The Regulation of β-catenin signalling by α-catenin

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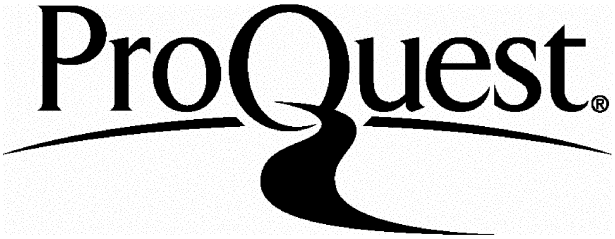

ABSTRACT

Alpha-catenin and β-catenin link cadherins to the cytoskeleton at sites of cell-cell contact. Beta-catenin also associates with Tcf/Lef-1 transcription factors, activating transcription of genes encoding proteins involved in cell cycle regulation and differentiation, such as cyclin D1 and c-myc. Mutations in β-catenin that lead to its stabilisation and accumulation in the nucleus are associated with increased proliferation of colon cancer cells.

This thesis examines the regulation of β-catenin signalling by α-catenin. It shows that overexpression of β-catenin results in formation of intranuclear rod-like structures. The rod-like structures are disrupted by co-expression of proteins that interact with the armadillo repeat domain of β-catenin. In contrast, exogenous α-catenin prevents nuclear entry of the rod-like structures. However α-catenin translocates to the nucleus in cells expressing β-catenin and Tcf/Lef-1.

Endogenous α-catenin is present both in the nucleus and in the cytoplasm of colon cancer cells. A variant cell line that lacks α-catenin shows a significant increase in β-catenin/Tcf-dependent transcription, compared to that in the α-catenin-positive parental cell line. A nuclear-targeted form of α-catenin inhibits β-catenin/Tcf activity to a similar extent as wild type α-catenin in these cells. These observations indicate that α-catenin can inhibit β-catenin/Tcf activity in the nucleus. *In vitro* experiments show that α-catenin inhibits the association of the β-catenin/Tcf complex with its target DNA.

While nuclear import of α-catenin is mediated by the β-catenin/Tcf complex, nuclear export of α-catenin is mediated by nuclear export signals

present at its amino-terminal domain. These lie within the β-catenin-binding domain, suggesting that their accessibility is regulated by binding to β-catenin. Thus the nuclear export of α-catenin may be linked to the regulation of β-catenin/Tcf activity in the nucleus.

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CHAPTER I- General Introduction

I.1- *Cell Adhesion:*

Multicellular organisms have developed mechanisms to maintain cells together to form tissues. This is achieved by secretion of an extracellular matrix that serves as a scaffold for the cells and by cell-cell interactions. To accomplish the latter, several classes of adhesion molecules have evolved. These molecules can be separated in two major groups: cadherins and members of the immunoglobulin (Ig) superfamily. The cadherins mediate Ca^{2+}-dependent cell-cell adhesion, while the Ig superfamily mediates Ca^{2+}-independent adhesion. The cadherins are particulary relevant to this thesis, since they bind to the cytoskeleton via the catenins.

I.2- *Cadherins*

The cadherin superfamily can be divided into five subfamilies: classical cadherins type I and type II; desmosomal cadherins; protocadherins and cadherin-related proteins. Classical cadherins are transmembrane glycoproteins that promote adhesion by homophilic interactions between the cadherin molecules present on the surface of one cell and those on the surfaces of adjacent cells, forming the adherens junction. Adhesion promoted by classical cadherins is mediated by five or six extracellular domains of approximately 110 residues (cadherin repeats), which contain Ca^{2+}-binding motifs.

I.2.1- *Homophilic binding of cadherins*

The amino-terminal domain of cadherins contains an HAV (histidine-alanine-valine) motif believed to be essential for adhesion. Synthetic peptides containing this motif inhibit physiological processes in which cadherins play essential roles, such as neurite outgrowth (Riehl et al., 1996) and compaction of eight-cell-stage mouse embryos (Blaschuk et al., 1990). Crystallographic studies of both E-cadherin and N-cadherin amino-termini show that each repeat forms a barrel-like structure resembling the domains present in Ig molecules (Overduin et al., 1995; Shapiro et al., 1995). Ca^{2+} ions are essential for the adhesion function of the cadherins because they intercalate between the cadherin repeats, making the molecule rigid and resistant to proteases (Pokutta et al., 1994). The specificity of homophilic binding seems to require both the first cadherin repeat (Nose et al., 1990), as well as other cadherin domains (Chappuis-Flament et al., 2001). Indeed, the cadherin repeat domains may function as a single structural unit, where the dimerisation of the first domain provides the first selective step, which precedes the overlap of the other cadherin domains. Multivalent interactions within these domains will then provide binding specificity.

I.2.2- *Cadherins in development and morphogenesis*

Cadherins are not only involved in maintaining adult tissues, but are also important for their development. E-cadherin is the first cadherin expressed in the mammalian embryo where it is essential for compaction of the cells at the morula stage. A similar process occurs during *Xenopus* development, where injection of EP-cadherin anti-sense mRNA blocks compaction. Later in development, the qualitative and quantitative expression of the cadherins is important in events involving cell migration

and segregation of specific tissues, such as in the formation of the neural tube, when loss of E-cadherin and gain of expression of N-cadherin precedes the migration of neural tube precursors from the ectoderm (reviewed by Gumbiner, 1996; Shapiro and Colman, 1998). These changes in expression allow the cells to dissociate from the ectoderm, migrate and then re-associate to form the neural tube (reviewed by Tepass, 2000).

I.2.3- ***Cadherin binding to the cytoskeleton***

The intracellular domain of classical cadherins is the most conserved region, suggesting its importance in promoting adhesion. Indeed deletions or mutations of this domain disrupt cell-cell adhesion. The first evidence that cadherins associate with the cytoskeleton came from studies that showed co-localisation of cadherins with actin-bundles at sites of cell-cell contacts (Hirano et al., 1987). More concrete evidence for the role of the cytoskeleton in cell-cell adhesion came from immunoprecipitation studies. These experiments led to the identification of the catenin molecules: α-catenin, β_catenin and γ-catenin (also known as plakoglobin). The carboxyl-terminal 25 amino acids of cadherins associate with β-catenin (Aberle et al., 1994; Jou et al., 1995). The latter provides the link between cadherins and the cytoskeleton via the interaction of its amino-terminal domain with α-catenin (Knudsen et al., 1995). Alpha-catenin has binding domains for several cytoskeletal proteins, including α-actinin, actin, vinculin and ZO-1 (Zonula occludens-1; Ozawa et al., 1990; Rimm et al, 1995; Knudsen et al., 1995). The juxtamembrane region of the cadherin cytoplasmic tail is involved in the binding to a catenin-related molecule, p120. This protein was first identified as a substrate for the tyrosine kinase pp60 c-src (Reynolds et al., 1989) and its phosphorylation status might play a role in regulating the

adhesiveness of the cell-cell adhesion complex (Aono et al. 1999).

I.2.4- ***Armadillo, β-catenin and plakoglobin***

Armadillo (the β-catenin homologue in *Drosophila*), β-catenin, and plakoglobin are highly homologous proteins, sharing 60-70% identity (McCrea et al., 1991). These proteins are characterised by the presence of the armadillo repeats, which were first identified in the *Drosophila* protein. These repeats are 42-43 amino acids long and are involved in protein-protein interactions. Similar repeats are found in other proteins with diverse functions, such as the yeast vacuolar membrane protein Vac8p, vertebrate p120, APC and importin-α (Peifer et al., 1994).

Structural studies revealed that each of these repeats forms three α-helices: a short one with two turns (H1), another with 2-3 turns (H2) and finally one with 3-4 turns (H3). H1 forms a 90^0 angle with a hairpin formed by H2 and H3 lined in an antiparallel form. This arrangement results in the formation of a superhelix with a long, positively charged groove (Huber et al., 1997), which provides a binding domain for associating proteins (Figure 1a) (Graham et al., 2000; Huber et al., 2001).

Beta-catenin and plakoglobin are often found at cell-cell junctions in a mutually exclusive manner. While β-catenin only associates with classical cadherins, plakoglobin also interacts with desmosomal cadherins. The amino and carboxyl termini of β-catenin seem to be responsible for this specificity, since when both the amino- and carboxyl-terminal domains of β-catenin are added to the plakoglobin armadillo repeats, association with the desmosomal cadherin, desmoplakin 2, is reduced (Wahl et al., 2000).

The generation of knockout mice or specific deletion of the catenins has provided some information about their participation in promoting cell-cell interactions. Deletion of the plakoglobin gene in mice, for

example, results in heart defects and skin blistering due to impairment of the desmosomes. In these mice, β-catenin associates with desmosomal cadherins (Ruiz et al., 1996; Bierkamp et al., 1996). Mice deficient in β-catenin do not develop mesoderm, but intercellular adhesion is preserved by association of plakoglobin with classical cadherins at adherens junctions (Huelsken et al., 2000).

I.2.5- ***Alpha-catenin***

Alpha-catenin provides the link between cadherins and the cytoskeleton, thus playing a key role in cell-cell adhesion. Using *in vitro* association assays, it was shown that amino acids 117-143, which are highly conserved in α-catenin from different species (see Figure 27b) are necessary and sufficient for the interaction of α-catenin with β-catenin and plakoglobin (reviewed by Kemler, 1993; Huber et al., 1997). Hydrophobic residues in this domain of α-catenin promote the formation of a α-helical secondary structure, which is necessary for the interaction with β-catenin and plakoglobin. Moreover, residues 82-279 present in this domain are involved in homodimerisation of α-catenin (Pokutta and Weis, 2000). The carboxyl-terminal 295 residues of α-catenin, which contain a ZO-1 binding site, are sufficient to promote strong adhesion when fused to E-cadherin (Imamura et al., 1999).

In MDA-MB-468 breast cancer cells, which lack α-catenin, vinculin can associate with the cadherin adhesion complex, apparently via β-catenin (Hazan et al., 1997). In cells that express α-catenin, vinculin is found in the adhesion complex, but through its association with α-catenin (Weiss et al., 1998). Since vinculin and α-catenin share some sequence similarity, it would be expected that, like vinculin, α-catenin could also localise to both cell-cell adhesion sites and cell-matrix adhesion sites.

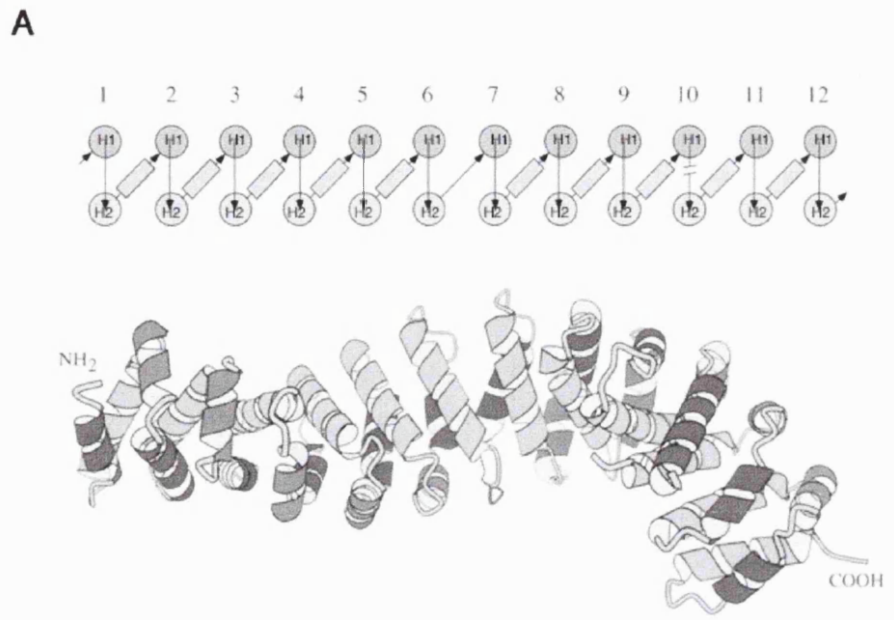
This apparently does not occur because α-catenin interacts strongly with β-catenin. Constructs of α-catenin that cannot bind to β-catenin are recruited to both cell-cell contacts and focal adhesions (Weiss et al., 1998). Cells that lack α-catenin not only cannot form adherens junctions, but also are incapable of organising tight junctions. The ability of α-catenin to associate with vinculin appears to be required for tight junction assembly (Watabe-Ushida et al., 1998).

The essential role of α-catenin in cell-cell adhesion has been clarified by expression of α-catenin in cells lacking this protein (Shimoyama et al., 1992; Watabe et al., 1998).

Also, analysis of mice with targeted-deletion of α-catenin in the skin has demonstrated the importance of α-catenin for intercellular adhesion and epithelial polarity (Vasioukhin et al., 2001). A schematic representation of α-catenin is shown in Figure 1b.

I.2.6- ***Regulation of cadherin function***

A balance between phosphorylation and dephosphorylation of members of the adhesion complex seems to regulate, or at least modulate, cell-cell adhesion. Treatment with growth factors such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF) disrupts cell-cell adhesion (reviewed by Gumbiner, 2000). This might involve tyrosine phosphorylation of β-catenin and/or p120. Direct association between β-catenin and the EGF receptor (Hoschuetzky et al., 1994; Shibamoto et al. 1994) and c-erbB-2 has been reported (Ochiai et al., 1994; Shibata et al., 1996). Phosphorylation of β-catenin by treatment of cells with IGF-1 (insulin-like growth factor type 1) disrupts the association of β-catenin with E-cadherin, and this might contribute to increased migration and metastasis (Playford et al., 2000).



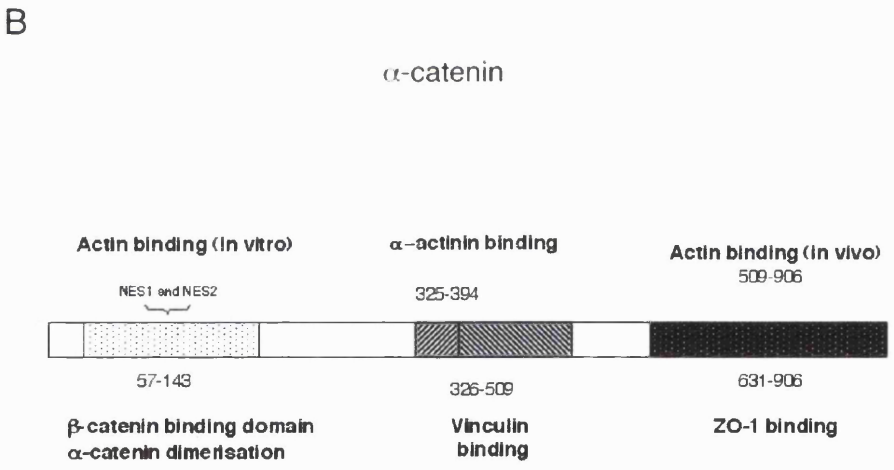


Figure 1.
(A) Armadillo repeat domain crystal structure.Topology (upper) and ribbon (lower) diagrams. Circles represent helices 1 and 2, as indicated, and rectangles represent helix 3 viewed end on and from the side, respectively. The arrows indicate the NH2- to COOH-terminal direction of the polypeptide. Arrows terminate at the circle perimeter when connected to the end of the helix farthest from the viewer and enter the circle when connected to the end of the helix nearest the viewer (taken from Huber et al., 1997).
(B) Schematic view of α-catenin. White dotted box contains α-catenin homodimerisation domain, α-actin and β-catenin binding domains as well as two NES signals. Hatched box includes α-actinin and vinculin binding domains. Black dotted box includes α-actin and ZO-1 binding domains.

Conversely, association of components of the adhesion complex with tyrosine phosphatases might neutralise the effects of tyrosine phosphorylation on adhesion (Brady-Kalnay et al., 1995; Kypta et al., 1996; Fuchs et al., 1996; Balsamo et al., 1996). However, all these observations only offer circumstantial evidence for the role of catenin tyrosine phosphorylation in the regulation of cell-cell adhesion. Adhesion promoted by chimaeras of E-cadherin linked directly to α-catenin, for instance, is still inhibited by pp60 v-*src*, even though β-catenin is not participating in the adhesion complex and the chimaera is not tyrosine-phosphorylated (Takeda et al., 1995).

The importance of p120 phosphorylation in regulating cadherin function is still controversial. While the phosphorylation status of p120 is important for its association with the cadherins (Aono et al., 1999; Reynolds et al., 1994), cadherin molecules lacking the p120-binding domain can still promote cell-cell adhesion (Yap et al., 1998; Ozawa and Kemler, 1998). This suggests that association of p120 with cadherins might not be necessary for cell-cell adhesion. For instance, in fibroblasts, cytoplasmic p120 inhibits Rho activity (Anastasiadis et al., 2000). Rho is involved in the organisation of actin stress fibres and in clustering of cadherins during adherens junction assembly. The inhibition of Rho activity and binding of p120 to cadherins are mutually exclusive processes (Anastasiadis et al., 2000), and the former seems to be a consequence of the interaction of cytoplasmic p120 with the Rho family exchange factor Vav2, which regulates small GTPases (Noren et al., 2000).

Another way of regulating adhesion relies on the dissociation of α-catenin from the adhesion complex. In this process, IQGAP1, a novel

RasGAP-related protein that is a target for the small GTPases Rac and Cdc42, may be involved. IQGAP1 localises to cell-cell adhesion sites in epithelial cells by binding to β-catenin and it can inhibit the binding of α-catenin to the adhesion complex, thereby decreasing cell-cell adhesion when Cdc42 is activated (Kuroda et al., 1998).

I.2.7- ***Cadherins and signal transduction***

An interesting question is whether cadherins regulate intracellular signals. In addition to forming the adherens junction, cadherins also seem to be involved in the formation of tight junctions and desmosomes (Gumbiner et al., 1988; Watabe et al., 1994). This could be explained by a cadherin-generated signal at adherens junctions leading to recruitment of other junction proteins. Alternatively, cadherins may mediate signalling by inducing the clustering of signalling molecules at the cell surface. As mentioned earlier, components of the adherens junction associate with growth factor receptor tyrosine kinases and receptor-like tyrosine phosphatases. Although these interactions might be important for the regulation of adhesion, it is also conceivable that they activate cadherin-dependent intracellular signals. For example, truncation of VE-cadherin blocks the ability of endothelial cells to respond to survival factors, such as the vascular endothelial growth factor type A (VEGF-A), resulting in cell-death. Interestingly this response is specific to VEGF-A, and cells expressing the truncated VE-cadherin molecules can still respond to survival cues mediated by basic fibroblast growth factor. These results suggest an intimate relationship between the cell-cell adhesion system and survival signals (Carmeliet et al., 1999).

The most convincing evidence linking cadherins with signal transduction comes from the presence of β-catenin in the adhesion

complex, since this protein is also a component of the Wnt-signalling pathway. Although cadherins might not be directly involved in this pathway, they are able to down regulate Wnt signalling by preventing β-catenin/Armadillo from entering the nucleus and functioning as a co-activator of transcription (Orsulic et al., 1999). For instance, overexpression of cadherins inhibits dorsal mesoderm induction and axis formation in *Xenopus* development (Heasman et al., 1994; Fagotto et al., 1996). Moreover, truncated E-cadherin proteins that bind to β-catenin but cannot promote adhesion still inhibit β-catenin-induced transformation, suggesting that the role of cadherin as a tumour suppressor is independent of its role in cell-cell adhesion (Gottardi et al., 2001).

I.3- ***Wnt signalling***

The Wnt signalling pathway is involved in determination of cell fate and cell polarity in worms and flies, and axis formation in vertebrates. Wnt signal leads the stabilisation and accumulation of β-catenin.

Wnts are a large family of secreted glycoproteins that bind to distinct classes of receptors. One class is defined by the seven-transmembrane receptor Frizzled (Fz), which transmits Wnt signals through G-proteins (Liu et al., 2001). In addition, cell surface heparan-sulphate proteoglycans act as co-receptors (Reichsman et al., 1996; Binari et al., 1997), possibly by promoting the assembly or membrane association of Wnt signalling complexes. More recently, it has been shown that LDL-receptor-related protein (LRP) function together with Fz for binding Wnt. For example, LRP-6 interacts with Fz, but only in the presence of soluble Wnt, suggesting that the binding of the Wnt ligand to the Fz receptor induces a conformational change, necessary for the interaction with LRP-6. LRP-6 is also a receptor for the Wnt pathway

inhibitor dickkopf (dkk). Dkk blocks LRP6-mediated Wnt signalling by interacting with domains that are distinct from those required for Wnt/Fz association (Mao, B. et al., 2001).

In a current model, Wnt associates with LRP/Fz, and this results in the association of axin with the cytoplasmic tail of LRP. Axin is necessary for β-catenin phosphorylation by GSK3 (glycogen synthase kinase-3), which will result in β-catenin degradation. The recruitment of axin by LRP causes destabilisation of axin and consequently stabilisation of β-catenin (Mao, J. et al., 2001). Wnt activation also leads to phosphorylation of Dishevelled (Dsh/dvl) (Yanagawa et al., 1995) by the Dsh associated kinases, DAK and PAR-1. The activities of these kinases enhance the Wnt signalling pathway, counteracting the function of Dsh in the JNK pathway (Sakanaka et al., 2000; Sun et al., 2001).

I.3.1- *Beta-catenin/Armadillo degradation by the proteasome*

Beta-catenin and Armadillo are essential components of the Wnt/Wg-signalling pathway in vertebrates and invertebrates, respectively. In the absence of Wnt, GSK3, a serine/threonine kinase, is active. In mammals there are two forms of GSK3 (α and β). Both are constitutively active and involved in glycogen metabolism, being inhibited by insulin and other growth factors (Stambolic and Woodgett, 1994). However, the regulation of GSK3 activity by Wnt and insulin involves different mechanisms and leads to distinct downstream events (Ding et al., 2000). Both axin and β-catenin contain consensus GSK3 phosphorylation sites. GSK3 phosphorylates β-catenin in a complex that also includes APC (Adenomatous polyposis coli) and axin. β-TrCP, a vertebrate homologue of an enzyme involved in ubiquitination (Slimb), recognises the phosphorylated β-catenin in this complex and promotes its ubiquitination

and degradation (Kitagawa et al., 1999) (see Figure 2a). Mutation of phosphorylation sites in β-catenin results in its stabilisation and subsequent activation of the Wnt signalling pathway (Yost et al., 1996).

I.3.2- ***APC and Axin***

The APC gene encodes a large multidomain protein that plays an essential role in the Wnt-signalling pathway. The APC protein contains seven armadillo repeat domains; 15 and 20 amino acids long repeat domains, which are involved in β-catenin binding; a basic domain responsible for microtubule binding; and domains at the carboxyl terminus involved in interactions with other proteins. In addition, the amino terminus contains an oligomerisation domain. A second APC gene, called APC2, lacks the 15 amino acid repeats domains, but still contains the 20 amino acids repeat domains. This protein associates with axin and inhibits β-catenin-dependent transcription (van Es et al., 1999). APC is mutated in most familial and spontaneous colon carcinomas. The mutations are concentrated in a cluster that results in truncated proteins lacking at least five of the seven 20 amino acid repeats, which are required for degradation of β-catenin (Polakis, 1995).

APC is also involved in other processes in the cell in addition to β-catenin degradation. APC clusters are found at protrusions of migrating cells, where it is associated with the tips of microtubules (Nathke et al., 1996); the association of APC with the plus ends of microtubules seems to be important for chromosome segregation (Fodde et al., 2001; Kaplan et al., 2001). In polarized epithelial cells, APC is found in two distinct pools associated with the plasma membrane. The localisation of APC at the lateral plasma membrane is dependent on the actin cytoskeleton while APC clusters at the basal membrane are associated with microtubules. In *Drosophila*, E-APC is

found at adherens junctions and this localisation is dependent on the actin cytoskeleton (Townsley and Bienz, 2000). The authors suggest that, in motile cells, APC preferably associates with microtubules. In polarized cells, however, APC associate with the actin cytoskeleton at the plasma membrane.

In *Drosophila*, mutations in APC2 result in defects in the syncytial development due to release of the mitotic spindle from the cortex, where wild-type APC2/Armadillo complexes normally co-localise with actin. This suggests that this complex may be the link between the spindle and the actin cortex at the plasma membrane (McCartney et al., 2001, reviewed by Allan and Nathke, 2001).

Axin and the related protein Axin2/Conductin are multidomain proteins that bind several other components of the Wnt signalling pathway. Mutations in axin cause axis duplication in the mouse embryo (Zeng et al., 1997). Axin directly binds to β-catenin, GSK3 and APC, suggesting that the role of axin in this complex is to provide a scaffold in which the other components bind (Hart et al., 1998; Itoh et al., 1998; Behrens et al., 1998). The presence of axin is indispensable for the degradation of β-catenin since it promotes phosphorylation of β-catenin by GSK3 (Ikeda et al., 1998; Hinoi et al., 2000). Axin and APC are also substrates for GSK3 phosphorylation (Yamamoto et al., 1999; Sakanaka et al., 1998) and the phosphorylation of axin by GSK3 stabilises axin (Yamamoto et al., 1999) and increases its affinity for β-catenin. Wnt signals induce axin dephosphorylation, reducing the association of axin with β-catenin (Willert et al., 1999).

I.3.3- ***Tcf/Lef-1 family of transcription factors***

Wnt signals activation or mutations in β-catenin, axin or APC result in accumulation of β-catenin in the cytoplasm. The excess β-catenin

translocates to the nucleus in mammalian cells (Huber et al., 1996; Rubinfeld et al., 1997) and also is found in the nuclei of Wnt-activated *Xenopus* oocytes (Yost et al., 1996). The significance of this nuclear localisation was demonstrated when it was discovered that β-catenin formed a complex with transcription factors of the HMG-family (high motility group) of Tcf/Lef-1 transcription factors that recognise and bind to the nucleotide sequence 5'CCTTTGACT3' in target genes (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1991). This interaction discovered using the yeast two-hybrid system was confirmed by co-immunoprecipitation, and it was further shown that the β-catenin/Tcf complex binds to a specific DNA probe in band-shift assays (Behrens et al., 1996; Molenaar et al., 1996). Lef-1 (Lymphocyte enhancer-binding factor), Tcf-1 (T-cell factor), Tcf-3, Tcf-4 and Pangolin (the *Drosophila* Tcf homologue) are all members of this family of transcription factors. They are thought to function as architectural proteins that bind to DNA causing it to bend (Giese and Grosscheld, 1993). Moreover, these transcription factors are not able to initiate transcription by themselves, but need to interact with other proteins to do so, possibly due to the lack of a transactivation domain (Roose et al, 1998). In fact, in the absence of β-catenin, they repress transcription (Cavallo et al., 1998; Roose et al., 1998). The amino terminus of Tcf/Lef-1 is responsible for its interaction with β-catenin, and isoforms that lack this region act as negative regulators of Wnt signalling (Molenaar et al., 1996; Behrens et al., 1996; Huber et al., 1996). In APC mutant cells, β-catenin and Tcf/Lef-1 form a constitutively active complex (Korinek et al., 1997). Post-translational modifications also seem to contribute to regulation of transcription. For example, phosphorylation of Tcf-4 by a NEMO-like kinase, down regulates β-

catenin signalling (Ishitani et al., 1999). In short, in the absence of a Wnt/Wg signal, Tcf/Lef-1 represses target genes in conjunction with other transcriptional repressors, such as Groucho (Cavallo et al., 1998; Roose et al., 1998). Upon Wnt-signalling, accumulation of β-catenin leads to its translocation to the nucleus, where it associates with Tcf/Lef-1 and activates transcription of gene targets.

I.3.4- Differences among armadillo family members

Although β-catenin and plakoglobin share some identity, they have distinct activities in cells. As mentioned earlier, targeted deletion of β-catenin and plakoglobin in mice results in different phenotypes suggesting that they have different roles during development (Haegel et al., 1995; Ruiz et al., 1996; Bierkamp et al., 1996; Huelsken et al., 2000).

Plakoglobin is more resistant to degradation by the proteasome than β-catenin (Sadot et al., 2000) and overexpression of plakoglobin results in the accumulation of β-catenin, most likely because it titrates proteins involved in the degradation of β-catenin. This accounts for the induction of double axis formation in *Xenopus* by expression of a membrane-tethered form of plakoglobin (Rubenstein et al., 1997). Both β-catenin and plakoglobin contain a domain at their carboxyl terminus that, when fused to the GAL4 DNA binding domain, activates transcription of a reporter plasmid containing a GAL4 recognition motif. However, plakoglobin is less active than β-catenin when a Lef-1 responsive reporter is used (Ben-Ze'ev and Geiger, 1998). Similarly, armadillo's carboxyl terminus contains a transactivation domain (Cox et al., 1999) that overlaps with the binding domain of the transcription factor Teashirt (Gallet et al., 1999). Nevertheless, neither β-catenin nor plakoglobin can rescue or complement Armadillo mutations in *Drosophila* (White et al., 1998), even

though β-catenin interacts with dTcf (Riese et al., 1997). This suggests that cooperation with species-specific co-activators is necessary for correct activation of target genes.

The amino terminus of β-catenin, which differs from those in Armadillo and plakoglobin, contains two regions that interact with TATA-box binding protein (TBP) and thus also functions as a transactivation domain. The same regions in plakoglobin do not associate with TBP (Hecht et al., 1999). One possibility is that the carboxyl-terminal domain functions as a general transactivation domain, while the amino-terminal domain provides specificity by binding to specific co-activators (Hecht et al., 1999).

The involvement of β-catenin in tumorigenesis is well documented. Mutations in β-catenin itself, or in genes encoding proteins that regulate its stability, have been identified in several kinds of cancer, especially of the colon and in melanomas (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). Plakoglobin levels, on the other hand, are usually low or absent in tumour cells (Ben-Ze'ev, 1997).

I.3.5- *Wnt gene targets*

Several Wnt target genes have been identified. For example, the *Xenopus* homeobox gene Siamois, which is involved in axis formation in the frog embryo (Brannon and Kimelman, 1996), Xnr3, a *Xenopus* nodal related gene (McKendry et al., 1997), and engrailed-2 (Mcgrew et al., 1999) are direct targets of β-catenin/Tcf. In *Drosophila*, Ultrabitorax (Ubx) (Riese et al., 1997) and Engrailed (Yu et al., 1998) are two examples of gene targets.

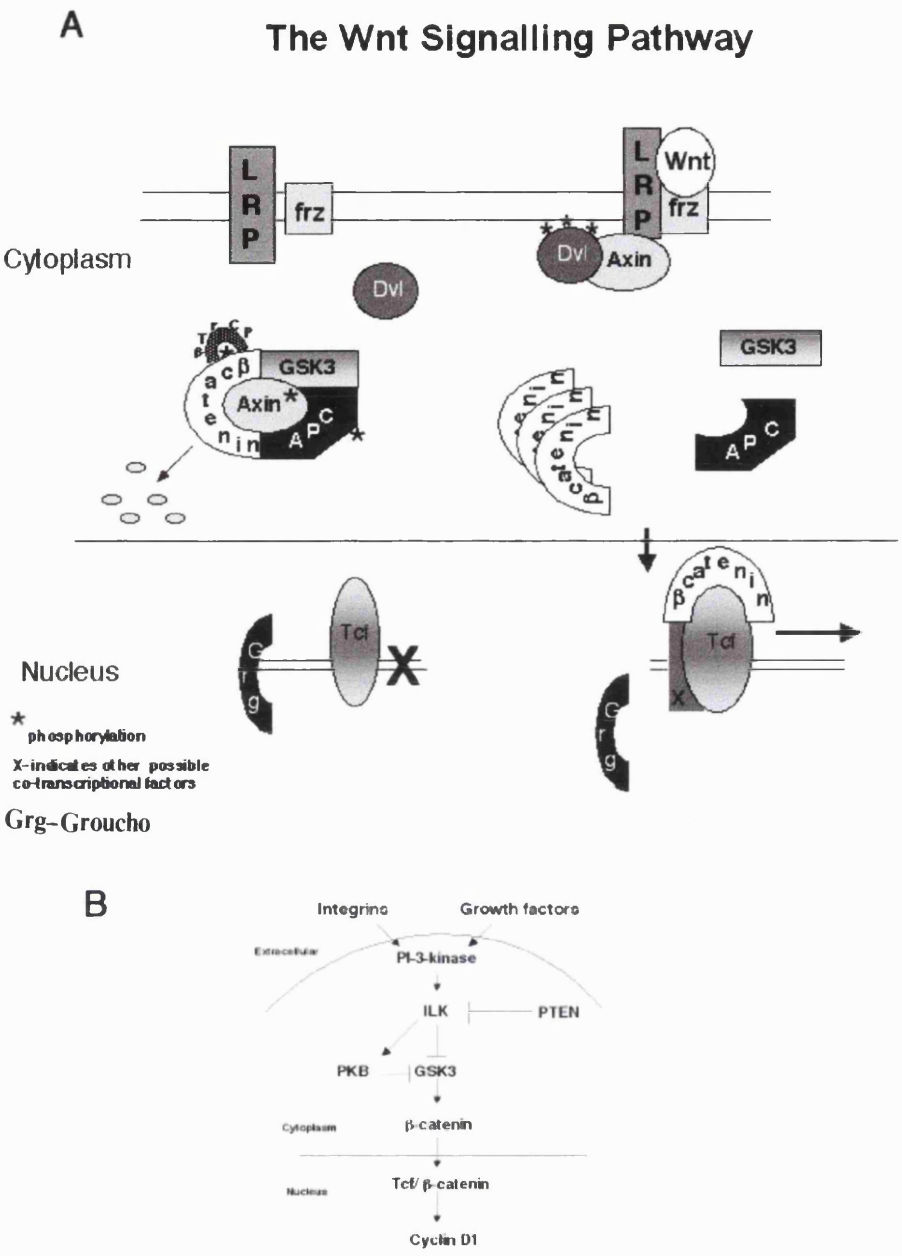


Figure 2.
(A)The Wnt Signalling pathway. The ligand Wnt binds to its receptors, Frizzled and LRP, in the cell membrane, initiating a cascade of events that will lead to the accumulation of β-catenin.GSK3 phosphorylates β-catenin when complexed to APC and axin. Phosphorylation of β-catenin targets it for degradation by the proteasome. Upon Wnt binding, β-catenin is released from the complex, it accumulates in the cytoplasm and enters the nucleus, where it binds to Tcf and activates transcription of gene targets. This activation might also require accessory proteins (X).
(B) Signal transduction pathways intersect and overlap.
Schematic representation of the regulation of β-catenin signalling by integrins and growth factors via ILK and GSK3. ILK activity is regulated in a PI-3 kinase-dependent manner. Stimulation of ILK results in a phosphorylation-mediated inhibition of GSK-3 activity. PTEN suppresses the expression of cyclin D1 by inhibiting PI-3K dependent activation of ILK (taken from Persad et al., 2001).

In mammalian cells, genes involved in cell cycle regulation and growth including c-myc (He et al., 1998), c-jun (Mann et al., 1999) and cyclin D1 (Tetsu et al., 1999 and Shutman et al., 1999) contain Tcf binding sites on their promoters and are activated by β-catenin/Tcf. Other examples in vertebrates are β-TrCP (Spielgelman et al., 2000) and Tcf-1 (Roose et al., 1999), both of which may provide a negative-feedback loop.

I.3.6- ***Other proteins involved in the transcription complex***

The Wnt-signalling cascade is now very complicated because several new activators and repressors of β-catenin/Tcf complex have been identified. Although this has made the pathway more intricate, it has provided explanations for the specific activation of gene targets in different tissues and organisms.

