DISSERTATION

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Presented by:
Diplom-Biologin Sandhya Singh
Born in: Mumbai, India

Wnt/Wingless Dysregulation in Small Intestinal Adenocarcinoma: Comparison with Colorectal Carcinoma

Referees: 1. Prof. Dr. Thomas Holstein

2. PD Dr. Hendrik Bläker

Abstract

Activation of the wnt/wingless pathway is one of the most important alterations associated with intestinal carcinogenesis. All tumor relevant mutations resulting in wnt/wingless activation cause a stabilization of its central downstream component, β-catenin. In this study we aimed in elucidating the molecular background of β-catenin stabilization in tumors that lack APC mutations, the most common reason for wnt/wingless activation in late age onset of large intestinal cancer. We therefore analysed tumors known to frequently lack APC mutations, 20 small bowel cancers and 20 early age of onset colon cancers, for alternative mechanisms of β-catenin stabilization. We demonstrate that in both tumor groups large genomic deletions in the β-catenin gene *CTNNB1* resulting in an in frame loss of large n-terminal domains contribute to tumorigenesis. We also show that mutations with a different extent of N-terminal deletions display a different accumulation pattern in the cellular compartments and that despite of identical mutational types; the accumulation pattern of β-catenin differs between tumors derived from the large bowel and the small bowel indicating both intestinal segments to differentially regulate β-catenin. The combination of these mutations with lack of general types of genomic instability further suggests an as yet poorly characterized tumorigenic pathway in which large scale β-catenin mutations play a dominant role.

To further characterize the mutations, we created mutant clones according to the sequence alterations found in the tumors. These were transfected into SW480 (Intestinal cell line) and MDCK (Kidney epithelial cell line) cell lines in order to determine their biologic properties in comparison to the wild type and missense point mutations in phosphorylation domains of β-catenin, the most frequent β-catenin mutations observed in human tumors. The large N-terminal deletion mutants were found to show a similar accumulation pattern in both cell lines as we saw in the tissue of small bowel adenocarcinoma. However, β-catenin overexpression (wild type less than mutant) was inducing apoptosis in SW480, a large intestinal adenocarcinoma cell line harbouring an APC mutation. This observation indicates that too large amounts of β-catenin negatively select tumor cells by inducing apoptosis, thus providing an explanation for the mutual exclusiveness of APC and β-catenin mutations in human tumors. β-catenin mutants, however, were oncogenic in

MDCK, a cell line with wild type APC and β-catenin. They were found to push cells into metastable phenotype with higher proliferation but no elevated migration potential, a characteristic of stem cells. The results for large deletion mutants were comparable to those of β-catenin point mutations indicating that if a functional difference between these two mutational types exists, it is related to a biological process other than proliferation or phenotypic switch. Further analysis on β-catenin deletion mutants may help to highlight such novel domain dependant functional interactions of the protein.

Zusammenfassung

Die arretierte Aktivierung des wnt/wingless Signalweges durch eine Mutation ist eine der häufigsten Veränderungen bei intestinalen Karzinomen. Unabhängig von der wnt/wingless Komponente, die von der Mutation betroffen ist, kommt es zu einer Stabilisierung des transkriptionell aktiven wnt/wingless Mediators β-catenin. Die Stablisierung des β-catenin beim Karzinom des Dickdarms beruht in der Regel auf Mutationen des degradierenden APC, dies ist bei Karzinomen des Dünndarms, die ebenfalls eine Stabilisierung des β-catenin aufweisen, nicht der Fall. Ziel dieser Arbeit war zum einen, die Ursache der β-catenin Stabilisierung bei Tumoren ohne APC Mutationen zu identifizieren. Hierzu wurden 20 Adenokarzinome des Dünndarms und 20 früh manifestierte Kolonkarzinome, die ebenfalls häufig keine APC Mutation aufweisen, auf Mutationen im β-catenin Gen (*CTNNB1*) untersucht. Auf diese Weise fanden wir in beiden untersuchten Gruppen einen ungewöhnlichen Mutationstyp, der zu einem Verlust von Teilen der für die Degradierung des β-catenin essentiellen n-terminalen Domäne führte. Der Umfang der Deletionen war hierbei variabel und hatte einen Einfluß weniger auf die Intensität der β-catenin Stabilisierung als auf die Lokalisation des β-catenin im Zellkern und im Zytoplasma. Darüber hinaus fand sich, daß gleichartige Deletions-Mutanten des β-catenin in Dünn- und Dickdarmkarzinomen zu unterschiedlichen Akkumulations Typen führten. Dies weist auf eine divergente Regulierung des β-catenin in Dünn- und Dickdarmmukosa hin.

Um die identifizierten, ungewöhnlichen Deletionsmutanten des β-catenin näher zu charakterisieren und einen Rückschluß auf die Auswirkungen von erweiterten Deletionen, die zusätzlich zu der Degradierungsdomäne noch c-terminal lokalisierte Domänen verloren haben, ziehen zu können, wurden entsprechende Mutanten mit Hilfe der „PCR-driven overlap extension“ Methode generiert und in die Zellkulturen SW480 (Kolonkarzinomzelllinie) und MDCK (Nierenepithel Zelllinie) transfiziert. Die Ergebnisse für die Deletionsmutanten wurden mit denen von Punktmutationen an Phosphorylierungsstellen der Degaradationsbox des β-catenin verglichen. Hierbei zeigte sich, daß das Akkumulationsverhalten von β-catenin im Zellkulturmodell den Befunden in den humanen Tumoren durchaus vergleichbar ist. In der Zelllinie SW480, die bereits eine APC Mutation besitzt, führte die Transfektion jedoch zur

Apoptose. Dies ist ein Hinweis für einen negativen Seletionsdruck einer zu starken β-catenin Akkumulation und erklärt, warum tumorassoziierte Mutationen entweder im APC oder im β-catenin, niemals aber in beiden Molekülen gleichzeitig gefunden werden. In der MDCK Zelllinie hingegen, einer Zelllinie ohne β-catenin oder APC Mutation, zeigte sich eine onkogene Wirkung der Transfektion der Mutanten. Diese ließ sich einerseits in Form einer erhöhten Proliferation nachweisen, andererseits fanden sich phenotypische Zellveränderungen die unter dem Begriff des „metastasierungsfähigen Phänotyps“ summiert werden. Die onkogenen Veränderungen zeigten sich jedoch in gleicher Weise für Deletionsmutanten und Punktmutationen des β-catenin, so dass eine höhere onkogene Potenz von Deletionsmutanten nicht postuliert werden kann. Die Tumordaten und die molekularen Daten weisen jedoch darauf hin, daß funktionelle Unterschiede zwischen den Mutationstypen bestehen und dass eine Ursache für die Häufung der Deletionsmutanten des β-catenin existiert. Zukünftige Analysen der Deletionsmutanten könnten dabei helfen, eine neue, Domänen-abhängige Interaktion des β-catenin aufzudecken.

Dedication

This thesis is dedicated to my late father. Incidentally he met his demise while I was far away from him in middle of this study. His words of inspiration and encouragement in pursuit of excellence, still linger on. May God rest his soul in peace.

Also, this thesis is dedicated to my mother and parents in law for their love, endless support and encouragement.

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ABBREVIATION INDEX

APC	Adenomatous polyposis coli
APS	Ammonium Persulphate
AJ	Adherens Junction
BMP	Bone Morphogenic Protein
Caspase	Cysteine aspartyl-specific protease
CBD	β-Catenin Binding Domain
CDK4	Cyclin dependent kinase 4
CIN	Chromosomal Instability
CKI	Casein Kinase I
CRC	Colorectal Cancer
CSS	Chromosomal Stable
CTNNA1	Alpha Catenin
CTNNB1	β catenin
DAPI	4'-6-Diamidino-2-phenylindole
Dkk	Dickkopf
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxyribonucleotides
Dsh	Dishevelled
DTT	Dithiothreitol
E. coli	Escherichia coli
ECL	Enhanced Chemiluminescense
EDTA	Ethylendiamintetraacetat
EMT	Epithelial Mesenchymal Transition
FACS	Fluorescence Activated Cell Sorter
FAP	Familial adenomatous polyposis
FBS	Fetal Bovine Serum
FCS	Fetal calf serum
Fz	Frizzled
GFP	Green Fluorescent Protein
GSK-3β	Glycogen Synthase Kinase 3β
HCC	Hepatocellular Carcinomas
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IHC	Immunohistochemistry
kD	Kilo-Dalton
kD	Kilo-Dalton
LB	Luria-Bertani medium
Lef	Lymphoid Enhancer Factor
LRP	Lipoprotein Receptor Related Protein

M	Molar
MACS	Microsatellite and Chromosomal Stable
MIN	Microsatellite Instability
mM	Millimolar
mRNA	Messenger RNA
MSS	Microsatellite Stable
ng	Nanogram
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PKC	Protein Kinase C
RNA	Ribonucleicacid
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulphate
SIC	Small Intestinal adenocarcinoma
TCF	T-cell factor
TEMED	N, N, N', N' –Tetramethylethylendiamin
Tris	Trishydroxy-methylaminomethan
UV	Ultraviolet
µ	Micro

M	Molar
MACS	Microsatellite and Chromosomal Stable
MIN	Microsatellite Instability
mM	Millimolar
mRNA	Messenger RNA
MSS	Microsatellite Stable
ng	Nanogram
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PKC	Protein Kinase C
RNA	Ribonucleicacid
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SDS	Sodium Dodecyl Sulphate
SIC	Small Intestinal adenocarcinoma
TCF	T-cell factor
TEMED	N, N, N', N' –Tetramethylethylendiamin
Tris	Trishydroxy-methylaminomethan
UV	Ultraviolet
µ	Micro

INTRODUCTION

1.1 **Biology of intestinal epithelium.**

1.1.1 **Anatomy of the intestines.**

The intestinal tract consists of a tube with four layers. The outer subserosal layer is made up of fatty tissue. It is followed by several sheets of innervated smooth muscles (lamina muscularis propria) that functions in peristalsis. The two inner layers consist of stromal tissue submucosa, and the mucosa layer which contains a small muscle layer (lamina muscularis mucosae), stromal cells and cuboidal epithelial cells at the surface. The latter process and absorb nutrients and compact stool. The small intestine is divided into the proximal duodenum, which makes up approximately 25-30 cm, the jejunum and ileum, both segments approximately 2-3 m in length. The large intestine follows the ileum and is 1 m in length. The absorptive surface in the small intestine is increased manifold by foldings of the inner two layers (Kerckring folds) and several luminal protrusions of the mucosa called villi (Figure 1.1). In the mucosa of large intestine there are no villi, only crypts, and instead of villi there is a flat epithelium (Figure 1.1)[1] [2] [3]. Villi of the small intestine and the flat luminal surfaces of the colon are populated by three different cell types, namely enterocytes, enteroendocrine and goblet cells. Enterocytes secrete hydrolases and absorb nutrient. Goblet cells are more in number in distal intestine and provide a protective mucous lining. The enteroendocrine cells are rare and secrete hormones including serotonine, substance P, and secretin [4] . A fourth cell type, the Paneth cell, resides at crypt bottoms of the small intestine. It secretes antimicrobial peptides and enzymes such as cryptidins, defensin, and lysosomes [5]. During embryologic development, the intestinal epithelium originates as a pseudo stratified layer of endodermal origin that proliferates vigorously, converts into a single layered epithelium, which starts forming invaginations. Cell division becomes restricted to pockets at the base of the mucosal invaginations (Figure 1.2). Smooth muscles and connective stromal tissue differentiate from mesoderm. The proliferative pockets of small intestine reshape into mature crypts in the first few weeks after

birth. In the large intestine, the fetal crypts become progressively deeper as the submucosa grows out, while the number of crypt units increases by crypts fission [6-9] .

1.1.2 Cell renewal and stem cells in the intestinal epithelium.

The intestinal mucosa contains a large number of invaginations termed the crypts of Lieberkühn. Epithelial cells are constantly being renewed in these crypts in a coordinated series of events involving proliferation, differentiation, cell migration, and cell shedding towards the intestinal lumen [10, 11]. Only Paneth cells and pluripotent stem cells localize at the bottom of crypts and escape this flow. From the stem cells, progenitors are generated that occupy the lower third of the crypt, the amplification compartment [12] . The crypt progenitors divide every 12- 16 hours [13], generating 200 cells per crypt every day, until their migration brings them to the mid crypt region where they cease proliferation and differentiate into one of the functional types of intestinal mucosa. At the surface epithelium, cells undergo apoptosis and/or extrusion into the lumen. Proliferation in the intestine is not a cell autonomous feature but is dictated by various factors of which wnt/wingless signaling (WNT) from mesenchymal cells surrounding the bottom of crypts is one of the most important (Figure 1.3). This switch on the WNT signaling in the progenitor proliferating cells leads to nuclear accumulation of β-catenin and increases expression of growth promoting β-catenin/TCF target genes such as Cyclin D and C myc [14]. As the cell reaches the mid crypt region, β-catenin/TCF activity is down regulated and this results in cell cycle arrest and differentiation [15].

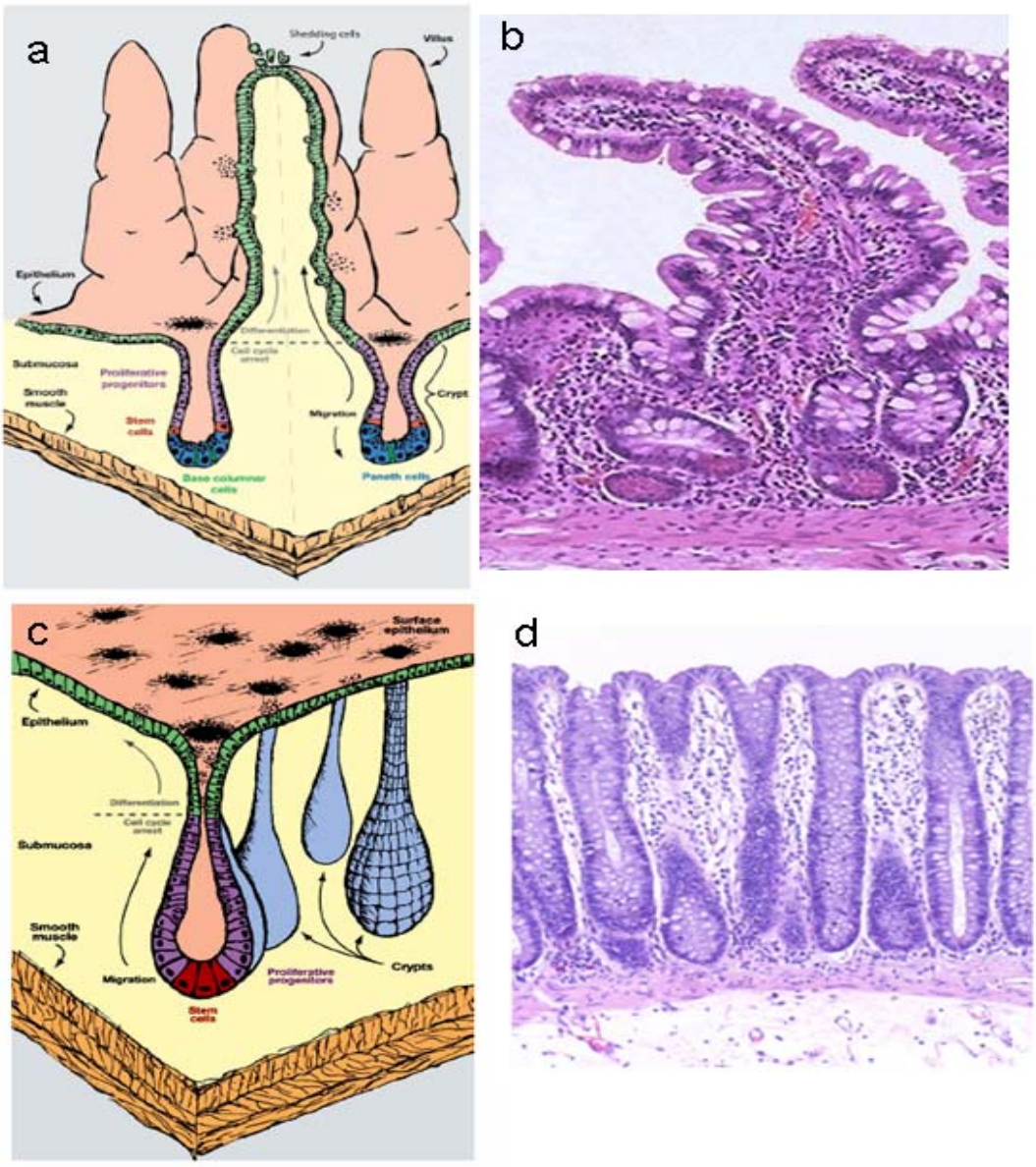


Figure 1.1 (a,b). The structure of the adult small intestine. Putative stem cells reside immediately above the Paneth cells. Base columnar cells, intermingled between the Paneth cells, may also behave as stem cells. Progenitors stop proliferating at the crypt-villus junction and express differentiation markers. Enteroendocrine, absorptive, and mucus secreting cells migrate upward, whereas Paneth cells migrate downward and localize at the bottom of the crypts. (c,d) Structure of the large intestine. Stem cells reside at the crypt bottom. Progenitors are amplified by constant division along the bottom two thirds of the crypts. Paneth cells are absent in the large intestine. Cell cycle arrest and differentiation occur when progenitors reach the top third of the crypts. (Reviewed by Sancho E et al. 2004)

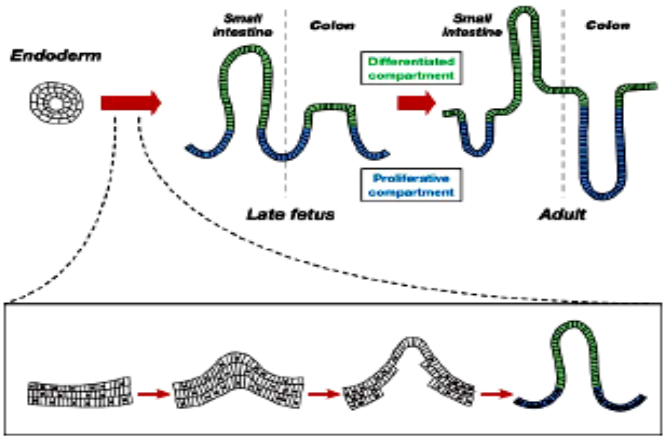


Figure-1.2 Structure of the early multilayered endoderm and the compartmentalized late fetal small and large intestines, and development into the final structure of the adult small and large intestine after invagination of the crypts and elongation of the villi. Inset shows detail of the morphogenetic movements that result in the formation of a simple epithelium with two compartments, from a multilayered endoderm. (Reviewed by Sancho E et al. 2004)

Four features define intestinal stem cells: retention of an undifferentiated phenotype, continuous production of all lineages, retention of self maintenance capabilities throughout life, and ability to regenerate upon injury. Colorectal stem cells (Figure 1.4b) occupy the crypt bottom whereas stem cells of small intestine (Figure 1.4a) reside either immediately above the Paneth cells compartment [16, 17] or intermingled with Paneth cells at the bottom of the crypts [6,7,8,9]. Intervillus pockets are initially polyclonal but rapidly become monoclonal through a poorly understood process of refinement[18].

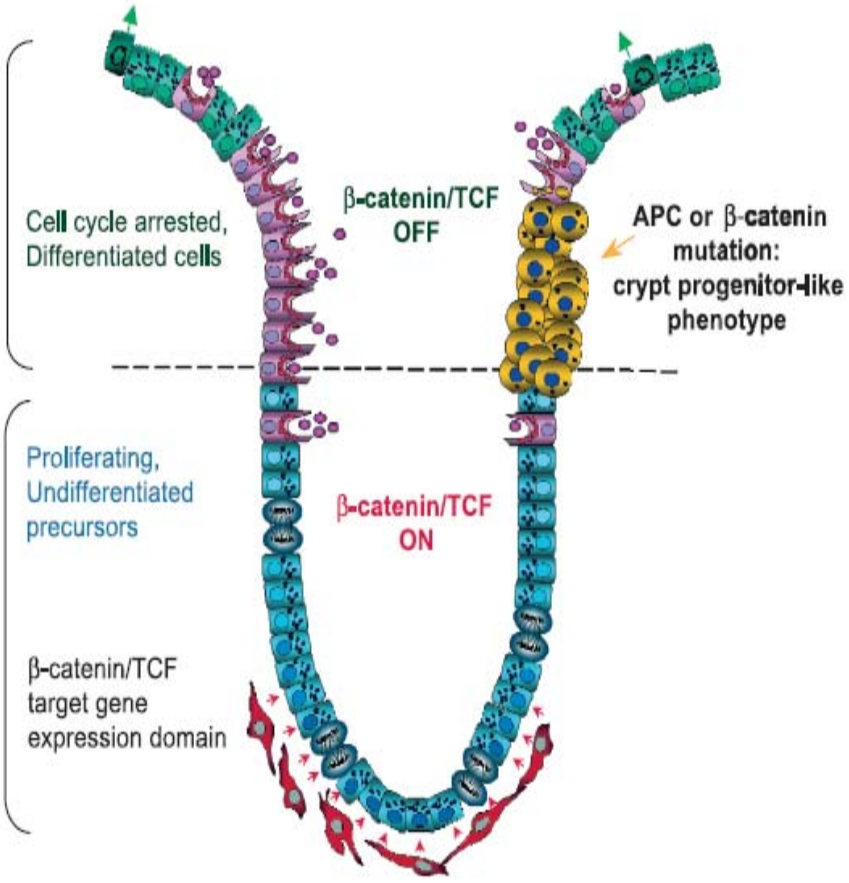


Figure 1.3 Schematic representation of a colon crypt and proposed model for polyp formation. At the bottom third of the crypt, the progenitor proliferating cells accumulate nuclear β-catenin. Consequently, they express β-catenin/TCF target genes. An uncharacterized source of WNT factors likely resides in the mesenchymal cells surrounding the bottom of the crypt, depicted in red. As the cells reach the mid-crypt region, β-catenin/TCF activity is downregulated and this results in cell cycle arrest and differentiation.(Reviewed by M. van de Wetering et al. 2002)

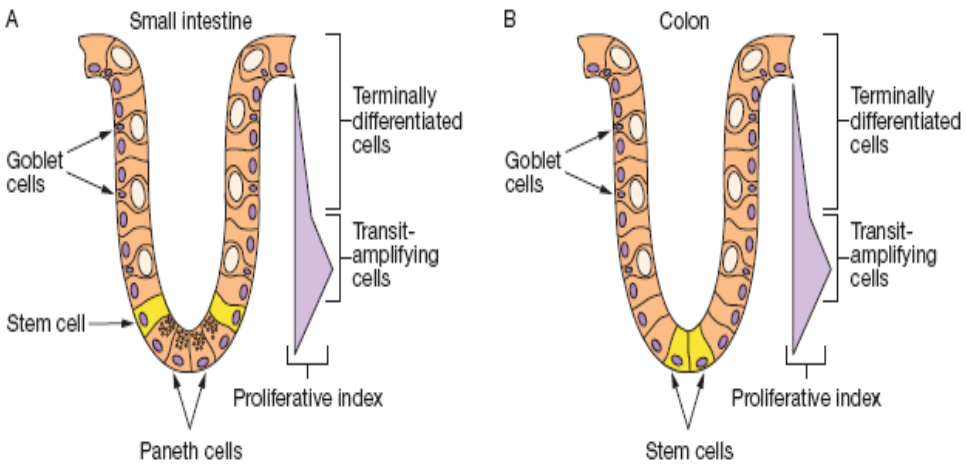


Figure 1.4 Schematic of the small intestinal and colonic crypts. (A) Within the small intestine, stem cells are thought to be located at position 4–5 distal to the Paneth cells. (B) Within the colon, they are at the base of the crypt. In the crypt, the majority of cell proliferation takes place in the transit-amplifying region and terminal differentiation usually occurs distal to this region. (Reviewed from Nat clin Gastroentrology Hepatol 2006 Nature Publishing Group)

Intestinal stem cells are extremely susceptible to apoptosis compared to other crypt progenitor cells [19]. Apoptosis is usually detected at the bottom of crypts after low dose irradiation which is compensated by surviving immediate descendents, which acquire stem cell features. Classical studies demonstrated existence of 4-6 stem cells at the bottom of crypts, even though up to the third generation of immediate descendents of these stem cells may retain clonogenic properties [19, 20]. The remaining approximately 100 cells in the crypts are rapidly proliferating, committed progenitors that can no longer regenerate the stem cell pool.

1.2 Intestinal cancer.

Cancers of gastrointestinal tract constitute a large group of malignant tumors. Among these, colorectal cancer is one of the most common and leading causes of cancer related morbidity and mortality in western countries. In Europe alone more then 210,000 new cases and 110,000 deaths are reported each year with approximately 5-6% risk of developing cancer during lifetime .Small intestinal

cancer is comparatively rare and accounts for only 1% of gastrointestinal malignancies despite of fact that the small intestine contains 90 % of the mucosal surface and is located between two areas of high cancer risk i.e. the stomach and the colon [21, 22]. Small intestinal adenocarcinoma is morphologically similar to colorectal carcinoma and both tumor types share hereditary and non hereditary risk factors, including the hereditary non polyposis colorectal cancer syndrome (HNPCC), familial adenomatous polyposis (FAP), Peutz–Jeghers syndrome (PJS) and Crohn`s disease[23]. Cancer is basically a genetic disease, caused by somatic DNA mutations initially affecting a single cell and resulting in autonomous growth. In general, small and large intestinal carcinogenesis can be described in terms of genetic instability that may effect either microsatellite sequences (microstatellite instability, MSI) [24, 25] or chromosome number and structure (chromosomal instability, CIN) [26]. MSI is caused by deficiency of the DNA mismatch repair system and usually found in carcinomas associated with hereditary non polyposis colorectal cancer syndrome (HNPCC) and approximately 15-20% of sporadic small and large intestinal adenocarcinoma [27-29]. Mismatch repair (MMR) was discovered in prokaryotes long ago but its involvement in cancer is known only within the last twenty years [22,23,24] Microsatellites are genomic regions where short DNA sequences or single nucleotides are repeated. During DNA replication, mutations occur through misalignment, which results either in contraction or elongation of the microsatellite sequences. These mutations are usually repaired by the mismatch repair system. Stable mutations occur due to deficiency in one of the mismatch repair genes. The MMR system includes six different genes (MSH2, MLH1, MSH6, PMS1, PMS2, MSH3) [30, 31]. MSI results in accumulation of mutations on a gene level, which become tumor relevant when growth regulating genes containing coding microsatellites (cMS) are mutated [27,28,29,30]. MSI tumors are normally with diploid karyotypes or with minimal chromosomal changes [32].

Chromosomal instability (CIN) describes an increased tendency to acquire chromosome aberrations when various processes involved in chromosome replication, repair, or segregation are dysfunctional during mitosis [33]. CIN is also found in majority of colorectal and small intestinal cancers. It is commonly linked to inactivation of the p53 and APC tumor suppersor in the colon but can

also be seen in tumors with wild type p53 [34]. CIN positive tumor cells show many chromosomal changes, like structural and numerical alternations of the chromosomes. The tumerogenic impact of CIN is linked mainly to physical gains and losses of tumor promoting oncogenes and tumor suppressor genes.
One of both types of genetic instability is found in most but not all small and large intestinal adenocarcinoma. A share of 10 – 20 % of tumors in both locations is microsatellite and chromosomal stable, known as MACS tumors. MACS tumors have a unique phenotype with distinct clinicopathologic and molecular characteristics [35]. Colon cancer with MACS phenotype is usually reported in young patients, indicating existence of a different molecular mechanism of carcinogenesis [36, 37] , consisting of few strong oncogenic potential mutations.

1.2.1 Hereditary intestinal cancer predisposition syndromes.

Approximately 20% of all patients with CRC shows some cancer predisposition, however, 3-5% are inherited in an autosomal dominant fashion [38, 39]. Hereditary cancer can be divide into two categories based on presence of polyposis as follows:-

Familial adenomatous polyposis (FAP) is characterized by initially benign neoplasm built from glandular type elements, called as intestinal adenomas [40]. FAP is diagnosed by presence of many (100-2500) colonic adenomas. A high number of polyps in FAP results in CRC in 100% of cases by the mean age of 40 years. FAP has an autosomal dominant mode of inheritance and is mainly caused by germline mutations in the tumor suppressor gene adenomatous polyposis coli (APC) [41]. However, its inactivation also occurs in a large proportion of sporadic colorectal cancer [42] . Colorectal adenomas arising in the background of FAP are usually associated with complete inactivation of the APC gene through somatic mutations in the remaining wild type allele. Later, these adenomas may aquire chromosomal instability and mutations in tumor suppressor genes and oncogenes such as K-ras, and p53. With these molecular changes adenomas may acquire aggressive behaviour and transform into carcinomas which behave aggressively in terms of invasiness and metastasis.

Heriditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant cancer syndrome which predisposes to multiple primary cancer without intestinal polyposis. HNPCC tumors are mainly located in the proximal colorectum with usually different pathological features [43, 44]. The average age of onset is approximately 40-45 years. Microstatellite instability (MSI) is the main feature of HNPCC [45]. MLH1 and MSH2 account for almost 90% of all identified mutations in MMR genes [46] . In HNPCC tumors, tumor relevant mutations caused by microsatellite instability have been identified in the transforming growth factor beta receptor II gene (TGFBR2), IGFR2, BAX, APC and CASP5 to name just a few [47, 48].

1.2.2 Sporadic colorectal cancer (CRC).

In western countries, the lifetime CRC risk is around 5% [49]. Approximately 50% of western population develops an adenoma by the age of 70. Only small proportions (between 3 and 5%) of CRCs are attributed to a hereditary cancer syndrome while the majority of them arise sporadically or on the background of as yet unknown hereditary predisposition. The fact that various stages of malignancy coexist from early benign lesion to fully metastatic tumors, have allowed the analysis of genetic alternations present at different stages leading to well defined adenoma-carcinoma model (figure 1.5) in colorectal cancer [50] proposing following points in its pathogenesis:-

1) CRC tumors occur as a result of the mutational activation of oncogenes coupled with inactivation of tumor suppressor genes.
2) Mutation in several genes is required to produce malignant tumors.
3) Genetic alternations occur in a preferred sequence but the total number of mutations defines tumor's phenotype rather then chronological order of mutation.

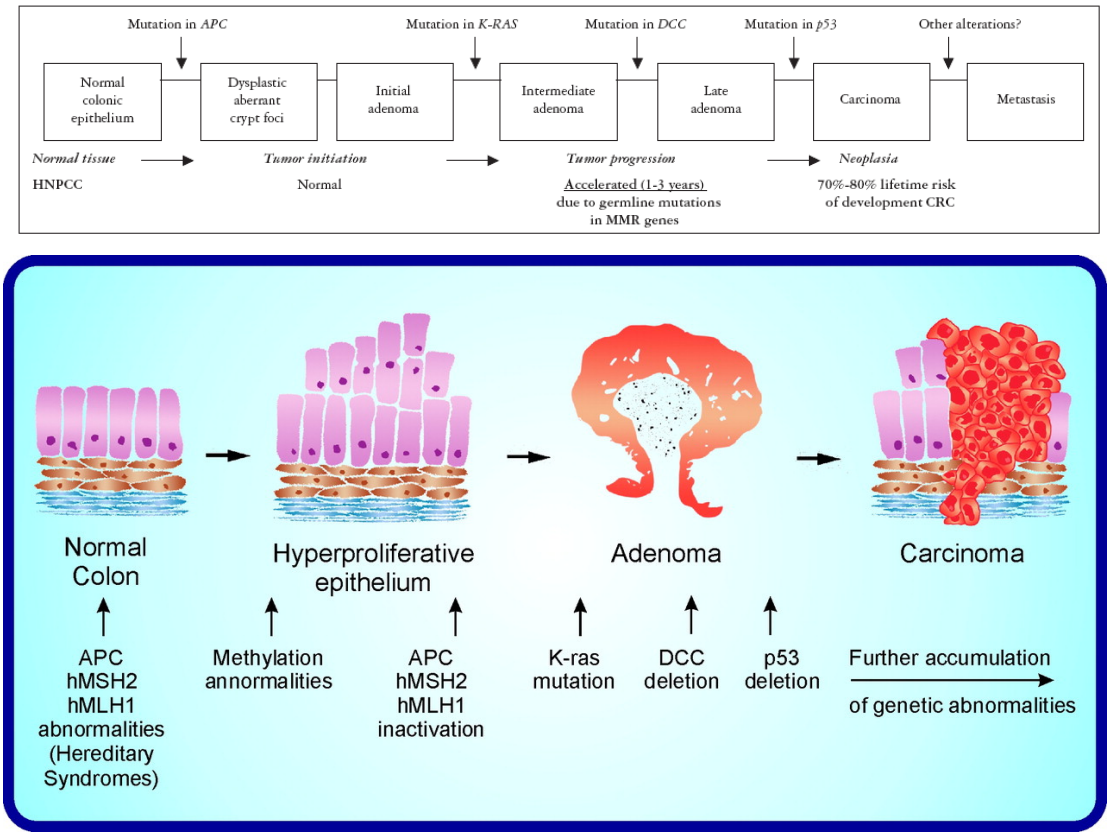


Figure 1.5 The adenoma carcinoma sequence in sporadic CRC and in HNPCC. (Reviewed by Freon and Vogelstein, 1990)

Adenoma - carcinoma sequence in colon cancer development involves an ordered succession of genetic changes that affects the genomes of normal colonic epithelial cells as they evolve progressively towards malignancy (Figure 1.5) Nevertheless, the specific sequence of genetic changes depicted in this figure is shown by only a small proportion of all colonic tumors. Even though, the majority (~70 %) of colon carcinomas suffer inactivation of the APC gene on chromosome 5q as an early step in this process, only 40 to 50 % acquire K-ras mutations, 50 to 70 % show loss of heterozygosity in p53, and about 60 % show a loss of heterozygosity on chromosome 18q [51]. Additionally, about 12 % tumors have mutation that leads to functional inactivation of the type 2 TGF-β receptor.

Therefore, most colon cancer will begin with inactivating APC mutations (or the functionally equivalent gain of β-catenin function mutations), but then will take alternative genetic pathways to malignant carcinoma. Cells undergoing mutation in APC or β-catenin become independent of physiological signals controlling β-catenin/ TCF downregulation. As a consequence, they continue to behave as crypt progenitor cells in the surface epithelium giving rise to aberrant foci (adenoma) perhaps providing an early mechanism of disease progression. An increased number of cells in the crypt–villus compartment (Figure 1.3) would preferably allow opportunities for a second hit.This second hit mutation might include mutation in K-ras or any other component of Ras signaling pathway giving the cell a growth advantage over normal colon epithelial cells.

