Preface

It has become clear that tumors arise from excessive cell proliferation and a corresponding reduction in cell death. Tumors result from the successive accumulation of mutations in key regulatory target genes over time. During the 1980s, a number of oncogenes were characterized, whereas from the 1990s to the present, the emphasis shifted to tumor suppressor genes (TSGs). It has become clear that oncogenes and tumor suppressor genes function in the same pathways, providing positive and negative growth regulatory activities. The signaling pathways controlled by these genes involve virtually every process in cell biology, including nuclear events, cell cycle, cell death, cytoskeletal, cell membrane, angiogenesis, and cell adhesion effects. Tumor suppressor genes are mutated in hereditary cancer syndromes, as well as somatically in nonhereditary cancers. In their normal state, TSGs control cancer development and progression, as well as contribute to the sensitivity of cancers to a variety of therapeutics. Understanding the classes of TSGs, the biochemical pathways they function in, and how they are regulated provides an essential lesson in cancer biology. We cannot hope to advance our current knowledge and to develop new and more effective therapies without understanding the relevant pathways and how they influence the present approaches to therapy. Moreover, it is important to be able to access the powerful tools now available to discover these genes, as well as their links to cell biology and growth control.

The scope of this two volume work, *Tumor Suppressor Genes*, *Volume 1: Pathways and Isolation Strategies* and *Volume 2: Regulation, Function, and Medicinal Applications*, is broad in the sense that it covers all the known tumor suppressor pathways and provides key information on the road to their discovery, analysis, and uses in cancer therapeutics. The aim of the first volume, *Pathways and Isolation Strategies*, is to educate the reader about known TSGs and the relevance of the biochemical pathways they regulate to human cancer. The reader has an opportunity in Volume 1 to access state-of-the-art protocols that have been successful in the identification of TSGs in the past, and that can be applied to isolate novel TSGs. With a novel TSG in hand, the reader has an opportunity in Volume 2, *Regulation, Function, and Medicinal Applications*, to explore the cell biology and biochemical function of the encoded protein, as well as its physiological role in vivo. Finally, in Volume 2, the reader is exposed to strategies for the use of information on TSGs to develop diagnostic and therapeutic strategies for cancer.

The two volumes of *Tumor Suppressor Genes* bring together many of the world's experts in the identification and characterization of TSGs. The work is intended to become the core reference and compilation of the emerging pathways and the growing number of molecules that suppress cancer. Importantly, it should also serve as a wide-ranging source of protocols useful in understanding and characterizing the function of

TSGs. One of the challenges facing cancer researchers and clinicians is to bring forward and develop active therapeutics. This book, by example, puts forward highly useful paradigms for rational drug design, based on our dramatic new understanding of molecular pathogenesis.

Tumor Suppressor Genes thus lays down a firm and timely foundation for understanding cancer. In this age of expression profiling and proteomics, there has already been revealed a remarkable complexity and interrelatedness of seemingly diverse processes and signal transduction pathways. For the student, this book provides a reference to the basics concerning the identity of the major TSGs and the signaling pathways they use to inhibit tumors. For the investigator, it provides not only a critical update, but also an extremely useful compendium of newly assembled research protocols, including both classical methods and state-of-the-art techniques. For the translational scientist, the book provides fertile ground for the development of therapeutic strategies based on understanding the mechanisms of action and appreciating the existing preclinical data. One of the criticisms of an effort leading to such a book is that the field is moving very quickly and material is likely to be outdated. However, with many of the world's leading experts providing a comprehensive overview of all tumor suppressing pathways, along with their detailed protocols, we believe we have provided an invaluable resource for continued learning and discovery. Finally, *Tumor Suppressor Genes* provides a bridge to those interested in translational research by giving examples of the rationale for many of the most promising manipulations that may lead to novel therapeutic agents.

Tumor Suppressor Genes is targeted at a broad audience including medical and graduate students, postdoctoral fellows, physician scientists, academics, and principal investigators. The text provides critical information to rapidly gain appreciation of important TSGs in human cancer, as well as modern methods for their discovery, analysis, and clinical application. The reader is enabled to learn the background and then access the literature in which studies designed to define their biology and biochemistry have been performed on known TSGs. Details of protocols with examples of their previous uses allow the researcher to apply current technologies to novel or known genes whose role has not yet been defined.

In summary, *Tumor Suppressor Genes* is a comprehensive compilation of the known tumor suppressing pathways, and the key molecular approaches to their discovery, analysis, and clinical applications. It should be of enduring value to students at all levels of experimental biology and medicine, as well as those clinicians who want to better understand the molecular biology of cancer.

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2

The APC Tumor Suppressor Pathway

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1. Introduction

1.1. Colon Cancer

Colon cancer is the second most common cause of cancer mortality among adults in the United States today ***(1)***. Colon cancer arises from a pathological transformation of the normal colonic epithelium to an adenomatous polyp, which can then progress to an invasive tumor. This progression is brought about by a number of genetic changes, which include the inactivation of tumor suppressor genes and activation of protooncogenes, the net result of which is to confer a proliferative advantage to the cancerous cell ***(2,3)***. These changes are best described in the colon cancer adenoma-carcinoma sequence model as outlined in **Fig. 1** ***(4)***.

