O-GLCNAC IS A GLUCOSE SENSOR IN THE PANCREATIC β CELL

by

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(Under the Direction of Lance Wells)

ABSTRACT

O-linked β-*N*-acetylglucosamine (O-GlcNAc) is a ubiquitous post-translational protein modification found on serine and threonine amino acid residues of intracellular proteins. This inducible and dynamic PTM is mediated by two cycling enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) in response to environmental stimuli. The nucleotide-sugar donor UDP-GlcNAc is the end product of the hexosamine biosynthetic pathway (HBP) and is responsive to glucose levels entering the cell. Provided its proposed role as a cellular nutrient sensor, O-GlcNAc has been implicated in contributing to the progression of type II diabetes. However, the molecular role for this PTM in the glucose-responsive, insulin secreting pancreatic β cell remains unclear. In this dissertation, I set out to study the role of O-GlcNAc in regulating molecular events in the β cell, specifically at the levels of insulin secretion and transcription. Using directed pharmacological approaches in the mouse insulinoma-6 (Min6) cell line, I demonstrate that elevating nuclear O-GlcNAc preserves glucose stimulated insulin secretion during chronic hyperglycemia. This observed secretory effect directly correlates with O-GlcNAc-induced elevation in perinuclear insulin under basal and prolonged hyperglycemic conditions. The molecular mechanism for these observed changes appears to be, at least in part, due to elevated O-GlcNAc-dependent increases in *Ins1* and *Ins2*

mRNA levels via elevations in histone H3 transcriptional activation marks. Further, whole transcriptome shotgun sequencing reveals that hyperglycemia altered gene transcription is restricted to a subset of genes and that the majority of genes regulated by inhibiting OGA levels are similarly regulated by a shift from euglycemic to hyperglycemic conditions. Thus, my work demonstrates a role for O-GlcNAc as a glucose sensor and modulator of gene transcription in pancreatic β cells.

INDEX WORDS: O-GlcNAc, OGT, OGA, HBP, Min6 pancreatic β cells, insulin secretion, insulin gene transcription, epigenetics, RNA-sequencing

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DEDICATION

I would like to first dedicate this thesis to my wife and best friend Brittany Durning. Thank you for all of your love, support and patience throughout this lengthy process, I could not have done it without you. I also want to dedicate this thesis to my parents, Charles and Lee-Ann Durning. Thank you for everything you have provided me throughout my life, but most importantly thank you for molding me into the person I am today. To my wife, Dad and Mom: I love you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

This dissertation is composed of four chapters.

The first chapter provides a brief summary of post-translational protein modifications with an emphasis on O-GlcNAc and its regulating enzymes. I also discuss previous research highlighting potential roles for this PTM in type II diabetes, epigenetic modulation and pancreatic β cells.

The second chapter is an invited review that I co-first authored for Critical Reviews in Biochemistry and Molecular Biology, entitled "Functional O-GlcNAc Modifications: Implications in molecular regulation and pathophysiology", that is currently under review. Specifically, I composed the: abstract, last two paragraphs of introduction, section 3, section 4, section 5 and concluding remarks. I also produced the following figures: 2.1, 2.3, 2.4, 2.5 and 2.6 A and B. This article focuses on data within the last five years connecting O-GlcNAc with specific biological processes and human disease. While most of this review does not directly pertain to my thesis research, it does touch on O-GlcNAcylation as an epigenetic regulator. As I will discuss in a latter section, O-GlcNAc appears to control insulin gene activation in pancreatic β cells through a chromatin-remodeling event.

Chapter 3 serves as the centerpiece of my dissertation. It is my first author manuscript that has been submitted for publication and is current under review. Several

groups had previously shown multiple proteins in the pancreatic β cell to be O-GlcNAc modified. However, none of these findings determined a functional role for this modification within this setting. My initial findings demonstrate that increasing O-GlcNAc through targeted pharmacological treatment can increase intracellular insulin levels in a mouse β cell line. Interestingly, this increase appears to regulate hormone biosynthesis in a non-glucose dependent manner and protects cells from chronic glucotoxicity commonly observed in type II diabetes. The remainder of this chapter details my work identifying O-GlcNAc as a nutrient sensor in mouse β cells, regulating insulin gene activation though an epigenetic mechanism. My results also suggest O-GlcNAc is a more global gene regulator in this cell line according to deep sequencing analysis.

The fourth and final chapter represents the overall conclusions from my thesis work. Here I discuss my scientific findings and how these results impact the scientific community. I also touch on future experiments required to determine the molecular mechanism for O-GlcNAc-mediated insulin gene control and how this knowledge may contribute to diabetes treatment.

2. Literature Review

Protein regulation in the cell is a complex process. A unique class of post-translation protein modifications (PTMs) immensely contributes to this coordination. Differing from conventional regulatory mechanisms, PTMs are non-template driven events that greatly enhance protein functional diversity within biological systems. Glycosylation represents a major component of this class and uses distinct amino acid-linked sugar additions to exert its affects [1]. Extensive efforts to identify the characteristics of these structures established numerous glycosylation subsets varying in their substrate attachment, chemical bond arrangement and saccharide composition. Specifically, this work established *N*-linked glycans (sugar additions to asparagine

residues), *O*-linked glycans (sugar additions to serine and threonine residues) and glycoaminoglycans (GAGs, free or bound polypeptide additions) as the predominant classes that have since evolved [1, 2]. In addition, the microheterogeneity of these glycans can be further expanded by phosphorylation [3, 4], acetylation [5] and sulfation [6] modifications on glycan structures. Improper glycan biosynthesis or regulation have been demonstrated to influence multiple classes of human disease [7, 8] and several infectious disease pathologies [9-11], illustrating the biological importance for O-GlcNAc.

Initially, the field of glycobiology was thought to consist primarily of cell-surface and extracellular moieties that trafficked through the secretory pathway. This was understandable at the time considering every identified glycan or glycoconjugate was extracellular or membrane-bound. However, in 1984 the Hart lab identified a novel protein-saccharide linkage, *O*-linked β-*N*-acetylglucosamine (O-GlcNAc), that was abundantly expressed inside lymphocytes [12]. Successive studies verified this finding and further demonstrated that intracellular proteins from organelles were highly O-GlcNAc modified [13]. Interestingly, this modification appeared to be a single-sugar moiety addition found in both the cytoplasm and nucleus that responded rapidly to signaling changes [14-16]. Then in 1996, investigation into a member of the small heat shock protein family, αB-crystallin, showed that the carbohydrate protein addition turned-over exceedingly faster than the protein backbone itself [17]. Together these reports portrayed O-GlcNAc as a dynamic, inducible, nucleocytoplasmic regulator that challenged the glycosylation central dogma.

In nearly thirty years since its discovery, countless groups have advanced our understanding of how O-GlcNAc is monitored and its functional role within the cell. Although identified in T lymphocytes, it has been observed in almost all eukaryotic organisms that range from single-cell pathogens to multicellular mammals [18-22]. Furthermore, the ambiguity of this PTM has proven to be seemingly limitless considering

the diverse molecular systems it affects [23-26]. Recent investigations are beginning to connect occupancy with function [27-29] and continue to define specific roles for O-GlcNAc in metabolic disease, including: diabetes [30], cardiomyopathy [31], Alzheimer's [32] and cancer [33]. In the remainder of this chapter I will discuss the cycling dynamics of O-GlcNAc, how it influences diabetes, its newly identified role in epigenetics and outline pancreatic β cell function.

The Hexosamine Biosynthetic Pathway (HBP) and O-GlcNAc Cycling Enzymes

Environmental responsiveness is crucial for cell regulation. The glucose-responsive HBP is a nutrient-sensing pathway that helps compensate for these changes [34]. A small portion of intracellular glucose (~4-5%) is directed into the HBP when fructose-6-phosphate is converted into glucosamine-6-phosphate by the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) [35, 36]. Apart from chemical conversion, free glucosamine can directly feed into the glucosamine-6-phosphate step of the HBP and proceed to substrate formation [37]. The pathway culminates in the nucleotide-sugar donor UDP-GlcNAc that serves as a substrate for complex glycosylation events, alternative nucleotide-sugar conversion and protein O-GlcNAcylation. Glucose is not the only metabolite influencing the HBP. Amino acids, free fatty acids and nucleotide availability have all been shown to impact levels of the substrate pool [36, 38, 39].

Serine and threonine residues are the targets for O-GlcNAc cycling, using the terminal hydroxyl group to add and remove the sugar moiety [40]. Unlike classical O-linked glycosylation that is synthesized by glycotransferases in the ER and Golgi [41], the O-GlcNAc enzymes reside in the cytoplasm and nucleus. *O*-GlcNAc transferase (OGT) and β-N-acetylglucosaminidase (OGA) are the only two enzymes in most eukaryotic systems responsible for O-GlcNAc addition and removal respectively (Figure 1.1) [42, 43]. Each enzyme is synthesized from a single gene, with *ogt* mapping to

chromosome Xq13.1 [44] and the OGA-encoding *mgea5* located on chromosome 10q24.1-24.3 [45].

OGT is in the class of glycosyltransferases that attach sugar molecules through a dehydration reaction. Early work described the enzyme as a heterotrimer consisting of two p110 subunits and a single p78 subunit, but was later revised since p78 was not localized in tissues exhibiting OGT activity [46]. The p110 subunit contains an *N*-terminal tetratricopeptide repeat (TPR) rich region and a *C*-terminal catalytic domain [46-48]. Comprehensive investigation using human cDNA samples revealed three distinct splice variants of OGT, differing within the TPR region. Gene splicing can give rise to nucleocytoplasmic OGT (ncOGT), mitochondrial-targeting OGT (mOGT) and a short OGT (sOGT) [49]. While sequence and substrate specificity for these enzyme variants remain elusive, their differing TPR repeat units appear to be crucial in facilitating interaction [50]. Because complete OGT knockout is embryonic lethal [51], knockdown and overexpression approaches are generally used as genetic tools. There are also several OGT-specific pharmacological compounds developed to block enzyme activity [52], but their effectiveness has not been fully validated.

OGA was originally thought to be part of the hyaluronidase family, but this was eventually discounted because it lacks the typical enzymatic activity exhibited by this group [53, 54]. It is a two-subunit protein with an *N*-terminal glycoside hydrolase catalytic domain [55] and a putative histone acetyltransferase (HAT) *C*-terminal region [56]. A caspase 3 cleavage site-containing region that is processed during apoptosis connects these amino and carboxyl termini [57]. Like its counterpart, OGA can also be alternatively spliced. However, to date only two isoforms have been identified: the predominantly cytoplasmic full length protein (OGA-L) and the shorter nuclear protein (OGA-S) that lacks the *C*-terminal domain [45]. Experiments directed at kinetics and substrate recognition have shown that these OGA isoforms display different *in vitro* characteristics [58, 59] and suggest they may interact with distinct protein groups [60].

Like OGT, several strategies exist to manipulate OGA levels. Enzyme knockdown or overexpression are common genetic modulations, but transfection efficiency differs drastically between cell types. There are several pharmacological inhibitors that specifically target OGA. O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc) was the first and most widely used inhibitor [61], but was found to display multiple off target affects. Recently, more potent inhibitors have been established that exhibit higher substrate specificity. These chemical compounds include: GlcNAcstatin [62], 1,2-dideoxy-2′-methyl-α-D-glucopyranoso-[2,1-d]-Δ2′-thiazoline (NButGT) [59] and Thiamet-G [63].

Initial studies displaying the importance of these cycling enzymes revealed that complete OGT knockouts were embryonic lethal [51] and OGA disruption caused neonatal death or severe growth defects [64]. Additional work in *Caenorhabditis elegans* showed that both OGT and OGA null organisms displayed striking metabolic changes, suppressed dauer larvae formation and caused insulin-mediated lifespan defects [19, 65, 66]. Even partial dysregulation of O-GlcNAc levels during embryogenesis proved to be problematic considering enzyme overexpression in zebrafish delayed epiboly and caused cytoskeletal disorganization [67]. But development is not the only biological process impacted by O-GlcNAc. Provided its nutrient and glucose sensing capabilities, this PTM is thought to contribute to the physiology of numerous metabolic diseases, with a heavy emphasis on diabetes.

Type II Diabetes Mellitus and O-GlcNAc: How a PTM influences this metabolic disease.

The Western world is currently in the midst of a type II diabetes (TIID) epidemic. As of 2011, 8.3% of the United States population was clinically diagnosed with the disease and 79 million people (or roughly 25.4%) were prediabetic [68]. TIID is a chronic illness that generally progresses through several pathophysiological events. The hallmark

symptoms are hyperglycemia and hyperinsulinemia that present due to insulin resistance at peripheral tissues [69-71].

Continuous circulating high glucose interferes with insulin-responsive pathways in the liver [72, 73], adipose tissue [74] and skeletal muscle [75]. At the molecular level, this resistance is caused by a dramatic reduction in binding between the secreted insulin hormone and its corresponding receptor [76, 77]. Disruption of this interaction dampens the insulin signaling cascade and in part blocks translocation of glucose transporters to the cell membrane [78]. Without this transporter present, circulating glucose is unable to enter the cells and remains in the blood. Prolonged hyperglycemia has been well documented in damaging multiple organ systems [79], including the pancreatic β-cell [80, 81]. In efforts to keep up with the hyperglycemic glucose demand, β-cells respond by synthesizing and secreting insulin. Unfortunately, the previously described non-responsive tissues cannot utilize the hormone and hyperinsulinemia ensues [82]. If these symptoms persist without therapeutic intervention, a phenomenon known as β-cell fatigue occurs.

Being a glucose-responsive cell type and the major producer of insulin, pancreatic β-cells are indispensible when it comes to controlling glucose concentrations. Within the hyperglycemic/hyperinsulinemic type II diabetes environment β cells experience non-relenting stimulation that inherently induces molecular stress. These unfavorable conditions cause glucotoxicity, attenuate insulin secretion and promote activation of apoptotic signaling networks [83, 84]. While significant pharmaceutical advancements have enabled disease treatment, no cure is currently available. This is most likely due to the incredibly complex nature of diabetes, underscoring the need to better understand the molecular basis of β cell biology [85].

O-GlcNAcylation has been reported to be involved in the establishment and progression of diabetes. Initial studies suggesting this connection showed that insulin sensitive-tissues expressed higher levels of OGT compared to their unresponsive counterparts [86, 87]. Further, there is an increase in O-GlcNAc-modification during insulin resistance [88]. Ensuing work verified this finding and showed that O-GlcNAc elevation directly correlated with insulin resistance in multiple insulin-responsive tissues. Specifically, increased nucleocytoplasmic O-GlcNAcylation in 3T3-L1 adipocytes promoted insulin resistance through defects in Akt signaling [89]. Increased HBP flux and O-GlcNAc levels strongly correlated with glucose transporter impairment and peripheral insulin resistance in skeletal muscle [90, 91]. O-GlcNAcylation is blocked under diabetic symptoms and alters transcription factor regulation of hepatic genes [92]. Additional studies showed that O-GlcNAc affects more than just insulin-responsive peripheral tissues. There is a long established notion that O-GlcNAc plays a functionally relevant role in the pancreatic β cell that will be discussed in a later section.

O-GlcNAc as an Epigenetic Regulator

While still in its adolescence, the field of epigenetics is already drastically changing the way we look at gene regulation. Early investigations examining gene expression patterns during development established that transcription factor binding to *cis*-acting DNA elements did not completely account for the observed changes. Instead, post-translational modification of nucleotides, histones and nucleosome complexes dramatically influenced gene activity through predisposed genetic inheritance or environmental influence [93, 94]. Epigenetic marks are a highly complex landscape of post-translational additions that differ from person to person, and even between cell types. There is a myriad of identified modifications that differentially modulate gene accessibility and activation, including: acetylation [95], methylation [96], ubiquitination [97], SUMOlation [98], citrullination [99] and phosphorylation [100]. To further

complicate matters, several of these PTMs can modify multiple amino acid residues sharing similar biochemical properties or exist as mono-, bi- or tri-moiety structures to impart distinct regulatory roles. This mechanism is extremely important for controlling gene expression, but molecular complexity has prevented researchers from understanding the hierarchical order. As expected, dysregulation of these epigenetic control mechanisms can result in multi-system disorders and disease [101-103]. In fact, considerable therapeutic efforts have been aimed at designing treatments that target epigenetic cycling enzymes as possible treatments [104, 105].

Extensive evidence suggests O-GlcNAc plays a major role in determining the epigenetic landscape within the cell. Initial studies found elevated O-GlcNAc levels at transcriptionally repressed chromosomal regions in *D. melanogaster* [21]. Within flies OGT is encoded by the Polycomb group (PcG) gene super, sex combs (sxc) and increases PcG-mediated gene silencing through an O-GlcNAc-derived mechanism [106]. More recent studies branching into mammalian systems verify these previous findings. Several groups demonstrate histone tails themselves are directly O-GlcNAc modified (Table 1.1) to regulate mitotic and cell cycle events [107-109]. While histone modification patterns remain relatively unknown, Ser112 O-GlcNAcylation on histone H2B appears to facilitate monoubiquitination at Lys120 for transcriptional activation [110]. Other works have shown that the O-GlcNAc influences components of chromatin-regulatory complexes (Table 1.2). This was first observed in studies showing OGT associates with mSin3A to cooperatively repress transcription in parallel with histone deacetylation [111, 112]. Additional data revealed OGT binds with ten eleven translocation (TET) enzymes at transcriptional start sites and mediates gene expression through O-GlcNAc addition to histone H2B [113]. Subsequent studies revealed OGT and TET enzyme interactions could regulate histone H3K4 methylation events [114] and embryonic stem cell differentiation [115, 116]. Similarly, O-GlcNAc modification of a histone lysine

methyltransferase MLL5 influenced H3K4 methylation events to facilitate retinoic-acid-induced granulopoiesis in human HL60 premelocytes [117]. Other work revealed OGT was essential for host cell factor 1 (HCF1) processing and promoted chromatin complex formation to regulate the cell cycle [114, 118, 119]. Together these findings implicate O-GlcNAc in controlling epigenetic gene regulation, but what about in the pancreatic β cell?

There is mounting evidence suggesting type II diabetes disease progression may be due to epigenetic deregulation [120-122]. With the overwhelming data implicating O-GlcNAc in the pathophysiology of this disease and its ability to influence epigenetics, the pursuit of investigating a functional role between the two is logical.

Insulin Dynamics in the Pancreatic β cell and a Potential Role for O-GlcNAc

Pancreatic islets of Langerhans are highly vascularized mini-organs made up of several cell types. Insulin-producing β cells and glucagon-producing α cells are the most well known due to their importance in regulating circulating glucose levels. The three more unfamiliar cell types are the somatostatin-producing δ cells, pancreatic polypeptide-producing PP cells and the most recently discovered ghrelin-producing ε cells [123]. The typical architecture in rodents consists of a core-mantle arrangement with centrally located β cells and all other islet cells surrounding the periphery [124-126]. Human islet organization is slightly different, where intermingling islet cell architecture exists [124, 127, 128]. While each plays a crucial role in maintaining metabolic homeostasis, the majority of pancreatic research has focused on β cells.

The primary role for pancreatic β cells is to produce insulin hormone. This powerful regulator influences the storage of multiple metabolites through actions in the liver, adipose tissue and skeletal muscle. Originally thought to be produced solely in the pancreas, recent data has revealed neuronal insulin production in the brain [129]. Levels

of circulating blood glucose are the primary regulators of insulin expression in the β cell by stimulating intracellular signaling events [130].

When increased glucose flux into the β cell occurs the first response is to secrete insulin (Figure 1.2). This occurs through a multi step process that begins with accelerated generation of cytosolic ATP via glycolytic and oxidative events [131]. Increasing the intracellular ATP/ADP ratio causes closure of the K_{ATP}-channel and leads to cell membrane depolarization [132, 133]. To correct for the negatively charged intracellular environment, voltage-gated Ca^{2+} channels allow for calcium ion influx [134, 135] that subsequently triggers insulin secretion from pre-docked membrane vesicles [136]. This represents the transient first phase of insulin release that typically occurs when glucose levels reach ~7mM and lasts for roughly ten minutes [137]. If this immediate hormone dump does not restore normal circulating glucose levels, a secondary phase of sustained release is activated at a much lower rate compared to the first. However, this requires insulin biosynthesis through a highly controlled transcriptional mechanism.

The insulin 5' promoter region has been extensively studied for decades. While the sequences differ slightly from human to rodent, the fundamental glucose-responsive elements share common homology [138]. Three transcription factor binding sites are located within 300 nucleotides of the transcriptional start site are primarily responsible for insulin gene activation in response to glucose. PDX-1 is a homeodomain-containing factor that interacts with TAAT nucleotide A elements at the insulin enhancer [139, 140]. The C1 *cis* element at the insulin promoter binds the DNA-binding component of RIPE3b1, termed MafA [141, 142]. The final required component is the basic helix-loop-helix protein NeuroD1/BETA2 that interacts with the insulin enhancer E element [143]. Mutational studies discovered that these transcription factors work in a synergistic manner in association with the p300/CBP initiation complex to activate insulin gene expression [144-146]. Interestingly, studies indicate that insulin gene control is also

regulated through histone methylation patterns within the proximal promoter region [144, 147]. Following transcription and translation, immature insulin hormone undergoes a series of proteolytic cleavages to produce the mature hormone [148] and refill granules in the reserve pool. These are the granules responsible to secondary insulin secretion during sustained hyperglycemia.

Several components within the pancreatic β cell network are post-translationally modified by O-GlcNAc. This PTM has been shown to influence insulin secretion dynamics [149, 150], proteins in the exocytosis pathway [151] and transcription factors controlling glucose-stimulated insulin gene activity [152-154]. There is also strong evidence that demonstrates altering O-GlcNAc levels is directly responsible for dysfunctional insulin secretion and β cell death observed in diabetes [155-159]. But the data is merely correlative and requires further validation.

Conclusive Remarks

Post-translational protein modifications are responsible for the majority of molecular diversity in eukaryotic organisms. O-GlcNAc attachment to serine and threonine residues represents one of these dynamic modifications that impacts extensive biological systems. Since its discovery almost thirty years ago, more than a thousand intracellular proteins have been shown to contain this moiety. However, it wasn't until recently that researchers began to link O-GlcNAc as a specific modulator of cellular mechanisms. Technological and scientific advancements have enabled us to discovery properties of this modification that will undoubtedly continue to move the field further. However, more work is needed to uncover how O-GlcNAc specifically impacts human disease and to determine if this PTM is potentially a logical therapeutic target.

