O-GLYCOSYLATION OF β-CATENIN REGULATES ITS NUCLEAR LOCALIZATION AND TRANSCRIPTIONAL ACTIVITY

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Abstract

β-catenin is a transcriptional co-activator in the Wnt signaling pathway. Upon Wnt stimulation, cytosolic β-catenin translocates into the nucleus where it forms complexes with members of the TCF/LEF family of transcription factors to activate gene transcription. Translocation into the nucleus is followed by transcriptional activation of β-catenin's target genes, which are involved in proliferation, angiogenesis and oncogenesis, is a crucial step in the progression of a subset of cancers. The cellular expression of β-catenin is known to be regulated by phosphorylation. However, the mechanisms(s) responsible for β -catenin nuclear entry is not well understood.

Recently, β-catenin was reported to be post-translationally modified by O-glycosylation in breast cancer cells. We investigated whether O-glycosylation regulates the signal transduction properties of the protein. Our results indicated that while there are higher levels of total β-catenin in the nucleus of two prostate cancer cell lines (DU-145 and LNCaP) compared to that in a normal prostate epithelial cell line (PNT1A), most of it was in the unglycosylated form. Also, the normal prostate cell line exhibited higher levels of O-glycosylated β-catenin in both the nucleus and cytosol than what was seen in the two prostate cancer cell lines. We carried out further experiments using PUGNAc, a non-cytotoxic reversible inhibitor of O-GlcNAcase, which causes a time dependent increase in cellular levels of O-glycosylated β-catenin. Treatment of prostate cancer cells with PUGNAc caused a decrease in the expression of β-catenin in the nucleus with increasing cellular O-glycosylation of the protein suggesting that O-glycosylation was

hindering β-catenin nuclear translocation. Additional studies showed that O-glycosylation of β-catenin decreased that transcriptional activity of a TopFlash reporter plasmid and the protein expression of two β-catenin target genes. Our results suggest that O-glycosylation of β-catenin may represent a novel mechanism important in the regulation of the nuclear localization and transcriptional activity of β-catenin.

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Chapter 1

Introduction

1.0 Introduction

Wnts are a large family of secreted glycoproteins whose genes are highly conserved among species (Cadigan and Nusse, 1997). They activate signalling pathways involved in embryonic development, morphogenesis, cell proliferation, stem cell fate and cell differentiation. Activation of Wnt pathways are initiated when secreted Wnts bind to their cognate receptors, Frizzled (Fzd) and low-density lipoprotein receptor related proteins 5 and 6 (LRP 5/6), on the plasma membrane (Bhanot et al., 1996; Pinson et al., 2000). Fzd are a class of seven-pass transmembrane receptors encoded by the frizzled genes and share homology to G-protein coupled receptors (GPCRs). LRP 5/6 are single pass transmembrane proteins that have relatively large extracellular and short cytosolic domains (He et al., 2004). There are three main branches of Wnt signalling.

1.1 Wnt/ β-catenin-independent Pathways

1.1.1 The Wnt/Ca^{2+} Pathway

The Wnt/Ca^{2+} pathway are activated by certain Frizzled receptor homologs that recruit in heterotrimeric GTP-binding proteins subsequently activating Phospholipase C (Kohn and Moon, 2005). Another method of Wnt/Ca^{2+} activation is through specific Wnt and Fzd homologs that stimulate protein kinase C (PKC) and calmodulin dependent protein kinase II (CAMKII), both of which play a role in regulating cell adhesion and motility (Kohn and Moon, 2005; Torres et al., 1996). Wnt stimulated CAMKII activity has been shown to participate in axis formation in early embryos of *Xenopus*, promoting

activity on the ventral side and antagonizing dorsal fate (Kuhl et al., 2000). This intracellular calcium release seems to be required for body plan specification in *Xenopus* development.

1.1.2 The Planar Cell Polarity Pathway

The second pathway is known as the planar cell polarity (PCP) pathway. PCP is the generation of a population of cells with uniform orientation within a single epithelial plane. It has been best studied in *Drosophila* as seen on structures such as bristles on the thorax, aligned hairs on wing cells and arrangement of ommatidium, modular building blocks, on facet eyes of insects (Jenny et al., 2006). Upon Wnt binding, Fzd receptor passes the signal to Dishevelled (DSH, Dvl in vertebrates), which has a structure typical of adaptor proteins and contains three protein-protein interaction domains (DIX, PDZ and DEP) which participate in the activation of different signalling events depending on which downstream effector it interacts with. The signal then travels via the small GTPases Rho and Rac stimulating the serine threonine kinase c-Jun N-terminal kinase (JNK) cascade eventually transmitting the signal to the nucleus (Fanto et al., 2000; Strutt et al., 1997; Boutros et al., 1998; Weber et al, 2000). Dvl mediates Wnt signalling during embryogenesis which is important in regulating development patterns, such as elongation of the dorsal-ventral axis, similar to PCP, termed convergent extension during gastrulation in vertebrates (Boutros et al. 1998; Heisenberg et al, 2000).

1.2 The Wnt/ β-catenin Canonical Pathway

The final pathway is the most well known of the three; it is the Wnt/Wingless β-catenin canonical pathway, which regulates cell proliferation and differentiation through a central mediator, β-catenin (Kuhl et al., 2000; Kuhl et al., 2001; Winklbauer et al, 2001; Sokol, 2000). β-catenin is a multifunctional protein; it was originally identified as the cytoplasmic component of adherence junctions, where it interacts with the cytoplasmic tail of the transmembrane protein E-cadherin and links it, via α catenin, to the actin cytoskeleton (Kemler 1993; Geiger et al., 1995). β-catenin was also thought to have signalling properties when the gene was cloned and found to be the mammalian homologue of armadillo, a segment polarity gene involved in the wingless signalling pathway in *Drosophila* (McCrea et al., 1991). β-catenin functions as a transcriptional co-activator regulated by the Wnt/ Wingless Canonical pathway. Cytosolic levels of β-catenin are tightly regulated by a multi-protein degradation complex that consists of the tumour suppressor protein adenomatous polyposis coli (APC) along with scaffolding proteins axin and conductin/axin2, which position β-catenin for phosphorylation at specific serine and threonine residues at the N-terminus of the protein by Casein Kinase 1 alpha (CK1α at serine 45) and Glycogen Synthase Kinase 3 beta (GSK3β at Threonine 41 and Serine 37 and 33) (Rubinfeld et al., 1993; Su et al., 1993; Behrens et al., 1998; Liu et al., 2002; Rubinfeld et al., 1996). Phosphorylation at these conserved sites targets the protein for ubiquitination and degradation by the 26S proteosome (Aberle et al., 1997) (Figure 1.1A).

Activation of the canonical pathway is initiated upon Wnt binding to Fzd. This promotes the phosphorylation of LRP 5/6 by GSK3β and CKIγ, which recruits axin to the plasma membrane where it is inactivated and/ or targeted for degradation through an unknown mechanism (Tamai et al., 2004; Davidson et al., 2005; Zeng et al., 2005). In addition, Wnt binding to Fzd results in hyperphosphorylation of Dvl (Willert et al., 2003; Rothbacher et al., 2000; Umbhauer et al., 2000; Takada et al., 2005). Though the mechanism is not clear, it has been reported by Swiatek et al., that CKIε can be activated by Wnt stimulation and several kinases such as CKIε and Par 1 that appear to be required for Wnt canonical signalling, can also phosphorylate Dvl (Swiatek et al., 2004; Peters et al., 1999; Sun et al., 2001; Gao et al., 2002; Hino et al., 2003; Cong et al., 2004; Ossipova et al., 2005). In turn, hyperphosphorylation of Dvl inhibits GSK3β (Yanagawa et al., 1995); however how it does so is still unknown. Dvl recruitment to the membrane and subsequent phosphorylation upon Wnt stimulation causes a conformational change in the protein resulting in the dissociation of a complex consisting of GSK3β, axin, the inhibitory protein Fratl and Dvl (Giles et al., 2003; Li et al., 1999; Fraser et al., 2002). Thus, the phosphorylation of Dvl prevents the phosphorylation of β-catenin by GSK3β (Giles et al., 2003). Both the degradation of axin and inhibition of GSK3β result in decreased phosphorylation and increased stabilization of β-catenin (Moon et al., 2004).

β-catenin accumulates in the cytosol, which subsequently leads to the localization of the protein into the nucleus. Its ability to interact with the T-cell Factor (TCF)/ lymphoid enhancer factor (LEF) family of transcription factors and activate transcription of Wnt target genes (Behrens et al., 1996; Molenaar et al., 1996) is dependent upon two

conserved nuclear proteins recently discovered in *Drosophila*, Pygopus (Pygo) and Legless/BCL-9 (Lgs) (Kramps et al., 2002; Thompson et al., 2002; Parker et al, 2002; Belenkaya et al., 2002)). Lgs/BCL-9 acts as an adaptor molecule connecting β-catenin to Pygo, which is constitutively localized to the nucleus and acts as a nuclear anchor. Studies done by Townsley et al. have shown that the nuclear localization of Lgs is entirely dependent on Pygo, and suggests that these proteins act primarily to target β-catenin to the nucleus (Townsley et al., 2004; Townsley et al., 2004) (Figure 1.1B).

In the absence of Wnts, DNA bound TCF/LEF interacts with the transcriptional repressor Groucho (Grg) (Roose et al., 1998; Brantjes et al., 2001; Chen et al., 1999.), which help to repress transcription of TCF genes by recruiting histone deacetylases. β-catenin contains a transactivation domain at its C-terminus, which has binding sites for transcriptional coactivators such as p300/CREB binding protein (CBP) (van de Wetering et al., 1997; Hecht et al., 2000; Takemura and Moon, 2000). CBP acts as a transcriptional coactivator by altering chromatin structure utilizing its intrinsic histone acetylase (HAT) activity to increase access to target gene promoters as well as acting as adaptor proteins that connect activators to the basal transcriptional machinery (Ogryzko et al 1996; Goldman, 1997). Once in the nucleus, β-catenin is able to interact with TCF/LEF elements bound to their promotor sites on the DNA, causing a conformational change that releases Grg, and through its transactivation domain and its ability to recruit other transcriptional co-activators, is able to drive the expression of Wnt/ β-catenin target genes.

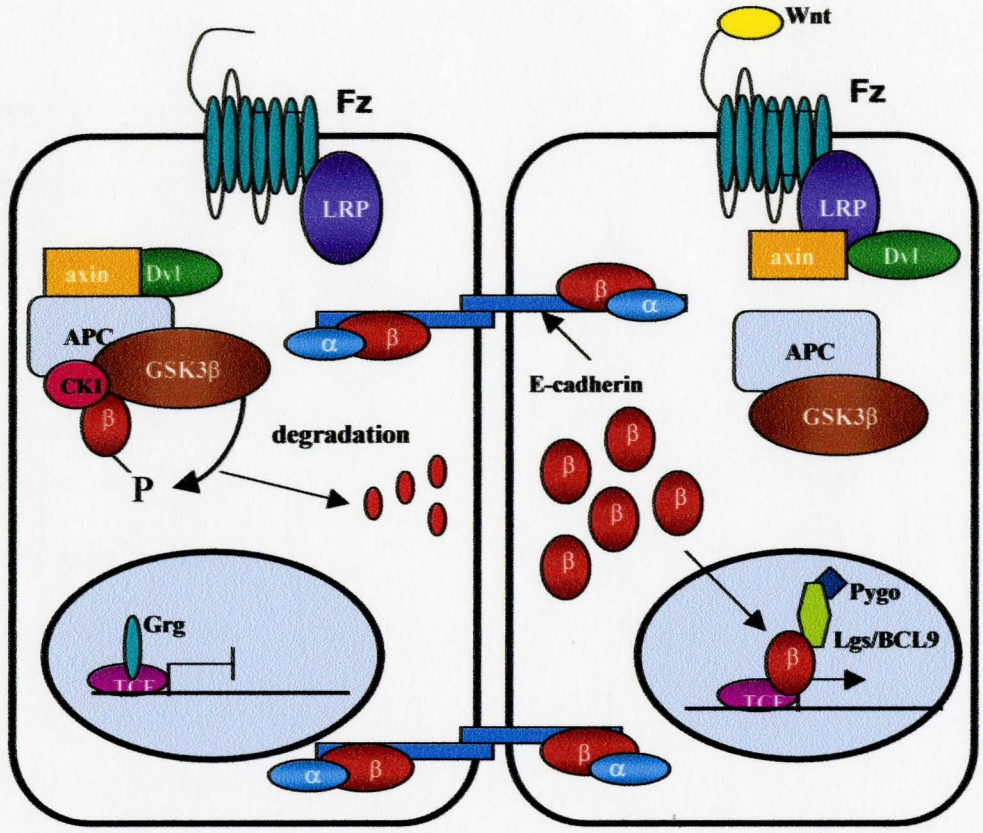


Figure 1.1: Wnt/β-catenin Signalling Pathway.

Figure 1.1A: In the absence of Wnt, β-catenin is degraded and regulation of prospective target genes are repressed.

Figure 1.1B: Wnts are secreted and bind to their receptor, which stabilizes β-catenin leading to its accumulation in the cytosol. β-catenin levels increase in the cytosol and subsequently β-catenin translocates into the nucleus where it interacts with T-cell Factor/Lymphoid Enhancer Factor (TCF/LEF) family of transcription factors, and activates transcription. The component shown are described in more detail in the text. β – β-catenin; Fz - Frizzled; LRP – Low density Lipoprotein receptor-related protein; Dvl - Dishevelled; APC – Adenomatous Polyposis Coli; GSK3β Glycogen Synthase Kinase 3beta; CK1 – Casein Kinase 1; α – alpha-catenin; Grg - Groucho; TCF – T-cell Factor; Pygo - Pygopus; Lgs/BCL9 – Legless/ B-cell Lymphoma-9 (Adapted from Moon et al. (2004) *Nature Rev.* **5**:689-99.)

1.2.1 The protein structure of β-catenin.

The central core region of β-catenin contains 12 repeating motifs called Armadillo repeats (Peifer et al., 1994; Huber et al., 1997) that are important in protein-protein interactions with many of β-catenin's interacting partners, as well has been demonstrated to be involved in the nuclear localization of the protein (Funayama et al., 1995; Koike et al., 2004). The N-terminus of the protein contains the regulatory region; also know as the "destruction box", which contains conserved serine and threonine residues that become phosphorylated by GSK3β subsequently targeting the protein for degradation. Mutations in this region, resulting in improper phosphorylation and degradation of the protein, have been found in many cancers (Morin et al., 1999). The transactivation domain of β-catenin is located at the C-terminus of the protein and contains binding sites for essential transcriptional co-activators (Hecht et al., 2000; Takamura and Moon, 2000). This region is also indispensable for Wnt signalling *in vivo* (van de Wetering et al., 1997; Cox et al., 1999) (Figure 1.2).

1.3 Target genes of Wnt/ β-catenin signalling pathway

The β-catenin/ TCF/ LEF bipartite complex targets transcription of numerous genes including those involved with proliferation, differentiation and apoptosis.

Cyclin D1 promotes the progression of cells through the G1 phase of the cell cycle. It phosphorylates and inactivates the cell-cycle inhibiting function of the retinoblastoma protein (pRb) in response to mitogenic signals. pRb serves as a gate keeper of the G1 phase, and passage through this checkpoint leads to DNA synthesis

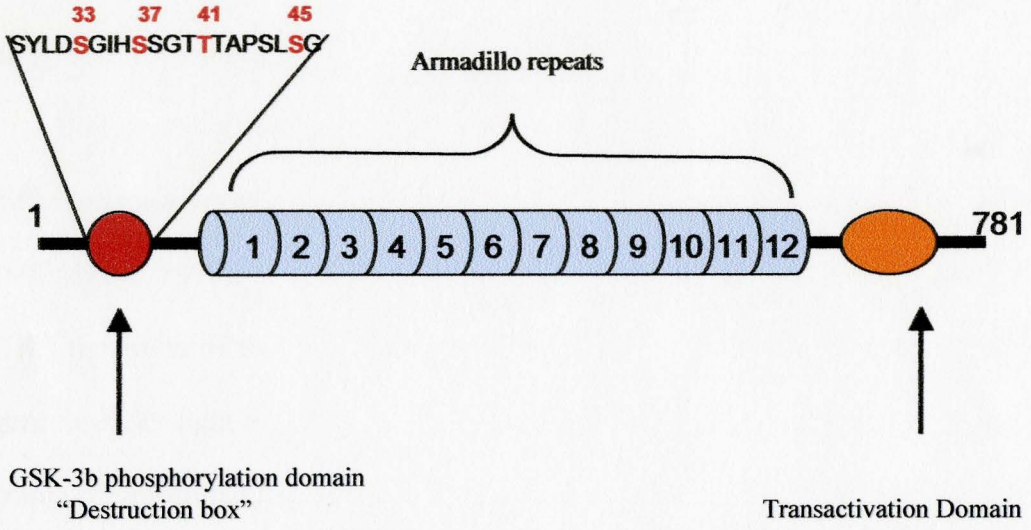


Figure 1.2: Schematic representation of the β-catenin domain structure.

The N-terminus (Red circle) contains four conserved serine/threonine phosphorylation sites (indicated in red) that are essential for mediating degradation of β-catenin. CK1 phosphorylates ser45 which primes the protein to be further phosphorylated by GSK3β at Ser33, Ser37 and Thr 41. The central core consists of 12 repeats of 42 amino acids (denoted Armadillo repeats 1-12 in gray) are important for mediating interactions between β-catenin and its binding partners. The C-terminal domain (Orange circle) contains transcriptional activation elements that are essential for the signalling activity of β-catenin. (Adapted from Morin PJ (1999) *Bioessays* **12:**1021-30)

(Baldin et al., 1993). Deregulated cyclin D1 expression is one of the most commonly observed alterations that cause unwarranted transit through the G1 phase (Bartkova et al., 1994). Overexpression of cyclin D1 is known to correlate with the risk of tumour progression and metastasis (Bartkova et al., 1994; Callanan et al., 1996).

C-myc is a transcription factor involved in cellular functions such as proliferation, differentiation, neoplastic transformation and apoptosis (Napoli et al., 2002; Hurlin et al., 1995; Dang, 1999). It has a central role in regulating cell proliferation specifically at the G1/S transition of the cell cycle (Dang et al., 1999). Normal functioning of the c-myc gene is under tight regulation by a variety of factors. Deregulation of this gene resulting from chromosomal translocation has been shown to promote the development of lymphoid malignancies (Shen-Ong et al., 1982; Dalla-Favera et al., 1982). The oncogenic properties of the c-myc gene and its product has been implicated in a variety of cancers where amplification of this gene is seen in lung, breast and cervical cancers and elevated levels of the protein are found in breast and colon cancers (Little et al., 1983; Munzel et al., 1991; Berns et al., 1996; Erisman et al., 1989).

Cyclin D1 and c-myc are over expressed in colorectal carcinomas, however both genes are rarely amplified or contain genetic alterations in colon cancers (Erisman et al., 1989). It was this unusual observation that prompted researchers to identify the cause of this over expression and lead to the identification of cyclin D1 and c-myc as target genes of β-catenin/TCF/LEF mediated transcription. Reintroduction of wild type APC into colon cancer cell lines that contain elevated levels of β-catenin resulting from mutations in APC, represses the expression of c-myc. β-catenin is able to activate c-myc expression

through TCF binding sites that have been identified in c-myc's promoter region (He et al., 1998). In addition the expression of cyclin D1, which contains TCF/LEF binding sites within its promoter, is strongly dependent on β-catenin/TCF signalling and has a direct effect on cell proliferation (Tetsu and McCormick, 1999; Shtutman et al., 1999).

Matrix metalloproteinases (MMPs) are a family of proteases that participate in invasion of growth whereby basal membranes and extracellular matrix are degraded (Brabletz et al., 1999). In cancer, these proteins are essential for the progression from benign adenomas to malignant carcinomas; initiating and maintaining the growth of primary tumours and metastasis. The expression of MMP-7 (also known as matrilysin) has been shown to be important for the invasive and metastatic potential of cancer cells (Powell et al., 1993; Yamamoto et al., 1995). It is expressed in tumour cells of colorectal carcinomas where overexpression of MMP-7 is shown in approximately 80% of colon cancer cells (Newell et al., 1994; Witty et al., 1994). Interestingly, a correlation of nuclear β-catenin and MMP-7 expression has been shown in human colorectal carcinomas (Brabletz et al., 1999) as well as in esophageal carcinomas (Saeki et al., 2002). Identification of TCF binding sites on the MMP-7 promoter is direct evidence that MMP-7 is a target gene of β-catenin/TCF/LEF signalling (Crawford et al., 1999).

Vascular endothelial growth factor-A (VEGFA) promotes the formation of new blood vessels and maintains their integrity by activating endothelial cell survival and antiapoptotic pathways (Xie et al., 2004; Baldwin et al., 2002; Nagy et al., 2002). This angiogenic protein is essential for tumour development and progression. VEGFA expression is correlated to tumour grade and depth of invasion and is also used as an

important prognostic factor in many human tumours (Hazelton et al., 2002; Lamszus et al., 2000; Dvorak, 2002; Toi et al., 2001). Studies done by Zhang et al. identified VEGFA as a target gene of β-catenin in HeLa and colon cancer cells (Zhang et al., 2001). Further studies identified seven TCF/LEF binding elements in the VEGFA promoter. In addition, β-catenin induces VEGFA mRNA and protein expression in human colon cancer cell lines (Easwaran et al., 2003).

Additional β-catenin target genes such as WISP-1, ARF and PPAR delta have been implicated as contributors to the progression of certain cancers when constitutively active (He et al., 1998; Xu et al., 2000; Damalas et al., 2001).

β-catenin also regulates important developmental genes such as siamois, twin and nodal-related 3 in Xenopus and Ultrabithorax (Ubx) in Drosophila (Gradl et al., 1999; Brannon et al., 1997; Laurent et al., 1997; McKendry et al., 1997; Riese et al., 1997) as well as mammalian genes involved in coordinating cell-cell and cell-substrate adhesion (Gradl et al., 1999) and regulating gap-junction communication (van der Heyden et al., 1998).

1.4 β-catenin signalling in Cancer

The deregulation of the Wnt/ β-catenin pathway and increased levels of β-catenin in the nucleus have been associated with tumorigenesis and the development of many types of cancers. Mutations in the components of the pathway involved in regulating β-catenin levels occur in a variety of cancers, and was first noted in colorectal cancers

(Brembeck et al. 2006; Vogelstein et al., 2004; Cottrell et al., 1992; Powell et al., 1992; Morin et al., 1997; Polakis, 2000; Bienz and Clevers, 2000).

Familial adenomatous polyposis (FAP) occurs due to a germline mutation in the tumour suppressor gene APC and results in an intestinal disorder involved with the development of colorectal polyps and increased risk of colon cancer (Henderson 2000; Miyoshi et al., 1992; Kinzler and Vogelstein, 1996). Inactivating mutations in APC typically result in a truncated protein found in 95% of FAP patients (Kinzler and Vogelstein, 1996; Laken et al., 1999). In some colon cancers where APC is not mutated, there are mutations in the amino terminus of the β-catenin protein. Mutations in the β-catenin gene, CTNNb1, at the GSK3β phosphorylation sites, result in an active form of the protein. This mutant form of the protein has been identified in two colon cancer cell lines HCT116 where there is a deletion at serine 45 and SW48 where there is a substitution at serine 33 (Morin et al., 1999). Interestingly, APC mutations and oncogeneic β-catenin mutations are mutually exclusive in colon cancer (Sparks et al., 1998). In a study done by Sparks et al. 50% of primary tumours with wild type APC, contain mutations affecting the phosphorylation sites at the regulatory domain of β-catenin. Moreover, tumours with mutated APC contained elevated levels of wild type β-catenin (Sparks et al., 1998).

Similarly, exclusivity between mutations in APC and β-catenin has also been found in numerous melanoma cell lines (Robbins et al., 1996; Rubinfeld et al., 1997). Melanomas with abnormally high levels of β-catenin are usually associated with mutations in CTNNb1, where as APC is altered in two melanoma cells lines with elevated

β-catenin expression but without CTNNb1 mutations (Rubinfeld et al., 1997). The common skin cancer known as pilomatricomas has the highest frequency of β-catenin mutations with 75% of tumours containing mutations in the N-terminal region. Deregulated β-catenin signalling is suggested to be the predominant cause of this cancer (Chan et al., 1999).

Mutations in Exon 3 of β-catenin gene, which affect N terminal phosphorylation sites, have also been found in 20% of primary human heptocellular carcinoma (HCC) tumours and hepatoma cell lines (Morin et al., 1999; Miyoshi et al. 1998; de la Coste et al., 1998). Polakis points out that two β-catenin target genes; c-myc and cyclin D1 are both amplified in a subset of HCC, though no overlap between their amplification and CTNNb1 mutations in HCC have been shown (Polakis, 2000).

