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Linking Colorectal Cancer to Wnt Signaling

Review

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Human cancer results from the accumulation of multiple independent genetic alterations. Studies of inherited and sporadic colorectal cancer have demonstrated that in the overwhelming number of cases the primary mutation targets a single signal transduction pathway. A story has rapidly unfolded in which the Wnt signaling pathway plays a central role in the etiology of this cancer. The sequence of discoveries provides a classic example of the synergy between insights gained from genetic studies of model organisms and cancer biology in man.

Introduction

Tumors of the colorectum are amongst the most common human neoplasms. Approximately 5% of the Western population will develop colorectal malignancies during their lifetime. A small fraction of these occur in an apparently inherited fashion. One of the best defined though rare forms of such cancer syndromes is familial adenomatous polyposis (FAP). Patients with FAP develop large numbers of benign adenomatous polyps of the colorectal epithelium in early adulthood. Almost invariably, some of these will progress into invasiveness and, ultimately, metastasize. With variable penetrance, FAP patients will also develop neoplastic lesions at extracolonic sites, for example desmoids, ampullary carcinomas, and hepatoblastomas (Kinzler and Vogelstein, 1996; Lal and Gallinger, 2000).

In 1991, the tumor suppressor Adenomatous polyposis coli (APC) encoded by the FAP locus was identified by molecular cloning (Groden et al., 1991; Kinzler et al., 1991). It turned out that, not only the tumors from FAP patients, but also most sporadic colorectal tumors have both *APC* alleles inactivated (Nagase and Nakamura, 1993). APC is a large protein whose sequence, at the time, was uninformative. Physical interactions of APC with several cellular proteins were tentatively identified. The discovery that APC binds to β-catenin (Rubinfeld et al., 1993; Su et al., 1993) provided the first significant clue as to the molecular function of APC, and identified what is now thought to be its most critical molecular target.

This discovery initially suggested a function of APC in cadherin-based cellular adhesion in which β-catenin plays a critical role (e.g., Hülsken et al., 1994; see below).

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However, a link between APC and Wnt signaling emerged shortly after this, when the Wnt pathway was discovered by genetic analysis in *Drosophila*. Specific mutant phenotypes led to the identification of critical Wnt signaling components, while epistasis experiments determined the functional order of these components in the pathway. This established that Armadillo, the fly homolog of β-catenin, mediates most if not all responses to signaling during normal development by Wingless, the archetypal Wnt ligand in *Drosophila* (Perrimon, 1994). Vertebrate homologs of all Wingless pathway genes were found. Injection of Wnt pathway components into early *Xenopus* embryos induced secondary body axes. Utilizing this versatile assay, it was demonstrated that the function of Wnt signal transducers is highly conserved between *Drosophila* and vertebrates (Miller and Moon, 1996).

Our review centers on the question of how the deregulation of β-catenin leads to malignant development in the mammalian gut epithelium. In focus will be the function of APC, still poorly understood at the molecular level. We shall present new insights into how APC controls β-catenin. We shall also summarize recent evidence suggesting that the loss of APC affects processes other than transcription, and that these may contribute to tumor progression. Our aim is to illustrate how many of the new discoveries that are relevant to colorectal cancer in humans have emerged from genetic and cell-biological studies in animal model systems.

The Canonical Wnt Pathway

Combined work in flies, frogs, and mammals produced the main outline of the canonical Wnt/β-catenin signaling pathway as we currently know it (Figure 1). This pathway plays a key role during normal animal development (Cadigan and Nusse, 1997). In the following, we shall attempt to sketch out the core issues regarding the transduction of the Wnt signal, focusing on aspects that are relevant to cancer. For more comprehensive information on this pathway, the reader is referred to the Wnt homepage (http://www.stanford.edu/rnusse/wntwindow.html).

In unstimulated cells, free cytoplasmic β-catenin/Armadillo is destabilized by a multiprotein complex containing Axin (or its homolog Conductin), glycogen synthase kinase 3β (GSK3, or Shaggy/Zeste-white 3 in *Drosophila*) and the APC tumor suppressor (Zeng et al., 1997; Behrens et al., 1998; Hart et al., 1998; Fagotto et al., 1999; Kishida et al., 1999a). Axin/Conductin has a scaffold function in this complex, binding to APC, GSK3, and β-catenin/Armadillo. Interaction between Axin and GSK3 in the complex facilitates efficient phosphorylation of β-catenin by GSK3 (Ikeda et al., 1998), most likely at critical serine and threonine residues in its N terminus (Aberle et al., 1997). This phosphorylation event earmarks β-catenin/Armadillo for ubiquitination by the SCF complex (containing the F box protein βTrCP/Slimb) (Jiang and Struhl, 1998; Marikawa and Elinson, 1998), and for subsequent degradation by the proteasome pathway (Aberle et al., 1997).

