

DOC2B ENHANCEMENT OF BETA CELL FUNCTION AND SURVIVAL

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## DEDICATION

This work is dedicated to my grandmother, Momena Reza Aslamy. A girl from a small village in Afghanistan with minimal formal education, Momena was wise beyond her years. Against the odds, Momena possessed unwavering courage, and became a revolutionary. I aspire to approach life the same way she lived hers - with compassion, wisdom, and fortitude.

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## DOC2B ENHANCEMENT OF BETA CELL FUNCTION AND SURVIVAL

Diabetes mellitus is a complex metabolic disease that currently affects an estimated 422 million people worldwide, with incidence rates rising annually. Type 1 diabetes (T1D) accounts for 5-10% of these cases. Its complications remain a major cause of global deaths. T1D is characterized by autoimmune destruction of  $\beta$ -cell mass. Efforts to preserve and protect  $\beta$ -cell mass in the preclinical stages of T1D are limited by few blood-borne biomarkers of  $\beta$ -cell destruction. In healthy  $\beta$ -cells, insulin secretion requires soluble n-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) complexes and associated accessory regulatory proteins to promote the docking and fusion of insulin vesicles at the plasma membrane. Two target membrane (t)-SNARE proteins, Syntaxin 1/4 and SNAP25/23, and one vesicle-associated (v)-SNARE protein, VAMP2, constitute the SNARE core complex. SNARE complex assembly is also facilitated by the regulatory protein, Double C2-domain protein  $\beta$  (DOC2B). I hypothesized that DOC2B deficiency may underlie  $\beta$ -cell susceptibility to T1D damage; conversely, overexpression of DOC2B may protect  $\beta$ -cell mass. Indeed, with regard to DOC2B abundance, my studies show reduced levels of DOC2B in platelets and islets of prediabetic rodents and new-onset T1D humans. Remarkably, clinical islet transplantation in T1D humans restores platelet DOC2B levels, indicating a correlation

With regard to protection/functional effects, DOC2B deficiency enhances susceptibility to T1D in mice, while overexpression of DOC2B selectively in  $\beta$ -cells protects mice from chemically induced T1D; this correlates with preservation of functional  $\beta$ -cell mass. Mechanistically, overexpression of DOC2B and the DOC2B peptide, C2AB, protects clonal  $\beta$ -cell against cytokine or thapsigargin-induced apoptosis and reduces ER stress; this is dependent on C2AB's calcium binding capacity. C2AB is sufficient to enhance glucose stimulated insulin secretion (GSIS) and SNARE activation in clonal  $\beta$ -cells to the same extent as full-length DOC2B. In summary, these studies identify DOC2B as a potential biomarker and novel therapeutic target for prevention/management of T1D.

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## LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMPK	Adenosine monophosphate kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Intracellular calcium concentration
DAG	Diacylglycerol
Doc2a	Double C2 domain protein $\alpha$
DOC2B	Double C2 domain protein $\beta$
Dox	Doxycycline
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GAD65	Glutamic acid decarboxylase 65
GSIS	Glucose-stimulated insulin secretion
GST	Glutathione s transferase
GLP-1	Glucagon-like receptor 1
GLUT4	Glucose transporter 4
GWAS	Genome-wide association study
HbA1c	Hemoglobin A1c
IA2	Islet antigen 2
IB	Immunoblot

IFN- $\gamma$	Interferon $\gamma$
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IL1 $\beta$	Interleukin 1 $\beta$
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
JNK	Jun N-terminal kinase
K <sub>ATP</sub>	ATP-dependent potassium channel
lncRNA	Long non-coding RNA
MAPK	Mitogen activated protein kinase
MKRBB	Modified Krebs-ringer bicarbonate buffer
MLD	Multiple low doses
mRNA	Messenger RNA
miRNA	Micro RNA
Munc13	Mammalian homolog of protein UNC-13
Munc18-1	Mammalian homolog of protein UNC-18-1/a
Munc18c	Mammalian homolog of protein UNC-18-3/c
NF- $\kappa$ B	Nuclear factor $\kappa$ B
NP-40	Non-ionic P-40 detergent
NOD	Non-obese diabetic
NOR	Non-obese diabetes-resistant

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
PIP	Phosphatidylinositol 4-phosphate
PM	Plasma membrane
PMSF	Phenylmethylsulfonylfluoride
pV	Pervanadate
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulfate
Ser	Serine
SNAP23/25	Synaptosome associated protein 23/25
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
STZ	Streptozotocin
t-SNARE	Target membrane SNARE protein
v-SNARE	Vesicle associated SNARE protein
STX1A	Syntaxin 1A
STX4	Syntaxin 4
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Tctex-1	Dynein light chain, tctex-type 1
Thr	Threonine

TNF- $\alpha$	Tumor necrosis factor $\alpha$
TRE	Tetracycline response element
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
Tyr	Tyrosine
VAMP2	Vesicle associated membrane protein 2
VAMP3	Vesicle associated membrane protein 3
ZnT8	Zinc transporter 8

## CHAPTER 1: INTRODUCTION

## **1.1. DIABETES AND CURRENT STANDARD OF CARE – WHAT’S NOT WORKING**

Diabetes mellitus is a complex disorder associated with increased risk of heart failure, stroke, blindness, neuropathy, and kidney disease, and is reaching epidemic proportions. Out of the estimated 422 million people currently afflicted with diabetes worldwide, about 5% have type 1 diabetes (T1D), which results from autoimmune destruction of beta cell function and mass. The remaining ~95% of diabetics have type 2 diabetes (T2D), a multi-tissue disease wherein peripheral insulin resistance is compounded by dysfunction and demise of beta cells.

Treatment options for patients with T1D are limited. Currently, the mainstay T1D treatment is exogenous insulin replacement. Cutaneous insulin pumps and closed-loop insulin pumps can also be used for the automatic administration of insulin under basal and postprandial conditions. The danger in using exogenous insulin replacement, however, is that normal, glucose-dependent biphasic release of endogenous insulin is lost and life-threatening hypoglycemic episodes are often affiliated with this therapy. Moreover, the demise of beta cell mass persists. Other T1D therapies currently under investigation are mostly immunosuppressant drugs, such as rituximab, teplizumab, and Cyclosporin A. The problem in using this approach is that the long-term safety of these therapies remains unknown. Islet transplantation is currently offered as an investigational therapy and has proven to be promising in treating T1D [1]. However, islet

transplantation procedures are limited by the number available donor islets, the need for immunosuppression, and the longevity of the grafted islets [2]. Thus, there is a need for novel T1D preventative therapies that can protect functional  $\beta$ -cell mass against destruction.

Unlike the limited monotherapy available for T1D, T2D by nature lends itself to various treatment approaches that aim to enhance insulin sensitivity, and others that aim to improve insulin secretion. Current T2D treatments range from diet and weight loss to various combinations of oral and injectable therapies (Table 1.1.). If diet and exercise are not sufficient to meet the accepted Hemoglobin A1c (HbA1c) threshold (typically  $< 7.5\%$ ), pharmacotherapy is initiated. The first line therapy, metformin, belongs to the family of biguanidine drugs and is thought to reduce blood glucose levels primarily by suppressing gluconeogenesis in the liver via activation of AMPK. However, the precise mechanism of action of metformin is poorly understood. Importantly, metformin is contraindicated in individuals with renal, liver, or cardiorespiratory failure, and is known to cause gastrointestinal side effects, which significantly limits its use. For those patients who cannot take metformin, or have no significant improvement in HbA1c, the second line therapy recommended for T2D patients is combination therapy of insulin and a sulfonylurea. Sulfonylureas work at the level of the beta cell to enhance insulin secretion via binding to the ATP-dependent potassium channel ( $K_{ATP}$ ). While sulfonylurea use is rapidly effective and has been shown to reduce microvascular complications [3], the risk of hypoglycemia and accompanying

weight gain associated with sulfonylurea therapy are major treatment-limiting factors.

Treatment	Target	Mechanism	Advantages	Disadvantages
<b>Tier 1: First-Second Line Therapies</b>				
Biguanide	Liver	↑ hepatic insulin sensitivity; ↓ gluconeogenesis and lipogenesis, via activating hepatic AMPK	↓ macrovascular events and mortality (UKPDS); Weight neutral; No hypoglycemia	GI side effects; Contraindicated in patients with kidney, liver, cardiorespiratory insufficiency, alcoholism or older age.
Insulin	Sk.Muscle Adipose  Liver	↑ glucose uptake in muscle, fat; ↓ hepatic glucose output	Rapidly effective; Improved lipid profile	Hypoglycemia; Daily injections; Constant monitoring.
Sulfonylurea	Pancreatic beta cell	↑ insulin secretion via binding to ATP-dependent K <sup>+</sup> channel	↓ microvascular complications (UKPDS); Rapidly effective	Severe hypoglycemia; GI issues, weight gain; CV safety issues; Hasten beta cell death.
<b>Tier 2: Lesser-Validated Therapies</b>				
Thiazolidine dione [TZDs]	Liver Adipose	↑ expression of genes that promote lipid storage and enhance hepatic insulin sensitivity	↓ loss of beta cell function; ↑ insulin sensitivity	↑ MI risk; Weight gain; Fluid retention; ↑ LDL, cholesterol; ↑ Bone fracture risk in women.
GLP-1 agonist	Pancreatic beta cell	↑ insulin secretion via binding to GLP-1 receptor	Weight loss; ↓ hypoglycemic episodes	↑ GI side effects; Requires injection; Fail if patients are insulinopenic.
<b>Other Therapies</b>				
DPP-4 inhibitor	Pancreatic beta cell	Inhibit cleavage of endogenous GLP-1	Weight neutral	Long-term safety ?; Risk of heart failure
SGLT2 inhibitor	Renal proximal tubule	Inhibits sodium-glucose cotransporters,	Weight loss; ↓ systolic blood pressure	urinary tract infections; Long-term safety ?
Alpha-glucosidase inhibitor	Small intestine	Delays carbohydrate digestion	Weight neutral; No hypoglycemia if taken alone	↑ GI side effects.
Meglitinide	Pancreatic beta cell	↑ insulin secretion via binding to K <sub>ATP</sub> channel	Rapidly effective	Hypoglycemia; Weight gain; Frequent dosing.

**Table 1.1. Current T2D treatments**

If sulfonylurea/insulin therapy is ineffective or causes side effects, the recommended Tier 2 therapy is the use of thiazolidinediones (TZDs). Tier 2 classification denotes therapies that are lesser-validated than Tier 1 therapies. TZDs lower blood glucose levels by increasing the expression of genes that promote lipid storage and enhance hepatic insulin sensitivity (Table 1.1.). However, the FDA has recently restricted TZDs due to affiliated severe risk of myocardial infarction, weight gain, and fractures. In lieu of prescribing TZDs, physicians may prescribe a glucagon-like receptor (GLP-1) agonist, a class of drugs that mimic the effects of the incretin, GLP-1, which causes increased insulin secretion, decreased glucagon release, increased satiety and reduced gastric motility. Although GLP-1 agonists have been shown to promote weight loss and have relatively lower risk of hypoglycemia, the frequent and often severe gastrointestinal side effects associated with GLP-1 agonists limit their use as well. Alarmingly, new evidence shows long term use of the GLP-1 agonist, Liraglutide, compromises human beta cell function in vivo [4]. Other therapies include dipeptidyl peptidase-4 (DPP-4 inhibitors), sodium-glucose cotransporter-2 (SGLT2) inhibitors, alpha-glucosidase inhibitors, and meglitinides; these act by increasing endogenous GLP-1 levels, inhibiting glucose reabsorption from the proximal tubule of the kidney, delaying carbohydrate digestion, and increasing insulin secretion, respectively. In addition, DPP-4 inhibitors can increase heart failure risk, alpha-glucosidase inhibitors cause gastrointestinal side effects, and meglitinides carry the risk of hypoglycemic episodes.

Taken together, it is clear that currently available therapy options for T2D are limited in terms of long-term safety, of achieving optimal glucose-dependent insulin secretion or promoting/protecting functional beta cell mass. Furthermore, none of these therapies directly promote skeletal muscle-mediated insulin sensitivity and action (i.e. glucose uptake), which accounts for ~80% of all excess glucose disposal by peripheral tissues [5, 6]. With 30-40% of the U.S. population predicted to be at risk for diabetes [7], this creates an urgent need for novel therapies that can dually protect functional beta cell mass and peripheral insulin sensitivity.

A review of the literature spanning the past 25 years reveals dually active factors that are required in both beta cell function and peripheral insulin action – SNARE proteins. Indeed, in the late 1990's, Nagamatsu and colleagues [8] demonstrated that diabetic GK rat islets were deficient in two t-SNARE proteins, Syntaxin 1A (STX1A) and SNAP25, and that replenishment of these factors resolved islet dysfunction. More recently, we demonstrated the capacity to restore normal function to dysfunctional T2D human islets by replenishing Syntaxin 4 (STX4), previously considered to be an inactive and redundant t-SNARE isoform in the  $\beta$ -cell [9]. Thus, my dissertation studies sought to explore emerging evidences that suggest there are deficiencies/defects in exocytosis proteins in diabetic rodents and humans, and to interrogate the consequences of overexpressing certain exocytosis factors in the context of maintaining glucose homeostasis. In doing so, I have assessed clues from other diseases, such as neurodegenerative disorders

and cancer, for which there is accumulating data on the benefits of replenishing deficient exocytosis proteins.

## **1.2. SNARE EXOCYTOSIS MACHINERY: THE “NUTS AND BOLTS”**

Exocytotic trafficking of proteins and lipids from the cytosol to the cell exterior constitutes one of the most important processes in the cell. Most eukaryotic cells achieve this via packaging of protein or lipid cargo in membrane-bound vesicles, which originate from the trans-Golgi network or recycling endosomes and are subsequently transported via cytoskeletal remodeling to the plasma membrane. Once at the membrane, vesicle docking, priming, and fusion depends on the high-affinity interaction of a complex of highly conserved proteins called SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors). This fundamental mechanism of regulated SNARE complex assembly is conserved in many different cells types, including neurons, exocrine, hematopoietic, and endocrine cells.

### **1.2.1. The SNARE core complex**

The SNARE complex consists of two target membrane (t)-SNARE proteins, Syntaxin (STX) and SNAP23 (or SNAP25), and one vesicle associated (v-SNARE) protein, VAMP [10-15]. STX proteins are ~35 kDa containing a carboxy-terminal transmembrane domain spanning the plasma membrane and an N-terminus oriented toward the cytoplasm [16]. The other t-SNARE type, SNAP-23/25 (23 or 25 kDa in size), is associated with the plasma membrane via palmitoylation of four cysteine residues in the central region of the protein [17].

The v-SNARE, VAMP, is a 18-20 kDa protein with a vesicle membrane-spanning carboxyl terminus and an N-terminus oriented away from the vesicle, toward the cytoplasm [15]. Ultrastructural evidence of the SNARE complex shows that one v-SNARE binds with two cognate t-SNARE proteins in a heterotrimeric 1:1:1 ratio [12, 14]. This SNARE core complex is extremely stable and is sufficient to withstand the energy barrier required to fuse the vesicle to the membrane [18]. Once fused, the SNARE complex is described as being in 'cis' configuration, as opposed to when the vesicle is previously docked or tethered and in 'trans' configuration (when the lipid bilayer of the vesicle is distinct from the plasma membrane, not yet having merged). The SNARE core complex is notoriously SDS-resistant, requiring boiling in SDS-containing buffer to dissociate into free monomers. Following vesicle fusion, the cis- complex SNARE proteins are bound by alpha-SNAP and NSF (N-ethylmaleimide sensitive factor) proteins to catalyze SNARE complex dissociation, allowing endocytosis of the v-SNARE and recycling of the individual t-SNAREs back to their respective plasma membrane compartments [12].

### **1.2.2. Regulation of the core complex -- SNARE accessory factors**

SNARE-mediated vesicle fusion is tightly regulated by accessory binding proteins, such as "SM" (Sec1/Munc18), Munc13, and DOC2 (double C2-domain containing proteins). Munc18 proteins, also called "syntaxin binding proteins (STXBP)", are ~66-68 kDa in size, are soluble, and do not contain a transmembrane domain [19]. These proteins are localized to the cytosol, and to the plasma membrane through direct interaction with their cognate Syntaxin partners [20, 21]. It is

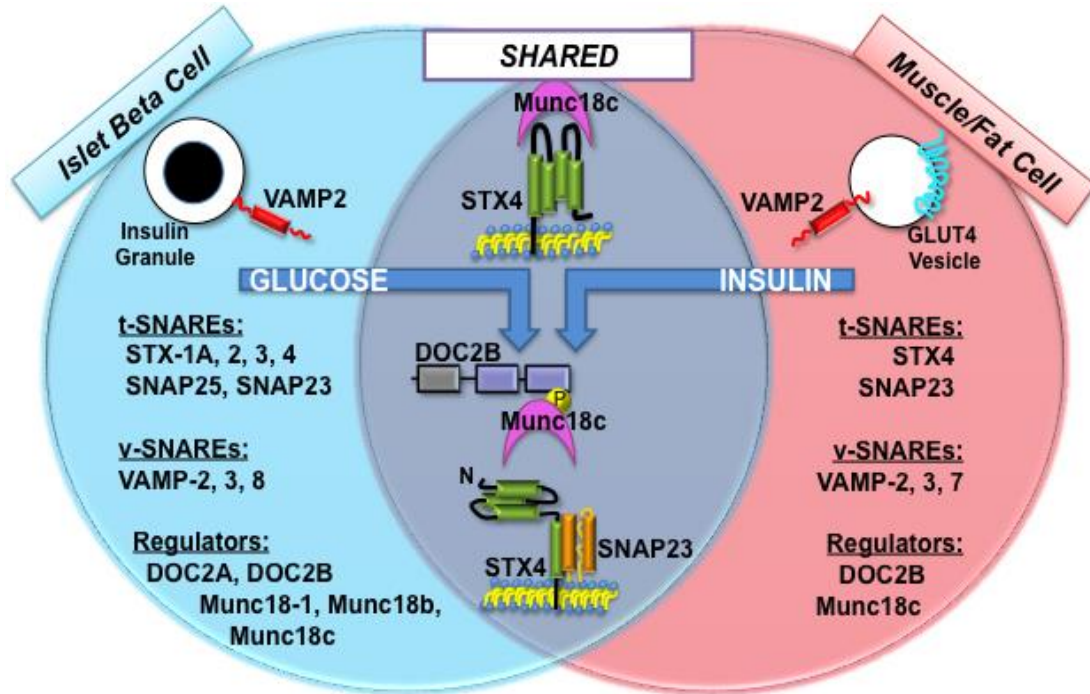
proposed that upon stimulation, Munc18 proteins assist Syntaxin's conformational change to its accessible, "open" or "active" conformation for its subsequent engagement as part of the SNARE core complex (i.e. VAMP, SNAP23/25, STX), and docking and fusion of vesicles [22, 23].

In addition to SM proteins, the calcium- and phosphoinositide binding protein isoform DOC2B has been shown to be essential for SNARE core complex assembly to occur in islet beta cells and muscle/fat cells [24, 25]. DOC2B is a ubiquitously expressed 46-50 kDa protein. In islet beta cells, DOC2B binds to the SNARE regulatory proteins Munc18-1 and Munc18c, via its C2A and C2B domains, respectively [26-28]. Other calcium-sensing C2-domain-containing proteins, including Munc13 and synaptotagmin, are implicated in accelerating SNARE complex formation and insulin release from beta cells via fostering STX1-based actions [29-32]. In skeletal muscle cells, DOC2B facilitates STX4-based actions [33]. Since many of these regulatory factors are implicated in T2D, I will discuss in detail their tissue-specific and conserved mechanisms required for insulin secretion and peripheral insulin action, in the following section.

### **1.3. TISSUE-SPECIFIC AND CONSERVED SNARE PROTEINS IN DIABETES-RELATED TISSUES**

Amongst the diabetes-related tissues (pancreatic beta cells, skeletal muscle cells, fat cells), there are exocytosis factors that are cell-type specific, and other factors that are conserved but that exhibit distinct mechanisms suited to the function of

that tissue in maintaining glucose homeostasis. As depicted in Figure 1.1., the set of SNARE core complex proteins required by beta cells, fat cells and muscle cells consists of STX4, SNAP23, and VAMP2. Islet beta cells, being neuroendocrine-derived, also express and utilize neuronal-specific isoforms of SNARE proteins, namely STX1A, SNAP25, DOC2A and Munc18-1. Hence, beta cells have some overlapping and functional redundancy in SNARE isoform usages. By contrast, skeletal muscle and fat cells have little to no redundancy, with only one t-SNARE of each type expressed (STX4 and SNAP23). Additional t- and v-SNAREs shown in Figure 1.1. are reportedly expressed and used in some cases but are not necessarily required for normal function (e.g. VAMP3). The following sections will explore the detailed mechanisms underlying beta cell insulin secretion and skeletal muscle glucose uptake. Of note, while the focus of this review is to highlight pre-diabetes and T2D through the lens of SNAREs levels, SNARE defects are one of a number of other defects (e.g. signaling) that contribute to T2D.



**Figure 1.1. SNARE and SNARE accessory protein isoforms utilized by islet beta cells and skeletal muscle cells for vesicle exocytosis: a common subset of factors.** Factors listed in the beta cell (left circle, blue) are known to facilitate glucose-stimulated insulin secretion, and those listed in the skeletal muscle cell (right circle, red) are required for insulin-stimulated glucose uptake. Those isoforms utilized by both beta cells and muscle cells are shown in the center of overlap.

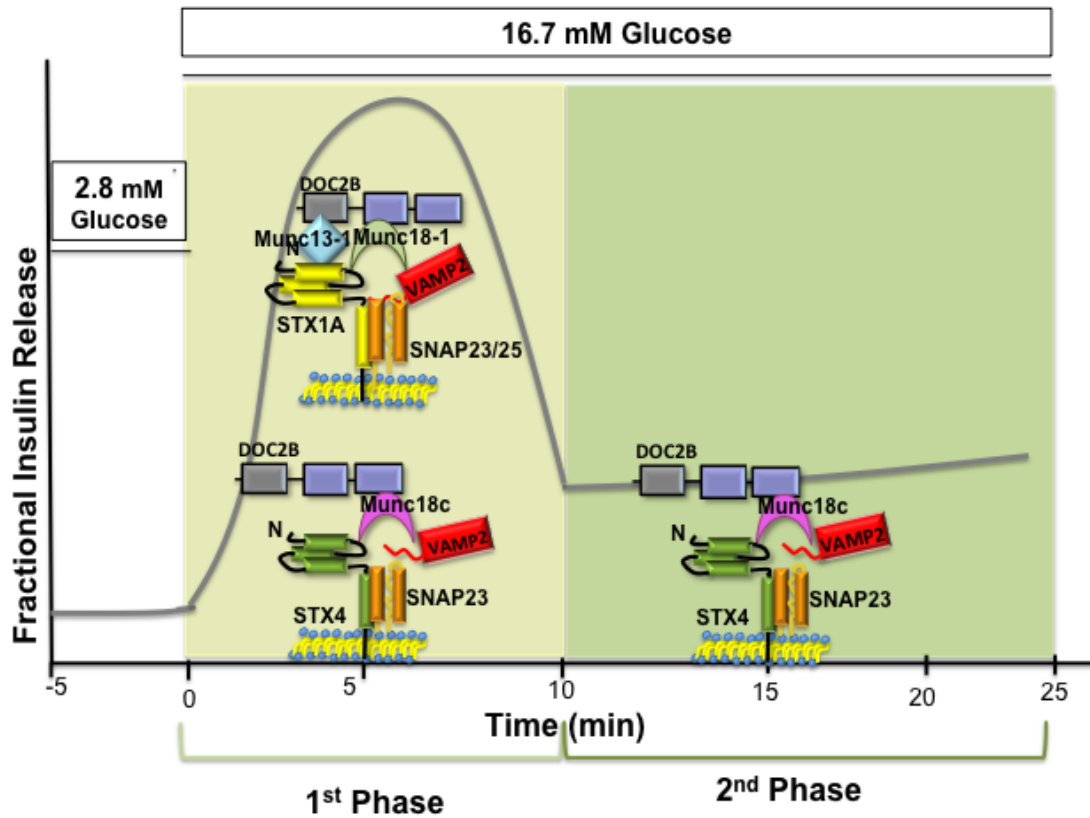
## **1.4. EXOCYTOSIS IN THE BETA CELL: BIPHASIC GLUCOSE STIMULATED INSULIN SECRETION**

Interestingly, although the majority of the SNARE isoforms were identified in the early 1990's [34], only the neuronal cluster of STX1A, SNAP25 and VAMP2 were initially studied for functionality in insulin release mechanisms. SNAP23 was found capable of substituting for SNAP25 in insulin secretion [35] shortly thereafter, but it was another decade before the functional requirements for other Syntaxin and SM isoforms such as Syntaxin 4 (STX4), Munc18b and Munc18c, were examined in the process of insulin release [36-38]. The beta cell is now known to express and use all four plasma membrane localized STX isoforms, STX1-4. At the plasma membrane, isoforms STX1-3 can bind to Munc18-1 (also called Munc18a) and Munc18-2 (Munc18b) [19, 39, 40], whereas only STX4 can bind to Munc18c [41, 42]. The following section discusses the ornate complexity of how these SNARE proteins are utilized that contributes to the biphasic pattern of insulin release.

### **1.4.1. Biphasic secretion and affiliated SNARE complexes in the beta cell**

Insulin granule exocytosis in the pancreatic beta cell is elicited in two discrete phases in response to a nutrient stimulus, commonly referred to as glucose-stimulated insulin secretion (GSIS). GSIS is evoked by sensing of the beta cell to a stimulatory level of extracellular glucose (16.7 mM most commonly used experimentally). This glucose enters the beta cell by passing through the constitutively-plasma membrane localized glucose transporter, GLUT2 (rodent)

or GLUT1 (human) [43]. Once inside the beta cell, glucose is metabolized to yield an increased ratio of cellular ATP:ADP. Increased ATP induces closure of the  $K_{ATP}$  channels, causing membrane depolarization and opening of voltage-dependent calcium channels, increasing intracellular calcium ( $[Ca^{2+}]_i$ ) levels. Elevated  $[Ca^{2+}]_i$  triggers the SNARE and regulatory proteins to facilitate priming and fusion of insulin-filled granules with the plasma membrane to prompt release of the insulin cargo from the cell. This chain of events constitutes the first-phase of GSIS (Figure 1.2.), occurring within 5-10 minutes of glucose stimulation, and uses STX1A- or STX4-based SNARE core complexes involving SNAP25 or SNAP23, and VAMP2 [35, 38, 44-46]. Second-phase insulin release, which is characterized by the mobilization of insulin granules from intracellular storage pools out to the plasma membrane, occurs beyond 10 minutes of glucose stimulation. Second-phase GSIS utilizes STX4, SNAP25 or SNAP23, and VAMP2 [38, 44, 47] (Figure 1.2.). Recent reporting has also shown that STX1A may be involved in second-phase GSIS as well [48]. Other Syntaxin isoforms, (e.g. STX2 and STX3) have also been shown to participate in beta cell exocytosis events, although are affiliated with more rare forms of exocytosis [49-51]. Another v-SNARE protein, VAMP8, also functions in insulin secretion but is required selectively for glucagon-like peptide (GLP-1) enhanced insulin release [52]. VAMP3 is also expressed in the  $\beta$ -cell, but knockout mouse models exhibited no secretion defects, suggesting that this isoform may be redundant [53].