Biallelic inactivation of Axin, originally identified as an inhibitor of Wnt signalling and a tumour suppressor, have been identified in HCC (Polakis, 2000; Peifer and Polakis, 2000, Satoh et al., 2000). Importantly, these mutations are found in HCCs that lacked mutations in CTNNb1. Mutations in the Axin gene result in a truncated protein that is unable to bind to β-catenin. Also, mutations in APC, which also result in a truncated form of the protein, lack Axin-binding sites. Mutations in these proteins, that are essential for the degradation and regulation of β-catenin, promote the protein's stabilization and induce its nuclear accumulation in cancers.

β-catenin mutations in the regulatory region of the protein have also been identified in other cancers such as ovarian cancer and medulloblastoma, and to a lesser

extent in prostate and uterine cancer (Polakis 1999; Palacios et al., 1998; Voeller et al., 1998; Kline et al., 1990; Itoh et al., 1993; Zurawel et al., 1998)

1.5 Nuclear translocation of β-catenin

The migration of nucleocytoplasmic proteins into the nucleus occurs via the conventional import pathway, which requires the protein to contain a nuclear localizing sequence (NLS), a short sequence of basic amino acids (Dingwall and Laskey, 1991). The NLS on the protein is recognized by importin α which then complexes with importin β, which interacts with nuclear pore complex (NPC) components and translocates through the nuclear pore by an energy dependent process involving the small soluble GTPase Ran (Gorlich and Mattaj, 1996; Nigg, 1997; Melchior et al., 1993; Moore et al., 1993).

β-catenin does not seem to follow the conventional pathway in that it does not contain an identifiable NLS, but was thought to be carried into the nucleus via its association with TCF/LEF, which does possess a typical basic-type NLS (Fagotto et al. 1998; Moolenar et al., 1996; Behrens et al., 1996; Huber et al., 1996). However studies done in Drosophila showed that a mutant form of Arm that was unable to bind to TCF/LEF, could still localize to the nucleus (Orsulic et al., 1996; van de Wetering et al., 1997). This suggested that β-catenin affects its own entry into the nucleus. This hypothesis was further supported by Yokoya et al. who showed that β-catenin can enter the nucleus without the aid of import receptors, and does not require Ran or GTP hydrolysis (Yokoya et al., 1999). It has been suggested that β-catenin nuclear translocation involves direct interaction with components of the nuclear pore complex

(NPC) however recent findings have challenged this view (Fagotto et al., 1998; Suh et al., 2003). To date, no nucleocytoplasmic binding partner of β-catenin has been identified to be mandatory for its nuclear import, although APC is known to bind and export β-catenin from the nucleus.

The classical export pathway is similar to the import pathway where proteins to be exported out of the nucleus contain nuclear exporting sequences and require specific exporting receptors that utilize Ran assisted GTP hydrolysis for translocation through the NPC to the cytoplasm. Initially, β-catenin's export was thought to be dependent on APC, which exits the nucleus via the classical export pathway. The nuclear cytoplasmic distribution of β-catenin was often correlated with APC, suggesting that APC physically escorted β-catenin out of the nucleus (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). However, studies done by Wiechens et al. suggest that β-catenin can also exit the nucleus independently of export receptors and energy requirements similarly as observed in import (Wiechens et al., 2001).

β-catenin's Armadillo repeats, involved in interaction with β-catenin's binding partners, along with the C-terminal transactivation domain have been shown to both be necessary and sufficient for the nuclear localization of the protein (Peifer et al., 1994; Huber et al., 1997; Funayama et al., 1995; Koike et al., 2004). Interestingly, the nuclear import receptor importin β, and its related proteins, possess similar motifs called HEAT motifs which are fundamentally similar in structure to ARM repeats and could also share functional similarities (Aitchison et al. 1996; Malik et al. 1997).

1.6 O-glycosylation of Nucleocytoplasmic proteins

O-linked β-*N*-acetylglucosamie (O-GlcNAc) is a post-translational modification found on nuclear and cytosolic proteins. It consists of the monosaccharide *N*-acetylglucosamine linked to the hydroxyl group of either serine or threonine residues (Haltiwanger et al. 1998). The nucleocytoplasmic enzymes O-β-*N*-acetylglucosaminyltransferase (OGT), which adds the O-GlcNAc and O-β-*N*-acetylglucosaminidase, which removes the sugar moiety (Kamemura and Hart, 2003), catalyze this dynamic modification. There are many proteins known to be O-glycosylated including tumour suppressor proteins, nuclear pore proteins, heat shock proteins and a variety of transcription factors (Hart, 1997).

O-glycosylation has been shown to regulate the function of many proteins. For example, the O-glycosylation of the C-terminal domain of RNA polymerase II may be involved in the formation of a pre-initiation complex (Kelly et al., 1993). Also, O-glycosylation of the c-myc protooncogene product promotes the stability and the subcellular localization of the protein (Kamemura et al., 2002). Moreover O-GlcNAc modification of the ubiquitous transcription factor Sp1, which plays a vital role in control of TATA-less house keeping gene transcription, modulates its transactivation capabilities by affecting its ability to interact with basal transcription machinery (Roos et al., 1997). Studies done by Majumdar et al. have also shown that O-glycosylation of Sp1 facilitates its migration into the nucleus and regulates the stability and activity of the protein

(Majumdar et al. 2004; Majumdar et al., 2006; Jackson et al., 1988; Keembiyehetty et al., 2002).

O-glycosylation is often compared to phosphorylation because both modifications have been shown to target the same residues on proteins and it has been suggested that O-GlcNAc competes with phosphate for the hydroxyl group on some proteins (Kamemura and Hart, 2003). Studies done by Comer et al. have demonstrated a reciprocal relationship between phosphorylation and O-GlcNac modification on the C terminal domain of RNA polymerase II (Comer et al., 2001). Phosphorylation of the C-terminal domain is required for transcriptional elongation. Glycosylation *in vitro* inhibits its phosphorylation and vice versa (Wells et al. 2001).

The phosphoprotein, c-myc, is degraded by the ubiquitin-proteosome pathway (Kamemura and Hart, 2003). Phosphorylation of the protein at Thr58 is required for its degradation. Post-translational modification of this residue regulates c-myc turnover and is known to be a mutational hot spot in lymphomas (Chou et al., 1995). As mentioned earlier, O-GlcNac regulates the stability of c-myc, it does this by specifically modifying the protein at Thr58 thus preventing phosphorylation and subsequent degradation.

Reciprocity between glycosylation and phosphorylation has also been reported for Sp1. Haltiwanger er al. has shown that increasing O-glycosylation in HT29 cells decreases the level of phosphate on Sp1 (Haltiwanger et al., 1998). Thus there is much evidence showing a reciprocal relationship in which the sites of glycosylation and phosphorylation occur at specific sites on particular proteins. On a broader scale, there are also studies that have shown that increasing global levels of O-glycosylation in cells

lead to decreased levels of phosphorylated proteins and vice versa (Griffith and Schmitz, 1999).

How nucleocytoplamic proteins may be functionally regulated by the analogous relationship between O-GlcNac glycosylation and phosphorylation and the mechanisms regulating this dynamic interplay are not well understood.

O-glycosylation of β-catenin was first observed in studies done by Zhu et al. These studies investigated whether cytoplasmic O-glycosylation regulated cell surfaced transport of E-cadherin during apoptosis. Treatment of breast cancer cells with an endoplasmic reticulum calcium pump inhibitor, thapsigargin (TG), resulted in O-glycosylation of both β-catenin and E-cadherin (Zhu et al., 2001). O-glycosylation did not alter β-catenin's adhesive properties, shown by its ability to still bind to E-cadherin, whether in the unglycosylated or glycosylated form.

1.7 Hypothesis and Objectives

Previous work done in breast cancer cell lines MCF 7 and MDA-MB-231 showed that there were higher basal levels of β-catenin, particularly in the nucleus, compared to a normal immortalized breast epithelial cell line, MCF 10A, however most of it being in the unglycosylated form. MCF 10A cells showed higher levels of O-glycosylated β-catenin than both of the breast cancer cell lines (unpublished data). Treatment of MCF 7 cells with the sarcoplasmic reticulum calcium pump inhibitor (TG) caused a time dependent increase in the O-glycosylation of β-catenin. TG treatment also caused a significant reduction in the mRNA and protein expression of cycling D1, a major target gene of β-

catenin transcriptional activity. However, this decrease may also have been attributed to other unspecific effects induced by TG.

Since O-glycosylation did not affect β-catenin's adhesive functions we sought to determine whether this modfication affected the protein's signalling properties. Based on the results seen in breast cancer cells we hypothesized that O-glycosylation of β-catenin regulates the protein's nuclear translocation and transcriptional activity.

Objective 1. Prostate cancer comparison

The basal levels of whole cell, nuclear and cytosolic O-glycosylated β-catenin in two prostate cancer cell lines DU145 and LNCaP will be compared to that in a normal immortalized epithelial prostate cell line, PNT1A.

Objective 2. PUGNAc effects on subcellular localization of β-catenin.

To modulate the levels of O-glycosylated β-catenin in the prostate cancer cell line DU145 with the potent, reversible inhibitor of the enzyme O-GlcNAcase, O-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc).

To determine the effects of increased O-glycosylation on the subcellular localization of β-catenin.

Objective 3. Determining changes in transcriptional activity of β–catenin and target gene protein expression.

To determine the changes that increasing O-glycosylation has on the transcriptional activity of β-catenin and the protein expression of two of its target genes.

Objective 4. Determine whether O-glycosylation can alter Wnt induced increases in nuclear β–catenin.

To investigate if O-glycosylation affects β-catenin's subcellular localization by using conditions known to affect nuclear localization and determining if increasing O-glycosylation under these conditions changes nuclear levels of β-catenin.

Chapter 2

Materials and Methods

2.1 Cell Culture

Human prostate cancer cells DU145 and LNCaP, mouse fibroblast cells lines NIH 3T3, L cells and L Wnt-3a cells were obtained from the American Type Culture Collection (ATCC) and were grown in Dulbecco's modified eagle's medium (DMEM; Sigma), supplemented with 10% fetal bovine serum (FBS; Hyclone), and 1% penicillin/streptomycin (P/S: Invitrogen). Human normal immortalized prostate cell line PNT1A were grown in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS; Hyclone), and 1% penicillin/streptomycin (P/S: Invitrogen). All cells were incubated in 5% CO2 at 37'C.

2.2 Cell Treatments

PUGNAc (Toronto Research Chemicals) was dissolved in sterile Milli-Q water to a final concentration of 20 mM, and diluted to final experimental concentrations in culture medium. DU145 cells were grown to 60-70% confluency in media containing 10% FBS, and then incubated in media containing 1% FBS for 18 hours before PUGNAc treatment.

Recombinant Wnt3a (R&D Systems) was dissolved in sterile Phosphate Buffered Saline to a final concentration of 10μg/ml, and diluted to final experimental concentrations in culture media.

2.3 Preparation of Conditioned Media

Conditioned media was collected from L cells and Wnt-3a-L-cells. These cells were seeded at a low density on 100mm culture plates and grown to 70 – 80% confluency in 10mL of media. If not used right away, conditioned media was stored at 4'C. All cells were treated with 10mL of conditioned media for various time periods.

2.4 Preparation of β-catenin Standard Curve

Recombinant human β-catenin standard (R&D Systems) was reconstituted with 500uL in 1mM EDTA, 0.5% Triton X-100 in PBS, pH 7.2-7.4 to a final concentration of 130ng/mL. A seven point standard curve using 2-fold dilutions with concentrations ranging from 130ng/mL to 2.03ng/mL was used. 5X SB was added to the samples, heated at 100'C for 5 minutes, loaded on to a tricine gel and immunoblotted for β-catenin. Corresponding protein/cells from DU-145 lysates were run on the same gel and immunoblotted for β-catenin. The densities of the total β-catenin bands on the Western blot were approximately the same values as densities taken from separate experimental β-catenin immunoblots indicating that the exposure times for the separate pieces of films were similar. Thus the standard protein band densities were suitable to produce a standard curve from which β-catenin amounts could be interpolated. The densities of the bands were quantified and graphed using Excel spreadsheet program. A regression line using the most linear part of the graph was used to interpolate relative amounts of β-catenin.

2.5 Preparation of Cell Lysates

Whole cell lysates were prepared by incubating with lysis buffer [10mM Tris-HCl pH 7.5, 1mM EDTA, 1mM EGTA, 0.5% Triton X-100, 2mM phenylmethylsulfonyl fluoride (PMSF), 80ng/ml aprotinin, 40ng/ml chymostatin, 40ng/ml antipain, 40ng/ml leupeptin, 40ng/ml pepstatin] for 15 minutes on ice and passed through a 20 gauge needle six times, then incubated for another 15 minutes on ice. Cellular debris and nuclei were removed by centrifugation at 16,000 rpm for 10 minutes at 4'C and the supernatant was collected.

Protein concentrations of whole cell lysates were determined for the supernatants using the Bradford protein assay (Bio-Rad) and read on a microplate reader at a wavelength of 570nM. The results were compared with bovine serum albumin (BSA) at known concentrations and the protein level (mg/mL) was extrapolated. If not used right away, all lysates were snap frozen in liquid nitrogen and stored at –80'C.

2.6 Cell counting on the Coulter Counter

A 100mm plate of cells was scraped into 11mL of PBS. After mixing thoroughly, 1mL of the cell suspension was taken and added to 10mL of Coulter counter isotone solution and mixed gently. An aliquot of the cells was counted using a Coulter counter (model Z2) set at a precalibrated threshold that was appropriate for the cell lines used. Each count was repeated three times and the mean value was used. The remaining 10mL of cell suspension was then spun down and used to prepare lysates.

2.7 Preparation of Nuclear and Cytosolic Extracts

Nuclear and cytosolic extracts were separated using the NE-PER kit as described by the manufacturers (Pierce). 10^6 cells (approximately 1x100mm plate of cells) were counted and incubated in 100μL of CERI and 10 μL of protease inhibitor cocktail (PIC) (Roche) for 10 minutes on ice, then 5.5μL of CERII was added, the lysate was vortexed for 5 seconds and incubated on ice for 1 minute. The cell lysates were centrifuged at 16,000 rpm for 10 minutes at 4°C. The supernatant containing the cytosolic fraction was collected and stored at -80 °C.

The remaining pellet containing the nuclei was resuspended in 50μL NER and 5 μL PIC (per 10^6 cells). The nuclei lysate was passed through a 20 gauge needle and vortexed in order to break open the nuclei and collect the inner contents. The samples were incubated on ice for 40 minutes, vortexing for 20 seconds every 10 minutes. The samples were centrifuged for 10 minutes at 16,000 rpm. The supernatant containing the nuclear fraction was collected and stored at -80 °C.

Protein concentrations of nuclear and cytosolic lysates were determined using the Bradford protein assay (Bio-Rad).

2.8 PUGNAc Treatment and WGA precipitation

DU145 cells were incubated in media containing 1% serum for 18 hours before PUGNAc treatment. Thereafter, cells were treated with PUGNAc (100μM) in the absence of any serum for different time intervals (4, 8, 14, 18 and 22 hours).

O-glycosylated proteins were isolated using the lectin Wheat Germ Agglutinin (WGA), which are proteins that bind to O-glycosylated proteins. 100μg of total protein or 10^6 cells from lysates (whole cell or cytosolic/nuclear) was mixed with 20 μL of WGA conjugated to Sepharose beads (Sigma), and topped off to 400-500uL with lysis buffer. The complexes were mixed by rotation for at least 2 to 4 hours at 4'C. Following, O-glycosylated protein complexes were pelleted by centrifugation at 16,000 rpm then washed 3-5 times with lysis buffer. The pellet was resuspended in 20uL of 5X sample buffer and the samples were heated at 100'C for 10 minutes. The samples were separated on either 10% SDS-PAGE or tricine acrylamide gels and the gels were then used for immunoblotting (Western blot) with various antibodies.

2.9 Western Blot

Protein and cell equivalence were resolved by SDS-PAGE and Tricine Gel electrophoresis, respectively. Protein concentrations were determined by using a Bradford assay (BioRad). Cell numbers were counted using a coulter counter. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Roche). The gels were transferred to the membrane at 85 volts fro 1 hour in transfer buffer (0.25M Tris, 2M Glycine, 20% 15% methanol), followed by blocking in 5% non-fat dry milk prepared in Tris Buffered Saline Tween (TBST: 1mM Tris Base, 15 mM NaCl, 0.1% Tween, pH 8.0; Sigma) overnight at 4'C. Membranes were probed for 1 hours and 30 minutes at room temperature or overnight at 4 'C with the relevant primary antibodies. The following antibodies were used in this study; anti-β catenin antibody (1:1000; mouse monoclonal;

Transduction Laboratories), anti-β actin (1:10 000; mouse monoclonal; Sigma), anti-Lamin B (1:1000; mouse monoclonal; Calbiochem), anti-α,β tubulin (1:1000; rabbit polyclonal; Cell Signaling), Cyclin D1 (1:200, mouse monoclonal; Sigma), VEGFa (1:200; rabbit polyclonal; Santa Cruz), anti-Sp1 (1:500; rabbit polyclonal; Santa Cruz), and anti-TCF4 (1:200; mouse monoclonal; Upstate). Membranes that were transferred from SDS-acrylamide gels were incubated in primary antibody diluted in 5% non-fat dry milk dissolved in TBST. Membranes that were transferred from tricine-acrylamide gels were incubated in primary antibody diluted in TBST with BSA. After incubation in primary antibody, washed with TBST and incubated with the relevant horseradish peroxidase conjugates secondary antibody (Santa Cruz) for 1 hour at room temperature. For SDS-acrylamide gels the secondary antibody was diluted in 5% non-fat dry milk, whereas for tricine acrylamide gels the dilutent was TBST and BSA. Secondary antibodies were then removed and membranes were washed three times for 10 minutes each in TBST. Western blot analysis was done using the enhanced chemiluminescence detection system (Perkin-Elmer) and developed on film.

2.10 Transfection of Mammalian cells with cDNA

Transfection of DU145 cells with the TopFlash Reporter construct was carried out using the ExGen Transfection Reagent (Fermentas). The optimal ratio of ExGen reagent:DNA was determine by transfecting the cells with GFP which contains a green fluorescent protein, and transfection efficiency was determine by monitoring the

expression of GFP with the fluorescent microscope. A 3:1 ratio of reagent to DNA was found to be optimal and was used for all experiements.

DU 145 cells were seeded in 60mm plates so that at the time of transfection they reach 50-60% confluency. Cells were transfected using ExGen 500 with reporter plasmid TopFlash. After 24 hours, media of transfected cells were changed to contain 1% FBS for approximately 18hours, then treated with PUGNAc (100mM) in the absence of serum for various time points. The effect of increasing O-glycosylation of β-catenin upon the TCF reporter gene activity was evaluated using the Luciferase assay system (Stratagene). Protein concentrations were determined by Bradford assay and the results were expressed as relative light units per microgram of protein. and the levels of luminescence were detected by luminometer (PE Applied Biosystems, Tropix TR717).

2.11 Pixel Analysis

Images of Western blots were captured using the Bio-Rad imaging device. The imaging software Quantity One was used to quantify the signal intensities (O.D.) of each band.

2.12 Statistical Analysis

A single ANOVA test was used to determine significance in the prostate cancer comparison experiments. A two-tailed T-test was used to determine significance for experiments with PUGNAc treatment and Wnt stimulation.

Chapter 3

Results

O-glycosylation of β-Catenin Regulates its Nuclear Translocation and Transcriptional Activity

3.0 Results

3.1 Comparison of basal O-glycosylated β-catenin levels in prostate cancer cells lines with normal prostate cell line.

The basal levels of O-glycosylated β catenin were measured in two prostate cancer cell lines, DU-145 and LNCaP, and compared to the levels in the normal immortalized prostate cell line, PNT1A, by Western blot (Figure 3.1). Results show that there were higher levels of total β-catenin in the two prostate cancer cell lines, DU-145 and LNCaP, compared to the PNT1A cell line, and most of it was in the unglycosylated (unmodified) form (Figure 3.2). O-GlcNAc proteins were detected by precipitation with the lectin wheat germ agglutinin (WGA), which binds unspecifically to O-glycosylated proteins. This was followed by immunoblotting with anti- β-catenin antibody. The unmodified β catenin was detected after initially precipitating out the O-glycosylated proteins; the remaining proteins in the sample were precipitated with trichloroacetic acid (TCA), then immunoblotted for β-catenin (Figure 3.2B).

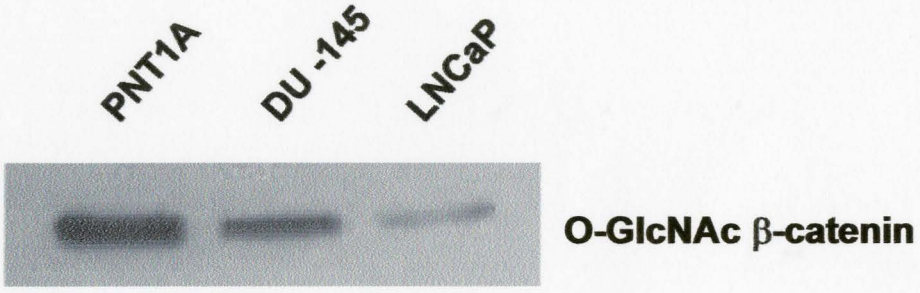
The basal levels of O-glycosylated β-catenin and total β-catenin in the nucleus and cytosol of both the prostate cancer cells lines (DU-145 and LNCaP) were compared to those found in the normal cell line (PNT1A). There were higher levels of O-glycosylated β-catenin in both the nucleus and cytosol of the normal cell line compared to the prostate cancer cell lines (Figure 3.3). While there seemed to be a higher level of cytosolic β-catenin in LNCaP cells, DU-145 cells had comparable amounts to those found

in the cytosol of PNT1A cells. Interestingly, there were higher levels of total nuclear β-catenin in both DU145 and LNCaP lines compared to the PNT1A cells (Figure 3.4).

Lamin B is an intermediate filament protein that provides structural integrity for the nucleus (Nigg, 1992). Detection of Lamin B from with specific antibodies can be used as a nuclear marker to show enrichment of this fraction. Results show that Lamin B was significantly expressed in the nuclear fractions with little to no expression in the cytosolic fraction as detected by Western blot using a mouse monoclonal antibody for the protein (Figure 3.5). α,β tubulin is the building block of microtubules, which are components of the cytoskeleton and can be used as a marker for cytosolic extracts (Westermann and Weber, 2003). α,β tubulin was detected using a mouse monoclonal antibody in the cytosolic fractions of the prostate and prostate cancer cell lines (Figure 3.5). There was no cytosolic contamination in the nuclear fraction as indicated by the absence of α,β tubulin expression in the nuclear fraction (Figure 3.5D). According to Western blot results, our nuclear/cytosolic fractionation procedure was adequate in separating and isolating the cellular compartments.