1.2.3 Intestinal cancer with stable genome (MACS tumor).

Most intestinal cancers usually show either microsatellite instability (MSI) or chromosomal instability (CIN). Recently, a subgroup (17%) of intestinal cancer was found to exist with apparently stable genome, with neither CIN nor MSI, known as microsatellite and chromosomal stable (MACS) tumors. Sporadic colorectal tumors without MSI or CIN exhibit distinct clinicopathologic features and genetic presentation, suggesting that these tumors might arise from another distinct pathway. [52-55].MACS was found to differ from CIN+ and MSI+ in three aspects. The clinicopathologic features of MACS were usually similar to MSI+ but different from CIN+. Comparatively, MACS preferred proximal location and poor differentiation. However, an immunohistochemical study demonstrates that MACS had a lower rate of loss of hMLH1 or BAX protein than MSI+ and less inactivation of APC protein than CIN+. In an epigenetic aspect, both MACS and MSI+ had a high rate of CpG island methylator phenotype with a difference for the presence of hMLH1 methylation. In comparison to CIN+, MACS had a more frequent CpG island methylator phenotype and MINT1 methylation. A MACS tumor shows divergent biological and behavioral features. MSI tumors arise predominantly in the proximal colon, are histologically mucinous or poorly differentiated, and are associated with a better prognosis [25, 56-58]. Whereas

CIN and MACS tumors are more common in the distal colon and rectum, moderately to well differentiated and carry a poorer prognosis than MSI tumors.

The research of Chan et al [55], reported a much greater rate of MACS tumors in early-onset as compared with late-onset tumors (64 versus 13%). These differences are very important to study mainly because of their direct revelance to prognosis. Some studies are performed to elucidate carcinogenesis mechanism of this subtype. Tang et al [59] found that stabilization of p53 protein in the absence of p53 gene mutation may play a critical role in the pathway of this subset. Nevertheless, MACS cancer may have a genetic basis different from either MSI or CIN, and further studies of these cancers may lead to discovery of new mechanisms of carcinogenesis and cancer susceptibility.

1.3 Wnt signaling pathway.

The wnt pathway plays key roles in development, tissue homeoatasis, and cancer. It was originally described in Drosophila as wingless pathway and is highly conserved among flies, frogs, and mammals [60]. Activation of the wnt/wingless pathway is one of the most important alterations associated with intestinal carcinogenesis. Nuclear or cytoplasmic accumulation of β-catenin is the hallmark of an active canonical Wnt pathway. β-catenin accumulation is present even in the smallest detectable lesion as well as in later stages. Complete nuclear translocation of β-catenin from the cytoplasm is observed at the invasion front providing an evidence for a major role of wnt signaling pathway in intitation, progression in situ, and finally metastasis. Other signaling pathways important in intestinal carcinogenesis include EGFR-MAPK, TGFβ, and TP53 signaling pathways. Interactions among various signaling pathways involved are being studied and will provide new understanding of tumorigenesis [61].

1.3.1 **Non-canonical Wnt pathway.**

There are many non-canonical pathways, but the two best-studied pathways are the Planar cell polarity (PCP) and Wnt/calcium pathways. Upon binding of Wnt to

its receptor, either Frizzled or a complex of Frizzled and RP5/6, a signal is transduced to the cytoplasmic phosphoprotein Dishevelled (Dsh). There are three Dsh proteins in mammals (Dsh-1, Dsh-2 and Dsh-3). The ligand/receptor interaction induce the phosphorylation of the Dsh family by casein kinase 1ε and -2 and PKCα., a common event of all Wnt-induced signaling pathways [62]. At the level of Dsh, the Wnt signal branches into roughly three separate pathways, the canonical pathway (Wnt/β-catenin pathway), planar cell polarity (PCP) pathway, and calcium pathway (Non Canonical pathway) (Figure 1.6).

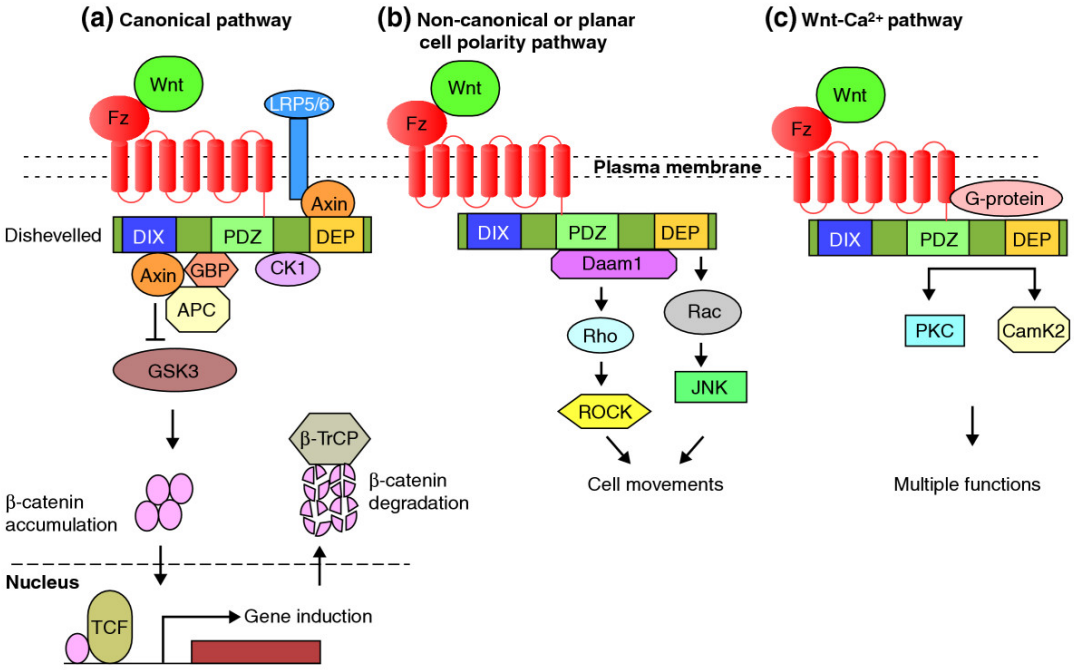


Figure 1.6 Wnt Signaling pathways (a) Canonical pathway (b) Non Canonical, Planar Cell Polarity pathway (c) Wnt-Ca^{2+} pathway. (Reviewed by Habas et al. 2005)

However, the way in which Dsh couples and distributes Wnt signaling into the three signaling branches is poorly understood. Furthermore, compared to the canonical pathway, PCP pathway and calcium pathway are largely unknown [63]. For non-canonical or planar cell polarity (PCP) signaling, Wnt signaling is

transduced through Frizzled independent of LPR5/6. Utilizing the PDZ and DEP domains of Dsh, this pathway mediates cytoskeletal changes through activation of the small GTPases Rho and Rac. For the Wnt-Ca2+ pathway, Wnt signaling via Frizzled mediates activation of heterotrimeric G-proteins, which engage Dsh, phospholipase C, calcium-calmodulin kinase 2 (CamK2), and protein kinase C (PKC). This pathway also uses the PDZ and DEP domains of Dsh to modulate cell adhesion and motility. In PCP and Ca2+ pathways Dsh is proposed to function at the membrane, whereas for canonical signaling Dsh has been proposed to function in the cytoplasm.

1.3.2 Canonical Wnt pathway (Wnt/β-catenin pathway).

“Canonical pathway” Wnt signaling is initiated when Wnt ligands binds to their receptor complex consisting of FZD family and a member of the LDL receptor family, LRP5/6. Signaling through the Frizzled (Fz) and LRP5/6 receptor complex induces the stabilization of β-catenin via the DIX and PDZ domains of Dishevelled (Dsh) and a number of factors including Axin, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). Stabilized β-catenin translocates into the nucleus where it complexes with members of the LEF/TCF family of transcription factors to mediate transcriptional induction of target genes. β-catenin is then exported from the nucleus and degraded via the proteosomal machinery. The central player here is a cytoplasmic protein termed β-catenin, the stability of which is regulated by the destruction complex [64]. When Wnt receptors are not engaged, two scaffolding proteins in the destruction complex, adenomatous polyposis coli (APC) and axin, bind newly synthesized β-catenin. CKI and GSK3, two kinases residing in the destruction complex, then sequentially phosphorylates a set of conserved Ser and Thr residues in the N-terminus of β-catenin. The resulting phosphorylated β-catenin recruits a β-TrCP-containing E3 ubiquitin ligase, which targets β-catenin for proteasomal degradation. In the presence of Wnt ligands, receptor occupancy inhibits the kinase activity of the destruction complex by an incompletely understood mechanism involving the direct interaction of axin with LRP5/6, and/or the actions of an axin-binding molecule, Dsh (Fig.1,7 (a))

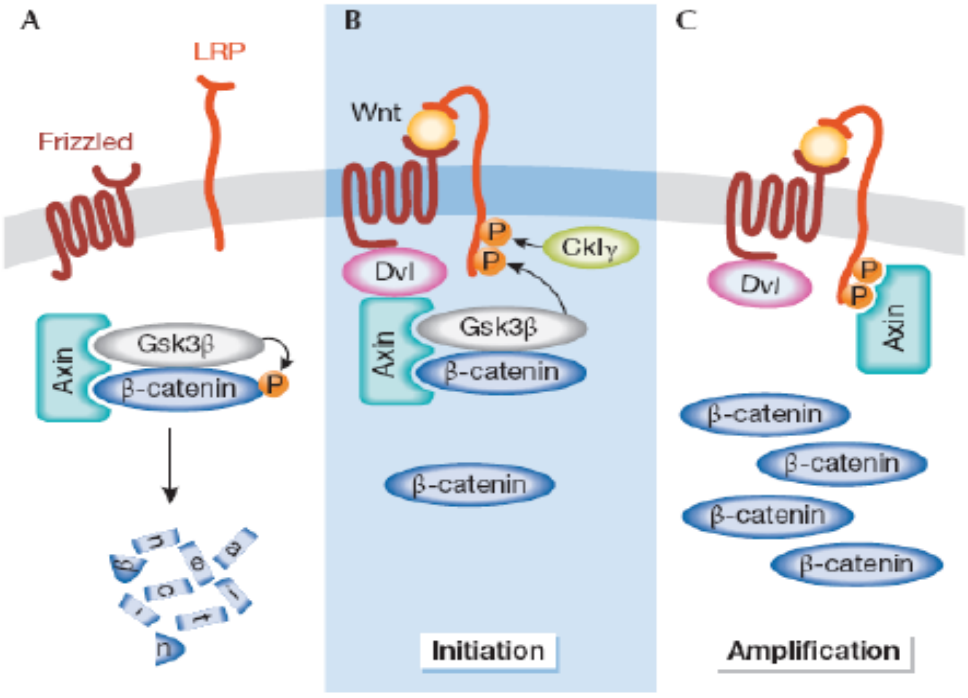


Figure 1.7 Model for the activation of the canonical Wnt/β-catenin pathway.(A) In absence of Wnt ligands, β-catenin is phosphorylated and degraded (B) On binding of Wnt to the receptors, FZD and LRP, Dvl binds to FZD and recruits the destruction complex through interaction with axin. Subsequently, LRP is phosphorylated and acts as docking site for axin. (C) Binding of axin to LRP leads to inhibition of the destruction complex and stabilization of β-catenin. (Reviewed by Fuerer, C and Nusse, R 2008)

As a consequence, β-catenin accumulates (Figure 1.7), travels into the nucleus where it engages the N-terminus of DNA-binding proteins of the Tcf/Lef family and initiates gene expression of target genes such as Cyclin D1 and c-myc [65] . The vertebrate genome encodes four highly similar Tcf/Lef proteins. It is also reported that in the absence of a Wnt signal, certain Tcfs repress target genes through a direct association with co-repressors such as Groucho. The interaction with β-catenin transiently converts Tcf/Lef factors into transcriptional activataors. As a consequence, the canonical pathway translates a Wnt signal into the transient transcription of a Tcf/Lef target genes.

1.4 Components of the Wnt pathway and their contribution to intestinal tumors.

1.4.1 Wnt factors, frizzled receptors and dishevelled.

Wnt proteins constitute a large family of at least 16 secreted cysteine rich glycoproteins, some of which are shown to promote neoplastic transformation in animal models and tissue culture but no direct involvement in human carcinogenesis is known [66, 67]. Wnt protein binds to extracellular domain of frizzled family of seven transmembrane receptors. 11 different frizzled genes are known but not much is known about their specific functions and lipid specificity. Low density lipoprotein receptor related proteins, LRP5 and LRP6 act as co-receptors for wnt signal transduction. In addition to membrane bound frizzled receptors, secreted frizzled receptors also exist and exert an antagonistic effect on Wnt signaling by binding to Wnt proteins. Classical membrane bound frizzled receptors are known to activate Wnt signaling. Their exact role in carcinogenesis is not known, but it has been reported that frizzled receptor E3 (FzE3) is expressed in many oesophageal cancers but not in matched normal tissues [68, 69]. Moreover, this expression of FzE3 correlates with nuclear translocation of β-catenin. Binding of Wnt ligand to a member of frizzled receptor family, results in its activation which recruit the cytoplasmic protein Dishevelled to the inner membrane and mediates its phosphorylation. Through distinct domains, dishevelled transduce Wnt signals and activates the Jun N-terminal kinase pathway [70]. Wnt signals are transduced by direct binding to dishevelled and axin which inhibits GSK-3β-dependent phosphorylation of β-catenin, mainly, due to destruction of APC/AXIN/GSK-3β complex. Additionally, protein kinase CK2 (casein kinase II), a protein serine/threonine kinase, is also able to phosphorylate dishevelled independent of Frizzled. The role of Dishevelled and CK2 in neoplastic transformation via β-catenin/TCF is not known and so far no mutations in these proteins are reported in any human cancers.

1.4.2 GSK-3β, Axin, Casein Kinase I, and β-TrCP: Regulators of β-catenin.

Tight regulation of free cytoplasmic β-catenin is the central switch of the Wnt pathway. In the absence of Wnt signal, degradation of β-catenin is initiated by promoting phosphorylation of serine 45 (S45) by casein kinase I (CK I). Phosphorylation of β-catenin is dependent on binding of CK1 to axin. Next, a multiprotein complex consisting of APC, Axin and serine/threonine kinase GSK-3β is formed and GSK-3β facilitates further phosphorylation of β-catenin amino terminus, starting at threonorine 41, and walking downstream to S37 and S33 [71, 72]. The aim of this phosphorylation is the generation of the canonical β-trCP recognition site around S33/S37 (DS*GXXS*; S* = phosphoserine). Phosphorylated β-catenin at all critical residues are bound by the F-box protein β-TrCP, a subunit of the SCF-type E3 ubiquitin ligae complex. This complex facilitates ubiquitination and subsequent proteasome degradation of phosphorylated β-catenin. β-catenin mutations at asparagin 32 or glycine 34 are also common in human cancer since they destroy the β-trCP recognition site, resulting in stabilization of β-catenin. Besides β-catenin, GSK-3β also phosphorylates axin and APC, thereby regulating the stability of axin and the binding efficiency of APC to ß-catenin, respectively. For phosphorylation of β-catenin by GSK-3ß, the presence of axin is required. Axin, or its homolog conductin (also called Axil or Axin-2), serves as a scaffold protein allowing assembly of the APC/Axin/GSK-3ß/β-catenin complex [73]. Interaction of axin proteins with APC, GSK-3β, and axin as well as with dishevelled occurs by non-overlapping regions. Binding to APC is via the RGS domain and binding to dishevelled occurs through a domain called DIX. Based on its function to downregulate oncogenic Wnt signaling axin could be viewed as a tumor-suppressor gene, a fact supported by many evidences [74]. In a subset of HCCs, axin is bialleically mutated leading to axin proteins lacking β-catenin binding sites and also in colorectal cancer with defective DNA mismatch repair and lacking mutation in β-catenin and APC [75]. No muations have been observed in GSK-3β gene as yet. This might be explained by the fact that GSK-3β also

phosphorylates other regulatory proteins outside the WNT pathway such as proteins in insulin and growth factor signaling pathways.

1.4.3 APC: Gatekeepers of large but not of small intestinal tumorigenesis.

The APC gene was identified on chromosome 5q by genetic analysis of familial adenomatous polyposis (FAP) families. Patients with FAP develop multiple adenomatous polyps of the colorectal epithelium, some of which progress to invasive carcinomas. Some FAP patients also suffer from extracolonic tumors, such as desmoid tumors, ampullary carcinomas, and hepatoblastomas [76]. The sequence of the APC gene spans 15 exons and encodes a 2,843-amino acid protein of 310 KD. APC plays a critical role in the pathogenesis of both, inherited and sporadic colorectal cancer and functions mainly as a regulator of free β-catenin [77]. While germline inactivation of APC occurs over the entire gene, somatic mutations are mainly found at 5' end of exon 15 between codons 1280 and 1500 (mutation cluster region, MCR) resulting in a frame shift or a premature stop codon and truncated APC protein [78] (Figure 1.8). Biallelic inactivation of APC usually results from a truncating mutation coupled with a deletion of the long arm of chromosome 5. Altogether, in ~50% of all colorectal cancers, APC function is inactivated by loss of APC expression or expression of a truncated protein [79]. The APC protein consists of multiple functional domains that mediate oligomerization and interaction with many cellular proteins including ß-catenin, γ catenin, GSK-3β, Axin/conductin, tubulin, EB1, hDLG, Asef, and Siah-1. However, the main function of APC appears to be the regulation of the free non-membrane bound pool of β-catenin in cooperation with GSK-3ß and Axin/conductin. Truncated APC proteins loose its ability to bind Axin which results in its inability to downregulate β-catenin resulting in its accumulation in the cytoplasm and the nucleus [80]

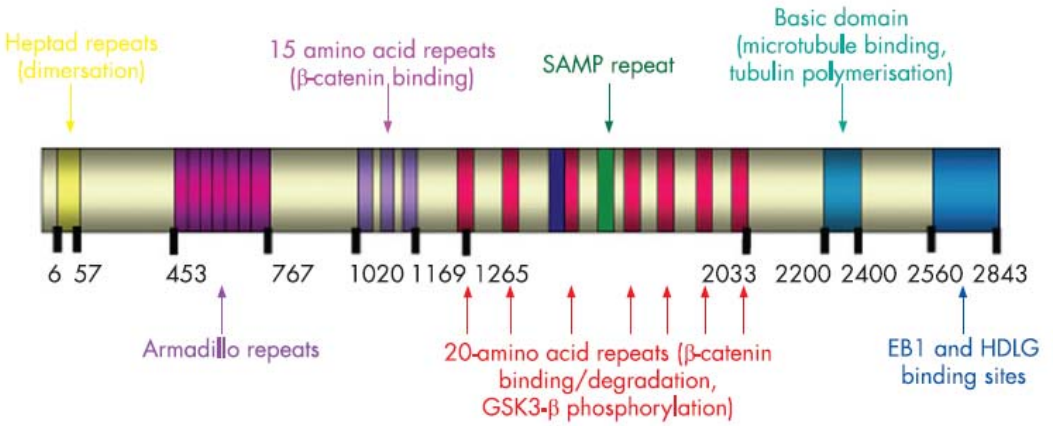


Figure 1.8 Schematic diagram of the adenomatous polyposis coli (APC) protein showing major functional domains by amino acid position. APC forms homodimers with itself by the oligomerization domain (oligo). The armadillo repeat region is made up of a 42- amino acid motif that is repeated 13 times. The regions for binding and downregulation of β-catenin and the binding region of Axin/conductin partly overlap. Microtubules bind to the basic region (basic) and the binding regions of Siah-1 and EB1 are confined to the carboxy terminus. The mutation cluster region (MCR) is located amino terminal to the Axin/conductin-binding sites. Most mutant APC proteins can no longer bind to Axin and are therefore incapable of downregulating ß-catenin. (Reviewed by Leedham and Wright 2008)

Recent studies proved a role of APC in the downregulation of non-phosphorylated, oncogenic forms of β-catenin which escape the ß-TrCP-dependent destruction. F-box protein, Ebi, is recruited in this alternative destruction pathway. This alternative pathway requires the interaction of APC with Siah-1, a p53-inducible gene, which is also involved in the regulation of the tumor-suppressor gene DCC. As Siah-1 binds to the carboxy terminus of APC and most colorectal cancers carry truncating mutations lacking the carboxy terminus, both the Axin/GSK-3β/β-TrCP and the Siah-1/Ebi destruction pathways are inactive. The Siah-1/Ebi system can have a functional role in regulating β-catenin only in tumors with β-catenin mutations and expressing wild-type APC [81]. Inspite of fact, APC inactivation and oncogenic activation of β-catenin exerts similar effect in terms of Tcf-transcriptional activation, APC mutations are found in the majority of colorectal cancers, whereas β-catenin mutations are only found in a colorectal cancers with wild-type APC, interestingly when both APC alleles need to be mutated versus only one β-catenin allele in order to deregulate Tcf

signaling. One hypothesis for this different frequency of APC and β-catenin mutations is that APC loss may provide the cell with a stronger growth advantage than activation of ß catenin, implying that APC has other vital functions like cell migration, adhesion, transcriptional activation and apoptosis besides promoting β-catenin degradation [82]. Moreover, β-catenin mutations are more common in small adenomas than in invasive cancers, and tumors carrying mutated β-catenin are less aggressive than tumors showing loss of APC. APC also has Wnt independent functions mediated through its carboxy terminus, the region commonly lost in colorectal cancer. APC directly associates with the microtubule cytoskeleton and binds to microtubule associated proteins of the EB/RP family. APC is also involved in the maintenance of chromosomal stability through localization to the kinetochore of metaphase chromosomes, a function most likely dependent on the interaction with EB1 [83].

1.4.4 β-catenin: central player of the Wnt pathway.

β-catenin is the mammalian orthologue of the drosophila Arm protein with dual role in cell adhesion and a key component of the Wnt signaling pathway. It functions both during development and tumorigenesis. β-catenin was first identified because of its binding to the cytoplasmic domain of the cell–cell adhesion protein E-cadherin. β-catenin protein can be distinguished into three main regions namely the N-terminal domain, the central core, and the C-terminal domain (Figure 1.9). The central core of β-catenin consists of 12 armadillo repeats (R1–R12) [84-86]. Each repeat forms three alpha-helices that are arranged together in a compact superhelix. Mutational analyses (deletions, point-mutations) have demonstrated that all three regions participate in both main functions of cell adhesion and cell signaling (Fig 1.9)[74, 87] β-catenin mediated cell adhesion depends primarily on its interactions with the cytoplasmic domain of cadherin and the N-terminal domain of α-catenin molecules. Sites of cadherin interaction are distributed throughout the 12 central repeat domains R1–R12 [88]. Interaction with α-catenin occurs at a well defined binding site spanning armadillo repeats R1–R2 and adjacent regions of the N-terminal domain [89-91]. The cell

signaling function of β-catenin depends on interaction with components of the destruction machinery and the transcription factors TCF/LEF-1. In the absence of wnt-signaling, β-catenin is efficiently phosphorylated at a site in the N-terminal domain. This phosphorylation event is facilitated by a large, multiprotein complex, including glycogen synthase kinase 3 beta (GSK-3β), Axin, and the tumor suppressor gene product adenomatous polyposis coli (APC)[73, 77]). Phosphorylated β-catenin is ubiquitinated and subsequently degraded by the proteosome [92]. In contrast, active wnt signaling efficiently decreases the destruction of β-catenin via inhibition of GSK-3β. At least two interactions of stabilized β-catenin are required for its function in transcriptional activation. β-catenin binds to TCF/LEF- 1 protein via R3–R10. The β-catenin /TCF complex can bind via a TCF binding domain to sites in the regulatory regions of wnt-target genes [93] . β-catenin itself contains redundant transactivation domains that have been located at R2–R4 and at the C-terminal region including R12[94].

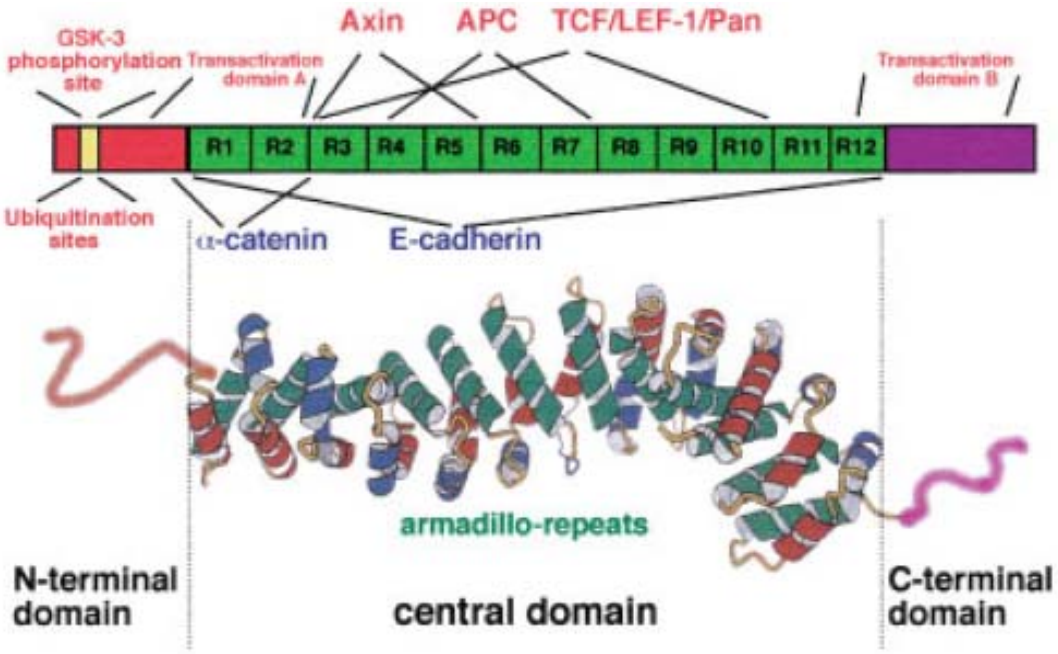


Figure 1.9 Functional and Structural domains of β-catenin involved in Wnt signaling and cell adhesion. (Reviewed by Andrew H. Huber, W.James Nelson and William I. Weis 1997)

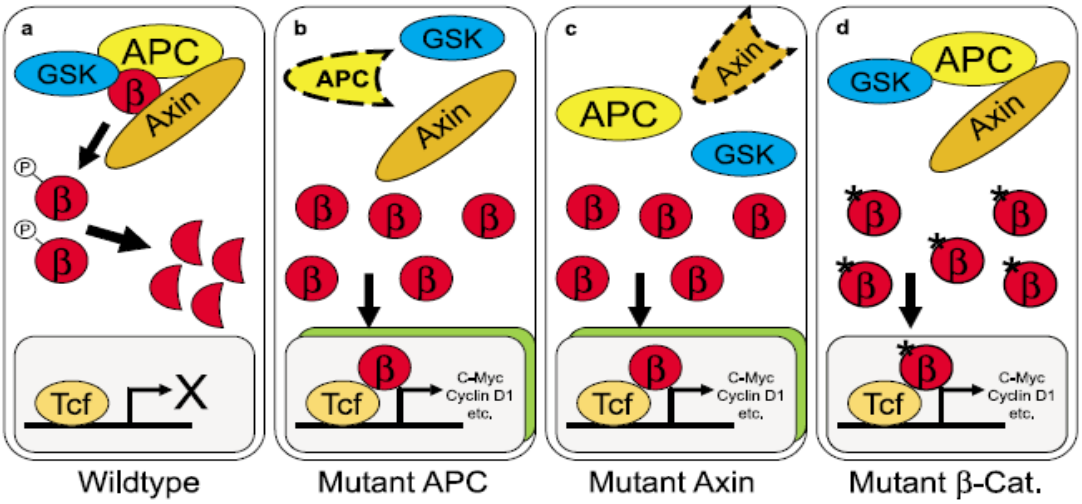


Figure 1.10 Deregulation of Wnt signaling in gastrointestinal tumors. a Under physiological conditions free β-catenin (ß)is rapidly degraded. Three different mechanisms can lead to deregulation of Wnt/ß-catenin/Tcf signaling. Inactivation of the tumor suppressor APC (b) or the scaffold protein Axin (c), and activating mutations of β-catenin itself (d)result in cellular accumulation of ß-catenin. After nuclear translocation and binding to Tcf, transcription of specific target genes is activated. (Reviewed by Burkhard Goke 2002)

Under physiological conditions most cellular β-catenin is bound to E-cadherin, a process regulated by tyrosine kinases and tyrosine phosphatases [95, 96]. In the absence of a Wnt signal free β-catenin is phosphorylated and degraded. β-catenin has been implicated in human cancer [97] and its oncogenic potential has been extensively studied in in vitro tissue culture models [98, 99] and in vivo animal models [100-102]. Three separate mechanisms have been found to lead to accumulation of β-catenin in the cytoplasm and nucleus of cancer cells: inactivation of the APC tumor suppressor gene in colorectal cancer; axin mutations in subsets of hepatocellular and colorectal cancers [103, 104], and mutations of ß-catenin's amino terminus in a variety of cancers (Figure 1.10) In half of colon tumors with intact APC, gain of function mutations in the β-catenin gene have been identified [105-109]. β-catenin mutations in colorectal tumors are usually present in the background of microsatellite instability [109, 110] while, inactivating APC mutations are mainly found in chromosomal instable cancer.

APC inactivating mutations and activating β-catenin mutations are mutually exclusive and have not been shown to coexist in a tumor. It indicates that coexistence of both mutations does not exert an additive effect on tumorigenic potential.

1.4.5 Activation of Tcf-dependent transcription.

Cytosolic accumulation of β-catenin leads to the formation of complexes with Tcf/Lef transcription factor which translocates to nucleus [111, 112]. Tcf/Lef factors facilitate gene-specific DNA binding and β-catenin serves as transcriptional activator. Tcf-4 is the only Tcf protein among all isoforms being consistently expressed in colorectal epithelial cells [113]. Mutation in Tcf-4 is known in a subset of colorectal cancer but can not substitute for APC or β-catenin mutations [114-116]. Moreover, Tcf-4 mutations are thought to have an additive rather than an initiating effect on neoplastic transformation. Once transformation of the colorectal epithelium has occurred, expression of Lef-1, a close homolog of Tcf-4, is upregulated. Only expression of the β-catenin sensitive isoform of Lef-1 is upregulated and can support the activation of Tcf/Lef target genes [117]. Many ß-catenin/Tcf target genes are known to contribute to tumor initiation and progression in colorectal adenocarcinoma from mice and humans [118]. For eg, c-myc and Cyclin D. c-myc is also overexpressed in other type of cancers due to its rearrangement or amplification whereas cyclin D overexpression was due to genetic alternations in the p16INK4a growth-inhibitory pathway, which includes Rb, CDK4, and Cyclin D1 [119, 120]. Matrilysin/MMP-7 is another target gene with critical functions in cancer promotion. In the absence of the metalloproteinase MMP-7, intestinal tumorigenesis is strongly suppressed in APC mutant mice [121-123]. WISP-1 is a ß-catenin/Tcf-4 target gene belonging to the CCN family of growth factors and cells overexpressing WISP-1 show characteristics of transformed cells including induction of tumor growth in nude mice [124]. Other genes proposed as targets of ß-catenin/Tcf include the gap junction protein connexin 43, peroxisome proliferator-activated receptor-δ, survivin, c-jun, fra-1, uPAR, ZO-1, NBL-4 , DRCTNNB1A , MDR1 , brachyury etc.

However, expression of target genes in respone to active Wnt signaling is tissue specific.

1.4.6 WNT signaling abnormalities in human cancers.

A wide range of human cancers carry mutations in at least one component of the canonical Wnt/β-catenin pathway leading to a ligand-independent stabilization of β-catenin. Approximately 50% of colorectal cancers (CRC) harbor loss-of-function mutations in the tumor suppressor gene APC. Loss of APC function is observed at an early stage in colorectal carcinogenesis and is believed to be the initiating event for formation of adenomatous polyps, and further progression of disease by conferring chromosomal instability in both inherited and sporadic cases of colon cancer. Inactivation of APC function leads to chromosomal instablity because of its important role in chromosomal processing. Interestingly, despite of fact, APC is widely expressed in other tissues, the incidence of its mutations is relatively rare in cancer outside the colon [76, 125]. Neverthless, mutation in β-catenin is a more common event in other types of human cancers including gastric, hepatocellular and ovarian cancers. Their incidence ranges from a few percent to as much as 60%. These mutations of β-catenin affect the N-terminal phosphorylation sites and thus render β-catenin resistant to phosphorylation and ubiquitination [126-128]. Mutations in Axin are also found in 5-10% of hepatocellular carcinomas and also in a small number of colorectal cancers lacking mutations in APC or β-catenin [103, 109]. Whereas, in ovarian cancer, nuclear β-catenin accumulation in approximately one third of cases indicates Wnt signaling activation, commonly associated with β-catenin point mutations [109].

A principle that emerges from these studies is that potentially any mutation that results in elevated β-catenin levels without decreasing cell viability may contribute to a premalignant condition or tumorigenesis. Aberrant activation of the other PCP and calcium pathways in malignant tissue is less well characterized and their relevance to human cancer is largely unknown. Wnt signaling dysregulation due to activating β-catenin mutation is seen in other gastrointestinal tumors like hepatoblastoma (52-89%), hepatocellular carcinoma

(20% and higher in hepatitis associated carcinoma) but in esophageal cancers no ß-catenin, APC or Axin mutations have been reported [129, 130] . Moreover, overexpression of FzE3 in squamous cell esophageal cancers correlates with nuclear translocation of β-catenin [69, 131]. In intestinal-type gastric cancer β-catenin mutations have been reported in some cases, but no β-catenin mutations were found in diffuse-type gastric cancer [131, [132]].

Small Intestinal adenocarcinoma is rare and morphologically similar to the colorectal adenocarcinoma. Although the adenoma to carcinoma pathway in colorectal cancer is well described, the mechanism of carcinogenesis in small intestine remains unclear [50]. Due to morphologic similarity and common predisposing etiological factors, it is rationale to presume similar genetic and molecular alterations in between small intestinal and colorectal adenocarcinoma. However, recent molecular studies have given new dimension to this discussion showing that molecular pathways of sporadic tumorigenesis differ in small intestine compared to large intestine [82, 83, 133, 134]. Aberrant expression of p53, RB and k-ras genes as well as microsatellite instability occurs at similar frequency in both small and large intestinal adenocarcinoma. Stabilization of β-catenin, a central oncogene activated in Wnt/wingless dysregulation, can be detected in 50% of sporadic small bowel carcinomas. However, an inactivation of the adenomatous polyposis coli (APC), the gatekeeper mutation and an indirect evidence of deregulated Wnt signaling pathway in colorectal cancer, is rarely observed in small bowel carcinomas [82, 83, 135, 136]. These findings suggest a differential regulation of cellular growth in the small intestine conferring higher resistance compared to colon.

These observations prompted us to hypothesize that activated Wnt signaling pathway plays an important role in small intestinal adenocarcinoma although the mechanism of its activation differs from colorectal carcinoma. Based on this hypothesis, the main aim of my research was to elucidate the molecular background of β-catenin stabilization in small intestinal adenocarcinoma and to compare the findings with early age onset of non hereditary colorectal cancers, usually displaying no APC mutations.

OBJECTIVES

The molecular background of small bowel adenocarcinomas is incompletely understood. Similar to colorectal cancer nuclear accumulation of β-catenin has been reported as a common phenomenon in this tumor type. In contrast to colorectal cancer, however, this feature can not be explained by inactivation of the APC tumor suppressor.

In this study we aimed in identifying the mechanisms of β-catenin accumulation in small bowel cancer by trying to answer the following questions.