This model of the genetic changes involved in the progression of colon cancer begins with the development of benign polyps. Inactivation of both copies (alleles) of the adenomatous polyposis coli (*APC*) gene located on chromosome 5 constitutes an early, and possibly the very first event in colorectal tumorigenesis ***(5)***. Consistent with this hypothesis, germline mutation of the *APC* gene causes the inherited familial adenomatous polyposis syndrome (FAP) (see below). Another early event in early colon carcinogenesis is the activation of the K-ras protooncogene through mutation of codon 12 or 13 ***(6–8)***. Interestingly, while *APC* and K-ras alterations are considered two of the earliest events in colon tumorigenesis, the order appears crucial. Indeed, while *APC* mutations were associated with early dysplasia, lesions exhibiting K-Ras mutations without *APC* alterations appeared mostly nondysplastic lesions with limited potential to progress to carcinoma ***(7)***.

Next in the progression from adenoma to carcinoma is the loss of heterozygosity of the long arm of 18q, near the *SMAD4* (*DPC4*) locus. *DCC* and *SMAD2* are also candidate tumor suppressor genes in this region. Interestingly, germline mutation of *SMAD4* was found to cause juvenile polyposis, implicating *SMAD4* further in colorectal cancer ***(9)***. Mutation of p53 on chromosome 17p appears to be a late-stage event. This mutation is thought to allow the growing tumor with multiple genetic alterations to evade cell cycle arrest and apoptosis, or programmed cell death ***(4)***. However, it is almost certain that there are additional, undiscovered genetic events that occur along the way as indi-

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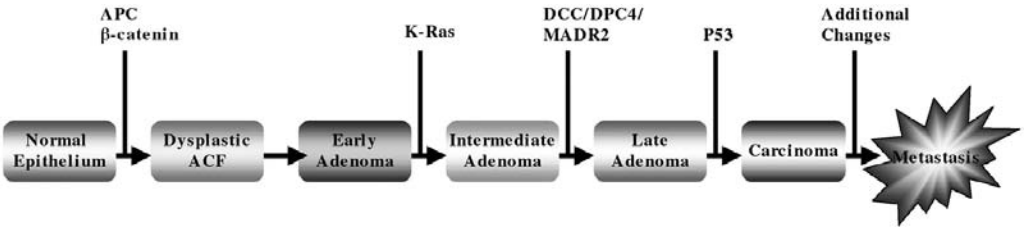

Fig. 1. A genetic model for colorectal tumorigenesis. Adapted from Fearon and Vogelstein ***(4)***.

cated by evidence of allelic loss on chromosomes 1q, 4p, 6p, 8p, 9q, and 22q in 25–50% of colorectal cancers ***(10)***.

Finally, genes involved in DNA repair have been implicated in colorectal cancer tumorigenesis. These genes include *hMSH2*, *hMLH1*, *hPMS1*, or *hPMS2* and are involved in DNA mismatch repair ***(11)***. The mutation of certain of these genes causes a colorectal cancer syndrome known as hereditary nonpolyposis colorectal cancer (HNPCC). Alteration of these genes is also prevalent in sporadic colon cancer, and may favor mutations of other crucial genes for colon cancer development such as *APC* and K-ras or may represent an alternative to the *APC* pathway for colon cancer development ***(12–14)***.

1.2. Familial Adenomatous Polyposis (FAP)

The identification of an interstitial chromosome 5q deletion in a patient with Gardner syndrome led to the approximate localization of the gene responsible for FAP ***(15)***, a rare, autosomal, dominantly inherited syndrome, characterized by the development of hundreds to thousands of colorectal adenomas ***(16)***. Some years later, several groups identified this gene as *APC* by performing linkage analysis of families with the syndrome, as well as positional cloning of the gene, localizing the gene to chromosome 5q21 ***(17–19)***. In addition to being a predominant germline mutation in many FAP patients, it is also mutated in approximately 80% of all sporadic colon cancer patients ***(5)***.

The age of onset of FAP is usually very young (20 years of age), and the disease affects approximately 1 in 10,000 individuals. Because of the large numbers of tumors as well as the fact that a subset of these adenomas tend to become malignant, the most common treatment for the disease is prophylactic resection of the colon. However, these adenomas appear outside the colon as well, and can be found in organs such as the stomach, pancreas, thyroid, periampullary region, and the central nervous system. In addition, the tumors can appear not just as adenomas, but also as adenocarcinomas, osteomas, desmoids, epidermal cysts, congenital hypertrophy of the retinal pigmented epithelium (CHRPE), and also as dental abnormalities. The pathological association of desmoid tumors and osteomas with FAP is clinically termed Gardner syndrome, and the association of CNS tumors, in particular medulloblastoma, is known as Turcot syndrome **(20).**

This diversity in the clinical profile of FAP patients is interesting, considering that virtually all these patients have mutations in the *APC* gene that result in C-terminally truncated proteins. This may be partially accounted for by the exact type and location of mutations in the *APC* gene. In fact, more than 1600 mutations, both germline and somatic, have been described for *APC*. A database is accessible online at http://perso.curie.fr/Thierry.Soussi/APC.html.

