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TGIF1 physiological levels limit β-cell distress and neonatal diabetes

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LIST OF ABBREVIATIONS:

Akt:	Protein Kinase B
Apaf-1:	Apoptotic Protease Activating Factor-1
ATF6:	Activating Transcription Factor 6
BID:	BH3 Interacting-Domain Death Agonist
CHOP:	C/EBP Homologous Protein
DALYs:	Disability-Adjusted Life Years
DISC:	Death-Inducing Signaling Complex
DM:	Diabetes Mellitus
DPP-4:	Dipeptidyl peptidase-4
EDEM:	ER Degradation-Enhancing Alpha-Mannosidase-Like Protein
ER:	Endoplasmic Reticulum
ERAD:	ER-Associated Degradation
FasL:	Fas Ligand
GDM:	Gestational Diabetes Mellitus
GLP-1:	Glucagon-like peptide-1
GWAS:	Genome-Wide Association Studies
HDACs:	Histone Deacetylases
HLA-DQ:	Human Leukocyte Antigen DQ
IDF:	International Diabetes Federation
INS:	Insulin Gene
IRE1:	Inositol-Requiring Enzyme 1
IRE1α:	Inositol-Requiring Enzyme 1 alpha
IRS-1:	Insulin Receptor Substrate-1
JNK:	c-Jun N-terminal Kinase
KCNJ11:	Potassium Voltage-Gated Channel Subfamily J Member 11
LADA:	Latent Autoimmune Diabetes in Adults
MAPK:	Mitogen-Activated Protein Kinase
NDM:	Neonatal Diabetes Mellitus
NF-κB:	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
Pdx1:	Pancreatic and duodenal homeobox 1
PERK:	PKR-like Endoplasmic Reticulum Kinase
PI3K:	Phosphoinositide 3-Kinase
PKR:	Protein Kinase R
PNDM:	Permanent Neonatal Diabetes Mellitus
PP-cells:	PP cells
ROS:	Reactive Oxygen Species
S1P:	Site-1 Protease
S2P:	Site-2 Protease

SGLT-2:	Sodium-glucose cotransporter-2
Smad2/3/4:	Mothers against decapentaplegic homolog 2/3/4
T1D:	Type 1 Diabetes
T2D:	Type 2 Diabetes
TβRI:	Type I serine/threonine kinase receptor
TβRII:	Type II transmembrane serine/threonine kinase receptor
TCF7L2:	Transcription Factor 7 Like 2
TGF-β:	Transforming Growth Factor-Beta
TGIF1:	TG-interacting factor 1
Th1/Th17:	T-helper 1/ T-helper 17 cells
TNF:	Tumor Necrosis Factor
TNFRSF1:	TNF Receptor Superfamily Member 1
tBID:	Truncated BID
TNDM:	Transient Neonatal Diabetes Mellitus
Tregs:	Regulatory T cells
TRAIL:	TNF-Related Apoptosis-Inducing Ligand
UPR:	Unfolded Protein Response
WHO:	World Health Organization
α-cells:	Alpha cells
β-cells:	Beta cells
δ-cells:	Delta cells
ε-cells:	Epsilon cells
IAPP:	Islet Amyloid Polypeptide (also known as amylin)

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ABSTRACT

TGIF1 belongs to the superfamily of homeodomain proteins, which regulate a wide variety of biological functions, including cell stemness and specification of cell fate during early development. Perhaps surprisingly, we found that enforced expression in pancreatic progenitor cells during embryogenesis resulted in severe diabetes, hinting at the possibility that TGIF1 might regulate pancreas development. Subsequent genetic experiments targeting β-cells showed that TGIF1 affected β-cell function and homeostasis. Transcriptomic analysis revealed that TGIF1 expression inhibits the expression of essential components of UPR signaling, underscoring a potential mechanism in which TGIF1 disrupts protein folding and secretion. Congruently, TGIF1 expression led to a dramatic disorganization of insulin within β-cells, accumulating as large aggregates, and was associated with decreased insulin secretion. Subsequent in vitro experiments showed that TGIF1 expression led to accumulation of insulin aggregates in the ER, thereby causing ER stress and concurrent impairment in insulin processing and secretion. In further support to these findings, conditional deletion of TGIF1 in pancreatic progenitor cells was also associated with hyperglycemia and diabetes, reinforcing the notion that TGIF1 physiological levels are instrumental to maintaining the balance between UPR and ER in β-cells. Finally, we serendipitously found that enforced expression of TGIF1 in β-cells recapitulated the cardinal hallmarks of

neonatal diabetes, shedding important insights into mechanistic paradigms of this enigmatic condition.

INTRODUCTION

1. Physiology and metabolic function of the pancreas

The pancreas is an elongated, lobulated organ situated in the retroperitoneal space, extending laterally from the duodenum to the spleen. It is comprised of a head, neck, body, and tail, with the head residing in the curvature of the duodenum and the tail extending towards the splenic hilum. The organ is enveloped by a thin connective tissue capsule, which invaginates to form septa, subdividing the gland into lobules (Ellis 2007). Histologically, the pancreas exhibits dual functional and structural roles, reflected in its specialized cells that differentiate to form either exocrine or endocrine compartments. The exocrine tissue consists predominantly of acinar cells, arranged in acini, which are spherical clusters that secrete digestive enzymes into an intricate network of duct that ultimately drain into the main pancreatic duct. The latter merges with the common bile duct, forming the ampulla of Vater that empties into the duodenum (Pandiri 2013). The endocrine portion is comprised of structures known as the Islets of Langerhans (also referred to as islets), which are disseminated throughout the exocrine tissue. Each islet is a micro-organ, containing multiple cell types that secrete specific hormones that are key regulators of metabolic homeostasis, including α-cells (secreting glucagon), β-cells (secreting insulin), δ-cells (secreting somatostatin), PP-cells (secreting pancreatic

polypeptide), and ε-cells (secreting ghrelin). Endocrine cells are richly vascularized, facilitating the direct and efficient secretion of hormones directly into the bloodstream under normal physiological conditions (Susan 2004, Pandiri 2013). Assisting in the execution of the both endocrine and exocrine pancreatic functions, pancreatic ductal cells are epithelial cells that comprise the complex ductal system ranging from intercalated ducts to intralobular and interlobular ducts, all of which culminate in the main pancreatic duct. Anatomically, these cells are primarily columnar or cuboidal and feature tight junctions, adherens junctions, and desmosomes, which all work to form tight, concentric cell connections that take the form of canonical duct structures. Functionally, ductal cells play a pivotal role in modulating the biochemical composition of pancreatic exocrine fluids. After initially secreted by acinar cells, the enzyme-rich exocrine fluid is altered as it transits through the ductal system. During the transportation of these fluids, ductal cells are responsible for secreting bicarbonate ions that assist in neutralizing the acidity of the stomach chime, a necessary step required for proper digestion (Pallagi, Hegyi et al. 2015). Any aberrations in these well-coordinated pancreatic functions or their structures due to injury, disease, or inflammation can significantly disrupt pancreatic homeostasis, leading to the onset of debilitating diseases including pancreatitis, pancreatic cancer, and diabetes (Karpińska and Czauderna 2022).

1.1 Diabetes Mellitus

1.1.1 Epidemiology

Globally, as of 2021, statistics indicated that approximately 537 million individuals (20-79 years old) were living with diabetes. Projections suggest that this number will climb to

over 643 million by the year 2030 and further expand to 783 million individuals by 2045, according International Diabetes Federation (IDF) (IDF, 2021). The prevalence is predominantly found in more modernized, first-world regions such as the Middle East, North America, and Western Pacific, where rapid urbanization, lifestyle, and dietary changes exacerbate the condition.

1.1.2 Mortality rates and disease burden

According to the World Health Organization (WHO), in 2019 diabetes was the seventh leading cause of death globally, responsible for an estimated 1.5 million deaths (WHO, 2019). This number is further compounded when taking into consideration the additional 2 million deaths associated with diabetes-related complications that result in various cardiovascular and kidney diseases. The quantifiable impact of diabetes extends beyond mere prevalence and mortality figures, as the overall disease burden, measured through Disability-Adjusted Life Years (DALYs), as well as other diabetes healthcare related cost statistics, revealed that diabetes-related healthcare expenditures were estimated to be nearly $966 billion in 2021, highlighting the significant financial and labor burden imposed by diabetes (IDF, 2021).

1.1.3 Subtypes of Diabetes Mellitus and their treatments

Diabetes Mellitus is a heterogeneous metabolic disorder classified by multiple subtypes, each characterized by a distinct etiological mechanism and clinical presentation. Highlighted below are the most common and prevalent forms of diabetes and their etiologies.

1.1.3.1 Type 1 Diabetes (T1D)

Type 1 Diabetes is predominantly an autoimmune condition characterized by the T-cell-mediated destruction of insulin-producing pancreatic β-cells. This autoimmune-driven loss of β-cells results in the direct and substantial loss of endogenous insulin expression, which disturbs glycemic regulatory pathways, eventually culminating in global dysglycemia (Atkinson, Eisenbarth et al. 2014). T1D most often manifests during childhood or adolescence, but it can also appear later in life, as a condition known as Latent Autoimmune Diabetes in Adults (LADA). Studies have revealed that this form of diabetes likely involves a complex combination of genetic susceptibility and environmental triggers such as diet, physical activity, and even severe infections that result in hyperactive immune responses (Knip, Veijola et al. 2005). Patients diagnosed with T1D or LADA often rely on life-long exogenous insulin administration in addition to continuous glucose monitoring. Immunotherapies and closed-loop insulin delivery systems are emerging therapies aimed at either mitigating the onset of T1D or more actively monitoring and influencing glycemic management, respectively.

1.1.3.2 Type 2 Diabetes (T2D)

Type 2 Diabetes Mellitus is the most common form of diabetes globally and primarily arises as a result of insulin resistance in peripheral tissues, i.e. skeletal muscle, liver, and adipose cells, but can also be accompanied and exacerbated by pancreatic β-cell dysfunction (Kahn, Cooper et al. 2014). Similar to T1D, genetic predisposition, along with environmental factors like diet and sedentary lifestyle, significantly contribute to its

pathogenesis. Peripheral tissue insulin resistance arises when pancreatic β-cells attempt to modulate hyperglycemic states that either frequently arise or chronically persist (often caused by high-fat, high-glucose diets), and overtime leads to the constitutive overcompensation of β-cells to continuously synthesize and secrete insulin (Kahn, Hull et al. 2006). Overtime, hyperinsulinemia denigrates the ability of cells in peripheral tissues to properly sense and detect insulin, leading to systemic insulin intolerance, and as a result, chronic dysglycemia. Concurrently, persistent hyperinsulinemic conditions can inflict irreversible damage on pancreatic β-cells, compromising their ability to secrete insulin, thereby amplifying dysglycemia further (Kahn 2003). The clinical presentation of T2D is highly variable, ranging from asymptomatic hyperglycemia to weight loss; though, many individuals with T2D or pre-diabetes remain asymptomatic for extended periods and may only be diagnosed during routine screenings or after the onset of complications like retinopathy, nephropathy, or cardiovascular events. Pharmacological strategies for T2D have expanded beyond metformin, the standard treatment for several decades, to include SGLT-2 inhibitors, GLP-1 receptor agonists, and DPP-4 inhibitors, each targeting different aspects of the gluco/insulin-regulatory pathways that govern metabolic homeostasis. SGLT-2 inhibitors facilitate glucose excretion via the urine by blocking its reabsorption in kidneys, lowering blood glucose levels. GLP-1 receptor agonists enhance insulin release, suppress glucagon secretion, slow gastric emptying, and promote satiety to control blood sugar. DPP-4 inhibitors prolong incretin hormone activity, which increases insulin secretion and decreases glucagon release in a glucose-dependent manner, aiding in glycemic regulation. (Zinman, Wanner et al. 2015, Drucker 2018).

1.1.3.3 Neonatal Diabetes Mellitus (NDM)

Neonatal Diabetes Mellitus (NDM) is a rare (1 in 90,000-160,000 births) monogenic form of diabetes that develops and clinically manifests within the first six months of postnatal life and is characterized by severe hyperglycemia and insulin deficiency. Two primary subtypes of neonatal diabetes exist: Transient Neonatal Diabetes Mellitus (TNDM) and Permanent Neonatal Diabetes Mellitus (PNDM), both distinguished by slightly different genetic etiologies, clinical manifestations, and therapeutic interventions. Additionally, TNDM often resolves within weeks to months but may recur later in life, whereas PNDM requires lifelong treatment (Temple, Gardner et al. 2000, Støy, Edghill et al. 2007). Clinically, NDM often presents with non-specific symptoms like failure to thrive, dehydration, and frequent urination. As such, diagnosis largely relies on a blood-glucose testing, often revealing severe dysglycemia, and is followed by genetic testing to identify if infants possess specific genetic mutations in the KCNNJ11, ABCC8, and several other genes implicated in NDM progression (Greeley, Naylor et al. 2011) Due to its early onset and the vulnerability of newborns, the risk of complications, such as developmental delay, malnutrition, and epilepsy, warrant early and accurate diagnosis followed by appropriate therapeutic intervention. Management strategies for NDM depend heavily on the underlying genetic etiology. For example, patients with KCNJ11 or ABCC8 mutations, often respond exceptionally well to sulfonylurea therapy, which can sometimes replace insulin therapy altogether (Pearson, Flechtner et al. 2006). While advances in molecular diagnostics and targeted therapies have substantially improved patient outcomes, the low incidence of NDM imposes constraints on the accumulation of robust clinical data. Given

its manifestation at a critically formative stage of human development, the necessity for focused study on NDM should be a priority among researchers and clinicians alike.

1.1.3.4 Gestational Diabetes Mellitus (GDM)

Gestational Diabetes Mellitus (GDM) is a dysglycemic condition that manifests during pregnancy and occasionally resolves postpartum, affecting approximately 2–10% of all mothers worldwide (Guariguata, Linnenkamp et al. 2014). Unlike other forms of diabetes, GDM is not the result of an absolute insulin deficiency but is characterized by insulin resistance and inadequate compensatory insulin secretion during pregnancy. If GDM presents during pregnancy, the clinical implications are twofold, as they affect both the mother and the unborn child. For the mother, there is an increased risk of developing T2D later in life, and for the fetus, there is an elevated risk of macrosomia, neonatal dysglycemia, and longer-term metabolic disturbances (Bellamy, Casas et al. 2009). Similar to other forms of diabetes, the etiology of GDM is multifactorial, involving both genetic and environmental factors, such as obesity and physical inactivity. Management usually includes lifestyle modification, glucose monitoring, and, in some cases, insulin therapy to maintain euglycemia (McIntyre, Catalano et al. 2019).

1.1.4 Pathophysiology

1.1.4.1 Insulin

In the overwhelming majority of cases, Diabetes is a metabolic disorder primarily characterized by the dysregulation of the peptide hormone insulin. In its mature form, Insulin is a small protein comprised of 51 amino acids, structured into two polypeptide

chains, A and B, linked by two disulfide bonds. These two chains originate from a single-chain precursor molecule, preproinsulin, which is synthesized in the ribosomes of pancreatic β-cells (Weiss 2009). Post-translational processing of preproinsulin begins in the endoplasmic reticulum (ER) after the nascent preproinsulin molecule, which contains an ER-localization signal sequence, enters into the lumen of the ER via translocon complexes. Immediately following its translocation, ER-resident peptidases excise the translocation signal peptides of preproinsulin from its N-terminus. As this cleavage event occurs, the preproinsulin polypeptide simultaneously undergoes crucial folding steps, assisted by ER-chaperones, and eventually forms three disulfide bonds—two between the A and B chains and one within the A chain itself (Liu, Weiss et al. 2018). Upon removal of the translocation signal peptides and proper folding, preproinsulin is converted to proinsulin and advances through the secretory pathway. Proinsulin is then trafficked from the ER and transported to the Golgi apparatus where it is sorted and packaged into secretory granules. These granules contain crucial enzymes that finally enzymatically cleave the C-peptide portion of the proinsulin polypeptide chain, yielding mature, biologically active insulin alongside equimolar amounts of C-peptide (Liu, Weiss et al. 2018). This maturation process is tightly regulated, with checkpoints at multiple steps to ensure that only properly folded and assembled precursor insulin molecules progress to the next stage. Secretion, too, is a meticulously orchestrated event. Insulin is released into the portal vein in a pulsatile manner, chiefly in response to elevated blood glucose levels. Other stimulants like amino acids, fatty acids, and hormones can also modulate insulin secretion, underscoring the relevance of dietary composition and feeding habits to insulin secretion (Newsholme, Bender et al. 2007).

Upon secretion into the bloodstream, insulin's primary molecular target is the insulin receptor, a transmembrane protein with intrinsic tyrosine kinase activity. Structurally, the receptor is organized as a heterotetrameric complex comprising two extracellular α subunits and two transmembrane-spanning β subunits, which are connected via disulfide bonds. Insulin binding occurs at the α subunits, which induces a conformational change that allows for autophosphorylation of specific tyrosine residues in the β subunits' intracellular domains (Hubbard, Wei et al. 1994). This autophosphorylation serves as the activation trigger for the receptor's tyrosine kinase activity, facilitating subsequent phosphorylation of downstream signaling pathways. One of the principal downstream pathways activated is the phosphoinositide 3-kinase (PI3K)/Akt pathway. Upon insulin receptor activation, insulin receptor substrates (IRS) like IRS-1 and IRS-2 are phosphorylated, providing a docking site for the p85 regulatory subunit of PI3K. This leads to activation of the p110 catalytic subunit of PI3K, resulting in the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 serves as a secondary messenger that finally activates Akt. Akt activation culminates in a series of cellular responses including increased glucose uptake via GLUT4 transporters, enhanced glycogen synthesis, and inhibition of apoptosis among other functions (Liu, Cheng et al. 2009). There are many other pathways and systems that are critically involved in the synthesis, processing, secretion, detection, and regulation of insulin, illustrating the complexity and integration of insulin regulation as a key driver of metabolic homeostasis.

1.1.4.2 Perturbation of insulin regulation

The dysregulation of insulin homeostasis, a central event often associated with the various subtypes of diabetes mellitus, is often the result of a confluence of interrelated cellular and molecular events. These factors, which can be both intrinsic and extrinsic to the β-cells involved in insulin synthesis, secretion, and action, can instigate the initial perturbations of early insulin dysregulation, and later on, serve to exacerbate existing imbalances (Kahn, Cooper et al. 2014). As such, they constitute key elements in the multifactorial etiology of diabetes and offer potential targets for therapeutic intervention.