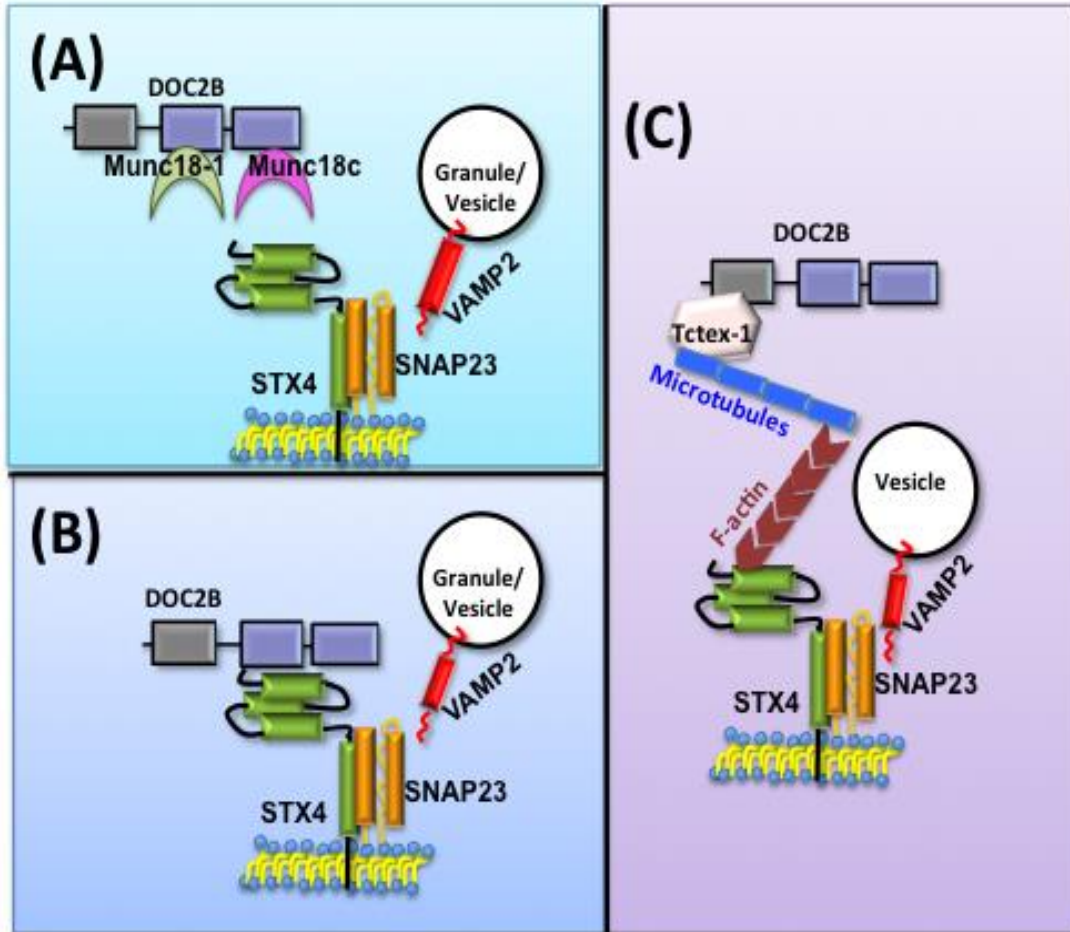
**Figure 1.2. Differential requirement of SNARE and SNARE accessory protein isoforms during each phase of glucose stimulated insulin secretion from islet beta cells.** While the first-phase of insulin release uses both STX1- and STX4-based complexes, the second phase of insulin release relies upon STX4-based SNARE complexes. Accessory factors shown to be affiliated with these complexes are also grouped with the corresponding t- and v-SNARE core complexes. Note: SNAP25/23 denotes that SNAP25 and SNAP23 both suffice in the affiliated complex.

#### **1.4.2. Regulation of Insulin Secretion -- SNARE accessory proteins**

While it is generally agreed that the Munc18 proteins facilitate the SNARE complex assembly process, the detailed protein-protein interaction changes in response to glucose stimulation are not agreed upon. For example, both Munc18-1 and Munc18c have been demonstrated to undergo phosphorylation and subsequent dissociation from their cognate STX partner [54-56]. However, other reports suggest that the Munc18 proteins remain bound or will reposition to become part of the final SNARE complex [57, 58]. In general, studies favoring the dissociation model stem from cell or tissue studies, whilst the other model stems from in vitro assays. Interestingly, Munc18c is only required for second-phase insulin secretion, whereas STX4 and DOC2B are required for both phases [24, 36, 38] (Figure 1.2.). Munc18-1 regulates first-phase secretion, and while initially this was presumed to occur via STX1A binding changes, it was later discovered that Munc18-1 overexpression enhances STX4 activation [59]; this has been since proposed as an explanation for why STX4 functions in first-phase insulin release.

Akin to the controversy described above regarding the molecular binding mechanisms of Munc18 proteins, the details regarding DOC2B's mechanism(s) of action are controversial. For example, Figure 1.3.A depicts DOC2B binding to Munc18-1 and Munc18c directly, via DOC2B's C2A and C2B domains, respectively, scaffolding both Munc18 isoforms concurrently in a heterotrimeric complex [26]. Alternatively, it has been suggested that DOC2B binds to STX4

rather than Munc18 proteins, involving a calcium-dependent mechanism [60] (Figure 1.3.B). The key difference between proposed mechanisms is the direct or indirect nature of DOC2B binding to STX4 to evoke its activation. While the details of the direct binding model (Figure 1.3.B) are still evolving, the Munc18-binding model (Figure 1.3.A) purports that glucose stimulation triggers rapid Munc18c phosphorylation, switching its affinity for binding to DOC2B and away from STX4, which permits STX4 opening for its engagement in SNARE core complexes [56]. As described below, some of these mechanistic concepts are also proposed in GLUT4-dependent glucose uptake in peripheral tissues.



**Figure 1.3. Proposed mechanisms underlying the benefits of DOC2B overexpression.** Overexpression of DOC2B has shown positive effects on whole body glucose homeostasis. The mechanism by which DOC2B overexpression exerts its positive effects in beta/muscle cells is still yet to be elucidated. (A) DOC2B proposed to function as a scaffold for Munc18-1 and Munc18c binding and subsequent activation of STX4. (B) DOC2B proposed to directly bind to STX4, allowing for STX4 activation and promotion of SNARE formation. (C) DOC2B proposed to bind the light chain (Tctex-1) of motor protein, Dynein, which functions along microtubules. DOC2B may act direct/indirectly on actin remodeling, subsequently allowing for translocation of vesicles to the plasma membrane.

## **1.5. EXOCYTOSIS OF GLUT4 VESICLES AND GLUCOSE UPTAKE IN SKELETAL MUSCLE AND FAT CELLS**

Following a meal, glucose uptake into peripheral skeletal muscle accounts for ~80% of whole body glucose clearance, whereas fat cells account for the remaining 20% via intracellular processes that promote trafficking of the vesicle containing the insulin-responsive glucose transporter, GLUT4 [61-65]. This process begins with insulin binding to the insulin receptor (IR) to induce its tyrosine autophosphorylation, which in turn activates the canonical PI3K→AKT signaling pathway, triggering the intracellularly localized GLUT4-containing vesicles to translocate to the sarcolemmal and T-tubule membranes, where the vesicles are docked and fused via SNARE proteins.

In the late 1990's numerous groups independently deduced that the t-SNARE isoforms STX4 and SNAP23, and v-SNARE VAMP2, comprised the machinery necessary and sufficient for GLUT4 vesicle docking/fusion [66-70]. This mechanism was later expanded to include a pivotal regulatory step by which the SNARE complex assembled: the insulin-activated IR directly phosphorylates Munc18c to trigger the activation of STX4, fostering SNARE complex formation [71, 72]. Since Munc18c phosphorylation occurs independently of PI3K, these results gave rise to the current model, wherein insulin elicits a coordinated response through activation of IR to evoke t-SNARE assembly in sync with PI3K-mediated vesicle mobilization to the plasma membrane, culminating in coordinate SNARE core complex formation and GLUT4 vesicle fusion. Given the ubiquitous

expression of this grouping of SNARE and SNARE accessory factors, this also provides a testable model for other exocytosis events initiating from pivotal extracellular signals.

#### **1.5.1. Regulation of GLUT4 Translocation/Glucose uptake – controversy surrounding SNARE accessory proteins**

While all groups agree fully that DOC2B plays a positive role in regulating insulin-stimulated GLUT4 vesicle exocytosis and SNARE complex formation, DOC2B's mechanism(s) of action in peripheral tissues, like those described in the beta cell, remain controversial, depending upon the experimental system used to derive the mechanism. For example, studies of primary mouse skeletal muscle or GLUT4myc-L6 myoblasts show that insulin stimulation increased DOC2B binding to phosphorylated Munc18c, coordinate with increased STX4 activation and SNARE complex formation [24, 33]. Consistent with this, DOC2B knockout (-/-) mouse skeletal muscle harbors abundant Munc18c-STX4 complexes and reduced SNARE complex formation [24]. In contrast, studies using 3T3L1 adipocytes or in vitro mixing assays using recombinant DOC2B report a direct STX4-DOC2B binding interaction [73, 74]; this interaction is not observed in primary tissues or L6 muscle cells [24]. Although this has yet to be experimentally reconciled, differences that might underlie these distinct mechanisms could be that the in vitro and 3T3L1 adipocyte studies assessed DOC2B association with STX4 using co-immunoprecipitations from cell lysates, a method that cannot distinguish direct from indirect binding interactions [73, 75]. In vitro studies also exclude additional DOC2B binding factors (such as microtubule

and/or actin cytoskeletal factors) that might otherwise influence how DOC2B associates with STX4. Indeed, DOC2B has been shown to interact with microtubule-associated Tctex-1 type proteins [75, 76]. Additionally, STX4 interacts with F-actin via the protein, Gelsolin [77]; this STX4-F-actin interaction may very well coordinate with microtubule-dependent trafficking of insulin, as insulin granules are known to require microtubule tracks for transport to the plasma membrane [78]. Hence, microtubule factors might bridge the DOC2B-STX4 interaction (as modeled in Figure 1.3.C). DOC2B also harbors an N-terminal Munc13 interacting domain (referred to as the MID domain), yet no Munc13 partners for DOC2B are yet identified in fat or skeletal muscle cells. Bridging factors remain relatively unexplored as potential targets for improving peripheral insulin sensitivity.

## **1.6. DEFICIENT, DEFECTIVE AND MIS-LOCALIZED EXOCYTOSIS FACTORS IN T2D TISSUES**

Following the discovery of important SNARE isoforms involved in insulin secretion and glucose uptake, several reports published data correlating deficiencies in SNARE and regulatory proteins to T2D and obesity in human subjects (as referenced in Table 1.2.). Several recent reports link polymorphisms in STX1A to impaired glucose metabolism in obese human subjects, and to the age of onset and insulin requirement in T2D individuals [79, 80]. Another report links decreased STX4 gene expression with T2D and psoriasis [81], and suggested that STX4 be considered a biomarker for T2D development in psoriasis cases. STX4, amongst other SNARE proteins such as STX1A, SNAP25 and VAMP2, have all been shown to be deficient in T2D human islets (Table 1.2.). Notably, the regulatory factors of these SNARE proteins, such as multiple Munc18 isoforms, DOC2B, Munc13-1, multiple synaptotagmin isoforms, and synaptophysin, are also deficient [8, 9, 31, 82-86]. Interestingly, SNAP23 was noted to be deficient at the site of exocytosis (the plasma membrane), but overall quantities were either unchanged or even increased [9, 87]. Modeling this in spontaneous rodent models of pre-diabetes and T2D, such as the obese and diabetic Zucker rats and the non-obese diabetic GK rat, revealed that many of these same exocytosis factor deficits are conserved (Table 1.2.). Although recent studies point to significant differences between human and mouse islets in terms of islet architecture and islet cell function, deficiencies in SNARE proteins under conditions of obesity and T2D are uniformly similar. Many SNARE deficiencies

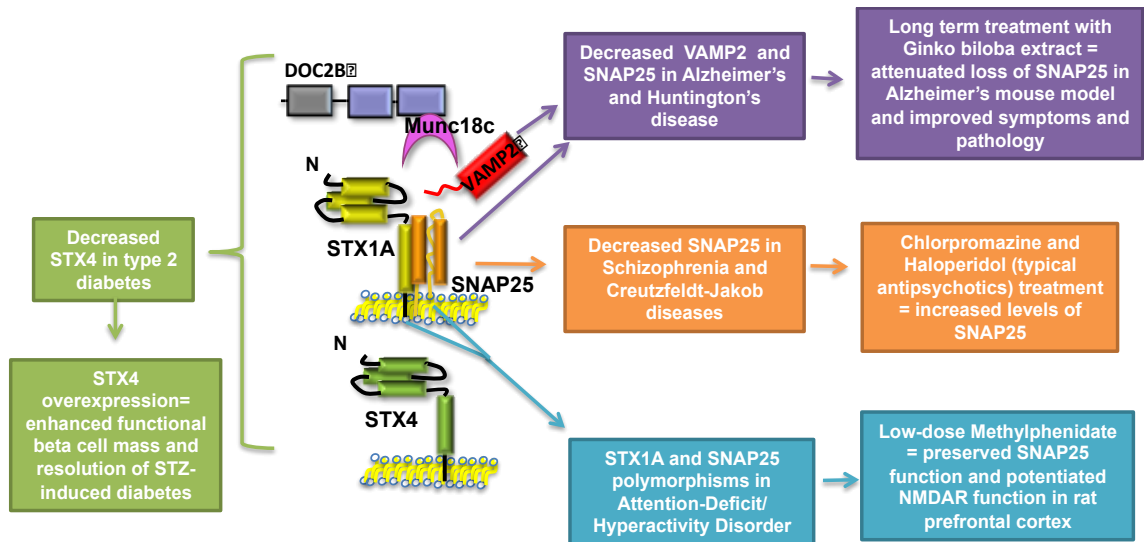
have been studied using gene-targeted ablation knockout mouse models, resulting in dysregulated metabolic phenotypes. This raises the question as to whether deficiencies of SNARE/regulatory proteins are a cause or a consequence of diabetes. The prevailing hypothesis is that deficiencies of SNAREs are likely a consequence of diabetes. However, STX1 gene mutations and polymorphisms have been linked with T2D, and SNAREs are noted as being targets of miRNA [88, 89] and lncRNAs [90, 91], suggesting that genetic and/or epigenetic links will need to be explored to fully address this question. Furthermore, whether exocytosis protein deficiency underlies  $\beta$ -cell dysfunction in T1D remains under explored. This is a particularly important point of interest, as the preclinical phase of T1D is marked by declining  $\beta$ -cell function [92, 93], and there exists a critical need for the ability to assess early loss of functional  $\beta$ -cell mass in predicting T1D onset.

Protein	Tissue	Protein Levels in T2D Humans	Protein Levels in T2D Rodents	References
DOC2B	Islets	Decreased	ND	[85]
Munc13-1	Islets	Decreased	Decreased in diabetic GK and obese Zucker fa/fa rats	[31, 82]
Munc18-1	Islets	Decreased	ND	[9, 82]
Munc18c	Islets; Sk. Muscle; Adipose	Decreased Decreased Decreased Decreased	Decreased in diabetic GK rats	[8, 82-84, 94]
SNAP23	Sk. Muscle	Decreased at site of exocytosis (PM)	ND	[87]
SNAP25	Islets	Decreased	Decreased in diabetic GK rats	[8, 82, 94]
STX1A	Islets	Decreased SNP (D68D, T to C) correlates to age at onset and insulin requirement in T2D	Decreased in hyperglycemic GK rats	[8, 9, 80, 82, 86]
STX4	Islets, Sk. Muscle	Decreased in both tissues	ND	[9, 84]
Synaptophysin	Islets	Decreased	ND	[82]
Synaptotagmin 4, 7, 11	Islets	Decreased	ND	[86]
VAMP2	Islets	Decreased	Decreased in diabetic GK rats	[82, 94]

**Table 1.2. Exocytosis protein expression in Type 2 diabetic humans and rodents.** Note: presented in alphabetical order of protein name. ND, not determined. Sk. Muscle, skeletal muscle; GK, Goto-Kakizaki.

## **1.7. CAN DEFICIENT/DEFECTIVE EXOCYTOSIS FACTORS BE REPLENISHED TO REMEDIATE DISEASE? CLUES FROM NEURODEGENERATIVE DISORDERS**

One of the earliest reports linking defects in exocytosis proteins to human disease involved senile plaques of Alzheimer subjects, which were found to be deficient in exocytosis proteins - namely STX1A, synapsin, snaptoophysin and synaptotagmin [95]. Akin to findings in islets described above, deficiencies in SNAP25 and STX1A were found in patients with Creutzfeldt-Jakob disease [96] (Figure 1.4.). Decreased abundances or functions in exocytosis proteins were also reported in subjects with Huntington's disease, Schizophrenia and Attention-Deficit/Hyperactivity Disorder [97-101]. In each disease, treatments involved restoration of SNARE proteins (Figure 1.4.). Related to this, STX4 replenishment to human T2D islet cells restored glucose-stimulated insulin secretory function equivalent to that of non-diabetic age-matched human islets [9], providing proof of concept for this as a restorative approach.



**Figure 1.4. Exocytosis proteins implicated in human neurological and metabolic diseases.** SNARE protein abundances (STX4, STX1A, SNAP25, and VAMP2) are decreased and genetic polymorphisms are implicated in a variety of neurodegenerative, autoimmune, and metabolic diseases. Therapies are proposed based on current studies that aim to replenish/preserve abundance of exocytosis proteins.

## **1.8. ARE EXOCYTOSIS PROTEINS IMPLICATED IN CELL SURVIVAL AND PROLIFERATION? CLUES FROM CANCER**

### **1.8.1. STX4 and DOC2B as Targets to Combat Cancer?**

Recent investigations have suggested that STX4 and DOC2B may act as tumor suppressors. For example, downregulation of STX4 was recently shown to be associated with cancer cell proliferation, as STX4 is required for acid sphingomyelinase translocation, a vesicle trafficking event needed for normal apoptotic mechanisms [102]. Similarly, DOC2B is required to regulate pro-apoptotic mechanisms in human cervical cancer cells, combating their proliferation; DOC2B transcription was found to be downregulated in several human cancer cell lines due to hypermethylation of the DOC2B promoter [103]. DOC2B replenishment in cervical cancer cells led to increased actin cytoskeleton remodeling, and inhibition of AKT and ERK hyper-phosphorylations, yielding attenuation of cell migration to decrease cancer cell growth [103]. Importantly, no increases in common mitogenic pathway proteins such as MAPK, ERK, or mTOR mitogenic pathways were reported with overexpression of STX4 or DOC2B. This suggests that efforts to overexpress STX4 or DOC2B as a means to treat  $\beta$ -cell and/or skeletal muscle dysfunction may be safe from tumor/cancer inducing side effects. This is a necessary consideration since efforts to increase  $\beta$ -cell mass are currently being scrutinized with concerns about inducing cancer.

### **1.8.2. STX4 and DOC2B – Potential Roles in Proliferation**

Despite studies suggesting that STX4 and DOC2B have tumor suppressor capabilities, other studies have implicated STX4 and DOC2B in playing roles in

proliferating cells during early embryogenesis. For example, STX4 homozygous (-/-) null mice died during early embryogenesis [69], and this was later linked to STX4's vital role in facilitating GLUT8 exocytosis to support glucose influx for the growing blastocysts [104]. In neurons, DOC2B expression occurs long before neurotransmitter release is functional, and this early expression pattern of DOC2B correlates with that of genes involved in neuronal proliferation and differentiation, such as the neuroepithelial stem cell marker, nestin [105]. Furthermore, studies have shown that DOC2B has a critical role in synaptic vesicle trafficking as early as embryonic day 18 (E18), as neurons from DOC2B deficient (-/-) knockout mice of this age exhibited impaired spontaneous release frequency [106]. This points to a putative role for DOC2B in the delivery of membrane proteins to the surface of proliferating neurons and/or to the tip of outgrowing axons. Whether overexpression of STX4 or DOC2B could be harnessed as a means to protect/promote beta cell proliferation will be an important area of future investigation.

### **1.9. EXOCYTOSIS PROTEIN REPLENISHMENT AND GLUCOSE HOMEOSTASIS: ISOFORM SPECIFICITY MATTERS**

The first demonstration that restoration of t-SNAREs to normal levels could recover insulin secretion in diabetic GK rat islets provided proof of concept for SNARE replenishment as an approach for restoring islet function [8]. The increasing evidences of exocytosis protein deficiencies in islets of obese and diabetic humans and rodents spurred the generation of several transgenic rodent models designed to test the effect of increased abundance of specific SNARE

isoforms on the regulation of islet function and glucose homeostasis in vivo (Table 1.3.).

Genotype	Alteration	Metabolic Phenotype	References
STX1A	Increased STX1A in pancreatic beta cells	Fasting hyperglycemia, reduced insulin secretion, insulin intolerance in male mice.	[107]
STX4	Increased STX4 in skeletal muscle, fat, and pancreas	Enhanced insulin sensitivity, GLUT4 translocation and skeletal muscle glucose uptake; Enhanced insulin secretion from islets; Increased healthspan, lifespan; Protected from age- and HFD-induced metabolic dysfunction.	[9, 38, 108, 109]
VAMP2	Increased VAMP2 in neurons	ND	[110]
Munc18-1	Increased Munc18-1 in neurons	ND	[111]
Munc18c	Increased Munc18c in fat, skeletal muscle and pancreas	Insulin resistant, glucose intolerant with impaired skeletal muscle glucose uptake; impaired insulin secretion from islets.	[112]
DOC2B	Increased DOC2B in skeletal muscle, fat, and pancreas	Enhanced insulin sensitivity, GLUT4 translocation and skeletal muscle glucose uptake; Enhanced insulin secretion from islets.	[33]

**Table 1.3. Transgenic mouse models of exocytosis protein overexpression**  
 ND, not determined.

### **1.9.1. STX1A and Munc18c - unexpectedly ineffective**

Despite a deficiency of STX1A and Munc18c in diabetic human and rodent islets (Table 1.2.), over-expression of either STX1A or Munc18c in transgenic mice did not improve glucose tolerance as expected – instead, both models exhibited profound glucose intolerance [107, 112]. Beta cell-specific STX1A overexpressing mice harbored beta cells that were functionally defective, with reduced depolarization-evoked membrane capacitance and reduced currents through the  $\text{Ca}^{2+}$  channels [107]. Years later, STX1A was shown to bind to the SUR1 regulatory subunit of islet beta cell  $\text{K}_{\text{ATP}}$  channels when overexpressed, inhibiting the activity of the channels and causing secretion defects [113]. Munc18c overexpression revealed a similar limitation – Munc18c transgenic mice, overexpressing Munc18c by as little as 2-3 fold in pancreas, skeletal muscle and adipose tissues, harbored dysfunctional islets as well as insulin-resistant skeletal muscle [112]. In both cell types, the additional Munc18c bound and sequestered endogenous STX4, reducing the formation of STX4-based SNARE complexes. These inhibitory actions leave STX1A and Munc18c with too narrow a window of efficacy for remediating islet function or skeletal muscle functions.

### **1.9.2. STX4 – an expandable hub for excitosomes?**

In contrast to mice overexpressing STX1A, mice overexpressing STX4 (simultaneously in pancreatic islets, skeletal muscle, and adipocytes) exhibited enhanced glucose homeostasis, resulting from increased skeletal muscle insulin sensitivity and islet function [108] (Table 1.3.). This is an important distinction between the two STX isoforms, and points to important structure-function

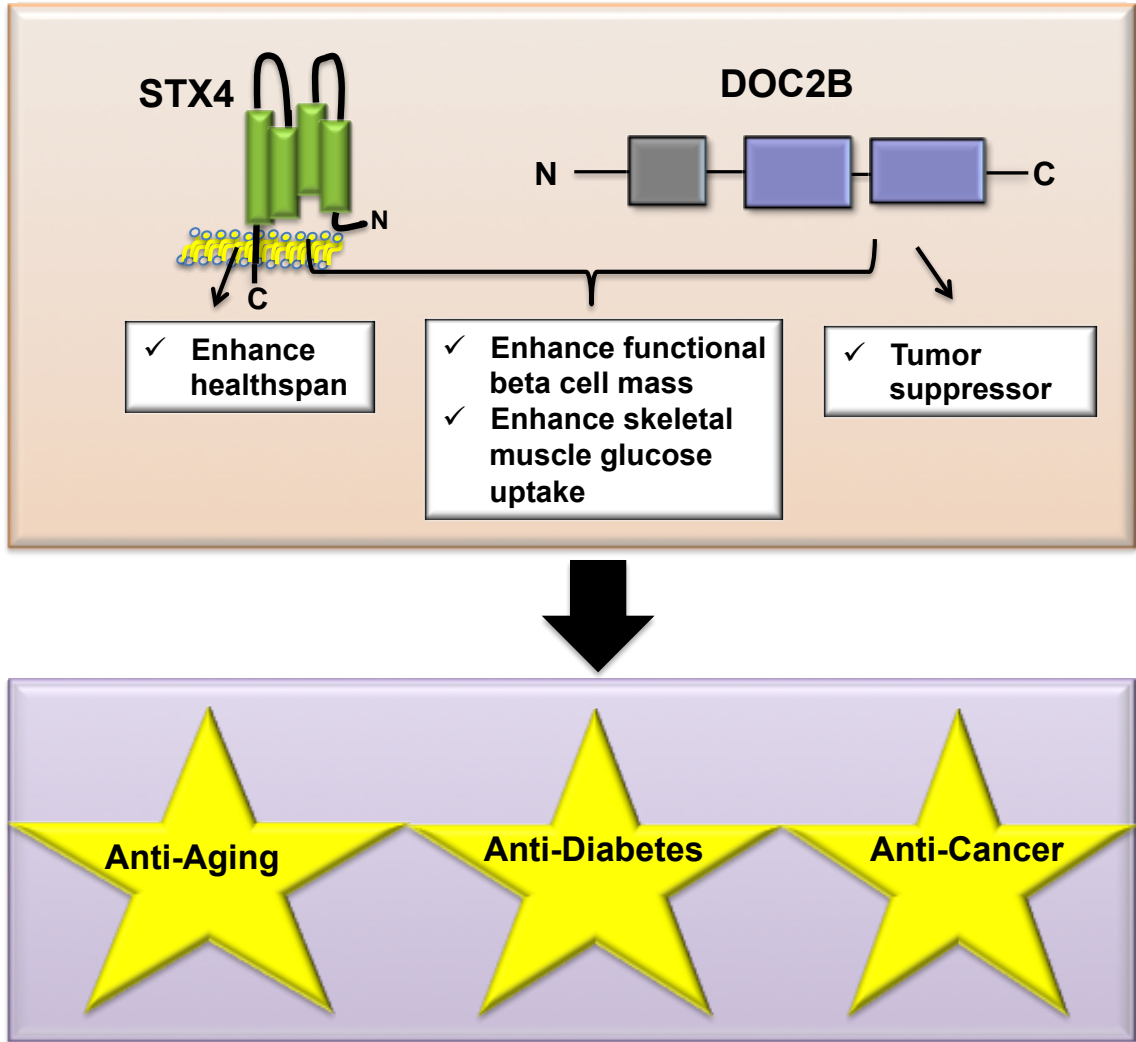
differences between these t-SNARE proteins. Amino acid alignment of STX1A and STX4 shows only 45% sequence similarity, supporting the concept that STX4 partners with significantly different factors. One such factor is filamentous actin (F-actin). Through its unique N-terminal alpha-spectrin like domain, STX4 is the only SNARE protein capable of direct F-actin binding [77, 114-116]. Moreover, in beta cells STX4 can associate with the actin binding and severing protein Gelsolin, and in 3T3-L1 adipocytes with the F-actin crosslinking protein alpha-fodrin [77, 117]. Both STX4-cytoskeletal protein associations are affiliated with positive effects upon vesicle exocytosis in beta cells and fat cells. Although insulin granule exocytosis from beta cells requires actin *depolymerization* [118-120], whilst GLUT4 vesicle exocytosis requires F-actin polymerization [121-123], both are considered F-actin remodeling events that may coordinate the timing of STX4 activation with the arrival of incoming granules/vesicles. If true, STX4's interaction with the actin cytoskeleton may constitute an "excitosome", a localized site at the plasma membrane with concentrated SNARE complex proteins and accessory proteins that promote granule docking to the membrane [124, 125]. Overexpression of STX4 may provide the basis for more excitosomes, i.e. more docking sites for exocytosis in skeletal muscle and beta cells.