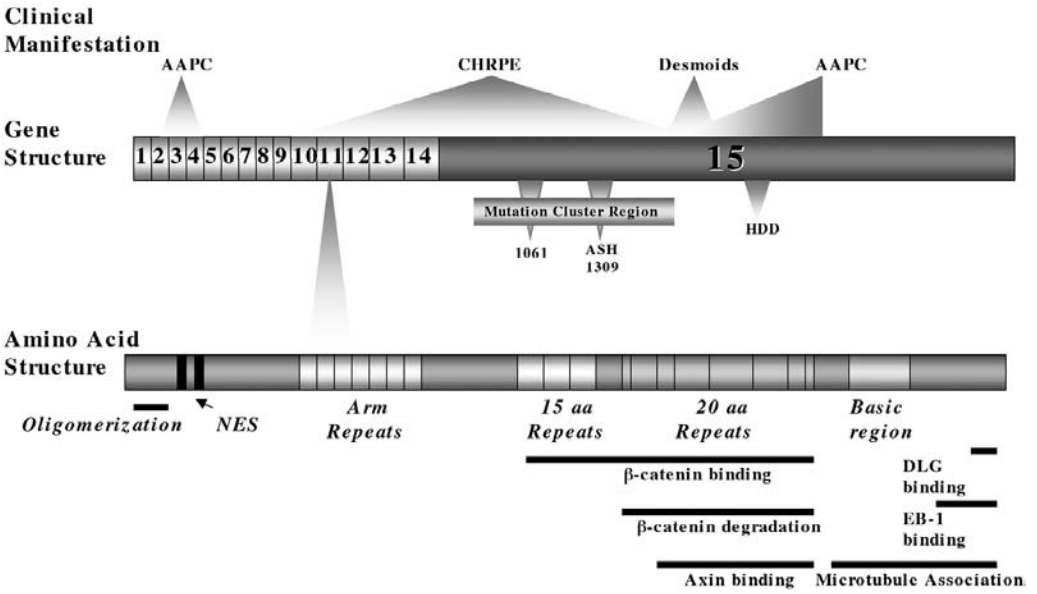

Fig. 2. The *APC* gene structure, amino acid structure, and disease profile.

2. APC Gene Structure

2.1. Gene Structure and APC Homologs

The structure of the *APC* gene is outlined in **Fig. 2**. The gene contains 21 exons which are within a 98-kb locus, and 16 of which are translated ***(21)***. The largest exon is exon 15, with a remarkable uninterrupted open reading frame of 6579 bp, which comprises more than 75% of the coding sequence for this gene. The open reading frame consists of 8538 bp, and codes for a protein of 2861 amino acids. The most abundantly occurring transcript of this gene lacks exon 10A (the smallest exon) and has a mass of 310 kDa, comprised of 2843 amino acids ***(22,23)***. Alternatively expressed exons of the gene encode alternate isoforms of the protein, ranging in molecular weight from 90 to 310 kDa. All these isoforms are expressed in different tissue-specific patterns, but their function is not as yet known, although the encoded proteins differ most dramatically in their N-termini and dimerization capacities ***(23–25)***. Another member of the *APC* family, *APC-2 /APC-L*, is located within a 50-kB genomic fragment on chromosome 19p13.3 ***(26)***. It is ubiquitously expressed during brain development, and closely resembles *APC* in its overall structure ***(27,28)***. However it does not contain the 15 amino acid repeats or the hDLG region and only has four 20 amino acid repeats, which will be described in the next section. There are several splice variants of this protein of which the function is unclear. It appears that *APC* can compensate for APC-2 loss but not vice versa, implying that there are overlapping functions of the two proteins. Overall, *APC* appears to have a wider range of function ***(29)***.

2.2. Amino Acid Sequence and Interacting Proteins

The N-terminal third of the APC protein contains seven Arm repeats (aa 453 to 767), named for an amino acid motif repeated in Armadillo, the *Drosophila* homolog of the protein β-catenin ***(30)*** (**Fig. 2**). This domain binds to APC-stimulated exchange

factor (ASEF), which allows for interaction with raf, and consequently with rac and rho, which mediate cell adhesion and motility via cytoskeletal changes ***(31)***. The region is referred to as the ARD domain, or armadillo repeat domain. Arm repeats are present in several proteins that are involved in protein–protein interactions, such as plakoglobin, plakophilin, p120cas, among others. Arm repeats in β-catenin are essential for APC binding, as well as E-cadherin association, and form a superhelix, with a positively charged groove, that is responsible for structural interactions with partnering proteins ***(32,33)***. The ARD is the most conserved part of APC and is the most homologous ARD to that of β-catenin, perhaps indicating that APC and β-catenin may interact with some of the same proteins. Recently, APC, via its ARD, has been found to bind to protein phosphatase 2A ***(34)***. This enzyme can also bind to Axin, another protein involved in the APC/β-catenin signal transduction pathway, implicating PP2A as a potential antagonist of the kinase GSK3β ***(35)***. These observations have yet to be confirmed, but the ARD does indeed seem to be important for survival, as it is retained in most mutations of the *APC* gene, and deletion of this region in mice causes early lethality ***(36)***.

The central third of APC contains three 15-aa repeats between amino acid residues 1020 and 1169 and seven 20-aa repeats, between residues 1262 and 2033. These repeats are comprised of the amino acids TPXXFSXXXSL ***(19)***. They are known as the β-catenin-binding repeats, but also associate with plakoglobin ***(37)***. These repeats are highly conserved among the species, and have great similarity to one another. The 20-aa repeats require phosphorylation by the protein GSK3β, before binding β-catenin ***(38)***. Interestingly, while overexpression of the 15-aa region has no effect, the 20-aa region downregulates β-catenin levels in vivo when expressed in *APC*-deficient cells ***(39)***. It is in the 20-aa portion of APC that the binding site for Axin can be found, a protein that is also part of the β-catenin/APC/GSK3β complex and that will be discussed in detail later in this chapter. The binding site for Axin appears to be crucial for the tumor suppressor role of APC and is comprised of three SAMP (Ser-Ala-Met-Pro) repeats ***(40,41)***.

