University of Alberta

β-catenin is O-GlcNAc modified at Serine 23:
Implications for β-catenin's Subcellular Distribution and Transcriptional Activity

by

Jacqueline Ra Young Ha

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A dedication to my students

You are the reason why I love what I do

Abstract

β-catenin is a potent oncoprotein that serves as a structural anchor at the adherens junctions and as a transcriptional co-activator of the Wnt Signaling pathway. β-catenin was identified to be post-translationally modified by O-linked β-D-N-acetyl-glucosamine (O-GlcNAc). This investigation was aimed to identify Serine 23 (Ser23) as a site for O-GlcNAc modification and to characterize the relevance of this site for β-catenin's function.

Serine 23 to Glycine mutant or wild-type β-catenin constructs were expressed in DU145 cell line and subsequently treated with PUGNAc—a drug that globally increases O-GlcNAcylation. O-GlcNAc-β-catenin levels were characterized by Wheat Germ Agglutinin (WGA)-HRP or WGA-agarose precipitation. Alterations in β-catenin's subcellular localization, interactions, and transcriptional activity were analyzed through confocal microscopy, immunoprecipitation, and, RT-qPCR and luciferase reporter assay, respectively.

This study demonstrated that Ser23 of β-catenin was a site for O-GlcNAcylation which increased β-catenin's localization to the plasma membrane and decreased its transcriptional activity within the nucleus.

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List of Abbreviations

AD	Androgen Dependent
AI	Androgen Independent
APC	Adenomatous Polyposis Coli
AR	Androgen Receptor
BCL9	B-cell CLL/lymphoma 9 protein
BSA	Bovine Serum Albumin
β-TrCP	β-Transducin repeat-Containing Protein
C-	Carboxyl
CaP	Prostate Cancer
CAM	Cell Adhesion Molecule
CBP	Cyclic AMP response element-binding protein
C.elegans	Caenohabditis elegans
CK1	Casein Kinase 1
CKII	Casein Kinase 2
CtBP	C-terminal Binding Protein
DMEM	Dulbecco's Modified Eagle's Medium
Dvl	Dishevelled
FBS	Fetal Bovine Serum
Fzd	Frizzled
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GFP	Green Fluorescent Protein
GSK3β	Glycogen Synthase Kinase 3 β
HAT	Histone acetyletransferase

HDAC	Histone deacetylase
HMG	High Mobility Group
HRP	Horse Radish Peroxidase
IF	Immunofluorescence
ICAT	Inhibitor of β-catenin and Tcf-4
LEF	Lymphoid Enhancer Factor
LRP5/6	LDL receptor related protein 5 and 6
MS	Mass Spectrometry
N-	Amino
NLS	Nuclear Localization Sequence
NES	Nuclear Export Sequence
NMR	Nuclear Magnetic Resonance
O-GlcNAc	O-linked β-D-N-acetylglucosamine
OGT	O-GlcNAc Transferase
OGA	β-N-acetylglucosamidase
PBS	Phosphate Buffered Saline
PCP	Planar Cell Polarity
PH	Pleckstrin Homology
PUGNAc	O-(acetamindo-2-deoxy-D-glucopyransylidene)-amino-*N*-phenylcarbamate
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
RTK	Receptor Tyrosine Kinase
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction

TBP	TATA Binding Protein
TBS	Tris Buffered Saline
TCF	T-Cell Factor
TLE	Transducin-Like Enhancer
TPR	Tetratricopeptide Repeat
UDP	Uridine diphosphate
VEGF	Vascular Endothelial Growth Factor
WGA	Wheat Germ Agglutinin

Chapter 1

Introduction

1.0 Introduction

1.1 β-catenin

β-catenin is a 781 amino acid protein encoded by the *CTNNB1* gene located on Chromosome 3p21. Its protein structure is composed of three distinct regions: the central armadillo domain, comprising 12 imperfect repeats of 42 amino acids, the amino (N) –terminal domain, containing phosphorylation sites vital for ubiquitin mediated proteosomal degradation, and the carboxyl (C) – terminal domain, housing the transactivation domain required for gene activation (Figure 1A) [1].

β-catenin serves two major functions within the cell. At the adherens junctions, β-catenin links E-cadherin to the actin cytoskeleton via α-catenin (Figure 1B). The presence of β-catenin at the cell surface ensures efficient cell-cell adhesion, the structural integrity of tissue architecture, and the maintenance of the epithelial phenotype. Within the nucleus, β-catenin exerts its oncogenic functions, where T-Cell Factor/ Lymphoid Enhance Factor (TCF/LEF) transcriptional factors complex with β-catenin to activate gene transcription of cancer promoting genes (Figure 1B). β-catenin's remarkable capability to partake in both cell signaling and adhesion can be explained by the existence of differing molecular forms of the same protein. Gottardi & Gumbiner, (2004) demonstrated that a TCF-specific form of β-catenin was generated after Wnt activation [2]. This selective form of β-catenin did not interact with the cadherin binding domain. Moreover, while organisms such as *Caenohabditis elegans* (*C. elegans*) utilize several forms of β-catenin to differentially control cell adhesion and signaling, vertebrates transform β-catenin to distinct structural configurations to maintain the same degree of coordination and regulation. The failure to do so, as frequently occurs in cancer, is a common mechanism by which carcinogenesis is facilitated. Therefore, it is key to fully unravel the complex machineries associated with β-catenin and to understand the structural basis of β-catenin's functionality; in particular, in terms of its subcellular interactions and its role as a co-activator of transcription.

1.1.1 The structure of β-catenin

1.1.1.1 Armadillo repeat domain

The long, positively charged armadillo domain is comprised of 12 armadillo repeats, forming β-catenin's ligand-recognition domain. This region hosts mutually exclusive interactions with β-catenin's numerous molecular partners. The positive charge of the groove and its negatively charged ligands assist in β-catenin interaction [3]. The armadillo repeats (residues 141-664), each repeat consisting of three α-helices, helices 1 (H1), 2 (H2), and 3(H3), are densely packed, forming an overall cylindrical conformation. The floor of this groove is made up of H3 helices. The proteolysis-resistant armadillo domain is also highly conserved and structurally rigid relative to the unstable terminal domains, which are sensitive to trypsin digestion [1, 3]. The inflexibility of the domain is caused by the extensive contacts between the 12 repeats, ensuring the stability of the continuous hydrophobic core [1].

Studies of three dimensional crystal structures of a variety of β-catenin complexes, including TCF, the cadherins, and APC, exposed a binding region (repeats 5-9) shared by the common ligands [4]. Specifically, armadillo repeats 6-8 forms a special part of the groove containing a series of asparagine (Asn) residues that engage the polypeptide backbone of a diverse cohort of ligands [5]. Ligands that recognize β-catenin all contain a conserved consensus sequence, containing Aspartic Acid (Asp) and Glutamic Acid (Glu) amino acids that form two disulfide bridges between Lysine (Lys) 435 and Lys312 of β-catenin, respectively [4]. Graham et al., (2000) dubbed these covalent bonds as "charged buttons" as they were required to affix the partners to β-catenin's armadillo domain [6]. Despite the commonalities, each ligand interacts with the groove in a distinct manner: E-cadherin's cytoplasmic domain interacts with the entire span of the armadillo domain and TCF interacts with mainly armadillo repeats 3-5 [4]. In each case, the ligand appears to undergo conformational adjustments to properly accommodate the rigid groove [5].

1.1.1.2 N- and C- terminal domains

Although the armadillo groove shows high binding affinity for β-catenin's various molecular partners, the full-length armadillo domain's interactions are rather weak, pointing to the significance of the terminal regions [7, 8]. The unstructured terminal tails flanking the armadillo repeat domain are highly flexible, and are proposed to regulate ligand binding. For instance, the interaction between the C-terminal (residues 696-781) and the armadillo repeats limit the binding of E-cadherin [7]. The N-terminal (residues 1-134) can also interact with the central domain, however, with low affinity when the C-terminus is absent. The deletion of the N-terminus resulted in a tighter binding of the C-terminal to the armadillo domain [8]. These results indicate that the two termini are interdependent and interact with the armadillo domain in a fold-back fashion.

The proximal regions of the C-terminal have also been shown to form an α-helix, designated as Helix C, which modulates Wnt-mediated transcription [3]. The significance of this particular helix is well documented: truncated *Drosophila armadillo* (fly homolog of β-catenin) lacking the Helix C failed to initiate transactivation, whereas truncation of the C-terminus up to the Helix C preserved signaling capacity [9]. Equally, Helix C was found on transcriptionally active forms of β-catenin in *C. elegans* but not on an adhesive form which preferred the cadherins [9, 10]. Moreover, experiments delivering a truncated *armadillo* constructs void of the N-terminus into the nucleus revealed an absence of β-catenin-TCF complexes, suggesting that the N-terminus influences, if not to a greater extent than the C-terminus, the gene transcription and chromatin remodeling functions possessed by β-catenin [11, 12]. Crystallographic and nuclear magnetic resonance (NMR) investigations suggested the dynamism of the unstructured tails distal to the Helix C: the negatively charged N- and C-tails respond to the positively charged groove in a highly variable manner and do not interact in a static conformation [3]. Hence, the tails may "shield" the armadillo repeat domain from any non-specific interaction, or act as "intramolecular

chaperones" of the armadillo repeat domain to facilitate ligand binding and to prevent self-aggregation of the repeats [3].

1.2 β-catenin and cell adhesion

The progression of many cancers involves the loss of cell adhesion and contact inhibition. This can be attributed to the aberrant regulation of cell adhesion molecules (CAMs), which comprise the cadherins, integrins, selectins and the super family of immunoglobulins (IgSF CAMs). E-cadherin is a calcium-dependent transmembrane glycoprotein responsible for mediating intercellular adhesion as well as the structural integrity of the cell. The cytoplasmic domain of E-cadherin interacts with the entire span of β-catenin's armadillo repeat domain, and features multiple, quasi-independent binding regions [13]. However, only the last 100 residues of the E-cadherin cytoplasmic domain make contact with the large binding interface on β-catenin [5, 13]. The cytoplasmic domain can be subdivided into five regions, I-V, based on their distinct interaction with β-catenin's armadillo repeats. Region IV hosts consensus sequences for casein kinase II (CK2) and GSK3β mediated serine phosphorylation. These sites are part of the extended PEST (Proline- Glutamic Acid- Serine- Threonine; Pro-Glu-Ser-Thr) sequence responsible for cadherin degradation; masking of the PEST domain when β-catenin binds consequently prevents degradation. The most functionally relevant are region II and IV, where certain phosphorylation events occur to affect binding affinity. In general, constitutive activation of tyrosine kinases contributes to abnormal growth, *in situ* carcinogenesis and metastasis [14]. Specifically, an α-helix in region II is packed against Tyrosine (Tyr) 654 of β-catenin's armadillo repeats 11-12 where the phosphorylation of Tyr654 by proto-oncogene tyrosine-protein kinase pp60c-src,was found to reduce the affinity of E-cadherin for β-catenin by 6-fold. Furthermore, the *in vitro* transfection of pp60c-src subsequently led to junctional instability and the gain of an invasive phenotype [13, 15, 16].

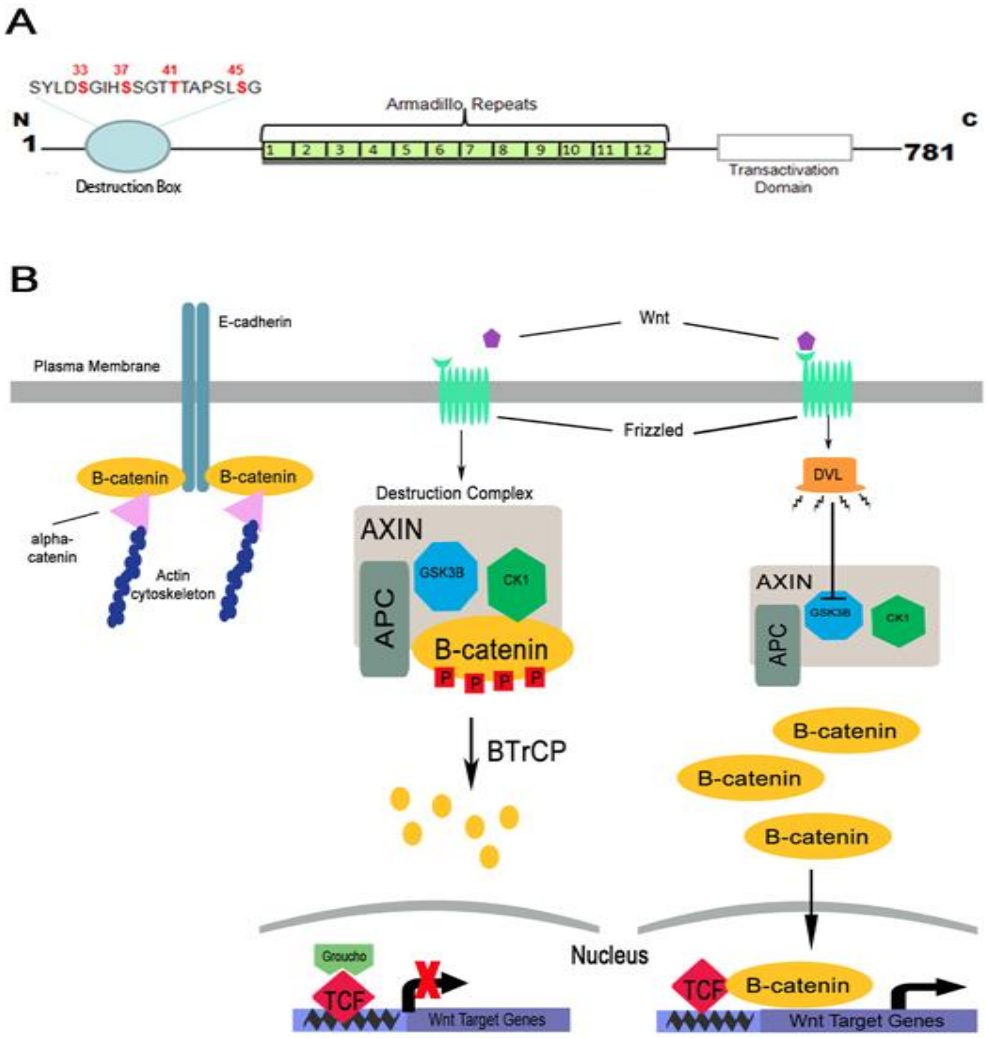

Figure 1—*Schematic representation of the protein structure of β-catenin and the canonical β-catenin dependent Wnt Signaling Pathway.* A) Illustration of β-catenin and its three primary domains: N-terminal (NT) domain containing the Destruction Box, central Armadillo Repeat Domain (Repeats 1-12), comprising a positively charged groove of 12 imperfect repeats of 42 amino acids, and C-terminal (CT) domain housing the transactivation domain. **B)** β-catenin serves two main functions within the cell. First, at the adherens junctions β-catenin serves as a structural anchor linking E-cadherin to the actin cytoskeleton. Secondly, β-catenin is an essential regulator of the Wnt Signal Transduction Pathway. In the absence of a Wnt signal, β-catenin levels are strictly regulated within the cell by a destruction complex which mediates β-catenin's phosphorylation and subsequent proteosomal degradation. Upon a Wnt signal, the destruction complex is inactivated, resulting in the accumulation of β-catenin within the cytoplasm and its subsequent localization to the nucleus. Here, β-catenin acts as the transcriptional co-activator of TCF/LEF transcription factor family of proteins.

Another key component of the adhesion complex is α-catenin, a protein linking the actin filaments to the E-cadherin bound β-catenin. The binding surface for α-catenin is N-terminal to the first armadillo repeat of β-catenin (residues 118-141) [13]. The conserved residue Tyr142 is a critical regulator of this region as it affects α-catenin-β-catenin interaction: phosphorylation of Tyr142 dissociated β-catenin from α-catenin with the simultaneous loss of cell adhesion [14, 17, 18]. Recent evidence suggested that the α- catenin to actin interaction requires α-catenin to be in its homodimeric form; however, the homodimerization interface located on α-catenin impedes the binding sites of β-catenin [19]. The monomeric form of α-catenin primarily binds to β-catenin but exhibits low affinity for actin. Thus, α-catenin cannot interact with both β-catenin and actin concomitantly. It seems, overall, α-catenin modulates actin dynamics in the presence of E-cadherin [19, 20].

1.3 The Wnt Signaling Pathway

The Wnt signaling pathway is activated by 19 Wnt isoforms (For a complete list of Wnt isoforms, refer to Reference 21). The Wnts are a family of secreted glycolipoproteins, which are conserved in all metazoan animals. Wnt ligands activate the Wnt pathway by binding to a seven-pass transmembrane frizzled (Fzd) receptor in conjunction with its co-receptors, LDL receptor related proteins 5 and 6 (LRP5/6). Signaling by these morphogens functions to direct cell proliferation, cell adhesion, tissue development, oncogenesis, tumor suppression, and cell-fate determination [22]. As a result, defective Wnt signal transduction plays a critical role in a range of hereditary diseases and cancers such as polycystic kidney disease [23], Alzheimer's disease [24], hepatocellular carcinoma [25], and colorectal cancer [26].

Wnt signaling can be divided into two categories: the canonical Wnt pathway and the non-canonical Wnt pathway. The former is activated by a certain subset of Wnt proteins that regulate β-catenin, while the latter operates independent of β-catenin signaling, namely the planar cell polarity (PCP) pathway and the

Wnt/Ca^{2+} pathway. This manuscript will focus on the canonical Wnt pathway; for a more comprehensive overview of the non-canonical Wnt Pathways (PCP and Wnt/ Ca^{2+} pathway) refer to References 27 and 28.

1.3.1 The canonical Wnt (β-catenin dependent) pathway: Brief Overview

The adhesive properties of β-catenin are juxtaposed by its oncogenic functions within the nucleus. Hence, the oncogenic potential of β-catenin mandates that its levels are strictly regulated within the cell. In the absence of a Wnt signal, β-catenin is targeted for degradation by the 'destruction complex' comprising Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3-beta (GSK3β) and casein kinase (CK1) [22]. β-catenin is recruited to the destruction complex by APC and targeted for degradation by the ubiquitin-proteosomal degradation system [22]. This involves the phosphorylation of Serine (Ser) 33, Ser37, Threonine (Thr) 41, and Ser45 by GSK3β and CK1 respectively, at the N-terminal domain's destruction box. Phosphorylation of β-catenin by CK1 at Ser45 primes the sequential phosphorylation at Ser33, Ser37, and Thr41 by GSK3β. Phosphorylation at Ser33 and Ser37 allows recognition of β-catenin by an E3 ubiquitin ligase subunit, β-TrCP (β-transducin repeat-containing protein), resulting in ubiquitination and subsequent proteasomal degradation [22]. Strict regulation of cytosolic β-catenin levels via the destruction complex ensures to some extent the nuclear availability of β-catenin. However, in the presence of a Wnt signal, β-catenin is stabilized to increase in cellular levels and subsequently translocate to the nucleus where it becomes transcriptionally active.

The Wnt signal involves the canonical Wnts, primarily Wnt3, Wnt3a, and Wnt6, binding to Fzd-LRP 5/6 complexes to activate Dishevelled (Dvl), which then disables GSK3β activity and stimulates LRP5/6 phosphorylation. Phosphorylation of LRP5/6 on its cytoplasmic tail leads to Axin docking at the plasma membrane, thus preventing the constitutive destruction of β-catenin [29, 30]. Consequently, β-catenin accumulates in the cytoplasm and ultimately translocates to the nucleus, where it acts as a co-activator of TCF/LEF family of DNA-binding proteins to

mediate *wnt* target gene transcription including c-myc, jun, Cyclin D1, and vascular endothelial growth factors (VEGF). For a comprehensive and updated overview of *wnt* target genes, refer to
http://www.stanford.edu/~rnusse/wntwindow.html.

1.3.2 β-catenin and the destruction complex

The 'destruction complex', responsible for β-catenin turnover, was described to encompass four major entities – the scaffold protein, Axin, the nuclear chaperone, APC, and, the kinases, GSK3β and CK1. The regulation of β-catenin by these components will be discussed here.