1.1.4.2.1 Oxidative stress and ER stress

Reactive oxygen species (ROS) are inherent byproducts of cellular metabolic activities such as mitochondrial oxidative phosphorylation. Under physiological conditions, their generation is counterbalanced by cellular antioxidant defense mechanisms, ensuring a tightly controlled redox homeostasis (Holmström and Finkel 2014). However, when the production of ROS overwhelms the cellular capacity for detoxification, a pathological state known as oxidative stress ensues. The role of oxidative stress in instigating diabetic conditions has been well studied, and many investigations have shown that it is a pivotal factor that directly impinges upon several pathways associated with normal insulin synthesis and processing, which ultimately results in the aberration of cellular glucose homeostasis and subsequent diabetic phenotypes (Giacco and Brownlee 2010). Oxidative stress has a particularly profound impact on the endoplasmic reticulum (ER), the organelle central to the synthesis, folding, modification, and transport of proteins within the cell. In the context of pancreatic β-cells, the ER plays a crucial role in the synthesis and folding of insulin. Excessive ROS can disrupt the ER redox balance,

leading to the accumulation of misfolded proteins within the ER lumen, a condition termed ER stress (Malhotra and Kaufman 2007). ER stress states activate the unfolded protein response (UPR), a cellular program that functions to restore ER homeostasis. The UPR is mediated through three primary signaling branches led by the ER resident proteins: inositol-requiring enzyme 1 (IRE1), protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) (Hetz 2012). These regulators alleviate ER stress by increasing the production of chaperone proteins, slowing down protein translation, and enhancing the degradation of misfolded proteins. However, if ER stress persists and the UPR fails to restore ER homeostasis, protein synthesis is severely diminished and eventually apoptotic pathways are triggered, leading to β-cell death and consequently, diminished insulin production.

Notably, under homeostatic conditions, ATF6 resides in the ER membrane, with its transcriptional activation domain held latent through interaction with the ER chaperone BiP (Shen, Chen et al. 2002). Upon the accumulation of misfolded proteins and resultant ER stress, BiP dissociates from ATF6, enabling the latter's translocation into the Golgi apparatus. In the Golgi, ATF6 undergoes regulated intramembrane proteolysis, as it is sequentially cleaved by Site-1 and Site-2 proteases (S1P and S2P) (Walter and Ron 2011). This cleavage event releases the N-terminal cytosolic domain of ATF6, which is a potent transcription factor. Once freed, this polypeptide fragment translocates to the nucleus where it activates the transcription of ER stress-response genes, which encode for molecular chaperones like BiP and ER degradation-enhancing alpha-mannosidase-like protein (EDEM), enzymes involved in protein folding and components of the ER-associated degradation (ERAD) pathway. If ATF6 is unable to adequately stymie the

accumulation of ER stress and ER stress instigators, in a last ditch effort to mitigate redox damage to nearby cells, ATF6 will upregulate C/EBP homologous protein (CHOP), a pro-apoptotic transcription factor. Induction of CHOP serves as a regulatory mechanism to eliminate cells that are unable to restore ER homeostasis, thereby preventing the propagation of cellular dysfunction (Yamamoto, Sato et al. 2007).

In the context of insulin regulation, ATF6-mediated UPR can have both adaptive and maladaptive consequences. On one hand, the upregulation of ER chaperones and folding enzymes facilitates the efficient folding and post-translational modification of proinsulin, promoting its maturation into biologically active and mature insulin. On the other hand, in cases where ER stress is rampant, chronic activation of ATF6 can lead to sustained upregulation of CHOP, ultimately triggering apoptotic pathways that contribute to pancreatic β-cell death. This loss of β-cell mass reduces the capacity of islets to synthesize and secrete insulin, contributing to hyperglycemia and the exacerbation of diabetes.

1.1.4.2.2 Inflammation

Inflammation is an essential component of the immune system's response capacity, yet when dysregulated or persistent, can contribute significantly to the pathogenesis of many diseases, including diabetes (Medzhitov 2008). Central to the process of inflammation are cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), which can be produced by activated macrophages and even adipocytes, especially in the context of obesity, a known risk factor for T2D (Hotamisligil 2006). At the molecular level, cytokines interact with their specific receptors to activate a

cascade of signaling events that modulate cell behaviors and responses, with a notable activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, acting as one of the more prolific regulators of inflammatory responses. Upon activation, NF-κB translocates to the nucleus and promotes the transcription of pro-inflammatory genes, further amplifying the inflammatory response (Lawrence 2009). Concurrently, these cytokines can instigate signaling pathways that impair insulin action, such as the c-Jun N-terminal kinase (JNK) pathway. Activation of JNK results in the phosphorylation of serine residues on insulin receptor substrate-1 (IRS-1), thereby inhibiting its function and attenuating the downstream phosphatidylinositol 3-kinase (PI3K)/Akt signaling cascade, which is crucial for insulin-mediated glucose uptake (Hirosumi, Tuncman et al. 2002).

Further compounding issues with insulin regulation, studies have revealed that inflammation can also induce ER dysfunction by instigating ER stress and by dampening ER stress response pathways. The interplay between inflammation and ER stress establishes a complex, self-perpetuating feedback loop that significantly undermines insulin regulatory pathways. For example, pro-inflammatory cytokines like TNF-α and IL-6 can instigate ER stress by disrupting ER and cell calcium homeostasis. The imbalance and disruption of calcium concentration within these compartments eventually leads to the activation of UPR pathway mediators IRE1, ATF6, etc. As discussed previously, these augment the expression of ER-resident chaperones and proteins involved in ERAD, further contributing to the deterioration insulin expression, and in severe cases, driving β cell apoptosis, debilitating the ability of islets to maintain metabolic homeostasis.

1.1.4.2.3 β cell apoptosis

β cell apoptosis represents yet another critical pathological event that contributes to the deterioration of insulin homeostasis and the subsequent manifestation of diabetes. β cells can be driven towards apoptosis by various factors such as oxidative stress, ER stress, and inflammation, among others, which we have previously discussed in depth. One major pathway implicated in β cell apoptosis is the intrinsic or mitochondrial apoptosis pathway, which involves the Bcl-2 family of proteins (Lightfoot, Chen et al. 2011). Under conditions of metabolic stress or elevated levels of pro-inflammatory cytokines, pro-apoptotic modulatory proteins like Bax and Bak are activated (White, Zhang et al. 2020). Their activation results in mitochondrial outer membrane permeabilization, allowing for the release of cytochrome c into the cytosol compartment. Once in the cytosol, cytochrome c forms a complex with Apaf-1 and caspase-9, working in concert to form the apoptosome (Yuan and Akey 2013). This complex further activates effector caspases like caspase-3 and caspase-7, culminating in cellular disassembly of major cellular compartments and ultimately apoptosis. Another noteworthy mechanism involves the activation of the death receptor pathway or extrinsic apoptosis pathway. This involves the binding of death ligands such as TNF-α, Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL) to their respective death receptors, TNFRSF1, Fas and DR4/DR5 (Chervonsky, Wang et al. 1997). The binding of these ligands triggers the formation of the death-inducing signaling complex (DISC), leading to the activation of the initiator caspase-8. Once activated, caspase-8 can either directly activate effector caspases like caspase-3 or cleave the BID protein to tBID, which then translocates to the mitochondria and triggers intrinsic apoptosis pathways (Maedler, Schumann et al. 2006). Additionally,

ER stress can also act as a strong inducer of beta-cell apoptosis. As elaborated previously, prolonged activation of ATF6 during ER stress augments the expression of genes that encode ER-resident chaperones and ERAD proteins. However, if ER stress remains unresolved, this pathway can also upregulate CHOP, a transcription factor that promotes apoptosis by repressing the expression of anti-apoptotic Bcl-2 while upregulating the expression of pro-apoptotic Bim (Song, Scheuner et al. 2008).

In summation, β cells apoptosis is a complex process influenced by various intracellular and extracellular factors, that could be considered a last ditch effort by β cells to mitigate ER stress and overcome cellular dysfunction. However, the collective impact of these apoptotic events on β cell poses significant challenges for the pancreas and its islets, as loss of β cell populations eventually reduce the efficiency of overall insulin production and thus global metabolic regulation.

1.2 Genetic alterations of diabetes and their animal models

Over the last 3 decades, it has become increasingly evident to researchers and clinicians that diabetes' etiology is enormously heterogeneous, requiring significant contributions from not only environmental factors, but also genetic mutations that affect the governance of β cell survival/maintenance and insulin regulatory pathways (Chung, Erion et al. 2020). Numerous studies have been conducted to elucidate the genetic architecture of diabetes, and through these advancements, several key genes have been identified, contributing directly or indirectly to diabetes susceptibility. Consensus within the diabetes research community indicates that mutation in INS (Insulin), KCNJ11 (Potassium Voltage-Gated Channel Subfamily J Member 11), TCF7L2 (Transcription Factor 7 Like 2), SLC30A8

(Solute Carrier Family 30 Member 8), and specific alleles of the HLA-DQ gene mutations are the most significant and common mutations found in patients with diabetes (Sladek, Rocheleau et al. 2007, Lemelman, Letourneau et al. 2018). It should be noted that these mutations are not all always found in every case of diabetes, and that each subtype may present with a singular or combination of these mutations.

While genome-wide association studies (GWAS) and candidate gene approaches have been instrumental in identifying these variants, their implication in the disease is still largely founded on animal model studies, particularly rodent (rat/mouse) models. These models allow the dissection of each gene alone or in combination, and their role in the regulation of glucose homeostasis, insulin synthesis, processing, and secretion, and global cellular responses to insulin. The use of gene knockout or transgenic approaches in mice has been particularly insightful, and we will explore in the next section the identification, validation, and the mechanism of these diabetic mutations using these genetic approaches.

1.2.1 INS

The identification and characterization of the INS gene, which encodes the insulin protein, has been seminal in our understanding of diabetes, especially T1D. Interestingly, this gene was one of the first ever to be cloned and sequenced, marking it as a monumental achievement in molecular biology. Its discovery opened avenues for the biotechnological synthesis of human insulin, a therapeutic replacement that drastically improved the management of diabetes and demonstrated the power of utilizing molecular techniques to modify disease outcomes (Goeddel, Kleid et al. 1979).

Mechanistically, mutations in the INS gene can manifest in various ways, yet all have the ultimate effect of disrupting the structure and function of the insulin molecule, thus influencing its synthesis, processing, storage, and secretion. For instance, point mutations that results in substitutions to its peptide sequence, such as cysteine for tyrosine at position 96 (C96Y), can lead to aberrant disulfide bond formation (Balboa, Saarimäki-Vire et al. 2018). Misfolding that occurs due to this mutation triggers UPR and commits the defective insulin molecule to proteasomal degradation through the ERAD pathway. The resulting reduction in functional insulin availability, as a consequence, compromises glucose homeostasis. One illustrative example of this can be found in the widely studied and used Akita mouse model, which carries the C96Y mutation in the insulin 2 gene (Ins2), a homolog of the human INS gene (Izumi, Yokota-Hashimoto et al. 2003). In these mice, the misfolded insulin aggregates within the ER lumen, activating all three major branches of the UPR, including ATF6, IRE1, and PERK. If unresolved, this ER stress can culminate in apoptosis of pancreatic β cells via activation of CHOP and JNK, thereby leading to insulin deficiency and hyperglycemia, mimicking a T1D-like phenotype. Similarly, a mutation, R6C, has also been implicated to instigate similar downstream insulin dysfunction, but in the context of NDM (Edghill, Flanagan et al. 2008).

Such mutations, as demonstrated both in human patients as well as diabetic animal mouse models, are evidence that INS gene defects play an instrumental and universal role in the pathophysiology of diabetes. Concurrently, it validates the notion that the pathological cascade of diabetes can be mechanistically attributed to misfolded insulin polypeptides, the triggering of UPR and ER stress, and even β cell apoptosis, which all

operate in concert to reduce the capacity of the pancreas and islets to maintain metabolic homeostasis.

1.2.2 KCNJ11

The KCNJ11 gene encodes the Kir6.2 subunit of the ATP-sensitive potassium (K-ATP) channel present in the plasma membrane of pancreatic β cells. Historically, mutations in the KCNJ11 gene were identified as a genetic locus implicated in the development of NDM, and less commonly, in some cases of T2D (Gloyn, Pearson et al. 2004). One of the most extensively studied mutations is E23K, identified through genome-wide association studies to be associated with increased risk for T2D (Sachse, Haythorne et al. 2021). Researchers discovered that this mutation alters the ATP-binding affinity of the K-ATP channel, making it less sensitive to inhibition by ATP. Consequently, pancreatic β cells are less responsive to increases in intracellular ATP concentrations that occur in response to elevated blood glucose, thereby diminishing insulin secretion during periods where increased insulin demand is critical (post-feeding). Additional mutations in the KCNJ11 genes, such as R201H, have been identified in human patients with NDM and have been extensively studied. As an activating mutation, the mechanism behind R201H is theorized to essentially "lock" the K-ATP channel in an open state, rendering the β cells electrically unresponsive to glucose (Quan, Barszczyk et al. 2011). This perturbation of the K-ATP channel effectively prevents β cells from properly transitioning between polarized and depolarized states thereby causing a deficit in insulin release. Remarkably, in some cases, patients with KCNJ11 mutations have been successfully transitioned from insulin therapy to sulfonylureas, a class of drugs that has been clinically proven to

modulate the activity of and directly close K-ATP channels, thereby stimulating insulin secretion and in turn ameliorating the diabetic phenotype (Gloyn, Pearson et al. 2004).

KCNJ11 mutations serve as yet another instructive model for understanding the mechanistic underpinnings of insulin dysfunction that characterizes various forms of diabetes. Investigations of this gene have provided valuable insights and have demonstrated that the modulation of K-ATP channel function and, by extension, glucose-mediated insulin secretion, is pivotal for maintaining euglycemia. Disruptions of this fine-tuned system often leads to aberrant β cell electrophysiology and compromised insulin release, and perturbations in cellular ion-channel activity due to altered K-ATP channel structure/function precipitate a cascade of events culminating in glucose intolerance, which ultimately manifest as diabetes.

1.2.3 TCF7L2

The transcription factor 7-like 2 (TCF7L2) gene encodes a transcription factor integral to the Wnt signaling pathway, and studies of this gene gained particular prominence following GWAS investigations that identified certain polymorphisms within this locus as high-risk factors for T2D (Grant, Thorleifsson et al. 2006). Given that Wnt signaling is a conserved pathway involved in a myriad cellular processes, including differentiation, proliferation, and survival, researchers suspected that mutations in TCF7L2 were responsible for perturbing pancreatic β cell homeostasis. At the time of their discovery, single nucleotide polymorphisms (SNPs) like rs7903146 in TCF7L2 were correlated with its increase in risk, though the direct mechanistic underpinning of their association with diabetes remained elusive (Cropano, Santoro et al. 2017). While its mechanisms are still

not entirely understood, recent studies suggest the role of TCF7L2 in β cells seems to be multifaceted, affecting the cells intracellular calcium ion dynamics and even their survival. Specifically, the silencing of TCF7L2 expression affects the transcription of genes encoding for calcium channel subunits, particularly Cacna1d. The Cacna1d gene encodes the Cav1.3 subunit of L-type voltage-gated calcium channels, which are integral to calcium ion influx in response to membrane depolarization (Reinbothe, Alkayyali et al. 2013). Functionally, these channels act as conduits for calcium ions, which under the right conditions, assist in triggering downstream signaling cascades essential for the exocytosis of insulin granules. The alteration in Cacna1d expression significantly alters global insulin availability, as it impacts the synchronized release of insulin granules essential for effective glucose homeostasis, offering an explanation for TCF7L2's association with increased diabetes risk (del Bosque-Plata, Martínez-Martínez et al. 2021).

1.3.4 HLA-DQ2 and HLA-DQ8

The Human Leukocyte Antigen (HLA) complex is a genomic locus harboring many genes that are indispensable for the regulation and function of adaptive immune responses. Specifically, these genes encode major histocompatibility complex (MHC) molecules that mediate antigen presentation to T lymphocytes, serving as key determinants in immunological self-tolerance and the initiation and modulation of immune responses (Trowsdale and Knight 2013). Mutations in HLA genes have been strongly implicated in the susceptibility to autoimmune disorders, most notably, T1D. In fact, up to 90% of T1D patients carry at least one of these specific alleles (Steck and Rewers 2011).

Mechanistically, the HLA-DQ2 and HLA-DQ8 alleles encode specific MHC class II molecules that are instrumental in the presentation of antigenic peptides to CD4+ T-helper cells. Canonically, upon assembly in the endoplasmic reticulum and subsequent trafficking to the plasma membrane, these MHC class II molecules engage with T-cell receptors on the surface of CD4+ T-helper cells, initiating an immune cascade (Neefjes, Jongsma et al. 2011). However, in the case of T1D, HLA-DQ2 and HLA-DQ8 MHC variants acquire the ability to present autoantigens derived from pancreatic islet cells, notably insulin and glutamic acid decarboxylase (GAD) peptides (Pugliese 2017). The erroneous presentation of these peptides leads to an autoimmune reaction against islets, with β cells becoming the principal target of T-cell mediated cell death. This immunologically mediated decline in β-cell populations effectively reduces the overall β-cell mass in islets, which ultimately results in impaired insulin production, culminating in persistent hyperglycemia, which is the hallmark of T1D. Studies have revealed that the polymorphisms in HLA-DQ2 and HLA-DQ8 result in the expression of MHC class II molecules with a unique peptide-binding cleft configuration that preferentially presents pancreatic islet-derived antigens such as insulin to autoreactive T-cells (Noble and Valdes 2011).

Animal models, such as the Non-Obese Diabetic (NOD) mouse, have provided vital insights into understanding the role of HLA-DQ2 and HLA-DQ8 in diabetes pathogenesis. In this model, mutations that are homologous to human HLA-DQ2 and HLA-DQ8 alleles were incorporated, which lead to an aggressive autoimmune phenotype that mirrors human T1D (Pearson, Wong et al. 2016). Furthermore, these mice also exhibited elevated levels of proinflammatory cytokines like IFN-γ and IL-2, which

exacerbate the autoimmune cascade and contribute to β-cell apoptosis. Similar to observations seen in humans, these polymorphisms resulted in a decrease in overall β-cell mass in NOD mice.

