DETERMINATION OF O-GLYCOSYLATION SITES OF β-CATENIN

DETERMINATION OF O-GLYCOSYLATION SITES OF β-CATENIN

BY

TIHANA GRUBAC
B.Sc. (McMASTER UNIVERSITY)

DETERMINATION OF O-GLYCOSYLATION SITES OF β-CATENIN

By

Tihana Grubac, B.Sc.

A Thesis
Submitted to the School of Graduate Studies
In Partial Fulfilment of the Requirements
For the Degree
Master of Science

McMaster University

MASTER OF SCIENCE (2008) McMaster University
(Biochemistry and Biomedical Sciences) Hamilton, Ontario

TITLE: Determination of O-glycosylation sites of β-catenin

AUTHOR: Tihana Grubac, B.Sc. (McMaster University)

SUPERVISOR: Dr. Sujata Persad

NUMBER OF PAGES: xi, 112

ABSTRACT

Cells respond to their environment through dynamic posttranslational modification of their existing proteins. There are more than 20 posttranslational modifications that occur on eukaryotic proteins. Several of these modifications, with phosphorylation being the hallmark, participate in signal transduction. Generally, glycosylation is not thought to participate directly in signaling. Complex N- and O-linked glycosylation occurs on membrane-bound or secreted proteins that are synthesized in the endoplasmic reticulum and Golgi apparatus. The lumenal or extracellular localization of these glycans restricts their potential for dynamic responsiveness to signals. In contrast, O-GlcNAc is a simple monosaccharide modification that is abundant on serine or threonine residues of nucleocytoplasmic proteins. An O-GlcNAc site consensus motif has not yet been identified. However, many attachment sites are identical to those used by serine/threonine) kinases, and a neural network program has been developed to predict O-GlcNAc sites. The dynamic glycosylation of serine or threonine residues on nuclear and cytosolic proteins by O-linked beta-N-acetylglucosamine (O-GlcNAc) is abundant in all multicellular eukaryotes. On several proteins, O-GlcNAc and O-phosphate alternatively occupy the same or adjacent sites, leading to the hypothesis that one function of this saccharide is to transiently block phosphorylation. Many proteins have been identified that carry this modification, including transcription factors, cytoskeletal proteins, nuclear pore proteins, oncogene products, and tumor suppressors. O-GlcNAc appears to modify a large number of nucleocytoplasmic proteins· One of important regulatory proteins on which this project concentrates is β-catenin.

Here, we examined where does this type of posttranslational modification takes place on the protein. Our results indicated that β-catenin is O-glycosylated on both the N-terminus and C-terminus, but not at the ARMADILLO segment. Further, we show that the known phosphorylation sites located at the N-terminal "destruction box" of this protein are not involved in O-glycosylation. Furthermore, we demonstrated that the threonines adjacent to phosphorylation-site Threonin41 are not essential in O-glycosylation process. In addition, treatment of prostate cancer lines with PUGNAc, a non-cytotoxic reversible inhibitor of O-GlcNAcase, caused a decrease in the expression of transfected β-catenin in the nucleus with increasing cellular O-glycosylation of the protein suggesting that O-glycosylation was hindering β-catenin's nuclear translocation. Additional studies showed that O-glycosylation of β-catenin decreased transcriptional activity of a TopFlash reporter plasmid.

In summary, our results show that β-catenin is O-glycosylated on the N- and C-terminus, but not on ARMADILLO segment, and that phosphorylation sites are not the critical for O-glycosylation. Furthermore, our data show 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.

Aknowledgements

I would like to express my gratitude to my supervisor, Dr. Sujata Persad, whose expertise, understanding, and patience, added considerably to my graduate experience.
I appreciate her vast knowledge and skill in many areas and her assistance in writing this thesis. I would like to thank the other members of my committee, Dr. Gurmit Singh, Dr. Geoff Werstuck and Dr. Yingfu Li for their guidance and expertise and the assistance they provided at all levels of the research project. I would like to thank my lab members, past and present, for their knowledge and experience.

I would also like to thank my family for the support they provided me through my entire life and in particular, I must acknowledge my future husband and best friend, Vojo Rusic, without whose love, encouragement and constant support, I would not be able to complete this journey.

In conclusion, I recognize that this research would not have been possible without the financial assistance of the Department of Biochemistry and Biomedical Sciences at the McMaster University and Juravinski Cancer Center, and express my gratitude to those agencies.

Table of Contents

List of Tables and Figures

Chapter 1

Introduction

1.0 Introduction

Signal transduction pathways are efficient systems that allow cells and other living organisms to amplify faint signals in order to create intense, vital responses. A key element of virtually any signaling pathway is the interaction and regulation of various intracellular intermediates within the pathway. Since its discovery as a protein associated with the cytoplasmic region of E-cadherin, β-catenin has been shown to perform two apparently unrelated functions: it has a crucial role in cell-cell adhesion in addition to a signaling role as a component of the Wnt pathway (Barker and Clevers, 2000). As a signaling molecule, β-catenin supplies an activating domain to the T-cell factor (TCF) and lymphoid enchancer (LEF) family of DNA-binding proteins, and activates gene transcription in the nucleus. Many of these target genes, including cyclin D1 and c-myc, play critical roles in cell growth and proliferation (Bhanot et al., 1996). Posttranslational stabilization of β-catenin, leading to elevated protein levels and constitutive gene activation, has been proposed as an important step in oncogenesis. Subsequently, anomalous β-catenin signalling has been associated with many different types of cancers.

1.1 The Wnts

Wnts are a large family of highly conserved secreted signaling glycoproteins that regulate cell-to-cell interactions during embryogenesis. Insights into the mechanisms of Wnt action have emerged from several systems: genetics in *Drosophila* and *Caenorhabditis elegans* and ectopic gene expression in *Xenopus* embryos (Cadigan and Nusse, 1997). During embryonic development, Wnts have diverse roles in governing

cell fate, proliferation, differentiation, migration, polarity, and death. In adults, Wnts function in homeostasis, and inappropriate activation of the Wnt pathway is implicated in a variety of cancers (Bhanot et al., 1996). The Wnt signaling cascade is initiated when secreted Wnts bind their cognate receptors, Frizzled (Fzd) and LRP5/6 (Low-density-lipoprotein, LDL-receptor-like protein 5 or 6), on the plasma membrane. Fzd are seven-fold transmembrane receptors having a cysteine-rich domain (CDR), which is essential for Wnt binding and signaling. Fzd are encoded by frizzled genes and share homology to G-protein coupled receptors (Pinson et al., 2000). At least three pathways exist for Wnt signaling: the canonical Wnt or dishevelled-dependent β-catenin pathway, which contributes to the establishment of the dorsal-ventral axis (Nusse and Willert, 1998), the planar cell polarity pathway, which is essential for cell polarization (Tada et al., 2000; Wallingford et al., 2000), and the dishevelled-independent protein kinase C-pathway (Fzd/PKC), which controls cell-sorting behavior in the mesoderm (Winklebauer et al., 2001).

1.1.1 The Wnt/β-catenin Canonical Pathway

This particular pathway is the most well known pathway of the three. In the dishevelled-dependent β-catenin pathway (Fig.1.0) the signaling cascade is initiated at the cell membrane by interaction between the Fzd receptor and the Wnt protein. The signal is then mediated inside the cell and further transduced to Dishevelled (DSH in Drosophila, Dvl in vertebrates) (Nusse et al., 1994), which becomes activated. This activation is followed by the inactivation of zeste-white 3/glycogen synthase kinase 3β (zw3/GSK3β) (Siegfried and Wilder, 1994) leading to the accumulation of β–catenin/Armadillo protein in the cytoplasm (van Leeuwen et al., 1994).

β-catenin/Arm enters the nucleus and modulates gene expression together with TCF/LEF proteins. The TCF/LEF-β-catenin complex comprises a bipartite positive acting transcription factor that targets transcription of numerous genes including the oncogenes c-myc and cyclin D1 (van de Wetering et al., 1997).

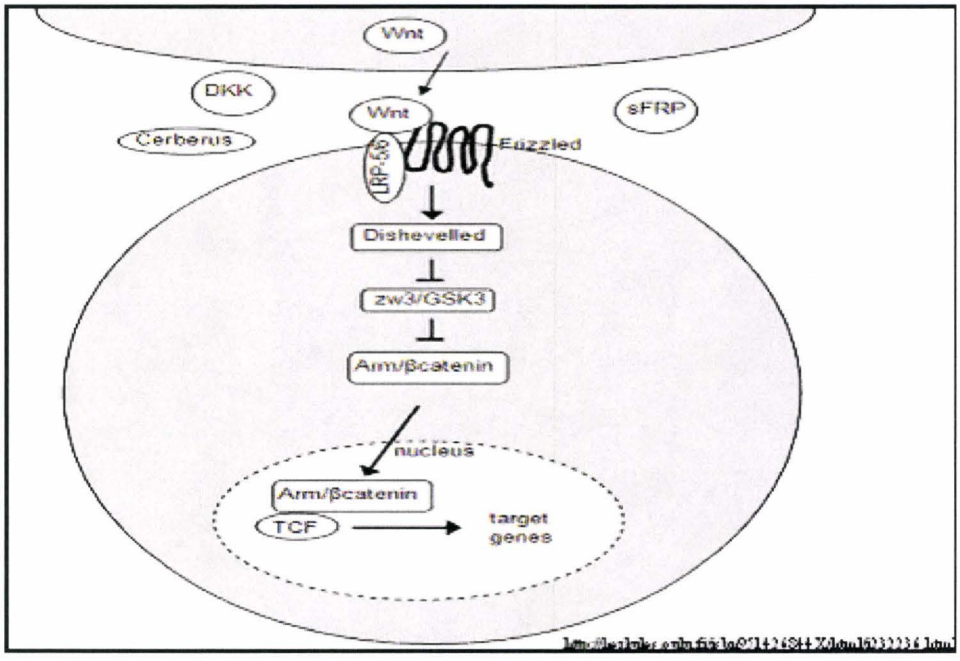


Figure 1.0 Simplified model of Wnt/β -catenin signaling pathway. Wnt ligand activates Frizzled/LRP-5/6 receptor complex, which is followed by signaling cascade leading to accumulation of β-catenin in the cytoplasm. β -catenin enters the nucleus, where it modulates gene expression together with TCF (Adapted from Brown at al; *Genome Biology* 2005 **6**:231).

1.1.2 The Planar Cell Polarity Pathway

The planar cell polarity (PCP) pathway is the generation of a population of cells with uniform orientation within a single epithelial plane. It is β-catenin independent pathway since it does not involve the components usually placed on Dsh, such as zw3/GSK3, Arm (β–catenin) or Tcf-3 (Wallingford et al., 2000). The Wnt binding to Fzd receptor stimulates generation of signal

to Dishevelled (Fig.2.0). The signal is than passed to the group of small GTPases such as RhoA and Rac, followed by the activation of the serine threonine kinase c-JUN N-terminal kinase (JNK) (Strutt et al., 1997; Boutros et al., 1998). The JNK cascade eventually transmits the signal to the nucleus where it is used in the regulation of important development patterns.

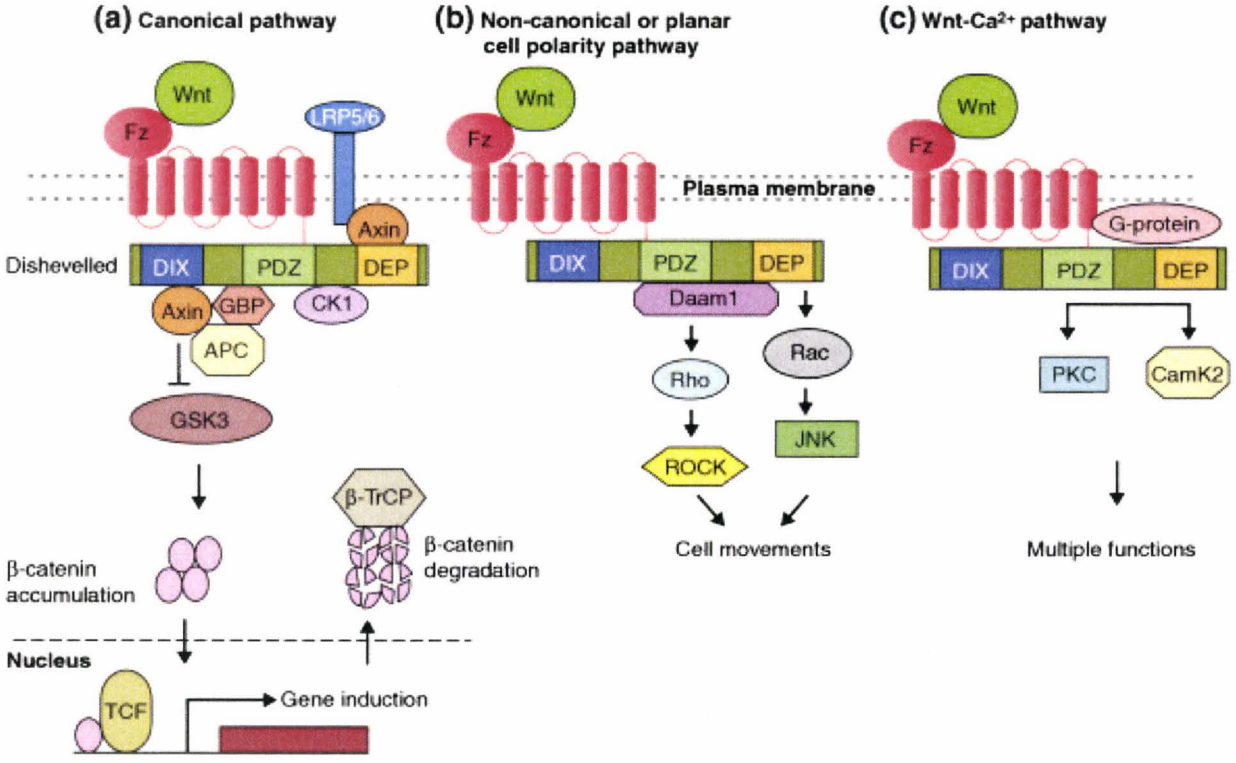


Figure 2.0: Wnt signaling Pathways – Canonical, Planar Cell Polarity, TheWnt/Ca $^{2+}$ Pathways (Adapted from Habas and Dawid *Journal of Biology* 2005 **4**:2).

1.1.3 The β-catenin-independent protein Kinase C/Ca²+ Pathway

TheWnt/Ca pathway differs from the other two in being independent of Dishevelled function. Fzds can discriminate between different Wnt ligands and this selectivity appears to be important for determining which downstream pathway is activated in the cell. In this pathway,

Wnt proteins function via cell surface receptors to stimulate an increase in intracellular Ca^{2+}release and the subsequent activation of protein kinase C (PKC) through the activation of heterotrimeric G proteins of the G_i class (Figure 2.0)(Winklebauer et al., 2001).

1.2.0 β-catenin

β-catenin contains 130 amino acid amino-terminal domain, 12 imperfect repeats of 42 amino acids (armadillo (ARM) repeats), and a carboxyl-terminal domain of 100 amino acids for the total of 781 amino acids (Figure 3.0) (Peifer et al., 1994; Huber et al., 1997). The N-terminus, "destruction box", is said to be important for regulating the stability of β-catenin, since it contains conserved serine and threonine residues that become phosphorylated by GSK3β subsequently targeting protein for degradation. The mutations of these sites are implicated in the development of many cancers (Rubinfeld et al., 1996). The C-terminus is most well known for functioning as a transcriptional activation domain. However, one of the most significant aspects of the structure of β-catenin is that its arm repeat binding domain allows for overlapping interaction between multiple binding partners, and also allows for nuclear localization of the protein (Funayama et al., 1995).

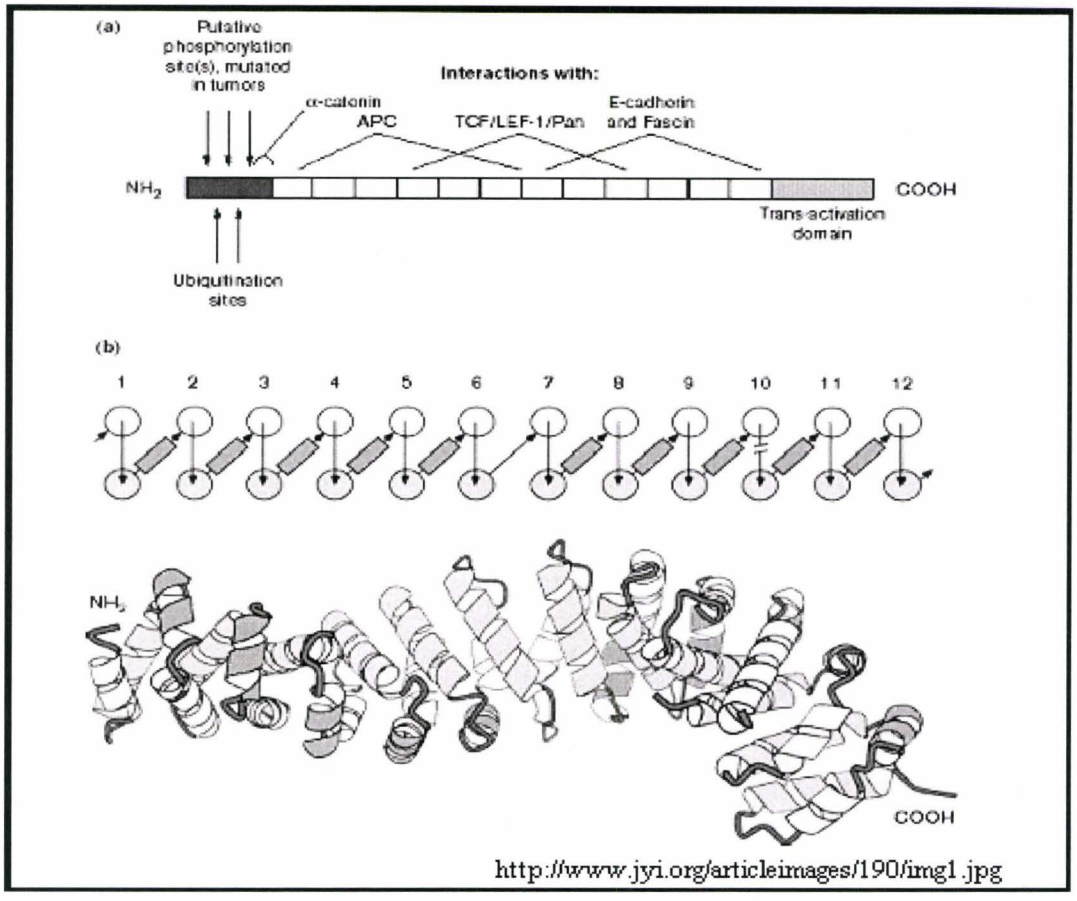


Figure 3.0. The structure of β-catenin. (a) The primary structure of β-catenin: central domain consisting of 12 imperfect repeats (ARM repeats) that interact with APC, TCF, E-cadherin, and fascin. (b) Topology (top) and ribbon structure (bottom) of the 12 imperfect repeats: circles and rectangles are alpha-helices viewed from the top and the side, accordingly; arrows run from amino-terminus to carboxyl-terminus (Adapted from Current Opinion in Genetics & Development).

One way of regulation of Wnt signaling pathway is to control for β-catenin levels through its phosphorylation. β-catenin is normally targeted for ubiquitin-dependent proteasomal degradation by phosphorylation from the degradation complex. In the cytoplasm, β-catenin interacts with the adenomatous polyposis coli (APC) and axin proteins; they recruit glycogen synthase kinase-3 (GSK-3) and casein kinase I to form a destruction complex that phosphorylates β-catenin at four conserved serine and threonine residues at the N terminus and targets it to the 26S proteasome (Figure 4.0) (Willert and Nusse, 1998; Rubinfeld et al., 1996; Rubinfeld et al., 1995).

Activation of Wnt signaling promotes stabilization of β-catenin by negatively regulating the activity of GSK-3 through binding of the Wnt ligand to a frizzled (Fz)/low-density lipoprotein receptor related protein (LRP) complex, activating the cytoplasmic protein Dishevelled (Dsh in Drosophila and Dvl in vertebrates) which inhibits GSK-3. In normal cells, the phosphorylation/dephosphorylation of β-catenin is tightly regulated.

However, in cancer cells abnormal Wnt signalling, mutations in the components of the degradation complex, or mutations in β-catenin itself, lead to an increase in β-catenin nuclear localization and transcriptional activity which has been shown to be an important event in the evolution of a number of malignancies, including colon cancer, melanoma, ovarian cancer, and prostate cancer .

4.0 A 4.0 B

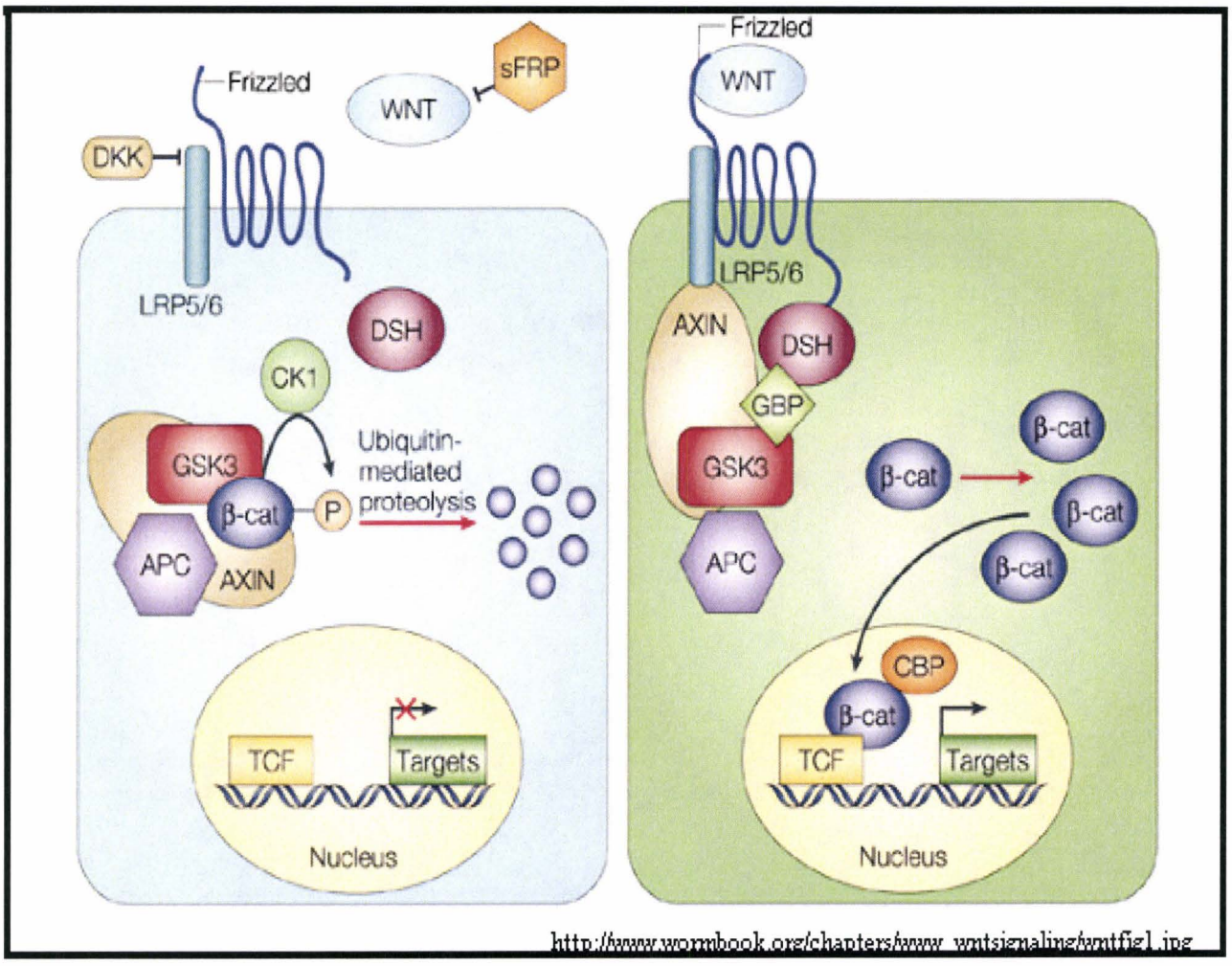


Figure 4.0 Wnt/ β-catenin canonical pathway.

4.0 A: Cytosolic levels of β-catenin are tightly regulated by a multi-protein degradation complex consisting of GSK3β, APC and Axin, which promote the proteosomal degradation of β -catenin (left).

4.0 B In the presence of Wnt (right), the degradation complex collapses allowing cytosolic β-catenin to accumulate and subsequently translocate into the nucleus where it then binds to TCF/LEF transcription factors and activates transcription of target genes. The components shown in the figure are more detailed described in the text. (Adapted from Moon et al. (2004) Nature Rev. 5: 689-99.)

1.2.1 Target genes of Wnt/β-catenin signaling pathway

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

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). 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). The oncogenic properties of the c-myc gene and its product have been implicated in a variety of cancers and elevated levels of the protein are found in breast and colon cancers (Little et al., 1983; Munzel et al., 1991; Erisman et al., 1989).

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 check point leads to DNA synthesis (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 tumor progression and metastasis (Bartkova et al., Callanan et al., 1996).

c-myc and cyclin D1 are overexpressed in colorectal carcinomas, however both genes are rarely amplified or contain genetic alteration in colon cancers (Erisman et al., 1989). It was this

unusual observation that prompted researchers to identify the cause of this overexpression and lead to identification of c-myc and cyclin D1 as target genes of β-catenin/TCF/LEF mediated transcription. Reintroduction of the 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 indentified 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 signaling and has a direct effect on cell proliferation (Ttesu and McCormick, 1999).

