IDENTIFICATION AND CHARACTERIZATION OF A MISSENSE MUTATION IN O-GLCNAC TRANSFERASE THAT SEGREGATES WITH DISEASE IN A FAMILY WITH X-LINKED INTELLECTUAL DISABILITY

by

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(Under the Direction of Dr. Lance Wells)

ABSTRACT

O-GlcNAc transferase (OGT) is responsible for the addition of the β-N-acetylglucosamine post-translational modification to serine/threonine residues of hundreds of nuclear and cytoplasmic proteins. In a focused X chromosome exome next generation sequencing of 30 probands with X-linked Intellectual disability (ID), a novel missense mutation in the OGT gene (Xq13.1) has been identified in a family with three affected males. The mutation occurs in the tetratricopeptide(TPR) region [762G>T (p.L254F)]of the transferase. The clinical phenotypes of these patients include hypospadia, clinodactyly, short stature, microcephaly, and- ID. To study the physiological role of this mutation, lymphoblastoid cell lines from two affected males, one mother and three unaffected related males were isolated. Steady-state OGT protein levels are decreased in the patient samples compared to the carrier and normal control in agreement with molecular modeling that predicts the mutation to be destabilizing. This was further validated by half-life studies that demonstrate a faster turnover of the L254F-OGT. We have generated a recombinant L254F-OGT that has allowed us to

perform activity studies and L254F-OGT is active in vivo against protein substrates and in vitro against a synthetic peptide. Surprisingly, steady-state global O-GlcNAc levels remain grossly unaffected in XLID. The same samples, however, show a decrease in steady-state O-GlcNAcase (OGA, the enzyme that removes O-GlcNAc from proteins) levels. These findings imply a compensation mechanism exists, although imperfect, given the phenotype of the patients, for maintaining global O-GlcNAc levels. L254F-OGT patients also show a decrease in OGA steady state mRNA levels and luciferase reporter expression. OGT has been previously shown to exist in a co-repressor complex to down regulate gene expression. We have observed that there is enrichment of OGT at the proximal promoter region of OGA leading us to hypothesize that OGT regulates OGA transcription in the patient lymphoblastoids. In parallel, global transcriptome analysis by performing RNA deep sequencing has revealed that there are disease specific changes in gene expression. Currently we are determining the mechanism of regulation of OGA gene repression by OGT as well as validating targets obtained by RNA deep sequencing analysis. Finally, we will generate induced pluripotent stem (iPS) cells from the fibroblast of affected patients and carriers in order to explore the imperfect compensatory mechanism in derived neural lineages due to the specific phenotypes observed. For the first time, we have identified and partially characterized a missense mutation in OGT that causes a disease, XLID.

INDEX WORDS: O-GlcNAc, O-GlcNAc transferase, O-GlcNAcase, XLID

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DEDICATION

I would like to dedicate this work to my grandmother and grandfather, who played a pivotal role in my early education and development. To my mother and father, without whom I would have never made it this far. Thank you for believing in me and dreaming with me. Thank you for teaching me how to fly and reach for the stars. To my best friend and husband, Viejay, who truly has been my pillar of strength since high school and continues to be my best man. Thank you for believing in me and teaching me patience and perseverance and helping me reach my goals.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Posttranslational modifications

Proteins are intricately regulated to augment functional diversity within the cell. Protein regulation can occur by several posttranslational modifications (PTMs) such as phosphorylation, ubiquitination, acetylation, sumoylation, etc. Several of these PTMs occur in combination with each other, further enhancing microheterogeneity of the modified proteins. PTMs of proteins by enzymatic glycosylation is the most widespread and complex co/posttranslational modification that is conserved evolutionarily ranging from eubacteria to eukaryotes [1]. More than half of the eukaryotic proteins are believed to be glycosylated [1, 2]. To date, 1% of all mammalian genes are estimated to be involved in glycosylation [3, 4]. Protein linked oligosaccharides are comprised of two major classes: O-linked glycosylation (where the sugar is attached to hydroxyl group of serines/ threonines) and N- linked glycosylation (where the sugar is attached to the amide linkage of asparagine). N-glycosylation is initiated in the endoplasmic reticulum (ER) and subsequently the added dolichol phosphate oligosaccharide precursor is trimmed and flipped to the medial golgi for further processing [5, 6]. Defects in assembly, trafficking or processing of N-glycans due to hypomorphic mutations in proteins involved in N-glycosylation lead to congenital disorders of glycosylation (CDGs- type I and II) [7, 8]. Other examples of N-glycosylation dysregulation are seen in cancers [9] and inflammatory diseases [10, 11]. Serine and threonine residues of proteins can be modified by a variety of sugar molecules. A few examples include O-

linked α-N- acetylgalactosamine (O-GalNAc) whose addition occurs in the secretory pathway (mainly the cis- golgi) by polypeptide GalNAc transferases [1], O-Mannosylation that occurs in the ER [12] by the enzymatic action of mannosyltransferases [5], and β-N-acetylglucosamine (O-GlcNAc) addition to nuclear and cytoplasmic proteins by O-GlcNAc transferase (OGT) [13-15]. Many disorders have been associated with O- glycosylation such as cancers [16], congenital muscular dystrophy [17], Alzheimer's disease [18-20], and type II diabetes [21]. This thesis will focus on O-GlcNAc modification and the enzymes OGT and O-GlcNAcase (OGA), which add and remove this modification on proteins respectively (Figure 1.1).

β-N-acetylglucosamine (O-GlcNAc)

O-GlcNAc is a single monosaccharide regulatory modification (Figure 1.1) that occurs on over two thousand intracellular proteins [15, 22, 23]. While this modification was originally discovered in lymphocytes in 1984 [15], O-GlcNAc has since been observed in almost all eukaryotes including unicellular parasites, plants and metazoans, with the latter being the most studied in the last thirty years since its discovery [24-28]. Owing to the fact that O-GlcNAc is added to hydroxyl groups of serines or threonines of proteins, which can also be phosphorylated, several studies have established the interplay between these two modifications, including their competition for the same site on a protein as well as the crosstalk between kinases and interaction between phosphatases and OGT [29-35] (Figure 1.3). O-GlcNAc is more analogous to phosphorylation rather than classical glycosylation in that the monosaccharide is not further extended, it occurs on serines and threonines and responds to environmental stimuli. While there is a myriad of at least 125 kinases [36] and about 8 phosphatases

[37] that specifically act on serines and threonines, interestingly, there is only one functional OGT and OGA in mammals [14, 38-41]. Kinases have evolved to diversify functionally via gene duplication [42]. OGT regulation is often likened to RNA polymerase II regulation [43, 44] wherein interacting partners and posttrasnslational modifications regulate enzyme activity. O-GlcNAc modified proteins are involved with many cellular processes in metazoans: cell cycle regulation [45], transcriptional control [46-49], signal transduction [50, 51], nutrient sensing [52, 53], stress responses [2] and chromatin remodeling [54-58]. In the more recent past, assignment of site occupancy of O-GlcNAc on specific proteins is continuing to shed light on defining its functional roles in disease. Taking into account the role of O-GlcNAc as a metabolic sensor (discussed in the next paragraph), there is compelling evidence implicating it in diabetes [59, 60], Alzheimer's disease [20, 61], cardiovascular disease [62, 63] and several cancers [64-68].

Hexosamine Biosynthetic Pathway (HBP)

Energy homeostasis maintenance by environmental cues is crucial to cellular regulation. HBP is a glucose responsive nutrient sensor that regulates glucose metabolism [52, 69]. Approximately 2-5% of circulating glucose enters as the nutrient sensing HBP as glucose-6-phosphate, before being converted into fructose-6-phosphate by G6P isomerase. The transaminase reaction of fructose-6-phosphate by glutamine fructose-6-phosphate aminotransferase (GFAT) to yield glucosamine-6-phosphate is the rate-limiting step of the pathway [70]. The nucleotide sugar donor UDP-GlcNAc is the end product of this pathway. UDP-GlcNAc is a substrate for complex glycosylation, production of other nucleotide sugars and as the obligate substrate for O-GlcNAc

transferase (OGT), giving rise to O-GlcNAc modified products. The levels of UDP-GlcNAc are regulated by glutamine, acetyl-CoA and uridine triphosphate (UTP) in addition to glucose availability [52, 69, 71] (Figure 1.2). This further strengthens the hypothesis that O-GlcNAc is a metabolic sensor that can read the status of the cellular metabolites and tightly regulate associated cellular processes.

The O-GlcNAc Cycling Enzymes, OGT and OGA

OGT catalyzes the transfer of the O-GlcNAc moiety to hydroxyl groups of serines and threonines of proteins. OGT was discovered as a cytosolic enzyme [14] and was later cloned and characterized in 1997 [38, 72, 73]. OGT has a C-terminal catalytic domain [38, 72] and an N-terminal tetratricopeptide repeat (TPR) domain [73]. TPRs are composed of a tandem array of degenerate 34 amino acid repeats that are duplicated between 3 and 16 times in a protein [74]. OGT exhibits as three isoforms: a nucleocytoplasmic isoform containing 13.5 TPRs, a mitochondrial isoform containing 9 TPRs and a small isoform that has only 3 TPRs [75]. OGT is encoded as a single gene and maps to chromosome Xq13.1 and the protein belongs to family GT41 GT-B glycosyltransferases [76-78]. Mammalian *ogt* knockouts are embryonic lethal, demonstrating its requirement for cell survival [77]. A consensus sequence for OGT substrate specificity remains elusive, however, there are many proposed methods for regulation of OGT. These include: substrate targeting via protein-protein interactions at the TPR region and phosphatidyl inositol phosphate (PIP)-binding domain, post-translational modifications like O-GlcNAc and phosphorylation, and substrate localization and availability [50, 79]. We have identified a subset of OGT interacting proteins (unpublished data Vaidyanathan, Zhao and Wells) (Table 1.1) that can help

illustrate the role of these partners in regulating OGT. OGT is also modified by PTMs like serine/ threonine phosphorylation [80, 81], SUMOylation [82], O-GlcNAcylation [83], and tyrosine phosphorylation [79, 83, 84] (Figure 1.4) (unpublished data Wells and Hart). Insulin mediated increase in tyrosine phosphorylation of OGT results in an increase in OGT activity [79]. In an IMAC enrichment assay followed by LC-MS/MS analysis, we identified a tyrosine phosphorylation on OGT (unpublished Vaidyanathan, Zhao and Wells) (Figure 1.4). However, there are 42 tyrosine sites in the full length OGT sequence with 15 potential Src tyrosine phosphorylation sites [38]. Further characterization is necessary to understand insulin stimulated tyrosine phosphorylation and the sites of occurrence.

OGA was purified and partially characterized in the 1990s, followed by cloning and further characterization in the early 2000s, when it was found to be ubiquitously expressed in all tissues [39, 40]. OGA is predominantly expressed in the cytoplasm, but has been demonstrated to exist in the nucleus as well as the mitochondria [40, 41, 85]. OGA is encoded as a single gene *mgea5* located on chromosome 10q24.1-24.3 [86]. While originally mistaken for a hyaluronidase, subsequent studies have established that OGA is in fact a beta-N-acetyl- glucosaminidase belonging to family GH 84 [87]. *Mgea5* is alternatively spliced to produce two isoforms: one full length that is localized to the cytoplasm and the other that lacks a C- terminal domain is nuclear localized [86, 90]. OGA has a catalytic N-terminal O-GlcNAcase domain and a C-terminal domain that has low sequence identity to histone acetyltransferase (HAT) domains, which are linked by a region containing a caspase 3 cleavage site that is processed during apoptosis [90, 91]. Current evidence has convincingly demonstrated that OGA lacks the previously

proposed HAT activity [92]. OGA is essential for development and *Oga* homozygous null mice are perinatal lethal [93]. Regulation of OGA remains elusive while a few studies implicate it in protein complexes [41, 94, 95]. In an OGA interactome analysis in our lab, we overexpressed and purified OGA in HEK293T cells and were able to identify several partners (Table 1.2)(unpublished data Vaidyanathan, Zhao and Wells). OGA is phosphorylated [80, 81, 96] as well as O-GlcNAc modified [97]. However, these sites have been identified in large-scale phosphoproteomics as well as quantitative glycosylation approaches and no further characterization of these sites have been elucidated. In an IMAC enrichment assay followed by LC-MS/MS experiment, we were able to identify a novel phosphorylation site T370 and three O-GlcNAc sites S422, T427, T430 (Figure 1.5). Further characterization using orthogonal approaches as well as mutational studies will provide a deeper insight into the regulation of OGA.

Aberrant O-GlcNAcylation and dysregulation of OGT and OGA in disease

Several studies have established that O-GlcNAc cycling enzymes are essential for life. As previously mentioned, *Ogt* knockouts are embryonic lethal and *Oga* null mutants are perinatal lethal in mice [77, 93]. Additionally, *Caenorhabditis elegans* studies demonstrated insulin mediated lifespan defects, suppression of dauer formation and dramatic metabolic changes in *Ogt* or *Oga* null mutants [98, 99]. *Xenopus laevis* studies showed that maintenance of O-GlcNAc levels within a narrow range is necessary for normal development [100]. A separate study by our laboratory further demonstrated that both addition and removal of O-GlcNAc caused similar defects in cell cycle survival and epiboly in zebrafish development [100]. Aside from a major role in developmental processes, O-GlcNAc modification affects several other biological processes. Increased

O-GlcNAcylation and reduced levels of OGA is seen in older mice compared to younger controls insinuating a role of O-GlcNAc in aging in mammals [93].

Dysregulation of O-GlcNAcylation and aberrant levels of OGA are causative of genomic instability [93]. Genomic instability is one of the hallmarks of cancer and there is overwhelming evidence that supports the involvement of O-GlcNAc and its cycling enzymes in this group of diseases. Recent literature encompasses studies on breast, lung, colon, pancreatic cancers and chronic lymphocytic leukemia. Increases in OGA activity have been observed in both breast and thyroid cancers with a concurrent decrease in O-GlcNAc levels [101, 102]. In contrast, O-GlcNAc levels as well as the cycling enzymes are upregulated in several cancers and most importantly, knock down of OGT leads to decreased tumor formation in mouse models [68].

Decreased glucose metabolism seen in Alzheimer's disease causes a downregulation of neurofilament M (NF-M) O-GlcNAc levels, subsequently causing an increase in NF-M phosphorylation levels [103] that correlates with disease. This is an example of the reciprocal nature of O-GlcNAc and phosphate in a disease state.

O-GlcNAc in transcription and epigenetic regulation

In the first decade of its existence, O-GlcNAc and its cycling enzymes were identified, isolated and purified. In the second decade, most of the modifications on specific proteins were established and a tremendous explosion of analytical techniques assisted in identification of several hundred modified proteins. In the third decade, functional roles of O-GlcNAc in specific cellular processes and disease states were brought to the forefront with studies particularly illuminating specific sites of modification on proteins. Now, entering the fourth decade, O-GlcNAc is a part of the histone code and new studies

are emerging with convincing evidence corroborating O-GlcNAc as an epigenetic regulator.

The role of O-GlcNAc in epigenetics is being meticulously explored in the field. As early as 1993, it was known that RNA polymerase II is O-GlcNAc modified [47] and subsequently OGT has been identified in the pre-initiation complex [49]. Recent studies have demonstrated that O-GlcNAc is part of the histone code [56, 104]. Previous work has demonstrated explicitly the presence of OGT in either transcriptional activation or repression complexes. OGT has been demonstrated to be in complex with the transcriptional co-repressor mSin3A/HDAC1, cooperatively repressing Sp1 mediated transcription and leading to a decrease in histone acetylation [105]. OGT is also shown to interact with the TET proteins and together they are responsible for mediating H3K4 methylation events and regulation of differentiation [57]. In our studies, we have identified a role for OGT in global transcriptional regulation in X-linked intellectual disability (XLID, described below), as well as a role in OGA gene repression. We hypothesize that OGT regulates OGA gene repression in XLID and causes global transcriptional changes in the disease state.

X-linked Intellectual Disability

Intellectual disability (ID) is the most common form of cognitive impairment and affects 1-3% of total population [16, 106, 107]. ID is the leading problem of socio-economic health care in Western countries as per the Centers for Disease Control and Prevention [108, 109] and is characterized by an intelligent quotient (IQ) of 70 or lower and the exhibition of two or more behavioral deficits in terms of social, conceptual or practical adaptation [16, 110]. Thus, people exhibiting at least 2 fold standard deviation less than

the mean, which is normally distributed to a mean set at 100, are classified as exhibiting ID. The severity of ID is commonly classified as mild (IQ 70-50) and severe (<30) [111].

Distribution of factors causing ID is heterogeneous and can range from malnutrition during pregnancy, neurotoxicity, perinatal brain ischemia, fetal alcohol syndrome to chromosomal abnormalities including deletions, aneuploidies to monogenic mutations [111]. Chromosomal aberrations that are cytogenetically visible account for 15% of all patients with severe ID [109]. However, submicroscopic deletions, duplications and missense mutations have recently emerged in the field. This is primarily due to previous limitations in identification technology and the more recent spotlight on the X-chromosome. Monogenic causes have been mainly attributed to genes found on the X-chromosome. Therefore, ID can be the cause of inherent genetic abnormalities and 5-10% of ID in males is inherited in an X-linked pattern [112]. Epidemiological studies consistently show 30-50% excess of males over females. At least 102 genes with mutations result in 81 of the known 160 XLID syndromes and over 50 families that exhibit non-syndromal XLID (NS-XLID)[112].

Fragile X Syndrome

The most common form of XLID and perhaps the best studied is the fragile X syndrome (FXS). FXS is an example of a conspicuous cytogenetic aberration, the “fragile sites”, wherein the region of trinucleotide CGG repeats are not stained and look like DNA breaks [113]. Most cases of FXS present with amplified CGG trinucleotide repeats in the 5’ untranslated region of the fragile X mental retardation 1 (FMR1) gene that is localized to the long arm of the X chromosome [114]. FMR1 encodes the fragile X mental retardation protein (FMRP). FXS patients typically have >200 repeats of the CGG

motif resulting in the methylation and silencing of the FMR1 gene [115]. In conjunction with severe ID, FXS patients exhibit a medley of features ranging from mild physical characteristics like large ears to autism and epilepsy [116]. Other than the CGG repeats, there are also known point mutations in FMR1 that can cause FXS. Both cytogenetic analyses as well as PCR of the FMR1 gene of individuals suspected to have FXS are used as diagnostic tools.

Glycosylation and NS-XLID

A missense variant in asparagine linked glycosylation 13 homolog (ALG13) was identified by X-chromosome exome sequencing in an Arabian with NS-XLID. ALG13 encodes a protein that heterodimerizes with asparagine linked glycosylation 14 homolog (ALG14) to form a functional UDP-GlcNAc transferase that catalyzes the addition of the second GlcNAc to the dolichol linked GlcNAc (GlcNAc-PP-Dol) molecule in the ER membrane [117]. ALG13 also interacts with atrophin I (ATN1) whose mutations have been implicated in Dentatorubral-pallidoluysian atrophy (DRPLA), a disease with variegated causes, most notably intellectual disability [118]. This study highlights the crossover of heterogeneous genetic disorders as it was previously shown that a patient manifested with CDG-I due to a missense de novo variant in ALG13 and did not display ID because of their early death at one year of age [119]. Nevertheless, these two separate reports may lead to the discovery of underlying biological processes of ALG13 in neurological development. Further biochemical characterization of the variant ALG13 is necessary to understand the direct role of N-glycosylation, if any in the NS-XLID patients. Lack of clear phenotypes in NS-XLID makes it more challenging to diagnose especially without segregation analysis.

Technologies and limitations in studying heterogeneous genetic diseases

Genetically multifarious disorders like XLID, CDG and CMD remain diagnostically evasive. This is mostly owing to the iniquitous overlapping of clinical features amongst the three diseases, effectively making specific gene targeting a challenge. Potent diagnostic tools are essential for proper molecular corroboration as well as efficacy of treatment and therapeutics. A correlation between genotype and phenotype in a robust manner is imperative to study complex traits that may be the result of the underlying biological process. Most pathogenic variants of genes were identified between 1980-2010 using linkage analysis in large pedigrees and subsequent testing, including breakpoint analysis, of candidate genes obtained[120]. The locus for Huntington disease was the first to be mapped to a specific chromosome as early as 1983 by employing recombinant DNA technology [121]. With the introduction of polymerase chain reaction (PCR), positional cloning, and expressed sequence tags (ESTs), momentum was gained in identification of pathogenic mutations in genes [122, 123]. Gene expression arrays, SNP arrays and genomic arrays are some other technologies that have been used since then to establish genetic variants in disease. In parallel, mouse knockout models began providing a powerful approach to illustrate gene function *in vivo* [124, 125] mainly due to the 85% similarity to the human genome. However, studying autism, ID, adaptive behavior etc. proves challenging using mouse models. This has led to the propagation of using immortalized patient cell lines to study XLID.

With the advent of the human genome project [37], possibilities were plentiful in terms of studying human disease. Whole genome sequencing has transitioned through

many stages to the high throughput next generation sequencing available currently. However, only 1% of the human genome contains protein coding genes and 95% of disease causing variants are nestled in this region [126]. Therefore, the ongoing focus is mainly diverted to exome sequencing. Exomes are faster to sequence and more cost efficient when compared to whole genome sequencing. Nonetheless, the inefficacy of exome sequencing in identifying structural or non-coding variants is a limitation that is overcome by whole genome sequencing [127]. Orthogonal approaches are essential to prove pathological molecular function or dysregulation, especially due to the smaller group of people analyzed by exome sequencing.

Conclusive remarks

Technological advancements and scientific pursuits have clearly allowed for our considerable knowledge of various diseases. In the context of human health, both Mendelian and non-Mendelian diseases are extensively studied. Speed of automated analyses and bioinformatics tools have opened up a plethora of target genes. However, biochemical characterization and uncovering molecular mechanism is still tedious but mandatory for targeted therapeutics as well as diagnosis. While the role of O-GlcNAc in many diseases has been elucidated as previously mentioned, the direct impact of mutations in either cycling enzyme has yet to be associated with a disease. This thesis will delve into the identification of a novel missense mutation in OGT that segregates with XLID.

