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The actin cytoskeleton and the nuclear translocation of -catenin in human oesophageal squamous carcinoma cell lines

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Chapter One: Literature review

1.1 Epithelial development and integrity

Cell adhesion is crucial for establishing epithelial cell polarity, organization and differentiation, which subsequently leads to the proper development of the epithelium (Wodarz, 2002). Epithelia either consists of a single layer forming a simple epithelium or of two or more layers forming stratified epithelium (Squier and Kremer, 2001). Stratified epithelia have an important role in development, such as the formation of the epidermis of the skin, or the lining of the gastrointestinal tract, where it serves as a protective function (Ganz, 2002; Squier and Kremer, 2001). The formation of multiple layers in stratified epithelia requires cell adhesion events, which involve the interaction of the epithelial cells with each other and/or with the extracellular matrix (ECM), and the association with the internal cytoskeleton (Nelson, 2003; Wodarz, 2002). Clearly, the disruption of such adhesive events underlies pathological phenomena such as invasion and metastasis (Hirohashi and Kanai, 2003). It is therefore important to determine the events fundamental to cell adhesion and the association with the internal cytoskeleton in order to fully understand pathological conditions such as the above.

1.1.1 Epithelial cell adhesion

Cell adhesion events include the interaction between cell adhesion receptors and molecules present in the ECM (Wodarz, 2002). The ECM is a complex network composed of glycoproteins, proteoglycans and non-glycosylated proteins to which the epithelial cells adhere (Roughley, 2001). Another aspect of cell adhesion is the cell-cell interaction, which is mediated between cell adhesion receptors on neighbouring cells (Gooding *et al*., 2004). Importantly, these cell adhesion events are associated with the internal cytoskeleton and are critical for initiating epithelial cell polarity and development of the epithelium (Nelson, 2003; Wodarz, 2002). Epithelial cell polarity

involves the differentiation of the plasma membrane of the epithelial cell into two structurally different domains known as the apical and basolateral domains (Nelson, 2003; Wodarz, 2002; Yeaman *et al*., 1999). The apical domain faces the lumen (unattached surface), while the basolateral domain faces the serosal surface to which it attaches (Nelson, 2003). These domains perform different biological functions in accordance with their environment (Nelson, 2003).

1.1.1.a Cell-ECM interactions

Attachment of the epithelial cells to the ECM is essential for maintaining tissue integrity, cell migration, cell cycle progression and regulating tissue growth and morphogenesis (Brakebusch *et al*., 2002). The main cell adhesion receptors involved in cell-ECM interactions are the integrins, which bind to molecules present in ECM (Brakebusch *et al*., 2002; Sage, 2001). The integrins are heterodimeric transmembrane proteins composed of an α and a β subunit from a possible 18 and 8 subunits respectively (Brakebusch *et al*., 2002). The interaction between the cytoplasmic tail of the β subunit with cytoskeletal associated proteins, such as α-actinin, vinculin and tensin, allows for the complete interaction between the epithelial cell and the ECM (Yeaman *et al*., 1999). Although it is necessary to mention the cell-ECM interaction, this field is not developed further in the following text, as this study focuses on a cell-cell adhesion based molecule.

1.1.1.b Cell-cell adhesion: The cadherins and the catenins

The specific formation of epithelial tissue requires the selective interaction and aggregation between epithelial cells, which is mediated by cell-cell adhesion (Pećina-Šlaus, 2003). One of the main cell-cell adhesion receptors required for the maintenance of solid tissues are the cadherins, which are transmembrane glycoproteins (Gooding *et al*., 2004; Koch *et al*., 2004). The cadherins belong to a superfamily consisting of 80 different proteins and are categorized according to the

following: the classical type I-, classical type II- and desmosomal- cadherins, protocadherins and cadherin-like proteins (Gooding *et al*., 2004; Koch *et al*., 2004). The classical type I cadherins are classified according to their tissue distribution, that is the placental (P), neuronal (N) and the epithelial (E)-cadherin, the latter being of direct interest to this study (Pećina-Šlaus, 2003). E-cadherin plays an important role in the recognition and sorting of epithelial cells during development of specific tissue architecture (Nelson, 2003).

E-cadherin, like all classical cadherins, is composed of an amino (N)-terminal extracellular domain, a transmembrane domain and a carboxyl (C)-terminal cytoplasmic tail domain (Gooding *et al*., 2004). The extracellular (EC) part has five domains (EC1- EC5), each containing 110 amino acid residues (Gooding *et al*., 2004). This EC region of E-cadherin is involved in the homotypic interaction with another cadherin, meaning that the E-cadherin binds only to an identical molecule on adjacent cell surfaces (Figure 1.1) (Koch *et al*., 2004). It is this precise interaction, and the expression of the different cadherins in separate cell populations, which allows for the formation of specific tissues and organs (Yeaman *et al*., 1999).

There are two models which illustrate the potential homotypic interaction between E-cadherins on individual cells: 1) the linear zip model which suggests that the homotypic interaction is mediated by EC1 to EC1 (Figure 1.1.a) and: 2) the "intercalation" model, which suggests that the interaction is mediated via EC1, EC2 and EC3 with EC5, EC4 and EC3 respectively (Figure 1.1.b) (Gooding *et al*., 2004; Koch *et al*., 2004; Renaud-Young and Gallin, 2002). Another point that should be noted is that cadherin mediated cell-cell adhesion is dependent on the presence of calcium (Ca^{2+}) with four of the EC domains containing Ca^{2+} binding sites (Chitaev and Troyanovsky, 1998; Ko *et al*., 2001). Calcium maintains the structure of cadherins and prevents their degradation by proteolytic enzymes (Koch *et al*., 2004).

In order for cell-cell adhesion to be functional (as described above), it is essential that there is a linkage between the cadherins and the cytoskeleton (Imamura *et al*., 1999; Ko *et al*., 2001). The cadherins are anchored in cells by associating with a group of proteins called the catenins, which link the cytoplasmic domain of the cadherin with the actin cytoskeleton (Chitaev and Troyanovsky, 1998, Imamura *et al*., 1999).

There are three major types of catenins involved in cell-cell adhesion namely the α−, β− and γ-catenins, which are 102 kilo-Daltons (kDa), 92 kDa and 83 kDa in size, respectively (Gooding *et al*., 2004). The cytoplasmic tail of E-cadherin binds either to β-catenin or γ-catenin (also known as plakoglobin) (Chitaev and Troyanovsky, 1998). The α-catenin has two actin binding sites and binds to either β- or γ-catenin and thus links either the β- or γ-catenin, bound to the cytoplasmic tail of E-cadherin, to the actin cytoskeleton (Figure 1.1c) (Adams *et al*., 1996; Chitaev and Troyanovsky, 1998; Obama and Ozawa, 1997). Although both β- and γ-catenin bind to E-cadherin and to α-catenin, the binding affinity of β-catenin with both α-catenin and E-cadherin is higher than that of γ-catenin (Adams *et al*., 1996; Aberle *et al*., 1994; Obama and Ozawa, 1997). β-catenin therefore provides the essential link between E-cadherin and the actin cytoskeleton, thus forming the basis of functional cell-cell adhesion (Adams *et al*., 1996; Imamura *et al*., 1999).

