Characterization of a Novel Transcription Factor, Transcription Factor 19 (TCF19), in Diabetes Pathogenesis

by

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Abstract

Diabetes is complex metabolic disorder characterized by defects in insulin secretion or insulin sensitivity, resulting in hyperglycemia. Type 1 diabetes (T1DM) is the result of an autoimmune mediated destruction of the insulin producing beta cells, whereas Type 2 diabetes (T2DM) is more commonly associated with insulin resistance. In both cases, an increase in apoptosis of the beta cells leads to a substantial decrease in beta cell mass. Transcription Factor 19 (TCF19), is a novel diabetes gene associated with both T1DM and T2DM in genome wide association studies (GWAS), suggesting that it plays a central role in diabetes pathogenesis. TCF19 has not been widely studied, but is proposed to regulate the cell cycle. Our lab has previously shown that TCF19 is necessary for proliferation and survival in the INS-1E beta cell line, and that TCF19 regulates DNA damage response (DDR) pathways. The role of DNA damage in the islet and beta cell has not been extensively studied.

In this thesis, I discuss what is known about the DDR in the diabetes field, and specifically, the islet and the beta cell. I also discuss the findings from our TCF19 overexpression model where we discovered novel pathways regulated by TCF19, including the DDR pathway. Additionally, I present findings from our whole body Tcf19 knockout (wbTcf19KO) mouse model and how knockout of Tcf19 affects glucose tolerance, beta cell function, gene expression, and DNA damage. I conclude my thesis with discussing the ongoing experiments and the development of tools that will help determine TCF19 binding partners, mechanism, and elucidate the impact of TCF19 variants on TCF19 function and its contribution to diabetes pathogenesis.

Introduction

Diabetes

Diabetes is a huge public health problem affecting over 37 million Americans with rates continuing to increase each year (Centers for Disease Control (CDC) National Diabetes Statistics Report https://www.cdc.gov/diabetes/basics/type2.html). Additionally, 88 million Americans have prediabetes, discounting individuals who do not know they have diabetes (CDC Diabetes Statistics Report). The majority of diabetes cases (~90%) are made up of Type 2 diabetes (T2DM)[1]. However, rates of Type 1 diabetes (T1DM) also continue to rise in the U.S [2].

Glucose sensing, uptake, metabolism, and production are finely tuned and tightly regulated processes that are dysfunctional in diabetes patients. This can lead to uncontrolled glucose levels. The pancreatic islet is composed of several cell types including the beta cell, alpha cell, delta cell, and pancreatic polypeptide (PP) cells[3]. The beta cells, which comprise approximately 70% of the islet, are responsible for sensing increases in blood glucose levels and releasing insulin in response to this rise[4]. However, numerous other stimuli such as nutrients, amino acids, and hormones can affect insulin secretion[5,6]. Diabetes can develop when glucose homeostasis is dysregulated, leading to high levels of glucose in the blood. Unchecked, high glucose levels can lead to glucotoxicity which can damage blood vessels that supply blood to vital organs and lead to serious health complications including microvascular complications such as retinopathy, neuropathy and nephropathy[7,8]. Macrovascular complications such as cardiovascular, cerebrovascular, and peripheral arterial diseases can also occur[9].

Type 1 Diabetes

In T1DM, pancreatic beta cells undergo autoimmune attack leading to the destruction of the majority of the beta cell population[10]. Genetic predisposition to the disease combined with environmental factors such as viral infections can lead to immune-mediated attack on the beta cells[11]. Interestingly, many candidate genes for T1DM such as *MDA5, PTPN2,* and *TYK2,* regulate antiviral responses in both beta cells and the immune system[12,13]. Upon viral infection, the unmasking of beta cell autoantigens by immune cell attack further promotes immune cell infiltration and beta cell destruction[11]. Viral infection can also promote the recruitment of natural killer cells and T cells to the islet and lead to the production of pro-inflammatory cytokines including interferon-alpha (IFN-alpha), interferon-gamma (IFN-gamma), and interleukin 1 beta (IL-1beta), all of which can induce endoplasmic reticulum (ER) stress and activation of the intrinsic apoptotic pathway leading to decreased beta cell mass[14]. The defective insulin secretion in T1DM reflects the progressive beta cell destruction of 60-100% of beta cell mass depending on disease duration, genetic factors, and functional defects[15,16]. Despite the progress in developing T1DM therapies, there is not yet a cure, and treatment for T1DM continues to include lifelong exogenous insulin injections[17].

Type 2 diabetes

On the other hand, T2DM is characterized by insulin resistance in the peripheral tissues[18]. This insulin resistance is caused by increased adiposity and obesity which leads to a decrease of glucose transport into the liver, muscle, and fat cells[19]. It is predicted that with current trends, approximately 50% of Americans will be obese by 2030 [20].

T2DM is caused primarily by lifestyle factors in addition to genetics. Genetic susceptibility genes including *TCF7L2, PPARG, KCNJ11,* and many more that have roles in regulating stress, glucose metabolism and inflammation among many others, can predispose an individual to developing T2DM[21]. Lifestyle factors such as physical inactivity, sedentary lifestyle, smoking, and excess consumption of alcohol and unhealthy foods can contribute to T2DM[22]. Specifically, factors that promote obesity lead to a chronic inflammatory response in adipose tissues and can lead to insulin resistance[23]. Therefore, weight loss has been one of the gold standards to reversing T2DM. In the face of insulin resistance, beta cells attempt to compensate by increasing insulin production and beta cell mass[24]. However, overtime, beta cells are unable to keep up with the high demands for insulin and leads to beta cell apoptosis and eventually, decreased beta cell mass[24]. Current therapeutic approaches for T2DM include analogues to glucagon-like peptide 1 (GLP-1), which is an incretin that promotes insulin release and glucagon suppression, and DPP-IV inhibitors which prevent the breakdown of endogenous GLP-1[25].

Beta cell proliferation and mass

A common trait of T1DM and T2DM is that they are diseases of reduced functional beta cell mass. Further understanding of mechanisms that lead to increased beta cell mass, such as proliferation, are crucial to preventing beta cell mass loss. Beta cells proliferate at a high rate during embryogenesis but decrease into adulthood where beta cells proliferate at rates as low as 0.1-0.3% a day[26]. However, adult beta cells are known to proliferate in response to stress in order to maintain euglycemia such as during times of pregnancy and obesity[27,28]. In particular, under settings of obesity, studies have shown that mice that fail to upregulate beta cell proliferation in

response to obesity eventually develop diabetes[29]. This suggests that defects in the adaptive beta cell proliferative response to obesity contributes to diabetes development.

While beta cells can be stimulated to proliferate under conditions of pregnancy or obesity, stimulating beta cell proliferation at basal levels proves to be challenging. Many studies have successfully stimulated rodent beta cells to proliferate through overexpression of growth factors such as insulin-like growth factors (IGFs), cell cycle genes, and certain hormones such as GLP-1 and parathyroid hormone-related protein (PTHrP), but scientists have not been successful in promoting human beta cell proliferation[30]. A reason may be that human beta cells are more resistant to proliferation. It was found that in response to overexpression of cell cycle genes, rodent cell lines translocated the cell cycle molecules to the nucleus to induce proliferation whereas human islets sequestered the cell cycle molecules in the cytoplasm[31]. Additionally, the only nuclear G1/S molecules found in human beta cells were cell cycle inhibitors and none of the cyclins or cyclin dependent kinases (CDK) necessary to drive proliferation[31]. These findings pose challenges to inducing human beta cell proliferation.

It has also been shown that epigenetic regulators and other transcription factors upstream of cell cycle genes and inhibitors such as polycomb repressive complexes (PRCs) and forkhead box protein M1 (FOXM1) are key regulators of G1/S cell cycle entry as well as later phases of the cell cycle[32,33]. Additionally, c-MYC controls the expression of many cell cycle genes and is able to direct both rodent and human beta cell proliferation[34–36]. Unfortunately, despite the growing literature studying mechanisms that lead to beta cell proliferation, there are currently no methods or

therapeutics that have successfully managed to reproducibly induce human beta cell proliferation.

Beta cell apoptosis

The other end of beta cell mass regulation includes beta cell mass loss through mechanisms such as apoptosis. Unlike beta cell proliferation, beta cell apoptosis occurs at very low rates during embryogenesis and increases with age[15,37]. Excessive rates of beta cell apoptosis compared to beta cell proliferation can lead to an overall decrease in beta cell mass and insufficient insulin production.

Many factors such as hyperglycemia, inflammation, ER stress and lipotoxicity promote beta cell death in diabetes[38–41]. In T2DM, ER stress can be caused by inflammation, amyloid deposits, and excess nutrients[42,43]. Additionally, in times of insulin resistance, the beta cell increases its production of insulin to compensate, and this response can lead to insulin misfolding thereby promoting the unfolded protein response[44].

Chronic increases of plasma free fatty acids (FFA) concentrations results in disturbances in lipid metabolism regulation which leads to decreased beta cell function and lipotoxicity[45]. Studies have shown that prolonged exposure of isolated islets or insulin-secreting cells to elevated FFA levels inhibits glucose stimulated insulin secretion (GSIS) and induces cell death through apoptosis[45]. Additionally, compared to untreated rat islets, rat islets cultured for 7 days in the presence of FFA had increased DNA fragmentation and expression of apoptotic genes[46]. FFAs can also induce inflammation and activate inflammasomes which in turn can lead to beta cell death[47]. The elevated cytokine or chemokine levels that accompany inflammation can lead to immune cell infiltration in the islets, a process that occurs in both T1DM and

T2DM, and can lead to beta cell apoptosis[47,48]. Specifically, the proinflammatory cytokines TNF-alpha, IL-1beta, and IFN-gamma have been shown to induce beta cell death[49].

Chronically high glucose levels can also contribute to beta cell death and have been observed in rat models of diabetes[50]. The mechanisms that lead to beta cell apoptosis include upregulation of Fas, and constitutively expressed Fas ligand is upregulated in response to IL-1beta expression in beta cells[51–53].

Taken together, these studies demonstrate that a multitude of factors can lead to beta cell apoptosis. However, one specific pathway, the DNA damage response (DDR) pathway, has gained attention in recent years as having a role in diabetes pathogenesis. While the mechanisms behind DNA damage in diabetes pathogenesis have not been well studied, numerous studies have shown that patients with diabetes have an increase in DNA damage and decreased ability to repair damage[54–57]. Many of studies were performed in other cell types such as lymphocytes and other organs such as kidney, lung, and epithelial cells. Studies specifically looking at DNA damage in the islet and beta cell are lacking and requires further investigation to determine the exact role of DNA damage in beta cell death and diabetes.

TCF19 background

Transcription factor 19 (TCF19) is a transcription factor that is associated with both T1DM and T2DM in genome wide association studies (GWAS)[58,59]. *Tcf19* is located on chromosome 6p31.1, which is near the human leukocyte antigen (HLA) locus. HLA class II genes are strong predictors of T1DM susceptibility, but several novel single nucleotide polymorphisms (SNPs) in the extended major histocompatibility complex (MHC) were also found to be uniquely associated with

T1DM[58,60]. To find potential causal SNPs within the coding regions of nearby genes, linkage disequilibrium (LD) analysis was performed and identified *TCF19* as a lead causal gene for this association. Three non-synonymous SNPs leading to amino acid changes within *TCF19* were shown to have moderate to strong LD with the associated SNPs. SNPs near TCF19 were also shown to be associated with T2DM[59,61]. The fact that *TCF19* was found to be associated with T1DM and T2DM suggests a role for *TCF19* in diabetes pathogenesis independent of autoimmunity.

The human TCF19 protein as well as the murine protein contains a proline-rich region and a forkhead associated (FHA) domain. Proline-rich regions are commonly found in transactivating proteins and can facilitate intermolecular interactions such as signal transduction, cell-cell communication, antigen recognition, and cytoskeletal organization[62]. The FHA domain is commonly found in regulatory proteins such as kinases, phosphatases, and transcription factors that are involved in the cell cycle and DNA damage response [63,64]. FHA domains recognize phophopeptide domains which are a motif that can play roles in protein-protein interactions and has been implicated in intracellular signaling pathways[65]. Two of the *TCF19* SNPs identified in GWAS are found in this domain, which may potentially affect the domain function such as binding to target genes to elicit transcriptional effects. The primary structure of human TCF19 but not the murine Tcf19 protein also includes a plant homeodomain (PHD) zinc finger domain. This domain can interact with chromatin via methylated histones, and specifically, the PHD finger of TCF19 has been shown to interact with H3K4me3 to recruit protein complexes to certain promoters[66,67]. The lack of PHD finger domain in the rodent protein may suggest different functions between the species and an important difference to keep in mind when working in different model species.

When the lab began working on *TCF19* in 2009, very little was known about this gene. There was only one publication in 1991 characterizing the novel gene[68]. The 1991 paper described *Tcf19* as a growth regulated gene first expressed at the G1/S transition of the cell cycle with its maximal expression coinciding with peak DNA synthesis[68]. Its expression correlated with other cell cycle genes that are maximally expressed at the G1/S transition and *Tcf19* expression is decreased in response to cycloheximide treatment, an inhibitor DNA synthesis, suggesting its role in growth regulation[68].

In later years, *Tcf19* was found in a coordinately regulated cluster of cell cycle genes in a microarray of pancreatic islet gene expression which were differentially expressed between diabetic and nondiabetic leptin-deficient ob/ob mouse models[29]. Leptin is a hormone that regulates satiety, and mutations in this gene lead to obesity[69]. On a C57BL/6J mouse background, these leptin mutant mice are able to maintain euglycemia by increasing beta cell mass and insulin secretion despite insulin resistance[70]. However, the leptin mutation in BTBR mice leads to severe T2DM and the mice are unable to maintain sufficient insulin production. *Tcf19* was found to be significantly increased in response to obesity in the C57BL/6J mouse strain, but not the BTBR[70]. This suggests that *Tcf19* may have a role in compensatory beta cell proliferation under settings of obesity.

While studies looking at the transcriptional regulatory mechanisms of *Tcf19* have been scarce, a paper published in 2013 showed that knockdown of the gene, nuclear protein transcription regulator 1 (*Nupr1),* led to increases in *Tcf19* and *Ccna2* levels[71]. Nupr1 is a stress activated protein that has been implicated in cell proliferation and apoptosis and is induced in response to glucose and TNF-alpha, both of which are elevated in T2DM [71]. Additionally, Nupr1 knockout mice were found to be

hyperinsulinemic due to increased beta cell mass[71]. The study also found that overexpressing *Tcf19* in a mouse beta cell line, Min6, led to a significant increase in cell proliferation as well as *Ccna2* mRNA levels[71]. Taken together, these data suggest that Tcf19 may have a role in regulation *Ccna2* expression.

Since first beginning work on *Tcf19*, the lab published a paper in 2013 describing the effects of *Tcf19* knockdown in the INS-1E beta cell line[61]. The lab showed that knockdown of *Tcf19* resulted in decreased proliferation and an increase in apoptosis[61]. Additionally, there was a significant reduction of expression of numerous cell cycle genes from the late G1 phase through the M phase, and the cells were found to be arrested at the G1/S checkpoint[61]. Knockdown of *Tcf19* also led to increased apoptosis and susceptibility to ER stress with a subsequent decrease in ER homeostasis genes. These results show that *Tcf19* is necessary for both proliferation and survival in INS-1 cells. Additionally, in more recent years, the lab showed that TCF19 impacts the DNA damage and inflammatory pathway.

The DNA damage pathway

The DNA damage response (DDR) pathway and regulation of the cell cycle are intricately linked[72]. The DDR is comprised of a set of signaling pathways for the detection and repair of DNA damage[73]. Upon DNA damage stress, the key DNA damage sensors, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR), phosphorylate a histone variant on Ser 139 (γH2AX)[74]. This phosphorylation plays a key role in the recruitment of DNA repair machinery to the site of damage and is one of the most sensitive markers used to assess for DNA damage[74]. ATM and ATR also phosphorylate an array of downstream proteins responsible for cell cycle arrest, DNA damage repair, and, under conditions where the DNA cannot be

repaired, apoptosis[75,76] A well-studied protein in cell cycle arrest is the tumor suppressor protein, p53, which is responsible for the transient arrest of cell cycle progression at the G1 phase[72]. This arrest is thought to give the cell time to repair its damage[72].

Tcf19 knockdown showed G1/S cell cycle arrest, and overexpression of TCF19 led to increases in DNA damage gene expression suggesting that TCF19 may have a role in the DDR pathway[61,77]. The DDR pathway is not very well studied in diabetes, and specifically, in the beta cell. Despite correlative studies showing increased DNA damage in patients with diabetes and decreased DNA repair ability, a large gap in knowledge exists regarding the role for DNA damage in contributing to diabetes pathogenesis.

Summary

Rates of diabetes continue to grow each year, and diabetes remains a huge public health problem and burden. Currently, there is no cure for diabetes, and expanding and maintaining beta cell mass continues to be a key area that holds therapeutic potential. Better understanding of pathways and mechanisms that can promote beta cell proliferation and mass, as well as those that decrease beta cell apoptosis can open new avenues for diabetes treatments.

My thesis focuses on delineating the role of a novel diabetes susceptibility gene, *TCF19*, in diabetes pathogenesis. Specifically, my thesis will focus on elucidating the role of *TCF19* in adaptive beta cell proliferation and the role of *TCF19* in modulating the DNA damage response pathway. In Chapter 1, I will provide a review on what is known about DNA damage, an understudied field, in diabetes pathogenesis. I will specifically focus on what is known about DNA damage in the beta

cell and how this may lead to diabetes. In Chapter 2, I will discuss the findings of our TCF19 overexpression model and how TCF19 modulates inflammatory and DNA damage gene expression. In Chapter 3, I will focus on the characterization of our whole body Tcf19 knockout (wbTcf19KO) mouse model and how Tcf19 KO affects glucose tolerance, insulin secretion, proliferation, and DNA damage processes in the islet. In Chapter 4, I will discuss the ongoing experiments and tools created to elucidate protein binding partners and mechanistic studies to determine the effects of TCF19 genetic variants and protein domains on TCF19 function. Chapter 5 will summarize the findings from the previous chapters and provide overall conclusions and future directions of the study.

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Chapter 1: DNA damage pathways in the beta cell and their implications for diabetes pathogenesis

The following chapter is a review article currently in preparation for submission to a journal.

The following literature review was conducted and written by Grace Yang.

Introduction

Our DNA is constantly under attack from endogenous or environmental factors that can lead to DNA strand breaks or mutations[1]. Lifestyle and environmental factors that can cause DNA damage include obesity, smoking, UV radiation, and genotoxic chemicals[2]. At the cellular level, simple intracellular metabolism and replication can result in DNA damage[3]. For example, oxidative DNA damage is an inevitable byproduct of cellular metabolism, yet it is a significant cellular stressor and can lead to the most abundant DNA lesion, 8-oxo-dG[4]. Other types of DNA damage including alkylation of bases, adduct formation, DNA crosslinking, and DNA single or double stranded breaks can lead to mutagenesis if left unrepaired[5].

Effective mechanisms for detecting and repairing damaged DNA are essential for maintaining genomic DNA integrity to prevent mutations and the transformation of healthy cells to cancers[6]. Defects in DNA repair mechanisms underlie a host of human diseases such as cancer, neurogenerative disorders, cardiovascular disease, and metabolic syndrome[1,7].

While DNA damage is well recognized as a critical factor in cancer development, aging, and neurodegeneration, the exact role of DNA damage in diabetes pathogenesis is not well studied. Most studies to date studying DNA damage in diabetes have been largely correlative. In this review article, we aim to address evidence supporting the role of DNA damage diabetes. Additionally, we will specifically address what is known about DNA damage in the islet and beta cell, and how it may affect beta cell mass and function. Finally, we will discuss potential molecular targets of the DNA damage pathway that may hold therapeutic potential for diabetes treatments.

DNA damage response and repair disturbances in obesity

The rising prevalence of obesity is a public health crisis. Excess body fat can lead to metabolic disorders and increase the risk for heart disease, diabetes, stroke, and cancer[8]. Patients who are obese have increased DNA lesions such as double strand breaks (DSB), single strand breaks (SSB), and oxidized bases[9]. Additionally, obese patients have DNA damage levels almost two times higher than their non-obese counterparts[9,10]. Phosphorylation of the Serine 139 residue on the histone variant H2AX (γH2AX) is an early and sensitive marker for monitoring DSB and is significantly elevated in overweight and obese children compared to lean controls[11,12]. Additionally, adipose tissue secretes a host of inflammatory adipokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-alpha), monocyte chemoattractant protein-1 (MCP-1), and resistin, which promotes an environment of chronic inflammation[13]. This inflammation leads to the production of reactive oxygen species (ROS), which further acts as a signaling molecule and mediator of inflammation[14] . Under physiological conditions, ROS has important roles in cell survival, differentiation, and the immune system[15] . However, overproduction of ROS, which is observed in obesity, can lead to endogenous DNA damage and cell cycle arrest[9,16]. Furthermore, the secreted cytokines such as TNF-alpha and IL-6 travel to different parts of the body and produce more proinflammatory molecules such as cyclooxygenase 2 (COX2), nitric oxide synthase (NOS), ROS, nitric oxide (NO) and superoxide, all of which can promote DNA lesions[17]. All together, these molecules contribute to the vicious cycle of inflammation and DNA damage[18].

Disturbances in DNA repair pathways are also observed with increasing adiposity[19]. Specifically, nucleotide excision repair (NER) capacity is decreased with increasing BMI in young females, and DSB repair mechanics are altered in obese

adolescents compared to their lean counterparts[20]. Oxidative stress can impair DNA repair enzymes, and high fat diets can disturb epigenetic mechanisms for DNA repair genes[21,22]. Taken together, there is substantial evidence that obesity can induce DNA damage and alter DNA repair mechanisms.

The accumulation of DNA damage may contribute to increased cancer risk. The number of cancer cases caused by increased weight sits at about 20%[23]. There is also strong evidence that being overweight or obese throughout adulthood increases the risk of several cancers including breast, colon, and prostate[24]. Additionally, prolonged DNA damage and DDR activation is associated with increased cellular senescence and cell death[25].

DNA damage in diabetes

Type 1 and Type 2 diabetes (T1DM, T2DM) patients have increased DNA damage in their tissues and cells[26,27]. The exact contribution of DNA damage to T1DM and T2DM has not been extensively studied, and literature dissecting the mechanisms and pathways involved are sparse.

However, there are a handful of correlative studies that show that high levels of oxidative DNA damage from ROS are observed in rodent and humans with diabetes[26,27]. Specifically, 8-hydroxy 2'-deoxy-guanosine (8-OHdG), a sensitive biomarker for oxidative DNA damage, is increased in obesity and in patients with T1DM and T2DM[27–29]. Comet assay, an assay that directly measures DNA strand breaks, showed a higher level of DNA damage strand breaks in individuals with T1DM compared to healthy controls[30]. Additionally, elevated levels of DNA strand breaks in T2DM are correlated with poor glycemic control[31]. Ciminera et al. showed that in HEK293T cells, chronically elevated glucose increases DNA advanced glycation end

product (AGE) levels, in particular, the DNA glycation adduct *N2*-(1-carboxyethyl)-2'-deoxyguanosine (CEdG)[32]. The elevated glucose also inhibited DNA repair[32]. While the majority of these experiments were performed in peripheral blood cells, urine, and serum samples, Kushwaha et al compared DNA strand breaks in lymphocytes, lung, liver, heart, aorta, kidney, and pancreas from diabetic and control rats and found that DNA damage was increased in all the tested tissues[33].

In addition to increased DNA damage, diabetic patients also have decreased DNA repair efficiency[31]. Kumar et al showed that experimental T1DM and T2DM impairs DNA repair, leading to senescence, inflammatory phenotypes, and fibrosis[34]. Additionally, diabetes patients displayed a higher susceptibility to DNA damage induced by hydrogen peroxide and doxorubicin as well as decreased efficacy at repairing the DNA damage induced by these agents compared to healthy controls[35].

Several pathophysiological factors in diabetes have been implicated in the increased DNA damage observed in patients with diabetes. High glucose concentrations (30mmol/L) have been reported to cause DNA damage in human endothelial cells and was also later replicated in studies using mouse and human renal cells[36,37]. Hyperglycemia promotes the formation of AGEs which have been shown to cause DNA damage in human liver and colon cells through ROS, NF-kappaB and AngII[38]. AGEs were also found to stimulate 8-oxo-dG, a DNA adduct, formation in multiple cell types[39,40].

Increased levels of circulating insulin are also hypothesized to be a DNA damage promoting factor. High insulin levels due to insulin resistance, often observed in patients with obesity and prediabetes, have been shown to cause base oxidation by triggering the production of ROS[41] .

Free fatty acids (FFA) are shown to cause DNA strand break in the RINm5F rat beta cell line and primary human fibroblasts[42]. Specifically, palmitic acid is a potent inducer of DNA damage[42]. Additionally, FFA have been shown to cause increased NO production leading to mitochondrial DNA (mtDNA) damage in INS-1 cells, and expression of a vector for the DNA repair enzyme, human 8-oxoguanine DNA glycosylase/apurinic lyase (hOGG1), into the INS-1 cellular genome significantly decreased FFA-induced mtDNA damage[43].

Taken together, these studies show that the pathophysiological factors that may precede full on diabetes development can promote DNA damage. In turn, this prolonged damage without adequate DNA repair can lead to further inflammation, fibrosis, or irreversible cell cycle arrest and apoptosis.

DNA damage in the islet and beta cell

The studies previously described have been performed in many different cell types, but there is a general lack of information studying the mechanisms of DNA damage in the islet and beta cell. However, it has been observed that exposure of islets to cytokines such as IL1-beta, TNF-alpha, and IFN-gamma induce islet DNA damage and apoptosis[44–46]. In more recent years, Oleson et al. showed a dual role for NO in regulating the response of beta cells to DNA damage and demonstrated that while NO induces cellular damage and impairs beta cell function, it can also promote beta cell survival[47,48].

The few publications that have attempted to elucidate how the DDR affects beta cells have shown involvement for cell cycle inhibitory proteins, the key signal-transducing kinases of the DDR, Ataxia telangiectasia mutated (ATM) and Ataxia

telangiectasia and Rad3 related (ATR), and their dependent phosphorylated downstream proteins such as p21 and p53.