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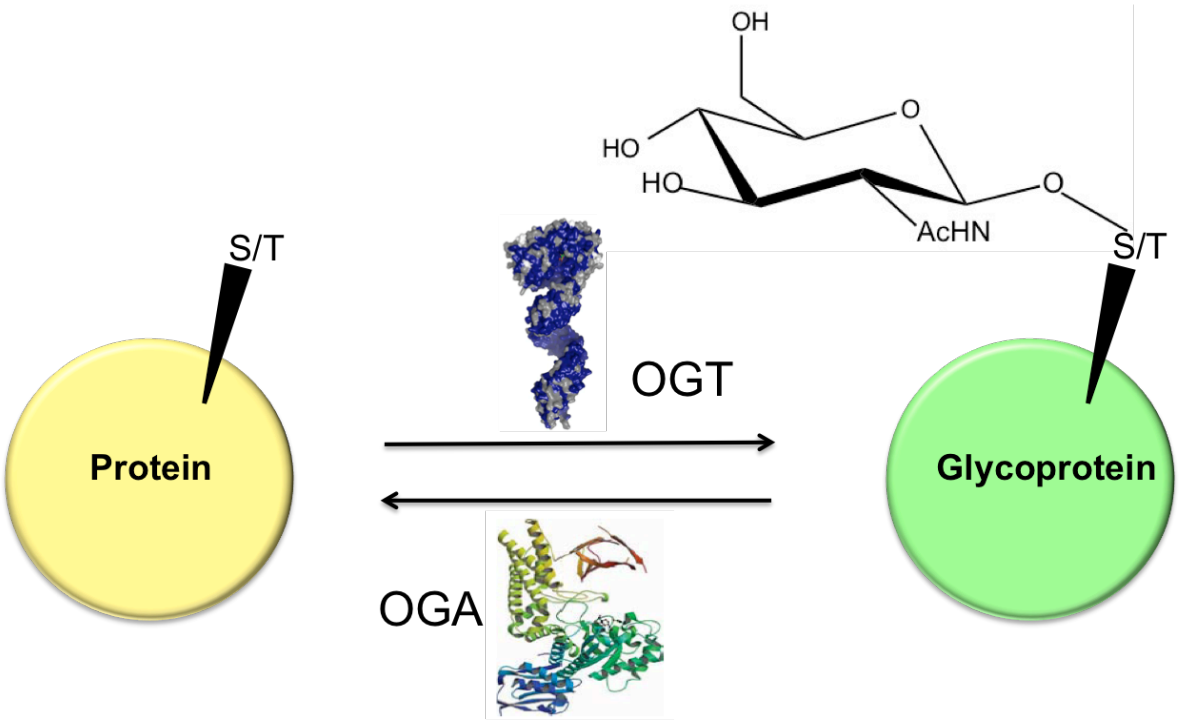

Figure 1.1: O-GlcNAc and O-GlcNAc cycling enzymes OGT and OGT. The dynamic and inducible posttranslational modification, O-GlcNAc, is cycled by OGT, catalyzing its addition, and OGA, catalyzing its removal. Structures of enzymes adapted from Clarke et al [128] and Dennis et al [87]

Figure 1.2: Hexosamine biosynthetic pathway. Once glucose enters the cell, 2-5% of it is shunted through the HBP. Flux through the HBP produces the sugar nucleotide donor UDP-GlcNAc that serves as the substrate for OGT. Additionally, UDP-GlcNAc can also be used in other types of complex glycosylation as well in the synthesis of other nucleotide sugars.

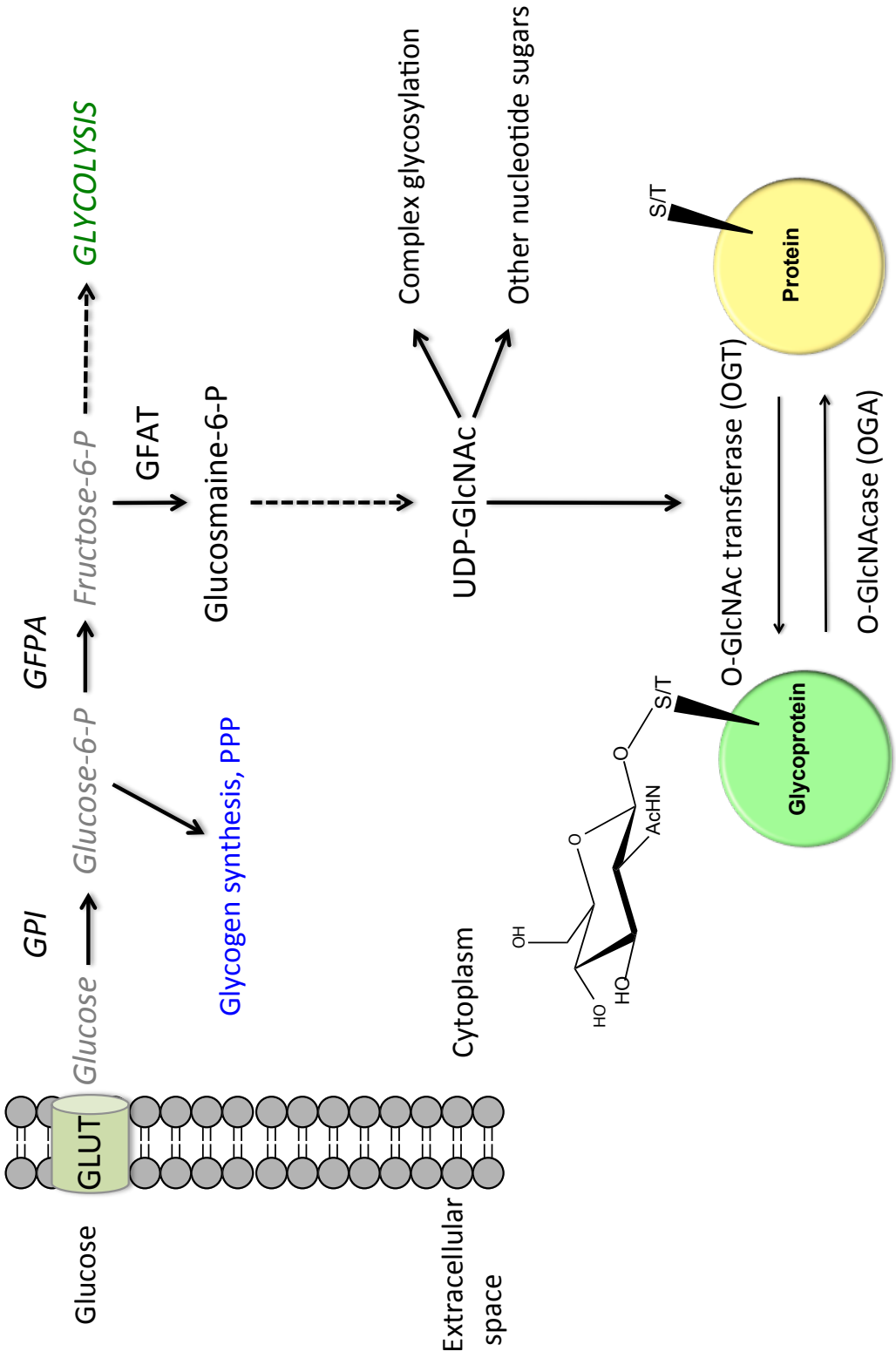
Glucose
GLUT
Extracellular space
Cytoplasm
GPI
Glucose
Glucose-6-P
GFPA
Fructose-6-P
GLYCOLYSIS
Glycogen synthesis, PPP
GFAT
Glucosmaine-6-P
UDP-GlcNAc
Complex glycosylation
Other nucleotide sugars
O-GlcNAc transferase (OGT)
O-GlcNAcase (OGA)
Glycoprotein
Protein
S/T
S/T
AcHN
OH
HO
HO

Figure 1.3: Dynamic interplay between O-GlcNAc and Phosphate. O-GlcNAc modification is akin to phosphorylation and they both occur on serines and threonines of proteins. O-GlcNAc can occupy the same site as a phosphate on a given protein resulting in a yin-yang relationship. The two modifications can also exist adjacent to each other on a given protein. This further diversifies the function of the protein modified.

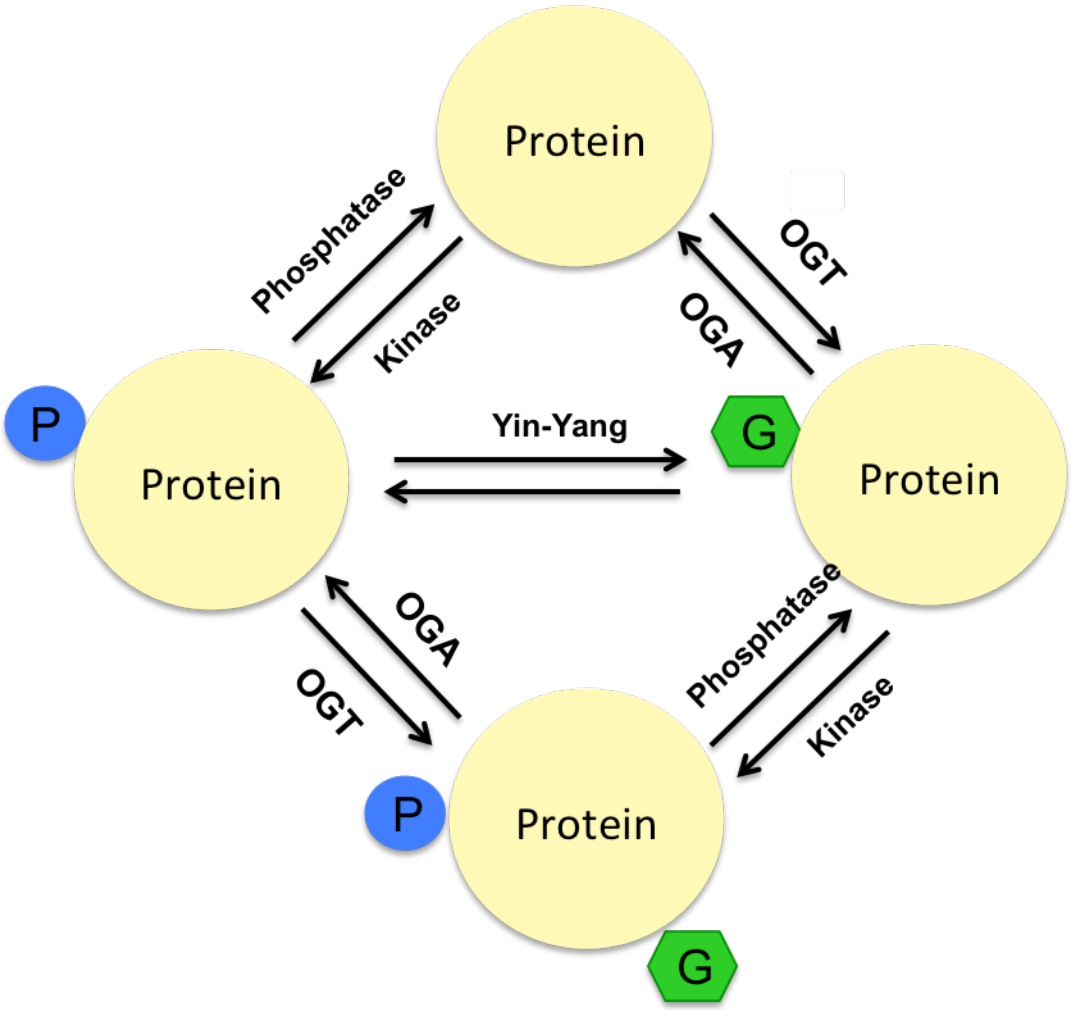
Protein
Phosphatase
Kinase
OGT
OGA
P
Protein
Yin-Yang
G
Protein
OGA
OGT
Phosphatase
Kinase
P
Protein
G

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Table 1.1: OGT interacting proteins identified in a mass spectrometry screen.

Unpublished data- Vaidyanathan, Zhao and Wells

GENE ID	SYMBOL	FULL NAME	BIOLOGICAL FUNCTION
23013	SPEN	SMART/HDAC1-associated repressor protein	Transcriptional regulation
3012	HIST1H2AB	Histone H2A type 1-B/E	Nucleosome assembly
121504	HIST1H4A	Histone H4	Nucleosome assembly
10155	TRIM28	Transcription intermediary factor 1-beta isoform 1	Transcription regulation
10419	PRMT5	Isoform 1 of protein arginine N-methyltransferase	Transcription regulation
10606	PAICS	Multifuntional protein ADE2	Purine biosynthesis
11304	DSTN	Destrin (Actin depolymerzing factor), isoform CRA_a	Actin binding
2896	GRN	Granulin isoform-1	Cytokine
7018	TF	Serotransferrin	Iron transport
79770	TXNDC15	Thioredoxin domain-containing protein 15	Cell redox homeostasis

Figure 1.4: Novel site of modification on OGT. Unpublished data-Vaidyanathan, Zhao and Wells, Wells and Hart. Following IMAC enrichment, we performed HCD-ETD to identify a medium confidence novel tyrosine phosphorylation site on OGT. Tyrosine phosphorylation studies can shed light on the regulation of OGT.

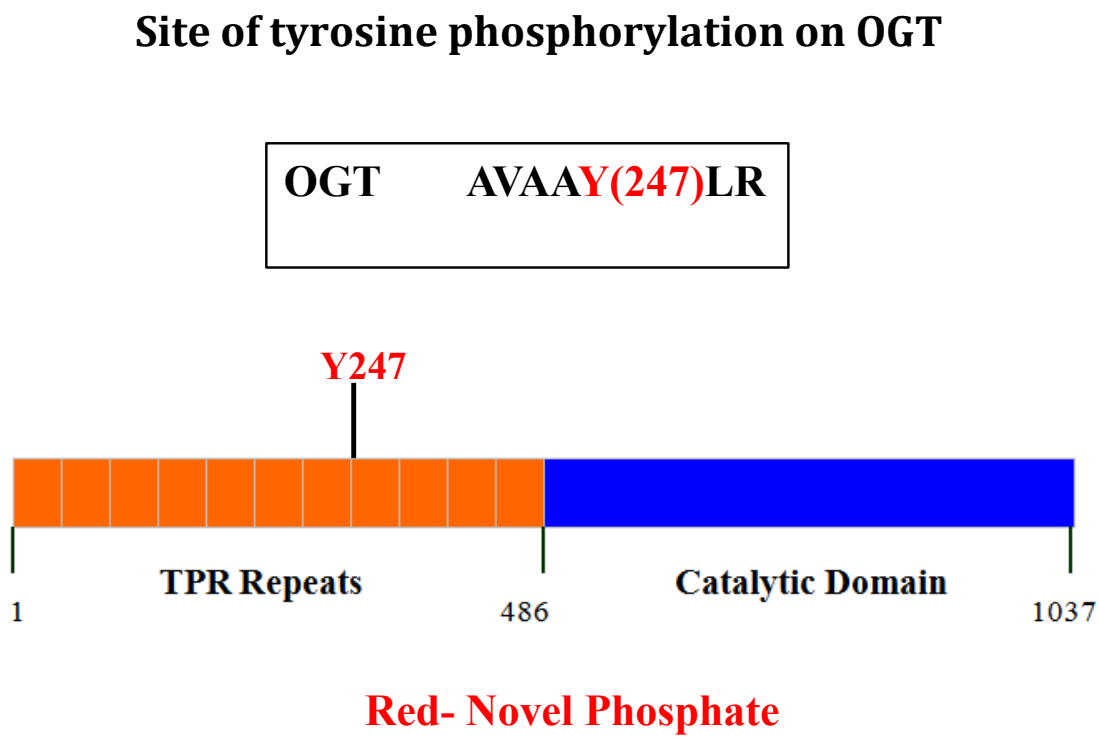


Table 1.2: OGA interacting proteins identified in a mass spectrometry screen.

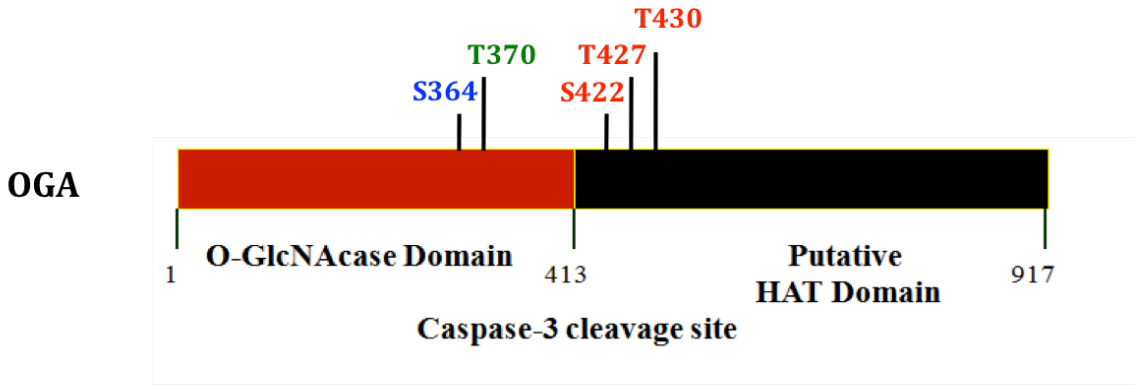
Unpublished data- Vaidyanathan, Zhao and Wells.

GENE ID	SYMBOL	FULL NAME	BILOGICAL FUNCTION
23524	SRRM2	Serine/arginine repetitive matrix protein	mRNA processing
10606	PAICS	Multifunctional protein ADE2	Purine synthesis
11304	TRIM28	Transcription intermediary factor 1-beta isoform 1	Transcription regulation
10419	PRMT5	Isoform 1 of protein arginine N-methyltransferase	Transcription regulation
5901	RAN	RAN, Ras oncogene family, isoform CRA_c	Transport
2896	VIM	Vimentin	Intermediate filament
5901	RAN	RAN, Ras oncogene family, isoform CRA_c	Transport
7168	TRAF2	TNF receptor-associated factor 2	Apoptosis
51231	VRK3	cDNA FLJ53256, highly similar to Homo sapiens vaccinia related kinase 3 transcript variant	Protein amino acid phopshorylation
55852	TEX2	Testis-expresses sequence 2 protein isoform 1	Signal transduction
57679	ALS2	Alsin	Cell survival

Figure 1.5: Novel PTMs identified on OGA. Unpublished data- Vaidyanathan, Zhao and Wells. Known sites are referenced in the Human protein reference database. Using IMAC enrichment and pseudo neutral loss- collision induced dissociation (pNL-CID), we were able to map one novel phosphorylation site. Following enrichment for O-GlcNAc and pNL-CID, we were able to map 3 sites on the same peptide sequence. PTMs and their role will shed light on OGA regulation.

Sites of phosphorylation and O-GlcNAc mapped on OGA

OGA	LENEGS(364)DEDIET(370)DVLYSPQMALK
OGA	ASVVDGTPLVAAPS(422)LNATT(427)VVT(430)TVYQEPIMSQGAALSGEPTTLTK



Blue- Known Phosphorylation
Green- Novel Phosphorylation
Red- Novel HexNAc

CHAPTER 2: FUNCTIONAL O-GLCNAC MODIFICATIONS: IMPLICATIONS IN MOLECULAR REGULATION AND PATHOPHYSIOLOGY

1

[1] Vaidyanathan. K, Durning. S, and Wells. L. Crit Rev Biochem Mol Biol. 2014 Mar-Apr;49(2):140-63. Epub 2014 Feb 14.
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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.

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 (Figure 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]. Recently, an extracellular OGT (eOGT) has been identified, which modifies serines and threonines of epidermal growth factor-like (EGF) repeats, like those found on *Drosophila* Notch [34, 35].

OGA was cloned and partially characterized in the early 2000s and is found ubiquitously expressed in all tissues [4, 36]. 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 [37]. 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 [38], but also affected the hexosaminadase enzyme family [39]. Recently, several highly selective OGA inhibitors have been generated that exhibit greater specificity for N-acetylglucosaminidases compared to hexosaminidase A/B (Figure 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 [40-42]. Several OGT inhibitors are also documented in the literature [43], but have not been widely evaluated or used in the field to date. In addition, several groups have established enrichment and detection strategies for O-GlcNAc modification on proteins [44, 45]

Since its discovery, O-GlcNAc has been shown to modify thousands of proteins in numerous cellular pathways. However, the transcriptional regulation of OGT and OGA remain to be elucidated. 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.

Epigenetic Regulation by O-GlcNAc

Chromatin is a highly dynamic structure that critically regulates transcription [46]. Chromatin is composed of DNA and histones that are condensed to form nucleosomes [47]. This higher order chromatin structure regulates gene transcription and repression [46, 47]. Chromatin is composed of transcriptionally active euchromatin that is gene-rich and heterochromatin which is gene-poor and transcriptionally silent [48]. Nucleosomal rearrangement is crucial for the movement of the transcription machinery along the DNA [47]. Chromatin remodeling is a complex process involving several known PTMs like acetylation, methylation, ubiquitination and phosphorylation [46, 49, 50].

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, 51]. RNA Polymerase II is O-GlcNAc modified [15] and more recently OGT was shown to be a member of the pre-initiation complex [52, 53]. Disruption of the activity of either OGT or OGA leads to

transcriptional defects and impaired pre-initiation complex formation [53]. 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 [54]. 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 [54].

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 [55] [Figure 2.2]. OGT and mSin3A act synergistically to repress basal and Sp1 mediated transcriptional activation [55]. Moreover, estrogen target genes are hyperglycosylated in the absence of estrogen in Mcf-7 cells [55]. mSin3A and HDAC1 are both known to be O-GlcNAc modified [55].

