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APC dosage effects in tumorigenesis and stem cell differentiation

CLAUDIA GASPAR and RICCARDO FODDE*

Dept. of Pathology, Josephine Nefkens Institute, Erasmus University Medical Center, Rotterdam, The Netherlands

ABSTRACT It is well established that concentration gradients of signaling molecules (the so-called "morphogens") organize and pattern tissues in developing animals. In particular, studies in *Drosophila* and different vertebrates have shown that gradients of the Wnt, Hedgehog (Hh) and transforming growth factor-beta (TGF-β) families of morphogens play critical roles in limb patterning. Morphogens are often expressed in organizing centres and can act over a long range to coordinate the patterning of an entire field of cells. These observations imply that exposure to different concentrations of these diffusible factors may trigger differential cellular responses. In order to study these dosage-dependent Wnt/β-catenin signaling effects, we have generated several hypomorphic mutant alleles at the mouse *Apc* locus and studied their cellular and phenotypic outcomes in stem cell renewal and differentiation, and in tumorigenesis. The results clearly show that *Apc* mutations differentially affect the capacity of stem cells to differentiate in a dosage-dependent fashion. Likewise, different *Apc* mutations (and the corresponding Wnt signaling dosages) confer different degrees of susceptibility to tumorigenesis in the corresponding mouse models. These results have implications for the understanding of the molecular and cellular basis of tumor initiation by defects in the Wnt pathway. We propose a model in which adult somatic stem cell compartments are characterized by tissue-specific β-catenin threshold levels for cell proliferation, differentiation and apoptosis. Different *APC* mutations will result in different levels of β-catenin signaling, thus conferring different degrees of tumor susceptibility in different tissues. Hence, β-catenin dosage - dependent effects may not only explain how a single pathway is involved in the development and homeostasis of different tissues, but also its pleiotrophic role in tumorigenesis.

KEY WORDS: *Wnt, APC, development, differentiation, colorectal tumorigenesis, dosage*

APC, Wnt signalling and colorectal cancer

In 1991 different research laboratories in the US and Japan isolated the adenomatous polyposis coli *(APC)* gene on chromosome 5q22 and identified *APC* germline mutations in patients affected by Familial Adenomatous Polyposis (FAP), an hereditary colorectal cancer syndrome characterized by the presence of hundreds to thousands of polyps in the colon and rectum (Groden *et al.,* 1991, Joslyn *et al.,* 1991, Kinzler *et al.,* 1991, Nishisho *et al.,* 1991). Even more importantly, *APC* was subsequently found to be mutated in the majority of sporadic colorectal cancers notwithstanding the histological stage of the neoplastic lesions analyzed (Miyoshi *et al.,* 1992, Powell *et al.,* 1992, Smith *et al.,* 1993). Thus, *APC* mutations represent one of the earliest event in the adenoma carcinoma sequence (Kinzler and Vogelstein 1996). Mutation analysis of the *APC* gene in sporadic and FAP polyps showed that both alleles are mutated in the majority of the cases (Ichii *et al.,* 1993, Levy *et al.,* 1994), in agreement with Knudson's two hit model of tumorigenesis for tumor suppressor genes (Knudson 1971).

During the last decade, several functional studies have been conducted on the *APC* gene and its protein product, especially aimed at the identification of its main tumor suppressing function (Polakis 2000, Fodde *et al.,* 2001). Notably, these studies have shown APC's involvement in a wide variety of cellular processes such as cell cycle regulation, apoptosis, cell adhesion and migration, microtubule assembly, cell fate determination and chromosomal stability (Fodde 2003). This multi-functionality is reflected by the presence of a large number of specific motifs and domains along the 312 kDa APC protein (Fig. 1). However, biochemical and genetic evidence was provided showing that APC's main suppressor

Abbreviations used in this paper: APC, Adenomatous Polyposis Coli; CRC, Colorectal Cancer; ES, Embryonic Stem; FAP, Familial Adenomatous Polyposis; Hh, hedgehog gene; TGF, transforming growth factor.

***Address correspondence to:** Dr. Riccardo Fodde. Dept. of Pathology, Josephine Nefkens Institute, Erasmus University Medical Center, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. Fax: +31-10-408-8450. e-mail: r.fodde@erasmusmc.nl

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activity resides in its ability to bind to and down-regulate β-catenin (Korinek *et al.,* 1997, Morin *et al.,* 1997, Smits *et al.,* 1999). β-catenin is a key player in the formation of adherens junctions of mammalian epithelia through its binding with the cell adhesion molecule E-cadherin. Moreover, it represents the central signaling molecule within the WNT signal transduction pathway.

Three different functional domains within APC allows it to regulate β-catenin in the cell: three 15 a.a. repeats bind β-catenin, whereas seven 20 a.a. motifs and three SAMP repeats (Ser-Ala-Met-Pro) trigger its down-regulation through the formation of the so-called destruction complex together with the scaffold proteins axin and conductin, and the glycogen synthase kinase 3β (GSK3β) (Rubinfeld *et al.,* 1993, Su *et al.,* 1993, Behrens *et al.,* 1998).
In the absence of the WNT signal, the destruction complex is assembled and GSK3β phosphorylates four critical Ser/Thr residues in β-catenin. This targets β-catenin for ubiquitination and subsequent proteasomal degradation (Rubinfeld *et al.,* 1996, Aberle *et al.,* 1997, Behrens *et al.,* 1998, Hart *et al.,* 1998). Extracellular ligands of the WNT family bind to the transmembrane receptor frizzled and the co-receptor lipoprotein-related proteins 5 and 6 (LRP-5/6) thus inactivating GSK3β in the destruction complex. This inactivation process is not well understood but involves the intracellular protein Dishevelled. As a consequence, β-catenin becomes stabilized, accumulates in the cytoplasm and is eventually shuttled to the nucleus where it binds to DNA-binding proteins of the T-cell factor (TCF/LEF) family, to serve as an essential co-activator of transcription (Behrens *et al.,* 1996, Molenaar *et al.,* 1996).

