Functional Characterization of Naturally Occurring Mutant Transglutaminase 2

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Physiology

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ABSTRACT

Transglutaminase 2 (TG2) is a functionally and structurally complex ubiquitous protein. TG2 has Ca^{2+}-dependent transamidating activity, can bind and hydrolyze ATP/GTP, function as a G-protein in intracellular signaling, and has reported kinase activity. TG2 knockout mice are observed to have impaired glucose-stimulated insulin secretion (GSIS). Three naturally occurring mutations, including Met330Arg, Ile331Asn, and Asn333Ser, have been reported in the TG2 protein and observed to be related with maturity onset diabetes of the young (MODY). In order to assess the role of the various domains of TG2 in GSIS, a series of constructs were generated using site-directed mutagenesis to express either wild-type or mutant TG2 protein including an additional peptide tag for identification purposes (Myc-tag). Overexpression of the naturally occurring Myc-tag mutants in INS-1E cells generated a loss in GSIS compared to wild type-TG2 overexpression. Each mutant was shown to have diminished transamidation and kinase activities, along with altered GTP-binding which was responsive to glucose stimulation. Naturally occurring mutations in TG2 impact the transamidation, kinase, and GTP-binding functions of TG2. The GTP-binding function of TG2 has a significant impact on GSIS from pancreatic beta cells in addition to its transamidating activity.

ACKNOWLEDGEMENTS

I would like to offer my most sincere thanks to my supervisor, Dr. Suresh Mishra, whom I am indebted to for granting me the privilege to join his laboratory and for the guidance and support he endowed unto me. The experience and education he has provided will prove invaluable and will not be forgotten.

I would like to thank the members of my student advisory committee, Dr. Janice Dodd, Dr. Grégoire Nyomba, and Dr. Leigh Murphy, for their honest criticism, patience, and encouragement. Leading by example, their integrity and outstanding enthusiasm for science, was a clear means of inspiration for me throughout my studies.

I would like to thank my past and current colleagues from the Diabetes Research Group. Dr. Suddu Ande was a superb mentor and greatly assisted me in several aspects of my project. Dr. Hoa Nguyen, with his great expertise in islet isolation, guided my animal model work forward. Dr. Jong Han Lee and Dr. Yuan Yuan Gu were dear friends and had given exemplary advice and support. Each and every member of the Diabetes Research Group has contributed in some way to the success of my study and I thank them deeply.

I wouldn't be where I am today if it weren't for the constant and unwavering support of my friends and family. With insurmountable patience and love, my parents have always encouraged me to push onward through my studies and attain my goals. They will always have my dearest gratitude. My friends and colleagues have made some of my most challenging years so far, some of the most enjoyable as well. I thank them all whole-heartedly and wish them the best.

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ABBREVIATIONS

$BPNH_2$	5-(Biotinamido)Pentylamine
5-HT	5-hydroxytryptamine, serotonin
A	Alanine
ACN	acetonitrile
ATP/ADP	adenosine triphosphate/diphosphate
ATPase	adenosinetriphosphatase
BSA	bovine serum albumin
bp	basepair(s)
C	Cysteine
CCK	cholecystokinin
cDNA	complementary deoxyribonucleic acid
CO2	carbon dioxide
°C	degree Celsius
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DM	Diabetes Mellitus
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum

Abbreviations

FADH2	flavin adenine dinucleotide, reduced
g	gram
G	glucose
GLP-1	glucagon-like peptide 1
GLUT2	glucose transporter 2
GSIS	glucose-stimulated insulin secretion
GTP/GDP	guanine triphosphate/diphosphate
GTPase	guanosinetriphosphatase
HBSS	Hank's balanced salt solution
HCl	hydrochloric acid
H_2O	water
HRP	horse radish peroxidase
IP_3	inositol triphosphate
I	Isoleucine
KRBH	Krebs-Ringer bicarbonate HEPES buffer
kg	kilogram
KCl	potassium chloride
kDa	kilo Daltons
M	molar
M	Methionine
MgCl2	magnesium chloride
mM	millimolar
min (s)	minute (s)

Abbreviations

ml	millilitre
MODY	maturity-onset diabetes of the young
mRNA	messenger RNA
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
N	Normal
N	Asparagine
nM	nanomolar
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSF	N-ethylmaleimide sensitive fusion proteins
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PLC	phospholipase C
R	Arginine
Rb	Retinoblastoma
RPMI	Roswell Park Memorial Institute culture medium
RT	room temperature
G	gravitational force
RNA	ribonucleic acid

Abbreviations

ROS	reactive oxygen species
s	second
S	Serine
SERT	serotonin transporter
siRNA	small interfering RNA
SDS	sodium dodecyl sulphate
SNARE	Soluble NSF Attachment Protein Receptor
TBS	tris buffered saline
TBS-T	tris buffered saline with 0.1% Tween 20
TCA	tricarboxylic acid cycle
TEMED	N,N,N',N-tetramethylethylenediamine
TFA	trifluroacetic acid
TG2, TGM2	Transglutaminase 2/tissue Transglutaminase
TMB	3,3′,5,5′-Tetramethylbenzidine
Tris	tris (hydroxymethyl) amino methane
Tween-20	polyxyethylene (20) sorbitan monolaurate
µl	microliter
µm	micrometer
µM	micromolar
µg	microgram
VMAT2	vesicular monoamine transporter 2
WT	Wild-type
%	percent

I. INTRODUCTION

Research Rationale

Diabetes Mellitus is a metabolic disease which has interrelated vascular, neuropathic, and nephropathic components. It encompasses a group of metabolic diseases which are characterized by hyperglycemia generated through the disruption of insulin secretion, insulin action, or a combination of the two [1]. Diabetes Mellitus consists of two main categories, type 1 and type 2 diabetes. Type 2 diabetes can arise primarily from either beta cell dysfunction or insulin resistance at peripheral tissues, or a combination of the two. [2]. From the 1980's and throughout the 1990's there has been a massive rise in the number of individuals diagnosed with type 2 diabetes [3] and this number is projected to continue to rise in the future [4]. There are several behavioural and lifestyle risk factors including obesity, physical inactivity, diet, and stress [5] as well as genetic risk factors, including the possession of alterations in the maturity onset diabetes of the young (MODY) genes that may induce beta cell dysfunction [6].

Transglutaminase 2 (TG2) is a functionally and structurally complex protein. The *TGM2* gene is situated on human chromosome 20, specifically within the 20q12 locus [7]. Chromosome 20q has been shown to contain a susceptibility locus for noninsulin-dependent type 2 diabetes (NIDDM) in linkage studies and is thought to possess multiple susceptibility genes [8-10]. TG2 knockout mice have been observed to be glucose intolerant, have partially abolished insulin secretion in response to glucose, and display a

phenotype analogous to patients with MODY [11]. Three heterozygous missense mutations in the *TGM2* gene have been discovered in individuals previously diagnosed with MODY. These mutations cause the TG2 protein to incur one of three amino acid substitutions, including methionine 330 to arginine (Met330Arg), isoleucine 331 to asparagine (Ile331Asn), and the asparagine 333 to serine (Asn333Ser) [11, 12]. A well characterized function of TG2 is its calcium-dependent transamidation activity, which catalyzes the generation of a γ-glutamyl-ε-lysine bond between the side chain of lysine and glutamine residues either within the same protein or between two separate proteins [13]. Recently, TG2 has been shown to modulate glucose-stimulated insulin secretion (GSIS) from beta cells by covalently coupling serotonin onto small G-proteins. The bonding of serotonin onto glutamine residues within these insulin secretion-modulating small GTPases, results in their constitutive activation thereby affecting GSIS [14].

TG2 possesses intrinsic kinase activity and phosphorylates a variety of proteins, including insulin-like growth factor binding protein-3 (IGFBP3) [15]. A study recently identified IGFBP3 as possessing a modulatory role in insulin secretion [16]. In addition, it was observed that Ca^{2+} and ATP regulate the kinase and transamidation activities of TG2 in an opposing manner [15]. ATP facilitates kinase activity, while exerting an inhibitory effect on transamidation activity, whereas Ca^{2+} has the opposite effect. GTP is able to bind to TG2, allowing TG2 to act as a G-protein in cell signaling pathways [17]. Similar to ATP binding, GTP binding and transamidation activity are inversely associated with each other. Despite the fact that the transamidation catalytic triad and putative GTP/ATP binding loops are located distantly from each other in the primary structure of

TG2, in the tertiary structure they are found in close proximity as seen in the crystal structure of TG2 [18, 19]. The naturally occurring mutations of TG2, Met330Arg, Ile331Asn, and Asn333Ser -TG2 are located within the region containing the transamidation catalytic triad, and similar to the triad, are found in close proximity to the GTP/ATP binding loops in the tertiary structure of TG2. The effects these naturally occurring mutations of TG2 have on its enzymatic functions, as well as its GTP binding ability, have not been elucidated. While a role for the transamidation activity of TG2 in GSIS from pancreatic beta cells has been postulated [12], there is a lack of evidence to support it. The prospect that more than one of TG2's functional activities is involved in GSIS has not yet been investigated. It is likely that these mutations, due to their respective locations on TG2, affect the transamidation, kinase, and GTP-binding/G-protein activities of this multi-functional protein. Therefore, the goal of this thesis project was to elucidate the functional impact of the naturally occurring mutations on TG2 functional activities, including GTP-binding, and their relationship with GSIS in pancreatic beta cells. The first objective was to determine the effect of the overexpression of each mutant on GSIS. The second objective was to evaluate the effect of these mutations on TG2's functional activities and determine if these effects on TG2's functional activities could be correlated with each mutant's influence on GSIS.

Literature Review

1. Pancreas: Structure and Physiology

Located in the posterior wall of the abdominal cavity of mammals, the pancreas is a gland with a blend of exocrine and endocrine constituents that has an essential role in metabolism and digestion. The endocrine segment of the pancreas influences metabolism through the secretion of hormones that modulate carbohydrate, lipid, and protein metabolism. The exocrine, or acinar, segment of the pancreas produces and secretes digestive enzymes and an alkaline pancreatic fluid into the pancreatic duct, which terminates in the lumen of the duodenum. The secretion of the bicarbonate-rich fluid, along with digestive enzymes, is stimulated by a variety of means, including the two peptide hormones released by the duodenum, cholecystokinin (CCK) and secretin. The alkaline fluid functions to buffer the acidic contents entering the small intestine from the stomach [20].

The endocrine pancreas is composed of minute masses of endocrine cells, known as the islets of Langerhans, dispersed among the exocrine tissue. The islets of Langerhans secrete two major hormones, insulin and glucagon [21]. These two hormones function by modulating the rate of catabolism and anabolism of endogenous carbohydrates, lipids, proteins, and other substrates to make certain that the energy requirements are guaranteed in the basal state and during exercise. Furthermore, these hormones direct the flow of nutrient input from meals. They achieve these roles mainly

through actions on the liver, muscle, and adipose tissue throughout the body. Additional secretory peptide products of islet cells include amylin, pancreastatin, somatostatin, and pancreatic polypeptide. There are roughly 1 million islets in the human adult pancreas, only comprising 1% to 1.5% of the total pancreatic mass. An islet consists of approximately 2500 cells, 60%-70% of which are the insulin secreting β cells, 20%-25% are the glucagon[21, 22] and pancreastatin secreting α cells [23], and 10% are the somatostatin producing δ cells and the pancreatic polypeptide secreting PP cells. Each islet is composed of a core of β cells surrounded by either α and/or δ cells or δ and PP cells [21, 22].

During a meal the nutrient inflow from the intestines, along with gastrointestinal secretagogues released in response to food intake, stimulate the secretion of insulin. Additionally, the enzyme products and alkaline fluid of the exocrine pancreas are released into the duodenum to aid digestion. The islet hormones flow from the pancreatic vein into the portal vein, where they unite with the blood containing the nutrients absorbed from digestion. The liver, being the central organ in substrate traffic, receives primary exposure to the islet hormones and in higher concentrations than peripheral tissues. During peak hormone secretion from the pancreas, up to 50% of the plasma glucagon and insulin content can be extracted by the liver on the first pass, thereby providing a means of regulating the availability of these hormones to other tissues [22].

2. Insulin: Structure, Synthesis, and Secretion

Mature insulin has a molecular weight of about 6000 Da and is composed of two straight peptide chains, known as the A and B chains, which are connected by two disulfide bridges. It is formed from the insulin precursor preproinsulin, which sequentially consists of an N-terminal peptide, the B chain peptide, a connecting peptide, known as C peptide, and the A chain peptide. Prior to the completion of synthesis, the N-terminal peptide is cleaved from the molecule. The resulting proinsulin molecule obtains disulfide linkages between the A and B chain while en route to the Golgi apparatus, forming a folded conformation. Once in the Golgi apparatus, the C peptide is slowly cleaved from the two chains by proconvertase-1 and carboxypeptidase-H. Insulin, along with equimolar quantities of C peptide, is packaged and stored in granules to be released by exocytosis [22].

A large variety of factors can stimulate or inhibit insulin secretion from pancreatic beta cells. When substrate availability is high, such as during a meal, insulin is secreted and transported throughout the body by the circulatory system. The hormone stimulates the use of incoming nutrients while concurrently inhibiting the mobilization of analogous endogenous substrates. Glucose is the insulin-secreting stimulant of greatest importance in humans. Since the primary function of insulin is to stimulate the uptake and use of glucose in peripheral tissue, the substrate-hormone pairing of insulin and glucose forms a feedback system that closely maintains plasma glucose levels [22]. Several nutrients, such as the amino acids alanine, glutamine, leucine, and free fatty acids such as palmitate can act as insulin secretagogues. Leucine is roughly one-third as potent as glucose in

stimulating insulin release, while glutamine and leucine in combination act to stimulate insulin to an equal degree as glucose [24-26]. Hormones are also known to influence insulin secretion, one of which is somatostatin, which acts through inhibitory G-proteins found on the cellular membrane of the beta cell to decrease insulin release [22].

A glucose transporter, known as GLUT-2 in humans, facilitates the movement of glucose into the beta cell, maintaining an intracellular glucose level almost identical to the interstitial fluid. It is apparent that the increasing levels of glucose are first "sensed" by the enzyme glucokinase. Glucokinase functions by phosphorylating glucose, which is the primary and rate-limiting step required for islet glucose utilization [20]. The resulting molecule, glucose-6-phosphate, is then able to enter the glycolytic pathway to subsequently produce two molecules of pyruvate. Pyruvate enters the mitochondria, where it is decarboxylated to form acetyl-CoA, which is utilized by the tricarboxylic acid cycle (TCA) to generate NADH and $FADH_2$. These two reducing agents are oxidized in the electron transport chain in order to promote ATP production [22]. The rapid increase of the cytoplasmic concentration of ATP results in the closure of the ATP-sensitive potassium channels (K_{ATP}) located on the plasma membrane, thereby depolarizing the beta cell. This depolarization triggers the opening of the voltage-gated Ca^{2+} channels, creating an influx of Ca^{2+} into the cytoplasm [27-29]. The elevation of cytoplasmic Ca^{2+} stimulates the recruitment of secretory granules to the plasma membrane, where they dock, fuse, and release their contents into the extracellular environment (Figure 1). Two pools of insulin secretory granules exist and are known as the reserve pool and the ready-releasable pool. The elevation in Ca^{2+} functions to promote the sudden exocytosis of the

granules in the ready-releasable pool, followed by the recruitment of additional granules from the reserve pool [30].

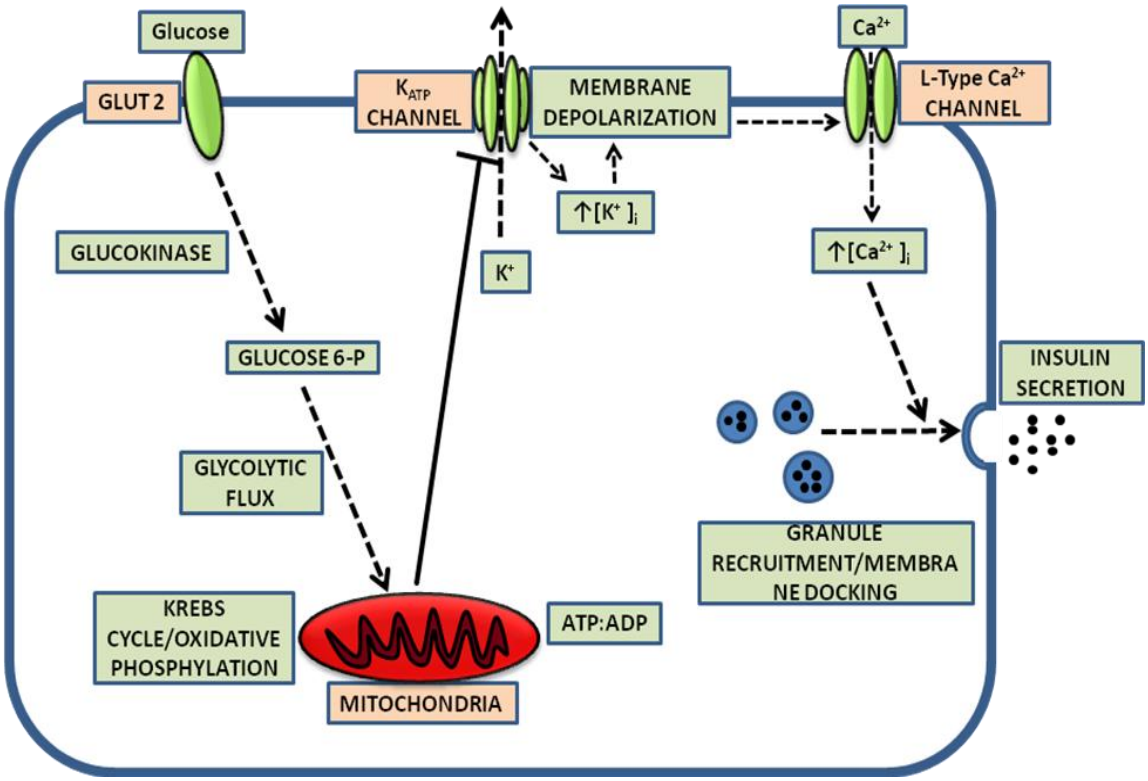


Figure 1. Glucose stimulated insulin secretion general pathway.
Glucose is transported into beta cells and metabolized, increasing intracellular ATP levels. K_{ATP} channels close, depolarizing the cellular membrane, opening voltage-gated L-type Ca^{2+} channels. A surge of intracellular calcium ions activates the mechanism for secretory granule movement and exocytosis.

The stimulatory amino acids, free fatty acids, and ketoacids enter the beta cells and contribute to the production of ATP to synergistically increase insulin secretion along with glucose. There are exceptions however, including arginine, which potentiates insulin release due to the cumulative effect its positive charge has on inducing membrane depolarization [31]. A secondary increase in cAMP levels following glucose exposure enhances insulin secretion through a cAMP-dependent protein kinase, known as Protein

Kinase A. PKA functions by phosphorylating proteins that may participate in exocytosis or granule recruitment [32]. Stimulatory G proteins facilitate the insulin-secretory effects of peptides such as glucagon and glucagon-like peptide-1 (GLP-1). GLP-1, an incretin hormone, enhances insulin release via activation of adenylyl cyclase, cAMP generation, PKA activation, and an increase in intracellular Ca^{2+} levels [33]. A stimulatory G protein associated with phospholipase C (PLC) mediates the capability of acetylcholine to enhance insulin release. This G protein produces the hydrolysis of phosphatidylinositol bisphosphate to inositol triphosphate (IP_3) and diacylglycerol (DAG). Through the interaction with receptors on the endoplasmic reticulum, IP_3 promotes the release of intracellular Ca^{2+} stores into the cytosol. The generation of DAG induces Protein Kinase C activation, which has a potentiatory effect on the secretory response aroused by glucose [31].

3. Diabetes Mellitus

3.1 General

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia due to an absolute or partial lack of insulin and/or insulin insensitivity at peripheral tissues [34]. DM was once thought to be a disease of minor significance, however in the past two decades there has been a dramatic increase in the number of people diagnosed worldwide. Diabetes is currently recognized as one of the greatest threats to human health in the 21st Century [3]. In 2006 roughly 177 million people were estimated to have diabetes worldwide, a number that was projected to increase to at least

300 million by the year 2025 [35]. Today, it has been estimated that the number of individuals with diabetes worldwide in 2008 was a staggering 347 million [36]. Individuals with diabetes have a much greater risk of developing several acute complications, including diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy. These complications can have a devastating impact on quality of life and generate significant costs to health care systems [35].

3.2 Type 1 and Type 2 Diabetes

There are two main types of diabetes, known as type 1 diabetes and type 2 diabetes. Type 1 diabetes is primarily caused by the autoimmune-mediated destruction of pancreatic beta cells. There is a known predisposition to the onset of Type-1 diabetes in individuals with specific highly polymorphic immune-system-recognition molecules or human leukocyte antigen (HLA) genotypes. HLA class I and class II molecules present antigenic peptides from the cellular surface to CD8 and CD4 T lymphocytes, respectively. The autoimmune cellular destruction may result in complete insulin deficiency, leaving patients dependent on exogenous insulin to manage glucose levels and to prevent ketoacidosis [37]. Type 2 diabetes arises through decreased insulin secretion by the beta cells and/or insulin resistance at the target site, such as skeletal muscle and liver and adipose tissue [34]. Beta cell dysfunction can involve a variety of issues, including disruption in the pattern of pulsatile and oscillatory release of insulin under basal conditions [38], inadequate ability of glucose to stimulate and alter insulin secretion [39], and loss of proinsulin to insulin conversion efficiency [40]. The frequency of type 2 diabetes is significantly higher than type 1, accounting for over 90% of cases on

a global scale. The factors widely accepted as the main contributors to the onset of diabetes are genetic susceptibility and environmental and behavioural factors including sedentary lifestyle, nutrition (high sugar and fat intake), and obesity [3].