Despite advances in understanding the role of HLA-DQ2 and HLA-DQ8 alleles in diabetes susceptibility, effective immunomodulatory therapies targeting the mechanism of these alterations remain out of reach. This gap underscores the need for continued research to improve treatment efficacy. Ongoing research aims at developing antigen-specific therapies that could alter the disease course by inducing antigen-specific tolerance, either through the introduction of specific peptides or modification of the MHC complex itself (Roep, Wheeler et al. 2019).

1.3 TGF-β signaling pathway and its link to glucose homeostasis

Transforming Growth Factor-Beta (TGF-β) was originally identified in 1981 for its ability to induce cellular transformation and potentiate oncogenesis in mesenchymal cells (Chang, Furth et al. 1982). TGF-β is an integral component of an extensive superfamily comprising more than 30 structurally related proteins, which includes activins, bone morphogenetic proteins, and growth and differentiation factors. The physiological impact of TGF-β and the pathways it modulates are underscored by its broad involvement in numerous cellular processes, ranging from cell differentiation to cell proliferation, to apoptosis and immune system regulation (Massagué 2012). Given the extensive influence of TGF-β signaling pathways, aberrations in its expression or function can lead to a multitude of pathological conditions, including neoplastic diseases, fibrotic disorders, and autoimmune dysregulation, and diabetes (Batlle and Massagué 2019).

The TGF-β family in mammals comprises three isoforms: TGF-β1, TGF-β2, and TGF-β3. These isoforms share between 70% to 80% sequence homology and are encoded by distinct genes. Each isoform is initially synthesized as a precursor molecule composed of a latency-associated peptide and a mature TGF-β peptide. A disulfide bond links these two elements, forming a small latent complex, and subsequent cleavage by proteases eventually separates the mature, dimeric, biologically-active form of TGF-β (Roberts 1998). The TGF-β signaling pathway is initiated when this mature dimeric ligand binds to the type II transmembrane serine/threonine kinase receptor (TβRII), which possesses intrinsic kinase activity. The initial TGF-β to TβRII ligand-receptor interaction then catalyzes the phosphorylation and activation of the type I serine/threonine kinase receptor (TβRI). The activated type I and type II receptors then coalesce to form a heterotetrameric complex that then serves as a platform for downstream signal propagation (Massagué 2012).

The intricacies of downstream TGF-β signaling are orchestrated through both canonical (Smad-dependent) and non-canonical (Smad-independent) pathways. In the canonical pathway, TβRI phosphorylates Smad2 and Smad3, and once phosphorylated, the Smad proteins form a complex with their co-mediator Smad4, and translocate to the nucleus where they modulate gene expression in concert with other transcription factors. In contrast, non-canonical pathways of TGF-β signaling activation involves the engagement of other signaling cascades including MAPK, PI3K, and JNK, which together, influence a wide variety of cellular processes (David and Massagué 2018).

Recent studies have begun to elucidate the role of TGF-β in metabolic disorders, particularly diabetes (Yan, Zhang et al. 2014). TGF-β has been shown to influence both

insulin secretion and sensitivity, thereby establishing it as a molecule of interest in the context of β-cell function and global metabolic homeostasis (Shi, Shi et al. 2018). For instance, it was discovered that TGF-β signaling regulates the expression of insulin and insulin-modulatory genes by directly altering the activity of transcription factors such as pancreatic and duodenal homeobox 1 (Pdx1), the master regulator of insulin promoter activity (Sayo, Hosokawa et al. 2000). Moreover, TGF-β has been found to impact insulin sensitivity through activation of the Akt, a serine/threonine-specific protein kinase that influences the uptake of glucose in cells throughout the body (Budi, Muthusamy et al. 2015).

In relation to pancreatic inflammation, investigations have revealed that TGF-β signaling has bifunctional roles, as it can either amplify or mitigate inflammatory responses in a context dependent manner. One scenario in which TGF-β signaling works to promote inflammation depends on its ability to upregulate the of expression of pro-inflammatory cytokines IL-6 and TNF-α in pancreatic islets. The upregulation of these cytokines can activate NF-κB signaling, thereby initiating a cascade of events that culminate in β-cell apoptosis and impaired insulin secretion (Wang, Wang et al. 2022). Conversely, TGF-β is also known to induce the differentiation of naïve CD4+ T cells into regulatory T cells (Tregs), which exert immunosuppressive functions. Tregs are well known to be crucial in maintaining self-tolerance and preventing autoimmune reactions by suppressing effector T cells, like T-helper 1 (Th1) and T-helper 17 (Th17) cells (Veldhoen, Hocking et al. 2006). Overall, it should be noted that the impact of TGF-β in pancreatic inflammation is not merely a function of its signaling, but also of the cellular context in which this signaling occurs. Factors such as the presence of other cytokines,

the metabolic state of the cells, and even the specific isoform of TGF-β involved, can tip the balance toward either an inflammatory or an anti-inflammatory state, which in turn either exacerbate or ameliorate metabolic irregularities, making it an attractive candidate for further studies related to diabetes (Li, Wan et al. 2006).

Interestingly, evidence also suggest that TGF-β signaling can play a crucial role in islet amyloid formation, which is considered a pathogenic feature of both T1D and T2D (Wyss-Coray, Lin et al. 2001). In the context of β cells, the significant molecular entity involved in aggregation is the islet amyloid polypeptide (IAPP), also known as amylin (Blencowe, Furterer et al. 2022). Normally co-secreted with insulin, IAPP acts to slow gastric emptying and inhibiting glucagon secretion, both crucial steps responsible for mitigating irregular changes in blood-glucose levels. The physiological consequence of increased IAPP levels result in the formation of insoluble amyloid aggregates within the pancreatic β-cell. These amyloid deposits have a significant cytotoxic effect, leading to cellular dysfunction and if left unchecked, eventually apoptosis (Akter, Cao et al. 2016). The cytotoxicity of IAPP aggregates is thought to occur through several mechanisms, including membrane disruption and activation of pro-apoptotic pathways (i.e., JNK) (Subramanian, Hull et al. 2012). Moreover, amyloid aggregation triggers a severe local inflammatory response, recruiting macrophages and pro-inflammatory cytokines to the islet, which further exacerbates β-cell dysfunction while also contributing to a self-perpetuating cycle of inflammation and cellular damage (Cosentino and Regazzi 2021). It should be noted that investigations have uncovered that accumulation of misfolded IAPP is accelerated by ER and oxidative stress conditions, which present frequently in T2D (Huang, Lin et al. 2007). Therefore, upregulated IAPP expression via aberrant TGF-

β signaling not only adds to the pool of aggregating peptides within the ER but also compounds the already stressed cellular machinery, making the role of TGF-β in islet amyloid formation particularly destructive.

1.4 TGIF1

TG-interacting factor 1 (TGIF1) is a transcriptional repressor initially identified for its inhibitory role in TGF-β signaling pathways. However, emerging research underscores TGIF1's ubiquity across various tissues, as it is capable of acting as a key regulator in a variety of different cellular activities, including cell cycle modulation, cellular differentiation, and even metabolic regulation. TGIF1 encodes a 272 amino acid protein, which contains highly conserved homeodomain essential for DNA binding. This homeodomain enables highly specific targeted binding, often in gene promoter regions, which facilitate TGIF1's role as a transcriptional repressor. TGIF1's versatility in controlling gene expression broadens its functional relevance in cellular processes beyond its repressive role in TGF-β signaling, allowing it to independently function as a multi-faceted regulator of various cellular activities, and in some circumstances, potentiate or exacerbate pathological states including metabolic and oncogenic diseases.

TGIF1 represses TGF-β function primarily by binding to Smad2 and Smad3, which are intracellular effectors activated by TGF-β signaling. Upon ligand binding, TGF-β receptors phosphorylate Smad2 and Smad3, which then typically translocate to the nucleus to activate transcription of TGF-β-responsive genes. TGIF1 interacts with these Smads and recruits corepressor complexes, including histone deacetylases (HDACs), to TGF-β responsive promoters. Consequently, TGIF1 modifies chromatin to a closed

conformation, inhibiting the transcription of genes involved in cell proliferation, differentiation, and extracellular matrix formation. This suppression is critical in maintaining cellular homeostasis and preventing TGF-β overactivity that can lead to pathological fibrosis and cancer.

Additionally, TGIF1 has been implicated in the modulation of other signaling pathways such as those involving Ant and TNF, which are critical in inflammatory and immune responses with implications in diabetes. In the context of diabetes, TGIF1 appears to influence pathways that regulate insulin sensitivity and inflammatory responses. Abnormal activity of TGIF1 could disrupt these pathways, contributing to the chronic inflammation and insulin resistance observed in metabolic disorders. The precise mechanisms by which TGIF1 regulates Ant and TNF signaling pathways and their downstream effects on metabolic processes require further elucidation but suggest a broader role for TGIF1 in the pathophysiology of diabetes beyond its established functions in TGF-β signaling.

Manuscript

TGIF1 PHYSIOLOGICAL LEVELS LIMIT B-CELL DISTRESS AND NEONATAL DIABETES

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Abstract

TGIF1 belongs to the superfamily of homeodomain proteins, which regulate a wide variety of biological functions, including cell stemness and specification of cell fate during early development. Perhaps surprisingly, we found that enforced expression of TGIF1 in pancreatic progenitor cells during embryogenesis resulted in severe diabetes, hinting at the possibility that TGIF1 might regulate pancreas development. Genetic experiments targeting b-cells showed that TGIF1 affects β-cell function and homeostasis. Transcriptomic analysis reveals that TGIF1 expression inhibits expression of essential components of UPR signaling, underscoring a potential mechanism by which TGIF1 affects protein folding and secretion. Congruently, TGIF1 expression led to a dramatic disorganization of insulin in β-cells, accumulating as large aggregates, and this was associated with decreased insulin secretion. Subsequent in vitro experiments showed that TGIF1 expression leads to the accumulation of insulin aggregates in ER, thereby causing ER stress and attendant impairment in insulin processing and secretion. In further support to these findings, conditional deletion of Tgif1 in pancreatic progenitor cells was associated with hyperglycemia and diabetes, reinforcing the notion that TGIF1 physiological levels are instrumental to maintaining the balance between UPR and ER needed for normal β-cell function. Finally, we serendipitously found that enforced of TGIF1 in β-cells can recapitulate the cardinal hallmarks of neonatal diabetes, shedding important insights into mechanistic paradigms of this enigmatic condition.

Introduction

Diabetes Mellitus is a metabolically debilitating health condition that impairs our body's capacity to adequately maintain normal body glucose levels (euglycemia) (Møller and Nair 2008). Etiologically, multiple forms of diabetes (e.g., type 1, type 2, gestational, and neonatal diabetes) are attributable to the dysfunction of insulin-producing β-cells, a specialized subset of cells located within the pancreatic islets of Langerhans (Ilonen, Lempainen et al. 2019, Roden and Shulman 2019). Islets of Langerhans (referred to hereafter as islets) are highly organized and complex multicellular sub-compartments embedded within the acinar parenchyma of the pancreas and are comprised primarily of five different hormone-producing cell types (Kim, Miller et al. 2009). Each cell type in pancreatic islets secretes a specific hormone: α-cells produce glucagon, β-cells produce insulin and amylin, δ-cells produce somatostatin, γ-cells produce pancreatic polypeptide, and ε-cells produce ghrelin (Da Silva Xavier 2018). Balanced production and secretion of each of these hormones is essential for maintaining homeostatic metabolism and energy utilization needs for the body. Dysfunction in any of these cell populations can lead to detrimental metabolic and health outcomes; however, specific deterioration/loss of β-cell function and the concomitant perturbation in insulin production has been found to account to all types of diabetes, which together represent the seventh leading cause of death globally, being responsible for an estimated 1.5 million death annually (Federation (2013), (Roden and Shulman 2019)).

Type 1 Diabetes (T1D) is predominantly an autoimmune condition characterized by the T-cell-mediated destruction of insulin-producing β-cells. This autoimmune-driven loss of β-cells results in the substantial loss of insulin expression, which disturbs glycemic regulatory pathways, eventually culminating in global dysglycemia. Type 2 Diabetes (T2D) is the most common form of diabetes globally and primarily arises as a result of insulin resistance in peripheral tissues (i.e. skeletal muscle, liver, adipose cells), but can also be accompanied and exacerbated by β-cell dysfunction. Unlike T1D or T2D, neonatal diabetes mellitus (NDM) is are rare form of diabetes that is diagnosed within the first six months of life, occurring in approximately 1 in 90,000-160,000 live births, and is a multifaceted disease with a heterogeneous genetic etiology Federation (2013), (Lemelman, Letourneau et al. 2018). In NDM patients, genetic defects in key genes such as KCNJ11, ABCC8, and INS, compromise the β-cell's intrinsic ability to properly synthesize, process, and secrete insulin, ultimately resulting in dysglycemia (Vaxillaire, Dechaume et al. 2007, Gole, Oikonomou et al. 2018). Beyond this genetic backdrop, a growing body of evidence has increasingly highlighted the potential role of chronic endoplasmic reticulum (ER) stress in NDM pathogenesis (Tahmasebi, Khoutorsky et al. 2018).

It is widely recognized that persistent ER stress leads to a variety of detrimental cellular outcomes, primarily the aggregation of unfolded proteins (Balchin, Hayer-Hartl et al. 2016). ER stress is primarily induced when there is an imbalance between the load of unfolded and folded nascent peptides within the ER lumen, often occurring when the protein-folding capacity of ER-resident chaperones is limited or insufficient to meet processing demands (Kozutsumi, Segal et al. 1988). To mitigate the accumulation of

unfolded peptides within the ER, cells initiate a series of molecular events in an attempt to remedy this imbalance in a process collectively termed unfolded protein response (UPR) (Kaufman 2005). The overall effects of UPR activation result in reduced burden of unfolded or misfolded peptides within the ER. For example, by reducing the overall load of proteins entering the ER, achieved by blocking de novo protein synthesis pathways and preventing protein translocation into the ERs luminal space, cells are able to reduce ER stress and prevent further accumulation of unfolded proteins (Bernales, Papa et al. 2006). Other arms of the UPR function to improve the ER's capacity to process and properly fold unfolded proteins, which is accomplished by the activation of UPR target genes, some of which directly lead to the synthesis of ER-chaperones responsible for protein folding (Pavitt and Ron 2012). If the initial wave of UPR is unable to sufficiently ameliorate unfolded protein levels within the ER, cells induce the activation of specific transcription factors, such as IRE1α, which in turn promote the expression of genes encoding proteins and enzymes that enable the ER-associated degradation (ERAD) of misfolded proteins (Lee, Iwakoshi et al. 2003). Under intense ER stress and persistent activation of protein synthesis, the UPR may not sufficiently ameliorate the buildup of deleterious proteins, leading to the formation of insoluble and cytotoxic aggregates (Balchin, Hayer-Hartl et al. 2016). Early pathological examinations of cadavers revealed an accumulation of unfolded, insoluble insulin and islet amyloid polypeptide (IAPP) aggregates in the pancreatic islets of T2D patients, suggesting that deposition of these aggregate species might be associated with diabetes progression (Johnson, O'Brien et al. 1989). Emerging evidence has clearly indicated that misfolded insulin and IAPP aggregate deposition in β-cells directly contribute to the prevalence and severity of

diabetes, underscoring the critical roles ER stress and UPR play in the maintenance of β-cell function and homeostasis (Haataja, Gurlo et al. 2008, Jurgens, Toukatly et al. 2011, Mukherjee, Morales-Scheihing et al. 2017). Notwithstanding this tremendous progress in unraveling the deleterious impacts of perturbed ER and UPR mechanisms in diabetes, the underlying molecular mechanisms behind these perturbations remain poorly understood.

TGIF1 belongs to a superfamily of homeodomain proteins, which regulate a plethora of biological functions, including specification of cell fate during development and cell stemness. Genetic ablation of *Tgif1* in mice resulted in several subtle phenotypes, including growth retardation, runting, and craniofacial abnormalities (Wotton, 2018). In adult mice with global knockout, TGIF1 has been shown to promote cholesterol synthesis and lipid metabolism, which prompted the first hypothesis that perturbations in TGIF1 function might drive or exacerbate certain metabolic diseases (Pramfalk, 2015). Yet, how and where deregulation of TGIF1 expression contributes to the etiology of these metabolic diseases remains elusive. To tackle this gap, we employed a variety of genetic approaches with conditional deletion or overexpression of Tgif1 in pancreas progenitor cells or distinct pancreatic cell populations to unravel whether TGIF1 could influence glucose metabolism and consequently whole-body metabolism. Overall, our comprehensive genetic approaches revealed that maintaining proper physiological levels of TGIF1 plays a key role in ensuring normal β-cell function and euglycemia, functioning as a rheostat to maintain suitable levels of ER and UPR needed for efficient insulin processing and secretion. Importantly, we also discovered that TGIF1 might contribute to

the etiology of neonatal diabetes, a debilitating disease for which only superficial mechanistic explanations have been reported.

Materials and Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University. Additionally, all animal metabolic experimentation was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The following mouse strains were used in this study:

-LSL-Tgif1

-Pdx1.Cre

-Ins2.Cre

-Ptf1a.Cre

-Pdx.Cre^{ESR1}

-LSL-Luc

-Tgif1$^{fl/fl}$

-Tgif1-/-: gTgif1^{KO}

-LSL:Tgif1.GFP:Pdx1.Cre: pTgif1^{OV}

-LSL:Tgif1:Ins2.Cre: iTgif1^{OV}

-LSL:Tgif1:Ptf1a.Cre: Ptf1aTgif1^{OV}

-LSL:Tgif1:LSL-Luc:Pdx.Cre^{ESR1}: pTgif1^{OVLuc}

-Tgif1fl/fl:Pdx.Cre: pTgif1^{CKO}

All mice were maintained on a mixed C57BL/6 and FVB/N genetic background. Mice were housed in a temperature (22 °C) and humidity-controlled facility with access to standard rodent chow and water ad libitum throughout the study unless otherwise noted. A twelve-hour light:dark cycle was also in place in the animal facility (6:00AM-6:00PM).

Determination of blood glucose, plasma insulin, and plasma c-peptide levels

To assess basal blood glucose levels, mice were fasted for 6 hours, and blood glucose levels were measured from tail blood samples using a ReliOn Premier Glucometer (Arkray USA, Minneapolis, MN, USA), with 3-4 blood glucose measurements recorded and averaged in order to achieve a more accurate glucose measurement. ALPCO mouse Insulin and mouse C-peptide ELISAs (80-INSMS-E01 & 80-CPTMS-E01, respectively) were used to measure serum insulin and C-peptide levels. Briefly, blood was collected from each mouse via tail snip into serum separator tubes and allowed to clot for 30 minutes at room temperature. The tubes were then centrifuged at 1000g for 10 minutes at 4°C, and serum was collected and stored at -80°C until analysis. For INS-1 in vitro cell line analysis, the cells were plated in 6-well plates (3x replicates each, Dox-/+) and treated as indicated. The supernatant was collected and stored at -80°C until analysis. Insulin

and C-peptide levels were measured according to the manufacturer's ELISA protocol and instruction sheet. Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (version 9.0.0). A two-tailed unpaired t-test was used to compare means between groups, and $p < 0.05$ was considered statistically significant.