The C-terminal end of APC is rich in basic amino acids (**Fig. 2**). This region is essential for microtubule binding, particularly residues 2219–2580, which bind nonassembled tubulin and co-localize with microtubules in vivo ***(42,43)***. EB1, a member of the EB1/RP family of tubulin-binding proteins first identified by the two-hybrid system as an APC-associated protein ***(44)***, binds this region as well. EB1 also associates with microtubules and tubulin in vitro and in vivo. Consistent with these findings, APC has been found in the leading edge of migrating epithelial cells where there is an increased concentration of microtubules, further implicating the protein in cell adhesion and migration ***(45)***.

Two other proteins have important interactions with the C terminus of the APC protein: the human homolog of the *Drosophila* tumor suppressor gene, discs large (DLG), which binds the C-terminal 72 amino acids ***(46)***, and the protein phosphatase PTP-BL ***(47)***. Both proteins contain a PDZ domain that binds to C-terminal XS/TXV domains. APC contains specifically the sequence VTSV, which matches this motif, and the interaction of these two proteins was identified through yeast-two-cell hybrid screening, and by co-localization studies. DLG and APC co-localize at areas of cell–cell contact both in epithelial cells, as well as neuronal junctions along synaptic processes.

Finally, APC interacts with itself. It is the tertiary structure of APC that may mediate its dimerization and oligemerization capacities. The first 170 amino acids are sufficient for APC dimerization in vitro ***(48,49)***. The N-terminal third of the protein contains a coiled-coil motif, marked by a series of heptad repeats of hydrophobic residues. The first of these heptad repeats involves the first 45 amino acids, which are crucial for APC homodimerization ***(49)***. Although not previously demonstrated, these regions may also mediate oligomerization between wild-type and mutant proteins, resulting in a dominant negative effect ***(50,51)***.

2.3. APC Mutations in Colon and Other Cancers

As previously mentioned, more than 90% of *APC* mutations result in a premature stop codon, and thus in the truncation of the C terminus ***(52)***. A 5-bp deletion at codons 1309 and 1061 are found in 18% and 12%, respectively, of all germline mutations (*see* **Fig. 2**). These deletions, which occur in short direct repeats within *APC*, have also been identified in patients without any family history, suggesting that they may also arise sporadically. Germline mutations can predict the phenotype of the disease they are likely to cause, by their location within the *APC* gene. A phenotype of multiple tumors (over 5000) is predicted when the mutation arises between codons 1249 and 1330, whereas mutations that are upstream or downstream of this region result in a phenotype of fewer than 1000 tumors ***(53)***. Attenuated adenomatous polyposis coli (AAPC) is caused by mutations at codons 78 to 163 (5' end), and this disease is characterized by the development of very few tumors (less than 100) at a later age. Similarly, germline 3' mutations in exon 15 are also associated with an attenuated phenotype ***(54)***. Germline mutations in codons 457–1444, as well as in exon 9, can code specifically for CHRPE ***(55)***, and germline mutations between codons 1395 and 1560 are associated with desmoid tumors and mandibular osteomas ***(56)***.

Unlike germline mutations, which are found all along the gene, sporadic mutations are clustered (about 805 of all sporadic mutations) at the 5' end of exon 15, between codons 1280 and 1500 ***(57)***. This region is known as the mutation cluster region (MCR). It overlaps with the 15-amino acid domain between residues 1020 and 1169, and the 20-aa repeats between residues 1324 and 2075 that are responsible for β-catenin interactions, as well as the SAMP repeats located within the 20-aa repeats that interact with Axin. In addition, three somatic mutational hotspots can be found at codons 1309, 1450, and 1554, and account for 7%, 8%, and 5% of all somatic mutations, respectively. In addition, the promoter regions of *APC* termed 1A and 1B may be targets of an epigenetic mechanism of silencing—hypermethylation. Although there is no evidence of 1B methylation, 1A is heavily methylated in colorectal cancers, and this is thought to be the second hit in colon tumorigenesis ***(58)***. Interestingly, no evidence for the hypermethylation of 1A in normal colonic mucosa can be found ***(59)***.

In Ashkenazi Jews, about 6% of the population expresses a germline variation of APC known as the I1307K variant ***(60)***. This polymorphism leads to a hypermutable A_8 tract that can undergo slippage to produce frameshift mutations. Accordingly, I1307K allele carriers are 50–70% more likely to develop colon cancer ***(61)***. Another variant is the E1317Q allele, which is associated with multiple colorectal adenomas ***(62)***. It has been suggested that E1317Q affects APC function by subtle effects on β-catenin sequestra-

tion or degradation. In fact, most germline and somatic mutations result in the loss or alteration of β-catenin binding, as most of these point mutations occur at CpG dinucleotides, generating stop codons by a C-to-T transition in a CGA sequence.

3. APC Function

APC plays important roles in cell cycle, motility, adhesion, and signaling. It is often referred to as a multitasking protein, and the disruptions of its interactions may lead to the inability of APC to perform these functions, thereby contributing to tumor formation. The main functions attributed to APC are downregulation of the Wnt pathway (through β-catenin), modulation of cell adhesion/migration, and maintenance of chromosomal stability.