Tay et al. found that 16 week db/db mice, a mouse model of T2DM, had significantly higher DSBs compared to 10 week old db/db mice, and that these mice had DSBs comparable to islets from their positive control STZ-treated (a T1DM mouse model) mice, suggesting increased DNA damage in beta cells of diabetic mice[49]. They observed elevated levels of p53, p21, caspase 3, and gadd45a, and further demonstrated that in their model, p21 inhibition led to increased beta cell apoptosis, and that overexpression of p21 was protective[49]. P21 has important roles in cell cycle arrest which may allow time for the cell to repair its damaged DNA[50]. Additionally, p21 has roles in DNA repair, and this study suggests an association between p21 upregulation during DSB and cell survival[50,51].

Focusing on another important protein in the DDR, Horwitz et al. showed that beta cell DDR led to activation of 53BP1 protein and accumulation of p53 in biopsy and autopsy material from patients with recently diagnosed T1DM as well as a rat model of T1DM[52]. Another group demonstrated a role for p53 by creating a transgenic mouse model lacking the transactivation domain of the full-length protein that modulates total p53 activity, and found that these mice had a dramatic decrease in beta cell mass and proliferation with an increase in the cell cycle inhibitor, p21[53]. The roles of p53 and p21 in the beta cell response to DNA damage, and whether their upregulation or downregulation may be beneficial towards beta cell survival under DDR stress conditions, require further studies. However, it is likely that the extent of DNA damage and type of stress that induces the DNA damage may play a role in the differential gene regulation and cell response.

The PARP-1 protein is a first responder that detects DNA strand breaks and contributes to efficient repair through modulation of chromatin factors and modification of DNA repair factors[54]. Andreone et al. showed that PARP-1 deficiency protects islets against cytokine-induced death[55]. However, PARP-1 deficiency failed to provide protection against the inhibitory actions of cytokines on insulin secretion or the DNA damaging actions on the islet[55]. This paradoxical finding highlights the complexity of the DDR, and careful attention to all aspects of beta cell function in DNA damage conditions are crucial to fully understanding the role of the DDR in diabetes.

Other studies have looked at the role of the key DDR kinases, ATM and ATR. It has been shown that while NO induced DNA damage promotes beta cell apoptosis, micromolar amounts of NO actually inhibit ATM and ATR and promote beta cell survival[56,57]. Uhlemeyer et al. investigated the role of ATM and p53 in T2DM models, and showed differential regulation of the two proteins in beta cell survival under four different pathophysiological types of diabetogenic beta cell stress[58]. Specifically, they found that ablation of both ATM and p53 protects against beta cell apoptosis induced by DNA damage and lipotoxicity whereas only p53 knockdown protected against cytokine induced cell death[58]. ATM and p53 differentially regulate cell death depending on the type of insult. These studies describe the complicated and various roles of DDR proteins under different types of stress conditions.

Other studies to date have looked at the role of DNA repair enzymes in beta cells. Several studies have shown that loss of DNA repair genes in the islet leads to decreased beta cell function and survival[59,60]. Tyrberg et al. demonstrated that expression of *OGG1*, a base excision enzyme, was increased in human T2DM islets[59]. Certain polymorphisms of base excision repair genes*, APE1* and *XPG*, were associated with T2DM in the Turkish population[60]. Guevara et al. used mice deficient

for the DNA excision-repair gene, *Ercc1*, and found that loss of this DNA repair gene led to a significant reduction in beta cell area, glucose stimulated insulin secretion (GSIS), and an increased susceptibility to apoptosis[61].

Taken together, these studies present evidence for increased DNA damage in the beta cells of models of T1DM and T2DM compared to their non-diabetic counterparts. Additionally, beta cell DNA damage can be a critical contributor to diabetes pathogenesis through its effects on beta cell function and cell death. However, the pathways and proteins involved in the regulation of the DDR are complicated in that the type of stress, extent of DNA damage, and length of exposure can lead to different cellular responses and the activation/inhibition of DNA damage proteins. Further understanding of these mechanisms and DNA damage conditions is an important step to understanding the role of the beta cell DDR in diabetes progression.

Molecular targets and pathways for potential diabetes therapeutics

Specific therapeutic targets that can alleviate DNA damage or increase DNA damage repair in the islet and beta cell have not been studied. While dozens of small molecule inhibitors targeting DDR pathways have been developed in the cancer field, it is important to note that many of these cancer therapies aim at promoting tumor cell apoptosis or cell cycle checkpoint arrest to inhibit proliferation. In diabetes, the opposite outcomes are usually desired, with therapies working towards decreasing beta cell apoptosis and promoting beta cell proliferation. Despite the different desired outcomes, current therapies in the cancer field may still provide information as to the proteins/genes in the DDR pathway that have been identified as targets.

Several studies have shown an increase in mTORC1 activity in human and mouse primary islets that are exposed to nutrient overload, and this is paralleled by an increase in apoptosis[62–64]. Additionally, mTORC1 signaling has been shown to be linked to the DNA damage response pathway. A commonly used mouse model of T2DM, obese mice and leptin receptor deficient db/db mice, were found to have an upregulation of mTORC1[65–67]. Additionally, islets isolated from patients with T2DM exhibited increased mTORC1 activation compared to nondiabetic individuals, and further analysis revealed that the pancreatic human beta cells, and not alpha cells, were the source of mTORC1 upregulation in these islets[65]. Exposure to chronically high glucose levels and lipids activated mTORC1, and mTORC1 inhibition prevented beta cell death and enhanced insulin secretion[62,64,68]. Additionally, IL-6, which is shown to induce protective autophagy and beta cell survival, inhibited mTORC1[69]. Inhibition of mTORC1-S6K1 with a selective S6K1 inhibitor restored insulin secretion in isolated human islets from patients with T2DM[65]. These studies demonstrate that increased mTORC1 activation under pathophysiological conditions present in diabetes is deleterious for the islets and beta cells and that inhibition of mTORC1 and/or S6K1 could potentially serve as a way to increase beta cell survival and function.

Despite the potential of mTORC1 inhibition for diabetes therapies, studies with mTOR inhibitors, such as rapamycin, show controversial results. Fraenkel et al. tested mTOR/S6K1 inhibition with rapamycin in Psammomys obesus (P. obesus), a model of nutrition-dependent T2DM, and unexpectedly found that rapamycin worsened hyperglycemia in diabetic P. obesus[70]. Rapamycin treated diabetic P. Obesus had an abolished increase in serum insulin compared to their nontreated diabetic counterparts[70]. GSIS and insulin biosynthesis were impaired in islets treated with

rapamycin[70]. Chronic inhibition of mTORC1 with rapamycin also caused glucose intolerance in mice[70,71]. Possible explanations for these findings include the fact that rapamycin also inhibits mTORC2, which is crucial for insulin-mediated suppression of hepatic gluconeogenesis[71]. There are currently no therapies that inhibit just mTORC1. However, blocking specific downstream mTORC1 signaling proteins using highly specific protein inhibitors, such as those used in mTORC1-S6K1 studies, may be an alternative therapeutic option.

Focusing on a different potential target, Ciminera et al. showed that NER capability was impaired under high glucose conditions[32]. They found that under high glucose conditions, HIF1-alpha protein attenuates the expression of hypoxia response elements (HRE) inducible genes, including may genes involved in NER[32]. Impaired NER allowed DNA lesions to remain in the genome leading to replication stress[32]. However, when HIF1-alpha was stabilized under high glucose conditions, DNA repair was increased and DNA damage was decreased[32]. Currently, there is a selective HIF1-alpha stabilizer, AKB-4924, that has been used for treatment of inflammatory bowel disease[72]. Whether or not this pharmacological agent will prove useful for diabetes will require further studies.

As patients with diabetes have increased DNA damage and decreased repair capacity, targeting the proteins involved in these pathways may hold therapeutic potential. Modulating p53, a downstream protein target of ATM and ATR, has been shown to protect against DNA damage. Specifically, deletion or inhibition of p53 restored mitochondrial function, increased glucose tolerance, and protected against the development of T1DM[73]. Directly targeting p53 through chemical inhibitors to p53 such as pifithrin – alpha- hydrobromide, which blocks p53 transcriptional activation and its induction of apoptosis, could hold therapeutic potential in preventing beta cell

apoptosis [74,75]. While this molecule has not reached clinical trials, it has been shown to protect against neuronal death in models of stroke and neurodegenerative disorders[74]. Additionally, pifithrin protected against DNA damage induced apoptosis in different cell lines[76].

The functions of p53 are very diverse and complex, and p53 also plays roles in preventing the initiation of tumor development. It also has roles in autophagy and antioxidant functions[77]. These other roles must be kept in mind when targeting this protein for therapeutic purposes, as decreased p53 expression has been shown to exist in tumors.

An interesting study in 2017 focused on targeting immune cells rather than beta cells[78]. The study showed that targeting p53 in immune cells for autoimmune disorders suppressed recently activated T cells and targeted pathological T-cells without compromising naïve regulatory, or quiescent memory T-cell pools[78]. This had clear benefits in models of CD8+ T cell driven autoimmune disorders. Targeting CD8+ autoimmune T-cells could be an effective therapy for T1DM.

Finding ways to increase beta cell DNA repair capacity in patients with diabetes could also be potential avenue of intervention. DNA repair proteins are already being studied as therapeutics in cancer[79]. While the cancer field is targeting the inhibition of DNA repair proteins in order to inhibit cancer cells from resisting cancer therapies, diabetes treatments will likely work to increase the beta cell's DNA repair capacity. Decreased expression of DNA repair genes and polymorphisms in DNA repair genes are associated with T2DM. Specifically, polymorphisms in *APE1* and *XPG*, which are involved in base excision repair (BER), were found to be correlated with T2DM[60]. OGG1, a protein responsible for repair of oxidative stress damage was also found to be decreased in diabetic patients. Finding ways to upregulate these repair proteins as

well as other DNA repair enzymes could serve as a means to increase DNA repair capacity in beta cells.

The relationship between beta cell DNA damage, DNA repair, and diabetes pathogenesis warrants further study. However, studies have shown that beta cells in patients with diabetes have increased DNA damage and decreased repair capacity that can negatively affect beta cell function and response to diabetogenic stress. Several pathways and targets may hold therapeutic potential and were discussed in this review. However, it is important to realize that many DDR and repair proteins have multiple other roles such as preventing cancer progression and promoting cell death of mutagenic cells. Therefore, a better understanding of the mechanisms of these pathways, specifically in the beta cell, and the conditions in which these proteins can decrease DNA damage while protecting against mutagenesis and cancer development are needed before developing therapies targeting the DDR pathway for diabetes treatment.

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Chapter 2: TCF19 impacts a network of inflammatory and DNA damage response genes in the pancreatic beta cell

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TCF19 Impacts a Network of Inflammatory and DNA Damage Response Genes in the Pancreatic β-Cell

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Abstract: Transcription factor 19 (TCF19) is a gene associated with type 1 diabetes (T1DM) and type 2 diabetes (T2DM) in genome-wide association studies. Prior studies have demonstrated that Tcf19 knockdown impairs β-cell proliferation and increases apoptosis. However, little is known about its role in diabetes pathogenesis or the effects of TCF19 gain-of-function. The aim of this study was to examine the impact of TCF19 overexpression in INS-1 β-cells and human islets on proliferation and gene expression. With TCF19 overexpression, there was an increase in nucleotide incorporation without any change in cell cycle gene expression, alluding to an alternate process of nucleotide incorporation. Analysis of RNA-seq of TCF19 overexpressing cells revealed increased expression of several DNA damage response (DDR) genes, as well as a tightly linked set of genes involved in viral responses, immune system processes, and inflammation. This connectivity between DNA damage and inflammatory gene expression has not been well studied in the β-cell and suggests a novel role for TCF19 in regulating these pathways. Future studies determining how TCF19 may modulate these pathways can provide potential targets for improving β-cell survival.

Keywords: DNA damage; inflammation; STRING; RNA-seq; PANTHER; diabetes; β-cell; TCF19

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1. Introduction

The pancreatic β-cells are endocrine cells whose primary role is to synthesize and secrete insulin. Insulin is required to maintain euglycemia. However, the pancreatic β-cell is susceptible to many different stressors including oxidative stress, endoplasmic reticulum (ER) stress, and inflammation [1,2]. These stressors are exacerbated in patients with obesity, insulin resistance, and diabetes [3–5]. This can lead to β-cell apoptosis and reduced β-cell mass [5,6]. Pancreatic islets from patients with T2DM have increased ER stress which can lead to β-cell dysfunction and apoptosis [7–9]. In addition, increased circulating cytokines and localized islet inflammation are characteristics of T2DM and can contribute to β-cell death [10]. Hyperglycemia, as well as metabolic abnormalities associated with diabetes can lead to oxidative stress, resulting in increased intracellular reactive oxygen species (ROS) that contribute to β-cell dysfunction [11,12]. While many of these sources of β-cell stress have been well studied, there are other factors that can lead to β-cell dysfunction and apoptosis that are less studied. In particular, DNA damage has started gaining attention in recent years as having a role in diabetes pathogenesis. The microenvironment in the islet during diabetes involves oxidative stress and inflammatory insults that can increase DNA damage [13–16]. Additionally, DNA damage in islets elicited by the β-cell toxin, streptozotocin (STZ), causes an elevation of proinflammatory cytokines [14]. However,

this inflammatory response is attenuated after inactivation of the master DNA repair gene, ataxia telangiectasia mutated (ATM) [14]. Horwitz et al. also demonstrated that the β-cell DNA damage response (DDR) was more frequent in islets infiltrated by CD45+ immune cells [14]. This brings to light a fascinating connection between DNA damage, inflammatory, and immune responses in the islet. A better understanding of the intersection between these processes will provide potential regulatory targets to reduce and resolve DNA damage and inflammatory stress on the β-cell that may serve to help maintain adequate β-cell mass and function in diabetes.

In humans, the gene TCF19 (transcription factor 19) is associated with both T1DM and T2DM in genome-wide association studies [17–20]. TCF19 is expressed in human islets and shows a positive correlation with BMI in nondiabetic subjects [21]. In mice, Tcf19 is widely expressed; however, its expression is highest in the pancreatic islet and increases with obesity when β-cells are known to increase proliferation [21]. Others have similarly identified Tcf19 as a gene upregulated in proliferating β-cells and found that knockdown of TCF19 impairs insulin secretion in a human β-cell line [22–26]. We have previously demonstrated that siRNA-mediated knockdown of Tcf19 in rat insulinoma INS-1 cells reduces β-cell proliferation and survival and impairs cell cycle progression beyond the G1/S checkpoint [21]. Additionally, Tcf19 knockdown increases apoptosis via reduced expression of genes involved in the maintenance of ER homeostasis and increased expression of proapoptotic genes [21].

The TCF19 protein contains a forkhead-associated (FHA) domain, which is a phosphopeptide recognition domain commonly found in many transcription factors that participate in DNA repair and cell cycle regulation [27]. The human TCF19 (hsTCF19) protein, but not the mouse protein, also harbors a plant homeodomain (PHD) finger, allowing it to interact with chromatin. PHD finger proteins are often considered "chromatin readers" that recognize modified histones and can recruit additional transcriptional machinery to these areas [28]. Specifically, the tryptophan residue at position 316 in hsTCF19 has been shown to bind to chromatin via tri-methylated histone H3 and to regulate cell proliferation in liver cells through this interaction [29,30]. Taken together, these characteristics support the role of TCF19 as a transcriptional regulator of β-cell proliferation and survival.

The purpose of this study was to determine the effect of TCF19 overexpression on proliferation and survival in the β-cell. In this study, we overexpressed TCF19 in INS-1 cells and found that TCF19 overexpression does not induce proliferation, cell cycle progression, or impact cell survival. Rather, there was significant upregulation of a tightly interconnected set of genes involved in cell stress, inflammation, and antiviral responses, alluding to a previously unexplored role for TCF19 in the β-cell. Additionally, we find that TCF19 overexpression in human islets leads to significant upregulation of several DDR genes. Using a novel analysis for potential transcriptional co-regulators on these upregulated genes, we identified STAT1, STAT2, and IRF1 as likely drivers of the tight transcriptional gene network. Interestingly, there was no measurable activation of these transcription factors, indicating alternate mechanisms of regulating the inflammatory and DDR gene expression. These findings not only identify an intriguing connection between DNA damage and inflammatory responses in the β-cell but elucidate a novel role for TCF19 in modulating these two pathways.

2. Results

2.1. Human TCF19 Overexpression Increases ^{3}H-Thymidine Incorporation in INS-1 Cells but Does Not Change Cell Cycle Gene Expression

Based on our original studies on Tcf19, we concluded that Tcf19 was necessary for normal β-cell proliferation, as Tcf19 knockdown led to impaired cell cycle progression, reduced ^{3}H-thymidine incorporation, and G1/S cell cycle arrest [21]. We next wanted to determine if increased levels of TCF19 could drive β-cell proliferation, and therefore, we overexpressed hsTCF19 in INS-1 rat insulinoma cells. The human TCF19 protein was chosen for overexpression as it contains the PHD finger domain that is known to mediate

interactions with methylated histones (specifically trimethylated histone 3 at lysine 4 (H3K4me3)) [29,30]. The PHD finger domain is not present in the rodent protein. As we have not yet identified a reliable and specific TCF19 antibody, we generated a C-terminal myc-tagged TCF19 to allow for probing on the western blot. TCF19 overexpression was confirmed at both the mRNA and protein level (Figure 1A,B).

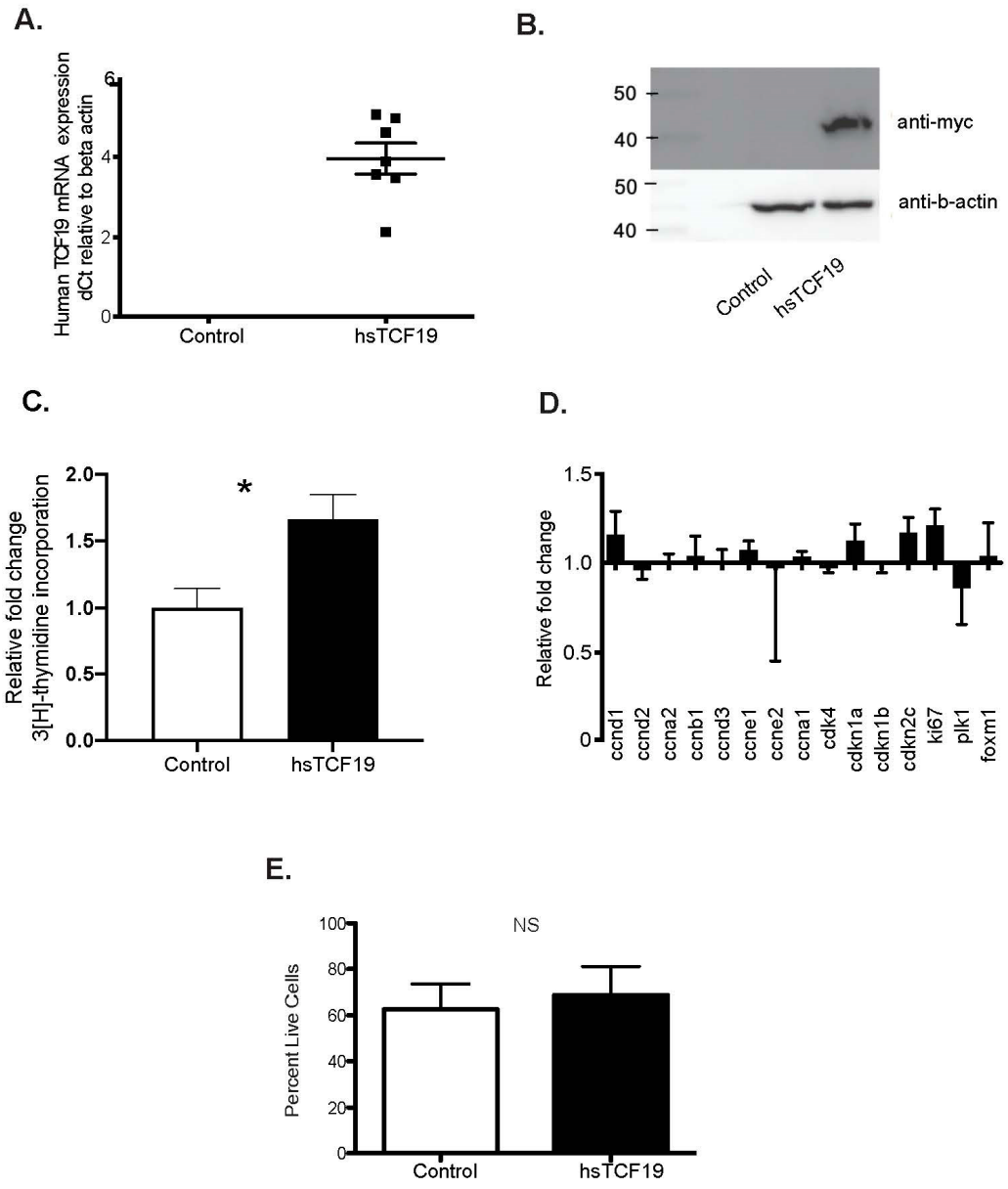


Figure 1. (**A**) Overexpression of human transcription factor 19 (TCF19) in INS-1 cells was confirmed by qRT-PCR. (**B**) Western blot against anti-myc tag confirms overexpression of human TCF19 (hsTCF19) (**C**) Overexpression of hsTCF19 in INS-1 cells leads to increased ^{3}H-thymidine incorporation (n = 5). (**D**) hsTCF19 overexpression does not lead to any significant changes in cell cycle gene expression (n = 5) (**E**) Overexpression of hsTCF19 in INS-1 cells does not affect cell viability (n = 5). Data are means ± SEM * p <0.05.

As an assay to assess proliferation, we measured ^{3}H-thymidine nucleotide incorporation in cells expressing hsTCF19 vs. empty vector control. INS-1 cells overexpressing hsTCF19 showed a significant two-fold increase in ^{3}H-thymidine nucleotide incorporation suggesting increased cell proliferation (Figure 1C). To confirm that the ^{3}H-thymidine nucleotide incorporation observed correlated with an increase in the expression of cell cycle

genes, as would be expected in a dividing cell, we assessed cell cycle gene expression with quantitative real-time PCR (qRT-PCR). Interestingly, there was no significant change in expression of cell cycle genes, including the proliferative marker, *Ki67* (Figure 1D). We concluded that overexpression of hsTCF19 in INS-1 cells does not lead to transcriptional activation of cell cycle genes, suggesting an alternate process for nucleotide incorporation that does not result in cell cycle progression. DNA repair may be an alternative pathway that leads to increased ^{3}H-thymidine nucleotide incorporation [31]. DNA damage and repair responses are important in preserving genome integrity, and an accumulation of DNA damage without sufficient repair can result in cell cycle arrest at the G1/S checkpoint [32]. However, qRT-PCR showed no significant change in cell cycle inhibitors *Cdkn2c (p18), Cdkn1a (p21),* and *Cdkn1b (p27)* with hsTCF19 overexpression, suggesting that there was no induction of substantial DNA damage leading to cell cycle arrest or activation of checkpoint inhibitors (Figure 1D). We next hypothesized that if hsTCF19 overexpression is affecting DNA repair, it may elicit a change in cell viability. However, after staining cells with trypan blue, we found that the percentage of live cells was not significantly affected by hsTCF19 overexpression (Figure 1E).

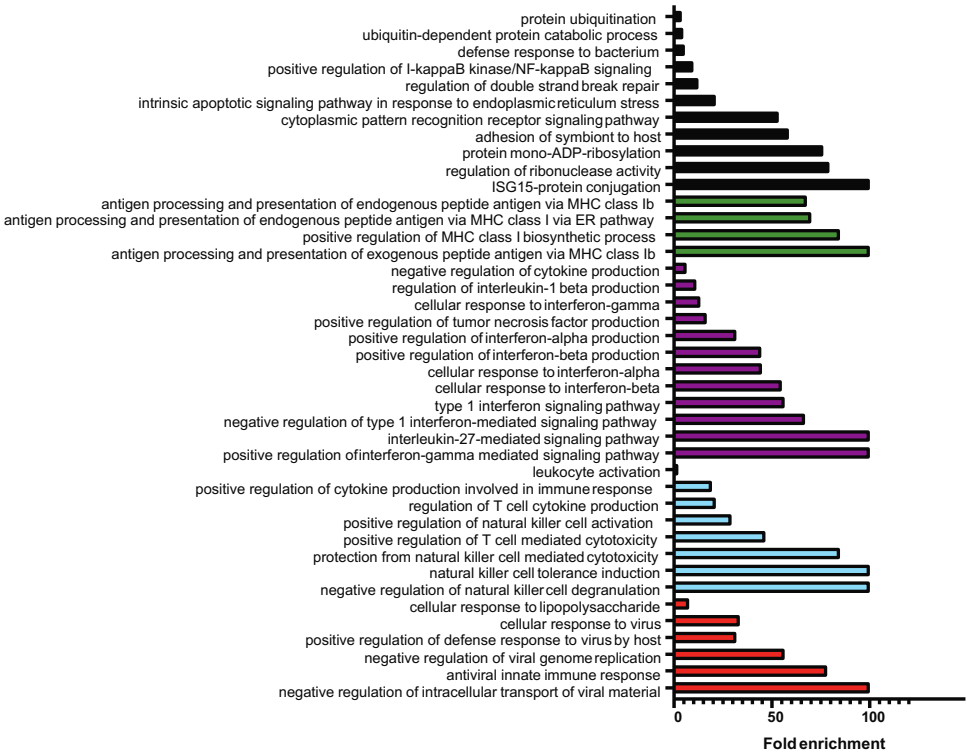
2.2. RNA-Seq Analysis Reveals a Role for TCF19 in Regulating Viral, Inflammatory, and DNA Damage Genes

To obtain a more global perspective on what genes TCF19 could be regulating, we performed RNA-seq analysis on INS-1 cells overexpressing hsTCF19. Notably, this revealed only a relatively small number of differentially expressed genes. Of the 160 genes differentially expressed between the groups (false discovery rate (FDR) < 5%), 136 genes were upregulated and 24 were downregulated (Table S1), suggesting that TCF19 likely acts as a positive regulator of transcription. 92 gene IDs were identified as being upregulated >2-fold from the original list of 136 upregulated genes, and of these, 85 were uniquely mapped and included in the PANTHER Fisher's Exact overrepresentation test [33–35]. This analysis revealed 199 significantly overrepresented Biological Process Gene Ontology (GO) terms (FDR < 5%). These GO terms were sorted hierarchically and the most specific subclasses of GO terms are listed in Figure 2A. This analysis shows that genes upregulated with hsTCF19 overexpression are highly enriched for biological processes relating to double-strand break repair, apoptosis in response to ER stress, antigen presentation, interferon signaling, immune system processes, and viral responses (Figure 2A).