The common result of both types of diabetes is hyperglycemia. Hyperglycemia has been shown to be associated with an increased production of reactive oxygen species (ROS). ROS are produced, mainly in the mitochondria, from either the reduction of molecular oxygen or the oxidation of molecular water [41]. The amounts of ROS generated can be amplified in circumstances where caloric intake exceeds energy expenditure, resulting in greater levels of NADH production and ultimately also ROS [42]. In high levels, ROS facilitate the development of insulin resistance, β-cell dysfunction, and mitochondrial dysfunction through damaging lipids, membranes, proteins, and DNA [41]. Beta cells are known to exhibit significantly lower levels of antioxidant enzyme gene expression, where the amounts of superoxide dismutase, catalase, and glutathione peroxidase expression in these cells are 5-30% of that observed in liver cells [43]. Beta cells are much more susceptible to the damage caused by ROS than other cell types.

3.3 MODY

Maturity-onset diabetes of the young (MODY) is a monogenic type of diabetes mellitus, characterized by an early age of onset and an autosomal dominant mode of inheritance. Thus far, six genes have been shown to be responsible for MODY, including the hepatocyte nuclear factor (HNF)-4 alpha gene (MODY1), glucokinase (GCK) gene

(MODY2), HNF-1 alpha gene (MODY3), insulin promoter factor-1 (IPF-1) gene (MODY4), HNF-1 beta gene (MODY5), and neurogenic differentiation factor 1 (NEUROD1) gene (MODY6). These genes are known to be critical for proper beta cell functionality and are involved in insulin gene transcription, glucose metabolism, and pancreatic development. The two most prevalent forms of MODY are MODY2 and MODY3 [44]. In a clinical study with 90 Caucasian families that were diagnosed with MODY, 63% were found to have mutations in the HNF-1 alpha gene and 20% had mutations in the GCK gene [45].

Glucokinase functions as a glucose sensor and mutations of the *GCK* gene can cause the impairment of beta cells' sensitivity to glucose. The three hepatocyte nuclear factors, HNF-4 alpha, HNF 1 alpha, and HNF-1 beta, function together to regulate insulin gene transcription as well as the expression of genes whose transcripts are involved in glucose transport and metabolism. The transcription factor IPF-1 also mediates these effects, while even possessing a role in pancreatic development. In general, patients living with mutations of transcription factors display more severe hyperglycemia than those with MODY2, due to compensatory mechanisms that increase insulin release. It has been noted that sequence variations of these six genes are responsible for 75-80% of MODY cases in Caucasians, and as little as 20-40% in Chinese, Japanese, and Korean families [46]. Therefore, a large percentage of patients diagnosed with MODY may possess one or a number of mutations of unknown genes.

4. Transglutaminases

4.1. General

The mammalian transglutaminases are a family of enzymes characterized by their ability to covalently modify proteins by cross-linking them through ε(γ-glutamyl)lysine bonds. They catalyze, in a Ca^{2+}-dependent manner, the transamidation of glutamine residues to lysine residues, which forms proteolytically resistant isopeptide bonds [47]. These bonds can form between amino acids of the same peptide or those of two separate peptides to form either intrachain or interchain isopeptide bonds respectively (Figure 2). Low molecular weight amines, such as serotonin (5-HT), may substitute for lysine in transamidation reactions. Alternatively, in the absence of suitable amines, water is able to participate as an acyl-acceptor substrate, which results in the deamination of protein-bound glutamine residues. To date, eight transglutaminase enzymes have been found in mammals; though only six have been characterized to a significant degree. These six transglutaminases include plasma Factor XIIIa, keratinocyte TG (type 1), tissue TG (type 2), epidermal TG (type 3), prostate TG (type 4), and TG5 [48].

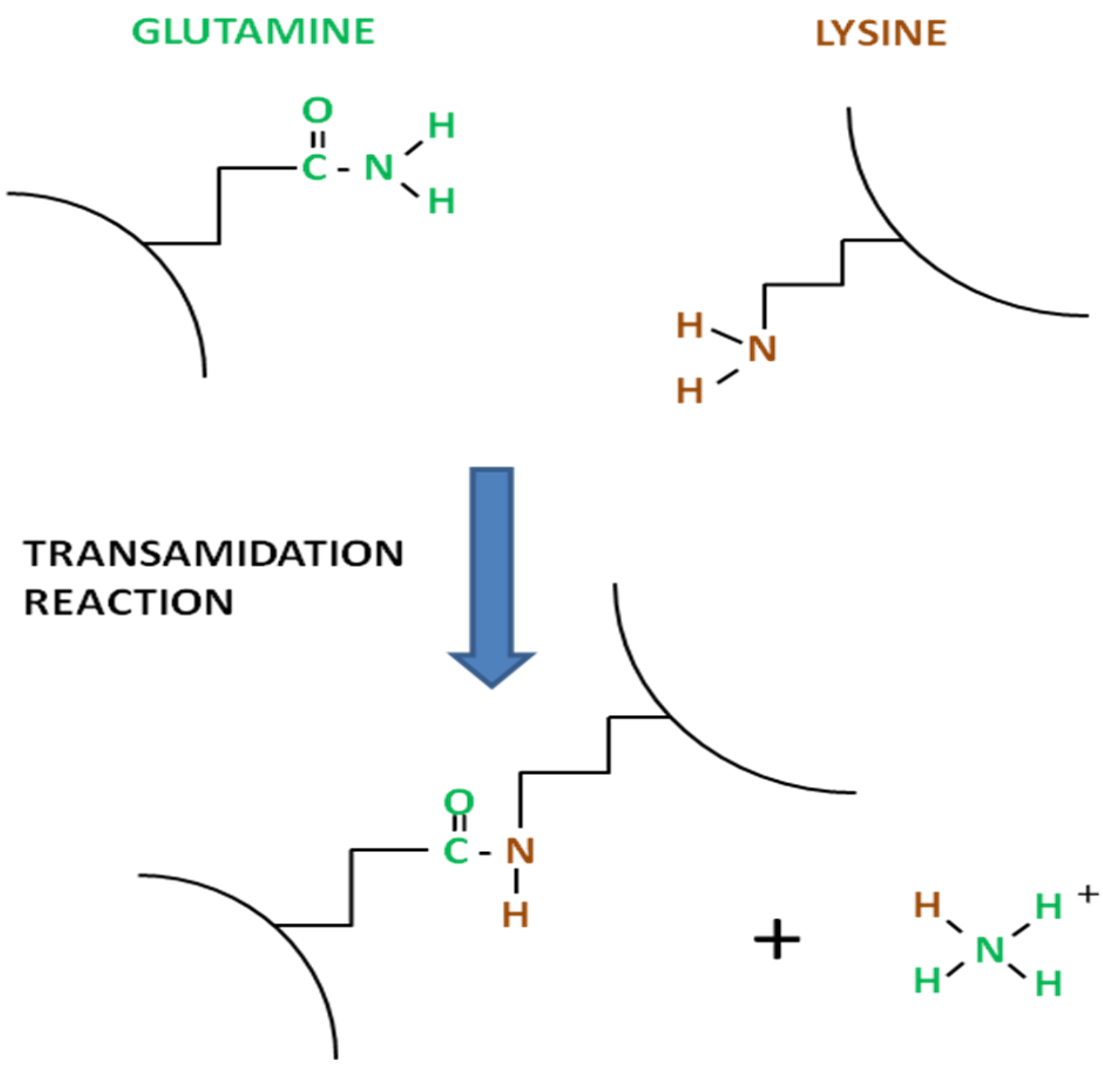


Figure 2. Diagram of transamidation reaction mechanism.
Transglutaminases crosslink glutamine and lysine residues through a ε(γ-glutamyl)lysine bond. This reaction is Ca^{2+}-dependent and the Lysine residue can be substituted by low molecular weight amines, such as serotonin.

The enzymatic activity of each transglutaminase member is differentially regulated. While TG2, TG3, TG4, and TG5 are inhibited by GTP, TG1 and factor XIIIa are not. An additional difference lies in the fact that TG3 requires the cleavage at a loop between its catalytic core and C-terminal beta-barrels in order for it to become

catalytically active [49], whereas for TG2 and factor XIIIa this same cleavage leads to their inactivation [50]. Regardless of the fact that the regulation and substrate specificity vary noticeably for each transglutaminase type, all of the catalytically active enzymes share a significant degree of sequence similarity, including strict conservation of key active-site residues such as the catalytic triad. Also, in order to allow access to protein substrates for crosslinking, each transglutaminase family member must undergo a major conformational change to expose the active-site residues [51]

4.2 Transglutaminase 2

4.2.1 TG2, A Multifunctional Enzyme

Transglutaminase 2 (TG2) is a multifunctional, ubiquitous member of a Ca^{2+}-dependent protein cross-linking family of enzymes. Aside from its well-characterized transamidation activity [52] TG2 can also bind and hydrolyze GTP [17], as well as function as a protein-disulfide isomerase [53], and a serine and threonine kinase [15]. The GTPase activity has been linked to the ability of TG2 to act as a G protein involved in signaling from $alpha_{1B/D}$ adrenergic receptors to downstream effectors including phospholipase Cδ1 [17]. TG2 has also been shown to have a high affinity binding site for fibronectin [47]. The transamidation and kinase activities of TG2 are inversely regulated by Ca^{2+} and ATP. While bound to Ca^{2+}, TG2 is present in an open conformation and is primarily functioning though its cross-linking activity [54], however in the presence of lower Ca^{2+} and higher ATP concentrations the kinase activity of TG2 is enhanced [15] (Figure 3).

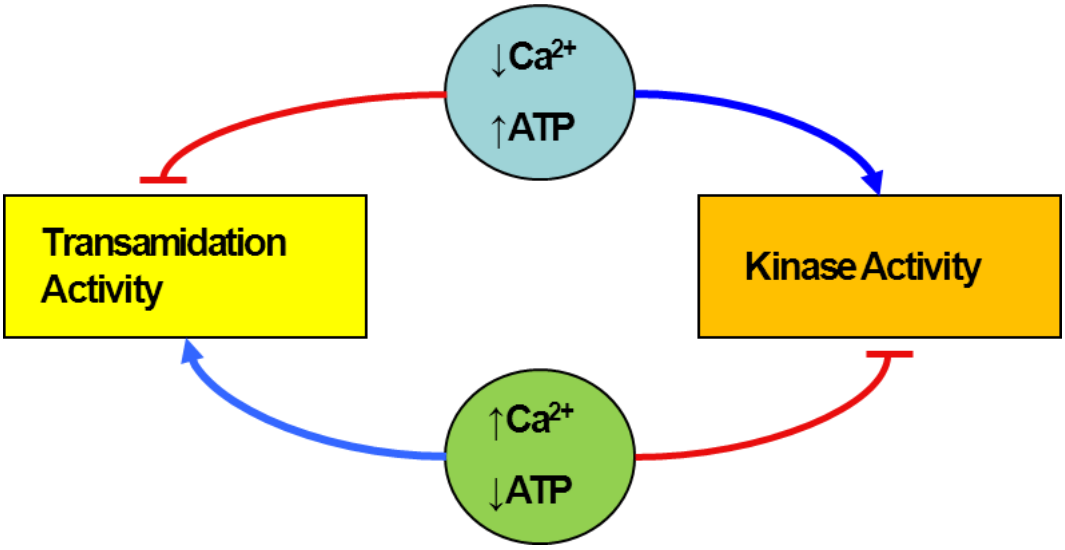


Figure 3. Reciprocal regulation of TG2's activities by ATP and Ca^{2+} levels.
ATP facilitates kinase activity and has an inhibitory effect on transamidation activity, while Ca^{2+} has an opposite effect.

4.2.2 Structure and Function

TG2 has a molecular weight of 78 kDa and a length of 687 amino acids. The protein possesses four domains, including an N-terminal β-sandwich, the core domain, and two C-terminal β-barrels. The active site for the transamidation activity is found in the core domain and is made up of a cysteine-histidine-aspartic acid catalytic triad (Cys^{277}-His^{335}-Asp^{358}) [51]. TG2 is located significantly in both the intracellular, specifically in the nucleus, cytosol, and cellular membrane, and the extracellular environment of numerous tissues. Therefore, it is acceptable to assume that its catalytic functions are tightly regulated to ensure cellular and tissue components aren't excessively modified. While TG2 is found in the extracellular matrix, it does not possess a signal sequence to direct it to the Golgi apparatus/endoplasmic reticulum and has never been observed within either structure. It is thought that TG2 is secreted through a non-

characterized non-classical secretory mechanism, most likely mediated by fibronectin binding via its N-terminal β-sandwich [47, 55].

The *in vitro* transamidating activity of TG2 requires millimolar concentrations of calcium ions, while GTP and GDP operate as allosteric inhibitors through the induction of a closed conformation state whereby the active site is buried [17]. Intracellular environments have calcium concentrations many times less than what has been shown to be required for TG2's activity *in vitro*. For example the intracellular concentration of calcium ions of β-cells in islets can be found in the range of 100nM to 300nM, under basal and stimulated conditions respectively [56]. Due to this, it is believed that intracellular TG2 is without enzymatic activity and that it functions in the cell through G-protein signalling [51]. However, it may also be possible that in the cellular environment TG2's transamidation activity, along with its other activities, is regulated by alternative means other than GTP/ATP and Ca^{2+} binding, including unknown post-translational modification.

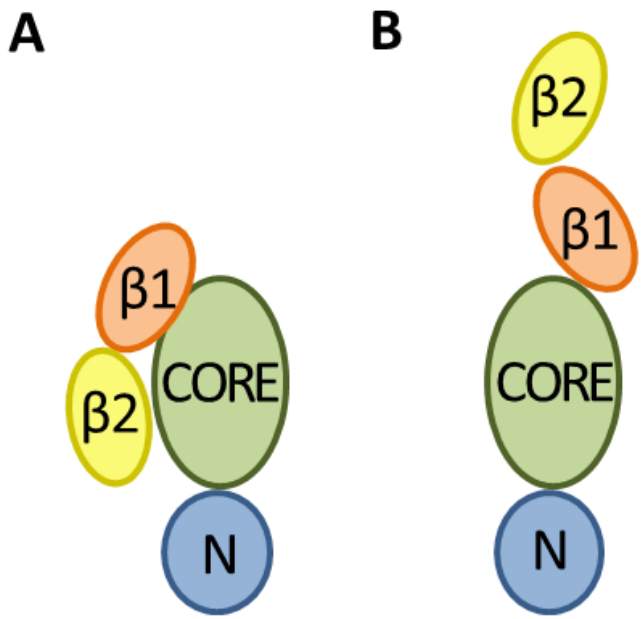


Figure 4. Simple illustration conformational change by TG2.
A. GDP-bound structure of TG2, closed conformation. B. Calcium ion-bound TG2, open conformation. The N-terminal beta sandwich is displayed in blue (N), the catalytic domain in green (CORE), the C-terminal β1 barrel in orange, and the C-terminal β2 barrel in yellow.

A recent study has proposed five sites that function as domains for calcium ion binding in TG2. The proposed amino acids involved in these sites are aspartic acid, glutamic acid, and asparagine. These amino acids are conserved amongst the tranglutaminase family and due to their polar nature, which increases their surface potential, and the negative charge of the acidic amino acids, they are deemed essential for Ca^{2+} binding. The amino acids of interest in each proposed calcium ion binding site is shown is Table 1. Site 1 binds tightly to Ca^{2+}, and while shown to have a significantly negative impact on TG2's transamidation activity when mutated, it is not the sole site required for the initiation of this activity. It was found that the mutation of either site 3 through 5 results in a reduction in overall calcium ion binding and a complete loss of

transamidating activity. The mutation of site 2 resulted in the least loss of activity, though the loss was still notable. From this study it is acceptable to infer that not one, but many sites upon TG2 are crucial for calcium's initiation of its transamidating activity and that it is highly likely that a sequential mechanism of site occupancy may be involved [57]. It has been observed that the Ca^{2+} affinities of C_2-domains, a type of protein Ca^{2+}-binding domain, are greater when the domains are in the presence of its substrate than when the substrate is absent [58]. It may be reasonable to postulate that TG2's affinity for Ca^{2+} may differ depending on which of its substrates is present or whether it is in contact with any interacting partners.

Table 1. Putative transglutaminase 2 Ca^{2+}-binding sites.

Proposed Sites Involved in Ca^{2+} Binding	Site 1	Site 2	Site 3	Site 4	Site 5
Amino Acids of Interest	229N 231N 232D 233D	396E 398N 400D 448E 452E 453E 455E	306D 308N 310N 329N	152D 154E 155E 156E 159E	434D 435E 437E 438D

The numbering of the sites is based upon the order of discovery. Sites 1 through 3 were previously identified on analogous regions in TG3 by Ahvazi et al in 2002 [59]. While Site 3 was a novel finding by Ahvazi el al., Site 1 and Site 2 were already shown to have calcium binding potential in analogous regions in factor XIII by Weiss et al in 1998. and Fox et al in 1999., respectively [60, 61]. Sites 4 and 5, novel sites identified by Kiraly et al. were chosen due to a significantly higher density of negatively charged amino acids. [57] Note: N, asparagine; D, aspartic acid; E, glutamic acid. Table adapted from Kiraly et al. Figure 2, permission to use copyrighted material was given on June 28, 2011 by John Wiley and Sons, provided by Copyright Clearance Center. [57]

With regard to kinase activity, TG2 was initially shown to be an insulin-like growth factor-binding protein-3 (IGF-BP3) kinase in breast cancer cells, phosphorylating both serine and threonine residues [15]. Prior to this discovery, it had already been noted that TG2 possessed a GTP binding domain and was able to hydrolyze both GTP and ATP [62]. Subsequently it was discovered that TG2 was able to phosphorylate the core histones H2A, H2B, H3, H4, as well as histone H1 *in vitro*. TG2 was also shown to phosphorylate H3 and H1 *in situ* [63]. The kinase activity of TG2 also extends to its ability to phosphorylate Rb. PKA was implicated as being able to phosphorylate TG2, particularly at Ser-216, which was shown to modulate TG2's activity, as well as alter protein binding with 14-3-3 protein [64]. The phosphorylation of TG2 by PKA reduces its transamidation activity, while enhancing its kinase activity [65]. While the currently known substrates for TG2 kinase activity implicates TG2 further in modulating gene expression and cell cycle regulation, it is possible that TG2 may also have substrates that are directly involved in insulin secretion from beta cells since it can also be localized in the cytosol and cellular membrane in addition to the nucleus.

GTP primarily binds to residues of the first and last strands (amino acids 476-482 and 580-583) of β-barrel 1 and to the two core domain residues, Lys-173 and Phe-174, which extend beyond a loop to meet β-barrel 1 [19, 66, 67]. It is believed that when GTP binds to these residues, it stabilizes two β-barrel 1 loops, which blocks substrate access to the catalytic site. One of these β-barrel 1 loops extends into the core domain, positioning Tyr-516 within hydrogen bonding distance of Cys-277, theoretically effectively inhibiting the interaction between Cys-277 with its substrate [54]. ATP, like GTP, also

involves the interaction between residues Arg-476 and Arg-478, which form ion pairs with the γ-phosphate of ATP, as well as Gly-480 and Val-479, which form hydrogen bonds with the β-phosphate. Tyr-583, another shared bonding residue for ATP and GTP, forms a hydrogen bond with the nitrogen of the adenine ring. However, the overall interaction between TG2 and ATP is significantly weaker than with GTP, as the TG2 residues Ser-482 and Arg -580 only participate in GTP binding. As a result, ATP binding confers noticeably less stability to TG2 than GTP binding, which not only inhibits the transamidation activity of TG2, but also decreases its susceptibility to protease digestion [68].

Interestingly, the five Ca^{2+} -binding sites discussed earlier (Table 1) that have been shown to affect the initiation of TG2's transamidation activity, seem to also have a regulatory role over TG2's GTPase activity and GTP/GDP binding. When either site 4 or 5 are unable to bind Ca^{2+} due to mutations, TG2's GTPase activity is no longer impacted negatively by increasing Ca^{2+} concentrations. In addition, mutations to either of these sites result in altered GTP/GDP binding and enhanced basal GTPase activity. Sites 4 and 5 in TG2's tertiary structure are positioned sterically close to the hydrophobic pocket for GTP/GDP binding [57, 66].