3.1. Downregulation of Wnt Signaling

The Wnt family of proteins is comprised of several members, all of which are cysteine-rich secreted proteins of about 38–45 kDa in size. Wnts were first identified in *Drosophila*, are highly conserved throughout the species, and are important in mediating cell–cell interactions during embryogenesis. In normal development, Wnts are expressed both tissue specifically and temporally ***(63)***. Deletions of Wnt in mice result in very specific phenotypes, and will be discussed in the section concerning transgenic animals. In general, the deletion of specific Wnts results in the lack of development of specific organs, stressing the importance of Wnts in development due to their signal transduction cascades during proliferation and differentiation.

3.1.1. β-Catenin Regulation

One of the main features of Wnt signaling is the regulation of β-catenin stabilization. Although β-catenin stability can be regulated by Wnt/APC-independent mechanisms, such as via integrin-linked kinase, GBP, and the c-met receptor tyrosine kinase ***(64)***, APC appears to be a key player. In the absence of Wnt signals, cytoplasmic β-catenin is phosphorylated at multiple residues in its N terminus by glycogen synthase kinase 3β (GSK3β) and then targeted for degradation (**Fig. 3**). The phosphorylation of β-catenin depends on a multiprotein complex that consists of APC, Axin/Conductin, and GSK3β ***(40,65)***. Phosphorylated β-catenin is recognized by the ubiquitin ligase β-TrCP/Slimb, ubiquitinated ***(66–68)*** and degraded by the proteasome pathway ***(69–71)***. Axin and APC are also phosphorylated by GSK3β, resulting in enhanced β-catenin binding to the complex and thus enhanced β-catenin degradation ***(72,73)***.

Wnt signaling leads to the stabilization of β-catenin and its accumulation in the cytoplasm. The free β-catenin in the cytoplasm can translocate into the nucleus and participate in the transcription of target genes through its association with T-cell factors (TCFs), which provide the DNA-binding moiety ***(74)***. Because β-catenin does not contain a nuclear localization sequence, it was originally suggested that β-catenin enters the nucleus through its association with TCF. More recent evidence suggests that β-catenin enters the nucleus through direct interactions with the nuclear pore complex in a manner similar to importin β ***(75,76)***.

The APC protein can be identified both in the cytoplasm and the nucleus and contains NLS ***(77–79)***. Sublocalization of APC can vary dramatically in epithelial cells of

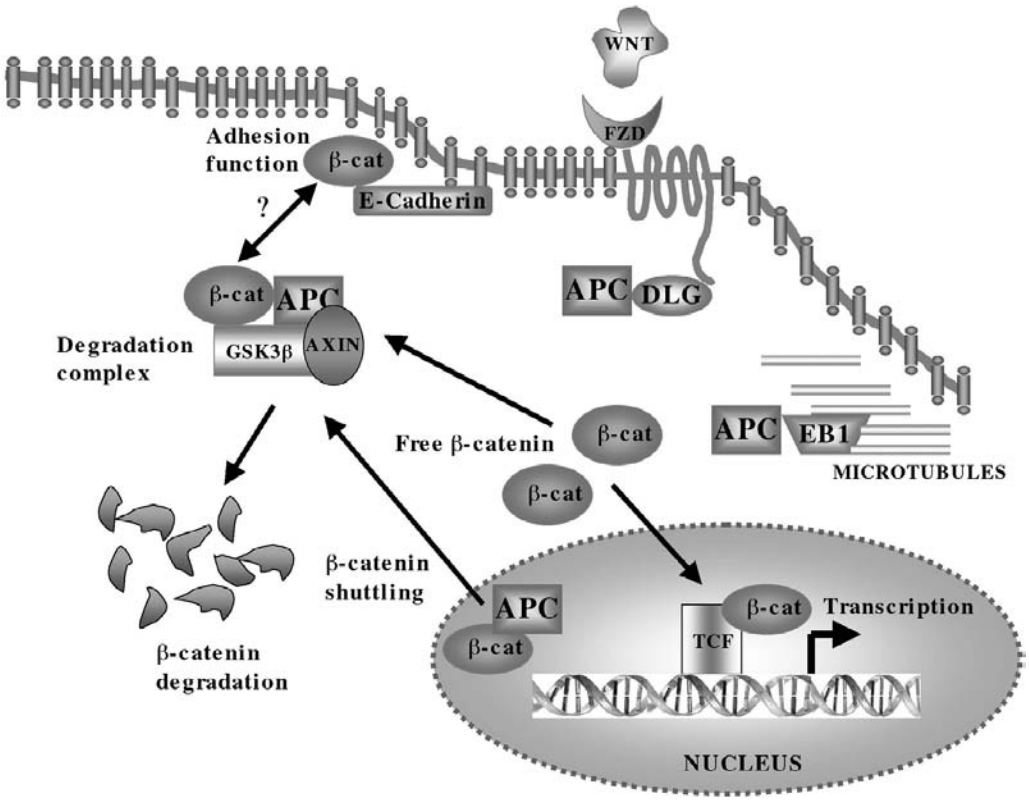

Fig. 3. The APC/β-catenin signaling pathway. See text for details.

the same lineage; for example, enterocytes at the base of the intestinal crypt are negative for APC, but the upper third of the crypt expresses some APC. The cells at the luminal surface of the crypt express even more APC, which is localized at their apical surface ***(77)***. At its N terminus, APC also contains two nuclear export sequences at amino acids 68–77 and 165–174, as well as a putative one at 1472–1481 ***(80)***. APC could thus downregulate the Wnt pathway by acting as a shuttle for β-catenin from the nucleus to the degradation complex in the cytoplasm. Mutant APC proteins lose the ability to exit from the nucleus leading to an excess nuclear β-catenin and possibly the activation of TCF/β-catenin transcriptional targets.