Matrix metalloproteinases (MMP) are a family of zinc-dependant proteases that are secreted by cells, and are responsible for much of the turnover of matrix components, such as basal membrane and extracellular matrix (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 tumors and metastasis. The expression of MMP-7 (matrilysin) has been shown to be important for the invasive and metastatic potential of cancer cells (Powel et al., 1993; Yamamoto et al., 1995). It is expressed in tumor cells of colorectal carcinomas where overexpression of MMP-7 is shown in approximately 80% of colon cancer cells (Newel 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 signaling (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 tumor development and progression. VEGFA expression is correlated to tumor grade and depth of invasion and is also used as an important prognostic factor in many human tumors (Lamszus et al., 2000; Toi et al., 2001; Hazelton et al., 2002; Dvorak et al., 2002). Studies done by Zhang et al identified VEGFA as target gene for 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).

1.2.2 Role of β-catenin in signal transduction and cancer

Both the deregulation of the Wnt/β–catenin pathway and increased levels of β-catenin in the nucleus have been implicated with the development of different types of cancers (Vogelstein et al.,2004; Cottrell et al., 1992; Powel et al., 1992). In normal, cells β-catenin functions to link E-cadherin with α-catenin at the adherens junctions (Reya et al., 2005). β-catenin also functions as a transcriptional factor acting as a co-activator of the TCF/LEF family of DNA-binding proteins. Binding of the TCF/β-catenin bipartite complex at TCF binding sites activates transcription of genes that control cell proliferation and invasiveness, including cyclin D1 and c-myc (Moon et al., 2004). Excessive transcription of these genes mediated by excess cellular expression of β-catenin is associated with the oncogenic properties of cancer cells (Hazelton et al., 2002). Free intracellular levels of β-catenin are strictly controlled by the ubiquitin-proteasome system activation of which requires the target phosphorylation of highly conserved serine-33, 37, 34 and threonine-41 residues at the N-terminal region of the protein by GSK-3β

(Aberle et al., 1997). Phosphorylation is the post-translational modification of β-catenin which is established in the regulation of its transcriptional activity. Familial adenomatous polyposis (FAP) occurs due to a germline mutation in the tumor suppressor gene APC and results in an intestinal disorder involved with the development of colorectal polyps and increased risk of colon cancer (Henderson et al., 2000; Kinzler and Vogelstein, 1996; Miyoshi et al., 1992). Inactivating mutation 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 cancers cell lines. HCT116 is a cell line with a deletion at serine 45 and SW48 with a substitution at serine 33 (Morin et al., 1999). Interestingly, APC mutation and oncogenic β-catenin mutations are mutually exclusive in colon cancer. Moreover, tumors with mutated APC contained elevated levels of wild-type β-catenin (Sparks et al., 1998).

Also, numerous melanoma cell lines posses this exclusivity between APC and β-catenin mutations (Robbins et al., 1996 Rubinfeld et al., 1997). Melanomas with abnormally high levels of β-catenin are usually associated with mutations in CTNNb1, whereas as APC is altered in two melanoma cell lines with elevated β-catenin expression (Rubinfeld et al., 1997). The common skin cancer known as pilomatricomas has the highest frequency of β-catenin mutations with 75% of tumors containing mutations in the N-terminus (Chan et al., 1999).

Biallelic inactivation of Axin, originally identified as inhibitor of Wnt signaling and a tumor suppressor, have been identified in human hepatocellular carcinomas (HCC) (Polakis et al.,

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 result in truncated version 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 been identified in other cancers such as ovarian cancer and medulloblastoma, and to a lesser extent in prostate and uterine cancer (Zurawl et al., 1998; Polakis et al., 1999; Voeller et al., 1998; Itoh et al., 1993).

1.2.3 O-glycosylation in the regulation of proteins

The reversible regulatory post-translational modification, O-linked β-N-acetylglucosamine (O-GlcNAc) involves a single O-linked attachment of monosaccharide N-acetyl-glucosamine to serine/threonine residues of proteins found exclusively in the nucleus and cytosoplasm (Fig5.0) (Haltiwagner 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 (Kanemura nad Hart et al., 2003), catalyze this dynamic modification.

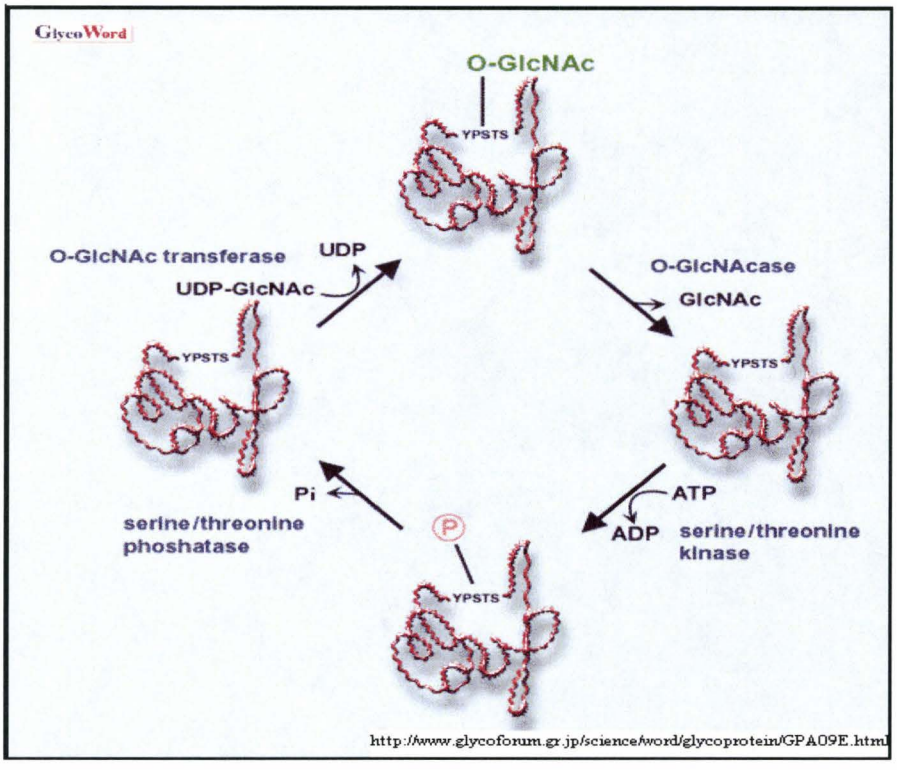


Figure 5.0. Reciprocal relationship between phosphorylation and O-GlcNAc modifications of hydroxyl group of serine or threonine residue. O-GlcNAc: O-linked N-acetylglucosamine. O-GlcNAcase: O-GlcNAc specific N-acetylglucosaminidase (Adapted from http://www.glycoforum.gr.jp/).

O-GlcNAc modifications are more analogous to phosphorylation than to other types of glycosylation since they 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). Some studies also suggested that a reciprocal relationship exists between phosphorylation and O-GlcNAc modification (Figure 6.0) (31).

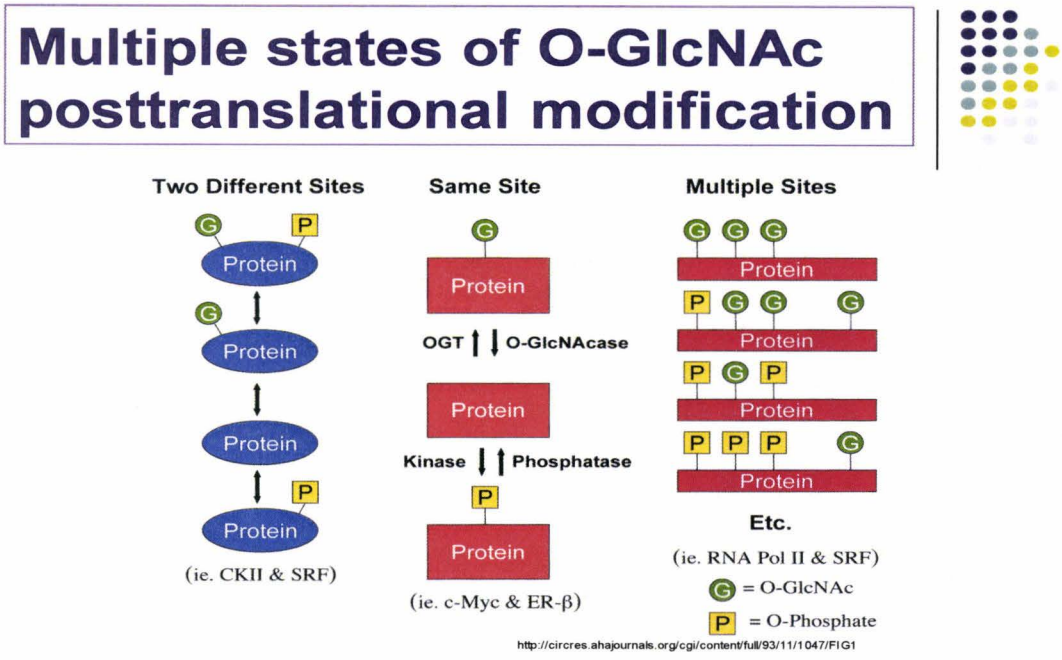


Figure 6.0 Multiple states of O-GlcNAc posttranslational modification. O-GlcNAc modification occurs on serine and threonine residues. O-GlcNAc modification may occur alone, adjacent to a phosphorylation site, at the same site as a phosphorylation site, or at multiple sites in any number of combinations. O-GlcNAc and phosphorylation in some cases have a reciprocal relationship where they compete for the same site or adjacent sites. Specific examples of adjacent-site O-GlcNAc modification are casein kinase II (CKII) and SRF; same site, c-Myc and estrogen receptor (ER-ß); and multiple sites, RNA Pol II and SRF. (Adapted from Stephen A. Whelan and Gerald W. Hart; Circulation Research. 2003; 93:1047-1058).

O-glycosylation has been shown to regulate the function of many proteins. RNA Polymerase II O-glycosylation at the C-terminus is involved in the formation of pre-initiation complex (Kelly et al., 1993). O-GlcNAc modification of the ubiquitous transcription factor Sp1, which plays a vital role in control of TATA-less housekeeping gene transcription, modulates its transactivation capabilities by affecting its ability to interact with basal transcription machinery (Ross 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 (Majumdra et al., 2004; Majumdra et al., 2004).

Also, O-glycosylation of the c-myc protooncogenic product promotes the stability and the subcellular localization of the protein (Kamemura eet al., 2002).

Furthermore, O-GlcNAc modification may be an alternative nuclear transport signal on some proteins (30-32). Thus O-glycosylation provides an additional level of regulation to signal transduction pathways that could allow for exquisite control of cell regulatory mechanisms beyond that provided by kinases and phosphatases.

1.3 O-glycosylation and β-catenin

Recently there has been compelling evidence that β-catenin is being post-translationally modified by O-glycosylation (Zhu et al., 2001). O-glycosylation, however, 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. The previous studies in our lab analyzed the status of O-GlycNAc modified β-catenin in several prostate cell lines (CaP) and found out that while a significant amount of the cellular β-catenin in the normal prostate cell line is in the O-glycosylated form, the prostate cancer cells, exhibit little to no O-glycosylated-β-catenin. Furthermore, the expression of O-glycosylated β-catenin can be induced in CaP cells upon treatment with the drug, O- (2-acetamido-2-deoxy-D-gluco-pyranosylidene) amino-N-phenylcarbamate (PUGNAc). PUGNAc has been shown to induce O-GlycNAc modification in a variety of proteins (Comert and Hart, 2000). O-GlcNacase is inhibited by this drug, resulting in an increase in the levels of O-glycosylated β-catenin. This indicates that O-glycosylation may negatively regulate the signal transduction properties of β-catenin.

1.4 Hypothesis and Objectives

As detailed above, β-catenin engages in multiple mutually exclusive protein-protein interactions. However, little is understood about factors that regulate the selective assembly of these protein complexes and proteins translocation into nucleus.

In seeking to explore these issues, we investigated possible sites for *O*-GlcNAcylation on the N and C terminus as well as ARMADILLO of β-catenin. This modification has the potential to add an additional level of control to the protein stability, junctional and cytoskeletal assembly, and signaling and transcriptional activity of β-catenin which we tried to investigate in this project.

Objective 1. Subcloning β-catenin into pEGFP-C2

Human full length, wild type β-catenin gene will be PCR amplified from the pcDNA3.1Zeo vector using specific set of primer designed by SECentral computer program. The β-catenin gene will be then ligated to the doubly digested humanized green fluorescent protein expression vector, pEGFP-C2. The GFP part of the fusion protein will function as a fluorescent tag that can be detected both in living and in formaldehyde fixed cells.

Objective 2. Generation of β-catenin constructs

Different β-catenin constructs will be made containing GFP fused to various portions of this protein. The same cloning techniques will be employed as in the previous objective, but different set of primers will be used. An attempt will be made to make ARMADILLO, as well as N- and C- GFP constructs containing either amino or carboxyl part of the β-catenin. Furthermore, mutants of β-catenin phosphorylation sites will be introduced into pEGFP-C2 vector in order to evaluate their importance on O-glycosylation activity of this protein. When the specific part of the protein is narrowed down, the importance of its O-glycosylation in β-catenin activity will be determined.

Objective 3. The optimization and evaluation of transcriptional efficiency of GFP-β-catenin construct

Upon creation of GFP-β-catenin construct, transient transfectional efficiency will be optimized. Lipofectamine, FuGENE 6 and ExGen transfection reagents mixed with varying DNA concentrations will be supplemented to the prostate cancer cells, DU145, in order to find the most suitable transfection method.

The phosphorylation status of GFP-β-catenin will be determined since the N-terminal fusion will be used to connect GFP and β-catenin.

Also, the O-glycosylation ability of the construct will be verified by precipitation of the protein with the lectin wheat germ-agglutinin (WGA). WGA has been used for enriching and detecting O-GlycNAc modifications. This experiment will be repeated and the transfected cells will be exposed to PUGNAc for variable time points.

Objective 4. O-glycosylation effect on regulation of nuclear translocation of β-catenin

β-catenin does not contain nuclear localization sequence (NLS) and very little is known about how nuclear trafficking of β-catenin is regulated. Our previous studies indicate that the expression of O-glycosylated β-catenin is inversely related to the anticipated transcriptional status of the prostate cancer cells, and it is possible that O-glycosylation may function to prevent nuclear localization of the protein.

DU145 cells will be transfected with the GFP-β-catenin vector or empty vector and will be subjected to PUGNAc treatment. The WGA precipitation of O-glycosylated proteins will be preformed and the levels of O-glycosylated β-catenin will be determined in both.

Chapter 2

Material and Methods

2.1 Constructs

Full length, wild-type β-catenin gene was isolated from pcDNA3.1/Zeo vector (Gift from Dr. using SECentral generated primers with incorporated KpnI (N-terminus) and BamHI (C-terminus) restriction sites (SigmaGenosys). SECentral computer program located suitable primers that would satisfy necessary conditions such as, length, GC content, melting/annealing temperature, complementarity, etc. KpnI/BamHI digested PCR product was ligated with T4 Ligase(New England Biolabs) to pEGFP-C2 vector. Max Efficiency DH5α E.Coli (Invitrogen) were used to grow the DNA on Kanamycin containing Luria-Bertani (LB) Media.

QIAGEN Maxi Prep Kit was used for the subsequent DNA isolation. The same procedure was followed when isolating specific β-catenin fragments (N-terminus, C-terminus, ARMADILLO, 1st and 2nd half of ARMADILLO) using appropriate primers (SigmaGenosys).

Also, the same set of steps was followed for the isolation of full length phosphorylation site mutants (N-terminus) of β-catenin from pBABE-hygro-ΔSer33,37,45ΔThr41 and pBABE-hygroΔThr41 vectors(obtained from Dr. Andrews lab). The mutants were successfully ligated to pEGFP-C2 and checked by DNA sequencing (Mobix).

Finally, Site-directed mutagenesis technique was employed to make additional N-terminus mutants of β-catenin (Stratagene). Threonine 40, Threonine 42, and both Threonine40/42 mutations (coding for Alanine) were introduced into a full length wild type β-catenin-pEGFP-C2.

2.2 Isolation of N-terminus mutants from a full-length β-catenin

SECentral was employed to find appropriate restriction sites that would allow for extraction of N-terminus mutants (ΔSer33,37,45ΔThr41, ΔThr40, ΔThr41, ΔThr42, ΔThr40/42) from full length mutated β-catenin-pEGFP-C2 vector. Restriction enzymes, SpeI and XbaI(New England Biolabs) isolated mutated portion of N-terminus together with some Armadillo segments. The new constructs were isolated with Gel extraction kit (Qiagen) and re-ligated using T4 Ligase(New England Biolabs). The transformed Max Efficiency DH5α were plated on 30μg Kanamycin LB plates and isolated using DNA Extraction Kit (Qiagen).

2.3 PCR (Polymerase Chain Reaction)

PCR was used to amplify DNA of interest containing the restriction sites needed for successful introduction into pEGFP-C2 vector. To perform several parallel reactions, master mix was prepared containing double distilled water, 10x PCR buffer, 10 Mm dNTP mix, 10 μM of forward and reverse primers and 50mM of MgCl2. Platinum *Taq* DNA Polymerase High Fidelity (1.25u/50μl, Invitrogen) and template DNA (1μg) were mixed with master mix into individual tubes. The samples were amplified for 25 cycles at different annealing temperatures to find the most optimal for generation of high quality and quantity DNA samples. The Large Scale PCR reaction was then repeated using appropriate conditions.

2.4 Restriction Endonuclease digestion

In order to avoid STAR activity of BamHI and KpnI enzymes used to digest template DNA and the acceptor vector, the pcDNA3.1-β-catenin and pEGFP-C2 were cut using BamHI (10 Units/20μL) and KpnI (10 Units/20μL) in BamHI Specific Buffer (New England BioLabs) for 4 hours (37°C).

2.5 Preparation of Buffers

Buffer compositions are given per liter of solution. Do not autoclave MOPS- or isopropanol containing buffers; sterilize by filtration instead. Buffer calculations are based on Tris base adjusted to pH with HCl (Tris·Cl). If using Tris·HCl reagent, the quantities used should be recalculated.

P1: Dissolve 6.06 g Tris base, 3.72 g Na2EDTA·2H2O in 800 ml distilled water. Adjust the pH to 8.0 with HCl. Adjust the volume to 1 liter with distilled water. Add 100 mg RNase A per liter of P1.

P2: Dissolve 8.0 g NaOH pellets in 950 ml distilled water, 50 ml 20% SDS (w/v) solution. The final volume should be 1 liter.

P3: Dissolve 294.5 g potassium acetate in 500 ml distilled water. Adjust the pH to 5.5 with glacial acetic acid (~110 ml). Adjust the volume to 1 liter with distilled water.

QBT: Dissolve 43.83 g NaCl, 10.46 g MOPS (free acid) in 800 ml distilled water. Adjust the pH to 7.0 with NaOH. Add 150 ml pure isopropanol and 15 ml 10%

QC: Dissolve 58.44 g NaCl and 10.46 g MOPS (free acid) in 800 ml distilled water. Adjust the pH to 7.0 with NaOH. Add 150 ml pure isopropanol. Adjust the volume to 1 liter with distilled water.

QF: Dissolve 73.05 g NaCl and 6.06 g Tris base in 800 ml distilled water and adjust the pH to 8.5 with HCl. Add 150 ml pure isopropanol. Adjust the volume to 1 liter with distilled water.

QN: Dissolve 93.50 g NaCl and 10.46 g MOPS (free acid) in 800 ml distilled water and adjust the pH to 7.0 with NaOH. Add 150 ml pure isopropanol. Adjust the volume to 1 liter with distilled water.

STE: Dissolve 5.84 g NaCl, 1.21 g Tris base, and 0.37 g Na2EDTA·2H2O in 800 ml distilled water. Adjust the pH to 8.0 with HCl. Adjust the volume to 1 liter with distilled water.

2.5 PCR Purification and Gel Extraction

DNA sample was isolated following protocol using QIAquick Purification Kit. . In order to purify PCR sample from primers, nucleotides, polymerases, and salt, 500 mL of Buffer PB was added and the sample was spun in a QIAquick spin column. 750μL of Buffer PE was used for washing the sample and 30 μL of Buffer EB for eluting clean DNA from the column. Gel extraction was done in a similar matter where DNA sample was extracted by dissolving the 1% agarose (100 mL TAE, 1g agarose) in Buffer QG and incubating at 50°C for 10 minutes. Isopropanol was added to the mixture and spun in the QIAquick column. The sample was washed and eluted with the same buffers used in PCR purification protocol.

2.6 T4 Ligation Experiment and DH5α E.Coli Transformation

The Ligation mixture containing 3:1 and 9:1 ratio of insert to vector were mixed with T4 Buffer and T4 Ligase (New England Biolabs) and left overnight at 16°C. The ligation mixture was then transformed into Max Efficiency DH5α (Invitrogen). 5μL of ligation reaction was mixed with 50μL of DH5α and incubated on ice for 30 minutes. The mixture was then heat-shocked for 45 seconds at 42°C and mixed with 500 mL of S.O.C (Super Optimal Broth) Medium and shaken at 225 rpm (37°C) for 1 hour. The entire reaction was spread onto Kanamycin (30μg/mL) LB plates and incubated overnight at 37°C. The colonies were picked up and 5 ml E.Coli culture was grown overnight in LB/Kan medium (10g tryptone, 5g yeast extract, 10g NaCl/per liter)

2.7 Plasmid DNA Purification Using QIAGEN Plasmid Mini and Maxi Kits

The E.Coli cells were harvested from the culture by centrifugation at 6000xg (15 minutes, 4°C) and resuspended in 10 mL of Buffer P1(50 mM Tris·Cl, pH 8.0; 10 mM EDTA; 100 μg/ml RNase A). 10mL of Buffer P2 (lysis buffer, 200 mM NaOH, 1% SDS (w/v)) was added followed by Buffer P3 (neutralization buffer; 3.0 M potassium acetate, pH 5.5) and incubated on ice for 20 minutes. The mixture was centrifuged at 20000xg (30 minutes, 4°C) and the supernatant was re-centrifuged again for 15 minutes. The QIAGEN-tip 500 was equilibrated with 10 mL Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0;15% isopropanol (v/v);0.15% Triton® X-100 (v/v)) and the supernatant was added to the column and washed two times with 30 mL of Buffer QC

(1.0 M NaCl; 50 mM MOPS, pH 7.0;15% isopropanol (v/v)). The DNA was eluted with 15 ml of Buffer QF (1.25 M NaCl; 50 mM Tris·Cl, pH 8.5;15% isopropanol (v/v)).

2.8 Site-directed Mutagenesis

The PCR reaction was divided into two sets containing each 25 ng of DNA template, 25 mM dNTP mix, 10x *Pfu* DNA Polymerase reaction buffer, dH2O, Pfu Turbo DNA Polymerase (2.5 units/50 μL) and 125 ng of sense and antisense primers separately into each mixture. The mutant plasmid was generated and amplified following parameters that have been optimized for use in Stratagene's QuickChange site-directed mutagenesis. The parental DNA was digested with DpnI (200 Units/μL) overnight and the mutant DNA was transformed into High Efficiency DH5α and plated on LB/Kan plates.

2.9 Cell Culture

Human prostate cancer cell line, DU145, was 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). All cells were incubated in 5% CO2 at 37°C.

2.10 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 16 hours before PUGNAc treatment.

2.11 Preparation of Cell Lysates

Whole cell lysates were prepared by incubating with lysis buffer [10 mM Tris-HCl pH 7.5, 1mM EDTA, 1mM EGTA, 0.5% Triton X-100, 2mM phenylmethylsulphonyl fluoride (PMSF), 80 ng/ml aprotinin, 40 ng/ml chymostatin, 40 ng/ml antipain, 40 ng/ml leupeptin, 40 ng/ml pepstatin] for 15 minutes on ice. Cellular debris and nuclei were removed by centrifugation at 16 000rpm 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 570 nM. The results were compared with bovine serum albumin (BSA) at known concentrations and the protein level (mg/ml) was extrapolated. The lysates were snap frozen in liquid nitrogen and stores at -80°C for the future use.

2.12 Preparation of Nuclear and Cytosolic Extracts

Nuclear and cytosolic extracts were separated using NE-PER kit as described by manufacturers (Pierce). 10^6 cells (approximately 1x100mm plate of cells) were counted and incubated in 100µL of cytoplasmic extraction reagent I (CERI) and 10µL of protease inhibitor cocktail (PIC; Roche) for 10 minutes on ice, then 5.5µL of cytoplasmic extraction reagent II (CERII) was added, the lysate was vortexed for 5 seconds and incubated on ice for 1 minute. The cell lysates were then 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 nuclear extraction reagent (NER) and 5µL of PIC (per 10^6 cells). The nuclei lysates was passed through a 20 gauge needle and vortexed in order to break open the nuclei and collect the inner contents. The samles 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 and the supernatant containing nuclear fraction was collected and stored art -80°C. Protein concentrations for cytosolic and nuclear fractions were determined using Bradford Protein Assay (Bio-Rad).

2.13 PUGNAc Treatment and WGA precipitation

DU145 cells were incubated in media containing 1% fetal bovine serum for 16 hours before treatment with PUGNAc. Thereafter, cells were treated with PUGNAc (100µM) in the absence of serum for different time intervals (0, 4, 8, and 12 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 was mixed with 20μL of WGA conjugated to Sepharose beads (Sigma), and topped off to 500μL with lysis buffer. The complexes were mixed by rotation for at least 3 hours at 4°C after which the samples were pelleted by centrifugation at 16 000rpm for 10 minutes. The centrifugation process was repeated 3-4 times with lysis buffer washes in between each cycle. The pellet was resuspended in 20μL of 5X sample buffer and the samples were boiled for 5 minutes at 100°C. The samples were separated on 10% SDS-PAGE gels which were then used for immunoblotting (Western blot) with various antibodies.