WNT signaling is tightly controlled by a number of regulators at different steps of the signal transduction cascade. Dickkopf1 and dickkopf2 have been identified as extracellular proteins that modulate the pathway in a positive and negative fashion, respectively (Wu *et al.,* 2000, Semenov *et al.,* 2001). Also, the activity of TCF is tightly controlled at the nuclear level, as TCFs can complex with co repressors such as groucho and chibby (Cavallo *et al.,* 1998, Takemaru *et al.,* 2003).

The WNT pathway is nowadays recognized to function in a variety of critical biological processes such as embryonic development, cell polarity and cell fate specification (Cadigan and Nusse 1997). Moreover, a large body of experimental evidence collected over the past decades clearly pinpoints its role in tumorigenesis. Loss of *APC* function or oncogenic β-catenin activation (Morin *et al.,* 1997, Sparks *et al.,* 1998) results in uncontrolled signaling and activation of downstream target genes. The first two identified downstream target genes of the APC/β-catenin pathway, *c-MYC* and cyclin D1, are clearly relevant in tumor formation because of their role in proliferation, apoptosis and cell-cycle progression (He *et al.,* 1998, Shtutman *et al.,* 1999, Tetsu and McCormick 1999). Changes in the normal expression pattern of these genes are likely to increase the overall proliferation rate. Other WNT target genes, such as matrilysin (Brabletz *et al.,* 1999, Crawford *et al.,* 1999), *CD44* (Wielenga *et al.,* 1999), and *MYC* itself (Brabletz *et al.,* 2000), appear more likely to play a role in tumor promotion rather than initiation.
(see also http://www.stanford.edu/~rnusse/pathways/targets.html).

Here, rather than presenting a comprehensive overview of the different members of the WNT pathway and the WNT downstream target genes thought to play key roles in homeostasis and cancer, we will discuss the issue of dosage of APC/β-catenin signaling and how it can affect stem cell renewal and differentiation during development and tumorigenesis. Though well known to cause not only developmental defects in model organisms but also inherited disorders in man, gene dosage effects have been overlooked in tumor biology. Cell proliferation, differentiation and apoptosis are modulated by gradients of WNT morphogens during development and in the adult organism. Hypomorphic alleles at tumor suppressor genes like *APC* are likely to cause WNT signaling dosage fluctuations below tissue-specific thresholds thus interfering with the control of fundamental cellular processes which either directly trigger tumorigenesis or modify the cellular environment so that additional mutations and/or epigenetic changes at other genes can successfully promote tumor growth.

APC dosage effects in tumorigenesis

As mentioned above, familial adenomatous polyposis is due to germline APC mutations that predict the truncation of the protein. A large number of different nonsense, frameshift, and splice site mutations spread throughout the *APC* gene have been characterized among FAP patients. This broad mutation spectrum is reflected by the clinical heterogeneity of the FAP phenotype: large variability of age of onset, polyp multiplicity and distribution along the GI tract, number and type of extra-colonic manifestations

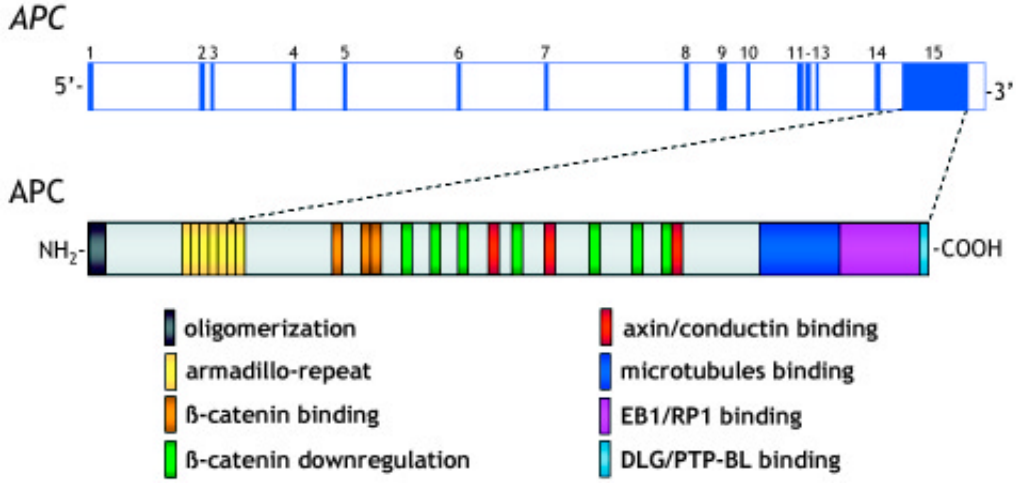


Fig. 1. Schematic representation of the *APC* tumor suppressor. *Upper bar shows the* APC *gene characterized by 15 exons. Exon 15 is the largest (6.6 kb) and encodes most of the protein's main functional domains. Along the APC protein (lower bar), conserved regions, such as the armadillo repeats, and regions that interact with other proteins, including tubulin, the microtubule-associated protein EB1, discs large (DLG), β-catenin and axin/conductin, are shown.*