The association of β-catenin with p300 and the related protein CBP (CREB-binding protein) provides yet another means of regulating transcription. CBPs have histone acetylase activity and can modify chromatin structure. They are also transcriptional co-activators, linking transcription complexes to the basal transcription machinery (Mannervik et al., 1999). Moreover, they potentiate the activity of other transcription factors such as p53, cyclin E and AP-1. Although in *Drosophila* CBP acts as a repressor of Wg (Waltzer and Bienz, 1998), in vertebrates these proteins seem to function as positive regulators of Wnt (Hecht et al., 2000). The activity of *Drosophila* CBP (dCBP) is also regulated by phosphorylation by MAP kinase, providing another way to regulate the Wg signal (Hecht and Kemler, 2000).

Repression by Lef-1 requires histone deacetylase (HDAC) activity and association of Lef-1 with HDAC1 has been shown to occur *in vivo* (Billin et al, 2000). Association of Lef-1 with β-catenin results in the

inhibition of histone deacetylase activity and activation of a reporter driven by a Lef-1 responsive promoter (Billin et al., 2000). Repressors such as Groucho (Cavallo et al, 1998; Roose et al., 1998) are also involved in the regulation of β-catenin/Tcf target genes, possibly due to their interactions with histone deacetylases (Branjes et al., 2001). The binding of β-catenin might displace these repressors or regulate the acetylation status of chromatin, allowing transcription to take place. On the other hand, displacement of repressors is not sufficient to trigger activation of transcription; association with other proteins is also required. Beta-catenin, for instance, can associate with the TATA-box binding protein TBP directly or indirectly via its association with Pontin-52. Pontin-52 is a conserved protein that might link β-catenin to the basal transcription machinery (Bauer et al., 1998). Counteracting the positive effect of Pontin-52 on Wnt-signalling is a protein named Reptin-52 (repressor of Pontin-52). Reptin-52 competes with Pontin-52 for the binding to amino acids 183/187-284 in β-catenin and it inhibits β-catenin dependent transcription (Bauer et al., 2000).

As mentioned above, the carboxyl terminus of Armadillo also interacts with the zinc-finger protein Teashirt, (that acts as a negative regulator of Wg-signalling Gallet et al., 1998). Wg induces phosphorylation of Teashirt, thereby inhibiting expression of Ubx in the fly midgut (Waltzer et al., 2001).

Finally, Suppressor of fused [Su (fu)], a negative regulator of Hedgehog signalling in the fly, binds to β-catenin in mammalian cells and downregulates its signalling activity by facilitating nuclear export of β-catenin (Meng et al., 2001).

I.4- *Nuclear Transport*

Eukaryotic cells are characterised by a nuclear membrane that segregates nucleic acids and proteins involved in DNA duplication and transcription from the cytoplasm. Because protein synthesis takes place in the cytoplasm, however, mechanisms have evolved to transport mRNA from the nucleus to the cytoplasm and transport nuclear proteins, synthesised in the cytoplasm, into the nucleus. Small proteins (up to ~40-50 kDa) can diffuse in and out of the nucleus in an independent manner, although there are small molecules, such as tRNAs, rRNAs, mRNAs and histones, which transit in a carrier-mediated and active way. Proteins that exceed the exclusion size and traffic in and out of the nucleus usually have nuclear localisation signals (NLS), consisting of a sequence of positively charged residues, and leucine rich sequences that form nuclear export signals (NES). These sequences guarantee transit to and from the nuclear compartment.

I.4.1- *Nuclear Import*

This process starts in the cytoplasm, by recognition of the NLS by importin-α and association of the cargo/importin-α complex with importin-β. The interaction of the whole complex with the nuclear pore complex (NPC) is mediated by importin-β. Entrance through the pore is an energy-dependent process involving the hydrolysis of GTP by the small GTPase Ran. This hydrolysis also results in the release of the cargo from the importin-α/β complex, and recycling of the importins to the cytoplasm, where more cargo will be assembled. The export of the importins uses a separate set of export receptors called CAS.

I.4.2- *Nuclear export*

The export of proteins from the nucleus also involves specific receptors. The major nuclear export pathway is mediated by the interaction between the NES sequence present in the cargo protein with the CRM1/exportin receptors. Similar to nuclear import, export requires the small GTPase Ran, but in this case, binding of RanGTP stabilises the cargo/CRM1 complex. There is a gradient of RanGTP between the nucleus (high levels) and cytoplasm (low levels), and, since RanGTP affects the binding of cargo with import and export factors in opposite ways, this gradient guarantees the directional transport of cargo through the NPC (Izaurralde et a., 1997). The antifungal antibiotic Leptomycin B (LMB) blocks export by interacting with CRM-1 and preventing passage through the NPC.

I.4.3- *Nuclear localisation of APC*

In cultured human epithelial cells, staining of APC shows that this protein is present in the cytoplasm, in the nucleus and also concentrated at the edges of migrating cells (Neufeld et al., 1997; Nathke et al., 1996). Recently, the role of APC in the regulation of Wnt-signalling has been expanded and this protein is considered important, not only in controlling β-catenin degradation, but also in the export of nuclear β-catenin (Henderson, 2000; Rosin-Arbesfeld et al., 2000; Zhang et al., 2000; Neufeld et al. 2000). These reports also show that the localisation of wild-type APC is LMB-sensitive, suggesting that it is mediated by CRM-1. These studies, however, disagree on the precise location of the NESs. Henderson (2000) showed that only one of the three putative NES identified at the amino terminus of APC was active and the results of Neufeld et al. (2000) support these findings. Another study, however, described NES signals at

the 20 amino acid repeat domains of APC (Rosin-Arbesfeld et al., 2000).

Two NLS signals have also been identified in APC, one located between residues 1767-1772 and the other at 2048-2053 (Zhang et al., 2000). However, since mutant APC lacking both the above NLSs is found in the nucleus of colon cancer cells, it is likely that there are other NLS, or that APC does not require an NLS to enter the nucleus (Henderson, 2000).

I.4.4- ***Nuclear localisation of β-catenin***

The accumulation of β-catenin that results from a Wnt signal or from mutations in β-catenin, axin and APC, leads to translocation of β-catenin into the nucleus (Rubinfeld et al., 1997; Korinek et al., 1997; Morin et al., 1997; Liu et al., 2000; Satoh et al., 2000). Since β-catenin lacks a canonical NLS, it was believed that β-catenin entered the nucleus by binding Tcf/Lef-1. However, β-catenin mutants that cannot associate with Tcf can still enter the nucleus (Orsulic et al., 1996; Miller et al., 1997). Indeed, β-catenin may use a similar mechanism as Importin-β to enter the nucleus, involving direct association with components of the NPC (Fagotto et al., 1998; Yokoya et al., 1999). It is unclear whether APC contributes to the export of β-catenin, since even in the absence of cytoplasmic factors and presence of LMB endogenous β-catenin can exit the nucleus (Eleftheriou et al., 2001). Another possibility is that nuclear export of β-catenin is regulated by the associating protein Su(fu) in a CRM-1 dependent manner. Repression of β-catenin transcription activity by Su(fu) is therefore reversed by the nuclear export inhibitor LMB (Meng et al., 2001).

I.5- ***Tumorigenesis***

Malignant transformation is a multi-step process, triggered by mutations in genes involved in cell proliferation. Tumorigenesis is

characterised by deregulation of cell growth and loss of contact inhibition. Malignancy involves, not only activation of transcription of specific genes, but also, changes in cell adhesion and cytoskeletal organisation, leading to changes in cell shape and migration.

I.5.1- ***Cadherins in tumorigenesis***

The role of the E-cadherin-catenin complex in tumour progression has been the focus of many studies, and these molecules might serve as prognostic markers for certain cancers. Reduced expression levels or loss of E-cadherin have been reported for several tumours from different tissues and organs including colon, stomach, liver, skin, pancreas, lung, ovary and bladder. There seems also to be an inverse correlation between the degree of malignancy and levels of E-cadherin expressed by the tumour cells, where more differentiated and less invading tumours express more of this protein, while more invasive, less differentiated ones have lost E-cadherin expression (Dorudi et al., 1995; Gamallo et al., 1993; reviewed by Shiosaki et al., 1996). Inactivation of the E-cadherin adhesion system in cancers might be achieved by mutation on the promoter region of the gene encoding for this protein or through other components of the adhesion system. Mutation or reduced expression of the catenins, changes in the phosphorylation of components of the cell-cell adhesion complex, and modification of the adhesion complex by proteins such as IQGAPI and Rho, can all modulate cell-cell adhesion. Activation of the oncogene product c-erbB-2, for example, leads to high phosphorylation of β-catenin and loss of cell-cell adhesion in cells that have intact E-cadherin (Ochiai et al., 1994).

The role of cadherins in controlling cell growth might be achieved by regulation of adhesion and/or by titrating signalling molecules, such as

β-catenin. There is evidence that both mechanisms can take place. For instance, in three-dimensional cultures, antibodies that block E-cadherin function prevent contact-dependent growth inhibition and induce proliferation of mammary carcinoma cells by reducing cellular levels of p27 (St.Croix et al., 1998). On the other hand, expression of E-cadherin constructs, which contain the cytoplasmic domain of this protein, reduces the growth of SW480 cells independently of cell adhesion. This inhibition is dependent on direct binding to β-catenin and inhibition of β-catenin-dependent transcription (Gotardi et al., 2001; Simcha et al., 2001). Cadherin constructs also function as negative regulators of Wnt-signalling in *Xenopus*, inhibiting dorsal mesoderm induction (Heasman et al., 1994).

I.5.2- ***Wnt signalling in tumorigenesis***

Malignant growth results from the accumulation of several, independent mutations and/or chromosomal alterations. FAP (familial adenomatous polyposis) patients will develop over the years, several benign polyps in the colon and rectum. In some cases, the benign polyps will progress and begin to invade, causing metastasis. Genetic analysis of FAP patients has revealed germline mutations in the tumour suppressor gene, APC. The discovery that the APC protein is a component of the Wnt-signalling pathway suggested this pathway was intimately involved in the development of colorectal cancer.

I.5.3- ***APC in tumorigenesis***

The intestinal epithelium is a tissue in constant renewal. Cells are continuously dividing at the base of the villi and being shed at the top. This results in a balance in the regenerating colon mucosa. Mutations in APC disturb this equilibrium resulting in the formation of intestinal polyps (Morin et al., 1996). In approximately 85% of cases of either

sporadic or hereditary colorectal tumours, APC function is lost (Kinzler and Volgelstein, 1996). Another effect of APC mutations might be altered cell migration in the intestinal villi, resulting from the inability of mutated APC to bind and stabilise microtubules (Nathke, 1996). Moreover, APC binds to an exchange factor (Asef), which activates the small G protein Rac regulating remodelling of the actin cytoskeleton (Kawasaki et al., 2000). The incidence of cancers with mutations in the APC gene exceeds those with mutations in β-catenin: 70-80% of cancers reported in the literature have APC mutations against 1-10% of cancers with mutations in β-catenin. This is intriguing, especially because for tumours to develop, mutations in β-catenin only need to occur in one allele, while in APC, both alleles must lose their function (Rowan et al., 2000, reviewed by Fearnhead et al., 2001). This suggests that APC might have other important functions in the cell in addition to its role in regulating β-catenin. Indeed, APC is involved in chromosome segregation, and it accumulates at the kinetochore during cell division, presumably helping microtubules to bind to this region. Truncations of the carboxyl-terminal microtubule-binding domain of APC are often found during tumour progression and contribute to chromosomal instability (Kuipers et al., 2001; Kaplan et al., 2001). In addition, APC not only down-regulates β-catenin, but also plakoglobin. Although no oncogenic mutations have been reported for plakoglobin, it can function as an oncogene when its expression is deregulated (Kolligs et al., 2000).

I.5.4- ***Beta-catenin in tumorigenesis***

As mentioned earlier, up regulation of the level of β-catenin is involved in development of cancer. Overexpression of β-catenin in the intestinal epithelium of mice results in an increase cell proliferation.

However, this is antagonised by higher levels of apoptosis, which prevents tumour formation (Wong et al., 1998). In the skin, however, overexpression of β-catenin results in increased development of hair follicles and formation of benign tumours of the hair matrix (Gat et al., 1998). Two cell cycle regulation genes, c-myc and cyclin D1, are targets of β-catenin-dependent transcription (He et al., 1998; Tetsu et al., 1999). C-myc is involved in cell cycle progression, transformation and apoptosis. In colon cancer cells that express mutant β-catenin, other mutations in oncogenes and proto-oncogenes, such as *K-ras*, are also found. These prevent apoptosis and result in cell multiplication. Mutations in β-catenin have also been identified in melanomas (Rubinfeld et al., 1997), prostate cancer (Voeller et al., 1998) and other cancer types, and they appear to be a crucial step in cancer progression in many cell types.

I.5.5- *Alpha-catenin in tumorigenesis*

Altered cell-cell adhesion in cancer cells results from impaired function of the cell-cell adhesion complex. Analysis of several human carcinoma samples indicates that, in some cases, where E-cadherin expression is unchanged, α-catenin expression is down regulated. In two studies, approximately 80% of the primary tumours analysed showed a reduction in α-catenin (Shiozaki et al., 1994; Kadowaki et al., 1994). Similar results have been obtained for prostate cancer (Morton et al., 1993). In colon cancer cell lines, poorly differentiated cells exhibit low Ca^{2+}-dependent cell-cell adhesion due, not to the lack of E-cadherin expression, but rather the absence of α-catenin. Re-introduction of α-catenin restores adhesion (Breen et al., 1993). The lack of α-catenin expression also correlates with non-epithelial morphology and an increase in invasiveness

in *in vitro* assays, and is linked to unfavourable prognosis for patients with colon cancer (Vermeulen et al., 1995; Raftopoulos et al., 1998; Vermeulen et al., 2000). *In vivo* assays, however, fail to show a correlation between invasiveness and lack of α-catenin, suggesting that environmental factors are also involved (Hoorde et al., 2000). Expression of α-catenin is linked to that of E-cadherin in L cells, which do not normally express cadherins. Forced expression of cadherin in these cells, promotes stabilisation of α-catenin, restoring cell-cell adhesion (Nagafuchi et al., 1987). Moreover, when overexpressed in this cadherin-deficient cell line, α-catenin inhibits Wnt 3a-induced transcription (Takahashi et al., 2000). So far, no tumours have been found in which α-catenin is present and E-cadherin is not, suggesting that alterations in the α-catenin gene affect only the expression of this protein without interfering with E-cadherin levels. These findings indicate that the absence of α-catenin, and not that of E-cadherin, correlates with the pathology of these tumours (Gofuku et al., 1999).

Ovarian cells lacking functional α-catenin also have impaired cell-cell adhesion, which is restored after artificial expression of this protein. More importantly, expression of α-catenin slows the growth rate of these cells and also decreases their ability to form tumours when injected into nude mice (Bullions et al., 1997). Taken together, these results indicate that down regulation of α-catenin may be important, not only for cell-cell adhesion, but also for cell growth and invasion.

I.6- *Other players in tumorigenesis*

I.6.1- *PI3K and PTEN*

The activity of lipid kinases, such as phosphatidylinositol 3 kinase (PI3K), is essential for many cellular processes. Mutations in the p110γ catalytic subunit of PI3Kγ have been identified in human primary

colorectal adenocarcinomas and in colon cancer cell lines. The overexpression of this subunit restores normal growth in cells expressing mutated APC, p53, β-catenin or *K-ras* (Sasaki et al. 2000).

Protection from apoptosis requires the activation of PKB/Akt and negative regulation of the tumour suppressor PTEN (phosphatase and tensin homologue deleted from chromosome 10). PTEN, by dephosphorylating phosphatidylinositol 3,4,5 triphosphate (PIP-3), antagonises the activity of PI3K, inhibiting cell growth and thus functioning as a tumour suppressor. The activation of PKB/Akt by PI3K inhibits apoptosis by preventing the release of cytochrome c from mitochondria. Activated Akt also phosphorylates BAD and caspase–9, inactivating these two pro-apoptotic factors (reviewed by Di Cristofano et Pandofili, 2000). Moreover, activated Akt inactivates GSK3, resulting in cyclinD1 expression (Sun et al., 1999). Cells that have lost PTEN expression have higher levels of cyclin D1, a β-catenin/Tcf target gene. Expression of PTEN in these cells results in inhibition of β-catenin/Tcf-dependent transcription, possibly by promoting degradation of β-catenin (Paramio et al., 1999; Persad et al., 2001).

I.6.2- *ILK*

Another player in the pathway described above is the Integrin-Linked Kinase (ILK). ILK is a serine/threonine kinase that transmits signals activated by cell-extracellular matrix interactions. Overexpression of ILK in epithelial cells results in nuclear localisation of β-catenin and activation of β-catenin/Tcf-dependent transcription (Novak et al., 1998). The activation of Wnt-signalling by ILK is a consequence of its ability to inactivate GSK3 (see Figure 2b).

I.7- *Cross talk between different signalling pathways*

I.7.1- *Wnt and TGF-β signalling*

Mutations in APC are not sufficient to trigger malignancy and further genetic alterations are needed for the development of invasiveness. Such alterations are represented for example, by loss of a response to TGF-β (transforming growth factor-β) in colon cancers cells (Thiagalingam et al., 1996; Grady et al., 1999). TGF-β inhibits cell growth and may be involved in maintaining the normal cycle of cell renewal in the intestinal epithelium. Activation of TGF-β induces the formation of a complex containing Smad 2/3 and Smad 4, which associates with transcription factors and activates specific gene targets. The Smad 4 and Smad 2 genes are lost in some colorectal tumours (reviewed by Bienz and Clevers, 2000). Further evidence for the involvement of this signalling pathway in tumorigenesis comes from mice doubly-heterozygous for mutations in APC and Smad 4, which develop large intestinal tumours that are both highly proliferative and invasive (Takatu et al., 1998).

Cooperation between Wnt and TGF-β signalling also occurs in the frog embryo, where both pathways are necessary for the establishment of the dorsal signalling centre (Spemann's organizer). Beta-catenin/Tcf complexes associate with Smad 4, leading to expression of homeobox XTwin gene (Nishita et al., 2000). The collaboration between Wnt and TGF-β signalling would result in differential expression of specific genes depending on whether or not Smad 4 is present in the β-catenin/Tcf complex. For instance, the promoter regions of Twin and Ubx contain both Wnt/Wg and TGF-β recognition elements (Hecht and Kemler, 2000).

I.7.2- *Involvement of Ras*

Point mutations that activate the *K-ras* proto-oncogne are found in at least 50% of human colorectal cancers and they play an essential role in tumorigenesis. Disruption of activated *K-ras* in colon cancer cell lines changes their morphology, abrogates their ability to evade contact inhibition, causes loss of anchorage-independent growth, slows the growth rate, inhibits the formation of tumours in nude mice and reduces expression of c-myc (Shirasawa et al., 1993). The *ras* pathway is also involved in the lack of response of colon cancer cells to TGF-β signals (Calonge et Massague, 1999).

I.7.3- *p53*

P53 is a tumour suppressor gene mutated in most cancers (reviewed by Damalas et al., 1999). Functional p53 helps to maintain cellular genomic stability by eliminating cells with DNA damage or by helping repair this damage. The cellular level of p53 is normally low, since p53 is degraded in the ubiquitin-proteasome pathway (Maki et al., 1996). In response to stress, p53 levels rise and it moves to the nucleus, causing cell cycle arrest or induction of apoptosis. Overexpression of β-catenin results in high levels of p53 protein (but not mRNA), due to competition between these two proteins for the components of the degradation pathway (Damalas et al., 1999). β-catenin also competes with p53 for the binding to the co-activator CBP/p300, which is necessary for activation of apoptosis by p53 (Miyagishi et al., 2000).

More recently p53 was shown to be involved in the activation of an alternative degradation pathway for β-catenin. A p53-inducible ubiquitin-conjugating enzyme, Siah, binds to the carboxyl terminus of APC and induces β-catenin degradation. Siah also binds to the F-box protein Ebi, which binds to

β-catenin. Unlike β-TrCP, Ebi interacts with β-catenin, independently of GSK3 phosphorylation (Matsuzawa and Reed, 2001). Thus, Siah downregulates β-catenin signalling in cells that express β-catenin lacking GSK3 phosphorylation sites, but does not downregulate this signalling pathway in cells that express truncated APC (Liu et al., 2001). These results indicate the existence of a degradation pathway that is APC-dependent, but GSK3-independent.

I.7.4- *Wnt and Retinoic acid signalling*

Retinoids (vitamin A derivatives) have a strong effect on cell proliferation, differentiation and carcinogenesis. Treatment of cells with retinoic acid (RA) decreases β-catenin-dependent transcription through a direct association between the retinoic acid receptor (RAR) and β-catenin. Moreover, this association induces transcription of RAR-responsive promoters, suggesting that β-catenin can work as a co-factor in the RA signalling pathway (Easwaran et al., 1999). More recently, it has been shown that treatment of colon cancer cells that express vitamin D receptor (VDR) with vitamin D3 results in down regulation of β-catenin-dependent transcription, promoting cell differentiation. This inhibition is due to the competition between ligand-activated VDR and Tcf for binding to β-catenin (Palmer et al., 2001).

The intimate connection between cell-cell adhesion and Wnt-signalling pathway has highlighted the importance of the proteins involved in regulating the levels of β-catenin, both during development and in tumour progression. Alpha–catenin is ideally placed to regulate these processes, since it is found in cell adhesion complexes and associates with β-catenin to influence Wnt signalling.

CHAPTER II- Materials and Methods

II.1- *Molecular Biology*

II.1.1- *DNA purification*

II.1.1.2- *Minipreps*

DNA was prepared from 2 ml of culture using the UltraClean Mini Plasmid Prep Kit (Cambio #12300-100) according to the manufacturer's instructions. This kit is based on lysis of cells by alkali. After purification, one tenth of the DNA obtained was digested with appropriate restriction enzymes to check its identity. Once a positive clone was identified, the respective colony was amplified by growth overnight at 37^0 C in 100 ml of LB (Luria-Bertani medium: bacto-tryptone 10 g/l; bacto-yeast extract 5 g/l; NaCl 10 g/l; pH 7.0) with appropriate antibiotic.

II.1.1.3- *Maxipreps*

The amplified colony was harvested at 6,000 *g* for 10 minutes at 4^0 C and the plasmid DNA was purified using the EndoFree Plasmid Maxi Kit (QIAGEN #12362). The kit is based on a modified alkaline lysis method followed by binding of plasmid DNA to an anion-exchange resin under controlled pH and salt conditions. Proteins, RNA and low-molecular impurities are removed by a medium-salt wash (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol). The plasmid DNA is eluted in a high-salt buffer (1.6M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol) and further precipitated with isopropanol. The salt is removed by a wash with 70% ethanol. The plasmid DNA isolated by this method is pure enough for use in transfections of mammalian cells.

II.1.2- *DNA Sequencing*

To confirm the identity of the DNAs, these were sequenced by MWG-Biotech, Ebersberg, Germany.

II.1.3- ***Agarose gels***

DNA fragments were routinely visualised on 1 x TAE agarose gels (1 x TAE: 40 mM Tris-acetate; 1 mM EDTA) stained with 0.8 μg/ml ethidium bromide (GibcoBRL #15585-011). Fragments between 500 and 5000 base pairs were separated in 1% agarose gels (SIGMA #A-9311). For fragments smaller than 500 base pairs, 1.5-2% agarose was used. The 1 Kb DNA Ladder (GibcoBRL #15615-016) was used as molecular weight marker to assess fragment sizes. DNA samples were loaded with 6 x loading buffer (SIGMA #G-7654) to a final concentration of 1 x and electrophoresis was carried out at 80 volts for about 1 hour.

II.1.4- ***Restriction digests and purification of DNA fragments***

As a general rule, approximately 1 μg of plasmid DNA was digested in with 10 U of restriction enzyme (all restriction enzymes used were obtained from GibcoBRL or New England Biolabs) in a final volume of 30 μl. To prevent re-ligation of vectors when only one restriction enzyme was being used, DNA was treated with Calf Instestinal Alkaline Phosphatase (GibcoBRL #18009-027) according to manufacturer's instructions. This enzyme was heat inactivated for 10 minutes at 75^0 C, prior to ligation. The fragments were separated in agarose gels as described above and once visualised, the DNA of interest was excised from the gel with a scalpel blade and purified from the agarose using QIAquick Gel Extraction Kit (QIAGEN #28704), following manufacturer's instructions. This method is based on the absorption of DNA by silica gel columns in the presence of high salt, while impurities are washed away. The DNA was then eluted from the columns using the elution buffer (10 mM Tris, pH 8.5) provided and used for ligation reactions.

II.1.5- *Ligation of DNA fragments*

Typically ligation reactions were carried out using a molar ratio of vector (gel-purified linear DNA) to insert of between 1:5 and 1:10. When oligonucleotides were used for the ligation the ratio vector:insert was 1:30. The total reaction volume was 10 μl and included 1 μl of T4 ligase (Promega #M1801) in the buffer system supplied with the enzyme. The reactions were performed at 15^0 C for 18 hours and one fifth of the ligation reaction was used to transform competent *E. coli* as described below.

II.1.6- *Transformation of E.coli*

Subcloning efficiency competent *E. coli* (DH5α) were purchased from GibcoBRL (#18265-017) and transformation followed instructions provided by the manufacturer. Briefly, a 50 μl aliquot of competent cells was thawed on ice, mixed thoroughly with 2 μl of the ligation reaction and left to incubate on ice for 30 minutes. Cells were heat-shocked for 30 seconds at 37^0 C and then placed on ice for two minutes. After the heat shock cells were grown in a shaking incubator set at 225 rpm in 0.8 ml of SOC media (GibcoBRL #15544-042) for 1 hour at 37^0 C. As a general rule, 0.2 ml of the transformation was plated onto LB-agar plates (LB medium plus 20 g/l bacto agar) containing 100 μg/ml Carbenicillin (SIGMA #C-3416) or 50 μg/ml of Kanamycin (SIGMA #K-0254). Plates were incubated overnight at 37^0 C to allow growth of the transformed cells. For DNA purification, colonies were picked and grown overnight in 2 mls of LB plus appropriate antibiotic for selection.

II.1.7- *DNA Plasmid constructs*

II.1.7.1- *Beta-catenin constructs*

GFP fusions were made using GFP derived from pEGFP vectors (Clontech, Palo Alto, CA). To make GFP-β-catenin, an EcoRI fragment encoding *Xenopus* β-catenin with a carboxyl-terminal myc-epitope tag (pMT23-βmyc) (Kypta et al., 1996) was cloned into the EcoRI site of pEGFP-C1. GFP-β-catenin-ΔR (which lacks the armadillo repeats) and GFP-β-catenin-ΔC were made in the same way using pMT23-βΔRmyc and pMT23-βΔCmyc, respectively. GFP-β-catenin-ΔN and GFP-β-catenin-ΔNΔC were made by cloning the XhoI-EcoRI fragments from pMT23-βmyc and pMT23-βΔCmyc, respectively into pEGFP-C1 that was cut with XhoI-EcoRI. Both plasmids encode GFP fused to residue 151 of β-catenin. To make GFP-β-catenin-ΔN2, pMT23-β-43-165 was first made by PCR. This encodes the amino acid sequence MEVMN followed by residues 43-165 of β-catenin. The XhoI-EcoRI fragment from pMT23-βmyc (containing residues 151-781) was then cloned into pMT23-β-43-165 cut with XhoI, to make pMT23-ΔN2-βmyc. The EcoRI fragment from pMT23-ΔN2-βmyc was then inserted into pEGFP-C1 resulting in a GFP fusion containing the sequence MEVMN followed by residues 43-781 of β-catenin and a myc-epitope tag. To make GFP-β-catenin-Δα, pMT23-β-120 was first made by PCR. This encodes residues 1-120 of β-catenin with a 3′ XhoI site. The XhoI fragment containing these residues was cloned into pMT23-βΔNmyc to make pMT23-βΔαmyc, which lacks residues 122-150 of βmyc. This was subcloned into pEGFP-C1 to make GFP-β-catenin-Δα. Beta-catenin-GFP encodes residues 1-761 with a carboxyl-terminal GFP tag. It was made by first inserting a BglII fragment containing residues 7-761 of β-catenin into the BamHI site of pEGFP-N1. The missing amino-terminal sequences of β-catenin were then replaced using the HindIII fragment encoding residues 7-

365 with the HindIII fragment from wild-type β-catenin encoding residues 1-365. To make GFP-β-1-420 (GFP-β-1/7), an EcoRI-PvuII fragment from pMT23-βmyc was inserted into pEGFP-C1 cut with EcoRI-SmaI. To make β-398-761-GFP (β-8/12-GFP), β-catenin-GFP was cut with HindIII and re-ligated to remove the sequence encoding residues 1-365. The fusion protein begins at the next methionine, residue 398.

II.1.7.2- ***APC, Tcfs, Axin and Reporter plasmids***

Myc-epitope tagged APC cDNA (Vleminckx et al., 1997) was kindly provided by Dr. Barry Gumbiner, Memorial Sloan-Kettering Cancer Center, NY. HA-tagged Tcf-4 cDNAs, p45 Tcf-1, p33 Tcf-1, pTOPFlash and pFOPFlash reporters (Korinek at al., 1997) were gifts from Dr. Marc van de Wetering and Hans Clevers, Utrecht University, The Netherlands. An EcoRI/SalI treated HA-Tcf-4 was cloned into pEGFP-N1 treated with the same restriction enzymes to create a GFP-Tcf-4 fusion protein. The Cyclin D1 reporter plasmid include the pA3 luciferase plasmid attached to the full length cyclin D1 promoter (1745-luc) and was a gift from Dr. Richard Pestell, Albert Einstein College of Medicine, Yeshiva University, New York. Axin DNA was a gift from Dr. Akira Kikuchi, Hiroshima University, Japan. Axin was excised with SmaI/EcoRV and sub-cloned into the pEGFP-C1 SmaI site to create a GFP-axin fusion protein, with the GFP tagged to the amino terminus of axin.

II.1.7.3- ***Cadherin, α-catenin, Vinculin and ADF/cofilin constructs***

Truncated myc-epitope tagged *Xenopus* N-cadherin cDNAs (Riehl et al. 1996) were provided by Dr. Christine Holt, University of California San Diego, CA, and cloned into pSG5 (Stratagene) for expression in COS-7 cells. Plakoglobin-GFP was made by inserting the EcoRI/XbaI fragment from pCS2mt-dG-XPKG-GFP (Rubenstein et al., 1997) (a gift from Dr. Mike Klymkowsky, University of Colorado, CO) into pEGFP-C1. It encodes

Xenopus plakoglobin lacking the first 39 residues (similar to the deletion made in β-catenin to produce GFPΔN2-β-catenin) with GFP at the carboxyl terminus. Human αN-catenin cDNA (Claverie et al., 1993) was provided by Drs. Jean-Pierre Hardelin and Christine Petit, Institute Pasteur, Paris. The coding sequence was removed using XbaI-NsiI and cloned into pMT23 cut with SpeI-PstI. Avian αN-catenin fused to GFP (Sehgal et al., 1997) was provided by Drs. Ravinder Sehgal and Louis Reichardt, (University of California, San Francisco). Plasmids encoding GST-α-catenin constructs (full-length, GST-α N-228 and GST-α C-447; Rimm et al., 1995) and respective purified fusion proteins were generously provided by Dr. Vania Braga (Imperial College, London), with permission from Dr. David Rimm (Yale University).

To make GFP-α-catenin-NLS (GFP-α-NLS), a double-stranded oligonucleotide (MWG Biotech, Germany) encoding the nuclear localisation signal from SV40 large T antigen (Fisher et al., 1988) and appropriate restriction sites was ligated into GFP-αN-catenin cut with AflII and HindIII. This resulted in the insertion of the sequence AARDPKKKRKV after residue 902 of avian αN-catenin, followed by a stop codon. GFP-αΔ-catenin-NLS (GFP-αΔ-NLS) was made by deleting internal sequences in GFP-α-catenin-NLS using XhoI, resulting in a fusion protein containing residues 1-214 of αN-catenin followed by the nuclear localisation signal.

GFP-vinculin consists of full-length chicken vinculin cDNA inserted into pGZ21 vector that contains a CMV promoter and enhanced GFP protein. This construct was a gift from Dr. Benjamin Geiger (The Weizmann Institute of Science, Rehovot, Israel).

XAC(A3)-GFP consists of *Xenopus* ADF (actin depolymerising factor) in which serine residue 3 has been mutated into an alanine. This

mutation prevents phosphorylation and consequent inactivation of ADF by LIM kinase (Abe et al., 1993 and 1996) and was a gift of Dr. James Bamburg (University of Colorado).

II.8- *Cloning of α-catenin NES into Rev(1.4)-GFP*

Oligonucleotides were synthesised that encoded the amino acid sequences of the two putative NES present in avian αN-catenin. The oligonucleotides were 5′ phosphorylated and had a 5′ BamHI site and a 3′ Age I site to facilitate cloning into the Rev(1.4)-GFP vector. The oligonucleotides were annealed by heating for 30 seconds at 95^0C and then cooling at 37^0C for 15 minutes. Annealing was checked by agarose electrophoresis. Purified vector and oligonucleotides were ligated as described above and the ligation used to transform competent *E.coli*.

II.8.1-*Oligonucleotide sequences*

αN-catenin-NES1

Amino acid sequence(126-143) AARA LLSA VTRLLILADM (NES in bold)

Oligonucleotide sequence:

5′-GATCCAGCTGCTCGTGCCTTGCTTTCTGCTGTTACCCGCTTGCTCATCCTGGCTGATATGA-3′

3′-GTCGACGAGCACGGAACGAAAGACGACAATGGGCGAACGAGTAGGACCGACTATACTGCC -5′

αN-catenin-NES2

Amino acid sequence(142-159) DMAD VMRLLSH LKIVEEA (NES in bold)

Oligonucleotide sequenc

5′-GATCCAGATATGGCGGACGTCATGAGGCTTCTTTCACATCTGAAAATTGTAGAGGAAGCA-3′

3′-GTCTATACCGCCTGCAGTACTCCGAAGAAAGTGTAGACTTTTAACATCTCCTTCGTGGCC-5′

These oligonucleotides were annealed and ligated to the Rev(1.4)-GFP to create Rev-α-NES1 and Rev-α-NES2, respectively. The insertion was confirmed by sequencing using specific pEGFP primers.

II.8.2- *Mutagenesis of α-catenin*

Oligonucleotides were synthesised to mutate two of the residues on each of the putative α-catenin-NES signals in GFP-α-catenin and in NES2 in the Rev-α-NES2 plasmid.

II.8.2.1- *Mutation of α-catenin-NES1*

Amino acid sequence (128-140) RALLSA ATRALIL (mutated residues in bold)

Oligonucleotide sequence

5′-CGTGCCTTGCTTTCTGCTGCTACCCGCGCGCTCATCCTGGC-3′

3′-GCACGGAACGAAAGACGACGCTGGGCGCGCGAGTAGGACCG-5

The introduction of this DNA sequence in the wild type α-catenin generates a BssHI site that was used for screening positive clones, which were then confirmed by sequencing.

II.8.2.2- *Mutation of α-catenin-NES2*

Amino acid sequence (144-158) ADVMRLLSH AKAVEE (mutated residues in bold)

Oligonucleotide sequence

5′-GCGGACGTCATGAGGCTTCTTTCACATGCGAAAGCTGTAGAGGAAGC-3′

3′-CGCCTGCAGTACTCCGAAGAAAGTGTACGCTTTCGACATCTCCTTCG-5′

These two residues were chosen because it has been shown that they should not affect binding to β-catenin if mutated (Huber et al., 1997). The

screening for positive clones in this case was done by digesting them with XbaI, which is lost in the mutated construct. Positive clones were confirmed by sequencing.

