Autophagy Dysregulation and Lipid Droplet Accumulation in Pancreatic β-cells under Metabolic Stress

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Dedicated to my family who supported me through everything

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Abstract

Obesity and type 2 diabetes mellitus (T2D) are metabolic disorders often accompanied by elevated blood glucose and fatty acid levels. Chronic hyperglycemia and increased free-fatty acid (FFA) concentrations lead to the build-up of reactive oxygen species and toxic metabolites that are harmful to beta-cells and eventually cause their progressive deterioration. Both T2D and obesity are characterized by an excess deposition of triacylglycerol (TAG) and subsequent lipid droplet (LD) accumulation in adipose tissue. Perilipin-2 (PLIN2) is a ubiquitously expressed LD-associated protein that regulates TAG metabolism and LD formation. However, PLIN2 expression in the pancreas and its potential role in the pathogenesis of T2D remain unknown.

We hypothesized that PLIN2 is upregulated in the islets of obese (NDO), type-2 diabetic (T2DN), and type-2 diabetic obese (T2DO) patients in response to lipid overload. Our immunolabelling shows increased PLIN2 in islet beta cells stained immunopositive for c-peptide in type-2 diabetic patients. Gene expression analysis (RT-PCR) confirmed an upregulation of PLIN2 expression in the pancreas of T2DN and T2DO, as well as significant changes in lipid metabolism, autophagy, and oxidant defense genes in T2DN and T2DO. A possible explanation for the increased LD buildup in T2DN and T2DO could be due to the suppression of autophagic systems which would decrease the rate of lipophagy. Transcription factor EB (TFEB) is a master regulator of autophagy and lysosome biogenesis. Immunolabeling revealed that TFEB is active in normal islets but is suppressed in islets from T2DN. Similarly, TFEB activation as well as lysosomal associated membrane protein 1 (LAMP1), Sequestosome (SQSTM1/p62), and microtubule-associated protein 1A/1B-light chain 3 (LC3) expression is significantly downregulated by the combination of hyperglycemia and elevated FFA in cultured cells.

Taken together, elevated glucose and fatty acids levels may inhibit autophagic processes from efficiently clearing lipids which accumulate and lead to lipotoxicity in type-2 diabetic and obese patients.

Résumé

L’obésité et le diabète sucré de type 2 (T2D) sont des troubles métaboliques souvent accompagnés par des niveaux élevés de glucose et d’acides gras dans le sang. L’hyperglycémie chronique et les acides gras libres (FFA) conduisent à une accumulation d’espèces d’oxygène réactives et de métabolites toxiques qui endommagent les cellules bêta et causent leur détérioration progressive. Le T2D et l’obésité sont caractérisés par un dépôt excédentaire de triacylglycérols (TAG) et l’accumulation subséquente de gouttelettes lipidiques (LD) dans les tissus adipeux. La périlipine-2 (PLIN2) est une protéine exprimée de manière ubiquiste en association avec les LD qui régule le métabolisme des TAG et la formation de LD. Cependant, l’expression de PLIN2 dans le pancréas et son rôle potentiel dans la pathogenèse du T2D restent inconnus.

Nous avons émis l’hypothèse que PLIN2 est régulée positivement dans les îlots de patients obèses (NDO), diabétiques de type 2 (T2DN) et diabétiques de type 2 obèses (T2DO) en réponse à une surcharge lipidique. Notre immunomarquage montre un niveau accru de PLIN2 dans les îlots de cellules bêta immunopositives pour le peptide c chez les patients de T2D. L’analyse d’expression génique (RT-PCR) confirme la régulation positive de PLIN2 dans le pancréas de patients T2DN et T2DO, en plus de changements significatifs dans le métabolisme lipidique, l’autophagie et les gènes de défense anti-oxydative. Une possible explication pour l’accumulation accrue de LD chez les patients T2DN et T2DO pourrait être la suppression des systèmes autophagiques, ce qui diminuerait le niveau de lipophagie. Le facteur de transcription EB (TFEB) est un régulateur maître de l’autophagie et de la biogenèse des lysosomes. L’immunomarquage révèle que TFEB est active dans les îlots normaux en réponse à une surcharge lipidique, mais est inhibé dans les îlots de patients T2DN. De plus, l’activation de TFEB et l’expression de la protéine membranaire lysosomale 1 (LAMP1), du Sequestosome (SQSTM1/p62) et de la protéine associée aux microtubules 1A/1B chaîne légère 3 (LC3) sont inhibées de façon significative par la combinaison de l’hyperglycémie et un niveau élevé de FFA chez les cellules en culture.

Globalement, l’hyperglycémie et l’hyperlipidémie pourraient empêcher les processus d’autophagie d’éliminer les lipides efficacement, entraînant la lipotoxicité chez les patients obèses et diabétiques de type 2.

List of figures and Tables

Contributions of Authors

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List of Abbreviations

Akt, Protein kinase B

Atg7, Autophagy related 7

ATP, Adenosine triphosphate

ADP, Adenosine diphosphate

AMPK, AMP-activated protein kinase

BAD, Bcl-2-associated death promoter

BAX, Bcl-2-associated X protein

Bcl-2, B-cell lymphoma 2

Bcl-xL, B-cell lymphoma-extra large

BID, BH3 interacting-domain death agonist

BMI, Body mass index

CAT, Catalase

CAV1, Caveolin-1

CoA, Coenzyme-A

CLEAR, Coordinated lysosomal expression and regulation

CPT1, Carnitine palmitoyltransferase 1

CYBA, Cytochrome B-245 alpha chain

EGFP, Enhanced green fluorescent protein

ER, Endoplasmic reticulum

FFA, Free fatty acids

FOXO1, forkhead box protein 1

FSP27, Fat-specific-protein 27

HMOX1, Heme oxygenase 1

G6P, Glucose-6-Phosphate

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

GCG, Glucagon

GCK, Glucokinase

GPX1, Glutathione peroxidase 1

GSIS, Glucose-stimulated insulin release

LAMP1, lysosome-associated membrane protein 1

LC3-I/II, Phosphatidylethanolamine conjugated light-chain 3-I/II

LD, Lipid droplet

IL1β/6/18, Interleukin-1 beta/6/18

IFy, Interferon gamma

IGF1, insulin-like growth factor 1

Ins1/2, Insulin 1/2

MCP-1, Monocyte chemoattractant protein-1

mTORC1, mammalian target of rapamycin complex 1

NFkB, Nuclear factor kappa beta

NDN, non-diabetic normal

NDO, non-diabetic obese

OA, Oleic Acid

PA, Palmitic Acid

PDX1, Insulin promoter factor 1

PLIN1/2/3/4/5, Perlipin-1/2/3/4/5

PPAR-α/y, Peroxisome proliferator-activated receptor alpha/gamma

ROS, Reactive Oxygen Species

SST, Somatostatin

SOD1/2, Superoxide dismutase 1/2

SQSTM1/p62, Sequestosome 1

SREBP1, Sterol regulatory element binding protein 1

T2DM/T2D, Type-2 diabetes mellitus/Type-2 diabetic

T2DN, Type-2 diabetic

T2DO, Type-2 diabetic obese

Tsc2, Tuberous sclerosis 2

TFEB, Transcription factor EB

TNF-α, Tumor necrosis factor-alpha

UCP2, uncoupling protein 2

VDAC, voltage-dependent anion channel

VEGF-A, Vascular endothelial growth factor A

Rationale and Objectives

Lipid droplets (LD) are micellar lipid organelles traditionally regarded as simple storage depots. However, recent evidence suggests they are dynamic organelles involved in many facets of lipid synthesis, enzymatic conversion and breakdown as well as other processes such as production of pro-inflammatory molecules. Abnormal LD size and accumulation is prevalent in many human metabolic diseases including: dyslipidemia, hepatic steatosis, atherosclerosis, obesity, and diabetes. Ectopic lipid accumulation is linked with inflammation and immune cell infiltration. One method cells have for breaking down excessive LD is through lipophagy, which sequesters portions of LD into autophagosomes that are eventually broken down in lysosomes. Lipophagy is a sub-process of autophagy, which is transcriptionally regulated by transcription factor EB (TFEB), a central regulator of autophagy and lysosomal biogenesis. TFEB and autophagy are suppressed by high cellular nutrient levels.

This thesis focuses on examining the role of LD accumulation and autophagy through their respective markers, perilipin-2 (PLIN2) and transcription factor EB (TFEB), in response to hyperglycemia and hyperlipidemia in both human samples and cell-based assays. We sought to clarify the connection between lipid accumulation and autophagic dysfunction seen in obese and diabetic patients.

Section 1: Literature Review

1.1 Obesity, T2DM, and the pancreatic islet

Obesity and type-2 diabetes mellitus (T2DM) are two of the most prevalent metabolic disorders in the western world. Broadly, obesity is defined as having a body mass index greater than 30kg/m^2 (Boyle, Thompson, Gregg, Barker, & Williamson, 2010), while patients who have a fasting blood glucose levels greater or equal to 7 mmol/L (Vijan, 2015) are diagnosed with T2DM. The rate of obesity and obesity have been growing at a worrying rate in Canada with estimated obesity prevalence reaching 21% by 2019 (Twells, Gregory, Reddigan, & Midodzi, 2014) and T2DM estimated prevalence reaching 12.1% by 2025 (Rowley & Bezold, 2012). This trend has lead researchers to describe the incoming tide as a “diabesity” epidemic. Obesity and T2DM are known to increase the risk of serious cardiovascular, hepatic, and renal diseases. Obesity is a major risk factor for the development of T2D and is often thought of as a stepping stone into the pre-diabetic stage (Khaodhiar, Cummings, & Apovian, 2009). Epidemiological studies have correlated increased adipose tissue mass to glucose intolerance (Despres, 1993). It is not surprising that obesity and T2DM are co-morbidities and lead to insulin resistance and islet dysfunction (Eckel et al., 2011; Kahn & Flier, 2000).

Islets of Langerhans, embedded in the pancreas, are the key regulators of glucose homeostasis in the body. Islets are composed of three major types of endocrine cells: glucagon-secreting α-cells, insulin-secreting β-cells, and somatostatin (SST)-secreting cells. β-cells comprise the majority of the islet (~70% of total cells) are responsible for the regulation of insulin production and secretion. Decreased insulin sensitivity typically involves two processes: (i) desensitization of peripheral insulin receptor signaling (Flier, 1983), (ii) increased insulin production due to β-cell expansion. β-cells can adapt to increased demands for insulin secretion which occur during normal physiological processes such as puberty (Moran et al., 1999) and pregnancy (Buchanan, Metzger, Freinkel, & Bergman, 1990). In obese patients, nutrient overload (increased glucose

and fatty acid consumption) reduces adipocyte and muscle insulin sensitivity (DeFronzo, Bonadonna, & Ferrannini, 1992). Consequently, β-cells compensate for insulin resistance in obese patients by increasing in cell number which allows for increased insulin production and release (Linnemann, Baan, & Davis, 2014). Although the initial adaptation is very successful and glucose levels can be controlled, this state cannot be sustained as chronic islet stress leads to β-cell injury and eventual death (Weir & Bonner-Weir, 2004). T2DM is diagnosed when β-cells can no longer compensate for the higher insulin demands and the body loses control of glucose homeostasis. β-cell death and islet atrophy is evident in late stage T2DM. Thus the transition from normal to obese and ultimately diabetes mirrors the transition of normal islets from adaptive expansion to degeneration (see Figure 1).

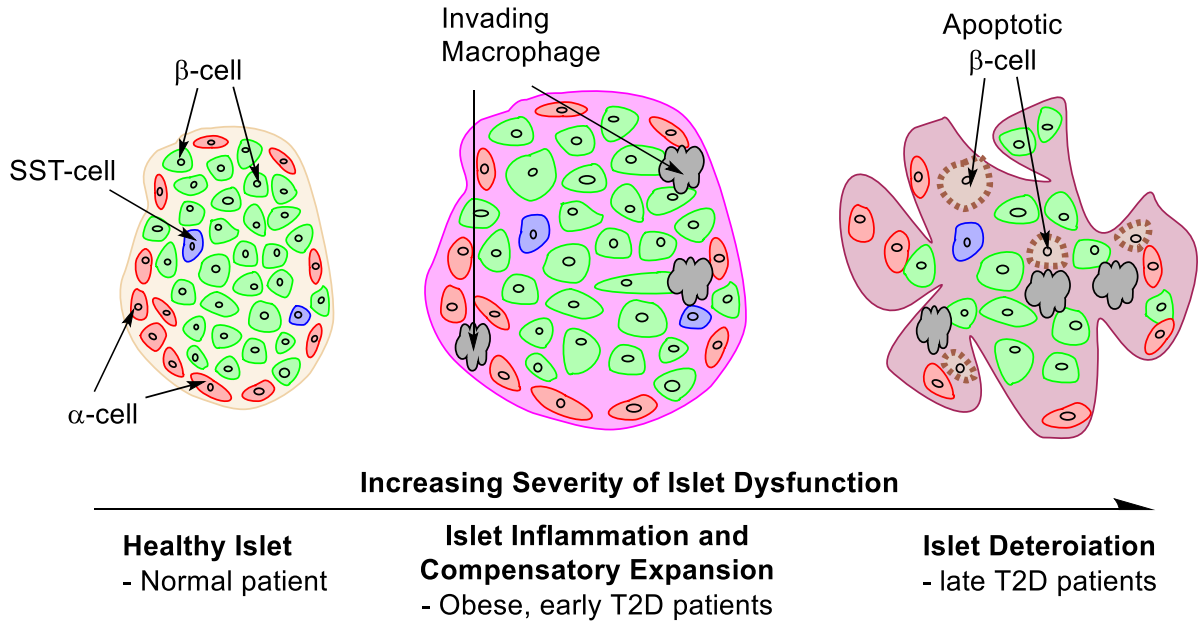

Figure 1) Pancreatic islet adaptation. Pancreatic islets can initially adapt to metabolic stress through expansion. However, chronic stress leads to β-cell loss and islet destruction. Stressed islets also secrete pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interferon gamma (IFγ), and interleukin-6 (IL-6) which can acts as chemoattractant to macrophages. Alpha cells (α-cells) secrete glucagon, beta cells (β-cells) secrete insulin, and somatostatin cells (SST-cells) secrete somatostatin.

Pancreatic inflammation is an important contributor to islet loss in T2DM. In obese patients, an excessive consumption of fatty acids increase adipocyte triglyceride

synthesis and storage. Adipocytes hypertrophy in response to increased demands for fatty acid storage (Rutkowski, Stern, & Scherer, 2015). Aside from its lipid storage roles, adipocytes are endocrine cells and are known to secrete hormones regulating appetite, metabolism, and various cytokines. Excessive lipid storage in adipocytes dysregulates cell metabolism and pushes them towards metaflammation (Hotamisligil, 2017); adipocyte hypertrophy/inflammation is associated with increased secretion of monocyte chemoattractant protein-1 (MCP-1) (Sartipy & Loskutoff, 2003), tumor necrosis factor-α (TNF-α), and interleukin-1 beta (IL1β) (Lagathu et al., 2006). Although adipocytes from obese patients secrete greater amount of cytokines compared to non-obese individuals under physiological conditions, circulating cytokine levels are still relatively low (Kahn & Flier, 2000). This suggests that cytokines produced by adipocytes are signaling in a paracrine fashion (Hotamisligil, 1999). Interestingly, high lipid levels are known to cause adipocyte infiltration into the pancreas (Pinnick et al., 2008) and ectopic lipid accumulation in the pancreas is associated with pancreatic dysfunction (van Herpen & Schrauwen-Hinderling, 2008). Related to adipocyte infiltration is the increased macrophage infiltration into pancreatic islets seen in T2DM (Ehses et al., 2007). Many factors contribute to creating the inflammatory milieu (TNF-α, IL-1β, IL-6,- IL-18, MCF-1) that attracts and activates macrophages in the islets including: (i) cytokines produced by infiltrating adipocytes, (ii) β-cell secreted IL-1β in response to stress (Maedler et al., 2017), (iii) activation of resident islet macrophages (Eguchi & Nagai, 2017), and (iv) production of pro-inflammatory amyloid species (Westwell-Roper, Ehses, & Verchere, 2014). It is important to note that the inflammatory environment is due to the cumulative effect of many complementary processes which mutually feedback towards increased inflammation and islet degeneration. Increased levels of IL-1β in particular have been found to directly stimulate β-cell apoptosis (Maedler et al., 2017). Elevated cytokines in the islet environment are known to lead to the overexpression of vascular endothelial growth factor-A (VEGF-A) which causes β-cell loss (Agudo et al., 2012). More globally, the activation of nuclear factor kappa beta (NFkB) by various cytokines has been associated with islet death (Melloul, 2008). Islet inflammation is an important factor contributing to β-cell dysfunction and islet loss in T2DM. Other factors such as

dysregulated metabolism due to hyperglycemia and hyperlipidemia also play a causative role in mediating islet degeneration.

1.2 Glucolipotoxicity: disruption of islet metabolism

Glucolipotoxicity can be defined as islet dysfunction due to elevated glucose and fatty acids (Prentki, Joly, El-Assaad, & Roduit, 2002). Excessive nutrient loading in β-cells can lead to alterations in glucose and fatty acid processing, mitochondrial dysfunction, endoplasmic reticulum stress, and transcriptional deregulation of metabolic genes (see Figure 2) (Poitout & Robertson, 2008).

β-cells function as both glucose sensors and glucose regulators in the body. Glucose transporter 2 (GLUT2) is an insulin-independent glucose transporter expressed exclusively in β-cell and hepatocytes which allows for the rapid uptake of glucose regardless of the extracellular glucose concentration (Schuit, Huypens, Heimberg, & Pipeleers, 2001). Unlike most other cell types, β-cells phosphorylate glucose to glucose-6-phosphate (G6P) using glucokinase (GCK) instead of hexokinase. Unlike hexokinase, G6P does not decrease GCK activity through negative-feedback. Increases in extracellular glucose therefore directly increases β-cell G6P levels, which increases glycolytic flux and elevates cellular ATP/ADP ratio. Increased ATP/ADP ratio inhibits the ATP-sensitive potassium channel which leads to depolarization of the cell (Schuit et al., 2001). Depolarization triggers calcium influx through voltage-dependent Ca^{2+} channels. Calcium entry acts as a trigger for the exocytosis of insulin granules near the plasma membrane (Fu, Gilbert, & Liu, 2013). GCK is the rate limiting step for glucose metabolism in β-cells and the enzyme concentration is kinetically tuned to maximize glucose sensitivity in the physiological range (Schuit et al., 2001). Consequently, GCKs expression is tightly controlled and changes in GCK expression compromises glucose sensitivity, and cell survival (H. Wang & Iynedjian, 1997).