The translational implication of targeting exocytosis proteins for diabetes remediation has been effectively demonstrated with STX4 in human pancreatic islets. Islets from human T2D cadaveric donors transduced to replenish STX4 levels to that of non-diabetic islets showed fully restored biphasic insulin

secretion [9]. Furthermore, consistent with STX4 being limiting for function, biphasic insulin release was enhanced to supranormal levels in non-diabetic human islets transduced to overexpress STX4, and transplantation of a very minimal number of these islets (200 per graft) was capable of attenuating STZ-induced diabetes [9]. This intriguing data support the concept of STX4 enrichment as a potential novel therapeutic target for enhancing beta cell function in humans.

### **1.9.3. STX4 Promotes Healthspan and Longevity**

Recent evidence has shown that beyond enhancing insulin secretion and glucose uptake, STX4 overexpression also promotes healthspan and longevity [109]. Transgenic mice overexpressing STX4 in pancreas, skeletal muscle, and adipocytes lived ~33% longer than wild-type control littermates [109]. Furthermore, when challenged with high fat diet-induced obesity, STX4 transgenic mice showed preserved islet insulin secretion and skeletal muscle GLUT4 translocation compared to high fat-fed wild type mice [109]. Microarray analysis of muscle from the STX4 transgenic mice showed changes in pathways of leptin and AMPK signaling, as well as reduced FoxO1 protein levels in pancreata. These intriguing results suggest that preservation of insulin sensitivity into old age, via retaining a rapid rate of glucose uptake/ clearance of excess circulating glucose, may protect against hyperglycemia-related damage to cells, diabetes, and improve overall healthspan and lifespan (Figure 1.5.).



**Figure 1.5. Translational implications for SNARE/accessory proteins?** Based on preliminary animal studies and in vitro analysis, targeting exocytosis proteins such as STX4 and DOC2B for enrichment appears to be beneficial in treatment strategies to deter aging/diminished healthspan, diabetes and cancer. N- denotes N-terminus; C- denotes C-terminus.

#### **1.9.4. DOC2B as a restorative?**

Mice overexpressing DOC2B simultaneously in beta cells, skeletal muscle and adipocytes show a significantly increased capacity for glucose-stimulated insulin granule exocytosis and insulin-stimulated GLUT4 vesicle translocation [33]. While overexpressing DOC2B by only ~3 fold compared to endogenous levels, these transgenic mice were exquisitely insulin sensitive, showing supranormal levels of glucose tolerance (Table 1.3.). Importantly, despite the capacity of the islets to secrete 30-50% more insulin in response to a glucose bolus, the mice never exhibited hypoglycemia. This may be due to the rapid response of the skeletal muscle to the insulin release during first phase, triggering rapid glucose clearance and rapid return to normoglycemia, with this return being the natural cue for the pancreas to decrease release of insulin back to resting/basal levels. With this exquisite coordination and because of its ability to promote STX4 activation, DOC2B over-expression presents an attractive target for restoring glucose homeostasis. The advantage to DOC2B is that it can enhance both phases of insulin release, and if delivered systemically, might enhance skeletal muscle insulin sensitivity concurrently. Whether DOC2B over-expression carries the capacity to protect against diabetogenic stimuli, remains to be tested.

#### **1.9.5. Stoichiometry**

Since overexpression of either STX4 or DOC2B potentiates selective exocytosis events in vivo and in clonal cells, STX4 and DOC2B are considered to be present in beta cells and muscle cells at limiting levels [28, 33, 60, 108, 126]. This concept is supported by quantification of stoichiometric ratios of STX4 and

SNAP23 in skeletal muscle from C57BL6 mice, where SNAP23 was found present in a 3-fold molar excess over that of STX4 [108], providing sufficient SNAP23 for appropriate stoichiometric ratios of t-SNARE binary complexes in the muscle of the STX4 overexpressing mice (STX4:SNAP23:VAMP2 assemble in a 1:1:1 ratio [14]. DOC2B is not a SNARE protein per se, but its overexpression in skeletal muscle increased the abundance of STX4-based SNARE complexes, coordinate with an increase in DOC2B-Munc18c complexes and increased STX4 activation [33]. This trio of effects is consistent with earlier cell culture findings pointing to DOC2B 'freeing' STX4 from Munc18c [56]. The only stoichiometric data reported on DOC2B comes from beta cells, wherein DOC2B was recently shown capable of binding in 1:1:1 ratio with Munc18-1 and Munc18c [26]. Stoichiometry of SNARE proteins and SNARE accessory factors still needs to be carefully quantified in primary tissue, although to date there is full consensus regarding beneficial effects of STX4 and DOC2B overexpression amongst primary islets and multiple different beta cell lines. Lastly, studies to investigate alterations in STX4 or DOC2B and their stoichiometry with other factors involved in proliferative and anti-tumorigenic mechanisms will require further investigation. Clearly, therapies designed to enhance STX4 and DOC2B could be clinically useful for a variety of metabolic aberrations (Figure 1.5.).

## **1.10. PERSPECTIVES: TARGETING EXOCYTOSIS PROTEINS FOR IDEAL DIABETES TREATMENT OUTCOMES**

Evidence suggesting that defects in exocytosis proteins precede onset of both T1D and T2D. Thus, these proteins may be ideal novel therapeutic targets for diabetes remediation. The ideal diabetes treatment outcomes for type 1 diabetes would be to enhance functional beta cell mass. T2D treatments ideally aim to enhance both functional beta cell mass and peripheral insulin sensitivity. It is thus plausible that proteins that can dually enhance functional beta cell mass and glucose uptake should be investigated for potential clinical relevance. Beyond functional enrichment, increasing evidence shows that exocytosis proteins may be involved in cell survival processes. For example, in neurons, it has been shown that STX1A and SNAP25 promote neuron survival via membrane recycling processes [127]. In lymphocytes, SNAP23 is limiting and necessary for B and T cell development and fibroblast survival [128]. SNAP25 has also been shown to play a role in neuronal spine morphogenesis and plasticity [129]. Protection of beta cell mass and promotion of survival are certainly ideal outcomes for a diabetes therapeutics, and should be kept in mind when developing novel drugs.

What are the potential perils to overexpressing SNARE/accessory proteins? With increasing evidence that exocytosis proteins may be involved in cell survival processes, there is controversy regarding exocytosis proteins involvement in tumorigenesis. Some studies have shown that certain SNAREs may facilitate

tumor cell migration, mediate inflammation involved in cancer development, and may be under regulation by oncogenes [130]. Despite this, both STX4 and DOC2B have been shown to possess pro-apoptotic/anti-cancer properties [102, 103], indicating that isoform-type and context-type specificities are crucial to determine when considering replenishment or over-expression strategies for treatment/prevention of diabetes and/or cancer.

### **1.11. RATIONALE AND CENTRAL HYPOTHESIS**

The rationale for investigating the role of exocytosis proteins as targets for preventing/treating T1D is that increasing evidences link deficiencies of exocytosis proteins to diabetes in humans and rodents [8, 79, 82, 84, 86, 94]. Furthermore, studies aimed to enhance exocytosis proteins such as DOC2B and STX4 has resulted in promising outcomes that point to the ability to maintain functional  $\beta$ -cell mass. This dissertation will hereto forth focus on DOC2B. I will also focus on T1D and the inflammatory aspects of  $\beta$ -cell dysfunction and demise that are common to both T1D and T2D. My central hypothesis is that DOC2B abundance in the islet  $\beta$ -cell is compromised during development of T1D, and that early  $\beta$ -cell specific enrichment of DOC2B will protect/improve functional beta cell mass and survival to delay/prevent T1D onset. I tested this hypothesis via two Specific Aims: 1) Determine the relationship between DOC2B abundance and new-onset T1D in humans and rodent model systems; and 2) Determine the effect of enriched DOC2B on functional  $\beta$ -cell mass in vivo, and resistance to diabetic stress in  $\beta$ -cell specific transgenic rodent models and human islets.

## **CHAPTER 2**

### **EXOCYTOSIS PROTEIN DOC2B AS A BIOMARKER OF TYPE 1 DIABETES**

## **2.1. SYNOPSIS**

Efforts to preserve and protect  $\beta$ -cell mass in the preclinical stages of type 1 diabetes (T1D) are limited by few blood-borne biomarkers of  $\beta$ -cell destruction. We demonstrate here that the  $\beta$ -cell exocytosis protein, Double C2 domain protein (DOC2B) abundance is substantially reduced in prediabetic NOD mouse platelets, and these changes are mirrored in the pancreatic islets from the same mice. Similarly, human DOC2B levels were reduced over 2-fold in platelets from new-onset T1D human subjects, and this reduction was mirrored in T1D human islets. Cytokine stimulation of normal islets reduced DOC2B expression ex vivo. Remarkably, platelet DOC2B levels increased after islet transplantation in T1D patients. These data suggest that reduction of DOC2B is an early feature of T1D, and DOC2B abundance may serve as a valuable in vivo indicator of  $\beta$ -cell mass and early biomarker of T1D.

## **2.2. INTRODUCTION**

Diabetes mellitus is a complex metabolic disorder that currently affects an estimated 422 million people worldwide, with incidence rates steadily rising [131]. Type 1 diabetes (T1D) accounts for 5-10% of these cases. Its complications remain a major cause of global morbidity and mortality. T1D is characterized by autoimmune destruction of  $\beta$ -cell mass, and the preclinical phase of T1D is marked by declining  $\beta$ -cell function [92, 93]. Studies of early intervention in T1D have shown limited effectiveness, yet have generally shown greater success in subjects that retain greater insulin secretory capacity, and in those with the

shortest time since clinical onset of disease [132, 133]. However, prevention efforts to protect  $\beta$ -cell mass are hindered by the limited availability of early biomarkers to accurately predict  $\beta$ -cell destruction and subsequent progression to clinical disease.

In healthy  $\beta$ -cells, insulin secretion requires soluble N-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins and associated accessory regulatory proteins to promote the docking, priming, and fusion of insulin vesicles at the plasma membrane. Two target membrane (t)-SNARE proteins, Syntaxin1/4 and SNAP25/23, and one vesicle-associated (v)-SNARE protein, VAMP2, constitute the SNARE core complex [134]. Assembly of the SNARE complex occurs when one v-SNARE binds two cognate t-SNARE proteins in a heterotrimeric ratio [14]. SNARE complex assembly is also facilitated by the accessory regulatory protein, Double C2-domain  $\beta$  (DOC2B) [28, 60]. Several studies have established that in animal models, deficiencies in DOC2B result in glucose intolerance and insulin secretion defects [24, 25]. Conversely, overexpression of DOC2B using a global transgenic mouse model enhances insulin secretion and peripheral glucose uptake [33]. Although DOC2B deficiency in rodents has been linked to type 2 diabetes [135], the association between DOC2B protein levels and T1D is still unknown.

Deficient first-phase insulin secretion is a hallmark of preclinical T1D [92, 93]; thus, the ability to assess early pancreatic  $\beta$ -cell destruction is critically important

for predicting disease onset. Currently, risk prediction for T1D relies heavily on family history, genetic screening, and the presence of antibodies against  $\beta$ -cell antigens that often appear relatively late in the progression of disease. The use of autoantibodies in evaluating T1D risk is limited, as >50% of autoantibody-positive patients remain disease-free, even at 5 years follow up [136]. Risk scores have been established [137], but remain insufficient to provide an accurate prognosis, nor an accurate measurement of  $\beta$ -cell health, as many autoantibody-positive individuals are slow to progress through the stages [138] of preclinical disease. To improve early prediction of T1D, ongoing studies seek to investigate the levels of circulating factors that reflect declining  $\beta$ -cell health, such as proinsulin [139], HSP-90 [140], and unmethylated insulin DNA [141] as potential biomarkers of T1D.

Another potential source of biomarkers is the blood-derived platelet, which is currently being investigated in diseases such as Alzheimer's disease [142] and cancer [143], and has been implicated in T1D. Changes in the platelet proteome and morphology have been noted in T1D; for instance, altered intracellular  $\text{Ca}^{2+}$  [144], enhanced formation of microparticles [145], and altered morphology [146] have been reported to result in platelet hyper-reactivity and development of vasculopathies. Importantly, platelets harbor many of the same exocytosis proteins as the pancreatic  $\beta$ -cell, including SNARE isoforms and accessory regulatory proteins [147].

Biomarkers of  $\beta$ -cell destruction in blood have more clinical potential than those in pancreatic islets, as islet procurement is not feasible for routine diagnosis; therefore, we investigated the correlation between DOC2B protein abundance in blood-derived platelets and pancreatic islets in NOD mice and T1D humans. We found that protein abundance of DOC2B is reduced in platelets and islets from prediabetic NOD mice, compared to control mice. Furthermore, DOC2B protein abundance is reduced in platelets and islets from humans with new-onset T1D, compared to matched controls. Notably, we also reveal that DOC2B levels are substantially increased in T1D human platelets after islet transplantation, when C-peptide levels were markedly increased.

## **2.3. MATERIAL, SUBJECTS, AND METHODS**

### **2.3.1. Animals**

Animals were maintained under protocols approved by the Indiana University Institutional Animal Care and Use Committee and following the National Research Council Guidelines for the Care and Use of Laboratory Animals. Female non-obese diabetic (NOD) NOD/ShiLtJ (RRID:IMSR\_JAX:001976) and major histocompatibility complex (MHC)-matched control non-obese diabetes resistant (NOR) (RRID:IMSR\_JAX:002050) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). We have observed that female NOD mice begin to convert to T1D at 17-18 weeks of age, with an average conversion rate of 78% by 20 weeks of age, as previously reported [148]. Random blood glucose

analysis was performed weekly to monitor conversion to T1D, which is characterized by non-fasting blood glucose levels >250 mg/dl for three consecutive days. To assess DOC2B levels before conversion to T1D, pancreatic islets were isolated, using a method as described previously [9], at 7 weeks (earliest time point for sufficient islet cell yield), 13 weeks (intermediate time point), and 16 weeks of age (latest time point before conversion to T1D). Islet isolation yield decreases in mice less than 8 weeks of age [149]. Islet lysates were then used for SDS-PAGE and immunoblotting. Mouse blood was collected (from 13 and 16 week old mice; blood was insufficient from 7 week old mice) and platelets were isolated as previously described [147]. Platelet lysates were then used for SDS-PAGE and immunoblotting.

### **2.3.2. Human Subjects**

This protocol was approved by the Indiana University Institutional Review Board. For evaluation of DOC2B levels in human platelets (new-onset T1D study), subjects aged 8-14 (11 males and 6 females) with new-onset T1D were recruited over an 18-month period. Consent was obtained from parents, with assent from the pediatric subjects. Subjects were diagnosed with T1D if they met the criteria of 1 or more positive autoantibodies with clinical features of T1D: hyperglycemia, weight loss, and normal body mass index (BMI), or those who were autoantibody negative but <10 years old at diagnosis. Exclusion criteria were as previously described [140]. For each visit, subjects received \$25. Subjects had blood drawn at diagnosis and at the first follow-up appointment 7-10 weeks after diagnosis. Insulin treatment of T1D subjects was started at time of diagnosis. Non-diabetic

control subjects (8 males and 6 females) were recruited from the community and matched to T1D subjects based on gender, age, and BMI (see Table 2.1. for demographic data).

Characteristic	Non-T1D controls	T1D subjects
<b>Number of subjects</b>	14	17
<b>Age (y), (range)</b>	11.4 y (8.0-14.3)	10.3 y (4.3-14.1)
<b>Sex (male)</b>	55%	57%
<b>BMI (Kg/m<sup>2</sup>)*</b>	20.2 ± 3.0	17.5 ± 2.8
<b>Number of autoantibodies positive †</b>	----	0 AutoAb positive: 1 1 AutoAb positive: 5 2 AutoAb positive: 9 3 AutoAb positive: 2
<b>Basal insulin requirement prior to hospital discharge (units/kg/d)</b>	----	0.30 ± 0.09
<b>C-peptide at diagnosis (pmol/l) ‡</b>		110 ± 169 (13-608)
<b>HbA1c at diagnosis (range)</b>	----	11.0 ± 1.7% (7.5 ± 14.2)
<b>HbA1c at first follow-up (range)</b>	----	7.7 ± 0.8% (6.4-9.0)

**Table 2.1. Pediatric T1D study demographics**

**Abbreviations:** *BMI*, body mass index; *HbA1c*, hemoglobin A1c; *T1D*, type one diabetes. Values displayed are means ± standard deviations unless otherwise noted. \*For BMI calculations, 1 T1D subject did not have a diagnosis height and 1 non-T1D control did not have a registration height. For these subjects heights from clinic follow-up were used to calculate BMI. †The following 3 diabetes-associated antibodies were tested: GAD, miAA, and IA-2A. ‡ For C-peptide at diagnosis, n=13.

Samples were de-identified and coded by the clinical team prior to distribution to the research lab for platelet isolation and analyses. Platelets were isolated by centrifugation from blood, as previously described [150], and lysed for SDS-PAGE and immunoblotting. Upon quantification of the data for each sample, the clinical team re-identified samples to permit grouping of data into T1D vs non-diabetic for statistical comparisons.

For evaluation of DOC2B levels in human islets (T1D islet transplantation study), samples were obtained from T1D islet transplantation recipients, as approved by the City of Hope Institutional Review Board. Two subjects, aged 43 and 52 years, were recruited for human islet transplantation based on the following criteria: T1D diagnosis with frequent or life-threatening hypoglycemia with or without unawareness symptoms. Blood was obtained from both subjects prior to transplantation (Day 0), and on Days 30, and 75 after islet transplantation (see Table 2.2. for demographic data). Platelets were isolated by centrifugation from blood, as previously described [150], and lysed for SDS-PAGE and immunoblotting.

	<b>Islet Transplant Recipients</b>	
Characteristics	<b>COH-027</b>	<b>COH-028</b>
<b><u>Recipient Characteristics</u></b>		
<b>Sex</b>	F	M
<b>Age at transplant (years)</b>	43	52
<b>Weight (kg)</b>	76.5	92
<b>BMI (kg/m<sup>2</sup>)</b>	28.93	29.77
<b>Duration of diabetes (years)</b>	33	34
<b>HbA1c (%)</b>	5.5	8.5
<b>Insulin intake (units/day)</b>	28	52
<b>Fasting/Glucagon-Stimulated C-peptide (ng/ml)</b>	0.03/0.02	<0.02/<0.02
<b>Autoantibodies</b>	GAD65-neg   IA-2-pos mIAA-pos   ZnT8-neg	GAD65-pos   IA-2-neg mIAA-pos   ZnT8-neg
<b>PRA Class I/Class II (%)</b>	0/0	0/0
<b><u>Islet Graft Characteristics</u></b>		
<b>Islet Dose (IEQ)</b>		
<b>Total IEQ</b>	240,133	482,755
<b>IEQ/kg</b>	3,139	5,247
<b>Islet Purity (%)</b>	50	68
<b>Packed Cell Volume (ml)</b>	1.9	2.8
<b>Islet Viability (%)</b>	91	94

**Table 2.2. Baseline adult islet transplant recipient and islet graft characteristics**

**Abbreviations:** *PRA, panel reactive antibody; IEQ, islet equivalent.*

### **2.3.3. Islet cell transplantation**

For the T1D islet transplant study, human pancreata were procured from ABO-compatible, cross-match negative cadaveric donors. The islets were isolated under cGMP conditions by the Southern California Islet Cell Resource Center at City of Hope using a modified Ricordi method. Islets were maintained in culture for up to 72 hours prior to transplantation. Islets were transplanted intraportally with heparinized saline (35 U/kg recipient body weight) using a transhepatic percutaneous approach.

### **2.3.4. Clinical//laboratory assays**

For the new-onset T1D study, autoantibodies to glutamic acid decarboxylase 65 (GAD-65), insulin, and Islet Antigen 2 (IA2) were assayed from peripheral blood at diagnosis at Mayo Medical Laboratories (Rochester, Minnesota). Glycated hemoglobin (HbA1c) was also measured at diagnosis and at first clinic follow-up (7-10 weeks after diagnosis) using the Bayer A1cNow system or the Bayer DCA2000 analyzer (Tarrytown, New York). C-peptide was measured in stored serum samples using the C-peptide ELISA kit (Alpco, Salem, New Hampshire; detection range 20-3000 pM).

For the T1D islet transplant study, plasma C-peptide measurements were performed by the Northwest Lipid Metabolism and Diabetes Laboratory (Seattle, WA) using the Tosoh C-Peptide II Assay (Tosoh Bioscience, Inc, San Francisco, CA; detection range 0.02-30 ng/ml). A fasting C-peptide < 0.2 ng/ml and 6-min glucagon-stimulated C-peptide < 0.3 ng/ml were used to confirm T1D diagnosis

prior to islet transplant. Autoantibodies (GAD-65, IA-2A, insulin [mIAA], and zinc transporter 8 [ZnT8]) were analyzed using radiobinding assays by the Autoantibody/HLA Service Center at the Barbara Davis Center for Diabetes (Aurora, CA).

### **2.3.5. Ex vivo islet preparations**

Non-T1D human cadaveric pancreatic islets were obtained through the Integrated Islet Distribution Program at City of Hope. The islets were prepared and treated with a cytokine mixture (10 ng/ml TNF- $\alpha$ , 100 ng/ml IFN- $\gamma$  and 5 ng/ml IL-1  $\beta$ ; ProSpec, East Brunswick, NJ, USA) for 72 hours, as previously described [151]. The islets were then used in qRT-PCR analysis or SDS-PAGE followed by immunoblotting.

### **2.3.6. Immunofluorescence**

Human paraffin-embedded pancreatic tissue sections were obtained from the Network for Pancreatic Organ Donors with Diabetes (nPOD). Five sections from formalin-fixed paraffin-embedded (FFPE) tissue samples were obtained from T1D (n=3) and age and BMI-matched non-diabetic (n=3) donors. Pancreas sections were immunostained with primary and secondary antibodies listed in Table 2.3. Slides were counterstained to mark the nuclei, using 4',6-diamidino-2-phenylindole (DAPI) (Vectashield; Vector Laboratories, Burlingame, CA) and viewed using a Keyence BZ X-700 fluorescence microscope (Keyence Corporation, Itasca, IL). All human T1D samples were prepared and processed at the same time; confocal images were taken with identical acquisition settings. Islet immunofluorescence was assessed by imaging 20-30 islets (grouping of

four or more insulin-positive cells) per subject. Analysis was performed in a blinded fashion using Image-Pro Software (Media Cybernetics, Rockville, MD, USA) to quantify fluorescence intensities using methods as previously described. [152] Defined regions of interest (ROIs) were used to delimit islets from adjacent acinar tissue and average intensity measurements of insulin and DOC2B were quantified by splitting the merged image into two color channels with the same ROI.

### **2.3.7. Immunoblotting**

Platelet and islet protein lysates for the NOD mouse study were resolved on a 10% SDS-PAGE gel and transferred to standard PVDF (Bio-Rad, Hercules, CA, USA). Platelet proteins from the new-onset T1D study were resolved on a 10% SDS-PAGE gel using an SE400 air-cooled 18 x 16 cm vertical protein electrophoresis unit (Hoefler, Inc. Holliston, MA) and transferred to standard PVDF (Bio-Rad). Platelet proteins from the T1D islet transplant study were resolved on a 12% SDS-PAGE gel using a Criterion™ 13.3 x 8.7 cm vertical electrophoresis unit (Bio-Rad) and transferred to standard PVDF. All blots were probed as outlined in Table 2.3.

<b>Protein Target</b>	<b>Source</b>	<b>Catalogue No.</b>	<b>RRID No.</b>
<b>Primary antibodies used in NOD mouse study/ex vivo islet study</b>			
DOC2B	Proteintech	20574-1-AP	AB_10696316
Tubulin	Abcam	ab56676	AB_945996
iNOS	Millipore	ABN26	AB_10805939
<b>Primary antibodies used in New-onset T1D/T1D transplant study</b>			
DOC2B	Abnova	H00008447-B01P	AB_10549446
GAPDH	Abnova	ab9485	AB_307275
<b>Primary antibodies used in immunofluorescence study</b>			
DOC2B	Proteintech	20574-1-AP	AB_10696316
Insulin	Abcam	ab7842)	AB_306130
<b>Secondary antibodies</b>			
goat anti-rabbit	Bio-Rad	1706515	AB_11125142
goat anti-mouse	Bio-Rad	1706516	AB_11125547
Alexa Fluor 568 goat anti-rabbit	Abcam	ab175471	AB_2576207
Alexa Fluor 488 goat anti-guinea pig	Thermo	A-11073	AB_2534117

**Table 2.3. Primary and secondary antibodies used in study**

### **2.3.8. Quantitative real-time PCR**

Total RNA was isolated from human islets using the Qiagen RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) and assessed using the QuantiTect SYBR Green RT-PCR kit (Qiagen). Primers used for the detection of hDoc2b are as follows: forward: 5'-CCAGTAAGGCAAATAAGCTC-3' and reverse: 5'-GGGTTTCAGCTTCTTCA-3'. Standard tubulin primers (Cat:QT00089775, Qiagen) were used for normalization.