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance tests (ipGTTs) were performed on mice to assess their glucose metabolism and tolerance. Mice were fasted for 6 hours and then injected intraperitoneally with glucose (2g/kg body weight). Blood glucose levels were measured from tail blood samples at baseline and 15, 30, 60, and 90 minutes post-injection using a glucometer (*ReliOn*, Premier). The area under the curve (AUC) for blood glucose levels was calculated for each group, and statistical significance was determined using a two-tailed Student's t-test with a significance level of $p < 0.05$.

Intraperitoneal insulin tolerance test

Intraperitoneal insulin tolerance tests (ipITTs) were performed on mice to evaluate their insulin sensitivity. Mice were fasted for 4 hours and then injected intraperitoneally with insulin (0.75U/kg body weight). Blood glucose levels were measured from tail blood samples at baseline and 15, 30, 60, and 90 minutes post-injection using a glucometer (*ReliOn*, Premier). The AUC for blood glucose levels was calculated for each mouse, and

statistical significance was determined using a two-tailed Student's t-test with a significance level of $p < 0.05$.

High-fat diet (HFD) feeding

To investigate the effect of HFD on glucose tolerance and insulin sensitivity in TGIF1.ko mice, a separate group of TGIF1.ko mice (n=6 males and n=6 females) and control mice (n=6 males and n=6 females) were fed a HFD (60% kcal from fat) for 6 months. Intraperitoneal glucose tolerance and insulin tolerance tests were performed on these mice at 3 and 6 months of HFD feeding, as described above. The AUC for blood glucose levels was calculated for each mouse, and statistical significance was determined using a two-tailed Student's t-test with a significance level of $p < 0.05$.

Immunoblotting

To extract their protein lysates, cells were treated with a lysis buffer cocktail of 1mM EGTA, 2.5% sodium pyrophosphate, 1ug/uL leupeptin, 150mM NaCl, 20mM Tris HCl (pH 7.5), 1% Triton, 1mM β-glycerophosphate, 1mM EDTA, phosphatase inhibitors (Sigma Aldrich, #P5726) and protease inhibitors (Sigma Aldrich, #P8340). The protein lysates were quantified using a Bradford assay, and 40µg of protein was combined with SDS sample buffer (BioRad, #1610747) and loaded into a 10-14% polyacrylamide gel (BioRad, #5671124) and electrophoretic ally separated at a constant voltage of 60mA. Precision PageRuler™ Dual Color Protein Ladder (BioRad, #1610374) was used as molecular weight markers. The protein contents of the gel were transferred onto nitrocellulose

membranes (BioRad, #1620115) using a semi-wet transfer system at 25V for 30 minutes at room temperature. The membranes were then blocked with 5% non-fat dry milk (NFDM) in TBS-T for 1 hour at room temperature. Primary antibodies were diluted in 5% NFDM in TBS-T and incubated with the membranes overnight at 4°C. The membranes were washed, and the appropriate peroxidase-conjugated secondary antibody was added and incubated for 1 hour at room temperature. Finally, the membranes were washed and developed using enhanced chemiluminescence (ECL) detection substrate (BioRad, #170-5061). Images of the membranes were acquired using the ChemiDoc MP Imaging System (BioRad).

Histology, Immunohistochemistry and Immunofluorescence

Tissue samples were fixed in 10% formalin and then embedded in paraffin wax to produce tissue blocks. Sections of 5 µm thickness were cut from the tissue blocks using a microtome and mounted on glass slides. The sections were then deparaffinized with xylene and rehydrated using a series of graded ethanol solutions. For hematoxylin and eosin staining (H&E), tissue sections were first deparaffinized and rehydrated as described above. Following deparaffinization, tissue sections were immersed in hematoxylin solution (Sigma-Aldrich, HHS128-4L) for 2 minutes, followed by a clarifier solution (Epredia, 7402L) for 15 seconds, and a blueing reagent solution (Epredia, 7301L) for 1 minute. Between each of the three steps, sections were immersed in water for 1 minute. Next, the sections were immersed in an eosin solution (Sigma-Aldrich, HT110280-2.5L) for 3 minutes, dehydrated in graded ethanol solutions (VWR, 89370-088), and cleared with xylene (Koptec, V1001).

For immunofluorescence staining, tissue sections were first deparaffinized and rehydrated as described above. Antigen retrieval was performed by incubating the sections in a citrate buffer at 95°C for 30 minutes. To block non-specific binding, sections were incubated with 5% goat serum for 1 hour at room temperature. Following washing steps, primary antibodies were then applied and sections were incubated overnight at 4°C. The primary antibodies used were [Cell Signaling: 8132s, 2760s]. After washing, the sections were incubated with fluorescently-labeled secondary antibodies, Alexa-Fluor®568 (Invitrogen, #A11011) or Alex-Fluor®488 (Invitrogen, #A11088), for 1 hour at room temperature. The sections were then counterstained with DAPI (Vector Laboratories,#H1800) and fixed with fluorescent mounting medium. For *in vitro* IF assays, cells were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 minutes at room temperature (RT) to preserve the cellular morphology and immobilize the proteins of interest. After fixation, the cells were washed with PBS three times for 5 minutes each to remove residual PFA. Following the wash, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at RT and subsequently washed with PBS three times for 5 minutes each. Cells were then blocked with 5% bovine serum albumin (BSA) in PBS for 1 hour at RT. The primary antibody was (diluted in 1% BSA in PBS) was added and the cells were incubated overnight at 4°C. After washing the cells with PBS, the secondary antibody was added and cells were incubated for 1 hour at RT and finally counterstained using DAPI to visualize the nuclei.

For immunohistochemistry staining, tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval as described above. Endogenous peroxidase activity was quenched by incubation with 5% hydrogen peroxide for 10 minutes at room temperature.

The sections were then blocked with 5% goat serum and incubated with primary antibodies overnight at 4°C. The primary antibodies used were [Cell Signaling 13092s, 82630s]. After washing, the sections were incubated with biotinylated secondary antibodies for 1 hour at room temperature. Following biotinylation, sections were incubated with streptavidin-HRP for 30 minutes at room temperature. Tissue section staining and colorization was achieved by using a DAB Peroxidase substrate kit (Vector Laboratories, #SK-4100). Finally, the tissues were counterstained with hematoxylin and mounted with a non-aqueous mounting medium.

Dual-Luciferase Reporter Assay

For the quantification of luciferase and Renilla activities, the Promega Dual-Luciferase Reporter Assay System (Catalog #E1910) was employed according to the manufacturer's instructions. Cos7 cells were seeded as triplicates in 12-well plates at a density of 5x10^4 cells/well and cultured in DMEM supplemented with 10% FBS, glutamine, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO2. After 24 hours, cells were transfected with 500 ng of cDNA of interest along with firefly luciferase-constructs and 50 ng of Renilla luciferase reporter plasmids using GeneJet (Thermo Scientific) following the manufacturer's protocol. Post-transfection (48 hours), cells were washed with PBS and lysed using 100 µl of Passive Lysis Buffer (PLB) per well. The lysate was collected and transferred to a 1.5 ml Eppendorf tube. Samples were prepared according to Promega Reporter kit and luminesce was measured via a BioTek luminometer at 540 nm.

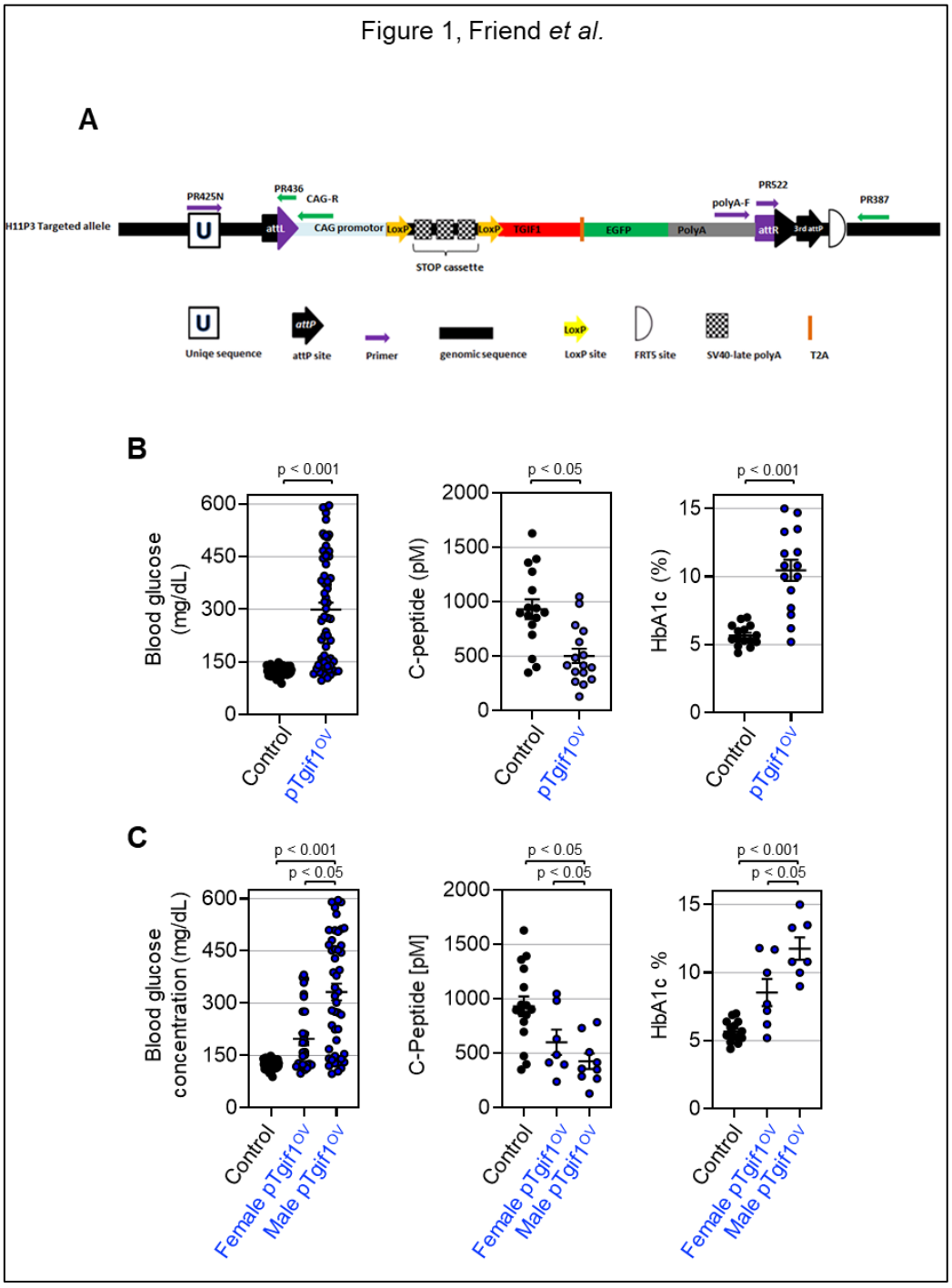
Results

Overexpression TGIF1 in pancreatic progenitor cells leads to diabetes

We have previously shown that TGIF1 is dispensable for pancreatic development, as assessed using mice with either pancreas-specific deletion of Tgif1 in pancreatic progenitor cells or global Tgif1 knockout. In efforts to further investigate the role TGIF1 in pancreas physiology and function, we sought to study the effect of increased TGIF1 expression in pancreas using a new mouse model in which a conditional allele of Tgif1 (LSL-CAG-Tgif1) was targeted to the Hipp11 locus using the TARGATT strategy (Figure 1A). We crossed LSL-CAG-Tgif1 mice with the Pdx1.Cre strain, which expresses Cre recombinase in all pancreatic progenitor cells that give rise to islet, ductal, and acini. For simplicity, this mouse model will be referred to hereafter as pTgif1^{OV}. pTgif1^{OV} mice were born at the expected Mendelian ratio and exhibited no overt physical or developmental defects at least until the age of 9 months. Intriguingly, initial indications that pTgif1^{OV} mice experience metabolic defects began to emerge at the age of 4 weeks, as exemplified by the excessive fecal diarrhea and urine deposits in cages housing pTgif1^{OV} mice as compared to the cages of their littermate control mice (Figure S1A). Comprehensive physical examinations of 4-week-old mice showed that the pTgif1^{OV} cohort weighed slightly but significantly less than their control siblings, a phenotype that persisted over a period of 9 months (Figure S1B). To assess the dietary welfare of pTgif1^{OV} animals, we monitored their solid chow and water consumption (ad libitum) over a period of 72 hours. As shown in Figure S1B, a significant increase in water consumption was evident exclusively in pTgif1^{OV} mice, in line with the excessive diarrhea and urine deposit

observation. Of note, we did not observe any difference in chow consumption across both groups (Figure S1B). The combined manifestations of diarrhea, polyuria, and excessive thirst in pTgif1^{OV} mice aligned closely with the typical clinical symptoms of diabetes in patients, prompting us to explore a possible diabetes phenotype in these mice. Quite remarkably, the vast majority of pTgif1^{OV} mice showed significantly higher fasting blood glucose concentrations (>200 mg/dL) while their littermate controls display the typical range of glucose concentrations (≤150 mg/dL) in healthy mice (Figure 1B). Noteworthy, more than 67% of pTgif1^{OV} mice displayed concentrations exceeding 300 mg/dL (Figure 1B), which is considered as a sign of severe diabetes. To substantiate this finding, we evaluated the concentration of insulin C-peptide and HbA1c in the plasma of both cohorts, and the results showed that pTgif1^{OV} mice exhibited levels consistent with a positive diabetic diagnosis, based on widely accepted clinical thresholds for diabetes (Figure 1B) (Jones and Hattersley 2013). Interestingly, one of the more perplexing aspects of our diagnostic studies was the highly variable range of blood glucose, insulin C-peptide, and HbA1c concentrations within our age-matched population of pTgif1^{OV} mice (Figure 1B). Exploring these disparities further, we found that when we stratified the pTgif1^{OV} population data by sex, females, though still dominantly within the diabetic range, displayed somewhat attenuated levels of each diabetic diagnostic marker compared to male pTgif1^{OV} mice (Figure 1C). A literature search revealed that the subdued diabetic phenotype in our female pTgif1^{OV} mice bore striking resemblances to observations made with the diabetic Akita mouse model, which have a mutation in the Ins2 gene that affects the processing of insulin and thereby culminates in ER stress (Yoshioka, Kayo et al. 1997, Wotton and Taniguchi 2018). Female Akita mice, much like pTgif1^{OV} females, tend to

have less increased blood glucose levels and other diabetic diagnostic markers compared to Akita males. The parallels between these two mouse models suggested a potential shared underlying mechanism that warrants further investigations as discussed later.



TGIF1-mediated ADM is dispensable for the diabetic phenotype

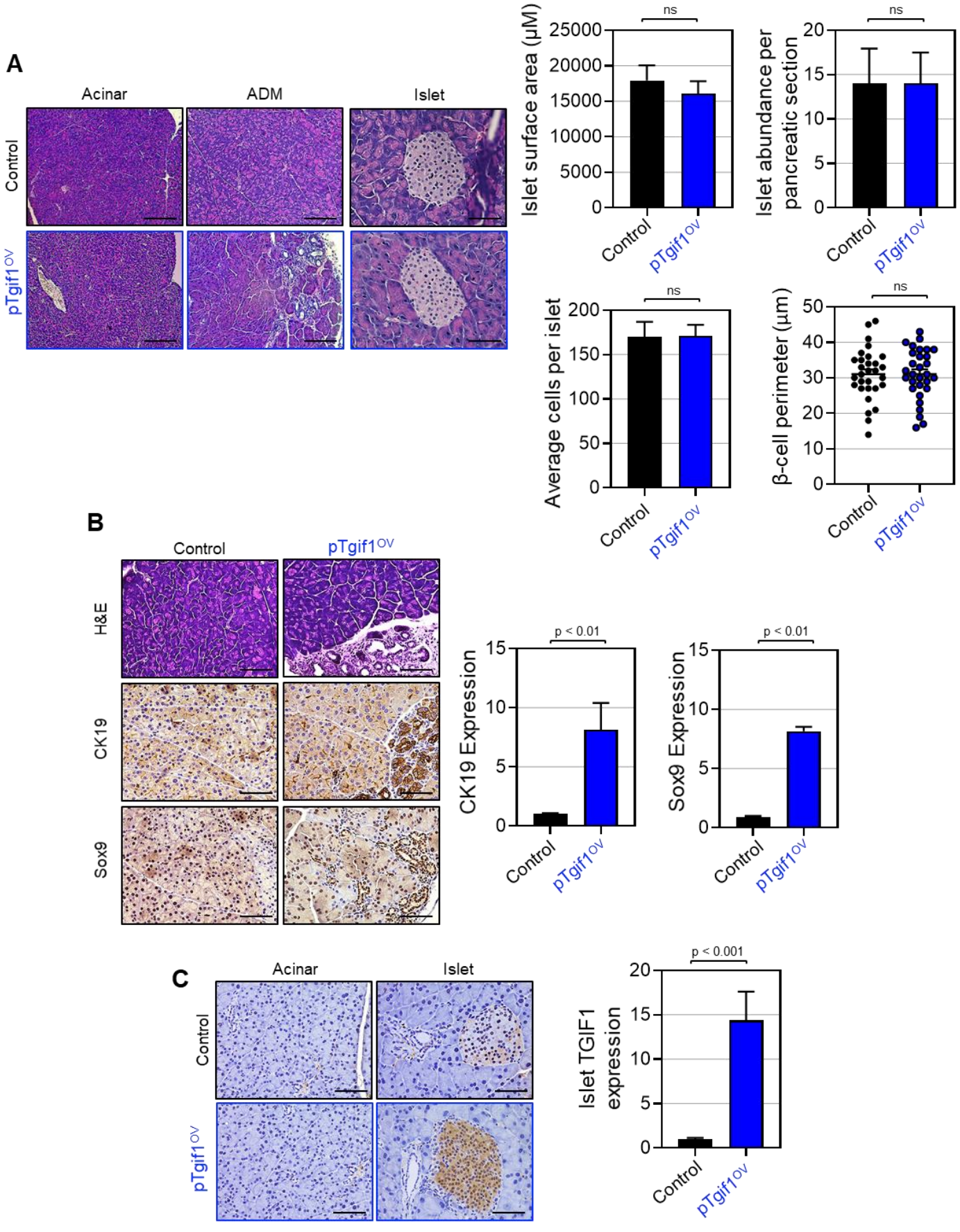
Having shown that pTgif1^{OV} mice develop a severe diabetes phenotype, we next set out to explore the underlying mechanisms. Accordingly, we conducted histology experiments using pTgif1^{OV} mice with high blood-glucose levels (>300mg/dL) and their euglycemic control littermates. Considering that the Pdx1 promoter in Pdx1.Cre mice enables TGIF1 overexpression in all pancreatic progenitor cells (e.g., acinar, ductal, islet), we characterized each of these pancreatic compartments individually by hematoxylin and eosin (H&E). While staining of pancreatic tissue sections revealed that islet and ductal structures in all mice were not perturbed by TGIF1 overexpression, the acinar parenchyma in 3 out of 12 pTgif1^{OV} mice (25%) exhibited morphological alterations reminiscent of acinar-to-ductal metaplasia (ADM), a pathological cellular differentiation process often driven by inflammatory responses such those driven by pancreatitis or pancreatic ductal adenocarcinoma (PDAC) (Figure 2A). Interestingly, serial sectioning analyses of the remaining 9 (75%) pTgif1^{OV} pancreatic samples reveled no evidence of ADM, suggesting this phenomenon was likely not the contributing feature instigating glycemic dysregulation in pTgif1^{OV} mice. To confirm this observation, we conducted immunohistochemistry (IHC) assays utilizing antibodies to Sox9 and Cytokeratin 19 (CK19), two markers of ADM. We detected lesions that stain positive for Sox9 and CK19 in sections from the three mice identified as having ADM by H&E, whereas the 9 others diabetic pTgif1^{OV} mice showed staining similar to that of wild-type mice.

