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Localisation and interactions of adenomatous polyposis coli and Beta-catenin in epithelial and colorectal cancer cells

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Summary

Adenomatous polyposis coli (APC) is a tumour suppressor protein that is a critical component of the Wnt signalling pathway. APC has been described as 'the gene for colon cancer', reflecting its importance in this disease. Mutations in APC have been reported in both hereditary and sporadic forms of colorectal cancer, and are seen at the earliest stages of colorectal cancer which can be observed.

There have been many reports of the subcellular localisation of both full length and truncated APC, many of these conflicting. Work presented here characterises the localisation and interactions of APC using a variety of antibodies directed to various epitopes within APC. Characterisation of a panel of APC antibodies shows that many of the conflicting reports of the localisation of APC resulted from use of antibodies which detect proteins other than full length APC.

Localisation of APC and β-catenin has been shown to be linked to cell density, cell type and mutation status of APC and β-catenin. The distribution of truncated APC and β-catenin is closely linked in sub-confluent SW480 cells, with both being localised to the nucleus. At high cell density nuclear localisation of β-catenin and truncated APC and co-localisation is lost. We postulated that in SW480 cells the decrease in nuclear β-catenin as cell density increases could be due to an increase in β-catenin bound to E-cadherin with formation of adherens junctions. In co-immunoprecipitation assays an increase in binding between β-catenin and E-cadherin, and a corresponding decrease in binding between β-catenin and APC, was observed at high cell density. Although an increase in β-catenin/E-cadherin interaction was seen with cell density, co-localisation of β-catenin and E-cadherin at the cell membrane was not seen in all cells, and membrane E-cadherin did not appear to be necessary for nuclear exclusion of β-catenin.

An apical protein has been identified, the localisation of which had previously been reported as being that of full length APC. Work presented here shows that this apical staining is not due to full length APC, but instead appears to be a 150 kDa protein identified as a potential novel isoform of APC. Unlike full length APC, this protein does not interact with β-catenin. This potential APC isoform shows variable distribution in epithelial and colorectal cancer cell lines, with similarities to the localisation of *Drosophila* E-APC, suggesting a similar role in spindle orientation.

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Abbreviations

AEBSF – 4(2-aminoethyl)benzenesulfonyl fluoride
APC – Adenomatous Polyposis Coli
APCL – Adenomatous Polyposis Coli Like
ACF – Aberrant Crypt Foci
BMP – Bone Morphogenetic Protein
CAPS – 3-(cyclohexylamino)-1-propane sulfonic acid
CKI – Casein Kinase I
CRM1 – Chromosome Maintenance Region 1
DEP – Dishevelled, Eg-10, plekstin
DIC – Differential Interference Contrast
DIX – Dishevelled and Axin
DLG – Discs Large
DMEM – Dulbecco's Modified Eagle's Medium
DMSO – Dimethlysulphoxide
DNA – Deoxyribonucleic Acid
DTT – Dithiothrietol
Dvl – Dishevelled
EC – Embryonal Carcinoma
ECACC – European Collection of Cell Cultures
EDTA – (Ethylenedinitrilo)tetra acetic acid
FAP – Familial Adenomatous Polyposis
FBS – Foetal Bovine Serum
GBP – GSK Binding Protein
GSK – Glycogen Synthase Kinase
HCC – Hepatocellular Carcinoma
HMBA – Hexamethylene Bisacetamide
HNPCC – Hereditary Non-Polyposis Coli Cancer
LEF – Lymphocyte Enhancer Factor
LMB – Leptomycin B
LRP – LDL (Low Density Lipoprotein) Receptor Related Protein
MCR – Mutation Cluster Region
MDCK – Madine Darby Canine Kidney
MEM – Minimum Eagle's Medium
MMP – Matrix Metalloproteinase
MMR – Mismatch Repait
mRNA – Messanger Ribonucleic Acid
NES – Nuclear Export Sequence
NLS – Nuclear Localisation Sequence
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PDZ – PSD-95/DLG/ZO-1
PP2A – Protein Phosphatase 2A
RNA – Ribonucleic Acid
RT – Reverse Transcription
SCF – SKP-1, Cdc53, F-box

SDS-PAGE – Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SSEA – Stage Specific Embryonic Antigen
TCF – T-Cell Factor
TLE – Transducing-Like Enhancer of split

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Chapter 1 - Introduction

Adenomatous polyposis coli (APC) is a tumour suppressor protein that is a critical component of the Wnt signalling pathway. APC has been described as 'the gene for colon cancer', reflecting its importance in this disease. Mutations in APC have been reported in both hereditary and sporadic forms of colorectal cancer, and are seen at the earliest stages of colorectal cancer which can be observed. APC functions within the Wnt signalling pathway to regulate the activity of β-catenin, and therefore the transcription of Wnt pathway target genes. Much attention has been focused on how APC regulates the activity of β-catenin, and how mutation of APC and β-catenin leads to inappropriate expression of Wnt pathway target genes and ultimately to cancer. APC also has additional functions outside the Wnt signalling pathway. APC has been reported to be localised to kinetochores and to have a role in maintaining chromosomal stability. APC has also been reported to be localised to the plasma membrane and microtubule tips, and may be involved in cellular adhesion and locomotion.

1.1 APC and β-catenin in the Wnt signalling pathway: Wnt signal transduction via APC and β-catenin

A key event in Wnt signalling is the stabilisation of β-catenin. As well as being a key component of the Wnt signalling pathway, β-catenin is also involved in intercellular adhesion. The primary structure of β-catenin consists of a 130 amino acid N-terminal domain, the armadillo repeat region of 12 imperfect repeats of 42 amino acids and a 100 amino acid C-terminal domain. The N-terminal domain has been shown to be important in regulating the stability of β-catenin, through a number of phosphorylation sites within this region. Deletions and certain point mutations of the N-terminal domain results in constitutively active forms of β-catenin (Yost *et al.*, 1996; Munemitsu *et al.*, 1996; Barth *et al.*, 1997).

In the absence of a Wnt signal, free cytoplasmic β-catenin is targeted for destruction by a multi-protein degradation complex consisting of axin, or its homologue conductin, GSK-3β and APC (reviewed in Bienz, 2002). A complex

of axin and casein kinase Iε induces phosphorylation of β-catenin at Ser45. Phosphorylation of Ser45 is necessary and sufficient to initiate phosphorylation of β-catenin at the Wnt-dependent phosphorylation sites (Amit *et al.*, 2002; Hagen & Vidal-Puig, 2002; Liu *et al.*, 2002; Sakanaka, 2002). Following phosphorylation of Ser45, interaction of β-catenin with the degradation complex leads to phosphorylation of β-catenin at the GSK-3β phosphorylation sites, Ser33, Ser37 and Thr41 (van Noort *et al.*, 2002; Sadot *et al.*, 2002).

The middle section of APC contains three repeats of 15 amino acids, followed by seven related but distinct repeats of 20 amino acids. Both of these sets of repeats are able to bind independently to β-catenin (Rubinfeld *et al.*, 1993; Rubinfeld *et al.*, 1995; Su *et al.*, 1993) but only the 20 amino acid repeats are involved in phosphorylation of β-catenin (Munemitsu *et al.*, 1995). A minimum of three of the 20 amino acid repeats are required for degradation of β-catenin (Polakis, 1997; Rubinfeld *et al.*, 1997). APC can be directly phosphorylated by GSK-3β (Rubinfeld *et al.*, 1996) with binding of APC to axin enhancing this phosphorylation (Ikeda *et al.*, 2000). Phosphorylation of APC appears to be important for degradation of β-catenin (Rubinfeld *et al.*, 1996; Rubinfeld *et al.*, 1997). The structure of APC and its function in normal and cancerous cells is discussed further in section 1.4.

Wnt-dependent phosphorylation of β-catenin marks it for destruction by the proteasome pathway (Aberle *et al.*, 1997; Orford *et al.*, 1997). Phosphorylated β-catenin interacts strongly with the F-box protein β-TrCP (Slimb in *Drosophila*), a subunit of the SCF ubiquitin ligase complex (Hart *et al.*, 1999; Winston *et al.*, 1999; Liu *et al.*, 1999). The WD40 repeat domain of β-TrCP interacts directly with phosphorylated β-catenin but not with unphosphorylated β-catenin. Phosphorylated β-catenin becomes ubiquitinated by this complex which targets it for destruction by the 26S proteasome (reviewed in Maniatis 1999).

In the absence of a Wnt signal free β-catenin is phosphorylated and degraded and is therefore not available to enter the nucleus. Under these conditions TCF/Lefs

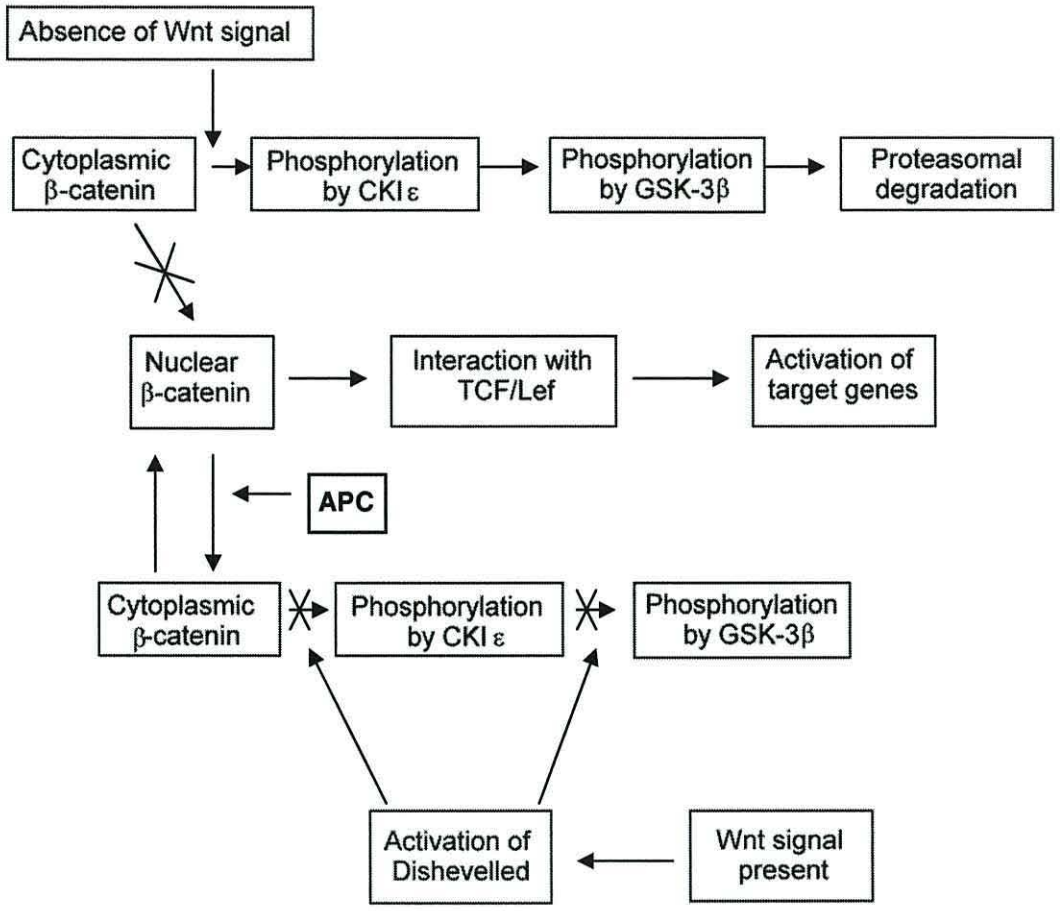


Figure 1.1
The Wnt signalling pathway. When no Wnt signal is present β-catenin is phosphorylated by casein kinase Iε (CKIε) and GSK-3β, which marks it for ubiquitination and proteasomal degradation. When a Wnt signal is present, dishevelled inhibits phosphorylation of β-catenin which leads to nuclear entry of dephosphorylated β-catenin and activation of Wnt pathway target genes.

bind to specific promoter/enhancer regions and, along with Groucho/TLE (Cavallo *et al.*, 1998; Roose *et al.*, 1998) and the histone deacetylase Rpd3 (Chen *et al.*, 1999), repress gene expression (reviewed in Bienz, 1998; Roose & Clevers, 1999).

Wnts act on target cells in a paracrine fashion through receptors of the Frizzled family (Bhanot *et al.*, 1996). Frizzled receptors are composed of an extracellular N-terminal domain, seven transmembrane spanning sequences and an intracellular C-terminal domain (Bhanot *et al.*, 1996). The mechanism of activation of the Frizzled receptors and the mechanism of transmission of the signal from the receptor to the cytoplasm are not known.

In the presence of a Wnt signal the destruction complex does not form, instead, a complex is formed between dishevelled, GBP/Frat1, axin and Zw3/GSK-3 (Li *et al.*, 1999; Salic *et al.*, 2000; Farr *et al.*, 2000). β-catenin is released from this complex without being phosphorylated and is therefore not marked for degradation (Salic *et al.*, 2000).

The binding of Wnt ligands to Frizzled receptors leads to inhibition of the degradation complex via dishevelled (Cadigan & Nusse, 1997; Dale, 1998). Dishevelled proteins have been shown to interact with a number of proteins involved in phosphorylation of β-catenin. Dishevelled has been shown by immunoprecipitation to interact directly with axin (Li *et al.*, 1999). Axin phosphorylation is reduced in the presence of a Wnt signal, which leads to a decrease in the affinity of β-catenin-axin interaction. The reduced affinity for binding to β-catenin leads to release of β-catenin from the degradation complex and therefore gives increased levels of free β-catenin (Willert *et al.*, 1999; Jho *et al.*, 1999). Dishevelled also interacts with casein kinase Iε (Peters *et al.*, 1999; Sakanaka *et al.*, 1999), which phosphorylates β-catenin at Ser45. Dishevelled (Dvl in vertebrates) has three conserved domains, an N-terminal DIX domain, which is also found in axin, a central PDZ domain and a DEP (Dishevelled, egl-10, plekstin) domain C-terminal to the PDZ domain (Li *et al.*, 1999). Three

human homologues of dishevelled have been identified (Dvl-1, Dvl-2, Dvl-3) (Semenov & Snyder, 1997).

Wnt-dependent binding of axin to the cytoplasmic tail of LRP may also have a role in rearranging the degradation complex in the presence of a Wnt signal (Mao *et al.*, 2001; Tolwinsky *et al.*, 2003). LRP/Arrow is required for Wnt signalling in both *Drosophila* and vertebrates and has been shown to bind to Wnt-Frizzled to form a ternary complex (Wehrli *et al.*, 2000; Tamai *et al.*, 2000; Pinson *et al.*, 2000). The cytoplasmic tail of LRP has been shown to bind axin in a Wnt dependent manner (Mao *et al.*, 2001; Tolwinsky *et al.*, 2003).

The presence of a Wnt signal results in dephosphorylated β-catenin being able to enter the nucleus (Staal *et al.*, 2002) and interact with TCF/Lefs. Interaction of β-catenin with TCF/Lefs causes them to act as transcriptional activators (reviewed in Bienz, 1998; Roose & Clevers, 1999) and leads to activation of Wnt pathway target genes.

1.2 APC and β-catenin in the Wnt signalling pathway: Nuclear-cytoplasmic shuttling of APC and β-catenin

β-catenin does not contain a nuclear localisation sequence but is able to enter the nucleus. β-catenin contains armadillo repeats similar to those seen in importin, a major component of the nuclear import machinery (Goerlich *et al.*, 1994). It was therefore suggested that when levels of β-catenin are higher than normal, such as when a Wnt signal is present, β-catenin may be able to interact directly with the nuclear import machinery and become translocated into the nucleus (Willert & Nusse, 1998).

It has been observed that β-catenin docks specifically to the nuclear envelope (Fagotto *et al.*, 1998). Such docking was observed to be specifically competed by importin-β/β-karyopherin indicating that both β-catenin and importin-β/β-karyopherin interact with the same nuclear pore component. A lack of β-catenin

import into the nucleus was observed in the presence of normal cytosol, suggesting nuclear import of β-catenin is regulated by cytosolic events in the Wnt signalling pathway.

As well as controlling the degradation of β-catenin, phosphorylation also controls the subcellular localisation of β-catenin. It has been shown that Wnt signals are transmitted though β-catenin dephosphorylated at Ser37 and Thr41 (Staal *et al.*, 2002). It is only dephosphorylated β-catenin that is able to enter the nucleus, accumulation of β-catenin in the cytosol, by inhibition of proteasomal activity, is not sufficient for nuclear accumulation of β-catenin.

As well as controlling nuclear entry of β-catenin through its role in phosphorylation of β-catenin, APC also has a role in nuclear export of β-catenin. Several studies have shown that APC has a CRM1-dependent nuclear export function for β-catenin that regulates the sub-cellular localisation and turnover of β-catenin (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a). Nuclear localisation of APC and β-catenin in cell lines which express only truncated APC is thought to be due to loss of nuclear export sequences which are found in the central domain of APC. A total of five nuclear export sequences have been identified in APC (Henderson 2000; Neufeld *et al.*, 2000a; Rosin-Arbesfeld *et al.*, 2000), two at the N terminus and three in the central region around the 20 amino acid repeats.

APC truncations in the majority of colorectal tumours (Miyaki *et al.*, 1994) and cell lines derived from colorectal tumours (Rosin-Arbesfeld *et al.*, 2000) result in loss of all three central nuclear export sequences suggesting that these nuclear export sequences are important in regulating APC and β-catenin localisation. It has been shown that the nuclear export sequence NES1506 (the closest to the N terminus of the central nuclear export sequences) appears to be critical in determining localisation. Cell lines which retain NES1506 but have lost the other two central nuclear export sequences show efficient nuclear exclusion of both APC and β-catenin (Rosin-Arbesfeld *et al.*, 2003).

It is not clear what role the N-terminal nuclear export sequences (Henderson 2000; Neufeld *et al.*, 2000a) have in nuclear export of APC as there are conflicting reports as to the activity of these N-terminal nuclear export sequences. It has been reported that an APC construct truncated at 1,309 amino acids was able to be exported from the nucleus, and export some β-catenin, in SW480 cells (Henderson, 2000), this suggests the N-terminal nuclear export sequences are active in this case. However, this data has been disputed by Rosin-Arbesfeld and co-workers (2003) who reported that an APC construct lacking all central nuclear export sequences, but having both N-terminal nuclear export sequences, was found to some extent in nuclei of cells and was not effective at reducing β-catenin dependent transcriptional activity (Rosin-Arbesfeld *et al.*, 2003). It has also been reported that APC fragments lacking the N-terminal nuclear export sequences are efficiently excluded from the nucleus and are able to reduce β-catenin-dependent transcriptional activity in SW480 cells (Rosin-Arbesfeld *et al.*, 2003; Heppner Goss *et al.*, 2002).

β-catenin has also been shown to undergo APC independent nuclear export (Eleftheriou *et al.*, 2001; Wiechens & Fagotto, 2001; Henderson & Fagotto, 2002), although it has not been determined when one mechanism may prevail over the other. It has been suggested that CRM1-independent nuclear export is relatively slow and that the rate of nuclear export is accelerated by APC (Rosin-Arbesfeld *et al.*, 2003).

In the absence of a Wnt signal, TCF/Lefs act as transcriptional repressors (Brannon *et al.*, 1997; Bienz, 1998; Riese *et al.*, 1997). When a Wnt signal is present dephosphorylated β-catenin enters the nucleus and forms a complex with TCF/Lefs. When complexed with β-catenin TCF/Lefs act as transcriptional activators (reviewed in Roose & Clevers, 1999). It has recently been reported that the rate of nuclear export of APC determines the transcriptional activity of β-catenin (Rosin-Arbesfeld et al., 2003). Therefore, APC is involved in controlling degradation, subcellular localisation and transcriptional activity of β-catenin.

1.3 Functions of APC and β-catenin outside the Wnt signalling pathway

As well as its role in the Wnt signalling pathway, β-catenin is also a component of the adherens junction complex. β-catenin interacts with the cytoplasmic domain of the transmembrane cell adhesion molecule E-cadherin (Aberle *et al.*, 1996; Kemler, 1993) and with α-catenin which connects the adherens junction complex with the actin cytoskeleton (Aberle *et al.*, 1994; Hulsken *et al.*, 1994; Jou *et al.*, 1995). E-cadherin is able to negatively regulate β-catenin/TCF-LEF signalling. The regulation of β-catenin/TCF-LEF signalling by E-cadherin is dependent on its β-catenin binding region but is not dependent on its intercellular adhesion function (Orsulic *et al.*, 1999; Gottardi *et al.*, 2001).

APC has also been shown to localise to the plasma membrane (Rosin-Arbesfeld *et al.*, 2001), where its association with the plasma membrane is actin dependent. In *Drosophila* expressing mutant E-APC defects in adhesion can be detected, which indicates a function for APC in cellular adhesion (Hamada & Bienz, 2002). *Drosophila* E-APC may also be involved in anchoring microtubules to the cellular cortex (McCartney *et al.*, 2001) in mitosis and orienting spindles within the epithelial plane (Lu *et al.*, 2001).

APC has been reported to be localised to the apical membrane (Miyashiro *et al.*, 1995; Reinacher-Schick & Gumbiner, 2001; Anderson *et al.*, 2002), however it seems likely that this observation is due to interaction of a number of antibodies with a protein other than full length APC (chapter 5).

APC has been reported to be localised to microtubule tips (Näthke *et al.*, 1996). The C-terminal basic domain of APC interacts with tubulin and APC is able to promote assembly of microtubules *in vitro* (Zumbrunn *et al.*, 2001; Nakamura *et al.*, 2001). APC is also able to protect microtubules from shrinking *in vitro* and from drug-induced depolymerisation *in vivo* (Munemitsu *et al.*, 1994). The majority of APC microtubule tip clusters are found at microtubule tips which meet at the plasma membrane in protrusions of actively migrating cells (Näthke

et al., 1996), which indicates APC may have a role in cellular migration (Näthke *et al.*, 1996; Barth *et al.*, 1997)

APC has also been reported to have a role in chromosome segregation. APC was shown to be localised to kinetochores in mitotic cells (Kaplan *et al.*, 2001; Fodde *et al.*, 2001). An increased incidence of abnormal mitoses and chromosomal aberrations have been observed in embryonic stem cells expressing only truncated APC compared to those expressing only wild type APC. Expression of a dominant negative construct in wild type APC cells also resulted in an increase in abnormal mitoses (Fodde *et al.*, 2001). APC has also been observed at the centrosomes, with particularly high levels of truncated APC seen at the centrosomes in cancer cells (Tighe *et al.*, 2001).

1.4 Structure and functional domains of APC

The APC gene, which has been mapped to chromosomal band 5q21 (Bodmer *et al.*, 1987), consists of 21 exons. 7 of the 21 exons are alternatively spliced, including a number of exons upstream of exon 1 (Santoro & Groden, 1997). Exon 15, the final exon of APC, is unusually large at 6574 base pairs. The most common isoform of APC is a 2843 amino acid, 312 kDa protein. Figure 1.2 shows the domain structure of APC.

The **oligomerisation domain**, which consists of a series of heptad repeats, is situated at the N-terminal end of APC and allows APC to form homo-dimers (Su *et al.*, 1993; Joslyn *et al.*, 1993). The oligomerisation domain allows full length APC to form dimers with both wild type and truncated APC, the lack of availability of wild type APC due to it being dimerised with truncated APC may account for the dominant negative effect of mutations in APC seen in many cases.

The **armadillo repeat region** consists of seven repeats, is highly conserved and is retained in mutant APC proteins (Miyoshi *et al.*, 1992a). The armadillo repeat region has been shown to bind the B56 regulatory subunit of protein phosphatase

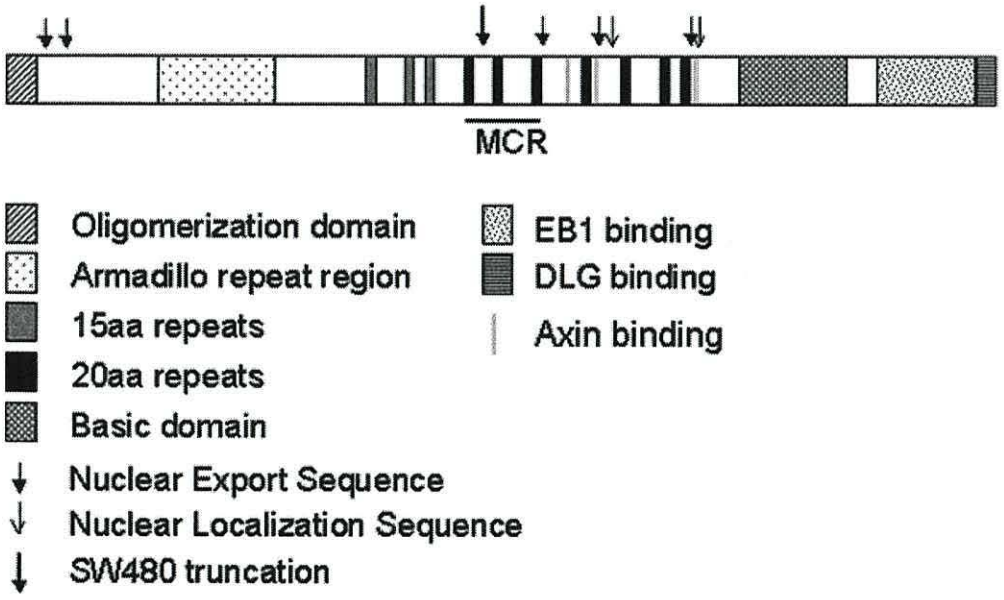


Figure 1.2
Functional domains of full length APC. "MCR" indicates the location of the mutation cluster region.

2A (PP2A), a heterotrimeric serine-threonine protein phosphatase which negatively regulates the Wnt signalling pathway (Seeling *et al.*, 1999). The armadillo repeat region also binds to the APC-stimulated guanine nucleotide exchange factor (Asef) (Kawasaki *et al.*, 2000). The armadillo repeat region may also allow APC to enter the nucleus independently of nuclear import seqences (Rosin-Arbesfeld *et al.*, 2000; Galea *et al.*, 2001).

The three **15 amino acid repeats** between amino acids 1020 and 1169 act as binding sites for β-catenin (Rubinfeld *et al.*, 1993; Su *et al.*, 1993). Unlike the 20 amino acid repeats, binding of β-catenin to the 15 amino acid repeats does not mark β-catenin for proteasomal degradation (Munemitsu *et al.*, 1995).

The central region of APC contains seven **20 amino acid repeats**, the sequence of these being TPXXFSXXXSL (Groden *et al.*, 1991). Only a single 20 amino acid repeat is necessary for binding of β-catenin, but at least three of the seven 20 amino acid repeats must be present for efficient downregulation of β-catenin (Rubinfeld *et al.*, 1997). The majority of truncations in APC eliminate most or all of the 20 amino acid repeats (Polakis, 1997).

APC contains three **axin binding sites** within the same region as the 20 amino acid repeats (Hart *et al.*, 1998; Kishida *et al.*, 1998). The axin binding sites of APC lie between the third and fourth, the fourth and fifth and after the seventh, and final, 20 amino acid repeat. Axin appears to act as a scaffold protein in the formation of the complex which phosphorylates β-catenin and marks it for proteasomal degradation (Ikeda *et al.*, 1998; Nakamura *et al.*, 1998; Kishida *et al.*, 1999).

The **basic domain** of APC lies between amino acids 2200 and 2400 at the C-terminal end of APC (Groden *et al.*, 1991). This region contains a large proportion of basic amino acids, and is thought to bind microtubules. An APC fragment consisting of amino acids 2219-2580 has been shown to bind to tubulin and promote assembly of microtubules *in vitro* (Deka *et al.*, 1998), and APC has

been shown to localise to microtubule plus ends *in vivo* (Näthke *et al.*, 1996; Mimori-Kiyosue *et al.*, 2000; Mogensen *et al.*, 2002).

The C-terminal end of APC contains an **EB1 binding** domain (Su *et al.*, 1995). The end-binding protein EB1 is closely associated with the centromere, mitotic spindle and plus ends of microtubules thoughout the cell cycle (Berruetta *et al.*, 1998; Morrisson *et al.*, 1998). Localisation of EB1 to the plus ends of microtubules occurs independently of APC (Berruetta *et al.*, 1998; Morrisson *et al.*, 1998). APC lacking the EB1 binding domain can still bind to microtubules but does not localise to plus ends, suggesting that EB1 has a role in directing APC to microtubule tips (Mimori-Kiyouse *et al.*, 2000).

The C-terminal end of APC contains a **DLG binding domain**, which binds to DLG, the human homologue of the *Drosophila* discs large tumour suppressor. The final 72 amino acids of APC are necessary for binding to DLG to take place, and an APC consisting of only the final 72 amino acids of APC binds strongly to DLG (Matsumine *et al.*, 1996).

Five **nuclear export sequences** have been identified within APC, two of these are at the N-terminal end (Neufeld *et al.*, 2000a) and three are close to the mutation cluster region (Rosin-Arbesfeld *et al.*, 2000).

Two **nuclear localisation sequences** have been identified within APC (Neufeld *et al.*, 2000b). These nuclear localisation sequences are located within the 20 amino acid repeat region, close to the central nuclear export sequences.

The majority of sporadic mutations in APC are truncating mutations within the **mutation cluster region (MCR)** which corresponds to codons 1,286-1,513 of full length APC (Miyoshi *et al.*, 1992b). Mutation within the mutation cluster region of APC usually results in expression of a truncated form of APC which lacks many, or all, of the 20 amino acid repeats, the central nuclear export sequences (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000) and the two nuclear localisation sequences (Neufeld *et al.*, 2000b).

1.5 Localisation of APC and β-catenin in normal human colon mucosa

Normal colonic mucosa consists of a large numbers of invaginations, named the crypts of Lieberkühn. A monolayer of epithelial cells lines these crypts. Stem cells are located at the base of the crypts and produce progenitor cells which occupy the lower third of the crypt. Epithelial cells towards the surface are constantly renewed as progenitor cells migrate upwards towards the surface and differentiate. The epithelial cells eventually slough off into the lumen to be replaced by differentiating cells moving up the crypt. The entire process of epithelial cell renewal takes 3-5 days (Potten & Loeffler, 1990)

β-catenin is often found in the nuclei of cells within the proliferating compartment at the bottom third of crypts, with nuclear staining being strongest at the base of the crypts (van de Wetering *et al.*, 2002). The most prominently expressed TCF family member in the intestinal epithelium is TCF-4 (Korinek *et al.*, 1997). The β-catenin/TCF-4 complex has been identified as the master switch which controls proliferation versus differentiation in healthy and malignant intestinal epithelial cells (van de Wetering *et al.*, 2002).

APC is localised to the cytoplasm in differentiated epithelial cells above the crypt, and is additionally localised to the nuclei of cells towards the base of crypts (Rosin-Arbesfeld *et al.*, 2003). Nuclear APC at the base of crypts may be related to Wnt pathway activation in the proliferative cells of the crypt (van de Wetering *et al.*, 2002). Like the proliferating cells at the base of crypts, adenocarcinomas with truncating mutation of APC and loss of heterozygosity expressed nuclear APC (Rosin-Arbesfeld *et al.*, 2003).

The localisation of APC in the intestinal epithelium has been described in a number of other studies, with many of these finding APC to be localised to the apical membrane (Miyashiro *et al.*, 1995; Midgley *et al.*, 1997; Reinacher-Schick & Gumbiner, 2001; Anderson *et al.*, 2002). However, many of these studies used antibodies that have been shown to be unreliable, or to detect proteins other

than full length APC (Rosin-Arbesfeld *et al.*, 2001; Mogensen *et al.*, 2002; Roberts *et al.*, 2003; chapters 3, 5).

1.6 The roles of APC and β-catenin in cancer: Mutations in APC or β-catenin are seen in the majority of sporadic colorectal tumours

Colorectal cancer is one of the most common cancers in both the United States of America and in Western Europe, and is one of the leading causes of cancer related morbidity and mortality in the Western world. In the USA approximately 140,000 new cases of colorectal cancer and over 50,000 deaths due to colorectal cancer are reported each year (Greenlee *et al.*, 2000). In Europe 213,000 new cases and 110,000 deaths due to colorectal cancer are reported each year (Pisani *et al.*, 1999). The lifetime risk of developing colorectal cancer within the population is approximately 6%. By the age of 70, approximately 50% of the Western population will have developed an adenoma (Pisani *et al.*, 1999).

The earliest stage of colorectal neoplasia which can be observed is aberrant crypt foci (ACF). These consist of abnormally formed crypts and can only be observed microscopically. Aberrant crypt foci can be composed of either cells of normal morphology (nondysplastic) or dysplastic cells. Aberrant crypt foci consisting of dysplastic cells are more likely to progress to a polyp: a benign mass which protrudes into the lumen of the colon. There are two categories of polyp: hyperplastic (nondysplastic) or adenomatous (dysplastic). Adenomatous polyps are also known as adenomas. Hyperplastic polyps have normal architecture and cellular morphology while adenomatous polyps are characterised by abnormalities in inter- and intra-cellular organisation. Progression of colorectal neoplasia and mutations commonly associated with colorectal cancer are shown in figure 1.3.

Mutations in APC and β-catenin have been reported in many types of cancer but are most frequently found in colorectal tumours. In non-hereditary cases of colorectal cancer, APC mutation has been reported in up to 85% of cases (Tucker

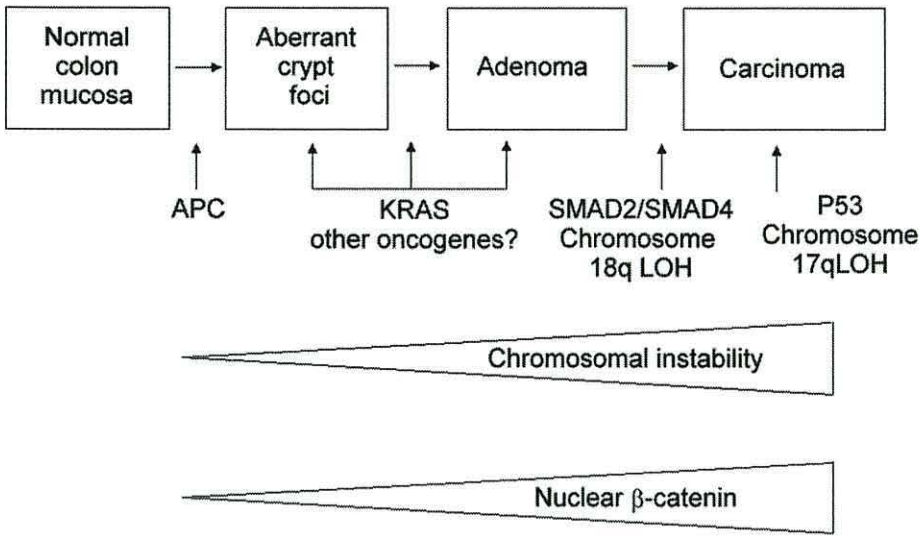


Figure 1.3
The progression of colorectal cancer. Mutation in APC is observed at the earliest observable stage of colorectal cancer, aberrant crypt foci. Some aberrant crypt foci may progress to give polyps (a benign mass which protrudes into the lumen), adenomatous (dysplastic) polyps (adenoma) may then progress to carcinoma of the colon. As colorectal carcinoma progresses, further mutations of oncogenes and tumour suppressor genes, and an increase in chromosomal instability are observed.

& Pignetelli, 2000). Mutations in APC have been found even in aberrant crypt foci: the earliest stage of colorectal cancer (Powell *et al.*, 1992; Jen *et al.*, 1994; Smith *et al.*, 1994a). Activating mutations in β-catenin, which affect functionally significant phosphorylation sites, are also common in colorectal cancer (e.g. Morin *et al.*, 1997).

1.7 The roles of APC and β-catenin in cancer: Familial Adenomatous Polyposis is a hereditary disease which leads to a high incidence of colorectal cancer, and is associated with mutation of APC

Familial adenomatous polyposis (FAP) is a hereditary condition which results in numerous adenomatous polyps in the colon and rectum of affected individuals. If these polyps remain untreated colorectal cancer develops, with the majority of patients developing colorectal tumours by the fourth decade of life. The incidence of FAP in the population is approximately 1 in 8000. Approximately 25% of all cases are novel germline mutations (Bisgaard *et al.*, 1994).

Germline mutations in APC have been identified in the majority of FAP patients (Cottrell *et al.*, 1992; Nagase & Nakamura, 1993; Laken *et al.*, 1999), with most of these being either nonsense or frameshift mutations which lead to truncation of APC. Colorectal tumours from FAP patients have been found to carry either additional somatic mutation of the second APC allele, or loss of heterozygosity at the APC locus (Miyoshi *et al.*, 1992a; Solomon *et al.*, 1987; Ichii *et al.*, 1993; Levy *et al.*, 1994; Lamlum *et al.*, 1999). Mutations at codons 1061 and 1309 account for almost a third of all germline mutations in APC. The majority of germline mutations occur between codons 200 and 1600. Mutations beyond codon 1600 are rare (Beroud & Soussi, 1996; Miyoshi *et al.*, 1992a). The location of the germline mutation within APC appears to determine the type of mutations which eventually affects the second allele. Germline mutations between codon 1194 and 1392 are associated with loss of heterozygosity, whereas germline mutations outside this region are associated with truncating mutations within the mutation cluster region (Lamlum *et al.*, 1999).

1.8 The roles of APC and β-catenin in cancer: Hereditary non-polyposis coli cancer (HNPCC)

Hereditary non-polyposis colon cancer (HNPCC) arises due to germline mutations in the DNA mismatch repair (MMR) complex (Kinzler & Vogelstein, 1996; Kolodner, 1996; Markowitz, 2000). Over 90% of cases of HNPCC arise from mutations in hMSG2 and hMLH1, two components of the mismatch repair complex (Yan *et al.*, 2000). Individuals carrying these mutations have an 80% lifetime risk of developing colon cancer and an increased risk of gastric and endometrial cancers. HNPCC tumours show somatic inactivation of the second allele of the mismatch repair gene mutated in the germline. This results in an approximately 1000-fold increase in spontaneous mutation rates, and accelerates the time taken for development of colon cancer to 36 months (Eshleman *et al.*, 1995).

Mutations in APC have been observed in 21% of HNPCC cases (Konishi *et al.*, 1996). 43% of HNPCC cases have mutations in β-catenin. In total 64% of HNPCC cases carry mutations in either APC or β-catenin, no cases of mutation in both β-catenin and APC have been observed (Miyaki *et al.*, 1999). Although HNPCC does not involve hereditary defects in APC, mutation of APC (or β-catenin) is likely to be an important step in carcinogensis in the colon of HNPCC patients.

1.9 The roles of APC and β-catenin in cancer: Mutations in APC and β-catenin have also been reported in a number of non-colorectal cancers

Mutations in APC and β-catenin are not isolated to colorectal cancer but have been found in a number of other, non-colorectal, tumours.

Of 26 analysed human melanoma cell lines, 7 were found to have abnormally high levels of free β-catenin (Rubinfeld *et al.*, 1997). In 6 of these cell lines stabilising mutations of the β-catenin gene were found. APC was mutated in two

of the cell lines. Expression of wild type APC in the two cell lines expressing mutated APC eliminated the excess β-catenin.

Stabilisation of β-catenin has also been seen in cases of aggressive fibromatosis (desmoid tumour), which can occur as a sporadic lesion or as part of Familial Adenomatous Polyposis (FAP) (Tejpar *et al.*, 1999). Of 42 sporadic aggressive fibromatoses analysed 9 tumours had mutations in APC and 22 had a point mutation of β-catenin at either codon 41 or 45 resulting in a stabilised form of β-catenin. Elevated levels of β-catenin were seen in all 42 tumours.

Hepatocellular carcinoma (HCC) is a common fatal cancer. β-catenin has been found to be mutated in around 20% of cases of HCC (Huang *et al.*, 1999). β-catenin has also been implicated in another form of liver cancer, hepatoblastoma, an embryonic liver tumour which occurs mainly in children under 2 years of age (Wei *et al.*, 2000). 12 of 18 tumours analysed were found to contain mutations in β-catenin, these were either deletions or mutations at the GSK-3 phosphorylation site. 11 tumours were analysed for localisation of β-catenin, all of these demonstrated nuclear and cytoplasmic accumulation of β-catenin.

APC and β-catenin mutations have been observed in some sporadic medulloblastomas (Huang *et al.*, 2000). Cerebellar medulloblastoma is a highly malignant and invasive tumour usually seen in children. 46 medulloblastomas were screened for mutations in APC and β-catenin. Three tumours contained miscoding mutations of APC and another four contained miscoding mutations in β-catenin.

1.10 The roles of APC and β-catenin in cancer: Mechanisms by which mutations of APC and β-catenin lead to colorectal cancer

Mutation of APC is an initiating event in many colorectal cancers, with mutations in APC being observed in aberrant crypt foci, the earliest stage of colon cancer which can be observed (Powell *et al.*, 1992; Jen *et al.*, 1994; Smith

et al., 1994a). There are a number of mechanisms by which mutations in APC can confer a selective advantage on tumour cells. These are: activation of the Wnt pathway and inappropriate expression of target genes; chromosomal instability and possibly defects in cellular adhesion.

It has been suggested that the endogenous mutation rate is not sufficient to allow the large number of genetic changes found in many human cancers, including colorectal cancers. Some form of genetic instability may be an essential requirement for tumour progression (Loeb, 2001; Loeb *et al.*, 1974). Two forms of genetic instability have been observed in colorectal cancers: microsatellite instability and chromosomal instability. Microsatellite instability arises due to defects in the DNA mismatch repair machinery and is seen in approximately 15% of cases of colorectal cancer (Thibodeau *et al.*, 1993; Parsons *et al.*, 1993; Bhattacharyya *et al.*, 1994; Eshleman *et al.*, 1995). Chromosomal instability is a more prominent form of genetic instability in colorectal cancers. Tumours with chromosomal instability have defects in chromosome segregation, leading to variation in chromosome number and other chromosomal defects (Lengauer *et al.*, 1997; Thiagalingam *et al.*, 2001). The mechanisms of chromosomal instability in colorectal cancers are not completely understood. A connection between APC mutation and chromosomal instability and localisation of APC to the kinetochores has been reported (Kaplan *et al.*, 2001; Fodde *et al.*, 2001). Therefore, mutation of APC as an early event in colorectal cancer may lead to chromosomal instability, allowing rapid accumulation of further mutations.

In well-differentiated colorectal adenocarcinoma with lymph node metastases it has been reported that cells at the invasive front are de-differentiated while cells in the central mass of the tumours are epithelial in appearance (Brabletz *et al.*, 2001). In the central mass, where cells have the appearance of differentiated epithelial cells, β-catenin is found at the membrane. In de-differentiated mesenchymal-like cells at the invasive front β-catenin was found to be nuclear in the majority of cells with little or no membrane staining. Many of these tumours were found to have mutations in APC and some had mutations in β-catenin. In cell lines expressing only mutated APC, both APC and β-catenin are found to

accumulate in the nucleus (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000). Mutation of APC may be important in allowing nuclear localisation of β-catenin at the invasive front. However, not all tumours analysed were found to express mutated APC. Therefore, while mutations in APC may be beneficial to tumour cells in terms of being able to form metastases, mutations in APC may not be essential for metastasis of colorectal tumours.

It seems that the most important consequence of mutation of APC or β-catenin in colorectal cancers is inappropriate activation of Wnt target genes. Mutations in β-catenin commonly affect the phosphorylation sites. Loss, or alteration, of the phosphorylation sites allows β-catenin to escape degradation and accumulate in the cell as it is phosphorylation of β-catenin which marks it for degradation (Amit *et al.*, 2002; Hagen & Vidal-Puig, 2002; Liu *et al.*, 2002; Sakanaka, 2002; van Noort *et al.*, 2002; Sadot *et al.*, 2002). Phosporylation also influences the sub-cellular localisation of β-catenin, with de-phosphorylated β-catenin being the form that accumulates in the nucleus (Staal *et al.*, 2002).

APC regulates the degradation (reviewed in Bienz, 1998) and localisation (Staal *et al.*, 2002) of β-catenin by its role in phosphorylation of β-catenin; regulates the localisation of β-catenin by removing it from the nucleus via the CRM1 nuclear export pathway (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a; Neufeld *et al.*, 2000b) and regulates the transcriptional activity of β-catenin (Rosin-Arbesfeld et al., 2003). The majority of mutations in APC observed in sporadic cases of colorectal cancer are truncating mutations within the mutation cluster region, which corresponds to codons 1,286-1,513 of full length APC (Miyoshi *et al.*, 1992b). Mutations within the mutation cluster region of APC usually result in expression of a truncated form of APC which lacks many, or all, of the 20 amino acid repeats and all of the central nuclear export sequences (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000). Loss of the 20 amino acid repeats leads to defects in control of phosphorylation, and subsequent degradation, of β-catenin. Loss of efficient phosphorylation of β-catenin also allows nuclear accumulation of β-catenin as it is β-catenin dephosphorylated at the Wnt-dependent phosphorylation sites Ser37 and Thr41

which is able to enter the nucleus (Staal et al., 2002). Loss of the central nuclear export sequences leads to nuclear accumulation of both APC and β-catenin (Rosin-Arbesfeld et al., 2000, Henderson, 2000). It is the rate of nuclear export of APC which determines transcriptional activity of β-catenin (Rosin-Arbesfeld *et al.*, 2003). Availability of β-catenin in the nucleus combined with inefficient nuclear export of truncated APC would lead to activation of Wnt pathway target genes.

APC is localised to the cytoplasm in differentiated epithelial cells above the crypt, and is additionally localised to the nuclei of cells towards the base of crypts (Rosin-Arbesfeld *et al.*, 2003). Nuclear APC at the base of crypts may be related to Wnt pathway activation in the proliferative cells of the crypt (van de Wetering *et al.*, 2002). Like the proliferating cells at the base of crypts, adenocarcinomas with truncating mutation of APC express nuclear APC (Rosin-Arbesfeld *et al.*, 2003). As nuclear localisation of APC in cells towards the base of crypts is associated with proliferation and may be due to Wnt pathway activation, nuclear accumulation of APC (and β-catenin) in colorectal cancer cells may also result in increased proliferation.

Truncating mutations in APC and mutation of phosphorylation sites within β-catenin, as described above, leads to defects in control of the degradation and localisation of β-catenin. This leads to inappropriate activation of Wnt pathway target genes in tissues or situations where these genes would not normally be transcriptionally active. The functions of Wnt pathway target genes in development and cancer gives an indication of how their regulation by the Wnt signalling pathway is important in development, and how mutations in components of the Wnt signalling pathway can lead to cancer by inappropriate expression of these genes. A large number of Wnt-responsive genes which are important in cancer and development have been identified, only a few examples relevant to colorectal tumours are discussed here.

The Cyclin D1 gene was shown to be a direct target of the Wnt signalling pathway through Lef-1 binding sites in the Cyclin D1 promoter (Shtutman *et al.*,

1999). Cyclin D1 protein levels were elevated in cells overexpressing β-catenin and reduced in cells overexpressing the cytoplasmic domain of E-cadherin, which sequesters β-catenin. In colon cancer cells expression of Cyclin D1 was reduced by inhibitors of β-catenin, wild-type APC, axin and the cytoplasmic tail of E-cadherin. Expression of a dominant negative TCF in colon cancer cells was shown to inhibit expression of cyclin D1 (Tetsu & McCormick, 1999). Increased β-catenin levels may therefore promote neoplastic conversion by triggering inappropriate expression of Cyclin D1, leading to uncontrolled progression through the cell cycle.

The oncogene c-MYC has been identified as a target of the Wnt signalling pathway (He *et al.*, 1998). Expression of c-MYC was shown to be activated by β-catenin and repressed by wild-type APC. The regulation of c-MYC is mediated by TCF-4 binding sites in the promoter. MYC family proteins promote proliferation, growth and apoptosis and inhibit terminal differentiation (reviewed in Grandori *et al.*, 2000).

Another target of the Wnt pathway which is important in human colorectal cancers is matrix metalloproteinase-7 (MMP-7) (Brabletz *et al.*, 1999). MMP-7 is overexpressed in 80% of human colorectal cancers and is an important factor in early tumour growth. MMP-7 is regulated by β-catenin/TCF-4.

Four Wnt/β-catenin target genes were identified in a screen for genes affected by β-catenin overexpression in colorectal cell lines (Mann *et al.*, 1999). These genes are c-jun, fra-1, urokinase-type plasminogen activator receptor (uPAR) and ZO-1. C-jun and fra-1 are components of the AP-1 transcription complex which activates transcription of uPAR. ZO-1 is involved in epithelial polarization. C-jun, fra-1 and uPAR are all upregulated by Wnt/β-catenin signalling. ZO-1 was observed to be downregulated by β-catenin in colorectal cancer cell lines.

Overexpression of the CD44 family of cell surface glycoproteins has been shown to be an early event in colorectal tumorigenesis (Wielenga *et al.*, 1999). CD44 expression in non-neoplastic intestinal mucosa of mice with defects in APC was

confined to the crypt epithelium. In both adenomas and invasive carcinomas CD44 was strongly overexpressed. Deregulated CD44 expression can be detected in the earliest detectable lesions of colorectal neoplasia, aberrant crypt foci with dysplasia (ACFs). ACFs of familial adenomatous polyposis (FAP) patients also overexpress CD44.

Expression of cyclooxygenase-2, an inducible prostaglandin synthase, is important in intestinal tumorigensis resulting from APC mutations. Wnt-1 expression in the mouse mammary epithelial cell lines RAC311 and C57MG stabilises cytoplasmic β-catenin, and leads to upregulation of cyclooxygenase-2 (Howe *et al.*, 1999).

Many of the target genes of the Wnt/β-catenin pathway are important in control of cell growth, proliferation and differentiation, and many are known to be upregulated in colorectal cancers (see above). Therefore an important consequence of mutation in APC (or β-catenin) in colorectal cancers is de-regulation and inappropriate expression of Wnt pathway target genes, leading to abnormal proliferation and de-differentiation.

The β-catenin/TCF-4 complex has been identified as the master switch which controls proliferation versus differentiation in healthy and malignant intestinal epithelial cells (van de Wetering *et al.*, 2002). In normal crypts β-catenin is localised to the nuclei of proliferating cells at the base of crypts, but not the differentiated cells towards the surface. Nulcear accumulation of β-catenin, due to mutation in either β-catenin or APC, could lead to an active β-catenin/TCF-4 complex and allow inappropriate expression of target genes. Mutations in APC, as an early event in colorectal cancer progression, would then allow inappropriate expression of a number of oncogenes and tumour suppressor genes with cells which would normally differentiate being able to retain a proliferative progenitor phenotype.

1.11 Cell lines: Colorectal cancer cell lines

Two colorectal cancer cell lines are used here: HCT116 and SW480.
HCT116 cells were derived from a human colorectal carcinoma and have a 3 base pair deletion in β-catenin resulting in loss of a serine residue at codon 45 (Ilyas *et al.*, 1997; Morin *et al.*, 1997; Sparks *et al.*, 1998). This serine residue is phosphorylated by the Casein Kinase Iε (CKIε) pathway and must be phosphorylated before Wnt-dependent phosphorylation of residues Ser33, Ser37 and Thr41 can occur (Amit *et al.*, 2002; Hagen & Vidal-Puig, 2002; Liu *et al.*, 2002; Sakanaka, 2002).

SW480 cells were derived from a human colorectal adenocarcinoma. The tumour from which the SW480 cell line was derived gave rise to lymph node metastases (Leibovitz *et al.*, 1976). SW480 cells have a single base pair mutation at codon 1338 of APC resulting in a stop codon and truncation of the protein at 1337 amino acids. This cell line also has loss of heterozygosity at the APC locus and therefore only expresses APC truncated at 1337 amino acids (Nishisho *et al.*, 1991).

1.12 Cell lines: Epithelial cell lines expressing wild type APC and β-catenin

Three epithelial cell lines, all expressing only wild type APC and wild type β-catenin were used. These were HEK293, C57MG and MDCK.

HEK293 is a cell line derived from human embryonic kidney and transformed by exposure to fragments of adenovirus type 5 DNA (Graham *et al.*, 1977). These cells are epithelial in morphology. No defects in the Wnt signalling pathway have been identified in this cell line.

C57MG is a mouse mammary epithelial cell line with no known defects in the Wnt signalling pathway. This cell line is responsive to Wnt-1, and shows transformation and deregulated growth in the presence of a Wnt signal (Brown *et al.*, 1986)

MDCK (Madine Darby Canine Kidney) cells are derived from the kidney of a healthy adult female cocker spaniel. The original MDCK cell line is heterogeneous, and epithelial in morphology. This cell line has no known defects in the Wnt signalling pathway. Two sublines have been derived from the MDCK parent line, MDCKI and MDCKII. These two MDCK sublines have the morphology of polarised epithelial cells and the MDCKII line has previously been used to study APC localisation in polarised epithelial cells (e.g. Näthke *et al.*, 1996; Rosin-Arbesfeld *et al.*, 2001). The parent MDCK line has also been used to analyse APC localisation and function (e.g. Zhang *et al.*, 2001) and it is this cell line which is used here.

1.13 Cell lines: Embryonal carcinoma cell line NTERA-2

NTERA-2 is a human embryonal carcinoma cell line. NTERA-2 is a sub-line derived from TERA-2, which was originally obtained by culturing embryonal carcinoma (EC) cells from a lung metastasis of a testicular teratocarcinoma (Fogh & Trempe, 1975). The TERA-2 cell line was passaged through a *nu/nu* nude mouse, a mouse which lacks a functional immune system. A well differentiated teratocarcinoma was formed which consisted of glandular structures, mesenchyme, neural elements and embryonal carcinoma cells. A number of sub-lines, including NTERA-2 Clone D1 (referred to here as NTERA-2) were derived from the EC cells of this teratocarcinoma (Andrews *et al.*, 1984).

Teratocarcinomas are generally composed of a variety of adult, embryonic and extra-embryonic tissues and EC cells (Andrews *et al.*, 1984). The embryonal carcinoma cells behave as pluripotent stem cells which give rise to all other cell types in the tumour. Embryonal carcinoma cells may originate from a displaced embryonic stem (ES) cell which somehow retains its pluripotency into postnatal life. Alternatively, they may originate from germ cells which manage to proliferate without entering meiosis.

When cultured in the presence of retinoic acid NTERA-2 cells differentiate to give neurones and other cell types (Andrews, 1984; Fenderson *et al.*, 1987; Thompson *et al.*, 1984). NTERA-2 embryonal carcinoma cells commit to differentiate within 1-2 days of exposure to retinoic acid. In the absence of retinoic acid very little spontaneous differentiation is seen, unless cells are grown at very low density (Andrews *et al.*, 1984). The mechanisms which regulate differentiation of NTERA-2 cells in response to retinoic acid are not well understood.

Wnt-13 expression has been detected in NTERA-2 cells differentiating in response to retinoic acid and hexamethyl bisacetamide (HMBA) (Wakeman *et al.*, 1998). Wnt-13 expression was detected in the early phases of differentiation in response to both retinoic acid and HMBA, and in non-neural differentiated cells for several weeks after induction of differentiation. No Wnt-13 expression was detected iN-terminally differentiated neurones or in undifferentiated embryonal carcinoma cells. Mouse Wnt-13 is expressed in the embryonic mesoderm during gastrulation (Zakin *et al.*, 1998). Later in development Wnt-13 is expressed in the dorsal midline of the diencephalon and mesencephalon, the heart primordia, the periphery of the lung bud and the otic and optic vesicles.

Differentiation of NTERA-2 can also be induced by treatment with 7.5mM lithium chloride, usually for 7 days. Treatment with lithium chloride mimics Wnt signalling by inhibiting GSK3 (Klein & Melton, 1996), leading to accumulation of free β-catenin. Large flat cells formed in response to treatment with lithium chloride are similar to those seen in cultures in which cells have differentiated due to low density (Giesberts *et al.*, 1999). The morphology of these large flat cells also resembles that of small patches of large flat cells that form in retinoic acid induced cultures. The pattern of antigen expression in lithium chloride treated cells is also similar to that seen in the large flat cells in retinoic acid induced cultures (Fenderson *et al.*, 1987). It is possible that Wnt-13 expression in NTERA-2 cells in response to retinoic acid or HMBA induction is an important step in differentiation in response to these inducers.