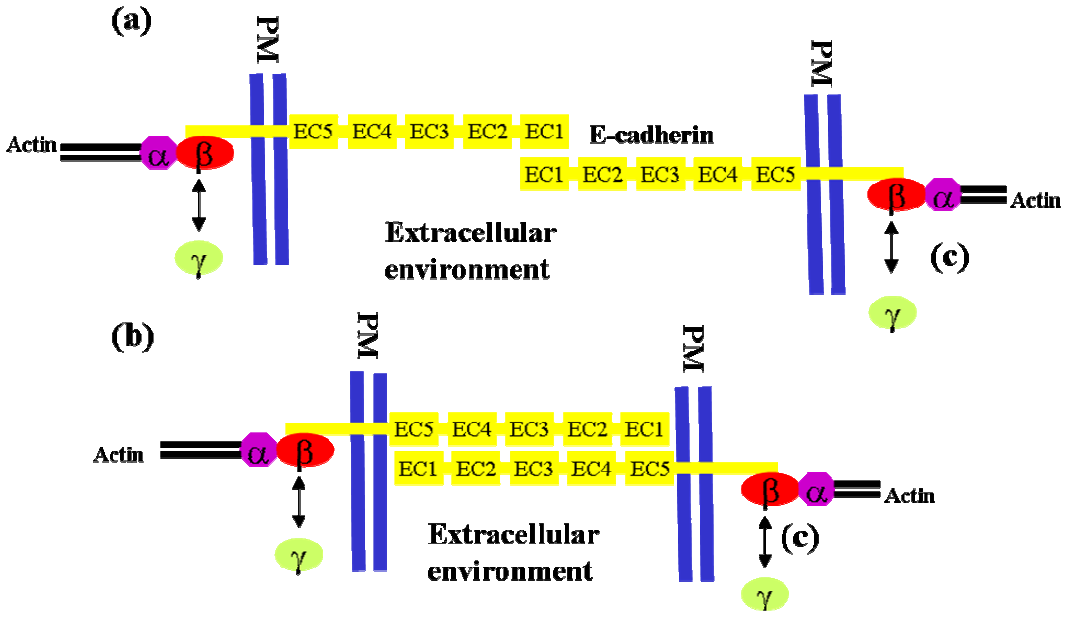


Figure 1.1 Cell-cell adhesion mediated by the E-cadherins. The cadherin extracellular (EC) portion is composed of 5 domains (EC1-EC5). **(a)** The linear zip model suggests that EC1 interacts with EC1 on cadherins present on neighbouring cells. **(b)** The intercalation model suggests that the interaction of cadherins between opposing cells is via EC1, EC2 and EC3 with EC5, EC4 and EC3 respectively. **(c)** The cytoplasmic portion of the cadherin binds either to β-catenin or γ-catenin. β-catenin or γ-catenin, binds to α-catenin, which in turn binds to the actin cytoskeleton. β = β-catenin; γ = γ-catenin; α = α-catenin; PM = plasma membrane.

1.1.2 E-cadherin, α- and β-catenin and the actin cytoskeleton

The relationship of β-catenin with E-cadherin and α-catenin is mediated by specific domains and amino acid residues present in these proteins. It is therefore necessary to briefly mention the primary structure of β-catenin although it is also discussed in the following text. β-catenin consists of an N-terminal domain of 149 amino acids, followed by the core domain of 515 residues composed of 12 copies of a 42 amino acid sequence motif, known as the armadillo (ARM) repeats, and a C-terminal 108 residue domain (Huber *et al*., 1997; Huber and Weis, 2001).

The β-catenin-E-cadherin interaction is mediated via the ARM repeats 1-12 (amino acid residues 120-683) of β-catenin and 100 amino acid residues present in the cytoplasmic domain of E-cadherin (Aberle *et al*., 1994; Huber and Weis, 2001). In a recent study it was suggested that this interaction entails the preferential binding of E-cadherin to a β-catenin-α-catenin heterodimer, rather than to a β-catenin monomer (Gottardi and Gumbiner, 2004). This β-catenin-α-catenin heterodimer is formed by the interaction between the start of the ARM repeat region of β-catenin (amino acid residues 120 and 151) with the junction of the N-terminal domain of α-catenin (Aberle *et al*., 1994; Graham *et al*., 2000; Koslov *et al*., 1997). The amino acid residues (121-141) of β-catenin form an α-helical structure, necessary for its interaction with three α–helices present within α-catenin to form a 4-helix bundle (Graham *et al*., 2000).

At the adherens junction, other components are present which are thought to play a role with the interaction of β-catenin and either α-catenin and E-cadherin. These components include the Rho family of small GTPses, such as Rac1 and Cdc42, as well as molecules involved in tyrosine phosphorylation of β-catenin (Piedra *et al*., 2003; Wheelock and Johnson, 2003).

1.1.2.a Rac1, Cdc42 and IQGAP1

Rac1 and Cdc42 are present at cadherin based cell contacts, and are thought to be involved in regulating actin assembly and organization (Wheelock and Johnson, 2003; Yap and Kovacs, 2003). Rac1 has a downstream effector, IQGAP1, which promotes polymerization and cross-linking of actin filaments thereby enhancing cell-cell adhesion (Briggs and Sacks, 2003; Noritake *et al*., 2004). However, IQGAP1 also binds to β-catenin, which results in the dissociation of α-catenin from β-catenin and therefore weakens cell-cell adhesion (Briggs and Sacks, 2003; Wheelock and Johnson, 2004). Noritake *et al*. (2004) hypothesized that this contradictory downstream effect of IQGAP1 depends on the state of Rac1, i.e. whether it is GTP-bound or GDP-bound, respectively (Figure 1.2.a).

1.1.2.b Tyrosine phosphorylation of β-catenin

Molecules such as epidermal growth factor receptor (EGF-R), Src, Fyn and Fer are all involved in the tyrosine (Tyr) phosphorylation of β-catenin, which is another mechanism to disrupt cell-cell adhesion (Fujii *et al*., 1996; Hazan and Norton, 1998; Piedra *et al*., 2003; Rosato *et al*., 1998; Tinsley *et al*., 2002). The Tyr phosphorylation of β-catenin at residues 142 and 654 destabilizes its interaction with α-catenin and E-cadherin respectively (Piedra *et al*., 2001; Piedra *et al*., 2003). There are several Tyr phosphatases (PTP), which compensate for this Tyr phosphorylation by dephosphorylating Tyr-phosphorylated β-catenin. The most well-known are Tyr phosphatase PTP-1B, Pez and SHP-1 (Figure 1.2.b) (Duchesne *et al*., 2003; Wadham *et al*., 2003; Xu *et al*., 2004).