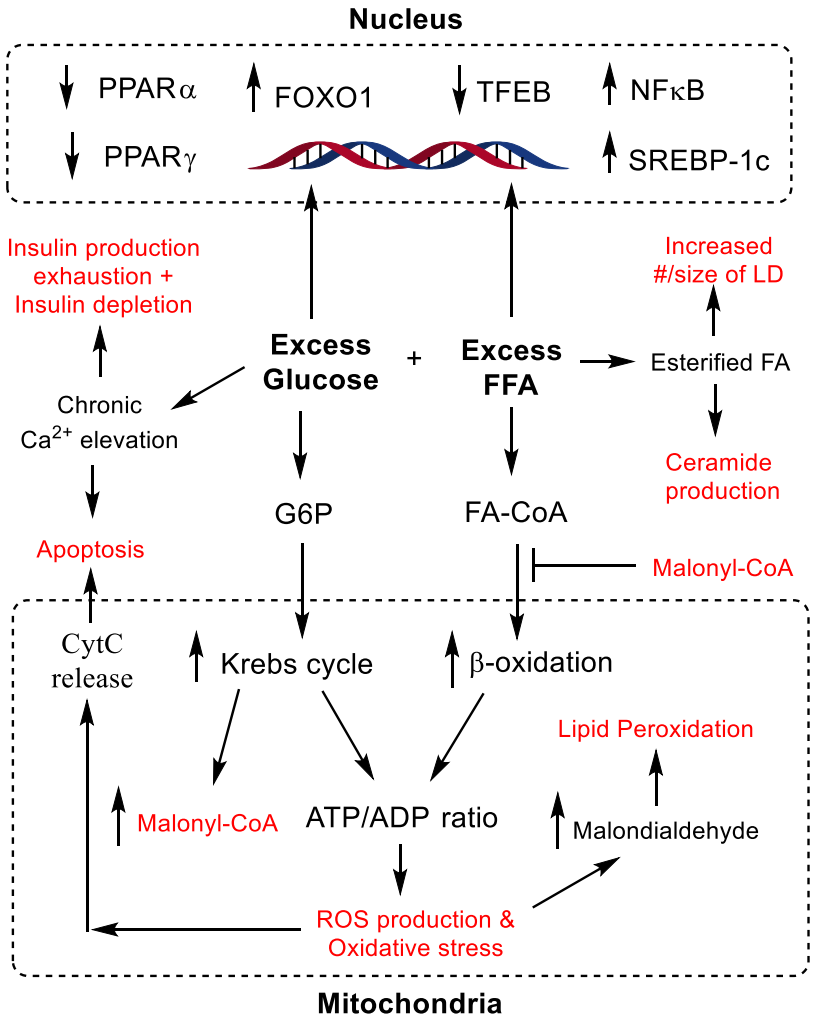

Figure 2) Pathways involved in glucolipotoxicity. In β-cells, glucose is converted to glucose-6-phosphate (G6P) by glucokinase. Since glucokinase is not regulated by G6P negative feedback, it is continuously used to fuel proton transport at the electron transport chain. High levels of mitochondria transmembrane potential and ATP generation increases ROS production due to the creation of free radicals which can damage the mitochondria through protein and lipid peroxidation. Damaged mitochondria can release Cytochrome C (CytC) into cytosol which triggers apoptosis. At the same time, increased glucose metabolism increases the production of malonyl-coenzyme A (Malonyl-CoA) which inhibits carnitine palmitoyltransferase 1 (CPT1) and prevents fatty acid coenzyme A (FA-CoA) from transporting to the mitochondria. As a result, additional free-fatty acids (FFA) will be shunted to lipid droplets or the ceramide synthesis pathways. High concentrations of glucose and FFA can alter the levels of many broad acting transcription factors including: peroxisome proliferator-activated receptor alpha/gamma (PPARα/γ), forkhead box protein O1 (FOXO1), transcription factor EB (TFEB), nuclear factor kappa-beta (NFkB), sterol regulatory element-binding protein 1c (SREBP-1c).

Chronically elevated glucose concentrations have been shown to impair β-cell function and survival (Jonas et al., 1999; Laybutt et al., 2002). A consequence of glucotoxicity is the increased generation of reactive oxygen species (ROS). High glucose concentration increases ROS-producing metabolites such as: (i) glyceraldehyde, which can autoxidize and generate α-ketoaldehydes and hydrogen peroxide (Wolff & Dean, 1987), (ii) methylglyoxal, which can form advanced glycation end-products and generate ROS through the Maillard reaction (Wellsknecht, Zyzak, Litchfield, Thorpe, & Baynes, 1995), and (iii) glucosamine, which can increase hydrogen peroxide production and down regulate insulin, GLUT2, GCK expression (Kaneto et al., 2001). Overproduction of ROS leads to decreased glucose-stimulated insulin release and inhibition of insulin gene expression by inhibiting insulin promoter factor 1 (PDX1) translocation (Olson et al., 1995). Elevated ROS can cause generalized cell damage such as lipid peroxidation, DNA oxidation, and protein damage. β-cells are especially vulnerable to oxidative stress due to inherently low anti-oxidant genes including superoxide dismutase 1-2 (SOD1-2), glutathione peroxidase 1 (GPX1), and catalase (CAT). Anti-oxidant treatment can reduce the deleterious effects of hyperglycemia (Tang et al., 2012). In order to combat the increased oxidative stress, islets can upregulate antioxidant genes including glutathione peroxidase 1 (GPX1) (Lacraz et al., 2009), cytochrome B-245 alpha chain (CYBA) (Hasnain et al., 2014), and heme oxygenase 1 (HMOX1) (Kjorholt, Akerfeldt, Biden, & Laybutt, 2005). HMOX1 overexpression haven been shown to protect β-cells against cell loss induced by inflammatory stimuli (Huang et al., 2010; Pileggi et al., 2001). Similarly, overexpression of GPX1 protects β-cells against oxidative stress and hyperglycemia in animal models (Harmon et al., 2009). Glucotoxicity can also overload the mitochondria by increasing mitochondrial electron transfer which elevates the proton gradient. Higher mitochondrial transmembrane potential increases proton leakage through mitochondrial uncoupling proteins (UCP), and oxidative phosphorylation increases the production of superoxides. Interestingly, β-cell UCP2 knockout have been show to protect against impaired insulin secretion caused by hyperglycemia (Krauss et al., 2003). UCP2 indirectly regulate insulin secretion by lowering the ATP/ADP ratio by dissipating the proton gradient without ATP generation. UCP2 levels are regulated by increased mitochondrial ROS levels (C. Y. Zhang et al., 2001). Chronically elevated

ROS and thus increased number of UCP2 levels could lower the sensitivity of glucose-induced insulin secretion.

Aside from high glucose levels, fatty acids levels are also elevated in both obese and diabetic patients. Non-esterified fatty acids primarily enters β-cells through diffusion and quickly equalize inside the cell (Hamilton & Kamp, 1999). Under physiological glucose concentrations, β-cells preferentially utilize fatty acids metabolism for energy generation (Malaisse, Best, Kawazu, Malaisselagae, & Sener, 1983). However, under hyperglycemic conditions, β-cells shift from predominantly lipid metabolism to glucose fueled respiration due to increased levels of malonyl-CoA which inhibits fatty acid mitochondrial transport (Prentki & Corkey, 1996). Excess fatty acids are sequestered to intracellular lipid storage pools called lipid droplets (see section 1.3). Inside the cell, non-esterified fatty acids are transported into the mitochondria via carnitine palmitoyltransferase I (CPT1) and metabolized through β-oxidation. Fatty acid transport through CPT1 can be a rate limiting step for fatty acid metabolism due to CPT1 inhibition by malonyl-CoA when glucose levels are high. Furthermore, loss of CPT1 malonyl-CoA inhibition have been show to protect against palmitate-induced toxicity (Henique et al., 2010). Fatty acids regulate insulin expression, and release in complex ways. Short-term fatty acid treatment did not directly trigger insulin secretion but potentiated glucose-stimulated insulin release (GSIS) (Gravena, Mathias, & Ashcroft, 2002). However, prolonged treatment with fatty acids reduced GSIS but increased basal insulin release (Carpentier et al., 1999). The length and saturation of fatty acids affects its potentiating effect on GSIS. Fatty acids with longer chain length and fewer double bonds have a greater insulintropic effect compared to fatty acids with shorter chain length and more double bonds (Stein et al., 1997). Long term fatty acid exposure had been shown to decrease insulin expression by downregulating the transcription factor PDX1 in rat pancreatic islets (Gremlich, Bonny, Waeber, & Thorens, 1997).

Apart from its regulatory effects on insulin, fatty acids in combination with elevated glucose facilitate β-cell death via glucolipotoxicity. Saturated fatty acids (such as palmitic acid) in combination with hyperglycemia reduce β-cell viability, whereas

unsaturated fatty acids (such as oleic acid) show reduced toxicity or may even protect against saturated fatty acid toxicity (Cnop, Hannaert, Hoorens, Eizirik, & Pipeleers, 2001; Maedler et al., 2001). The mechanisms of cell death induced by saturated fatty acids such as palmitic acid are multifactorial and not entirely understood. Prominent contributors of palmitic acid toxicity include (i) increased ceramide generation (Boslem, Meikle, & Biden, 2012), (ii) ER stress (Mirmira, 2012). De novo ceramide synthesis involves the condensation of serine and palmitate by serine palmitoyl transferase (Weiss & Stoffel, 1997). Elevated ceramide induces apoptosis by increasing ROS, cytochrome-c release, and inhibition of protein kinase B (Akt) (Galadari, Rahman, Pallichankandy, Galadari, & Thayyullathil, 2013). Palmitate triggers ER stress by triggering calcium release from the ER (Cui et al., 2013) and activating the unfolded protein response due to ER calcium depletion and increased protein misfolding (Cnop, Igoillo-Esteve, Cunha, Ladriere, & Eizirik, 2008).

Glucose and fatty acids modulate many transcription factors which can shift the cellular metabolic profile. Peroxisome proliferator-activated receptors (PPAR) are a class of transcription factors regulating many facets of metabolism including lipid uptake, carbohydrate and lipid breakdown. PPAR-α is a key regulator of β-oxidation in β-cells and is transcriptionally downregulated by hyperglycemia (Ravnskjaer, Boergesen, Dalgaard, & Mandrup, 2006). Interestingly, PPAR-α expression is critical for maintaining islet heath as PPAR-α knockout in ob/ob mice dramatically lowered islet mass (Lalloyer et al., 2006). PPAR-γ plays an important role in the adaptation of islets in diabetic conditions; PPAR-γ knockout animals are resistant to islet hyperplasia when given a high-fat diet, suggesting decreased adaptive capabilities (Rosen et al., 2003). PPAR-γ agonist thiazolidinediones improve insulin sensitivity and are used clinically for the treatment of T2DM (Gupta, Kono, & Evans-Molina, 2010). In diabetic rats, PPAR-γ gene expression is downregulated in cardiac cells but can be normalized by the thiazolidinedione pioglitazone which also improves glucose control (Pelzer et al., 2005). Another important transcription factor in islet neogensis and survival is the forkhead box protein 1 (FOXO1). FOXO1 is a known regulator of β-cell proliferation and differentiation. FOXO1 is regulated by a host of kinases and its phosphorylation leads to

cytosol sequestration and inactivation. Interestingly, FOXO1 nuclear translocation inhibited PDX1 expression which inhibited β-cell proliferation (Kitamura et al., 2002). In obese, hyperglycemic mice, FOXO1 is dephosphorylated which inhibited the expression of PDX1 and lead to β-cell death (Kluth et al., 2011). Other important transcription factors implicated in T2DM and obesity include NFkβ and TFEB. NFkβ is involved in the islet death mediated by inflammation (Imai, Dobrian, Morris, & Nadler, 2013). TFEB is discussed extensively in section 1.4.

The overall effect of glucolipotoxicity in increasing glucose-insensitivity, oxidative stress, ER stress, and gene dysregulation all taxes the adaptive capacity of β-cells to the limit and contribute to their eventual apoptotic death in T2DM (Poitout et al., 2010). Apoptosis is regulated by a balance of pro-apoptotic proteins including: Bcl-2-associated X protein (BAX), BH3 interacting-domain death agonist (BID), Bcl-2-associated death promoter (BAD), and anti-apoptotic gene B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra-large (Bclx). BAX is a pro-apoptotic cytosolic protein which binds to the mitochondrial voltage-dependent anion channel (VDAC). Binding of BAX to VDAC triggers cytochrome-c release from the mitochondria which leads to the activation of the intrinsic apoptotic pathway. Chronic hyperglycemia in mouse pancreatic β-cells causes BAX oligomerization, cytochrome-c release, and caspase-3 activation (Kim et al., 2005). Bcl-2 is an anti-apoptotic protein located on the mitochondria. Bcl-2 binds to the BH-3 domain of pro-apoptotic proteins BAX, BID, BAD and inhibit them from releasing cytochrome-c from the mitochondria (Llambi et al., 2011). Exposure of human β-cells to a mixture of free fatty acids decreased Bcl-2 expression (Lupi et al., 2002). Thus glucolipotoxicity tends to shift β-cells towards the apoptotic state by tipping gene expression in favor of the pro-apoptotic proteins.

1.3 Lipid droplets in obesity and T2DM

Lipid droplets (LDs) are intracellular organelles classically relegated to inert lipid storage depots, but they are dynamic and have much broader functions (Walther & Farese, 2012; Welte, 2015). For instance, recent findings reveal that these dynamic organelles

act as signaling and enzymatic hubs for a variety of cellular proteins involved in physiological and pathological processes in lipid metabolism, steroid synthesis, protein storage, protein degradation, autophagy, and inflammation (Arrese, Saudale, & Soulages, 2014; Walther & Farese, 2012; Welte, 2015). LDs (see Figure 3) are micelles composed of a hydrophobic core surrounded by a monolayer of phospholipids and LD-associated proteins.

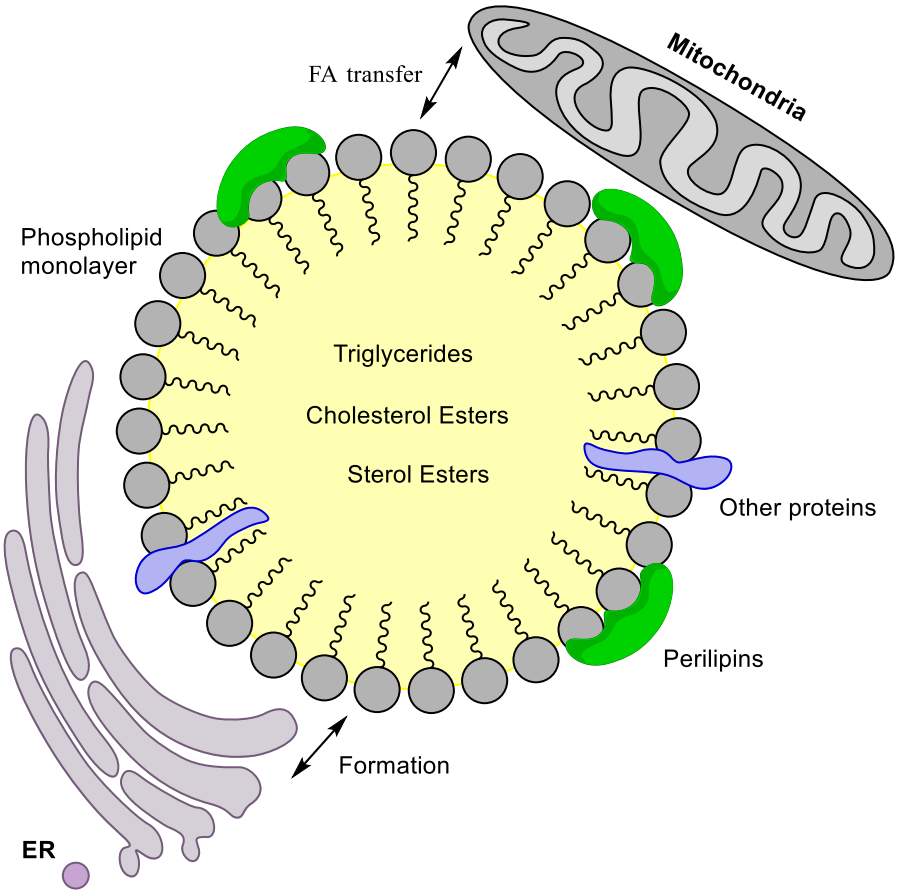

Figure 3) Lipid droplet structure and organelle interactions. Lipid droplets are composed of a phospholipid monolayer surrounding a hydrophobic core composed of triglycerides, cholesterol esters, and sterol esters. The surface is composed of a family of structural proteins named perilipins as well as other proteins such as caveolins, lipases, and inflammatory enzymes such as cyclooxygenase-2. Lipid droplets are thought to be hatched in the endoplasmic reticulum (ER) through a budding mechanism. Lipid droplets can dock to mitochondria and provide a supply of fatty acids (FA) for β-oxidation. Lipid droplets can also transiently associate with autophagosomes during macrolipohagy to provide fuel during nutrient deprivation.