II.8.2.3- *Mutation of the β-catenin binding domain in α-catenin*

The β-catenin binding domain in GFP- α-catenin was mutated using the oligonucleotide below. This altered the residues 130, 134 and 137 to alanines.

Amino acid sequence(128-140) RA ALSA ATRALIL (mutated residues in bold)
Oligonucleotide sequenc
5′-GGGCTGCTCGTGCCGCGCTTTCTGCTACCCGGGCGCTCATCCTGGCTG-3′
3′-CCCGACGAGCACGGCGCGAAAAGACGACGATGGGCCCGCGACTAGGACCGAC-5′

According to Huber et al. (1997) mutations at residue 130 reduced 50% of the β-catenin binding to α-catenin. Positive clones were confirmed by sequencing.

II.2- *Mammalian tissue culture*

II.2.1-*Tissue culture:*

Cells were grown in DMEM containing 10% foetal bovine serum (FBS) plus 100 U/ml of penicillin and 0.1 mg/ml of streptomycin (GibcoBRL #61965-026; #10108-157; SIGMA #P-4333, respectively) in a humidified 10% CO_2 incubator at 37^0 C. In general, the cells were passaged every two to three days by treatment with 0.5 x Trypsin/EDTA solution [0.5 % of porcine trypsin and 0.1 mM EDTA in HBSS (137 mM NaCl; 2.68 mM KCl; 1.76 mM KH_2PO_4; 8 mM Na_2HPO_4; pH 7.4)] into new 90 mm tissue culture dishes (Falcon # 353003) at 1:5 or 1:10 dilution.

Table 1. *Cell lines*

Cell line	*Origin*	*Significant Characteristics*	*Source*
A431 cells	Human cervical epidermoid carcinoma cell line	Used for induction of ADF rods since these cells have a maximal response after treatment with DMSO.	Dr. L.Reichardt, UCSF
Caco-2 cells	Human colorectal adenocarcinoma cell line	Heterozygous G-to-C missense mutation in codon 245,exon 5 in β-catenin leading to a change from glycine to alanine in the third arm repeat. APC with a non-sense mutation at codon 1367, generating a truncated protein of 170kDa.	Dr.M.Vivanco, ICR
COS-7 cells	African Green Monkey kidney cells	Derivative of CV1 cells that express SV40 large T antigen	Dr.M.Vivanco, ICR
DLD-1, *1b, **DLDα- and ***DLDα+ cells	Human colorectal adenocarcinoma cell line	Truncated form of APC (1-1427) *ras* mutation. * DLD-1 cells from a different source ** Variant from the DLD-1 cell line which has lost expression of α-catenin. *** These cells do not form a cobblestone monolayer, but still express α-catenin.	Dr.M.Vivanco, ICR, **Dr.S.T.Suzuki Institute for Developmental Research, Aichi Jap *** Isolated in our laboratory

HCT116 cells	Human colorectal carcinoma cell line	Poorly differentiated cells with mutations in codon 45 of exor 3 of β-catenin causing the loss of a serine residue in GSK3 phosphorylation site; *K-ras* mutation	Dr.M.Vivanco, ICR
HEK 293 cells	Human embryona kidney cell line	These cells express the transforming gene of humar adenovirus 5.	Dr. S. Moss MRC, LMCB
HepG2 cells	Human hepatocellular carcinoma cell line	These cells express one allelic mutant form oβ-catenin, that lacks the α-catenin binding domain	Dr.M.Vivanco, ICR
MDCK cells	Madin-Darby canine kidney cells	These cells express components of the adherens junctions and form a cobblestone monolaye in culture	Dr.M.Vivanco, ICR
Neuro-2A cells	Mouse neuroblastoma cell line	These cells express very low levels of endogenous catenins.	Dr. L.Reichardt, UCSF
SW480 cells	Human colorectal adenocarcinoma cell line	Poorly differentiated cells with mutant form of APC (1-1337); *K-ras* mutation; two mutate endogenous p53 alleles	Dr.M.Vivanco, ICI
RKO cells	Human colorectal tumour cell line	These cells express wild type β-catenin and APC	Dr.M.Vivanco, ICR

II.2.2- *Mammalian cell transfections*

Cells were plated the day before transfection at 60-70% confluence onto a tissue culture 6-well plate (Falcon #353046). Transfections were performed using the Lipofectamine PLUS reagent (GibcoBRL #10964-013) and the optimal conditions were determined for each cell type. Usually, for each well, 2 μg of plasmid DNA was diluted in Optimem (GibcoBRL # 51985-026) and then pre-mixed with 4 μl of PLUS reagent and incubated for 15 minutes in a final volume of 100 μl. After this incubation, 100 μl of Optimem containing 2 μl of Lipofectamine was added to the DNA-PLUS complex, mixed and incubated for a further 15 minutes at room temperature. While this incubation was taking place, the cells were washed with 1 ml of Optimem. The DNA-PLUS-Lipofectamine complex mixture was then added to the cells and incubated for 3 hours in a 10% CO_2 incubator at 37^0 C. After this, the mixture was removed and DMEM with 10% FBS was added to the cells. For co-transfection studies using GFP-fusion constructs, the ratio of non-GFP plasmids to GFP-plasmids was 20:1, except when using APC, which was used at 100:1 because APC was poorly expressed. For experiments in which HATcf-4, β-catenin and α-catenin were co-transfected, the ratios of DNAs used were 20:1:1. Immunocytochemical labelling experiments indicated that under these conditions more than 90% of cells expressing GFP fusion proteins also expressed the non-GFP proteins.

For transcription assays the following amounts of DNA were added to each well of a 6-well plate: 25 ng RSV β-gal, 150 or 200 ng pTOPFlash (or pFOPFlash as a negative control) or 200 ng cyclin D1 promoter reporter, 200 ng Tcf-4, 100 ng pMT23 β-catenin, and 200 ng pMT23 vector, pMT23 α-catenin, GFP-α-catenin constructs or GFP plasmid.

II.2.3- *Colony formation assay*

For the colony formation assay, cells were transfected as described above and 24 hours post-transfection they were transferred from the 6-well plates onto a 90 mm tissue culture dish and allowed to attach overnight. Selection medium containing 500 μg/ml of Neomycin Sulfate (G418, Calbiochem #34812) was then added. The medium was changed every two days for approximately 2 weeks, until all the cells from the untransfected control had died. Colonies were then stained using 1% Crystal Violet (SIGMA #C3886) in 2% ethanol for 15 minutes. Colonies greater than 1 mm were counted.

II.2.4- *Preparation and analysis of cell extracts*

For western blotting, cells were washed in ice cold TBS (20 mM Tris, pH 7.5; 150 mM NaCl), lysed in 1 ml of modified RIPA buffer on ice [150 mM NaCl; 50 mM NaF; 20 mM Hepes, pH 8.0; 1% Triton X-100; 0.1% SDS (Research genetics #750008); 0.5% Sodium Deoxycholate] containing 0.1 mM sodium orthovanadate, 1 mM EDTA, pH 8.0 and protease inhibitors Leupeptin and Aprotinin at 10 μg/ml. All reagents were from SIGMA, unless specified otherwise. Lysed cells were collected by scraping with a rubber policeman and transferred to a 1.5 ml eppendorf tube and incubated for 10 minutes on ice. Lysates were cleared by centrifugation at 12,000 *g* at 4^0C for 10 minutes. An aliquot of the lysates was heated for 5 minutes at 95^0C with the same volume of 2 x Laemmli sample buffer (SIGMA #S-3401) to denature proteins prior to separation in polyacrylamide gels, which were prepared according to Sambrook et al. (1989). The separated proteins were transferred to nitrocellulose membrane (0.2 μm pore size; Scheleicher and Schuell #10401396) in transfer buffer (39 mM Glycine; 48 mM Tris base; 0.037% SDS; 20% methanol) using a BIORAD semi-dry apparatus (BIORAD #170-3949).

After transfer the blots were washed once in TBS-T buffer (100 mM Tris, pH 7.5; 200 mM NaCl; 0.1% Tween) and then blocked for one hour in blocking buffer (3% BSA Fraction V; 1% ovalbumin in TBS-T). Western blots were probed for 2 hours with the appropriate primary antibodies diluted in blocking buffer. After incubation, blots were washed 6 times in TBS-T, for 10 minutes each. This was followed by incubation for 1 hour with secondary antibodies diluted in blocking buffer. Then blots were washed again and were developed using Super Signal chemiluminescence substrate kit (PIERCE #343080) following instructions provided by the manufacturer. Blots were exposed to film (Amersham #RPN3103K) and developed using a X-Ograph compact X2 X-ray developer. The blots were subjected to densitometry using the BIORAD Fluor-S MultiImager and X-ray films were subjected to densitometry using the BIORAD GS-710 Calibrated Imaging Densitometer.

II.2.5- ***Immunoprecipitations***

For immunoprecipitation experiments, cells were lysed as described earlier, but using NP-40 lysis buffer (150 mM NaCl; 50 mM NaF; 20 mM Tris pH 8.0; 1% NP-40) containing 0.1 mM sodium orthovanadate, 1 mM EDTA, Leupeptin and Aprotinin, both at 10 μg/ml, to preserve interactions between β-catenin and α-catenin (NP-40 from Amresco #9036-19-5). Usually 1-2 μg of monoclonal antibody, or 5 μl of rabbit anti-serum was added to 1 mg of extract, followed by 2 hours incubation on ice. In order to precipitate the antibody-protein complexes, protein A-Sepharose (used when polyclonal antibodies were used for the immunoprecipitation; Pharmacia #17-0780-01) or anti–mouse IgG agarose beads (used when monoclonal antibodies were used for the immunoprecipitation; SIGMA #A-6531), were incubated with the extracts for 30 minutes at 4^0C in a mixer tube rotator. Non-specific proteins were removed by four washes in cold NP-40 buffer and one wash with cold

TBS. Beads were then pelleted, resuspended in 2 x Laemmli sample buffer, heated for 5 minutes at 95^{0}C. Immunoprecipiated proteins were separated by SDS-PAGE and transferred to nitrocellulose for western blotting analysis.

II.2.6- *Isolation of α-catenin/β-catenin/Tcf complexes*

To immunoprecipitate α-catenin/β-catenin/Tcf complexes, lysates enriched for nuclear proteins from transfected Neuro-2A or untransfected SW480 cells were prepared as follows: cells were lysed in NP-40 lysis buffer for 10 minutes on ice and centrifuged for 10 minutes at 500 *g*. The pellet containing the nuclei was washed 2 times in the same buffer. To extract the nuclear proteins, this pellet was incubated in NP-40 lysis buffer containing 400 mM NaCl for 30 minutes on ice. The lysates were then centrifuged for 10 minutes at 15,000 *g*. The supernatant was diluted with an equal volume of NP-40 lysis buffer without NaCl prior to immunoprecipitation.

II.2.7- *Immunofluorescence*

For immunofluorescence, cells were plated onto glass coverslips pre-coated with 30 μg/ml collagen (Cohesion #PC0701). Untransfected cells or cells expressing GFP fusion proteins were washed in PBS (Phosphate Buffered Saline; 137 mM NaCl; 2.68 mM KCl; 1.76 mM KH_2PO_4; 10.1 mM Na_2HPO_4) and fixed for 15 minutes using 3% paraformaldehyde in 1 x PBS. In order to improve detection of nuclear rods formed by myc-epitope tagged β-catenin, fixed cells were treated with cold methanol (at -20^{0}C) for 1 minute prior to washing in PBS and blocking in 1% BSA (Fraction V, SIGMA #A-7906) for 30 minutes. Alternatively, fixed cells were permeabilised for 1 minute in 0.2% Triton X-100; 150 mM NaCl; 50 mM Tris, pH 7.5, prior to blocking. Fixed cells were incubated with primary antibodies for two hours. Cells were then washed 3 times for 10 minutes in blocking buffer, and incubated for 1 hour

with Texas Red-, FITC- or Cy5-conjugated anti-mouse or anti-rabbit IgG and then washed again as above. After staining, slides were mounted in Mowiol (Calbiochem #475904).

For induction of ADF nuclear rods, 24 hours post-transfection DMEM/10%FBS medium containing 10% dimethyl sulfoxide (DMSO, SIGMA #D-5879) was added to the cells for 10 minutes. Cells were then fixed with 4% paraformadehyde: 0.1% glutaraldehyde solution for 1 hour to help preserve ADF rods. Cytochalasin D (SIGMA #C-8273) or nocodazole (SIGMA #M-1404) were added to the cells at 0.3 μM and 1 μg/ml, respectively for 3 hours. Cells were fixed and mounted as described above.

Confocal images were obtained using a laser scanner (MRC 1024, BIORAD) attached to a Nikon microscope (Optiphot 2) and processed using Adobe Photoshop.

II.3 *Antibodies*

Table 2. ***Primary Antibodies***

Antibody	*Dilution for Immunostaining*	*Dilution for Western blotting*	*Dilution for Immunoprecipitations*	*Source or reference*
Monoclonal anti-α-catenin	1:500	1:1000	n.a.	Zymed # 13-9700
Monoclonal anti-β-catenin	1:500	1:1000	1-2 μg/mg of cell extract	Transduction labs #C19220
Monoclonal anti-cyclin D1	n.a.	1:1000	n.a.	Santa Cruz #sc-8396
Monoclonal anti-GFP	n.a.	1:1000	1-2 μg/mg of cell extract	Boehringer Mannheim #1814460
Monoclonal anti-myc-epitope 9E10	1:500	1:1000	1-2 μg/mg of cell extract	SIGMA # M-5546
Monoclonal anti-Tcf-1	n.a.	1:1000	n.a.	Gift from Dr.Nick Barke
Monoclonal anti-Tcf-4	1:500	1:1000	n.a.	Upstate Biotech. #05-511
Monoclonal anti-vinculin	n.a.	1:1000	n.a.	SIGMA # V4505
Polyclonal anti- α-catenin (CME)	1:300	1:1000	5 μl/ mg of extract	Kypta et al., 1996

Polyclonal anti-HA	1:500	1:1000	n.a.	Santa Cruz #sc-805
Polyclonal anti-GFP	n.a.	1:5000	5 µl/ mg of extract	Seghal et al., 1997
Polyclonal anti-pan-cadherin	n.a.	1:2000	5 µl/ mg of extract	SIGMA # C3678
Polyclonal anti-NFκB p65 (C-20)-G	1:1000	n.a.	n.a.	Santa Cruz #sc372-G

Table 3. ***Secondary Antibodies***

*Antibody**	*Dilution for Immunostaining*	*Dilution for Western blot*	*Source*
Cy5-conjugated sheep anti-mouse IgG	1:1000	n.a.	Jackson ImmunoResearch labs #515-175-003
FITC-conjugated goat anti-mouse IgG	1:400	n.a.	Jackson ImmunoResearch labs #115-096-006
FITC-conjugated goat anit-rabbit IgG	1:400	n.a.	Jackson ImmunoResearch labs #111-095-003
HRP-conjugated donk anti-mouse IgG	n.a.	1:5000	Jackson ImmunoReseach labs #715-035-150

HRP-conjugated don anti-rabbit IgG	n.a.	1:10000	Jackson ImmunoReseach labs #711-035-152
TexasRed-conjugated goat anti-mouse IgG	1:400	n.a.	Calbiochem #401246
TexasRed-conjugated goat anti-rabbit IgG	1:400	n.a.	Calbiochem #401355

n.a- not applicable.

*- Anti-rabbit antibodies had minimal cross-reaction to bovine, chicken, goat, guinea pig, Syrian hamster, horse, human, mouse,rat and sheep serum proteins. Anti-mouse antibodies had minimal reaction to bovine, chicken, goat, guinea pig, Syrian hamster, horse, human, rabbit, rat and sheep serumprotein.

II.4- *Transcription assays*

For transcription assays, transiently transfected cells were washed twice in ice-cold PBS and collected in 1.25 ml of ice-cold PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. Cells were pelleted by centrifugation at 10,000 *g* for 30 seconds and processed for luciferase and β-galactosidase activities. Cell pellets were lysed in 200 µl of Reporter Lysis Buffer provided with the Luciferase Assay System (Promega #E4530), incubated 15 minutes, then frozen and thawed. Lysed cells were centrifuged for 10 minutes at 12.000 *g* and supernatants were used for luciferase assays.

II.4.1- *Luciferase assays*

To determine luciferase activity, a 20 µl aliquot of the cleared extract was measured using luciferase substrate provided in the Luciferase Assay System in an Autolumat LB 953 (Berthold) according to the manufacturer's protocols. Luciferase activity of each sample was then normalised against β-

galactosidase activity to correct for transfection efficiency.

II.4.2- ***Beta-galactosidase assays***

To determine β-galactosidase activity a 20 µl aliquot of the cleared extract (see Luciferase assay) was added to 180 µl of substrate solution (60 mM Na_2HPO_4; 60 mM NaH_2PO_4; 10 mM KCl; 1 mM $MgSO_4$; 0.0014% BME; 500 µM CPRG) in a 96-well plate. Absorbance at 570 nm was measured every minute over a 1 hour period at 37^0C using a colorimetric plate-reader (MRX). The average change in absorbance per unit time was taken as the relative β-galactosidase activity.

II.5- ***Gel shift assays***

Neuro-2A cells transfected with 1.5 µg of p45 Tcf-1 DNA were lysed in NP-40 buffer containing 300 mM NaCl; 1 mM DTT, Leupeptin and Aprotinin (10µg/ml). Approximately 2 µg of protein was used for each binding reaction. HPSF-purified double-stranded oligonucleotide probes (MWG-Biotech) containing a Tcf-binding site ACTCTGGTACTGGCCCTTTGATCTTTCTGG or a mutated Tcf-binding site ACTCTGGTACTGGCCCGGGGATCTTTCTGG (Barker et al., 1999) were labelled using T4 polynucleotide kinase (Promega #M4101) and radiolabel (γ^{32}P)ATP (Amersham #AA0018), following manufacturer's instructions. Unincorporated (γ^{32}P)ATP was removed using Micro Bio Spin 30 chromatography columns (BIORAD #732-6223). Each binding reaction (15 µl) contained 10 mM Tris, pH 7.5; 0.5 mM EDTA; 50 mM NaCl; 1 mM $MgCl_2$; 4% glycerol; 0.5 mM DTT; 1 µg of poly(dI-dC); 1 µg of salmon sperm DNA and approximately 0.4 ng of radiolabelled DNA probe. The following purified proteins were used: 0.25 µg of histidine-tagged β-catenin (Kypta et al., 1996); 2 µg of GST-α-catenin; 1 µg of αN (GST fused to α-catenin residues 1-228) or 4 µg of αC (GST fused to the carboxyl-

terminal 447 residues of α-catenin). Extract from Tcf-1 transfected Neuro-2A cells was incubated with the purified proteins in binding buffer for 5 minutes at room temperature. The labelled probe was then added, and the mixture was incubated for 15 minutes at room temperature. For antibody "supershift" experiments, 0.5-1 μg of antibody was added, and the incubation was continued for a further 10 minutes. Gel loading buffer (10 x: 250 mM Tris, pH 7.5; 0.2% bromophenol blue and 40% glycerol) was added, and the complexes were separated on a nondenaturing 5% polyacrylamide gel (National Diagnostics #EC-852) in 0.25 x TBE buffer (23 mM Tris; 1 mM EDTA) and processed for autoradiography.

II.6- ***Nuclear export assays***

II.6.1- ***Principle***

HIV-1 Rev protein contains both a nuclear localisation signal (NLS) and a nuclear export signal (NES). The nuclear import mediated by its NLS can be specifically inhibited by Actinomycin D treatment. This drug prevents association of Rev with the nucleoli. Similarly, NES-mediated export is inhibited by treatment with Leptomycin B (LMB), which binds to the CRM-1 receptor specifically inhibiting CRM-1-mediated nuclear export (Kudo et al., 1998). Fusion of the carboxyl-tail of Rev to GFP does not interfere either with nuclear import or export of this protein. Taking advantage of this fact, a nuclear export assay has been developed in which a putative NES is cloned into a plasmid that contains an alternative Rev protein fused to GFP [Rev(1.4)-GFP]. This alternative protein has a mutated NES that no longer promotes export, but its NLS is left intact. A putative NES can be tested for its export capabilities by insertion into Rev(1.4)-GFP.

II.6.2- *Assay procedure*

For studies of nuclear export, COS-7 cells were transfected and 24 hours later they were treated simultaneously with cycloheximide at 15 μg/ml (to ensure that any cytoplasmic GFP was the result of nuclear export rather than newly synthesised protein) and either LMB at 5-10 ng/ml (Gorlich and Kutay, 1999) or Actinomycin D at 5 μg/ml for 3 hours. After drug treatment the cells were fixed with 3% paraformadehyde and examined by fluorescence microscopy. The localisation of the Rev(1.4)-GFP constructs containing putative NES was analysed by counting number of cells with the GFP-protein either only in the nucleus, both in the nucleus and cytoplasm, or only in the cytoplasm. The results were compared those of the wild-type Rev-NES (Rev-GFP) and mutant Rev(1.4)-GFP.

CHAPTER III- *Subcellular localisation of β-catenin constructs*

III.1- *Introduction*

Beta-catenin is a multifunctional protein involved in several cellular processes. The subcellular localisation of β-catenin is vital for this protein to exert its various functions. When in the membrane, β-catenin participates in the cell-cell adhesion process. The cellular levels of β-catenin are controlled by its degradation in the cytoplasm and when this process is inhibited, β-catenin translocates to the nucleus where it co-operates with transcription factors to activate gene transcription. The diverse functions of β-catenin may therefore be linked to its levels of expression, cellular localisation and/or its various binding partners (Figure 3a). In this chapter I describe experiments using GFP-β-catenin fusion proteins to study the localisation of β-catenin in the cell, and the effects of co-expression of β-catenin binding partners on this localisation.

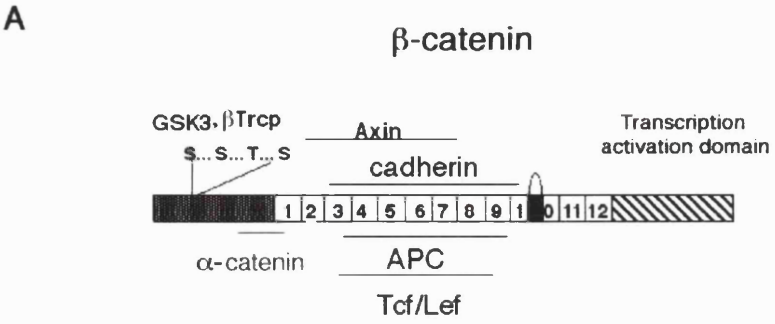
III.2- *Beta-catenin forms rod-like structures in the nucleus*

A schematic view of the fusion proteins examined is shown in Figure 3b. Unless otherwise specified, all the experiments were performed using COS-7 cells, which efficiently co-express multiple plasmids. First, the expression levels and apparent molecular masses of the proteins expressed by these constructs were determined. Total cell lysates from transiently transfected cells were prepared 24 hours after transfection. These were subjected to western blotting and probed using a monoclonal antibody raised against GFP (Figure 4a). The different constructs yielded similar amounts of protein and these were of the predicted molecular mass.

The subcellular localisation of the different β-catenin proteins varied according to which domains of β-catenin the constructs encoded. In cells transiently expressing full-length β-catenin, with either a GFP tag at the amino terminus (Figure. 5a) or the carboxyl terminus (Figure 5b), the fusion protein localised to the nucleus, where it formed fine punctate spots in some cells and rod-like structures in others. Intranuclear rod-like structures were observed more frequently using GFP-β-catenin than using β-catenin-GFP. The presence of β-catenin fusion proteins in the nucleus, and not in the membrane, may be because COS-7 cells are fibroblasts, which express a low level of cadherin. To explore this possibility, MDCK cells, which are of epithelial origin and express a high level of cadherin, were transiently transfected with GFP-β-catenin (Figure 5c). In these cells, tagged β-catenin localised at the sites of cell-cell contact, but it was also present in the nucleus in some cells, forming rod-like structures such as the ones found when using COS-7 cells.

To determine the level of expression of GFP-β-catenin required for formation of intranuclear rods, lysates of cells transfected with increasing amounts of GFP-β-catenin plasmid (from 0 ng to 100 ng) were probed in western blots with anti-β-catenin monoclonal antibody. This antibody recognises both wild-type and GFP-tagged β-catenin, allowing the bands corresponding to both endogenous and exogenous protein to be quantified by densitometry. Simultaneously, cells were monitored for transfection efficiency and for appearance of GFP-β-catenin rods.

A



B

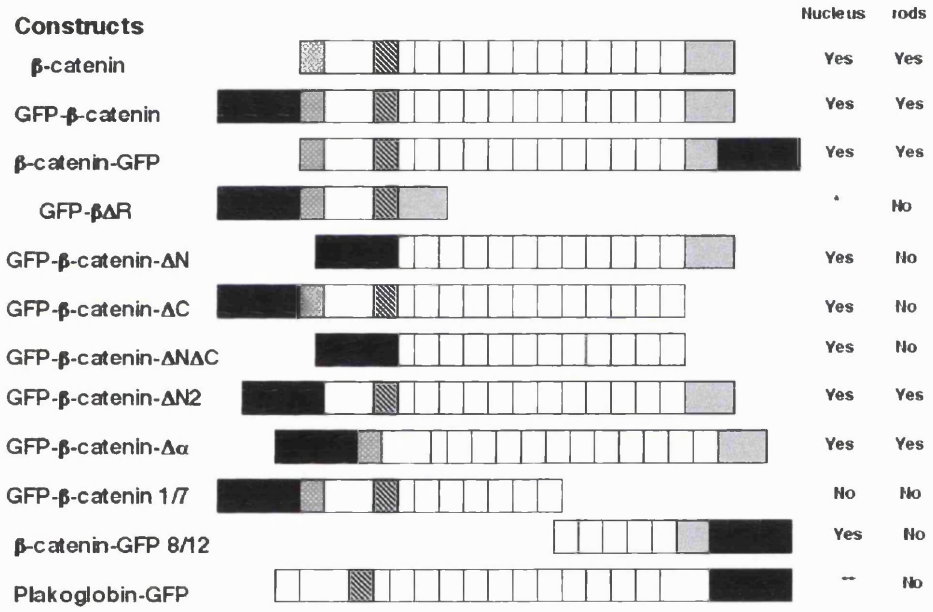


Figure 3.
(A) Schematic view of β-catenin showing the domains for binding to α-catenin, Tcf, APC, axin and β-TrCP, as well as the GSK3 phosphorylation sites. Dotted box and hatched box represent amino- and carboxyl-terminus, respectively. Armadillo repeats are indicated by numbers.
(B)Schematic diagram of the constructs described in the text, and a summary of the results obtained using COS-7 cells.The top construct includes the positions of the armadillo repeats, as defined by Huber et al. (1997). For GFP fusions, GFP is in black, the grey dotted domain contains the GSK3 phosphorylation sites (N2), the striped domain contains the α-catenin binding site, and the light grey domain contains sequences in the carboxyl-terminus that are necessary for transcriptional transactivation.
* Similar to GFP, i.e. found in nucleus and cytoplasm.
** Enters nucleus when co-expressed with Tcf-1.

The amount of exogenous β-catenin was also compared to that of endogenous β-catenin in SW480 colon cancer cells. These cells have high levels of β-catenin due to impairment of the β-catenin destruction pathway (Munemitsu et al., 1995). In a typical experiment (16 hours after transfection of COS-7 cells with GFP-β-catenin), 20% of cells detected were green and, of these, 10% expressed enough protein to form rods (equivalent to 2% of all cells). The amount of GFP-β-catenin made in these cells (estimated by densitometry) was 5% of total endogenous β-catenin, suggesting that the maximal level of GFP-β-catenin in a cell with rods was 2.5-fold more than the level of endogenous β-catenin (2% of cells make 5% protein) (Figure 4b). This estimate does not take into account green cells, which do not make rods, so the relative level of expression required for rod formation may be lower than estimated.

To eliminate the possibility that GFP was responsible for the nuclear accumulation and formation of β-catenin rods, the localisation of a construct encoding a carboxyl-terminal myc-tag fused to β-catenin was examined. Cells expressing this fusion protein were visualised using an anti-myc-tag monoclonal antibody. Myc-tagged β-catenin also formed nuclear rods (Figure 5d), indicating that the ability of β-catenin to localise to the nuclear compartment and form rods was independent of GFP.

The dynamics and kinetics of rod formation were analysed by time-lapse video microscopy of cells transfected with GFP-_-catenin. The spots were already present in some cell nuclei 16 hours after transfection. During a four-hour period these spots fused gave rise to the rods (Figure 6).

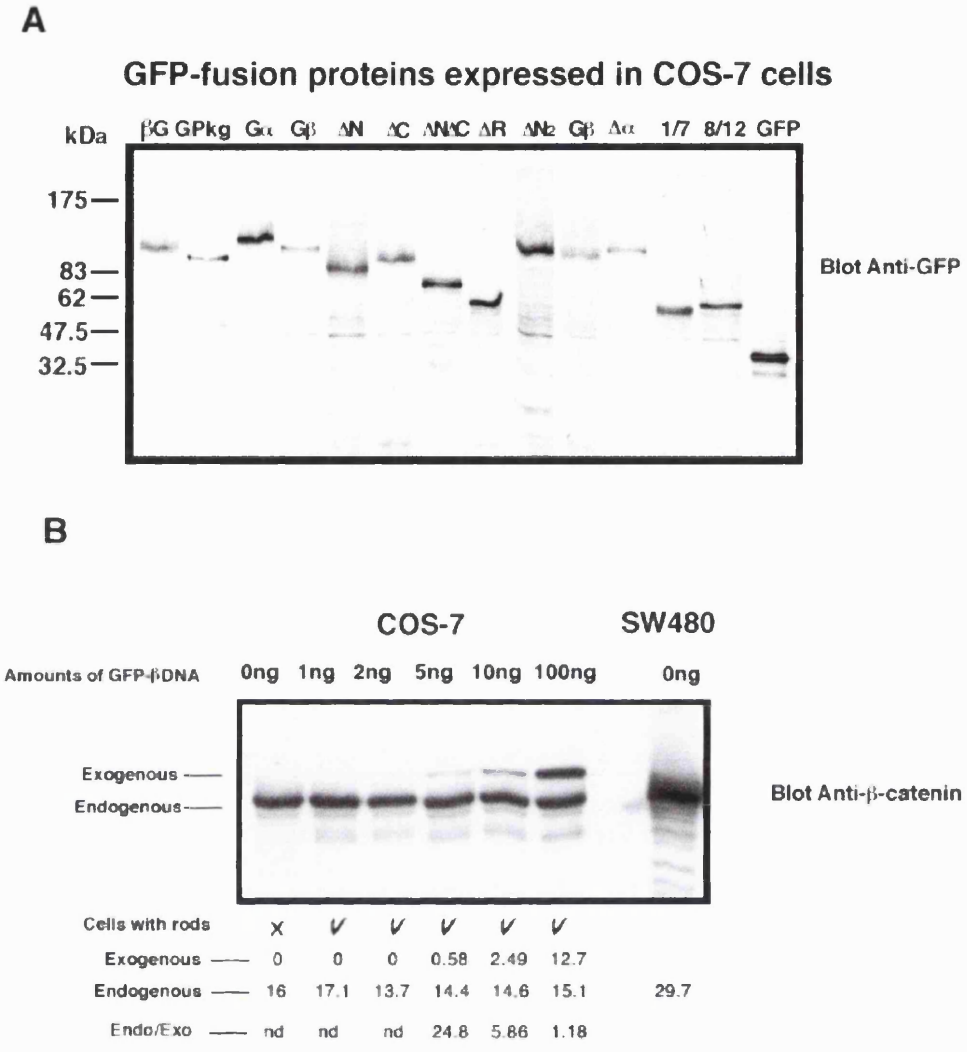


Figure 4.
(A) Anti-GFP western blot of extracts from COS-7 cells transfected with the different GFP-fusion proteins.
βG-β-catenin-GFP; **GPkg**-Plakoglobin-GFP; **Gα**-GFP-α-catenin; **Gβ**-GFP-β-catenin; **ΔN**-GFP-β-cateninΔN; **ΔC**-GFP-β-cateninΔC; **ΔNΔC**-GFP-β-catenin-ΔNΔC; **ΔR**-GFP-β-catenin-ΔR; **ΔN2**-GFP-β-catenin-ΔN2; **Δα**-GFP-β-catenin-Δα; **1/7**-GFP-β-1/7; **1/8**-β-8/12-GFP; **G**-GFP. The positions of molecular weight markers are indicated on the left.
(B)Anti-GFP-β-catenin western blot of extracts from COS-7 cells (lanes 1 to 6) or SW480 cells (lane 7). COS-7 cells were transfected with a total of 1 μg of empty vector/GFP-β-catenin, containing increasing amounts of GFP-β-catenin (0 ng, 1 ng, 2 ng, 5 ng, 10 ng and 100 ng). Densitometry was used to measure the relative expression levels of GFP-β-catenin (lanes 3 to 5) and endogenous β-catenin. Endo/Exo values are the ratios of endogenous β-catenin/ exogenous β-catenin.

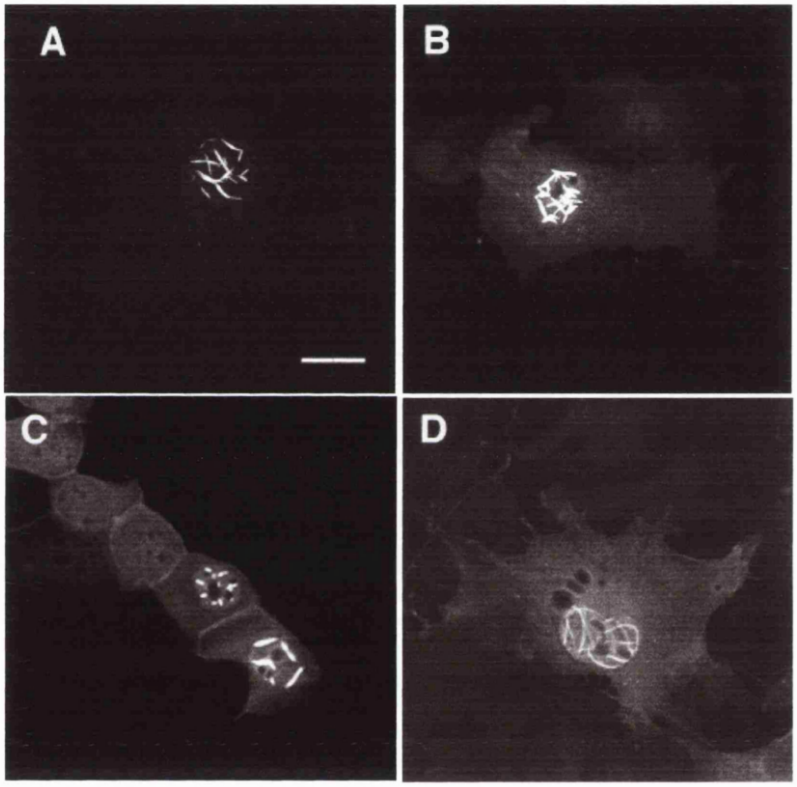


Figure 5.
β-catenin forms rods in the nucleus.
COS-7 cells (A, B and D) or MDCK cells (C) expressing the following proteins were visualised by immunocytochemistry and confocal microscopy: (A and C) GFP-β-catenin, (B) β-catenin-GFP, (D) β-catenin-myc stained with 9E10 mAb. Scale bar 7 μm.