To determine the relationship between the significantly upregulated genes, we performed Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis on the 92 genes that were upregulated >2-fold. STRING uses an algorithm to reflect co-occurrences of genes in literature to predict associations for a particular group of genes [36]. STRING analysis revealed highly significant connectivity between almost all input genes (enrichment p-value $< 1 \times 10^{-16}$), suggesting that TCF19 may be regulating one cluster of interconnected genes (Figure 2B). We hypothesized that this cluster of genes may have roles in viral and interferon responses, as well as the DDR.

Among the upregulated genes (Table S1), several are known to be involved in DDR and repair pathways *(Parp9, Parp10, Parp12, Parp14)* [37]. In particular, *Parp9* and another gene from the dataset, *Dtx3l*, have been shown to work as a complex to promote DNA repair [38]. Other significantly upregulated genes include those from the oligoadenylate synthase (Oas) family *(Oas1i, Oasl2, Oas2, Oas1a, Oas1g, Oas1f*), which are stimulated by type 1 interferons in response to viral infections [39]. However, they can also be activated by DNA damage, where they may have roles in Poly(ADP-ribose) (PAR) synthesis and interacting with *PARP1* during DNA repair [39,40]. *Mx1*, *Ddx60,* and *Usp18* are genes with known antiviral roles and were also significantly upregulated [41–43]. These observations suggest that TCF19 may play a previously unreported role in the DDR and viral and inflammatory response pathways.

A.



B.

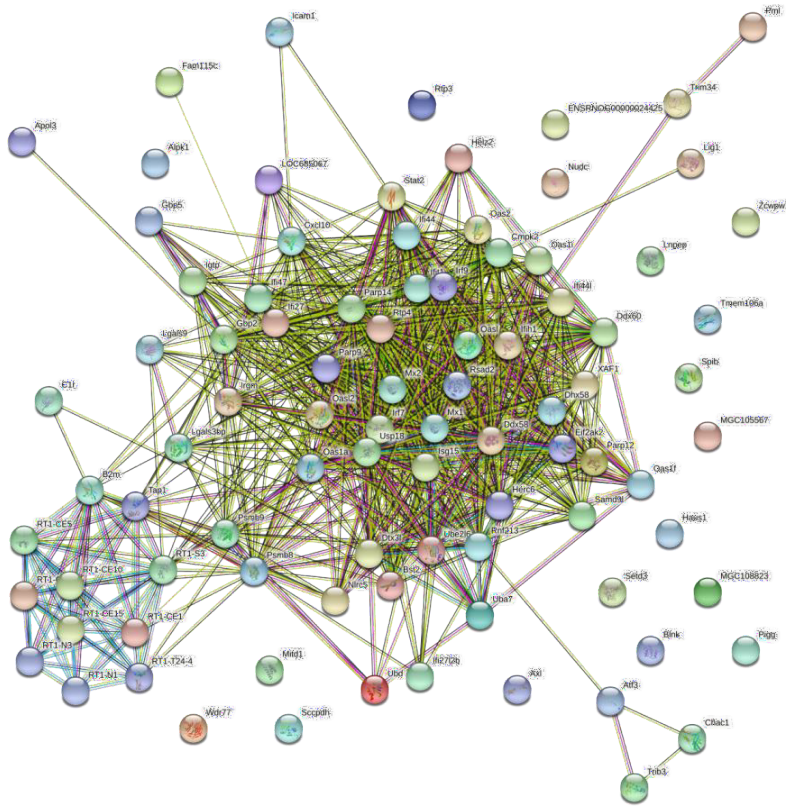


Figure 2. RNA-seq analysis of INS-1 cells overexpressing hsTCF19 identifies upregulated genes that form a tight node of interconnected genes with roles in viral, immune, and interferon response pathways (**A**) PANTHER overrepresentation test

for GO biological processes on >2-fold upregulated genes show enrichment for genes involved in viral responses (red), regulation of immune response processes (blue), regulation of cytokines (purple), and antigen presentation (green). Additionally, other processes such as regulation of double strand break repair, apoptotic signaling pathway in response to ER stress, and positive regulation of NF-κB signaling were also overrepresented (black) (FDR < 5%). (**B**) STRING analysis on differentially expressed genes with >2-fold upregulation shows tightly interconnected network of genes.

To assess the extent to which the findings in this overexpression model could be translated to human islets, we overexpressed hsTCF19 in human islets (Figure 3A) and assessed several of the differentially expressed genes from the RNA-seq dataset (Figure 3B). Notably, transcript levels for DDR genes *PARP9* and *DTX3L* were significantly upregulated in human islets overexpressing hsTCF19 compared to the empty vector control islets (Figure 3B). Antiviral genes *MX1* and *DDX60* were also significantly upregulated (Figure 3B).

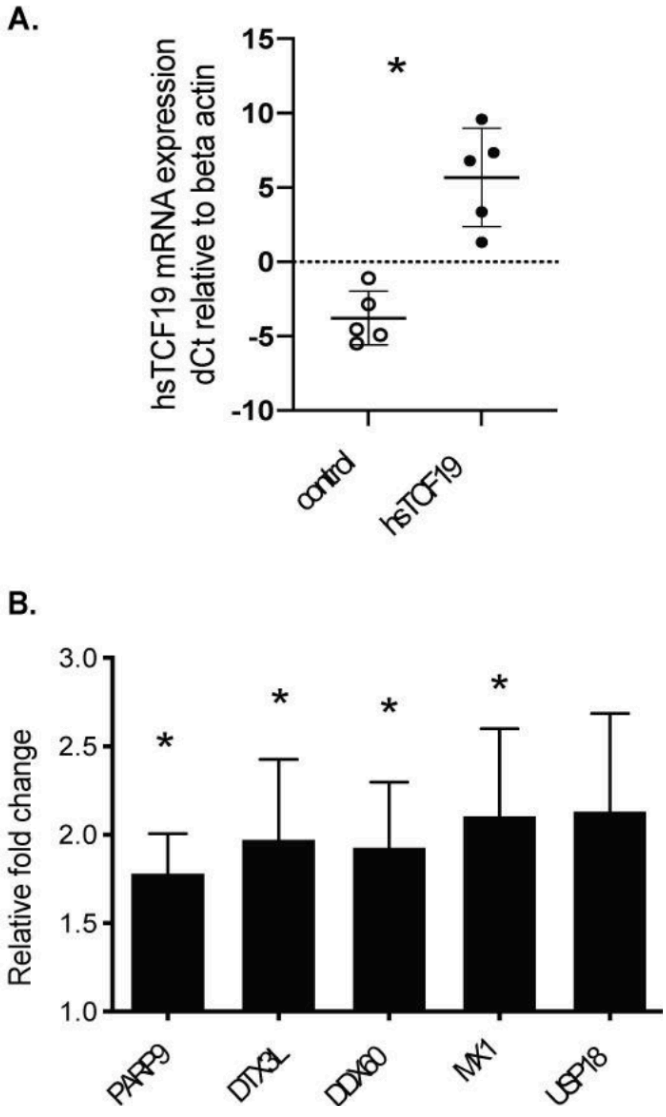


Figure 3. Human TCF19 overexpression in human islets upregulates key DNA damage repair genes as well as viral response genes. (**A**) hsTCF19 overexpression in human islets was confirmed with qRT-PCR. Control represents human islets transfected with an empty vector and hsTCF19 represents human islets transfected with TCF19 plasmid. (**B**) hsTCF19 overexpression in human islets leads to upregulation of genes (qRT-PCR) that were also upregulated in INS-1 cells (n = 5). Gene names are shown on the horizontal axis. Data are means ± SEM * $p < 0.05$.

2.3. Mining Algorithm for GenetIc Controllers (MAGIC) Analysis for Common Transcriptional Regulators

To look for common transcriptional regulators associated with the promoters of the 92 genes that were upregulated >2-fold in INS-1 cells overexpressing hsTCF19, we performed MAGIC analysis [44]. MAGIC compares input gene lists to ChIP seq tracks archived in Encyclopedia of DNA Elements (ENCODE) and predicts which nuclear proteins are enriched at the promoters/regulatory regions of the input gene list [44]. These analyses revealed Signal Transducer and Activator of Transcription (STAT)1 and STAT2 as positive drivers of the gene set (Figure 4). The associations were striking with p-values of 7.81×10^{-19} and 3.23×10^{-20}, respectively. Specifically, STAT1 and STAT2 are known to interact with the promoter of 17 genes out of the 92 from the gene set. There was strong enrichment within the gene set compared to overall promoter interactions for these STAT proteins across the genome, suggesting that TCF19 leads to upregulation of genes that can also be regulated by the STAT proteins. However, these associations in ENCODE were not determined in β-cells or islets and were often based on experiments involving interferon stimulation. Additionally, Interferon Response Factor (IRF)1, a transcription factor important in both innate and adaptive immunity, also showed striking enrichment for promoter interactions with the upregulated gene set ($p = 2.77 \times 10^{-16}$).

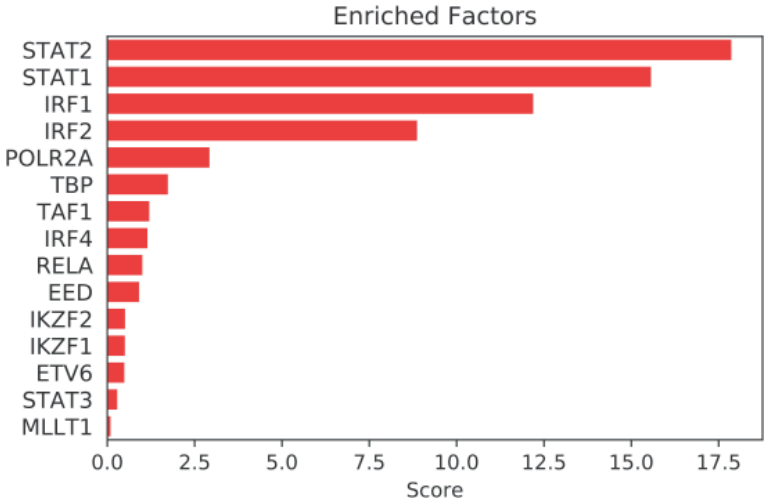


Figure 4. MAGIC analysis on the list of upregulated genes after TCF19 expression in INS-1 cells identifies significant enrichment for genes with known ChIP signals for STAT1, STAT2, and IRF1 in their promoters (FDR < 10%). A higher score reflects increased likelihood that the factor is enriched for binding gene regions in the list of upregulated genes. Genes included in analysis were those that were upregulated more than 2-fold with an associated FDR < 5%.

As these transcription factors could be potential regulators of the upregulated genes in this dataset, we assessed the activation level of these transcription factors. Full activation of STAT1 involves both phosphorylation at tyrosine (Y701) and serine (S727) residues [45]. Y701 phosphorylation is required for nuclear accumulation of STAT1, while full transcriptional activity of STAT1 requires phosphorylation at S727 [45]. STAT1 is phosphorylated at the S727 residue in response to interferons [45,46]. We did not observe a significant difference in S727 levels (Figure 5A). Interestingly, we observed a significant decrease in Y701 phosphorylation (Figure 5B). There was no change in IRF1 levels (Figure 5C). STAT2 was not detectable in the INS-1 cells. Taken together, this suggests that TCF19 does not directly modulate the levels of STAT1, STAT2, or IRF1 in β-cells, nor does it impact the phosphorylation of S727 on STAT1 that is required for full activity. We actually see a decrease in Y701 STAT1 phosphorylation, suggesting that less STAT1 is capable of moving to the nucleus for transcriptional activity in the presence of TCF19.

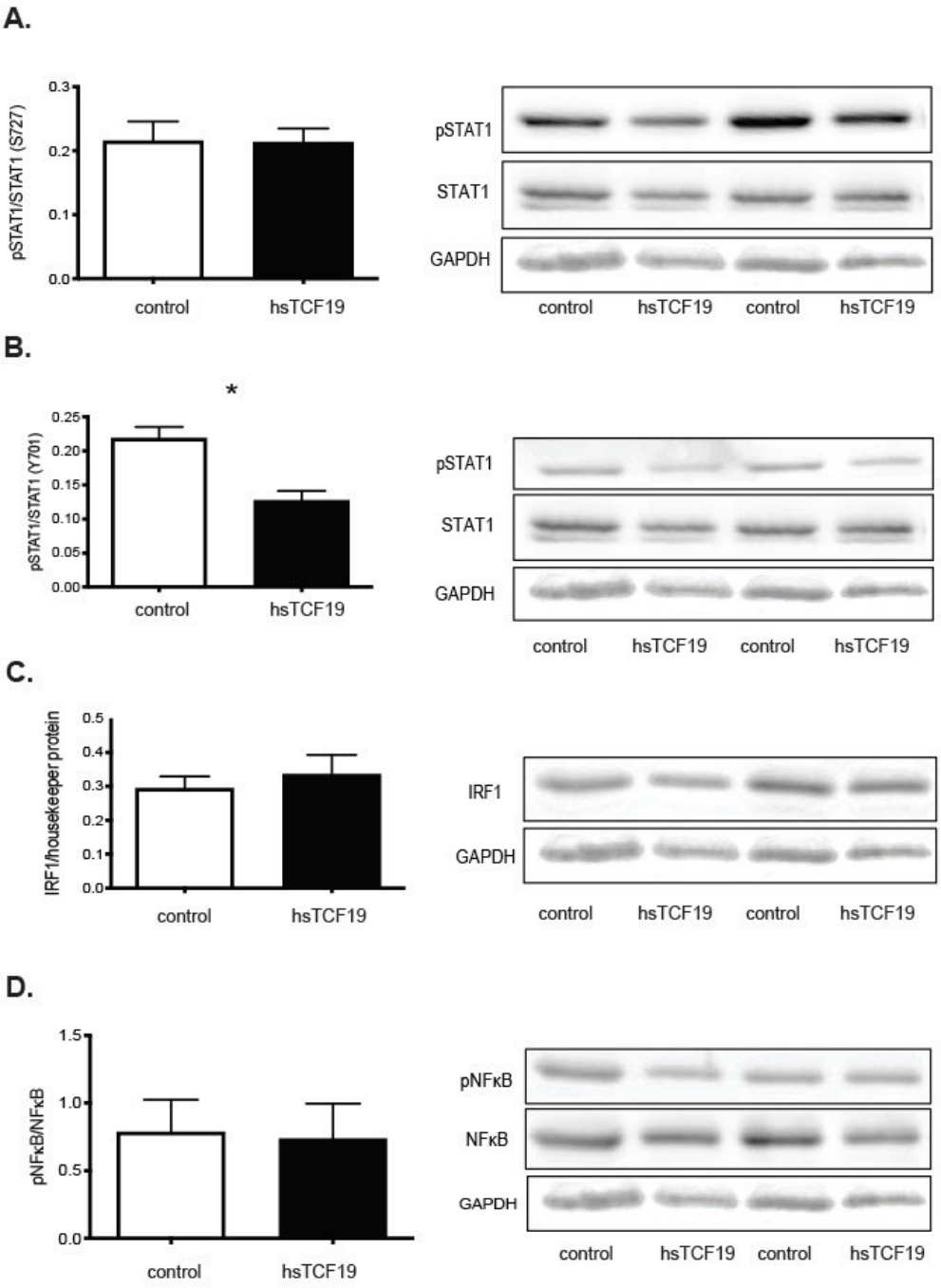


Figure 5. Overexpression of TCF19 in INS-1 cells does not lead to increased activation of transcription factor targets but leads to decreased activation of STAT1 Y701 phosphorylation. Representative images of two western blot replicates along with analysis of all replicates are shown. Densitometry with Image J 1.44o was used to quantify the bands on the western blots, which were then normalized to the housekeeper protein band. (**A**) Serine 727 phospho-STAT1/STAT1 protein expression does not show a statistically significant difference between control and hsTCF19 overexpressing cells (n = 5) (phospho-STAT1~91 kDa, STAT1~84, 91 kDa). (**B**) Tyrosine 701 phospho-STAT1/STAT1 protein expression shows statistically significant decrease in hsTCF19 overexpressing cells compared to control (n = 3) (phospho-STAT1~84, 91 kDa). (**C**) IRF1 protein levels are not significantly different. Representative western blots in the figure have IRF1 levels normalized to GAPDH. Three other replicates are normalized to beta tubulin (n = 5) (IRF1~48 kDa). (**D**) There is no difference in phospho NF-κB/NF-κB levels with hsTCF19 overexpression (n = 3) (phospho NF-κB~65 kDa, NF-κB~65 kDa). All data are means ± SEM * $p < 0.05$.

Although not identified as a potential co-regulator in MAGIC analysis, Nuclear Factor Kappa-B (NF-κB) has a well-characterized role in mediating inflammation and is also activated by the cyclic GMP-AMP synthase (cGAS)-Stimulator of Interferon Genes (STING) pathway, which is a component of the innate immune system that functions to detect cytosolic DNA and leads to the production of type 1 interferons [47]. Additionally, positive regulation of the NF-κB pathway signaling was a biological process that was significantly overrepresented in PANTHER GO analysis (Figure 2A). Therefore, we predicted that NF-κB may be a possible regulator of the upregulated gene set. However, we found no increase in the phosphorylation of NF-κB with TCF19 overexpression (Figure 5D).

3. Discussion

Inflammation is a pathophysiological state associated with both T1DM and T2DM. In T1DM, immune cells are critical mediators of islet inflammation through their secretion of cytokines such as interleukin 1 beta (IL-1beta) and tumor necrosis factor-alpha (TNF-alpha) [48]. Additionally, substantial evidence suggests that triggering events such as a viral infection may initiate the β-cell damaging process [49]. In T2DM, obesity induces chronic, low grade inflammation which activates inflammatory pathways and the release of pro-inflammatory cytokines and adipokines [50,51]. Inflammation not only exacerbates insulin resistance and promotes β-cell death but can also contribute to DNA damage [15,52].

In this study, we delineate a role for TCF19, in the inflammatory and DNA damage pathways. We find that TCF19 overexpression significantly increases expression of inflammatory and DDR genes, suggesting a novel role for TCF19 in regulating these two pathways. We find that the significantly upregulated genes from TCF19 overexpression are tightly associated, and we describe potential transcription factor co-regulators of these genes. This brings to light an interesting crosstalk between the inflammatory and DNA damage pathways in the β-cell and suggests how alterations in TCF19 expression or function may contribute to diabetes pathogenesis in both T1DM and T2DM. Although one of many genes associated with diabetes in GWAS, TCF19 is unusual in having associations with both types of diabetes. This shared association suggests that TCF19 may regulate a mechanism involving shared pathophysiology in both T1DM and T2DM, such as β-cell damage or inflammatory responses.

Knockdown of Tcf19 has been shown to result in cell cycle arrest [21]. While we show here that overexpression of hsTCF19 does not result in significant changes in cell cycle genes, hsTCF19 overexpression does result in increased expression of DDR genes. The DDR is made up of DNA damage sensing proteins, transducers, and effectors [53]. Once an aberrant DNA structure is recognized, downstream phosphorylation cascades within the DDR network are initiated with many of the downstream effector proteins having roles in promoting cell cycle arrest [53]. This allows time for the cell to repair the damaged DNA. Other effector proteins upregulate DNA damage repair genes or promote senescence or apoptosis in the face of unrepairable DNA damage [32]. With TCF19 overexpression, we find an increase in genes involved in the DDR but no decrease in cell viability, suggesting that these cells are not undergoing apoptosis. Additionally, the lack of significant change in cell cycle genes including cell cycle inhibitors suggests there is no DNA damage-induced cell-cycle arrest. Notably, these experiments were all performed in the absence of any inducers of DNA damage or interferons, yet we observed upregulation of classic interferon-response genes. Therefore, enhanced TCF19 expression alone is sufficient to independently activate these pathways. Since overexpression of TCF19 led to upregulation of many DNA damage repair genes, this suggests that within the DDR network TCF19 most likely plays a role as a transcriptional regulator that may promote DNA damage repair.

Tcf19 knockdown leads to cell cycle arrest at the G1/S transition [21]. This is consistent with the cell cycle arrest that occurs upon DNA damage to cells in the G1 phase to prevent entry into the S phase [32]. Sustained DNA damage can eventually result in cellular apoptosis [54]. We previously showed that 3–7 days of Tcf19 knockdown led to an increase in cells undergoing apoptosis and a decrease in cell viability. Combining these prior

results with current data, we propose that cells lacking TCF19 are inefficient at repairing DNA damage, ultimately leading to cell cycle arrest or cell death due to accumulated DNA damage. We hypothesize that with TCF19 overexpression, DNA damage repair is upregulated.

Interestingly, many of the genes upregulated by TCF19 overexpression are also involved in interferon and immune responses. Additionally, GO analysis revealed an overrepresentation of genes involved in viral, DNA damage, and stress response processes. This signature of viral, inflammatory, and DNA damage responses brings to light an interesting and emerging field connecting DNA damage and the interferon response. Treatment of cells with etoposide, an agent that induces double stranded DNA breaks, leads to the induction of interferon-stimulated genes regulated by NF-κB [55]. The cGAS-STING pathway is a component of the innate immune system that functions to detect cytosolic DNA and, upon activation of STING, results in the production of type 1 interferons [56]. However, after etoposide treatment, there is noncanonical activation of the STING pathway by the DNA repair proteins, ATM and PARP1 [56]. Additionally, the DNA sensor, cGAS, has been shown to be shuttled to the nucleus under conditions of DNA damage [57]. Given these connections, we also looked for an increase in phospho-STING after TCF19 overexpression but did not see any significant changes (data not shown). Further exploration of possible connections between the cGAS-STING pathway and DNA damage and inflammatory responses in the β-cell remain intriguing new directions for future study.

While these studies show that DNA damage can lead to inflammatory gene expression, inflammation can also induce DNA damage. Chronic inflammation can lead to the production of ROS, which are capable of DNA damage through the formation of free radicals and DNA lesions [58]. In further support of the coordinate regulation between these two pathways, viruses can activate the DDR network and also inhibit several DDR proteins [59]. As viral infection is an important initiating factor in T1DM, this could serve as a potential link between the two pathways where the immune system's viral response may trigger DNA damage and progression to T1DM. It is likely that the DDR and inflammatory pathways are part of a positive feedback loop [60]. We see a dual response gene signature of viral/interferon and DNA damage processes with TCF19 overexpression, suggesting that TCF19 may regulate both these processes. However, we acknowledge the possibility that TCF19 may regulate just one of these processes, and in turn, be indirectly affecting the other.

STRING analysis further supports the tight association between the DNA damage and inflammatory genes in this dataset. MAGIC analysis revealed STAT1, STAT2, and IRF1 as common regulators of this gene set. These transcription factors have well-characterized roles in response to interleukins and interferons, specifically type 1 interferons [61]. However, there have also been studies showing a role for these transcription factors in the DDR and repair pathway [59]. A few of these transcription factors have been found to be responsible for the induction of interferon alpha and gamma genes in response to DNA damage or have roles in regulating DNA damage repair proteins [59,62].

While we did not observe direct increases in phosphorylation or protein levels of these transcription factors, phosphorylation events can be transient and tightly regulated. It is possible that the time point of harvest (48 h post-transfection) may have been too late to capture the phosphorylation event. We did observe a significant decrease of STAT1 Y701 phosphorylation which is required for STAT1 dimerization, nuclear translocation, and DNA binding [45]. This alludes to the possibility that TCF19 may actually be attenuating STAT1 activity. However, there has been debate that dimerization of STAT1 may not be necessary for initiation of interferon-dependent signaling, and that positive and negative transcriptional control may also be modulated by unphosphorylated STAT1 [63]. Additionally, studies have shown that S727 of STAT1 can be phosphorylated independently of Y701, and that Y701 is necessary but not sufficient for interferon-induced S727 phosphorylation [45]. The time scale by which each phosphorylation event reaches its maximal activity is different, with Y701 reaching its maximal level earlier than S727, after cytokine

stimulation [45]. This demonstrates the complexity of STAT1 regulation and bodes for further investigation as to how TCF19 overexpression may be modulating its different phosphorylation status.

While we chose to look at phosphorylation events for activation of the transcription factors identified through MAGIC analysis, other types of post-translational modifications, such as those that may work to alter chromatin structure or recruit histone modifiers cannot be ruled out. Notably, TCF19 has been shown to interact with H3K4me3 through its PHD finger to repress gluconeogenic gene expression and to modulate proliferation in HepG2 cells [29,30]. Therefore, it is likely that TCF19 is not directly activating these transcription factors through phosphorylation events, but instead may bind to H3K4me3 at a transcriptionally active promoter and thereby impact transcriptional activation. Additionally, the TCF19 protein harbors an FHA domain, which may allow binding to phosphor-epitopes on proteins [64,65]. FHA domains are often found in proteins that are critical in the cell cycle and regulated through phosphorylation events but are also found in proteins that are involved in the DDR [64]. The FHA domain of TCF19 contains a serine residue at position 78 (Ser78) that has been shown to be phosphorylated after DNA damage [66]. Ser78 in TCF19 is located within a Ser-Gln motif, which is recognized by kinases involved in the DDR such as ATM and ATR [66]. Therefore, it is possible that TCF19 is a downstream target of ATM or can alter gene expression by acting as a co-regulator to other kinases.

We hypothesize that TCF19 affects DDR gene expression through interactions with modified histones via the PHD finger and/or acts as a co-activator to DNA damage proteins by recruiting other DNA damage transcription factors to areas of active chromatin. While we did not directly measure an interaction of any of the transcription factors from the MAGIC analysis with relevant promoters in response to TCF19 overexpression, our data suggest that TCF19 either modulates their ability to activate transcription or may in fact simply be regulating the expression of these genes independently of these transcription factors. The exact mechanism of how TCF19 modulates these inflammatory and DDR genes to promote diabetes susceptibility requires further investigation.

Overall, our work highlights the complexity of regulation of gene expression involved in DNA damage and inflammatory response genes and alludes to the interesting crosstalk between these processes in the context of TCF19. With respect to diabetes susceptibility, individuals with genetic variants of TCF19 may be unable to properly regulate β-cell responses to DNA damage and inflammatory insults, therefore predisposing them to increased β-cell apoptosis. Future experiments will explore the nature by which TCF19 modulates DNA damage repair and inflammatory genes under conditions of stress. Furthermore, this will provide for potential therapeutic targets to prevent or attenuate DNA damage and inflammation to preserve functioning β-cells in at-risk individuals.