It has been suggested that NTERA-2 cells are committed neuronal precursor cells (Pleasure & Lee, 1993). However, they express characteristic features of human EC cells (Andrews *et al.*, 1996). NTERA-2 embryonal carcinoma cells can also be induced to differentiate into non-neural cells by treatment with hexamethylene bisacetamide (HMBA) (Andrews *et al.*, 1990) and bone morphogenetic protein 7 (BMP7) (Andrews *et al.*, 1994). They have been observed to form mesenchyme rather than neurones in response to retinoic acid treatment when the gamma retinoic acid receptor is overexpressed (Moasser *et al.*, 1994; Moasser *et al.*, 1995). Therefore they are not likely to be restricted to neuronal differentiation.

Interaction between the retinoic acid and Wnt signalling pathways has been observed in colon epithelial cells (Easwaran *et al.*, 1999). The retinoic acid receptor RARβ negatively regulates Wnt target genes by competing with TCF/Lefs for binding to β-catenin. However, it is the alpha and gamma forms of the retinoic acid receptor that are expressed in NTERA-2 cells (Andrews, 1998) therefore the effect of retinoic acid on NTERA-2 may be very different to that seen in colon epithelial cells. Overexpression of RARγ resulted in mesenchymal differentiation in preference to neural differentiation on retinoic acid induction, suggesting that the various retinoic acid receptors have a role in influencing the direction of NTERA-2 differentiation (Moasser *et al.*, 1994, Moasser *et al.*, 1995).

1.14 Aims, objectives and overview

Analysis of the Wnt signalling pathway in differentiation of NTERA-2 cells

One of the initial aims of this project was to investigate expression of Wnt pathway components during retinoic acid induced differentiation in NTERA-2, and attempt to identify novel homologues of Wnt pathway components. To do this a degenerate primer RT-PCR screen was carried out targeting three Wnt pathway components, TLE, dishevelled and TCF. At the time this work was carried out the human genome project had not yet been completed. This work is discussed in chapter 7. As no novel homologues of Wnt pathway components

were identified in this screen, and no changes in localisation of β-catenin were seen in response to retinoic acid treatment,this part of the project was not pursued further.

Another of the initial aims of this project was to study localisation and interactions of two known Wnt pathway components, APC and β-catenin, in colorectal cancer cell lines and epithelial cell lines. As described above, mutations in β-catenin and APC are commonly seen in colorectal cancers. A panel of antibodies were used to study the subcellular localisation of APC and β-catenin in a variety of cell lines. This included normal epithelial cell lines (HEK293, C57MG, MDCK), colorectal cancer cell lines with mutation in either APC (SW480) or β-catenin (HCT116) and the embryonal carcinoma cell line, NTERA-2. At the outset of this study, little was known of the localisation of APC and much of the work describing localisation of APC (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a; Neufeld *et al.*, 2000b) had not yet been published.

Characterisation of reactivity of a panel of APC antibodies

It became apparent that different APC antibodies detected APC at varying locations within the cell. Validation of reactivity of antibodies was therefore of paramount importance. A panel of antibodies to APC were used and western blots carried out as a first step in characterising reactivity of these antibodies. Western blots enabled us to determine whether antibodies were able to detect full length and truncated APC, and potentially whether antibodies cross-reacted with other proteins. As well as western blotting, localisation of APC was determined by immunofluoresecence and data was compared for a variety of antibodies. These data gives an indication of whether these antibodies can reliably be used for immunodetection of APC or if they are detecting other protein species. This work is discussed in chapter 3.

Analysis of variations in localisation and interactions of APC and β-catenin with cell density

In immunofluroescence staining experiments with the APC antibodies anti-APC (M-APC) and anti-APC (N-15) the localisation of truncated APC was observed to vary with cell density. The localisation of β-catenin also varied with cell density in the two colorectal cancer cell lines (SW480 & HCT116) studied. The aims of this section of the project was to further characterise cell density dependent variation in localisation and seek explanations for the variation seen. To attempt to meet these aims co-immunofluorescence for APC and β-catenin was carried out at low and high cell density in a variety of cell lines, two colorectal cancer cell lines (SW480, HCT116), three epithelial cell lines (HEK293, C57MG, MDCK) and the embryonal carcinoma cell line NTERA-2. To seek explanations for the observations made in immunofluorescence studies, attempts were made to correlate localisation of APC and β-catenin to junctional integrity via E-cadherin expression, and proliferative status of the cells by examining Ki67 expression. Blocking of CRM1-dependent nuclear export was used to examine nuclear-cytoplasmic shuttling of APC and β-catenin in a variety of cell lines at varying densities. Western blots and co-immunoprecipitation were used to examine expression levels and interactions of APC, β-catenin and E-cadherin at various cell densities. This work is discussed in chapter 4.

Characterisation of an apical protein detected by multiple APC antibodies

Antibody characterisation (chapter 3) revealed that a number of antibodies detect an apical protein in a number of cell lines. This protein could not be full length APC, as it was detected by C-terminal directed APC antibodies in SW480 cells, which only express truncated APC. Also, this apical staining was not seen with the APC antibody M-APC, which is known to reliably detect full length APC. We sought to further characterise the localisation seen with these APC antibodies in a variety of cell lines and to determine the molecular basis for this apical staining. To attempt to determine the molecular basis of this apical staining western blots were carried out to determine the size of a likely candidate protein and immunoprecipitation and mass spectrometry analysis was carried out to attempt to identify the protein. RT-PCR and northern blots were carried out to

attempt to identify the position of a potential alternative splice site within exon 15 of APC. This work is discussed in chapter 5.

A screen for novel interacting partners of APC and the 150 kDa potential isoform of APC

To identify novel interacting partners of full length APC and the 150 kDa potential isoform of APC (described in chapter 5), a screen involving co-immunoprecipitation with a variety of APC antibodies and mass spectrometry analysis was carried out. As little is known of this 150 kDa protein, which is detected by multiple APC antibodies, information concerning its interacting partners would give useful clues to its function. This work is discussed in chapter 6.

Chapter 2 – Materials and Methods

2.1 Source of cell lines

C57MG were a gift from A.M.C Brown (Cornell University Medical College, New York), NTERA-2 (clone D1) were a gift from P.W. Andrews (University of Sheffield). All other cell lines (SW480, HCT116, HEK293, MDCK) were obtained from the European Collection of Cell Cultures (ECACC).

2.2 Routine cell culture

NTERA-2, SW480 and C57MG cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma/Invitrogen) supplemented with 10% foetal bovine serum (FBS) (Invitrogen). MDCK and HEK293 were cultured in minimum essential medium (Eagle) (Sigma/Invitrogen) supplemented with 10% FBS and 0.1mM non-essential amino acids (Sigma). HCT116 were cultured in McCoy's 5A medium (modified) (Sigma/Invitrogen) supplemented with 10% FBS. NTERA-2 were maintained in a 37°C incubator with 10% CO_2, all other cell lined were maintained in a 37°C incubator with 5% CO_2. NTERA-2 were routinely passaged with 3mm glass beads or Trypsin-EDTA solution (Sigma/Invitrogen), all other cell lines were routinely passaged with trypsin-EDTA.

2.3 Cell density experiments

For immunofluorescence cells were grown in 40mm tissue culture dishes. HCT116 and SW480 cells were seeded at a density of $5x10^5$ cells/dish, MDCK cells were seeded at $5x10^4$ cells/dish, HEK293 cells were seeded at $1x10^6$ cells/dish, C57MG cells were seeded at $1x10^5$ cells/dish and NTERA-2 were seeded at $5x10^5$ cells/dish. Cells were fixed and stained (see section 2.8) after being allowed to grow for 12-24 hours (sub-confluent), 3 days (confluent) or 5 days (super-confluent).

For western blot sub-confluent cells were grown for 12-24 hours in a T75 cell culture flask, super-confluent cells were allowed to grow for 48 hours after reaching confluence (typically 4-5 days total growth time) in a T25 flask. Western blot was carried out as described in section 2.9. Seeding densities for a T25 flask were: SW480, HCT116, NTERA-2 – $1.5x10^6$ cells/flask; MDCK – $1.5x10^5$ cells/flask; HEK293 – $3x10^6$ cells/flask; C57MG – $3x10^5$ cells/flask.

For immunoprecipitation one T75 flask of super-confluent cells and two T150 flasks of sub-confluent cells were used. Growth time was as for western blot (above). Immunoprecipitation was carried out as described in section 2.11.

2.4 Leptomycin B treatment

Cells were seeded into 40mm tissue culture dishes as described above for cell density experiments and allowed to grow to the required density. Cells were then treated with Leptomycin B (LMB) (Sigma) at a concentration of 10ng/ml for 4 or 16 hours. Untreated controls were grown alongside LMB treated cells. Indirect immunofluorescence was carried out as described below.

2.5 Nocodazole treatment

HCT116 cells were seeded at a density of $5x10^5$ cells/dish in 40mm tissue culture dishes and allowed to become confluent (3 days). Cells were then incubated for 15 minutes on ice with 30μM nocodazole (Sigma) followed by 60 minutes at 37°C in the same media. Control cells were treated similarly with an equivalent volume of di-methylsulphoxide (DMSO), the solvent for the nocodazole stock solution. Completely untreated cells were also stained alongside the treated cells and control cells. Immunofluorescence was carried out as described in section 2.8.

2.6 Cell cycle experiments

For mimosine synchronisation HCT116 and SW480 cells were seeded at a density of $3x10^4$ cells/dish in 40mm cell culture dishes or $2x10^5$ cells/T25 flask in normal media (10% FBS) and allowed to grow for 2 days. Cells were then incubated in reduced FBS (0.1%) media for 48 hours followed by 12-16 hours in normal 10% FBS media with 300μM mimosine (stock: 5mM dissolved in media). Following mimosine treatment cells were returned to normal media and allowed to progress through the cell cycle until they reached the required stage of the cell cycle (as determined by propidium iodide staining and flow cytometry, section 2.7).

For synchronisation by double thymidine block HCT116 and SW480 cells were seeded at a density of at a density of $3x10^4$ cells/dish in 40mm cell culture dishes or $2x10^5$ cells/T25 flask in normal media (10% FBS) and allowed to grow for 1 day. Cells were then treated with 2.5mM thymidine for 16 hours, returned to normal media for 10 hours and again treated with 2.5mM thymidine for 16 hours. Cells were then returned to normal media and allowed to progress through the cell cycle until they reached the required stage of the cell cycle (as determined by propidium iodide staining and flow cytometry, section 2.7).

40mm dishes were used for indirect imunofluorescence staining as described below. For G1 analysis unsynchronised cultures were used as the majority of unsynchronisd cells were seen to be in G1. For S phase HCT116 cells were allowed to progress through the cell cycle for 4.5 hours following mimosine synchronisation. For G2 HCT116 cells were allowed to progress through the cell cycle for 13 hours following mimosine synchronisation. SW480 cells did not synchronise well enough for G1/S/G2 synchronisation. For mitosis SW480 and HCT116 cells were either fixed 7.5 hours after return to normal media following double thymidine block or unsynchronised cultures were used. For estimation of the proportion of abnormal mitoses untreated cells were used. Synchronisation was assessed by propidium iodide staining and flow cytometry analysis as described in section 2.7.

2.7 Flow cytometry analysis of cell cycle

One T25 flask was used for each time-point to confirm cells used for cell cycle experiments were at required stage of the cell cycle. Cells were harvested by trypsinisation, washed twice in PBS/2%FBS and resuspended at 1-2x10^6 cells/ml. 3 volumes of cold absolute ethanol was added to the cell suspension and cells were fixed at 4°C for 1 hour. Cells were then washed twice in PBS and incubated in propidium iodide staining solution (3.8mM sodium citrate, 50µg/ml RNaseA, 50µg/ml propidium iodide) for 3 hours at 4°C. Cells were analysed on a Partec PASIII flow cytometer.

2.8 Immunofluorescence

Cells were washed in phosphate buffered saline (PBS) then fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were then washed in PBS before being permeabilised in 0.2% Triton-X-100 for 15 minutes at room temperature. After a further PBS wash cells were blocked in PBS-5%FBS for 1 hour at room temperature. Cells were then incubated with primary antibodies (see table 2.1) diluted in PBS-5%FBS for 30-60 min at 37°C. Cells were then rinsed in PBS three times for 5 minutes before incubation for 30-60 minutes at 37°C in secondary antibodies (see table 2.2) diluted in PBS-5%FBS. Following secondary antibody incubation cells were again washed three times in PBS and stored in PBS until they were imaged using a Zeiss Axioplan 2 confocal microscope. For propidium iodide staining 50µg/ml RNaseA was added to the primary antibody solution and 1µg/ml propidium iodide added to the PBS in the second wash step after secondary antibody incubation.

A control immunofluorescence labelling was carried out simultaneously which lacked primary antibody. To control for cross-reactivity in multiple labelling experiments, immunofluorescence staining was carried out as described above

with each of the primary antibodies used, and all the relevant secondary antibodies.

2.9 Western blot

Cells were washed twice with PBS and detached from the flask surface using a scraper. Cells were then centrifuged at 2000g for 5 minutes and resuspended in lysis buffer (50mM Tris-HCl pH7.4, 100mMKAc, 1mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride), 10μM E-64, 2μg/ml aprotinin, 1μM pepstatin, 10μM bestatin, 100μM leupeptin, 1mM sodium ortho-vanadate). An equal volume of Laemmli buffer (20%(v/v) glycerol, 0.2%(w/v) bromophenol blue, 4%(w/v) SDS (sodium dodecyl sulphate), 200mM DTT (Dithiothreitol), 100mM Tris-HCl pH6.8) was added and lysate was boiled for 5 minutes before being loaded on a 0.75mm thick 7.5% SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis). For size determination Precision Protein™ prestained broad range protein standards (BioRad) or Prestained protein ladder 10-180KDa (MBI Fermentas) were run alongside the protein samples. Gels were run at 110 volts for 1 hour followed by 200 volts for 2 hours. Proteins were transferred to Hybond P PVDF membrane (Amersham) at 600mA for 15-18 hours in either 10mM CAPS (3-[cyclohexylamino]-1-propane sulfonic acid) (pH11.0) with 1% methanol or 2x Towbin buffer (380mM Glycine, 50mM Tris) with 0.02% SDS. Immunodetection was carried out in 10% milk in PBS with 0.1% Tween 20 for monoclonal primary antibodies and 0.5% Tween 20 for polyclonal primary antibodies. Incubation with antibody solutions was at room temperature for 1 hour, with a wash step of 15 minutes in milk solution between incubation with primary and secondary antibodies. Antibody details can be seen in tables 2.1 and 2.2. Following incubation with secondary antibody membranes were washed once for 10 minutes in milk solution, followed by two 10 minute washes in PBS/0.1% Tween 20 (for monoclonal primaries) or PBS/0.5% Tween 20 (for polyclonal primaries). Bands were visualised on Kodak X-OMAT AR film with Western Blotting Luminol Reagent (Santa-Cruz) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Table 2.1 Primary Antibodies

Antibody	Clone no. / ID no.	Source	Host	Stock conc.	IF	Western	IP
APC (ALI 12-28)	ALI 12-28	Upstate Biotech.	Mouse IgG_1	500µg/ml	1/250	1/2500	-
APC (C-20)	C-20	Santa-Cruz	Rabbit	200µg/ml	1/75	1/1000	-
APC (H-290)	H-290	Santa-Cruz	Rabbit	200µg/ml	1/75	1/3000	-
APC (N-15)	N-15	Santa-Cruz	Rabbit	200µg/ml	1/75	1/1000	A/G
APC (Ab1)	FE9	CN Biosciences	Mouse IgG_1	100µg/ml	1/50	1/100	-
APC (Ab2)	IE1	CN Biosciences	Mouse IgG_1	100µg/ml	1/50	1/100	A/G
APC (Ab3)	AC4	CN Biosciences	Mouse IgG_1	100µg/ml	1/50	1/100	-
APC (Ab4)	HG2	CN Biosciences	Mouse IgG_1	100µg/ml	1/50	1/100	-
APC (Ab5)	CF11	CN Biosciences	Mouse IgG_{2b}	100µg/ml	1/50	1/100	A/G
APC (Ab6)	DB1	CN Biosciences	Mouse IgG_1	100µg/ml	1/50	1/100	A/G
APC (Ab7)	CC-1	CN Biosciences	Mouse IgG_{2b}	100µg/ml	1/50	1/100	-
APC (Ab120)	c-APC 28.9	AbCam	Mouse IgG_1	1mg/ml	1/150	1/2500	-
APC (M-APC)	-	I. Nathke	Rabbit (crude)	-	1/50	-	-
APC (M-APC)	-	I. Nathke	Rabbit (affinity purified)	1.3mg/ml	1/500	-	-
APC2 (A-17)	A-17	Santa-Cruz	Goat	200µg/ml	1/75	-	-
β-catenin	E-5	Santa-Cruz	Mouse IgG_1	200µg/ml	1/75	1/1000	A/G
β-catenin	H-102	Santa-Cruz	Rabbit	200µg/ml	1/75	1/1000	A/G
β-catenin p33,37,41	-	Cell Signalling	Rabbit	-	-	1/1000	-
β-catenin p41,45	-	Cell Signalling	Rabbit	-	-	1/1000	-
Drebrin	254.2	W.W. Franke	Guinea Pig	-	1/500	1/2500	A
E-cadherin	36	Transduction labs.	Mouse IgG_{2a}	250µg/ml	1/250	1/2500	-
Ki-67	PP-67	Sigma	Mouse IgM	-	1/500	-	-
Lamin A+C	JoL2	Chemicon	Mouse IgG	-	-	1/50	-
MyosinIIa	-	Covance	Rabbit	1mg/ml	1/1000	1/5000	A/G
αTubulin	B-5-1-2	Sigma	Mouse IgG_1	-	-	1/10,000	-
αTubulin	YOL1/34	AbCam	Rat IgG_{2a}	1mg/ml	1/100	-	-
Tubulin	-	Sigma	Rabbit	-	-	1/500	-

Table 2.2 Secondary Antibodies

Antibody	Conjugate	Stock conc.	Use	Source
Donkey anti-goat	Cy3	1mg/ml	IF (ML) 1/500	Jackson Immunoresearch
Donkey anti-guinea pig	Cy3	1mg/ml	IF (ML) 1/400	Jackson Immunoresearch
Goat anti-mouse	AlexaFlour 488	2mg/ml	IF (ML) 1/400	Molecular Probes
Goat anti-mouse	AlexaFluor 568	2mg/ml	IF (ML) 1/400	Molecular Probes
Goat anti-rabbit	AlexaFluor 488	2mg/ml	IF (ML) 1/200	Molecular Probes
Goat anti-rabbit	AlexaFlour 568	2mg/ml	IF (ML) 1/200	Molecular Probes
Goat anti-rabbit	Cy5	1.5mg/ml	IF (ML) 1/400	Jackson Immunoresearch
Goat anti-rat	AlexaFluor 568	2mg/ml	IF (ML) 1/100	Molecular Probes
Donkey anti-guinea pig	HRP	1mg/ml	W 1/40,000	Jackson Immunoresearch
Goat anti-mouse	HRP	400µg/ml	W 1/1000	Santa-Cruz
Goat anti-rabbit	HRP	400µg/ml	W 1/2000	Santa-Cruz

Abbreviations for tables 2.1 & 2.2: "IP": Immunoprecipitation "W": Western "IF": Immunofluorescence "-": not known/not used "A": protein A agarose "G": Protein G agarose "ML": Suitable for multiple labelling (Highly cross-adsorbed) "HRP": Horseradish peroxidase

2.10 Fractionation of cell lysates

Cells were washed twice with PBS and detached from the flask surface using a scraper. Cells were then centrifuged at 2000g for 5 minutes and resuspended in hypotonic buffer (50mM Tris-HCl pH7.4, 0.1M sucrose, 1mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride), 10μM E-64, 2μg/ml aprotinin, 1μM pepstatin, 10μM bestatin, 100μM leupeptin, 1mM sodium ortho-vanadate). An equal volume of lysis buffer C was added (1% NP-40, 10mM magnesium chloride, 1mM AEBSF, 10μM E-64, 2μg/ml aprotinin, 1μM pepstatin, 10μM bestatin, 100μM leupeptin, 1mM sodium ortho-vanadate). Lysates were then centrifuged at 6000g for 2 minutes, the supernatant (cytoplasmic fraction) was removed and an equal volume of Laemmli buffer added. Lysis buffer N (50mM Tris-HCl pH7.4, 100mMKAc, 1mM AEBSF, 10μM E-64, 2μg/ml aprotinin, 1μM pepstatin, 10μM bestatin, 100μM leupeptin, 1mM sodium ortho-vanadate) and an equal volume of Laemmli buffer was added to the remaining nuclei. Samples were boiled and western blot carried out as described above.

2.11 Immunoprecipitation

Cells were washed twice with PBS and detached from the flask surface using a scraper. Cells were then centrifuged at 2500g for 5 minutes and resuspended in lysis buffer 200-500μl (50mM Tris-HCl pH7.4, 200mM potassium acetate, 0.5% Triton-X-100, 1mM AEBSF, 10μM E-64, 2μg/ml aprotinin, 1μM pepstatin, 10μM bestatin, 100μM leupeptin, 1mM sodium ortho-vanadate). Cells were then lysed in a FastPrep FP120 for 45 seconds on speed 6.5 and centrifuged at 20,000g at 4°C for 20 minutes. Supernatants were collected to fresh tubes and 20μg/ml antibody added, the lysates were then incubated at 4°C for 2-3 hours. Negative controls were processed alongside, normal IgG from the relevant species was added to these instead of antibody.

Approximately 30μL protein A or Protein G linked to agarose beads was then added to the lysates, which were then incubated on ice with shaking for 1 hour.

For pre-blocking the protein A/G agarose beads were incubated on ice for 1 hour with shaking in a 4% milk/PBS solution before being washed 5 times with PBS. Following incubation with the lysate/antibody mix the protein A/G beads were washed three times in either lysis buffer or a more stringent wash buffer (50mM Tris-HCl pH7.4, 400mM KAc, 0.5%(v/v) Triton-X-100, 0.5%(w/v) deoxycholic acid, 1mM AEBSF, 10μM E-64, 2μg/ml aprotinin, 1μM pepstatin, 10μM bestatin, 100μM leupeptin, 1mM sodium ortho-vanadate). The more stringent wash buffer was used only in situations where one of the proteins involved in the immunoprecipitation gave a large amount of non-specific interaction with the protein A/G agarose beads. 20μl Laemmli buffer was then added to the protein A/G beads. Following boiling for 5-10 minutes samples were loaded onto SDS-PAGE gels.

For western blotting SDS-PAGE gels and transfers were performed as described in section 2.9. For mass spectrometry analysis gels were run at 110 volts for 1 hour followed by 200 volts for 3-4 hours. For size determination Precision Protein^{TM} prestained broad range protein standards (BioRad) or Prestained protein ladder 10-180KDa (MBI Fermentas) were used. Gels were stained using Sliver Stain Plus (BioRad). Bands were cut from the gel using a scalpel and prepared for mass spectrometry using the MassPREP^{TM} automated digestion system (Micromass). Samples were analysed with a Q-Tof2 tandem mass spectrometer (Micromass).

2.12 Retinoic acid and lithium chloride treatment of NTERA-2

NTERA-2 cells for retinoic acid treatment were seeded at a density of $2x10^6$ cells/T75 flask or $2x10^5$ cells/40mm dish with retinoic acid at a concentration of 10μM. Cells were treated for 2, 4, 7, and 14/15 days. Differentiation status was analysed by flow cytometry as described in section 2.14.

For lithium chloride treatment NTERA-2 cells were seeded at a density of $2x10^5$ cells/dish in 40mm cell culture dishes and treated with 7.5mM lithium chloride for 7 days.

2.13 Immunofluorescence (β-catenin in NTERA-2)

Immunofluorescence for β-catenin in retinoic acid and lithium chloride treated NTERA-2 cells was performed essentially as described above, with some changes. Cells were permeabilised in 0.5% Triton-X-100, the primary antibody used for these experiments was β-catenin (H-102) at a dilution of 1/50, the secondary antibody used was rhodamine conjugated swine anti-rabbit (Dako) at a concentration of 1/100.

2.14 Flow cytometry

NTERA-2 cells for flow cytometry analysis were grown in T75 cell culture flasks as described in section 2.12. Cells were harvested using trypsin-EDTA and resuspended in PBS. Cells were counted using a haemacytometer, $2x10^6$ cells were used for each flow cytometry preparation. Cells were incubated for 1 hour at 4°C in antibody solution (primary antibody (SSEA4-1/5, A2B5-1/5, Tra-1-60-1/5, SSEA1-1/5, SSEA3-1/10, HA control – 1/10), 0.1% sodium azide, 5% FBS, PBS). Cells were then washed three times in wash buffer (0.1% sodium azide, 5% FBS, PBS) before being incubated in secondary antibody (FITC conjugted anti-mouse (Santa-Cruz) 1/20, 0.1% sodium azide, 5% FBS, PBS) at 4°C for 1 hour. Cells were washed twice in wash buffer and then fixed in 100µl PBS, 100µl 2% formaldehyde in PBS, 300µl PBS in that order.

2.15 mRNA purification

NTERA-2 cells were treated with retinoic acid as described above for 2, 4, 7 and 15 days. mRNA was purified using the Mini Message Maker kit (Mini Message Maker instruction manual, Novagen, sections 11a, 15-17). RNA quality was checked on a 1% agarose gel and quantity determined by spectrophotometry.

2.16 Reverse transcription

Approximately 300ng purified mRNA was mixed with 1µl 0.5µg/µl Oligo$(dT)_{12-18}$ and 12µl autoclaved distilled water, this was incubated at 70°C for 10 minutes followed by 1 minute on ice. This was then mixed with 2µl First-Strand buffer (250mM Tris-HCl (pH 8.3), 375mM KCl), 2µl 25mM magnesium chloride, 1µl 10mM dNTP mix and 0.1M DTT and incubated at 42°C for 5 minutes. 200 units SuperScriptII reverse transcriptase (Invitrogen) was added and reactions incubated at 42°C for 50 minutes, 70°C for 15 minutes and 5 minutes on ice. 2 units RNaseH was then added and reactions incubated at 37°C for 20 minutes.

2.17 Degenerate primer PCR

Alignments were produced using ClustalX (Thompson *et al.* 1997) and primers chosen within well conserved regions. PCR Master Mix (AbGene) (1.25U *Taq* polymerase, 75mMTris-HCl (pH8.8), 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2mM each dATP, dCTP, dGTP and dTTP) with 4µL cDNA and 2pmol/µl primers was used for all degenerate primer PCR reactions. Reactions were incubated at 94°C for 5 minutes followed by 35 cycles of 1 minutes at 94°C, 1 minute at annealing temperature (see table 2.3, below), 1 minute at 72°C and a final extension of 10 minutes at 72°C.

PCR products were analysed on agarose gels and purified from the gel using the QIAquick Gel Extraction Kit (QIAquick Spin Handbook, Qiagen, January 1997, page 24-25). Purified PCR products were then cloned into the pCR2.1-TOPO vector using the TOPO TA Cloning kit (TOPT TA Cloning Instruction Manual, Version L, Invitrogen, page 5-6) and transformed into TOP10 *E. coli*.

Clones were analysed by colony PCR using the primers originally used for the degenerate primer PCR. Clones were categorised by restriction digests, MapDraw (DNA*, DNASTAR Inc.) was used to predict restriction sites. Plasmids were purified from clones using the QIAprep Miniprep kit (QIAprep Miniprep Handbook, Qiagen, July 1999, page 18-19) and sent to MWG biotech for sequencing

Table 2.3: Degenerate primers used in NTERA-2 PCR

Primer Pairs	**Primer Sequence**	**Annealing temp.**
Dsh810	ctcaayatcrtcacdgtcackctm	50°C
Dsh1370	tratcttgagccacatgcggtcvc	
Dsh810	ctcaayatcrtcacdgtcackctm	50°C
Dsh1190	acagccatcaggcaaagacgatcca	
TLE1420	gcdgayggkcagatgcagccbgt	55°C
TLE1950	gtcccagsascksacygtgttgtcca	
TLE1820	ctcmtgctgcagcgayggsaacat	55°C
TLE2330	gtrgccttcttrtcmccmgagcc	
Tcf1200	gsmatyccbcaycckgccathgt	50°C
Tcf1420	ccvagratytggttratggchgc	
Wnt13LW	ctgggtaacacgggtgactcg	50°C
Wnt13RW	ggttcctgtactctggcacctg	

r =a.g; y=c.t; m=a,c; k=t,g; s=c,g; w=a,t; h=a,t,c; b=t,c,g; d=a,t,g; v=a,c,g; n=a,c,t,g.

2.18 RT-PCR of mRNA purified from SW480 cells

mRNA from SW480 cells was purified using the Oligotex™ Direct mRNA Mini Kit (Oligotex Handbook, Qiagen, First Edition, July 1999, page 37-42).

5µl purified mRNA was mixed with 1µl 50µg/µl random hexamers and 12µl autoclaved distilled water, this was incubated at 70°C for 10 minutes followed by 1 minute on ice. This was then mixed with 2µl First-Strand buffer (250mM Tris-HCl (pH 8.3), 375mMKCl), 2µL 25mM magnesium chloride, 1µL 10mM dNTP mix and 0.1M DTT and incubated at 25°C for 5 minutes. 200 units SuperScriptII reverse transcriptase (Invitrogen) was added and reactions incubated at 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes and 5 minutes on ice. 2 units RNaseH was then added and reactions incubated at 37°C for 20 minutes.

PCR primers were designed using PrimerSelect (DNA*, DNASTAR Inc.). PCR Master Mix (AbGene) (1.25U *Taq* polymerase, 75mMTris-HCl (pH8.8), 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2mM each dATP, dCTP, dGTP and dTTP) with 4µL cDNA and 2pmol/µl primers was used for all PCR reactions. Reactions were incubated at 94°C for 5 minutes followed by 35 cycles of 1 minutes at 94°C, 1 minute at annealing temperature (see table 2.4, below), 1-2 minutes at 72°C and a final extension of 10 minutes at 72°C.

Table 2.4: APC and APCL primers

Primers	Sequence	Position	Annealing temp.
APC1	atactccccggtgattgac		50°C
APC2	tctcgcttctttgtgttgttattc		
APC3	gcgttggcacttatctattc		50°C
APC4	agtgtcagtttatttttccttcag		
APCS1	ccaatatgtttttcaagatgtagt		50°C
APCS2	cagtaggtgctttatttttaggta		
APC13	gcactaccatccagcacagaaa		55°C
APC15	gcagaagacgacgcagatg		
APC15a	ttagttttacaccgggggatgata		50°C
APC15	gcagaagacgacgcagatg		
APC13b	gtttgcagatctccaccact		50°C
APC15	gcagaagacgacgcagatg		
APCL1	ggggagcgtggtggcagcgatgat		60°C
APCL2	cagatggggccgggtgaggtaatg		
APCL3	cgtgctgagcgccctgtgga		60°C
APCL4	tggcgatcatcttgtgcttggagt		

2.19 Sequence analysis

Sequence analysis was carried out using BLAST (Altschul *et al.*, 1997), ClustalX (Thompson *et al.*, 1997) and the DNA* software package (DNASTAR Inc.).

2.20 Northern analysis

Cells (6-7x10^7 cells/prep) were washed in ice cold PBS, lysed in denaturing solution (26mM sodium citrate (pH4.0), 0.5% N-lauryl sarcosine, 125mM β-mercaptoethanol, 4M guanidine thiocyanate) and vortexed for 30-60 seconds. Sodium acetate was added to a final concentration of 0.25M and a volume equal to the volume of denaturing solution of phenol:chloroform added. Following vigorous mixing and incubation on ice for 15 minutes lysates were centrifuged at 10,000g for 20 minutes at 4°C. The aqueous phase was removed to a fresh tube and an equal volume of isopropanol added. Preps were incubated at -20°C for 30 minutes then centrifuged 10,000g for 10 minutes at 4°C. The pellet was washed in 10ml ice cold 75% ethanol and again centrifuged 10,000g for 10 minutes at 4°C. After drying the RNA was then resuspended in sterile nuclease-free distilled water. RNA concentration was determined by spectrophotometry, and quality of RNA checked on a 1% agarose gel.

mRNA was purifed from total RNA (250µg total RNA per prep) using the NucleoTrap mRNA purification kit (NucleoTrap mRNA instruction manual, Machery-Nagel, January 2000, page 5-6). mRNA was precipitated with 0.25M sodium acetate and 2.5 volumes ethanol, centrifuged 10,000g for 10 minutes at 4°C, washed in 70% ethanol and centrifuged again as above. RNA was resuspended in 5µl distilled nuclease-free water and 15µl loading buffer (50% formamide, 2.6M formaldehyde, 1x MOPS, 0.2% brompohenol blue, 15% glycerol, 20µg/ml ethidium bromide), mRNA was then heated to 65°C for 15 minutes followed by a minimum of 2 minutes on ice before being separated on a denaturing gel.

3-4μg mRNA per lane was separated on a denaturing 1% agarose MOPS gel (1% agarose, 2.2M formaldehyde, 40mM MOPS acid (pH 7.0), 10mM sodium acetate, 1mM EDTA) at 80 volts for 4-5 hours. The gel was then washed twice in distilled water for 10 minutes and in 10x SSC (1.5M sodium chloride, 0.15M sodium citrate, pH 7.0) for 10 minutes. mRNA was transferred to Hybond N+ membrane (Amersham) by overnight capillary transfer in 10x SSC. mRNA was crosslinked by UV irradiation for 60 seconds at 254nm.

Probes were labelled by random priming with ^{32}P-dCTP using the Megaprime™ kit (Amersham) (Megaprime™ DNA labelling systems instruction manual RPN1604, Standard Megaprime protocol, pages 11-14). Probes used were the PCR products from the following primers: APC1 & APC2; APCS1 & APCS2 (table X). Radiolabelled probes were purifed using the QIAquick Nucleotide Removal kit (QIAquick Spin Handbook, Qiagen, January 1997, page 22-23). Membranes were pre-hybridised in Rapid-hyb buffer (Amersham) for 30-60 minutes at 42°C, radiolabelled probes were then added and membranes incubated overnight at 42°C. Membranes were washed in 2-0.1x SSC, 0.1% SDS at 42-55°C until background levels were sufficiently reduced. Blots were then visualised on Kodak X-OMAT AR film. Size of RNA was determined by comparison with 2μg/well 0.28-6.58kb markers (Promega).

Chapter 3 – Results: Characterisation of a panel of antibodies raised to adenomatous polyposis coli

3.1 Introduction

Analysis of the localisation of a protein within the cell is an important step in understanding its function. Accordingly many groups, including ours, have an interest in defining the cellular localisation of APC. This work has resulted in a variety of localisations being described for APC, much of this being conflicting data.

APC has been found to shuttle between the nucleus and cytoplasm (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a; Neufeld *et al.*, 2000b); to be localised to microtubule tips (Näthke *et al.*, 1996; Rosin-Arbesfeld *et al.*, 2001; Mogensen *et al.*, 2002); to be localised to the plasma membrane in an actin-dependent manner (Rosin-Arbesfeld *et al.*, 2001); and it has been found at the kinetochore during mitosis (Fodde *et al.*, 2001; Kaplan *et al.*, 2001). APC has also been reported at the apical membrane (Miyashiro *et al.*, 1995; Reinacher-Schick & Gumbiner, 2001; Anderson *et al.*, 2002). Other studies have shown full length APC to be predominantly localised to the cytoplasm (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000) and a contrasting study reported APC to be localised to the nucleus (Zhang *et al.*, 2001; Anderson *et al.*, 2002). As mentioned above, APC has been reported to be localised to microtubule tips (Näthke *et al.*, 1996; Rosin-Arbesfeld *et al.*, 2001; Mogensen *et al.*, 2002) with no apical staining, while others report apical localisation of APC with no staining seen at microtubule tips (Reinacher-Schick & Gumbiner, 2001; Anderson *et al.*, 2002).

One explanation for the differing localisations of APC, even within the same cell lines, may be due to the use of different antibodies. It is possible that some of these conflicting reports of the localisation of APC could be due to different functional isoforms or modifications of APC, with different antibodies detecting different localisations depending on which isoforms or modifications expose the relevant epitopes. Use of unreliable antibodies may also account for some of the different localisations seen.

We set out to determine the cellular localisation of APC using a variety of APC antibodies. At the outset of this study, little was known of the localisation of APC and many of the papers describing localisation of APC (see above) had not yet been published. We used a panel of antibodies to APC (table 3.1) and carried out western blotting as a first step in characterising reactivity of these antibodies. Western blots enabled us to determine whether antibodies were able to detect full length and truncated APC, and potentially whether antibodies also cross-reacted with other proteins. As well as western blotting, localisation of APC was determined by immunofluoresecence and data was compared for a variety of antibodies. These data gives an indication of whether these antibodies can reliably be used for immunodetection of APC or if they are detecting other protein species.

The results of this study are presented for each of the antibodies listed in table 3.1. This is presented as a summary and includes data published from other laboratories for a comprehensive listing of antibody reactivities.

Table 3.1
Summary of the APC antibodies used in this study. Clone number, epitope and the host the antibody was raised in are shown. More information on these antibodies is available in table 2.1 in materials and methods.

Antibody	**Clone no. / ID no.**	**Epitope**	**Host**
APC (ALI 12-28)	ALI 12-28	aa135-433	Mouse IgG_1
APC (C-20)	C-20	C-term. 20aa	Rabbit
APC (H-290)	H-290	aa1-289	Rabbit
APC (N-15)	N-15	aa2-16	Rabbit
APC (Ab1)	FE9	exon 1	Mouse IgG_1
APC (Ab2)	IE1	C-term. 300aa	Mouse IgG_1
APC (Ab3)	AC4	exon 3	Mouse IgG_1
APC (Ab4)	HG2	C-term. 300aa	Mouse IgG_1
APC (Ab5)	CF11	exon 2	Mouse IgG_{2b}
APC (Ab6)	DB1	C-term. 300aa	Mouse IgG_1
APC (Ab120)	c-APC 28.9	C-term. 300aa	Mouse IgG_1
APC (M-APC)	-	aa1043-2130	Rabbit

3.2 Anti-APC (M-APC) detects nuclear shuttling of APC in full length and truncated APC cell lines

Anti-APC (M-APC) is a rabbit polyclonal antibody raised to a large central region of full length APC (Näthke *et al.*, 1996). This antibody has been shown to detect both full length and truncated APC at the expected sizes in a variety of cell lines (Mogensen *et al.*, 2002), and is able to reliably detect transfected APC-GFP constructs (Rosin-Arbesfeld *et al.*, 2001). This antibody detects APC shuttling between nucleus and cytoplasm with exact localisation varying with mutation status of APC (Rosin-Arbesfeld *et al.*, 2000). It also detects APC associated with the plasma membrane in an actin-dependent manner (Rosin-Arbesfeld *et al.*, 2001) and at microtubule plus ends (Näthke *et al.*, 1996; Mogensen *et al.*, 2002). During mitosis this antibody detects APC at the kinetochores (Kaplan *et al.*, 2001). The localisation of APC detected by this antibody in a variety of cell lines is discussed further in chapter 4.

As this antibody has been shown to reliably detect APC on immunofluorescence staining, the staining seen with other APC antibodies was compared to this antibody to determine whether they are efficiently detecting full length and truncated APC on immunofluorescence.

3.3 Anti-APC (N-15) cross reacts with Ku80 and is unreliable for western blot and immunoprecipitation

Anti-APC (N-15) is a rabbit polyclonal antibody raised to amino acids 2-16 at the N terminus of APC. The reliability of anti-APC (N-15) has been questioned (Mogensen *et al.*, 2002, Rosin-Arbesfeld *et al.*, 2001; Roberts *et al.*, 2003).

Anti-APC (N-15) does not detect APC by western blot and immunoprecipitation, but instead cross-reacts with a number of other proteins, the strongest band being identified as Ku80 (Roberts *et al.*, 2003). The staining seen with anti-APC (N-15) does not appear to co-localise to that seen with a

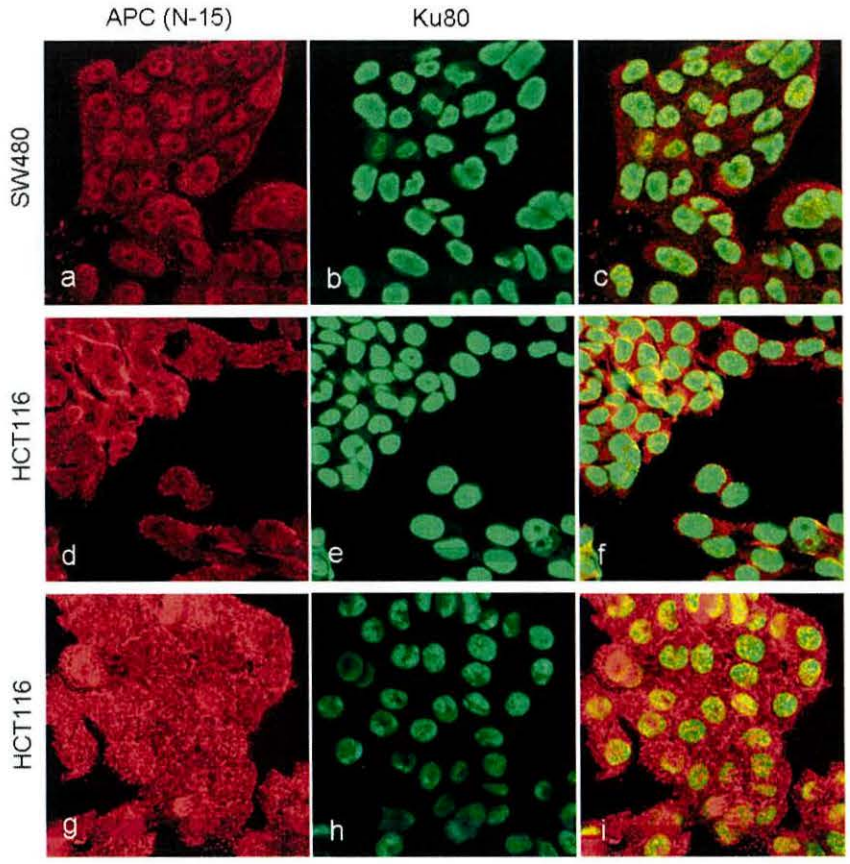


Figure 3.1
Anti-APC (N-15) cross-reacts with Ku80 in immunoprecipitation and western blot but does not co-localise with Ku80 in immunofluorescence. Ku80 is localised to the nucleus in both cell lines (b,e,h). Anti-APC (N-15) gives predominantly nuclear staining in SW480 cells (a), which express only truncated APC, and predominantly cytoplasmic staining in HCT116 cells which express only wild type APC (d). Apical staining is seen in both these cell lines and does not co-localise with Ku80 (g, data not shown).

Ku80 antibody in immunofluorescence. Ku80 is localised to the nucleus in all cell lines studied, nuclear staining with N-15 varied between cell lines (figure 3.1a,d,g). Ku80 did not co-localised with the apical staining detected by N-15 (figure 3.1g-i). Published work shows that anti-APC (N-15) detects APC in cell lines with either wild type or truncated APC (Henderson, 2000). Anti-APC (N-15) does give similar localisation to anti-APC (M-APC) on immunofluorescence, with both detecting predominantly nuclear APC in truncated APC cell lines and MDCK cells and cytoplasmic APC in most wild type APC cell lines (Henderson, 2000; Rosin-Arbesfeld *et al.*, 2000; chapter 4). N-15 does not detect APC localised to microtubule tip clusters (Rosin-Arbesfeld *et al.*, 2001), but this may be due to alternative splicing leading to a lack of exon 1 (which N-15 is directed to) in the APC population which localises to microtubule tip clusters. N-15 detects an apical protein which is not detected by M-APC. This apical protein is detected by a number of other APC antibodies (chapter 5), and may not be due to cross-reactivity with an unknown protein. In one study anti-APC (N-15) failed to detect transfected APC-GFP constructs (Rosin-Arbesfeld *et al.*, 2001). The localisation of APC detected by this antibody in a variety of cell lines is discussed in chapter 4. The staining seen with anti-APC (N-15) is lost when the antibody is pre-incubated with the appropriate blocking peptide (data not shown), this shows that any staining seen with this antibody is specific rather than general background staining. However, it does not remove the possibility that this antibody binds specifically to a protein other than APC.

While anti-APC (N-15) may detect APC in immunofluorescence staining, it does not detect APC in western blot or immunoprecipitation. Due to the poor results with this antibody in western blot and immunoprecipitation and its questionable reliability in immunofluorescence it must be concluded that this antibody should not be relied upon for immunodetection of APC.

3.4 APC (ALI12-28) detects APC by western blot, but also detects other proteins

Anti-APC (ALI12-28) is a mouse monoclonal antibody directed to an epitope within amino acids 135-433 of APC. To confirm that this antibody does

effectively detect APC, a western blot was carried out on whole cell lysates from two cell lines which express wild type APC, HCT116 and HEK293, and SW480 which express only truncated APC. Figure 3.2a shows that while this antibody is able to detect both full length and truncated APC, on longer exposures many other bands are also seen.

Western blot of fractionated SW480 lysates demonstrated that truncated APC expressed in this cell line was found in both the nucleus and the cytoplasm, with a greater amount in the nucleus (figure 3.2b). Controls with lamin and tubulin demonstrated that fractionation was efficient (Figure 3.2c,d). Lysate from an equal amount of cells was loaded into the cytoplasmic and nuclear lanes. The localisation as seen by fractionation is consistent with the localisation seen with anti-APC (M-APC) (section 4.3) and is consistent with previous reports of the localisation of truncated APC in SW480 cells (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000).

3.5 Anti-APC (ALI12-28) detects APC only in the cytoplasm in both truncated and wild type APC cell lines

Staining with anti-APC (ALI12-28) was seen in the cytoplasm only in all cell lines, both in those expressing full length APC and in SW480 cells, which express only truncated APC (figure 3.3). No variation in localisation with cell density was observed with anti-APC (ALI12-28). This contrasts with observations using anti-APC (M-APC) and anti-APC (N-15), where in SW480 cells APC is observed to be predominantly nuclear in subconfluent cells (Figure 3.3b) with some loss of nuclear staining as cell density increases (figure 3.3e). In double labelling experiments where the cells were stained with anti-APC (ALI12-28) and anti-APC (N-15) it could be seen that SW480 cells showing nuclear staining for APC with anti-APC (N-15) only had cytoplasmic staining with anti-APC (ALI 12-28) (Figure 3.3a-f).

APC has been observed to accumulate in the nucleus in response to Leptomycin B (LMB), an inhibitor of CRM1-dependent nuclear export (Rosin-Arbesfeld *et*

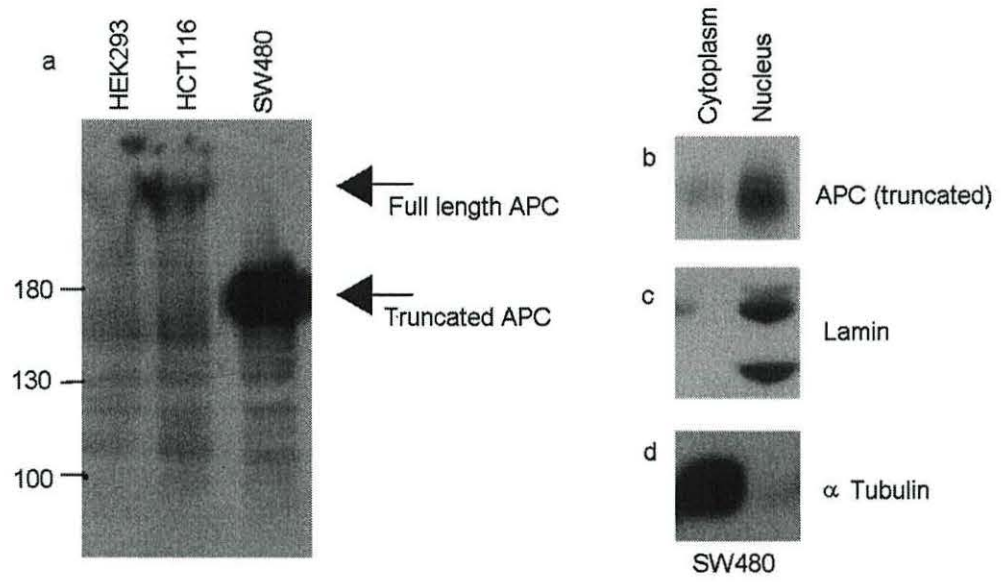


Figure 3.2
Anti-APC (ALI12-28) does detect both full length and truncated APC on western blot and is able to detect nuclear APC in fractionated lysates. Anti-APC (ALI12-28) detects full length APC in HCT116 and HEK293 lysates and truncated APC in SW480 lysate (a). Molecular weight markers (kDa) are shown to the left of the image. Fractionation shows that this antibody detects truncated APC in both the nucleus and cytoplasm in SW480 cells (b). Lamin (c) and tubulin (d) western blots show that fractionation was successful.

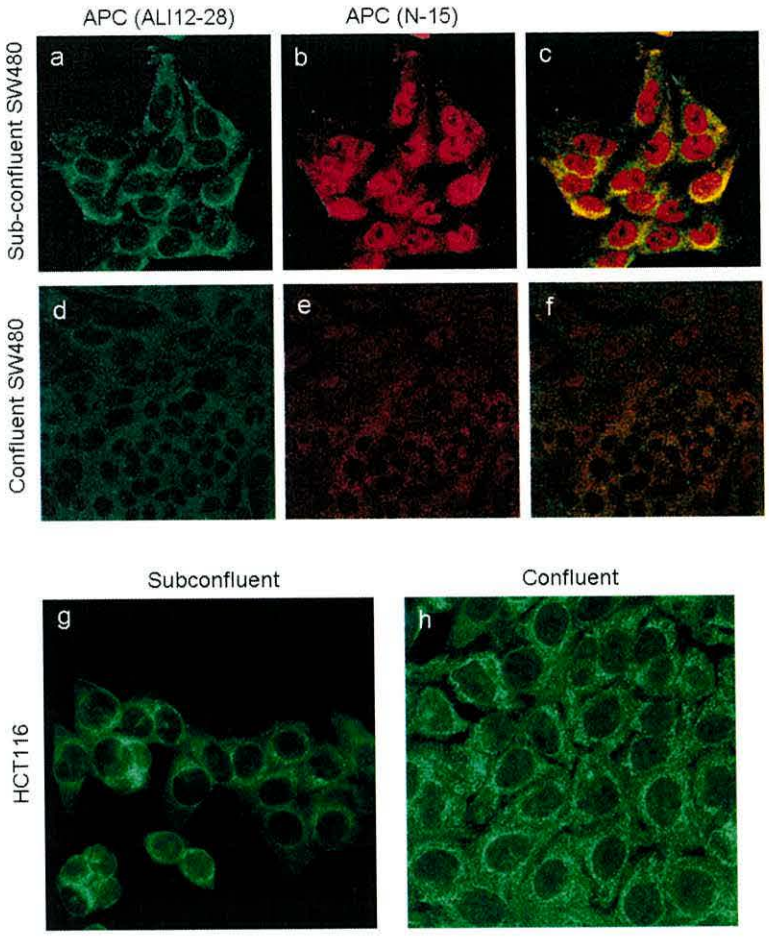


Figure 3.3
Anti-APC (ALI12-28) detects APC only in the cytoplasm in all cell lines. In SW480 cells truncated APC is found in the nucleus (b,e) but Anti-APC (ALI12-28) gives only cytoplasmic staining (a,d). Staining in HCT116 is again cytoplasmic (g,h), the same as in SW480 cells. No variation in staining with cell density was seen with Anti-APC (ALI12-28).

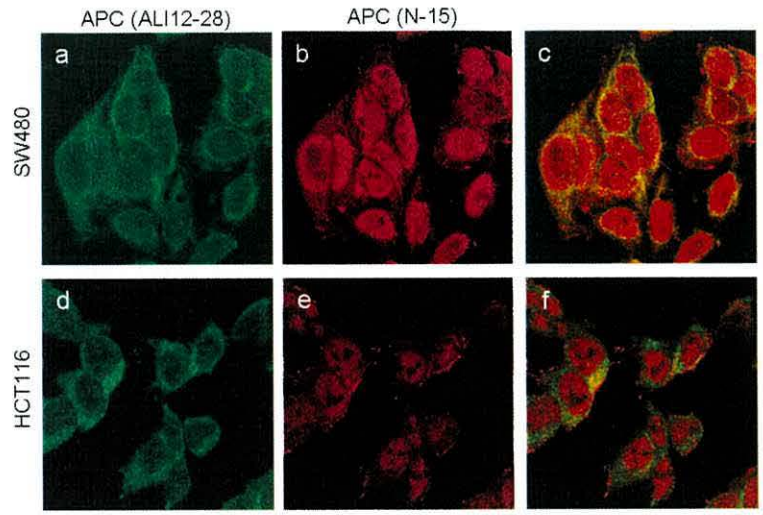


Figure 3.4
Anti-APC (ALI12-28) does not detect nuclear APC in Leptomycin B treated cells. APC is nuclear in Leptomycin B treated SW480 cells (b) and in Leptomycin B treated HCT116 cells (e). Anti-APC (ALI12-28) does not detect nuclear APC in either of these cell lines when treated with Leptomycin B (a,d).

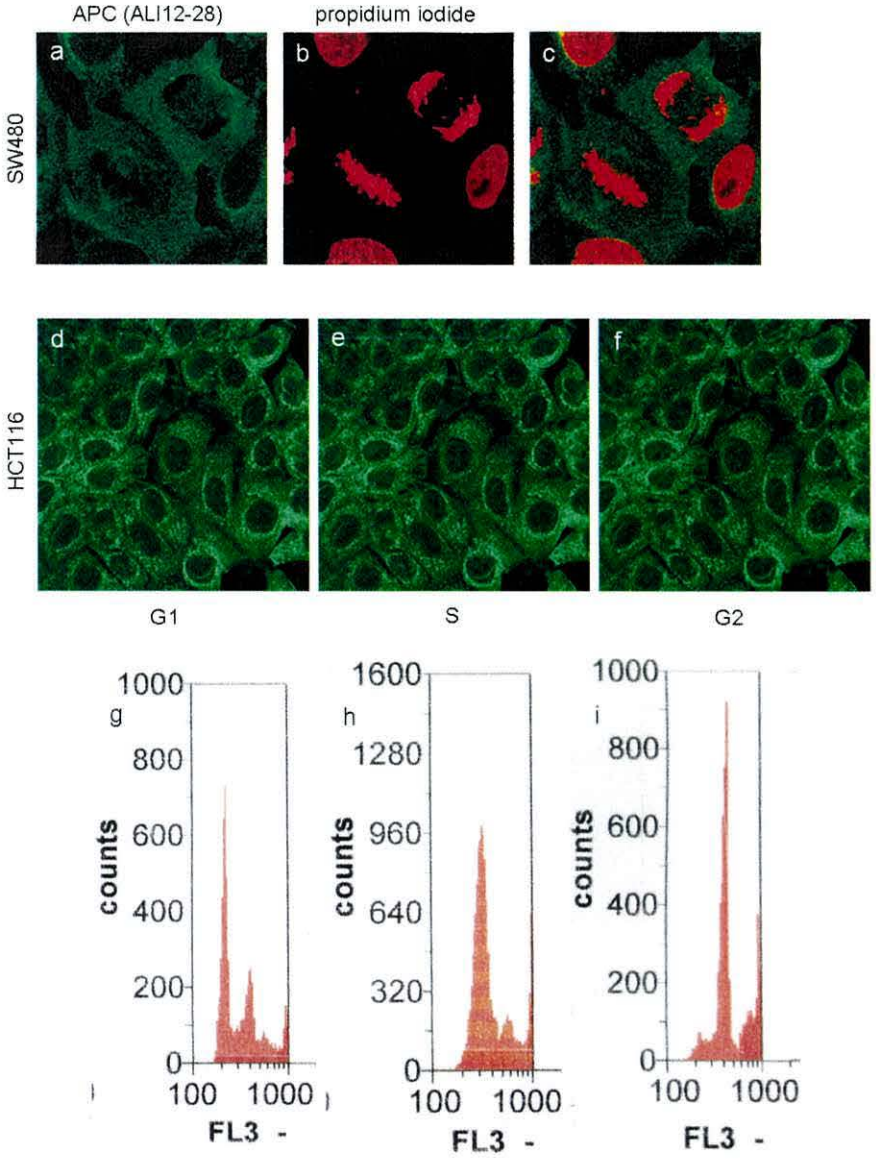


Figure 3.5
Anti-APC (ALI12-28) shows APC to be localised to the cytoplasm throughout the cell cycle with some decoration of the spindle during metaphase in SW480 cells. In unsynchronised HCT116 cells, which are seen to be mostly G1 cells (g), APC is cytoplasmic (d). In mimosine synchronised S phase (e,h) and G2 (f,i) cells APC is again cytoplasmic. In mitotic cells APC is localised to the cytoplasm and excluded from chromosomes with some spindle decoration in SW480 cells (a-c). This spindle decoration was not seen in HCT116 cells (data not shown). Propidium iodide staining and flow cytometry analysis confirmed cells to be at the required stage of the cell cycle (g-i).

al., 2000; Henderson, 2000; Neufeld *et al.*, 2000a; Neufeld *et al.*, 2000b). Treatment of HCT116 and SW480 with LMB and staining with anti-APC (ALI12-28) did not show any nuclear accumulation of APC (Figure 3.4a,d). The same cells labelled with anti-APC (N-15) were seen to have nuclear accumulation of APC in response to LMB in HCT116 cells (Figure 3.4e) and nuclear staining in both treated (Figure 3.4b) and untreated (Figure 3.3b) cells in SW480.

