/6J and mixed genetic backgrounds whereas this difference was only observed in male *Slc30a8* KO mice on the mixed genetic background (Table 5.1) [247]. In male *Slc30a8* KO mice on a mixed genetic background this decrease in fasting insulin was associated with reduced GSIS from isolated islets [247] whereas in female *Slc30a8* KO mice on the C57BL/6J genetic background it was not (Fig. 5.4B). While this may indicate that a defect that exists in female *Slc30a8* KO islets *in vivo* is not maintained in isolated islets, this apparent

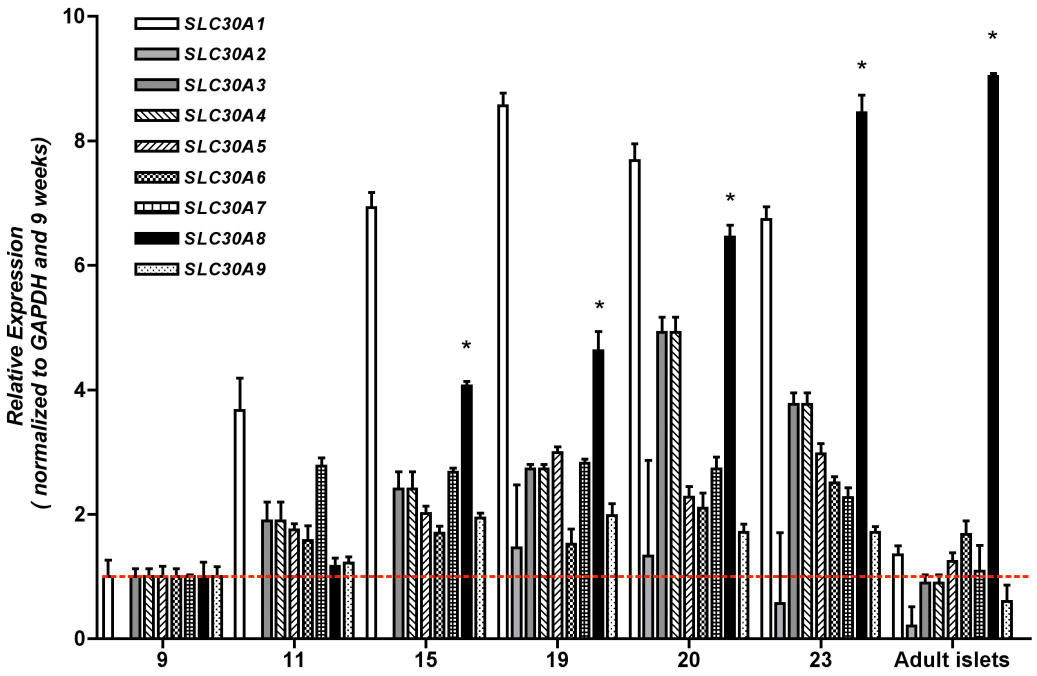


Figure 5.8. Normalized expression of selected *SLC30* genes by quantitative RT-PCR in 9-23 week old human fetal pancreas and adult human islets. Data in triplicate (mean ± S.E.M.) is normalized to endogenous *GAPDH* and quantified and expressed relative to 9 week old fetal samples. *SLC30A2* expression was detectable in the fetal pancreas from 19 weeks. *p<0.05 from 9 weeks for *SLC30A8* (black bars).

discrepancy more likely simply reflects the fact that the decrease in fasting insulin in male *Slc30a8* KO mice on a mixed genetic background is much greater than seen in fasting C57BL/6J female *Slc30a8* KO mice (50% vs 20%; Ref. [247] and Table 5.1). Therefore many more isolated islet preparations would have to be studied to uncover the expected small change in GSIS, which is not feasible given the inherent variability between isolated islet preparations. Most strikingly, no change in glucose tolerance was observed in male or female *Slc30a8* KO mice on either a mixed [247] or pure (Figs. 5.1 & 5.3) genetic background. Overall these results suggest a minor role for *Slc30a8* and β cell zinc in the regulation of glucose metabolism. The small alteration in proinsulin secretion in male *Slc30a8* KO mice does suggest a role for zinc in insulin processing (Table 5.1), though even this effect is also influenced by genetic background (Fig. 5.5).

The conclusion from this study that ZnT-8 plays a minor role in the regulation of glucose metabolism is consistent with our previous conclusion [247] and that of Lemaire et al. [244]. In contrast, Nicholson et al. [194] observed an impairment in glucose tolerance and insulin secretion in *Slc30a8* KO mice during IPGTTs. All these studies were performed in mice with a global *Slc30a8* deletion on a mixed genetic background. In more recent studies in which the *Slc30a8* gene was deleted specifically in beta cells, the same group observed a more mild impairment in glucose tolerance during OGTTs but this was associated with no impairment of insulin secretion [243]. In humans the rs13266634 polymorphism in the *SLC30A8* gene has been linked to not only type 2 diabetes but also impaired glucose tolerance [193], impaired proinsulin to insulin conversion [240] and reduced first phase insulin secretion [249]. It is therefore apparent that a change in ZnT-8 function in humans leads to an increased susceptibility to the

development of impaired β-cell function, making it easy to envisage how a functionally impaired β-cell would be more prone to the action of other environmental factors that promote β-cell failure and lead to type 2 diabetes [250]. In contrast, overall, the studies of ZnT-8 function in mice fail to clearly establish why this protein would be associated with altered susceptibility to the development of type 2 diabetes. There is clear evidence for altered zinc levels [194, 243, 244, 247], GSIS [243, 247] and proinsulin conversion (Fig. 5.5; Ref. [243]) in these mouse models but the effects are influenced by gender and modifier genes and there is no consistent change in glucose tolerance. Potential explanations for this difference between the human and mouse data include the possibility that ZnT-8 is more important for islet function in humans than mice or that mice can more readily compensate for changes in ZnT-8 expression. With respect to the latter, future studies in which *Slc30a8* is deleted in adult mice rather than during development might be informative as might studies in older mice. In contrast to many mouse models [251], high fat feeding does not appear to unmask a diabetic phenotype in *Slc30a8* KO mice, though only 2 mice were studied [194].

The physiological basis for observed reduction in fasting insulin levels in female mice on the pure C57BL/6J background (Table 5.1), and both male and female mice on the mixed genetic background [247], without a concommitant change in fasting glucose levels remains unknown. This phenomenon does not appear to be explained by altered insulin sensitivity (Fig. 5.6) or a major change in proinsulin secretion (Table 5.1). The results do provide weak evidence for an offsetting change in glucagon secretion. Thus, though no statistically significant changes in fasting glucagon levels were observed in male or female C57BL/6J *Slc30a8* KO mice, female C57BL/6J *Slc30a8* KO mice do

display a trend toward reduced glucagon secretion (Table 5.1). Furthermore, this trend is also observed in male mice on a mixed genetic background and reaches significance in the comparison of KO and heterozygous mice, presumably due to the larger sample size [247]. This might suggest that our inability to detect a difference in fasting glucagon levels in the aforementioned groups may be due to a lack of power. The hypothesis that glucagon secretion is impaired in *Slc30a8* KO mice would be consistent with the demonstration that ZnT-8 is expressed in α cells [247]. In addition, it has been previously shown that the zinc released from the insulin hexamers following insulin release by the β cell can affect glucagon secretion by the α cell, though the mechanism by which it does this is disputed [132, 177, 242]. This suggests that deletion of *Slc30a8* could affect glucagon secretion from α-cells by both direct and indirect mechanisms. However, in fasting female *Slc30a8* KO mice on a mixed genetic background there is no evidence for a trend toward reduced glucagon secretion despite the clear reduction in fasting insulin [247], which implies that altered glucagon secretion cannot explain the normal fasting glucose levels in the presence of reduced fasting insulin levels.

In vitro studies have suggested that the rs13266634 non-synonymous SNP within the *SLC30A8* gene, which results in an arginine to tryptophan conversion at the C-terminus of the protein, alters the ZnT-8 dimer interface and thereby affects zinc uptake and accumulation into granules [194]. Cells transfected with a ZnT-8 expression vector encoding the tryptophan variant show a reduction in zinc uptake and consequent granular zinc levels as compared to cells transfected with a ZnT-8 expression vector encoding the arginine variant [194]. Furthermore, deletion of *Slc30a8* results in a marked reduction in islet zinc content [194, 243, 244, 247]. Given the postulated critical role of zinc in

insulin secretion [243], it is logical that SNPs within the *SLC30A8* gene would be associated with impaired β-cell function. It was surprising, therefore, given the decrease in zinc observed in *Slc30a8* KO islets, that the observed phenotype in both our study and in other reports was mild and did not result in significant perturbations in whole body glucose metabolism. Consistent with observations in *Slc30a8* KO mice *in vivo*, no significant differences between the two ZnT-8 variants described above were observed in tissue culture studies with respect to their affect on GSIS, despite significant differences in their effects on granular zinc levels [194].

Overall, these observations suggest that the complete absence of ZnT-8 is not enough to lead to impaired glucose tolerance in mice. It is conceivable, however, that additional disturbances are required to unmask a more significant phenotype in *Slc30a8* KO mice, such as the presence of mutations in other type 2 diabetes-associated genes or zinc-deficient diets. Future studies will address these possibilities. In addition, the results suggest that either very low levels of zinc are sufficient for proper insulin crystallization and secretion or that zinc is not as important for insulin secretion as previously hypothesized. Indeed, guinea-pig insulin lacks a histidine residue in the B10 position of the molecule, which normally binds zinc in hexamer formation, and the zinc content of guinea pig islets is very low compared to mice and rats [167].

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Thesis Summary

This dissertation describes experiments that provide insight into the roles of both G6pc2 and ZnT-8 *in vivo*. Both the *G6PC2* and *SLC30A8* genes had been previously identified in GWA studies to be associated with FPG levels and type 2 diabetes, respectively. Thus, our studies sought to provide evidence that the identified SNPs affect the gene in which they are located, as opposed to affecting expression of a neighboring gene, as well as to better understand how changes in expression of these genes would result in altered susceptibility to elevated FPG levels and type 2 diabetes. Using static islet incubations, IPGTTs and pancreas perfusion studies, we demonstrated that deletion of *G6pc2* on the pure C57BL/6J genetic background results in a leftward shift in the dose-response curve of GSIS, resulting in enhanced insulin secretion at submaximal glucose concentrations. Surprisingly, however, we also demonstrated that the phenotype appears to be more complex at higher glucose concentrations. *G6pc2* KO mice displayed impaired glucose tolerance, perhaps as a result of impaired calcium resequestration in the ER. In addition, while high fat feeding did exacerbate the phenotype, this effect was both genetic background and gender dependent.

Both male and female *Slc30a8* KO mice display reductions in fasting plasma insulin levels with no change in FBG concentrations on the mixed C57BL/6J X 129SvEv genetic background. Male mice also display impaired GSIS during static islet

incubations but have normal glucose tolerance during an IPGTT. We sought to clarify this phenotype further on the C57BL/6J genetic background. In this case, female, but not male, mice display reduced fasting plasma insulin levels with no change in FBG. Female *Slc30a8* KO mice, however, exhibited normal glucose tolerance following either an IPGTT or an OGTT and islets isolated from these mice showed no change in GSIS in static islet incubations. The female mice do, however, display reductions in proinsulin levels, indicating that these mice possess an insulin processing defect.

In summary, the studies described here attempted to provide insight into how two genes identified in the GWA studies, *G6PC2* and *SLC30A8*, may be contributing to FPG and type 2 diabetes, respectively. The data derived from the study of *G6pc2* KO mice strongly support a role for G6pc2 in the regulation of FBG. In contrast, the data derived from the study of *Slc30a8* KO mice revealed that, depending on the genetic background, insulin secretion can be unaffected despite a major reduction in granule zinc content. Furthermore, these KO mouse studies did not uncover the reason why the *SLC30A8* gene is associated with risk for the development of type 2 diabetes.

Though the phenotype of the *G6pc2* KO mice is consistent with the results of the GWA studies, the *Slc30a8* KO mouse phenotype differs from what would be expected based on observations in humans. This may be explained by the fact that the GWA study approach only indicates altered susceptibility to the given condition. Thus, the variant identified within the *SLC30A8* gene may not individually lead to impaired β-cell function. Rather, it is likely that a combination of additional risk variants and environmental components may play an important role in the contribution of rs13266634 to type 2 diabetes. This may, therefore, explain the discrepancy between the human

GWA studies and the observed phenotype in the *Slc30a8* KO mice. Future studies will address this possilibity. Alternatively, it is also possible that rather than merely serving to alter *SLC30A8* function, the rs13266634 risk variant may instead result in protein misfolding and, subsequently, ER stress.

Further studies to elucidate the role of *G6pc2*

These studies have begun to clarify the biological role of both proteins; however, a number of additional studies could be performed to further clarify their function *in vivo*. Though initial attempts by both our laboratory [49] and by Chou and colleagues [252] to demonstrate glucose-6-phosphatase activity of G6PC2 and G6pc2 were unsuccessful, Petrolonis and colleagues were able to successfully demonstrate that G6PC2 could hydrolyze G6P [45]. In collaboration with John Hutton, we have since modified the assay conditions and have demonstrated that both G6PC2 and G6pc2 display hydrolytic activity (Fig. 1.2). Thus, using these novel conditions in which cells are mildly permeabilized, it will be important, first and foremost, to demonstrate that while we can detect glucose-6-phosphatase activity in islets isolated from WT mice, glucose-6-phosphatase activity is either reduced or absent in islets isolated from *G6pc2* KO mice.

In addition to demonstrating altered glucose-6-phosphatase activity in islets isolated from WT and KO mice, the use of WT and KO islets will definitively answer whether G6pc2 is responsible for glucose cycling in the islet. In collaboration with Dr. Jamey Young we can use a stable isotope strategy [253] to more accurately assess whether there is a decrease in glucose cycling in the knockout islets.

Finally, to confirm the aforementioned studies as well as the data described in Chapter III, it will be important to measure additional parameters in isolated islets such as G6P, ATP and NAD(P)H. Because overexpression of glucokinase has been shown to result in increased intracellular levels of both G6P and ATP along with a leftward shift in the $S_{0.5}$ of GSIS [254, 255] and because deletion of *G6pc2* results in a similar leftward shift in the $S_{0.5}$, we would expect to observe elevated G6P and ATP levels in the *G6pc2* KO mouse islets. Loss of G6pc2 would be predicted to leave phosphorylation of glucose unopposed and would eliminate the futile cycle and, therefore, the consumption of ATP. Furthermore, intracellular NAD(P)H levels would be predicted to be elevated in *G6pc2* KO mouse islets due to increased glucose metabolism, changing in parallel with enhanced insulin secretion at low glucose concentrations.