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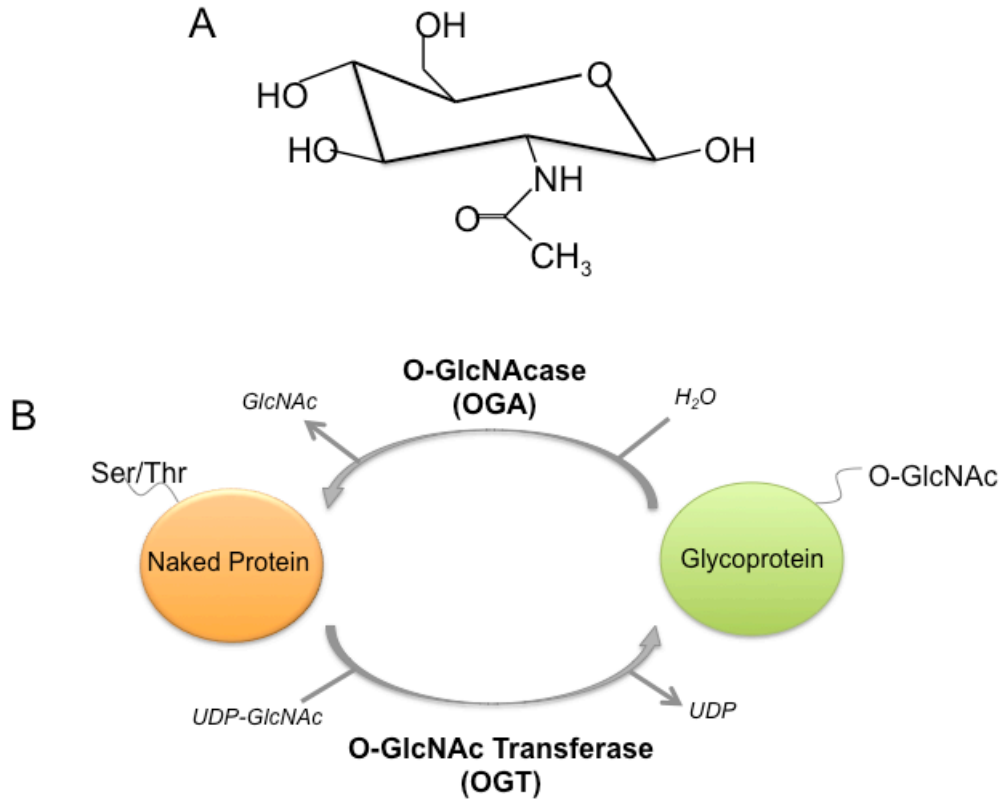
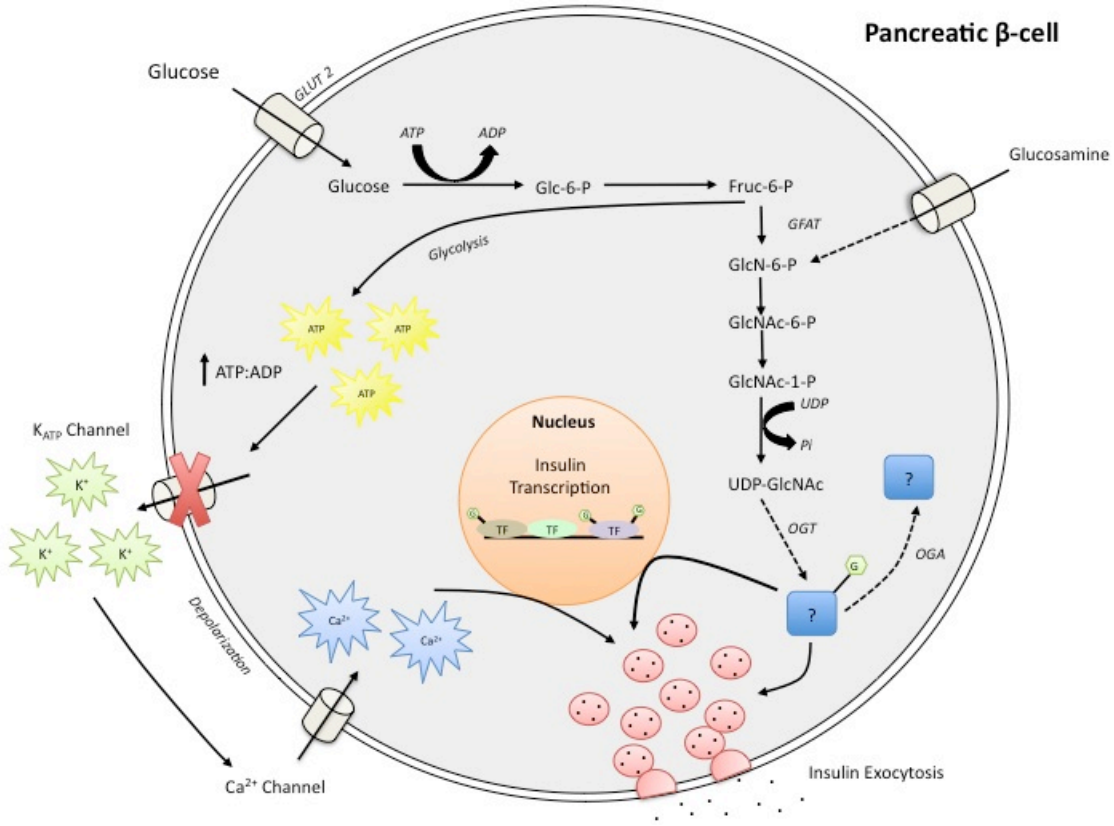
Figure 1.1



O-GlcNAc is a post-translational modification cycling on and off proteins in response to environmental stimuli. (A) The single sugar moiety is chemically similar to glucose, but contains an *N*-acetyl group at the C2 position. Attachment occurs through a β-linkage connecting the C1 with the hydroxyl group on serine and threonine residues. (B) O-GlcNAcylation is an enzymatic reaction requiring the activity of two distinct proteins. O-GlcNAc transferase (OGT) is responsible for adding the modification, while O-GlcNAcase (OGA) removes the PTM. These enzymes respond to changing nutrient levels in a dynamic and inducible manner in the cytoplasm and nucleus of the cell.

Figure 1.2



The pancreatic β cell is responsible for maintaining normal circulating glucose levels through the synthesis and secretion of insulin. Increasing cellular glucose flux triggers intracellular signaling events that culminate in first phase insulin release. To refill the exocytosis granules, insulin gene transcription and translation synthesize the preproinsulin protein that is processed into its mature form. Known as the reserve insulin pool, these hormone-containing vesicles traffic to the membrane if glucose levels remain elevated and release additional insulin during a second phase secretion event. Several proteins in the secretory pathway and transcription factors involved in insulin gene transcription are known to be O-GlcNAc modified (green circles). However, their direct functional role is yet to be established.

Table 1.1

Verified sites of O-GlcNAc modified histones. Displayed above are the identified O-GlcNAcylated histones and the specific amino acid residues that are modified. The third column displays other histone PTMs affected by O-GlcNAc addition. (Ub: ubiquitin, Me: methylation, Ac: acetylation, P: phosphorylation, x: no data available)

Histone	Modified Sites	PTMs Influenced	References
H2A	Thr101	x	Sakabe *et al.*, 2010
H2B	Ser36; Ser112	x; Increases Lys120 Ub	Sakae *et al.*, 2010; Chen *et al.*, 2013; Fujiki *et al.*, 2011
H3	Ser10	Increases Lys9 Me, Ac; Decreases Lys9 P	Fong *et al.*, 2012; Zhang *et al.*, 2011
H4	Ser37	x	Sakabe *et al.*, 2010

Table 1.2

O-GlcNAc cycling enzymes interact with epigenetic complexes to influence biological events. Displayed above are the complexes that OGT and/or OGA are known to impact at an epigenetic level. This regulation is exerted by the O-GlcNAc status of these complexes to either promote or prevent molecular activities.

O-GlcNAc in Epigentic Complexes	Biological Function	References
Repressive		
mSin3,HDAC,OGT	Blocks transcription	Yang *et al.*, 2002; Cox *et al.*, 2013; Hwang *et al.*, 2013
TET1, mSin3A, NURD, OGT	Blocks ECS Differentiation	Shi *et al.*, 2013; Vella *et al.*, 2013
PRCL1/2,OGT/OGA	Blocks transcription	Sinclair *et al.*, 2009
Sirt1,OGT	Inactive Hcrt gene	Hayakawa *et al.*, 2013
Activating		
$HCF1_N$, OGT	Enables cell cycle complex formation, promotes G1 to S phase entry	Capotosti *et* al., 2011; Daou *et* al., 2011; Deplus *et al.*, 2013
MLL5. OGT	HKMT and gene activation	Fujiki *et al.*, 2009; Nimura *et al.*, 2009; Chikanishi *et al.*,2010
p300, CBP, OGA	Active Hcrt gene	Hayakawa *et al.*, 2013
TET2/3, OGT	Active $H3K4me^3$ enriched promoters	Duplus *et al.*, 2013; Chen *et al.*, 2013

CHAPTER 2

FUNCTIONAL O-GLCNAC MODIFICATIONS: IMPLICATIONS IN MOLECULAR REGULATION AND PATHOPHYSIOLOGY[#]

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Preface

This chapter is an invited review that I co-authored. Specifically, I composed the: abstract, last two paragraphs of introduction, section 3, section 4, section 5 and concluding remarks. I also produced the following figures: 2.1, 2.3, 2.4, 2.5 and 2.6 A and B.

Abstract

O-linked β-N-acetylglucosamine (O-GlcNAc) is a regulatory post-translational modification of intracellular proteins. The dynamic and inducible cycling of the modification is governed by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) in response to UDP-GlcNAc levels in the hexosamine biosynthetic pathway (HBP). Due to its reliance on glucose flux and substrate availability, a major focus in the field has been on how O-GlcNAc contributes to metabolic disease. For years this PTM has been known to modify thousands of proteins implicated in various disorders, but direct functional connections have until recently remained elusive. New research is beginning to reveal the specific mechanisms through which O-GlcNAc influences cell dynamics and disease pathology including clear examples of O-GlcNAc modification at a specific site on a given protein altering its biological functions. The following review intends to focus primarily on studies in the last half decade linking O-GlcNAc modification of proteins with chromatin-directed gene regulation, developmental processes, and several metabolically–related disorders including: Alzheimer's, heart disease and cancer. These studies illustrate the emerging importance of this post-translational modification in biological processes and multiple pathophysiologies.

1. Introduction

Post-translational protein modifications (PTMs) are critical for imparting microheterogeneity and increasing protein functional diversity in biological systems.

Several classes of PTMs have been identified, including: phosphorylation, ubiquitination, acetylation, SUMOylation, glycosylation, etc. Phosphorylation is the most established regulatory moiety, but interestingly, it took nearly twenty-five years after its discovery before groups began determining its functional roles [1, 2]. A similar evolutionary timeframe is taking shape for O-GlcNAc. Initial studies investigating O-GlcNAc were aimed at determining its regulation and identifying processes it affected. Over the last several years, technological advancements have enabled the field to ask and begin to answer complex questions regarding O-GlcNAc's mechanistic role in human disease.

O-GlcNAc: A Post-translational Protein Modification

O –GlcNAc is a single monosaccharide regulatory modification occurring on nucleocytoplasmic proteins [3-5]. Approximately 2-5% of cellular glucose enters the nutrient sensing hexosamine biosynthetic pathway (HBP). The transaminase reaction of fructose-6-phosphate by glutamine fructose-6-phosphate amidotransferase (GFAT) to yield glucosamine-6-phosphate is the rate-limiting step of the pathway [6, 7]. The end product of the pathway is the nucleotide sugar donor UDP-GlcNAc that is used as the substrate for O-GlcNAc modification. UDP-GlcNAc can also be incorporated into complex glycosylation pathways and in the production of other nucleotide sugars (Fig 2.1)[8]. The levels of the nucleotide sugar donor are regulated by amino acid, free fatty acid, nucleotide and glucose availability [7-10].

First reported in 1984 (Torres and Hart), the addition of O-GlcNAc [11] occurs on serine and threonine residues of nuclear and cytosolic proteins and is described as being analogous to phosphorylation. These modifications are both regulated by cycling enzymes in response to environmental stimuli and compete for similar amino acid residues. In fact, a dynamic interplay between the two PTMs has been described in several cases [12, 13]. However, O-GlcNAc and phosphate can occur at adjacent and

distal sites, suggesting additional regulatory roles for O-GlcNAcylation than just blocking phosphorylation. O-GlcNAc modified proteins regulate many cellular processes: cell cycle progression [14], transcriptional control [15, 16], signal transduction [17, 18], nutrient sensing [8, 19] stress responses [20] and chromatin remodeling [21-24].

The O-GlcNAc Cycling Enzymes

Two genes in mammals encode the enzymes governing O-GlcNAc cycling: O-GlcNAc transferase (OGT) and β-N-acetylglucosaminidase (OGA), which add and remove the O-GlcNAc moiety respectively [4, 25-27].

OGT, whose activity was initially characterized in 1992 [25], was cloned and partially characterized in the late 1990s [26, 28, 29]. Mammalian OGT knockouts are embryonic lethal, demonstrative of its importance in cell survival [30]. OGT has an N-terminal tetratricopeptide repeat (TPR) domain and a C-terminal catalytic domain [26, 28]. No clear consensus sequence has been identified for OGT substrate specificity, but several factors are proposed to regulate OGT activation. These include: protein-protein interactions mediated by the TPR region, localization in part by a phosphatidyl inositol phosphate (PIP)-binding domain, post-translational modifications and substrate availability [17, 31]. The gene encoding OGT can be alternatively spliced to produce three isoforms differing at their N-terminal TPR region [32, 33].

OGA was cloned and partially characterized in the early 2000s and is found ubiquitously expressed in all tissues [4, 34]. OGA has a catalytic N-terminal O-GlcNAcase domain, and a C-terminal domain that has sequence similarity to histone acetyltransferase (HAT). Recently, work has convincingly demonstrated this enzyme lacks previously proposed HAT activity [35]. In mammals, OGA is encoded as a single gene that can be alternatively spliced producing two isoforms and differ at their C-terminal ends (Toleman 2004).

Methods for Studying Cellular Regulation via O-GlcNAc

Manipulating HBP flux through glucose exposure, glucosamine (GlcN) addition or using the amidotransferase inhibitors 6-diazo-5-oxonorleucine (DON) or *O*-diazoacetyl-L-serine (Azaserine), can indirectly modulate O-GlcNAc levels [8]. More specific strategies modulating global O-GlcNAc levels can also be implemented to directly target the cycling enzymes. Overexpressing or knocking down OGA and OGT are commonly used genetic manipulation approaches, while specific OGA inhibitors can also be used to investigate O-GlcNAC-specific affects. O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc) was the first established OGA inhibitor widely used in the field [36], but also affected the hexosaminadase enzyme family [37]. Recently, several highly selective OGA inhibitors have been generated that exhibit greater specificity for N-acetylglucosaminidases compared to hexosaminidase A/B (Fig 2.1). These inhibitors include: GlcNAc-configured nagstatin derivative (GlcNAcstatin), 1,2-dideoxy-2′-methyl-α-D-glucopyranoso-[2,1-d]-Δ2′-thiazoline (NButGT) and Thiamet-G [38-40]. Several OGT inhibitors are also documented in the literature [41], but have not been widely evaluated or used in the field to date.

Since its discovery, O-GlcNAc has been shown to modify thousands of proteins in numerous cellular pathways. However, recent work has begun to unravel the molecular importance of this PTM on specific sites of given proteins involved in diverse biological processes. The following sections will highlight this movement by presenting data published within the last several years, with an emphasis on epigenetics and several metabolically influenced diseases.

2. Epigenetic Regulation by O-GlcNAc

Chromatin is a highly dynamic structure that critically regulates transcription [42]. Chromatin is composed of DNA and histones that are condensed to form nucleosomes [43]. This higher order chromatin structure regulates gene transcription and repression [42, 43]. Chromatin is composed of transcriptionally active euchromatin that is gene-rich and heterochromatin which is gene-poor and transcriptionally silent [44]. Nucleosomal rearrangement is crucial for the movement of the transcription machinery along the DNA [43]. Chromatin remodeling is a complex process involving several known PTMs like acetylation, methylation, ubiquitination and phosphorylation [42, 45, 46].

The first studies implicating O-GlcNAc in epigenetic regulation were done in *D. melanogaster*. The findings identified elevated O-GlcNAc levels in transcriptionally repressed regions of polytene chromosomes and significantly lower levels in "puff" regions, indicative of active transcription [23, 47]. RNA Polymerase II is O-GlcNAc modified [15] and more recently OGT was shown to be a member of the pre-initiation complex [48, 49]. Disruption of the activity of either OGT or OGA leads to transcriptional defects and impaired pre-initiation complex formation [49]. Drosophila super *sex combs* (*sxc*) is a polycomb group (PcG) gene located in chromosome 2R that maps to the same region as *OGT [21, 23]*. PcG's form a multiprotein complex to orchestrate epigenetic regulation of target genes involved in developmental regulation, pluripotency and cancer (Schuettengruber 2007, Ringrose 2007, Pietersen 2008, Schwartz 2008). Mutations in *sxc* affect OGT protein expression and activity *in vivo* and both human and *Drosophila* OGT can rescue *sxc* mutations [21] convincingly establishing that OGT is in fact *sxc*. O-GlcNAc modification and PcG binding regions overlap at the polytene chromosomes [21]. *Sxc/OGT* null mutants in *Drosophila* exhibit a loss of polycomb repression, providing further evidence for OGT involvement in gene silencing [21]. The polycomb repressive complex 2 (PRC2) is also O-GlcNAc modified [50]. In fact, PRC2 mutations in mouse embryonic stem cells (mESC) cause deregulated OGT

and O-GlcNAcylation levels on proteins associated with the chromatin-remodeling complex [50].

O-GlcNAc and Chromatin: Transcriptional Repression

A breakthrough in identifying OGT in complex with mSin3A/HDAC1 revealed a potential role for OGT in gene silencing [51] [Fig 2.2]. OGT and mSin3A act synergistically to repress basal and Sp1 mediated transcriptional activation [51]. Moreover, estrogen target genes are hyperglycosylated in the absence of estrogen in Mcf-7 cells [51]. mSin3A and HDAC1 are both known to be O-GlcNAc modified [51].

Many tissue-dependent differentially methylated regions (T-DMRs) have been identified in mammalian embryonic stem cells (ESC), where hyper- and hypomethylation play a role in silencing and activating loci respectively [52-54]. In combination with histone modifications, these regions are vital in regulating gene activity at developmental stages in ESC [55, 56]. Investigation into ManNAc-stimulated *hypocretin neuropeptide precursor* (*Hcrt*) gene regulation revealed OGA and OGT are localized within the before mentioned T-DMRs [57]. ChIP experiments illustrate higher O-GlcNAc signal within the *Hcrt* promoter region (regions 1 and 2) during gene inactivity [57]. Enzymatic inhibition studies show a repressive role for O-GlcNAcylation in *Hcrt* expression. This is further strengthened by OGT association with repressive factors Sirt1 and Ezh2 at hypoacetylated T-DMR regions of non-neuronal differentiation cells [57].

Histones 2A, 2B, 3 and 4 (H2A, H2B, H3, H4) are O-GlcNAc modified [24, 58] when assessed orthogonally by both click chemistry and immunoblotting methods [24]. These findings are further verified in histone overexpression and O-GlcNAc immnunoblot studies using Hela cells [24]. Click chemistry studies reveal the following O-GlcNAc modified histone sites: Thr101 on H2A, Ser36 on H2B and Ser47 on H4 [24]. Alanine mutants of the three identified sites did not completely abrogate reactivity of the

histones to O-GlcNAc specific antibodies [24] suggesting additional O-GlcNAc sites on each of the histones exist.

Glucosamine addition increases O-GlcNAc serine 10 (Ser10) of histone H3, subsequently decreasing the phosphorylation of the same residue [58, 59]. Interestingly, when H3 Ser10 is O-GlcNAcylated, its neighboring residue lysine 9 (K9) presents with decreased acetylation [58]. Acetylation of H3K9 is a mark of active transcription [45, 60] which further validates H3 Ser10 O-GlcNAcylation as a repressive mark. Consistent with this, the transcriptional repression marks H3K9me3 and H3K27me3 are elevated upon increases in H3 O-GlcNAcylation, while the activation mark H3K4me3 decreases [58]. These data collectively describe the repressive role mediated by the O-GlcNAc modification of H3 Ser10.

O-GlcNAc and Chromatin: Transcriptional Activation

Another study also identified O-GlcNAc sites on H2B and mapped three sites on this protein: Ser91, Ser112 and Ser123 of H2B [61]. Alanine mutations of Ser112 significantly reduced O-GlcNAcylation by OGT *in vitro* [61]. H2B modification at Ser112 is shown to be glucose dependent since 24-hour starvation results in its deglycosylation in Hela cells [61]. Glucose replenishing restores the S112 O-GlcNAcylation gradually within a 24-hour period [61]. This O-GlcNAc modification also influences H2B Lys120 monoubiquitination as highlighted by the replenishment of glucose facilitating this histone addition [61]. This notion is validated considering OGT knockdown leads to diminished modification of Lys120 [61]. HBP inhibitors attenuate the effect of glucose responsiveness as indicated by the loss of both Ser112 O-GlcNAcylation and Lys120 monoubiquitination [61]. Further, Ser112Ala and Thr122Ala H2B mutations revealed the absence of K120 monoubiquitination even in the presence of extracellular glucose [61]. However, mutating H2B Lys120Arg did not affect the O-

GlcNAcylation at H2B Ser112 [61]. This leads to the logical conclusion that Ser112 O-GlcNAcylation mediates Lys120 monoubiquitination of H2B. H2B monoubiquitination is an activation mark that has been previously described to be induced by glycolysis [62]. H2B Ser112 O-GlcNAc is located within euchromatin of polytene chromosomes in fly (Fujiki 2011) and co-localizes with H3K4me2, an activation mark rather than the H3K9me2/ H3K27me3 repressive marks [61]. Glycogen synthase kinase 3β (GSK3β) transcription was induced by Ser112-O-GlcNAcylated H2B, but totally ablated by OGT knockdown [61]. These results suggest a potential role for Ser112-O-GlcNAc on H2B as a nutrient sensor to facilitate transcription of genes involved in gluconeogenesis. In pluripotent stem cells differentiating into orexin neurons, OGA is found to interact with the transcriptional activation machinery components p300 and CBP at the T-DMR of *Hrct [57]*. These events directly correlate with observed elevations in histone H3 and H4 acetylation marks during gene activation [57].

Ten-eleven translocation (TET) proteins are Fe^{2+} and 2-oxoglutarate-dependent dioxygenases that oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [63, 64]. TET proteins mainly associate with CpG rich promoter regions [65-67]. Histone 3 lysine 4 trimethylation (H3K4me3), an activation mark, also marks CpG rich promoter regions [68]. Interestingly, most Tet1-bound promoters are marked by H3K4me3 [65, 66]. Mammals contain three TET proteins, namely TET1, TET2 and TET3. TET1 and TET2 colocalize with OGT in ESC, with TET1 being O-GlcNAc modified at residue Thr535 [69, 70]. TET1 in particular has been suggested to impart transcriptional regulation by interacting with chromatin remodeling and histone modification complexes Sin3a and NuRD [71]. In addition, OGT and TET1 association in ESC appears to preferentially bind at unmethylated CpG-rich promoter regions in close proximity to the transcriptional start site [69]. OGT siRNA-directed knockdown studies reduce Tet1 targeting and 5hmC enrichment on TET1 regulated genes [69, 70].

Using affinity purification and MS techniques, OGT was found associated with TET2 and TET3 *in vitro* [72] [Fig 2.2]. Moreover, in mESCs, TET2 interacts with OGT endogenously [72]. The C-terminal catalytic double-strand beta-helix (DSBH) region of TET2 and TPRs 5 and 6 of OGT are essential for this interaction [72]. OGT and TET2 interaction occurs at the chromatin with TET2 being necessary for OGT recruitment. This is verified by shRNA TET2 knockdown studies that totally ablate chromatin associated OGT levels [72]. However, knockdown of OGT did not significantly alter TET2 retention at the chromatin [61, 72]. Both OGT and TET2 knockdowns impair histone O-GlcNAcylation with TET2 reduction dramatically reducing H2B Ser112 O-GlcNAc modification levels [72]. TET2 knockout mice display impaired OGT activity and decreased global O-GlcNAcylation that parallel decreased H3K4me3 [73]. Genome-wide ChIP-Seq analysis provides insight on the distribution of OGT, TET2 and H2B Ser112 at transcription start sites (TSSs) with promoters that are H3K4me3 positive [72, 73]. This study implicates the recruitment of OGT by TET2 to the chromatin to mediate transcriptional activation.

MS analysis and size-exclusion chromatography assays identify the existence of a larger complex consisting of OGT, TET1, TET2, mSin3A and host cell factor (HCF1) [69, 70, 73]. Interestingly, mSin3A and HDAC1 were shown to co-purify with OGT [51] and with TET1 [65, 74]. OGT binding at H3K4me3- positive promoters directly corresponds with observed TET1 ChIP-Seq signal [69, 73]. As previously described, OGT and the mSin3A/HDAC1 complex are involved in gene silencing in HepG2 cells as well as in *in vitro* studies [51] [Fig 2.2]. HCF-1 is a known interacting and substrate partner of OGT [75-77]. OGT O-GlcNAcylates HCF-1 and is proposed to function as a protease to cleave HCF-1 [77]. HCF-1 is also a component of the SET1/COMPASS H3K4 methyl transferase (MT) complex [75]. OGT and TET2/3 have been identified in a complex with all members of the SET1/COMPASS H3K4 MT family including the

methyl transferase SETD1A [73]. OGT and TET protein activities are required for the SETD1A-chromatin binding event facilitating transcriptional activation of hematopoietic genes [73] [Fig 2.2]. OGT inhibition reduces OGT interaction with HCF-1 [73, 77] and concomitantly decreases the association with SET1DA MT [73]. These data together suggest that HCF-1 interaction is required for the TET2/3-OGT mediated transcriptional activation by SET1/COMPASS H3K4 MT [Fig 2.2]. A separate study highlights that OGT association with the histone lysine methyl transferase MLL5 is necessary to induce differentiation of promyelocytes by retinoic acid (RA) [22]. OGT O-GlcNAc modifies MLL5 and activates its histone lysine methyl transferase (HKMT) activity to cause di-methylation of H3K4 [22]. This causes RA stimulation leading to the expression of the differentiation promoting transcription factor C/EBPε [22]. Given the role of OGT and O-GlcNAc in chronic lymphocytic leukemia (CLL) (discussed in the last section), further investigation could shed light on the role of TET2/3, OGT, and *MLL* genes in leukemia.