This shows that this antibody is unable to detect nuclear APC by immunofluorescence. It is possible that this antibody can only detect a sub-population of APC due to conformational changes or protein-protein interactions masking the antibody binding site in much of the APC in the cell, including nuclear APC. The denaturing conditions used in western blotting would then allow detecting of nuclear APC by this antibody. The possibility of cross-reactivity with an unrelated protein also exists.

3.6 Anti-APC (ALI12-28) detects APC in the cytoplasm throughout the cell cycle, with some spindle decoration at metaphase in SW480 cells

To test whether localisation of APC varied during the cell cycle, HCT116 cells were synchronised by treatment with mimosine (as described in materials and methods, section 2.6). No variation in localisation was observed during G1/S/G2, with cells showing cytoplasmic localisation throughout (figure 3.5d-f). Propidium iodide staining and flow cytometry was carried out to confirm cells were at the required stage of the cell cycle (figure 3.5g-i).

Mitotic HCT116 and SW480 cells were stained with anti-APC (ALI12-28) and propidium iodide. In both cell lines APC was observed throughout the cytoplasm and excluded from the chromosomes during mitosis (figure 3.5a-c). In SW480 cells some spindle decoration of APC was observed during metaphase (figure 3.5a-c), this was not seen in any HCT116 metaphase cells.

As spindle decoration with anti-APC (ALI12-28) is only seen in SW480 cells, this may reflect an abnormal localisation due to truncation. Wild type APC has been observed at kinetochores during mitosis (Fodde *et al.*, 2001; Kaplan *et al.*, 2001). Spindle decoration of APC has not previously been observed. It may be that only a sub-population of APC, detected by anti-APC (ALI12-28), decorates the spindle at metaphase. Other antibodies may fail to detect this spindle decoration due to brighter staining of the surrounding cytoplasm masking the small amount of APC localised to the spindle. However, the possibility remains that the spindle decoration seen with this antibody is due to cross-reactivity.

In summary, by immunofluorescence staining with anti-APC (ALI12-28) APC is predominantly cytoplasmic throughout the cell cycle and does not accumulate in the nucleus in response to LMB. However, fractionation demonstrates that this antibody is able to detect nuclear APC. A possible explanation for the lack of nuclear staining in immunofluorescence with this antibody is that the nuclear shuttling population of APC lacks the binding site of this antibody. The ability of this antibody to detect denatured APC extracted from the nuclei of cells excludes this possibility. It is a possibility that for some reason the denatured protein on a western blot is detectable by this antibody but that nuclear APC in its native form is not detectable, either due to a conformational change or an interacting protein blocking the antibody-binding site. However, as there are a number of bands other than those of the correct size for APC on western blot it remains a possibility that when used for immunofluorescence this antibody is detecting a protein other than APC.

3.7 A number of APC antibodies detect an apical protein in immunofluorescence and/or a 150 kDa protein in western blot

APC antibodies Ab4, Ab5, Ab6 and Ab120 detect an apical protein in immunofluorescence staining of cell lines expressing wild type APC and SW480 cells, which express only truncated APC. None of these antibodies appear to detect full length APC in immunofluorescence (section 5.3), as determined by comparison to staining seen with anti-APC (M-APC). APC antibodies Ab2,

Ab5, Ab6, Ab120, and H-290 all detect a 150 kDa protein, as well as full length APC on western blot (section 5.4). The N-terminal directed antibodies, Ab5 and H-290, also detect truncated APC on western blots (section 5.4). This apical protein and 150 kDa protein are discussed further in chapter 5.

Anti-APC (Ab4) detects an apical protein on immunofluorescence but does not detect a 150 kDa protein or full length APC on western blot (figure 3.6). This antibody detects proteins of approximately 200 kDa and 100 kDa, with many other fainter bands (figure 3.6). The manufacturer's datasheet for Ab4 states it is only suitable for immunofluorescence (Ab4 datasheet, CN Biosciences). The inability of anti-APC (Ab4) to detect full length APC in western blot may be due to it only being suitable for use with native protein, rather than the denatured protein encountered in western blots. As a number of other antibodies which do reliably detect full length APC also detect this apical staining, we suggest that Ab4 is reliable for use in immunofluorescence even though it is unable to detect APC on western blot.

Ab2 detects both full length APC and a 150 kDa protein on western blot (section 5.4), but does not appear to be suitable for use in immunofluorescence. Immunofluorescence staining using this antibody detects only very low levels of cytoplasmic staining (data not shown). Ab2 is sold for use in western blots (Ab2 datasheet, CN Biosciences). It may only be able to detect denatured APC, as in western blots, and not the native protein in immunofluorescence staining.

Anti-APC (H-290) also detects both full length APC and a 150 kDa protein on western blot but does not detect an apical protein on immunofluorescence. Anti-APC (H-290) appears to be unable to detect the nuclear shuttling population of APC as it does not give any nuclear staining in SW480 cells, only cytoplasmic staining is seen with this antibody (data not shown). Anti-APC (H-290) is a rabbit polyclonal raised to amino acids 1-289 of APC, so the exact antibody binding sites are not known. The peptide this antibody is raised to overlaps the epitope for anti-APC (ALI 12-28) (section 3.4) so it may be that both these antibodies detect the same APC population on immunofluorescence. Alternatively, anti-APC (H-290) may be unable to detect APC or the apical

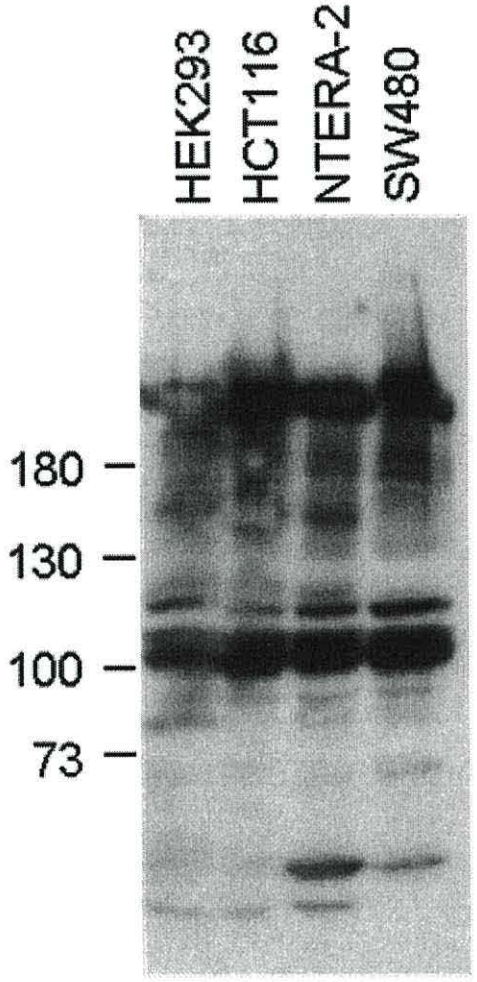


Figure 3.6
Anti-APC (Ab4) is not able to detect full length APC by western blot but instead detects a number of other proteins. Anti-APC (Ab4) detects protein at approximately 200 kDa and 100 kDa in all cell lines, both those expressing wild type APC and those expressing truncated APC. A number of other fainter bands are also seen. Successful transfer of full length and truncated APC was confirmed by stripping the membrane and re-probing with anti-APC (ALI12-28). Markers show molecular weight in kDa.

protein on immunofluorescence due to conformational changes or protein-protein interactions blocking antibody binding. However, this antibody may be cross-reacting with another protein in immunofluorescence, as it does detect proteins other than full length APC and the 150 kDa protein on western blot (figure 5.9).

3.8 Anti-APC (Ab3) is unable to detect full length APC on western blot and does not give staining consistent with other APC antibodies in immunofluorescence

Ab3 is an APC antibody directed to an epitope within exon 2, at the N terminus of APC. On western blot this antibody gives two bands at around 200 kDa, one band at approximately 140 kDa, a particularly dark band at below 73 kDa and a number of other faint bands (figure 3.7a). These bands are present in all cell lines studied, including SW480 cells, which express only truncated APC. This antibody does not seem to be able to detect full length APC, or truncated APC in SW480 lysates. In SW480 cells immunofluorescence staining with anti-APC (Ab3) gives a similar localisation to that seen in these cells with anti-APC (M-APC). In subconfluent SW480 cells APC is predominantly localised to the nucleus with fainter cytoplasmic staining with this nuclear localisation being progressively lost as cells grow to higher density (figure 3.7a,e). This is similar to the localisation seen with anti-APC (M-APC) (section 4.3). However, the localisation seen with anti-APC (Ab3) in HCT116 cells is not consistent with the localisation seen with anti-APC (M-APC). Ab3 gives nuclear staining as well as cytoplasmic staining (figure 3.7h) whereas M-APC gives predominantly cytoplasmic staining. Anti-APC (Ab3) does not appear to detect microtubule tip clusters of APC or apical staining (data not shown).

It has been suggested that the 200 and 140 kDa bands seen on western blot with this antibody are splice variants of APC which lack exon 15 (Kraus *et al.*, 1996). It seems more likely that these bands are due to cross-reactivity of anti-APC (Ab3) with other proteins as other APC antibodies do not appear to consistently detect the same bands. Also, as exon 15 of APC is unusually large at 6574 base

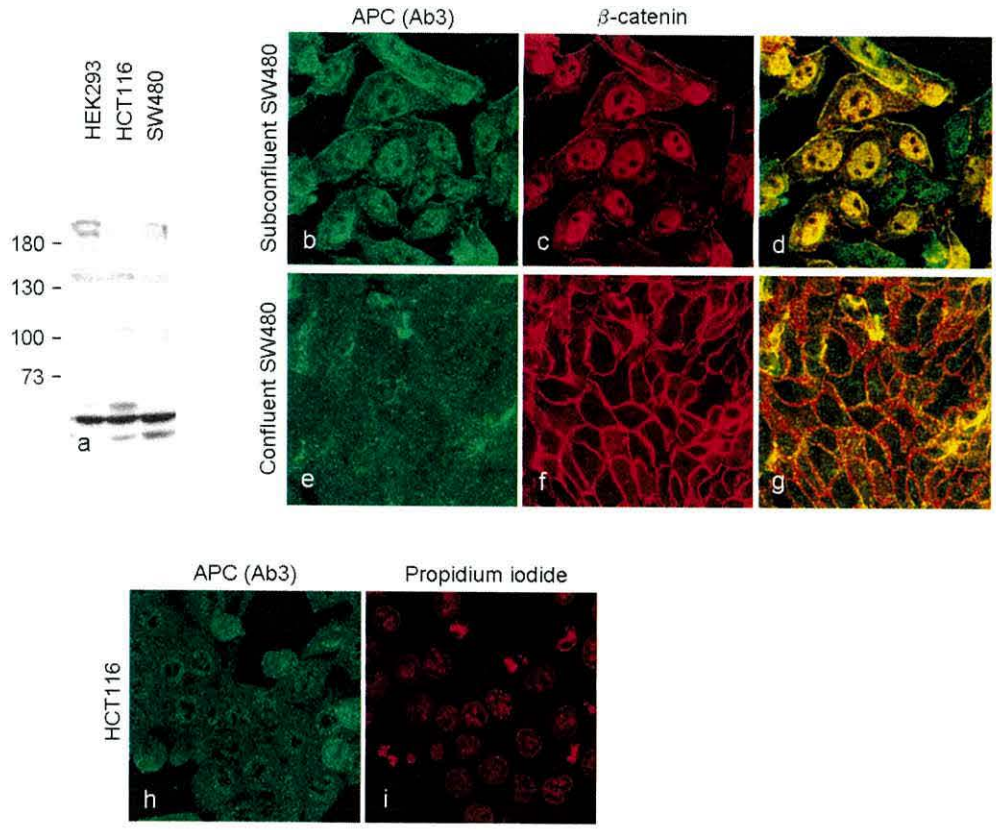


Figure 3.7
Anti-APC (Ab3) is not able to detect full length or truncated APC on western blot and may detect proteins other than APC in immunofluorescence. In western blot anti-APC (Ab3) is unable to detect full length or truncated APC but instead detects a number of other proteins (a). Markers show molecular weight in kDa. Successful transfer of full length and truncated APC was confirmed by stripping the membrane and re-probing with anti-APC (ALI12-28). Immunofluorescence staining with this antibody in SW480 cells, which express only truncated APC, is consistent with that seen with anti-APC (M-APC) with nuclear staining in sub-confluent cells (b) being lost as cells reach higher density (e). However, the localisation seen with anti-APC (Ab3) in HCT116 cells is predominantly nuclear which contrasts the predominantly cytoplasmic staining seen with anti-APC (M-ACP).

pairs, which is over 75% of the total APC sequence, it would seem unlikely that an isoform lacking exon 15 could be as large as 140-200 kDa.

In conclusion, anti-APC (Ab3) appears to be unreliable for immunodetection of APC both by western blot and immunofluorescence. It does not appear to be able to detect full length APC by western blot and does not give a localisation consistent with any other antibodies on immunofluorescence.

3.9 Anti-APC (Ab1) detects APC and additional proteins on western blot but does not appear to reliably detect APC in immunofluorescence staining

Anti-APC (Ab1) is a mouse monoclonal antibody directed to an epitope within exon 1 of APC. This antibody has been used for detecting of APC both on western blot (e.g Neufeld *et al.*, 2000a; Reinacher-Schick & Gumbiner, 2001; Rosin-Arbesfeld *et al.*, 2003) and immunofluorescence (Anderson *et al.*, 2002; Zhang *et al.*, 2001). This antibody does appear to reliably detect both full length and truncated APC (Reinacher-Schick & Gumbiner, 2001; figure 3.8a) but also strongly detects a protein of around 60 kDa and another protein at below 37 kDa (figure 3.8a). Anti-APC (Ab1) appears to detect far greater amounts of this 60 kDa protein than either full length or truncated APC (figure 3.8a).

In immunofluorescence anti-APC (Ab1) detects strong nuclear staining with fainter granular cytoplasmic staining in all cell lines studied (figure 3.8b,c). The staining seen with this antibody is the same in both cell lines expressing truncated APC and in those which express wild type APC (figure 3.8b,c). The staining pattern seen with anti-APC (Ab1) is not consistent with that seen with anti-APC (M-APC), or with any other antibody used here. This, coupled with the fact that it strongly detects other bands on western blot, indicates that this antibody may not reliably detect APC by immunofluorescence staining. However, this antibody does appear to be reliable for western blot as it does appear to detect both full length and truncated APC and only additionally detects a few much smaller proteins. It is unusual to find an APC antibody which does not give a number of bands on western blots: compared to many APC antibodies

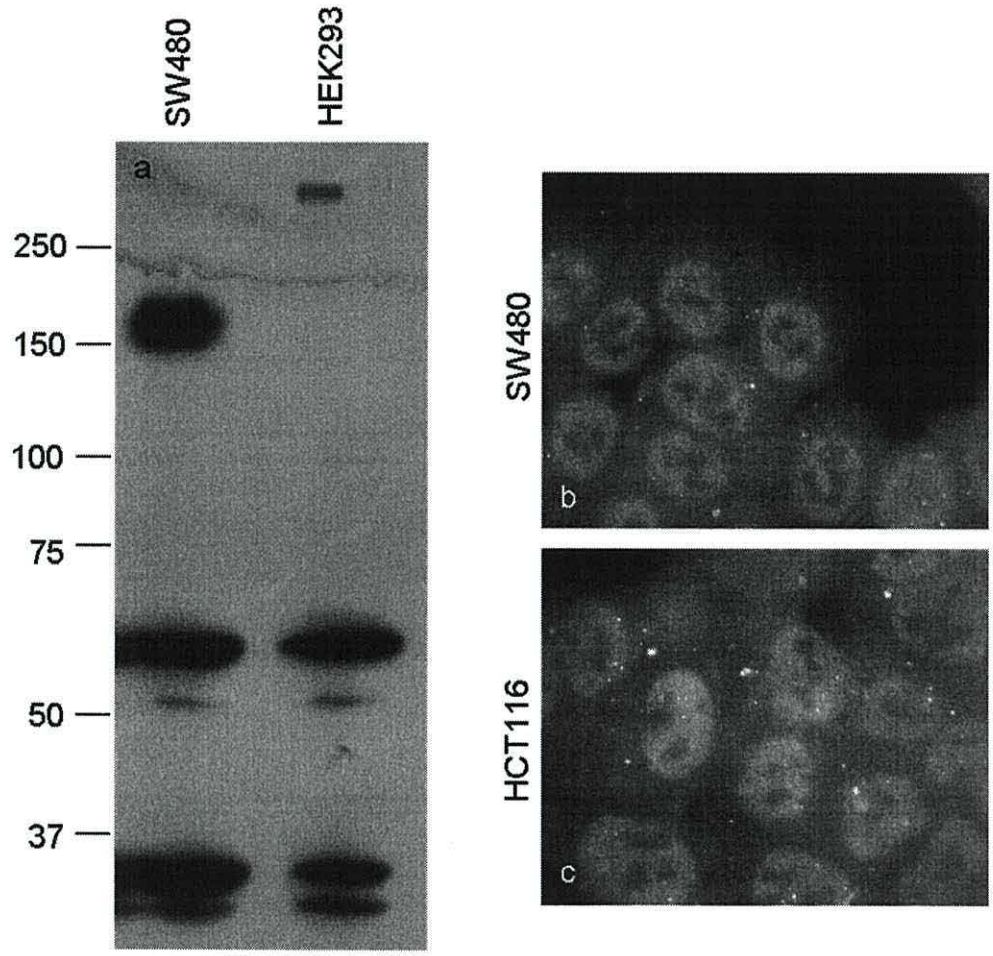


Figure 3.8
Anti-APC (Ab1) does detect full length and truncated APC but also detects other proteins in both western blot and immunofluorescence. Anti-APC (Ab1) detects truncated APC in SW480 lysate and full length APC in HEK293 (G.T. Roberts) (a). As well as detecting APC anti-APC (Ab1) also detects a protein at approximately 60 kDa and another at below 37 kDa (a). Markers show molecular weight in kDa. In immunofluorescence staining anti-APC (Ab1) detects nuclear APC and granular cytoplasmic staining in both wild type APC (c) and truncated APC (b) expressing cell lines. This staining is not consistent with anti-APC (M-APC) or any other APC antibody used and is likely to be due to cross-reactivity.

the number of other bands detects by anti-APC (Ab1) on western blot is quite low.

3.10 Anti-APC (C-20) does not appear to reliably detect APC by either western blot or immunofluorescence

Anti-APC (C-20) is a rabbit polyclonal antibody raised to a peptide corresponding to the C-terminal 20 amino acids of APC. APC (C-20) has previously been used to detect APC by immunofluorescence in both cultured cells and colon tissue (Anderson *et al.*, 2002; Zhang *et al.*, 2001). However, this antibody does not appear to detect APC in immunofluorescence.

In immunofluorescence anti-APC (C-20) gives nuclear staining with granular staining appearing to be concentrated in the nucleoli (figure 3.9a-f). The staining seen with this antibody was not similar to that seen with anti-APC (M-APC) or any other APC antibody used here. Interestingly, the localisation seen with anti-APC (C-20) was similar to that seen with an APCL antibody (A-17) from the same supplier (figure 3.9g). The staining seen with anti-APC (C-20) is lost when the antibody is pre-incubated with the appropriate blocking peptide (data not shown), this shows that any staining seen with this antibody is specific rather than general background staining. However, it does not remove the possibility that this antibody binds specifically to a protein other than APC.

Western blots were carried out to confirm whether anti-APC (C-20) was able to detect APC by western blot. This antibody did appear to be able to detect full length APC but also detected a number of other bands, which were present in both HCT116 (full length APC) and SW480 (truncated APC) (G.T. Roberts, unpublished data). One of the proteins this antibody detects is approximately 250 kDa in size. This is close to the size of human APCL which is a 254 kDa protein (Nakagawa *et al.*, 1999; van Es *et al.*, 1998) and is expressed in HCT116 and SW480 cells (section 7.3).

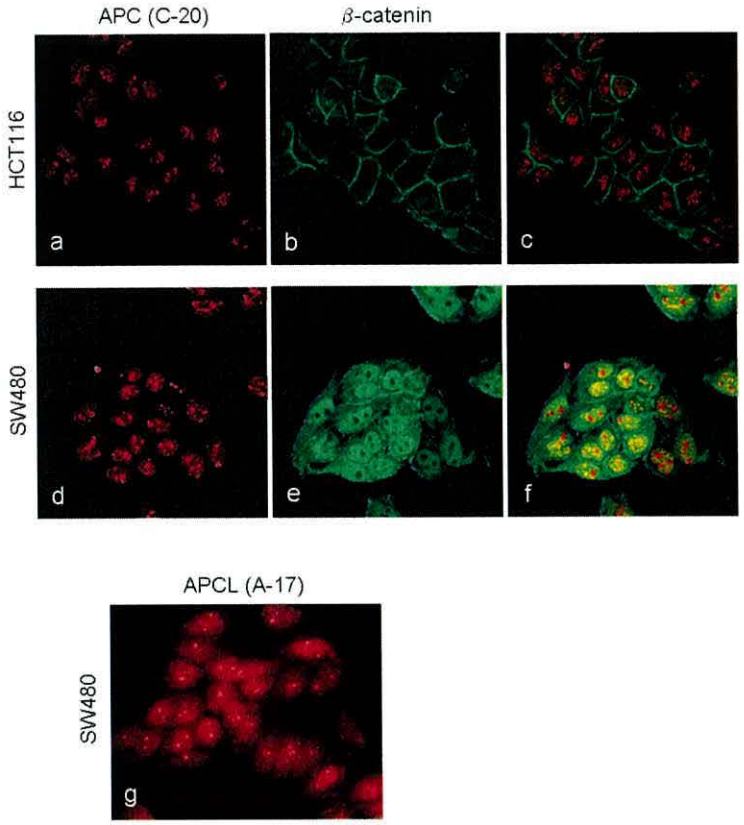


Figure 3.9
Anti-APC (C-20) gives nuclear staining with granular staining concentrated in the nucleoli in both HCT116 (a) and SW480 (d) cells. As this is a C-terminal directed antibody it cannot be detecting full length APC in SW480 cells. Similar staining is observed with anti-APCL (A-17) (g) which indicates this antibody may be cross-reacting with APCL.

Anti-APC (C-20) detects a protein of approximately 250 kDa as well as full length APC on western blot and gives the same localisation as that seen with an antibody directed to APCL on immunofluorescence. This seems to indicate that anti-APC (C-20) may be cross-reacting with APCL. However, the localisation seen with these two antibodies differs from that previously reported for APCL (Jarrett *et al.*, 2001), so it remains a possibility that both these antibodies are cross-reacting with another protein.

3.11 Discussion of results chapter 3: Characterisation of a panel of antibodies raised to adenomatous polyposis coli

A total of twelve antibodies raised to various regions of APC have been used here. All these antibodies have been raised to different regions of the same protein and give a surprising variety of localisations for APC (summarised in table 3.2). A surprising number of antibodies were found to be unreliable for immunodetection of APC by particular techniques. There have been a number of conflicting descriptions of the localisation of APC. Full length APC has been reported to be predominatly localised to the cytoplasm (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000) but has been reported by others to be localised to the nucleus (Zhang *et al.*, 2001; Anderson *et al.*, 2002). Some report APC to be localised to microtubule tips (Näthke *et al.*, 1996; Rosin-Arbesfeld *et al.*, 2001; Mogensen *et al.*, 2002) with no apical staining, while others claim apical localisation of APC with no staining seen at microtubule tips (Reinacher-Schick & Gumbiner, 2001; Anderson *et al.*, 2002). These conflicting descriptions of the localisation of APC can be accounted for by the use of different antibodies, which may not reliably detect APC. The ability of these antibodies to detect APC and the localisation seen with these antibodies is summarised in table 3.2.

Table 3.2
Summary of western blot and immunofluorescence observations for a panel of APC antibodies. Table show whether antibodies are able to detect full length APC at approximately 310 kDa, a 150 kDa protein (see section 5.4), and the localisation seen with these antibodies in a full length APC cell line, HCT116, and a truncated APC cell line, SW480.

Antibody	**detects APC on western blot**	**Detects 150 kDa protein**	**Localisation in HCT116**	**Localisation in SW480**
APC (ALI 12-28)	Yes	No	Cytoplasmic	Cytoplasmic
APC (C-20)	Yes	No	Nuclear/Nucleolar	Nuclear/Nucleolar
APC (H-290)	Yes	Yes	Cytoplasmic	Cytoplasmic
APC (N-15)	No	No	Apical/cytoplasmic	Nuclear/apical
APC (Ab1)	Yes	No	Nuclear	Nuclear
APC (Ab2)	Yes	Yes	-	-
APC (Ab3)	No	No	Nuclear	Nuclear
APC (Ab4)	No	No	Apical/cytoplasmic	Apical/cytoplasmic
APC (Ab5)	Yes	Yes	Apical/cytoplasmic	Apical/cytoplasmic
APC (Ab6)	Yes	Yes	Apical/cytoplasmic	Apical/cytoplasmic
APC (Ab120)	Yes	Yes	Apical/cytoplasmic	Apical/cytoplasmic
APC (M-APC)	Yes	-	Cytoplasmic	Nuclear

Of the antibodies characterised here the majority appear to be unable to detect APC by at least one technique, or to cross react with other proteins as well as APC. Many APC antibodies detect and apical protein, which appears to correspond to a 150 kDa protein rather than full length APC, this is discussed further in chapter 5.

Localisation with anti-APC (M-APC) (and anti-APC (N-15)) is dependent on both mutation status of APC and cell density. This is discussed further in chapter 4.

Chapter 4 – Results: Variation of localisation and interactions of APC and β-catenin with mutation status and cell density

4.1 Introduction

Several studies have shown that APC has a CRM1-dependent nuclear export function for β-catenin that regulates the sub-cellular localisation and turnover of β-catenin (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a). In sub-confluent colorectal cancer cell lines which express only truncated APC, both APC and β-catenin are localised to the nucelus with fainter cytoplasmic staining, and some membrane staining for β-catenin. This contrasts with colorectal cancer cell lines which express wild type APC but have a mutation in β-catenin, in these cell lines APC is predominantly cytoplasmic and β-catenin is predominantly localised to the membrane. When these cells are treated with Leptomycin B, an inhibitor of CRM1-dependent nuclear export, nuclear accumulation of both APC and β-catenin is observed.

β-catenin has also been shown to undergo APC-independent nuclear export (Eleftheriou *et al.*, 2001; Weichens & Fagotto, 2001; Henderson & Fagotto, 2002), although it has not been determined when one mechanism may prevail over the other. It has been suggested that CRM1-independent nuclear export is relatively slow and that the rate of nuclear export is accelerated by APC (Rosin-Arbesfeld *et al.*, 2003).

Nuclear localisation of APC and β-catenin in cell lines which express only truncated APC is thought to be due to loss of nuclear export sequences which are found in the central domain of APC. APC truncations in the majority of colorectal tumours (Miyaki *et al.*, 1994) and cell lines derived from colorectal tumours (Rosin-Arbesfeld *et al.*, 2000) result in loss of all three central nuclear export sequences suggesting that these nuclear export sequences are important in regulating APC and β-catenin localisation.

The nuclear export sequence NES1506 (the closest to the N terminus of the central nuclear export sequences) appears to be critical in determining localisation. Cell lines which retain NES1506 but have lost the other two central

nuclear export sequences show efficient nuclear exclusion of both APC and β-catenin (Rosin-Arbesfeld *et al.*, 2003).

Although other putative nuclear export sequences have been discovered at the N-terminal end of APC (Henderson 2000; Neufeld *et al.*, 2000a) it is not clear what role these have in nuclear export of APC. It has been reported that an APC construct truncated at 1,309 amino acids was able to be exported from the nucleus, and export some β-catenin, in SW480 cells (Henderson 2000), this suggests the N-terminal nuclear export sequences are active in this case. However, these data has been disputed by Rosin-Arbesfeld and co-workers (2003) who claim that APC fragments lacking the N-terminal nuclear export sequences are efficiently excluded from the nucleus and are able to reduce β-catenin-dependent transcriptional activity in SW480 cells (Rosin-Arbesfeld *et al.*, 2003; Heppner Goss *et al.*, 2002). Also, an APC construct lacking all central nuclear export sequences but having both N-terminal nuclear export sequences was found to some extent in nuclei of cells and was not effective at reducing β-catenin dependent transcriptional activity (Rosin-Arbesfeld *et al.*, 2003).

It has been reported that both APC and β-catenin undergo cell density-dependent redistribution (Brabletz *et al.*, 2001; Zhang *et al.*, 2001; Brocardo *et al.*, 2001; Dietrich *et al.*, 2002). This may account for some of the differences seen in activity of nuclear export sequences, as it is possible that different nuclear export sequences are active as cell density varies. Also, much of this work has been carried out using transfected contructs, this may result in levels of protein different from endogenous levels. Overexpression may result in the apparent functions of the protein being different from that which would be seen with endogenous protein.

Co-localisation of APC and β-catenin in the nuclei of colorectal cancer cell lines with truncating mutations in APC has been described (Rosin-Arbesfeld, et al 2000; Henderson, 2000). It has also been reported that both APC and β-catenin undergo cell density-dependent redistribution (Brabletz *et al.*, 2001; Zhang *et al.*, 2001; Brocardo *et al.*, 2001; Dietrich *et al.*, 2002). No study to date has carried

out a direct comparison of APC and β-catenin localisation within the cell as cell density varies. Also, as all the colorectal cancer cell lines used in these studies either express truncated APC or mutated β-catenin the localisation of these proteins in normal epithelial cell lines which have both wild type APC and wild type β-catenin has not been described.

In normal colon mucosa APC is detected in the cytoplasm in normal cells above the crypt and is detected nuclei in cells at the base of crypts (Rosin-Arbesfeld *et al.*, 2003). Cells at the base of crypts are proliferating cells while cells above the crypt are differentiated epithelial cells. β-catenin has been observed in the nuclei of cells at the base of crypts and at the membrane in all cells with exclusion from the nucleus in differentiated epithelial cells above the crypt (van de Wetering *et al.*, 2002). In polarised epithelial MDCK cells APC has been reported to be localised to the cytoplasm and microtubule tip clusters in sub-confluent cells or to the lateral membrane in confluent cells (Rosin-Arbesfeld *et al.*, 2001). APC has also been reported to be localised to the nucleus in MDCK cells (Zhang *et al.*, 2001).

In this study we further examine localisation of APC and β-catenin in a variety of cell lines at a variety of cell densities, and seek explanations for the changes observed in localisation of APC and β-catenin with cell density. Localisation of APC and β-catenin are linked in sub-confluent colorectal cancer cell lines, and both proteins are seen to shuttle between nucleus and cytoplasm. Localisation of APC and β-catenin has not previously been correlated in normal epithelial cells or high density colorectal cancer cells. Localisation of APC and β-catenin are linked in sub-confluent colorectal cancer cell lines, and both proteins are seen to shuttle between nucleus and cytoplasm. We investigate whether the localisation of APC and β-catenin is still correlated in confluent colorectal cancer cell lines and whether the observations made in colorectal cancer cell lines are also seen in normal epithelial cells and attempt to find explanations for changes in localisation of APC and β-catenin observed.

Two colorectal cancer cell lines are studied here: HCT116 which has a 3 base pair deletion in β-catenin resulting in loss of a serine residue at codon 45 (Ilyas *et al.*, 1997; Morin *et al.*, 1997; Sparks *et al.*, 1998) and SW480 which has a mutation in APC resulting in truncation at 1338 amino acids and only one allele of APC due to loss of heterozygosity (Nishisho *et al.*, 1991). Three normal epithelial cell lines are studied: HEK293 (human embryonic kidney), C57MG (mouse mammary epithelial) and MDCK (canine kidney epithelial). In addition one embryonal carcinoma cell line (NTERA-2) which has one truncated (approximately 120 kDa) and one wild type allele of APC was observed (G.T. Roberts, unbublished data).

4.2 Antibodies

Two antibodies are used in this study anti-APC (M-APC) and anti-APC (N-15) (see figure 4.1 for location of antibody epitopes relative to the structure of full length APC). Anti-APC (M-APC) is directed to amino acids 1034-2130 (Näthke *et al.*, 1996), this antibody and has been show to reliably detect APC (Rosin-Arbesfeld et al., 2001; Mogensen et al., 2002). Anti-APC (N-15) has been used to detect APC and gave similar localisation to M-APC in cell lines with both wild type and truncated APC (Henderson, 2000). However, questions have been raised as to the reliability of anti-APC (N-15) (Mogensen *et al.*, 2002, Rosin-Arbesfeld *et al.*, 2001; Roberts *et al.*, 2003). In western blot and immunoprecipitation anti-APC (N-15) does not detect APC and shows a strong cross-reactivity to Ku80 (Roberts *et al.*, 2003). In immunofluorescence staining anti-APC (N-15) does give a similar localisation to anti-APC (M-APC) with regards to nuclear shuttling of APC in colorectal cancer cell lines. However, anti-APC (N-15) fails to detect APC at microtubule tip clusters and detects an apical protein which is not detected by anti-APC (M-APC) (Rosin-Arbesfeld *et al.*, 2001). Although questions have been raised as to the reliability of anti-APC (N-15) data for this antibody is presented here as it is similar to the localisation seen for anti-APC (M-APC) in this case.

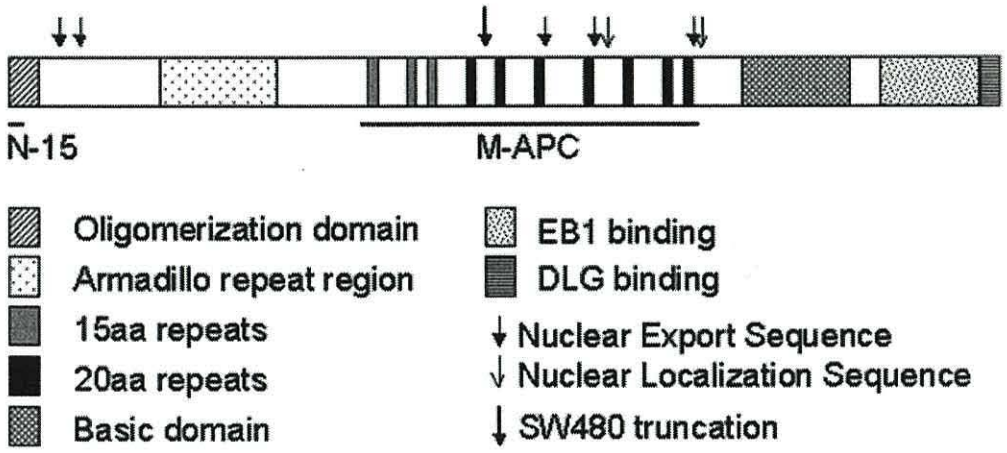


Figure 4.1
Epitopes of anti-APC (N-15) and anti-APC (M-APC) relative to the structure of full length APC. Anti-APC (N-15) is directed to a peptide corresponding to amino acids 2-16 of full length APC. Anti-APC (M-APC) is directed to a peptide corresponding to amino acids 1034-2130 of full length APC.

4.3 Localisation of β-catenin and truncated APC varies with cell density in colorectal carcinoma cell line SW480

In well-differentiated colorectal adenocarcinoma with lymph nose metastases it has been reported that cells at the invasive front are de-differentiated while cells in the central mass of the tumours are more epithelial in appearance (Brabletz *et al.*, 2001). In the central mass, where cells appear to be differentiated epithelial cells, β-catenin is found at the membrane. In de-differentiated mesenchymal-like cells at the invasive front β-catenin was found to be nuclear in the majority of cells with little or no membrane staining. SW480 cells, which are derived from a colorectal adenocarcinoma which gave rise to a lymph node metastasis (Leibovitz *et al.*, 1976), are found to give similar changes in localisation of β-catenin between low and high density cells. Low density cells are mesenchymal in appearance and a similar localisation of β-catenin and E-cadherin to de-differentiated cells at the invasive front (Brabletz *et al.*, 2001). High density SW480 cells appear similar to cells found in the central mass of invasive tumours, with membrane bound β-catenin and E-cadherin and a more epithelial appearance. It has also been shown that down-regulation of β-catenin expression by antisense-oligonucleotides inhibits *in vitro* proliferation and invasiveness of APC mutant colon carcinoma cells (Roh *et al.*, 2001). This indicates SW480 cells are likely to be a useful model for this type of tumour, with sub-confluent cells representing cells at the invasive front and super-confluent cells representing the differentiated epithelial cells seen in the central mass.

In sub-confluent SW480 cells truncated APC is localised to the nucleus with fainter cytoplasmic staining (figure 4.2b,k). β-catenin is also predominantly localised to the nucleus with fainter cytoplasmic staining and some membrane staining. This co-localisation of APC and β-catenin is in accordance with previous observations in cell lines expressing only truncated APC (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000).

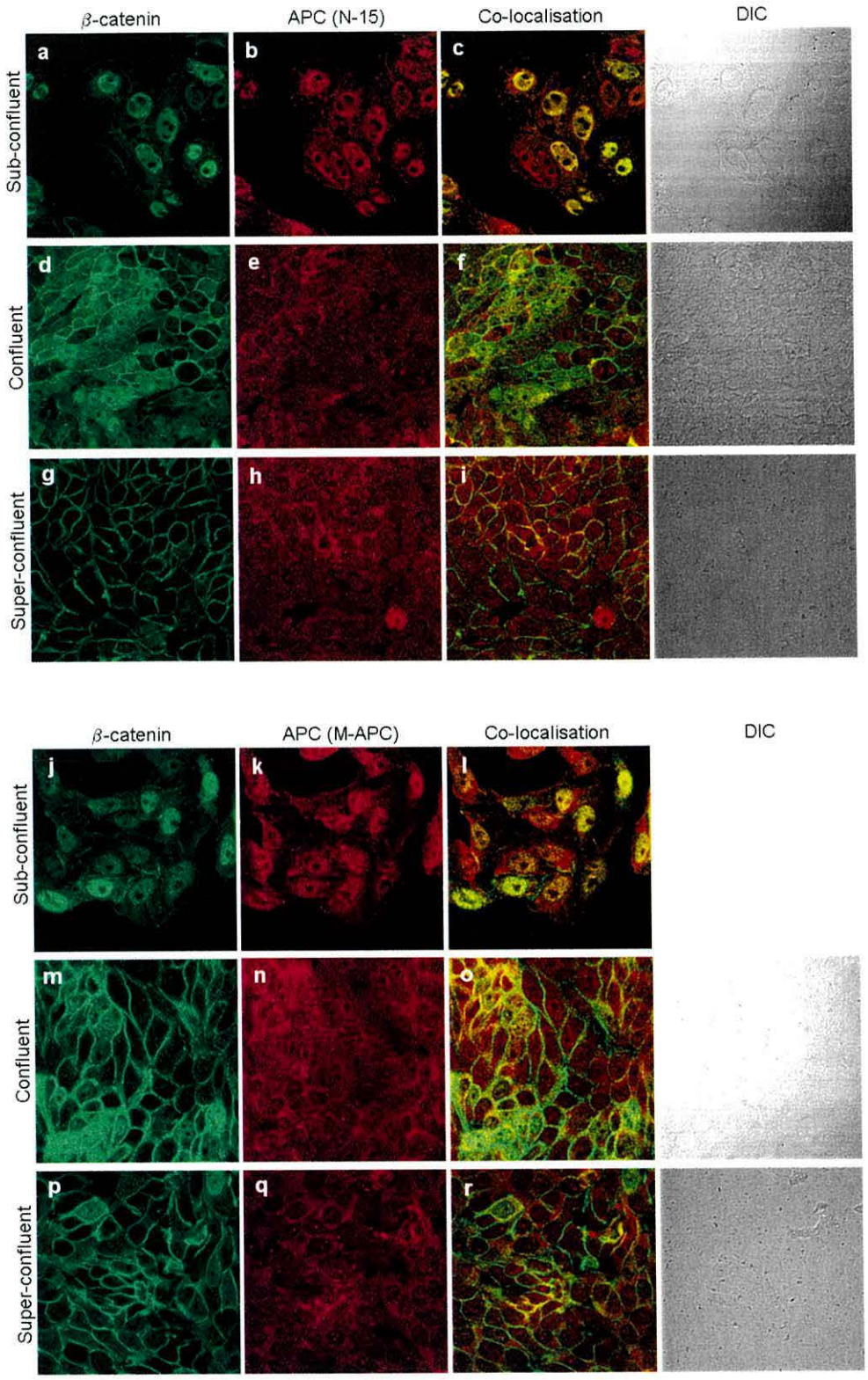


Figure 4.2
Localisation of APC is dependent on cell density in SW480 cells and becomes uncoupled from β-catenin distribution as observed by confocal immunofluorescence microscopy. In sub-confluent cells β-catenin is localised

predominantly in the nucleus with fainter cytoplasmic staining and some membrane staining (a, j). APC is located similarly, predominantly in the nucleus with fainter cytoplasmic staining (b, k). In confluent cells β-catenin shows two localisations, nuclear (with membrane and some cytoplasmic staining) and membrane only (with no nuclear staining) (d, m). Truncated APC in confluent cells is observed in three distribution categories, cytoplasm staining greater than nucleus, cytoplasm staining equal to nucleus and cytoplasm staining less than nucleus (e, n). In super-confluent cells the majority of cells have only membrane β-catenin with some cells having faint nuclear staining (g, p), in the majority of super-confluent cells APC is either predominantly cytoplasmic or equally stained (h, q). Localisation was similar with anti-APC (N-15) and anti-APC (M-APC). In sub-confluent cells APC and β-catenin co-localise well (a-c, j-l), this co-localisation is lost in confluent (d-f, m-o) and super-confluent (g-i, p-r) cells.

As cell density increases nuclear localisation of both APC and β-catenin is progressively lost. In confluent SW480 cells three categories for localisation of APC were observed: nucleus more brightly stained than cytoplasm (as in the majority of sub-confluent cells); evenly stained and cytoplasm more brightly stained than nucleus (figure 4.2e,n). β-catenin localisation was divided into two categories: nuclear (membrane staining and some cytoplasmic staining was also seen in these cells) or membrane only (with no nuclear staining) (figure 4.2d,m). Variation in brightness of staining for β-catenin was observed but the reason for this was not clear. In super-confluent cells a similar distribution of APC to that seen in confluent cells was observed (figure 4.2h,q). β-catenin in super-confluent cells was mostly localised to the membrane with faint nuclear staining in some cells (figure 4.2g,p), none of the brightly stained cells observed in sub-confluent and confluent cultures were seen in super-confluent cultures.

Localisation of APC and β-catenin was quantified by classifying each cell according to localisations of the two proteins, as described in the previous paragraph. For β-catenin cells were classified by localisation regardless of brightness of staining. In sub-confluent cells the majority of cells have nuclear staining for β-catenin (92.6%) (figure 4.3a), this corresponds to predominantly nuclear staining for APC (80.7%). In confluent cells (figure 4.3b) a much larger proportion of cells have only membrane β-catenin (42.4%). The majority of cells are evenly stained for APC (56.6%), some cells have greater cytoplasmic staining (28.3%) and some have greater nuclear staining (15.1%). The co-localisation of APC and β-catenin seen in sub-confluent cells is lost with no correlation between APC and β-catenin staining seen in confluent cells.

Similar results are seen with anti-APC (N-15). Figure 4.4 follows the same set of cultures over 6 days and shows how localisation of both APC and β-catenin change with cell density. In this case cells with nuclear β-catenin are divided into two categories, those that are brightly stained and those that have only faint nuclear staining. It can be seen from the graphs in figure 4.4 that there is a general decrease in nuclear β-catenin and a decrease in cells stained more

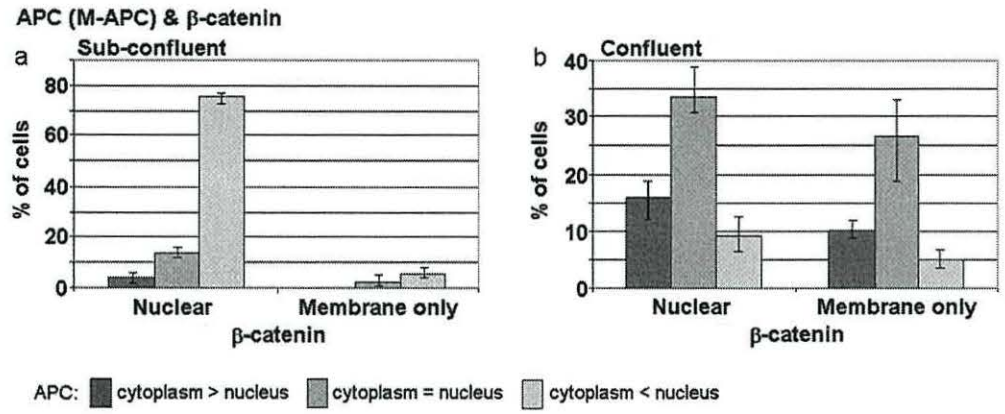


Figure 4.3
The relationship between APC and β-catenin localization at low cell density is lost at high cell density in SW480 cells. In sub-confluent cells (a,c) the majority of cells show nuclear staining for both β-catenin and APC. In confluent cultures (b,d) the co-localization between APC and β-catenin seen in sub-confluent cultures is lost. These graphs show the mean of three repeats with error bars representing the range of the data. β-catenin is split into two categories of localisation, nuclear and membrane only. APC is split into three categories of localisation, nuclear staining greater than cytoplasmic staining (cytoplasm < nucleus), nucleus and cytoplasm equally stained (cytoplasm = nucleus) and cytoplasmic staining greater than nuclear staining (cytoplasm > nucleus).

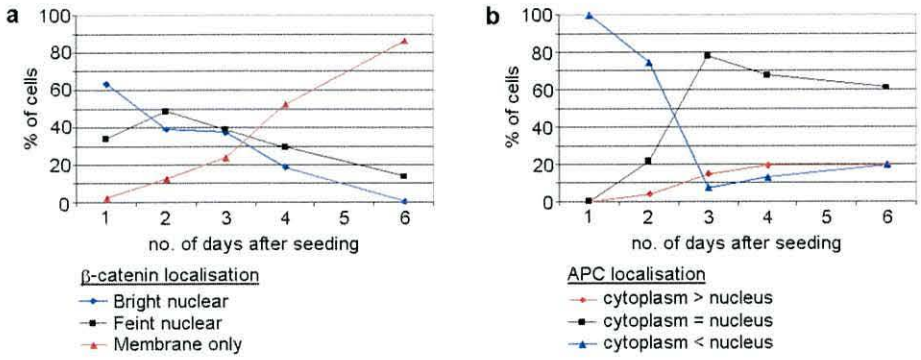


Figure 4.4
Changes in localisation of APC and β-catenin as cell density increases. Data shown here is for one repeat using anti-APC (N-15). Day 1 represents sub-confluent cells, day 3 confluent cells and day 5 super-confluent cells. At day 1 the majority of β-catenin is nuclear with almost no cells having only membrane staining (a). As cell density increases there is a decrease in cells having nuclear β-catenin and a corresponding increase in cells which have only membrane staining. At day 1 APC is predominantly nuclear, as cell density increases there is a general decrease in cells with nuclear localisation of APC and a corresponding increase in cells which are either evenly stained or have predominantly cytoplasmic staining (b).

brightly for β-catenin. Figure 4.4a shows that the number of cells having nuclear β-catenin decreases from almost 100% at 1 day (sub-confluent) to less than 15% at day 6 (super-confluent). Both bright and feint nuclear staining decrease as cell density increases. In high density cells only faint nuclear staining is seen. Correspondingly, the proportion of cells having only membrane β-catenin increases from barely above zero at day 1 to almost 90% at day 6. Figure 4.4b shows that APC localisation also changes as cell density increases, with a general decrease in proportion of cells having predominantly nuclear APC and a corresponding increase in the proportion of cells having either predominantly cytoplasmic staining or even staining. As with anti-APC (M-APC) the change in localisation as cell density increases is associated with a loss of co-localisation of APC and β-catenin (data not shown).

The change in localisation of APC and β-catenin as cells progress from low to high cell density appears to be reversible. SW480 cells were allowed to grow for 7 days, 2 days beyond the density used for super-confluent cells and seeded as usual. The 7 day culture appeared similar to super-confluent cells with membrane β-catenin and predominantly cytoplasmic APC. The sub-confluent cells seeded from this culture appeared similar to normal sub-confluent cells, with nuclear APC and β-catenin (data not shown).

4.4 NTERA-2 cells, which have one truncated and one wild type copy of APC also show cell density-dependent variation in APC localisation

NTERA-2 is a human embryonal carcinoma cell line derived from a lung metastasis of a testicular teratocarcinoma (Fogh & Trempe, 1975; Andrews *et al.*, 1984). A similar APC localisation is seen in this cell line to that seen in SW480, which is derived from a colorectal tumour which gave rise to a lymph node metastasis (Leibovitz *et al.*, 1976). NTERA-2 cells have one wild type APC allele and one mutated allele giving a truncated protein of approximately 120 kDa (G.T.

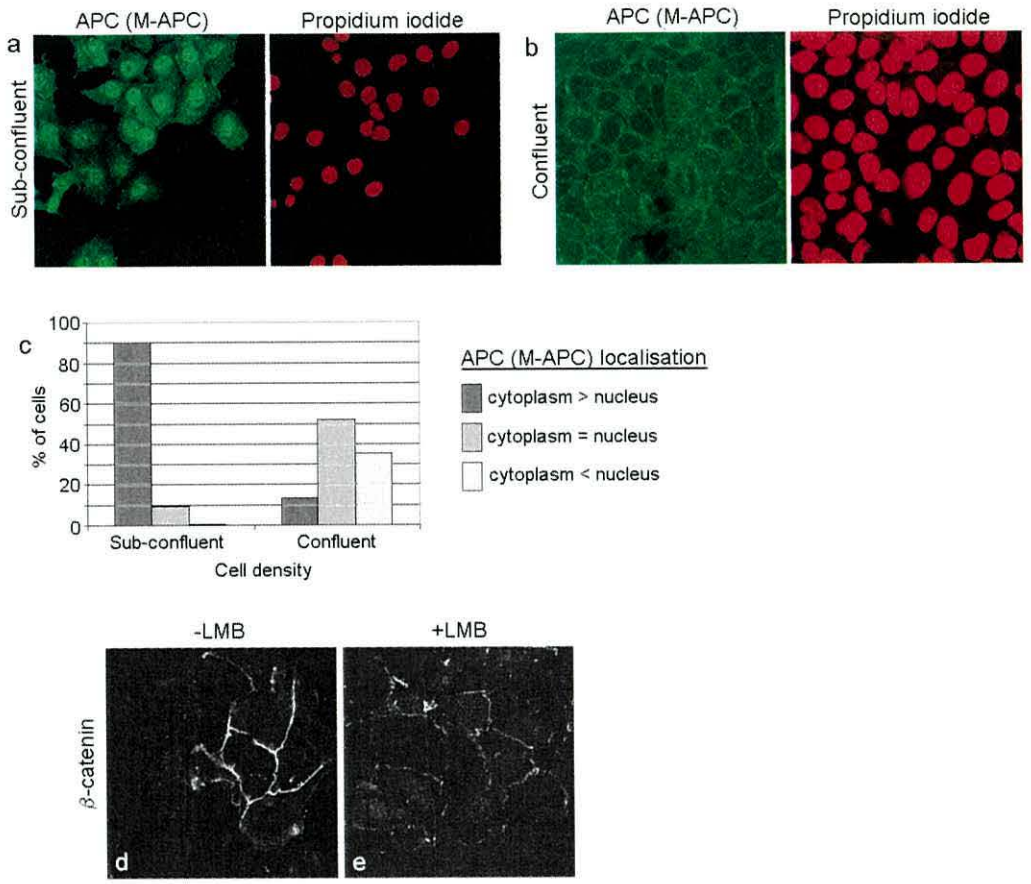


Figure 4.5
Localisation of APC in NTERA-2 embryonal carcinoma cells is similar to that seen in SW480. In sub-confluent cells APC is found in the nucleus with fainter cytoplasmic staining (a). In confluent cells a mixture of localisations is seen (b), this is similar to localisations observed in SW480. There is also an increase in membrane staining for APC in confluent cells compared to sub-confluent cells. The graph (c) quantifies the localisations of APC seen in cubconfluent and confluent NTERA-2 cells. β-catenin is localised to the membrane in NTERA-2 cells, with a small amount of nuclear accumulation of β-catenin in some cells when treated with Leptomycin B.

Roberts, unpublished data). In sub-confluent NTERA-2 cells APC (M-APC) is nuclear in approximately 90% of cells (figure 4.5a,c). Confluent NTERA-2 cells have a similar APC localisation to confluent SW480 cells with some cells being evenly stained, some cells being more brightly stained in the nucleus and some being more brightly stained in the cytoplasm. Many cells in a confluent NTERA-2 culture have lateral membrane staining for APC (as seen in normal epithelial cells (Rosin-Arbesfeld *et al.*, 2001)), this was not seen in SW480 cultures and is likely to be due to the remaining wild type allele of APC in NTERA-2. In NTERA-2 β-catenin is predominantly membrane bound with no nuclear staining (figure 4.6d), although nuclear staining is seen when cells are treated with lithium (a Wnt signal mimic) (section 7.5).