We have also considered the possibility that loss of *G6pc2* during development results in compensatory responses such that the effect of the deletion is minimized. For this reason, it will be interesting to perform similar experiments to those described in Chapter III using an inducible gene targeting approach in the mouse with the Cre/loxP system in order to remove *G6pc2* during adulthood.

The role of G6pc2 during exercise: preliminary data and future directions

Although initially deletion of *G6pc2* had appeared beneficial and thus the evolutionary conservation of G6pc2 function had been unclear, the importance of G6pc2 function became apparent at high glucose levels. Our data suggest that G6pc2 activity may be necessary for the resequestration of ER calcium following insulin secretion. However, we questioned whether G6pc2 had any additional functions in different

settings. We hypothesized the G6pc2 activity may be important under conditions in which insulin secretion needs to be rapidly shut off, such as during the "fight-or-flight" response. Thus, we performed preliminary exercise studies on the mice at various running speeds to determine whether we could detect a defect in the ability of the *G6pc2* KO mice to exercise. During exercise, various neural inputs are activated which lead to the inhibition of insulin secretion possibly through mechanisms involving hyperpolarization of the islet β-cell membrane or by inhibition of the exocytotic machinery [256, 257]. If these neural inputs were able to regulate the glucokinase/G6pc2 futile cycle, however, this would present a novel mechanism by which insulin secretion is shut off during exercise. Though no obvious defect was apparent at lower running speeds (Fig. 6.1A,B), *G6pc2* KO mice become severely hypoglycemic at faster speeds (Fig. 6.1C,D). Future studies will follow up on these results with a larger cohort of mice. In addition, the mechanism by which exercise conditions could modulate the G6pc2 protein will be investigated. A first step will be to measure GSIS in islets isolated from both WT and KO mice following adrenergic stimulation.

To further address these preliminary findings, we have developed a novel assay to look at putative phosphorylation sites (Fig. 6.2) regulated either through the neural circuitry during exercise or by other unknown mechanisms under alternative conditions. The assay is based on the observation that in the glucose responsive rat 832/13 insulinoma cell line, which lacks endogenous G6pc2 [49], expression of a transiently transfected rat *G6PC-luciferase* fusion gene is stimulated in the presence of glucose in the media [258]. Addition of a G6pc2 expression vector, however, blunts this response, presumably due to its ability to reduce G6P levels, thus inhibiting the signaling pathway

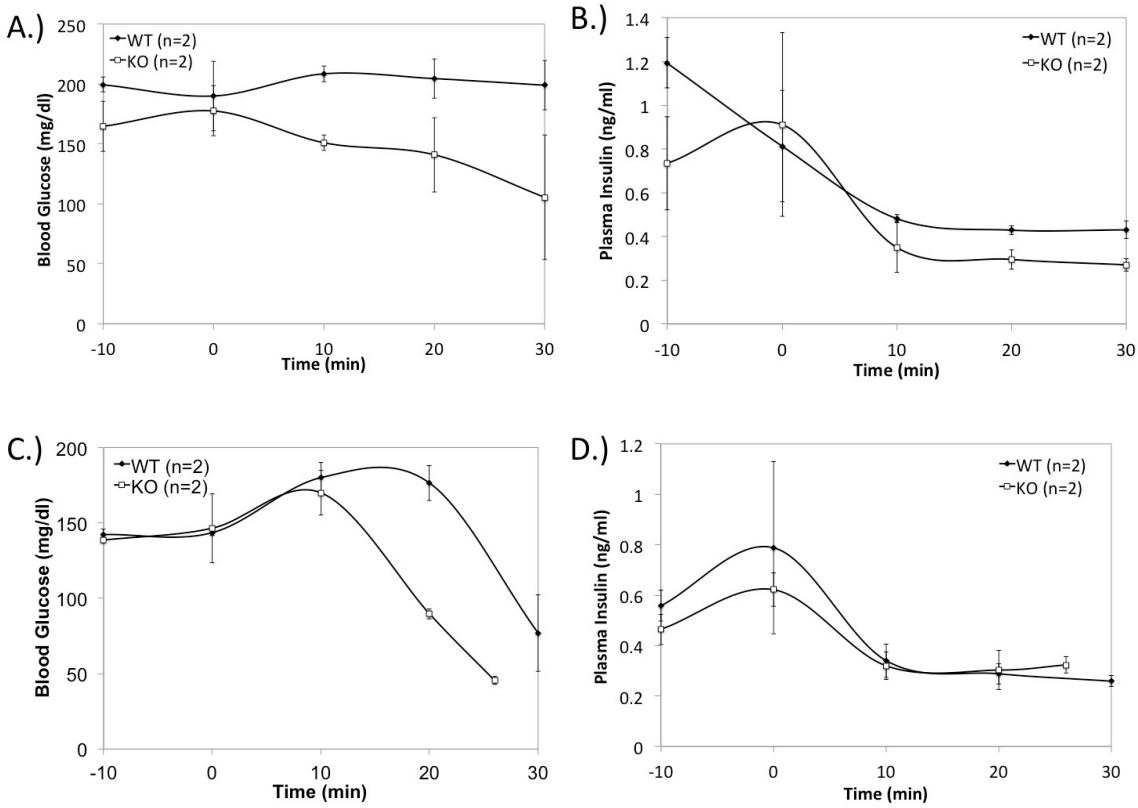


Figure 6.1. Analysis of exercise tolerance in *G6pc2* KO mice. Arterial blood glucose (A and C) and plasma insulin (B and D) during exercise in 13 week old male wild type (WT, closed diamonds) and knockout (KO, open squares) mice. Following a 5 hour fast, chronically catheterized mice performed 30 min of running at 14 (A and B) or 18 (C and D) m/min on a motorized treadmill and arterial blood was sampled at the indicated time points. Data are expressed as means ± S.E.M.

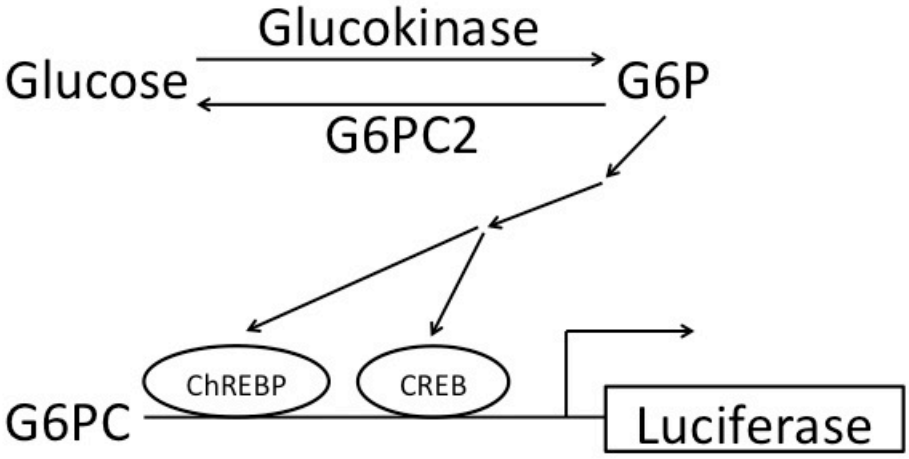


Figure 6.2. A novel transcriptional assay to assess G6PC2 enzyme activity in intact insulinoma cells. In the glucose-responsive 832/13 cell line, the rat *G6pc* promoter is activated by glucose in the media through transcription factors ChREBP and CREB. In the presence of G6pc2, however, G6P levels are reduced and glucose-stimulated *G6pc-luciferase* fusion gene expression is blunted.

that stimulates *G6PC-luciferase* expression. We have begun to mutate serine and threonine residues conserved in both G6pc and G6pc3 and located on the cytoplasmic domain of the protein to either alanine (preventing phosphorylation) or glutamate and aspartate (constitutively active phosphomimetics). Preliminary analyses indicate that mutation of residues 142, 203, 281, and 354 to alanine results in inactivation of the G6pc2 protein (Fig. 6.3). Mutation of residues 142, 203, and 281 to the phosphomimetic glutamate, however, also inactivated the protein (Fig. 6.4). It is possible that glutamate is not acting as a true phosphomimetic in this case. Alternatively, the mutation may be altering the structure and thus disrupting the catalytic activity of the protein. Future studies will also look at mutation of this site to the alternative phosphomimetic aspartate. Mutation of residue 354 to glutamate did result in a small activation of the protein suggesting that this may be a potential site of phosphorylation (Fig. 6.4). Additional putative sites of phosphorylation will also be investigated. Furthermore, we can investigate whether the mutants affect the K_M and/or the V_{MAX} of G6P hydrolysis by using varying G6P concentrations as previously described [44].

The effect of a high fat diet on the phenotype of G6pc2 *KO mice: preliminary data and future directions*

The G6pc2 protein contains a phosphatidic acid phosphatase domain that is conserved among the other two G6PC family members. It has been previously shown that lipid products of phosphatidylinositol 3-kinase activity as well as unsaturated fatty acids and fatty acyl-CoA esters can inhibit G6pc activity [259]. Thus, we hypothesized that lipid products derived from high fat feeding may similarly inhibit G6pc2 activity and

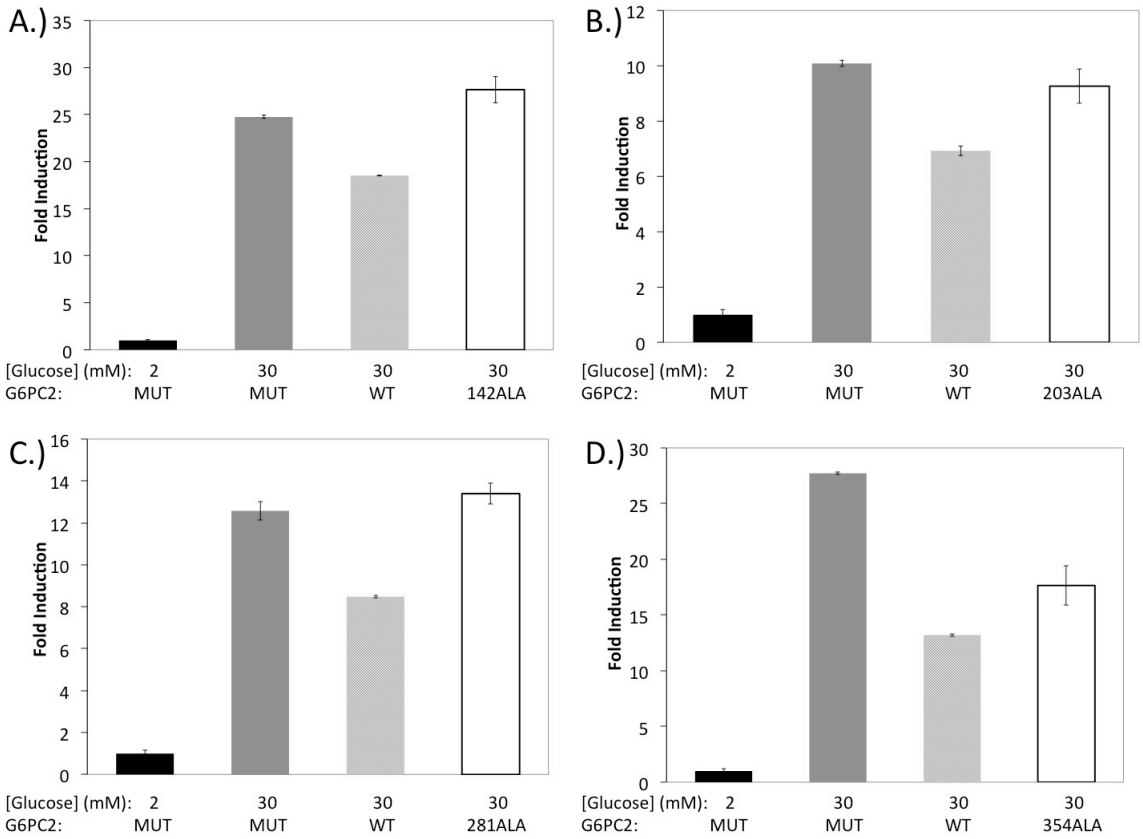


Figure 6.3. Analysis of the effect of alanine mutation on putative phosphorylation sites in G6pc2. 832/13 insulinoma cells were transiently transfected with a *G6pc*-firefly *luciferase* fusion gene and expression vectors encoding *Renilla* luciferase and either WT, catalytically inactive (MUT) or a mutated form of G6pc2 in which the indicated amino acid had been switched to alanine. Because overexpression of G6pc2 has been shown to result in ER stress, the catalytically inactive G6pc2 is used to control for this possibility. Following transfection, cells were incubated for 18-20 hours in serum-free medium containing 30mM glucose (unless otherwise specified). Cells were harvested and luciferase activity was assessed. Results are displayed as means ± S.E.M. of a single experiment using three independent preparations of each G6pc2 variant.