3. Stem Cells and Development

Eukaryotic embryogenesis is a complex orchestration of molecular and environmental events working in concert at precise times. Glucose plays a vital role in determining many aspects of early development. [11, 78]. Given the direct connection between glucose and the HBP, investigation into how O-GlcNAc impacts development has been widely studied.

OGT gene deletions in mESC provided the initial data suggesting O-GlcNAc plays an important role in development. Notably, complete knockout resulted in loss of embryonic stem cell viability and embryonic lethality due to incomplete embryogenesis [30]. Hyperglycemia was also shown to perturb blastocyst formation within the developing mouse through an HBP-directed mechanism [79]. O-GlcNAc appears to be the cause considering *OGT* inhibition prevented the hyperglycemia-induced

complications observed during development [79]. Additional supporting evidence demonstrated mouse OGA knockouts were perinatally lethal [80]. OGT and OGA targeted morpholino injection or enzyme overexpression studies results in stalled epiboly, preventing gastrulation and increasing embryonic death in zebrafish [81]. Furthermore, disturbing the balance of O-GlcNAc during development in zebrafish significantly reduces body size and tissue disorganization in ectoderm, mesoderm and endoderm germ layers [81]. These findings confirm the importance of precisely regulating OGT, OGA and O-GlcNAc during embryonic development and preempted further investigation into how this PTM influences developmental regulation of ESC and germ cell differentiation.

O-GlcNAc Regulates ESC Self-Renewal

Self-renewal and pluripotency are hallmark characteristics of ESC and several studies have been conducted to determine how O-GlcNAc is involved in these processes (Fig 2.3). Integrin adhesion complexes are known to regulate embryonic development through the integrin β4 cytosolic domain and plectin interaction [82]. GlcN treated mESC contain decreased levels of integrin β4 mRNA and protein levels. Interestingly, these reductions disrupt the complex formation between integrin β4 and plectin necessary for proper development [83, 84]. Elevating O-GlcNAc levels through both GlcN flux and OGA inhibition increases mESC migration, while OGT inhibition blocks this action [84]. Several mESC proteins essential for self-renewal are O–GlcNAc modified, including Oct4, Sox2 and Zpf281 [50, 81, 85]. Additionally, mSin3a is O-GlcNAc modified and is clearly demonstrated to be involved in epigenetic regulation during development [50, 51]. Elevating O-GlcNAc in mESC inhibits their self-renewal capacity and prevents somatic cell reprogramming into induced pluripotent stem cells (iPSC) [85]. Oct4 and Sox2 are components of the core pluripotency network and part of somatic cell reprogramming cocktails to generate iPSC [86-88]. Both of these transcription factors are O-GlcNAc modified and Oct4 O-GlcNAcylation promotes mESC self-renewal and reprogramming

through a transcriptionally regulated mechanism [85]. In depth expression analysis reveals O-GlcNAc addition on Oct4 subsequently induces many pluripotency-related genes, including *Klf2, Klf5, Nr5a2, Tbx3* and *Tcl1* [85]. This work establishes direct O-GlcNAc involvement in regulating key pluripotency and self-renewal proteins.

The previously discussed TET and T-DMRs are also shown to influence ESC fate determination through O-GlcNAc control (Fig 2.3). Increasing O-GlcNAc levels during development prevents the transition of ESC into germ cells provided OGT interacts with several epigenetic repressive members, including: TET1/2, mSin3a, Sirt1 and Ezh2 [57, 69, 70]. This is further supported by data demonstrating that OGA interacts with members of the transcriptional activation complex, p300 and CBP, at hypermethylated T-DMR region of *Hrct [57]*

Work in mouse embryonic fibrolblasts (MEFs) demonstrate that O-GlcNAc plays a role in the cell cycle control [58, 59, 89-92]. Because OGA null mice rarely reach maturity, MEFs can be isolated from mid-gestation embryos for investigation prior to glycosylation-linked lethality [80]. In agreement with previous work [93], O-GlcNAcylation fluctuates throughout the cell cycle stages, but constitutively increased O-GlcNAc levels in OGA null MEFs causes aberrant cell cycle progression [80]. The observed loss of normal cell cycle control results in genomic instability as indicated by various abnormal nuclear morphologies that increases the number of senescent MEFs [80]. Together these findings suggest that fluctuations in O-GlcNAc levels influence the self-renewal and pluripotent characteristics of ESC, but more investigation is needed to establish direct roles.

O-GlcNAc Regulates Differentiation into Specialized Cell-types

Upon stimulation by lineage-specific growth factors, multipotent stem cells differentiate into specialized cells during later development [94]. Recent work implicates

O-GlcNAc plays a major role in mesoderm germ cell differentiation to an even higher degree than in ESC pluripotency (Fig 2.3).

O-GlcNAc has long been associated with modulating many molecular aspects within adipose cells [18, 19]. It has since been identified as one of the main transcriptional regulatory modifications dictating adipocyte differentiation. Studies using the 3T3-L1-adipocyte cell line reveal protein O-GlcNAcylation increases during adipocyte differentiation [95, 96]. As expected, an increase in OGT and GFAT-1 protein levels, as well as GFAT-1 mRNA, directly correlate with observed O-GlcNAc elevations [95, 96]. OGT and GFAT inhibition decreases O-GlcNAc levels and prevents preadipocyte differentiation in 3T3-L1 cells [95].

Two basic leucine zipper transcription factors belonging to the CCAAT/enhancer-binding protein family (C/EBP) are implicated in O-GlcNAc-directed adipocyte differentiation. C/EBPα and C/EBPβ are critically important for controlling adipocyte differentiation [97-99] and respond directly to changes in O-GlcNAc (Fig 2.3)[95, 96, 100, 101]. Elevating O-GlcNAc levels increases C/EBPα expression along with another adipose-related mesoderm marker, PPARγ, during differentiation [101]. Additionally, blocking glucose flux through the HBP in 3T3-L1 cells prevents lipid droplet formation during preadipocyte differentiation and correlates with deceased C/EBP α/β and PPARγ protein expression [95, 96]. A separate study looking at C/EBPβ identified two amino acid residues as being O-GlcNAc modified: Ser180 and Ser181 [100]. Interestingly, increasing O-GlcNAc occupancy at these sites in 3T3-L1 preadipocytes prevents subsequent phosphorylation at adjacent residues, decreases C/EBPβ DNA binding and transactivation and delays the adipocyte differentiation program [100]. Considering these antagonistic roles for O-GlcNAc modification on C/EBPβ, further investigation is required to understand the molecular connection. However, it is clear that O-

GlcNAcylation of C/EBPα and C/EBPβ directly influence adipocyte differentiation events.

While the primary focus on O-GlcNAc-mediated adipose differentiation has centered on C/EBPα and β, other factors involved in the developmental process have been identified. MS analysis confirms that vimentin, nucleoporin p62 and p98, Ewing sarcoma, long chain fatty acid-CoA ligase 1 and pyruvate carboxylase proteins are all more O-GlcNAc modified during preadipocyte differentiation [96]. Along with C/EBP α and β, elevated O-GlcNAc increases the expression of the adiponectin, angiotensinogen, resistin and visfatin adipocytokines in 3T3-L1 cells to facilitate differentiation [95, 101, 102]. While the precise mechanisms for O-GlcNAc regulation on these factors remains unknown, this PTM has been shown to be critical for adipocyte differentiation.

O-GlcNAcylation appears to be instrumental in spontaneously differentiating cardiac precursor cells as evident by O-GlcNAc reduction during embryoid body transition [103]. This shift is likely due to a decrease in OGT protein levels during this developmental stage, which can be augmented by elevating HBP flux with GlcN addition and OGA inhibition to selectively increase O-GlcNAc [103]. In a similar vein, work has been done to address whether changes in O-GlcNAc affect myoblast differentiation events. Myogenic stimulation queues activation of the skeletal myogenic program and the induction of multinucleated myotubes starting at day 1 and progressing thereafter [104, 105]. Protein observation during this time frame shows that O-GlcNAc levels in C2C12 myoblasts dramatically decrease between days 1 and 2 of myotubule formation, in parallel with increasing OGA and OGT mRNA and protein levels [106]. OGA reduction using several pharmacological inhibitors or siRNA's perturbs myoblast differentiation from day 1 through day 5 as indicated by the persistence of mononucleated cells [106]. Terminal differentiation of myoblasts is regulated by the activation of muscle-specific genes including: *myogenin, myosin heavy chain* (*MHC*) and *muscle regulatory factor 4*

(*mrf4*) [104, 107]. OGA inhibition in C2C12 myoblasts significantly decreases the number of myogenin- and MHC-positive cells as well as *myogenin, MHC,* and *mrf4* gene expression, suggesting that O-GlcNAc reduction is critical during myogenesis (Fig 2.3)[106]. Therefore, O-GlcNAc modulation is crucial for the temporal expression of genes during cardiac cell differentiation.

Although still in its infancy, new work demonstrates that O-GlcNAc may also be involved in chondrocyte differentiation and bone formation [108, 109]. Insulin and insulin like growth factor-I (IGF-1) are strong stimulators of chondrogenesis and endochondral ossification (EO) during growth plate cartilage differentiation into bone [110, 111]. During insulin-induced differentiation of ATCD5 pre-chondrogenic cells, O-GlcNAc levels are significantly increased and persist for the duration of development [109]. These results are also seen during ascorbic acid-induced ATCD5 differentiation, which is not directly related to the glucose metabolism pathway and insulin to suggest O-GlcNAc may independently regulate this transition [109, 112]. OGA inhibition studies in the absence of insulin causes the activation of several pre-chondrogenic genes required for differentiation, indicating elevations in O-GlcNAc alone can regulate ATCD5 development [109]. This is further validated considering that reduction in HBP flux ablates insulin-stimulated differentiation and blocks the expression of these chondrogenic genes [109]. Additionally, matrix metalloproteinase (MMP) proteases 3 and 9, that are vital in ECM remodeling during chondrocyte differentiation [113, 114], are also upregulated during OGA inhibition to the same degree as with insulin stimulation [109]. OGA inhibition also influences several proteins that regulate CREB- and RUNX2-mediated gene expression during osteoblast differentiation, including CREB-binding protein (CBP) and TFGβ-activated kinase 1 and 2 (TAB1/TAB2) (Fig 2.3)[103, 108]. As of now, the regulatory importance O-GlcNAc imparts in these proteins is unknown. In

total, these findings demonstrate a clear role for O-GlcNAc in regulating the terminal differentiation of adipocytes, cardiac muscle, cartilage and bone.

4. The Brain and Central Nervous System

The eukaryotic central nervous system (CNS) is an intricately intertwined signaling network controlling cognitive processing, emotional responsiveness and interpretive and integrative functions. The brain and spinal cord represent the main contributors to CNS function and enable whole system communication through synaptic stimulation [115-117]. While only constituting a small portion of an organism's mass the CNS requires a significant amount of metabolic fuel, utilizing approximately 50% of the total glucose load [118]. Provided its well-documented dependency on glucose flux, it is logical to speculate O-GlcNAc plays a major role in CNS regulation. To this end, proteomic analysis through a variety of mass spectrometry techniques identifies a large number of O-GlcNAc proteins within the CNS, some of which are pivotal in neuronal processes [18, 119-127]. In fact, the presynaptic zone proteins Bassoon and Piccolo are two of the most heavily O-GlcNAc modified proteins ever observed [125]. Recent studies have examined how O-GlcNAc contributes to synaptic signaling and have illustrated its involvement towards the establishment of Alzheimer's disease as described below.

A Neuroprotective Role for O-GlcNAc in Alzheimer's Disease

Alzheimer's disease is a neurodegenerative disorder that typically presents with aging. The hallmark phenotype includes: dementia, neurofibrillary tangles (NFTs), amyloid plaque accumulation, nerve cell degeneration and related brain physiological changes [128, 129]. Considering the accelerated decline of glucose utilization in the Alzheimer's disease brain [130-134], many groups have investigated the role O-GlcNAylation plays in disease progression.

One of the defining pathological features in Alzheimer's is the oligomerization of the microtubule-associated protein tau, ultimately producing NFTs. This progression is controlled at the molecular level by hyperphosphorylation of tau, causing conformational rearrangements [135, 136]. Given the extensive crosstalk between protein phosphorylation and O-GlcNAcylation [125, 137-139], tau O-GlcNAcylation has been investigated. Indeed, tau is shown to be O-GlcNAc modified at Thr123, Ser208, Ser333, Ser400 and Ser692, with Ser400 representing the primary functional site [122, 126, 138, 140, 141].

O-GlcNAc levels in the brain during Alzheimer's progression appear to decrease as hyperphopsphorylation increases [122, 126, 142]. This may directly coincide with decreasing glucose metabolism observed in the aging brain [143, 144]. Frontal cerebral cortex samples from deceased Alzheimer's patients display significant reduction in global O-GlcNAc levels, but increased tau hyperphosphorylation as compared to wild-type controls [145]. Immunofluorescent studies on human brain samples reveal a yin-yang relationship between tau O-GlcNacylation and phosphorylation [145]. Non-hyperphosphorylated tau from patient brain samples are heavily O-GlcNAcylated compared to the hyperphosphorylated pool [145]. This data suggests the global decrease in O-GlcNAc may contribute to the hyperphosphorylated tau phenotype in Alzheimer's diseased brains (Fig 2.4B). It also introduces OGA inhibition as a potential therapeutic target for disease treatment.

Manipulation of HBP flux and O-GlcNAc cycling enzymes directly influences Alzheimer's disease. GFAT-1 inhibition in rat brains not only reduces the amount of O-GlcNAc, but also correlates with drastic elevation of tau phosphorylation to imply reducing glucose metabolism, and subsequently O-GlcNAc, induces hyperphosphorylation of tau [142]. Studies using mouse models mimicking tauopathy show that inhibiting OGA decreases phosphorylation of tau at several residues and

protects against tau-driven neurodegeneration [126]. It also partially reduces the number of NFT-like structures in the brainstem, spinal cord, hypothalamus and cerebral cortex, while slowing tau aggregation and oligomerization (Fig 2.4A)[126]. Conversely, shOGT addition to HEK-293 cells transfected with human tau increases phosphorylation [142].

Another morphological feature of Alzheimer's disease is the formation of amyloid plaques due to amyloid-β (Aβ) peptide accumulation. Plaque generation is caused by the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretase respectively (Fig 2.4B)[146]. APP is recognized as the first plasma membrane protein identified to be O-GlcNAc modified [147], but the functional role of this modification was not thoroughly investigated until recently. Experiments in mice suffering from Aβ aggregation-induced Alzheimer's reveal that elevation in O-GlcNAc via OGA inhibition significantly reduces Aβ plaque load and decreases neuroinflammation in the brains of these animals [148]. Active γ-secretase is a complex containing four protein subunits, including nicastrin (NCT) required for substrate recognition and binding [149, 150]. Mass spectrometry and mutational analysis confirms NCT is modified by O-GlcNAc at Ser708 and this PTM addition attenuates γ-secretase activity and prevents APP cleavage (Fig 2.4A)[148].

The main proteolytic processing pathway for APP uses α- and γ-secretase to produce a secreted sAPPα fragment and prevents Aβ plaque aggregation [151, 152]. Due to the observed neuroprotective properties of sAPPα [153] and the fact that APP is O-GlcNAc modified, investigation into a functional role for O-GlcNAylation in the non-amyloidogenic processing pathway has recently been elucidated. Cell culture experiments using human neuroblastoma cells show that elevations in O-GlcNAc levels via pharmacological inhibition of OGA increase the amount of sAPPα and prevents Aβ load [154]. Genetic and pharmacological manipulation studies targeting the O-GlcNAc

cycling enzymes in SH-SY5Y human neuroblastoma cells confirm O-GlcNAcylation promotes sAPPα [154].

Ubiquitin is a post-translational protein modification known to accumulate at Aβ plaques and NFTs in Alzheimer's [155-157]. This PTM is crucial in regulating protein turnover via the proteasome [158, 159] and is proposed to be dysfunctional in neurodegenerative diseases [160]. Extensive research has established functional connections between O-GlcNAc, ubiquitination and the proteasome [61, 124, 141, 161-165]. Interestingly, mass spectrometry experiments identify an O-GlcNAc site on the 26S proteasome complex ubiquitin receptor subunit RPN13 (also known as ADRM1/ARM1). This protein recruits the deubiquinating enzyme UCH37 to the proteasome and serves as a ubiquitin receptor [124, 166, 167]. Combined with the seemingly neuroprotective role O-GlcNAcylation plays in the brain, O-GlcNAc modification of RPN13 may decrease the ubiquitination status of Aβ and NFTs and diminish the Alzheimer disease phenotype. However, further investigation into this area is needed since a direct functional connection is yet to be established. These results collectively demonstrate that O-GlcNAc imparts neuroprotection in the aging brain and its decline exacerbates Alzheimer's progression.

Synaptic Signaling and Memory

Cre-recombinase-expression experiments targeting OGT in both neonatal wild type and hemizygous female mice reveals significant changes in hypothalamic gene activity and the epigenetic microRNA environment [168]. Functional clustering analysis shows enrichment for genes involved in energy utilization, protein regulation and synapse formation to suggest O-GlcNAc does more than protect against Alzheimer's in the mammalian CNS [168]. Several independent studies reveal that O-GlcNAc appears to

modulate synaptic communication at the signaling and trafficking stages, ultimately controlling long-term memory formation.

One of the more influential transcription factors determining the expression of genes in neuronal processes is cAMP-response element binding protein (CREB) [169, 170]. It is long established that phosphorylation aids in regulating CREB activity within the nervous system, but is not the sole regulatory PTM [171-173]. CREB is now known to be O-GlcNAc modified at Ser40, whose induction increases in response to calcium- and kinase-dependent neuronal activation [174, 175]. The major functionally relevant phosphorylation site of CREB is located at Ser133 [176]. Contrary to most instances, mutational studies demonstrate a cooperative role for O-GlcNAc and phosphorylation in mediating CREB activity [174]. Both OGA overexpression and Ser40Ala mutations illustrate that CREB glycosylation represses both basal transcription and activity-dependent CREB-induced gene expression in neurons [174]. In addition, obstructing Ser40 O-GlcNAc modification of CREB accelerates dendrite and axon elongation, while concurrently deregulating basal and activity-induced dendritic growth [174].

Nerve cell communication in the CNS is a chemically regulated process requiring synaptic vesicle endocytosis. Clatherin-coated vesicles represent one specific type of trafficking molecule taking part in this process, promoting signal transmission following the removal of several inhibitory phosphorylation sites [177, 178]. AP180 is an important adapter protein mediating lipid and clatherin binding interaction during neurotransmitter release [179]. Mass spectrometry reveals that AP180 can be O-GlcNAcylated at Thr310 and extensively phosphorylated at numerous residues in rodent brains [121, 180, 181]. Surprising results indicate that Thr310 of AP180 can be modified by a unique O-GlcNAc-phosphate moiety that is flanked by Ser306 and Ser313 phosphosites [121]. Since both O-GlcNAc and phosphorylation events increase hydrophilicity and solubility, these adjacent PTMs on AP180 may hinder vesicle endocytosis by inhibiting protein-

protein interactions [121]. In contrast, these modifications may potentially serve as docking sites for specific substrate interaction [121]. While enticing possibilities, neither has been confirmed experimentally to this point. This is not the first time O-GlcNAc sites have been found on synaptic vesicles involved in neurotransmitter signaling. Bassoon and Piccolo proteins vital for synapse assembly and vesicle docking have also been shown to be extensively O-GlcNAc modified, but impact on function has yet to be established [125]

As briefly mentioned, O-GlcNAc is suspected to contribute to nerve cell growth and elongation. Experiments in developing chicken forebrains show that O-GlcNAc localizes strongly in the cell bodies of axonal filopedia, lamellipodia protrusions and the growth cone [182]. Elevating O-GlcNAc by OGA inhibition increases axon branching events in neurons, while attenuating axonal filopodial numbers [182]. These results, together with the observation that elevating O-GlcNAc blocks forskolin-induced phosphorylation required for branching, suggest a repressive role for O-GlcNAc in axon branching and neuronal morphogenesis [182]. Because nerve cell growth and plasticity are important in cognitive behavior, investigation into an O-GlcNAc-directed role in learning and memory is ongoing. Mek2, a kinase stimulating Erk 1/2 signaling via phosphorylation, is an important regulator in synaptic plasticity, learning and memory [183]. This protein can be O-GlcNAc modified (Ser396) as well as phosphorylated (Ser394) to trigger negative feedback inhibition and block the MEK pathway [124, 184-186]. Reciprocity is likely to occur between these proximal sites on Mek2 to influence cognition through neuronal cell signaling control. Additionally, o-glycosylation of the previously discussed CREB protein appears to modulate long-term memory formation and consolidation [174]. In a somewhat similar study, the *Drosophila* PERIOD protein (dPER) is O-GlcNAcylated and temporally regulated in Schneider 2 cells [187]. This protein interacts with several others to form a transcriptional feedback loop controlling

circadian rhythms; the daily oscillations in behavioral and physiobiochemical processes [188, 189]. OGT siRNA knockdown experiments dramatically shorten normal bimodal morning and evening behavior, while overexpressing OGT increased this behavioral period [187]. Specifically, manipulation of OGT regulates dPER nuclear/cytoplasmic entry into pacemaker neurons to most likely account for the altered rhythms [187]. Results strengthening this notion demonstrate that O-GlcNAc modification of dPER delays its phosphorylation-driven degradation, likely through the commonly observed reciprocal PTM relationship [187]. While more work is needed to understand the specific functions for O-GlcNAc in the CNS, it is clear that this modification regulates synaptic signaling proteins in the circadian clock network and during memory formation.

5. O-GlcNAc in the Heart: Cardiac Function and Inflammatory Signaling

O-GlcNAc has been implicated in pathogenesis and end-stage complications of type II diabetes for more than a decade [18, 190-193]. Because heart disease represents the largest group of diabetes-related problems, many studies have been aimed at identifying how O-GlcNAc impacts the molecular events leading to cardiac complications [194-197].

Post-injury Cardiac Protection by O-GlcNAc Enrichment

Heart disease-related complications are responsible for the highest rate of annual deaths in the Western world [198]. Arterial blockage restricts blood flow from reaching tissues, starving them of oxygen and glucose required for normal cellular metabolism. This condition, also known as ischemia, is of major concern in the heart where myocardial damage attenuates physiological function. Cardiac injury is often exacerbated when normal blood supply returns to the site in an event called reperfusion. The rapid restoration of oxygen and nutrient supplies causes an inflammatory response and often leads to oxidative stress-induced tissue damage that can culminate in cellular apoptosis

[199, 200]. Since O-GlcNAc levels are induced by stress and glucose flux, both of which occur during reperfusion, experimentalists have recently investigated whether this PTM may be involved in the process of ischemia-reperfusion injury.

Left ventricle myocardial biopsies from human patients displaying aortic stenosis have elevated O-GlcNAc levels compared to normal control samples [201]. Further analysis reveals that OGA and OGT protein levels are higher in these patients, coinciding with increased gene expression profiles for these cycling enzymes [201]. Rat models recapitulating the pathophysiology in the failing heart display similar results, suggesting O-GlcNAc signaling increases under cardiac stress [201]. Interestingly, manipulating O-GlcNAc levels in cardiomyocytes under basal conditions does not significantly impact heart function [202]. However, animals subjected to ischemia and reperfusion display considerable elevations in O-GlcNAc in damaged ventricle cells that can be augmented by increasing HBP flux with GlcN pre-supplementation [203]. Together these findings insinuate strong correlation between elevated O-GlcNAcylation and cardiac complications, but does this synergism convey negative or positive effects within the heart?