4. Materials and Methods

4.1. Human Islets and INS-1 Cell Culture

INS-1E rat insulinoma cells were cultured in RPMI 1640 supplemented with 1% antibiotic-antimycotic (Gibco, 15240–062), 1% L-glutamine, 1% sodium pyruvate, and 10% fetal bovine serum. 2-Mercaptoethanol was added to a final concentration of 50 μM to supplemented media before each use. Human islets were obtained from nondiabetic organ donors through the Integrated Islet Distribution Program. An exemption was granted for human islet work by the Institutional Review Board at the University of Wisconsin. Human islets were cultured in uncoated petri dishes with RPMI 1640 containing 8mM glucose, 10% heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin. INS-1 cells and islets were cultured at 37 ˚C and 5% CO_2 in a humidified atmosphere.

4.2. Creation of TCF19 Overexpression Vector

The human TCF19 clone HsCD00002769 was purchased in the pDNR-Dual vector backbone (DNASU Plasmid repository). The pcDNA4-TO-myc/his B backbone vector (Invitrogen) was chosen for overexpression. This vector utilizes a CMV promoter, which

ensures robust expression of the inserted gene of interest. Following the inserted TCF19 sequence is both a C-terminal c-myc tag as well as six histidine residues to allow for identification of the overexpressed protein in the absence of reliable TCF19 antibodies. The hsTCF19-pcDNA4 vector was created with In-Fusion HD cloning (Clontech) following kit instructions. Colonies were screened with PCR for insert size and then sequenced to confirm TCF19 insertions and sequence integrity.

4.3. Transfection with hsTCF19-His/Myc-pcDNA4

INS-1 cells and islets were transfected with either hsTCF19 or pcDNA4 control, using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA). INS-1 cells were trypsinized and resuspended in transfection medium (RPMI 1640 supplemented with 1% L-glutamine, 1% sodium pyruvate, and 10% fetal bovine serum). Cells in transfection medium were then added to an hsTCF19 or control plasmid-Lipofectamine mixture at 2–5 µg DNA/5 × 10^6 cells and plated. Transfection medium was removed 12–18 h post-transfection and replaced with complete growth medium. These conditions were the same for all INS-1 overexpression studies, including RNA-Seq sample preparation.

Human islets were washed in 1x PBS and resuspended in Accutase (Sigma, St. Louis, MO, USA) dissociation solution for 3 min at 37 °C, with tube inversions every 30 s. Islets were then resuspended in 2 mL transfection medium and plated into dishes. hsTCF19 or control plasmid-Lipofectamine 2000 mixture was added at 2 µg DNA/1000 islets. Transfection medium was removed 12–18 h post-transfection and replaced with complete growth media.

4.4. Western Blotting

INS-1 cells were harvested 48 h after transfection and washed in ice-cold PBS. Cells were lysed in protein lysis buffer (0.05 M HEPES, 1% NP-40, 2 mM activated sodium orthovanadate, 0.1 M sodium fluoride, 0.01 M sodium pyrophosphate, 4 mM PMSF, 1 mM leupeptin, 2 µM okadaic acid and Sigma Protease inhibitor cocktail). Cells were incubated in the lysis buffer on ice for 15 min with vortexing every 5 min. The protein concentrations were determined using Bradford protein assay. The protein samples were run on 4–10% SDS-PAGE gradient gel and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature and were incubated overnight in primary antibody, washed 3X in TBST and incubated 1 h in secondary antibody. Blots were developed with Pierce ECL Western Blotting Substrate (Thermo Fisher, Waltham, MA, USA) or Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher), imaged with a GE Image-Quant charge-coupled device camera, and then quantified by densitometry with Image J 1.44o (http://imagej.nih.gov/ij, accessed 28 February 2020). Primary antibodies and dilutions were as follows: Myc antibody (9E10:sc-40, Santa Cruz Biotechnology, 1:1000), Beta actin (8H10D10, Cell Signaling, 1:1000), STAT1 (#9172, Cell Signaling, 1:1000), phospho-STAT1 Ser727 (#9177, Cell Signaling, 1:1000), phospho-STAT1 Tyr701 (D4A7 #7649, Cell Signaling, 1:1000), phospho- NF-κB p65 Ser536 (93H1 #3033, Cell Signaling, 1:1000), NF-κB p65 (D14E12 XP #8242, Cell Signaling, 1:1000), IRF1 (D5E4, Cell Signaling, 1:1000), GAPDH (14C10, Cell Signaling, 1:1000), Beta-tubulin (#2146, Cell Signaling, 1:1000) in 5% BSA-TBST or 5% non-fat milk. Secondary antibodies and dilutions were as follows: Anti-rabbit IgG, HRP-linked antibody (#7074, Cell Signaling, 1:2000), anti-mouse IgG, HRP-linked antibody (#7076, Cell Signaling, 1:2000).

4.5. Quantitative Real-Time PCR

RNA was isolated from INS-1 and human islets 48 h post-transfection using RNeasy cleanup kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Concentration and purity of RNA were determined using a NanoDrop ND-2000c Spectrophotometer, and 100–250 ng of RNA was reverse transcribed to make cDNA with Applied Biosys- tem High-Capacity cDNA synthesis kit. Quantitative real-time PCR (qRT-PCR) reactions

were carried out using Power SYBR green PCR Master Mix (Applied Biosystems) and the StepOnePlus Real Time PCR System (Applied Biosystems). Reverse transcriptase free samples were used as negative controls. All samples were run in triplicates with Cycle threshold (Ct) values normalized to β-actin to yield ΔCt. Fold changes were then calculated between experimental and control samples: fold change $2^{(\Delta Cexperimental—\Delta Ctcontrolt)}$. For gene expression in INS-1 cells, results were analyzed by non-paired *t*-test of the ΔCt t values, while human islets were analyzed by paired *t*-test. Significance was determined by $p < 0.05$. Primer sequences used are in Table S2.

4.6. Viability

Transiently transfected INS-1 cells were harvested at 48 h post-transfection by using a cell scraper to dislodge all cells, and 10 µL of cells were collected from each well. Cell viability was determined using trypan blue (Corning, Corning, NY, USA) staining using the TC-10 Automated Cell Counter (BioRad, Hercules, CA, USA). Comparisons were made by paired *t*-test, including all technical and biological replicates; statistical significance was determined by $p < 0.05$.

4.7. Proliferation/^{3}H-Thymidine Incorporation

To measure cell proliferation, transiently transfected INS-1 cells were incubated with ^{3}H-thymidine (Perkin Elmer, Waltham, MA, USA, NET0270001MC) at a final concentration of 1 µL ^{3}H-thymidine/mL of supplemented RPMI media for 4 h. Cells were then trypsinized and washed three times with ice-cold PBS. DNA and protein were precipitated by the addition of ice-cold 10% trichloroacetic acid (TCA) and incubated for 30 min on ice. The precipitate was then pelleted at 18,000×*g* for 10 min at 4 °C. Pelleted precipitate was solubilized in 0.3 N NaOH and vortexed for 15 min. Radioactivity was measured using a liquid scintillation counter, and a fraction of the solubilized product was kept to measure total protein by the Bradford assay. Sample counts per minute were individually normalized to protein, and an average for each transfection was determined. Results were analyzed by unpaired *t*-test, and statistical significance was determined by $p < 0.05$.

4.8. RNA Sequencing

INS-1 cells were transfected with either hsTCF19-pcDNA4 or pcDNA4 control vector as stated in the methods above. Cells were cultured 48 h post-transfection before being collected for RNA using the RNeasy Kit (Qiagen). Total RNA was verified for concentration and purity using a NanoDrop ND-2000c Spectrophotometer and Agilent 2100 BioAnalyzer. Samples that met the Illumina TruSeq Stranded Total RNA (Human/Mouse/Rat) (Illumina Inc., San Diego, CA, USA) sample input guidelines were prepared according to the kit's protocol. Cytoplasmic ribosomal RNA reduction of each sample was accomplished by using complementary DNA probe sequences attached to paramagnetic beads. Subsequently, each mRNA sample was fragmented using divalent cations under elevated temperature, and purified with Agencourt RNA Clean Beads (Beckman Coulter, Pasadena, CA, USA). First strand cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers. Second strand cDNAs were synthesized using DNA Polymerase I and RNAse H for removal of mRNA. Double-stranded cDNA was purified using Agencourt AMPure XP beads (Beckman Coulter, Pasadena, CA, USA). cDNAs were end-repaired by T4 DNA polymerase and Klenow DNA Polymerase and phosphorylated by T4 polynucleotide kinase. The blunt ended cDNA was purified using Agencourt AMPure XP beads. The cDNA products were incubated with Klenow DNA Polymerase to add an 'A' base (Adenine) to the 3^t end of the blunt phosphorylated DNA fragments and then purified using Agencourt AMPure XP beads. DNA fragments are ligated to Illumina adapters, which have a single 'T' base (Thymine) overhang at their 3^{t}end. The adapter-ligated products are purified using Agencourt AMPure XP beads. Adapter ligated DNA was amplified in a Linker Mediated PCR reaction (LM-PCR) for 12 cycles using Phusion™ DNA Polymerase and Illumina's PE genomic DNA primer set

followed by purification using Agencourt AMPure XP beads. Quality and quantity of finished libraries were assessed using an Agilent DNA1000 series chip assay (Agilent Technologies, Santa Clara, CA, USA) and Invitrogen Qubit HS cDNA Kit (Invitrogen, Carlsbad, CA, USA), respectively. Libraries were standardized to 2 nM. Cluster generation was performed using the Illumina cBot. Paired-end, 100bp sequencing was performed, using standard SBS chemistry on an Illumina HiSeq2500 sequencer. Images were analyzed using the standard Illumina Pipeline, version 1.8.2. RNA Library preparation and RNA Sequencing was performed by the University of Wisconsin-Madison Biotechnology Center.

Sequencing reads were adapted, and quality trimmed using the Skewer trimming program [67]. Quality reads were subsequently aligned to the annotated reference genome (Rnor_6.0) using the STAR aligner [68]. Quantification of expression for each gene was calculated by RSEM [69]. The expected read counts from RSEM were filtered for low/empty values and used for differential gene expression analysis using EdgeR [70] using a FDR cut off of <5%.

4.9. GO Term Enrichment

Upregulated genes with fold change >2 were input into PANTHER 16.0 and compared against Rattus norvegicus reference genome list using the Overrepresentation Test to test for enrichment using the GO biological process complete Ontology database DOI:10.5281/zenodo.4735677 (Released 2021-05-01, http://www.pantherdb.org, accessed 8 June 2021) [33–35]. Fisher's exact test and FDR correction was used to determine statistical significance. We had 92 gene IDs on the initial input list, and 85 were uniquely mapped to their corresponding PANTHER ID.

4.10. Protein-Protein Interaction Network Construction

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database was used to construct the protein-protein interaction network on genes that were upregulated >2-fold (https://string-db.org/, access date 8 June 2021) [36]. Interaction score of >0.4 was used as the cutoff criterion.

4.11. Mining Algorithm for GenetIc Controllers (MAGIC) Analysis

MAGIC analysis uses Encyclopedia of DNA Elements (ENCODE) ChIPseq data to look for statistical enrichment of transcription factors (TFs) that are predicted to bind to regions in a gene set. It determines if genes in a list are associated with higher ChIP values than expected by chance for a given transcription factor or cofactor based on ENCODE data. Detailed methods are found in Roopra et al. [44] All genes that were induced more than 2-fold with an associated FDR < 5% were used as input and tested against the 5Kb_Gene.mtx matrix.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/metabo11080513/s1, Table S1: Differentially expressed genes with FDR < 5% from RNA-seq on hsTCF19 overexpression in INS-1 cells. Table S2: Primer sequences used for quantitative real-time PCR experiment.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The Python script and information on how to access required Matrix files are available at https://github.com/asroopra/MAGIC.

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Chapter 3: Characterization of a whole body Tcf19 KO (wbTcf19KO) mouse model

The following chapter is a manuscript currently in preparation.

Jeeyoung Han and Joseph Blumer performed mouse breeding and phenotyping studies. Joseph Blumer performed immunofluorescence studies and ImageJ analysis. RNA-seq of islets was performed by UW Biotechnology Center. Sukanya Lodh and Grace Yang performed RNA-seq analysis and Grace Yang performed further RNA-seq data interpretation and data representation with PANTHER overrepresentation tests. All remaining studies were performed by Grace Yang.

ABSTRACT

Transcription Factor 19 (*TCF19*) is a gene found to be associated with both Type 1 and Type 2 diabetes (T1DM, T2DM). Recent studies have shown a role for TCF19 in beta cell survival, proliferation, and impacting inflammatory and DNA damage response gene expression. However, the functional role of TCF19 in glucose homeostasis and how it may relate to the diabetic phenotype has not been studied. Here, we generated a whole body Tcf19 knockout (wbTcf19KO) mouse model and show that knockout of Tcf19 does not lead to any significant phenotypic differences compared to the wild type (WT) mice. However, wbTcf19KO mouse islets showed a significant increase in DNA damage as well as an increase in cell stress, DNA damage, and pro-apoptotic genes. These islets also had a decrease in genes related to proliferation and beta cell identity. When wbTcf19KO mice were placed on a short term high fat diet (HFD), their islets had impaired adaptive proliferative ability compared to islets from WT mice. When wbTcf19KO mice were placed on a long term HFD, these mice were significantly more glucose intolerant than WT mice and had a significant increase in DNA damage in their islets. Taken together, our data suggests that Tcf19 has a role in the DNA damage response (DDR) pathway in the islet and specifically, loss of Tcf19 leads to increased DNA damage. Additionally, our data show that Tcf19 is necessary for maintaining proliferative capacity and glucose tolerance in mice under conditions of HFD induced stress.

INTRODUCTION

The insulin-producing beta cells within pancreatic islets are critical for maintaining glucose homeostasis. Inability of beta cells to compensate through proliferation in the

face of increased insulin demand such as during times of insulin resistance or increased apoptosis that occurs due to endoplasmic reticulum stress (ER) or cytokine and oxidative stress, can lead to dysglycemia[1–3]. Chronic exposure to these stressors promotes beta cell apoptosis and decreased beta cell mass [4,5] .

A decrease of up to 60% of beta cell mass has been reported in Type 2 diabetes (T2DM) patients [6]. Additionally, several Genome Wide Association Studies (GWAS) have identified variants of specific cell cycle genes that may influence beta cell mass during development [6,7]. These variants may serve as genetic predisposing factors to diabetes. Pro-inflammatory cytokines such as interleukin-1 beta (IL-1beta), tumor necrosis factor-alpha (TNF-alpha), and interleukin-6 (IL-6) which are released by infiltrating immune cells in the islet can induce beta cell apoptosis in Type 1 diabetes (T1DM) and lead to decreased beta cell mass [4]. Another contributor of decreased functional beta cell mass is beta cell dedifferentiation where beta cells revert to progenitor - like cells [8]. Overall, there is sufficient evidence showing increased beta cell apoptosis and decreased beta cell mass in patients with diabetes.

While extensive research has been done studying the role of pro-inflammatory cytokines, ER stress, and dedifferentiation as players in decreased beta cell mass and diabetes pathogenesis, an emerging area of interest is the contribution of the DNA damage response (DDR) pathway to decreased functional beta cell mass. Many components of the diabetic milieu such as pro-inflammatory cytokines and reactive oxygen species (ROS) can induce DNA damage in beta cells and promote apoptosis [9–12]. Studies also show that patients with diabetes have increased DNA damage and

decreased repair capacity compared to their non-diabetic counterparts, alluding to a less well-studied role for DNA damage in diabetes development[13] .

In this study, we focus on the contribution of a novel transcription factor, Transcription Factor 19 (TCF19), in diabetes pathogenesis. GWAS reveals *TCF19* as a potential causal gene for both T1DM and T2DM, with genetic polymorphisms predicted to have functional effects on diabetes risk [14,15]. *TCF19* is expressed in both humans and rodents with highest expression in the pancreatic islet[16]. Additionally, *Tcf19* is upregulated in mouse models of non-diabetic obesity [17] . The TCF19 protein contains several protein domains including a forkhead-associated (FHA) domain, which is found in many proteins involved in the DDR, and also contains a plant homeodomain (PHD) finger domain which allows it to interact with tri-methylated histone H3 [18–20]. Our lab has previously shown that *Tcf19* is necessary for beta cell proliferation and survival in the INS-1 beta cell line and also elucidated a role for TCF19 in the DDR and inflammatory pathway[16,21]. These characteristics indicate a role for TCF19 as a transcriptional regulator of the DDR, inflammation, and cell proliferation and survival pathways.

In this study, we utilize a germline whole-body knockout mouse model of Tcf19 (wbTcf19KO) to explore the role of Tcf19 in beta cell mass regulation through the proliferative and DDR pathway. We find that while these mice have normal beta cell function and glucose tolerance, wbTcf19KO mouse islets have altered islet size distribution, decreased proliferative capacity, decreased beta cell identity gene expression, and increased DNA damage compared to wild-type (WT) controls. Furthermore, wbTcf19KO mouse islets have increased DNA damage upon cytokine and high fat diet (HFD) induced stress. We also show that wbTcf19KO islets have impaired

compensatory proliferative ability in response to a short term HFD. Taken together, these findings identify key roles for Tcf19 in maintaining beta cell proliferative capacity and regulating DNA damage responses.

METHODS

Mice

We generated a whole body knockout (wbTcf19KO) mouse using embryonic stem (ES) cells from the International Knockout Mouse Consortium (IKMC). Briefly, ES cells with the targeted allele was obtained from the IKMC. These ES cells are from male C57BL/6N mice and are heterozygous for the mutation. The main components of this vector are a 5' and 3' homology arms that mediate homologous recombination, and a central targeting cassette that disrupts gene function and reports gene expression using a LacZ reporter. The cassette is flanked by FRT recombination sites to allow removal by breeding by breeding with a Flp recombinase mouse. The vector also contains a pair of loxP recombination sites around exon 3 of *Tcf19.* The targeting vector also contains a neo resistance gene driven by the beta-actin promoter for clone selection. Chimeras with the ES cells (in the C57BL/6N background) were initially mated these with C57BL/6N wild type mice to obtain germline transmission. We then mated with an EIIa-Cre mouse which generates LoxP site recombination in the germline for a complete genetic knockout. A diagram outlining these steps can be found in Figure 1A.

Immunohistochemistry

Pancreata from WT and wbTcf19KO mice were frozen in Optimal Cutting Temperature (OCT). The frozen tissue was sectioned using cryostat Leica CM 1950. Four male mice per genotype were used. Sections were seven microns thick. First, excess OCT was removed with a delicate task wiper wet with 1xPBS. A hydrophobic slide marker was used to circle the tissue sections. Slides were then washed in 1xPBS twice for 5 minutes each. Next, the tissue was permeablized with 0.1% Tritonx100 in 1xPBS for 15 minutes. Slides were then washed in 1xPBS twice for 5 minutes each. Dako protein block (Dako x0909) was added to the sections for 30 minutes followed by washing the slides twice in 1xPBS for 5 minutes each. Primary antibody (Insulin: Dako Polyclonal Guinea Pig Anti-Insulin A0564; Ki67: CST Ki-67 mAb D3B5 Rabbit) was diluted in Dako antibody diluent (Dako S3022) (1:400 and 1:100 respectively) and added to the sections. Slides were placed in a humidified chamber and incubated overnight at 4C. The next day, sections were washed with 0.05% Tween-20 in 1xPBS for 5 minutes followed by a 5 minute wash in 1xPBS. Secondary antibody (Cy3-anti guinea pig and FITC-anti rabbit) was diluted in Dako antibody diluent (Dako S3022) (each at 1:400) and added to the sections for 30 minutes at room temperature. Sections were washed with 1xPBS three times for 5 minutes each. Vectashield with Dapi (Vector H-1200) was added to the sections and covered with a glass coverslip and sealed with clear nail polish. Sections were imaged using EVOS FL Auto microscope, individual islet images were captured at 20X magnification and pancreas section scans were captured at 10X magnification. All images were analyzed using ImageJ software. Statistical significance was determined with non-paired t-test and significance determined by $p<0.05$.

TUNEL staining

To measure apoptosis, Promega DeadEnd™ TUNEL Fluorometric TUNEL System (G3250) was used according to manufacturer's protocol. Briefly, sections were equilibrated with Equilibration Buffer for 10 minutes. Then the sections were labeled with TdT reaction mix. The slides were placed in humidified chamber and incubated at 37C for one hour. Slides were kept in the dark with minimal light exposure for this point forward. After the hour, 2xSSC was added to the section to stop the reaction for 15 minutes. Slides were then washed in 1xPBS twice for 5 minutes each. The sections then followed immunofluorescence beginning with protein block for co-staining. Statistical significance was determined with non-paired t-test and significance determined by $p<0.05$.

Freezing pancreas

Mice were anesthetized by intraperitoneal injection of avertin (500mg/kg). A fully anesthetized mouse was verified by loss of righting reflex and no reaction to both tail and toe pinch. The abdominal and thoracic cavities were opened and a syringe containing sterile 1xPBS was inserted into the left ventricle and a small nick was made in the right atrium. 1xPBS was circulated until all blood was cleared, then the syringe was replaced with one containing 4% paraformaldehyde and this was then circulated until all tissues had been fixed. Next, the whole pancreas was removed and placed in 4% paraformaldehyde for at least 30 minutes. Pancreas was then transferred to a 1xPBS wash for 5 min. The pancreas was then transferred to a 30% sucrose solution until the

tissue sank to the bottom, approximately 45 min. Next, pancreas was placed in a 1:1 ratio of 30% sucrose and OCT compound until it sank to the bottom, approximately 30 min. Then, pancreas was placed in OCT for approximately 10 min. Lastly, a thin layer of OCT was added to a tissue block and the pancreas was placed on top and covered with OCT and placed on dry ice to freeze and then stored in -80C.

ImageJ analysis

All immunofluorescence images were analyzed using ImageJ software. Beta cell area was calculated by measuring the total area of the insulin positive portion of all islets within a pancreas section and divided by the total area of the pancreas section time 100. Beta cell and total pancreas area were hand traced in ImageJ using a Wacom trackpad and pen and using the measure option under the analyze tab, which provided the areas in pixels. The area was then converted to um^2 with a pixel to um conversion factor determined by imaged ruler. Rates of apoptosis and proliferation were generated from individual islet images taken at 20X magnification. A macro was used to automatize total nuclei per islet and individual TUNEL and Ki67 foci were hand counted using the cell counter tool within ImageJ. If the macro was unable to appropriately count all nuclei due to inability to separate multiple nuclei, the islet would be hand counted using the same cell counter tool.

Pyruvate Tolerance Test

A 30% solution of sodium pyruvate (Sigma P5280) was made up in 0.9% saline solution and filter sterilized. Mice were weighed and an intraperitoneal (IP) injection was

administered with 15% pyruvate of body weight. Blood glucose was measured using tail prick at T:0, T:15, T:30, T:45, T:60, T:90, and T:120. Statistical significance was determined with non-paired t-test and significance determined by $p<0.05$.

Glucose Tolerance Test

Mice were weighed and an IP injection of a 50% solution of dextrose for a total concentration of 2g of dextrose/kg of body weight was administered. Blood glucose was measured using tail prick at T:0, T:15, T:60, and T:120. Statistical significance was determined with non-paired t-test and significance determined by $p<0.05$.

Insulin Tolerance Test

Mice were weighed and an IP injection of 0.75mU/g of insulin (Humalin) in 0.9% sterile saline was administered. Blood glucose was measured using tail prick at T:0, T:15, T:30, T:45, T:60, T:90. Statistical significance was determined with non-paired t-test and significance determined by $p<0.05$.

Comet Assay

The measurement of DNA strand breaks was examined using a CometAssay Kit (Trevigen) About 100 islets were counted out and treated with accutase (1mL/700 islets) to disperse them into single cells. Islets in accutase were then inverted every 30 seconds for 8 minutes while at 37C. 100,000 cells were then counted out and resuspended in 10uL PBS and combined with 50 uL LMAgarose (at 37C) and immediately spread onto a well of comet slides with a pipette tip. This procedure was repeated for all samples. Slides

were then placed at 4C in the dark for 10 minutes and then immersed in 4C Lysis Solution for 30 min. Slides were then immersed in filter sterilized 1xTBE buffer at 4C for 15 minutes and then placed on gel electrophoresis platform in 1XTBE and run at 4C at 25V for 20 minutes. After electrophoresis, slides were immersed in ddH20 for 5 minutes and then immersed in 70% ethanol for 5 minutes. Samples were then placed at room temperature to dry. After drying, samples were stained with 20ug/mL of ethidium bromide and allowed to stain for 20 minutes. Slides were then rinsed in ddH20 for 5 minutes. 15uL of Vectashield (Vector Laboratories H-1000-10) was pipetted onto the samples. Samples were sealed with coverslip and nail polish. Quantitative analyses were performed using CometScore 2.0 (http://rexhoover.com/index.php?id=cometscore). Intensities of tail DNA/whole comet intensity was a readout from CometScore 2.0 and represented %tail DNA. A fold change was calculated relative to the average tail intensity of the control group. Statistical significance was determined with non-paired t-test and significance determined by $p<0.05$.

Cytokine treatment

Islets isolated from mice were allowed to incubate for 24 hours before treatment. Cytokine cocktail containing 50ng/mL of TNF alpha (Miltenyi Biotec, #139-101-687) ,10 ng/mL IL-1beta (Miltenyi Biotec, #130-101-680), and 50 ng/mL IFN-gamma (Mitenyi Biotec, #130-105-785) were added to the cells. Islets were harvested 24 hours later and collected for protein. Statistical significance was determined with non-paired t-test and significance determined by $p<0.05$.

High Fat Diet

Mice were fed a high fat diet (HFD) with 60% of kcals coming from fat (Envigo TD.06414). Fasting blood glucose and body weights were measured weekly. After 10-weeks of HFD mice were subjected to a glucose tolerance test. After 10-weeks of HFD feeding, mice were euthanized and either islets were collected for RNA and protein or whole pancreas (N=4) were collected for immunofluorescence staining. Statistical significance was determined with non-paired t-test and significance determined by $p<0.05$.

Quantitative real-time PCR

RNA was isolated from mouse islets 24 hours after islet isolation and hand picking. RNA was extracted with RNeasy cleanup kit (Qiagen) according to manufacturer's protocol. Concentration and purity of RNA was determined using a NanoDrop ND-2000c Spectrophotometer, and 100-250 ng of RNA was reverse transcribed to make cDNA with Applied Biosystem High Capacity cDNA synthesis kit. Quantitative real-time PCR reactions were carried out using Power SYBR green PCR Master Mix (Applied Biosystems) and the StepOnePlus Real Time PCR System (Applied Biosystems). Reverse transcriptase free samples were used as negative controls. All samples were run in triplicates with Cycle threshold (C*t*) values normalized to β-actin to yield ∆C*t*. Fold changes were then calculated between experimental and control samples: fold change $2^{(\Delta Ct\text{experimental} - \Delta Ct\text{control})}$. Results were analyzed by non-paired *t*-test of the ∆C*t* values, and significance was determined by $P< 0.05$. Primer sequences used for experiments are in

Table 1.