The disulfide bonds formed between the side chains of adjacent cysteine residues are believed to function structurally, by stabilizing the local backbone conformation, and facilitate a redox role by mediating electron exchanges [69]. A redox sensitive cysteine triad consisting of Cys230, Cys370, and Cys371 has been identified in TG2. TG2 is

known to be sensitive to oxidative conditions to the point of being functionally inactivated in the presence of oxidizing agents. It is also known that the addition of a reducing agent, such as DTT, can restore TG2's activity. This loss of activity under oxidative conditions may be due to the oxidation of cysteine residues, resulting in the formation of disulfide bonds. Within the redox sensitive cysteine triad, Cys370 is involved in two disulfide bonds, either with Cys371 or Cys230. It is proposed that the Cys230-Cys370 bond forms under less oxidizing conditions than the Cys370-371 bond, and that Cys230, which is unique to TG2, functions as a redox sensor that initiates oxidation and the inactivation of TG2 [51, 70]. TG2's activity appears to be modulated by the presence of oxidizing and reducing agents, suggesting that the metabolic state of its surroundings may direct its functionality as well as conformation.

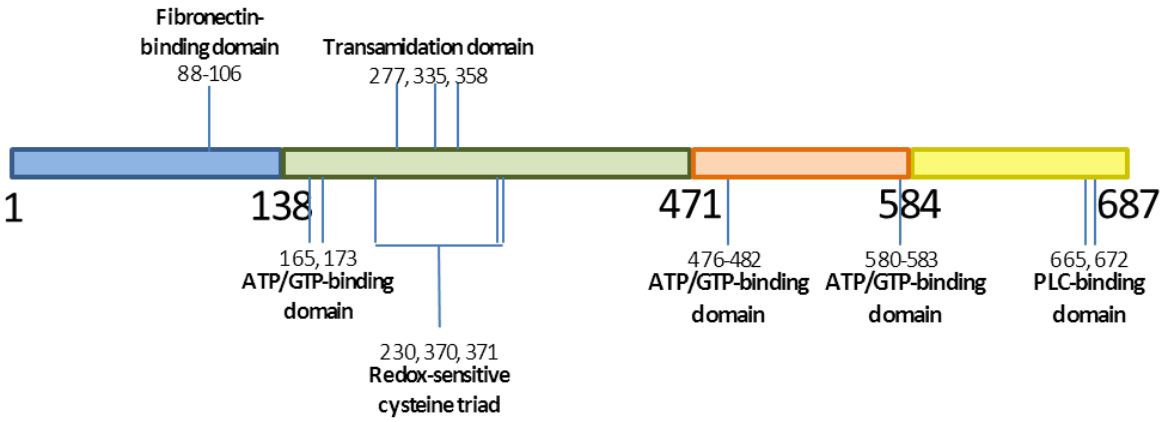


Figure 5. Diagrammatic representation of TG2 and its functional domains.
The N-terminal beta sandwich is displayed in blue (N), the catalytic domain in green (CORE), the C-terminal β1 barrel in orange, and the C-terminal β2 barrel in yellow. Although the transamidation catalytic and putative GTP/ATP binding loops are located distantly from each other in the primary structure of TG2, they are very close to each other in the secondary structure as revealed by the crystal structure of TG2. Adapted from Antonyak et al. [71], figure 1a. © 2006 by The National Academy of Sciences of the USA.

4.2.3 TG2 and GSIS, Growing Insight into Their Relationship

Since several decades ago, transglutaminases have been implicated in possessing a role in GSIS from pancreatic beta cells. This role was suggested since the transamidation activity of transglutaminases is sensitive to the environmental Ca^{2+} concentration and that agents documented as transglutaminase inhibitors, such as monodansylcadaverine, were shown to abrogate glucose-stimulated insulin secretion from isolated rat pancreatic islets in a dose-dependent manner [72, 73]. Much more recently, a study observed that TG2 was the transglutaminase with the greatest and most significant presence within pancreatic islets and that in the absence of TG2, insulin secretion was significantly reduced compared to the control. During a glucose tolerance test, TG2 knockout mice were shown to have almost half the level of insulin in the plasma as wild-type mice. Similarly, during an acute insulin release test by glucose stimulation, TG2 knockout mice displayed a 42% reduction in insulin secretion in comparison to wild-type [11].

As discussed previously, depending on the genetic background, 25-80% of individuals who are diagnosed with MODY do not possess a mutation in one of the six well characterized MODY genes [46]. Three heterozygous missense mutations in the TG2 gene that leads to the generation of TG2 protein with Met330Arg, Ile331Asn, or Asn333Ser amino acid substitutions respectively, were discovered in patients previously diagnosed with MODY [11, 12]. These mutations were deemed significant due to their location within the catalytic domain of TG2 and proximity to members of the catalytic

triad. It was indeed shown that each of the naturally occurring mutations in TG2 resulted in a loss of transamidation activity *in vitro* [12]. However, the impact of these mutations on the other functional activities of TG2, including its kinase activity, ATP/GTP binding, and ATP/GTP hydrolysis was not assessed. The three naturally occurring mutation sites in TG2 used for this study are not only located amongst the transamidation catalytic triad, but are also found in close proximity to the putative GTP/ATP binding loops in TG2's tertiary structure [54] (Figure 6).

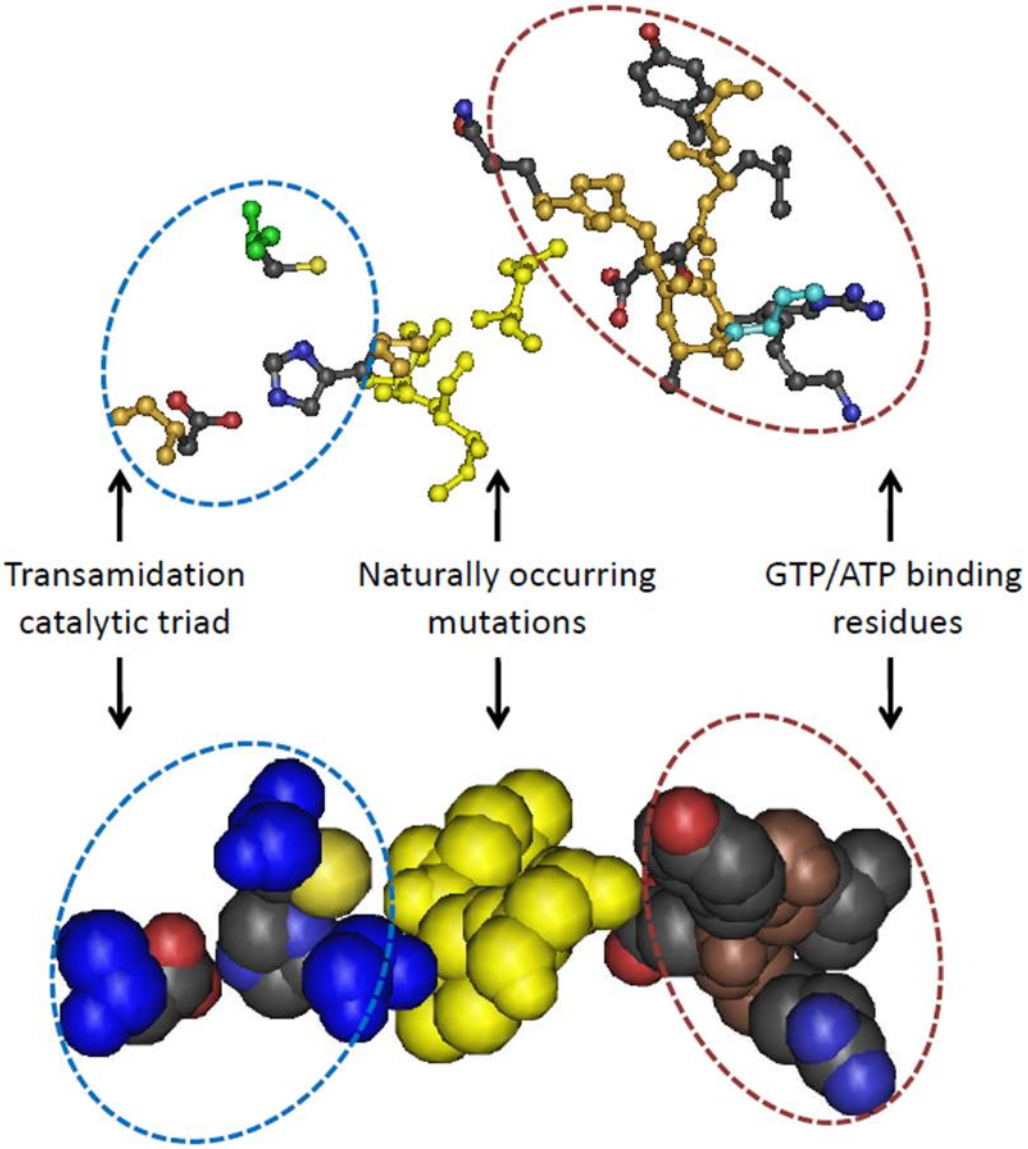


Figure 6. Naturally occurring mutations in TG2 are flanked between transamidation catalytic triad and GTP binding residues.
Relative position of transamidation catalytic triad (Cys^{277}, His^{335}, and Asp^{358}), naturally occurring mutation sites (Met^{330}, Ile^{331}, and Asn^{333}) and GTP/ATP binding loops (^{169}Gln-Gly-Ser-Ala-Lys^{173} and ^{580}Arg-Asp-Leu-Tyr^{583}) in TG2 are shown in ball and stick model (upper panel) and in a space filled model (lower panel).

Serotonin (5-HT), a monoamine with a molecular weight of 176Da, is commonly known as a neurotransmitter in the central nervous system (CNS), which regulates mood and cognition (Villalon 2007). Serotonin has also been implicated as a hormone with numerous functions outside of the central nervous system, acting as a strong mitogen and influencing the growth of several tissues, including the liver [74] and the mammary gland [75]. Platelets (thrombocytes) transport serotonin in the blood and secrete it in response to tissue injury where it promotes vasoconstriction. Until quite recently, the biological functions of serotonin have been solely attributed to its action on one of the many 5-HT receptors ($5\text{-}HT_1$-$5\text{-}HT_7$) located in the CNS and peripheral tissues [76]. A study found that transglutaminases were able to covalently link serotonin to small GTPases through its transamidation activity, leaving the GTPases constitutively active [77]. These small GTPases, RhoA and Rab4, once active inside the platelets, interact with downstream effectors, generating cytoskeletal rearrangements [78] and the exocytosis of α-granules [79], respectively. This finding suggested that serotonin could act through a receptor-independent intracellular signaling pathway mediated by transglutaminases.

For several decades it has been evident that serotonin is present in the beta cells of pancreatic islets. In addition, the beta cells are able to synthesize serotonin and store it in the same secretory β-granules as insulin [80, 81]. A more recent study determined that

tryptophan hydroxylase 1 knockout mice (*Tph1* -/-), which are specifically deficient in peripheral 5-HT only, displayed higher blood glucose levels and significantly lower serum insulin levels than wild-type mice. Tph1 is the rate-limiting enzyme in the biosynthesis of 5-HT from tryptophan.

It has also been recently observed that serotonin in beta cells functions through a similar mechanism as in platelets, where through the action of transglutaminase 2, it is covalently bound to small GTPases directly involved in vesicle recruitment and transport. Serotonin is covalently linked to a glutamine residue, substituting for lysine in the transamidation reaction [14]. The small GTPases implicated were Rab3a and Rab27a, regulating replenishment of the ready releasable pool of β-granules and functioning in the targeting of β-granules from the resting pool to the ready releasable pool, respectively (Figure 7). Rab3a is able to form a complex with calmodulin, limiting the release of insulin from secretory vesicles. The formation of this complex was found to be favoured when Rab3a was constitutively active and bound to GTP [82]. Active Rab27a facilitates the glucose-promoted replenishment of a pool of membrane-docked vesicles, possibly through the interaction with the principal Rab27a effector in beta cells, granuphilin, which binds directly to the membrane-anchored SNARE (Soluble NSF Attachment Protein Receptor) , syntaxin 1a [83].

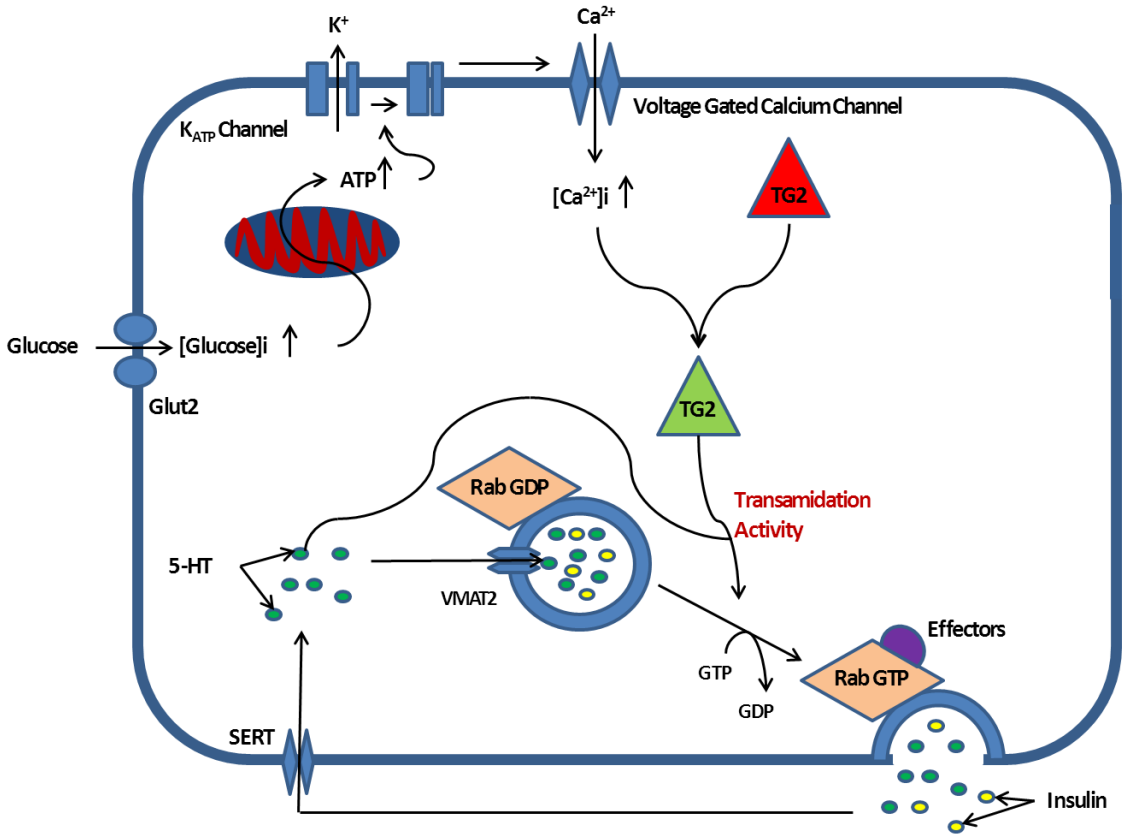


Figure 7. Previously proposed model of TG2's modulation of insulin secretion in beta cells.
Glucose enters beta cells through Glut2 transporter, is metabolized through the glycolytic pathway, TCA cycle, and the electron transport chain to produce ATP. ATP closes ATP-sensitive K^+ channels, which depolarizes the membrane, opening voltage-gated Ca^{2+} channels. The increase in intracellular calcium ions promotes insulin release from secretory vesicles. TG2's transamidation activity is stimulated by calcium ion binding. VMAT2, vesicular monoamine transporter 2. SERT, serotonin transporter.

II. HYPOTHESIS AND OBJECTIVES

Hypothesis

Transglutaminase has been shown to have an important role in GSIS in pancreatic beta cells. To date, the influence TG2 has upon insulin secretion has been attributed to its transamidation function. Naturally occurring mutations of TG2 implicated in MODY have been previously shown to have reduced TGase activity. In addition, inhibition of TG2's TGase activity through nonspecific inhibitors has been observed to abrogate insulin release from beta cells. The impact of these mutations on TG2's other functional activities on GSIS, including its kinase activity and GTP-binding ability, have not been investigated. While the naturally occurring mutations of TG2 are located within the transamidation domain, they are also found in close proximity to the ATP/GTP-binding sites in TG2's tertiary structure. We hypothesize that these mutations will affect TG2's other functional activities, in addition to its TGase activity, and the impact of these mutations on each of the functional activities of TG2 is collectively affecting GSIS.

Objectives

This study will seek to determine which functional activities of TG2 are involved in GSIS from pancreatic beta cells. To aptly do so, the following will be determined:

(a) the localization of naturally occurring mutants within beta cells

(b) the effect of the naturally occurring mutations on TG2's transamidation, kinase, and ATP/GTP-binding ability.

(c) the effect of the overexpression of the naturally occurring mutations on GSIS in beta cells

(d) the connection between protein serotonylation and insulin secretion by beta cells

III. MATERIALS AND METHODS

A. Materials

Chemicals used in this thesis were purchased from the companies listed in Table 1.

Table 2: Materials used in Study

Product Name	Company Purchased From
(Monoclonal)-anti-Myc tag, rabbit	Cell Signalling (Danvers, MA, USA)
(Monoclonal)-anti-serotonin, rat	Pierce (Rockford, IL, USA)
(Monoclonal)-anti-serotonin, rat	Santa Cruz (Santa Cruz, CA, USA)
(Polyclonal)-anti-goat IgG-HRP, donkey	Santa Cruz (Santa Cruz, CA, USA)
(Polyclonal)-anti-transglutaminase type II, goat	Sigma-Aldrich (Mississauga, CA, USA)
Anti-rabbit HRP-linked	Cell Signalling (Danvers, MA, USA)
Acetonitrile (ACN)	Fisher Scientific (Ottawa, CA)
Acrylamide, 99%	Sigma-Aldrich (Mississauga, CA, USA)
Albumin, Bovine	Sigma-Aldrich (Mississauga, CA, USA)
Alexa Fluor 488 anti-rabbit, goat	Invitrogen (Burlington, CA)
Ammonium Bicarbonate	Sigma-Aldrich (Mississauga, CA, USA)
Ammonium Persulfate	Sigma-Aldrich (Mississauga, CA, USA)
Anhydrous Ethanol	Commercial Alcohols (Brampton, ON, CA)
Bio-Safe Coomassie	Bio-Rad (Hercules, CA, USA)
5-(Biotinamido)Pentylamine	Sigma-Aldrich (Mississauga, CA, USA)
Calcium Chloride	Fisher Scientific (Ottawa, CA)
CL-Xposure Film	Thermo Scientific (Rockford, IL, USA)
Collagenase	Sigma-Aldrich (Mississauga, CA, USA)
Crosslink IP Kit	Pierce (Rockford, IL, USA)

CultureSlides	BD BioSciences
Dimethyl Sulfoxide (DMSO)	Fisher Scientific (Ottawa, CA)
Dithiothreitol (DTT)	Sigma-Aldrich (Mississauga, CA, USA)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich (Mississauga, CA, USA)
Enhanced chemiluminescence kit	Amersham (Manassas, NJ, USA)
Enhanced chemiluminescence kit	Promega (Sunnyvale, CA, USA)
Fetal Bovine Serum (FBS)	Invitrogen (Burlington, CA)
Formalin	Fisher (Kalamazoo, MI, USA)
FuGENE HD transfection system	Roche (Penzburg, Germany)
Glucose	Sigma-Aldrich (Mississauga, CA, USA)
Glycerol	Fisher Scientific (Ottawa, CA)
Glycine	Fisher Scientific (Ottawa, CA)
Hanks' Balanced Salts	Sigma-Aldrich (Mississauga, CA, USA)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich (Mississauga, CA, USA)
Histone 4, Human Recombinant	Upstate (Lake Placid, NY, USA)
Histopaque 1077	Sigma-Aldrich (Mississauga, CA, USA)
Histopaque 1119	Sigma-Aldrich (Mississauga, CA, USA)
Hydrochloric Acid (HCl)	Fisher Scientific (Ottawa, CA)
Insulin Growth Factor-Binding Protein 3 (IGF-BP3)	Mediagnost (Germany)
Insulinoma-1E cell line (INS-1E), rat	Provided by Universities of Toronto and Geneva
Iodoacetamide	Sigma-Aldrich (Mississauga, CA, USA)
Kodak MR Biomax Film	Sigma-Aldrich (Mississauga, CA, USA)
Magnesium Chloride	Fisher Scientific (Ottawa, CA)
Mammalian Cell Lysis Kit	Sigma-Aldrich (Mississauga, CA, USA)

2-Mercaptoethanol	Sigma-Aldrich (Mississauga, CA, USA)
Mouse insulinoma-6 cell line (MIN6)	Provided by University of Manitoba
Non-fat dry milk	Bio-Rad (Hercules, CA, USA)
OPTI-MEMI reduced serum medium (1X), liquid	Invitrogen (Burlington, CA)
pCMV6 Entry (Myc/Flag tagged) vector	Origene Technologies (USA)
Penicillin/Streptomycin	Invitrogen (Burlington, CA)
Phosphate Buffered Saline (PBS)	ATCC (Burlington, ON, CA)
Potassium Chloride	Fisher Scientific (Ottawa, CA)
Potassium Phosphate	Fisher Scientific (Ottawa, CA)
Profound c-Myc IP/CO-IP kit	Pierce (Rockford, IL, USA)
Quick Start Bradford Dye Reagent, 1X	Bio-Rad (Hercules, CA, USA)
Recombinant human TGM2	R&D Systems (Minneapolis, MN, USA)
Retinoblastoma (amino acids 773-928)	Upstate (Temecula, CA, USA)
RPMI 1640 medium without glucose	Invitrogen (Burlington, CA)
Site Directed Mutagenesis Kit	Stratagene (CA, USA) (CA, USA)
Sodium Bicarbonate	Sigma-Aldrich (Mississauga, CA, USA)
Sodium Chloride	Fisher Scientific (Ottawa, CA)
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich (Mississauga, CA, USA)
Sodium Hydroxide (NaOH)	Fisher Scientific (Ottawa, CA)
Sodium Pyruvate	Sigma-Aldrich (Mississauga, CA, USA)
Trifluroacetic Acid (TFA)	Fisher Scientific (Ottawa, CA)
Tris(hydroxymethyl)aminomethane (Tris)	Fisher Scientific (Ottawa, CA)
Trypsin-EDTA	Invitrogen (Burlington, CA)
Trypsin, Sequence Grade	Promega (Madison, WI, USA)

TWEEN-20	Sigma-Aldrich (Mississauga, CA, USA)
Ultra sensitive mouse insulin ELISA kit	Crystal Chem Inc. (Downers Grove, IL, USA)
Ultra sensitive rat insulin ELISA kit	Crystal Chem Inc. (Downers Grove, IL, USA)

B. Methods

1. Cell Culture

The rat insulinoma cell line, INS-1E, was a generous gift from the University of Toronto and mouse MIN6 cells were provided by Dr. Eisenstat, University of Manitoba. INS-1E cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, $3.4x10^{-4}$% Beta-mercaptoethanol, and 100 µg/mL penicillin-streptomycin. The pH of the media was equilibrated with 1N sodium hydroxide solution (NaOH). MIN6 cells were cultured in DMEM containing 10% FBS and 100 µg/mL penicillin-streptomycin. Cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C.