The hydrophobic core is composed of sterol esters, cholesterol esters and triglycerides. The surface is coated with phospholipids such as: phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol and the coating proteins including: perilipin-1/2/3/4/5 (PLIN1-5), fat-specific-protein 27 (FSP27), and caveolin-1 (CAV1) (Yang et al., 2012). LDs are cell-type specific in-respect to their size, content, and function. In adipocytes, LDs are large lipid storage depots taking up the majority of the cell's volume. PLIN1 and FSP27 are adipocyte specific LD proteins. Interactions between FSP27 and PLIN1 promote fusion of smaller LD, leading to the large LD found in adipocytes (Grahn et al., 2013). Genetic model of FSP27 depleted mice show smaller adipocyte LD as well as increased mitochondrial activity and greater insulin sensitivity (Toh et al., 2008). CAV1 is an important mediator of LD lipolysis in adipocytes as CAV1 knockout leads to decreased rate of lipolysis as well as lipid accumulation (Cohen et al., 2004). CAV1 likely enables the phosphorylation of PLIN proteins by protein kinase A which allows lipase access to LD. Aside from adipocytes, LDs are found in almost all cell types. For example, in microglia, LDs are formed in-response to inflammatory stimuli and participate in the synthesis of eicosanoids (Khatchadourian, Bourque, Richard, Titorenko, & Maysinger, 2012). Concomitant with increased number of LDs is the upregulation of the ubiquitously expressed LD-coating protein PLIN2. During high cellular lipid uptake (i.e. postprandial period), PLIN2 and PLIN3 are upregulated and recruited to the surface of the LD whereby they could restrict the size of the LD and reduce lipase access (Bell et al., 2008). Interestingly, studies have reported PLIN2 as an important regulator of islet ER stress and autophagic flux in diabetic mouse models (Chen et al., 2017). The functions of the ubiquitously expressed PLIN3 are less explored but are likely similar to that of PLIN2 (Sztalryd et al., 2006).

The incorporation of fatty acids into LD is also dependent on the chain length and degree of saturation (number of double bonds) (Plotz, Hartmann, Lenzen, & Elsner, 2016). Unsaturated fatty acids such as oleic acid readily convert to triglycerides which promote LD formation. In contrast, saturated fatty acids, e.g. palmitic acid do not promote LD formation but induce toxicity (see figure 4). Studies have shown unsaturated fatty acids can reduce the toxicity of saturated fatty acids by shunting

saturated fatty acids into LD (Listenberger et al., 2003). Sequestering fatty acids into LD would temporarily reduce the cytosolic FFA concentration and the cells metabolic burden. However, chronic lipid overload could lead to the formation of abnormally large, unstable LD that cannot be sufficiently contained by PLIN proteins, lipid leakage results which contribute to lipotoxicity (see Figure 5).

A

Induction of LD Formation	Name	Category	Chemical Structure	# double bonds	Found in
1 (lowest)	Lauric Acid	Saturated FA	C12H24O2	0	Coconut Milk
2	Palmitic Acid	Saturated FA	C16H32O2	0	Meats, Butter
3	α-linolenic acid	Polyunsaturated FA	C18H30O2	3 (C-9,12,15)	Nuts, Seeds
4	Oleic Acid	Monounsaturated FA	C18H34O2	1 (C-9)	Olive oil
5 (highest)	Linoleic Acid	Polyunsaturated FA	C18H32O2	2 (C-9,12)	Nuts, Seeds

B

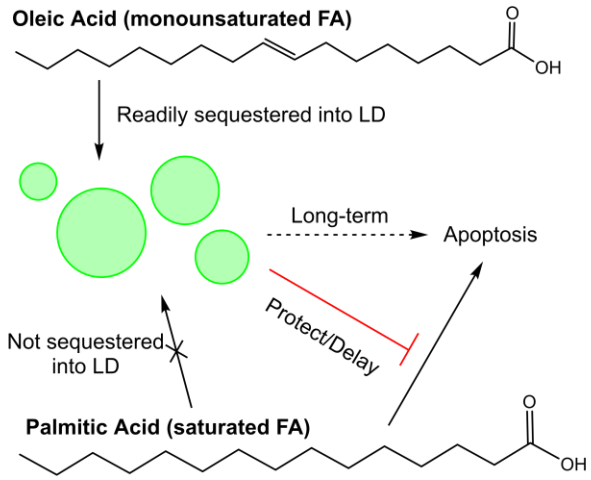

Figure 4) Saturated and unsaturated fatty acids are differentially incorporated into lipid droplets. (A) Table of common of fatty acids (FA) ordered by their ability to induce lipid droplet (LD) formation. (B) While monounsaturated FA such as oleic acid are readily converted to triglycerides and sequestered into LD, saturated FA such as palmitic acid are not and exert a toxic effect by elevating mitochondrial ROS production and inducing ER stress. Co-treatment of oleic acid with palmitic acid is known to reduce the short-term toxicity of palmitic acid. However, long term treatment with oleic acid compromises β-cell survival. LD likely blunts the short-term toxicity of FA.

LD are mobile structures that communicate and interact with other intracellular organelles (Schuldiner & Bohnert, 2017). Current research suggest that LD are formed from a mechanism involved budding off of the ER. Fatty acid, cholesterol, and sterol

molecules are processed in the ER and transferred to LD as esters for storage. It is well known that LD contains traces of ER-associated proteins (Yang et al., 2012). Increased formation of LD could be a protective mechanism against ER stress by shunting away misfolded or unfolded proteins (Ploegh, 2007). In yeast, agents which induce ER stress are known to stimulate LD formation (Fei, Wang, Fu, Bielby, & Yang, 2009). However, recent studies suggest a deleterious effect of LD on ER-stress where PLIN2 downregulation in β-cells from diabetic animals leads to decreased ER stress, enhanced autophagy, and decreased β-cell apoptosis (Chen et al., 2017). Akin to lipid storage, temporarily sequestering unfolded proteins into LD may lead to a greater unfolded protein response in the long run due to the accumulation and instability of large, leaky LD. LD-mitochondria interaction is involved in lipid metabolism where lipid droplets shuttle fatty acid to the mitochondria for β-oxidation. PLIN5 is a key mediator of LD-mitochondria binding (H. Wang et al., 2011). Interestingly, mitochondria association with LD is more pronounced during starvation which suggests that the activation of autophagic pathways triggers LD-mitochondria driven lipolysis (Nguyen et al., 2017; Rambold, Cohen, & Lippincott-Schwartz, 2015). In the context of obesity and T2DM, greater number of LD could overload the metabolic demands on the mitochondria due to the increased influx of fatty acid, leading to increased ROS formation and oxidative stress. LD association with autophagosomes and lysosomes during lipophagy will be discussed in section 1.5.

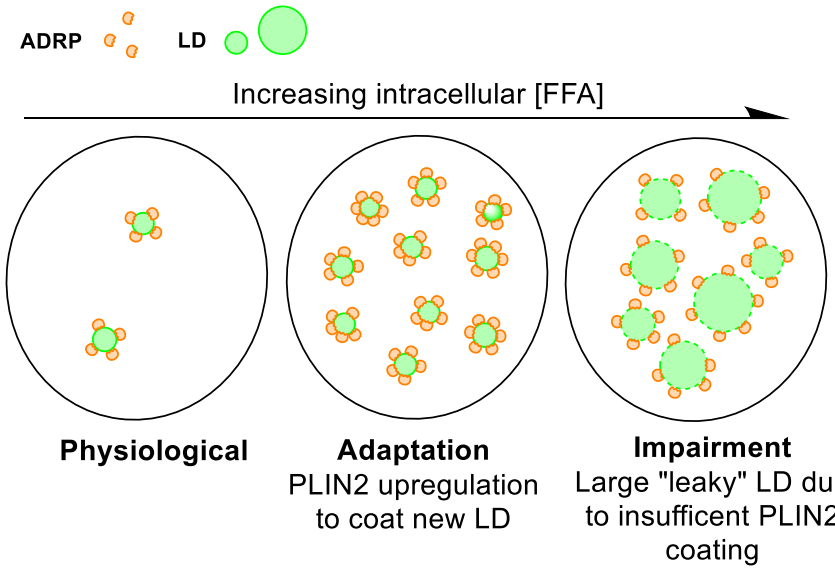

Figure 5) Large lipid droplets are not adequately protected. At physiological concentrations of FFA, cells have few lipid droplets that are covered with perlipin-2 (PLIN2). As the cell is challenged with increasing concentrations of FFA, the cell can initially adapt by coating additionally formed LD with PLIN2. However, as existing LD are enlarged, these large lipid droplet becomes "leaky" and lipotoxic due to insufficient PLIN2 coating.

1.4 Autophagy in obesity and T2DM

A functional autophagy system is crucial for maintaining islet homeostasis. Targeted β-cell inactivation or repression of autophagy in animal models leads to the development of obesity and/or diabetes. Autophagy is the regulated degradation of cellular components and has several classical functions: (i) removal of damaged organelles, (ii) clearance of misfolded proteins, and (iii) provision of energy during starvation. Briefly, macroautophagy involves the enclosure of a part or whole of an organelle by a double-membrane vesicle known as an autophagosome (see Figure 6). The autophagosome then merges with a lysosome, forming an autolysosome which can degrade the vesicular content. The autophagic process and the entire molecular mechanisms of autophagy is extensively reviewed elsewhere (Kliosnky, 2016). Regulatory mechanisms of autophagy include numerous factors, some of which are highlighted here and are particularly relevant in the present study. Mammalian target of rapamycin complex 1 (mTORC1) is a central kinase responsible for regulating many aspects of metabolism,

energy utilization, and cell growth. mTORC1 activity is regulated by nutrient abundance in the cell and is inhibited by nutrient deprivation or absence of growth factors such as insulin (Vander Haar, Lee, Bandhakavi, Griffin, & Kim, 2007). mTORC1 inhibition leads to the formation of an autophagosome initiation complex which leads to the recruitment of phosphatidylethanolamine conjugated light-chain 3 (LC3-II) to what would be the lumen of the autophagosome. In the cytosol, the adaptor protein p62/SQSTM1 binds to ubiquinated proteins and organelles and subsequently binds to LC3-II. The autophagosome closes with the LC3-II-p62-ubuquinated cargo in the lumen of the vesicle. An increased LC3-II level is used as an indicator for active autophagy. An increased p62 accumulation is a crude marker for the inhibition of autophagy (Kliosnky, 2016). Lipophagy is a sub-process of autophagy by which lipid droplets are sequestered to autophagosomes and eventually degraded by lysosomes. In hepatocytes, autophagosomes form around a LD and pinch-off portions of both the membrane and hydrophobic lipids. BODIPY493/503-stained neutral lipids and LD surface proteins PLIN2 and PLIN5 co-localize with LAMP1 (Singh et al., 2009). In enterocytes, LDs are immediately sequestered into autophagosomes upon emergence from the ER (Khaldoun et al., 2014). Interestingly, LD could shuttle neutral lipid stores to already formed autophagosomes through a PNPLA5 (patatin like phospholipase domain containing 5) dependent mechanism (Dupont et al., 2014). LDs are likely sequestered to autophagosomes through several interrelated mechanisms depending on the cell type and cellular environment.

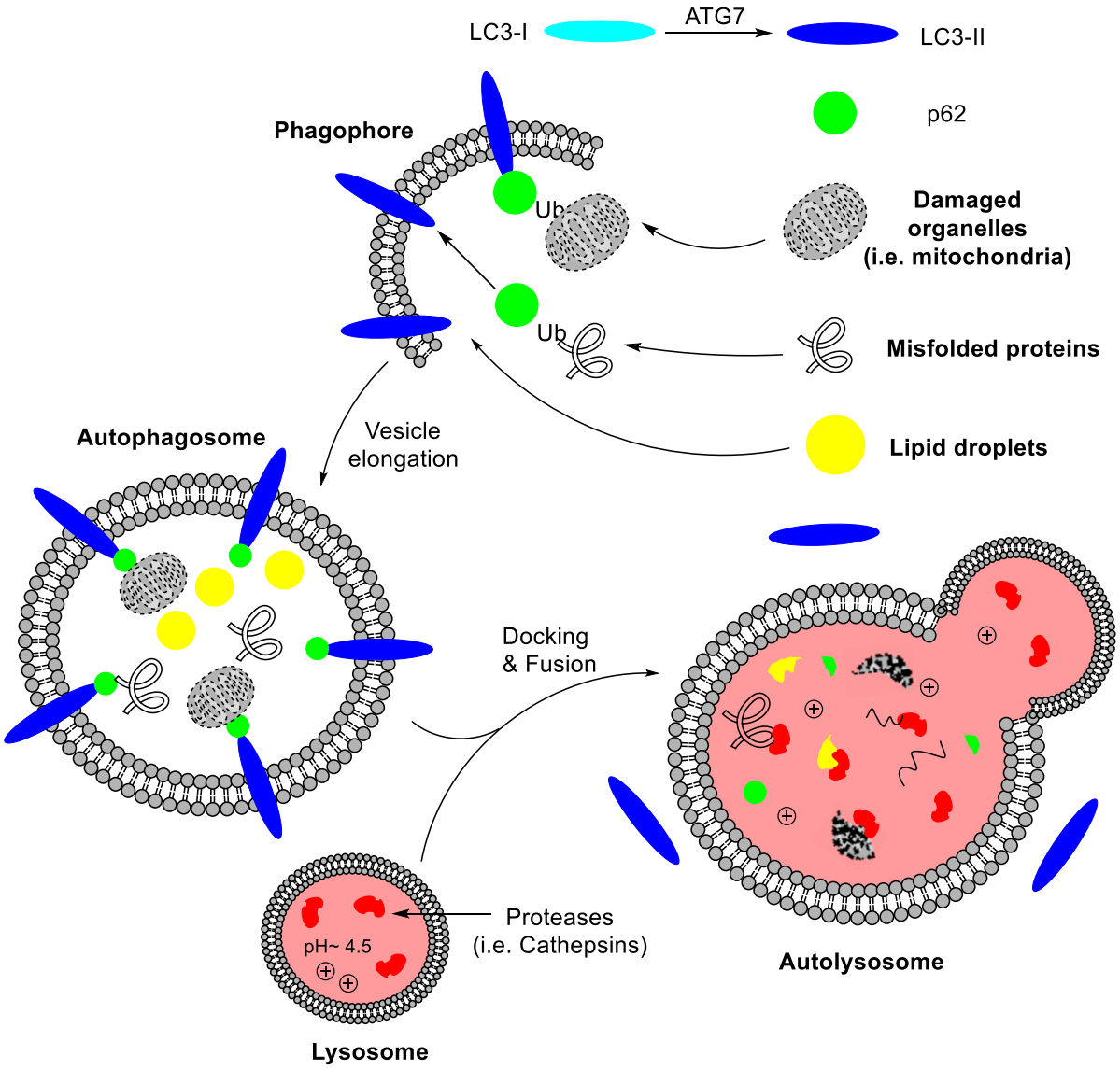

Figure 6) Simplified Schematic of autophagosome formation and lysosome fusion. Several important autophagic processes are highlighted above. Microtubule-associated protein 1A/1B (LC3-I) is conjugated to phosphatidylethanolamine through an ATG7 (autophagy related 7) dependent process. Damaged organelles and proteins are tagged by ubiquitin and shuttled to LC3-II by the adaptor protein p62 (sequestosome). Pieces of lipid droplets are incorporated into autophagosomes through partial engulfment of LD during the nucleation process. After completion of vesicle elongation, the mature autophagosome fuses with lysosomes to form autolysosomes which houses an acidic environment containing proteases, and esterases to breakdown cellular structures.

Autophagy is crucial in maintaining β-cell health; especially during periods of high nutrient load when β-cells accrue mitochondrial damage from increased metabolic demands and low innate anti-oxidant defense, ER stress due to the demand for insulin production, and LD accumulation. In the healthy cell, autophagy can recycle worn-out/damaged organelles such as the mitochondria through mitophagy which prevents the initiation of apoptosis, degrade misfolded proteins which lower ER stress, and reduce LD buildup through lipophagy. Electron microscopy and immunohistochemistry from T2DM human pancreatic islets sections showed autophagic vacuole accumulation and increased p62 staining in β-cells (Masini et al., 2009; Mizukami et al., 2014). Genetic ablation of key autophagy proteins in genetic animal models lead to β-cell dysfunction. β-cell specific *Atg7* knockout led to islet degeneration, accumulation of protein aggregates, and decreased glucose-stimulated insulin production (H. S. Jung et al., 2008). *Atg7* knockout mice fed a high-fat diet showed markedly higher blood glucose levels compared to WT animals in the glucose tolerance test (Ebato et al., 2008). Inhibition of autophagy mirrored symptoms present in many diabetic animal models including: insulin-resistance, increased glucose levels, and β-cell abnormalities (Gonzalez et al., 2011). It is therefore possible that obesity and T2DM causes autophagic impairments which contribute to the development of β-cell failure. In-vitro studies suggested that hyperglycemia and fatty acid treatment of human β-cells lead to the blockade of autophagic flux (Mir et al., 2015). In the same study, high glucose and fatty acid treatment increased the expression of mTORC1. As previously mentioned mTORC1 acts as the cell's nutrient sensor and is active during high energy load to promote anabolic processes and inhibits autophagy or cell catabolism. In obesity and T2DM, chronic high energy levels may prevent mTORC1 from inactivating. Nutrient overload of β-cells with glucose and FFA synergistically activated mTORC1 (Vernier et al., 2012). Notably, β-cell specific Tsc-2 knockout, which hyperactivated mTORC1 and repressed autophagy, led to β-cell failure due to increased mitochondrial and ER stress (Bartolome et al., 2014). Inhibition of mTORC1 with rapamycin improved β-cell survival in the Akita diabetic mouse model (Bachar-Wikstrom et al., 2013). Figure 7 illustrate the regulation of mTORC1 by Tsc2.