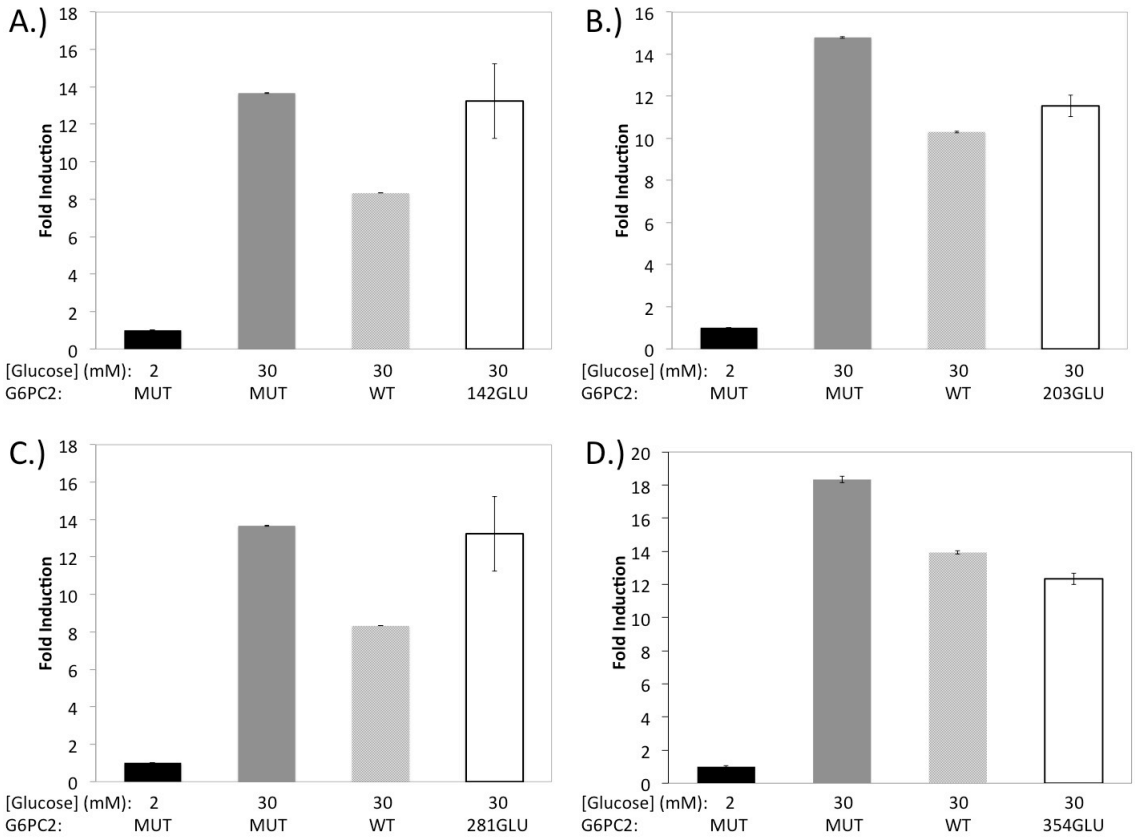


Figure 6.4. Analysis of the effect of glutamate mutation on putative phosphorylation sites in the G6pc2 protein. 832/13 insulinoma cells were transiently transfected with a *G6pc*-firefly *luciferase* fusion gene and expression vectors encoding *Renilla* luciferase and either WT, catalytically inactive (MUT) or a mutated form of G6pc2 in which the indicated amino acid had been switched to glutamate. Because overexpression of G6pc2 has been shown to result in ER stress, the catalytically inactive G6pc2 is used to control for this possibility. Following transfection, cells were incubated for 18-20 hours in serum-free medium containing 30mM glucose (unless otherwise specified). Cells were harvested and luciferase activity was assessed. Results are displayed as means ± S.E.M. of a single experiment using three independent preparations of each G6pc2 variant.

thus blunt/negate the observed differences in fasting blood glucose between WT and KO mice.

Table 6.1 shows that the difference in FBG between male WT and KO mice on a mixed, C57BL/6J x 129SvEv, genetic background is surprisingly accentuated by high fat feeding. While this difference in FBG might initially be caused by an unexpected activation of G6pc2 or stimulation of G6pc2 expression by lipids, the data show a surprising difference in body weight between WT and KO mice. This weight difference is presumably associated with a secondary difference in insulin sensitivity, consistent with the difference in fasting plasma insulin levels, that may further accentuate a difference in FBG. The differences in body weight were gender specific; they were not seen between high fat fed female WT and KO mice and the difference in FBG was not statistically significant.

Interestingly, when the study was repeated with mice on a pure, C57BL/6J, genetic background, the opposite result was observed. There was no difference in weight between high fat fed male WT and KO mice and the difference in FBG (Table 6.2) was similar to that observed in chow fed mice (Table 3.1). In contrast, a difference in weight was observed between high fat fed female WT and KO mice though, as with males, the difference in FBG (Table 6.2) was similar to that observed in chow fed mice (Table 3.1).

Overall, these observations suggest that high fat feeding does not activate G6pc2 since a consistent accentuation of the difference in FBG would have been expected regardless of gender or genetic background. Rather the data suggest that G6pc2 can influence the magnitude of diet-induced obesity but in a manner that is gender and genetic background dependent. These observations do leave open the possibility that

Table 6.1. Phenotypic characterization of high fat fed *G6pc2* KO mice on a C57BL/6J x 129SvEv genetic background. At 21 weeks of age, after 13 weeks on a high fat diet mice were fasted for 5 hours and then weighed. Mice were anesthetized 1 hour later, their length was measured and blood isolated. Blood glucose and plasma cholesterol, triacylglycerol, glycerol, insulin and glucagon levels were determined as described in Chapter II. Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. WT=wild type; -/+=heterozygous; KO=knockout.

Gender & Genotype	Weight (g)	Length (mm)	Glucose (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	Glycerol (mg/dl)	Insulin (ng/ml)
Female WT	26.7 ± 1.6 (8)	100.4 ± 0.8 (8)	132.1 ± 10.8 (8)	97.3 ± 7.0 (8)	46.3± 3.8 (8)	3.1 ± 0.2 (8)	0.44 ± 0.14 (7)
Female -/+	26.5 ± 1.3 (11)	100.5 ± 1.0 (11)	136.1 ± 5.2 (10)	100.5 ± 5.0 (11)	40.3 ± 2.7 (11)	2.8 ± 0.2 (11)	0.43 ± 0.05 (11)
Female KO	27.4 ± 2.1 (8)	99.9 ± 0.6 (7)	125.3 ± 6.3 (8)	100.0 ± 4.8 (7)	48.6 ± 4.4 (8)	3.4 ± 0.4 (8)	0.60 ± 0.22 (8)
Male WT	40.6 ± 0.9 (12)	103.9 ± 0.8 (12)	218.6 ± 13.1 (12)	160.6 ± 9.0 (11)	58.7 ± 2.9 (10)	3.2 ± 0.3 (11)	3.15 ± 0.35 (11)
Male -/+	35.7 ± 0.8 (23) *	102.7± 0.5 (23)	165.2 ± 6.4 (23) *	133.3 ± 4.9 (22) *	61.1 ± 2.2 (22)	3.0 ± 0.1 (23)	1.43 ± 0.19 (23) *
Male KO	33.9 ± 1.1 (12) **	102.2 ± 0.7 (12)	137.8 ± 9.8 (12) **,***	114.1 ± 5.2 (11) **,***	54.7 ± 3.1 (11)	2.6 ± 0.2 (12)	1.28 ± 0.21 (12) **

1. Weight: *p<0.001 for male WT v Het, **p<0.0001 for male WT v KO
2. Glucose: *p<0.001 for male WT v Het, **p<0.05 for male Het v KO, ***p<0.0001 for male WT v KO
3. Cholesterol: *p<0.01 for male WT v Het, **p<0.05 for male Het v KO, ***p<0.001 for male WT v KO
4. Insulin: *p<0.0001 for male WT v Het, **p<0.0001 for male WT v KO

Table 6.2. Phenotypic characterization of high fat fed *G6pc2* KO mice on a C57BL/6J genetic background. At 21 weeks of age, after 13 weeks on a high fat diet mice were fasted for 5 hours and then weighed. Mice were anesthetized 1 hour later, their length was measured and blood isolated. Blood glucose and plasma cholesterol, triacylglycerol, glycerol, insulin and glucagon levels were determined as described in Chapter II. Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. WT=wild type; -/+=heterozygous; KO=knockout.

Gender & Genotype	Weight (g)	Length (mm)	Glucose (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mmol/l)	Glycerol (mmol/l)	Insulin (ng/ml)
Female WT	35.1 ± 1.0 (17)	101.4 ± 0.3 (18)	151.7 ± 4.6 (18)	129.9 ± 3.9 (14)	48.5 ± 2.6 (15)	3.6 ± 0.2 (15)	0.72 ± 0.37 (2)
Female -/+	34.3 ± 1.3 (30)	101.6 ± 0.3 (28)	150.4 ± 4.2 (30)	125.3 ± 4.2 (28)	50.2 ± 1.4 (26)	3.8 ± 0.1 (27)	1.08 ± 0.14 (11)
Female KO	30.4 ± 1.1 (23) *, **	100.8 ± 0.2 (23)	130.0 ± 3.3 (24) *, **	118.8 ± 3.6 (23) *	50.0 ± 1.6 (22)	3.6 ± 0.1 (23)	0.76 ± 0.17 (7)
Male WT	47.7 ± 0.5 (24)	105.4 ± 0.3 (24)	225.2 ± 7.4 (24)	196.5 ± 6.6 (13)	56.9 ± 2.7 (13)	3.0 ± 0.2 (12)	3.82 ± 0.61 (6)
Male -/+	47.3 ± 0.4 (37)	104.9 ± 0.3 (41)	211.7 ± 5.1 (40)	184.6 ± 2.4 (36) **	55.8 ± 1.0 (38)	3.3 ± 0.1 (37) *	5.30 ± 0.53 (10)
Male KO	48.0 ± 0.6 (17)	105.9 ± 0.3 (17) *	205.8 ± 5.9 (17) ***	179.5 ± 4.3 (7)	60.2 ± 4.8 (7)	3.5 ± 0.3 (7)	6.33 ± 1.44 (3)

1. Weight: *p<0.05 for female Het v KO, **p<0.05 for female WT v KO
2. Length: *p<0.05 for male Het v KO
3. Glucose: *p<0.001 for female Het v KO, **p<0.001 for female WT v KO, ***p=0.056 for male WT v KO
4. Cholesterol *p<0.05 for female WT v KO, **p<0.05 for male WT v Het
5. Glycerol: *p<0.05 for male WT v Het.

high fat feeding stimulates G6pc2 expression but in a manner that depends on strain-specific modifier genes or promoter differences.

The small difference observed in weight in female chow fed C57BL/6J mice (Table 3.1) and larger difference in high fat fed mice (Table 6.2) suggests that in this context the effect of G6pc2 on body weight is accentuated by, but is not dependent on, the presence of high fat feeding. Thus, additional studies were performed to gain insight into the mechanism whereby female C57BL/6J *G6pc2* KO mice are protected from diet-induced obesity.

Growth curves show that a difference in weight is already apparent in female mice after 3 weeks of high fat feeding (Fig. 6.5A) whereas no differences are detected in males (Fig. 6.5B). An analysis of body composition using NMR reveals that female G6pc2 KO mice have decreased fat mass and increased muscle mass, when expressed as a percentage of body weight, relative to WT mice (Table 6.3). Again no differences were detected in males (Table 6.3). Although female WT mice were 4.64 g heavier than KO mice after 12 weeks of high fat feeding (Table 6.2), calculations indicated that this would only amount to a 0.08 gram difference in daily food intake, a difference too small to measure given the inherent variability in food intake between mice. An analysis of food intake during the initial three weeks of high fat feeding, a time during which body weights have not significantly diverged (Fig. 6.5A), confirmed that a difference in food intake between female WT and KO mice could not be detected (Fig. 6.6).

A key experiment that remains is to perform an analysis of the effect of a high fat diet on the phenotype of *G6pc2* KO mice on a 129SvEv genetic background. Key differences were observed between the effect of the high fat diet on the mixed C57BL/6J

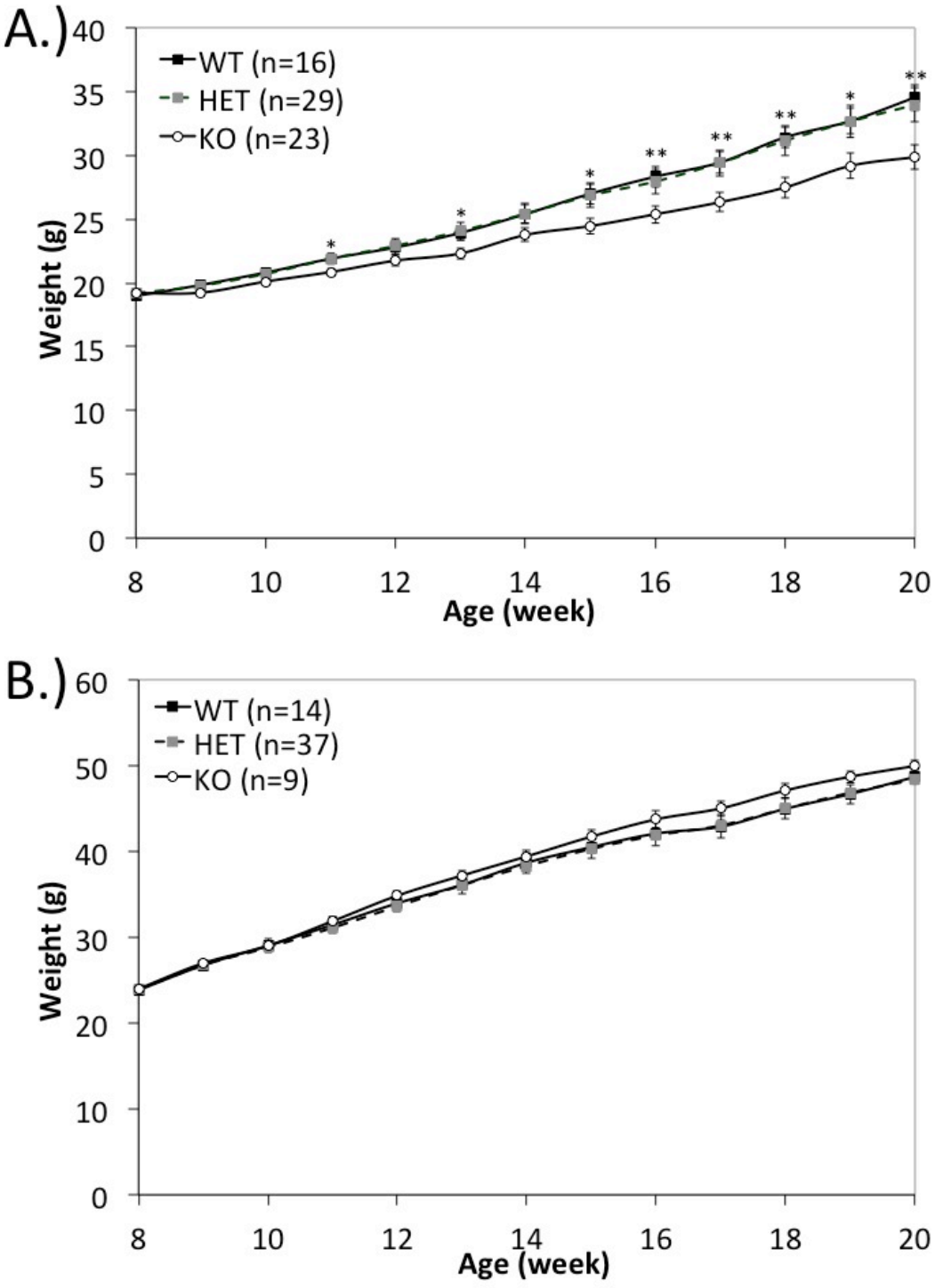


Figure 6.5. Analysis of the growth curves of high fat fed *G6pc2* KO mice on a C57BL/6J genetic background. A high fat diet (60% fat calories; Mouse Diet F3282; BioServ) study was initiated at 8 weeks of age and maintained for 12 weeks. Fed body weights were measured weekly in female (Panel A) and male (Panel B) wild type (WT), heterozygous (HET) and knockout (KO) animals. Results show the mean glucose concentrations ± S.E.M. *p<0.05; **p<0.01.