Next, we focused our efforts on the islet, a compartment that hosts α-cells and β-cells, which secrete glucagon and insulin, respectively, hormones with critical roles in maintaining glycemic homeostasis. H&E analysis failed to reveal any disparities in the

frequency, size, morphology, or number of cells in the islets of pTgif1^{OV} mice as compared to islets of control mice, irrespective of whether the pTgif1^{OV} mice presented with ADM or not (Figure 2A). As such, these findings clearly showed that TGIF1 overexpression does not affect islet survival or morphology, and further reinforce the notion that ADM per se was not sufficient to disrupt islet functionality leading to hyperglycemia in pTgif1^{OV} mice.

Given that structural perturbations in pancreatic tissues were not sufficient to explain the severe diabetic phenotype of pTgif1^{OV} mice, we then extended our efforts to characterize the expression and localization of TGIF1 within the entire pancreas parenchyma. Performing IHC experiments, we found that control mice expressed appreciable but relatively low levels of TGIF1 within the islets and negligible expression in acinar cells. pTgif1^{OV} mice displayed the same TGIF1 localization patterns as the control group, yet they showed a 4-fold increase in TGIF1 expression levels specifically in the islet (Figure 2C). Based on these findings, it was becoming evident that the diabetic phenotype in pTgif1^{OV} mice dif not hinge on structural or morphological aberrations in the islets, but rather on other alternative mechanisms, most conceivably direct perturbations in insulin homeostasis in β-cells.

Figure 2, Friend *et al.*



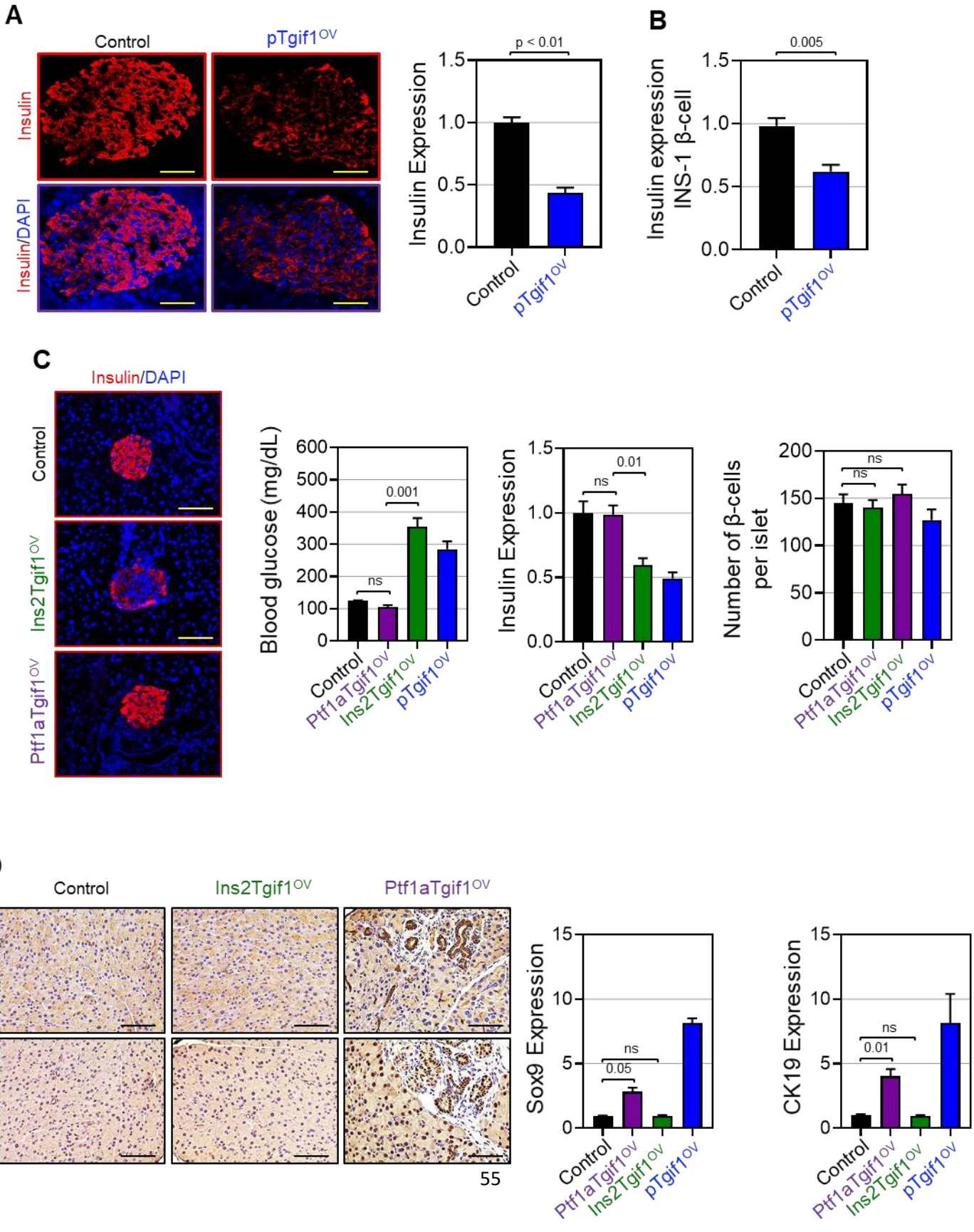
TGIF1 overexpression in pancreatic β-cells perturbs insulin production

To delve more deeply into how elevated TGIF1 expression in islets leads to diabetes, we conducted immunofluorescence (IF) assays to assess insulin and glucagon expression. Compared to wild-type sibling mice, pTgif1ov mice displayed a marked reduction in insulin expression in β-cells, whereas glucagon levels in α-cells remained largely unchanged (Figure 3A). To determine if such decline in insulin expression was a result of β-cell loss, β-cell dysfunction, or both, we quantified the average number of β-cells in the islets of both groups of mice. Quantification of the surface area of islets based on this parameter revealed no apparent variation in β-cell populations between either group, suggesting that any loss of insulin expression in pTgif1^{OV} mice might originate as a result of β-cell dysfunction rather than β-cell death or impaired growth. This observation is consistent with our earlier study that pTgif1ov mice display decreased circulating levels of insulin C-peptide. To corroborate these observations further, we carried out in vitro studies using the INS-1 β-cell line that we engineered to express doxycycline-inducible TGIF1, and found here again that increased TGIF1 expression led to decreased insulin expression (Figure S3A).

In light of the substantial evidence that elevated TGIF1 overexpression in pancreatic progenitor cells (i.e., Pdx1.cre) impaired insulin production in β-cells, it became imperative to ascertain whether this phenomenon could also occur if TGIF1 expression is confined to β-cells. To probe this possibility, and further rule out the possibility that decreased insulin expression originate owing to the inflammatory milieu engendered by ADM, we generated mouse models with targeted TGIF1 overexpression in β-cells

(ins2Tgif1^{OV}) or acinar cells (Ptf1aTgif1^{OV}) using the Ins2.Cre and Ptf1A.Cre (also known as p48.Cre) strains, respectively. Remarkably, Ins2Tgif1O^{V} mice exhibited severe hyperglycemia (72% of mice > 200mg/dL), which was mirrored again by a significant drop in insulin expression and no changes in β-cell number, thus recapitulating the diabetic phenotype seen in pTgif1^{OV} mice (Figures 3C and 3D). In contrast, Ptf1aTgif1^{OV} mice did not manifest any significant reduction in insulin expression or β-cell number, and all mice were found to have normal glycemic levels (Figure 3C). These mice, however, did exhibit lesions reminiscent of ADM, consistent with earlier observations made using pTgif1^{OV} mice. Sox9 and CK19 staining confirmed the ADM phenotype in Ptf1aTgif1^{OV} mice (Figure 3D). Collectively, these data strengthen the notion that the TGIF1-mediated diabetes is primarily contingent on its overexpression within β-cells, and not merely a general feature of its overexpression in acinar cells or pancreas as a whole. It is also tempting to speculate that TGIF1 might modulate β-cell's ability to effectively produce insulin.

Figure 3, Friend *et al.*



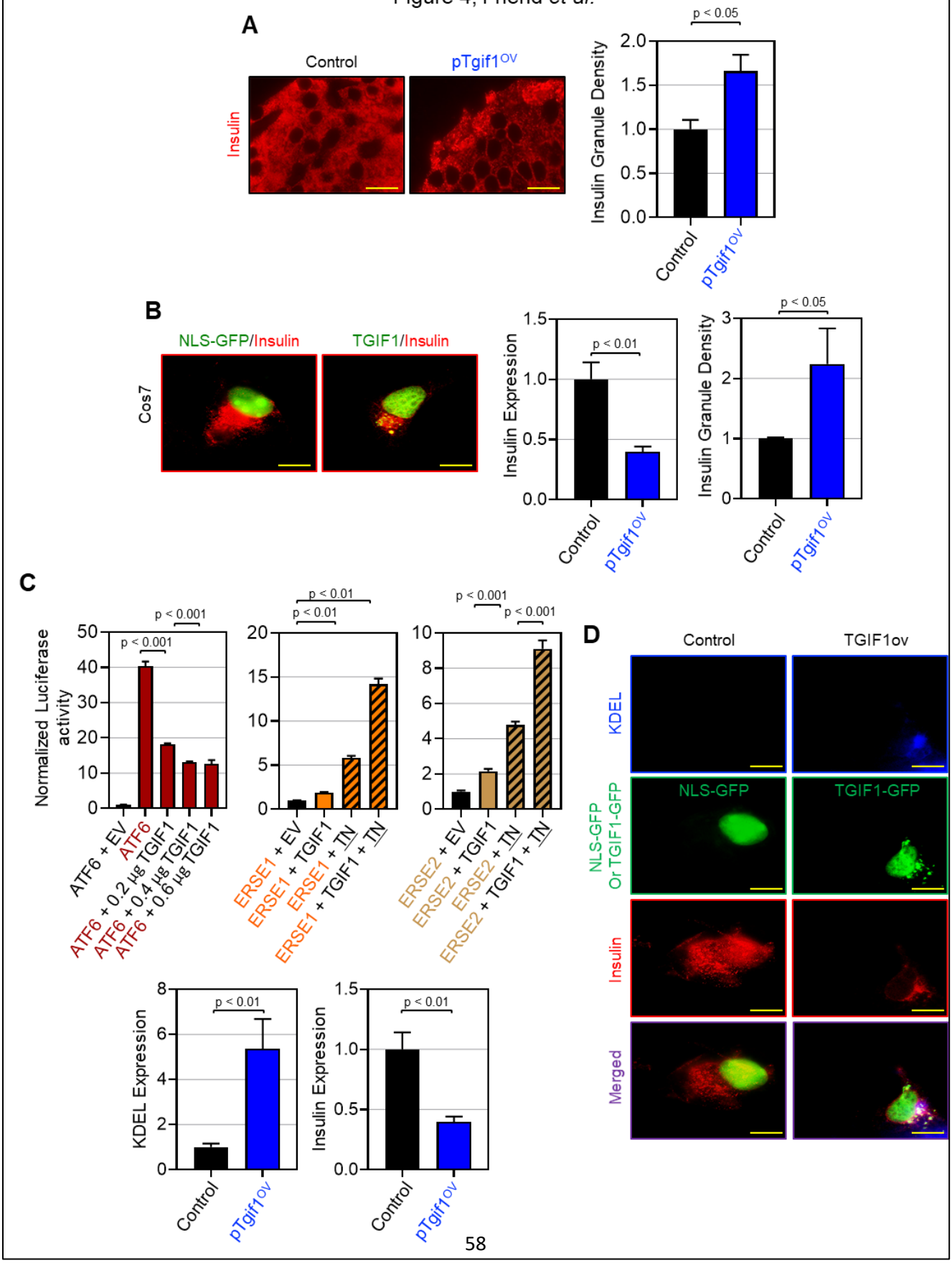
TGIF1 overexpression in pancreatic β-cells perturbs insulin homeostasis

In the course of our immunofluorescence experiments, we serendipitously discovered an additional anomaly in pTgif1^{OV} and Ins2Tgif1^{OV} β-cells that is abnormal spatial organization of insulin within β-cells. To understand this phenomenon further, we utilized high-resolution microscopy to compare the spatial distribution of insulin in pTgif1^{OV} mice and their wild-type siblings. As shown in Figure 4A, insulin spatial architecture in pTgif1^{OV} β-cells appeared to be highly disordered, being very compact and sometimes forming relatively large aggregates along the cytoplasm in β-cells. In stark contrast, wild-type siblings exhibited a more dispersed and homogenous pattern of insulin distribution, consistent with previous studies (Figure 4A). We next used a computational algorithm designed to assess pixel intensity variance to quantify insulin dispersion and compaction within the entire surface area of β-cell. This analysis clearly showed that insulin was extensively aggregated in pTgif1^{OV} β-cells despite the fact that the overall insulin expression was diminished as compared to control mice (Figure 4A). Based on these findings, we postulate that enforced TGIF1 expression in β-cells might lead to diabetes at least through mechanisms that perturb insulin spatial distribution as well as its expression.

To corroborate these predictions, we conducted in vitro experiments using pancreatic cells co-transfected with a constitutive plasmid encoding insulin in the absence or presence of a TGIF1 cDNA, with the aim to focus on insulin spatial distribution while avoiding any interference that could result from variation in its gene expression. In contrast to cells expressing insulin only, which showed homogeneous insulin distribution, cells with concomitant TGIF1 overexpression accumulate large insulin aggregates exhibiting high pixel intensity, congruent with our in vivo findings. Also congruent with our

in vivo finding, the overall pixel intensity significantly decreased in cells co-expressing insulin together with TGIF1 as compared to cells receiving insulin alone (Figure 4B). In parallel immunoblotting experiments confirmed that enforced TGIF1 expression was associated with decreased insulin protein expression (Figure S4A). We also conducted gene reporter assays using luciferase under the promoter of Ins2, and the results showed that enforced TGIF1 expression failed to repress Ins2 gene expression. As a positive control, Ins2 gene expression was significantly induced upon expression of Pdx1, the master transcription factor regulating insulin expression (Figure S4B). Based on these findings, one would conclude that enforced expression of TGIF1 perturbs insulin spatial distribution, which could conceivably account for its accumulation in aggregates, and in turn compromises it expression and secretion to an extent that culminates in severe diabetes.

Figure 4, Friend *et al.*



TGIF1 restricts UPR in β-cells

To investigate the molecular mechanisms by which TGIF1 drives insulin mislocalization and aggregation, we conducted global transcriptomic analysis (RNA-Seq) to identify genes that are differentially expressed in pTgif1^{OV} mice relative to their wild-type siblings. Preliminary gene enrichment analysis results highlighted a statistically significant repression of pathways associated with protein export, protein processing, and even more specifically, protein processing and folding pathways within the ER (Figure S4A). Interestingly, among the most significantly repressed genes in pTgif1ov mice stood out key regulators of the Unfolded Protein Response (UPR) pathway, including Ero1, XBP1, and ATF6, among other. These data prompted us to speculate that TGIF1-mediated downregulation of UPR signaling in pTgif1ov mice might affect proper folding of insulin, which could conceivably lead to its aggregation and concomitant degradation. There is precedent to suggest this scenario, for instance in Akita mice in which a mutation in insulin that affects its folding leads to its accumulation in intracellular aggregates and destabilization. Additionally, misfolding of insulin is a general hallmark of human T2D, being considered as the first phase of aggregation that engenders a first wave of ER stress in β-cells to escalate the aggregation of unfolded /misfolded insulin peptide. Taking advantage of these observations, we endeavored to assess the effects of TGIF1 overexpression on both UPR and ER stress responses. We initially conducted gene reporter assays with constructs featuring luciferase under the control of UPR responsive elements (ATF6-Luc), a target genes involved in UPR. We found that ectopic expression of ATF6, a self-inducer of ATF6-luc promoter (UPR) activity, led to a dramatic increase in ATF6-Luc luciferase levels, and this effect was severely mitigated upon TGIF1

expression, in line with our in vivo transcriptomic data in pTgif1^{OV} mice showing that TGIF1 inhibits UPR signaling (Figure 4C). Next, we reasoned that if TGIF1 indeed compromises the general canonical UPR response, its enforced expression should result in ER stress, as observed in Akita and other mouse models of diabetes as well. Here again, we used constructs in which the expression of luciferase is under the control of ER stress-responsive elements, specifically ERSE1, ERSE2. Upon TGIF1 overexpression, we observed a significant increase in ERSE1-Luc and ERSE1-Luc expression, revealing an ability of TGIF1 to induce ER stress at steady states conditions (Figure 4C). To determine whether TGIF1 could exacerbate ER stress initiated in response to other stimuli, we utilized Tunicamycin, an antibiotic that is known to induce overwhelming ER stress through suppression of N-glycosylation in the endothelium reticulum. We detected a massive increase in ERSE1-Luc and ERSE1-Luc expression, and this effect was further increased upon TGIF1 overexpression, indicating that TGIF1 can also exacerbate ER stress even under the most deleterious responses (Figure 4C). In a more direct approach, we examined the expression and localization of a fluorescent reporter gene (BFP) fused to the KDEL sequence, which is found in the vast majority of proteins residing in the ER and serves as an ER retention signal. We generated cells stably expressing BFP-KDEL and tested its behavior in response to TGIF1 overexpression. Interestingly, we observed a marked increase in the aggregation of BFP-KDEL in the ER, providing further evidence that TGIF1 induces ER stress, consistent with its ability to suppress UPR signaling. We also attempted to harness the aggregation of the BFP-KDEL reporter to investigate whether TGIF1 could lead to the aggregation of insulin in the ER, performing co-immunofluorescence assays to visualize the spatial organization of BFP-KDEL and

insulin. As shown in Figure 4D, enforced expression of TGIF1 led to the formation of insulin aggregates, which predominately co-localize with BFP-KDEL aggregates, strongly suggesting that TGIF1 affects insulin homeostasis in the ER compartment. The collective observations support a model in which TGIF1 functions to inhibit UPR signaling, thereby initiating a series of events leading to heightened ER stress, altered intracellular distribution and availability of insulin, and ultimately diabetes.