2.14 Western Blot

Protein concentrations were determined using Bratford assay (Bio-Rad) and the protein equivalence were resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Roche). The gels were transferred to the membrane at 85 volts for 1 hour in transfer buffer (0.25 M Tris, 2 M Glycine, 20% methanol), followed by blocking in 5% non-fat dry milk prepared in Tris Buffered Saline Tween (TBST: 1 mM Tris Base, 15 Mm NaCl, 0.1% Tween, pH 8.0; Sigma) overnight at 4°C. Membranes were probed for 2 hours at room temperature or overnight at 4°C with the primary antibody of interest. The following antibodies were used in this study; anti-β-catenin antibody (1:1000; mouse monoclonal; Transduction Laboratories), anti-β-actin (1:10000; mouse monoclonal; Sigma), anti-Lamin B (1:1000; mouse monoclonal; Calbiochem), anti-amino-β-catenin (1:1000; rabbit polyclonal; Cell Signaling), anti-carboxy-β-catenin(1:1000; rabbit polyclonal, Cell Signaling), anti-active-β-catenin (1:500; mouse monoclonal;Upstate), anti-armadillo, anti-GFP (1:500; mouse monoclonal; Cell Signaling), anti-Phospho-β-catenin (Ser33/37/Thr41) (1:500; polyclonal rabbit; Cell Signaling)

Membranes that were transferred from SDS-acrylamide gels were incubated in primary antibody diluted in 5% non-fat dry milk dissolved in TBST. The membranes were washed in TBST before incubation in the mixture of secondary antibody and 5% milk-TBST. After 1 hour at room temperature incubation in secondary antibody the 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.

2.15 Immunoprecipitation

DU145 cells were transfected with ARMADILLO, 1stHALF and 2nd HALF constructs using ExGen Transfection reagent. After 48-72 hours the cells were lysed and Bradford assay was employed to determine protein concentration. 100μg of the total protein was mixed with GFP primary antibody (1:250,) overnight at 4°C.

2.16 Transfection of Mammalian cells with cDNA

Transfection of DU145 cells with DNA constructs and TOPFlash Reporter was carried out using ExGen Transfection Reagent (Fermentas). The optimal ratio of ExGen reagent:DNA was determined by transfecting cells with DNA constructs expressing GFP (green fluorescence protein) and monitoring the expression signal through the fluorescence microscope. It was determined that the optimum Transfection is obtain when using 3μg of DNA and 9.87μL of ExGen Reagent.

DU145 cells were plated in 60 mm plates and transfected once they reached 60-70% confluency. After 48-72 hours cells were either lysed for Western blot or the media was changed to 1% in order to start the PUGNAc experiment. The effect of increasing O-glycosylation of β-catenin upon 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. The levels of luminescence were detected by luminometer (PE Applied Biosystems, Tropix TR717).

Chapter 3

Results

3.0 RESULTS

3.1 Subcloning β-catenin into pEGFP-C2 vector

Appropriate primers complementary to the start and the end of the protein containing restriction sites of interest (BamHI and KpnI) (Figure 3.1) were mixed with pcDNA3.1/Zeo-β-catenin vector in a small scale PCR (Polymerase Chain Reaction) amplification which was done at different annealing temperatures in order to find optimal amplification conditions for large scale PCR. All PCR reactions at each temperature set were successful (55°C, 56.2°C, 57.4°C, 58°C), as the bands appeared at correct β-catenin size(~ 2400 base pairs(bp))(Figure 3.2). The large scale PCR product was extracted from agarose gel using QIAquick Gel Extraction Kit and both PCR product and pEGFP-C2 were digested with KpnI and BamHI restriction enzymes which would cut specifically designed restriction sites on β-catenin DNA and multiple cloning sites of pEGFP-C2 vector.

3.1A

PRIMER DESIGN

FORWARD PRIMER

5' AATGGTGGGTACCTC-ATGGCTACTCAAGCTGATT 3' Length: 33 nucleotides

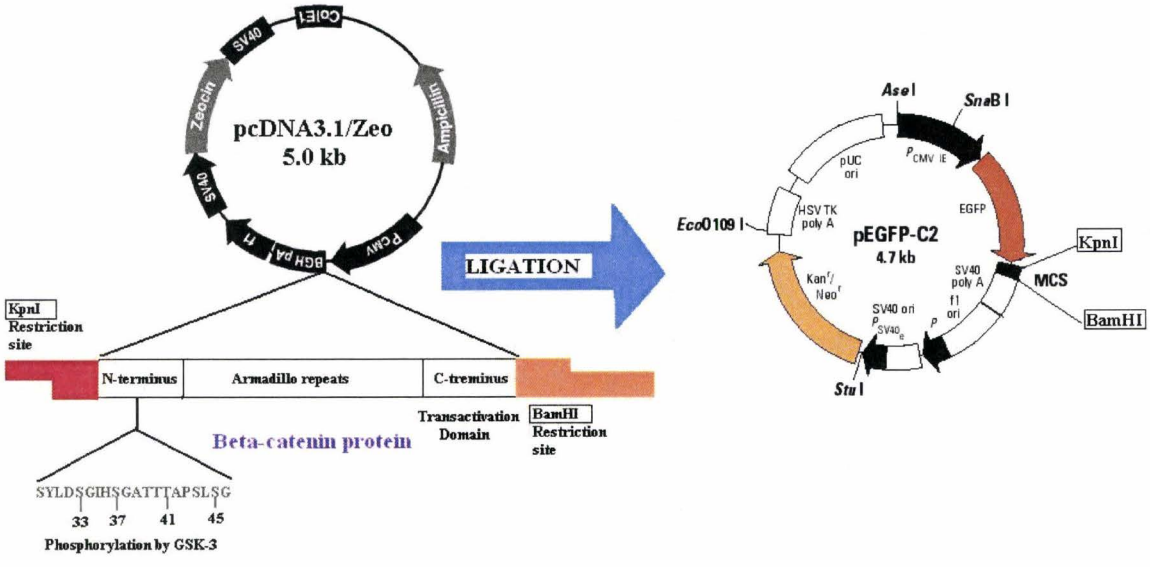
KpnI

REVERSE PRIMER

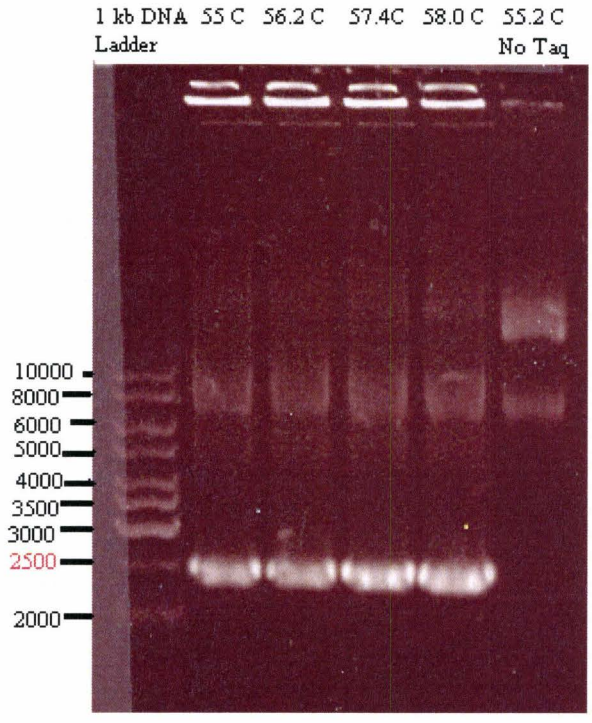
5' CGCGGATCCTTA-CAGGTCAGTATCAAACCAG 3' Length: 29 nucleotides

BamHI

3.1B



3.1C


1 kb DNA Ladder
55 C
56.2 C
57.4C
58.0 C
55.2 C No Taq
10000
8000
6000
5000
4000
3500
3000
2500
2000

3.1D

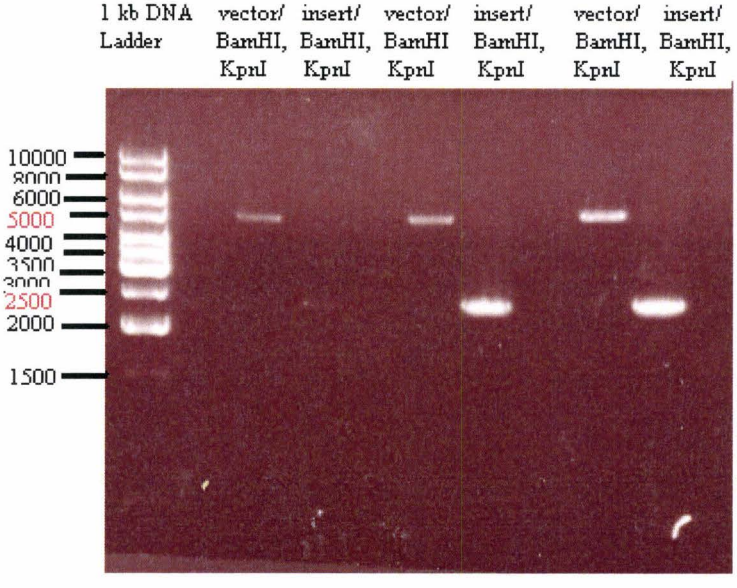

1 kb DNA Ladder
vector/ BamHI, KpnI
insert/ BamHI, KpnI
vector/ BamHI KpnI
insert/ BamHI, KpnI
vector/ BamHI, KpnI
insert/ BamHI, KpnI
10000
8000
6000
5000
4000
3500
3000
2500
2000
1500

Figure 3.1. Ligation of β-catenin from pcDNA3.1/Zeo vector into a pEGFP-C2 fluorescence vector.

Figure 3.1A SECentral was used to generate primers containing specific BamHI and KpnI restriction sites in the β-catenins N- and C-terminus, respectively. The forward primer is complementary to the beginning of the β-catenin protein and contains the ATG start codon, and the reverse primer is complementary to the very end having the stop codon followed by the BamHI restriction site.

Figure 3.1B Outline of Subcloning process that involves transfer of β-catenin gene from pcDNA3.1/Zeo vector into a green fluorescence vector via specifically designed primers

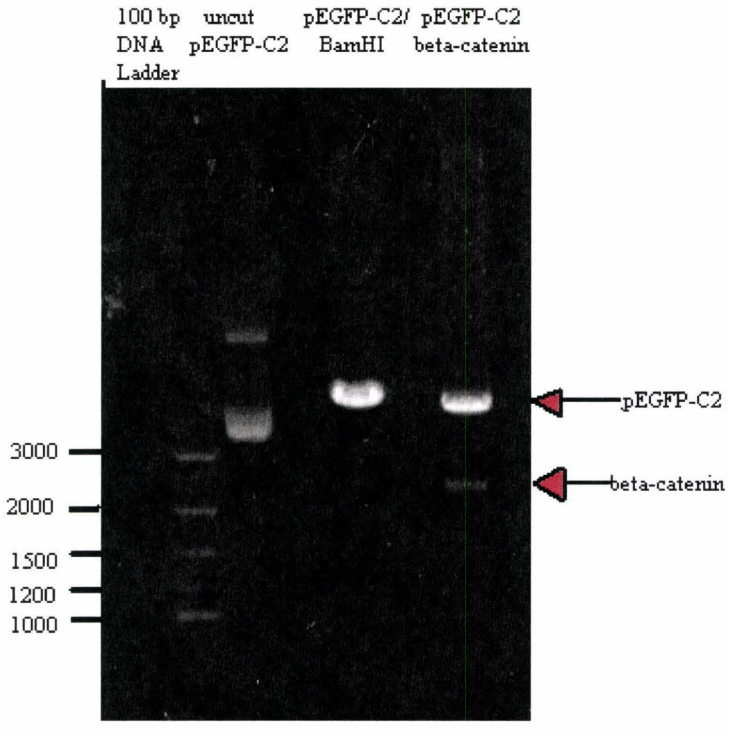
Figure 3.1C 1% Agarose Gel of Small Scale PCR Amplification of β-catenin gene at different annealing temperatures. At every set temperature, band corresponding to β-catenin (~2500 bp) is visible, except at lane containing negative control (no Taq polymerase).

Figure3.1D pEGFP-C2 vector (~5000bp) and PCR product (~2500) digested with KpnI and BamHI restriction enzymes and run on 1% agarose gel

3.2 Ligation and Isolation of pEGFP-C2-β-catenin plasmid

Following digestion and gel extraction of vector and insert the ligation reaction was done as described in the materials and methods section. The ligation reaction was transformed and plated on kanamycin resistant media and DH5α E.Coli colonies were picked up and subjected to mini-prep (QIAGEN) to verify a successful insertion of β-catenin into the GFP vector. Digestion reaction was done using endonucleases (BamHI and KpnI) utilized for cloning β-catenin into pEGFP-C2 vector. Qiagen Plasmid Maxi Kit was used to isolate DNA of interest and, DNA sequencing, as well as double digestion method were employed to confirm the nature and orientation of the cloned product. The band corresponding to the insert-β-catenin appeared at approximately 4500 base pairs preceded by an acceptor vector-pEGFP-C2 that is about 5000 base pairs long (Figure 3.2 A). After the length of an insert was verified the entire DNA of interest was sequenced in order to check for mutation introduced by DNA Polymerase during PCR reaction (Figure 3.2 B).

3.2 A


100 bp DNA Ladder
uncut pEGFP-C2
pEGFP-C2/ BamHI
pEGFP-C2 beta-catenin
3000
2000
1500
1200
1000
pEGFP-C2
beta-catenin

3.2 B

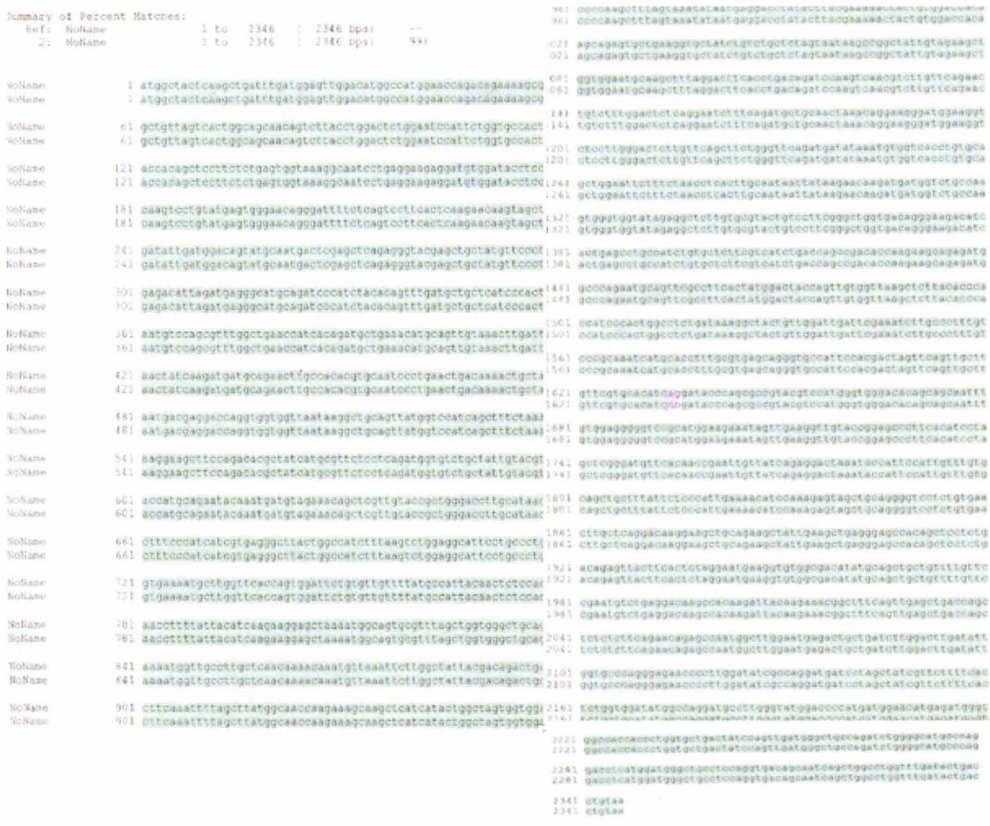


Figure 3.2A Agarose Gel of Digested Plasmid DNA extracted from kanamycin resistant DH5α E.coli. Positive clones were selected by analytical digestion of plasmid DNA by KpnI and BamHI restriction endonucleases. Digested samples were run on a 1% agarose gel with 2.0µl of ethidium bromide for visualization at 110 volts.

Figure 3.2B Final Sequencing results done by Mobix. In order to verify that the β-catenin gene was successfully isolated from pcDNA3.1 vector without any mutation introduced by High Fidelity Taq Polymerase three different primers were designed with SECentral. Each primer was specific and complementary for a certain position in the β-catenin gene to allow for a full length DNA sequencing

3.3 Generation of ARMADILLO β-catenin construct

The same subcloning experimental procedure was followed as in pEGFP-C2-β-catenin subcloning experiment as described in materials and methods. Appropriately designed primers for Armadillo were constructed using SECentral computer program. The forward primer was complementary to the beginning of Armadillo segment at approximately 400 base pairs from the start of β-catenin gene, and the reverse primer had binding specificity for the end of Armadillo located at approximately 2013 bp from the start of β-catenin gene (Figure 3.3 A). The PCR was done effectively and Armadillo segment was isolated from the rest of the β-catenin gene as seen in the Figure 3.3 B. The insert together with the GFP vector went through acknowledged sequence of cloning step used. The final product was a new construct consisting of pEGFP-C2 vector and ARMADILLO segment. The band corresponding to ~1500 bp in size is the construct of interest which was then sequenced to check for mutation (Figure 3.3 C)

3.3 A

PRIMER DESIGN-ARMADILLO

FORWARD PRIMER

5' AATGGTGGGTACCTC-CATGCAGTTGTAAAAC 3'

KpnI

Length: 30nucleotides
Position: 400 bp

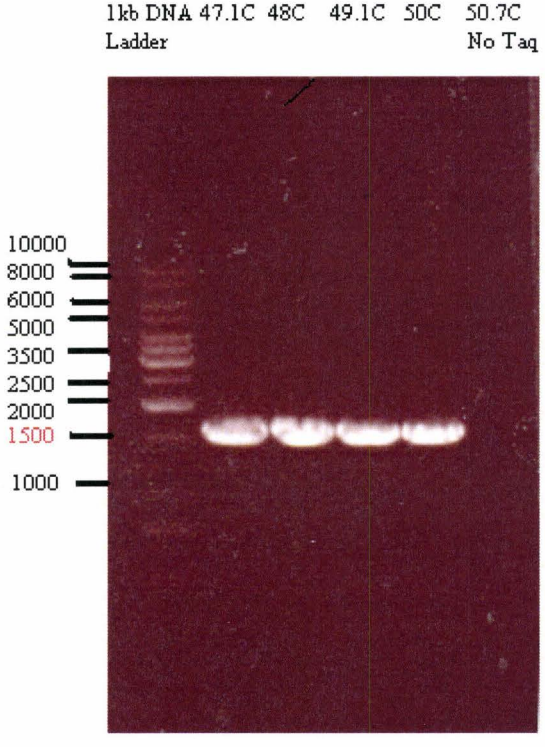
REVERSE PRIMER

5' CGCGGATCCTTA-CTTGTAATCTTGTGGC 3'

BamHI

Length: 28 nucleotides
Position: 2013bp

3.3 B



3.3 C

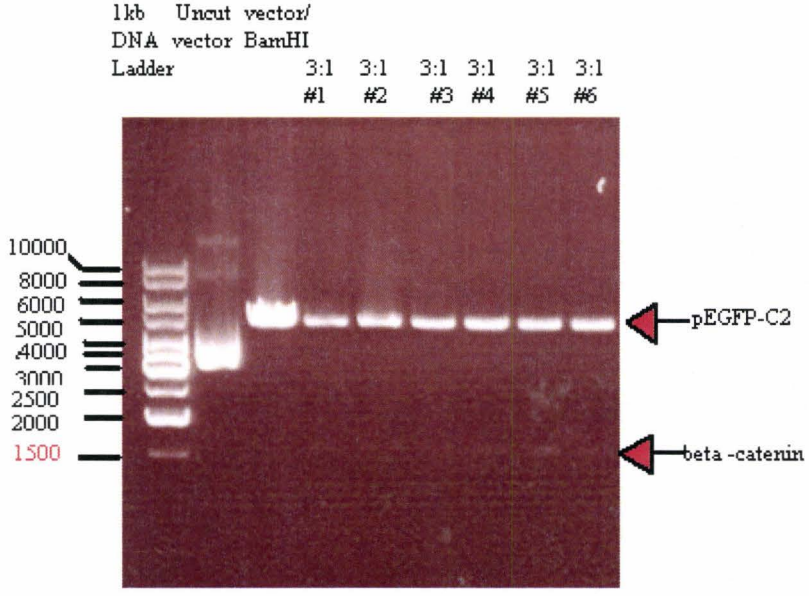


Figure 3.3 A Subcloning of ARMADILLO construct into pEGFP-C2 vector. Specific primers designed by SECentral complementary to the beginning and the end of Armadillo segment. Each primer contains a restriction site of interest (BamHI/KpnI) and amplified the 1684 base pairs of ARMADILLO including the primers.

Figure 3.3 B Analytical PCR was done with appropriate primers and at specific temperatures. The *Taq* Polymerase was successful in amplifying only Armadillo segment using the specific primers. The band appeared at ~1500 bp which is the approximate size of Armadillo segment by itself.

Figure 3.3 C BamHI and KpnI digested vector (pEGFP-C2) and ARMADILLO were used in ligation reaction in the presence of T4 Ligase. Positive clones identify after double digestion with BamHI and KpnI enzymes confirm the successful cloning of Armadillo into GFP vector

3.4 Generation of mutated β-catenin constructs

Two mutated versions of pBABE-hygro-β-catenin constructs were obtained from Dr. David W. Andrews's lab. Both, β-catenin with all four phosphorylation sites mutated (Serine 33, 37, 45 and Threonine 41) and β-catenin with only Threonine 41 mutation, all coding for Alanine, were subjected to PCR and ligated to pEGFP-C2 vector. Primers used in this experiment were the same as used for isolation of a wild-type β-catenin from pcDNA3.1/Zeo vector and insertion in pEGFP-C2 vector (Figure 3.4). The same subcloning procedure was used as explained in the material and methods. The sequencing of the clones generated confirmed that the mutations were consistent with the protein sequence.

3.4

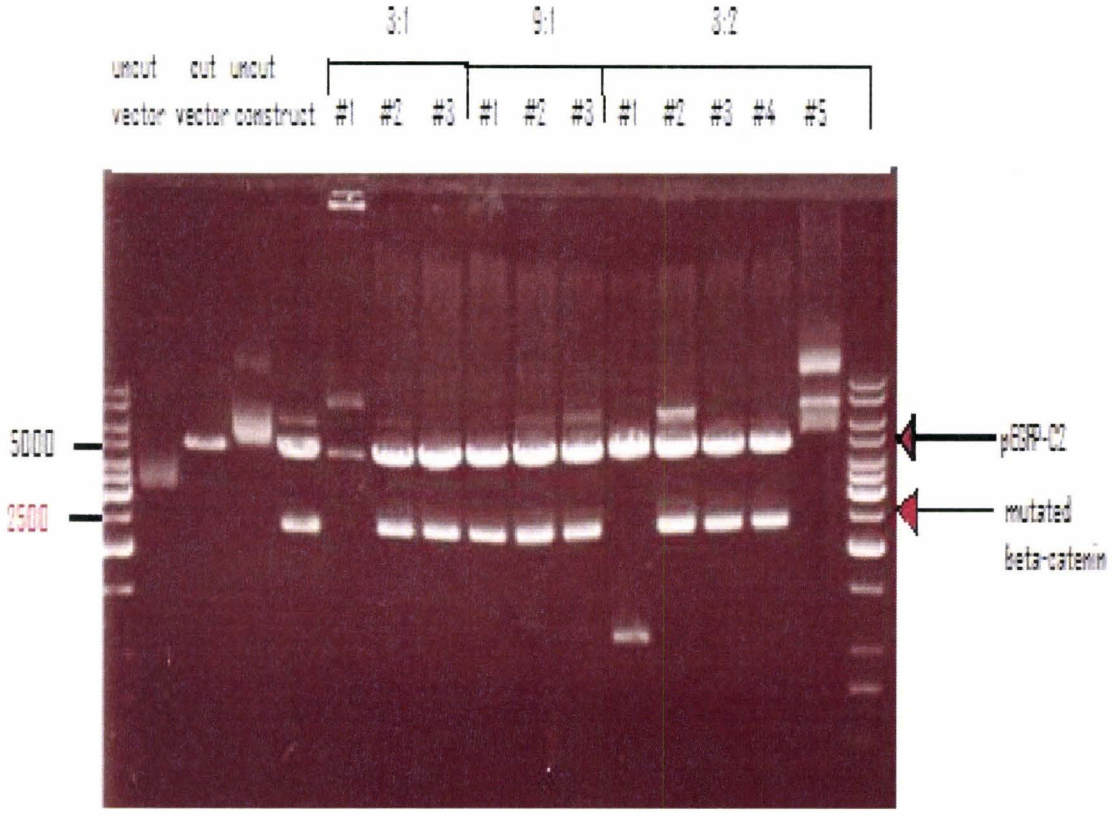
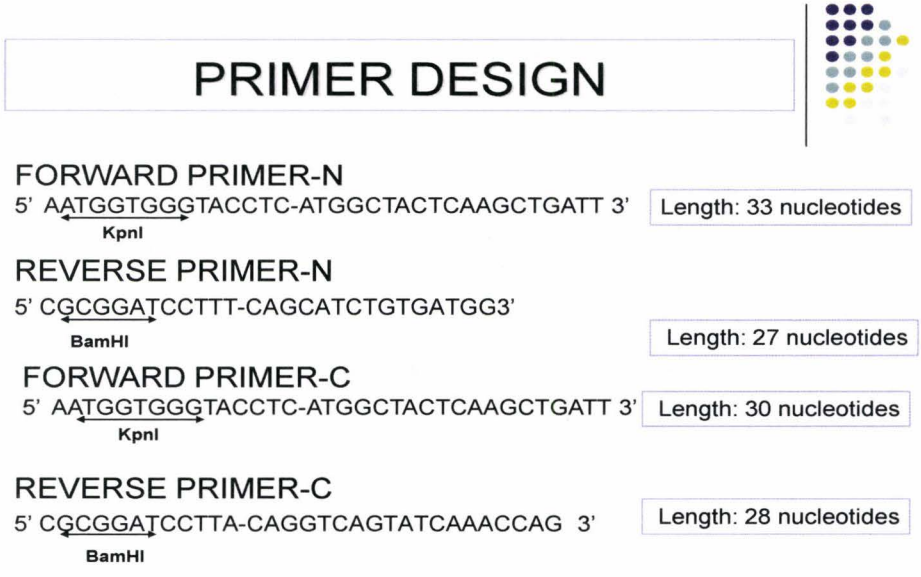


Figure 3.4: Subcloning of mutated β-catenin constructs into pEGFP-C2 vector. Positive clones after double digestion with BamHI and KpnI enzymes were running at the same size as the wild-type β-catenin, ~2500 base pairs. The MOBIX sequencing confirmed the clones containing the mutated phosphorylation sites (ΔSer33,37,45ΔThr41 and ΔThr41). The same subcloning procedure was used as in obtaining wild-type β-catenin and ARMADILLO.