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 [56-58]. In

combination with histone modifications, these regions are vital in regulating gene activity at developmental stages in ESC [59, 60]. Investigation into ManNAc-stimulated *hypocretin neuropeptide precursor* (*hcrt*) gene regulation revealed OGA and OGT are localized within the before mentioned T-DMRs [61]. ChIP experiments illustrate higher O-GlcNAc signal within the *hcrt* promoter region (regions 1 and 2) during gene inactivity [61]. 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 [61].

Histones 2A, 2B, 3 and 4 (H2A, H2B, H3, H4) are O-GlcNAc modified [24, 62] 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 [62, 63]. Interestingly, when H3 Ser10 is O-GlcNAcylated, its neighboring residue lysine 9 (K9) presents with decreased acetylation [62]. Acetylation of H3K9 is a mark of active transcription [49, 64], 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 [62]. 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 [65]. Alanine mutations of Ser112 significantly reduced O-GlcNAcylation by OGT *in vitro* [65]. H2B modification at Ser112 is shown to be glucose dependent since 24-hour starvation results in its deglycosylation in HeLa cells [65]. Glucose replenishing restores the S112 O-GlcNAcylation gradually within a 24-hour period [65]. This O-GlcNAc modification also influences H2B Lys120 monoubiquitination as highlighted by the replenishment of glucose facilitating this histone addition [65]. This notion is validated considering OGT knockdown leads to diminished modification of Lys120 [65]. HBP inhibitors attenuate the effect of glucose responsiveness as indicated by the loss of both Ser112 O-GlcNAcylation and Lys120 monoubiquitination [65]. Further, Ser112Ala and Thr122Ala H2B mutations revealed the absence of K120 monoubiquitination even in the presence of extracellular glucose [65]. However, mutating H2B Lys120Arg did not affect the O-GlcNAcylation at H2B Ser112 [65]. 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 [66]. 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 [65]. Glycogen synthase kinase 3β (GSK3β) transcription was induced

by Ser112-O-GlcNAcylated H2B, but totally ablated by OGT knockdown [65]. 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 [61].* These events directly correlate with observed elevations in histone H3 and H4 acetylation marks during gene activation [61].

Ten-eleven translocation (TET) proteins are Fe^{2+} and 2-oxoglutarate-dependent dioxygenases that oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [67, 68]. TET proteins mainly associate with CpG rich promoter regions [69-71]. Histone 3 lysine 4 trimethylation (H3K4me3), an activation mark, also marks CpG rich promoter regions [72]. Interestingly, most Tet1-bound promoters are marked by H3K4me3 [69, 70]. 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 [73, 74]. TET1 in particular has been suggested to impart transcriptional regulation by interacting with chromatin remodeling and histone modification complexes Sin3a and NuRD [75]. 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 [73]. OGT siRNA-directed knockdown studies reduce TET1 targeting and 5hmC enrichment on TET1 regulated genes [73, 74].

Using affinity purification and MS techniques, OGT was found associated with TET2 and TET3 *in vitro* [76] [Figure 2.2]. Moreover, in mESCs, TET2 interacts with OGT endogenously [76]. The C-terminal catalytic double-strand beta-helix (DSBH) region of

TET2 and TPRs 5 and 6 of OGT are essential for this interaction [76]. 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 [76]. However, knockdown of OGT did not significantly alter TET2 retention at the chromatin [65, 76]. Both OGT and TET2 knockdowns impair histone O-GlcNAcylation with TET2 reduction dramatically reducing H2B Ser112 O-GlcNAc modification levels [76]. TET2 knockout mice display impaired OGT activity and decreased global O-GlcNAcylation that parallel decreased H3K4me3 [77]. 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 [76, 77]. 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) [73, 74, 77]. Interestingly, mSin3A and HDAC1 were shown to co-purify with OGT [55] and with TET1 [69, 78]. OGT binding at H3K4me3- positive promoters directly corresponds with observed TET1 ChIP-Seq signal [73, 77]. As previously described, OGT and the mSin3A/HDAC1 complex are involved in gene silencing in HepG2 cells as well as in *in vitro* studies [55] [Figure 2.2]. HCF-1 is a known interacting and substrate partner of OGT [79-81]. OGT O-GlcNAcylates HCF-1 and is proposed to function as a protease to cleave HCF-1 [81]. HCF-1 is also a component of the SET1/COMPASS H3K4 methyl transferase (MT) complex [79]. 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 [77]. OGT and TET protein activities are required for the SETD1A-chromatin binding event facilitating transcriptional activation of hematopoietic genes [77] [Figure 2.2]. OGT inhibition reduces OGT interaction with HCF-1 [77, 81] and concomitantly decreases the association with SET1DA MT [77]. These data together suggest that HCF-1 interaction is required for the TET2/3-OGT mediated transcriptional activation by SET1/COMPASS H3K4 MT [Figure 2.2]. 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 and OGT in hematopoiesis and leukemia.

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, 82]. 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 [83]. O-GlcNAc appears to be the cause considering OGT inhibition prevented the hyperglycemia-induced complications observed during development [83]. Additional supporting evidence demonstrated mouse *oga* knockouts were perinatally lethal [84]. OGT and OGA targeted morpholino injection or enzyme overexpression studies results in stalled epiboly,

preventing gastrulation and increasing embryonic death in zebrafish [85]. 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 [85]. 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 (Figure 2.3). Integrin adhesion complexes are known to regulate embryonic development through the integrin β4 cytosolic domain and plectin interaction [86]. 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 [87, 88]. Elevating O-GlcNAc levels through both GlcN flux and OGA inhibition increases mESC migration, while OGT inhibition blocks this action [88]. Several mESC proteins essential for self-renewal are O–GlcNAc modified, including Oct4, Sox2 and Zpf281 [54, 85, 89]. Additionally, mSin3a is O-GlcNAc modified and is clearly demonstrated to be involved in epigenetic regulation during development [54, 55]. Elevating O-GlcNAc in mESC inhibits their self-renewal capacity and prevents somatic cell reprogramming into induced pluripotent stem cells (iPSC) [89]. Oct4 and Sox2 are components of the core pluripotency network and part of somatic cell reprogramming cocktails to generate iPSC [90-92]. Both of these

transcription factors are O-GlcNAc modified and Oct4 O-GlcNAcylation promotes mESC self-renewal and reprogramming through a transcriptionally regulated mechanism [89]. In depth expression analysis reveals O-GlcNAc addition on Oct4 subsequently induces many pluripotency-related genes, including *klf2, klf5, nr5a2, tbx3* and *tcl1* [89]. This work establishes direct O-GlcNAc involvement in regulating key pluripotency and self-renewal proteins.

The previously discussed TETs and T-DMRs are also shown to influence ESC fate determination through O-GlcNAc control (Figure 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 [61, 73, 74]. 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 [61]*

Work in mouse embryonic fibrolblasts (MEFs) demonstrate that O-GlcNAc plays a role in the cell cycle control [62, 63, 93-96]. Because OGA null mice rarely reach maturity, MEFs can be isolated from mid-gestation embryos for investigation prior to glycosylation-linked lethality [84]. In agreement with previous work [97], O-GlcNAcylation fluctuates throughout the cell cycle stages, but constitutively increased O-GlcNAc levels in OGA null MEFs causes aberrant cell cycle progression [84]. 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 [84]. 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 [98]. Recent work implicates O-GlcNAc plays a major role in mesoderm germ cell differentiation to an even higher degree than in ESC pluripotency (Figure 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 [99, 100]. As expected, an increase in OGT and GFAT-1 protein levels, as well as GFAT-1 mRNA, directly correlate with observed O-GlcNAc elevations [99, 100]. OGT and GFAT inhibition decreases O-GlcNAc levels and prevents preadipocyte differentiation in 3T3-L1 cells [99].

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 [101-103] and respond directly to changes in O-GlcNAc (Figure 2.3)[99, 100, 104, 105]. Elevating O-GlcNAc levels increases C/EBPα expression along with another adipose-related mesoderm marker, PPARγ, during differentiation [105]. 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 [99, 100]. A separate study looking at C/EBPβ identified two amino acid residues as being O-GlcNAc modified: Ser180 and Ser181 [104]. 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 [104]. 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 [100]. 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 [99, 105, 106]. 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 [107]. 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 [107]. 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 [108, 109]. Protein observation during this time frame shows that O-GlcNAc levels in C2C12 cells dramatically decrease between days 1 and 2 of myotubule formation, in parallel with increasing OGA and OGT mRNA and protein levels [110]. 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 [110]. 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*) [108, 111]. OGA inhibition in C2C12 cells 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 (Figure 2.3)[110]. Based on this data, O-GlcNAc modulation appears to influence temporal gene expression 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 [112, 113]. 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 [114, 115]. During insulin-induced differentiation of ATCD5 pre-chondrogenic cells, O-GlcNAc levels are significantly increased and persist for the duration of development [113]. 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 [113, 116]. 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 [113]. This is further validated considering that reduction in HBP flux ablates insulin-stimulated differentiation and blocks the expression of these chondrogenic genes [113]. Additionally, matrix metalloproteinase (MMP) proteases 3 and 9, that are vital in ECM remodeling during chondrocyte differentiation [117, 118], are also upregulated during OGA inhibition to the same degree as with insulin stimulation [113]. OGA inhibition also influences several proteins that regulate CREB- and RUNX2-mediated gene expression during osteoblast differentiation, including CREB-binding protein (CBP) and TGF β-activated kinase 1 and 2 (TAB1/TAB2) (Figure 2.3)[107, 112]. 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.

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 [119-121]. 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 [122]. 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, 123-131]. In fact, the presynaptic zone proteins Bassoon and Piccolo are two of the most heavily O-GlcNAc modified proteins ever observed [129]. 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 [132, 133]. Considering the accelerated decline of glucose utilization in the Alzheimer's disease brain [134-138], 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 [139, 140]. Given the extensive crosstalk between protein phosphorylation and O-GlcNAcylation [129, 141-143], 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 [126, 130, 142, 144, 145].

O-GlcNAc levels in the brain during Alzheimer's progression appear to decrease as hyperphopsphorylation increases [126, 130, 146]. This may directly coincide with decreasing glucose metabolism observed in the aging brain [147, 148]. 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 [149]. Immunofluorescent studies on human brain samples reveal a yin-yang relationship between tau O-GlcNacylation and phosphorylation [149]. Non-hyperphosphorylated tau from patient brain samples are heavily O-GlcNAcylated compared to the hyperphosphorylated pool [149]. This data suggests the global decrease in O-GlcNAc may contribute to the hyperphosphorylated tau phenotype in Alzheimer's diseased brains (Figure 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 [146]. Studies using mouse models mimicking tauopathy show that inhibiting OGA decreases phosphorylation of tau at several residues and protects against tau-driven neurodegeneration [130]. 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 (Figure 2.4A)[130]. Conversely, shOGT addition to HEK-293 cells transfected with human tau increases phosphorylation [146].

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 (Figure 4B)[150]. APP is recognized as the first plasma membrane protein identified to be O-GlcNAc modified [151], 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 [152]. Active γ-secretase is a complex containing four protein subunits, including nicastrin (NCT) required for substrate recognition and binding [153, 154]. 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 (Figure 2.4A)[152].

. The main proteolytic processing pathway for APP uses α- and γ-secretase to produce a secreted sAPPα fragment and prevents Aβ plaque aggregation [155, 156]. Due to the observed neuroprotective properties of sAPPα [157] 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 [158]. Genetic and pharmacological manipulation studies targeting the O-GlcNAc cycling enzymes in SH-SY5Y human neuroblastoma cells confirm O-GlcNAcylation promotes sAPPα [158].

Ubiquitin is a post-translational protein modification known to accumulate at Aβ plaques and NFTs in Alzheimer's [159-161]. This PTM is crucial in regulating protein turnover via the proteasome [162, 163] and is proposed to be dysfunctional in neurodegenerative diseases [164]. Extensive research has established functional connections between O-GlcNAc, ubiquitination and the proteasome [65, 128, 145, 165-169]. 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 [128, 170, 171]. 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 [172]. 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 [172]. 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) [173, 174]. It is long established that phosphorylation aids in regulating CREB activity within the nervous system, but is not the sole regulatory PTM [175-177]. CREB is now known to be O-GlcNAc modified at Ser40, whose induction increases in response to calcium- and kinase-dependent neuronal activation [178, 179]. The major functionally relevant phosphorylation site of CREB is located at Ser133 [180]. Contrary to most instances, mutational studies demonstrate a cooperative role for O-GlcNAc and phosphorylation in mediating CREB activity [178]. Both OGA overexpression and Ser40Ala mutations illustrate that CREB glycosylation represses both basal transcription and activity-dependent CREB-induced gene expression in neurons [178]. In addition, obstructing Ser40 O-GlcNAc modification of CREB accelerates dendrite and axon elongation, while concurrently deregulating basal and activity-induced dendritic growth [178].

Nerve cell communication in the CNS is a chemically regulated process requiring synaptic vesicle endocytosis. Clathrin-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 [181, 182]. AP180 is an important adapter protein mediating lipid and clathrin binding interaction during neurotransmitter release [183]. Mass spectrometry reveals that AP180 can be O-GlcNAcylated at Thr310 and extensively phosphorylated at numerous residues in

rodent brains [125, 184, 185]. 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 [125]. 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 [125]. In contrast, these modifications may potentially serve as docking sites for specific substrate interaction [125]. 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 [129]

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 [186]. Elevating O-GlcNAc by OGA inhibition increases axon branching events in neurons, while attenuating axonal filopodial numbers [186]. 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 [186]. 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 [187]. This protein can be O-GlcNAc modified (Ser396) as well as phosphorylated

(Ser394) to trigger negative feedback inhibition and block the MEK pathway [128, 188-190]. Reciprocity is likely to occur between these proximal sites on Mek2 to influence cognition through neuronal cell signaling control. Additionally, O-GlcNAcylation of the previously discussed CREB protein appears to modulate long-term memory formation and consolidation [178]. In a somewhat similar study, the *Drosophila* PERIOD protein (dPER) is O-GlcNAcylated and temporally regulated in Schneider 2 cells [191]. This protein interacts with several others to form a transcriptional feedback loop controlling circadian rhythms; the daily oscillations in behavioral and physiobiochemical processes [192, 193]. OGT siRNA knockdown experiments dramatically shorten normal bimodal morning and evening behavior, while overexpressing OGT increased this behavioral period [191]. Specifically, manipulation of OGT regulates dPER nuclear/cytoplasmic entry into pacemaker neurons to most likely account for the altered rhythms [191]. Results strengthening this notion demonstrate that O-GlcNAc modification of dPER delays its phosphorylation-driven degradation, likely through the commonly observed reciprocal PTM relationship [191]. 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.

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, 194-197]. 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 [198-201].

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 [202]. 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 [203, 204]. 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 [205]. Further analysis reveals that OGA and OGT protein levels are higher in these patients, coinciding with increased gene expression profiles for these cycling enzymes [205, 206]. Rat models recapitulating the pathophysiology in the failing heart display similar results, suggesting O-GlcNAc signaling increases under cardiac stress [205, 206]. Interestingly, manipulating O-GlcNAc levels in cardiomyocytes under basal conditions does not significantly impact heart function [207]. 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 [208]. 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 artrial and aortic vascular reactivity [209]. Other studies inhibiting OGA demonstrate augmented cardiac contraction and relaxation, while significantly attenuating the appearance of arrhythmic activity during reperfusion [207]. Work using conditional cardiomyocyte-specific *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 [206]. 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 [206]. Interestingly, there is no significant difference in myocyte hypertrophy and survival rate between cmOGT and WT mice post-infarction [206]. 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 [206]. Very recently, additional cmOGT studies showed that early deletion during cardiomyocyte maturation drastically increased

perinatal mortality and induced severe dilated cardiomyopathy in survivors [210]. Strikingly, *ogt* deletion in mature cardiomyocytes only exhibited gradual cardiomyopathic symptoms and were otherwise normal [210]. While this suggests O-GlcNAc signaling is crucial during cardiomyocyte development, the need for this PTM in the adult heart may not be as necessary as previously thought.

One of the major concerns of prolonged ischemia is irreversible myocyte infarction. 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 [211]. Various exogenous metabolites can trigger preconditioning, as can anesthetic treatment typically referred to as anesthetic preconditioning (APC) [211, 212]. Mice subjected to APC through isoflurane supplementation express elevated O-GlcNAc levels within the heart compared to untreated controls [213, 214]. APC mice display decreased myocardial infarction in the area at risk that can be reversed with OGT inhibitor pretreatment [213]. OGT inhibition combined with APC also significantly enhances myocyte viability following stimulated ischemia-reperfusion [213]. Isoflurane-initiated APC protects against ischemic injury at least in part by regulating mitochondrial ion flow through voltage-dependent anion channels (VDAC) [215, 216]. Previous studies reveal that O-GlcNAc modification of VDAC is essential for myocardial survival [214], 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 [217, 218]. Because VDAC is a proposed 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 (Figure 2.5A)[213].

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 [219]. Rise in pressure can be caused by a number of events, including partial blood vessel occlusion, and if untreated can lead to myocardial infarction [220]. 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 [209]. DOCA-salt and OGA inhibited rats display decreased cardiac relaxation in response to acetylcholine and decreased phosphorylation of cardiovascular homeostatic proteins eNOS and Akt [209]. 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 [209]. Other work demonstrates that increasing O-GlcNAc via OGA inhibition reduces endothelial nitric oxide synthase activity to attenuate nitric oxide production [221] and appears to impair vasodilator activity in DOCA-salt models [209]. Endothelin-1 (ET-1) is a peptide that induces vasoconstriction and has shown to be elevated in the vasculature of DOCA-salt hypertensive rats [222]. Interestingly, in hypertensive

conditions ET-1 also activates transcription factors governing inflammation, oxidative stress and tissue damage [223, 224]. Rat aortas incubated with ET-1 peptide display elevations in stimulated vasoconstriction in combination with increased vascular O-GlcNAcylation [225]. OGT inhibition blocks this ET-1 induced effect on vascular activity, suggesting that O-GlcNAc in part mediates this ET-1 response [225]. ET_A receptor agonist supplementation diminishes vascular O-GlcNAc levels and augments vascular contractile function typically observed upon ET-1 stimulation [225]. Together these results suggest O-GlcNAc plays a role in both cardioprotection following ischemic and reperfusion injury and vascular dysfunction during salt-induced hypertension, highlighting the need for additional investigation into how this PTM directly influences these systems.

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 [226-228]. Considering its 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 [229, 230]. 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 [231]. 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 [231]. Further studies indicate that O-GlcNAc reduction decreases ANP mRNA by blunting NFAT signaling and specifically prevents its nuclear translocation [231]. 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) [232]. Strikingly, hypertrophic increases in O-GlcNAc directly correlate with a GLUT1 and GLUT4 expression imbalance, while OGA overexpression restores normal transporter proportions [231]. 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 [233]. 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 [233]. 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 [234-236]. Overproduction of ROS through activated ROS-enzymes, including inducible nitric oxide synthase (iNOS), is mediated by TNFα

stimulation of the nuclear factor kappa B (NF-κB) pathway [237, 238]. Rat aortic rings treated with TNFα display impairment in depolarization-induced contractile responses that is reversed with GlcN or OGA inhibitor addition [239]. 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 [239]. 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 [240]. Several studies implicate O-GlcNAc involvement in regulating NF-κB transduction [241-243], but more recent work provides a clear link in rat aortic smooth muscle cells. Phosphorylation of NF-κB is essential in determining its transcriptional activity [244-247]. Aortic smooth muscle cells incubated with GlcN or an OGA inhibitor limits inflammatory NF-κB p65 DNA binding typically seen in TNFα stimulation [248]. GlcN supplementation or OGA inhibition increases O-GlcNAc modification of NF-κB p65 and prevents its concurrent nuclear phosphorylation at Ser536 (Figure 2.5C)[248]. This reduction of phosphorylated p65 coincides directly with its enhanced interaction with the inhibitory complex protein nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) and the reduction in TNFα triggered inflammatory signaling [248].

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 [249]. 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 [250]. 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 [251, 252]. Two major interaction components in this system are Beclin-1 and Bcl-2, the pro- and anti-apoptosis promoting factors respectively [253]. Dissociation of Bcl-2 from Beclin-1 induces autophagic events and is linked to pressure overload stress-induced cardiac hypertrophy [254]. Both interacting partners can be O-GlcNAcylated and phosphorylated to differentially control their interaction [255]. 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 [255]. Moreover, blocking HPB flux significantly increases the autophagic response in diabetic mice and OGA inhibition greatly reduces Beclin-1 expression [255]. 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 [208]. 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 [208]. 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 (Figure

2.5B)[208]. 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.

O-GlcNAc Regulates Transcriptional Activity in Cancer

Pancreatic Cancer

NF-κB is a transcription factor known to play a role in various cellular processes like inflammation, cell survival, tumorigenesis and apoptosis [256, 257]. 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 [258]. The nuclear localization signal on NF-κB is uncovered in this state to allow for its nuclear translocation and facilitating transcription of downstream genes [259]. 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 [241, 260] [Figure 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) [260]. 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 [260]. OGT siRNA mediated knockdown in HEK293 cells display decreased mRNA levels of the NF-κB regulated genes for IL-8 and BCL2A1 [Figure 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 [261] [Figure 2.6 A and B]. Attenuation of NF-κB signaling pathway can result in pancreatic ductal adenocarcinoma (PDAC) cell apoptosis [262], while constitutive NF-κB signaling is a hallmark of several cancers including PDAC [263].