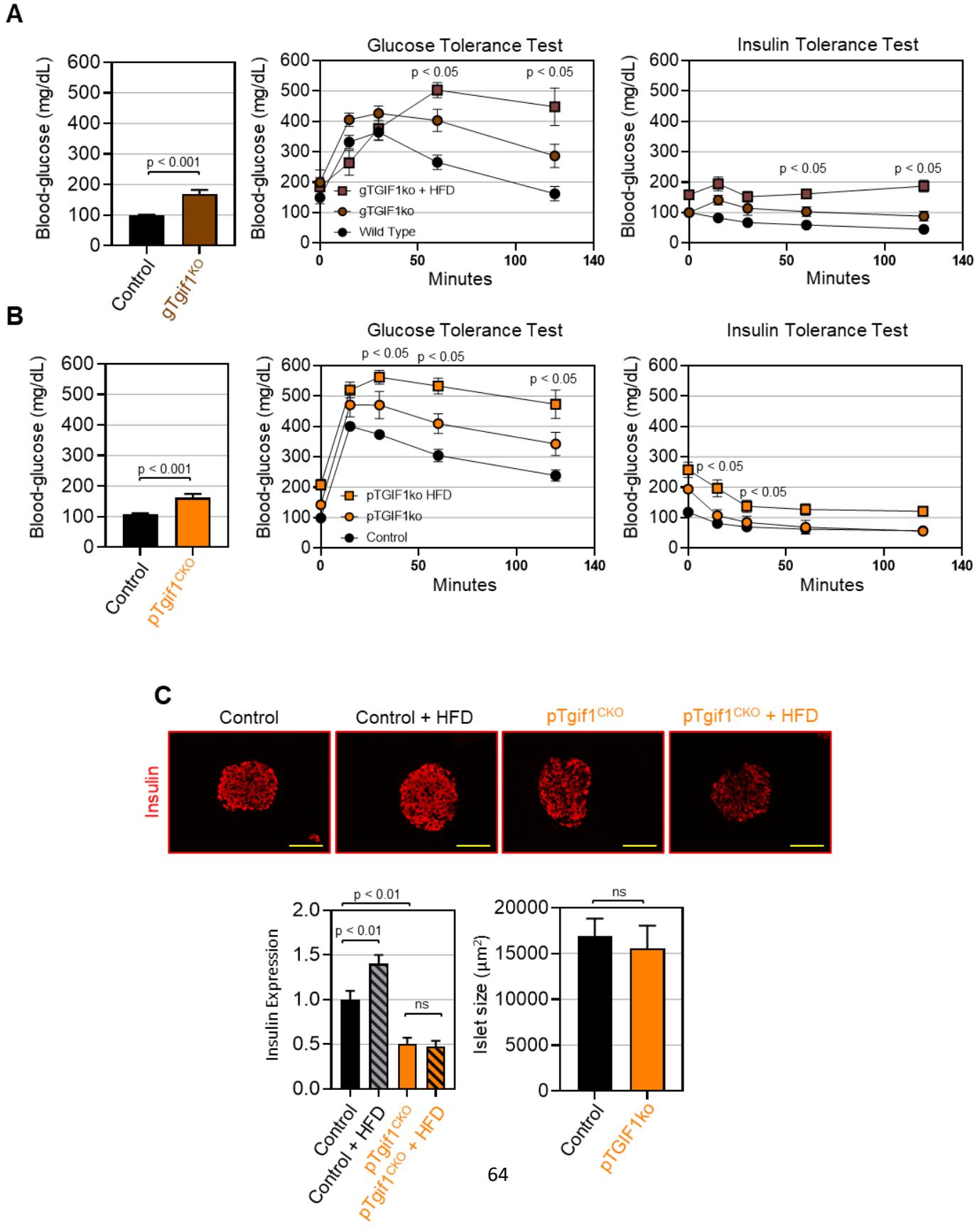
TGIF1 deficiency leads to diabetes

In light of our findings that TGIF1 overexpression compromises insulin folding and integrity, we turned our attention on the potential impact endogenous TGIF1 could play in pancreatic function and metabolic regulation under steady state or high-fat diet-induced hyperglycemia. To accomplish this, we generated Tgif1 global knockout (gTgif1^{KO}) in the C57BL/6 background, which become obese, insulin resistant, and glucose intolerant following prolonged high-fat diet feeding (Figures 5A and S5A). Surprisingly, preliminary assessments of gTgif1^{KO} mice fed regular chow revealed a suboptimal glycemic profile characterized by modest but significant hyperglycemia under fasting conditions (Figure 5A). Congruent with this observation, glucose tolerance test (GTT) showed that gTgif1^{KO} mice displayed impaired glucose tolerance relative to wild-type mice (Figure 5A). Under high-fat diet feeding conditions, these defects not only persisted but also continued to worsen as animals gained weight, prompting us to speculate that gTgif1^{KO} mice might also suffer from inadequate insulin production, given our previous data with mice overexpressing TGIF1 (pTgif1^{OV}) (Figure S5A). It is noteworthy however, that gTgif1^{KO} mice gained more weight than their wild-type littermates, raising the concern as to

whether the impaired glucose could stem from insulin resistance in peripheral tissues owing to obesity-mediated general inflammation rather defects in insulin production. To discriminate between these two possibilities, we generated mice with pancreas-specific Tgif1 knockout (Tgif1fl/fl;Pdx1.Cre, referred to hereafter as pTgif1^{CKO}) and performed the same experiments in the absence or presence of high-fat diet. Several lines of evidence support the notion that Tgif1 ablation directly affects insulin availability in β-cells. First, pTgif1^{CKO} mice fed regular chow consistently manifested a suboptimal glycemic profile characterized by modest hyperglycemia, similar to what was observed in mice with Tgif1 global knockout (Figure 5B). Second, pTGIF1^{CKO} mice displayed impaired glucose tolerance, which tends to worsen with high-fat diet feeding, similar to what observed in mice with Tgif1 global knockout. Third, administration of insulin to pTgif1^{CKO} mice was able to normalize their blood glucose to an extent approaching that of wild-type mice irrespective of whether mice were fed regular or high-fat diet, suggestive of a defect in insulin secretion (Figure 5B). This also indicated that pTgif1^{CKO} mice have normal response to insulin in peripheral tissue. Fourth, relative to wild-type siblings, pTgif1^{CKO} mice displayed decreased circulating insulin levels, reinforcing the idea that these mice might have a defect in insulin availability, secretion, or both. Fifth, histological analysis showed that the size of islets in pTgif1^{CKO} mice was indistinguishable from those of control mice. Finally, immunofluorescence staining revealed a striking phenomenon of irregular insulin expression throughout out the islets, with the majority of cells displaying attenuated expression of insulin or even complete absence of insulin, consistent with the low circulating levels of insulin. During the course of this genetic study, we also sought to rule out potential compensation from perturbed expression of TGIF2, which shares structural

and functional homology with TGIF1. Accordingly, we generated mice with pancreas-specific double knockout of Tgif1 and Tgif2. These mice did not display any signs of abnormal physical or behavioral activity, yet they displayed a metabolic phenotype similar to that of pTgif1^{KO} mice, characterized by mild hyperglycemia, glucose intolerance, and decreased circulating insulin levels (Figure S5B). Based on this finding, as well as earlier observations of TGIF1 inhibits UPR signaling in β-cells, the most plausible explanation is that Tgif1 ablation culminates in hyperactive UPR signaling, thereby leading to sustained folding of insulin to an extent that exceeds demand, in turn leading to insulin rapid clearance. The notion that overly-efficient insulin folding and processing leading to diabetes is quite coherent and not new, as it has been reported in other landmark studies that β-cells often engage proteasome and/or autophagosome machineries to clear surplus or excess insulin, emanating due to hyperactivation of UPR signaling.

Figure 5, Friend *et al.*



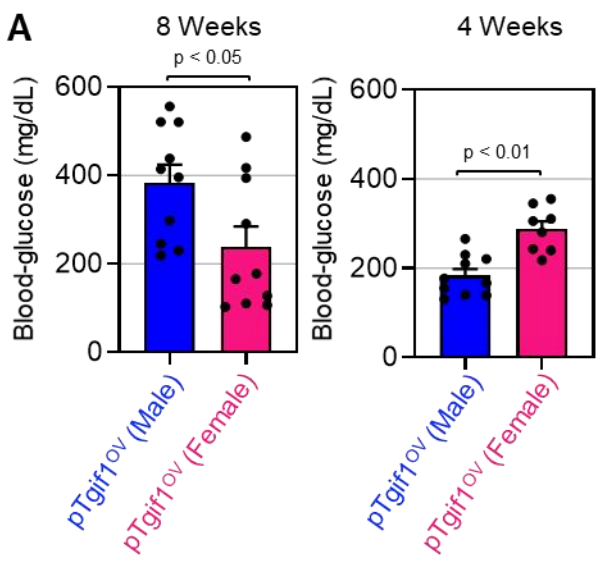
TGIF1 overexpression drives neonatal diabetes

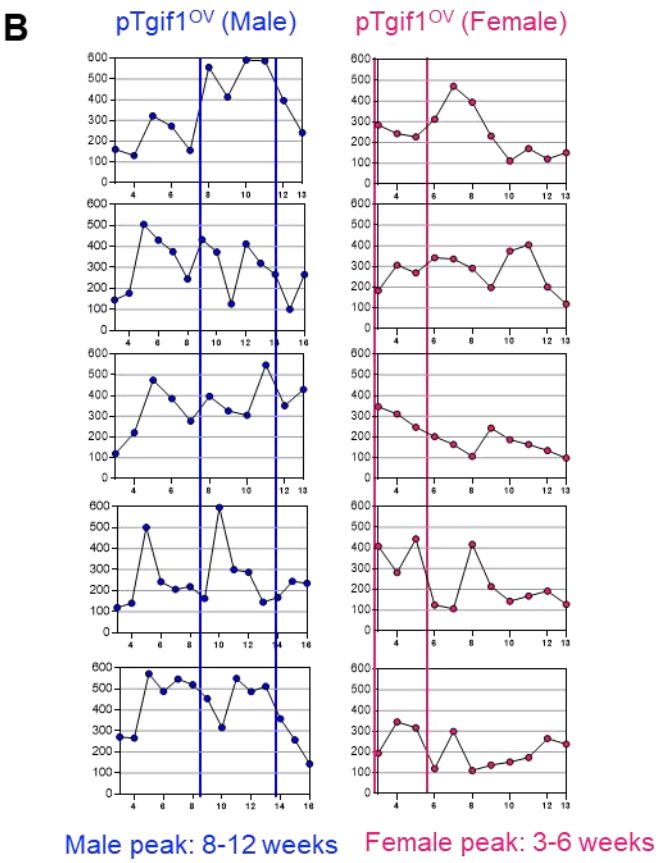
As mentioned earlier, the hyperglycemia phenotype in 8-week-old pTgif1^{OV} mice was very heterogeneous among and between male and female groups, as gauged by blood-glucose measurements. Most notably, approximately 30% of males displayed blood-glucose concentrations ranging from 160 to 250 mg/dL, which is considered mildly diabetic in mice, while more than 50% of mice displayed concentrations exceeding 260 mg/dL, representative of a more severe and advanced presentation of diabetes. Conversely, while more than 25% of female mice displayed concentrations between 160 to 250 mg/dL, only a small fraction of less than 10% presented with concentrations beyond 260 mg/dL (Figure 6A). Intriguingly, when we conducted similar measurements with the youngest cohorts of mice, the trend was opposite, in that more female mice were severely hyperglycemic than male mice (i.e., 50% versus 10%), with mean blood glucose levels exceeding 260 mg/dL (Figure 6A). Given that pTgif1^{OV} mice were generated on a pure FVB/N background, we initially interpreted these findings as experimental variations due to cohorts being obtained from different progenitor mice. To explore this discrepancy further, we monitored fasting blood glucose variations between the age of three weeks (after weaning to avoid interference from mother feeding) and 4 months, measuring fasting blood-glucose once a week. We detected a very rapid, sharp (often exceeding 300 mg/dL), and transient increase in blood glucose in the fast majority of female mice, peaking at the age of 3 or 4 weeks, and then declining to mild hyperglycemic levels or even to normal glycemic levels after 6 weeks (Figure 6B). Male mice also experienced the same trend but with delayed kinetics, attaining the peak level between the age of 5 and 6 weeks, before declining sharply between the age of 10 and 12 weeks. Nonetheless,

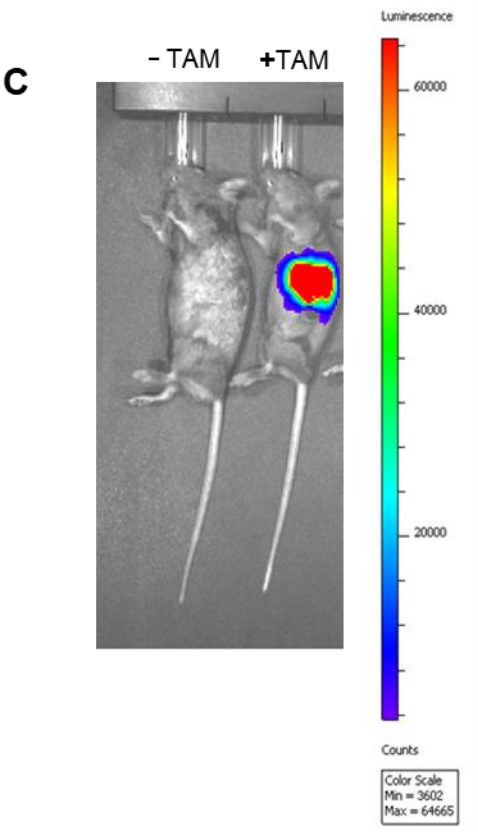
a small fraction of pTgif1^{OV} mice (10% for female and 25% for male) did not follow this transient pattern and instead continued to exhibit a severe hyperglycemia phenotype lasting at least until the termination of the experiment (Figure 6B). This transient and sometimes permanent diabetic phenotype closely resembles the neonatal form of diabetes human, a poorly understood disorder that arises in infants within the first six months of life and then either disappears later during the infant age or persists lifelong.

In terms of disease etiology, many reports have associated a variety of genetic, epigenetic, and in utero factors with neonatal diabetes, though most of the observations remain somewhat speculative. One of the well-established causes of neonatal diabetes is perturbation in insulin folding, due to mutations in the insulin gene itself or alterations that disrupt UPR signaling. Direct evidence supporting this notion came from genetic studies using mice with deletion of EIF2AK3, the gene encoding PERK (Protein Kinase RNA-Like ER Kinase), which governs an essential branch of the UPR signaling. Genetic deletion of EIF2AK3 in β-cells during early development leads to severe diabetes, while its ablation in the adulthood is dispensable for normal β-cell function and glucose homeostasis. Based on this literature, we reasoned that if TGIF1 overexpression in pTgif1^{OV} mice during early development indeed drives neonatal diabetes due to its ability to impair UPR signaling, then enforcing TGIF1 expression in adult mice should be void of any harmful effects on β-cells. To probe this possibility, we crossed our LSL-CAG-Tgif1 with Ins2-CreERT2 mice, which provides temporal activation of Cre recombinase by the administration of tamoxifen. To monitor the activation of Cre recombination, we also included a conditional luciferase (LSL-Luc) targeted to the Rosa26 mice. As anticipated, administration of tamoxifen to this compound mice (iTgif1$^{OV\text{-}Luc}$) at the age of three months

led to efficient activation of Cre recombinase, as assessed by bioluminescence imaging using live animals (Figure 6C). Despite several attempts during a period exceeding 6 months following Cre recombinase activation, we were never able to see any significant changes in blood glucose levels irrespective of the sex, weight, or age of the cohorts (Figure 6C). To the best of our knowledge, this is the first demonstration that TGIF1 plays a causal role in the pathogenesis of neonatal diabetes, which is congruent with and reinforces its function in restricting UPR signaling in pancreatic β-cells.