3.5 Generation of N/C constructs

SECentral computer program was used to generate two sets of specific primers necessary for isolation of amino (N) and carboxyl(C) region of wild-type β-catenin (Figure 3.5 A). The primers isolated the N-terminus (1-399 nucleotides) and C-terminus (2017-2346 nucletides). Digestion and subsequent ligation of PCR products generated pEGFP-C2-N and pEGFP-C2-C positive clones which were confirmed by MOBIX sequencing.

3.5 A



3.5 B

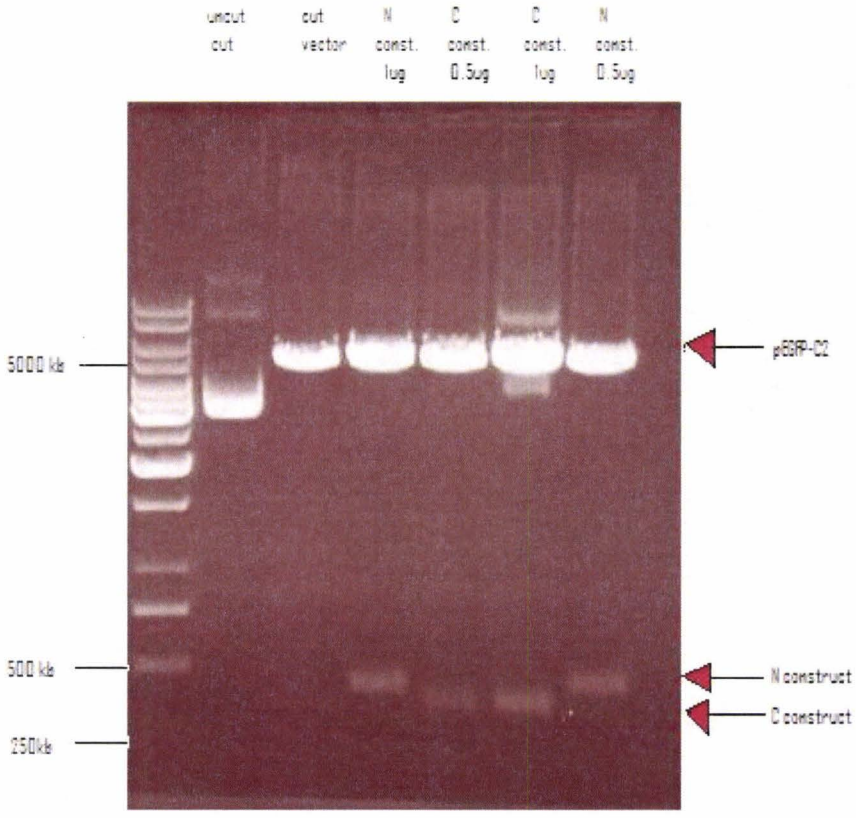


Figure 3.5 A SECcentral generated primers for isolation of amino and carboxyl terminus. Specific primers containing appropriate restriction sites for Bam HI and KpnI were designed to satisfy condition of successful PCR procedure and to allow for isolation of N and C terminus portions of β-catenin

Figure 3.5 B Subcloning of N and C constructs into pEGFP-C2 vector. Positive clones of N and C constructs after double digestion with BamHI and KpnI enzymes. C terminus running lower since it is shorter (386 base pairs) in size followed closely by N-terminus (453 bp) which is slightly larger in size. Note that the size is larger because of the added primers at the ends of N and C terminus.

3.6 Generation of individual Armadillo fragments

The first (400-1150bps) and the second (1151-2016bps) halves of Armadillo segment were generated using cloning techniques already employed for obtaining other β-catenin constructs. First step was designing appropriate primers which would allow for successful isolation of a specific DNA via PCR experiment. The PCR product was then digested together with the acceptor vector (pEGFP-C2) with two same restriction enzymes (BamHI and KpnI) after which the T4 Ligase was employed to join the vector and insert together. Successful clones were then transformed into DH5α and DNA was amplified and isolated using wide variety of techniques already described in the materials and methods section. The nucleotide sequence of these newly formed constructs was verified by Mobix.

3.6 A

PRIMER DESIGN
1ST & 2ND HALF ARMADILLO

FORWARD PRIMER-ARM HALF

5' AATGGTGGGTACCTC-CTCCTTGGGACTCTTGTTC 3' Length: 34 nucleotides

KpnI

REVERSE PRIMER-ARM HALF

5' CGCGGATCCTTA-ACCTTCCATCCCTTCCTG 3'

BamHI

Length: 30 nucleotides

3.6 B

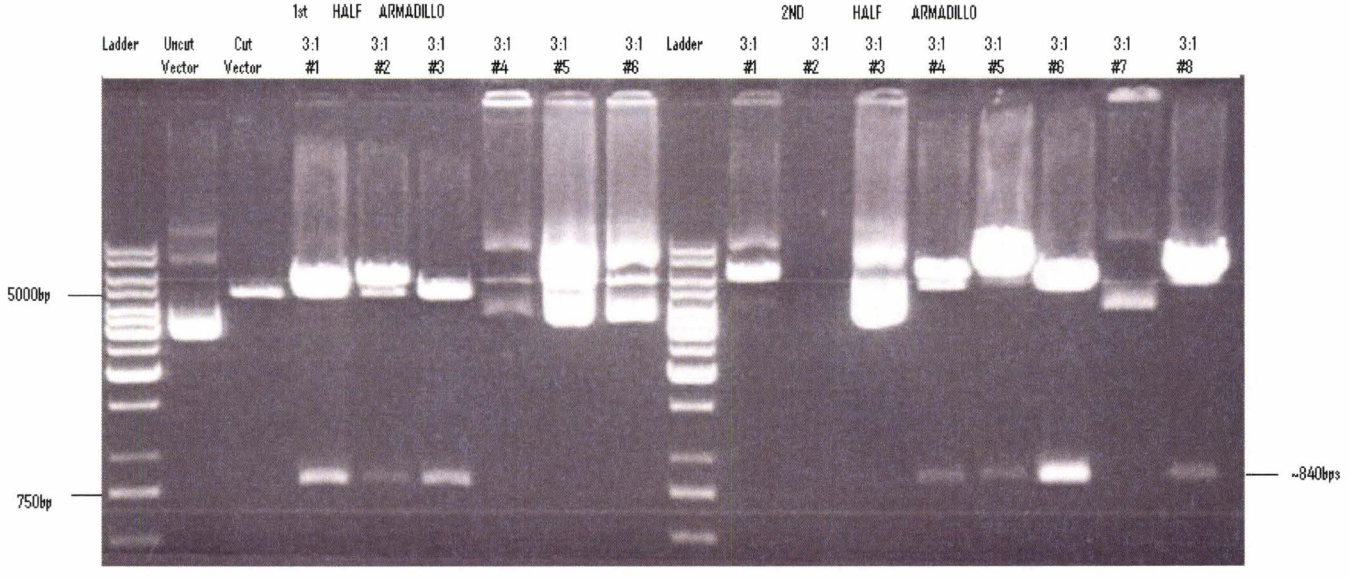


Figure 3.6A Primers used for isolation of specific portions of ARMADILLO

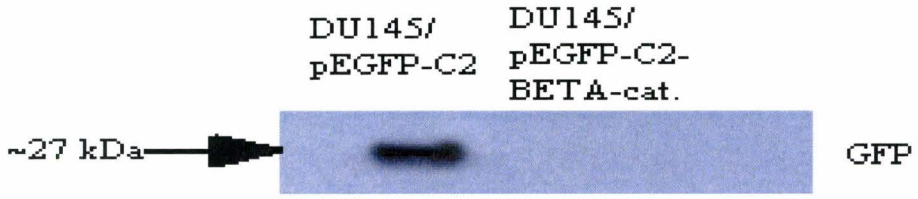
Primers used for the isolation of the first half of ARMADILLO were specifically complementary to the beginning of the ARMADILLO (used forward primer generated when full length ARMADILLO was isolated) and a primer complementary to the middle of ARMADILLO. The second pair of primers were matched together so they specifically amplify second half of ARMADILLO (forward newly created and reverse used the same as reverse primer used for the isolation of full length ARMADILLO).

Figure 3.6B Generation of first and second halves of β-catenin's ARMADILLO. Positive clones were detected by digesting newly created DNA with Bam HI and KpnI restriction enzymes. The 1st half and the 2nd half are approximately the same size and as on the gel they are running together.

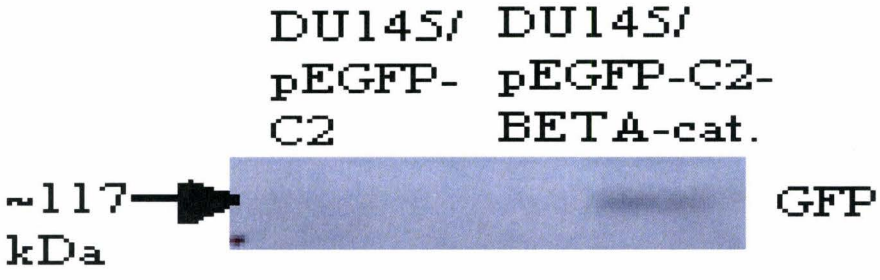
3.7 The optimization and evaluation of transcriptional efficiency of GFP-β-catenin construct

Prostate cancer cells, DU145, were transfected with pEGFP-C2-β-catenin vector or empty vector using ExGen Transfection Reagent (Fermentas). 3μg, 5μg, and 7μg of DNA were used to find the appropriate ratio of DNA to ExGen Transfection Reagent which would increase transfection efficiency. The recommended volume and concentrations were followed according to ExGen protocol appropriate for the 60 mm cell plates. It has been determined that using 5μg of DNA with 9.87 μL of ExGen provided the most optimal transfection efficiency of the DNA of interest as detected by monitoring expression of GFP with the fluorescence microscope. 8, 16, 24, 48, and 72 hours after transfection, GFP fluorescence was detected using conofocal microscope. It has been determined that the transfection is greatest after 48-72 hours after ExGen/DNA addition to DU145 cells. Cells were lysed in RIPA buffer and subjected to the Western Blotting Experiment (Figure 3.7 A-C). GFP protein was detected in DU145 transfected with empty vector at the appropriate size of approximately 27 kDa (Figure 3.7 A). Also, much higher band was detected in DU145 transfected with GFP-β-catenin vector (~117kDa) (Figure 3.7B). Furthermore, the phosphorylated β-catenin was observed at the same height using anti-phospho β-catenin antibody suggesting that the construct is getting phosphorylated. This experiment demonstrates the functionality of newly created β-catenin construct, which is getting expressed in prostate cancer cells, DU145.

3.7A


DU145/
pEGFP-C2
DU145/
pEGFP-C2-
BETA-cat.
~27 kDa
GFP

3.7B


DU145/
pEGFP-
C2
DU145/
pEGFP-C2-
BETA-cat.
~117
kDa
GFP

3.7C

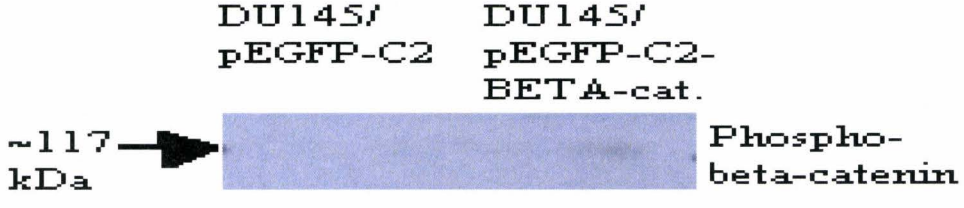

DU145/
pEGFP-C2
DU145/
pEGFP-C2-
BETA-cat.
~117
kDa
Phospho-
beta-catenin

Figure 3.7 Western Blots expressing levels of :

(A) Green Fluorescence Protein, DU145 , prostate cancer cells, transfected with pEGFP-C2 vector only expressed the green fluorescence protein after 72 hours. The protein was detected at the appropriate size of ~27 kDa but it was not detected in DU145 cells transfected with GFP-β-catenin construct suggesting that the two proteins are translated together and are fused together.

(B) β-catenin. Full length, transfected, wild type GFP-β-catenin was detected at the higher band size then its endogenous counter part because the protein is translated with additional ~27 kDa from the GFP protein.

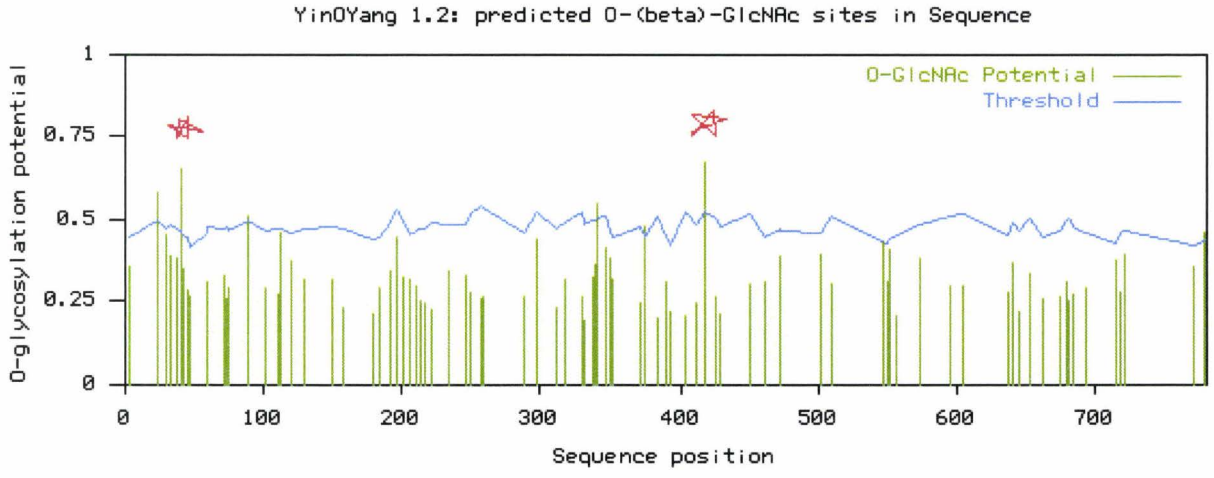
(C) Phosphorylated β-catenin in prostate cancer cell lines, DU145, transfected with empty vector (pEGFP-C2) or vector + insert (pEGFP-C2-β-catenin). The band was detected at ~117 kDa suggesting that the GFP-β-catenin is post-translationally modified by phosphorylation.

3.8 β-catenin O-glycosylation in DU145 cells

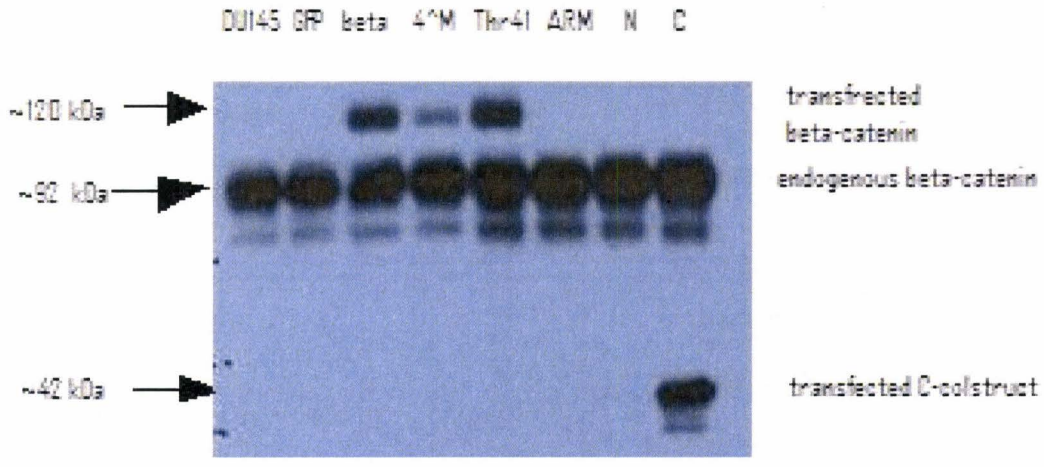
The Center for Biological Sequence Analysis Server (CBS) (YINGOYANG) was used to predict possible O-glycosylation attachment sites in eukaryotic protein sequences (Figure 3.8 A). Based on this program, the most possible glycosylation sites are probable in ARMADILLO segment, followed by N-terminus, at Threonine–41, which is the amino acid involved in phosphorylation and regulation of β-catenin. The next highest prediction is Serine 23 located also in the N-terminus. 5μg of β-catenin DNA (full length, components and mutated full length) was transfected using ExGen into DU145 prostate cancer cells. After 72 hors of transfection, the expression of the GFP-β-catenin protein was detected using conofocal microscope and the cells were RIPA lysed and subjected to Bradford protein assay. Western blot of total amount of β-catenin was done first to detect that the successful transformation was completed. This was followed by immunoblotting with anti-β-catenin antibody. (Figure 3.8 B). The total levels of endogenous β-catenin appeared at the appropriate protein size of approximately 92 kDa. The full length GFP-β-catenin constructs are visualized at 120 kDa mark because of additional weight of GFP protein (~27 kDa). While the C-terminal portion of β-catenin showed up at a lower size of ~ 42 kDa, the N-terminus failed to be observed because the anti-β-catenin antibody did not possess an appropriate epitope necessary for the detection of this part of the protein. The immunoblotting experiment was done using anti-amino-β-catenin antibody that contains an appropriate epitope for N-terminus and the total levels of N-terminus (~44 kDa) construct and endogenous β-catenin (~92 kDa) were successfully detected (Figure 3.8 C).

3.8A

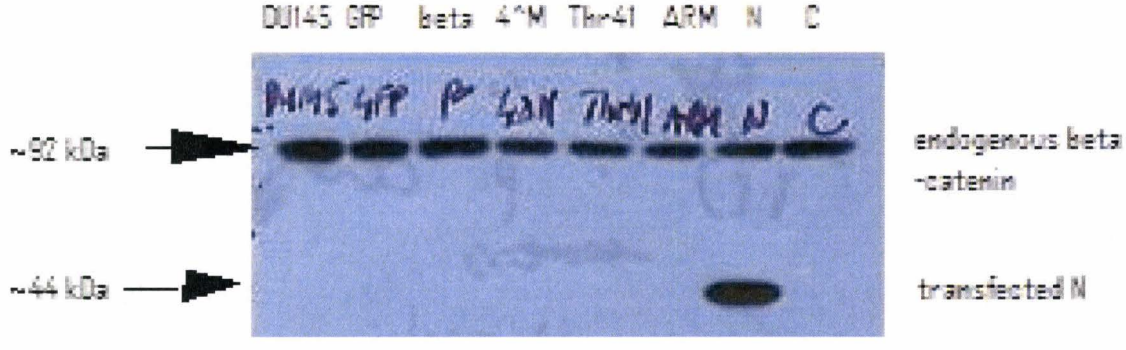
SeqName	Residue	O-GlcNAc result	Potential	Thresh. (1)	Thresh. (2)
Sequence	23 S	+	0.5827	0.4991	0.6232
Sequence	41 T	++	0.6577	0.4609	0.5716
Sequence	89 T	+	0.5133	0.4995	0.6236
Sequence	341 S	+	0.5493	0.5041	0.6299
Sequence	374 S	+	0.4822	0.4493	0.5560
Sequence	418 T	++	0.6768	0.5274	0.6613
Sequence	779 T	+	0.4630	0.4346	0.5362



3.8 B β-CATENIN



3.8 C N-β-CATENIN



3.8 D

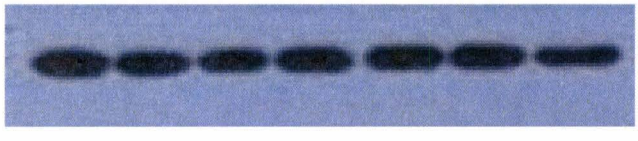


Figure 3.8 Western blot of DU145 cells transfected with different GFP-β-catenin constructs.

(A) The YinOYang WWW server produces neural network predictions for O-ß-GlcNAc attachment sites in eukaryotic protein sequences. This server can also use NetPhos, to mark possible phosphorylated sites and hence identify "Yin-Yang" sites.

(B) Depicts the Western blot using **β-catenin** primary antibody. Endogenous β-catenin appeared in all DU145 cells, transfected and non-transfected.

(C) DU145 lysates blotted with carboxy-β-catenin primary antibody. The size of the C terminus by itsel is approximately 42 kDa which corresponds to the appropriate size on the blot.

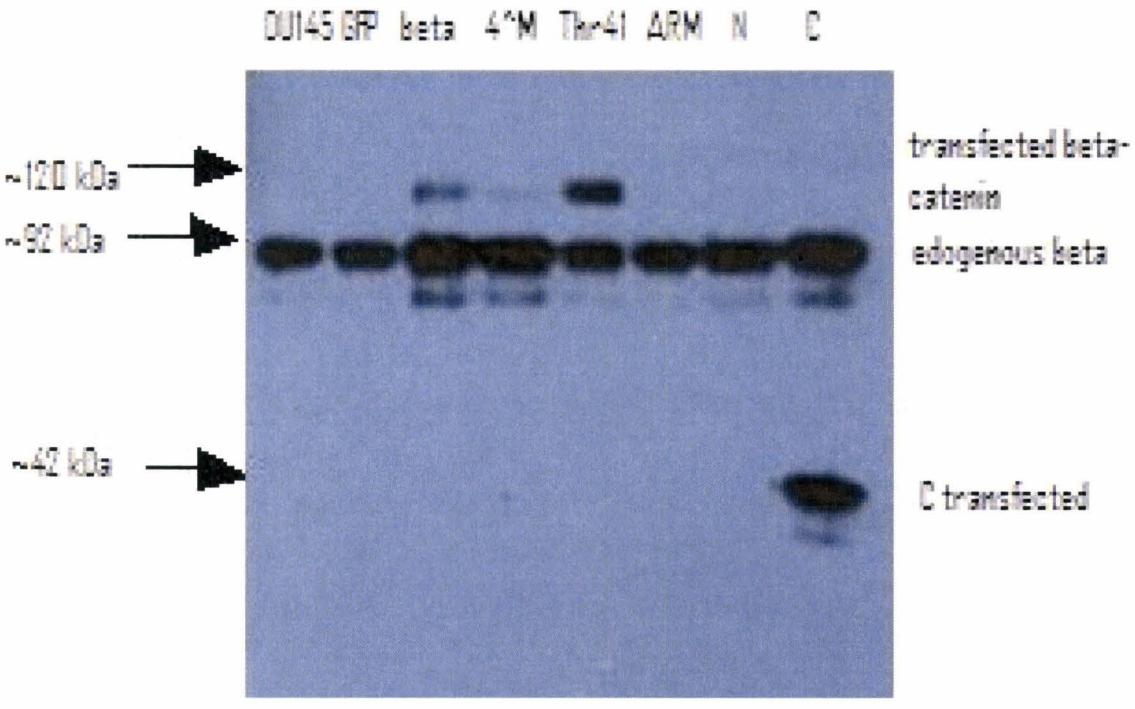
(D) The blots were immunoblotted for total β-catenin and were reprobed with anti-actin antibody as a control for loading

3.9 Wheat Germ Agglutinin experiment

The basal levels of O-glycosylated β-catenin were measured in DU145, prostate cancer cell line. 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, anti-amino-β-catenin and anti-GFP antibody. Results show that full length wild-type and mutated versions of β-catenin are getting post-translationally modified by O-glycosylation. Also, C-terminal and N-terminal wild-type full length constructs are getting O-glycosylated.