- How important is β-catenin accumulation for small bowel adenocarcinoma and are the findings comparable to those in early age onset of non hereditary colorectal cancer?
- Is β-catenin accumulation caused by inactivation of the degradation machinery or is it associated with alterations of the β-catenin itself?
- If β-catenin alterations occurred did they affect single amino acids or did gross alterations occur?
- What is the genetic mechanism behind β-catenin alterations?

By answering these questions we identified large scale deletions in the *CTNNB1* gene in small intestinal carcinomas and in some early age of onset non hereditary colon cancers. Small intestinal carcinomas with large scale β-catenin deletions displayed strong homogenous nuclear β-catenin accumulation throughout the tumor areas, whereas colon cancers showed patchy areas of homogenous nuclear accumulation. We analysed the functional effect of these large scale deletion mutations and compared the effects to to those of point mutations in order to find out the following :

- What are the functional consequences of these large scale mutations in cell culture models?
- Do the large scale mutations exhibit a similar effect on proliferation and migration like single amino acid substitutions?
- What is the cellular sub localization of the mutated β-catenin?

Therefore, we analysed paraffin embedded tumor material from a total of 20 small and 20 large intestinal adenocarcinomas, prepared mutant β-catenin clones, corresponding to the mutations found in the tumors, and transfected these mutants into two cell lines for functional analysis.

3. MATERIAL AND METHODS

3.1 MATERIALS

3.1.1 Solutions, chemicals and kits

Qproteome FFPE Tissue Kit	Qiagen (Hilden)
DNeasy Blood & Tissue Kit	Qiagen (Hilden)
RNeasy FFPE Kit	Qiagen (Hilden)
Enzyme and Buffer	Peqlab (Erlangen)
dNTPs Set	Roche (Mannheim)
Primers	Thermo Scientific (Braunschweig)
SuperScript® III One-Step RT-PCR	Invitrogen (Karlsruhe)
Big Dye Termination sequencing Kit	Applied Biosystems (Warrington, UK)
High Pure PCR Product Purification Kit	Roche (Mannheim)
Filme (Western Blotting)	Amersham Bioscience
ECL plus Western Blotting Detection System	(Frieburg)
Immobilon PVDF membrane (0.45 µm)	Millipore (Schwalbach)
Acrylamide gel solution (30%)	Serva Electrophoresis(Heidelberg)
TEMED	
Ammonium persulfate	
Standard Chemicals	Bio-Rad (Munich)
(not mentioned separately)	BD Bioscience (Heidelberg)
	Merck (Darmstadt)
	Roche (Mannheim)
	Roth (Karlsruhe)
	Sigma (Deisenhofen)
Restriction Enzymes and Buffers	New England Biolabs (Frankfurt)
	MBIFermentas (Bad Durrenberg)
Rapid Ligation Kit	Roche (Mannheim)
UltraPureTM RNase free Water	Invitrogen (Karlsruhe)

DH5α Competent cell	Invitrogen (Karlsruhe)
QIAprep Spin Miniprep Kit	Qiagen (Hilden)
QIAfilter Plasmid Midi Kit	Qiagen (Hilden)
Pipette, Tips and Eppendorf	Greiner (Nürtinger)
	Eppendorf (Hamburg)
	ABImed (Langenfeld)
Media and additionals for the Cell Culture	PAA Laboratories (Cölbe)
Plasticware Cell Culture	Greiner (Nürtinger)
	Falcon (Heidelberg)
	NuncTM (Wiesbaden)
	TPP (Basel, Schweiz)
Sodium Butyrate	Millipore (Schwalbach)
Lipofectamine 2000 and Geneticin	Invitrogen (Karlsruhe)
BCA Protein Assay Kit	Pierce (Braunschweig)
VenorGeM, Mycoplasma Detection Kit	Minerva Biolabs (Berlin)

3.1.2 Devices

ABI – 377 DNA Sequencer	AppliedBiosystems (Darmstadt)
Agarose Gel Electrophoresis System	Keutz (Reiskirchen)
CO2- Incubator (Hera cell 150)	Heraeus (Hanau)
Cell Culture centrifuge Universal R32	Hettich Centrifuge (Tuttlingen)
Centrifuge Varifuge 3.0/3.0 R	Heraeus/Kendro (Hanau)
ELISA-Reader (Multiscan Ascent)	Thermo Electron Corporation
FACS CaliburTM	BD Bioscience (Heidelberg)
Gel Documentation System (Alpha ImagerTM)	Biozym (Oldendorf)
Fluorchem-Imaging System WB	Alpha Innotech/Biozym (Oldendorf)
Film Developer Machine (Optimax Typ TR)	MS Laborgeräte (Heidelberg)
Fluorescence Microscope	Leica (Wetzlar)
Inverse Microscope Olympus CKX41	Olympus (Hamburg)
Inverse Microscope (Axiovert 25)	Zeiss (Jena)

Camera Altra 20 (Soft Imaging System)	Olympus (Hamburg)
Laminar Flow (Microflow)	NuncTM (Wiesbaden)
Microfuge Micro 200	Hettich centrifuge (Tuttlingen)
Microwave	Panasonic (Heidelberg)
Nanodrop ND-1000 UV-VIS Spectrophotometer	PeqlabBiotechnologie (Erlangen)
PAA Gel Electrophoresis System	Biozym (Oldendorf)
PCR Block (Multi Cycler PTC)	Biozym (Oldendorf)
(pH 210 Microprocessor pH Meter)	Hanna Instruments (Kehl am Rhein)
Photometer	Eppendorf (Hamburg)
Orbital Shaker (Biometra WT17)	Biometra (Göttingen)
Shaking- Thermoblock	Eppendorf (Hamburg)
Western Blot Apparatus (LKBMultiphorII)	Pharmacia/Pfizer Pharma (Karlsruhe)
Western Blot Apparatus (Wetblot)	Biozym (Oldendorf)

3.1.3 Software

Analysis getIT Vers. 5.0	Olympus (Hamburg)
Axio Vision Rel. 4.6	Zeiss (Jena)
CellQuest Pro	BD Bioscience (Heidelberg)
Multicycle program	Phoenix Flow Systems (San Diego, CA)
Endnote	Thomson Reuters, USA

3.1.4 Antibody

The following primary antibodies were used: Mouse β-catenin (BD, Heidelberg, Germany), Rabbit α catenin and mouse Flag M2 clone (Sigma, Deisenhofen, Germany), Rabbit GFP (Abcam, Heidelberg, Germany), Mouse cyclin D (DCS-6 SC20044) and mouse β-actin (Santacruz, Heidelberg, Germany) . As secondary antibodies we used anti-rabbit,anti-mouse (cell signaling, Heidelberg, Germany)

coupled to horse raddish peroxidase (HRP) for western blot . For immunofluorescence, we used goat anti-mouse ALEXA 568 and goat anti rabbit Alexa 466 (Molecular probes, OR, USA). For visualization in IHC, the labelled immunoperoxidase method with AEC as a chromogen was applied in an automated staining system(Techmate Horizon; Dako, Hamburg, Germany).

3.1.5 Tumor tissues.

Twenty non-FAP-associated small intestinal and 20 non hereditary early age of onset colorectal adenocarcinomas were investigated (Table 2.1). Small intestinal adenocarcinoma includes eight duodenal, three jejunal and two ileal adenocarcinomas, as well as seven carcinomas in segmented small intestinal resections without specification of jejunal or ileal origin. Fifteen patients were male, five female. Patient age was in the range 35–90 (median 71) years. Colorectal adenocarcinoma patient's age was in the range 22-35. The clinical data and tumor location for small intestinal and colorectal adenocarcinoma are given in Table 1. The study was approved by the institutional ethics committee (Application No. 206/05) at the Medical Faculty at Heidelberg University.

Table 2.1: Clinical and pathological data for patients tumor tissues.

ID	Age	Gender	Tumor Location/Differentiation
Small Intestinal Adenocarcinoma			
T3	71	Male	Ileum/adenocarcinoma
T7	66	Male	Jejunum/ileum/adenocarcinoma
T12	75	Male	Jejunum/ileum/mucinous adenocarcinoma
T13	83	Male	Ileum/adenocarcinoma
T18	74	Male	Duodenum/mucinous adenocarcinoma
T19	68	Male	Jejunum/mucinous adenocarcinoma
T24	74	Female	Jejunum/adenocarcinoma
T30	71	Male	Duodenum/mucinous adenocarcinoma
T160	79	Female	Jejunum/ileum/adenocarcinoma
T172	84	Male	Jejunum/adenocarcinoma
T174	61	Male	Duodenum/adenocarcinoma
T180	64	Male	Duodenum/adenocarcinoma
T206	90	Female	Jejunum/ileum/adenocarcinoma
T235	44	Female	Duodenum/adenocarcinoma
T255	53	Male	Jejunum/ileum/adenocarcinoma
T256	35	Male	Duodenum/adenocarcinoma
T320	37	Male	Duodenum/adenocarcinoma
T410	74	Female	Duodenum/mucinous adenocarcinoma
T716	54	Male	Duodenum/adenocarcinoma
T820	72	Male	Jejunum/ileum/adenocarcinoma
Colorectal Adenocarcinoma			
T542	33	Male	Right colon/adenocarcinoma
T128	32	Male	Liver met/adenocarcinoma
T272	28	Female	Periton met/adenocarcinoma
T084	27	Female	Pancreas metast/mucinous adenocarcinoma
T123	34	Female	Sigma/adenocarcinoma
T097	22	Female	Rectum/adenocarcinoma
T353	32	Male	Colon nos/adenocarcinoma
T576	33	Female	Sigma/adenocarcinoma
T353	35	Female	Coecum/adenocarcinoma
T320	33	Male	Sigmoid/adenocarcinoma
T273	33	Female	Sigmoid/adenocarcinoma
T409	28	Female	Right colon/adenocarcinoma
T316	31	Male	Coecum/adenocarcinoma
T240	34	Male	Metast/adenocarcinoma
T115	25	Female	Rectum/mucinous adenocarcinoma
T183	35	Male	Perit metast/adenocarcinoma
T173	34	Male	Colon nos/adenocarcinoma
T117	32	Female	Ascending Colon
T832	30	Male	Right colon
T218	31	Male	Right colon

3.2 METHODS

3.2.1 In vivo paraffin tumor analysis.

3.2.1.1 Immunohistochemistry.

Immunohistochemistry was performed on 5 µm thick paraffin sections, pretreated by 10 min microwave boiling in a 0.2 mM citrate buffer. A monoclonal antibody against amino acids 571–781 of human β-catenin (1:200; clone 14, BD Heidelberg, Germany) was incubated for 30 min at room temperature. For visualization purposes, the labelled immuno peroxidase method with AEC as a chromogen was applied in an automated staining system (Techmate Horizon; Dako, Hamburg, Germany). The staining pattern and intensity were compared between tumor cells and normal mucosa in individual samples and this ratio was compared to the other investigated cases. A significant increase of β-catenin staining in the cytoplasm was scored as ‘stabilization’. In cases with stabilization, two different patterns were distinguished: (a) cytoplasmic accumulation with only focal nuclear translocation in the invasion front of the tumors was scored as cytoplasmic predominant’; and (b) accumulation in all tumor nuclei was scored as ‘nuclear-predominant’.

3.2.1.2 Protein extraction from paraffin fixed tumor tissue.

In order to achieve protein isolation from paraffin embedded tissues (nine slides with 5 µm each per sample), the Qproteome FFPE tissue kit was used according to the manufacturer’s instructions (Qiagen, Hilden, Germany). Total protein extracts were quantified (280 nm, Nanodrop photometer), separated on 10% SDS–PAGE (40 µg/lane), electro-transferred to a PVDF membrane for western blotting.

3.2.1.3 DNA and RNA extraction from paraffin fixed tumor tissue.

Sections (5 µm thick) from paraffin-embedded tumor tissues were de-waxed in xylene for 10 min, washed in 100%, 90% and 80% ethanol and rehydrated in distilled water. Unstained sections were analysed under the microscope to scrape tumor areas with at least 70% tumor cells. DNA and RNA, was isolated using the DNeasy and the RNeasy FFPE kit (Qiagen). Isolation was performed according to the manufacturer's instructions.

3.2.1.4 Genomic DNA analysis.

For the mutational analysis of exon 3, intra-exonic primers were used as previously described [107]. For the mutational analysis of exon 2 and 4 as well as for intron 2 and 3, new primers were synthesized (Table 2.2), based on the nucleic acid sequences given in the genomic database (RNA: NM 001098209, DNA: NT 022517). For the investigations on long-distance deletions, forward primers from intron 2 were combined with reverse primers from exon 3 and intron 3. PCR reactions were performed for 35 cycles under standard temperature conditions.

3.2.1.5 cDNA analysis.

For the cDNA-analysis, a one-step RT–PCR procedure (Superscript^{TM}III, Invitrogen, CA, USA) was applied according to the manufacturer's instructions. cDNA primers flanking the boundaries of exon 2–5 were applied (Table 2.2). A forward primer in exon 2 was combined with a reverse primer from exon 3 to check RNA quality. The same exon 2 forward primer was combined with reverse primers in exon 4 and 5 in order to identify large deletions of coding sequences.

3.2.1.6 Sequencing.

Genomic and RT–PCR fragments were purified using the High Pure PCR Purification kit (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer, subjected to direct cycle sequencing using a bigDye termination kit (Applied Biosystems, Warrington, UK) and automated sequencing by ABIPrism 377 (Applied Biosystems). Sequencing was performed in forward and reverse directions using primers identical to those for primary PCR (cDNA, exon 2–exon 5; DNA, previously published primers [107]). For deletion mutants, primers generating the shortened fragments in primary PCR at the DNA level were used for sequencing and the case-corresponding RNA was sequenced using forward and reverse cDNA primers of exon 2-exon 4 in cases T3, T13 and T820, as well as forward primer for cDNA exon 2 and reverse primer for exon cDNA exon 5 for case T256.

3.2.2 Generation of mutated coding sequences.

3.2.2.1 Overlap extension polymerase chain reaction (PCR).

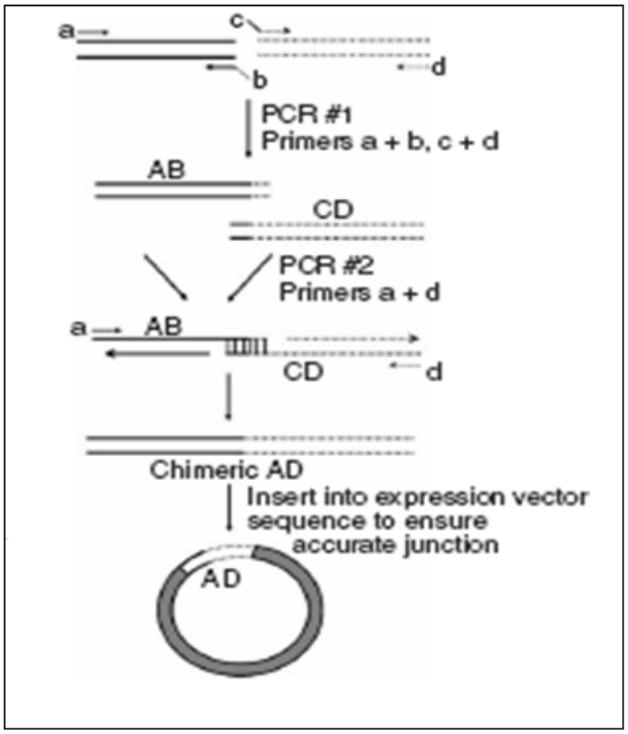
Extension of overlapping gene segments by PCR is a simple, versatile technique for site-directed mutagenesis and gene splicing [137]. We used this method to create large in frame deletion mutant constructs of β-catenin. Initial PCRs generate overlapping gene segments (AB, CD) as seen in Figure (2.1), are then used as template DNA for another PCR to create a full-length product. Internal primers generate overlapping, complementary 3' ends on the intermediate segments to introduce deletions for site-directed mutagenesis. For gene splicing, internal primers encode the nucleotides found at the junction of adjoining gene segments. Overlapping strands of these intermediate products hybridize at this 3' region in a subsequent PCR and are extended to generate the full-length product, amplified by flanking primers that can include restriction enzyme sites for inserting the product into an expression vector for cloning purposes.

Table 2.2 Primers for gDNA and cDNA analysis in SIC and CRC

Region	Forward Primer	Reverse Primer
cDNA Exon 2–Exon 3	5'-cctgttcccctgagggtatt-3'	5'-ctgtggtagtggcaccagaa-3'
cDNA Exon 2–Exon 4	5'-cctgttcccctgagggtatt-3'	5'-tgcatgccctcatctaatgt-3'
cDNA Exon 3–Exon 5	5'-tggatacctcccaagtcctg-3'	5'-gcatgatagcgtgtctggaa-3'
DNA Intron 2A	5'-gggtatttgaagtataccatacaactg-3'	5'-gctggtggcttgtttgcta-3'
Intron 2B	5'-ccttttgctccattttctgc-3'	5'-tcaaatctgaaagacagccaag-3'
Intron 2C	5'-ctgagctaaccctggctatca-3'	5'-aacagccgcttttctgtctg-3'
Intron 3A	5'-tggatacctcccaagtcctg-3'	5'-tccacagttcagcatttacct-3'
Intron 3B	5'-aaatgttgtggtgaagaaaagaga-3'	5'-ggatgagcagcatcaaactg-3'
Exon 4	5'-gctgaactgtggatagtgagtggt-3'	5'-tgaaactactccccttgagca-3'

3.2.2.2 Mutagenic primers.

A list of primers used for construction of the wild and mutated β-catenin constructs are given in Table (2.3). The primers were designed in a way to create desired large in frame deletion. Primers 'b' and 'c' consist of overlapping regions whereas flanking primers 'a' and 'd' contains restriction enzymes to facilitate cloning of modified PCR product into two different mammalian expression vector. The primers 'a' and'd' were constructed to introduce unique KpnI restriction endo nuclease sites at the 5'-end and BamHI sites at the 3' end of the amplified fragments. The primers were used also to screen the cloned DNA of β-catenin constructs for the presence of mutant inserts (Hybaid PCR Express Thermal Cycler). Flag tag was added at C-terminus of all constructs by using reverse primers 'd' with flag tag followed by BamHI restriction site. Forward primer also consists of Kozak sequence after KpnI restriction site.



AB : mutated cDNA fragment of β-cat from tumors.
CD : cDNA fragment amplified from CTNNB1 cDNA clone (IMAGE:6151332) containing wild type β- catenin in pCMV-SPORT6.
AD : Complete β-cat mutated gene in frame.

Figure 2.1 PCR-mediated overlap extension method to generate a chimeric gene products. Chimeric gene products were generated by two PCRs using two internal primers b and c and flanking primers (a and d) containing restriction sites KpnI and BamHI (Primer d also consist of FLAG tag) to generate intermediate PCR products AB and CD with an overlapping fragments including nucleotides that span the junction of segments AB (solid line) and CD (dashed line). Products AB and CD are denatured and used as template DNA for the second PCR; strands of each product hybridize at their overlapping, complementary regions containing the desired mutation (here the mutation from patient sample) .The second PCR generates the hybrid gene product AD with restriction sites which is then digested and ligated into mammalian expression vector pcDNA3. (Reviewed by Heckman and Pease 2007)

3.2.2.3 Plasmid construction.

Human β-catenin cDNA clone (IMAGE: 6151332) in pCMV-SPORT6 (ATCC, Wesel, Germany) was used to generate deletion mutants (Fig 4.1). Mutations observed in tumor samples (T256 and T3) were generated by joining mutated cDNA fragment from paraffin fixed patient tissue to later wild type β-catenin obtained from cDNA clone (IMAGE:6151332) by PCR driven overlap extension method. Mutant T256 lacked ∆(A17 – A128) and mutant T3 lacked ∆(A5 – A80). Mutant T60 lacking ∆(A60-A110) was prepared by joining initial PCR fragments AB containing nucleotide sequence from codon 1 to 60 and second PCR fragment CD from codon 110 to 780. Primer 'b' was designed to contain few overlapping nucleotide sequence from fragment CD. Point mutant ΔS45 & S33Y was obtained by RT-PCR from HCT116 (Colon Carcinoma Cell Line) and SW48 know to harbour deletion of serine at 45 and a point mutation at codon 33, respectively. All primers contained restriction site to facilitate cloning into pcDNA3 vector (Invitrogen). The β-catenin cDNA insert sequences were PCR-amplified using forward and reverse primers (Table 2.3) containing KpnI site (in forward primer) and BamH1 (in reverse primer) restriction sites and cloned in-frame into pcDNA3.

FLAG tagged β-catenin pcDNA3 plasmids were used as templates to PCR amplify wild type and mutant β-catenin products without the FLAG tag but with restriction sites KpnI and BamH1 (Table 2.3) to tag all of these constructs with GFP tag at the C-terminus. PCR product were digested and ligated in mammalian expression plasmid pEGFP-N1 (Clontech). The β-catenin cDNA insert sequences were PCR-amplified using forward and reverse primers containing KpnI site (in forward primer) and BamH1 (in reverse primer) restriction sites. β-catenin cDNA PCR products were then cloned in-frame into pEGFP-N1 vector. All clones were confirmed by sequencing.

Table 2.3 Primers used for generating wild type and mutant β-catenin and further cloning into pcDNA3 and pEGFP-N1 with Flag and GFP tag respectively.

Construct FLAG TAG at C-terminus in pcDNA3 vector	**Forward Primer**	**Reverse Primer**
β-catenin(WT)	5' taatgcggccgcatggctac tcaagctgatttgatggagttg 3'	5' attaggatccctacttgtcatcgtcatccttg taatccaggtcagtatcaaaccaggccagc 3'
β-catenin AB	5' cctgttcccctgagggtatt3'	5'gcatgatagcgtgtctgga 3'
β-catenin CD	5' ctgctaaatgacgaggaccag3'	5' ccaatcacaatgcaagttcag 3'
β-catenin(T256) AD	WT (Forward)	WT(Reverse)
β-catenin(T3) AD	5' taatgcggccgcatggctactcaag Atattgatggacagtatgc 3'	WT(Reverse)
β-catenin(T60) AB	WT (Forward)	5'catcaaactgtgtagaggaggt atccacatcctcttcctcagg 3'
β-catenin(T60) CD	5' tctacacagtttgatgctgctcatcccac 3'	WT(Reverse)
β-catenin(T60) AD	WT (Forward)	WT(Reverse)
β-catenin S33Y	WT (Forward)	WT(Reverse)
β-catenin ΔS45	WT (Forward)	WT(Reverse)
Construct GFP TAG at C-terminus in pEGFP-N1 Vector	Forward Primer	Reverse Primer
β-catenin (all construct excepet T3)----GFP	5'gtcgacggtacccatggct actcaagctgatttgatg 3'	5'accggtggatcccgcagg tcagtatcaaaccag 3'
β-catenin(T3)---GFP	5' gtcgacggtacccatggcta ctcaagatattgatggacagtatgc 3'	5'accggtggatcccgcaggt cagtatcaaaccag 3'

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3.2.3 Standard DNA manipulation techniques.

3.2.3.1 Digestion with restriction endonucleases.

Cleavage by restriction enzymes (or restriction endonucleases) plays a key role in all the gene manipulation (cloning) procedures. These enzymes, part of the bacterial restriction/modification (or restriction/methylation) systems, allow bacteria to monitor the origin of incoming DNA and to destroy the molecules recognized as foreign. In particular, the enzymes termed type II restriction endonucleases are important in all aspects of molecular biology. They recognize specific sequences (usually 4 – 6 bp in length) and of specific base composition, and cleave the DNA into fragments in a defined manner. The sequences recognized are usually palindromic, that is they read the same in both directions on each strand, and when cleaved, they leave a cohesive-ended fragment. The ends produced from different molecules but by the same enzyme are complementary (also 'cohesive' or 'sticky') and consequently, anneal to each other. The standard reaction mixture contained DNA to be cut, the appropriate enzyme, enzyme buffer (from 10 × stock) and sterile distilled water. All digests were done at 37°C. To double digest pcDNA3 and PEGFPn1 vector for cloning PCR products, 15 µl (5 µg) of vector DNA was digested for 2-3 hours with 1 µl (20 units) of BamHI and 4 µl (40 units) of KpnI in 1x BamHI buffer in a total reaction volume of 250 µl. Wild type and mutated β-catenin PCR product were gel purified and eluted in 30 µl elution buffer for double digestion with BamHI and KpnI. For digestion, 30 µl of eluted PCR product was digested overnight with 1 µl (20 units) of BamHI and 4 µl (40 units) of KpnI in 1x BamHI buffer in a total reaction volume of 60 µl. Both digested vector backbone and PCR products were gel purified after excising desired band and used for setting up the ligation reaction. However, vector backbone was de-phosphorylated too to avoid background colonies, formed of incomplete restriction digestion and ligation.

3.2.3.2 Dephosphorylation.

To reduce the background in cloning experiments, phosphatases which remove the 5'-terminal phosphate groups from a nucleic acid strand are used. By dephosphorylating the restricted cloning vector they prevent its re-ligation, eliminating any possibility of dimmer formation or re-circularization of the vector. The restricted, linearized vector DNA was treated with calf intestine alkaline phosphatase before ligation. For dephosphorylation of restricted vector, 1 – 3 µg of DNA was treated with 0.25 – 0.5 units of enzyme for 30 min at 37°C in the final volume of 50 µl.

3.2.3.3 DNA purification from gel and solution.

Usually, DNA at subsequent stages of a cloning procedure requires purification to remove the contaminants from the previous step, e.g. enzymes, buffer components, etc. Purification of DNA from solution or gel during cloning different β-catenin constructs, generated by PCR was performed using High pure PCR product purification kit from Roche. This kit is designed for the efficient and convenient isolation of PCR products from amplification reactions for removing primers, mineral oil, salts, unincorporated nucleotides, and the thermostable DNA polymerase which may inhibit subsequent enzymatic reactions involved in cloning of the PCR products. Moreover, nucleic acids from other modification reactions (e.g., restriction endonuclease digests, alkaline-phosphatase treatment, or kinase reactions) were also purified using this kit. Briefly, 1 volume of the DNA sample mixed with 5 volumes of binding buffer (allowing efficient recovery of DNA fragments starting from 100 bp) was applied to the high pure filter tube and centrifuged (microcentrifuge, 13,000 rpm) for 1 min. Flow-through was discarded and the column washed twice with wash Buffer (centrifuged as before, flow through discarded and centrifuged again). DNA was eluted with Buffer EB (up to 50 µl) or sterile distilled water (for optimum yield, water pH was adjusted to 7.0 – 8.5). The purification of DNA fragments from agarose gels was performed similarly after dissolving gel containing DNA fragment in binding buffer at 56 degree in heating block. The procedure followed enzymatic reactions and was

done to separate digested DNA from its uncut fraction, but mostly to isolate an appropriate DNA fragments prior to the ligation reaction.

3.2.3.4 Ligation.

The cohesive ends produced by certain restriction enzymes anneal and can be joined covalently by DNA ligase to create artificially recombinant molecules. The ligase forms a covalent bond between the 5'-phosphate at the end of one strand and the 3'-hydroxyl of the adjacent one. Ligation of the specific DNA fragment with a specialized DNA carrier molecule (vector) is necessary in order to be able to propagate the DNA of interest. Ligation was performed using rapid ligation kit from Roche as recommended in the manufacturers instruction. Briefly, 1 µl (5 units) of T4 DNA ligase was used and the vector/insert ratio of 1:5 for all β-catenin constructs (total DNA about 100 ng) in a final volume of 20 µl was used. A control ligation reaction with vector but no insert DNA present was set up at the same time to assess the likelihood of vector re-ligation (reaction background). The reaction was performed at room temperature for five minutes.

3.2.3.5 Bacterial transformation.

The recombined (cut and ligated) DNA can be replicated indefinitely (cloned) within microbial cells. Bacterial transformation, the process by which bacterial cells take up naked DNA molecules, is an essential step in many cloning experiments. Bacterial cells are made competent by treatment with calcium chloride at low temperature (0 – 5°C) in the early logarithmic phase of growth. The bacterial cell membrane is permeable to chloride ions, but not to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA, however the exact mechanism of this process is unknown. Calcium chloride treatment has to be followed by a brief increase in temperature, termed heat shock (optimum at 42°C). E. coli cells survive at this temperature due to the expression of the heat shock genes. We used competent DH5α bacterial strain (Invitrogen) for transformation. DH5α, derivative of E. coli is

usually used for maintenance and storage of bacterial clones. DH5α is recA- (lacking the recombinase A gene) for insert stability of recombinants (and endA-, lacking the endonuclease A gene, for improving the quality of plasmid DNA). For transformation, 50 µl aliquots of competent cells were pipeted into pre-chilled tubes, mixed gently with about 1 µl of plasmid DNA preparation or up to 4 µl (40 ng of DNA) of ligation mixture, and left on ice for 5 min. The tubes were then heated for 30 s in a 42°C water bath and placed on ice for 2 min. Following the addition of 80 µl of LB medium at room temperature, the transformation mixtures were incubated with shaking for 60 min at 37°C. Cells were plated onto LB agar plates containing ampicillin or Kanamycin. Ten to twenty colonies were screened for each constructs by colony PCR and further validated by restriction digestion after mini prep for presence of insert. Finally, the clones were sequenced to avoid existence of any point or misense mutation that can effect the reading frame and eventually the protein sequence.

3.2.3.6 Colony PCR (Polymerase chain reaction).

Colony PCR was performed with single colonies (colony forming units) of transformed DH5α cells containing cloned plasmid to confirm that the correct gene was inserted into the vector. The transformed cells were grown on Luria-Bertani (LB) agar plates containing the appropriate antibiotics. Forward and reverse primers used for colony PCR had overlapping regions from the plasmid vector that would produce a PCR product containing the gene if it had been positively cloned. Single colonies were picked from the LB plate and resuspended in 6 µL of double distilled water (ddH2O). Four microliters of the resuspended colony were added to a PCR mixture with a final volume of 25 µL. The reaction volume consisted of 1 unit (U) of Taq DNA polymerase, 100 µM of each dNTP, 1X Reaction Buffer, 1 µM of each of the primers. The DNA containing the inserted gene was amplified by a PCR Thermocycler using the following cycles: 94°C for 2 min; 25 cycles of 94°C for 45 sec, 58°C for 1 min, 72°C for 1 min; 72°C for 10 min. The PCR products were resolved with a 1% agarose gel and the DNA bands were viewed under UV light in the GelDoc.

3.2.3.7 Mammalian expression plasmids.

Mammalian expression plasmid pcDNA3 (Invitrogen) and pEGFP-N1 (Clontech) were used as vector for cloning β-catenin WT and mutant constructs. pcDNA3 was used for cloning β-catenin constructs with FLAG tag at C-terminus whereas pEGFP-N1 vector generates GFP tag at C-terminus. All constructs were generated by cloning PCR product (β-catenin WT and mutant) into above mentioned vector using KpnI and BamH1 restriction site. Constructs were used for transient and stable transfection in SW480 and MDCK cell culture.

pcDNA3 (Fig 2.2) is an expression vector widely used for constitutive, high level expression in mammalian cells. It is useful for studying protein expression by transient transfection as well as by stable integration into the genome. It consists of CMV (Immediate-early Cytomegalovirus virus promoter) for high-level expression in a wide variety of mammalian cell lines. Flag tag was attached at the C-terminus while generating different inserts by PCR. Primer sequences were designed to contain the nucleotide sequence for generating Flag tag at the C-terminus of the protein upon expression after transfection. pcDNA3 vectors were used for transient transfection of SW480 cells.

pEGFP-N1 (Fig 2.2) is also a mammalian expression vector with a CMV promoter. We generated β-catenin wild type and different mutant form pcDNA3 constructs by PCR. Primers were designed to contain restriction sites for KpnI and BamHI but lacking flag nucleotide sequence. GFP sequence after BamHI restriction site, adds GFP tag at C-terminus of β-catenin. These clones were used to generate stable cell pool in MDCK cell after transfection and selection with G418. Addition of GFP tag facilitates the selection of MDCK cells stably transfected with β-catenin wild type and mutants.

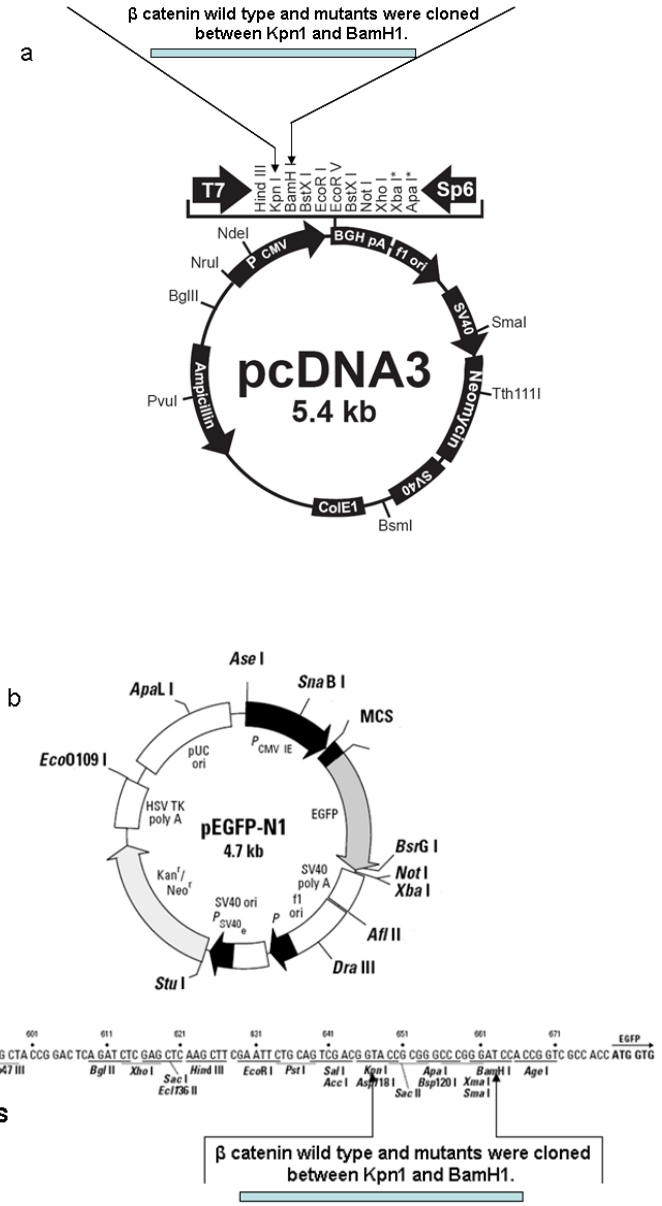


Figure 2.2 Vector Map of Plasmids used in cloning: a) pcDNA3 vector map, used for generating Flag Tag constructs using KpnI and BamHI site of MCS region. B) pEGFP-N1 vector map, used for generating GFP tag constructs using KpnI and BamHI site of MCS region.