4.5 Localisation of wild type APC does not change significantly with cell density while β-catenin does show some cell density-dependent redistribution in colorectal carcinoma cell line HCT116

HCT116 is a cell line derived from a colorectal carcinoma. This cell line has wild type APC but mutated β-catenin which has a serine residue at codon 45 deleted (Morin *et al.*, 1997; Sparks *et al.*, 1998). β-catenin is localised to the membrane in all cells in both sub-confluent and confluent cells. In sub-confluent cultures 5.3% of cells have nuclear staining for β-catenin and many cells have cytoplasmic staining (figure 4.7a,g). In confluent HCT116 cultures β-catenin is localised to the membrane in all cells with nuclear staining in only 0.1% of cells. APC is either evenly stained or predominantly cytoplasmic in both sub-confluent and confluent cells. The nuclear APC localisation seen in the two cell lines, NTERA-2 and SW480, with truncated APC was not seen in HCT116. Anti-APC (N-15) and anti-APC (M-APC) gave similar localisation with the exception that additional strong apical and edge staining was seen with N-15 but not with M-APC and membrane staining was occasionally seen with M-APC but not with N-15. This is consistent with previous reports (Rosin-Arbesfeld, et al 2000; Henderson, 2000; Neufeld, 2000b) with the exception of the small amount of nuclear staining observed in sub-confluent cells.

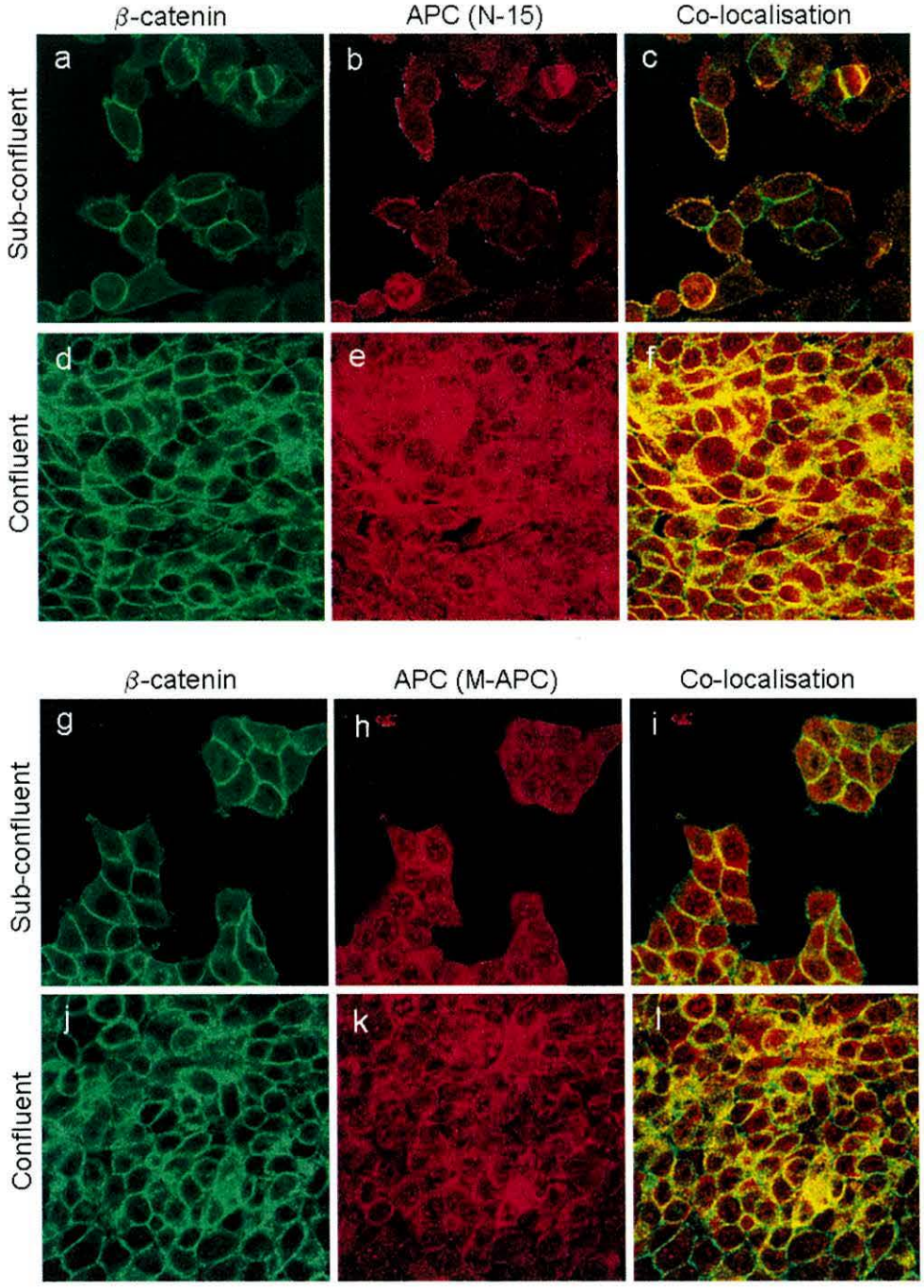


Figure 4.6

Localisation of APC and β-catenin in sub-confluent and confluent HCT116 colorectal carcinoma cells using confocal immunofluorescence microscopy. In sub-confluent cells APC is observed either predominantly in the cytoplasm or equally distributed between the cytoplasm and nucleus (b,h). In confluent cells a similar distribution of APC is observed (e,k). In sub-confluent cells β-catenin is predominantly located at the membrane with some cytoplasmic staining (a,g) with 5.3% of cells also showing nuclear staining. Localisation of β-catenin is similar in confluent cells with the exception that only 0.1% of cells have nuclear staining. The two antibodies used give similar localisation with the exception that edge and apical staining is seen with N-15 which is not seen with M-APC.

4.6 Neither APC nor β-catenin show significant cell density-dependent redistribution in most normal epithelial cells

In HEK293 cells (a normal human epithelial embryonic kidney cell line). β-catenin is seen at the membrane and weakly in the cytoplasm in both sub-confluent and confluent cells (figure 4.7a,d,g,j), no nuclear staining of β-catenin was observed. APC is either evenly stained or more brightly stained in the cytoplasm with both anti-APC (N-15) (figure 4.7b,e) and anti-APC (M-APC) (figure 4.7h,k), a similar distribution to that seen in HCT116 cells (figure 4.6). There does sometimes appear to be more pronounced cytoplasmic staining in confluent cells than in sub-confluent cells with anti-APC (M-APC), however the overall pattern of localisation does not change.

C57MG, a normal mouse mammary epithelial cell line, also shows either even or predominantly cytoplasmic staining of APC in both sub-confluent (figure 4.8b,h) and confluent (figure 4.8e,k) cells with both APC antibodies. β-catenin is localised to the membrane and cytoplasm in both sub-confluent (figure 4.8a,g) and confluent cells (figure 4.8d,j) cells. MDCK, a normal canine kidney epithelial cell line also has a similar localisation of β-catenin. In both sub-confluent (figure 4.9a,g) and confluent (figure 4.9d,j) β-catenin is localised to the membrane with a small amount of cytoplasmic staining. Unlike HCT116 cells, which had nuclear β-catenin in some sub-confluent cells, no nuclear β-catenin was observed in either HEK293, C57MG or MDCK cells.

The data presented here shows that localisation of APC and β-catenin in normal epithelial cell lines is similar to the localisation in wild type APC colorectal cancer cell lines. However, two differences were observed between the normal epithelial cell lines and wild type APC colorectal cancer cells. Firstly, in a small proportion of HCT116 cells nuclear β-catenin is observed in sub-confluent cells, nuclear β-catenin was not observed in any of the normal epithelial cell lines. Secondly, HCT116 cells appear to be much more brightly stained for β-catenin than the normal epithelial cell lines. While confocal microscopy is not a quantitative technique this observation is confirmed by western blot on whole

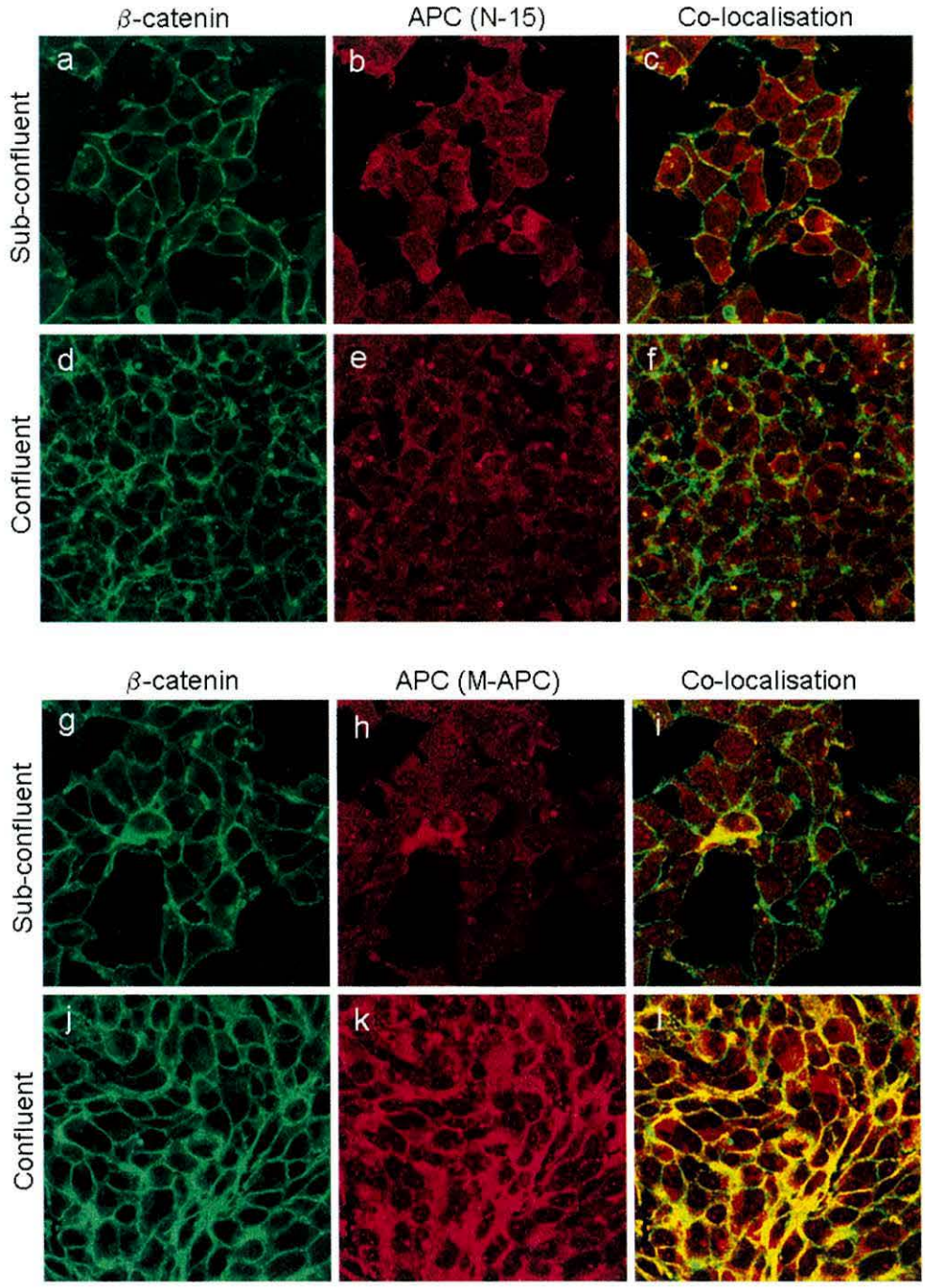


Figure 4.7
Localisation of APC and β-catenin in sub-confluent and confluent HEK293 human embryonic kidney epithelial cells. In sub-confluent cells APC is observed either predominantly in the cytoplasm or equally distributed between the cytoplasm and nucleus (b,h). In confluent cells a similar distribution of APC is observed (e,k). In sub-confluent (a,g) and confluent (d,j) cells β-catenin is predominantly located at the membrane with some cytoplasmic staining. No nuclear β-catenin was observed in sub-confluent or confluent cells.

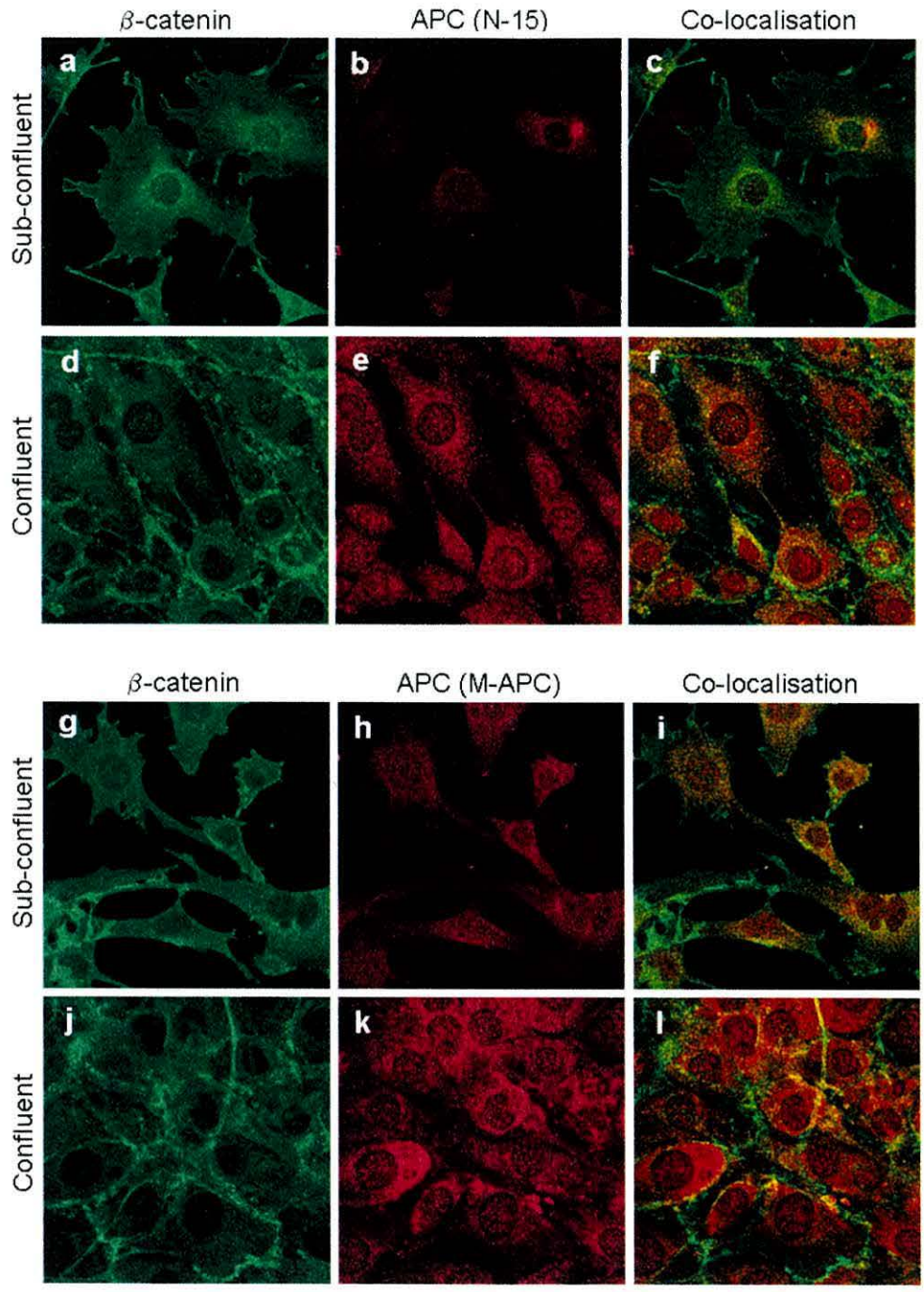


Figure 4.8
Localisation of APC and β-catenin in sub-confluent and confluent C57MG mouse mammary epithelial cells. In sub-confluent cells APC is observed either predominantly in the cytoplasm or equally distributed between the cytoplasm and nucleus (b,h). In confluent cells a similar distribution of APC is observed (e,k). In sub-confluent (a,g) and confluent (d,j) cells β-catenin is predominantly located at the membrane with some cytoplasmic staining. No nuclear β-catenin was observed in sub-confluent or confluent cells.

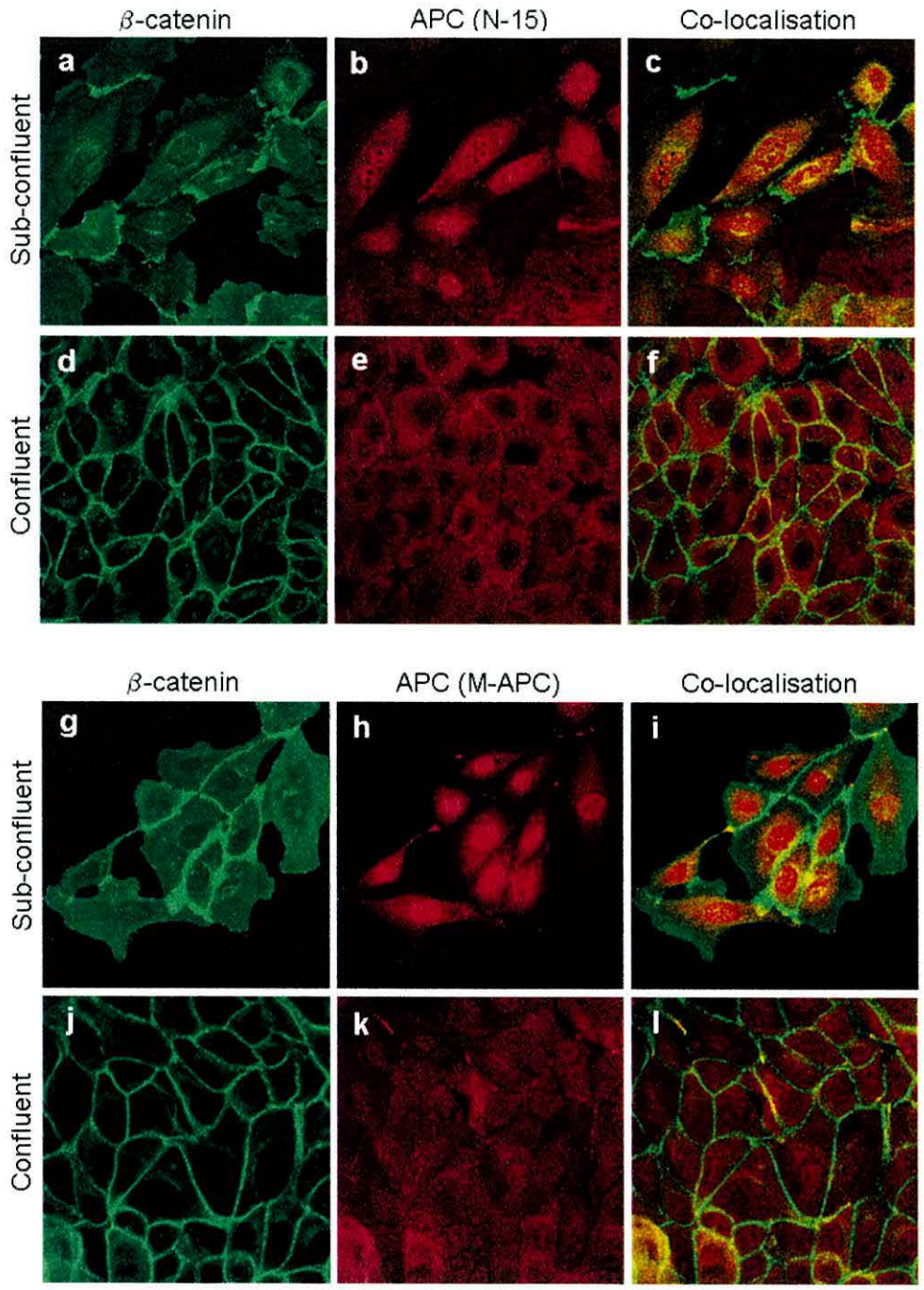


Figure 4.9
Localisation of APC and β-catenin in sub-confluent and confluent MDCK cells. In sub-confluent cells APC staining is predominantly nuclear with fainter cytoplasmic staining (b,h). In confluent cells a reduction in nuclear APC is observed (e,k). This is a global change seen in all cells, unlike SW480 and NTERA-2 in which a variety of localisations was observed in. In sub-confluent (a,g) and confluent (d,j) cells β-catenin is predominantly located at the membrane with some cytoplasmic staining. No nuclear β-catenin was observed in sub-confluent or confluent cells.

cell lysate (not shown). This may reflect reduced degradation of β-catenin in the colorectal cancer cell lines, which express mutant β-catenin, compared to the normal epithelial cell lines, which express wild type β-catenin. However, these cell lines are derived from different tissues leaving the possibility that difference in expression of β-catenin may be linked to tissue type rather than cancer/non-cancerous cells.

4.7 MDCK cells express only wild type APC but do show cell density-dependent redistribution of APC

MDCK, a normal canine kidney epithelial cell line, was similar to the other normal cell lines (HEK293, C57MG) in β-catenin localisation (section 4.6) but differed from other normal epithelial cell lines in APC localisation. In sub-confluent cells APC was observed to be nuclear (figure 4.9b,h). As cell density increased APC was seen to become more cytoplasmic (figure 4.9e,k). Unlike the two cell lines carrying mutations in APC, SW480 and NTERA-2, the reduction in nuclear APC appeared to be a global change rather than a mosaic pattern with a variety of localisations. It is not clear why cell density-dependent redistribution of APC is seen in this cell line, interestingly a sub-line derived from MDCK, MDCKII, does not have nuclear localisation of APC even in sub-confluent cells.

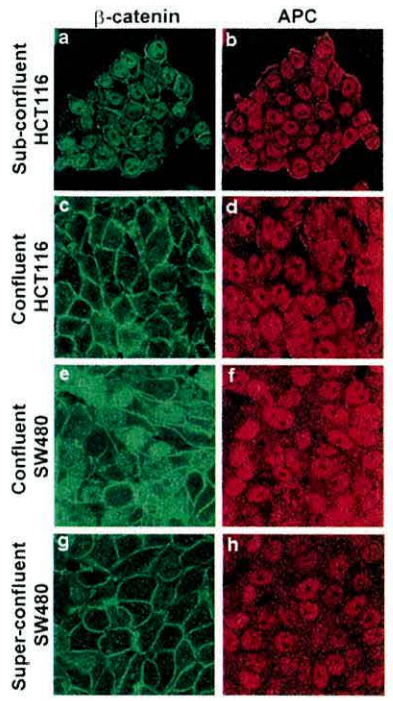
Rosin-Arbesfeld and co-workers (2001) described localisation of APC in MDCK cells as predominantly cytoplasmic, while Zhang and co-workers (2001) have described nuclear localisation of APC reducing with cell density as described here. This difference in localisation seen in MDCK cells has been cited as evidence of unreliability/cross-reactivity of anti-APC (N-15). Two factors have resulted in the variety of localisations reported in MDCK cells. Firstly, there are three MDCK lines: the original cell line MDCK, and two sub-lines, MDCKI and MDCKII, all of which are commonly referred to as MDCK. Cytoplamic localisation of APC with antibody M-APC (Rosin-Arbesfeld *et al.*, 2001) has been reported in the sub-line MDCKII whereas Zhang and co-workers (2001)

reported nuclear localisation in the original MDCK line. Figure 4.9 shows that in the original MDCK line both M-APC and N-15 detect nuclear APC in sub-confluent cells with a cell density-dependent redistribution to the cytoplasm. As well as the different sub-lines of MDCK used a variety of antibodies have been used to detect APC in this cell line, not all of these antibodies reliably detect APC (chapter 3).

4.8 Extent of CRM1-dependent nuclear export of β-catenin varies with cell type and cell density while APC is continuously exported in all cell lines at all densities

APC and β-catenin are known to shuttle between the nucleus and cytoplasm in many colorectal cancer cell lines (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a; Neufeld *et al.*, 2000b). Cells were treated with Leptomycin B (LMB), an inhibitor of CRM1-dependant nuclear export, to investigate whether the cell density-dependent changes in localisation of APC and β-catenin were associated with a change in CRM1-dependent nuclear export. APC was seen to respond to LMB by accumulating in the nucleus in both SW480 and HCT116 at all densities tested (figure 4.10b,d,f,h). In the normal epithelial cell lines tested APC again accumulated in the nucleus in response to LMB in all cell lines in both sub-confluent and confluent cells (figure 4.11b,d,f,h,j,l). This demonstrates that APC shuttles continuously between the nucleus and cytoplasm in all cell lines studied at all densities.

These observations are consistent with previous observations in sub-confluent cells (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a; Neufeld *et al.*, 2000b) but contradict observations that cell density-dependent redistribution of APC does not depend on sustained nuclear export (*Zhang et al.*, 2001). Zhang and co-workers (2001) use anti-APC (Ab1) in their LMB experiments. There is doubt whether this antibody detects APC effectively in immunofluorescence experiments. Anti-APC (Ab1) cross-reacts strongly with a



	+LMB	Untreated
Nuclear β-catenin	83.4% n=489	5.3% n=942

	+LMB	Untreated
Nuclear APC	95.3% n=414	2.1% n=1024

	+LMB	Untreated
Nuclear β-catenin	14.0% n=662	0.1% n=1376

	+LMB	Untreated
Nuclear APC	95.2% n=662	0.4% n=1376

	+LMB	Untreated
Nuclear β-catenin	81.7% n=732	47.9% n=594

	+LMB	Untreated
Nuclear APC	89.9% n=732	19.4% n=594

	+LMB	Untreated
Nuclear β-catenin	63.5% n=468	19.3% n=378

	+LMB	Untreated
Nuclear APC	88.2% n=468	13.5% n=378

Figure 4.10
APC shuttles continuously between the nucleus and cytoplasm and accumulates in the nucleus in response to Leptomycin B (LMB) in colorectal carcinoma cells whereas nuclear accumulation of β-catenin in response to LMB decreases as cell density increases. Sub-confluent HCT116 show nuclear accumulation of β-catenin in response to LMB in 83.4% of cells (a), however in confluent cells only 14% show nuclear accumulation (c). Only 81.7% of LMB treated confluent SW480 cells (e) and 47.9% of untreated control cells show nuclear accumulation of β-catenin. There is a further decrease in nuclear β-catenin in super-confluent cells with only 63.5% of LMB treated cells (g) and 19.3% of untreated control cells showing nuclear localisation of β-catenin. In both cell lines at all densities observed APC accumulates in the nucleus in response to LMB. Images show only treated cells, tables show data for both LMB treated cells and the untreated control cells.

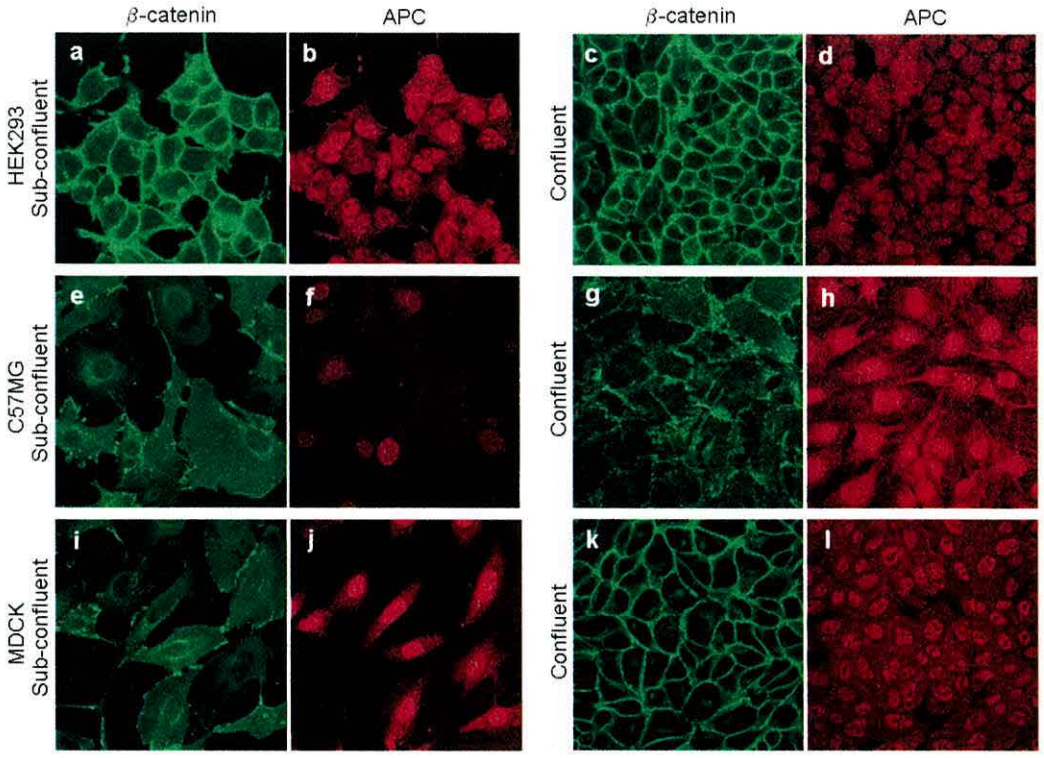


Figure 4.11
β-catenin does not accumulate in the nucleus in response to Leptomycin B (LMB) treatment in normal epithelial cells. As in the colorectal carcinoma cells APC also accumulates in the nucleus in response to LMB at all densities tested in normal epithelial cells (b,d,f,h,j,l). β-catenin does not accumulate in the nucleus in response to LMB treatment in any of the normal epithelial cell lines tested (a,c,e,g,i,k).

protein of 60-70 kDa on western blot and gives nuclear staining in all cell lines studied, including those in which M-APC detects only cytoplasmic APC (section 3.9). In untreated super-confluent MDCK cells anti-APC (Ab1) shows APC to be predominantly nuclear which contradicts the localisation seen with anti-APC (M-APC) or anti-APC (N-15).

The observation that APC is shuttling continuously at all densities indicates that the density dependent redistribution of truncated APC to the cytoplasm in the truncated APC cell lines is not due to decreased nuclear entry of APC. It has been shown that transfecting constructs with only the N-terminal nuclear export sequences into SW480 cells does not efficiently exclude APC from the nucleus or reduce β-catenin-dependent transcriptional activity, while the central nuclear export sequences are sufficient to reduce β-catenin-dependent transcriptional activity and exclude APC from the nucleus (Rosin-Arbesfeld *et al.*, 2003). These experiments were carried out in sub-confluent SW480 cells. One possibility is that these N-terminal nuclear export sequences are not active in nuclear exclusion of APC in sub-confluent cells but become more active as cell density increases. However, it is also a possibility that APC is exiting the nucleus by a CRM1-independent mechanism in high density cells.

Localisation of β-catenin in LMB treated cells varies with cell line and cell density. In sub-confluent HCT116 cells β-catenin accumulates in the nucleus in response to LMB (figure 4.10a) as previously described (Henderson 2000). 83.4% of sub-confluent LMB treated HCT116 cells were seen to have nuclear β-catenin, compared to 5.3% of untreated cells. In confluent HCT116 cells this decreased, with 14% of LMB treated cells (figure 4.10c) and only 0.1% of untreated cells having nuclear β-catenin. In sub-confluent SW480 cells the majority of cells have nuclear localisation of β-catenin even without LMB treatment (figure 4.2a,j). Nuclear localisation decreased in confluent cells with 81.7% of LMB treated cells and 47.9% of untreated control cells having nuclear β-catenin. This decreased further in super-confluent SW480 cells with 63.5% of LMB treated cells and 19.3% of untreated cells having nuclear β-catenin.

In contrast to the colorectal cancer cell lines studied, β-catenin was not seen to accumulate in the nucleus in response to LMB in any of the normal epithelial cell lines studied here at either low or high cell density. This indicates that in normal epithelial cell lines, which express wild type APC and β-catenin, either β-catenin exits the nucleus independently of APC/CRM1 or nuclear entry of β-catenin is restricted. It has previously been shown that β-catenin is able to exit the nucleus by an LMB-insensitive mechanism (Yokoya *et al.*,1999; Eleftheriou *et al.*,2001; Fagotto *et al.* 1998; Wiechens & Fagotto, 2001). This indicates that the LMB-dependent nuclear accumulation of β-catenin seen in colorectal cancer cell lines is as a result of abnormality in control of β-catenin localisation in these cell lines, possibly due to mutation in APC or β-catenin. In cases of mutation, β-catenin phosphorylation sites are often affected, and it has been reported that it is de-phosphorylated β-catenin which enters the nucleus in response to a Wnt signal in normal epithelial cells (Staal *et al.*, 2002). The observation that truncation of APC also allows β-catenin to enter the nucleus may indicate that as well as removing β-catenin from the nucleus APC may also have a function in regulating nuclear entry of β-catenin. Alternatively, it may be that in cells with defects in degradation of β-catenin it is the extremely high levels of β-catenin which is allowing to evade control of its nuclear entry.

As mentioned earlier, it has been suggested that sub-confluent cells may be a model for cells at the invasive front of a tumour whereas high density cells may be more similar to the central mass of a tumour (Brabletz *et al.*, 2001). This is supported by the observation that super-confluent cells are more similar to differentiated epithelial cells, whereas sub-confluent cells are similar to de-differentiated mesenchymal cells. The reduction in LMB-dependent nuclear accumulation of colorectal cancer cells as they become more confluent further supports this theory in that they become more similar to differentiated epithelial cells, which do not show any LMB-dependent nuclear accumulation of β-catenin.

In NTERA-2 cells, which express both truncated and wild type APC, a small amount of β-catenin is seen to accumulate in the nuclei when CRM1-dependent nuclear export is blocked by treatment with Leptomycin B. However, despite

90% of sub-confluent cells having nuclear APC (figure 4.5c) not all cells accumulate β-catenin in their nuclei in response to LMB (figure 4.5e). This is in contrast to SW480 cells, which express only truncated APC, where the majority of sub-confluent cells have nuclear localisation of β-catenin even without LMB treatment (figure 4.2a,l, figure 4.3a,c). This may be due to the remaining wild type APC having an effect on control of nuclear entry of β-catenin, possibly by controlling total levels of β-catenin. Once the Wnt pathway is activated in these cells (by lithium treatment (section 7.5)) they appear similar to SW480 cells in that β-catenin localises to the nucleus. In wild type APC cells treated with lithium, nuclear accumulation of β-catenin is at a low enough level that it is not detected by a total β-catenin antibody but only by an antibody specific to de-phosphorylated β-catenin (Staal *et al.*, 2002). This indicates that accumulation of large amounts of β-catenin in the nucleus in response to Wnt signalling is not a normal response and may be a result of the truncated APC expressed in NTERA-2.

4.9 Cell density-dependent redistribution of truncated APC and β-catenin is not linked to proliferative status of the cells

One potential explanation for the changes in localisation of truncated APC and β-catenin with cell density is that in confluent cultures there is a reduction in cell proliferation. This was tested by staining SW480 cells grown for 4 days (confluent is 3 days, super-confluent is 5 days) for either APC or β-catenin and Ki-67. Ki-67 is a proliferating cell marker which stains proliferating cells in all stages of the cell cycle but does not stain G_0 cells which have ceased to proliferate. Figure 4.12 shows there is no correlation between Ki-67 expression and localisation of either APC or β-catenin in SW480 cells. This shows there is no link between cell proliferation and APC and β-catenin localisation.

This contrasts observations that cell density-dependent redistribution of APC in MDCK cells is linked to proliferative status (Zhang *et al.*, 2001). MDCK cells express wild type APC whereas SW480 cells express only truncated APC which

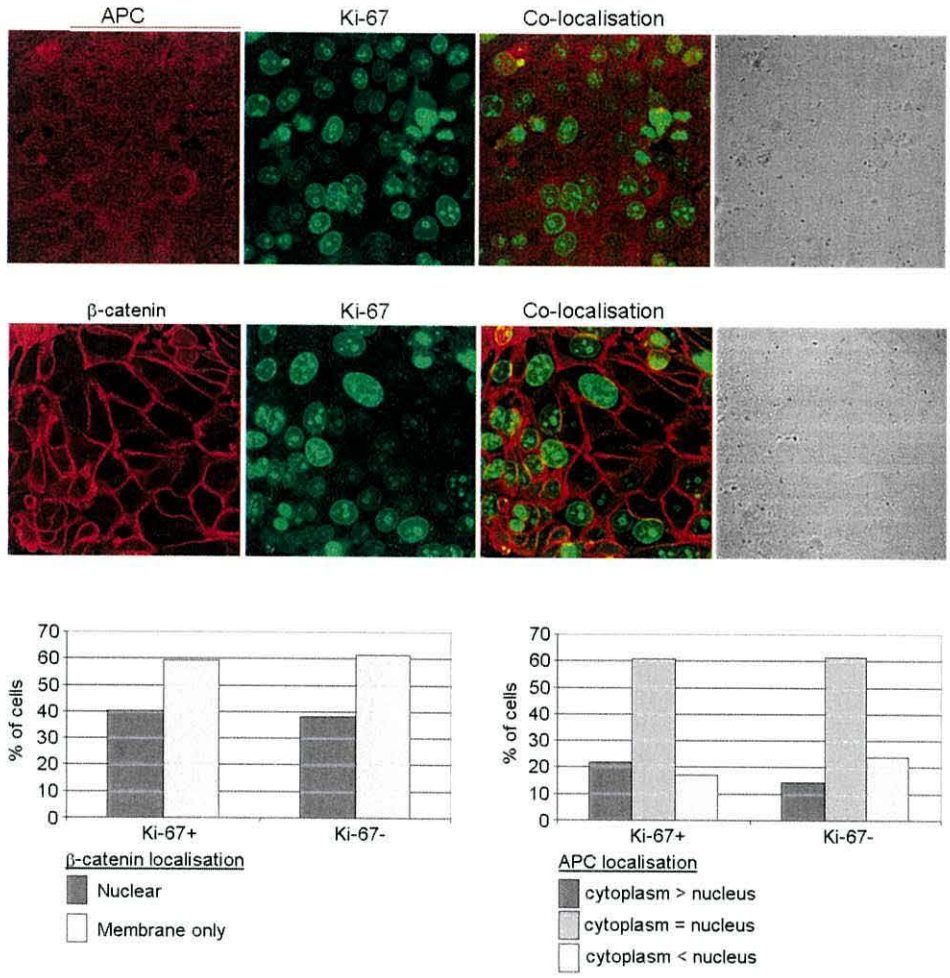


Figure 4.12
No correlation is seen between APC or β-catenin localisation and cell proliferation in SW480 as seen by Ki-67 expression. All the localisations observed for both β-catenin and APC are observed in both Ki-67 positive and Ki-67 negative cells. This indicates no correlation between proliferative status and localisation of either APC or β-catenin.

may account for this difference. However, Zhang and co-workers (2001) used FACS analysis to indicate proliferative status and concluded that a global decrease in proliferating cells was associated with a global decrease in nuclear localisation of APC. However, they assumed all super-confluent cells are in G_0 whereas FACS data shows a significant proportion in G_2 indicating that at least some cells are still proliferating. This would indicate that a sizeable proportion of super-confluent cells should have a similar appearance to proliferating sub-confluent cells whereas observations indicate that the change in localisation with cell density in MDCK cells is a global change.

4.10 Phosphorylated β-catenin levels change with cell density but total levels of β-catenin do not change

β-catenin localisation and degradation is dependent on phosphorylation (Sadot *et al.*, 2002; Staal *et al.*, 2002), therefore it was tested whether there was a change in either total β-catenin levels or in levels of phosphorylated β-catenin with cell density. Two antibodies were used. One, anti-p41,45-β-catenin is directed to the phosphorylation site at serine 45, phosphorylation of this site is necessary before Wnt dependent phosphorylation to occur (Amit *et al.*, 2002; Hagen & Vidal-Puig, 2002; Liu *et al.*, 2002; Sakanaka, 2002). The other, anti-p33,37,41-β-catenin is directed to the Wnt dependent phosphorylation sites (van Noort *et al.*, 2002; Sadot *et al.*, 2002). No density-dependent change was seen in total β-catenin levels in any of the cells lines studied (figure 4.13, table 4.1). In SW480 cells it was observed that many sub-confluent and confluent cells were more brightly stained with these brightly stained cells being lost from super-confluent cultures. Despite this reduction in brightly stained cells no global decrease in β-catenin levels was observed by western blot. A decrease was observed in p33,37,41-β-catenin (the Wnt dependent phosphorylation sites) in super-confluent cells compared to sub-confluent cells in both the cancer and the normal epithelial cell lines studied. It was not clear whether this drop was due to a decrease in phosphorylation of β-catenin or due to increased speed of degradation once it had been phosphorylated. p41,45-β-catenin levels decreased

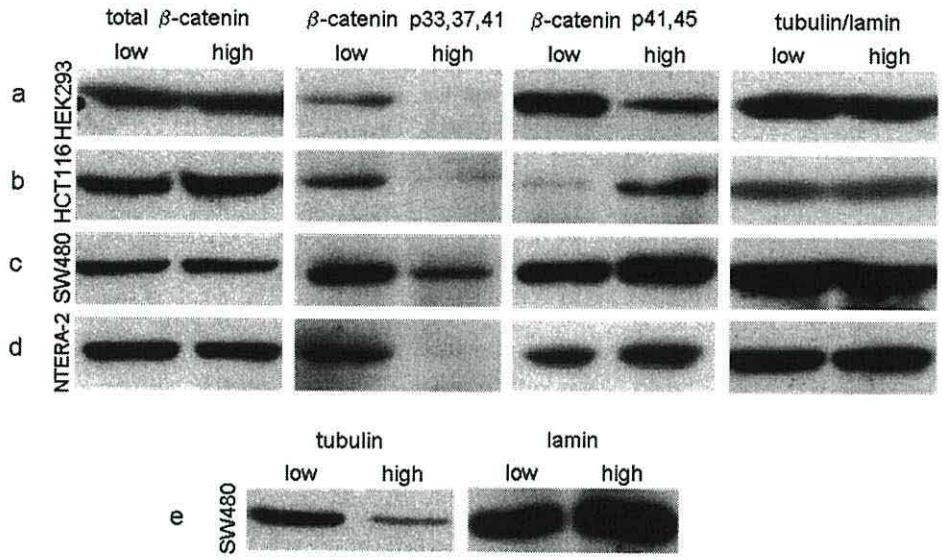


Figure 4.13
Total β-catenin expression does not change with cell density but levels of phosphorylated β-catenin changes with cell density. Densitometry analysis is shown in table 4.1. Going from low (sub-confluent) to high (super-confluent) cell density no change is seen in β-catenin expression by western blot. Phosphorylated β-catenin detected by an antibody directed to β-catenin phosphorylated at aa 33,37 or 41 decreases as cell density increases. Phosphorylated β-catenin detected by an antibody directed to β-catenin phosphorylated at aa 41 or 45 increases as cell density increases in colorectal cancer cells but decreases as cell density increases in normal epithelial cells. A decrease in α-tubulin levels as cell density increases is observed in SW480 (e), a lamin western of the same blot shows the decrease in α-tubulin levels is not due to a decrease in the total amount of protein loaded.

in all the cancer cell lines, but either did not change or increased in normal epithelial cells (figure 4.13, table 4.1), however there is not a sufficient number of repeats for normal epithelial cells to show this conclusively.

Table 4.1
Densitometry analysis of western blots shown in figure 4.13. β-catenin and phospohrylated β-catenin (p33,37,41 or p41,45) were normalised for either α-tubulin or total β-catenin (show in brackets). All data is shown as an x-fold increase in expression levels, therefore numbers of less than one indicate a decrease. Decrease in expression levels are shown in red and increases in black. "-" indicates a decrease to levels to low to be quantified. Lamin was used as a loading control for SW480 instead of α-tubulin.

Cell line/repeat	total β-catenin (tubulin)	p33,37,41 (tubulin)	p33,37,41 (total)	p41,45 (tubulin)	p41,45 (total)
293 1	1.03	-	-	0.38	0.37
293 2	2.52	-	-	0.94	0.37
293 3	1.38	0.43	0.31	0.40	0.29
HCT116 1	0.88	-	-	0.78	0.88
HCT116 2	0.67	0.27	0.40	4.04	6.06
HCT116 3	1.35	0.20	0.15		
SW480 1			-		0.27
SW480 2	0.56	0.24	0.43	0.93	1.66
SW480 3			0.32		1.20
Nt2/D1 1	4.48	0.12	0.02	1.08	0.24
Nt2/D1 2	0.83	-	-	0.97	1.17
Nt2/D1 3	1.45	0.69	0.47	1.59	1.10

While interesting changes in phosphorylation are shown here, it was not possible to correlate these changes to the changes in β-catenin localisation observed, as there is not a sufficient amount of phosphorylated β-catenin in the cells for immunofluorescence staining. Also, none of the phosphorylated β-catenin changes seen here correlated with a particular change in localisation. While phosphorylation/dephosphorylation may still have a role in determining localisation it is not possible to determine this from the data presented here.

It was observed that α-tubulin levels appeared to reduced in super-confluent SW480 relative to sub-confluent cells using anti- α-tubulin (B-5-1-2) (figure 4.13e). Coomassie blue stain and western with an anti-lamin antibody showed

loading was consistent and this appeared to be a genuine change. It seems unlikely that there is a genuinely very low level of α-tubulin expression in this cell line and microtubules are still clearly visible by immunofluorescence staining with anti- α-tubulin (YOL1/34) in high density cells. The reason for this change observed in α-tubulin was not clear but where possible SW480 western data was normalised for lamin rather than α-tubulin.

4.11 β-catenin is bound to truncated APC in higher levels at low cell density and to E-cadherin in higher levels at high cell density

Another possible explanation for the changes in localisation seen with cell density was increased binding of β-catenin to E-cadherin at the membrane, leaving less β-catenin available for entry into the nucleus. This was tested by immunofluorescence staining for both E-cadherin and β-catenin, and immunoprecipitation for β-catenin and western blot for β-catenin, APC (ALI12-28) and E-cadherin in sub-confluent and super-confluent SW480 cells. In sub-confluent cells there is only very low levels of cytoplasmic E-cadherin and no detectable membrane E-cadherin (data not shown). β-catenin in sub-confluent cells is localised mostly to the nucleus and cytoplasm with some faint membrane staining (figure 4.2a,j). In super-confluent cells all cells have membrane localisation of β-catenin, with some also having faint nuclear staining, and many, but not all, cells also have membrane localisation of E-cadherin (figure 4.14e). In westerns of whole cells lysate no detectable E-cadherin is seen at low density (sub-confluent), whereas at high density (super-confluent) substantial expression of E-cadherin is seen (figure 4.14a). An 18-fold increase is seen in E-cadherin co-immunoprecipitated with β-catenin (figure 4.14a). From low to high cell density a change in total expression and co-immunoprecipitation with β-catenin is also seen for APC (figure 4.14b). A 2.3-fold decrease in total APC expression and a 10.7-fold decrease in co-immunoprecipitation with β-catenin was seen from low to high cell density in SW480 cells. Densitometry results were normalised for β-catenin.

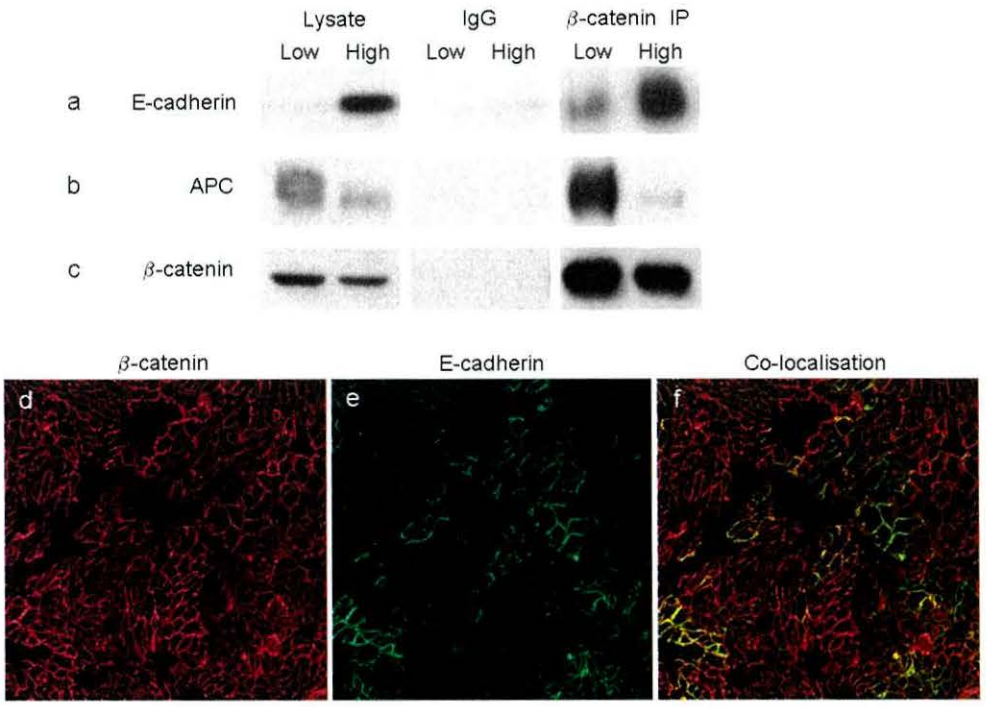


Figure 4.14
The total amount of E-cadherin and the amount interacting with β-catenin increases as cell density increases in SW480 cells, a decrease in APC expression and interaction with β-catenin is also observed. There is an 18-fold increase in the amount of E-cadherin found interacting with β-catenin by immunoprecipitation and western blot between sub-confluent and super-confluent cells, there is also a corresponding increase in total expression levels of E-cadherin (a). There is a 2.3-fold decrease in total amount of APC expressed between sub-confluent and super-confluent cells and a 10.7-fold decrease in the amount of APC found interacting with β-catenin by immunoprecipitation and western blot (b). Whole cell lysate and immunoprecipitations are normalised for β-catenin (c). Low density is sub-confluent, high density is super-confluent. All cells in a confluent SW480 culture express membrane β-catenin (d) but not all cells express membrane E-cadherin (e).

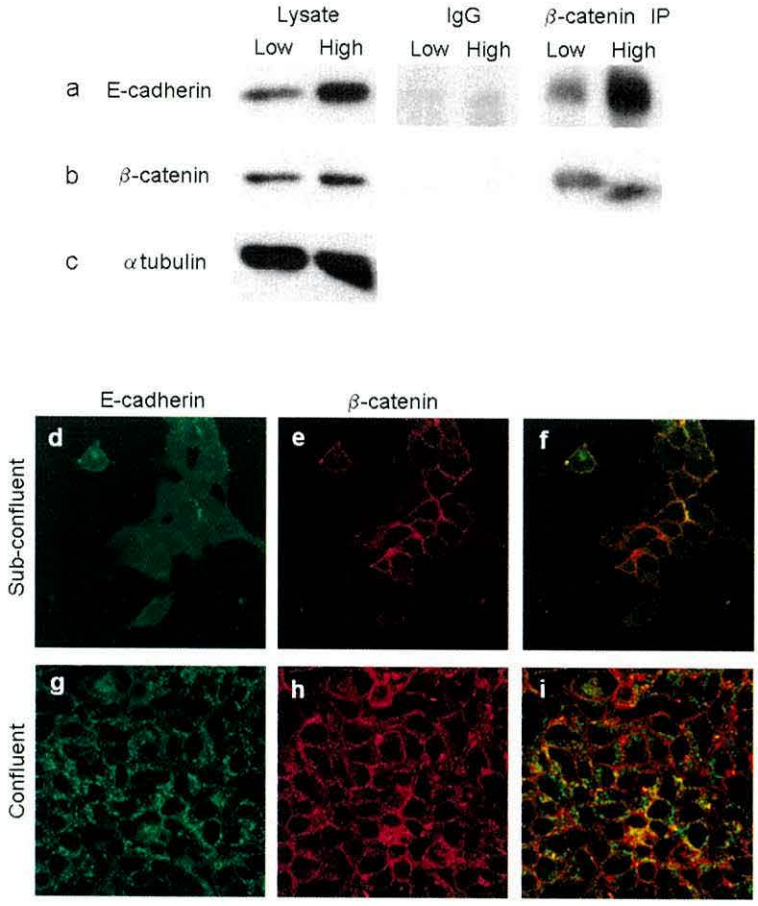


Figure 4.15
The normal epithelial cell lines HEK293 shows increased E-cadherin expression and E-cadherin/β-catenin interaction as cell density increases. A 2-fold increase in E-cadherin in whole cell extract and a 2.1-fold increase in E-cadherin in β-catenin immunoprecipitations was observed when comparing sub-confluent cells to super-confluent cells. This data was normalised for β-catenin. Whole cell extract normalised for α-tubulin demonstrated no change in β-catenin expression (1.05-fold increase) and a 2.1-fold increase in E-cadherin expression. Low density is sub-confluent, high density is super-confluent. This corresponds to an increase in E-cadherin co-localising with β-catenin at the membrane in confluent (g-i) cells compared to sub-confluent cells (d-f).

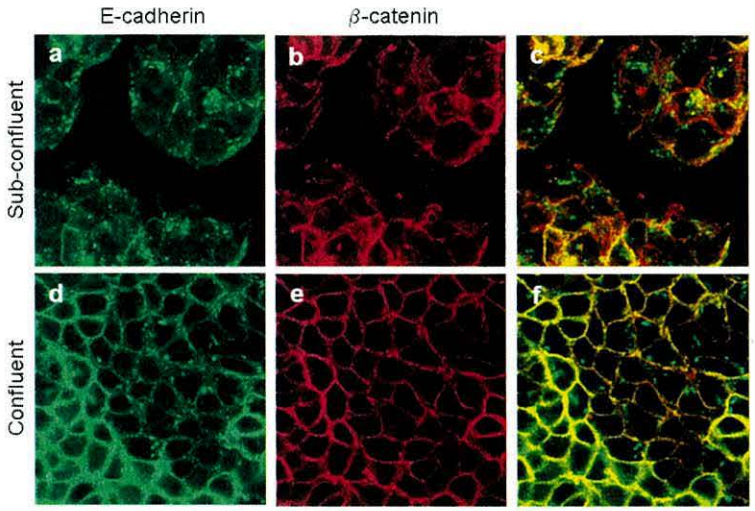


Figure 4.16
An increase in membrane localisation of E-cadherin with increase in cell density is observed in embryonal carcinoma cell line NTERA-2. In sub-confluent NTERA-2 cells the majority of E-cadherin is found in the cytoplasm (a) while β-catenin is localised to the membrane. In confluent cells an increase in membrane staining of E-cadherin is seen with some cells being more brightly stained than others (d). β-catenin is localised to the membrane (e) and co-localises well with E-cadherin (f).

Therefore as SW480 cells progress from low to high cell density an increase in E-cadherin levels and E-cadherin interaction with β-catenin is seen. Also, from low to high cell density there is a decrease in APC expression and in its interaction with β-catenin. This can be related to the change in β-catenin localisation from low to high cell density. In sub-confluent SW480 cells APC and β-catenin co-localise well (figure 4.2 a-c,j-l, figure 4.3a,c), this co-localisation is lost in confluent (figure 4.2d-f,m-o, figure 4.3b,d) and super-confluent (figure 4.2g-i,p-r) SW480 cells. So as loss of co-localisation is observed by immunofluorescence a corresponding reduction in interaction is seen by co-immunoprecipitation and western blot. Moreover, for E-cadherin and β-catenin an increase in interaction as seen by co-immunoprecipitation is associated with an increased membrane localisation of the two proteins (figure 4.14). However, while an increase in junctional (E-cadherin interacting) β-catenin, and potentially an increase in junctional integrity, may play a part in the localisation changes seen, not all changes can be explained by this alone. In confluent cultures many cells have no detectable nuclear β-catenin even when treated with LMB but do not express junctional E-cadherin (not shown).

The change in E-cadherin levels and its interaction with β-catenin is not unique to SW480 cells, a similar but smaller change is also seen in HEK293 cells. Comparison of whole cell lysate from low and high density cultures demonstrated a 2-fold increase in E-cadherin levels. There was a corresponding 2.1-fold increase in E-cadherin/β-catenin interaction by co-immunoprecipitation and western blot. E-cadherin was normalised for β-catenin (figure 4.15a,b). When whole cell lysates were normalised to α-tubulin (figure 4.15c) no change was observed in β-catenin expression with cell density (1.05-fold increase). A 2.1 fold increase in E-cadherin expression was seen when normalised to α-tubulin. A change in E-cadherin localisation is also seen by immunofluorescence. In sub-confluent cells there is very little membrane E-cadherin with only very faint staining cytoplasmic staining in the majority of cells (figure 4.15d), β-catenin is localised to the membrane (figure 4.15e). In confluent cells there appears to be a general increase in the brightness of the

staining for E-cadherin and also an increase in E-cadherin co-localising with β-catenin at the membrane (figure 4.15g-i).