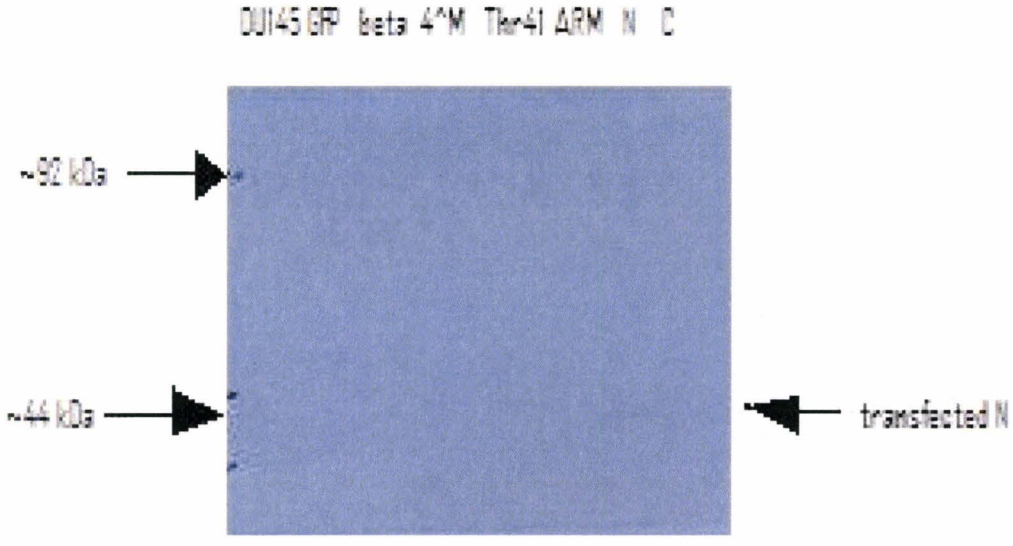
3.9 A

WGA-β-CATENIN



3.9 B

WGA-N-β-CATENIN



3.9 C

WGA-GFP

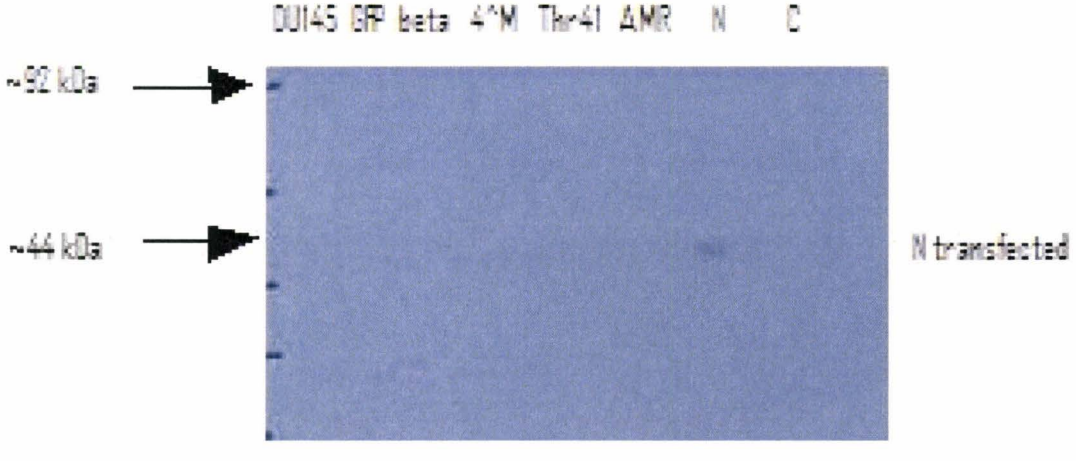


Figure 3.9 Wheat Germ Agglutinin (WGA) pulldown of DU145 lysates.

(A) WGA blotted with anti-β-catenin primary antibody. Full length, wild-type and mutated β-catenins are getting O-glycoslylated. Also C-terminus is getting post-translationally modified as well.

(B) WGA blotted with anti-**amino**-β-catenin antibody. N-terminus, wild-type construct is getting O-glycosylated as well exhibited by the band presence at the ~44 kDa mark.

(C) Figure showing the WGA of DU145 samples blotted with anti-GFP primary antibody which detects and reconfirms that N-terminus is getting O-glycosylated.

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

3.10 PUGNAc caused an increase in O-glycosylated β-catenin in DU145 cells

The prostate cancer cell line, DU145, 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.

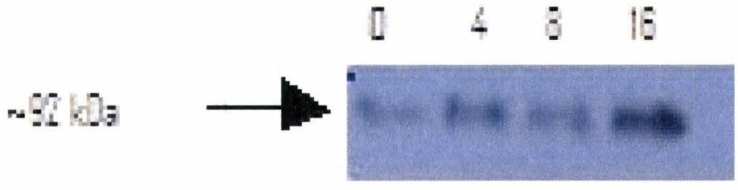
Optimal treatment conditions required that PUGNAc treatment of DU145 cells be done in the absence of serum in order to prevent factors in the serum from interfering with the activity of the drug. Also treatment intervals had to be chosen so that viability of the cells was not compromised by the removal of serum from the media.

DU145 cells were grown in the presence of full serum (10% FBS) and then in the media containing 1% serum for 16 hours before treatment with PUGNAc for various time points in the absence of serum. A dose response with PUGNAc in DU145 cells under these treatment conditions was done and a working concentration of 100 μM was determined as optimal.

PUGNAc caused a significantly large increase in O-glycosylated β-catenin, detected by WGA precipitation, at time points 4, 8, 16 hours (Figure 3.10 A). The expression of total β-catenin remained constant throughout PUGNAc treatment indicating that PUGNAc caused an increase in O-glycosylation without having any effect upon expression of the protein itself (Figure 3.10 C). Also, treatment with PUGNAc and subsequent immunoblotting with anti-ACTIVE-β-catenin showed that the amount of dephosphorylated β-catenin decreases with an increased time exposure to PUGNAc (Figure 3.10 B).

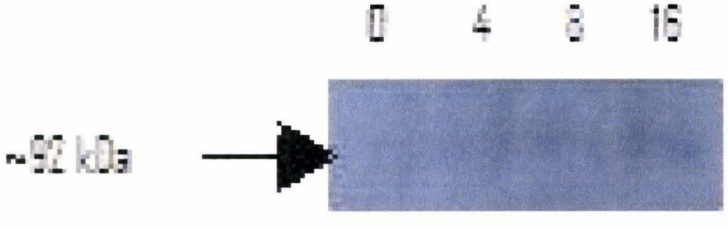
3.10 A

WGA-β-CATENIN

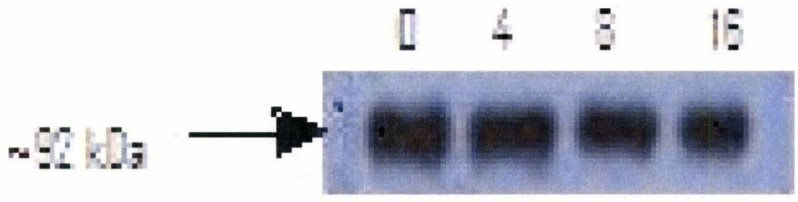


3.10 B

ACTIVE-β-CATENIN



3.10 C β-CATENIN



3.10 D

β-ACTIN

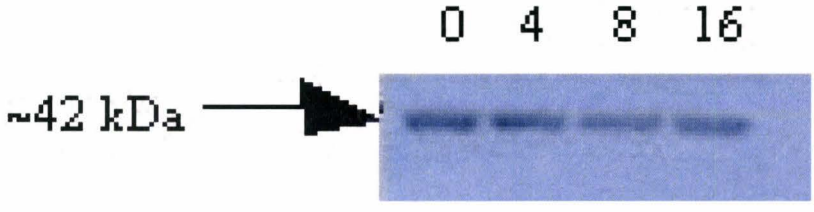


Figure 3.10 Transfected DU145 cells treated with PUGNAc at various time points and subjected to western blotting.

A. Shows PUGNAc treated DU145 lysates subjected to WGA and blotted with β-catenin primary antibody. The amount of O-glycosylated endogenous β-catenin increases with time and treatment with PUGNAc

B. PUGNAc treated DU145 lysates blotted with ACTIVE-anti-β-catenin. ACTIV form of β-catenin is dephosphorylated version of this protein and it also increases in total cell lysates with time and PUGNAc

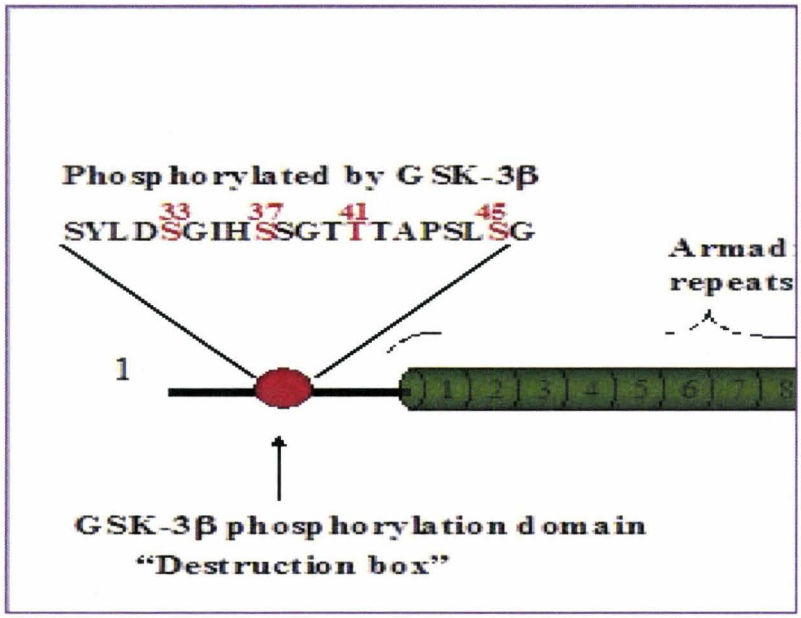
C. PUGNAc treated DU145 lysates blotted with anti-β-catenin antibody. Endogenous levels of β-catenin stay constant with time and treatment of PUGNAc

D. Figure shows loading control-β-actin.

3.11 Site-directed mutagenesis

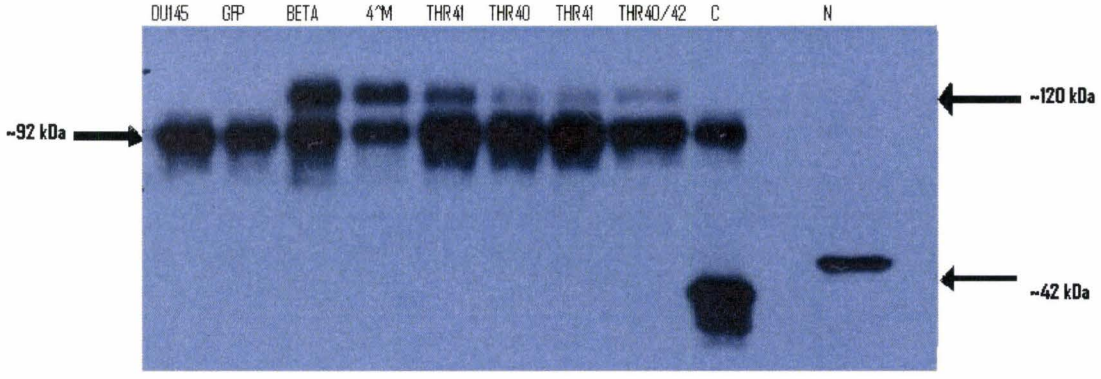
Three additional full-length β-catenin mutants were made using QuickChange site-directed mutagenesis method (Stratagene). Sites Threonine 40 and Threonin 42 located upstream and downstream of a phosphorylation site (Threonine 41) were mutated to Alanine either as a single mutation or as a double mutation. Mobix sequencing was employed to distinguish between the positive and negative clones. These newly created mutants were then used in the WGA experiment as well as TOPFlash and Nuclear/Cytosolic localization experiments to see if these sites could have an effect on the O-glycosylation of the protein.

3.11 A

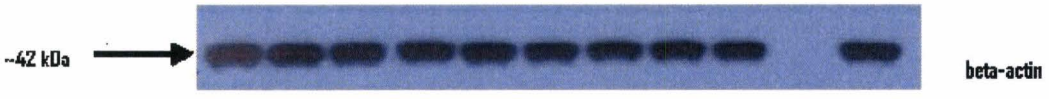


3.11 B

β-CATENIN N-β-CATENIN



3.11 C



3.11 D

WGA-β-CATENIN WGA-N-β-CATENIN

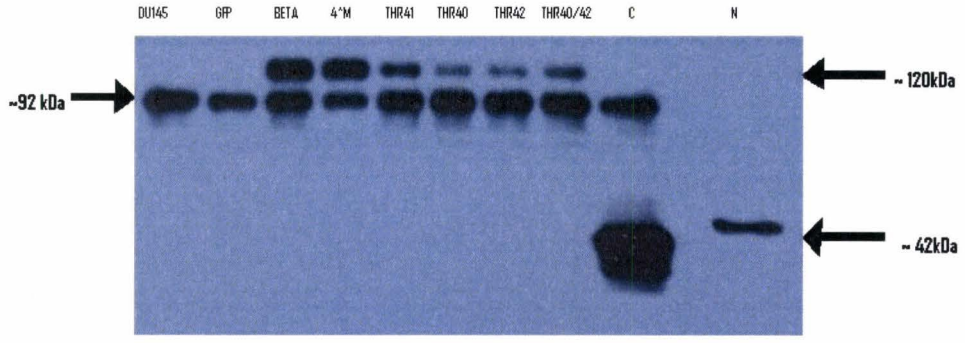


Figure 3.11: Western blot of DU145 cells transfected with different pEGFP-C2-β-catenin constructs.

(**A**) Illustration of N-terminal amino acids sites that were mutated, either by itself or together, in order to check their effect on O-glycosylation of β-catenin

(B) Depicts the Western blot using β-catenin and amino-β-catenin primary antibodies. This blot shows that all of the full length and different segments of β-catenin were transfected successfully into DU145 using ExGen transfection reagent

(C) Figure showing the loading control-β-actin

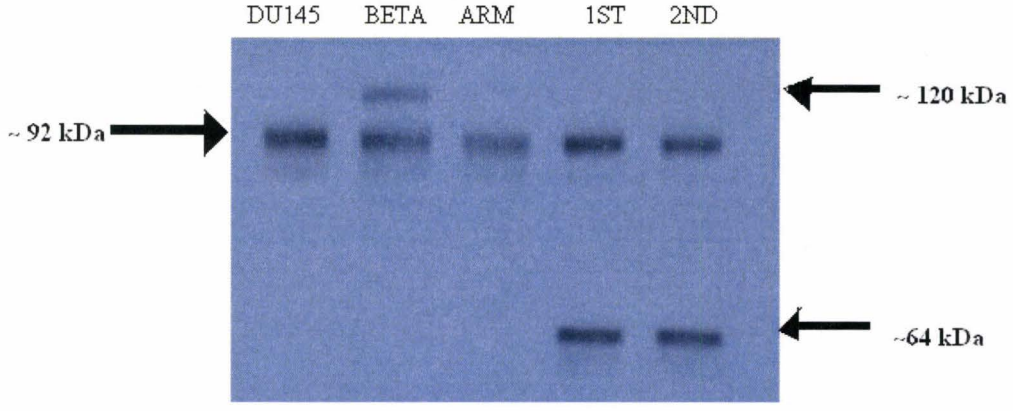
(D) WGA experiment done on the same samples from **B.** β-catenin and N-β-catenin primary antibodies were used, respectively. The blot shows that the full length wild-type and mutated versions (4ΔM, Thr40,41,42,40/42) are getting O-glycosylated. Also it shows that the constructs, N-terminus and C-terminus are getting O-glycosylated.

3.12 Determination of ARMADILLO O-glycosylation status

O-glycosylation properties of Armadillo and its constructs (1st and 2nd half) were examined. First, regular immunoblotting experiments were done using Armadillo antibody (Santa Cruz) in order to confirm the presence of the protein constructs. Next, to re-confirm, immunoprecipitation experiment was done using Green Fluorescence Protein monoclonal antibody and Armadillo antibody, respectively. Finally, WGA was done to clarify the O-glycosylation capacity of Armadillo

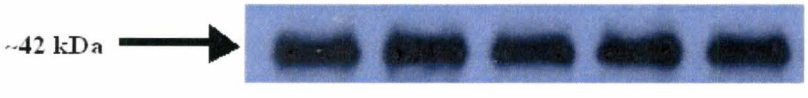
3.12 A

ARMADILLO



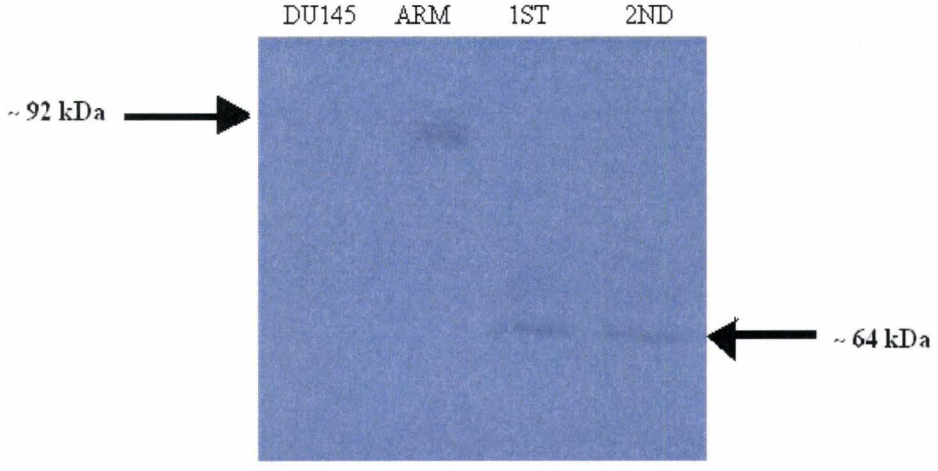
3.12 B

β-ACTIN



3.12 C

IP-ARMADILLO



3.12 D **WGA-ARMADILLO**

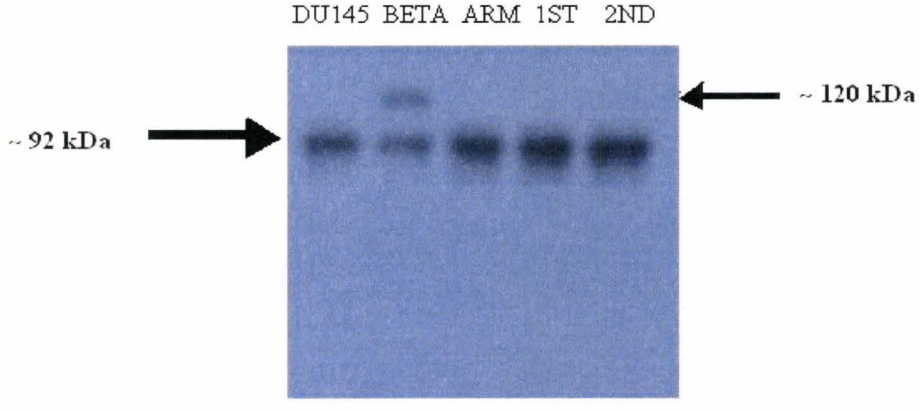


Figure 3.12: Western blot of DU145 cells transfected with different GFP-β-catenin constructs.

(A) Depicts the Western blot using **ARMADILLO** primary antibody. ARMADILLO antibody picks up endogenous levels as well as transfected β-catenin constructs.

(B) Also, loading control, β-actin is pictured below the blot A.

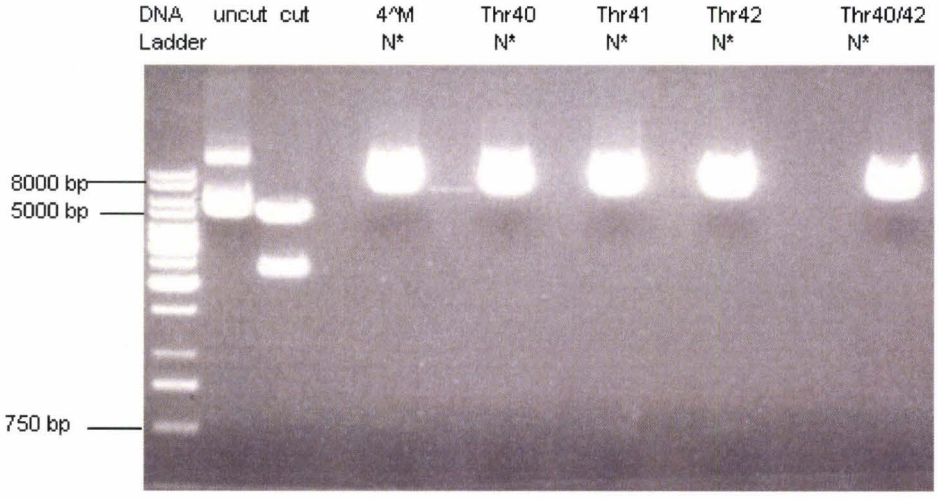
(C) Immunoprecipitation (IP) experiment done on the same samples from **A.** using GFP and Armadillo antibody. In order to eliminate endogenous β-catenin from the lysates, IP was done with GFP antibody and then the blot was washed in ARMADILLO antibody. Only transfected construct appeared on the blot after development of the film.

(D) Wheat Germ Agglutinin **(WGA)** pulldown of DU145 lysates. WGA blotted with **ARMADILLO** antibody. This shows that ARMADILLO segment of β-catenin does not get O-glycosylated

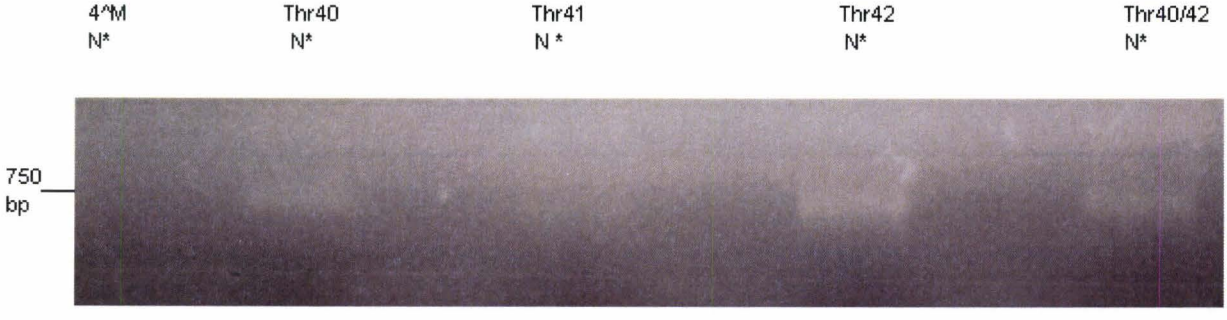
3.13 Determination of N terminus O-glycosylation properties

In order to clearly distinguish if the mutations of the specific sites (Thr40,41,42,40/42) have an effect on the O-glycosylation capacity of the N-terminus, the mutated N-terminus was isolated from full length mutated β-catenin-pEGFP-C2 vector by cutting it with SpeI (cuts at 1605bp) and XbaI (cuts at1406 of pEGFP-C2 MCA) restriction enzymes and re-ligating with T4 Ligase. The fragment left behind is 753 nucleotides and it contains the entire C-terminus and a portion of ARMADILLO. This furthermore clarified the involvement of theses specific sites in the O-glycosylation of the N-terminus.

3.13 A



3.13 B



3.13 C

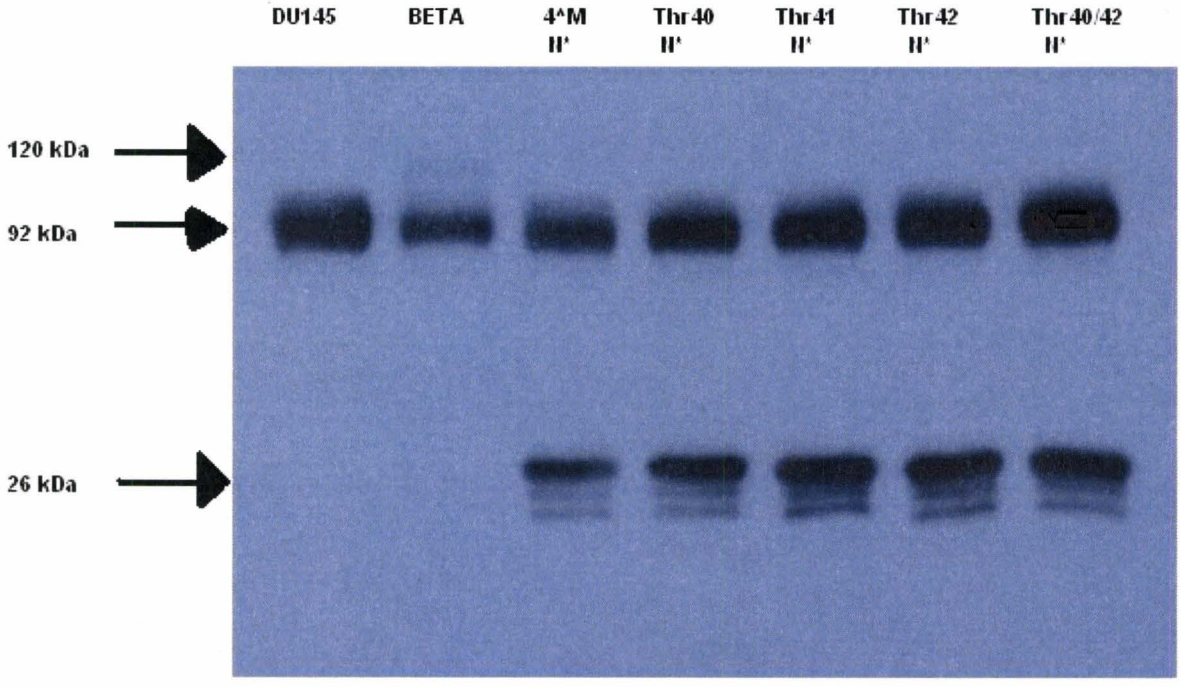

DU145
BETA
4^M II'
Thr40 II'
Thr41 II'
Thr42 II'
Thr40/42 II'
120 kDa
92 kDa
26 kDa

Figure 1.13 Determination of N-terminus mutants on the O-glycosylation properties of N-terminus

(A) Specific restriction enzymes (SpeI and XbaI) were used to isolate mutated N-terminus from the rest of the protein. This figure depicts final stage in cloning experiment which allowed us to separate our DNA of interest (pEGFP-C2-N-mut) from the full-length β-catenin in pEGFP-C2.