3.2.4 Isolation and purification of plasmid DNA.

Isolation of plasmid DNA is a standard requirement for any DNA analysis and/or manipulation purposes. In this work the alkaline lysis procedure was used, the most common method of DNA isolation. This technique exploits the difference in topology and consequently, in denaturation and renaturation characteristics of small, covalently closed circular plasmid DNA molecules and much larger chromosomal DNA. Under alkaline conditions (pH 11) both DNAs are denatured. High pH changes the condition of ionizable groups (ionizing certain groups and deionizing others), while SDS (ionic detergent) disrupts cell membranes and destabilizes all hydrophobic interactions holding various macromolecules in their native conformation. Rapid neutralization of the alkaline medium with a high salt potassium acetate buffer causes the chromosomal DNA, being too large to renature correctly and form an insoluble aggregate. Small plasmid DNA molecules remain in solution due to their covalently closed nature promoting interstrand rehybridization. Rapid neutralization also precipitates denatured proteins (and cell debris) along with insoluble potassium dodecyl sulphate. This allows the entrapment and precipitation of the high molecular weight chromosomal DNA. Soluble and insoluble materials are separated by centrifugation. The scale of the procedure may vary, depending on the amount of DNA that is required.

3.2.4.1 Small scale isolation (‘Miniprep’).

The Plasmid Mini Kit by Qiagen was used according to the manufacturer’s instructions. Qiagen plasmid DNA isolation protocols are based on the modified alkaline lysis procedure. However, the deproteinisation step of phenol/chloroform extraction is replaced by the column chromatography and the supernatant material (after centrifugation following the neutralisation step) is applied to the Qiagen Anion-Exchange Resin. The resin binds the plasmid DNA under

appropriate low-salt and pH conditions, while all the contaminants (RNA, proteins, dyes, low-molecular-weight impurities) are removed by a medium-salt wash. DNA is eluted in a high-salt buffer and then desalted and concentrated by isopropanol precipitation. DNA, isolated from 10 ml overnight bacterial culture and isopropanol precipitated from the lysate, is redissolved in TE buffer and loaded onto the Qiagen resin. Then, it is purified according to the general protocol. This procedure yielded up to 7 µg of DNA from 10 ml of overnight culture.

3.2.4.2 Large scale isolation ('Maxiprep').

Two different Qiagen kits were used for the isolation of DNA in bigger quantities. These were the QIAfilter Plasmid Maxi and HiSpeed Plasmid Maxi Kit. Both were used according to the manufacturer's instructions. Both protocols rely on the modified alkaline lysis as a cell disruption method. Qiagen QIAfilter Plasmid Maxi Kit was used e.g. for DNA isolation from DH5α clones for the sequencing purposes. The purification protocol follows essentially the Mini Kit one but uses larger culture and reagent volumes. Additionally, QIAfilter Maxi Cartridges are used to clear (filter) bacterial lysates without centrifugation. The protocol yielded up to about 50 µg from about 250 ml of overnight culture. Qiagen HiSpeed Plasmid Maxi Kit was used to prepare plasmid DNA for transient transfection and generation of stable cell pools. This kit prepares plasmid of high purity which improves upon transfection efficiency. The purification procedure follows essentially the Maxi Kit one. Similarly, it employs Maxi Cartridges to clear bacterial lysate instead of centrifugation. However, use of the HiSpeed Tip packed with Qiagen Resin HS, in place of the conventional Qiagen-tip, reduces the time of DNA binding, washing and eluting. Eluted DNA, mixed with isopropanol, is applied to the QIAprecipitator Module where it is bound and dried and then released with an appropriate buffer. The HiSpeed Maxi Kit yielded up to about 70 µg of DNA from about 200 ml of overnight culture.

3.2.4.3 Glycerol stocks of bacterial strains.

For long-term storage 8% glycerol stocks of DH5α clones (different mutants) were prepared. A single colony of an appropriate clone was inoculated into 50 ml of LB broth with ampicillin (0.1 mg/ml) or kanamycin (0.05 mg/ml) and incubated with vigorous shaking at 37°C until the OD600 reached 0.6 – 0.9. 0.9 ml was removed from the culture and mixed by vortexing with 0.1 ml of sterile 80% glycerol. The stocks were stored frozen at -70°C. Before use, the strains were grown from a single colony on freshly prepared agar plates and incubated at 37°C overnight. Plates were stored at 4°C for up to 1 month.

3.2.5 Mammalian cell culture.

3.2.5.1 Cell lines.

All cultured cells were grown at 37° C in 5 % CO2 humidified atmosphere. MDCK (ATCC CCL-34, Normal kidney epithelial cells,) and SW480 (ATCC CCL-228, human colorectal adenocarcinoma cells) cells were grown in DMEM and RPMI medium respectively, supplemented with penicillin, streptomycin, and 10 % serum. Continuous cell culture was maintained in T75 flask and different experiments were performed in other well plate's format. MDCK stable cell pools over expressing β-catenin wild type and mutants were generated by transfection of pEGFP-N1 constructs, followed by selection in G418, were maintained in complete DMEM medium supplemented with 500 µg/ml of G418.

3.2.5.2 Cell culture media and solutions.

The products used for cell culture and transfection are mentioned below, mostly obtained from PAA (Colbe) if mentioned otherwise.

Product	Ingredients, Usage
DMEM, Dulbecco's modified Eagle's Medium Mit L-Glutamine	Glucose (4.5 g/l), mit L-Glutamin MDCK cell cultivation
DMSO (Sigma)	Cell Cryopreservation
FCS, Fetal Calf Serum	Additive for cell culture medium
RPMI, Roswell Park Memorial Institute	With L-Glutamin, SW480 cell Cultivation
Lipofectamine 2000 (Invitrogen)	Cationic Lipid Formulation for transfecting Plasmid DNA
OptiMEM (Gibco)	Medium for Plasmid DNA Transfection
Pencillin-Streptomycin-Antibiotic solution	10,000 U/ml Pencillin and 10mg/ml Streptomycin, as an antibacterial additive in cell culture medium.
Geneticin	500 µg/ml for selecting stable cell pool of MDCK cells
Trypsin EDTA solution	0.5 g/l Trypsin and 0.2 g/l EDTA, For Cell Passage

3.2.5.3 Cell line cultivation and passages.

The cell lines were maintained in culture by harvesting and passages. Harvesting of the cells was performed as followed:
The cells were shortly washed with 0.05 % trypsin/0.02 % EDTA in PBS solution and subsequently incubated with fresh trypsin/EDTA solution at 37°C. After the detachment of the cells trypsin was inhibited by addition of serum containing growth medium. EDTA was removed by centrifugation at 180 g to 500 g and

subsequently uptake of the cell pellet in fresh growth medium. The cell pellet was suspended in complete growth medium and passages into a split ratio of 1:5 or 1:10 to maintain the stock. The cells were counted by hematocytometer and seeded with different cell number depending on experiment to be performed.

3.2.5.4 Cell line preservation and thawing.

Cell lines in continuous culture are prone to genetic drift, finite cell lines are fated for senescence; all cell cultures are susceptible to microbial contamination. Moreover, we generated stable cell pool of MDCK cell over expressing β-catenin mutants, known to loose expression over 8-10 passages due to many different mechanism involved in gene silencing. It was vitally important to freeze the initial stable cell pool and get back to it in case cell pool in culture losses expression. For cryopreservation and thawing of cells, cultures should be healthy with a viability of >90% and no signs of microbial contamination. Cultures should be in log phase of growth (this can be achieved by using pre-confluent cultures i.e. cultures that are below their maximum cell density and by changing the culture medium 24 hours before freezing). Freezing medium containing 90% of serum and 10 % DMSO was prepared and maintained at 2 – 8 degree centrigrade. DMSO was used as a cryoprotectant to protect the cells from rupture by the formation of ice crystals. Cells were harvested by trypsinization. Harvested cell pellet was resuspended in cold freezing medium at the recommended viable cell density for the specific cell type. Cells were gently mixed to maintain a homogeneous cell suspension and freezed at –80°C overnight after keeping them in ice for 1 hour. Cells were thawed rapidly and then diluted slowly into warm growth medium. The best way to do this is to transfer the contents of the thawed vial to a 50 ml centrifuge tube and then add dropwise, with continual swirling 10 ml of warm medium. Then the cells were put on a 100 ml dish. This way the cells don't suffer osmotic shock. Some cells seem to benefit from being diluted into medium, spun down, and then resuspended in warm medium. This gets rid of the DMSO, but may disturb fragile cells. If the cells are seeded without spinning, the medium was changed as soon as the cells have attached.

3.2.5.5 Transient transfection of plasmid DNA.

SW480 were transiently transfected with different Flag tagged β-catenin constructs to study effects of mutations on β-catenin localization and oncogenic potential. SW480 cells were transfected with the use of Lipofectamine 2000 reagent (Invitrogen). Cells were trypsinized briefly 1 d before transfection and plated on 35-mm-diameter dishes so that they were 50-80% confluent on the day of transfection. 2 µg of DNA and 6 µl of Lipofectamine 2000 reagent was diluted in 100 µl of serum-free medium separately and incubated for five minutes. Later, DNA- Lipofectamine 2000 mixture was mixed and incubated at room temperature for 20 min. While complexes were forming, cells were washed with serum-free medium twice and 800 µl of medium without serum and antibiotics was added. The DNA-Lipofectamine reagent complexes were applied to the cells and incubated at 37°C at 5% CO_2 for 3 h. After incubation, recovery medium with 10% FBS was added to bring the final volume to 2 ml. After overnight incubation, the recovery medium was replaced with fresh, complete medium containing serum and antibiotics. Expression of flag tagged β-catenin wild type and mutants were analysed by western blotting from protein extracts, prepared after 48 hours of transfection. Fluorescence microscopy for localization studies were performed after similar transfection of cells initially seeded on coverslips placed in 35 mm cell culture dishes.

3.2.5.6 Generation of MDCK stable cell pool.

To obtain MDCK cell lines that stably express the constitutive, GFP-tagged constructs of wild type and mutant β-catenin, stable pool of cells were generated. The plasmids with wild type and mutant β-catenin were amplified in E. Coli and purified using the Maxi Prep kit (Qiagen). Sub-confluent MDCK cells were transfected on 10 cm plates with 2 µg DNA : 6 µl of DNA using the Lipofectamin 2000 reagent (Invitrogen). Two days post transfection, cells were split 1:20 into selection medium containing 500 µg/ml G418. Most of the cells died within two three days after addition of selection medium, colonies started appearing eleven

days after transfection. An approximately equal number of colonies grew up for each transfected plasmid. For each transfection, all of the colonies were trypsinzed and combined to give stable pools. Stable cell pools were checked for expression of GFP tagged wild type and mutant β-catenin by immunofluorescence and western blotting. Stable cell pools were maintained in G418 supplemented growth media to prevent loss of expression of the gene over multiple passages of the clones.

3.2.6 Functional analysis.

Protein analysis was done in transiently transfected SW480 cells and stable cell pool of MDCK cells to check the expression of various β-catenin constructs and how it effects expression of other genes involved in various other functional pathway. Proliferation and migration assays were performed on MDCK stable cell pool and 30 hours after transient transfection of SW480 with different β-catenin constructs.

3.2.6.1 Preparation of total protein lysate from cultured cells.

Cells were washed 3 times with ice cold PBS and lysed directly on the plates or flasks after addition of RIPA protein lysis buffer [50 mM TRIS-HCI pH 7.4, 150 mM NaCI, 1 % nonidet P-40 (NP- 40), 0.5 % sodium deoxycholate, 0.1 % SDS, 1 mM EDTA, 1 mM PMSF, 10 µM sodium orthovanadate 10 mM, 20 µg/ml, leupeptin, 10 µg/ml pepstatin A, 20 µg/ml of aprotinin]. The cells were scraped and lysed by sonication for 20 seconds on ice using UW70 type dounce homogenizer and kept on ice for 20 min and centrifuged at 10.000 x g for 12 min.

3.2.6.2 Determination of protein concentration.

The method employing bicinchoninic acid (BCA) was used as an alternative for the colourimetric detection and quantification of total protein in 17AATR and pET11c control crude extracts. The method depends on the reduction of Cu2+ to Cu1+ by protein under alkaline conditions (the biuret reaction) and the highly

sensitive colorimetric detection of the cuprous cation (Cu1+) in the reaction with BCA. Chelation of two molecules of BCA with one cuprous ion gives an intense purple coloured product with a strong absorbance at 562nm. The method seems to be more reliable than the Bradford one as the amount of dye binding does not depend purely on the content of Arg and Lys (Bradford method) but the macromolecular structure of the protein, the number of peptide bonds and the presence of four aminoacids (Cys, cystine, Trp and Tyr). The detection limit is about 20 µg of protein per ml. The protein concentration was determined using BCA protein assay kit (PIERCE) according to the manufacturer's instructions. A set of protein standards was prepared by diluting the 2 mg/ml BSA stock in water to get a range from 25 to 1000 µg of protein. 25 µl of each standard or unknown sample replicate was added to a microplate well. 200 µl of working reagent was added to each well and mixed thoroughly on a plate shaker for 30 seconds. Absorbance was measured at 560 nm after incubation at 37°C for 30 minutes.The readings were corrected for the A560 of the control sample, which contained 25µl of the water in place of the protein. The amount of protein in unknown samples was determined from standard curve based on known BSA concentrations.

3.2.6.3 SDS-polyacrylamide gel electrophoresis.

10-12 % SDS Polyacrylamide gel was prepared according to the following recipe: for separating gel of 10 ml volume: 4 - 3.3 ml of distilled water, 2.5 ml of 1.5 M Tris-HCl (pH 8.8), 3.3 – 4.0 ml of 30 % Polyacrylamide mix (37.5 : 1 ratio of mono and bis acrylamide), 100 µl of 10 % SDS, 100 µl 10 % ammonium persulphate (APS) and 4 µl N, N, N', N' tetramethylethylenediamine (TEMED). For 5 ml of stacking gel: 3.4 ml of distilled water, 630 µl of 1 M Tris-HCl (pH 6.8), 830 µl 30 % polyacrylamide mix, 50 µl 10% SDS, 50 µl 10 % APS and 5 µl TEMED. The gel was cast in MiniProtean III apparatus (BioRad, Germany). The protein samples were mixed with SDS sample solution (Rotiload, Roth, Germany) and boiled for 5 minutes for complete denaturation. This mixture was loaded onto the gel and electrophoresed.

3.2.6.4 Immunoblotting.

Immunoblotting was performed utilizing the semi-dry transfer method. Nitrocellulose membrane (Schleich and Schuell, Germany) and 6 layers of Whatman 3 paper were cut exactly to the size of the gel and soaked in transfer buffer along with the gel. A stack was made with 3 sheets on the top and bottom of the gel and membrane and placed in between the graphite plates of the transfer apparatus (Biometra, Germany). Transfer was performed at 0.8 mA/cm^2 current for 1 hour. The efficiency of the transfer was confirmed by staining the membrane with a 0.1 % solution (containing 1 % acetic acid) of Ponceau S (Sigma, Germany). The excess stain was removed with water. After visualisation of the protein, the blot was completely washed off the stain with water and put into blocking solution (5 % nonfat milk powder in PBS containing 0.05 % tween 20) for 1 h at room temperature or over night at $4^{o}C$. The blots were then incubated in the blocking solution containing 1: 500 –1:1000 dilution of the primary antibody for 1 h at RT. After washing with wash buffer (PBS containing 0.05 % tween 20) for 10 minutes, the blot was incubated with the secondary antibody (against the species of the primary antibody and linked to horse radish peroxidase) (1:2000 dilution) for 1 h at RT. After 3 washes of 5 minutes each with wash buffer, the specific bands were developed using a chemiluminiscent system (Santa Cruz, Germany). Equal volumes of solution A and B were mixed and the blot was incubated in this solution for 1 minute. The blot was immediately exposed to an X-Ray film in dark for varying periods of time ranging from 5 sec to 2 min and developed automatically. The specific bands were scanned and quantified densitometerically using TINA version 2.09 g. All immunoblots were sequentially incubated with anti-β-actin as control, and specific signals adjusted in relation to the expression of this house-keeping gene.

3.2.6.5 Immunofluorescence.

MDCK stable cell pools were seeded on sterile coverslips at 1.25x105 cells per well on a 24-well plate, overnight, in the appropriate growth medium. SW480 cells were transiently transfected on coverslips with different β-catenin constructs, incubated for 30 hours. Coverslips in all cases were gently rinsed three times (10 sec) with PBS before fixation in 3% paraformaldehyde for 20 min. Coverlips were then washed three times in PBS before permeabilization in 0.2% Triton X-100 in PBS for 5 min. Following three more 5 min washes in PBS, cells were blocked with 0.3% bovine serum albumin (BSA) in PBS for 20 min. Coverslips were incubated with primary antibodies at indicated concentrations for 1 hr in a humidity chamber. Cells were then washed three times for 5 min in wash reagent before incubation in the appropriate fluorescent-conjugated secondary antibody or stain (Table) for 30 min in a humidity chamber. Following a final three 5 min washes in wash reagent, coverslips were mounted on glass slides using 4ul of either DAKO or Mowiol mounting media, and visualized under a Leica TCS confocal microscope. Images were processed using Adobe Photoshop and Leica Lite software.

3.2.6.6 Determination of proliferation – “Flow cytometry”.

Analyses were performed using a Galaxy pro flow cytometer (Partec, Münster/ FRG) equipped with a mercury vapor lamp (100 W) and filter combination for the detection of cells stained with 2,4-diamidino-2-phenylindole (DAPI) and a 488 nm laser with filter combination for FITC. For high-resolution flow cytometry, single cells were isolated from native sampled tissues using 2.1% citric acid/0.5% tween 20 according to the method of Ehemann [138, 139] followed by slight shaking at room temperature. For staining cell suspensions were incubated in phosphate buffer (7.2g Na_2HPO_4 x $2H_2O$ in 100ml H_2O dist.) of pH 8.0 containing DAPI. Each histogram represents 30.000-100.000 cells for measuring DNA-index and cell cycle. Histogram analysis was performed with the Multicycle program (Phoenix Flow Systems, San Diego, CA).

3.2.6.7 Determination of migration – “Scratch assay”.

Cells were plated onto 6-well tissue culture dishes at near confluence in complete tissue culture medium. Confluent cell layer were treated for 3 hours with 5 µg/ml of mitomycin C medium containing antibiotic but no FCS to block cell proliferation. Cell layers were scratched using a 20 µl eppendorf micropipette tip. Wells were washed gently with medium and immediately replaced with complete medium. Spontaneous cell migration was monitored over 24 hours. Phase contrast images were captured with Altra 20 Olympus camera and documented, and wound width was measured at three independent wound sites per group. Wound width corresponding to each time point was expressed as the mean of three values +/- SE.

4. Results

4.1. β-catenin analysis in paraffin embedded tumor samples.

In this study, we investigated 20 small intestinal adenocarcinoma and 20 early age of onset colon cancers to elucidate the molecular background of β-catenin stabilization for comparison with colon cancer from elderly patients. Unlike small bowel cancers and some early age of onset colon cancers, colon carcinomas from elderly patients commonly show stabilization of β-catenin through APC mutations. To answer the question if other causes of β-catenin stabilization are found in early age of onset colon cancers, we compared the findings in the small bowel with 20 cases of colorectal cancer. Paraffin embedded tumor sample were analysed for β-catenin alterations by immunohistochemistry, western blot analysis, and sequence analysis on the RNA and DNA levels.

4.1.1 β-catenin expression pattern in small intestinal and colorectal adenocarcinoma.

In the non neoplastic mucosa of the small and the large bowel, the epithelial cells displayed a membranous staining, reflecting the role of β-catenin in formation of adherens junctions (Figure 4.1 A). Only few cells in the bottom of crypts displayed nuclear accumulation of β-catenin, these cells likely reflect progenitor cells, the differentiating cells towards the luminal surface displayed lack of nuclear β-catenin reactivity (Figure 4.1 B). Approximately 50% of small bowel cancers and 75 % of early age of onset colon cancer cells showed increased reactivity towards the β-catenin antibody. It was restricted to the cell membrane (Figure 4.1C) in approximately 50% of cases while two different patterns of strong β-catenin accumulation in the nucleus were observed in the remaining carcinomas. In the small and the large bowel, one pattern was associated with nuclear accumulation in the carcinoma cells at the invasion front but not in the differentiating areas of the tumor (Figure 4.1D)

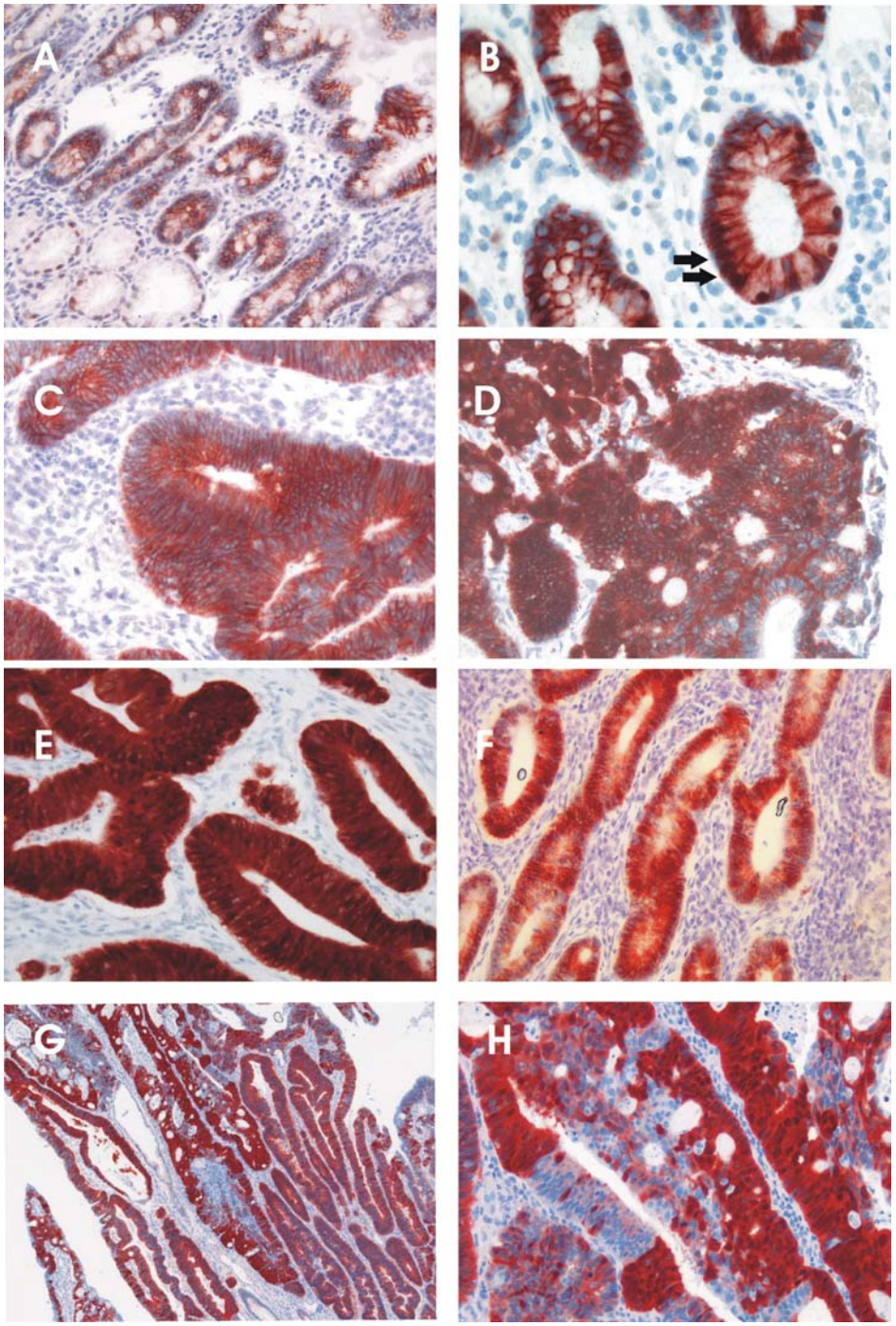


Figure 4.1: β-Catenin immunohistochemistry showing different types of protein accumulation. (A, B) Normal mucosa with membranous staining and nuclear staining for progenitor cells at crypt (arrow); (C) Increased membranous accumulation in both SIC and CRC; (D) nuclear accumulation only at the invasive front in both SIC and CRC; (E) homogeneous nuclear accumulation in small intestinal cancer; (F, G, H) heterogeneous nuclear accumulation at either differentiating or invasive front of colon cancer.

A second pattern was identified as a homogeneous nuclear accumulation throughout tumor areas affecting each cell in small bowel cancers (Figure 4.1E) and nuclear accumulation in patchy areas at either differentiating and invasive carcinoma cells in the colon (Figure 4.1 F,G,H).

4.1.2 Stabilization of mutated β-catenin.

Immunohistochemical analysis of paraffin embedded tumor tissues showed some cases with an abnormal nuclear β-catenin localization and stabilization, in comparison to normal mucosa. In order to analysis the basis of β-catenin stabilisation, we isolated total protein from paraffin embedded tumor samples and performed western blot analyses. Shortened β-catenin isoforms were identified in four and three cases out of 20 cases of small intestinal and early age onset colorectal adenocarcinoma, respectively (Figure 4.2, Table 4.1A, 4.1B). In addition to the wild type β-catenin with a molecular weight of 88 kDa, tumors T3, T820 of small intestinal adenocarcinoma and T117,T218 from colorectal adenocarcinoma showed a tumor-specific β-catenin isoforms of approximately 80 kDa. Different β-catenin isoforms were found for T13 and T256 of approximately 81and 75 kDa, respectively (Figure 4.2). Smallest β-catenin isoforms of 71 kDa was observed for T832 from colorectal adenocarcinoma. While only the aberrant β-catenin was detected in T13, aberrant and normal β-catenin was seen for all other tumors. The presence of both normal and mutated β- catenin is most likely a result from an admixture of non-tumorous epithelial cells while protein isolation. This observation correlated with the concentration of tumor cells on the whole paraffin sections prepared for protein isolation.

All tumors with shortened β-catenin isoforms displayed aberrant nuclear accumulation of β-catenin by immunohistochemistry. While homogenous nuclear accumulation was noticed in small bowel cancer, patchy areas of nuclear β-catenin accumulation were found in the corresponding colon cancers.

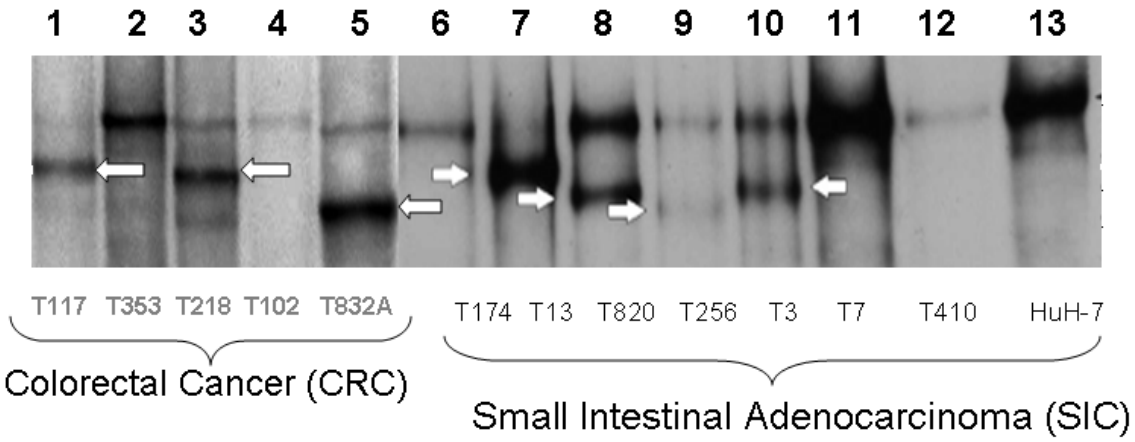


Figure 4.2 Western blot analysis displaying differently shortened β-catenin proteins in lanes 1, 3, 5 of colon cancer corresponding to tumors 117 (lane 1), 218 (lane 3), 832A (lane 5) and lane (7-10) of small intestinal cancer corresponding to tumors T13 (lane 7), T820 (lane 8), T256 (lane 9) and T3 (lane 10) Normal length β-catenin is seen in tumors loaded in lanes 2, 4,6,11 and 12. Lane 13, normal length β-catenin (HuH-7 cell line).

4.1.3 Molecular basis of shortened β-catenin, the RNA level

Immunohistochemical analysis confirmed the Wnt signaling dysregulation and western blot data indicated that this dysregulation is primarily due to mutation of β-catenin itself and not a secondary effect to mutation of proteins involved in degradation machinery of β-catenin. Western blot analysis displayed four differently shortened β-catenin proteins. Thus, the possibility of deletion mutants is given. We therefore first tested the mRNA of β-catenin for deletion mutants using different primer sets which flank exon boundaries. Mutational analysis of exon 3 of β-catenin excluded any point mutation at or near phosphorylation sites. However, in four cases of small bowel cancer (Figure 4.4) and 3 cases of colon cancer (Figure 4.3, 4.4), all of which displaying aberrantly shifted bands in western blot and nuclear accumulation of β-catenin, shortened RNA fragment at the N-coding area of the mRNA were noticed. The most common finding was a complete lack of the coding segments of exon 3 found in 2 small and 2 large bowel carcinomas, corresponding to the most common shortened β-catenin isoform. In tumor T13 an in frame insertion of 15 bp beginning after codon 21

was seen. This was combined with a deletion of codons 22-80 of the of β-catenin mRNA. T256 showed a deletion from codon 17 to codon 128. In T823 a large deletion lacking codons starting from base -48 of the start codon and ending at codon 130 was found. The deletion results in an alternative start codon at codon 131, as indicated by the open reading frame analysis (ORF finder, NCBI) and the deduced molecular weight of this shortened β-catenin isoforms corresponds well to the length identified by western blot analysis.

4.1.4 Molecular basis of shortened β-catenin, the DNA level

Mutation in small intestinal adenocarcinoma.

T13 harboured a 247 bp insertion in exon 3 of the *CTNNB1* gene (codon 22). This mutation introduced coding sequences of the serinc gene on chromosome 9. On a transcriptional level, the mutation was found to result in an in-frame insertion of 15 bp of serinc RNA followed by a de novo splicing and fusion to exon 4 of *CTNNB1* (Figure 4.3, 4.4, 4.5). The resulting mutated protein lacks amino acids 22–80 of β-catenin and five novel amino acids were inserted. Two carcinomas displayed large genomic deletions, including the complete intron 2 sequence (T820) or a deletion of exon 3 combined with partial deletions of introns 2 and 3 (T3). At transcriptional level, both mutations were found to result in a skipping of exon 3 and in-frame fusions of exons 2 and 4 (Figure 4.3, 4.4, 4.5). The resulting mutated proteins lack amino acids 5–80 of β-catenin. T256 showed a deletion from codon 17 in exon 3 to codon 128 in exon 4, including the complete intron 3 sequences. At the RNA level the mutation resulted in an in frame fusion of codon 16 with codon 129, leading to a lack of amino acids 17–128 of β-catenin. Agarose gel picture in Figure 4.3 shows clearly band of higher molecular weight of 223 bp from tumor T13 in comparison to molecular weight of 157 base pair for exon3 deletion and 295 base pair for novel mutation observed in T256.

Mutations in colorectal adenocarcinoma

These mutations were checked at transcriptional level only. Quality of genomic DNA isolated for these tissues was good enough to analyse presence or absence of long stretches of intron 2 and 3 at genomic level.

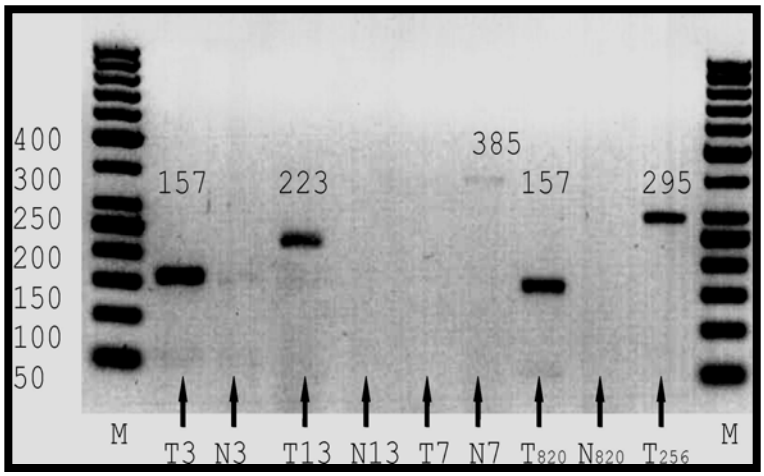


Figure 4.3 RT–PCR analyses for *CTNNB1* mutation in SIC (A) Primers amplifying from exon 2 to exon 4 for small intestinal adenocarcinoma. The expected fragment length including the entire exon 3 is 385 bp (faint band N7). A tumor-specific band of 157 bp corresponding to a complete lack of exon 3 is seen in T3 and T820. T13 shows a tumor-specific band of 223 bp. Primers from exon 2 to exon 5 produce a 295 bp fragment in T256, while the expected size of WT-cDNA would be 631 bp.

Figure 4.4 shows the chromatogram from sequence analysis from all of the mutants seen in tumor tissue. Figure 4.5 shows the graphical overview for wild type RNA and protein for β-catenin along with deletion mutants observed in tumor tissues. It also shows the different domains involved in its phosphorylation, degradation and overlapping binding region for certain proteins involved in different functional aspects of cell adhesion like E-cadherin and wnt signaling like TCF/Lef 1 and APC.