An increase in E-cadherin co-localising with β-catenin at the membrane as cell density increases is also seen in the embryonal carcinoma cell line NTERA-2. In sub-confluent cells β-catenin is localised to the membrane while the majority of E-cadherin staining is cytoplasmic (figure 4.16a-c). In confluent cells there is an increase in membrane E-cadherin (figure 4.16d). β-catenin is again localised to the membrane and co-localises well with E-cadherin. Variation was seen in the brightness of membrane staining for E-cadherin in confluent cells (figure 4.16d). Unlike SW480 and HEK293 no increase in E-cadherin expression was observed on western blot comparing whole cell lysate in sub-confluent and super-confluent cells (data not shown).

4.12 Discussion of results chapter 4: Variation in localisation and interactions of APC and β-catenin with mutation status and cell density

It has been shown that E-cadherin is able to negatively regulate β-catenin/TCF-LEF signalling. This regulation of β-catenin/TCF-LEF signalling is dependent on the β-catenin binding region but is not dependent on the intercellular adhesion function of E-cadherin (Orsulic *et al.*, 1999; Gottardi *et al.*, 2001). Transfection of E-cadherin into SW480 cells reduces TCF-LEF-dependent transcription (as shown by reporter constructs) and significantly inhibits growth. Orsulic and co-workers (1999) observed a reduction in nuclear localisation of β-catenin in SW480 cells transiently transfected with E-cadherin. In addition an increase in nuclear localisation and a decrease in membrane localisation of β-catenin was observed in E-cadherin-/- embryonic stem (ES) cells compared to wild type ES cells. However, Gottardi and co-workers (2001) found no significant change in localisation in stably transformed SW480 cells with either wild type E-cadherin or any E-cadherin constructs. This difference may be explained by the different transfection methods, possibly by different expression levels of E-cadherin. It was shown that only a small proportion of β-catenin in SW480 cells was able to bind E-cadherin and TCF-LEFs (Gottardi *et al.*, 2001), so E-cadherin can reduce transcriptional activity of β-catenin without affecting the localisation of the large pool of β-catenin which interacts with neither E-cadherin or TCF-LEFs. It is possible that sufficiently high levels of E-cadherin may bind this pool of β-catenin which would not normally interact with E-cadherin.

Endogenous E-cadherin may affect β-catenin localisation in two ways. Increased E-cadherin at cell-cell junctions as cells reach higher density is associated with increased membrane β-catenin and increased β-catenin/E-cadherin interaction. Therefore it is possible there is less β-catenin available to enter the nucleus in high density cells. However, in confluent cells many cells have no detectable nuclear β-catenin but do not express membrane β-catenin so this cannot account for all localisation changes observed. Another possibility is that some of the increased E-cadherin expression with cell density observed is cytoplasmic E-cadherin. This would allow increased E-cadherin/ β-catenin interaction,

potentially leaving that β-catenin unable to enter the nucleus but not bound at the membrane, this would account for membrane E-cadherin not being necessary for nuclear exclusion of β-catenin.

Increased expression, membrane localisation and β-catenin interaction of E-cadherin as cell density increases is a theme seen in many cell lines, both those derived from tumours and the 'normal' epithelial cell line HEK293 (section 4.11). Normal cells in the body are not generally found in a 'subconfluent' state, while cells at the invasive front of tumours have a great advantage in being able to break away from the original tumour and metastases. In invasive tumours loss of membrane E-cadherin and nuclear accumulation of β-catenin is observed at the invasive front, while in the central mass both proteins are localised to the membrane (Brabletz *et al.*, 2001). The changes in E-cadherin and β-catenin expression and localisation may be a mechanism which is present to an extent in normal epithelial cells and goes awry in the case of metastatic tumours. A similar mechanism is seen during follicular morphogenesis, with Wnt pathway activation, downregulation of E-cadherin and changes in cellular adhesion leading to downgrowth from a layer of epithelial stem cells to initiate follicle formation (Jamora *et al.*, 2003).

It has been suggested that truncated APC senses cell-cell contacts and accumulates in the nucleus in response to their disruption (Brocardo *et al.*, 2001). APC in subconfluent cells lacking membrane E-cadherin is nuclear and much of that nuclear localisation is lost in super-confluent cells which have membrane E-cadherin. However, the results presented here indicate that in confluent SW480 cells a significant proportion of cells have APC predominantly localised to the cytoplasm or evenly distributed between cytoplasm and nucleus without any membrane localisation of E-cadherin. This suggests that membrane E-cadherin, and fully established cell-cell junctions, is not required for loss of the nuclear localisation of truncated APC.

It has been shown that it is the rate of nuclear export of APC that determines the transcriptional activity of β-catenin (Rosin-Arbesfeld *et al.*, 2003). It is likely

that the decreased nuclear localisation of APC in high density cells is due to increased nuclear export of APC, it cannot be due to a large decrease in nuclear entry of APC as APC is still seen to accumulate in the nucleus in response to LMB. As it is the nuclear export of APC which is important in determining transcriptional activity of β-catenin, it is possible that although there is no correlation between APC localisation and β-catenin localisation in confluent cells, there may be a correlation between APC localisation and transcriptional activity of β-catenin. In confluent SW480 cells increased nuclear export of APC, due to transfection of APC constructs containing the central nuclear export sequences, results in decreased TCF-LEF transcriptional activity (Rosin-Arbesfeld *et al.*, 2003). It may be that N-terminal nuclear export sequences of endogenous truncated APC become more active in high density cells and have a similar effect. While SW480 cells have a large pool of nuclear β-catenin it is likely that not all of this is transcriptionally active (Gottardi *et al.*, 2001). This explains why the steady-state levels of β-catenin are not correlated with its transcriptional activity (Rosin-Arbesfeld *et al.*, 2003).

As well as regulating transcriptional activity of β-catenin, APC also has a nuclear export function for β-catenin (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a; Neufeld *et al.*, 2000b). In sub-confluent SW480 cells both APC and β-catenin accumulate in the nucleus, this is likely to be due to the inability of truncated APC (lacking central nuclear export sequences) to shuttle from the nucleus and remove β-catenin (Rosin-Arbesfeld *et al.*, 2003). Although it has been reported that truncated APC can undergo nuclear export (Henderson, 2000), this has been disputed (Rosin-Arbesfeld *et al.*, 2003). It may be that N-terminal nuclear export sequences are only active under certain circumstances (such as high cell density), or that some of these conflicting results are due to abnormal high levels of protein expressed from transfected constructs. As cells become more confluent there is a reduction in nuclear localisation of both APC and β-catenin, this may be due to N-terminal nuclear export sequences becoming more active and allowing APC to exit the nucleus and carry β-catenin with it. However, no correlation is seen between nuclear APC and nuclear β-catenin in confluent cells.

Two mechanisms for the reduction of nuclear β-catenin with cell density have been suggested here: increased nuclear export of APC as cell density increases and increased expression of E-cadherin and increasing E-cadherin/β-catenin interaction resulting in less nuclear entry of β-catenin. Neither of these mechanisms alone would account for the changes in localisation of β-catenin seen in SW480 as cell density increases. It may be that it is a combination of the two mechanisms which determines localisation of β-catenin.

In normal epithelial cells wild type APC is efficiently exported from the nucleus at all cell densities. The majority of β-catenin in the cell is localised to the membrane and is not available to enter the nucleus. Any free cytoplasmic β-catenin is efficiently targeted for destruction. In cells carrying mutations in β-catenin, such as HCT116, degradation of β-catenin is likely to be affected due to mutations affecting phosphorylation sites. In HCT116 the phosphorylation site affected, Ser45, is a priming site which must be phosphorylated by Casein Kinase Iε before the Wnt-dependent phosphorylation sites can be phosphorylated (Amit *et al.*, 2002; Hagen & Vidal-Puig, 2002; Liu *et al.*, 2002; Sakanaka, 2002). Defects in phosphorylation of β-catenin would allow free β-catenin to accumulate and enter the nucleus. Mutations in β-catenin can also affect its interaction with E-cadherin (Chan *et al.*, 2002) which would also allow free β-catenin to accumulate rather than being sequestered by E-cadherin. Due to efficient nuclear export of β-catenin by APC/CRM1 HCT116 cells appear similar to normal epithelial cells in terms of β-catenin localisation until they are treated with LMB. On LMB treatment β-catenin is no longer removed from the nucleus by APC via CRM1 and can be seen to accumulate in the nucleus, this accumulation is not seen in normal epithelial cells where β-catenin is efficiently excluded from the nucleus. Nuclear entry of β-catenin is reduced in confluent cells, which may be due to an increase in expression of E-cadherin resulting in less free β-catenin being available to enter the nucleus.

SW480 cells express only truncated APC which leads to defects in nuclear export of APC and β-catenin and in degradation of free β-catenin. As a result of this large amounts of APC and β-catenin accumulate in the nucleus in SW480 cells. However, this changes with cell density. The decrease in nuclear β-catenin with cell density may be due to increased nuclear export of APC and increased interaction with E-cadherin, as described above. NTERA-2 cells have one wild type APC allele and one truncated allele. APC is localised to the nucleus in sub-confluent cells while β-catenin is localised to the cell membrane. Nuclear localisation of APC is lost as cell density increases, as in SW480. It may be that the remaining wild type allele of APC is able to efficiently target the majority of free β-catenin for proteasomal degradation so in untreated cells nuclear localisation is not seen despite there being one truncated allele of APC. This is consistent with the very small amount of nuclear accumulation of β-catenin in response to LMB seen in these cells, as very little β-catenin would be available for entry into the nucleus. When NTERA-2 are treated with lithium a large amount of nuclear β-catenin is seen. One possibility is that once a Wnt signal allows β-catenin to enter the nucleus the truncation in one allele of APC is dominant in giving defective nuclear export, this is consistent with the nuclear localisation of APC in sub-confluent cells.

MDCK cells are unusual in that they are 'normal' epithelial cells which express both wild type APC and wild type β-catenin but have nuclear localisation of APC in sub-confluent cells. Like other normal epithelial cells β-catenin does not localise to the nucleus even when cells are treated with LMB. As one of the sub-lines derived from this cell line, MDCKII, does not show nuclear localisation of APC in sub-confluent cells (Rosin-Arbesfeld *et al.*, 2001) it is unlikely that nuclear localisation of APC in this cell line is due to an undiscovered mutation in APC. In normal colon mucosa APC is detected in the cytoplasm in epithelial cells above the crypt and is detected in nuclei in cells at the base of crypts (Rosin-Arbesfeld *et al.*, 2003). Cells at the base of crypts are proliferating cells while cells above the crypt are differentiated epithelial cells. This shows that APC can be nuclear for reasons other than truncation and may offer an explanation for the nuclear APC seen in MDCK cells. The original MDCK line

is a heterogenous cell line, MDCKII cells are a cell type which dominates in high passage number MDCK cultures, they polarise well and appear to be epithelial cells. It is possible that nuclear staining in MDCK cells is due to them being de-differentiated proliferating cells, while MDCKII cells are a more differentiated epithelial cell type which lack nuclear APC.

In cancer cells reduced degradation would allow β-catenin to accumulate and enter the nucleus independently (Fagotto *et al.*, 1998). An excess of β-catenin and/or mutations in β-catenin would result in E-cadherin being unable to sequester all the available β-catenin and in cell lines expressing only truncated APC defects in APC-dependent nuclear export of β-catenin would allow large amounts of β-catenin to accumulate in the nucleus. Increased E-cadherin expression and increased nuclear export of APC, possibly due to N-terminal nuclear export sequences would then change the overall localisation of β-catenin as cells reached higher density.

Chapter 5 – Results: Identification of a 150 kDa potential APC isoform

5.1 Introduction

APC has been reported to be composed of up to 21 exons, with seven of these being alternatively spliced (Santoro & Groden, 1997). Exon 15, the last exon is unusually large at 6574 base pairs. Many different form of APC are known to exist as a result of alternative splicing and post-translational modifications (Thliveris *et al.*, 1994; Samowitz *et al.*, 1995; Sulekova *et al.*, 1995; Bala *et al.*, 1996; Kraus *et al.*, 1996; Santoro & Groden, 1997; Pyles *et al.*, 1998; reviewed by Polakis, 1997). The roles of each isoform within the cell has not been determined. A large multi-functional protein such as APC is an excellent candidate for the existence of functional variants with different roles in various tissues both during development and in the adult organism. APC has been found to shuttle between the nucleus and cytoplasm (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a; Neufeld *et al.*, 2000b), is localised to microtubule tips (Näthke *et al.*, 1996; Rosin-Arbesfeld *et al.*, 2001; Mogensen *et al.*, 2002), is localised to the plasma membrane in an actin-dependent manner (Rosin-Arbesfeld *et al.*, 2001) and has been found at the kinetochore during mitosis (Fodde *et al.*, 2001; Kaplan *et al.*, 2001). APC has also been observed at the apical membrane (Miyashiro *et al.*, 1995; Reinacher-Schick & Gumbiner, 2001; Anderson *et al.*, 2002). However, the apical localisation of APC is not consistent with the staining observed with anti-APC (M-APC) (Rosin-Arbesfeld *et al.*, 2001), which has been shown to reliably detect full length APC (Näthke *et al.*, 1996; Rosin-Arbesfeld *et al.*, 2001; Mogensen *et al.*, 2002). It is possible that the detection of an apical protein with a variety of APC antibodies is reflecting detection of a functionally distinct population of APC.

At the outset of this study, the localisation of full length and truncated APC had not been fully reported (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000). We therefore carried out immunofluorescence staining with a variety of APC antibodies. It became apparent that different antibodies detected APC at varying locations within the cell. Validation of reactivity of antibodies was therefore of paramount importance (chapter 3). A number of antibodies detected APC at the apical membrane. This included C-terminal directed APC antibodies in cell lines expressing only truncated APC, which indicates that this apical protein is not full

length APC. We sought to further characterise the localisation seen with these APC antibodies in a variety of cell lines. To attempt to determine the molecular basis of this apical staining western blots were carried out to determine the size of a likely candidate protein, immunoprecipitation and mass spectrometry analysis was carried out to attempt to identify the protein. RT-PCR and northern blots were carried out to attempt to identify the position of a potential alternative splice site.

5.2 Antibodies

A panel of six antibodies directed to APC were used (figure 5.1). These antibodies were selected based from a larger panel of antibodies based on the localisation of APC detected with these antibodies, and their ability to detect a 150 kDa protein on western blot. The full panel of APC antibodies used are discussed in chapter 3. Anti-APC (N-15) also appears to detect this apical protein but has not been included here due to questions raised about the reliability of this antibody (Mogensen *et al.*, 2002, Rosin-Arbesfeld *et al.*, 2001; Roberts *et al.*, 2003). Two N-terminal directed antibodies (H-290 and Ab5) and four C-terminal directed antibodies are used (Ab2, Ab4, Ab6, Ab120). Anti-APC (H-290) is a rabbit polyclonal directed to amino acids 1-289 or APC. All other antibodies used are mouse monoclonal antibodies. The epitope for anti-APC (Ab5) is amino acids 60-73, which is within exon 2 of APC. The C-terminal antibodies were raised to the last 300 amino acids of full length APC, their exact epitopes have not been characterised.

5.3 An apical protein is detected by a number of N- and C-terminal directed APC antibodies in wild type and truncated APC cell lines

The C-terminal directed APC antibody Ab4 detects an apical staining protein in the colorectal cancer cell lines HCT116 and SW480 (Figure 5.2b,c). This is a particularly interesting observation as this antibody is directed to the C-terminus of APC and it is not possible that it is detecting full length APC in SW480 as

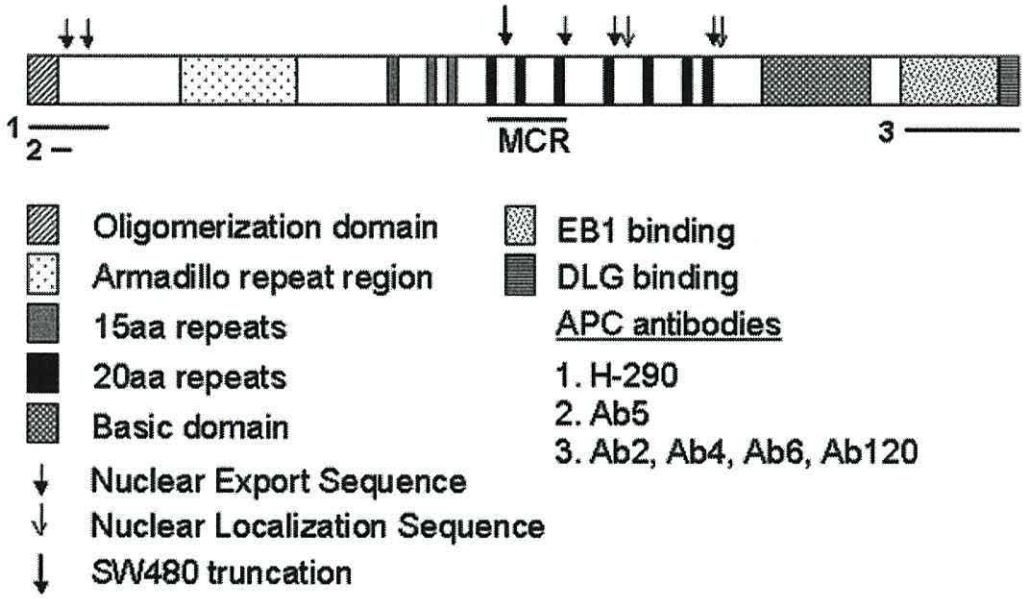


Figure 5.1
Figure 1 shows the regions of APC the antibodies used here are directed to. H-290 is a rabbit polyclonal antibody directed to amino acids 1-289 at the N-terminal end of APC. Ab5 is a mouse monoclonal directed to an epitope within exon 2 at the N-terminal end of APC. Ab2, Ab4, Ab6 and Ab120 are mouse monoclonal antibodies directed to the last 300 amino acids at the C-terminal end of APC.

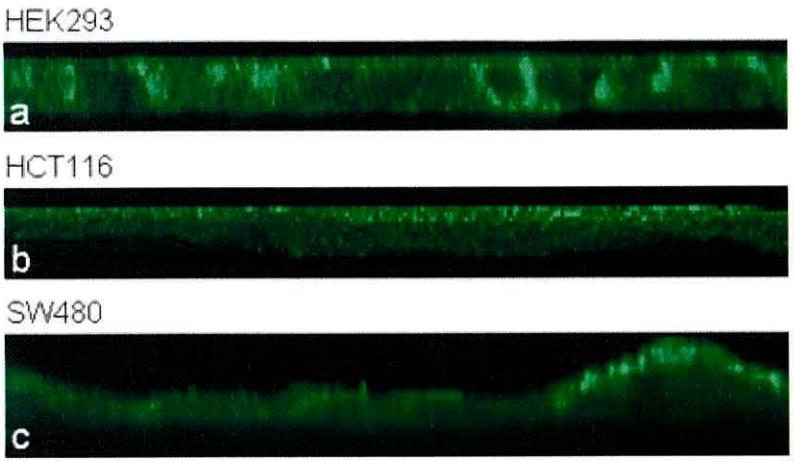


Figure 5.2
The C-terminal directed APC antibody Ab4 detects an apical protein in HCT116 (b) and SW480 (c) colorectal cancer cell lines. In the normal epithelial cell line HEK293 (a) Ab4 stains the lateral membranes and occasionally the basal membrane as well as the apical staining seen in the colorectal cancer cell lines. Images show side views generated from confocal Z-stacks, the apical region of the cells is towards to top of the images.

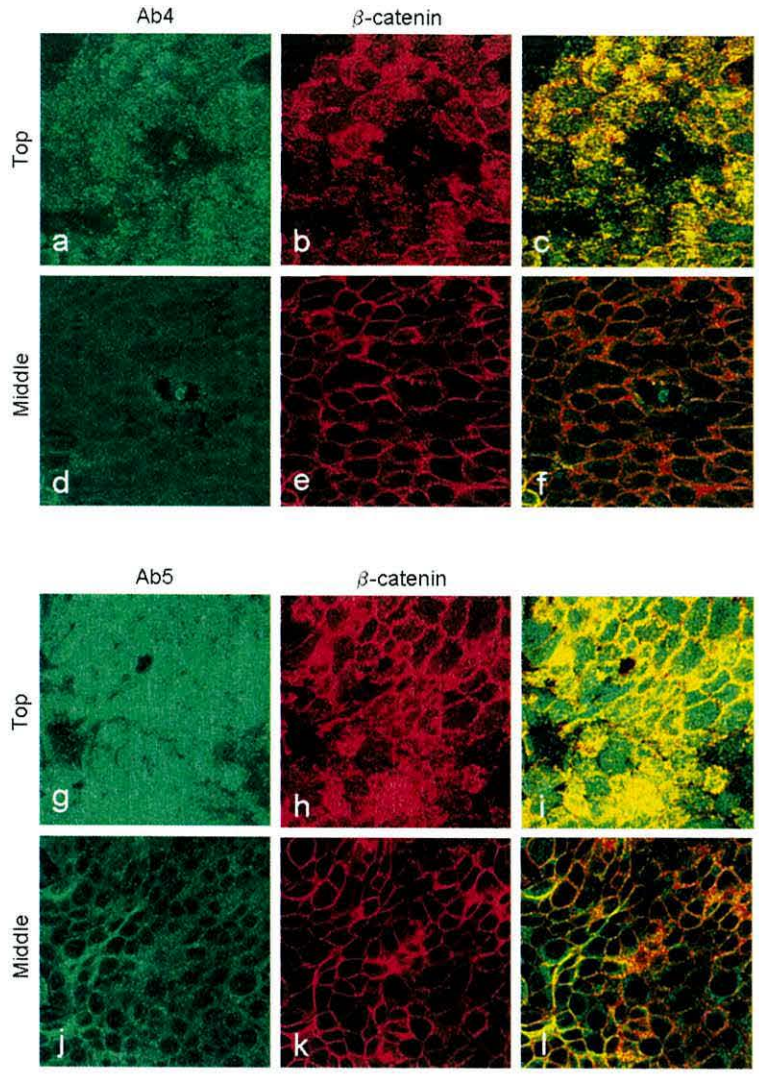


Figure 5.3
APC antibodies Ab4 and Ab5 stain the cytoplasm and the apical membrane in HCT116 cells. Apical and middle sections of confocal Z-stacks are shown. β-catenin staining is shown to mark the position of the cell membrane. Staining with both Ab4 (a-f), C-terminal, and Ab5 (g-l), N-terminal, is similar. Both antibodies detect a protein which is localised to the cytoplasm and concentrated at the apical membrane.

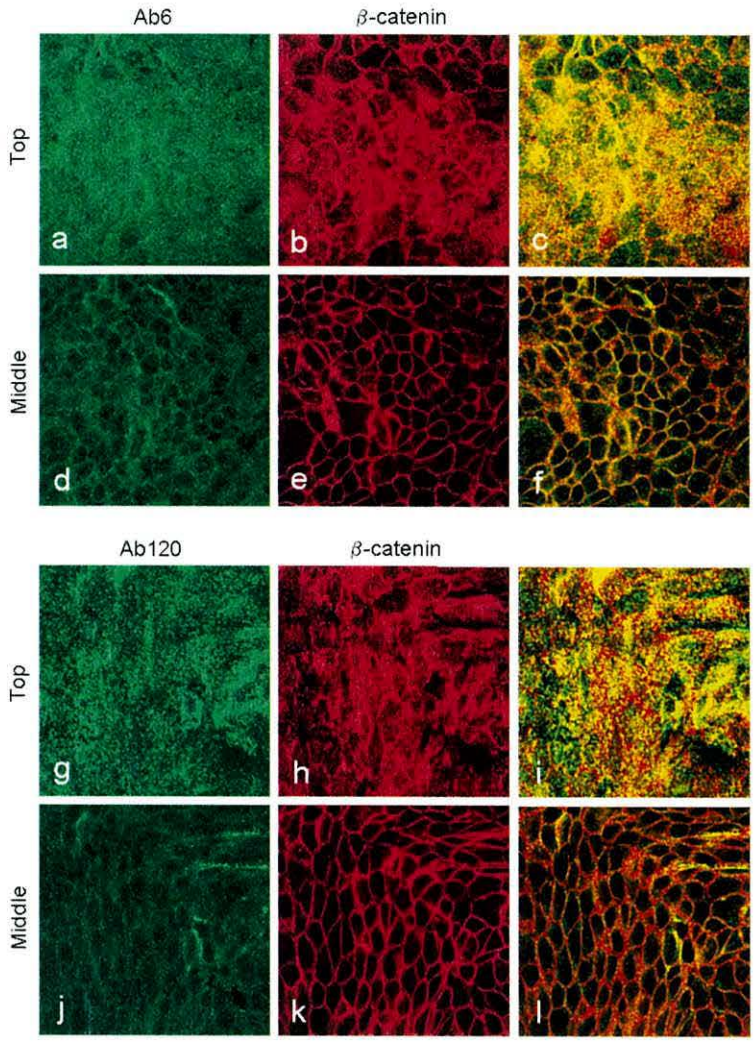


Figure 5.4
Ab6 and Ab120, two antibodies directed to the C-terminal 300 amino acids of APC, also detect a protein which is present in the cytoplasm and concentrated at the apical membrane. Apical and middle sections of confocal Z-stacks are shown. The staining pattern seen with Ab6 and Ab120 is the same as that seen with Ab4 and Ab5 (figure 3).

SW480 cells only express APC which is truncated at 1337 amino acids. In addition to apical staining, edge staining, where cells are not in contact with other cells, and fainter cytoplasmic staining (figure 5.3a,d) was also observed. This staining pattern was the same in colorectal cancer cell lines with full length APC or truncated APC. In sub-confluent colorectal cancer cell lines the localisation of this protein was similar to that seen in confluent cells, with 'apical' and edge staining and fainter cytoplasmic staining, although the apical staining was less pronounced than in confluent cells (data not shown).

Two other C-terminal directed APC antibodies (Ab6 and Ab120) (figure 5.4), and one N-terminal directed APC antibody (Ab5) (figure 5.3g,j) also detect this apical protein in HCT116. These antibodies give identical staining to Ab4, and like Ab4 these three antibodies also detect edge staining and fainter cytoplasmic staining (figure 5.3, figure 5.4). All these antibodies also detect an apical protein in SW480 cells. Ab6 and Ab120 are C-terminal directed APC antibodies so, like Ab4, should not be able to detect any of the truncated APC expressed in SW480 cells.

As these C-terminal directed APC antibodies appear to detect protein in SW480 cells, which should express only truncated APC, PCR and sequencing was carried out to confirm that this cell line is SW480. Sequencing revealed a C-T point mutation resulting in a stop codon at 1338 amino acids, which confirms that this cell line is SW480. Of the five cloned PCR products analysed all had this point mutation (data not shown).

In the normal epithelial cell line HEK293 anti-APC (Ab4) detects a protein at the apical and lateral membranes (figure 5.2a). Staining of the basal membrane was also occasionally observed. This contrasts the colorectal cancer cell lines where staining was concentrated at the apical membrane and no lateral membrane staining was observed (figures 5.2-4). Like the colorectal cancer cell lines, edge and cytoplasmic staining were also observed in HEK293.

In confluent NTERA-2 embryonal carcinoma cells localisation is similar to that seen in the colorectal cancer cells with the exception that the apical staining is

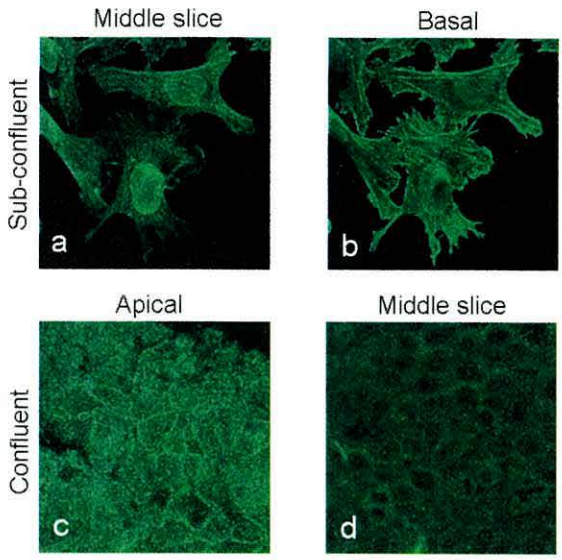


Figure 5.5
Anti-APC (Ab4) detects a protein localised to the cytoplasm, nucleus, apical membrane and cytoplasmic filaments in the embryonal carcinoma cell line NTERA-2 at low density (a,b). In confluent NTERA-2 cells this protein is localised to the cytoplasm (e) and apical membrane and is particularly concentrated at the apico-lateral membrane (d). Apical and middle sections of confocal Z-stacks are shown.

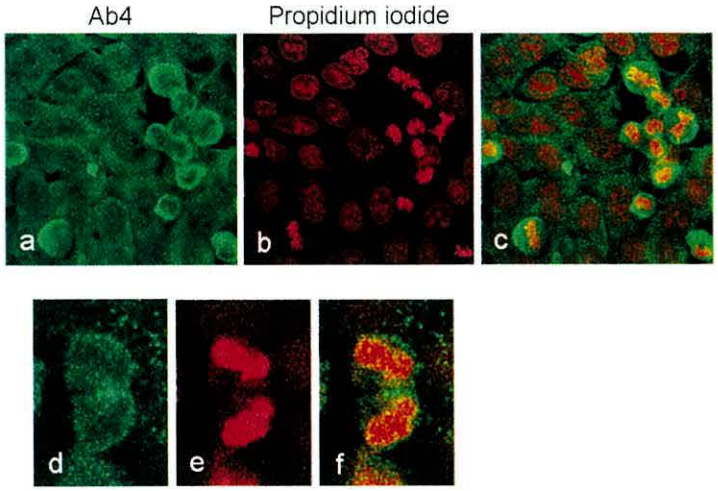


Figure 5.6
During mitosis anti-APC (Ab4) detects a protein localised to the cell membrane and cytoplasm, and excluded from the chromosomes. Unlike interphase cells the staining is not concentrated at the apical membrane of mitotic cells but is all around the cell membrane (a-c). During cytokinesis this protein is concentrated at the new membrane forming between daughter cells (d-f).

concentrated to the apico-lateral membrane (figure 5.5c). Like the colorectal cancer cell lines there was also edge staining and fainter cytoplasmic staining (figure 5.5d). In sub-confluent colorectal cancer cell lines the localisation of this protein was similar to that seen in confluent cells. This contrasts with NTERA-2 where the localisation in low-density cells is very different from that seen in high-density cells and in the colorectal cancer cell lines. There is a small patch of 'apical' staining above the nucleus and quite pronounced edge staining. Unlike the other cell lines observed, sub-confluent NTERA-2 cells have nuclear staining for this protein (figure 5.5a) and staining concentrated along cytoplasmic filaments, particularly at the base of cells (figure 5.5b). These filaments did not co-localise with α-tubulin (data not shown).

In mitotic cells the staining with all these antibodies is concentrated around the plasma membrane (Ab4 in HCT116 is shown in figure 5.6, staining was similar with Ab5, Ab6 and Ab120 in both HCT116 and SW480 cells (data not shown)). At cytokinesis staining was concentrated along the new membrane forming between daughter cells (figure 5.6d-f).

APC has been shown to accumulate in the nucleus in response to Leptomycin B (LMB) treatment (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a; Neufeld *et al.*, 2000b; chapter 4.8), which inhibits CRM1-dependent nuclear export. HCT116 cells were treated with LMB to test whether the protein detected by these APC antibodies could detect nuclear APC in LMB-treated cells. Three antibodies, Ab4, Ab5 and Ab6 were used to stain HCT116 cells and as a positive control cells were also stained with a β-catenin antibody (figure 5.6). β-catenin was seen to accumulate in the nucleus in response to LMB treatment which shows LMB treatment was successful (figure 5.7g). None of the three antibodies used (Ab4, Ab5 and Ab6) detected nuclear APC in LMB treated cells (figure 5.7a,c,e). Unlike full length APC the protein detected by these antibodies does not undergo CRM1-dependent nuclear export.

The apical staining given with these antibodies can be disrupted by treatment with nocodazole, a microtubule disrupting agent (figure 5.8). Confluent HCT116

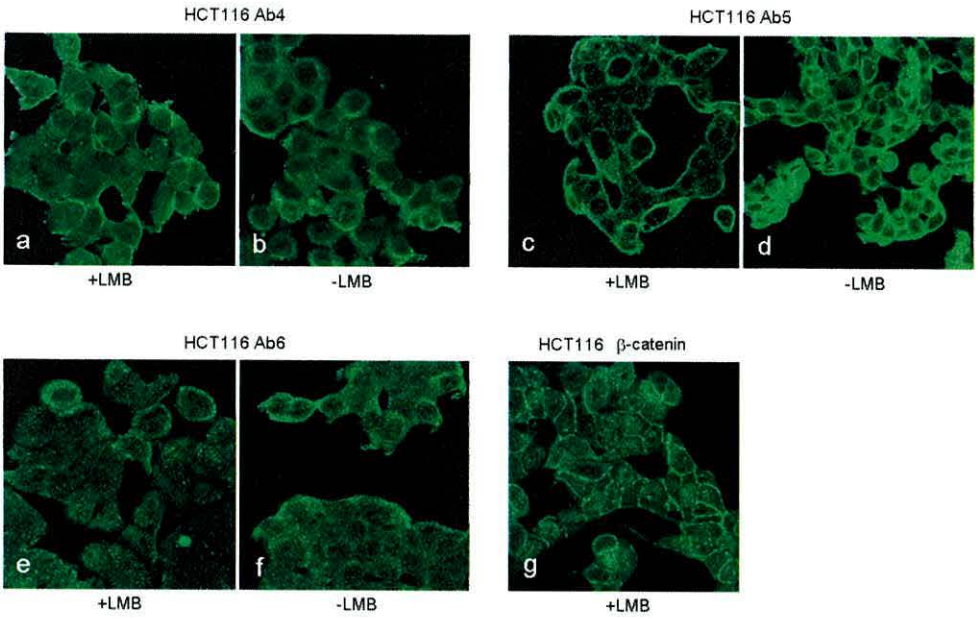


Figure 5.7
The protein detected by APC antibodies Ab4, Ab5 and Ab6 in HCT116 does not shuttle continuously between the nucleus and cytoplasm as full length APC does. Treatment with Leptomycin B (LMB) and staining with Ab4 (a,b), Ab5 (c,d) or Ab6 (e,f) does not give any nuclear staining. Staining of cells with b-catenin confirms that blocking of CRM1 dependent nuclear export by LMB treatment was successful (g).

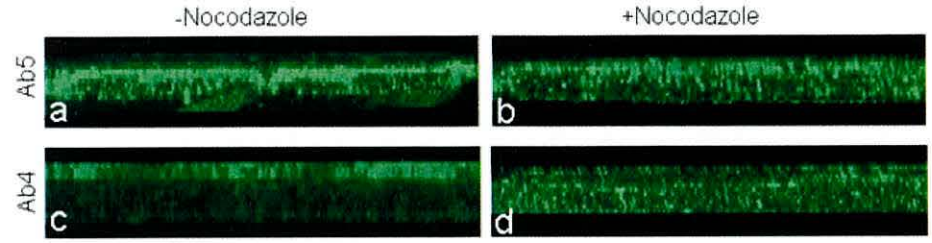


Figure 5.8
Apical localisation of the protein detected by APC antibodies Ab4 and Ab5 in HCT116 cells is dependent upon an intact microtubule network. In untreated cells (a,c) strong apical staining is seen. In nocodazole treated cells (b,d) this apical staining is lost. Treatment with DMSO, the solvent for nocodazole, did not result in loss of apical staining (not shown). Disruption of microtubules was confirmed by staining with an α-tubulin antibody (not shown). Images shown are side views generated from confocal Z-stacks, the apical surface of the cells is towards the top of the image.

cells were treated with either nocodazole, or with DMSO as a negative control and stained with either Ab4 or Ab5 (see materials and methods, section 2.5). After nocodazole treatment a dramatic reduction in apical staining was seen with both antibodies. Cells were stained for α-tubulin to confirm microtubules had been disrupted (data not shown).

All the antibodies used here are directed to epitopes within the C- or N-terminal end of APC, and all detect a protein localised to the apical membrane. This localisation is seen in SW480 cells with C-terminal antibodies therefore this apical protein cannot be full length APC as SW480 cells express only APC truncated at 1337 amino acids. None of these antibodies seem to detect full length or truncated APC in immunofluorescence staining, as determined by comparison to staining patterns using the M-APC antibody. APC has been shown to accumulate in the nucleus in response to LMB treatment, none of these antibodies detect nuclear APC in LMB treated HCT116 cells. If Ab5, an N-terminal directed APC antibody, were able to detect APC it would also be expected to give nuclear staining in SW480 but it does not. APC has been seen localised to clusters at the plus ends of microtubules (Näthke *et al.*, 1996; Rosin-Arbesfeld *et al.*, 2001; Mogensen *et al.*, 2002), these microtubule tip clusters are not detected by any of the antibodies which detect the apical staining seen here (data not shown).

5.4 A panel of N- and C-terminal directed APC antibodies detect an approximately 150 kDa protein on western blot

A panel of C- and N-terminal directed APC antibodies (Ab4, Ab5, Ab6, Ab120) all detect an apical protein, which does not appear to be full length APC. To attempt to clarify what these antibodies are detecting, whole cells lysate western blots were carried out. All but Ab4 were able to efficiently detect full length APC on western blot (figure 5.9b,c,d). The manufacturers of anti-APC (Ab4) only recommend this antibody for use in immunofluorescence (Ab4 datasheet, CN Biosciences). The poor results with this antibody in western blot may be due

to it only being suitable for use with native protein rather than the denatured protein used in western blots (chapter 3.7). Ab5, an N-terminal directed antibody, was able to detect truncated APC in SW480 whole cell lysate (figure 5.9d). All these antibodies also detect a protein of around 150 kDa. A number of other bands are also seen on longer exposures, which are often necessary to visualise full length APC (APC is notoriously difficult to transfer in western blot). The only band observed with all these antibodies, other than that for full length APC, is the one at approximately 150 kDa.

Two other antibodies, which were not suitable for immunofluorescence staining, also detect a protein at 150 kDa on western blot. One of these is the C-terminal directed monoclonal APC antibody Ab2 (figure 5.9a), the other is an N-terminal directed rabbit polyclonal APC antibody, H-290 (figure 5.9e). Both of these efficiently detect full length APC and the N-terminal antibody H-290 is also able to detect truncated APC in SW480 cells (figure 5.9a,e).

5.5 Immunoprecipitation and western blot show that different APC antibodies all recognise the same 150 kDa protein

In total, five APC antibodies detect an approximately 150 kDa protein on Whole cell lysate western blots. While these proteins all detect a band of the same size relative to markers, this does not prove that these antibodies are all detecting the same protein: it may be they are all detecting different proteins at approximately the same size. To confirm that these antibodies are detecting the same protein immunoprecipitation and western blot was carried out. Immunopreciptation of HCT116 and SW480 whole cell lysate with Ab5 (N-terminal) and Ab6 (C-terminal) followed by western blot with the N-terminal directed APC antibody H-290 gave a band at approximately 150 kDa (figure 5.10a,b). Immunoprecipitation of SW480 lysate with Ab5 and western blot with H-290 (both N-terminal directed APC antibodies) detects truncated APC at 150 kDa (figure 5.10a). This transfer was not optimised for transfer of full length APC, which is notoriously difficult to transfer in western blot, so no band

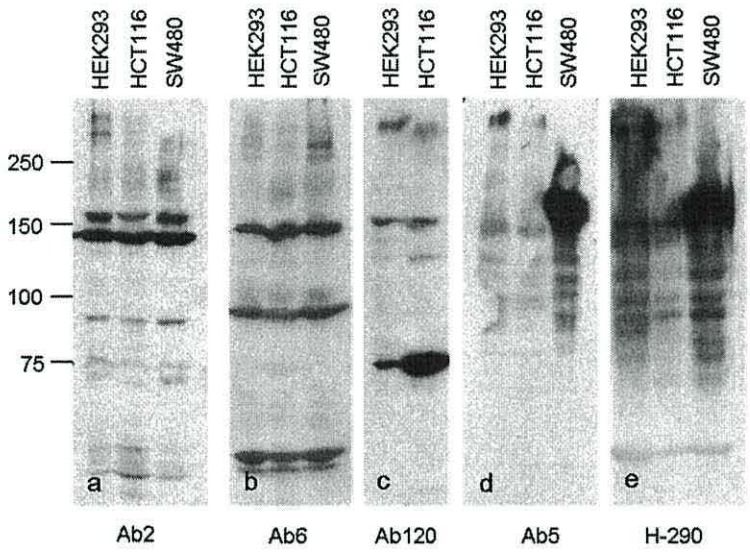


Figure 5.9
The C-terminal APC antibodies Ab2 (a), Ab6 (b) and Ab120 (c) and the N-terminal APC antibodies Ab5 (d) and H-290 (e) all detect a protein at 150 kDa as well as full length APC (310 kDa) in HCT116 and HEK293 whole cell lysate western blots. The N-terminal antibodies also detect truncated APC at approx. 150 kDa in SW480 lysates (d,e). The C-terminal APC antibodies detect a 150 kDa protein in SW480 cells, which express only truncated APC (a-c).

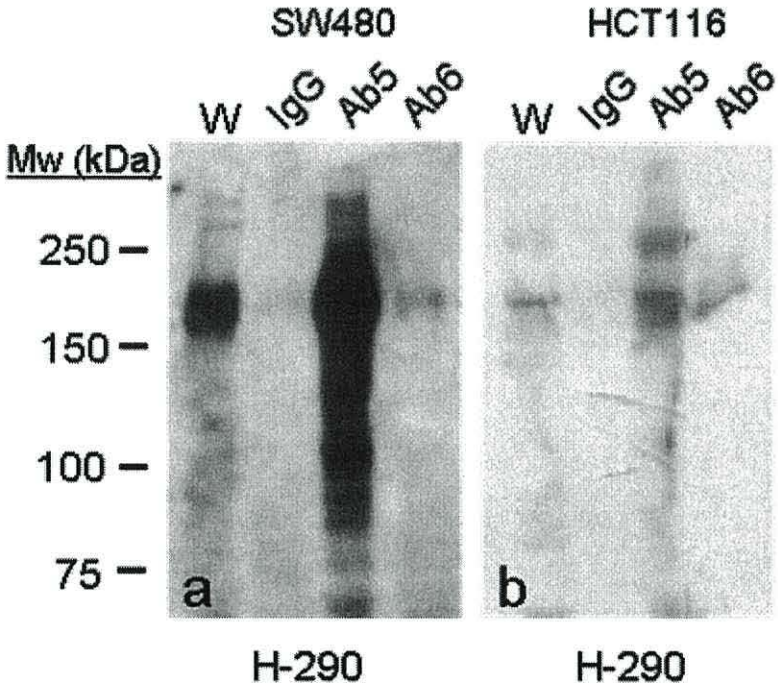


Figure 5.10
Immunoprecipitation with anti-APC (Ab5) (N-terminal) and anti-APC (Ab6) (C-terminal) and western blot with anti-APC (H-290) (N-terminal) gives a band at 150 kDa for both HCT116 (b) and SW480 (a) cells. "W" indicates whole cell lysate, "IgG"is a negative control immunoprecipitation in which the antibody has been replaced by normal IgG. The strong band at approx. 150 kDa in SW480 Ab5 immunoprecipitation is due to truncated APC. Full length APC is not detected on these western blots as transfer conditions were not optimised for transfer of full length APC.

corresponding to full length APC was observed. Immunoprecipitation of SW480 whole cell lysate with Ab2, Ab6, Ab120 and 5B2 (a mouse monoclonal antibody directed to the last 226 amino acids of APC), all of which are C-terminal directed APC antibodies, and western blot with Ab5, an N-terminal APC antibody, also gave bands at 150 kDa (G.T. Roberts, unpublished data). This indicates that these antibodies are all detecting the same protein rather than different proteins which happen to be of around the same size.

Four antibodies detect this apically localised protein in immunofluorescence and five antibodies detect a 150 kDa protein on western blot. Immunoprecipitation and western blot shows that many of these antibodies, including pairs of C- and N-terminal directed antibodies, are detecting the same 150 kDa protein. APC antibodies Ab5, Ab6 and Ab120 all detect both apical staining on immunofluorescence and a 150 kDa band on western blot. It therefore seems likely that the 150 kDa protein observed on western blot corresponds to the apical protein seen in immunofluorescence staining. If the apical staining corresponds to the 150 kDa protein this is a total of seven APC antibodies which detect this protein. Anti-APC (N-15) may also detect this apical protein (Reinacher-Schick & Gumbiner, 2001; Rosin-Arbesfeld *et al.*, 2001), though this antibody is not thought to be reliable (Mogensen *et al.*, 2002, Rosin-Arbesfeld *et al.*, 2001; Roberts *et al.*, 2003). Anti-APC (5B2) also appears to detect this 150 kDa protein on western blot and immunoprecipitation (G.T. Roberts, unpublished data). Apical staining has been observed in colon sections with Ab4 (Anderson *et al.*, 2002; Reinacher-Schick & Gumbiner, 2001), N-15 (Reinacher-Schick & Gumbiner, 2001) and a polyclonal antibody directed to the C-terminal 14 amino acids of full length APC (Miyashiro *et al.*, 1995). Another two polyclonal APC antibodies, directed to amino acids 8-347 (N-terminal) and amino acids 2813-2827 (C-terminal) have also been reported to detect APC at apical membranes in a variety of tissues including normal colon (Midgley *et al.*, 1997).

One possible explanation for the apical localisation seen is that it is antibody cross-reactivity with an unrelated protein. One of the antibodies which detects this apical protein, N-15, does seem to be unreliable, at least for immunoprecipitation and western blot (Mogensen *et al.*, 2002, Rosin-Arbesfeld

et al., 2001; Roberts *et al.*, 2003). In this case the apical staining could reasonably be put down to cross-reactivity. However, none of the other antibodies which detect apical staining or a 150 kDa protein have been questioned. All these antibodies appear to be able to detect full length APC on western blot, which supports their reliability. It seems very unlikely that this many independently produced antibodies could cross-react with the same protein, although this does remain a possibility.

Another possible explanation for the apical staining is that these antibodies are all detecting APCL rather than APC. APCL is expressed in both HCT116 and SW480 cells (chapter 7.3), it is not know whether APCL is expressed in HEK293 cells. Human APCL is a 254 kDa protein, of all the antibodies used here only Ab6 shows a band close to 254 kDa (figure 5.9b), this band is very faint and only visible on longer exposures. Immunoprecipitation with APC antibodies Ab2, Ab5 and Ab6 and western blot with an antibody raised to APCL antibody does not give a band at 254 kDa as would be expected if these antibodies were cross-reacting with APCL (G.T. Roberts, unpublished data). APCL has been reported to be localised to the cytoplasm, golgi apparatus, along the membrane and in filamentous structures (Jarrett *et al.*, 2001). There have been no reports of apical localisation of APCL. The localisation seen with the APC antibodies used here does have some similarity to that described for APCL, most notably the filamentous structures seen in NTERA-2. However, there are also significant differences between staining seen with these antibodies and that described for APCL.

Other potential explanations for this protein is that it is either the result of an alternative splicing event within exon 15 of APC, or that it another protein which has extensive homology to APC at both the N- and C-terminal ends, possibly a new APC homologue. BLAST searches (human genome, BLASTN, BLASTP) (Altschul *et al.* 1997) to the last 300 amino acids of APC (the region many of these antibodies are directed to), both protein and nucleotide, did not find any significant matches. Therefore, it seems unlikely that this is a protein with extensive homology to APC in this region, such as a novel APC homologue.

This leaves the possibility that this 150 kDa protein is an alternative splice variant of APC. Exon 15, the last exon of APC, is unusually large at 6574bp, this would mean that the splice event would have to take place within exon 15. This type of alternative splicing, where part of an exon is spliced out as an intron, has previously been observed in other proteins (reviewed by Graveley, 2001). Alternative splicing within APC has also previously been described (Thliveris *et al.*, 1994; Samowitz *et al.*, 1995; Sulekova *et al.*, 1995; Bala *et al.*, 1996; Kraus *et al.*, 1996; Santoro & Groden, 1997; Pyles *et al.*, 1998), though not within exon15. It has also been shown that there is at least one alternative start site for translation within APC, at AUG 184 downstream of the start site in exon 1 (Heppner Goss *et al.*, 2002). There are also a number of potential alternatively spliced exons upstream of exon 1, many of which contain start sites (Santoro & Groden, 1997). A large multi-functional protein such as APC is an excellent candidate for the existence of functional variants with different roles in various tissues both during development and in the adult organism. It seems quite possible that this 150 kDa protein may be a splice variant of APC.

5.6 Denatured immunoprecipitation with the C-terminal directed APC antibody Ab6 in SW480 gives a 150 kDa band

Immunoprecipitation was carried out on SW480 whole cell lysate to attempt to isolate and identify the 150 kDa protein described above. SW480 cells were used as they express only truncated APC so no full length APC, containing C-terminal sequences, would be available to interact with the C-terminal directed antibodies used. Immunoprecipitation under non-denaturing conditions gave a large number of bands, which made it difficult to distinguish which precipitated proteins are directly interacting with antibodies and which are a result of co-immunoprecipitation. To overcome this difficulty whole cell lysate was boiled before immunoprecipitation, this results in complexes being disrupted and all proteins being denatured. Immunoprecipitation was carried out in this manner with the APC antibodies Ab2, Ab5 and Ab6 on SW480 whole cell lysates. Immunoprecipitation with Ab5 did not give any proteins not present in the control lane (figure 5.11a). Ab2 and Ab6 gave bands at approximately 60 kDa

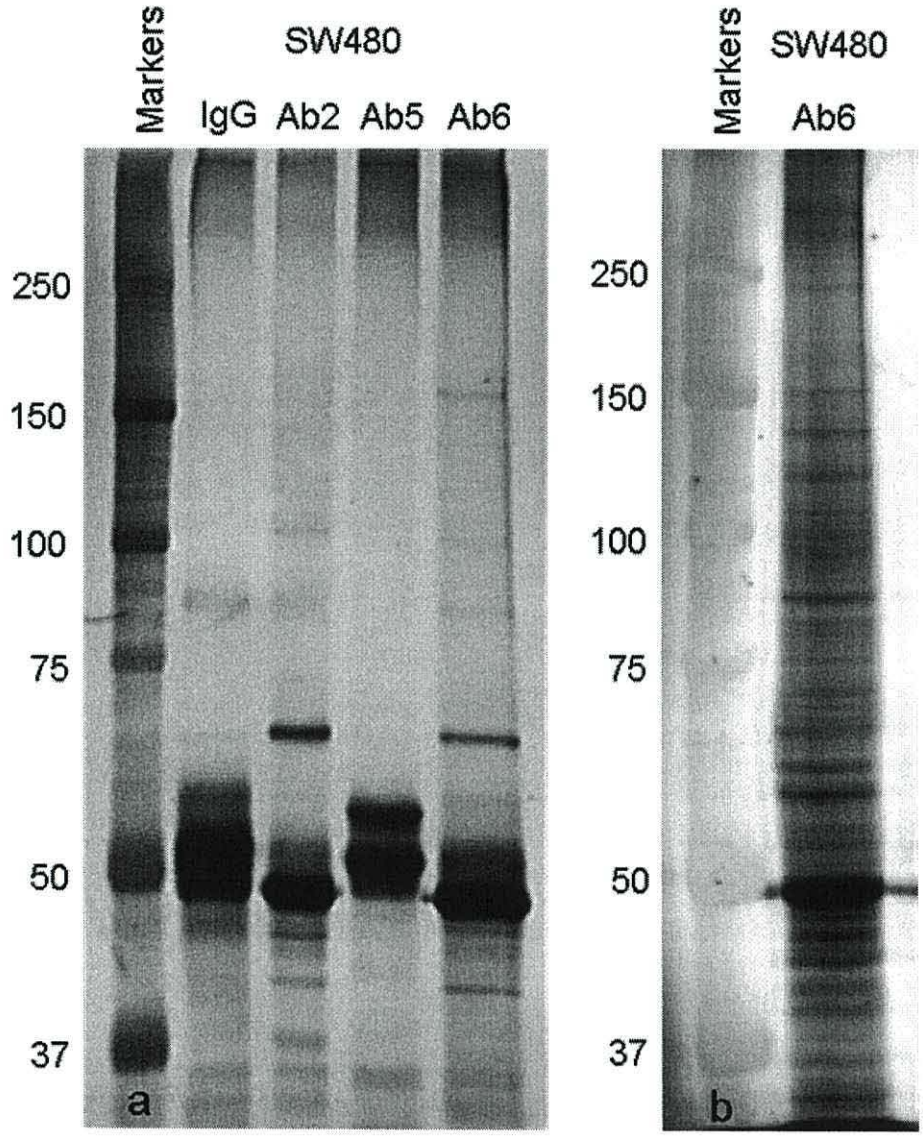


Figure 5.11
Denatured immunoprecipitation of SW480 lysate with APC antibody Ab6 gives a band at 150 kDa (b) not present in the negative control lane (IgG). Immunoprecipitation with Ab2 and Ab6 on HCT116 and SW480 lysates gives a band at 100 kDa and approx. 60 kDa (a) which are not present in the negative control lane (IgG). These proteins have not yet been identified.

and just above 100 kDa (figure 5.11a). These bands did not correspond to any bands seen reproducibly on western blot so were not analysed further. A band was present at approximately 150 kDa on immunoprecipitation of SW480 whole cell lysate with Ab6 (figure 5.11a), this was analysed by mass spectrometry but the amount of protein present was not sufficient for identification. To attempt to purify a greater quantity of this protein for analysis the immunoprecipitation of SW480 whole cell lysate with Ab6 was repeated with a greater amount of cells. Again, a band was seen at 150 kDa (figure 5.11b), additional bands are seen on this repeat compared to the repeat shown in figure 5.11a as in this immunoprecipitation the protein G-agarose was blocked with milk prior to incubation with lysate/antibody so milk proteins bound to the protein G-agarose were visible on the gel. The 150 kDa band was analysed by mass spectrometry, but again not enough protein was available for conclusive identification.