### **2.3.9. Statistical analysis**

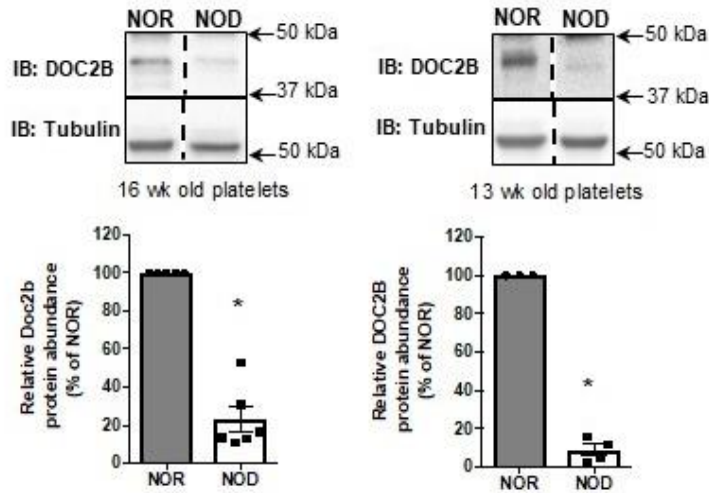
Data were evaluated for statistical significance using Student's *t* test for comparison of two groups; ANOVA and Tukey's post-hoc test (GraphPad Software, La Jolla, CA, USA) were used for comparison of more than two groups. Data are expressed as the average  $\pm$  SEM.

## **2.4 RESULTS**

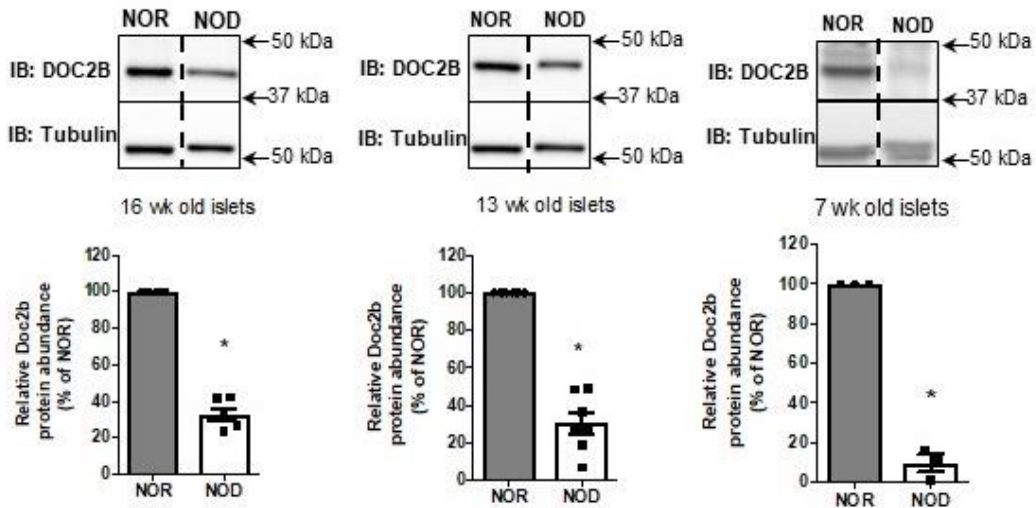
### **2.4.1. Low DOC2B levels in pre-diabetic NOD mouse platelets and islets**

To investigate whether DOC2B protein levels are altered in the blood prior to onset of T1D, we examined platelet DOC2B abundance in young pre-diabetic NOD mice and MHC-matched NOR mice. Immunoblotting revealed that platelets from 16- and 13-week old NOD mice exhibited up to a 90% reduction in DOC2B protein levels (Figure 2.1.A) compared to NOR platelets. Furthermore, islets from 16- and 13-week old NOD mice showed at least a 65% reduction in DOC2B protein levels (Figure 2.1.B.) compared to NOR islets. NOD islets from as early as 7 weeks of age showed a 90% reduction in DOC2B protein (Figure 2.1.B). The average blood glucose levels from random blood testing of NOD and NOR mice were below 250 mg/dL at 7, 13, and 16 weeks (Table 2.4.), indicating that the mice had not yet converted to diabetes. These data show that DOC2B protein abundance is reduced in both islets and platelets of prediabetic mice.

### (a) Mouse platelet DOC2B



### (b) Mouse islet DOC2B



**Figure 2.1. DOC2B protein abundance is reduced in platelets of pre-diabetic NOD mice.** Platelets were isolated from 16 or 13 wk old group-housed female NOD and age-matched NOR mice and proteins were resolved on SDS-PAGE for immunoblotting. DOC2B levels were quantified relative to tubulin immunoblotting in the same lane. Dashed vertical lines indicate splicing of lanes from within the same gel exposure. Islets were isolated from 16, 13 or 7 wk old group-housed female NOD and age-matched NOR mice and proteins were resolved on SDS-PAGE for immunoblotting. DOC2B levels were quantified relative to tubulin loading in the same lane. Dashed vertical lines indicate splicing of lanes from within the same gel exposure. Data are shown as means  $\pm$  SEM (n=3-6 mice per group); \*p<0.05.

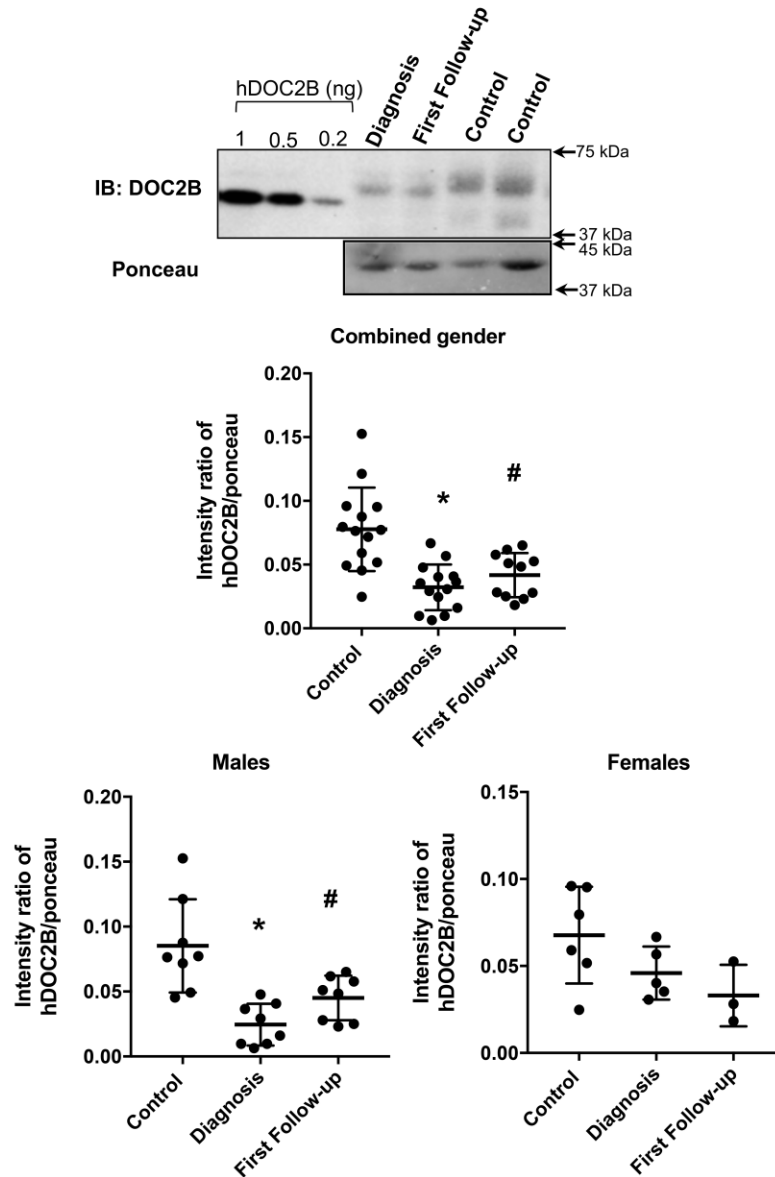
<b>Avg. Non-fasting Blood Glucose (mg/dL)</b>			
	<b><u>16 wks</u></b>	<b><u>13 wks</u></b>	<b><u>7 wks</u></b>
<b>NOR</b>	197 ± 17	183 ± 10	130 ± 6
<b>NOD</b>	196 ± 19	194 ± 22	127 ± 5

**Table 2.4.: Average blood glucose levels of NOD and NOR mice at 16, 13, and 7 weeks.** Data represent the average ± S.EM; n=6 per group for mice at 16 and 13 weeks; n=5 per group at 7 weeks. Random non-fasting blood glucose was measured for NOR and NOD female mice at 13 and 16 weeks of age. No statistical differences were seen.

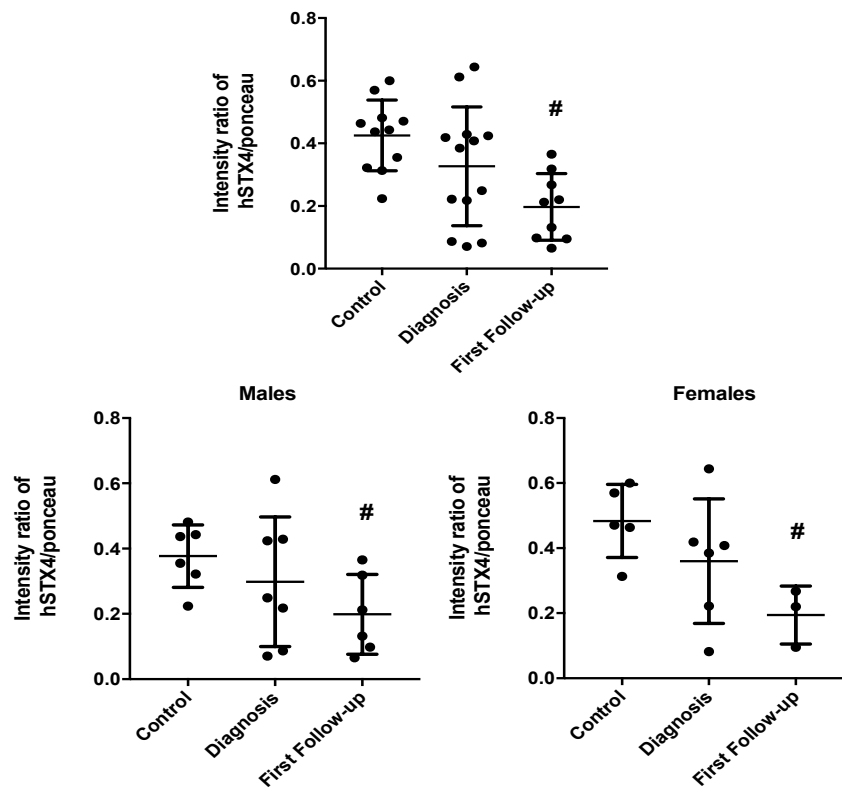
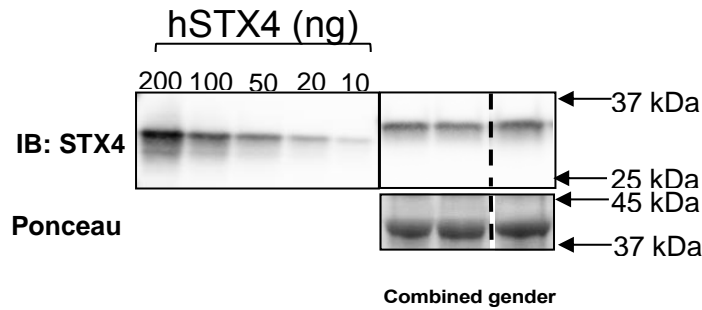
#### **2.4.2. Low DOC2B levels in new-onset T1D human platelets**

In the new-onset T1D study, we quantified protein content of DOC2B using platelets from new-onset T1D subjects in comparison to controls (Table 2.1.). Platelets from new-onset T1D subjects exhibited reduced protein levels of DOC2B for both sexes, both at diagnosis and at first clinic follow-up 7-10 weeks later. When males and females were assessed separately, DOC2B levels were reduced in males by >60% compared to non-diabetic control subjects, persisting even after insulin treatment of the patient and reduction of HbA1c (Figure 2.2.). The significant loss of DOC2B at T1D diagnosis was selective for DOC2B compared to another exocytosis protein, syntaxin 4 (STX4) (Figure 2.3.). These data indicate that DOC2B is decreased in T1D platelets independent of glycemic control, relative to non-diabetic human platelets, and that platelet DOC2B levels are already diminished at T1D diagnosis.

## Human platelet DOC2B



**Figure 2.2.** Platelets were isolated from new-onset T1D patients at the time of diagnosis (“Diagnosis”) and 7-10 weeks later (“First Follow-up”), and from matched controls (“Control”). Platelet proteins were resolved on SDS-PAGE for immunoblotting. Standard curves were generated using recombinantly-expressed and purified human DOC2B protein on each gel to confirm that the the band intensities of DOC2B in human platelets fell within the dynamic range of the curve on the same gel. DOC2B was quantified relative to protein loading determined by Ponceau S staining in the same lane (37-68 kDa segment). Dashed vertical lines indicate splicing of lanes from within the same gel exposure. Data are shown as means  $\pm$  SEM for DOC2B (n=11-14 per group [gender-combined group, 8 males per group, 3-6 females per group]); \*p<0.05, Diagnosis vs. Control.; #p<0.05 Follow-up vs. Control).

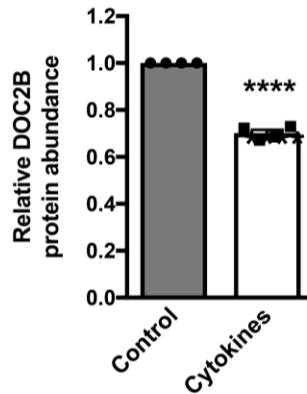
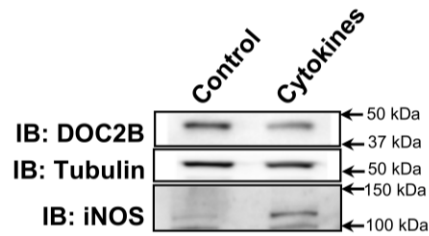


**Figure 2.3. Human Platelet STX4.** Platelet proteins from children with T1D and age/gender/BMI matched controls were isolated at Diagnosis and First Follow-up 7-10 weeks later, then resolved on SDS-PAGE for immunoblotting for STX4. Standard curves were included using recombinantly-expressed and purified human STX protein on each gel with band intensities of STX4 in human platelets falling within the dynamic range of the curve on the same gel. Dashed vertical lines indicate splicing of lanes from within the same gel exposure. Data are shown as means  $\pm$  SEM.  $n=10-13$  per gender-combined group, 5-7 males per group, 3-6 females per group); \* $p<0.05$ , Diagnosis vs. Control.; # $p<0.05$  Follow-up vs. Control.

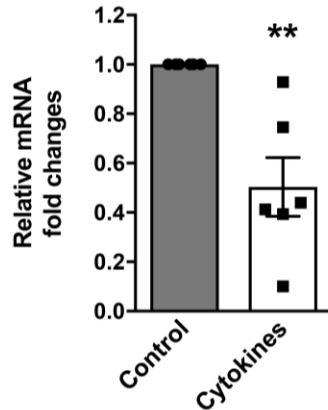
### **2.4.3. Ex vivo pro-inflammatory cytokine treatment reduces human islet DOC2B levels**

T1D is associated with elevated circulating pro-inflammatory cytokines, which damage  $\beta$ -cells [153]. Because obtaining pancreatic islets from living T1D subjects is virtually impossible, we evaluated the relationship between T1D and DOC2B levels by treating human cadaveric non-diabetic islets (Table 2.5.) ex vivo with pro-inflammatory cytokines in effort to simulate the circulating milieu. Cytokine treatment (IL-1 $\beta$ , TNF- $\alpha$ , INF- $\gamma$ ) elevated the levels of islet iNOS (Figure 2.4.A), consistent with the reported effects of cytokine exposure [154]. Correspondingly, DOC2B protein and mRNA levels were reduced by 30% and 50%, respectively (Figure 2.4.A-B). These data suggest that a T1D-like milieu can decrease DOC2B levels in human islets.

(a) Human islet DOC2B Protein



(b) Human islet Doc2b mRNA



**Figure 2.4. DOC2B protein and mRNA abundance is reduced in adult human islets subjected to treatment with pro-inflammatory cytokines.** Human adult cadaveric islets were incubated under control conditions or with pro-inflammatory cytokines for 72 h at 37°C. Islet protein lysates were resolved by SDS-PAGE for immunoblotting (a) or for RNA extraction and qRT-PCR analysis (b). In addition to hDOC2B, tubulin and iNOS levels were also evaluated by immunoblotting. Bars represent mean  $\pm$  SEM for 4 or 5 independent sets of human islets evaluated for protein and mRNA analyses, respectively; \*\*\*\* $p$ <0.0001, \*\* $p$ <0.002.

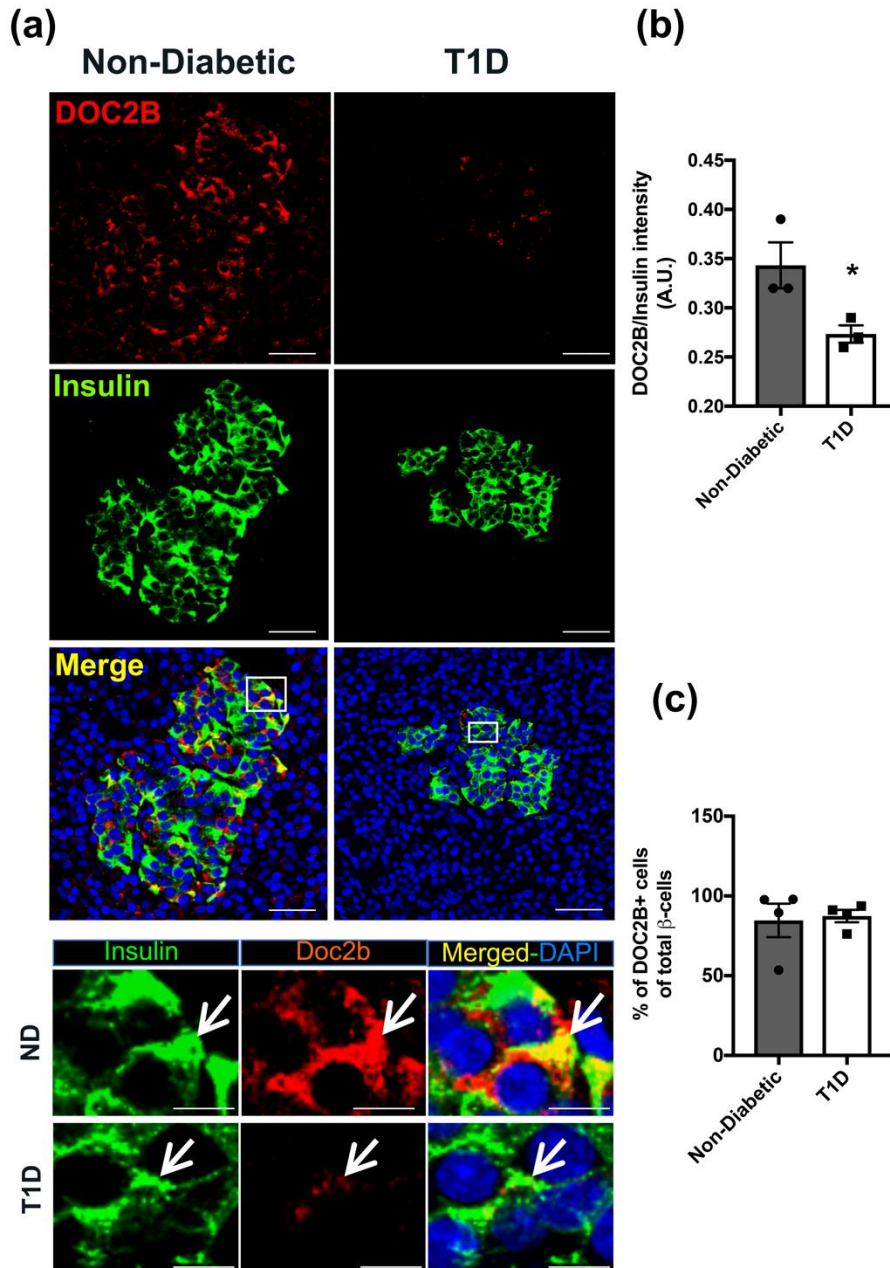
Unos/COH ID no.	Sex	Age	BMI	Race	Islet purity	Islet viability	Exp. use
ACIN402	M	49	40.0	Hispanic	N/A	N/A	protein
ACIY103	M	24	24.6	Caucasian	78%	N/A	protein
Hu966	M	20	30.6	African American	88%	N/A	protein
ADBL	F	53	23.8	Caucasian	90%	90%	protein
ADFE489	F	45	23.1	Asian	N/A	N/A	mRNA
ADDV480	M	52	25.4	Caucasian	N/A	N/A	mRNA
AEFU443	F	49	29	Caucasian	95%	95%	mRNA
Hu1000	M	49	29	Hispanic	N/A	N/A	mRNA
Hu966	M	20	30.6	African American	88%	N/A	mRNA
ACIY103	M	24	24.6	Caucasian	78%	N/A	mRNA

**Table 2.5. Non-diabetic human islet donor characteristics**

**Abbreviations:** NA= data not available.

#### **2.4.4. Reduced DOC2B protein in human early-onset T1D islets.**

To investigate changes in DOC2B levels in T1D human pancreata, we used paraffin-embedded slides (obtained from nPOD [Network for Pancreatic Organ Donors with Diabetes]) from cadaveric donors for DOC2B immunofluorescence evaluation in early-onset pediatric T1D (5 years or less with T1D) (n=3) versus matched non-diabetic (ND) controls (n=3) (Figure 2.5.A, and Table 2.6.). Measuring relative immunofluorescent intensities, we observed a decrease in DOC2B abundance in T1D islets versus than in non-diabetic controls (Figure 2.5.B). Although the relative number of DOC2B-positive  $\beta$ -cells in non-diabetic and T1D islets were similar (Figure 2.5.C), DOC2B intensity was reduced in T1D  $\beta$ -cells.



**Figure 2.5. DOC2B protein levels are reduced in islets of pediatric T1D humans.** Slides obtained from nPOD, comprised of early-onset T1D and age-matched non-diabetic human pancreata, were immunostained for the presence of DOC2B or insulin in DAPI-positive cells. A) representative images, low power images scale bar=100  $\mu$ m, higher magnification images scale bar=25  $\mu$ m. B) tabulated relative intensities; n = 3 donors, \* $p < 0.05$ . C) Number of DOC2B-positive  $\beta$ -cells  $p =$  not significant, (N.S.).

nPOD ID no.	Sex	Age	BMI	Race	Years of T1D
6113	F	13.1	24.7	Caucasian	1.5
6342	F	14	24.3	Caucasian	2
6243	M	13	21.3	Caucasian	5
6386	M	14	23.9	Caucasian	ND
6392	M	14.1	23.6	Caucasian	ND
6340	M	9.7	20.3	Caucasian	ND

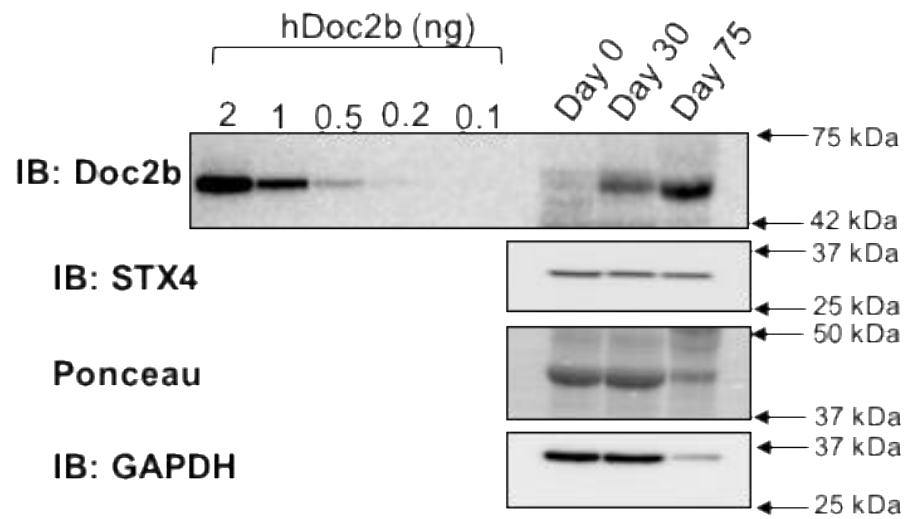
**Table 2.6. nPOD sample human pancreata donor characteristics.**

**Abbreviations:** *ND*; non-diabetic

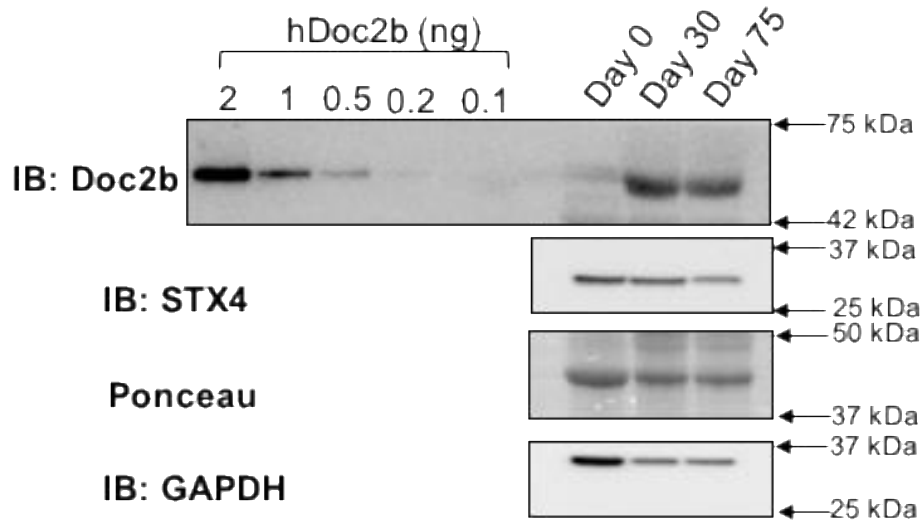
#### **2.4.5. DOC2B levels restored after clinical islet transplantation.**

In the T1D islet transplantation study (Table 2.2.), we found that the pre-transplant platelet DOC2B levels were very low in both subjects relative to a hDOC2B protein standard curve (Figure 2.6.A-B, Day 0). Notably, within 30 days of transplantation, each T1D islet recipient showed a robust increase in platelet DOC2B protein, which persisted to 75 days after transplantation (Figure 2.6.A-B, Days 30 and 75). These data coincide with changes in C-peptide levels in these subjects: while each subject had low to almost undetectable fasting/glucagon-stimulated C-peptide levels before transplantation, the C-peptide levels were substantially increased by 30 days after transplantation (Table 2.6.). As C-peptide levels are indicative of overall islet function, these data suggest that in humans, DOC2B levels in platelets correlate with relative functional  $\beta$ -cell mass.