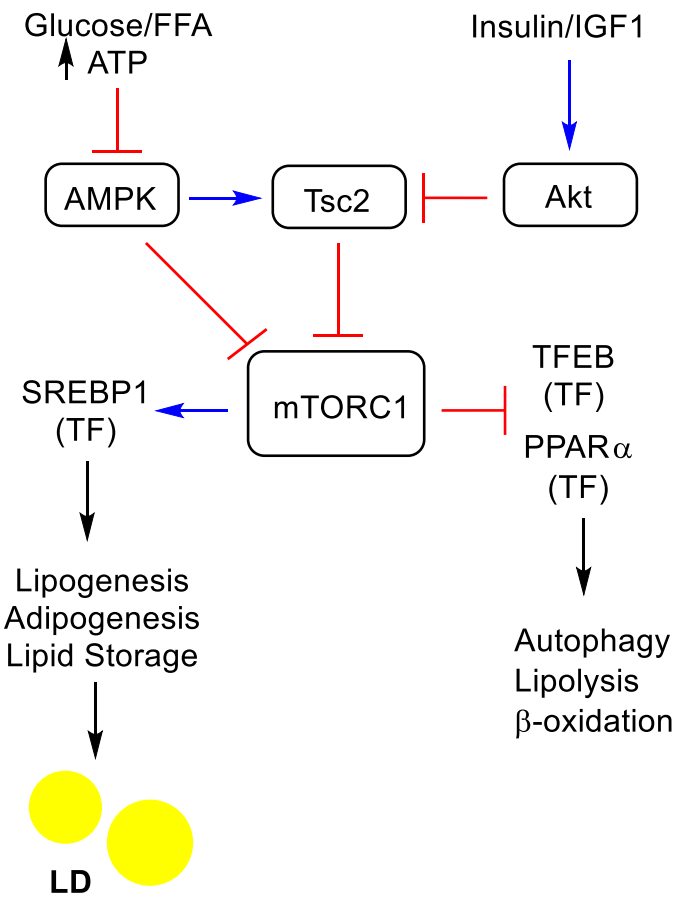

Figure 7) mTORC1 as a central regulator for cellular lipid status. Activation of mammalian target of rapamycin complex 1 (mTORC1) promotes anabolic processes such as protein synthesis, fatty acid storage, and increasing cellular energy levels. Active mTORC1 activates sterol regulatory element binding protein 1 (SREBP1) to increase lipid synthesis and storage while repressing autophagy and β-oxidation by inhibiting transcription factor EB (TFEB) and peroxisome proliferator-activated receptor alpha (PPARα). mTORC1 is repressed by active tuberous sclerosis (Tsc2). Tsc2 is regulated by two opposing process. AMP-activated protein kinase (AMPK) is activated at low energy levels (low ATP) to stimulate energy production by decreasing protein and lipid synthesis in-favor of β-oxidation. AMPK activates Tsc2 to repress mTORC1 and also directly suppresses mTORC1 activity. On the other hand, protein kinase B (akt) is activated by growth hormone such as insulin and insulin-like growth factor 1 (IGF-1) and suppresses Tsc2 to activate mTORC1. In the context of T2D and obesity, excess glucose and fatty acid suppresses the AMPK pathway while a high extracellular insulin level activates Akt. As a result mTORC1 becomes highly active and promotes excessive lipid storage.

mTORC1 regulates autophagy through phosphorylation of Transcription factor EB (TFEB). TFEB is a master regulator of autophagy that co-ordinates lysosomal biogenesis, autophagosome formation, and mitochondrial energetics. In the normal state, TFEB is phosphorylated at Ser211/142 by mTORC1 which enables the binding of protein 14-3-3 that sequesters TFEB in the cytoplasm (Roczniak-Ferguson et al., 2012). However, in response to starvation or mTORC1 inhibition by rapamycin/torin-1, TFEB phosphorylation at Ser211/142 is removed and TFEB translocate to the nucleus and activates the transcription genes involved in autophagic activity and lysosomal biogenesis (Settembre et al., 2011) (see Figure 8). TFEB is necessary for lipid degradation in the liver (Settembre et al., 2013).

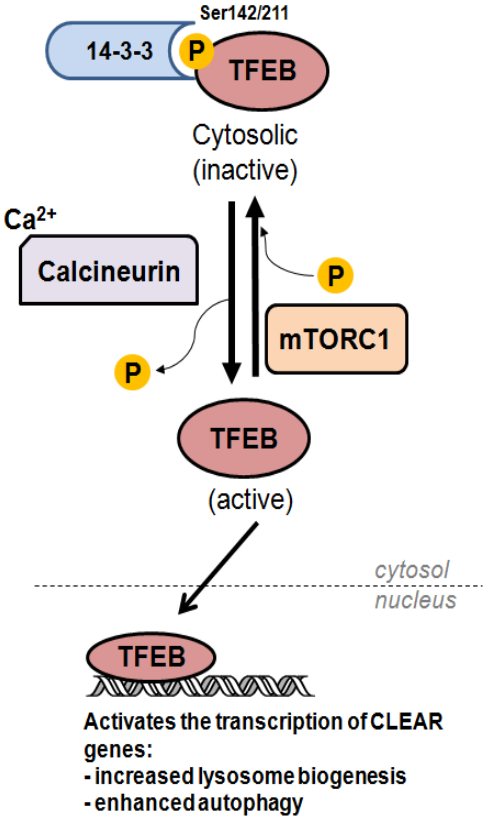

Figure 8) TFEB regulatory pathway. Transcription factor EB (TFEB) is inactive when phosphorylated by mTORC1 due to the inhibitory binding by 14-3-3 and is sequestered in the cytoplasm. Repression of mTORC1 or activation of the phosphatase calcineurin removes the blockade on TFEB which then translocates to the nucleus and activates the transcription of genes in the CLEAR (coordinated lysosomal expression and regulation) network.

Overabundance of nutrients could cause chronic mTORC1 hyperactivation and TFEB repression which limits β-cell autophagy due to the suppression of autophagic gene expression and lysosome biogenesis. Results from transcription analysis in islets from T2DM patients showed decreased expression of TFEB-regulated genes such as LAMP2, Cathepsin B and D (Masini et al., 2009). Based on these findings, current studies were designed to reveal differences in TFEB expression and location in human islets from human obese or lean subjects with or without T2D.

1.5 Connecting nutrient overload and autophagy

Fatty acid spillover from the large, leaky lipid droplets leads to ER stress, induction of apoptosis, and insulin resistance in β-cells. The timely removal of excess LD is therefore necessary to prevent lipotoxicity in β-cells. Aside from cytosolic lipase activity, lipophagy is emerging as an important mechanism for lipid droplet degradation by autophagy. In the liver, inhibition of autophagy or knockouts of key autophagy proteins have been show to increase lipid droplet accumulation due to decreased lipolysis (Singh et al., 2009). Studies of lipophagy in diverse cell populations suggest that lipophagy may be a general mechanism for degrading LD (Hubbard et al., 2010; Kaushik et al., 2011; Ouimet et al., 2011; Singh et al., 2009). Aside from clearing excess lipids inside the cell, lipophagic degradation of LD may represent a key pathway for the clearance of misfolded ER proteins temporarily stored on the surface of LD (Klemm, Spooner, & Ploegh, 2011). Inadequate clearance of LD could therefore further increase ER stress (X. Zhang & Zhang, 2012). The mechanism of lipophagy is extensively reviewed elsewhere (Singh & Cuervo, 2012; Ward et al., 2016). Furthermore, Atg7 knockdown in MIN6 or 3-methyladenine treatment in primary mouse islets increased insulin secretion and increased lipid droplet accumulation. In the same study, inhibition of lysosomal acid lipase with Lalistat mirrored the increase in insulin secretion and increase in lipid droplet number after inhibition of autophagy (Pearson et al., 2014).

mTORC1 hyperactivation due to hyperglycemia and fatty acid treatment was associated with lipid droplet accumulation (Vernier et al., 2012). However, the mechanism by which

mTORC1 hyperactivation increases LD accumulation in β-cells is not known. We hypothesized that nutrient overload in obesity and/or diabetes causes LD accumulation by increasing LD formation and inactivation of TFEB through mTORC1 hyperactivation. Studies have shown that TFEB is necessary for lipid degradation in the liver (Settembre et al., 2013). The role of TFEB in pancreatic islets has not yet been published. In this study, we asked if T2DM and obesity altered (1) islet structure, function and induced macrophage infiltration (section 3.1), (2) PLIN2 protein as well as other LD-associated genes (section 3.2), (3) TFEB expression and localization (section 3.3), (4) metabolism, oxidative stress and apoptosis related genes (section 3.4) in pancreatic islets, (5) whether hyperglycemia an hyperlipidemia is responsible for these changes (section 3.5-7). To answer these questions, we used the methods described in the following section.

Section 2: Methods

2.1 Samples, cell culture, and reagent preparation

Human pancreatic tissue

Following tissue extraction, adult human pancreatic tails were immersed in RNAlaterTM (Qiagen, #76104) for RNA extraction or fixed in 10% neutral buffered formalin solution (Fisher Scientific, SF100) and paraffinized for sectioning and immunolabelling. Patients were divided into four groups based on patient body mass index (BMI) and diabetes status: NDN (non-diabetic normal, BMI<25), NDO (non-diabetic obese, BMI>30), T2DN (type-2 diabetic, BMI<25), T2DO (type-2 diabetic obese, BMI>30). Patient information is summarized in table 1. Pancreata samples were provided by Quebec Transplant with prior consent from patients for use in research.

Cell Cultures

INS-1 rat insulinoma cells (AddexBio #C0018007) were cultured in RPMI-1640 media containing 11.1mM glucose, 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 2 g/L sodium bicarbonate, 10% FBS, 50 uM 2-mercaptomethanol, 1% penicillin-streptomycin and maintained at 37°C with 5% CO_2 and 99% relative humidity. Cells were sub-cultured every 3-4 days. The culturing glucose concentration of 11.1mM (based on manufacturer recommendations) is much greater than the physiological level found in humans (~ 5mM). This was expected since most immortalized cell lines require high glucose media for optimal survival. Thus the cancerous nature of INS-1 cells likely explain why they survival optimally at supraphyiological glucose concentrations. In order to apply a glucose challenge, we defined 30mM glucose as the hyperglycemia in culture. Although 30mM of blood glucose is unlikely to be found in human patients, it is a common concentration used in-vitro to study the effects of hyperglycemia.

Stable EGFP-TFEB Transfection

INS-1 cells were seeded at 2 million cells per well in 6-well plates. Cells were transfected with 1.65ug/well pEGFP-N1-TFEB (containing CMV promoter and neomycin resistant gene) using Lipofectamine 2000 (FischerScientific, #11668-019). After 48h, the

cell media was changed and supplemented with 400 ug/ml geneticin (Sigma, G418) to select for resistant cells. The selection media was changed every 3 days and maintained for 3 weeks. After 3 weeks, resistant cells were trypsinized and seeded into 96-well plates at a density of 0.5 cells per well to select for single colonies. After two weeks, resistant colonies bearing GFP tag were reseeded into 6-well plates for further expansion. Several colonies were tested for functional translocation response by starving cells in HBSS for 1h at 37˚C. Several monoclonal colonies were tested and colony 15 was selected on the basis of high percentage of stably transfected cells and active TFEB-EGFP nuclear translocation upon starvation. INS-1-EGFP-TFEB cells were cultured as wild-type INS-1 cells except for additional 200 ug/ml geneticin supplementation in culturing media. pEGFP-N1-TFEB was a gift from Shawn Ferguson (Addgene plasmid #38119).

Fatty Acid/BSA Complex Preparation

Oleic acid (Sigma #1008), and palmitic acid (Sigma # P0500) were dissolved in 1X Krebs-Ringer bicarbonate buffer (KRBH) complexed with 5% fatty-acid free BSA (Sigma #A8806) under gentle heating and stirring for 10 min. and sterile filtered through a 0.22 um filter. Free-fatty acid concentration was quantified using Wako HR series NEFA-HR(2) according to manufacturer instructions.

Table 1) Patient information

Sample	Age	Sex	Weight (kg)	Height (cm)	BMI	Medical History	Medication	Cause of death	Used for qPCR	Used for IHC
NDN1	74	-	61	165	22.4	na	na	Cranial trauma	Yes	No
NDN2	50	M	70	178	22.1	na	na	Stroke	Yes	No
NDN3	35	F	48	148	21.8	na	na	Stroke	Yes	No
NDN4	26	F	70	170	24.2	na	na	Anoxia	Yes	No
NDN5	52	F	53	162	20.2	na	na	Cerebral hemorrhage	Yes	No
NDN6	72	F	59	163	22.3	na		Trauma	No	Yes
NDN7	14	F	50	162	19.1	na	na	Trauma	No	Yes
NDN8	56	F	52	160	20.3	na	na	Cerebrovascular accident	No	Yes
NDN9	40	M	59	180	18.2	na	na	Trauma	No	Yes
NDN10	63	F	56	158	22.4	na	na	-	No	Yes

NDO1	62	F	82	160	31.8	na	na	Cerebral hemorrhage	Yes	No
NDO2	51	M	118	180	36.4	na	na	Cerebral hemorrhage	Yes	No
NDO3	70	F	90	160	35.2	na	na	Cerebral hemorrhage	Yes	No
NDO4	60	F	90	157	36.5	na	na	Stroke	Yes	No
NDO5	48	F	74	157	30	na	na	Anoxia	Yes	No
NDO6	57	F	95	160	37.1	na	na	Cerebral hemorrhage	Yes	No
NDO7	12	-	52	168	18.4	na	na	Cerebral hemorrhage	No	Yes
NDO8	55	M	75	165	27.5	na	na	Cerebral edema	No	Yes
NDO9	35	F	122	165	44.8	na	na	Subarachnoid hemorrhage	No	Yes
NDO10	68	M	95	168	33.7	na	na	Sub-dural hemorrhage	No	Yes
NDO11	64	F	90.5	150	40.2	na	na	Cerebral hemorrhage	No	Yes
NDO12	71	M	94	188	26.6	na	na	Trauma	No	Yes
T2DN1	54	F	59	158	23.6	T2D, hypertension, COPD, hyperlipidemia, smoker	Glyburide, metformin	Cerebral hemorrhage	Yes	No
T2DN2	54	M	80	178	25.2	T2D, hypertension, hyperlipidemic	Anti-hyperglycemic, diet	Trauma	Yes	No
T2DN3	63	M	70	170	24.2	T2D, smoker, alcoholic	Diet	Trauma	Yes	No
T2DN4	60	M	73	170	25.3	T2D, hypertension, hyperlipidemic, smoker, alcoholic	Diet	Anoxic enchephalopathy	Yes	No
T2DN5	64	M	64	170	22.1	T2D	Metformin	Cerebral hemorrhage	Yes	No
T2DN6	63	M	70	170	24.2	T2D, smoker, alcoholic	-	Trauma	No	Yes
T2DN7	64	M	64	170	22.1	T2D	Metformin	Cerebral hemorrhage	No	Yes
T2DN8	54	F	59	158	23.6	2D, hypertension, COPD, hyperlipidemia, smoker	Glyburide, metformin	Cerebral hemorrhage	Yes	Yes
T2DO1	54	F	83	165	30.5	T2D	Insulin	Abdominal trauma	Yes	No
T2DO2	69	F	138	177	44.0	T2D	-	Cerebral hemorrhage	Yes	No
T2DO3	60	M	113	173	37.9	T2D, hypertension, smoker	Metformin	Cerebral anoxia	Yes	No
T2DO4	66	F	81	154	34.2	T2D, manic depression, smoker	Metformin, glyburide	Sub-arachnoid hemorrhage	Yes	No
T2DO5	66	M	95	170	32.8	T2D	Metformin	Anoxia	Yes	Yes (CD68 only)
T2DO6	54	F	77	160	30.1	T2D, hypertension	-	Cerebrovascular accident	No	Yes
T2DO7	75	M	76	166	27.58	T2DM, Smoker,	Glyburide	Cerebral hemorrhage	No	Yes

T2DO8	53	M	106.4	156	43.7	T2D	Norepinephrine bitartrate	AVC hemorrhage	No	Yes
T2DO9	65	F	86.7	157	35.2	T2D	Metformin	Anoxia	No	Yes

2.2 Assays

RT-PCR

Samples frozen at -80°C in RNAlater were homogenized in RLT buffer. Homogenized tissues were processed in Quacube^{TM} (Qiagen) using RNEasy mini kit. RNA concentration was assessed by measuring UV absorbance at 260nm and 280nm using a spectrophotometer and RNA purity was measured by taking the OD260/280 (close to 2.0). RNA integrity was measured by 1.5% agarose gel electrophoresis for the appearance of 18S and 28S band. RNA was reverse transcribed to cDNA using Omniscript Reverse Transcription Kit (Qiagen) and oligo-dT primers (Qiagen). qPCR were performed using IQ^{TM} SYBR® Green Supermix (Bio-Rad) in CFX96^{TM} Real-Time System (Bio-Rad), CFX Manager Software 1.1. Primers used are listed in table 2. α-actin was used as the reference gene in human samples and GAPDH, β-actin, β-tubulin was used as reference genes in rat INS-1 samples. Genes of interest were analyzed for relative quantities (ΔCt) and fold change in gene expression ($\Delta\Delta$Ct) using the data analysis software CFX Manager 2.0.