O-GlcNAc and OGT levels are elevated in several different pancreatic cancer cell lines corresponding with decreased OGA levels [264]. This observed increase in OGT and concomitant decrease in OGA is seen in other cancers, such as lung and colon [265]. 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 [264]. 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 [264]. OGT inhibition [266] leads to reduced O-GlcNAcylation and inhibits both colony formation and cell proliferation [264]. This is recapitulated *in vivo* by using OGT silenced orthotopic xenografts [264]. Immunocompromised mice injected with OGT shRNA display smaller tumors in weight compared to scrambled shRNA [264]. OGT shRNA mediated suppression of hyper O-GlcNAcylation induces caspase-3 and caspase-9 cleavage, indicative of apoptosis [264]. Conversely, elevating O-GlcNAc levels by inhibiting OGA decreases caspase-3 cleavage and rescued cells from suspension-induced apoptosis [264]. 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 [267] in PDAC cells [264]. 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 [245]. Reduction in PDAC hyper O-GlcNAcylation decreases p65 nuclear localization and transcriptional activity [264], 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 [264]. Furthermore, OGA inhibition mediated increase in O-GlcNAc lead to increased p65 O-GlcNAcylation [264]. Additionally, anchorage-independent growth induced by p65 overexpression is ablated in OGT knockdown PDAC cells [264]. 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 [268]. FOXM1 is shown to upregulated in several cancers [269] with some examples being breast and prostate cancers [270, 271]. Furthermore, FOXM1 is clearly implicated in cell migration, invasion, angiogenesis, metastasis and inflammation [269, 272]. Another protein of the Forkhead family, FOXO1 is a known O-GlcNAc modified protein [273]. The functional impact of this modification is still unclear.

It is documented that OGT downregulation inhibits cell cycle progression [94, 97] [274, 275]. Consistent with other studies [264, 271], 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 [270]. 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 [270]. Consistent with this data, targets of FOXM1 like Survivin, Nek2, PLK1 are also decreased in OGT knockdown in both cell lines [270]. FOXM1 is a known transcriptional activator of Skp2 [276], which regulates the degradation of $p27^{Kip1}$ during the G1/S transition [277] [Figure 2.6D]. Interestingly, levels of $p27^{Kip1}$ are increased in OGT knockdown in both MDA-MB-231 and MCF-10A-Erb2 cells [270]. Furthermore, reduction in OGT causes accumulation of cells in G1 phase [270] [Figure 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 [278, 279] 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 [270].

OGT knockdown studies also implicate O-GlcNAcylation in breast cancer metastasis via E-Cadherin/catenin complex [280]. E-cadherin is pivotal for cell-cell adhesion, which is mediated by its interaction with β-catenin and p120 [281-283]. OGT silencing in 4T1 breast cancer cells causes an elevation in E-Cadherin and β-catenin protein expression while p120 remains unaltered [280]. In murine 4T1 cells which recapitulate human breast cancer phenotype, only p120 and β-catenin are O-

GlcNAcylated [280] unlike E-Cadherin that is found O-GlcNAcylated in several other breast cancer cell lines [284]. 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 [280]. Interestingly, OGT and E-cadherin double knockdown of cells cannot inhibit cell migration as efficiently as OGT single knockdown in the 4T1 cells [280]. O-GlcNAc modification of E-cadherin by endoplasmic stress inducing agents block cell surface transport and cell adhesion capacity [284]. Given that loss of E-cadherin is associated with breast cancer transformation and metastases [285, 286], 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 [271]. 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 [271]. 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 [271]. FOXM1 is shown to play a role in angiogenesis by the regulation of VEGF in several cancers [287-289]. 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 [271]. OGT regulates FOXM1 expression via protesosomal

degradation and a non-degradable FOXM1 can rescue the angiogenic potential of shOGT expressing PC3-ML cells [271]. 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 [290, 291]. PC3-ML cells expressing shOGT have decreased ability to invade as observed by matrigel transwell assays [271]. Additionally, these cells have a significant reduction in their MMP2 and MMP9 mRNA and protein expression when compared to control PC3-ML cells [271]. 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 [271] 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 [292]. c-Myc, a proto-oncogene, is O-GlcNAcylated at T58 in its N-terminal transactivation domain [16, 293]. 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 [294]. O-GlcNAc modification of β-catenin in normal cells is higher than in cancer cell lines like LNCap [295]. 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 [295]. 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 [296]. Cancerous cells exhibit the "Warburg effect" whereby the cells display significantly increased glucose consumption and aerobic glycolysis [297, 298]. 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 [299]. CLL patient samples display higher O-GlcNAc levels when immunoblotted with RL2 antibody in comparison to peripheral blood mononuclear cells (PBMCs) [300]. Targets of OGT like p53, c-Myc, Akt and OGT itself are O-GlcNAcylated in CLL patients [300]. Employing OGT inhibitor strategies, it is evident that Akt T308 phosphorylation is increased in CLL when O-GlcNAc is decreased [300]. Conversely, elevation of O-GlcNAc levels by addition of uridine and GlcNAc attenuates Akt T308 phosphorylation and decreases its activity [18, 300]. Increasing O-GlcNAc levels in CLL patient cells impairs c-Jun N-terminal kinase (JNK) phosphorylation thereby affecting IκB phosphorylation [300]. This defective phosphorylation of JNK is observed in normal B cells, as well as CLL when incubated overnight with uridine and glucosamine [300]. Elevated O-GlcNAc levels affect JNK signaling to retard cell division and activation signals possibly describing the observed RL2 index of less severe CLL [300]. 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 [300]. 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 [301] and is stabilized by O-GlcNAc modification [302]. p53 loss of function is associated with an increase in glycolysis [303] via IKK-NF-κB pathway [304]. 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 [267]. p53 deficient MEFs display higher O-GlcNAcylated IKKβ [267]. Additional studies confirm that p65-NF-κB is necessary for $p53^{-/-}$ mediated enhanced glycolysis [304]. Moreover, p65-NF-κB knockdown in $p53^{-/-}$ MEFs leads to decreased O-GlcNAcylated IKKβ and activating phosphorylation of IKKβ [267]. 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 [267]. O-GlcNAc on S733 is important for enhanced glycolysis as mutating the serine to a glutamate or alanine both lead to lower glucose consumption [267]. TNFα stimulation of p53 deficient MEFs activates IKKβ and NF-κB in comparison to WT MEFs [267]. 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 [305]. Elevated O-GlcNAc levels also lead to reduction in the activity of

phosphofructokinase 1 (PFK1) activity [305], serving as a major player in and regulating the flux through glycolysis [306]. 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 [305] at the residue S529 [305]. S529 is the highly conserved residue on PFK1 that allows for allosteric regulation by fructose 2,6- bisphosphate (F-2,6 BP) [307]. 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 [305]. 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 [305]. No change in either glycolysis or lactate production is observable in S529A PFK knock in under OGT overexpression [305]. Inhibiting flux through glycolysis can shift the levels of pentose phosphate pathway (PPP) [305]. OGT overexpression increases PPP flux in WT PFK1 knock in demonstrating the deregulation of glycolysis [305]. Consistent with PPP flux, NADPH and reduced glutathione (GSH) are increased in WT PFK1 knock in cells with OGT overexpression under hypoxia [305]. S529A knock in cells demonstrate significantly lower levels of NADPH and GSH suggesting that blocking glycosylation may potentially restore glycolysis [305]. Immunocompromised mice injected with WT PFK1 knock in cells with OGT overexpression display more tumorous growths while the S529A mice exhibit smaller tumors [305]. PFK1 O-GlcNAcylation at S529 is required for enhanced tumor growth and this can be exploited for therapeutics against cancerous cells.

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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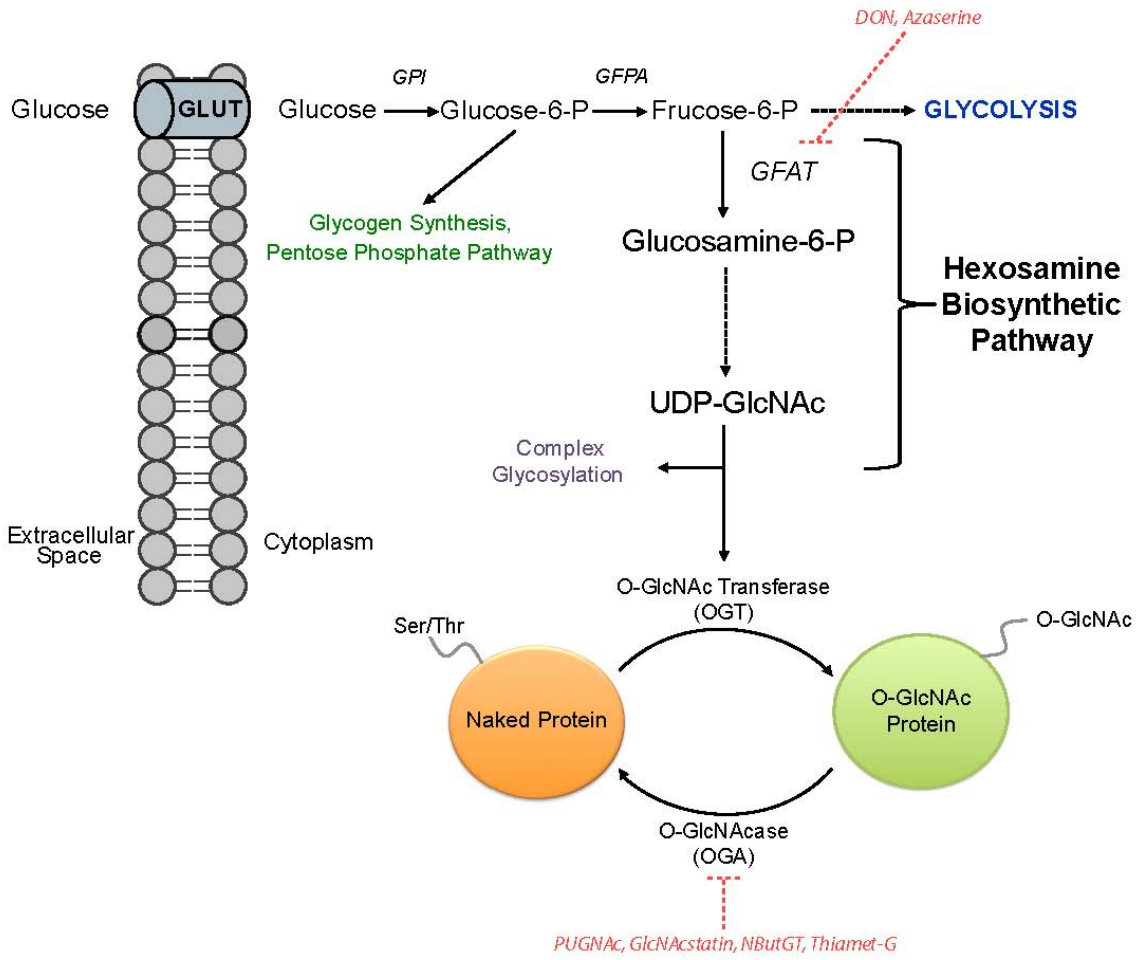
Figure 2.1: The HBP and the O-GlcNAc Modification. The majority of glucose entering the cell is used in glycolysis, glycogen synthesis or the pentose phosphate pathway. However, a small portion is shunted into the HBP, whose end product is the nucleotide sugar donor UDP-GlcNAc. UDP-GlcNAc serves as a 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.


DON, Azaserine
Glucose
GLUT
Glucose
GPI
Glucose-6-P
GFPA
Frucose-6-P
GLYCOLYSIS
GFAT
Glycogen Synthesis,
Pentose Phosphate Pathway
Glucosamine-6-P
Hexosamine
Biosynthetic
Pathway
UDP-GlcNAc
Complex
Glycosylation
Extracellular
Space
Cytoplasm
O-GlcNAc Transferase
(OGT)
Ser/Thr
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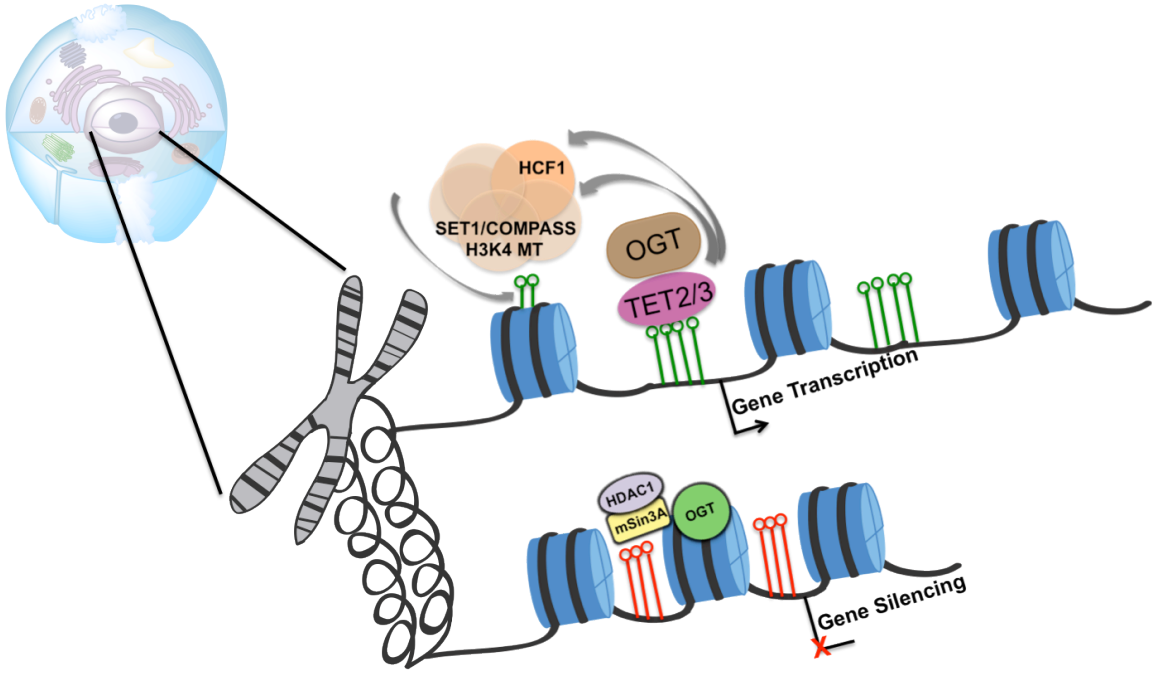


Figure 2.2: OGT associates with chromatin remodeling complexes. OGT associates with both transcriptional coactivator and corepressor complexes. OGT association with TET2/3 is necessary for the chromatin binding event of SETD1A methyl transferase. This facilitates the transcription of hematopoietic genes possibly in a HCF-1 dependent manner. OGT can also interact with mSin3A along with HDAC1 to functionally repress transcription including Sp1 activated genes.

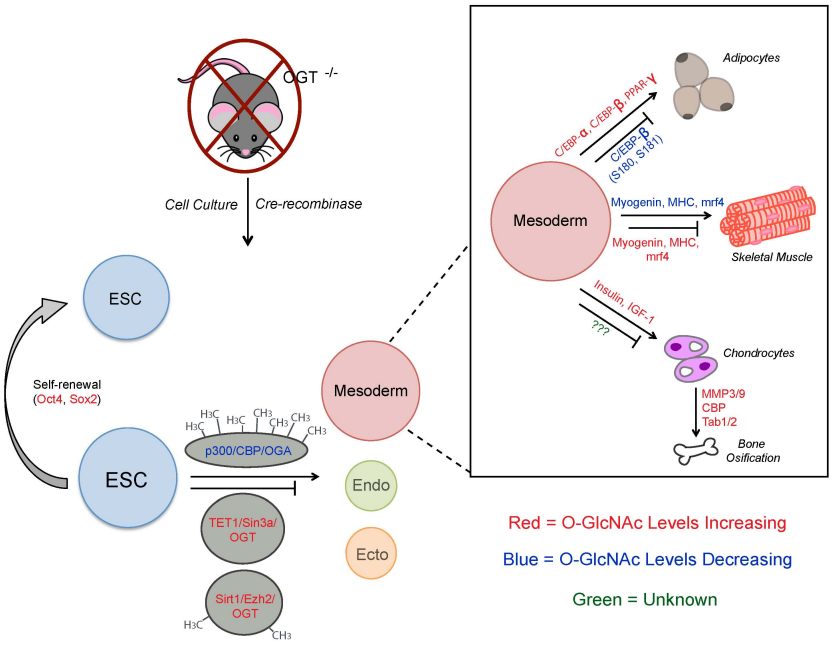


Figure 2.3: O-GlcNAc levels regulate ESC characteristics and mesoderm differentiation. Complete OGT gene knockout is embryonic lethal, but studies in cell culture or the *Cre*-recombinase system enables O-GlcNAc investigation during differentiation and development. O-GlcNAc appears to influence ESC self-renewal 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.

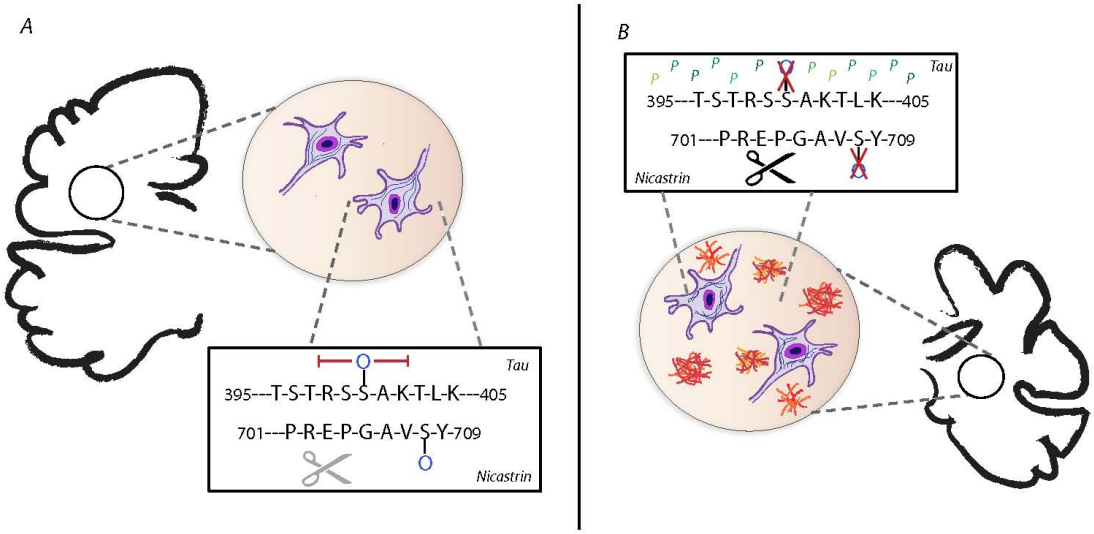


Figure 2.4: O-GlcNAc protects against symptoms of neurodegeneration in the Alzheimer's brain. (A) The microtubule-associated protein Tau can be O-GlcNAc modified at Ser400 and inhibit its subsequent hyperphosphorylation in Alzheimer's brain samples and models. The nicastrin subunit of the γ secretase 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 myocardial ischemia limit oxidative stress through a mitochondrial VDAC-1 mechanism. It has been suggested that 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.