A
8 Weeks
4 Weeks
p < 0.05
p < 0.01
Blood-glucose (mg/dL)
600
400
200
0
pTgif1OV (Male)
pTgif1OV (Female)


B
pTgif1OV (Male)
pTgif1OV (Female)
Male peak: 8-12 weeks
Female peak: 3-6 weeks


C
- TAM
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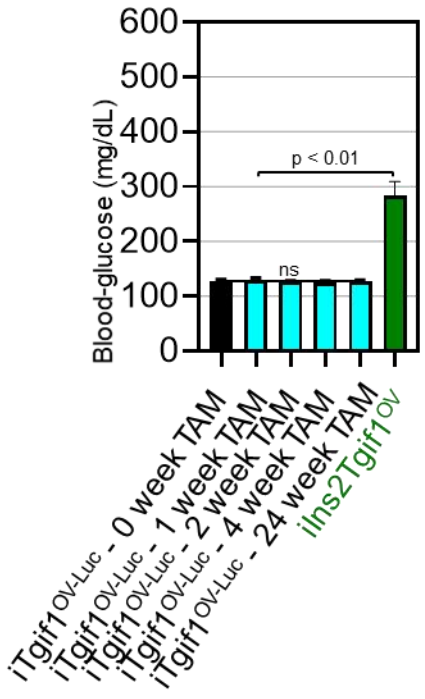
Luminescence
60000
40000
20000
Counts
Color Scale
Min = 3602
Max = 64665


600
500
400
300
200
100
0
Blood-glucose (mg/dL)
p < 0.01
ns
iTgif1OV-Luc - 0 week TAM
iTgif1OV-Luc - 1 week TAM
iTgif1OV-Luc - 2 week TAM
iTgif1OV-Luc - 4 week TAM
iTgif1OV-Luc - 24 week TAM
iIns2Tgif1OV

Discussion and future directions

Overall, our study provides the first evidence that TGIF1 plays an instrumental role in diabetes pathogenesis, affording a tremendous advance in our understanding of β-cells, while adding a critical dimension to the elucidation of the molecular mechanisms governing insulin homeostasis. Diabetes typically results from impaired insulin secretion, insulin resistance, or a combination of both (DeFronzo, Ferrannini et al. 2015), and is associated with a variety of indolent or detrimental complications that ultimately hinder the quality of life and survival of patients. Our new pTgif1^{OV} mouse model with enforced expression of TGIF1 in pancreatic progenitor cells during early development exhibits numerous clinical features emblematic of diabetes, including polyuria, polydipsia, and a reduction in body mass (Karpińska and Czauderna 2022). The discovery that pTgif1^{OV} mice present with diminished levels of insulin both in the circulatory system and within β-cells reinforces the notion that TGIF1 overexpression impairs glucose homeostasis through mechanisms affecting β-cells functions, most specifically, their capacity to properly process insulin. Therefore, our findings provide new insights into mechanistic paradigms of diabetes mellitus, which could ultimately pave the way for innovative therapeutic strategies to curb this debilitating disease.

The absence of overt anatomical or developmental pancreatic abnormalities in pTgif1^{OV} mice indicates that the primary impact of TGIF1 overexpression in pancreatic cells is likely a result of metabolic defects rather than alterations in early development processes, which is particularly relevant given the established roles of TGIF1 in early development (Wotton and Massagué 2001). Thus, our data challenge the conventional

perception that TGIF1's role is confined to specifying cell fate during development, reframing it as a critical regulator of metabolism homeostasis, functioning in pancreatic β-cells to regulate insulin processing and thereby its availability. Furthermore, the observation that pTgif1^{OV} mice maintain an intact pancreatic anatomy implicates TGIF1's overexpression in impairing β-cell functions rather than inducing structural pancreatic defects.

Interestingly, the sex-specific variability in the diabetic phenotype observed in the six-week-old pTgif1^{OV} mice, with females showing either no or somewhat attenuated levels of hyperglycemia compared to males, echo observations widely reported in the Akita model of diabetes (Izumi, Yokota-Hashimoto et al. 2003). In Akita mice, a mutation in the Ins2 gene leads to the misfolding of insulin, creating ER stress this is deemed responsible for the diabetic phenotype (Zraika, Hull et al. 2009). This resemblance provided us with the first hint that TGIF1 may intersect with molecular pathways implicated in the ER stress, perhaps thereby affecting insulin integrity and ensuing β-cell dysfunction in a sex-differential manner. Such a mechanism is perfectly consistent with literature emphasizing the differential ER stress and UPR signaling between sexes (Hodeify, Megyesi et al. 2013). The consistency across pTgif1^{OV} and Akita models reinforces the concept that sex-specific factors may influence differential regulatory mechanisms of TGIF1 in male and female, or perhaps differing susceptibilities to metabolic disturbances caused by TGIF1 dysregulation. Regardless, these previous findings and our data underscore the critical role of a tightly regulated TGIF1 expression for normal β-cell function and insulin homeostasis.

Seeking mechanisms, although it was intriguing that a subset of pTgif1^{OV} mice developed ADM, the majority of hyperglycemic mice were free of these inflammatory-related lesions, ruling out the possibility that the effects of TGIF1 is mediated through a non-cell autonomous mechanisms. Direct support comes from Ins2Tgif1^{OV} mice, which phenotypically mimic the hyperglycemia and insulin deficits seen in pTgif1^{OV} mice, but do not develop ADM, unambiguously demonstrating that TGIF1 dysregulation in β-cell-specific was the primary cause of the diabetic phenotype. On the other hand, Ptf1aTgif1^{OV} mice had normal glycemia despite the presence of abundant ADM in the acinar compartment. This is a critical distinction that separates the hyperglycemic effects of TGIF1 overexpression from the ADM-associated pathology often observed in inflammatory pancreatic diseases and pancreatic ductal adenocarcinoma (PDAC) (Kopp, von Figura et al. 2012). Besides ADM, the apparent normal histological integrity of islet architecture in pTgif1^{OV} mice also implies that TGIF1-mediated hyperglycemia is not a consequence of morphological alterations or altered islet cellularity, both of which are shown to drive diabetes under certain circumstances. Instead, our data point towards β-cell dysfunction as the underlying mechanism, in apparent agreement with the growing body of evidence that β-cell functional impairment can precipitate diabetes in the absence of significant β-cell loss (Halban, Polonsky et al. 2014).

Given the relatively high levels of TGIF1 in islets, it was tempting to envision that TGIF1 may act as a key regulator of β-cell function, probably influencing insulin synthesis, processing, or secretion. This assumption prompted a shift in our efforts towards intrinsic islet mechanisms, focusing on insulin and glucagon, the major regulators of glucose metabolism. The results revealed a marked decrease in insulin expression in β-cells while

the expression of glucagon was not affected. But most appealing was the fact that TGIF1 induced the accumulation of insulin in large aggregates, providing us with the first indication that TGIF1 might affect the folding of insulin. Of note, our histological analyses failed to show any loss of β-cell populations or β-cell mass. The aberrant aggregation of insulin observed in both pTgif1^{OV} mice and echoed in Ins2Tgif1^{OV} mice emphasizes a striking distinction with the typical insulin homogeneous spatial architecture seen in healthy β-cells. This anomalous spatial organization of insulin was also coupled with a reduction in its overall expression, indicating that TGIF1 overexpression may perturb post-translational processing of insulin. Similar aggregation and decreased expression of insulin were observed in cell systems in vitro, suggesting that the effects of TGIF1 are mediated through a cell autonomous mechanism. Under these conditions, TGIF1 had no effect on insulin gene expression, reiterating the possibility that TGIF1 might influence the processing of insulin, either during its maturation or vesicular trafficking, but with the net effect insulin misfolding and aggregation in β-cells. These effects are likely to be driven through TGIF1's ability to suppress UPR signaling, as gauged by global transcriptomic as well as molecular approaches. As defects in UPR signaling culminates in ER stress and concomitant protein aggregation and degradation, we went on to perform experiments to demonstrate that enforcement of TGIF1 expression was sufficient to drive ER stress, thereby providing a mechanistic explanation as to its ability to drive insulin aggregation and decreased expression. These innovative insights into the perturbation of insulin homeostasis offer new perspectives on the β-cell dysfunction in diabetes that could be harnessed for mechanistic or therapeutic purposes.

Our observations are physiologically relevant as ablation of the Tgif1 gene was also deleterious to β-cell function and homeostasis. In fact, using mice with global (gTgif1^{KO}) or pancreas-specific (pTgif1^{CKO}) knockout of Tgif1, we consistently detected substantial hyperglycemia at steady states and very pronounced glucose intolerance when mice were challenged with glucose. High-fat diet feeding exacerbated these metabolic defects, clearly indicating that maintaining proper TGIF1 physiological levels is essential for preserving normal β-cell function. Interestingly, challenging mice with exogenous insulin almost completely normalized the blood glucose levels in pTgif1^{CKO} mice, lending support the notion that the primary defect in these mice is likely related to insulin production rather than peripheral insulin sensitivity. Drawing from these observations and earlier studies linking TGIF1 to UPR signaling, it is plausible to speculate that the absence of TGIF1, as much as its overexpression, might lead to dysregulated UPR signaling in β-cells. In the case of TGIF1 absence, the UPR is instead hyperactive, and paradoxically, results in an overly efficient insulin folding process that oversaturate the β-cell's capacity to maintain homeostatic insulin levels. Landmark literature reinforces this model wherein an extensive UPR folding behavior triggers cellular responses to limit insulin processing, including through proteasomal degradation and autophagy. This intricate mechanism underscores a delicate balance in β-cell, where both deficient and excessive UPR activity can culminate in diabetes, therefore reinforcing the general paradigm that not only is the failure to produce or fold insulin detrimental but also an overly efficient folding system that exceeds the cellular capacity for insulin storage and secretion yields a similar outcome.

The most intriguing finding in this study is the discovery that TGIF1 might be intimately linked to neonatal diabetes, an enigmatic form of diabetes whose etiology and mechanisms are still unfolding. The transient hyperglycemic patterns together with the persistent, lifelong diabetes in a fraction of pTgif1^{OV} mice draw a striking parallel to human neonatal diabetes, which encompasses both transient (TNDM) and permanent (PNDM) neonatal diabetes mellitus. The transient elevation in blood glucose levels observed in our model, which subsequently resolves to milder hyperglycemia or returns to normoglycemia, mirrors TNDM, where hyperglycemia appears shortly after birth and remits within a few weeks to months. In contrast, the sustained hyperglycemia seen in a subset of both pTgif1^{OV} mice suggests a condition akin to PNDM, where diabetes is diagnosed within the first six months of life and persists without remission. This discovery is unprecedented, as none of the etiological genes identified so far in TNDM or PNDM and tested in genetically engineered mouse model fulfill the criteria of a candidate for TNDM and PNDM alike. The mechanistic insights from our model resonate with the literature on neonatal diabetes, where mutations in the INS gene itself that disrupt its folding, lead to PNDM. Additionally, mutations that disrupt UPR signaling, such as those found in EIF2AK3, result in PNDM. Our observations suggest that TGIF1 functions during a critical developmental window to fine-tune the UPR signaling, and any perturbation in this balance could lead to diabetes phenotypes resembling both TNDM and PNDM. It is important to highlight that enforced expression of TGIF1 in mature, adult animals did not affect their blood glucose levels even following prolonged overexpression. As such, the differential impact of TGIF1 on neonatal and adult β-cells offers a novel perspective on β-cells, both in terms of the resilient traits and vulnerabilities during early development and

adult life, thus shedding new light on mechanistic paradigms of TNDM, PNDM, and perhaps diabetes mellitus in general. Future investigations to unravel TGIF1's exact role in the evolving β-cells could reveal targets for therapeutic intervention during critical periods of β-cell life with the potential to mitigate or even prevent the onset of neonatal diabetes.

Figure S1, Friend *et al.*

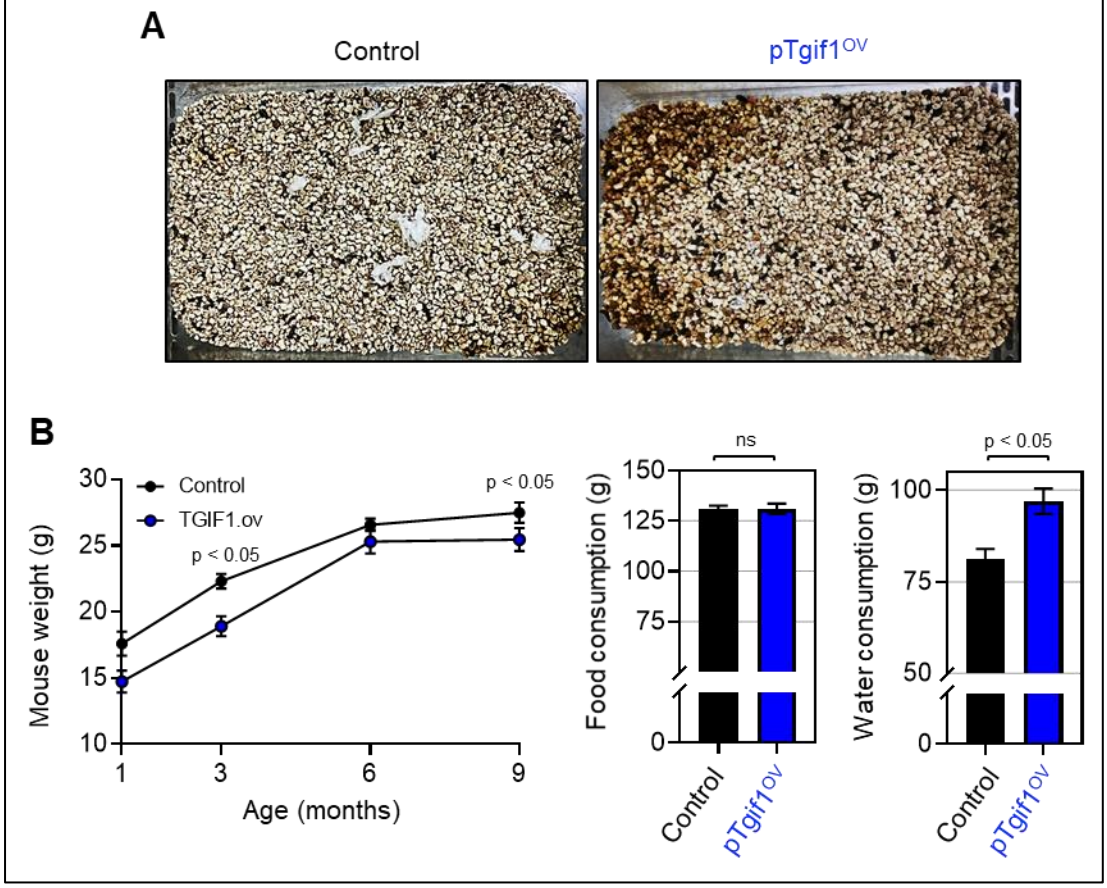
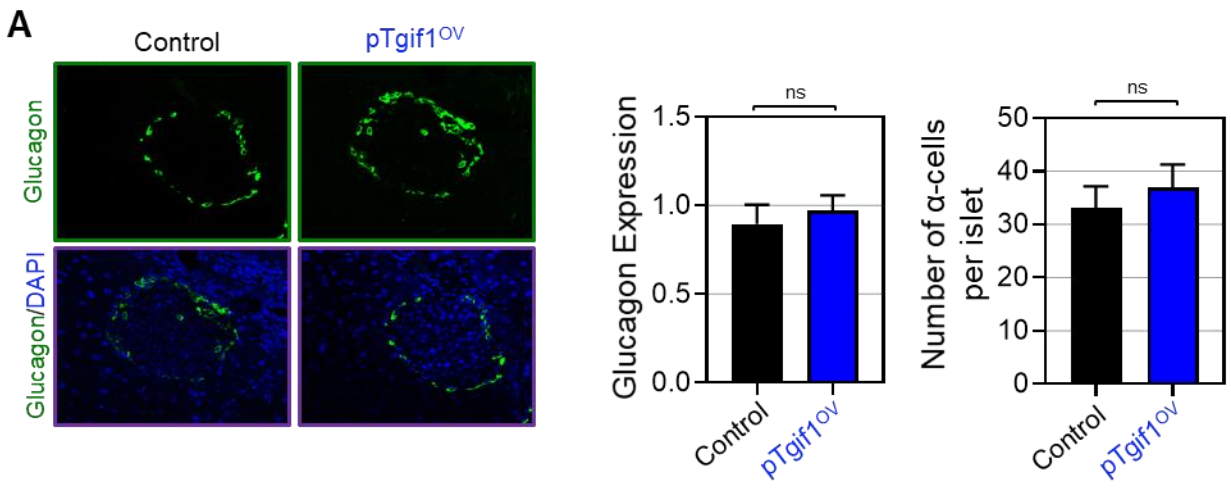


Figure S3, Friend *et al.*



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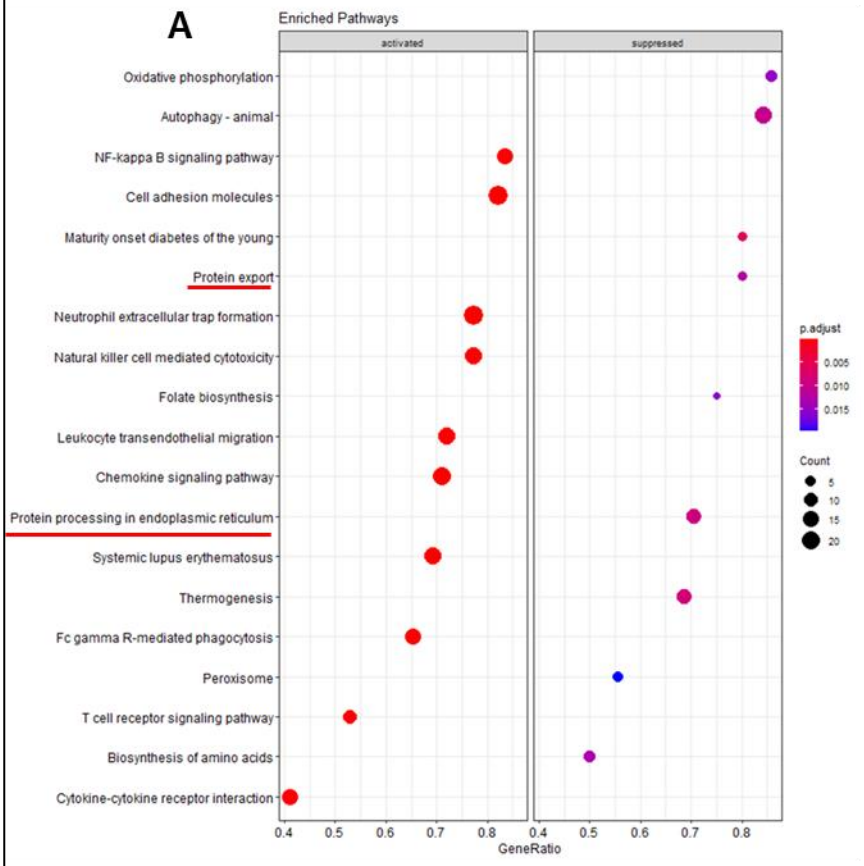