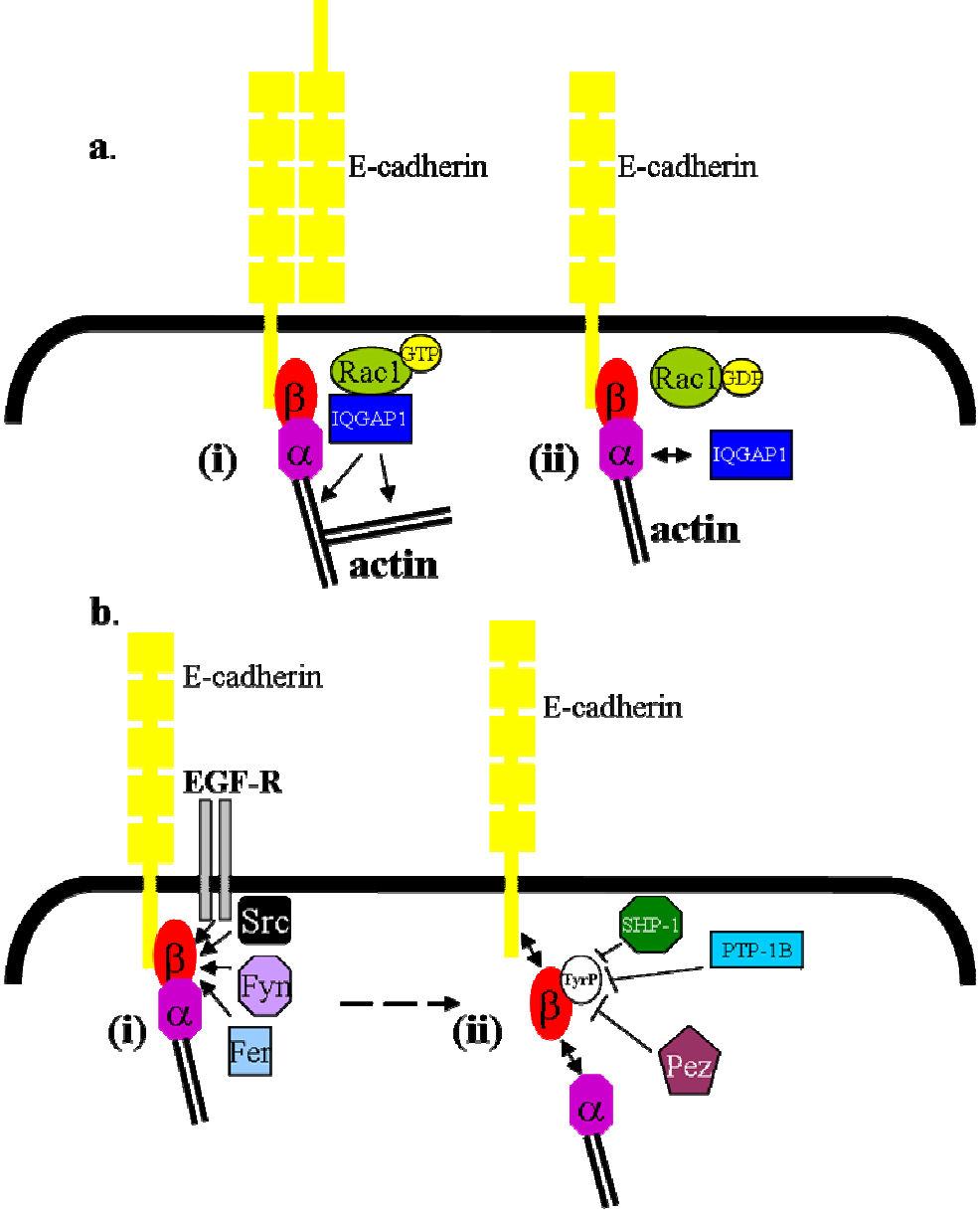


Figure 1.2 The interaction of β-catenin with E-cadherin and α-catenin at the adherens junction. **(a)** The effect of Rac1 and IQGAP1 on β-catenin at the adherens junction based on recent findings by Noritake *et al*., (2004). **(i)** GTP-bound Rac1 binds to IQGAP1, which promotes the actin polymerisation and crosslinking the adherens junction. **(ii)** When levels of GDP-bound Rac1 increases, IQGAP1 binds to β-catenin and alters its interaction with α-catenin. **(b)** Tyr phosphorylation of β-catenin at adherens junction. **(i)** The EGF-R, Src, Fyn and Fer promote Tyr phosphorylation of β-catenin. **(ii)** This results in disruption of the interaction between β-catenin and either E-cadherin or α-catenin. The PTPs such as SHP-1, Pez and PTP-1B are responsible for dephosphorylating Tyr phosphorylated β-catenin and therefore stabilising the β-catenin interaction with E-cadherin and α-catenin. β = β-catenin; α = α-catenin; TyrP = tyrosine phosphorated.

1.2 β-catenin

β-catenin has a dual role in the cell: as well as being associated at the sub-membrane region, where it is essential for cell-cell adhesion, it is also present in the cytoplasm and the nucleus (Mann *et al*., 1999). In the nucleus it forms a transcription factor complex with lymphoid enhancer factor/T-cell factor (LEF/TCF) to enhance transcription of target genes with LEF/TCF consensus sequence in their promoter region e.g. *cyclin D1* (Li *et al*., 2005). The concentration of β-catenin in the cytoplasm and the nucleus is determined and regulated by a number of different mechanisms, which are discussed below.

1.2.1 The β-catenin gene and protein

Since β-catenin is the protein in question for this study it is necessary to talk about the β-catenin gene, as its expression is a determinant for the cellular level of β-catenin protein. The human full-length β-catenin gene, *CTNNB1*, is located on chromosome 3p22-p21.3 and according to the latest sequencing analysis is 41 kilobase-pairs (kb) in size, excluding the promoter region (GenBank: accession no. AY463360, Livingston *et al*., 2003; Kraus *et al*., 1994 cited in: Nollet *et al*., 1996). *CTNNB1* is composed of 16 exons and 15 introns, which range between 61 to 790 base pairs (bp) and 84 to 23451 bp respectively (Figure 1.3.a) (Livingston *et al*., 2003; Nollet *et al*., 1996).

The major transcription initiation site of *CTNNB1* is at the start of exon 1, which is an A residue located 214 nucleotides upstream of the ATG translation initiation codon located on exon 2 (Figure 1.3.a) (Nollet *et al*., 1996). The promoter region is 2760 bp upstream of exon 1, until 27 bp downstream of the start of exon 1 (Li *et al*., 2004). This promoter region has a TATA box (position –29 to the transcription initiation site) and various consensus sequences which are recognised and bound by the

transcription factors: nuclear factor (NF)-κB, specificity protein (SP1)-1, activator protein (AP)-2, AP-1, LEF/TCF and early growth response-1 (EGR-1) (Li *et al*., 2004; Nollet *et al*., 1996).

The transcription of *CTNNB1* leads to the formation of the β-catenin mRNA (GenBank: Accession no. NM_001904, Hulsken *et al*., 1994). The mRNA is composed of a coding sequence (2346 bases) as well as the 5′ (214 bases) and 3′ (802 bases) untranslated repeat regions (Figure 1.3.b) (Nollet *et al*., 1996). The translation of the β-catenin mRNA and therefore the formation of the β-catenin protein begins with the translation initiation site (ATG) on exon 2 and ends at the termination codon (TAA) on exon 15 (Figure 1.3.c) (Livingston *et al*., 2003; Nollet *et al*., 1996).

The primary structure of β-catenin, as described above, consists of an N-terminal domain, the core domain composed of the ARM repeats, and a C-terminal domain (Huber and Weis, 2001). Each ARM repeat consists of a 42 amino acid sequence motif, which forms 3 helices (Huber *et al*., 1997). These helices subsequently form a superhelix, featuring a long positively charged groove (Huber *et al*., 1997). This ARM repeat domain of β-catenin mediates the binding of β-catenin to cadherins, adenomatous polypsis coli (APC), axin, LEF/TCF family of transcription factors and allows for the entry of β-catenin to the nucleus (discussed in more detail in the following text) (Giannini *et al*., 2000; Gottardi and Gumbiner, 2004; Graham *et al*., 2000; Huber and Weis, 2001; Hulsken *et al*., 1994; Eklof Spink *et al*., 2001).