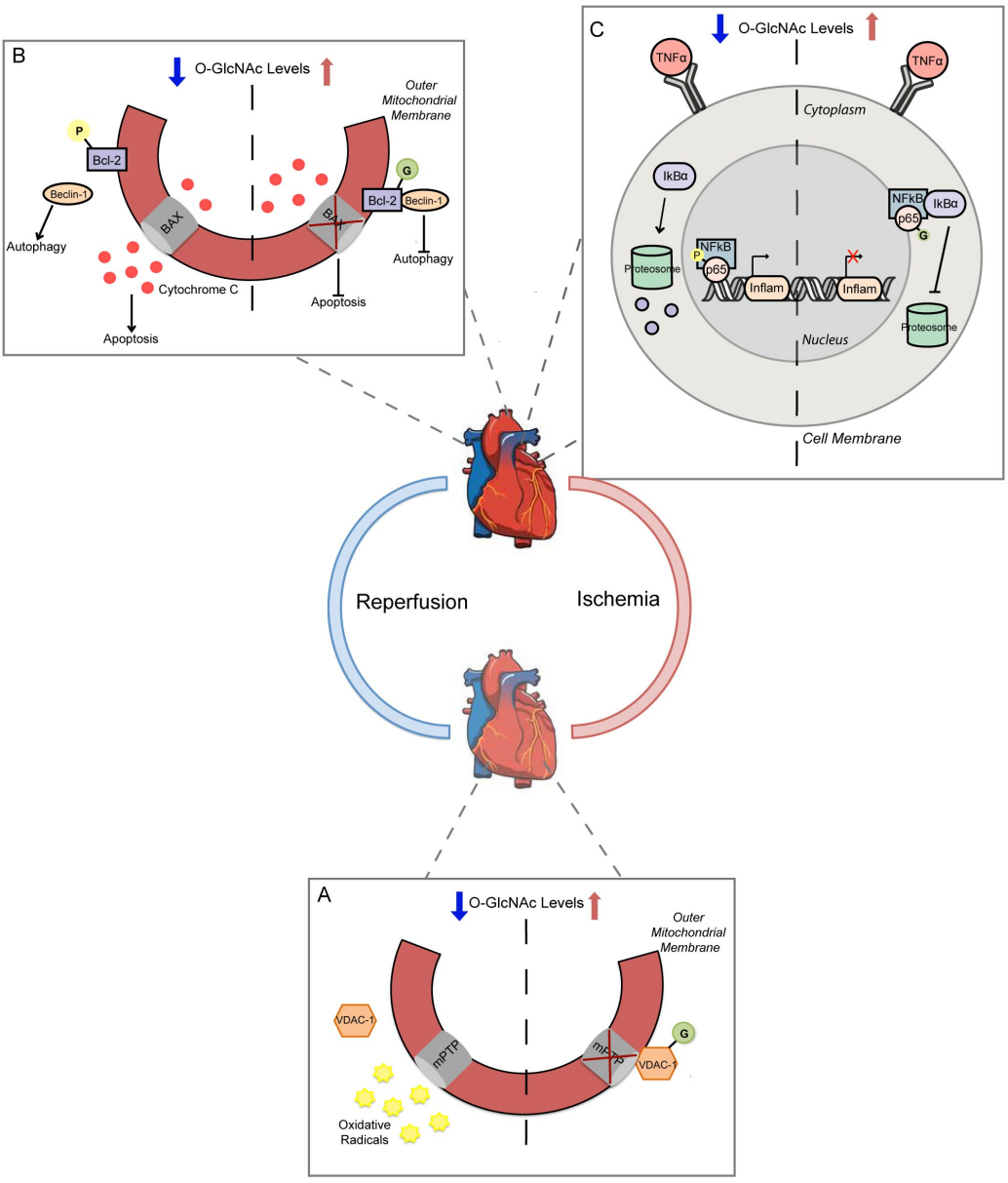
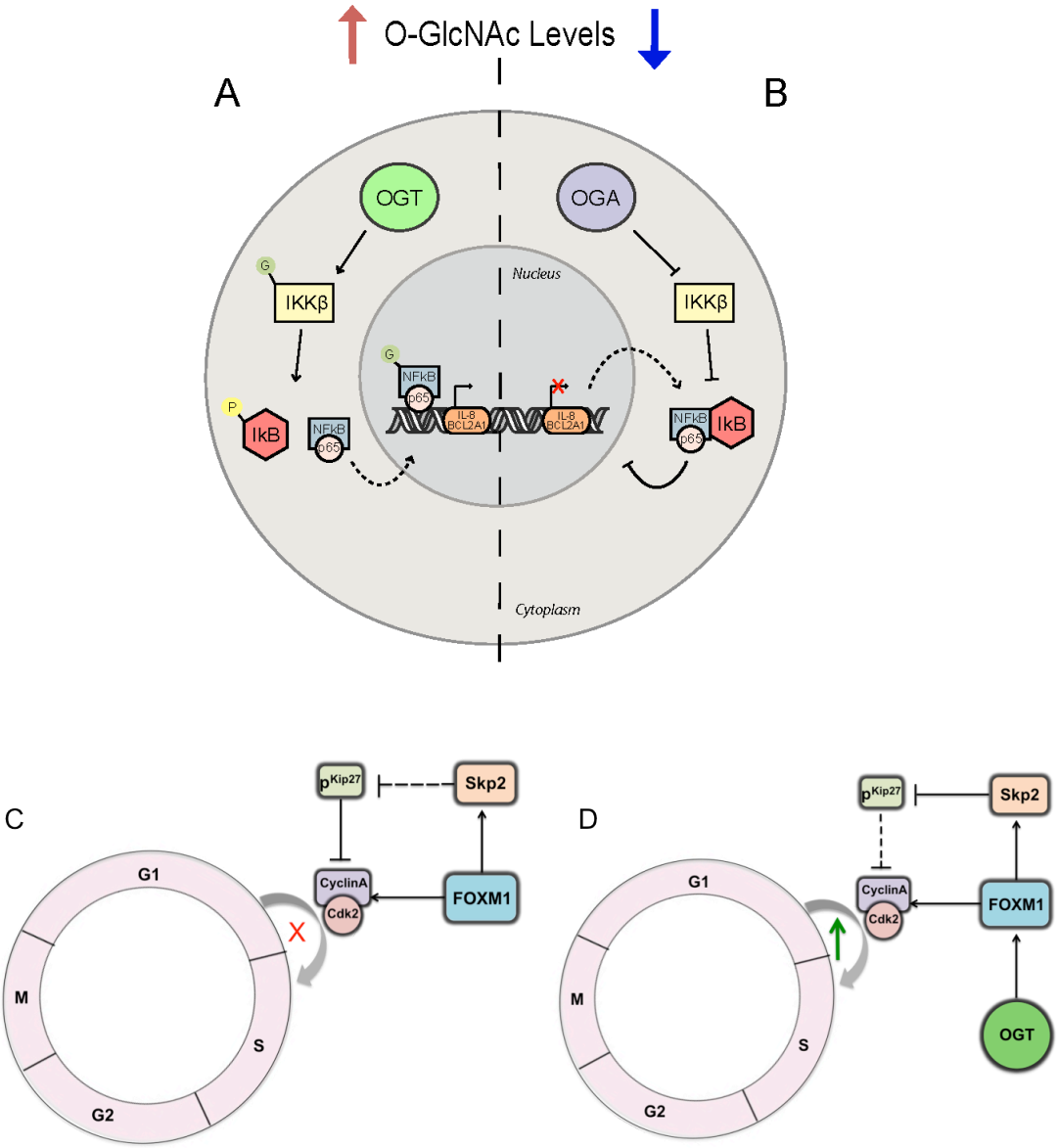

B
O-GlcNAc Levels
Outer Mitochondrial Membrane
P
Bcl-2
Beclin-1
Autophagy
BAX
BAX
Bcl-2
G
Beclin-1
Autophagy
Cytochrome C
Apoptosis
Apoptosis
C
O-GlcNAc Levels
TNFα
TNFα
Cytoplasm
IkBα
NFkB
p65
IkBα
G
Proteosome
NFkB
P
p65
Inflam
Inflam
Proteosome
Nucleus
Cell Membrane
Reperfusion
Ischemia
A
O-GlcNAc Levels
Outer Mitochondrial Membrane
VDAC-1
mPTP
mPTP
G
VDAC-1
Oxidative Radicals

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.


O-GlcNAc Levels
A
B
OGT
OGA
IKKβ
IKKβ
Nucleus
NFkB
p65
IL-8
BCL2A1
IkB
Cytoplasm
C
D
pKip27
Skp2
CyclinA
Cdk2
FOXM1
G1
S
G2
M
OGT

CHAPTER 3: IDENTIFICATION AND CHARACTERIZATION OF A MISSENSE MUTATION IN O-GLCNAC TRANSFERASE THAT SEGREGATES WITH DISEASE IN A FAMILY WITH X-LINKED INTELLECTUAL DISABILITY

2

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To be submitted to AJHG

1. Abstract

Beta-N-acetylglucosamine (O-GlcNAc) is a dynamic post-translational modification that is covalently attached to serines and threonines of nuclear and cytoplasmic proteins and has been recently implicated in epigenetic regulation. Single genes encode enzymes for its attachment [O-GlcNAc transferase (OGT)] and removal [O-GlcNAcase (OGA)]. An X-chromosome exome screen identified a missense mutation in the tetratricopeptide repeat (TPR) region [762G>T (p.L254F)] of OGT that segregates with X-linked intellectual disability (XLID) in a family. Patients exhibit hypospadia, clinodactyly, short stature, microcephaly, and ID. A decrease in steady-state OGT protein levels was observed in isolated lymphoblastoid cell lines from two patient samples harboring L254F-OGT, compared to a carrier or control males from the same family. We have demonstrated that this decrease is in part due to the shorter half-life of the L254F-OGT. Surprisingly, steady-state global O-GlcNAc levels remained grossly unaffected. The same samples, showed a decrease in steady-state OGA levels, implying that a compensation mechanism exists, albeit imperfect given the phenotype of the patients, for maintaining global O-GlcNAc levels. L254F-OGT patient samples also showed a decrease in OGA mRNA levels and luciferase reporter expression, suggesting that OGT regulates the transcription of OGA. We observed an enrichment of OGT at the proximal promoter region of OGA and are currently examining the mechanism of the OGA gene repression. In parallel, we have performed global transcriptome analysis of L254F-OGT lymphoblasts compared to WT that demonstrates several changes in gene expression that are specific to the disease. Future studies will focus on the generation of induced pluripotent stem (iPS) cells from affected patients and unaffected relatives in order to

explore the imperfect compensatory mechanism in cell type specific contexts, with a focus on neural lineages due to the specific phenotypes observed.

2. Significance

O-GlcNAc and the cycling enzymes have been studied in extensive detail for their role in several human diseases [1-4]. However, this study is the first example of a mutation in OGT segregating with a disease. We demonstrate in this study that the mutation produces an active unstable but OGT enzyme. Surprisingly, despite lowered OGT protein levels, global O-GlcNAc levels remain unaltered in both patients and controls. However, we observed a significant decrease in the steady state levels of the other cycling enzyme OGA. This suggests that a compensation mechanism is in play to maintain global O-GlcNAc levels. The observed decrease is due to the regulation of the OGA gene expression. Additionally, we also examined the global transcriptome regulation of the mutant OGT and observed several disease specific changes in gene expression when compared to the WT.

3. Introduction

1-3% of the world population is affected by Intellectual disability (ID) [5-7]. ID is the leading problem of socio-economic health care in Western countries as per the Centers for Disease Control and Prevention [8, 9] owing to the lifetime support required by the affected individuals. ID is characterized by an intelligent quotient (IQ) of 70 or lower. In addition to ID, affected individuals exhibit two or more behavioral deficits in terms of social, conceptual or practical adaptation [7, 10]. A variety of factors cause ID and can range from malnutrition during pregnancy, fetal alcohol syndrome to chromosomal abnormalities including deletions, aneuploidies to monogenic mutations

[11]. Recently, submicroscopic deletions, duplications and missense mutations have been the focus in the field that is primarily due the advent of the human genome [12] as well as the subsequent explosion in sequencing technologies. Monogenic causes have been mainly attributed to genes found on the X-chromosome.5-10% of ID in males is inherited in an X-linked pattern [13] and epidemiological studies consistently show a 30-50% excess of males over females diagnosed with ID. Mutations in at least 102 genes result in 81 of the known 160 XLID syndromes to date [13].

Proteins can be modified with O-linked β-N-acetylglucosamine (O-GlcNAc) at their terminal hydroxyl groups of serines or threonines in order to influence various intracellular processes [14-19]. O-GlcNAc modification is dynamic and inducible in that it is a single moiety addition cycling on and off intracellular proteins in response to the cellular environment [20-22]. This property makes O-GlcNAc modification more akin to phosphorylation than classical glycosylation. In mammals, a single gene encodes for the enzymes responsible for the addition and removal of O-GlcNAc, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), respectively [23-25]. The sugar nucleotide UDP-GlcNAc, which is the end product of the hexosamine biosynthetic pathway (HBP), is the donor substrate for OGT [26, 27]. The O-GlcNAc modification has been observed on over two thousand proteins involved in various processes in the cell [3], implicating O-GlcNAc in the etiology of several diseases [1, 28-30].

OGT is encoded as a single gene and maps to chromosome Xq13.1 [31]. OGT was cloned and characterized in 1997 [23, 32, 33] and is shown to encompass a C-terminal catalytic domain [23, 32] and an N-terminal tetratricopeptide repeat (TPR) domain that varies in length [33, 34]. Mammalian *ogt* knockouts are embryonic lethal,

demonstrating its requirement for cell survival [31]. A consensus sequence for OGT substrate specificity remains elusive; however, protein-protein interactions, splice variants, PTMs, substrate localization and availability [29, 35] have been proposed to regulate OGT activity. OGA, the enzyme that removes the O-GlcNAc modification, is ubiquitously expressed in all tissues [24, 36]. OGA is encoded as a single gene *mgea5* located on chromosome 10q24.1-24.3 [37]. OGA has a catalytic N-terminal O-GlcNAcase domain and a C-terminal domain with low sequence identity to histone acetyltransferase (HAT) domains, which are linked by a region containing a caspase 3 cleavage site that is processed during apoptosis [38, 39]. Current evidence has convincingly demonstrated that OGA lacks the previously proposed HAT activity [40]. OGA is essential for development and *Oga* homozygous null mice are perinatal lethal [41]. Regulation of OGA has remained elusive, but a few studies have implicated a regulatory role via its presence in various protein complexes [42-44]. Of the two cycling enzymes, OGA has been described in less detail in the literature. Our data suggests a role for the regulation of OGA by OGT in the setting of XLID.

In this study, we have identified a missense mutation in OGT (L254F) that segregates with disease in a family with XLID. This mutation occurs in the TPR domain and results in an unstable but active protein. Reduced OGA protein levels compensate for lowered OGT protein levels in the patients. The function of this compensatory mechanism is likely to maintain global O-GlcNAc levels. We have also demonstrated that OGA protein levels are decreased by means of transcriptional regulation, as both OGA mRNA and luciferase reporter expression were decreased in XLID lymphoblastoid cells. OGT, in addition to mSin3A and HDAC1, are enriched at the OGA promoter in XLID.

Together, these results suggest that L254F-OGT regulates OGA gene expression in XLID lymphoblastoids perhaps in a co-repressor dependent fashion. In parallel, RNA sequencing has revealed a variety of genes that are regulated in a disease specific context, including several histones and other transcriptional regulators. Our findings support the role of OGT as a transcriptional regulator in multiple settings in the cell.. Specifically, our work suggests a role for OGT in epigenetic regulation of XLID.

Results

A single nucleotide polymorphism in O-GlcNAc Transferase segregates with disease in a family with XLID. An X-chromosome exome sequencing screen identified a family with a missense mutation in OGT gene at 762 G>T (p.L254F) (Figure 3.1). Three family members displayed ID. Two of them also exhibited small head circumference, 5th finger clinodactyly and hypospadias (Table 3.1). Lymphocytes from patients and controls were isolated for further studies and immortalized to create lymphoblastoids as described in the materials and Methods section. Bioinformatic analyses using various servers (listed in Table 3.2) predicted an unstable protein as a result of the mutation. Two other mutations in OGT have been identified in two separate families. These mutations are E339G that also occurs in the 9th TPR and T560A that occurs in the catalytic pocket. The T560A mutation is previously described to be involved in the UDP-GlcNAc binding [128, 402] and ablation of the residue reduced activity of the enzyme towards a protein substrate [128]. This further strengthens the proposed causal role of OGT in XLID.

OGT protein is unstable but active in XLID lymphoblastoids. Since the L254F-OGT protein is predicted to be unstable, we examined steady state levels of OGT in both

patient and control lymphoblastoids. Immunoblotting analyses of XLID patients (P1 and P2) exhibited significantly lower OGT steady state levels of patient lymphoblastoids by at least 50% as demonstrated by densitometry (Figure 3.2A and B) compared to heterozygous female carrier (CA) as well as unaffected WT males (C1, C2 and C3). We also compared lymphoblastoid OGT levels from the patients against an unrelated WT male and observed a similar difference in OGT levels (data not shown). We used 4 different OGT specific antibodies (AL28, DM17, H300 and anti-OGT from PTGlabs) to eliminate antibody specific artifacts (Figure 3.2A, B). We also used two different loading controls, β-Actin and α- tubulin, as they are both O-GlcNAc modified [45-47]. When recombinant L254F-OGT is exogenously introduced into HEK293T cells, it is expressed and capable of modifying nucleocytoplasmic proteins *in vivo* and is comparable to WT OGT overexpression (Figure 3.3A). Immunoprecipitation and *in vitro* activity assays of both HA-tagged L254F-OGT and WT OGT demonstrated an active mutant enzyme (Figure 3.3B) capable of transferring radioactive UDP-GlcNAc to an acceptor peptide substrate. The decrease in OGT half-life is mediated by faster protein turnover as observed in XLID patient P1 when compared to controls C1 (Fig 3.4A). Comparison of C2 and P2 showed a similar trend (data not shown). The half-life of WT OGT in normal control C1 was in the range of 12-14 hours (n=2) compared to the L254F-OGT in the patient P1 whose half-life was in the range of 5.5-7 hours (n=2). (Figure 3.4B) following the same trend in two biological replicates. β-Actin was used as the loading control due to its extended half life of 2-3 days [48].

Global O-GlcNAc levels remain unaltered in XLID lymphoblastoids despite reduced OGT protein levels. Considering that steady state levels of OGT are decreased

in XLID patient lymphoblastoids, we expected to observe a global decrease in O-GlcNAc levels. Surprisingly, global O-GlcNAc levels remained unaltered in patients (P1 and P2) when compared to both female carrier (CA) as well as unaffected male relatives (Figure 3.5). We also compared the patients against an unrelated WT male and observed no alteration in global O-GlcNAc levels (data not shown). We probed for O-GlcNAc by using anti O-GlcNAc antibodies 110.6 and mAb10 antibodies. We observed similar results using mAb3 and RL2 antibodies (data not shown).

Steady state OGA protein levels are decreased in XLID patient lymphoblastoids. As a result of global O-GlcNAc levels persisting at normal levels, we examined the other cycling enzyme of this dynamic modification. Interestingly, OGA protein levels were diminished in XLID patients (P1 and P2) when compared to a female carrier (CA) and unaffected male relatives (Figure 3.6). Densitometric analysis revealed that the XLID afflicted patients had at least 90% reduction in their OGA protein levels (Figure 3.6). We also compared the patients against an unrelated WT male and observed that the reduced patient OGA levels were recapitulated similar to other WT controls (data not shown). Thus, we have uncovered a compensation mechanism that is in play in the XLID lymphoblastoids. This mechanism might be in place to maintain steady state global O-GlcNAc levels in the cell; however, it is imperfect owing to the clinical phenotype of the XLID patients.

OGA mRNA and promoter luciferase expression are decreased in XLID lymphoblastoids. OGT has been implicated in the transcriptional regulation of several genes and is known to modify RNA Pol II as well as exist in the pre-initiation complex [49, 50]. To investigate whether the decrease in OGA steady state levels was due to a

decrease in transcription, we performed quantitative RT-PCR to assess mRNA levels of OGA.. XLID patient lymphoblastoids exhibited a significant decrease in OGA mRNA (Figure 3.7A). We also performed cycloheximide half-life assessment and ruled out the fact that lowered OGA protein levels maybe due to protein turnover (data not shown), as there was no change in protein stability during the 24 hour treatment. To further strengthen our hypothesis that OGA is transcriptionally down regulated, we transfected a 2kb proximal promoter region of OGA tagged to a luciferase reporter into XLID and control lymphoblastoids. After 48 hours of transfection, we observed that there was significantly lower expression of the promoter in the XLID lymphoblastoids when compared to the controls (Figure 3.7B). Together with the decreased mRNA levels and unaltered protein turnover rates, this led us to hypothesize that OGA is transcriptionally regulated by OGT.

OGT protein is enriched at the OGA promoter in XLID. OGA down regulation in XLID in a transcriptional dependent manner led us to further probe the OGA promoter region. ChIP was performed using OGT and O-GlcNAc specific antibodies. Enrichment at the promoter using qPCR with primers designed to amplify the proximal promoter region of OGA. OGT was significantly enriched while O-GlcNAc displayed a similar trend of enrichment at the XLID OGA promoter (Figure 3.8A and B). These results suggest that while there is less OGT protein, there is more enrichment at the OGA promoter in XLID lymphoblastoids. It has been established previously that OGT exists in co-repressor complexes that down regulate gene expression [51]. This led us to hypothesize that OGT might exist in a similar co-repressor complex at the OGA promoter. We then tested for mSin3A and HDAC1 at the OGA promoter by performing ChIP and using the same

proximal promoter primers as described above. We observed an increase in both HDAC1 and mSin3A at the OGA promoter in XLID compared to controls (Figure 3.8Cand D). The study mentioned previously also demonstrated that both mSin3A and HDAC1 are substrates of OGT [51]. Probing into the activity of HDAC1 and its O-GlcNAc modified status, we can assess whether this result is a direct dependence on O-GlcNAc or OGT.

Global transcriptome analysis reveals segregation of a subset of genes by disease. Due to the previously fortified role of OGT in transcription and our combined knowledge of the regulation of OGA being down regulated in XLID, we elected to perform an Illumina Hiseq 2000 RNA sequencing. We chose to compare two related unaffected males (C1 and C2) to the patients (P1 and P2) afflicted with XLID. Using the R software to perform Spearman correlation [52], we demonstrated that the transcripts segregate perfectly with the disease and not by generation (Fig 3.9 A). The patient, P1 is the nephew of controls, C1 and C2 as well as the other patient P2. A plethora of genes involved in various cellular processes exhibited differential expression pattern in the patients compared to the controls. Following the stringency applied to our dataset described in the Methods section, we were able to quantify 8800 genes. When we applied fold filters, there were 349 genes that were differentially expressed 2-fold, 89 genes that changed 3-fold and 38 genes that changed 4-fold. When we compared the "mock" set to the real data, we observed a 1.3 fold enrichment of disease over natural variation in the human subjects. Of note, there was a 3.9-fold enrichment of disease in the 3-fold group of genes. In total, we saw 1.01% of genes being differentially regulated in the patients versus the controls in the 3-fold group (Figure 3.9B). Of the 89 genes in

the 3-fold group, 67% of the genes were upregulated in disease while 33% were downregulated (Figure 3.9 C). Panther Gene list analysis software was used to assign gene ontology based grouping [53]. Together this data in conjunction with its previously described role in transcriptional regulation under various settings [49, 54, 55] strongly suggests that OGT influences the expression of a subset of genes in the XLID cells. In particular, sin-3A associated protein 25 kd (SAP25) was observed to be increased in the patients compared to the controls. SAP25 is shown to be an integral member of the Sin3A repressor complex [56] that is involved in gene repression in an mSin3A- dependent mechanism. Current studies are aimed at identifying the entire repressor complex present on the OGA promoter as well as using the XLID lymphoblastoids as a model to study the OGA promoter region. We have orthogonally validated HIST14B (histone, H4) to be upregulated and HIST1H3A (histone, H3.1) to be downregulated in the XLID samples as seen in the transcriptome data. Histones are posttranslationally modified to confer epigenetic marks on the genome [57, 58]. Further investigation will shed light on the H3- and H4- epigenetic marks and their potential role in XLID and regulation of OGA gene transcription.

5. Discussion

While both XLID and O-GlcNAc have been studied for several decades, we have for the first time observed a mutation in OGT that segregates with XLID. O-GlcNAc modification of almost two thousand proteins impacts several crucial cellular processes. A novel SNP has been identified in OGT by a focused X-chromosome exome sequencing in XLID patients. The observation that a missense mutation in OGT segregates with disease is the first example of its kind illuminating an important functional role for OGT. In

addition to the novel missense mutation in OGT, an alteration was observed in another gene, *PRICKLE3,* which is in the linkage region. However, this gene is not expressed in brain and therefore eliminated as having any direct effect.