2. Overexpression of Wild-type TG2 and TG2 Mutants

The pCMV6 Entry (c-Myc/FLAG tagged) vector containing the TG2 gene was obtained from Origene Technologies, USA. The c-Myc-tag and FLAG-tag are both short amino-acid peptides with a length of 11 and 8 residues, respectively. C-Myc-tagged and FLAG-tagged proteins have been used extensively as a detection system and a means of affinity purification, due to the availability of antibodies that bind to their epitope [84]. The Cysteine 277 to Alanine (Cys277Ala) mutant-TG2, Methionine 330 to Arginine (Met330Arg) mutant-TG2, Isoleucine 331 to Asparagine (Ile331Asn) mutant-TG2, and the Asparagine 333 to Serine (Asn333Ser) mutant-TG2 were made using a site-directed mutagenesis kit (Stratagene, USA) following the manufacturer's instructions. The primers used to generate each mutant TG2 are listed in Table 3. Authenticity of all the constructs was confirmed by DNA sequencing. Transfections with TG2 wild-type, (Cys277Ala)

mutant-TG2, (Met330Arg) mutant-TG2, (Ile331Asn) mutant-TG2, (Asn333Ser) mutant-TG2, and empty pCMV6 Entry (Myc/Flag tagged) vectors were performed using FuGene HD transfection system. A ratio of two micrograms of plasmid DNA to three microlitres of FuGene HD transfection reagent was mixed in OPTI-MEMI reduced serum medium. The mixture was vortexed and incubated at RT for twenty min. The mixture was then allocated evenly and directly into the medium of the INS-1E cells, followed by gentle shaking of the dish. The cells were incubated with the vectors for 48 h, before being treated and harvested.

Table 3. List of primers utilized.

Primers	Sequences (5'→3')
Cysteine 277 to Alanine (Cys277Ala) mutant-TG2	
Forward	GTCAAGTATGGCCAGGCCTGGGTCTTCGCCGC
Reverse	GCGGCGAAGACCCAGGCCTGGCCATACTTGAC
Methionine 330 to Arginine (Met330Arg) mutant-TG2	
Forward	GTGACAAGAGCGAGAGGATCTTGAACTTCCAC
Reverse	GTGGAAGTTCCAGATCCTCTCGCTCTTGTCACC
Isoleucine 331 to Asparagine (Ile331Asn) mutant-TG2	
Forward	GACAAGAGCGAGATGAACTGGAACTTCCACTGC
Reverse	GCAGTGGAAGTTCCAGTTCATCTCGCTCTTGTC
Asparagine 333 to Serine (Asn333Ser) mutant-TG2	
Forward	GCGAGATGATCTGGAGCTTCCACTGCTGGGT
Reverse	ACCCAGCAGTGGAAGCTCCAGATCATCTCGC

3. Immunofluorescence

INS-1 cells were grown on CultureSlides to 60% confluency and were transfected with vectors expressing Myc-tagged TG2 wild-type and TG2-mutants accordingly. After transfection, cells were incubated for 48 hours. Cells were glucose-sensitised in glucose free media for 4 hours, and then incubated with either 3mM or 16.7mM glucose for 1 hour. Cells were washed with TBS-T and fixed with 10% formalin for 20 minutes at room temperature. After two five minute washes with TBS-T, cells were blocked with 1% BSA in TBS-T for two hours at room temperature or overnight at 4°C. Blocking solution was aspirated, followed by incubation of cells with Myc-antibody in 1% BSA in TBS-T for 2 hours at room temperature or overnight at 4°C. Cells were then washed three times with TBS-T, and incubated for 1 hour at room

4. Immunoprecipitation

The Pierce ProFound™ c-Myc Tag IP/Co-IP Kit was used according to provided manufacturer's instructions to immunoprecipitate Myc-tagged TG2 mutants and TG2 wild-type proteins expressed in Ins-1E cell line. The lysates of the cells expressing the Myc-tagged TG2 mutants and TG2 wild-type proteins were incubated overnight with anti-c-Myc agarose beads in a spin column. The beads were then washed with 0.5 mL TBS-T four times, followed either by resuspension of the beads with 50 mM Tris buffer, pH 7.4, or elution of the protein with elution buffer. Either samples were collected and stored at -20°C until use. For samples to be used in the kinase and transamidation activity assays, rather than subjecting to elution, the protein-bead complexes were resuspended in 50 mM Tris buffer pH 7.4 to preserve enzyme functionality.

5. Western Blotting

Cells were rinsed in phosphate-buffered saline (PBS) and collected in lysis buffer, containing Igepal CA-630 (1%), tris-EDTA buffer (25 mM, pH 7.6), sodium chloride (150 mM), deoxycholic acid sodium salt (1%), sodium lauryl sulfate (0.1%), and protease inhibitor cocktail (104 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, 80μM Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin, 1.4 mM Pepstatin A). Samples were incubated on ice, with intermittent vortexing, for 30 minutes, followed by centrifugation at 13,000RPM for 10 minutes. Protein concentrations of supernatants were determined by Bradford protein assay with bovine serum albumin (BSA) as a standard. SDS-PAGE sample buffer was added to the samples, and the samples were then boiled for 5 minutes. Proteins were resolved on 10-16% SDS-PAGE, according to target protein size, and transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat dry milk in TBS-T for one hour at room temperature and then incubated with primary antibodies for 1 hour at room temperature or overnight at 4°C. Membranes were washed three times in TBS-T wash buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0), then incubated with either horseradish peroxadise (HRP)-conjugate secondary antibodies or streptavidin-HRP conjugate for 1 hour at room temperature or overnight at 4°C. Membrane washing steps with TBS-T were repeated, followed by addition of ECL reagent. Membranes were exposed to Kodak Biomax MR Film and analyzed. If necessary, the membranes were subjected to 15mL strip buffer (100mM 2-mercaptoethanol, 10mM TBS-T) at 30°C for 30-45 minutes, with three subsequent

washes for 10 minutes in TBS-T to remove the 2-mercaptoethanol. The membrane was then reprobed with the desired primary and secondary antibodies.

Table 4: List of antibodies used along with dilution factors.

Antibody	Dilution factor
c-Myc	1:1000
Serotonin	1:500
Streptividin-HRP	1:3000
Pan-Actin	1:1000
TG2	1:1500
Goat-HRP	1:5000
Rabbit-HRP	1:5000
Rat-HRP	1:5000
Mouse-HRP	1:5000

6. In Vitro Kinase Assay

Either recombinant histone (H4) protein or insulin-like growth factor-binding protein 3(IGF-BP3) were incubated separately with affinity purified wild type TG2 and TG2 mutants in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM Magnesium Chloride, [γ-^{32}P]ATP (25μCi/mL) for 30 minutes at 30°C. The reaction was stopped by the addition of SDS-PAGE sample buffer and samples were boiled for 5 minutes. Samples were resolved on SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by autoradiography. Determination of TG2 protein content in samples was determined by probing nitrocellulose membranes with primary Myc antibody, which were detected with

horseradish peroxidise-conjugated secondary anti-rabbit IgG followed by exposure to ECL.

7. Transglutaminase Transamidating Activity Measurement

The Transglutaminase Assay Kit (Sigma) was used according to provided manufacturer's instructions to evaluate the transamidating activity of the Myc-tagged TG2 mutants and TG2 wild-type proteins expressed in Ins-1E cell line. In the assay, the free amine group of Poly L-Lysine, which is covalently attached to the plate surface, is covalently bound to the γ-carboxamide group of biotin-TVQQEL-OH substrate present in the assay buffer. The amount of immobilized biotin was quantified using streptavidin-peroxidase and TMB (3,3′,5,5′-Tetramethylbenzidine) substrate. TG2 protein samples used in the assay were purified with a Myc-tag immunoprecipitation kit. TG2 protein levels of each sample were compared by Western Blot to normalize final activity values.

8. In Situ Transglutaminase Activity Assay

INS-1E cells were cultured in 60 mm tissue culture plates to 60% confluency and were transfected with vectors expressing Myc-tagged TG2 wild-type and TG2-mutants accordingly and were incubated for 48 hours. Cells were preincubated for 1 hour with 1 mM 5-(biotinamido)pentylamine($BPNH_2$) in standard culture medium. Cells were rinsed in PBS, and were sensitised to glucose for 30 minutes in glucose-free standard medium. Cells were rinsed again in PBS, and incubated in medium containing either 3 mM or 16.7 mM glucose for 30 minutes. Cells were lysed and supernatant was collected for western blot analysis with streptavidin HRP antibody(1:2000).

9. In Vitro Transglutaminase Assay

INS-1E cells were cultured in 60 mm tissue culture plates to 60% confluency and were transfected with vectors expressing Myc-tagged TG2 wild-type and TG2-mutants accordingly and were incubated for 48 hours. Myc-tagged TG2 mutants and TG2 wild-type proteins expressed in Ins-1E cell line used in the assay were purified from the cell lysate with a Myc-tag immunoprecipitation kit. Agarose bead-bound proteins were incubated at 37°C for 2 h in a 50 mM HEPES buffer, pH 9.0, containing 10 mM DTT, 5 mM $CaCl_2$, 0.25µg recombinant Rb protein, and 1 mM $BPNH_2$. In control samples, $CaCl_2$ was replaced by 5 mM EGTA. The reaction was stopped by the addition of SDS-PAGE sample buffer and samples were boiled for 5 minutes. Samples were then analyzed by Western blot using the streptavidin HRP antibody (1:2000).

10. ATP/GTP Binding Assay

INS-1E cells were cultured in 60 mm tissue culture plates to 60% confluency and were transfected with vectors expressing Myc-tagged TG2 wild-type and TG2-mutants accordingly and were incubated for 48 hours. Cells were rinsed in PBS, and were sensitized to glucose for 30 minutes in glucose-free standard medium. Cells were rinsed again in PBS, and incubated in medium containing either 3.3 mM or 16.7 mM glucose for 30 minutes. Cells were lysed and supernatant was collected. 100 µg of total protein of lysate was incubated with 20 µL of either GTP-agarose resin (50% (v/v) slurry in 10 mM Tris/300 mM NaCl/1 mM EDTA, pH 8.0) or ATP-agarose resin diluted in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl) to a total volume of 300 µL for 2 hours

at 4ºC. The resin was subjected to five cycles of washing and centrifugation using ice cold TBS-T before the agarose beads were resuspended in 50 mM Tris-HCl buffer, pH 7.5. The resuspension was analyzed by Western Blot utilizing anti-Myc antibody to determine the quantity of Myc-tagged TG2 bound to the ATP/GTP agarose beads.

11. Transglutaminase-Mediated Serotonylation Assay

INS-1E cells were cultured in 60 mm tissue culture plates to 60% confluency and were transfected with vectors expressing Myc-tagged TG2 wild-type and TG2-mutants accordingly and were incubated for 48 hours. Cells were rinsed in PBS, and were sensitized to glucose for 30 minutes in glucose-free standard medium. Cells were rinsed again in PBS, and incubated in medium containing either 3 mM or 16.7 mM glucose for 30 minutes. Cells were lysed and supernatant was collected for western blot analysis with serotonin antibody.

12. Insulin Secretion Assays

INS-1E cells were cultured in 60mm tissue culture plates to 60% confluency and were transfected with vectors expressing Myc-tagged TG2 wild-type and TG2-mutants accordingly and were incubated for 48 hours. Cells were rinsed in PBS, and were sensitised to glucose for 30 minutes in glucose-free Krebs Ringer bicarbonate (KRB) buffer solution (118.4 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 25 mM $NaHCO_3$ (equilibriated with 5% CO_2-95% O_2, pH 7.4), 0.2% BSA, and 10 mM HEPES). Cells were rinsed again in PBS, and incubated with KRB buffer containing either 3.3 mM or 16.7 mM glucose for 30 minutes. KRB buffer was collected

and analyzed for insulin content with the Ultra Sensitive Rat Insulin ELISA kit, according to provided instructions. Lysates of cells were collected and total protein content was determined. Insulin content of KRB buffer was normalized with total protein content. Protein content was determined by Bradford Assay.

13. Animals and Diets

TG2 knockout C57BL/6 mice, male and female, were generously provided by Dr. Nikolaos Frangogiannis (Baylor College of Medicine, Houston, Texas, USA) with permission from Dr. Gerry Melino (University of Leicester, UK). Wild-type C57BL/6 mice, male and female, were provided by the University of Manitoba Central Animal Care Services. Mice were housed under controlled temperature (21°C) and light conditions (12-h light/dark cycle) with access to water and standard diet. Experiments involving mice were executed as approved by the Animal Care Committee of the University of Manitoba.

14. Isolation of Pancreatic Islets

Islets were isolated by collagenase digestion of the mouse pancreas (Lacy and Kostianovsky 1967) with slight modification [85]. Prior to injection of the collagenase into the pancreas, the lower end of the bile duct was clamped to prevent the movement of collagenase solution into the duodenum. Upon injection of the collagenase solution (3 mL of 6 mg/mL collagenase in Hank's Balanced Salt Solution (HBSS)) with a 30 gauge angled needle, the pancreas was relocated to a tube which is immediately placed on ice. The pancreatic material was then incubated in the HBSS solution for 17-20 minutes at

37°C. Next, the pancreatic tissue was gently syringed through a 14 gauge needle into a continual texture, suspended in a total of 9 mL HBSS solution, centrifuged at 1500 rpm for 1 minute, and supernatant discarded. The pellet was resuspended in HBSS solution and gently syringed, followed by a repetition of the wash step just prior. In total, four washes of this nature were performed before the supernatant was once again discarded and 3 mL each of 1077 and 1119 Histopaque was added, mixed, followed by the addition of another 3 mL of 1077 Histopaque and 3 mL HBSS layered in such order. The density gradient containing the tissue was centrifuged for 20 minutes at 2000 rpm at 4°C. The tissue collected from the upper two layers after spinning was then placed in a Petri dish. Islets were handpicked by choosing rounded, opaque islets through a dissecting microscope at a magnification of 10X and placed into RPMI 1640 medium containing 11 mM glucose, 10% heat-inactivated fetal bovine serum, and 100µg/mL penicillin-streptomycin. The islets were then relocated to a fresh Petri dish of the same medium and incubated for 24 hours in a humidified atmosphere containing 5% CO_2 at 37°C prior to experimental use.

15. Batch incubations

Islets were selected on the basis of consistent morphology, color, and size (50 µm diameter). Batches of 4 islets were pre-incubated in Krebs Ringer bicarbonate (KRB) buffer solution containing 3.3 mM glucose for 30 minutes. Batches were transferred to tubes containing 3.3 mM glucose and incubated for 30 minutes. The batches were then transferred to tubes containing 16.7 mM glucose and incubated for 30 minutes, while the previous buffer was collected. After the 30 minute incubation period the 16.7 mM

glucose buffer was collected. The total insulin content of the islets was measured following acid-ethanol (0.1%HCl in 75% ethanol) extraction of collected batches. The insulin content of each sample was analyzed by an Ultra Sensitive Mouse Insulin ELISA kit, according to provided instructions. For the ELISA to be completed it was necessary to dilute the 16.7 mM glucose buffer ten times and the islet insulin content extract one thousand times.

16. In Gel Digestion of Proteins

Coomassie Blue-stained bands of interest were excised from the gel and cut into 1 mm^3 cubes. The gel pieces were washed in 100 mM ammonium bicarbonate (NH_4HCO_3) for 10 minutes, spun, and the supernatant was discarded. Next, they were washed in 40% acetonitrile (ACN) in 100 mM NH_4HCO_3 for 10 minutes, spun, and the liquid was discarded. The fragments were subsequently bathed in a volume of ACN that was 5X the gel volume for 5 minutes, spun, and the ACN was removed. These washing steps were repeated until all visible dye in the gel disappeared. The pieces were vacuum-dried briefly, then bathed in 3X the gel volume of 10mM dithiothreitol (DDT) in 100 mM NH_4HCO_3 for 45 minutes at 55°C, spun down, and the liquid was discarded. The difference between the volume of liquid added and discarded was the volume of trypsin used for digestion. Next, the gel pieces were incubated in 3X the gel volume of 55 mM Iodoacetamide in 100 mM NH_4HCO_3 for 30 minutes in the dark, spun down, and the supernatant was discarded. The previous washing step was repeated. 5 ng/µL of trypsin in 50 mM NH_4HCO_3 containing 5 mM $CaCl_2$ was added to the gel fragments and incubated at 37°C overnight. Peptides were extracted through liquid collection and two

washing steps, repeated thrice, using 0.01% trifluoroacetic acid in 50% ACN and neat ACN. Liquid collections from gel pieces of the same band were pooled and vacuum dried to a suitable concentration. The sample was then centrifuged at 13000 rpm for 10 minutes, the supernatent collected, and frozen at -80°C until sent for mass spectrometry analysis.

17. Statistical Analysis

The data were analyzed by one way ANOVA with Dunnett's multiple comparison or Student's *t*-test, and are presented as the mean ± SEM and $P < 0.05$ was considered significant.

IV. RESULTS

1. INS-1E and MIN6 express TG2 and are able to express wild-type and mutant TG2 through transfection.

It was first necessary to determine that the cell lines to be used expressed TG2 and were capable of being transfected with wild-type, and the selected mutant varieties, of TG2. To determine whether TG2 is expressed in MIN6 (data not shown) and INS-1 cell lines we collected whole lysates and analyzed them by western blot. TG2 was found to be present in both cell lines (Figure 8A). Subsequently we transfected the cells with wild-type and mutant TG2 expressing vectors and analyzed by western blot. The control vector did not produce a signal and the TG2-expressing vectors were successful in generating TG2 in both cell lines (Figure 8B). The affinity of the Myc antibody was observed to be greater than that of TG2 antibody (Figure 8A,8B). We used immunocytochemistry to determine the localization and expression levels of the wild-type and mutant TG2 vectors in the MIN6 and INS-1 cell lines. In the literature TG2 has been shown to be localized in several intracellular locations, including the nucleus, cytosol, and cellular membrane. We sought to rule out the possibility that TG2 is influencing insulin secretion through transcriptional regulation in the nucleus and observe the nature of TG2's localization within the cytosol. Each TG2 variant was found to be ubiquitously positioned in the cytosol, while relatively absent in the nucleus for both MIN6 (Figure 9) and INS-1 (Figure 10).