5.7 RT-PCR and sequencing reveals a potential splice site within exon 15 of APC

The apical staining described here is detected using C-terminal APC antibodies and is present in colorectal cancer cell lines with truncating mutations in APC (section 5.3; Rosin-Arbesfeld *et al.*, 2001; Reinacher-Schick & Gumbiner, 2001). It seems likely that a large portion, or all, of the mutation cluster region would be removed if this were due to an APC splice variant. To test this primers spanning the mutation cluster region were designed and RT-PCR carried out on mRNA isolated from HCT116 and SW480 cells (figure 5.12a). Primers APC13 & APC15 were used for the initial PCR, APC13b & APC15 and APC15a & APC15 were used to confirm PCR products were APC. Two PCR products were initially observed with primers APC13 & APC15, one at between 2600 base pairs and one at slightly less than 500 base pairs (figure 5.12b), there was also a very faint product at around 500 base pairs (data not shown). Sequencing of the 2600 base pair RT-PCR product confirmed that this product was amplified from full length APC mRNA (data not shown). The brighter band below 500 base pairs consisted of a mixture of products (as seen by restriction digests and further PCR). One clone could be amplified by primers APC15a & APC15. Sequencing of this

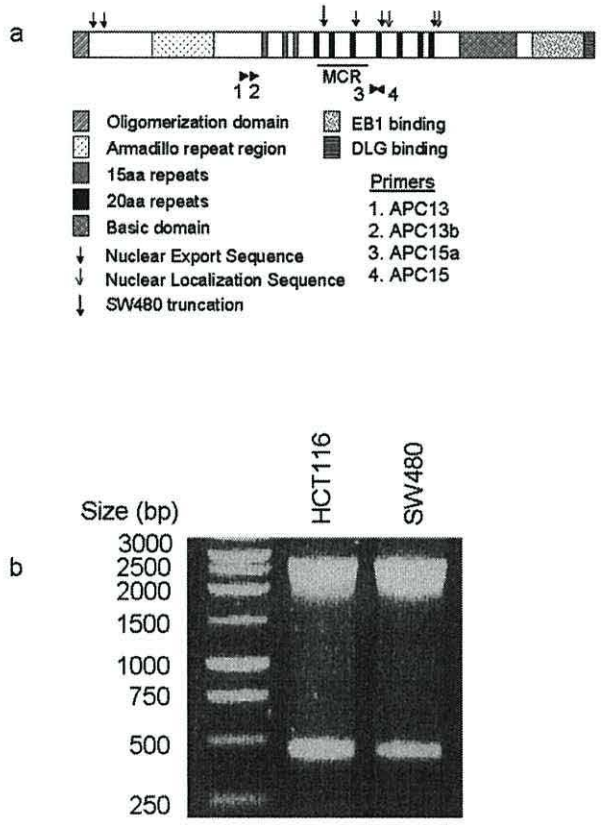


Figure 5.12
Primers were designed either side of the mutation cluster region (a) in an attempt to identify a splice variant resulting in the 150 kDa protein detected by many APC antibodies. A PCR product corresponding to full length APC was seen at approx. 2600 base pairs. The product below 500 base pairs was found to be due to mis-priming at a site within APC that the primer APC13 was not expected to recognise. A fainter product at 500 base pairs (not visible in this image) was found to contain APC sequences, as well as other unrelated sequences due to cross-reactivity of primers.

product confirmed it was APC but this product appeared to have arisen due to mis-priming at a site within APC rather than a splice event (data not shown). The less abundant PCR product at 500 base pairs was cloned and analysed. This PCR product also gave a mixture of different sequences, the majority of which were not APC. One clone was found which could be amplified by both APC13b & APC15 and APC15a & APC15, this clone was sequenced and found to match APC and give a potential splice site. Protein prediction showed that the sequence of this product was in frame both before and after the potential splice. This splice site would remove, the entire mutation cluster region, all three 15 amino acid repeats, the first three 20 amino acid repeats, one axin binding site and one nuclear export sequence.

The sequence of this cloned product does contain some intron-exon boundary consensus sequences. The branch site usually consists of a consensus sequence of Py-N-Py-Py-Pu-A-Py (N=any nucleotide, Py=pyrimidine, Pu=purine) typically at –40 to –18 from the acceptor site (reviewed in Hastings & Krainer, 2001), this potential splice site has a perfect match to this consensus at –41 from the acceptor site (figure 5.13a). The acceptor site is also a perfect match to the consensus of CCAG (figure 5.13a). There is usually a pyrimidine rich tract between the branch site and acceptor site, which is not seen here, however this is not always present and it often not necessary for splicing to occur. The donor site in this potential splice product is AT (figure 5.13b), the donor site AT is much rarer than GT and is usually paired with the acceptor site AC rather than AG. Structural predictions of full length APC mRNA did not indicate any stem-loops, which could possibly give rise to RT-PCR artefacts, in this region (data not shown).

The sequence of this potential splice variant is in frame, has almost all the consensus sequences of a splice site and does not have any indications of mRNA structure which could give rise to artefacts, so it seems possible that it is a real splice variant. However, only one clone was found, all other clones analysed from this PCR reactions appeared to be cross-reactivity of primers with unrelated mRNAs (data not shown). A second primer pair was designed and RT-PCR carried out in an attempt to verify the single clone found with the original

Figure 5.13a
Intron-Exon Boundary of potential APC splice variant identified by RT-PCR and sequencing. The acceptor site is a perfect match to the consensus of CCAG. A branch site is found at –41 from the acceptor site. No pyrimidine rich tract, which is usually found between the branch and acceptor sites, is seen here.

```
      -40        -30        -20        -10       -1
CCATGCCAACAAAGTCATCACGTAAAGCCAAAAAGCCAGCCCAGACTGCTTCAAAATTACCTCCA
```

Consensus:

Py-N-Py-Py-Pu-**A**-Py (-40 to -18 from AG) Py rich tract-N-C_{65}-**AG**

Actual:

T G C C A **A** C (-41 from AG) $^{11}/_{30}$ ($^{4}/_{12}$) Py -C-C-**AG**

Figure 5.13b
Exon-Intron Boundary of potential APC splice variant identified by RT-PCR and sequencing. The donor site AT is rarer than GT and is usually paired with an acceptor sequence of AC. Here the AT donor is paired with the acceptor site AG.

AGCGAGGTTTGCAGATCTCCACCACTGCAGCCCAG**AT**TGCCAAGATGGAAGAAGTGTCAGCCATT
CAT

Consensus:

NNNNNNNNNNNNNNNNNN-**GT**_____**AG**-NNNNNNNNNNNNNNNNNNN

OR

NNNNNNNNNNNNNNNNNN-**AT**_____**AC**-NNNNNNNNNNNNNNNNNNN

Actual:

NNNNNNNNNNNNNNNNNN-**AT**_____**AG**-NNNNNNNNNNNNNNNNNNN

primers. Four clones, two of them identical, were found to have APC sequence. However, none of these clones matched the original potential splice variant. None of these new clones gave predicted protein sequence which was in-frame, all had stop codons and none matched splice site consensus sequences. Although the original potential splice variant identified by RT-PCR is in-frame and appears to match well to splice site consensus sequences, it could not be confirmed by further RT-PCR experiments so the possibility that it is an RT-PCR artefact could not be eliminated.

5.8 Northern analysis with probes directed to the mutation cluster region and C-terminal end of APC

Northern analysis was carried out in an attempt to identify an mRNA corresponding to the 150 kDa protein, and confirm the potential splice site seen by RT-PCR. As SW480 cells express only truncated APC due to a stop codon at 1338 amino acids and the potential 150 kDa splice variant is detected by C-terminal directed antibodies, it would be reasonable to expect that the region spliced out would include the truncation site in SW480. Two probes were used for northern analysis, one spanning the truncation in SW480 (APCS1-S2: the cloned PCR product from primers APCS1 & APCS2) and one at the C-terminal end of APC (APC1-2: the cloned PCR product from APC1 & APC2) (figure 5.14c), within the region the panel of C-terminal antibodies used here are directed to. Both probes were approximately 400 base pairs in size. The exact sequence of this 150 kDa protein is not known and so it was not possible to predict the size of the mRNA, an estimate of size of the mRNA is approximately 4,000 bases of translated sequence with additional untranslated sequence of up to 2,000 bases.

Both probes detected the full length APC mRNA (figure 5.14a,b), both also cross-reacted with the ribosomal RNA, even under high stringency conditions (figure 5.14a,b: 28S sub-unit close to 4,981 base marker; 18S sub-unit close to 1,908 base marker). Probe APCS1-S2 detects additional bands, either side of the 6,583 base marker, and below the 2,604 base marker. The identity of these bands

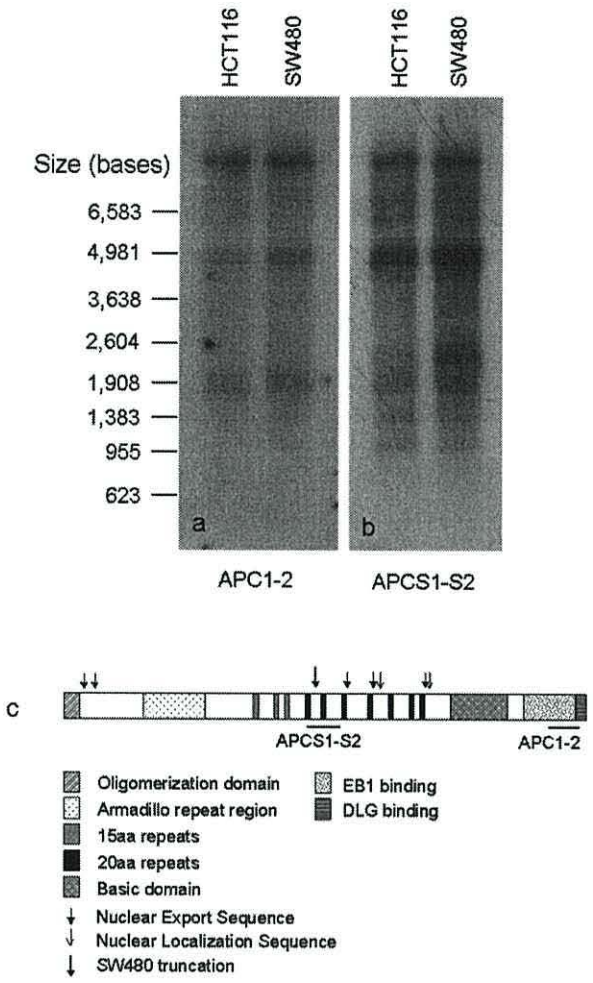


Figure 5.14
Northern blot of mRNA from both HCT116 and SW480 with probes directed to the mutation cluster region of APC (b,c) and the binding site of the C-terminal directed APC antibodies used here (a,c). Both probes detect full length APC mRNA, but do not detect any APC isoform consisting of the far C-terminal end but not the mutation cluster region.

was not clear. As they were detected with the mutation cluster region probe but not the C-terminal probe, it is not possible that these mRNAs correspond to the 150 kDa protein or apical staining described here. These bands may be due to other isoforms of APC or may be cross-reactivity of the probe with other, non-APC, mRNAs.

There are two potential explanations for the lack of mRNA corresponding to the 150 kDa protein on northern blot. Firstly, the predicted size of this mRNA is 4,000-6,000 bases, within this region the probes used both cross-react to the 28S ribosomal sub-unit (figure 5.14a,b), this may mask any APC splice variant close to this size. Secondly the immunofluorescence staining seen with these antibodies is quite faint compared to the staining with antibodies which detect full length and truncated APC. While immunofluorescence is not a quantitative technique, this fainter staining may indicate low abundance of the 150 kDa protein, which may also suggest low abundance of the corresponding mRNA. In many western blots a greater amount of 150 kDa protein is seen compared to full length APC (figure 5.9). However, as transfer of full length APC to membranes is problematic the amount of full length APC seen on western blot may not reflect the levels of APC in the cells. There does seem to be a much greater amount of truncated APC (SW480) than the 150 kDa protein with N-terminal directed APC antibodies (figure 5.9d,e).

5.9 The 150 kDa potential APC isoform identified here does not interact with β-catenin

As full length APC interacts with β-catenin, co-immunopreciptation and western blot was carried out on SW480 lysates to examine whether the 150 kDa protein described here interacts with β-catenin. As SW480 cells express only truncated APC the C-terminal directed APC antibody should only be able to immunoprecipitate the 150 kDa potential splice variant in this cell line. Immunoprecipitation with Ab5 and western blot with a β-catenin antibody, and immunoprecipitation for β-catenin and western blot with H-290, shows that there

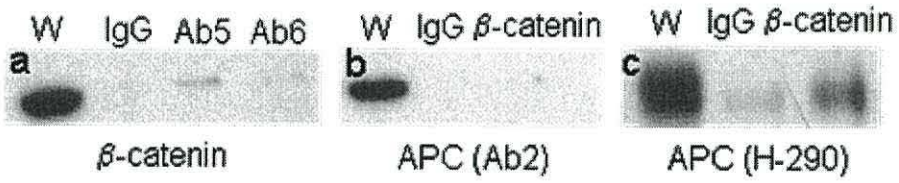


Figure 5.15
There is no significant interaction between the 150 kDa potential APC isoform and β-catenin. Immunoprecipitation with Ab5 (N-terminal) and western blot with a β-catenin antibody shows there is a very small amount of interaction between truncated APC and β-catenin (a). The reciprocal co-immunoprecipitation for β-catenin and Western blot with H-290 confirms this (c). Immunoprecipitation with Ab6 (C-terminal) and Western blot with a β-catenin antibody shows there is no significant interaction between truncated APC and β-catenin (a). The reciprocal co-immunoprecipitation for β-catenin and Western blot with Ab2 confirms this (b). The blot shown in b has been stripped and re-probed for c.

is a small amount of interaction between truncated APC and β-catenin (figure 5.15a,c). Immunoprecipitation with Ab6 and western blot with a β-catenin antibody, and immunoprecipitation of β-catenin and western blot with Ab2, shows there is no significant interaction between β-catenin and the 150 kDa potential APC isoform (figure 5.15a,b).

It is the 20 amino acid repeats within APC that bind to β-catenin (Rubinfeld *et al.*, 1995), a single 20 amino acid repeat can be sufficient for binding of β-catenin to APC (Rubinfeld *et al.*, 1997). This suggests that many, if not all, of the 20 amino acid repeats are missing from this 150 kDa protein. The potential splice site identified by RT-PCR (section 5.7) would account for removal of three 20 amino acid repeats. As this potential APC isoform is 150 kDa in size whereas full length APC is 310 kDa in size, there would need to be more alternative splicing events than the one potentially identified in exon 15. There may be further splice events within exon 15 resulting in removal of most, or all, of the 20 amino acid repeats from the 150 kDa isoform.

5.10 Discussion of results chapter 5: Identification of a 150 kDa potential APC isoform

An apical protein is detected by four APC antibodies, three of these are directed to the C terminus and one is directed to the N terminus. Three of these antibodies detect a 150 kDa band on western blot. A further two antibodies not used for immunofluorescence also detect a 150 kDa protein on western blot giving a total of two N-terminal directed and three C-terminal directed antibodies which detect a 150 kDa band as well as full length APC on western blot. Co-immunoprecipitation and western blot shows that many of these antibodies, including pairs of C- and N-terminal directed antibodies, are detecting the same protein.

Anti-APC (5B2) also appears to detect this 150 kDa protein on western blot and immunoprecipitation (G.T. Roberts, unpublished data). Apical staining has been observed in colon sections with Ab4 (Anderson *et al.*, 2002; Reinacher-Schick & Gumbiner, 2001), N-15 (Reinacher-Schick & Gumbiner, 2001) and a polyclonal antibody directed to the C-terminal 14 amino acids of full length APC (Miyashiro *et al.*, 1995). Another two polyclonal APC antibodies, directed to amino acids 8-347 (N-terminal) and amino acids 2813-2827 (C-terminal) have also been reported to detect APC at apical membranes in a variety of tissues including normal colon (Midgley *et al.*, 1997). Anti-APC (N-15) may also detect this apical protein (Reinacher-Schick & Gumbiner, 2001; Rosin-Arbesfeld *et al.*, 2001), however the reliability of this antibody has been questioned (Mogensen *et al.*, 2002, Rosin-Arbesfeld *et al.*, 2001; Roberts *et al.*, 2003).

It seems very unlikely that all these APC antibodies could be cross-reacting with the same protein. Immunoprecipitation with many of these APC antibodies and western blot with an APCL antibody shows that these antibodies do not appear to cross-react with APCL. BLAST searches do not find any proteins that may have extensive homology to APC in the regions these antibodies are directed to. The most likely explanation for this 150 kDa protein and apical staining seems to be that it is a splice variant of APC. RT-PCR did give a clone which could be due to a splice event in exon 15, however, this could not be confirmed by further RT-

PCR or northern blot. Immunoprecipitation gives a band at 150 kDa with Ab6 in SW480, but as yet it has not been possible to isolate sufficient amounts of this protein for identification by mass spectrometry.

Immunological reactivity show that this splice variant must consist of at least exon 2 and the C-terminal 300 amino acids of APC. As C-terminal antibodies can detect this protein in truncated APC cell lines a large section of the mutation cluster region must be absent.

Alternative splicing of APC has previously been described (Thliveris *et al.*, 1994; Samowitz *et al.*, 1995; Sulekova *et al.*, 1995; Bala *et al.*, 1996; Kraus *et al.*, 1996; Santoro & Groden, 1997; Pyles *et al.*, 1998). Two of these studies described a 150 kDa APC isoform (Pyles *et al.*, 1998; Kraus *et al.*, 1996). The 150 kDa isoform described by Kraus and co-workers (1996) is unlikely to be the 150 kDa isoform described here but is more likely to be cross-reactivity of antibody anti-APC (Ab3/AL4) (section 3.8). It is unclear whether the 150 kDa isoform described by Pyles and co-workers (1998) corresponds to the 150 kDa isoform described here. Both appear to consist of exon 2 and at least part of exon 15, the 150 kDa isoform described by Pyles and co-workers (1998) also has the alternatively spliced exon BS (brain specific) but not exon 1. The 150 kDa isoform described by Pyles and co-workers (1998) was brain specific and not expressed in mitotically active cells whereas the 150 kDa protein described here was observed in mitotically active cultured cells. The BS containing APC isoform (Pyles *et al.*, 1998) interacts with β-catenin whereas the isoform identified here does not appear to have a significant interaction with β-catenin (section 5.9).

The 150 kDa APC isoform is detected at the apical membrane in the two colorectal cancer cells studied, SW480 and HCT116, and at the apical and lateral membranes in the normal epithelial cell line HEK293. It is not clear whether this is an inherent difference in cell lines or whether it is a difference between cancerous and non-cancerous cells. Anderson and co-workers (2002) described apico-lateral membrane staining in normal colon and apical staining in carcinoma

and adenoma of the colon with the APC antibody Ab4, so it may well be a difference between cancerous and non-cancerous cells rather than a difference between different cell lines.

The apical staining seen here is dependent on an intact microtubule network, disruption with nocodazole results in loss of apical membrane staining. The basic domain at the C-terminal end of APC is known to interact directly with microtubules (Munemitsu *et al.*, 1994; Smith *et al.*, 1994b). Unlike full length APC this protein does not accumulate in the nucleus in response to LMB which shows that it does not shuttle continuously between nucleus and cytoplasm as full length APC does (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a; Neufeld *et al.*, 2000b; section 4.8). This potential APC variant is absent from the nucleus in all differentiated cells, such as epithelial cells and colorectal cancer cells, but is nuclear in the embryonal carcinoma cell line NTERA-2. Nuclear staining is also seen in embryonic stem cells (data not shown) so this may reflect a difference between pluripotent cells and differentiated cells.

In *Drosophila* E-APC has been shown to be concentrated at apico-lateral adherens junctions, and often appears to be concentrated at the apical cell surface of epithelial cells (McCartney *et al.*, 1999; Yu & Bienz, 1999; Yu *et al.*, 1999). *Drosophila* E-APC has been shown to be involved in determination of spindle orientation, it is required to keep mitotic spindles oriented within the epithelial plane (Lu *et al.*, 2001). In normal epithelia the APC isoform described here is found at apical and lateral membranes whereas in the two colorectal cancer cell lines, a type of cancer derived from epithelial cells, the localisation is apical only. Similar observations have been made in colon epithelia compared to colorectal tumours (Anderson *et al.*, 2002). *Drosophila* E-APC is required for correct spindle orientation, which determines whether epithelial cells grows laterally or becomes stratified. An early step in many colorectal tumours is cell division along an incorrect plane resulting in loss of an epithelial monolayer and expansion of epithelial cells away from the plane of epithelial cells. The difference in localisation of this apical staining APC isoform between normal and cancerous cells, and similarity to the localisation of *Drosophila* E-APC, suggests it may have a role in spindle orientation.

Chapter 6 – Results: A co-immunoprecipitation screen for interacting partners of full length APC and a 150 kDa potential APC isoform

6.1 Introduction

To date many proteins have been identified which interact with APC. These include components of the Wnt signalling pathway, is addition to proteins relating to other functions of APC (reviewed in Fearnhead *et al.*, 2001). As APC is a large protein with a number of functions, it is a good candidate to apply a screen to isolate novel interacting partners.

A potential APC isoform of 150 kDa has been identified (chapter 5). In contrast to full length APC, this protein does not interact with β-catenin, so is unlikely to function in the Wnt signalling pathway. Knowledge of proteins which interact with this APC isoform may give clues to its function.

To identify novel APC interacting proteins co-immunoprecipitation was carried out on HCT116 cell lysates, and precipitated proteins identified by mass spectrometry. Due to the identification of the potential APC isoform, described in chapter 5, co-immunoprecipitation of SW480 lysate using the C-terminal directed antibody anti-APC (Ab6) was carried out to identify interactions of this 150 kDa protein.

6.2 Myosin IIA, drebrin, BAF155 and clathrin were identified as potential APC interacting proteins by co-immunoprecipitation

Co-immunoprecipitations of HCT116 cell lysates using the N-terminal APC antibody Ab5, resulted in a band at slightly below 250 kDa on SDS-PAGE. This band was enriched in the co-immunoprecipitation lane compared to the control lane (figure 6.1) and was present in multiple repeat experiments. This band was excised from the gel and analysed by Q-Tof mass spectrometry, which showed this protein to be myosin IIA (table 6.1).

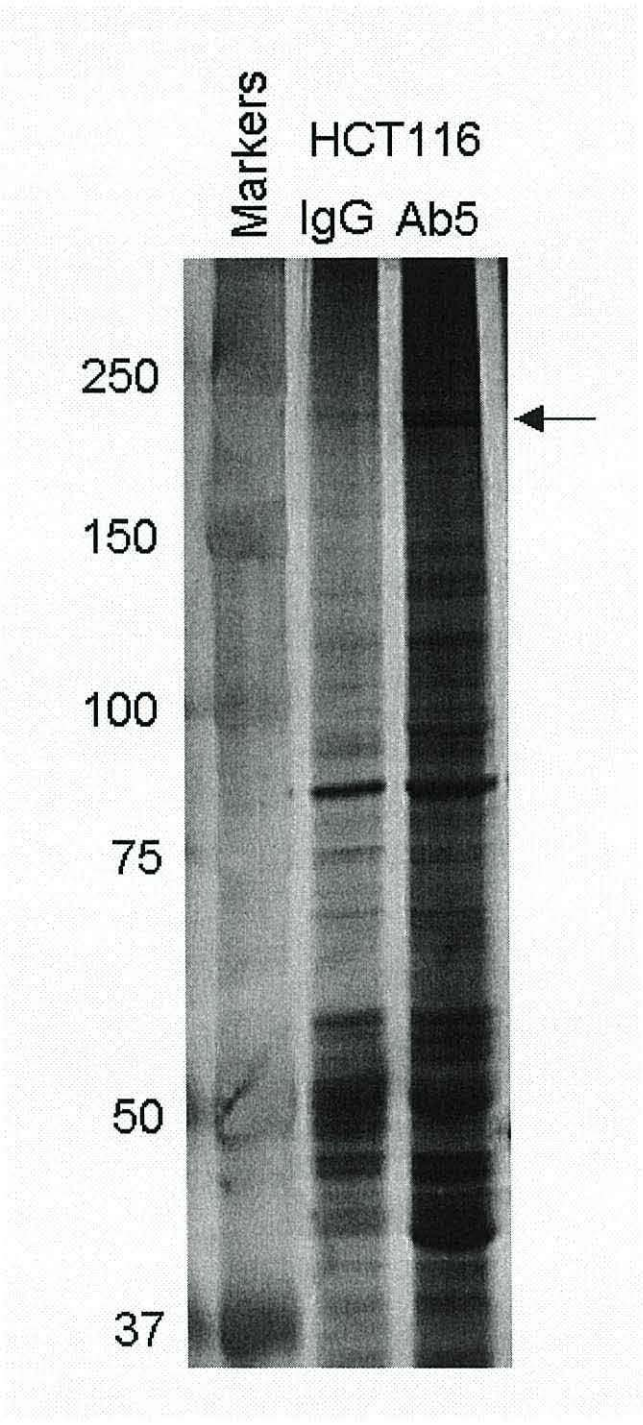


Figure 6.1
Co-immunoprecipitation with N-terminal directed APC antibody Ab5 precipitates a protein below 250 kDa which was found, by Q-Tof mass spectrometry, to be myosin IIA. The arrow shows the protein identified as myosin IIA. Although a protein of a similar size is present in the control lane (IgG) it is greatly enriched in the APC co-immunoprecipitation lane (Ab5).

Table 6.1
Myosin IIA was identified as an APC interacting protein by co-immunoprecipitation and mass spectrometry. Seven peptides were matched to non-muscle myosin, form IIA with a total score of 271.

Query	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Rank	Peptide
10	597.32	1192.62	1192.61	0.02	0	31	1	ALELDSNLYR
11	603.33	1204.65	1204.63	0.01	0	17	9	TDLLLEPYNK
12	606.80	1211.58	1211.58	0.00	0	24	2	DLEAHIDSANK
15	653.34	1304.66	1304.66	-0.00	0	40	1	EQADFAIEALAK
16	659.88	1317.75	1317.74	0.01	0	27	1	LDPHLVLDQLR
19	783.35	1564.69	1564.67	0.02	0	65	1	ELEDATETADAMNR
20	1017.50	2023.98	2023.94	0.03	0	77	1	ELESQISELQEDLESER

gi|12667788 **Mass:** 227646 **Total score:** 271 **Peptides matched:** 7
myosin, heavy polypeptide 9, non-muscle [Homo sapiens]

gi|625305 **Mass:** 227799 **Total score:** 271 **Peptides matched:** 7
myosin heavy chain non-muscle form A - human

Co-immunoprecipitation was carried out on SW480 cell lysate (a cell line which expresses only truncated APC) with anti-APC (Ab6) (a C-terminal directed APC atibody). This immunoprecipitation resulted in a band at below 125 kDa which was not present in the control immunoprecipitation. This protein was analysed by Q-Tof mass spectrometry and identified as drebrin (G.T. Roberts, unpublished data). Since drebrin has been co-immunoprecipitated with a C-terminal directed antibody in a cell line which expressed only truncated APC, drebrin cannot be interacting with full length APC. It seems likely that drebrin is interacting with the 150 kDa potential APC isoform (chapter 5).

Co-immunoprecipitation of SW480 cell lysates with anti-APC (Ab6) also precipitated a protein of approximately 150 kDa (figure 6.2), and one at above the 150 kDa marker at approximately 180 kDa. Both these bands were enriched in the Ab6 immunoprecipitation lane compared to the control lane and were present in multiple repeat experiments. The 150 kDa protein was identified by mass spectrometry as the BAF155 sub-unit of the SWI/SNF chromatin remodelling complex (table 6.2) and the 180 kDa protein was identified as clathrin (table 6.3). Due to time constraints of this project, interaction of these

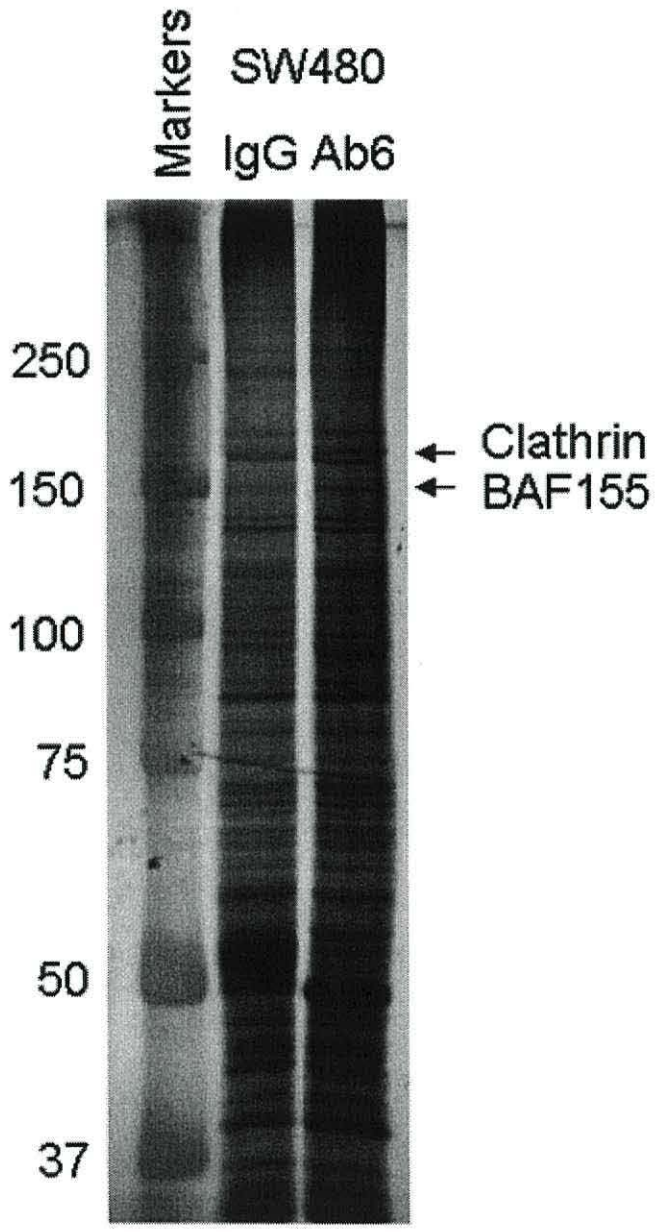


Figure 6.2
Co-immunoprecipitation with C-terminal directed antibody, Ab6, in SW480 cells precipitated two proteins identified, by mass spectrometry, as clarthrin and BAF155. Although many proteins are present in the control lane (IgG), these two proteins are enriched in the co-immunoprecipitation lane (Ab6). As this co-immunoprecipitation used a C-terminal antibody in SW480 cells, which express only truncated APC, only proteins interacting with the 150 kDa potential isoform, and not those interacting with truncated APC, would be co-immunoprecipitated.

proteins with the potential APC isoform of 150 kDa could not be confirmed by co-immunoprecipitation and western blot.

Table 6.2
BAF155 was identified as a potential interacting partner of the 150 kDa isoform of APC by co-immunoprecipitation and mass spectrometry, with 9 peptides matched and a total score of 264.

Query	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Rank	Peptide
7	503.12	502.11	501.32	0.80	0	5	1	LEIK
58	507.78	1013.54	1013.53	0.02	0	59	1	HVTNPAFTK
72	538.26	1074.50	1074.48	0.02	0	14	10	NFMIDTYR +oxidation (m)
118	672.80	1343.59	1343.57	0.03	0	51	1	MEADPDGQQPEK
130	710.85	1419.69	1418.65	1.04	0	23	2	HFEELETIMDR
180	889.96	1777.90	1778.85	-0.95	0	(19)	1	FWESPETVSQLDSVR
181	890.45	1778.89	1778.85	0.04	0	34	1	FWESPETVSQLDSVR
222	996.47	1990.93	1990.90	0.03	0	87	1	GGTVADLDEQDEETV TAGGK
229	1019.53	2037.04	2037.00	0.04	0	16	1	DMEDPTPVPNIEEVVL PK + oxidation (m)

gi|21237802 **Mass:** 123157 **Total score:** 264 **Peptides matched:** 9
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin c1; mammalian chromatin remodelling complex BRG-1 associated factor 155; SWI/SNF 155 kDa subunit; chromatin remodelling complex BAF155 subunit [*Homo sapiens*]

Table 6.3
Clathrin was identified as a potential interacting partner of the 150 kDa isoform of APC by co-immunoprecipitation and mass spectrometry. The peptides were also matched to clathrin from a number of species, only matches to human protein are shown here.

Query	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Rank	Peptide
46	472.27	942.53	942.49	0.04	0	18	3	HELIEFR
64	528.30	1054.58	1054.57	0.01	0	11	3	YIEIYVQK
65	535.28	1068.55	1068.54	0.01	0	39	1	AHIAQLCEK
77	555.84	1109.67	1109.66	0.01	0	14	2	LLLPWLEAR
112	648.85	1295.68	1295.66	0.02	0	18	2	LLYNNVSNFGR
114	652.85	1303.69	1303.65	0.03	0	49	1	NNLAGAEELFAR
121	667.84	1333.66	1333.63	0.04	0	34	1	IYIDSNNNPER
128	696.36	1390.70	1390.66	0.04	0	45	1	LECSEELGDLVK
140	726.36	1450.70	1450.66	0.04	0	16	1	AHTMTDDVTFWK
153	754.40	1506.79	1506.75	0.04	0	37	1	VIQCFAETGQ
172	815.92	1629.83	1629.78	0.05	0	27	1	FNALFAQGNYSEAAK
198	937.99	1873.96	1873.92	0.04	0	9	2	DPELWGSVLLESNPYR
219	982.02	1962.03	1962.00	0.03	0	12	2	AFMTADLPNELIELLE K +oxidation (m)
223	657.70	1970.07	1970.02	0.05	0	25	1	LASTLVHLGEYQAAV DGAR
235	1021.06	2040.11	2040.08	0.03	0	21	1	LPVVIGGLLDVDCSED VIK
250	1061.05	2120.09	2120.03	0.05	0	27	1	DTELAEELLQWFLQEE K
283	785.11	2352.30	2352.24	0.06	0	22	1	ISGETIFVTAPHEATAG IIGVNR
284	1178.11	2354.20	2354.14	0.06	0	23	1	SVNESLNNLFITEEDY QALR
293	961.20	2880.58	2880.51	0.07	0	30	1	RPLIDQVVQTALSETQ DPEEVSVTVK

gi|4758012 **Mass:** 193260 **Total score:** 449 **Peptides matched:** 19 clathrin heavy chain; clathrin, heavy polypeptide-like 2 [*Homo sapiens*]

gi|30353925 **Mass:** 189538 **Total score:** 449 **Peptides matched:** 19 CLTC protein [*Homo sapiens*]

6.3 Co-immunoprecipitation and western blot shows that myosin IIA interacts with either full length/truncated APC or the 150 kDa APC isoform

Non-muscle myosin II is involved in cytokinesis (DeLozanne & Spudich, 1987; Knecht & Loomis, 1987), capping of cell surface components (Pasternak *et al.*, 1989) and polarisation of cell locomotion (Wessels & Soll, 1990; Wessels *et al.*, 1988). Non-muscle myosin II consists of multiple isozymes with two different genes producing the heavy chains (Katsuragawa et al., 1989). Myosin IIA is non-muscle myosin containing the MHC-A heavy chain.

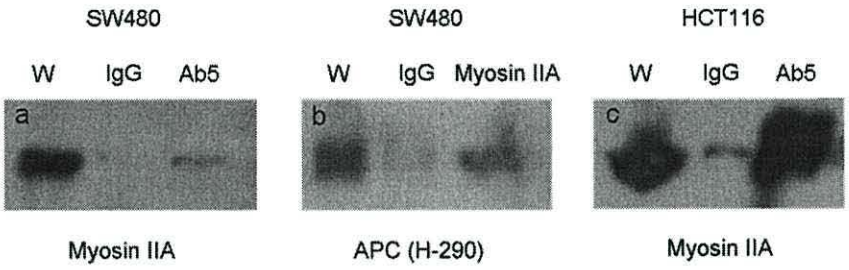


Figure 6.3
Co-immunoprecipitation with N-terminal APC antibody, Ab5, precipitates myosin IIA in both HCT116 and SW480 (a,c). This indicates that either myosin IIA is able to interact with both full length and truncated APC, or that it interacts with the 150 kDa isoform of APC. The reciprocal co-immunoprecipitation also shows that myosin IIA interacts with either truncated APC or the 150 kDa variant (b). However as N-terminal directed APC antibodies were used for both immunoprecipitations and western blots, and SW480 cells express only truncated APC of approximately 150 kDa, it was not possible to confirm whether myosin IIA interacts with truncated APC or the 150 kDa variant.

Myosin IIA was identified as a potential interacting partner of APC by co-immunoprecipitation in HCT116 cells using anti-APC (Ab5). HCT116 cells express both full length APC and the 150 kDa protein identified as a potential isoform of APC (chapter 5). Anti-APC (Ab5) is able to detect both full length APC and the 150 kDa variant on western blot. To confirm interaction of myosin IIA with either full length APC or the 150 kDa isoform, co-immunoprecipitation and western blot were carried out.

Immunoprecipitation of HCT116 cell lysate with anti-APC (Ab5) and western blot with an antibody directed to myosin IIA gives a band at approximately 200 kDa (figure 6.3c), the expected size for myosin heavy chain form A. This indicates that a protein precipitated by anti-APC interacts with myosin IIA. However, as anti-APC (Ab5) is able to detect both full length APC and the 150 kDa isoform on western blot, it is not possible to determine whether myosin IIA interacts with full length APC or with the 150 kDa isoform. Co-immunoprecipitation with anti-APC (Ab5) on SW480 cell lysate and western blot with an antibody raised to myosin IIA also gave a band at approximately 200 kDa (figure 6.3a). As SW480 cells express both truncated APC and the 150 kDa protein, both of which could be immunoprecipitated by anti-APC (Ab5), it is not possible to determine whether it is truncated APC or the 150 kDa potential APC isoform which interacts with myosin IIA.

The reciprocal co-immunoprecipitation with anti-myosin IIA and western blot with anti-APC (H-290) gives a band at 150 kDa in SW480 cells (figure 6.3b). SW480 cells express both truncated APC, which runs at around 150 kDa on SDS-PAGE (predicted size147 kDa), and the 150 kDa isoform. Therefore it is not possible to determine from this data whether myosin IIA is interacting with truncated APC or the 150 kDa protein.

Co-immunoprecipitation with anti-myosin IIA and western blot with an APC antibody which detects both full length APC and the 150 kDa protein in a full length APC-expressing cell line, such as HCT116, would show which protein myosin IIA is interacting with. This experiment was carried out, poor transfer of

full length APC made it problematic to determine which of these proteins myosin IIA interacts with (data not shown).

6.4 Myosin IIA and the apical protein detected by APC antibodies co-localise well in a variety of cell lines

Co-localisation of myosin IIA with either full length APC or the apical protein detected by APC antibodies would give and indication of which of these proteins myosin IIA is interacting with. Staining with anti-APC (Ab4), one of the antibodies which detects the apical protein, and a myosin IIA antibody in HCT116 cells shows that the apical protein and myosin IIA co-localise well at the apical membrane(figure 6.4a-e), with myosin IIA also being localised to the lateral membranes. Anti-myosin IIA and anti-APC (Ab4) also show co-localisation in the normal epithelial cell line HEK293, with both proteins localised to the apical and lateral membranes (figure 6.4f-h). During cytokinesis in HCT116 (figure 6.4i-l) and SW480 (not shown), myosin IIA and APC (Ab4) antibodies detect protein at the new membrane forming between daughter cells.

In the embryonal carcinoma cell line NTERA-2 anti-APC (Ab4) stains filaments throughout the cytoplasm. These filaments did not co-localise with α-tubulin (section 5.3). Staining with anti-myosin IIA and anti-APC (Ab4) shows that the filaments detected by anti-APC (Ab4) co-localise with myosin IIA (figure 6.5a-c). This indicates that it is likely that the 150 kDa apical protein is interacting with myosin IIA, as they co-localise extensively in all cell lines studied. Similar localisation of myosin IIA along stress fibres has been reported in locomoting endothelial cells (Kolega, 1998).

Anti-APC (M-APC) detects full length and truncated APC but not the apical protein (sections 3.2, 3.7). It seems unlikely that full length APC is interacting with myosin IIA in HCT116 and HEK293 cells due to lack of co-localisation at the apical membrane. However, full length APC is also localised to the lateral plasma membrane in some cell lines (Rosin-Arbesfeld *et al.*, 2001). Myosin IIA

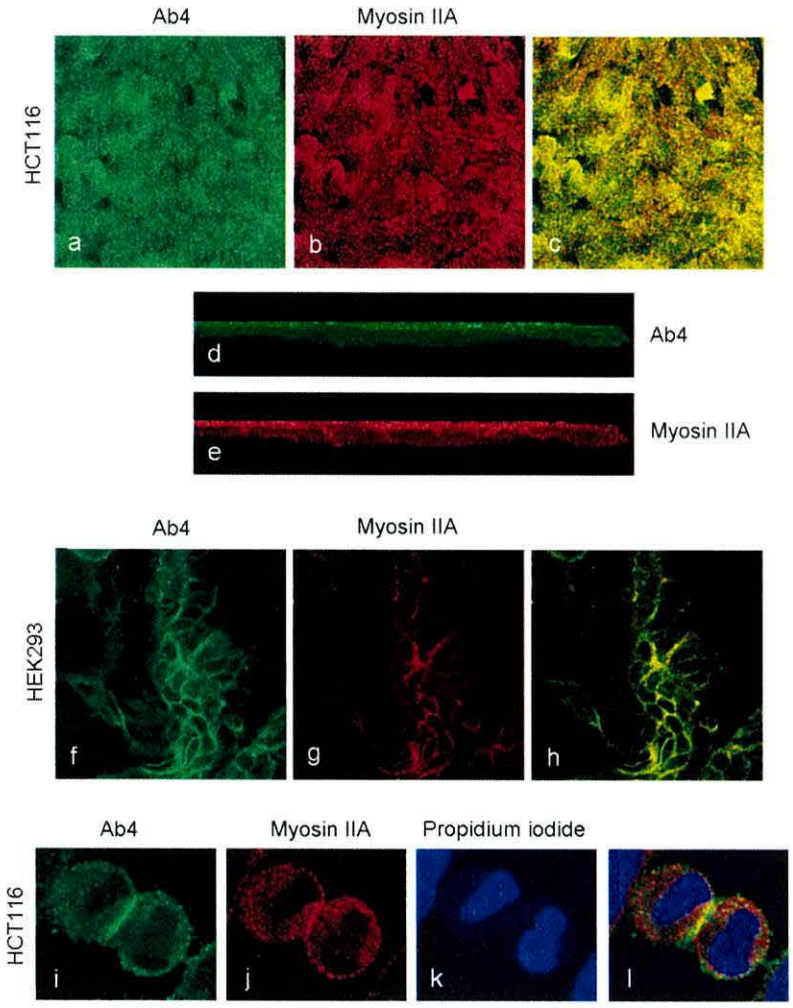


Figure 6.4
Myosin IIA and the 150 kDa APC variant co-localise at the apical membrane in colorectal cancer cell lines HCT116 (a-e) and also at the lateral membrane in normal epithelial cell line HEK293 (f-h). During cytokinesis, both myosinIIa and the 150 kDa APC variant localise between the two daughter cells (i-l). Side views of cells, generated from confocal Z-stacks (d,e), are shown with the apical surface towards the top of the image.

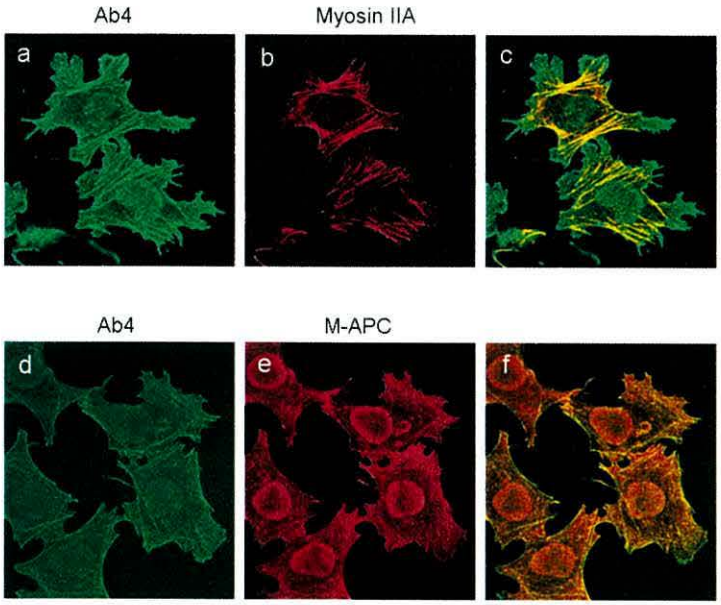


Figure 6.5
Myosin IIA co-localises with the cytoplasmic filaments seen with APC antibody Ab4 in NTERA-2 cells (a-c). This indicates that it may be the 150 kDa protein, a potential APC isoform which is interacts with myosin IIA. However, full length APC, as detected by anti-APC (M-APC) (e) is also localised to these filaments (d-f). Therefore, localisation of both full length APC and the 150 kDa protein is consistent with an interaction with myosin IIA.

is also localised to the lateral plasma membrane (figure 6.4) suggesting that full length APC and myosin IIA do co-localise in epithelial cells and are potentially able to interact with each other.

Myosin IIA is localised to cytoplasmic filaments in low density NTERA-2 cells. To test whether full length APC also localises to these filaments NTERA-2 cells were double labelled with anti-APC (M-APC) and anti-APC (Ab4) (to detect the 150 kDa protein). As both M-APC and the myosin IIA antibody used here are raised in rabbit it was not possible to label both myosin IIA and APC with M-APC directly. Anti-APC (Ab4) detects the cytoplasmic filaments so co-localisation of staining with anti-APC (Ab4) and anti-APC (M-APC) was used as an indication of whether full length APC localises to these filaments. The APC antibodies M-APC and Ab4 do not give identical localisation in NTERA-2, but both these antibodies do detect the cytoplasmic filaments to an extent, although they are more pronounced with anti-APC (Ab4). This shows that, as in epithelial cell lines, myosin IIA and full length APC co-localise in NTERA-2, so again have the potential to interact with each other.

6.5 Reciprocal co-immunoprecipitation and western blot detects and interaction between drebrin and the 150 kDa protein (a potential APC isoform)

Drebrin (**d**evelopmentally **re**gulated **br**ain prote**in**) is an actin-interacting protein of 70 kDa which was originally identified as a brain specific protein thought to be involved in the regulation and maintenance of the nervous system (Shirao & Obata, 1986; Shirao *et al.*, 1990; Shirao, 1995; Hayashi *et al.*, 1996; Sasaki *et al.*, 1996). It has since been found that the E2 isoform of drebrin is expressed in a wide variety of non-neuronal cells (Shirao *et al.*, 1994; Peitsch *et al.*, 1999; Keon *et al.*, 2000). Although drebrin is 70 kDa in size it has an unusual mobility on SDS-PAGE and runs at approximately 120 kDa. Little is known about the function of drebrin in non-neuronal cells but it is known that it interacts with actin (Sasaki *et al.*, 1996) and may have a role in regulation of actin dynamics (Asada *et al.*, 1994). Drebrin has been observed to be associated with adherens

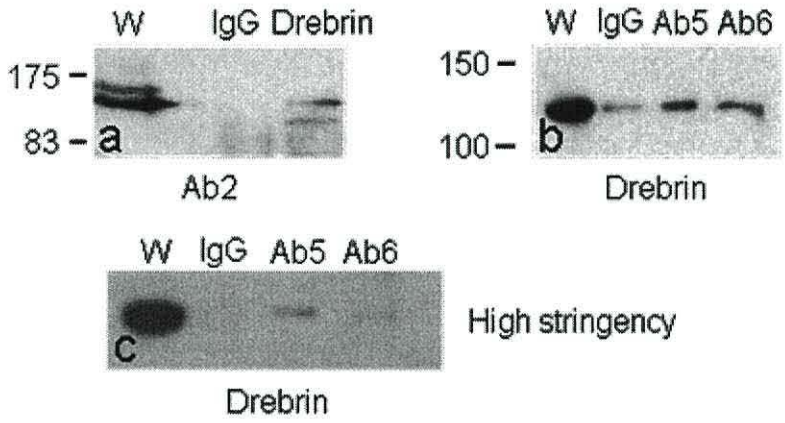


Figure 6.6
Reciprocal co-immunoprecipitation and western blot shows interaction between drebrin and the 150 kDa APC variant. Co-immunoprecipitation with a drebrin antibody and western blot with a C-terminal directed antibody in SW480, shows a 150 kDa protein interacts with drebrin (a). As this co-immunoprecipitation was done using SW480 cells, which express only truncated APC, this 150 kDa band detected by a C-terminal directed APC antibody must correspond to the 150 kDa isoform and not truncated APC. The reciprocal co-immunoprecipitation with N- and C-terminal directed APC antibodies and western blot with a drebrin antibody also shows interaction between the 150 kDa APC isoform and drebrin (b,c). b and c show similar co-immunoprecipitations, with c being a higher stringency immunoprecipitation than b.

junctions in some epithelial and endothelial cells with its distribution being mutually exclusive to that of vinculin (Peitsh *et al.*, 1999).

To confirm the interaction of drebrin with the 150 kDa APC variant reciprocal co-immunoprecipitation and western blots were carried out. This was carried out in SW480 cells, as immunoprecipitation with C-terminal directed antibodies in this cell line can only precipitate the 150 kDa variant as this cell line only expresses truncated APC. Immunoprecipitation with a drebrin antibody and western blot with anti-APC (Ab2) in SW480 cells gave a band at 150 kDa which was not present in the control immunoprecipitation (figure 6.6a). Another band was also present, running below the band at 150 kDa, the identity of this protein is not known. The reciprocal co-immunprecipitation with APC antibodies Ab5 (N-terminal) and Ab6 (C-terminal) followed by western blotting with a drebrin antibody gave a band at approximately 120 kDa, the expected size for drebrin (figure 6.6b,c). However, a band of the same size was also present in the control immunoprecipitation, although this protein was enriched in the APC immunoprecipitation lanes compared to the control lanes (figure 6.6b). A second, higher stringency, immunoprecipitation was carried out, in this case drebrin was co-immunoprecipitated with both APC antibodies (Ab5, Ab6) and was absent from the control lane (figure 6.6c).

6.6 Drebrin and the 150 kDa APC variant co-localise in a variety of cell lines

Immunofluorescence staining for drebrin and the 150 kDa APC variant was carried out in a variety of cell lines to confirm whether drebrin and the 150 kDa APC variant co-localise. Drebrin localises to the plasma membrane, with some concentration at the apical surface in SW480 and HCT116 cells (figure 6.7b,c). Drebrin co-localises with the 150 kDa APC variant at the apical membrane in colorectal cancer cell lines HCT116 and SW480 (figure 6.7a,b) and at the lateral membranes in normal epithelial cell line HEK293 (figure 6.7c). During cytokinesis the 150 kDa APC variant and drebrin co-localises at the membrane between daughter cells (figure 6.7d).

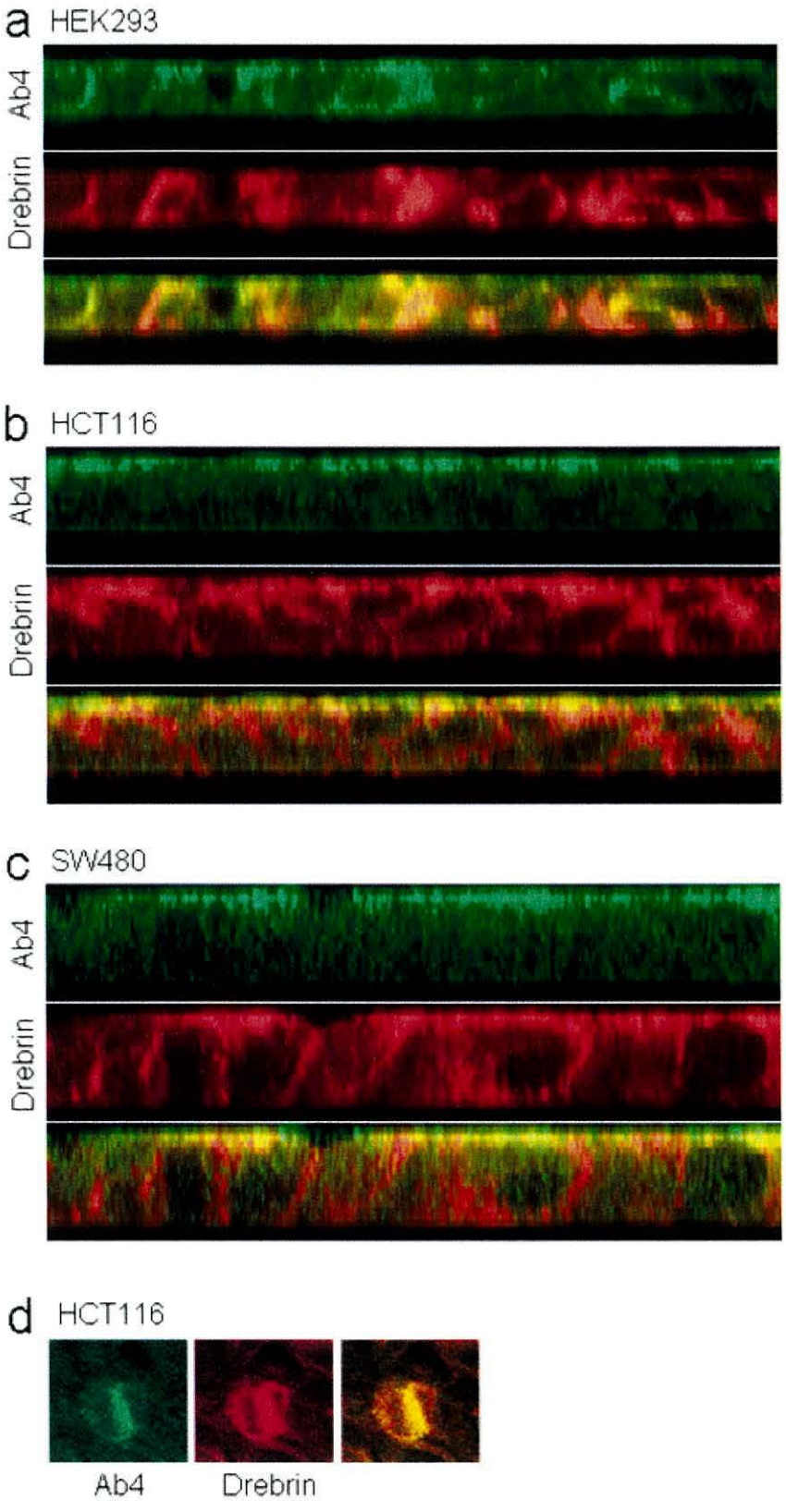

a HEK293
Ab4
Drebrin
b HCT116
Ab4
Drebrin
c SW480
Ab4
Drebrin
d HCT116
Ab4
Drebrin

Figure 6.7

Drebrin and the 150 kDa potential APC isoform co-localise at the apical membrane in colorectal cancer cell lines HCT116 (b) and SW480 (c) and additionally at the lateral membrane in normal epithelial cell line HEK293 (a). Drebrin and the 150 kDa APC isoform localise between the two daughter cells at cytokinesis.

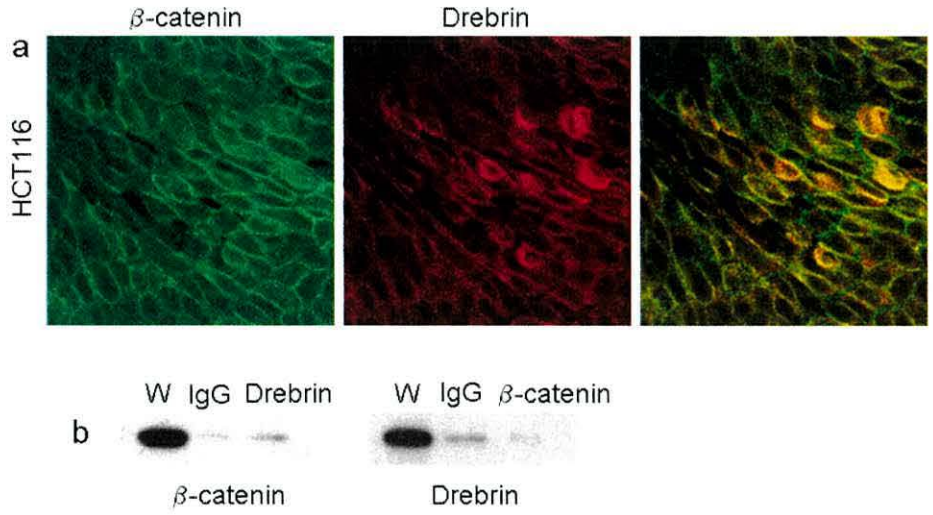


Figure 6.8
Drebrin and β-catenin co-localise at the cell membrane (a) but no evidence of interaction between the two proteins was found by co-immunoprecipitation and western blot (b). Western blots shown are long exposures. Faint bands are seen in the control and co-immunoprecipitation lane in both western blots. The immunoprecipitation lane is not significantly enriched compared to the control lane in both parts of the reciprocal co-immunoprecipitation and western blot, so it must be concluded that there is no significant interaction between these two proteins.