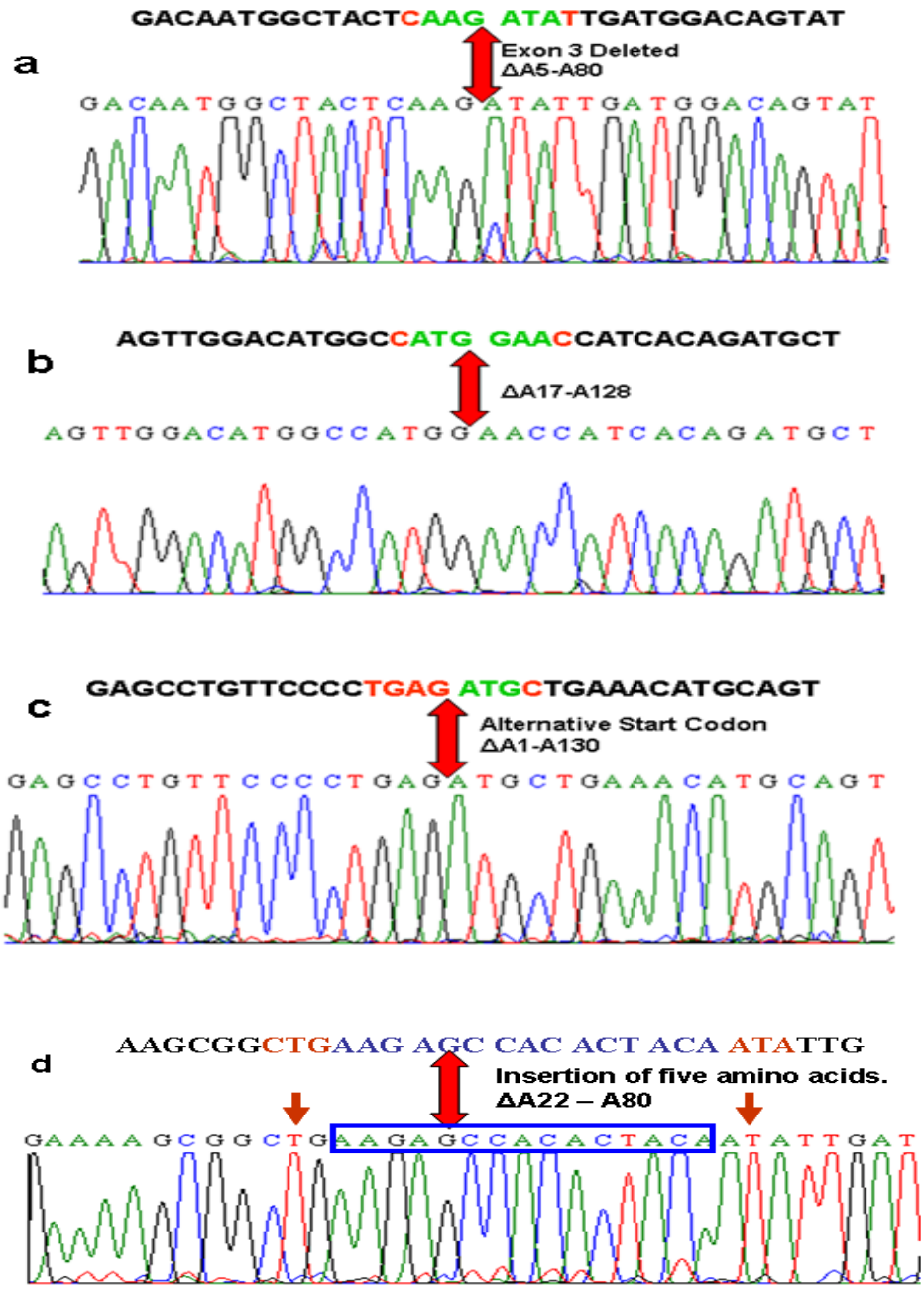


Figure 4.4 DNA sequence chromatogram of stabilized three differently shortened β-catenin.(a) In frame deletion of exon3 at cDNA leads to deletion of amino acids 5 to 80, corresponding to tumors (T3, T820, and T13) of SIC, and tumors (T117, T218) of CRC (b) Tumor T256 from SIC, with in frame deletion of amino acids 17 to 28. (C) Tumor T832 from CRC, with alternative start codon, in frame deletion of amino acid 1 to 130.(D) T13 Insertion of 15 bp associated with a deletion of codons 22 to 80.

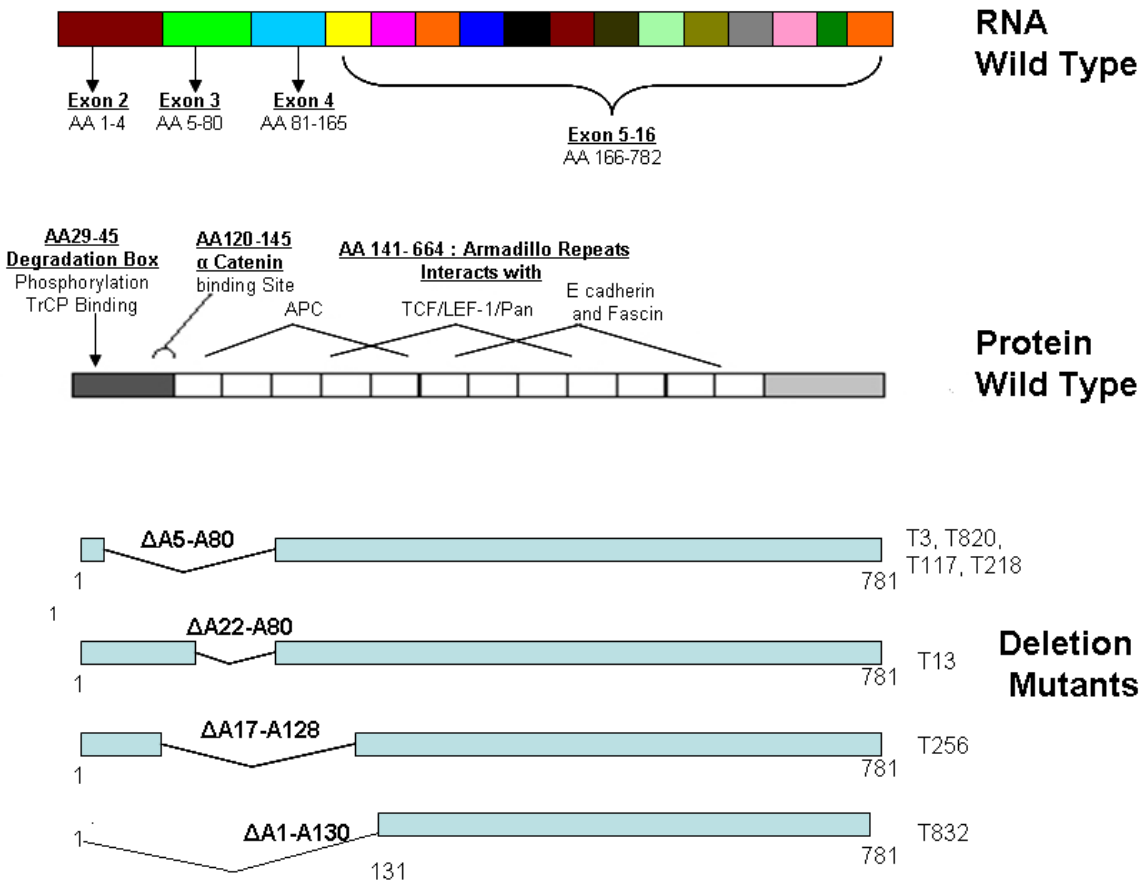
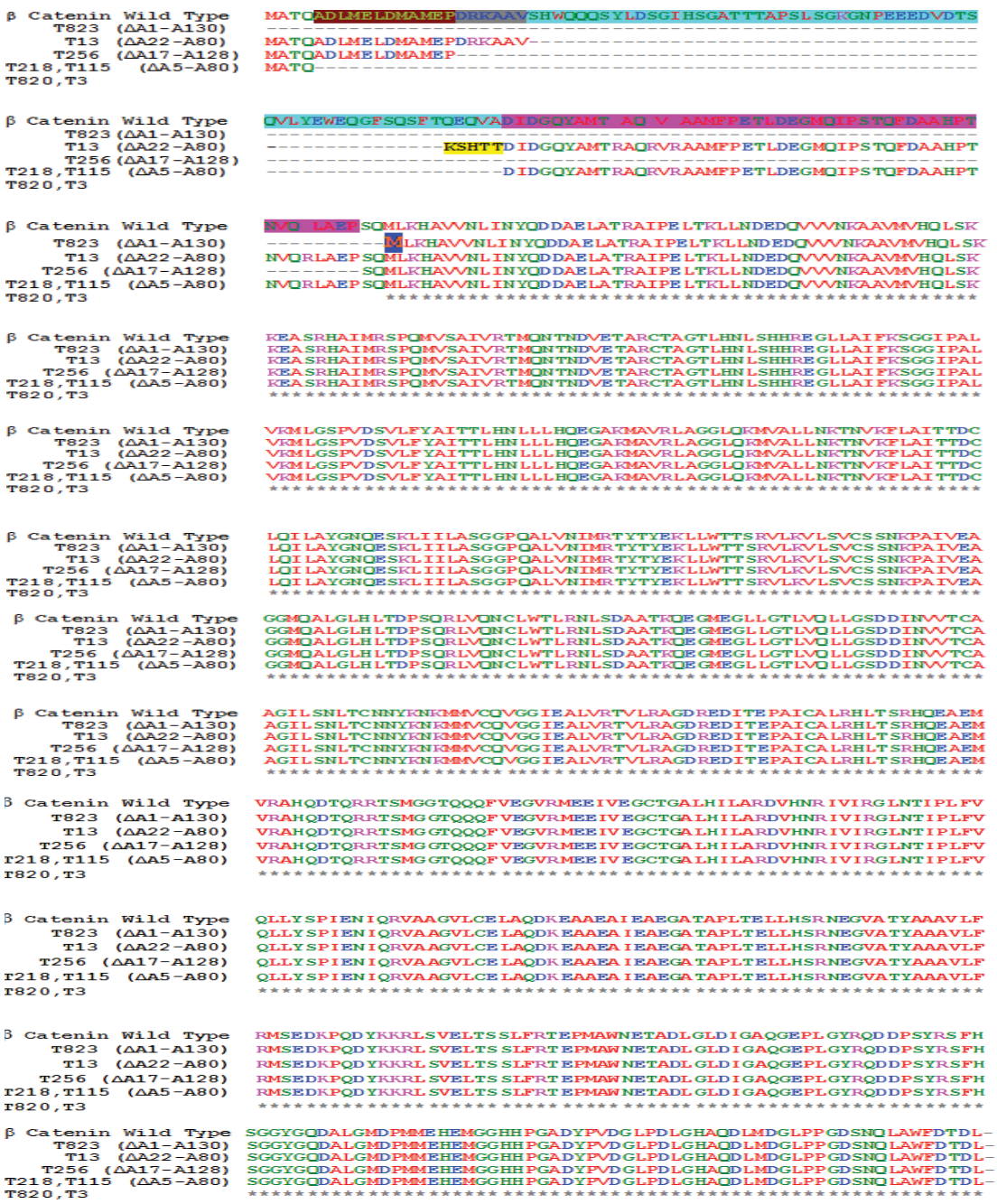


Figure-4.5: Graphical overview of β catenin wild type and deletion mutants, showing the N-terminal domains affected by losses.


β Catenin Wild Type
T823 (ΔA1-A130)
T13 (ΔA22-A80)
T256 (ΔA17-A128)
T218,T115 (ΔA5-A80)
T820,T3

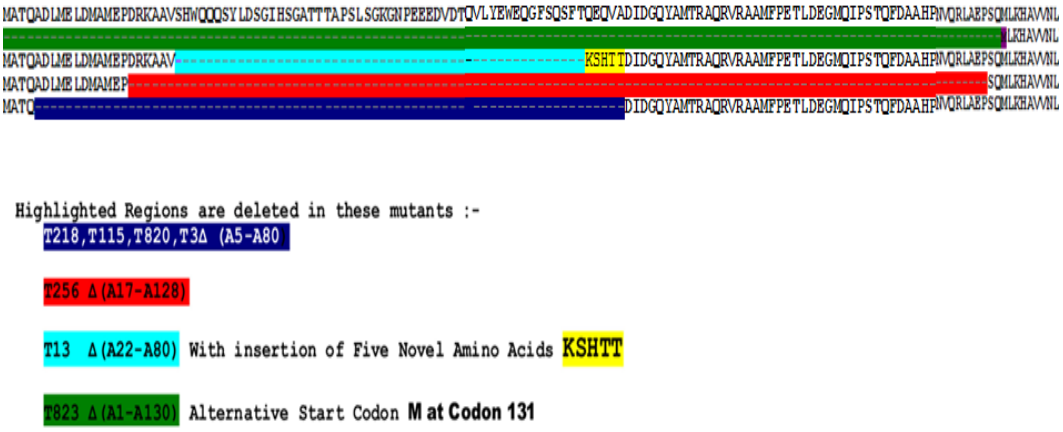


Figure 4.6 Four novel large in frame deletion mutations of β-catenin encodes an in frame fusion protein with N-terminal lacking degradation box but with identical C-terminal. (A) Multiple Sequence alignment of the mutated β-catenin found in tumor tissues with wild type β-catenin. Regions lost in different mutants are highlighted in different colours. (B) Schematic representation of the regions lost in different deletion mutants. Different mutants have some overlapping regions that are common in different mutants. Mutant T13 shows an insertion of five novel amino acids (highlighted in yellow) along with deletion of amino acids 22 to 80. Mutant T823 lost complete N-terminal and start with an alternative start codon

Figure 4.6 shows the multiple alignment of deduced primary amino acid sequence of different deletion mutants observed in all investigated tumor tissues and wild type β-catenin. All mutations affected the N-terminal of β-catenin lacking the complete degradation box, some part of α-catenin binding site or the complete N-terminus with alternative start codon but with identical C-terminal region (Figure 4.6 a, b). However, while these mutants lack only N-terminal regions the large deletions might induce conformational changes in tertitary structure affecting their binding affinity to other known binding partners. Additionally, homogenous complete nuclear localization of β-catenin for T256 all over the tumor area suggests complete loss of regulation over its nucleo-cytoplasmic shuttling which is still maintained in other deletion mutants. This interesting observation suggests loss or gain of known or unknown binding partner interaction with a very important role in nuclear cytoplasmic shuttling of β-

catenin and further strengthen the existing hypothesis, that β-catenin nuclear translocation is an active process and not merely based on passive diffusion based on its stabilization.

Table 4.1 (A) Immunohistochemical, western blot and sequence analyses for small intestinal adenocarcinoma.

ID	IHC	WB	DNA	RNA	Protein
T3	Nuclear	Mutant	IVS2-1 IVS3+56del	c.14-251del	A5-A80del
T7	Cytoplasmic	WT	No	No	WT
T12	Cytoplasmic	WT	No	No	WT
T13	Nuclear	Mutant	65–66ins247bp	c.65–251delins15bp	V22–A80delins5AA
T18	Normal	WT	No		
T19	Cytoplasmic	WT	No	No	WT
T24	Normal	WT	No	No	WT
T30	Normal	WT	No	No	WT
T160	Normal	WT	No	No	WT
T172	Cytoplasmic	WT	No	No	WT
T174	Cytoplasmic	WT	No	No	WT
T180	Normal	WT	No	No	WT
T206	Cytoplasmic	WT	No	No	WT
T235	Normal	WT	No	No	WT
T255	Normal	WT	49–385del	No	WT
T256	Nuclear	Mutant	No	c.49–385del	D17–P128del
T320	Normal	WT	No	No	WT
T410	Normal	WT	No	No	WT
T716	Normal	WT	IVS2 + 1–IVS2–1del	No	WT
T820	Nuclear	Mutant		c.14–251del	A5–A80del

Table 4.1 (B) Immunohistochemical, western blot and sequence analyses for colorectal carcinoma. Nd: not determined

ID	IHC	WB	DNA N.d.	RNA	Protein
T542	Cytoplasmic	Nd		No	Nd
T218	nuclear	Mutant		c.14-251del	A5-A80del
T275	Cytoplasmic	Nd		no	Nd
T084	Cytoplasmic	Nd		no	Nd
T123	Cytoplasmic	Wt		no	WT
T097	Cytoplasmic	Nd		no	Nd
T458	Cytoplasmic	Nd		no	Nd
T576	Cytoplasmic	Nd		no	Nd
T353	Cytopl/Nuclear	Wt		no	WT
T320	Cytoplasmic	Wt		no	WT
T273	Cytoplasmic	Nd		no	Nd
T409	Cytoplasmic	Nd		no	Nd
T316	Cytoplasmic	Nd		no	Nd
T240	Cytoplasmic	Nd		no	Nd
T115	Cytoplasmic	Nd		no	Nd
T183	Cytoplasmic	Nd		no	Nd
T173	Cytoplasmic	Nd		no	Nd
T117	Cytoplasmic	Nd		no	Nd
T218	nuclear	Mutant		c.14-251del	A5–A80del
T832	Nuclear	Mutant		c-48-c390	A1-130del
-					

4.1.5. Comparison of *CTNNB1* findings with general genetics.

Microsatellite and chromosomal instability (MIN and CIN) are two distinct mechanisms underlying intestinal tumor development. One of both types of genetic instability can be identified in the majority of intestinal cancers. Recent studies have shown, a subset of intestinal cancers (varying from 17 to 50 %), especially those diagnosed at young age [52, 53], were found to be microsatellite- and chromosomal-stable (MACS) tumors [55]. It is hypothesized

that they represent a distinctive group that may arise from an uncharacterized unique molecular pathway [59]. Since development of cancer requires multiple somatic mutations in a diversity of genes, microsatellite and chromosomally stable tumors must have accumulated such somatic mutations due to a mechanism different from CIN and MSI. Second possibility might be one or two hit hypothesis where mutation occurs in only one or two gene with high oncogenic potential.

Murata et al [140, 141] described this large deletion mutation in carcinoma with microsatellite instability and our data from tumor tissue showed large deletion mutation in β-catenin from young aged patients. Therefore, we checked the microsatellite and chromosomal stability for all the small intestinal carcinoma tumor tissues with differential β-catenin stabilization and localization. However, we only analysed microsatellite instability for colon cancer cases with aberrant β-catenin accumulation. All the tumors from small intestinal carcinomas except T3 were found to be microsatellite and chromosomal stable (Table 4.2). T3 was microstatellite stable but showed chromosomal instability. All colon cancer cases were microstatellite stable. 3 out of 4 cases (75%) of small intestinal carcinomas were microsatellite stable and chromosomal stable, thus giving us room to hypothesize that β-catenin large deletion mutation might activates its oncogenic potential to much higher level to induce tumorigenesis in absence of any general type of genomic instablity.

Table 4.2: Genomic stability data for the tumors with β-catenin mutation.

Small Intestinal Adenocarcinoma			**Colorectal Cancer**		
ID	**MSS/MSI**	**CSS/CSI**	**ID**	**MSS/MSI**	**CSS/CSI**
T3	MSS	CSI	T117	MSS	ND
T820	MSS	CSS	T218	MSS	ND
T13	MSS	CSS	T832	MSS	ND
T256	MSS	CSS			

4.2 Creating β-catenin constructs to generate an in vitro model system.

Our data with tumor tissue showed large in frame genomic deletions without any point mutation in β-catenin. It showed strong stabilizing potential in 20% of small intestinal adenocarcinoma and 15% of non hereditary early age of onset colorectal cancer. Three out of four tumors from small intestinal adenocarcinoma harbouring large deletion mutations, showed aberrant homogenous nuclear accumulation throughout tumor tissues, were exclusively found with MACS genotype. Therefore, we hypothesized that large N-terminal deletion mutants in our tumor samples in the background of genomic stability may have higher oncogenic potential in comparison to β-catenin point mutants.
On the basis of these observations, we plan to generate an in vitro model system by transfecting Flag tagged and GFP tagged β-catenin mutants and express them in SW480 (intestinal epithelial from colorectal adenocarcinoma) and MDCK (Normal kidney epithelial cell from dog) cell line. SW480 is known to harbour an APC mutation as well as chromosomal instability whereas MDCK cells are normal immortalized kidney epithelial cells. Specific aims were:-

- Competitive analysis of oncogenic potential of point mutation with large deletion β-catenin mutants and how it differs in SW480 and MDCK.
- Determining the fate of different β-catenin mutations on its subcellular localization in SW480 and MDCK.

To assay and compare the oncogenic potential, we studied the impact of β-catenin point and large deletion mutation on proliferation and invasion potential of SW480 cells and MDCK cells. Therefore, to analyze the effect of different β-catenin mutations in vitro, we constructed wild type and different large deletion mutants of β-catenin (Fig 4.7) using PCR driven overlap extension method (Fig 3.1. 4.8). All the mutants were tagged by FLAG or GFP at the C-terminus to differentiate it from endogenous β-catenin. Large deletion mutants corresponding to T3 and T256 (tumor sample number) lacking amino acids Δ (A5-A80) and Δ

(A17-A128) were generated to delete the complete degradation box including all phosphorylation sites. Mutant Δ (A17-A128) lacks some part of alpha catenin binding site also. A novel large deletion mutant was designed, lacking amino acids 60 (coded by exon 3) to amino acid 110 (coded by exon 4) and referred as Δ(A60-A110). It lacks conserved regions between the degradation box and the α-catenin binding site with an unknown function and never reported in any studies so far. For functional comparison to point mutation, we utilized the Δ S45 and S33Y point mutant, which lacks the serine residue at position 45 and 33, mutations mainly found in colon carcinoma. The WT construct represent the complete functional β-catenin. .

FLAG tagged β-catenin constructs were generated in pcDNA3 plasmids (Figure 4.9). GFP tagged constructs were cloned in pEGFP-N1. The β-catenin cDNA insert sequences were reamplified from pcDNA3 construct without FLAG tag using forward and reverse primers containing KpnI site (in forward primer) and BamH1 (in reverse primer) restriction sites and cloned in-frame into pEGFP-N1. All clones were confirmed by sequencing.

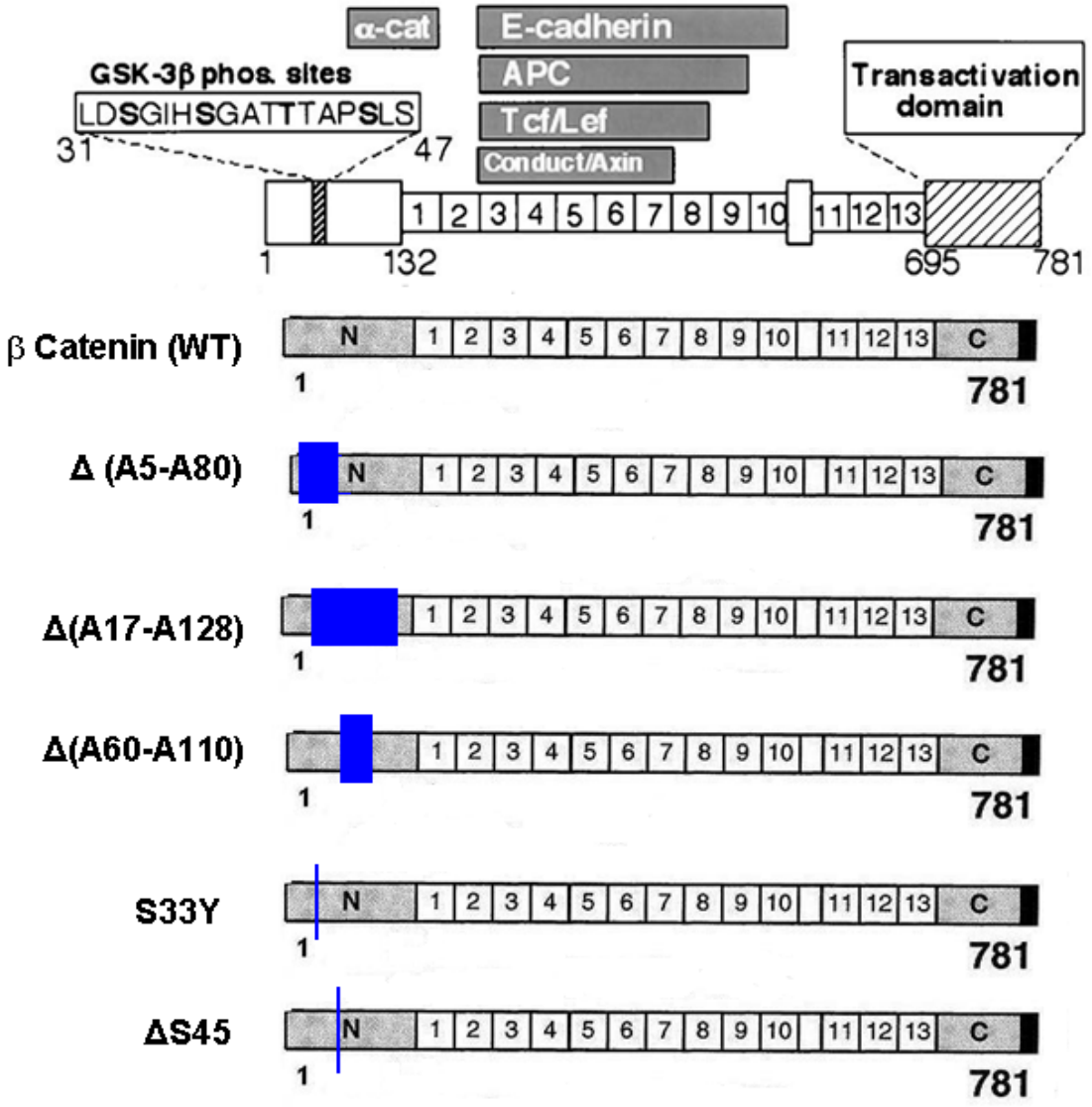


Figure 4.7 Schematic outline of β-catenin domains and proteins encoded by expression constructs. Area marked in blue was mutated. GSK3β phosphorylation sites are shown in the β-catenin N-terminal region; armadillo repeats in the central region; the C-terminal transcriptional activation domain; and the regions required for interaction with α catenin, E-cad, APC, Tcf/Lef factors, and conductin/axin. In addition to wild-type (WT) β-catenin, the structures of mutated proteins are indicated. Mutants were FLAG tagged and GFP tagged at C-terminus when cloned in pcDNA3 and Pegfp-n1 respectively.

Figure 4.8 β-catenin PCR fragments with mutation and restriction sites , generated using PCR driven overlap extension method for cloning into pcDNA3 mammalian vector.

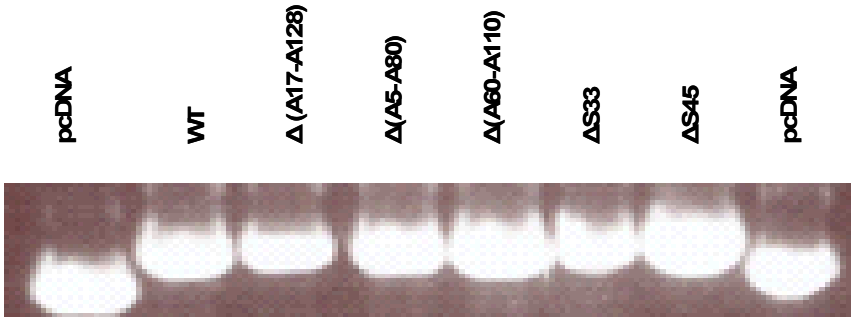


Figure 4.9 pcDNA3 vectors cloned with β-catenin PCR fragments, obtained from PCR driven overlap extension method.

4.3 Transfection and functional assay in SW480 cells.

To examine domains in β-catenin that are important for regulating its subcellular distribution and its role in activating oncogenic potential, we overexpressed wild type and different β-catenin mutants in SW480 cells. SW480 is a cell line from intestinal origin established from a primary colon adenocarcinoma. There is a G -> A mutation in codon 273 of the p53 gene resulting in an Arg -> His substitution and a C -> T mutation in codon 309 resulting in a Pro -> Ser substitution. These cells expresses elevated levels of p53 protein and is positive for expression of c-myc, K-ras, H-ras, N-ras, myb, sis and fos oncogenes. Additionally, it has a

truncation mutation of APC that delete the axin binding sites to prevent β-catenin degradation, resulting in abnormally high levels of cytoplasmic and nuclear β-catenin in these tumor cells.

4.3.1 Transient overexpression of FLAG tagged β-catenin constructs.

Wild type and mutant β-catenin proteins lacking large NH2 terminal areas and point mutants (S33Y and ΔS45) were expressed in SW480 cells. The sequence for FLAG tag epitope recognized by monoclonal Flag antibody M2 (F3165, sigma) was added to the 3' termini of all cDNA constructs to distinguish these protein products from endogenous β-catenin. All constructs were prepared in pcDNA3 to constitutively express the β-catenin transgene and transfected transiently into SW480. SW480 cells transfected with an empty pcDNA3 vector served as a negative control. Following transient transfection, expression of flag tagged wild-type and mutated forms of β-catenin were monitored by Western blotting (Fig 4.10 A). Flag immunoblot showed expression of all flag tagged wild type and mutated β-catenin in correct size range. However, band intensity was lightest for Δ (A17-A128) followed by mutant Δ (A5-A80). Negative control cells showed no signals with the Flag antibody. Flag Tag Epitope inaccessibility in T256 and T3 can be the reason for lighter band intensity. Large deletion mutation in T256 and T3 might cause adoption of different three dimensional protein structure which can lead to inaccessibility of Flag epitope.

Immunoblotting with β-catenin antibody (Fig 4.10) showed two bands for cells transfected with different β-catenin constructs. One band is an endogenous β-catenin while other one was the flag tagged β-catenin . Large deletion mutant Δ (A17-A128), Δ (A5 - A80) and Δ (A60-A110) showed shortened mutant β-catenin bands along with one band of higher molecular weight corresponding to endogenous β-catenin . Two separate bands can not be observed for WT, S33Y and ΔS45, since exogenous wild type β-catenin and point mutants were not always well separated by SDS-PAGE because of similarity in their electrophoretic mobilities, due to very less difference in their molecular weight. A thick band of mutants in contrast to negative control clearly confirms the presence of mutated exogenous form of flag tagged β-catenin.

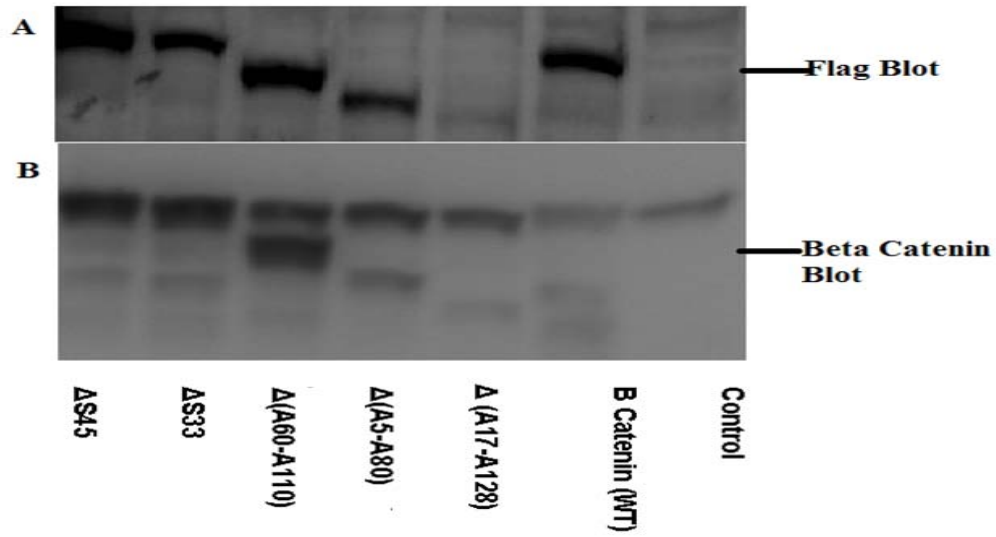


Figure 4.10 Expression of Flag taged wild type and mutant β-catenin in transiently transfected SW480 cells. A, Equal protein from cytoplasmic extract of con, wt, Δ (A17-A128), Δ (A5-A80), Δ (A60-A110), S33Y and ΔS45 was immunoblotted with Flag antibody. B, Immunoblot with a β catenin antibody.

4.3.2 Differential subcellular localization of β-catenin constructs.

To examine domains of β-catenin important for regulating its subcellular distribution, we overexpressed wild type and different β-catenin mutant constructs in SW480 cells and checked their localization by immunofluorescence. SW480 cells normally have β-catenin in the cytoplasm and the nucleus due to an existing APC truncation mutation. SW480 cells were transiently transfected with FLAG tagged β-catenin constructs and immunostained with anti Flag antibody. The results in Fig 4.11 show complete nuclear accumulation of mutant Δ (A17-A128) with no accumulation in the cytoplasm or at the membrane. Δ (A5-A80), S33Y, and ΔS45 showed both nuclear and cytoplasmic accumulation similar to wild type SW480 cells with little higher nuclear accumulation in Δ (A5-A80). Mutant Δ (A60 – A110) showed cytoplasmic accumulation with no nuclear accumulation. Mutant Δ (A17-A128) showed similar subcellular localization both

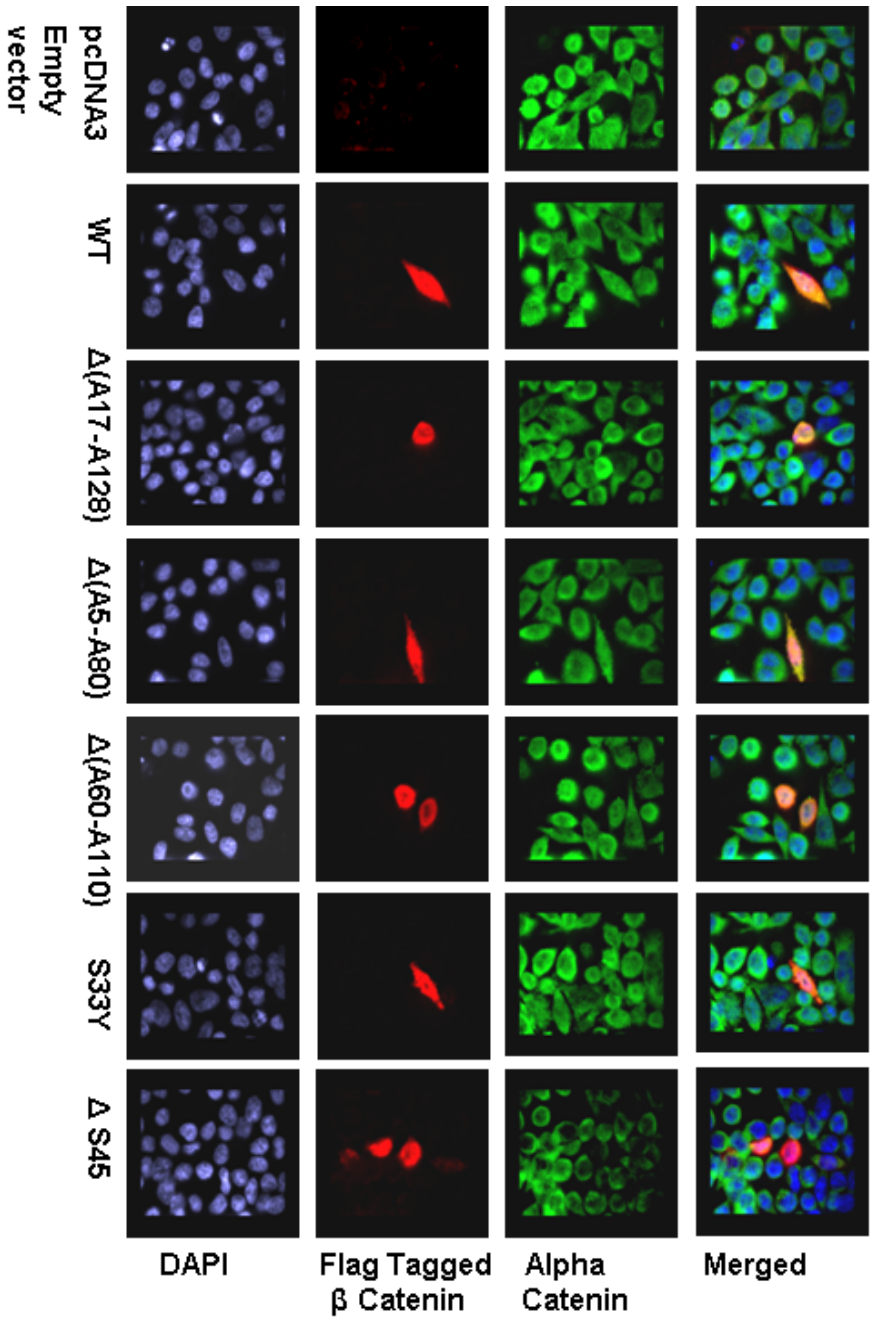


Figure 4.11 Effect of β-catenin mutation on its accumulation pattern; Δ(A17-A128) shows complete β-catenin nuclear accumulation without cytoplasmic or membranous accumulation and co-localizes with nuclear α-catenin similar to as seen in tumor sample. SW480 cells transiently transfected with different Flag tagged β-catenin clones and double stained with mAb Flag antibody and antiserum against α-catenin. β-catenin (wt), S33Y, ΔS45 localizes in the cytoplasm and nucleus but Δ(A60-A110) localizes mainly in the cytoplasm. All the mutants co localize with α-catenin with nuclear translocation in Δ(A17-A128).

in tumor tissue (in vivo) and cell culture (in vitro). However, APC was not mutated in the corresponding tumor sample which had a microsatellite and chromosomal stable genotype whereas SW480 cells are colon tumor cells with (APC $^{mut/mut}$) a truncated form of APC and also present with a genomic instability.