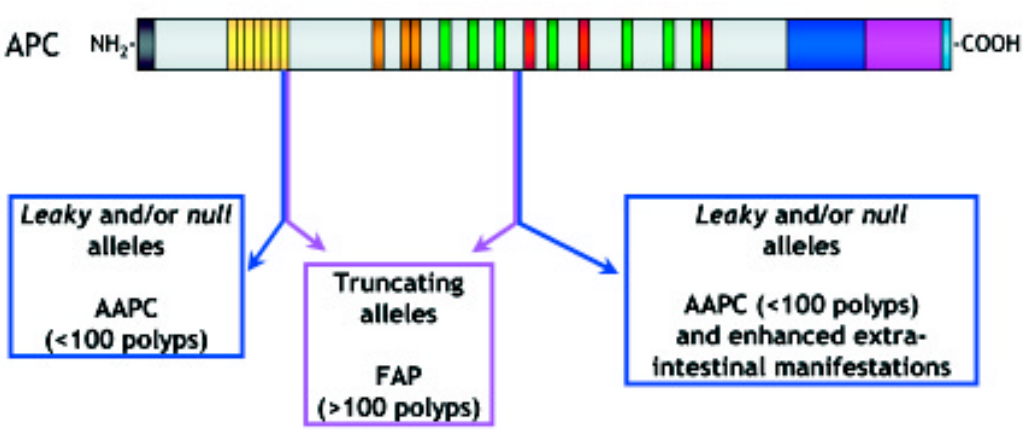


Fig. 2. Human genotype-phenotype correlations at the *APC* gene. *Summary of the relationship between site of the* APC *mutations, their consequences for the stability of the corresponding truncated proteins, and the FAP clinical features (polyp multiplicity and extra-colonic manifestations). In general, mutations located close to the 5' end and in the 3' half of the gene (beyond codon 1600) result in unstable mRNAs and/or polypeptides, and a mild and variable FAP phenotype, termed attenuated adenomatous polyposis coli (AAPC). Consistent correlations between germline mutations at the 3' half of the* APC *gene and FAP extra-intestinal manifestations such as desmoid tumors, CHRPE's and osteomas have also been reported (Fodde and Khan, 1995). Mutations in the central part of the gene (codon 450-1450) result in* in vivo *stably truncated APC proteins and in classical FAP phenotypes characterized by early onset and high polyp multiplicity (>100 and up to several thousands) in the large bowel (Fodde and Khan 1995, Fodde et al., 1999).*

has been reported among different FAP families. This combination of genetic and phenotypic heterogeneity has allowed the establishment of genotype-phenotype correlations (Fodde and Khan 1995) (Fig. 2). In general, mutations that lead to the expression of an *in vivo* stably truncated APC protein result in classical FAP phenotypes characterized by early onset and high polyp multiplicity (>100 and up to several thousands) in the large bowel. On the other hand, truncating *APC* mutations which, due to the instability of the corresponding mRNAs and/or proteins, do not lead to the expression of stably truncated polypeptides are often associated with atypical or attenuated forms of FAP often characterized by delayed age of onset, lower tumor multiplicity and enhanced extra-colonic manifestations (Fodde and Khan 1995). However, the observed FAP intra-familial phenotypic variability, presumably due to genetic and environmental modifiers, does not allow the establishment of more precise cause-effect correlations between different dosages of WNT signaling and their phenotypic outcomes. The generation and analysis of mouse models carrying different *Apc* mutations in the same inbred genetic background and in controlled environmental (dietary) conditions, have been instrumental to our understanding of the mechanisms underlying multi-organ tumorigenesis due to uncontrolled WNT signaling (Fodde and Smits 2001) (Fig. 3).

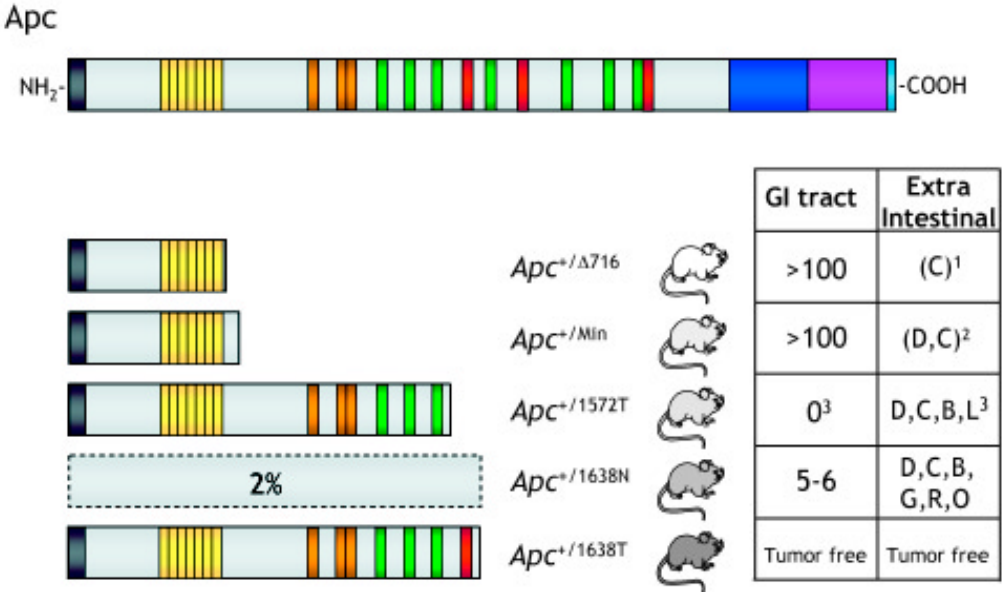
The *Apc^{Min}* mouse model is due to an A to T transversion resulting in a nonsense mutation at codon 850. Heterozygous Apc$^{+/Min}$ mice display a severe intestinal phenotype with more than 100 upper GI tumors in the C57BL/6J (B6) genetic background"(Su *et al.,* 1992). The wild-type *Apc* allele is lost together with the entire chromosome 18 in almost 100% of the lesions (Levy *et al.,* 1994). Furthermore, *Apc$^{+/Min}$* mice have a low penetrance of extracolonic-manifestations such as mammary tumors, desmoids or cysts (Moser *et al.,* 1993, Smits *et al.,* 1998, Halberg *et al.,* 2000). Homozygosity for this mutation leads to embryonic lethality at very early stages of gestation (6.5 dpc) (Moser *et al.,* 1995). Accordingly, a similar mouse model, Apc$^{\Delta 716}$, carrying a targeted truncated mutation at codon 716 is also characterized by multiple intestinal tumors and no extra-intestinal manifestations (Oshima *et al.,* 1995). Both Apc^{Min} and Apc$^{\Delta 716}$ mutations result in stable truncated Apc proteins retaining the N-terminal oligodimerization and Arm domains but are deprived of all the β-catenin binding and down-regulation domains.