There are other examples of proteins that form rod-like structures. For instance, heat shock or DMSO treatment of A431 cells induces formation of intranuclear rods composed of actin and cofilin/ADF (Nishida et al., 1987). To investigate if β-catenin nuclear rods were related to ADF/actin rods, a GFP-tagged form of ADF was co-expressed with myc-tagged β-catenin. After 24 hours the cells were treated with DMSO to induce formation of ADF intranuclear rods (Figure 7b), and were analysed by immunofluorescence using anti-myc antibody to visualise β-catenin (Figure 7a). Beta-catenin rods were longer than the ones formed by ADF and they did not co-localise with ADF nuclear rods (Figure 7c).

III.3- ***Mutational analysis of β-catenin nuclear localisation and rod formation***

Beta-catenin can be separated into three domains: the amino-terminal domain, the armadillo repeat domain and the carboxyl-terminal domain. In order to investigate if the armadillo repeat domain of β-catenin was important for rod formation, I examined the localisation of a GFP-β-catenin fusion protein lacking the armadillo repeat domain (βΔR). The distribution of this protein (Figure 8a) was similar to that of GFP alone (Figure 8b). Both proteins appear evenly spread throughout the cell nucleus and cytoplasm, most likely because they are below 50 kDa and can passively diffuse into the nuclear compartment (reviewed by Nigg, 1997). No rod-like structures were observed with βΔR, indicating that the armadillo repeats are required for rod formation.

Plakoglobin, also known as γ-catenin, has armadillo repeats with 83% homology to those in β-catenin (McCrea et al, 1991). Therefore, I examined the localisation of a plakoglobin-GFP fusion protein (Rubenstein et al, 1997). Plakoglobin-GFP accumulated in the cytoplasm of many cells,

sometimes forming aggregates (Figure 8c). In some cells it was also found in the nucleus, but in either case no rods were found (Figure 8d).

Thus plakoglobin and β-catenin appear to differ in that β-catenin has a higher propensity to accumulate in the nuclei of cells.

Beta-catenin and plakoglobin sequences diverge predominantly in the amino and carboxyl-terminal domains (57% and 15% identity, respectively; McCrea et al., 1991). Therefore, the significance of these regions for the nuclear localisation pattern of β-catenin was assessed. Constructs encoding GFP fused to β-catenin with an amino-terminal deletion of residues 1-151 (Figure 9a) or with a carboxyl-terminal deletion of residues 699-781 (Figure 9b), or deletions of both these domains (containing residues 151 to 698; Figure 9c), accumulated and formed aggregates in the nucleus without forming rods. This indicates that, for the rod structures to form, the armadillo repeat and sequences in the amino- and carboxyl-terminal domains are required.

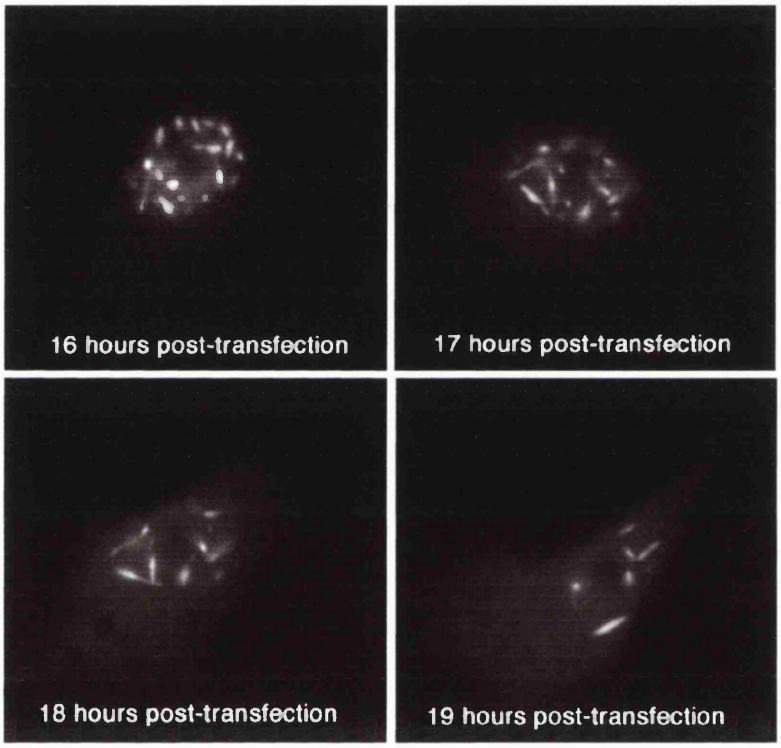


Figure 6.
Dynamics of rod-formation.
COS-7 cells expressing GFP-β-catenin were filmed for a four-hour period using a time-lapse fluorescent microscope. The time post-transfection is indicated in each panel of the figure.

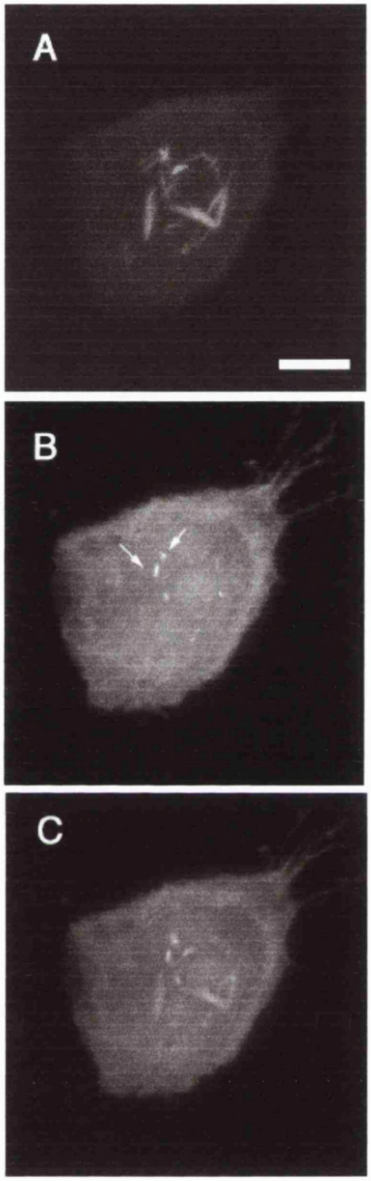


Figure 7.
Beta-catenin and ADF rods do not co-localise.
A431 cells transfected with both β-catenin-myc and GFP-ADF were treated with DMSO. (A) β-catenin-myc stained with 9E10 mAb, (B) GFP-ADF, indicated by arrows, (C) Overlay of (A) and (B). Scale bar 7 μm.

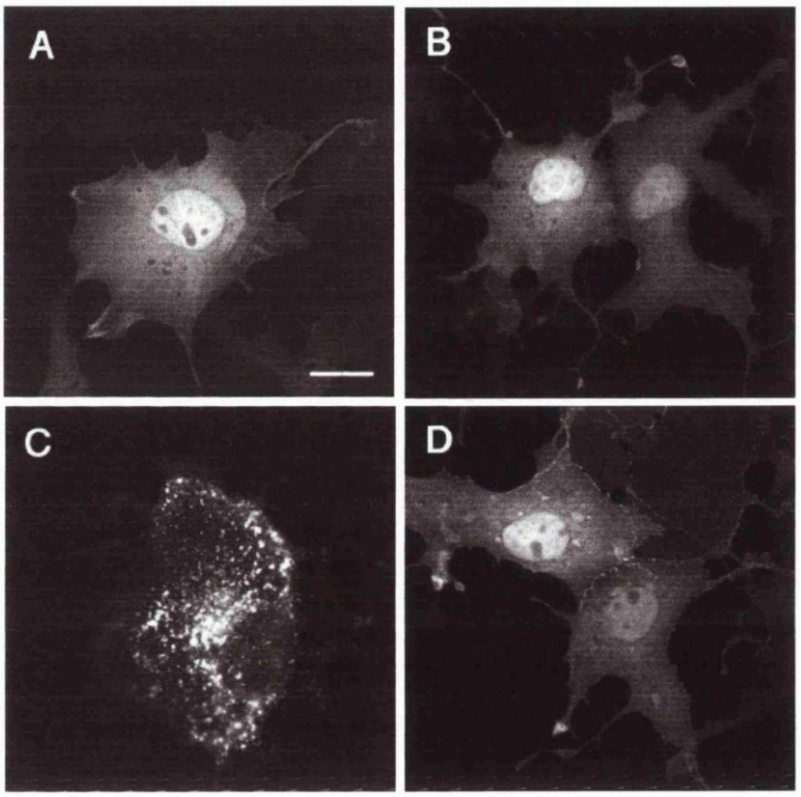


Figure 8.
The β-catenin armadillo repeat domain is required for rod formation.
COS-7 cells expressing the following GFP fusion proteins were visualised by confocal microscopy: (A) GFP-β-catenin-ΔR, (B) GFP, (C and D) Plakoglobin-GFP. Scale bar 7 μm.

Two further mutants were made: one containing the amino-terminal half of β-catenin (residues 1-420, which include the amino-terminal domain and armadillo repeats 1-7), and the other comprising the carboxyl-terminal half of β-catenin (residues 398-761, which include armadillo repeats 8-12 and the carboxyl-terminal domain of β-catenin). When expressed in cells these constructs had very distinct distributions. The amino-terminal half of β-catenin accumulated in the cytoplasm and was never detected in the nucleus (Figure 9d). The carboxyl-terminal construct, on the other hand, localised to the nucleus where it formed spherical aggregates (Figure 9e). These results indicate that the sequences necessary for nuclear localisation of β-catenin lie within the carboxyl-terminal half of β-catenin. In addition, they show that although the amino-terminal half of β-catenin is not required for nuclear entry, it is necessary for formation of the rods.

The amino-terminal domain of β-catenin contains serine and threonine residues that can bind to β-TrCP after phosphorylation by GSK3 (Hart et al., 1999; Liu et al., 1999). One possibility is that the phosphorylation status of β-catenin affects rod formation. The amino-terminal domain of β-catenin also contains the α-catenin binding site (Aberle et al., 1994; 1996). To determine if either of these regions of β-catenin is involved in rod formation, further amino-terminal deletion mutants were analysed. Both β-catenin lacking residues 1-43 (GFP-βΔN2, Figure 9f), which contain the GSK3 phosphorylation sites, or lacking residues 120-150 (GFP-β-Δα-catenin, Figure 9g), which contain the α-catenin-binding site, are able to form rods. These results discard the possibility that interactions of β-catenin with either β-TrCP or with α-

catenin are involved in the formation of the nuclear rods. In addition, they suggest that residues 43-120 are important for rod formation.

III.4- *Beta-catenin nuclear rods are disrupted by interactions with proteins that bind to the armadillo repeats*

Since the armadillo repeats are essential for interaction of β-catenin with several proteins, including cadherins (Aberle et al., 1997; Jou et al., 1995), there was a possibility that these interactions were involved in rod formation. To test this, the effects of co-expressing β-catenin with a construct encoding the transmembrane and cytoplasmic domains of N-cadherin (N-cadΔE/myc) were examined. Beta-catenin-GFP was used in these experiments instead of GFP-β-catenin because the latter also has a myc-epitope tag. N-cadΔE/myc localises predominantly to internal membranes (Riehl et al., 1996; Figure 10b). When expressed with β-catenin-GFP, N-cadΔE/myc abolished rod formation and also redirected β-catenin-GFP from the nucleus to the cytoplasm (Figure 10a). As a control, a mutated version of N-cadherin was used. This construct, called N-cadΔEΔC/myc, cannot bind β-catenin because most of the cadherin cytoplasmic domain has been deleted. Although the localisation of N-cadΔEΔC/myc (Figure 10d) was similar to that of N-cadΔE/myc, expression of N-cadΔEΔC/myc did not affect the localisation of β-catenin-GFP (Figure 10c).

The tumour suppressor gene product APC also associates with the armadillo repeats of β-catenin (Rubinfeld et al, 1993; Su et al., 1993).

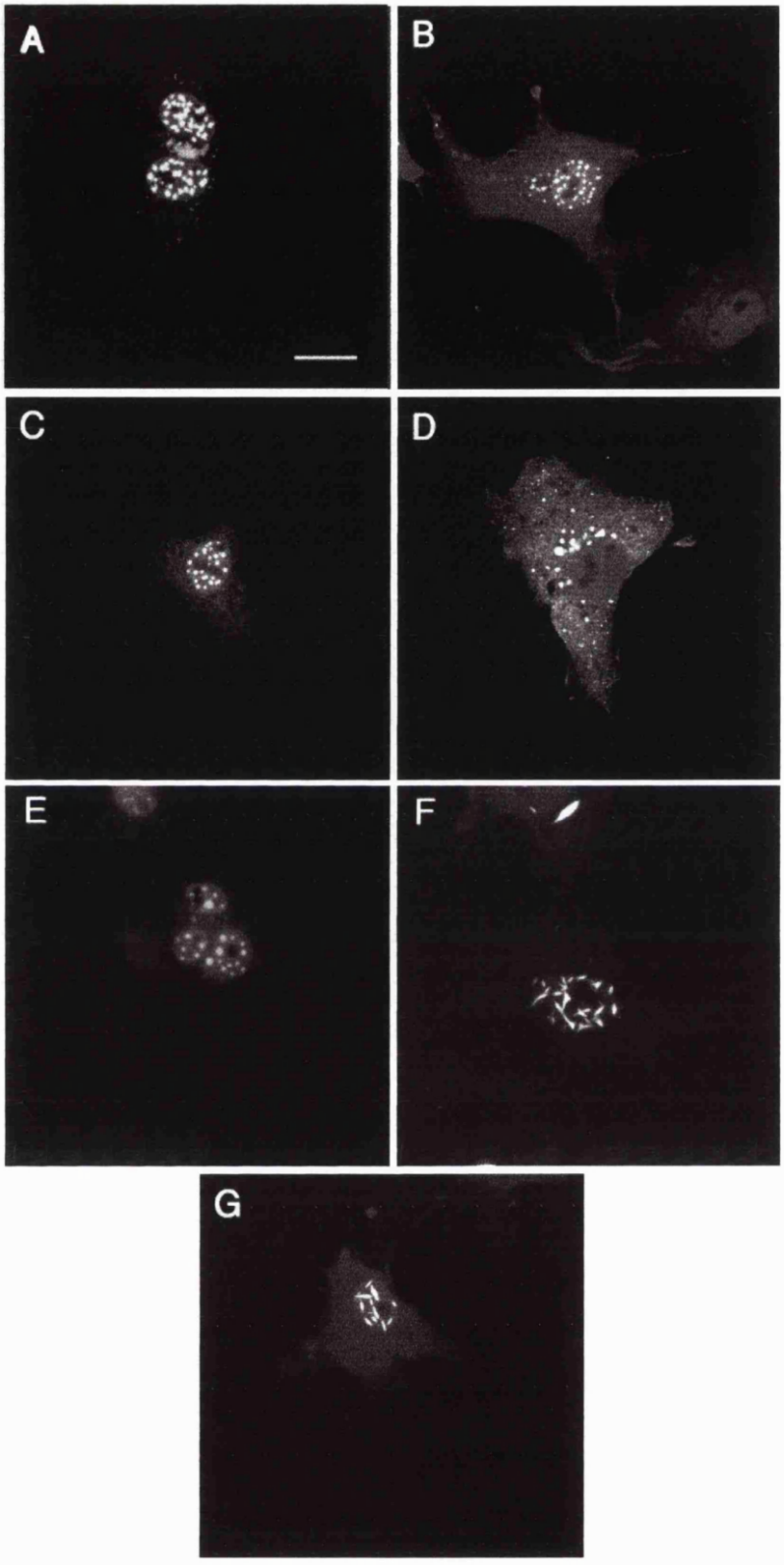


Figure 9.
Analysis of domains required for nuclear localisation and rod formation.
COS-7 cells expressing the following GFP fusion proteins were visualised by confocal microscopy: (A) GFP-β-catenin-ΔN, (B) GFP-β-catenin-ΔC, (C) GFP-β-catenin-ΔNΔC, (D) GFP-β-1/7 (amino-terminal half), (E) β-8/12-GFP (carboxyl-terminal half), (F) GFP-β-catenin-ΔN2, (G) GFP-β-catenin-Δα. Scale bar 7 μm.

Therefore the effect of co-expressing a myc-tagged version of APC (Vleminckx et al., 1997) on the subcellular localisation of β-catenin-GFP was analysed. As shown for co-expression of β-catenin-GFP with N-cadΔE/myc, the presence of myc-epitope tagged APC prevented nuclear localisation of β-catenin-GFP. Both APC (Figure 10f) and β-catenin-GFP (Figure 10e) accumulated in amorphous cytoplasmic aggregates. Therefore, expression of both N-cadherin and APC, which associate with β-catenin armadillo repeats, can prevent nuclear entry and rod formation of β-catenin. These results, and those that will be presented below using another binding partner, Tcf-4, suggest that, for rod formation to take place, the armadillo repeat domain must be free of interacting proteins.

III.5- *Beta-catenin forms cytoplasmic rods in the presence of α-catenin*

As shown above, a β-catenin fusion protein lacking one or both terminal domains can still localise to the nucleus, but is unable to form rods. This suggests that there might be two distinct events: the first being β-catenin nuclear import, and the second being its aggregation in the form of rods. To try to separate these two events, the effect of α-catenin expression on GFP-β-catenin localisation was analysed.

Alpha-catenin is a cytoplasmic protein that links the cadherin/β-catenin complex to the actin cytoskeleton by binding to the distal part of the amino-terminal domain of β-catenin, a region that is not required for rod formation (see Figure 9g). Expression of α-catenin with GFP-β-catenin resulted in the retention of the latter in the cytoplasm in the form of elongated rod-like structures (Figure 11a) that co-localised with α-catenin (Figure 11b). Converse experiments were also performed using a GFP-α-catenin fusion protein and myc-tagged β-catenin. When GFP-α-catenin was co-expressed with myc-tagged β-catenin, it also formed elongated

rod-like structures in the cytoplasm (Figure 11c), which co-localised with β-catenin (Figure 11d). As a control, GFP-α-catenin was co-expressed with the deletion mutant, β-Δα-catenin, which lacks the α-catenin binding site. Like GFP-β-Δα-catenin (Figure 9g), β-Δα-catenin formed rods in the nucleus, as visualised by anti-myc antibody staining (Figure 11f). In the same cells GFP-α-catenin was cytoplasmic and did not sequester β-Δα-catenin in the cytoplasm (Figure 11e).

Vinculin is a cytoskeletal protein that shares some homology with α-catenin (Herrenknecht et al., 1991). Like α-catenin, vinculin can also bind α-actinin and actin, and in some cell types it associates with cell-cell adhesion complexes (Hazan et al., 1997). To determine whether vinculin would co-localise with β-catenin rods, GFP-vinculin was co-expressed with myc-tagged β-catenin. GFP-vinculin and β-catenin did not co-localise and GFP-vinculin (Figure 11 g) did not affect the localisation of β-catenin, which still formed intranuclear rods (Figure 11h). Therefore vinculin differs from α-catenin with respect to its effects on β-catenin localisation.

In addition to binding to β-catenin, α-catenin binds actin. Since the rods formed by ADF contain actin, it was interesting to determine if cytochalasin D (which is an actin-destabilising drug) would affect the β-catenin/α-catenin cytoplasmic rods. Therefore, cells were transfected with GFP-β-catenin and α-catenin and GFP was visualised under fluorescence microscopy. Untreated cells are shown in Figure 12a.

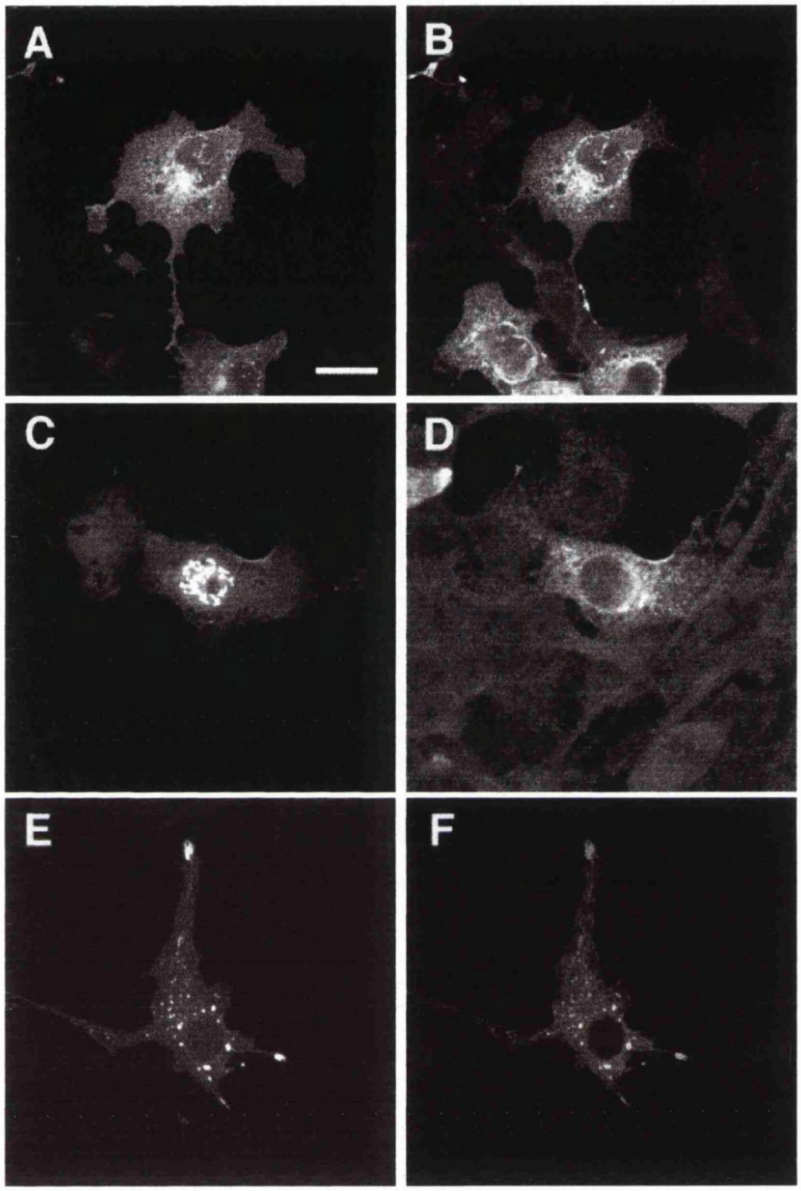


Figure 10.
N-cadherin and APC prevent formation of GFP-β-catenin nuclear rods.
COS-7 cells expressing GFP-β-catenin together with the following proteins were visualised by confocal microscopy: (A and B) β-catenin-GFP plus N-cadΔE/myc (with full-length cytoplasmic tail), (C and D) β-catenin-GFP plus N-cadΔEΔC/myc (mutant with 6 residue cytoplasmic tail), (E and F) β-catenin-GFP plus APC.
(A, C and E) visualising GFP, (B, D and F) myc-tag proteins stained with 9E10 mAb.
Scale bar 7 μm.

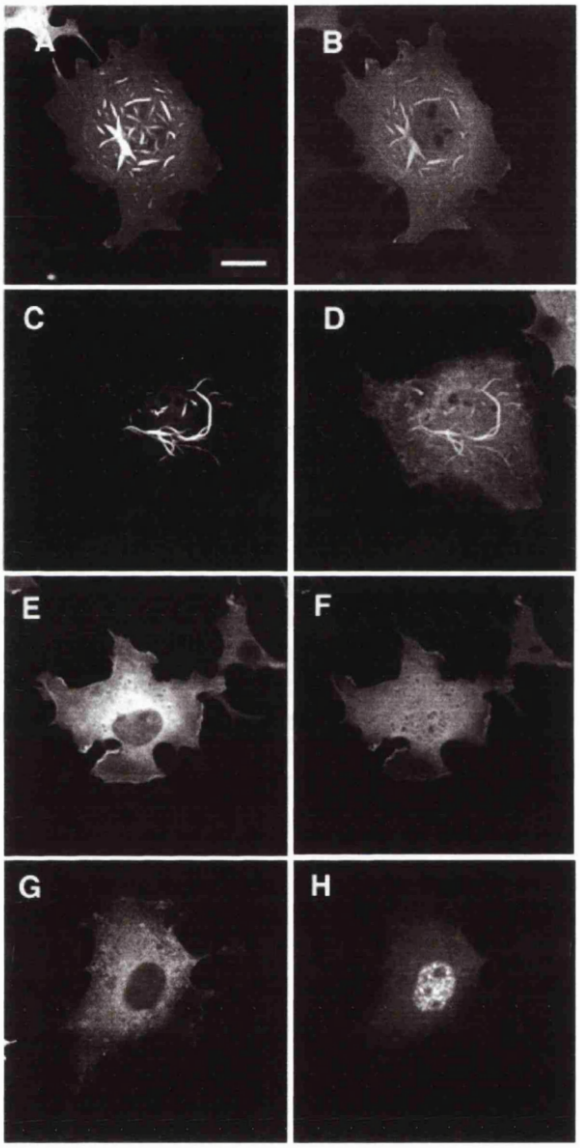


Figure 11.
Alpha-catenin and β-catenin, but not vinculin, associate to form rods in the cytoplasm.
COS-7 cells expressing the following proteins were visualised by confocal microscopy: (A and B) GFP-β-catenin plus α-catenin, (C and D) GFP-α-catenin plus β-catenin, (E and F) GFP-α-catenin plus β-catenin-Δα, (G and H) GFP-vinculin plus β-catenin. (A, C, E and G) Visualising GFP. (B) Stained for α-catenin, with CME polyclonal antibody, (D, F and H) stained with 9E10 mAb. Scale bar 7 μm.

The β-catenin/α-catenin cytoplasmic rods were not disrupted by cytochalasin D treatment (Figure 12b). Therefore, F-actin does not appear to be involved in the formation of β-catenin/α-catenin cytoplasmic rods.

Cells transfected with GFP-β-catenin and α-catenin were also treated with nocodazole. This drug, which depolymerises microtubules, had no effect on the cytoplasmic rods formed by GFP-β-catenin and α-catenin (Figure 12c). This suggests that polymerised microtubules are not involved in the formation or maintenance of the β-catenin/α-catenin cytoplasmic rods.

III.6- *Tcf-4 overrides the effects of α-catenin on β-catenin localisation*

Beta-catenin interacts with transcription factors of the Tcf/Lef-1 family and this interaction is essential for its role in the Wnt signalling pathway. Since the armadillo repeat domain of β-catenin is also involved in this association, the effect of co-expressing HA-tagged Tcf-4 on β-catenin localisation was examined. When cells expressed both Tcf-4 (visualised by staining with an anti-HA monoclonal antibody, Figure 13b) and GFP-β-catenin (Figure 13a), both proteins appeared evenly distributed in the nuclear compartment and did not form aggregates. This confirms the importance of the armadillo repeat domain for the constitution of the β-catenin rods. As a control, a mutant form of Tcf-4 (HA-ΔNTcf-4) that lacks the β-catenin binding site was used. The expression of HA-ΔNTcf-4 (Figure 13d) did not impede the aggregation of GFP-β-catenin in the nucleus (Figure 13c). Expression of GFP-tagged plakoglobin with HA-Tcf-4 also resulted in homogeneous nuclear localisation of both fusion proteins (Figures 13e and 13f, respectively), confirming that these two proteins can interact *in vivo*. It was shown earlier that the majority of the

cells did not express plakoglobin-GFP in the nucleus (Figure 8c). In the presence of Tcf-4, however, this was not the case, indicating that although plakoglobin lacks the intrinsic ability to enter the nucleus, it can be transported there by Tcf-4 (Figure 13e).

Alpha-catenin and the Tcf/Lef-1 family of transcription factors interact with different domains of β-catenin (the distal part of the amino-terminal domain and the armadillo repeats, respectively) and have different effects on β-catenin localisation. In order to determine which of these binding proteins had a stronger influence on β-catenin localisation, the effect of expressing α-catenin in cells expressing both β-catenin and Tcf-4 was examined. When HA-tagged Tcf-4, GFP-tagged α-catenin and myc-tagged β-catenin were co-expressed, all three proteins were found in the nucleus (Tcf-4, Figure 14a; α-catenin, Figure 14b; β-catenin Figure 14c). As a control, the cells were transfected with HA-tagged ΔN-Tcf-4, GFP-α-catenin and myc-tagged β-catenin (ΔNTcf-4, Figure 14d; α-catenin, Figure 14e; β-catenin Figure 14f). In this case, GFP-α-catenin remained in the cytoplasm (Figure 14e) and redirected some of the β-catenin to the cytoplasm (Figure 14f). ΔNTcf-4 (Figure 14d) did not affect the localisation of GFP-α-catenin (Figure 14e).

The localisation of GFP-vinculin, when co-expressed with β-catenin and Tcf-4, was also examined. Unlike GFP-α-catenin, GFP-vinculin (Figure 14h) remained cytoplasmic in the presence of the β-catenin/Tcf-4 complex (Tcf-4, Figure 14g; β-catenin, Figure 14i). These results indicate that transfected α-catenin, but not vinculin, can be carried into the nucleus by the β-catenin/Tcf-4 complex.

In order to determine if the localisation of endogenous α-catenin was similarly affected by expression of β-catenin or β-catenin/Tcf-4, cells

were transfected with GFP-β-catenin or GFP-β-catenin and Tcf-4. The presence of GFP-β-catenin in the nucleus (Figure 15a) did not appear to affect the subcellular localisation of endogenous α-catenin (Figure 15b). In contrast, endogenous α-catenin was found in the nuclei of cells expressing GFP-β-catenin and HA-Tcf-4 together (Figure 15d, the localisation of GFP-β-catenin is shown in Figure 15c).

The nuclear localisation of GFP-α-catenin in the presence of the β-catenin/Tcf-4 complex was also investigated biochemically by cell fractionation. Nuclear and cytosolic extracts were prepared from cells co-expressing GFP-α-catenin, HA-ΔNTcf-4 and β-catenin, or GFP-α-catenin, HA-Tcf-4 and β-catenin. The extracts were probed using a GFP antibody (Figure 15 e). The intensities of the bands representing GFP-α-catenin were measured by densitometry. When the cells were expressing HA-ΔNTcf-4, which cannot bind to β-catenin, the cytoplasmic fraction contained most of the GFP-α-catenin (Figure 15e, lanes 1 and 2). On the other hand, when co-expressed with wild-type Tcf-4, GFP-α-catenin was also present in the nuclear fraction (Figure 15e, lanes 3 and 4). As a control for the enrichment of the nuclear fraction with nuclear proteins, a GFP-tagged version of Tcf-4 was used. The nuclear fraction contained almost all of the GFP-Tcf-4 (Figure 15e, lane 5).

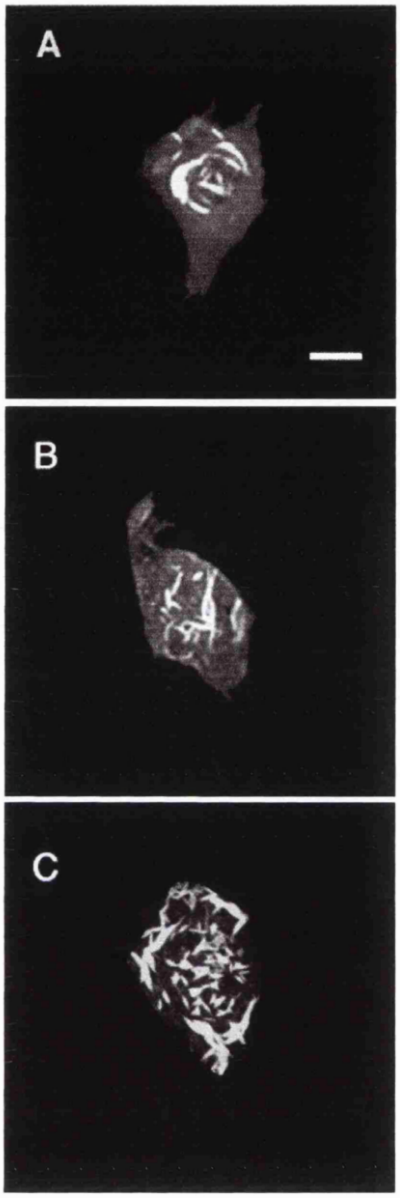


Figure 12.
Cytochalasin D and nocodazole do not affect the cytopalsmic rods formed by GFP-β-catenin and α-catenin.
COS-7 cells expressing GFP-β-catenin plus α-catenin, visualising GFP.
(A) Untreated cells, (B) treated with cytochalasin D, (C) treated with nocodazole.
Scale bar 7 μm.

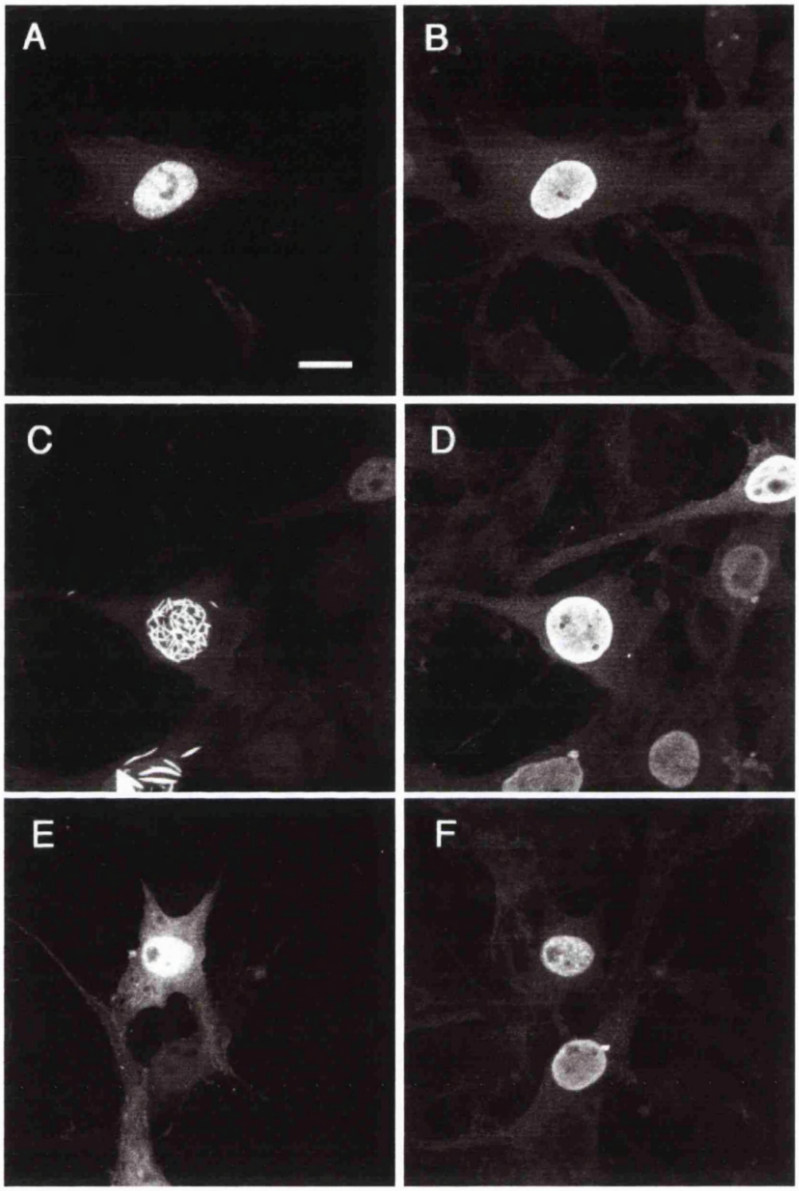


Figure 13.
Tcf-4, but not ΔN-Tcf-4, disrupts formation of GFP-β-catenin nuclear rods. COS-7 cells expressing the following proteins were visualised by confocal microscopy: (A and B) GFP-β-catenin plus HA-Tcf-4, (C and D) GFP-β-catenin plus HA-ΔN-Tcf-4, (E and F) Plakoglobin-GFP plus HA-Tcf-4. (A, C and E) visualising GFP, (B, D and F) stained with anti-HA polyclonal antibody. Scale bar 7 μm.