Results indicate that the two prostate cancer cells line investigated here, DU-145 and LNCaP, showed higher levels of total β-catenin in whole cell lysates and nuclear extracts than the normal prostate cell line, PNT1A, however most of it was in the unglycosylated form. Also, the PNT1A cells expressed significantly higher levels of O-glycosylated β-catenin in both the nucleus and cytosol than the two prostate cancer cell line

3.1A


PNT1A
DU -145
LNCaP
O-GlcNAc β-catenin

3.1B

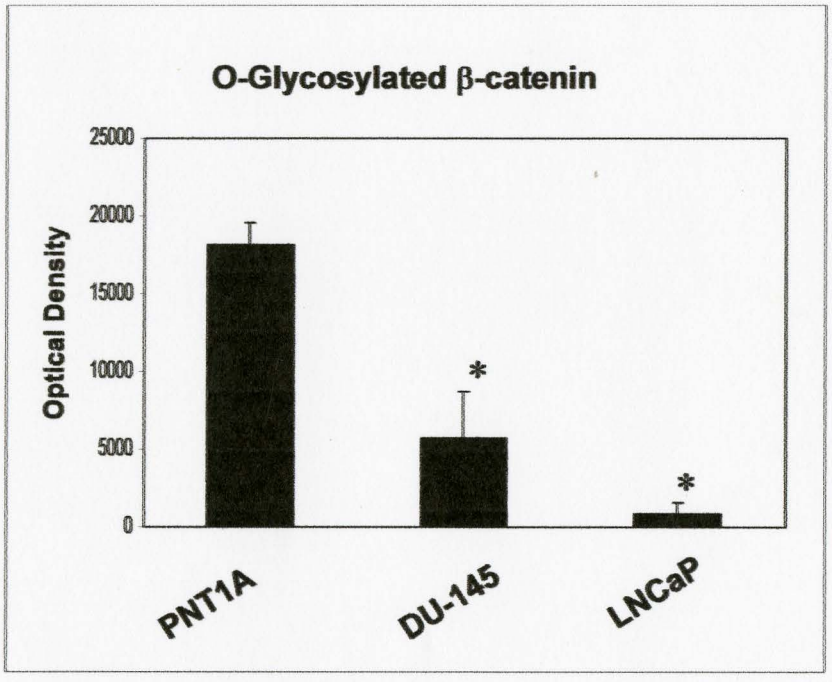
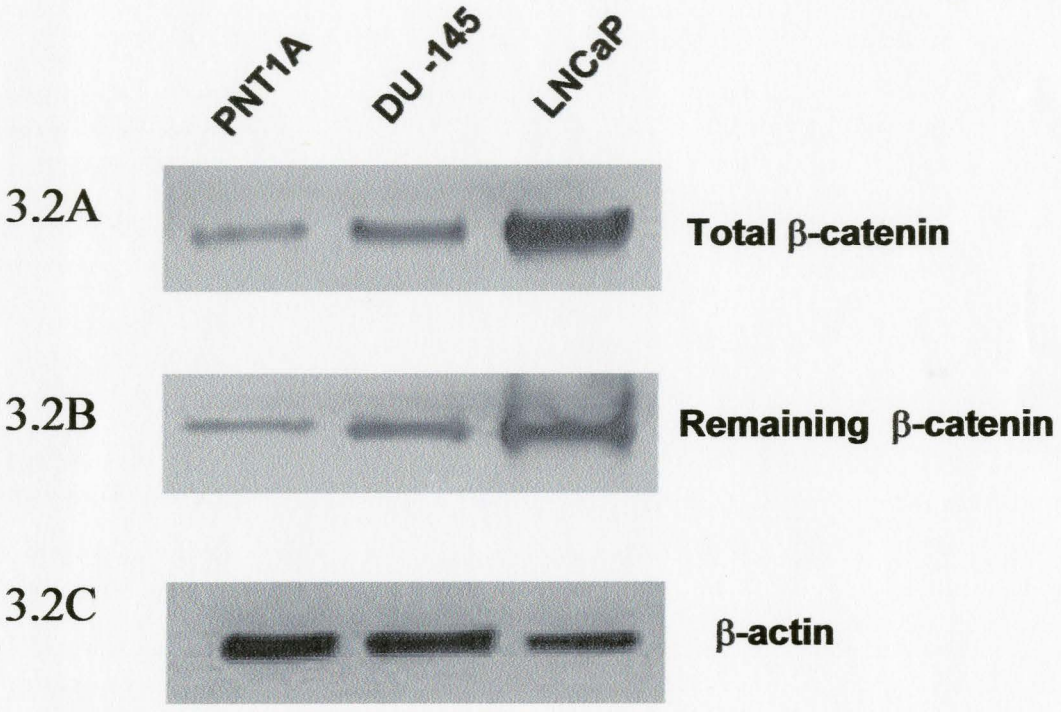

O-Glycosylated β-catenin
Optical Density
25000
20000
15000
10000
5000
0
*
*
PNT1A
DU-145
LNCaP

Figure 3.1: Western blots showing levels of O-GlcNAc β-catenin in normal immortalized prostate cell line compared to prostate cancer cell line.

Figure 3.1A: There are higher levels of O-glycosylated β-catenin in the normal prostate cell line (PNT1A) compared to the prostate cancer cell lines **(DU-145, LNCaP). O-glycosylated β-catenin was evaluated by precipitation of O-GlcNAc proteins with WGA and immunoblotted with anti-β-catenin** antibody.

Figure 3.1B:Densitometric quantification of O-glycosylated β-catenin levels. * $p < 0.005$ compared to PNT1A

Values are means of three independent experiments and error bars are the standard error of the mean


PNT1A
DU -145
LNCaP
3.2A
Total β-catenin
3.2B
Remaining β-catenin
3.2C
β-actin

3.2D

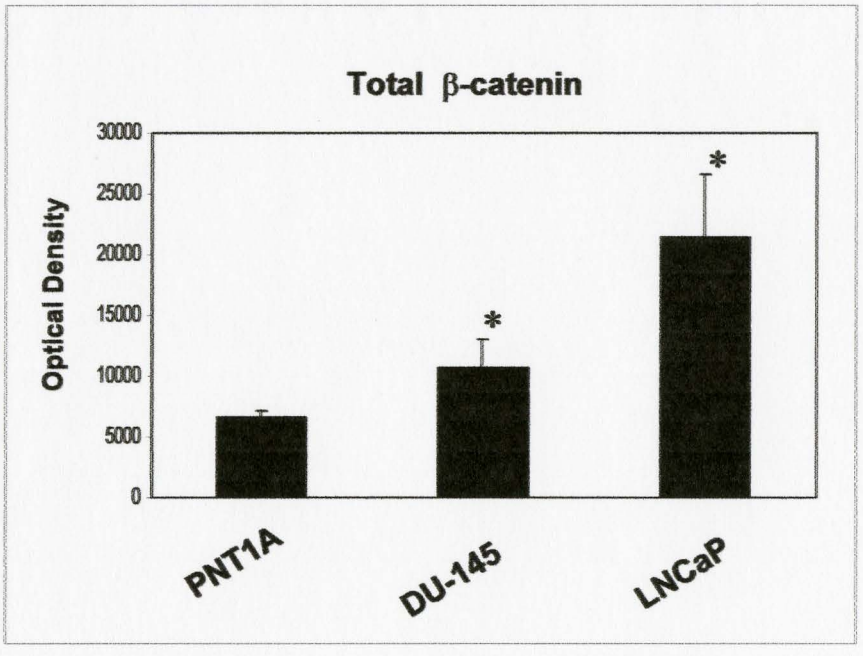

Total β-catenin
Optical Density
30000
25000
20000
15000
10000
5000
0
*
*
PNT1A
DU-145
LNCaP

Figure 3.2: Western blots showing levels of total β-catenin in normal immortalized prostate cell line (PNT1A) compared to prostate cancer cell lines (DU-145 and LNCaP).

Figure 3.2A: There are higher levels of total β-catenin in the cancer cell lines compared to the normal cell line.

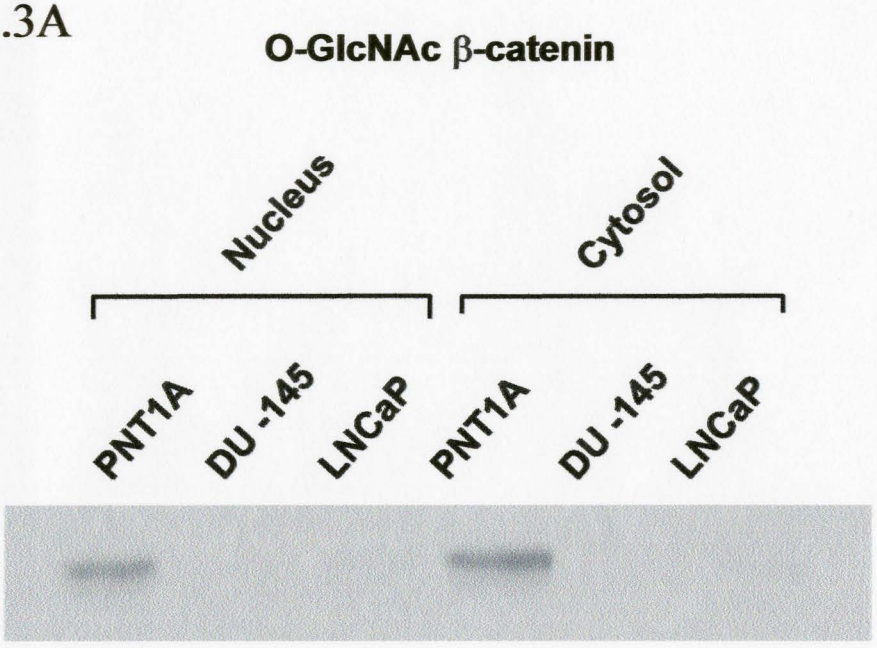
Figure 3.2B: Remaining β-catenin was isolated by precipitating remaining proteins with trichloroacetic acid (TCA) after WGA precipitation, then immunoblotted for β-catenin.

Figure 3.2C: The blots were immunoblotted for total β-catenin and were reprobed with anti-actin antibody as a control for loading.

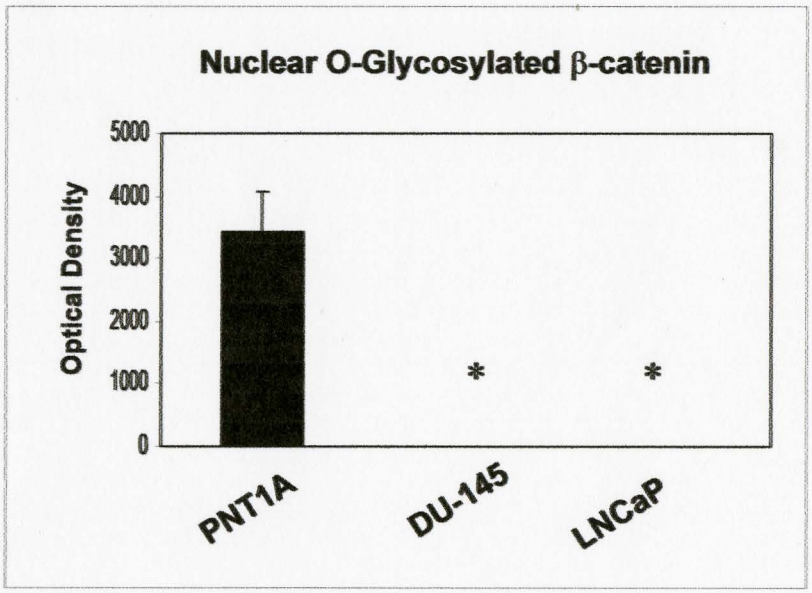
Figure 3.2D: Densitometric quantification of total β-catenin protein levels. * $p < 0.05$ compared to PNT1A

Values are means of three independent experiments and error bars are the standard error of the mean

3.3A


O-GlcNAc β-catenin
Nucleus
Cytosol
PNT1A
DU -145
LNCaP
PNT1A
DU -145
LNCaP

3.3B


Nuclear O-Glycosylated β-catenin
Optical Density
5000
4000
3000
2000
1000
0
*
*
PNT1A
DU-145
LNCaP

3.3C

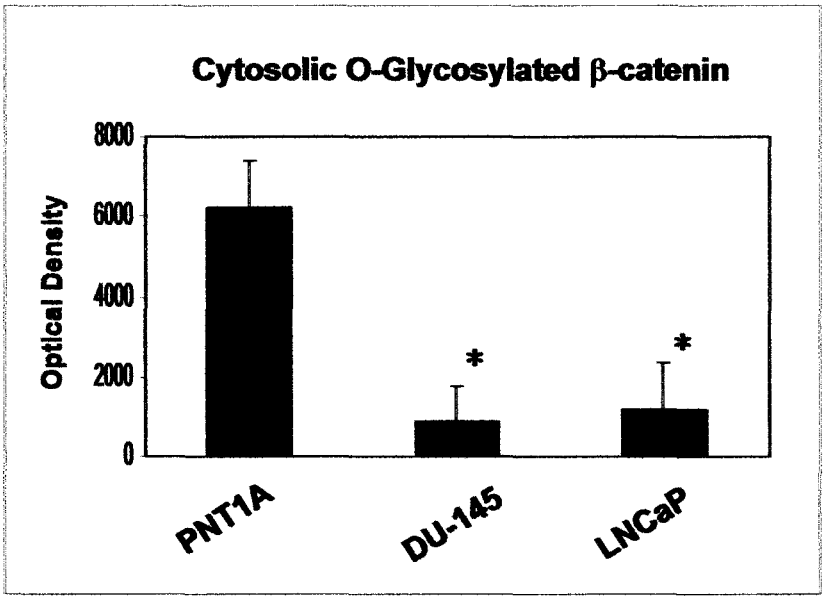


Figure 3.3: Western blot showing lower levels of O-glycosylated β–catenin in the nucleus and cytosol of prostate cancer cell lines versus normal prostate cell line.

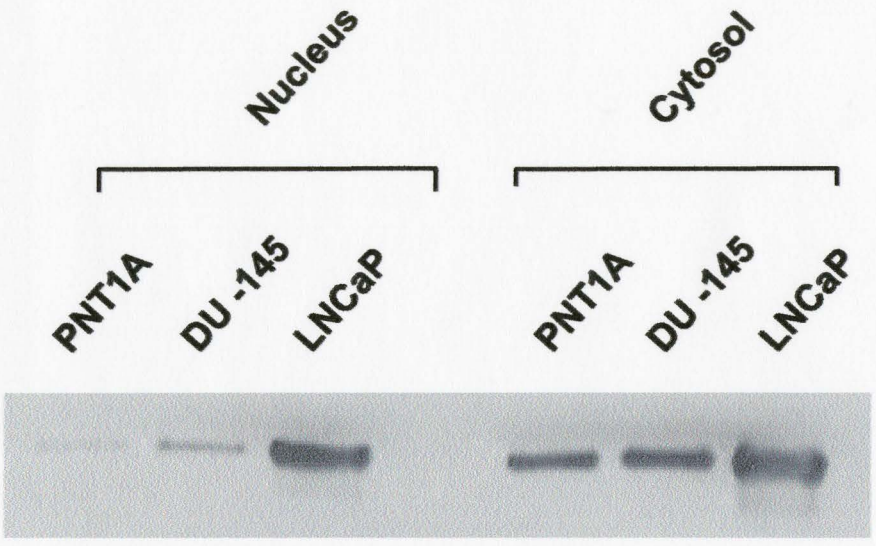
Figure 3.3A: O-glycosylated proteins were precipitated out from nuclear and cytosolic fractions with WGA. β-catenin immunoblots show higher levels of O-glycosylated β-catenin in the nucleus and cytosol of PNT1A cells compared to DU-145 and LNCaP cells.

Figure 3.3B: Densitometric quantification of nuclear O-glycosylated β-catenin protein levels. * $p < 0.005$ compared to PNT1A

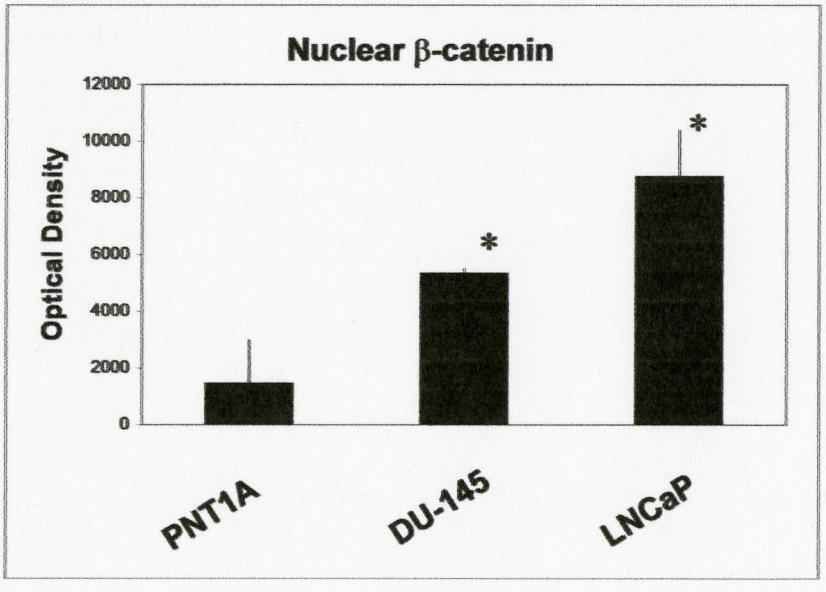
Figure 3.3C: Densitometric quantification of cytosolic O-glycosylated β-catenin protein levels. * $p < 0.05$ compared to PNT1A

Values are means of three independent experiments and error bars are the standard error of the mean

3.4A


Total β-catenin
Nucleus
Cytosol
PNT1A
DU -145
LNCaP
PNT1A
DU -145
LNCaP

3.4B


Nuclear β-catenin
Optical Density
12000
10000
8000
6000
4000
2000
0
*
*
PNT1A
DU-145
LNCaP

3.4C

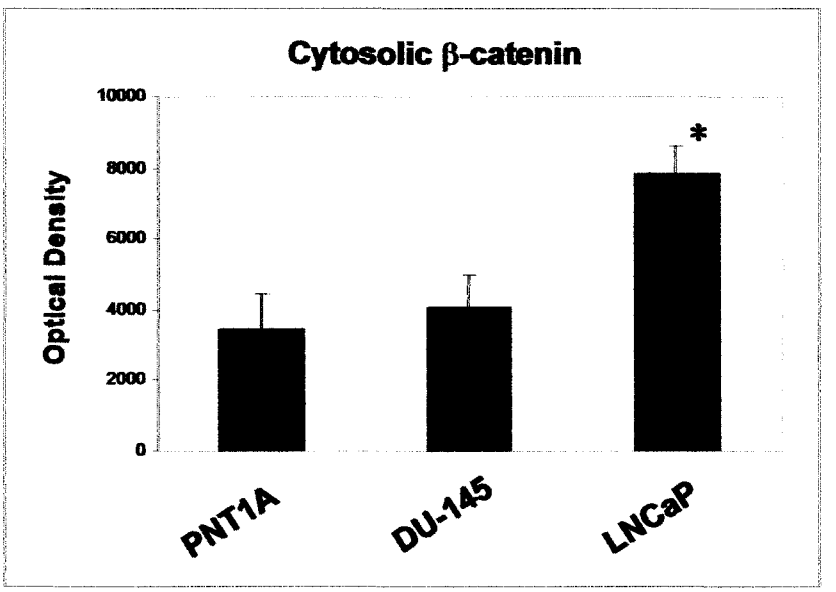


Figure 3.4: Western blot showing levels of total β–catenin in the nucleus and cytosol of prostate cancer cell lines versus normal prostate cell line.

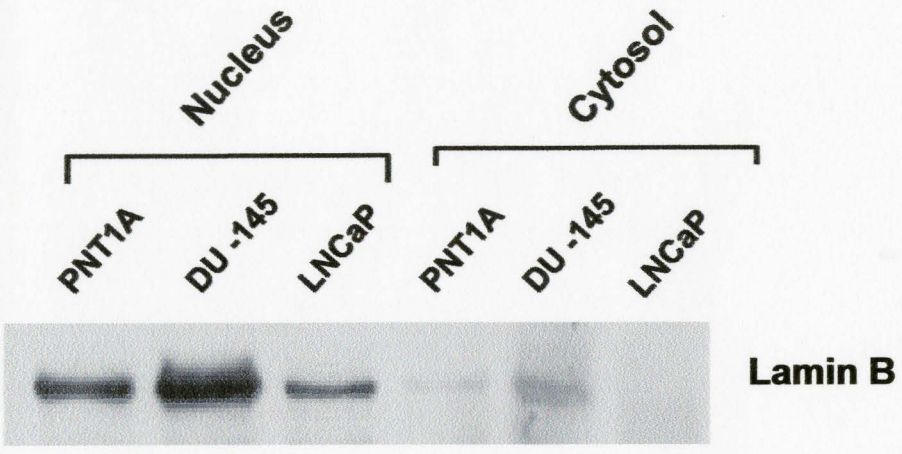
Figure 3.4A: There are higher levels of β-catenin in the nucleus of DU-145 and LNCaP cells compared to PNT1A but only higher levels in the cytosol of LNCaP cells compared to PNT1A cells

Figure 3.4B: Densitometric quantification of nuclear β-catenin protein levels. * $p < 0.05$ compared to PNT1A

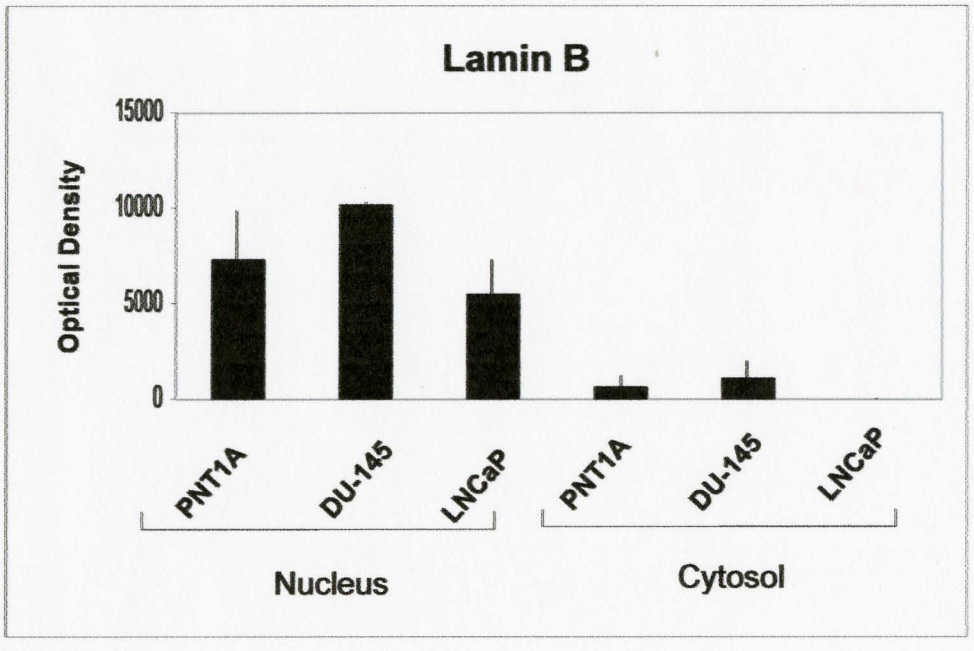
Figure 3.4C: Densitometric quantification of cytosolic β-catenin protein levels. * $p < 0.05$ compared to PNT1A

Values are means of three independent experiments and error bars are the standard error of the mean

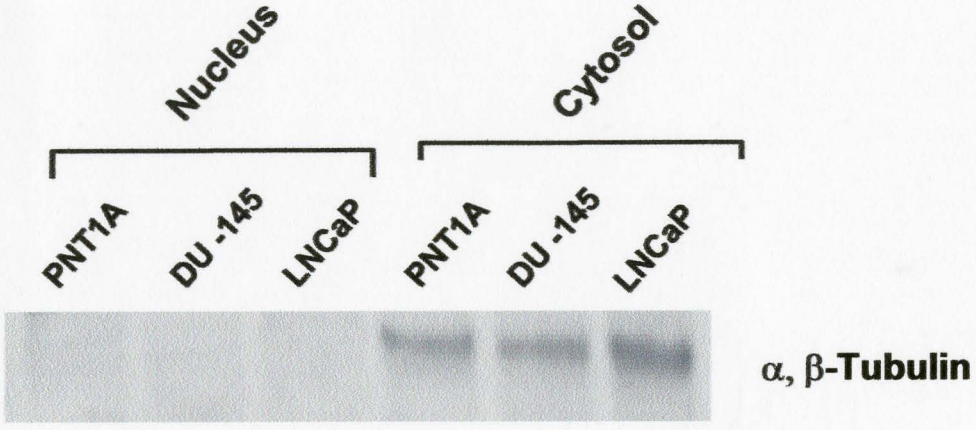
3.5A


Nucleus
Cytosol
PNT1A
DU -145
LNCaP
PNT1A
DU -145
LNCaP
Lamin B

3.5B


Lamin B
Optical Density
15000
10000
5000
0
PNT1A
DU-145
LNCaP
PNT1A
DU-145
LNCaP
Nucleus
Cytosol

3.5C


Nucleus
Cytosol
PNT1A
DU -145
LNCaP
PNT1A
DU -145
LNCaP
α, β-Tubulin

3.5D

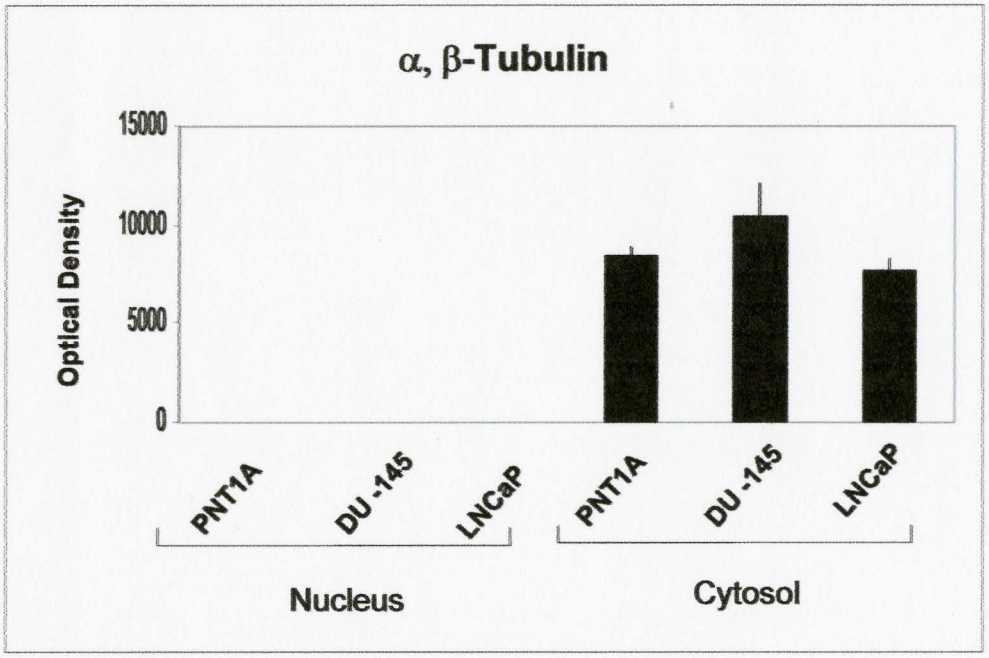

α, β-Tubulin
Optical Density
15000
10000
5000
0
PNT1A
DU -145
LNCaP
PNT1A
DU -145
LNCaP
Nucleus
Cytosol

Figure 3.5: Markers for nuclear and cytosolic fractions.