Table 2) Primers used in this study

Primers for Human Genes				
Gene	**Reference**	**Forward**	**Reverse**	**Amplicon(bp)**
PLIN2	NM_001122	GCTGAGCACATTGAGTCACG	GCATTGCGGAACACTGAGTAGA	172
ACTB	NM_001101	TTCCTGGGCATGGAGTCCTGT	CTTGATCTTCATTGTGCTGGGTGC	188
BAX	NM_138761	TGCTTCAGGGTTTCATCCAGGA	CTGTCCAGTTCGTCCCCGAT	138
BCL2	NM_000633	TGCACCTGACGCCCTTCAC	TTCCACAAAGGCATCCCAGCC	244
CAV1	NM_001753	AGACTCGGAGGGACATCTCTACA	GTCATCGTTGAGGTGTTTAGGGTC	165
CPT1A	NM_001876	CGATGTTACGACAGGTGGTTTGAC	CGGAATGTTCGGATTGATGTCGC	200
GAPDH	NM_002046	AAGATCATCAGCAATGCCTCCTG	TGACCTTGCCCACAGCCTT	228
FSP27	NM_022094	ATCAGAACAGGGGACAAGGCA	CTTCACGTTCAGGCAGCCAATG	129
FOXO1	NM_002015	GACCTCATGGATGGAGATACATTGGA	GTGTAACCTGCTCACTAACCCTCA	128
GCK	NM_000162	GCTGAGATGCTCTTCGACTAC	CTTGGTCCAGTTGAGAAGGATG	152
GLUT2	NM_000340	GTGCTGCTGAATAAGTTCTCTTGG	GTCCACAGAAGTCCGCAATGTA	225
GCG	NM_002054	GACTACAGCAAGTATCTGGACTCC	TCTGGGAAATCTCGCCTTC	220
GPX1	NM_000581	GAATGTGGCGTCCCTCTGAG	TGCGTTCTCCTGATGCCCA	131
HMOX	NM_002133	GCAACAAAGTGCAAGATTCTGCC	TGGCATAAAGCCCTACAGCAACT	140
INS	NM_000207	CCATCAAGCAGATCACTGTCCT	GTGTAGAAGAAGCCTCGTTCC	180
PPARA	NM_001001928	ATTTGCTGTGGAGATCGTCCTGG	GCATCCGACTCCGTCTTCTTGAT	214
PPARG	NM_138712	TATCCGAGGGCCAAGGCTTC	AAACCTGGGCGGTCTCCACT	177
SST	NM_001048	CCAGACTCCGTCAGTTTCTG	GCCATAGCCGGGTTTGAGTTA	204
UCP2	NM_003355	GATACCAAAGCACCGTCAATGCCTAC	GGCAAGGGAGGTCATCTGTCAT	185
SQSTM1	NM_003900	ATCTCCCGCCAGAGGCTGA	GGATGCTTTGAATACTGGATGGTGTC	156
CYBA	NM_000101	ATCTACCTACTGGCGGCTGTG	TCCTCCTCGCTGGGCTTCTT	154
HPRT	NM_000194	CCTGGCGTCGTGATTAGTGATGAT	CAGAGGGCTACAATGTGATGGC	181
SDHA	NM_004168	GCAGAACCTGATGCTGTGTG	TTCCAGAGTGACCTTCCCAGTG	216
Primers for Rat Genes (INS1)				
Gene	**Reference**	**Forward**	**Reverse**	**Amplicon(bp)**
PLIN2	NM_001007144.1	AGCCACAGATTGCGGTCG	GTCACTACATCCTTTGCCCCAG	141
PLIN3	XM_001061015.2	CAGCAGCAGAGACAGGAAAGGA	TCACAGATTCCATCAGGCTAAGCAC	168
FSP27	NM_001024333.2	CTGGGCAAGGTCCAAGACAT	CTGTTCTGATGGGGACTTCCAC	167
TFEB	NM_001025707.1	GCTAACAGATGCTGAGAGCCG	CTTGAGGATGGTGCCCTTGT	174
LAMP1	NM_012857.2	GTTCAGCACCTCCAACTATTCCCT	CACTCTTCCACAGACCCAAACC	174
p62	NM_175843.4	TGAGTCGGCTTCTGCTCCAT	TCTTATCTTCTGTGCCTGTGCTGG	184
LC3	AY310156.1	ACTAACCACTGCCTCTCAACCTG	TCGCTCTATAATCACCTGGGTCG	204
INS1	NM_019129.3	CATCAGCAAGCAGGTCATTGTTCC	AGGTACAGAGCCTCCACCAG	148
INS2	NM_019130.2	CATCAGCAAGCAGGTCATTGTTCC	GGGTGTGTAGAAGAATCCACGC	182
PPARα	NM_013196.1	GTCATCACAGACACCCTCTCTCC	AGCCTTCACATGCGTGGAC	165
PPARδ	NM_013124.3	GTCTCACAATGCCATCAGGTTTGG	TTTGGTCAGCGGGAAGGAC	165
UCP2	NM_019354.2	CATGACAGACGACCTCCCTTG	GGTGACAAACATTACTACGTTCCAGG	243

Immunohistochemistry

Samples were taken from the tail of the pancreas before isolation and stored at 4 °C in phosphate-buffered formalin. Paraffin-embedded tissue sections (5-µm) were deparaffinized in xylene, dehydrated in ethanol and then gradually rehydrated. Antigen retrieval was achieved by heat-induced epitope retrieval using citrate buffer (10 mM, 0.05% Tween-20, pH 6.0). After reaching boiling temperature, slides were maintained at sub-boiling temperature for 40 min. Sections were blocked in blocking buffer (2% horse serum/10% goat serum/1mM HEPES/0.1% sodium azide/0.3% triton x-100 in HBSS) for 2 h and incubated with primary antibodies targeting TFEB (rabbit polyclonal, Bethyl Laboratories #A303-673) (1:200 dilution), CD68 (mouse monoclonal, Dako #M0814) (1:20 dilution), C-peptide (mouse monoclonal, Meridian Life Science # E54094M) (1:100 dilution) and perilipin-2 (guinea pig polyclonal, Fitzgerald Industries #20R-AP002) (1:200 dilution) at 4°C (overnight). After removing the primary antibodies and washing the slides, sections were incubated with secondary antibodies conjugated to Alexa Fluor 488 (goat anti-rabbit IgG, Invitrogen, A32731), Alexa Fluor 488 (goat anti-mouse IgG, Invitrogen, A-11029), Alexa Fluor 647 (goat anti-guinea pig IgG, Invitrogen, A-21450). Slides (Fisher Scientific, 12-550-14) were mounted using Aqua-Poly/Mount (Polysciences, Inc, #18-606).

Lipid Droplet Staining and Quantification

INS-1 cells were seeded onto poly-d-lysine coated coverslips at 20,000 cells per coverslip and incubated for 48 hours before treatment. Cells were treated with 500 uM oleic acid under normoglycemic (5mM glucose) or hyperglycemic (30mM glucose) culturing media for 24 hours before fixation in 2% PFA diluted in PBS+ for 10 min. at room temperature. Cells were washed in PB + (PBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$. Cells were labelled with 20 uM BODIPY 493/503 and 10 uM Hoechst 33342 for 10 min. at RT and washed 3x with PBS+. Coverslips were mounted onto microscope slides with non-hardening EverBrite Mounting medium (Biotium #23001) and the edges sealed with commercial nail polish. Images were captured using a fluorescence microscope and the LD count was done using ImageJ.

TFEB Translocation Test

We tested whether hyperglycemia and FFA decrease the rate of TFEB nuclear translocation. INS1-TFEB-EGFP were seeded in 24-well plates at 500,000 cells per well. 24h post seeding, cells were treated in 5mM/30mM [GLU] in the presence or absence of 500uM oleic acid, palmitic acid, or oleic acid + palmitic acid for 48h. After treatment, cells were starved for 1h in HBSS and fixed in 2% PFA for 10 min. at room temperature and washed twice with PBS. Cell nuclei were labelled with 10uM Hoechst 33342 for 10min. at room temperature and washed once with PBS. Cells were imaged by fluorescence microscopy. EGFP-TFEB nuclear translocation was assessed by taking the ratio of the area normalized EGFP fluorescence intensity in the nucleus over the cytosol. A higher ratio indicates greater nuclear EGFP-TFEB translocation, image analysis were performed in ImageJ.

Measurement of mitochondrial metabolic activity (MTT Assay)

INS-1 and INS-1-EGFP-TFEB were seeded at 500,000 cells per well in 24-well plates and incubated for 24h before treatment. Cells were treated as indicated in the figure legend. After treatment, the media was replaced with serum-free culturing media containing 0.5mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide (MTT) and incubated for 1h at 37°C. The media was aspirated and replaced with 400ul dimethyl sulfoxide (DMSO) to solubilize the formazan crystals. Aliquots of 100ul were transferred in triplicates to 96-well plates (Sarstedt) and the absorbance was measured at 595nm using a Benchmark microplate reader (Bio-Rad, ON, Canada).

2.3 Equipment and statistical analysis

Microscopy

Confocal imaging was performed with Zeiss LSM 510 NLO inverted confocal microscope using a Plan Achromat 63X/1.4 Oil DIC objective. Fluorescent detection of Alexa Fluor 647-conjugated antibody was achieved by using a HeNe 633 nm laser and a long pass (LP) 650 filter. Alexa Fluor 488-conjugated secondary antibody was imaged using Argon 488 nm excitation laser and a 500-550 band pass (BP) filter. All images

were acquired at a resolution of 1024 × 1024 pixels (x, y). Fluorescence microscopy was performed with a Leica D6MI4000 inverted microscope using a 63X Oil objective captured using a Leica DFC345 FX camera.

Statistical analysis

All data are expressed as means ± standard error of the mean (SEM). Statistical differences were analyzed by one way analysis of variance (ANOVA) followed by post-hoc Tukey's test. Statistical differences are indicated by * $p<0.05$ or ** $p<0.01$.

3.1 Islet and cell function

Islets of Langerhans are scattered throughout the pancreas as individual islands composed of three major cell types. We performed immunohistochemistry to identify the three types: glucagon secreting cells (α-cells), insulin secreting cells (β-cells), and somatostatin secreting cells (SST-cells) (Figure 9A).

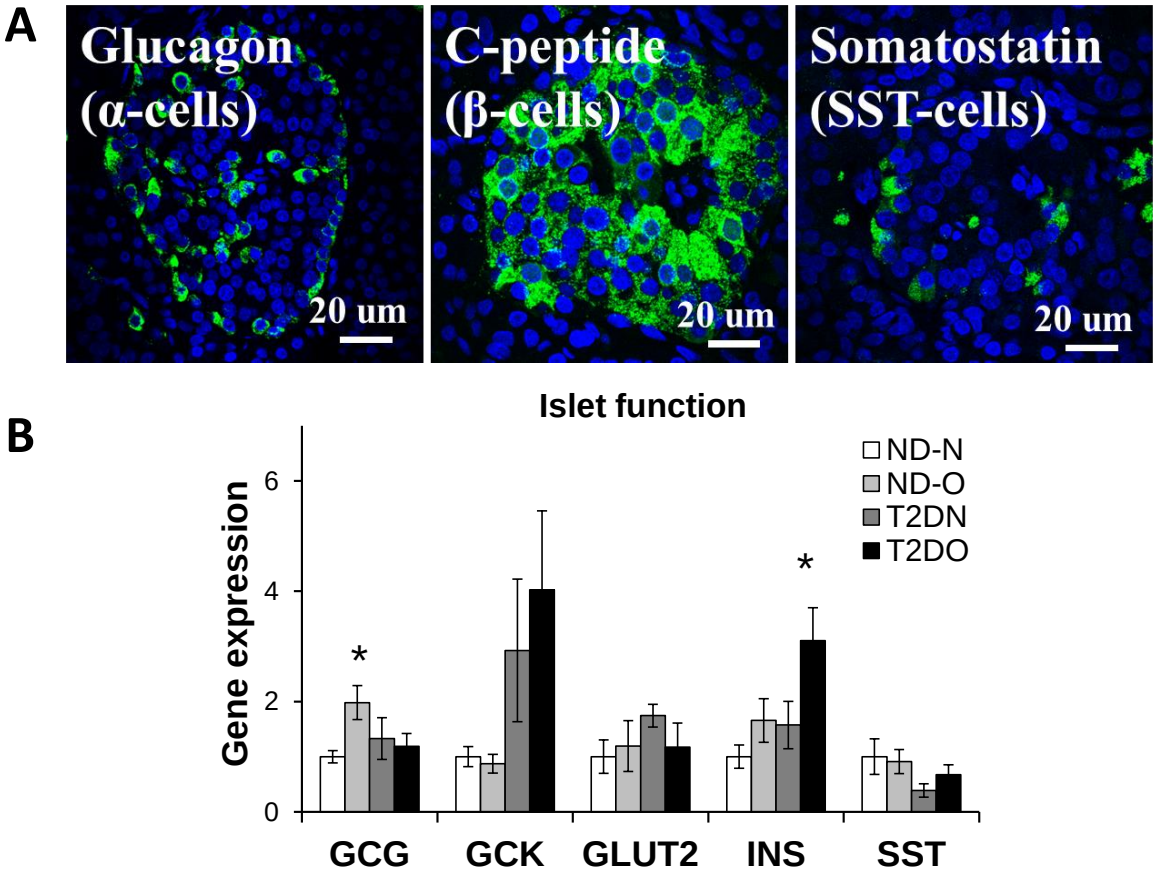

Figure 9) Islet cell types and qPCR of islet function genes in patients. (A) Representative images of islet cell types. (B) Gene expression analysis of islet function genes from NDN (normal non-obese), NDO (normal obese), T2DN (type-2 diabetic normal), T2DO (type-2 diabetic obese). GCG=glucagon, GCK=glucokinase, GLUT2=glucose transporter type 2, INS=insulin, SST=somatostatin. Shown are average fold increase ± standard error of the mean (SEM) in gene expression between groups over control (NDN). N=5 for all groups. Statistical significance is indicated by * (compared to NDN), * $p<0.05$.

In order to compare the overall health and function of the islet, we performed qPCR to quantify the changes in GCG, GCK, GLUT2, INS, and SST in different donor groups since dysregulation in these genes would represent major alterations in the control of islet-released hormones or glucose control (Figure 9B). GCG was significantly upregulated in NDO compared to NDN (NDO-NDN: -0.6±0.15, $p<0.05$). GCK was elevated in the diabetic donors compared to non-diabetic donors but did not reach statistical significance (T2DN-NDN: +3±1.2, T2DO-NDN: +4±0.7). INS was significantly upregulated in T2DO compared to NDN (T2DO-NDN: +3.2±0.3). GLUT2 and SST gene expression were not significantly different between the donor groups.

One salient feature in rodent models of obesity and diabetes is the infiltration of macrophages into the islet environment. To assess whether macrophage infiltration occurred due to obesity, diabetes, and obesity + T2D, we used antibodies raised against CD68 and labeled macrophages in human islets. Immunohistochemistry data revealed an increased macrophage infiltration into or surrounding islets both in pancreata of obese and T2D patients (Figure 10AB). Interestingly, the combination of obesity and T2D did not additively increase macrophage infiltration. However, it should be noted that despite the significant increase, the overall number of macrophages were quite limited in the islets from obese and T2D groups. As a positive control, we labeled macrophages from human glioblastoma specimen which contain a large number of CD68-positive cells (Figure 10C). Considering the relatively small number of infiltrating macrophages in pancreatic samples, they are unlikely to contribute to gene expression changes in pancreatic samples analyzed by qPCR. Under inflammatory conditions, macrophages and hepatocytes contain a large number of lipid droplets. Our next objective was to analyze the LD status in pancreata of obese and lean patients with and without T2D.

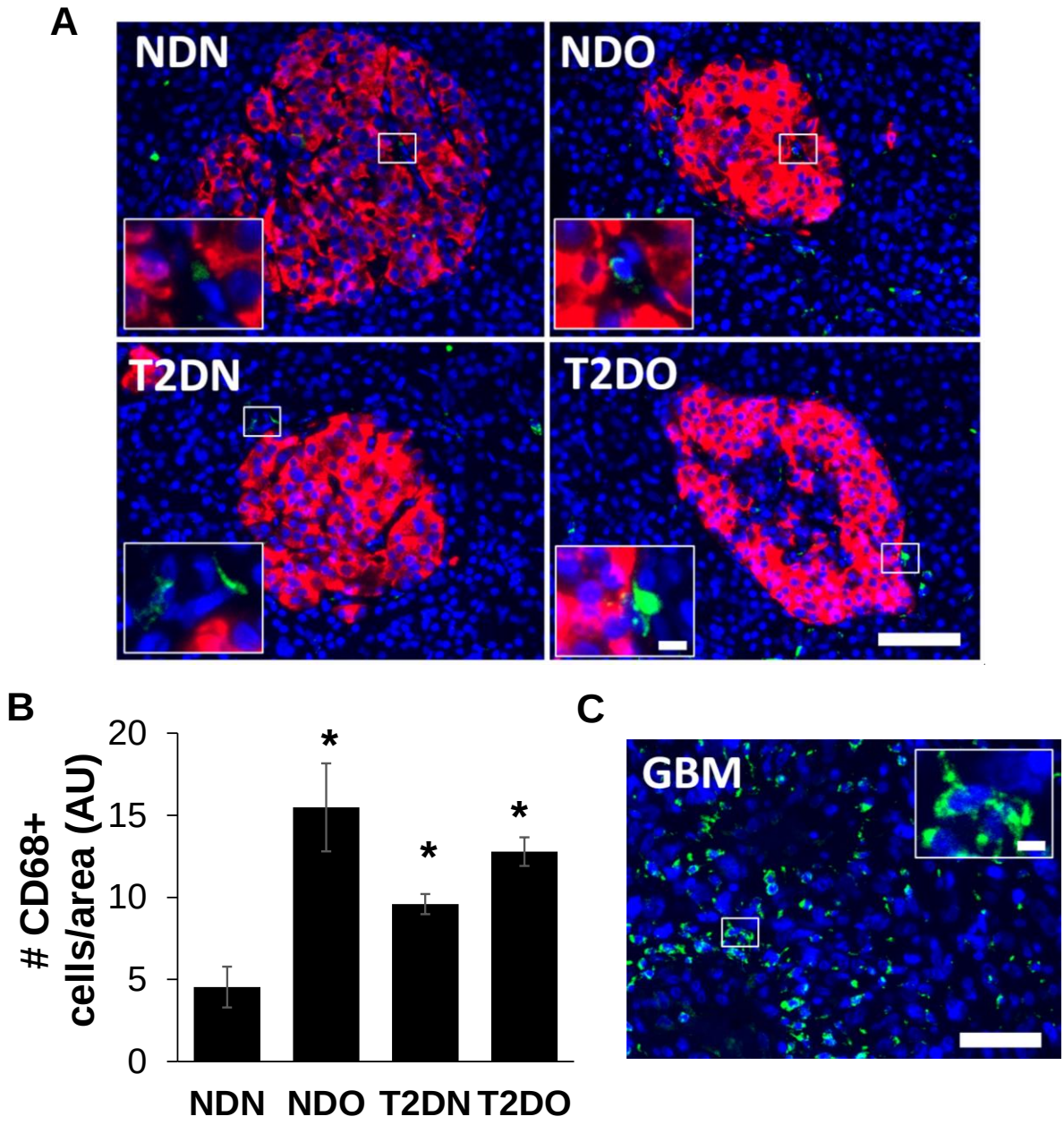

Figure 10) Invasion of CD68 positive macrophages in pancreatic islets of non-T2 diabetic and T2 diabetic subjects. (A) Immunofluorescent staining of CD68 in islets in human pancreatic sections from different categories of donors. Fluorescence images show triple labelling of CD68 (green), insulin (red), and nucleus (blue) in ND-N, ND-O, T2DN, and T2DO donors. Arrows point to CD68-positive cells, inset shows a zoomed-in image of a CD68-positive cell. Main scale bar = 100 um, inset scale bar = 10 um. (B) Quantification of number of CD68-positive macrophages surrounding or infiltrating islets from ND-N (N=5), ND-O (N=5), T2DN (N=3), T2DO (N=3) patients. The number of CD68-positive cell was normalized over the counted area. Statistically significant compared to NDN are indicated by * $p<0.05$, error bars represent the SEM. (C) Positive control for CD68 staining in glioblastoma.