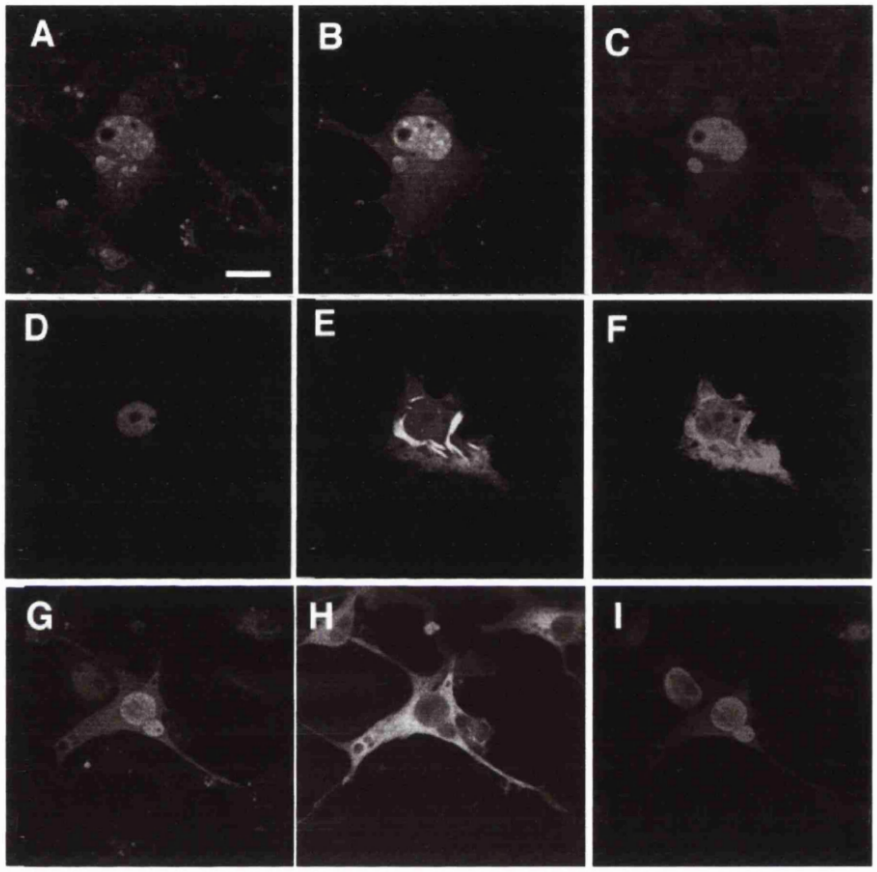


Figure 14.
GFP-α-catenin enters the nucleus in the presence of β-catenin and Tcf-4. COS-7 cells expressing the following proteins were visualised by fluorescence microscopy: (A, B and C) HA-Tcf-4, GFP-α-catenin and β-catenin-myc, (D, E and F) HA-ΔN-Tcf-4, GFP-α-catenin and β-catenin-myc. (G, H and I) HA-Tcf-4, GFP-vinculin and β-catenin-myc. (A, D and G) stained with anti-HA polyclonal antibody, (B, E and H) visualising GFP, (C, F and I) stained with 9E10 mAb. Scale bar 7 μm.

Taken together, the experiments in Figures 14 and 15 suggest that the β-catenin/Tcf-4 complex can transport both exogenous and endogenous α-catenin into the nucleus.

Studies using other cell types lead to the suggestion that α-catenin sequesters β-catenin in the cytoplasm (Simcha et al., 1998). This sequestration has been proposed to account for the ability of α-catenin to inhibit β-catenin-dependent transcription. My observation that α-catenin enters the nucleus with the β-catenin/Tcf complex cells does not support this model. In order to determine whether α-catenin inhibits β-catenin/Tcf dependent transcription in my experiments, I performed transcription assays using a luciferase reporter plasmid driven by a promoter recognised by Tcf/Lef-1 transcription factors. The cells were transfected with Tcf-4, β-catenin, the Tcf-responsive reporter plasmid and either control (empty vector), α-catenin or vinculin expression vectors. The results, shown in Figure 16a, indicate that both α-catenin and GFP-α-catenin were able to reduce the transcriptional activity of the β-catenin/Tcf-4 complex to a similar extent as N-cadherin, a known inhibitor of β-catenin signalling. GFP-vinculin did not inhibit the transcriptional activity of the β-catenin/Tcf-4 complex.

GFP-α-catenin also repressed luciferase activity in colon cancer cell lines, which have high endogenous β-catenin/Tcf-4-dependent transcription (Figure 16b). The extent of inhibition varied among the different cell lines, ranging from 50% in HCT116 cells to 70% in SW480, Caco-2 and DLD-1 cells.

Taken together these observations suggest that α-catenin enters the nucleus in cells expressing β-catenin and Tcf-4, where it inhibits the transcriptional activity of the β-catenin/Tcf-4 complex.

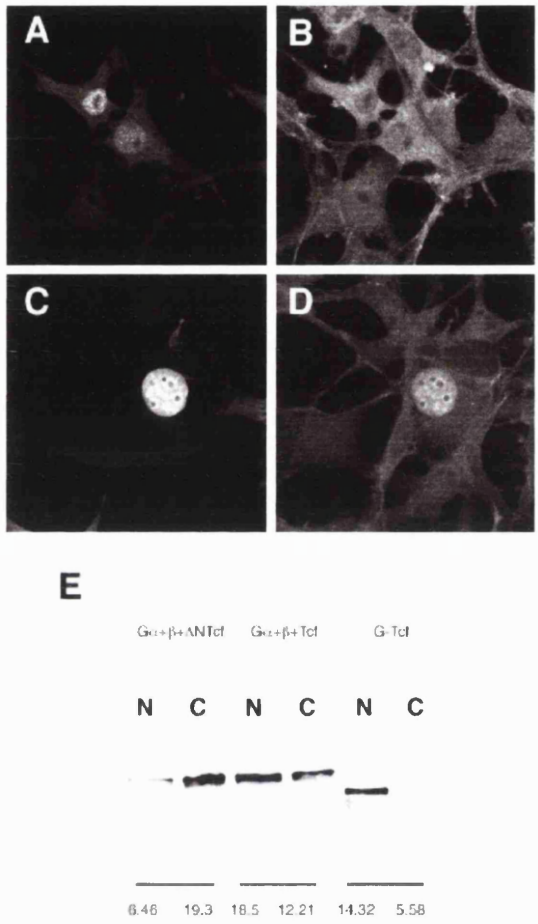


Figure 15.
Endogenous α-catenin enters the nucleus in the presence of β-catenin and Tcf-4. COS-7 cells expressing the following proteins: (A and B) GFP-β-catenin, (C and D) GFP-β-catenin plus HA-Tcf-4, were visualised directly for GFP-fusion proteins (A and C) or stained for endogenous α-catenin (B and D) using CME polyclonal antibody. Scale bar 7 μm.
(E) Nuclear and cytoplasmic extracts from COS-7 cells expressing GFP-α-catenin, β-catenin-myc and HA-ΔN-Tcf-4 (lanes 1 and 2); GFP-α-catenin, β-catenin-myc and HA-Tcf-4 (lanes 3 and 4) or GFP-Tcf-4 (lanes 5 and 6) were blotted with anti-GFP polyclonal antibody. N-nuclear and C-cytoplasmic fractions. Numbers at the bottom of each lane indicate relative intensities obtained by densitometry.

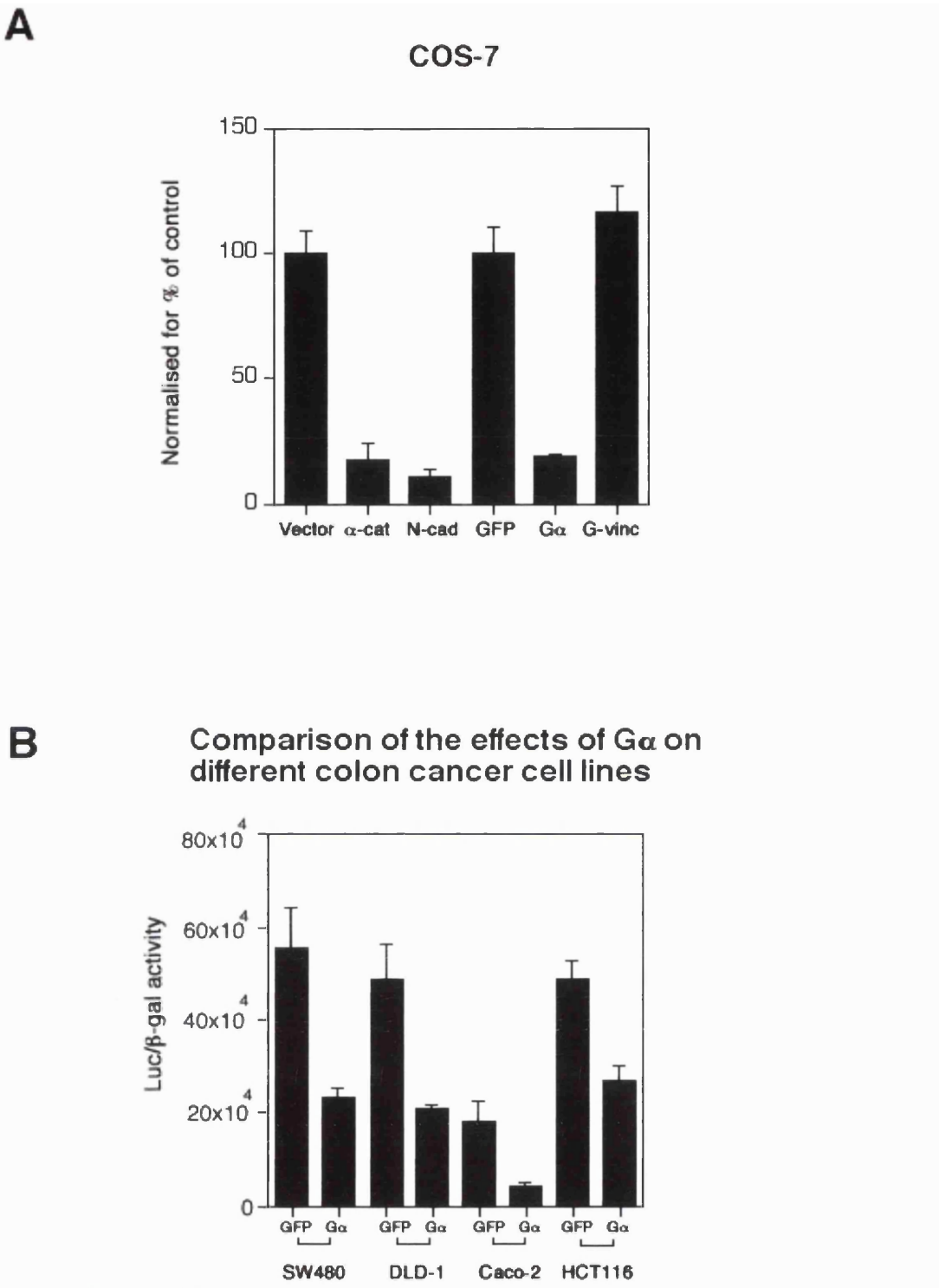


Figure 16a

Alpha-catenin inhibits β-catenin-dependent transcription. (A) Cells were transfected with β-catenin-myc, HA-Tcf-4, pTOPFlash, RSV β-gal, plus pMT23 empty vector, α-catenin, N-cadherin, GFP, GFP-α-catenin or GFP-vinculin as indicated in the figure. Luciferase activity values were normalised for β-gal activity and are presented as % of the control (vector or GFP). (B) GFP-α-catenin inhibits endogenous β-catenin-dependent transcriptional activity in colon cancer cells. SW480, DLD-1, Caco-2 and HCT116 cells were tranfected with pTOPFlash and RSV β-gal, plus GFP or GFP-α-catenin. Luciferase activity was normalised for transfection efficiency in each cell line.

III.7- *Discussion*

In this chapter I have shown, using GFP-fusion proteins, that overexpression of β-catenin leads to its accumulation in the nucleus and formation of rod-like structures. I have also shown that α-catenin enters the nucleus in cells expressing β-catenin and Tcf-4.

The three-dimensional structure of the armadillo repeat domain of β-catenin has been solved (Huber et al., 1997). Each armadillo repeat is composed of a sequence of three α-helices folding β-catenin into a superhelix of α-helices featuring a positively charged groove. Many of the β-catenin binding proteins associate within this groove.

It is not known how the amino- and carboxyl-terminal domains of β-catenin fit into this structure, but they appear to regulate both intramolecular and intermolecular interactions. For example, the carboxyl-terminal domain of β-catenin can interact with the armadillo repeat domain, competing with E-cadherin and the TATA-box binding protein, TBP, for binding to the armadillo repeat domain (Piedra et al., 2001). The carboxyl-terminal domain of *Drosophila* Armadillo has also been shown to interact with the armadillo repeat domain in yeast two-hybrid experiments (Cox et al., 1999). The terminal domains also appear to be involved in preventing β-catenin from interacting with desmoglein-2 in the desmosome (Wahl et al., 2000). Moreover, these domains can inhibit the association of β-catenin/Lef-1 with DNA (Zhurinsky et al., 2000). Considering this evidence, it is possible that the rods I have described are a result of the association between intact β-catenin monomers, forming an array, where the acidic-terminal domains of one monomer interact with the positive grooves in adjacent monomers. This would account for the inability of β-catenin ΔN, ΔC and ΔNΔC constructs to form rods and the

more efficient rod formation by GFP-β-catenin compared to β-catenin-GFP (which lacks carboxyl-terminal residues 761 to 781). The fact that nuclear plakoglobin did not form rods could also result from differences between the amino- and/or carboxyl-terminal domain sequences of β-catenin and plakoglobin.

The β-catenin binding domains in Tcf/Lef-1, APC, cadherin and axin, although unrelated in sequence, contain acidic residues that interact with the positively charged groove of β-catenin. Serine/threonine phosphorylation of APC, cadherin and axin further increases the negative charge in the β-catenin binding domains of these proteins, thereby increasing their association with β-catenin (Hulsken et al., 1994; Rubinfeld et al., 1995; Behrens et al., 1996; Molenaar et al., 1996; Orsulic and Peifer, 1996; Pai et al., 1996; Ikeda et al., 1998). Conversely, phosphorylation of β-catenin in the armadillo repeat domain impairs E-cadherin binding (Roura et al., 1999; Piedra et al., 2001), possibly because it reduces the positive charge in the groove.

The assembly of the cadherin/catenin complex has been analysed by pulse-chase and co-sedimentation experiments (Hinck et al., 1994). β-catenin associates with cadherins immediately after synthesis, when E-cadherin is still in the endoplasmic reticulum. Alpha-catenin is recruited to the complex later, when E-cadherin reaches the plasma membrane. The crystal structure of the β-catenin/E-cadherin complex indicates that the binding of cadherin to β-catenin prevents cadherin degradation. This results from an overlap of PEST motifs, which are associated with rapid protein turnover, with the β-catenin binding domain in E-cadherin (Huber et al., 2001). The binding of β-catenin to E-cadherin may not only prevent cadherin degradation, but it may also prevent β-catenin molecules from

aggregating to form rods.

The crystal structure of the complex formed between β-catenin and the β-catenin binding domain of Tcf-3 has been solved. It shows that the binding domain of Tcf-3 forms an elongated structure that interacts at three sites within the positively charged groove of β-catenin (Graham et al., 2000). Although I used Tcf-4 rather than Tcf-3, the β-catenin binding domain in this family of transcription factors is highly conserved. For instance Tcf-3 and Tcf-4 share two conserved residues (Asp-16 and Leu-48) that are critical for β-catenin binding (Omer et al., 1999). Tcf-3 and E-cadherin interact with some of the same residues in β-catenin, but they acquire different secondary structures to do so. Moreover, Tcf-3 contains residues that mimic those found in the β-catenin binding domain of E-cadherin (Huber et al., 2001). This explains why cadherin and Tcf compete for β-catenin binding.

Taken together with my results, the structural data are consistent with a model in which proteins that interact with the armadillo repeat domain of β-catenin impairs the formation of the rods by preventing interactions between the armadillo repeats and the terminal domains of β-catenin. Although plakoglobin also contains armadillo repeats, overexpression of this protein in COS-7 cells does not lead to formation of rods. Moreover, plakoglobin-GFP is found in the cytoplasm in the majority of transfected cells, unless it is co-expressed with Tcf (Figures 8c and 13c). These results contradict what was reported by Simcha et al. (1998), who overexpressed plakoglobin in an epithelial cell line, and also what was observed by Rubenstein et al. (1997) by injecting this protein into *Xenopus* fertilized eggs. The reason for these discrepancies is unknown, but may be due to differences in the cells lines used. For

instance, COS-7 cells do not normally express significant levels of plakoglobin (Kowalczyk et al., 1997). Moreover, in developing *Xenopus* embryos, the Wnt signalling pathway is active, so plakoglobin can associate with XTcf-3 and enter the nucleus.

Simcha et al. (1998) also reported that β-catenin formed nuclear rods, which contained Lef-1 and vinculin. However, in my experiments, the β-catenin rods were disrupted by co-expression of Tcf-4 (similar results were obtained in our laboratory for Lef-1 and Tcf-1). Moreover, GFP-vinculin did not co-localise with β-catenin, and it did not affect the formation of the β-catenin rods. Finally, co-expression of β-catenin, Tcf-4 and GFP-vinculin did not lead to accumulation of GFP-vinculin in the nucleus. Therefore, my results suggest that if the rods do contain Lef-1 and/or vinculin, these proteins are probably at levels that are too low to disrupt the assembly of the rods.

Although β-catenin can be transported into the nucleus through its association with Tcf/Lef-1, in certain situations this is not required. In fact, β-catenin nuclear localisation is believed to involve direct association with components of the nuclear pore in a similar way to importins which contain repeats related in structure to armadillo repeats (Conti et al., 1998; Fagotto et al., 1998, Yokoya et al., 1999; Wiechens and Fagotto; 2001). My results indicate that the sequences necessary for β-catenin nuclear localisation lie within armadillo repeats 8-12, since the construct containing these repeats and the carboxyl-terminal tail is nuclear, but the removal of the tail alone does not affect β-catenin nuclear localisation.

In cells that are not overexpressing β-catenin, stabilisation of this protein through activation of the Wnt signalling pathway leads to β-catenin accumulation in the nucleus. However, endogenous β-catenin rods

have not been reported in cells responding to Wnt. The reason for this might be that stabilised β-catenin associates with Tcf/Lef-1 and any excess of free β-catenin would result in cell death (Kim et al., 2000). The apoptosis induced by overexpressed β-catenin is independent of β-catenin-dependent transcription, and it might be a physiological mechanism to eliminate cells that have high levels of β-catenin from the population.

In this context, several neurodegenerative diseases are a consequence of aggregation of proteins in the nucleus. Brains of Alzheimer's patients, for example, contain not only amyloid deposits, but also ADF/cofilin/actin inclusions (Minamide et al., 2000). The appearance of these inclusions, emerging from endogenous mutated proteins or overexpressed ones, is somehow involved in the loss of neuronal function. Mutations in presenilins are the major cause of familial Alzheimer's disease. The presenilins are involved in the breakdown of the β-amyloid precursor protein, but also appear to down regulate Wnt signalling through direct interactions with GSK3 and β-catenin. Thus, loss of presenilin function can lead to accumulation of β-catenin, which, together with other events would culminate in the neurodegeneration observed in Alzheimer's brains (Soriano et al., 2001). It may be informative to examine diseased neuronal tissues for the presence of nuclear β-catenin aggregates.

Similar to N-cadherin and APC, α-catenin prevented nuclear accumulation of β-catenin. However, α-catenin could not prevent the formation of β-catenin rod-like structures. The sequestration of β-catenin in the cytoplasm by α-catenin required direct interaction of these proteins, since it did not occur when the α-catenin binding site was deleted in β-catenin. It is likely that the inability of GFP-α-catenin to disrupt β-catenin

rods is because the α-catenin binding domain in not involved in rod formation. Since β-catenin rods could be found both in the cytoplasm and in the nucleus, nuclear localisation of β-catenin is not required for rod formation. This makes it unlikely that other specific nuclear proteins are required for the formation of these structures.

In the presence of Tcf-4, α-catenin was unable to sequester β-catenin in the cytoplasm. The domains involved in the interaction of these two proteins with β-catenin are distinct. One possibility is that the interactions of α-catenin with components of the cytoskeleton are sufficient to prevent nuclear localisation of β-catenin. In the presence of Tcf-4, which contains a canonical nuclear localisation sequence, the interactions of α-catenin with the cytoskeleton may not be sufficient to anchor the complex in the cytoplasm. This would result in α-catenin being transported to the nucleus with the β-catenin/Tcf-4 complex. Since GFP-α-catenin was present in the nucleus and it inhibited β-catenin/Tcf-4 dependent transcription, this inhibition may take place in the nucleus.

Although α-catenin and vinculin share many binding partners, including the catenins (Huber et al., 1997; Weiss et al., 1998) they behave differently in the experiments presented here. GFP-vinculin was unable to co-localise with the β-catenin rods and it did not affect the formation of these structures or their localisation. Moreover, GFP-vinculin did not inhibit β-catenin signalling. Thus, the interaction of vinculin with the catenins is not strong enough to affect β-catenin function in the nucleus.

To conclude, these experiments suggest that α-catenin inhibits β-catenin signalling, not by sequestration of β-catenin in the cytoplasm, but rather by some other mechanism in the nucleus. The mechanism of inhibition will be addressed in Chapter IV.

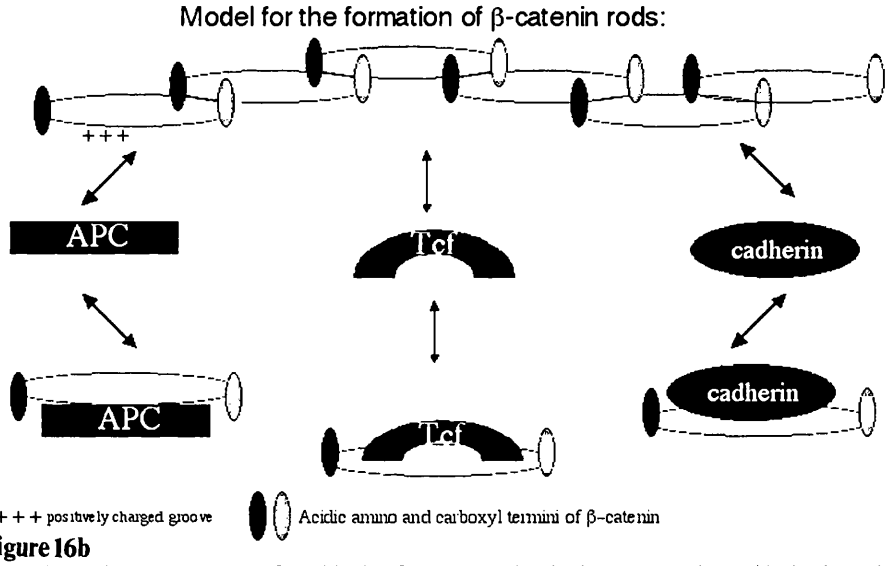


Figure 16b

The amino and carboxyl-termini of neighboring β-catenin molecules interact with the positively charged groove formed by the armadillo repeat domain of β-catenin. APC, Tcf and cadherin molecules also bind to the armadillo repeat domain, hence in the presence of these proteins, β-catenin rods do not form.

CHAPTER IV-*Alpha-catenin inhibits β-catenin-dependent transcription in the nucleus*

IV.1- *Introduction*

The results described in Chapter III suggest that α-catenin can inhibit β-catenin signalling and that this is achieved by means other than sequestration of β-catenin in the cytoplasm. Although overexpression of α-catenin has been shown to inhibit β-catenin signalling (Sehgal et al., 1997; Simcha et al., 1998), the role of endogenous α-catenin in this process has not been investigated. In this chapter, I will describe experiments addressing the physiological relevance of α-catenin nuclear localisation and the mechanism by which this protein inhibits β-catenin signalling.

IV.2- *Endogenous α-catenin is present in the nucleus in colon cancer cells*

The majority of colon cancer cell lines contain high levels of β-catenin due, either to mutations in β-catenin itself or to mutations in the APC protein, both of which prevent β-catenin degradation by the proteasome. In either case, the result is the activation of β-catenin/Tcf dependent transcription (Korinek et al., 1997; Morin et al., 1997).

In non-transformed epithelial cells, endogenous α-catenin is present at cell-cell junctions and in the cytoplasm (Hinck et al., 1994; Nathke et al., 1994). Overexpression of α-catenin in COS-7 cells results in its accumulation in the cytoplasm. When expressed in the presence of β-catenin and Tcf-4, α-catenin localises to the nucleus, suggesting that α-catenin can be transported to the nucleus by the β-catenin/Tcf complex. Since colon cancer cells contain endogenous β-catenin/Tcf complexes, I examined the localisation of endogenous α-catenin in a panel of colon cancer cells lines by immunocytochemistry.

SW480 and DLD-1 cells express a truncated form of APC, HCT116 cells express a mutated form of β-catenin and RKO cells contain wild-type APC and β-catenin (reviewed by Ilyas et al., 1997; Rowan et al., 1999; da Costa et al., 1999). Beta-catenin was present in the nucleus of SW480 (Figure 17a), HCT116 (Figure 17c) and DLD-1 (Figure 17e) cells. Beta-catenin also localised to the plasma membrane in HCT116 and DLD-1 cells, which express cadherins. There was no β-catenin in the nucleus and little in the membrane of RKO cells (Figure 17g), which do not express cadherins. Two other cell lines were analysed, both of which are non-adhesive variants of DLD-1 cells that have been selected as floating cells from DLD-1 cell cultures. DLDα+ cells do not form a cobblestone monolayer with defined cell-cell junctions, although they do express α-catenin, while DLDα- cells have the same morphology as DLDα+, but have lost α-catenin expression (Ozawa, 1998). Beta-catenin was found in the cytoplasm and nucleus in both DLDα+ (Figure 17i) and DLDα- (Figure17k) cells.

Alpha-catenin had the same distribution as β-catenin in SW480 cells being present both in the cytoplasm and the nucleus (Figure 17b). In HCT116 cells α-catenin co-localised with β-catenin at the cell membrane, at cell-cell junctions and also in the cytoplasm (Figure 17d). In DLD-1 cells, α-catenin co-localised with β-catenin at cell-cell junctions, in the cytoplasm and in cell nuclei (Figure 17f). RKO cells had low levels of α-catenin, which was found only in the cytoplasm (Figure 17h).

In DLDα+ cells α-catenin localisation was similar to that of β-catenin, being both in the cytoplasm and the nucleus (Figure 17j). In DLDα- cells, α-catenin staining was negative since these cells do not express this protein (Figure 17l).

The levels of cell-cell adhesion complex proteins among these different cell lines were compared by western blotting of cell extracts (Figures 18a, 18b and 18c). Cadherins were present in all the cells, but at relatively low levels in SW480 cells, where it is unstable (Efstathiou et al., 1998). Similar amounts of β-catenin and vinculin were expressed in the DLD-1 variants, while more β-catenin was present in SW480 cells (Figure 18a, lane 5). This might account for the stronger staining of this protein in the nuclei of these cells (Figure 17a). All the cell lines, apart from DLDα-, which do not express α-catenin, expressed similar amounts of α-catenin (Figure 18a, lane 4). To analyse the cadherin/catenin complexes in these cells, NP-40 extracts were prepared and subjected to immunoprecipitation using α-catenin antibody (Figure 18b). The immunoprecipitates were probed for cadherin (upper panel) or β-catenin (lower panel). Alpha-catenin immunoprecipitates from SW480 cells contained low levels of cadherin and high levels of β-catenin, reflecting the levels of these proteins in this cell line (Figure 18b, lane 5). Although the variant DLDα+ cells cannot form a cobblestone monolayer, cadherins and catenins were still able to co-immunoprecipitate in these cells (Figure 18b, lane 3). DLDα- cells also cannot form a cobblestone monolayer owing to the absence of α-catenin. However β-catenin immunoprecipitates from DLDα- cells still contained cadherin (Figure 18c), indicating that the absence of α-catenin does not affect the formation of β-catenin/cadherin complex.

A parental DLD-1 cell line (DLD-1b) from a distinct source was also used to control for clonal differences. These cells expressed similar amounts of β-catenin, α-catenin, cadherin and vinculin as DLD-1 cells (Figure 18a). In these cells, α-catenin co-immunoprecipitated with both β-catenin and cadherin (Figure 18b, lane 1).

IV.3- ***Loss of endogenous α-catenin correlates with higher β-catenin/Tcf transcription activity***

In order to assess the influence of endogenous α-catenin on endogenous β-catenin signalling, β-catenin/Tcf-dependent transcription was measured in DLD-1 parental cell lines (DLD-1 and DLD-1b), DLDα+ and DLDα- using TOP/FOPFlash plasmids. Cells were transiently transfected with the TOP/FOPFlash reporter plasmids and a β-galactosidase expression plasmid in order to normalise for the efficiency of transfection. The ratio between TOPFlash and FOPFlash luciferase values represents a measure of β-catenin signalling in these cells. When the transcriptional activity was compared among these cell lines, DLDα- exhibited a β-catenin-dependent transcriptional activity that was 30% higher than the activities in the α-catenin-positive cell lines (Figure 18d). This suggests that a consequence of the absence of α-catenin is an increase in β-catenin-dependent transcriptional activity.

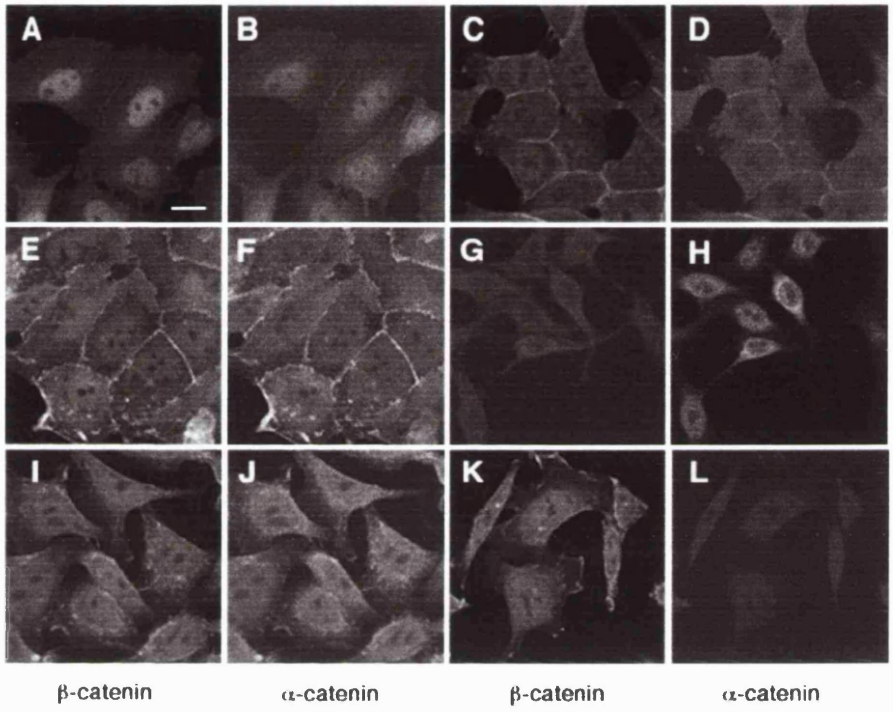


Figure17.
Alpha-catenin is present in the nuclei of colon cancer cells.
The localisation patterns of β-catenin (A, C, E, G, I and K) and α-catenin (B, D, F, H, J and L) were determined using specific antibodies and the following cell lines: SW480 (A and B), HCT116 (C and D), DLD-1 (E and F), RKO (G and H), α-catenin positive DLD-1 variant (DLDα+, I and J) and α-catenin negative DLD-1 variant (DLDα-, K and L).
Scale bar 7 μm.

IV.4- *Nuclear-targeted α-catenin inhibits β-catenin signalling*

The observation that endogenous α-catenin is present in the nuclei of some colon cancer cells, taken together with the results described above, suggest that inhibition of β-catenin dependent transcription occurs in the nucleus. To investigate this possibility, a nuclear localisation signal (NLS) was added to GFP-α-catenin (GFP-α-NLS). Assuming that α-catenin inhibits β-catenin signalling by sequestration of the latter in the cytoplasm, a nuclear-targeted version of α-catenin would not be efficient in repressing β-catenin/Tcf activity. First, the localisation of both GFP-α-catenin and GFP-α-NLS was analysed in COS-7 and SW480 cells. In COS-7 cells GFP-α-catenin was cytoplasmic (Figure 11e) and GFP-α-NLS was exclusively nuclear (Figure 19a). When GFP-α-NLS was co-transfected with β-catenin, it was unable to disrupt the rods formed by the latter in the nuclei of COS-7 cells, instead it co-localised with β-catenin in the nuclear rods (Figure 19b). In SW480 cells, GFP-α-catenin, like endogenous α-catenin, was found both in the nucleus and cytoplasm (Figure 19d; Figure 20a), while GFP-α-NLS was exclusively nuclear (Figure 19c; Figure 20b). The localisation of GFP-α-catenin was also examined in other colon cancer cells. GFP-α-catenin was present both in the nucleus and cytoplasm of DLD-1 (Figure 20d), similar to the localisation of endogenous α-catenin these cells. The same result was obtained with DLDα- cells (Figure 20g).