This study has demonstrated for the first time that the half-life of OGT, both WT as well as the L254F-OGT is implicated in XLID. We demonstrated that OGT steady state protein levels are decreased in the patient lymphoblastoids as a result of lowered half-life of the L254F-OGT when compared to WT. This data validates the bioinformatic predictions for the mutant version of OGT. Complete loss of OGT is lethal in mammals [31]. Therefore, we proceeded with the knowledge that OGT would be active in XLID cells and validated that L254F-OGT is active and can transfer O-GlcNAc moieties to both protein substrates as well as synthetic peptide substrates just as well as the WT. Surprisingly, global O-GlcNAc levels remained unaltered in XLID lymphoblastoids compared to WT. This led us to evaluate the other cycling enzyme of the O-GlcNAc modification, OGA. Steady state OGA protein levels were lowered in XLID leading to the observation that a compensation mechanism exists in the XLID lymphoblastoids. This result suggests that there is a requirement for maintenance of global O-GlcNAc levels despite the disease state in lymphoblastoids. Further evaluation is required to elucidate if O-GlcNAc levels are altered in the brains of XLID patients.

Reduction of OGA protein levels in XLID lymphoblastoids was due to a decrease in OGA transcription as seen by reduced transcript levels in XLID compared to WT. To further validate this finding, we performed promoter luciferase reporter assays, which further validate reduced transcription of OGA mRNA in XLID. Anomalous O-GlcNAc levels have been sustained in diseased states by elevated OGT expression and

decreased OGA expression [59]. The compensation of OGA may be a cell type specific effect and further exploration into patient neuronal lineages could demonstrate if this is recapitulated in the neurons which could address the phenotypes of the XLID afflicted patients.

Fragile X syndrome (FXS) is characterized by the CGG repeats in the promoter of the fragile mental retardation 1 (FMR1) leading to hypermethylation and subsequent gene silencing [60]. In addition to DNA methylation like in the case of FXS, histone modifications and chromosome remodeling are also observed in XLID [61, 62]. Specifically, mutations in α-thalassemia X-linked mental retardation syndrome (ATRX), a chromatin remodeling protein, are linked to methylation of repetitive DNA sequences causing gene repression [63]. Mutations in methyl CpG binding protein 2 (MeCP2) cause histone H4 hyperacetylation indicative of gene activation which may lead to over expression of target genes involved in the pathogenesis of Rett syndrome, that affects females [64]. In a similar fashion, we have observed enrichment of OGT, mSin3A and HDAC1 at the OGA promoter. This complex has previously been described to regulate gene repression [51]. This effect may exist dynamically in normal cells but is captured at a steady state level due to the mutation in OGT for the first time. Furthermore, global histone acetylation marks and methylation marks would provide supporting evidence for our model (Figure 3.10) that OGT transcriptionally regulates OGA by tethering a co-repressor complex at the OGA promoter.

O-GlcNAc has joined the ‘histone code’ and has been demonstrated to modify the core histones [65-67]. O-GlcNAc modification of the histones is shown to add further complexity to the epigenetic landscape by occurring at both transcriptional activation

and repression sites [65, 67]. Both histones H3 and H4 are involved in nucleosome assembly and transcriptional regulation. Chromatin remodeling is a complex process involving several known PTMs like acetylation, methylation, ubiquitination and phosphorylation [68-70]. Histone marks or PTMs can be both active and repressive [71]. In addition to the validation of our RNA sequencing data, analyzing the H3- and H-4 repressive and activation marks respectively in the setting of XLID is necessary to delineate the mechanism of chromatin remodeling involved in XLID patients with the L254F-OGT. Deep sequencing analysis has opened up a number of target genes to study to describe XLID in this family. More importantly, there is a subset of genes that are preferentially regulated with disease as observed in the spearman correlation analysis. Both up and down regulated genes are involved in similar biological processes

For the first time in the literature, this study implicates the involvement of a missense mutation in OGT that segregates with a disease. Our results suggest that the mutant, unstable but active OGT regulates OGA in a transcription dependent manner by recruiting a co-repressor complex. Furthermore, regulation of the OGA promoter has not been described previously in the literature. Future studies can describe the impact of this regulation under different biological settings. More specifically in the context of XLID, a relevant cell line like neuronal cells can be explored by using iPSC technology to create neuronal lineages from patient fibroblasts.

6. Material and Methods

Materials and Reagents. Tissue culture media, serum, and antibiotics were purchased from Gibco. O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenyl carbamate (PUGNAc) was purchased from Toronto Research Chemicals Inc. Anti O-

GlcNAc (CTD 110.6) was previously generated in Dr. Gerald W. Hart's Laboratory (JohnsHopkins University). Anti O-GlcNAc (RL2) antibody was from Enzo Life Sciences. Protein A/G beads, normal sera, and agarose conjugated beads were purchased from Santa Cruz Biotechnology. Anti OGT (DM17) antibody was from Sigma Aldrich. Anti OGT (H300) antibody was from Santa Cruz Biotechnology. Anti-OGT (AL28) antibody was previously generated in Dr. Gerald W. Hart's Laboratory (JohnsHopkins University). Anti-OGT (11576-2-AP) was from Proteintech. mAb 10 was generated previously in Dr. Geert Jan Boons' laboratory (CCRC, University of Georgia). Anti-OGA antibody was purchased from Proteintech.

X-chromosome Exome sequencing and analysis. Genetic DNA samples from 82 XLID male samples (30 unrelated, and 52 related [26 sib-pairs]) were used to generate Illumina sequencing library using TruSeq DNA sample Preparation kits. The X chromosome exome was enriched using an Agilent SureSelect X chromosome exome kit. Individual libraries were uniquely barcoded and sequenced on the Illumina HiSeq2000 platform using either 75-bp or 100-bp pair-end read modules. INDEL realignment and base recalibration were conducted using GATK. Unified Genotyper (GATK) was used for variant calling [parameters: -ploidy 1, all else at default]. The final variant output was pre-processed by removing variants with zero coverage from one strand and by removing variants in close proximity (<10 bp) to another variant in the same sample. Such variants are most likely to be erroneous.

Potential XLID variants were identified using a sib-pair comparison filter. Given the rarity of XLID, our filter assumes that unique XLID mutations are rare and shared only between affected family members. Variant output from sequencing was filtered by

retaining only those variants shared between related samples and by removing variants shared between unrelated samples in our cohort. Additionally, variants shared with 162 male samples (unaffected control) from the 1000 Genomes project were removed. This filter effectively reduced the total number of variants from 1774 ± 240 variants per samples to 31 ± 5 variants per sample, easing identification of potential disease-causing mutations.

Bioinformatic analysis. We used Eris protein stability server [72] to predict stability of L254F-OGT. Eris calculates the free energy change affected by a point mutation (ΔΔG).

Creation of Lymphoblastoid cell line. Cell lines were obtained by immortalization of lymphocytes from blood samples using Epstein-Barr virus using standard protocols [73].

Tissue culture, transfection and cycloheximide treatment. Cells were cultured in RPMI 1640 medium containing 15% fetal bovine serum and 1% antibacterial/ antimycotics. Cells were passaged every week and grown in suspension to desired density for assays at 37°C in 10% CO_2.

5ug of L254F-OGT or WT OGT were transfected in HEK293T cells using JetPrime reagent. eGFP transfection was used as a both a transfection control as well as vector control. We followed manufacturers' protocol for transfection.

Cycloheximide was added to cells at a concentration of 50 μM for 0, 1, 2, 4, 8, 16 and 24 hours.

Cell lysis and Immunoblotting. Lymphoblastoids from both patients (P1 and P2) and controls (CA, C1, C2 and C3) lysed using 50mM Tris (pH-8), 150mM NaCl, 1mM EDTA, 1% Tritin-X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1X protease and phosphatase

inhibitors, 10uM PUGNAc and 1mM DTT, a protocol modified from Brumbaugh et al [74]. The nucleocytoplasmic proteins obtained were resolved on SDS-PAGE gel by electrophoresis and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked for 1 hr at room temperature in 1X TBST, 0.2%Tween20 supplemented with 3.7% BSA for O-GlcNAc antibody 110.6. For all other antibodies, 1X TBST, 0.1% Tween20 supplemented with 3% BSA (HA tag, mAb14, mAb 10) or 5% milk (OGT, OGA). OGT and OGA antibodies were purchased from ProteinTech Group lab (Cat# 11576-2-AP and Cat # 14711-1-AP). Following blocking, membranes were incubated overnight at 4°C with the different antibodies. Membranes were washed four times with 1X TBST, 0.1% Tween20 and incubated at room temperature for 1 hr with a secondary horseradish peroxidase-conjugated antibody. Then the membranes were washed four times with 1X TBST supplemented with Tween20. Proteins were detected using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

Site-directed Mutagenesis. To create the recombinant L254F-OGT, we designed primers using the QuikChange Primer Design program available through Agilent Technologies. Mutagenesis was set up using QuickChange Site-Directed Mutagenesis kit (Cat# 200523) as per the manufacturer's protocol. Sanger sequencing was done at Johns Hopkins Synthesis and Sequencing facility to validate the mutant DNA sequence.

OGT activity assay. L254F-OGT and WT OGT were assayed in vitro using a synthetic CK II peptide (H2N-PGGSTPVSSANMM-COOH) substrate. H^3-UDP-GlcNAc at 100,000 CPM was added to each reaction along with 10mM Tris pH-7.5, 2.5ul of CKII peptide, 1ug OGT and 50uM GlcNAc, in that order. The samples were incubated overnight at 37C. The reaction was halted using 1% formic acid. Samples were then cleaned up using C18

reverse phase columns. The eluates from the columns were then counted using scintillation fluid. The data was normalized to OGT protein as observed in an anti-HA western blot.

RNA isolation and Quantitative RT-PCR. Total RNA was extracted from patient and control lymphoblastoids using the Qiagen RNeasy Plus Minikit (Cat# 74134) by following the manufacturer's protocol. cDNA was prepared using the Bio-Rad iScript cDNA synthesis kit (Cat# 170-8890) as per the manufacturer's instructions. The resulting cDNA was used as a template for amplification in a Bio-Rad 96-well MyIQ Single Color Real-Time PCR Detection Instrument using the SYBR protocol. All qPCR primers were purchased from Qiagen Quantitect primer assays and used with the Bio-Rad iQ SYBR Green Supermix (Cat# 170-8880). Changes in gene expression of OGA (QT), OGT (QT), HIST1H4B (QT00207207) and HIST1H3A (QT00246764) were normalized using the RPL4 (QT), GAPDH, CYCG and GUS as the housekeeping genes. Quantification was done using the ΔΔCt method [75].

Reporter Luciferase Assays. Lymphoblastoids from both patients (P1 and P2) were transfected with pGL4.10 luciferase vectors using the Roche X-tremeGENE HP DNA transfection reagent (Cat# 06366244001). The constructs used were a 2 kb proximal promoter region of OGA that was determined using Promoter 2.0 prediction and checked by sequencing. The promoter region was introduced in luciferase vector constructs from Promega. Controls used were the Renilla luciferase (pGL4.74 hRluc/TK) [Promega Cat# E6921], SV40 promoter (pGL4.13 luc2/SV40) [Promega Cat# E6681] and the pGL4.10 luc empty vector [Promega Cat# E6651]. After 48 hours of

transfection, cells were pelleted followed by lysis and detection using Promega Dual-Glo Luciferase Assay System (Cat# E2980) as per the manufacturers protocol.

Chromatin Immunoprecipitation (ChIP). ChIP was performed as previously described [76]. Briefly, DNA and protein were cross-linked using 2% formaldehyde and quenched with glycine. Sonicated DNA extract was precleared using protein A/G agarose beads and mouse or rabbit IgG linked to agarose conjugate. Chromatin from $3x10^6$ cells were used for each immunoprecipitation (IP). Lysates were incubated with anti-OGT (Abcam Cat# 50273) or anti-O-GlcNAc Mab14 antibody at 5 and 3 µg respectively 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 primers to the proximal OGA promoter (Forward 5' aggggaaacagcggaagac 3' and Reverse 5' tgccacctctgcgggt 3'). The primers were designed using the UCSC In-Silico PCR free tool.

RNA sequencing analysis and bioinformatics. RNA from lymphoblastoids from both patients (P1 and P2) and controls (C1 and C2) were extracted using the Qiagen RNeasy Plus Minikit (cat# 74134) by following the manufacturer's protocol. Samples were submitted to Genomics Services Lab (GSL) at Hudson Alpha Institute of Biotechnology, Huntsville, Alabama for PolyA mRNA library preparation and further sequencing and

analysis. 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 samples with a RNA integrity Number (RIN) value of at least 9.5 or higher was used for further processing. From each the 4 samples, 1000ng RNA was used for PolyA mRNA library prep using NEBNext PolyA magnetic Isolation module New England BioLabs Inc., Ipswich, MA, USA) according to manufacturer's protocol. Samples were individually barcoded with unique in-house genomics service lab (GSL) primers and amplified through 10 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 of the prepared mRNA libraries for downstream 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. Cluster generation was carried out on a cBot v1.4.36.0 using the Truseq Paired-end (PE) Cluster Kit v3.0 (Illumina, Inc., San Diego, CA, USA). Paired End (PE) sequencing was performed 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 analysis and base calling was performed using the standard Illumina Pipeline consisting of Real time Analysis (RTA) version v1.13. Raw reads were

demultiplexed using a bcl2fastq conversion software v1.8.3 (Illumina, Inc., San Diego, CA, USA) with default settings.

Post processing of the sequencing reads from RNA-seq experiments from each sample was performed as per our unique in-house pipeline. Briefly, quality control checks on raw sequence data from each sample was performed using FastQC (Babraham Bioinformatics, London, UK). Raw reads were mapped to the reference human genome hg19/GRCh37 using TopHat v1.4 [77, 78] with two mismatches allowed and other default parameters. TopHat is a splice junction mapping tool for RNA-seq reads that utilizes an ultra-fast high-throughput short read aligner Bowtie [77]in the background and then takes the mapping result and identifies the splice junctions. The alignment metrics of the mapped reads was estimated using SAMtools [79]. Aligned reads were then imported onto the commercial data analysis platform, Avadis NGS (Strand Scientifics, CA, USA). After quality inspection, the aligned reads were filtered on the basis of read quality metrics where reads with a base quality score less than 30, alignment score less than 95, and mapping quality less than 40 were removed. Remaining reads were then filtered on the basis of their read statistics, where missing mates, translocated, unaligned and flipped reads were removed. The reads list was then filtered to remove duplicates. To reduce noise from low signal reads, the minimum intensity was set to 8, and reads were removed if neither the average of patient values or control values were above 16. The intent of this filter was to ensure conservative interpretation of fold-change for low signal values. The final list was created by removing reads where the relative standard deviation (%RSD) of the patient values (P1 and P2) or control values (C1 and C2) was greater than 25%. Differential expression of

genes was calculated on the basis of fold change (using thresholds of ±2.0, ±3.0, and ±4.0) observed between defined conditions. To assess data quality, 2 "mock" versions of the final reads list were created by grouping P1 with C1 and P2 with C2 (mock1) and P1 with C2 and P2 with C1 (mock2) and filtering as described above (from noise reduction on).

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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Figure 3.1: Identification of a novel missense mutation in O-GlcNAc transferase by X-chromosome exome sequencing. A. In a focused X-chromosome exome sequencing screen, a missense mutation in OGT was identified at 762G>T by illumina sequencing. B. This nucleotide change occurs in the TPR region and leads to an amino acid change from L254>F. C. 3 patients from a single family exhibit the mutant allele. L stands for males with the WT allele coding for L254 while F stands for the mutant allele that encodes the L254F mutation and L/F stands for heterozygous females.

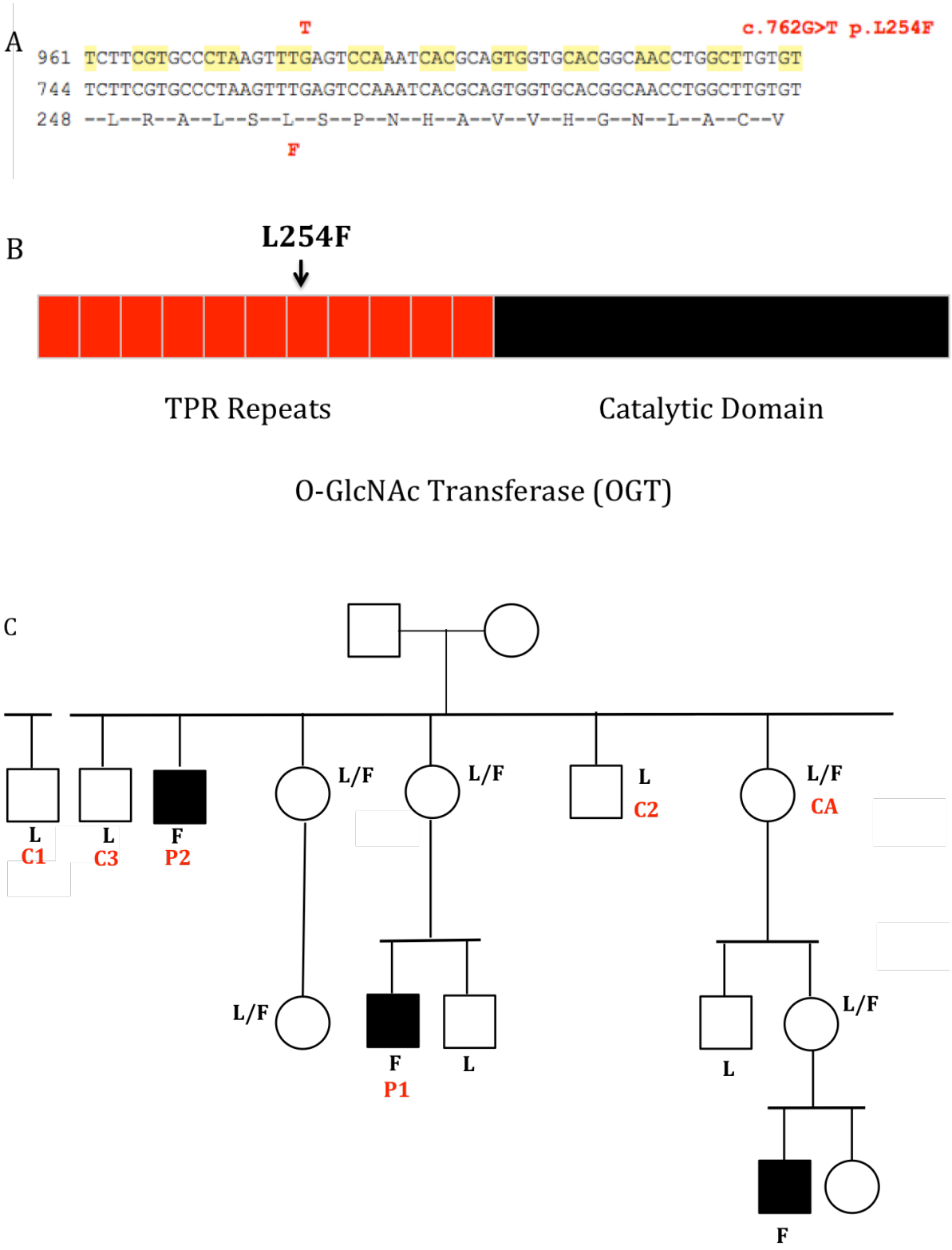

A
T
c.762G>T p.L254F
961 TCTTCGTGCCCTAAGTTTGAGTCCAAATCACGCAGTGGTGCACGGCAACCTGGCTTGTGT
744 TCTTCGTGCCCTAAGTTTGAGTCCAAATCACGCAGTGGTGCACGGCAACCTGGCTTGTGT
248 --L--R--A--L--S--L--S--P--N--H--A--V--V--H--G--N--L--A--C--V
F
B
L254F
TPR Repeats
Catalytic Domain
O-GlcNAc Transferase (OGT)
C
L
C1
L
C3
F
P2
L/F
L/F
L
C2
L/F
CA
L/F
F
P1
L
L/F
L
F

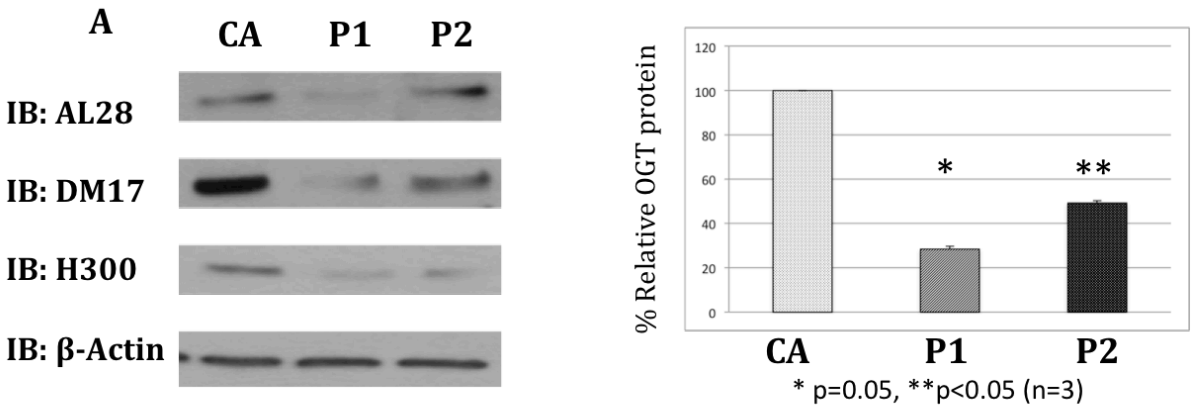
Table 3.1: Phenotypes of the patients used in this study.

Patient	P1	P2
Age	56.5 years	14.75 years
Phenotype	small head cirumference mild 5th finger clinodactyly small testes	small head cirumference 5th finger clinodactyly mild 1st degree hypospadia

Table 3.2: Bioinformatics analyses on L254F-OGT using different serves listed in the table below.