GSK3β and CK1 mediated phosphorylation has a major impact on the functionality of β-catenin. Unmodified β-catenin at GSKβ residues Ser33, Ser37 and Thr41 has been characterized as an active pool of β-catenin [31, 32]. In other words, transactivation by β-catenin can be altered by phosphorylation. Using monoclonal antibodies detecting β-catenin specifically unmodified at Ser37 and Thr41 (active β-catenin), Maher et al., (2010) indicated that active β-catenin exists in a monomeric form and was found in far fewer proportions relative to the total pool of β-catenin [32]. That being said however, the low levels of active β-catenin were almost exclusively located in the nucleus. Furthermore, Maher et al., (2010) observed that β-catenin phosphorylated at Thr41/Ser45 was spatially uncoupled from β-catenin phosphorylated at Ser33/Ser37/Thr41. This suggested that phosphorylation at Ser45 by CK1 extended beyond a simple priming gesture [32]. Since the majority of the Thr41/Ser45 phosphorylated β-catenin translocated to the nucleus, it is entirely possible that phosphorylation at Ser45 configures an active form of β-catenin [32]. In contrast, β-catenin phosphorylated at Ser33/Ser37/Thr41 was generally cytoplasmic and was ultimately subjected to protein degradation. The proteosomal degradation of β-catenin is initiated by the F-box protein, β-TrCP, which recognizes the double phosphorylated N-terminal destruction motif (Ser33 and Ser37) and binds β-catenin (residues 20-31) [33] to

cause the ubiquitination of β-catenin at specific lysine residues by the larger ^{SCF}β-TrCP complex [34].

Interestingly, the mechanism underlying the nuclear localization of β-catenin remains unclear as β-catenin does not contain a nuclear localization sequence (NLS) nor does it utilize the conventional importin nuclear transport system [35]. It is likely that NLS containing chaperones such as APC, Axin, and RanBP3 (Ran binding protein 3) shuttle β-catenin into the nucleus [35]. APC is of particular interest, as it has been proposed to participate in a range of roles within the destruction complex and nucleus. As a nuclear exporter of β-catenin, APC is able to restrict TCF interaction and thus gene transcription [36-38]. This model can be partly supported by the nuclear accumulation of β-catenin in colorectal cancer cells (SW480) expressing mutated APC, as well as elevated β-catenin levels due a complete loss of the APC gene observed in *Drosophila* [39]. More directly, transient transfection of wild type APC into SW480 diminished nuclear levels of β-catenin and increased the degradation of β-catenin [36]; in turn, treatment with leptomycin B, a nuclear export inhibitor of APC, or the mutagenesis of the nuclear export signal (NES) on APC, abolished these effects, respectively, as well as increased the levels of β-catenin within the nucleus [40]. Taken together, these data support the role of APC as a nuclear chaperone of β-catenin.

Structurally, APC binds to β-catenin by either its three 15-amino acid repeats (15 aa) or seven 20-amino acid repeats (20 aa) at its central domain [41, 42]. Despite their sequence similarities, the different β-catenin-binding repeats have crucial differences. The 15 aa repeats are not modified by phosphorylation and bind to armadillo repeat 5-8 of β-catenin, overlapping the regions of TCF binding site [43]. In spite of this physical arrangement, the 15 aa repeats cannot hinder TCF-β-catenin interaction [43]. The 20 aa repeats are highly conserved and can be phosphorylated on the SXXSSLSXLS (S is Serine; L is Leucine) consensus motif [20]. Phosphorylation at this motif by GSK3β and CK1 drastically increases APC interactions with β-catenin by 300- to 500- fold [38]. In fact, phosphorylation of

the third 20 aa repeat has by far, the tightest binding affinity for β-catenin [44]. Interestingly, the deletion of this site accounts for the majority of APC mutations in colorectal cancer [20, 45-47]. Crystal structures of the complex between the 20 aa repeats and the armadillo groove of β-catenin by Xing et al., (2004), demonstrated that the phosphorylated 20 aa repeats of APC bound to armadillo repeats 1-5 of β-catenin with high affinity. Indeed, binding competition assays have confirmed phosphorylated APC disrupts β-catenin-TCF interaction, in part, due to APC residues N-terminal to the 20 aa repeat which adopt a conformation identical to that of TCF and E-cadherin [38].

Axin facilitates the phosphorylation-dependent degradation of β-catenin by anchoring β-catenin, APC, CK1 and GSK3β to specific binding sites. Axin interacts with APC through its regulator of G-protein signaling (RGS) [48], while APC requires the Ser-Ala-Met-Pro (SAMP) repeats, in conjunction with the 15 aa and 20 aa repeats, in its central domain to effectively interact with Axin [39, 49, 50]. The β-catenin binding domain of Axin includes a highly conserved helical region that interacts with armadillo repeats 3 and 4 of β-catenin's positively charged groove [51].The helical region on Axin is C-terminal to the GSK3β binding site and runs roughly parallel to the superhelix formed by β-catenin's armadillo repeats. This places GSK3β at the N- terminus of β-catenin to augment the phosphorylation efficiency of GSK3β by >20,000 fold [52]. Alternatively, the anchored CK1 and GSK3β can phosphorylate Axin to increase its affinity for β-catenin [53].

Although APC cannot independently induce GSK3β-dependent phosphorylation of β-catenin, the synergy between APC and Axin considerably increased levels of GSK3β modified β-catenin [39, 48]. This suggests APC, along with the kinases, are essential to forming complexes with Axin to mediate the degradation of β-catenin. The current models hold that APC sustains the efficiency of the 'destruction complex' by controlling the release and the recruitment of β- catenin. The basis of these models is that the phosphorylated 20 aa repeats on APC

competitively inhibits β-catenin-Axin interaction [51]. Phosphorylated APC first transports β-catenin to the 'destruction complex', where dephosphorylation of APC reduces its binding affinity, causing β-catenin to bind preferentially to Axin [39]. A candidate dephosphorylation agent is protein phosphatase 2A (PP2A). This multimeric phosphatase promotes β-catenin turnover and causes β-catenin stabilization when inhibited [51]. In *in vitro* studies, PP2A was shown to directly dephosphorylate APC [51].

1.3.3 β-catenin and transactivation

Nuclear β-catenin activates gene transcription by forming a complex with the TCF/LEF family of DNA binding proteins to mediate the transcription of Wnt target genes. Although the exact sequence of events that occur once β-catenin has translocated into the nucleus remains elusive, there are several models explaining the role of β-catenin in gene activation. The simplest explains β-catenin as a co-activator by providing a transcriptional activation domain to TCF/LEF [54]. Another model proposes that β-catenin heterodimerizes with TCF/LEF to displace repressor proteins, Groucho/ transducing-like enhancer (TLE), C-terminal binding protein (CtBP) or histone deacetylases (HDAC), and thereby switching TCF/LEF from a quiescent state into its transcriptionally active form [44, 54]. Since TCF is DNA bound, changes in chromatin structure are also necessary to lift the transcriptional blockade imparted by these repressor proteins [56, 57]. β-catenin was found to interact with numerous other chromatin modifying proteins such as histoneacetyltransferases (HATs), cyclic AMP response element-binding protein (CBP) or its close relative p300, TATA binding protein (TBP) and Brg-1 to assemble a multimeric complex in conjunction with TCF/LEF [57].

The TCF/LEF family consists of Tcf-1, Tcf-3, Tcf-4 and Lef-1, and may function to either activate or repress the transcription of a plethora of genes depending on the availability of β-catenin in the nucleus [58]. While Tcf-1 and Tcf-4 may play dual roles as both an activator and repressor, Lef-1 exists predominately as an activator whereas Tcf-3 is often a repressor [22]. Upon Wnt activation,

approximately 50 residues of Tcf-4 within the N-terminal interact with β-catenin in two distinct binding surfaces [20]: an extended region (residues 13-25) that interacts with armadillo repeats 4-9 and an α-helix formed by residues 40-50 that binds to armadillo repeats 3-5. The second binding interface involves TCF's N-terminal DNA-binding high mobility group (HMG) domain which overlaps the binding interface for transcriptional repressor Groucho/TLE [20]. Thus, these repressors are displaced when β-catenin binds with to TCF with higher affinity.

Along with β-catenin, many other co-activators of TCF have been identified. B-cell CLL/lymphoma 9 (BCL9) protein is an adaptor protein proposed to aid transactivation by providing docking sites for other transcriptional machinery such as pygopus [59], a transcription factor containing a plant homeodomain (PHD) finger for chromatin remodeling. BCL9 has not only been found to interact with β-catenin/TCF complex to activate transcription, but has also been found to sequester β-catenin in the nucleus [85]. The crystal structure of a β-catenin/BCL9/Tcf-4 complex revealed that BCL9 interacted with β-catenin at a region N-terminal to the structural groove of the armadillo repeat domain. The β-catenin binding domain on BCL9 forms an α-helix, but unlike other co-activators, the helix does not overlap the binding sites of other β-catenin partners. This helix can be mutated to prevent proper β-catenin-BCL9 binding without compromising the integrity of other indispensable interactions [59]. Sampietro et al., (2006) demonstrated that simultaneous mutations within hydrophobic pockets of the first armadillo repeat, especially on residues L156A and L159A (A is Alanine), effectively abolished BCL9 binding but not that of E-cadherin and α-catenin, the only two known proteins that bind to the same region on β-catenin [59]. This suggests that BCL9 interacts with β-catenin through unique, hydrophobic contact and underscores the therapeutic potential of small molecule inhibitors to prevent the transcription of *wnt* target genes via precise interferences of the BCL9-β-catenin complex.

A variety of antagonists functions to further regulate β-catenin/TCF-mediated transcription. The nuclear antagonist, ICAT (inhibitor of β-catenin and Tcf-4), inhibits binding of β-catenin to Tcf-4 *in vitro* and has been shown to decrease Tcf-4-induced reporter activity [57, 61, 62]. This inhibitory attribute is due to its high affinity for the armadillo repeats 5-10, which are shared between TCFs, APC, and cadherins.

1.4 β-catenin and post translational modifications

Post-translational modifications have been found to occur on β-catenin including serine/threonine phosphorylation, tyrosine phosphorylation, ubiquitylation, acetylation, and O-GlcNAc glycosylation. The aforementioned modifications can occur on the N-terminus, the C-terminus, or the surface of the armadillo repeat domain to further regulate β-catenin's cellular activity. Notably, tyrosine phosphorylation, in addition to its role in disassembling adherens junction as discussed earlier (Section 1.2), has long been implicated to affect the transcriptional activity of β-catenin. For example, phosphorylation of Tyr654 can increase β-catenin's interaction with the basal transcriptional machinery such as TBP, by disassociating the C-terminal from the armadillo repeat domain. Mutation of Tyr654 to glutamate released β-catenin from cadherins and enhanced its activity as a co-activator of transcription, although there is no data suggesting that nuclear β-catenin is phosphorylated at this site [7, 14]. Interestingly, phosphorylation of Tyr142 by c-Met acts as a molecular switch that transforms the adhesive form of β-catenin into one that preferentially binds to BCL9-2, which increased the transcription of *wnt* target genes. β-catenin that contained a mutated Tyr142 did not efficiently bind to BCL9-2 resulting in a dramatic decrease in *wnt* target gene transcription [63]. Similar outcomes were mimicked by the CK2-mediated phosphorylation of Thr393, which potentiated Wnt signaling by instilling β-catenin with resistance to proteosomal degradation and an elevated co-transcriptional function [64].

Levy et al., (2004) showed that lysine acetylation positively modulates β-catenin's transcriptional activity [65]. Specifically, acetylation at residue Lys345 located in armadillo repeat 6, increased binding affinity of β-catenin for Tcf-4, and required the acetyltransferase activity of co-activator, p300. Mutation on Lys345 severed the co-opertivity between p300 and β-catenin, which served to reduce β-catenin's co-activator function.

β-catenin has also been recently demonstrated to be post translationally modified by O-linked β-D-N-acetylglucosamine (O-GlcNAc) in breast cancer (MCF-7) cell line upon exposure to thapsigargin, a drug that induces cytoplasmic O-GlcNAcylation by activating a pathway localized at the endoplasmic reticulum [66, 67]. The O-GlcNAc modification of β-catenin was thereafter supported by various groups [66-69], although, the functional significance of this modification as well as the specific site(s) of O-GlcNAcylation of β-catenin were not established. Sayat et al., (2008) was the first to demonstrate the functional implications of O-GlcNAc modified β-catenin, where they established that increased O-GlcNAcylation attenuated the nuclear levels of β-catenin, increased its cytosolic levels and consequently decreased its transcriptional activity [67]. O-GlcNAcylation was a novel regulatory modification that was identified for β-catenin in addition to its well characterized regulation by phosphorylation.

1.5 O-GlcNAcylation

O-GlcNAcylation involves the single O-linked attachment of β-N-acetyl-glucosamine to the hydroxyl moiety of serine and/or threonine residues of proteins found in the nucleus and cytoplasm [70]. The nucleo-cytoplasmic enzymes O-GlcNAc-transferase (OGT) and β-N-acetyl-glucosaminidase (O-GlcNAcase, OGA) mediate the addition and removal of O-GlcNAc groups from proteins, respectively [71] (Figure 2). The attachment O-GlcNAc to nuclear and cytoplasmic proteins has only recently been recognized as a post translational modification fundamental to the regulation of cellular processes involving, nutrient sensing/metabolism (See Appendix A4.1), stress response, cell

cycle/division, transcription/translation (See Appendix A4.2) and cell signaling [72].

O-GlcNAcylation is exclusively found on cytoplasmic and nuclear proteins and is not modified or elongated to more complex structures. This makes O-GlcNAcylation distinct from classical glycosylation which mediates extracellular and luminal localization signals of proteins [73-76]. To date, over one thousand proteins have been identified to be O-GlcNAc modified and this number is rapidly growing as the technology for detecting O-GlcNAcylation improves [77]. Protein targets of O-GlcNAcylation are those involved in a wide range of functions including transcription factors, cytoskeletal proteins, nuclear pore proteins, chromatin proteins, molecular chaperones, as well as cytosolic and glycolytic enzymes, and, proteins of the proteosome [summarized in 78]. As such, O-GlcNAcylation has been established to be an additional regulatory mechanism, alongside phosphorylation, for site-specific modulation of protein function and behaviour including 1) protein stability/degradation, 2) subcellular localization, 3) protein-protein interactions, and 4) transcriptional activity [summarized in 70].

O-GlcNAcylation occurs on a timescale similar to phosphorylation [79] and has been reported to competitively occupy the same and/or adjacent sites of phosphorylation. Only recently has O-GlcNAcylation signaling and its cross talk with phosphorylation cascades become recognized as having important roles in cancer related processes.

1.5.1 Immunological detection of the O-GlcNAc modification

O-GlcNAcylation is generally undetected by commonly used analytical protein methods such as gel electrophoresis, as the addition of the sugar does not affect the migration of a polypeptide. The lectin, wheat germ agglutinin (WGA) continues to be the most useful tool to probe for O-GlcNAc [77, 80]; WGA has been used for the enrichment and detection of O-GlcNAc including O-GlcNAc groups on low copy number proteins [81]. However, the sensitivity of this method

is limited as proteins with multiple O-GlcNAc residues are readily detected. To date, there are no O-GlcNAc-specific lectins that have been identified. Antibodies have also been developed to react with O-GlcNAc in the context of protein structure including the monoclonal RL2 [82] and CTD 110.6 [83] antibodies. However, these antibodies are somewhat restricted in their target specificity [84]. The RL2 antibody is the only antibody that was raised against mammalian O-GlcNAc modified glycoproteins [85] which recognizes and binds estrogen induced O-GlcNAc modified protein [86]. Similarly, the limitations of the CTD 110.6 antibody was demonstrated by Isono (2011), who showed that glycosylation detected by CTD 110.6 cross reacted with N-GlcNAc$_2$ modified glycoproteins [87]. Although the available tools are limited, the use of WGA, RL2, CTD 110.6 have been reported in literature and generally accepted for the immunological detection of O-GlcNAcylation [87].

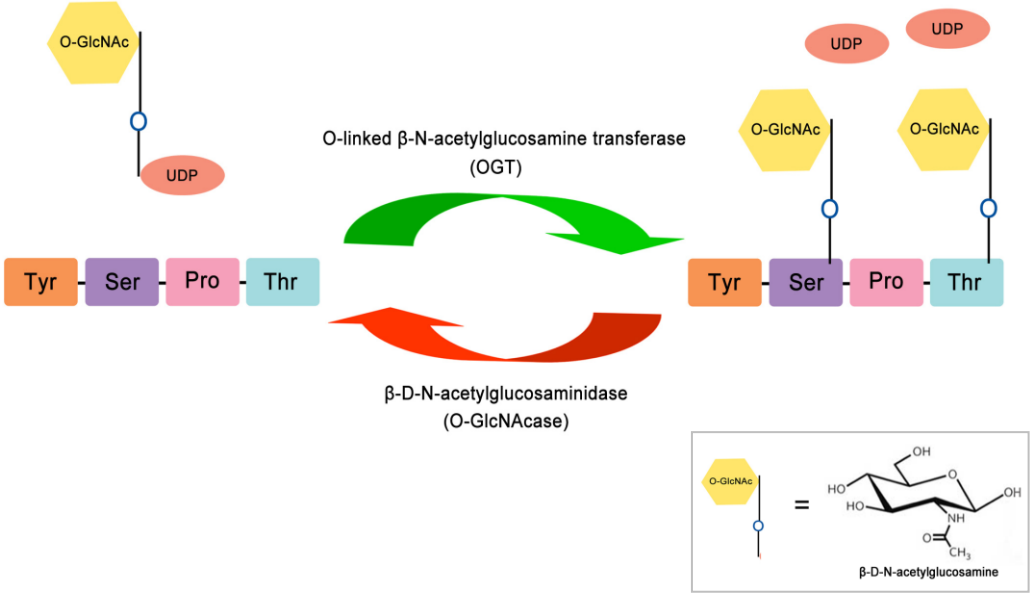

Figure 2—*Schematic representation of O-GlcNAcylation*. O-GlcNAcylation involves the single O-linked attachment of β-N-acetyl-glucosamine to the hydroxyl moiety of serine and/or threonine residues of proteins found in the nucleus and cytoplasm. The nucleocytoplasmic enzymes O-GlcNAc-transferase (OGT) and β-N-acetyl-glucosaminidase (O-GlcNAcase, OGA) mediate the addition and removal of O-GlcNAc groups from proteins, respectively.

1.5.2 The enzymes of O-GlcNAc cycling

The O-GlcNAcylation of nuclear and cytoplasmic proteins has been described in bacteria [77, 88, 89], and has also been documented in all multi-cellular organisms, including *C.elgans,* insects and plants [90]. Deletion of the OGT gene is embryonically lethal in mice suggesting the critical role of O-GlcNAcylation in cell survival [91]. Unlike phosphorylation, where a multitude of kinases and phosphatases regulate its addition and removal, O-GlcNAcylation relies only on the functions of OGT and O-GlcNAcase [78]. The very fact that OGT and O-GlcNAcase are single gene sets suggests that there are tightly controlled regulatory mechanisms in place to ensure substrate specificity.

1.5.2.1 OGT

Uridine diphospho-*N*-acetlyglucosamine: polypeptide-β-N-acetylglucosaminyltransferase (O-GlcNAc-transferase or OGT) is expressed in all cell types where its gene is found near the centromere of the X-chromosone (Xq13.1) [78]. Cloning of the OGT gene in rat, *C. elegans*, human, and plants demonstrated multiple splice variants, two of which are characterized to be mitochondrial OGT (mOGT) and nucleocytoplasmic OGT (ncOGT). Mitochondrial OGT is an active transferase that is tightly associated with the mitochondrial inner membrane [92] and has different substrate specificity than that of ncOGT [93]. Currently, mOGT is thought to be a splice variant of ncOGT; however, the functional implications for mOGT are unknown as studies have been limited [92].

Nucleocytoplasmic OGT is the most studied variant and is found within the nuclear and cytoplasmic compartments of the cell. It exists as a 110 kDa or 78 kDa isoforms which interact to form a 1) homotrimer comprising identical 110 kDa subunits or 2) heterotrimer consisting of two 110 kDa subunits and one 78 kDa subunit [78, 94]. The ncOGT isoform will be referred to as OGT.

OGT consists of a catalytic C-terminal domain containing the Uridine diphosphate (UDP)-GlcNAc (where UDP serves as the energy donor; See Appendix A4.1) binding site, and an N-terminal protein: protein interaction tetratricopeptide repeat (TPR) domain. These two domains are separated by a bipartite nuclear localization sequence [70]. The TPR motif consist of 34 amino acid repeat arranged in serial arrays [78, 95, 96] that fold into an elongated superhelix. Mammalian OGT contains 11.5 TPRs essential for inter-subunit interactions [70, 97-99], substrate recognition [70, 97-99] and protein-protein interactions [70, 100-103].

To date, there has been no absolute consensus sequence identified for directing OGT to its various substrates. Based on proteomics studies, OGT was identified to have a preference for Pro-Val-Ser-Thr and Thr-Thr-Ala containing sequences [78, 104]. Approximately one half of O-GlcNAc sites contain a Pro-Val-Ser (PVS) motif, while the other half does not have any commonalities except for their serine or threonine moieties [77]. The mechanism by which this single OGT has such a high degree of sequence specificity for its peptide substrates is still unclear [70]. Many of OGT interactions act to anchor and/or target OGT to signaling and transcriptional complexes. In effect, these interactions may modulate the activity of OGT by affecting its localization, regulating its binding partners, or targeting it to complexes specifically activated by signal transduction events.