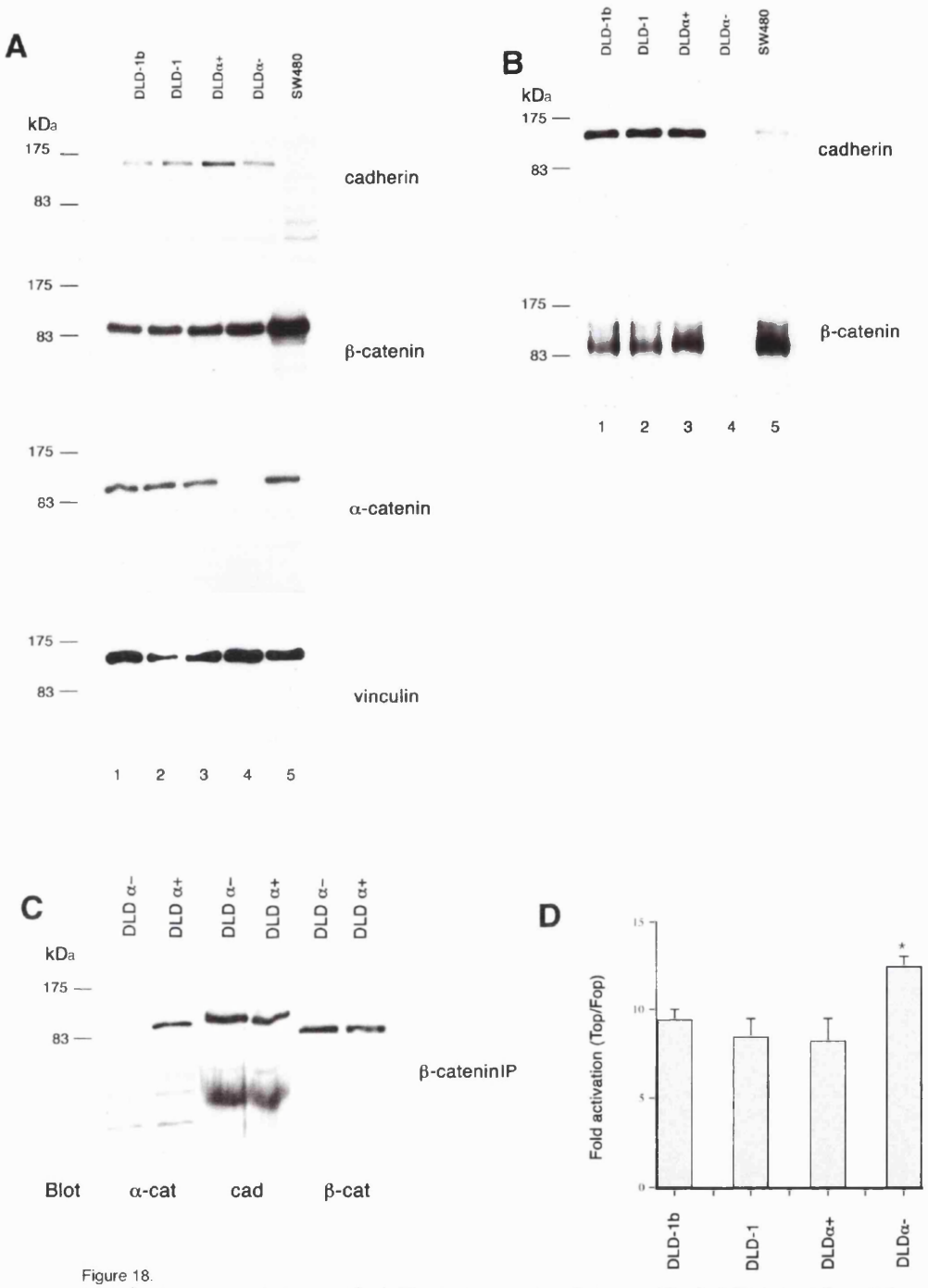


Figure 18.
Loss of endogenous a-catenin expression in DLD-1 cells correlates with increased β-catenin/Tcf transcription activity. (A) Total cell extracts from two clones of parental DLD-1 cells (lanes 1 and 2), α-catenin positive variant DLD-1 cells (DLDα+, lane 3), α-catenin negative variant DLD-1 cells (DLDα-, lane 4) and SW480 cells (lane 5) were probed using the antibodies indicated. (B) α-catenin immunoprecipitates from NP-40 extracts of parental DLD-1 cells (lanes 1 and 2), α-catenin positive variant DLD-1 cells (DLDα+, lane 3), α-catenin negative variant DLD-1 cells (DLDα-, lane 4) and SW480 cells (lane 5) were probed with the antibodies indicated. The lines on the left of the blots indicate positions of molecular weight markers (175 kDa and 83 kDa).(C) β-catenin immunoprecipitates from NP-40 extracts of α-catenin positive variant DLD-1 cells (DLDα+, lanes 2, 4 and 6) and α-catenin negative variant DLD-1 cells (DLDα-, lanes 1, 3 and 5) were probed with the antibodies indicated. The lines on the left of the blots indicate positions of molecular weight markers (175 kDa and 83 kDa).(D) The following cells were transfected with plasmids for RSV-β-galactosidase and either pTOPFlash or pFOPFlash. Two clones of parental DLD-1 cells (columns 1 and 2), α-catenin positive variant DLD-1 cells (DLDα+, column 3) and α-catenin negative variant DLD-1 cells (DLDα-, column 4). Luciferase activities were normalised for transfection efficiency and fold activation was calculated as the ratio of pTOPFlash and pFOPFlash luciferase activities. Experiments were repeated in triplicate at least three times. * $p < 0.01$ in the Student's 't' test (compared to with the activity in each of the cell lines expressing α-catenin.

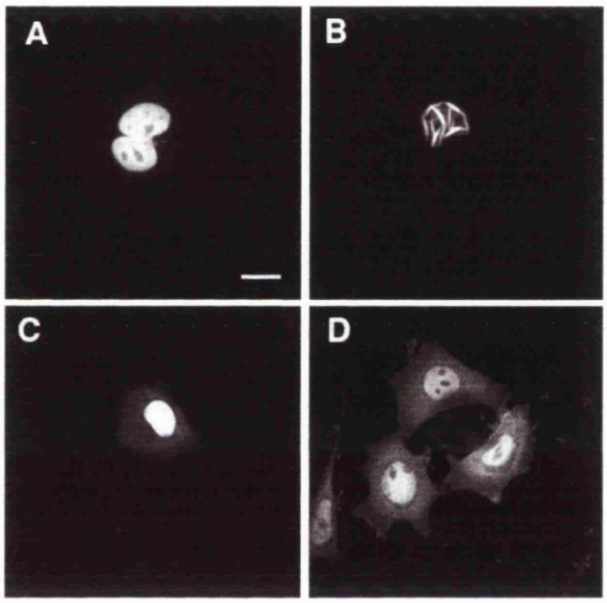


Figure 19.
Subcellular localisation of GFP-α-NLS and GFP-α-catenin in COS-7 and SW480 cells. Cells were transfected with constructs encoding the following proteins and visualized for GFP by fluorescence microscopy: (A) GFP-α-NLS in COS-7 cells, (B) GFP-α-NLS plus β-catenin in COS-7 cells, (C) GFP-α-NLS in SW480 cells, (D) GFP-α-catenin in SW480 cells. Scale bar 7 μm.

In HCT116 cells, GFP-α-catenin was mainly in the cytoplasm and was excluded from the nucleus in ~ 70% of the cells (Figure 20 j). GFP-α-NLS was mainly nuclear in DLD-1 (Figure 20e), DLDα- (Figure 20h) and HCT116 cells (Figure 20k).

The effect of GFP-α-NLS on β-catenin/Tcf activity was examined. GFP-α-NLS inhibited β-catenin signalling by 70% in COS-7 cells co-expressing myc-tagged β-catenin and Tcf-1. This was similar to the level of inhibition achieved both by GFP-axin and GFP-α-catenin (Figure 21a). GFP-α-NLS inhibited endogenous β-catenin/Tcf activity in SW480 cells by 60%, also similar to the inhibition by GFP-α-catenin (Figure 21b). GFP-α-NLS also inhibited β-catenin signalling in other colon cancer cell lines tested, including DLD-1 (Figure 22b), DLDα- (Figure 22c) and HCT116 (Figure 22d) to a similar extent as in SW480 cells (Figure 22a).

HepG2 is a liver cancer cell line that predominantly expresses a mutant form of β-catenin that lacks the α-catenin binding site (de la Coste et al., 1998). In HepG2 cells, neither GFP-α-catenin nor GFP-α-NLS reduced β-catenin/Tcf dependent transcription (Figure 22e). However, GFP-axin, which can still bind to the mutant β-catenin expressed by HepG2 cells, inhibited transcription by approximately 50%. Taken together, these results suggest that inhibition of β-catenin signalling by α-catenin can take place in the nucleus, and that α-catenin must bind to β-catenin in order to inhibit its signalling function.

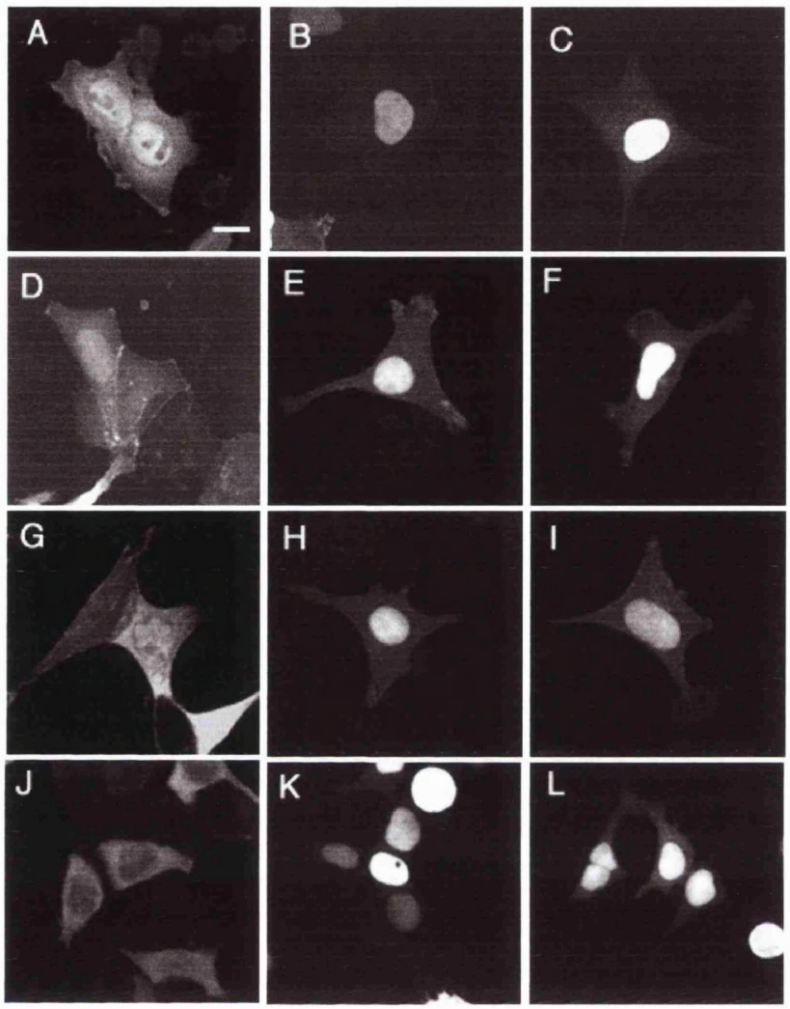


Figure 20.
Subcellular localisation of GFP-α-catenin, GFP-α-NLS and GFP-αΔ-NLS in colon cancer cells.
Cells were transfected with constructs encoding the following proteins and visualised by fluorescence microscopy: (A, D, G and J) GFP-α-catenin, (B, E, H and K) GFP-α-NLS and (C, F, I and L) GFP-αΔ-NLS. (A, B and C) SW480 cells, (D, E and F) DLD-1 cells, (G, H and I) DLDα- cells, (J, K and L) HCT116 cells. Scale bar 7 μm.

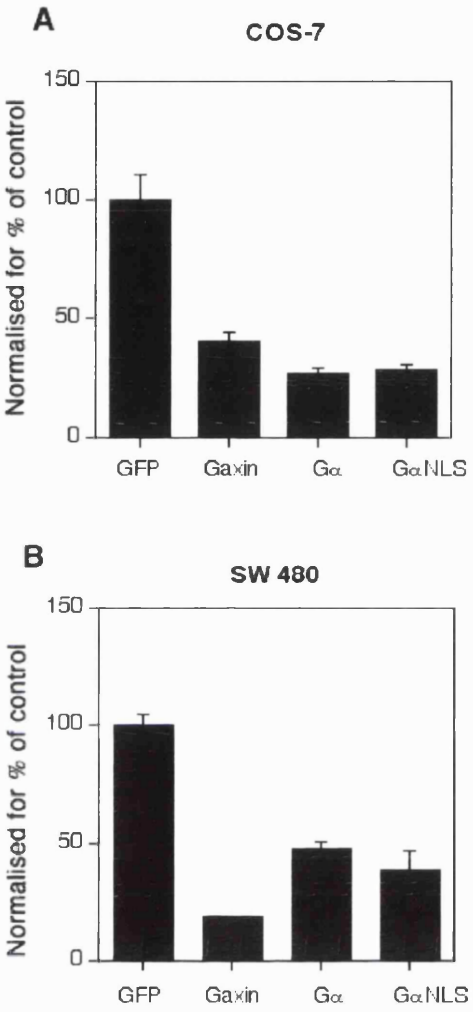


Figure 21.
Targeted expression of GFP-α-catenin in the nucleus represses β-catenin/Tcf transcriptional activity.
(A) COS-7 cells and (B) SW480 cells were transfected with plasmids encoding β-catenin and Tcf-1 (COS-7 cells only), pTOPFlash, RSV-β-galactosidase and the GFP fusion proteins indicated. Experiments were repeated at least three times. The values shown are the mean of a typical experiment, normalised for β-galactosidase activity, and with the mean of the relevant controls set at 100%. Each transfection was done in triplicate, error bars show standard deviation.

Several cytoskeletal proteins have been shown to interact with α-catenin, including vinculin, α-actinin, actin and ZO-1 (Weisset al., 1998; Watabe-Uchida et al., 1998; Nieset et al., 1997; Itoh et al., 1997; Rimm et al., 1995). In order to determine whether these interactions are required for α-catenin to inhibit β-catenin function as a transcriptional co-activator, I made a truncated construct encoding the first 214 amino acids of α-catenin, with a NLS sequence fused to GFP (GFP-αΔ-NLS). This protein retains the β-catenin and the homodimerisation domains of α-catenin (Pokutta and Weiss, 2000), but lacks the binding sites for vinculin, α-actinin and ZO-1. GFP-αΔ-NLS reduced β-catenin-dependent transcription activity to a similar extent as GFP-α-NLS in SW480 (Figure 22a), DLD-1 (Figure 22b), DLDα- (Figure 22c) and HCT116 cells (Figure 22d), but did not reduce β-catenin-dependent transcription in HepG2 cells (Figure 22e). These results suggest that interactions with vinculin, α-actinin and ZO-1, or dimerisation with endogenous α-catenin, are not required for α-catenin to inhibit β-catenin signalling.

IV.5- ***Inhibition of an endogenous target of β-catenin signalling by GFP-αΔ-NLS***

The cyclin D1 gene has been shown to be an endogenous target of Wnt signalling (Tetsu and McCormick, 1999). Until now, the transcriptional activity of β-catenin/Tcf has been measured using an artificial promoter that contains Tcf binding sites fused to a luciferase reporter. To test if α-catenin would be able to inhibit an endogenous target of Wnt signalling, a reporter plasmid containing the cyclin D1 promoter was used. HEK 293 cells were chosen for these experiments, since it has been reported that expression of β-catenin in this cell line

increases the level of endogenous cyclin D1 (Shtutman et al., 1999). For the transcription assays HEK 293 cells were transfected with a reporter plasmid containing the cyclin D1 promoter and a stable form of β-catenin (β-ΔN2) together with GFP (negative control), GFP-axin (positive control for repression) or GFP-αΔ-NLS. Both GFP-αΔ-NLS and GFP-axin inhibited the transcription of the reporter plasmid by more than 50% (Figure 23a). The effects of GFP-αΔ-NLS on β-catenin signalling were also tested by measuring the levels of cyclin D1 protein in HEK 293 cells transfected with the same β-catenin construct (β-ΔN2) used for the transcription assays (Figure 23b). These cells were transfected with empty vector pMT23 and GFP, pMT23-β-catenin and GFP, pMT23 and GFP-αΔ-NLS, or pMT23-β-catenin and GFP-αΔ-NLS. Cell extracts were prepared 48 hours post-transfection and probed for cyclin D1 protein by western blotting. Cells transfected with vector and GFP or with GFP-αΔ-NLS contained only basal levels of cyclin D1 (Figure 23b, lanes 1 and 3, respectively). Cells transfected with β-catenin had increased levels of cyclin D1, and this increase did not occur in cells transfected with β-catenin and GFP-αΔ-NLS (Figure 23b, lanes 2 and 4, respectively). These results indicate that GFP-αΔ-NLS is able to inhibit an endogenous target of β-catenin/Tcf signalling.

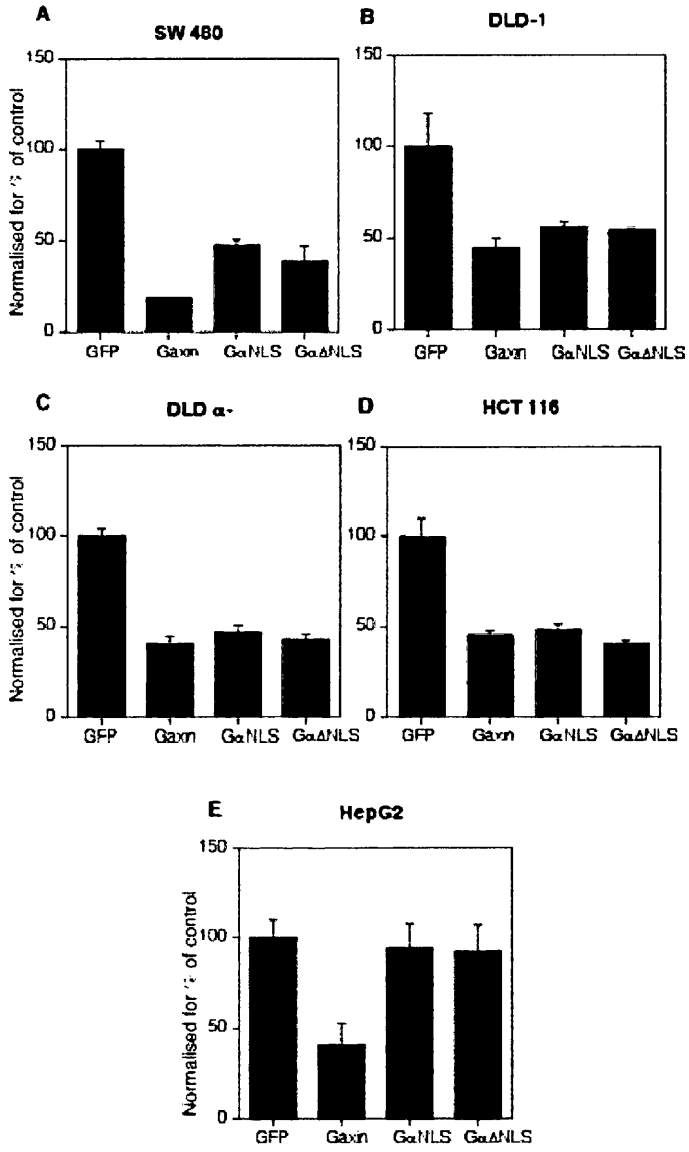


Figure 22.
Targeted expression of the amino-terminal domain of α-catenin in the nucleus represses endogenous β-catenin/Tcf transcriptional activity. (A) SW480, (B) DLD-1, (C) DLDa-, (D) HCT116 and (E) HepG2 cells were transfected with pTOPFlash, RSV-β-galactosidase and plasmids encoding the GFP fusion proteins indicated. Experiments were repeated at least three times. The values shown are the mean of a typical experiment, normalised for β-galactosidase activity, and with the mean of the control (GFP) set at 100%. Each transfection was done in triplicate, error bars show standard deviation.

IV.6- *Inhibition of colony formation by GFP-αΔ-NLS*

Since GFP-α-catenin constructs repressed β-catenin/Tcf signalling in the reporter assay and also inhibited cyclin D 1 expression, I sought to examine if this inhibition would correlate with an *in vivo* inhibition of cell growth. To do that I choose HCT116 cells, since it was already shown that repression of β-catenin/Tcf signalling in these cells, by a dominant negative form of Tcf, inhibits cell growth in a colony formation assay (Tetsu et al., 1999). Cells were transfected with GFP (positive control), GFP-axin (negative control), GFP-α-catenin or GFP-αΔ-NLS and then selected using neomycin sulphate. Untransfected cells died within 2 weeks of growing in selective media (Figure 24a shows representative plates after the colonies were stained). Cells transfected with GFP, on the other hand, grew and formed several colonies. Colonies bigger than 1 mm were counted and their number was set as 100% of control (Figure 24b). GFP-α-catenin and GFP-αΔ-NLS repressed growth by approximately 40%, which is comparable with their ability to repress β-catenin/Tcf signalling (Figure 22d) in this cells line. GFP-axin had a higher ability to inhibit growth of these cells, which was comparable to the inhibition obtained using the reporter plasmid as a measurement of β-catenin/Tcf signalling.

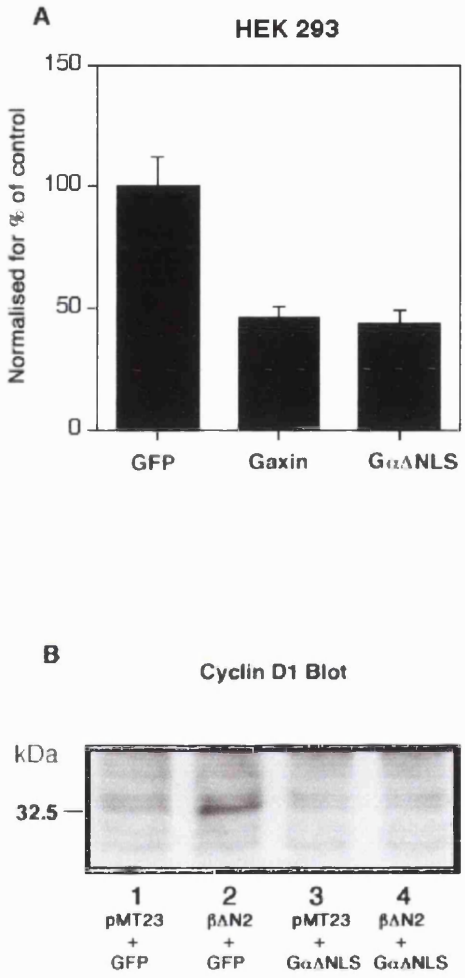


Figure 23.
Inhibition of an endogenous target of β-catenin signalling by GFP-αΔ-NLS.
(A) HEK 293 cells were transfected with cyclin D1 reporter plasmid, RSV-β-galactosidase, pMT23-β-catenin-ΔN2 and the GFP proteins indicated. Experiments were repeated three times and each transfection was done in triplicate. The values shown are the mean of a typical experiment, normalised for β-galactosidase activity, and with the mean of the control (GFP) set at 100%, error bars show standard deviation.
(B) Extracts of HEK 293 cells transfected with pMT23 and GFP (lane 1), pMT23-β-catenin-ΔN2 and GFP (lane 2), pMT23 and GFP-αΔ-NLS (lane 3) or pMT23-β-catenin-ΔN2 and GFP-αΔ-NLS (lane 4) were probed for cyclin D1. The position of the molecular weight marker (32.5 kDa) is indicated on the left.

IV.7- *Alpha-catenin forms a complex with β-catenin and Tcf family members*

The results obtained so far suggest that α-catenin inhibits β-catenin/Tcf signalling in the cell nucleus. One possibility is that α-catenin disrupts the β-catenin/Tcf complex in the nucleus. Alternatively, α-catenin may alter the interaction between the β-catenin/Tcf complex and its DNA target or the interaction between the β-catenin/Tcf complex and other transcription factors, such as the components of the basal transcription machinery (Hecht et al., 1999).

In order to distinguish these possibilities, I first determined whether α-catenin could form a stable complex with β-catenin and Tcf-1. Neuro-2A cells were chosen for these experiments because they express low levels of endogenous catenins. These cells were transfected with β-catenin and α-catenin in the presence of two different Tcf-1 isoforms, either p45, which can interact with β-catenin or p33, which lacks the β-catenin binding site. As a control, α-catenin was transfected with p45 Tcf-1 in the absence of β-catenin. Cells were lysed in NP-40 buffer to prevent loss of interactions between α-catenin and β-catenin. The lysates were then immunoprecipitated with an antibody against α-catenin and probed with a Tcf-1 antibody. Similar amounts of p45 Tcf-1 (Figure 25a, lane 1) and p33 Tcf-1 (Figure 25a, lane 3) were detected in the total extracts of transfected Neuro-2A cells. Alpha-catenin was unable to associate with p45 Tcf-1 in the absence of β-catenin (Figure 25a, lane 5). In addition, α-catenin did not associate with p33 Tcf-1, even in the presence of β-catenin (Figure 25a, lane 6). Alpha-catenin co-immunoprecipitated with p45 Tcf-1 when β-catenin was co-expressed in Neuro-2A cells, indicating that the three proteins can form a complex (Figure 25a, lane 4). These results suggest that the β-

catenin/Tcf complex is not disrupted by α-catenin. In order to determine if an endogenous complex containing α-catenin, β-catenin and Tcf could be detected, similar experiments were performed using SW480 cells. Nuclear extracts from SW480 cells were prepared and subjected to immunoprecipitation with β-catenin, cadherin or α-catenin antibodies and then blotted for Tcf-4. SW480 cells express two different Tcf-4 isoforms, Tcf-4B and Tcf-4E (Figure 25b, lane 1; Korinek et al., 1997). Both of these isoforms associated with β-catenin (Figure 25b, lane 2), but not with cadherin (Figure 26b, lane 3). Alpha-catenin immunoprecipitates contained both Tcf-4B and Tcf-4E, indicating that a stable nuclear complex containing endogenous α-catenin, β-catenin and Tcf is present in SW480 cells (Figure 25b, lane 4).

IV.8- ***Alpha-catenin disrupts the β-catenin/Tcf/DNA complex***

In order to determine whether α-catenin interferes with the interaction between the β-catenin/Tcf complex and its DNA target, I performed DNA band shift assays. A radiolabelled oligonucleotide containing a Tcf binding site was used as a probe and an oligonucleotide with a mutation in this site was used as a control. When cell extracts from Neuro-2A cells transfected with p45 Tcf-1 were incubated with the Tcf probe, one major protein-DNA complex was detected (Figure 26, lane 3). The migration of this complex could be further retarded by addition of a Tcf-1 antibody, indicating that it contains Tcf-1 (Figure 26, lane 4). Addition of purified β-catenin reduced the amount of the Tcf-1/DNA complex and created a second, slower migrating complex (Figure 26, lane 5). This second complex is likely to be formed by β-catenin, Tcf-1 and DNA since it was supershifted by addition of a β-catenin antibody (Figure 26, lane 6). A control GFP monoclonal antibody did not interfere with the migration of

either complex (Figure 26, lane 10). Addition of purified GST-α-catenin abolished formation of the β-catenin/Tcf-1/DNAcomplex (Figure 26, lane 7) but did not interfere with the Tcf-1/DNA complex. To identify which domain of α-catenin is responsible for this effect, two different regions of this protein fused to GST were used in the band-shift assay. The first 228 amino acids of α-catenin, which comprise the β-catenin binding domain, had the same effect as full-length α-catenin, disrupting the β-catenin/Tcf-1/DNA complex without interfering with the Tcf-1/DNA complex (Figure 26, lane 9). On the other hand, a fusion protein composed of the 448 carboxyl-terminal amino acids of α-catenin fused to GST had no effect on the interaction of either complex (Figure 26, lane 8). Together, these results validate the possibility that α-catenin prevents the association of β-catenin/Tcf-1 with DNA. Furthermore, these results indicate that the amino-terminal domain of α-catenin is sufficient to disrupt the β-catenin/Tcf-1/DNA complex, suggesting a mechanism for its ability to inhibit β-catenin dependent transcription.

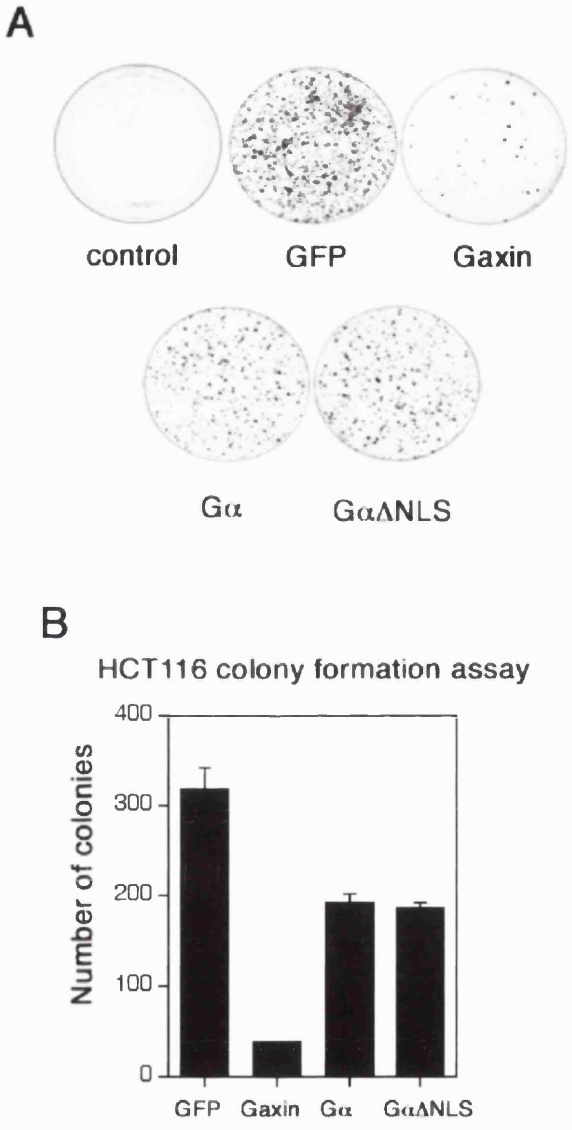


Figure 24.
GFP-α-catenin, GFP-α-NLS and GFP-αΔ-NLS inhibit HCT116 cell colony formation.
(A) HCT116 cells were transfected with the indicated GFP constructs and allowed to grow under neomycin selection. After 2 weeks colonies were stained and counted.
(B) Quantification of the colony growth assay. Experiments were done twice in duplicate, error bars show standard deviation.

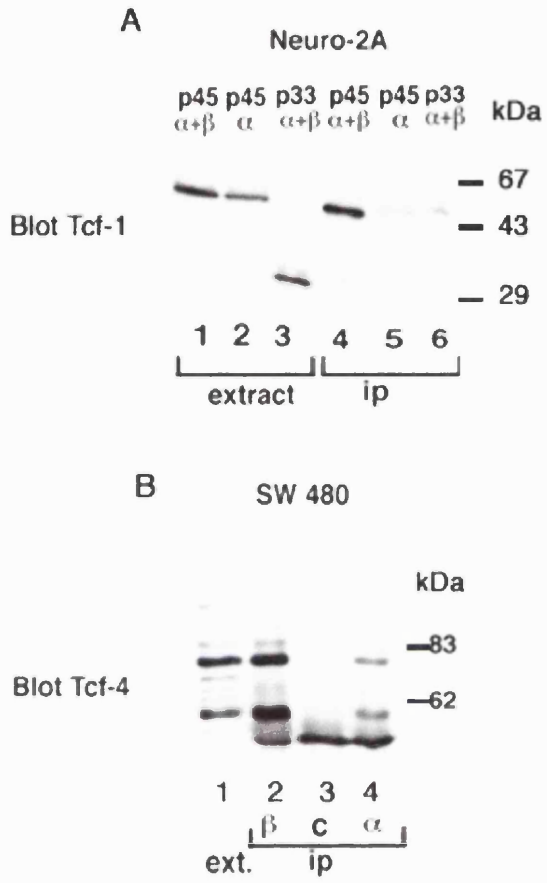


Figure 25. Alpha-catenin does not disrupt the β-catenin/Tcf complex. (A) NP-40 extracts from Neuro-2A cells transfected with p45 Tcf-1 and GFP-α-catenin (lanes 2 and 5), p45 Tcf-1, GFP-α-catenin and GFP-β-catenin (lanes 1 and 4) or p33 Tcf-1, GFP-α-catenin and GFP-β-catenin (lanes 3 and 6) were probed with an anti-Tcf-1 mAb. Total extracts in lanes 1, 2 and 3 and α-catenin immunoprecipitates in lanes 4, 5 and 6.
(B) Nuclear extracts from SW480 cells were immunoprecipitated with the following antibodies: anti-β-catenin (β), anti-cadherin (c) and anti-α-catenin (α) and probed with an anti-Tcf-4 mAb. Total extract is in lane 1 and immunoprecipitates are in lanes 2, 3 and 4. The lines on the right indicate positions of molecular weight markers.

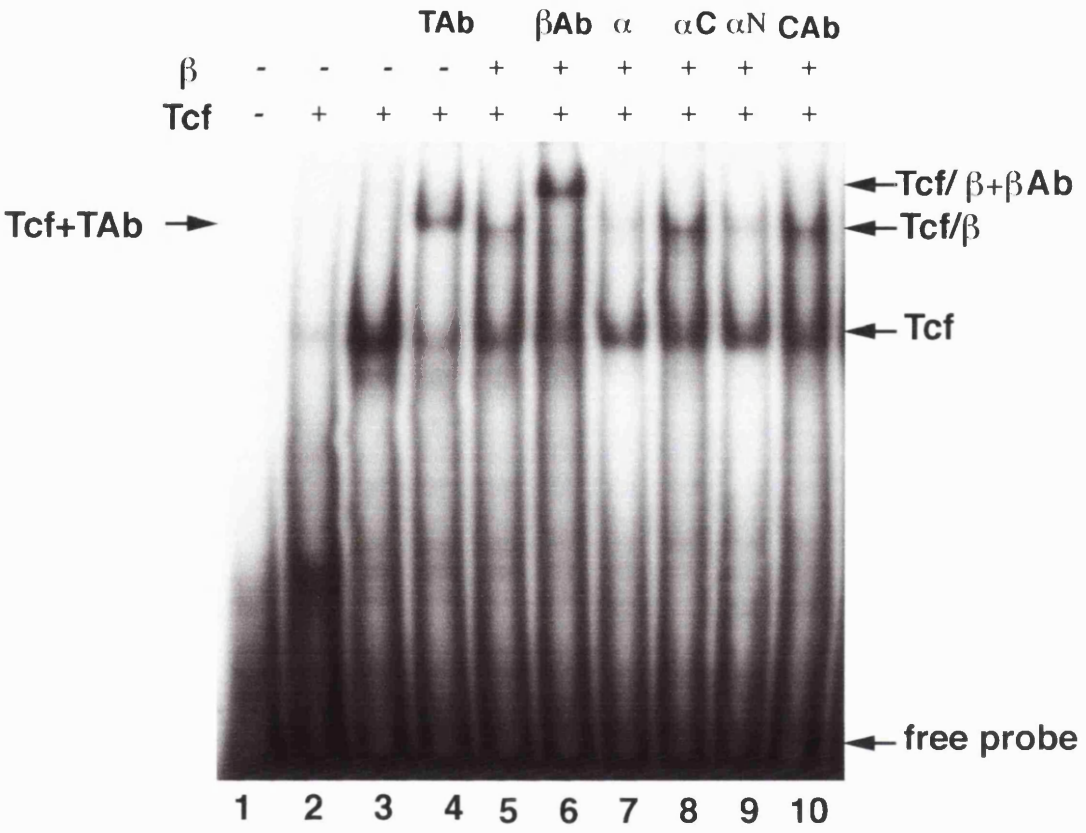


Figure 26.
Alpha-catenin blocks the interaction between the β-catenin/Tcf complex and DNA. Gel retardation assays were conducted using a double-stranded oligonucleotide probe containing a Tcf-binding site (the probe used in lane 2 has a mutation in the Tcf-binding motif) and extracts from Neuro-2A cells transfected with p45 Tcf-1 (lanes 2-10). Lane 1 contains free probe only. The following proteins were incubated with cell extracts: anti-Tcf-1 mAb (TAb, lane 4), β-catenin (lanes 5-10), anti-β-catenin mAb (βAb, lane 6), GST-α-catenin (α, lane 7), GST-α C-447(αC, lane 8), GST-α N-228 (αN, lane 9) and anti-GFP mAb as a control antibody (Cab, lane 10). Arrows on the left and right of the figure indicate the positions of DNA/protein complexes.

IV.9- *Discussion*

The tumour suppressor function of α-catenin is believed to be related to its role in cell-cell adhesion. However, based on the results presented here, it is possible that α-catenin also regulates tumour progression via the pool of β-catenin involved in signalling. The results presented in this Chapter and in Chapter III suggest that the nuclear localisation of α-catenin is linked, at least in part, to the localisation of β-catenin. Moreover, they suggest a role for α-catenin in the nucleus.