3.1.2. TCF/β-Catenin Transcriptional Activity and Targets of the Pathway

TCF/Lef transcription factors were originally identified as lymphoid-specific DNA-binding proteins that recognize a specific sequence- 5′CTTTGWW3′, where W = A or T ***(81–83)***. TCF/Lef bind to DNA via their high-mobility group (HMG), which induces a sharp bend in the DNA helix, but have no transactivation domains ***(74,84)***. As such, TCF/Lef are considered "silent" transcription factors, as they can activate gene transcription upon the binding of β-catenin, which contains the transactivation domains that TCF lacks, and these are contained within the C- and N-terminal ARD ***(85-87)***.

In the absence of Wnt signaling, TCFs can actively mediate the repression of Wnt-regulated genes via binding of the co-repressors TLE/Groucho ***(88–91)***. Groucho, for

example, can repress transcription via the recruitment of repressive chromatin through interactions with histone H3 ***(88)***. CBP was reported to antagonize Wnt signaling by interacting directly with TCF, and acetylating a lysine residue in the β-catenin region of dTCF ***(92)***. On the other hand, CBP was also found to interact directly with β-catenin and act as a transcriptional coactivator to increase expression from TCF sites ***(93–95)***. The reason for these two apparently opposite functions of CBP on the Wnt pathway are currently unknown. TCF transcriptional activity can also be modified by phosphorylation. For example, a MAP kinase-related pathway involving a TGF-β-activated kinase (TAK1) and NEMO-like kinase can antagonize β-catenin/TCF signaling by phosphorylating TCF ***(96)***.

Many putative targets of the TCF/β-catenin transcription factors have been identified in tumorigenesis. The first two genes to be identified as targets of TCF/β-catenin, c-Myc, and Cyclin D1, provided a molecular basis for the oncogenic property of the pathway ***(97–99)***. The transcription of c-Myc can be repressed by overexpressing APC ***(97)***, and transfecting cells with a dominant negative TCF can result in the arrest of colon cancer cells in the G1 phase of the cell cycle, by interfering with the production of cyclin D1 (*98*). Other targets of this pathway include PPARδ ***(100)***, the matrix metalloproteinase matrilysin ***(91,101)***, TCF1 ***(102)***, and AP-1, another transcription factor, as well as c-jun and fra1 ***(103)***. Inappropriate activation of one or some of these genes may be responsible for the oncogenic properties of the pathway, although this has not been convincingly proven ***(104)***.

3.1.3. Mutations of the Wnt Pathway in Colon and Other Cancers

APC mutations account for 80% of sporadic colorectal cancers and have been found at low frequencies in a few other cancers ***(105–108)***. The general importance of the Wnt pathway in human cancer has been emphasized by the identification of mutations of various members of the pathway in a large number of human malignancies. The case of β-catenin is particularly interesting. As described above, APC is crucial for the downregualtion of β-catenin-mediated transcriptional activity. Interestingly, activating mutations that make β-catenin resistant to ubiquitination and proteasome degradation have been identified in about half the colon cancers with wild-type APC ***(109,110)***. These cancers presumably have no selective pressure for APC mutations, since they already have the pathway activated through a β-catenin-activating mutation. These mutations typically arise at the N-terminal GSK3β phosphorylation site, essential for β-catenin degradation ***(64,73)***. Mutations in β-catenin have now been identified in many malignancies, including melanoma ***(111)***, ovarian cancer ***(112)***, prostate cancer ***(113)***, skin cancer ***(114)***, medulloblastoma ***(115)***, liver cancer ***(116,117)***, and endometrial cancer ***(118)***. Finally, AXIN1 mutations have also been identified in hepatocellular carcinomas ***(119)***. These findings emphasize the importance of this pathway in human cancer and its relevance to the function of APC.

3.2. Adhesion and Migration

One of the interesting observations of tumors in the intestinal epithelium was their abnormal tissue architecture, and concomitant defects in cell migration and adhesion. As these tumors arise from the loss of functional APC, the role of APC in the change of the morphology of these cells was examined. In addition to its well-known function as

a β-catenin regulator, and thus the regulation of β-catenin/ E-cadherin interactions in cell–cell adhesion, APC also has strong links with other molecules involved in cell migration. As mentioned previously, APC accumulates at the crypt/villus boundary, and this location is critical to the enterocyte migration from the crypt ***(120)***. When a C-terminus-truncated form of APC is introduced into mice, heterozygotes show an increase in their β-catenin levels in the enterocytes, which accumulate at the crypt/villus boundary ***(121)***. In addition, forcibly expressing the full-length gene also results in a deregulation of cell migration ***(122)*** and expression of an oncogenic β-catenin in the mouse intestine lead to abnormal architecture of the villi ***(123)***. APC-rich membranes are actively involved in cell migration in response to wounded epithelial monolayers, upon addition of scatter factor /HGF ***(124)***.

In addition, as mentioned earlier, APC binds to a rac-specific guanine nucleotide exchange factor termed ASEF ***(31)***. The rac/rho pathway has been shown to be involved in the regulation of the actin cytoskeletal network and is responsible for phenomena such as membrane ruffling, cell flattening, and motility. Microtubule reorganization may also be a target of APC signaling. These dynamic polymers of α and β subunits are involved in cell migration and polarized cellular morphogenesis, and their functions may be regulated by APC. APC has been found to be localized at the end of microtubules, regions known as puncta, where microtubules have reassembled to form protrusions, an important part of migration ***(125)***. APC has in-vivo microtubule-binding capacities and can nucleate microtubules in vitro.