(B) Focused and zoomed in agarose gel picture showing the bands corresponding to the insert (N-terminus mutated)

(C) WGA pulldown of N-terminus mutated protein together with full-length β-catenin (control). The blot shows that the mutated amino acids (Thr40, 42, 40/42) are not involved in O-glycosylation of the protein.

3.14 Nuclear and Cytosolic Experiment

DU145 cells were transfected with full-length β-catenin constructs using ExGen Transfection Reagent. After 48 hours of transfection, prostate cancer cell line, DU145, was treated with a drug (PUGNAc) in order to manipulate levels of O-glycosylation to determine how this modification affects the subcellular localization of β-catenin. After specific time points, 0, 4, 8, and 12 hours, DU145 cells were lysed and nuclear and cytosolic extracts were separated using the NE-PER kit (Pierce). Protein concentration of nuclear and cytosolic lysate was determined using the Bradford protein assay (BioRad). 100μg of total protein was subjected to WGA precipitation to detect the change in the O-glycosylation levels over time. Also, markers for nuclear and cytosolic fractions, Lamin B and α/β Tubulin, were used to make sure there is no contamination between these two samples.

Lamin B is an intermediate filament protein that provides structural integrity for the nucleus (Nigg, 1992). Detection of Lamin B 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 no detectable expression in the cytosolic fraction as detected by Western blot using a mouse monoclonal antibody for the protein (Figure 3.14 C).

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 cancer cell line (Figure 3.14 D). There was no cytosolic contamination in the nuclear fraction as indicated

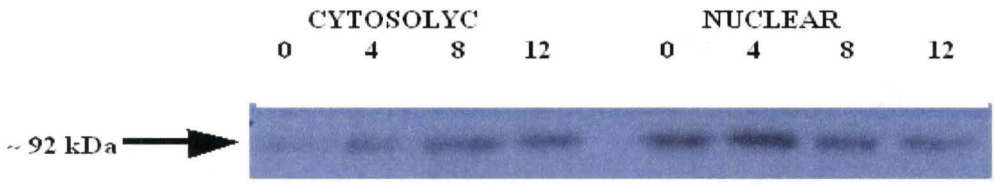
by the absence of α,β tubulin expression in the nuclear fraction (Figure 3.14 D). According to Western blot results, our nuclear/cytosolic fractionation procedure was adequate in separating and isolating the subcellular compartments.

Results show that PUGNAc caused significant increase in O-glycosylated β-catenin in DU145 cells (Figure 3.14 A). 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 cytosolic β-catenin (Figure 3.14 A). One explanation for this significant result is that O-glycosylated β-catenin was blocked from the entry into the nucleus while its export 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.

Furthermore, wheat germ agglutinin pull down was done on the PUGNAc treated and β-catenin construct transfected DU145 cells and the same pattern of subcellular localization was observed for the transfected constructs (Figure 3.14 B).

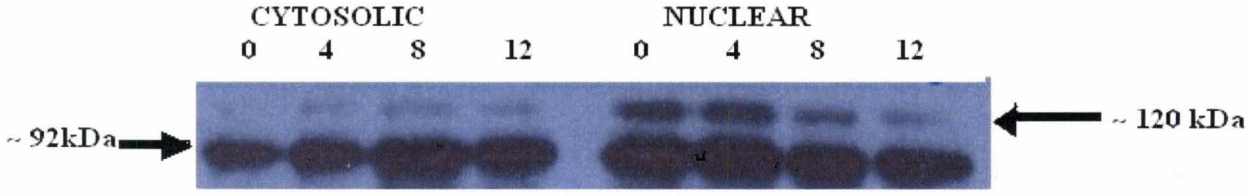
3.14 A

β-CATENIN



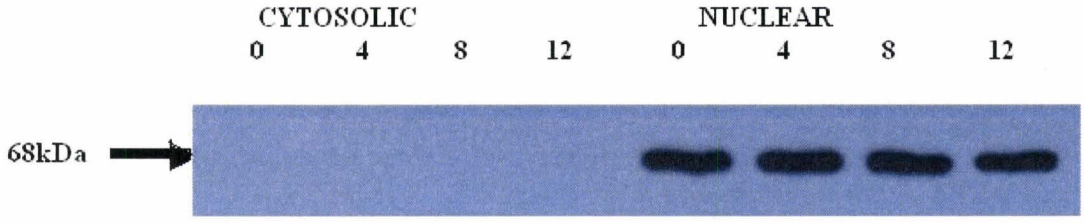
3.14 B

WGA-β-CATENIN



3.14 C

LAMIN B



3.14 D

α/β TUBULIN

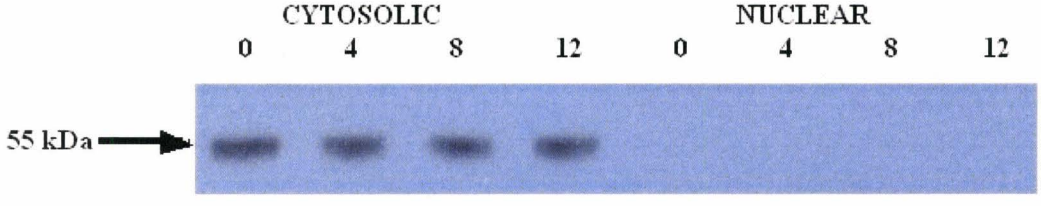


Figure 3.14: Levels of cytosolic O-glycosylated β-catenin and β-catenin's constructs increased with PUGNAc treatment of DU145 cells. Levels of nuclear O-glycosylated β-catenin and β-catenin's constructs decreased with PUGNAc treatment.

A. Nontransfected DU145 control registering O-glycosylated levels of endogenous β-catenin.

B. Transfected β-catenin constructs behave in a similar manner in terms of O-glycosylation. The levels of O-glycosylated, transfected β-catenin increase with time and PUGNAc treatment

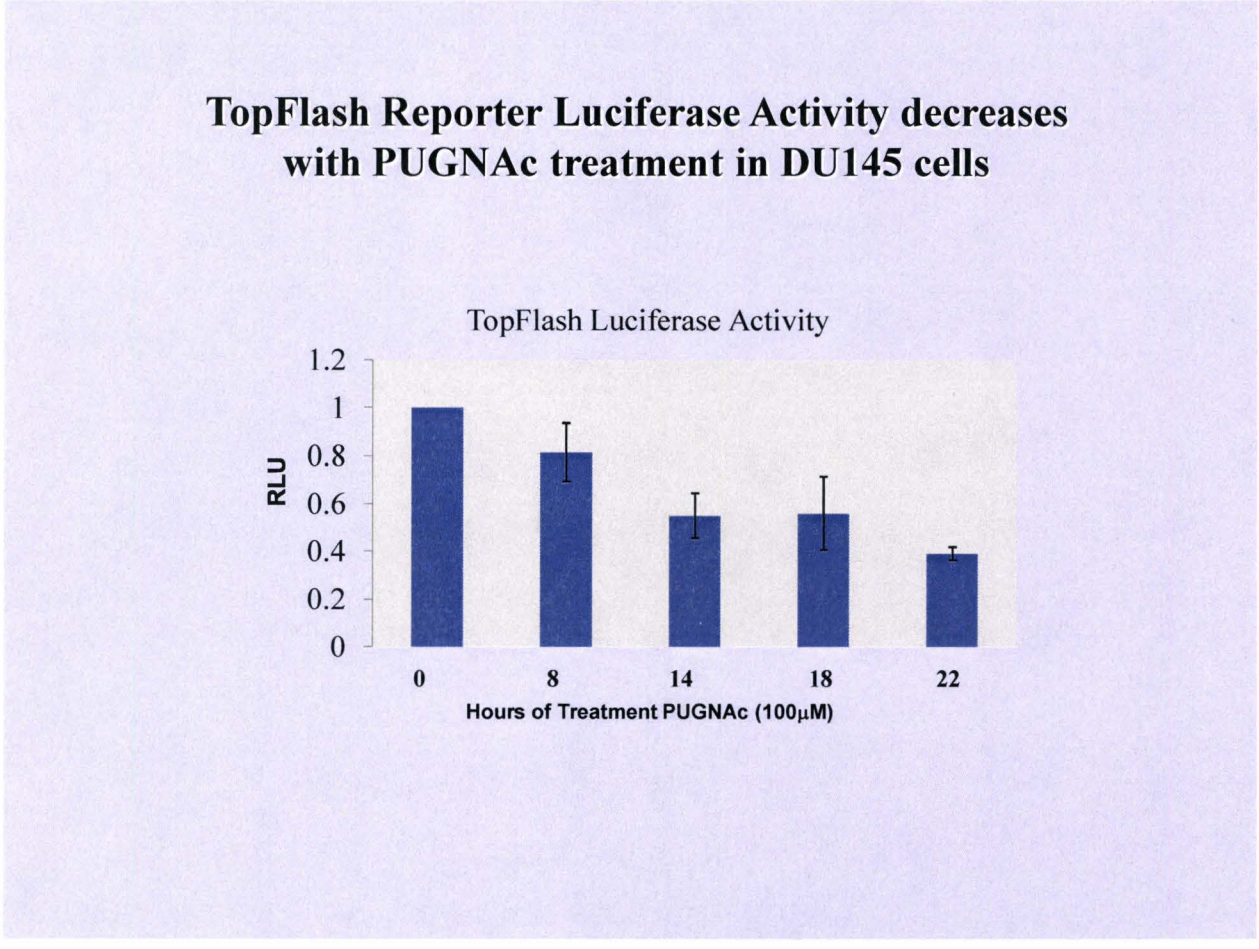
C. Lamin B is used as a nuclear marker to show nuclear enrichment. There is no nuclear contamination in the cytosolic fractions. Lamin B levels are detected in the nucleus.

D. α/β Tubulin is used as a cytosolic marker indicating that there was no cytosolic contamination in the nuclear fraction. α/β Tubulin levels are detected in the cytosol.

O-glycosylation regulates the transcriptional activity of β-catenin

3.15 TopFlash Reporter assay

In order to determine if O-glycosylation affects β-catenin's mutants transcriptional activity, 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. In the previous studies our lab determined that when DU145 cells were transfected with the reporter plasmid and treated with PUGNAc for the indicated time points luciferase activity decreased.



Here, the primary goal is to see if the Luciferase Activity changes when the full-length mutants of β-catenin are transfected.

TopFlash Reporter vector was co-transfected with different β-catenin full-length constructs using ExGen Transfection Reagent. After 48 hours of transfection DU145 cells were treated with PUGNAc for 0, 4, 8, 12 hours and were RIPA lysed. Protein concentration was determined with Bradford assay and the levels of luminescence were detected by luminometer (PE Applied Biosystems). Results show a decreased luciferase activity after 8 hours of PUGNAc treatment, which remained decreased throughout the treatment (Figure 3.15). The same trend was observed for all full length constructs used, with no differences observed between the wild type and mutated versions of β-catenin suggesting that full length N-terminal mutants did not affect O-glycosylation efficiency and its subcellular localization into the nucleus.

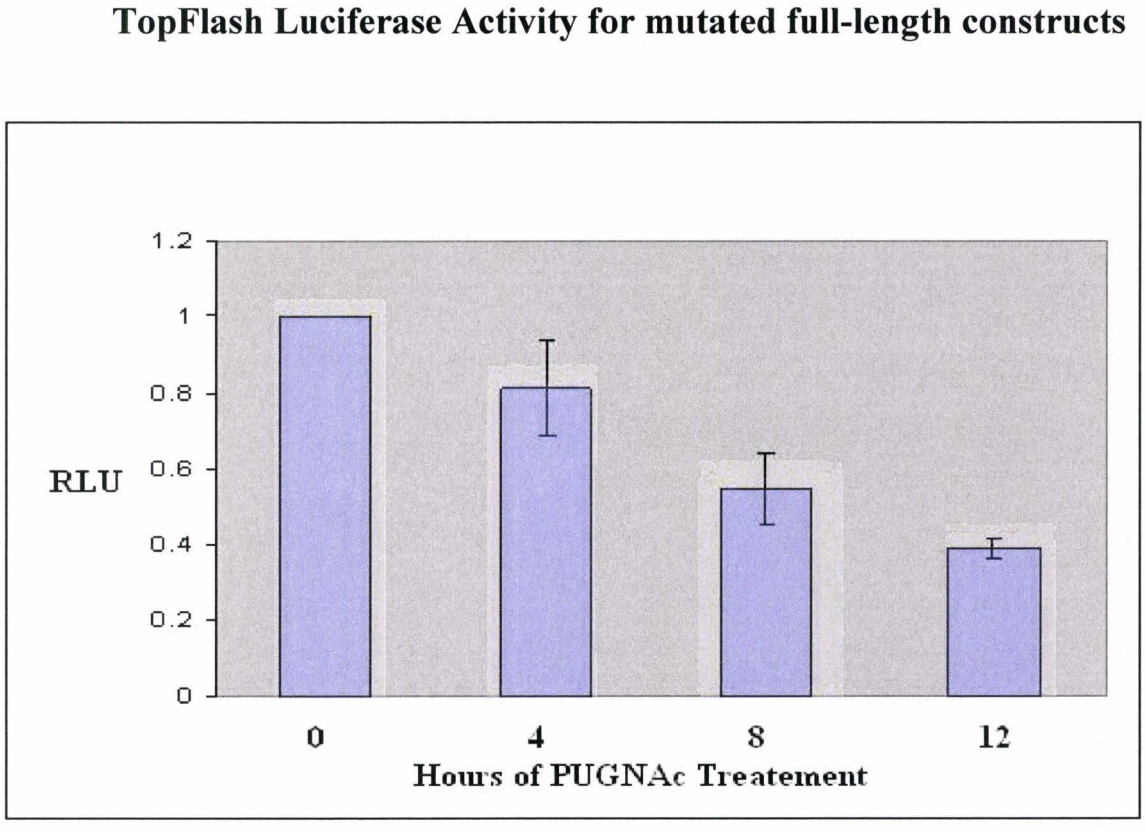


Figure 3.15: Decreased relative Luciferase activity after PUGNAc treatment.

DU145 cells were co-transfected with TopFlash reporter vector and full length β-catenin mutated constructs and were treated with PUGNAc for the indicated time points. Cells were lysed with Luciferase lysis buffer and transcriptional activity was assessed. A decrease in luciferase activity was seen at 8 and 12 hours compared to a 0hr control

Chapter 4

Disscussion, Concluding Remarks and Future Directions

4.1 Discussion

β-catenin signaling has been implicated in the progression of many types of cancers. Its oncogenic properties are attributed to its ability to enter the nucleus 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 as 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 adherens junctions. Thus we asked the question: "Which part/s of β-catenin is/are involved in O-glycosylation?"

pcDNA3.1/Zeo vector containing full length, wild type β-catenin was used to transfer the DNA of interest into a green fluorescence protein expressing vector, pEGFP-C2. Full length, wild-type β-catenin gene was isolated from pcDNA3.1 vector using SECentral generated primers with incorporated KpnI (N-terminus) and BamHI (C-terminus) restriction sites. KpnI/BamHI digested PCR product was ligated with T4 Ligase to pEGFP-C2 vector. Max Efficiency DH5α E.Coli were used to grow the DNA and QIAGEN Maxi Prep Kit was used for the subsequent DNA isolation. The same procedure was followed when isolating specific β-catenin fragments (N-terminus, C-terminus, ARMADILLO, 1st and 2nd half of ARMADILLO) using appropriate primers.

Also, the same set of steps was followed for the isolation of full length phosphorylation site mutants (N-terminus) of β-catenin from pBABE-hygro-ΔSer33,37,45ΔThr41 and pBABE-hygroΔThr41 vectors. The mutants were successfully ligated to pEGFP-C2 and checked by DNA sequencing.

Finally, Site-directed mutagenesis technique was employed to make additional N-terminus mutants of β-catenin. Threonine 40, Threonine 42, and both Threonine40/42 mutations (coding for Alanine) were introduced into a full length wild type β-catenin-pEGFP-C2. All generated construct were transfected into a prostate cancer cell (DU145) using ExGen transfection reagent and the protein expression was assayed after 72hrs. Wheat Germ Agglutinin pull down was employed to isolate only O-glycosylated proteins, subsequently using Western blot with β-catenin, amino-β-catenin, or armadillo antibody to detect O-glycosylated β-catenin. It has been determined that armadillo segment is not getting post-translationally modified by O-glycosylation. However, both C-terminus and N-terminus of β-catenin are being O-glycosylated, while the nature of N-terminus mutants is still not comprehensible. In order to clearly distinguish if the mutations of the specific sites (Thr40,41,42,40/42) have an effect on the O-glycosylation capacity of the N-terminus, the mutated N-terminus constructs were isolated from full length mutated β-catenin-pEGFP-C2 vector by cutting it with SpeI and XbaI restriction enzymes and re-ligating with T4 Ligase. This clarified the involvement of theses specific sites in the O-glycosilation of the N-terminus. It showed that these specific mutations do not show apparent involvement in O-glycosylation property of β-catenin.

In order to investigate whether O-glycosylation of β-catenin and β-catenin constructs affect the nuclear localization of the protein, we manipulated the levels of O-GlcNAc in the prostate cancer cell line, DU145, by treatment of these cells with the chemical inhibitor PUGNAc.

PUGNAc, O-(2-acetamido-2-deoxy-d-glucopyranosylidene) amino-*N*-phenylcarbamate, is 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 in cells (Haltiwanger et al., 1998).

DU145 cell 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 DU145 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. Also the levels of total dephosphorylated β-catenin increased with time of PUGNAc treatment. One possible explanation for these observations is that O-glycosylation of β-catenin may prevent it from entering the nucleus. As a result, the export from the nucleus is not hindered and the build up of β-catenin in the cytosol is seen.

β-catenin is able to enter the nucleus on its own, without the aid of any know nucleocytoplasmic binding partner. 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 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 and its full length constructs is affecting the protein's ability to enter the nucleus.

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. B-catenin interacts with APC and axin, which enhances its export to the cytoplasm (Barth et al., 1997).

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 full length, both wild type and mutated, β-catenin's nuclear import is hindered by O-glycosylation, and 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 DU145 cells indicating that O-glycosylation was affecting the transcriptional activity of β-catenin.

4.2 Concluding Remarks

The dynamic glycosylation of serine or threonine residues on nuclear and cytosolic proteins by O-linked beta-N-acetylglucosamine (O-GlcNAc) is abundant in all multicellular eukaryotes. On several proteins, O-GlcNAc and O-phosphate alternatively occupy the same or adjacent sites, leading to the hypothesis that one function of this saccharide is to transiently block phosphorylation. The diversity of proteins modified by O-GlcNAc implies its importance in many basic cellular and disease processes. Here we systematically examined where this modification taks place and if the phosphorylation sites are indeed involved also in O-glycosylation. In conclusion, the results from this study show that both N- and C-terminus of β-catenin protein are getting O-glycosylated. The Armadillo segment is not post-translationally modified by O-glycosylation. The phosphorylation sites are not vital for O-glycosylation and also that O-glycosylation has an effect on the protein's subcellular localization and transcriptional activity.

4.3 Future Directions

F9 teratocarcinoma cells in which β-catenin gene is knocked-out were generated by Dr. FUKUNAGA YOSHITAKA (Yoshitaka et al, 2005) . F9 teratocarcinoma cells in which β-catenin and/or plakoglobin genes are knocked-out were generated and investigated in an effort to define the role of β-catenin and plakoglobin in cell adhesion. They showed that loss of only β-catenin expression did not affect cadherin-mediated cell adhesion activity. However, loss of both β-catenin and plakoglobin expression severely affected the strong cell adhesion activity of cadherin. These data confirmed that plakoglobin can compensate for the absence of β-catenin. These β-catenin defficient cells could be used in the future experiments in order to eliminate any interference of endogenous protein with the generated constructs and do more in-depth TOPFlash experimrnts.

Immunofluorescence experiments using GFP constructs to see localization of different constructs within the cell. This would allow of the *in vivo* monitoring of β-catenin transportation in cytosol, nucleus and cellular membrane.

Alternatively, we should also determined levels of O-GlcNAcylated proteins by immunoprecipitation of the proteins with their respective antibodies (anti-β-catenin) and identification of the O-GlcNAc-modification with WGA analyzed by Western blot for O-GlcNAc with horseradish peroxidase labeled WGA (WGA-HRP).

Determine specific amino acid in the C-terminus and N-terminus that is getting O-glycosylated and mutate it in order to determine O-glycosylation sites in the N-terminus. This could be done by making sequential constructs of either N or C terminus and checking their O-glycosylation potential. Once the shorter construct with O-GlycNAc potential is identified the site will be defined by making even shorter constructs until the specific Serine or Threonine is recognized.

Specifically examine, Serine 23, which was predicted by YINGOYANG server and has been shown to be the site of O-glycosylation for plakoglobins. Site-directed Mutagenesis could be employed to specifically mutate Serine 23 and check for O-glycosylation via Wheat Germ Agglutinine or /and some additional methods.

Also, it would be important to identify the sites of O-glycosylation in order to produce specific antibodies to O-glycosylated β-catenin and replace the less specific methods of detecting the glycosylated form of the protein.

Furthermore, it would be interesting to determine if cytoplasmic O-glycosylation regulates β-catenin's association with cellular interactors.

References

Aberle H, Bauer A, Stappert J, Stappert J, Kispert A and Kemler R (1997) β-catenin is a target for the ubiquitin–proteosome pathway. EMBO J 1997;16:3797–3804.

Baldin, V., Lukas, J., Marcote, M.J., Pagano, M. & Draetta, G (1993) Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev.* **7**, 812–821.

Baldwin ME, Stacker SA and Achen MG (2002) Molecular control of Lymphanogiogenesis. *Bioessays* **24:** pp 1030-1040.

Barker N, Clevers H. Catenins (2000) Wnt signaling and cancer. Bioessays 22:961-965.

Barth, A. I.M., Pollack, A. L., Altschuler, Y., Mostov, K. E., Nelson, W. J. (1997). NH2-terminal Deletion of beta -Catenin Results in Stable Colocalization of Mutant beta -Catenin with Adenomatous Polyposis Coli Protein and Altered MDCK Cell Adhesion. *J. Cell Biol.* 136: 693-706.

Bartkova J, Lukas J, Strauss M and Bartek J (1994) The PRAD-1/cyclin D1 oncogen product accumulates aberrantly in a subset of colorectal carcinomas. *Int J Cancer* **58:** pp 568-73

Behrens, J., Jerchow, B.A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D. and Birchmeier, W. (1998) Functional interaction of an axin homolog, conductin, with β-catenin, APC, and GSK3β.Science **280**, 596–599

Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosshеld R and Birchmeier W (1996) Functional interactions of β-catenin with the transcription factor LEF-1. *Nature* **382:** pp 638-642.

Belenkakya TY, Han C, Standley HJ, Lin X et al. (2002) Pygopus encodes a nuclear protein essential for Wingless/Wnt signaling. *Development* **129:** pp 4089-4101.

Berns EM, Klijn JG, Smid M, van Staveren IL, Look MP, van Putten WL and Foekens JA (1996) TP53 and MYC gene alterations independently predict poor prognosis in breast cancer patients. *Genes Chromosomes Cancer* **16:** pp 170-179.

Bhanot P, Brink M, Samos CH, et al. A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature 382:225-230, 1996.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., Nusse, Nusse (1996). A new member of the frizzled family from Drosophila functions as a wingless receptor. Nature **382(6588)**: 225-230.

Bhavanandan, V. P., and Katlic, A. W. (1979) The interaction of wheat germ agglutinin with sialoglycoproteins. The role of sialic acid. J Biol Chem. **254**,4000-8.

Bienz M and Clevers H (2000) Linking colorectal cancer to Wnt signaling. *Cell* **103:** pp 311-320.

Boutros, M., Paricio, N., Strutt, D.I., Mlodzik, M. (1998). Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell **94(1)**: 109--118.

Brabletz, T., Jung, A., Reu, S., Porzner, M., Hlubek, F., Kunz-Schughart, L. A., Knuechel, R., Kirchner, T. (2001). Variable beta -catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc. Natl. Acad. Sci. USA* 98: 10356-10361

Brannen M, Gomperts M, Sumoy L, Moon RT and Kimelman D (1997) A β-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. *Gene Dev.* **11:** pp 2359-2370.

Brantjes H, Roose J, van der Wetering M, Clevers H (2001) All Tcf HMG box transcription factors interact with Groucho-related co-repressor. *Nucleic Acid Research* **29:** pp 1410-1419.

Cadigan KM and Liu YI (2005). Wnt signaling: complexity at the surface. *Jour cell Sci.* **199:** 395-402.

Callanan M, Leroux D, Magaud JP and Rimokh R (1996) Implication of cyclin D1 in malignant lymphoma. *Crit Rev Oncog.* **7:** pp 191-203.

Chan EF, Gat U, McNiff JM and Fuchs E (1999) A common human skin tumor is caused by activating mutations in β-catenin. *Nature Genet.* **21:** pp 410-413.

Chen G, Fernandez J, Mische S, Courey AJ (1999) A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes Dev.* **13:** pp 2218-2230.

Chesire DR, Ewing CH, Sauvageot J, Bova GS and Isaacs WB (2000) Detection and analysis of β-catenin mutations in prostate cancer. *Prostate* **4:** pp 323-334.

Chesire DR, Isaacs WB (2003). β-catenin signaling in prostate cancer: an early perspective. *Endoc. Relat. Cancer.* 10: 537-560.

Chou TY, Heart GW and Dang CV (1995) c-Myc is glycosylated at threonine 58, a known phosphorylation site and a mutational hot spot in lymphomas. *J Biol Chem.* **270:** pp 18961-18965.

Comer FI, Hart GW. (2001) Reciprocity between O-GlcNAc and O-phosphate on the carboxyl terminal domain of RNA polymerase II. *Biochemistry.* 40:7845-7852.

Comer, F. I., and Hart, G. W. (2000). O-Glycosylation of nuclear. and cytosolic proteins. Dynamic interplay between O-GlcNAc. and O-phosphate. *J Biol Chem* **275**, 29179-29182

Cong F, Schweizer L and Varmus H (2004) Wnt signals across the plasma membrane to activate the β-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP *Development* **131:** pp 5103-5115.