Table 6.3. NMR analysis of body composition of *G6pc2* KO mice on a C57BL/6J genetic background. Body composition was assessed using an mq10 NMR analyzer (Bruker Optics) on 6 hour fasted animals at 20 weeks of age following 12 weeks of high fat feeding (60% fat calories; Mouse Diet F3282; BioServ). Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. WT=wild type; -/+=heterozygous; KO=knockout.

Gender & Genotype	Fat (g)	Muscle (g)	Free Fluid (g)	Fat (%)	Muscle (%)	Water (%)
Female WT	13.0 ± 0.6 (14)	19.4 ± 0.2 (14)	0.64 ± 0.02 (14)	37.8 ± 1.1 (14)	56.9 ± 0.8 (14)	1.90 ± 0.09 (14)
Female -/+	11.9 ± 1.1 (29)	19.0 ± 0.3 (29)	0.59 ± 0.02 (29)	34.1 ± 2.2 (29)	59.9 ± 1.6 (29)	1.84 ± 0.06 (29)
Female K/O	10.2 ± 1.1 (24)	18.5 ± 0.2 (24) *	0.60 ± 0.02 (24)	31.2 ± 2.4 (24) *	61.7 ± 1.7 (24) *	1.99 ± 0.07 (24)
Male WT	20.6 ± 0.3 (11)	25.2± 0.4 (11)	0.70 ± 0.05 (11)	42.9 ± 0.5 (11)	52.3 ± 0.4 (11)	1.44 ± 0.09 (11)
Male -/+	20.1 ± 0.2 (33)	24.8 ± 0.2 (33)	0.74 ± 0.02 (33)	42.6 ± 0.3 (33)	52.5 ± 0.2 (33)	1.57 ± 0.05 (33)
Male K/O	20.9 ± 0.5 (9)	25.5 ± 0.3 (9) **	0.74 ± 0.04 (9)	42.8 ± 0.6 (9)	52.3 ± 0.4 (9)	1.52 ± 0.07 (9)

1. Muscle: *p<0.05 for female WT v KO, **p<0.05 for male het v KO
2. Fat %: *p<0.05 for female WT v KO
3. Muscle %: *p<0.05 for female WT v KO.

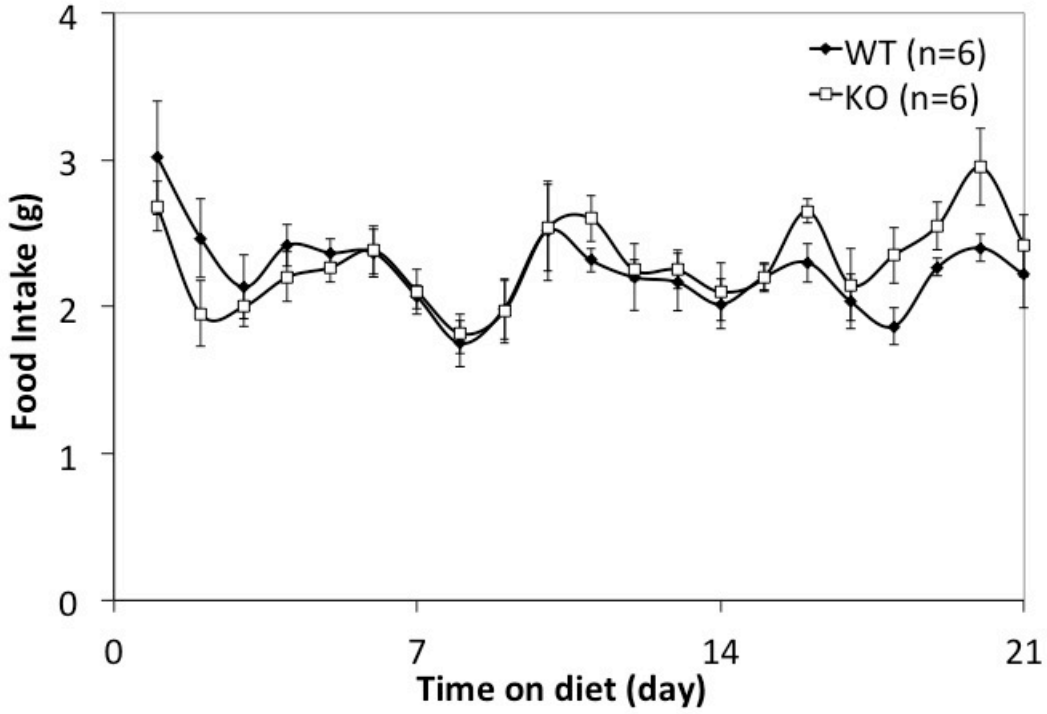


Figure 6.6. Analysis of food intake in female *G6pc2* KO mice on a C57BL/6J genetic background. At 8 weeks of age, individually housed females were given a high fat diet (60% fat calories; Mouse Diet F3282; BioServ) for three weeks. Food intake was measured daily. Results are means ± S.E.M. obtained from the number of animals indicated. WT=wild type; KO=knockout.

x 129SvEv genetic background (Table 6.1) and the pure C57BL/6J genetic background (Table 6.2). We have attributed these differences to both gender and the effect of modifier genes. An analysis of high fat feeding on the 129SvEv genetic background will help to elucidate how genetic background and modifier genes may have been playing a role in the differences we observed. In addition, we have observed a number of SNPs in the C57BL/6J and 129SvEv *G6pc2* promoter sequences. These polymorphisms may also be contributing to the differences observed between genetic backgrounds.

G6PC2 as a therapeutic drug target

The studies described here provide evidence that G6PC2 may be an intriguing drug target for lowering blood glucose levels in humans. The safe and effective long-term pharmacological treatment of type 2 diabetes has remained challenging. Recently, many pharmaceutical companies turned to glucokinase activators as a potential target [260]. Long thought to be the primary glucose sensor of the pancreatic β-cell, glucokinase performs the first step in glycolysis, the phosphorylation of glucose [232], and thus would oppose the action of G6PC2. Glucokinase is also present in the α- and δ-cells of the islet and in a number of other organs including the liver, enteroendocrine cells, hypothalamus, and gonadotropes and thyrotropes of the pituitary [261-263]. Inactivating mutations in glucokinase can result in MODY2 [264, 265] or permanent neonatal diabetes mellitus (PNDM) [266, 267] while activating mutations can lead to persistent hyperinsulinemic hypoglycemia in infancy (PHHI) [268-271]. The activating mutations of the latter occur within an allosteric activator site that has since been used as the target site of therapeutic glucokinase activators (GKA) [272]. All of the GKAs have

been shown to result in a lowered $S_{0.5}$ of GSIS, though the effect on the catalytic constant (K_{CAT}) and on the Hill coefficient (n_H) has been varied [260].

Significant limitations exist, however, when considering glucokinase as a drug target. Most importantly perhaps is the concern that increased insulin secretion and insulin action may result in hypoglycemia. As discussed above, activating mutations in glucokinase can cause PHHI [268-271]. Similarly, though some GKAs, such as Piragliatin, have been shown to be effective in lowering blood glucose in both healthy control subjects and patients with type 2 diabetes, they pose the risk of mild to moderate hypoglycemia [273]. Thus, careful dosing is critical to avoid hypoglycemia. Futhermore, glucokinase is widely expressed which allows for a greater possibility for negative side effects. The effect of the GKA on the various tissues where glucokinase is expressed must therefore be carefully assessed. Finally, glucokinase activation may lead to fatty-liver syndrome and hyperlipidemia. Recent GWA studies found polymorphisms within the *GKRP* gene to be associated with variations in serum triglyceride levels [274-276]. Because GKAs interfere with GKRP inhibition of glucokinase, it is reasonable that GKAs may similarly increase hepatic lipid storage and serum triglyceride levels due to increased flux through the glycolytic and TCA pathways and insulin stimulated lipid synthesis.

Similar to GKAs, it is reasonable to expect that G6PC2 could be a viable target given that it is expected to oppose the action of glucokinase and, as suggested by our data, also affect the $S_{0.5}$ of GSIS. Interestingly, using high throughput screening of a small molecule library, Petrolonis and colleagues were able to identify a specific inhibitor of G6PC2 that did not affect G6PC [45]. In addition, it is possible that G6PC2 may

actually be a superior target to glucokinase. Polymorphisms within the gene result in mild variations in blood glucose [8, 95, 216, 217] while deletion of *G6pc2* in mice results in a relatively mild phenotype and, despite the complete loss of the protein, glucose levels, though significantly lowered, are still within a normal range. This suggests that inhibition of the gene would not result in the hypoglycemia seen with inhibition of glucokinase. Furthermore, unlike glucokinase, G6PC2 has only been detected in the islet [44] and thus targeting G6PC2 may avoid some of the negative side effects that might occur with GKAs. One obvious limitation that deserves further investigation is the observation that at high glucose doses, the presence of G6pc2 appeared to be beneficial. In addition to this caveat, it is unclear what level of suppression that would be required to affect blood glucose levels *in vivo*. *G6pc2* heterozygous mice display normal glycemia, suggesting that G6pc2 may need to be suppressed greater than 50% in order to observe an effect. In humans, however, though G6PC2 levels have not been measured in patients with distinct *G6PC2* polymorphisms, studies performed *in vitro* suggested that, for example, the rs13431652 G allele which is associated with a 25% reduction in promoter activity is associated with a significant reduction in blood glucose [96].

In summary, if our hypothesis that G6PC2 is an important inhibitory component of the β-cell glucose sensor is correct, then suppression of G6PC2 will represent a novel therapy to lower blood glucose.

Further studies to elucidate the role of ZnT-8

Surprisingly, our data as well as studies performed by other laboratories have indicated that deletion of *Slc30a8* results in a relatively mild phenotype despite

significant loss of islet zinc and the postulated importance of zinc for proper insulin secretion [194, 243, 244, 247]. Though a nonsynonymous polymorphism within the *SLC30A8* gene was shown to be associated with increased risk of type 2 diabetes [9], diabetes is multifactorial and results from a combination of both genetic and environmental factors. Thus, future studies will address whether a more drastic phenotype could be unmasked following deletion of *Slc30a8* in combination with additional stresses such as deletion of another type 2 diabetes susceptibility gene (Table 1.2) or following high fat feeding. Interestingly, Nicolson and colleagues did analyze *Slc30a8* KO mice following 12 weeks of a high fat diet and observed an increase in body weight and increases in both fasting blood glucose and plasma insulin though they observed no significant impairments in glucose tolerance [194]. This study was limited, however, by very low sample sizes (n=2). It will be important to follow up on these studies with much larger cohorts, especially due to the variation in the chow-fed phenotypes observed between colonies both within this study [194] and compared to other studies [243, 244, 247].

In addition to the aforementioned studies, it will be interesting to determine whether a low zinc diet will exacerbate the observed phenotype. Though we were unable to detect islet zinc in the *Slc30a8* KO mice, the method we used only detects free and loosely bound zinc [247]. It is conceivable then that there are low levels of bound zinc that are sufficient for proper islet function. Thus, a zinc deficient diet, which has been previously been shown to result in impaired GSIS and exacerbate the db/db phenotype [173] may lower islet zinc levels below a critical threshold level. Furthermore, zinc

deficiency has previously been shown to exacerbate the phenotype of other zinc transporter knockout mice [277, 278].

One final key aspect that remains to be addressed is the transcriptional regulation of *SLC30A8* and whether SNPs located within regulatory regions of the gene may also impact type 2 diabetes susceptibility. Little is currently known about the regulation of *SLC30A8* gene expression. It has been previously demonstrated that treatment of either MIN6 cells or primary murine islets with inflammatory cytokines interleukin 1β (IL1β) and tumor necrosis factor α (TNFα) results in a reduction in *Slc30a8* mRNA expression, suggesting that ZnT-8 may contribute to changes in β-cell function under inflammatory conditions [279]. In addition, it has also been shown that in INS1E cells, but not in murine islets, 16 mM glucose down-regulates *Slc30a8* mRNA [280]. Furthermore, zinc depletion also caused a reduction in *Slc30a8* expression levels [280].

Our lab has investigated the transcriptional regulation of both human *SLC30A8* and mouse *Slc30a8* gene expression. We were unable to demonstrate promoter activity in transient transfections, however, the use of stable transfections demonstrated that the region spanning -6154 to -1 in humans and -1803 to -1 in mice, relative to the translation start site, shows promoter activity in βTC3 cells. Furthermore, in contrast to the studies suggesting that zinc regulates the mouse *Slc30a8* gene, expression of a human *SLC30A8* promoter-luciferase fusion gene was unaffected by zinc treatment following stable transfection in βTC3 cells. This suggests that zinc regulation of *SLC30A8* expression may occur through a different region, such as through one of the enhancers we identified in the gene (see below) or through a post-transcriptional mechanism [281].

In addition, studies performed in our lab have identified two highly conserved enhancer regions, designated A and B, located within introns 2 and 3 of the mouse *Slc30a8* and human *SLC30A8* genes [248]. While enhancer A appears to confer fusion gene expression selectively in β-cell lines, enhancer B confers fusion gene expression in both α- and β-cell lines [248]. Furthermore, we demonstrated that mutation of either of two Pdx1 binding sites within enhancer A significantly reduces enhancer activity and that both electrophoretic mobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP) assays confirm Pdx1 binding to the enhancer *in vitro* and *in situ*, respectively [248]. Similarly, the equivalent enhancer A region within the human *SLC30A8* intron 2 also represents a β-cell specific transcriptional enhancer [281].