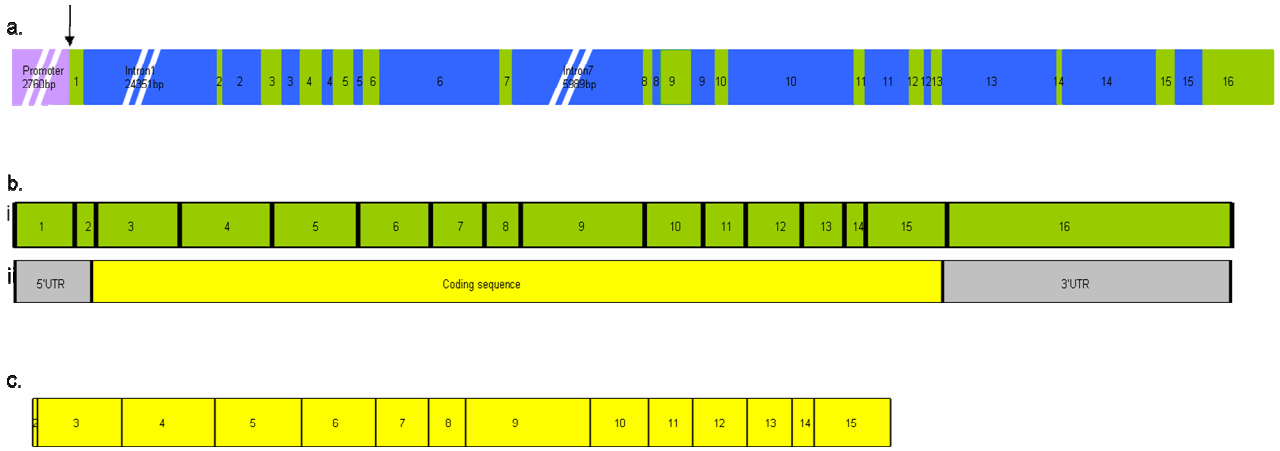


Figure 1.3 *CTNNB1* gene, mRNA and coding sequence. **(a)** The genomic sequence of *CTNNB1* includes the promoter region (purple), 16 exons (green) and 15 introns (blue). Each number represents the respective exon or intron. The arrow indicates the transcriptional start site. **(bi)** The mRNA of *CTNNB1* is formed by the splicing of the exons. Each number represents the respective exon which forms part of the mRNA. **(bii)** The *CTNNB1* mRNA consists of the coding region (yellow), a 5′ and 3′ UTR regions (grey). The coding region starts 12 bp from the end of exon 2 up to and including 208 bp from exon 15. **(c)** The coding sequence of *CTNNB1*, with the numbers corresponding to the relative exon which forms part of the coding sequence.

1.2.2 β-catenin in the cytoplasm

In the cytoplasm, β-catenin together with glycogen synthase kinase-3β (GSK-3β) and APC form a complex with axin scaffolding protein (Dajani *et al*., 2003; Eklof Spink *et al*., 2001). The Casein Kinase (CK) I and CKII also binds to axin and phosphorylate β-catenin at serine (Ser) 29, Ser 45, threonine (Thr) 102 and Thr 112 (Amit *et al*., 2002; Bek and Kemler, 2002). In addition, GSK-3β phosphorylates β-catenin at Ser 33, Ser 37 and Thr 41, as well as axin and importantly APC, which increase their binding affinity to β catenin (Figure 1.4) (Dajani *et al*., 2003; Doble and Woodgett, 2003). It has been postulated that phosphorylated APC displaces β-catenin from axin and moves away from GSK-3β active site (Xing *et al*., 2003). This leads to the dephosphorylation of APC, which decreases its binding affinity to β-catenin and results in the release of Ser/Thr phosphorylated β-catenin to the cytoplasm (Xing *et al*., 2003).

The Ser/Thr phosphorylated β-catenin, now a target protein for ubiquitination, is recognized by β-transducin repeat-containing protein (β-TrCP) (Liu *et al*., 1999). This is followed by the recruitment of the ubiquitin proteosome complex which leads to the degradation of β-catenin (Liu *et al*., 1999). This, however, is not the only ubiquitin proteosome pathway involved with β-catenin, as unphosphorylated β-catenin is also degraded by pathways, such as the ones mediated by tumour protein 53 (p53) or agonist of retenoid-x-receptors, RxR (Figure 1.4) (Liu *et al*., 2001; Matsuzawa and Reed, 2001; Xiao *et al*., 2003).

On the other hand, there are positive regulators of β-catenin which increase its stable form in the cytoplasm, with the most acknowledged being the canonical wingless-type (wnt) pathway (Moon *et al*., 2004). The wnt molecules are cysteine-rich glycoproteins, and there are 19 known members of the wnt family in mammalian cells (Doble and Woodgett, 2003; Moon *et al*., 2004). These molecules bind to the

cell surface receptors which consist of a member of the frizzled family and a member of the low-density lipoprotein (LDL)-receptor related protein 5 or 6 (LRP5/6) (Cong *et al*., 2004; Mao *et al*., 2001). Consequently, the cytoplasmic protein dishevelled (dvl) is phosphorylated and activated (Cong *et al*., 2004). Activated dishevelled phosphorylates GSK-3β thereby inhibiting its activity (Figure 1.5) (Moon *et al*., 2004).

Additional factors involved in phosphorylation of GSK-3β are protein kinase C (PKC) and phosphoinositide-3-kinase (PI3-K) downstream effectors: integrin linked kinase (ILK) and Akt (Figure 1.5) (Christian *et al*., 2002; Delcommenne *et al*., 1998, Troussard *et al*., 2003). Since the phosphorylation of β-catenin by GSK-3β leads to its degradation, the inhibition of GSK-3β activity potentially leads to an accumulation of stabilized cytoplasmic β-catenin. However, factors such as Dickkopf (DKK) and phosphatase and tensin homolog (PTEN) inhibit the binding of wnt molecule to LRP5/6 and the activation of ILK by PI3-K respectively, and thereby compensate for this GSK-3β phosphorylation (Kuhnert *et al*., 2004; Persad *et al*., 2001).

The propyl isomerase (Pin1) is another positive regulator of β-catenin, which binds to the Ser 246-Proline motif of β-catenin (Ryo *et al*., 2001). Subsequently, Pin1 isomerizes and induces a conformational change of β-catenin in such a way, as to prevent its interaction with APC and therefore its degradation (Ryo *et al*., 2001).

The balance between all the various regulators of β-catenin governs its level in the cytoplasm. An accumulation of cytoplasmic β-catenin level leads to its subsequent translocation to the nucleus (Salomon *et al*., 1997; Simcha *et al*., 1998).

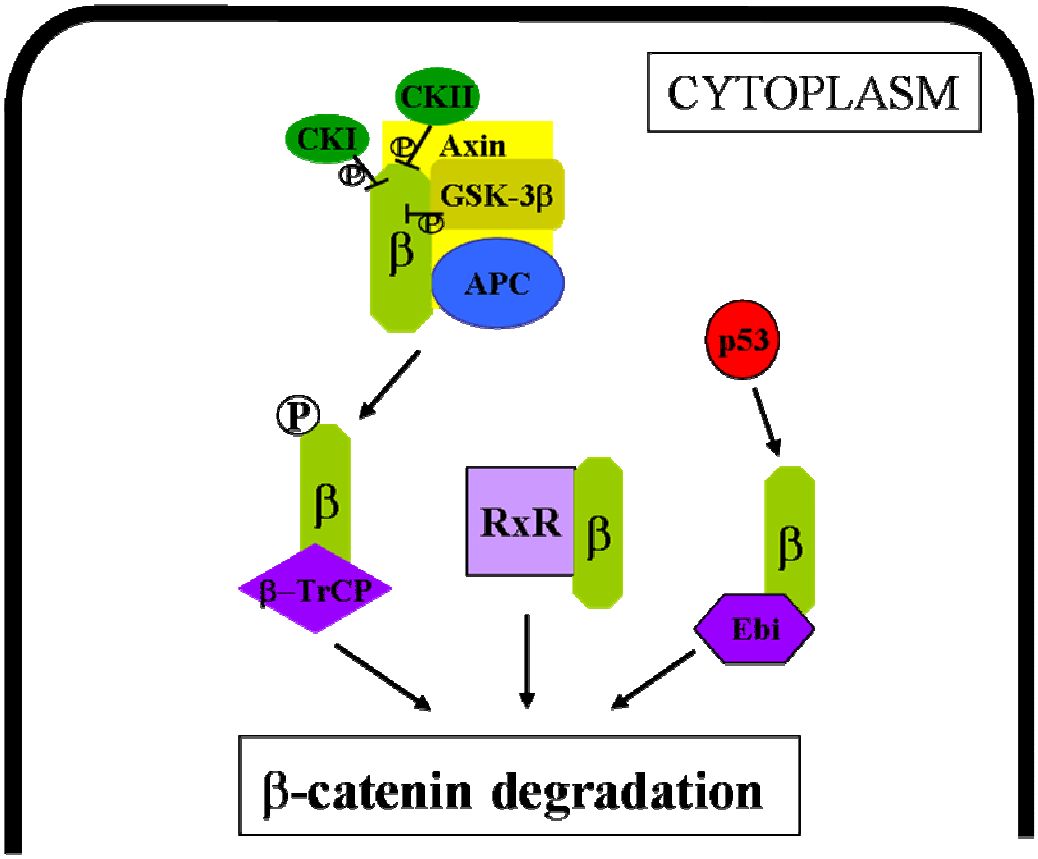


Figure 1.4 Negative regulators of β-catenin in the cytoplasm. β-catenin forms a complex with APC, GSK-3β and axin. CKI , CKII and GSK-3β phosphorylate β-catenin at serine and threonine residues. The phosphorylated β-catenin is recognised by β-TrcP, which leads to the degradation of β-catenin by the ubiquitin proteosome pathway. Other pathways are involved in degradation of β-catenin such as the ones mediated by the RxR and p53. β = β-catenin; APC = Adenomatous polypsis coli; GSK-3β = Glycogen synthase kinase-3β; CKI and CKII = Casein Kinase I and II, respectively. RxR = retenoid receptors.