Experiments investigating cardiac function in animals following ischemia/reperfusion show that OGA inhibition increases arterial and aortic vascular reactivity [204]. Other studies inhibiting OGA demonstrate augmented cardiac contraction and relaxation, while significantly attenuating the appearance of arrhythmic activity during reperfusion [202]. Work using conditional OGT knockout mice (cmOGT) show that disrupting cardiomyocyte O-GlcNAc levels does not significantly influence cardiac function within the unstressed heart since there are no signs of increased hypertrophy, apoptosis or collagen accumulation compared to WT controls [205]. However, cmOGT mice subjected to infarction exhibit worsening symptoms of heart failure, specifically: exaggerated left ventricular dilation in diastole, aggravated fractional

shortening, impaired left ventricle contraction and relaxation and increased cases of pulmonary edema [205]. Interestingly, there is no significant difference in myocyte hypertrophy and survival rate between cmOGT and WT mice post-infarction [205]. However, noninfarcted myocardium in the hearts of cmOGT mice display greatly elevated levels of apoptosis and decreased expression of nutrient signaling molecules that together implies a veritable metabolic collapse when OGT is absent from the infarcted heart [205].

One of the major concerns of prolonged ischemia is irreversible myocyte infarction [206]. A preventive measure to reduce tissue death is ischemic preconditioning, where periods of coronary artery occlusion are delicately interspersed with reperfusion events to establish an acute memory phase to prevent myocardial injury [207]. Various exogenous metabolites can trigger preconditioning, as can anesthetic treatment typically referred to as anesthetic preconditioning (APC) [207, 208]. Mice subjected to APC through isoflurane supplementation express elevated O-GlcNAc levels within the heart compared to untreated controls [209]. APC mice display decreased myocardial infarction in the area at risk that can be reversed with OGT inhibitor pretreatment [209]. OGT inhibition combined with APC also significantly enhances myocyte viability following stimulated ischemia-reperfusion [209]. Isoflurane-initiated APC protects against ischemic injury at least in part by regulating mitochondrial ion flow through voltage-dependent anion channels (VDAC) [210, 211]. Previous studies reveal that O-GlcNAc modification of VDAC is essential for myocardial survival [212], but were never tested under ischemic conditions. APC treatment prevents the opening of the mitochondrial permeability transition pore in cardiac myocytes during ischemia, prohibiting the translocation of pro-apoptotic molecules [213, 214]. Because VDAC is one of the structural components regulating pore opening and is O-GlcNAcylated, it is possible that this modification helps impart oxidative mitochondrial protection. Indeed, APC adult cardiac mitochondria

displayed higher levels of O-GlcNAc modified VDAC compared to unconditioned controls, while OGT inhibition reverses this effect and abolishes APC oxidative protection (Fig 2.5A)[209].

Multiple lines of research have established that O-GlcNAc offers cardioprotection in the heart, but there is also some evidence indicating a potential problematic role for the PTM. Arterial hypertension is a chronic elevation in blood pressure that significantly increases the heart's workload [215]. Rise in pressure can be caused by a number of events, including partial blood vessel occlusion, and if untreated can lead to myocardial infarction [216]. Provided the degree of O-GlcNAc involvement after ischemia and reperfusion, it is reasonable that it may influence molecular aspects of hypertension. Deoxycortacosterone acetate (DOCA)-salt induced hypertension is a common mineralocorticoid model that elevates O-GlcNAc in treated rats compared to WT controls [204]. DOCA-salt and OGA inhibited rats display decreased cardiac relaxation in response to acetylcholine and decreased phosphorylation of cardiovascular homeostatic proteins eNOS and Akt [204]. Further experiments show that DOCA hypertension elevates O-GlcNAc-modified eNOS in the rat aorta, while decreasing levels of OGA, OGT and the HBP rate-limiting enzyme GFAT expression [204]. Other work demonstrates that increasing O-GlcNAc via OGA inhibition reduces endothelial nitric oxide synthase activity to attenuate nitric oxide production [217] and appears to impair vasodilator activity in DOCA-salt models [204]. Endothelin-1 (ET-1) is a peptide that induces vasoconstriction and has shown to be elevated in the vasculature of DOCA-salt hypertensive rats [218]. Interestingly, in hypertensive conditions ET-1 also activates transcription factors governing inflammation, oxidative stress and tissue damage [219, 220]. Rat aortas incubated with ET-1 peptide display elevations in stimulated vasoconstriction in combination with increased vascular O-GlcNAcylation [221]. OGT inhibition blocks this ET-1 induced effect on vascular activity, suggesting that O-GlcNAc

in part mediates this ET-1 response [221]. ET_A receptor agonist supplementation diminishes vascular O-GlcNAc levels and augments vascular contractile function typically observed upon ET-1 stimulation [221]. Together these results implicate O-GlcNAc as a possible culprit in cardiac dysfunction during salt-induced hypertension, although additional research is required to substantiate this claim. In all, these findings show that O-GlcNAc is essential for cardioprotection following ischemic and reperfusion injury, but additional studies are needed to determine it's contribution during hypertension.

O-GlcNAc in Cardiac Inflammatory Signaling

Hypertrophy and oxidative stress impinge on cardiovascular function by influencing the state of cellular inflammation. Acute vascular injury, as discussed previously, activates inflammatory signaling cascades to recruit primary immune system mediators as the initial protective response [222-224]. Considering it's vital role in responding to cellular stress, many studies have been aimed at determining the role of O-GlcNAc in cardiac inflammation and the purpose for this PTM within this process.

Phenylephrine (PE) stimulation is a commonly used model to recapitulate cardiac hypertrophy through activation of the neural factor of activated T-cells (NFAT) signaling cascade [225, 226]. During hypertrophic events there is an observed increase in arterial natriuretic peptide (ANP) levels that appears to directly correlate with O-GlcNAc signaling. Not only does PE treatment elevate O-GlcNAcylation and OGT protein levels in neonatal rat cardiomyocytes, but also induces higher expression of ANP mRNA [227]. Under conditions where HBP flux is blocked or OGA levels are elevated, both O-GlcNAc and ANP mRNA levels are significantly reduced in response to PE incubation [227]. Further studies indicate that O-GlcNAc reduction decreases ANP mRNA by blunting NFAT signaling and specifically prevents its nuclear translocation [227].

Previous work suggests that myocardial hypertrophy is at least partially caused by dysregulation of glucose uptake and utilization, wherein the insulin-dependent glucose transporter (GLUT1) is preferentially favored over its non-insulin dependent counterpart (GLUT4) [228]. Strikingly, hypertrophic increases in O-GlcNAc directly correlate with a GLUT1 and GLUT4 expression imbalance, while OGA overexpression restores normal transporter proportions [227]. In contrast, cardiomyocytes from diabetic mice lack augmented ANP levels versus controls during PE supplementation, along with the reduction in other early markers of cardiac hypertrophy [229]. These findings may be in connection with O-GlcNAc signaling seeing in that GFAT inhibition in diabetic mice causes significantly elevated ANP expression and OGA inhibition completely blocks the observed increase in WT controls [229]. Although these results imply a possible protective role for O-GlcNAc in regards to hypertrophic cardiac signaling, it is important to consider the other metabolic irregularities at play in the diabetic phenotype that may be influencing this pathway.

Activation of the inflammatory signaling cascade in shown to impart arterial epithelial dysfunction through T lymphocyte-induced elevation in tumor necrosis factor (TNF) α [230-232]. Overproduction of ROS through activated ROS-enzymes, including inducible nitric oxide synthase (iNOS), is mediated by TNFα stimulation of the NFκB pathway [233, 234]. Rat aortic rings treated with TNFα display impairment in depolarization-induced contractile responses that is reversed with GlcN or OGA inhibitor addition [235]. Increasing O-GlcNAc also appears to drastically decrease TNFα-induced iNOS protein expression and the accumulation of free radical forming nitrotyrosine radicals often seen during oxidative stress [235]. O-GlcNAc-induced iNOS attenuation is also observed in rats subjected to trauma-hemorrhage followed by full resuscitation and directly correlates with their significantly increased survival rate [236]. Several studies implicate O-GlcNAc involvement in regulating NFκB transduction [237-239], but more

recent work provides a clear link in rat aortic smooth muscle cells. Phosphorylation of NFκB is essential in determining its transcriptional activity [240-243]. Aortic smooth muscle cells incubated with GlcN or an OGA inhibitor limits inflammatory NFκB p65 DNA binding typically seen in TNFα stimulation [244]. GlcN supplementation or OGA inhibition increases O-GlcNAc modification of NFκB p65 and prevents its concurrent nuclear phosphorylation at Ser536 (Fig 2.5C)[244]. This reduction of phosphorylated p65 coincides directly with its enhanced interaction with the inhibitory complex protein IκBα and the reduction in TNFα triggered inflammatory signaling [244].

Genetically programmed cell death, or apoptosis, contributes to cell destruction following cardiac infarction and ischemia/reperfusion injury. OGT overexpression significantly reduces the ER stress response in cardiomyocytes subjected to hypoxia and reoxygenation and ultimately protects against unfolded protein response (UPR)-induced cell death [245]. But until recently, little was known at a molecular level as to how increasing O-GlcNAc augments this cell survival. Autophagy is essential for cellular protection, but if constitutively activated can promote apoptosis [246]. This process is extremely active in the injured cardiovascular system and its maladaptive control is thought to be primarily responsible for cell death in heart failure [247, 248]. Two major interaction components in this system are Beclin-1 and Bcl-2, the pro- and anti-apoptosis promoting factors respectively [249]. Dissociation of Bcl-2 from Beclin-1 induces autophagic events and is linked to pressure overload stress-induced cardiac hypertrophy [250]. Both interacting partners can be O-GlcNAcylated and phosphorylated to differentially control their interaction [251]. Interestingly, upon glucose starvation in the diabetic model pro-apoptotic protein Beclin-1 levels are reduced in cardiomyocytes to suggest a potential role for the HBP and O-GlcNAc [251]. Moreover, blocking HPB flux significantly increases the autophagic response in diabetic mice and OGA inhibition greatly reduces Beclin-1 expression [251]. Neonatal rat ventricular myocytes treated with

GlcN and, to lesser extents OGA inhibition, display increased mitochondrial Bcl-2 that correlates with decreased post-ischemia and reperfusion cell injury during OGT overexpression [203]. Along with these findings, GlcN and OGT overexpression also prevent the loss of cytochrome c after cardiac damage, which serves as an apoptotic cell identifier when secreted from the mitochondria [203]. siRNA OGT-directed knockdown experiments verify these pharmacological findings by causing greatly reduced mitochondrial Bcl-2, exhibiting markedly higher cytochrome c secretion and disrupting mitochondrial membrane potential to promote higher cellular apoptosis after ischemia and reperfusion (Fig 2.5B)[203]. This set of studies clearly indicates the cardiac protection provided by O-GlcNAc occurs within cell signaling networks to prevent oxidative damage, apoptosis and uncontrolled autophagy.

6. O-GlcNAc Regulates Transcriptional Activity in Cancer

Pancreatic Cancer

Nuclear factor kappa B (NF-κB) is a transcription factor known to play a role in various cellular processes like inflammation, cell survival, tumorigenesis and apoptosis [252, 253]. In its inactive state NF-κB is sequestered in the cytoplasm by binding to inhibitory kB (IκB). Following extracellular stimulation, IκB is phosphorylated by IκB kinase and subsequently ubiquitinated to facilitate proteosomal degradation [254]. The nuclear localization signal on NF-κB is uncovered in this state to allow for its nuclear translocation and facilitating transcription of downstream genes [255]. NF-κB is known to interact with OGT and contains several O-GlcNAc modification sites in lymphocytes with mutational analysis confirming T352 is required for NF-κB translocation and activation [237, 256] [Fig 2.6A]. Hyperglycemia causes increased transcriptional activation of NF-κB due to nuclear translocation by decreased interactions between NF-κB and IκB in vascular smooth muscle cells (VSMCs) [256]. Interestingly, OGA

overexpression under hyperglycemic conditions inhibits nuclear translocation of NF-κB while increasing O-GlcNAc with OGT overexpression is required for NF-κB activation in VSMCs [256]. OGT siRNA mediated knockdown in HEK293 cells display decreased mRNA levels of the NF-κB regulated genes *IL-8* and *BCL2A1* [Fig 2.6B]. OGT overexpression in HEK293 cells increase transcription of these genes while conversely, OGA overexpression reduces their transcription suggesting OGT and O-GlcNAc cycling are required for the transcriptional activation of NF-κB [257] [Fig 2.6A and B]. Attenuation of NF-κB signaling pathway can result in pancreatic ductal adenocarcinoma (PDAC) cell apoptosis [258], while constitutive NF-κB signaling is a hallmark of several cancers including PDAC [259].

O-GlcNAc and OGT levels are elevated in several different pancreatic cancer cell lines corresponding with decreased OGA levels [260]. This observed increase in OGT and concomitant decrease in OGA is seen in other cancers, such as lung and colon [261]. The observed hyper O-GlcNAcylation in many cancers like breast (Caldwell 2010), pancreatic (Ma 2013), prostate (Lynch 2012), liver (Zhu 2012), lung and colorectal (Mi 2011, Yehezkel 2012) maybe attributed to the expression pattern of the cycling enzymes. Notably, UDP-GlcNAc levels are elevated in pancreatic cancer cell (Ma 2013). OGT knockdown in PDAC cell line, MiaPaCa-2, led to an observed decrease in cell proliferation in both 2-and 3- dimensional cultures as well as colony formation [260]. However, non-transformed human pancreatic epithelial cells (HPDE) did not display reduced cell proliferation when OGT was silenced to the same extent as PDAC cells [260]. OGT inhibition [262] leads to reduced O-GlcNAcylation and inhibits both colony formation and cell proliferation [260]. This is recapitulated *in vivo* by using OGT silenced orthotopic xenografts [260]. Immunocompromised mice injected with OGT shRNA display smaller tumors in weight compared to scrambled shRNA [260]. OGT shRNA mediated suppression of hyper O-GlcNAcylation induces caspase-3 and caspase-

9 cleavage, indicative of apoptosis [260]. Conversely, elevating O-GlcNAc levels by inhibiting OGA decreases caspase-3 cleavage and rescued cells from suspension-induced apoptosis [260]. Collectively, these data establish a role for hyper O-GlcNAcylation in PDAC cell survival via inhibition of apoptosis. The p65 subunit of NF-κB and its kinase, IKKβ, are O-GlcNAc modified [263] in PDAC cells [260]. OGT knockdown studies in PDAC cells display reduced O-GlcNAcylation and IKKβ mediated phosphorylation at S536 of p65 that prevent its nuclear translocation and activation [241]. Reduction in PDAC hyper O-GlcNAcylation decreases p65 nuclear localization and transcriptional activity [260], while also decreasing NF-κB targets Cyclin D1, Vimentin and Bcl-xL protein expression levels. Conversely, E-cadherin levels, normally inhibited by NF-κB, are increased in OGT knockdown PDAC cells [260]. Furthermore, OGA inhibition mediated increase in O-GlcNAc lead to increased p65 O-GlcNAcylation [260]. Additionally, anchorage-independent growth induced by p65 overexpression is ablated in OGT knockdown PDAC cells [260]. These results show that increased O-GlcNAc levels seen in PDAC cells correspond to their increased proliferative capacity. This provides evidence to suggest that targeting OGT may be therapeutically useful to increase caspase-mediated apoptosis in these cells.

Breast Cancer

Forkhead Box M1 (FOXM1) is a proliferation specific transcription factor controlling the cell cycle at the S phase, M phase, G1/S and G2/M phase [264]. FOXM1 is shown to upregulated in several cancers [265] with some examples being breast and prostate cancers [266, 267]. Furthermore, FOXM1 is clearly implicated in cell migration, invasion, angiogenesis, metastasis and inflammation [265, 268]. Another protein of the Forkhead family, FOXO1 is a known O-GlcNAc modified protein [269]. The functional impact of this modification is still unclear.

It is documented that OGT downregulation inhibits cell cycle progression [90, 93] [270, 271]. Consistent with other studies [260, 267], OGT is required for *in vivo* tumorigenesis as evidenced by a four-fold reduction in tumor volumes in *Nu/Nu* mice injected with OGT shRNAs compared to scrambled control [266]. FOXM1 protein expression is diminished in the breast cancer cell line MDA-MB-231 and oncogene over-expressing cell line MCF-10A-Erb2 when OGT is knocked down [266]. Consistent with this data, targets of FOXM1 like Survivin, Nek2, PLK1 are also decreased in OGT knockdown in both cell lines [266]. FOXM1 is a known transcriptional activator of Skp2 [272], which regulates the degradation of $p27^{Kip1}$ during the G1/S transition [273] [Fig 2.6D]. Interestingly, levels of $p27^{Kip1}$ are increased in OGT knockdown in both MDA-MB-231 and MCF-10A-Erb2 cells [266]. Furthermore, reduction in OGT causes accumulation of cells in G1 phase [266] [Fig 2.6C]. Another target of FOXM1, matrix metalloproteinase 2 (MMP2) is down regulated in OGT knockdown MCF-10A-Erb2 cells. MMP2 is a major player in angiogenesis and metastasis [274, 275] that is regulated by OGT levels through a possible mechanism via FOXM1. Inhibiting OGT pharmacologically decreases FOXM1 protein levels in MCF-10A-Erb2 cells, reducing their proliferation and invasion capacities in response to lower O-GlcNAc levels [266].

OGT knockdown studies also implicate O-GlcNAcylation in breast cancer metastasis via E-Cadherin/catenin complex [276]. E-cadherin is pivotal for cell-cell adhesion, which is mediated by its interaction with β-catenin and p120 [277-279]. OGT silencing in 4T1 breast cancer cells causes an elevation in E-Cadherin and β-catenin protein expression while p120 remains unaltered [276]. In murine 4T1 cells which recapitulate human breast cancer phenotype, only p120 and β-catenin are O-GlcNAcylated [276] unlike E-Cadherin that is found O-GlcNAcylated in several other breast cancer cell lines [280]. Immunofluorescence detection portrays a significant increase in E-Cadherin, β-catenin and p120 on the cell surface in OGT silenced cells

while OGA inhibition displays lowered levels of E-Cadherin, β-catenin and p120 at the cell surface [276]. Interestingly, OGT and E-cadherin double knockdown of cells cannot inhibit cell migration as efficiently as OGT single knockdown in the 4T1 cells [276]. O-GlcNAc modification of E-cadherin by endoplasmic stress inducing agents block cell surface transport and cell adhesion capacity [280]. Given that loss of E-cadherin is associated with breast cancer transformation and metastases [281, 282], this data suggests that OGT deregulates E-Cadherin function in breast cancer cell line. Collectively, OGT is involved in breast cancer proliferation and metastases through its regulation of FOXM1 as well as E-Cadherin.

Prostate Cancer

Prostate carcinoma cell lines exhibit higher OGT mRNA, protein and O-GlcNAc levels compared to normal prostate cell that directly coincide with lower OGA protein levels [267]. Lentiviral knockdown of OGT in PC3-ML prostate carcinoma cell line leads to an 80% reduction in anchorage independent growth compared to PC3-ML control cells [267]. Both shOGT treatment and OGT inhibition display decrease in PC3-ML ability to grow in 3D culture and lower FOXM1 expression and elevated $p27^{Kip1}$ expression [267]. FOXM1 is shown to play a role in angiogenesis by the regulation of VEGF in several cancers [283-285]. Vascular endothelial growth factor (VEGF) mRNA is decreased by 50% in shOGT expressing PC3-ML cells and correlates with decreased VEGF mRNA by FOXM1 knockdown [267]. OGT regulates FOXM1 expression via protesosomal degradation and a non-degradable FOXM1 can rescue the angiogenic potential of shOGT expressing PC3-ML cells [267]. This suggests that OGT levels and its regulation of FOXM1 are crucial for the angiogenic potential of prostate cancer cells.

MMP2 and matrix metalloproteinase 9 (MM9) have been previously described to in prostate cancer metastasis [286, 287]. PC3-ML cells expressing shOGT have

decreased ability to invade as observed by matrigel transwell assays [267]. Additionally, these cells have a significant reduction in their MMP2 and MMP9 mRNA and protein expression when compared to control PC3-ML cells [267]. Non-degradable FOXM1 mutant can restore MMP2 levels completely and MMP9 levels partially further reiterating the role of OGT mediated FOXM1 regulation of invasiveness in PC3-ML cells (Lynch 2012). Moreover, PC3-ML cells expressing shOGT have reduced bone metastatic potential when introduced in immunocompromised mice, compared to control shRNA animals [267] identifying OGT as a potential target for prostate cancer therapy.

OGT inhibition in LNCap, VCap and PC3 cancer cell lines causes loss of c-Myc protein expression [288]. c-Myc, a proto-oncogene, is O-GlcNAcylated at T58 in its N-terminal transactivation domain [16, 289]. C-Myc is a nuclear phosphoprotein containing a basic Helix-loop-Helix zipper domain that is a well-established transcriptional regulator involved in several cellular processes such as proliferation, differentiation and apoptosis [290]. O-GlcNAc modification of β-catenin in normal cells is higher than in cancer cell lines like LNCap [291]. O-GlcNAcylation negatively regulates the transcriptional activity of β-catenin through cytoplasmic sequestration, confirmed in OGA inhibition studies that decrease its nuclear accumulation and augments its cytoplasmic pool in DU-145 and LNCap prostate cancer cells [291]. The mechanism of dysregulating β-catenin O-GlcNAcylation and its nuclear localization is yet to be elucidated. This study highlights that O-GlcNAc levels can play a protective role against disease and antagonists of OGA can be exploited for prostate cancer therapy.

O-GlcNAc Modulates Metabolism in Other Cancers

Altered metabolism is a hallmark of cancer cells [292]. Cancerous cells exhibit the "Warburg effect" whereby the cells display significantly increased glucose consumption and aerobic glycolysis (Dang 1999). Given that HBP is regulated by glucose flux and its

end product is the substrate for OGT, the potential role of HBP, O-GlcNAc and OGT in cancers is being intensively studied.

CLL is characterized by the aberrant responses to microenvironment [293]. CLL patient samples display higher O-GlcNAc levels when immunoblotted with RL2 antibody in comparison to peripheral blood mononuclear cells (PBMCs) [294]. Targets of OGT like p53, c-Myc, Akt and OGT itself are O-GlcNAcylated in CLL patients [294]. Employing OGT inhibitor strategies, it is evident that Akt T308 phosphorylation is increased in CLL when O-GlcNAc is decreased [294]. Conversely, elevation of O-GlcNAc levels by addition of uridine and GlcNAc attenuates Akt T308 phosphorylation and decreases its activity [18, 294]. Increasing O-GlcNAc levels in CLL patient cells impairs c-Jun N-terminal kinase (JNK) phosphorylation thereby affecting IκB phosphorylation [294]. This defective phosphorylation of JNK is observed in normal B cells, as well as CLL when incubated overnight with uridine and glucosamine [294]. Elevated O-GlcNAc levels affect JNK signaling to retard cell division and activation signals possibly describing the observed RL2 index of less severe CLL [294]. Stage IV CLL patients have a lower RL2 index in comparison to a milder CLL phenotype suggesting that higher O-GlcNAc levels are indicative of indolent CLL phenotype [294]. However, the mechanism leading to reduction in O-GlcNAcylation in the more aggressive CLL phenotypes is still unclear.

p53 is a tumor suppressor that is the target of many mutations in several cancers [295] and is stabilized by O-GlcNAc modification [296]. p53 loss of function is associated with an increase in glycolysis [297] via IKK-NF-κB pathway [298]. MCF-7 cells with p53 knockdown consume more glucose in comparison to control and exhibit elevated levels of O-GlcNAcylated IKKβ and activating phosphorylated IKKβ [263]. p53 deficient MEFs display higher O-GlcNAcylated IKKβ [263]. Additional studies confirm that p65-NF-κB is necessary for $p53^{-/-}$ mediated enhanced glycolysis [298]. Moreover,

p65-NF-κB knockdown in $p53^{-/-}$ MEFs leads to decreased O-GlcNAcylated IKKβ and activating phosphorylation of IKKβ [263]. Transformed Tig-3 human primary fibroblasts also display increased glucose consumption as well as concomitant elevation of O-GlcNAcylated IKKβ and activation phosphorylation of IKKβ [263]. O-GlcNAc on S733 is important for enhanced glycolysis as mutating the serine to a glutamate or alanine both lead to lower glucose consumption [263]. TNFα stimulation of p53 deficient MEFs activates IKKβ and NF-κB in comparison to WT MEFs [263]. These data suggest that O-GlcNAcylation of IKKβ may mediate the constitutive NF-κB activation as seen in several cancers.