4.3.3 Nuclear co accumulation of α and β-catenin.

In the initial study with tumor tissue, we described large deletions and one insertion in the β-catenin gene, *CTNNB1*. Mutation-induced generation of abnormal transcripts was shown by cDNA analysis. Stabilization of mutated protein was demonstrated by immunohistochemistry and western blot analysis, respectively. Three tumors (T3, T820, and T13) with a lack of exon 3 sequences displayed strong homogenous nuclear accumulation of β-cateninin in the background of cytoplasmic accumulation. Tumor (T256) with an additional loss of exon 4 showed a nuclear-predominant accumulation of β-catenin with little cytoplasmic protein concentration. Tumors with cytoplasmic β-catenin stabilization and nuclear translocation solely at the invasion front did not show comparable mutations. In accordance with our previous analysis, no point mutations within exon 3 were observed in these cases [120]. Most of mutations reported here resulted in a loss of N-terminal β-catenin sequences from residues 5–80. These mutant proteins lost all phosphorylation sites encoded by exon 3 along with the recognition site for β-TrCP. β-Catenin degradation via phosphorylation through the APC–AXIN–GSK3β degradation complex and subsequent β-TrCP-mediated ubiquitination is likely to be largely impaired in these mutants making it more stabilized but its nuclear/cytoplasmic shuttling is still regulated. Accumulation of the largest deletion mutant (D17–P128del in T256) was mainly restricted to the nucleus (Fig 4.11), indicating that the additional loss of residues 81–128 destabilizes β-catenin in the cytoplasm or deregulate the nuclear–cytoplasmic shuttling of the protein. The D17–P128del mutant has lost parts of the α-catenin binding site of β-catenin located between residues 120–147 and also some part of conserved region with unknown function between β-catenin degradation box and α-Catenin binding site. α-catenin is known to sequester β-catenin in the cytoplasm and it can also inhibit the

transcriptional activity of β-catenin. Thus, our data indicate that the stability of the cytoplasmic β-catenin pool depends on an interaction of α- and β-catenins. Loss of the binding motives in either β- or α-catenin may enhance nuclear concentrations of β-catenin and α-catenin either through direct interaction or through some known or unknown partner. Therefore, we checked for α-Catenin localization in all tumor tissues with differential subcellular localization of β-catenin by Immunohistochemistry. Tumor tissue T256 showed homogenous predominant nuclear colocalization of β-catenin and α-catenin (Figure 4.11, 4.12). Therefore, we hypothesized that this deletion mutant lacks binding site for some known or unknown binding partner with a very specific role in regulating nucleo cytoplasmic shuttling of β-Catenin. Since, tumor tissue T256 showed nuclear co-accumulation of β-catenin and α-catenin, we checked for α and β-catenin colocalization in SW480 cell culture by double staining with Flag Tag and alpha catenin antibody after transient transfection with flag tagged β-catenin mutants. As seen in the results (Fig 4.11) all of the mutant's co localize with α-Catenin and show similar accumulation pattern as β-catenin. Interestingly, we observed exactly similar nuclear co accumulation pattern for α-catenin and β-catenin for SW480 cells transfected with Δ(A17-A128) as seen in vivo for tumor sample T256 (Figure 4.12). The deletion mutant Δ (A17-A128) lacks the initial few amino acids of α-Catenin binding domain (120-147) of β-catenin, shows no effect on its binding potential with α-catenin. Mutant T60 lacks the conserved domain (amino acid 60-110) spares the nucleus and stabilizes in the cytoplasm. As seen on western blot (Fig 4.10), it has a high stabilization potential comparable to mutant S33Y and ΔS45, although not seen in any tumor tissues so far. However, deletion mutant Δ (A17-A128) also has deregulated nucleo-cytoplasmic shuttling without stabilization in the cytoplasm and accumulates into the nucleus. Therefore, this nuclear accumulation might be due to efficient degradation in the cytoplasm or active import into the nucleus or poor export from the nucleus. Therefore, our data provide evidence that β-catenin nuclear accumulation is an active process, based not merely on its stabilization, but involving a potential binding partner not known so far.

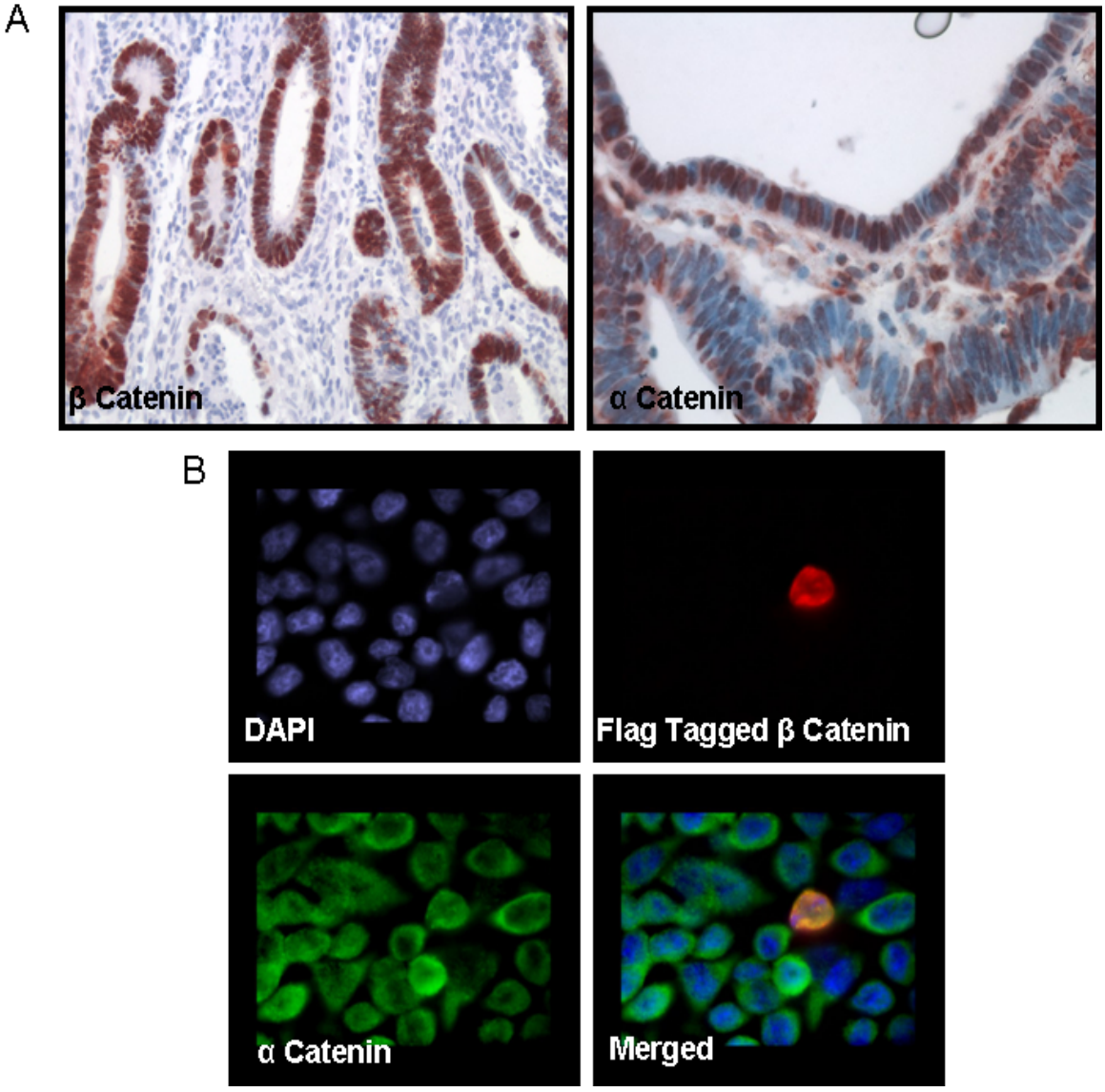


Figure 4.12 Nuclear co-accumulation of α- and β-catenin in vivo and in vitro for β-catenin mutant Δ(A17 – A128) A) Immunohistochemistry for α- and β-catenin in tumor tissue (T256). B) Flag tagged mutant β-catenin co localizes with α-catenin in transiently transfected SW480 cells.

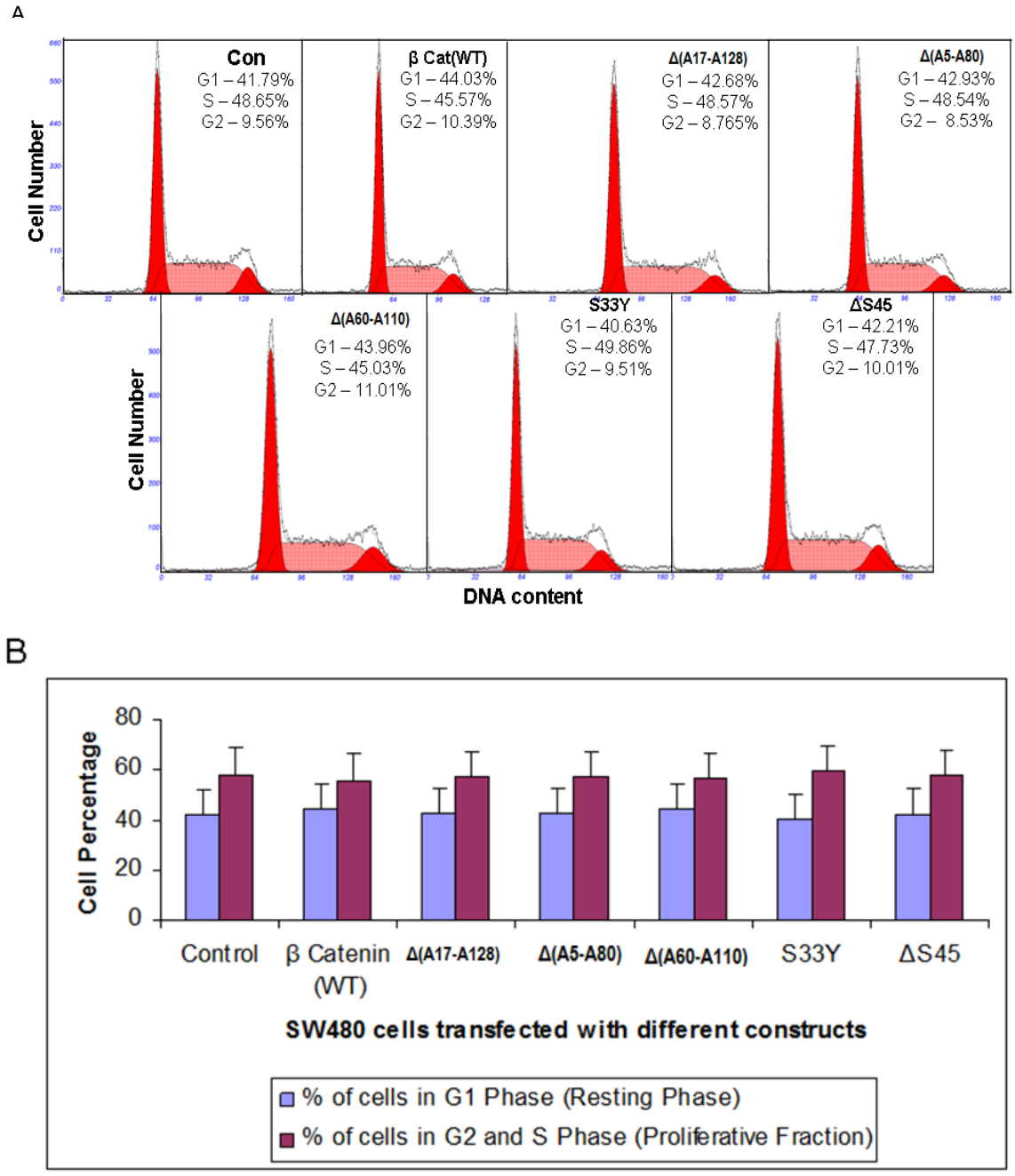


Figure 4.13 β-catenin overexpression shows no effect on proliferation in SW480 cells. A, DNA/flow cytometry analysis of the wild type and mutant β-catenin transfected cells have approximately equal number of cells in G1, S and G2 phase. B, Bar graph for percentage of cells in resting phase and proliferative phase for SW480 cells transfected with different constructs.

4.3.4 β-catenin mutants show no effect on proliferation.

β-catenin stabilization activates Wnt/β-catenin signaling pathway, known to influence cell proliferation due to expression of certain target genes like cyclin D and c myc. Therefore, we performed flow cytometric analysis to check the effect of β-catenin mutation on alterations in cellular proliferation. We characterized the distribution of mock transfected and β-catenin (WT) and mutant expressing SW480 cells in the cell cycle analysis during exponential growth phase. Expression of β-catenin mutants had no significant effect on cell proliferation (Fig 4.13) over mock transfected control cells, with approximately equal proportion of cells in G1, S and G2 phase. Thus, we concluded that proliferation is not affected by expression of any of β-catenin mutant transfected SW480 cells.

4.3.5 β-catenin mutants have no influence on cell migration.

β-catenin stabilization may negatively regulate cell adhesion by translocating from membrane to cytoplasm where it is involved in its signaling function. β-catenin nuclear translocation at the invasive front of cancers indicates it as an important event in the transition from a benign tumor to an invasive, metastatic cancer. Therefore we expected mutant T256 to be more invasive because of its complete homogenous nuclear localization. We performed migration assay on mock (empty vector) and β-catenin (WT) and different mutant transfected cells. Scratch assays were performed to examine the effect of β-catenin mutation over wild type on the invasiveness of SW480 cells. In this assay, the tumor cells have to invade through the scratch made in cell monolayer mimicking the basal lamina. Cells were treated with mitomycin for three hours at 10μg/ml before making scratch to inhibit cell proliferation and prevent filling up of scratch due to cell proliferation rather then invasiveness. Comparing mock transfected cells, and β-catenin (WT) and mutant over-expressing cells, we did not observe any differences between them with regard to their invasiveness (Fig 4.14).

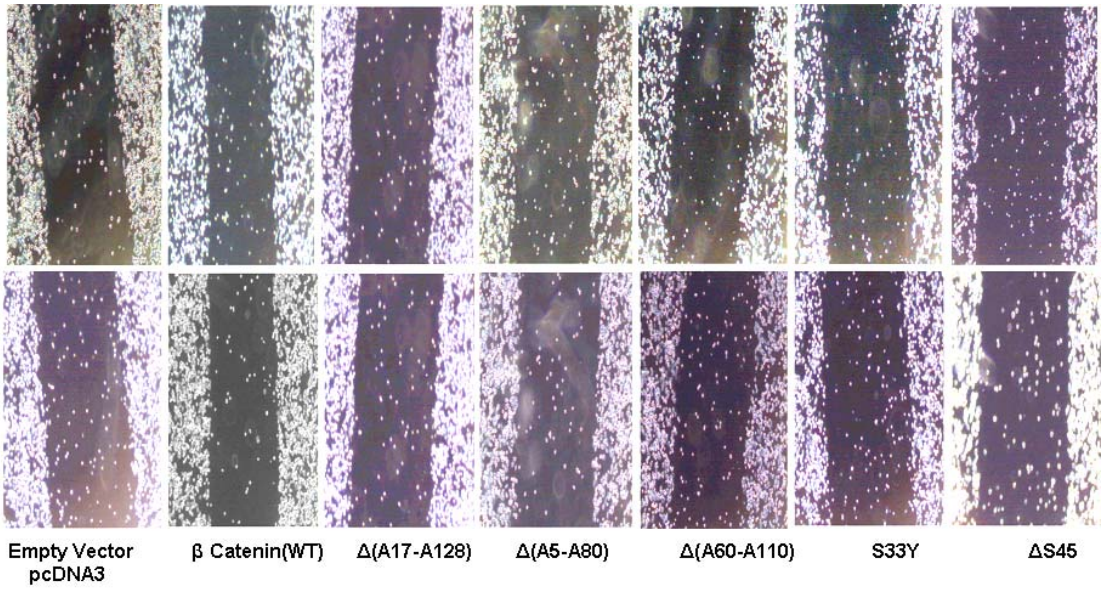


Figure 4.14 β-catenin shows no effect on migration of Sw480 cells. Scratch wound assays of SW480 cells over expressing β-catenin (WT and mutants) and empty vector transfected control SW480 cells. The photographs were taken 24 h after the wounds were made.

4.3.6 Expression of GFP tagged wild type and mutant β-catenin constructs.

Transient transfection and expression of Flag tagged wild type and mutant β-catenin showed difference in their localization but with no functional effect in migration and proliferation assay in SW480. Stable cells could not be established in SW480 cells with Flag Taged constructs and showed poor transfection efficiency of about 10-20%. Therefore, poor transfection efficiencies for different β-catenin constructs might be responsible for no significant effect in functional assays. Therefore, we transfected SW480 cells with GFP tagged β-catenin constructs and checked migration for positively transfected cells. The sequence for GFP tag epitope recognized by polyclonal GFP antibody (ab6556, Abcam) was added to the 3' termini of all cDNA constructs to distinguish these protein products from endogenous β-catenin. All constructs were prepared in pEGFP-N1 to constitutively express the β-catenin transgene, and transiently transfected into SW480 using empty vector as a negative control. Following transient transfection, expression of wild-type and mutated forms of β-catenin was

monitored by Western blotting (Fig 4.15) with a GFP epitope tag present at the C-terminus of each protein. We could identify the exogenous β-catenin by both GFP antibody and β-catenin antibody in correct size range. However, contrary to FLAG tagged β-catenin we could observe two bands for all β-catenin constructs. The green fluorescent protein (GFP) is a protein composed of 238 amino acid residues of 27 Kda that exhibits bright green fluorescence when exposed to blue light. Therefore, endogenous β-catenin and GFP tagged β-catenin are well separated on SDS page, and identifies two bands on western blotting with β-catenin antibody.

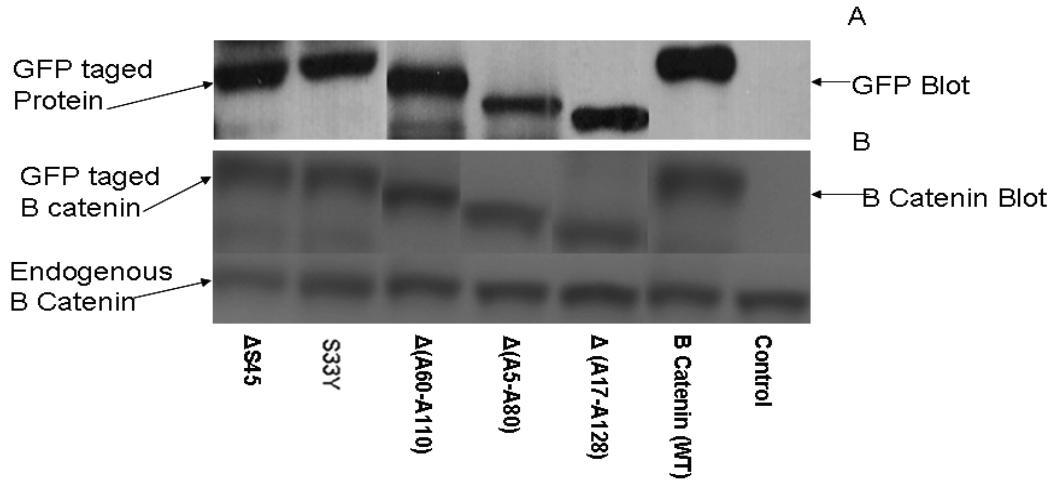


Figure 4.15 Expression of GFP tagged wild type and mutant β-catenin in transiently transfected SW480 cells. A, Equal protein from cytoplasmic extract of con, wt, Δ(A17-A128), Δ(A5-A80), Δ(A60-A110), S33Y and ΔS45 was immunoblotted with GFP antibody. B, Immunoblot with a β catenin antibody.

4.3.7 Overexpression of β-catenin induces apoptosis.

Expression of different forms of GFP tagged β catenin showed different localization as seen for flag tagged constructs (Fig 4.11). Scratch assays were performed to examine the effect of β-catenin mutation over wild type on the invasiveness of SW480 cells like it was performed for Flag tagged constructs. Migration was checked for GFP positive cells immediately after making a scratch

and after 24 hours. As seen in Figure 4.16, we didn't observe any effect on migration potential of positively transfected green cells over control cells. Cells in pink are wild type cells and positively transfected cells are green. However, cells over expressing GFP tagged exogenous β-catenin disappears in time (7-10 days), it seems possible that β-catenin overexpression causes cell death.

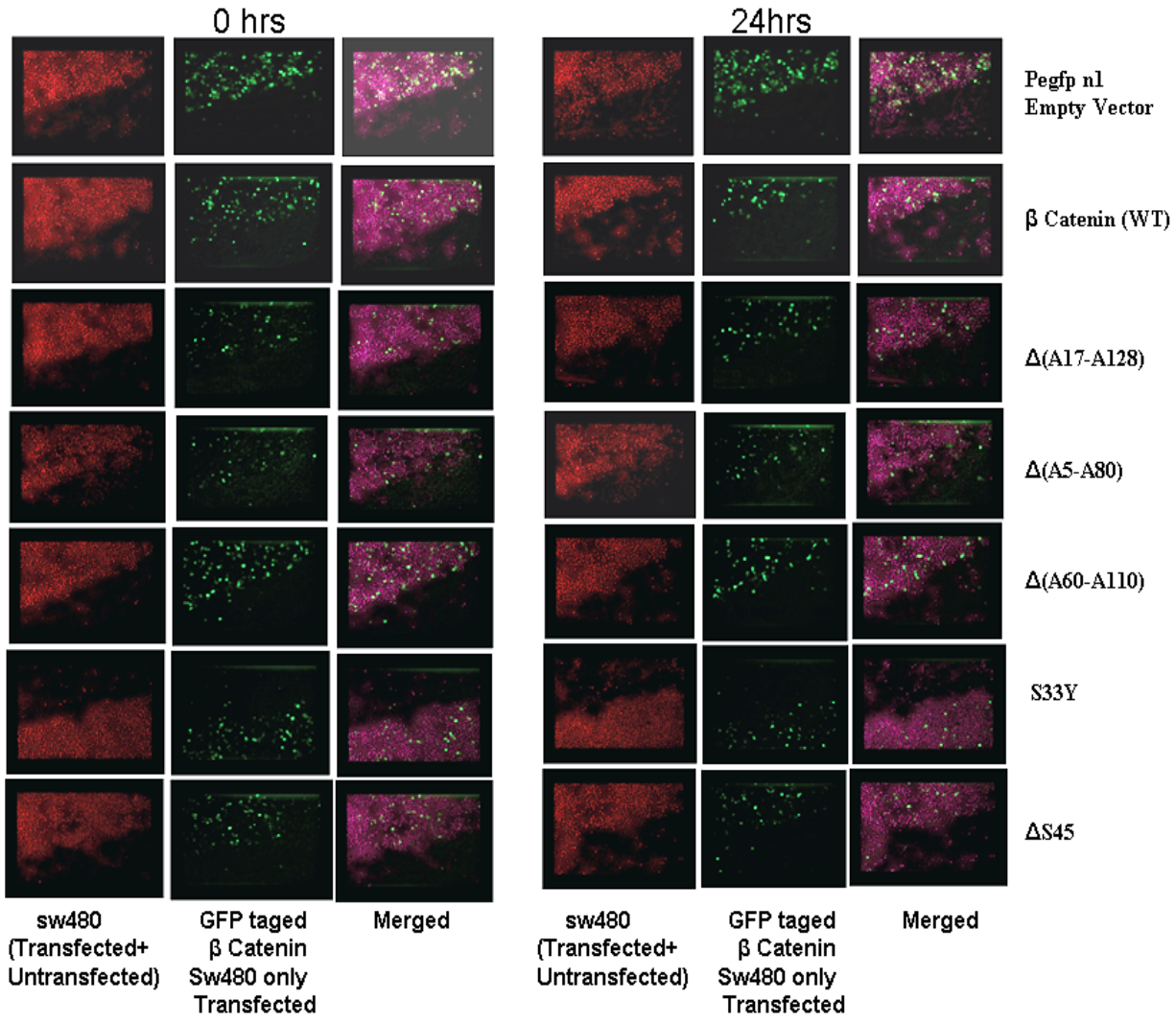


Figure 4.16 Scratch Assay for SW480 cells transiently transfected with GFP tagged β-catenin constructs. Cells were evaluated for their ability to migrate into a cell free area following physical disruption of the monolayer. Overexpression of β-catenin has no effect on migratory potential of SW480 cells.

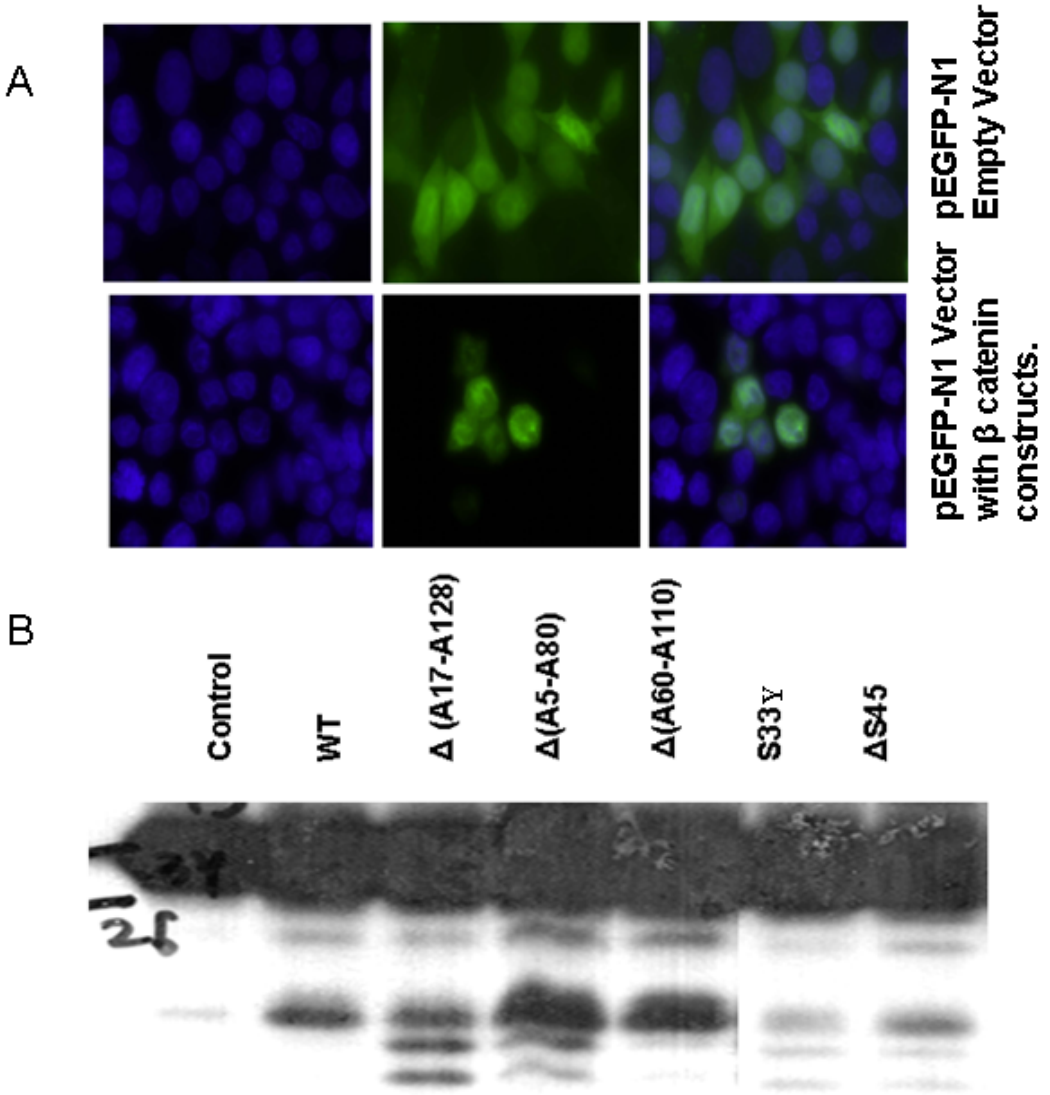


Figure 4.17 β-catenin overexpression in SW480 leads to apoptosis; (A) SW480 cells transfected with β-catenin constructs lose their spindle-like morphology, shrink, and detach from the culture well bottom; in contrast, cells transfected with empty vector are fully protected against apoptotic death. (B) Caspase 3 immunoblot to show its cleavage in SW480 cells transfected with β-catenin constructs. No cleaved caspase 3 is present in control cells transfected with empty vector.

Moreover, SW480 cells expressing GFP tagged or even Flag tagged wild type and mutant β-catenin constructs showed shrinked morphology with shrinked

nuclei as seen in DAPI stained nuclei (Fig 4.17). Shrinked nuclei are one of hallmark of apoptotic cell death. To further confirm that SW480 cells over expressing β-catenin undergo apoptosis and not necrosis, we performed immunoblotting for caspase 3. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspase 3 is a caspase protein that interacts with caspase 8 and caspase 9. Caspase 3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways. As an executioner caspase, the caspase 3 has virtually no activity until it is cleaved by an initiator caspase after apoptotic signaling events have occurred. As seen in Figure 4.17, we can see the presence of caspase 3 cleavages in wild type and mutant β-catenin constructs. Cleaved product is higher in mutants Δ (A17-A128) and Δ (A5-A80). This provides evidence that these mutants lead cells into apoptosis earlier then wild type, Δ (A60-A110), S33Y, and ΔS45. Cells over expressing mutants T256 and T3 die earlier than other mutants. This explains the presence of lighter band on western blot and presence of few cells with positive FLAG staining in immunofluorescence. This also explains the inability to generate the stable cells with FLAG tag β-catenin constructs. Since, at end we were left only with neomycin resistant cells since all the cell over expressing FLAG tagged β-catenin died.

SW480 is known to harbor an APC truncation mutation [142], which initiates transformation, by effects on cell adhesion as well as the known effects on β-catenin signaling. Further overexpression of β-catenin and different stabilized form, guides them in physiological stress situation that leads to apoptosis and hence no conclusion can be made on oncogenic transformation potential of different form of β-catenin. APC and β-catenin mutation both affects the Wnt signaling pathways. Literature also shows APC and β-catenin mutation are mutually exclusive of each other and never found to coexist [105, 143, 144]. Our data with SW480 cell culture somehow explains these finding. Therefore, we planed to study the β-catenin oncogenic potential in non transformed immortalized cell line with no mutation in other genes and having intact components of Wnt signaling pathway.

4.4 Transfection and functional analysis in MDCK cell culture.

MDCK cells were used to study the oncogenic potential of different β-catenin constructs since they contain intact cadherin and Wnt signaling components. We used MDCK normal kidney epithelial cell line from canine and β-catenin is shown to function as oncogene in it [99, 145]. Additionally, the presence of endogenous WT β-catenin mimics human tumors, which exhibit both mutant and WT protein, and thus no attempt was made to alter the endogenous protein. We performed experiments with MDCK stable cell pool overexpressing different β-catenin mutants, since MDCK cell are usually prone to clonal morphological variations. Therefore, stable cell pools were used to avoid the phenotypic artifacts that can result from selection and propagation of clones derived from single transfected cells.

4.4.1 MDCK stable cell pool expressing GFP tagged β-catenin constructs.

The non transformed, epithelial cell line MDCK was used to generate stable cell pools harboring cells stably transfected with β-catenin constructs in pEGFP-N1 mammalian expression vector, constitutively expressing β-catenin (WT) and different mutants. β-catenin WT and mutants were GFP tagged at the C-terminus to distinguish it from endogenous β-catenin. All the plasmids included the neomycin resistance cassette for selection. Cells were transfected with plasmids, 48 hours later, the cells were split 1 to 20 and cultured for two weeks in the presence of 600µg/ml of geneticin. An approximately equal number of colonies grew up for each transfected plasmid. For each transfection, all the colonies were trypsinized and combined to give stable pools. As a negative control, a stable cell pool was generated from transfecting empty vector. All stable cell pools were treated with histone deacetylase inhibitor sodium butyrate at 5mM concentration overnight to non specifically increase transgene expression. Generation of stably transfected cell by providing selection pressure using geneticin G418 sometimes leads to complete cessation of gene expression over time. This transgene expression silencing is usually caused by epigenetic downregulation and occurs via DNA methylation, covalent modification of histones, resulting in formation of

repressive chromatin structure. Sodium butyrate reverses methylation and increase the transgene expression. MDCK stable pools over expressing different GFP tagged β-catenin mutants showed presence of GFP positive cells with strong green fluorescence on examination by fluorescence microscope.