3.3. Role of APC in Chromosomal Stability/Mitotic Checkpoint

Both the microtubule-binding domain and the EB1-binding region of APC implicate it in the regulation of chromosomal instability ***(44,126–128)***. Both microtubules and EB1 are associated with mitosis and cell division and spindle formation in dividing cells ***(129)***. EB1 is also associated with centrosomes and required for a microtubule-dependent checkpoint in yeast ***(130)***. These suggestive data have been supported recently by a study that shows that mouse embryonic stem cells that are homozygous for the min or APC1638T (truncated APC) alleles display extensive chromosome and spindle aberrations, implicating APC as an important player in the mitotic checkpoint ***(131,132)***. During these processes, APC associates with the checkpoint proteins Bub1 and Bub3 and can be found accumulated at the kinetochore. These results provide evidence that the C-terminal end of APC is crucial for chromosomal stability and may contribute to the observed chromosomal instability in colon cancer. Interestingly, the functions of APC as a regulator of β-catenin and as a cell-cycle checkpoint protein are likely independent, since ES cells from APC1638T mice also exhibit chromosomal instability. APC1638T homozygous mice contain a C-terminally deleted APC protein capable of degrading β-catenin but deficient in its ability to interact with EB1.

3.4. Consequences of APC Loss of Function

The exact physiologic role of the APC tumor suppressor pathway in tumorigenesis remains to be elucidated. Overexpression of APC has been reported to increase apoptosis in an APC-deficient colon cell line, suggesting that β-catenin may act as a survival factor ***(133)***. Interestingly, mice lacking *TCF4,* a *TCF* family member implicated in colon cancer ***(134)***, are characterized by a lack of intestinal stem cells ***(135)***. These find-

ings suggest that the β-catenin/TCF pathway may be important for the maintenance of stem cell characteristics including longevity. Thus, the first step in colon tumorigenesis may be the activation of the APC/β-catenin pathway in order to inhibit differentiation and preserve stem cell characteristics. *APC* expression has also been reported to cause a G1/S block in NIH 3T3 cells ***(136)***, and this block is associated with the interaction of APC with hDLG ***(137)***. Consistent with this finding expression of a dominant negative TCF protein in a colon cancer line containing an activated β-catenin was shown to cause a G1 block ***(98)*** and overexpression of an activated β-catenin promotes G1/S transition ***(138)***. The effect of β-catenin activation on cell proliferation has been controversial, with some studies demonstrating increased proliferation of cells expressing activated β-catenin ***(138,139)*** and other studies finding an effect on tumor size but not on proliferation ***(140)***. β-Catenin expressing cells were also found to exhibit reduced contact inhibition and an increased ability to grow in soft agar ***(138)***.

As is the case for other oncogenes, β-catenin activation can in some cases have negative effects on cell proliferation. Indeed, it has been shown that APC inactivation in *Drosophila* leads to retinal defects similar to those occasionally observed in FAP patients ***(141)***. The *Drosophila* retinal defects are due to increased levels of apoptosis in retinal neurons, and this phenotype appears to be dependent on Armadillo upregulation. These findings demonstrate that activation of the APC/β-catenin pathway may have very different consequences in different tissue types and further implicates this pathway in tissue homeostasis.

4. Animal Models

Animal models have contributed significantly to our understanding of the APC tumor suppressor pathway. The previous sections have already mentioned several of these models. Here, we will describe in more details these models with deficiencies or overexpression of the various proteins important in the pathway.

4.1. Apc

The first Apc-deficient mice were generated by using ethylnitrosurea to create a point mutation in a mouse germline. One line of mutagenized mice was termed the min mouse, which has been briefly described earlier in this chapter. These mice display multiple intestinal neoplasia and have an anemic phenotype caused by multiple bleeding polyps in the intestine, very similar to the phenotype seen in patients with FAP. The Min mice were found to carry a nonsense mutation in mouse *Apc* ***(142)***. The homozygous mice are not viable *in utero,* and the heterozygous mice rarely lived past 5 mo. The tumor profile of these animals differs slightly from FAP patients in that tumors develop in the small rather than large intestine and about 10% of animals develop mammary tumors. There is recent evidence, based on a highly sensitive technique, that inactivating mutations of APC may be involved in breast cancer ***(108)***, but this finding requires confirmation. Interestingly, the crossing of these animals with other strains revealed the interaction of *APC* with modifier loci. When min mice were crossed with C57BL/6 mice (their parent strain), they developed high numbers of polyps, but when they were crossed with other strains (AKR, for example), they appeared resistant to tumor development. The gene controlling this tumor profile was identified as *Mom1* (modifier of Min 1) and was mapped to the distal end of mouse chromosome 4 ***(143)***. The human homolog to this gene has been identified as a se-

cretory type II phospholipase A2 (*sPLA2*) and is located at 1p35 a chromosomal region frequently lost in human cancer. However, this gene was not found mutated in human colorectal cancer and is not believed to play a major role in this disease ***(144)***. The extent of environmental influences and epigenetic effects on FAP patients remains unknown.