First, the presence of endogenous and ectopically expressed α-catenin in the nuclei of cells that have high levels of nuclear β-catenin, such as SW480 and DLD-1 cells, indicates that α-catenin can be transported to the nucleus, most likely through its association with the β-catenin/Tcf complex. It is possible that β-catenin, when unregulated either by Wnt signals or by defects in the degradation pathway, functions as a chaperone, taking α-catenin into the nucleus. There is evidence suggesting that Armadillo functions as a chaperone for the transcription factor Teashirt, which is found in the nuclei, but does not have a NLS (Gallet et al., 1998 and 1999). Beta-catenin/Tcf complexes that are not associated with endogenous α-catenin could transport overexpressed GFP-α-catenin into the nucleus. This may explain the presence of exogenous α-catenin in the nuclei of some colon cancer cells, but not others. This model however, fails to explain why α-catenin is not nuclear in HCT116 cells, which contain nuclear β-catenin. The reason for the difference in localisation of α-catenin among the colon cancer cell lines examined is unknown at present, but it suggests that additional mechanisms exist to regulate α-catenin localisation. However, it is not a result of different mutations present in these cell lines since α-catenin is also excluded from the nucleus in Caco-2

cells, which express a mutant APC similar to the APC mutants expressed in SW480 and DLD-1 cells (results shown by others in the laboratory). Alpha-catenin and β-catenin cellular localisation might be a consequence of the levels of these proteins in the cells and also the levels of associating proteins, such as APC, Tcf-family members and cytoskeletal components. In addition, Wnt signalling has been implicated in actin re-organisation, and it is possible that disorganisation of actin fibres might release α-catenin, allowing it to enter the nucleus (Shibamoto et al., 1998; Torres and Nelson, 2000).

The lack of α-catenin in the DLDα- cells correlates with higher β-catenin signalling activity. This supports a role for endogenous α-catenin in the regulation of β-catenin-dependent transcription. In DLDα- cells β-catenin and cadherin still form a complex (Figure 18c), discarding the possibility that the β-catenin signalling activity is higher in these cells because of a change in the interaction between β-catenin and cadherin, which could result in higher levels of nuclear β-catenin. The fact that DLDα+ cells also had lower β-catenin-dependent transcriptional activity than DLDα- cells suggests that the higher activity measured in the latter, does not correlate with the cell morphology, since both DLDα+ and DLDα- cells have a similar disorganised morphology, being unable to form a cobblestone monolayer. Taken together, these results indicate that endogenous α-catenin can inhibit endogenous β-catenin signalling.

Despite the fact that DLDα+ cells express α-catenin, they have altered cell-cell adhesion, and do not form a cobblestone monolayer. Although I have not investigated the reasons for this, experiments in our laboratory indicate that DLDα+ cells express a slower migrating isoform of p120. Since this protein has been shown to bind to the juxtamembrane

region of cadherins and is involved in the modulation of cell-cell adhesion, it is possible that the isoform expressed by DLDα+ accounts for their altered cell-cell adhesion (Ohkubo and Osawa, 1999).

The evidence for a role of nuclear α-catenin is strengthened by the use of a nuclear-targeted form of α-catenin, which inhibited β-catenin signalling to the same extent as non-targeted α-catenin in all the cell lines tested. The extent of repression by the GFP-α-catenin constructs was greater in DLDα- cells than in DLDα+ cells. This might be because the β-catenin/Tcf complex is partially inhibited by endogenous α-catenin in the latter cell type. In addition, the ability of GFP-αΔ-NLS to inhibit signalling in α-catenin negative cells indicates that the mechanism of inhibition does not involve interactions between exogenous and endogenous α-catenin.

Cytoskeletal proteins that have been shown to interact with α-catenin do not seem to be necessary for the repression of β-catenin signalling, since the G-αΔ-NLS, which lacks binding sites for most of these proteins, still repressed β-catenin signalling. This was shown to be true both for transfected COS-7 cells and for colon cancer cell lines using the TOP/FOPFlash reporter assays. Moreover, GFP-αΔ-NLS inhibited β-catenin-dependent transcription in the cyclin D1 reporter assay and it repressed β-catenin-induced expression of the cyclin D1 protein. Alpha-catenin contains two domains that bind actin *in vitro,* a major site in the carboxyl-terminal domain and a second site close to the β-catenin-binding site (Rimm et al., 1995). Although GFP-αΔ-NLS does not contain the major site, it retains the second actin-binding site. It thus remains possible that the effects of α-catenin on β-catenin-dependent transcription involve actin. Actin is normally rapidly exported from the nucleus, but actin mutants that accumulate in the nucleus inhibit cell proliferation (Wada et al., 1998).

Furthermore, it has been shown that actin is involved in serum induction of SRF (serum responsive factor) target genes (Sotiropoulos et al., 1999).

The mechanism by which α-catenin inhibits β-catenin signalling does not involve disruption of the complex formed by β-catenin and Tcf, since it was possible to co-immunoprecipitate these three proteins both from transfected Neuro-2A cells and from nuclear extracts of SW480 cells. Although the Tcf-4 isoforms expressed by SW480 cells were present in both the β-catenin and α-catenin immunoprecipitates, the amounts of both these isoforms co-immunoprecipitated with β-catenin were higher than the amounts co-immunoprecipitated with α-catenin (Figure 25b, compare lanes 1 and 4). Therefore, SW480 cells most likely have two pools of β-catenin/Tcf complex: one pool is bound to α-catenin and is inactive and the other pool is α-catenin-free and active. This would explain how these cells maintain high β-catenin signalling, despite the presence of α-catenin in their nuclei. The results shown so far suggest that the relative levels of β-catenin/Tcf and α-catenin/β-catenin/Tcf complexes in the nucleus will determine the overall transcription activity. Thus, in SW480 cells, where the amount of β-catenin far exceeds that of α-catenin, one would predict that endogenous nuclear α-catenin would not significantly affect transcription. In contrast, in DLD-1 cells, which contain lower levels of β-catenin than SW480 cells (Figure 18a), one would predict that loss of α-catenin would result in higher β-catenin/Tcf-dependent transcription activity. This appears to be the case, since in DLDα- cells β-catenin/Tcf activity is 30% higher than in the α-catenin expressing DLD-1 cells.

Although endogenous β-catenin, Tcf and α-catenin can interact, the presence of α-catenin perturbs the association of β-catenin/Tcf complex with DNA, as shown by gel-shift experiments. The amino terminus of α-

catenin, which contains the β-catenin binding domain, is sufficient to prevent the interactions between the β-catenin/Tcf complex and DNA. Since this truncated α-catenin does not contain the binding domains for other cytoskeletal proteins, we can rule out the involvement of vinculin, α-actinin and ZO-1 in this process. However, it remains possible that actin is somehow involved in this inhibition, as discussed above. The effects of α-catenin on the disruption of the β-catenin/Tcf-1/DNA *in vivo* complex may also involve displacement of other proteins involved in transcription.

While GFP-α-catenin was found in the nucleus of SW480 and DLD-1 cells, this was not the case in HCT116 cells, where only 30% of transfected GFP-α-catenin was nuclear. This suggests that the 50% inhibition of β-catenin signalling by GFP-α-catenin in HCT116 cells may be mediated, in part, by sequestration of β-catenin in the cytoplasm.

Using a colony formation assay as another way of measuring the inhibitory effects of α-catenin on β-catenin signalling, I showed that the inhibition of colony formation by both GFP-α-catenin and GFP-αΔ-NLS seems to correlate with the repression obtained using the reporter assay as a measure of β-catenin signalling. Both GFP-α-catenin and GFP-αΔ-NLS inhibited the number of colonies exceeding 1 mm, possibly due to arrest of cell growth. In addition, when dominant-negative Tcf was used in the colony formation assay, the authors report the same number of colonies in the plates of cells infected with dominant-negative Tcf or control DNA. However, the colonies in the dominant-negative Tcf plate were significant smaller when compare to the ones in the control plate, suggesting that cell viability was not being affected (Tetsu and McCormick, 1999).

CHAPTER V- *Nuclear export of α-catenin*

V.1- *Introduction*

The correct localisation of β-catenin is vital for its dual function in the membrane and in the nucleus. However, as I have shown, although necessary, the nuclear localisation of β-catenin per se is not sufficient for its function in promoting transcription.

Alpha-catenin can regulate β-catenin in two ways. First, in the absence of Tcf, α-catenin sequesters β-catenin in the cytoplasm. Second, in the presence of Tcf, α-catenin disrupts the β-catenin/Tcf/DNAcomplex in the nucleus.

Recent work indicates that β-catenin nuclear localisation can be regulated by APC. Thus, APC is not only involved in the sequestration and degradation of β-catenin, but may also contribute to the export of β-catenin from the nucleus. APC contains both nuclear localisation signals (NLS) and nuclear export signals (NES). A canonical NES, which is required for CRM-1-dependent export of most proteins, contains the consensus sequence $LX_{(1-3)}$ $LX_{(2-3)}$ LXL, where L (leucine) can be substituted by other large hydrophobic residues and X is any amino acid (Nix and Beckerle, 1997; Figure 27a). Although some reports suggest that APC exports β-catenin from the nucleus (Neufeld et al; 2000; Rosin-Arbesfeld et al.; 2000; Henderson; 2000), others suggest that β-catenin moves in and out of the nucleus independently of CRM-1 (Fagotto et al., 1998; Yokoya et al., 1999; Wiechens and Fagotto, 2001). In this chapter I describe work in which I have investigated whether α-catenin nuclear localisation is regulated and whether this might provide an additional

way to regulate the localisation or function of β-catenin.

V.2- *Alpha-catenin contains nuclear export signals*

Comparative analysis of α-catenin and αN-catenin sequences from different species revealed two putative nuclear export signals, which I have called NES1 and NES2. These sequences are conserved in both α-catenin and αN-catenin and also in α-catenin from different species (Figure 27b). Moreover, these sequences are not present in vinculin.

To assess if the putative NES signals in α-catenin could promote nuclear export *in vivo,* oligonucleotides encoding each one, were cloned into the Rev(1.4)-GFP plasmid. This plasmid encodes a Rev-GFP fusion protein that lacks a functional NES and has been used by others to evaluate nuclear export activities of diverse NES (Henderson and Eleftheriou, 2000). As a positive control, I used Rev-GFP with a wild-type NES. In the majority of transfected COS-7 cells, Rev-GFP is found both in the nucleus and in the cytoplasm, owing to the presence of both a NLS and a NES (Figure 28a). Treatment of cells with Actinomycin D inhibits nucleolar association of Rev, thereby preventing its retention in the nucleus and thus indirectly promoting export. As a result, the number of cells with Rev-GFP both in the nucleus and cytoplasm is reduced, and in many cells Rev-GFP is exclusively cytoplasmic.

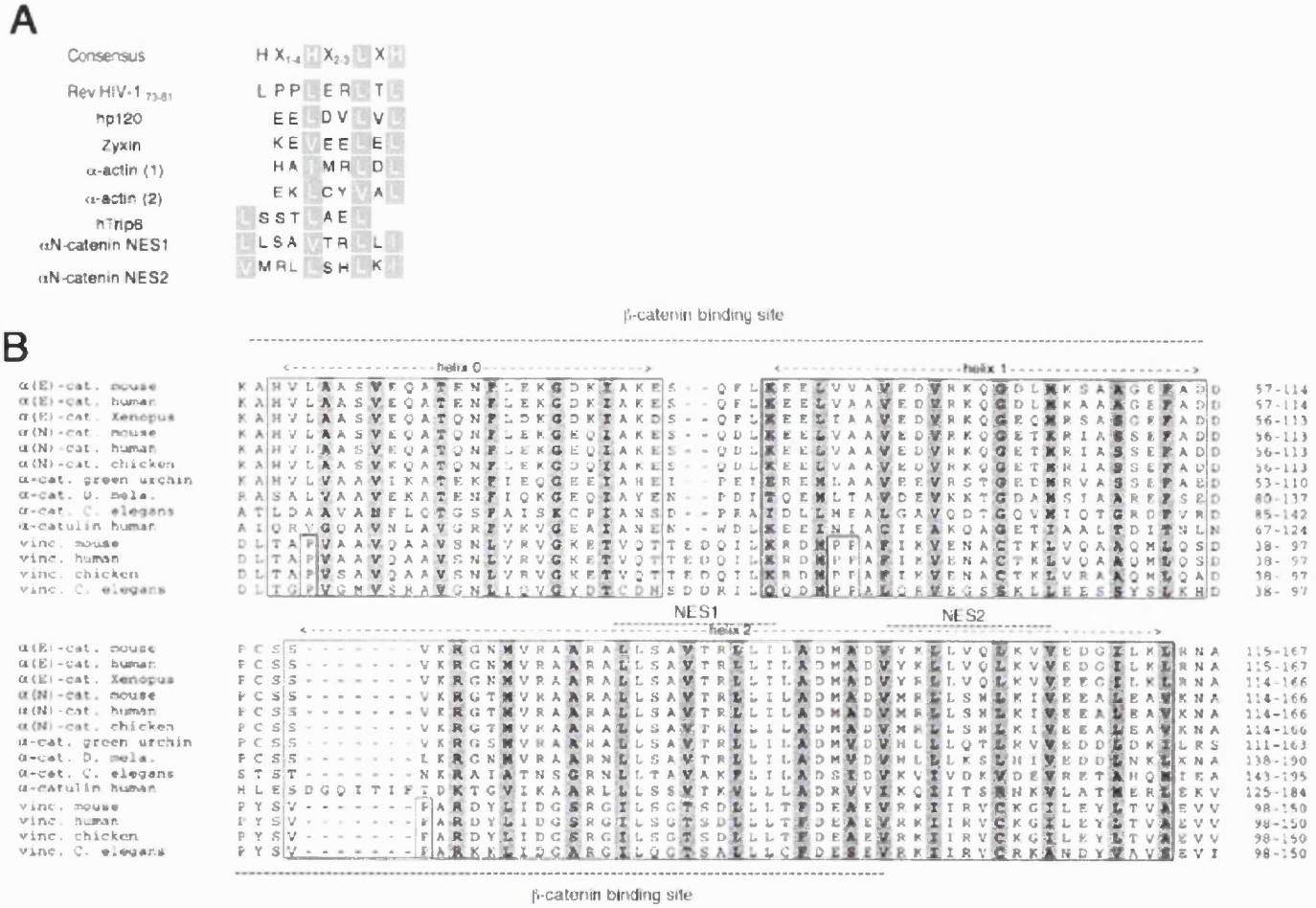


Figure 27.
Two putative NES sequences are present in α-catenin.
(A) Consensus sequence for NES present in other proteins mentioned in the text. In the consensus NES, H is large hydrophobic residue, such as leucine, valine or isoleucine; L is leucine and X is any amino acid.
(B) Structure-based sequence alignment of α-catenin, α-catulin and vinculin. Numbers on the right indicate amino acid positions; secondary structure elements based on the β/α-catenin structure are indicated at the top and by the boxed areas, where the hydrophobic core residues are shaded in light grey (Pokutta and Weiss, 2000). NES1 and 2 and β-catenin binding domain are indicated.

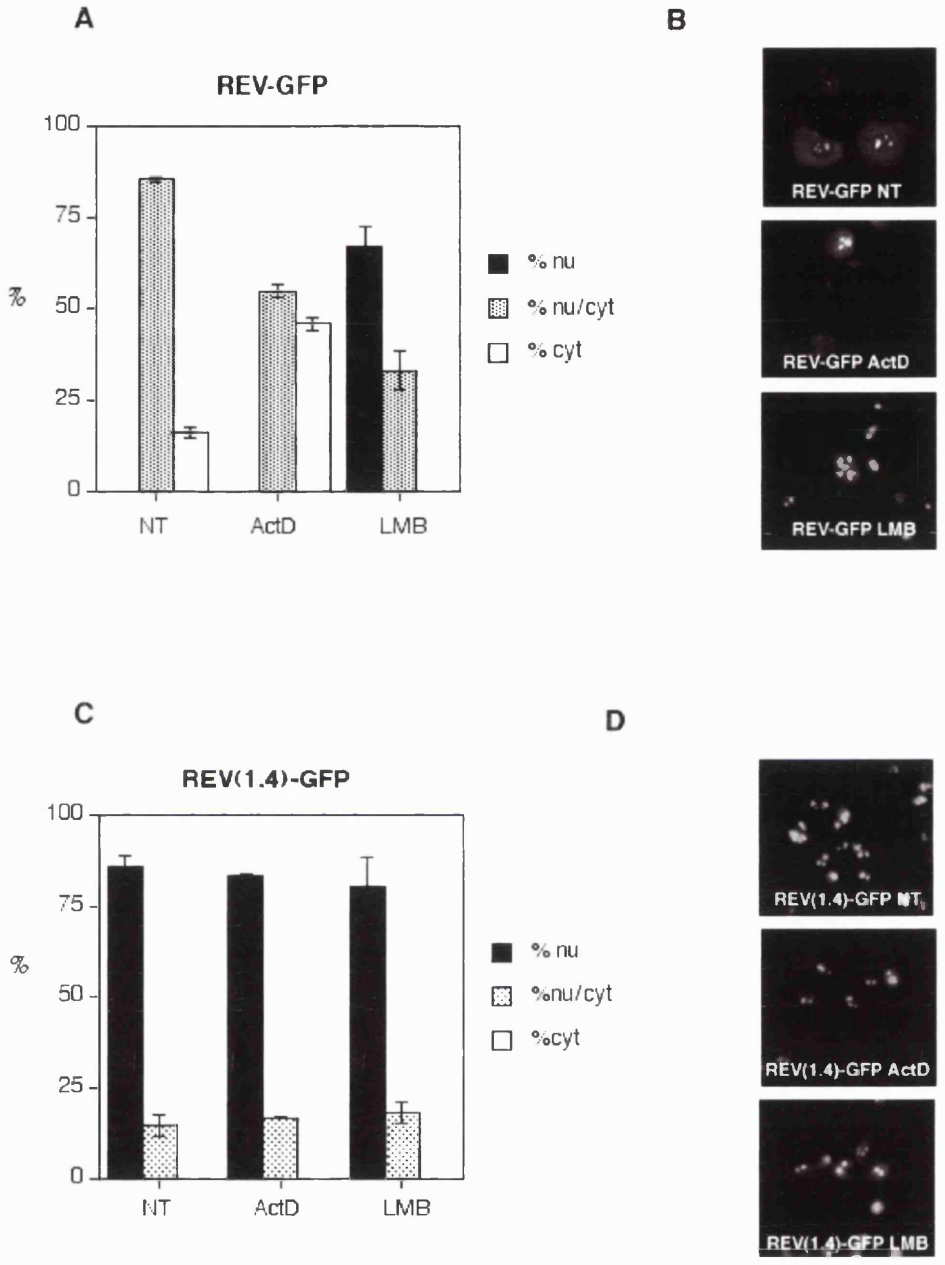


Figure 28.
Nuclear export assay (i) Controls.
Cells transfected with Rev-GFP (A and B) or Rev(1.4)-GFP (C and D) were treated with cycloheximide at 15 μg/ml for 3 hours, either untreated (NT), in the presence Actinomycin D (5 μg/ml) (ActD) or LMB (10 ng/ml). (A and C) Quantification of the cellular localisation of each construct after drug treatment. Each experiment was done at least three times and approximately 500 cells were counted for each condition. Error bars indicate standard deviations. (B and D) Representative images of the cells under each indicated treatment.

Treatment of cells with Leptomycin B (LMB) causes a dramatic change in the distribution of Rev-GFP, and 3 hours after treatment, Rev-GFP is exclusively nuclear in the majority of the cells. Representative images of cells expressing Rev-GFP after each treatment are shown in Figure 28b. The drugs do not affect the localisation of Rev(1.4)-GFP plasmid, which remains mainly nuclear in the transfected cells (Figure 28 c and d).

When NES1 was tested in the nuclear export assay (Rev-α-NES1) it was exclusively nuclear in 35% of the cells (Figure 29a), in the remaining cells, it was found both in the nucleus and cytoplasm. Treatment with Actinomycin D resulted in an increase in the number of cells where Rev-α-NES1 was exclusively cytoplasmic. This suggests that α-NES1 has weak export activity. Consistent with this, LMB did not affect the localisation of Rev-α-NES1, which remained predominantly nuclear. Representative images of cells expressing Rev-α-NES1 after each treatment are shown in Figure 29b.

When NES2 was tested is the nuclear export assay (Rev-α-NES2), its distribution was similar to that of Rev-GFP. In untreated cells, Rev-α-NES2 was found either both in the nucleus and cytoplasm (~75% of cells) or exclusively in the cytoplasm (Figure 29c). Treatment with Actinomycin D resulted in a shift to the cytoplasm in many of the cells. In addition, treatment with LMB resulted in a shift of Rev-α-NES2 to the nucleus in the majority of the cells (Figure 29c). These results indicate that the α-NES2 is a strong NES. Images of cells expressing Rev-α-NES2 after each treatment are shown in Figure 29d.

In order to confirm that α-NES2 is a canonical NES, I generated Rev-α-NES2mut, which contains a form of α-NES2 in which two key

residues in the predicted NES, have been mutated to alanine. These changes completely abrogated nuclear export activity NES2 (Figure 30a). Moreover, treatment with Actinomycin D or LMB did not affect the localisation of Rev-α-NES2mut. Indeed, Rev-α-NES2mut behaved similarly to Rev(1.4)-GFP, which contains a non-functional NES. Images of cells expressing Rev-α-NES2mut after each treatment are shown in Figure 30b.

The relative nuclear export activities of various NES from different proteins have been measured by Henderson and Eleftheriou (2000). In the scoring system described by these authors, the NES present in HIV-Rev protein receives a score of +7 (where the maximal score is +9). The score is based on the percentages of cells containing the Rev protein exclusively in the nucleus or in the nucleus and cytoplasm, and on the changes in these percentages after treatment with Actinomycin D. When I compared the export activities of α-NES1, α-NES2 and Rev-NES in my experiments, α-NES1 scores +3, while α-NES2 scores +7. As expected, Rev-α-NES2mut, similar to Rev(1.4)-GFP, scores 0.

V.3- ***Effects of LMB on the localisation of endogenous and exogenous α-catenin***

The results presented above suggest that at least one of the NES sequences found in α-catenin is a strong NES when assayed in the context of Rev-GFP. To investigate the possibility that α-catenin localisation is regulated by nuclear export, I examined the effect of LMB on the localisation of endogenous α-catenin in colon cancer cells.

First, DLD-1 cells were either untreated or treated with 10 ng/ml LMB for 16 hours, fixed and then stained for endogenous α-catenin and β-catenin (Figure 31). There was no detectable difference in the nuclear

levels of either α-catenin (compare Figures 31a and 31c) or β-catenin (compare Figures 31b and 31d) after LMB treatment. As a control, parallel DLD-1 cultures were stained for NFκB p65. NFκB/IκBα complexes shuttle between the cytoplasm and the nucleus owing to the presence of both a NLS and a CRM-1-dependent NES (Huang et al., 2000). Addition of LMB caused a complete shift of NFκB from the cytoplasm (Figure 31e) to the nucleus (Figure 31f), indicating that DLD-1 cells do respond to LMB. The lack of effect of LMB on α-catenin and β-catenin localisation may be because these proteins are already nuclear in these cells, making it difficult to detect an increase in nuclear localisation.

As shown in Chapter IV, HCT116 cells do not normally contain endogenous nuclear α-catenin (Figure 17d). Therefore I examined the effects of LMB on endogenous catenin localisation in HCT116 cells (Figure 32). However, LMB did not affect the localisation of α-catenin (compare Figures 32a and 32c), or β-catenin (compare Figures 32b and 32d) in HCT116 cells. This suggests that there are processes, other than nuclear export, controlling the localisation of the catenins in these cells. One possibility is that the catenins are sequestered either in the cytoplasm by the cytoskeleton or in the nucleus by transcription factors.

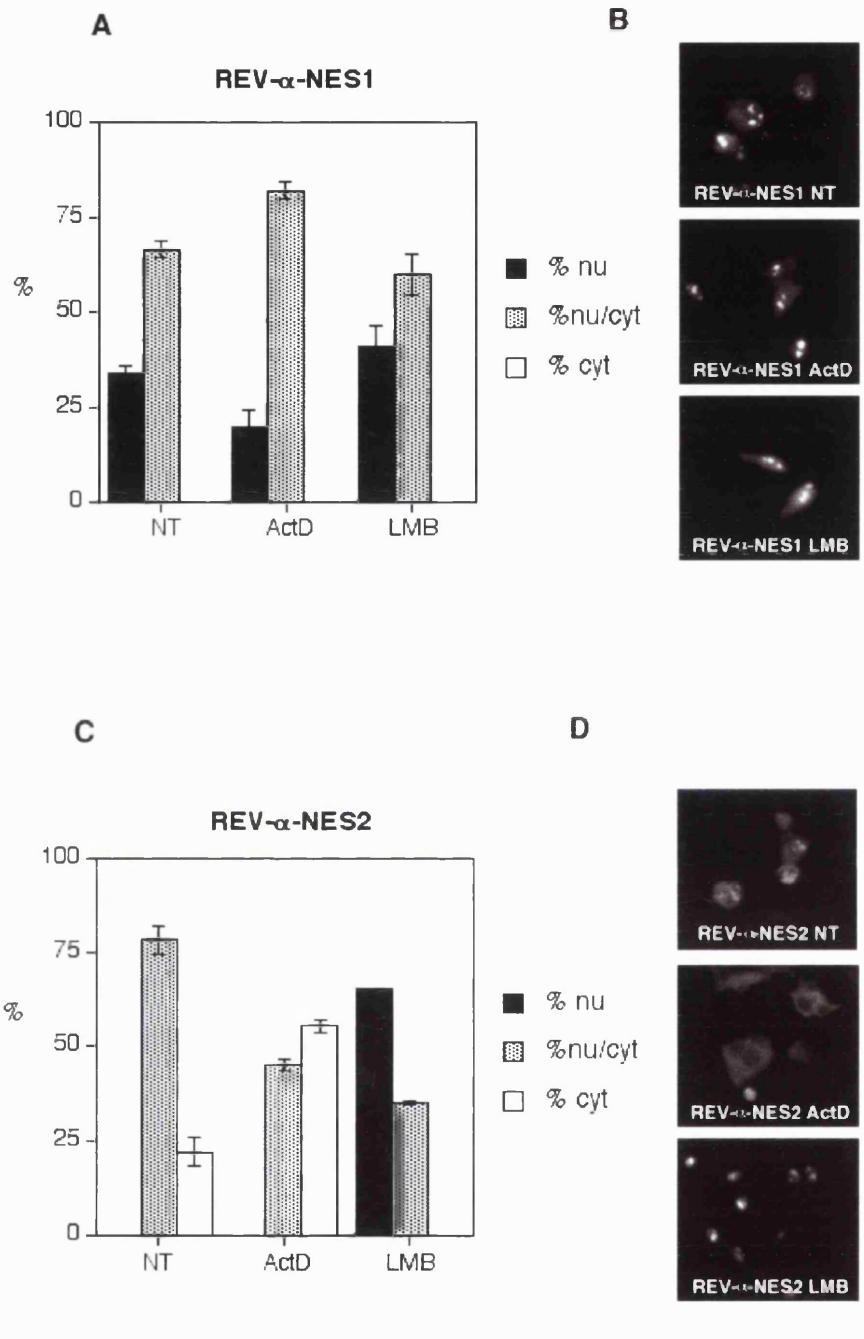


Figure 29.
Nuclear export assay (ii) Alpha-NES constructs.
Cells transfected with Rev-α-NES1 (A and B) or Rev-α-NES2 (C and D) were treated with cycloheximide at 15 μg/ml for 3 hours, either untreated (NT), in the presence Actinomycin D (5 μg/ml) (ActD) or LMB (10 ng/ml). (A and C) Quantification of the cellular localisation of each construct after drug treatment. Each experiment was done at least three times and approximately 500 cells were counted for each condition. Error bars indicate standard deviations. (B and D) Representative images of the cells under each indicated treatment.

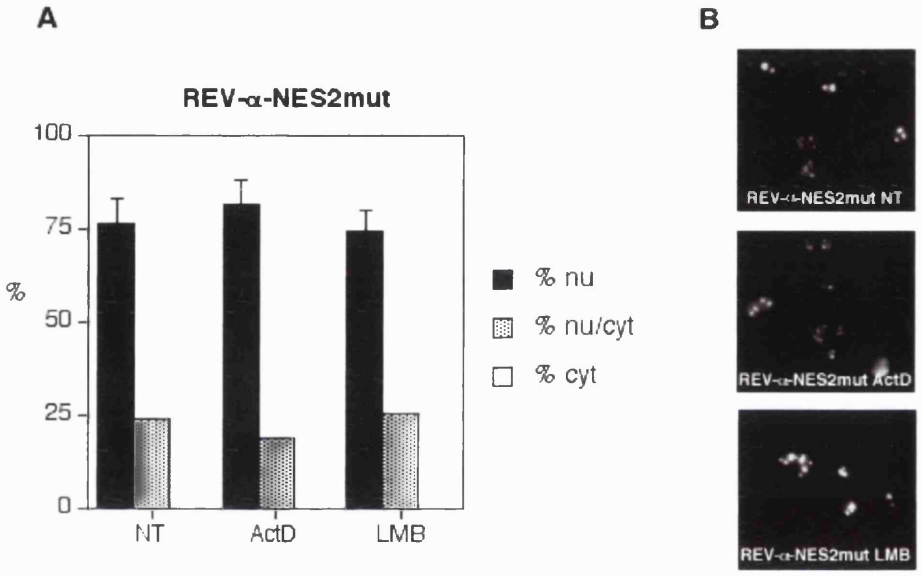


Figure 30.
Nuclear export assay (iii) Mutated α-NES2 construct.
Cells transfected with Rev-α-NES2mut were treated with cycloheximide at 15 μg/ml for 3 hours, either untreated (NT), in the presence Actinomycin D (5 μg/ml) (ActD) or LMB (10 ng/ml).
(A) Quantification of the cellular localisation of Rev-α-NES2mut after drug treatment.
Each experiment was done at least three times and approximately 500 cells were counted for each condition. Error bars indicate standard deviations.
(B) Representative images of the cells under each indicated treatment.

A sequestration mechanism may not be sufficient to maintain the localisation of exogenously expressed catenins, which may saturate such a system. Indeed, in HCT116 cells approximately 30% of the cells, which express GFP-α-catenin, do not exclude this protein from the nucleus (Figure 20 j). Therefore HCT116 cells were transfected with GFP-α-catenin and allowed to express this protein for 24 hours. These cells were then treated for 3 hours with cycloheximide, which blocked further protein synthesis, either in the presence or absence of LMB (Figure 33). Under these conditions, LMB caused a significant increase in the number of cells with nuclear GFP-α-catenin (Figure 33c). A representative image of the transfected cells after each treatment is shown in Figures 33a and 33b. Thus, ectopically expressed GFP-α-catenin localisation is, in part, determined by nuclear β-catenin and in part by CRM-1-dependent nuclear export. Since GFP-α-catenin did not shift from the cytoplasm to the nucleus in all LMB-treated cells, additional mechanisms may exist to control α-catenin localisation.

V.4- ***Mutagenesis of the putative NES in GFP-α-catenin***

In order to determine whether NES1 or NES2 were involved in the export of GFP-α-catenin, they were mutated, and the mutant constructs were expressed in HCT116 cells. Western blotting of cell extracts (Figure 34a) indicated that GFP-α-NES1mut (lane 4) and GFP-α-NES2mut (lane 5) were expressed to the same level as GFP-α-catenin (lane 2). The ability of these forms of GFP-α-catenin to bind to β-catenin was also examined.

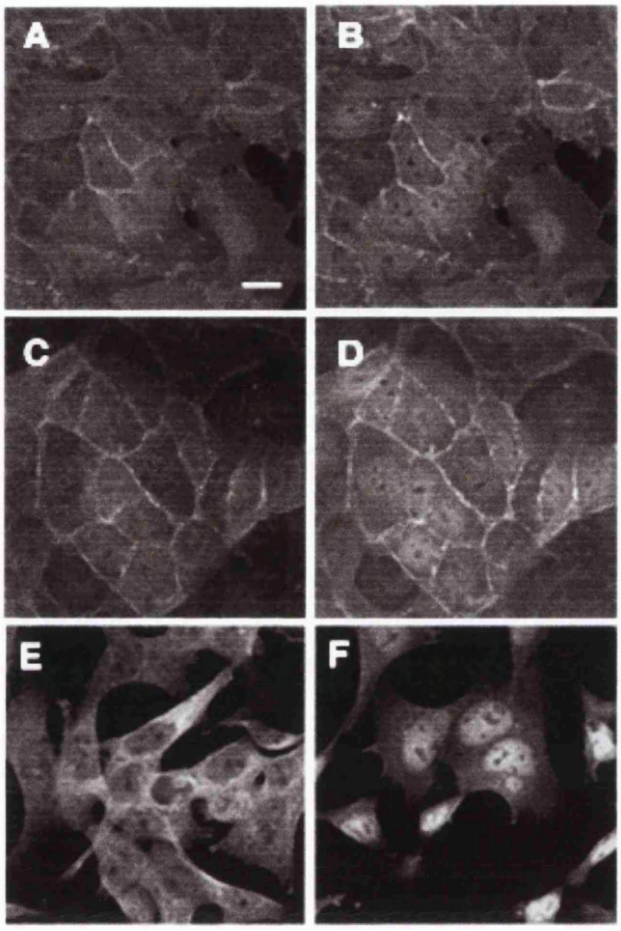


Figure 31.
Effect of LMB on the localisation of endogenous α-catenin and β-catenin on DLD-1 cells.
DLD-1 cells treated with LMB at 10 ng/ml for 16 hours were stained for endogenous α-catenin (A and C), endogenous β-catenin (B and D) or NFκB (E and F). (A, B and E) untreated cells, (C, D and F) LMB treated cells. Scale bar 7 μm.

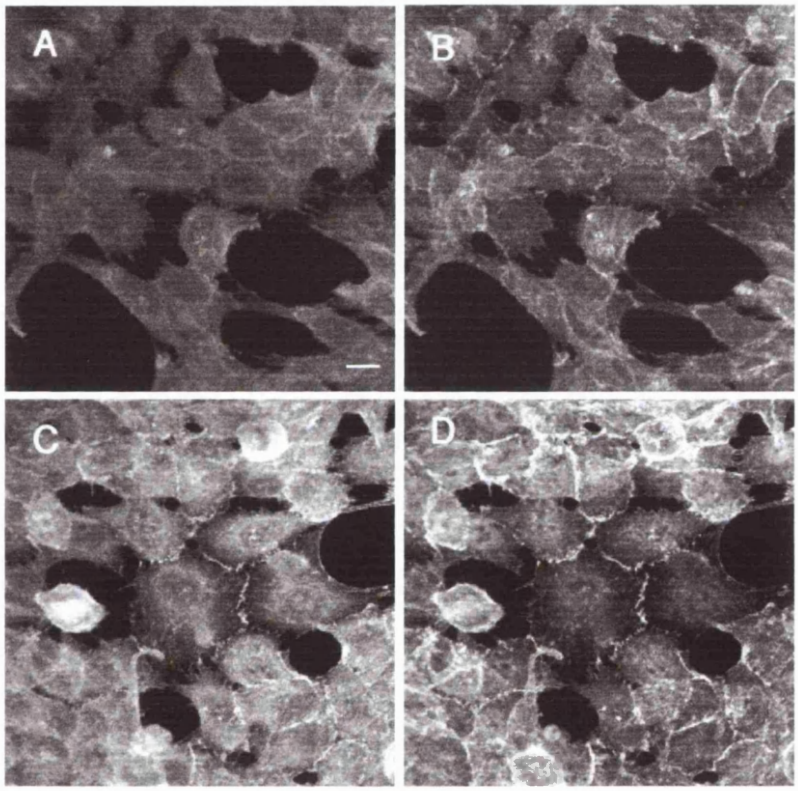


Figure 32.
Effect of LMB on the localisation of endogenous α-catenin and β-catenin on HCT116 cells. HCT116 cells treated with LMB at 10 ng/ml for 16 hours were stained for endogenous α-catenin (A and C) or endogenous β-catenin (B and D). (A and B) untreated cells, (C and D) LMB treated cells. Scale bar 7 μm.