MDCK stable pools expressing different mutants were also examined by immunoblotting after treatment with sodium butyrate, to evaluate GFP tagged β-catenin in transfected cells and compare it with control cells. Western blot with GFP antibody showed GFP tagged β-catenin in all of β-catenin (WT) and mutant stable clones, but not in the vector only control cells. Western blot with β-catenin antibody showed two bands for all stable clones, but only one band in vector only control cells. The upper band with high molecular weight is the GFP tagged β-catenin and the lower band is endogenous β-catenin with lower molecular weight (Fig 4.18 A)

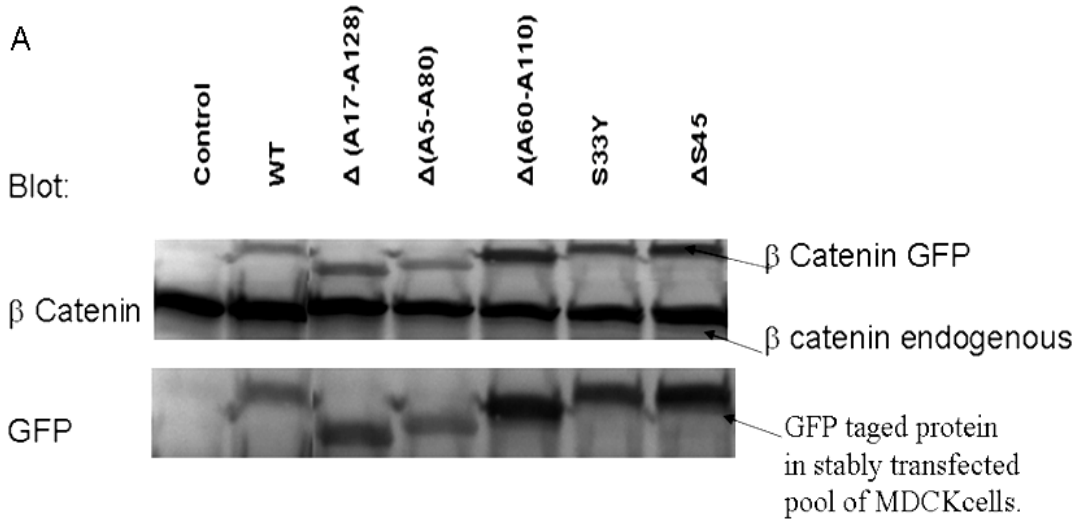
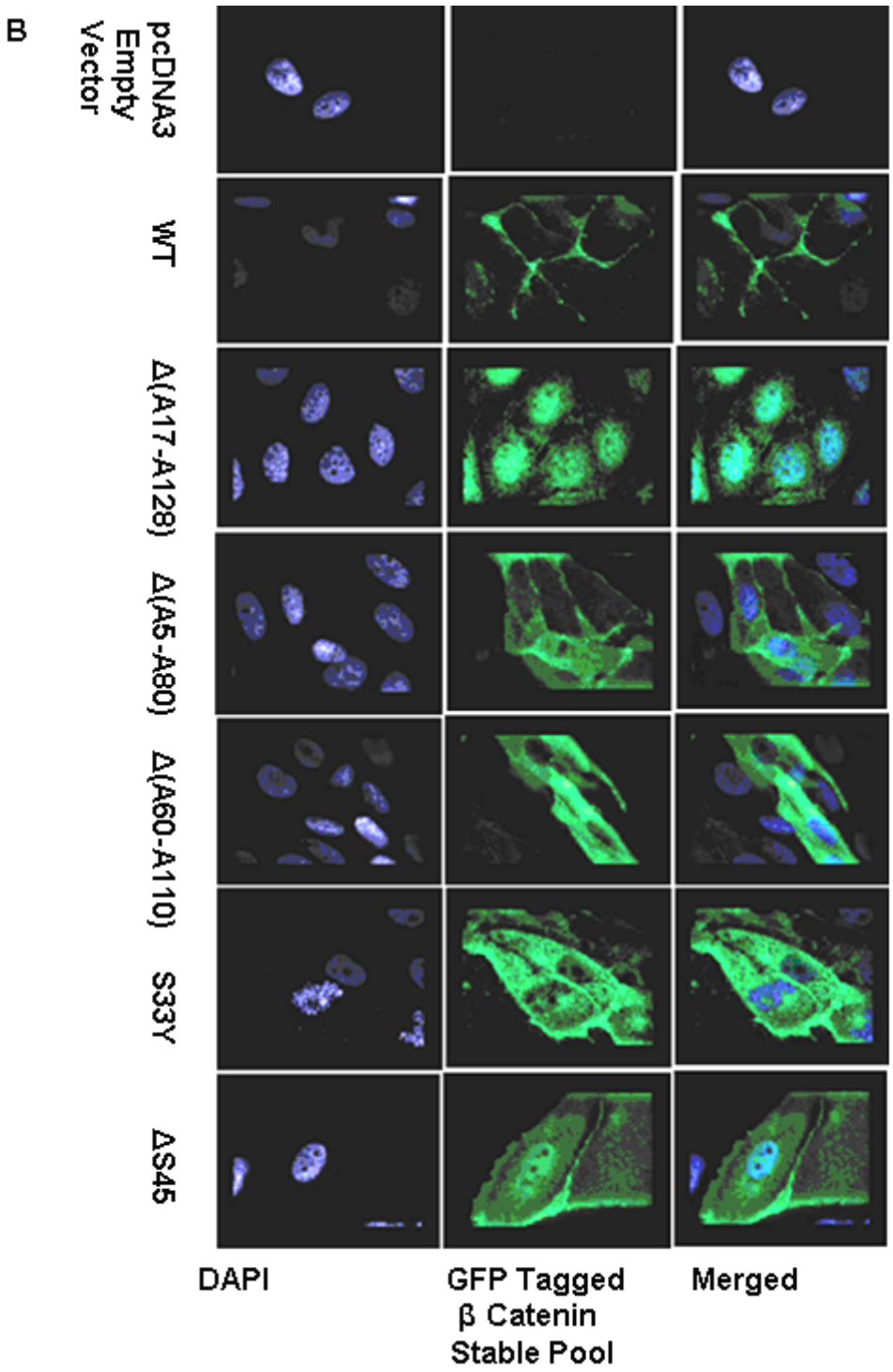


Figure 4.18 Generation of MDCK stable clones (A) Western blot analysis to check expression of β-catenin and GFP tagged β-catenin in stably transfected pool of MDCK cells. SDS-PAGE separate GFP tagged β-catenin from endogenous β-catenin. The upper band is GFP tagged β-catenin and lower band is endogenous β-catenin. One lower band for endogenous β-catenin is there in Vector only transfected control cells. Triton lysate was also western blotted with GFP antibody and showed GFP- β-catenin in all stable clones (B) Immunofluorescence of GFP- β-catenin in stable clones. WT and Mutant stable pools are detected by immunofluorescence using GFP antibody. The GFP tagged protein was absent in vector only transfected control cells


B
pcDNA3 Empty Vector
WT
Δ(A17-A128)
Δ(A5-A80)
Δ(A60-A110)
S33Y
ΔS45
DAPI
GFP Tagged β Catenin Stable Pool
Merged

4.4.2 Differential subcellular localization of wild type and mutant β-catenin in MDCK cells.

We checked the effect of β-catenin mutation on its localization in SW480 cells after transient transfection. Stable cells could not be established in SW480, since β-catenin overexpression leads them into apoptosis. Moreover, transient transfection usually results in very high and non physiological level of expression for the transfected transgene. Therefore, to obtain information on the effect of β-catenin mutation on its localization that is more physiologically relevant, we generated stable pools of MDCK cells expressing wild type β-catenin and different point and large deletion mutants that were considerably more stable than wild type in tumor tissues. As seen in Fig 4.18 (B), β-catenin wild type is mainly located at membrane with no accumulation in the cytoplasm or in the nucleus. Mutant Δ(A60–A110) and S33Y show strong cytoplasmic accumulation with little nuclear translocation whereas mutant Δ (A5 – A80) and ΔS45 show nuclear accumulation in the background of cytoplasmic accumulation. However, the largest deletion mutant Δ (A17 – A128) showed complete nuclear accumulation without cytoplasmic or membranous accumulation. Deletion mutant Δ (A17 – A128) and Δ (A5 – A80) showed similar accumulation patterns as observed in the corresponding tumor tissues T256 and T3.

SW480 has an APC $^{mut/mut}$ genotype that effects the wild type β-catenin localization by mainly concentrating it in cytoplasm and nucleus. APC has an important role to play in nuclear cytoplasmic shuttling of β-catenin. Therefore, we observed difference in localization pattern of wild type β-catenin in SW480 cells and MDCK. MDCK cell with wild type APC showed strong membranous localization of wild type β-catenin, compared to cytoplasmic and nuclear accumulation in SW480. Similarly other mutants also showed slight variation in accumulation pattern. However, mutant Δ (A17 – A128) had a similar complete nuclear localization as seen in SW480 transiently transfected cells and tumor tissue T256. Mutants Δ (A17 – A128) complete nuclear localization in MDCK, a normal epithelial cell line with wild type APC and intact components of Wnt signaling pathways indicate this mutation to lack components regulating its nucleo cytoplasmic shuttling.

4.4.3 β-catenin alters cell morphology.

β-catenin is part of protein complex that constitute adherens junctions (AJs). AJs are necessary for the creation and maintenance of epithelial cell layers by regulating cell growth and adhesion between cells. Any form of β-catenin mutation that affects its stability at adherens junction will disturb the epithelial cellular phenotype. Overexpression of β-catenin in MDCK cells was shown to alter cell morphology previously [146]. Therefore, we examined the morphology and colony formation of MDCK stable pool expressing different mutants. The morphology of cells expressing β-catenin wild type and mutants was different compared to control cells (Fig 4.19). The MDCK stable cell pools used in our study also showed somewhat similar morphology as the MDCK cells expressing an inducible form of β-catenin mutants in stable clones [146]. The WT and mutants were less efficient at forming tight colonies of cells compared to control cells. Moreover, the cells along the edges of WT and mutants colonies tend to extend projections giving them a mesenchymal phenotype. However, in low density culture, the morphology of cells expressing mutant Δ (A17–A128) and to some extent mutant Δ(A5–A80) and WT, was different from cells expressing mutant Δ(A60–A110), S33Y, ΔS45 (Fig 4.19). Cells at the edges of colonies, from WT, Δ (A17–A128) and Δ(A5–A80) extend few and show less pointed projections onto surrounding cell free surfaces. Moreover, many single cells were not found associated into colonies for mutant Δ(A60–A110), S33Y, and ΔS45, giving them more mesenchymal morphology than control cells. However, WT, Δ(A17–A128) and Δ(A5–A80) showed more mesenchymal phenotype then control cells but less than mutants Δ(A60–A110), S33Y, and ΔS45, giving them a metastable phenotype. The metastable phenotype is acquired by the cell by its ability to express attributes of both epithelial and mesenchymal phenotype.

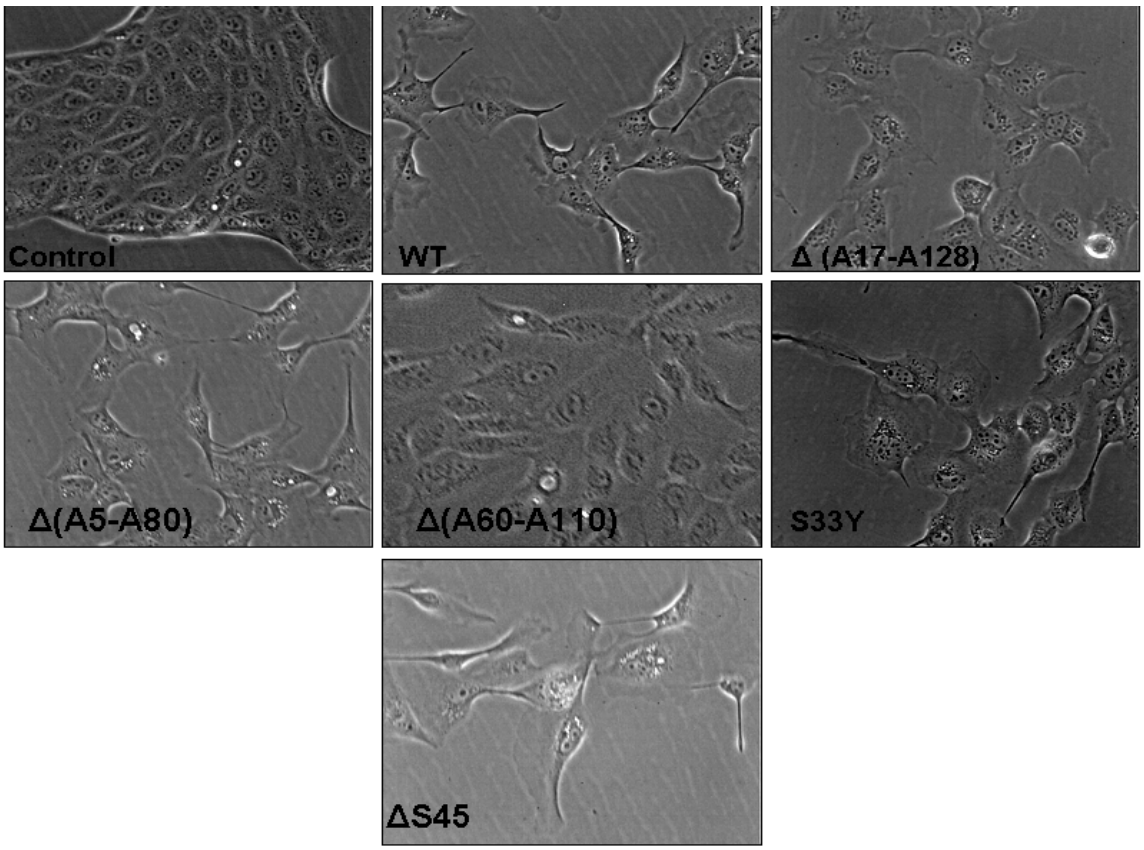


Figure 4.19 Effect of β-catenin (wt) and Mutants overexpression in stable pool effects colony morphology. WT and mutant cell pools ere less efficient at forming tight colonies of cells as compared to only vector transfected control cells.

β-catenin immunohistochemistry in tumor tissue (Fig 4.20) also displays a role of β-catenin in epithelial mesenchymal transition. As seen in figure 4.20(A), normal intestinal mucosa displays nuclear accumulation in few cells at the bottom of crypts mainly representing progenitor cells, whereas differentiating cells towards the luminal surface display membranous staining with a role in maintenance of adherens junction.

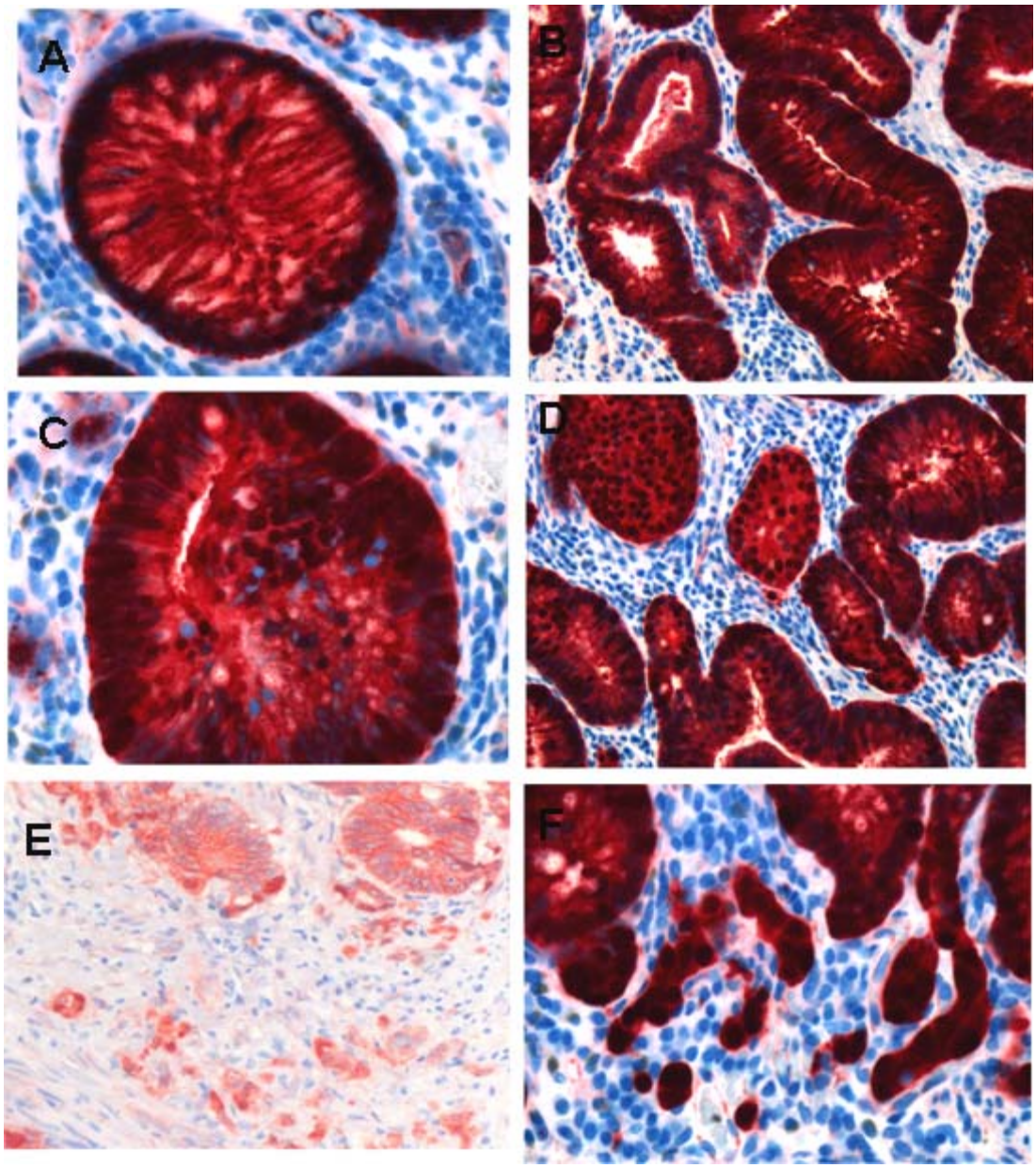


Figure 4.20: β-catenin Immunohistochemistry shows different pattern of protein accumulation for epithelial mesenchymal transition in tumor tissue. A) Normal crypts with nuclear staining at base and membranous staining at lumen. (B,C) Increased nuclear reactivity with loss of membranous staining in differentiating cells. (D,E,F) Cells dissociating from tumor tissue into the neighboring stromal tissue with higher nuclear accumulation of β-catenin and mesenchymal transformation.

Tumor tissue in figure 4.20 (B,C,D) displays higher nuclear reactivity with loss of membranous staining even in the differentiating cells but still confined to intestinal crypts. However, tumor tissue in figure 4.20 (E, F) displays higher nuclear β-catenin reactivity in completely dissociated cell, reflecting an important role in epithelial mesenchymal transition leading to invasion and metastasis.

4.4.4 Effect of β-catenin mutation on its migratory and proliferation potential.

β-catenin overexpression alters cellular morphology by giving it more fibroblastic, mesenchymal appearance. Alterations in cellular morphology turn an epithelial cell into a mesenchymal cell with altered adhesion and migration capacity. Phenotypic markers for an epithelial to mesenchymal transition (EMT) include an increased capacity for migration and three-dimensional invasion. EMT is a characteristic feature of cells undergoing proliferation. EMT has been shown to occur in proliferating cells (e.g. stomach epithelium) when pathways such as wnt signaling known to be involved with EMT are altered. Therefore, we checked the migratory and proliferative potential of MDCK stable pools expressing different β-catenin constructs by scratch assay and flow cytometric analysis respectively. Mutants Δ(A60–A110), S33Y, and ΔS45 were found to be more fibroblastic in appearance in comparison to WT, Δ(A17–A128) and Δ(A5–A80). As expected, wounded monolayers of cells grown on tissue culture plate reveled an increased migratory potential for mutants Δ (A60–A110), S33Y and ΔS45 with more fibroblastic appearance, compared to WT and mutants Δ(A17–A128) and Δ(A5–A80) (FIG 4.21) with metastable phenotype. WT transfected cells have their β-catenin localized at the membrane giving it higher adhesive property compared to signaling, however it is still actively degraded in cytoplasm.

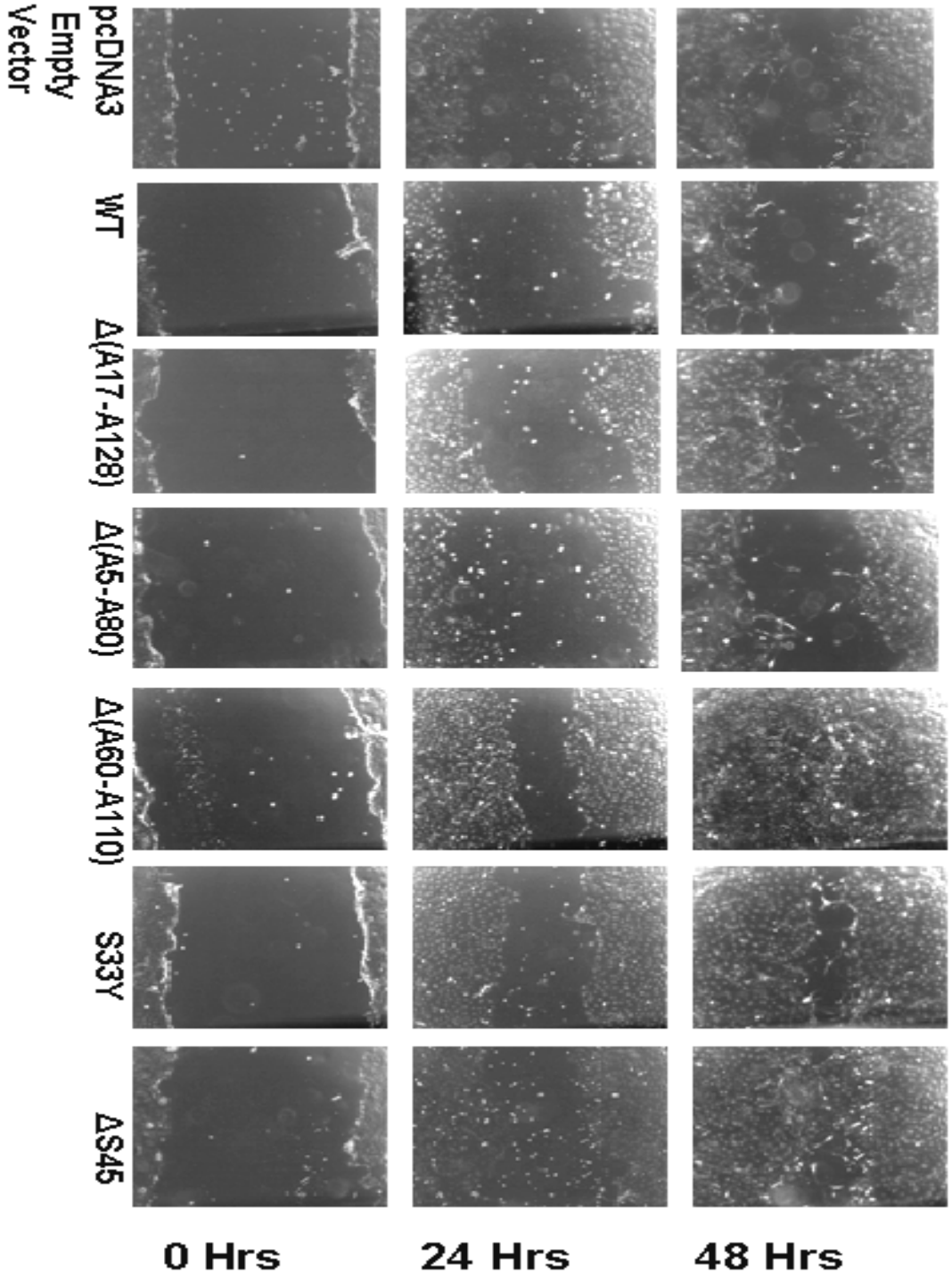


Figure 4.21 Scratch Assay. Cells were evaluated for their ability to migrate into a cell free area following physical disruption of the monolayer. T60, S33Y and ΔS45 showed relatively higher migration then WT, T256 and T3.

Mutant Δ (A17–A128) and Δ (A5–A80) were localized away from the membrane but still have a poor migratory potential. This can be due to metastable phenotype since metastability is consistent with the expression of stem cell markers in intestinal cells undergoing EMT and such plasticity is found in the progenitor cells in various other organs. Cell plasticity can also explain the poor EMT in Δ(A17–A128) and Δ(A5–A80). Sustained β-catenin dependent Wnt signaling in the crypt epithelial cells results in the persistence of the stem/progenitor phenotype; that is continued proliferation, and a failure to migrate up the crypt and terminally differentiate. [17]. Chronic persistence of stem/progenitor phenotype through mutation of intracellular components of the canonical Wnt pathway leads to formation of benign tumors as a initiating event, which can later progress to carcinomas [147]. Mutant Δ (A17–A128) and Δ (A5–A80) showed homogenous nuclear β-catenin accumulation and nuclear translocation was not only restricted at invasive front. Moreover, existence of these mutations in microsatellite stable tumors from relatively younger colon cancer patients, provides strong evidence about its involvement in initiating tumorigenesis, and implies strong pathogenic effect in generating genomic instability later on [140, 141].

Persistence of stem/progenitor phenotype is also marked by increased proliferation. Therefore, we examined the distribution of MDCK stable cell pools in different cell cycle phases by flow cytometry. As seen in result (Fig 4.22 A,B), all stable pools expressing different β-catenin mutants showed increase in proliferation, with more number of cells in S and G2 phase then empty vector transfected control cells. Stable pool Δ (A17–A128) showed highest percentage of cell in proliferative phase followed by Δ (A5–A80) and then other mutants. However, it is interesting to note that the increase in signaling above control levels and the differences between various mutants were relatively small compared to other published results [98, 99]. It may be explained by the fact that we worked with stable pool of cells that stably express a constitutively active transgene. G418 selection pressure to select stable pool, results in production of

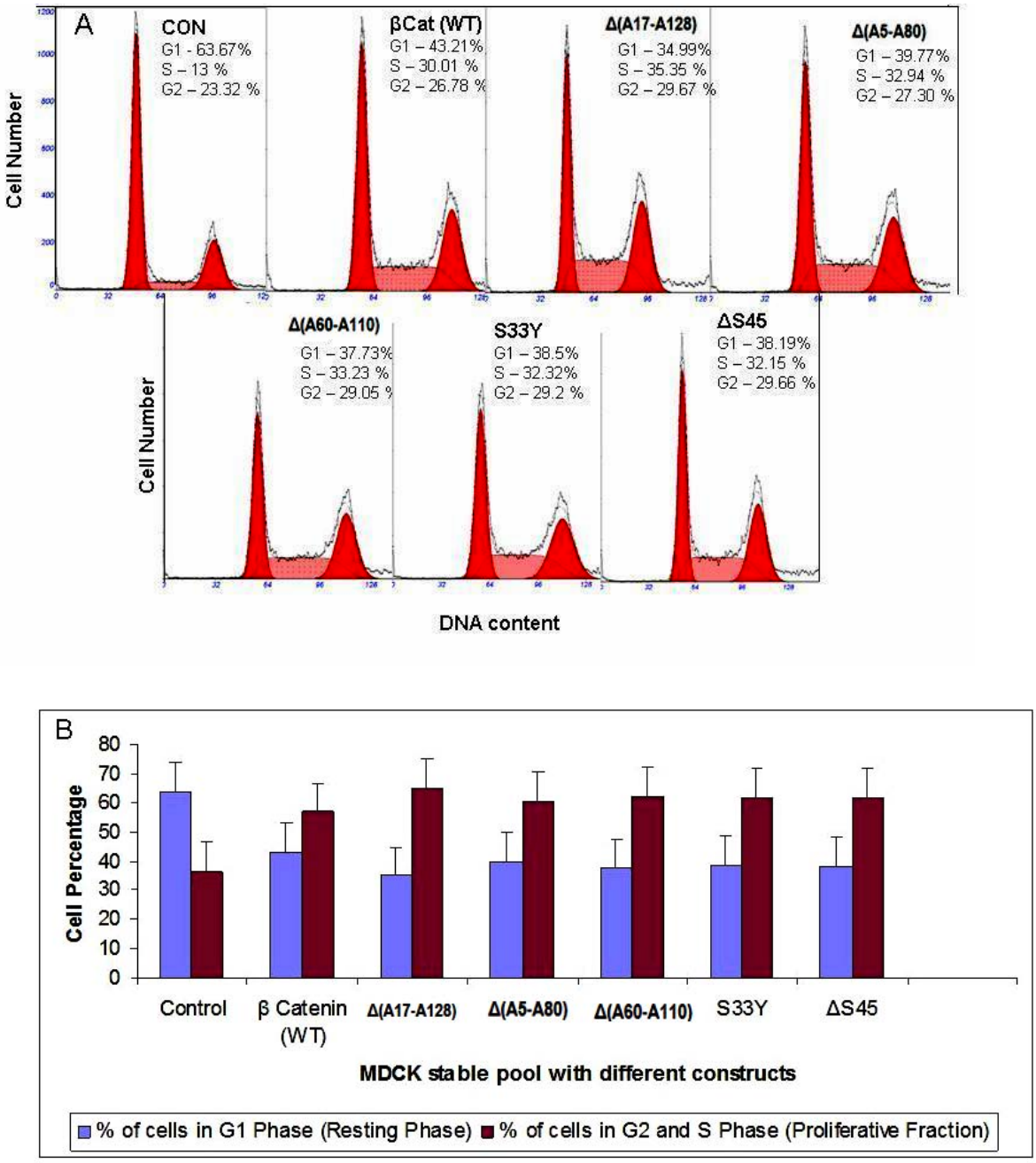


Figure 4.22 β-catenin overexpression alters Cell proliferation. A, DNA/flow Cytometry analysis of cell pools demonstrates stable pools with β-catenin mutants have significantly higher percentage of cells in S and G2 phase and lower percentage of cells in G1 phase. B, Quantification of cell cycle analysis in MDCK stable pool. Bar graph for percentage of cells in resting phase and proliferative phase for stable cell pool transfected with different constructs.

cells expressing only moderately elevated β-catenin protein. Nevertheless, MDCK stable cell pools also consist of G418 resistant cells not expressing mutant β-catenin and hence not showing any functional effect in these cells.

4.4.5 Analysis of Wnt target gene in MDCK stable cell pools.

β-catenin (WT) and various mutants showed different accumulation pattern along with an effect on proliferation, migration and morphology. Cyclin D acts as cell cycle regulator and is one of potential target gene of β-catenin activation. Therefore, we analysed Cyclin D expression level in MDCK stable pools expressing different constructs by immunoblotting with Cyclin D. However, no significant difference among missense mutation and wild type β-catenin in comparison to control (Fig 4.23) was detected in stable cell pool at confluence. Mutant Δ (A17–A128) showed a slight decrease in Cyclin D expression compared to other mutants. Earlier studies [145] have shown differences in Cyclin D level at RNA level but not at protein level. This can be explained by presence of some posttranslational regulation which might down regulate their levels. However, we didn't check their expression at RNA level. Moreover, MDCK is a kidney epithelial cell line and hence their β-catenin target gene might be different from the cells from intestinal origin. A technical aspect could explain these differences. We worked with stable pools of cells, including fractions of G418 resistant cells not expressing our β-catenin constructs. Therefore, we can never be sure of an equal number of positively transfected cells in the mutants studied here. Moreover, we could observe a larger number of GFP positive cells in point mutants and Δ(A60 – A110) compared to WT, Δ(A17–A128) and Δ(A5 – A80) by fluorescence microscope indicating the efficiency of transfection to differ between the mutants and thus providing an explanation for the varying results in target gen expression analysis.

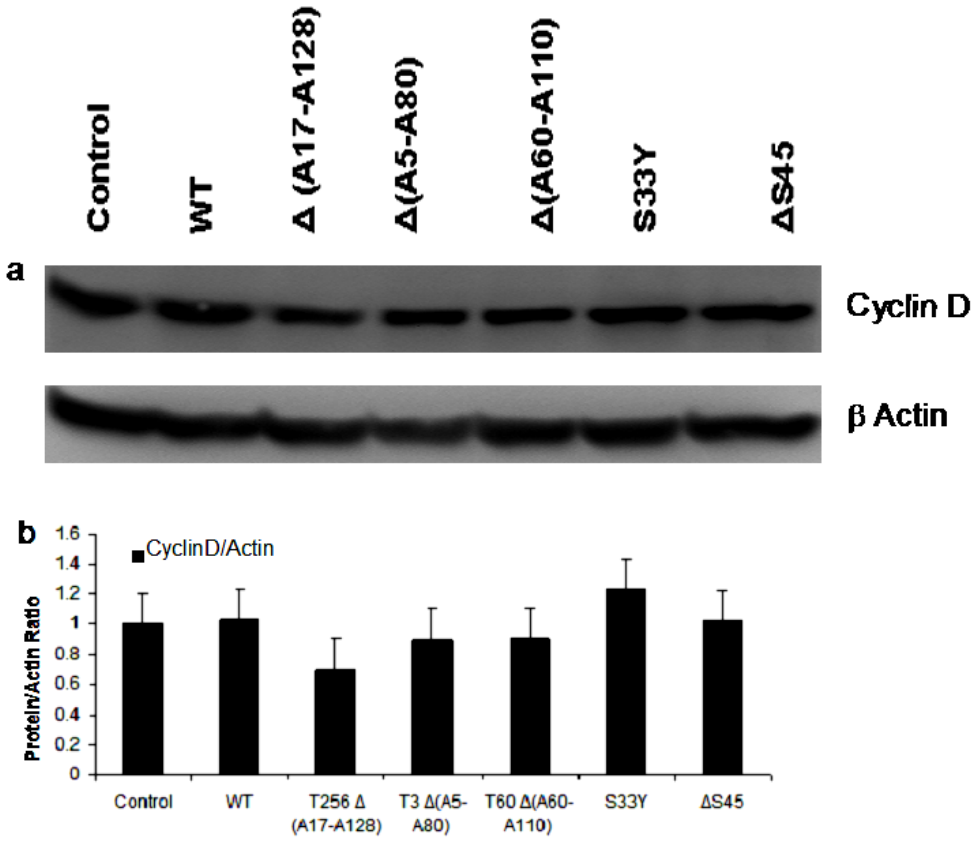


Figure 4.23 Cyclin D Expression in MDCK stable cell pools. a) Total protein lysate was immunoblotted with Cyclin D antibody. Blot of β actin demonstrates equal protein loading. b) Mutant T256 shows slight decrease in Cyclin D expression as identified by densitometric analysis. The blot was normalized to β actin band.

5. DISCUSSION

Carcinogenesis is a sequential process following a series of mutations that result in uncontrolled growth and invasive behaviour of cells. The mutations usually occur in components of pathways essential for the control of normal growth. It is not surprising, therefore, that malignant transformation of cells in organs, in which a given pathway plays a dominant role in homeostasis associate with mutations in this pathway. However, when the same pathway is affected in two different tumor types, the mutated pathway components are frequently different. This is the case for example in the epidermal growth factor (EGF) signaling cascade. While lung cancers, frequently carry activating mutations in the EGF-Receptor [121] carcinomas of the pancreatobiliary tract harbor mutations in the k-ras oncogene, an intracellular downstream component of EGF signaling [148].The reason for this difference between tumor types is not clear. Among the potential explanations exposure towards different carcinogens that preferentially induce a certain type of mutation and organ specific mechanisms of pathway control are the most plausible.

One of the remarkable features of the small intestine is the fact that although the crypt stem cells rapidly divide and although the small intestine contains by far the most stem cells in the gastrointestinal tract, malignancies are exceedingly rare in this location. This must mean that the small intestinal cells, unlike those in the colon, where cancer is highly frequent, have evolved some very effective mechanisms protecting against genetic damage.

The wnt/wingless pathway is one of the most frequently altered signaling cascades in in a variety of human tumors. Several mechanisms of constitutive activation of wnt/wingless exist but stabilization of β-catenin is the common dominator of all these alterations. Mutations can occur in members of the β-catenin degradation complex, namely APC and Axin, as well as in β-catenin itself, while mutation in the wingless receptor and its ligands have not been reported [149]. It is interesting to note, that APC mutations dominate the reason for β-catenin stabilization in colorectal cancer but do not play a significant role for tumorigenesis outside the colon. Similarly, Axin mutations have as yet only been reported for hepatocellular carcinomas [103], while in all the remaining tumors, in

which wingless activation plays a role, like papillary thyroid cancer, pancreatic pseudopapillary carcinomas, skin tumors, β-catenin is mutated [106-109].