Gene Ontology (GO) term analysis

Upregulated and downregulated genes with fold change >1.5 or <1.5 were input into PANTHER 16.0 and compared against the Mus Musculus reference genome. To test for gene enrichment, overrepresentation test using the GO biological processes complete Ontology database in PANTHER with Fisher's exact test and FDR correction was used to determine statistical significance.

RNA Sequencing

Islet cells were cultured 24 h in 37C incubator post isolation before being collected for RNA using the RNeasy Kit (Qiagen). Total RNA was verified for concentration and purity using a NanoDrop ND-2000c Spectrophotometer and Agilent 2100 BioAnalyzer. Samples that met the Illumina TruSeq Stranded Total RNA (Human/Mouse/Rat) (Illumina Inc., San Diego, CA, USA) sample input guidelines were prepared according to the kit's protocol. Cytoplasmic ribosomal RNA reduction of each sample was accomplished by using complementary DNA probe sequences attached to paramagnetic beads. Subsequently, each mRNA sample was fragmented using divalent cations under elevated temperature, and purified with Agencourt RNA Clean Beads (Beckman Coulter, Pasadena, CA, USA). First strand cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers. Second strand cDNAs were synthesized using DNA Polymerase I and RNAse H for removal of mRNA. Double-stranded cDNA was purified using Agencourt AMPure XP beads

(Beckman Coulter, Pasadena, CA, USA). cDNAs were end-repaired by T4 DNA polymerase and Klenow DNA Polymerase and phosphorylated by T4 polynucleotide kinase. The blunt ended cDNA was purified using Agencourt AMPure XP beads. The cDNA products were incubated with Klenow DNA Polymerase to add an 'A' base (Adenine) to the 3′ end of the blunt phosphorylated DNA fragments and then purified using Agencourt AMPure XP beads. DNA fragments are ligated to Illumina adapters, which have a single 'T' base (Thymine) overhang at their 3′end. The adapter-ligated products are purified using Agencourt AMPure XP beads. Adapter ligated DNA was amplified in a Linker Mediated PCR reaction (LM-PCR) for 12 cycles using Phusion™ DNA Polymerase and Illumina's PE genomic DNA primer set followed by purification using Agencourt AMPure XP beads. Quality and quantity of finished libraries were assessed using an Agilent DNA1000 series chip assay (Agilent Technologies, Santa Clara, CA, USA) and Invitrogen Qubit HS cDNA Kit (Invitrogen, Carlsbad, CA, USA), respectively. Libraries were standardized to 2 nM. Cluster generation was performed using the Illumina cBot. Paired-end, 100bp sequencing was performed, using standard SBS chemistry on an Illumina HiSeq2500 sequencer. Images were analyzed using the standard Illumina Pipeline, version 1.8.2. RNA Library preparation and RNA Sequencing was performed by the University of Wisconsin-Madison Biotechnology Center.

Sequencing reads were adapted, and quality trimmed using the Skewer trimming program[22] . Quality reads were subsequently aligned to the annotated reference genome (Rnor_6.0) using the STAR aligner[23]. Quantification of expression for each gene was calculated by RSEM[24] . The expected read counts from RSEM were filtered for low/empty

values and used for differential gene expression analysis using EdgeR [25] using a FDR cut off of <5%.

Western blotting

Mouse islets were picked day of isolation and placed in 37C incubator for 24 hours. They were then washed in ice-cold PBS and lysed in cold RIPA buffer (ThermoFisher, 8990) containing a protease inhibitor cocktail (Roche, #4693116001) and phosphatase inhibitor cocktail (Roche, 4906845001). Islets were incubated in the RIPA buffer on ice for 15 min with vortexing every 5 min. The protein concentrations were determined using Bradford protein assay. Protein samples were run on 10% SDS-PAGE gel and then were dry transferred onto a 0.2µm PVDF membrane (Biorad, 1704156) using Trans Blot turbo dry transfer system (Biorad, 1704150). Membranes were blocked in 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature and were incubated overnight in primary antibodies. Membranes were then washed 3X in TBST and incubated 1 h in secondary antibody. Blots were developed with Pierce ECL Western Blotting Substrate (Thermo Fisher, Waltham, MA, USA) or Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher), imaged with a GE ImageQuant charge-coupled device camera, and then quantified by densitometry with Image J 1.44o (http://imagej.nih.gov/ij, accessed 28 February 2020). Primary antibodies and dilutions were as follows: Beta actin (8H10D10, Cell Signaling, 1:1000), Phospho-histone H2A.X(Ser139) (20E3, Cell Signaling, 1:1000). Secondary antibodies and dilutions were as follows: Anti-rabbit IgG, HRP-linked antibody (#7074, Cell Signaling, 1:2000), anti-

mouse IgG, HRP-linked antibody (#7076, Cell Signaling, 1:2000). Statistical significance was determined with non-paired t-test and significance determined by $p<0.05$.

RESULTS

Young, lean wbTcf19KO mice maintain normal glucose homeostasis

Tcf19 is highly expressed in adult islets, suggesting that basal expression of Tcf19 may be necessary for maintaining beta cell mass and function [16]. We hypothesized that knockout of Tcf19 would lead to beta cell dysfunction and impaired glucose homeostasis. To test this, we generated germline whole body Tcf19 knockout (wbTcf19KO) mice (Figure 1A & B) and found that wbTcf19KO mice are fertile, viable, and had normal litter sizes.

Fasting blood glucose levels at 15 weeks of age were not different between male and female wbTcf19KO mice and WT (6NTac) controls (Figure 2A). Additionally, an insulin tolerance test (ITT) at 12 weeks of age revealed no difference in insulin sensitivity (Figure 2B). A pyruvate tolerance test (PTT) at 13 weeks of age revealed no difference in hepatic glucose output, and a glucose tolerance test (GTT) performed at 15 weeks of age revealed no difference in glucose tolerance (Figure 2C & 2D). *Ex vivo* perifusion assay on the islets revealed no difference in insulin secretion between wbTcf19KO islets and WT islets (Figure 2E).

WbTcf19KO mouse islets have downregulation of genes involved in beta cell identity, vesicle transport, and proliferation

While we did not see any differences in glucose phenotype between the

wbTcf19KO mice and WT controls, we wanted to determine the genes Tcf19 could be regulating. RNA-seq on wbTcf19KO mouse islets revealed many significantly differentially expressed genes (FDR<0.05) compared to WT islets, and out of these genes, 763 were downregulated. The downregulated genes were enriched for Gene Ontology (GO) terms such as “synaptic vesicle localization” and “regulation of transport” (Figure 3A). These included genes important in insulin secretion, notably synaptotagmin 7 (*Syt7)*, and sortilin 1 (*Sort1)* (Figure 3B). In addition, there was a decrease in *Ki67* expression as well as several beta cell function and identity genes including *Pdx1*, *Nkx6.1*, *and Nkx2.2* (Figure 3B).

WbTcf19KO mice have decreased expression of Ki67 and altered islet size distribution

To determine if the decreased beta cell identity and *Ki67* gene expression would result in any changes in islet morphology, we measured and quantified beta cell area in wbTcf19KO mouse pancreas. No difference was observed in beta cell area (Figure 4A). However, there was an intriguing pattern of altered islet size distribution. Specifically, wbTcf19KO mice had a significantly greater number of very small islets, with a trend towards fewer larger islets (Figure 4B).

Additionally, immunofluorescence on pancreas sections from wbTcf19KO mice revealed a 30% decrease in Ki67+ beta cells (fig 4C). These data demonstrate morphological changes in wbTcf19KO islet size and a decrease in proliferative marker expression.

WbTcf19KO mouse islets have decreased adaptive beta cell proliferative response on a 1-week HFD

Given that wbTcf19KO islets had decreased markers for proliferation, we wanted to determine if lack of Tcf19 would impair compensatory beta cell proliferative response. Mice fed a short term HFD (1-week) have been shown to stimulate expansion of beta cell mass and upregulate cell cycle genes prior to the onset of insulin resistance[26,27]. Therefore, we placed wbTcf19KO mice placed on a 1-week HFD. wbTcf19KO mice on a 1-week HFD failed to upregulate cell cycle and proliferative genes, *Cyclin D2* and *Ki67*, to the levels seen in control mice (Figure 5A & 5B). The inability of wbTcf19KO mouse islets to increase proliferative genes in response to a short term HFD demonstrates that Tcf19 is necessary for the islet adaptive proliferative response to acute HFD feeding.

WbTcf19KO mouse islets have upregulation of genes involved in cell stress, apoptosis, and DNA damage

Our wbTcf19KO RNA-seq data showed an upregulation of 733 genes. PANTHER GO biological process overrepresentation test showed upregulation of pathways such as “defense response to virus”, “inflammatory response”, and processes involved in immune cell chemotaxis and interferon and interleukin signaling pathways (Figure 3C). Additionally, real time PCR showed a significant upregulation of *Chop* and *Bak,* which are genes involved in apoptosis (Figure 3B). We also found that genes involved in the DDR such as *Gadd45a* and *Dtx3l* were also significantly upregulated (Figure 3B). These gene changes suggest that wbTcf19KO mouse islets have an increase in inflammatory pathway responses and increased cell stress and DNA damage.

For a more direct approach to determine the extent of DNA damage, we assessed wbTcf19KO mouse islets for the DNA damage marker, γH2AX, and found that islets from wbTcf19KO mice had increased γH2AX compared to their WT counterparts (Figure 6A). To directly visualize DNA strand breaks, we performed a neutral comet assay on dissociated wbTcf19KO islets. Comet assay showed a modest increase in DNA damage strand breaks (Figure 6B).

WbTcf19KO mouse islets do not have increased apoptosis

DNA damage has been implicated to induce beta cell apoptosis[10,28]. Given that wbTcf19KO mouse islets have increased DNA damage and pro-apoptotic gene expression, we assessed the levels of apoptosis in wbTcf19KO mice pancreas with TUNEL staining. There was no difference in the percent of TUNEL positive beta cells between wbTcf19KO mice pancreas and the WT mice pancreas (Figure 7). This indicates that while wbTcf19KO mice have increased markers of DNA damage, the extent of actual DNA damage is not sufficient to induce apoptosis.

WbTcf19KO mice have impaired glucose homeostasis and increased DNA damage after chronic HFD feeding

Long-term HFD feeding is a commonly used model to induce obesity and insulin resistance to mimic type 2 diabetogenic conditions in mice [29,30]. We fed male wbTcf19KO and WT control mice a high fat diet (HFD) comprised of 60% of kcal from fat for 10 weeks. By nine weeks on the diet, wbTcf19KO mice had higher fasting blood glucose levels than WT controls (Figure 8A). A glucose tolerance test (GTT) was performed 10 weeks post diet initiation, and this showed significantly higher glucose levels in wbTcf19KO mice at

timepoints 60 and 120 minutes. Area under the curve (AUC) analysis for each mouse was significantly higher in wbTcf19KO mice (Figure 8B). We observed signs of glucose intolerance in wbTcf19KO mice under HFD stress conditions, and hypothesized that this glucose intolerance may lead to DNA damage. We found that wbTcf19KO mouse islets had increased γH2AX, suggesting increased DNA damage in response to HFD (Figure 8C)

WbTcf19KO mouse islets have increased DNA damage under ex vivo cytokine exposure

Inflammatory cytokines such as TNF-alpha, IL-1beta, and IFN-gamma have been shown to have a critical role in beta cell dysfunction and apoptosis in T1DM [31,32]. T2DM is also characterized by an increase in circulating cytokines that can inhibit insulin secretion and promote apoptosis[31,33]. Additionally, cytokines can induce irreparable DNA damage in beta cells leading to increased apoptosis[10,34]. We treated isolated wbTcf19KO mouse islets with the cytokines, TNF-alpha, IL-1beta, and IFN-gamma and measured γH2AX. wbTcf19KO mouse islets treated with cytokines *ex vivo* had increased γH2AX compared to WT islets, suggesting increased DNA damage in response to cytokines (Figure 9)

Islets from an ob/ob mouse model and human islets from diabetic donors have increased DNA damage

To assess the translational relevance of DNA damage to human diabetes, we assessed γH2AX in islets from diabetic donors. We found that islets from diabetic donors

had significantly increased γH2AX (Figure 10B). Additionally, γH2AX was significantly increased in ob/ob islets, indicating that obesity also increases DNA damage (Figure 10A). Taken together, these results suggest a correlation between DNA damage and obesity and diabetes.

DISCUSSION

T1DM and T2DM involve a complex interplay between genetic, epigenetic, and environmental factors[4]. Alone, genetic risk may only confer a slight increase in diabetes risk[35]. However, when in combination with environmental risk factors such as obesity, viral infection, and stress, this may trigger the development of diabetes in those who are more genetically susceptible[36–39]. *TCF19* was found to be a T1DM and T2DM diabetes susceptibility gene. *TCF19* contains coding variants that may confer increased diabetes risk[14,15]. Therefore, an understanding of how *TCF19* functions alone and under a diabetes related stress environment will elucidate the function of this diabetes gene, and provide a foundation for the development of more personalized diabetes therapies.

In this study, we utilized a whole body Tcf19 knockout (wbTcf19KO) mouse model and find that while these mice have no significant defects in beta cell function, as measured by multiple tolerance tests, wbTcf19KO mice have significantly decreased expression of the proliferative marker, *Ki67.* We further show that wbTcf19KO mice have an impaired adaptive proliferation response under acute stress. Additionally, these mice have decreased beta cell identity gene expression and an altered islet size distribution accompanied by increased islet DNA damage gene expression. The DNA damage marker, γH2AX, was increased in wbTcf19KO islets at baseline with further increases in

expression observed after HFD and cytokine stress. To demonstrate the translational significance of DNA damage in diabetes pathogenesis, we also showed that levels of γH2AX are increased in islets from ob/ob mice as well as human islets from diabetic donors.

TCF19 is known for its roles in regulating cell proliferation, specifically in liver and cancer cell lines[19,40]. Yet, *TCF19* is most highly expressed in the pancreatic islet, suggesting a significant role for this gene in the islet[16]. Studies from our lab focusing on the islet have shown that *Tcf19* is necessary for proliferation and survival in the INS-1 rat beta cell line[16]. Additionally, TCF19 overexpression in INS-1 cells impact the DNA damage response (DDR) and inflammatory pathways[21]. These studies highlight involvement for TCF19 in cell proliferation and stress response pathways. To better understand the functional role of TCF19 and how it may affect beta cell function, we generated a germline whole body Tcf19 knockout (wbTcf19KO) mouse model.

The basic metabolic phenotypes in young, lean adult mice show no difference between wbTcf19KO and WT female and males. This was not surprising, as Tcf19 expression correlates in proliferating islets and increases expression in a non-diabetic *ob/ob* mouse model[17]. This indicates that Tcf19 most likely exerts its effect under conditions of islet stress, such as the *ob/ob* setting, or under conditions where beta cell proliferation is necessary. This may be the reason we did not observe any difference in phenotypes in lean, healthy mice, where there is not necessarily any induction of islet stress.

However, transcriptomic analysis with RNA-seq revealed that wbTcf19KO islets have decreased gene expression of beta cell identity (*Pdx1*, *Nkx6.1*, *Nkx2.2*), insulin

vesicle exocytosis (*Syt7)*, and intracellular protein trafficking (*Sort1*). These genes are relevant for maintaining beta cell mass, beta cell identity, and insulin secretion[41,42]. *Nkx6.1* and *Pdx1,* key beta cell identity genes, are also important regulators of glucose stimulated insulin secretion (GSIS), and their expression is decreased during beta cell dedifferentiation and transdifferentiation[43–45]. *Sort1* is a member of the VPS10 protein family with well-studied roles in intracellular protein trafficking and sorting [41]. Additionally, *Sort1* has receptors on the beta cell, and the protective effect of *Sort1* on beta cells involves key regulatory mechanisms in glucose homeostasis[46]. *Sort1* expression is also decreased in insulin resistance and obesity, implicating a downregulation of intracellular trafficking and decreased glucose homeostasis capability[47]. *Syt7* is a mediator of GSIS and has roles in replenishing insulin granules for exocytosis[48]. Beta cell dedifferentiation and transdifferentiation are also proposed contributors to decreased beta cell mass in T2DM, and the decreased expression of beta cell identity genes in wbTcf19KO islets along with the decreased expression of genes involved in insulin secretion and vesicle trafficking may indicate that wbTcf19KO beta cells are on a path towards reduced maturity and decreased function. We hypothesize that the beta cells in our baseline, lean wbTcf19KO mouse model are moving towards a threshold where, if placed under stress, will lead to their inability to adapt and maintain proper functioning.

Perhaps the most striking gene expression change was the decrease in the proliferative marker, *Ki67.* This decreased expression was confirmed by immunohistochemistry staining of Ki67+ beta cells in wbTcf19KO pancreas sections. wbTcf19KO mice also had an altered islet size distribution with an increase in very small islets. Pathological changes of the pancreas in patients with T2DM have been observed

in cadaveric pancreatic sections, namely a decrease in beta cell fraction in large islets compared to their nondiabetic subject counterparts[49]. This change was accompanied by a reciprocal increase in alpha cell fraction[49]. However, total alpha cell area was still decreased along with beta cell area in T2DM[49]. Future studies will have to be performed to determine whether alpha cell area is changed in wbTcf19KO pancreas. However, we hypothesize that while wbTcf19KO mice are still able to maintain glucose homeostasis, these morphological changes in the islet such as reduced islet size and decreased proliferative rate may predispose wbTcf19KO mice to decreased beta cell mass over time and eventually lead to diabetes.

Adult beta cells proliferate at an extremely low rate (0.5%) with essentially no replication under physiological conditions[50]. However, increased rates of proliferation have been observed under conditions of beta cell stress such as pregnancy and recent onset of T1DM[51,52]. Additionally, this compensatory increase in beta cell mass is also observed in obese nondiabetic patients in the face of insulin resistance[53,54]. While the exact timing of when the compensatory response occurs in humans is not known, and likely varies among individuals, several studies in mice have shown enhanced beta cell proliferation after only 1-week of high fat diet feeding, with significant increases in proliferative genes such as *Ki67*, *CyclinA2*, *CyclinB1*, and *Foxm1*[26,27].

We tested the compensatory beta cell proliferative ability of our wbTcf19KO mice by placing them on a 1-week HFD. In response to 1-week HFD, wbTcf19KO mice failed to upregulate *CyclinD2* and were not able to increase *Ki67* to levels seen in the WT HFD fed mice. This indicates a necessary role for Tcf19 in compensatory beta cell proliferation to acute HFD feeding. Additionally, the compensatory role of Tcf19 in beta cell

proliferation in response obesity is also supported by a previous study that showed a significant upregulation of Tcf19 in a model of nondiabetic obesity[17].

While our RNA-seq data from wbTcf19KO islets revealed a decreased expression of proliferation, vesicle transport, and beta cell identity genes, the genes that were significantly upregulated represented processes such as “defense response to virus”, “inflammatory response”, and processes involved in immune cell chemotaxis and interferon and interleukin signaling pathways. Immune and inflammation are processes that have been well studied in diabetes. The innate immune response signaling pathway is a key player in T1DM progression, and chronic activation of macrophages and immune cells which secrete proinflammatory cytokines can lead to beta cell apoptosis[55]. In T2DM, chronic low-grade inflammation and hyperglycemia cause oxidative stress through regulation of the stress activated signaling pathways NF-kappaB, p38, and JNK/SAPK, which can then promote the release of proinflammatory cytokines[56]. Additionally, excess body weight or adiposity as seen in obesity, can promote the release of adipokines[57,58]. The increased expression of inflammatory genes in wbTcf19KO mice suggests an elevation of inflammatory processes that can be deleterious to the beta cell.

TCF19 has been shown to impact expression of DDR genes[21]. We observed that *Dtx3l* and *Gadd45a*, which are DDR genes, are upregulated in our wbTcf19KO RNA-seq data. While the DDR pathway is not well studied in the context of diabetes pathogenesis and the beta cell, a handful of papers have shown correlations between diabetes and DNA damage. Patients with T2DM have increased DNA damage and a decreased ability to repair DNA damage[13]. Furthermore, diabetic patients have inefficient DNA repair mechanisms which may increase diabetes risk[13].

The DDR is a highly integrated, tightly regulated network. Damage severity affects downstream pathways including those involved in promoting DNA repair, cell cycle arrest, senescence, or apoptosis[59,60]. The phosphoinositide-3-kinase-related protein kinase (PIKK) family controls the cellular response to DNA damage and is comprised of the DNA damage sensing kinases, Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3-related (ATR)[61,62]. Upon activation, ATM and ATR phosphorylate the histone 2 variant, H2AX, at Ser-139, known as γH2AX [62]. This is the first step to signal DNA repair protein assembly at the damaged site, and γH2AX has been established as a sensitive and specific marker for monitoring DNA double strand breaks[63]. Western blot for γH2AX revealed a significant increase in this marker in wbTcf19KO islets compared to WT controls, suggesting significantly elevated activation of DDR in wbTcf19KO islets. To directly visualize DNA strand breaks, we performed comet assay on wbTcf19KO islets. Comet assay showed a modest but insignificant increase in fragmented DNA in wbTcf19KO islets, indicating that the increased γH2AX did not directly correlate with observable strand breaks. However, several studies have recognized the importance of using multiple parameters to analyze DNA damage. A comparative study between comet assay and γH2AX foci showed disparity between the two methods' ability to detect DNA damage[64]. Whereas comet assay did not detect significant amounts of DNA damage, γH2AX formation was detectable[64]. While comet assay on wbTcf19KO mouse islets does not directly show a significant increase in strand breaks, our other data, DDR gene changes and γH2AX expression, indicate that the DDR is elevated in wbTcf19KO mouse islets.

The key DNA damage proteins, ATM and ATR, phosphorylate various proteins, such as Checkpoint kinase 1 (Chk1), Checkpoint kinase 2 (Chk2), and p53, which are involved in checkpoint arrest, apoptosis, and DNA repair[65–67]. These proteins can enhance transcription of downstream genes such as *Gadd45a* and *Dtx3l* which can also directly participate in cell cycle arrest, DNA repair, and apoptosis[67–69]. *Dtx3l* and *Gadd45a* expression were increased in wbTcf19KO islets. *Gadd45a* functions in arresting cell cycle progression and can also induce apoptosis when there is irreparable DNA damage[70]. *Dtx3l* is well studied in its role in DNA damage repair[69,71]. Combining these data with the overrepresentation of inflammatory and immune response process seen in our RNA-seq GO analysis, we hypothesize that TCF19 functions as a DNA damage repair protein, or coregulator to a repair protein, and that in its absence, results in increased levels of DNA damage and inflammation. If left unchecked this can lead to cellular apoptosis.

While we did not find any significant difference between levels of apoptosis between wbTcf19KO and WT mice as measured by TUNEL staining, it is important to note that TUNEL staining detects cells primarily undergoing late stage apoptosis where there is extensive DNA degradation[72]. Therefore, it is possible that the wbTcf19KO beta cells may either be in early stage apoptosis or an arrested cell cycle state. Future experiments focusing on the earlier stages of apoptosis will need to be performed to answer this question. However, based on the normal glucose tolerance and beta cell function of wbTcf19KO mice, it is likely that the level of DNA damage sustained in wbTcf19KO mouse islets is not yet irreparable or at the extent where it will severely impact beta cell function and mass. This could explain the lack of observed TUNEL staining.

Several studies indicate that obesity leads to increased DNA damage and decreased DNA repair[28,73]. Specifically, increased caloric and fat intake lead to DNA damage through oxidative stress and p53 activation[74,75]. HFD feeding is a commonly used mouse model of obesity and has been used to study the effects of impaired glucose tolerance and T2DM [76,77]. We placed wbTcf19KO mice on a HFD for 10 weeks to assess how lack of Tcf19 would affect DNA damage and beta cell function under T2DM conditions. wbTcf19KO mice have impaired glucose tolerance after 10 weeks HFD feeding, indicating impaired beta cell function. We found that wbTcf19KO mouse islets had a significant increase in γH2AX after 10 weeks on a HFD compared to the controls. These data indicate a necessary role for Tcf19 in maintaining beta cell function and DDR in times of nutrient overload and HFD stress.

Other key sources of beta cell stress during of T1DM and T2DM development include cytokines[78]. The proinflammatory cytokines, which can be secreted by immune cells, islet cells, and adipose tissue, include IFN-gamma, IL-1beta and TNF-alpha, and can cause DNA damage[79–82]. After *ex vivo* cytokine treatment, wbTcf19KO mouse islets sustained significantly more DNA damage compared to WT controls. Collectively, these data indicate that mice lacking Tcf19 have greater DNA damage under diabetogenic stressors compared to their WT counterparts.

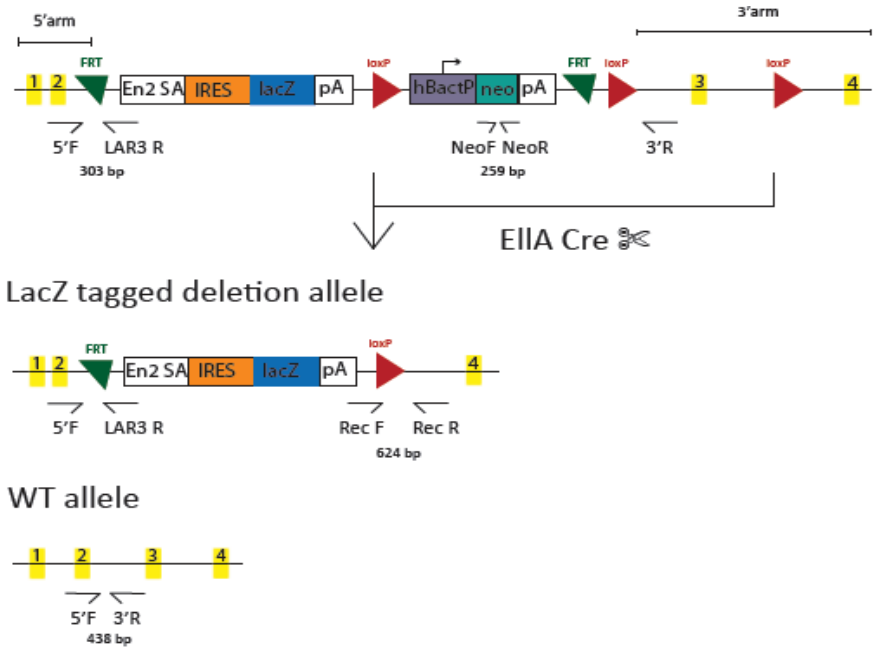
The process of DNA damage in the beta cell and its contribution to diabetes is a new and emerging field. To determine how applicable our DNA damage findings would be to human diabetes, we assessed DNA damage levels in donor islets from patients with T2DM. We found that donor islets from T2DM had significantly increased γH2AX

compared to their nondiabetic counterparts. This implicates differential regulation of DDR and repair pathways in diabetic individuals compared to their nondiabetic counterparts.