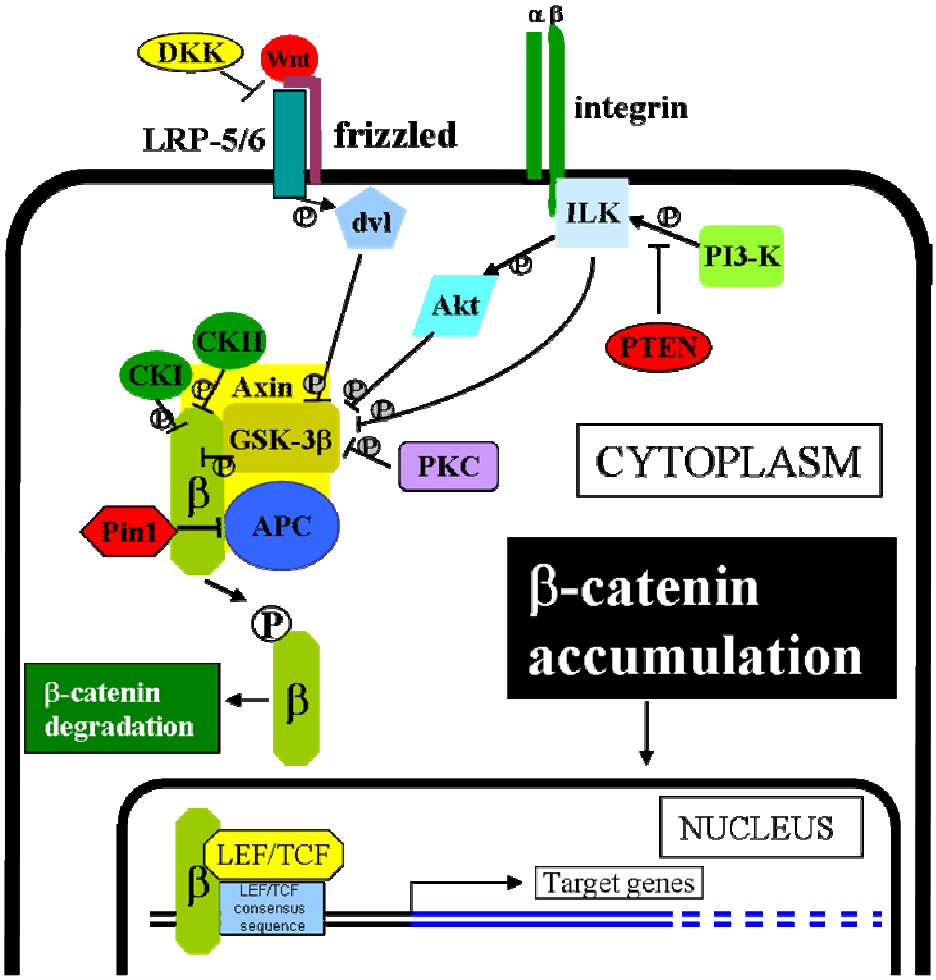


Figure 1.5 Positive regulators of β-catenin in the cytoplasm. Wnt signal binds to receptor frizzled and LRP 5/6 and phosphorylate and therefore activates dvl, which phosphorylates and inhibits GSK-3β activity. PI3-K phosphorylates and activates ILK, which in turn phosphorylates and activates Akt. Akt, ILK and PKC phosphorylate and inhibit GSK-3β activity. Pin1 leads to a conformational change in β-catenin structure in such a way as to decrease its binding with APC. An accumulation of β-catenin in the cytoplasm leads to its translocation to the nucleus, where it binds to LEF/TCF to enhance transcription of target genes with LEF/TCF consensus sequence. β = β-catenin; APC = Adenomatous polypsis coli; GSK-3β = Glycogen synthase kinase-3β; dvl = dishevelled; PI3-K = phosphoinositide-3 kinase; ILK = integrin linked kinase; DKK = Dickkopf; LRP 5/6 = LDL-receptor related protein; PKC = protein kinase C; LEF/TCF = lymphoid enhancer factor/T-cell factor; Pin1 = propyl isomerase.

1.2.3 Association of β-catenin with LEF/TCF in the nucleus

In the nucleus, β-catenin forms a complex with LEF/TCF family of transcription factors (Graham *et al*., 2000). LEF/TCF belongs to the homeobox genes and consists of the following isoforms: LEF-1, TCF-1, TCF-3 and TCF-4 (Hurlstone and Clevers, 2002). A study of the β-catenin-TCF interaction by using *Xenopus* TCF (xTCF) revealed that the β-catenin binding region of xTCF is divided into three modules: N-terminal β-hairpin, an extended region that contains acidic residues and an α-helix. Graham *et al*. (2000) proposed that the primary step in the β-catenin/TCF recognition involves the interaction between the extended region of TCF with the ARM repeats (3-10) of β-catenin (Gottardi and Gumbiner, 2004). The subsequent binding of the α-helix of TCF to β-catenin is essential for high-affinity binding (Graham *et al*., 2000).

LEF/TCF contain a high mobility group (HMG) box DNA-binding domain, which induce sharp bends in DNA, suggesting that LEF/TCF is an architectural transcription factor (Bustin, 1999; Hurlstone and Clevers, 2002). The HMG domain of the LEF/TCF alone is insufficient to stimulate transcription and β-catenin is required as a transcription cofactor for LEF/TCF (Behrens *et al*., 1996; Hurlstone and Clevers, 2002). Behrens *et al*. (1996) demonstrated that LEF-1 induces a 120^{o} bend in the DNA and β-catenin decreases this bend by 40^{o} to enhance expression of genes with LEF/TCF consensus sequence. These genes include genes involved in cell proliferation such as *c-myc*, *cyclin D1, c-jun* and *fra-1* and genes involved in migration and invasion such as urokinase type plasminogen activator (*uPA*), matrix metalloproteases (*MMP*) *7* and *MMP 26* (Hiendlmeyer *et al*., 2004; Li *et al*., 2005; Mann *et al*., 1999; Marchenko *et al*., 2002; Saeki *et al*., 2002).

1.2.4 Regulation of β-catenin induced transcriptional activity in the nucleus

In the nucleus there are several activators as well as repressors of the β-catenin-LEF/TCF mediated gene expression. The transcriptional activators of β-catenin-LEF/TCF such as Brahma related gene-1 (Brg1), CREB-binding protein (CBP) and pontin52 modify the chromatin structure in such a way as to facilitate gene expression (Barker *et al*., 2001; Bauer *et al*., 1998; Feng *et al*., 2003; Takemaru and Moon, 2000). Pontin52 is also able to bind with β-catenin and the TATA binding protein (TBP) (Bauer *et al*., 1998). It is therefore a possibility that pontin52 may link β-catenin-LEF/TCF to the basic transcriptional machinery (Bauer *et al*., 1998; Feng *et al*., 2003).