1.5.2.2 O-GlcNAcase/OGA

β-N-acetylglucosamidase (O-GlcNAcase or OGA) is encoded by the meningioma expressed antigen 5 (MGEA5) gene located on chromosome 10 at position 10q24.1-q24.3 [84, 105]. It is a 104 kDa protein that catalyzes the removal of O-GlcNAc groups [77]. O-GlcNAcase exists as a nuclear cytosolic monomer with two functional domains, an N-terminal hexosaminidase catalytic domain and the C-terminal histone acetyltransferase (HAT) domain [70].

The structure of O-GlcNAcase is often over-simplified despite the recent advances in understandings of O-GlcNAcase's structure. As such, how O-GlcNAcase may interact with its substrates is currently not well understood. Schimpl et al., (2010) stated that there may be a putative domain comprising a stretch of over 300 amino acids between the hexosaminidase and HAT domains that is important for substrate recognition. This particular region was a conserved substrate binding groove which required distinct surface residues for binding its targets [106]. Using point mutation of residues lining this binding groove, Schimpl et al., (2010) demonstrated that certain residues were not required for the hydrolysis of 4MU-GlcNAc pseudosubstrates, but were necessary for the hydrolysis of specific glycoprotein substrates. The study by Schimpl et al., (2010) was the first reported evidence that elucidated specific interactions between OGA and its substrates proteins beyond the sugar moiety.

Over the past several years, the inhibition of O-GlcNAcase has been a topic of interest as these inhibitors can be valuable research tools. To experimentally increase O-GlcNAc levels within the cell, selective inhibitors of O-GlcNAcase have been developed which include streptozotocin (*N*-methyl-*N*-nitrosoureido D-glucosamine (STZ) [78, 107], 1, 2-dideoxy-2'-methyl-α-D-glucopyranoso-[2,1-d]-Δ2'-thiazoline (NAG-thiazoline), and O—(acetamido-2-deoxy-D-glucopyransylidene)-amino-*N*-phenylcarbamate (PUGNAc) [78, 108] (variants of these inhibitors have been developed in recent years). STZ was shown to be only a modest inhibitor of OGA *in vitro* and also induced apoptosis of pancreatic β-cells. PUGNAc, on the other hand, was able to increase cellular levels of O-GlcNAc without cell death. Currently, STZ is discouraged from use as an inhibitor of O-GlcNAcase as its efficacy has been questioned in literature. NAG-thazoline inhibits O-GlcNAcase with a K_i of 70nM however showed little selectivity for O-GlcNAcase [78]. In contrast, PUGNAc was determined to be a potent inhibitor of O-GlcNAcase with the K_i value of approximately 50nM; however, this was with poor selectivity as it could also inhibit functionally related human β-hexosaminidase which hydrolyses N-acetylgalactosamine (GalNAc)

residues [78]. Nevertheless, O-GlcNAcase has been determined to have absolute selectivity for GlcNAc-containing substrates and have no affinity for GalNAc containing compounds. PUGNAc's high potency somewhat facilitated avoiding non-specificity [78].

1.5.3 O-GlcNAcylation and phosphorylation

Many proteins are reciprocally modified by O-GlcNAc or O-phosphate under varying conditions. This includes O-GlcNAc or O-phosphate occupancy at the same, distant or proximal serine and threonine amino acid residue sites. Hart et al., (2011) state that this competitive interplay between these two post translational modifications is likely a result from either the large size of the O-GlcNAc residue (Stokes radius four to five times larger than a phosphate), the negative charge of the phosphate moiety, or by the induction of conformational changes in the protein by either modifications [77, 109, 110]. Yet, on some proteins, both modifications have been found to exist simultaneously at different sites. Thus, by altering the pattern of serine and/or threonine sites available for phosphorylation, O-GlcNAcylation can alter phosphorylation patterns and protein function [70]. In support of this, Wells et al., (2004) demonstrated that OGT and protein phosphatase 1 (PP1) exist together in a complex [70, 111], indicating that this enzyme complex can both remove phosphate and add O-GlcNAc on the same protein.

The dynamic cross talk between O-GlcNAcylation and phosphorylation is quite extensive. Wang et al., (2007) quantified the phosphorylation dynamics at a single site level by globally increasing O-GlcNAcylation by the inhibition of O-GlcNAcase using a proteomic approach followed by Mass Spectrometry (MS) analysis [112]. Upon examining 711 phosphorylation sites, increasing global O-GlcNAcylation by inhibiting O-GlcNAcase, virtually increased or decreased every actively cycling phosphorylation site (as determined by sensitivity to phosphatase inhibitor okadaic acid) [77, 112]. Additionally, in a large scale proteomic study, lithium chloride (LiCl) inhibition of a single kinase, GSK3β,

increased O-GlcNAcylation of different protein substrates [73, 77, 113]. However, on others, the levels of O-GlcNAcylation decreased, indicating that the relationship between these two modifications was not simply reciprocal [73].

The data obtained from high through-put analyses suggest an extreme complex crosstalk between O-GlcNAcylation and phosphorylation. This molecular diversity is one way in which cells may regulate and adjust to the constant changes within the cellular environment. Given the interplay between phosphorylation and O-GlcNAcylation in the regulation of protein function as well as the known roles of phosphorylation in the mechanisms underlying cancer, it is no surprise that O-GlcNAcylation is involved in cancer etiology [77].

1.5.4 O-GlcNAcylation of oncogenes and tumor suppressors

The dynamic interplay between phosphorylation and O-GlcNAcylation in the regulation of protein function has been described for various tumor suppressors and oncoproteins in multiple cancers. For example, the levels of the tumor suppressor p53, a major gatekeeper of genomic stability and a critical component to the cellular stress response is tightly regulated by phosphorylation at Thr155 which targets the protein for proteosomal degradation. However, when p53 is O-GlcNAcylated at Ser149, this antagonizes its phosphorylation at Thr155, ultimately inhibiting the degradation of p53 [77, 114]. More often than not, p53 is mutated or functionally inactivated in tumors. Paradoxically, cells that have lost p53 switch to a highly glycolytic metabolism resulting in increased glucose consumption and elevated global protein O-GlcNAcylation [73].

In addition, several studies have also demonstrated a role for O-GlcNAcylation in cell cycle progression. C-Myc, a potent oncogene that regulates cell growth and division, is O-GlcNAcylated at Thr58 which is also a GSK3β phosphorylation site. Reciprocal modification of Thr58 of c-Myc by phosphorylation and O-GlcNAcylation was observed in rapidly dividing and non-dividing cells, respectively [77]. Also, the tumor suppressor, retinoblastoma protein (pRb) is O-

GlcNAc modified when it suppresses E2F-1 transcription, a transcription factor that mediates gene expression required for entry into S phase of the cell cycle. The suppression of E2F-1 transcription by pRb is blocked when pRb is phosphorylated during mid-to late-G1 of the cell cycle [77, 115].

The emerging functions of O-GlcNAcylation as key player of cancer relevant processes was further substantiated by its regulatory role in the epithelial-mesenchymal transition. Under normal conditions, SNAIL1, a negative regulator of E-cadherin gene expression, is phosphorylated by CK1 and GSK3β to subsequently promote its ubiquitination and proteosomal degradation [116, 117]. Upon O-GlcNAcylation, however, phosphorylation of GSK3β is blocked [117], resulting in the stabilization of SNAIL1 and in turn, leading to increased SNAIL1 expression and repression of E-cadherin [116].

Currently, our knowledge of the mechanisms underlying O-GlcNAcylation and its role in malignancy is limited. Inconsistencies in O-GlcNAc signaling have been observed for various tumour samples as well as in multiple pathways important for cell proliferation and metastasis. However, it is evident that O-GlcNAcylation provides an additional level of regulation in signal transduction allowing for the precise control of cell regulatory mechanisms. Therefore, understanding O-GlcNAcylation and its regulation on the mediators of tumorigenesis will offer greater insight into the complexities and contexts of cancer progression.

1.5.4.1 β-catenin and O-GlcNAcylation

The cross regulation of β-catenin function by phosphorylation and O-GlcNAc modification is quite evident, with phosphorylation regulating its degradation, and, as mentioned previously (Section 1.4), with O-GlcNAcylation regulating its localization and transcriptional activity. Specifically, Sayat et al., (2008) found that upon increasing O-GlcNAcylation using the O-GlcNAcase inhibitor, PUGNAc, cellular levels of O-GlcNAc-β-catenin increased in prostate cancer (CaP) cell lines [67]. This was accompanied by a decrease in the nuclear levels of

β-catenin and a corresponding increase in its cytoplasmic levels. TOPflash luciferase reporter assays (a direct measure of β-catenin transcriptional activity) indicated that the transcriptional activity of β-catenin was inversely related to its O-GlcNAcylated levels [67]. TCF-β-catenin interactions were significantly reduced upon O-GlcNAcylation while O-GlcNAcylated β-catenin-APC interactions remained unchanged. Taken together, these data suggested that O-GlcNAcylation of β-catenin negatively regulated its nuclear localization and transcriptional activity. Although the functional implications of O-GlcNAcylated β-catenin were established by Sayat et al., (2008), the specific sites of O-GlcNAc modification that may be mediating these effects were yet to be identified.

Recently, plakoglobin, a homologue of β-catenin, was found to be O-GlcNAcylated at Thr14 near its N-terminal 'destruction box' [69]. Plakoglobin and β-catenin share 65% sequence homology at the armadillo domain but are divergent at the N-terminal domain with the exception of some highly conserved phosphorylations sites [69]. Sequence alignment indicated that Thr14 was homologous to Serine 23 (Ser23) of β-catenin [69].

1.6 Hypotheses

i. Serine 23 is a site for O-GlcNAcylation.

ii. O-GlcNAcylation of β-catenin at Serine 23 alters its association with cytosolic and nuclear interactors to regulate its subcellular distribution and transcriptional activity.

1.7 Objectives

i. To identify Serine 23 as a putative site for O-GlcNAc modification of β-catenin.

ii. To determine the functional relevance of the O-GlcNAcylation of Serine 23 in regulating β-catenin's subcellular localization and transcriptional function within the cell.

Chapter 2
Materials and Methods

2.0 Materials and Methods

2.1 Constructs and transformation

To elucidate the site(s) of O-GlcNAc modification, a Serine 23 to Glycine (S23G) mutation was introduced to the full length or the N-terminal (NT) domain of recombinant β-catenin subloned into a green fluorescent protein (GFP) expression vector (pEGFP-C2) using site directed mutagenesis as per manufacturer's protocols (Stratagene). Contrary to Hatsell et al., (2003) in which Thr14 was substituted to alanine, Ser23 of β-catenin was substituted with glycine. The decision to do so was based on the observation that a serine to alanine substitution required two point mutations (AGT to ACG) to introduce this missense mutation, whereas a serine to glycine mutation only required one (AGT to GGT). Furthermore, a glycine substitution is recognized to have the same effect as an alanine substitution, as they both remove the side chain beyond the β-carbon of an amino acid residue while not altering the main-chain conformation [118, 119].

Recombinant wild type NT, S23G NT mutant (S23G-NT), Wild Type full length (WT), S23G mutant full length (S23G), Armadillo repeats 1-12 (AF), Armadillo repeats 1-6 (A1-6), Armadillo repeats 7-12 (A7-12), wild type C-terminal (CT) , and Ser33A, Ser37A, Ser45A,Thr41A quadruple full length mutant (4M) (a gift from Dr. D.W. Andrews) β-catenin constructs subcloned into pEGFP-C2 expression vector, were introduced to Max Efficiency DH5α E.coli (New England Biolabs) according to manufacturer's transformation procedures. Positive colonies were amplified in Luria-Bertani (LB) media containing Kanamycin. Plasmid constructs were purified using Qiagen Plasmid Purification Kit (Qiagen) as per manufacturer's protocols.

2.2 Cell culture and transfection of plasmid constructs

Human cancer cell lines, DU145 CaP, and, SAOS2 and U2OS osteosarcoma (American Type Culture Collection) were grown in Dulbecco's

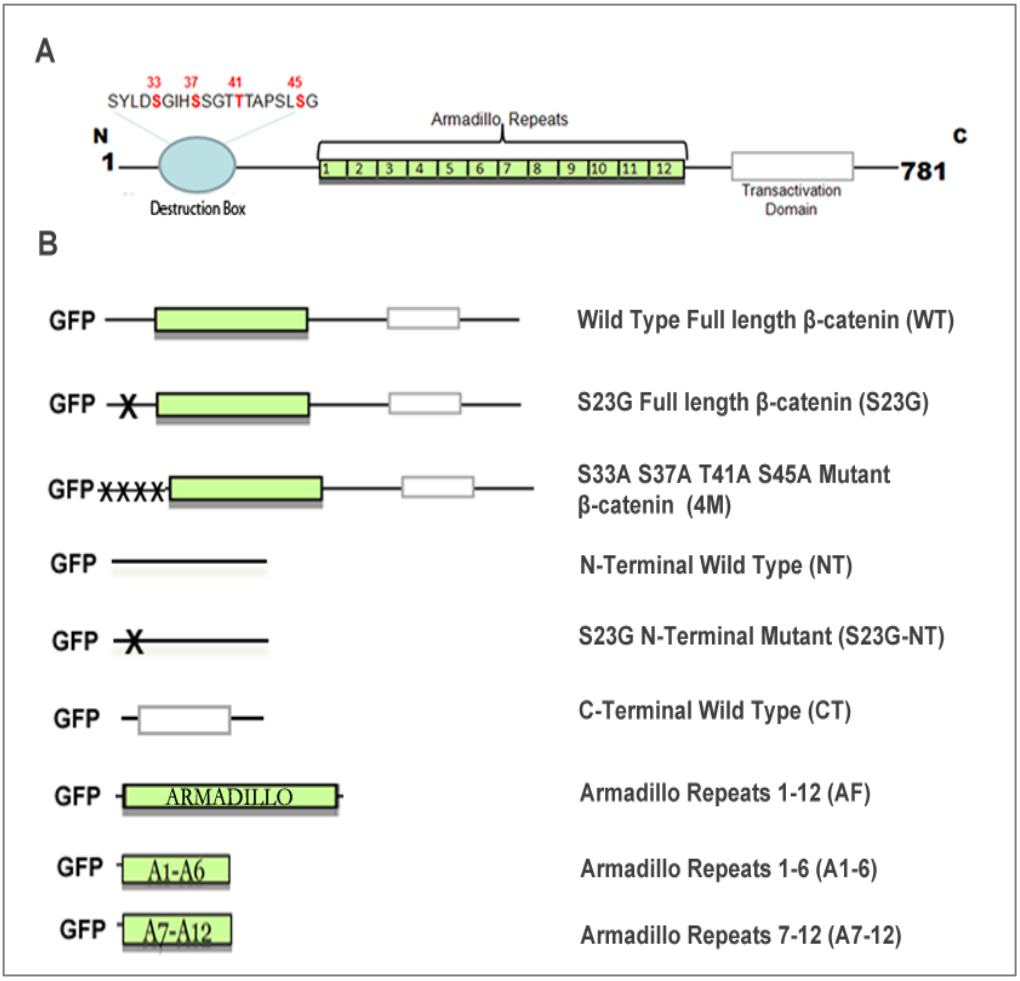

Table 1— *Schematic representation of β-catenin and β-catenin constructs.* A) Illustration of the protein structure of β-catenin and its three primary domains. **B)** Representation of GFP tagged β-catenin constructs used within this study.

Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomyacin. All cells were maintained in 5% CO_2 at 37°C. Cells were grown to 70% confluency and transfected with pEGFP-C2·h-NT, pEGFP-C2·h-S23G-NT, pEGFP-C2·h-CT, pEGFP-C2·h-WT, pEGFP-C2·h-S23G, pEGFP-C2·h-AF, pEGFP-C2·h-A1-6, pEGFP-C2·h-A7-12, pEGFP-C2·h-4M or pEGFP-C2 empty vector control (3μg). All transfections utilized Lipofectamine2000 (Invitrogen) according to manufacturer's protocols.

2.3 PUGNAc treatment

O-(2-acetamido-2-deoxy-D-gluco-pyranosylidene)-amino-N-phenylcarbamate (PUGNAc) (Toronto Research Chemicals) was dissolved in sterile water to a concentration of 20 mM, and diluted to a final concentration of 100 μM. Prior to PUGNAc treatment, DU145, SAOS2 and U2OS cells were incubated for 18 hours with media containing DMEM 1% FBS. Thereafter, cells were treated with 100 μM PUGNAc in the absence of serum for 14 hours.

2.4 Whole cell lysis and nuclear extracts

Whole cell lysates were obtained by incubating transfected cells with 100 μl of lysis buffer (NP-40-DOC Buffer: 10 mM Tris–HCl pH 7.5, 1% NP-40, 0.5% Sodium Deoxycolate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 80 ng/ml aprotinin, 40 ng/ml chymostatin, 40 ng/ml antipain, 40 ng/ml leupeptin, 40 ng/ml pepstatin) on ice for 10 minutes. Cellular debris was removed by centrifugation for 5 minutes at 16,000 *x g*. Nuclear and cytosolic extracts were separated using the NE-PER Nuclear Cytoplasmic Extraction Kit as described by the manufacturer's protocols (ThermoScientific). Protein concentrations were determined using Bicinchoninic Protein Determination Assay (ThermoScientific).

2.5 Immunoblotting

Equivalent protein quantities were resolved by 8% Tricine polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked in 1% Bovine Serum Albumin

(BSA) and incubated with WGA-Horsh Radish Peroxidase (HRP) or blocked with 3% BSA and incubated with various antibodies at 4˚C overnight. Western blots were visualized using Western Lightning® Plus-ECL (PerkinElmer, LAS Inc.). Antibodies to the following proteins were used for this study: β-Catenin (Cell Signalling), E-cadherin (Cell Signalling), TCF (Cell Signalling), α-β-Tubulin (Cell Signalling), Lamin B (Calbiochem), Actin (Santa Cruz Biotechnology Inc.), GFP (AbCam; Santa Cruz Biotechnology Inc.) followed by peroxidase-conjugated secondary antibodies (GE Healthcare UK Limited). Densitometric analysis was performed using IMAGEJ Software. Histograms are representative of three or more independent experiments and are reported as a fold change of control set at 1.0. Statistical analysis was performed by Student's t-test (*$p<0.05$) using SigmaPlot Software.

2.6 Isolation and determination of O-GlcNAcylated proteins

O-GlcNAcylated β-catenin was determined by one of two methods. O-GlcNAcylated proteins were isolated by precipitation with WGA-agarose beads (Vector Laboratories). WGA-agarose beads were incubated with 100 µg of cell lysate in 200 µL of lysis buffer overnight at 4˚C. Thereafter, beads were precipitated by centrifugation at 4˚C and samples were washed with phosphate buffered saline (PBS). Beads were then eluted by SDS PAGE sample buffer. Complexes were separated on 8% Tricine polyacrylamide gels and proteins were immunoblotted with anti-β-catenin (Cell Signalling) or anti-GFP (AbCam; Santa Cruz Biotechnology Inc.) antibodies. Alternatively, levels of O-GlcNAcylated β-catenin was determined by immunoprecipitation of 100 µg of cell lysates with either anti-β-catenin (Cell Signalling) or anti-GFP (AbCam) antibody preabsorbed to Protein A/G agarose beads (Santa Cruz Biotechnology Inc.). Samples were washed with PBS and eluted by SDS PAGE sample buffer. Isolated complexes were separated on 8% Tricine polyacrylamide gels and characterized by Western Blot analysis. O-GlcNAc modified proteins were identified using WGA-HRP (Vector Laboratories) at a dilution of 1µg/ml in 1% BSA. Each sample condition (-/+PUGNAc) was normalized to their internal controls to ensure equal immunoprecipitation of the

protein of interest. Alterations in O-GlcNAcylation was based on comparisons between -/+ PUGNAc conditions. Where indicated, input was 25% of initial load.

2.7 Immunoprecipitation

100 µg of protein lysate were incubated with anti-β-catenin (Cell Signalling), anti-E-cadherin (Cell Signalling), anti-TCF (Cell Signalling) or anti-GFP (AbCam) antibodies preabsorbed to Protein A/G agarose beads (Santa Cruz Biotechnology Inc.). Beads were precipitated by centrifugation at 4˚C. Samples were washed with PBS and eluted by SDS PAGE sample buffer. Isolated complexes were separated on Tricine polyacrylamide gels and characterized by Western Blot analysis.