We have also identified an additional SNP, rs62510556, within a conserved enhancer located in *SLC30A8* intron 2 [281]. Although we demonstrated that the polymorphism modulates enhancer activity, it was not found to be associated with type 2 diabetes in a French case-control cohort. Additional studies will be necessary to determine whether this SNP is associated with type 2 diabetes in other populations. A lack of association with type 2 diabetes would suggest that either rs62510556 does not change *SLC30A8* expression sufficiently *in vivo* to have a biological effect or, more interestingly, that the presence of a variant form of ZnT-8, as manifest by the rs13266634 SNP, increases type 2 diabetes risk in some populations whereas changes in *SLC30A8* expression do not. If correct, this latter possibility would imply that it is the presence of a mutant form of ZnT-8 that causes problems with β-cell function whereas the absence or reduction of ZnT-8 is less deleterious. Finally, the identification of other rare SNPs

located within *SLC30A8* regulatory regions will help to address whether polymorphisms that markedly affect gene expression are sufficient to alter diabetes susceptibility.

REFERENCES

1. Billings, L.K. and J.C. Florez, *The genetics of type 2 diabetes: what have we learned from GWAS?* Ann. N.Y. Acad. Sci., 2010. **1212**: p. 59-77.
2. Vaxillaire, M. and P. Froguel, *Genetic basis of maturity-onset diabetes of the young.* Endocrinol. Metab. Clin. North Am., 2006. **35**: p. 371-384.
3. Grant, S.F., et al., *Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes.* Nat Genet, 2006. **38**(3): p. 320-3.
4. Altshuler, D., et al., *The common PPARγ Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes.* Nature Genetics, 2000. **26**: p. 76-80.
5. Gloyn, A.L., et al., *Large-scale association studies of variants in genes encoding the pancreatic beta-cell KATP channel subunits Kir6.2 (KCNJ11) and SUR1 (ABCC8) confirm that the KCNJ11 E23K variant is associated with type 2 diabetes.* Diabetes, 2003. **52**(2): p. 568-72.
6. Florez, J.C., et al., *Haplotype structure and genotype-phenotype correlations of the sulfonylurea receptor and the islet ATP-sensitive potassium channel gene region.* Diabetes, 2004. **53**(1360-68).
7. Billings, L.K. and J.C. Florez, *The genetics of type 2 diabetes: what have we learned from GWAS?* Annals of the New York Academy of Sciences, 2010. **1212**: p. 59-77.
8. Bouatia-Naji, N., et al., *A polymorphism within the G6PC2 gene is associated with fasting plasma glucose levels.* Science, 2008. **320**(5879): p. 1085-8.
9. Sladek, R., et al., *A genome-wide association study identifies novel risk loci for type 2 diabetes.* Nature, 2007. **445**(7130): p. 881-5.
10. Cori, G.T., C.F. Cori, and G. Schmidt, *The role of glucose-1-phosphate in the formation of blood sugar and synthesis of glycogen in the liver.* J Biol Chem, 1939. **129**: p. 629-639.
11. Mithieux, G., *New knowledge regarding glucose-6 phosphatase gene and protein and their roles in the regulation of glucose metabolism.* European journal of endocrinology / European Federation of Endocrine Societies, 1997. **136**(2): p. 137-45.
12. Foster, J.D., B.A. Pederson, and R.C. Nordlie, *Glucose-6-phosphatase structure, regulation, and function: an update.* Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine, 1997. **215**(4): p. 314-32.
13. Lei, K.J., et al., *Mutations in the glucose-6-phosphatase gene that cause glycogen storage disease type 1a.* Science, 1993. **262**(5133): p. 580-3.
14. Shelly, L.L., et al., *Isolation of the gene for murine glucose-6-phosphatase, the enzyme deficient in glycogen storage disease type 1A.* The Journal of biological chemistry, 1993. **268**(29): p. 21482-5.
15. Gluecksohn-Waelsch, S., *Genetic control of morphogenetic and biochemical differentiation: lethal albino deletions in the mouse.* Cell, 1979. **16**(2): p. 225-37.
16. Ruppert, S., et al., *Two genetically defined trans-acting loci coordinately regulate overlapping sets of liver-specific genes.* Cell, 1990. **61**(5): p. 895-904.

17. Chatelain, F., et al., *Development and regulation of glucose-6-phosphatase gene expression in rat liver, intestine, and kidney: in vivo and in vitro studies in cultured fetal hepatocytes.* Diabetes, 1998. **47**(6): p. 882-9.
18. Lin, B., et al., *Cloning and characterization of cDNAs encoding a candidate glycogen storage disease type 1b protein in rodents.* The Journal of biological chemistry, 1998. **273**(48): p. 31656-60.
19. van de Werve, G., et al., *New lessons in the regulation of glucose metabolism taught by the glucose 6-phosphatase system.* Eur J Biochem, 2000. **267**(6): p. 1533-49.
20. Van Schaftingen, E. and I. Gerin, *The glucose-6-phosphatase system.* Biochem J, 2002. **362**(Pt 3): p. 513-32.
21. Puskas, F., et al., *Conformational change of the catalytic subunit of glucose-6-phosphatase in rat liver during the fetal-to-neonatal transition.* The Journal of biological chemistry, 1999. **274**(1): p. 117-22.
22. Gerin, I., et al., *Sequence of a putative glucose 6-phosphate translocase, mutated in glycogen storage disease type Ib [see comments].* FEBS Lett, 1997. **419**(2-3): p. 235-8.
23. Arion, W.J., et al., *The specificity of glucose 6-phosphatase of intact liver microsomes.* J. Biol. Chem., 1972. **247**: p. 2558-65.
24. Maloney, P.C., et al., *Anion-exchange mechanisms in bacteria.* Microbiol. Rev., 1990. **54**: p. 1-17.
25. Chen, S.Y., et al., *The glucose-6-phosphate transporter is a phosphate-linked antiporter deficient in glycogen storage disease type 1b and 1c.* FASEB J., 2008. **22**: p. 2206-2213.
26. Chou, J.Y. and B.C. Mansfield, *Molecular genetics of type I glycogen storage disease.* Trends Endocrinol Metabol, 1999. **10**: p. 104-113.
27. Veiga-da-Cunha, M., et al., *The putative glucose 6-phosphate translocase gene is mutated in essentially all cases of glycogen storage disease type I non-a.* Eur J Hum Genet, 1999. **7**(6): p. 717-23.
28. Mithieux, G., et al., *Glucose-6-phosphatase mRNA and activity are increased to the same extent in kidney and liver of diabetic rats.* Diabetes, 1996. **45**(7): p. 891-6.
29. Li, Y., M.C. Mechin, and G. van de Werve, *Diabetes affects similarly the catalytic subunit and putative glucose-6- phosphate translocase of glucose-6-phosphatase.* J Biol Chem, 1999. **274**(48): p. 33866-8.
30. Ashmore, J., A.B. Hastings, and F.B. Nesbett, *The effect of diabetes and fasting on liver glucose-6-phosphatase.* Proc Natl Acad Sci U S A, 1954. **40**: p. 673-678.
31. Streeper, R.S., et al., *A multicomponent insulin response sequence mediates a strong repression of mouse glucose-6-phosphatase gene transcription by insulin.* J Biol Chem, 1997. **272**(18): p. 11698-701.
32. Barthel, A., et al., *Differential regulation of endogenous glucose-6-phosphatase and phosphoenolpyruvate carboxykinase gene expression by the forkhead transcription factor FKHR in H4IIE-hepatoma cells.* Biochem Biophys Res Commun, 2001. **285**(4): p. 897-902.
33. Schmoll, D., et al., *Regulation of Glucose-6-phosphatase Gene Expression by Protein Kinase Balpha and the Forkhead Transcription Factor FKHR.*

EVIDENCE FOR INSULIN RESPONSE UNIT-DEPENDENT AND -INDEPENDENT EFFECTS OF INSULIN ON PROMOTER ACTIVITY. J Biol Chem, 2000. **275**(46): p. 36324-36333.
34. Streeper, R.S., et al., *Hepatocyte nuclear factor-1 acts as an accessory factor to enhance the inhibitory action of insulin on mouse glucose-6-phosphatase gene transcription.* Proc Natl Acad Sci U S A, 1998. **95**(16): p. 9208-13.
35. Vander Kooi, B.T., et al., *The Three insulin response sequences in the glucose-6-phosphatase catalytic subunit gene promoter are functionally distinct.* J Biol Chem, 2003. **278**(14): p. 11782-93.
36. Tang, E.D., et al., *Negative regulation of the forkhead transcription factor FKHR by Akt.* J Biol Chem, 1999. **274**(24): p. 16741-6.
37. Nakae, J., B.C. Park, and D. Accili, *Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway.* J Biol Chem, 1999. **274**(23): p. 15982-5.
38. Rena, G., et al., *Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B.* J Biol Chem, 1999. **274**(24): p. 17179-83.
39. Guo, S., et al., *Phosphorylation of serine 256 by protein kinase B disrupts transactivation by FKHR and mediates effects of insulin on insulin-like growth factor-binding protein-1 promoter activity through a conserved insulin response sequence.* J Biol Chem, 1999. **274**(24): p. 17184-92.
40. Brunet, A., et al., *Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.* Cell, 1999. **96**(6): p. 857-68.
41. Biggs, W.H., 3rd, et al., *Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1.* Proc Natl Acad Sci U S A, 1999. **96**(13): p. 7421-6.
42. Kops, G.J., et al., *Direct control of the Forkhead transcription factor AFX by protein kinase B.* Nature, 1999. **398**(6728): p. 630-4.
43. Takaishi, H., et al., *Regulation of nuclear translocation of forkhead transcription factor AFX by protein kinase B.* Proc Natl Acad Sci U S A, 1999. **96**(21): p. 11836-41.
44. Arden, S.D., et al., *Molecular cloning of a pancreatic islet-specific glucose-6-phosphatase catalytic subunit-related protein.* Diabetes, 1999. **48**(3): p. 531-42.
45. Petrolonis, A.J., et al., *Enzymatic characterization of the pancreatic islet-specific glucose-6-phosphatase-related protein (IGRP).* J Biol Chem, 2004. **279**: p. 13976-13983.
46. Boustead, J.N., et al., *Identification and characterization of a cDNA and the gene encoding the mouse ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein.* J Mol Endocrinol, 2004. **32**(1): p. 33-53.
47. Hutton, J.C. and R.M. O'Brien, *Glucose-6-phosphatase catalytic subunit gene family.* The Journal of biological chemistry, 2009. **284**(43): p. 29241-5.
48. Martin, C.C., et al., *Identification and Characterization of a Human cDNA and Gene Encoding a Ubiquitously Expressed Glucose-6-Phosphatase Catalytic Subunit-Related Protein.* J Mol Endocrinol, 2002. **29**: p. 205-22.

49. Martin, C.C., et al., *Cloning and Characterization of the Human and Rat Islet-Specific Glucose-6-Phosphatase Catalytic Subunit-Related Protein (IGRP) Genes.* J Biol Chem, 2001. **276**(27): p. 25197-207.
50. Shieh, J.J., et al., *The islet-specific glucose-6-phosphatase-related protein, implicated in diabetes, is a glycoprotein embedded in the endoplasmic reticulum membrane.* FEBS Lett, 2004. **562**(1-3): p. 160-4.
51. Guionie, O., et al., *Identification and characterisation of a new human glucose-6-phosphatase isoform.* FEBS Lett, 2003. **551**(1-3): p. 159-64.
52. Shieh, J.J., et al., *A glucose-6-phosphate hydrolase, widely expressed outside the liver, can explain age-dependent resolution of hypoglycemia in glycogen storage disease type Ia.* J Biol Chem, 2003. **278**(47): p. 47098-103.
53. Wang, Y., et al., *Deletion of the gene encoding the ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein (UGRP)/glucose-6-phosphatase catalytic subunit-beta results in lowered plasma cholesterol and elevated glucagon.* J Biol Chem, 2006. **281**(52): p. 39982-9.
54. Cheung, Y.Y., et al., *Impaired neutrophil activity and increased susceptibility to bacterial infection in mice lacking glucose-6-phosphatase-beta.* J Clin Invest, 2007. **117**(3): p. 784-93.
55. Boztug, K., et al., *A syndrome with congenital neutropenia and mutations in G6PC3.* The New England journal of medicine, 2009. **360**(1): p. 32-43.
56. Khan, A., C. Hong-Lie, and B.R. Landau, *Glucose-6-phosphatase activity in islets from ob/ob and lean mice and the effect of dexamethasone.* Endocrinology, 1995. **136**(5): p. 1934-8.
57. Taljedal, I.B., *Presence, induction, and possible role of glucose-6-phosphatase in mammalian pancreatic islets.* Biochem Journal, 1969. **114**: p. 387-394.
58. Jensen, M.V., et al., *Metabolic cycling in control of glucose-stimulated insulin secretion.* American journal of physiology. Endocrinology and metabolism, 2008. **295**(6): p. E1287-97.
59. Ashcroft, F.M., D.E. Harrison, and S.J. Ashcroft, *Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells.* Nature, 1984. **312**(5993): p. 446-8.
60. Cook, D.L. and C.N. Hales, *Intracellular ATP directly blocks K+ channels in pancreatic B-cells.* Nature, 1984. **311**(5983): p. 271-3.
61. Henquin, J.C., et al., *Hierarchy of the beta-cell signals controlling insulin secretion.* European journal of clinical investigation, 2003. **33**(9): p. 742-50.
62. Shiota, C., et al., *Sulfonylurea receptor type 1 knock-out mice have intact feeding-stimulated insulin secretion despite marked impairment in their response to glucose.* The Journal of biological chemistry, 2002. **277**(40): p. 37176-83.
63. Nenquin, M., et al., *Both triggering and amplifying pathways contribute to fuel-induced insulin secretion in the absence of sulfonylurea receptor-1 in pancreatic beta-cells.* The Journal of biological chemistry, 2004. **279**(31): p. 32316-24.
64. Szollosi, A., et al., *Glucose stimulates Ca2+ influx and insulin secretion in 2-week-old beta-cells lacking ATP-sensitive K+ channels.* The Journal of biological chemistry, 2007. **282**(3): p. 1747-56.