Figure 3.5A: Lamin B is used as a nuclear marker to show nuclear enrichment. There was no significant nuclear contamination in the cytosolic fraction.

Figure 3.5B Densitometric quantification of Lamin B protein levels in the nuclear and cytosolic fractions.

Figure 3.5C: α,β Tubulin is used as a cytosolic marker indicating that there was no cytosolic contamination in the nuclear fraction.

Figure 3.5D: Densitometric quantification of α,β Tubulin protein levels in the nuclear and cytosolic fractions.

Values are means of three independent experiments and error bars are the standard error of the mean

3.2 Effect of O-glycosylation on the subcellular localization of β-catenin.

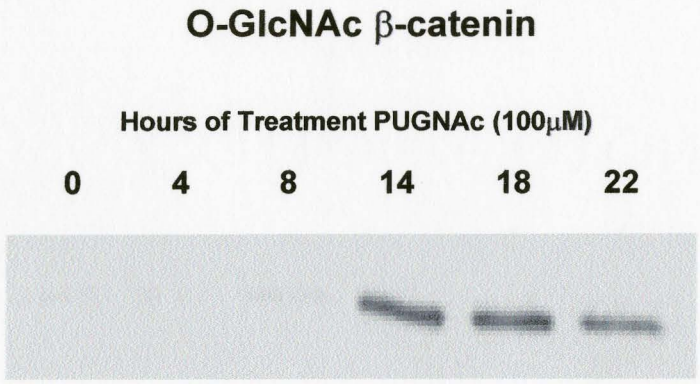
3.2.1 PUGNAc caused an increase in O-glycosylated β-catenin in DU-145 cells.

The prostate cancer cell line, DU-145, was treated with a drug (PUGNAc) in order to manipulate the levels of O-glycosylation to determine how this modification affects the subcellular localization of β-catenin.

Optimal treatment conditions required that PUGNAc treatment of DU-145 cells be done in the absence of serum in order to prevent factors in the serum from interfering with the activity of the drug. Also, treatment intervals had to be chosen so that the viability of the cells was not compromised by the removal of serum from the media. DU-145 cells were grown in the presence of full serum (10%FBS) and then in media containing 1% serum for 18 hours before treatment with PUGNAc for various times points in the absence of serum. A dose response with PUGNAC in DU-145 cells under these treatment conditions was done and a working concentration of 100μM was determined as optimal.

PUGNAc caused a significantly large increase in O-glycosylated β-catenin, detected by WGA precipitation, at times points 14, 18 and 22 hours (Figure 3.6). The expression of total β-catenin remained constant throughout PUGNAc treatment indicating that PUGNAc caused an increase in O-glycosylation without having any effects upon the expression of the protein itself (Figure 3.7).

3.6A



3.6B

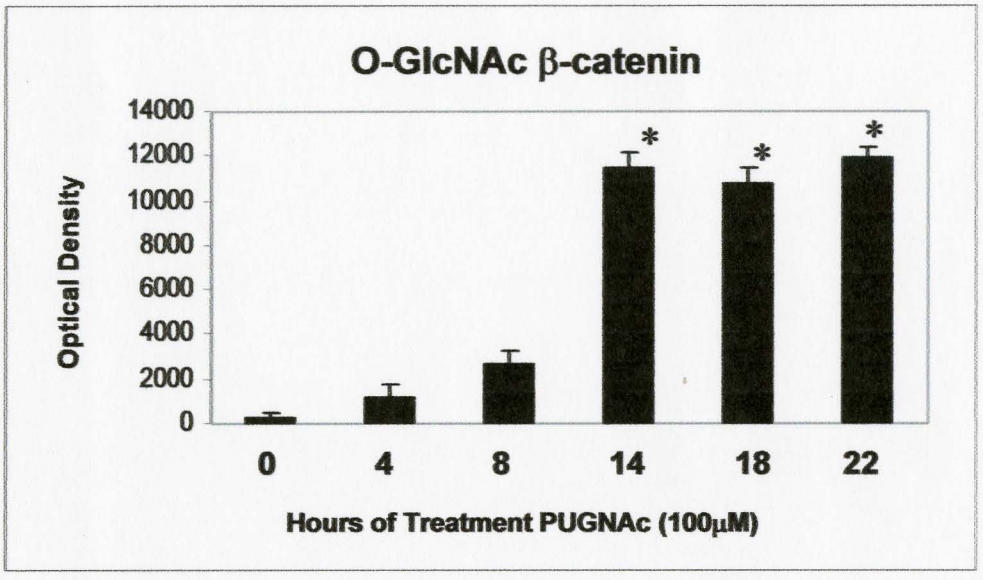


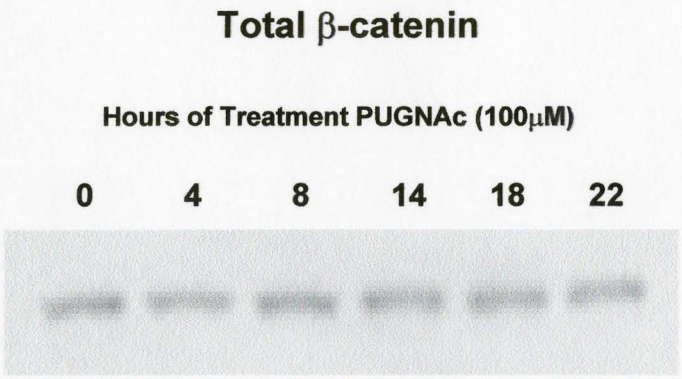
Figure 3.6: Western blot showing PUGNAc induction of O-glycosylation of β-catenin in DU-145 cells.

Figure 3.6A: DU-145 cells were grown to 70% confluency in 10% (vol/vol) FBS, the media was then changed to 1% FBS for approximately 18 hours. **Following, cells were treated with 100μM of PUGNAc, in the absence of serum, for the indicated time periods. An induction in O-glycosylated β-catenin** was seen at 14, 18 and 22 hours

Figure 3.6B: Densitometric quantification of O-glycosylated β-catenin protein levels. * $p < 0.005$ compared to 0hrs control (lane 1)

Values are means of three independent experiments and error bars are the standard error of the mean

3.7A



3.7B

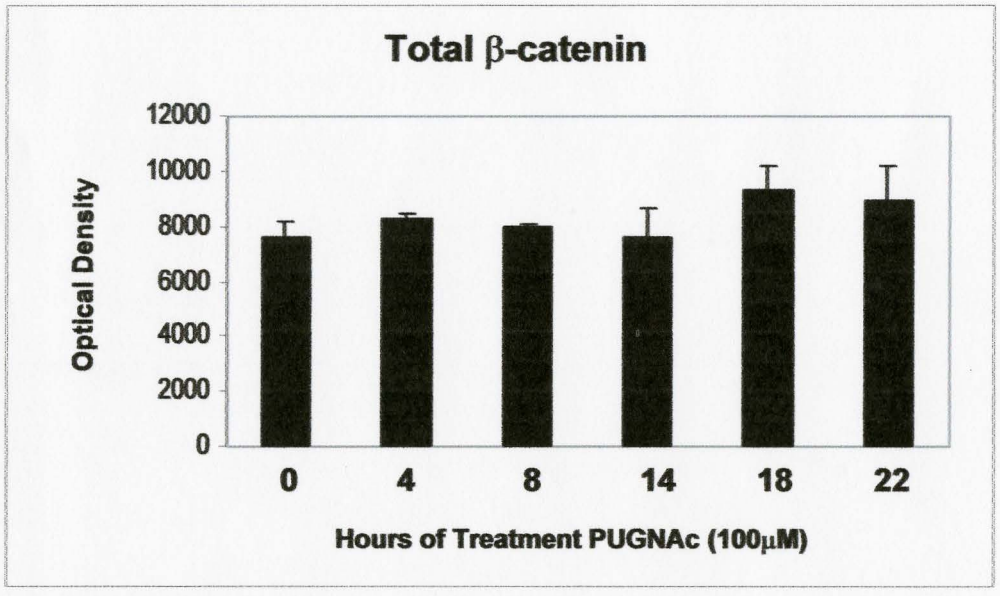


Figure 3.7: Western blot showing total β-catenin levels in DU-145 cells treated with PUGNAc.

Figure 3.7A: As the levels of O-glycosylated β-catenin increased, the levels of total β-catenin remain unchanged for the indicated treatment time points.

Figure 3.7B: Densitometric quantification of total β-catenin protein levels.

Values are means of three independent experiments and error bars are the standard error of the mean

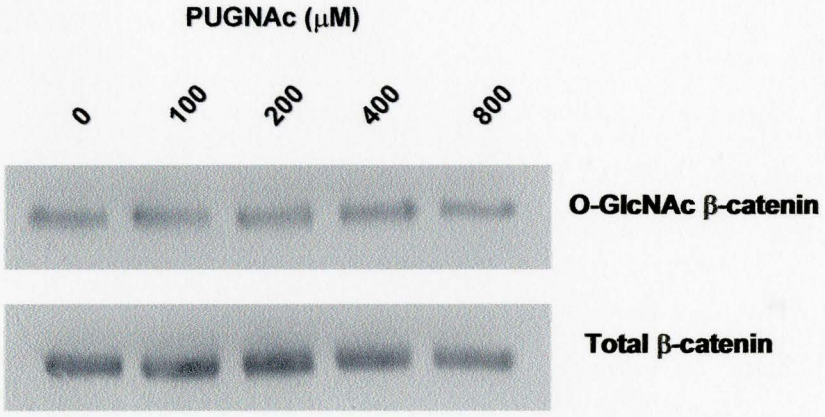
3.2.2 PUGNAc treatment of normal immortalized prostate cell line, PNT1A.

The basal expression of O-glycosylated β-catenin in the normal prostate cell line, PNT1A, was considerably higher compared to the levels in the prostate cancer cell lines, LNCaP and DU-145. Experiments were done to determine if treatment with PUGNAc could increase the already high basal levels of β-catenin in PNT1A cells (Figure 3.8). A dose response with PUGNAc was done in PNT1A cells at different serum concentrations in order to determine the optimal serum concentration that would not interfere with PUGNAc activity while maintaining the viability of the cells. Treatment of the cells at 100, 200, 400 and 800 μM concentrations in media containing 10% FBS showed no increase in O-glycosylated β-catenin compared to untreated cells (Figure 3.8A). Similar results were seen when cells were treated at 100, 200 and 400 μM in media containing 1% and 5% serum (Figure 3.8B, 3.8C). The cells were not able to survive in the absence of serum for long periods of time, thus treatment with PUGNAc in the absence of serum was not done. Based on these results, PUGNAc could not cause an increase in O-GlcNac β-catenin above normal levels in PNT1A cells.

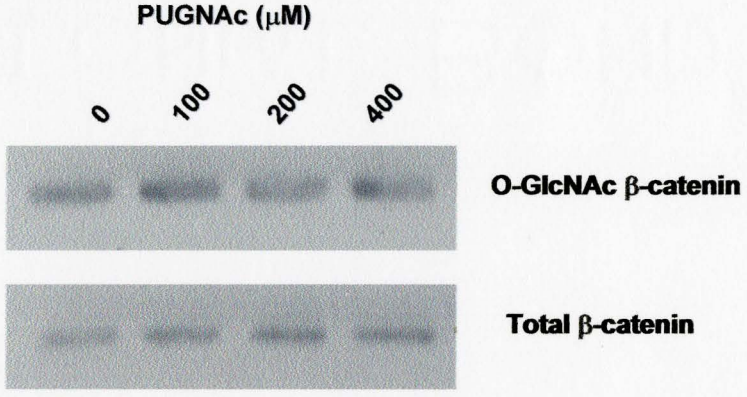
3.2.3 Nuclear and Cytosolic levels of β-catenin changed with increasing O-glycosylation in DU-145 cells.

PUGNAc caused a significant increase in O-glycosylated β-catenin in DU-145 cells (Figure 3.6). Interestingly, as the expression of O-glycosylated β-catenin increased, there was a corresponding decrease in the levels of nuclear β-catenin and an increase in

3.8A **10% Serum**



3.8B **1% Serum**



3.8C **5% Serum**

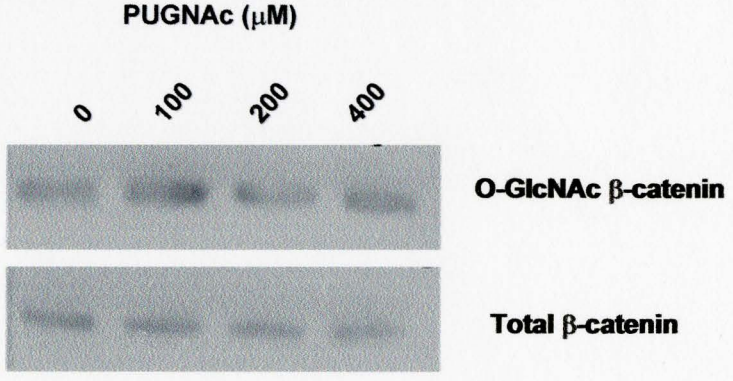


Figure 3.8: PUGNAc dose response in PNT1A cells.

Figure 3.8A: Cells were treated with increasing concentrations of PUGNAc in media containing 10% serum for 24 hours. Western blot of O-glycosylated proteins immunoblotted for β-catenin.

Figure 3.8B: Western blot of total β-catenin levels.

Figure 3.8C: Cells were treated with increasing concentrations of PUGNAc in media containing 1% serum for 8 hours. Western blot of O-glycosylated proteins immunoblotted for β-catenin.

Figure 3.8D: Western blot of total β-catenin levels.

Figure 3.8E: Cells were treated with increasing concentrations of PUGNAc in media containing 5% serum for 16 hours. Western blot of O-glycosylated proteins immunoblotted for β-catenin.

Figure 3.8 F: Western blot of total β-catenin levels.

cytosolic β-catenin (Figure 3.9, 3.11). One explanation for this significant result is that O-glycosylated β-catenin was blocked from entry into the nucleus while its exit from the nucleus remained unhindered. Although the exact mechanism is unknown at this time, our results suggest that O-glycosylation prevents β-catenin's localization in the nuclear compartment.

In order to quantify the amounts of β-catenin in the nuclear and cytosolic compartments during PUGNAc treatment and the amounts of nuclear and cytosolic β-catenin being O-glycosylated we carried out western blot analysis of serial dilutions of a known concentration of purified β–catenin protein. Densitometric analysis of the serial diluted β-catenin protein produced a standard curve from which amounts could be interpolated (Appendix I). The Western blot of the diluted standard protein was done separately from the Western blots for nuclear and cytosolic O-glycosylated and total β-catenin. The amounts of nuclear β-catenin decreased approximately 50 percent from 0 hours to 22 hours of PUGNAc treatment (Figure 3.9C). While the amount of β-catenin in the cytosol increased to slightly less than two-fold from 0 to 22 hours of PUGNAc treatment (Figure 3.11C).

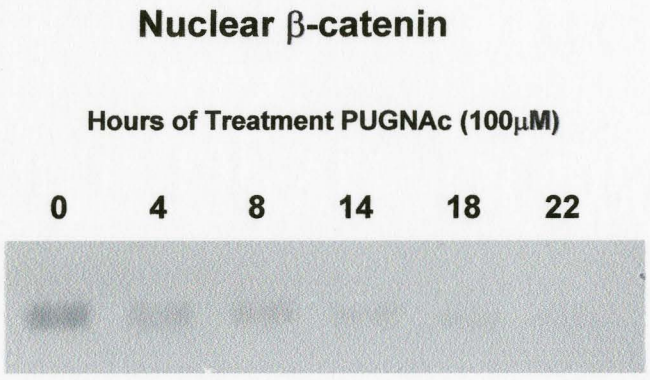
The levels of cytosolic O-glycosylated β-catenin clearly increased as the expression of the protein increased in the cytosol (Figure 3.12C). While, the ratio of β-catenin that became O-glycosylated in the cytosol to the amount of total cytosolic β-catenin remained relatively constant at approximately 50 percent throughout the treatment. Thus half of the total β-catenin in the cytosol was consistently becoming O-

glycosylated during PUGNAc treatment. There does not seem to be a consistent pattern with the amounts of nuclear O-glycosylated β-catenin in relation to the decrease in nuclear β-catenin seen during PUGNAc treatment though a three fold increase is seen at 14 and 18 hours compared to no treatment (Figure 3.10C). The standard error of the amounts of nuclear O-glycosylated β-catenin was too large to make any significant conclusions.

In order to confirm that the quantities of O-glycosylated β–catenin in the nucleus and cytosol truly represented all of the O-glycosylated β–catenin present in those compartments a second WGA precipitation, following procedures identical to the first, was done to the remaining lysate sequentially following the initial WGA precipitation. Our results showed that all of the O-glycosylated β–catenin was being precipitated out during the first WGA precipitation. There was no O-glycosylated β–catenin detected by the second WGA precipitation (Appendix II).

Nuclear and cytosolic markers were detected in these extracts in order to verify that the levels of nuclear and cytosolic β-catenin during PUGNAc treatment were being observed correctly. The nuclear marker Lamin B remained constant in the nucleus throughout the treatment indicating that the levels of nuclear β-catenin were indeed decreasing with increasing O-glycosylation. α,β tubulin was not present in the nuclear fraction indicating that there was no cytosolic contamination in the nucleus and levels of the protein in the cytosolic fraction remained constant (Figure 3.13).

3.9A


Nuclear β-catenin
Hours of Treatment PUGNAc (100μM)
0
4
8
14
18
22

3.9B

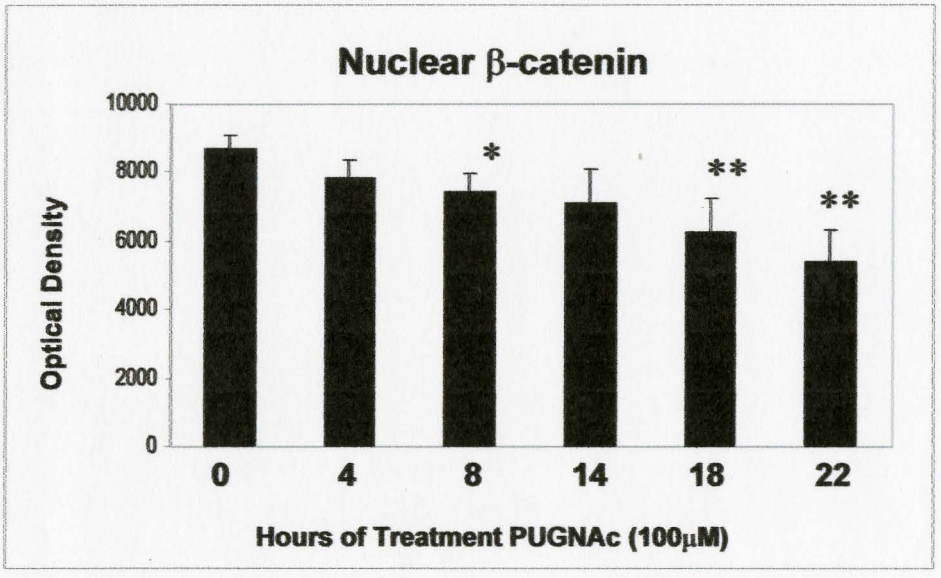

Nuclear β-catenin
Optical Density
10000
8000
6000
4000
2000
0
*
**
**
0
4
8
14
18
22
Hours of Treatment PUGNAc (100μM)

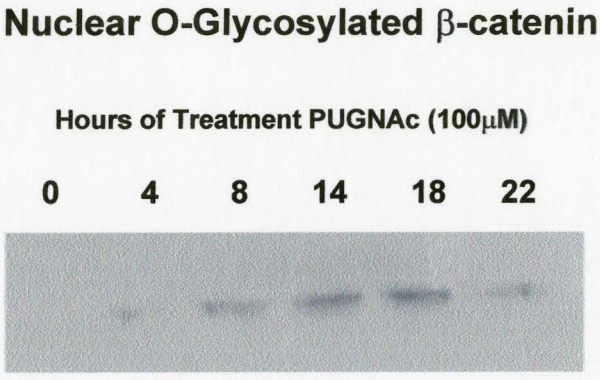
Figure 3.9: Levels of nuclear β-catenin decreased with PUGNAc treatment in DU-145 cells.

Figure 3.9A: DU-145 cells were incubated in media containing 1% FBS for **approximately 18 hours before they were treated with PUGNAc (100μM) in** the absence of serum for the indicated time periods. Cells were then collected **in PBS and counted using a Coulter counter. For nuclear β-catenin,** approximately $6x10^4$cells were loaded on a tricine gel and immunoblotted with anti-β-catenin antibody.

Figure 3.9B: Densitometric quantification of nuclear β-catenin protein levels in nuclear fraction. * $p < 0.1$ compared to 0hrs control (lane 1), ** $p < 0.05$ compared to 0hrs control (lane 1)

Values are means of three independent experiments and error bars are the standard error of the mean

3.10A


Nuclear O-Glycosylated β-catenin
Hours of Treatment PUGNAc (100μM)
0
4
8
14
18
22

3.10B

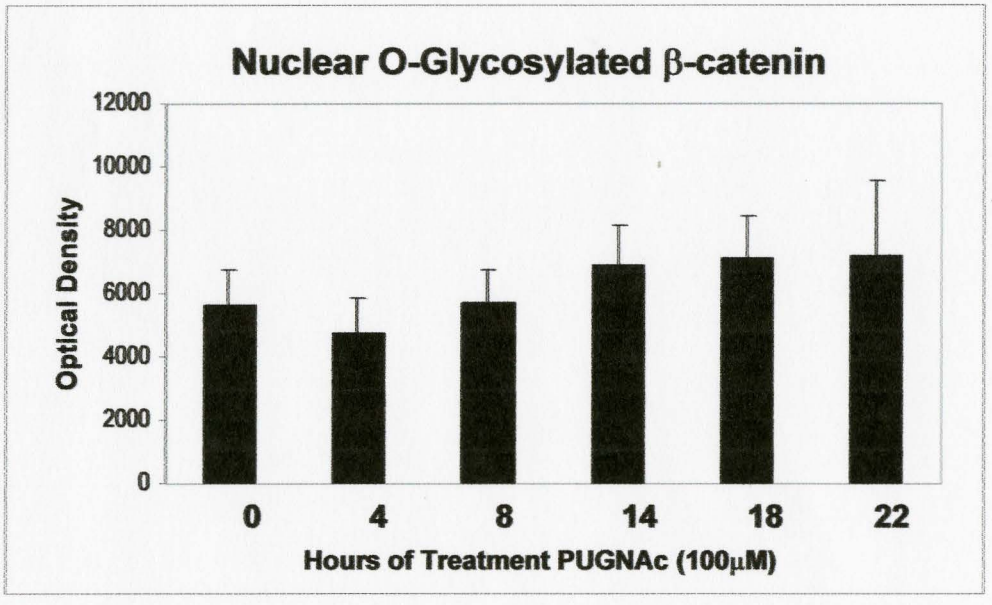

Nuclear O-Glycosylated β-catenin
Optical Density
12000
10000
8000
6000
4000
2000
0
0
4
8
14
18
22
Hours of Treatment PUGNAc (100μM)

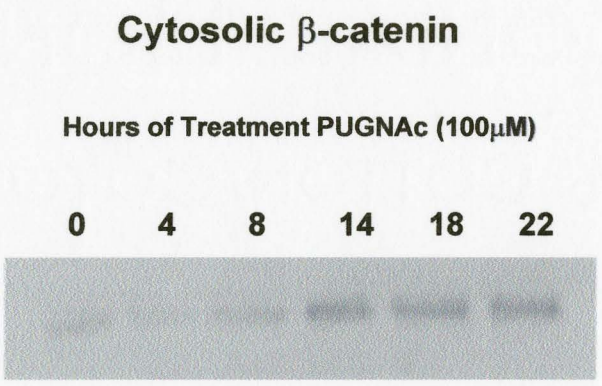
Figure 3.10: Levels of nuclear O-glycosylated β-catenin increased with PUGNAc treatment of DU-145 cells.

Figure 3.10A: For O-glycosylated nuclear β-catenin, approximately $5x10^5$ cells were used for WGA precipitation, sample buffer was added and 20 μL of the sample was loaded on a tricine gel and immunoblotted with anti-β-catenin antibody.

Figure 3.10B: Densitometric quantification of O-glycosylated β-catenin protein levels in the nuclear fraction.