Bioinformatics Server	**L254F**
ipTree [80]	destabilizing
Panther [81]	deleterious
Mustab [82]	decreased stability
MuPro [83]	decrease
I-Mutant [84]	decrease stability
Mutation tasting [85]	disease causing
PolyPhen [86]	probably damaging
Eris Protein Stability Server [7272]	destabilizing

Figure 3.2: Steady state OGT levels are decreased in XLID lymphoblastoids. A. Immunublotting of crude lysates from XLID lymphoblastoids when compared to a control female carrier(CA) using three different anti-OGT antibodies. A representative densitometry graph is shown(n=3, $p \leq 0.05$) using DM-17 antibody. B. Immunublotting of crude lysates from XLID lymphoblastoids when compared to a control normal related males C1, C2 and C3 using anti-OGT (PTGlab). Both β-Actin and α- tubulin serve as the loading controls (n=3)


A
CA
P1
P2
IB: AL28
IB: DM17
IB: H300
IB: β-Actin
% Relative OGT protein
120
100
80
60
40
20
0
*
**
CA
P1
P2
* p=0.05, **p<0.05 (n=3)

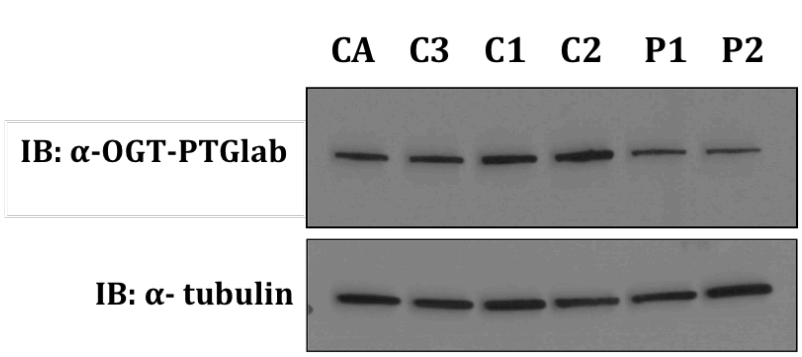
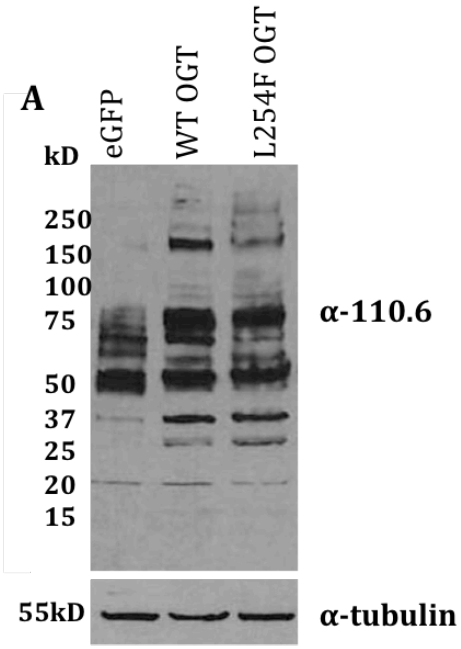

B
CA
C3
C1
C2
P1
P2
IB: α-OGT-PTGlab
IB: α- tubulin

Figure 3.3: L254F-OGT is active towards protein substrates *in vivo* as well as a synthetic peptide *in vitro*. Recombinant L254F-OGT was transiently transfected in HEK293T cells and compared to WT OGT transfected cells. eGFP was used as a mock and transfection control. A. Immunoblotting of crude lysates from L254F-OGT and WT OGT transfected HEK293T cells using anti O-GlcNAc antibody, 110.6, with α- tubulin serving as the loading control. B. Overexpressed L254F-OGT and WT OGT were immunopurified from HEK293T cells using anti-HA antibody. Purified L254F-OGT was assayed for activity using a synthetic CKII peptide and compared to WT OGT activity, which was set to 100%.


A
eGFP
WT OGT
L254F OGT
kD
250
150
100
75
50
37
25
20
15
α-110.6
55kD
α-tubulin

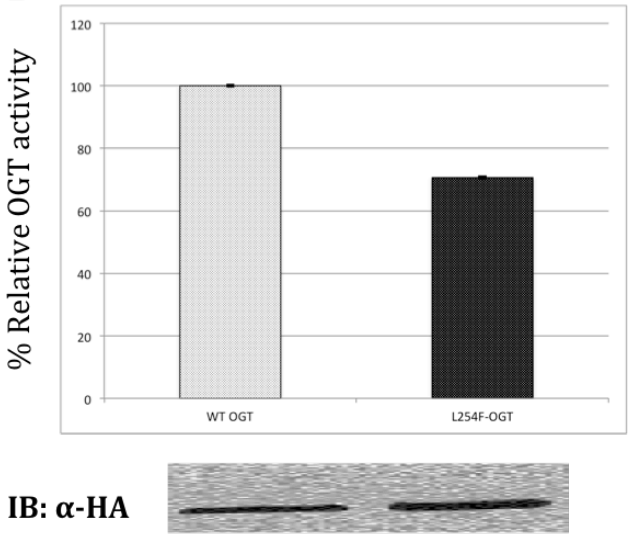
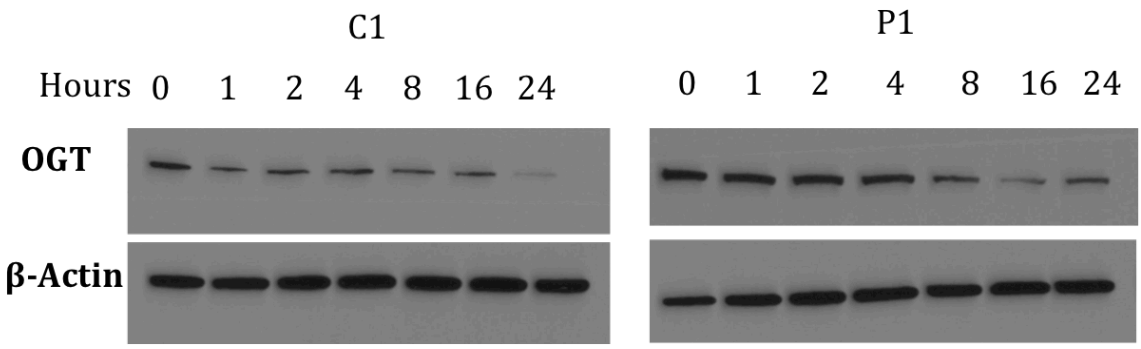

B
% Relative OGT activity
120
100
80
60
40
20
0
WT OGT
L254F-OGT
IB: α-HA

Figure 3.4: L254F-OGT has a shorter half-life than WT OGT in lymphoblastoids cell lines. XLID and WT lymphoblastoids were treated with cycloheximide as described in the Materials and Methods. A. Immunoblotting of crude lysates from XLID and control lymphoblastoids using anti-OGT antibody from PTGlabs and β-Actin as the loading control. B. A graphical representation of biological replicates using densitometry (n=2).

A


C1
P1
Hours 0 1 2 4 8 16 24
0 1 2 4 8 16 24
OGT
β-Actin

B

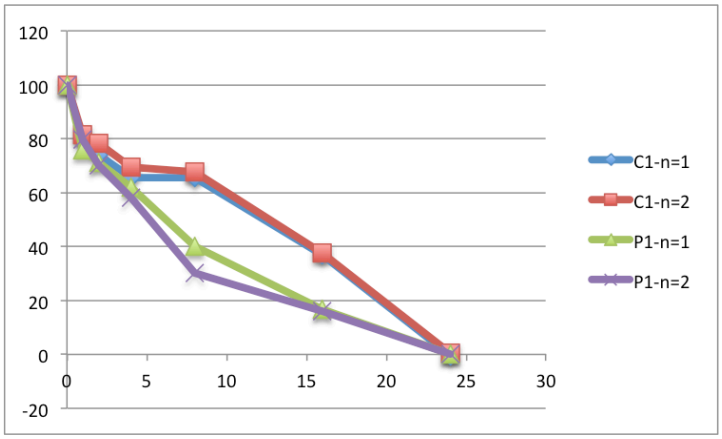
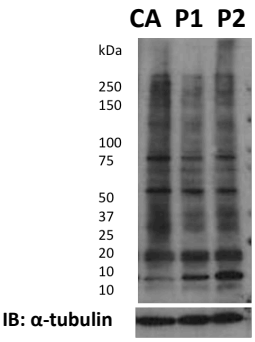
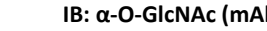

120
100
80
60
40
20
0
-20
0
5
10
15
20
25
30
C1-n=1
C1-n=2
P1-n=1
P1-n=2

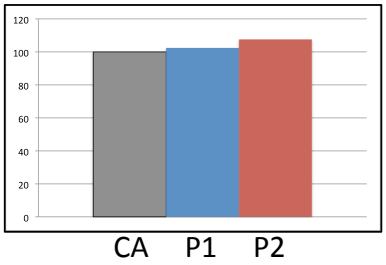
Figure 3.5: Global O-GlcNAc levels remain unaltered in XLID lymphoblastoids. Lymphoblastoids were compared to both female carrier CA and control normal related males C1, C2 and C3. Immunoblotting crude lysates from XLID and control lymphoblastoids using mAb 10 and 110.6. XLID

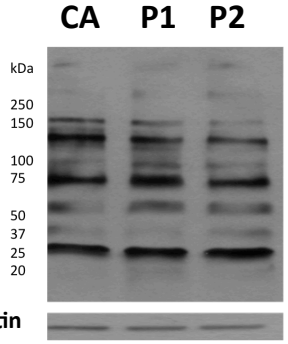




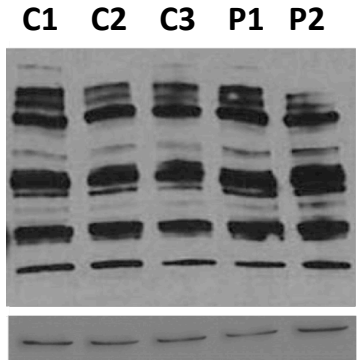
IB: α-O-GlcNAc (mAb14)

Relative O-GlcNAc modified proteins (mAb14)





IB: α-O-GlcNAc (110.6)



IB: α-O-GlcNAc (110.6)

Relative O-GlcNAc modified proteins (110.6)

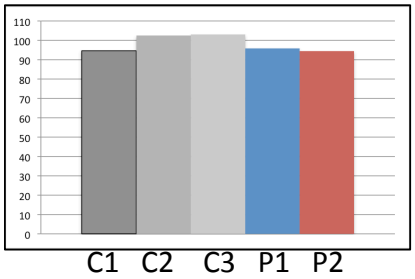
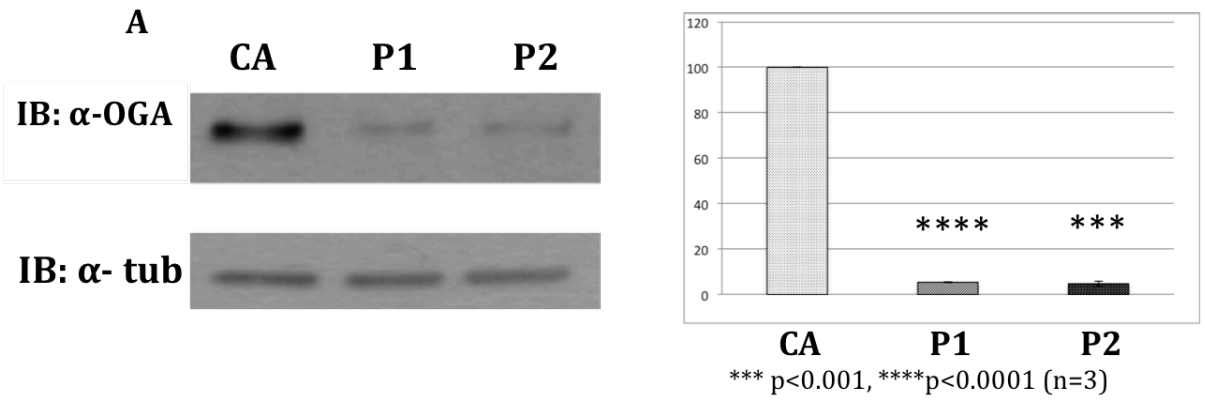


Figure 3.6: Steady state OGA protein levels are lowered in XLID lymphoblastoids. A. Crude lysates from XLID lymphoblastoids were immunoblotted with 2 different OGA antibodies. OGA levels were significantly decreased in XLID lymphoblastoids compared to female carrier, CA ($n=3$, $p \leq 0.001$) as shown by the densitometry graph. B. A similar decrease was observed in XLID when compared to normal related males, C1, C2 and C3 ($n=3$).



*** p<0.001, ****p<0.0001 (n=3)

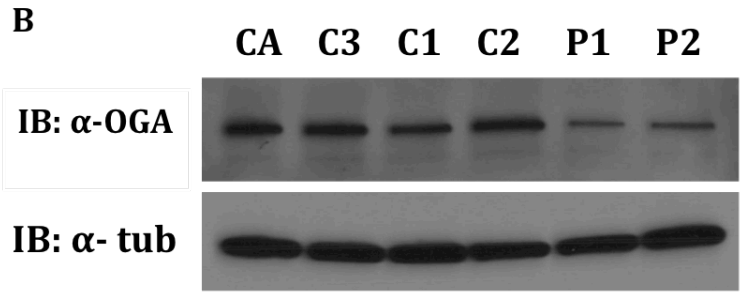
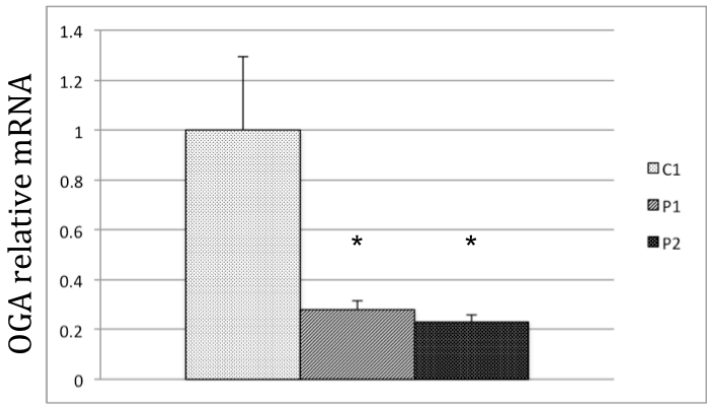


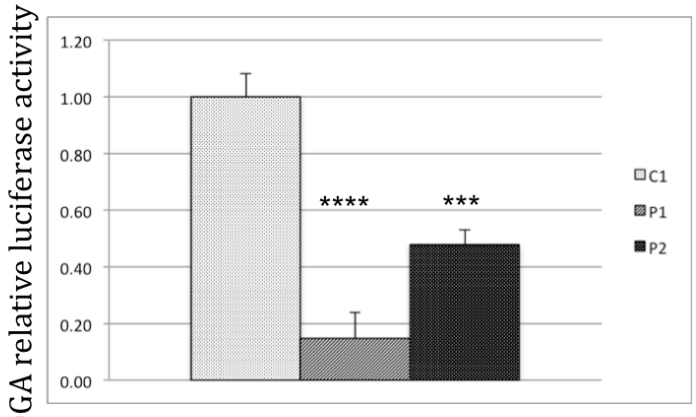
Figure 3.7: OGA steady state mRNA and luciferase promoter expression levels are decreased in XLID lymphoblastoids. A. Steady state OGA mRNA levels assayed by quantitative RT-PCR show decreased transcript levels in both patient samples, P1 and P2 compared to a normal male, C1 ($n=3$, $p < 0.05$). B. A 2 kb proximal promoter region of OGA was introduced into pGL4.10 luciferase vector and transiently co-transfected with pGL4.74 Renilla into XLID or control lymphoblastoids. 48 hours post transfection, the cells were harvested and measured for relative light units and normalized to Renilla expression value ($n=3$, $p \leq 0.001$).

A



*p<0.05 (n=3)

B



****p<0.0001 ***p<0.001 (n=3)

Figure 3.8: OGT is enriched at the OGA proximal promoter. Patient (P1 and P2) and control (C1 and C2) lymphoblastoids were fixed and prepared for Chromatin immunoprecipitation (ChIP) analyses as described in the materials and methods. A. ChIP was performed using OGT, B. mAb14, C. HDAC1and D. mSin3A antibodies. All values were determined using qPCR relative to the % input and are presented as percent input (OGT, n=3, mSin3A, mAb14 and HDAC1, n=1).

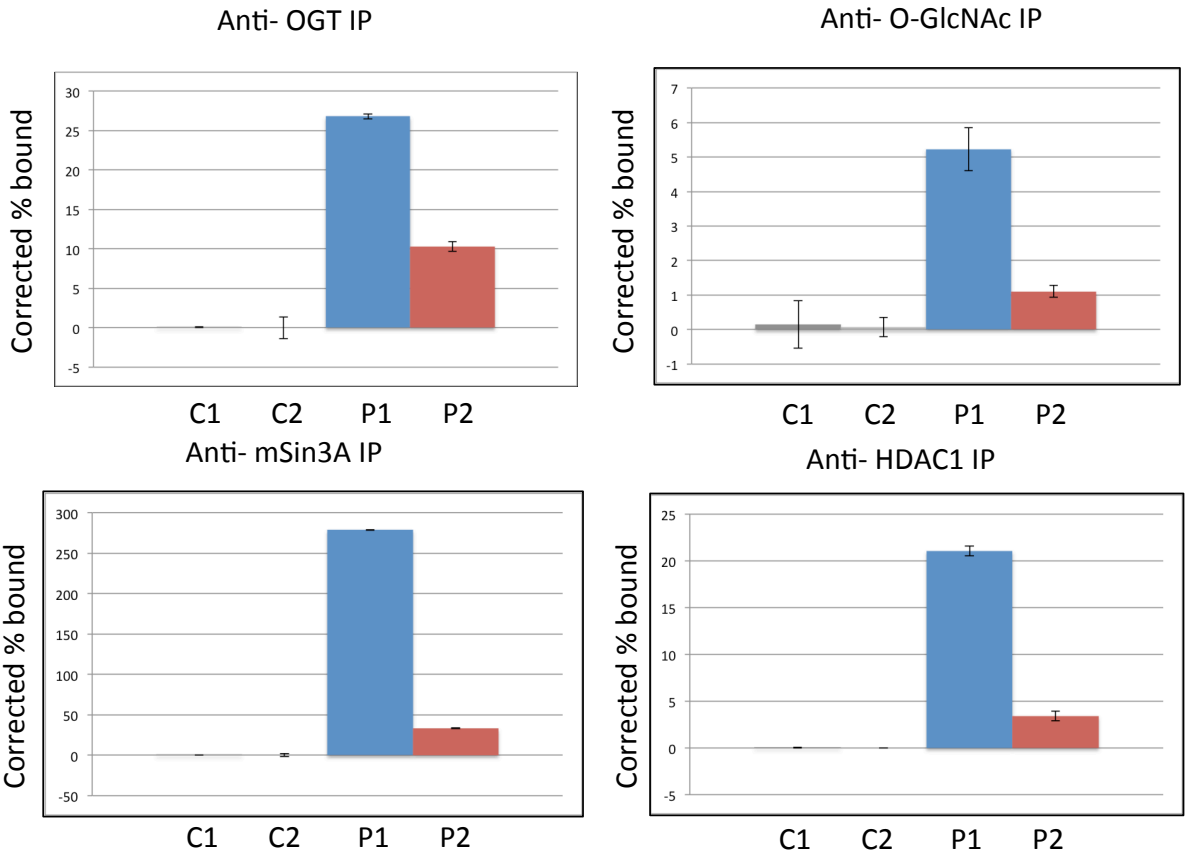
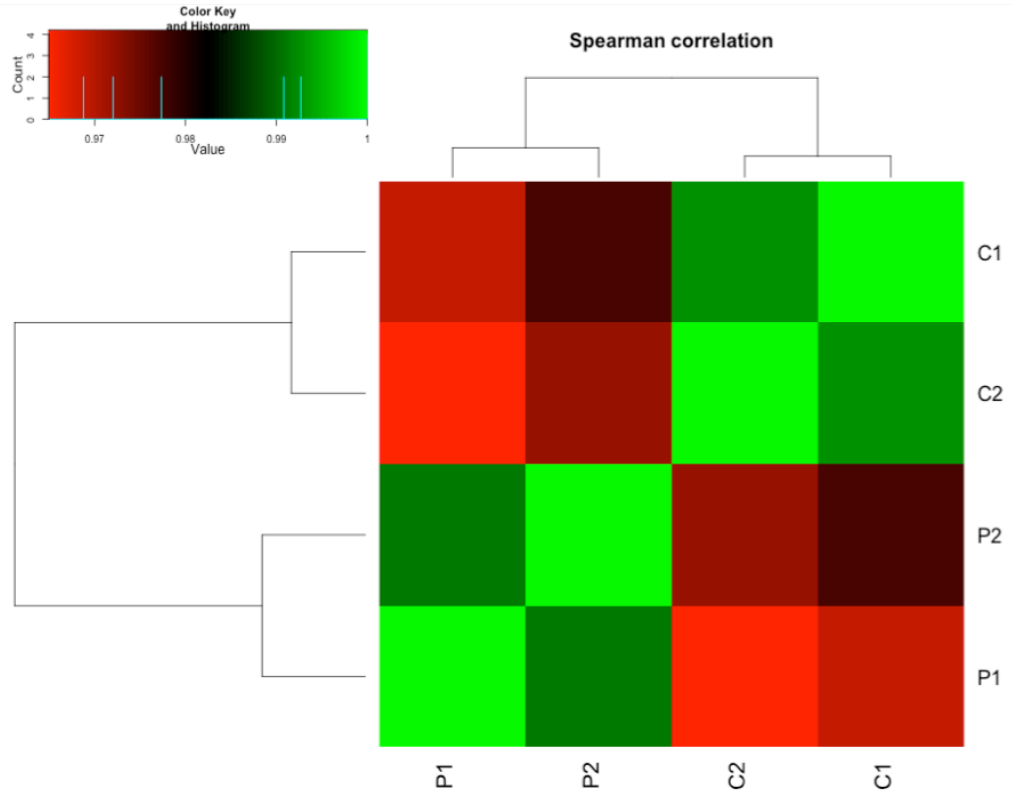
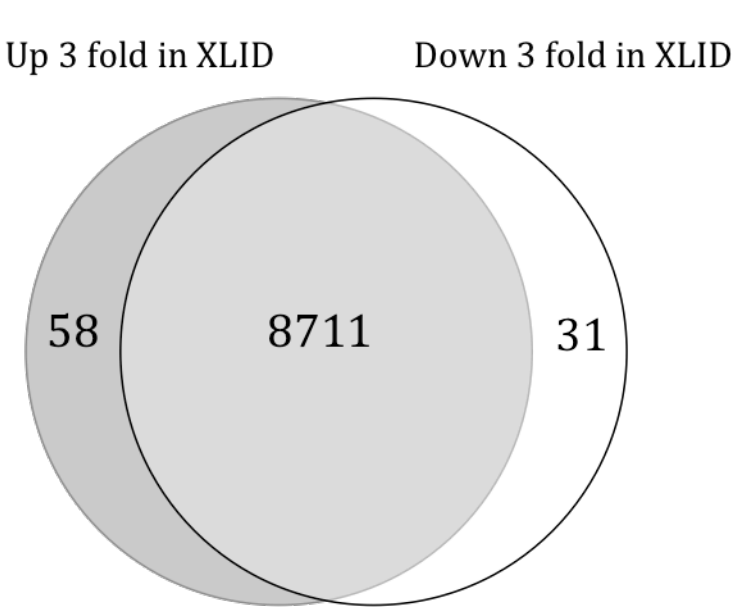

Anti- OGT IP
Corrected % bound
30
25
20
15
10
5
0
-5
C1
C2
P1
P2
Anti- O-GlcNAc IP
Corrected % bound
7
6
5
4
3
2
1
0
-1
C1
C2
P1
P2
Anti- mSin3A IP
Corrected % bound
300
250
200
150
100
50
0
-50
C1
C2
P1
P2
Anti- HDAC1 IP
Corrected % bound
25
20
15
10
5
0
-5
C1
C2
P1
P2

Figure 3.9: OGT regulates a subset of genes in XLID compared to WT. A. Spearman correlation analysis shows segregation of the disease. B. About 1% of genes are being differentially regulated in the patients versus the controls in the 3-fold group. C. Stringency was applied to filter out any data less than 3- fold with the red dotted lines showing our selection. In the 3-fold regulated list, multiple genes are up and downregulated. D and E. Validation of up and down regulated targets from RNA-Seq, HIST1H4B and HIST1H3A respectively.