65. Remedi, M.S., et al., *Hyperinsulinism in mice with heterozygous loss of K(ATP) channels.* Diabetologia, 2006. **49**(10): p. 2368-78.
66. Joseph, J.W., et al., *The mitochondrial citrate/isocitrate carrier plays a regulatory role in glucose-stimulated insulin secretion.* The Journal of biological chemistry, 2006. **281**(47): p. 35624-32.
67. Ronnebaum, S.M., et al., *A pyruvate cycling pathway involving cytosolic NADP-dependent isocitrate dehydrogenase regulates glucose-stimulated insulin secretion.* The Journal of biological chemistry, 2006. **281**(41): p. 30593-602.
68. Bell, G.I., et al., *Molecular biology of mammalian glucose transporters.* Diabetes Care, 1990. **13**: p. 198-208.
69. Thorens, B., M.J. Charron, and H.F. Lodish, *Molecular physiology of glucose transporters.* Diabetes Care, 1990. **13**(3): p. 209-18.
70. Meglasson, M.D. and F.M. Matschinsky, *New perspectives on pancreatic islet glucokinase.* Am J Physiol, 1984. **246**: p. E1-13.
71. Meglasson, M.D. and F.M. Matschinsky, *Pancreatic islet glucose metabolism and regulation of insulin secretion.* Diabetes Metab Rev, 1986. **2**(3-4): p. 163-214.
72. Johnson, J.H., et al., *The high Km glucose transporter of islets of Langerhans is functionally similar to the low affinity transporter of liver and has an identical primary sequence.* J Biol Chem, 1990. **265**(12): p. 6548-51.
73. Efrat, S., M. Tal, and H.F. Lodish, *The pancreatic beta-cell glucose sensor.* Trends in biochemical sciences, 1994. **19**(12): p. 535-8.
74. Epstein, P.N., et al., *Expression of yeast hexokinase in pancreatic beta cells of transgenic mice reduces blood glucose, enhances insulin secretion, and decreases diabetes.* Proc Natl Acad Sci USA, 1992. **89**(24): p. 12038-42.
75. Efrat, S., et al., *Ribozyme-mediated attenuation of pancreatic beta-cell glucokinase expression in transgenic mice results in impaired glucose-induced insulin secretion.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(6): p. 2051-5.
76. Tal, M., et al., *[Val12] HRAS downregulates GLUT2 in beta cells of transgenic mice without affecting glucose homeostasis.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(13): p. 5744-8.
77. Khan, A., et al., *Evidence for the presence of glucose cycling in pancreatic islets of the ob/ob mouse.* The Journal of biological chemistry, 1989. **264**(17): p. 9732-3.
78. Khan, A., et al., *Glucose cycling in islets from healthy and diabetic rats.* Diabetes, 1990. **39**(4): p. 456-9.
79. Sweet, I.R., et al., *Measurement and modeling of glucose-6-phosphatase in pancreatic islets.* The American journal of physiology, 1997. **272**(4 Pt 1): p. E696-711.
80. Argaud, D., et al., *Stimulation of glucose-6-phosphatase gene expression by glucose and fructose-2,6-bisphosphate.* J Biol Chem, 1997. **272**(19): p. 12854-61.
81. Massillon, D., et al., *Glucose regulates in vivo glucose-6-phosphatase gene expression in the liver of diabetic rats.* J Biol Chem, 1996. **271**(17): p. 9871-4.

82. Massillon, D., et al., *Carbon flux via the pentose phosphate pathway regulates the hepatic expression of the glucose-6-phosphatase and phosphoenolpyruvate carboxykinase genes in conscious rats.* J Biol Chem, 1998. **273**(1): p. 228-34.
83. Frigeri, C., et al., *The Proximal Islet-Specific Glucose-6-Phosphatase Catalytic Subunit Related Protein (IGRP) Autoantigen Promoter is Sufficient to Initiate but not Maintain Transgene Expression in Mouse Islets In Vivo.* Diabetes, 2004. **53**: p. 1754-1764.
84. Ebert, D.H., et al., *Structure and promoter activity of an islet-specific glucose-6-phosphatase catalytic subunit-related gene.* Diabetes, 1999. **48**(3): p. 543-51.
85. Wang, Y., et al., *Long-range enhancers are required to maintain expression of the autoantigen islet-specific glucose-6-phosphatase catalytic subunit-related protein in adult mouse islets in vivo.* Diabetes, 2008. **57**(1): p. 133-41.
86. Bischof, L.J., et al., *Characterization of the Mouse Islet-Specific Glucose-6-Phosphatase Catalytic Subunit-Related Protein Gene Promoter by In Situ Footprinting. Correlation with Fusion Gene Expression in the Islet Derived bTC-3 and Hamster Insulinoma Tumor Cell Lines.* Diabetes, 2001. **50**: p. 502-514.
87. Martin, C.C., et al., *Upstream Stimulatory Factor (USF) and NeuroD/BETA2 Contribute toIslet-Specific Glucose-6-Phosphatase Catalytic Subunit Related Protein (IGRP)Gene Expression.* Biochem J, 2003. **371**: p. 675-686.
88. Martin, C.C., J.K. Oeser, and R.M. O'Brien, *Differential regulation of islet-specific glucose-6-phosphatase catalytic subunit-related protein gene transcription by Pax-6 and Pdx-1.* J Biol Chem, 2004. **279**(33): p. 34277-89.
89. Martin, C.C., et al., *Foxa2 and MafA regulate islet-specific glucose-6-phosphatase catalytic subunit-related protein gene expression.* J Mol Endocrinol, 2008. **41**(5): p. 315-28.
90. Sander, M. and M.S. German, *The beta cell transcription factors and development of the pancreas.* J Mol Med, 1997. **75**(5): p. 327-40.
91. Melloul, D., S. Marshak, and E. Cerasi, *Regulation of insulin gene transcription.* Diabetologia, 2002. **45**(3): p. 309-26.
92. Hay, C.W. and K. Docherty, *Comparative analysis of insulin gene promoters: implications for diabetes research.* Diabetes, 2006. **55**(12): p. 3201-13.
93. Hu, C., et al., *A genetic variant of G6PC2 is associated with type 2 diabetes and fasting plasma glucose level in the Chinese population.* Diabetologia, 2009. **52**(3): p. 451-6.
94. Chen, W.M., et al., *Variations in the G6PC2/ABCB11 genomic region are associated with fasting glucose levels.* The Journal of clinical investigation, 2008. **118**(7): p. 2620-8.
95. Dos Santos, C., P. Bougneres, and D. Fradin, *A single-nucleotide polymorphism in a methylatable Foxa2 binding site of the G6PC2 promoter is associated with insulin secretion in vivo and increased promoter activity in vitro.* Diabetes, 2009. **58**(2): p. 489-92.
96. Bouatia-Naji, N., et al., *Genetic and functional assessment of the role of the rs13431652-A and rs573225-A alleles in the G6PC2 promoter that are strongly associated with elevated fasting glucose levels.* Diabetes, 2010. **59**(10): p. 2662-71.

97. Khaw, K.T., et al., *Glycated haemoglobin, diabetes, and mortality in men in Norfolk cohort of european prospective investigation of cancer and nutrition (EPIC-Norfolk).* BMJ, 2001. **322**(7277): p. 15-8.
98. Lawes, C.M., et al., *Blood glucose and risk of cardiovascular disease in the Asia Pacific region.* Diabetes Care, 2004. **27**(12): p. 2836-42.
99. Abdul-Ghani, M.A. and R.A. DeFronzo, *Plasma glucose concentration and prediction of future risk of type 2 diabetes.* Diabetes Care, 2009. **32 Suppl 2**: p. S194-8.
100. Sarwar, N., et al., *Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies.* Lancet, 2010. **375**(9733): p. 2215-22.
101. Abdul-Ghani, M.A., et al., *Minimal contribution of fasting hyperglycemia to the incidence of type 2 diabetes in subjects with normal 2-h plasma glucose.* Diabetes Care, 2010. **33**(3): p. 557-61.
102. Heni, M., et al., *The impact of genetic variation in the G6PC2 gene on insulin secretion depends on glycemia.* The Journal of clinical endocrinology and metabolism, 2010. **95**(12): p. E479-84.
103. Saxena, R., et al., *Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels.* Science, 2007. **316**(5829): p. 1331-6.
104. Dupuis, J., et al., *New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk.* Nature Genetics, 2010. **42**(2): p. 105-16.
105. Iynedjian, P.B., *Molecular physiology of mammalian glucokinase.* Cellular and molecular life sciences : CMLS, 2009. **66**(1): p. 27-42.
106. Matschinsky, F., et al., *Glucokinase as Pancreatic β Cell Glucose Sensor and Diabetes Gene.* J. Clin. Invest., 1993. **92**: p. 2092-2098.
107. Froguel, P., et al., *Familial hyperglycemia due to mutations in glucokinase. Definition of a subtype of diabetes mellitus.* The New England journal of medicine, 1993. **328**(10): p. 697-702.
108. Rose, C.S., et al., *A variant in the G6PC2/ABCB11 locus is associated with increased fasting plasma glucose, increased basal hepatic glucose production and increased insulin release after oral and intravenous glucose loads.* Diabetologia, 2009. **52**(10): p. 2122-9.
109. Ingelsson, E., et al., *Detailed physiologic characterization reveals diverse mechanisms for novel genetic Loci regulating glucose and insulin metabolism in humans.* Diabetes, 2010. **59**(5): p. 1266-75.
110. Li, X., et al., *Additive effects of genetic variation in GCK and G6PC2 on insulin secretion and fasting glucose.* Diabetes, 2009. **58**(12): p. 2946-53.
111. Lieberman, S.M., et al., *Identification of the beta cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8384-8.
112. Anderson, M.S. and J.A. Bluestone, *The NOD mouse: a model of immune dysregulation.* Annu Rev Immunol, 2005. **23**: p. 447-85.
113. Yang, J., et al., *Islet-specific glucose-6-phosphatase catalytic subunit-related protein-reactive CD4+ T cells in human subjects.* J Immunol, 2006. **176**(5): p. 2781-9.

114. Jarchum, I.T., T. Takaki, and T.P. Dilorenzo, *Efficient culture of CD8(+) T cells from the islets of NOD mice and their use for the study of autoreactive specificities.* J Immunol Methods, 2008. **339**(1): p. 66-73.
115. Serreze, D.V., M.P. Marron, and T.P. Dilorenzo, *"Humanized" HLA transgenic NOD mice to identify pancreatic beta cell autoantigens of potential clinical relevance to type 1 diabetes.* Ann N Y Acad Sci, 2007. **1103**: p. 103-11.
116. Takaki, T., et al., *HLA-A*0201-restricted T cells from humanized NOD mice recognize autoantigens of potential clinical relevance to type 1 diabetes.* J Immunol, 2006. **176**(5): p. 3257-65.
117. Krishnamurthy, B., et al., *Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP.* The Journal of clinical investigation, 2006. **116**(12): p. 3258-65.
118. Nakayama, M., et al., *Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice.* Nature, 2005. **435**(7039): p. 220-3.
119. Wang, Y., et al., *Deletion of the gene encoding the islet-specific glucose-6-phosphatase catalytic subunit-related protein autoantigen results in a mild metabolic phenotype.* Diabetologia, 2007. **50**(4): p. 774-8.
120. Shameli, A., et al., *Endoplasmic reticulum stress caused by overexpression of islet-specific glucose-6-phosphatase catalytic subunit-related protein in pancreatic Beta-cells.* The review of diabetic studies : RDS, 2007. **4**(1): p. 25-32.
121. Chimienti, F., et al., *Zinc homeostasis-regulating proteins: new drug targets for triggering cell fate.* Curr Drug Targets, 2003. **4**(4): p. 323-38.
122. Jansen, J., W. Karges, and L. Rink, *Zinc and diabetes--clinical links and molecular mechanisms.* The Journal of nutritional biochemistry, 2009. **20**(6): p. 399-417.
123. Bergman, B. and R. Soremark, *Autoradiographic studies on the distribution of zinc-65 in mice.* The Journal of nutrition, 1968. **94**(1): p. 6-12.
124. Vallee, B.L. and K.H. Falchuk, *The biochemical basis of zinc physiology.* Physiological reviews, 1993. **73**(1): p. 79-118.
125. Wellinghausen, N., H. Kirchner, and L. Rink, *The immunobiology of zinc.* Immunology today, 1997. **18**(11): p. 519-21.
126. Coleman, D.L., *The influence of genetic background on the expression of mutations at the diabetes (db) locus in the mouse. VI: Hepatic malic enzyme activity is associated with diabetes severity.* Metabolism, 1992. **41**(10): p. 1134-6.
127. Emdin SO, D.G., Cutfield JM, Cutfield SM, *Role of zinc in insulin biosynthesis.* Diabetologia, 1980. **19**(3): p. 174-82.
128. Zalewski, P.D., et al., *Video image analysis of labile zinc in viable pancreatic islet cells using a specific fluorescent probe for zinc.* J Histochem Cytochem, 1994. **42**(7): p. 877-84.
129. Dodson, G. and D. Steiner, *The role of assembly in insulin's biosynthesis.* Current opinion in structural biology, 1998. **8**(2): p. 189-94.
130. Coyle, P., et al., *Importance of storage conditions for the stability of zinc- and cadmium-induced metallothionein.* Biological trace element research, 2001. **81**(3): p. 269-78.