Increasing O-GlcNAc levels by over-expressing OGT in lung cancer cell line H1299 leads to decreased glucose consumption along with lower lactate and ATP levels [299]. Elevated O-GlcNAc levels also lead to reduction in the activity of phosphofructokinase 1 (PFK1) activity [299], serving as a major player in and regulating the flux through glycolysis [300]. PFK1 is O-GlcNAcylated in a variety of cell lines including LNCap, MDA-MB-231 and MCF-7 (Yi 2012). Under hypoxia and glucose deprivation, normally associated with tumorigenesis, PFK is O-GlcNAcylated in H1299 cells [299] at the residue S529 [299]. S529 is the highly conserved residue on PFK1 that allows for allosteric regulation by fructose 2,6- bisphosphate (F-2,6 BP) [301]. O-GlcNAcylation of S529 of PFK1 causes formation of low molecular weight complex while S529A is unperturbed and runs as a higher molecular weight complex [299]. Overexpressing OGT in H1299 cells containing Flag-tagged knock in of WT PFK1 reduces lactate production and glycolysis, a key feature of cancer cell metabolism [299]. No change in either glycolysis or lactate production is observable in S529A PFK knock in under OGT overexpression [299]. Inhibiting flux through glycolysis can shift the levels of pentose phosphate pathway (PPP) [299]. OGT overexpression increases PPP flux in WT PFK1 knock in demonstrating the deregulation of glycolysis [299].

Consistent with PPP flux, NADPH and reduced glutathione (GSH) are increased in WT PFK1 knock in cells with OGT overexpression under hypoxia [299]. S529A knock in cells demonstrate significantly lower levels of NADPH and GSH suggesting that blocking glycosylation may potentially restore glycolysis [299]. Immunocompromised mice injected with WT PFK1 knock in cells with OGT overexpression display more tumorous growths while the S529A mice exhibit smaller tumors [299]. PFK1 O-GlcNAcylation at S529 is required for enhanced tumor growth and this can be exploited for therapeutics against cancerous cells.

7. Concluding Remarks

Extensive understanding into how O-GlcNAc influences biological systems has grown considerably in recent years. This dynamic and inducible nutrient sensor is a well-established regulator of metabolic- and stress-induced cellular activities. Unfortunately, direct functional connections have proven difficult due to technological limitations in combination with the field's adolescence. More recent studies are beginning to substantiate previous claims that O-GlcNAc is essential in controlling molecular events. Epigenetics has exploded onto the scene as of late, providing an intricate model for environmental gene regulation. Although its biological introduction within this area was delayed compared to other PTMs, it is now clear that O-GlcNAcylation is a major part of the histone code. Various works demonstrate histone proteins themselves can carry the O-GlcNAc moiety, while the cycling enzymes interact with numerous chromatin-associated complexes to affect nucleosome accessibility. Stem cell biology is extremely promising in terms of therapeutics, but details of the signaling pathways dictating cellular fates remain elusive. O-GlcNAc is now known to regulate ESC pluripotency and self-renewal, along with mesodermal differentiation into several cell types. Deregulated metabolism represents a common phenotype in many disease pathologies. Earlier works flirted with the notion that O-GlcNAc contributed substantial molecular regulation in

these ailments, but were unable to show this decisively. Thanks to extensive investigation over the last several years, this PTM is definitively shown to influence the progression of multiple diseases, including: Alzheimer's, diabetes, ischemic and reperfusion cardiac injury, hypertension and cancer. In all of these areas O-GlcNAc appears to exert its control at the cell cycle or transcriptional levels, further cementing it as a vital molecular component. While these new findings are exciting and encouraging, there is still much work to be done to validate these results and establish clear functional roles for site-specific O-GlcNAc modification on particular proteins. But science is a discipline that becomes more complicated with discovery and it appears that O-GlcNAc will only continue to beneficially confound our understanding for years to come.

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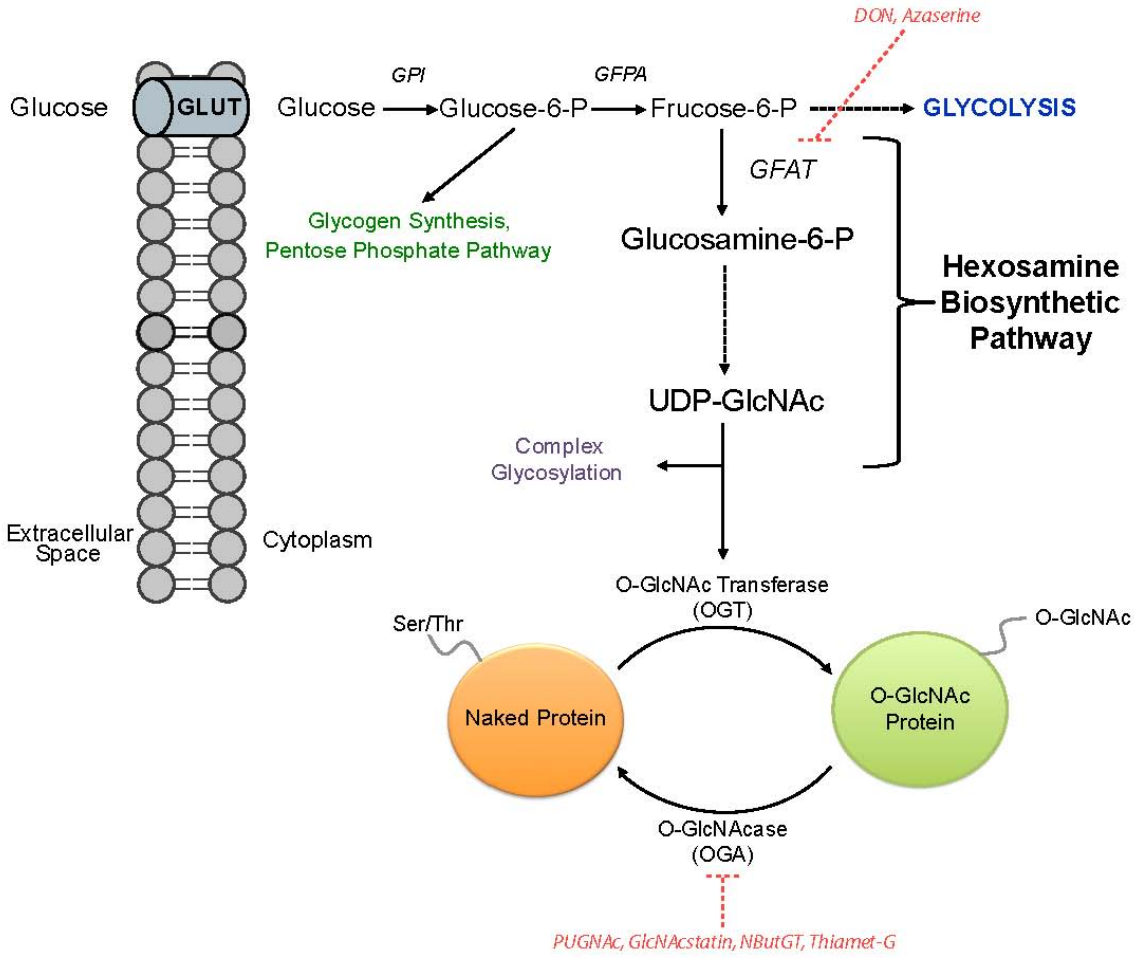
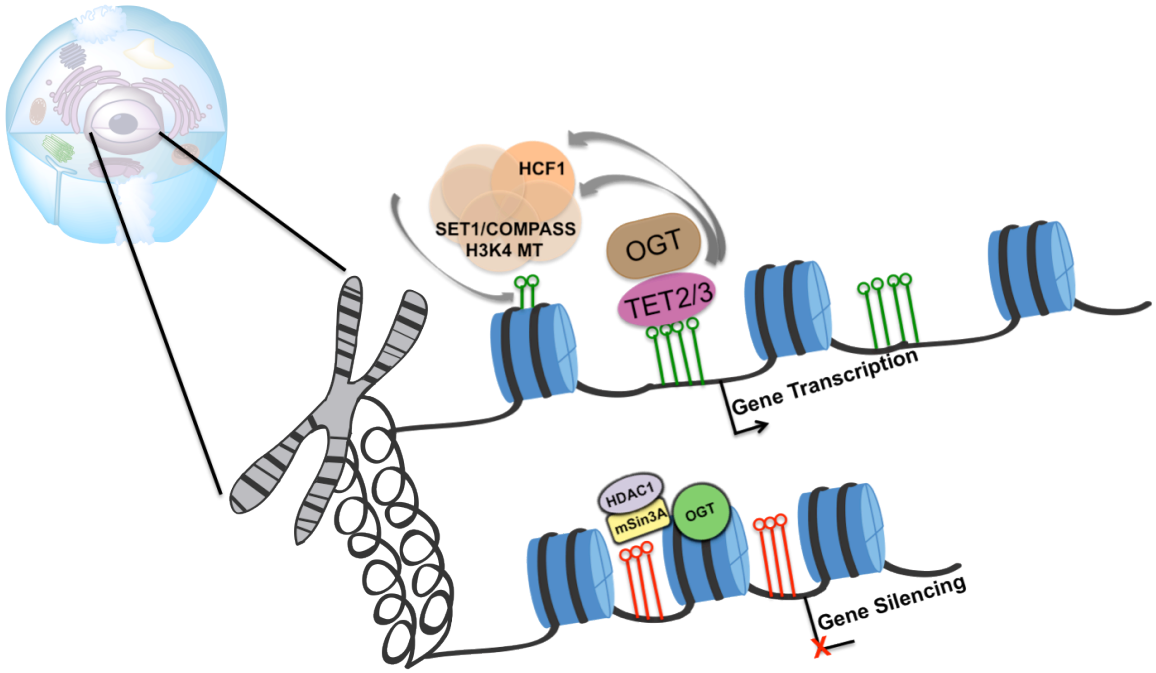
Figure 2.1



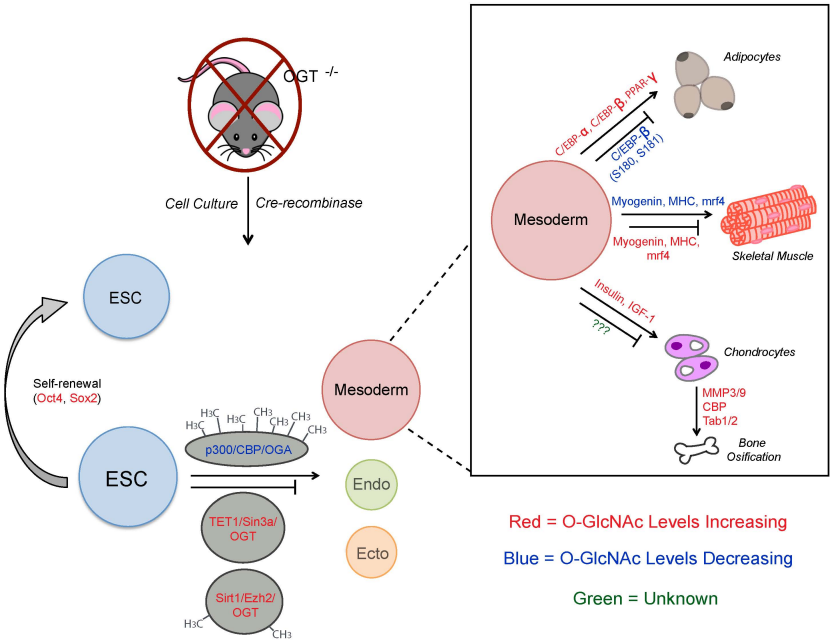
The HBP and the O-GlcNAc Modification. The majority of glucose entering the cell is used in glycolysis, glycogen synthesis or the pentose phosphate pathway. However, a small portion is shunted into the HBP, whose end product is the nucleotide sugar donor UDP-GlcNAc. UDP-GlcNAc serves as a donor for several downstream events, including the synthesis of other nucleotide sugar donors, complex glycosylation events and the post-translational modification of nuclear and cytosolic proteins with O-GlcNAc. OGT is responsible for the enzymatic addition of this sugar moiety to the hydroxyl groups of serine and threonine residues, whereas OGA is the enzyme that removes the PTM. Altered flux through the HBP is one mechanism of attenuating O-GlcNAc cycling that influences numerous molecular events in the cell. Both GFAT and OGA inhibitors are highlighted in red and indicate the stage at which they function.

Figure 2.2



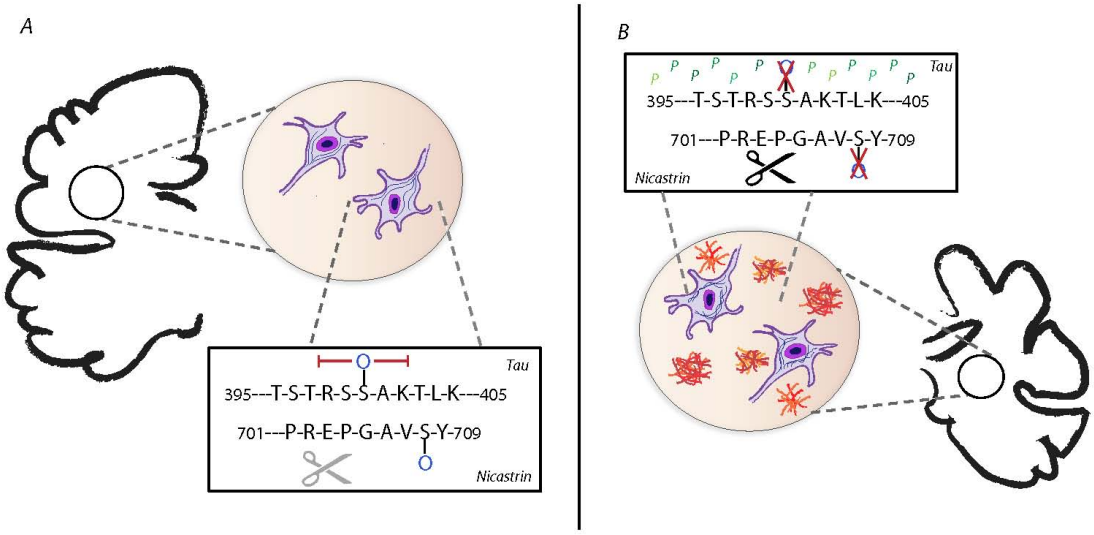
OGT associates with chromatin remodeling complexes. OGT associates with both transcriptional coactivator and corepressor complexes. OGT association with TET2/3 is necessary for the chromatin binding event of SETD1A methyl transferase. This facilitates the transcription of hematopoietic genes possibly in a HCF-1 dependent manner. OGT can also interact with mSin3A along with HDAC1 to functionally repress transcription including Sp1 activated genes.

Figure 2.3



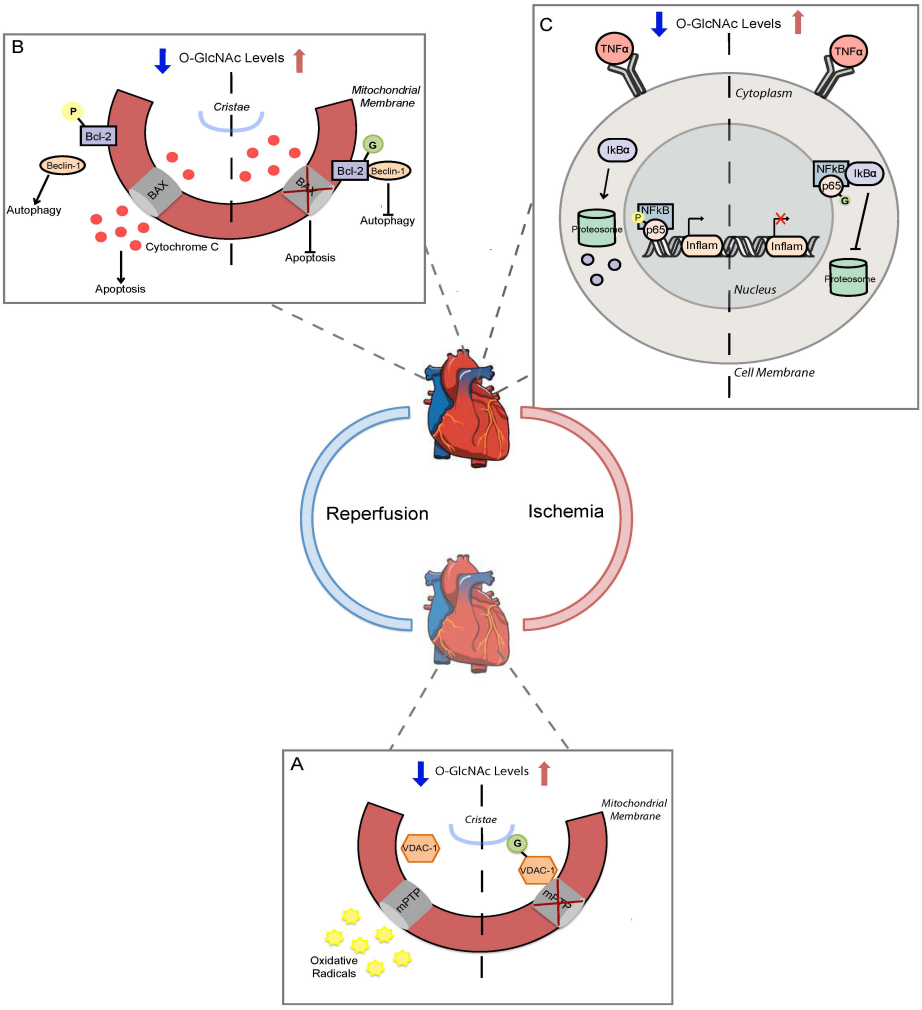
O-GlcNAc levels regulate ESC characteristics and mesoderm differentiation. Complete OGT gene knockout is embryonic lethal, but studies in cell culture or the *Cre*-recombinase system enables O-GlcNAc investigation during differentiation and development. O-GlcNAc appears to influence ESC self-renewal that directly correlates with modulation of several embryonic transcription factors, including Oct4 and Sox2. The cycling enzymes OGT and OGA also interact with the chromatin remodeling and preinitiation complexes to control ESC pluripotency. Mesodermal cell fate is also regulated in response to O-GlcNAc levels, specifically affecting adipocyte, muscle, chondrocyte and bone differentiation. Blue font indicates reduced O-GlcNAc levels; red font indicates elevated O-GlcNAc levels; green font represents currently unknown O-GlcNAc affects.

Figure 2.4



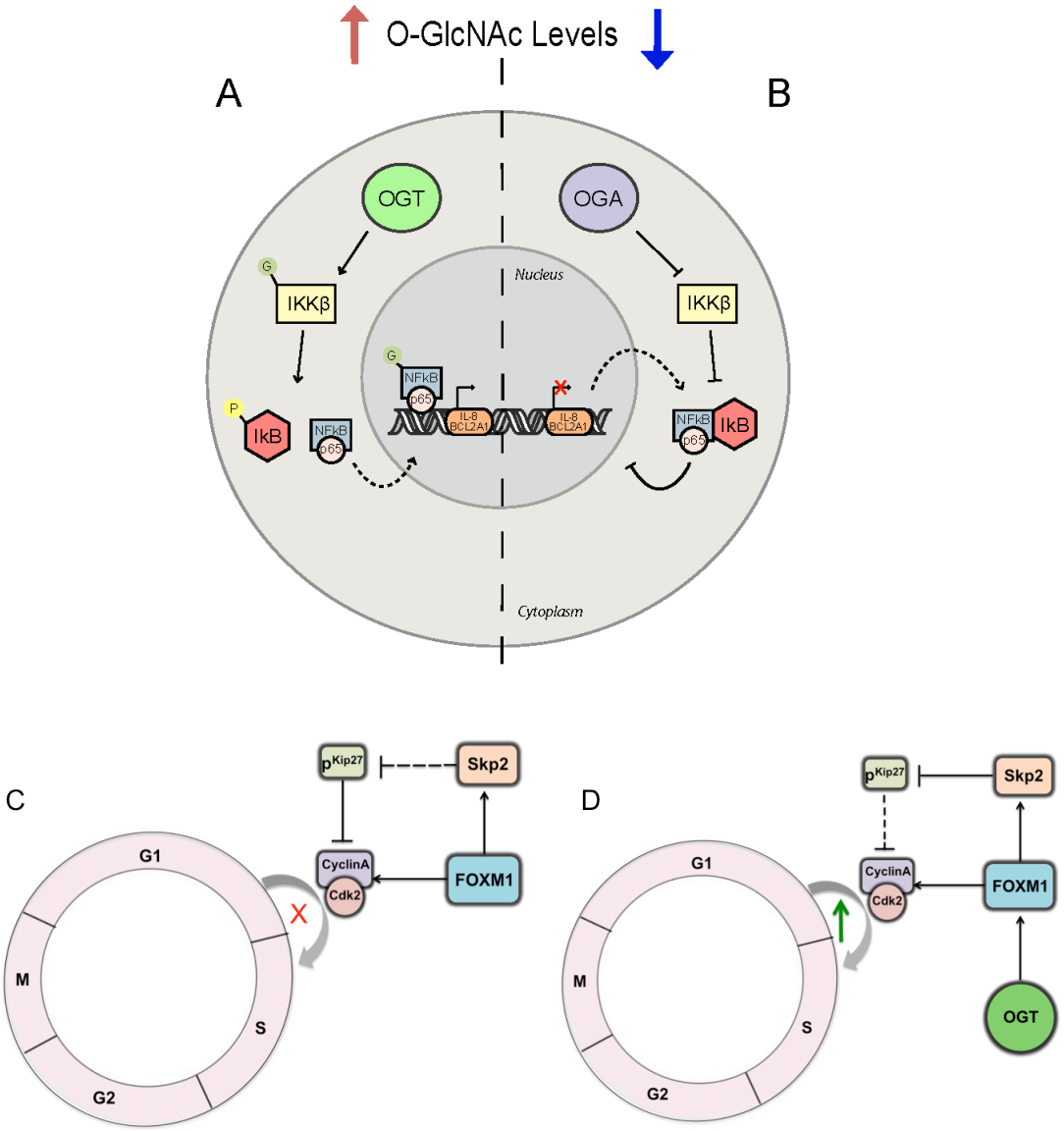
O-GlcNAc protects against symptoms of neurodegeneration in the Alzheimer's brain. (A) The microtubule-associated protein Tau can be O-GlcNAc modified at Ser400 and inhibit its subsequent hyperphosphorylation in Alzheimer's brain samples and models. The nicastrin subunit of the γ secretase complex can also be O-GlcNAcylated at Ser708, preventing APP cleavage and aggregation observed during Alzheimer's progression. (B) Reducing O-GlcNAc levels on both tau and nicastrin alleviates these protective affects, resulting in neurofibrillary tangles and amyloid β plaque accumulation.

Figure 2.5



Increased O-GlcNAcylation offers cardioprotection following ischemia-reperfusion injury. (A) Elevations in O-GlcNAc after vascular ischemia limit oxidative stress through a mitochondrial VDAC-1 mechanism. O-GlcNAc modification of VDAC-1 increases its interaction with the mitochondria permeability transition pore (mPTP) and prevents radical release. When VDAC-1 is unmodified, the mPTP can open and release harmful radical species into circulation. B Upon cardiac reperfusion the pro-autophagic protein Beclin-1 dissociates from its inhibitor Bcl-2 and stimulates constitutively active autophagy. Phosphorylation of Bcl-2 prevents its interaction with Bcl-2 associated X protein (BAX) in the mitochondrial membrane, causing cytochrome c release and apoptosis signal initiation. Bcl-2 O-GlcNAcylation during reperfusion promotes its interaction with Beclin-1 and BAX to inhibit downstream activation of autophagy and apoptosis pathways. (C) NFkB signaling is common following reperfusion in the heart. Decreasing O-GlcNAc promotes phosphorylation of the NFkB DNA binding subunit p65 and restricts IkBα protein inhibition. This enables p65 nuclear translocation where it can stimulate inflammatory gene activation. O-GlcNAc modified p65 subsequently blocks its phosphorylation to promote IkBα-mediated NFkB inhibition and prevents inflammatory gene activation.

Figure 2.6



OGT regulates transcription factors in the cancerous state. A IKKβ phosphorylates IκB facilitating its dissociation from NFκB. Elevating O-GlcNAc by overexpression of OGT or inhibition of OGA O-GlcNAcylates IKKβ and NFκB. NFκB that is O-GlcNAc modified can translocate to the nucleus. In cancer cells, there is an upregulation in this process allowing for increased gene transcription of NFκB targets. B Lowering O-GlcNAc levels by overexpressing OGA or using OGT inhibitors leads to deglycosylation of NFκB and its subsequent expulsion into the cytoplasm. Here it can stay sequestered with IκB, and affects NFκB downstream signaling. C In normal cells, G1/S transition is tightly regulated by p^{Kip27} via inhibition of CyclinA/Cdk2. Skp2 negatively regulates p^{Kip27} to allow for G1/S transition. D In cancer cells, upregulation of OGT levels cause an increase in FOXM1 and thereby Skp2 which inhibits p^{Kip27}. This simulates a constituitive G1/S transition that allows for proliferative capacity of the cells.