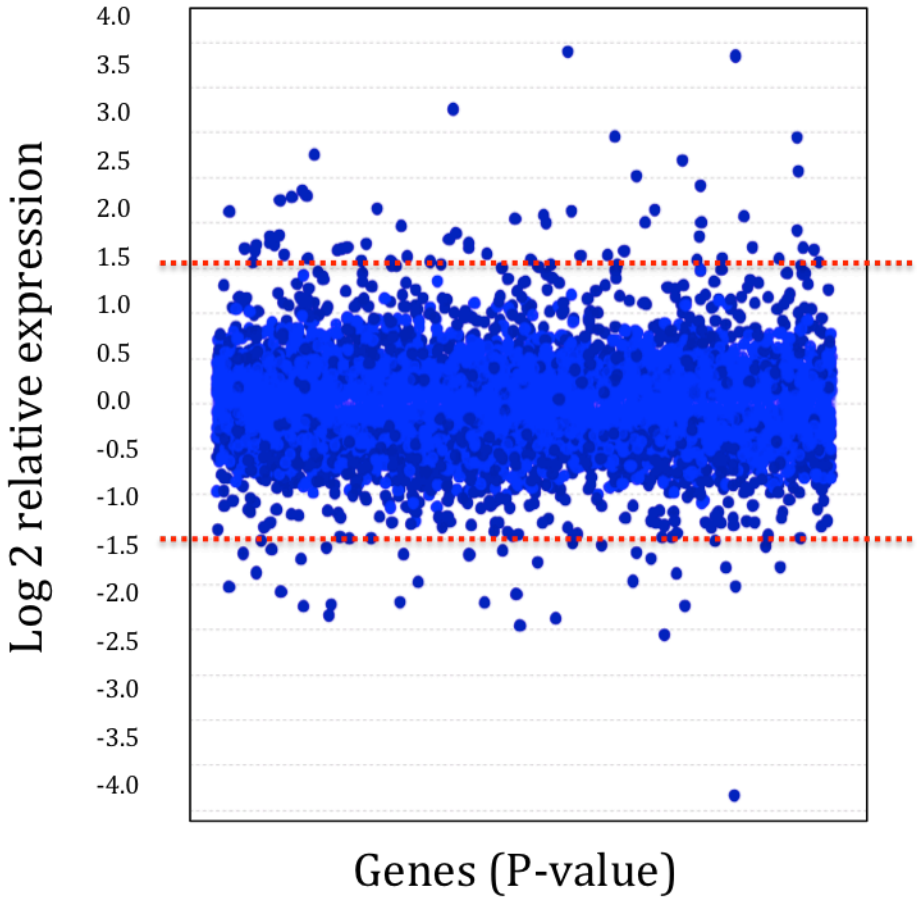
A


Color Key
and Histogram
Count
0
1
2
3
4
0.97
0.98
0.99
1
Value
Spearman correlation
C1
C2
P2
P1
P1
P2
C2
C1

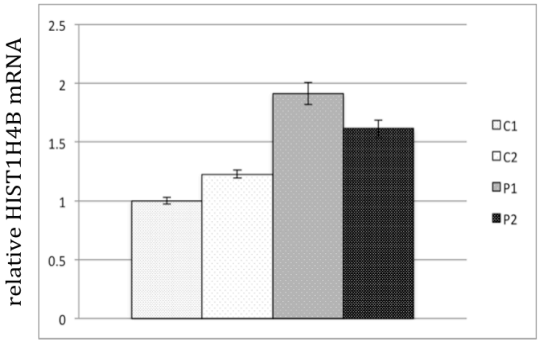
B


Up 3 fold in XLID
Down 3 fold in XLID
58
8711
31

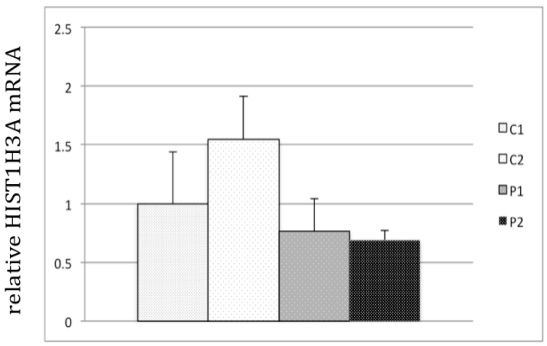
C


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
Log 2 relative expression
Genes (P-value)

D


relative HIST1H4B mRNA
2.5
2
1.5
1
0.5
0
C1
C2
P1
P2

E


relative HIST1H3A mRNA
2.5
2
1.5
1
0.5
0
C1
C2
P1
P2

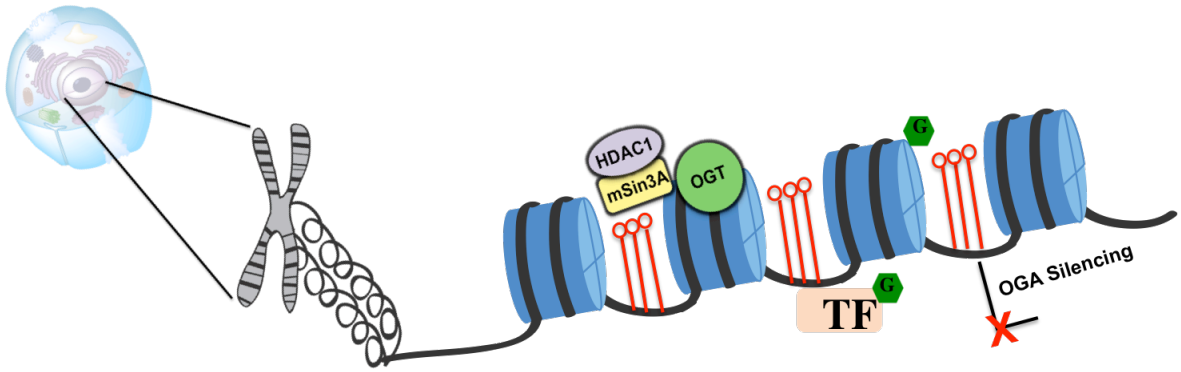


Figure 3.10: Model- OGA gene expression in XLID is regulated by OGT and the mSin3A co-repressor complex. Further characterization of the OGA promoter is required to identify all the elements associated with the repressor complex as well as to identify whether the O-GlcNAc enrichment is due to the histones or transcription factors involved that are modified.

CHAPTER 4: DISCUSSION

Significance of Research Findings and Future Directions

The X-chromosome only contains 4% of all human genes, however, 10% of all Mendelian diseases are assigned to the X-chromosome [1]. The pattern of inheritance associated with X-linked genes was identified as early as the 18th century as in the case of hemophilia [1]. X-linked inheritance pattern was observed in intellectual disability (ID) as early as 1930s [2] and it was only in the 1980s that the first successes were seen in gene mapping and causal gene identification[3, 4]. Subsequent studies have consistently illustrated a 30% excess in males with X-linked intellectual disability (XLID) [5]. Diverse causes such as hereditary and genetic complexities, faulty embryogenesis, inborn errors of metabolism and maternal dependent prenatal and perinatal complications are involved in the pathogenesis of the disease [6]. Several studies paved way for the current technologies available to examine the X- chromosome and elucidate the involvement of X-linked genes in the etiology of ID [2, 7, 8]. 1: 600- 1: 1000 males of the total world population are afflicted with XLID [5, 9] and only 50% of the cases have been assigned a defective gene [5] and the remaining cases are of unknown etiology. These individuals present with intellectual and learning disabilities in addition to dysmorphic, metabolic, autistic, epileptic and neuromuscular features [10]. The heterogeneity of the XLID and the overlapping of its symptoms with CMD and CDGs have remained a challenge in medical genetics. Nonetheless, identification of

monogenic causes of XLID has gained momentum in the last decade owing to the emergence of .the sequence of the human X-chromosome [1]. Discovery of such monogenic variants is vital in our understanding of biological processes both fundamental and new. Thus far, the genetic landscape of XLID as well as the newly identified epigenetic landscape makes it an intricate group of disorders to study for understanding the molecular mechanism of the disease [8, 11].

The O-GlcNAc transferase (OGT) enzyme catalyzes the addition of O-GlcNAc moieties to terminal hydroxyl groups of serines and threonines of proteins [12-14]. While OGT has been implicated in various diseases, a direct causal connection has remained elusive until this study. For the first time, we have identified a mutation in OGT that segregates with disease in a family with XLID. This mutation occurs in the tetratricopeptide (TPR) region of the enzyme. This region is necessary for interaction with substrates as well as with interacting partners to target a subset of substrates [15, 16]. A previous study has reported a SNP in the intronic region of OGA [17].

Previous studies have established O-GlcNAc as a metabolic sensor [18] and one of the many causes of XLID is inborn errors of metabolism [6]. While our studies in the XLID lymphoblastoids demonstrate unaltered global O-GlcNAc levels, this may not be the case in neural cell types where the phenotypes are observed in these patients. Aberrant O-GlcNAc levels are indicative of disease in other settings like cancer [19] and hyperglycemia [20]. Studies investigating the levels of O-GlcNAc in cell type specific contexts like the neural lineages will provide evidence for a metabolic involvement of OGT in XLID. OGT is highly expressed in the brain [13] and a recent study has demonstrated that placental OGT is crucial for hypothalamic gene expression in the

mouse neonatal brain [21]. Mouse fetal XY placentas exhibit substantially lowered OGT protein and mRNA levels when compared to the XX placentas [21]. Subsequently a knock down in mouse XX placental OGT led to hemizygous females and was compared to WT females to understand dosage specific effects and changes in neurodevelopment specific gene expression were observed [21]. Our data demonstrates that the XLID males (L254F-OGT) have lowered steady state OGT levels when compared to the unaffected male relatives (WT OGT). This raises a valid question as to what maybe the global gene expression patterns in XLID in the human brain? To address this, XLID fibroblasts can be obtained to create induced pluripotent stem cells (iPSCs) that can then be differentiated into neural lineages. The neural cells can be used to test gene expression patters by RNA deep sequencing. Furthermore, mouse knock in studies can provide an understanding of spatiotemporal OGT gene regulation during development that could explain the clinodactyly, hypospadia and microcephaly. Current work is also focused on the O-GlcNAcome of the L254F-OGT. We are employing this approach to compare O-GlcNAc modified proteins between the XLID and WT cells to qualitatively assess if there are changes in certain proteins versus others. Metabolically labeling XLID and WT lymphoblastoids with N-azidoacetylgalactosamine-tetraacylated (Ac4GalNAz) [22] will allow for its incorporation onto proteins as O-GlcNAz that can be further enriched using 4-dibenzocyclooctynol (DIBO) resin to capture the azide moiety via copper free click chemistry. This enriched eluate fraction can be analyzed by shotgun proteomics to identify the O-GlcNAc modified proteins in XLID and WT lymphoblastoids.

The decreased steady state levels of L254F-OGT are due to its lowered half-life as seen by the cycloheximide treatment assay. This mechanism alters available protein

levels of OGT, however, it does not affect enzyme activity as seen by the unaltered global O-GlcNAc levels in the patient lymphoblastoids in comparison to WT. In parallel, recombinant L254F-OGT is also active and is capable of transferring O-GlcNAc to both protein and peptide substrates. This is demonstrated *in vivo* by overexpression in HEK293T cells and followed by immunoblotting with anti-O-GlcNAc antibody and in vitro by immunopurification and activity assays. Therefore, in these patients the mutation in OGT does not affect activity of the enzyme in the lymphoblastoids. Examining the interactome profile maybe useful to elucidate if there are any differences in the binding partners of OGT of the L254F-OGT compared to the WT cells due to the mutation occurring in the essential TPR domain of the full length enzyme. Both endogenous as well as recombinant L254F OGT transfected into the XLID as well as control cells can be co-immunoprecipitated to identify interacting partners using shotgun proteomics. If the mutation affects interactions with a subset of proteins, this approach will allow us to identify targets that we can study to elucidate regulation of L254F-OGT. We have been able to identify a tyrosine phosphate on OGT at Y247. Its proximity to the L254F mutation is an interesting angle to pursue. Does the phenylalanine affect the Y247 phosphorylation? To address this we can use the WT versus the L254F-OGT mutant and perform acute insulin stimulation followed by this we can perform LC-MS/MS on these samples that are enriched for phosphates as we did previously to identify the Y247 site. Orthogonally we can performImmunoblotting with pan-phosphotyrosine antibodies.

In order to maintain global O-GlcNAc levels, the levels of O-GlcNAcase (OGA, the enzyme that removes the O-GlcNAc modification), in XLID lymphoblastoids are

significantly decreased. This has prompted us to uncover a compensation mechanism in this cell type that exists, although imperfect because the patients display a clinical phenotype. We believe that this mechanism is in play in this cell line because the patients do no exhibit any phenotype in their lymphocytes, the precursor of the immortalized lymphoblastoids. Previous studies have demonstrated that pharmacological manipulation leading to perturbed O-GlcNAc levels affect the protein levels of the cycling enzymes [23, 24]. Antagonists of OGA cause a decrease in OGT protein levels; possibly to compensate for the elevated O-GlcNAc levels while a concurrent increase is seen in OGA protein levels [24]. Conversely, inhibiting OGT leads to decreased OGA protein levels and increased OGT protein levels. [23]. This suggests that maintenance of homeostatic O-GlcNAc levels is important for normal cellular activities.

The observed decrease in OGA protein levels could be indicative of a transcriptional or translational defect. We were able to confirm that the decrease in OGA protein was due to a significant decrease in the steady state levels of OGA mRNA in the XLID cells when compared to a WT control. Decreases in steady state mRNA levels could be a result of posttranscriptional mRNA degradation or through regulation of the gene promoter. To test this, we expressed a 2kb proximal promoter region of OGA in a luciferase construct in XLID and WT lymphoblastoids. We observed a significant decrease in the expression of the promoter construct in the XLID cells when compared to a normal control. While the transfection of this episomal promoter cannot recapitulate the chromatin environment of the endogenous promoter, we are performing parallel assays to describe the mode of OGA gene repression that is

occurring in these XLID cells when compared to control. To eliminate the effect of posttranslational processing, we also assayed for protein stability via cycloheximide treatments and were unable to detect changes in protein levels up to 24 hours. This strengthens our hypothesis that OGA is regulated by OGT in the XLID lymphoblastoids at the level of transcription.

To elucidate the role of OGT in regulating OGA transcription, we performed chromatin immunoprecipitation (ChIP) and detected an enrichment of OGT at the proximal OGA promoter region. We also observed the same trend with O-GlcNAc at the OGA promoter. This suggests a direct role for OGT in the regulation of OGA gene expression. With the knowledge that OGT exists in the mSin3A/HDAC1 co-repressor complex to downregulate gene expression [25], we propose that a similar mechanism maybe in play with regard to OGA gene regulation. Future studies are aimed at identifying the other components of this complex that are present at the OGA promoter in XLID. Currently, we are evaluating the role of the mSin3A/HDAC1 complex as well as global histone activation and repressive marks.

We also examined the global transcriptome of the XLID patients in comparison to normal controls and observed by unbiased Spearman correlation that our samples segregated by disease and not by relation of the samples. Both controls and one patient are brothers while the other patient is a nephew. We did notice some changes that maybe due to natural variation in using four different human cell lines or their familial generation. Current studies are focused on validating some of the targets obtained from the RNA deep sequencing analysis. Of interest to us are the genes that are involved in transcriptional regulation. We observed an upregulation in sin3A-associated protein,

25kD (SAP25) in the patients when compared to the WT controls where it was not detected. This gene is shown to be a member of the mSin3A/HDAC1 complex and necessary for gene repression in an mSin3A- dependent manner [26]. We also observed a down regulation in histone H3 subunit and an increase in histone H4 subunit in the patients compared to the controls. Histones are posttranslationally modified and this confers complexity to the epigenetic landscape of the genome [27, 28]. As previously mentioned, specific histone marks indicative of either activation or repression will further improve our understanding in the context of specific genes. In addition, An O-GlcNAc and an OGT ChIP followed by deep sequencing may provide a subset of genes that are directly affected by either the levels of O-GlcNAc or the mutant OGT in XLID. Overlap of the genes obtained by this method and the RNA Seq data may provide an insight into the different regulatory processes due to the genes involved in disease specific context can be further explored. Due to the obvious ethical issues in obtaining neural samples from patients, we have performed all our assays in the lymphoblastoid cell lines available. In the XLID field, this is the most used and a well-accepted model to study the effect of anomalous genetic variation. Future studies are aimed at obtaining neural lineages from patient fibroblasts by creating iPSCs. These studies will further enhance our understanding of XLID and the causal role of OGT. In addition to studying XLID, these studies will also serve to illuminate the regulation (dysregulation) of OGT, OGA and O-GlcNAc and their importance in maintaining normal cellular function. While mouse models may not be ideal to recapitulate a phenotype such as ID, knock in studies could allow for examining the developmental defects observed in the human patients.

While this thesis is focused on the L254F-OGT family, two other mutations in XLID in two different families were also identified. One of these mutations also occurs in the TPR region (E338G) while the other occurs in the catalytic region (T560A). The T560A mutation is previously described to be involved in the UDP-GlcNAc binding [29, 30] and ablation of the residue reduced activity of the enzyme toward the protein substrate TGFβ activated kinase 1 (TAB1) but is still capable of autoglycosylation [30]. The identification of more than one mutation in OGT associating with XLID families gives probable cause for its involvement in the disease as well as a potential causal role.

Furthermore, characterizing the E338G and T560A mutation both in a recombinant system as well as in relevant cell types will allow us to ascertain if the molecular mechanism of these mutations recapitulate the L254F mutation. We have one lymphoblastoids cell line available from the E338G family and an unrelated normal male control. Most likely, the other two mutations have their own mode of regulation in disease due to the residues mutated as well as the region of the enzyme the mutation occurs in. That is, the L254F is a fairly well conserved change but it still affects enzyme stability. The E338G mutation is a more dramatic change in terms of the residue change from a negatively charged amino acid to a more neutral amino acid and this occurs in the TPR region. Similarly, the change from the threonine to an alanine results in the loss of a hydroxyl moiety that is necessary for sugar nucleotide binding [30]. Preliminary bioinformatics analyses predict that both the E338G and T560A mutation could result in the destabilization of the protein. Whether these mutations play a role in affecting stability, activity, or substrate targeting by binding partners would be the first step toward characterization. Furthermore, as in the case of the L254F family, neural

lineages will be essential to understand phenotype specific effects in relevant cell lines. Molecular mechanisms driving a disease state are crucial for any diagnostic and therapeutic options.

Finally, to summarize our findings, we have identified a SNP in OGT for the first time that segregates with disease in a family with XLID. Since the first identification, two other families with XLID have also exhibited mutations in OGT, in both cases a single nucleotide variant. This illustrates the first example of a disease associated with a mutation in the coding region of OGT. Thirty years after identification of O-GlcNAc in the Hart laboratory [12] we have characterized a mutation in one of the cycling enzymes. We were also able to identify a cell type specific compensatory mechanism that maintains global O-GlcNAc levels by regulation of OGA. We demonstrate that this is due to the regulation of OGA gene expression by OGT. For the first time, this study also elucidates the regulation of the human promoter region of OGA that occurs to maintain steady state O-GlcNAc levels by downregulating OGA gene expression in response to a defect in OGT steady state protein levels.

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