Overall, our work highlights an important role for the novel diabetes gene, *Tcf19*, in beta cell proliferation and DDR pathways as well as a role in maintaining beta cell function under conditions of HFD stress.

Tcf19 has several coding mutations in humans that are associated with diabetes, and we believe that these variants may inhibit its function in DNA damage repair and beta cell function[14,15]. This is significant because genetic disruption of DNA damage repair genes have been shown to lead to beta cell apoptosis and are associated with T2DM [83–85]. We believe that Tcf19 has a role in DNA damage repair, and that lack of Tcf19 leads to an increase in DNA damage which can affect beta cell function and adaptation to stress. Overtime, this will lead to diabetes. Future studies will explore the nature by which variants of TCF19 affect proliferative capacity, function, and DNA damage responses in the beta cell.

A.



B.

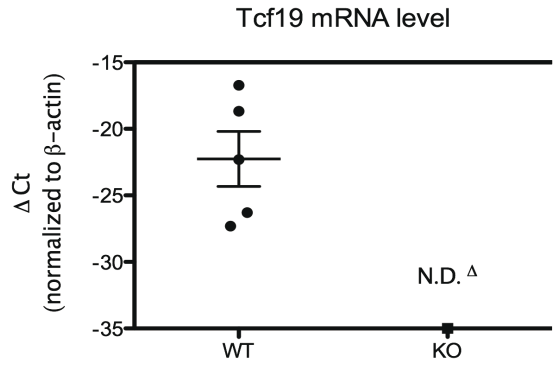


Figure 1: Diagram of generation of wbTcf19KO mice. **A.** A targeted cassette obtained through IKMC had FRT sites and loxP sites available for deletion of selected portions of the allele. LacZ expressing beta-galactosidase is preceded by an internal ribosomal entry site (IRES) to allow translation of the lacZ gene from transcriptional activity driven by the Tcf19 promoter. Neomycin resistance (Neo) is present for the initial selection of positive ES cell clones, and removed after recombination at FRT or loxP sites. **B.** RT-qPCR confirms no mouse Tcf19 expression level in wbTcf19KO mouse islets.

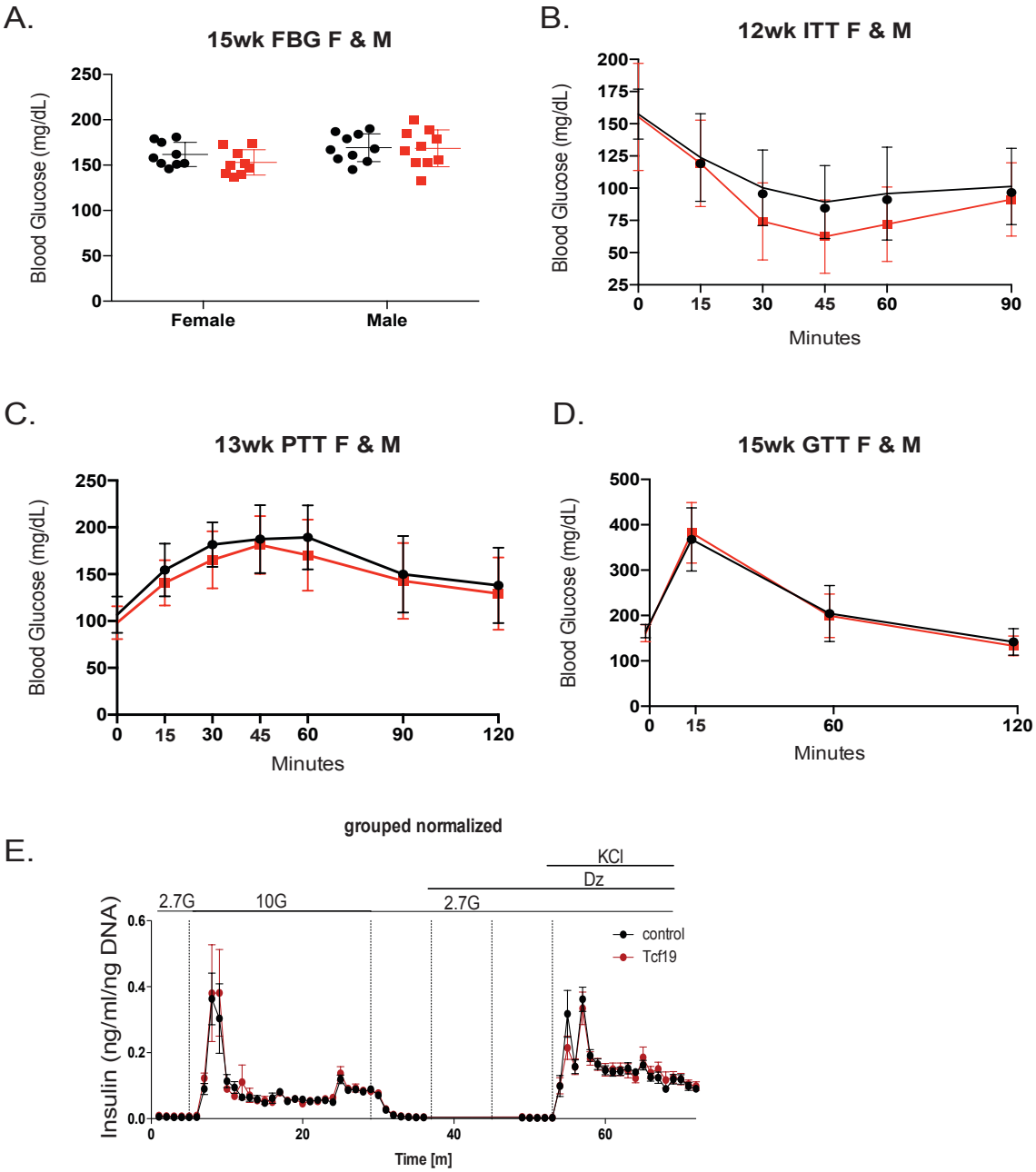


Figure 2: wbTcf19KO mouse phenotypes including glucose tolerance, insulin sensitivity, insulin secretion, and hepatic glucose output are not significantly different between wbTcf19KO and WT mice. **A.** Fasting glucose levels were not different between WT and KO mice at 15 weeks of age. **B.** Insulin tolerance test (ITT) on WT and wbTcf19KO mice at 12 weeks of age shows no difference in insulin sensitivity. **C.** Pyruvate tolerance test (PTT) revealed no difference in hepatic glucose output **D.** Glucose tolerance test (GTT) shows no difference in glucose tolerance between WT and KO mice. **E.** *Ex vivo* perifusion assay on islets reveal no difference in insulin secretion between wbTcf19KO islets and WT islets in female mice. N=6.

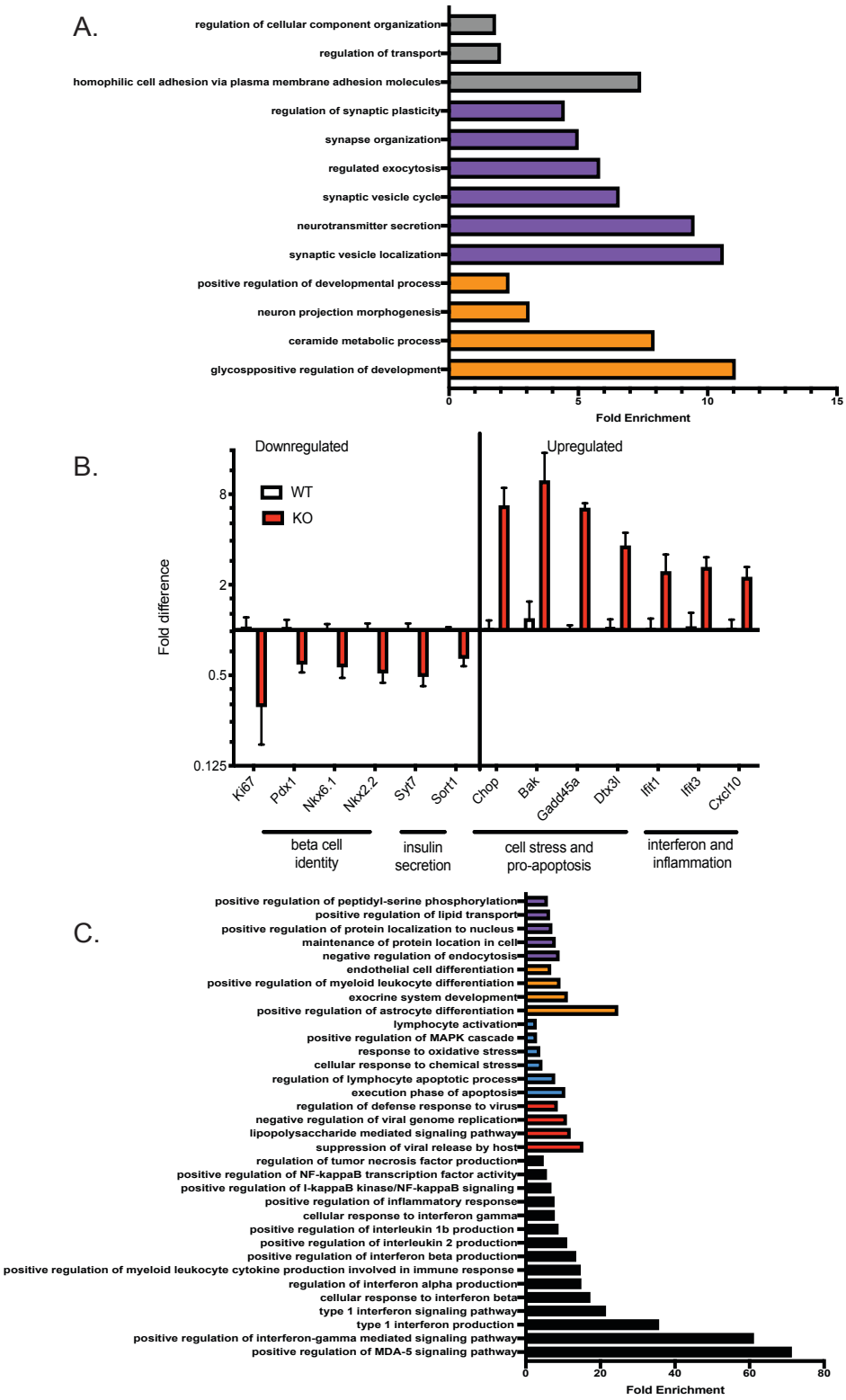


Figure 3: RNA-seq and PANTHER GO analysis show several pathways significantly overrepresented among our upregulated and downregulated genes. **A.** PANTHER overrepresentation test for GO biological processes on >1.5 fold downregulated genes

show enrichment for processes involved in morphogenesis and developmental processes (orange), synaptic vesicle localization and exocytosis (purple) and regulation of transport and cellular organization (grey). **B.** RT-qPCR on differentially expressed genes confirmed downregulation of genes involved in proliferation (Ki67), beta cell identity and insulin secretion, and an upregulation of genes involved in cell stress, apoptosis, and inflammation. **C.** PANTHER overrepresentation test for GO biological processes on >1.5 fold upregulated genes show enrichment for processes involved in immune, interferon, inflammation and cytokine pathways (black), viral defense responses (red), pathways involved in stress and apoptosis (blue), cellular differentiation (orange), and regulation of localization, transport, and phosphorylation processes (purple). N=5 for RNA-seq experiments.

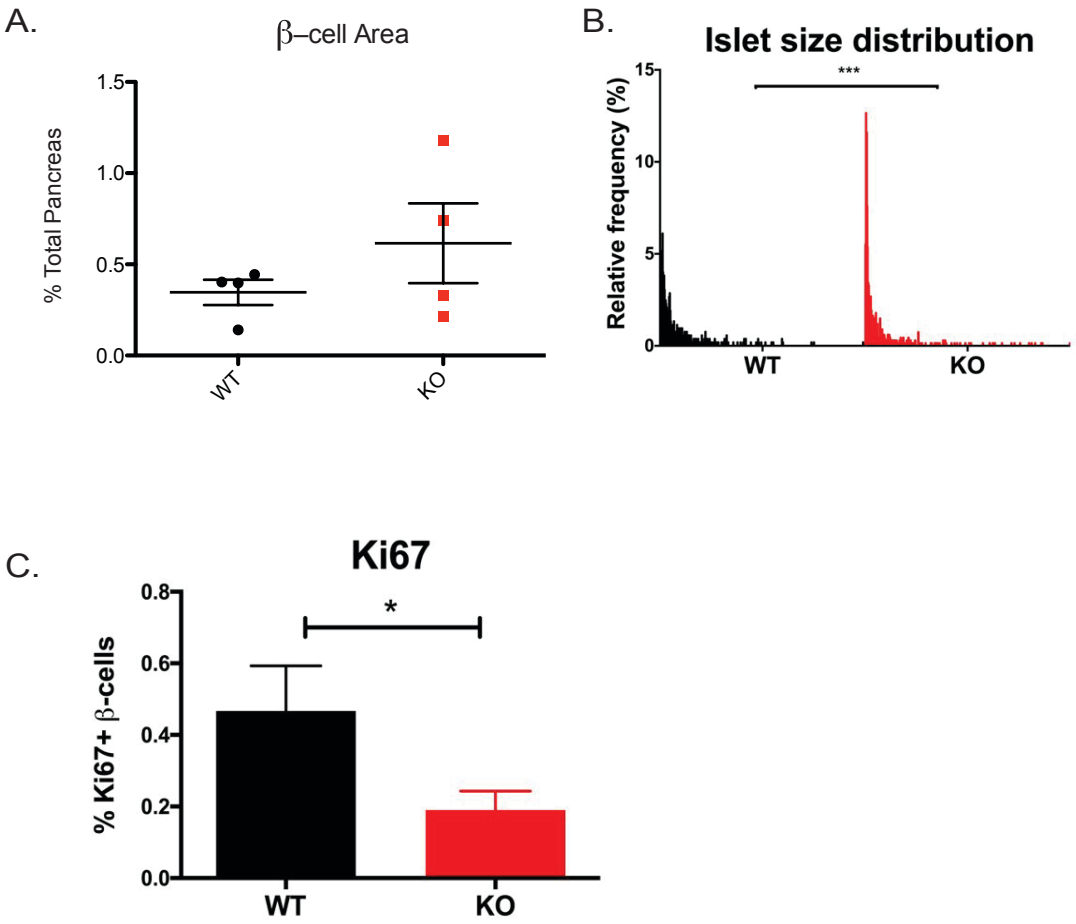


Figure 4: Quantification of beta cell area and islet size shows altered islet size distribution with a decrease in proliferative marker expression and no difference in beta cell apoptosis. **A.** Beta cell area is not significantly different between wbTcf19KO mice pancreas compared to WT. N=4 **B.** wbTcf19KO mouse islets have a greater number of very small islets and fewer large islets compared to WT mice. N=4 **C.** wbTcf19KO mice pancreas have a significant decrease in Ki67 staining compared to WT pancreas. N=3. Data are means ± SEM * $p < 0.05$

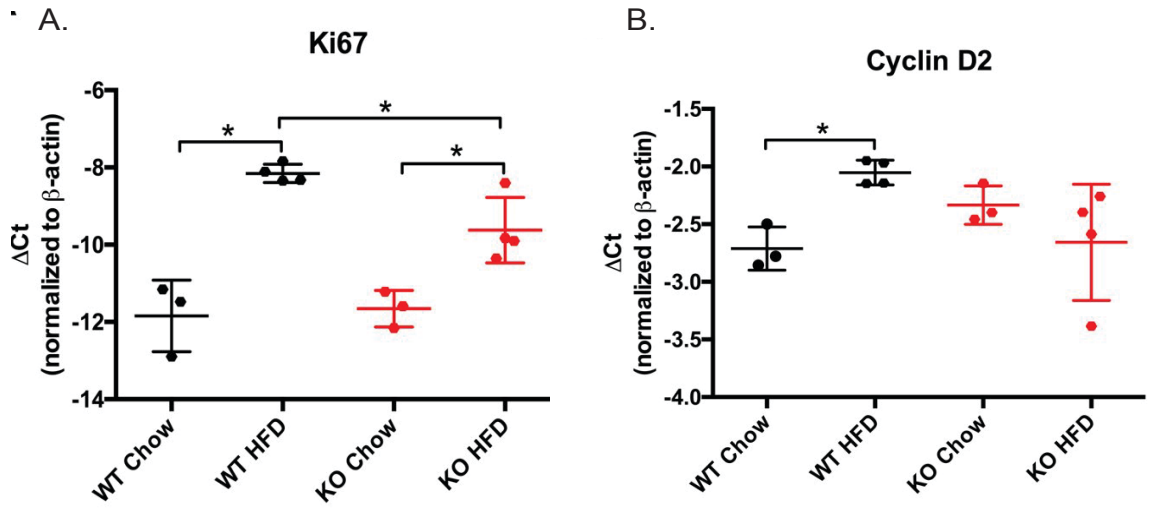


Figure 5: wbTcf19KO mice fail to upregulate cell cycle genes to similar levels as seen in WT mice after 1- week HFD. **A.** After 1-week HFD, wbTcf19KO mice are not able to upregulate *Ki67* expression at similar levels as those seen in WT mice. **B.** After 1-week HFD, wbTcf19KO mice have a slight decrease in *CyclinD2* levels while WT mice significantly upregulate *CyclinD2* levels in islets. N=4 for all experiments. Data are means ± SEM * $p < 0.05$.

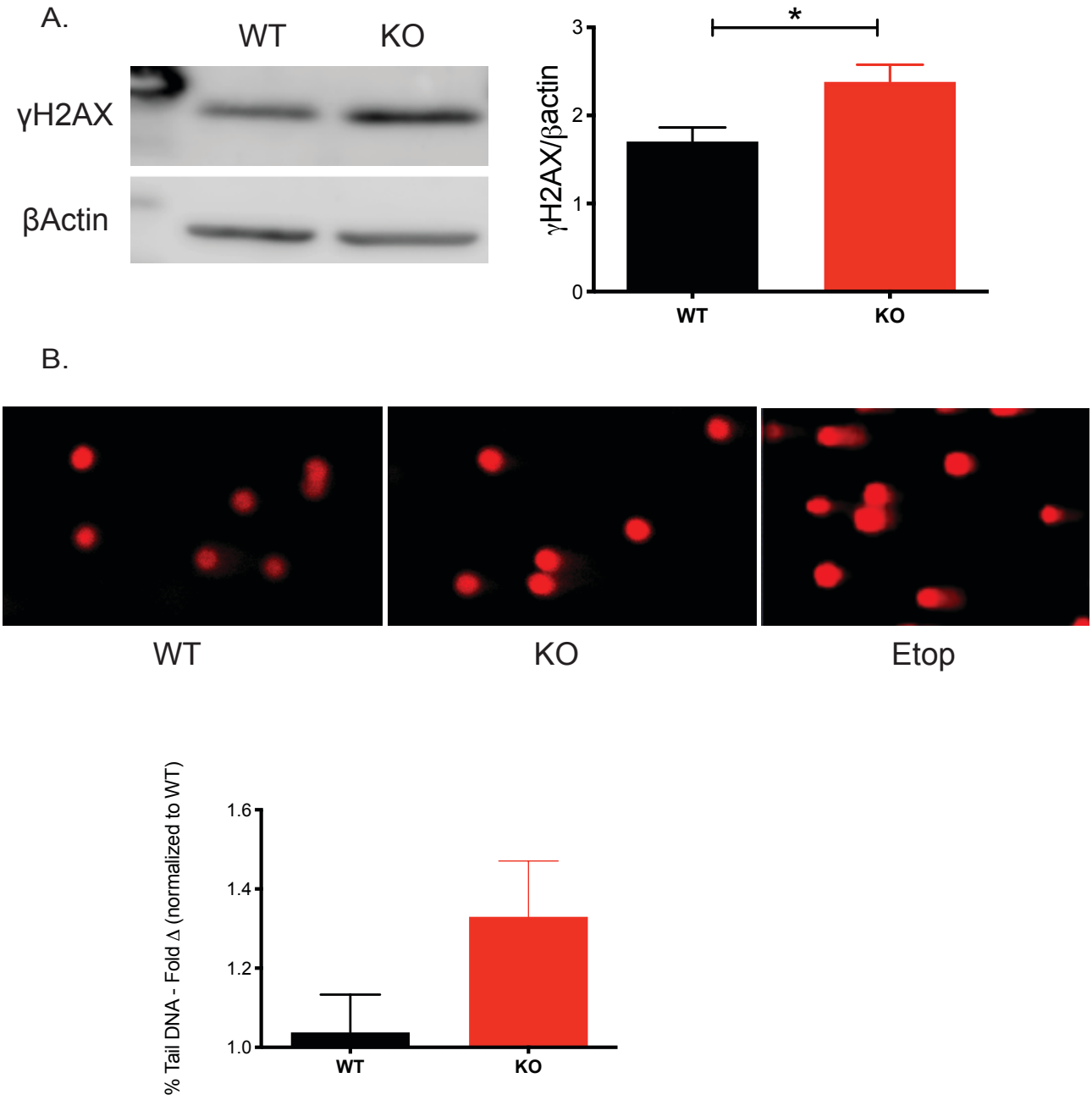


Figure 6: wbTcf19KO mouse islets have increased DNA damage marker, γH2AX, and slightly increased DNA strand breaks. **A.** Western blot and quantification show that wbTcf19KO mouse islets have increased γH2AX compared to WT islets. N=3 **B.** Comet assay and quantification of % tail DNA shows wbTcf19KO have a slight increase in DNA strand breaks compared to WT islets. Etoposide treated islets were used as a control. N=4. Data are means ± SEM * $p < 0.05$.

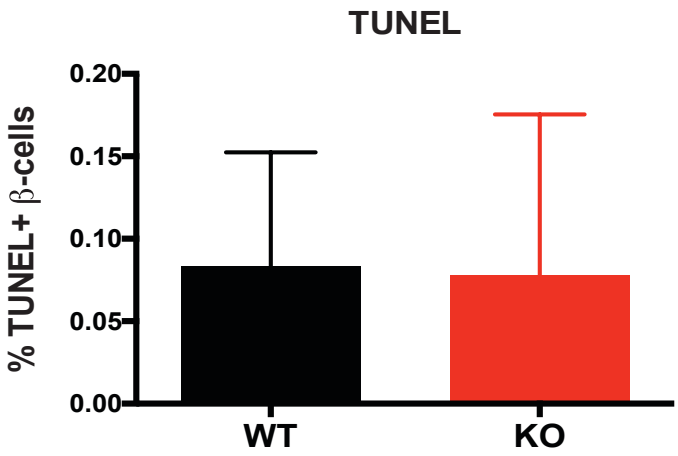


Figure 7: TUNEL staining on mouse pancreas sections show that wbTcf19KO mouse pancreas have similar levels of apoptosis as WT mouse pancreas. N=4.

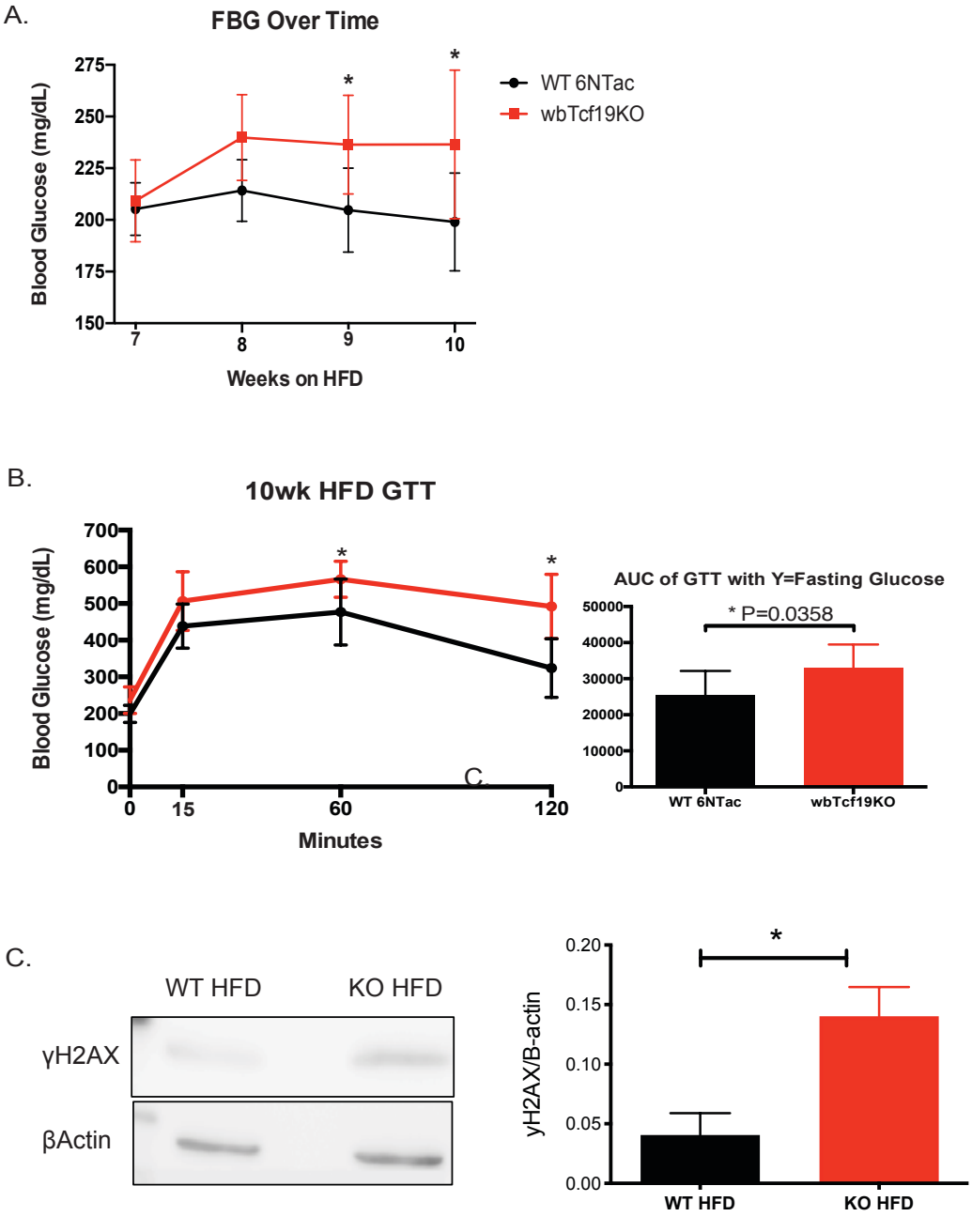


Figure 8: wbTcf19KO mice were fed a HFD and assessed for glucose tolerance and DNA damage. **A.** At 9 weeks, wbTcf19KO mice have higher fasting blood glucose than WT mice. N=8 **B.** Glucose tolerance test (GTT) after 10 weeks HFD feeding show that wbTcf19KO mice have impaired glucose tolerance compared WT mice at time points 60 min and 120 min and also have increased area under the curve (AUC) measurement. N=8 **C.** γH2AX western blotting and quantification show that wbTcf19KO mice have a significant increase in γH2AX compared to WT islets. N=3. Data are means ± SEM * $p < 0.05$.