Cottrell S, Bicknell D, Kaklamanis L, Bodmer WF (1992) Molecular analysis of APC mutations in familial adenomateous polyposis and sporadic colon carcinoma. *Lancet* **340:** pp 626-630.

Cox RT, Pai LM, Miller JR, Orsulic S, Stein J, McCormick CA et al. (1999) Membrane-tethered Drosophila Armadillo cannot transducer Wingless signal on its own. *Development* **126:** pp 1327-1335.

Crawford HC, Fingelton BM, Rudolph-Owen LA, Hepner-Gross KJ et al. (1999) The metalloproteinase matrilysin is a target of β-catenin transactivation in intestinal tumors. *Oncogene* **18:** pp 2883-2891.

Dang CV (1999) c-Myc target genes involved in cell growth, apoptosis and metabolism. *Mol Cell Biol.* **19:** pp 1-11.

Dang CV, Resar LMS, Emison E, Kim S, Li Q et al. (1999) Function of the c-Myc oncogenic transcription factor. *Exp. Cell Res.* **253:** pp 63-77.

Davidson G, Wu W, Shen J, Bilic J, Fenger U, Stannek P, Glinka A,Niehrs C (2005) Casein kinase 1γ couples Wnt receptor activation to cytoplasmic signal transduction. Nature 438: 867–872.

de la Coste A, Romangolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, Fabre M, Chelly J, Beldjord C, Khan A and Perret C (1998) Somatic mutations of the β-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proct. Natl. Acad. Sci. USA* **95:** pp 8847-8852.

de Nigris F, Sica V, Herrmann J, Condorelli G, Chade AR, Tajana G, Lerman A, Lerman LO, Napoli C (2003) c-Myc oncoprotein: cell cycle-related events and new therapeutic challenges in cancer and cardiovascular diseases. *Cell Cycle.* ;**2:**325-328. Review.

Dingwall C and Laskey RA (1991) Nuclear targeting sequence-a consensus? *Trends Biochem. Sci.* **16:** pp 478-481.

Dvorak HF (2002) Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol.* **20:** 4368-4380.

Eswaran V, Lee SH, Inge L, Guo L, Goldbeck C et al. (2003) β-catenin regulates vascular endothelial growth factor expression in colon cancer. *Cancer Research* **63:** 3145-3153.

Erisman MD, Scott JK, Astrin SM. (1989) Evidence that the familial adenomatous polyposis gene is involved in a subset of colon cancers with a complementable defect in c-Myc regulation. *Proc Natl Acad Sci USA.* **86:** 4264-4280.

Erisman MD, Scott JK, Watt RA, Astrin SM. (1988) The c-myc protein is constitutively expressed at elevated levels in colorectal carcinoma cell lines. *Oncogene.* **2:** 367-378.

Fagotto F, Gluck U and Gumbiner BM (1998) Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin. *Curr Biol.***8:** 181-190.

Fanto M, Weber U, Strutt DI, Mlodzik M (2000) Nuclear signaling by Rac and Rho GTPases is required in the establishment of epithelial planar polarity in the Drosophila eye.*Curr Biol.* **10(16):** pp 979-988.

Funayama N, Fagotto F, McCrea P and Gumbiner BM (1995) Embryonic axis induction by the armadillo repeat domain of β-catenin: evidence for intracellular signaling. *J. Cell Biol.* **128:** pp. 959–968.

Gao Y, Wells L, Comer FI, Parker GJ, Hart GW (2001) Dynamic O-glycosylation of nuclear and cytosolic proteins: cloning and characterization of a neutral, cytosolic β-N-acetylglucosaminidase from human brain. *J Biol Chem* **276**: 9838-9845.

Gavert, N., Conacci-Sorrell, M., Gast, D., Schneider, A., Altevogt, P., Brabletz, T., Ben-Ze'ev, A. (2005). L1, a novel target of β-catenin signaling, transforms cells and is expressed at the invasive front of colon cancers. *J. Cell Biol.* 168: 633-642

Geiger B, Yehuda-Levenberg S and Bershadsky AD (1995) Molecular interactions in the submembrane plaque of cell-cell and cell-matrix adhesions. *Acta Anat.* **154:** 46-62.

Gerstein AV, Almeida TA, Zhao G, et al. (2002) APC/CTNNB1 (β-catenin) pathway alterations in human prostate cancers. *Genes Chromosomes.Cancer* **34:** 9-16.

Giles RH, van Es JH, Clevers H (2003) Caught up in a Wnt storm: Wnt signalling in cancer. *Biochem. Biophys. Act.* **1653(1):** pp 1-24.

Goldman MA. (1997) Executive decision: chromatin structure and gene regulation. *Trends Genet.* **13:** pp 387-388.

Gorlich D and Mattaj IW (1996) Nucleocytoplasmic transport. *Science* **271:** pp 1513-1518.

Gradl D, Kuhl M and Wedlich D (1999) The Wnt/Wg signal tranducer β-catenin controls fibronectin expression. *Mol. Cell Biol.* **19(8):** pp 5576-5587.

Griffith LS and Schmitz G (1999) O-linked N-acetylglucosamine levels in cerebellar neurons respond reciprocally to perturbations of phosphorylation. *Eur. J Biochem.* **262:** pp 824-831.

Haltiwanger RS, Grove K, Philipsberg GA (1998) Modulation of O-linked N-acetylglucosamine levels on nuclear and cytoplasmic proteins in vivo using the peptide O-GlcNAc-beta-N-acetylglucosaminidase inhibitor O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate.*J Biol Chem.* **273(6):**3611-3617.

Harris TJ, Peifer M (2005) Decisions, decisions: β-catenin chooses between adhesion and transcription. *Trends Cell Biol* **15:** 234–237

Hart GW (1997) Dynamic *O*-linked glycosylation of nuclear and cytoskeletal proteins. *Annu. Rev. Biochem.*, **66:** 315–320.

Hao XP, Pretlow TG, Rao JS, Pretlow TP. β-catenin expression is altered in human colonic aberrant crypt foci. Cancer Res 2001; 61: 8085-8088.

Hazelton D, Nicosia RF and Nicosia SV (1999) Vascular endothelial growth factor levels in ovarian cyst fluid correlate with malignancy. *Clin. Cancer Res.* **5:** pp 823-829.

He TC, Sparks AB, et al. (1998). Identification of c-myc as a target of the APC pathway. *Science.* **281:** 1509-1512.

He X, Semenov M, Tamai K and Zeng X (2004) LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. *Development.* **131(8):**1663-1677.

Hecht A, Vleminckx K, Stemmler MP, van Roy F and Kemler R (2000) The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *EMBO J.* **19(8):**1839-1850.

Heisenberg CP, Tada M, Rauch GJ, Saúde L, Concha ML, Geisler R, Stemple DL, Smith JC, Wilson SW (2000) Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature.* **405(6782):** 76-81.

Henderson BR, Fagotto F (2002) The ins and outs of APC and β-catenin nuclear transport. *EMBO Rep* **3:** 834–839.

Hinck L, Nelson WJ and Papkoff J (1994) Wnt-1 modulates cell-cell adhesion in mammalian cells by stabilizing beta-catenin binding to the cell adhesion protein cadherin. *J Cell Biol.* **124(5):** 729-41.

Hino S, Michiue T, Asashima M and Kikkuchi A (2003) Casein kinase I epsilon enhances the binding of Dvl-1 to Frat-1 and is essential for Wnt-3a-induced accumulation of beta-catenin. *J Biol Chem.* **278:** 14066-73.

Huber AH, Nelson WJ and Weis WI (1997) Three-dimensional structure of the armadillo repeat region of β-catenin. *Cell* **90(5):** pp 871-882.

Huber O, Korn R, McLaughlin J, Ohsugi M, Herrmann BG, Kemler R (1996) Nuclear localization of β-catenin by interaction with transcription factor LEF-1. *Mech Dev.* **59(1):** 3-10.

Hurlin PJ, Quéva C, Koskinen PJ, Steingrímsson E, Ayer DE, Copeland NG, Jenkins NA, Eisenman RN (1995) Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-myc dependent transformation and are expressed during neural and epidermal differentiation. *EMBO J.* **14(22):** 5646-5659.

Itoh H, Hirata K and Ohsato K (1993) Turcot's syndrome and familial adenomatous polyposis associated with brain tumor: review of related literature. *Int J Colorectal Dis.* **8(2):** 87-94.

Jackson SP and Tijan R (1988) O-glycosylation of eukaryotic transcription factor: implication for mechanisms of transcriptional regulation. *Cell* **55:** pp 125-33.

Jenny A and Mlodzik M (2006) Planar cell polarity signalling: A common mechanism for cellular polarization. *The Mount Sinai Journal of Medicine* **73:** pp 738-748.

Jessen S, Gu B, Dai X (2008) Pygopus and the Wnt signaling pathway: a diverse set of connections. *Bioessays.* **30(5):** 448-56.

Jung, A., Schrauder, M., Oswald, U., Knoll, C., Sellberg, P., Palmqvist, R., Niedobitek, G., Brabletz, T., Kirchner, T. (2001). The Invasion Front of Human Colorectal Adenocarcinomas Shows Co-Localization of Nuclear β-catenin, Cyclin D1, and p16INK4A and Is a Region of Low Proliferation. *Am. J. Pathol.* 159: 1613-1617.

Kamemura K and Hart GW (2003) Dynamic interplay between O-glycosylation and O-phosphorylation of nucleocytoplasmic proteins: a new paradigm for metabolic control of signal transduction and transcription. *Prog Nucleic Acid Res Mol Biol.* **73:**107-136.

Kamemura K, Hayes BK, Comer FI and Hart GW (2002) Dynamic interplay between O-glycosylation and O-phosphorylation of nucleocytoplasmic proteins: alternative glycosylation/phosphorylation of THR-58, a known mutational hot spot of c-Myc in lymphomas, is regulated by mitogens. *J Biol Chem.* **277(21):**19229-19235.

Kawasoe T, Furukawa Y, Daigo Y, Nishiwaki T, Ishiguro H, Fujita M, Satoh S, Miwa N, Nagasawa Y, Miyoshi Y, Ogawa M, Nakamura Y (2000) Isolation and characterization of a novel human gene, DRCTNNB1A, the expression of which is down-regulated by β-catenin. *Cancer Res.* **60:**3354-3358.

Keembiyahatty CN, Candelaria RP, Majumdra G, Raghow et al (2002) Paradoxical regulation of Sp1 transcription factor by glucagon. *Endocrinology* **143:** pp 1512-1529.

Kelly WG, Dahmus ME and Hart GW (1993) RNA polymerase II is a glycoprotein. Modification of the COOH-terminal domain by O-GlcNAc. *J Biol. Chem.* **268:** pp 10416-10424.

Kemler R (1993) From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* **9:** pp 317-321.

Kikuchi A (2003) Tumor formation by genetic mutations in thecomponents of the Wnt signaling pathway. *Cancer Sci* **94:** 225–229.

Kim K, Pang KM, Evans M, Hay ED (2000) Overexpression of beta-catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. Mol Biol Cell; 11: 3509-3523.

Kinzler KW and Vogelstein B (1996) Lessons from hereditary colorectal cancer. *Cell* **87:** pp 159-170.

Kline RC, Wharton, Atkinson EN, Burke TW, Gershenson DM, and Edwards CL (1990) Endometriod carcinoma of the ovary: retrospective review of 145 cases. *Gynecol. Oncol.* **39:** pp 337-346.

Klymkowsky MW (2005) β-catenin and its regulatory network. *Hum Pathol* **36:** 225–227.

Kohn AD and Moon RT (2005) Wnt and calcium signalling: β-catenin independent pathways. *Cell Calcium* **38:** pp 439-446.

Koike M, Kose S, Furuta M, Taniguchi N, Yokoya F, Yoneda Y, Imamoto N (2004) β-catenin shows an overlapping sequence requirement but distinct molecular interactions for its bidirectional passage through nuclear pores. *J Biol Chem.* **279(32):** 34038-47.

Kramps T, Peter O, Bunner E, Nellen D et al. (2002) Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear β-catenin-TCF complex. *Cell* **109:** pp 47-60.

Kreppel LK, Blomberg MA, Hart GW. (1997) Dynamic glycosylation of nuclear and cytosolic proteins. Cloning and characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats. *J Biol Chem* **272**: 9308-9315.

Kuhl M, Sheldahl LC et al. (2001)Antagonistic regulation of convergent extension movements in Xenopus by Wnt/beta-catenin and Wnt/Ca2+ signaling. *Mech Dev.* **106:** 61-76.

Kuhl M, Sheldahl LC, Park M, Miller JR and Moon RT (2000) The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. *Trends Genet.* **16:** 279-83.

Laken et al. (1999) Analysis of masked mutations in familial adenomatous polyposis. *Proc. Natl. Acad. Sci. USA.* **96:** pp 2322-2326.

Lamszus K, Lengler U, Schmidt NO, Stavrou D, Ergün S, Westphal M (2000) Vascular endothelial growth factor, hepatocyte growth factor/scatter factor, basic fibroblast growth factor, and placenta growth factor in human meningiomas and their relation to angiogenesis and malignancy. *Neurosurgery* **46:** pp 938-947.

Laurent MN, Blitz IL, Hashimoto C, Rothbächer U, Cho KW (1997) The Xenopus homeobox gene twin mediates Wnt induction of goosecoid in establishment of Spemann's organizer. *Development* **124:** 4905-4916.

Lee JS, Ishimoto A and Yanagawa S (1999) Characterization of mouse dishevelled (Dvl) proteins in Wnt/Wingless signalling pathway. *J. Biol. Chem.* **274:** pp 21464-21470.

Li L, Yuan H, Weaver CD, Mao J, Farr GH 3rd , Sussman DJ, Jonkers J, Kimelman D, Wu D (1999) Axin and Frat1 interact with dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. *EMBO J.* **18(15):**4233-40.

Little CD, Nau MM, Carney DN, Gazdar AF, Minna JD (1983) Amplification and expression of the c-myc oncogene in human lung cancer cell lines. *Nature* **306:** pp 194-196.

Li C., Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X, and He X (2002) Control of the β-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* **108**: pp 837–847

Majumdar G, Harrington A, Hungerford J, Martinez-Hernandez A, Gerling IC, Raghow R, Solomon S (2006) Insulin dynamically regulates calmodulin gene expression by sequential O-glycosylation and phosphorylation of sp1 and its subcellular compartmentalization in liver cells. *J Biol Chem.* **281(6):** 3642-50.

Majumdar G, Wright J, Markowitz P, Martinez-Hernandez A, Raghow R, Solomon SS (2004) Insulin stimulates and diabetes inhibits O-linked N-acetylglucosamine transferase and O-glycosylation of Sp1. *Diabetes* **53:** pp 3184-3192.

Malik HS, Eickbush TH and Goldfarb DS (1997) Evolutionary specialization of the nuclear targeting apparatus. *Proc. Natl. Acad. Sci. USA.* **94:** PP 13738-13742.

Mann B, Gelos M, Siedow A, Hanski ML, Gratchev A, Ilyas M, Bodmer WF, Moyer MP, Riecken EO, Buhr HJ, Hanski C. Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-

factor signaling in human colorectal carcinomas. *Proc Natl Acad Sci U S A.* 1999; 96: 1603–1608.

McCrea PD, Turck CW, Gumbiner B (1991) Homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin. *Science* **254:** pp 1359-1361.

McKendry R, Hsu SC, Harland RM, Grosschedl R (1997) LEF-1/TCF proteins mediate wnt-inducible transcription from the Xenopus nodal-related 3 promoter. *Dev Biol.* **192:** pp 420-431.

Melchior F, Paschal B, Evans J and Gerace L (1993) Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J Cell Biol.* **123:**1649-1659.

Miyoshi Y, Ando H, Nagase H, Nishisho I, Horii A, Miki Y, Mori T, Utsunomiya J, Baba S, Petersen G, et al. (1992) Germ-line mutations of the APC gene in 53 familial adenomatous polyposis patients. *Proc Natl Acad Sci U S A.* **89(10):**4452-4456.

Miyoshi Y, Iwao K, Nagasawa Y, Aihara T, Sasaki Y, Imaoka S, Murata M, Shimano T, Nakamura Y (1998) Activation of the beta-catenin gene in primary hepatocellular carcinomas by somatic alterations involving exon 3. *Cancer Res.* **58:**2524-7.

Molenaar M, van de Wetering M, Oosterwegel M, Peterson-Maduro J, Godsave S, Korinek V, Roose J, Destrée O, Clevers H (1996) XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell.* **86(3):**391-399.

Moon RT, Kohn AD, De Ferrari GV, Kaykas A (2004) WNT and β-catenin signalling: diseases and therapies. *Nat Rev Genet.* **5(9):**691-701.

Moore MS and Blobel G (1993) The GTP-binding protein Ran/TC4 is required for protein import in the nucleus. *Nature* **365:** pp 661-663.

Morin PJ. (1999) β-catenin signaling in cancer. *Bioessay.* 21:1021-1030.

Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, et al. (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275:**1787-1790.

Nagafuchi A, Ishihara S, Tsukita S (1994) The roles of catenins in the cadherin-mediated cell adhesion: functional analysis of E-cadherin-alpha catenin fusion molecules. *J Cell Biol.* **127:**235-245.

Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ, Lawitts JA, Benjamin L, Tan X, Manseau EJ, Dvorak AM, Dvorak HF (2002) Vascular permeability factor/vascular

endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J Exp Med.* **196:**1497-1506.

Napoli C, Lerman LO, de Nigris F, Sica V (2002) c-Myc oncoprotein: a dual pathogenic role in neoplasia and cardiovascular diseases? *Neoplasia.* **4(3):**185-190. Review.

Nelson WJ, Nusse R (2004) Convergence of Wnt, β-catenin, and cadherin pathways. *Science* **303:** 1483–1487.

Neufeld KL, Nix DA, Bogerd H, Kang Y, Beckerle MC, Cullen BR, White RL (2000) Adenomatous polyposis coli protein contains two nuclear export signals and shuttles between the nucleus and cytoplasm. *Proc Natl Acad Sci U S A.* **97:**12085-12090.

Nigg EA (1997) Nucleoplasmic transport: signals, mechanism and regulation. *Nature* **386:** pp 779-787.

Nigg EA (1992) Assembly and cell cycle dynamics of the nuclear lamina. *Semin Cell Bio.* **3(4):** pp 245-253.

Noordermeer, J., Klingensmith, J., Perrimon, N., Nusse, R. (1994). dishevelled and armadillo act in the wingless signalling pathway in Drosophila. Nature **367(6458)**: 80--83.

Nusse R & Varmus HE (1992) Wnt genes. Cell. 69: 1073-1087.

Orsulic S and Peifer M (1996) An in vivo structure-function study of armadillo, the beta-catenin homologue, reveals both separate and overlapping regions of the protein required for cell adhesion and for wingless signaling. *J Cell Biol.* **134:**1283-1300.

Ossipova O, Dhawan S, Sokol S, Green JB (2005) Distinct PAR-1 proteins function in different branches of Wnt signaling during vertebrate development. *Dev Cell.* **8:**829-841.

Palacios J and Gamallo C (1998) Mutations in the β-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. *Cancer Res.* **58:**1344-7.

Papkoff J, Rubinfeld B, Schryver B, Polakis P (1996) Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. *Mol Cell Biol.* **16:**2128-2134.

Parker DS, Jemison J, Cadigan KM (2002) Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. *Development.* **129:**2565-2576.

Peifer M, Polakis P (2000) Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus. *Science.* **287:**1606-1609.

Peifer M, Berg S, Reynolds AB (1994) A repeating amino acid motif shared by proteins with diverse cellular roles. *Cell.* **76:**789-791.

Peters JM, McKay RM, McKay JP and Graff JM (1999) Casein Kinase I transduces Wnt signals. *Nature* **401:** pp 345-350.

Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J. and Skarnes, W. C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 407,535 –538.

Polakis P (2000) Wnt signaling and Cancer. *Genes Dev.* **14:** pp 1837-1851.

Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW (1992) APC mutations occur early during colorectal tumorigenesis. *Nature.* **359:**235-237.

Powell WC, Knox JD, Navre M, Grogan TM, Kittelson J, Nagle RB, Bowden GT (1993) Expression of the metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. *Cancer Res.* **53(2):**417-422.

Riese J, Yu X, Munnerlyn A, Eresh S, Hsu SC, Grosschedl R, Bienz M (1997) LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. *Cell.* **88:**777-787.

Robbins PF, El-Gamil M, Li YF, Kawakami Y, Loftus D, Appella E, Rosenberg SA (1996) A mutated β-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. *J Exp Med.* **183:**1185-1192.

Roos MD, Su K, Baker JR, Kudlow JE (1997) O-glycosylation of an Sp1-derived peptide blocks known Sp1 protein interactions. *Mol Cell Biol.* **17:**6472-6480.

Roose J, Molenaar M, Peterson J, Hurenkamp J, Brantjes H, Moerer P, van de Wetering M, Destrée O, Clevers H (1998) The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature.* **395:**608-12.

Rosin-Arbesfeld R, Townsley F and Bienz M (2000) The APC tumor suppressor has a nuclear export function. *Nature* **406:** pp 1009-1012.

Rothbächer U, Laurent MN, Deardorff MA, Klein PS, Cho KW, Fraser SE (2000) Dishevelled phosphorylation, subcellular localization and multimerization regulate its role in early embryogenesis. *EMBO J.* **19(5):**1010-1022.

Rubinfeld B, et al. (1996). Binding of GSK3b to the APC- β-catenin complex and regulation of complex assembly. Science. 272:1023-6.

Rubinfeld B, et al. (1995). The APC protein and E-cadherin form similar but independent complexes with a-catenin, β -catenin, and plakoglobin. J Biol Chemistry. 270:5549-55.

Rubinfeld B, Robbins P, El-Gamil M, Albert I, Porfiri E, Polakis P (1997) Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science.* **275:**1790-1792.

Saeki H, Tanaka S, Sugimachi K, Kimura Y, Miyazaki M, Ohga T, Sugimachi K (2002) Interrelation between expression of matrix metalloproteinase 7 and β-catenin in esophageal cancer. *Dig Dis Sci.* **47:**2738-2742.

Satoh S, Daigo Y, Furukawa Y, Kato T, Miwa N, Nishiwaki T, Kawasoe T, Ishiguro H, Fujita M, Tokino T, Sasaki Y, Imaoka S, Murata M, Shimano T, Yamaoka Y, Nakamura Y (2000) AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. *Nat Genet.* **24:**245-50.

Shimizu H, Julius MA, Giarré M, Zheng Z, Brown AM, Kitajewski J (1997) Transformation by Wnt family proteins correlates with regulation of beta-catenin. *Cell Growth Differ.***8:**1349-1358.

Shtutman M, Zhurinsky J, Simcha I, Albanese C et al. (1999) The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. USA.* **96:** pp 5522-5527.

Siegfried, E., Wilder, E.L., Perrimon, N. (1994). Components of wingless signalling in Drosophila. *Nature* **367**: 76-80.

Sokol S (2000) A role for Wnts in morpho-genesis and tissue polarity. *Nat Cell Biol.* **2(7):** pp E124-5.

Sparks AB, Morin PJ, Vogelstein B, Kinzler KW (1998) Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Res.* **58(6):**1130-1134.

Strutt, D.I., Weber, U., Mlodzik, M. (1997). The role of RhoA in tissue polarity and Frizzled signalling. Nature **387**: 292-295.

Su LK, Johnson KA, Smith KJ, Hill DE, Vogelstein B, Kinzler KW (1993) Association between wild type and mutant APC gene products. *Cancer Res.* **53(12):**2728-31.

Su LK, Vogelstein B and Kinzler KW (1993) Association of the APC tumor suppressor proteins with catenins. *Science* **262:** pp 1734-1737.

Suh EK, Gumbiner BM (2003) Translocation of beta-catenin into the nucleus independent of interactions with FG-rich nucleoporins. *Exp Cell Res.* **290(2):**447-456.

Swiatek W, Tsai IC, Klimowski L, Pepler A et al. (2004) Regulation of casein kinase I ε activity by Wnt signaling. *J. Biol. Chem.* **279:** pp 13011-13017.

Tada, M. and Smith, J.C. (2000) Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. Development 127: 2227-2238.

Takada R, Hijikata H, Kondoh H, Takada S (2005) Analysis of combinatorial effects of Wnts and Frizzleds on beta-catenin/armadillo stabilization and Dishevelled phosphorylation. *Genes Cells.* **10(9):**919-928.

Takemaru KI, Moon RT (2000) The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. *J Cell Biol.* **149(2):**249-254.

Tamai K, Zeng X, Liu C, Zhang X, Harada Y, Chang Z, He X (2004) A mechanism for Wnt coreceptor activation. *Mol Cell.* **13(1):**149-56.

Takayasu, H., Horie, H., Hiyama, E., Matsunaga, T., Hayashi, Y., Watanabe, Y., Suita, S., Kaneko, M., Sasaki, F., Hashizume, K., Ozaki, T., Furuuchi, K., Tada, M., Ohnuma, N., Nakagawara, A. (2001). Frequent Deletions and Mutations of the β-Catenin Gene Are Associated with Overexpression of Cyclin D1 and Fibronectin and Poorly Differentiated Histology in Childhood Hepatoblastoma. *Clin. Cancer Res.* 7: 901-908

Takayama T, Shiozaki H, Shibamoto S, Oka H, Kimura Y, Tamura S, et al. β-catenin expression in human cancers. Am J Pathol 1996; 148: 39-46.

Tetsu O, McCormick. (1999) β-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 398:422-426.

The Center for Biological Sequence Analysis Prediction Server. YinOYang 1.2. October 2003. February 2007. < http://www.cbs.dtu.dk/services/YinOYang/>

Thompson B, Townsley F, Rosin-Arbesfeld R, Musisi H, Bienz M (2002) A new nuclear component of the Wnt signalling pathway. *Nat Cell Biol.* **4(5):**367-373.