6.7 β-catenin and drebrin co-localise, however no interaction is observed by co-immunoprecipitation and western blot

Drebrin has been reported to be localised to adherens junctions in epithelial cells (Peitsch *et al.*, 1999) and co-localises with β-catenin on immunofluorescence (figure 6.8a). Therefore reciprocal co-immunoprecipitation and western blot was carried out to test whether β-catenin and drebrin interact. In both reciprocal co-immunoprecipitations no interaction between β-catenin and drebrin was observed (figure 6.8b). Overnight exposures are shown so a very small amount of protein is visible in both the control and co-immunoprecipitation lanes. However, there is no significant difference between the control and co-immunoprecipitation lane which would indicate interaction between the two proteins.

6.8 Discussion of results chapter 6: A co-immunoprecipitation screen for interacting partners of full length APC and a 150 kDa potential APC isoform

Four proteins: drebrin, myosin IIA, BAF155 and clathrin, were identified as potential interacting partners of either APC or a 150 kDa APC isoform by an immunoprecipitation based screen for APC-interacting proteins. Interaction of BAF155 and clathrin with the 150 kDa APC isoform has not yet been cofirmed.

Drebrin was confirmed to interact with the 150 kDa protein, identified as a potential isoform of APC. In epithelial cells drebrin is localised to adherens junctions (Peitsh *et al.*, 1999). Little is known about the function of drebrin but it is known that it interacts with actin (Sasaki *et al.*, 1996) and may have a role in regulation of actin dynamics (Asada *et al.*, 1994). Interaction of drebrin with full length APC has not yet been examined.

APC – myosin IIA interaction was also confirmed, however it was not possibly to determine whether it is full length APC or the 150 kDa protein which interacts with myosin IIA. Co-localisation by immunofluorescence staining is consistent with either of these proteins, or both, interacting with myosin IIA.

Drebrin has been shown to interact with myosin IIB but not myosin IIA in dendritic spines (Cheng *et al.*, 2000). Here, the APC variant of 150 kDa is shown to interact with both myosin IIA and drebrin, this is inconsistent with the report above where myosin IIB but not myosin IIA interacts with drebrin. Myosin/drebrin interaction may vary with cell type, or the 150 kDa protein may interact with these two proteins independently.

Although drebrin and β-catenin both localise to adherens junctions and co-localise in a variety of cell lines, they were not seen to interact with each other. Drebrin has been reported to only localise to one particular type of adherens junction, those lacking vinculin (Peitsch *et al.*, 1999) so drebrin and β-catenin may localise to separate, different types of, adherens junctions.

In *Drosophila* E-APC has been shown to be concentrated at apico-lateral adherens junctions, and often appears to be concentrated at the apical cell surface of epithelial cells (McCartney *et al.*, 1999; Yu & Bienz, 1999; Yu *et al.*, 1999). In normal epithelia the 150 kDa APC isoform is found at apical and lateral membranes whereas in the two colorectal cancer cell lines, a type of cancer derived from epithelial cells, the localisation is apical only. *Drosophila* E-APC is required for correct spindle orientation (Lu *et al.*, 2001), which determines whether epithelial cells grows laterally or becomes stratified. An early step in many colorectal tumours is cell division along an incorrect plane resulting in loss of an epithelial monolayer and expansion of epithelial cells away from the original plane of division. Like *Drosophila* E-APC, the 150 kDa protein is localised to the apical and lateral membranes. This suggests it may have a similar role in spindle orientation to that of *Drosophila* E-APC. In normal epithelial cells the 150 kDa protein is localised to the lateral membrane as well as the apical membrane, this lateral membrane staining is lost in colorectal carcinoma cells. In colorectal tumours cell division takes place in various directions, rather than being confined to the epithelial plane. If the 150 kDa protein does have a function in aligning the spindle, loss of this protein from the lateral membranes may be an important step in tumour expansion.

It has been shown here that this apical protein interacts with drebrin and may interact with myosin IIA, two actin-interacting proteins. Localisation and interactions can give clues as to the function of this 150 kDa apical staining protein. The microtubule dependent localisation and its interaction with actin binding proteins suggest a role related to the cytoskeleton.

Chapter 7 – Results: The Wnt signalling pathway in retinoic acid-induced differentiation of NTERA-2 embryonal carcinoma cells

7.1 Introduction

NTERA-2 is an embryonal carcinoma cell line derived from a lung metastasis of a testicular teratocarcinoma (Fogh & Trempe, 1975; Andrews *et al.*, 1984). NTERA-2 is a sub-line derived from TERA-2, which was originally obtained by culturing embryonal carcinoma (EC) cells from a lung metastasis of a testicular teratocarcinoma (Fogh & Trempe, 1975). The TERA-2 cell line was passaged through a *nu/nu* nude mouse, a mouse which lacks a functional immune system. A well differentiated teratocarcinoma was formed which consisted of glandular structures, mesenchyme, neural elements and embryonal carcinoma cells. A number of sub-lines, including NTERA-2 Clone D1 (referred to here as NTERA-2) were derived from the EC cells of this teratocarcinoma (Andrews *et al.*, 1984).

Teratocarcinomas are generally composed of a variety of adult, embryonic and extra-embryonic tissues and EC cells (Andrews *et al.*, 1984). The embryonal carcinoma cells behave as pluripotent stem cells which give rise to all other cell types in the tumour. Embryonal carcinoma cells may originate from a displaced embryonic stem (ES) cell which somehow retains its pluripotency into postnatal life. Or, they may originate from germ cells which manage to proliferate without entering meiosis.

When cultured in the presence of retinoic acid NTERA-2 cells differentiate to give neurones and other cell types (Andrews 1984; Fenderson *et al.*, 1987; Thompson *et al.*, 1984). NTERA-2 embryonal carcinoma cells commit to differentiate within 1-2 days of exposure to retinoic acid. In the absence of retinoic acid very little spontaneous differentiation is seen (Andrews *et al.* 1984). Wnt-13 expression has been detected in NTERA2 cells differentiating in response to retinoic acid (Wakeman *et al.*, 1998). Wnt-13 expression was detected in the early phases of differentiation in response retinoic acid and in non-neural differentiated cells for several weeks after induction of differentiation. No Wnt-13 expression was detected in terminally differentiated neurones or in undifferentiated EC cells. Differentiation of NTERA-2 can also be induced by treatment with 7.5mM lithium chloride, usually for 7 days. Treatment with lithium chloride mimics Wnt signalling by inhibiting GSK-3

(Klein & Melton, 1996). It is possible that Wnt-13 expression in NTERA-2 cells in response to retinoic acid induction is an important step in differentiation.

The aim of the work presented here was to investigate expression of Wnt pathway components during retinoic acid induced differentiation in NTERA-2, and attempt to identify novel homologues. To do this a degenerate primer RT-PCR screen was carried out targeting three Wnt pathway components, TLE, Dishevelled and Tcf. At the time this work was carried out the human genome project had not yet been completed.

7.2 Expression of Wnt pathway components during differentiation of NTERA-2

Messenger RNA (mRNA) was purified from retinoic acid induced NTERA-2 cells 2, 4, 7 and 15 days after start of retinoic acid induction, and from NTERA-2 embryonal carcinoma cells (see materials and methods). To confirm retinoic acid induced differentiation of NTERA-2 flow cytometry analysis for cell surface markers was carried out in untreated embryonal carcinoma cells and after 7 days retinoic acid treatment (table 7.1). Expression of cell surface markers was consitent with previously described expression in NTERA-2 for both EC cells and retinoic acid induced cells (Andrews, 1984). The one exception was that A2B5 expression was lower than expected in retinoic acid treated cells, this was later found to be due to a poor batch of A2B5 antibody. Therefore it seems that retinoic acid induced differentiation of the NTERA-2 cells used in these experiments was successful.

Table 7.1
Flow cytometry analysis of cell surface markers in NTERA-2 embryonal carcinoma cells and retinoic acid treated cells used for mRNA purification. Table shows percentage of cells expressing cell surface markers, numbers in brackets give expected percentage of cells expressing markers (Andrews, 1984).

	Embryonal carcinoma cells	Retinoic acid treated cells
SSEA4	95% (90-100%)	60% (~50%)
SSEA1	0% (~0%)	21% (~20%)
A2B5	4% (~0%)	18% (80-90%)
Tra-1-60	96% (90-100%)	70% (~60%)

Three Wnt pathway members were targeted in an RT-PCR screen to determine which homologues are expressed and to attempt to identify novel homologues. These three Wnt pathway components were Dishevelled (Cadigan & Nusse, 1997; Dale, 1998), TLE (Parkhurst, 1998; Cavallo *et al.*, 1998), and Tcf (reviewed in Bienz & Clevers, 2000).

Degenerate primers to Tcf (figure 7.1), TLE (figure 7.2) and Dishevelled (figure 7.3) were designed from ClustalX (Thompson *et al.*, 1997) alignments of human homologues (TLE, Dishevelled) or human and mouse homologues (Tcf). Degenerate primers were places in well-conserved regions with some unconserved sequence within the resulting PCR products. Primers to Wnt-13, APC and APCL were designed using DNA* software (DNASTAR Inc.). Where possible primers were designed so that the PCR product covered multiple exons, this was to eliminate the possibility that any PCR product seen was due to genomic DNA contamination of the mRNA.

RT-PCR was carried out on mRNA from embryonal carcinoma and retinoic acid induced NTERA-2 cells. To confirm the previous observation that Wnt-13 expression is induced in retinoic acid treated cells (Wakeman *et al.*, 1998) RT-PCR was carried out on mRNA from embryonal carcinoma and retinoic acid treated cells (figure 7.4a). Wnt-13 was expressed in retinoic acid induced cells at all time-points (2-15 days) but not in embryonal carcinoma cells.

Dishevelled (Dvl) acts to inhibit GSK-3 when a Wnt signal is present, this results in stabilisation of β-catenin (Cadigan & Nusse, 1997; Dale, 1998). Dishevelled has three conserved domains, an N-terminal DIX domain, which is also found in Axin, a central PDZ domain and a DEP (Dishevelled, egl-10, plekstin) domain C-terminal to the PDZ domain (Li *et al.*, 1999). Three human homologues of Dishevelled have been identified (Dvl-1, Dvl-2, Dvl-3) (Semenov & Snyder, 1997).

Figure 7.1: CLUSTAL X (1.64b) multiple sequence alignment of a number of human and mouse Tcf homologues. Primers are shown in grey, forward primer is Tcf1200, reverse primer is Tcf1420. "*" indicates conserved sequence.

```
Mouse tcf4B   CTGTCCTCTAGGTTCCCTCCCCATATGGTCCCTCCCCATCACACTCTGCACACGACCGGCATCCCTCACCCGGCCATCGTCACACC
Mouse tcf-4   -----------GTTCCCTCCCCATATGGTCCCTCCCCATCACACTCTGCACACGACCGGCATCCCTCACCCGGCCATCGTCACACC
Human tcf-4   -----------GTTCCCTCCCCATATGGTCCCACCACATCATACGCTACACACGACGGGCATTCCGCATCCGGCCATAGTCACACC
Mouse tcf-3   GTCTCAAGCCGGTTCCCACACATGGTGGCTCCTGCCCATCCTGGTCTGCCCACCTCAGGAATCCCCCACCCTGCCATCGTCCCCTC
Mouse tcf-1   ------TTCACCCACCCATCCTTGATGCTGGGATCTGGTGTACCTGGACACCCAGCAGCCATCCCTCACCCGGCCATTGTGCCCTC
Human tcf-7   ------TTCACCCACCCATCCTTGATGCTAGGTTCTGGTGTACCTGGTCACCCAGCAGCCATCCCCCACCCGGCCATTGTGCCCCC
                     ***   *    **       *   *         * * *  * *  ** ** ** ** ***** **  *  *

Mouse tcf4B   GACAGTCAAGCAGGAATCCTCCCAGAGTGACGTCGGCTCACTCCAC--AGCTCAAAGCATCAGGACTCCAAAAAGGAAGAAGAGAA
Mouse tcf-4   GACAGTCAAGCAGGAATCCTCCCAGAGTGACGTCGGCTCACTCCAC--AGCTCAAAGCATCAGGACTCCAAAAAGGAAGAAGAGAA
Human tcf-4   AACAGTCAAACAGGAATCGTCCCAGAGTGATGTCGGCTCACTCCAT--AGTTCAAAGCATCAGGACTCCAAAAAGGAAGAAGAAAA
Mouse tcf-3   CATTGTGAAGCAGGAGCCAGC--AGCCCCCAGCCTGAGCCCTGCAGTGAGTGCGAAATCCCCAGTTACGGTGAAGAAGGAAGAGGA
Mouse tcf-1   CTCAGGGAAGCAGGAG-CTGC--AGCCATATGATAGAAACCTGAAA--A-C-----ACAGGCAGAACCCAAGGCAGAGAAGGAGGC
Human tcf-7   CTCAGGGAAGCAGGAG-CTGC--AGCCCTTCGACCGCAACCTGAAG--A-C-----ACAAGCAGAGTCCAAGGCAGAGAAGGAGGC
                  *  ** *****  *  *  **      *  *     **  *   *              *   *        *  * **

Mouse tcf4B   GAAGAAGCCCCACATAAAGAAGCCCCTTAATGCATTCATGTTGTATATGAAAGAGATGAGAGCGAAGGTGGTGGCCGAATGCACAT
Mouse tcf-4   GAAGAAGCCCCACATAAAGAAGCCCCTTAATGCATTCATGTTGTATATGAAAGAGATGAGAGCGAAGGTGGTGGCCGAATGCACAT
Human tcf-4   GAAGAAGCCCCACATAAAGAAACCTCTTAATGCATTCATGTTGTATATGAAGGAAATGAGAGCAAAGGTCGTAGCTGAGTGCACGT
Mouse tcf-3   GAAGAAACCTCACGTGAAAAAGCCCCTGAATGCCTTCATGTTGTATATGAAGGAGATGAGGGCCAAGGTGGTGGCCGAGTGTACCC
Mouse tcf-1   TAAGAAGCCAGTCATCAAGAAACCCCTCAATGCGTTCATGCTTTACATGAAGGAGATGAGAGCCAAGGTCATTGCTGAGTGCACAC
Human tcf-7   CAAGAAGCCAACCATCAAGAAGCCCCTCAATGCCTTCATGCTGTACATGAAGGAGATGAGAGCCAAGGTCATTGCAGAGTGCACAC
               ***** **   * * ** ** ** ** ***** ****** * ** ***** ** ***** ** *****  * ** ** ** **

Mouse tcf4B   TGAAAGAGAGTGCAGCCATCAACCAGATTCTCGGGCGCAGGTGGCACGCCCTGTCCAGGGAAGAACAGGCAAAATATTACGAGCTG
Mouse tcf-4   TGAAAGAGAGTGCAGCCATCAACCAGATTCTCGGGCGCAGGTGGCACGCCCTGTCCAGGGAAGAACAGGCAAAATACTACGAGCTG
Human tcf-4   TGAAAGAAAGCGCGGCCATCAACCAGATCCTTGGGCGGAGGTGGCATGCACTGTCCAGAGAAGAGCAAGCGAAATACTACGAGCTG
Mouse tcf-3   TGAAGGAAAGTGCAGCCATTAACCAAATCCTGGGAAGAAAGTGGCACAACCTGTCAAGAGAAGAACAGGCCAAATACTATGAGCTT
Mouse tcf-1   TCAAGGAGAGCGCTGCCATCAACCAGATCCTGGGTCGCAGGTGGCATGCACTATCTCGAGAAGAGCAGGCCAAGTACTATGAACTG
Human tcf-7   TTAAGGAGAGCGCTGCCATCAACCAGATCCTGGGCCGCAGGTGGCACGCGCTGTCGCGAGAAGAGCAGGCCAAGTACTATGAGCTG
              * ** ** ** ** ***** ***** ** ** **  * * ******    ** **  * ***** **  ** ** ** ** ** **
```

Figure 7.2: CLUSTAL X (1.64b) multiple sequence alignment of four human TLE homologues. TLE1420 and TLE1950 are shown in grey, TLE1820 and TLE2330 are shown in bold. "*" indicates conserved sequence.

```
TLE1    ACTGCAGACGGTCAGATGCAGCCTGTCCCTTTTCC-CCCGACGCCCCTCATCGGACCCGGAATCCCCCGGCATGCTCGCCAGATCAACACC
TLE2    TCTGCGGACGGGCAGATGCAGCCGGTTCCCTTCCC-CTCGGATGCACTGGTAGACGCGGGCATCCCGCGGCACGCCCGGCAGCTGCACACG
TLE3    AGTGCTGATGGGCAGATGCAGCCCGTGCCCTTCCCCCACGAC-GCCCTGGCAGGCCCCGGCATCCCGAGGCACGCCCGGCAGATCAACACA
TLE4    AGCGCAGATGGTCAGATGCAGCCTGTCCCTTTTCCACCCGAC-CCCCTCATCGGACCTGGAATCCCCCGGCATGCTCGCCAGATCAACACC
           ** ** ** *********** ** ** ** ** * **    * **    *   * ** *****  **** ** ** *** *  ****

TLE1    CTCAACCACGGGGAGGTGGTGTGCGCTGTGACCATCAGCAACCCCACGAGACACGTGTACACAGGCGGGAAGGGCTGCGTCAAGGTCTGGG
TLE2    CTGGCCCATGGCGAGGTGGTCTGCGCGGTCACCATCAGCGGCTCCACACAGCATGTGTACACGGGCGGCAAGGGCTGTGTGAAGGTGTGGG
TLE3    CTCAGCCACGGGGGGGTGGTGTGTGCCGTGACCATCAGCAACCCCAGCAGGCACGTCTACACAGGTGGCAAGGGCTGCGTGAAGATCTGGG
TLE4    CTCAACCACGGGGAGGTGGTGTGCGCGGTGACCATCAGCAACCCCACGAGACACGTGTACACGGGTGGGAAGGGCGCGGTCAAGGTCTGGG
        **  *** ** * ****** ** ** ** *********  * ***     **  ** ***** ** ** ******   ** *** * ****

TLE1    ACATCAGCCACCCTGGCAATAAGAGCCCTGTCTCCCAGCTCGACTGTCTGAACAGAGACAATTATATCCGTTCCTGTAAATTGCTACCCGA
TLE2    ACGTGGGCCAGCCTGGGGCCAAGACGCCCGTGCGCCAGCTCGACTGCCTGAACCGAGACAACTACATTCGTTCCTGCAAGTTGCTGCCGGA
TLE3    ACATCAGCCAGCCAGGCAGCAAGAGCCCCATCTCCCAGCTGGACTGCCTGAACAGGGACAATTACATGCGCTCCTGCAAGCTGCACCCTGA
TLE4    ACATCAGCCACCCAGGCAATAAGAGTCCTGTCTCCCAGCTCGACTGTCTGAACAGGGATAACTACATCCGTTCCTGCAGATTGCTCCCTGA
        ** *  **** ** **    ****  **  *   ****** ***** ****** * ** ** ** ** ** ***** *   ***  ** **

TLE1    TGGCTGCACTCTCATAGTGGGAGGGGAAGCCAGTACTTTGTCCATTTGGGACCTGGCGGCTCCAACCCCGCGCATCAAGGCGGAGCTGACG
TLE2    TGGCCGGAGTCTGATCGTGGGCGGTGAGGCCAGCACCTTGTCCATTTGGGACCTGGCGGCGCCCACCCCCCGTATCAAGGCCGAGCTGACT
TLE3    TGGGCGCACGCTCATCGTGGGCGGCGAGGGCAGCACGCTCACCATCTGGGACCTGGCCTCGCCCACGCCCCGCATCAAGGCCGAGCTGACG
TLE4    TGGTCGCACCCTAATTGTTGGAGGGGAAGCCAGTACTTTGTCCATTTGGGACCTGGCGGCTCCAACCCCACGCATCAAGGCAGAGCTGACA
        ***  * *  ** ** ** ** ** ** * *** **  *  **** **********  * ** ** ** ** ******** ********

TLE1    TCCTCGGCCCCCGCCTGCTACGCCCTGGCCATCAGCCCCGATTCCAAGGTCTGCTTCTCATGCTGCAGCGACGGCAACATCGCTGTGTGGG
TLE2    TCCTCAGCCCCAGCCTGCTACGCCCTGGCCGTCAGCCCCGACGCCAAGGTTTGCTTCTCCTGCTGCAGCGATGGCAACATTGTGGTCTGGG
TLE3    TCCTCGGCTCCCGCCTGTTATGCCCTGGCCATTAGCCCTGACGCCAAAGTCTGCTTCTCCTGCTGCAGCGATGGGAACATTGCTGTCTGGG
TLE4    TCCTCGGCCCCCGCCTGCTATGCCCTGGCCATCAGCCCCGATTCCAAGGTCTGCTTCTCATGCTGCAGCGACGGCAACATCGCTGTGTGGG
        ***** ** ** ***** ** ********* * ***** **  **** ** ******** ********** ** ***** *  ** ****

TLE1    ATCTGCACAACCAGACACTAGTGAGGCAATTCCAGGGCCACACAGACGGAGCCAGCTGTATTGACATTTCTAATGATGGCACCAAGCTCTG
TLE2    ACCTGCAGAATCAGACTATGGTCAGGCAGTTCCAGGGCCACACGGACGGCGCCAGCTGCATTGATATTTCCGATTACGGCACTCGGCTCTG
TLE3    ACCTGCACAACCAGACCCTGGTCAGGCAGTTCCAGGGCCACACAGATGGGGCCAGCTGCATAGACATCTCCCATGATGGCACCAAACTGTG
TLE4    ATCTGCACAACCAGACCTTGGTGAGGCAATTCCAGGGCCACACAGATGGAGCCAGCTGTATTGACATTTCTAATGATGGCACCAAGCTCTG
        * ***** ** *****  * ** ***** ************** ** ** ******** ** ** ** **  ** * *****    ** **
```

```
TLE1    GACGGGTGGTTTGGACAACACAGTCAGGTCCTGGGACCTGCGCGAGGGGCGGCAGCTGCAGCAGCACGACTTCACCTCCCAGATCTTCTCC
TLE2    GACAGGGGGCCTGGACAACACGGTGCGCTGCTGGGACCTGCGGGAGGGCCGCCAGCTGCAGCAGCATGACTTCAGCTCCCAGATTTTCTCC
TLE3    GACAGGGGGCCTGGACAACACGGTGCGCTCCTGGGACCTGCGGGAGGGCCGACAGCTACAGCAGCATGACTTCACTTCCCAGATCTTCTCG
TLE4    GACAGGTGGTTTGGACAACACGGTCAGGTCCTGGGACCTGCGGGAGGGGCGGCAGCTGCAGCAGCACGACTTCACCTCCCAGATCTTTTCT
        *** ** **  ********** **  * * ************ ***** ** ***** ******** *******  ******** ** **

TLE1    CTGGGGTACTGCCCCACCGGGGAGTGGCTGGCAGTGGGCATGGAGAGCAGCAATGTGGAGGTGCTGCACGTGAACAAGCCTGACAAGTACC
TLE2    CCCTGCCACTGCCCTAACCAGGACTGGCTGGCGGTCGGAATGGAGAGTAGCAACGTGGAGATCCTGCACGTCGGCAAGCCGGAGAAATACC
TLE3    CTGGGCTACTGCCCCACTGGGGAGTGGCTGGCTGTGGGCATGGAGAGCAGCAACGTGGAGGTGCTGCACCACACCAAGCCTCACAAGTACC
TLE4    CTGGGCTACTGCCCAACTGGAGAGTGGCTTGCAGTGGGGATGGAGAACAGCAATGTGGAAGTTTTGCATGTCACCAAGCCAGACAAATACC
        *   *  ******* *     ** ***** ** ** ** *******  ***** *****  *  ****      ******  * ** ****

TLE1    AGCTGCACCTGCATGAGAGCTGCGTGCTGTCCCTGAAATTTGCTTACTGTGGTAAATGGTTTGTGAGTACTGGAAAAGATAACCTCCTCAA
TLE2    AGCTGCACCTCCACGAGAGCTGCGTGCTGTCCCTGAAGTTTGCCCCTTGCGGACGGTGGTTTGTGAGCACCGGGAAGGACAACCTGCTCAA
TLE3    AGCTGCACCTGCACGAGAGCTGCGTGCTCTCCCTCAAGTTCGCCTACTGCGGCAAGTGGTTCGTGAGCACTGGGAAAGATAACCTTCTCAA
TLE4    AACTACATCTTCATGAGAGCTGTGTGCTGTCGCTCAAGTTTGCCCATTGTGGCAAATGGTTTGTAAGCACTGGAAAGGACAACCTTCTGAA
        * ** ** ** ** ******** ***** ** ** ** ** **    ** **    ***** ** ** ** ** ** ** ***** ** **

TLE1    TGCTTGGCGGACCCCCTATGGAGCCAGCATATTCCAGTCCAAAGAGTCCTCGTCAGTGCTTAGCTGTGACATCTCTGTGGATGATAAGTAC
TLE2    CGCCTGGAGGACGCCGTACGGGGCCAGCATTTTCCAGTCCAAGGAGTCGTCCTCAGTCCTGAGTTGTGACATCTCCAGAAATAACAAATAC
TLE3    CGCCTGGAGGACGCCTTATGGAGCCAGCATATCCCAGTCTAAAGAATCCTCGTCTGTCTTGAGTTGTGACATTTCAGCGGATGACAAATAC
TLE4    TGCCTGGAGAACGCCTTACGGGGCCAGTATATTCCAGTCCAAAGAATCCTCATCGGTGCTTAGCTGTGACATCTCCGTGGACGACAAATAC
         ** *** * ** ** ** ** ***** ** * ****** ** ** ** ** ** **  * ** ******** **     *  * ** ***

TLE1    ATAGTCACT[GGCTCGGGGGACAAGAAGGCTAC]AGTCTATGAAGTCATCTACTGAAAACATTATGTGGT----------------
TLE2    ATTGTGACA[GGCTCGGGGGACAAGAAGGCCAC]CGTGTATGAGGTGGTCTACTGAAGACATGACCCCCC----------------
TLE3    ATTGTAACA[GGCTCTGGTGACAAGAAGGCCAC]AGTTTATGAGGTCATCTACTAA--ACAAGAACTCCAGCAGG------------
TLE4    ATTGTCACT[GGCTCTGGGGATAAGAAGGCCAC]AGTTTATGAAGTTATTTATTAAAGACAAATCTTCATGCAGACTGGACTTCTCC
        ** ** ** ***** ** ** ******** ** ** ***** **  * ** * *  ***
```

Figure 7.3: CLUSTAL X (1.64b) multiple sequence alignment of human Dishevelled homologues. Primers are shown in grey. Forward primer is Dsh810, reverse primer is Dsh1370. "*" indicates conserved sequence.

```
Dvl-1  AAACGCCGGCGGAGGAAGCAGCGCCTTCGGCAGGCGGACCGGGCCTCCTCCTTCAGCAGCATAACCGACTCCACCATGTCCCTCAACATCG
Dvl-2  ---CGGCGGCGAAGGAAGCAGAGGCCGCCCCGCCTGGAGAGGACGTCATCCTTCAGCAGCGTCACAGATTCCACAATGTCTCTCAATATCA
Dvl-3  AAGCGGCGGCGGCGGAAGCAGAAGGTTTCTCGGATTGAGCGGTCCTCGTCCTTCAGCAGCATCACGGACTCCACCATGTCACTCAACATCA
          ** *****  ********         *     **  ** * ** ************ * ** ** ***** ***** ***** ***

Dvl-1  TCACTGTCACGCTCAACATGGAAAGACATCACTTTCTGGGCATCAGCATCGTGGGGCAGAGCAACGACCGTGGAGACGGCGGCATCTACAT
Dvl-2  TCACAGTCACGCTAAACATGGAGAAGTACAACTTCCTGGGTATCTCCATTGTTGGCCAAAGCAATGAGCGGGGAGACGGAGGCATCTACAT
Dvl-3  TCACGGTCACTCTCAACATGGAAAAATATAACTTCTTGGGCATCTCCATTGTGGGCCAAAGCAACGAGCGTGGTGACGGCGGCATCTACAT
       **** ***** ** ******** *   *  ****  **** ***  *** ** ** ** ***** ** ** ** ***** ***********

Dvl-1  TGGCTCCATCATGAAGGGCGGGGCTGTGGCCGCTGACGGCCGCATCGAGCCCGGCGACATGTTGCTGCAGGTGAATGACGTGAACTTTGAG
Dvl-2  TGGCTCCATCATGAAGGGTGGGGCTGTGGCGGCCGACGGGCGCATTGAGCCAGGGGACATGCTTTTGCAGGTGAATGACATGAACTTTGAG
Dvl-3  TGGCTCTATCATGAAGGGTGGGGCCGTGGCTGCTGATGGACGCATCGAGCCAGGAGATATGTTGTTACAGGTAAACGAGATCAACTTTGAG
       ****** ********** ***** ***** ** ** ** ***** ***** ** ** *** *  * ***** ** **  * *********

Dvl-1  AACATGAGCAATGACGATGCCGTGCGGGTGCTGCGGGAGATCGTTTCCCAGACGGGGCCCATCAGCCTCACTGTGGCCAAGTGCTGGGACC
Dvl-2  AACATGAGCAACGATGACGCTGTGCGGGTGCTGAGGGACATTGTGCACAAGCCTGGCCCCATTGTGCTGACTGTGGCCAAGTGCTGGGATC
Dvl-3  AACATGAGTAATGACGATGCAGTCCGGGTACTGCGGGAGATTGTGCACAAACCGGGGCCCATCACCCTGACTGTAGCCAAGTGCTGGGACC
       ******** ** ** ** ** ** ***** *** **** ** **   * *  * ** *****    ** ***** ************** *

Dvl-1  CAACGCCCCGAAGCTACTTCACCGTCCCACGGGCTGACCCGGTGCGGCCCATCGACCCCGCCGCCTGGCTGTCCCACACGGCGGCACTGAC
Dvl-2  CCTCTCCTCAGGCCTATTTCACTCTCCCCCGAAATGAGCCCATCCAGCCAATTGACCCTGCTGCCTGGGTGTCCCATTCCGCGGCTCTGAC
Dvl-3  CAAGTCCACGTGGTTGCTTCACATTGCCCAGGAGCGAGCCCATCCGGCCCATTGACCCTGCGGCCTGGGTCTCCCACACTGCAGCCATGAC
       *    ** *     *  *****  * **  *    ** **  * * *** ** ***** ** ****** * *****  * ** **  ****

Dvl-1  AGGAGCCCTGCC----------------C--CGCTACGAGC-----------------------------------------------------
Dvl-2  TGGCACCTTCCCAGCCTATC-CAGGTTCCTCCTCCATGAGCACCATTACATC----------TGGATCG-----TCTTTGCCT------GA
Dvl-3  CGGCACCTTCCCTGCATACGGCATGAGCC-CCTCCCTGAGCACCATCACCTCCACCAGCTCCTCCATCACCAGTTCCATCCCTGACACAGA
        **  ** * **                *  * *   ****

Dvl-1  -----TGGAAGAGGCGCCGCTGACGGTGAAGAGTGACATGAGCGCCGTCGTCCGGGTCATGCAGCTGCCAGACTCGGGACTGGAGATCCGC
Dvl-2  TGGCTGTGAAGGCCGGGGTCTCTCCGTCCATACGGACATGGCATCGGTGACCAAGGCCATGGCAGCTCCAGAGTCTGGACTGGAAGTCCGG
Dvl-3  GCGCCTAGACGACTTCCACTTGTCCATCCACAGTGACATGGCTGCCATCGTAAAAGCCATGGCCTCCCCTGAATCAGGGTTGGAGGTCCGT
              ** *         *  *  *  * *  ******    *  *       * ****      ** ** ** **  ****  ****

Dvl-1  GACCGCATGTGGCTCAAGATCACCATCGCCAATGCCGTCATCGGGGCGGACGTGGTGGACTGGCTGTACACACACGTGGAGGGCTTCAAGG
Dvl-2  GACCGCATGTGGCTCAAGATCACCATCCCTAATGCCTTTCTGGGCTCGGATGTGGTTGACTGGCTCTACCATCACGTGGAGGGCTTTCCTG
Dvl-3  GACCGCATGTGGCTCAAGATTACCATCCCTAATGCTTTCATCGGCTCAGATGTGGTGGACTGGCTGTACCACAATGTGGAAGGCTTCACGG
       ******************** ****** * *****  *  * **  * ** ***** ******** ***    * ***** *****    *
```

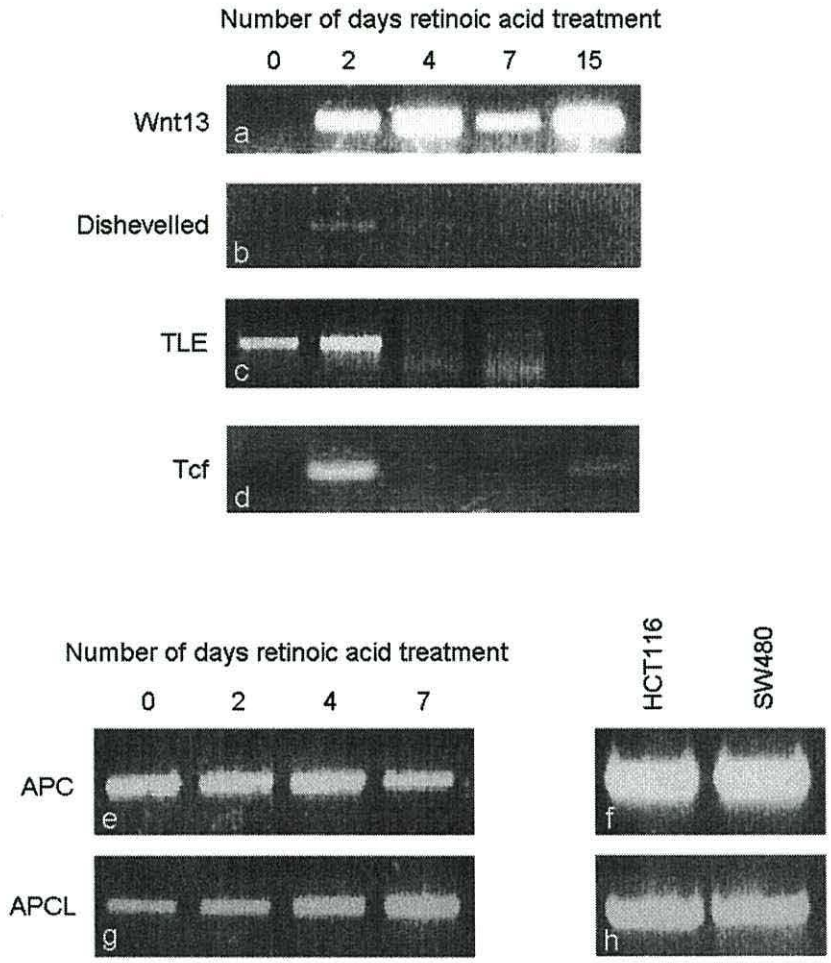


Figure 7.4
RT-PCR of Wnt pathway components during differentiation of NTERA-2.
Expression of Wnt signalling pathway components during retinoic acid induced differentiation of NTERA-2 was analysed by RT-PCR. Wnt-13 was expressed in all retinoic acid treated cells as previously described (Wakeman *et al.*, 1998) (a). Dishevelled expression appears to peak at 2 days and then decrease as retinoic acid treatment progresses (b). TLE is expressed more strongly in embryonal carcinoma cells and during the early stages of retinoic acid induction (c). Tcf was seen most strongly at 2 days retinoic acid treatment (d). APC was expressed in all cells, both retinoic acid induced and embryonal carcinoma NTERA-2 cells (e), and in two colorectal cancer cell lines, SW480 and HCT116 (f). APCL was also expressed in all cells analysed (g,h), there appeared to be some increase as retinoic acid treatment progressed (g). RT-PCR products from 2 days retinoic acid treatment was cloned and analysed.

Dishevelled expression peaked at 2 days retinoic acid treatment and decreased gradually to 15 days, no dishevelled expression was detected in embryonal carcinoma cells (figure 7.4b). This would be consistent with a role in stabilising β-catenin as Wnt-13 is expressed during retinoic acid induced differentiation of NTERA-2 (Wakeman *et al.*, 1998) which would be predicted to result in stabilisation of β-catenin. However, RT-PCR is not a truly quantitative technique so any changes in expression seen by RT-PCR would need to be confirmed by northern blot, western blot or quantitative PCR. Degenerate RT-PCR product from the 2 day time-point was cloned and sequenced. Of a total of 40 clones the majority were Dvl-2 and Dvl-3. No Dvl-1 was seen (table 7.2), RT-PCR with Dvl-1 specific primers also failed to give product at any time-point, however no positive control was available to confirm these primers were able to amplify Dvl-1. One clone gave a PCR product that appeared to be a combination of Dvl-2 and Dvl-3 sequences (see section 7.4). No novel homologues of Dishevelled were found.

The Groucho/TLE family of basic helix-loop-helix proteins are transcriptional co-repressors of Wnt target genes (Parkhurst, 1998; Cavallo *et al.*, 1998). Grouchos do not bind to DNA directly but instead bind to transcription factors, including TCF/Lefs. Expression of TLE was seen at 2 days retinoic acid treatment and in untreated embryonal carcinoma cells (figure 7.4c), a very small amount of RT-PCR product was also visible at 4 and 7 days retinoic acid treatment. The primers used here were TLE1820 and TLE2330 (figure 2), TLE 1420 and TLE1950 gave RT-PCR product at all time-points but cloning and sequening revealed that the majority of these products were not TLE homologues. RT-PCR product from 2 days retinoic acid treatment was cloned and sequenced. 80 clones were analysed, only TLE1 and TLE4 PCR products were found (table 7.2). Reduction in TLE expression with retinoic acid treatment would be consistent with de-repression of β-catenin signalling due to retinoic acid induced Wnt-13 expression. However, RT-PCR is not a truly quantitative technique so any changes in expression seen by RT-PCR would need to be confirmed by northern blot, western blot or quantitative PCR. No novel homologues of TLE were found.

Tcfs are part of the Tcf/Lef family of proteins. In the absence of a Wnt signal Tcf/Lefs act as transcriptional repressors. When a Wnt signal is present nuclear entry of β-catenin and its interaction with Tcf/Lefs convert Tcf/Lefs into transcriptional activators (reviewed in Bienz & Clevers, 2000). Tcf expression was seen most strongly at 2 days following the start of retinoic acid induced differentiation (figure 4d). RT-PCR products from 2 days retinoic acid treatment were cloned, 20 clones were analysed. Clones sequenced matched to Tcf1, Tcf3 and Tcf4 (table 7.2). Tcf expression was seen most strongly after 2 days retinoic acid treatment, however only one repeat of RT-PCR was carried out for Tcf and no western or northern blots were done to confirm this. Lef1 (another member of the Tcf/Lef family) expression was not analysed as these primers would not be expected to amplify Lef1. No novel homologues of Tcf were found.

Table7.2
RT-PCR analysis of Wnt pathway components during NTERA-2 differentiation. TLE, Tcf and Dishevelled expression was analysed during retinoic acid induced differentiation of NTERA-2. Table shows homologues used in generating an alignment for primer design, the number of clones analysed and the homologues expressed at 2 days following start of retinoic adic induction of differentiation.

	Alignment	**No. of clones analysed**	**Homologues expressed after 2 days retinoic acid treatment**
TLE	TLE1,2,3,4	80	TLE1, TLE4
Tcf	Tcf1,3,4 (human and mouse)	20	Tcf1, Tcf3, Tcf4
Dishevelled	Dvl1,2,3	40	Dvl-2, Dvl-3, translocation?

7.3 APC and APCL are expressed at all time-points during retinoic acid induced differentiation of NTERA-2

APCL (or APC2) was initially identified as a brain specific homologue of APC (Nakagawa *et al.*, 1998; van Es *et al.*, 1999). However, APCL has more recently been reported to be expressed in a variety of cell types (Jarrett *et al.*, 2001) so may not be brain specific. Expression or APC and APCL during retinoic acid induced differentiation of NTERA-2 was analysed by RT-PCR. APC and APCL

were seen to be expressed at all time-points and in untreated embryonal carcinoma cells (figure 7.4e,g). Two sets of primers were used to analyse APC and APCL expression: APC1 & APC2 and APC3 & APC4 for APC; APCL1 & APCL2 and APCL3 & APCL4 for APCL. APC1 & APC2 and APCL3 & APCL4 are shown in figure 7.4, the other sets of primers gave similar results (data not shown).

As APCL were brain specific it would not be expected to be expressed in undifferentiated embryonal carcinoma cells. There is an increase in RT-PCR product as cells differentiate, however RT-PCR is not quantitative so this change is not conclusive. It has been suggested that NTERA-2 cells are committed neuronal precursor cells (Pleasure & Lee, 1993). However they express characteristic features of human EC cells (Andrews *et al.*, 1996). NTERA-2 EC cells can also be induced to differentiate into non-neural cells by treatment with hexamethylene bisacetamide (HMBA) (Andrews *et al.*, 1990) and bone morphogenetic protein 7 (BMP7) (Andrews *et al.*, 1994). They have been observed to form mesenchyme rather than neurones in response to retinoic acid treatment when the gamma retinoic acid receptor is overexpressed (Moasser *et al.*, 1994 & 1995). Therefore they are clearly not restricted to neuronal differentiation. Expression of a brain specific protein in NTERA-2 embryonal carcinoma cells would support their being committed neuronal precursors. However, the possibility that APCL is not actually brain specific is more likely. APCL primers were designed across an intron which removes the possibility that any PCR product seen is due to genomic DNA contamination of the mRNA.

To test whether APCL is expressed in tissues other than brain RT-PCR was carried out on two colorectal cancer cell lines, SW480 and HCT116. Both these cell lines were seen to express both APC and APCL (figure 7.4f,h). This shows that APCL can be expressed in tissues not derived from the brain.

7.4 A potential translocation within Dvl-2/Dvl-3 has been identified by RT-PCR

Of the 40 Dishevelled clones analysed one, Dsh34, did not match any human Dishevelled homologue. Sequence analysis shows that this clone may reflect a translocation within Dvl-2/Dvl-3 in NTERA-2.

Dsh34 was aligned to the three known human Dishevelled homologues. Its sequence did not match Dvl-1 (figure 7.5), but did partially match Dvl-2 (figure 7.6) and Dvl-3 (figure 7.7). While it was initially thought that this may be a novel Dishevelled homologue, it seems more likely from the exact matches to Dvl-2 and Dvl-3 and the abrupt change from matching one to matching the other (figure 7.8) that this clone is due to a translocation.

A human genome BLAST search found two matches to Dsh34, these were on chromosomes 3 and 17 (figure 7.9). Dsh34 matches to three exons of Dvl-3 on chromosome 3 (figure 7.10) and to two exons of Dvl-2 on chromosome 17 (figure 7.11). There are 10 base pairs of sequence which match to both Dvl-2 and Dvl-3 in between the sequence that matches Dvl-2 and the sequence that matches Dvl-3 (figure 7.8). The change from sequence matching Dvl-3 to that matching Dvl-3 is very close to an intron/exon boundary, but due to 10 base pairs which match exactly to Dvl-2 and Dvl-3 it is not possible to determine where exactly the boundary is. It is possible that the translocation is actually within the intron, if so PCR of genomic DNA may reveal where exactly this translocation event has occurred. If this were a novel homologue of Dishevelled rather than a translocation event it would be expected that the sequence would be represented within the human genome database which it is not.

To eliminate the possibility that this clone was an RT-PCR artefact a second pair of primers were designed which would only amplify Dvl-2 and Dsh34 (Dsh1040) and another which would only amplify Dvl-3 and Dsh34 (Dsh1190) (figure 7.12). These primers did give an RT-PCR product of the expected size but due to the small size of this PCR product it was not possible to clone and sequence it. RT-PCR with Dsh810, a degenerate primer which would amplify any Dishevelled mRNA, and Dsh1190, a primer specific to Dsh34 and Dvl-2 gave a

Figure 7.5: CLUSTAL X (1.64b) multiple sequence alignment of clone 34 from Dishevelled RT-PCR screen and human Dvl-1. Primers are shown in bold print. This alignment shows that Dsh34 sequence does not match to Dvl-1.

```
Dsh34   ----------------------CTCAATATCGTCACTGTCACGCTAAACATGGAAAAATATAACTTCTTGGGCATCTCCATTGTGGGCCAAC
Dvl-1   ATAACCGACTCCACCATGTCCCTCAACATCGTCACTGTCACGCTCAACATGGAAAGACATCACTTTCTGGGCATCAGCATCGTGGGGCCAC
                         ***** ***************** ********** * ** ****  ********  *** ***** * **

Dsh34   TTCTTGGGCATCTCCATTGTGGGCCAAAGCAACGAGCGTGGTGACGGCGGCATCTACATTGGCTCTATCATGAAGGGTGAGGCCGTGGCTG
Dvl-1   TTTCTGGGCATCAGCATCGTGGGGCAGAGCAACGACCGTGGAGACGGCGGCATCTACATTGGCTCCATCATGAAGGGCGGGGCTGTGGCCG
        **  ********  *** ***** ** ******** ***** ********************** *********** * *** ***** *

Dsh34   CTGATGGACGCATCGAGCCAGGAGATATGTTGTTACAGGTAAACGAGATCAACTTTGAGAACATGAGTAATGACGATGCAGTCCGGGTACT
Dvl-1   CTGACGGCCGCATCGAGCCCGGCGACATGTTGCTGCAGGTGAATGACGTGAACTTTGAGAACATGAGCAATGACGATGCCGTGCGGGTGCT
        **** ** *********** ** ** ****** * ***** ** **  * ***************** *********** ** ***** **

Dsh34   GCGGGAGATTGTGCACAAACCGGGGCCCATCACCCTGACTGTAGCCAAGTGCTGGGACCCAAGTCCACGTGGTTGCTTCACATTGCCCAGG
Dvl-1   GCGGGAGATCGTTTCCCAGACGGGGCCCATCAGCCTCACTGTGGCCAAGTGCTGGGACCCAACGCCCCGAAGCTACTTCACCGTCCCACGG
        ********* **   * *  *********** *** ***** ******************  ** **  * * ******  * **  **

Dsh34   AGCGAGCCCATCCAGCCAATTGACCCTGCTGCCTGGGTGTCCCATTCCGCGGCTCTGACTGGCACCTTCCCAGCCTA-----TCCAGGTTC
Dvl-1   GCTGACCCGGTGCGGCCCATCGACCCCGCCGCCTGGCTGTCCCACACGGCGGCACTGACAGGAGCCCTGCCCCGCTACGAGCTGGAAGAGG
           ** **  * * *** ** ***** ** ****** *******  * ***** ***** **  ** * **   ***     * * *

Dsh34   CTCCTCCAT--GAGCACCATTACATCTGGATCGTCTTT-GCCTGATGGCTGTGA-AGGCCGGGGTCTCTCCGTCCATACGGACATG-GCAT
Dvl-1   CGCCGCTGACGGTGAAGAGTGACATGAGCGCCGTCGTCCGGGTCATGCAGCTGCCAGACTCGGGACTGGAGATCCGCGACCGCATGTGGCT
        * ** *     * * *   * ****  *   **** *  *  * ***    **  ** *  *** **     ***       **** *  *

Dsh34   C--GGTGACCAAGGCCATGGCAGCTCCAGAGTC--------TGGACTGGAAGTCCGCGACCGCATGTGGCTCAAGATTA---
Dvl-1   CAAGATCACCATCGCCAATGCCGTCATCGGGGCGGACGTGGTGGACTGGCTGTACACACACGTGGAGGGCTTCA-AGGAGCG
        *  * * ****  ****  ** *     * * *        ********  ** * *   **     ****  * *  *
```

Figure 7.6: CLUSTAL X (1.64b) multiple sequence alignment of clone 34 from Dishevelled RT-PCR screen and human Dvl-2. Primers are shown in bold print. This alignment shows that Dsh34 sequence does not completely match to Dvl-2, but does match to one part of Dvl-2 sequence exactly.

```
Dsh34   ----------CTCAATATCGTCACTGTCACGCTAAACATGGAAAAATATAACTTCTTGGGCATCTCCATTGTGGGCCAAAGCAACGAGCG
Dvl-2   TCCACAATGTCTCTCAATATCATCACAGTCACGCTAAACATGGAGAAGTACAACTTCCTGGGTATCTCCATTGTTGGCCAAAGCAATGAGCG
                 ********* **** ***************** ** ** ****** **** *********** *********** *****

Dsh34   TGGTGACGGCGGCATCTACATTGGCTCTATCATGAAGGGTGAGGCCGTGGCTGCTGATGGACGCATCGAGCCAGGAGATATGTTGTTACAGG
Dvl-2   GGGAGACGGAGGCATCTACATTGGCTCCATCATGAAGGGTGGGGCTGTGGCGGCCGACGGGCGCATTGAGCCAGGGGACATGCTTTTGCAGG
         ** ***** ******************************** *** ***** ** ** ** ***** ******** ** *** * ** ****

Dsh34   TAAACGAGATCAACTTTGAGAACATGAGTAATGACGATGCAGTCCGGGTACTGCGGGAGATTGTGCACAAACCGGGGCCCATCACCCTGACT
Dvl-2   TGAATGACATGAACTTTGAGAACATGAGCAACGATGACGCTGTGCGGGTGCTGAGGGACATTGTGCACAAGCCTGGCCCCATTGTGCTGACT
        * ** ** ** ***************** ** ** ** ** ** ***** *** **** *********** ** ** *****    ******

Dsh34   GTAGCCAAGTGCTGGGACCCAAGTCCACGTGGTTGCTTCACATTGCCCAGGAGCGAGCCCATCCAGCCAATTGACCCTGCTGCCTGGGTGTC
Dvl-2   GTGGCCAAGTGCTGGGATCCCTCTCCTCAGGCCTATTTCACTCTCCCCCGAAATGAGCCCATCCAGCCAATTGACCCTGCTGCCTGGGTGTC
        ** ************** **   *** *  *  *  ***** * *** * *  ************************************

Dsh34   CCATTCCGCGGCTCTGACTGGCACCTTCCCAGCCTATCCAGGTTCCTCCTCCATGAGCACCATTACATCTGGATCGTCTTTGCCTGATGGCT
Dvl-2   CCATTCCGCGGCTCTGACTGGCACCTTCCCAGCCTATCCAGGTTCCTCCTCCATGAGCACCATTACATCTGGATCGTCTTTGCCTGATGGCT
        ********************************************************************************************

Dsh34   GTGAAGGCCGGGGTCTCTCCGTCCATACGGACATGGCATCGGTGACCAAGGCCATGGCAGCTCCAGAGTCTGGACTGGAAGTCCGCGACCGC
Dvl-2   GTGAAGGCCGGGGTCTCTCCGTCCATACGGACATGGCATCGGTGACCAAGGCCATGGCAGCTCCAGAGTCTGGACTGGAAGTCCGGGACCGC
        ********************************************************************************** ******

Dsh34   ATGTGGCTCAAGATTA--------------
Dvl-2   ATGTGGCTCAAGATCACCATCCCTAATGCC
        **************
```

Figure 7.7: CLUSTAL X (1.64b) multiple sequence alignment of clone 34 from Dishevelled RT-PCR screen and human Dvl-3. Primers are shown in red. This alignment shows that Dsh34 sequence does not completely match to Dvl-3, but does match to one part of Dvl-3 sequence exactly.

```
Dsh34   ----------CTCAATATCGTCACTGTCACGCTAAACATGGAAAAATATAACTTCTTGGGCATCTCCATTGTGGGCCAAAGCAACGAGCG
Dvl-3   TCCACCATGTCACTCAACATCATCACGGTCACTCTCAACATGGAAAAATATAACTTCTTGGGCATCTCCATTGTGGGCCAAAGCAACGAGCG
                 ***** *** **** ***** ** ************************************************************

Dsh34   TGGTGACGGCGGCATCTACATTGGCTCTATCATGAAGGGTGAGGCCGTGGCTGCTGATGGACGCATCGAGCCAGGAGATATGTTGTTACAGG
Dvl-3   TGGTGACGGCGGCATCTACATTGGCTCTATCATGAAGGGTGGGGCCGTGGCTGCTGATGGACGCATCGAGCCAGGAGATATGTTGTTACAGG
        **************************************** ************************************************

Dsh34   TAAACGAGATCAACTTTGAGAACATGAGTAATGACGATGCAGTCCGGGTACTGCGGGAGATTGTGCACAAACCGGGGCCCATCACCCTGACT
Dvl-3   TAAACGAGATCAACTTTGAGAACATGAGTAATGACGATGCAGTCCGGGTACTGCGGGAGATTGTGCACAAACCGGGGCCCATCACCCTGACT
        ********************************************************************************************

Dsh34   GTAGCCAAGTGCTGGGACCCAAGTCCACGTGGTTGCTTCACATTGCCCAGGAGCGAGCCCATCCAGCCAATTGACCCTGCTGCCTGGGTGTC
Dvl-3   GTAGCCAAGTGCTGGGACCCAAGTCCACGTGGTTGCTTCACATTGCCCAGGAGCGAGCCCATCCGGCCCATTGACCCTGCGGCCTGGGTCTC
        ************************************************************ *** *********** ******** **

Dsh34   CCATTCCGCGGCTCTGACTGGCACCTTCCCAGCCTATC-CAGGTTCCTCCTCCATGAGCACCATTACATC----------TGGATCG-----
Dvl-3   CCACACTGCAGCCATGACCGGCACCTTCCCTGCATACGGCATGAGCC-CCTCCCTGAGCACCATCACCTCCACCAGCTCCTCCATCACCAGT
        ***  * ** **  **** *********** ** **   ** *  ** ***** ********** ** **          *  ***

Dsh34   TCTTTGCCTGATG------GCTGTGAAGGCCGGGGTCTCTCCGTCCATACGGACATGGCATCGGTGACCAAGGCCATGGCAGCTCCAGAGTC
Dvl-3   TCCATCCCTGACACAGAGCGCCTAGACGACTTCCACTTGTCCATCCACAGTGACATGGCTGCCATCGTAAAAGCCATGGCCTCCCCTGAATC
        **  * *****        **   ** * *       * *** **** *  ********  *  *    ** ********  * ** ** **

Dsh34   TGGACTGGAAGTCCGCGACCGCATGTGGCTCAAGATTA-------------
Dvl-3   AGGGTTGGAGGTCCGTGACCGCATGTGGCTCAAGATTACCATCCCTAATGC
         **  **** ***** **********************
```

Figure 7.8: CLUSTAL X (1.64b) multiple sequence alignment of Dishevelled clone 34 and human Dishevelled homologues Dvl-2 and Dvl-3. Sequence in red matches Dvl-2, sequence in blue matches Dvl-3, sequence in black matches both and sequence in grey matches neither. "*" indicates sequence which matches both Dvl-2 and Dvl-3, the number underneath the sequence indicates which Dishevelled homologue the sequence matches where it does not match both. This shows that the early part of the sequence matches almost exactly to Dvl-3 while the most 3' end of the sequence matches Dvl-2 exactly, there are 10bp in between these two regions that match both Dvl-2 and Dvl-3.

```
Dsh34   ------------------TCTCAATATC TCAC GTCACGCTAAACATGGAAAAATATAACTTCTTGGGCATCTCCATTGTGGGCCAAAGCA
Dvl-2                     TCTCAATATC TCAC GTCACGCTAAACATGGAGAAGTACAACTTCCTGGGTATCTCCATTGTTGGCCAAAGCA
Dvl-3                     ACTCAACATC TCAC GTCACTCTCAACATGGAAAAATATAACTTCTTGGGCATCTCCATTGTGGGCCAAAGCA
                           *****2*** **** *****2**2********3**3**3******3****3**********3**********

Dsh34   ACGAGCGTGGTGACGGCGGCATCTACATTGGCTCTATCATGAAGGGTG GGCCGTGGCTGCTGATGGACGCATCGAGCCAGGAGATATGTTG
Dvl-2   ATGAGCGGGGAGACGGAGGCATCTACATTGGCTCCATCATGAAGGGTG GGCTGTGGCGGCCGACGGGCGCATTGAGCCAGGGGACATGCTT
Dvl-3   ACGAGCGTGGTGACGGCGGCATCTACATTGGCTCTATCATGAAGGGTG GGCCGTGGCTGCTGATGGACGCATCGAGCCAGGAGATATGTTG
        *3*****3**3*****3*****************3************ ***3*****3**3**3**3*****3********3**3***3*3

Dsh34   TTACAGGTAAACGAGATCAACTTTGAGAACATGAGTAATGACGATGCAGTCCGGGTACTGCGGGAGATTGTGCACAAACCGGGGCCCATCAC
Dvl-2   TTGCAGGTGAATGACATGAACTTTGAGAACATGAGCAACGATGACGCTGTGCGGGTGCTGAGGGACATTGTGCACAAGCCTGGCCCCATTGT
Dvl-3   TTACAGGTAAACGAGATCAACTTTGAGAACATGAGTAATGACGATGCAGTCCGGGTACTGCGGGAGATTGTGCACAAACCGGGGCCCATCAC
        **3*****3**3**3**3*****************3**3**3**3**3**3*****3***3****3***********3**3**3*****333

Dsh34   CCTGACTGTAGCCAAGTGCTGGGACCCAAGTCCACGTGGTTGCTTCACATTGCCCAGGAGCGAGCCCATCCAGCCAATTGACCCTGCTGCCT
Dvl-2   GCTGACTGTGGCCAAGTGCTGGGATCCCTCTCCTCAGGCCTATTTCACTCTCCCCCGAAATGAGCCCATCCAGCCAATTGACCCTGCTGCCT
Dvl-3   CCTGACTGTAGCCAAGTGCTGGGACCCAAGTCCACGTGGTTGCTTCACATTGCCCAGGAGCGAGCCCATCCGGCCCATTGACCCTGCGGCCT
        3********3**************3**333***3*33333*33*****33*3***3*3*33**********2***2**********2****

Dsh34   GGGTGTCCCATTCCGCGGCTCTGACTGGCACCTTCCCAGCCTATC-CAGGTTCCTCCTCCATGAGCACCATTACATC----------TGGAT
Dvl-2   GGGTGTCCCATTCCGCGGCTCTGACTGGCACCTTCCCAGCCTATC-CAGGTTCCTCCTCCATGAGCACCATTACATC----------TGGAT
Dvl-3   GGGTCTCCCACACTGCAGCCATGACCGGCACCTTCCCTGCATACGGCATGAGCC-CCTCCCTGAGCACCATCACCTCCACCAGCTCCTCCAT
        ****2*****22*2**2**22****2**********2**2**222**2*22**2*****2**********2**2**2222222222*22**

Dsh34   CG-----TCTTTGCCTGATG------GCTGTGAAGGCCGGGGTCTCTCCGTCCATACGGACATGGCATCGGTGACCAAGGCCATGGCAGCTC
Dvl-2   CG-----TCTTTGCCTGATG------GCTGTGAAGGCCGGGGTCTCTCCGTCCATACGGACATGGCATCGGTGACCAAGGCCATGGCAGCTC
Dvl-3   CACCAGTTCCATCCCTGACACAGAGCGCCTAGACGACTTCCACTTGTCCATCCACAGTGACATGGCTGCCATCGTAAAAGCCATGGCCTCCC
        *222222**22*2*****22222222**222*22*2*2222222*2***2****2*22********22*22*2222**2********22*2*
```

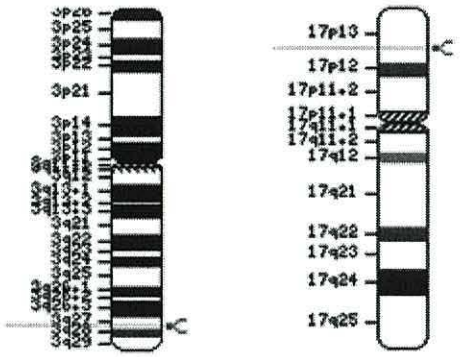


Figure 7.9
Dishevelled clone 34 human genome BLAST search gives two hits, one on chromosome 3 (3q27) which corresponds to Dvl-3, and one on chromosome 17 (17p13) which corresponds to Dvl-2.