CHAPTER 3

O-GLCNAC EPIGENETICALLY REGULATES THE INSULIN GENE IN MIN6 PANCREATIC β CELLS[#]

[1]Durning S.P., Flanagan-Steet H. and Wells L. To be submitted to *PNAS*

1. Abstract

The post-translational protein modification O-linked β-*N*-acetylglucosamine (O-GlcNAc) is a proposed nutrient sensor that has been shown to regulate multiple biological pathways. This dynamic and inducible enzymatic modification to intracellular proteins utilizes the end product of the nutrient sensing hexosamine biosynthetic pathway (HBP), UDP-GlcNAc, as its substrate-donor. Type II diabetic patients have elevated O-GlcNAc modified proteins within pancreatic β-cells due to chronic hyperglycemia-induced glucose overload, but a molecular role for O-GlcNAc within β-cells remains unclear. Using directed pharmacological approaches in the mouse insulinoma-6 (Min6) cell line, we demonstrate that elevating nuclear O-GlcNAc preserves glucose stimulated insulin secretion during chronic hyperglycemia. This observed secretory effect directly correlates with O-GlcNAc-induced elevation in perinuclear insulin under basal and prolonged hyperglycemic conditions. The molecular mechanism for these observed changes appears to be, at least in part, due to elevated O-GlcNAc-dependent increases in *Ins1* and *Ins2* mRNA levels via elevations in histone H3 transcriptional activation marks. Further, RNA-sequencing reveals that this mechanism of altered gene transcription is restricted and that the majority of genes regulated by elevated O-GlcNAc levels are similarly regulated by a shift from euglycemic to hyperglycemic conditions. These findings implicate the O-GlcNAc modification as a mechanism for hyperglycemia regulated gene expression in the β cell and specifically for O-GlcNAc levels modulating insulin gene expression and promoting long-term insulin release under chronic hyperglycemic conditions.

2. Significance Statement:

O-GlcNAc modification of intracellular proteins is implicated in type II diabetes. While the field has primarily focused on O-GlcNAc altering signaling in insulin-

responsive tissues, we investigated this putative glucose-responsive modification in the insulin-secreting β cell. Here we demonstrate that elevation in O-GlcNAc augments sustained secretion of insulin in response to chronic hyperglycemia. The molecular mechanism involves O-GlcNAc-dependent increases in histone activating marks at the insulin promoter resulting in increased insulin mRNA and protein. Finally, we demonstrate that elevation in O-GlcNAc levels alters the expression of many of the same genes observed upon a shift from euglycemia to hyperglycemia. These results suggest a role for O-GlcNAc as a glucose sensor and modulator of transcription in pancreatic β cells.

3. Introduction

Type II diabetes related complications continue to be a leading cause of death in the United States [1]. Characterized by elevations in circulating blood glucose, two distinct pathways within the mammalian system are at fault. Chronic hyperglycemia results in insulin resistance in muscle, liver and adipose tissues responsible for glucose clearance. The inability of peripheral tissues to absorb glucose causes hyperinsulinemia and glucose toxicity, serving as main contributors toward disease progression [2]. In attempts to cope with the elevated glucose levels, pancreatic β-cells respond by increasing insulin hormone production and secretion. However, continual stimulation via elevated glucose levels eventually leads to β-cell fatigue and decreased insulin release [3].

Numerous environmental factors act in combination to influence insulin regulation both at the transcriptional and translational levels [4-7]. Within the last decade post-translation protein modifications have emerged as key regulatory avenues dictating diverse cellular activities. Terminal hydroxyls of serine and threonine residues can be modified by O-linked β-N-acetylglucosamine (O-GlcNAc) to influence intracellular

processes [8-12]. This dynamic and inducible PTM more closely mimics phosphorylation in that it is a single molecule addition cycling on and off intracellular proteins in response to the cellular environment [13-15]. The donor substrate for O-GlcNAc is the sugar nucleotide UDP-GlcNAc, which is the end product of the HBP [16, 17]. In mammals, only a single gene encodes for each enzyme responsible for addition and removal of O-GlcNAc, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) respectively [18-20]. Due to the involvement of UDP-GlcNAc in multiple metabolic pathways and the diversity of proteins modified by O-GlcNAc within these systems, O-GlcNAc has been implicated in the etiology of several metabolic diseases, including diabetes [21-23].

High glucose stimulation in cultured mouse insulinoma 6 (Min6) cells is known to elevate O-GlcNAc levels [24], while direct modulation of either the HBP or O-GlcNAc regulatory enzymes appear to influence various pancreatic β-cell functions [24-27]. Despite this link, the molecular mechanism through which this modification works remains elusive. Here we use pharmacological manipulation approaches to show that O-GlcNAc enables Min6 cells to secrete higher levels of insulin in response to chronic glucose stimulation. O-GlcNAc also elevates intracellular insulin levels even when glucose flux is low. We also show that O-GlcNAc increases insulin 1 (*Ins1*) and insulin 2 (*Ins2*) mRNA levels in Min6 cells above physiological conditions in a glucose-independent manner. The observed elevation in insulin mRNA is caused, at least in part, by histone H3 regulation as demonstrated by elevations in epigenetic marks promoting transcriptional activation at the *Ins2* promoter. O-GlcNAc also appears to modulate the expression of an abundance of genes in a parallel manner to high glucose. Together these results suggest that O-GlcNAc serves as a glucose sensor in Min6 cells, regulating glucose-mediated gene expression in the pancreatic β cell.

4. Results

Elevated O-GlcNAc levels extend the capacity of Min6 cells to secrete insulin under chronic glucose exposure. Increased glucose flux through the HBP increases O-GlcNAc protein modification in the nucleus of Min6 cells [24]. To examine the effects of elevating O-GlcNAc levels in Min6 cells we elevated HBP flux using high glucose stimulation and through two different pharmacological inhibitors. O-(2-acetamido-2-deoxy-D-glucopyranosylidine)amino-N-phenylcarbamate (PUGNAc; PUG) and GlcNAcstatin (GNS) are OGA inhibitors used to elevate intracellular O-GlcNAc. These inhibitors work by preventing the enzymatic removal of O-GlcNAc by OGA. Previous studies have shown that while PUG is a global hexosaminidase inhibitor, GNS is highly specific for OGA [28, 29]. O-GlcNAc levels in Min6 cells following treatment were unchanged between our conditions in whole cell extracts (Fig 3.6). LG media supplemented with PUG or GNS increased nuclear O-GlcNAc compared to LG alone (2.7-fold each ± 0.31, 0.18), as did cells exposed to HG (2.1-fold ± 0.53) to a lower amount (Fig 3.1A) using a subcellular fractionation approach.

Insulin secretion within the mammalian system follows a pulsatory pattern. The first phase consists of the immediate release of pre-loaded insulin from membrane-docked granules, followed by an environmentally stimulated second phase dependent on gene transcription and new protein synthesis [30, 31]. To address whether increasing O-GlcNAc influenced β cell secretion, media from pharmacologically treated Min6 cells was harvested and tested for secreted mature insulin levels. Using the standard insulin radioimmunoassay in the field, acute insulin secretion from Min6 cells was not altered by elevations in O-GlcNAc alone, but was altered by HG treatment (Fig 3.1B). The combination of HG stimulation and OGA inhibition appeared to increase release compared to HG at 1hr (PUG=1.5-fold ± 0.46, GNS=1.2-fold ± 0.37), although GNS failed to reach statistical significance (Fig 3.2B).

It has been reported that hyperglycemia-induced insulin secretion from pancreatic β cells diminishes over time [32, 33], so we examined whether O-GlcNAc affects more prolonged secretory events. Min6 cells exposed to both HG and GNS for extended periods of time demonstrated the capacity to secrete higher levels of insulin compared to HG alone 3h post- stimulation (1.9-fold ± 0.07, Fig 3.2B). Together these data suggest that elevating O-GlcNAc levels enables Min6 cells to maintain a higher glucose-stimulated insulin secretion response over time.

Intracellular insulin content is higher in Min6 cells when elevating O-GlcNAc. To explain this prolonged secretion of insulin over time and investigate whether increased O-GlcNAc affects insulin hormone biosynthesis, Min6 cells were pharmacologically stimulated for 1 hr, harvested and lysed for intracellular mature insulin quantification via radioimmunoassay. As expected, the amount of intracellular insulin was higher in cells treated with HG compared to LG (1.9-fold ± 0.61, Fig 3.2A). OGA inhibition using PUG or GNS raised intracellular insulin levels significantly above LG alone (2.6-fold each ± 0.77, 0.48) and GNS was 1.36-fold ± 0.48 higher in comparison to HG treatment alone (Fig 3.2A). These results were confirmed by immunohistochemical staining for intracellular insulin. Following treatment cells were fixed, stained and subsequently analyzed by confocal microscopy (Fig 3.2B). In contrast to cells treated with LG, cells incubated in either HG or in LG plus PUG or GNS showed increases in the number of insulin positive puncta. These results suggest that increasing O-GlcNAc in Min6 cells elevates intracellular insulin content.

To further determine whether elevating O-GlcNAc levels increases the capacity of Min6 cells to secrete insulin, cells were treated for 1 or 3 hr in LG, HG, or HG+GNS. Following treatment cells were fixed, stained and subsequently analyzed by confocal microscopy. After 1 hr, no differences in the content of intracellular insulin were detected between HG and HG+GNS (Fig 3.2C). However, 3 hr chronic treatment with HG+GNS

increased intracellular insulin levels compared to HG alone (Fig 3.2C). In addition, although the majority of insulin staining after HG was present at the plasma membrane, in cells treated with HG+GNS significant staining was evident both at the plasma membrane and throughout the cytoplasm (Fig 3.2C). These likely represent both the ready releasable and reserve insulin granule pools, although specific localization was not confromed. These findings imply that elevations in O-GlcNAc help maintain glucose sensitivity and insulin secretion in Min6 cells under chronic hyperglycemia.

Elevating O-GlcNAc increases mouse *Ins1* and *Ins2* steady state mRNA. Since O-GlcNAc increased insulin hormone levels in Min6 cells, we next asked whether this was due to insulin gene regulation. RNA was extracted from Min6 cells following 1 hr treatment and used for steady-state mouse *Ins1* and *Ins*2 quantitative mRNA measurements. Incubating Min6 cells in HG elevates both *Ins1* (2.0-fold ± 0.24) and *Ins2* (1.5-fold ± 0.14) mRNA levels compared to LG treated cells (Fig 3.3A and 3.3B). When O-GlcNAc is elevated using PUG and GNS, mouse *Ins1* mRNA levels are significantly higher versus LG (5.2 ± 1.62 and 6.3-fold ± 1.85, Fig 3.3A). HG also contains substantially less *Ins1* mRNA compared to PUG (2.6-fold ± 0.48) and GNS (3.2-fold ± 0.53) conditions. A similar trend is observed when examining the mouse *Ins2* gene, where PUG and GNS treated cells express more *Ins2* mRNA than LG (1.8 ± 0.22 and 2.2-fold ± 0.24, Fig 3.3B). Unlike *Ins1* measurements, there was no statistical difference on *Ins2* mRNA levels between HG, PUG and GNS conditions (Fig 3.3B). This varying gene responsiveness may be due to the fact that *Ins1* originated from a reverse-transcribed partially processed mRNA of *Ins2* [34, 35]. While both genes contain the regulatory region required for preproinsulin mRNA synthesis [34], the majority of insulin in the rodent system is produced through *Ins2* gene activation, making it more homologous to the human insulin gene [36]. This agrees with our insulin synthesis and secretion data where we saw a modest O-GlcNAc-correlated increase in both experiments

that is more representative of the *Ins2* mRNA results versus *Ins1*. Chronic elevations in O-GlcNAc show similar trends to that of prolonged insulin secretion results in which Min6 cells stimulated in LG+GNS for 3 hr contain significantly higher *Ins2* mRNA levels than both LG (3.1-fold ± 0.37) and HG (1.5-fold ± 0.37, Fig 3.3C). Interestingly, under chronic hyperglycemia Min6 cells failed to express significantly higher *Ins2* transcript compared to LG, suggesting increasing O-GlcNAc alone may protect against β cell fatigue (Fig 3.3C). Combined these results demonstrate that the elevation of intracellular insulin levels is caused by an O-GlcNAc-mediated insulin gene regulatory mechanism.

Increasing O-GlcNAc directly correlates to elevated histone H3 epigenetic marks promoting transcriptional activation. Recent studies demonstrate that O-GlcNAc can directly modify histone proteins and several other components of the nucleosome complex [37]. To investigate whether the increases in steady state *Ins2* mRNA was due to chromatin remodeling events, we performed chromatin immunoprecipitation (ChIP) experiments in combination with quantitative PCR towards the mouse *Ins2* promoter sequence. Histone H3 acetylation and trimethylation are epigenetic modifications serving as global marks to indicate active areas of gene transcription [38, 39]. We used antibodies designed against histone H3 bi-acetylated lysine 9 and 14 (H3K9,14Ac) and histone H3 trimethylated lysine 4 ($H3K4me^3$) to probe for O-GlcNAc associated chromatin changes at the mouse *Ins2* promoter.

LG+GNS treatment significantly increases the amount of O-GlcNAc levels at the Ins2 promoter region, while HG only shows a modest increase (Fig 3.4A). When investigating $H3K4me^3$ at the *Ins2* promoter, HG samples always seemed to contain more marks than LG, but never quite reached statistical significance (Fig 3.4B). This has been previously observed when investigating genome-wide histone modifications in human pancreatic islets and may be true for the more homologous mouse *Ins2* gene [40].

Conversely, GNS treated cells displayed abundantly more H3K4me^3 marks (3.2-fold ± 0.42) at the *Ins2* promoter versus LG (Fig 3.4B). Min6 cells incubated in HG conditions trend toward having more H3K9,14Ac marks (1.9-fold ± 0.74) at the mouse *Ins2* promoter compared to LG incubated cells (Fig 3.4A). GNS treatment significantly increases the amount of H3K9,14Ac (1.91-fold ± 0.18) at the *Ins2* promoter versus LG alone (Fig 3.4C). Histone H3 data revealed there was no significant change in the amount of H3 at the *Ins2* promoter between our conditions, ensuring the O-GlcNAc directed increases in H3 activation marks was not due to nucleosome degradation (Fig 3.4D).

We also wanted to investigate the degree to which increasing O-GlcNAc levels contributed to global histone modification. Western blot analysis investigating global H3K4me3, H3K9,14Ac and histone H3 levels showed no significant differences between pharmacological conditions in acid-extracted histone samples (Fig 3.4E-G). Together, these results show that O-GlcNAc accumulation at the *Ins2* gene promoter directly correlates with elevations in H3K9,14Ac and H3K4me^3 chromatin-associated transcriptional activating marks, but does not appear to affect these marks on H3 on a whole genome scale.

O-GlcNAc regulates a subset of genes in Min6 cells. Considering global histone activating marks were unaffected between our conditions, we next investigated whether O-GlcNAc only regulated a subset of genes in Min6 cells. Illumina Hiseq 2000 RNA sequencing confirmed numerous genes exhibited altered expression patterns in response to hyperglycemia and/or OGA inhibition (Fig 3.5A). Comprehensive bioinformatics showed that 2657 genes (16%) were affected in at least one of our conditions out of a total of 16,638 expressed. Based on this data, 84% of the genes in Min6 were unchanged by our pharmacological conditions, which is likely the reason we were unable to see changes in total H3 activating marks (Fig 3.4 E-G). HG treatment altered the expression of 1987 (11.9%) of the total genes expressed and LG+GNS influenced 1369 (8.4%). If

the two conditions (hyperglycemia and O-GlcNAcase inhibition) were independent, one would expect approximately 1% overlap in gene expression changes based on these two datasets. However, bioinformatics analysis revealed that 726 genes were similarly affected (changed in the same direction by at least two-fold in both cases) in both HG and LG+GNS treated samples, representing 27.3% of the total altered subset of genes (Fig 3.5A). Over half of all gene expression profiles altered by OGA inhibition overlapped with hyperglycemia-induced changes and more than 1/3 of all hyperglycemia-induced changes overlapped with OGA inhibition (Fig 3.5A). Bioinformatics analysis showed that the same protein classes were either up or downregulated during OGA inhibition and hyperglycemia compared to LG alone (Fig 3.5B). Together this data strongly suggests that O-GlcNAc influences the expression of a subset of genes in Min6 cells and appears to account for a majority of the altered gene activity caused by hyperglycemia through its glucose sensing ability.

5. Discussion

Using various pharmacological strategies manipulating intracellular O-GlcNAcylation, we are able to show that this PTM serves as a glucose sensor in the pancreatic β cell and influences gene expression in Min6 cells. Nuclear extracts clearly display elevated O-GlcNAc during OGA enzyme inhibition, while hyperglycemia slightly elevates modification levels (Fig 3.1A). This difference could be attributed to the relatively small amount of intracellular glucose entering the HBP (3-5%) compared to other metabolic pathways [16]. PUG and GNS treatment on the other hand specifically target OGA, directly influencing the O-GlcNAc status within the cell. Elevating O-GlcNAc in the absence of glucose stimulation does not mediate acute insulin secretory events (Fig 3.1B). This suggests that while proteins within the exocytosis pathway are known to be O-GlcNAc modified [41, 42], it does not alone affect acute insulin hormone release in Min6 cells. However, we were able to show that when inhibiting OGA and

chronically stimulating insulin release, Min6 cells remained glucose sensitive compared to HG (Fig 3.1C).

It was clear by our prolonged secretion experiments that O-GlcNAc was influencing insulin within Min6 cells, but not through direct secretory pathway modulation. We were able to demonstrate that increasing levels of O-GlcNAc in Min6 cells drastically elevates the intracellular insulin content in a glucose-independent manner (Fig 3.2A and 3.2B). We also noticed that OGA inhibition resulted in higher levels of insulin production when compared to HG (Fig 3.2A). Furthermore, inhibiting OGA under prolonged glucose stimulation enabled Min6 cells to maintain high levels of evenly dispersed intracellular insulin levels compared to membrane localized levels observed in HG treatment alone (Fig 3.2C). These results indicate that elevating O-GlcNAc levels in Min6 cells

Insulin production is governed by gene transcription in response to environmental stimuli [43] and since O-GlcNAcylated proteins are heavily involved in nuclear events [8], it was logical to think that the intracellular insulin elevation was due to transcriptional control. Our data clearly displayed that increasing O-GlcNAc in Min6 cells directly correlates with elevations in mouse *Ins1* and *Ins2* steady-state mRNA levels during acute stimulation (Fig 3.3A and 3.3B). We also show that elevating O-GlcNAc levels enable Min6 cells to maintain higher levels of *Ins2* transcripts under prolonged glucose exposure compared to HG treatment along. This finding importantly indicates that β cell glucose sensitivity is at least partially maintained by increasing O-GlcNAc. Insulin mRNA synthesis and translation can be regulated through a variety of mechanisms [3, 44, 45]. Considering the half-life of *Ins2* mRNA in Min6 cells is greater than 24 hrs [46], the time frame of our experiment (8 hr) and the fold increases observed, transcript stabilization would be unable to account for the observed changes. Several glucose-responsive transcription factors that bind the insulin promoter are O-GlcNAc

modified [24-26]. But we found that O-GlcNAc did not impact *Ins1* promoter activity in luciferase-reporter assays targeting the promoter region upstream of the transcriptional start site (Fig 3.7) and prompted us to investigate alternative molecular mechanisms.

Recently groups have shown that O-GlcNAc plays a role in epigenetics, either by directly modifying histone tails [47] or through OGT and OGA incorporation into chromatin complexes [48, 49]. We used several histone H3 transcriptional activation marks to demonstrate that the O-GlcNAc mediated increase in insulin mRNA is through a chromatin mechanism. An O-GlcNAc specific antibody indicated that there is more O-GlcNAc modification at the *Ins2* promoter when OGA is inhibited and only slightly higher upon elevated glucose exposure (Fig 3.4A). OGA inhibition dramatically increases the amount of H3K4me^3 and H3K9,14 bi-acetyl marks at the *Ins2* promoter compared to LG (Fig 3.4B and 3.4C). Hyperglycemia also trended towards elevating these activation marks (Fig 3.4B and 3.4C). These results suggest that a modest increase in O-GlcNAc levels (hyperglycemia) result in a trending toward activation marks on H3, while a more significant increase in the PTM results in a significant increase on H3 activation marks. Importantly, we also demonstrate that the observed elevation in H3 transcriptional activating marks is not simply due to O-GlcNAc-directed nucleosome degradation, since all three conditions contain similar histone H3 levels at the *Ins2* promoter (Fig 3.4D). Of interest, immunoblot experiments display that O-GlcNAc does not influence H3K9,14 bi-acetyl and H3K4me^3 marks at a global level (Fig. 3.4E-4G), leading us to investigate the gene specificity of hyperglycemia and O-GlcNAc elevation. Deep sequencing revealed numerous genes that were modulated by O-GlcNAc apart from *Ins1* and *Ins2,* many of which greatly overlap with HG stimulation (Fig 3.5A). Bioinformatics analysis revealed that both hyperglycemia and elevated O-GlcNAc levels similarly regulated the same protein classes (Fig 3.5B). In all the sequencing results suggest O-GlcNAc greatly

contributes to the hyperglycemic-induced gene expression changes in pancreatic β cells and serves as a molecular glucose sensor to regulate gene activity.

Our results suggest that glucose-stimulated insulin gene activation in the Min6 cell line utilizes the O-GlcNAc protein modification in an epigenetic mechanism to mediate transcription. We also propose that many of the hyperglycemia-mediated transcriptional changes in type II diabetes occur through an O-GlcNAc mechanism, implicating this PTM as a glucose-sensor in the pancreatic β cell. Future experiments will be aimed at determining the precise mechanism through which O-GlcNAc epigenetically regulates insulin gene activity and to investigate if the results in our cell culture model are recapitulated in vivo. Of significant future interest is the large overlap in regulated genes by both hyperglycemia and O-GlcNAcase inhibition and determining whether our hypothesis that the O-GlcNAc modification is one of the major effectors of hyperglycemia-induced transcriptome regulation. These answers will determine the extent to which O-GlcNAc contributes in diabetic β cell fatigue and hyperinsulinemia.

6. Materials and Methods

Tissue Culture. Mouse insulinoma 6 (Min6) cells were a generous gift from Gerald Hart (John's Hopkins University). Cells were cultured in Dulbecco' modified Eagle's media containing 25 mM glucose, 15% (v/v) fetal bovine serum, 2 mM glutamine and 100 μM β-mercaptoethanol. Upon reaching 70% confluency, cells were harvested using 0.25% trypsin-PBS solution (1:1) and split at a 1 to 3 ratio. All experiments were performed using cells between passages 20-30.

Pharmacological Incubation Conditions. Min6 cells were introduced to either low (2 mM) or high (25 mM) glucose media and pre-treated with the following chemicals: 25 μM *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amido *N*-phenylcarbamate (PUGNAc) (Toronto Research Chemicals Inc.); 500 pM GlcNAcstatin (Dr. Daan Van

Aalten, Univeristy of Dundee). Cells were incubated in conditions for 6 hrs before a 1X PBS wash and 1 hr starvation (0.5 mM glucose) period with the above-mentioned inhibitors. Afterwards, cells were again washed with 1X PBS and returned to pharmacological conditions as outlined above for a 1 hr acute treatment before harvesting media and/or cells for experimental procedures. Experiments requiring chronic 3 hr pharmacological exposure were washed with 1X PBS and fed fresh conditioned media at 1hr increments until conclusion.

Western Blotting. Min6 cells from pharmacological conditions were separated into cytoplasmic and nuclear fractions as outlined previously [50]. Proteins were resolved on SDS-PAGE gel by electrophoresis and transferred to PVDF membrane (Bio-Rad). Membranes were blocked for 1 hr at room temperature in 1X TBST, 0.1% Tween20 supplemented with BSA. Following blocking, membranes were incubated overnight at 4°C using the O-GlcNAc-specific monoclonal 10 (Mab10), anti-histone H3 tri-methyl K4 (Abcam, ab8580), acetyl-histone H3 Lys9/Lys14 (Cell Signaling, 9677L) or anti-histone H3 (Abcam, ab1791) antibody. Membranes were washed three times with 1X TBST, 0.1% Tween20 and incubated at room temperature for 1 hr with a secondary horseradish peroxidase-conjugated antibody. Afterwards, membranes were washed four times with 1X TBST, 0.1% Tween20 and subjected to ECL chemiluminescence (Thermo Scientific) via autoradiography for detection.