## A) COH-027



## B) COH-028



**Figure 2.6. DOC2B levels in adult T1D human platelets are increased after clinical islet transplantation.** Platelets obtained from two clinical islet transplant recipients prior to (Day 0) islet infusion, or on Days 30 and 75 post-infusion, were evaluated by quantitative immunoblotting for DOC2B protein content: (a) subject COH-027, (b) subject COH-028. Ponceau S staining and GAPDH show the relative protein loading of the membranes used for immunoblotting.

<b>Islet Transplant Recipients</b>		
	<b>COH-027</b>	<b>COH-028</b>
Immunosuppression Regimen	<u>Induction:</u> rATG, etanercept, anakinra <u>Maintenance:</u> tacrolimus, MMF, +/- sirolimus	<u>Induction:</u> rATG, etanercept, anakinra, <u>Maintenance:</u> tacrolimus, MMF +/- sirolimus
Additional Immunosuppression for suspected islet graft rejection	Solumedrol, Plasmapheresis, IVIg & Rituxan for suspected islet graft rejection	NA
HbA1c (%) Pre-Tx	5.5	8.5
Day 30	ND	ND
Day 75	5.3	6
Insulin intake (units/day) Pre-Tx	28	52
Day 30	13	28
Day 75	15	8
Fasting/Glucagon-Stimulated C-peptide (ng/ml)		
Pre-Tx	0.03/0.02	<0.02/<0.02
Day 30	2.78/3.56	1.71/3.39
Day 75	0.84/1.42	1.23/2.40
Mixed Meal Tolerance Test (MMTT) C-peptide at 0/90min		
Pre-Tx	ND	ND
Day 30	ND	ND
Day 75	0.63/2.65	1.62/2.95
Oral Glucose Tolerance Test (OGTT) BG (mg/dl)   C-pep (ng/ml), Fasting/120min		
Pre-Tx	ND	ND
Day 30	BG: 102/197   C-pep: 1.49/6.72	BG: 127/228   C-pep: 1.65/4.81
Day 75	BG: 101/199   C-pep: 0.71/3.52	BG: 148/310   C-pep: 1.46/3.22
Autoantibodies Pre-Tx	GAD65-neg / IA-2-pos mIAA-pos / ZnT8-neg	GAD65-pos / IA-2-neg mIAA-pos / ZnT8-neg
Day 75	GAD65-pos* / IA-2-pos mIAA-neg* / ZnT8-neg	GAD65-pos / IA-2-neg mIAA-pos / ZnT8-neg

**Table 2.6. Islet transplant recipient treatment and outcome summary.**

**Abbreviations:** NA, Not applicable; ND, Not done; Pre-Tx: Pre-Transplant (baseline);

\*Denotes change in auto or allo-antibodies status from baseline

## 2.5. DISCUSSION

The ability to detect  $\beta$ -cell destruction is critical in accurately predicting prognosis during the preclinical phase of T1D, hence the current need for additional early biomarkers. We show that DOC2B protein levels are substantially reduced in platelets and islets from pre-diabetic NOD mice vs. NOR control mice. Furthermore, we reveal that levels of human DOC2B are significantly lower at the time of diagnosis in platelets of new-onset T1D pediatric patients than platelets from matched control subjects. Notably, DOC2B levels were reduced at 7-10 weeks post-diagnosis, despite therapeutic remediation of hyperglycemia in the human subjects. Consistent with this, islet DOC2B protein levels were reduced in early onset T1D pancreatic tissue samples compared to matched controls. Loss of DOC2B protein and mRNA could be recapitulated by exposure of non-diabetic human islets to pro-inflammatory cytokines *ex vivo*, suggesting that the inflammatory milieu in pre-diabetic and T1D humans may cause DOC2B loss. Remarkably, clinical islet transplant recipients exhibit a restoration of DOC2B levels in platelets, compared with their own nearly undetectable levels of platelet DOC2B prior to receiving the transplanted islets. These data suggest that DOC2B protein is a candidate biomarker of pre-diabetes and T1D, with the levels possibly reporting relative functional  $\beta$ -cell mass.

To our knowledge, this is the first study to establish an association between T1D and levels of an exocytosis protein in blood-derived platelets and pancreatic islets. Reduced DOC2B in islets is indicative of deficient islet functional health

[24]. Strikingly, platelet DOC2B levels in islet transplant recipients correlated with the presence of a functional islet mass. This correlative finding supports the possibility that the platelet DOC2B stems not necessarily from the pancreas per se, since islets are grafted into the liver in these human recipients, but that the platelets and/or precursor megakaryocytes may be sampling DOC2B from the islets irrespective of islet location. It also remains possible that the increased DOC2B content stems from 'rested' native residual islets of the transplanted patients. However, this is inconsistent with our pediatric platelet data showing that even after insulin therapy to ameliorate new-onset hyperglycemia, DOC2B levels remained deficient. Mechanistically, questions arise as to if and how platelets and islets communicate to determine DOC2B levels. Supporting the concept of platelet-islet communication, it has been demonstrated that islet transplantation in T1D patients stabilizes platelet abnormalities, as transplant recipient platelets show normal volume and activation [155]. Indeed,  $\beta$ -cells release exosomes as a way of shuttling various miRNAs, mRNAs, and proteins to targeted peripheral cells [156].  $\beta$ -cell exosomes were also recently shown to carry proteins, such as GAD-65, IA-2, and proinsulin, to dendritic cells, which then become activated [157]. Could islets similarly release DOC2B mRNA or protein, which is subsequently taken up by megakaryocytes or by the platelets themselves? Furthermore, platelets can selectively absorb proteins from the blood [158]. In fact, platelet sequestration of tumor-specific proteins was detected in animals harboring small tumors [158]. Notably, a direct interaction between platelets and pancreatic  $\beta$ -cells has been reported, and protein from platelets

was shown to be transferred to  $\beta$ -cells [159]. Is it possible that the platelets are sampling the islets to mirror islet DOC2B levels? As the data presented herein are correlative in nature, future studies will be required to determine the detailed molecular mechanism regulating changes in DOC2B protein abundance in human T1D samples.

The concept of DOC2B as a biomarker is appealing because DOC2B levels in platelets and islets are significantly decreased in normoglycemic NOD mice months before their conversion to T1D. Female NOD mice typically convert to T1D between 18-24 weeks of age, but as early as 5 weeks of age, NOD mouse islets show signs of insulinitis, resulting from an initial phase of pancreatic inflammation that reduces  $\beta$ -cell function and mass [160]. Given that DOC2B content in human islets decreased upon islet exposure to pro-inflammatory cytokines, which was sufficient to evoke iNOS expression, it is possible that the cytokine-induced drop in islet DOC2B signals reduced islet viability. Although it has been demonstrated by multiple groups that whole-body DOC2B knockout mice show deficient glucose-stimulated insulin secretion [24, 25],  $\beta$ -cell mass was not evaluated. While it is also possible that DOC2B expression is genetically repressed in NOD mice, the genetics of NOD mice have been well studied and DOC2B was not identified as deviating from control [161]. DOC2B mRNA expression was also decreased in response to pro-inflammatory cytokine exposure in non-diabetic human islets, suggesting that DOC2B might undergo transcriptional repression during T1D development. Indeed, promoter methylation

and silencing of DOC2B gene have been reported to occur in cancer cell types [103]. Future studies will be required to determine the detailed molecular mechanism regulating changes in DOC2B protein abundance in human T1D samples.

While novel findings were obtained, there are also some limitations to the conclusions that can be derived from this study. First, this work derives from two pilot studies with relatively small sample sizes of human pediatric subjects (N=14 controls and N=17 T1D patients; new-onset T1D study) and adult T1D islet transplant subjects (N=2; T1D islet transplantation study). Secondly, the pediatric cohort was evaluated at clinical diagnosis of T1D, so these patients already have full-blown disease. A larger future study would benefit from a prospective design that includes subjects who are significantly at risk, but who are normoglycemic. We are also limited in that only the first clinical follow-up samples were obtained, and we do not have further follow-up data for a longitudinal evaluation of changes in DOC2B. Lastly, the number of adult T1D clinical islet transplantation subjects followed was limited by our initial requirement for collection of fresh blood for platelet isolation, to be consistent with our pediatric T1D cohort. Given our focus on DOC2B as an early predictor of T1D, future studies will examine serum and plasma, both of which contain abundant and detectable levels of DOC2B. This will permit studies of larger numbers of T1D transplant recipients and evaluation of stored samples from a variety of repositories, to enable more generalized conclusions.

In summary, we demonstrate reduced abundance of the exocytosis protein, DOC2B, in prediabetes (NOD mice) or the early onset of T1D (humans). In addition, we show a novel correlation between the platelet and islet DOC2B levels, suggesting that platelet evaluation may provide prognostic information about T1D risk and progression. Furthermore, transplantation of healthy, functional islets increased the levels of DOC2B in T1D human platelets. Given the need to accurately assess  $\beta$ -cell destruction at an early stage, DOC2B may be a viable biomarker for T1D.

## **CHAPTER 3**

# **DOC2B PROTECTS $\beta$ -CELLS AGAINST INFLAMMATORY DAMAGE AND ENHANCES FUNCTION**

### 3.1. SYNOPSIS

Loss of functional  $\beta$ -cell mass is an early feature of type 1 diabetes. Functional  $\beta$ -cells require soluble n-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) complexes to release insulin, as well as SNARE complex regulatory proteins like the Double C2-domain protein  $\beta$  (DOC2B). We hypothesized that DOC2B deficiency or overabundance may confer susceptibility or protection, respectively, to functional  $\beta$ -cell mass. Indeed, *Doc2b*<sup>-/+</sup> heterozygous mice show an unusually severe response to multiple low dose streptozotocin (MLD-STZ) and have more apoptotic  $\beta$ -cells and less  $\beta$ -cell mass. In addition, inducible  $\beta$ -cell specific DOC2B overexpressing transgenic ( $\beta$ Doc2b-dTg) mice resist MLD-STZ-induced  $\beta$ -cell damage, fasting hyperglycemia, and impaired glucose tolerance as compared with control MLD-STZ mice; similarly, human  $\beta$ -cells overexpressing DOC2B resist cytokine-induced apoptosis. Mechanistically, DOC2B enrichment was found to enhance glucose-stimulated insulin secretion (GSIS) and SNARE activation. Furthermore, expression of a DOC2B peptide, comprised of its tandem C2A and C2B domains, is sufficient to confer the GSIS- and SNARE-enhancement effects of full-length DOC2B; expression of either C2 domain alone was without benefit. DOC2B and C2AB enrichment confer protection from cytokine or thapsigargin-induced  $\beta$ -cell apoptosis. These studies identify a DOC2B peptide that confers the beneficial functional effects of DOC2B and may be a target to protect functional  $\beta$ -cell mass.

### 3.2. INTRODUCTION

In the United States, type 1 diabetes (T1D) currently affects an estimated 1.25 million people, and this prevalence is predicted to rise to 5 million Americans by 2050 [162-164]. T1D is characterized by hyperglycemia caused by autoimmune destruction of islet  $\beta$ -cells, leading to a decline in  $\beta$ -cell function and mass. Increasing evidence shows that  $\beta$ -cell dysfunction precedes clinical onset of type 1 diabetes [92, 165]. Indeed multiple clinical trials show that T1D patients who display even minimal  $\beta$ -cell function at the onset of study exhibit decreased microvascular complications, including retinopathy, and neuropathy [166, 167]. Currently available therapy for T1D patients is limited to exogenous insulin replacement. The danger in this approach, however, is that normal, glucose-dependent release of endogenous insulin is lost, and that life-threatening hypoglycemic episodes and destruction of  $\beta$ -cell mass persists. Thus, there is a high demand for alternate/adjuvant therapies that can promote the preservation of  $\beta$ -cell mass and function.

Normal insulin secretion is dependent on an intact insulin secretory process machinery, termed “SNARE (soluble n-ethylmaleimide-sensitive fusion protein-attachment protein receptor) proteins”. The SNARE complex consists of two target membrane (t)-SNARE proteins, Syntaxin and SNAP25, and one vesicle associated (v-SNARE) protein, VAMP [10, 11, 14, 46]. Upon glucose stimulation, one v-SNARE binds with two cognate t-SNARE proteins in a 1:1:1 heterotrimeric ratio [14]. SNARE proteins facilitate the biphasic manner of insulin secretion

using different requisite SNARE protein isoforms [134, 168]. The first phase of insulin secretion (first 5-10 minutes following glucose stimulation) uses syntaxin 1A (STX1A), syntaxin 4 (STX4), SNAP25 or SNAP23, and the v-SNARE VAMP2, whereas second-phase (>10 minutes following glucose stimulation) utilizes STX4, SNAP25 or SNAP23, and VAMP2 [35, 38, 44, 46, 47, 169, 170]. Another v-SNARE protein, VAMP8, also functions in insulin secretion but is required selectively for glucagon-like peptide (GLP-1) enhanced insulin release [52].

Assembly of the SNARE complex in the  $\beta$ -cell is regulated by accessory binding proteins, such as “SM” (Sec1/Munc18) and DOC2B (double C2-domain containing protein). Munc18 proteins localize to the cytosol and plasma membrane via direct binding to cognate Syntaxin partner proteins [37, 59, 171]. Upon glucose stimulation, Munc18 proteins allow for the “activation” of Syntaxin to its VAMP2-accessible “open” conformation, and subsequent assembly of the SNARE core complex, and fusion of insulin granules with the plasma membrane [26, 56]. DOC2B, via interactions with Munc18 proteins and Syntaxins, is essential for this mechanism to occur [56, 60, 74].

DOC2B belongs to the family of double C2 domain proteins. It is a ubiquitously expressed, 46-50 kDa protein, and has been shown to localize to the plasma membrane in  $\beta$ -cells and muscle/fat cells [28, 33, 56]. DOC2B is composed of an N-terminal Munc13-interacting domain (MID) linked to C-terminal tandem C2 domains (C2A and C2B), motifs known to bind  $\text{Ca}^{2+}$  and phospholipids. The C2A

and C2B domains of DOC2B also bind Munc18-1 and Munc18c, respectively, to regulate the SNARE complex and facilitate the “activation” of STX4, allowing for SNARE complex assembly and subsequent insulin secretion [26-28]. Several laboratories have shown that overexpression of DOC2B enhances insulin secretion by 30-40% in vitro [28, 60]. Moreover, transgenic mice overexpressing DOC2B (simultaneously in the pancreas, skeletal muscle, and adipose tissue) show enhanced whole body glucose homeostasis and insulin sensitivity, with enhanced islet glucose-stimulated insulin secretion (GSIS) ex vivo, but have normal fasting insulinemia [33]. These data suggest DOC2B enrichment to be beneficial, although detailed mechanisms for DOC2B actions in the  $\beta$ -cells are limited by the use of a global transgene expression system. Doc2b null knockout mice harbor dysfunctional islets [24, 25], consistent with observed low DOC2B transcript levels in islets of diabetic rodents [135]. However, it remains unknown whether DOC2B deficiency is causal or consequential to the loss of  $\beta$ -cell function and diabetes onset.

In this study, we tested the hypothesis that DOC2B deficiency underlies  $\beta$ -cell dysfunction and susceptibility to diabetes, and that Doc2b enrichment selectively in the  $\beta$ -cell is sufficient to confer protection against pro-inflammatory stimuli. Data gained from DOC2B heterozygous knock out mice, inducible  $\beta$ -cell specific transgenic DOC2B over-expressing ( $\beta$ Doc2b-dTg) mice, and DOC2B-boosted rodent  $\beta$ -cells, provide strong support for this hypothesis. Furthermore, these  $\beta$ Doc2b-dTg mice also show enhanced glucose tolerance under standard

conditions, associated with a robust capacity for robust insulin release, prompting investigations into the molecular mechanism by which DOC2B enrichment boosts GSIS. Furthermore, we show that enrichment of a peptide comprised of the DOC2B's tandem C2 domains (C2AB) is sufficient to confer the GSIS boosting effect of full-length DOC2B and also confers protection against thapsigargin-induced  $\beta$ -cell apoptosis.

### **3.3. RESEARCH DESIGN AND METHODS**

#### **3.3.1. Materials**

Rabbit anti- DOC2B antibody used for detection of endogenous DOC2B levels and detection of h DOC2B -DDK was purchased from Proteintech (1:1000 dilution, Rosemont, IL, USA, cat# 20574-1-AP). Rabbit anti-cMyc antibody was purchased from Santa Cruz Biotechnology (1:1000 dilution, Dallas, TX, USA, cat# sc-40). The rabbit anti-STX4 antibody used in the pull-down binding assay was generated in-house [172]. Cleaved caspase 3 antibody was purchased from Cell Signaling (1:1000 dilution, Danvers, MA, USA, cat# 9661). The CHOP antibody used was purchased from Cell Signaling (1:1000 dilution, Danvers, MA, USA, cat# 2895). The PARP antibody was purchased from Cell Signaling (1:1000 dilution, Danvers, MA, USA, cat# 9532). Phospho-elf2a and elf2a antibodies were purchased from Cell Signaling (1:1000 dilution, cat#9721 and #9722). Tubulin antibody was purchased from Abcam (1:5000 dilution, Cambridge, MA, USA, cat#ab56676). Goat anti-rabbit- and anti-mouse-horseradish peroxidase secondary antibodies were purchased from Bio-Rad

(Hercules, CA, USA, cat# 1706515; 1706516). Thapsigargin was purchased from Sigma (cat #T9033). Glutathione sepharose 4B agarose beads were obtained from GE Life Sciences (Pittsburgh, PA, USA). Enhanced chemiluminescence (ECL) was purchased from Amersham Biosciences (Pittsburg, PA, USA). Humulin R was obtained from Eli Lilly and Co. (Indianapolis, IN, USA).

### **3.3.2. Animals and in vivo experiments**

Animals were maintained under protocols approved by the Indiana University School of Medicine and City of Hope Institutional Animal Care and Use Committees and followed the Guidelines for the Use and Care of Laboratory Animals. Doc2b<sup>-/+</sup> heterozygous mice were generated as described previously [24]. Rat Doc2b cDNA was subcloned into the 5' Pme1 and 3' BamH1 restriction sites in the pTRE-pIRES-eGFP vector (gift from Drs. Solomon Afelik and Jan Jenssen, Cleveland Clinic) downstream of the TRE (tetracycline response element) to provide tetracycline/doxycycline (Dox) inducibility. Linearized plasmid was microinjected into C57BL6 oocytes (Transgenic Core, Indiana University School of Medicine). Of two founders, one transmitted the transgene to offspring, and was shipped to the City of Hope Animal Research Center for subsequent colony expansion. TRE-Doc2b<sup>-/+</sup> offspring crossed to rat insulin promoter (RIP)-rtTA<sup>-/+</sup> mice (purchased from Jax Labs) produced Dox-inducible  $\beta$ -cell selective expression in the double transgenic offspring ( $\beta$ Doc2b-dTg) mice. Mice were generated and maintained on the C57BL/6J genetic background. Female  $\beta$ Doc2b-dTg mice and single transgenic controls were fasted 6 h (08:00-14:00 hours) before intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal

insulin tolerance test (IPITT) analyses as previously described [24]. 2 mg/ml of glucose, and 1U/kg insulin were administered to mice for IPGTT and IPITT analyses, respectively. Streptozotocin (STZ) was freshly prepared in cold saline and injected intraperitoneally in multiple low doses (MLD; 35 mg/kg body weight, as described [173]) into male Doc2b-/+ and wildtype littermates (aged 8 weeks) for 5 consecutive days and assessed by IPGTT on day 10. Male mice were used for STZ experiments as this sex is reportedly more sensitive to STZ treatment, as previously described [174]. Male  $\beta$ Doc2b-dTg mice (aged 9-10 weeks) were provided Dox-treated (2 mg/ml) or standard drinking water for 3 weeks prior to MLD-STZ (35-40 mg/kg doses); IPGTT was assessed on day 24. On day 25 serum samples and tissues were collected, with pancreata fixed and paraffin-embedded for TUNEL staining. Non-STZ-treated male and female mice were used for tissue harvest and evaluation of expression of SNARE and SNARE accessory proteins. Serum insulin levels were measured via radioimmunoassay (Millipore).

### **3.3.3. Immunofluorescence and cell death assay**

Paraffin-embedded pancreatic tissue sections were prepared and assessed as previously described [59]. To assess  $\beta$ -cell specificity, pancreatic sections were immunostained with guinea pig anti-insulin (1:100 dilution, Dako/Agilent, Santa Clara, CA, USA cat#A0564), rabbit anti- DOC2B (1:200 dilution, Proteintech, Rosemont, IL, USA, cat# 20574-1-AP), and mouse anti-glucagon (1:200 dilution, Santa Cruz Biotechnology, Dallas, TX, USA, cat# sc-13091). Alexa Fluor 555 goat anti-rabbit, 647 goat anti-guinea pig, and 405 goat anti-mouse IgG (H+L)

were used to detect DOC2B, insulin, and glucagon, respectively. To assess apoptosis, pancreatic sections were immunostained with guinea pig anti-insulin (1:100 dilution, Dako/Agilent, Santa Clara, CA, USA cat#A0564). The TUNEL In Situ Cell Death Detection Kit Fluorescein (Roche, Mannheim, Germany) was used to stain apoptotic cells. Alexa Fluor 488 goat anti-guinea pig IgG (H+L) (1:500 dilution, Invitrogen) secondary antibody was used for detection of insulin. Sections were scanned using Zeiss Axio Observer and LSM 700 microscopes and analyzed using Image-Pro Software (Media Cybernetics, Rockville, MD, USA). Results were expressed as the percentage of cells positive for TUNEL staining relative to the total number of insulin-positive cells.

#### **3.3.4. Cell culture, transfections, GSIS and binding assays**

Human DOC2B adenovirus was generated by insertion of the hDoc2b-mycDDK cDNA from the pCMVhDoc2bmycDDK plasmid (purchased from OriGene Technology, Inc. MD, USA) into the pAd5CMVmpA adenoviral vector. Adenoviruses were packaged with EGFP to enable visualization of infection efficiency, amplified, and purified for use by Viraquest, Inc. (North Liberty, IA, USA). Rat INS-1 832/13  $\beta$ -cells (gift from C.B. Newgard, Duke University, Durham, NC, USA and Patrick Fueger, City of Hope, Duarte, CA, USA) were cultured in RPMI 1640 medium as previously described [175] and transduced with Ad5-CMV-human (h)Doc2b prior to treatment with a cytokine mixture (10 ng/ml TNF- $\alpha$ , 100 ng/ml IFN- $\gamma$  and 5 ng/ml IL-1 $\beta$ ; ProSpec, East Brunswick, NJ, USA) for 16 h, as previously described [151]. Cells were harvested in 1% NP-40 lysis buffer and cleared detergent lysates were used for immunoblotting for

apoptosis/ER stress markers. Mouse MIN6  $\beta$ -cells were cultured as described [55]. Cells were pre-incubated in serum-free media for 2 h followed before cells were harvested in 1% NP-40 lysis buffer and cleared detergent lysates were used for glutathione S transferase (GST)-VAMP2 interaction assays, as described previously [172]. Rat INS-1 832/13  $\beta$ -cells (passage 70-80) and MIN6  $\beta$ -cells (passage 3-7) were used for GSIS analyses performed using the K2 Transfection System (Biontix, Munchen, Germany). Doc2b-GFP and C2AB-GFP (gift from U. Ashrey, Tel-Aviv University, Ramat-Aviv, Israel).GSIS analyses were performed as previously described. INS-1 832/13 cells were placed in low glucose/low serum medium overnight prior to starvation in KRBB for 1 hr and glucose treatment (basal, 2.8 mM or glucose stimulated, 20 mM) for 30 minutes. MIN6 cells were starved in MKRBB for 1 hr prior to glucose treatment (basal-2.8 mM or glucose stimulated-20 mM). Supernatants were collected for insulin levels to be analyzed using the Rat Insulin ELISA (Mercodia, Uppsala, Sweden) or mouse Insulin ELISA (Alpco, Salem, NH, USA), respectively.

### **3.3.5. Islet Morphometry**

Islet morphometry was evaluated using anti-insulin immunohistochemical stained pancreatic sections as described [151]. Percentage of  $\beta$ -cell area was calculated using Keyence BZX-Analyzer software (Keyence Corporation, Itasca, IL, USA). Data shown are representative of three pancreatic sections per mouse.  $\beta$ -cell mass was calculated by multiplying percentage of  $\beta$ -cell area with pancreas weight.

### 3.3.6. Statistical analysis

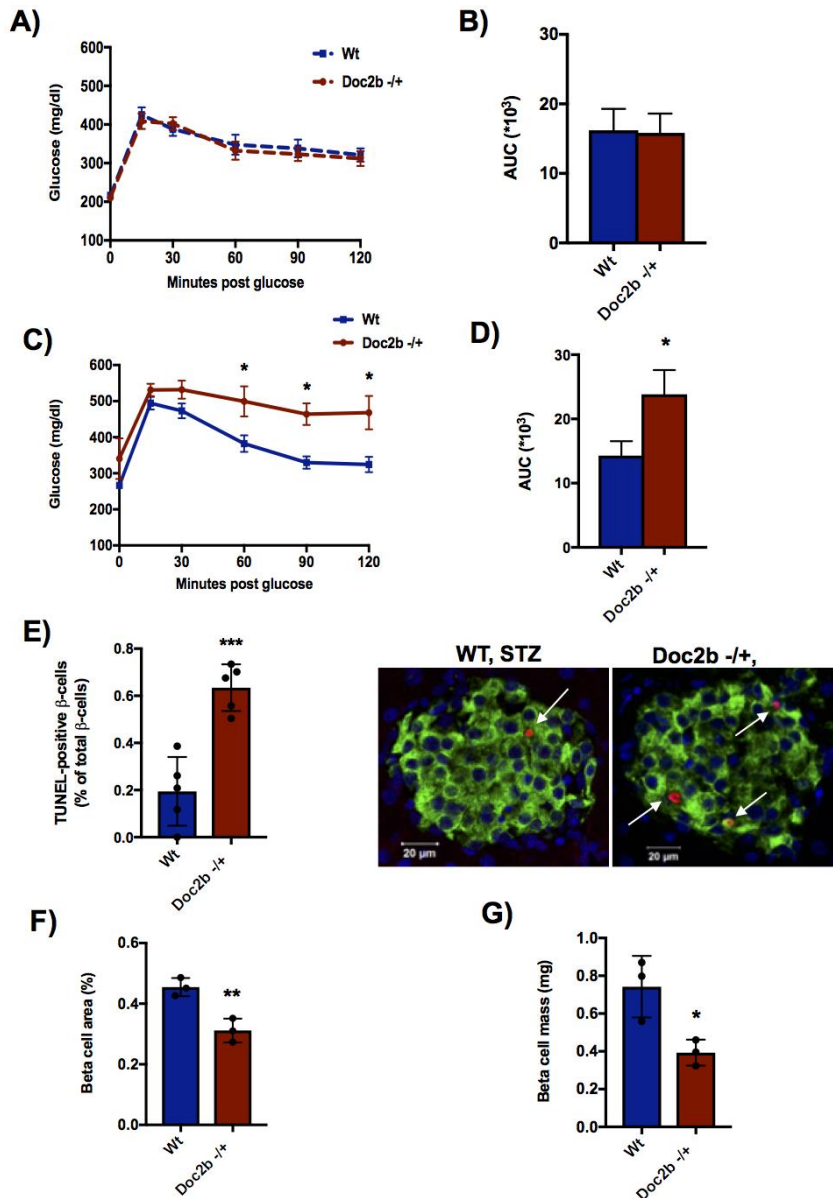
All data are expressed as the mean  $\pm$  SD for all figures except those representing IPGTT/ITT or Area under the curve (mean  $\pm$  SEM). Data were evaluated for statistical significance using Student's *t* test for comparison of two groups, or ANOVA and Tukey's posthoc test (GraphPad Software, La Jolla, CA, USA) for more than two groups.