In the past years, our laboratory has generated different FAP mouse models due to hypomorphic *Apc* mutations (Fodde *et al.,* 1994, Smits *et al.,* 1998, Smits *et al.,* 1999). Two of them, Apc^{1638N} and Apc^{1638T} carry similar targeted mutations at codon 1638 which differ in their ability to express the corresponding 182 kDa truncated protein encompassing all 3 β-catenin binding domains, three of the 7 β-catenin down-regulating repeats, and only one of the 3 SAMP axin/conductin binding motifs. Whereas the Apc^{1638T} mutation does express the expected 182 kDa truncated protein at normal levels readily detected by western analysis, residual (1-2%) amounts of the same protein are detectable in Apc^{1638N} only after immunoprecipitation assays (Smits *et al.,* 1999, Kielman *et al.,* 2002). Notably, Apc$^{+/1638T}$ mice are tumor free and homozygous Apc$^{1638T/1638T}$ animals are viable though with growth retardation and specific developmental defects (Smits *et al.,* 1999).

Apc$^{+/1638N}$ mice do develop intestinal tumours though significantly fewer (5-6) when compared with Apc^{Min} and Apc$^{\Delta 716}$. Apart from its attenuated intestinal tumor phenotype, the Apc$^{+/1638N}$ mouse model is characterized by a wide spectrum of extra-intestinal manifestations ranging from desmoid tumors, epidermal cysts, mammary tumors, abnormalities of the retinal pigment epithelium, gastric tumors and osteomas (Fodde *et al.,* 1994, Marcus *et al.,* 1997, van der Houven van Oordt *et al.,* 1997, Smits *et al.,* 1998, Smits *et al.,* 2000b). Apc$^{1638N/1638N}$ homozygous animals are embryonic lethal and die during gastrulation at 7.5 dpc (Kielman *et al.,* in preparation).

More recently, we have generated the Apc^{1572T} mouse model by targeting a nonsense codon at residue 1572 (Smits *et al.,* 1999)(Gaspar *et al.,* in preparation). The Apc^{1572T} mutation results in a stable truncated protein encompassing all the β-catenin binding domains, 3 of the 7 β-catenin downregulating repeats, but, unlike Apc^{1638T}, none of the SAMP axin/conductin binding motifs. Surprisingly, Apc$^{+/1572T}$ mice develop invasive and metastasizing mammary cancer, desmoids, epidermal cysts, and hepatic tumors, but no intestinal adenomas. An overview of the above Apc mouse models, their intestinal and extra-intestinal phenotype, and their corresponding truncated proteins is depicted in Fig. 3.

The striking phenotypic differences among Apc mouse models strongly correlates with specific dosages of transcriptionally active nuclear β-catenin (and thus of WNT signal transduction) measured



	GI tract	Extra Intestinal
$Apc^{+/\Delta 716}$	>100	(C)[1]
$Apc^{+/Min}$	>100	(D,C)[2]
$Apc^{+/1572T}$	0[3]	D,C,B,L[3]
$Apc^{+/1638N}$	5-6	D,C,B, G,R,O
$Apc^{+/1638T}$	Tumor free	Tumor free

Fig. 3. Mouse genotype-phenotype correlations at the *Apc* gene. *Pre-clinical mouse models for FAP. The full-length APC protein is shown with the major functional domains characterized to date (see key in Fig. 1). The main five models are depicted next to the truncated APC proteins they encode. Please note that Apc^{1638N} and Apc^{1638T} truncated proteins are virtually identical as far as the position of the termination codon is concerned. However, whereas Apc^{1638T} is present in a 1:1 ratio with wild-type Apc, in Apc^{1638N} only 'leaky' amounts (1–2%) of the predicted protein are generated. Tumor multiplicity in the gastro-intestinal tract and elsewhere are listed in the table. These phenotypic data have been obtained from inbred C57BL/6J mice.* Abbreviations*: C, cysts; D, desmoids; B, breast tumors; L, liver tumors; O, osteomas; R, abnormalities of the retinal pigment epithelium; G, gastric tumors.* Notes*: [1]Apart from the rare occurrence of epidermal cysts (1 in 200 mice), extra-intestinal manifestations have not been reported for the $Apc^{\Delta 716}$ mouse model (Dr. M. Taketo, personal communication). [2]Apc^{Min} mice develop on average less than a single desmoid tumor per animal and 1-2 cutaneous cysts invariably located in the skin covering the neck region (Smits et al., 1998, Halberg et al., 2000). [3]Gaspar* et al., *manuscript in preparation.*

by the TOPFLASH reporter assay (Korinek *et al.,* 1997) in ES cells carrying the Apc^{Min}, Apc^{1638N},–Apc^{1638T}, and Apc^{1572T} targeted mutations in various genetic combinations (Smits *et al.,* 1999, Kielman *et al.,* 2002). We observed an increasing gradient of β-catenin regulatory activity among the different genotypes with $Apc^{Min/Min}$ and $Apc^{1638N/1638N}$ ES cells showing the highest reporter activity, followed by $Apc^{1638N/1572T}$, $Apc^{1638N/1638T}$, and $Apc^{1638T/1638T}$, the latter being comparable to wild type cells (Fig. 4).

A clear trend in the correlation between WNT signaling and tumor phenotype is visible: severe truncation of the β-catenin regulating domains as in the case of Apc^{Min}, results in high dosages of transcriptionally active β-catenin and in the highest tumor multiplicity in the GI tract. In the case of more hypomorphic alleles as Apc^{1638N}, Apc^{1638T} and Apc^{1572T}, the decrease in β-catenin signaling is accompanied by a lower polyp incidence in the intestine. On the other hand, the type and incidence of extra intestinal manifestations seems to be inversely correlated with WNT activity and the severity of the intestinal phenotype. Desmoid tumors and cysts, virtually absent in Apc^{Min} (Smits *et al.,* 1998, Halberg *et al.,* 2000), are common in both Apc^{1638N} and Apc^{1572T}, while the latter is also characterized by spontaneous and aggressive mammary adenocarcinomas unique to this strain (Gaspar *et al.,* in preparation) (Fig. 3).