3.2 Lipid droplet markers in islets

We hypothesized that obesity and T2D diabetes would lead to lipid overload in the islets due to increased glucose and fatty acid storage. To assess LD in the pancreas from NDN, NDO, T2DN, T2DO donors, we immunolabeled pancreatic sections for PLIN2, a protein constitutively expressed on the surface of LD. There was a marked increase in PLIN2 signal in the islet from both the T2DN and T2DO donors and a subtle increase in the NDO (Figure 11). PLIN2 strongly co-localized with β-cells (C-peptide positive) and were mainly localized in the islets. To assess the expression of LD-associated genes, we performed qPCR to analyze the levels of CAV1, PLIN2, and FSP27. In accordance with our PLIN2 IHC, PLIN2 gene expression was significantly upregulated in T2DN (3.7±0.3) compared to NDN (1±0.1). FSP27 was significantly upregulated in NDO (5.6±1.3) compared to NDN (1±0.2) but not in the T2DO (2.3±1.3). CAV1 expression levels were not significantly different between any of the groups.

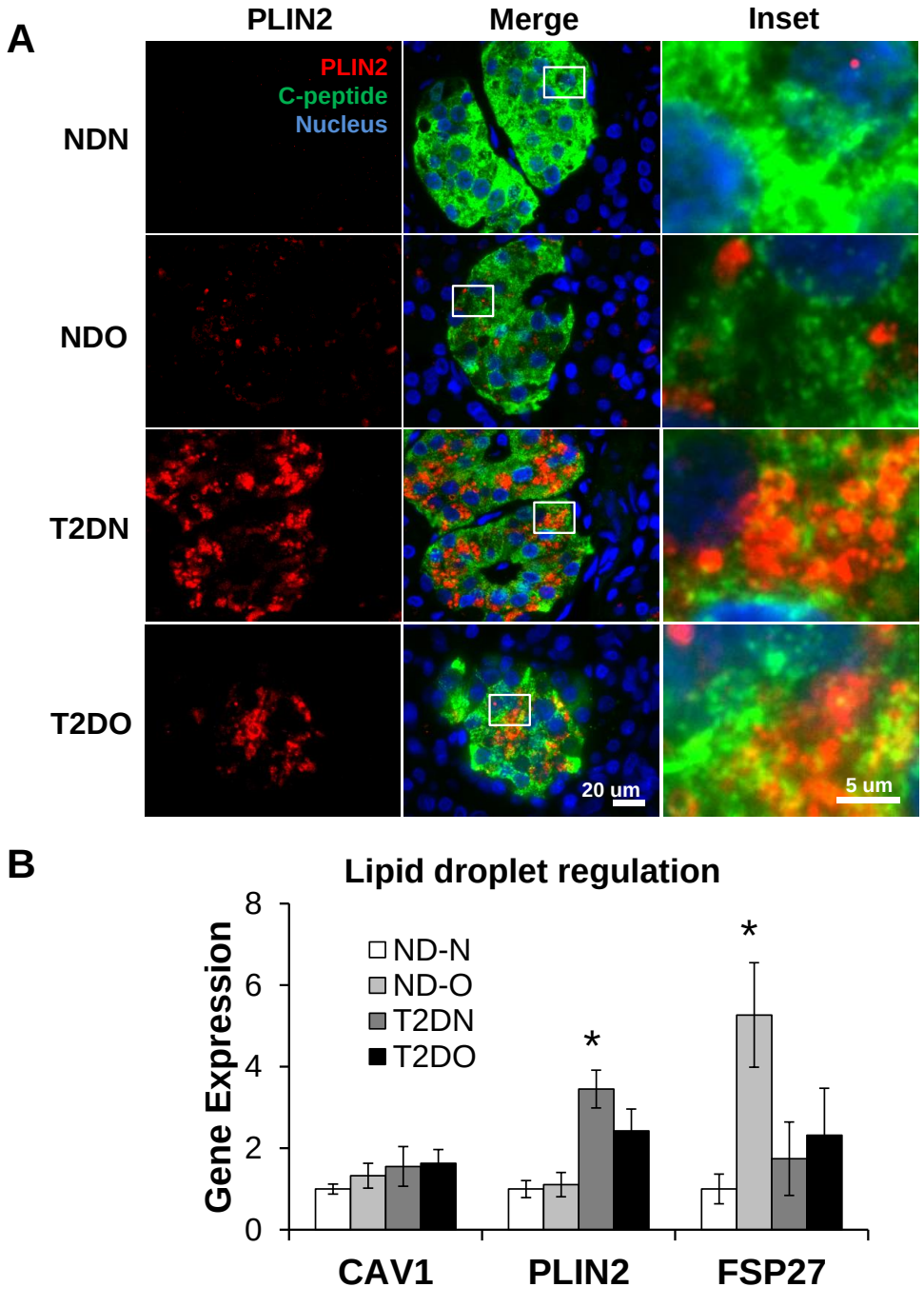

Figure 11) PLIN2 labelling and LD-associated gene analysis in patients. (A) Pictomicrograph of sections double immunolabeled with PLIN2 (red), C-peptide (green), and stained for Hoechst 33342 (blue). Images are representative from 4 islets slices per group. (B) Gene expression analysis of lipid droplet regulation genes (CAV1=caveolin 1, PLIN2=perilipin 2, FSP27=fat specific protein 27). Shown are average fold increase ± standard error of the mean (SEM) in gene expression between groups over control (NDN). N=5 for all groups. Statistically significance were indicated by * (compared to NDN), * $p<0.05$.

3.3 TFEB nuclear translocation is suppressed in T2D

Having seen an accumulation of LD in the islets of T2D donors, we hypothesized that the excessively nutrient rich environment in obesity and T2D inhibits the autophagic clearance of lipids. The transcription factor TFEB is a master regulator of autophagy and lysosome biogenesis. We compared TFEB status in islets from normal, obese, T2D, and obese T2D donors (Figure 12). In the non-diabetic sections, we detected TFEB nuclear translocation in 3/8 sections stained (NDN 2/4 sections, NDO 1/4 sections with TFEB nuclear translocation) whereas there was no TFEB nuclear translocation from any of the diabetic sections (T2DN 0/3, T2DO 0/3 sections with TFEB nuclear translocation). In islets from T2D donors, TFEB is localized to the perinuclear region and the total stain appears less abundant. Overall, T2D corresponded with reduced islet TFEB nuclear translocation.

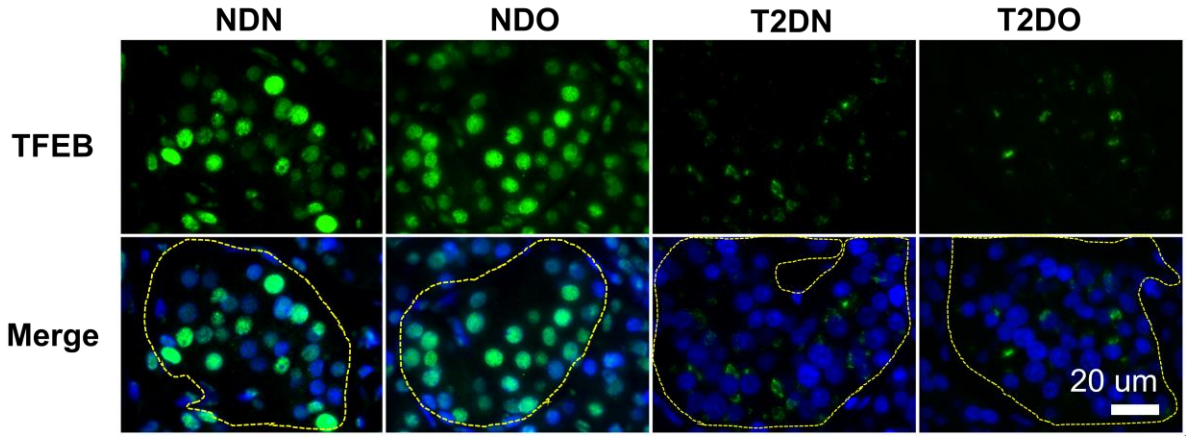

Figure 12) TFEB nuclear translocation in T2DN and T2DO
Representative images of TFEB immunolabelling in NDN, NDO, T2DN, and T2DO. Islets regions (in yellow) are traced from bright-field image based on morphology. Images represent at least 3 images from N=4 NDN, N=4 NDO, N=3 T2DN, N=3 T2DO patients.

3.4 T2D and obesity alter islet metabolism, Redox, and apoptosis related gene expression

Given the role TFEB exerts on global cell metabolism and the importance of autophagy in maintaining homeostasis, we examined the effects of increased lipid load and inhibited autophagy on pancreatic genes regulating metabolisms, apoptosis, and oxidative stress.

T2D and obesity lead to significant changes in the expression of transcription factors FOXO1, PPARα, PPARy. FOXO1 was significantly upregulated compared to NDN in both NDO and T2DO. Interestingly, PPARα and PPARy had opposing patterns of regulation. PPARα was significantly upregulated in NDO, T2DN, and T2DO while PPARy was downregulated in T2DN and T2DO. There were significant mitochondrial changes in the pancreas from T2D patients including highly upregulated CPT1A in T2DN and T2DO compared to NDN (Figure 13). CPT1A is a crucial enzyme on the surface of the mitochondria which facilitates beta-oxidation. Upregulation of CPT1A mRNA suggested an upregulation of beta-oxidation to combat the increased lipid load due to increased LD. Increased beta-oxidation would increase the transmembrane potential of the mitochondria by increasing oxidative phosphorylation and driving up proton transport. The uncoupling protein UCP2 was upregulated in NDO, T2DN, and T2DO suggesting an increase in non-ATP coupled proton leak (Figure 13). Increased proton leak has been shown to increase oxidative stress. There was significant upregulation of anti-oxidant defense genes GPX1 and HMOX1 in the pancreas from T2D donors. Since ROS is reported as a primary contributor of islet degeneration, we examined the gene expression of BAX and BCL2 as indicators of apoptosis. While there was a moderate increase in the pro-apoptotic gene BAX, there was 6-8 fold upregulation of the anti-apoptotic gene BCL2 (Figure 13) in T2DN and T2DO. These changes at mRNA level in mitochondria, anti-ROS defense, and apoptosis-associated events in T2D and pancreata of obese subjects, suggest a chain of events linking metabolic stress to oxidative stress to cell death or adaptive compensation. Moreover, adaptive changes revealed by qPCR analyses suggest that the effects of diabetes are

still within the control of compensatory mechanisms and have not reached the stage of irrevocable damage. This is supported by the lack of obvious morphological deterioration of the islets (Figure 9A) from obese and T2D donors.

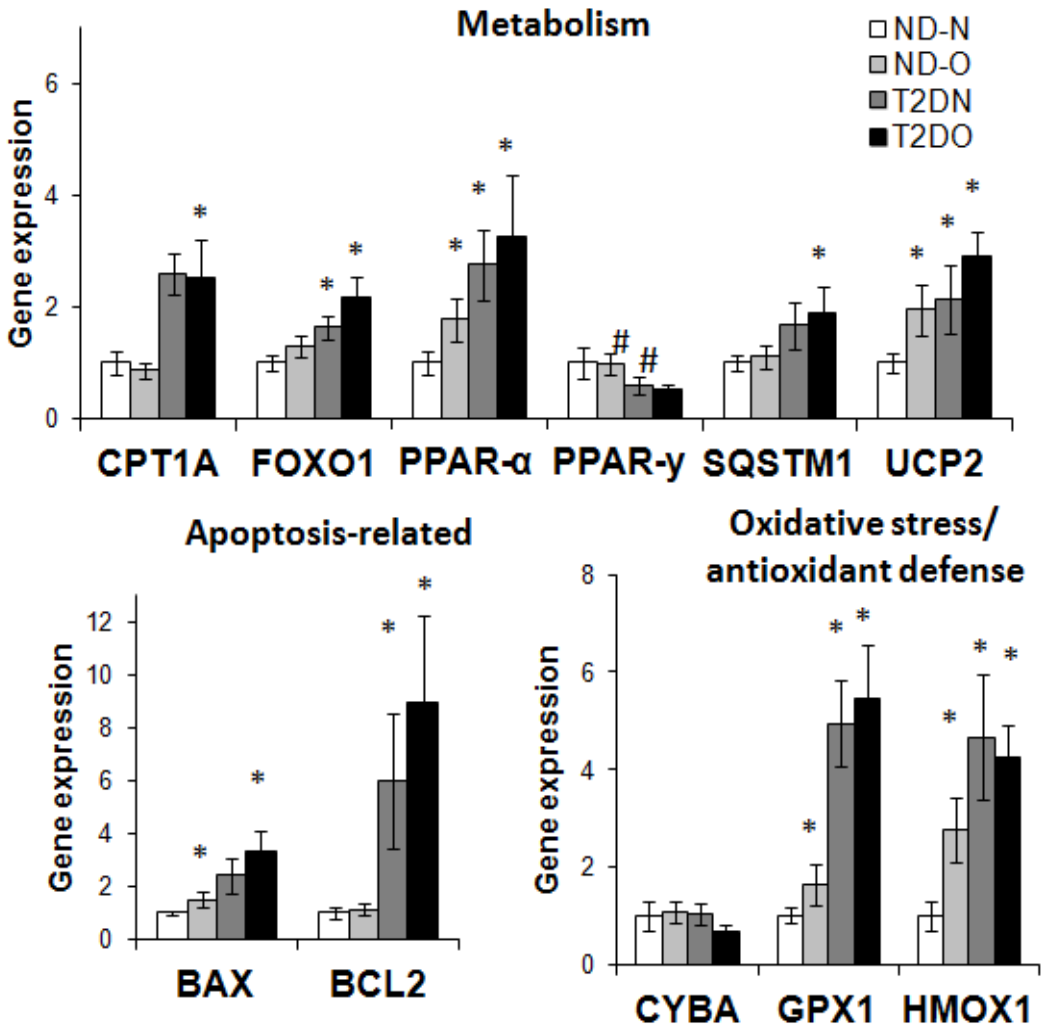

Figure 13) Gene expression analysis of islet function genes in patients from NDN (normal non-obese), NDO (normal obese), T2DN (type-2 diabetic normal), T2DO (type-2 diabetic obese) groups. CPT1A=carnithine palmitoyltransferase 1A, FOXO1=forkhead box O1, PPAR-α=peroxisome proliferator activated receptor α, PPAR-y=peroxisome proliferator activated receptor y, SQSTM1=sequestome 1, UCP2=uncoupling protein 2, BAX=BCL2 associated X, BCL2=BCL2, apoptosis regulator, CYBA=cytochrome B-245 α-chain, GPX1=glutathione peroxidase 1, HMOX1=heme oxygenase 1). Shown are average fold increase ± standard error of the mean (SEM) in gene expression between groups. N=5 for all groups. Statistical significance is indicated by * (compared to NDN) # (compared to NDO), * or # $p<0.05$.

3.5 Effect of hyperglycemia and hyperlipidemia on LD in INS-1

Although pancreatic dysfunction due to T2D and obesity is multifactorial and involves a host of metabolic and hormonal changes, two major contributors are hyperglycemia and hyperlipidemia. We examined the direct effects of hyperglycemia and hyperlipidemia on LD and autophagy in-vitro using INS-1 as a cell-based model. We defined normoglycemia as culturing medium containing 5mM glucose and hyperglycemia as medium containing 30mM glucose. Hyperlipidemia was defined as medium containing: 500uM oleic acid (OA), 500uM palmitic acid (PA), or 250uM oleic acid + 250uM palmitic acid (OA + PA). Hyperglycemia in combination with hyperlipidemia drastically increased the number of LD compared to either alone (Figure 14). Hyperglycemia and hyperlipidemia synergistically upregulated LD accumulation in INS-1.

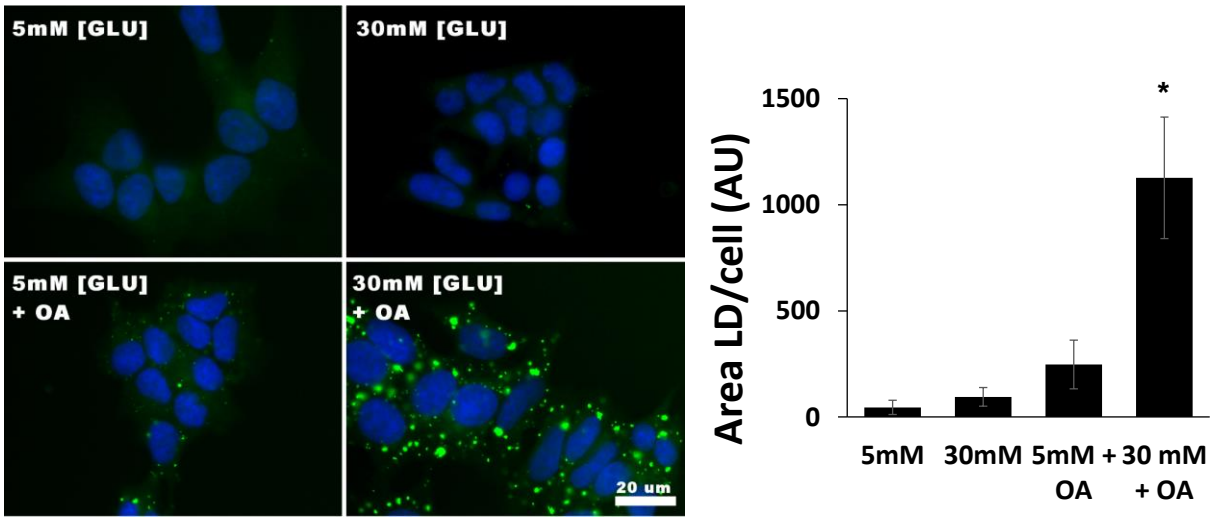

Figure 14) Hyperglycemia and hyperlipidemia synergistically upregulate LD in INS-1
INS-1 incubated in 5mM [GLU]/30mM [GLU] medium +/- 500uM oleic acid (OA) for 24h and stained for LD with BODIPY 493/503 (green) and Hoechst 33342 (blue) and imaged using fluorescence microscopy. The left panel shows representative images while the right panel shows the quantification for each condition. LD was quantified as the total area of LD per cell, experiments represent the mean from 3 independent experiments, error bars represent SEM, * $p < 0.05$.