2.8 Immunocytochemistry

Cells were cultured onto coverslips for immunocytochemical analysis of the expression and localization of specific proteins. Briefly, cells were washed with PBS and fixed with 4% formaldehyde and subsequently incubated in cold 100% methanol at -20°C for 10 minutes for cell membrane permeabilization. Cells were stained with anti-β-catenin antibody (Cell Signalling) followed by incubation with AlexaFluor ® 488 conjugated secondary antibody (Invitrogen) for visualization. GFP tagged β-catenin proteins constructs were visualized by monitoring GFP fluorescence. Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). Cells were viewed by Carl Zeiss Laser Scanning Microscope and data was analyzed using LSM510 software.

2.9 RT-qPCR

DU145 cells were allowed to reach 70% confluency in DMEM 10% FBS media. Cells were transfected with plasmid constructs and subsequently treated with PUGNAc (100 µM). Total RNA was isolated from DU145 cells using RNeasy Qiagen Kit according to the manufacturer's instructions and quantified spectrophotometrically. 1 µg of total RNA was reverse-transcribed using SuperScript® II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantification of *wnt* target gene expression, specifically Cyclin D1 and

VEGF, were assessed using Power SYBR Green PCR Master Mix (Applied Biosystems). Samples were amplified with a precycling hold at 95 °C, 15 seconds, 30 cycles of annealing and extension at 60 °C for 1 minute. Primer oligonucleotides used for this experiment are as follows:

Cyclin D1	
Sense	5′ – CTGGCCATGAACTACCTGGA – 3′
Anti-Sense	5′ – GTCACACTTGATCACTCTGG – 3′
VEGF	
Sense	5′ – GCAGAATCATCACGAAGTGG – 3′
Anti-Sense	5′ – GCATGGTGATGTTGGACTCC – 3′
GAPDH	
Sense	5′ – ACCTGGTGCTCAGTGTAGCC – 3′
Anti-Sense	5′ – CAATGACCCCTTCATTGACC – 3′

Table 2— Select primer oligonucleotides for Cyclin D1, VEGF and GAPDH used for RT-qPCR analysis of *wnt* target gene expression.

Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) gene served as the endogenous control. Each measurement was performed in triplicate with Rotor-Gene 3000 instrument (Montreal Bio-tech Inc.) and analysed using ROTOR-GENE-6 Software. Gene expression was determined using the relative standard curve method normalized to GAPDH binding protein expression. Histograms are reported as a fold change of control which was set at 1.0. Statistical analysis was performed by Student's t-test (*$p<0.05$) using SigmaPlot Software.

2.10 TOPflash/FOPflash luciferase reporter assay

DU145 cells were co-transfected with 2 µg of GFP tagged WT or S23G full length β-catenin and 2 µg of β-catenin reporter construct containing consensus wild type (TOPflash) or mutated (FOPflash) TCF binding site upstream of a luciferase reporter gene [120]. After 24 hours, transfectants were treated with

PUGNAc as mentioned previously and luciferase activity was measured. pRL-TK *renilla* luciferase was the internal transfection control. Reporter assays were performed using the Dual-Luciferase Reporter Kit (Promega). Luminescence was quantified using FLUOstar OMEGA Multi-mode microplate reader (BMG Labtech). Histograms are representative of three independent experiments.

Chapter 3

Results

3.0 Results

3.1 β-catenin is O-GlcNAc modified at its N-terminal domain.

In order to decipher the putative domain at which β-catenin was actively cycling O-GlcNAc groups, DU145 CaP cell line was transfected with GFP tagged N-terminal (NT), C-terminal (CT), Armadillo repeats 1-12 (AF), Armadillo repeats 1-6 (A1-6), or Armadillo repeats 7-12 (A7-12) domains and treated with or without 100 μM PUGNAc (as described in Materials and Methods). Alterations in the levels of O-GlcNAcylation were characterized by WGA-agarose precipitation, followed by Western Blot analysis.

As shown in Figure 3A, there was a 30% induction of O-GlcNAcylation of β-catenin within the NT domain upon PUGNAc treatment. This indicated that there was active cycling of O-GlcNAc modification occurring at the NT domain. On the other hand, the CT domain was observed to have high levels of base line O-GlcNAcylation; however, O-GlcNAcylation was not inducible despite PUGNAc treatment which suggested that there was no active cycling of O-GlcNAc modification occurring at the CT domain (Figure 3A). In contrast, there was no O-GlcNAcylation present for the AF, A1-6 and A1-7 domains of β-catenin upon PUGNAc treatment (Figure 3B). The pEGFP empty vector served as a control; there was no alteration in O-GlcNAc modification of the GFP protein upon PUGNAc treatment.

3.2 β-catenin is O-GlcNAc modified at Serine 23.

As the NT domain was found to be the region of active O-GlcNAc cycling, a Serine 23 to Glycine mutation was introduced to the NT domain (S23G-NT) in order to characterize Ser23 as a potential site for O-GlcNAc modification.GFP-S23G-NT and GFP-wild type NT domains were transfected into DU145 cells and treated with or without 100 μM PUGNAc. Alterations in the levels of O-GlcNAcylation were characterized by WGA-agarose precipitation.

Introduction of a S23G mutation to the NT resulted in no change in O-GlcNAcylation relative to GFP-wild type NT domain upon PUGNAc treatment (Figure 4A). This suggested that Ser23 was a site for O-GlcNAcylation. The presence of baseline detection of O-GlcNAc modification with an S23G mutation suggests that alternative sites of O-GlcNAcylation may exist within the NT domain.

In order to assess the functional impact of Ser23 on β-catenin regulation, a S23G mutation was introduced to a GFP tagged full length β-catenin construct (S23G). Subsequently, *full length* GFP-S23G and *full length* GFP-Wild Type (WT) β-catenin were transfected into DU145 cells and treated with or without PUGNAc. Alterations in the levels of O-GlcNAcylation were characterized by both WGA-agarose precipitation (Figure 4B) and WGA-HRP (Figure 4C). Similar to our NT construct, levels of O-GlcNAc in full length GFP-WT β-catenin was increased relative to the untreated group (Figure 4B and 4C); the full length GFP-S23G β-catenin had no significant induction of O-GlcNAc modification upon PUGNAc treatment (Figure 4B and 4C).

3.3 N-terminal phosphorylation sites of β-catenin are not O-GlcNAc modified.

As the NT is key to β-catenin's stabilization, it was necessary to determine whether the O-GlcNAcylation of β-catenin affected the phosphorylation status of β-catenin. DU145 cells were transfected with a GFP tagged Ser33A, Ser37A, Thr41A and Ser45A quadruple mutant (4M) β-catenin construct in which all four phosphorylation sites of the destruction box were mutated, and, subsequently treated with 100 μM PUGNAc for a set time course (Figure 5). Upon PUGNAc treatment, GFP-4M β-catenin demonstrated increased O-GlcNAcylation in a time dependent manner. This increase in O-GlcNAcylation was in parallel to endogenous β-catenin, indicating that the NT phosphorylation sites of β-catenin were not sites of O-GlcNAc modification and that these sites did not affect the dynamics of O-GlcNAc modification of β-catenin.

3.4 Serine 23 regulates the subcellular distribution of β-catenin.

To observe the effects of the O-GlcNAc modification of Ser23 on the subcellular distribution of β-catenin, immunofluorescence (IF) analysis of endogenous β-catenin, and, recombinant full length GFP-WT and GFP-S23G β-catenin constructs were performed in the presence or absence of PUGNAc treatment. Figure 6 shows that there was cytoplasmic and nuclear distribution of endogenous, GFP-WT and GFP-S23G β-catenin in untreated DU145 CaP cells. Interestingly, when cells were treated with 100 μM PUGNAc, both endogenous and GFP-WT β-catenin preferentially translocated to the plasma membrane; however, localization of the GFP-S23G mutant β-catenin remained unchanged upon PUGNAc treatment. This suggested that Ser23 was not only an important site for O-GlcNAc modification but also played a role in regulating the subcellular distribution/localization of β-catenin.

3.5 Serine 23 regulates β-catenin's interaction with E-cadherin.

The observation that O-GlcNAcylation of endogenous and GFP-WT β-catenin resulted in a preferential relocalization of the protein to the plasma membrane, there was question to whether this modification played a role in regulating β-catenin's interactions with its major cellular interactors at the cell membrane, namely E-cadherin. First, endogenous β-catenin was immunoprecipitated from PUGNAc treated DU145 whole cell lysates using anti-β-catenin antibody. Figure 7A indicates that there was an increase in the interaction of endogenous β-catenin with E-cadherin upon PUGNAc treatment. Next, it was necessary to observe whether Ser23 was a potential site that altered this interaction. In accordance with IF, immunoprecipitation of GFP-WT and GFP-S23G β-catenin constructs from DU145 whole cell lysates using anti-GFP antibody showed that GFP-WT β-catenin had increased interactions with E-cadherin upon O-GlcNAcylation (Figure 7B). However, immunoprecipitation of GFP-S23G β-catenin demonstrated no alterations in its interactions with E-cadherin relative to untreated cells. These observations were validated with reciprocal immunoprecipitation. WGA-agarose precipitation or WGA-HRP analysis

confirmed increased O-GlcNAcylation of endogenous and recombinant β-catenin, respectively (Figure 7A and 7C). pEGFP empty vector and total non-specific IgG antibody served as the internal controls.

3.6 Serine 23 regulates β-catenin's nuclear localization.

By IF and Western blot analysis, the redistribution of β-catenin to the plasma membrane indicated that nuclear levels of β-catenin would correspondingly be altered. In order to confirm this distribution, nuclear lysates of DU145 CaP cells transfected with either full length GFP-WT or GFP-S23G β-catenin were treated with or without PUGNAc. Upon PUGNAc treatment, there was a significant decrease in the nuclear levels of GFP-WT β-catenin, while nuclear levels of GFP-S23G mutant remained unaltered (Figure 8). These results reinforced that the O-GlcNAcylation of Ser23 regulated the subcellular distribution and nuclear availability of β-catenin.

3.7 O-GlcNAcylation at Serine 23 decreases β-catenin's interaction with TCF.

The decrease in nuclear β-catenin suggested that TCF-β-catenin interactions would similarly be reduced. As such, immunoprecipitation analysis was performed with nuclear lysates of PUGNAc treated DU145 cells transfected with GFP-WT and GFP-S23G β-catenin. In accordance with Figure 8, preliminary results in Figure 9 show that TCF and GFP-WT β-catenin interactions decreased upon PUGNAc treatment. On the other hand, GFP-S23G β-catenin and TCF interactions remained unaltered despite PUGNAc treatment. The presence of increased O-GlcNAcylation of β-catenin was confirmed with WGA-agarose precipitation.

3.8 O-GlcNAcylation of β-catenin decreases the transcription of Wnt target genes, VEGF and Cyclin D1.

Decreased levels of β-catenin together with decreased β-catenin-TCF interactions within the nucleus would suggest a decrease in TCF-β-catenin mediated

transactivation of *wnt* target genes. Using RT-qPCR, *wnt* target gene expression, specifically the transcriptional activation of Cyclin D1 and vascular endothelial growth factor (VEGF), were analyzed. RT-qPCR analysis indicated significant decreases in mRNA copy number of Cyclin D1 (~40%) and VEGF (~40%) upon PUGNAc treatment in GFP-WT β-catenin transfected DU145 cells (Figure 10). This was expected as nuclear levels of β-catenin and TCF-β-catenin interactions were decreased with increased O-GlcNAcylation (Figure 8 and 9). In contrast, levels of Cyclin D1 and VEGF mRNA copy number in DU145 cells transfected with GFP-S23G mutant β-catenin was higher relative to GFP-WT β-catenin and unaltered despite PUGNAc treatment.

3.9 O-GlcNAcylation decreases the transcriptional activity of β-catenin.

To further confirm the observed decrease in target gene expression was in fact due to the altered nuclear availability of β-catenin, β-catenin's transcriptional activity was investigated using TOP/FOPflash luciferase reporter assay. TOP/FOPflash reporter assay is recognized to be a direct representation of β-catenin's transcriptional activity. Luciferase activity was quantified in the presence or absence of PUGNAc treatment. pRenilla served as the transfection control.

Luciferase activity was decreased upon PUGNAc treatment in GFP-WT β-catenin transfected DU145 cells (Figure 11). In contrast, TOPflash luciferase activity was greater and also unaltered in DU145 cells transfected with GFP-S23G β-catenin. These results are consistent with the RT-qPCR data previously mentioned. Although there was a trend in decreased transcriptional activity of GFP-WT β-catenin with PUGNAc treatment, these results were not statistically significant. Collectively, the lower levels of nuclear localization of β-catenin upon O-GlcNAc modification suggested that O-GlcNAcylation played a role in regulating the transcriptional activity of the protein.

3.10 O-GlcNAcylation of β-catenin is cell line specific: O-GlcNAcylation regulates the subcellular localization of β-catenin in U2OS Osteosarcoma cell line, but not SAOS2.

The observed effect of the O-GlcNAc modification on β-catenin's localization and transcriptional activity brought perspective to its role in CaP tumorigenesis. In order to extrapolate this mechanism to other cancers, the effect of O-GlcNAcylation of β-catenin in osteosarcoma cell lines, U2OS and SAOS2 was elucidated. Like DU145, both U2OS and SAOS2 cell lines expressed endogenous β-catenin (Figure 12A and 12B) and with low levels of the O-GlcNAcylated form of the protein. PUGNAc treatment resulted in an increase in the O-GlcNAcylation of endogenous β-catenin in U2OS cell line (Figure 12A); interestingly, the SAOS2 cell line had minimal to undetectable levels of O-GlcNAcylation and no observable induction of O-GlcNAc modification (Figure 12B).

To characterize whether this induction of O-GlcNAcylation regulated the subcellular localization of β-catenin in a comparable manner to that of DU145 CaP cell line, immunofluorescence analysis of endogenous β-catenin in U2OS and SAOS2 was performed. Immunofluorescence analysis indicated that β-catenin was distributed both within the cytosol and nucleus in both U2OS and SAOS2 cell lines (Figure 12C). Upon PUGNAc treatment, there was a redistribution of β-catenin from the nucleus to the plasma membrane in the U2OS cells; however there seemed to be no significant change in the subcellular distribution of endogenous β-catenin in the SAOS2.

3.11 Figures and Figure Legends

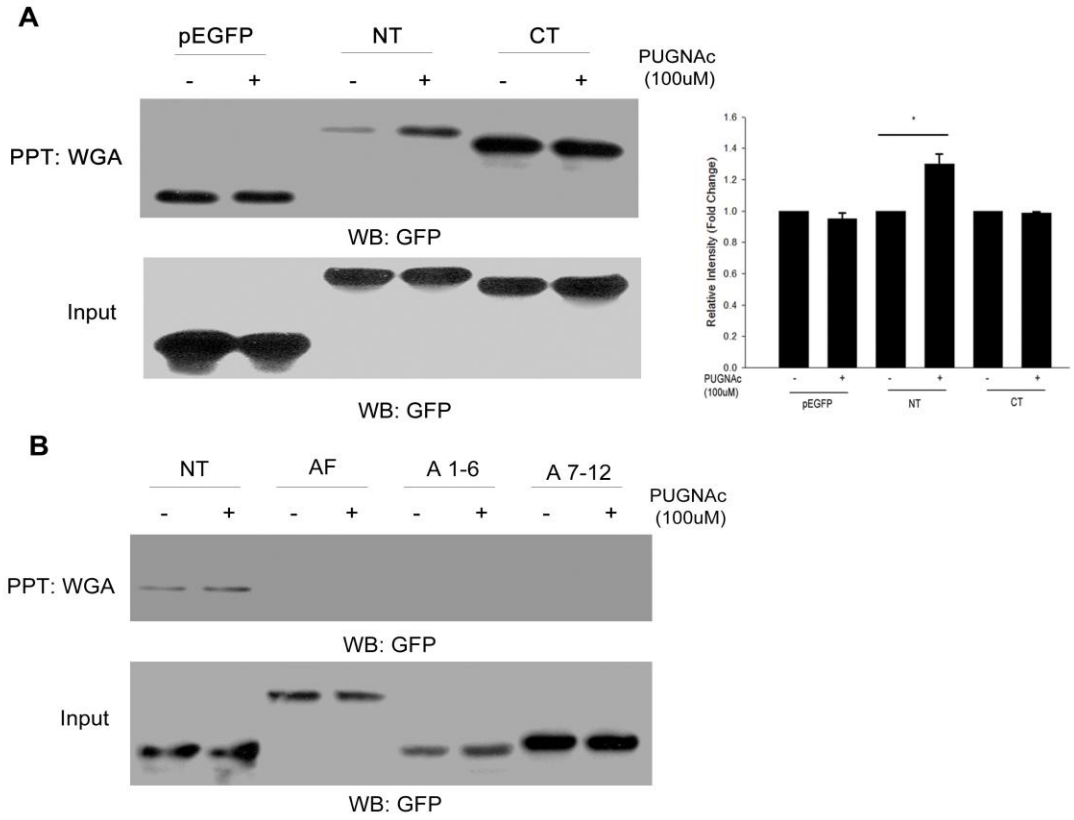

Figure 3—*N-terminal but NOT the Armadillo and C-terminal domain of β-catenin is O-GlcNAc modified.* Whole cell lysates of DU145 cells transfected with GFP tagged N-terminal (NT), C-terminal (CT), Armadillo repeats 1-12 (AF), or, Armadillo repeats 1-6 (A1-6) and 7-12 (A7-12) were treated with or without 100 µM PUGNAc, precipitated with WGA-agarose beads, and characterized by Western blot analysis. **A)** There was a 30% induction of O-GlcNAcylation at the NT domain upon PUGNAc treatment, suggesting the presence of active O-GlcNAc cycling within this region. High baseline levels of O-GlcNAc modification was observed for the CT domain; however, O-GlcNAcylation at the CT domain was not inducible with PUGNAc treatment. **B)** NT domain was O-GlcNAc modified while Armadillo domain was not. *$p<0.05$.

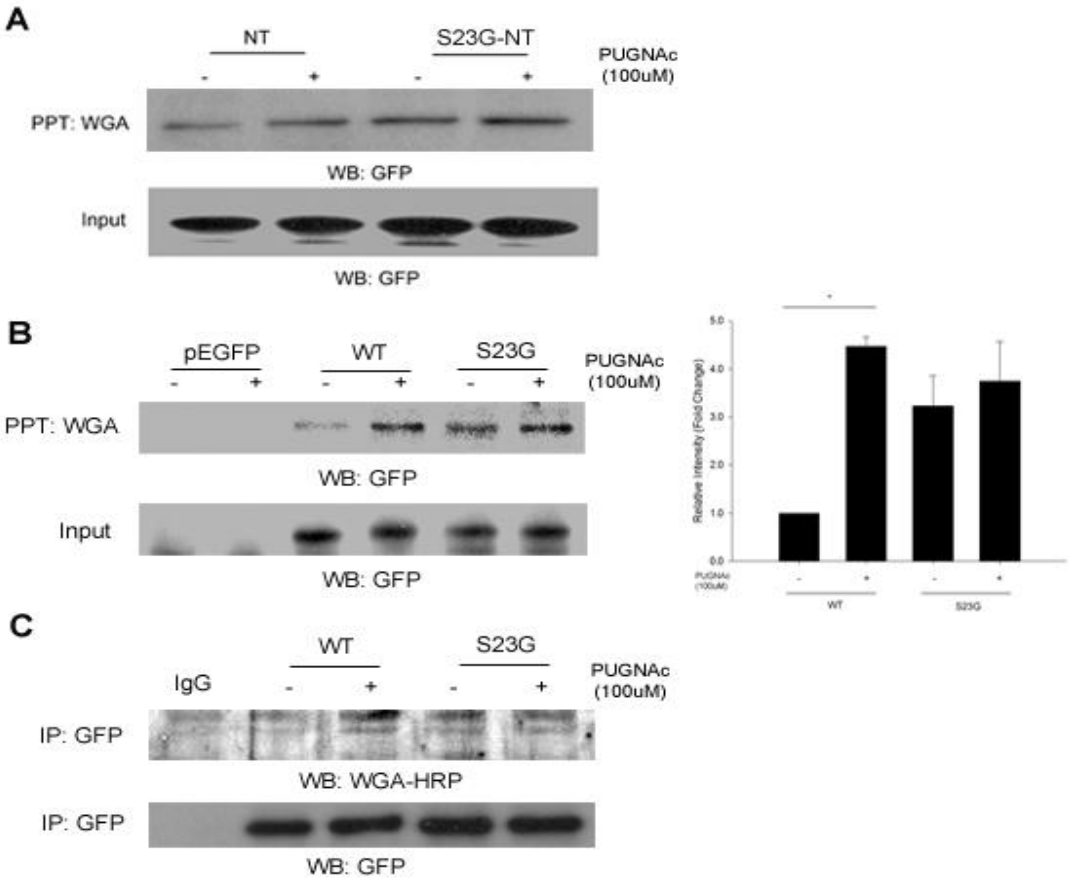

Figure 4—*Serine 23 is a site for O-GlcNAc modification.* A) DU145 cells transfected with wild type GFP-NT or mutant GFP-S23G-NT β-catenin constructs treated with or without 100 μM PUGNAc. Levels of O-GlcNAcylation at the NT domain increased upon treatment with PUGNAc. There was no change in O-GlcNAc levels of S23G-NT despite PUGNAc treatment. **B) and C)** DU145 cells transfected with GFP tagged *full length* WT or *full length* S23G mutant β-catenin with or without PUGNAc treatment. Levels of O-GlcNAcylated full length GFP-WT β-catenin increased with PUGNAc treatment. O-GlcNAc levels of full length GFP-S23G mutant β-catenin were not altered upon PUGNAc treatment. Alterations in O-GlcNAc levels were characterized by **B)** WGA-agarose precipitation of whole cell lysates followed by Western Blot analysis with anti-GFP antibody or **C)** immunoprecipitation of whole cell lysates with anti-GFP antibody followed by Western Blot analysis with WGA-HRP. *$p<0.05$.