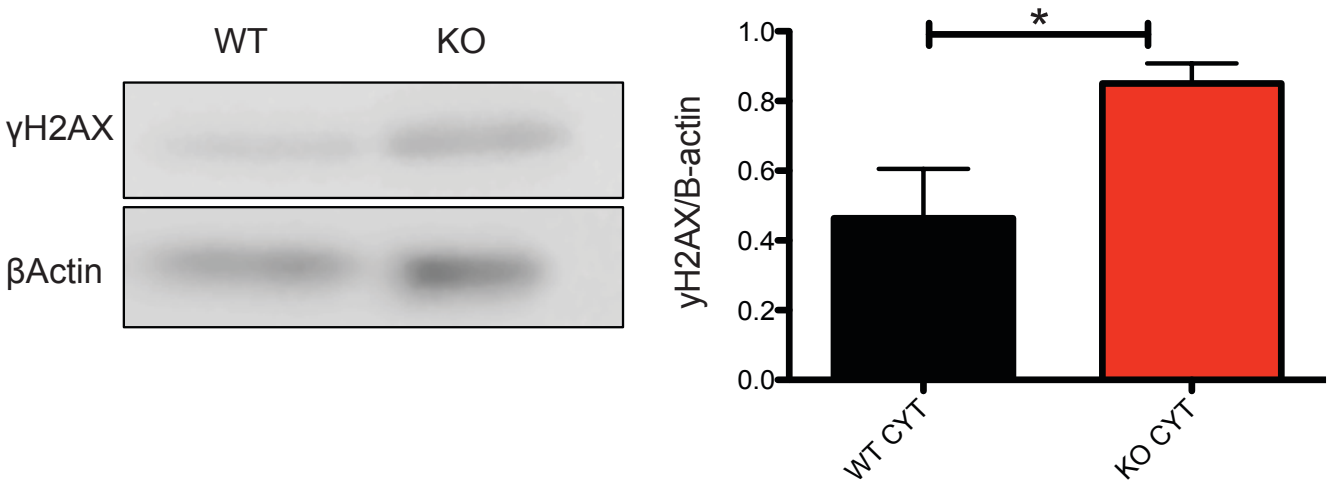


Figure 9: Western blot for γH2AX and quantification show that wbTcf19KO mouse islets treated with cytokines *ex vivo* have significantly increased γH2AX compared to WT mouse islets. N=4. Data are means ± SEM * $p < 0.05$.

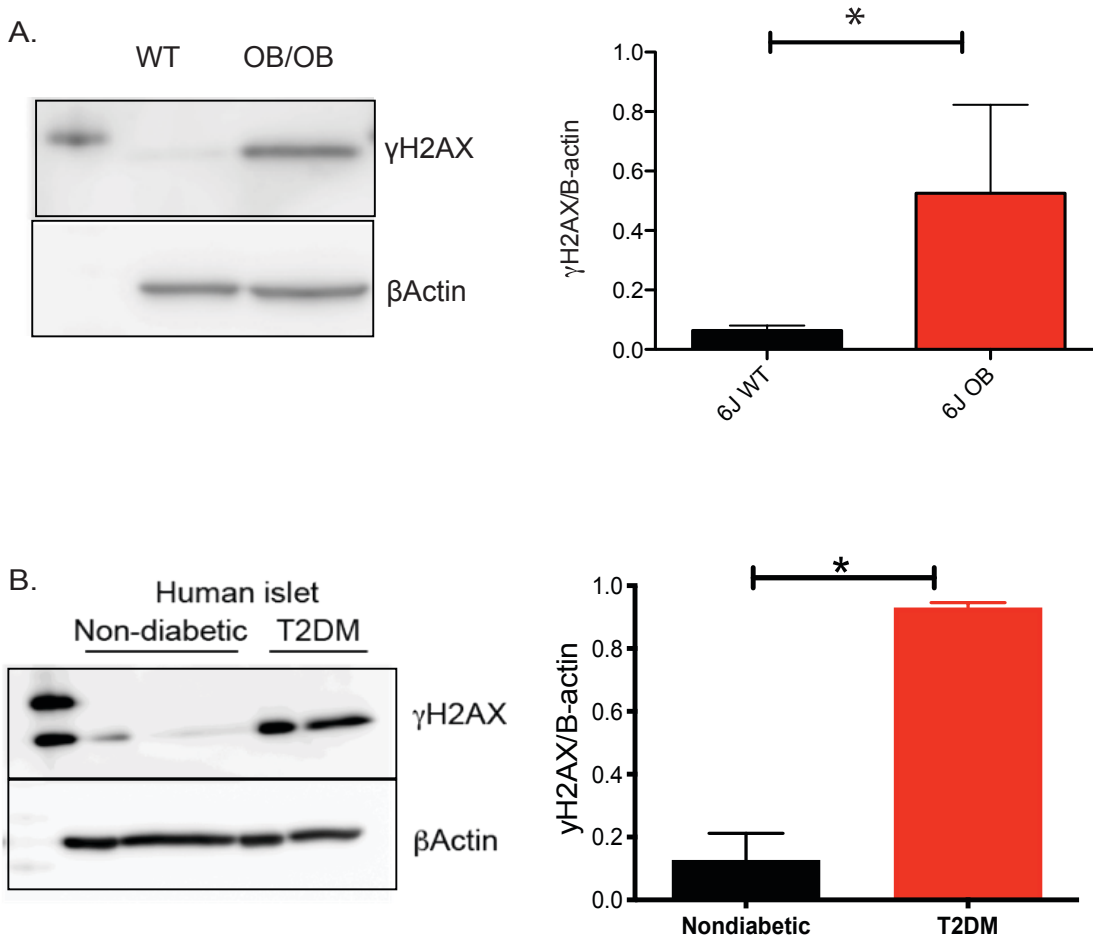


Figure 10: Western blot and quantification for γH2AX from (**A**) ob/ob mouse islets and (**B**) human T2DM donor islets show a significant increase in γH2AX levels compared to WT or nondiabetic counterparts. N=4. Data are means ± SEM * $p < 0.05$.

Table 1: Table of primer sequences used for real-time PCR experiments

Gene	5’ Primer	3’ Primer
Mouse Ki67	CTGCCTGCGAAGAGAGCATC	AGCTCCACTTCGCCTTTTGG
Mouse cyclinD2	GGATGCTAGAGGTCTGTGAGG	CAGCGGGATGGTCTCTTTCA
Mouse Tcf19	CAGCGGGATGGTCTCTTTCA	AGGTCAGAAGGTCACCATCACTCA
Mouse Nkx6.1	CCCCATCAAGGATCCATTTTGT	CTCTCCGTCATCCCCAGAGA
Mouse Nkx2.2	CTTGGGATGGAATTGCCTGCT	TTGGAGAAGAGCACTCGGCG
Mouse Syt7	ACTGGGCAAACGCTACAAGA	AGTCCTCGAAATTGATGGCTTT
Mouse Sort1	CTGGGGTTATTCTCGTCCTGACCA	CGGACACCTCCGCTGTTAGTA
Mouse Chop	AGTCCTCGAAATTGATGGCTTT	TCTGCTTTCAGGTGTGGTGGTGTA
Mouse Bak	GGACCTGGGTGCAGTTCCTC	CCGAGGAGGACCTAGGCAGA
Mouse Gadd45a	TGGTGACGAACCCACATTCA	CGGGAGATTAATCACGGGCA
Mouse Dtx3l	CGGGAGATTAATCACGGGCA	TTTCAACACTTTCTCCTTATCTGC
Mouse Ifit1	GCATTCTGAATGCAGCTCACCTC	CTTGGGATGGAATTGCCTGCT
Mouse Ifit3	CCTGGGGAAACTACGCCTG	TTCTGGGCATTCCATGCTGT
Mouse Cxcl10	TGAGATCATTGCCACGATGAAA	TGGCTAAACGCTTTCATTAAATTCT

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Chapter 4: TCF19 mechanism, binding partners, and localization studies

Sukanya Lodh purified his-tagged TCF19 protein samples for mass spectrometry. Mass spectrometry was performed by the Human Proteomics Program at UW-Madison. Grace Yang performed the remaining work presented.

INTRODUCTION

The role of DNA damage in the beta cell has not been well studied, and while several studies allude to a role for DNA damage in diabetes pathogenesis, there is yet a mechanism to be discovered on how the DNA damage pathway contributes to pathogenesis. Correlative studies have shown that patients with diabetes have increased DNA damage and a decreased ability to repair the damage[1]. Knockdown and knockout of DNA damage genes have been shown to lead to impaired ability to regulate glucose levels[2,3]. Additionally, individuals and animal models with loss of function mutations in DNA repair genes eventually develop diabetes[2,4]. These studies support an important role for DNA damage response (DDR) pathways in diabetes pathogenesis. They also highlight a role for DNA damage in impairing glucose regulation.

In humans, Transcription Factor 19 (*TCF19*) is a novel gene associated with both Type 1 and Type 2 diabetes (T1DM, T2DM)[5,6]. *TCF19* contains Single Nucleotide Polymorphisms (SNPs) leading to amino acid changes which may increase diabetes risk[5,6]. *TCF19* expression is the highest in the islet and its levels are increased in non-diabetic obesity[7]. Recently, our lab has shown that TCF19 impacts a network of genes involved in inflammation and DNA damage[8]. Taken together, these studies suggest that TCF19 may work through DNA damage and inflammatory pathways in the islet, and that SNPs within TCF19 may disrupt TCF19 function and lead to diabetes.

Our studies in whole body Tcf19 knockout (wbTcf19KO) mice reveal that Tcf19 knockout leads to increased DNA damage and DNA strand breaks as well as a decrease in proliferation markers in the islet. wbTcf19KO mouse islets also have a downregulation of genes involved in beta cell identity and vesicle exocytosis, and the islets have a

significant upregulation of genes involved in the DDR, apoptosis, inflammation, and cell stress pathways. Additionally, wbTcf19KO mice on a long term (10 week) high fat diet (HFD) have decreased glucose tolerance and increased DNA damage. In response to an acute HFD, wbTcf19KO mice have a defect in the adaptive proliferative response. These studies suggest a role for TCF19 in modulating the DDR pathway in the islet, and that lack of Tcf19 results in increased DNA damage and cell stress which may ultimately affect beta cell function.

The primary protein structure of TCF19 contains a forkhead-associated (FHA) domain and a plant homeodomain (PHD) finger. The FHA domain is found in a variety of proteins involved in cell cycle regulation and the DDR[9,10]. Specifically, TCF19 contains a serine residue at position 78 (Ser78) that is phosphorylated in response to DNA damage[11]. Additionally, Ser78 is located in a Ser-Gln motif that is recognized by a key DNA damage sensing kinase, Ataxia telangiectasia mutated (ATM)[11]. The PHD finger domain of TCF19 has been shown to bind to modified histones and specifically can interact with trimethylated lysine 4 of histone H3 (H3K4me3)[12,13]. These findings illuminate the potential functional significance of TCF19 protein domains.

This chapter focuses on the ongoing studies and tools developed to determine the mechanism and protein binding partners of TCF19. We have created tools that can be used for future studies to assess the effects of mutations within key residues of the FHA and PHD finger domain within TCF19. We have also created TCF19 SNP mutations found in GWAS to determine how these point mutations may increase genetic susceptibility to DNA damage. I will also discuss our experiments to identify TCF19 protein binding

partners as well as localization studies with halo-tagged TCF19 to identify subcellular localization of TCF19 in response to DNA damage stress.

Overall, the studies in this chapter will provide the tools and basis for future studies determining the role of TCF19 protein domains, genetic variants, and binding partners, with the ultimate goal of elucidating the molecular mechanism for TCF19 in diabetes. While many of these results are preliminary and require further experiments to reach definite conclusions, I included these data in my thesis to document the generation of these tools and the initial experimental data that I have generated.

METHODS AND MATERIALS

AD293 cell culture

AD293 human kidney cells were cultured in Dulbecco's Modified Eagle Medium (DMEM Gibco 11965092) supplemented with 1% antibiotic-antimycotic (Gibco, 15240-062) and 10% fetal bovine serum. AD293 cells were cultured at 37C and 5% CO_2 in a humidified atmosphere.

Transfection of hsTCF19 and etoposide treatment for mass spectrometry experiments

AD293 cells were transfected with either hsTCF19 or pcDNA4 control using Lipofectamine 2000 (Invitrogen). AD293 cells were trypsinized and resuspended in transfection medium (DMEM supplemented with 10% FBS). Cells in transfection medium were then added to a hsTCF19 or control plasmid-Lipofectamine mixture at 5 µg/5x10^6 cells and plated. Transfection medium was removed 12-18 hours post transfection and

replaced with complete growth medium. For samples that were exposed to DNA damage, 60 μM etoposide (Sigma E1383) was added to the cells for 3 hours.

Nickel column affinity purification of hsTCF19 his-tagged protein

Nickel column purification was performed according to kit protocol (Thermo Fisher HisPur Ni-NTA Purification kit #88227). Briefly, spin columns were equilibrated with Equilibration Buffer and protein extract was mixed with Equilibration Buffer before adding to the column. The column was centrifuged at 700xg for 2 min and the resin was washed with Wash Buffer. The column was centrifuged again at 700xg for 2 min and each fraction was collected into separate centrifuge tube. His-tagged protein was eluted with Elution Buffer. Protein elution was measured by absorbance with Nanodrop ND-2000c Spectrophotometer at 280 nm and then analyzed with Coomassie. Eluates were sent in for mass spectrometry analysis at the Human Proteomics Program at UW-Madison.

Transfection of halo-tagged TCF19 overexpression and etoposide treatment for localization studies

AD293 cells were transfected with either haloTCF19 or pcDNA4 control using Lipofectamine 2000 (Invitrogen). AD293 cells were trypsinized and resuspended in transfection medium (DMEM supplemented with 10% FBS). Cells in transfection medium were then added to a hsTCF19 or control plasmid-Lipofectamine mixture at 3 μg/1x10^6 cells and plated. Transfection medium was removed 12-18 hr post transfection and replaced with complete growth medium. Cells that were to receive etoposide treatment were treated with 60 μM etoposide (Sigma E1383) for 6 hours.

Immunofluorescence of HaloTag TMR fluorescent stain

Halo-TCF19 staining was performed according to manufacturer's protocol for HaloTag TMR ligand (Promega Cat No. G8252). Briefly, a 1:200 dilution of HaloTag TMR ligand was prepared and then added to the cell culture plate at a volume of 1/5 the existing volume. Ligand containing medium was replaced and cells were rinsed with 1x PBS. Vectashield antifade mounting medium with DAPI (Vector laboratories H-1200-10) was then added to the slides and sealed with a coverslip and nail polish. Immunofluorescence was performed with Nikon A1R HD Confocal Microscope.

Protein-protein interaction network analysis (STRING)

Search Tool for Retrieval of Interacting Genes/Proteins (STRING) database was used to construct the protein-protein interaction network of proteins extracted from mass spectrometry protein list (https://string-db.org/). Interaction score of >0.4 was used as the cutoff criterion.

Creating TCF19 mutant constructs through In-fusion site directed mutagenesis

Site-directed mutagenesis was performed using InFusion HD cloning Plus (Clontech Cat no. 638909). Briefly, primers were designed to overlap by 15 bp at their 5' ends and incorporate the desired mutation. The vector was then linearized by inverse PCR using a three step PCR protocol and CloneAmp HiFi PCR Premix by running the reaction in a thermocycler at 98C for 10sec, 55C for 5 sec, and 72 C for 5sec/kb for 30 cycles. The reaction was then treated with Cloning Enhancer to remove the circular double stranded template from the reaction. DNA was then run out on a gel and the PCR

sample was excised and cleaned up with Gel PCR cleanup kit according to manufacturer's protocol (Clontech Cat no. 638911). The purified DNA was then used as template for the In-fusion reaction (100 ng of DNA) and then incubated at 50C for 15 minutes with In-fusion enzyme.

Transformation into stellar competent cells

Transformation was performed according to manufacturer's protocol (Protocol PT5055-2 Clontech, Cat no. 636763). Briefly, Stellar Competent Cells were thawed and 50uL were pipetted into a falcon tube. 2.5uL of InFusion reaction was used for transformation. Tubes were then placed on ice for 30 min and then heat shocked for exactly 45 sec at 42C. Tubes were then placed on ice for 1-2 minutes. SOC medium was added to bring final volume to 500uL and then incubated to shake for 1 hr at 37C at 225 rpm. 100 uL were pipetted on ampicillin LB agar plates to incubate overnight at 37C. After overnight incubation, single colonies were picked and grown overnight in LB broth for 18 hours before MIDI prep.

MIDI prep of overnight culture

MIDI prep was performed using QIAgen plasmid MIDI kit according to manufacturer's protocol. Briefly, bacterial culture was harvested and pelleted and then resuspended in Buffer P1. Buffer P2 was then added until lysate became viscous and then Buffer P3 was added and vigorously inverted 4-6 times and incubated on ice for 5 min. The mixture was then centrifuged at maximum speed for 10 min, supernatant containing plasmid DNA was removed, and then the supernatant was applied to a

QIAGEN-tip and allowed to gravity flow through resin. QIAGEN-tip was washed with Buffer QC and then the DNA was eluted with BufferQF. The DNA was then precipitated with isopropanol, centrifuged, and then the DNA pellet was washed with 70% ethanol and centrifuged once more. DNA pellet was allowed to air dry and DNA was re-dissolved in TE buffer. DNA concentration and purity was determined with Nanodrop ND-2000c Spectrophotometer.

DNA sequencing sample preparation

Sample preparation with BigDye Terminator v1.1 5x Sequencing Buffer (Thermo Fisher Cat no. 4336697) was performed according to manufacturer's protocol. Briefly, PCR primer mix with sequence specific primers, BigDye, 5X Buffer, and deionized water were combined in a single reaction and 100ng DNA added to the reaction. The reaction was then run for the following cycling conditions: 10 min at 95C; 35 cycles of 3sec at 95C, 15 sec at 62C, 30 sec at 68C; and 3 min hold at 72C. Samples were then sent in to UW-Madison Biotechnology Center DNA sequencing facility.

Analysis of sequencing data

A plasmid Editor (ApE) was used to align DNA sequencing reads from mutant hsTCF19 Sanger sequencing reads to hsTCF19 DNA sequence reads to confirm correct mutation was incorporated at correct location.

Immunoprecipitation of Halo-tag TCF19 protein

Immunoprecipitation of Halo-tagged TCF19 proteins were performed with Chromotek Halo-trap affinity beads (Chromotek otak-20). Briefly, cell pellet from halo-TCF19 overexpressing AD293 cells were lysed with 200uL RIPA buffer (Thermo Fisher Cat no. 89900), spun down and then transferred to pre-cooled tube with 300uL dilution buffer supplemented with protease inhibitor cocktail (Roche, #04693116001) and phosphatase inhibitor (Roche, 4906845001). Halo-trap A beads were then resuspended, washed, and added to the lysate and then tumbled end-over-end for 1 hour at 4C. The tube was then spun down and supernatant discarded. Halo-Trap A beads were resuspended and washed twice. Beads were resuspended in 2x SDS sample buffer and boiled to dissociate immunocomplexes from beads. Beads were spun down and then supernatant collected and analyzed on Western Blot.

Western Blot

30uL of protein samples from immunoprecipitation experiment was run on 10% SDS-PAGE gel and were dry transferred onto a 0.2um PVDF membrane (Biorad,1704156) using a Trans-Blot Turbo dry transfer system (Biorad, 1704156). Membranes were blocked in 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 hr at room temperature and were incubated overnight in primary antibody, washed 3X in TBST and incubated 1 hr in secondary antibody. Blots were developed with Pierce ECL Western Blotting Substrate (Thermo Fisher Cat no. 32106) and imaged with a GE ImageQuant charge-coupled device camera. Primary antibodies and dilutions are as follows : Anti-HaloTag pAb (Promega G928A, 1:1000), c-Jun (Cell Signaling 60A8, 1:50). Secondary antibodies and dilutions are as follows: Anti-rabbit IgG, HRP-linked antibody (#7074 Cell Signaling, 1:2000).

RESULTS

Affinity purification-mass spectrometry proteomics of hsTCF19 overexpressing AD293 cells reveals potential binding partners

We have previously elucidated a role for TCF19 in DNA damage responses. We observed significant increases in DDR gene expression levels after TCF19 overexpression and increased DNA damage markers in wbTcf19KO mice. Identifying TCF19 protein interactions is a critical next step in understanding its biological function and how it may work under DNA damage conditions.

In order to determine how TCF19 is modulating DDR gene expression, we performed preliminary analysis with affinity-purification mass spectrometry (AP-MS) on TCF19 overexpressing AD293 cells. Briefly, AD293 cells were split into three transfection groups - pcDNA (empty vector control), hsTCF19 overexpressing cells (hsTCF19), and hsTCF19 overexpressing cells treated with etoposide, an agent that induces DNA double strand breaks (hsTCF19 + etop) (Figure 1A). 48 hours after transfection and then 3 hours +/- etoposide treatment, cells were harvested and protein lysate was collected. Because the expression construct generated TCF19 protein with a 6x histidine tag at the C-terminus, we were able to perform affinity purification using an activated nickel column. Bottom-up proteomics was performed on the eluted samples and protein identifications were obtained by analyzing the data via MASCOT database search. This generated a list of proteins that were also eluted from the nickel column with TCF19, which we consider as putative TCF19 binding partners. TCF19 was by far the most abundant protein identified, and this indicated successful purification. Analysis of these datasets allowed us to extract the unique proteins from each condition. Specifically, we extracted the

unique proteins from the hsTCF19 and hsTCF19+etop list that were not found in the pcDNA control list. Because of our hypothesis that TCF19 acts as a transcriptional regulator, we specifically focused on transcription factors or other transcriptional regulators identified in the proteomics analysis. This revealed several transcription factors that were only identified in the etoposide treated cells which represent candidate proteins that interact with TCF19 specifically under conditions of DNA damage (Fig 1B). Search Tool for Retrieval of Interacting Genes/Proteins (STRING) analysis to look at functional interactions in the dataset revealed a small network of proteins with C-JUN being the center node with the most connections. This highlights C-JUN as a potential common interactor or regulator of the proteins in the dataset (Fig 1C).

To confirm C-JUN as a binding partner, co-immunoprecipitation was performed on haloTCF19 after etoposide treatment. Despite successful immunoprecipitation of haloTCF19, we did not detect C-JUN as a co-precipitant (Fig 2A and B). While this does not confirm an interaction between TCF19 and C-JUN, there are several caveats that render this conclusion inconclusive. As different affinity-purification tags were used (Halo vs. histidine) it is possible that the halo-tag inhibited the interaction. If the interaction is weak, we may need to further optimize co-immunoprecipitation conditions to allow detection. Future experiments will be necessary to definitively determine whether TCF19 and C-JUN interact.

HaloTCF19 localization studies reveal nuclear and cytoplasmic localization with increased TCF19 localization in a punctate manner after etoposide treatment

The subcellular localization of TCF19 can provide insight as to what role it may play in cellular function, as well as its function under different stress conditions. To assess TCF19 subcellular localization, we created a Halo-tagged TCF19 construct for direct visualization of TCF19 protein. The fluorescent Halo-Tag can be visualized directly in live cells or detected in fixed tissue with an anti-Halo antibody. We transfected haloTCF19 into AD293 cells, fixed the cells, and then performed confocal microscopy. We find that under a non-stressed baseline conditions, haloTCF19 localizes to both the nucleus and cytoplasm (Fig 3A). However, after 6 hour treatment with the DNA damaging agent, etoposide, we see a shift toward more punctate staining, including in the nucleus (Fig 3B). This preliminary experiment indicates a change in TCF19 subcellular localization in response to DNA damage, and the distinct punctate staining pattern may indicate TCF19 localization to specific sites of the DNA. It also demonstrates that TCF19 is not always localized to the nucleus, despite its likely role as a transcriptional regulator, and opens the possibility that TCF19 may have other non-transcriptional functions and/or its nuclear localization may be regulated by signaling pathways or protein-protein interactions.

Creation of TCF19 mutants

The PHD finger domain of TCF19 has been shown to bind to the H3K4me3 histone modification[12,13]. Specifically, TCF19 binding to H3K4me3 led to the recruitment of protein complexes that could then alter gene expression and increase cell proliferation in liver cells[13]. Site-directed mutagenesis of a key residue in the PHD finger, mutating the W316 residue to tyrosine (W316Y), resulted in no binding activity and significantly decreased cell growth[13]. In order to determine the role of the TCF19 PHD finger in the islet and beta

cell, we generated TCF19 plasmids with W316Y point mutations (Fig 4B). This TCF19 mutant will be of use in future studies to determine if specific functions of TCF19 depend on its ability to interact with H3K4me3.

Additionally, TCF19 contains a FHA domain which is phosphorylated in response to DNA damage and likely interacts with other proteins[11]. Specifically, after treatment with UV radiation, TCF19 was phosphorylated on the serine 78 residue which is located in a Ser-Gln motif that is recognized by kinases involved in the DDR such as ATM and ATR[11]. Therefore, it is possible that TCF19 is a downstream target of ATM/ATR. Serine 78 point mutations were designed into TCF19 constructs to either inhibit phosphorylation (S78A) or mimic phosphorylation (phosphomimetic) (S78Glu) (Fig 4C). These TCF19 mutants will be useful in future studies to determine if this phosphorylation event is critical for TCF19 function in response to DNA damage.