In addition, we assessed the expression of LD associated genes under hyperglycemic and hyperlipidemic conditions. Hyperglycemia lead to significant upregulation of PLIN2 compared to normoglycemia (Figure 15). While PLIN2 expression remains unchanged with fatty acid (FA) treatments under normoglycemic conditions, FA treatment under hyperglycemia tended to lower PLIN2 expression. PLIN3 was generally lower under hyperglycemia compared to normoglycemia with 30mM [GLU] PA significantly lower compared to 5mM [GLU] PA. FSP27 is significantly downregulated in hyperglycemia compared to normoglycemia.

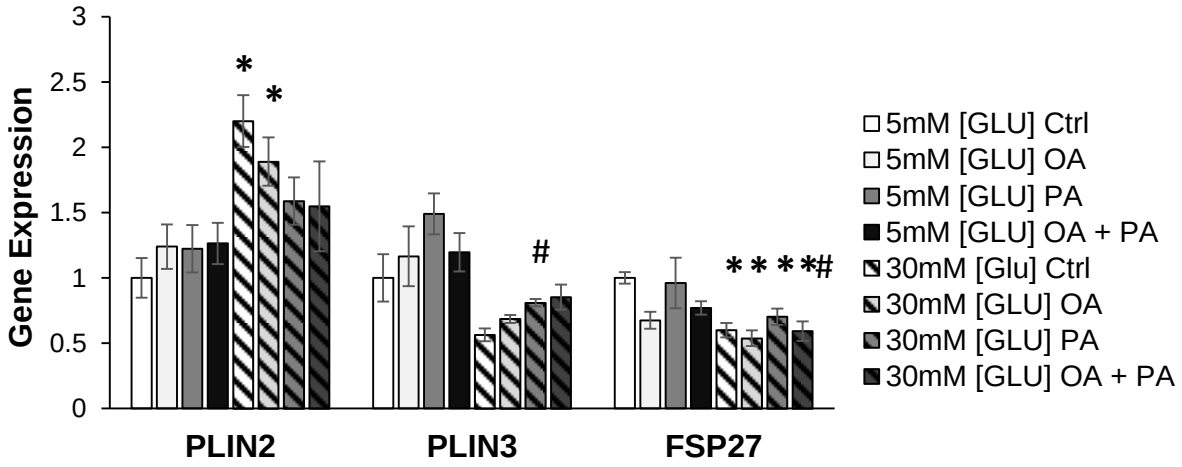

Figure 15) Gene expression analysis in INS-1 of LD-associated genes under normoglycemic (5mM [GLU]), hyperglycemic (30mM [GLU]), treated with 500uM oleic acid (OA), 500 uM palmitic acid (PA), or 250uM oleic acid (OA) + 250uM palmitic acid (PA). Shown are average fold increase ± standard error of the mean (SEM) in gene expression between groups from 3 independent experiments. Statistically significance were indicated by * (compared to 5mM [GLU] Ctrl) # (30mM [GLU] compared to the respective 5mM [GLU] group), * or # $p<0.05$.

Section 3.6 Hyperglycemia downregulates autophagy and inhibits TFEB nuclear translocation

Given that hyperglycemia and hyperlipidemia increases LD and PLIN2 in INS-1 cells, we hypothesized that nutrient overload would downregulate and inhibit TFEB activation. We assessed the gene expression of autophagy-associated genes TFEB, LAMP1, p62, LC3 under normoglycemia, hyperglycemia, and in-combination with fatty acids. Hyperglycemia treatment lead to a general reduction in the expression of TFEB, LAMP1, and LC3 although the differences did not reach statistical significance ($p<0.05$) (Figure 16). Autophagy appears to be downregulated at the transcriptional level (downregulation of TFEB), lysosomal level (downregulation of LAMP1), and autophagosome assembly (downregulation of p62 and LC3). Interestingly, fatty acid treatment tended to upregulate autophagy under normoglycemia; p62 was significantly upregulated with 5mM [GLU] OA + PA compared to control. However, hyperglycemia tended to blunt the upregulation of autophagy genes by fatty acids; p62 was significantly upregulated under normoglycemia treated with FFA compared to control but was not upregulated under hyperglycemic with FFA.

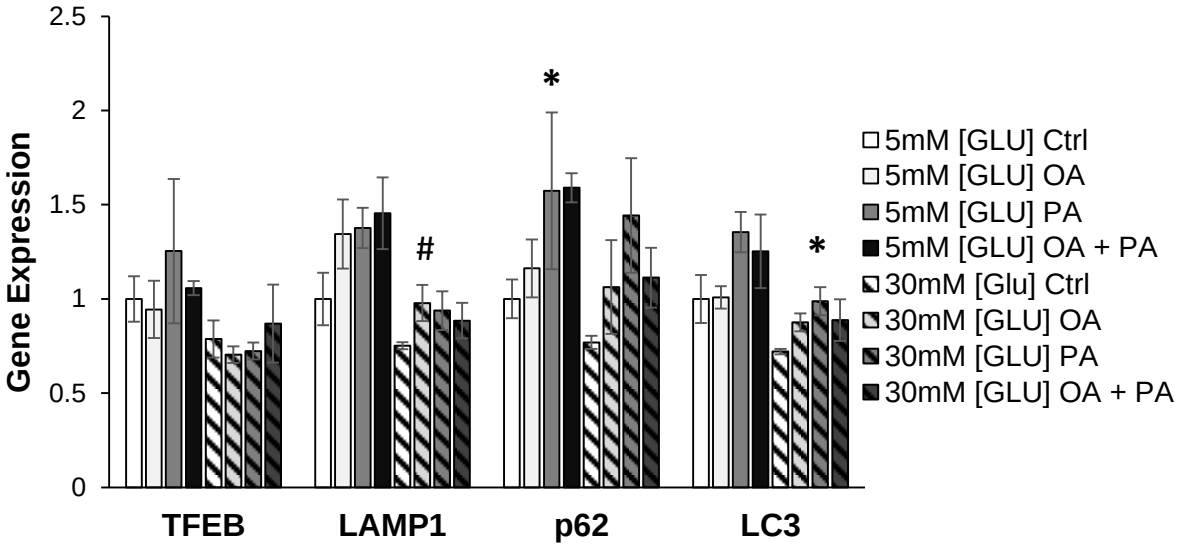

Figure 16) Gene expression analysis in INS-1 of autophagy-associated genes under normoglycemic (5mM [GLU]), hyperglycemic (30mM [GLU]), treated with 500uM oleic acid (OA), 500 uM palmitic acid (PA), or 250uM oleic acid (OA) + 250uM palmitic acid (PA). Shown are average fold increase ± standard error of the mean (SEM) in gene expression between groups from 3 independent experiments. Statistically significance were indicated by * (compared to 5mM [GLU] Ctrl) # (30mM [GLU] compared to the respective 5mM [GLU] group), * or # $p<0.05$.

One of the consequences of hyperglycemia and hyperlipidemia is hyperactivation of mTORC1. We hypothesized that hyperglycemia and hyperlipidemia would inhibit TFEB nuclear translocation due hyperactivity of mTORC1. INS-1 stably transfected with TFEB-EGFP were exposed to hyperglycemia and hyperlipidemia for 48h and we measured the starvation-induced TFEB nuclear translocation. There was a significant reduction in TFEB nuclear translocation in 30mM [GLU] OA+PA compared to 5mM control conditions (Figure 17), suggesting that the combination of glucose and lipid overload inhibits TFEB activation.

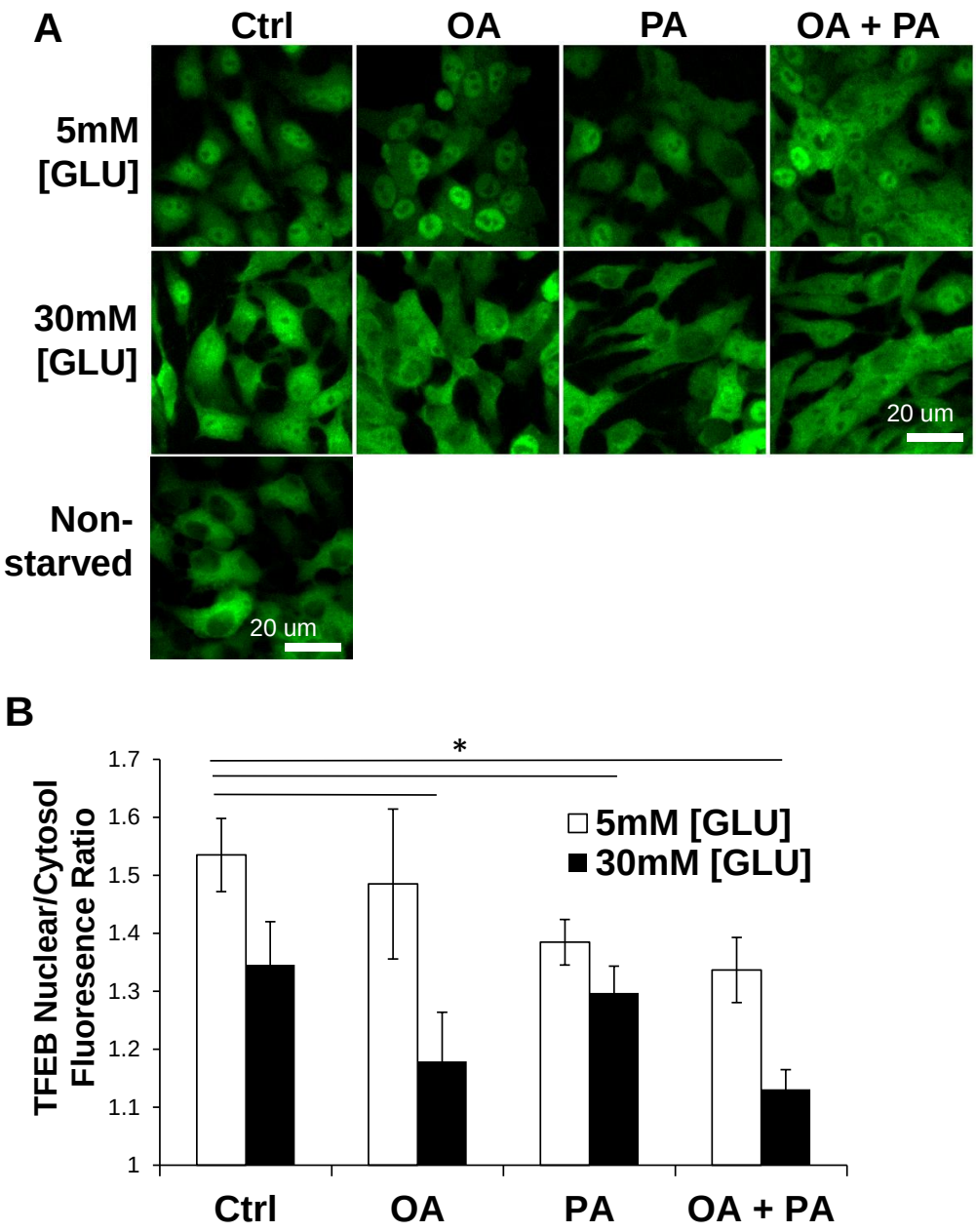

Figure 17) TFEB translocation assay in TFEB-EGFP stably transfected INS-1 cells. INS-1-TFEB-EGFP was cultured for 48h in normoglycemia (5mM [GLU]), hyperglycemia (30mM [GLU]), and treated with 500uM OA, 500uM PA, or 250uM OA + 250uM PA and starved for 1h in HBSS to induce TFEB nuclear translocation. (A) Representative images, (B) quantification of nuclear/cytosolic TFEB fluorescence ratio. n>50 cells from two-independent experiments, error bars represent the SEM, * $p<0.05$.

Section 3.7 Hyperglycemia and hyperlipidemia disrupt islet metabolism

We investigated what effects increased lipid accumulation and reduced autophagy would have on INS-1 metabolism and function. We performed qPCR on ins1/2, PPAR-α, PPAR-β, UCP2 on INS-1 cells exposed to 5/30mM [GLU] and OA, PA, OA + PA. Rats have two insulin coding genes termed Ins1 and ins2 which are under similar gene regulation. Hyperglycemia lead to a general downregulation of insulin expression (Figure 18); Ins1 and Ins2 was downregulated under 30mM [GLU] control compared to 5mM [GLU] control, although this did not reach statistical significance ($p<0.05$). Interestingly, FA treatment lead to significant increase in ins2 expression in normoglycemia + PA or OA + PA but was blunted under hyperglycemia + PA or OA + PA. PPAR-α, PPAR-δ, and UCP2 expression was not significantly changed in response to hyperglycemia or FA treatment.

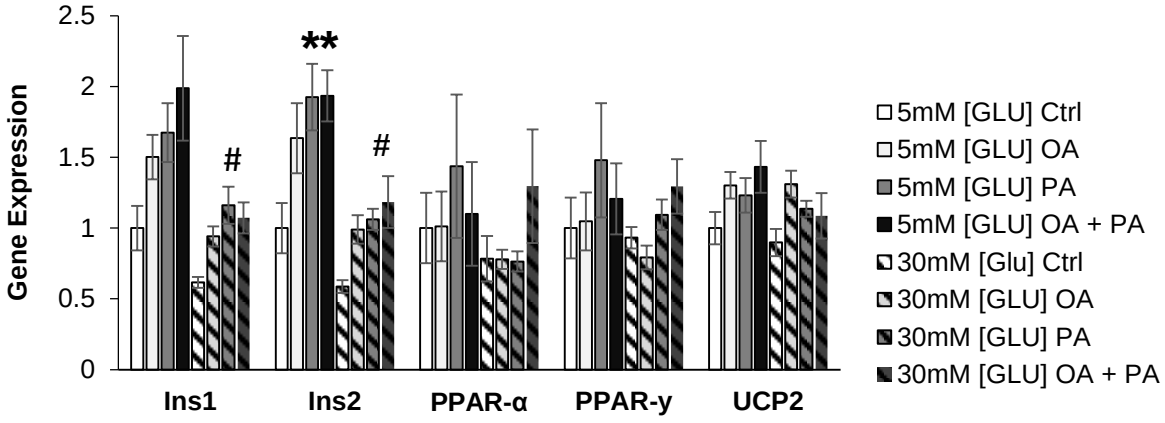

Figure 18) Gene expression analysis in INS-1 of metabolism-associated genes under normoglycemic (5mM [GLU]), hyperglycemic (30mM [GLU]), treated with 500uM oleic acid (OA), 500 uM palmitic acid (PA), or 250uM oleic acid (OA) + 250uM palmitic acid (PA). Shown are average fold increase ± standard error of the mean (SEM) in gene expression between groups from 3 independent experiments. Statistically significance were indicated by * (compared to 5mM [GLU] Ctrl) # (30mM [GLU] compared to the respective 5mM [GLU] group), * or # $p<0.05$.

Finally, we hypothesized that hyperglycemia and hyperlipidemia would reduce INS-1 viability due to glucolipotoxicity and that TFEB overexpression would protect against nutrient overload. We performed MTT assay to test the mitochondrial metabolic activity (MMA) of INS-1 (WT) and INS-1 overexpressing TFEB (INS-1-TFEB-EGFP) exposed to 5/30mM [GLU] with OA, PA, OA+PA. Contrary to our expectations, hyperglycemia increased MMA in both WT and INS-1-TFEB-EGFP cells (Figure 19).

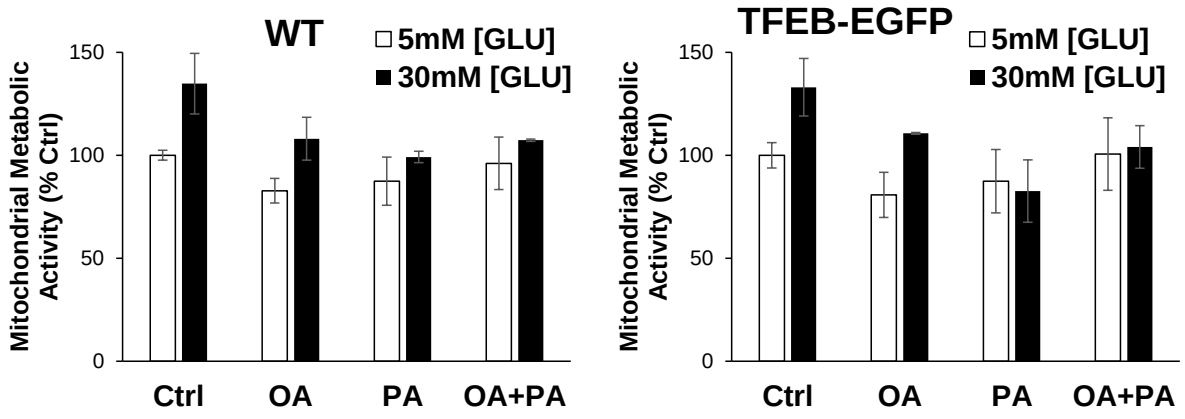

Figure 19) MTT assay for cell viability in INS-1 and INS-1-TFEB-EGFP exposed to 5mM [GLU], 30mM [GLU] in the presence and absence of 500uM OA, 500uM PA, or 250uM OA + 250uM PA for 48h. Error bars represent SEM, n=6 from two independent experiments. .