Tolwinski NS, Wieschaus E (2004) A nuclear escort for beta-catenin. *Nat Cell Biol.* **6(7):**579-580.

Tolwinski NS, Wieschaus E (2004) A nuclear function for armadillo/beta-catenin. *PLoS Biol.* **2(4):**E95.

Tolwinski NS, Wieschaus E (2001) Armadillo nuclear import is regulated by cytoplasmic anchor Axin and nuclear anchor dTCF/Pan. *Development.* **128(11):**2107-2117.

Trent JM, Wiltshire R, Su LK, Nicolaides NC, Vogelstein B, Kinzler KW (1995) The gene for the APC-binding protein β-catenin (CTNNB1) maps to chromosome 3p22, a region frequently altered in human malignancies. *Cytogenet Cell Genet.* **71(4):**343-4.

Umbhauer M, Djiane A, Goisset C, Penzo-Méndez A, Riou JF, Boucaut JC, Shi DL (2000) The C-terminal cytoplasmic Lys-thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/beta-catenin signalling. *EMBO J.* **19(18):**4944-4954.

Utsunomiya T, et al. (2001) Correlation of β-catenin and cyclin D1 expression in colon cancers. *Oncology* **61:** 226–233.

van Leeuwen, F., Samos, C.H., Nusse, R. (1994). Biological activity of soluble wingless protein in cultured Drosophila imaginal disc cells. Nature **368(6469)**: 342--344.

van de Wetering, M., Bejsovec, A., Peifer, M., Mortin, M., Clevers, Mortin, Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell **88**: 789-799.

van de Wetering M., Sancho E., Verweij C., de Lau W., Oving I., Hurlstone A., van der Horn K., Batlle E., Coudreuse D., Haramis A. P., Tjon-Pon-Fong M., Moerer P., van den Born M., Soete G., Pals S., Eilers M., Medema R., Clevers H. (2002) The ß-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111:** 241-250.

van der Heyden MA, Rook MB, Hermans MM, Rijksen G, Boonstra J, Defize LH, Destrée OH (1998) Identification of connexin 43 as a functional target for Wnt signalling. *J Cell Sci.* **111:**1741-1749.

Verras M, Sun Z (2006) Roles and regulation of Wnt signaling and beta-catenin in prostate cancer. *Cancer Lett.* **237(1):**22-32.

Vleminckx K, Kemler R, Hecht A (1999) The C-terminal transactivation domain of beta-catenin is necessary and sufficient for signaling by the LEF-1/beta-catenin complex in Xenopus laevis. *Mech Dev.* **81(1-2):** 65-74.

Voeller HJ, Truica CI, Gelmann EP (1998) Beta-catenin mutations in human prostate cancer. *Cancer Res.* **58(12):**2520-3

Vogelstein B and Kinzler KW (2004) Cancer genes and the pathways they control. *Nature Med.* **10:** pp 789-799.

Wallingford JB, Rowning BA, Vogeli KM, Rothbacher U, Fraser SE & Harland RM (2000) Dishevelled controls cell polarity during Xenopus gastrulation. Nature 405: 81-85.

Weber U, Paricio N, Mlodzik M (2000) Jun mediates Frizzled-induced R3/R4 cell fate distinction and planar polarity determination in the Drosophila eye. *Development.* **127(16):**3619-3629

Wells, L., Y. Gao, J. Mahoney, K. Vosseller, C. Chen, A. Rosen, and G.W. Hart. 2001. Dynamic O-glycosylation of nuclear and cytosolic proteins: Further characterization of the nucleocytoplasmic β -N-acetylglucosaminidase, O-GlcNAcase. *J. Biol. Chem.* **277:** 1755-1761.

Westerman S and Weber K (2003) Post-translational modifications regulates microtubule function. *Nat. Rev. Mol. Cell Biol.* **4:** pp 938-947.

Willert K, Nusse R (1998) β-catenin: a key mediator of Wnt signaling. *Curr Opin Genet Dev* **8:** 95-102.

Winklbauer R, Medina A, Swain RK & Steinbeisser H (2001) Frizzled-7 signalling controls tissue separation during Xenopus gastrulation. Nature 413: 856-860.

Witty JP, McDonnell S, Newell KJ, Cannon P, Navre M, Tressler RJ, Matrisian LM (1994) Modulation of matrilysin levels in colon carcinoma cell lines affects tumorigenicity in vivo. *Cancer Res.* **54(17):**4805-4812.

Yanagawa S, van Leeuwen F et al. (1995) The disheveled protein is modified by wingless signaling in Drosophila. *Genes Dev* **9(9):** pp 1087-1097.

Yang W, Yan HX, Chen L, Liu Q, He YQ, Yu LX, Zhang SH, Huang DD, Tang L, Kong XN, Chen C, Liu SQ, Wu MC, Wang HY (2008) Wnt/β-catenin signaling contributes to activation of normal and tumorigenic liver progenitor cells. *Cancer Res.* **68(11):** 4287-4295.

Yokoya F, Imamoto N, Tachibana T and Yoneda Y (1999) β-catenin can be transported into the nucleus in a Ran-unassissted manner. *Mol. Biol. Cell.* **10:** pp 1119-1131.

Yoshitaka Fukunaga, Huijie Liu, Masayuki Shimizu, Satoshi Komiya, Michio Kawasuji and Akira Nagafuchi; "Defining the Roles of β-catenin and Plakoglobin in Cell-cell Adhesion: Isolation of β-catenin/plakoglobin-deficient F9 Cells". *Cell Struct. Funct.*, Vol. **30**, 25-34 (2005).

Zeng X, Tamai K, Doble B, Li S, Huang H, Habas R, Okamura H, J, He X (2005) A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* **438:** 873–877.

Zhang X., Gaspard J. P., Chung D. C. (2001) Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. *Cancer Res.* **61:** 6050-6054.

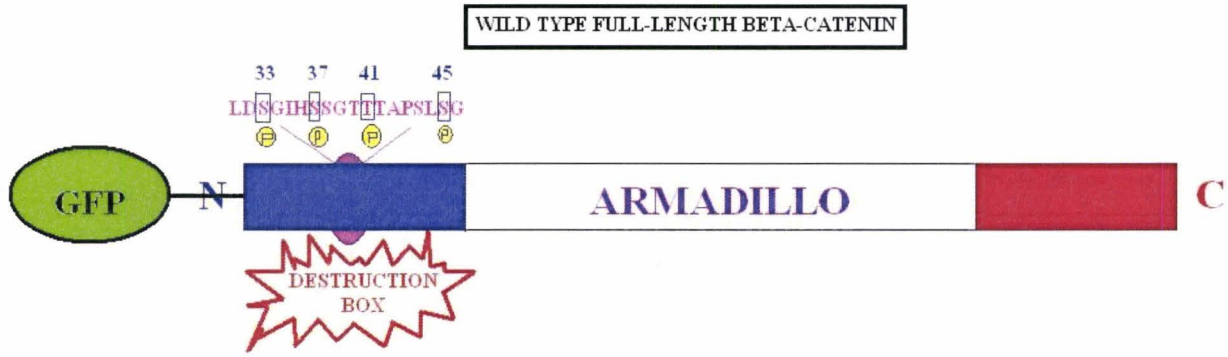
Zhu, W., Leber, B., and Andrews, D.W. (2001.). Cytoplasmic O-glycosylation prevents cell surface transport of E-cadherin during apoptosis. *EMBO J.* 20, 5999-6007.

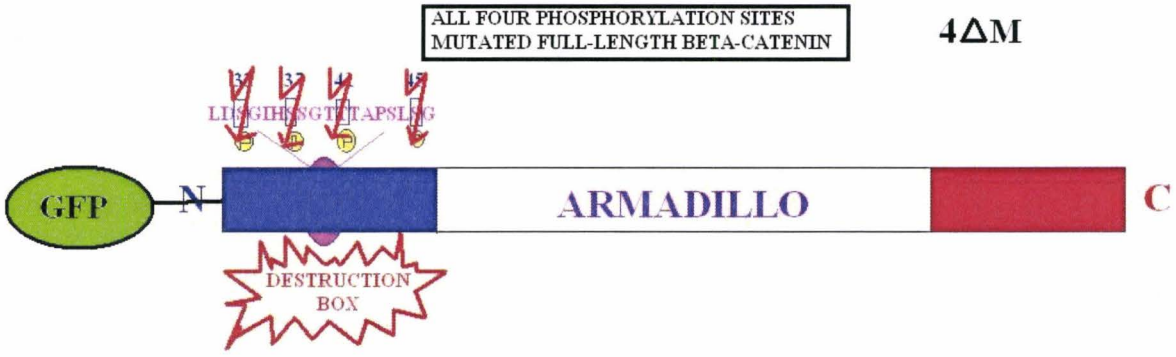
APPENDIX

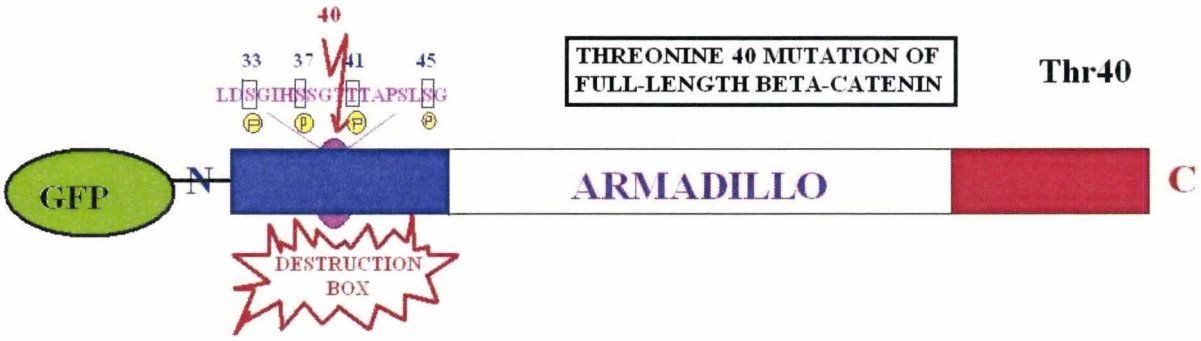
PRIMER SEQUENCE	NAME	LENGTH	POSITION
5' AATGGTGGGTACCTC ATGGCTACTCAAGCTGAT 3'	FRW-β Full-length	33	1
5' CGCGGATCCTTA-CAGGTCAGTATCAAACC 3'	REVERSE-β Full length	29	2346
5' AATGGTGGGTACCTC-ATGCATGCAGTTGTAAAAC 3'	FRW-ARMADILLO	33	400
5' CGCGGATCCTTA-CTTGTAATCTTGTGGC 3'	REVERSE-ARMADILLO	28	2013
5' AATGGTGGGTACCTC GCTACTCAAGCTGAT 3'	FRW-4ΔM Full length mutant	30	1
5' CGCGGATCCTTA-CAGGTCAGTATCAAACC 3'	Reverse-4ΔM Full length mutant	29	2346
5' AATGGTGGGTACCTC ATGGCTACTCAAGCTGAT 3'	FRW-Thr41 Full length mutant	33	1
5' CGCGGATCCTTA-CAGGTCAGTATCAAACC 3'	Reverse-Thr41 Full length mutant	29	2346
5' AATGGTGGGTACCTC-ATGGCTACTCAAGCTGAT 3'	FRW-N N-terminus	33	1
5' CGCGGATCCTTA-CAGCATCTGTGATGG 3'	REVERSE-N N-terminus	27	399
5' AATGGTGGGTACCTC-CGGCTTTCAGTTGAG 3'	FRW-C C-Terminus	30	2017
5' CGCGGATCCTTA-CAGGTCAGTATCAAACC 3'	REVERSE-C C-Terminus	29	2346
5' AATGGTGGGTACCTC-ATGCATGCAGTTGTAAAAC 3'	FRW-1ST HALF ARMADILLO	33	400
5' CGCGGATCCTTA-ACCTTCCATCCCTTCCTG 3'	REV- 1ST HALF ARMADILLO	30	1200

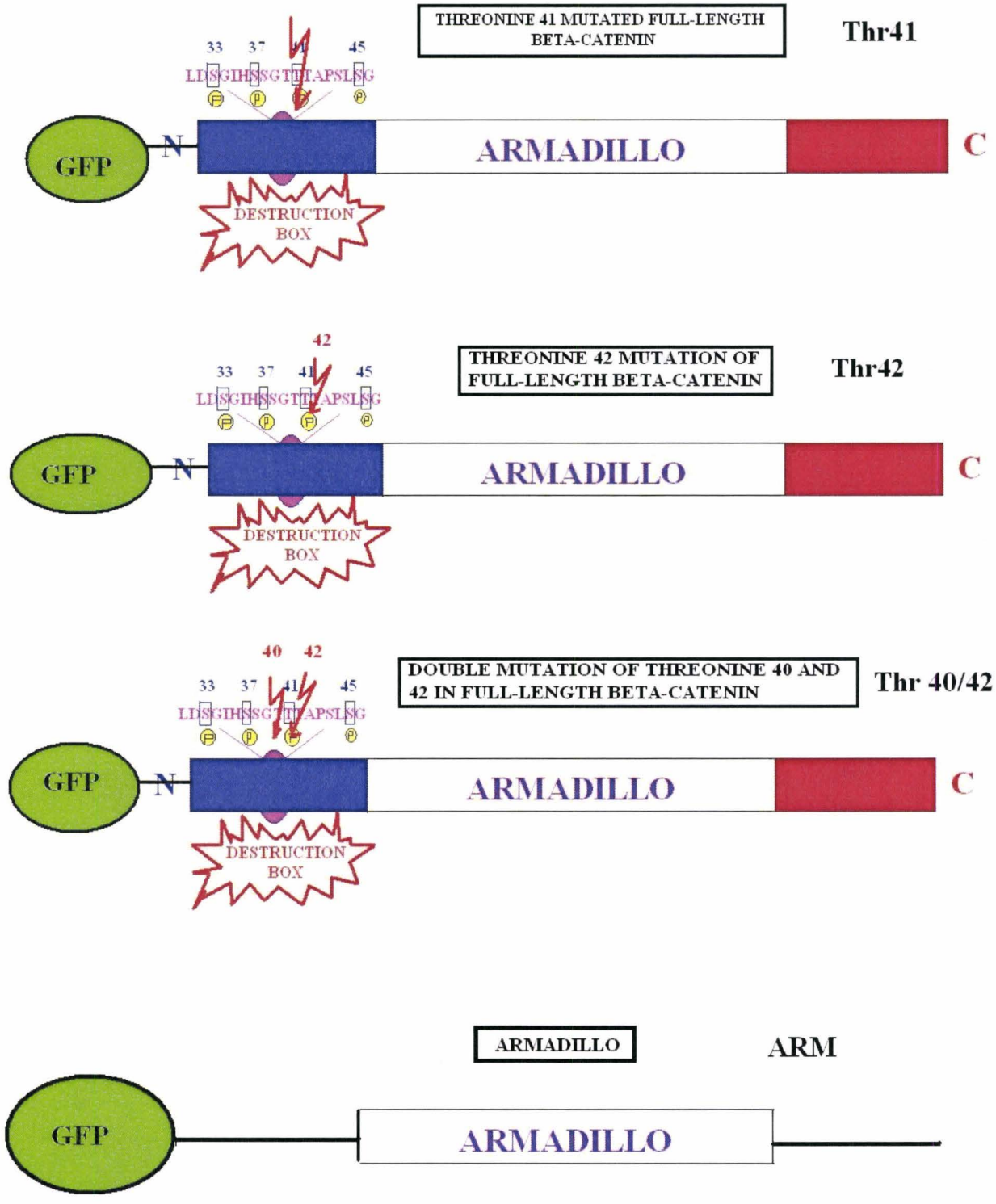
5' AATGGTGGGTACCTC-CTCCTTGGGACTCTTGTTC 3'	FRW-2ND HALF ARMADILLO	34	1201
5' CGCGGATCCTTA-CTTGTAATCTTGTGGC 3'	REV-2ND HALF ARMADILLO	28	2013

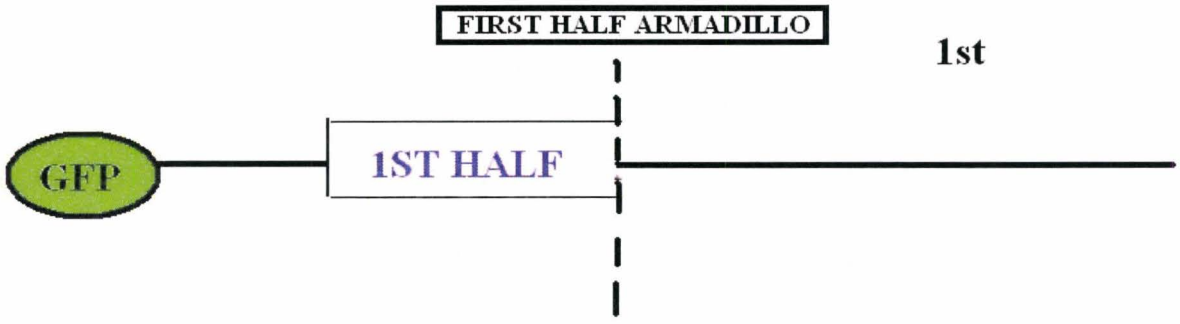
CONSTRUCTS

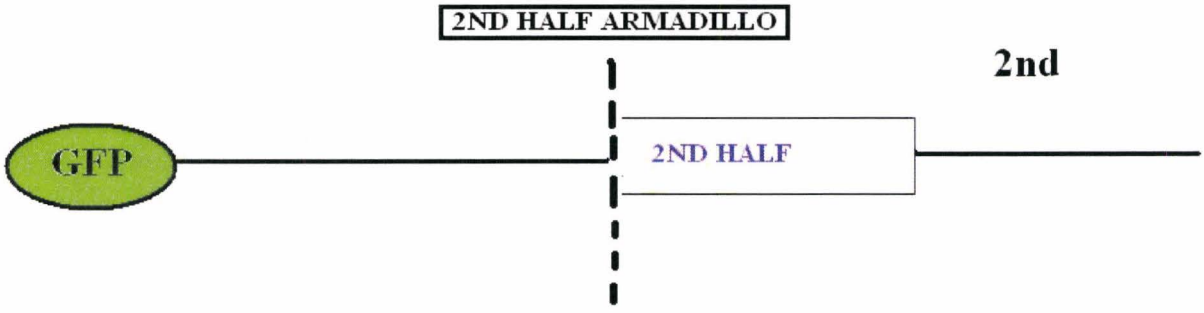

WILD TYPE FULL-LENGTH BETA-CATENIN
33 37 41 45
GFP
N
ARMADILLO
C
DESTRUCTION BOX

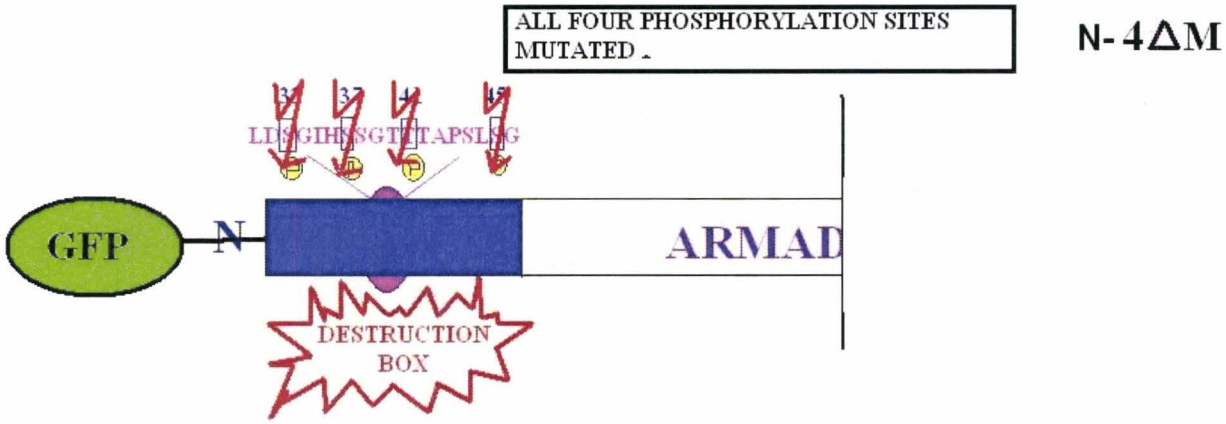

ALL FOUR PHOSPHORYLATION SITES MUTATED FULL-LENGTH BETA-CATENIN
4ΔM
GFP
N
ARMADILLO
C
DESTRUCTION BOX

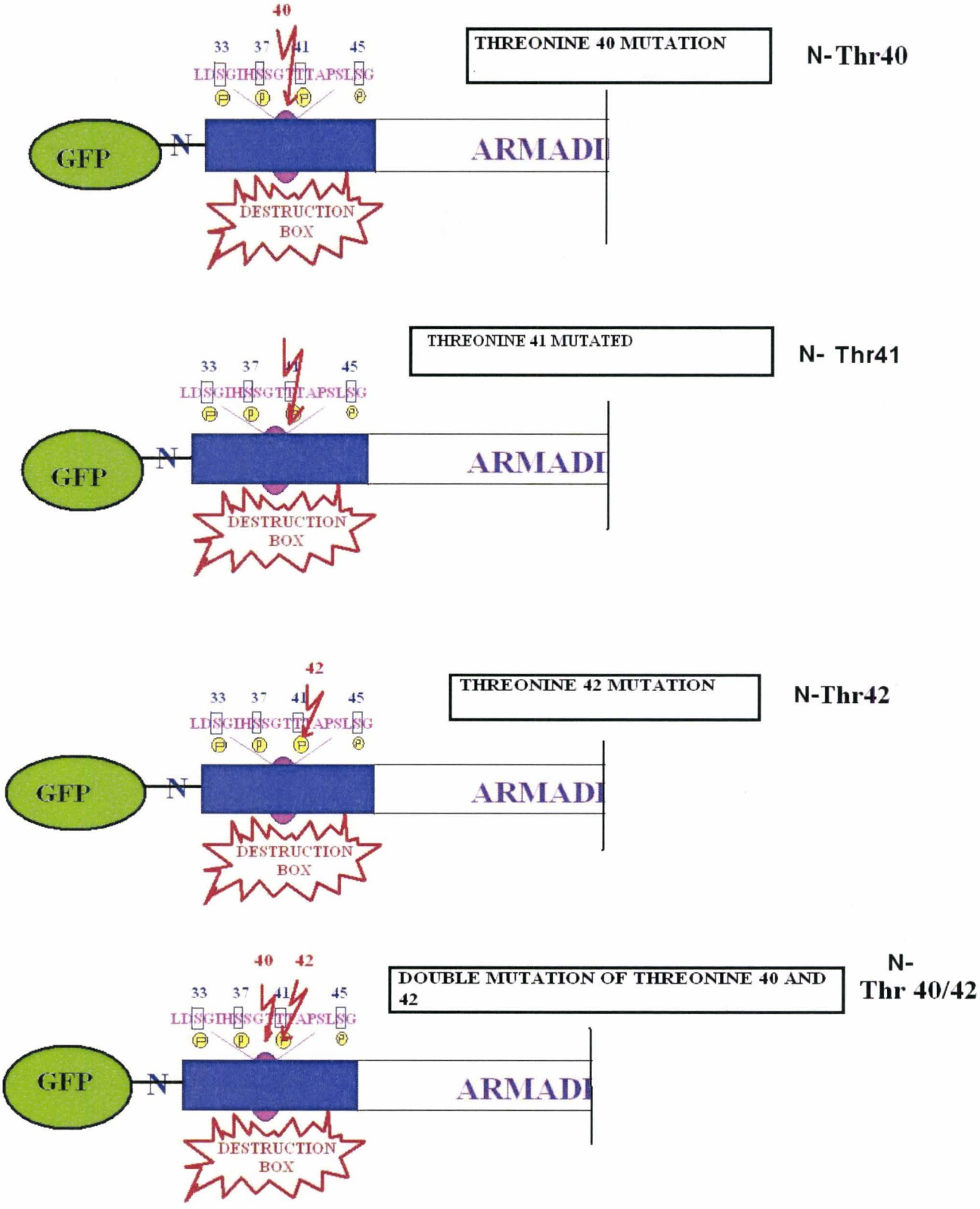

40
33 37 41 45
THREONINE 40 MUTATION OF FULL-LENGTH BETA-CATENIN
Thr40
GFP
N
ARMADILLO
C
DESTRUCTION BOX


THREONINE 41 MUTATED FULL-LENGTH BETA-CATENIN
Thr41
33 37 41 45
GFP
N
ARMADILLO
C
DESTRUCTION BOX
42
THREONINE 42 MUTATION OF FULL-LENGTH BETA-CATENIN
Thr42
33 37 41 45
GFP
N
ARMADILLO
C
DESTRUCTION BOX
40 42
DOUBLE MUTATION OF THREONINE 40 AND 42 IN FULL-LENGTH BETA-CATENIN
Thr 40/42
33 37 41 45
GFP
N
ARMADILLO
C
DESTRUCTION BOX
ARMADILLO
ARM
GFP
ARMADILLO


FIRST HALF ARMADILLO
1st
GFP
1ST HALF


2ND HALF ARMADILLO
2nd
GFP
2ND HALF


ALL FOUR PHOSPHORYLATION SITES MUTATED
N-4ΔM
GFP
N
ARMAD
DESTRUCTION BOX


40
33 37 41 45
LDSGIHSSGTTTAPSLSG
THREONINE 40 MUTATION
N-Thr40
GFP
N
ARMADI
DESTRUCTION BOX
THREONINE 41 MUTATED
N- Thr41
42
THREONINE 42 MUTATION
N-Thr42
40 42
DOUBLE MUTATION OF THREONINE 40 AND 42
N- Thr 40/42