Values are means of three independent experiments and error bars are the standard error of the mean

3.11A


Cytosolic β-catenin
Hours of Treatment PUGNAc (100μM)
0
4
8
14
18
22

3.11B

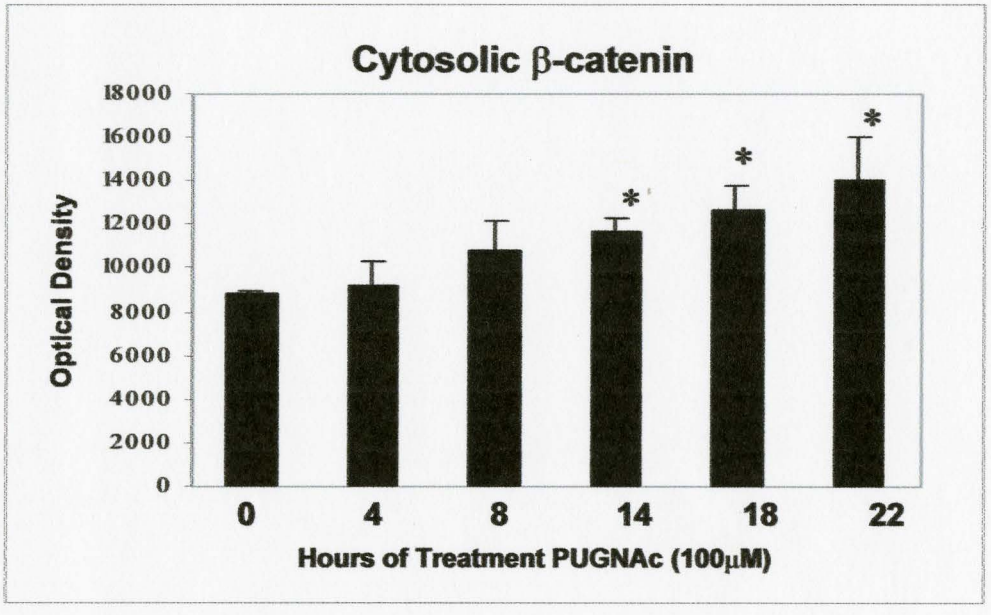

Cytosolic β-catenin
Optical Density
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
*
*
*
0
4
8
14
18
22
Hours of Treatment PUGNAc (100μM)

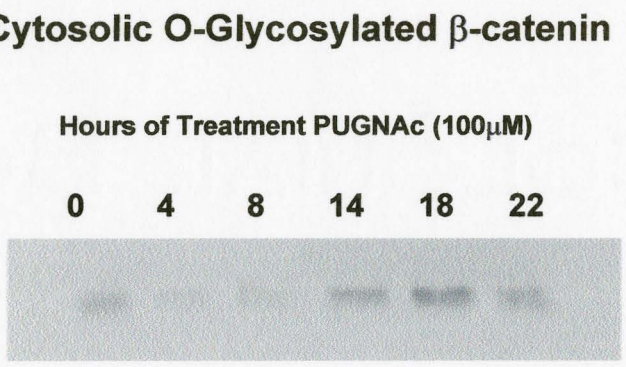
Figure 3.11: Levels of cytosolic β-catenin increased with PUGNAc treatment of DU-145 cells.

Figure 3.11A: DU-145 cells were incubated in media containing 1% FBS for approximately 18 hours before they were treated with PUGNAc (100μM) in the absence of serum for the indicated time periods. Cells were then collected in PBS and counted using a Coulter counter. For cytosolic β-catenin, approximately $3x10^4$ cells were loaded on a tricine gel and immunoblotted with anti-β-catenin antibody.

Figure 3.11B: Densitometric quantification of β-catenin protein levels in cytosolic fraction. * $p < 0.05$ compared to 0hrs control (lane 1)

Values are means of three independent experiments and error bars are the standard error of the mean

3.12A


Cytosolic O-Glycosylated β-catenin
Hours of Treatment PUGNAc (100μM)
0
4
8
14
18
22

3.12B

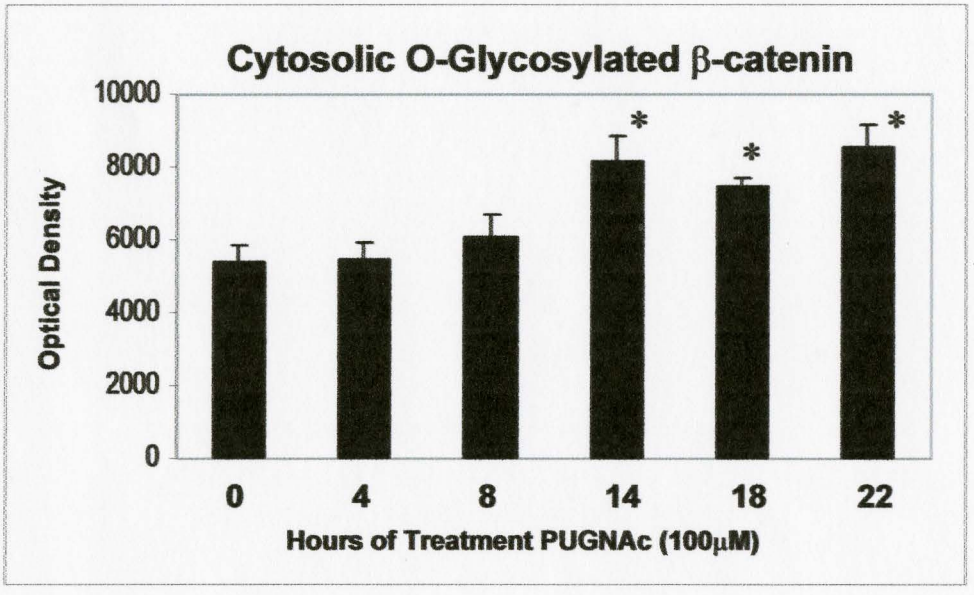

Cytosolic O-Glycosylated β-catenin
Optical Density
10000
8000
6000
4000
2000
0
*
*
*
0
4
8
14
18
22
Hours of Treatment PUGNAc (100μM)

Figure 3.12: Levels of cytosolic O-glycosylated β-catenin increased with PUGNAc treatment of DU-145 cells.

Figure 3.12A: For O-glycosylated cytosolic β-catenin, approximately $5x10^5$ cells were used for WGA precipitation, sample buffer was added and 20 μL of the sample was loaded on a tricine gel and immunoblotted with anti-β-catenin antibody.

Figure 3.12B: Densitometric quantification of O-glycosylated β-catenin protein levels in the cytosolic fraction. ** $p < 0.05$ compared to 0hrs control (lane 1)

Values are means of three independent experiments and error bars are the standard error of the mean

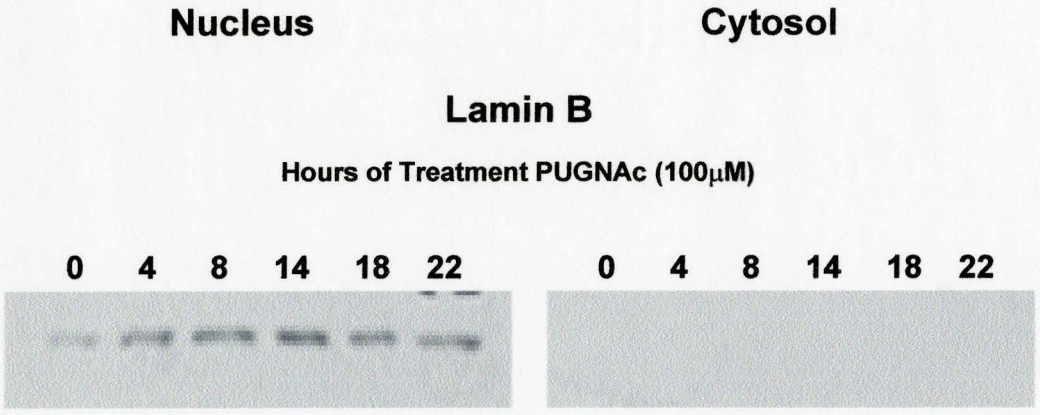
Hours of PUGNAc Treatment	O.D.	Nuclear β-catenin ng/mL	O.D.	Nuclear O-GlcNAc β-catenin ng/mL	O.D.	Cytosolic β-catenin ng/mL	O.D.	Cytosolic O-GlcNAc β-catenin ng/mL
0	8689.02	11.94 +/- 0.77	3039.86	1.13 +/- 2.14	8755.74	12.07 +/- 0.46	5374.09	5.60 +/- 0.84
4	7810.52	10.26 +/- 1.10	2563.75	0.22 +/- 2.15	9210.82	12.94 +/- 2.21	5453.74	5.75 +/- 0.95
8	7443.14	9.56 +/- 0.95	3044.39	1.14 +/- 1.99	10778.12	15.94 +/- 2.58	6080.61	6.95 +/- 1.12
14	7081.55	8.87 +/- 1.95	4046.06	3.06 +/- 2.49	11705.63	17.72 +/- 1.13	8178.17	10.97 +/- 1.33
18	6245.35	7.27 +/- 1.85	4306.44	3.56 +/- 2.50	12603.78	19.44 +/- 2.15	7473.09	9.62 +/- 0.43
22	5402.61	5.66 +/- 1.71	3296.49	1.62 +/- 4.50	14086.62	22.27 +/- 3.69	8523.24	11.63 +/- 1.27

Figure 3.13: Amounts of total and O-glycosylated β-catenin interpolated from a Standard Curve.

Amounts from nuclear and cytosolic fractions of DU-145 cells treated with PUGNAc. The Western blot of the diluted standard protein was done separately from the Western blots for nuclear and cytosolic O-glycosylated and total β-catenin.

Values are means of three independent experiments and error bars are the standard error of the mean

3.14A



3.14B

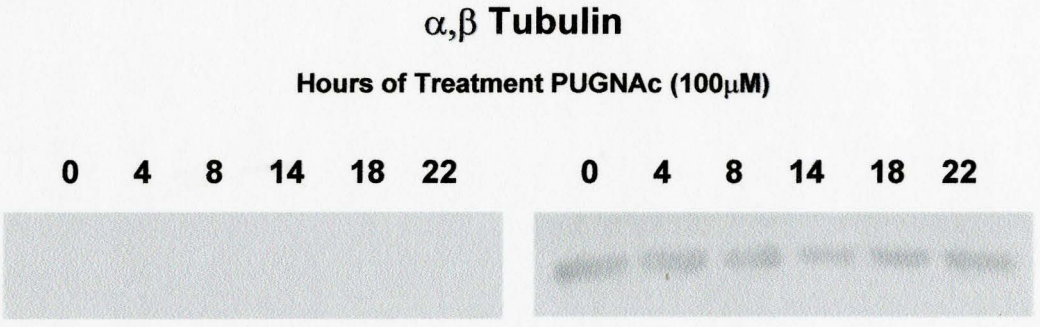


Figure 3.14: Lamin B and α,β tubulin levels.

DU-145 cells were incubated in media containing 1% FBS for approximately 18 **hours before they were treated with PUGNAc (100μM) in the absence of serum** for the indicated time periods. Cells were then collected in PBS and counted using a Coulter counter.

Figure 3.14A: Approximately $6x10^4$ cells were loaded on a tricine gel and immunoblotted with anti-Lamin B antibody.

Figure 3.14B: Approximately $6x10^4$ cells were loaded on a tricine gel and immunoblotted with anti- α,β Tubulin antibody.

α,β tubulin blots show no cytosolic contamination in the nuclear fraction, while Lamin B blots show enrichment in the nuclear fraction.

3.2.4 Observation of the subcellular localization of transcription factors Sp1 and TCF4 with increasing O-glycosylation in DU145 cells.

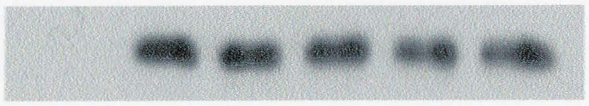
Sp1 is a transcription factor that interacts with a variety of promoters that contain G-C box elements. Insulin has been shown to induce O-glycosylation of Sp1, which facilitates its migration into the nucleus (Majumdar et al., 2006). We investigated whether O-glycosylation caused by PUGNAc treatment and its effects on protein localization could be seen in this transcription factor, which is also O-glycosylated. DU145 cells were treated with PUGNAc at varying time points, WGA precipitation of whole cell lysates showed that PUGNAc caused O-glycosylation of Sp1 as early as 4 hours and remained high throughout the treatment (Figure 3.14A). Immunoblots of nuclear extracts showed Sp1 levels increase at 8 and 14 hours of PUGNAc treatment, but then decrease at later time points, 18 and 22 hours (Figure 3.14C). We did not detect Sp1 in the cytosol. Thus as expected, increasing O-glycosylation of Sp1 transiently increased the transcription factor's localization into the nucleus.

The nuclear pore complex (NPC) contains proteins that can become heavily O-glycosylated. In order to confirm that O-glycosylation caused by PUGNAc was not disrupting normal movement through the nuclear pore complex via these nuclear pore components, the nuclear localization of another transcription factor was investigated. T-cell factor 4 (TCF4) migrates into the nucleus and binds to specific TCF binding elements on DNA. This transcription factor is not post-translationally modified by O-glycosylation as was seen by the fact that PUGNAc did not cause O-glycosylation of TCF4. Further, PUGNAc treatment of DU145 cells did not alter the nuclear localization of the protein as

Hours of Treatment PUGNAc (100μM)

0 4 8 14 18 22

3.15A



O-GlcNAc Sp1

0 4 8 14 18 22

3.15B

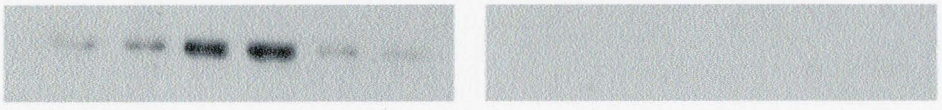
Total Sp1

Nucleus Cytosol

3.15C

Hours of Treatment PUGNAc (100μM)

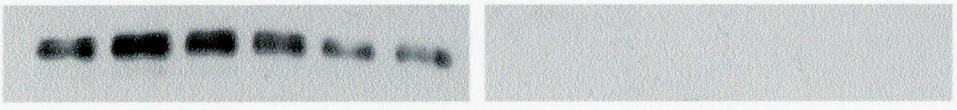
0 4 8 14 18 22 0 4 8 14 18 22



Total Sp1

3.15D

0 4 8 14 18 22 0 4 8 14 18 22



O-GlcNAc Sp1

Figure 3.15: PUGNAc induction of O-glycosylated Sp1 in DU145 cells.

Figure 3.15A: An induction in O-glycosylated Sp1 was seen after 4 hours and remained constant throughout treatment in whole cell lysates.

Figure 3.15B: The levels of total Sp1 remained relatively constant throughout treatment, except for slight decreases at 14 and 22 hours.

Figure 3.15C: Nuclear levels of total Sp1 increased after 8 hours and decreased after 18 hours. There was no Sp1 detected in the cytosol.

Figure 3.15D: Nuclear O-glycosylated Sp1 levels increased after 4 hours and remained high compared to 0 hours. There was no O-glycosylated Sp1 detected in the cytosol.

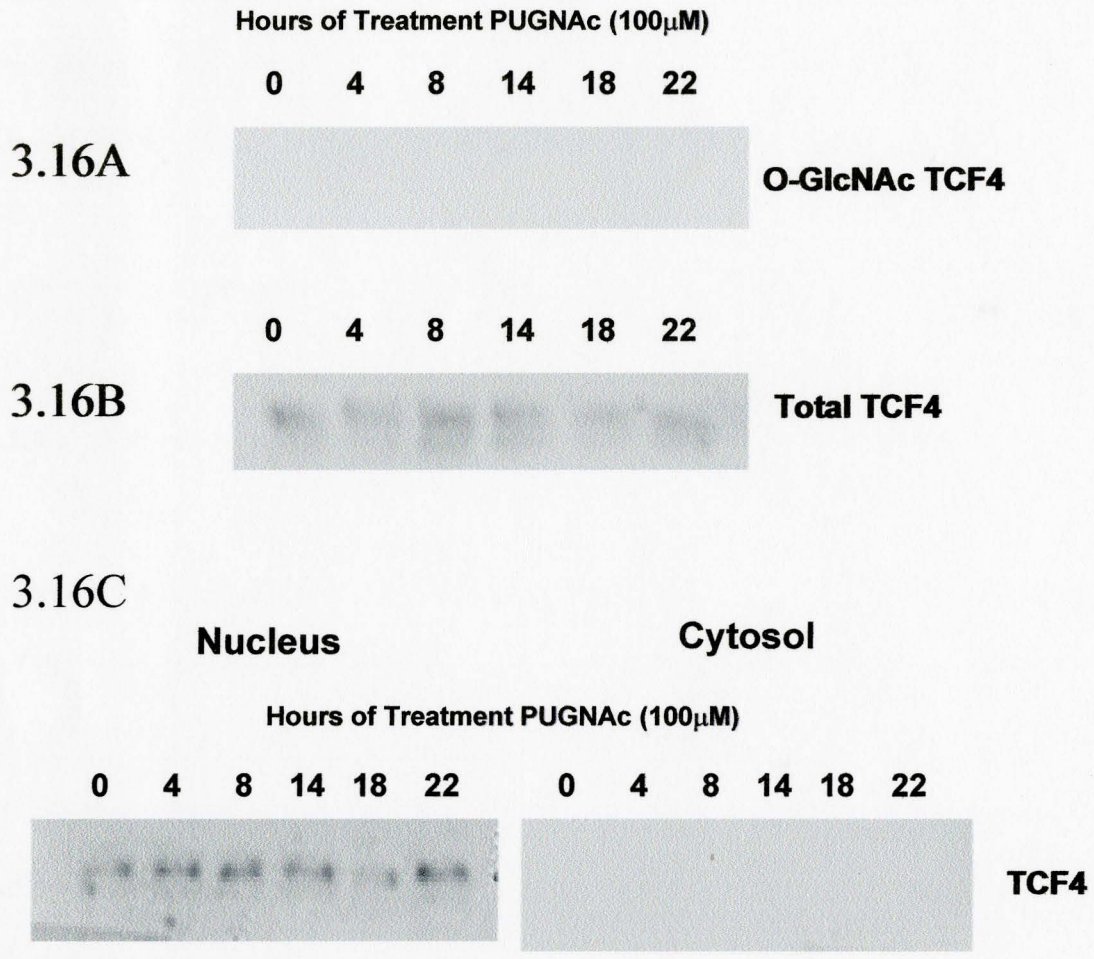


Figure 3.16: PUGNAc did not induce O-glycosylation of TCF4 in DU145 cells.

Figure 3.16A: O-glycosylated proteins from whole cell lysates were precipitated with WGA, immunoblots for TCF4 did not detect a signal.

Figure 3.16B: The levels of total TCF4 remained relatively consistent throughout treatment, except for slight increases at 8 and 14 hours.

Figure 3.16C: Nuclear levels of TCF4 remained constant with PUGNAc treatment, cytosolic TCF4 was not detected.

indicated by the steady levels of nuclear TCF4 (Figure 3.15). These results indicate that the effect of PUGNAc upon the subcellular localization of β-catenin is quite specific and is not a result of global alterations in transport across the nuclear membrane.

3.3 O-glycosylation regulates the transcriptional activity of β-catenin.

3.3.1 TopFlash Reporter

In order to determine if O-glycosylation affects β-catenin's transcriptional activity, a luciferase reporter assay was performed using transfection grade T-cell factor 4 (TCF4) reporter plasmid TopFlash. TopFlash contains two sets of three copies of the TCF binding site upstream of the Thymidine Kinase (TK) minimal promoter and Luciferase open reading frame. DU-145 cells were transfected with this reporter plasmid, allowed to recover for 24 hours and then treated with PUGNAc for various time intervals. A decreased luciferase activity was seen after 14 hours of PUGNAc treatment, which remained decreased throughout the treatment (Figure 3.16). However, as treatment with PUGNAc is done in the absence of serum it is possible that this decrease in reporter activity seen may be due to the reduced amounts of serum in the media during treatment. A reduction in serum for long enough periods would induce cells to conserve energy and slow down the processes that require energy, such as transcription. To address this issue, transfected DU-145 cells were incubated in the absence of serum without PUGNAc treatment for the indicated time points. Luciferase activity did not decrease significantly

3.17A

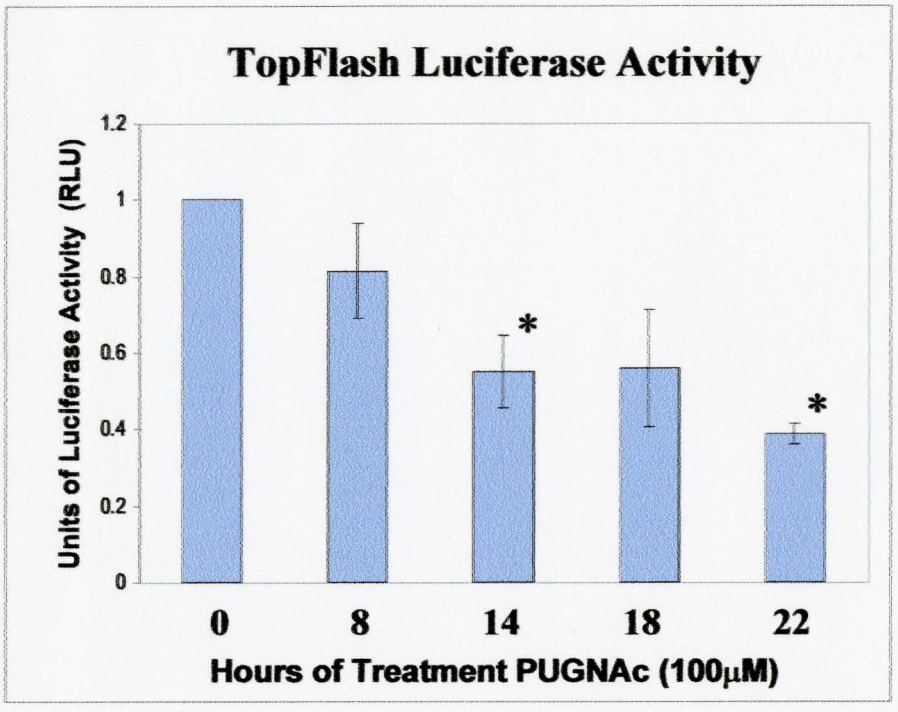


Figure 3.17: Decreased relative Luciferase activity after PUGNAc treatment.

Figure 3.16A: TopFlash transfected DU-145 cells were treated with PUGNAc for the indicated time points, after which cells were lysed and transcriptional activity was assessed. A decrease in luciferase activity was seen at 14, 18 and 22 hours * $p < 0.05$ compared to 0hrs control

(data are mean +/- s.e. and represent three independent experiments).

3.17B

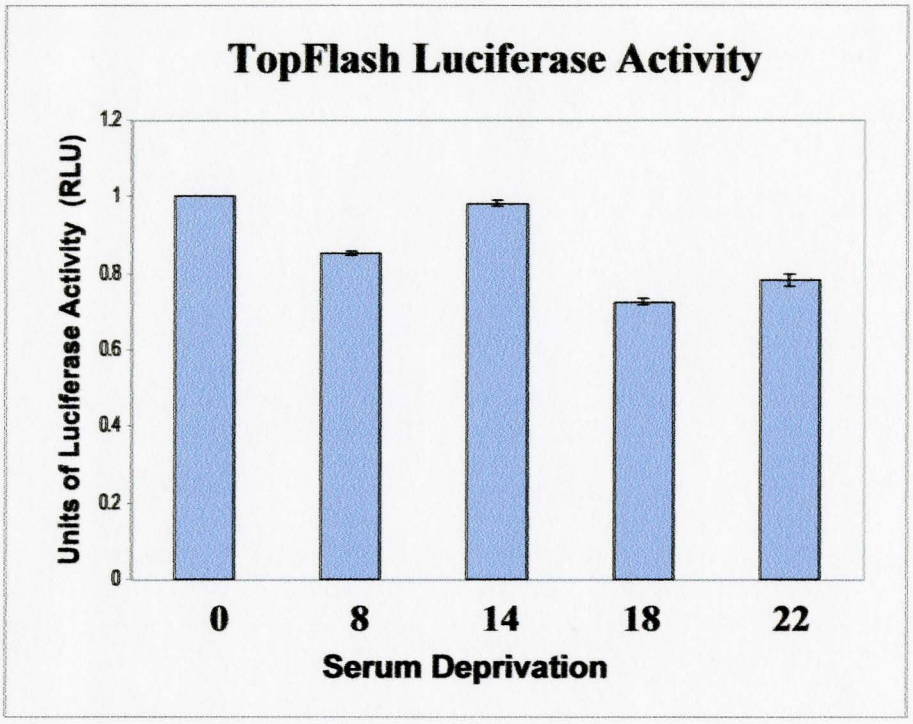


Figure 3.17B: TopFlash transfected DU-145 cells were in the absence of serum (and without PUGNAc treatment) for the indicated time intervals (data are mean +/- s.e. of triplicate repeats).

with serum removal indicating that the decrease in reporter activity seen previously was due to PUGNAc treatment.

3.4 O-glycosylation of β-catenin decreased the protein expression of Cyclin D1 and VEGFA

The effects of O-glycosylation of β-catenin on the protein expression of two target genes were determined. Whole cell lysates were immunoblotted for Cyclin D1 and VEGFA. The protein expression of both Cyclin D1 and VEGFA decreased temporally with PUGNAc treatment (Figure 3.17, 3.18). Results show that both Cyclin D1 and VEGFA protein levels remain relatively constant in the absence of serum even with the longest time interval of 22 hours (Figure 3.18C). Thus, the decrease in expression of these two genes can be attributed to the increased O-glycosylation of β-catenin caused by PUGNAc and not due to the absence of serum in the media.