Cancer is basically a genetic disease [50]. A cell acquires mutations during life time and if these mutations alter growth relevant genetic information, transformation may be the consequence. In intestinal cancer, two main different types of genomic alteration are seen. On the one hand, the genome of a tumor can be characterized by mutations affecting whole chromosomes and resulting in gains and losses which are visible by cytogenetic analysis, this type of genomic instability, also termed the chromosomal instability type contrasts with the microsatellite instability type, where mutations at a gene level occur in the presence of normal karyotypes. The latter instability follows inactivation of the DNA mismatch repair machinery, which allows for the generation of multiple mutations in a cell [150-152]. Chromosomal instability is more frequent than microsatellite instability, taken together one of both genomic instability types is found in 80% of intestinal cancers. 20% of tumors, however, lack chromosomal and microsatellite instability [55]. The pathogenesis of these so called MACS tumors (microsatellite and chromosomally stable) is poorly understood.

Small intestinal adenocarcinoma is an uncommon neoplasm morphologically similar to colorectal carcinoma, sharing many risk factors with colorectal carcinoma. However, small intestinal adenocarcinoma occurs 50 times less frequent than colorectal carcinoma, even though the small intestine occupy approximately about 75% of the length and 90 % of the mucosal surface of complete gastrointestinal tract [21]. The histological progression to invasive colorectal cancer termed as “adenoma-carcinoma sequence”, involves a series of defined genetic changes mainly in activation of oncogenes and inactivation of tumor suppressor genes [50, 123]. At present, there have been few studies regarding genetic pathways involved in small intestinal cancer. However, based on similarities between the two cancer types it is suggested that they may share many of genetic changes involved in carcinogenesis. Recent molecular studies have shown, however, that the molecular pathways of sporadic tumorigenesis differ in small intestine compared to large intestine [82, 120, 124, 125] Mutations in the P53 and k-ras genes and microsatellite instability occur at approximately similar frequency in both cancers. Inactivation of the Adenomatous polyposis coli

(APC) gene is usually not seen in small intestinal adenocarcinoma [82, 83, 126] but is a hallmark genetic change in colon cancer. Nevertheless, abnormal stabilization of β-catenin was found in approximately 50% of sporadic small intestinal adenocarcinoma indicating an alternative mutation to surrogate for APC mutation in this pathway [124, 125, 127]. These findings indicate that the control of regular cell growth in the small intestine is based on different mechanisms and resistant to genetic events that occur in colorectal cancer. Furthermore, if a mechanism for any potential differences can be identified, this may have clinical implications for future.

In this study we aimed to elucidate the molecular background of β-catenin stabilization in 20 cases small intestinal cancer. Additionally, we also included 20 cases of early age of onset non hereditary colorectal cancer, since these tumors may lack APC mutations and their pathogenesis can not be explained by an age related accumulation of mutations, the common finding in late age of onset colon cancers. By searching for alterations in β-catenin itself, we identified large deletions and insertions in the β-catenin gene, *CTNNB1*, in (4 out of 20) 20% of small intestinal adenocarcinoma and (3 out of 20) 15% of sporadic colorectal carcinoma. Mutation-induced generation of abnormal transcripts was shown by cDNA analysis and stabilization of mutated protein was demonstrated by immunohistochemistry and western blot analysis, respectively. 3 out of 4 small intestinal adenocarcinoma, with large deletions in *CTNNB1* were found in microsatellite stable and chromosomal stable (MACS) carcinomas, with nuclear-predominant accumulation in all areas of the tumors. In colorectal carcinomas, all three tumors with large *CTNNB1* deletions were microsatellite stable with patchy areas for nuclear accumulation. In small intestinal adenocarcinoma, three tumors with a lack of exon 3 sequences displayed strong cytoplasmic accumulation in addition to the nuclear accumulation of β-catenin. One tumor with an additional loss of residues coded by exon 4 showed a nuclear-predominant β-catenin accumulation with only little cytoplasmic protein concentration, a pattern recently observed in a subset of small intestinal adenocarcinoma [124, 127].

In colorectal carcinoma, two tumors lacked the complete exon 3 coding sequences while one tumor lacked the whole N-terminal region with an alternative start codon 131, resulting in a protein lacking amino acid residues

from 1 to 130. Interestingly, the tumor displayed patchy areas of nuclear β-catenin accumulation by immunohistochemistry and no homogeneous accumulation in the nucleus as seen in small bowel cancer. Tumors with cytoplasmic β- catenin stabilization and nuclear translocation solely at the invasion front did not show comparable mutations. Most of the mutations reported here resulted in a loss of N-terminal β-catenin sequences at residues 22–80. These mutant proteins have not only lost all phosphorylation sites encoded by exon 3, they also lack the recognition site for β-TrCP[153]. β-catenin degradation via phosphorylation through the APC–AXIN–GSK3β degradation complex and subsequent β-TrCP-mediated ubiquitination is likely to be largely impaired in these mutants. It is interesting to note, however, that an accumulation of the largest deletion mutant in the small intestine (D17–P128del in T256) was mainly restricted to the nucleus, while an even larger deletion (D1-130) in a large intestinal carcinoma resulted in cytoplasmic and nuclear accumulation, indicating that the loss of residues 81–128 destabilizes β-catenin in the cytoplasm or alters the nuclear–cytoplasmic shuttling of the protein in the small but not in the large intestine. Both the mutants, D17–P128del in small bowel cancer and D1-130del in the large intestine have lost parts of the α-catenin binding site of β-catenin located between residues 120–147[87, 128]. α-catenin has not only been shown to sequester β-catenin in the cytoplasm [129] but can also inhibit the transcriptional activity of β-catenin [130, 131]. Thus, our data indicate that the stability of the cytoplasmic β-catenin pool is differentially regulated in the small and the large intestine and depends on interaction of α- and β-catenin in the small intestinal cells. In the D17-128del mutant we could show, that beside β-catenin alpha catenin was accumulated in the nucleus. This phenomenon indicated a role of α-catenin in the nuclear export of β-catenin, a concept recently postulated also in other studies [154]. Loss of the binding motives in either β or α-catenin may enhance nuclear concentrations of β-catenin and support the oncogenic potential of β-catenin stabilization [155]. While large-scale deletions were only found in microsatellite-stable tumors in our study, Murata et al [140, 141] described this mutational type in a carcinoma with replication error phenotype, indicating a pathogenic impact of this alteration also in tumors arising in the background of microsatellite instability. Earlier studies, have also described

large-scale deletions in β-catenin for colon cancer and hepatocellular carcinoma [128, 141, 156, 157] but in contrast to small intestinal adenocarcinoma, this mutational type is infrequent compared to missense mutations at β-catenin phosphorylation site [107, 109, 127]. While exon 3 deletion mutation was described earlier, we identified two novel mutations, a A17-A128del in a small intestinal adenocarcinoma and an A1-A130del with an alternative start codon in colon cancer. The latter mutant with alternative start codon lacks the complete N-terminal transactivation domain containing amino acid residues 1 to 130.

In the herein investigated cohort of small bowel cancers, we did not find any tumor with a β-catenin missense mutation, resulting in an amino acid change at the conserved phosphorylation sites. Lack of β-catenin missense and APC mutations in small intestinal adenocarcinoma is of particular interest. At the molecular level, point mutations at β-catenin phosphorylation sites and inactivation of APC are considered to exert similar effects on activation of wnt/wingless signaling [76, 158]. This hypothesis is strengthened further by the observation of mutual exclusiveness of APC and β-catenin mutations in sporadic fundic gland polyps, desmoid tumors and colorectal carcinomas [159-162]. β-catenin stabilization in the cytoplasm with nuclear translocation of β-catenin in the invasion front is seen in most colon cancers [133] and almost all colon cancers have inactivating mutations in APC or point mutations in *CTNNB1* [109, 134].

A significant role of APC inactivation has been excluded for sporadic small intestinal adenocarcinoma [82, 120, 124]]. Previous data [125] and the data reported herein also exclude β-catenin point mutations as an important mechanism of small bowel carcinogenesis. Lack of both mutational types which are considered to exert similar effects on wnt-signaling in small bowel adenocarcinoma indicates that the initial gate keeping mutational events in sporadic colorectal and small intestinal carcinogenesis differ fundamentally with existence of some as yet unknown resistance mechanism that prevents occurrence of β-catenin point mutation and APC mutation in small intestinal cell. Idenfication of large deletion mutants of β-catenin in the absence of APC or β-catenin misense mutations with a MSI negative genotype in small bowel cancers and in colon cancers from young patients indicates the existence of an

alternative pathway involved in the pathogenesis of early age onset sporadic tumor development.

We describe here large-scale β-catenin deletions with a stabilizing potential as an alternative pathway for intestinal tumorogensis in absence of APC mutation and β-catenin point mutation which further excluded the possibility that *CTNNB1* point mutations play a significant role in small intestinal carcinogenesis. The existing data on mutations in APC and β-catenin to date contrast with the findings in colon cancer, which indicates different mechanisms of wnt/wingless control in the small and the large intestine. Moreover, the existence of large deletion mutants in colon cancers from young patients, which show a different pattern of β-catenin accumulation in the tumor cells, suggests an alternative pathway for tumorigenesis in the colon.

Usually, one of both types of genomic instability is found in most but not all small and large intestinal adenocarcinoma. According to the few recent studies performed, a share of approximately 20 % of colon cancer is negative for either CIN or MIN showing MACS phenotype. These MIN and CIN negative tumors were found in patients of young age [55]. Our study from small intestinal adenocarcinoma and colon cancer, also included tumors that were MIN and CIN negative (MACS) from patients younger than 35 years of age at the time of diagnosis.

In a subset of small intestinal and colon cancers we identified large in frame deletion deletions of β-catenin including the complete degradation box. The mutation result in an aberrant protein not only lacking all 4 phosphorylation sites, essential for degradation but also the recognition site for β –TrCp mediated ubiquitination. In the small bowel, these mutation lead to peculiar β-catenin accumulation pattern, with homogenous nuclear stabilization all over the tumor areas and not restricted to invasive front only, a phenotype mostly seen in colon cancer. The large N-terminal in frame deletions of β-catenin is expected to have stronger impact on β-catenin stabilization than a single mutation at phosphorylation sites. It was postulated that sporadic early age of onset intestinal cancer with lack of microsatellite and chromosomal instability (MACS) may present a distinctive group that may arise from an as-yet uncharacterized unique molecular pathway. Our finding of *CTNNB1* large scale deletions could present

one possible mechanism of tumorigenesis in young patients. The existence of unusual oncogenic mutations like those we found for β-catenin in single genes would provide an explanation for development of cancer with MACS genotype in young patients. Few studies have already been performed to probe into the carcinogenesis of this subtype [59, 163].

Therefore, we performed an in vitro study to investigate and compare the oncogenic potential of β-catenin large N-terminal deletions to point mutations. After having established the mutant clones using a previously described PCR-based method [164], we transfected Flag tagged and GFP tagged β-catenin wild type and different mutants into SW480 and MDCK cells. We analyzed the effect on morphology, proliferation and migration of cells after transfection to compare the oncogenic potential for wild type β-catenin and different mutant construct. SW 480 is a colon cancer cell line with an APC mutation that already results in β-catenin stabilization. We worked with transiently transfected SW480 cells since we were not able to establish stable cell pool or clone over expressing β-catenin. We could get neomycin resistant SW480 cells after selection, weekly expressing different constructs but the expression was lost after a period of three to four weeks of culturing the cells. Our experiments with transiently transfected SW480 cells showed no significant effect on proliferation or migration of cells over control. Nevertheless, positively transfected cells over expressing exogenous β-catenin disappear in time and showed shrunken apoptotic morphologies. We observed similar results with both flag tagged and GFP tagged constructs. Therefore, it seems likely that β-catenin overexpression causes cell death. Cell death was due to apoptosis and not necrosis since we could see the caspase 3 cleavage in cells over expressing β-catenin over mock transfected control cells. Our result was qualitative but still it can be observed that the extent of cleavage was more in case of mutant T3 and T256 compared to S33Y, ΔS45. β-catenin wt and T60 showed cleavage to a lesser extent compared to other mutants.

A comprehensive survey of primary colon tumors has shown a strict exclusivity between mutation in APC and β-catenin [105] for Wnt pathway activation. None of the tumors with mutant APC contains mutant β-catenin whereas tumors with wild type APC may contain mutations in amino terminal regulatory domain of β-catenin. The latter tumors, however, contain missense mutations or infrequently

exon 3 interstitial deletions that eliminated all of the regulatory serine/threonine residues. The overall frequency of β-catenin mutations in colon tumors is not very high probably because APC is mutated in many of these cancers. Nevertheless, large deletion mutation in β-catenin in absence of APC might act as initial driving force for tumorogenesis in the intestines. Our SW480 cell culture data provide an explanation for mutual exclusivity of APC and β-catenin mutation in human tumors. All β-catenin mutants, Δ (A17-A128), Δ (A5-A80), S33Y & ΔS45 are normally observed in human cancers. In the APC mutated cell line SW480 these mutations result in apoptosis. Therefore, coexistence of mutations in APC and β-catenin may associate with a negative selective pressure, a reason why both mutational types are not found in combination.

Mutant Δ (A60-A110) is not observed in any of human cancers and showed relatively less apoptosis even though it was highly stabilized like other β-catenin mutants. Similarly, apoptosis was significantly less in wild type β-catenin expressing cells compared to cells expressing well characterized mutations found in human cancers. We therefore decided that although SW480 is a cell line from intestinal origin established from a primary adenocarcinoma of the colon, it is not a good model system to study and compare oncogenic potential of β-catenin. SW480 has a bialleic truncating mutation of APC that delete the axin binding sites and prevent β-catenin degradation. This results in abnormally high levels of cytoplasmic and nuclear β-catenin in colon tumor cells. Therefore, in SW480 no conclusion can be made on oncogenic transformation potential of different mutant form of β-catenin. Many controversial data are published regarding whether or not β-catenin induces apoptosis and, if so, whether this is a direct or indirect effect [99, 100, 136, 137] . The mutants detected in the present study, however, may be used for further studies to dissect the mechanism involved in apoptosis induced by β-catenin overexpression not only in SW480 but also in other cell types.

Given the above mentioned phenomenon of apoptosis in SW480, we planed to study the β-catenin wild type and mutant's oncogenic potential in non transformed immortalized epithelial cell line with no mutation in any other gene. We used MDCK cells, a non transformed kidney epithelial cell line from canine with intact cadherin and Wnt signaling components. Previous studies [99, 145],

used this cell line to study the oncogenic potential for different missense β-catenin mutations. To assess the functional implications of large in frame N-terminal deletion mutants in comparison to missense mutation (S33Y, ΔS45) and wild type β-catenin, we created stable cell pool expressing various GFP tagged β-catenin constructs. We found that β-catenin wild type and all mutants contained some level of transforming ability as assayed by increased proliferation and migration over control cells. Mutant Δ (A17-A128), showed highest proliferation followed by other mutants. β-catenin wild type showed increased proliferation over mock transfected control cells but it was relatively less compared to other mutants. In migration assays for MDCK cells, mutant Δ (A60-A110), displayed highest migration potential followed by ΔS45 and then S33Y. Interestingly, mutants Δ (A17-A128) and Δ (A5-A80) were less migrative, slightly greater then β-catenin wild type, even though Δ (A17-A128), displayed relatively highest proliferation. As expected, large N-terminal deletion mutants were oncogenic and lead to a transformed phenotype. However, it was interesting to note that the difference among β-catenin wild type, missense mutation and large deletions were relatively small, contrary to our hypothesis and compared to other published data [106, 165, 166]. It might be explained by the fact that we performed these studies with cells stably expressing a constitutively active GFP tagged transgene. In line with our data on SW480 and previous studies [138] showing high levels of β-catenin expression and signaling that can be achieved by transient transfection is not conducive for cell survival and propagation. Therefore, selection pressure against very high expression results in production of stable pool of cells expressing only moderately elevated β-catenin protein which in turn limits the extent to which more stabilized mutant (large deletion mutants) could stimulate signaling with significant functional effect above wild type β-catenin or missense mutants. All mutants showed transformed phenotype over mock transfected control cells. However, large deletion mutant Δ (A17-A128) and Δ (A5-A80) displayed higher proliferation with relatively lower migration potential in contrast to Δ (A60-A110) and missense mutations. This might be due to leakiness in the assay or because of involvement of other pathways involved in regulation of β-catenin. Especially, the p53 activated Siah/SIP/SCF/Ebi pathway has been shown to degrade β-catenin, but does not

rely on phosphorylation of its N-terminus, [139, 140]. However, we didn't check our stable pool expressing different mutant for any p53 activation or Siah mediated β-catenin degradation. Future studies can be performed for analyzing the influence of these mutations on its turnover. Our novel mutant Δ (A60-A110), is not observed in tumor tissues so far. The mutant lacks codon 60 to 110 while its phosphorylation sites are completely intact. The mutant was found to be expressed and highly stabilized with transforming ability in in vitro studies. It might be due to exemption of this mutant from other pathway involved in β-catenin regulation. The region from codon 60 to 110 is conserved in β-catenin but its function is still unknown. Our data provides some clue that codons 60 to 110 might have some role to play in maintaining the cellular pool of β-catenin.,It might be hypothesized, that large deletion β-catenin mutants can mediate transformation by additional mechanisms like effecting interactions among different pathway than mere protein stabilization. However, this hypothesis requires further experimental validation, where our in vitro model system could be used to answer these basic questions.

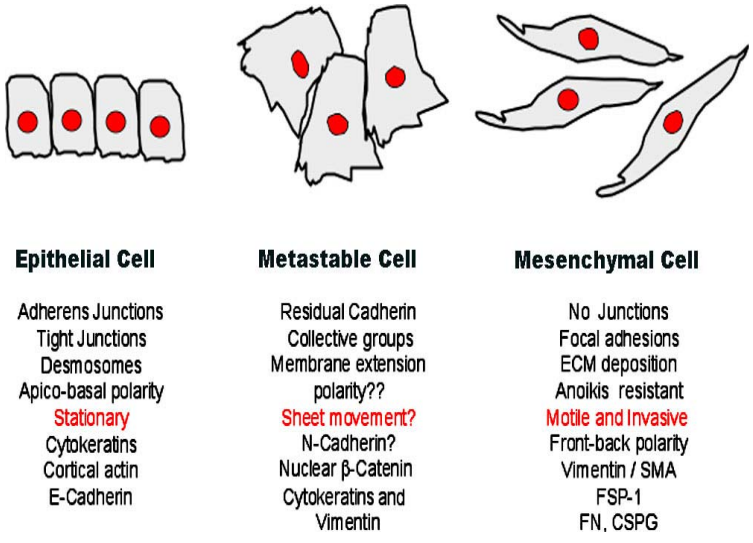


Figure 5.1 The metastable cell phenotype. Several studies have identified a hybrid cell showing both epithelial and mesenchymal traits. These cells are summarized here, in conjunction with their epithelial and mesenchymal counterparts. (Reviewed By Erik W. Thompson 2006)

β-catenin overexpression has a notable effect on cell morphology. The MDCK cell line is a non transformed epithelial line with strong intercellular adhesion that extends cell membrane only to limited degree. β-catenin overexpression converts MDCK into more mesenchymal cell type [141]. At low cell density, cell cell adhesion is reduced and the cells take on a more spindly, stretched shape. This change in morphology is reminiscent of an epithelial to mesenchymal transition (EMT) [142]. EMT is a developmentally important cellular process, especially during gastrulation. Moreover, if dysregulated in adult tissue, EMT has been suggested to play an important role in progression from benign tumor to metastatic carcinoma[143],[167]. β-catenin wild type, Δ (A60-A110) and missense mutants showed a mesenchymal phenotype, however large deletion mutants Δ (A17-A128) and Δ (A5-A80), displayed the so called “metastable phenotype”. The ability of cells to express attributes of both epithelial and mesenchymal phenotypes is referred as a “metastable phenotype” (Figure 5.1).

Metastability is consistent with the expression of stem cell markers in colorectal cells undergoing EMT and such plasticity may be found in progenitor cells in various organs [168]. One of the hallmark of stem cell is their ability to self renew and differentiate. Many studies have been performed to explore the similarities and differences that exist between normal stem cell maintenance within tissues and organs and the uncontrolled proliferation of cancer [147]. Tumors can be considered as small aberrant organ containing a hierarchy of progenitor cells and differentiated cells (Figure 5.2). However, dysfunctional in comparison to a physiologically functioning organ they still maintain their own survival and proliferation[169]. In the cellular hierarchy of tumor cells, there is a differentiation mechanism from tumor stem cells to tumor progenitor cells to mature tumor cells which ends in apoptosis and turnover [148]. Abnormal cellular behavior in this tightly controlled system can occur via genetic alterations, such as tumor suppressor loss or gene destabilization, which result in incremental neoplastic gains and disruption of the homeostatic system [149] leading to aggressive cancer.

Wnt signaling pathway plays an important role in maintenance of normal stem cells. If dysregulated it can result in an expanded progenitor cell population and later to cancer. The large N-terminal β-catenin deletion mutations Δ (A5-A80) and

Δ (A17-A128)) lead to homogenous nuclear accumulation both in vitro and in vivo. Additionally, these mutations were found in colon cancer patients of young age with no genomic instability providing evidence, that this type of mutation might act as initiating point in tumorigenesis by transforming stem cells from obedient to deviant cells having altered management. Nuclear accumulation of β-catenin is one of the characteristic features of stem cells [150, 151]. Our in vitro and in vivo experiments confirmed the nuclear localization of these large deletion mutants. Stably transfected MDCK stable cell pool can be used to generate stable clones for further studies on expression of other stem cell markers like Lgr5 (leucin rich repeat containing G protein coupled receptor 5-positive) and Bmi1 [170].

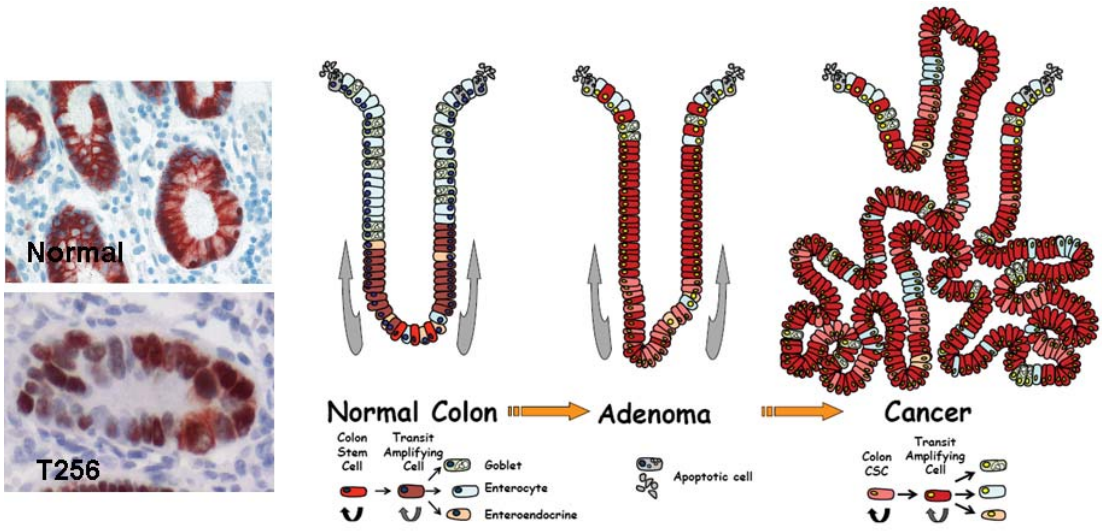


Figure 5.2 Model for colon cancer initiated by stem cells. Colon stem cells are located at the base of the crypt in normal colon and will diff erentiate while moving up the crypt in about 5 days. Adenoma will develop upon deregulation of stem cell homeostasis. Upon further neoplastic injuries, stem cells will transform into cancerous stem cells (CSCs) with some limited ability to differentiate. Homogenous nuclear accumulation in tumor tissue T256 might suggest cancer formation by disruption of stem cell homeostasis. (Reviewed by, Julie M Chandler and Eric Lagasse 2010).

To further investigate the downstream elements of wnt/wingless activation we analysed the Cyclin D expression by western blot analysis. There was no significant increase in its expression for large N-terminal deletion mutations, with

slight upregulation in case of missense mutants compared to mock transfected control cells. Provost et al. [145], examined and compared the effect of overexpression of different β-catenin missense mutations found in benign and malignant tumors. Their data did not reveal any detectable difference at protein level but a striking overexpression for S45A over other missense mutants at RNA level by northern blot. Therefore, our large deletion mutants and missense mutations can be compared at both RNA and protein level for activation of different downstream target gene activation of β-catenin. Nevertheless, cyclin D1 might not be the likely target genes with an essential role in the transformation of MDCK by β-catenin since target genes for Wnt signaling are very tissue specific.

Existing literature is full of inconsistent and conflicting data on the oncogenic potential of β-catenin. Positive as well as negative results are reported for NIH3T3 cells transfected with stabilized mutant forms of β-catenin [98]. L cells are transformed by these constructs [154, 155] whereas Rat 1 cells are not[165], although the latter are transformed by Wnt-1 which usually functions by activating β-catenin-LEF mediated transcriptional activation [165]. Rat kidney epithelial cells RK3E, immortalized by EIA, are transformed by stabilized β-catenin [98], but TEC-18 rat intestinal epithelial cells or 1811 human epithelial cells are not. MDCK cells are sensitive to transformation by wild type and stabilized β-catenin constructs [99] but other β-catenin mutants known to produce a stabilized protein failed to transform MDCK cells [171]. It can be concluded from these data that stabilized β-catenin can transform mammalian fibroblast and epithelial cells but the fact that different sublines of the same cell can vary in their susceptibility to transformation suggests the requirement for additional genetic changes not present in every cell line tested. All of the mammalian cells are from continuous lines and have gone through genetic changes leading to loss of growth regulatory mechanism.

In conclusion, based on our in vitro studies in SW480 and MDCK cells, β-catenin acts as an oncogene in MDCK. Large N term deletion mutants were oncogenic like missense mutants. However, the results were more of qualitative nature. Oncogenic potential can be more significantly compared if similar studies will be performed with a stable clone of these mutants in an inducible system rather than a constitutive active one. Other possibility might be to check these mutants in

primary cell line like CEF, non immortal cells that are tested just after a single subculture, a time interval that is insufficient for selection of genetic variants. Nevertheless, we conclude that the mechanism of transformation is not the same for large deletion mutants in comparison to missense mutants. Interestingly, mutants Δ (A17-A128) and Δ (A5-A80) appear to be less stabilized inspite of deletion of complete degradation box and recognition site for β-TrCP as seen on western blot. Moreover, expression of these mutants pushes cells into "metastable phenotype" with higher proliferation but lower migration potential, characteristic features of stem cells. All these evidences might suggest the expression of these mutants maintains intestinal stem cell characteristics, whose expression might get switched off iN-terminal differentiating cells. Whereas, missense mutation are more involved in epithelial to mesenchymal transition in later stages involved in metastasis and invasion. Further studies on exploring the exact mechanism of transformation by large deletion mutants can unravel the different kinase and pathways that play a role in β-catenin mediated signaling, and will be essential in understanding the tissue specific behavior of β-catenin. Nevertheless, large β-catenin mutations are suggestive of an alternative pathway with a role in initiating transformation in small intestine adenocarcinoma and non hereditary colon cancer with early age onset of disease, in the absence of APC mutations.

Finally, we analysed localization of different β-catenin mutants in cell culture by immunofluorescence since our tumor samples with large scale β-catenin mutations showed homogenous nuclear accumulation. SW480 has a truncation mutation in APC, which leads to defective β-catenin degradation and its accumulation in the cytoplasm and the nucleus. In SW480 cells, wild type β-catenin was localized in cytoplasm and nucleus while mutant Δ (A17-A128) localize completely in nucleus. Mutants Δ (A5-A80), S33Y and ΔS45 showed both nuclear and cytoplasmic accumulation similar to wild type β-catenin. Mutant Δ (A5-A80) displays a little higher nuclear accumulation whereas Δ (A60-A110) shows cytoplasmic accumulation with no nuclear accumulation.

Whereas in MDCK, normal non transformed epithelial cells, β-catenin wild type is mainly located at membrane with no accumulation in cytoplasm or nucleus. Mutant Δ (A60-A110) and S33Y show strong cytoplasmic accumulation with little

nuclear translocation whereas mutant Δ(A5-A80) and ΔS45 show nuclear accumulation in the background of cytoplasmic accumulation. However, Mutant Δ(A17-A128) showed complete nuclear accumulation similar to SW480, without accumulation in the cytoplasm or at the membrane.

Mutant Δ (A17-A128) displayed lower stabilization potential as observed on western blot but still translocated completely to the nucleus. Therefore, our localization studies indicate that β-catenin nuclear translocation is not merely based on its stabilization. Moreover, β-catenin has a molecular mass of 90 kDa, which should prevent its passive diffusion through the nuclear pores. It does not contain nuclear localization signal (NLS) or nuclear export signal (NES) sequences which are required for nucleo cytoplasmic transport by the importin/exportin system but still efficiently enter and exit the nucleus [157, 158]. On the basis of its structural similarities to armadillo repeats of importin β HEAT repeats, it has been suggested that β-catenin can directly interact with nuclear pore proteins although a later study challenged this view point [159]. However, some recent studies provide evidence that the transcriptional activity of β-catenin is modulated by direct regulation of its subcellular localization by variety of interaction partners. LEF/TCF transcription factor can enrich β-catenin in the nucleus [111, 142], and a complex of B-cell lymphoma 9 (BCL9) and its nuclear interactor Pygopus can also strongly recruit β-catenin to nuclear compartment [160, 161]

However, APC, axin and axin 2/conductin shifts β-catenin to the cytoplasm [162, 163], in addition to their role in the destruction complex. As TCF, BCL9, APC and axin shuttles between nucleus and cytoplasm[164-166], [161]it is suggested that TCFs and BCL9 may act as nuclear import factors for β-catenin, whereas APC and axin may actively export β-catenin into the cytoplasm. However, it has not been shown directly that these factors regulate β-catenin subcellular localization by active transport of β-catenin across the nuclear envelope. Our data showing complete nuclear localization for mutant Δ (A17-A128) in SW480 and MDCK indicate that the cytoplasmic and the nuclear pool of β-catenin can be differentially regulated. SW480 has an inactive truncated APC while MDCK has a complete functional wild type APC. Based on evidence from cell transfection experiments [165, 166], APC binds to the CRM1 export receptor via its nuclear

export signals and carries β-catenin from nucleus to cytoplasm. Later studies [172], demonstrated that APC dependent export of β-catenin is dominant in normal cells, whereas the high level of free stabilized β-catenin in tumor cells exit the nucleus via unidentified pathway independent of APC and CRM1 exporter. Nuclear localization of mutant Δ (A17-A128) in both MDCK and SW480, further proves existence of such APC independent pathway for nuclear export of β-catenin that might be deregulated. However, Δ (A17-A128) has an intact binding site for APC, but large N-terminal deletions might affect its binding efficiencies to interacting partners. Our in vitro model system can be used effectively to study the binding affinities of these deletion mutants to various known interacting partners of β-catenin.

α-catenin sequesters β-catenin in the cytoplasm [129, 166], and is also known to inhibit the transcriptional activity of β-catenin [130, 131]. The mutant Δ(A17-A128) has lost parts of the α-catenin binding site of β-catenin located between residues 120–147[87,128].Therefore, we performed α- and β-catenin colocalization studies in tumor tissues and in vitro for SW480 cells. Our in vivo and in vitro colocalization data shows complete nuclear translocation of both α- and β-catenin. β-catenin wild type and missense mutants, with no deletion in alpha catenin binding site, also co localize with β-catenin. In mutant Δ(A17-A128) with deletion of some residues in the α-catenin binding site binding efficiency to α-catenin may not be affect. However, expression of mutant Δ (A17-A128) translocated α-catenin along with it completely to nucleus. Loss of the binding motives in either β- or α-catenin may enhance nuclear concentrations of β-catenin. Thus, the complete nuclear accumulation of mutant T256 over other wild type and missense mutation may reflect a combination of nuclear retention and possibly higher rate of nuclear import with blocked export. Mutant Δ(A17-A128) lacks amino acids (D17–P128) which might leads to loss of binding partner involved in β-catenin nuclear export. This binding partner can be known or unknown. Recently, galectin-3 [167], a novel β-catenin binding partner in the N-terminal region from amino acids 1 -131 was identified. Galectin-3 (gal-3) is a pleiotrophic protein and an important regulator of tumor metastasis, which like β-catenin shuttles between the nucleus and the cytosol in a phosphorylation dependent manner. Moreover, β-catenin stimulation of Cyclin D1 and c-myc

expression is gal-3 dependent [173]. Therefore, Galectin along with other known β-catenin binding partners (APC, Axin etc) can be checked in vitro for their binding efficiency for wild type and mutant Δ(A17-A128) and followed finally for their role in nuclear cytoplasmic shuttling. However, yet unknown binding partners can also be screened using these mutants by yeast two hybrid assay, and later confirm there binding by invitro and in vivo assay. This new binding partner might be involved in yet to identify CRM1 and APC independent pathway involved in nuclear export of β-catenin.

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DECLARATION

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

Heidelberg, 12 July, 2011

Sandhya Singh

SANDHYA SINGH

Institute Of Pathology, University Klinikum, Im NeuenheimerFeld 220/221 D 69120, Heidelberg / Germany, sandhya_singh1@yahoo.com

PERSONAL DETAILS

Nationality: Indian
Citizenship: Indian

EDUCATION

Advance Postgraduate Diploma in Bioinformatics, 2002, University of Pune, India with Ist division (65.08%).

Master of Sciences in Biotechnology, 2001, Indian Institute of Technology, Roorkee, India with honours Ist divison (76.2 %)

Bachelor of Sciences in Bioscience, 1999, Jamia Millia Islamia, New Delhi, India with Ist division 73.3% (73.3%).

AWARDS

2004	Qualified CSIR-UGC National Eligibility Award.
2001	Qualified ICMR JRF Examination.
2001	DBT scholarship Award for bioinformatics course duration.

RESEARCH EXPERIENCE

Present -	Graduate Student Researcher Institute of Pathology, University Klinikum, University of Heidelberg, Heidelberg, Germany
Feb 2005 - Nov 2005	Project Fellow at Eicosanoid Research Division, Department of Experimental Gynaecology UKBF, Charite, Berlin, Germany.
Jan 2004 - Jan 2005	Project Fellow at National Brain Research Centre Gurgaon, Haryana, India
Aug 2002 - Oct 2003	Junior Research Fellow at Institute of Genomics and Integrated Biology, Delhi, India

PUBLICATIONS

1). Breuhahn K, **Sandhya Singh**, Schirmacher P, Bläker H: Large-scale N-terminal deletions but not point mutations stabilize β-cateninin small bowel carcinomas, suggesting divergent molecular pathways of small and large intestinal carcinogenesis. Journal of Pathology, 2008; 215: 300-7**.(Shared First Authorship)**

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POSTER PRESENTATION

1. Poster Presentation in “Wnt symposium 2010” held at DKFZ, Communication Center, Im Neuenheimer Feld 280, Heidelberg from October 25 – 26, 2010.

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- Ciccoli, R., Sahi, S., Singh, S., Deva, R., Zafiriou, M.P., Ishdorj, G., Kock, L., and Nigam, S. 3-hydroxyeicosanoids: novel bioactive compounds obtained from the interaction of 3-HETE, a fungal mimetic of AA, with COX-2 Abstract No: P-149.