A possible explanation for these genotype-phenotype correlations can be found in the central role of the APC/β-catenin signalling in stem cell renewal and differentiation during development and in adult tissues (Ridanpaa *et al.,* 2001, Batlle *et al.,* 2002, Kielman *et al.,* 2002, van de Wetering *et al.,* 2002). We hypothesize that homeostasis of several adult stem cell niches is regulated by tissue-specific WNT signaling levels. Specific β-catenin activity levels resulting from different *APC* mutations may affect proliferation, differentiation and apoptosis in a tissue-specific fashion, thus triggering tumorigenesis in a subset of organs throughout the body.

Notably, the absence of tumors and the post-natal viability characteristic of mice homozygous for the Apc^{1638T} allele allow us to delineate the critical domains of the APC protein involved in tumorigenesis and development: a truncated protein encompassing three of the seven 20 amino acid repeats and one SAMP motif, but missing all of the carboxy-terminal domains previously thought to be associated with tumorigenesis, is sufficient to ensure in utero development and prevent tumorigenesis in the adult (Smits *et al.,* 1999). A gene dosage effect is the cause of the phenotypic differences between Apc^{1638T} and Apc^{1638N} mouse models. Although these two alleles encode for the same truncated protein, whereas in Apc^{1638T} expression levels are comparable to those of the full-length protein and sufficient to prevent tumor formation, in Apc^{1638N} the presence of only residual amounts (1-2%) of this otherwise functional truncated protein underlies this model's susceptibility to multi-organ tumorigenesis.

A different example of the relevance of Wnt dosage effects in tumorigenesis came from the analysis of germline (1st hit) and somatic mutations (2nd hit) at the *APC* gene from FAP intestinal adenomas. Lamlum *et al.,* (1999) showed that polyps originating from patients with germline mutations around codon 1300 are characterized by allelic loss as the preferred second hit mechanism. On the other hand, polyps from patients harboring germline mutations outside this region show preferentially somatic point mutations and only very rare allelic losses as second hit mechanism (Lamlum *et al.,* 1999). In our laboratory, a similar approach was applied to both mouse and human polyps. Apc^{1638N} or Apc^{Min} mice when bred in a mismatch repair (Msh2) deficient background undergo somatic inactivation of the wild type *Apc* allele by point mutation rather than by complete chromosomal loss as observed in the majority of tumors from *Apc* mutant mouse models. Somatic mutations at the *Apc* gene in the $Apc^{+/+}$/$Msh2^{-/-}$ mice are predominantly dinucleotide deletions at simple sequence repeats

leading to truncated Apc polypeptides that partially retain some of the 20 a.a. beta-catenin downregulating motifs. In the presence of a germline *Apc* mutation ($Apc^{+/-}$/$Msh2^{-/-}$ mice) the spectrum of somatic *Apc* mutations is shifted to the 5' end, thereby completely inactivating Apc's beta-catenin downregulating activity (Smits *et al.,* 2000a). These results indicate that somatic *Apc* mutations are selected during intestinal tumorigenesis and that specific combination of *Apc* alleles encoding for specific dosages of β-catenin signaling represent the main selective factor. Also, analysis of the type and distribution of 2nd hit mutations in polyps from FAP patients revealed a clear interdependence between germline and somatic mutations (Albuquerque *et al.,* 2002). Germline mutations that do not retain any of the β-catenin downregulating domains are usually associated with somatic point mutations encompassing one or less frequently two downregulating motifs. In contrast, the majority of polyps from FAP patients with germline mutations retaining one downregulating motif are characterized by somatic loss of the wild type *Apc* allele, though few point mutations that remove all the downregulating motifs were also observed. Accordingly, among tumors where the germline defect retained 2 downregulating domains, the most common somatic hits are point mutations that remove all 20 a.a. domains, or, less frequently, allelic losses. It should be noted that most truncated APC proteins retaining one or more of the 20 a.a. repeats, as found in colorectal tumors, do not represent null alleles and encode for residual β-catenin regulating activity (Albuquerque *et al.,* 2002).

According to the "just-right" signaling model (Albuquerque *et al.,* 2002), APC regulation of Wnt/β-catenin signal transduction must be impaired at specific levels to successfully trigger tumorigenesis. A too low signal will not provide sufficient transcriptional response and selective advantage to allow clonal expansion, whereas a too strong signaling activity might trigger an apoptotic response (Kim *et al.,* 2000).

Another model has been suggested to describe the interdependence between 1st and 2nd hit in APC-driven tumorigenesis. According to the "loose fit" model, although the majority of the cases seem to retain at least two downregulating domains, margins of variation in the number of remaining functional domains exist (Crabtree *et al.,* 2003). Additional studies on tumors other than those of the colon-rectum, revealed tissue-specific thresholds of β-catenin mediated signaling. In desmoid tumours the majority of somatic mutations retain two or three downregulation domains (Miyaki *et al.,* 1993, Palmirotta *et al.,* 1995, Giarola *et al.,* 1998). Upper gastrointestinal tumours also seem to follow the same trend (Groves *et al.,* 2002).

APC dosage effects on differentiation

As mentioned before the Wnt pathway is better known for its role in differentiation and cell fate determination in a wide range of organisms, from Drosophila to mammals (Cadigan and Nusse 1997). In the early mouse embryo Wnt signaling is involved in the differentiation of pluripotent embryonic stem (ES) cells present in the inner cell mass into the three main germ layers: ectoderm, mesoderm and definitive endoderm. ES differentiation is not exclusively regulated by WNT but also by other factors like BMP's, FGF's, Smad's and Mixl1. Complex co-activation and interactions among these signaling pathways give rise to the different lineages (Loebel *et al.,* 2003).