131. Ohly, P., et al., *Zinc sulphate induces metallothionein in pancreatic islets of mice and protects against diabetes induced by multiple low doses of streptozotocin.* Diabetologia, 2000. **43**(8): p. 1020-30.
132. Gyulkhandanyan, A.V., et al., *Investigation of transport mechanisms and regulation of intracellular Zn2+ in pancreatic alpha-cells.* The Journal of biological chemistry, 2008. **283**(15): p. 10184-97.
133. Richards, M.P., *Recent developments in trace element metabolism and function: role of metallothionein in copper and zinc metabolism.* The Journal of nutrition, 1989. **119**(7): p. 1062-70.
134. Nath, R., et al., *Molecular aspects, physiological function, and clinical significance of metallothioneins.* Critical reviews in food science and nutrition, 1988. **27**(1): p. 41-85.
135. Sadhu, C. and L. Gedamu, *Regulation of human metallothionein (MT) genes. Differential expression of MTI-F, MTI-G, and MTII-A genes in the hepatoblastoma cell line (HepG2).* The Journal of biological chemistry, 1988. **263**(6): p. 2679-84.
136. Stennard, F.A., et al., *Characterisation of six additional human metallothionein genes.* Biochimica et biophysica acta, 1994. **1218**(3): p. 357-65.
137. Coyle, P., et al., *Metallothionein: the multipurpose protein.* Cellular and molecular life sciences : CMLS, 2002. **59**(4): p. 627-47.
138. Masters, B.A., et al., *Metallothionein III is expressed in neurons that sequester zinc in synaptic vesicles.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 1994. **14**(10): p. 5844-57.
139. Hoey, J.G., et al., *Expression of MT-3 mRNA in human kidney, proximal tubule cell cultures, and renal cell carcinoma.* Toxicology letters, 1997. **92**(2): p. 149-60.
140. Moffatt, P. and C. Seguin, *Expression of the gene encoding metallothionein-3 in organs of the reproductive system.* DNA and cell biology, 1998. **17**(6): p. 501-10.
141. Quaife, C.J., et al., *Induction of a new metallothionein isoform (MT-IV) occurs during differentiation of stratified squamous epithelia.* Biochemistry, 1994. **33**(23): p. 7250-9.
142. Liang, L., et al., *Activation of the complete mouse metallothionein gene locus in the maternal deciduum.* Molecular reproduction and development, 1996. **43**(1): p. 25-37.
143. Miles, A.T., et al., *Induction, regulation, degradation, and biological significance of mammalian metallothioneins.* Critical reviews in biochemistry and molecular biology, 2000. **35**(1): p. 35-70.
144. Moffatt, P. and F. Denizeau, *Metallothionein in physiological and physiopathological processes.* Drug metabolism reviews, 1997. **29**(1-2): p. 261-307.
145. Cherian, M.G. and M.D. Apostolova, *Nuclear localization of metallothionein during cell proliferation and differentiation.* Cellular and molecular biology, 2000. **46**(2): p. 347-56.

146. Ogra, Y. and K.T. Suzuki, *Nuclear trafficking of metallothionein: possible mechanisms and current knowledge.* Cellular and molecular biology, 2000. **46**(2): p. 357-65.
147. Cousins, R.J., *Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin.* Physiological reviews, 1985. **65**(2): p. 238-309.
148. Bremner, I. and J.H. Beattie, *Metallothionein and the trace minerals.* Annual review of nutrition, 1990. **10**: p. 63-83.
149. Hamer, D.H., *Metallothionein.* Annual review of biochemistry, 1986. **55**: p. 913-51.
150. Laychock, S.G., J. Duzen, and C.O. Simpkins, *Metallothionein induction in islets of Langerhans and insulinoma cells.* Molecular and cellular endocrinology, 2000. **165**(1-2): p. 179-87.
151. Chen, H., et al., *Overexpression of metallothionein in pancreatic beta-cells reduces streptozotocin-induced DNA damage and diabetes.* Diabetes, 2001. **50**(9): p. 2040-6.
152. Cai, L., *Metallothionein as an adaptive protein prevents diabetes and its toxicity.* Nonlinearity in biology, toxicology, medicine, 2004. **2**(2): p. 89-103.
153. Yang, L., et al., *Polymorphisms in metallothionein-1 and -2 genes associated with the risk of type 2 diabetes mellitus and its complications.* American journal of physiology. Endocrinology and metabolism, 2008. **294**(5): p. E987-92.
154. Beattie, J.H., et al., *Obesity and hyperleptinemia in metallothionein (-I and -II) null mice.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(1): p. 358-63.
155. Lichten, L.A. and R.J. Cousins, *Mammalian zinc transporters: nutritional and physiologic regulation.* Annual review of nutrition, 2009. **29**: p. 153-76.
156. Gitan, R.S., et al., *A cytosolic domain of the yeast Zrt1 zinc transporter is required for its post-translational inactivation in response to zinc and cadmium.* The Journal of biological chemistry, 2003. **278**(41): p. 39558-64.
157. McClelland, R.A., et al., *Oestrogen-regulated genes in breast cancer: association of pLIV1 with response to endocrine therapy.* British journal of cancer, 1998. **77**(10): p. 1653-6.
158. Taylor, K.M., et al., *Structure-function analysis of LIV-1, the breast cancer-associated protein that belongs to a new subfamily of zinc transporters.* The Biochemical journal, 2003. **375**(Pt 1): p. 51-9.
159. Taylor, K.M., *A distinct role in breast cancer for two LIV-1 family zinc transporters.* Biochemical Society transactions, 2008. **36**(Pt 6): p. 1247-51.
160. Kagara, N., et al., *Zinc and its transporter ZIP10 are involved in invasive behavior of breast cancer cells.* Cancer science, 2007. **98**(5): p. 692-7.
161. Kury, S., et al., *Identification of SLC39A4, a gene involved in acrodermatitis enteropathica.* Nature Genetics, 2002. **31**(3): p. 239-40.
162. Wang, K., et al., *A novel member of a zinc transporter family is defective in acrodermatitis enteropathica.* American journal of human genetics, 2002. **71**(1): p. 66-73.

163. Wang, K., et al., *Homozygosity mapping places the acrodermatitis enteropathica gene on chromosomal region 8q24.3.* American journal of human genetics, 2001. **68**(4): p. 1055-60.
164. Foster, M.C., et al., *Elemental composition of secretory granules in pancreatic islets of Langerhans.* Biophys. J., 1993. **64**: p. 525-532.
165. Blundell, T.L., et al., *Insulin: the reflection in the crystal and its reflection in chemistry and biology.* Adv Protein Chem, 1972. **26**: p. 280-422.
166. Grant, P.T., T.L. Coombs, and B.H. Frank, *Differences in the nature of the interaction of insulin and proinsulin with zinc.* Biochem J, 1971. **126**: p. 433-440.
167. Havu, N., G. Lundgren, and S. Falkmer, *Zinc and manganese contents of micro-dissected pancreatic islets of some rodents. A microchemical study in adult and newborn guinea pigs, rats, Chinese hamsters and spiny mice.* Acta endocrinologica, 1977. **86**(3): p. 570-7.
168. Peterson, J.D., et al., *Structural and crystallographic observations on hagfish insulin.* Nature, 1974. **251**(5472): p. 239-40.
169. Zimmerman, A.E. and C.C. Yip, *Guinea pig insulin. I. Purification and physical properties.* The Journal of biological chemistry, 1974. **249**(13): p. 4021-5.
170. Quarterman, J., C.F. Mills, and W.R. Humphries, *The reduced secretion of and sensitivity to insulin in zinc-deficient rats.* Biochem Biophys Res Commun, 1966. **25**(3): p. 354-8.
171. Boquist, L., et al., *Insulin biosynthesis, storage and secretion.* Lakartidningen, 1968. **65**: p. 3603-3607.
172. Begin-Heick, N., et al., *Zinc supplementation attenuates insulin secretory activity in pancreatic islets of the ob/ob mouse.* Diabetes, 1985. **34**: p. 179-184.
173. Simon SF, T.C., *Dietary Zinc Supplementation Attenuates Hyperglycemia in db/db Mice.* Exp Biol Med, 2001. **226**(1): p. 43-51.
174. Scott, D.A. and A.M. Fisher, *The insulin and the zinc content of normal and diabetic pancreas.* J Clin Invest, 1938. **17**: p. 725-28.
175. Ripa, S. and R. Ripa, *Zinc and diabetes mellitus.* Minerva Med, 1995. **86**: p. 415-21.
176. Gupta, R., et al., *Oral zinc therapy in diabetic neuropathy.* J Assoc Physicians India, 1998. **46**: p. 939-42.
177. Franklin, I., et al., *Beta-cell secretory products activate alpha-cell ATP-dependent potassium channels to inhibit glucagon release.* Diabetes, 2005. **54**(6): p. 1808-15.
178. Bloc, A., et al., *Zinc-induced changes in ionic currents of clonal rat pancreatic -cells: activation of ATP-sensitive K+ channels.* The Journal of physiology, 2000. **529 Pt 3**: p. 723-34.
179. Crouch, R.K., et al., *Localization of copper-zinc superoxide dismutase in the endocrine pancreas.* Experimental and molecular pathology, 1984. **41**(3): p. 377-83.
180. Wijesekara, N., F. Chimienti, and M.B. Wheeler, *Zinc, a regulator of islet function and glucose homeostasis.* Diabetes, obesity & metabolism, 2009. **11 Suppl 4**: p. 202-14.

181. Ho, E., et al., *Dietary zinc supplementation inhibits NFkappaB activation and protects against chemically induced diabetes in CD1 mice.* Experimental biology and medicine, 2001. **226**(2): p. 103-11.
182. Chen, M.D., Y.M. Song, and P.Y. Lin, *Zinc effects on hyperglycemia and hypoleptinemia in streptozotocin-induced diabetic mice.* Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme, 2000. **32**(3): p. 107-9.
183. Kim, B.J., et al., *Zinc as a paracrine effector in pancreatic islet cell death.* Diabetes, 2000. **49**(3): p. 367-72.
184. Bray, T.M. and W.J. Bettger, *The physiological role of zinc as an antioxidant.* Free radical biology & medicine, 1990. **8**(3): p. 281-91.
185. Seve, M., et al., *In silico identification and expression of SLC30 family genes: an expressed sequence tag data mining strategy for the characterization of zinc transporters' tissue expression.* BMC genomics, 2004. **5**(1): p. 32.
186. Chimienti, F., A. Favier, and M. Seve, *ZnT-8, a pancreatic beta-cell-specific zinc transporter.* Biometals, 2005. **18**(4): p. 313-7.
187. Fu, Y., et al., *Down-regulation of ZnT8 expression in INS-1 rat pancreatic beta cells reduces insulin content and glucose-inducible insulin secretion.* PLoS ONE, 2009. **4**(5): p. e5679.
188. Chimienti, F., et al., *In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion.* J Cell Sci, 2006. **119**(Pt 20): p. 4199-206.
189. Scott, L.J., et al., *A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants.* Science, 2007. **316**(5829): p. 1341-5.
190. Zeggini, E., et al., *Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes.* Science, 2007. **316**(5829): p. 1336-41.
191. Lauenborg, J., et al., *Common type 2 diabetes risk gene variants associate with gestational diabetes.* J. Clin. Endocrinol. Metab., 2009. **94**(1): p. 145-150.
192. Stancakova, A., et al., *Association of 18 confirmed susceptibility loci for type 2 diabetes with indices of insulin release, proinsulin conversion, and insulin sensitivity in 5327 non-diabetic Finnish men.* Diabetes, 2009. **[Epub ahead of print]**.
193. Xu, K., et al., *Association between rs13266634 C/T polymorphisms of solute carrier family 30 member 8 (SLC30A8) and type 2 diabetes, impaired glucose tolerance, type 1 diabetes--a meta-analysis.* Diabetes research and clinical practice, 2011. **91**(2): p. 195-202.
194. Nicolson, T.J., et al., *Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants.* Diabetes, 2009. **58**(9): p. 2070-83.
195. Wenzlau, J.M., et al., *A common nonsynonymous single nucleotide polymorphism in the SLC30A8 gene determines ZnT8 autoantibody specificity in type 1 diabetes.* Diabetes, 2008. **57**(10): p. 2693-7.
196. Wenzlau, J.M., et al., *The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(43): p. 17040-5.

197. Clee, S.M. and A.D. Attie, *The genetic landscape of type 2 diabetes in mice.* Endocrine reviews, 2007. **28**(1): p. 48-83.
198. Lee, S.K., et al., *Defective glucose-stimulated insulin release from perifused islets of C57BL/6J mice.* Pancreas, 1995. **11**(2): p. 206-11.
199. Wencel, H.E., et al., *Impaired second phase insulin response of diabetes-prone C57BL/6J mouse islets.* Physiology & behavior, 1995. **57**(6): p. 1215-20.
200. Freeman, H., et al., *Nicotinamide nucleotide transhydrogenase: a key role in insulin secretion.* Cell metabolism, 2006. **3**(1): p. 35-45.
201. Wattler, S., M. Kelly, and M. Nehls, *Construction of gene targeting vectors from lambda KOS genomic libraries.* Biotechniques, 1999. **26**(6): p. 1150-6, 1158, 1160.
202. Serreze, D.V., et al., *B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice.* J Exp Med, 1996. **184**(5): p. 2049-53.
203. Markel, P., et al., *Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains.* Nature Genetics, 1997. **17**: p. 280-4.
204. Sambrook, J., Fritsch, EF and Maniatis EF, *Molecular Cloning: A Laboratory Manual*. 2nd ed1989, Plainview, NY: Cold Spring Harbor Laboratory Press.
205. Zhang, H., et al., *The FoxM1 Transcription Factor is Required to Maintain Pancreatic ß-Cell Mass.* Mol. Endocrinol., 2006. **20**(8): p. 1853-1866.
206. Bonnevie-Nielsen, V., M.W. Steffes, and A. Lernmark, *A major loss in islet mass and B-cell function precedes hyperglycemia in mice given multiple low doses of streptozotocin.* Diabetes, 1981. **30**(5): p. 424-9.
207. Shiota, C., et al., *Impaired glucagon secretory responses in mice lacking the type 1 sulfonylurea receptor.* American journal of physiology. Endocrinology and metabolism, 2005. **289**(4): p. E570-7.
208. Brissova, M., et al., *Reduced PDX-1 expression impairs islet response to insulin resistance and worsens glucose homeostasis.* American journal of physiology. Endocrinology and metabolism, 2005. **288**(4): p. E707-14.
209. Brissova, M., et al., *Intraislet endothelial cells contribute to revascularization of transplanted pancreatic islets.* Diabetes, 2004. **53**(5): p. 1318-25.
210. Jacobson, D.A., et al., *Calcium-activated and voltage-gated potassium channels of the pancreatic islet impart distinct and complementary roles during secretagogue induced electrical responses.* The Journal of physiology, 2010. **588**(Pt 18): p. 3525-37.
211. Jahanshahi, P., et al., *Evidence of diminished glucose stimulation and endoplasmic reticulum function in nonoscillatory pancreatic islets.* Endocrinology, 2009. **150**(2): p. 607-15.
212. Danscher, G., et al., *Immersion autometallography: histochemical in situ capturing of zinc ions in catalytic zinc-sulfur nanocrystals.* J. Histochem. Cytochem., 2004. **52**: p. 1619-1625.
213. McGuinness, O.P., et al., *NIH experiment in centralized mouse phenotyping: the Vanderbilt experience and recommendations for evaluating glucose homeostasis in the mouse.* American journal of physiology. Endocrinology and metabolism, 2009. **297**(4): p. E849-55.