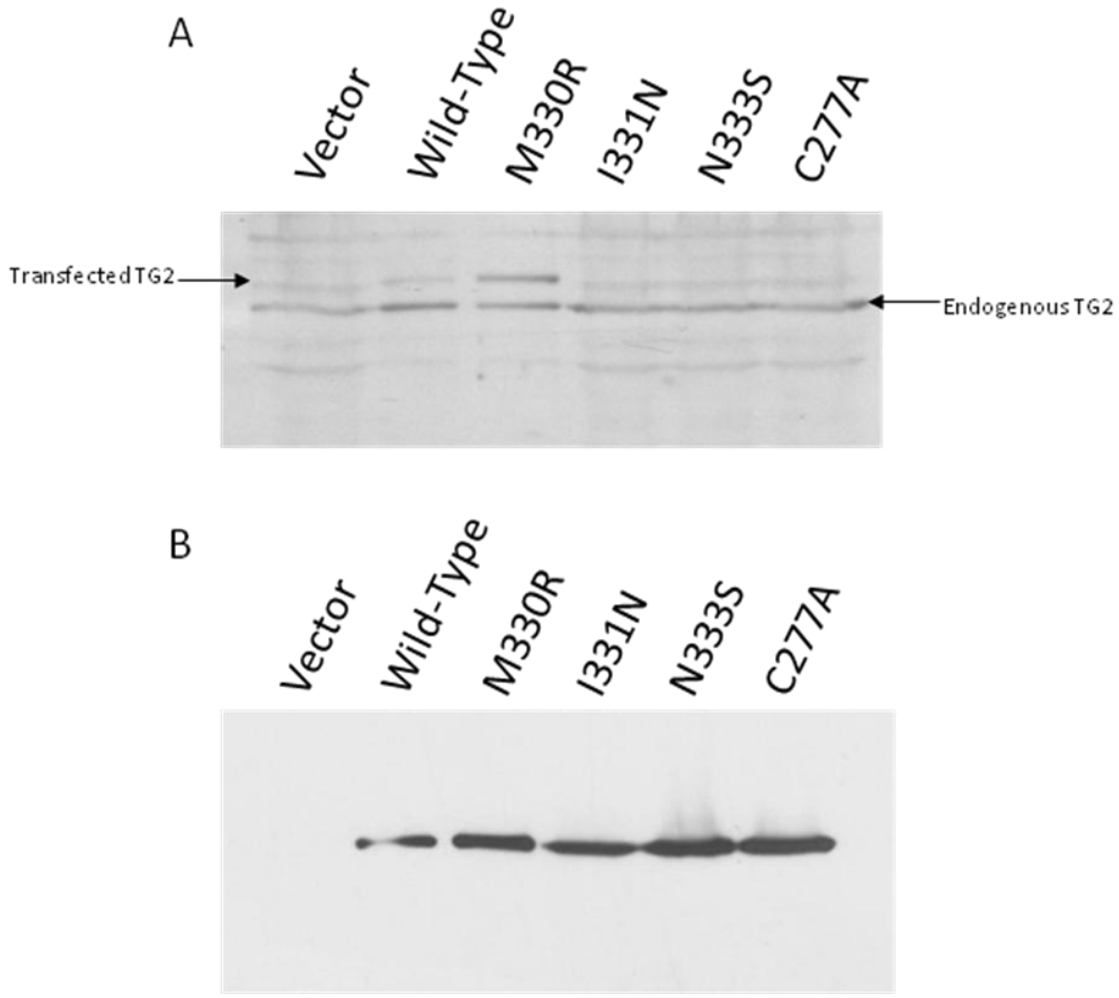


Figure 8. Expression of transfected and endogenous TG2 in INS-1E cell line.
Cells were transfected with vector control, wild-type, and mutant varieties of TG2. Lysate was collected and analyzed by Western blot. A. Membrane probed with TG2 antibody. Wild-type and mutant varieties of TG2 are observed at a higher molecular weight due to Myc-tag and Flag-tag on C- and N-terminals respectively. B. Membrane re-probed with c-Myc antibody.

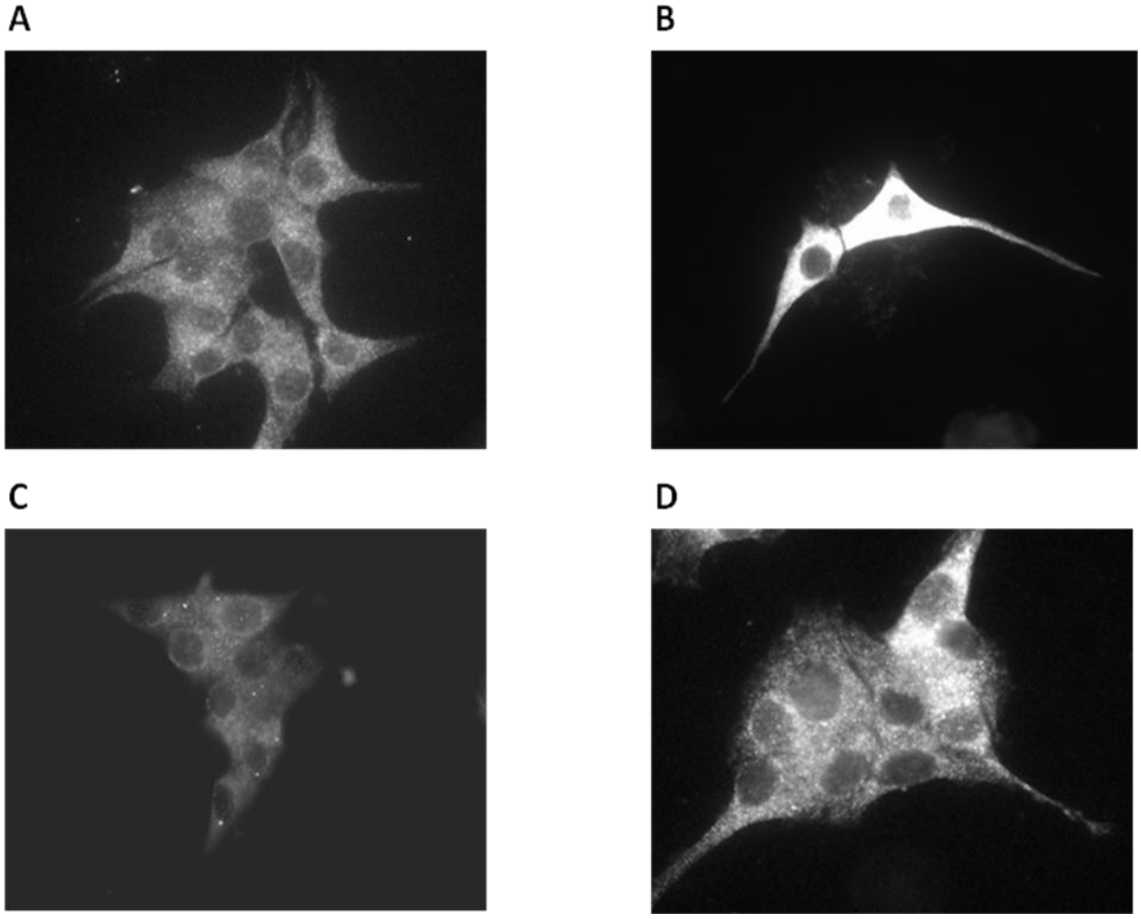


Figure 9. Expression and Localization of Myc-tagged wild-type and TG2 mutants in MIN6.
MIN6 cells were transfected with vectors expressing Myc-tagged TG2 wild-type and TG2-mutants accordingly and incubated for 48 hours. Cells were incubated with either 3.3 mM or 16.7 mM glucose for 30 minutes after glucose sensitization. Fluorescent probe used was anti-rabbit Alexa ®Fluor 488. A. WT TG2. B. Met330Arg TG2. C. Ile331Asn TG2. D. Asn333Ser TG2

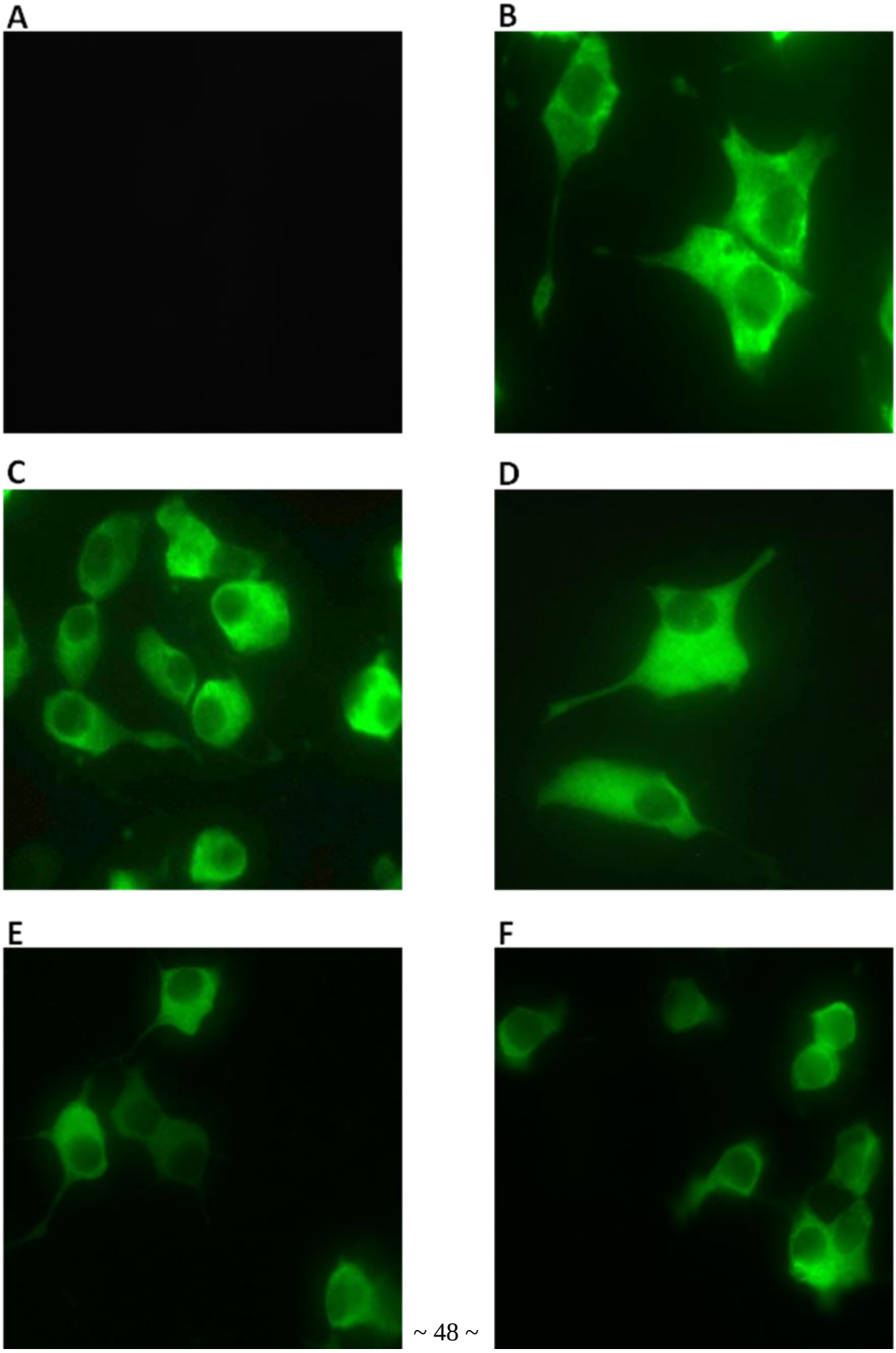

A
B
C
D
E
F

Figure 10. Expression and Localization of Myc-tagged WT and TG2 mutants in INS-1E.
INS-1E cells were transfected with vectors expressing Myc-tagged TG2 wild-type and TG2-mutants accordingly and incubated for 48 hours. Cells were incubated with either 3.3 mM or 16.7 mM glucose for 30 minutes after glucose sensitization. Fluorescent probe used was anti-rabbit Alexa ®Fluor 488. A. Negative Control. WT TG2 transfected cells without primary antibody. Contrast for imaging was kept constant with that which was used for non-controls. B. WT TG2. C. Met330Arg TG2. D. Ile331Asn TG2. E. Asn333Ser TG2. F. Cys277Ala TG2.

2. INS-1E and MIN6 respond to stimulating levels of glucose by releasing insulin.

It was necessary to ensure that both cell lines secreted insulin in response to stimulating levels of glucose before proceeding. In addition, it was crucial that the amount of insulin secreted under stimulating conditions was greater than under basal conditions. The cells were subjected to both basal and stimulating levels of glucose and the supernatents were collected and analyzed for the amount of insulin released. Both cell lines responded to higher levels of glucose by elevating their insulin secretion in relation to their basal outputs. However, in comparison to the MIN6 cells, the INS-1E cells had a substantially greater elevation in insulin secretion in the presence of high levels of glucose and also had a lower output of insulin in response to basal levels of glucose (Figure 11). Combined, both of these responses by the INS-1E cells provided a more substantial contrast to compare the change of GSIS responses produced by introducing an overexpression of TG2 or the expression of the mutant variants. For this reason, only INS-1E cells were used for the subsequent studies.

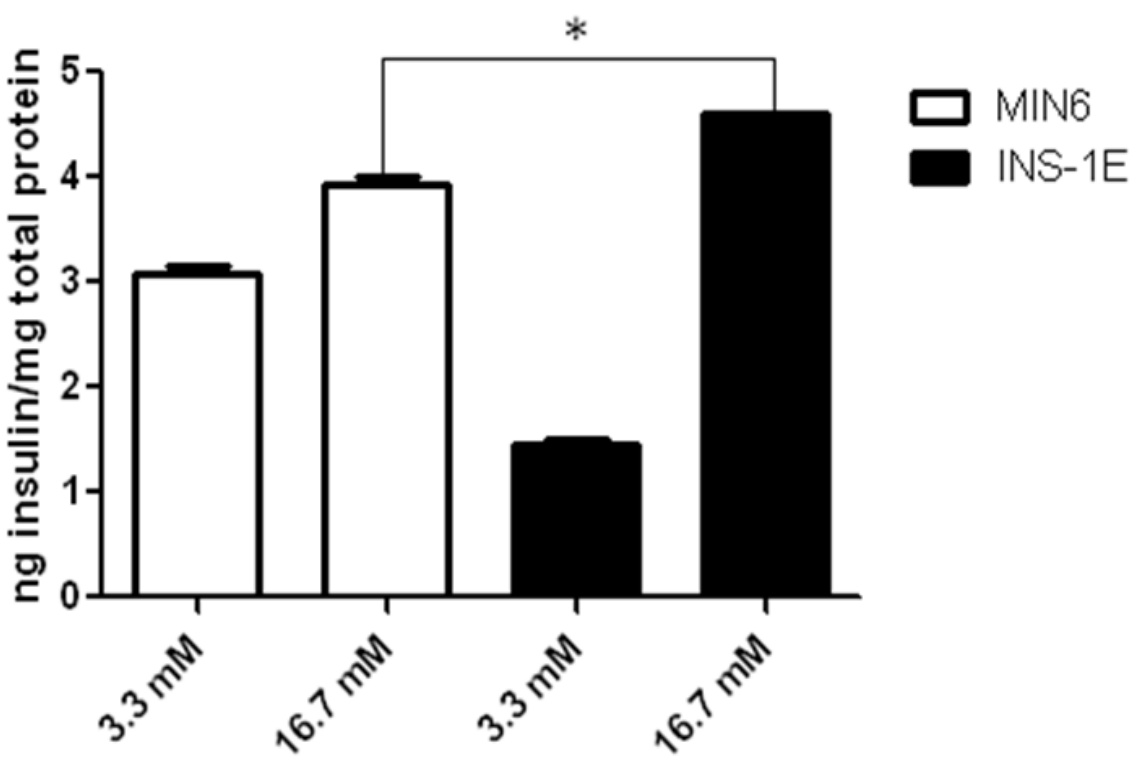


Figure 11. Comparison of GSIS between MIN6 and INS-1 cell lines.
Cells were sensitized to glucose for 30 minutes in glucose-free KRB. Cells were rinsed in PBS, and incubated with KRB buffer containing either 3.3 mM or 16.7 mM glucose for 30 minutes. Bars show endogenous insulin released expressed as the mean ± SEM ng/mL, Student's *t*-test, two tailed (n = 3). * Means significantly different ($P < 0.05$)

3. TG2 knockout mice islets have reduced GSIS when compared to wild-type mice islets.

TG2 knockout mice have been shown to exhibit reduced GSIS from their pancreatic islets, resulting in glucose intolerance [11]. To confirm the generalized impact of TG2's function in insulin secretion, experiments were performed with islets isolated by collagenase digestion of the pancreas of wild-type and TG2 knockout mice. These experiments were designed to elucidate whether the reduced insulin secretion from TG2-/- mice is a result of the loss of TG2 function in the pancreatic islets and not due to other

peripheral factors. The islets, once isolated, were subjected to basal and stimulating levels of glucose, followed by the immediate collection and analysis of the KRB solution for insulin content. TG2 knockout mice islets were found to secrete less than half of the amount of insulin in response to a stimulating concentration of glucose in relation to wild-type mice islets ($p<0.01$). While there was a trend, there was no statistically significant difference between GSIS of TG2 knockout islets and wild-type in basal conditions (Figure 12).

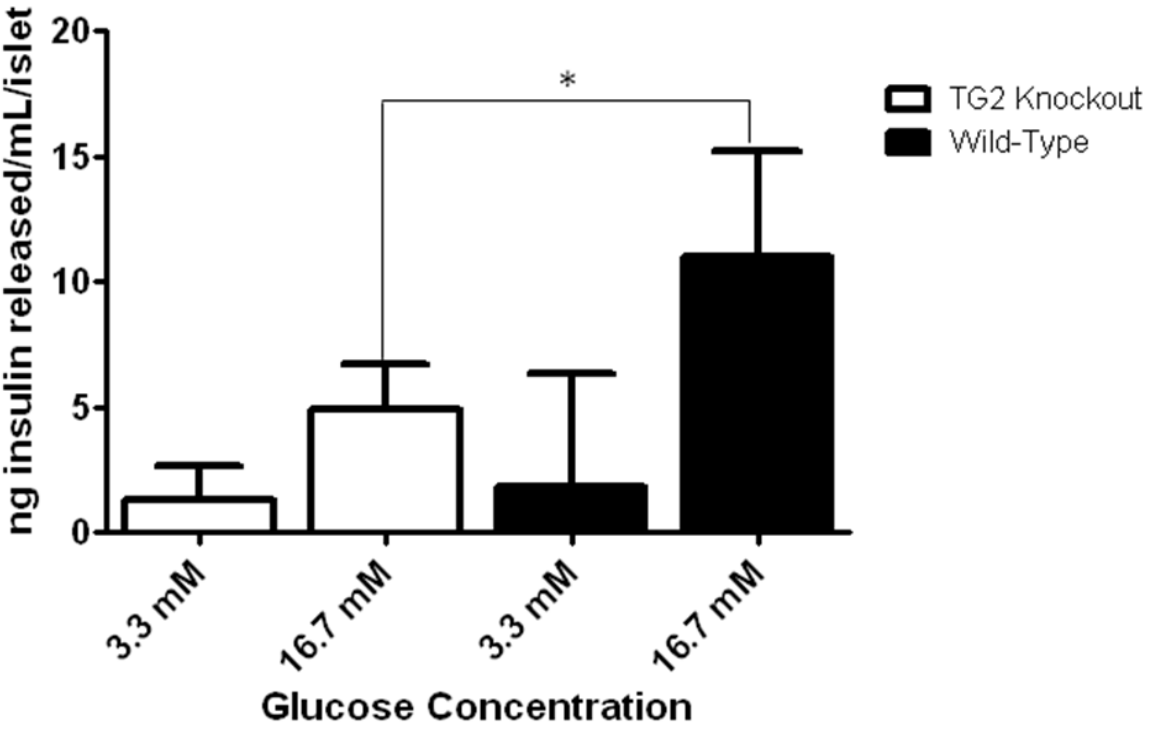


Figure 12. Comparison of TG2 knockout and wild-type mouse islets for GSIS.
Islets were sensitized to glucose for 30 minutes in 3.3 mM glucose KRB. Cells were rinsed in PBS, and incubated with KRB buffer containing either 3.3 mM or 16.7 mM glucose for 30 minutes. Bars show endogenous insulin released expressed as the mean ±SEM ng/mL/islet Student's *t*-test, two tailed (n = 12). * Means significantly different ($P < 0.01$)

4. The overexpression of mutant TG2 varieties in beta cells results in significantly lower GSIS compared to wild-type.

Pancreatic islets contain a number of endocrine cell types, which are known to affect each other through paracrine means. After confirming with islet studies that the loss of TG2, particularly in the pancreatic islets, results in a substantial reduction in GSIS, it was important to elucidate whether this loss of TG2 specifically and directly impacted the insulin-secretory functionality of pancreatic beta cells. The GSIS of INS-1 cells that were transfected with vectors expressing TG2 wild-type and TG2-mutants were compared. The expression of the wild-type and mutant varieties of TG2 in the beta-cells did not produce a noticeable effect on insulin secretion in response to basal levels of glucose. An overexpression of wild-type TG2 generated an increase in GSIS when compared to the control vector. In comparison to wild-type TG2, expression of the Cys277Ala and Ile331Asn mutants resulted in significantly less GSIS ($P < 0.05$). Ile331Asn-TG2 expression resulted in the most significant decrease in GSIS compared to wild-type TG2, followed by Cys277Ala-TG2. While the expression of the Ile331Asn and Cys277Ala mutant caused the greatest decrease in GSIS compared to wild-type (32% and 22% respectively), Met330Arg-TG2 and Asn333Ser-TG2 expression caused a notable decrease as well (20% and 7% respectively) (Figure 13).