The transcriptional repressors include groucho, chibby, inhibitor of β-catenin and TCF (ICAT), and reptin52 (Brantjes *et al*., 2001; Bauer *et al*., 2000; Behrens and Lustig, 2004; Satoh *et al*., 2004). Groucho regulates β-catenin-LEF/TCF by interacting with histone deacetylase-1, which promotes repression of gene expression (Brantjes *et al*., 2001). Chibby competes with LEF/TCF for β-catenin, while ICAT interferes with the interaction of β-catenin and LEF/TCF (Behrens and Lustig, 2004). Reptin52 interacts with pontin52 and represses β-catenin-LEF/TCF induced gene activation (Figure 1.6) (Bauer *et al*., 2000).

Another possible mechanism for regulating β-catenin induced transcriptional activity in the nucleus is by exporting β-catenin out of the nucleus. β-catenin is exported from the nucleus by APC and axin (Cong and Varmus, 2004; Rosin-Arbesfeld *et al*., 2003). APC enters the nucleus as it has nuclear localisation signal (NLS) (Neufeld *et al*., 2000). However, it also contains five different nuclear export signals (NES), which allow for its export mediated by the chromosome maintenance region-1 (CRM-1)/exportin receptor pathway (Fabbro and Henderson, 2003; Rosin-Arbesfeld *et al*., 2003; Zhang *et al*., 2001).

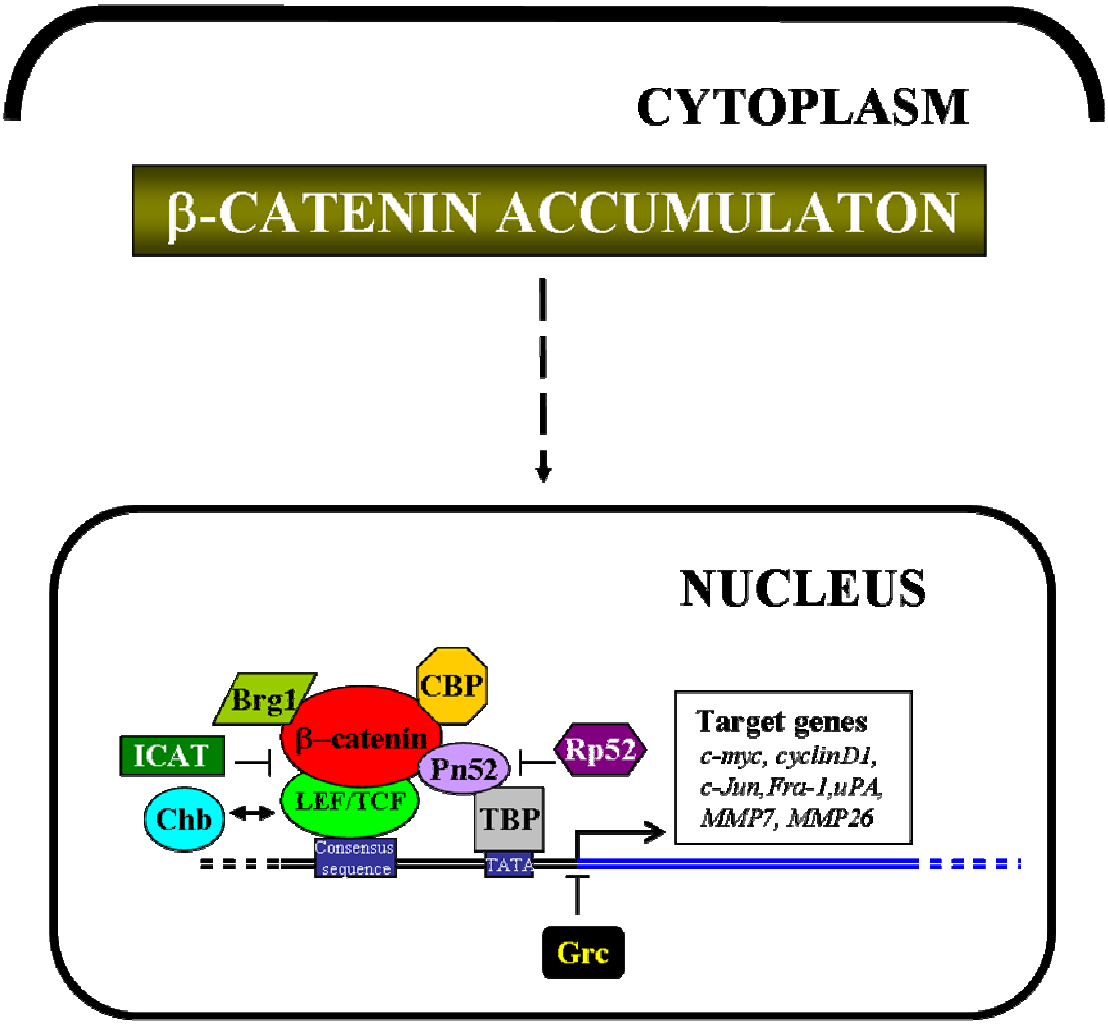


Figure 1.6 β-catenin in the nucleus. Once the levels of β-catenin increase in the cytoplasm, it translocates to the nucleus where it binds to LEF/TCF to induce transcription of target genes. Gene expression mediated by β-catenin-LEF/TCF is either enhanced by Brg1, CBP, Pontin52 or negatively regulated by Groucho, Reptin52, Chibby and ICAT. Pn52 = pontin52; Rp52 = Reptin52; Chb = Chibby; Grc = Groucho; Consensus sequence = LEF/TCF consensus sequence; uPA = urokinase type plasminogen activator.

The overexpression of APC shifts β-catenin from the nucleus to the cytoplasm and it is suggested that mutant APC, which is incapable of binding β-catenin, is unable to export β-catenin from the nucleus (Henderson and Fagotto, 2002). This results in the nuclear accumulation of β-catenin (Henderson and Fagotto, 2002).

Cong and Varmus (2004) demonstrated that axin was able to export β-catenin from the nucleus to the cytoplasm via the CRM receptor. The mechanism by which axin enters and exits the nucleus is likely due to the potential NLS and NES sequences identified on axin (Cong and Varmus, 2004).

1.3 β-catenin and its role in pathogenesis

Since β-catenin plays such important physiological roles in the cell, it follows that it is implicated as a significant factor in tumour development. A disruption in the regulation of β-catenin expression could possibly inhibit cell-adhesion events (mediated by β-catenin) or increase β-catenin in the cytoplasm and nucleus, which potentially leads to a state of abnormal cell proliferation, tumour invasion and ultimately metastasis.

Metastasis involves the formation of secondary tumours at different sites of the body to its primary location and is therefore the most dangerous aspect of cancer (Fidler, 2002; Fidler and Ellis, 1994). The detachment of the cells from the primary tumour may result in their subsequent invasion of blood vessels and surrounding tissues (Fidler, 2002; Keleg *et al*., 2003). Disruption of β-catenin mediated cell-adhesion events is responsible for the invasive properties of the tumour, and indeed this was observed in cancers such laryngeal squamous cell carcinoma (Lopez-Gonzalez *et al*., 2004). β-catenin may also contribute to the tumour invasion by increasing the expression of invasion related genes (see above).

The focus of this study is on the implication of the nuclear translocation of β-catenin, as it is critical to understand how β-catenin enters the nucleus, especially since an increase of nuclear β-catenin has been connected to the development of several tumours such as: oropharyngeal squamous cell carcinoma (SCC), gastrointestinal carcinoid tumours, prostate cancer and colorectal cancer (De la Taille *et al*., 2003; Fujimori *et al*., 2001; Pukkila *et al*., 2001; Wong *et al*., 2003).

1.4 Proposed translocation of β-catenin to the nucleus

Two models have been proposed to explain the translocation of β-catenin to the nucleus. The first model suggests that β-catenin binds to LEF/TCF in the cytoplasm which leads to its translocation to the nucleus. LEF/TCF contains classical NLS, which allows for its entry to the nucleus (Prieve *et al*., 1996; Prieve *et al*., 1998). The second model suggests that β-catenin, which does not contain the NLS sequences, is able to translocate to the nucleus via its ARM repeat region in a similar mechanism as importin-β (Giannini *et al*., 2000).