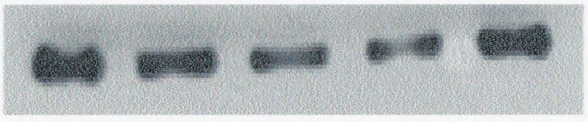
3.5 Determining whether O-glycosylation can alter Wnt induced increases in nuclear β–catenin

To further determine if O-glycosylation affects β-catenin's subcellular localization, conditions that are known to affect β catenin's localization into the nucleus, Wnt3a stimulation, was used in combination with PUGNAc in order to see if O-glycosylation affects Wnt induced nuclear localization

Treatment with PUGNAc (100μM)

0 8 14 18 22

3.18A  **Cyclin D1**

3.18B  **actin**

No PUGNAc Treatment

0 8 14 18 22

3.18C  **Cyclin D1**

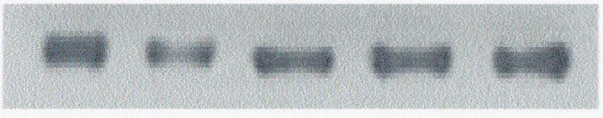
3.18D  **actin**

Figure 3.18: Cyclin D1 protein expression decreased with PUGNAc treatment in DU-145 cells.

Figure 3.18A: Cells were treated with PUGNAc in the absence of serum for the indicated time points. Cyclin D1 expression decreased.

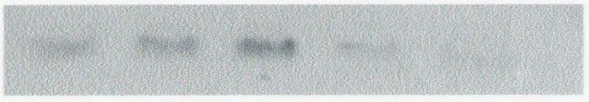
Figure 3.18B: The membrane was stripped and re-probed with anti-Actin antibody as a loading control.

Figure 3.18C: Cells were not treated with PUGNAc but put in media in the absence of serum for the indicated time points. Cyclin D1 expression remained constant.

Figure 3.18D: The membrane was stripped and re-probed with anti-Actin antibody as a loading control.

Treatment with PUGNAc (100μM)

0 8 14 18 22

3.19A  **VEGFA**

3.19B  **Actin**

No PUGNAc Treatment

0 8 14 18 22

3.19C  **VEGFA**

3.19D  **Actin**

Figure 3.19: VEGFA protein expression decreased with PUGNAc treatment in DU-145 cells.

Figure 3.19A: Cells were treated with PUGNAc in the absence of serum for the indicated time points. VEGFA expression decreased.

Figure 3.19B: The membrane was stripped and re-probed with anti-Actin antibody as a loading control.

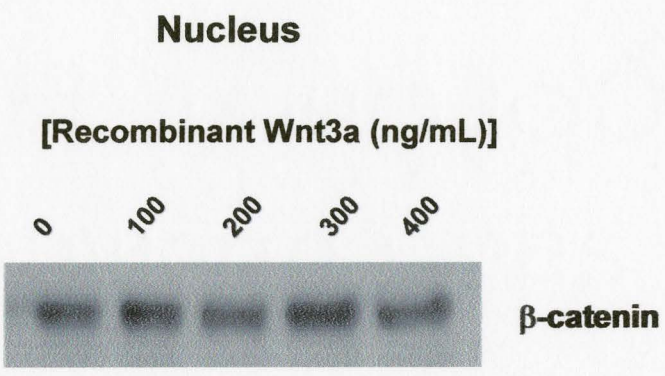
Figure 3.19C: Cells were not treated with PUGNAc but put in media in the absence of serum for the indicated time points. VEGFA expression remained constant.

Figure 3.19D: The membrane was stripped and re-probed with anti-Actin antibody as a loading control.

3.5.1 Wnt3a did not increase levels of nuclear β-catenin in DU-145 cells.

Since PUGNAc treatment conditions had already been optimized in DU-145 cells, this cell line was used to establish optimal Wnt3a conditions. First, cells were treated with a commercially available purified mouse Wnt3a recombinant protein at different concentrations. Wnt3a treatment showed no increase in β-catenin in either the nucleus of cytosolic fraction (Figure 3.19. 3.20). Alternatively, conditioned media from Wnt3a-L cells containing a stably transfected Wnt3a expression vector was used. Conditioned media from these cells contained secreted Wnt protein and was used to treat DU-145 cells. As a negative control, DU-145 cells were also incubated with conditioned media from the parental cell line that lacked the Wnt3a expression vector in order to show that there were no factors secreted from the parental cell line or factors in the serum that was increasing β-catenin levels. Treatment of DU-145 cells with Wnt3a conditioned media increased nuclear β-catenin at 8 hours (Figure 3.22). However this time point did not correspond with the time points established for PUGNAc treatment of these cells where induction was apparent at later time intervals. Wnt3a conditioned media did stimulate an increase in cytosolic β-catenin (Figure 3.23), however because of the lack of induction of nuclear β-catenin, this cell line was not used to study the combined effects of Wnt3a conditioned media and PUGNAc treatment on β-catenin's nuclear localization.

3.20A



3.20B

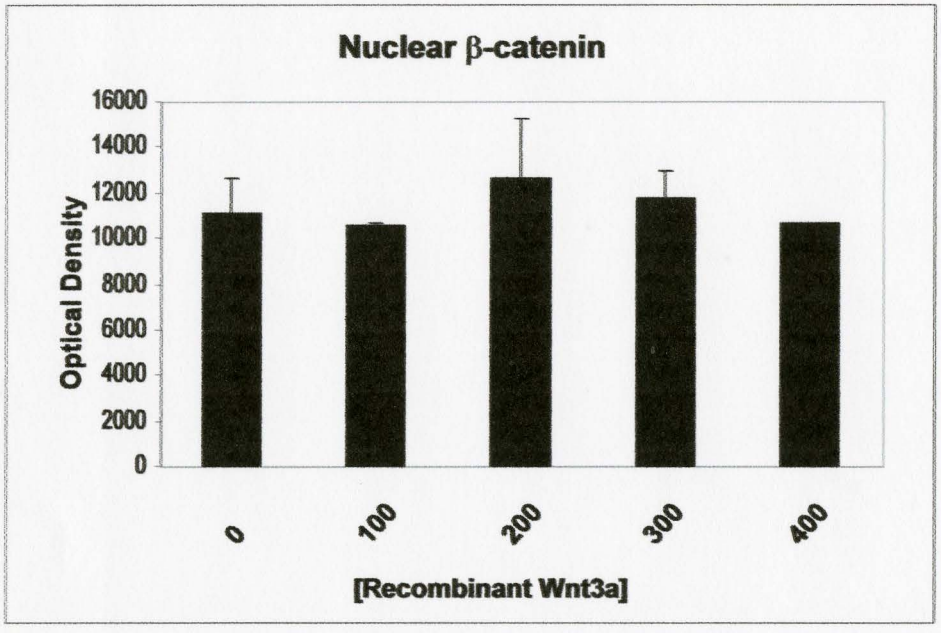


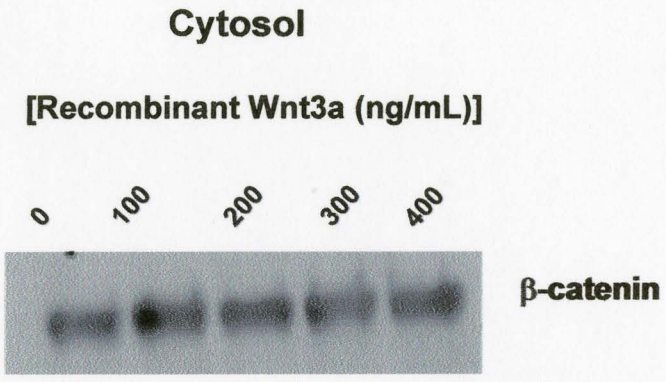
Figure 3.20: Recombinant Wnt3a dose response in nuclear fraction of DU-145 cells.

Figure 3.20A: Cells were treated with increasing concentrations of **recombinant Wnt3a protein for 24 hours. The levels of nuclear β-catenin** remained unchanged with treatment.

Figure 3.20B: Densitometric quantification of β-catenin protein levels in the nuclear fraction.

Values are means of three independent experiments and error bars are the standard error of the mean

3.21A



3.21B

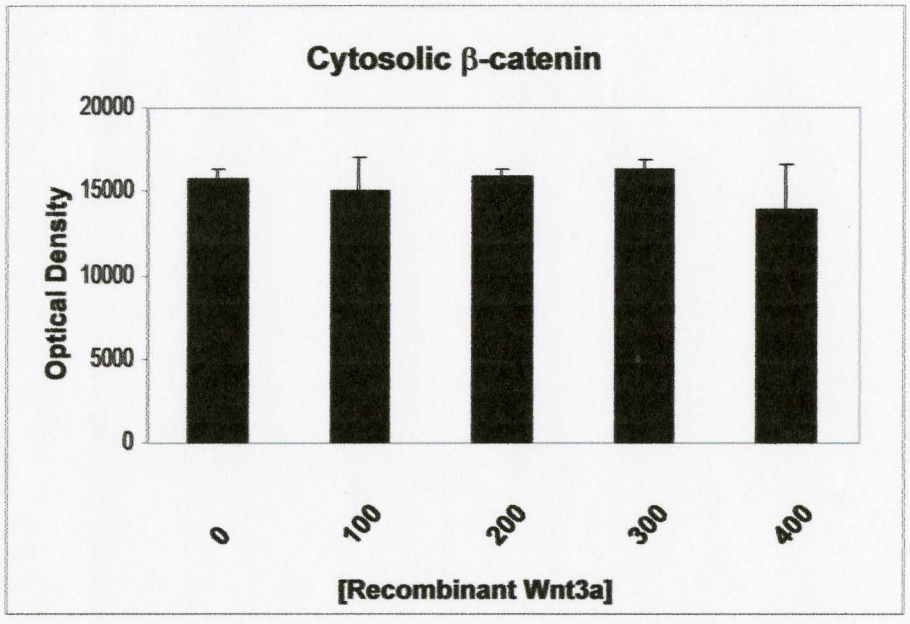


Figure 3.21: Recombinant Wnt3a dose response in cytosolic fraction of DU-145 cells.

Figure 3.21A: Cells were treated with increasing concentrations of **recombinant Wnt3a protein for 24 hours. The levels of cytosolic β-catenin** remained unchanged with treatment.

Figure 3.21B: Densitometric quantification of β-catenin protein levels in the cytosolic fraction.

Values are means of three independent experiments and error bars are the standard error of the mean

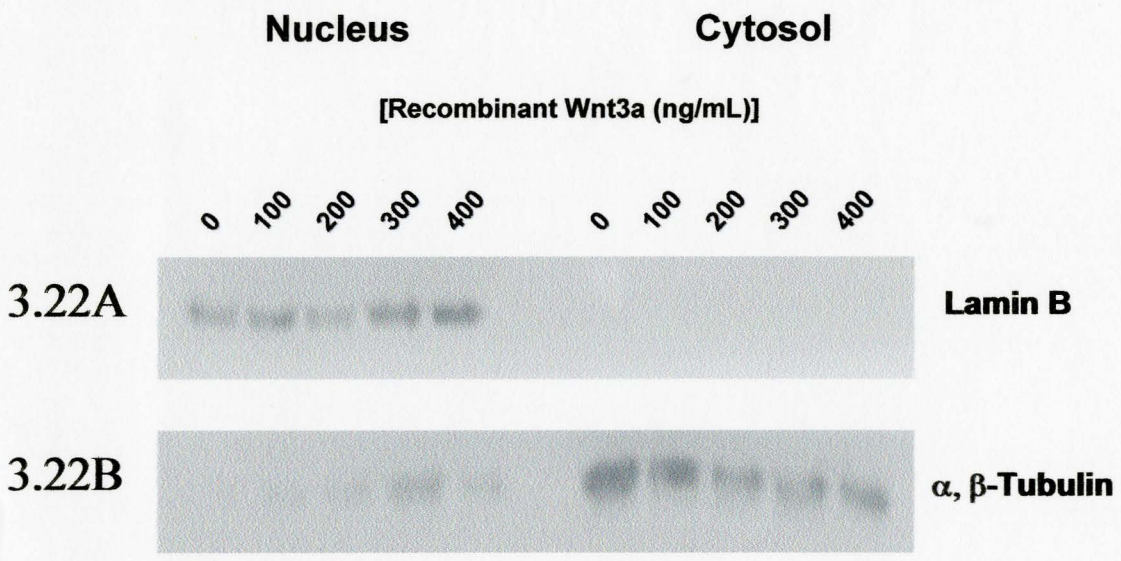
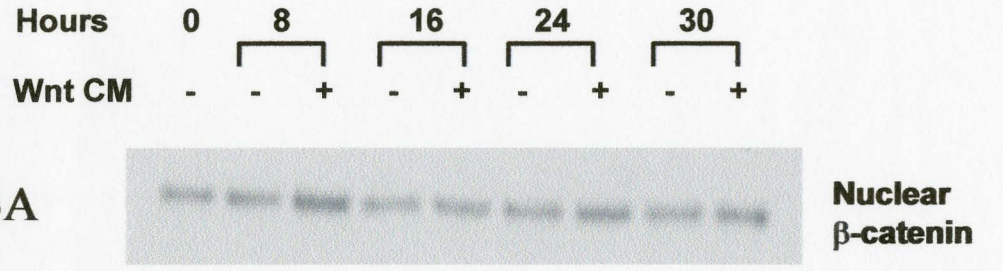


Figure 3.22: Markers for nuclear and cytosolic fractions in DU-145 cells treated with Recombinant Wnt3a.

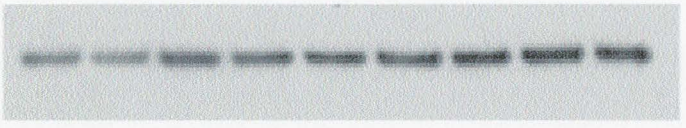
Figure 3.22A: Lamin B is used as a nuclear marker to show nuclear enrichment. There is no significant nuclear contamination in the cytosolic fraction.

Figure 3.22B: α,β Tubulin is used as a cytosolic marker indicating that there was no cytosolic contamination in the nuclear fraction.



3.23A

3.23B



3.23C

3.23D

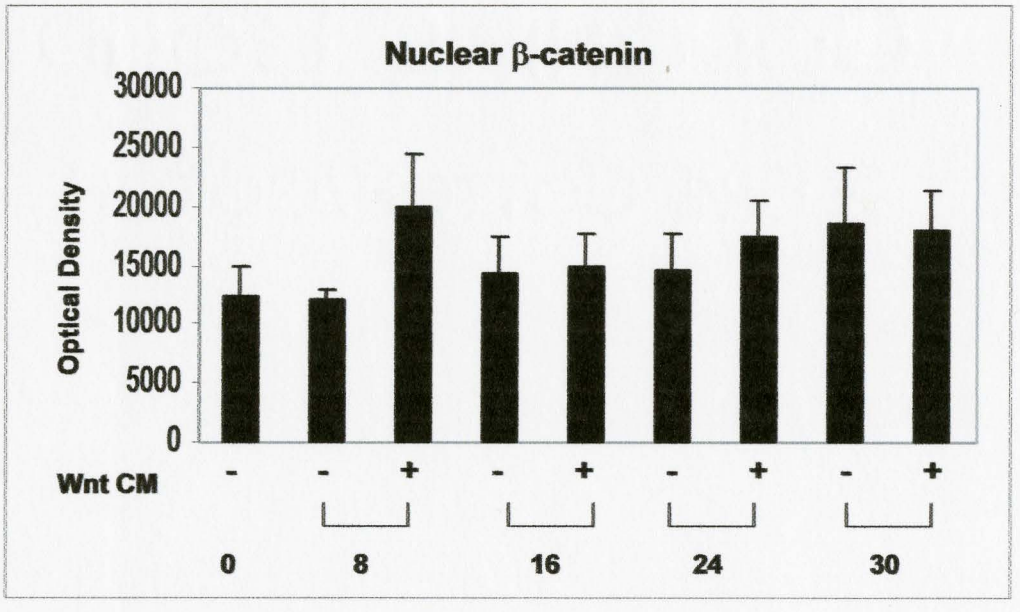


Figure 3.23: Nuclear β-catenin levels remained constant with Wnt3a CM treatment in DU145 cells.

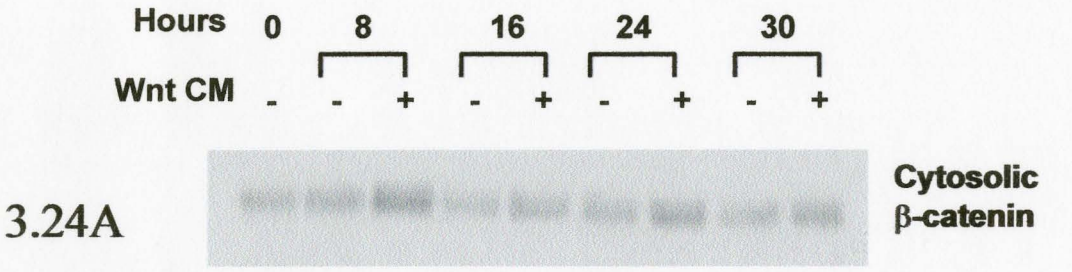
Figure 3.23A: Cells were treated with L cell and Wnt3a -L cell conditioned media for the indicated time periods. Levels of nuclear β-catenin did not change during treatment.

Figure 3.23B: Immunoblots were stripped and re-probed with anti-Lamin B as a loading control and to show enrichment of the nuclear enrichment.

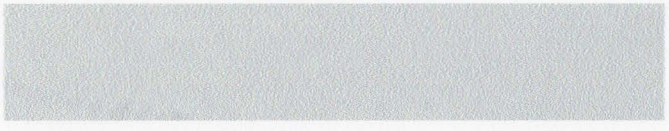
Figure 3.23C: Immunoblots were re-stripped and re-probed with anti-α,β tubulin antibody showing no cytosolic contamination in the nuclear fraction.

Figure 3.23D: Densitometric quantification of β-catenin protein levels in the nuclear fraction.

Values are means of three independent experiments and error bars are the standard error of the mean



3.24A



3.24B

3.24C

3.24D

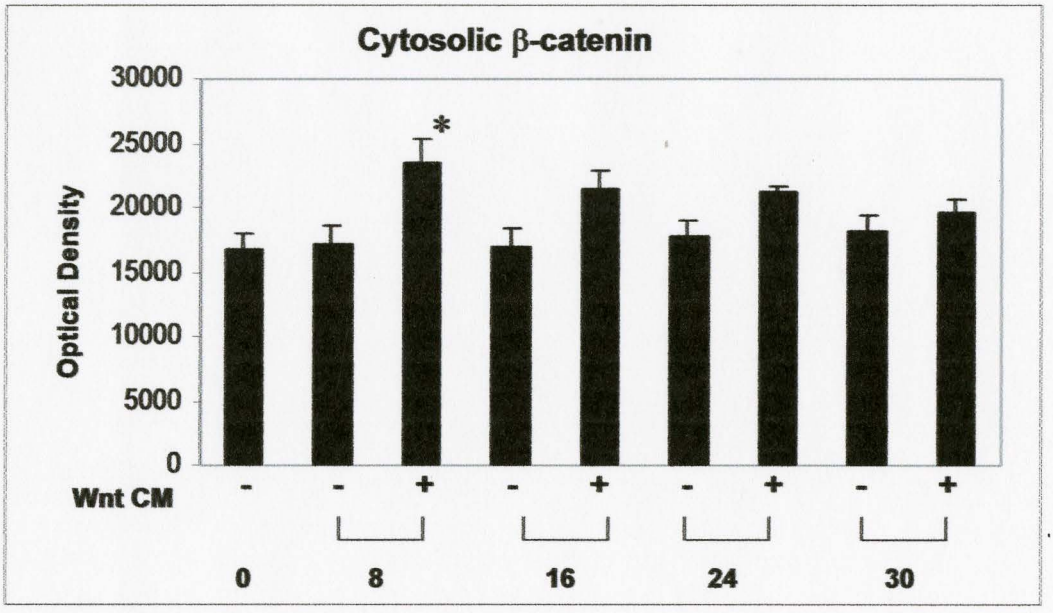


Figure 3.24: Cytosolic β-catenin levels increased with Wnt3a CM treatment in DU145 cells.

Figure 3.24A: Cells were treated with L cell and Wnt3a -L cell conditioned **media for the indicated time periods. Levels of cytosolic β-catenin** increased during treatment.

Figure 3.24B: Immunoblots were stripped and re-probed with anti-Lamin B antibody showing no nuclear contamination in the cytosolic fraction.

.Figure 3.24C: Immunoblots were re-stripped and re-probed with anti-α,β tubulin as a loading control.

Figure 3.24D: Densitometric quantification of β-catenin protein levels in the cytosolic fraction. * $p < 0.10$ compared to L-cell CM 8 hr control (lane2)

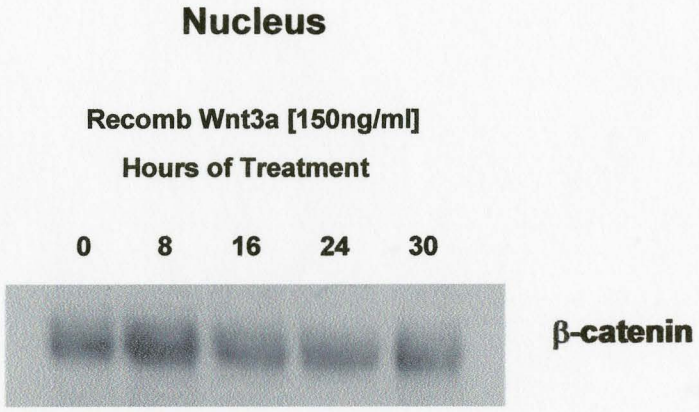
Values are means of three independent experiments and error bars are the standard error of the mean

3.5.2 Wnt3a conditioned media induced increases in nuclear β-catenin in NIH 3T3 cells.

Studies done by Yun et al. have shown increased total β-catenin levels after treatment with a commercially available Wnt3a ligand in the murine fibroblast cell line NIH 3T3 (Yun et al., 2005). Thus, using this cell line, a time course was done with the Wnt3a recombinant protein. NIH 3T3 cells were grown to 70% confluency in media containing 10% serum and treated with the recombinant protein. An increase in nuclear β-catenin was seen at 8 hours with treatment, however the levels returned back to basal after 16 hours (Figure 3.24). The cytosolic levels of β-catenin remained unchanged (Figure 3.25).

NIH 3T3 cells were also treated with Wnt3a and L cell conditioned media for various time points. β-catenin immunoblots of nuclear and cytosolic fractions clearly showed increased β-catenin in both compartments (Figure 3.27, 3.28). These results clearly indicated that using Wnt3a conditioned media rather than Wnt3a recombinant protein was the optimal treatment to increase β-catenin levels in these cells.

3.25A



3.25B

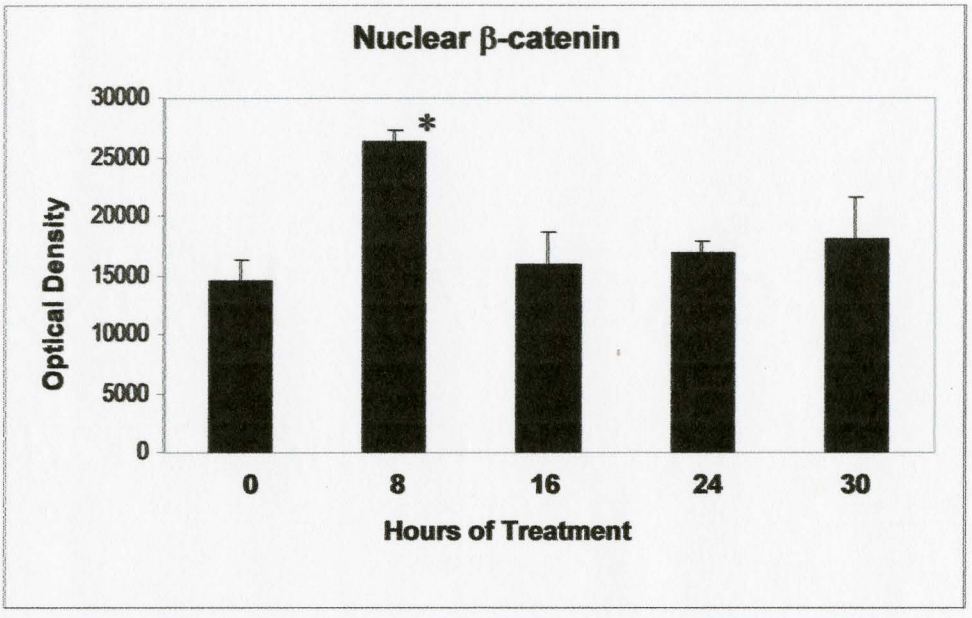


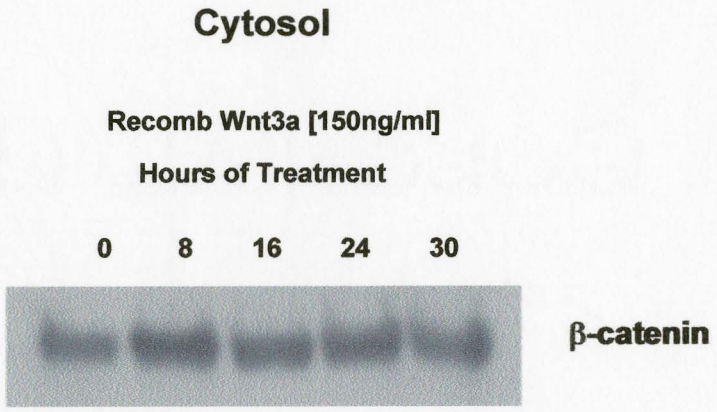
Figure 3.25: Levels of nuclear β-catenin in NIH 3T3 cells treated with Recombinant Wnt3a.