Apc-deficient mice have also been developed using gene targeting. The Apc Δ716 knockout mice are heterozygotes, which develop intestinal adenomas at a high rate ***(145)***. These mice develop tumors also along the small intestine, characterized by an abnormal bulging or pocketing of the intestinal epithelium at the crypt/villus boundary. However, there was no increase on the rate of proliferation of polyps, which are polyclonal in origin. In both the mice strains described above, animals homozygous for the deletion die *in utero*. To circumvent this, a conditional gene targeting system was used. The *Apc* gene was inactivated by the insertion of *loxP* sites into the introns on either side of exon 14 of the *Apc* gene, and then introducing this gene into the mouse germline. These mice bearing the *Apc-loxp* gene developed normally. By then using an adenovirus encoding the *cre*-recombinase gene to infect the colorectal region, the *Apc* allele could be specifically inactivated, once *Cre* recombined with *loxP*-deleting exon 14. Mice developed adenomas within 4 wk, and these adenomas showed deletion of exon 14 of *Apc* ***(146)***. The loss of APC in these animals provides us with many insights into how FAP may develop, and what kind of role APC plays in development, as the loss of APC leads to malformation of the intestinal crypts, due to defects in many process ranging from inappropriate activation of TCF to defects in adhesion, migration, and even apoptosis and growth arrest. Finally, Apc1638T mice have contributed significantly to our understanding of APC function ***(36)***. These mice are viable as homozygotes, and the adults do not develop tumors. The Apc1638T allele can downregulate β-catenin (albeit with a lower efficiency that wild-type APC), but appears to be deficient in its ability to bind microtubules (*see* **Subheading 3.3.**).

4.2. β-Catenin

Transgenic mice engineered to express intestinal β-catenin with a truncated N terminus develop polyps due to outpocketing of the intestinal epithelium ***(123)***. This epithelium remains intact even after villi development and fusion of adjacent villi, and the crypt compartment expands abnormally, perhaps due to the β-catenin/TCF4 signaling, as the crypt cells continue to proliferate without differentiating. When oncogenic β-catenin is expressed in the skin, however, there is increased morphogenesis at the hair follicle, resulting in mice with abundant fur ***(114)***. In addition, several tumors known as pilomatricomas, tumors of the hair matrix, form, and this has been supported in humans by the observation of β-catenin mutations in pilomatricomas. Nuclear overexpression of LEF1 was found to accompany these tumors in transgenic mice, indicating that these effects were due to APC/β-catenin deregulation.

4.3. TCFs and Other Wnt Pathway Proteins

Animals engineered to have defects in the other members of the APC/β-catenin signaling pathway also provide useful information as to the role of these proteins in disease and development. TCF1 knockouts do not develop T cells, but do develop adenomas in the gut and mammary glands, and the addition of mutant APC increases these tumors, and this implicates *TCF1* as a repressor of target genes in the absence of activated β-catenin, perhaps in cohort with *APC* ***(102)***. TCF4 knockouts lead to abnor-

malities of the small intestine, where the stem cell compartment of the crypts is missing, implicating Wnt signaling in the proliferation of these crypt stem cells ***(135)***. Lef1 knockouts exhibit defects in the formation of teeth, hair, and mammary glands, and this is thought to be mediated via disruptions of the signaling of these epithelial cells with their underlying mesenchyme ***(147)***. Mice that are deficient for both Lef1 and Tcf1 do not form paraxial mesoderm and develop an excess of neural tubes.

Mutations of the Wnt genes can sometimes mimic these findings, indicating an overlap of functions for the different members of this pathway. Wnt3a knockouts for example, mimic the Lef1/Tcf1 knockouts ***(148)***. Most Wnt knockouts, however, have significant effects on development. The ablation of Wnt-1 results in the abolition of a part of the mid-brain ***(149)***, Wnt4 and Wnt7a ablation result in the malformation of the kidney and limbs ***(150,151)***, and Wnt 5/Frizzled 5 disruption can have effects on placental angiogenesis ***(152)***. Disheveled gene abolition appears to have no effect on development, but some data suggest that they may have effects on behavior in the adult mice. Other transgenic animals containing mutations in targets of the β-catenin, such as c-myc, which develop hepatocellular carcinomas, demonstrate concomitant mutations in the GSK3β phosphorylation site of β-catenin ***(117)***, perhaps suggesting that targets other than c-Myc are critical for tumorigenesis through the APC pathway.

5. Conclusions

APC is a large protein that wears many hats. It is involved not only in cell signaling and control of gene transcription, but also in cell cycle regulation, cell proliferation, cell migration, cell adhesion, cytoskeletal reorganization, and chromosomal instability. The loss or deregulation of APC can thus have many consequences for a cell, including tumorigenesis. Which of these functions are interrelated and which are independent is a matter of intense investigation. Another important question is whether all of these functions are important for the tumor suppressive function of *APC*. Overall based on several independent lines of evidence, it appears that the downregulation of β-catenin is the major pathway by which APC suppresses tumorigenesis. This is supported by the following evidence: 1) mutations in *APC* and β-catenin tend to be mutually exclusive in colon cancer, 2) mice containing mutant alleles of *APC* that can downregulate β-catenin do not develop tumors, and 3) β-catenin mutations are found in many cancer types. It is possible and even likely that other functions of *APC* are important in tumor development. Consistent with this hypothesis is the fact that tumors containing β-catenin appear less likely to progress to malignancy than APC deficient tumors ***(153)***. This is also consistent with an important function of APC in chromosomal stability. In any event, the rapid advances of the past 10 years have allowed a deep understanding of the most important pathway deregulated in colon tumorigenesis. There is no doubt that future studies will clarify the many remaining questions and will provide a clear view of the molecular mechanisms important in this devastating disease.

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