1.4.1 The translocation of β-catenin to the nucleus by LEF/TCF

Behrens *et al*. (1996) and Kim and Hay (2001) demonstrated that overexpression of LEF-1 increased the nuclear level of β-catenin in Neuro 2A cells, NIH3T3 fibroblast and MDCK epithelial cells. Molenaar *et al*. (1996) also demonstrated that microinjection of xTCF-3 mRNA results in nuclear translocation of β-catenin in pre-midblastula transition blastomeres. However, co-injection with an xTCF-3 mutant, which is incapable of binding to β-catenin, did not result in the nuclear translocation of β-catenin (Molenaar *et al*., 1996).

The experiments described above illustrate the role that LEF/TCF plays in the translocation of β-catenin. However, as mentioned above, β-catenin can also enter the nucleus on its own via its ARM repeat (Giannini *et al*., 2000). Interestingly, although

Kim and Hay (2001) showed that LEF-1 increases endogenous β-catenin in the nucleus, they also noticed that exogenous expression of β-catenin leads to the nuclear translocation of β-catenin, independently of LEF-1.

In order to elucidate a possible mechanism of β-catenin translocation to the nucleus, it is necessary to understand the different modes of nuclear translocation. This involves proteins such as importin-α and -β, or transport mediated by the cytoskeleton, which is discussed below.

1.4.2 Importin-α and -β and the nuclear pore complex

Proteins can enter the nucleus via the action of importin-α and -β (Macara, 2001). Importin-α binds to a nuclear protein with an NLS sequence, to form a complex, which is recognised by importin-β (Figure 1.7.a) (Gorlich, 1998). Following this, importin-β docks the importin α–nuclear protein complex to the nucleus by binding to the nuclear pore complex (NPC) (Figure 1.7.b) (Gorlich, 1998). The NPC spans the double membrane of the nuclear envelope (NE) and comprises 30-50 different proteins or nucleoporins, which allows for the exchange of molecules between the cytoplasm and the nucleus (Jaggi *et al*., 2003; Lyman and Gerace, 2001).

In the nuclear pore complex, importin-β binds to Ran GTPase, which uses GTP and hydrolyses it to GDP, leading to the dissociation of importin-α and the attached nuclear protein from the NPC and into the nucleus (Figure 1.7.c) (Gorlich, 1998; Laskey *et al*., 1996). The concentration of Ran-GTP in the nucleus is higher than the Ran-GTP in the cytoplasm, which ensures the directional migration of importin-β/importin-α-nuclear protein complex into the nucleus (Izaurralde *et al*., 1997).

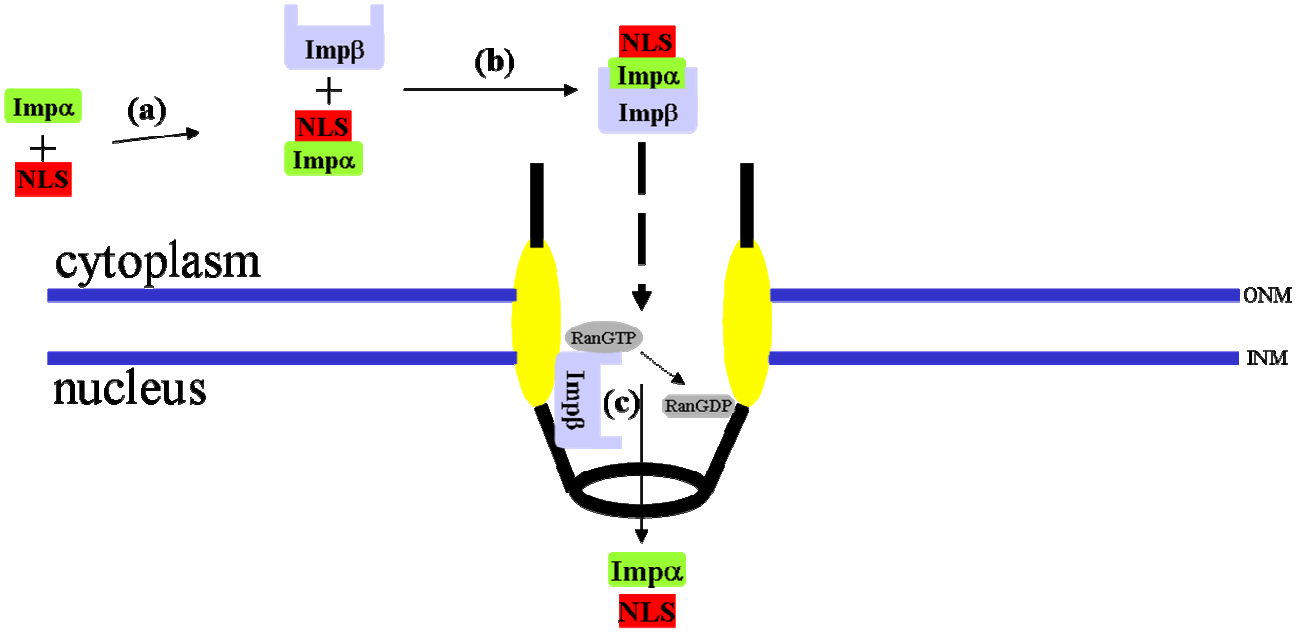


Figure 1.7 The translocation of nuclear proteins from the cytoplasm to the nucleus via importin-α/importin-β. **(a)** Importin-α recognises nuclear proteins with classical nuclear localisation signals (NLS) and binds to these proteins. **(b)** Importin-β binds to the importin-a-nuclear protein complex and docks the complex to the nuclear pore complex (NPC). **(c)** Importin-β binds to the NPC and RanGTP, which subsequently hydrolyses GTP to GDP leading to the release of importin-α and nuclear proteins into the nucleus, while importin-β remains bound to the NPC. ONM = outer nuclear membrane; INM = inner nuclear membrane; Impα = Importin-α; Impβ = Importin-β.

From previous investigations, it was established that β-catenin translocates to the nucleus in a process that is independent of either importin-α or -β (Fagotto *et al*., 1998 cited in: Yokoya *et al*., 1999). Fagotto *et al.* (1998) also demonstrated that β-catenin binds to phenylalanine-glycine (FG) repeat in the yeast nucleoporin Nup1, which is a common feature of many nucleoporins and is essential for efficient nucleocytoplasmic transport (Fagotto *et al*., 1998 cited in: Yokoya *et al*., 1999; Jaggi *et al*., 2003). This possibly allows for the entry of β-catenin to the nucleus (Yokoya *et al*., 1999).

Although β-catenin does not require importin-β for its nuclear translocation, it has been proposed that β-catenin enters the nucleus in a way similar to that of importin-β (Yokoya *et al*., 1999). This stems from the observation that β-catenin translocation to the nucleus is inhibited by wheat germ agglutinin (WGA) treatment, which binds to nucleoporins and is an inhibitor of importin-β transport (Yokoya *et al*., 1999). Furthermore, Koike *et al*. (2004) further showed that importin-β inhibited both import and export of β-catenin and that WGA inhibited the export of β-catenin. This suggests that β-catenin competes with importin-β for binding to the NPC (Koike *et al*., 2004; Yokoya *et al*., 1999).

However, Suh and Gumbiner (2003) showed that WGA treatment or excess importin-β did not affect the nuclear translocation of β-catenin. Suh and Gumbiner (2003) suggested that the observed discrepancies in these experiments could be that β-catenin has two nuclear targeting domains, one of the domains is prone to denaturation and allows for the nuclear translocation of β-catenin via a WGA insensitive pathway. The other nuclear targeting domain allows for the nuclear translocation of β-catenin via a WGA sensitive pathway (Suh and Gumbiner, 2003).