Gene dosage effects are well known to control differentiation and cell fate. Lack of expression of the transcription factor OCT4 (Pou5f1) results in trophectodermal ES cell differentiation; its increased expression induces endodermal and mesodermal differentiation whereas intermediate expression levels are needed for maintenance of ES cells pluripotency (Niwa *et al.,* 2000).

Different dosages of Apc/β-catenin signalling modulate stem cell renewal and differentiation. Mouse embryonic stem cells can easily be genetically manipulated and cultured *in vitro* without affecting their differentiation potential. There are several possibilities to assess the ability of these cells to differentiate: *in vitro* assays where culture conditions are modulated to induce ES differentiation into specific cell types or lineages, and teratoma formation assays where ES cells are injected in isogenic mice to allow *in vivo* differentiation and formation of benign teratomas.

Our collection of *Apc*-mutant ES cell lines shows a gradient of β-catenin regulatory activity and represents therefore a unique tool to study Wnt dosage-dependent consequences on stem cell differentiation (Fig. 4). We have therefore investigated the effect of different Apc dosages on the differentiation potential of mouse ES cells by teratoma formation and microarray expression profiling (Kielman *et al.,* 2002). We have provided genetic and molecular evidence that the ability and sensitivity of ES cells to differentiate into the three germ layers is inhibited by increasing dosages of β-catenin signaling ranging from a severe differentiation blockade in severely truncated *Apc* alleles, to more specific neuroectodermal, dorsal mesodermal and endodermal defects in more hypomorphic alleles. Accordingly, a targeted oncogenic mutation in the β-catenin gene recapitulates the differentiation defects observed in *Apc* mutant ES cells (Kielman *et al.,* 2002). From these results we

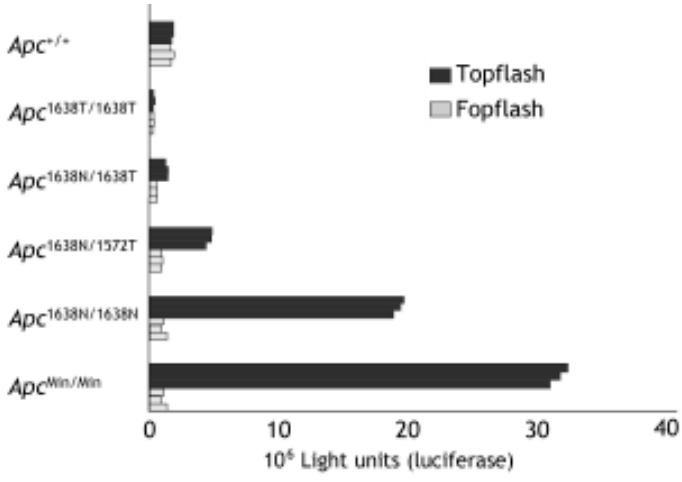


Fig. 4. Wnt (β-catenin/Tcf) reporter assay analysis of the *Apc* allelic series. *Embryonic stem cells carrying different allelic combinations of targeted* Apc *mutations were tested by the TOP-Flash reporter assay(Korinek et al., 1997) for their ability to activate luciferase expression under the control of TCF binding sites, (TOP, dark gray bars). Baseline expression of the vector was tested using mutated TCF binding sites (FOP, light gray bars). Measurements are the average of triplicate measurements of three independent experiments. All ES cell lines here employed are inbred 129/Ola, with the only exception of* $Apc^{Min/Min}$ *(C57BL/6J). Please note that this figure represents a composition derived from two different sets of previously reported assays (Smits* et al., *1999, Kielman* et al., *2002).*

can conclude that specific levels of Apc/β-catenin signaling differentially affect stem cell differentiation.

Differentiation into paraxial mesoderm requires a higher induction of Wnt signalling while differentiation into neuroectoderm and endoderm require lower levels or even inhibition of the Wnt signal (Loebel *et al.,* 2003). These results are in agreement with experiments in Wnt3a null mouse models where the epiblast cells of homozygous embryos divert into neuroectodermal differentiation instead of differentiating into mesodermal cells (Yoshikawa *et al.,* 1997). Moreover, mouse models lacking both Lef1 and Tcf1 are also defective in paraxial mesoderm differentiation (Galceran *et al.,* 1999). Sfrp2, an antagonist of Wnt signalling, promotes *in vitro* neuronal differentiation when transfected into ES cells (Aubert *et al.,* 2002).

Expression profiling of wild type and *Apc*-mutant teratomas supports the differentiation defects at the molecular level and pinpoints a large number of downstream structural and regulating genes. Hierarchical clustering analysis correctly clustered the *Apc*-mutant teratomas according to β-catenin signalling dosage and to differentiation potential, even in those cases histologically indistinguishable from wild type teratomas. Notably, the distribution between up and downregulated genes in the ES-derived teratomas is approximately 50-50. The latter observation indicates that Wnt/β-catenin activity is not exclusively associated with transcriptional activation of target genes but also with their repression (Kielman *et al.,* 2002). More recently, microarray analysis of undifferentiated ES cells revealed that the expression profile of wild type stem cells is distinguishable from that of *Apc* mutant cells even before differentiation is induced (unpublished data). Thus, differential transcriptional regulation of Wnt/β-catenin downstream targets precedes the differentiation defect.

During development, the organism is able to accommodate stem cells in specific niches that preserve and restrict their renewal and differentiation. Although thought to be derived from ES cells through a progressive restriction of their pluripotency during development, adult stem cells still possess ample cell-renewal capacity and differentiation potential (Jiang *et al.,* 2002). They play an important role in the homeostasis of several self-renewing tissues like bone marrow, gut and skin. The dosage effects mentioned for ES cells also seem to hold true for adult stem cells. Mesodermal stem cells have the capability of differentiating among others to adipocytes and myocytes; Ross and colleagues have elegantly shown that activation of the Wnt pathway inhibits adipogenesis by downregulating the expression of transcription factors like C/EBPα and PPARγ. On the other hand, constitutive Wnt signaling is essential for myocyte differentiation since withdrawal of the Wnt signal by a dnTCF4 triggers the trans-differentiation of myoblasts into adipocytes (Ross *et al.,* 2000). Hence, Wnt signalling seems to control the differentiation program of mesenchymal adult stem cells in a dosage dependent way.