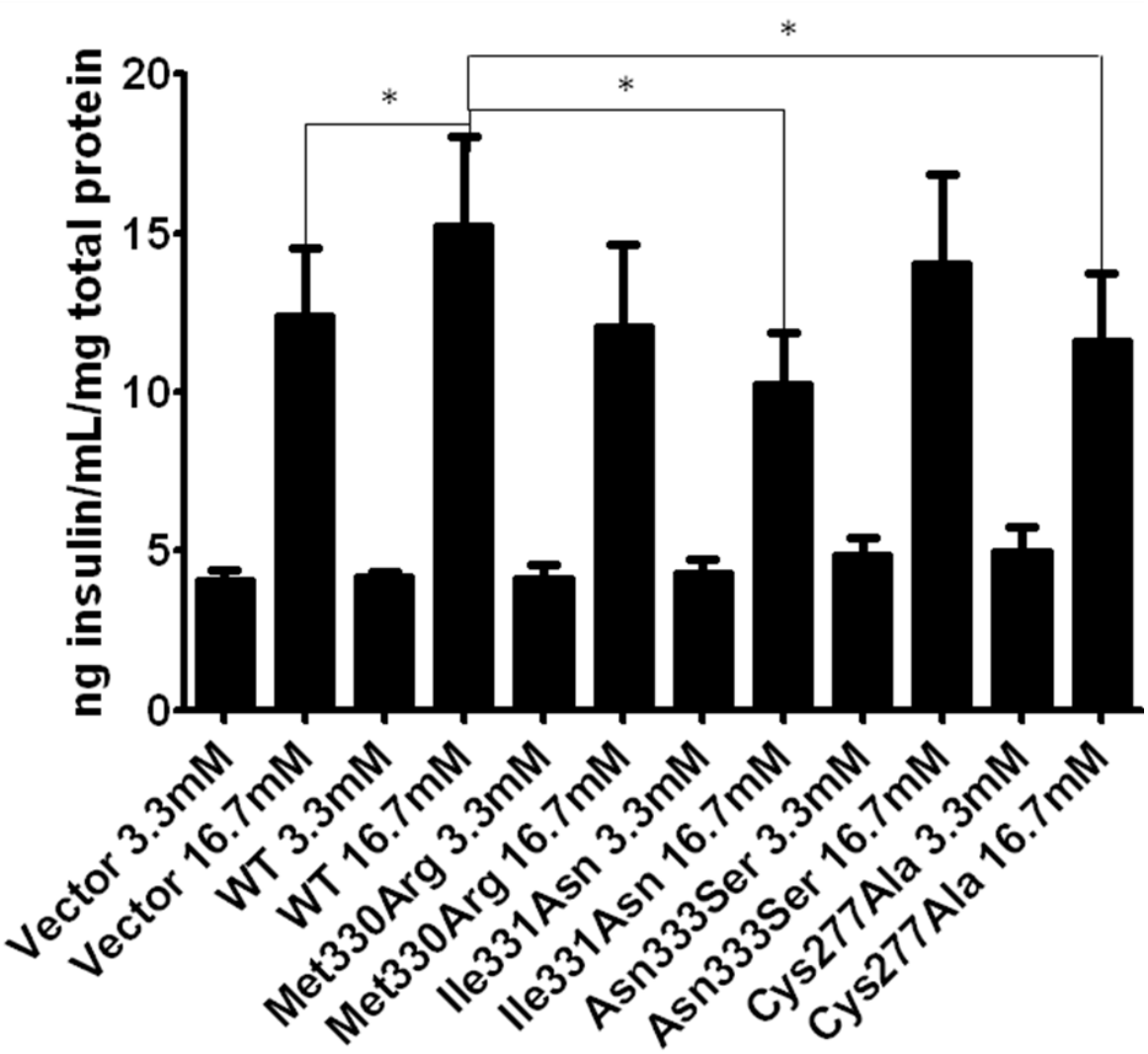


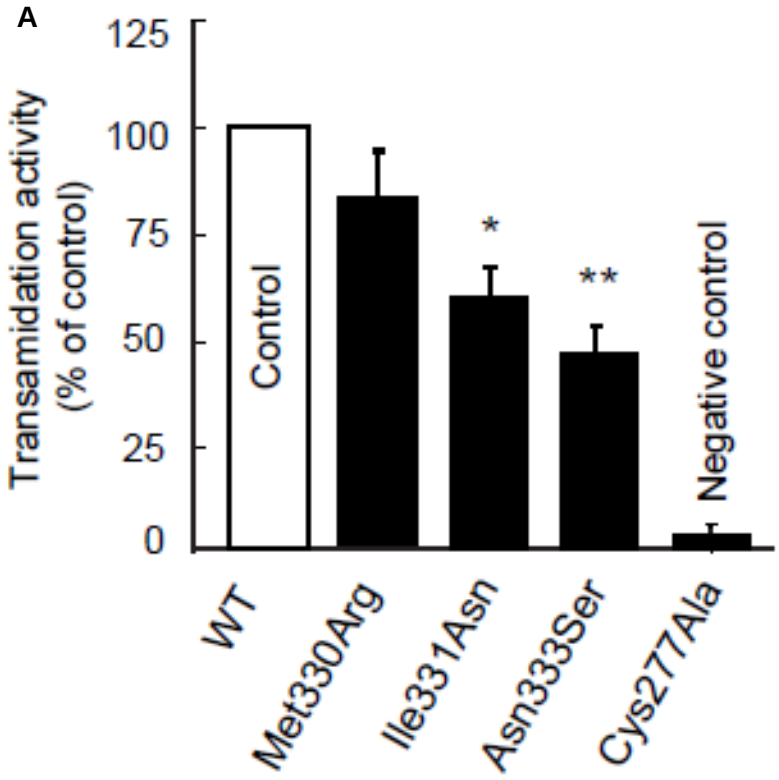
Figure 13. GSIS comparison of wild-type and mutant TG2 transfected INS-1E cells. INS-1 cells were sensitized to glucose for 30 minutes glucose-free KRB. Cells were incubated with KRB buffer containing either 3.3 mM or 16.7 mM glucose for 30 minutes at 37ºC. Bars show endogenous insulin released normalized with total protein content expressed as the mean ±SEM ng/mL/mg total protein, one way ANOVA with Dunnett's multiple comparison, (n = 5). * Means significantly different ($P < 0.05$)

5. Naturally occurring TG2 mutants have reduced transamidation activity in comparison to wild-type.

In order to characterize which functions of TG2 are influenced by the select mutations, and how these functional changes affect GSIS of pancreatic beta cells, INS-1E

cells were transfected with wild-type and mutant varieties of Myc-TG2. The lysates of these cells were subjected to affinity purification using a Myc-IP kit to isolate the Myc-TG2, which was utilized in experiments designed to analyze transamidation and kinase activities. Purified wild-type TG2 and TG2 mutants were incubated with biotin peptides in lysine coated plates. The amount of biotin crosslinked with lysine was quantified by a colorimetric assay. These data were calculated as a percentage of wild-type TG2's crosslinking activity. The amino acid Cys^{277} is one of three amino acids forming TG2's transamidation catalytic triad. The Cys277Ala mutant has been used extensively to inactivate the transamidation function of TG2 [52] and has been used in this thesis project as a transamidation activity negative control. A greater reduction in *in vitro* transamidation activity amongst the three naturally occurring mutations was found in Asn333Ser -TG2 ($p<0.01$) and Ile331Asn -TG2 ($p<0.05$) respectively. There was no statistically significant change observed for the Met330Arg-TG2 mutant in comparison to wild-type. The transamidation activity-dead Cys277Ala-TG2 produced negligible levels of biotin-lysine crosslinking, as expected (Figure 14A). These data were comparable to what was observed in previous studies [12]. In addition, we examined the *in situ* transamidation activity of TG2 mutants in INS-1E cells through the utilization of the amine substrate $BPNH_2$. A similar pattern of reduced transamidation activity in TG2 mutants compared to wild-type TG2 was observed that was comparable to the *in vitro* transamidation assay, particularly in the lower half of the blots where the biotinylated protein bands are relatively distinct from one another. While the vector-control, wild-type, and Met330Arg-TG2 lanes show similar, strong levels of biotin incorporation, the

Ile331Asn-TG2, Asn333Ser-TG2, and transamidation activity dead Cys277Ala-TG2 lanes displayed relatively reduced biotin-protein crosslinking (Figure 14B).



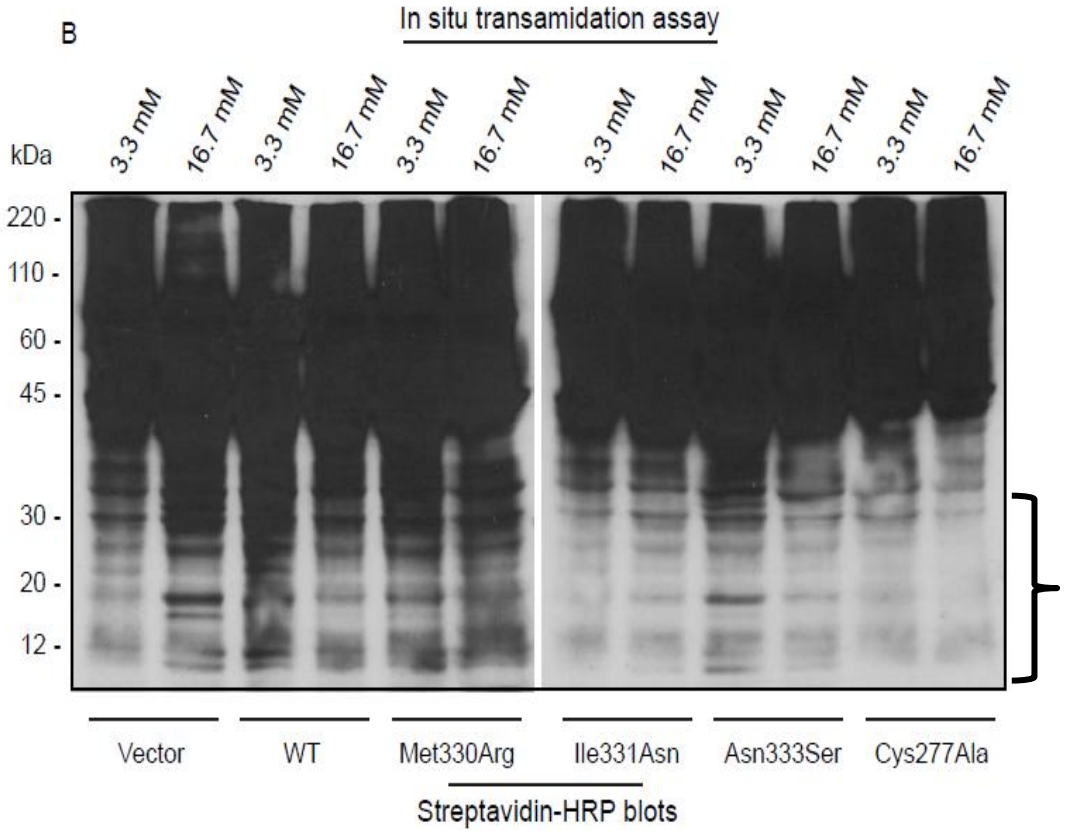


Figure 14. Comparison of transamidation activities amongst wild-type and mutant TG2.
A. Recombinant TG2 was expressed in INS-1E cell line and purified by Myc-immunoprecipitation kit. Purified samples were analyzed through use of a transamidation assay kit. Histogram displaying relative transamidation activity of WT and mutant TG2 as determined by *in vitro* transamidation colorimetric assay, which quantified the ability of TG2 to crosslink biotin onto lysine coated plates. (Mean ± SEM, Student's *t*-test, two tailed, n=6). * $p<0.05$; ** $p<0.01$ versus WT control. B. Western blot displaying *in situ* transamidation activity using 5-(biotinamido)pentylamine ($BPNH_2$) as a substrate. Streptavidin-HRP used as 1:3000 dilution (n=1).

6. TG2 GTP-binding, not ATP-binding, is sensitive to changes in beta cell glucose stimulation.

To measure the GTP and ATP binding abilities of TG2, cell lysates containing Myc-tagged wild-type and naturally occurring TG2 mutants were incubated with either GTP-agarose or ATP-agarose affinity resin in kinase buffer. GTP-agarose resin is

comprised of GTP attached to agarose beads through its γ-phosphate and is resistant to phosphatases present in crude cell/tissue extracts [86]. The binding abilities were determined by immunoblotting against the TG2 bound to the affinity resin with anti-Myc antibody. Vector control exhibited a lack of GTP-binding for both basal and stimulatory conditions. Wild-type TG2 displayed low GTP-binding under basal conditions, though the activity increased in the presence of stimulatory glucose concentrations. Met330Arg -TG2 had similar GTP-binding affinity as wild-type TG2 under basal glucose concentration; however under stimulatory conditions Met330Arg -TG2's GTP binding affinity was substantially greater than wild-type TG2. The GTP-binding ability of Ile331Asn was minimal in both conditions in comparison to wild-type. Surprisingly, Asn333Ser -TG2 exhibited greater GTP-binding under basal conditions, which was then reduced in response to stimulatory glucose concentrations (Figure 15A,B).

Vector control exhibited a lack of ATP-binding for both basal and stimulatory conditions. In contrast to the GTP-binding affinity results, the ATP-binding affinity for wild-type and naturally occurring mutant TG2 proteins did not change substantially between basal and stimulatory glucose conditions. Wild-type TG2 and Met330Arg -TG2 were both observed to have the highest ATP-binding ability. A similar ATP-binding pattern was observed between Ile331Asn and Asn333Ser -TG2. Taken together, the GTP-binding function was found to be modulated by glucose stimulation and its loss more closely correlated with significantly reduced insulin secretion (see Figure 13) from INS-1E cells than ATP-binding, as observed with the expression of Ile331Asn -TG2 (Figure 15A,B).

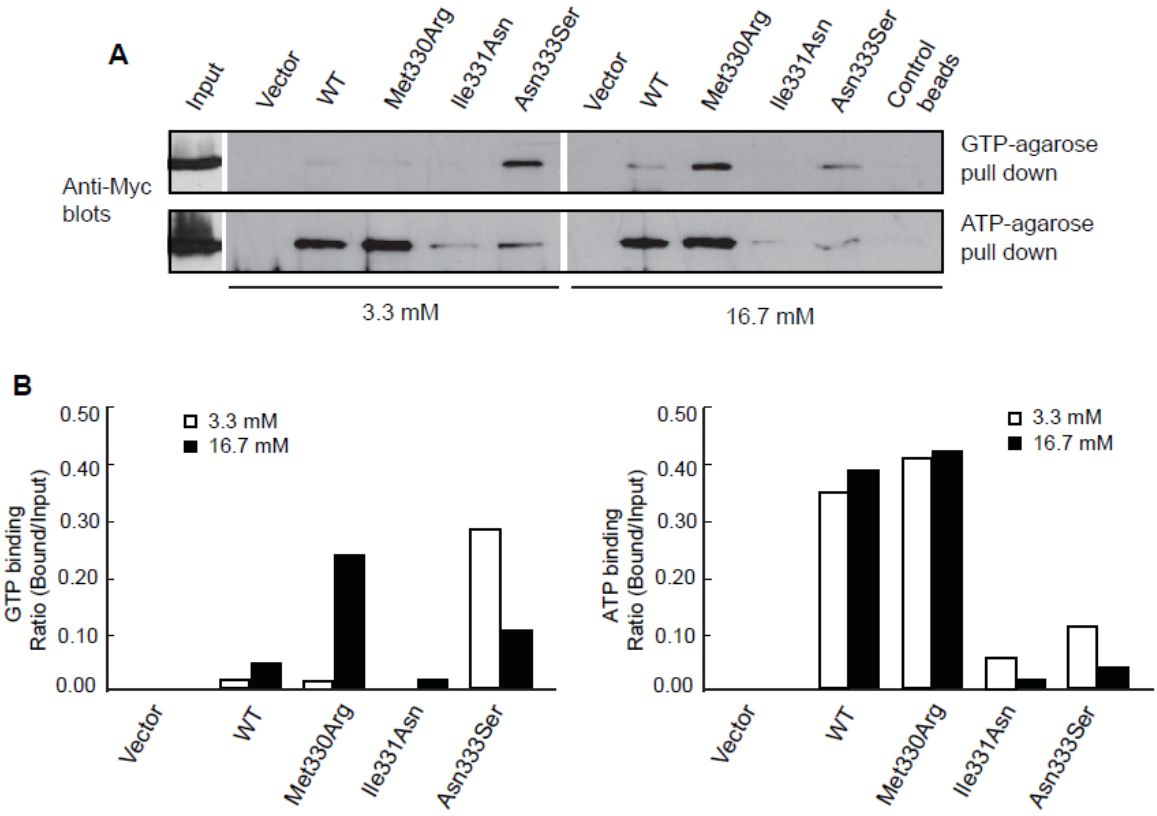
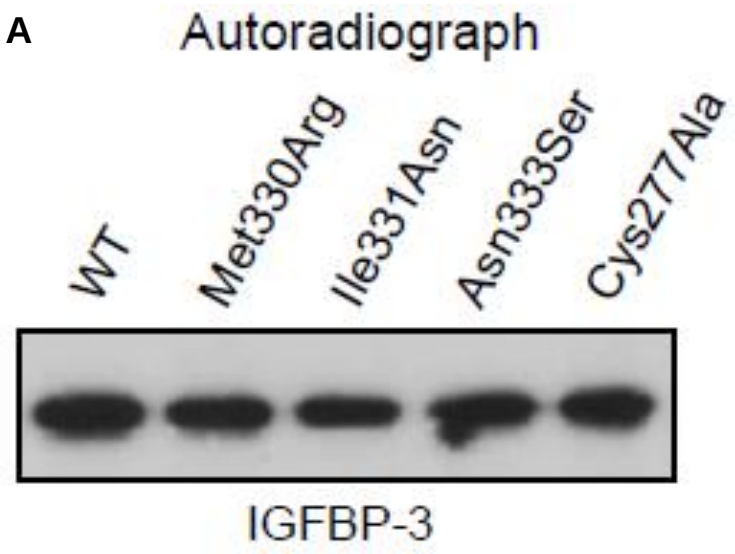


Figure 15. GTP/ATP-binding assay for wild-type and naturally occurring TG2 mutants.
Samples were incubated with GTP/ATP-agarose affinity resin for 2 hours at 4ºC, followed by immunoblot with Myc antibody (1:1000). A. ATP/GTP-binding assay. Respective Western Blots for lysates of cells grown in either 3.3 mM and 16.7 mM glucose. B. Histograms showing relative changes in GTP and ATP binding in WT and mutant TG2 as shown in panel A. Representative of n=2 experiments. Pattern of glucose-sensitive changes in binding was reproducible.

7. Naturally occurring TG2 mutants have altered kinase activity.

The greatest relative reduction in kinase activity was observed in Ile331Asn -TG2, followed by Asn333Ser -TG2 and Met330Arg -TG2 respectively. The Cys277Ala -TG2 mutation was also shown to have a reduced kinase activity, similar to the naturally occurring mutants. All mutant varieties of TG2 had kinase activities that were roughly

75% of that observed with WT-TG2, although this was only significant in Ile331Asn - TG2. While there seemed to be differences between each of the mutants in their ability to phosphorylate IGFBP3, these differences were minimal (Figure 16). The *in vitro* kinase assay was also carried out with recombinant H4 as the substrate. A similar pattern for reduced kinase activity of mutant and wild-type TG2 was seen. The level of phosphorylation by TG2 was however significantly less than that seen by IGFBP3 (results not shown).