This was important because, to enter the nucleus, α-catenin must associate with β-catenin. Since both NES1 and NES2 lie within the β-catenin-binding domain of α-catenin, their mutation may interfere with this binding. To do this, NP-40 extracts from COS-7 cells expressing myc-tagged β-catenin and the mutant GFP-α-catenin fusion proteins were prepared. Anti-GFP immunoprecipitates were probed for β-catenin and then reprobed for GFP, and the intensities of the bands corresponding to GFP-α-catenin fusion proteins and co-immunoprecipitated β-catenin were quantified by densitometry. This provided an estimate of the affinity of each construct for β-catenin. GFP-α-catenin binding to β-catenin was set at 100% (Figure 34b, lane 1).

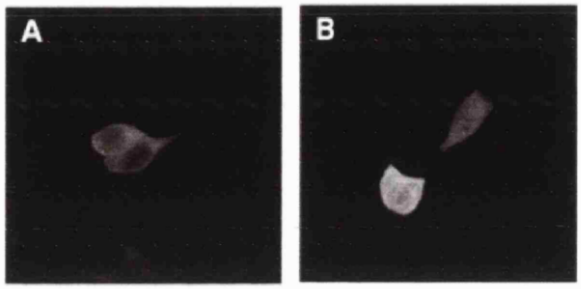
As a control I used GFP-αβmut, a form of GFP-α-catenin that contains mutations at residues known to be involved in β-catenin binding. GFP-αβmut bound β-catenin less efficiently. The binding ranging from 8 to 20% in the immunoprecipitation assays (Figure 34b, lane 2). GFP-α-NES1mut and GFP-α-NES2mut bound 50% and 70% of β-catenin, respectively. Therefore, these mutations in the NES sequences do interfere with binding of GFP-α-catenin to β-catenin.

Next, the localisation of the mutant GFP-α-catenin proteins was examined in HCT116 cells. Cells were scored for the presence of the fusion proteins exclusively in the cytoplasm or both in the cytoplasm and in the nucleus. A representative image of the localisation of each construct is shown in Figure 35a. The results, which are quantified in Figure 35b, show that GFP-α-catenin was excluded from the nucleus in approximately 70% of the cells. GFP-αβmut was excluded from the nucleus in a higher proportion of the cells, most likely because it has a lower affinity for β-catenin and as a result, enters the nucleus less efficiently. Mutation of

NES1 did not significantly affect the proportion of cells with GFP-α-catenin in the nucleus. Mutation of NES2, however, did affect the localisation of GFP-α-catenin, resulting in a 22% reduction in the number of cells with GFP-α-NES2mut in the cytoplasm, suggesting that NES2 promotes export of GFP-α-catenin. To summarise, this mutational analysis suggests that the localisation of GFP-α-catenin in HCT116 cells is controlled, in part, by nuclear export mediated by NES2, but not through NES1.

V.5-*Inhibition of β-catenin signalling by α-catenin NES mutants*

If the α-catenin inhibits β-catenin signalling in the nucleus, GFP-α-NES2mut would be predicted to be a better repressor of β-catenin signalling than GFP-α-catenin in HCT116 cells. To test this possibility, β-catenin/Tcf transcription assays were performed using GFP, GFP-α-catenin, GFP-αβmut, GFP-α-NES1mut or GFP-α-NES2mut (Figure 36). Normalised luciferase activity in cells expressing GFP was set at 100%. GFP-α-catenin reduced β-catenin-dependent transcription by 55%. GFP-αβmut reduced transcription by only 15%, confirming that an intact β-catenin binding site is necessary for repression of transcription. GFP-α-NES1mut reduced transcription to a similar extent (~18%) as GFP-αβmut, also reflecting the lower affinity of this construct for β-catenin. In contrast, despite its lower affinity for β-catenin, GFP-α-NES2mut reduced β-catenin-dependent transcription to a similar extent as GFP-α-catenin.



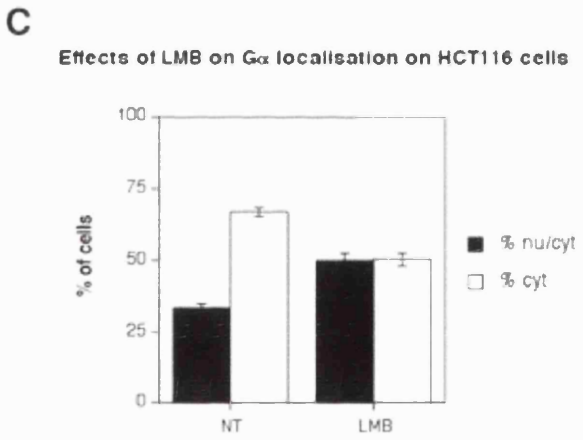


Figure 33.
Effect of LMB on the localisation of GFP-α-catenin on HCT116 cells. HCT116 cells were transfected with GFP-α-catenin and, after 24 hours, were treated with cycloheximide at 15 μg/ml for 3 hours in the absence (A) or presence (B) of LMB at 10 ng/ml, and then visualised by fluorescence microscopy. Scale bar 7 μm.
(C) Quantification of GFP-α-catenin cellular localisation before and after LMB treatment in HCT116 cells. The graph represents the mean values from three different experiments. Approximately 500 cells were counted for each condition. Error bars indicate standard deviations.

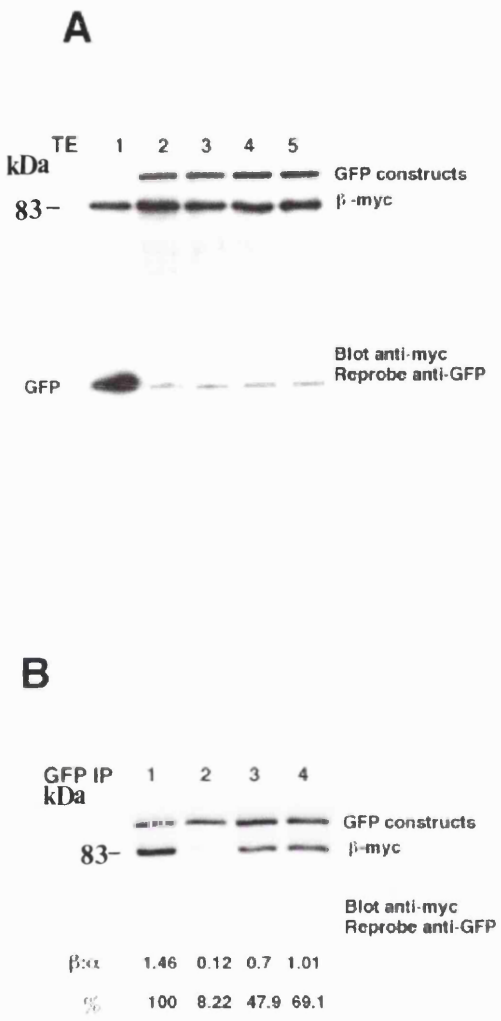
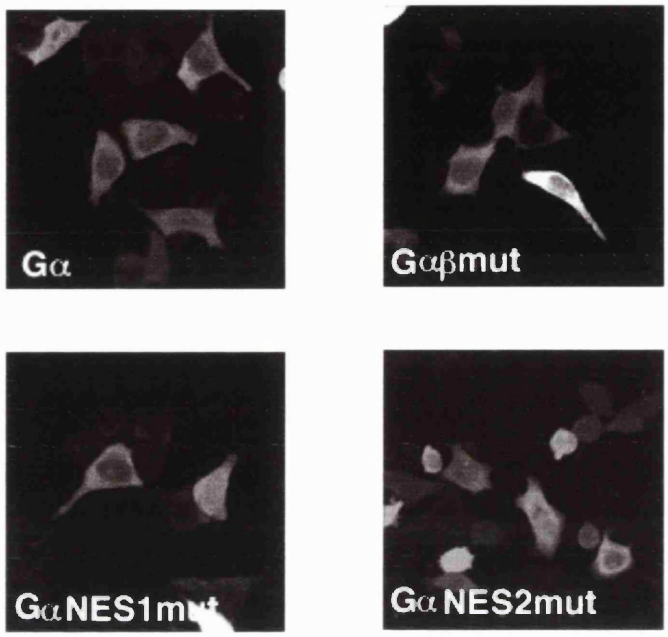


Figure 34.
Characterisation of the different GFP-α-catenin NES constructs.
COS-7 cells were transfected with GFP constructs and β-catenin-myc.
(A) NP-40 extracts were probed sequentially with anti-myc and anti-GFP antibodies. The identities of the bands and the molecular weight marker (83kDa) are indicated. GFP (lane 1), GFP-α-catenin (lane 2), GFP-αβmut (lane 3), GFP-α-NES1mut (lane 4), GFP-α-NES2mut (lane 5).
(B) Anti-GFP immnuoprecipitations from these extracts were probed sequentially with anti-myc and anti-GFP antibodies. GFP-α-catenin (lane 1), GFP-αβmut (lane 2), GFP-α-NES1mut (lane 3), GFP-α-NES2mut (lane 4). The identity of the bands and the 83kDa molecular weight marker are indicated. The intensities of the bands were measured by densitometry. b:a is the ratio between β-catenin-myc and GFP-α-catenin. % indicates an estimate of the affinity of each construct for β-catenin, with the value for GFP-α-catenin set at 100%.

A



B

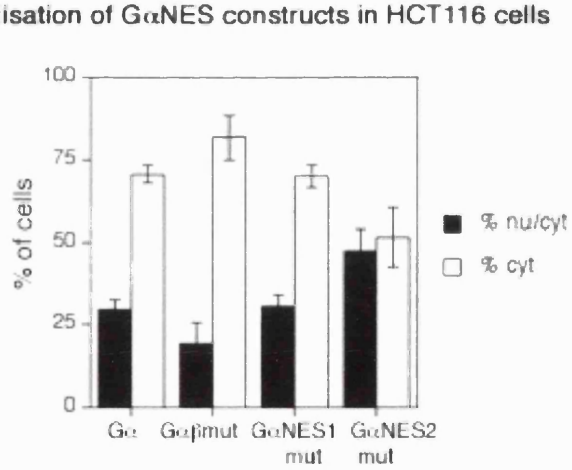


Figure 35.
Subcellular localisation of GFP-α-catenin constructs in HCT116 cells.
(A) HCT116 cells were transfected with the different GFP-α-catenin NES constructs as indicated and, after 24hours, were treated with cycloheximide at 15 μg/ml for 3 hours. Representative pictures of the subcellular localisation of each construct in HCT116 cells. Scale bar 7 μm.
(B) The subcellular localisation of each GFP-α-catenin construct was quantitated by counting. The graph represents the means of three different experiments using approximately of 500 cells. Error bars indicate standard deviations.

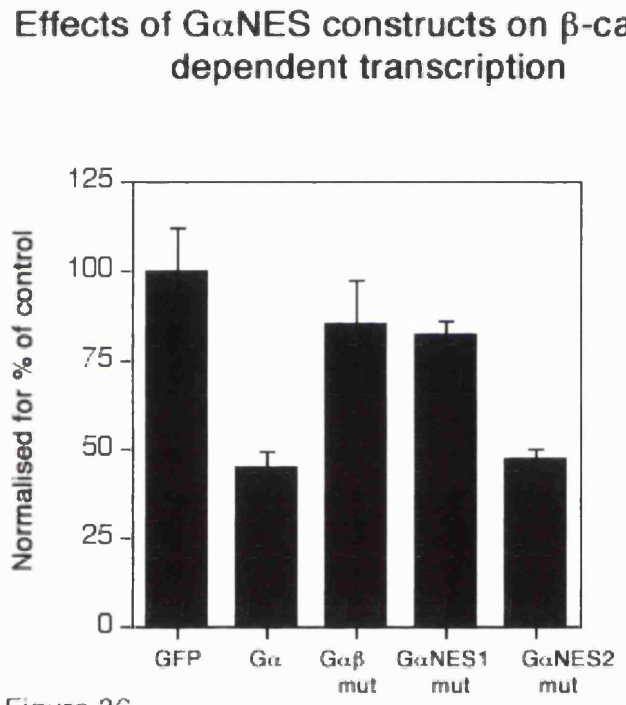


Figure 36.
Effect of GFP-α-catenin-NES constructs on β-catenin-dependent transcription.
HCT116 cells were transfected with pTOPFlash, RSV β-galactosidase and the different GFP constructs as indicated. Experiments were repeated three times, error bars indicate standard deviation.

This might be because GFP-α-NES2mut is not exported from the nucleus as efficiently as GFP-α-catenin, thereby compensating for its reduced affinity for β-catenin.

V.6- Discussion

In this chapter, I have identified and characterised a strong NES in α-catenin that may influence the ability of α-catenin to regulate β-catenin signalling in the nucleus. Although unexpected, the regulation of α-catenin localisation by nuclear export has precedent. There are now several cytoplasmic proteins with roles in cell adhesion that also have a function in the nucleus.

One example is another member of the catenin family, p120. There are several p120 isoforms that result from alternative-splicing. One of these contains a NES at its carboxyl terminus (van Hengel et al., 1999). When in the nucleus, p120 interacts with the transcription factor Kaiso (Daniel and Reynolds, 1999), a methylation-dependent transcriptional repressor (Prokhortchouk et al., 2001). A second example is the tight-junction protein, ZO-1, which localises to the nucleus in sub-confluent epithelial cells and shifts to the plasma membrane upon maturation of cell-cell contacts (Gottardi et al., 1996). ZO-1 associates with the Y-box transcription factor, and together these two proteins regulate the expression of Erb-2 in a cell density-dependent manner (Balda and Matter, 2000). The LIM domain subfamily of proteins, which includes Zyxin, Trip6 and Ajuba, are found primarily at focal plaques. However, they can also be found in the nucleus, and they contain both NLS and NES sequences suggesting that they shuttle in and out of the nucleus.

Cytoskeletal proteins such as actin have also been found in the nucleus (reviewed by Rando et al., 2000). Monomeric actin, in contrast to

α-catenin, is small enough to diffuse into the nucleus, but the presence of two LMB-sensitive NES indicate that actin nuclear localisation can be regulated by nuclear export. Although the nuclear function of actin is not known, there appears to be a link between nuclear levels of G-actin and SRF (serum responsive factor)-dependent transcription (Sotiropoulos et al., 1999).

In contrast to the examples described above, which either have a NLS or can diffuse into the nucleus, α-catenin must associate with another protein, β-catenin, in order to enter the nucleus. The observation that α-catenin is cytoplasmic in HCT116 cells but predominantly nuclear in other colon cancer cell lines that also have nuclear β-catenin, suggests that the presence of nuclear α-catenin is not only regulated at the level of nuclear export, but also at the level of nuclear import. Since nuclear import of α-catenin requires binding to β-catenin, the regulation of this interaction as well as the rate of nuclear import/export of β-catenin may determine α-catenin localisation.

Although two putative NES were identified in α-catenin, only NES2 was a strong NES, both in the Rev-GFP export assay and in the context of α-catenin itself. However, it remains possible that NES1 and NES2 both function together *in vivo*. It is likely that nuclear export of α-catenin, in contrast to its nuclear import, occurs independently of β-catenin. However, the situation might be a bit more complicated, since the crystal structure of a α-catenin/β-catenin heterodimer indicates that both α-catenin NES1 and NES2 overlap with the β-catenin binding domain. This overlap might provide an additional mechanism for regulating α-catenin nuclear localisation. For example, the binding of β-catenin to α-catenin could mask the NES sites in α-catenin, thereby preventing export.

Mutation of both NES1 and NES2 reduced α-catenin binding to β-catenin, at least as determined in immunoprecipitation assays from NP-40 extracts. For NES1, mutation prevented α-catenin from inhibiting β-catenin-dependent transcription because it could no longer efficiently bind to β-catenin. With regard to inhibition of β-catenin-dependent transcription by the NES2 mutant, it is likely that the opposing effects of a reduction in the affinity of the NES2 mutant for β-catenin, and the reduced nuclear export of the NES2 mutant, resulted in a molecule that inhibits β-catenin-dependent transcription to a similar extent as wild-type α-catenin. To conclude, since α-catenin lacks any consensus NLS it is likely that it moves into the nucleus only when bound to β-catenin. In the presence of Tcf/LEF-1, the three proteins form a nuclear complex, the localisation of which is maintained by the NLS present in Tcf/Lef-1. In addition, α-catenin may remain in the nucleus while bound to β-catenin, since this binding would mask the NES found in α-catenin. It is likely that, in addition to nuclear import/export, α-catenin localisation is regulated by sequestration in the cytoplasm.

Table 4. Summary of results obtained with the different GFP-α-constructs.

Constructs	Localization in the cell	Inhibition of transcription in colon cancer cells	Binding to β–catenin in IP assay
GFP- α	SW480- Nuclear DLD-1- Nuclear HCT116-cytoplasmic	50-60% in these cell lines	Set as 100%*
GFP- α-NLS	Nuclear in all the above cell lines	50-60% in these cells lines	Not tested
GFP- αΔ-NLS	Nuclear in all the above cell lines	50-60% in these cells lines	Not tested
GFP- αβmut	HCT116- cytoplasmic in 80%of the cells. Not tested in other cell lines	15% inhibition in HCT116 cells. Not tested in other cell lines	8% * (sd ±10%)
GFP- αNES1mut	HCT116- cytoplasmic in 70%of the cells. Not tested in other cell lines	15% inhibition in HCT116 cells. Not tested in other cell lines	50% * (sd±10%)
GFP- αNES2mut	HCT116- cytoplasmic in 50%of the cells. Not tested in other cell lines	50-60% inhibition in HCT116 cells. Not tested in other cell lines	70% * (sd±10%)

* These values were obtained by densitometry of immunoprecipitations assays. The value obtained by the binding of GFP-α to β-catenin was set as 100%.

CHAPTER VI- *Final Discussion*

Several mechanisms have evolved to regulate β-catenin signalling. They include the sequestration of β-catenin in the plasma membrane by binding to cadherins, the degradation of β-catenin in the cytoplasm by the proteasome, apoptosis of cells that inappropriately express β-catenin, acetylation of Tcf, binding of co-repressors to Tcf, binding of co-activators to β-catenin, nuclear export of β-catenin by proteins such as Su(Fu), and inhibitory feedback loops, such as when Wnt signals increase the expression of β-TrCP, resulting in increased degradation of β-catenin. The large number of regulatory mechanisms emphasises the importance of keeping the β-catenin signalling pathway under strict control. Loss of control has detrimental consequences, both during embryonic development and for adult tissue homeostasis, such as in the intestinal epithelium.

Taking into consideration the results I have obtained, I propose a further regulatory mechanism, which involves the regulation of β-catenin nuclear function by α-catenin. The model I present involves nuclear sequestration of α-catenin by β-catenin/Tcf complexes. Alpha-catenin forms a complex with β-catenin/Tcf when these proteins are overexpressed in cells, and in an endogenous situation. The complex formed by α-catenin/β-catenin/Tcf, however, is not able to promote transcription, possibly because it cannot associate with its DNA target.

The importance of endogenous α-catenin in repression of β-catenin-dependent transcription is supported by the observation that α-catenin negative cells have higher β-catenin-dependent transcription in Tcf-reporter assays (Figure 18d). Moreover, the expression of α-catenin is

often reduced during tumour progression (Hirohashi, 1998), and several cancer-derived cell lines have mutations in the α-catenin gene. Reintroduction of α-catenin into such lines reduces cell growth (Watabe et al., 1994) and attenuates tumour formation (Bullions et al., 1997). DLD-1 cells that have lost α-catenin expression are more invasive than the parental cell line (Vermeulen et al., 1995). This increased invasiveness might be due to a reduction in cell adhesion or to an increased β-catenin/Tcf-dependent transcription, or both. Interestingly, one study of colon cancer samples showed that many tumours analysed had reduced expression of either E-cadherin (29%) or of α-catenin (56%), but increased tumour invasion and metastasis correlated with reduced expression of α-catenin (Gofuku et al., 1999). The tumour suppressor activities of both E-cadherin and α-catenin might result from their association with β-catenin, which prevents nuclear localisation (in the case of E-cadherin) and β-catenin-dependent transcription (for both E-cadherin, which competes with Tcf, and for α-catenin, which prevents DNA binding). An alternative explanation is that binding of E-cadherin or α-catenin to β-catenin affects cell-cell adhesion, facilitating juxtacrine signalling via other transmembrane receptors that regulate growth (Watabe-Ushida et al., 1998; Carmeliet et al., 1999). However, some recent reports do not support a role for cell adhesion on suppression of cell growth. For example, E-cadherin constructs that cannot promote adhesion, but can bind to β-catenin, reduce cell growth in SW480 cells (Gottardi et al., 2001). In accordance with these results, the inhibition of β-catenin-dependent transcription by GFP-α-catenin is also independent of its ability to promote adhesion, since it does not require the domains that associate with the actin cytoskeleton, which are required for cell-cell adhesion.

The nuclear localisation of α-catenin must be regulated in some way, since it clearly differs among colon cancer cell lines that have nuclear β-catenin. For example, α-catenin is nuclear in SW480 (Figure 17b) and DLD-1 cells (Figure 17f), but not in HCT116 cells (Figure 17d).

It is possible that the degree of differentiation of the cell lines plays a role in the regulation of nuclear α-catenin. For example, in Caco-2 cells, differentiation can be achieved *in vitro* by growing the cells at confluence. This results in a down regulation of β-catenin-dependent transcription and complex formation with Tcf, possibly due to a decrease in Tcf expression (Mariadason et al., 2001). It may be informative to examine the localisation of α-catenin in differentiating Caco-2 cells.

In addition to the regulation of β-catenin signalling at the level of β-catenin degradation, there are several mechanisms in place to inhibit β-catenin function in the nucleus. Duplin is a nuclear protein that competes with Tcf for the binding to the armadillo repeats of β-catenin, hence inhibiting β-catenin-dependent transcription (Sakamoto et al., 2000). Groucho proteins are also transcriptional repressors, that can bind to Tcf. Binding of full length or truncated forms of α-catenin to β-catenin in the nucleus might also displace co-activators of transcription, such as Pontin-52, which forms a tripartite complex with β-catenin and Tcf (Bauer et al., 1998; 2000). Although the Pontin-52 binding domain does not overlap with the α-catenin binding domain, their proximity might result in mutually exclusive binding of Pontin-52 or α-catenin with β-catenin, something that remains to be tested. Another co-activator of β-catenin-dependent transcription is the nuclear protein p300 (Miyagishi et al., 2000). This acetyltransferase also participates in activation of transcription in other signalling pathways, suggesting that its activity is necessary for

alleviating repression imposed by chromatin structure. In addition dCBP, a p300 related protein in *Drosophila*, acetylates dTcf, reducing Armadillo binding (Waltzer and Bienz, 1998). In SW480 and HCT116 cells, endogenous p300 and β-catenin co-immunoprecipitate (Miyagishi et al., 2000; Sun et al., 2000). However, the identity of the domain in β-catenin responsible for the association with p300 is still a matter to be resolved. One report shows that the carboxyl terminus of p300/CBP can interact with armadillo repeats (residues 152-423 and 424-709, respectively; Miyagishi et al., 2000). However, a second report uses pull-down assays to show that residues 630-781 of β-catenin interact with p300 (Hecht et al., 2000), and a third report shows that β-catenin lacking amino-terminal residues does not associate with p300 (Sun et al., 2000). It is possible that, being a general transcriptional activator, p300 independently associates with both of the transcriptional activation domains in β-catenin. The discrepancies might also reflect differences in the activities of these proteins in different cell types and organisms.

The α-catenin binding site in β-catenin also overlaps with the binding domains of TBP (residues 47-159). TBP might help to keep the β-catenin/Tcf bound to DNA, and displacement of TBP from this complex by binding of α-catenin to the β-catenin/Tcf complex might destabilise the interactions of β-catenin/Tcf with DNA.

The regulation of protein nuclear localisation occurs in different ways. First, it can rely on the presence of both nuclear localisation signals (NLS) and nuclear export signals (NES). The presence of both signals in the same molecule provides a way to independently control nuclear localisation. Second, nuclear localisation of proteins without NLS or NES can be mediated by binding to other proteins that contain these signals.

Alternatively, the masking and unmasking of the NES signal offers an additional way to control nuclear export.

In the context of the Wnt/Wg signalling pathway, there are examples of all of the above mechanisms. APC localisation in the cell illustrates the first mechanism, in which nuclear localisation is dependent on both NLS and NES signals. An example of the second mechanism is given by the transcription factor Teashirt, which does not have a NLS but translocates to the nucleus bound to Armadillo (Gallet et al., 1999). The third mechanism might be illustrated by the nuclear localisation of α-catenin. This protein localises to the nucleus when bound to the β-catenin/Tcf complex. In this complex, α-catenin NES signals are masked, since their positions overlap, with the β-catenin binding domains.

The positions of the α-catenin NES signals also overlap with the α-catenin homodimerisation domain. In the presence of β-catenin, however, α-catenin dimers are not favoured (Koslov et al., 1997). In both β-catenin and α-catenin the binding domains form an amphipathic helix where the hydrophobic residues are present at the interacting interface between the two proteins (Aberle et al., 1996; Huber et al., 1997b; Pokutta et al, 2000). The crystal structure of a chimeric protein formed by fusing the amino-terminal region of β-catenin (residues 118-151) to the amino terminus of α-catenin (residues 57-264) has been determined, and it reinforces the importance of hydrophobic residues for the interaction between α-catenin and β-catenin (Pokutta et al., 2000). The important hydrophobic residues are marked in light grey in Figure 27b. It is interesting to note that in NES1, apart from residue I-139, all the other key residues for the NES are involved in β-catenin binding. In NES2, at least two residues, possibly three (V-146, L-149 and L-153) are also involved in binding to β-catenin.

This suggests that when α-catenin is bound to β-catenin in the nucleus, the NES signals are masked, and this prevents the export of α-catenin. It is tempting to speculate that other co-activators of transcription present in the nucleus compete with α-catenin for binding to the amino-terminal domain of β-catenin. This would result in displacement of α-catenin from the β-catenin/Tcf complex, exposure of its NES signals, and consequently nuclear export of α-catenin. Once in the cytoplasm, α-catenin might be sequestered by associating with components of the actin cytoskeleton. Thus transcription of β-catenin/Tcf target genes could resume. In cells where the β-catenin signal is constitutively active, α-catenin nuclear entry would be a continuous process, dependent on both sequestration of this protein in the cytoplasm and also on a balance between import and export of α-catenin. By adding LMB, hence blocking CRM1-dependent nuclear export, one might expect to see higher levels of α-catenin in the nucleus. This was not the case for endogenous α-catenin in DLD-1 and HCT116 cells, suggesting that the localisation of a majority of endogenous α-catenin is controlled by sequestration in the cytoplasm or that nuclear export of a-catenin is independent of CRM1. However, localisation of exogenous α-catenin in HCT116 cells was affected by this LMB, supporting the possibility that α-catenin can utilise CRM1 to move continuously in and out of the nucleus in these cells. It is still possible that endogenous α-catenin is also affected by LMB, but immunostaining is not sensitive enough to detect it. If this model is correct, one might predict that LMB would inhibit β-catenin-dependent transcription in DLD-1 α-catenin positive cells, but have no effect on the transcription in the DLD-1 α-catenin negative cells. However, since proteins such as APC and Su(fu) also regulate β-catenin nuclear export, these experiments would be

difficult to interpret. Nevertheless, since β-catenin can move out of the nucleus independently of other factors (Eleftheriou et al., 2001), the contribution of endogenous α-catenin to the inhibition of endogenous β-catenin-dependent transcription is unlikely to involve nuclear export directly. Rather, it appears to involve the ability of α-catenin to block the interaction of the β-catenin/Tcf complex with DNA.

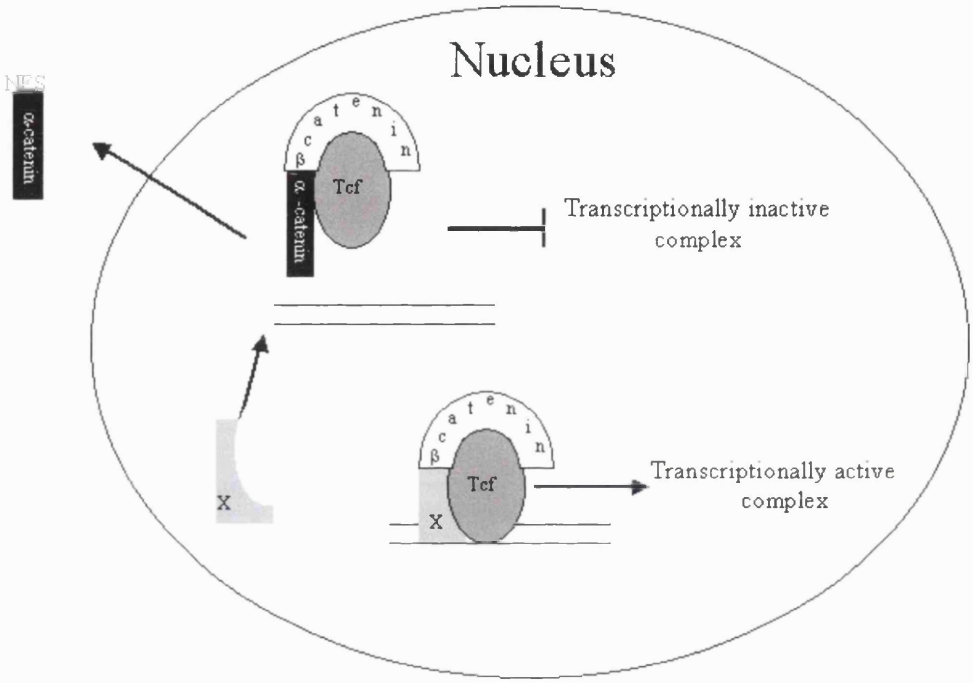
Targeted ablation of αE-catenin in the skin causes the expected defects in cell adhesion, but in addition, epithelial polarity is altered. Interestingly, these α-catenin-null keratinocytes proliferate more quickly than wild-type cells, owing to a sustained activation of the Ras-MAP kinase pathway. Moreover, these keratinocytes are more invasive. Comparison of α-catenin-null keratinocytes with desmoplakin-null keratinocytes showed that, although both present severe adhesion defects, only α-catenin-null keratinocytes are hyperproliferative. However, α-catenin null keratinocytes do not appear to have any obvious increase in β-catenin-signalling activity, and hyperproliferation may result from these cells responding better to growth factor stimulation (Vasioukhin et al., 2001).

Re-introduction of α-catenin into α-catenin-defective cells, such as the lung carcinoma PC9 cell line (Bullions et al., 1997) and the ovarian carcinoma OV2008 cell line (Watabe et al., 1994) inhibits their growth. In contrast, a recent report (Matsubara et Ozawa, 2001) showed no inhibition of growth induced by re-introduction of α-catenin into α-catenin-negative DLD-1 colon cancer cells. Although I showed that α-catenin represses β-catenin-dependent transcription in this cell line, this inhibition is clearly not sufficient to inhibit cell growth.

This is most likely because DLD-1 cells have a number of other mutations that deregulate their growth, including a mutation in *K-ras*.

To conclude, my results suggest that α-catenin enters the nucleus bound to the β-catenin/Tcf complex. In the nucleus, α-catenin reduces β-catenin-dependent transcription, possibly by promoting dissociation of the β-catenin/Tcf complex from its DNA target. Competition between α-catenin and other nuclear proteins for binding to β-catenin may displace α-catenin from the β-catenin/Tcf complex, resulting in the exposure of the α-catenin NES and the nuclear export of α-catenin (Figure 37).

Model for α-catenin regulation of β-catenin in the nucleus



Model for the regulation of beta-catenin-dependent transcription by alpha-catenin. Alpha-catenin is transported into the nucleus by the beta-catenin/Tcf complex. The alpha-catenin/beta-catenin/Tcf complex is transcriptionally inactive because it does not bind to DNA. Other transcriptional co-activators compete with alpha-catenin for binding to beta-catenin. Displacement of alpha-catenin from the beta-catenin/Tcf complex exposes the NES on alpha-catenin. As a result alpha-catenin is exported from the nucleus, the beta-catenin/Tcf complex binds to DNA and gene transcription resumes.

List of Abbreviations

Act D	Actinomycin D
ADF	Actin depolymerising factor
ATP	Adenosine triphosphate
APC	Ademato us poliposis coli protein
BME	2-Mercaptoethanol
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
$CaCl_2$	Calcium chloride
CBP	CREB-binding protein
CIAP	Calf-intestinal alkaline phosphatase
CPRG	Chlorophenol red-b-D-galactopyranoside
CREB protein	cAMP responsive element-binding
CRM-1	Chromosome region maintenance
DATP	2'-Deoxyadenosine 5'triphosphate
DNA	Deoxyribonucleic acid
CDNA	Complementary DNA
dNTP	Deoxynucleotide triphosphate
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulfoxide
Dsh	Dishevelled
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Disodium ethylenediaminetetra-acetate
EGF	Epidermal growth factor
FAP	Familial adenomatous polyposis
Fz	Frizzled
IGF-1	Insulin-like growth factor type 1
Hepes	N-[-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HGF	Hepatocyte growth factor
FITC	Fluorescein-5-isothiocyanate
GAP	GTPase-activating protein
GFP	Green fluorescent protein

GSK3	Glycogen synthase kinase-3
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HA	Hemagglutinin
HDAC	Histone deacetylase
HBSS	Hank's buffered saline solution
HIV	Human immuno-deficiency virus
IgG	Immunoglobulin type G
ILK	Integrin-linked kinase
JNK	C-jun amino-terminal kinase
KCl	Potassium chloride
KDa	Kilodalton
KH_2PO_4	Potassium phosphate, monobasic
LDL	Low density lipoprotein
LMB	Leptomycin B
Lef-1	Lymphoid enhancer factor-1
LRP	LDL receptor-related protein
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MOPS	3-(N-Morpholino)propanesulfonic acid
MRNA	messenger RNA
Na_2HPO	Sodium phosphate, dibasic
NaCl	Sodium chloride
NaF	Sodium fluoride
NaH_2PO_4	Sodium phosphate, monobasic
NES	Nuclear export signal
NLS	Nuclear localisation signal
NPC	Nuclear pore complex
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3 kinase
PIP-3	Phosphatidylinositol 3,4,5 triphosphate
PKB	Protein kinase B
Poly(dIdC)	Polydeoxyguanylic-polydeoxycytidylic acid

PTEN	Phosphatase and tensin homologue deleted from chromosome 10
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Rotation per minute
RSV	Rous sarcoma virus
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SRF	Serum responsive factor
TAE	Tris-acetate buffer
TBE	Tris-borate buffer
TBP	TATA-box binding protein
TGF-β	Transforming growth factor -β
Tcf	T cell factor
TRNA	Transfer RNA
Tween	Polyethylene sorbitan monolaurate
U	Units
VDR	Vitamin D receptor
VEGF-A	Vascular endothelial growth factor type A
ZO-1	Zonula occludens-1 protein

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