4.1 General discussion

Results from this study show: (1) an upregulation of LD and LD-associated protein PLIN2 in the pancreatic islets from T2DM patients. (2) PLIN2 co-localization with C-peptide labelled β-cells in pancreata from T2DN and T2DM. (3) PLIN2 increased expression in T2D is accompanied by an inhibition of autophagy, (4) TFEB, a master transcriptional activator of autophagy, is translocated in the subset of pancreas from both NDN and NDO patients but not in T2DN or T2DO patients and (5) Several genes implicated in lipid metabolism, beta cells survival and autophagy are dysregulated in T2D.

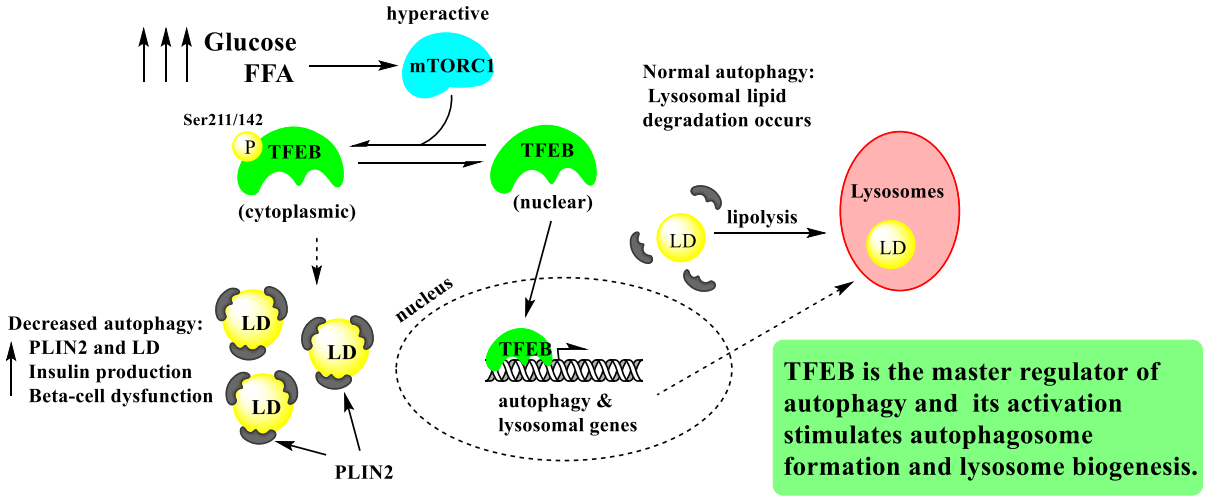

Figure 20) Schematic for dysregulated TFEB and lipid droplet accumulation during hyperglycemia and hyperlipidemia

Based on these findings we propose that in T2D, elevated glucose and FFA overload leads to: (i) increased LD accumulation and PLIN2 expression due to increased intracellular fatty acids, (ii) suppressed lipophagy due to greater inhibition of TFEB activity by mTORC1. Consequently, the lack of lipid degradation and decreased maintenance of cellular components through autophagy in T2D patients could increases mitochondrial and ER stress which ultimately contributes to β-cell apoptosis (Figure 20).

Hyperglycemia increases the expression of PLIN2 and decreases the expression of TFEB, LC3, LAMP1, and p62. These reductions in gene expressions and their cellular distribution are associated with the increased lipid accumulation and decreased autophagy contributing to the β-cell loss of function and eventually death.

4.2 Lipid regulation in T2D

β-cells have two solutions in response to elevated intracellular fatty acid: (i) increase lipid metabolism by β-oxidation, or (ii) temporarily store fatty acid as triglycerides inside LD. Fatty acids are an important energy source for β-cells. Under physiological conditions, fatty acids are used over glucose in β-cells (Malaisse, Best, Kawazu, Malaisse-Lagae, & Sener, 1983). However, under pathological conditions such as hyperglycemia in T2D, the ATP/ADP ratio is significantly increased due to elevated glucose and further increases in ATP production could jeopardize mitochondrial function. To reduce the high ATP/ADP generated, mitochondria can adapt by increasing proton leakage through uncoupling proteins to reduce ATP generation. We saw an increase in UCP2 expression in the pancreas of both T2DN and T2DO patients (Figure 13). Elevated CPT1A expression in T2DN and T2DO (Figure 13) suggest an increase in fatty acid transport across the mitochondria and thus increased rate of β-oxidation. However, given that high glucose levels increases the generation of malonyl-CoA, the major inhibitor of CPT1A (Prentki & Corkey, 1996), it is unlikely that increased β-oxidation alone is sufficient to metabolize the excess FFA.

Our results suggest that T2D is associated with increased PLIN2 expression which indirectly supports an accumulation of LD. PLIN2 is a constitutively expressed LD-associated protein whose expression is tightly linked to increases in LD (Imamura et al., 2002). PLIN2 is rapidly degraded when not on the surface of LD (Greenberg et al., 2011). Our immunohistochemistry analysis revealed PLIN2 forming prominent circular structures co-localized with C-peptide labelled β-cells (Figure 11A). Interestingly, T2D but not obesity seems to be the principle factor in the upregulation of PLIN2. Hyperglycemia may be required for PLIN2 upregulation in conjunction with free fatty

acids. In INS-1 cells, oleic acid only induced substantial lipid droplet formation in conjunction with hyperglycemia (Figure 14). Similarly, hyperglycemia significantly upregulated PLIN2 mRNA expression in INS-1 (Figure 15). PLIN2 participates in both the formation, reorganization and the degradation of lipid droplets. PLIN2 knockout in mouse models decreased high fat diet-induced hepatic LD accumulation (Najt et al., 2016). In the same study, PLIN2 ablation exerted a protective effect by decreasing hepatic inflammation and liver steatosis compared to wild-type mice. Although most studies are in agreement that PLIN2 stabilizes LD, whether PLIN2 plays a protective or destructive during high-fat load is unclear. We propose that PLIN2 can be protective and destructive depending on the cell type, LD size, and state of the mitochondria. For example, PLIN2 knockdown in AML12 hepatocytes reduces insulin sensitivity (Bell et al., 2008) while PLIN2 overexpression in muscle improved insulin sensitivity (Bosma et al., 2012). In INS-1 cells, PA or OA + PA together reduced PLIN2 expression under hyperglycemic conditions (see Figure 15) compared to hyperglycemia control or with oleic acid. This suggests that although oleic acid can be stored into LD, palmitic acid is poorly incorporated into or could further destabilize LD. Sequestering fatty acids as triglycerides inside of LD could temporarily reduce the lipotoxicity. In isolated rat β-cells, triglyceride accumulation was inversely proportional to the cytotoxicity for a given fatty acid, e.g. palmitic acid lead to the lowest triglyceride accumulation and produced the greatest toxicity (Cnop et al., 2001). Similarly, PLIN2 overexpression in INS-1 cells protected against palmitic acid induced toxicity by increasing triglyceride storage (Borg et al., 2009). On the other hand, PLIN2 knockdown in β-cells decreased LD formation for all fatty acid treatments but did not further increase cytotoxicity (Plotz et al., 2016). Collectively, PLIN2 is likely upregulated in β-cells in-response to lipid challenge under hyperglycemic conditions and is a controller of lipid storage and lipolysis. Like PLIN2, PLIN3 is also a ubiquitously expressed LD-associated protein and acts as a stabilizer (Miura et al., 2002). Unlike PLIN2, PLIN3 protein is stable in the cytosol and total protein levels are not controlled by LD formation (Bulankina et al., 2009). Downregulation of PLIN3 expression under hyperglycemia (Figure 15) could lead to decreased LD stability and the generation of large, leaky LD. Both PLIN2 and PLIN3 regulate chaperone-

mediated autophagic lipolysis (Kaushik & Cuervo, 2015), the mechanisms of which will be discussed in section 4.3.

Aside from promoting LD formation, fatty acids are known inducers of cytokine production in adipocytes (U. J. Jung & Choi, 2014). In human β-cells, palmitate treatment increased the production of IL-1β, TNF-α, IL-6, and IL-8 (Igoillo-Esteve et al., 2010). Fatty acid induced cytokines can attract infiltrating adipocytes and macrophages. We found a significant upregulation of FSP27 expression, an adipocyte-specific LD-associated protein, in NDO (Figure 11). Interestingly, FSP27 upregulation correlated with obesity not with T2D and FSP27 was lower in T2DO compared to NDO. A previous study has reported the greatest islet adipocyte infiltration in obese mouse-models and in T2D human pancreas is associated with increased triglyceride accumulation (Pinnick et al., 2008). Our results suggest that while obesity and T2D both increase islet LD content, LD in islets from obese patients are largely in FSP27 expressing adipocytes, whereas PLIN2-associated LDs are found in β-cells. The cause and cell type-dependent upregulation of FSP27 remains to be determined. We found that FSP27 expressed in INS-1 cells was significantly downregulated by hyperglycemia even in the presence of fatty acids (Figure 15). There are no reports on the expression of FSP27 in β-cells, so far. However, a study in drosophila has shown opposing roles for PLIN1 and PLIN2 in LD mobilization (Bi et al., 2012). Given the similar expression profiles between PLIN1 and FSP27 (Moreno-Navarrete et al., 2014), it could be argued that FSP27 and PLIN2 are oppositely regulated in response to hyperglycemia. FSP27 is a transcriptional target of PPARG (Matsusue et al., 2008); decreased PPARG expression in T2DO (Figure 11) could explain the decrease in FSP27 in T2DO. Since PLIN2 is a transcription target of PPARA (Janssen et al., 2015), increased PPARA expression in T2DN and T2DO (Figure #3) could partly explain the increase in PLIN2 in T2D. Following the pattern of FSP27 expression, immunolabelling for CD68 revealed the greatest macrophage infiltration in NDO pancreata (Figure 10). Given the crosstalk between macrophages and adipocytes (Lau, Ye, Xu, & Lam, 2007), similar chemokines likely lead to the islet infiltration of both adipocytes and macrophages in human islets.

4.3 Inhibition of autophagy in T2D

Autophagy is emerging as a prominent pathway in the cellular degradation of lipid stores (C. W. Wang, 2016). Having established the crucial role of autophagy in maintaining islet health, we asked whether autophagy is suppressed in pancreatic islets in obese, T2D, and obese T2D patients. Interestingly, TFEB was translocated to the nucleus in a greater proportion of NDN and NDO patients compared to T2DN and T2DO (Figure 12). As previously mentioned, TFEB is a master regulator of autophagic genes and lysosome biogenesis. Genes under TFEB transcriptional regulation include: LAMP1 (lysosome associated membrane protein 1), MCOLN1 (mucolipin 1), BCL2 (beclin 2), and SQSTM1 (p62), and Rab7A (Palmieri et al., 2011). Repression of TFEB activity in islets from T2D patients would lead to decreased autophagy and a reduction in lysosomal activity. A recent study in cardiomyocytes has found reduced TFEB expression in both a mouse model of type-1 diabetes (Akita mice) and in obese patients (Trivedi et al., 2016). Furthermore, glucolipotoxicity synergistically reduced TFEB expression in cultured cell which decreased autophagic flux, lysosomal number and cathepsin-B activity (Trivedi et al., 2016). Our study in β-cells recapitulates similar findings in that TFEB, LC3, p62 and LAMP1 was reduced by hyperglycemia (Figure 16). Puzzlingly, SQSTM1 mRNA expression was elevated in the pancreas from T2D patients (Figure 13) which we anticipated to be reduced given decreased TFEB activity. However, SQSTM1 expression is also regulated by Nrf2, an oxidative stress-response transcription factor (Jain et al., 2010). Given the increase in anti-oxidant defense genes in T2D (Figure 13), increased p62 expression may be driven by a cellular response to ROS.

TFEB nuclear translocation is controlled by mTORC1. When active, mTORC1 phosphorylates TFEB which sequesters TFEB in the cytoplasm. mTORC1 responds to changes in cellular energy levels and is positively regulated by increases in glucose, oxygen, and amino acids. Active mTORC1 upregulate anabolic processes and suppresses autophagy. Vernier et al have demonstrated hyperglycemia and hyperlipidemia treatment leads to increased mTORC1 activity. In support of this finding,

we found decreased rate of starvation-induced TFEB nuclear translocation in β-cells pre-treated with elevated glucose and FFA (Figure 17). Importantly, increased autophagy enhances LD breakdown in β-cells as rapamycin, a classical inhibitor of mTORC1, treatment greatly diminished LD accumulation (Vernier et al., 2012). In β-cell specific Tsc2 knockout animals, which have hyperactive mTORC1, led to inhibition of p62 (decreased autophagic flux), increased oxidized mitochondrial proteins, and reduced Akt phosphorylation in response to insulin (increased insulin resistance) (Bartolome et al., 2014). In hyperglycemia, in addition to the inhibition of TFEB and autophagic gene expression, we observed a decrease in insulin gene expression (Figure 18). Hyperactivation of mTORC1 is not limited to β-cells and may be an overarching metabolic dysregulation due to nutrient overload in T2D. mTORC1 suppresses insulin signaling by inhibitory phosphorylation of serine residues of insulin-receptor substrate 1 (IRS1) (Tremblay et al., 2005). Rapamycin inhibition of mTORC1 reversed insulin resistance in adipose tissue, skeletal muscle, and liver in hyperinsulinaemic rats (Ueno et al., 2005). Upregulation of autophagy by either inhibiting mTORC1 using inhibitors such as rapamycin or increasing TFEB activity seem attractive for the treatment of T2D in restoring insulin sensitivity or β-cell health. Although abnormally active mTOR leads to deleterious diabetic-like symptoms, normal mTOR function is still critical for β-cell proliferation and metabolic function. Long-term rapamycin treatment leads to worsened glucose intolerance, liver lipid accumulation, and β-cell loss in diabetic animals (Fraenkel et al., 2008; Schindler, Partap, Patchen, & Swoap, 2014; Y. Wang et al., 2015). Whether increased TFEB activation can rescue β-cell loss and improve insulin sensitivity has yet to be explored. However, TFEB overexpression in the liver increases lipid catabolism through autophagy. Moreover, in mouse fed a high-fat diet, TFEB overexpressing mice had lower blood glucose levels, improved glucose tolerance, and decreased fat gain compared to WT while TFEB-knockout mice increased fat gain (Settembre et al., 2013). Interestingly, TFEB activity can be increased through physical exercise which improves mitochondrial function and energy utilization in skeletal muscle (Mansueto et al., 2017). Future studies should explore whether increased TFEB activity could improve obese, and diabetes related β-

cell dysfunctions by facilitating organelle renewal and increasing the degradation of lipid depots.

LD may inhibit autophagy through the actions of PLIN2. A recent study reported PLIN2 upregulation in Akita mice (genetic model for T1D) due to increased ER stress (Chen et al., 2017). PLIN2 knockout in Akita mice increased autophagic flux as seen by increased LC3II/LC3I ratio and decreased p62 levels. PLIN2 knockout Akita mice have reduced hyperglycemia, improved insulin production, and decreased β-cell death (Chen et al., 2017). The mechanism by which PLIN2 inhibit autophagic flux has not been elucidated, but given that PLIN2 promotes the storage of FFA inside lipid depots, inhibition of lipophagy would be in-line with its directive. However, PLIN2 is also required for LD lipolysis through chaperone-mediated autophagy by recruiting ATGL and macroautophagy associated proteins to the surface of LD (Kaushik & Cuervo, 2015). Interestingly, chaperone-mediated autophagy was greatly enhanced by starvation. Consolidating the results from these studies suggest PLIN2 inhibits LD lipophagy under nutrient rich conditions to store fuel, but facilitates LD degradation when nutrient levels are low to restore energy balance. Nutrient oversaturation is a new phenomenon at the evolutionary timescale and in this context Neel proposed a thrifty gene hypothesis (Neel, 1999), to explain adaptation of β-cells in oscillating periods of low and high energy load. Periods of fasting would enable β-cells to upregulate autophagy, through TFEB nuclear translocation, and recycle worn-out organelles and accumulating lipids. A recent study on islet regeneration has shown that fasting in diabetic mice lowers mTOR activity, promotes β-cell neogenesis, and reverses diabetic symptoms (Cheng et al., 2017). Intermittent fasting proved beneficiary in other disease associated with impaired autophagy including Alzheimer's disease, excitotoxicity, and aging (Anson et al., 2003; Cheng et al., 2017; Halagappa et al., 2007). Taken together, previously reported and our current results suggest that pharmacological interventions and acquisition of healthy nutritional regiments should be tailored for patients with T2D. Combined therapeutic approaches targeting TFEB and mTORC1 with physical activity could be a rewarding direction in the intervention of T2D and other metabolic disorders. Metformin, the current standard therapy for T2D and a potent AMPK activator, also

inhibits the activity of mTORC1 (see Figure 7) and thus promotes autophagy (Feng et al., 2014; Xiao et al., 2017). The combination of metformin with selective mTOR inhibitor (torin-2) synergistically reduces cellular energy levels in pancreatic carcinomas (Soliman, Steenson, & Etekpo, 2016). Whether metformin in combination with mTORC1 inhibition or TFEB activation can reduce islet dysfunction in T2D remains an interesting but unexplored topic.

Section 5: Conclusions

The results from the experiments conducted in this thesis suggest (1) PLIN2, the structural protein on the surface of LD, are upregulated in both T2DN and T2DO patients, (2) TFEB, the master regulator of lysosomal biogenesis, expression and nuclear localization is decreased in T2DN, and T2DO patients, (3) mitochondrial dynamics in pancreatic islets was significantly altered by type-2 diabetes, as evidenced by regulatory changes in genes involved in mitochondrial transport (CPT1A, UCP2), anti-oxidant defense (GPX1, HMOX1), and apoptosis (BAX, BCL2). Our in-vitro experiments confirm hyperglycemia as the mediator for lipid droplet accumulation, and inhibition of TFEB activation, and autophagy. We propose that future studies should explore TFEB activation as an intervention strategy against islet dysfunction in diet-induced metabolic disorders.

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