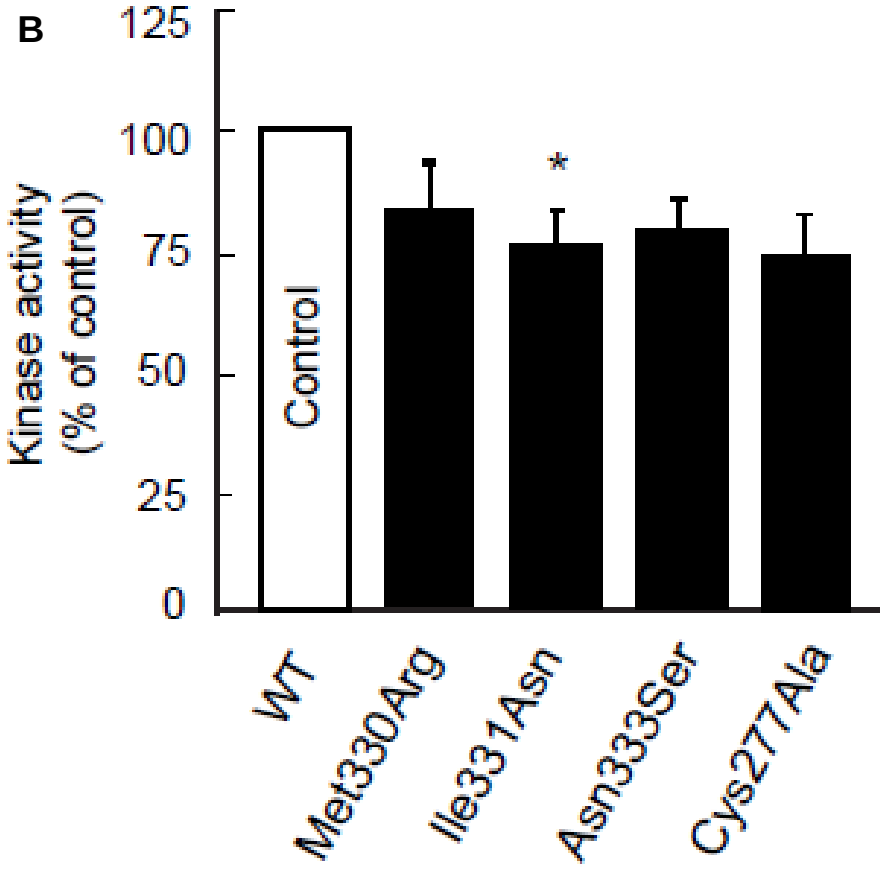


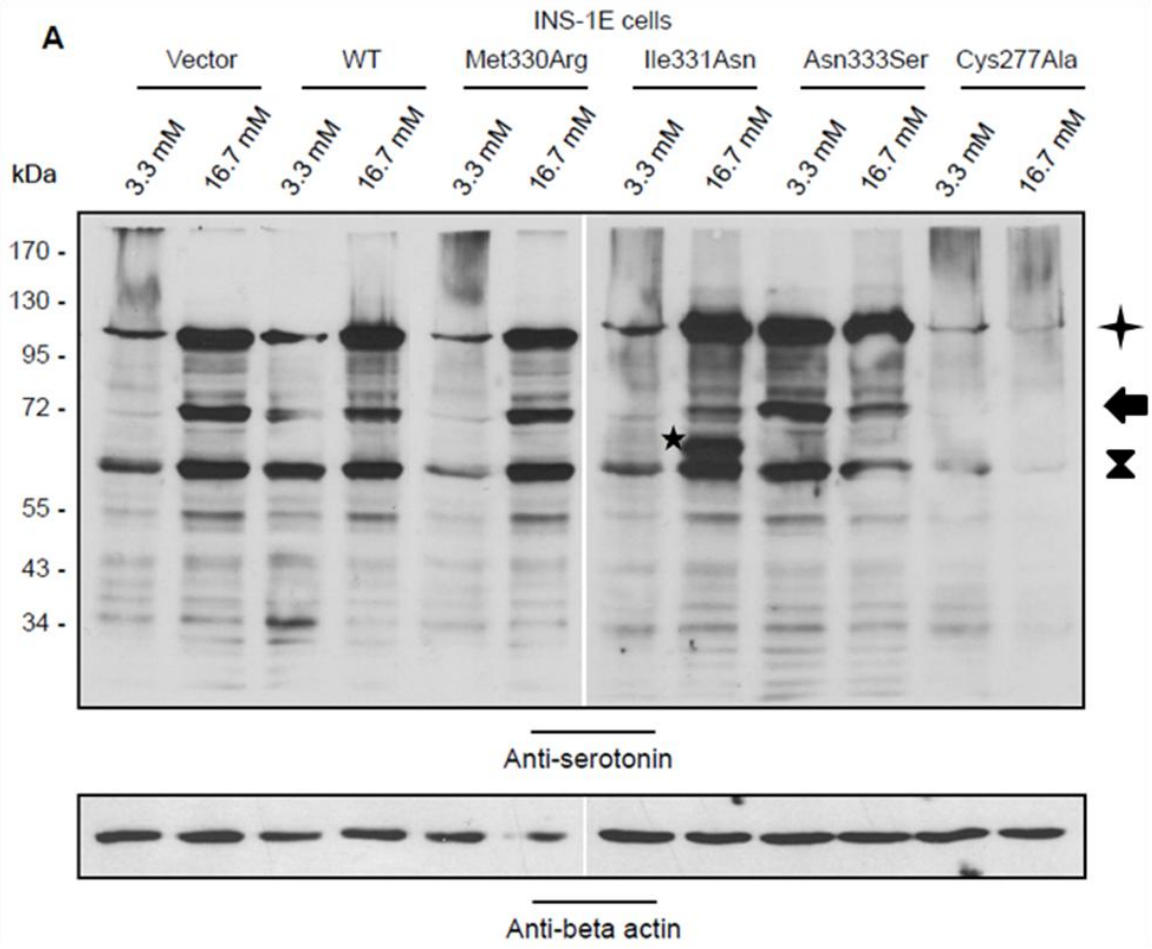
Figure 16. Impact of naturally occurring mutations in TG2 on its kinase activity. A. IGFBP3 protein was incubated separately with TG2 mutants and TG2 wild-type protein bound to agarose beads in kinase buffer for 30 minutes at 30°C. Samples were resolved on SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by autoradiography. TG2 protein content was determined by probing nitrocellulose membranes with primary Myc antibody (1:1000) and horseradish peroxidase-conjugated secondary anti-rabbit (1:5000) IgG followed by exposure to ECL. B. Histogram shows the relative changes in kinase activity versus wild-type control as determined by densitometric analysis of phosphorylated IGFBP3. (Mean ± SEM, Student's *t*-test, two tailed, n=3) * $p<0.05$.

8. A 72 kDa band is differentially serotonylated by the expression of naturally occurring TG2 mutants. Serotonylation by TG2 is glucose-sensitive.

It was recently observed that serotonin in beta cells is covalently bound, through TG2's transamidation activity, to small GTPases that are directly involved in vesicle recruitment and transport [14]. The different variations of the loss of transamidation

activity between the three mutations prompted our interest in determining the effect of these mutations on protein serotonylation in INS-1E cells. In comparison to vector-control the overexpression of wild-type TG2 resulted in an overall increase in protein serotonylation, including a noticeable enhancement of protein serotonylation under basal conditions (Figure 17A). Expression of each of the three mutations generated substantially diverse, yet lower levels of serotonylation. Interestingly, akin to TG2's GTP-binding, the level of protein serotonylation was responsive to changes in glucose concentrations. While vector control, wild-type TG2 and Met330Arg-TG2 overexpression displayed an increase in protein serotonylation under stimulatory conditions compared to basal, the remaining mutations, along with Cys277Ala-TG2, each produced unique responses to changes in glucose concentration. Asn333Ser-TG2 expression, while generating comparably lower serotonylation in response to stimulatory glucose than vector control, yielded levels of serotonylation under basal conditions similar to that observed for wild-type expression under stimulating conditions. Cys277Ala-TG2 expression resulted in little to no protein serotonylation in both basal and stimulatory glucose concentrations. Interestingly, out of three major serotonylated bands, two were downregulated in response to glucose stimulation (Figure 17A ⬅,⧗), while one remained unchanged in cells expressing Asn333Ser-TG2 (Figure 17A✦). Under stimulating conditions Ile331Asn-TG2 expression induced a novel band to appear (Figure 17A★), while a common band observed in all other lanes was diminished. The presence of this novel band may be an indication of proteolytic cleavage of the 72 kDa band (Figure 17A). A similar band pattern of serotonylation proteins was observed in an anti-serotonin immunoblot of WT and TG2-/- mice islets exposed to stimulatory glucose

concentrations. While the visible bands were similar in size, a difference in protein serotonylation was apparent between WT and TG2-/- mice. Out of the three prominently serotonylated bands, two were upregulated in pancreatic islets from TG2 null mice, while a decrease in serotonylation of the 72 kDa band was observed (Figure 17B).



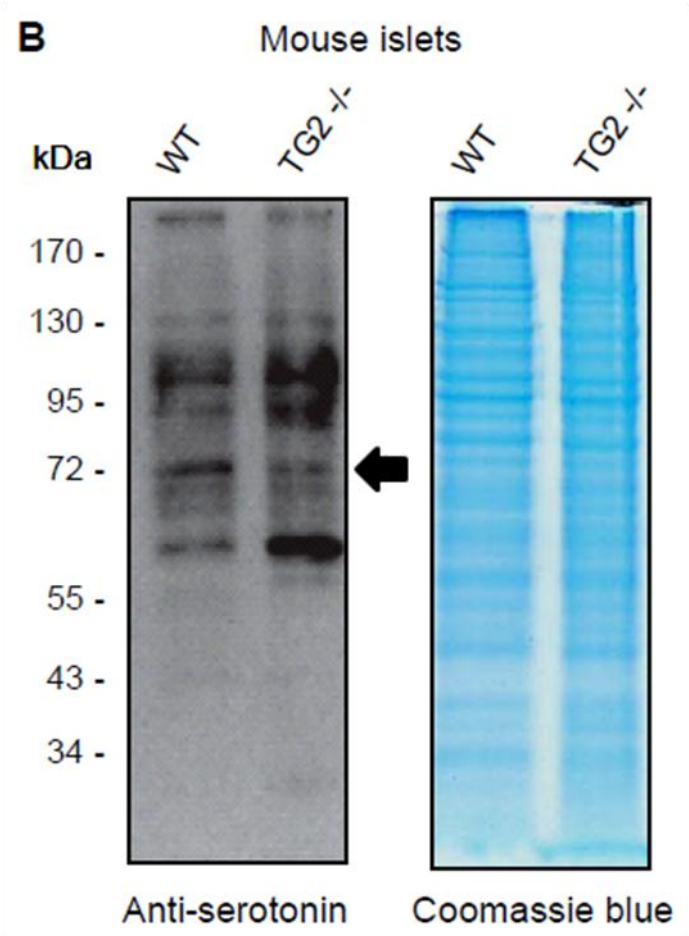


Figure 17. Comparison of whole cell lysate serotonylation.
A. INS-1E cells were cultured in 60 mm tissue culture plates, transfected, treated, and analyzed according to materials and methods section. B. Anti-serotonin immunoblot showing protein serotonylation in pancreatic islets in response to glucose stimulation from wild type (WT) and TG null mice (TG2-/-). Coomassie blue stained gel is shown as loading control. ⬅ represents 72 kDa band of interest (n=1).

9. TG2 has a large body of potential substrates for serotonylation.

To examine the possible substrates for serotonylation by TG2, INS-1E cell lysate was immunoblotted with anti-serotonin to identify bands of interest. The identity of the 72 kDa protein band apparent in the former protein serotonylation results shown in Figure 17 was investigated. Identical gels were stained with Coomassie Blue and the band was excised from the gel, digested with trypsin, and peptides were analyzed through mass

spectrometry. Several GTPases were identified, along with other secretory-associated proteins (vesicle/cytoskeletal associated proteins) (Table 5). These data are consistent with a recent report of an important role of serotonylation of GTPases in insulin secretion from beta cells.

Table 5. Mass spectrometry analysis of excised 72 kDa serotonylated band from INS-1E cell lysate.

log(e)	log(I)	% (measured)	% (corrected)	Unique	Mr	Description
-67.7	3.01	45	56	9	30	Mapre1, Microtubule-associated protein RP/EB family member 1 (APC-binding protein EB1)
-45.5	2.58	33	39	7	22.4	Rab1, Ras-related protein Rab-1A.
-40.3	2.45	21	33	5	26.9	Vapb, Vesicle-associated membrane protein-associated protein B (VAMP- associated protein B)
-37.9	2.35	21	24	4	23.5	Rab7a, Ras-related protein Rab-7a (Ras-related protein p23) (Ras-related protein BRL-RAS)
-29.3	2.33	22	30	4	23.5	Rab2a,
-22	2.39	7.5	9	1	22.2	Rab1b-ps1, Ras-related protein Rab-1B. Source: Uniprot/SWISSPROT P10536
-21	2.22	10	17	2	27.8	Vapa, Vesicle-associated membrane protein-associated protein A (VAMP- associated protein A)
-14.3	2.06	10	15	2	25.2	RAB5C, member RAS oncogene family Source: RefSeq_peptide NP_001099310
-11.8	2.13	13	22	2	24.7	Sec22b, Vesicle-trafficking protein SEC22b (SEC22 vesicle-trafficking protein homolog B)
-9.5	1.92	8.7	12	2	24.5	Rab11b, Ras-related protein Rab-11B. Source: Uniprot/SWISSPROT O35509
-6.7	1.92	6	8	2	34.4	actin related protein 2/3 complex, subunit 2 Source: RefSeq_peptide NP_001100389
-5.1	1.82	6	7	1	23.7	Rab5b, RAB5B, member RAS oncogene family Source: RefSeq_peptide NP_001073405
-3.2	1.65	4.8	5	1	23.4	Rab6b, similar to Ras-related protein Rab-6B [Source: RefSeq_peptide (XP_343460)]
-1.7	1.58	4.1	5	1	22.1	Rhob, Rho-related GTP-binding protein RhoB precursor.
-1.6	1.57	5.4	7	1	20.8	Rap1b, Ras-related protein Rap-1b precursor (GTP-binding protein smg p21B).
-1.5	1.9	4.5	5	1	25	Rab3a, Ras-related protein Rab-3A.
-59.4	2.68	40	47	7	23	Rab18, Ras-related protein Rab-18.

The masses determined for the proteolytic peptides are compared with masses calculated for theoretically possible enzymatic cleavage products for every sequence in a protein sequence database. The program used for data analysis was The Global Proteome Machine mass spectrum analysis system (GPM). The protein is identified based on an evaluation of this comparison and ranked by the number of positive matches. % - sequence coverage, Unique – number of unique peptides identified of particular protein in sample, Mr – molecular weight of protein. Log(e) Values <-3 indicates an acceptable level of confidence. Proteins with values >-3 were included for sake of discussion.

V. DISCUSSION

1. TG2 is expressed in pancreatic beta cells.

In a previous study, the expression levels of six tranglutaminase genes, TGM1 through TGM5 along with TGM7, in the human endocrine pancreas were analyzed. TGM2 was the only gene of those studied that had significant expression levels. TGM1 expression was the closest to that of TGM2, though that said, was barely detectable and largely dwarfed by TGM2's expression, which was observed to be several hundred fold greater [12]. Since this particular study focused on the expression of these genes in the endocrine pancreas as a whole, it was necessary to determine whether TG2 is expressed specifically in beta cells. INS-1E and MIN6 cell lysates were immunoblotted against anti-TG2 antibody. Both cell lines were found to naturally express TG2. It was then deemed essential to ensure that the cell lines were capable of being successfully transfected with wild-type and the naturally occurring mutants of TG2. The transfection of the INS-1E cell lines with the variants of TG2 was achieved and was able to be repeated with reliable outcomes.

TG2 has been shown to have a significant and contrasting role in apoptosis, depending upon the stimuli and the cell type in which it is present. Inhibition of TG2 expression in promonocytes was shown to suppress apoptosis [87]. A separate study utilizing fibroblast and neuronal cell lines indicated that overexpression of TG2 can sensitize cells to undergo apoptosis in response to certain stimuli [88]. In contrast to that finding another study reported that expression of TG2 in a human leukemia cell line imparted a survival advantage compared with those where TG2 activity was inhibited

[89]. It seemed fundamental to determine whether the effect of TG2 on beta cell function was due to its impact on cell survival. Also, TG2 has been observed to localize in the nucleus, where it can associate with transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) proteins. These associations have been proposed as a means by which TG2 can impart cell survival [90]. It was therefore important to illuminate whether TG2 localizes to specific intracellular compartments in pancreatic beta cells and if its cellular position changes in response to beta cell stimulation by glucose. In addition, we sought to validate the possibility that the functional differences of the mutant varieties of TG2 may alter their cellular localization.

Through the immunofluorescent study of wild-type, naturally occurring mutants, and transamidation function-dead Myc-TG2 transfected INS-1E cells, as well as MIN6 cells, not only were we able to analyze protein localization, but we were able to determine whether the overexpression of functional and non-functional TG2 had an impact on cell survival. No differences between the localization of the wild-type and mutant Myc-TG2 were observed, under both basal and stimulating concentrations of glucose in MIN6 and INS-1E cells. The Myc-tagged proteins were found to be positioned ubiquitously in the cytoplasm, while the nucleus appeared almost devoid of transfected TG2. No significant change in cell morphology or rate of apoptosis was apparent through visual inspection of the transfected cells. This finding is supported by a pancreatic islet TG2 knockout study that observed that in comparison to wild-type islets, $TG2^{-/-}$ islets displayed no differences in either size or morphology and that total

pancreatic insulin and glucagon content remained similar to that seen in wild-type islets [11].

2. GSIS is significantly altered by changes in TG2 levels and functionality.

TG2 has long since been implicated as having an impact on GSIS from pancreatic beta cells. TG2's TGase activity is sensitive to environmental Ca^{2+} and in addition, the inhibition of TG2 by nonspecific cross-linking inhibitors partially abolishes GSIS from isolated islets [72, 73]. TG2 knockout mice are glucose intolerant and possess reduced glucose-stimulated insulin secretion and display a phenotype analogous to MODY. [11]. TG2 has also been observed to modify the functionality of insulin secretion-modulating small GTPases through serotonylation [14]. However, due to TG2's reputation for exhibiting a diverse set of roles in response to altered environments, much remains to be elucidated regarding its role in GSIS.

We first sought to replicate the observation that TG2 knockout mice have impaired GSIS from pancreatic islets. Our own experimental results, indicating more than a 50% reduction in insulin secretion by isolated TG2 knockout mice islets under stimulating glucose concentrations in comparison to wild-type, was consistent with the previous study. Moreover, in the presence of basal glucose concentrations both the TG2 knockout and wild-type islets had similar GSIS. These results suggest that TG2's functional role in insulin secretion revolves around the beta cell's response to heightened stimulation by glucose and does not significantly impact the basal state and functionality of the beta cells.

Discussion

The pancreatic islets are composed of a number of cell types that alter each other's functioning through forms of intercellular signalling, such as paracrine signaling [22]. We felt it was crucial to determine whether the substantial loss in GSIS from pancreatic islets lacking TG2 function was, in actuality, due to the loss of TG2 function in the beta cells and not just the altered communication from its neighbouring cell types. It was also deemed essential to determine whether the expression of naturally occurring mutants, Met330Arg, Ile331Asn, and Asn333Ser -TG2 would generate a change in GSIS and if so, compare the degree of change for each mutant to that produced through the overexpression of wild-type and expression of loss-of-transamidation mutant Cys277Ala -TG2. We hypothesized that the expression of Cys277Ala -TG2 would have a high probability of generating a decrease in GSIS when compared to wild-type.

Overexpression of wild-type TG2, as well as expression of the Met330Arg and Asn333Ser mutant TG2, generated an increase in GSIS when compared to the control vector. Wild-type TG2 overexpression resulted in the greatest increase in GSIS, followed by Asn333Ser and Met330Arg respectively. In contrast, the expression of the Cys277Ala and Ile331Asn mutants resulted in equal or less GSIS when compared to the control vector. The expression of the Ile331Asn mutant caused the greatest decrease in GSIS compared to wild-type TG2, followed by Cys277Ala, Met330Arg, and Asn333Ser respectively (Figure 13). The fact that the expression of Ile331Asn -TG2 mutant consequently lowered GSIS to a greater degree than Cys277Ala in response to a stimulating glucose concentration warranted curiosity. If the transamidation activity of

TG2 was solely responsible for its functional role in insulin secretion, it would be suspected that the expression of loss-of-function mutant Cys277Ala -TG2 would generate the lowest GSIS from beta cells. However it should be noted that recently the Cys277Ala -TG2 mutant was observed to not only have a loss of transamidation function, but its GTP-binding ability is reduced almost 20% in comparison to wild-type TG2 [91]. The fact that the Ile331Asn -TG2 generates a lower level of GSIS than Cys277Ala -TG2 suggests that the transamidation activity may not be the only activity of TG2 involved in insulin secretion. The analysis of the functional activities of the mutants, particularly the transamidation activity, kinase activity, and GTP-binding ability, provided insight into the crucial TG2 activities in regards to insulin secretion. It should also be noted that overexpressing these mutant varieties of TG2 may actually be increasing the required level of stimulatory glucose to produce maximal insulin secretion. Further study into whether higher levels of glucose could raise the GSIS of beta overexpressing TG2 mutants to levels similar to cells overexpressing wild-type TG2 could be an asset.

3. Naturally occurring TG2 mutants each possess altered functional activities.

The stimulatory effect mediated by glucose on the release of insulin secretion is due to the intracellular increase of ATP, generated through the metabolism of glucose, followed by the intracellular increase in calcium ions through the action of ATP on ATP-sensitive potassium ion channels, the subsequent membrane depolarization, and the opening of voltage-gated Ca^{2+} channels. TG2's transamidation activity is initiated and maintained by increasing calcium ion concentrations, while it is inhibited in the presence of GTP [17, 92] and ATP [65]. Conversely, increasing concentrations of Ca^{2+} inhibit the

kinase activity of TG2 [65], along with its GTP-binding ability [93]. Since beta cells are exposed to an intracellular increase in ATP, GTP, and Ca^{2+} during glucose stimulated insulin release, it is acceptable to hypothesize that the kinase, GTP-binding, and transamidation activities of TG2 may each be affected, imparting changes in signaling patterns or protein-protein interactions that affect insulin secretion. We sought to determine the changes in these activities for each of the naturally occurring mutants and contrast these differences with wild-type TG2. Once this step in the study was completed it was possible to compare these changes in activities to resulting changes in GSIS of transfected cells.

3.1 Naturally occurring TG2 mutants have reduced transamidation activity in comparison to wild-type.

Porzio et al. had previously attempted to elucidate whether the naturally occurring mutants, Met330Arg, Ile331Asn, and Asn333Ser, possessed altered transamidation activity. The group observed a substantial reduction in the activity for each mutant, roughly by 50%, compared to wild-type TG2 [12]. When the difference in the endogenous levels of TG2 in COS7 and INS-1E cells in taken into account, a similar reduction in transamidation activity of the TG2 mutants was seen in our hands. We observed that the Asn333Ser mutation had the lowest transamidation activity, followed by Ile331Asn and Met330Arg respectively. The naturally occurring mutants are each positioned within the core domain of TG2 and in close proximity to the 335-histidine residue of the transamidation catalytic triad. In addition, one of the proposed Ca^{2+} binding sites by Kiraly et al. [57] is located directly adjacent to the mutants. One of the

asparagine residues in this Ca^{2+} binding site, Asn-329, is in fact adjacent to the mutation Met330Arg [57]. There is great potential that these mutations may be altering either the Ca^{2+}-binding affinity of TG2 or the transamidation-substrate interface through the induction of conformational changes or altered affinity for post-translational modifications. The alteration of the overall electrical charge in binding sites may also be occurring, as in the case of the Met330Arg mutation, where the uncharged methionine is replaced with the positively charged arginine amino acid. Common to all Ca^{2+} binding sites, whether typical or atypical, is a high negative surface potential, derived mainly from asparagine and glutamine residues [57]. A change in the surface potential of these sites, for example by the substitution of methionine with arginine, may affect their Ca^{2+}-binding potential.