Quantification of Insulin Secretion. Media samples harvested from pharmacologically manipulated Min6 cells were diluted 1:100 in 1X PBS the same day. Insulin levels in diluted samples were determined using the Rat Insulin RIA Kit (Millipore) following directions outlined in the manufacturer's protocol. Radioisotope quantification was performed using a Packard Cobra Quantum E5002 Gamma Counter (Perkin-Elmer).

Quantification of Intracellular Insulin Levels. Harvested Min6 cell samples were resuspended in lysis buffer (50mM Tris-HCl, pH 7.5, 26.5 mM NaCl, 1% NP-40, 1% SDS, 50mM EDTA, 0.1% protease inhibitor cocktail (Calbiochem) and 0.1% phosphotase inhibitor cocktail (Calbiochem)) and syringe ruptured with a 23-gauge needle. Samples were centrifuged at 14,000 RPM for 30 min at 4°C. Whole cell extracts were then diluted 1:1200 in 1X PBS and used for insulin determination with the rat Insulin RIA Kit as described above. Radioisotope quantification was performed using a Packard Cobra Quantum E5002 Gamma Counter (Perkin-Elmer).

Immunofluorescence and Confocal Microscopy. Pharmacologically treated Min6 were fixed using 3.7% paraformaldehyde at room temperature for 20 minutes. Following fixation, cells were washed three times with 1X PBS and incubated in anti-insulin (Dako, A0564) primary antibody supplemented with 1mg/mL BSA and 0.2% Triton X-100 for 1 hr. Primary antibody was removed and cells were washed three times in 1X PBS and incubated in Alexa-fluor 488 fluorophore conjugated secondary antibody and TO-PRO 3 nuclear stain (Invitrogen) supplemented with 1mg/mL BSA and 0.2% Triton X-100 for 45 minutes. Cells were washed three times with 1X PBS, mounted onto glass coverslips using Prolong Gold Antifade Reagent (Invitrogen) and allowed to dry overnight at 4°C. Samples were imaged with a 60X oil immersion objective (N.A. 1.4) on an Olympus FV1000 Laser scanning Confocal microscope. Multiple images were acquired through a z-series, with a z-step of .45μM. The resulting images were analyzed and compressed with Image J software. In most cases the presented images are a maximum intensity projection that includes the entire z-stack.

RNA Isolation and Quantitative RT-PCR. Total RNA was isolated from Min6 cells using the RNeasy Plus Minikit (Qiagen, 170-8840) as described in manufacturer's protocol. First-strand cDNA synthesis was performed using the iScript Reverse Transcription Supermix Kit (BioRad) according to manufacturer's instructions. The

resulting cDNA was used as a template for PCR amplification of the mouse insulin 1 (Qiagen, QT01660855), mouse insulin 2 (Qiagen, QT00114289) and TATA binding protein 1 (Qiagen, QT00198443) genes. Insulin gene mRNA levels were quantified using MyIQ Single Color Real-Time PCR Detection Instrument (BioRad) and normalized to Tbp1 expression levels.

Chromatin Immunoprecipitation (ChIP). ChIP was performed as previously described [51]. Briefly, DNA and protein were cross-linked using 2% formaldehyde. Sonicated DNA extract was precleared using protein A/G agarose beads and the corresponding agarose conjugate linked IgG. Chromatin from 3 X 10^6 cells were used for each immunoprecipitation (IP). Lysates were incubated with previously mentioned anti-histone H3 tri-methyl K4, acetyl-histone H3 Lys9/Lys14, anti-histone H3 or anti-O-GlcNAc Mab10 antibody at 2 μg per reaction overnight at 4°C with rotation. Protein-DNA complexes were incubated with protein agarose A/G beads for 2 h and washed 3 times using buffers containing 0.1% SDS, 1& Triton X-100, 2 mM EDTA, 20 mM Tris, 150 to 500 mM NaCl and protease inhibitors. DNA was eluted from beads using elution buffer containing 0.1% SDS and 100 mM $NaHCO_3$. Cross-linking was reversed by addition of NaCl to a final concentration of 325 mM and DNA was incubated overnight at 65°C. DNA was extracted using phenol-chloroform after RNase and proteinase K treatment and analyzed by quantitative real-time PCR (RT-PCR) against the mouse insulin 2 promoter region (*Ins2* Forward: 5'TGACCTACCCCACCTGGAGC3'; *Ins2* Reverse: 5'CTGGTGGTTACTGGGTCCCC3').

RNA-sequencing analysis and bioinformatics. RNA extraction was performed using the RNeasy Plus Minikit (Qiagen, 170-8840) from LG, HG and LG+GNS samples after previously described 1 hr incubation. Samples were sent to HudsonAlpha Genomic Services Lab (GSL, Huntsville, AL) for RNA-Seq library prep and sequencing. Briefly. the concentration and integrity of the extracted total RNA was estimated by Qubit® 2.0

Fluorometer (Invitrogen, Carlsbad, California, USA), and Agilent 2100 Bioanalyzer (Applied Biosystems, Carlsbad, CA, USA), respectively. RNA-Seq library prep was performed with 500 ng total RNA from each sample, followed by enrichment for polyadenylated RNA sequences using the poly(A) selection technique. Each sample was individually barcoded with unique in-house GSL primers and amplified through 8 cycles of PCR using KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Inc., Woburn, MA, USA). The quality of the libraries were assessed by Qubit® 2.0 Fluorometer , and the concentration of the libraries was estimated by utilizing a DNA 1000 chip on an Agilent 2100 Bioanalyzer, respectively.

Accurate quantification for sequencing applications was determined using the qPCR-based KAPA Biosystems Library Quantification kit (Kapa Biosystems, Inc., Woburn, MA, USA). Each library was then diluted to a final concentration of 12.5nM and pooled equimolar prior to clustering. Paired End (PE) sequencing was performed to generate approximately 25 million reads per sample using a 200 cycle TruSeq SBS HS v3 kit on an Illumina HiSeq2000, running HiSeq Control Software (HCS) v1.5.15.1 (Illumina, Inc., San Diego, CA, USA). Image Raw reads were demultiplexed using a bcl2fastq conversion software v1.8.3 (Illumina, Inc., San Diego, CA, USA) with default settings. Following RNA-Seq, raw reads were mapped to reference mouse genome mm9 using TopHat v2.0. Aligned reads were imported onto the Avadis NGS data analysis platform (Strand Life Sciences, San Francisco, CA, USA). Reads were first filtered on their quality metrics, then duplicate reads were removed. Normalized gene expression was quantified using the TMM (Trimmed Mean of M-values) algorithm [52]. The transcriptional profile from each sample group (LG, HG and LG+GNS) was compared by principle component analysis (PCA) and hierarchal clustering analysis to determine the layout and spread of the expression data. Differential expression of genes were calculated on the basis of fold change (using default cut-off $\geq \pm 2.0$) observed between defined conditions, and the *p-value* of the differentially expressed gene list was estimated by *z*-

score calculations using a default cut off of 0.05 as determined by Benjamini Hochberg FD correction.

Gene Ontology (GO) analysis was performed on the list of differentially expressed mRNAs between sample groups. Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/) was used for this analysis. prediction of affected protein classes from up and down regulated genes sets were made on Panther gene list analysis.

Statistical Analysis. Data are expressed as mean ± SEM. The differences between means and the effects of treatments were analyzed using paired Student's t-distribution ($P<0.05$). All statistically significant values are included in the figures and figure legends. Analysis was performed using GraphPad software.

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8. References

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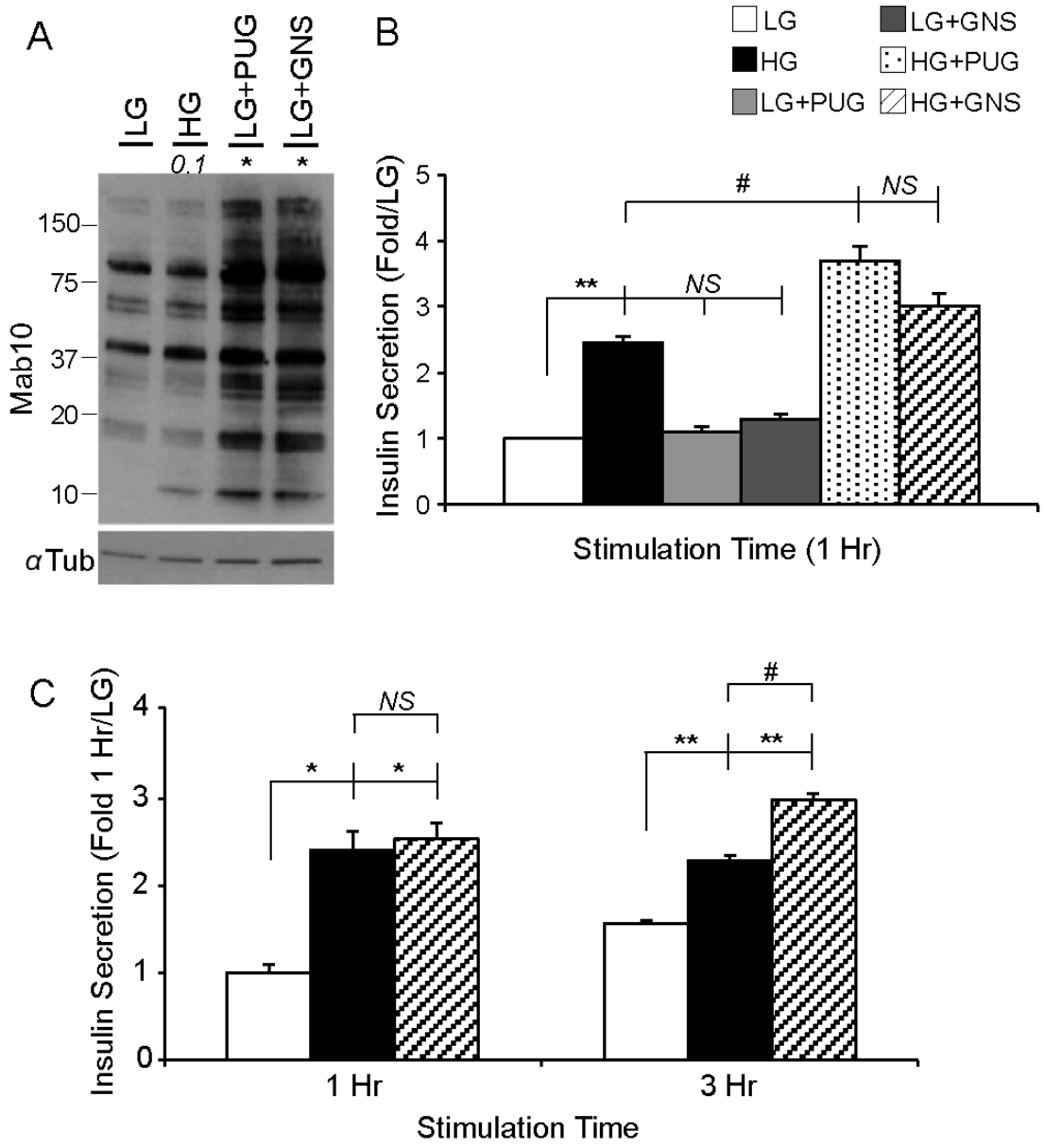
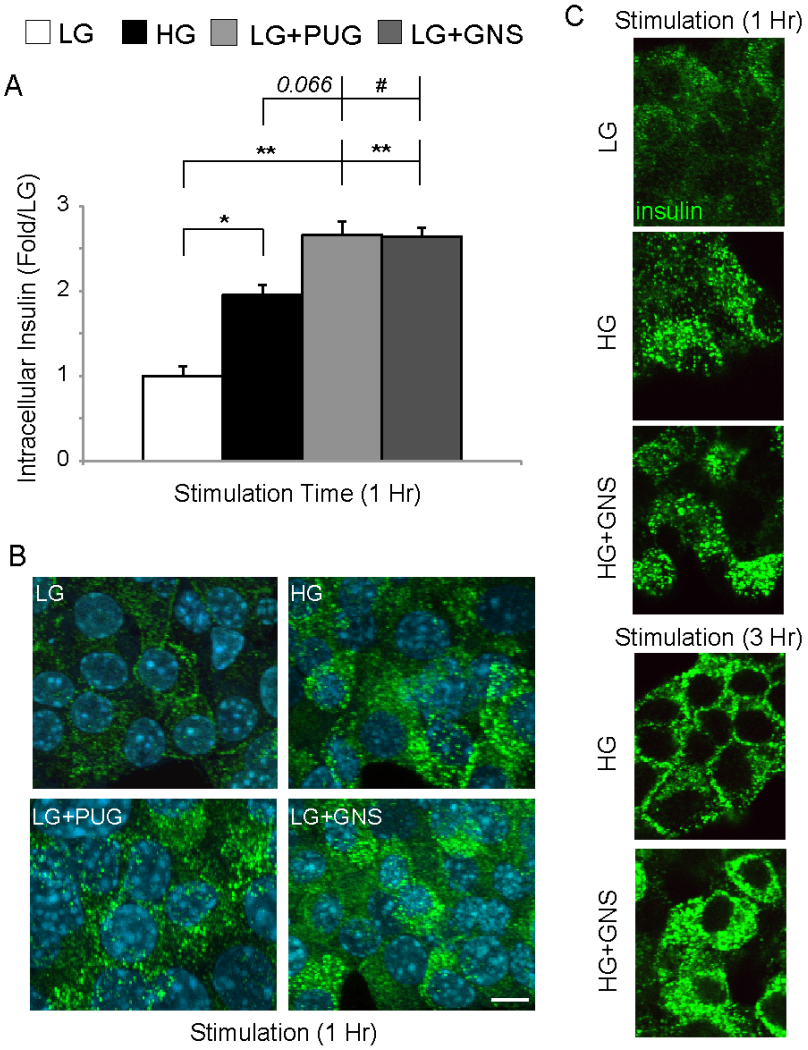
Figure 3.1



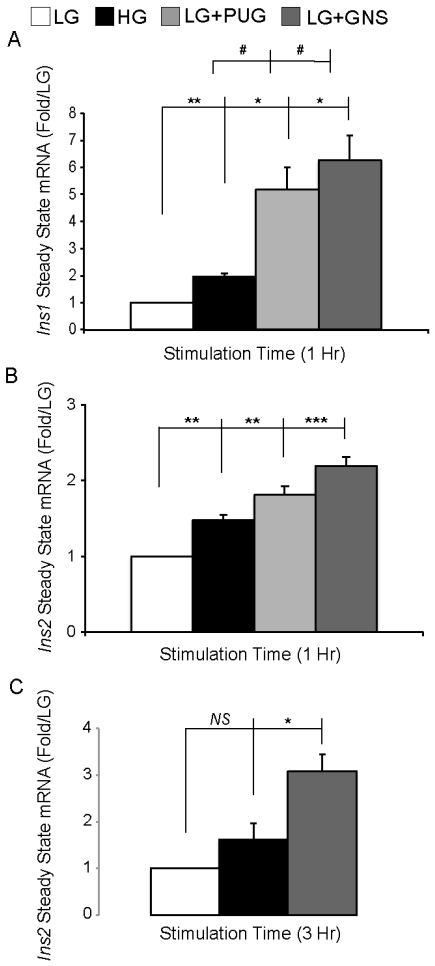
O-GlcNAc preserves chronic glucose-stimulated insulin secretion in Min6 cells. (A) Nuclear extracts from 1 hr pharmacologically treated Min6 cells were immunoblotted with O-GlcNAc antibody Mab10. α Tubulin serves as the loading control (n=3; *P<0.05). (B) Insulin secretion levels following 1 hr pharmacological stimulation from Min6 cells, quantified using a rat RIA. All values are presented as fold change to LG (*n*=6; ***P*<0.01 vs. LG, #*P*<0.05 vs. HG). (C) Acute and chronic insulin secretion in response to LG, HG and HG+GNS treatments in the media from Min6 cells and quantified using a rat RIA. All values are presented as fold change to 1 hr LG (*n*=3; **P*<0.05 vs. LG, ***P*<0.01 vs. LG, #*P*<0.05 vs. HG). All data is shown as the mean ± SEM.

Figure 3.2



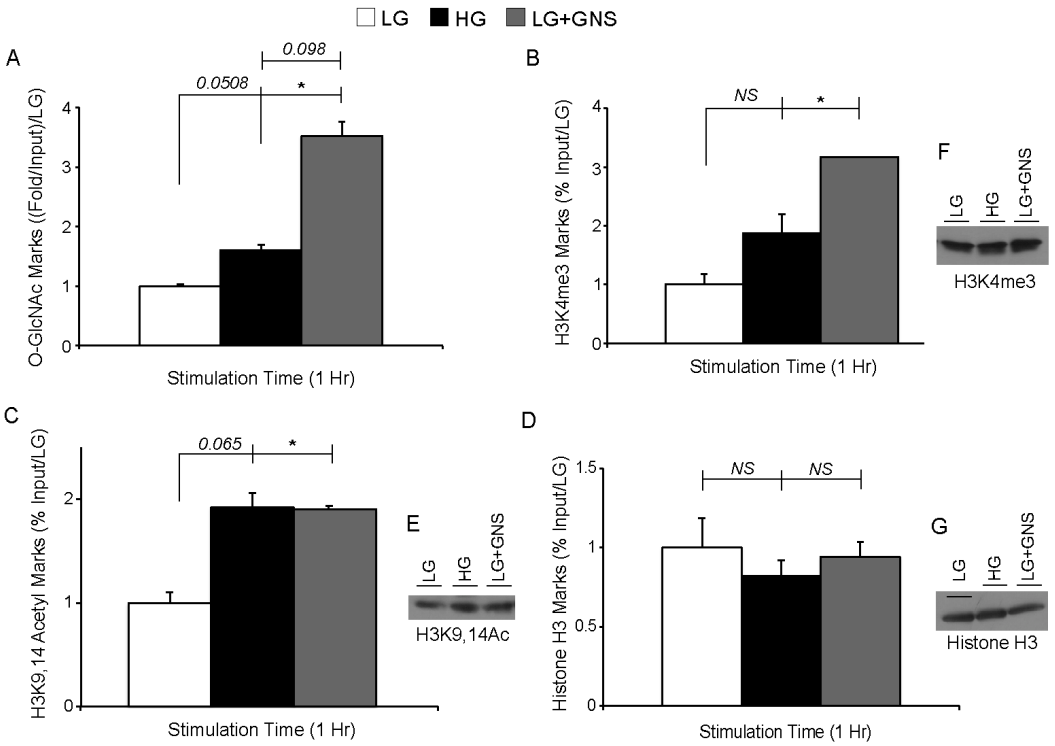
O-GlcNAc increases Min6 intracellular insulin levels. (A) Cells treated in pharmacological conditions for 1 hr were harvested and lysed for intracellular insulin measurements using a rat RIA. All values are presented as fold change to LG and data shows the mean ± SEM (n=6; *P<0.05 vs. LG, **P<0.01 vs. LG, #P<0.05 vs. HG). (B) Min6 were incubated in conditions for 1 hr and fixed for immunohistochemical and confocal microscopy analysis. Prepro-, pro- and mature insulin are stained green and the nucleus is stained blue (n=3). (C) Min6 cells treated in LG, HG and HG+GNS were fixed for immunohistochemical and confocal microscopy analysis after 1 hr incubation (top panel). Min6 cells treated for 3 hr in HG or HG+GNS were prepared and analyzed as described (bottom panel). Prepro-, pro- and mature insulin are again stained green, while the secretory granule protein rSec6 is stained red (n=3).

Figure 3.3



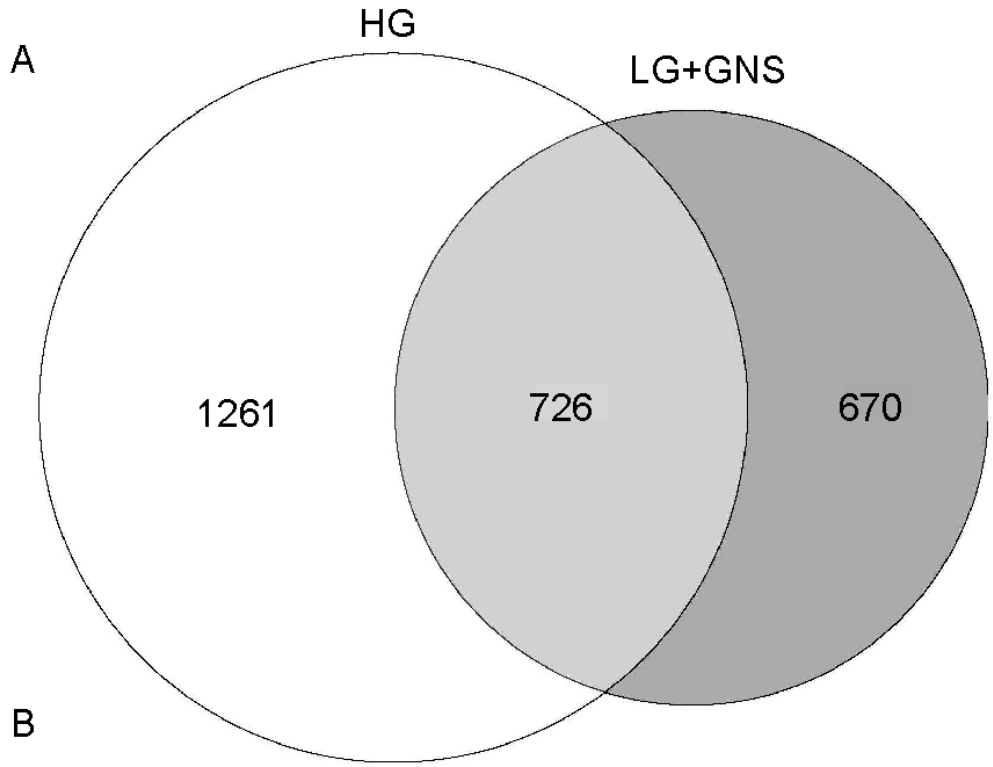
Elevations in O-GlcNAc increase Ins1 and Ins2 steady-state mRNA levels in Min6 cells. (A) Extracted RNA from Min6 cells following 1 hr treatment was used for mouse Ins1 mRNA quantification. All values are presented as fold change to LG (n=10; *P<0.05 vs. LG, **P<0.01 vs. LG, #P<0.05 vs. HG). (B) RNA extraction was performed from Min6 cells as described above, but used for mouse Ins2 mRNA quantification. All values are presented as fold change to LG (n=10; **P<0.01 vs. LG, ***P<0.001 vs. LG). All data is shown as mean ± SEM.

Figure 3.4



Histone H3 activation marks and O-GlcNAc levels increase at the Ins2 promoter. After 1 hr treatment, Min6 cells were fixed and prepared to analyze binding occupancy of several marks within the Ins2 promoter. (A) Mab10 was used in chromatin immunoprecipitation (ChIP) experiments and values were determined using sample qPCR fold change over IgG control and presented as fold change to LG (n=3; *P<0.05 vs. LG). Chromatin immunoprecipitation (ChIP) was performed for H3K4me^3 (B), H3K9,14Ac (C) and histone H3 (D) levels. All values were determined using qPCR relative to the % input and are presented as fold change to LG (all n=3; *P<0.05 vs. LG). Corresponding H3K4me^3 (E), H3K9,14Ac (F) and histone H3 (G) immunoblot analysis on global histone extracts are displayed next to ChIP data and was normalized to histone protein quantities within samples (n=3). All data is shown as mean ± SEM.