## 3.4. RESULTS

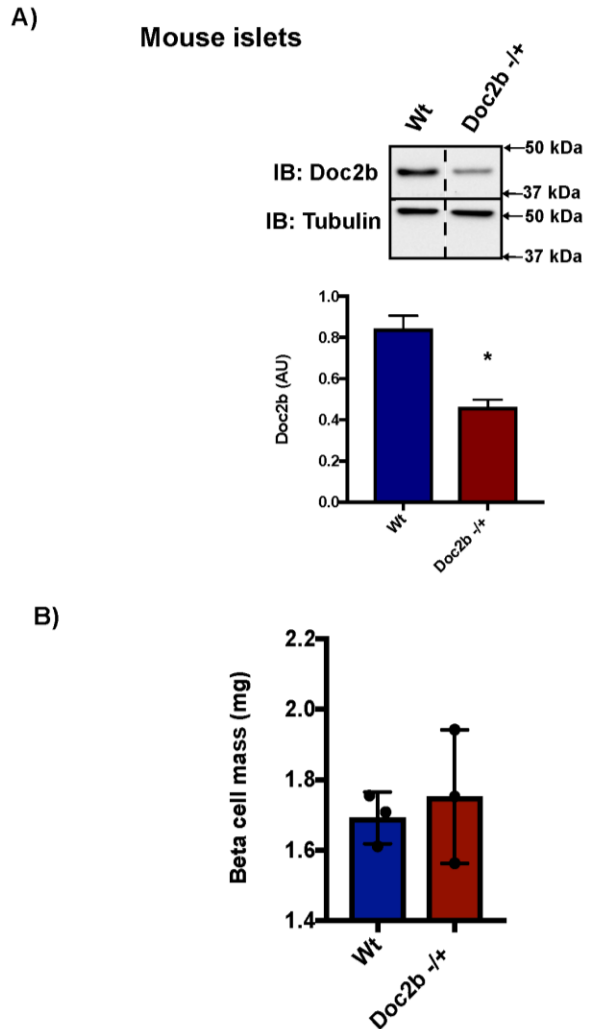
### 3.4.1. DOC2B<sup>-/+</sup> deficient mice are highly susceptible to STZ-induced glucose intolerance

To assess whether DOC2B abundance impacts  $\beta$ -cell susceptibility to T1D insults, we performed IPGTT assays prior to- and following a multiple low dose streptozotocin (35 mg/kg body weight) protocol (MLD-STZ), as previously described [173]. Prior to MLD-STZ treatment, young 7 wk old male Doc2b<sup>-/+</sup> and littermate wildtype (Wt) mice showed no differences in blood glucose levels (Figure 3.1.A-B). Ten days after the first STZ injection, Doc2b<sup>-/+</sup> mice showed significantly higher blood glucose levels than Wt mice (Figure 3.1.C-D). One day later all pancreata were collected and processed for TUNEL staining, which showed Doc2b<sup>-/+</sup> mice to have >3-fold higher levels of  $\beta$ -cell apoptosis as compared to Wt mice (Figure 3.1.E). Although the number of islets was similar in Doc2b<sup>-/+</sup> and Wt mice, the percentage of  $\beta$ -cell area was decreased in the Doc2b<sup>-/+</sup> mice, which also resulted in lower  $\beta$ -cell mass (Figure 3.1.F-G). DOC2B levels were ~50% reduced in islets from Doc2b<sup>-/+</sup> mice (Figure 3.2.A) and before STZ

treatment,  $\beta$ -cell mass was similar in Doc2b<sup>-/+</sup> vs Wt mice (Figure 3.2.B). Body weights of Doc2b<sup>-/+</sup> mice were equivalent to those of Wt mice over a 7- to 9-week period (Table 3.1.).



**Figure 3.1. DOC2B<sup>-/+</sup> deficient mice are more susceptible to STZ-induced glucose intolerance and  $\beta$ -cell apoptosis.** A) Prior to treatment with STZ, young 8 wk old male Wild type (Wt) and DOC2B<sup>-/+</sup> mice were fasted 6 h and subjected to intraperitoneal glucose tolerance test (IPGTT); n=13 mice/group. B) Quantification of area under the curve (AUC) for Pre-STZ IPGTT. C) DOC2B<sup>-/+</sup> and wild type littermates were then injected daily days 0-5 with STZ (35 mg/kg BW), and on day 10 were fasted 6 h and subjected to IPGTT; n=8 mice/group. D) Quantification of AUC for Post-STZ IPGTT. E) TUNEL immunofluorescent staining and quantification of TUNEL-positive  $\beta$ -cells (indicated by arrows in images, expressed as % of total  $\beta$ -cells) were conducted on fixed pancreases of STZ-treated DOC2B<sup>-/+</sup> and Wt mice. Data represent mean  $\pm$  SD; n=5 mice/group. Bar=20  $\mu$ m. F) Islet  $\beta$ -cell area and G) islet  $\beta$ -cell mass were calculated from fixed DOC2B<sup>-/+</sup> and Wt pancreases immunostained for insulin content; Data represent mean  $\pm$  SD; n=3 mice/group, 3 sections/mouse. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 DOC2B<sup>-/+</sup> vs. Wt mice.



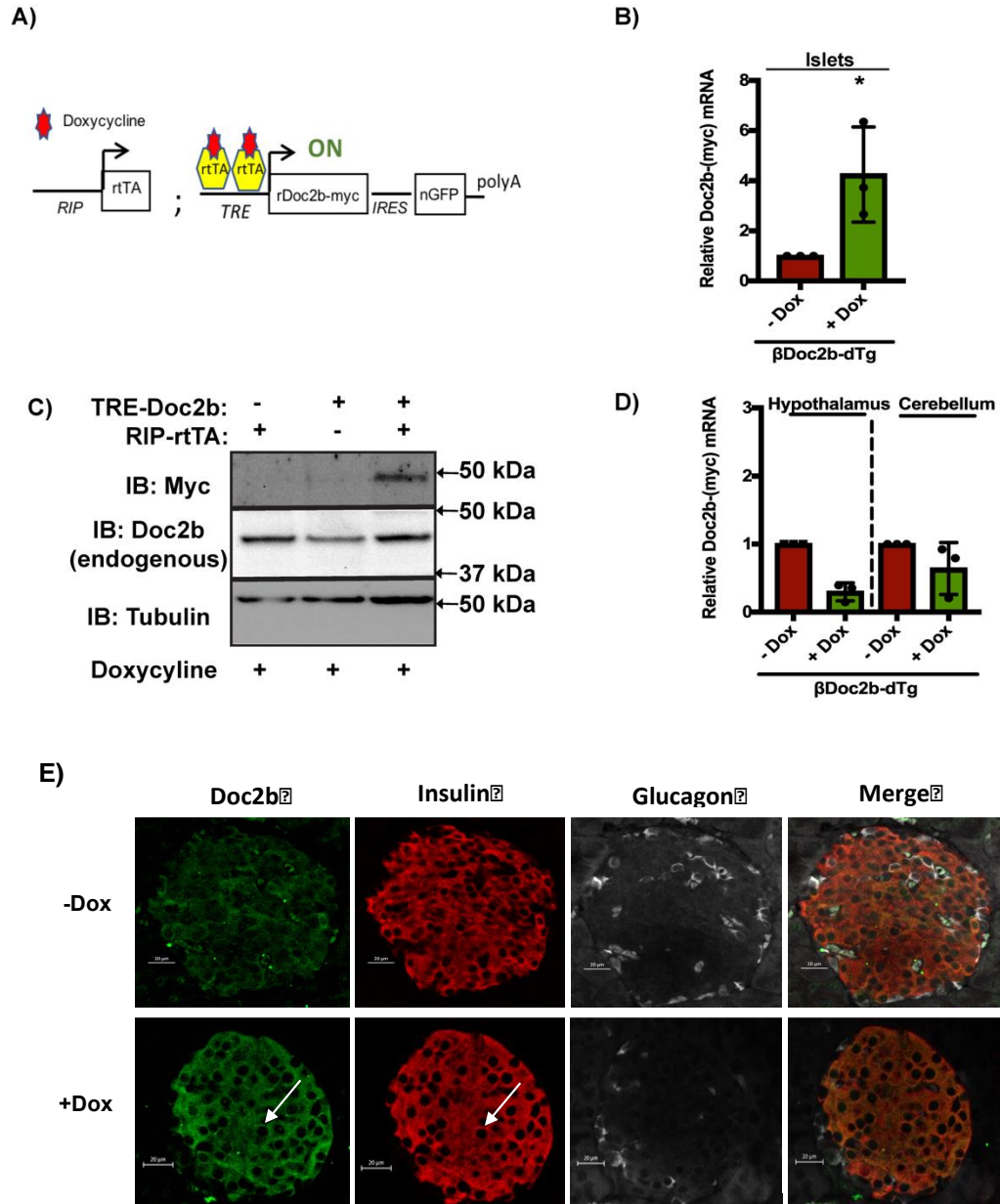
**Figure 3.2. Islets from Doc2b +/- mice are reduced in DOC2B, but have similar beta cell mass prior to STZ treatment.** (A) Islets from Doc2b +/- and Wt mice were harvested at 7 weeks of age and prepared for SDS-PAGE and immunoblotting with DOC2B and Tubulin. N=3 per group, \*p<0.05. (B) Pancreata from 7 week old Doc2b +/- and Wt were fixed in 4% paraformaldehyde prior to tissue sectioning and staining via immunohistochemistry for assessment of beta cell mass. N=3 per group, \*p<0.05.

<b>Body Weight (g)</b>	<b>Wild-type</b>	<b>Doc2b -/+</b>
<b>Pre-STZ</b>	22.8 ± 0.7	23.8 ± 0.6
<b>Post-STZ</b>	24.4 ± 0.4	24.2 ± 0.7

**Table 3.1. Body weights of Wild-type and Doc2b -/+ mice.** Data represent the average ± S.E. Body weights were measured for Wild-type and Doc2b -/+ male littermate mice Pre-STZ treatment at 7 weeks of age and Post-STZ treatment at 9 weeks of age (n=5 for WT and Doc2b +/- ) for determination of parameters shown. No statistical differences were seen.

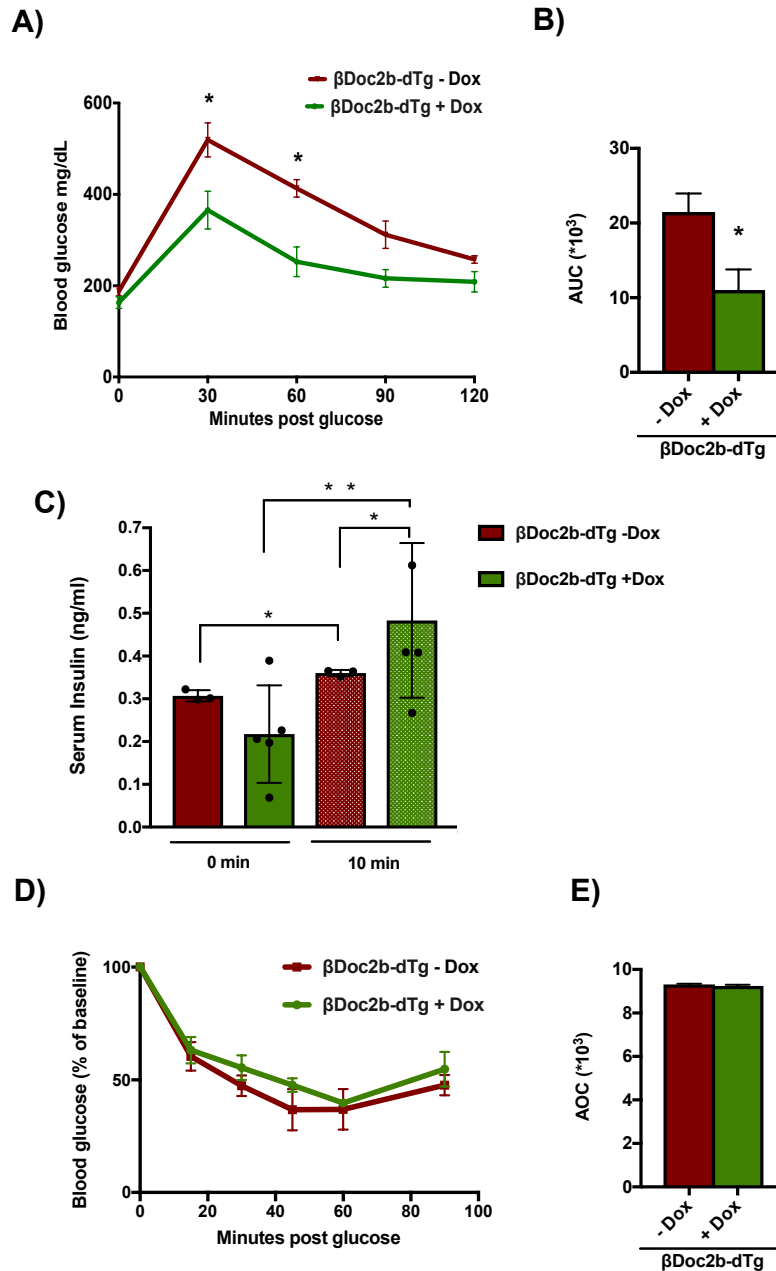
### **3.4.2. Enhanced glucose tolerance in doxycycline-inducible $\beta$ -cell specific DOC2B overexpressing transgenic mice**

To determine whether  $\beta$ -cell specific overexpression of DOC2B is sufficient to enhance whole-body glucose tolerance, we generated a doxycycline-induced,  $\beta$ -cell specific double transgenic mouse model ( $\beta$ Doc2b-dTg) (Figure 3.3.A). Doc2b-myc mRNA abundance in islets isolated from female mice was ~4-5-fold higher in Dox-induced  $\beta$ Doc2b-dTg mice as compared with non-Dox treated  $\beta$ Doc2b-dTg control mice (Figure 3.3.B), and the presence of the myc-tagged DOC2B protein was clearly detectable (Figure 3.3.C). No transgene overexpression was detected in hypothalamus or cerebellum (Figure 3.3.D). Immunofluorescent analysis of  $\beta$ Doc2b-dTg islets show  $\beta$ -cell specific overexpression of DOC2B compared with levels in non-Dox treated  $\beta$ Doc2b-dTg mice (Figure 3.3.E).



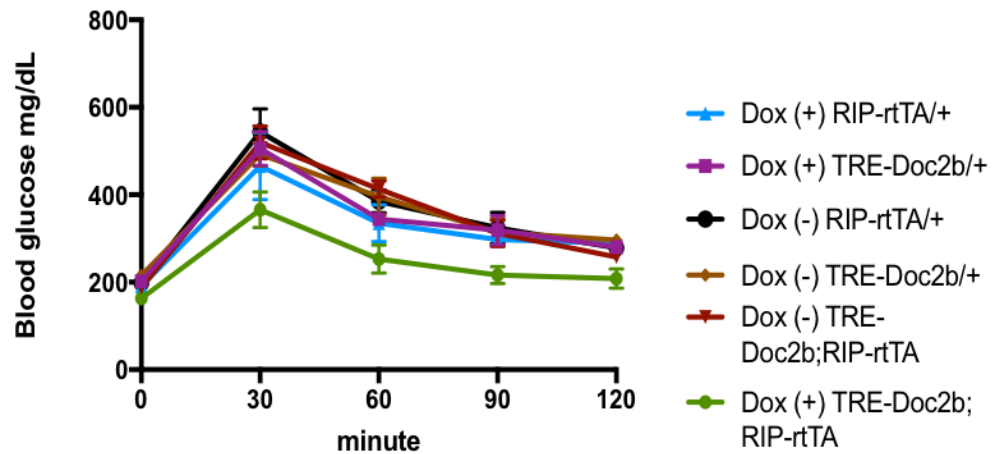
**Figure 3.3. Generation of  $\beta$ -cell specific Dox-inducible DOC2B-overexpressing mice.** A) Schematic design of RIP-rtTA<sup>+/+</sup> mice crossed to new TRE-Doc2b<sup>+/+</sup> mice. B) Doc2b mRNA, in primary islets isolated from female double transgenic ( $\beta$ Doc2b -dTg) mice. C) Dox-induced expression of DOC2B-myc protein in isolated islets, and D) lack of overexpression of rDoc2b mRNA in hypothalamus or cerebellum of the  $\beta$ Doc2b -dTg mice. E) Immunofluorescent imaging of DOC2B overexpression in the islets of the  $\beta$ Doc2b -dTg mice induced with or without Dox. Each panel of data represent the mean  $\pm$  SD of at least three mice per group; \*\* $p < 0.01$   $\beta$ Doc2b -dTg + Dox vs -Dox treated mice. Bar=20  $\mu$ m.

Changes to whole body glucose homeostasis were assessed by IPGTT in the  $\beta$ Doc2b-dTg mice. Dox-induced dTg mice showed significantly lower blood glucose levels at all time points after glucose injection; basal fasting blood glucose levels were comparable to that of non-Dox treated dTg control mice (Figure 3.4.A-B) or single transgenic mice (Figure 3.5.). Consistent with this, serum insulin levels in Dox-treated  $\beta$ Doc2b-dTg mice were 34% higher than controls within the first 10 min of glucose injection during IPGTT, but not statistically different under basal (time 0 in the IPGTT) conditions (Figure 3.4.C). No differences were seen between Dox-treated or control  $\beta$ Doc2b-dTg mice in intraperitoneal insulin tolerance tests, suggesting no changes to peripheral insulin sensitivity (Figure 3.4.D-E). No significant differences were seen in body/tissue weights of double and single transgenic mice (Table 3. These data suggest that enrichment of DOC2B selectively in adult pancreatic islet  $\beta$ -cells is sufficient to improve whole body glucose homeostasis.



**Figure 3.4.  $\beta$ -cell specific Dox-inducible DOC2B-overexpressing mice have enhanced glucose tolerance and glucose-stimulated serum insulin content.**

A) Female  $\beta$ Doc2b-dTg mice at 12-13 weeks of age were fasted 6 h and subjected to an intraperitoneal glucose tolerance test (IPGTT); n=5 mice/group. B) Quantification of area under the curve (AUC) for IPGTT. C) Serum insulin content in mice fasted for 6 h (0 min) and 10 min after a glucose injection. Data shown as mean  $\pm$  SD for three to five sets of mice. \* $p < 0.05$ , \*\* $p < 0.01$   $\beta$ Doc2b-dTg +Dox vs -Dox treated mice. D) Female  $\beta$ Doc2b-dTg mice were fasted 6 h and subjected to an intraperitoneal insulin tolerance test (IPITT); n=4 -Dox, n=3 +Dox treated  $\beta$ Doc2b-dTg mice. E) Area over the curve analyses of the IPITT. \* $p < 0.05$   $\beta$ Doc2b-dTg +Dox vs -Dox treated mice.



**Figure 3.5.  $\beta$ -cell specific Dox-inducible DOC2B-overexpressing mice have enhanced glucose tolerance versus single transgenic mice.** IPGTT following a 6 h fast for female  $\beta$ Doc2b-dTg (TRE-Doc2b; RIP-rtTA) and single transgenic controls (TRE-Doc2b/+; RIP-rtTA/+) with and without Dox treatment. Data shown are representative of three mice per group, average  $\pm$  SEM.

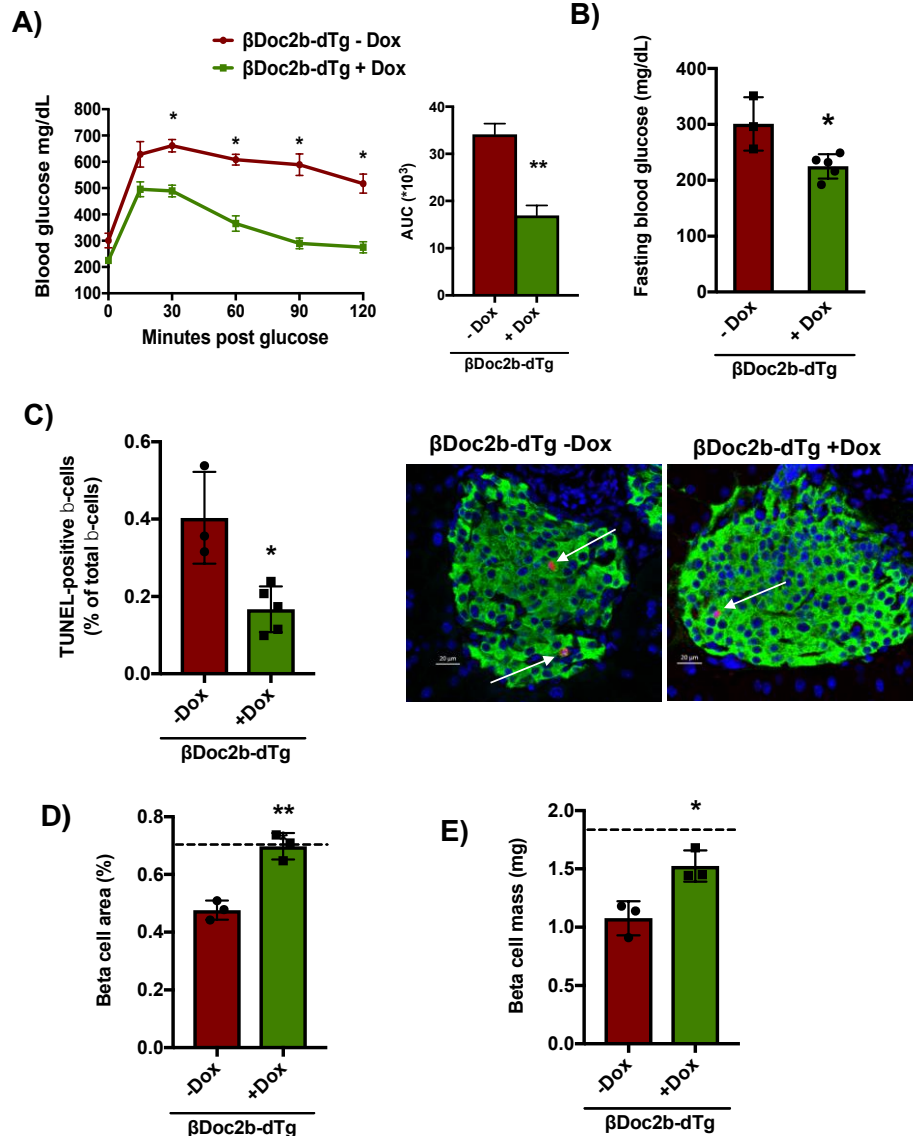
	Dox (-) RIP-rtTA/+	Dox (+) RIP-rtTA/+	Dox (-) TRE-Doc2b/+	Dox (+) TRE-Doc2b/+	Dox (-) TRE-Doc2b;RIP-rtTA	Dox (+) TRE-Doc2b;RIP-rtTA
<b>Body weight (g)</b>	23.9±0.90	23.0±0.83	24.0±0.90	24.2±1.10	23.6±0.60	23.9±0.20
<b>Heart (g)</b>	0.09±0.03	0.17±0.08	0.25±0.01	0.10±0.02	0.14±0.01	0.11±0.03
<b>Liver (g)</b>	1.19±0.11	1.18±0.14	1.48±0.10	1.19±0.10	1.17±0.06	1.29±0.09
<b>Spleen (g)</b>	0.07±0.01	0.08±0.03	0.07±0.05	0.07±0.01	0.06±0.02	0.05±0.01
<b>Brain (g)</b>	0.37±0.03	0.43±0.04	0.56±0.01	0.40±0.02	0.40±0.02	0.38±0.02
<b>Hypothalamus (g)</b>	0.05±0.02	0.04±0.02	0.08±0.03	0.04±0.01	0.07±0.04	0.03±0.01
<b>Fat (g)</b>	0.38±0.17	0.32±0.08	0.37±0.02	0.29±0.02	0.34±0.10	0.22±0.10
<b>Muscle (g)</b>	0.27±0.01	0.27±0.01	0.25±0.03	0.22±0.01	0.21±0.01	0.20±0.01
<b><i>Tissues (% of body weight)</i></b>						
<b>Heart (%)</b>	0.38±0.13	0.74±0.35	1.04±0.04	0.41±0.08	0.59±0.04	0.46±0.13
<b>Liver (%)</b>	4.97±0.46	5.13±0.61	6.16±0.41	4.92±0.41	4.95±0.25	5.39±0.38
<b>Spleen (%)</b>	0.29±0.04	0.35±0.13	0.30±0.20	0.29±0.04	0.25±0.08	0.21±0.04
<b>Brain (%)</b>	1.54±0.08	1.86±0.17	2.33±0.04	1.65±0.08	1.69±0.08	1.58±0.08
<b>Hypothalamus (%)</b>	0.21±0.08	0.17±0.08	0.33±0.13	0.17±0.04	0.30±0.17	0.13±0.04
<b>Fat (%)</b>	1.58±0.71	1.39±0.35	1.54±0.08	1.20±0.08	1.44±0.42	0.92±0.41
<b>Muscle (%)</b>	1.12±0.04	1.17±0.04	1.45±0.13	0.91±0.04	0.89±0.04	0.84±0.04

**Table 3.2. Body/tissue weights of Transgenic mice.** Data represent the average ± S.E. Body and tissue weights were measured for Dox (-) RIP-RtTA/+, Dox (+) RIP-rtTA/+, Dox (-) TRE-Doc2b/+, Dox (+) TRE-Doc2b/+, Dox (-) TRE-Doc2b;RIP-rtTA, and Dox (+) TRE-Doc2b;RIP-rtTA female littermate mice at 15-18 weeks of age (n=3 for each group) for determination of parameters shown. No statistical differences were seen.