Another classic example of adult stem cell is located in the intestinal crypts of Lieberkhun. The intestinal mucosa is specialized for absorption of nutrients and consists of a simple columnar epithelium the surface area of which is vastly increased via evaginating villi and invaginating crypts. Throughout the intestine, large numbers of cells are daily shed into the lumen and must be replaced by new cells differentiating from multipotent stem cells. These stem cells have the ability to differentiate into five different intestinal types: columnar absorptive enterocytes, mucus-secreting Goblet cells, hormone-secreting enteroendocrine cells, the antimicrobial peptide producing Paneth cells, and the M cell. The first three cell types can be found throughout the whole intestine, Paneth cells are restricted to the small intestine, and M cells can only be found in the crypts adjacent to the Peyer's patches (Marshman *et al.,* 2002).

A consistent body of experimental evidence supports a role for Wnt signaling in the regulation of intestinal stem cell differentiation. First, $Tcf4^{-/-}$ mice cannot sustain an intestinal stem cell compartment due to premature onset of differentiation, strongly suggesting that activation of Wnt downstream targets are required to maintain proliferative capacity (Korinek *et al.,* 1998). Thus, Wnt signaling is needed for the maintenance of the intestinal stem cell compartment. Further evidence was provided with the colon cancer cell line Caco-2 as a model. *In vitro* differentiation of Caco-2 cells along the absorptive cell lineage coincides with downregulation of Wnt signaling (Mariadason *et al.,* 2001). More recently, it was shown that inhibition of β-catenin/TCF-4 transcriptional activity induces a rapid G1 arrest and interferes with the physiological proliferation program active in the lower third of the colon crypt (van de Wetering *et al.,* 2002). Coincidently, an intestinal differentiation program is triggered. The Wnt target gene *c-MYC* plays a central role in this switch by direct repression of p21(CIP1/WAF1). Following disruption of β-catenin/TCF-4 activity, the decreased expression of c-MYC releases p21(CIP1/WAF1) transcription, which in turn mediates G1 arrest and differentiation (van de Wetering *et al.,* 2002). Thus, the β-catenin/TCF-4 complex seems to represent the master switch that controls the equilibrium between proliferation and differentiation in the intestinal epithelium.

In the small intestine, the progeny of stem cells follow specific differentiation patterns. Absorptive, enteroendocrine, and goblet cells migrate upwards toward the villus while Paneth cells occupy the bottom of the crypts. Batlle *et al.,* showed that β-catenin and TCF couple proliferation and differentiation to the sorting of cell populations in the intestinal epithelium by controlling the expression levels of the EphB receptors and their ligand ephrin-B along the crypt-villus axis (Batlle *et al.,* 2002). Accordingly, in the intestine of EphB-null mice the proliferative and differentiated populations intermingle, and Paneth cells do not follow their downward migratory path, but are scattered along crypt and villus.

A model for Wnt-mediated regulation of intestinal homeostasis and its consequences for tumour initiation and progression

Different observations from our and other laboratories are indicative of a central role of the Wnt signal transduction pathway in controlling the renewal and differentiation of stem cell compartments both during development and in the adult organism (Korinek *et al.,* 1998, Batlle *et al.,* 2002, Kielman *et al.,* 2002, van de Wetering *et al.,* 2002). Cell renewal and differentiation are regulated by β-catenin in a dosage-dependent fashion. It is now well established that concentration gradients of signaling molecules known as morphogens organize and pattern tissues in developing animals. In particular, studies of patterning of Drosophila and vertebrate limbs have demonstrated the critical roles of gradients of the Wnt, Hedgehog (Hh) and transforming growth factor-beta (TGF-β) families of morphogens. These molecules are often expressed in organizing centres, and can act over a long range to

coordinate the patterning of an entire field of cells. Importantly, their transcriptional and cellular effects are, as in the case of β-catenin, dose-dependent, which identifies them as the 'morphogens' first postulated in 1924 (Spemann and Mangold 2001, Kielman *et al.,* 2002). The latter nicely fits in a model where a Wnt organizing centre is located in the stroma underlying the intestinal epithelial lining that generates a gradient of Wnt activity along crypt-villus axis. Due to the organization of the intestinal epithelium in invaginations in the stroma (crypts) and protrusions into the lumen (villi), the stem cell niche at the bottom of the crypt is located at the shortest physical distance from a putative source of Wnt diffusible ligands. Accordingly, in the colon nuclear β-catenin localization has been shown in normal intestinal cells located at the bottom of the crypt (Batlle *et al.,* 2002, van de Wetering *et al.,* 2002) (Fig. 5). In the small intestine, Paneth cells migrate downwards and share the lower third of the crypt with the putative stem cells. Both Paneth cells and other intestinal cells located in the bottom third of the crypt are characterized by nuclear β-catenin. Therefore, physiological β-catenin signaling occurs in lower crypt cells including the Paneth cells (Batlle *et al.,* 2002). The downward migration pattern of Paneth cells results from the presence of the EphB3 receptor as shown in EphB3$^{-/-}$ mice where Paneth cells are scattered along crypt and villus. Notably, Paneth cells can correctly differentiate when positioned outside the bottom third of the crypt but fail to show nuclear β-catenin (Batlle *et al.,* 2002). Thus, while induction of β-catenin nuclear translocation appears to result from a cell non-autonomous process and depends on the position of the epithelial cells along the crypt-villus axis, the differentiation program of Paneth cells occurs independently of Wnt signalling. Accordingly, several Paneth-specific cryptdin/defensin-related genes were found to be upregulated in a dosage-specific fashion in teratomas derived from *Apc*-mutant ES cells (Kielman *et al.,* 2002).