It is interesting that β-catenin translocation to the nucleus depends on its ARM repeats, as these repeats are similar to the tandem repeat motif, known as HEAT motif, present in importin-β (Malik *et al*., 1997). Importin-α also has ARM repeats

and it is able to translocate to the nucleus on its own (Miyamoto *et al*., 2002). Further, Giannini *et al.* (2000) illustrated that the translocation of β-catenin to the nucleus was disrupted by the co-expression of APC and N-cadherin, which bind to the ARM repeat of β-catenin. This illustrates the importance of the ARM repeat in the translocation of β-catenin to the nucleus.

1.4.3 The cytoskeleton

The cytoskeleton, which plays an important role in cell-cell adhesion, is composed of three types of protein filaments: the intermediate filaments, the microtubules and the microfilaments (Alberts *et al*., 1994; Ku *et al*., 1999). Each type of filament is formed from different subunits: actin forms the microfilaments; tubulin forms the microtubules; and various fibrous proteins form the intermediate filaments (Ku *et al*., 1999).

The intermediate filaments, which are usually associated with the desmosomal cadherins, are predominantly involved in resisting mechanical stress applied to the cell (Alberts *et al*., 1994). On the other hand, microtubules and the actin cytoskeleton are the major components of the cytoskeleton involved in transportation of molecules, vesicles and trafficking of organelles (Apodaca, 2001; Huang *et al*., 1999; Schmalz *et al*., 1996). In this study, however, our attention is restricted to the actin mediated transport of proteins, as this possibly forms the basis to the nuclear translocation of β-catenin.

Most important to this study, actin has been implicated in the translocation of signalling proteins such as extracellular signal-related kinase (ERK) and PKC-α, which is developed in more detail in the following text (Aplin *et al*., 2001; Schmaltz *et al*., 1996). This generated the question of whether actin, or actin-related mechanisms, are responsible for the translocation of β-catenin from the cytoplasm to the nucleus. Since β-catenin binds indirectly with the actin cytoskeleton, we wanted to examine whether the actin cytoskeleton plays a role in the nuclear translocation of β-catenin.

1.5 Human oesophageal squamous cell carcinoma

Human oesophageal squamous cell carcinoma (HOSCC) is one of the leading forms of cancer with a high mortality rate in South Africa. HOSCC is a metastatic disease of the epithelium in the oesophagus and is usually in an advanced stage when diagnosed (Gore,1997). HOSCC histopathological changes in epithelium are associated with esophagitis, atrophy, mild to severe dysplasia, carcinoma *in situ* and finally invasive carcinoma (Kuwano *et al*., 2003; Mandard *et al.,* 2000). Patients with HOSCC also have symptoms such as weight-loss, difficulty in swallowing, cachexia, hoarseness, chest pains, hiccups, chronic cough and bleeding (Gore*,* 1997, Koshy *et al*., 2004).

The occurrence of HOSCC is due to a combination between environmental and genetic factors (Lu, 2000; Stoner and Gupta, 2001). Environmental factors characterized as high risk in HOSCC are: 1) a diet lacking in riboflavins, proteins, vitamins such as Vitamin A, C, E and minerals such as magnesium and zinc; 2) use of alcohol and tobacco; 3) consumption of high salt food that may be contaminated with microbial toxins, nitrosamines and hot beverages (Gore *et al*., 1997; Koshy *et al*., 2004; Kuwano *et al*., 2003; Lu, 2000; Saeki *et al*., 2000; Stoner and Gupta, 2001). Examples of genetic factors which contribute to HOSCC include increased *cyclin D1* expression, mutation in p53, reduced Retinoblastoma gene expression, overexpression of EGF-R, increased telomerase expression, elevated expression of cyclooxygenase-2 and nitric oxide synthase (Gore *et al*., 1997; Lu, 2000; Mandard *et al*., 2000; Robert *et al*., 2000; Shamma *et al*., 2000).

There are certain geographical locations which have high incidents of HOSCC such as China, Japan, Iran, France, Italy, Uruguay and South Africa (Lu, 2000; Stoner and Gupta, 2001). In South Africa, the occurrence of oesophageal cancer has increased since 1950 and is common among ethnic groups such as the Xhosa and in areas such as Transkei, Kimberly, Port Elizabeth, East London and the Western Cape, but has recently shown a dramatic increase in the major cities such as Johannesburg and

Pretoria (McGlashan, 1988; McGlashan *et al*., 2003; Pacella-Norman *et al*., 2002). In South Africa, the ratio of male to female with HOSCC is significantly high, and the risk of acquiring HOSCC increases with age (McGlashan, 1988; Pacella-Norman *et al*., 2002). HOSCC is a big health problem in South Africa and it is therefore essential to understand the events involved in the development of HOSCC.

1.5.1 Invasion and HOSCC

Given that HOSCC is a highly metastatic disease, events must occur during tumour development to permit the decrease in adhesion seen in invasion and metastasis. The decrease of E-cadherin expression is associated with progression of HOSCC (Lin *et al*., 2004; Zhao *et al*., 2003). Even though the EGF-R is overexpressed in most HOSCC, which can result in Tyr phosphorylation of β-catenin and impairs cell-adhesion (Fujii *et al*., 1996; Gibson *et al*., 2003; Hazan and Norton, 1998; Veale and Thornley, 1989), Andl *et al*. (2003) showed that EGF-R does not alter the phosphorylation statues of β-catenin in HOSCC. However, as Andl *et al*. (2003) pointed out, EGF-R mediates the up regulation of MMP 1, which degrades several ECM components, thereby enhancing invasion.

1.5.2 β-catenin and HOSCC

While the literature has expanded rapidly on the individual topics of β-catenin and cancer, there are a few instances where an attempt has been made to forge a link between β-catenin and HOSCC in particular. Wnt-1, but not wnt-5 and wnt-7, increases the amount of β-catenin in the cytoplasm of HOSCC (Mizushima *et al*., 2002). In addition, an increase of the frizzled receptor expression and reduced axin expression was implicated with the progression of HOSCC (Nakajima *et al*., 2002; Tanaka *et al*., 1998).

The accumulation of β-catenin in the cytoplasm could subsequently result in an increase of β-catenin concentration in the nucleus (Salomon *et al*., 1997; Simcha *et al*., 1998). This, in turn, could lead to a significant change in the expression of genes involved in cell proliferation and invasion, which potentially leads to metastasis (Simcha *et al*., 1998). An increased presence of β-catenin in the nucleus has been observed in HOSCC, which potentially increases expression of β-catenin target genes such as *MMP 7* (Saeki *et al*., 2002). β-catenin in the nucleus could also lead to the expression of other possible target genes such as *cyclin D1* and *c-myc*, which contribute to cell proliferation, whereas *MMP 7* further contributes to invasion and metastasis.

Since the presence of β-catenin in the nucleus is linked with HOSCC and has been shown to have far-reaching effects, we wanted to examine actin cytoskeletal involvement in β-catenin translocation from the cytoplasm to the nucleus in HOSCC cells.

1.6 Aims and objectives

In this project, the role of the actin cytoskeleton in the cytoplasmic/nuclear translocation of β-catenin in moderately differentiated HOSCC cell lines, derived from metastatic tumours, was examined (Veale and Thornley, 1989). The aims of this project are:

1) To investigate the role of the actin cytoskeleton in the translocation of β-catenin in cultured HOSCC cells. This is achieved by using cytochalasin D (cytoD) to block actin mediated translocation in the HOSCC lines and thereafter determine whether this treatment affected the concentration of β-catenin in the nucleus or cytoplasmic/membrane region.

2) To overexpress β-catenin, by transfecting a HOSCC line with full-length β-catenin cDNA, and stimulate its expression in an attempt to increase the cytoplasmic β-catenin protein concentration which could lead to the nuclear translocation of β-catenin. Thereafter, determine the role of the actin cytoskeleton in the nuclear translocation of β-catenin in the HOSCC line overexpressing β-catenin.