214. Ayala, J.E., et al., *Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice.* Disease models & mechanisms, 2010. **3**(9-10): p. 525-34.
215. Sarkar, S.A., et al., *Global gene expression profiling and histochemical analysis of the developing human fetal pancreas.* Diabetologia, 2008. **51**(2): p. 285-97.
216. Chen, W.M., et al., *Variations in the G6PC2/ABCB11 genomic region are associated with fasting glucose levels.* J Clin Invest, 2008. **118**(7): p. 2620-8.
217. Hu, C., et al., *A genetic variant of G6PC2 is associated with type 2 diabetes and fasting plasma glucose level in the Chinese population.* Diabetologia, 2009. **52**(3): p. 451-6.
218. Waddell, I.D. and A. Burchell, *The microsomal glucose-6-phosphatase enzyme of pancreatic islets.* The Biochemical journal, 1988. **255**(2): p. 471-6.
219. Perales, M.A., A. Sener, and W.J. Malaisse, *Hexose metabolism in pancreatic islets: the glucose-6-phosphatase riddle.* Molecular and cellular biochemistry, 1991. **101**(1): p. 67-71.
220. Trandaburu, T., *Fine structural localization of glucose-6-phosphatase activity in the pancreatic islets of two amphibian species (Salamandra salamandra L. and Rana esculenta L.).* Acta histochemica, 1977. **59**(2): p. 246-53.
221. Roe, M.W., et al., *Thapsigargin inhibits the glucose-induced decrease of intracellular Ca2+ in mouse islets of Langerhans.* The American journal of physiology, 1994. **266**(6 Pt 1): p. E852-62.
222. Roe, M.W., et al., *Voltage-dependent intracellular calcium release from mouse islets stimulated by glucose.* The Journal of biological chemistry, 1993. **268**(14): p. 9953-6.
223. Simonen, P.P., H.K. Gylling, and T.A. Miettinen, *Diabetes contributes to cholesterol metabolism regardless of obesity.* Diabetes Care, 2002. **25**(9): p. 1511-5.
224. Benedetti, A., R. Fulceri, and M. Comporti, *Calcium sequestration activity in rat liver microsomes. Evidence for a cooperation of calcium transport with glucose-6-phosphatase.* Biochimica et biophysica acta, 1985. **816**(2): p. 267-77.
225. Gilon, P., et al., *Uptake and release of Ca2+ by the endoplasmic reticulum contribute to the oscillations of the cytosolic Ca2+ concentration triggered by Ca2+ influx in the electrically excitable pancreatic B-cell.* The Journal of biological chemistry, 1999. **274**(29): p. 20197-205.
226. Chen, P.Y., et al., *Glucose-6-phosphate and Ca2+ sequestration are mutually enhanced in microsomes from liver, brain, and heart.* Diabetes, 1998. **47**(6): p. 874-81.
227. Korge, P. and K.B. Campbell, *Local ATP regeneration is important for sarcoplasmic reticulum Ca2+ pump function.* The American journal of physiology, 1994. **267**(2 Pt 1): p. C357-66.
228. Reiling, E., et al., *Combined effects of single-nucleotide polymorphisms in GCK, GCKR, G6PC2 and MTNR1B on fasting plasma glucose and type 2 diabetes risk.* Diabetologia, 2009. **52**(9): p. 1866-70.
229. Hu, C., et al., *Effects of GCK, GCKR, G6PC2 and MTNR1B variants on glucose metabolism and insulin secretion.* PLoS ONE, 2010. **5**(7): p. e11761.

230. Malik, T. and D.L. Trence, *Treating diabetes using oral agents.* Primary care, 2003. **30**(3): p. 527-41.
231. Edgerton, D.S., K.M. Johnson, and A.D. Cherrington, *Current strategies for the inhibition of hepatic glucose production in type 2 diabetes.* Frontiers in bioscience : a journal and virtual library, 2009. **14**: p. 1169-81.
232. Matschinsky, F.M., *Glucokinase as glucose sensor and metabolic signal generator in pancreatic beta-cells and hepatocytes.* Diabetes, 1990. **39**(6): p. 647-52.
233. Matschinsky, F.M., *Banting Lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm.* Diabetes, 1996. **45**(2): p. 223-41.
234. Niswender, K.D., et al., *Effects of increased glucokinase gene copy number on glucose homeostasis and hepatic glucose metabolism.* The Journal of biological chemistry, 1997. **272**(36): p. 22570-5.
235. Chimienti, F., et al., *Identification and cloning of a beta-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules.* Diabetes, 2004. **53**(9): p. 2330-7.
236. Katz, A., et al., *Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans.* J. Clin. Endocrinol. Metab., 2000. **85**: p. 2402-10.
237. Murgia, C., et al., *Diabetes-linked zinc transporter ZnT8 is a homodimeric protein expressed by distinct rodent endocrine cell types in the pancreas and other glands.* Nutrition, metabolism, and cardiovascular diseases : NMCD, 2009. **19**(6): p. 431-9.
238. Ward, W.K., et al., *Disproportionate elevation of immunoreactive proinsulin in type 2 (non-insulin-dependent) diabetes mellitus and in experimental insulin resistance.* Diabetologia, 1987. **30**: p. 698-702.
239. Wareham, N.J., et al., *Fasting proinsulin concentrations predict the development of type 2 diabetes.* Diabetes Care, 1999. **22**: p. 262-270.
240. Kirchhoff, K., et al., *Polymorphisms in the TCF7L2, CDKAL1 and SLC30A8 genes are associated with impaired proinsulin conversion.* Diabetologia, 2008. **51**(4): p. 597-601.
241. Ciaraldi, T.P. and D. Brady, *Comparisons of insulin and biosynthetic human proinsulin actions in cultured hepatocytes. Kinetics and biologic potencies.* Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme, 1990. **22**(5): p. 283-8.
242. Zhou, H., et al., *Zinc, not insulin, regulates the rat alpha-cell response to hypoglycemia in vivo.* Diabetes, 2007. **56**(4): p. 1107-12.
243. Wijesekara, N., et al., *Beta cell-specific Znt8 deletion in mice causes marked defects in insulin processing, crystallisation and secretion.* Diabetologia, 2010. **53**(8): p. 1656-68.
244. Lemaire, K., et al., *Insulin crystallization depends on zinc transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(35): p. 14872-7.

245. Rutter, G.A., *Think zinc: New roles for zinc in the control of insulin secretion.* Islets, 2010. **2**(1): p. 49-50.
246. van de Bunt, M. and A.L. Gloyn, *From genetic association to molecular mechanism.* Current diabetes reports, 2010. **10**(6): p. 452-66.
247. Pound, L.D., et al., *Deletion of the mouse Slc30a8 gene encoding zinc transporter-8 results in impaired insulin secretion.* The Biochemical journal, 2009. **421**(3): p. 371-6.
248. Pound, L.D., et al., *The pancreatic islet beta-cell-enriched transcription factor Pdx-1 regulates Slc30a8 gene transcription through an intronic enhancer.* The Biochemical journal, 2010. **433**(1): p. 95-105.
249. Boesgaard, T.W., et al., *The common SLC30A8 Arg325Trp variant is associated with reduced first-phase insulin release in 846 non-diabetic offspring of type 2 diabetes patients--the EUGENE2 study.* Diabetologia, 2008. **51**(5): p. 816-20.
250. Talchai, C., et al., *Genetic and biochemical pathways of beta-cell failure in type 2 diabetes.* Diabetes, obesity & metabolism, 2009. **11 Suppl 4**: p. 38-45.
251. Young, G.S. and J.B. Kirkland, *Rat models of caloric intake and activity: relationships to animal physiology and human health.* Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme, 2007. **32**(2): p. 161-76.
252. Shieh, J.J., et al., *In islet-specific glucose-6-phosphatase-related protein, the beta cell antigenic sequence that is targeted in diabetes is not responsible for the loss of phosphohydrolase activity.* Diabetologia, 2005. **48**(1851-1859).
253. Noguchi, Y., et al., *Effect of anaplerotic fluxes and amino acid availability on hepatic lipoapoptosis.* The Journal of biological chemistry, 2009. **284**(48): p. 33425-36.
254. Wang, H. and P.B. Iynedjian, *Acute glucose intolerance in insulinoma cells with unbalanced overexpression of glucokinase.* The Journal of biological chemistry, 1997. **272**(41): p. 25731-6.
255. Wang, H. and P.B. Iynedjian, *Modulation of glucose responsiveness of insulinoma beta-cells by graded overexpression of glucokinase.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(9): p. 4372-7.
256. Ahren, B., *Autonomic regulation of islet hormone secretion--implications for health and disease.* Diabetologia, 2000. **43**(4): p. 393-410.
257. Sharp, G.W., *Mechanisms of inhibition of insulin release.* The American journal of physiology, 1996. **271**(6 Pt 1): p. C1781-99.
258. Pedersen, K.B., et al., *The promoter for the gene encoding the catalytic subunit of rat glucose-6-phosphatase contains two distinct glucose-responsive regions.* Am J Physiol Endocrinol Metab, 2007. **292**(3): p. E788-801.
259. Mithieux, G., et al., *Liver microsomal glucose-6-phosphatase is competitively inhibited by the lipid products of phosphatidylinositol 3-kinase.* The Journal of biological chemistry, 1998. **273**(1): p. 17-9.
260. Matschinsky, F.M., *Assessing the potential of glucokinase activators in diabetes therapy.* Nature reviews. Drug discovery, 2009. **8**(5): p. 399-416.
261. Jetton, T.L., et al., *Analysis of upstream glucokinase promoter activity in transgenic mice and identification of glucokinase in rare neuroendocrine cells*

in the brain and gut. The Journal of biological chemistry, 1994. **269**(5): p. 3641-54.
262. Zelent, D., et al., *A glucose sensor role for glucokinase in anterior pituitary cells.* Diabetes, 2006. **55**(7): p. 1923-9.
263. Sorenson, R.L., et al., *Immunohistochemical evidence for the presence of glucokinase in the gonadotropes and thyrotropes of the anterior pituitary gland of rat and monkey.* The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society, 2007. **55**(6): p. 555-66.
264. Froguel, P., et al., *Close linkage of glucokinase locus on chromosome 7p to early-onset non-insulin-dependent diabetes mellitus.* Nature, 1992. **356**(6365): p. 162-4.
265. Hattersley, A.T., et al., *Linkage of type 2 diabetes to the glucokinase gene.* Lancet, 1992. **339**(8805): p. 1307-10.
266. Njolstad, P.R., et al., *Neonatal diabetes mellitus due to complete glucokinase deficiency.* The New England journal of medicine, 2001. **344**(21): p. 1588-92.
267. Njolstad, P.R., et al., *Permanent neonatal diabetes caused by glucokinase deficiency: inborn error of the glucose-insulin signaling pathway.* Diabetes, 2003. **52**(11): p. 2854-60.
268. Glaser, B., et al., *Familial hyperinsulinism caused by an activating glucokinase mutation.* The New England journal of medicine, 1998. **338**(4): p. 226-30.
269. Christesen, H.B., et al., *The second activating glucokinase mutation (A456V): implications for glucose homeostasis and diabetes therapy.* Diabetes, 2002. **51**(4): p. 1240-6.
270. Gloyn, A.L., *Glucokinase (GCK) mutations in hyper- and hypoglycemia: maturity-onset diabetes of the young, permanent neonatal diabetes, and hyperinsulinemia of infancy.* Human mutation, 2003. **22**(5): p. 353-62.
271. Cuesta-Munoz, A.L., et al., *Severe persistent hyperinsulinemic hypoglycemia due to a de novo glucokinase mutation.* Diabetes, 2004. **53**(8): p. 2164-8.
272. Matschinsky, F.M., et al., *The network of glucokinase-expressing cells in glucose homeostasis and the potential of glucokinase activators for diabetes therapy.* Diabetes, 2006. **55**(1): p. 1-12.
273. Bonadonna, R.C., et al., *Piragliatin (RO4389620), a novel glucokinase activator, lowers plasma glucose both in the postabsorptive state and after a glucose challenge in patients with type 2 diabetes mellitus: a mechanistic study.* The Journal of clinical endocrinology and metabolism, 2010. **95**(11): p. 5028-36.
274. Vaxillaire, M., et al., *The common P446L polymorphism in GCKR inversely modulates fasting glucose and triglyceride levels and reduces type 2 diabetes risk in the DESIR prospective general French population.* Diabetes, 2008. **57**(8): p. 2253-7.
275. Orho-Melander, M., et al., *Common missense variant in the glucokinase regulatory protein gene is associated with increased plasma triglyceride and C-reactive protein but lower fasting glucose concentrations.* Diabetes, 2008. **57**(11): p. 3112-21.
276. Sparso, T., et al., *The GCKR rs780094 polymorphism is associated with elevated fasting serum triacylglycerol, reduced fasting and OGTT-related insulinaemia, and reduced risk of type 2 diabetes.* Diabetologia, 2008. **51**(1): p. 70-5.

277. Kambe, T., et al., *Slc39a1 to 3 (subfamily II) Zip genes in mice have unique cell-specific functions during adaptation to zinc deficiency.* American journal of physiology. Regulatory, integrative and comparative physiology, 2008. **294**(5): p. R1474-81.
278. Dufner-Beattie, J., et al., *Mouse ZIP1 and ZIP3 genes together are essential for adaptation to dietary zinc deficiency during pregnancy.* Genesis, 2006. **44**(5): p. 239-51.
279. Muayed, M.E.I., et al., *Acute cytokine-mediated downregulation of the zinc transporter ZnT8 alters pancreatic β-cell function.* J Endocrinol, 2010. **206**: p. 159-169.
280. Smidt, K., et al., *SLC30A3 responds to glucose- and zinc variations in beta-cells and is critical for insulin production and in vivo glucose-metabolism during beta-cell stress.* PLoS ONE, 2009. **4**(5): p. e5684.
281. Pound, L.D., et al., *Characterization of the human SLC30A8 promoter and intronic enhancer.* Journal of molecular endocrinology, 2011.