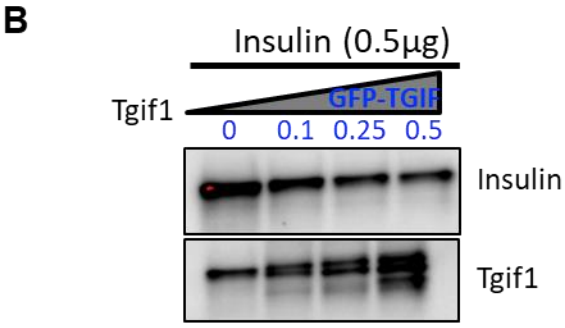

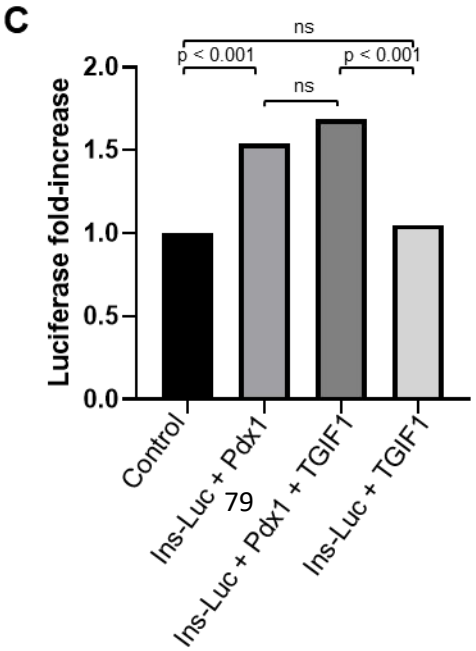

Figure S5, Friend *et al.*

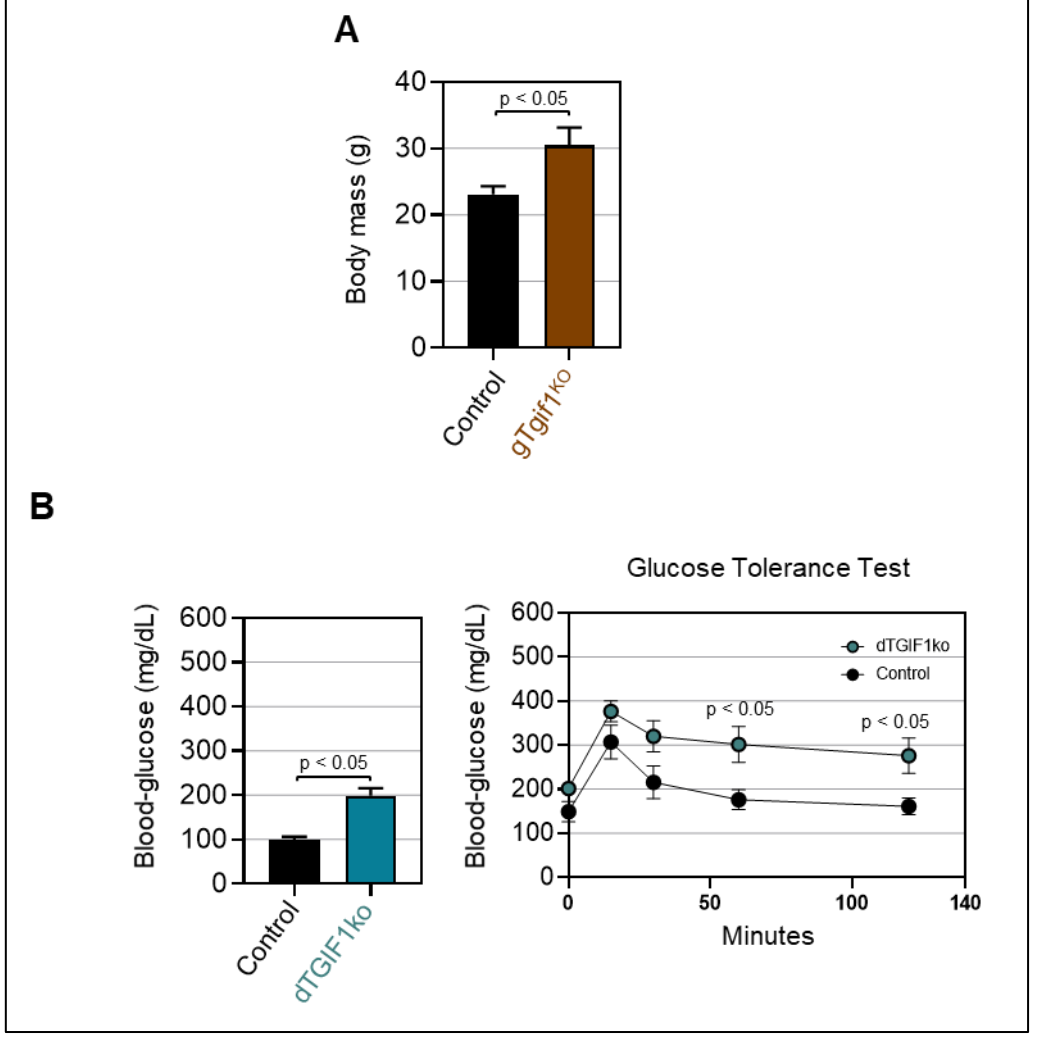

Figure 6, Friend *et al.*

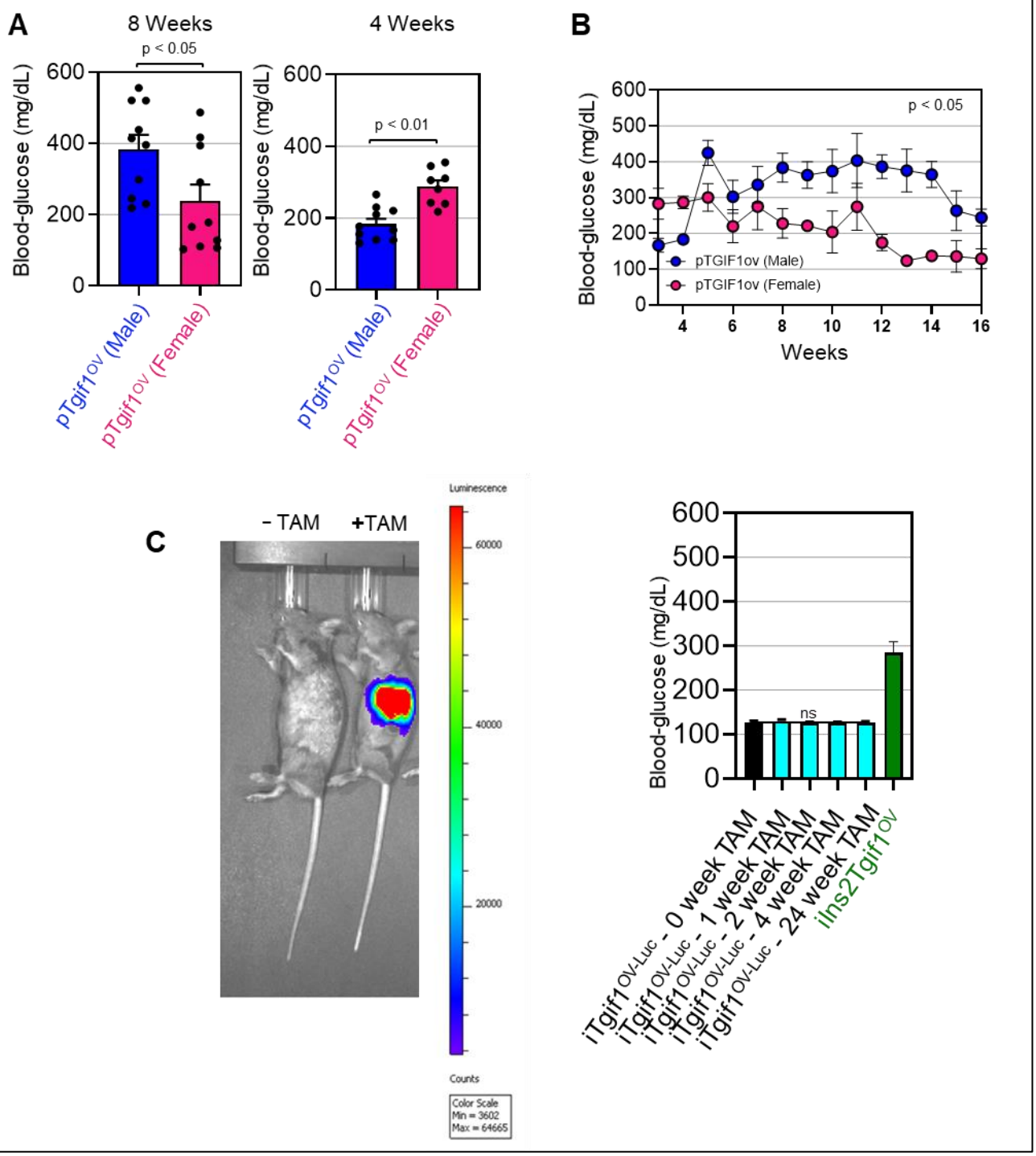

Figure Legends

Figure 1: Overexpression TGIF1 in pancreatic progenitor cells leads to diabetes

A: Pictographic schematic of TGIF1 cassette insertion in pTGIF1ov mice showing conditional TGIF1 overexpression upon Cre-loxP mediated recombination.

B: Comparative fasted blood-glucose concentrations in pTGIF1ov mice and wild-type littermates was measured and recorded (n=33). Serum C-peptide levels measured by ELISA in pTGIF1ov mice and control littermates, presented in picomolar (pM) concentrations (n=17). Relative HbA1c measurements were assayed from whole blood and serum samples from pTGIF1ov mice (n=16). Quantitative measurements are presented as mean ± SEM.

C: Fasting blood-glucose levels, C-peptide concentrations, and HbA1c percentages in control vs pTGIF1ov mice, with pTGIF1ov readings stratified by sex, providing a comparative analysis across the metabolic parameters assessed (n=8). Data are expressed as mean ± SEM.

Figure 2: TGIF1-mediated ADM is dispensable for the diabetic phenotype

A: FFPE pancreatic sections from 1-month-old control and pTGIF1ov mice (n=20) were stained with H&E. Representative pictures of normal and ADM regions areas are shown. Scale bars: 50 µm (left). Islet surface area, islet abundance, average islet cell count were all quantified for both control and pTGIF1ov groups. Quantitative measurements are presented as mean ± SEM.

B: FFPE pancreatic sections from 1-month-old control and pTGIF1ov mice (n=8) were stained with H&E or immunostained with anti-CK19 or Sox9 antibody and subjected to IHC. Representative pictures of normal and ADM regions areas are shown. Scale bars: 50 µm (left). CK19 and Sox9 expression were both quantified and presented as bar graphs. Quantitative measurements are presented as mean ± SEM.

C: FFPE pancreatic sections from 1-month-old control and pTGIF1ov mice (n=8) were immunostained with anti-TGIF1 antibody and subjected to IHC. Representative images of pancreatic acinar and islets cells are shown for each group. Scale bars: 50 µm (left). TGIF1 expression was quantified and presented as a bar graph. Quantitative measurements are presented as mean ± SEM.

Figure 3: TGIF1 overexpression in pancreatic β-cells perturbs insulin production

A: FFPE pancreatic sections from 1-month-old control and pTGIF1ov mice (n=25) were immunostained with anti-insulin antibody and subjected to IF. Representative islet regions of the pancreas are shown. Scale bars: 50 μm (left). Mean fluorescence intensity was quantified and presented as a bar graph. Quantitative measurements are presented as mean ± SEM.

B: Expression of insulin protein in INS-1 β-cells transfected with TGIF1 cDNA was measured via direct ELISA of insulin from cell lysate. Quantitative measurements are presented as mean ± SEM.

C: FFPE pancreatic sections from 1-month-old control, ins2TGIF1ov, and ptf1aTGIF1ov, mice (n=11) were immunostained with anti-insulin antibody and subjected to IF. Representative islet regions of the pancreas are shown. Scale bars: 50 μm (left). Blood-glucose measurements, mean fluorescence intensity of insulin, and the number of insulin positive β-cells were quantified and presented as bar graphs. Quantitative measurements are presented as mean ± SEM.

D: FFPE pancreatic sections from 1-month-old control, ins2TGIF1ov, and ptf1aTGIF1ov, mice (n=11) were immunostained with anti-CK19 or Sox9 antibody and subjected to IHC. Representative images of normal pancreatic acinar or ADM regions are shown. Scale bars: 50 μm (left). Sox9 and CK19 expression was quantified and shown as bar graphs. Quantitative measurements are presented as mean ± SEM.

Figure 4: TGIF1 overexpression in pancreatic β-cells perturbs insulin homeostasis

A: FFPE pancreatic sections from 1-month-old control and pTGIF1ov mice (n=25) were immunostained with anti-insulin antibody and subjected to IF. Representative islet regions of the pancreas are shown. Image is a resized image of an image acquired at a magnification of 100x. Scale bars: 50 µm (left). Insulin granule density was quantified and presented as a bar graph. Quantitative measurements are presented as mean ± SEM.

B: Expression of insulin and either NLS-GFP or TGIF1-GFP protein in Cos7 cells was assessed via IF assays cell lysate. Cells were probed with anti-insulin antibody and appropriate secondary antibody. Quantitative measurements of insulin and insulin granule density are presented as mean ± SEM.

C: Cos7 cells were co-transfected with ATF6-luc or ERSE1-luc or ERSE2-luc reporters and TGIF1, either with or without Tunicamycin (TN) (except for ATF6 experiment). Luciferase activity was measured and quantified and finally normalized and represented as a bar graph. Data presented as mean ± SEM.

D: Cos7 cells were co-transfected with plasmids encoding for a blue fluorescent protein (BFP) tagged with the KDEL sequence and TGIF1. Post-transfection, cells were cultivated under standard conditions to allow for stable expression and integration. KDEL expression and insulin expression were quantified and presented as a bar graph. Quantitative measurements are presented as mean ± SEM.

Figure 5: TGIF1 deficiency leads to diabetes

A: Comparative fasted blood-glucose concentrations in gTgif1KO mice and control littermates was measured and recorded (n=14). Glucose and insulin tolerance test were carried out on fasted gTgif1KO, gTgif1KO fed HFD, or control mice on independent days following 3 days of recovery from each assay (n=15). Quantitative measurements are presented as mean ± SEM.

B: Comparative fasted blood-glucose concentrations in pTgif1CKO mice and control littermates was measured and recorded (n=14). Glucose and insulin tolerance test were carried out on fasted pTgif1CKO, pTgif1CKO fed HFD, or control mice on independent days following 3 days of recovery from each assay (n=12). Quantitative measurements are presented as mean ± SEM.

C: FFPE pancreatic sections from control, control +HFD, pTgif1CKO, or pTgif1CKO + HFD mice (n=6) were immunostained with anti-insulin antibody and subjected to IF. Representative islet regions of the pancreas are shown. Scale bars: 50 μm (left). Mean fluorescence intensity of insulin, and the size of all islets were quantified and presented as bar graphs. Quantitative measurements are presented as mean ± SEM.

Figure 6: TGIF1 overexpression drives neonatal diabetes

A: Comparative fasted blood-glucose concentrations of pTgif1OV mice stratified by sex at either 8 or 4 weeks (n=10). Quantitative measurements are presented as mean ± SEM.

B: Comparative longitudinal fasted blood-glucose concentrations of pTgif1OV mice stratified by sex after observation over a 16 week period (n=10). Quantitative measurements are presented as mean ± SEM.

C: Depiction of Cre recombinase activity monitoring in Rosa26 mice via a conditional luciferase reporter (LSL-Luc) with or without Tamoxifen (TAM). Following TAM administration to iTgif1OV-Luc mice at three months of age, Cre-mediated recombination efficacy was evaluated using in vivo bioluminescence imaging. Comparative fasted blood-glucose concentrations of iTgif1OV-Luc mice at either 0, 1, 2 or 24 weeks (n=10). Quantitative measurements are presented as mean ± SEM.

Supplemental Figure Legends

Supplemental Figure 1: Overexpression TGIF1 in pancreatic progenitor cells leads to diabetes

A: Photographic images of bed-lining from cages housing either pTgif1OV or control mice over a 48-hour period. 6 mice housed per group per cage.

B: Comparative mass measurements in grams of pTgif1OV or control mice over the course of 9 months (n=18). Quantitative measurements are presented as mean ± SEM.

C: Comparative consumption of chow (grams) and water (grams) between pTgif1OV and control mice over a 48 hour period. Quantitative measurements are presented as mean ± SEM.

Supplemental Figure 3: TGIF1 overexpression in pancreatic β-cells perturbs insulin production

A: FFPE pancreatic sections from pTgif1OV or control mice (n=15) were immunostained with anti-glucagon antibody and subjected to IF. Representative islet regions of the pancreas are shown. Scale bars: 50 μm (left). Mean fluorescence intensity of glucagon, and number of glucagon positive cells were quantified and presented as bar graphs. Quantitative measurements are presented as mean ± SEM.

Supplemental Figure 4: TGIF1 overexpression in pancreatic β-cells perturbs insulin homeostasis

A: Gene set enrichment analysis of either pTgif1OV or control based on RNA-sequencing reads (n=6). Depiction of either activated or suppressed pathways are represented as dots along the plot, larger dots representing higher relative activation or suppression. See color scale for significance determinations.

B: Representative Western Blotting images of Cos7 cells co-transfected with Insulin (Top) and Tgif1 (Bottom). Insulin cDNA remained consistent across each sample, while increasing amounts of Tgif1 cDNA were transfected in sequential order across each sample.

C: Cos7 cells were co-transfected with Ins1-Luc reporters and either TGIF1 or Pdx1, or both together. Luciferase activity was measured and quantified and finally normalized and represented as a bar graph. Data presented as mean ± SEM.

Supplemental Figure 4: TGIF1 overexpression in pancreatic β-cells perturbs insulin homeostasis

A: Gene set enrichment analysis of either pTgif1OV or control based on RNA-sequencing reads (n=6). Depiction of either activated or suppressed pathways are represented as dots along the plot, larger dots representing higher relative activation or suppression. See color scale for significance determinations.

Supplemental Figure 5: TGIF1 deficiency leads to diabetes

A: Comparative mass measurements in grams of gTgif1KO or control mice at 6 weeks (n=20). Quantitative measurements are presented as mean ± SEM.

B: Comparative fasted blood-glucose concentrations in dTgif1KO mice and control littermates was measured and recorded (n=7). Glucose test were carried out on fasted dTgif1KO or control mice (n=7). Quantitative measurements are presented as mean ± SEM.

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