In the progenitor intestinal stem cells, Wnt/β-catenin signaling activates a genetic program of cell proliferation by c-MYC activation and p21(CIP1/WAF1) repression, and consequent 'stemness', i.e. the maintenance of multi-potency, by a broad range of downstream effectors. Moving upward along the crypt-villus axis, the exposure to the Wnt ligands will progressively decrease thus triggering p21-mediated growth arrest and terminal differentiation (Batlle *et al.,* 2002, Kielman *et al.,* 2002).

APC expression along the crypt villus also shows an inverse gradient suggesting an increased level of expression with maturation (Smith *et al.,* 1993, Midgley *et al.,* 1997). However, how this expression gradient is determined is still unclear. A similar regional organization of β-catenin expression and intracellular localization has been observed in the hair follicle (Ridanpaa *et al.,* 2001) and is likely to apply to a broad spectrum of adult stem cell compartments.

Enhanced β-catenin/TCF4 activity is common to normal intestinal stem cell compartments and colorectal cancer. This indicates that colorectal tumors may result from excessive proliferation and/or lack of differentiation within the stem cell compartment. Computer simulations of proliferation, migration and differentiation rates in the colonic crypt showed that only an enlargement of the stem cell compartment efficiently predicts polyp formation (Boman *et al.,* 2001). These observations well agree both with a model where tumor formation occurs in the crypt, but also with a top-down mechanism where well-differentiated cells in the villi may acquire *APC* or β-catenin mutations leading to Wnt deregulation, trans-differentiation and regression to a more proliferative, progenitor cell-like phenotype (Shih *et al.,* 2001). This central role of Wnt-mediated 'stemness' in tumor formation is also in agreement with the observation according to which specific degrees of beta-catenin signaling are selected during polyp formation (Albuquerque *et al.,* 2002). These tumor-selected signaling dosages will promote stem cell proliferation, inhibition of differentiation, and/or trans-differentiation in already committed cell types, as shown by the epithelial-mesenchymal transitions characteristic of colorectal cancer cells with nuclear β-catenin localization (Kirchner and Brabletz 2000). Tissue-specific thresholds in Wnt signaling dosages may exist among different stem cell niches, as indicated by the mammary and desmoid tumor phenotype of Apc^{1572T} and Apc^{1638N} mice (Smits *et al.,* 1998) (Gaspar *et al.,* manuscript in preparation). Accordingly, the Wnt pathway has been involved in a broad spectrum of tumor types arising from all three embryonic lineages in gene-specific (*APC, β-catenin, Axin, Pin1*) (Polakis 2000, Ryo *et al.,* 2001), and dosage-specific fashions (Smits *et al.,* 1999, Fodde and Smits 2001, Kielman *et al.,* 2002). It is likely that different mutations in a subset of Wnt-related genes confer specific signaling dosages to allow tumor formation in specific tissues by uncontrolled expansion of the corresponding stem cell compartment.

Notably, although *APC (* or *β-catenin)* mutations predict the nuclear accumulation of β-catenin throughout colorectal tumors, only

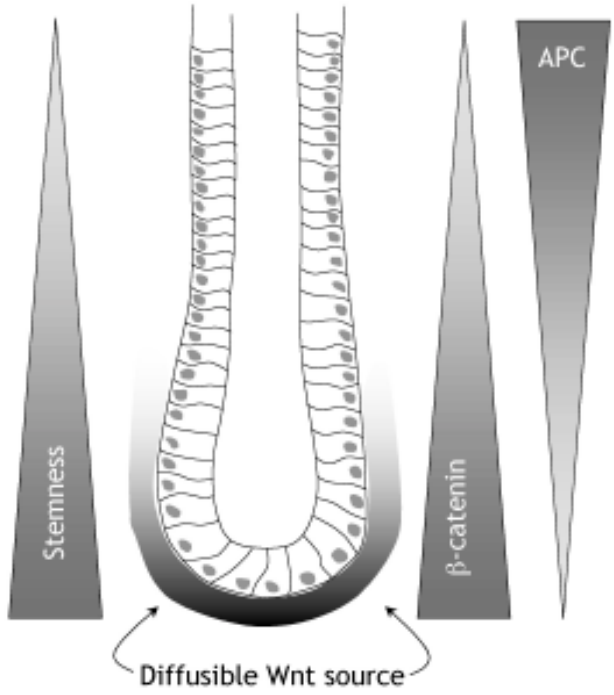


Fig. 5. Wnt mediated control of intestinal stem cell renewal and differentiation along the crypt villus axis. *The putative presence of a source of Wnt ligands within the stroma compartment underlying the intestinal mucosa generates a concentration gradient of Wnt morphogens reflected by the gradient of β-catenin expression (nuclear at the bottom of the crypt, more membrane-associated when moving towards the lumen (Batlle et al., 2002)) and by a gradient of 'stemness', i.e. the pluripotency of intestinal progenitor cells. APC expression along the crypt villus shows an inverse gradient (Smith et al., 1993, Midgley et al., 1997) suggesting an increased level of expression with maturation. However, how this expression gradient is determined is still unclear.*

a subset of the cancer cells are positive after immunohistochemistry (Kirchner and Brabletz 2000). This 'active' subset of alleged cancer stem cells increases with tumor size but not with the grade of dysplasia of the colon adenoma (Brabletz *et al.,* 2000). Presumably, *APC* mutations alone are not sufficient for nuclear translocation of β-catenin and other synergistic factors trigger constitutive Wnt signaling in more advanced stages of colorectal cancer. Further studies are needed to understand the molecular mechanisms underlying the dose-dependent transcriptional response in Wnt signaling, its primary downstream targets, and the likely cross-talk with other signal transduction pathways and cellular functions in homeostasis and in tumor formation and progression.

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