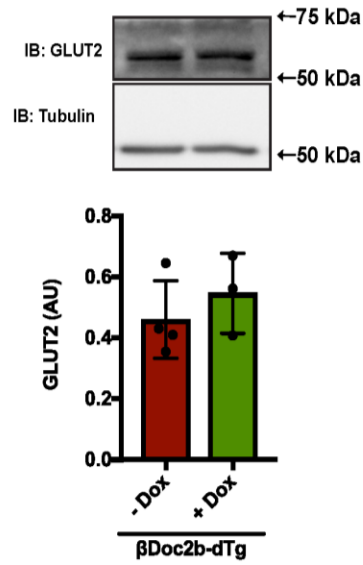
### **3.4.3. $\beta$ Doc2b-dTg mice are protected from STZ-induced glucose intolerance and $\beta$ -cell apoptosis.**

Male  $\beta$ Doc2b-dTg mice were subjected to the MLD-STZ protocol to determine if DOC2B enrichment in the  $\beta$ -cell provides protection from T1D-simulated destruction. IPGTT assessments at 24 days post-initiation of the protocol (14 days longer than used with Doc2b<sup>-/+</sup> mice) induced substantially increased blood glucose levels, with fasting levels ~300 mg/dl in non-dox-treated dTg mice (Figure 3.6.A-B). In contrast, Dox-induced  $\beta$ Doc2b-dTg mice were largely protected, even with the extended time after STZ treatment for maximal destruction.

To determine if protection from STZ-induced glucose intolerance and fasting hyperglycemia in the  $\beta$ Doc2b-dTg mice was related to changes in  $\beta$ -cell apoptosis, pancreata from the STZ-treated dTg mice were assessed by TUNEL staining. Islets from Dox-treated  $\beta$ Doc2b-dTg mice had ~58% fewer apoptotic cells than non-Dox-treated dTg mice (Figure 3.6.C). Dox-treated  $\beta$ Doc2b-dTg mice had higher  $\beta$ -cell area, corresponding to increased  $\beta$ -cell mass, compared with non-dox treated dTg mice (Figure 3.6.D-E). Additionally, GLUT2 expression was unchanged in Dox+ and Dox- dTg mice (Figure 3.7). Body weights of dTg -/+ Dox were similar pre and post-STZ treatment (Table 3.3.). These data suggest that overexpression of DOC2B protects islet  $\beta$ -cells against STZ-induced  $\beta$ -cell death.



**Figure 3.6.  $\beta$ -cell specific Dox-inducible DOC2B-overexpressing mice are protected from STZ-induced glucose intolerance, fasting hyperglycemia, and  $\beta$ -cell apoptosis.** A) Male  $\beta$ DOC2B-dTg mice aged 12-13 weeks old were injected daily on days 0-5 with STZ (35-40 mg/kg body weight), and on day 24 were fasted 6 h and subjected to an IPGTT, also shown: AUC quantification for IPGTT; n=3 -Dox, n=5 +Dox  $\beta$ DOC2B-dTg mice. B) Fasting blood glucose of STZ-treated  $\beta$ DOC2B-dTg mice; data represent mean  $\pm$  SD; n=3 -Dox, n=5 +Dox  $\beta$ DOC2B-dTg mice. C) Immunofluorescent staining and quantification of TUNEL-positive  $\beta$ -cells (indicated by arrows in images, expressed as % of total  $\beta$ -cells) were conducted on fixed pancreases of -Dox and +Dox-treated  $\beta$ DOC2B-dTg mice; data represents mean  $\pm$  SD; n=3 -Dox, n=5 +Dox. Bar=20  $\mu$ m. D) Islet  $\beta$ -cell area and E) islet  $\beta$ -cell mass were calculated from fixed  $\beta$ DOC2B-dTg whole pancreases immunostained for insulin content. Dotted line indicates the  $\beta$ -cell area and mass of non-STZ treated wild type mice in our colony [151]; Data represent mean  $\pm$  SD; n=3 mice/group, 3 sections/mouse. \*p<0.05, \*\*p<0.01  $\beta$ DOC2B-dTg +Dox vs -Dox treated mice.



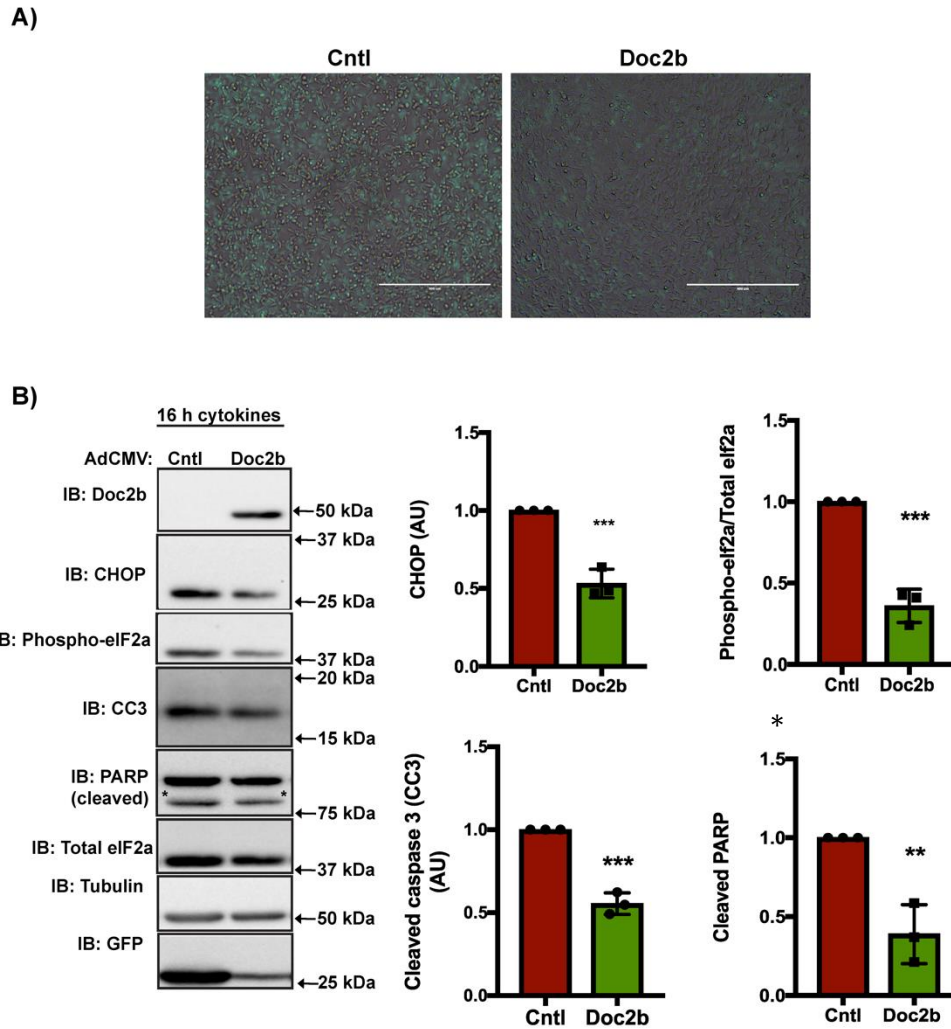
**Figure 3.7. GLUT2 levels are unchanged in Dox- and Dox+ dTg mice.** Islets from 12 week old female Dox- and Dox+  $\beta$ Doc2b-dTg mice were harvested and prepared for SDS-PAGE and immunoblotting for the  $\beta$ -cell specific glucose transporter, GLUT2. N=3, \*p<0.05.

<b>Body Weight (g)</b>	<b>dTg - Dox</b>	<b>dTg + Dox</b>
<b>Pre-STZ</b>	27.3 ± 1.2	29.4 ± 0.9
<b>Post-STZ</b>	28.3 ± 0.7	29.4 ± 1.1

**Table 3.3. Body weights of dTg-Dox and dTg+Dox mice, Pre/Post-STZ.** Data represent the average ± S.E. Body weights were measured for dTg-Dox and dTg+Dox male littermate mice Pre-STZ treatment at 12-13 weeks of age and Post-STZ treatment at 15-16 weeks of age (n=3 for dTg-Dox and N=5 for dTg+Dox ) for determination of parameters shown. No statistical differences were seen.

#### **3.4.4. Doc2b overexpression protects against cytokine-induced apoptosis and reduces ER stress.**

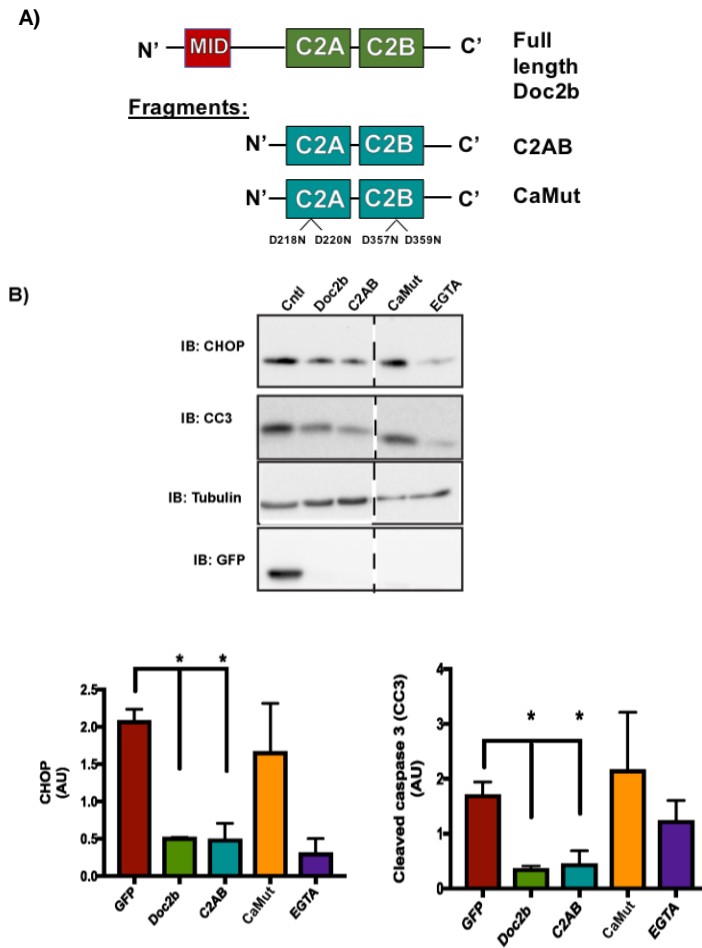
To date all studies of DOC2B overexpression in islet and in vivo function have used rat DOC2B; human (h) DOC2B has yet to be tested. Toward this, we generated adenoviral particles encoding hDoc2b tagged C-terminally with FLAG-DDK. Ad-hDoc2b-DDK was packaged with GFP for detection of  $\geq 70\%$  of rat INS-1 832/13 cells being virally-transduced (Figure 3.8.A). Treatment of Ad-hDOC2B-expressing INS-1 832/13 cells with pro-inflammatory cytokines for 16 h resulted in a 45% reduction in cleaved caspase-3 (CC3) and  $>50\%$  reduction in cleaved PARP as compared with Ad-control (Cntl) treated cells (Figure 3.8.B). Additionally, levels of ER stress markers, CHOP and Phospho-eIF2a were reduced by  $\sim 50\%$  and  $\sim 60\%$ , respectively, in Doc2b overexpressing  $\beta$ -cells (Figure 3.8.B). These data confirm that Doc2b promotes protection from apoptotic stimuli and reduces ER stress.



**Figure 3.8. DOC2B-overexpression in rat INS-1 832/13 cells protects against pro-inflammatory cytokine-induced  $\beta$ -cell apoptosis and ER stress.** A) Rat INS-1 832/13 were transduced with Ad5-CMV-hDOC2B-DDK or control virus (Cntl, Ad5-CMV) and subsequently treated with a cocktail of pro-inflammatory cytokines (10 ng/ml TNF- $\alpha$ , 100 ng/ml IFN- $\gamma$  and 5 ng/ml IL-1 $\beta$ ). B) Cleared detergent cell lysates were assessed for hDOC2B-DDK (~51 kDa, endogenous is 46 kDa) overexpression and effect upon cleaved caspase-3 (CC-3) levels. Asterisk indicates phospho-eIF2a band used for analysis. Tubulin and GFP served as controls for protein loading and cell line transduction, respectively. Vertical dashed line indicates splicing of a lane from within the same gel exposure. Data represent the mean  $\pm$  SD of three independent sets of homogenates for each cell line; \* $p$ <0.05, \*\*\* $p$ <0.001 for Ad-CMV-DOC2B vs Ad-CMV-Cntl treated cells. Bar=400  $\mu$ m.

### **3.4.5. A Doc2b peptide (C2AB) is sufficient to protect against thapsigargin-induced ER stress via calcium coordination.**

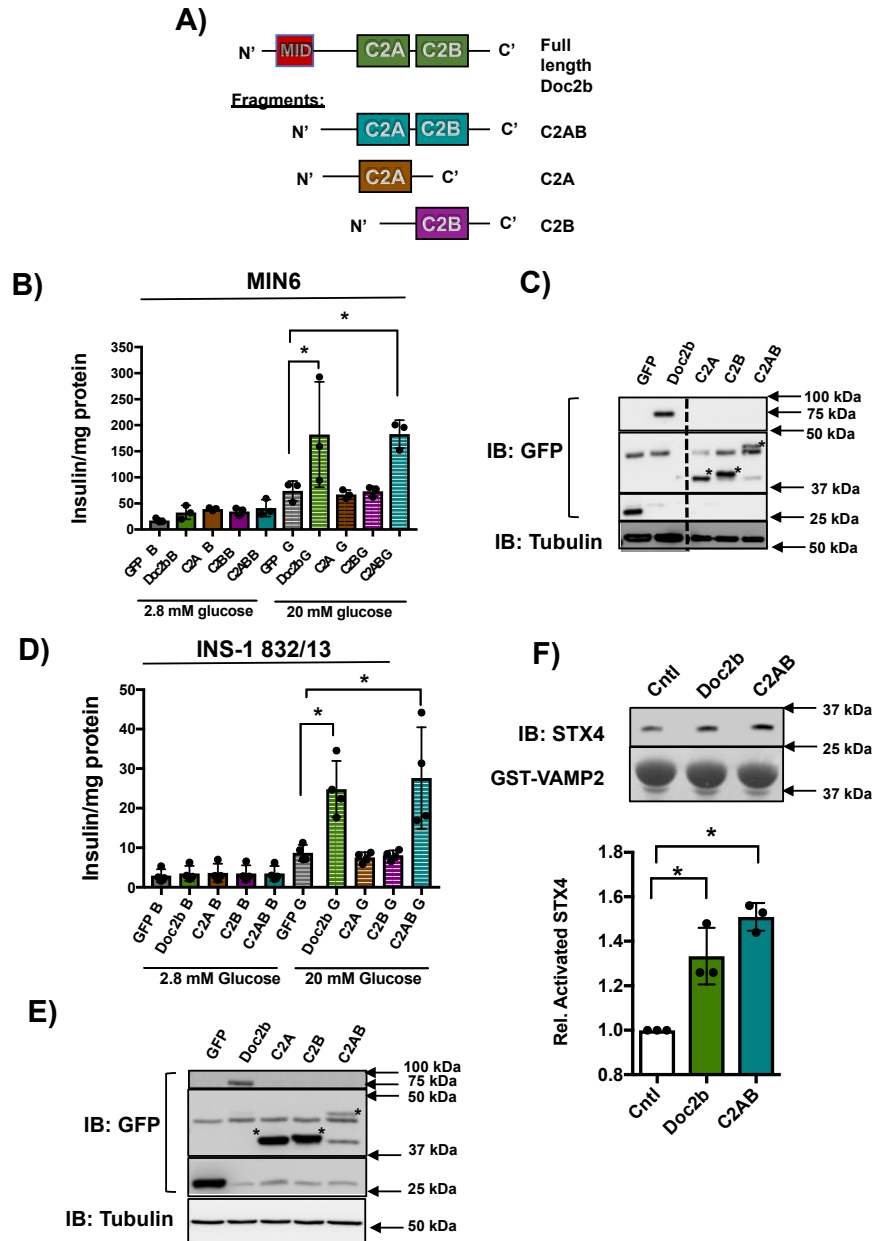
The DOC2B protein is composed of three primary domains, MID, C2A and C2B (Figure 3.9.A); the C2 domains are known calcium-sensing domains, binding 2 calcium ions each, and contributing to DOC2B's function. To further investigate DOC2B's anti-apoptotic actions and the role of C2 tandem domains in alleviating ER stress, plasmids encoding rDOC2B and an N-terminal truncation of the MID domain (referred to as C2AB), and a calcium ligand mutant form of C2AB (CaMut), which harbors 4 aspartic acid residue mutations (D218N, D220N, D357N, D359N), all with C-terminal GFP tags were generated (Figure 3.9.A). INS-1 832/13 cells were treated with a commercially available ER stress inducer and SERCA2b pump inhibitor, Thapsigargin. Treatment of DOC2B-GFP and C2AB-GFP overexpressing INS-1 832/13 cells with 1 $\mu$ M Thapsigargin for 6 h resulted in decreased levels of CHOP and CC3 compared to control (GFP) treated cells (Figure 3.9.B). These levels were similar to levels of CHOP and CC3 in cells treated with the calcium chelator, EGTA (Figure 3.9.B). This effect was abolished when cells were transfected with CaMut, as levels of CHOP and CC3 were elevated close to that seen in control (GFP) transfected cells (Figure 3.9.B). These data suggest that Doc2b's calcium binding capacity within the C2AB tandem domains is responsible for its protective effect against ER stress and apoptosis.



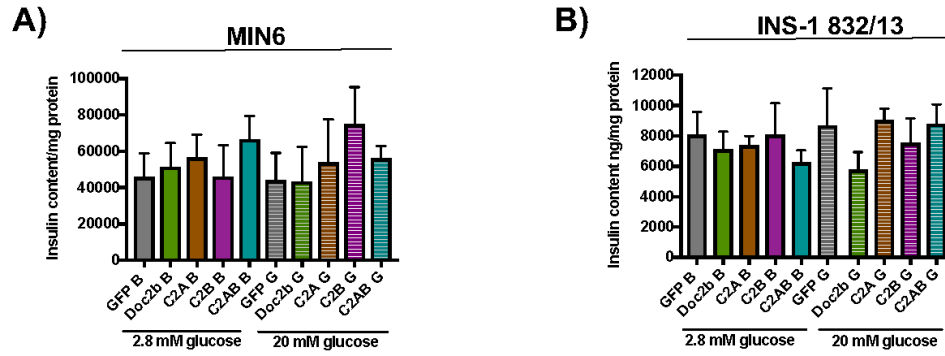
**Figure 3.9. C2AB is sufficient to protect against thapsigargin-induced ER stress via calcium coordination.** Rat INS-1 832/13 cells were transfected with rDoc2b, rC2AB, or CaMut (calcium ligand mutant of C2AB), and treated with Thapsigargin (1 $\mu$ M) for 6 hr prior to harvest with 1% NP40 lysis buffer. EGTA was used as a calcium chelator negative control. Whole cell lysates were run on SDS-PAGE gel for immunoblotting and assessed for levels of CHOP and CC3 proteins. Data represents average of independent 3 sets, \* $p$ <0.05.

### **3.4.6. DOC2B peptide C2AB is sufficient to enhance insulin exocytosis.**

The C2AB peptide was sufficient to protect  $\beta$ -cell against thapsigargin-induced apoptosis; we also wanted to investigate the capacity of C2AB and individual C2 domains (C2A and C2B) (Figure 3.10.A) to recapitulate DOC2B's functional enhancing properties. Full length rDOC2B-GFP expression in MIN6 cells recapitulated the enhancement of GSIS seen with rDOC2B in prior reports [28, 33, 60]. Remarkably, C2AB enhanced GSIS by the same ~2-3-fold level as did full length DOC2B (Figure 3.10.B). Neither C2A or C2B expression individually resulted in enhanced GSIS, despite confirmed expression of each to levels comparable to DOC2B-GFP; if anything, C2AB-GFP expression levels were slightly lower than the other proteins (Figure 3.10.C). Insulin content was also similar amongst all treatment groups (Figure 3.11A.). These results were recapitulated when constructs were tested in INS-1 832/13 cells (Figure 3.10.D-E, and Figure 3.11B.). DOC2B has been shown to be required for SNARE complex formation in  $\beta$ -cells, possibly via increasing STX4 activation and interaction with its cognate v-SNARE partner VAMP2 [33]. To determine if C2AB carries the capacity to replicate the underlying mechanism, C2AB or DOC2B proteins (untagged) were tested for the ability to evoke an increase in STX4 binding to GST-VAMP2 (Figure 3.10.F). Notably, C2AB protein fully recapitulated this increase in STX4 activation. These data suggest that C2AB constitutes a minimal region of DOC2B that is sufficient to confer enhanced GSIS and STX4 activation.



**Figure 3.10. Ability of the N-terminal truncation product C2AB to mimic full-length DOC2B function in glucose-stimulated insulin secretion.** A) Schematic of the rDOC2B-GFP truncation constructs. B) GSIS in MIN6 cells expressing full length rDOC2B, the N-terminal truncation mutant C2AB, and individual C2A or C2B domains. C) Expression was confirmed by immunoblotting; asterisks denote presence of the C2A, C2B, and C2AB bands by their expected molecular weights. Vertical dashed line indicates splicing of lanes from within the same gel exposure. D-E) GSIS and protein expression of rDOC2B-GFP truncation mutants in INS-1 832/13 cells; asterisks denote C2A, C2B, and C2AB bands. F) C2AB mimics the enhancement effect of full length DOC2B upon STX4 activation in MIN6 cells. Data represent the mean  $\pm$  SD of three independent sets of homogenates. \* $p < 0.05$  DOC2B or C2AB vs. GFP.



**Figure 3.11. Insulin content is similar in all transfected groups.** A) Insulin content/mg protein for MIN6 and B) INS-1 832/13 transfected cells with full length or C2 domains. Data shown are representative of three to four independent sets of tissue homogenates, average  $\pm$  SEM. No significant differences were detected.

### 3.5 DISCUSSION

The data presented here demonstrate an important role for DOC2B in maintaining  $\beta$ -cell mass and function. We propose that Doc2b deficiency leaves  $\beta$ -cells more susceptible to diabetogenic damage, and that overexpression of DOC2B in the  $\beta$ -cell enhances whole body glucose homeostasis and protects  $\beta$ -cell mass in an anti-apoptotic manner. We also show that C2AB comprises a minimal Doc2b peptide that is required for enhanced insulin secretion, and that its anti-apoptotic effects may involve calcium coordination within the C2AB peptide. Hence, the C2AB may provide a new target for promoting and protecting functional  $\beta$ -cell mass.

To our knowledge, this study is the first report of DOC2B having protective, anti-diabetic properties. Data presented herein reveals that Doc2b deficient mice (-/+ ) show greater susceptibility to MDL-STZ-induced  $\beta$ -cell destruction and worsened glucose tolerance. Contrasting this, our  $\beta$ -cell specific double transgenic DOC2B mice show enhanced whole-body glucose tolerance and resist MLD-STZ-induced glucose intolerance. TUNEL analyses and islet morphometry show the DOC2B-overexpressing mice have protected  $\beta$ -cell mass. INS-1 832/13  $\beta$ -cells transduced to overexpress human DOC2B also exhibit protection from pro-inflammatory cytokine and thapsigargin-induced apoptosis, indicating that DOC2B protection occurs at the level of the  $\beta$ -cells, and the protective effect is not dependent upon inter-islet non- $\beta$ -cell communication nor due to a circulating factor in vivo. The role of DOC2B or other exocytosis proteins in  $\beta$ -cell survival

mechanisms still remains largely unexplored, however recent reports have shown involvement of exocytosis proteins, such as VAMP8 involved in regulating  $\beta$ -cell proliferative events [52]. In neurons, DOC2B has been implicated in proliferation and differentiation during early embryogenesis [105, 106]. Yet, islet mass by morphometry in mouse islets with increased or decreased DOC2B levels isolated from mice under standard conditions, unchallenged by diabetogenic stimuli, is reportedly unchanged from that of wild-type littermate mice [24, 25, 33]; these data do not support the concept that DOC2B overexpression is acting via promoting proliferation or differentiation in the  $\beta$ -cell per se. Data reported here point to the action of DOC2B overexpression in promoting  $\beta$ -cell survival and protecting  $\beta$ -cell mass in the face of inflammatory and ER stress, with the protection occurring at the level of the  $\beta$ -cell itself. This is supported by our data showing the anti-apoptotic actions of h DOC2B overexpression in rat  $\beta$ -cell lines.

DOC2B is a C2-domain containing protein, in the family of proteins including Munc13, synaptotagmins, Ferlins, Rabphilin 3A and RIM1/2 [176-178]. These proteins have in common two aspects: all are calcium regulated/calcium sensing, and all are implicated in vesicle trafficking/exocytosis. The role of DOC2B as a calcium sensor in  $\beta$ -cells has been reported [60] and the tandem C2 domain region of DOC2B, referred to here as the C2AB, has been shown to be sufficient to support membrane fusion in cell-free assays, and shows stronger liposome binding than either of the isolated C2A and C2B domains [106]. Indeed, our data

show that C2AB can fully recapitulate the anti-apoptotic effects of full-length DOC2B in a calcium coordinating fashion. The calcium mutant form of C2AB has been previously reported to abolish DOC2B's calcium binding capacity [179]. ER stress mechanisms are known to be calcium-dependent events, and thus we propose that DOC2B overexpression may lead to sequestration of calcium ions, resulting in reduced cleaved caspase 3, PARP, CHOP and P-eIF2a.

C2AB also recapitulated GSIS enhancement seen with full-length DOC2B, whereas the C2 domains individually did not. Interestingly, the C2AB protein was able to recapitulate the activating effect of full-length DOC2B upon STX4 activation, consistent with prior work showing DOC2B to promote SNARE protein assembly [26, 60, 73, 74]. C2AB may confer its SNARE complex- promoting effect, in part, by acting directly on the membrane, as recent work shows that C2AB is sufficient to assemble in a ring-like oligomer [180], and act directly on membranes in regulating hemifusion events in neurons [181]. These findings are important starting points for further mechanistic investigation of DOC2B function in  $\beta$ -cells.

In summary, the data presented here show a novel, anti-apoptotic role for DOC2B in the  $\beta$ -cell. We propose that overexpression of DOC2B is sufficient to enhance whole body glucose homeostasis, and to protect  $\beta$ -cell mass against T1D-related stress. Furthermore, we show that the tandem C2AB domain is sufficient to enhance insulin secretion and protect against apoptosis, and that this

may be due to calcium coordination. DOC2B thus carries the potential to be a therapeutic target for prevention/management of T1D.

**CHAPTER 4**  
**DISCUSSION**

## **4.1. OVERVIEW**

The objective of my dissertation was to investigate whether DOC2B abundance was compromised during the development of T1D, and whether early,  $\beta$ -cell specific enrichment of DOC2B would protect functional beta cell mass against T1D insults. Toward this, DOC2B protein abundance was assessed in islets and blood borne platelets of new-onset T1D rodents and humans, as well as in the platelets of T1D islet transplant recipients. Additionally,  $\beta$ -cell specific transgenic mice were used to investigate the effect of DOC2B overexpression on whole body glucose homeostasis and  $\beta$ -cell mass under diabetogenic conditions. In the following sections, I will highlight findings from each chapter and future studies necessary to further elucidate the mechanisms of the phenomena described herein. Finally, I will discuss the overall significance and translational potential of the current studies in the context of preventing/treating T1D.