Figure 3.25A: Cells were treated with recombinant Wnt3a protein for the indicated time periods. An increase in nuclear β-catenin was seen at 8 hours then drops back to baseline for the remaining time points

Figure 3.25B: Densitometric quantification of β-catenin protein levels in the nuclear fraction. * $p < 0.05$ compared to 0hrs control (lane 1)

Values are means of three independent experiments and error bars are the standard error of the mean

3.26A



3.26B

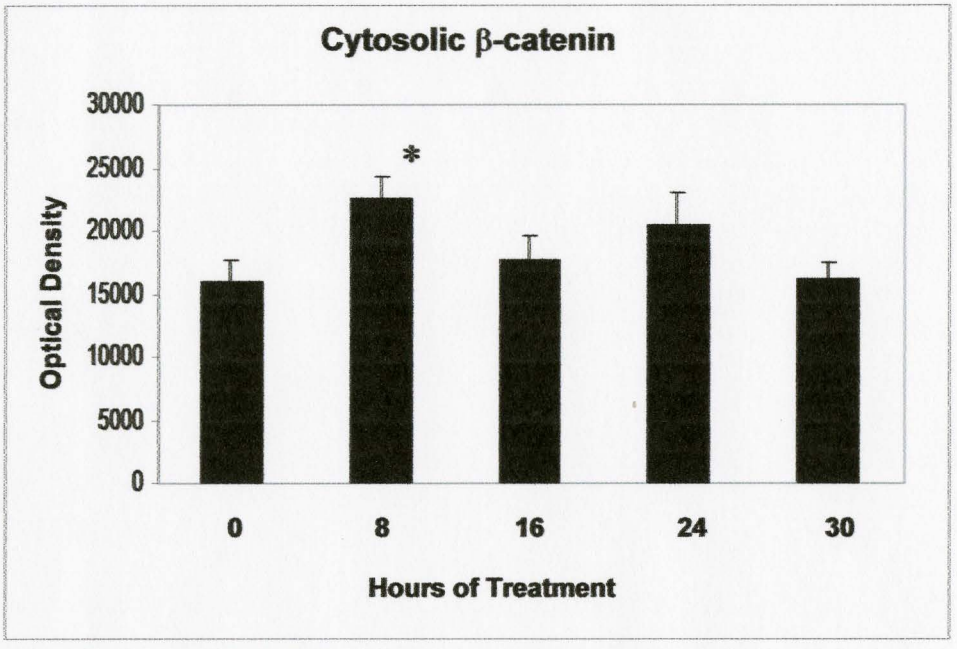


Figure 3.26: Levels of cytosolic β-catenin in NIH 3T3 cells treated with Recombinant Wnt3a.

Figure 3.26A: Cells were treated with recombinant Wnt3a protein for the indicated time periods. No significant increases in cytosolic β-catenin wasseen with treatment.

Figure 3.26B: Densitometric quantification of β-catenin protein levels in the cytosolic fraction. * $p < 0.10$ compared to 0hrs control (lane 1)

Values are means of three independent experiments and error bars are the standard error of the mean

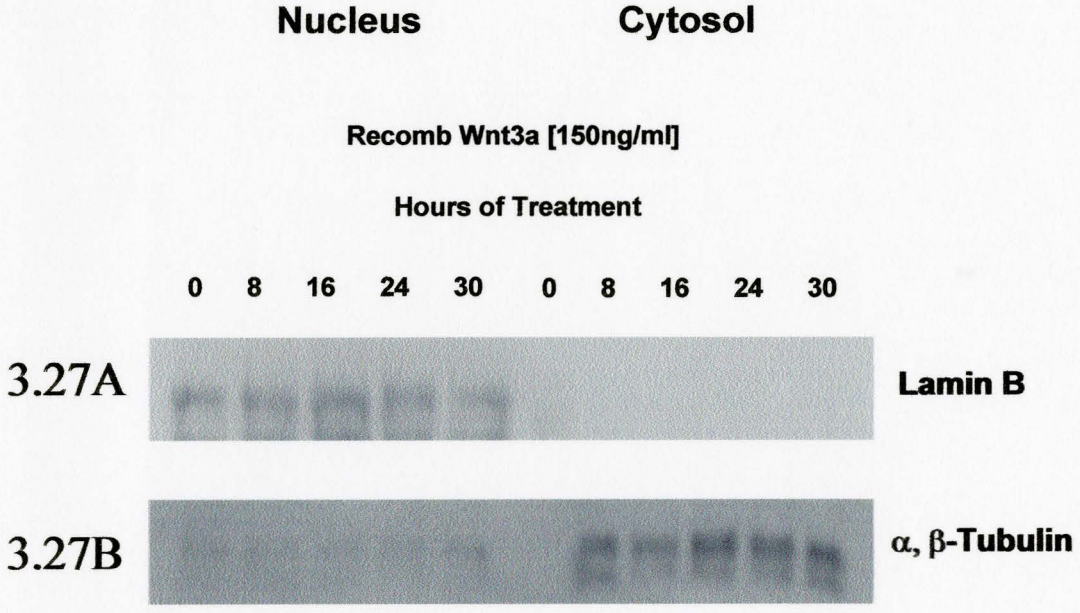
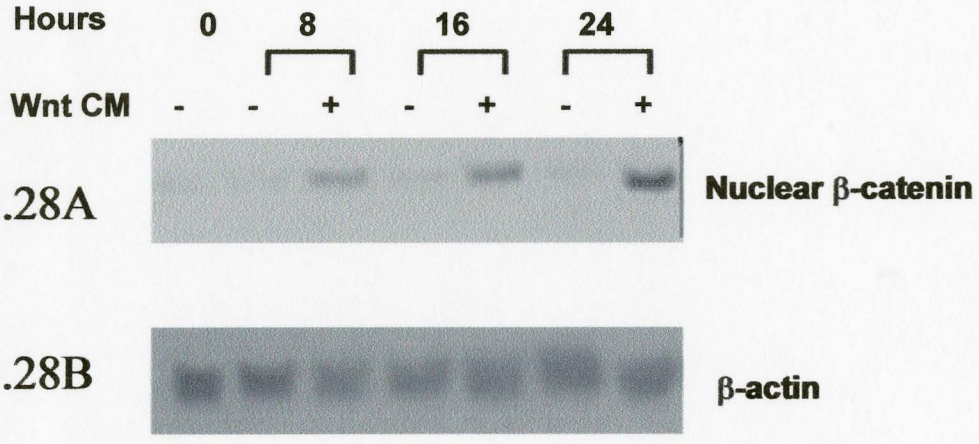


Figure 3.27: Markers for nuclear and cytosolic fractions in NIH 3T3 cells treated with Recombinant Wnt3a.

Figure 3.27A: Lamin B is used as a nuclear marker to show nuclear enrichment. There is no significant nuclear contamination in the cytosolic fraction.

Figure 3.27B: α,β Tubulin is used as a cytosolic marker indicating that there is no cytosolic contamination in the nuclear fraction.



3.28A

3.28B

3.28C

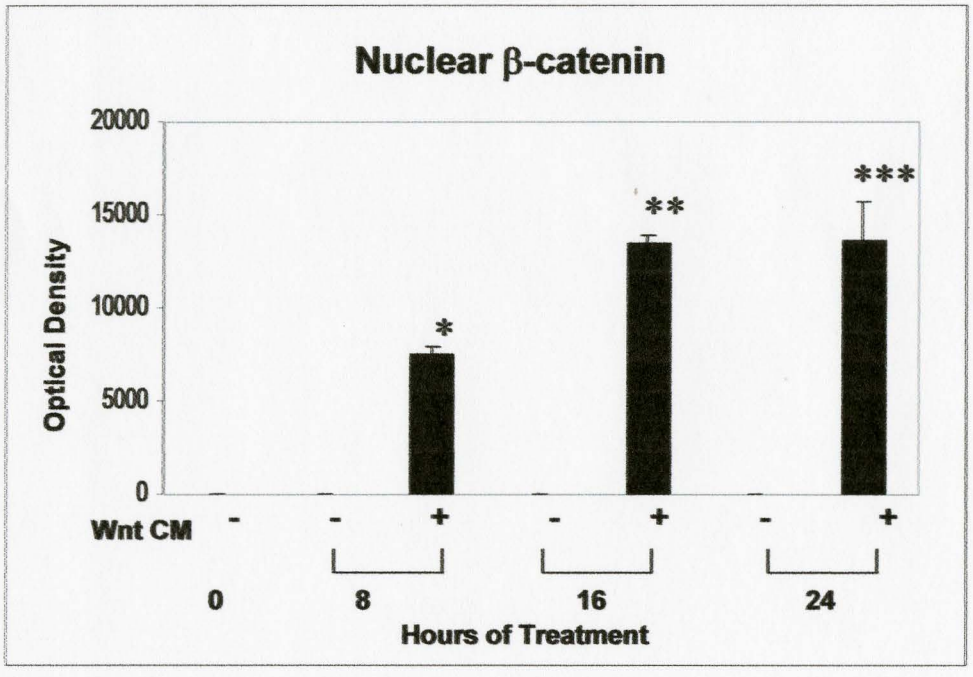


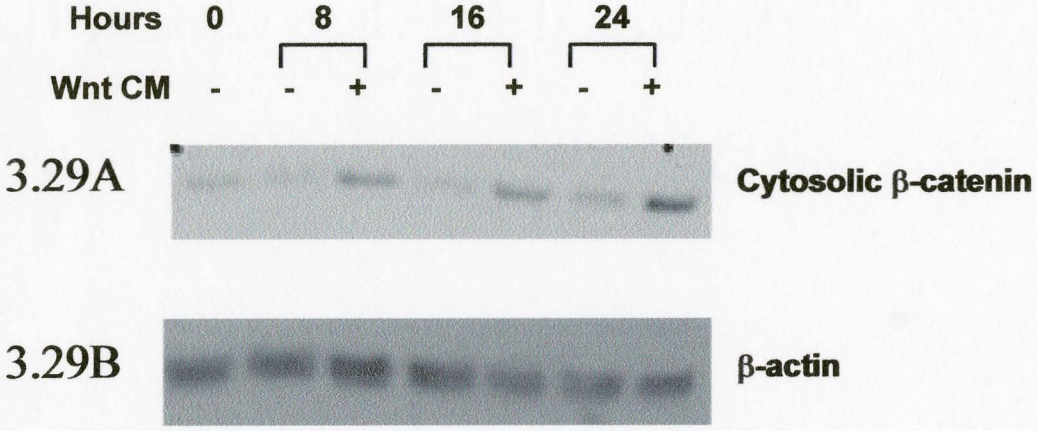
Figure 3.28: Wnt3a Conditioned media induced an increase in nuclear β-catenin levels in NIH 3T3 cells.

Figure 3.28A: Cells were treated with L cell and Wnt3a -L cell conditioned media for the indicated time periods. Levels of nuclear β-catenin increaseed with Wnt3a CM treatment, and remained the same as basal with L cell CM.

Figure 3.28B: Immunoblots were stripped and re-probed for Actin as a loading control.

Figure 3.28C: Densitometric quantification of β-catenin protein levels in the nuclear fraction.
* $p < 0.05$ compared to L-Cell CM 8hrs Control (lane 2)
** $p < 0.005$ compared to L-Cell CM 16hrs Control (lane 4)
*** $p < 0.005$ compared to L-Cell CM 24hrs Control (lane 6)

Values are means of three independent experiments and error bars are the standard error of the mean


Hours 0 8 16 24
Wnt CM - - + - + - +
3.29A
Cytosolic β-catenin
3.29B
β-actin

3.29C

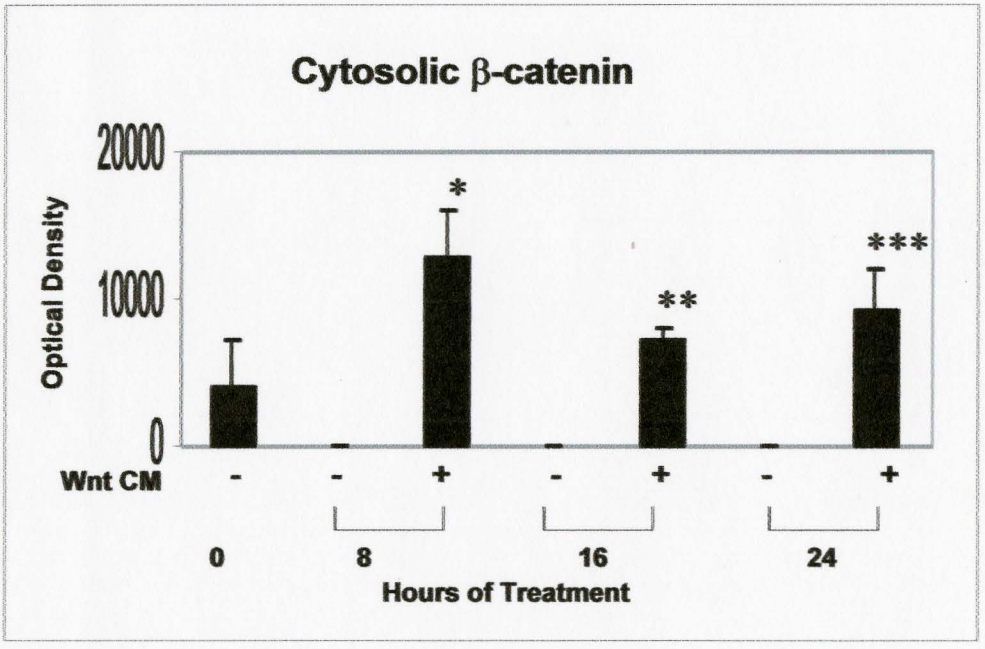

Cytosolic β-catenin
Optical Density
20000
10000
0
*
**
***
Wnt CM - - + - + - +
0 8 16 24
Hours of Treatment

Figure 3.29: Wnt3a Conditioned media induced an increase in cytosolic β-catenin levels in NIH 3T3 cells.

Figure 3.29A: Cells were treated with L cell and Wnt3a -L cell condition ed **media for the indicated time periods. Levels of cytosolic β-catenin increased** with Wnt3a CM treatment, and remained the same as basal with L cell CM.

Figure 3.29B: Immunoblots were stripped and re-probed for Actin as a loading control.

Figure 3.29C: Densitometric quantification of β-catenin protein levels in the cytosolic fraction.
* $p < 0.10$ compared to L-Cell CM 8hrs Control (lane 2)
** $p < 0.05$ compared to L-Cell CM 16hrs Control (lane 4)
*** $p < 0.10$ compared to L-Cell CM 24hrs Control (lane 6)

Values are means of three independent experiments and error bars are the standard error of the mean

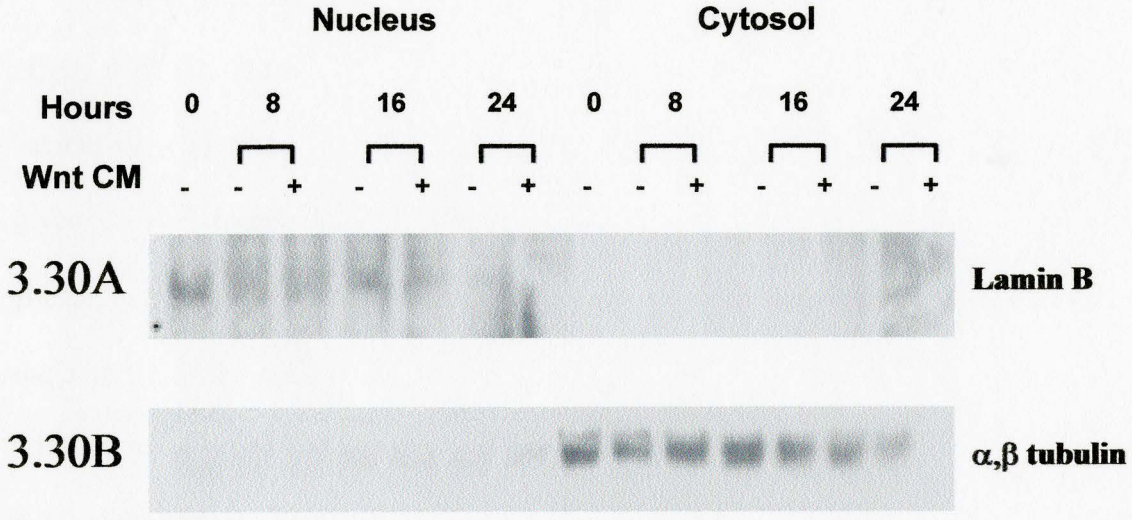


Figure 3.30: Markers for nuclear and cytosolic fractions in NIH 3T3 cells treated with conditioned media.

Figure 3.30A: Lamin B is used as a nuclear marker to show nuclear enrichment. There is no significant nuclear contamination in the cytosolic fraction.

Figure 3.30B: α,β Tubulin is used as a cytosolic marker indicating that there is no cytosolic contamination in the nuclear fraction.

3.5.3 Increased O-glycosylation of β-catenin by PUGNAc was not seen in NIH 3T3 cells.

Since Wnt3a stimulation in NIH 3T3 cells was established, we determined the effect of PUGNAc treatment conditions in this cell line. NIH 3T3 cells were treated with increasing concentrations of PUGNAc for 24 hours. No apparent increase in O-glycosylated β-catenin was seen (Figure 3.30). This lack of increased O-glycosylation of β-catenin by PUGNAc may be due to serum factors in the media that may interfere with the activity of the inhibitor however, serum could not be taken out of the culture media without compromising the viability of the cells. NIH 3T3 cells did not survive in media containing 5% FBS for more than 1 hours without detaching from the plate.

3.5.4 Wnt3a and PUGNAc did not increase nuclear β-catenin or O-glycosylation in L cells, respectively.

A third cell line was used to conduct these experiments. L cells, the parental cell line of Wnt3a-L cells, was treated with Wnt3a conditioned media for the indicated time points. An increase in nuclear and cytosolic β-catenin was seen at 8 hours and remained at that level throughout the entire time course (Figure 3.31). Next, L cells were treated with PUGNAc at increasing concentrations for 24 hours and WGA precipitation was done, however an increase in O-glycosylated β- catenin was not seen (Figure 3.32A). Total β-catenin blots indicate that there are very low levels of the protein in L cells; this could be due to the fact that these cells lack E-cadherin (Nagafuchi et al., 1994). Thus, L cells were treated with Wnt3a conditioned media in combination with PUGNAc in order

to increase the levels of total β-catenin in the cell available to become O-glycosylated. However, results showed that increasing total β-catenin levels did not increase O-glycosylated β-catenin levels (Figure 3.32C).

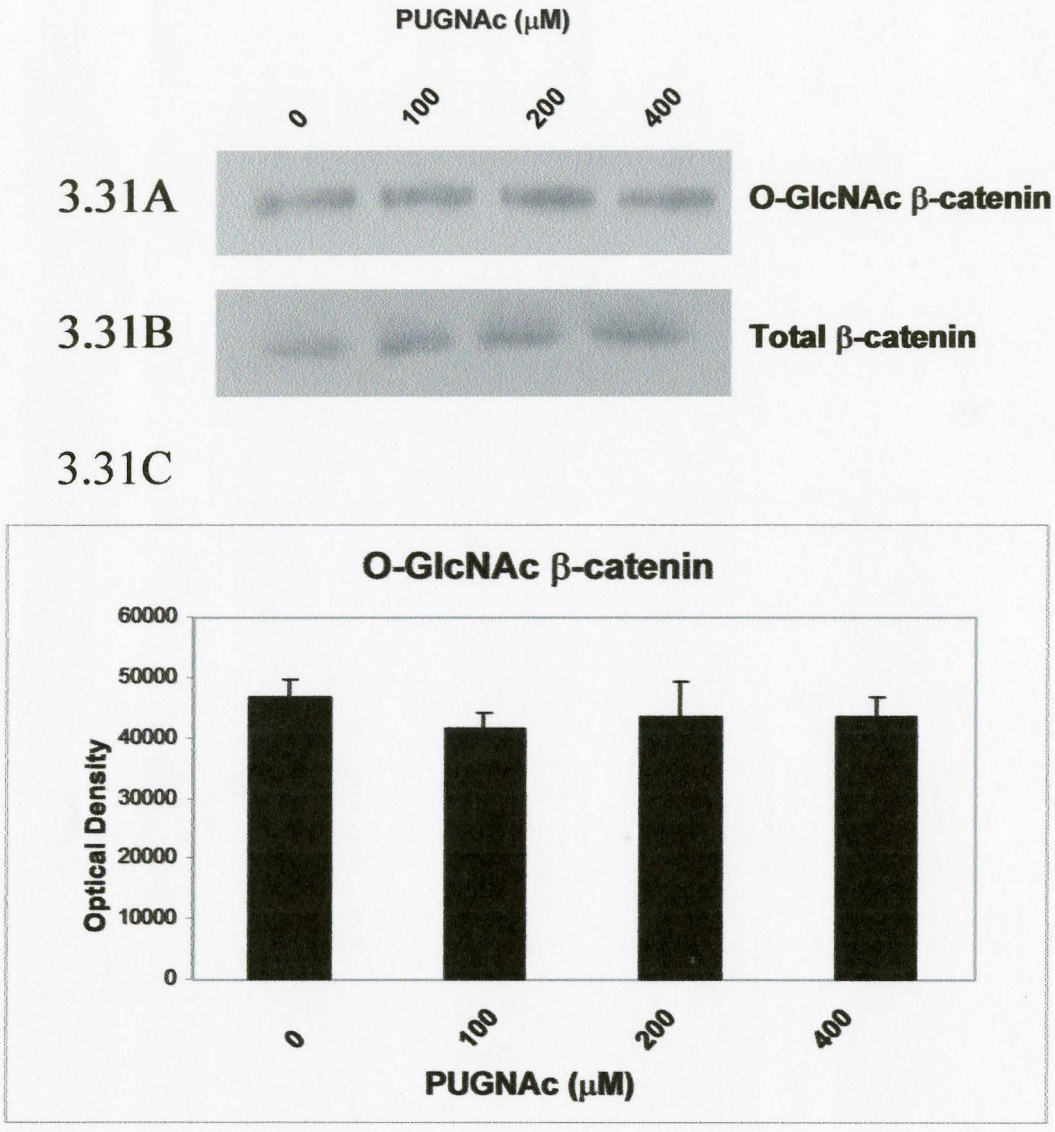


Figure 3.31: PUGNAc dose response treatment in NIH 3T3 cells.

Figure 3.31A: Cells were treated with increasing concentrations of PUGNAc in media containing 10% serum for 24 hours. O-glycosylated β-catenin was precipitated with WGA. Levels do not increase with PUGNAc treatment.

Figure 3.31B: Levels of total β-catenin remain constant with PUGNAc treatment.

Figure 3.31C: Densitometric quantification of O-glycosylated β-catenin protein levels.

Values are means of three independent experiments and error bars are the standard error of the mean

3.32

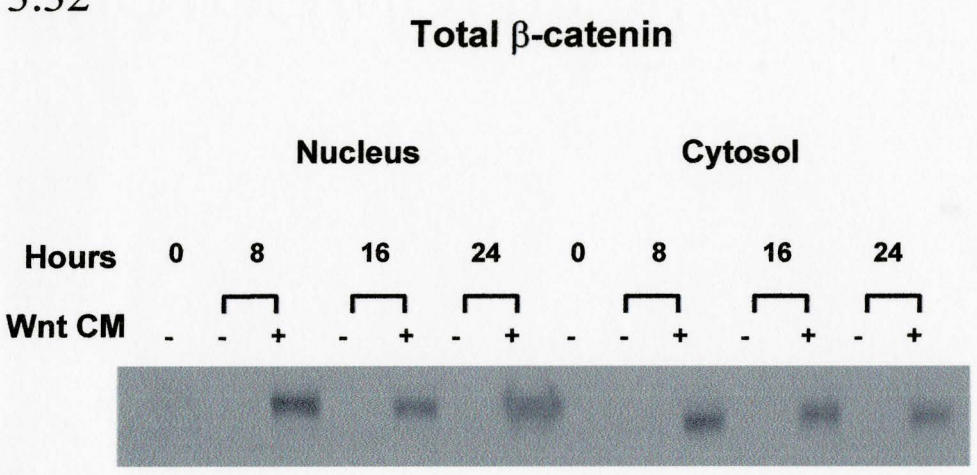


Figure 3.32 Wnt3a conditioned media induces increased nuclear and cytosolic β-catenin in L cells.