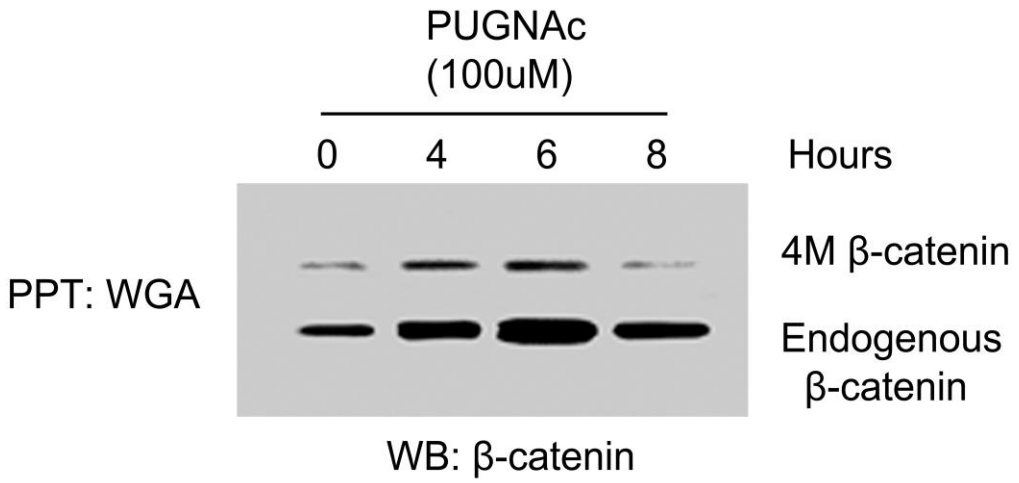

Figure 5—*Ser33, Ser37, Thr41, Ser45 of β-catenin are not sites of O-GlcNAc modification.* DU145 cells transfected with GFP tagged Ser33A, Ser37A, Thr41A, Ser45A quadruple mutant (4M) β-catenin were treated with 100 μM PUGNAc for a set time course. Whole cell lysates were precipitated with WGA-agarose beads and characterized with anti-β-catenin antibody to detect any alterations in O-GlcNAcylation. O-GlcNAcylation of the GFP-4M β-catenin increased in a time dependent manner parallel to endogenous β-catenin. The observed increase in O-GlcNAcylation indicated that Ser33, Ser37, Thr41, Ser45 were not sites of O-GlcNAc modification.

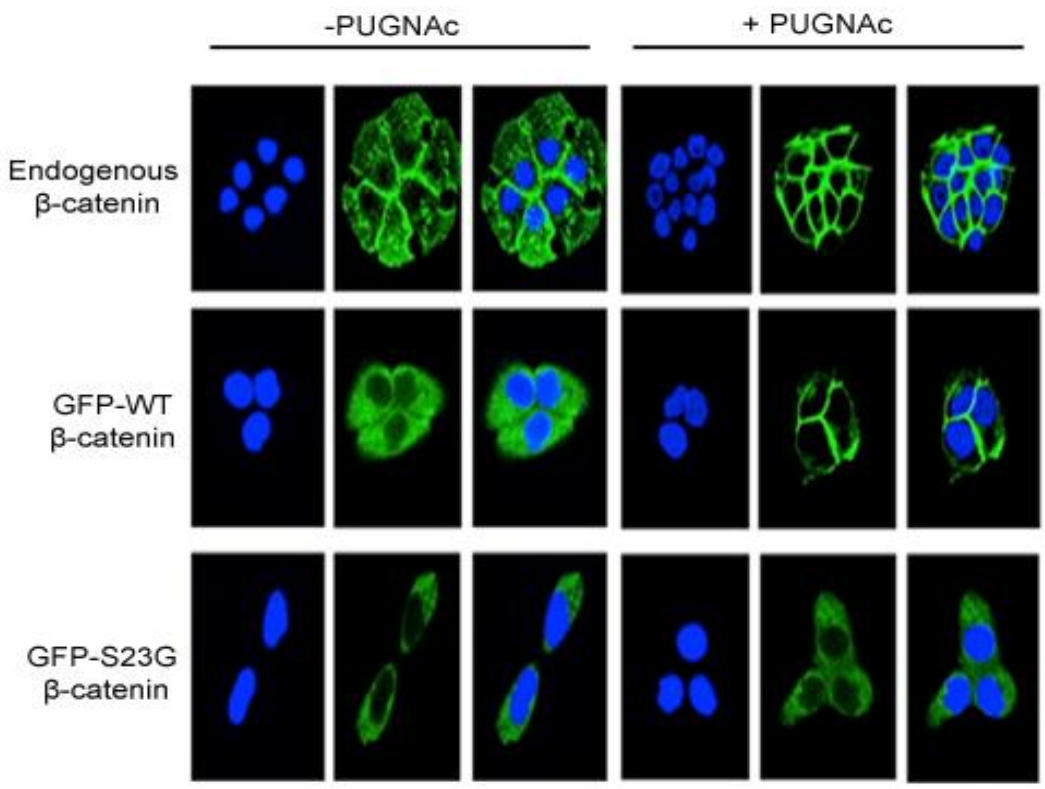

Figure 6—*Serine 23 regulates β-catenin's subcellular localization.* Immunofluorescence analysis of the subcellular localization of endogenous, and, full length GFP tagged WT and S23G mutant β-catenin in DU145 cells with or without 100 μM PUGNAc treatment. Endogenous β-catenin is dispersed throughout the cytoplasm and the nucleus (Panel 1). Upon PUGNAc treatment, β-catenin localized to the plasma membrane, characteristic of the stable epithelial phenotype. The GFP-WT β-catenin behaved similarly to endogenous β-catenin (Panel 2). PUGNAc treatment had no effect on the GFP-S23G mutant β-catenin as it remained dispersed throughout the cell (Panel 3). Nucleus-Blue, was stained with DAPI. Endogenous β-catenin was visualized using AlexaFluor ® 488 secondary antibody to anti-β-catenin primary antibody. GFP-WT and GFP-S23G mutant β-catenin constructs were followed by their GFP tag.

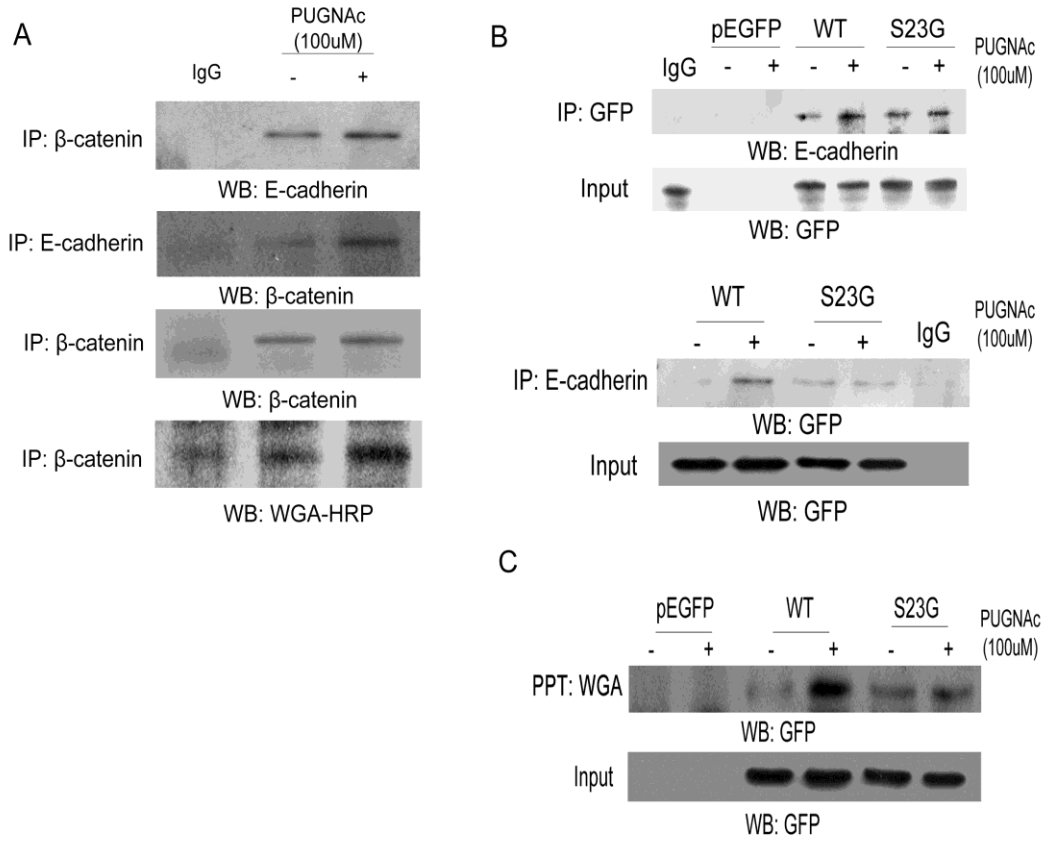

Figure 7*—β-catenin's interaction with E-cadherin is regulated by O-GlcNAc modification at Serine 23.* **A)** Immunoprecipitation of PUGNAc treated DU145 whole cell lysates resulted in increased binding of endogenous β-catenin with E-cadherin. Increased β-catenin-E-cadherin interaction was confirmed by reverse immunoprecipitation. Presence of O-GlcNAcylation was detected by WGA-HRP. **B)** Immunoprecipitation of PUGNAc treated DU145 lysate resulted in increased binding of full length GFP-WT β-catenin with E-cadherin while there was no change in interactions observed with the full length GFP-S23G mutant β-catenin. This was confirmed through reverse immunoprecipitation. Total non-specific IgG antibody served as the control. **C)** Using the same lysates as B), whole cell lysates of DU145 cells transfected with full length GFP-WT and GFP-S23G mutant β-catenin treated with or without PUGNAc and whole cell lysates were precipitated with WGA-agarose beads to detect the presence of O-GlcNAcylation. O-GlcNAcylation of GFP-WT β-catenin was induced upon PUGNAc treatment. As previously shown, the O-GlcNAcylation of GFP-S23G mutant β-catenin was unchanged despite PUGNAc treatment.

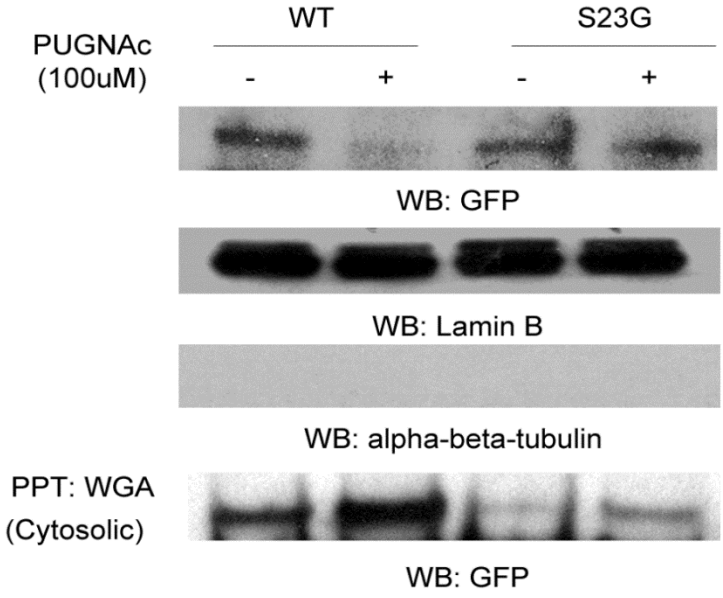

Figure 8—*Nuclear localization of β-catenin is regulated by O-GlcNAcylation at Serine 23.* Nuclear lysates of DU145 cells transfected with full length GFP-WT or GFP-S23G mutant β-catenin and treated with 100 μM PUGNAc were characterized by Western Blot Analysis. Levels of GFP-WT β-catenin decreased within the nucleus upon PUGNAc treatment. No change was observed in GFP-S23G mutant β-catenin. The presence of Lamin B ensured an enriched nuclear fraction. The absence of alpha-beta tubulin indicated a nuclear fraction free from cytosolic contamination. Cytosolic fractions were precipitated for O-GlcNAc-β-catenin with WGA-agarose beads to ensure the presence of O-GlcNAcylation.

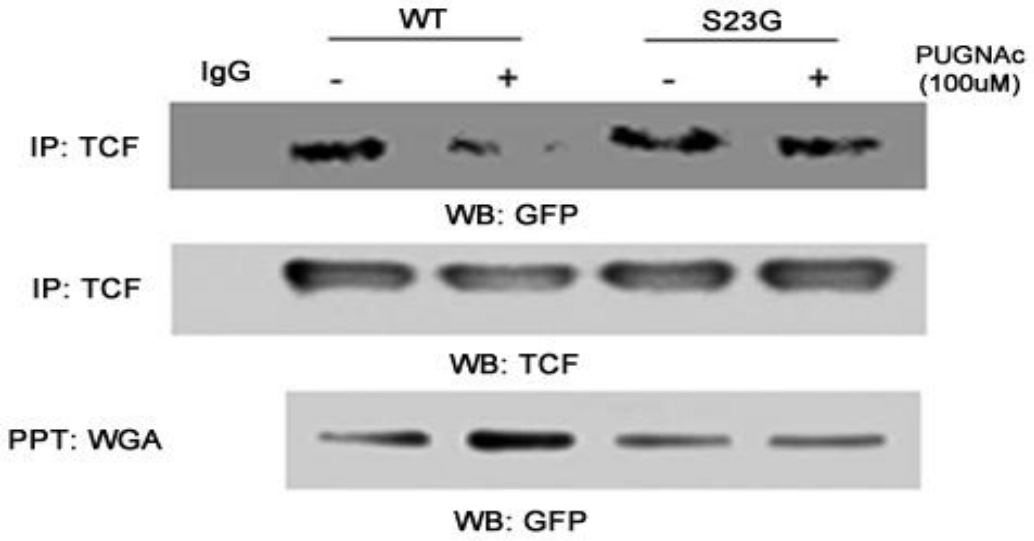

Figure 9—*β-catenin's interaction with TCF is regulated by O-GlcNAc modification at Serine 23.* Nuclear lysates of DU145 cells transfected with full length GFP-WT or GFP-S23G mutant β-catenin and treated with 100 μM PUGNAc were immunoprecipitated with anti-TCF antibody. Alterations in GFP-WT and GFP-S23G interactions with TCF were characterized by Western Blot Analysis using anti-GFP antibody. GFP-WT β-catenin-TCF interactions decreased upon PUGNAc treatment. No change was observed in GFP-S23G β-catenin-TCF interactions. The presence of O-GlcNAcylation was confirmed through WGA-agarose precipitation.

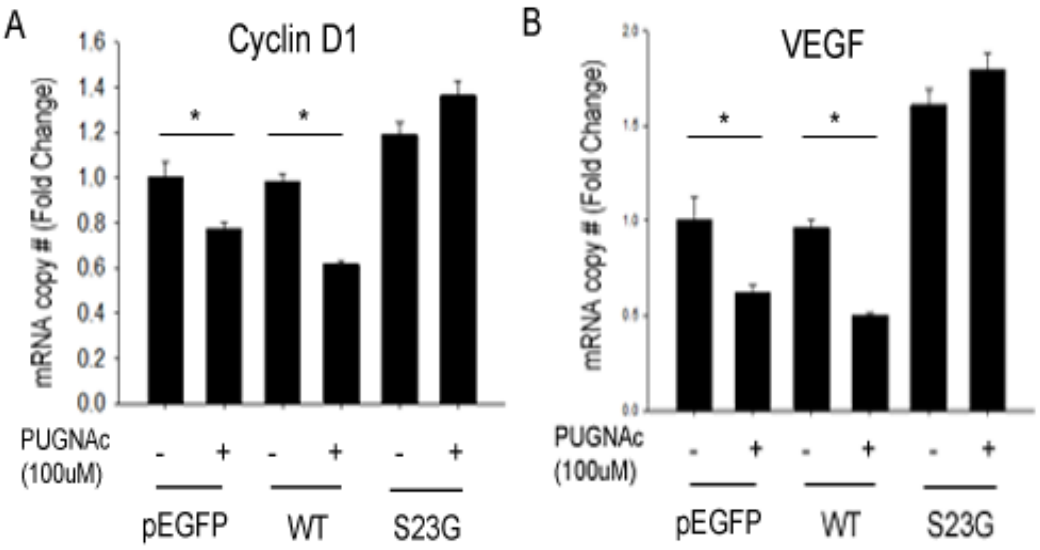

Figure 10—*O-GlcNAcylation of β-catenin decreases Wnt target gene expression. S23G mutation maximizes target gene expression.* RNA was extracted from DU145 cells transfected with full length GFP-WT or GFP-S23G mutant β-catenin and treated with or without 100 μM PUGNAc. pEGFP empty vector served as the control. RT-qPCR was performed on 1μg of total RNA extract. mRNA copy number of **A)** Cyclin D1 and **B)** VEGF decreased for GFP-WT and control conditions upon PUGNAc treatment. GFP-S23G mutant β-catenin transfected cells showed elevated levels of **A)** Cyclin D1 and **B)** VEGF mRNA expression and demonstrated no change in mRNA copy number of **A)** Cyclin D1 and **B)** VEGF in the presence or absence of PUGNAc treatment. *$p<0.05$.

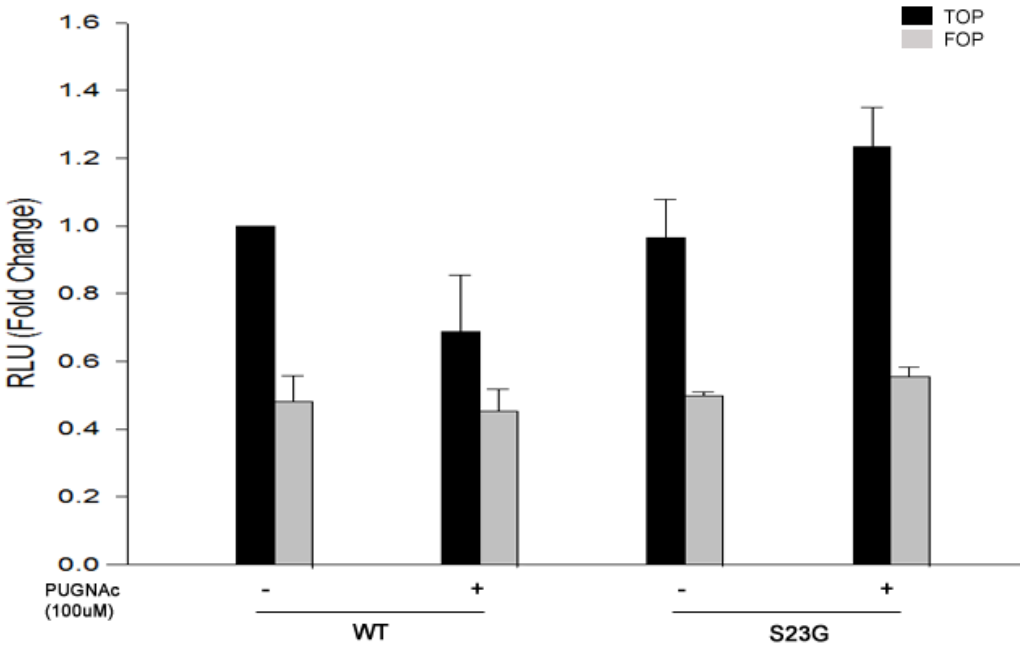

Figure 11—*O-GlcNAcylation of β-catenin decreases its transcriptional activity while S23G mutation negates this change.* DU145 cells were co-transfected with TOPflash or FOPflash reporter constructs, and, GFP-WT or GFP-S23G mutant full length β-catenin constructs. pRenilla served as the transfection control. Data sets were normalized to pRenilla. Transcriptional activity of GFP-WT β-catenin decreased upon PUGNAc treatment. There was increased overall transcriptional activity of GFP-S23G mutant β-catenin relative to GFP-WT β-catenin and no significant alterations between -/+ PUGNAc conditions.