### **4.1.1. Major findings**

DOC2B is one of three isoforms ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) belonging to the family of Double C2 domain proteins, and is known to regulate SNARE-mediated secretion both in neurons and  $\beta$ -cells. Previously, our laboratory and others have demonstrated that DOC2B overexpression enhances GSIS in vitro, and in vivo via a whole body transgenic model [28, 33]. My studies validate these findings, and furthermore, establish a correlation between T1D and DOC2B abundance in islets and platelets. My studies also provide evidence that DOC2B overexpression protects  $\beta$ -cell mass against T1D insults. In addition to establishing a correlation between T1D and DOC2B, the following major findings

were resultant of the current study: 1) DOC2B abundance is reduced in platelets of non-obese diabetic mice before conversion to T1D, and this reduction was mirrored in islets of the same mice; 2) DOC2B abundance is reduced in platelets of new-onset T1D human subjects, as well as in islets of early-onset T1D humans and islets treated with pro-inflammatory cytokines in vitro; 3)  $\beta$ -cell specific overexpression of DOC2B results in enhanced whole body glucose homeostasis in the absence and presence of T1D diabetogenic stress; 4) DOC2B overexpression protects  $\beta$ -cell mass against T1D diabetogenic stress in an anti-apoptotic manner; 5) the truncated C2AB domain recapitulates enhanced GSIS, STX4 activation, and mutations of DOC2B residues implicated in calcium handling ablate the functional and protection action of DOC2B and C2AB, respectively.

**4.1.2. DOC2B abundance is reduced in platelets and islets from prediabetic rodents and new onset T1D human subjects; DOC2B abundance is restored after clinical islet transplantation.**

My studies aimed to investigate whether DOC2B was a viable biomarker of  $\beta$ -cell destruction in T1D. Platelets were used as a blood-borne source of biomarkers because they harbor many of the same exocytosis proteins as the pancreatic  $\beta$ -cell, including DOC2B, and changes in platelet proteome and morphology have been noted in T1D [146]. My data established that prior to onset of T1D, and while still exhibiting normoglycemia, NOD mice have reduced abundance of DOC2B in platelets, and this reduction is mirrored in NOD islets versus NOR control mice, even as early as 7 weeks of age. I next sought to investigate

whether reduced platelet DOC2B levels was translatable in humans. My research revealed that DOC2B protein levels are reduced in platelets of new-onset T1D subjects, and remain low 7-10 weeks post-diagnosis, despite therapeutic intervention and remediation of hyperglycemia. DOC2B abundance is also reduced in cadaveric T1D human islets compared to non-diabetic controls. In vitro, treatment of non-diabetic human islets with pro-inflammatory cytokines results in decreased DOC2B protein and mRNA abundance, suggesting that the inflammatory milieu is causal to DOC2B loss. Remarkably, within 30 days of islet transplantation in T1D subjects, robust increase in DOC2B protein is evident, and persisted to the 75-day sampling. Increases in platelet DOC2B levels coincided with increased plasma C-peptide levels in both transplant subjects, suggesting that increased platelet DOC2B levels mirror increased infused functional islet mass.

To our knowledge, this is the first study to establish an association between T1D and levels of an exocytosis protein in blood-derived platelets and pancreatic islets. Reduced DOC2B in islets is indicative of deficient islet functional health [24]. Strikingly, platelet DOC2B levels in islet transplant recipients correlated with the presence of a functional islet mass. This correlative finding supports the possibility that the platelet DOC2B stems not necessarily from the pancreas per se, since islets are grafted into the liver in these human recipients, but that the platelets and/or precursor megakaryocytes may be sampling DOC2B from the islets irrespective of islet location. It also remains possible that the increased

DOC2B content stems from 'rested' native residual islets of the transplanted patients. However, this is inconsistent with our pediatric platelet data showing that even after insulin therapy to ameliorate new-onset hyperglycemia, DOC2B levels remained deficient. Mechanistically, questions arise as to if and how platelets and islets communicate to determine DOC2B levels. Supporting the concept of platelet-islet communication, it has been demonstrated that islet transplantation in T1D patients stabilizes platelet abnormalities, as transplant recipient platelets show normal volume and activation [155]. Indeed,  $\beta$ -cells release exosomes as a way of shuttling various miRNAs, mRNAs, and proteins to targeted peripheral cells [156].  $\beta$ -cell exosomes were also recently shown to carry proteins, such as GAD-65, IA-2, and proinsulin, to dendritic cells, which then become activated [157]. Could islets similarly release DOC2B mRNA or protein, which is subsequently taken up by megakaryocytes or by the platelets themselves? Furthermore, platelets can selectively absorb proteins from the blood [158]. In fact, platelet sequestration of tumor-specific proteins was detected in animals harboring small tumors [158]. Notably, a direct interaction between platelets and pancreatic  $\beta$ -cells has been reported, and protein from platelets was shown to be transferred to  $\beta$ -cells [159]. Is it possible that the platelets are sampling the islets to mirror islet DOC2B levels? Previously, our lab and others established that reduced levels of DOC2B proteins in islets is indicative of deficient islet functional health overall [24, 25]. Thus, reduced levels of DOC2B in blood-borne platelets may mirror reduced functional  $\beta$ -cell mass.

#### **4.1.3. DOC2B deficiency increases susceptibility to T1D insults; DOC2B overexpression protects beta cell mass in an anti-apoptotic manner.**

DOC2B deficiency renders pancreatic islets dysfunctional [24, 25]. Additionally, low DOC2B transcript levels have been reported in islets of diabetic rodents [135]. Thus, my studies aimed to investigate the hypothesis that DOC2B deficiency underlies  $\beta$ -cell dysfunction and susceptibility to diabetes. My research revealed that following multiple low dose STZ treatment, DOC2B heterozygous (-/+) mice show worsened glucose tolerance compared to wild type littermate control mice. Isolated pancreata from MLD-STZ treated DOC2B<sup>-/+</sup> mice show ~3 fold higher levels of  $\beta$ -cell apoptosis, which corresponded with lower  $\beta$ -cell mass than Wt control mice.

Given that DOC2B deficiency increases susceptibility to T1D damage, my research next aimed to investigate whether overexpression of DOC2B had a protective effect on  $\beta$ -cell mass under the same diabetogenic conditions. Thus, we generated a doxycycline-inducible,  $\beta$ -cell specific DOC2B overexpressing transgenic mouse ( $\beta$ DOC2B-dTg). My studies revealed that  $\beta$ DOC2B-dTg mice show enhanced whole-body glucose tolerance. After MLD-STZ treatment,  $\beta$ DOC2B-dTg mice displayed normal glucose tolerance. Furthermore,  $\beta$ DOC2B-dTg mice show lower levels of  $\beta$ -cell apoptosis, corresponding with protected  $\beta$ -cell mass, compared to control mice. In vitro, human and rodent  $\beta$ -cells transduced to overexpress human DOC2B also exhibit protection from pro-

inflammatory cytokine-induced apoptosis, indicating that DOC2B protection occurs at the level of the  $\beta$ -cells, and the protective effect is not dependent upon inter-islet non  $\beta$ -cell communication nor is it due to a circulating factor in vivo.

The role of DOC2B in  $\beta$ -cell survival mechanisms is still largely unexplored, however, in neurons, DOC2B has been implicated in proliferation and differentiation during early embryogenesis [105]. Yet, islet morphometry in mouse islets with increased or decreased DOC2B levels isolated from mice under standard conditions, unchallenged by diabetogenic stimuli, is reportedly unchanged from that of wild-type mice [24, 25, 33], which does not support the concept that DOC2B overexpression is acting via proliferative or differentiation mechanisms in the  $\beta$ -cell per se. My data rather point to the action of DOC2B overexpression in promoting  $\beta$ -cell survival and protecting  $\beta$ -cell mass against inflammatory stress, with the protection occurring at the level of the  $\beta$ -cell itself.

**4.1.4. DOC2B overexpression protects  $\beta$ -cells against cytokine induced apoptosis and reduces ER stress; The DOC2B peptide, C2AB, is sufficient to protect  $\beta$ -cells against Thapsigargin-induced ER stress.**

To further investigate the mechanism by which DOC2B confers anti-apoptotic effects, I show that cytokine-treated INS 832/13  $\beta$ -cells overexpressing DOC2B have lower levels of cleaved caspase 3, CHOP, and Phospho-eIF2a. Furthermore, overexpression of DOC2B or the DOC2B peptide, C2AB, is able to

protect  $\beta$ -cells from Thapsigargin-induced ER stress in a calcium coordinating fashion that is dependent on C2AB's calcium binding ability.

DOC2B is a C2-domain containing protein, in the family of proteins including Munc13, synaptotagmins, Ferlins, Rabphilin 3A and RIM1/2 [176-178]. These proteins have in common two aspects: all are calcium regulated/calcium sensing, and all are implicated in vesicle trafficking/exocytosis. The role of DOC2B as a calcium sensor in  $\beta$ -cells has been reported [60] and the tandem C2 domain region of DOC2B, C2AB, has been shown to be sufficient to support membrane fusion in cell-free assays, and shows stronger liposome binding than either of the isolated C2A and C2B domains [106]. Indeed, our data show that C2AB can fully recapitulate the anti-apoptotic effects of full-length DOC2B in a calcium coordinating fashion. The calcium mutant form of C2AB has been previously reported to abolish DOC2B's calcium binding capacity [179]. ER stress mechanisms are known to be calcium-dependent events, and thus we propose that DOC2B overexpression may lead to sequestration of calcium ions, resulting in reduced cleaved caspase 3, PARP, CHOP and P-eIF2a.

#### **4.1.5. The truncated C2AB domain confers the same GSIS enhancing properties as full length DOC2B**

Previous work has shown that overexpression of DOC2B enhances GSIS, although the mechanism underlying this beneficial action remains unresolved. DOC2B's three primary domains, MID, C2A and C2B, each bind to a variety of proteins that might contribute to DOC2B function. My data revealed that the

tandem C2 domain region of DOC2B, C2AB, is the minimal region sufficient to fully recapitulate enhanced enhanced GSIS and SNARE complex formation, as seen with full length DOC2B. Neither of the individual C2 domains (C2A or C2B) are able to recapitulate the activating effect of full-length DOC2B. My data is consistent with prior work showing C2AB promoting SNARE protein assembly [180, 182]. C2AB may confer its SNARE complex-promoting effect by acting directly on the membrane [181]. Thus, C2AB may be a viable small molecule that can be used to promote and protect functional  $\beta$ -cell mass.

## **4.2. FUTURE STUDIES**

### **4.2.1. Assess DOC2B abundance in platelets and islets from autoantibody positive, prediabetic T1D human subjects**

My data demonstrate reduced DOC2B levels in new-onset T1D human subjects. However, the current studies used relatively small sample sizes of pediatric subjects (N=14 controls and N=17 T1D) who already had clinical diabetes. Future investigations using subjects who are significantly at risk for T1D (presence of one or more autoantibodies), but not yet diagnosed, are necessary to validate the potential for DOC2B to be an early biomarker/indicator of pre-T1D in humans. My studies were also limited in that only the first clinic follow-up samples were obtained; further follow-up longitudinal evaluations of DOC2B changes for longer periods of time would be important to investigate any correlations between DOC2B levels and overall glycemic control. Future studies should also examine DOC2B levels in serum and plasma, both of which contain

abundant and detectable levels of DOC2B. This will permit studies of larger numbers of pre-/T1D subjects, as well as permit evaluation of stored samples from a variety of repositories. Yet, since platelets 'sample' proteins and mRNA from other cells, they may be the unique depot to detect changes in DOC2B levels corresponding to changes in functional islet mass. In order to accommodate a large cohort of samples, it would also be beneficial to create a DOC2B enzyme-linked immunosorbant assay (ELISA) designed for high sensitivity, high throughput assays using a well-validated, reliable human DOC2B antibody.

#### **4.2.2. Assess DOC2B levels in circulating beta cell specific exosomes**

Notably, my studies revealed that islet infusion raised levels of platelet DOC2B in two T1D transplant recipients. This finding supports the concept that platelets and/or precursor megakaryocytes may be sampling DOC2B from the islets. Supporting the concept of islet-platelet 'communication', it has been shown that islet transplantation in T1D patients stabilizes platelet abnormalities [155]. Indeed  $\beta$ -cell specific exosomes have been identified carrying various miRNAs, mRNAs, and proteins to targeted peripheral cells [156]. It is possible that DOC2B mRNA or protein is shuttled to platelets via exosomes. Exosomes can be isolated from serum [157], using various isolation techniques. Future studies could then investigate changes in DOC2B levels in exosomes from blood samples of T1D subjects prior to and following islet transplantation. Exosomal release of DOC2B

can be further validated in vitro using human islets and rodent cell lines subjected to cytokine treatment to simulate T1D inflammation.

#### **4.2.3. Beta cell specific DOC2B overexpressing transgenic NOD mice**

My data revealed DOC2B to have protective, anti-diabetic properties in rodents and in vitro. STZ is a commonly used reagent to model T1D in vivo, however, there are limitations to its use, as STZ is a chemical that causes  $\beta$ -cell destruction via alkylating mechanisms that damage  $\beta$ -cell DNA. While STZ is a useful chemical for pilot studies, it is not translatable to mechanisms involved in onset of T1D in humans. Future studies should aim to investigate effects of DOC2B overexpression in rodent models that are genetically susceptible to T1D. The NOD mouse is one of the most commonly used strains of spontaneously converting, T1D rodents.  $\beta$ DOC2B-dTg mice should be introgressed into the NOD strain to generate doxycycline-inducible, beta cell specific overexpressing NOD mice. Overexpression of DOC2B can be induced before and after conversion to T1D to further assess DOC2B's potential to protect  $\beta$ -cell mass against onset of disease or to protect residual  $\beta$ -cell mass, respectively. Islets from the DOC2B overexpressing NOD mice can also be used in cytotoxic T lymphocyte (CTL) killing assay to quantify  $\beta$ -cell death and protection against lymphocytic destruction, as previously described [183].

#### **4.2.4. Transplantation of DOC2B/C2AB overexpressing human islets**

The C2AB region of DOC2B has been shown to promote SNARE assembly in neurons [181]. My studies demonstrated that the C2AB region of DOC2B is the minimal region required for enhanced GSIS and SNARE complex assembly in

the  $\beta$ -cell. Future studies should focus on the translatable potential to target C2AB as a peptide-based therapy to protect functional  $\beta$ -cell mass in a human islet transplantation model. An adenovirus should be generated to similarly overexpress C2AB, and perfusion analyses can be conducted to evaluate changes in first and second-phase insulin secretion. If C2AB similarly enhances insulin secretion in human islets, adenovirally transduced human islets overexpressing C2AB and/or full length DOC2B can be transplanted in diabetic mice. Our lab has recently used a minimal islet transplant paradigm to test the ability of STX4 upregulation in transplanted islets to reverse diabetes in STZ-induced diabetic mice [9]. Similarly, the minimal islet transplant model should be used in recipient NSG (NOD scid gamma immunodeficient) mice prior to treatment with high dose STZ (180 mg/kg body weight), as previously described [9] and blood glucose measurements obtained to assess glucose homeostasis and the ability of C2AB and/or DOC2B to protect islet grafts from diabetic insults.

#### **4.2.5. Investigate DOC2B's $\text{Ca}^{2+}$ handling and protection from apoptosis.**

DOC2B's calcium sensing properties have been extensively studied in neurons [106, 176, 179], and to a lesser extent in the  $\beta$ -cell [60]. Furthermore, my studies are the first to establish that DOC2B can protect  $\beta$ -cells from diabetic insults, specifically via calcium coordination and reduction of ER stress. I showed that a mutant form of C2AB (calcium ligand binding mutations at 4 residues) was unable to protect against thapsigargin-induced ER stress and apoptosis. This should be further investigated. Isothermal titration calorimetry (ITC) should be

used to validate the ability of DOC2B, C2AB, and the calcium mutant (CaMut) to bind  $\text{Ca}^{2+}$  in the  $\beta$ -cell, using methods as previously described [179]. Intracellular calcium levels should be quantified using a reagent such as Fura-2 AM (using techniques as described [179]) in DOC2B or C2AB overexpressing  $\beta$ -cells that are subjected to cytokine or thapsigargin treatment in order to further prove that DOC2B/C2AB is sequestering  $\text{Ca}^{2+}$  stores, leading to protection against ER stress. RNA sequencing should be performed on cytokine treated-human islets overexpressing DOC2B or C2AB in order to identify further potential transcriptional “hits” involved in DOC2B’s anti-apoptotic mediating effects. Immunoprecipitation experiments using DOC2B overexpressing EndoC- $\beta$ H1 subjected to cytokine treatment should also be performed followed by mass spectrometry to investigate the presence of novel DOC2B interacting binding partners involved in its anti-apoptotic effects.

#### **4.2.6. Investigate DOC2B overexpression in context of T2D**

DOC2B transcript levels are reportedly reduced in islets of pre-T2D mice. These data suggest that DOC2B deficiency may also underlie susceptibility to T2D-related islet damage. Given that DOC2B overexpression protected  $\beta$ -cells against T1D-related insults, a high fat diet paradigm (as previously described [151]) should be used on  $\beta$ DOC2B-dTg mice to assess DOC2B’s protection of  $\beta$ -cell mass under glucolipotoxic and insulin resistant conditions. Human islets overexpressing DOC2B should also be treated with glucolipotoxic conditions to investigate the translatable nature of DOC2B’s proposed protective/anti-apoptotic effects.

#### **4.2.7. How to target overexpression of exocytosis factors**

Potential delivery options for increasing cellular levels of exocytosis proteins, such as DOC2B, are likely to vary in an isoform-type and context-specific manner. Based on current therapeutic options being explored in neurons, a recent study has utilized a truncated form of SNAP25 conjugated to human Hph-1, a protein transduction domain which allows penetration of various macromolecules into the cytoplasm and nucleus both in vitro and in vivo through local or systemic administrations [184]. This approach may be useful for treating neurodegenerative disorders, as Hph-1 conjugated proteins can cross the blood brain barrier. Additional delivery options involve gene therapy approaches using adeno-associated virus vectors (AAV), a delivery system currently being investigated in clinical trials for several diseases [185-187]. AAV vectors have recently been implicated in treating neurodegenerative disorders, as characterization of several novel AAV serotypes has shown that a single intravenous injection in adult mice leads to transduction of neural cells throughout the entire central nervous system [188]. Recent characterization of several novel AAV serotypes has revealed ideal serotypes for targeting skeletal muscle and pancreas [186, 189-191], and could potentially be utilized for gene therapy to target exocytosis proteins in treating pre-/T2D. Other options include enhancing human pancreatic islets with DOC2B slated for subsequent clinical transplantation. This would be most applicable to individuals with T1D, although clinical trials using transplantation procedures in T2D are currently being pursued [192, 193]. Notably appealing for clinically effective druggable targets is that

achieving high levels of overexpression of DOC2B would not be necessary, and that DOC2B does not trigger fasting hypoglycemic episodes, culminating in a safe and effective means to treat T1D.

### **4.3. CONCLUSIONS**

Overall, my studies revealed that DOC2B protein abundance is reduced in platelets and pancreatic islets during development of T1D in rodents, and in platelets of new-onset T1D pediatric subjects and islets of T1D cadaveric human donors. Remarkably, transplantation of islets correlated with normalized platelet levels of DOC2B in T1D human subjects, as abundance of DOC2B was elevated to the same level seen in non-diabetic individuals. DOC2B deficiency enhances susceptibility to T1D damage in mice, and overexpression of DOC2B protects  $\beta$ -cell mass against T1D insults in an anti-apoptotic and anti-ER stress manner. Furthermore, the C2AB region of DOC2B is sufficient to resist thapsigargin-induced ER stress, and requires intact calcium binding residues therein. C2AB is also the minimal domain required for enhanced GSIS, as seen with full-length DOC2B. In sum, these studies are significant because they are the first to establish an association between T1D and levels of DOC2B in blood-borne platelets and pancreatic islets in rodents and humans, and this can be exploited in further biomarkers studies for clinical applications. Furthermore, these studies show a novel, anti-apoptotic role for DOC2B in the  $\beta$ -cell, which is the initial step in establishing DOC2B as a potential therapeutic target for prevention/management of T1D.

**CHAPTER 5**  
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## Curriculum Vitae

## Arianne Aslamy

### Education

MD 2020  
Indiana University School of Medicine; Indianapolis, IN

PHD 2014-2018  
Department of Cellular and Integrative Physiology  
Indiana University; Indianapolis, IN

BS 2007-2011  
Department of Biochemistry  
University of Washington; Seattle, WA  
*Cum Laude*  
Cumulative GPA: 3.83/4.00

### Research Experience

Graduate Student, Thurmond Lab, June 2014-March 2018  
Department of Cellular & Integrative Physiology, Indiana University School of Medicine  
Ph.D. studies under Dr. Debbie Thurmond on the regulation of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and Double C2 domain beta (DOC2B) proteins in promoting insulin secretion in pancreatic beta cells.

Research Scientist Assistant, Li Lab, August 2009-May 2012  
Department of Biological Structure, University of Washington  
Conducted an independent research project under Dr. Weiqing Li on the role of tyrosine degradation in insulin signaling and longevity in *Caenorhabditis elegans*. Extensive experience in handling *C. elegans* and in the following molecular genetic procedures: RNAi application and screenings, Dauer/diapausal assays, Lifespan assays, RNA extraction, Quantitative real-time PCR analysis, DNA amplification, Genetic crossings.

Amgen Scholars Summer Research Student, Quinlan Lab, June -August 2010  
Department of Chemistry and Biochemistry, University of California, Los Angeles  
As an Amgen Scholar, conducted an independent research project under Dr. Margot Quinlan and Dr. Christina Vizcarra on the biochemical interactions between the *Drosophila* proteins profilin, cappuccino, and spire in actin nucleation. Experience with the following biochemical and molecular biological techniques: Site directed mutagenesis, Protein purification using Co<sup>2+</sup>-affinity and anion exchange chromatography, Pyrene fluorescence, Light scattering assays.

Biotechnology Boot Camp, San Diego, CA June 2009  
Forty hours of hands-on experience with Amgen Laboratories techniques including: Recombinant DNA technology, Spectrophotometry, Acid/Base extraction of compounds, ELISA assay, Gas/thin layer chromatography.

### **Honors & Achievements**

Indiana CTSI Predoctoral Award July 2015-July 2017  
Mary Gates Foundation Undergraduate Research Scholarship December 2010  
Amgen Scholars Program-UCLA Scholarship June 2010  
University of Washington Dean's List September 2007-June 2011

### **Membership**

Phi Beta Kappa Honors Organization  
Howard Hughes Medical Institution Biology Fellows  
University of Washington Afghan Student Association (Afghans at UW)

### **Publications, Abstracts & Research Presentations**

#### **Publications:**

1. **Aslamy A**, Thurmond DC. "Exocytosis proteins as novel targets for diabetes prevention and/or remediation?" Am J Physiol Regul Integr Comp Physiol. 2017 May 1;312(5): R739-R752.
2. Tunduguru R, Zhang J, **Aslamy A**, Brozinick JT, Elmendorf JS, Thurmond DC. "The actin-related p41ARC subunit contributes to p21-activated kinase-1 (PAK1)-mediated glucose uptake into skeletal muscle cells". J Biol Chem. 2017 Sep 25. pii: jbc.M117.801340. doi: 10.1074/jbc.M117.801340.
3. **Aslamy A**, Oh E, Olson E, Zhang J, Ahn M, Tunduguru R, Salunkhe V, Thurmond DC. "DOC2B protects  $\beta$ -cells against inflammatory damage and enhances function." [In Revision].
4. **Aslamy A**, Oh E, Ahn M, Olson E, Moin A, Thurmond DC. "Exocytosis protein DOC2B as an early biomarker of Type 1 Diabetes." [In Revision].
5. Zhang J, Oh E, **Aslamy A**, Tunduguru R, Veluthakal R, Salunkhe V, Merz K, Ahn M, Thurmond DC. "DOC2B enhanced muscle insulin sensitivity by interacting with cytoskeletal proteins" [Submitted].

#### **Abstracts:**

1. **Aslamy A**, Ahn M, Oh E, Olson E, Tunduguru R, Thurmond DC. Boosting DOC2B Expression in the Pancreatic Beta Cell is Sufficient to Enhance Whole-Body Glucose Tolerance – Translational Science Conference 2017, April 2017.
2. **Aslamy A**, Ahn M, Oh E, Olson E, Tunduguru R, Thurmond DC. Boosting DOC2B Expression in the Pancreatic Beta Cell is Sufficient to Enhance

Whole-Body Glucose Tolerance – 2017 Rachmiel Levine-Arthur Riggs Symposium, March 2017.

3. **Aslamy A**, Oh E, Thurmond DC. Is DOC2B an Early Biomarker of Type 1 Diabetes? [abstract]. Journal of Clinical and Translational Science. April 2016.
4. **Aslamy A**, Oh E, El-Zein K, Thurmond DC. Is DOC2B an Early Biomarker of Type 1 Diabetes? [abstract]. Diabetes 65: A536, 2016.

**Presentations:**

1. Title: “Boosting DOC2B Expression in the Pancreatic Beta Cell is Sufficient to Enhance Whole-Body Glucose Tolerance”. Translational Science 2017, Washington DC, April 2017. Poster Presentation
2. Title: “Boosting DOC2B Expression in the Pancreatic Beta Cell is Sufficient to Enhance Whole-Body Glucose Tolerance”. 2017 Rachmiel Levine-Arthur Riggs Symposium, Orlando, FL, March 2017. Poster Presentation
3. Title: “Is DOC2B an Early Biomarker of Type 1 Diabetes?” Indiana Clinical and Translational Sciences Institute Annual Meeting and Watanabe Prize Lecture, Indianapolis, IN, September 2016. Poster Presentation
4. Title: “Is DOC2B an Early Biomarker of Type 1 Diabetes?” American Diabetes Association 76<sup>th</sup> Scientific Sessions, New Orleans, LA, June 2016. Poster Presentation
5. Title: “DOC2B Enhancement of Beta Cell Function and Survival”. Translational Science Conference, Washington DC April 2016. Poster Presentation
6. Title: “DOC2B Enhancement of Beta Cell Function and Survival”. City of Hope Annual Poster Sessions, Duarte, CA, October 2015. Poster Presentation
7. Title: “DOC2B Enhancement of Beta Cell Function and Survival”. Indiana University Department of Cellular and Integrative Physiology Seminar, IUSM, May 2015. Oral Presentation
8. Title: “Synergistic Interactions between Profilin, Cappuccino, and Spire in Actin Nucleation”. CARE Summer Programs for Undergraduate Research Poster Presentation, UCLA, June 2011. Poster Presentation
9. Title: “Tyrosine Degradation, Insulin Signaling, and Aging in Caenorhabditis elegans”. University of Washington Undergraduate Research Symposium, UW, May 2011. Oral Presentation