A reduction in transamidation activity of TG2 mutants and a consistent decrease in insulin secretion from INS-1E cells overexpressing these mutants suggest a role for transamidation activity in insulin release. These results compliment earlier reports on the reduction of insulin secretion from rat beta cells treated with inhibitors of transglutaminase transamidation activity [72, 73]. However, the increase in Ca^{2+} concentration that leads to insulin secretion in beta cells in response to glucose is significantly lower (~150-250 nM) than the level of Ca^{2+} (~150-500 µM) shown to be required for TG2's transamidation activity [56, 94]. In addition, intracellular levels of GTP, which are known to inhibit transamidation activity, are many fold higher (~100 µM) than Ca^{2+} levels [95]. It is possible that ATP/GTP-binding function or other modifications of TG2, such as phosphorylation, alter the Ca^{2+} sensitivity of TG2, resulting in the activation of the transamidation function. However, the relatively lower

reduction in insulin secretion from cells overexpressing the transamidation loss-of-function Cys277Ala-TG2 mutant when compared to Ile331Asn -TG2 overexpression, suggests that TG2 transamidation activity, in itself, is not the sole activity involved in this process. Further supporting this notion is the observation that overexpression of the Asn333Ser -TG2 mutant, while possessing the greatest loss of transamidation activity compared to the other naturally occurring mutants, results in the least reduction in insulin secretion.

3.2 GTP and ATP-binding of naturally occurring mutations are differentially affected. TG2 GTP-binding, not ATP-binding, is sensitive to changes in beta cell glucose stimulation.

Prior to this study, the relationship between the ATP and GTP-binding activities of TG2 and GSIS in pancreatic islets had yet to be explored. The three naturally occurring mutation sites in TG2 used for this study are found in close proximity to the putative GTP/ATP binding loops in TG2's tertiary structure, in addition to its transamidation catalytic triad [54] (Figure 6). We hypothesized that one or more of these mutations may induce a change in the ATP or GTP-binding ability of TG2, in conjunction with alterations in transamidation activity. Our results supported this hypothesis, suggesting that the naturally occurring mutations in TG2 differentially affect GTP and ATP binding ability of TG2. Interestingly, the GTP-binding ability of TG2, and not the ATP-binding ability, was observed to be responsive to differing glucose concentrations. The GTP-binding ability of WT-TG2 was increased in response to glucose stimulation, while Ile331Asn -TG2 showed almost a complete loss of activity under both basal and stimulatory conditions. Ile331Asn -TG2's marked loss of function

in this regard correlated closely with its marked reduction of insulin secretion when compared to WT-TG2. The GTP-binding ability of Met330Arg -TG2 under stimulatory conditions is greater than that of Asn333Ser -TG2 (see Figure 15B), and Met330Arg -TG2 also has greater transamidating activity as well. It warrants investigation into why Asn333Ser -TG2 overexpression could generate higher GSIS than Met330Arg -TG2 (see Figure 13), if GTP-binding and transamidation activity do indeed have a GSIS enhancing effect. Furthermore, our finding of impaired/altered GTP binding function of naturally occurring TG2 mutants is consistent with an earlier report by Murthy et al [18] in which substitution of a conserved tryptophan residue at position 332 (which is flanked between two naturally occurring mutations in TG2) has been found to significantly reduce GTP binding.

A previous study has compiled evidence supporting the concept that increasing levels of GTP can confer stability and inhibit the spontaneous loss of transamidation activity when stimulated with Ca^{2+}. GDP and ATP were shown to also provide a similar effect, however, not as substantially as GTP [96]. In addition, this study found that the expression of a TG2 mutant, S171E-TG2, which does not bind or hydrolyze GTP and still displays normal transamidation activity *in vitro* [67], did not display transamidation activity *in vivo*. In fact, the level of transamidation exhibited by the S171E-TG2 mutant *in vivo* was comparable to that of the Cys277Ala -TG2 mutant [96]. GTP-binding ability may protect against proteosomal degradation and may provide protection against inhibitory post-translational modifications that may impede TG2's transamidation activity. The enhanced GTP-binding ability of Asn333Ser -TG2 under basal conditions

may very well impart an advantage that in part makes up for the substantial loss in transamidation activity. Moreover, the greater GTP-binding of Met330Arg -TG2 under stimulatory conditions may be competing with its transamidation activity, resulting in the relative reduction of insulin secretion in comparison to Asn333Ser -TG2 expressing cells. Apart from the concept that TG2's GTP-binding is altering its transamidation activity, the GTP-binding in itself may be directly influencing TG2's function as a G-protein. PLC activation by TG2's G-protein activity [17] could possibly be enhancing GSIS through the generation of IP_3 and DAG. The fact that the GTP-binding devoid Ile331Asn -TG2 mutant results in a greater reduction in GSIS than TGase loss of function Cys277Ala -TG2 mutant clearly highlights the possibility that another function of TG2 is involved, separate from its TGase activity. Additional signaling pathways involved in modulating insulin release from beta cells may be influenced by TG2's G-protein activity apart from PLC, which warrants further investigation. We propose that TG2 may be directly modulating insulin secretion through effector proteins as a G-protein, in addition to the transamidation-mediated serotonylation mechanism proposed by Paulmann *et al* [14] (Figure 18).

The variations in ATP-binding of the naturally occurring mutant TG2's did not show any correlation to GSIS. Met330Arg -TG2 expression exhibited GSIS quite lower than that of Asn333Ser -TG2, despite the fact that its ATP-binding affinity was significantly greater than that of Asn333Ser -TG2 and comparable to WT-TG2. In addition, Ile331Asn -TG2 expression resulted in a greater reduction in insulin secretion than Asn333Ser -TG2, even though their ATP-binding abilities were very similar. If the

ATP-binding ability of TG2 was closely involved in modulating GSIS, it would have been expected that the expression of Met330Arg -TG2 would generate an enhancement of GSIS akin to WT-TG2. The lack of regulation of this activity by the presence or absence of stimulatory conditions further suggests that the ATP-binding ability of TG2 is not involved in the beta cells response to elevated glucose. However, since none of the naturally occurring mutations resulted in a complete loss of this activity, one cannot completely rule out some contribution to function, as it may be possible that only a small fraction of this ability is required for normal beta cell operation.

While ATP and GTP share similar binding sites, GTP-binding utilizes two exclusive sites, Ser^{482} and Arg^{580}. It is believed that these two sites are responsible for the relatively stronger ligand-protein binding interaction of GTP to TG2 compared with ATP [68]. This weaker binding strength of ATP may be responsible for the lesser degree of stability it provides to TG2 in relation to GTP. In addition, the GTP-binding exclusive sites on TG2 could conceivably be linked to the modulation of TG2's GTP-binding ability in response to stimulatory glucose in beta cells. Whether these sites are contributing to this affect through the strengthening of the GTP binding or are influencing GTP-binding through their selective post-translational modification in response to altered environmental stimuli remains to be determined.

Earlier reports have shown alterations in the transamidation activity of TG2 depending on which of the two nucleotide states it is associated with. The nonhydrolyzable GTP analog, GTPγS, functioned as a potent inhibitor of

transglutaminase activity, to an even greater extent than GTP. GDP was shown to have the least significant impact upon transglutaminase activity [92, 97]. Subtle differences in the conformations induced by GTP versus GDP-binding could possibly be accountable for this effect. TG2 is capable of hydrolyzing GTP with a turnover number comparable to the intrinsic rates of GTP hydrolysis determined for Gα subunits, and the GTPase-activating protein (GAP)-catalyzed hydrolytic rates of small G proteins [98]. In addition to hydrolyzing GTP, TG2 is also capable of hydrolyzing ATP at a similar rate [62]. A past study implicated the core domain of TG2 in possessing the hydrolytic sites for ATP and GTP. It was thought that these hydrolysis sites were found within a 47 amino acid region at the start of the core domain, including residues 138 to 185 of TG2 [86]. Further investigation observed an impairment of GTP-binding and hydrolysis, without loss of TGase activity, as a result of the substitution of Lys^{173}, supporting the involvement of this region in TG2's hydrolytic activity [67]. It may be entirely possible that the naturally occurring mutations analyzed in this thesis, not only affect GTP-binding, but the hydrolytic activity as well, thereby altering TG2's conformation and activation state. The elevated GTP-binding observed by mutant Asn333Ser -TG2 during basal conditions may be due to a moderate reduction in GTPase activity and not an increased affinity for GTP. The inverse may apply for the mutant Ile331Asn -TG2, where a moderate enhancement of GTPase activity leads to lowered levels of bound TG2. Two proposed Ca^{2+}-binding sites of TG2 that are positioned sterically close to the hydrophobic pocket for GTP/GDP binding in its tertiary structure, when mutated, were shown to enhance GTP-binding and basal GTPase activity of TG2 [57, 66].

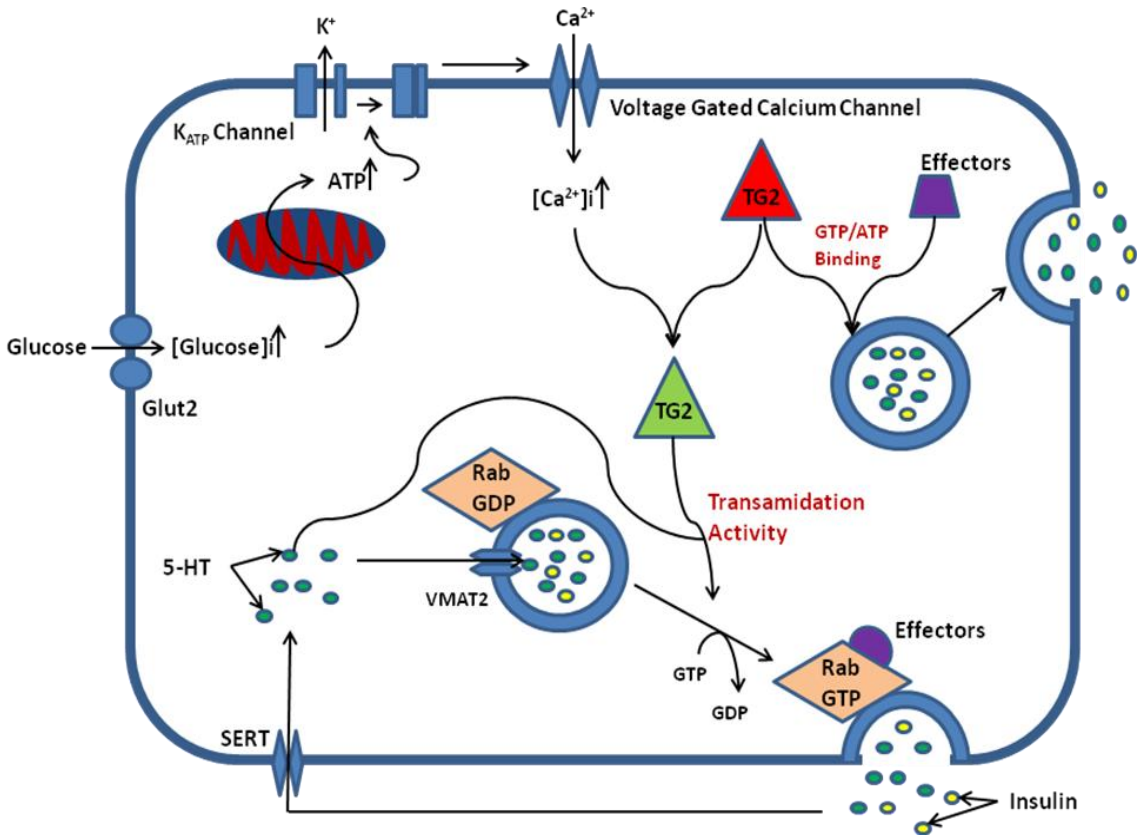


Figure 18. Newly proposed model of TG2's modulation of insulin secretion in beta cells.
Glucose enters beta cells through Glut2 transporter, is metabolized through the glycolytic pathway, TCA cycle, and the electron transport chain to produce ATP. ATP closes ATP-sensitive K^+ channels, which depolarizes the membrane, opening voltage-gated Ca^{2+} channels. The increase in intracellular calcium ions promotes insulin release from secretory vesicles. TG2's transamidation activity is stimulated by calcium ion binding. TG2's GTP/ATP binding (G-protein/kinase function) may be activated by protein-protein interactions promoted by increased intracellular Ca^{2+}. VMAT2, vesicular monoamine transporter 2. SERT, serotonin transporter.

3.3 Naturally occurring TG2 mutants have altered kinase activity.

TG2 has been previously shown to phosphorylate a number of proteins, including IGFBP3 [15] Rb [65], and the tail and core histones H1, H2A, H2B, H3, and H4 [63]. IGFBP3 has recently been observed to have a role in insulin secretion from beta cells,

where overexpression of WT and IGF-binding-deficient mutants were shown to negatively affect GSIS, ATP content, and Akt phosphorylation [16]. The naturally occurring mutations of TG2 each generated a reduction in IGFBP3 and H4 phosphorylation in relation to WT-TG2. Whereas the reduction was noteworthy (~25%), no statistically significant difference of kinase function was present amongst the mutants, except for Ile331Asn-TG2. While we cannot ignore the possibility that this consistent reduction in kinase activity in all of the mutants is somehow impacting insulin secretion, the data do not show a clear correlation with the changes observed in GSIS due to the expression of the mutants in the INS-1E cells. Additionally, since the concept of TG2 having kinase function is rather novel, the identification of proven substrates to implicate this activity in the modulation of insulin secretion from beta cells is limited. In regards to the source of the reduction of the activity by the mutations, there are two possibilities worth discussing. Firstly, the mutations may be directly impacting the availability of the kinase sites to the substrates due to their close proximity in the tertiary structure. Secondly, the mutations may be resulting in the alteration of TG2's ability to undergo post-translational modifications that affect its functional activities. It has been shown that the phosphorylation of TG2 by PKA can enhance the kinase activity of TG2 [65], implying that the loss of kinase function detected in these mutants could result from a loss of this particular modification of TG2. Another observed means of post-translational modification is the Ca^{2+}-regulated S-nitrosylation of TG2 by the nitric oxide (NO) carrier, S-nitrosocysteine (CysNO), which results in the inhibition of its transamidation activity [99].

3.4 A 72 kDa band which is differentially serotonylated by the expression of naturally occurring TG2 mutants is investigated. Serotonylation by TG2 is glucose-sensitive.

Recently, the covalent coupling of serotonin to small G-proteins, mediated by TG2, has been implicated in GSIS from pancreatic beta cells [14]. The two GTPases that were identified to be serotonylated by TG2 were Rab3a and Rab27a, which are both involved in regulating vesicular transport and docking within the beta cells [82, 83]. The serotonylation of these proteins has been observed to constitutively activate them, inhibiting their GTPase activity, while leaving the GTP-binding ability intact [100]. The glutamine residue that is serotonylated is found within a sequence conserved amongst all Rab proteins, DTAGQE [101]. In addition to Rab3a and Rab27a, studies have shown that transglutaminases are also able to serotonylate Rho [100] and Rab4 [77]. We analyzed the change in the serotonylation pattern in whole cell lysates of pancreatic beta cells as a result of the expression of the naturally occurring mutants, in response to basal and stimulatory conditions. A serotonylated protein band at approximately 72 kDa was dynamically altered especially in the pancreatic islets from TG2-/- mice and in the INS-1E cells expressing the Ile331Asn -TG2 mutant, under stimulatory conditions. Since the Ile331Asn -TG2 mutation represents the form of TG2 that produces the greatest reduction in GSIS, this suggested that the 72 kDa protein band may be an important target for serotonylation in regards to GSIS modulation. Analysis of the 72 kDa serotonylated band by mass spectrometry identified a number of Rab GTPases. It should be noted that the molecular masses of the proteins identified by mass spectrometry are lower than the molecular mass of the serotonylated band excised from the Coomassie blue stained reducing gel. This would imply that these proteins are not only serotonylated, but also

crosslinked. The unique serotonylated band generated by the expression of Ile331Asn -TG2 in response to glucose stimulation may be due to perturbed crosslinking of serotonylated proteins. Alternatively, it is now adequately established that the constitutive activation of small GTPases targets them for proteasomal degradation via ubiquitination [102]. Paulmann et al., also observed an enhancement of cycling between activation through serotonylation and inactivation by proteosomal degradation only in the presence of stimulatory levels of glucose in beta cells [14]. The expression of the Ile331Asn -TG2 mutant may contribute to an escalation of proteasomal degradation.

As observed for the *in situ* transamidation assay, there was a lack of correlation between insulin secretion and the serotonylation levels of the 72 kDa band in the vector and Cys277Ala -TG2 control groups. The 72 kDa band, which is present under stimulatory conditions for vector control, is completely absent when Cys277Ala -TG2 is overexpressed under stimulatory conditions (Figure 17A). If protein serotonylation was the sole contributor to TG2's modulation of GSIS, it would be reasonable to expect a greater loss of GSIS than what was observed while overexpressing Cys277Ala -TG2 in comparison to vector control (Figure 13). Protein serotonylation *per se* is not adequate to fully elucidate the insulin secretory phenotype displayed by INS-1E cells overexpressing TG2 mutants. Alongside the numerous Rab GTPases identified by mass spectrometry, several actin, tubulin, and vesicle-associated proteins were found in the 72 kDa serotonylated band as well. As Rab GTPases regulate many steps of membrane traffic, including vesicle formation [103], vesicle movement along actin and tubulin networks [104], and vesicle membrane fusion [105], it is reasonable to consider that serotonylation

of these cytoskeletal and vesicle membrane network proteins is involved in GSIS from beta cells.

VI. CONCLUSIONS

The data presented in this thesis demonstrates that the naturally occurring mutations in TG2 differentially affect transamidation, kinase, and ATP/GTP binding functions of TG2. This implicates the transamidation, kinase, and ATP/GTP binding functions of TG2 as being associated with each other and regulated in a coordinated manner. It is expected that more than one activity of TG2 is impacting insulin release. Prior to this thesis, the role of TG2 in the modulation of GSIS from pancreatic beta cells was only believed to involve its transamidation activity. While data in this thesis supports this notion, particularly in regard to TG2's ability to modify the activity of GSIS-modulating small GTPases through serotonylation, it is clear that its transamidation activity alone is not fully responsible for its role in beta cells. We present new evidence that identifies the GTP-binding function of TG2 of having a significant impact on GSIS from pancreatic beta cells in addition to TG2's TGase activity.

VII. FUTURE DIRECTIONS

The research reported in this thesis supports the concept that the TGase activity of TG2 is involved in GSIS from beta cells, while also illuminating the importance of TG2's other functional activities in GSIS as well, particularly its kinase and GTP-binding activities. While this finding can effectively provide further insight into the means by which TG2 modulates insulin secretion from beta cells, further study is required to elucidate the exact interactions that are mediating its effect on GSIS. Novel substrates for protein serotonylation by TG2 need to be identified that function in altering insulin secretion. Selectively mutating serotonylation sites on prospective proteins, and expressing these mutant proteins within beta cells, will reveal which substrates have the greatest relative impact on insulin secretion. There are several small GTPases that we identified in a serotonylated band from INS-1E beta cells that have been previously shown to influence vesicle formation, movement, and membrane fusion that may be of interest. Some examples include Rab5, which has been implicated in ligand sequestration in clathrin-coated pits [103], Rab6, which was shown to interact with a kinesin-like protein and is thought to regulate directional membrane transport of intracellular organelles [104], and Rab1, which was observed to recruit an effector capable of promoting a cis-SNARE complex formation that promotes vesicle targeting to the Golgi-apparatus [105].

Aside from further delving into possible serotonylation substrates, it will be quite important to analyze whether the observed impact that the naturally occurring mutations have on TG2's GTP-binding ability arose from changes in GTP-binding or GTP

hydrolysis. This would be possible by subjecting the purified mutants to a simple GTPase colorimetric assay, which would determine the rate of conversion of GTP to GDP by TG2. While the data presented in this thesis support the concept that other activities of TG2 are involved in insulin secretion in addition to its transamidation activity, it would be beneficial to perform a study that would definitively prove this is indeed the case. This may be accomplished through the generation of several TG2 mutants, by site-directed mutagenesis, that are each deficient in only one functional activity of TG2, which can be analyzed for their effect on GSIS. Murthy *et al.*, found a mutation (W241A-TG2) that lacked transamidation activity, while maintaining its GTP-binding function [18]. In addition, Ruan *et al*., observed that an arginine mutation (R580A-TG2) resulted in a maximal loss of GTP-binding with no significant loss of TGase function [91].

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