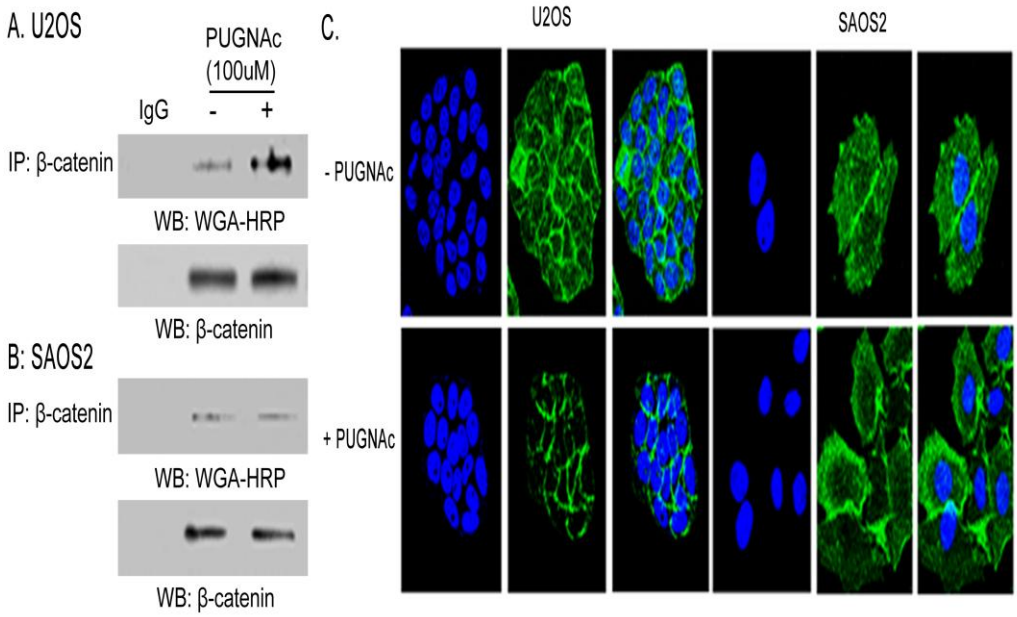

Figure 12—*O-GlcNAcylation of endogenous β-catenin alters its localization in U2OS but not SAOS2*. A) and B) U2OS and SAOS2 whole cell lysates treated with or without 100 µM PUGNAc were immunoprecipitated for β-catenin. O-GlcNAcylation of β-catenin was detected using WGA-HRP. There was minimal O-GlcNAc-β-catenin in both U2OS and SAOS2 cell line. Induction of O-GlcNAcylation of β-catenin upon PUGNAc treatment was present in U2OS but not SAOS2 cell line. **C)** Immunofluorescence analysis of the subcellular localization of endogenous β-catenin in U2OS and SAOS2 osteosarcoma cell line. In both U2OS and SAOS2, endogenous β-catenin was dispersed throughout the cytoplasm and the nucleus. Upon PUGNAc treatment, β-catenin localized to the plasma membrane in U2OS, similar to the observation in DU145. Alternatively, localization of endogenous β-catenin in SAOS2 upon PUGNAc treatment did not affect its localization, similar to the response of DU145 transfected with GFP-S23G mutant β-catenin. Nucleus-Blue stained with DAPI. Endogenous β-catenin was visualized using AlexFluor ® 488 secondary antibody to anti-β-catenin primary antibody.

Chapter 4

Discussion

4.0 Discussion

The present study is the first report to identify site specific regulation of β-catenin by the O-GlcNAc modification. Specifically, Ser23 of β-catenin's NT domain is a site for O-GlcNAc modification (Figure 3 and 4) which was observed to be an essential regulator of β-catenin's subcellular distribution and transcriptional activity in DU145 CaP cell line. In summary, O-GlcNAcylation of Ser23 resulted in the relocalization of β-catenin from the nucleus to the cytoplasm (Figure 6) leading to its increased interaction with E-cadherin (Figure 7). Additionally, increased O-GlcNAcylation concomitantly decreased β-catenin levels within the nucleus (Figure 8), TCF-β-catenin interactions (Figure 9), and β-catenin-TCF mediated transcriptional activity (Figure 10 and 11).

The location of Ser23 brings interesting functional prospects for the O-GlcNAc modification as it lies in close proximity to the destruction box, raising the possibility that the NT domain is the primary region regulating the stabilization and nuclear localization of β-catenin. This is particularly interesting as both the armadillo and CT domains are free from actively cycling O-GlcNAc modification (Figure 3A and 3B).

The NT and CT domains enable differential regulation of the armadillo domain for β-catenin's various partners. The NT domain's role in modulating localization and transcriptional activity of β-catenin can be achieved through its direct influence on ligand binding within the armadillo domain itself. Experiments delivering truncated armadillo domain void of the N-terminus into the nucleus revealed an absence of β-catenin-TCF complexes, suggesting that the N-terminus influences gene transcription and chromatin remodeling functions of β-catenin [11, 12]. Moreover, the presence of CT-Armadillo domain interactions was shown to enhance the stability of β-catenin by preventing its binding to the axin scaffolding complex; however, the impact of the CT domain upon β-catenin signaling at the level of the NT destruction box is yet unknown [53, 121].

As the interactions within the armadillo domain are largely dependent on ionic charge where phosphorylation events modulate this binding affinity, it is likely then, that site occupancy of O-GlcNAc at Ser23 may induce conformational changes of the protein to hinder or facilitate its interactions with molecular chaperones. For example, in the case of phosphorylation, tyrosine phosphorylation of the armadillo domain decreased β-catenin-E-cadherin interactions while concomitantly reducing CT-Armadillo domain interactions and opening its binding affinity to TBP, a key player in the basal transcriptional machinery for *wnt* target genes [7, 14]. Here, we have shown that in DU145 CaP cells, O-GlcNAcylation of β-catenin increased its interaction with E-cadherin at the plasma membrane (Figure 7). The addition of a missense mutation, S23G, decreased this interaction upon PUGNAc treatment, suggesting that Ser23 is a key site to stabilize the β-catenin-E-cadherin interaction at the adherens junctions. The implication for any structural alterations that may occur in order to regulate this interaction upon O-GlcNAcylation is unknown. It can be speculated, that the stabilization of the adherens junction observed upon O-GlcNAcylation, may contribute functionally in decreasing the metastatic potential of CaP cells.

The "Yin Yang" hypothesis establishes the relationship between O-GlcNAcylation and O-phosphorylation as a binary regulatory system for proteins. Previously, Van Noort et al., (2002) identified β-catenin to be phosphorylated at Ser23 by GSK3β [122]. This finding indicated that phosphorylation and O-GlcNAcylation modifications were in direct interplay at Ser23, suggestive of possible cross regulation of β-catenin's function by these two modifications. However, Van Noort et al, (2002) have also stated that the phosphorylation of Ser23 did not influence β-catenin's interactions with its cellular interactors or its function as a transcriptional activator [122]. Although phosphorylation at Ser23 was suggested to not have any functional impact on β-catenin, our data, alternatively, supports a role for O-GlcNAcylation of Ser23 in regulating β-catenin's subcellular localization and transcriptional activity. Specifically, we observed that the introduction of the missense mutation S23G to β-catenin

prevented O-GlcNAc modification associated effects that were observed with WT β-catenin. The nuclear localization of β-catenin (Figure 8), β-catenin-TCF interaction (Figure 9), β-catenin transcriptional activity (Figure 11) and *wnt* target gene expression (Figure 10) were unaltered in S23G mutant β-catenin transfectants despite increased O-GlcNAcylation. Based on this, it can then be hypothesize that O-GlcNAcylation and phosphorylation may indirectly work hand in hand to regulate the function of β-catenin, with O-GlcNAc regulating its localization and phosphorylation regulating its stabilization. The fact that many substrates of OGT are often also substrates of GSK3β suggests the possibility of OGT as a component of the destruction complex. It can be postulated that OGT, indirectly via O-GlcNAc modification of adjacent sites (Ser23) to phosphorylation sites (Ser33, Ser37, Thr41, Ser45), may spatially and temporally impose regulatory effects on β-catenin's cellular partners, such as APC at the level of the destruction complex.

APC, in its role to export β-catenin from the nucleus, sustains the efficiency of the destruction complex, by controlling the release and recruitment of β-catenin. Given that the interaction between APC and β-catenin was found to be unaltered upon O-GlcNAc modification [67], we can speculate that O-GlcNAcylation at Ser23 acts as a regulatory switch for stabilizing β-catenin from degradation within the cytoplasm. Specifically, β-catenin's increased localization to and restriction within the cytoplasm/plasma membrane may be regulated through its interaction with APC at the destruction complex. A study by Ikeda et al., (2000) demonstrated that the presence of β-catenin modulated the phosphorylation of APC by GSK3β in the presence of Axin. Specifically, β-catenin did not affect the activity of GSK3β, but enhanced/stimulated APC phosphorylation [123]. APC becomes phosphorylated by GSK3β to enhance its binding with β-catenin. Upon the dephosphorylation of APC, β-catenin is released for its own phosphorylation at the destruction complex. Based on this argument, O-GlcNAc modification of Ser23 at the N-terminal of β-catenin can putatively restrict GSK3β phosphorylation of APC, impeding adequate delivery of β-catenin to the

destruction complex and hence its phosphorylation. As stated by Mondoux et al., (2010), GSK3β would be the likely candidate to regulate β-catenin through this interplay between phosphorylation and O-GlcNAcylation [124].

The attenuation in mRNA expressions of *wnt* target genes with increased O-GlcNAcylation is likely the direct consequence of the decreased levels of β-catenin within the nucleus (Figure 8) and the decrease in β-catenin-TCF interaction (Figure 9). Yet, a consistent observation within this study was the correspondingly elevated transcriptional activity of β-catenin with the introduction of the S23G mutation to β-catenin (Figure 11) as reflected by the increased *wnt* target gene expression of Cyclin D1 and VEGF (Figure 10). It is unlikely that this increased transcriptional output is due to any alterations in TCF, as TCF was not modified by O-GlcNAcylation, nor was its nuclear levels changed by PUGNAc treatment [67]. TCF/LEF transcription factors are actively repressed by transcriptional co-repressors such as Groucho/TLE proteins. The presence of β-catenin within the nucleus displaces Grouch/TLE proteins and recruits an array of co-activator proteins including BCL9. Additionally, the observed co-localization of OGT and O-GlcNAcase within chromatin associated repressor complexes supports the possibility of these two enzymes in the regulation of transcriptional silencing [77]. This brings into question then, the ability of O-GlcNAcylation to regulate β-catenin co-activator function at the level of the transcriptional machinery by possibly altering its affinity to other transcriptional co-activators. The ability of post translational modifications to have such an effect was shown by Brembeck et al., (2004) who demonstrated phosphorylation of Tyr142 of β-catenin by c-Met acted as a molecular switch which transformed the adhesive form of β-catenin into one that preferentially bound to BCL9-2 resulting in a dramatic decrease in *wnt* target gene transcription [63]. In this case, it is then possible to hypothesize that O-GlcNAcylation may limit the capacity of β-catenin to alleviate TCF repression through its inability to compete with TCF-

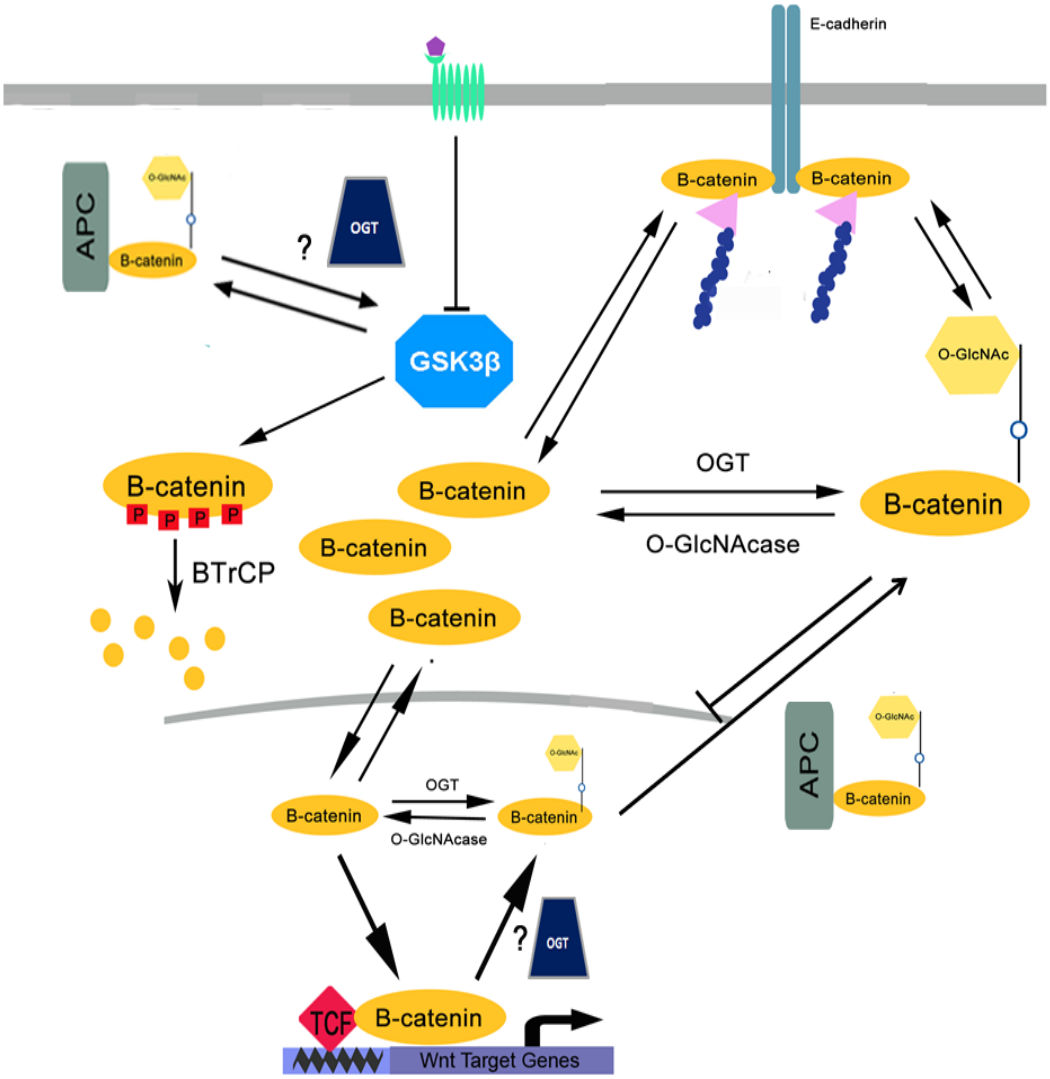

Figure 13— ***Schematic overview of the O-GlcNAcylation of β-catenin.*** With increased O-GlcNAc modification there is re-localization of β-catenin from the nucleus to the cytosol, mediated by the nuclear exporter APC. Within the cytoplasm, there is increased E-cadherin-β-catenin interaction and a consequent decrease in nuclear TCF-β-catenin interaction. The mechanism underlying this phenomenon is currently unknown. Furthermore, how the O-GlcNAc modification may be regulating β-catenin at the level of the transcriptional machinery (nucleus) and at the destruction complex (cytoplasm) remains to be investigated.

Groucho/TLE complexes. Hence, by introducing S23G mutation this inhibitory effect is removed allowing for more efficient transcriptional activation. As such, how the O-GlcNAc modification may affect TCF-β-catenin and TCF-Groucho competition at the level of the transcriptional machinery still remains to be answered.

The O-GlcNAc modification of certain proteins has more often than not, been found to be cell line specific. Hatsell et al, (2003) observed that plakoglobin and β-catenin were differentially O-GlcNAc modified in a cell context-specific manner. Indeed this was the case for the U2OS and SAOS2 cell line (Figure 12). Endogenous β-catenin was O-GlcNAc modified in U2OS osteosarcoma cell line, presenting with similar alterations in endogenous and GFP-WT β-catenin's localization in DU145 cells upon PUGNAc treatment. However, the SAOS2 osteoscarcoma cell line did not show this same pattern of alterations. Moreover, the baseline O-GlcNAc levels in SAOS2 cells were considerably lower relative to U2OS and DU145. This discrepancy may be explained by the possibility of O-GlcNAc regulatory mechanisms, either OGT or O-GlcNAcase, to be dysfunctional in SAOS2 cells. Previous reports show significant nuclear accumulation of β-catenin in osteosarcoma which implicates β-catenin as a key player in the pathogenesis of this malignant phenotype [125]. As such, further investigation on the differential regulation of O-GlcNAc-β-catenin within these two cell lines will be required to elucidate alternative mechanisms that may modulate the O-GlcNAc modification of β-catenin in various cancers.

In conclusion, this study demonstrated that Ser23 is a site for O-GlcNAcylation which also played a role in mediating β-catenin's subcellular localization and transcriptional activity. This regulatory effect may hinge on differentially altering β-catenin's affinity for its cellular interactors such as E-cadherin, and TCF. Although the molecular mechanism underlying this phenomenon remains to be established, it can be proposed that a tripartite system exits for the regulation of β-catenin, where cellular β-catenin is either in a phosphorylated, O-GlcNAcylated,

or unmodified state, which in turn modulates the various intracellular pools of β-catenin (membrane, cytosolic, nuclear) centering on the status of Ser23.

Chapter 5
Future Directions

5.0 Future Directions

5.1 Improvements for current study

Although the present study provides substantial insight into O-GlcNAc regulation of β-catenin, this investigation may be further strengthened by addressing the following areas:

5.1.1 Optimization of TOPFlash/FOPflash luciferase reporter assay

The current data demonstrated a decrease in TOPflash luciferase activity of WT β-catenin transfected DU145 cells upon PUGNAc treatment (Figure 11). This trend was consistently observed throughout the study. The data shown here is representative of three independent experiments. Although there is a decrease observed, statistical analysis has determined this decrease to be insignificant. Currently, further experimentation as well as additional optimizations of experimental procedures is in progress.

5.1.2 Experimentation in normal prostate cell line

Normal prostate cells (PNT1A) were observed to have greater levels of O-GlcNAc modified β-catenin relative to CaP cell lines [67]. Accordingly, studies which may characterize the behaviour of O-GlcNAc β-catenin in a non-transformed cell line such as the PNT1A would be beneficial. Similar experiments should be performed using GFP-WT and GFP-S23G mutant constructs in the presence and absence of PUGNAc to determine any alterations in the distribution and function of β-catenin in a normal cell phenotype.

5.1.3 Alternative approaches to increase O-GlcNAcylation

Chemical inhibitors such as PUGNAc are likely to have non-specific cellular effects. An alternative approach to address this issue is to either introduce cancer cell lines with shRNA/siRNA to O-GlcNAcase or an overexpression vector of OGT. Both conditions will effectively increase O-GlcNAcylation within the cell. These cell lines can then be used to re-evaluate alterations in β-catenin localization and interactions with its major partners.

5.1.4 Development of a β-catenin Serine 23 to Aspartic Acid (S23D) mutant

The study by Van Noort et al., (2002) demonstrated that Ser23 was a site for phosphorylation, although phosphorylation of Ser23 was reported not to interfere with the binding interactions and transcriptional activity of β-catenin. Thus, it is necessary to confirm this by creating an N-terminal and full length S23D mutant β-catenin and to re-evaluate any alterations in β-catenin function. An S23D mutation will mimic constitutive phosphorylation at Ser23.

5.2 Prospective Studies

Currently, the upstream regulatory pathway(s) that may modulate O-GlcNAc-β-catenin are unknown. As such, elucidating the intracellular signaling pathways that regulate the O-GlcNAcylation of β-catenin will provide insights to how this modification may halt or contribute to disease progression. Moreover, while it is crucial to examine aspects of O-GlcNAcylation at the molecular level, overall assessment of cell transformation and oncogenic potential is also necessary to further assess the importance of O-GlcNAcylation in cancer etiology. Specifically, the accumulating evidence supporting the involvement of β-catenin in CaP progression has redefined androgen receptor (AR) signaling in CaP tumorigenesis. Therefore, prospective studies in which this current investigation may positively contribute to this project are discussed below.