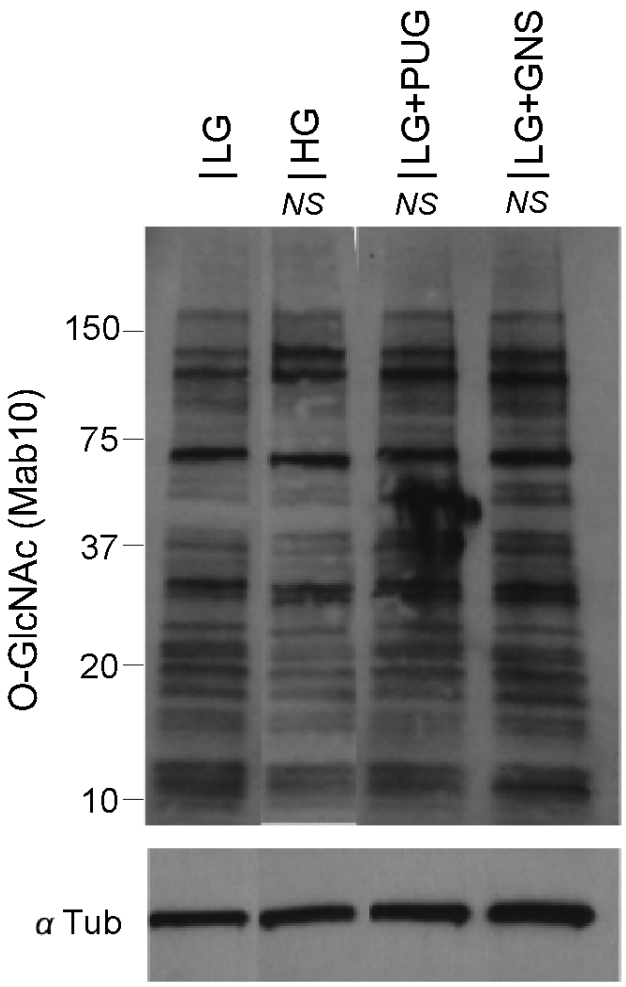
Figure 3.5



Protein Classes Affected	HG (Total Genes)	LG+GNS (Total Genes)
Receptor (PC00197)	299	227
Nucelic Acid Binding (PC00171)	215	151
Transcription Factor (PC00218)	211	140
Signaling Molecule (PC00207)	173	134
Hydrolase (PC00121)	166	110
Transporter (PC00227)	166	122

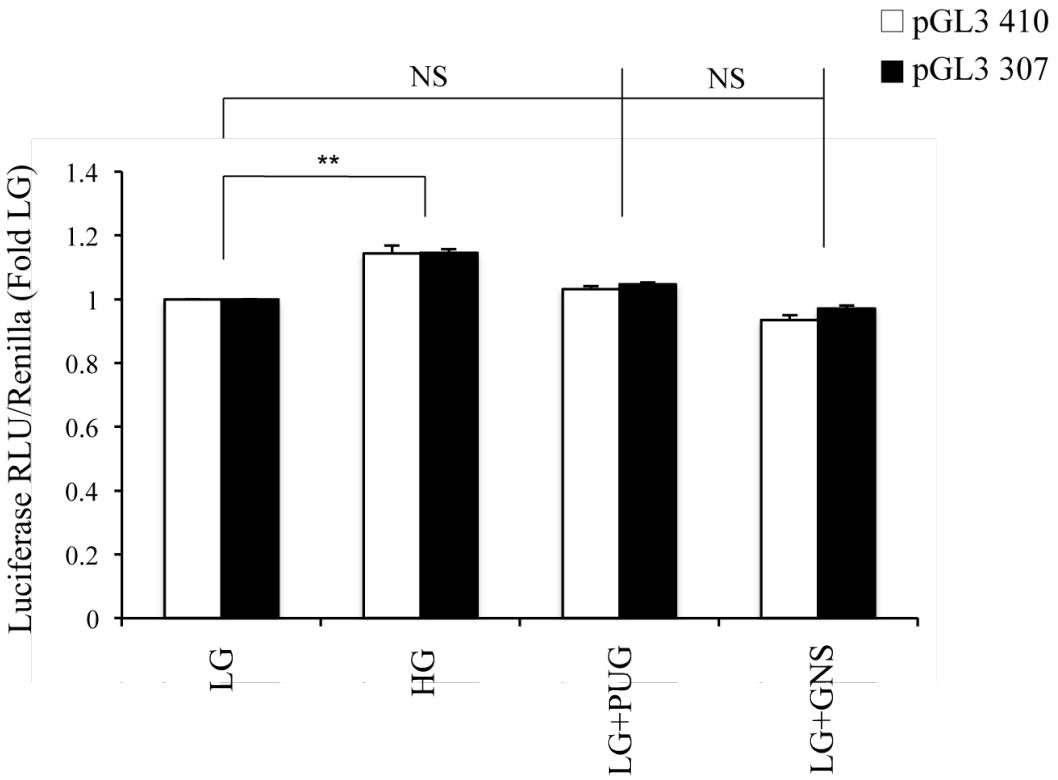
HG and O-GlcNAc stimulation regulate numerous gene expression profiles in Min6 cells. (A) In all, the expressions of 2657 genes were altered by at least 2-fold either by HG or LG+GNS treatment compared to LG. HG alone affected 1261 genes in Min6 cells, while LG+GNS influenced 670. Combined, HG and LG+GNS changed the expression of 726 common genes that represents approximately 27.3% of total genes affected. (B) The top six affected protein classes as measured by the total number of genes changing are the same in both HG and LG+GNS treatments. Values were calculated from RNA-sequencing results performed as outlined in the supplemental materials and methods section (n=1).

Figure 3.6



Pharmacological treatment of Min6 cells has no affect on global O-GlcNAc levels. Whole cell extracts from 1 hr pharmacologically treated Min6 cells were immunoblotted with O-GlcNAc antibody Mab10. α tubulin serves as the loading control (*n*=3). All data is shown as the mean ± SEM.

Figure 3.7



***Ins1* promoter activation is unaffected by O-GlcNAc.** Min6 cells were co-transfected with pGL3 luciferase constructs containing a 410 or 307 nucleotide sequence from the insulin 1 gene promoter or pGL3 Renilla. 48 hours post-transfection, cells were treated in pharmacological conditions as outlined in Chapter 3 and measured for relative light units (RLU). Values were normalized to Renilla value expression. (n=6; $^{**}P$<0.01)

CHAPTER 4

CONCLUSION

1. **Research Findings and Significance**

The pancreatic β cell plays a critical role in maintaining glucose homeostasis. As the predominant producer of insulin, this cell type enables circulating glucose uptake at peripheral tissues for fuel and energy storage [1]. The hexosamine biosynthetic pathway (HBP) utilizes a small percentage of cellular glucose to facilitate UDP-GlcNAc substrate synthesis. This sugar-nucleotide is the donor for nucleotide sugar biosynthesis, complex glycosylation and O-GlcNAc modification. O-GlcNAc is a dynamic and inducible post-translational modification (PTM) cycling on and off proteins in response to the environment [2]. Multiple studies have shown that this single sugar moiety is highly expressed in the pancreatic β cell [3, 4]. Additionally, the enzyme responsible for O-GlcNAc addition (O-GlcNAc transferase, OGT) is extremely abundant in these cells [5]. This data suggested a functional role for O-GlcNAc in the β cell, but a direct connection was never established.

Transcriptional control of the insulin gene has been well studied for decades. The 5' proximal promoter region mediates glucose-stimulated activation through synergistic transcription factor binding. Specifically, PDX-1, NeuroD1 and MafA interaction at evolutionarily conserved *cis* sequences recruits the preinitiation complex to the TATA to initiate insulin gene transcription [6, 7]. Interestingly, all three of these factors are known

to be O-GlcNAc modified [8-10]. Within the last decade, an alternate mechanism for transcriptional control has emerged. Covalent histone marks are epigenetic modifications regulating DNA accessibility and chromatin-associated complex recruitment. Pancreatic β cells also implement this transcriptional system at the insulin gene to regulate activity through nucleosome changes [11-14]. More recent investigation reveals O-GlcNAc is part of the histone code and appears to influence the epigenetic landscape [15]. However, there is no direct evidence linking the PTM to β cell gene control.

We set out to investigate whether manipulating O-GlcNAc influences pancreatic β cell activity, and if so, at what molecular level? To address these questions, we used directed pharmacological strategies to alter O-GlcNAcylation in the mouse insulinoma 6 (Min6) cell line. Our initial work was aimed at insulin secretion events. Elevating O-GlcNAc levels alone does not influence exocytosis in Min6 cells. However, when glucose stimulation and OGA inhibtion are combined, cells seemed to secrete more insulin when compared to high glucose treatment alone. Subsequent studies reveal O-GlcNAc augments insulin secretion in response to prolonged glucose exposure. This is representative of the hyperinsulinemic phenotype often observed in type II diabetes. Together these results suggest that increasing O-GlcNAc alone fails to influence insulin exocytosis, but enables Min6 cells to secrete more insulin under hyperglycemic conditions.

Considering glucose stimulation was required to illustrate an O-GlcNAc affect during exocytosis, we hypothesized that the PTM may affect biosynthesis of the hormone. Using a variety of techniques, we are able to demonstrate increasing O-GlcNAc results in elevated intracellular insulin accumulation to higher extents than low and high glucose (Fig 4.1). This result is interesting considering the O-GlcNAc-mediated insulin increase did not require glucose stimulation. Additionally, these results indicate the increased hormone levels localize within the perinuclear region of Min6 cells. Insulin secretion is a

biphasic event, consisting of first and second phase release [16]. The second phase requires reserve vesicles at the perinuclear region to be refilled with hormone before translocating to the membrane for secretion [17]. Since O-GlcNAc-induced insulin biosynthesis appears to affect this reserve pool, we thought that perhaps O-GlcNAcylation influences insulin gene activity.

Indeed, quantitative PCR (qPCR) analysis verifies elevations in O-GlcNAc alone can increase *Ins1* and *Ins2* steady state mRNA levels in Min6 cells. The mouse genome consists of two insulin genes. *Ins2* is more homologous to the human insulin gene and is responsible for the majority of insulin production in mice [18]. Its *Ins1* relative was evolutionarily established from a reverse-transcribed *Ins2* mRNA segment [19]. While both genes are functional, rodent mutational studies revealed *Ins2* knockouts alone led to hyperglycemia and diabetes development [20]. This may explain the difference in O-GlcNAc regulation between *Ins1* and *Ins2*. Pharmacological elevation of O-GlcNAc increases mRNA levels significantly above low glucose treatment in both insulin genes. However, the *Ins1* gene is slightly more responsive to O-GlcNAc elevations compared to the *Ins2* gene. Provided the information above, the O-GlcNAc mediated mRNA difference may be due to the amount of activity and comparable hormone production between these two genes. Based on these results, we can confidently claim that increasing O-GlcNAc levels in Min6 cells mediates *Ins1* and *Ins2* mRNA levels.

Gene transcription and downstream translation can be regulated by various mechanisms. We next attempted to determine the level at which O-GlcNAc exerts its affect. As previously mentioned, several glucose-responsive transcription factors binding at the insulin gene promoter are O-GlcNAcylated [8-10]. We wanted to test whether O-GlcNAc modification of these factors increased insulin promoter activation in Min6 cells. Using multiple insulin promoter luciferase constructs we were able to determine this was

not the case. As previously seen [21, 22], glucose stimulation causes a subtle increase in *Ins1* promoter activation, but O-GlcNAc failed to influence the promoter.

Immediately following transcription, pre-mRNA undergoes splicing to produce the functional mRNA template used in translation [23]. Alternative splicing has been shown to give rise to multiple protein isoforms based on selective intron removal during this process [24, 25]. While *Ins2* mRNA contains several intron regions, insulin 1 is intronless [26, 27]. Therefore, the steady state *Ins1* mRNA increase observed in qPCR cannot be due to alternative splicing. If we assume the mRNA increases for both insulin genes are under similar O-GlcNAc control, the increase in *Ins2* mRNA would not be due to alternative splicing either. However, this mechanism cannot be fully discredited, as we did not investigate this experimentally.

After splicing, mRNA molecules are processed into polypeptides via translation [28, 29] or degraded by the nucleosome [30]. Another possible mechanism through which O-GlcNAc may influence insulin synthesis is through mRNA stability. Perhaps increasing O-GlcNAcylation in the β cell occurs at the insulin mRNA level to increase its half-life and prevent degradation. Previous studies showed that the half-life of *Ins1* and *Ins2* mRNA in our cell line is greater than 24 hours [31]. Considering our longest incubation experiments last for 10 hours, it is extremely unlikely that O-GlcNAc affects this process to increase insulin biosynthesis.

Epigenetic regulation has proven to be a highly organized and powerful molecular mechanism controlling gene transcription. Several histone modifications are known to influence nucleosome structure, including: acetylation [32], methylation [33], ubiquitination [34], phosphorylation [35], etc. Recently, several groups have shown that O-GlcNAc modifies histones or chromatin complexes to mediate gene transcription (reviewed in [36]. We wanted to examine if our qPCR results were possibly due to O-

GlcNAc-directed chromatin remodeling events. We decided to use chromatin immunoprecipitation (ChIP) strategies to measure the amount of epigenetic marks localized at the *Ins2* promoter. Both histone H3 lysine 9, 14 bi-actylation (H3K9,14Ac) and histone H3 lysine 4 trimethylation ($H3K4me^3$) are transcriptional activating marks [37-39]. We demonstrate increasing O-GlcNAc leads to elevated H3K9,14Ac and $H3K4me^3$ marks at the *Ins2* promoter (Fig 4.1). High glucose treatment also increased these activating histone H3 marks, but not to a statistically significant level. We also confirmed that these epigenetic increases were not due to histone degradation at the *Ins2* promoter considering histone H3 levels were similar in each sample. We also used an immunoblotting approach to investigate these histone marks between our conditions. Using the same antibodies as in ChIP, we measured global levels of H3K9,14Ac, $H3K4me^3$ and histone H3 in crude histone extracts. Immunoblots with all three antibodies show no change between our different pharmacological conditions. Together, these results suggest that O-GlcNAc modulates insulin gene activation by influencing histone H3 activating marks at the promoter, but does not affect these modifications on a global scale.

Because O-GlcNAc has been shown to modify various transcription factors in multiple biological systems, we wanted to see if that was the case in Min6 cells. To do so, we used deep RNA-sequencing analysis and bioinformatics to determine every gene affected by O-GlcNAc. Our results indicate that O-GlcNAc modulates a large subset of genes in Min6 cells that are similarly affected during high glucose stimulation. These findings propose that O-GlcNAc may be an important transcriptional regulator in Min6 cells and suggests increases in this PTM heavily contributes to the transcriptional impact of hyperglycemia. However, additional studies quantifying these expression changes are needed to substantiate this claim.

We believe that our findings provide important information in terms of pancreatic β cell function and O-GlcNAc driven gene regulation. While previous reports provided evidence that O-GlcNAc was important in β cells, we show for the first time that O-GlcNAc directly impacts insulin gene activation in Min6 cells. Moreover, our findings provide additional support for this PTM as an epigenetic regulator of transcription. Our results also suggest O-GlcNAc serves as a glucose sensor in Min6 cells to initiate hormone synthesis. O-GlcNAc stimulated insulin production enables cells to secrete insulin under prolonged high glucose exposure (Fig 4.1). However, this may also be problematic when it comes to facilitating hyperinsulinemia and β cell fatigue. During type II diabetes, peripheral tissues become insulin resistant and force the β cell into overload in attempts to restore normal glucose levels. Unfortunately, this results in hyperinsulinemia because insulin sensitive tissues are unable to respond to the circulating hormone. If these symptoms persist, the β cell undergoes fatigue and attenuates insulin release. O-GlcNAc may help establish these hyperinsulinemic and β cell fatigue phenotypes in combination with chronic hyperglycemia by maximally driving insulin gene expression.

Our RNA-sequencing data reveals O-GlcNAc regulated a subset of genes in Min6 cells. Interestingly, bioinformatics analysis shows that the same protein classes are influenced in both hyperglycemic and elevated O-GlcNAc conditions. Furthermore, a large portion of the same genes in the high glucose stimulated and OGA inhibited samples were similarly affected. Based on these results we believe a large portion of hyperglycemia-induced gene expression changes in Min6 cells work through an O-GlcNAc mediated transcriptional mechanism.

2. Future Investigation

Although we believe these results provide evidence advancing both the β cell and O-GlcNAc fields, further investigation remains. First and foremost, while Min6 cells are a great system to study glucose-responsive insulin dynamics, it is a hybridoma-based line that may not recapitulate primary β cell function. In order to determine if our findings are applicable in *vivo*, these experiments need to be performed in isolated rodent β cells and examined in a type II diabetic mouse model. Additionally, since it has already been established that β cells from type II diabetic models express higher O-GlcNAc levels compared to WT controls [40], it would be interesting to compare insulin gene expression patterns and epigenetic marks between these groups.

We clearly show that O-GlcNAc increases insulin gene activity through nucleosome regulation, but the precise mechanism remains unknown. Additional experiments need to determine if O-GlcNAc regulates insulin gene transcription by directly modifying histones themselves or chromatin regulatory complexes. There is evidence for both of these mechanisms in the recent literature (reviewed in [36]), but this has never been studied in pancreatic β cells. To help in these studies, mass spectrometry strategies could be used to identify the O-GlcNAc sites on histone H3, along with other PTMs modifying proximal and distal residues under our pharmacological conditions in Min6 cells. To investigate whether O-GlcNAc impacts the nucleosome through protein complex regulation, immunoprecipitation studies using O-GlcNAc, OGT and OGA primary antibodies could be applied to nuclear and/or chromatin extracts. Subsequent mass spectrometry could be used to identify histone H3 interacting proteins, map their O-GlcNAc sites and compare key differences between each pharmacological condition. Narrowing the potential mechanism of action would allow for mutational investigation to see if specific amino acid changes attenuate the *Ins2* qPCR and ChIP results and reveal the specific O-GlcNAc-mediated mechanism.

Of particular interest in the field of transcriptional control is determining the epigenetic landscape in different cell types. Work has very recently been aimed at advancing tools to look at these changes in the pancreas [41] and determine if these epigenetic marks change in different disease states [42]. Interpretation of the specific histone H3 marks required for *Ins2* gene activation in Min6 cells could provide more detailed understanding into how environmental changes regulate epigenetic fluidity. It would also be very useful to determine the sequential order of PTM addition and removal that enable gene activation or silencing and compare the status of these marks during fluctuating O-GlcNAc levels. Addressing these questions will advance our understanding in how O-GlcNAc contributes to epigenetics and may also identify a potential therapeutic target to treat diabetes.

Using RNA-sequencing, we show that O-GlcNAc regulates a large number of genes in Min6 cells. To validate these results, additional qPCR is required to ensure these altered expression values are true. As previously mentioned, genes can be regulated by various mechanisms. We show that O-GlcNAc modulates the insulin gene through chromatin remodeling events. Further analysis is needed to determine if the observed changes in these additional genes also occurs through epigenetic mechanisms or if an alternative O-GlcNAc-mediated process is at work.

Several caveats remain in terms of understanding the epigenetic hierarchy in Min6 and other cell types. Our results reveal that only a small percentage of genes are being affected during glucose stimulation and elevations in O-GlcNAc. We were able to show that insulin gene activity is dramatically altered under these conditions through an epigenetic mechanism, but were unable to notice any significant changes at a global level. The major problem remaining is how to precisely focus on genes of interest regulated through nucelosomal control, while ignoring those that are unaltered. Currently, ChIP-grade antibodies are designed to target both histone and non-histone proteins and

modified histone tails. However, unless you know the protein or gene of interest these studies are futile because they lack gene specificity. Techniques or reagents able to investigate the epigenetic landscape of a particular gene or set of genes would help to revolutionize the field.

Pharmacological manipulation strategies are vital in determining cause and affect molecular events within a sample. Our utilization of two separate OGA inhibitors that produce similar results help to ensure that what we see is real, but there is always a chance that some of these findings may be due to pharmacological artifacts. Genetic studies focused on OGA and OGT would provide additional support for our observed inhibitor changes. Protein overexpression or knockdown studies would directly influence intracellular O-GlcNAc levels and dismiss the possibility for off-target inhibitor affects. Several different RNA interference (RNAi) approaches are available, including: small interfering RNA (siRNA) [43, 44], micro RNA (miRNA) [45], short hairpin RNA (shRNA) [46], etc. However, each repressor class may act differently between cell types and requires technical validation in the Min6 setting. Unfortunately, overexpression of the O-GlcNAc cycling enzymes have been problematic within the field. This could be attributed to a number of technical and/or molecular possibilities that make genetic interpretations difficult. While it may be more time and energy intensive, investigating the affects of O-GlcNAc in the pancreatic β cell using genetic approaches would be beneficial in verifying our pharmacological inhibition results.

3. Concluding Remarks

All together, my thesis work demonstrates that O-GlcNAc acts as a glucose-sensor in the Min6 β cell line. This PTM regulates insulin gene activity through a currently undescribed epigenetic mechanism that may contribute to hyperinsulinemia and β cell fatigue in the progression of type II diabetes.

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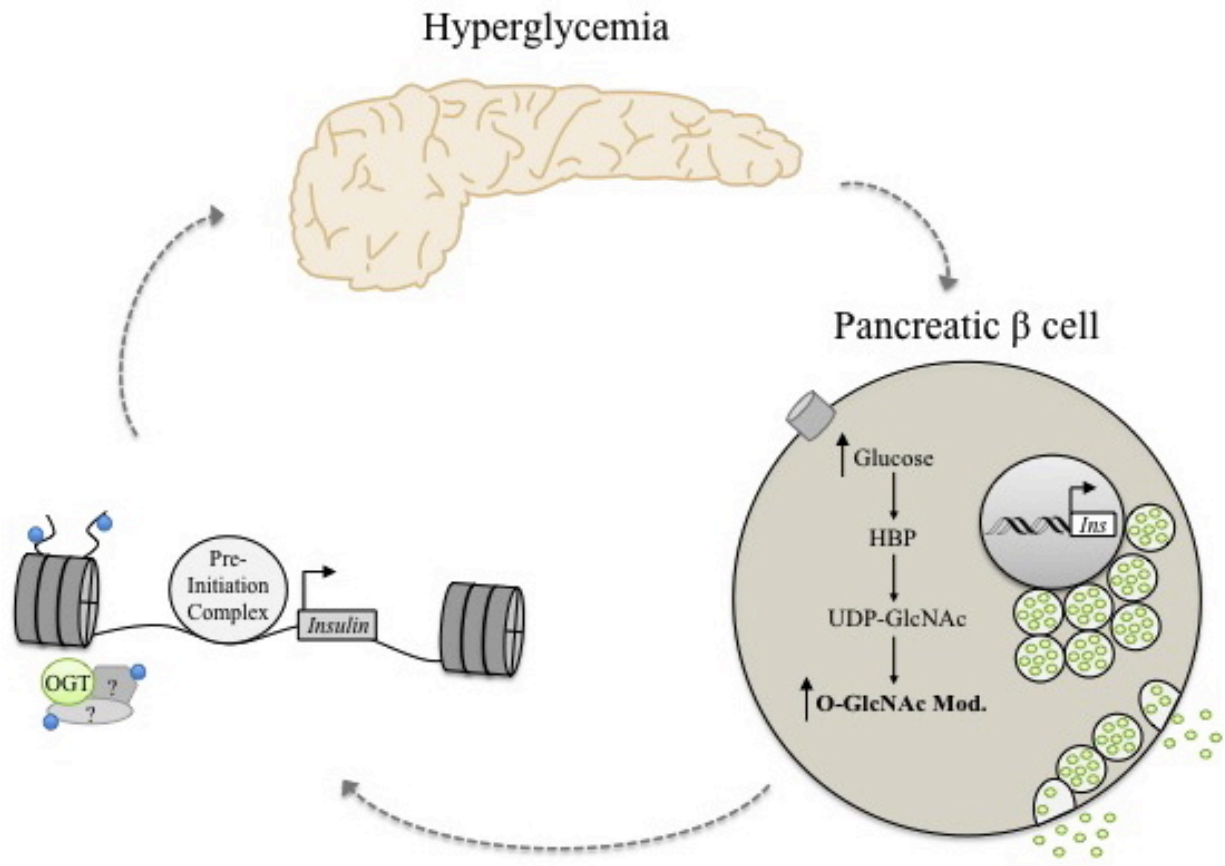
Figure 4.1



Epigenetic regulation of insulin gene transcription by O-GlcNAc. Chronic hyperglycemia increases HBP flux in pancreatic β cells, increasing O-GlcNAc modification levels. Elevations in O-GlcNAc triggers insulin hormone biosynthesis and augments sustained secretion under prolonged glucose exposure. This O-GlcNAc-induced insulin increase is due to an epigenetic mechanism, wherein increasing O-GlcNAc leads to chromatin remodeling events by either direct histone modification or influencing nucleosome regulatory complexes. In type II diabetes, this cycle is persistent and elevated O-GlcNAc may facilitate the hyperinsulinemic and β cell fatigue phenotypes commonly observed. Blue circles represent O-GlcNAc; green circles represent insulin.