L cells were treated with L cell and Wnt3a-L cell conditioned media for the indicated time periods. Nuclear and cytosolic fractions were collected as previously.

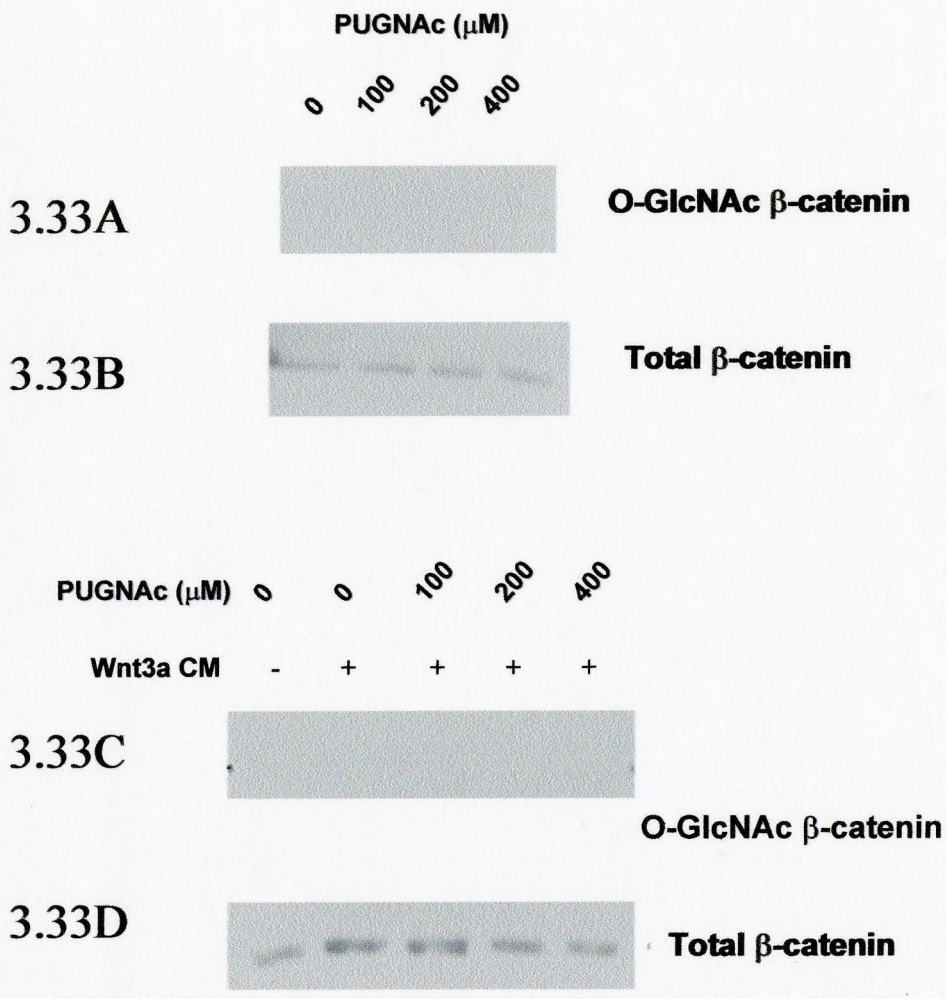


Figure 3.33 PUGNAc (and Wnt3a conditioned media) dose response treatment in L cells.

Figure 3.33A: Cells were treated with increasing concentrations of PUGNAc for 24 hours. No O-glycosylated β-catenin was detected by WGA precipitation.

Figure 3.33B: Levels of total β-catenin did not change with PUGNAc treatment.

Figure 3.33C: L cells were treated with 10mL of Wnt3a condition media (per 100mm plate) that contained 0% FBS, in combination with increasing **concentrations of PUGNAc for 24 hours. No O-glycosylated β-catenin was** detected by WGA precipitation.

Figure 3.33D: Levels of total β-catenin did not change with CM and PUGNAc combination treatment.

Chapter 4

Discussion, Concluding Remarks and Future Directions

4.1 Discussion

β-catenin signalling has been implicated in the progression of many types of cancers. Its oncogenic properties are attributed to its ability to enter the nuclear and activate transcription of target genes involved in proliferation, angiogenesis, invasion and metastasis. However, the mechanisms involved in the regulation of β-catenin nuclear translocation are not well understood.

β-catenin is known to undergo phosphorylation post-translational modification. This is important in regulating the stability of the protein and hence its cellular expression levels. β-catenin has also been shown to undergo O-glycosylation (Zhu et al., 2001). Previous work done in breast cancer cells suggested that O-glycosylation did not prevent β-catenin's interactions at adherence junctions. Thus we asked the question: "Does O-glycosylation modification affect the transcriptional properties of the protein?"

Using prostate cancer cells as a model we compared the basal levels of O-glycosylated β-catenin in two prostate cancer cells lines to basal levels found in a normal immortalized prostate epithelial cell line. Higher levels of O-glycosylated β-catenin was observed in the normal prostate cells compared to the prostate cancer cells lines, however there were higher levels of total β-catenin in the prostate cancer cells and most of it was in the unglycosylated form. Furthermore, nuclear and cytosolic fractions showed that there was little to no O-glycosylated β-catenin in the nucleus of the prostate cancer cell lines compared to the normal prostate cell line that had significant levels in the nucleus. There were higher levels of total nuclear β-catenin in the prostate cancer cell lines

compared to the normal cell line which was not surprising because of immunohistological studies done in prostate cancer tumours showing β-catenin accumulation and nuclear localization, as well as *in vitro* studies demonstrating increased levels of β-catenin (Chesire et al., 2000). However, low levels of O-glycosylated β-catenin in the nucleus of prostate cancer cells lines compared to a normal immortalized prostate cell line indicates that the mechanisms that regulated the O-glycosylation of this protein may be aberrant in prostate cancer cells resulting in higher levels of unglycosylated β-catenin in the nucleus.

In order to investigate whether O-glycosylation of β-catenin affects the nuclear localization of the protein, we manipulated the levels of O-GlcNAc in the prostate cancer cell line DU-145 cells by treatment of these cells with the chemical inhibitor PUGNAc. PUGNAc, O-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate, is a potent, non-toxic, irreversible inhibitor of the enzyme O-GlcNAcase. Since O-GlcNAcase is responsible for removing the monosaccharide from O-glycosylated proteins, its inhibition by PUGNAc prevents the cycling of GlcNAc on nucleocytoplasmic proteins. Treatment with this inhibitor results in a global increase in O-glycosylated protein in cells (Haltiwanger et al., 1998).

DU-145 cells were treated with the chemical inhibitor PUGNAc and an increase in O-glycosylated β-catenin was seen. Separation of the nuclear and cytosolic compartments of PUGNAc treated DU-145 cells showed that as levels of O-glycosylated β-catenin increased, the levels of total cytosolic β-catenin increased and total nuclear β-catenin decreased. One possible explanation for these observations is that O-glycosylation of β catenin may prevent it from entering the nucleus. As a result, an

emptying out of β-catenin from the nucleus and a build of β-catenin in the cytosol is seen. β-catenin is able to enter the nucleus on its own, without the aid of any known nucleocytoplasmic binding partners. The Armadillo repeats within the central core of β-catenin are necessary and required for the protein's import into the nucleus (Funayama et al., 1995; Koike et al., 2004). The mechanism of how these repeats are involved in import is still not clear, however, Fagotto et al., have suggested that β-catenin enters the nucleus independently by interacting with components of the nuclear pore complex (Fagotto et al., 1998). Thus, the protein may interact with these components via its Armadillo repeats, which have been shown to be important in interacting with many of β-catenin's binding partners. Our results suggest that O-glycosylation of β-catenin is affecting the protein's ability to enter the nucleus. Thus, it is possible that O-glycosylation may occur at these Armadillo repeats thus interfering with the ability of β-catenin to interact with these nuclear pore components. However, studies done by Suh et al. negate any interactions with nuclear pore proteins that are involved in translocation of β-catenin (Suh et al., 2003).

Alternatively, O-glycosylation may affect the protein's conformation, allowing it to fold in a manner that renders the protein more favorable or less favorable for binding to its interacting binding partners. β-catenin interacts with APC and axin, which enhances its export to the cytoplasm (Barth al., 1997; Tolwinski and Wieschaus, 2001). O-glycosylation may increase β-catenin's ability to bind to these cytosolic proteins, thus sequestering β–catenin in the cytosol. On the other hand, O-glycosylation may make β–catenin unfavorable for binding to TCF/LEF and BCL9, which have been shown to

recruit β-catenin to the nucleus (Behrens et al., 1996; Huber et al., 1996; Kramps et al., 2002; Townsley et al., 2004).

Another scenario is that O-glycosylation may target β-catenin for export out of the nucleus. Though β-catenin can be exported out of the nucleus via APC, it is also able to exit the nucleus independent of export receptors and energy requirements. O-glycosylated β-catenin may be fully able to enter the nucleus, however, once inside, its mechanism of export, whether it includes additional carrier proteins or not, quickly exports the protein back into the cytosol. Thus O-glycosylation may promote the export of β-catenin. O-glycosylation may still occur at the Arm repeats subsequently making β-catenin more attractive for interaction with binding partners involved in its nuclear export, such as APC.

Increased O-glycosylation by PUGNAc also affects the nuclear localization of the transcription factor Sp1. Studies done by Majumdar et al. have shown that insulin induced O-glycosylation of Sp1 is important in regulating the nuclear translocation and transcriptional activity of the protein (Majumdar et al., 2006). PUGNAc was able to increase O-glycosylated Sp1 in DU-145 cells, which increased the level of Sp1 in the nucleus. Thus, the resulting effect of O-glycosylation seems to be quite protein specific as it promotes increased nuclear localization of the transcription factor Sp1 while nuclear localization of β-catenin is prevented by O-glycosylation.

The import of proteins into the nucleus occurs through the NPC. Protein components of the NPC contain many residues that can be O-glycosylated. The question arises as to whether PUGNAc interferes with normal import via the nuclear pore proteins

that become O-glycosylated. Based on our results, nuclear translocation of transcription factor TCF4 is not affected by PUGNAc treatment indicating that movement through the NPC into the nucleus remains normal.

The ability of β-catenin to activate transcription of its target genes is dependent upon the protein's ability to enter the nucleus. Our results indicate that β-catenin's nuclear import is hindered by O-glycosylation, thus its ability to activate transcription may also be affected. The TOPFlash luciferase construct provides a way to measure the transcriptional capability of β-catenin in cells. Thus, if there are high levels of nuclear β-catenin in cells, this corresponds to high luciferase activity. The luciferase activity of TOPFlash decreased with PUGNAc treatment in DU-145 cells indicating that O-glycosylation was affecting the transcriptional activity of β-catenin.

We also investigated the effects of O-glycosylation on the protein expression of two β-catenin target genes. The protein expression of Cyclin D1 and VEGFA decreased with increasing O-glycosylation of β-catenin in DU-145 cells. The decrease in expression seen was not due to lack of serum in the media during PUGNAc treatment as shown by steady protein levels of both genes when cells are treated under the same serum conditions in the absence of PUGNAc.

Wnts are secreted glycoproteins involved in cell-cell communication and play an important role in key developmental and morphogenetic processes. Stable expression of several Wnt genes, including Wnt1 and Wnt3a, into mammalian cells stabilizes β-catenin and increases levels of the protein (Hinck et al., 1994; Papkoff et al., 1996; Shimizu et al., 1997). Furthermore, Wnt3a increases nuclear β-catenin in cells by signaling through the

canonical pathway (Kohn and Moon, 2005). In order to determine if O-glycosylation affects the nuclear translocation of β- catenin, Wnt3a was used in combination with PUGNAc, to see if O-glycosylation could change the increase in nuclear β-catenin levels.

DU-145 cells were treated with a purified Wnt3a recombinant protein for 24 hours. However, no increase in β-catenin was seen in either the nucleus or cytosol. Secreted Wnt3a from condition media was also used on DU-145 cells to try to stimulate an increase in nuclear β-catenin, however, like the recombinant protein; no increases were seen in either compartment. As seen in comparing this prostate cancer cell line with a normal epithelial prostate cell line, PNT1A, there seems to be high basal levels of the protein in DU-145 cells. Mutations in β-catenin or components of the degradation complex are rare in prostate cancer cells, however other regulatory mechanism may play a dominant role in β-catenin activation which consequently lead to increased levels of the protein (Verras and Sun, 2006). Studies done by Wissman et al. identified multiple components of the pathway, Wnt ligands, inhibitors and other co-regulators that were abnormally expressed in prostate cancer cell lines (Wissmann et al., 2003). Thus, it is possible that Wnt3a was not able to stimulate a further increase in the levels of β-catenin. Alternatively, Wnt3a may have been able to stimulate a small increase but because the levels of β-catenin were already too high, this increase was not significant enough to be detected.

The murine fibroblast cell line NIH 3T3 was treated with a purified Wnt3a recombinant protein, which had previously been shown to increase levels of nuclear β-catenin in these cells, however an increase in nuclear β-catenin was only seen at 8 hours,

then levels returned to basal for longer treatment points (Yun et al, 2005). Wnt3a conditioned media has previously been shown to induce accumulation of total and cytosolic β-catenin in NIH 3T3 cells (Yun et al., 2005; Lee et al., 1999). Our results show that treatment of NIH 3T3 cells with conditioned media caused increases in β-catenin in both the nucleus and cytosol. However, PUGNAc was not able to cause an increase in O-glycosylated β-catenin in this cell line. Serum factors in the media during treatment may have interfered with the activity of the inhibitor. However, removing serum from the media was not an option since NIH 3T3 cells could not remain viable in the absence of serum for more than 1 hour.

It is also possible that PUGNAc is indeed increasing O-glycosylated β–catenin levels however because the basal level of O-glycosylated β–catenin is already high the increase is not significant enough to be detected. This may also be the case in the normal prostate epithelial cell line PNT1A where, similar to NIH 3T3 cells, there are high basal levels of O-glycosylated β–catenin and PUGNAc was not able to cause any significant increases in O-GlcNAc β–catenin. This may be the trend seen in other normal epithelial and fibroblast cell lines where homeostasis within the cell requires there to be a high level of O-glycosylated β–catenin and subsequently low nuclear levels of the protein. Unfortunately, in prostate cancer cells, the balance between the addition and removal of O-GlcNAc may be disrupted and high levels of O-glycosylated β–catenin are no longer maintained.

The murine fibroblast cell line, L cells, only express trace amount of β–catenin, although they have a normal levels of its mRNA; it is suggested that this occurs because

these cells are devoid of any cadherins (Nagafuchi et al., 1994). As a result, the lack of the cadherin-based cell adhesion activity subsequently leads to low levels of β–catenin in cells. When we treated L cells with Wnt3a conditioned media there was a significant increase in nuclear and cytosolic β–catenin, as was seen in previous studies (Shibamoto et al., 1998). However, PUGNAc was not able to cause an increase O-glycosylation of β–catenin in these cells. In fact, the L cells exhibited no detectable O-glycosylated β–catenin probably due to the low levels of the protein in these cells. To circumvent this, the L-cells were treated with Wnt3a conditioned media in combination with PUGNAc. The Wnt3a conditioned media increased the levels of β–catenin making the protein more available to become O-glycosylated. In addition, the Wnt3a conditioned media did not contain serum in order to remove any interference serum factors could have on the activity of PUGNAc. Unfortunately, increasing levels of β–catenin did not increase O-glycosylation of the protein. We were still unable to detect any O-glycosylated β–catenin.

4.2 Concluding Remarks

In conclusion, the results from this study show that O-glycosylation of β-catenin regulates the nuclear translocation and transcriptional activity of the protein.

A comparison of O-glycosylated β-catenin expression in two prostate cancer cell lines, DU-145 and LNCaP, to that in a normal prostate epithelial cell line (PNT1A) showed that the normal cell line had higher levels of O-glycosylated β-catenin than the two cancer cell lines. Nuclear and cytoslic fractions showed that while the cancer cell lines contained more nuclear β-catenin than the normal most of it was in the unglycosylated form.

Treatment of DU145 cells with PUGNAc caused an increase in O-glycosylated β-catenin, which subsequently lead to a decrease in nuclear expression of the protein and increased levels in the cytosol. These results suggested that O-glycosylation was preventing β-catenin's nuclear translocation. Additional evidence that supported this hypothesis came from studies using the TopFlash reporter construct where a decrease in luciferase activity was seen with increasing O-glycosylation of β-catenin caused by PUGNAc in DU-145 cells. Similarly, a decrease in the protein expression of two β-catenin target genes, Cyclin D1 and VEGFA, was seen with increasing O-glycosylation of β-catenin.

Finally, to further investigate O-glycosylation's affect on β-catenin's nuclear translocation Wnt3a conditioned media was used to increase levels of nuclear β-catenin and compared to conditions where PUGNAc was used in combination in order to see if O-glycosylation would affect Wnt3a induced nuclear β-catenin levels. However, optimal treatment conditions could not be determined.

Overall, these findings are important, as no studies have shown a role for O-glycosylation in regulating the signalling properties of the protein. The data presented in this study indicate that O-glycosylation of β-catenin regulates the subcellular localization and transcriptional activity of the protein.

4.3 Future Directions

Based on our results, O-glycosylation of β-catenin prevents its migration into the nucleus thus further work is necessary to identify the mechanism(s) by which this occurs. Furthermore, determining how O-glycosylation affects β-catenin's ability to bind to its interacting partners, such as TCF/LEF and APC, may provide information as to how O-glycosylation affects the proteins nuclear localization.

Additional studies should be done to ensure that these results are not a single cell phenomenon. Thus, these experiments should be repeated in another prostate cancer cell line, such as LNCaP cells. It would also be interesting to repeat these experiments in a different type of cancer cell line, we have done so in breast cancer cells, in order to show that our results are not cancer specific and that the regulation of β-catenin by O-glycosylation is a general phenomenon seen in other types of cancers, not only prostate and breast.

It would be important to identify the sites of O-glycosylation of β-catenin. In doing so, the production of specific antibodies to O-glycosylated β-catenin would become available and replace the less specific methods of detecting the glycosylated form of the protein.

Finally, the regulation of O-glycosylation of β–catenin and identification of the signalling pathways involved in this regulation are important aspects to investigate in order to determine how these mechanisms become disrupted in prostate cancer and in

other types of cancers. This will help in the identification of key regulatory targets that could potentially be intercepted to limit cancer progression.

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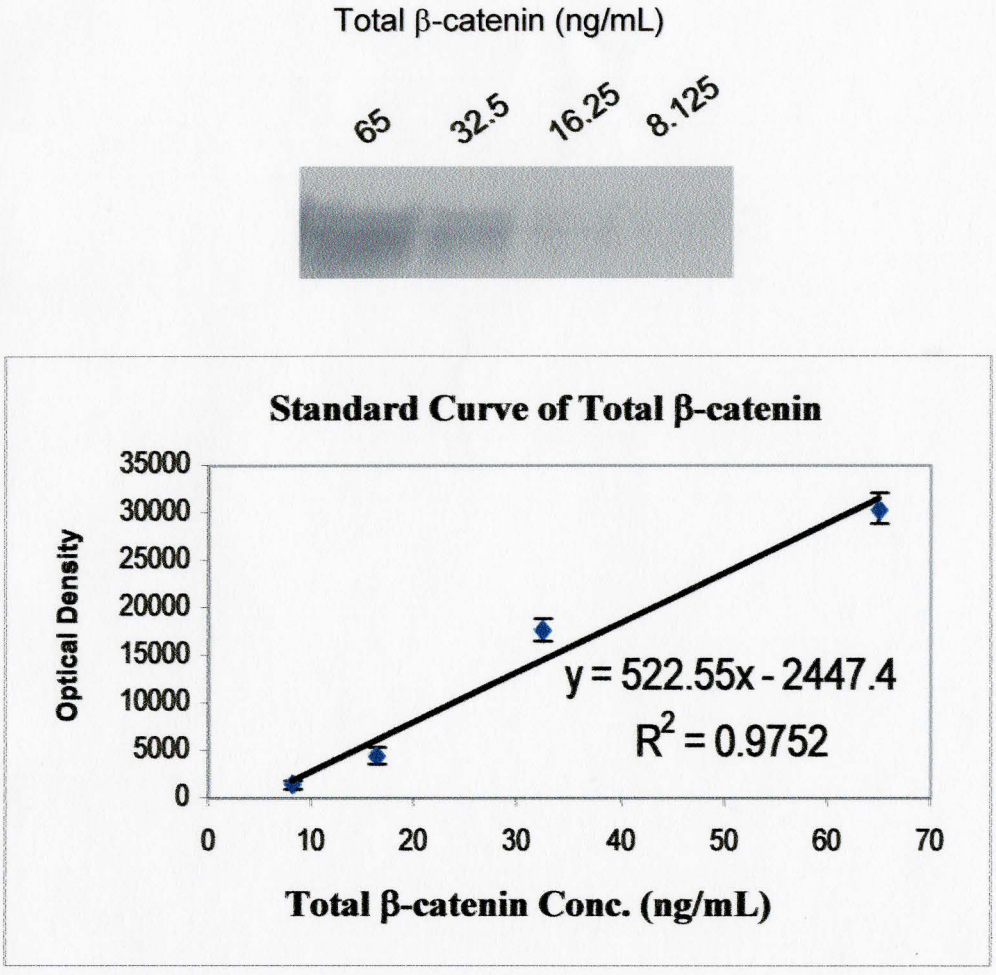
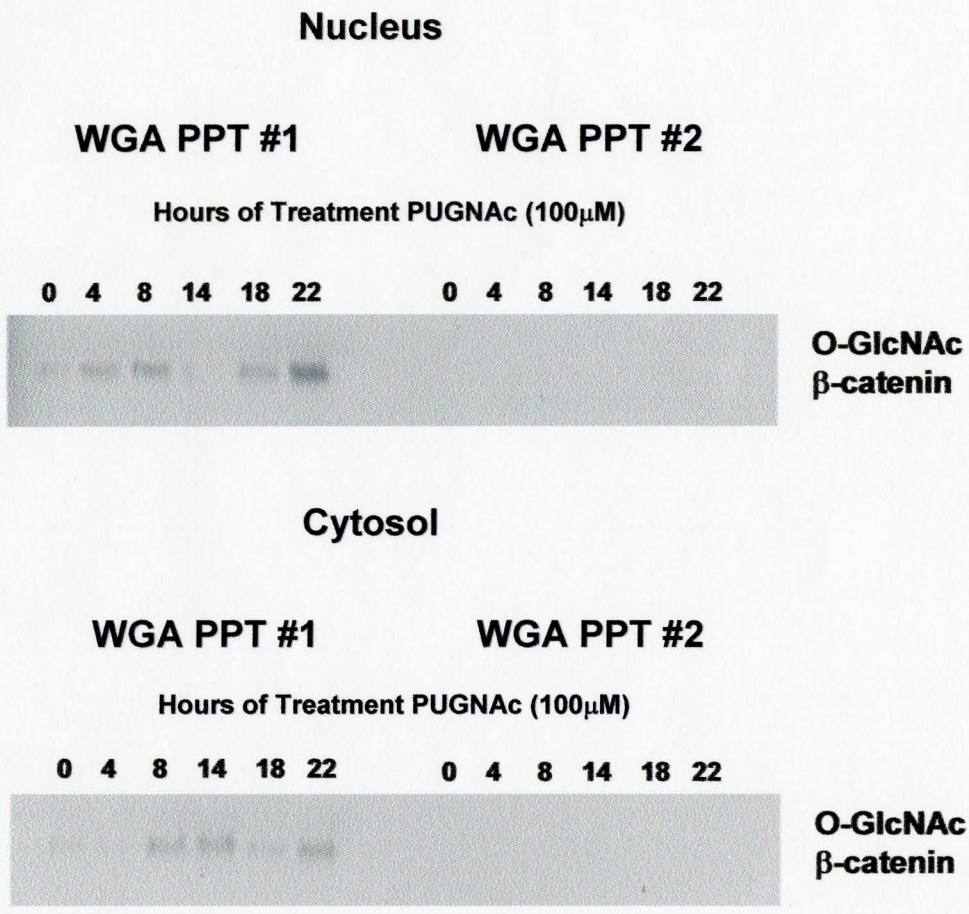


Appendix I: Standard curve of densitometric quantification of Total β-catenin protein in two-fold serial dilutions.

The graph was used to interpolate relative amounts of total and O-glycosylated β-catenin in the nucleus and cytosol.



Appendix II: WGA bound to all O-glycosylated β–catenin in first precipitation.

DU-145 cells were grown to 70% confluency in 10% (vol/vol) FBS, the media was then changed to 1% FBS for approximately 18 hours. Following, cells were treated with 100μM of PUGNAc, in the absence of serum, for the indicated time periods. The cells were lysed, nuclear and cytosolic fractions were collected. A significant amount of O-glycosylated β-catenin was precipitated out from the nuclear and cytosolic fraction. The remaining proteins were precipitated with WGA for a second time, and there was no O-glycosylated β-catenin detected.