5.2.1 Regulation of O-GlcNAc-β-catenin

Studies have demonstrated that there is interplay between the Wnt and PI3K signaling pathways in the regulation of β-catenin's stabilization; specifically through the common factor, GSK3β. Growth factor stimulation of receptor tyrosine kinases (RTK) and/or non-RTKs results in the activation of PI3K and the conversion of phosphatidylinositol-bis-phosphate (PIP_2) to phosphatidylinositol-tri-phosphate (PIP_3). The formation of PIP_3 recruits the serine and threonine kinase, Akt/PKB to the plasma membrane by its pleckstrin homology (PH) domain. At the plasma membrane, Akt/PKB is activated by its phosphorylation by PDK1/PDK2 at Thr308 and Ser473 [126]. Activated Akt (phosphorylated Akt [P-

AKT]) is the central effector of many downstream signaling pathways regulating protein synthesis, cell cycle, cell death, cell growth, and cell survival [summarized in 127]. GSK3β is phosphorylated at Ser9 by Akt, leading to its inhibition. In effect, the association between Akt-GSK3β and GSK3β-β-catenin brings the PI3K and Wnt pathways, respectively, at a junction where β-catenin's stability and nuclear availability may be regulated.

Reports have established the cycling of O-GlcNAc as a necessary entity for the correct transduction and amplification of signals, particularly within the PI3K pathway. For example, insulin stimulation of insulin responsive cells demonstrated that OGT was able to associate with the plasma membrane by binding to phosphoinositides [128]. The constitutive activation of the PI3K/Akt pathway drives the localization of OGT from the nucleus to the plasma membrane where it can bind to PIP_3. Here, OGT can then associate with the insulin receptor to become tyrosine phosphorylated causing increased OGT activity [73, 129]. The redistribution and activation of OGT allows for dynamic modification of downstream targets by O-GlcNAcylation which include IR-β, insulin receptor substrate 1 [130, 131], Akt, and OGT itself [68, 73, 128, 129]. O-GlcNAcylation of Akt inhibits its phosphorylation at Thr308 which shuts down its kinases activity to ultimately downregulate signaling [68, 73, 128].

The objective of this portion of the study will then be to determine the regulatory pathways responsible for the O-GlcNAc modification of β-catenin, particularly pertaining to the Wnt and PI3K pathway. Preliminary results were obtained for this aim of the study (See Appendix A1.1 for Materials and Methods). Specifically, DU145 cells were either treated with 150 ng/ml or 300 ng/ml Wnt3a at 0, 4, 8, 12 hours to activate the Wnt signalling pathway. Increased total levels of β-catenin (hence, its stabilization) was an indicator of an activated Wnt system (Appendix A2.1 Figure A2.1A). WGA-agarose precipitation of whole cell lysates demonstrated a steady increase in the levels of O-GlcNAc-β-catenin with 150 ng/ml Wnt3a stimulation (Figure A2.1B). Interestingly, 300 ng/ml Wnt3a

treatment of DU145 cells resulted in a time dependent decrease in O-GlcNAc-β-catenin by 12 hours. These results indicated that the Wnt pathway regulated the levels of O-GlcNAcylated β-catenin in a dose and time dependent manner. The implications for this finding raise the possibility of Wnt mediated regulation of OGT at the level of the destruction complex. Upon Wnt activation there is deactivation of the destruction complex. Therefore, if OGT were to be placed here, the very inactivation of the destruction complex would limit the accessibility of OGT to β-catenin, thereby decreasing O-GlcNAcylation of β-catenin as observed by Figure A2.1B. Therefore, as a future direction for this study, it would be interesting to investigate the role of the Wnt pathway in regulating OGT at the level of the destruction complex.

In order to further elucidate the possibility of cross regulation of O-GlcNAc-β-catenin, the next steps would involve the manipulation of the PI3K pathway. This can be achieved by directly inhibiting the enzyme, PI3K, through the chemical inhibitors Wortmannin or LY294002. Alternatively, growth factor stimulation by Insulin or Insulin Growth Factor-1 (IGF-1) is another means to activate the PI3K pathway. Once this has been characterized, GSK3β inhibition through LiCl may also be introduced. Further progress into this study will provide novel evidence of upstream regulation of the O-GlcNAc modification of β-catenin by OGT at the plasma membrane. Refer to Figure 14 for an overall schematic representation.

5.2.2 Functional implications of O-GlcNAc-β-catenin in prostate cancer progression

Elevated cellular expression of β-catenin is a marker associated with poor prognosis in many human cancers including adenocarcinoma of the colon, liver, ovary, breast and prostate [132-135]. Moreover, increased transcriptional activity of β-catenin is known to regulate tumorigenesis and tumor invasiveness by activating target genes such as Cyclin D1, c-Myc, and matrix metalloproteinases (MMP).

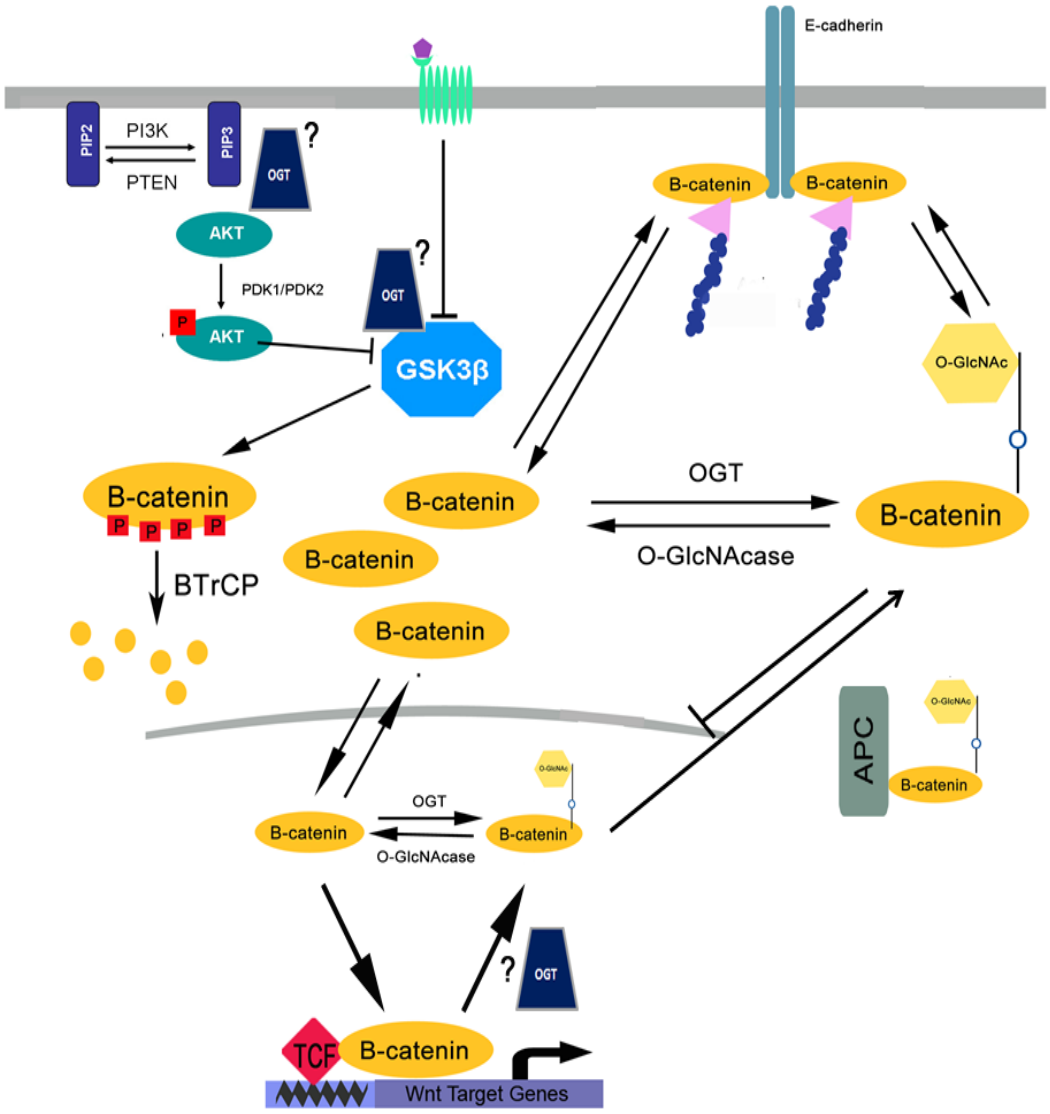

Figure 14—*Schematic overview of the putative upstream regulation of the O-GlcNAcylation of β-catenin.* Studies have demonstrated that there is interplay between the Wnt and PI3K signaling pathways in the regulation of β-catenin's stabilization; specifically through the common factor, GSK3β. Future prospects of this study would involve elucidating the possible dual regulation of O-GlcNAcylation of β-catenin by the Wnt and PI3K pathways at the level of the destruction complex and plasma membrane, respectively.

Preliminary results for this objective of the study have been obtained. In order to assess the biological implications of O-GlcNAcylation in DU145 cells, preliminary experiments using the scratch assay and matrigel invasion assay were performed (See Appendix A1.2 and A1.3 for Materials and Methods). The scratch assay, assessing the migratory potential of cells, demonstrated that there was a greater rate of wound closure upon PUGNAc treatment (Appendix A2.2 Figure A2.2; representative of 2 independent experiments). Additionally, the matrigel invasion assay showed that the invasive potential of DU145 cells increased upon PUGNAc treatment (experiment was performed in triplicates) (Appendix A2.3 Figure A2.3). In order to consolidate these findings, these assays must be repeated alongside parallel immunoblotting procedures to ensure that increased O-GlcNAcylation has been achieved.

In accordance with these preliminary results, increased O-GlcNAcylation with CaP progression has been supported by several reports. For example, Lynch et al., (2012) used the Oncomine ™ microarray database to demonstrate that OGT was overexpressed in prostate cancer tissue compared to normal prostatic epithelium. Moreover, elevated OGT was associated with poor clinical outcome. In addition, reduction in O-GlcNAcylation through RNAi of OGT in metastatic prostate cancer cell line, PC3-ML, reduced MMP-2 and MMP-9 expression [136]. The overarching effect of increased global O-GlcNAcylation is a combined effect of many factors which collectively contribute to the tumor phenotype. Hence, the phenotypic change observed upon increased global O-GlcNAcylation cannot be attributed to the effects of one particular protein. In order to decipher how β-catenin itself may be contributing to tumor cell phenotype may be difficult to establish as the global effects of O-GlcNAcylation may override the effects of β-catenin alone. An alternative option may be to use colon cancer cell lines which express high levels of β-catenin due to a deregulated Wnt signaling system. As stabilization of β-catenin is one of the major characteristics contributing to colon cancer, PUGNAc treatment can be speculated to have drastic effects on the properties of these cells. However, caution must be taken in interpreting these

results since the Wnt pathway, as shown previously, seems to regulate the O-GlcNAcylation of β-catenin in a dose and time dependent manner (Appendix A2.1 Figure A2.1). Furthermore, in some colon cancer cells, β-catenin itself may also be mutated.

5.2.3 Prostate cancer progression: β-catenin, Androgen Receptor, and O-GlcNAcylation

The progression of CaP from an androgen-dependent (AD) to a more lethal androgen-independent (AI) phenotype is characteristic of metastatic disease. The unpredictable nature of CaP progression has made the search for reliable markers for CaP diagnosis a pertinent one. Contributing factors that play a role in prostate tumorigenesis include the androgen receptor (AR), which plays a key role in prostate cell growth and differentiation [137]. Alterations in β-catenin distribution and expression have also been reported in patient CaP samples with a general trend of increased β-catenin levels in AI-CaP and a greater nuclear presence of β-catenin with increased Gleason grade [138]. The evidence is clear that increased AR expression results in sensitivity to androgens and that increased AR expression alone is sufficient to transform primary CaP into a more aggressive AI phenotype. The recent identification of a physical interaction between AR and β-catenin and AR and TCF was an exciting new development for understanding the mechanism underlying CaP progression. In effect, the observed accumulation of β-catenin and increase in AR activation are likely in interplay to arbitrate selective gene expression programs that potentiate prostate carcinogenesis.

The notion that there was a direct interaction between the AR and β-catenin was first established by Truica et al., (2000), who demonstrated that β-catenin was able to enhance AR transactivation, alter the sensitivity of AR to ligands and relieve the repression of anti-androgens on AR mediated transcription [139]. Alongside its modulatory role in increasing AR directed transcriptional activity, β-catenin also had an effect in decreasing TCF mediated gene expression. TCF

has also been found to bind the AR to provide co-operative regulatory control over β-catenin. Moreover, gene expression studies indicated that there was a decrease in Wnt transcription factors—Tcf-3 and LEF, Wnt target genes—MYC and CCND1, and, β-catenin inhibitors—CSNK2B, CSK1E, GSK3B, TP53, WNT5A and PLCB4 [140]. Therefore, in AI disease progression, the cytoplasmic pool of β-catenin is increased while the downstream effects of β-catenin-TCF transcriptional activity are suppressed [140]. Taken together, identifying the mechanism by which β-catenin differentially regulates and divides its role between AR and Wnt signaling pathways will provide novel insight into CaP tumorigenesis. Moreover, how the recently identified O-GlcNAc modification of β-catenin may facilitate or hinder β-catenin-AR interaction will further elucidate the mechanism underlying prostate tumorigenesis.

Chapter 6
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6.0 Bibliography

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Appendix

Appendix 1

Prospective Studies: Materials and Methods

A1.0 Appendix 1: Prospective Studies: Materials and Methods

A1.1 Prospective Studies 5.2.1: *Wnt3A stimulation of DU145 cells*

DU145 cells were seeded onto 6 well plates in DMEM 10% FBS 1% Penicillin/Streptomyacin. Cells were then serum starved for 18 hours, and subsequently treated with 150 ng/ml or 300 ng/ml recombinant human Wnt3A (R & D Systems) dissolved in sterile water for 0, 4, 8 and 12 hours. Cells were lysed using NP40-DOC lysis buffer and protein concentrations were determined by Bicinchoninic Acid Protein Determination Assay. O-GlcNAcylated β-catenin was precipitated using WGA-agarose beads and any alterations in the levels of O-GlcNAc β-catenin and other proteins of interest were characterized by Western Blot Analysis. Antibodies included anti-β-catenin (Cell Signalling), and anti-Actin (Santa Cruz Biotechnology Inc.).

A1.2 Prospective Studies 5.2.2: *Scratch Assay*

DU145 cell were seeded onto culture dishes in DMEM 10% FBS 1% Penicillin/Streptomyacin. Cells were set to rest and grow until 95% confluency was achieved. The medium was then replaced to DMEM 1% FBS for 24 hours. Subsequently, a scratch was made on a confluent monolayer of DU145 cells using a P200 pipette tip. The cells were then treated with 100 µM PUGNAc in DMEM 0.1% FBS. Scratch/Wound closure was viewed by light microscopy, and photographed at 0, 24, 48 hours. Every 12 hours, growth medium was changed with the addition of fresh PUGNAc.

A1.3 Prospective Studies 5.2.3: *Matrigel Invasion*

10^4 DU145 cells in serum free DMEM were seeded into Matrigel invasion inserts (BD Bioscience). These inserts were then placed in 24 well plate containing DMEM 10% FBS. Cells within each insert were treated with 100 µM PUGNAc. Untreated cells served as controls. DU145 cells were incubated at 37ºC, 5% CO_2 for 24 hours. Invaded cells were stained with 0.1% Crystal Violet solubilized in 4% Paraformaldehdyde in PBS. Membranes were extracted according to manufacturer's protocols. Invaded cells were visualized using light microscopy.

Appendix 2

Prospective Studies: Preliminary Data

A2.0 Appendix 2 Prospective Studies: Preliminary Data

A2.1 Prospective Studies 5.2.1: *Wnt3A Activation*

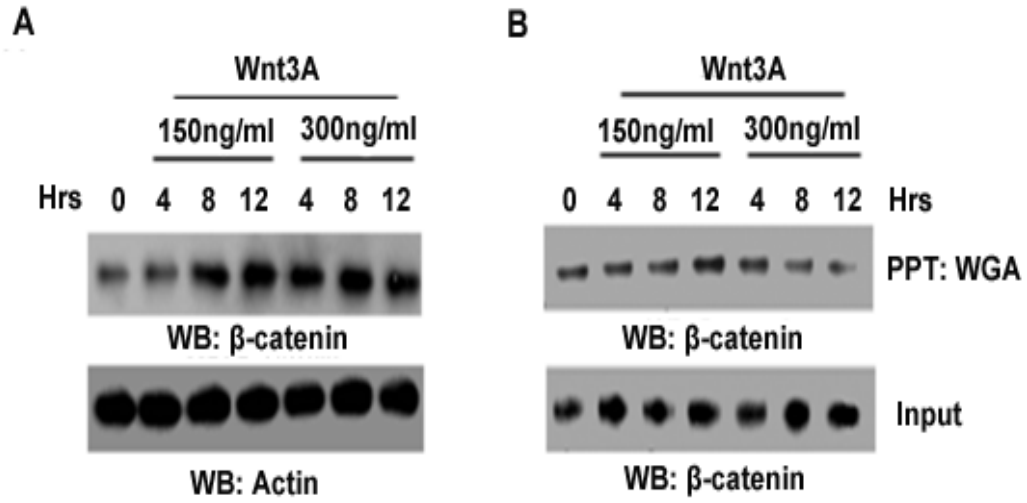

Figure A2.1—*Wnt activation increases β-catenin levels and decreases O-GlcNAcylation of β-catenin in a time and dose dependent manner.* DU145 cells treated with 150 or 300 ng/ml recombinant human Wnt3A for 4, 8, 12 hour time points. **A)** Wnt3A stimulation of DU145 cells increased the levels of β-catenin in a time dependent manner. **B)** DU145 cells treated with 150 or 300 ng/ml of recombinant Wnt3A were precipitated with WGA-agarose. At 150 ng/ml, Wnt3A treatment cased a steady increase in O-GlcNAc modified β-catenin (12 hour) while treatment of DU145 cells with 300 ng/ml resulted in a decrease (12 hour). There seems to be a threshold level of Wnt activation required for the regulation of O-GlcNAc-β-catenin.

A2.2 Prospective Studies 5.2.2: *Scratch Assay*

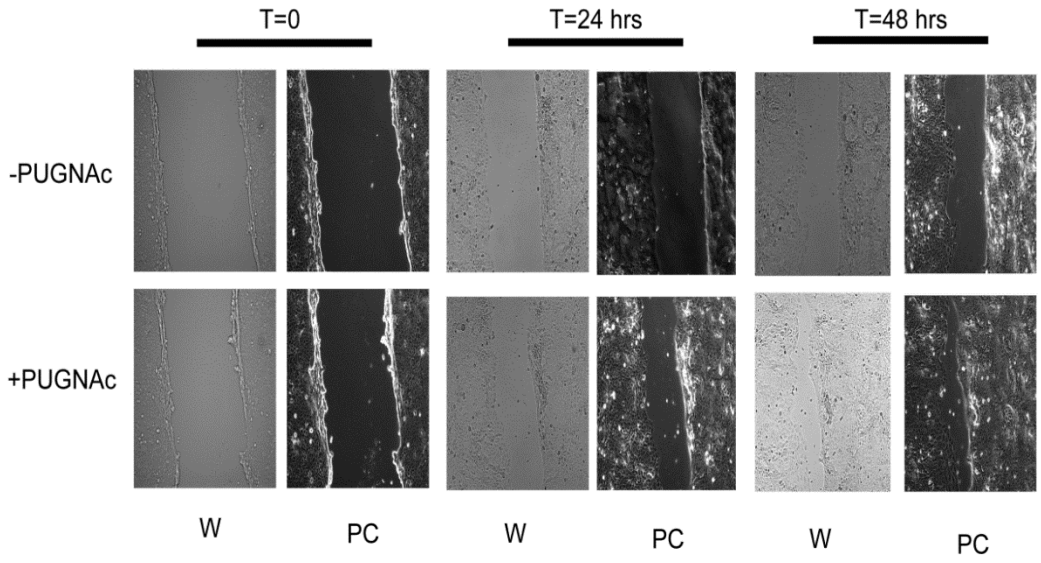

Figure A2.2—*Global O-GlcNAcylation increases migratory potential of DU145 CaP cells.* A scratch was made on a confluent monolayer of DU145 cells using a P200 pipette tip. Cells were then treated with 100 µM PUGNAc in DMEM 0.1% FBS. Wound closure was photographed at 0, 24, 48 hours. Rate of wound closure seems to be greater in PUGNAc treated DU145 cells. W-white light, PC-phase contrast.