TCF19 also has several functional variants that have been identified through GWAS. We looked at all the SNPs in TCF19 in the dbSNP database, and we specifically identified those SNPs that were coding variants. These coding variants were the ones that we generated for downstream studies to determine to effect on TCF19 function. A heat map showing the predicted functional effects of these variants revealed that the P109S amino acid substitution is non-conservative and likely to result in a function effect on the protein (Fig 4A). On the other hand, the V211M mutation is a conservative mutation and predicted to not have any effect. The P241L mutation is also predicted not to have an effect on protein function. We generated mutations of these variants, and specifically, are interested in the P109S mutation (Fig 4D). The effects of these variants on the DDR pathway can be assessed by overexpressing the mutant constructs into cell lines and

then performing functional experiments after DNA damage treatment to the cells. In this way, we will be able to connect human genetic variants directly to changes in TCF19 protein function which may help us to explain how the *TCF19* gene associations with T1DM and T2DM actually translate to pathogenic changes in beta cell biology.

DISCUSSION

In this chapter, we described several preliminary studies that were initiated during my PhD thesis work and can be followed up in future projects to advance our understanding of the function of TCF19 and its link to diabetes pathogenesis. We identified several potential TCF19 interacting partners that will need to be further validated with optimized pull-down studies. We further show that TCF19 localizes to both the nucleus and cytoplasm with an increase in punctate staining after etoposide treatment. We have created GWAS mutations in TCF19 plasmids and also point mutations within the FHA and PHD finger domain of TCF19 to help determine their effects on protein function.

Affinity purification mass spectrometry (AP-MS) produces large amounts of information and has become a standard method for discovering protein-protein interactions[14]. We chose to use our his-tagged TCF19 construct to perform purification on a nickel column because of the lack of a reliable TCF19 antibody. His-tagged TCF19 protein can then be purified on a nickel column by washing with high concentrations of imidazole. Analysis of proteins co-purified with TCF19 revealed a small list of transcription factors that were unique to either basal conditions or DNA damage conditions. The putative TCF19 interacting proteins under etoposide treatment have roles in chromatin

regulation and interacting with chromatin such as the BCL-6 corepressor (BCOR). Other transcription factors have roles in immune defense against viruses (Tripartite Motif Containing 25 (TRIM25)), regulators of CREB- activated and co-activators of CREB (CRTC1, CRTC2), embryonic stem cell maintenance (CCR4-NOT transcription complex subunit 1 (CNOT1)), and regulation of cell proliferation and apoptosis (Cellular Jun (C-JUN). Interestingly, STRING analysis on these putative interacting proteins revealed a small node of proteins with JUN (C-JUN) at the center, indicating its potential role in regulating this small network of proteins.

C-JUN is a subunit of the protein complex, Transcription Factor AP-1, which binds to AP-1 recognition elements found in many DDR genes[15]. Several DDR genes differentially expressed in our wbTcf19KO mouse model (*Gadd45a, Chop*), are also transcriptional targets of AP-1[16,17]. Preliminary co-immunoprecipitation experiments for C-JUN with haloTCF19 did not confirm a direct interaction between TCF19 and C-JUN. However, the conditions of this assay will require optimization especially if the interaction between TCF19 and C-JUN is weak or transient. Further steps such as cross-linking or optimization of buffer conditions, wash steps, and centrifugation will need to be done to ensure protein-protein interactions are not disrupted. HaloTCF19 and not the his-tagged TCF19 construct was chosen as the tagged protein to use for pull-down experiments in order to decrease nonspecific binding of other proteins in the cell that may have histidine residues. However, the HaloTag is a large tag at 33kDa, and therefore, we cannot discount the possibility that the large tag could be disrupting protein function or interfering with protein-protein interactions. The HaloTag was also placed on the N-terminus of TCF19, which may specifically disrupt protein-protein interactions with the FHA domain.

Therefore, future pull-down studies may utilize a smaller tag, such as a tetracysteine tag (~20kDa), to ensure the tag does not affect protein binding or function. Ultimately, these experiments are limited by the lack of availability of a high-quality antibody against TCF19 itself, and may be revisited in the future if such an antibody is developed.

To further learn about the role of TCF19 in cellular function, we performed subcellular localization studies which showed that TCF19 can localize to both the cytoplasm and the nucleus. Interestingly, in a study done in 2010, it was shown through a mammalian two-hybrid system that TCF19 localizes with a protein called LZTR1, which is a transcriptional regulator, and also localizes to the cytoplasmic surface of the Golgi network[18]. More recent studies have demonstrated that TCF19 promotes cell proliferation in HepG2 cells through its PHD finger which binds to H3K4me3[12,13]. Another study showed that TCF19 directly interacts with H3K4me3 to recruit a nucleosome remodeling complex to the promoters of certain genes[13]. These studies indicate a role for TCF19 in modulating gene expression in the nucleus. It is very likely that TCF19 localizes to both the nucleus and cytoplasm under different conditions, perhaps under conditions that require regulation of certain cell cycle or stress response genes in the presence of select stimuli. More careful quantitative analysis of TCF19's subcellular localization, particularly under relevant stress conditions and with the use of co-staining to clearly identify specific subcellular compartments, will be necessary to fully understand how TCF19 functions and the regulatory pathways that impact its localization.

In the wbTcf19KO mouse model and cell line overexpression model, we see an overrepresentation of genes involved in the DDR. Combining this with the subcellular localization data where we see cytoplasmic and nuclear localization of TCF19 at baseline

and then a stronger more punctate localization after DNA damage, we hypothesize that TCF19 may be shuttling into the nucleus after DNA damage stress to help activate the DNA damage response transcriptional pathways. Future experiments to validate the nuclear and cytoplasmic localization of TCF19 under different conditions could include western blotting for TCF19 in either nuclear and cytoplasmic extracts. Additionally, co-localization studies for binding partners validated from co-immunoprecipitation experiments will be useful for determining the function of the protein-protein interactions. Similar to the experiments described above, these studies may be confounded by the impact of large tags on the TCF19 protein and therefore ideal studies would involve native TCF19 when a reliable antibody is available.

TCF19 is a unique gene in that it is associated with both T1DM and T2DM in GWAS[5,6]. This is important because it suggests a role for *TCF19* in diabetes susceptibility outside of autoimmunity (as the association with T2DM would not be related to autoimmunity). *TCF19* is most likely regulating a mechanism common to both T1DM and T2DM. *TCF19* has three non-synonymous SNPs in linkage disequilibrium with lead T1DM SNPs[5]. Two of the SNPs are found in the proline rich region of TCF19. The proline rich region can facilitate intermolecular reactions and play roles in signaling events involving SH3 domains[19]. It is possible that the *TCF19* SNPs in this domain disrupt domain function.

A heat map showing the predicted functional effects of these SNPs revealed that the variant P109S will have an effect on function whereas the other variant in this domain was predicted to have no effect on function. The P109S mutant will be the first used for future experiments such as overexpression experiments looking at gene expression for

DNA damage genes, specifically after some form of stress treatment. Other readouts such as apoptosis, cell viability, and proliferation can be used to determine the functional effects of these SNPs.

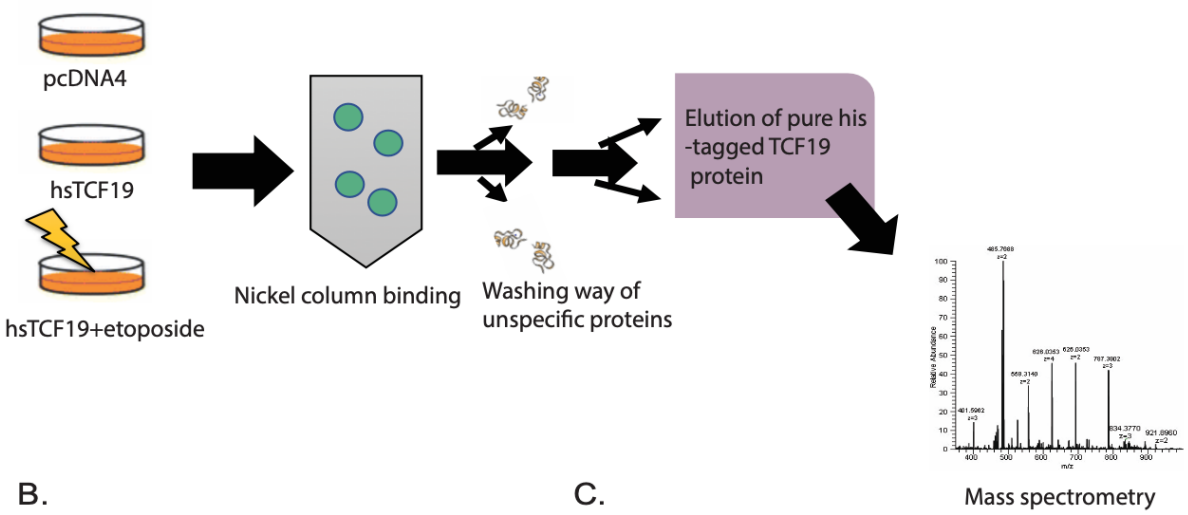
TCF19 also contains a PHD finger domain that has been shown to bind to H3K4me3[12,13]. Specifically, a mutation of the residue leading to a point mutation (W316Y) resulted in no binding to H3K4me3[13]. The PHD finger of TCF19 has been shown to bind to this specific residue to recruit histone modifiers and affect gene expression[13]. We hypothesize that TCF19 may be binding to this specific histone modifications in the beta cell to recruit activators or repressors to the promoters of DDR genes in response to DNA damage. We have created a TCF19 construct with a W316Y mutation so that future studies, including overexpression of this mutant construct, can be used to determine the proteins that TCF19 recruits after binding to H3K4me3 in response to DNA damage treatment.

Another domain within TCF19, the forkhead-associated (FHA) domain, is a phosphopeptide recognition domain that is commonly found in many regulatory proteins and recognizes phosphothreonine epitopes[9]. Proteins with FHA domains include those involved in regulating the cell cycle and DNA repair[20]. Specifically, the FHA domain in TCF19 contains a serine residue at position 78 (Ser78) which is phosphorylated after DNA damage[11] We hypothesize that TCF19 may be a downstream target of DNA damage proteins, or serve as a coregulator to DNA damage kinases[11]. In order to assess the function of this serine residue in TCF19, we generated a phosphomimetic mutation, which is an amino acid substitution that mimics the effect of a phosphorylated residue[21,22]. We generated a TCF19 construct with the serine mutated to glutamic acid to mimic a

constitutively active phosphorylation state. Additionally, we also generated a TCF19 constructs with the serine mutated to alanine, which inhibits phosphorylation[22]. These constructs can be used for future studies to help determine the effect of TCF19 serine phosphorylation after DNA damage on DDR gene expression and the functional effects of this domain such as proliferation, apoptosis, and DNA damage levels.

Overall, the work in this chapter provides the tools and preliminary experiments for future studies aimed at determining the functional and mechanistic role of TCF19 in DNA damage stress. Additionally, as a T1DM and T2DM susceptibility gene, the generation of TCF19 plasmids with SNPs will be a valuable tool to help elucidate how functional genetic variants of TCF19 may contribute to diabetes.

A.



B.

NON-STRESSED	STRESSED
BCLAF	BCOR
CNOT1	TRIM24
TCF20	TRIM25
ILF2	CRTC1
TRIM28	CRTC2
YAP1	CNOT1
SP1	JUN

C.

Figure 1: Mass spectrometry of his-tagged TCF19 pull down experiment reveal several potential TCF19 interacting partners. **A.** Schematic of affinity purification-mass spectrometry (AP-MS) treatment groups (pcDNA4, hsTCF19, hsTCF19 with etoposide) and experimental design. **B.** Transcription factors unique to hsTCF19 overexpressing cells (non-stressed column) and hsTCF19+etoposide (stressed column) cells. **C.** STRING analysis on potential transcription factor binding partners reveals a small network with JUN at the center.

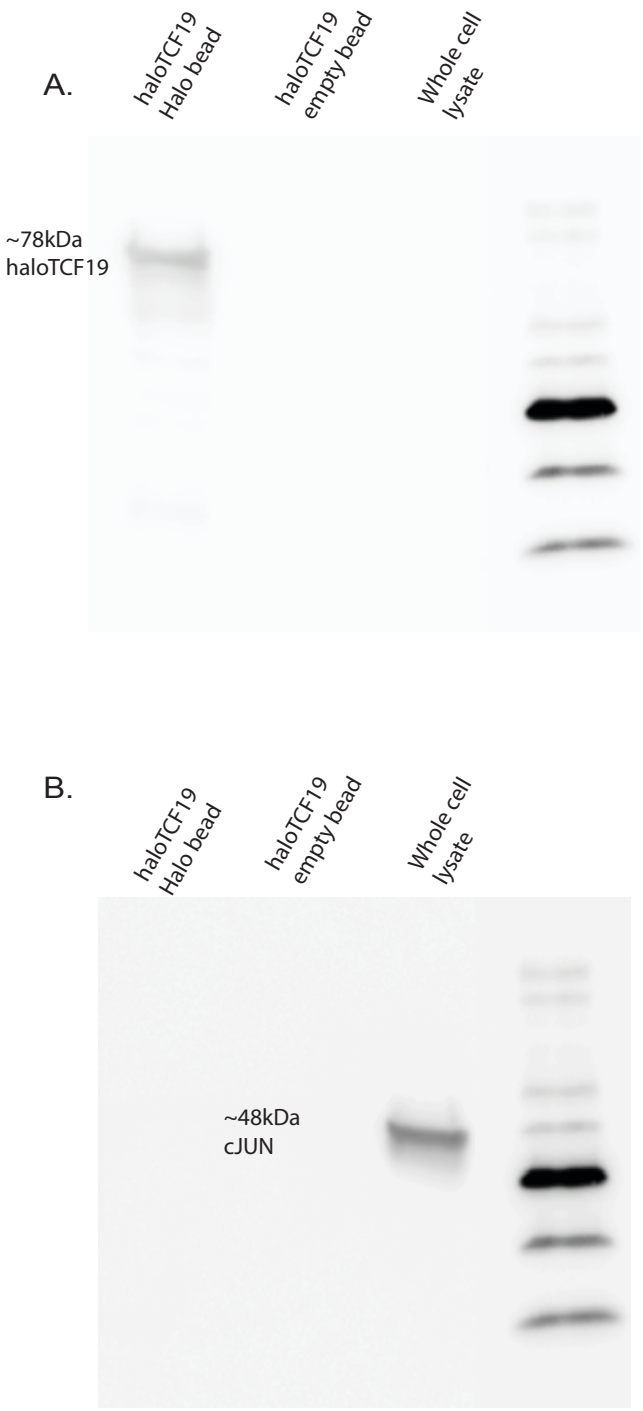
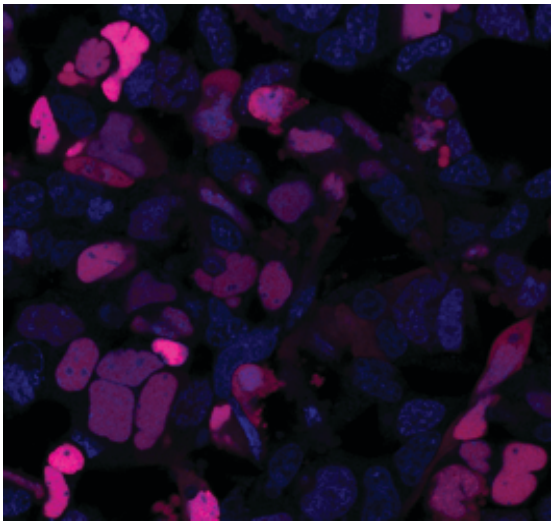


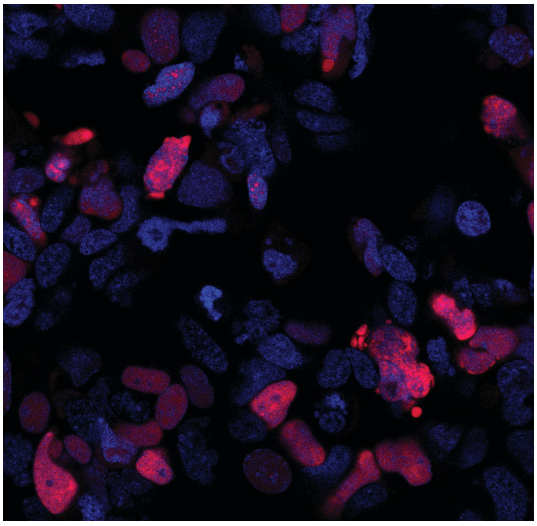
Figure 2: HaloTCF19 co-immunoprecipitation for cJUN experiments. **A.** HaloTCF19 is successfully pulled down with Halo-tag Nanobody/VHH conjugated to agarose beads and is specifically detected at the correct molecular weight. **B.** Western blot for cJUN shows that cJUN did not co-precipitate with haloTCF19.

A.



No Treatment

B.



6 hr etoposide treatment

Figure 3: Confocal microscopy experiments on haloTCF19 overexpressing AD293 cells to assess localization. **A.** haloTCF19 localizes to the nucleus and cytoplasm in untreated cells. **B.** After 6 hours etoposide treatment to induce DNA damage, haloTCF19 localizes in less diffuse, more punctate manner.

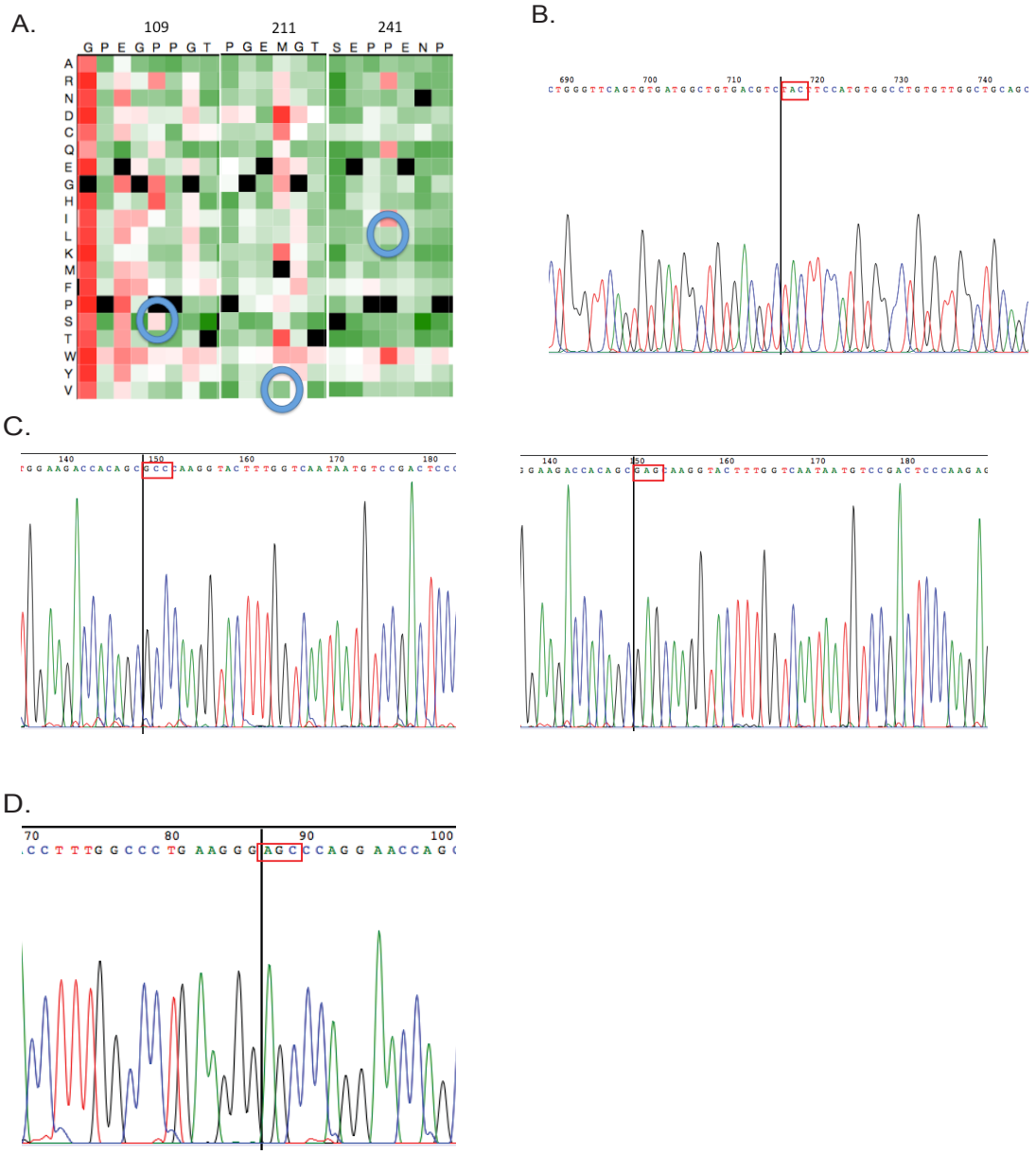


Figure 4: Heat map and site directed mutagenesis generating point mutations for PHD finger mutation, serine78 mutations and TCF19 variant mutation. **A.** Heat map describing functional effect of point mutants in TCF19. Red indicates substitution is predicted to have a positive effect, green indicates prediction of no effect. The mutations P109S, M211V and P241L are indicated with blue circles. **B.** Confirmation of DNA sequence from Sanger sequencing showing successful incorporation of W316Y PHD finger mutation (boxed in red). **C.** DNA base reads showing successful incorporation of serine 78 phosphorylation inhibition mutation (alanine mutation in left panel) and phosphomimetic mutation (glutamic acid mutation in right panel). **D.** DNA base reads showing successful incorporation of TCF19 variant predicted to have functional effect (P109S).

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Chapter 5: Conclusions and future directions

The following chapter was written by Grace Yang.

Conclusions

Despite the medical advancements in the diabetes field, diabetes continues to be a huge global health problem. To this day, there is yet a cure for diabetes, and cases continue to rise annually. Both Type 1 diabetes (T1DM) and Type 2 diabetes (T2DM) are diseases of reduced functional beta cell mass. Therefore, a better understanding of the factors that lead to beta cell proliferation/expansion and beta cell apoptosis are crucial to the development of therapies aimed to maintain adequate beta cell mass. In this thesis, I discussed the factors that affect beta cell mass in both T1DM and T2DM and focus on reviewing the literature about what is known about the DNA damage response in diabetes pathogenesis. Additionally, I characterize an overexpression model of TCF19, delineate pathways of regulation, and discuss ongoing experiments and tools that are being done to elucidate mechanistic targets of TCF19. I also discussed the findings from our whole body Tcf19 knockout (wbTcf19KO) mouse model and the gene expression changes relating to DNA damage, proliferation, beta cell identity, and insulin granule exocytosis. Additionally, I discussed the fact that wbTcf19KO mouse islets had a significant increase in the DNA damage marker, γH2AX, at baseline and after *ex vivo* islet treatment with cytokines and how this relates to diabetes.

We also found phenotype differences in glucose tolerance and beta cell function in wbTcf19KO mice after a long term HFD. wbTcf19KO mouse islets from mice on the HFD had increased γH2AX at baseline. We were also able to show that wbTcf19KO mice had impaired adaptive beta cell proliferative response to a 1-week HFD.

Future directions

The next step of this project will involve further experiments in the wbTcf19KO model on the HFD model including *ex vivo* experiments on islets to determine the extent of DNA damage using a comet assay and determining expression levels of DNA repair genes after treatment of islets with DNA damaging agents such as etoposide or hydrogen peroxide (as a model of oxidative stress). Additionally, immunohistochemistry of pancreas sections to determine beta cell mass, apoptosis and proliferation from HFD wbTcf19KO mice will help determine functional significance of Tcf19 under HFD induced stress.

Additionally, future experiments will need to be performed to specifically look at DNA repair capacity. These experiments can initially be performed by knocking down TCF19 in human beta cell lines, and later translated to human islets. After knockdown, DNA repair capacity can be assessed under different types of beta cell stress such as cytokines or hydrogen peroxide. DNA repair capacity can be determined with comet assay or γH2AX staining before, during, and after stress treatments. Cell viability and cell death can be determined with a caspase 3 assay or TUNEL staining. Additionally, assessing for activation of DNA damage pathways by looking at key DNA damage response proteins such as ATM, CHK1, CHK2, and p53 can be performed to determine the effect of TCF19 knockdown on these pathways.

Another next step in this project will be to determine TCF19 interacting partners which will help with mechanistic and functional insights to how TCF19 is regulating DNA damage response gene expression. We have already performed a preliminary round of affinity purification – mass spectrometry (AP-MS) on TCF19 overexpressing cells to determine potential TCF19 binding partners and the list revealed several candidate

transcription factors that bound to TCF19 after treatment with the DNA damaging agent, etoposide. Validation of these proteins by co-immunoprecipitation will be the next step, and will also include optimization of binding, washing, centrifugation, and incubation conditions to make sure the protein-protein interactions are not disrupted. Cross-linking may also be necessary if the interaction between proteins are weak. Additionally, ChIP-seq may also be performed to determine what DNA sequence TCF19 binds to (either indirectly or directly). Specifically, TCF19 has been shown to bind to H3K4me3 through its PHD finger, and therefore crosslinking of TCF19 to this particular histone modification and then performing ChIP-seq can give insight as to what DNA sequences TCF19 “binds” to indirectly. Additionally, ChIP-seq experiments overexpressing a mutant version of the PHD finger which inhibits its binding to H3K4me3 can be performed to determine the DNA sequences that are no longer bound compared to those from the WT TCF19 ChIP-seq data. These experiments can elucidate the genes TCF19 is directly regulating.

Additionally, co-localization experiments with haloTCF19 can be performed to demonstrate protein interaction with candidate binding partners *in vivo*. Co-localization experiments can be performed under conditions of DNA damage stress and recovery from damage. If the interactions are more transient, proximity ligation assays can be performed to visualize direct protein-protein interactions.

After determining TCF19 binding partners, these data can be used to extrapolate function and mechanism, and downstream experiments can work to elucidate specific pathways that TCF19 may be activating and regulating. We have created TCF19 mutants which are plasmid constructs that include the nonsynonymous SNPs identified from

GWAS. Overexpression of these constructs can be used to determine whether any of these mutations affect TCF19 downstream signaling pathway activation.

Taken together, we have shown that TCF19 is a gene that has roles in the DDR and is important for adaptive beta cell proliferation. Lack of Tcf19 leads to increased DNA damage. Our overall hypothesis is that TCF19 modulates the DNA damage repair pathway in order to prevent beta cell apoptosis and to allow the cell to proliferate and adapt under conditions of beta cell stress.