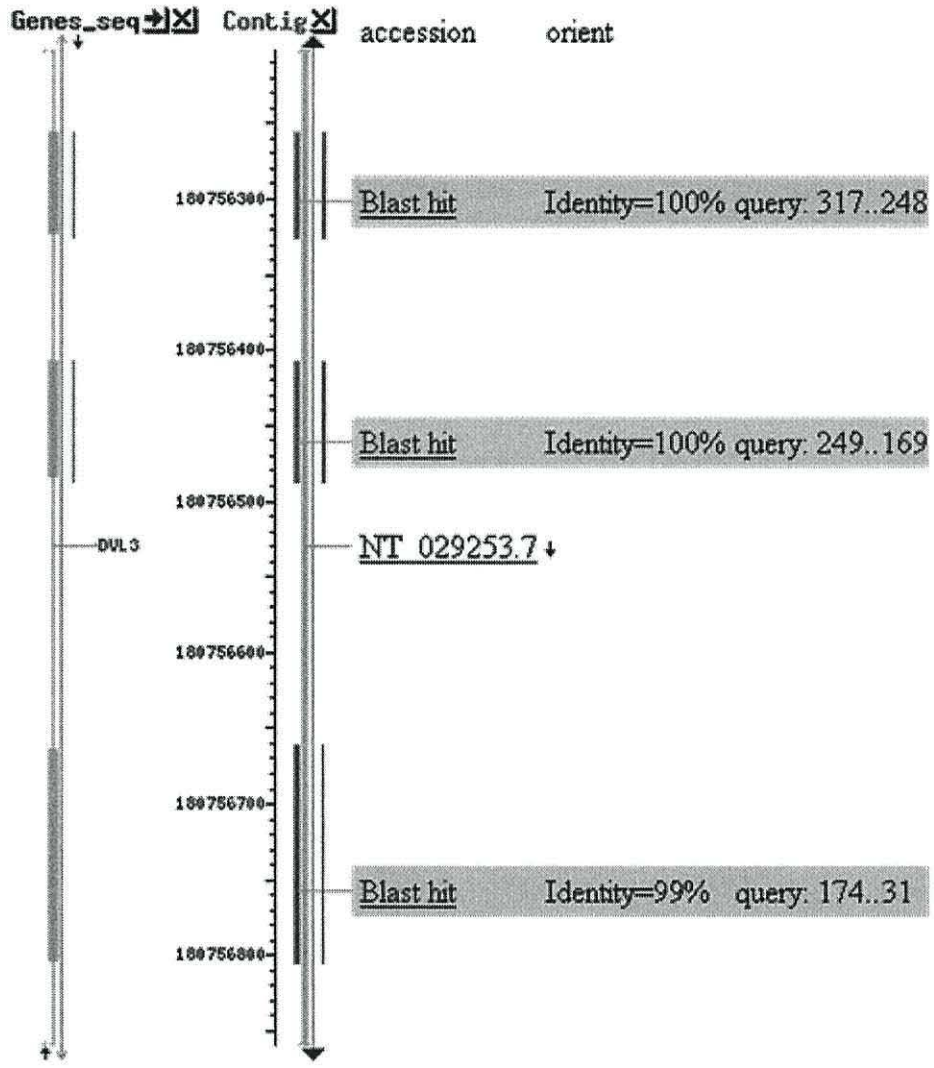


Figure 7.10
Dsh34 matches to three exons of human Dvl-3 sequence. This sequence is from chromosome 3, accession number NT_029256.

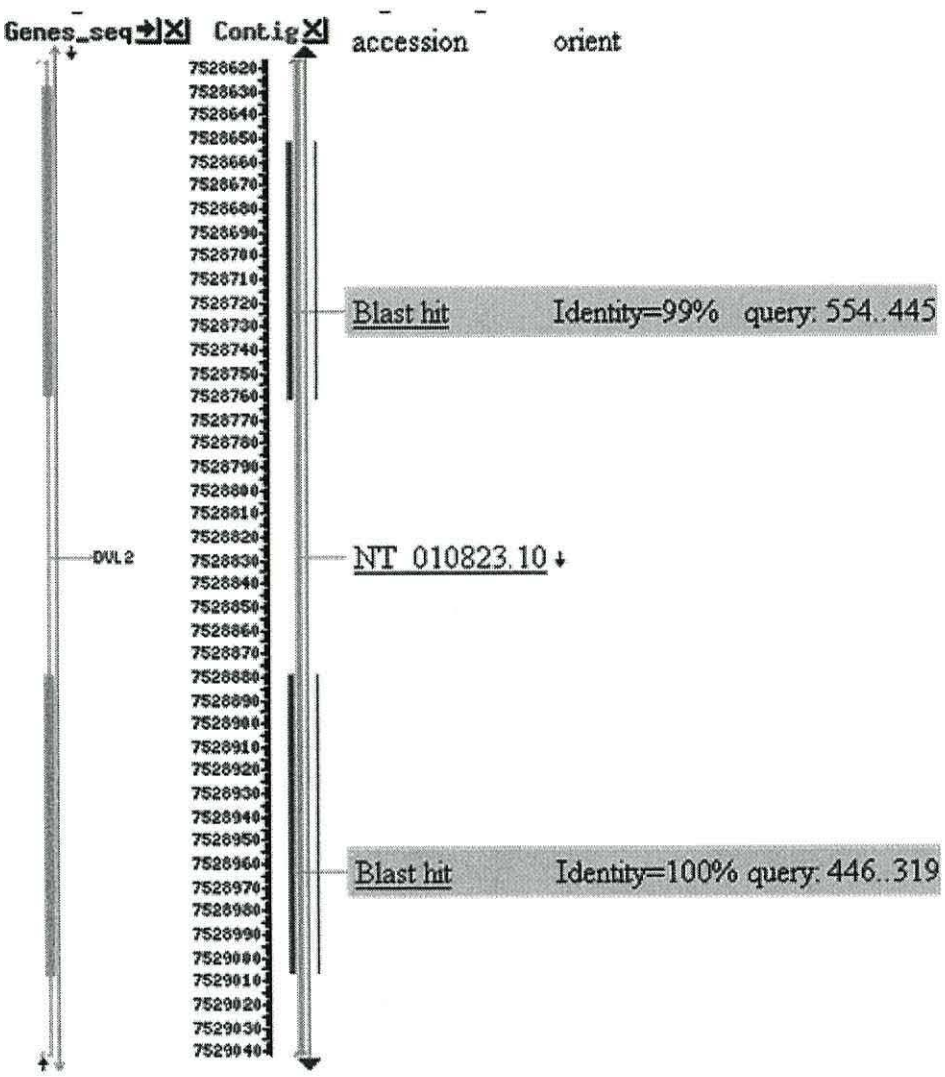


Figure 7.11
Dsh34 matches to two exons of human Dvl-2 sequence. This sequence is from chromosome 17, accession number NT_010823.

Figure 7.12: CLUSTAL X (1.64b) multiple sequence alignment of Dsh34 and the three human Dishevelled homologues: Dvl-1, Dvl-2 and Dvl-3. The original primers are shown in red (Dsh810 and Dsh1370). Two additional primers were designed (shown in blue) Dsh1040, which matches only Dvl-3 and Dsh34, and Dsh1190, which matches only Dvl-2 and Dsh34.

```
Dsh34   ----------------------------CTCAATATCGTCACTGTCACGCTAAACATGGAAAAATATAACTTCTTGGGCATCTCCATTGT
Dvl-1   TCAGCAGCATAACCGACTCCACCATGTCCCTCAACATCGTCACTGTCACGCTCAACATGGAAAGACATCACTTTCTGGGCATCAGCATCGT
Dvl-2   TCAGCAGCGTCACAGATTCCACAATGTCTCTCAATATCATCACAGTCACGCTAAACATGGAGAAGTACAACTTCCTGGGTATCTCCATTGT
Dvl-3   TCAGCAGCATCACGGACTCCACCATGTCACTCAACATCATCACGGTCACTCTCAACATGGAAAAATATAACTTCTTGGGCATCTCCATTGT
                                    ***** *** **** ***** ** ******** *   *  ****  **** ***  *** **

Dsh34   GGGCCAAAGCAACGAGCGTGGTGACGGCGGCATCTACATTGGCTCTATCATGAAGGGTGAGGCCGTGGCTGCTGATGGACGCATCGAGCCC
Dvl-1   GGGGCAGAGCAACGACCGTGGAGACGGCGGCATCTACATTGGCTCCATCATGAAGGGCGGGGCTGTGGCCGCTGACGGCCGCATCGAGCCC
Dvl-2   TGGCCAAAGCAATGAGCGGGGAGACGGAGGCATCTACATTGGCTCCATCATGAAGGGTGGGGCTGTGGCGGCCGACGGGCGCATTGAGCCC
Dvl-3   GGGCCAAAGCAACGAGCGTGGTGACGGCGGCATCTACATTGGCTCTATCATGAAGGGTGGGGCCGTGGCTGCTGATGGACGCATCGAGCCC
         ** ** ***** ** ** ** ***** ***************** *********** * *** ***** ** ** ** ***** ******

Dsh34   AGGAGATATGTTGTTACAGGTAAACGAGATCAACTTTGAGAACATGAGTAATGACGATGCAGTCCGGGTACTGCGGGAGATTGTGCACAAA
Dvl-1   CGGCGACATGTTGCTGCAGGTGAATGACGTGAACTTTGAGAACATGAGCAATGACGATGCCGTGCGGGTGCTGCGGGAGATCGTTTCCCAG
Dvl-2   AGGGGACATGCTTTTGCAGGTGAATGACATGAACTTTGAGAACATGAGCAACGATGACGCTGTGCGGGTGCTGAGGGACATTGTGCACAAG
Dvl-3   AGGAGATATGTTGTTACAGGTAAACGAGATCAACTTTGAGAACATGAGTAATGACGATGCAGTCCGGGTACTGCGGGAGATTGTGCACAAA
         ** ** *** *  * ***** ** **  * ***************** ** ** ** ** ** ***** *** **** ** **   * *

Dsh34   CCGGGGCCCATCACCCTGACTGTAGCCAAGTGCTGGGACCCAAGTCCACGTGGTTGCTTCACATTGCCCAGGAGCGAGCCCATCCAGCCAA
Dvl-1   ACGGGGCCCATCAGCCTCACTGTGGCCAAGTGCTGGGACCCAACGCCCCGAAGCTACTTCACCGTCCCACGGGCTGACCCGGTGCGGCCCA
Dvl-2   CCTGGCCCCATTGTGCTGACTGTGGCCAAGTGCTGGGATCCCTCTCCTCAGGCCTATTTCACTCTCCCCCGAAATGAGCCCATCCAGCCAA
Dvl-3   CCGGGGCCCATCACCCTGACTGTAGCCAAGTGCTGGGACCCAAGTCCACGTGGTTGCTTCACATTGCCCAGGAGCGAGCCCATCCGGCCCA
         * ** *****    ** ***** ************** **    ** *     *  *****  * **  *    ** **  * * *** *

Dsh34   TTGACCCTGCTGCCTGGGTGTCCCATTCCGCGGCTCTGACTGGCACCTTCCCAGCCTATC-CAGGTTCCTCCTCCATGAGCACCATTACAT
Dvl-1   TCGACCCCGCCGCCTGGCTGTCCCACACGGCGGCACTGACAGGAGCCCTGCCCCGCTACG--AG-------CTGGAAGAG-----GCGC--
Dvl-2   TTGACCCTGCTGCCTGGGTGTCCCATTCCGCGGCTCTGACTGGCACCTTCCCAGCCTATC-CAGGTTCCTCCTCCATGAGCACCATTACAT
Dvl-3   TTGACCCTGCGGCCTGGGTCTCCCACACTGCAGCCATGACCGGCACCTTCCCTGCATACGGCATGAGCC-CCTCCCTGAGCACCATCACCT
        * ***** ** ****** * *****  * ** **  **** **  ** * **    **    *        **    ***        *
```


Dsh34 C----------TGGATCG-----TCTTTGCCTGATG------GCTGTGAAGGCCGGGGTCTCTCCGTCCATACGGACATGGCATCGGTGAC
Dvl-1 ----------CG----------CTGACG---------GTGAAG--------------------AGTGACATGAGCGCCGTCGT
Dvl-2 C----------TGGATCG-----TCTTTGCCTGATG------GCTGTGAAGGCCGGGGTCTCTCCGTCCATACGGACATGGCATCGGTGAC
Dvl-3 CCACCAGCTCCTCCATCACCAGTTCCATCCCTGACACAGAGCGCCTAGACGACTTCCACTTGTCCATCCACAGTGACATGGCTGCCATCGT
Dsh34 CAAGGCCATGGCAGCT-CCAGAGTCTGGACTGGAAGTCCGCGACCGCATGTGGCTCAAGATTA----------
Dvl-1 CCGGGTCATG-CAGCTGCCAGACTCGGGACTGGAGATCCGCGACCGCATGTGGCTCAAGATCACCATCGCCAATGCCGTCATCGGGGCGGAC
Dvl-2 CAAGGCCATGGCAGCT-CCAGAGTCTGGACTGGAAGTCCGGGACCGCATGTGGCTCAAGATCACCATCCCTAATGCCTTTCTGGGCTCGGAT
Dvl-3 AAAAGCCATGGCCTCC-CCTGAATCAGGGTTGGAGGTCCGTGACCGCATGTGGCTCAAGATTACCATCCCTAATGCTTTCATCGGCTCAGAT

product of the expected size. This product was cloned and clones analysed by restriction digest and sequencing. Of the ten clones analysed two were Dsh34 and eight were Dvl-2 (data not shown). With the Dsh34 product appearing at this frequency it would be unlikely to be a PCR artefact, and it would be very unusual to see an RT-PCR artefact that combines sequence from two separate mRNAs.

One possibility is that Dsh34 has arisen due to a translocation event. Both Dvl-2 and Dvl-3 are seen expressed in NTERA-2 cells, both of these mRNAs are intact in the region where Dsh34 switches from matching one to matching the other. This means there is at least one intact allele of each Dishevelled, Dvl-2 and Dvl-3, in NTERA-2. One allele of either Dvl-2 or Dvl-3 could have undergone a translocation event resulting in an mRNA, and potentially a protein, which is composed of a mixture of Dvl-2 and Dvl-3 sequence. This would allow expression of Dvl-2, Dvl-3 and Dsh34 in these cells. The existence of protein expressed from this translocation could be confirmed by western blot, this was not done due to lack of availability of suitable antibodies.

7.5 β -catenin accumulates in the nucleus in response to lithium treatment but not in response to retinoic acid treatment

It has been shown that Wnt-13 is expressed during retinoic acid induced differentiation of NTERA-2 (Wakeman *et al.*, 1998). To confirm that Wnt-13 expression is having an effect on NTERA-2 cells during differentiation localisation of β-catenin was observed in untreated embryonal carcinoma cells and in retinoic acid treated cells. As a positive control NTERA-2 cells were treated with 7.5mM lithium chloride, which mimics a Wnt signal (Klein & Melton, 1996). To confirm differentiation of NTERA-2 cells FACS analysis was used (table 7.3). FACS analysis confirmed that retinoic acid induction was successful. A2B5 expression was lower than expected, this was due to a poor batch of antibody (see section 7.2). SSEA1 expression was higher than expected in both embryonal carcinoma cells and retinoic acid treated cells, however the

change in expression on retinoic acid treatment was approximately 20% which is as expected.

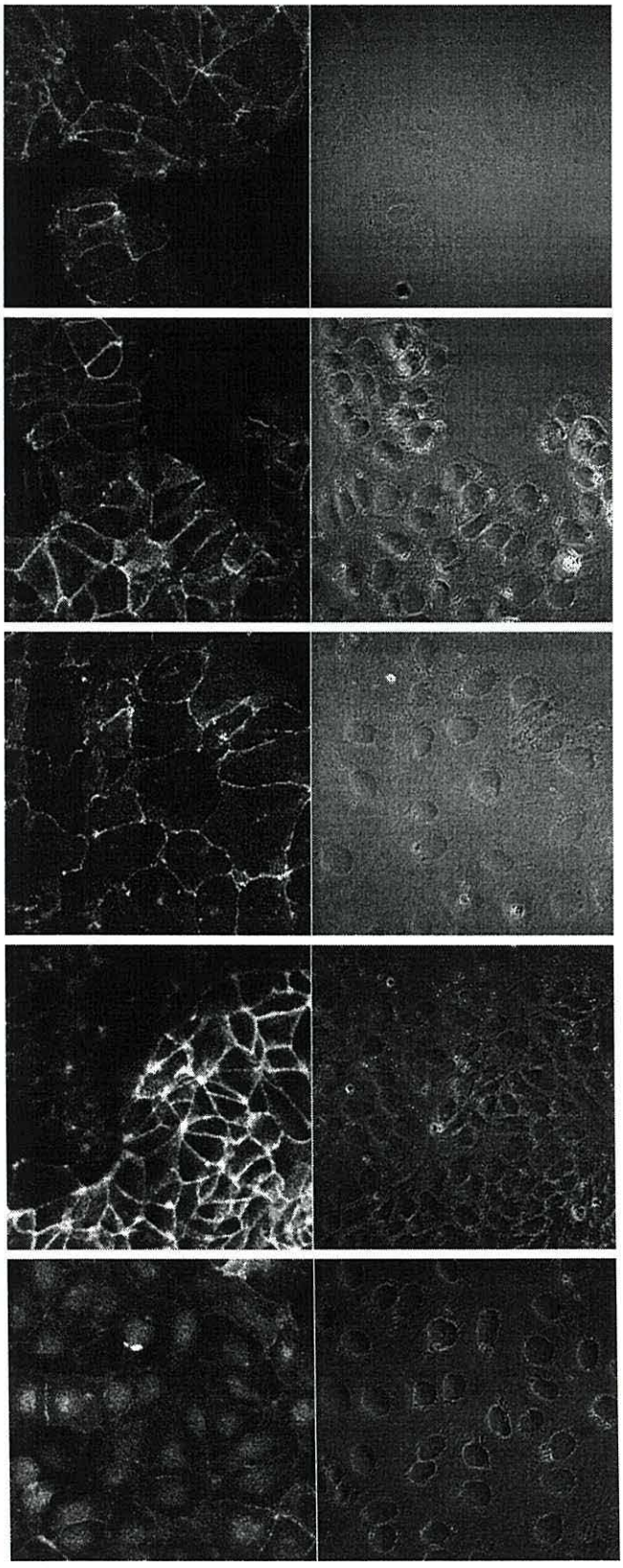
Table 7.3
Flow cytometry analysis of cell surface markers in NTERA-2 embryonal carcinoma cells and retinoic acid treated cells used for immunofluorescence staining for β-catenin. Table shows percentage of cells expressing cell surface markers, numbers in brackets give expected percentage of cells expressing markers (Andrews, 1984).

	Embryonal carcinoma cells	Retinoic acid treated cells
SSEA4	64% (90-100%)	45% (~50%)
SSEA1	16% (0%)	37% (~20%)
A2B5	1% (0%)	31% (80-90%)
Tra-1-60	86% (90-100%)	29% (~60%)

In untreated NTERA-2 embryonal carcinoma cells and retinoic acid induced NTERA-2 cells β-catenin was localised to the cell membrane (figure 7.13a-d). In NTERA-2 cells treated with 7.5mM lithium chloride β-catenin was localised to the membrane and nucleus (figure 7.13e). Retinoic acid treatment, and associated Wnt-13 expression, does not give nuclear localisation while lithium treatment shows that a Wnt signal in these cells does result in nuclear localisation of β-catenin. This indicates Wnt-13 expression during NTERA-2 differentiation is not having an effect via β-catenin. It is possible that lithium treatment is a much stronger Wnt signal than the Wnt-13 expressed in these cells, it is then possible that Wnt-13 is having an effect on β-catenin during NTERA-2 differentiation but that immnuofluorescence isn't sensitive enough to detect that change.

It has been shown that β-catenin localisation and degradation is dependent on phosphorylation (Sadot *et al.*, 2002; Staal *et al.*, 2002). Phosphorylation of the serine residue at codon 45 by Casein kinase Iε is necessary before Wnt dependent phosphorylation can occur (Amit *et al.*, 2002; Hagen & Vidal-Puig, 2002; Liu *et al.*, 2002; Sakanaka, 2002). Phosphorylation sites at residues 33, 37

Figure 7.13
Localisation of β-catenin in NTERA-2 In untreated embryonal carcioma cells and in retinoic acid cells β-catenin is found at the membrane with no nuclear staining. In lithium treated cells nuclear accumulation of β-catenin is seen. β-catenin is shown on the left with DIC or phase contrast image alongside.



a. Untreated embryonal carcinoma NTERA-2 cells

b. NTERA-2 cells treated with 10μM retinoic acid for 2 days

c. NTERA-2 cells treated with 10μM retinoic acid for 7 days

d. NTERA-2 cells treated with 10μM retinoic acid for 14 days

e. NTERA-2 cells treated with 7.5mM lithium chloride for 7 days

and 41 of β-catenin are phosphorylated in a Wnt dependent manner (van Noort *et al.*, 2002; Sadot *et al.*, 2002), it is β-catenin de-phosphorylated at these sites which enter the nucleus in response to a Wnt signal (Staal *et al.*, 2002). Antibodies to phosphorylated β-catenin are now available and these could be used in western analysis to more sensitively detect changes in β-catenin due to Wnt pathway activation.

7.6 Lithium treatment does not have a similar effect to retinoic acid on cell surface markers in NTERA-2

Lithium treatment only affects one of the cell surface markers used in characterising embryonal carcinoma cell differentiation. The percentage of cells expressing SSEA3 decreases from 20-30% in embryonal carcinoma cells to less than 5% in lithium chloride treated cells (figure 7.15). SSEA1, SSEA4, Tra-1-60 and A2B5 show no significant change (figure 7.15). There does appear to be some upregulation of SSEA1, however the error bars overlap so the change appears not to be significant across a number of repeats in this case. These figures contrast those for retinoic acid treated cells where SSEA3, SSEA1, SSEA4, Tra-1-60 and A2B5 all show significant change (Andrews, 1984). This shows that the differentiation resulting from retinoic acid treatment is not the same as that resulting from lithium chloride treatment. While Wnt-13 expression may play a part in retinoic acid induced differentiation the changes in cell surface markers seen in retinoic acid induction can not all be explained by Wnt pathway stimulation.

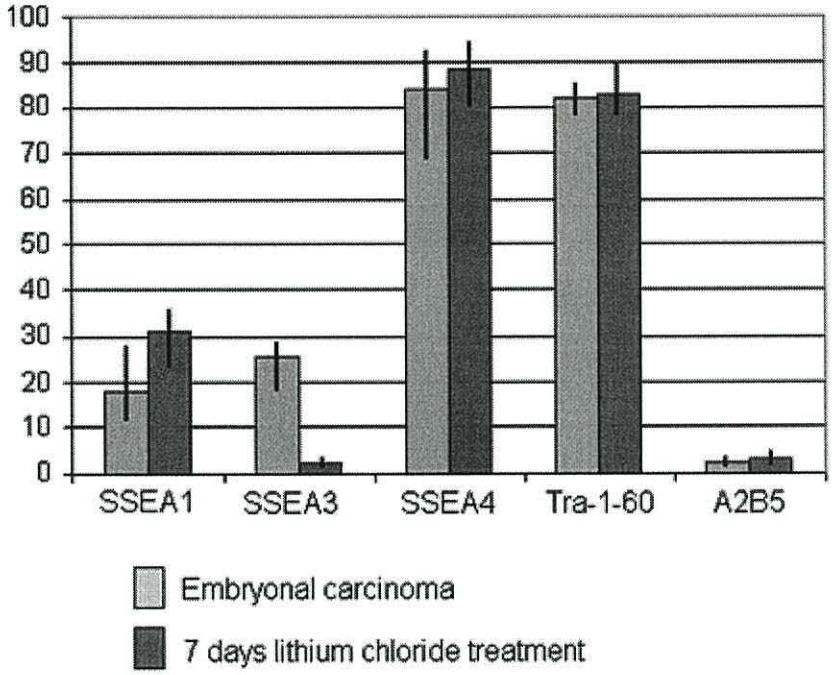


Figure 7.14
Cell surface marker expression in embryonal carcinoma (untreated) and lithium chloride treated NTERA-2 cells. Data from four repeats is presented here. SSEA1, SSEA4, Tra-1-60 and A2B5 show no significant change in lithium treated cells compared to untreated controls. SSEA3 shows a significant decrease when cells are treated with lithium chloride.

7.7 Discussion of results chapter 7: Results: The Wnt signalling pathway in retinoic acid-induced differentiation of NTERA-2 embryonal carcinoma cells

Induction of expression of Wnt-13 has previously been reported in NTERA-2 cells in response to retinoic acid treatment (Wakeman *et al.*, 1998). Expression of a number of Wnt pathway components was analysed in NTERA-2 embryonal carcinoma cells and in retinoic acid treated NTERA-2. A number of potentially interesting changes in expression of Wnt pathway components in response to retinoic acid treatment was seen (as shown by RT-PCR). However, these changes were not confirmed by truly quantitative methods such as northern or western blot or quantitative PCR.

Degenerate primers were used to attempt to identify novel homologues of Wnt pathway components. No novel homologues were identified by this method, however, an interesting potential translocation site between dishevelled homologues Dvl-2 and Dvl-3 was identified.

To confirm activation of the Wnt signalling pathway in response to retinoic acid-induced Wnt-13 expression, the localisation of β-catenin was observed in untreated cells and retinoic acid treated cells. As a positive control, cells were treated with lithium chloride, which mimics a Wnt signal by inhibiting GSK-3β. Nuclear localisation of β-catenin was seen in lithum chloride treated cells but not in untreated embryonal carcinoma cells or retinoic acid treated cells. As the localisation of β-catenin does not change in response to retinoic acid treatment, but does change in the control cells treated with lithium chloride, it seems unlikely that retinoic acid treatment of NTERA-2 leads to activation of the Wnt pathway.

FACS analysis of cell surface markers show that the differentiation resulting from retinoic acid treatment is not the same as that resulting from lithium chloride treatment. This indicates that while Wnt-13 expression may play a part in retinoic acid induced differentiation, the changes in cell surface markers seen in retinoic acid induction can not all be explained by Wnt pathway stimulation.

Chapter 8 - Discussion

8.1 Where is APC? – Characterisation of localisation of APC and of reactivity of APC antibodies

A variety of localisations of APC have been reported, many of these conflicting with each other. APC has been found to shuttle between the nucleus and cytoplasm (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a; Neufeld *et al.*, 2000b); to be localised to microtubule tips (Näthke *et al.*, 1996; Rosin-Arbesfeld *et al.*, 2001; Mogensen *et al.*, 2002); to be localised to the plasma membrane in an actin-dependent manner (Rosin-Arbesfeld *et al.*, 2001); and it has been found at the kinetochore during mitosis (Fodde *et al.*, 2001; Kaplan *et al.*, 2001). APC has also been reported at the apical membrane (Miyashiro *et al.*, 1995; Reinacher-Schick & Gumbiner, 2001; Anderson *et al.*, 2002). Some studies have shown full length APC to be predominantly localised to the cytoplasm in epithelial cells (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000), while a contrasting study reported APC to be localised to the nucleus in epithelial cells (Zhang *et al.*, 2001; Anderson *et al.*, 2002). As mentioned above, APC has been reported to be localised to microtubule tips (Näthke *et al.*, 1996; Rosin-Arbesfeld *et al.*, 2001; Mogensen *et al.*, 2002) with no apical staining, while others report apical localisation of APC with no staining seen at microtubule tips (Reinacher-Schick & Gumbiner, 2001; Anderson *et al.*, 2002).

Comparison of a large number of antibodies raised to APC in western blot and immunofluorescence reveals that many of these conflicting descriptions of the localisation of APC are due to the use of antibodies which detect proteins other than full length/truncated APC (chapter 3). Many of the APC antibodies characterised here detect a variety of proteins on western blot. Some of these proteins may be functional variants of APC. However, due to the variety of bands detected, and the lack of consistency between antibodies, it seems likely that many of the proteins detected by these antibodies are protein species not derived from APC. Comparison of immunofluorescence staining with a panel of APC antibodies shows that many of these antibodies give a staining pattern which is not consistent with the staining seen with anti-APC (M-APC) (an antibody which has been shown to reliably detect APC (Näthke *et al.*, 1996; Mogensen *et al.*, 2002; Rosin-Arbesfeld *et al.*, 2001)), or with any other APC

antibodies. This suggests that many APC antibodies detect proteins other than APC in immunofluorescence.

Comparison of the localisation of APC detected by a number of antibodies raised to APC demonstrated that only two localisations of APC were consistently observed with a variety of antibodies. Anti-APC (M-APC) detects a nuclear shuttling population of APC, as well as APC localised to microtubule tip clusters (section 3.2 & chapter 4). Anti-APC (N-15) also appears to detect the nuclear shuttling population of APC but does not detect APC localised to microtubule tip clusters (section 3.3 & chapter 4). The nuclear shuttling population of APC is discussed in chapter 4 and section 8.2. A number of APC antibodies detect an apical protein in immunofluorescence staining. Comparison of western blots for a panel of APC antibodies shows that as well as detecting full length APC, many antibodies also detect a 150 kDa protein. Many of the antibodies which detect this 150 kDa protein also detect apical localisation of APC, therefore it seems likely that the apical staining corresponds to the 150 kDa protein seen on western blot. This 150 kDa apical protein is discussed in chapter 5 and section 8.3.

8.2 The localisation and interactions of APC and β-catenin vary with mutations status, cell type and cell density

Several studies have shown that APC has a CRM1-dependent nuclear export function for β-catenin that regulates the sub-cellular localisation and turnover of β-catenin (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a). In sub-confluent colorectal cancer cell lines which express only truncated APC, both APC and β-catenin are localised to the nucelus with fainter cytoplasmic staining, and some membrane staining for β-catenin. This contrasts with colorectal cancer cell lines which express wild type APC but have a mutation in β-catenin, in these cell lines APC is predominantly cytoplasmic and β-catenin is predominantly localised to the membrane. When these cells are treated with Leptomycin B, an inhibitor of CRM1-dependent nuclear export, nuclear accumulation of both APC and β-catenin is observed. β-catenin has also been

shown to undergo APC-independent nuclear export (Eleftheriou *et al.*, 2001; Weichens & Fagotto, 2001; Henderson & Fagotto, 2002), although it has not been determined when one mechanism may prevail over the other. It has been suggested that CRM1-independent nuclear export is relatively slow and that the rate of nuclear export is accelerated by APC (Rosin-Arbesfeld *et al.*, 2003).

It has been reported that both APC and β-catenin undergo cell density-dependent redistribution (Brabletz *et al.*, 2001; Zhang *et al.*, 2001; Brocardo *et al.*, 2001; Dietrich *et al.*, 2002). The mechanisms for this are poorly understood, and one aim of the work presented here was to further characterise cell density-dependent redistribution of APC and β-catenin and to seek explanations for this.

It has previously been reported that truncated APC and β-catenin localise to the nucleus in cells expressing only truncated APC (Rosin-Arbesfeld *et al.*, 2000; Henderson 2000). It has also been reported that both APC and β-catenin undergo cell density-dependent redistribution (Brabletz *et al.*, 2001; Zhang *et al.*, 2001; Brocardo *et al.*, 2001; Dietrich *et al.*, 2002). While it has been shown that truncated APC and β-catenin co-localise in the nuclei of sub-confluent cells and that both proteins are redistributed as cell density increases, the localisation of APC and β-catenin during cell density-dependent redistribution of the two proteins had not previously been correlated. In contrast to previous reports, where there was a correlation between nuclear localisation of truncated APC and nuclear localisation of β-catenin, it was found that this correlation is lost as cell density increases (section 4.3). Also, two cell lines, MDCK (section 4.7) and NTERA-2 (section 4.4), were found to have nuclear localisation of APC without nuclear localisation of β-catenin. Therefore, it seems that nuclear co-localisation of APC and β-catenin is only seen in low density cells expressing only truncated APC.

Several studies have shown that APC has a CRM1-dependent nuclear export function for β-catenin that regulates the sub-cellular localisation and turnover of β-catenin (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a). Previous studies examining CRM1-dependent nuclear export of endogenous

APC and β-catenin were all carried out in cell lines expressing either truncated APC or mutated β-catenin. Inhibition of CRM1-dependent nuclear export confirms that full length APC shuttles continuously between the nucleus and cytoplasm in all cell lines examined, at both low and high cell density (section 4.8). Also, in high density SW480 cells truncated APC appears to shuttle between the nucleus and cytoplasm (section 4.8).

As previously reported (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000), β-catenin was seen to accumulate in the nucleus of sub-confluent colorectal cancer cell lines on inhibition of CRM1-dependent nuclear export (section 4.8). However, as cell density increases nuclear accumulation of β-catenin on inhibition of CRM1-dependent nuclear export decreases, with the majority of cells showing no nuclear accumulation of β-catenin (section 4.8). In all cell lines expressing wild type APC and β-catenin, no nuclear accumulation of β-catenin was observed in response to inhibition of CRM1-dependent nuclear export (section 4.8). There are two potential explanations for these observations: β-catenin may be excluded from the nucleus in normal epithelial cell lines and higher density colorectal cancer cell lines; or β-catenin may be able to exit the nucleus in an APC/CRM1-independent manner in these cells.

One possible explanation for the changes in distribution of APC and β-catenin with cell density was that changes in localisation are associated with a reduction in cell proliferation as cells reach higher density. Immunofluorescence staining for Ki-67, a proliferating cell marker, and APC or β-catenin, showed no correlation between localisation of APC or β-catenin and proliferative status of the cells (section 4.9). This constrasts with a previous study where it was reported that density-dependent redistribution of APC was linked to proliferative status of the cells (Zhang *et al.*, 2001). These contrasting observations may be due to the previous study using a cell line expressing full length APC, whereas this study observed a cell line, SW480, which expressed only truncated APC. However, Zhang and co-workers (2001) used FACS analysis to indicate proliferative status and concluded that a global decrease in proliferating cells was associated with a global decrease in nuclear localisation of APC. As this does

not look at localisation of APC in individual cells and correlate it to the proliferative status of each cell, this FACS method can only show that there is a decrease in proliferation and in nuclear localisation of APC, but does not correlate the two.

Nuclear localisation of APC and β-catenin in cell lines which express only truncated APC is thought to be due to loss of nuclear export sequences which are found in the central domain of APC. APC truncations in the majority of colorectal tumours (Miyaki *et al.,* 1994) and cell lines derived from colorectal tumours (Rosin-Arbesfeld *et al.*, 2000) result in loss of all three central nuclear export sequences suggesting that these nuclear export sequences are important in regulating APC and β-catenin localisation. Although other putative nuclear export sequences have been discovered at the N-terminal end of APC (Henderson 2000; Neufeld *et al.*, 2000a) it is not clear what role these have in nuclear export of APC. There have been conflicting reports of the activity of the N-terminal nuclear export sequences. In one study the N-terminal nuclear export sequences were found to be sufficient for nuclear export in the absence of the central nuclear export sequences (Henderson 2000). However, another study found that the N-terminal nuclear export sequences were not necessary for nuclear export of APC and that the N-terminal nuclear export sequences were not able to give efficient nuclear export of APC in the absence of the central nuclear export sequences (Rosin-Arbesfeld *et al.*, 2003).

A potential explanation for the conflicting data concerning activity of the N-terminal nuclear export sequences is that these nuclear export sequences are only active under certain conditions. If the N-terminal nuclear export sequences were active in confluent cells but not in sub-confluent cells this would explain the cell density-dependent redistribution of APC in SW480 cells. SW480 cells express only truncated APC which lacks the central nuclear export sequences (Nishisho *et al.*, 1991). If the N-terminal nuclear export sequences were only active in higher density cells this would explain why low density SW480 cells have predominantly nuclear APC localisation, which is progressively lost in higher density cells. APC has been shown to have a CRM1-dependent nuclear export

function for β-catenin (Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000; Neufeld *et al.*, 2000a). However, as there is no correlation between localisation of APC and β-catenin in confluent cells, increased APC/CRM1-dependent nuclear export of β-catenin as cells reach higher density cannot explain the changes in localisation of β-catenin seen.

It has been shown that E-cadherin is able to negatively regulate β-catenin/TCF-LEF signalling. This regulation of β-catenin/TCF-LEF signalling is dependent on the β-catenin binding region but is not dependent on the intercellular adhesion function of E-cadherin (Orsulic *et al.*, 1999; Gottardi *et al.*, 2001). A reduction in nuclear localisation of β-catenin has been reported in SW480 cells transiently transfected with E-cadherin (Orsulic *et al.*, 1999). Increased expression, membrane localisation and β-catenin interaction of E-cadherin as cell density increases is a theme seen in many cell lines, both those derived from tumours and the 'normal' epithelial cell line HEK293 (section 4.11). Increased binding of β-catenin to E-cadherin as cells reach higher density may sequester β-catenin and prevent its nuclear entry. However, while an increase in β-catenin /E-cadherin interaction and an increase in membrane localisation of the two proteins is seen in SW480 cells as density increases, membrane E-cadherin was not necessary for exclusion of β-catenin from the nucleus (section 4.11). One possibility is that some of the increased E-cadherin expressed as cell density increases, is cytoplasmic E-cadherin. This would allow increased E-cadherin/β-catenin interaction, potentially leaving that β-catenin unable to enter the nucleus but not bound at the membrane. This would account for membrane E-cadherin not being necessary for nuclear exclusion of β-catenin.

One possible model for the cell density-dependent redistribution of β-catenin is that a combination of nuclear export of β-catenin via the APC/CRM1 pathway, combined with sequestration of β-catenin by E-cadherin determines the localisation of β-catenin. In normal epithelial cells efficient degradation of free β-catenin would ensure that only β-catenin bound to E-cadherin, and therefore unable to enter the nucleus, would escape degradation. In HCT116 cells, which

carry a deletion at Ser45 of β-catenin, mutated β-catenin would escape phosphorylation and interact only weakly with E-cadherin at low cell density. Due to HCT116 cells expressing wild type APC, the majority of cells would not have nuclear β-catenin due to efficient export by the APC/CRM1-dependent pathway. As cell density increases, increased expression of E-cadherin would result in more of the free β-catenin being sequestered and therefore unable to enter the nucleus. SW480 cells express only truncated APC and show predominantly nuclear localisation of both APC and β-catenin in sub-confluent cells. Lack of nuclear export of APC and low expression of E-cadherin would result in nuclear accumulation of β-catenin in sub-confluent cells. As cells reach higher density nuclear β-catenin is lost, but is not correlated with loss of nuclear APC or with membrane localisation of E-cadherin. A combination of nuclear export of APC and sequestration of β-catenin by both cytoplasmic and membrane bound E-cadherin would determine the localisation of β-catenin.

It has been shown that presenilin is able to phosphorylate β-catenin independently of axin (Kang *et al.*, 2002). Increased activity of presenilin at high cell density may be a possible alternative explanation for changes in localisation of β-catenin. However, if β-catenin were being phosphorylated by presenilin at high cell density, this would not only decrease nuclear entry of β-catenin but would also increase degradation of β-catenin. No reduction in total β-catenin levels with cell density is observed (section 4.10). Therefore, this is unlikely to be the mechanism resulting in decreased nuclear localisation of β-catenin as cell density increases.

Another potential mechanism for redistribution of β-catenin with cell density in SW480 cells, may be activity of APCL at high cell density. APCL is expressed in SW480 cells (section 7.3) and may be able to downregulate β-catenin (Nakagawa *et al.*, 1998; van Es *et al.*, 1999). Due to lack of availability of suitable antibodies to APCL, localisation of APCL in SW480 has not been characterised. In future, downregulation of β-catenin by APCL at high cell density could be confirmed or excluded by RNAi (RNA interference) to remove expression of APCL.

The cell line NTERA-2 is unusual in that it has one wild type allele of APC and one carrying a truncating mutation resulting in a protein of approximately 120 kDa (G.T. Roberts, unpublished data). As in SW480 cells (which express only truncated APC), APC in NTERA-2 cells is localised to the nucleus in sub-confluent cells, with loss of nuclear staining at high cell density (section 4.4). In contrast to SW480 cells, no nuclear localisation of β-catenin is seen in NTERA-2 cells (section 4.4), and only a very small amount of nuclear accumulation seen when CRM1-dependent nuclear export is inhibited (section 4.8). It may be that the remaining wild type allele of APC is able to efficiently target the majority of free β-catenin for proteasomal degradation, resulting in lack of availability of β-catenin for nuclear entry. When NTERA-2 are treated with lithium a large amount of nuclear β-catenin is seen (section 7.5). A possible explanation is that once a Wnt signal allows β-catenin to enter the nucleus the truncation in one allele of APC is dominant in giving defective nuclear export. To summarise, in cases of trucation of one allele of APC, the truncated allele appears to be dominant for defective CRM1-dependent nuclear export but does not appear to prevent the remaining wild type allele giving efficient degradation of free cytoplasmic β-catenin.

MDCK cells are also different to the majority of cell lines observed both here and in other studies (e.g Rosin-Arbesfeld *et al.*, 2000; Henderson, 2000) in that they have nuclear localisation of wild type APC at low cell density (section 4.7). Nuclear APC has not been reported in other cell lines expressing full length APC. As one of the sub-lines derived from this cell line, MDCKII, does not show nuclear localisation of APC in sub-confluent cells (Rosin-Arbesfeld *et al.*, 2001) it is unlikely that nuclear localisation of APC in this cell line is due to an undiscovered mutation in APC. In normal colon mucosa, APC is detected in the cytoplasm in epithelial cells above the crypt and is additionally detected in nuclei in proliferating cells at the base of crypts (Rosin-Arbesfeld *et al.*, 2003). Cells at the base of crypts are proliferating cells while cells above the crypt are differentiated epithelial cells. The original MDCK line is a heterogenous cell line. MDCKII cells are a cell type which dominates in high passage number

MDCK cultures and have the morphology of polarised epithelial cells. It is possible that nuclear staining in MDCK cells is due to them being de-differentiated proliferating cells, while MDCKII cells are a more differentiated epithelial cell type which lack nuclear APC.

How do these observations in cell lines relate to the situation in normal colon mucosa and in colorectal neoplasia? In normal colon mucosa APC is localised to the nuclei of proliferating cells at the base of crypts, with nuclear staining being lost as cells move up the crypt and differentiation to produce epithelial cells (Rosin-Arbesfeld *et al.*, 2003). This is similar to the situation in MDCK cells, where wild type APC is localised to the nucleus in sub-confluent cells, with nuclear staining being lost as cells reach higher density, and lost in the MDCKII sub-line. Similar mechanisms to those present in epithelia, such as that of colon mucosa, may control localisation of APC in MDCK cells. Like SW480 cells, many colorectal tumours show nuclear localisation of APC (Rosin-Arbesfeld et al., 2003). Nuclear accumulation of β-catenin has also been observed in de-differentiated cells at the invasive front of well-differentiated colorectal carcinoma (Brabletz *et al.*, 2001). This contrasts cells in the central mass of such tumours which have predominantly membrane bound β-catenin and no nuclear accumulation on β-catenin. The changes in β-catenin localisation between the differentiated cells of the central mass and the de-differentiated cells at the invasive front is similar to the density-dependent redistribution of β-catenin seen in SW480 cells. The SW480 cell line was derived from a colorectal adenocarcinoma which gave rise to lymph node metastases (Leibovitz *et al.*, 1976). The SW480 cell line is likely to be a useful model for de-differentiation of cells at the invasive front of colorectal tumours.

SW480 has similarities to colorectal tumours, with sub-confluent cells representing those at the invasive front, and super-confluent cells being similar to those of the central mass. MDCK cells may represent differentation of normal epithelial cells such as those in the crypt. Understanding the mechanisms which lead to density-dependent redistribution of APC and/or β-catenin in these cell

lines is likely to give useful clues to the mechanisms present in normal colon mucosa and in colorectal neoplasia.

8.3 A panel of antibodies raised to APC detect a 150 kDa protein which is localised to the apical membrane

APC has also been reported to be localised to the apical membrane (Miyashiro *et al.*, 1995; Reinacher-Schick & Gumbiner, 2001; Anderson *et al.*, 2002). However, the apical localisation of APC is not consistent with the staining observed with anti-APC (M-APC) (Rosin-Arbesfeld *et al.*, 2001), which has been shown to reliably detect full length APC (Näthke *et al.*, 1996; Rosin-Arbesfeld *et al.*, 2001; Mogensen *et al.*, 2002). This apical protein is detected by a number of C-terminal directed APC antibodies in SW480 cells, which express only truncated APC, therefore it is not possible that this apical protein is full length or truncated APC (section 3.7 & chapter 5). It is possible that the detection of an apical protein with a variety of APC antibodies is reflecting detection of a functionally distinct population of APC.

Many antibodies raised to APC detect a 150 kDa protein on western blots, as well as full length APC (section 5.4). Three antibodies have been shown to detect both the apical protein in immunofluorescence and the 150 kDa protein on western blot (sections 5.3 & 5.4). Therefore, it seems likely that the 150 kDa protein corresponds to the apical protein. Immunoprecipitation and western blot shows that many of these antibodies detect the same protein (section 5.5). As a number of independently produced antibodies, directed to various regions of APC, detect this protein it is unlikely to be due to cross-reactivity of antibodies with an unrelated protein. Immunoprecipitation and western blot does not show any cross-reactivity of these antibodies with APCL (G.T. Roberts, unpublished data). Immunological reactivity of this 150 kDa apical protein shows that, if it is a novel APC isoform, it must contain at least exon 2 and the C-terminal 300 amino acids of APC (chapter 5).

The apical staining seen here is dependent on an intact microtubule network, disruption with nocodazole results in loss of apical membrane staining (section 5.3). The basic domain at the C-terminal end of APC is known to interact directly with microtubules (Munemitsu *et al.*, 1994; Smith *et al.*, 1994b). This is consistent with evidence from immunological reactivity suggesting that the C-terminal end of APC is present in this potential APC isoform. A potential splice event has been identified within exon 15, but has not yet been confirmed. Evidence gathered so far points to this apical protein being an isoform of APC, although it has not yet been conclusively identified as such. Another possibility is that this protein is a novel APC homologue, or other protein with extensive homology to APC. However, database searches have failed to find any candidate sequences with sufficient homology to APC.

Alternative splicing of APC has previously been described (Thliveris *et al.*, 1994; Samowitz *et al.*, 1995; Sulekova *et al.*, 1995; Bala *et al.*, 1996; Kraus *et al.*, 1996; Santoro & Groden, 1997; Pyles *et al.*, 1998). Two of these studies described a 150 kDa APC isoform (Pyles *et al.*, 1998; Kraus *et al.*, 1996). The 150 kDa isoform described by Kraus and co-workers (1996) is unlikely to be the 150 kDa isoform described here but is more likely to be cross-reactivity of antibody anti-APC (Ab3/AL4) (section 3.8). It is unclear whether the 150 kDa isoform described by Pyles and co-workers (1998) corresponds to the 150 kDa isoform described here. Both appear to consist of exon 2 and at least part of exon 15, the 150 kDa isoform described by Pyles and co-workers (1998) also has the alternatively spliced exon BS (brain specific) but not exon 1. The 150 kDa isoform described by Pyles and co-workers (1998) was brain specific and not expressed in mitotically active cells whereas the 150 kDa protein described here was observed in mitotically active cultured cells. The BS containing APC isoform (Pyles *et al.*, 1998) interacts with β-catenin whereas the isoform identified here does not appear to have a significant interaction with β-catenin (section 5.9).

This apical protein does not have any significant interaction with β-catenin (section 5.9), so does not appear to function in the Wnt signalling pathway as full

length APC does. In *Drosophila* E-APC has been shown to be concentrated at apico-lateral adherens junctions, and often appears to be concentrated at the apical cell surface of epithelial cells (McCartney *et al.*, 1999; Yu & Bienz, 1999; Yu *et al.*, 1999). *Drosophila* E-APC has been shown to be involved in determination of spindle orientation, it is required to keep mitotic spindles oriented within the epithelial plane (Lu *et al.*, 2001). In normal epithelia the APC isoform described here is found at apical and lateral membranes, whereas in the two colorectal cancer cell lines the localisation is apical only (section 5.3). Similar observations have been made in normal colon crypts compared to colorectal tumours (Anderson *et al.*, 2002). *Drosophila* E-APC is required for correct spindle orientation, which determines whether epithelial cells grows laterally or becomes stratified. An early step in many colorectal tumours is cell division along an incorrect plane resulting in loss of an epithelial monolayer and expansion of epithelial cells away from the plane of epithelial cells. The difference in localisation of this apical staining APC isoform between normal and cancerous cells, and similarity to the localisation of *Drosophila* E-APC, suggests it may have a role in spindle orientation.

A screen for interacting partners of this 150 kDa protein identified drebrin and myosin IIA as interacting partners of this protein (chapter 6). It should be noted that it has not yet been confirmed that myosin IIA interacts with the 150 kDa protein rather than full length/truncated APC. Although immunofluorescence staining of a variety of cell lines does show these proteins co-localise and therefore have the potential to interact.

In epithelial cells drebrin is localised to adherens junctions (Peitsh *et al.*, 1999). Little is known about the function of drebrin but it is known that it interacts with actin (Sasaki *et al.*, 1996) and may have a role in regulation of actin dynamics (Asada *et al.*, 1994). Non-muscle myosin II is involved in cytokinesis (DeLozanne & Spudich, 1987; Knecht & Loomis, 1987), capping of cell surface components (Pasternak *et al.*, 1989) and polarisation of cell locomotion (Wessels & Soll, 1990; Wessels *et al.*, 1988). The two proteins identified as interacting partners of the 150 kDa potential APC isoform are both involved in the

cytoskeleton. This is consistent with the role proposed for the 150 kDa protein above.

This potential APC variant is absent from the nucleus in all differentiated cells, such as epithelial cells and colorectal cancer cells, but is nuclear in the embryonal carcinoma cell line NTERA-2. Nuclear staining is also seen in embryonic stem cells (data not shown) so this may reflect a difference between pluripotent cells and differentiated cells. BAF155 was identified as a potential interacting partner of this 150 kDa protein (section 6.2), although this has not yet been confirmed. BAF155 is a component of the SWI/SNF chromatin remodelling complex, which is involved in activation of transcription (Imbalzano *et al.*, 1996). This suggests this 150 kDa protein may have a role in chromatin remodelling/transcriptional control in pluripotent cells, although this is rather speculative until interaction between the 150 kDa protein and BAF155 is confirmed.

8.4 Summary

There have been many reports of the subcellular localisation of both full length and truncated APC, many of these conflicting. Characterisation of commercially available antibodies raised to APC shows that many of these conflicting observations resulted from use of antibodies which appear to detect proteins other than full length APC.

The subcellular localisation of APC and β-catenin has been shown to be linked to cell density, cell type and mutation status of APC and β-catenin. A potential mechanism for density-dependent redistribution of APC has been identified as variable activity of N-terminal nuclear export sequences, although further work is necessary to confirm this. Localisation of β-catenin may be controlled by a combination of APC/CRM1-dependent nuclear export and sequestration of β-

catenin by E-cadherin. An alternative mechanism in cell lines expressing only truncated APC may be regulation of β-catenin localisation by APCL.

An apical protein has been identified, the localisation of which has previously been reported as the localisation of full length APC. Work presented here shows that this apical staining is not due to full length APC, but instead appears to be a 150 kDa protein identified as a potential novel isoform of APC. Unlike full length APC, this protein does not interact with β-catenin. This potential APC isoform shows variable distribution in epithelial and colorectal cancer cell lines, with similarities to the localisation of *Drosophila* E-APC, which suggests it may have a spindle orientation role similar to that of *Drosophila* E-APC.

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