A2.3 Prospective Studies 5.2.2: *Matrigel Invasion Assay*

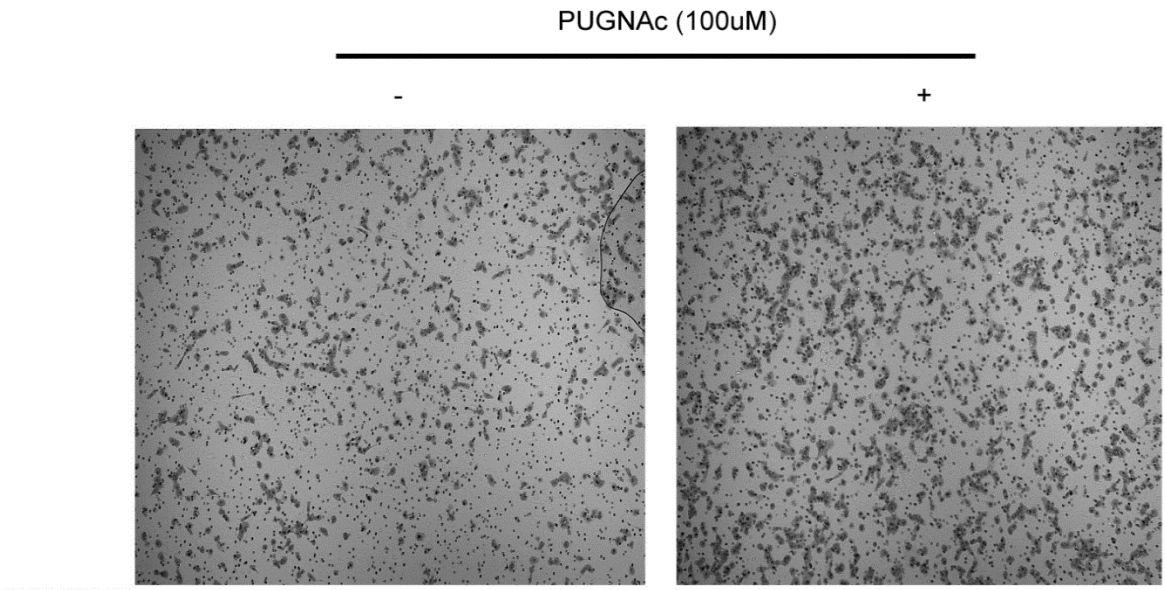

Figure A2.3—*O-GlcNAcylation increases invasive potential of DU145 CaP cells.* 10^4 DU145 cells in serum free DMEM were seeded into Matrigel invasion chambers (BD Bioscience). Cells were treated with 100 µM PUGNAc. Untreated cells served as controls. DU145 cells were incubated at 37ºC, 5% CO_2 for 24 hours. Invaded cells were stained with 0.1% Crystal Violet solubilized with 4% Paraformaldehdyde in PBS. Invaded cells were visualized by light microscopy. Number of invaded DU145 CaP cells increased upon PUGNAc treatment.

Appendix 3

Trial and Error: Optimization of the Immunological Detection of O-GlcNAc-β-catenin

A3.0 Appendix 3: Trial and Error: Optimization of the Immunological Detection of O-GlcNAc-β-catenin

A3.1 PUGNAc Treatment

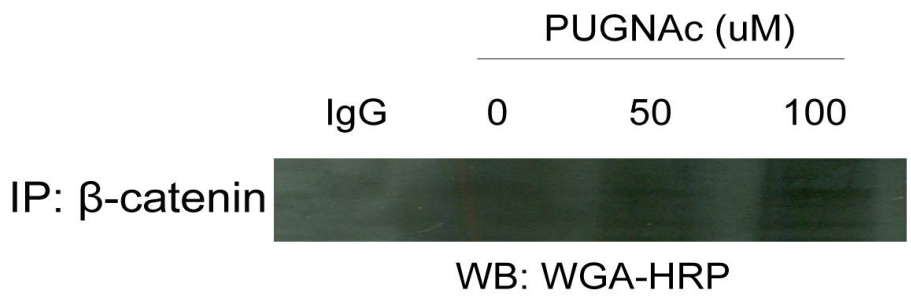

Figure A3.1—*100 μM PUGNAc is sufficient to induce O-GlcNAcylation of β-catenin.* DU145 cells were grown to 80% confluency in DMEM 10% FBS 1% Penicillin/Streptomyacin. Growth medium was changed to 1% FBS for 18 hours. Cells were then treated with 0, 50, or 100 μM PUGNAc in serum free medium for 14 hours. PUGNAc treatment procedures were adapted from Sayat et al., (2008) [67]. Cells were lysed with NP40-DOC lysis buffer and whole cell lysates were subsequently immunoprecipitated with anti-β-catenin antibody (1:50) and alterations in O-GlcNAcylation was characterized by Western Blot Analysis using 1:10000 WGA-HRP diluted in 1% BSA. IgG non-specific antibody served as the control.

The optimal concentration of PUGNAc treatment was first characterized in order to achieve sufficient O-GlcNAc modification of β-catenin. Alterations in O-GlcNAcylation were analyzed by Western Blot analysis using WGA-HRP. Although difficult to visualize, there is an increased induction of O-GlcNAc-β-catenin with 100 μM PUGNAc relative to 50 μM PUGNAc treatment. In effect, a final concentration of 100 μM PUGNAc was used throughout this study.

A3.2 Immunoblotting: Detection of the O-GlcNAc modification

As observed in Figure A3.1 the non-specific staining due to the WGA-HRP was a deterrent for the adequate visualization of the protein band of interest. This was problematic as the high background hindered imaging and further characterization of O-GlcNAcylation of β-catenin. In order to optimize the Western Blot

procedure several modifications to previously reported protocols were established as follows:

A3.2.1 Alterations in the concentration of WGA-HRP

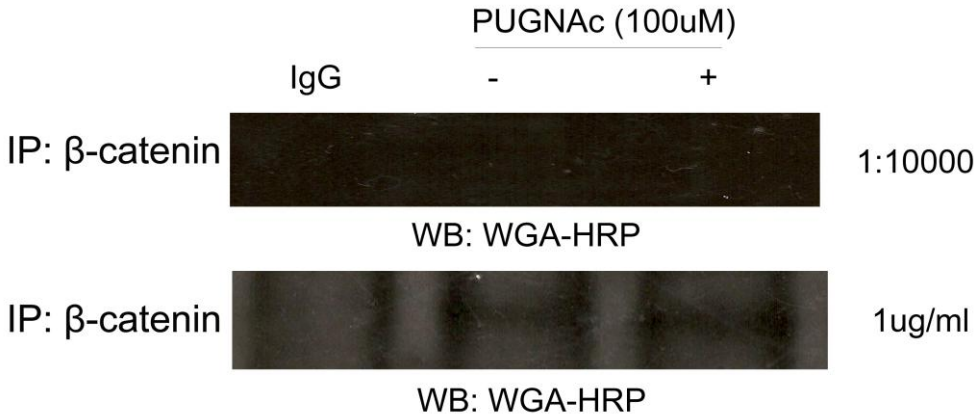

Figure A3.2.1—*1 µg/ml WGA-HRP decreases non-specific and background staining*. Whole cell lysates of DU145 cells treated with 100 µM PUGNAc were immunoprecipitated using anti-β-catenin antibody and characterized by Western Blot Analysis. PVDF membranes were incubated in either 1:10000 WGA-HRP diluted in 1% BSA or 1µg/ml WGA-HRP diluted in 1% BSA.

The immunoblotting procedure for the detection of O-GlcNAcylation has proven to be difficult as this procedure, unlike standard Western Blot procedures, does not require a primary antibody which targets the antigen of interest. Due to the absence of a primary antibody, the visualization is based solely on the binding of and concentration of WGA-HRP. As such, the signal to background ratio is compromised. In order to minimize non-specific binding, the concentration of WGA-HRP was modified through various trials. The stock concentration of WGA-HRP is 40 mg/ml. Optimal concentration of WGA-HRP for Western Blot procedures was found to be 1 µg/ml.

The signal by which protein bands of interest are visualized is through the reaction of HRP with a specified chemiluminescent reagent. The reaction between luminol and HRP causes the oxidation of luminol in basic conditions. The luminol

is in an excited state and upon its decay to ground state, light is emitted, and subsequently detected by autoradiography. Here, the level of noise is extremely high causing the autoradiograph to become overloaded. Hence, by adjusting the concentration of luminol, light emission can be correspondingly optimized. To further optimize for WGA-HRP concentrations, the Chemiluminescent reagent Western Lightning ® Plus-ECL (PerkinElmer, LAS Inc.) was prepared according to manufacturer's protocols, and diluted 1:10 with water.

A3.2.2 Alterations in the stringency of PVDF membrane washing procedures

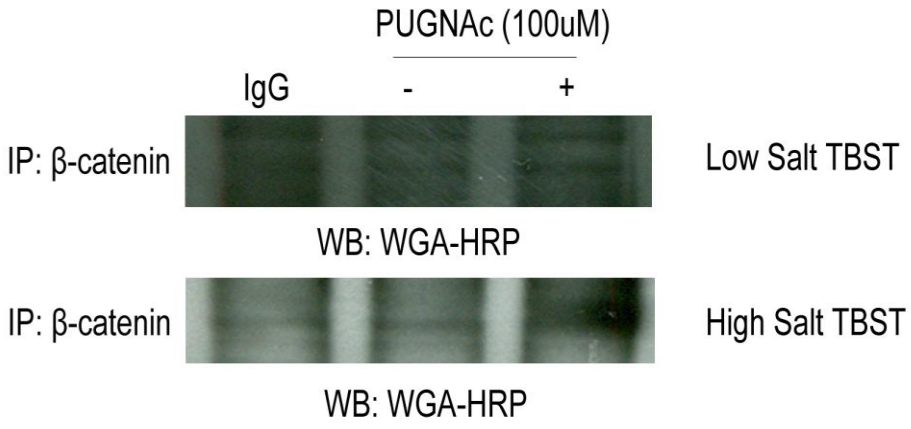

Figure A3.2.2—*High Salt Tris Buffered Saline-0.05% Tween20 (HS-TBST) minimizes non-specific binding*. Whole cell lysates of DU145 cells treated with 100 µM PUGNAc were immunoprecipitated using anti-β-catenin antibody and characterized by Western Blot Analysis using WGA-HRP. PVDF membranes were incubated in 1 µg/ml WGA-HRP diluted in 1% BSA for 45 minutes at room temperature. Membranes were washed with either Low Salt TBST or High Salt TBST.

PVDF membranes were washed in either High or Low salt wash buffers subsequent to probing with WGA-HRP. Increased salt concentrations modulate affinity of the proteins and non-specific protein-protein or protein-surface interactions. High Salt (HS) Tris buffered saline (TBS)-0.05% Tween20 (TBST) was made at a concentration of 10 mM Tris, 1 M NaCl, 0.05% (v/v) Tween 20, pH 7.5. Low Salt (LS) TBST was made at a concentration of 10 mM Tris, 150

mM NaCl, 0.05% (v/v) Tween20, pH 7.5. Figure A3.2.2 demonstrates that High Salt wash better accentuated the protein band of interest relative to LS-TBST.

In effect, immunoblotting procedures were modified to incorporate more stringent wash methods. Upon incubation of PVDF membranes with WGA-HRP, membranes were washed with LS-TBST 0.1% Tween20 for 60 minutes and subsequently washed with HS-TBS-0.05% Tween20 for an additional 60 minutes. Thereafter, membranes were rinsed in water for 30 minutes. Chemiluminescent reagent was applied at a 1:10 diluted concentration prior to exposure on autoradiograph.

A3.2.3 Alterations in PVDF membrane blocking procedures

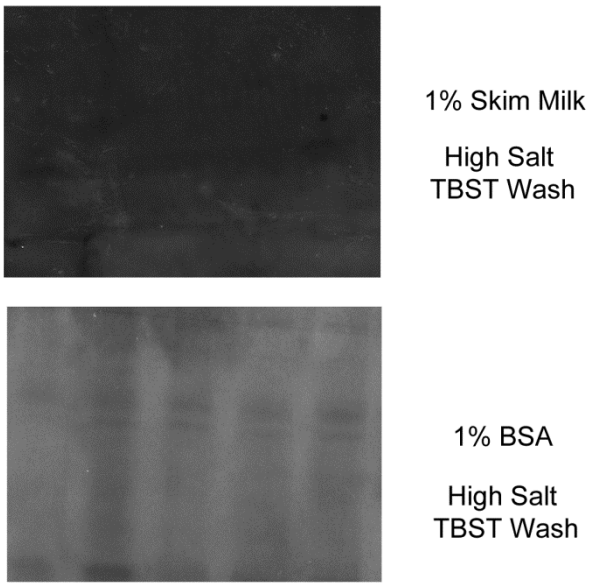

Figure A3.2.3—1% ***BSA not 1% non-fat milk minimizes background staining of PVDF membranes*****.** Characterization of PVDF membranes blocked with either 1% skim milk or 1% BSA prior to incubation with WGA-HRP.

Although altering wash stringency greatly minimized non-specific binding of WGA-HRP, the experimental procedures were further modified to analyze various blocking reagents. PVDF membranes were blocked in either 1% skim

milk (w/v), 1% BSA (w/v), or 1% Fish Gelatin (w/v) solubilized in LS-TBS-0.1% Tween20. As shown in Figure A3.2.3, BSA was the preferred blocking reagent for this particular procedure. Fish gelatin also demonstrated to be an optimal blocking reagent (data not shown). The concentration of 1% was determined to be adequate as any less would increase the non-specific binding and any more would fully block the membrane.

Although the application of these procedures significantly optimized the detection of O-GlcNAc-β-catenin, each trial/independent experiment had inconsistencies despite the controlled methodology. Furthermore, as the levels of O-GlcNAc are not present at high levels, slight deviations in background noise hindered visualization as well as imaging of the western blot. As such, this investigation used both methods of WGA-agarose precipitation as well as WGA-HRP to characterize the O-GlcNAcylation of β-catenin.

Appendix 4

Additional Information

A4.0 Appendix 4: Additional Information

A4.1 O-GlcNAcylation and the Hexosamine Biosynthetic Pathway

The O-GlcNAc modification is under unique metabolic regulatory control through the Hexosamine Biosynthetic Pathway (HBP), permitting for the modulation of various cellular pathways in response to nutrients and stress [77, 79, 141]. As a nutrient sensor, O-GlcNAcylation underlies fundamental mechanism of chronic metabolic diseases, such as diabetes/insulin resistance and cancer [77].

The HBP integrates glucose, glutamine, nitrogen, fatty acid, and nucleic acid metabolic pathways to produce uridine diphosphate (UDP)-GlcNAc, the high energy donor substrate for OGT [71]. In particular, approximately 2-3% of total cellular glucose enters the HBP where fluctuating glucose concentrations influence the concentration of UDP-GlcNAc [71].

The HBP shares its first two steps with glycolysis where hexokinase phosphorylates glucose to produce glucose-6 phosphate, where it is then converted into fructose 6-phophate by the enzyme phosphoglucose isomerase. Fructose 6-phosphate enters the HBP to be converted into glucosamine 6-phosphate by the rate limiting enzyme, glutamine:fructose -6-phosphate aminotransferase (GFAT1). Subsequent to a series of enzymatic steps, UDP-GlcNAc is produced. GFAT1 is the rate limiting step of the HBP as it receives feedback inhibition by both glucosamine 6- phosphate and the final product UDP-GlcNAc [71].

The potential for increased UDP-GlcNAc as a nutrient sensor lies in the ability of OGT to respond to varying UDP-GlcNAc concentrations [71]. OGT has a low apparent K_m for UDP-GlcNAc (545nM) allowing the enzyme to be active in times of decreased UDP-GlcNAc concentrations [70, 71, 77]. Interestingly, increased UDP-GlcNAc concentrations have been reported to change the apparent K_m of OGT for its peptide substrates, where peptides can become better substrates upon increased UDP-GlcNAc [71]. Therefore, even slight increases in UDP-GlcNAc

concentrations were able to cause differential regulation of protein O-GlcNAcylation.

A4.1.1 HBP and Cancer

One hallmark of tumor metabolism is the use of aerobic glycolysis by cancer cells (Warburg Effect) rather than oxidative phosphorylation to produce ATP [71]. In highly proliferating tumor cells, aerobic glycolysis can uphold the rapid bioenergetic and biosynthetic demands required by the cell. Aerobic glycolysis within a nutrient rich environment is an inefficient process for energy production; however, for rapidly proliferating cancer cells, this is less imperative than the requirement for biosynthesis of macromolecules and organelles [71]. Alternatively, tumor cells consume high levels of glutamine, a primary energy source, at a rate 10 fold higher than normal cells [71]. Glutamine is converted to glutamate by glutaminase then into alpha-ketogluterate in the mitochondria and can funnel into the HBP flux to increase O-GlcNAcylation [71].

The concentration of UDP-GlcNAc in cancer cells is currently unknown; however, N-linked glycosylation which uses UDP-GlcNAc as a donor substrate generates larger and more highly branched oligosaccharides in tumor cells, suggesting an increased need for this metabolite [71]. There is no general consensus to whether there is increased or decreased O-GlcNAcylation with the progression of cancer, or whether the O-GlcNAc modification is a reliable marker for metastatic potential [77]. For example, clinical data has demonstrated that solid breast, lung and colon tumours had higher levels of O-GlcNAcylation in more aggressive tumors while in chronic lymphocytic leukemia O-GlcNAcylation levels were increased in lymphocytes which corresponded to a better overall prognosis [71, 77]. However, the site specific regulatory control that O-GlcNAcylation may have on proteins is likely masked by analyzing only global cellular levels of O-GlcNAc [77]. Although no human cancers are directly linked to mutations in OGT or OGA (likely due to the lethality of such mutations), alterations in the regulation of these two enzymes have profound effects on

cellular functions [71]. Moreover, the tight regulation of O-GlcNAcylation by OGT and OGA on its protein substrates has clear implications for the functional modulation of these proteins.

A4.2 O-GlcNAcylation and Transcription

There is growing body of evidence that demonstrates the O-GlcNAc modification to generally inhibit gene expression by increasing DNA compaction [116]. Kelly et al., (1989) showed that chromosomes contain numerous glycoproteins with terminal O-GlcNAc moieties upon visualization by FITC-WGA staining. Alternatively, puff regions- the active sites of RNA synthesis were less concentrated with O-GlcNAc [142, 143]. The observed co-localization of OGT and O-GlcNAcase within chromatin associated repressor complexes supports the possibility of these two enzymes in the regulation of transcriptional silencing. OGT is known to associate with histone deacetylase (HDAC) complexes [70, 101] suggesting then, that together with O-GlcNAcase's HAT domain may dynamically regulate transcriptional complexes by O-GlcNAcylation. As such, Sakabe et al, (2010) demonstrated that histones themselves were modified with O-GlcNAc within the nucleosomal core. DNA is wound around an octamer of histones comprising a tetramer of histone 3 (H3) and histone 4 (H4) and two dimers of histone 2 H2A and H2B [144]. O-GlcNAc sites were mapped to Thr101 of H2A, Ser36 of H2B, and Ser47 of H4. Histone O-GlcNAcylation increased with heat shock and was concomitant with DNA condensation.

More recently, studies have established that *Drosophila OGT* was encoded by a polycomb group gene *super sex combs* (sxc) [143]. These genes are master regulators of repressing the expression of a subset of genes during development, stem cell maintenance, and genomic imprinting [77, 143-148]. Major sites of O-GlcNAc modification on polytene chromosomes corresponded to polycomb group binding sites, linking O-GlcNAcylation to polycomb group mediated gene silencing.

In vitro and *in vivo* binding assays demonstrated that mammalian Sin3a (mSin3a), a core component of a large multi-protein co-repressor complex, and OGT co-operatively repressed transcription [70]. The interaction was mapped to OGT's TPR repeats 1-6 and the PAH4 domain of mSin3a [70, 101]. Upon further investigation, it was found that silenced estrogen responsive genes, pS2, EB1 and CatD, were bound to hyperglycosylated proteins, also in association with mSin3a [70, 101]. This suggested a model by which mSin3a recruited OGT to promoters to repress gene transcription through increased O-GlcNAcylation.

On the other hand, evidence has shown that the formation of the pre-initiation complex by RNA polymerase II at promoter sites required the presence of O-GlcNAc modification at the C-terminal of RNA polymerase II [77, 149]. Once this complex was formed, O-GlcNAc groups needed to be removed prior to the phosphorylation of its C-terminal domain to allow for transcriptional elongation [77, 149]. Given the contrasting data, the role of O-GlcNAc on transcription factors is likely via a gene-specific manner.