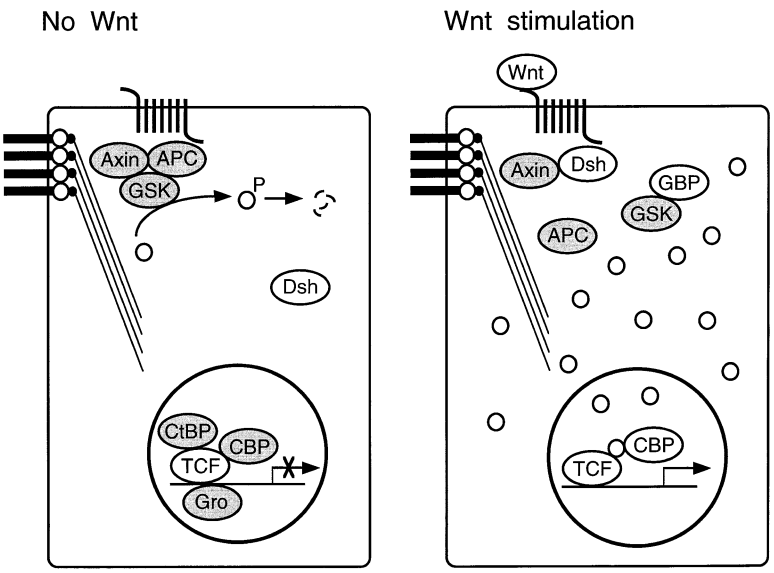

Figure 1. The Canonical Wnt Pathway

Activators of the pathway are white; negative regulators are gray. Left, in the absence of Wnt stimulation, the Axin complex actively earmarks β-catenin/Armadillo (white circles) for degradation by the proteasome. The levels of cytoplasmic β-catenin/Armadillo are low, and TCF is repressed. Right, after Wnt stimulation of the Frizzled receptor (arbitrarily drawn to be in the apical membrane), Dsh is recruited to the membrane where it binds to Axin to inhibit the Axin complex. β-catenin/Armadillo accumulates and, after translocation into the nucleus, binds to TCF to coactivate Wnt target genes. Inhibition of the Axin complex by GBP/Frat appears to be an alternative to Wnt-mediated inhibition. CBP switches from being a negative regulator to being a coactivator, apparently depending on the stimulation status of the cell. Note also the apicolateral adherens junctions to which the Axin complex appears to be anchored. These junctions are formed by the transmembrane protein E-cadherin (black bars) which is linked to the actin cytoskeleton (thin lines) by β-catenin/Armadillo and α-catenin (black dots).

When cells are stimulated by Wnt ligands, the cytoplasmic protein Dishevelled is recruited to the membrane (Axelrod et al., 1998; Boutros et al., 2000). Dishevelled appears to inhibit the Axin complex by direct binding to Axin (Kishida et al., 1999a; Smalley et al., 1999). Whether Dishevelled binds directly to Frizzled, the seven transmembrane receptors for the Wnt ligands (Bhanot et al., 1996), or whether intermediary proteins are involved in the signal transmission between Frizzled and Dishevelled is unknown at present. Also, the mechanism of Dishevelled-mediated inhibition of the Axin complex is not understood, but one suggestion has been that Dishevelled may cause disintegration of this complex (Kishida et al., 1999b). Alternatively, dorsal cells in the *Xenopus* embryo cells seem to inactivate the Axin complex in a process that does not apparently involve the stimulation of Frizzled receptors by Wnt ligands: in these cells, GBP/Frat can inhibit GSK3 by preventing its binding to Axin (Yost et al., 1998; Farr et al., 2000).

Wnt signaling triggers the inhibition of a constitutive cellular activity, namely the destabilization of free cytoplasmic β-catenin by the Axin complex. How this complex is inhibited rapidly and comprehensively in response to ligand-bound Frizzled remains a mystery. One suggestion has been that the efficacy of the inhibitory process may be achieved by anchorage of the Axin complex at the membrane, in the vicinity of the Frizzled receptor (Yu et al., 1999) (Figure 1). The basis for this was the observation that *Drosophila* APC is associated with adhesive membrane junctions; evidence suggested that this junctional association is essential for APC's function in destabilizing Armadillo (Yu et al., 1999; McCartney et al., 1999). The subcellular localization of Axin in the apical submembrane compartment of embryonic *Xenopus* cells is consistent with this anchorage model (Zeng et al., 1997; Fagotto et al., 1999).

Stabilized β-catenin/Armadillo is somehow released from the Axin complex and translocates into the nucleus by virtue of an intrinsic ability that does not depend on the Ran/importin machinery (Fagotto et al., 1998; Yokoya et al., 1999). Once in the nucleus, β-catenin/Armadillo binds to TCF (T cell factor) proteins and serves as a coactivator of TCFs to stimulate transcription of Wnt target genes (Behrens et al., 1996; Molenaar et al., 1996). Interestingly, TCFs are context-dependent transcription factors with an architectural role in enhancer complex assembly: often, TCF function depends on partner proteins and on simultaneous signaling by other pathways (Carlsson et al., 1993; Giese and Grosschedl, 1993; Riese et al., 1997). Thus, TCF plays a key role in a cell's task of integrating multiple positional inputs.

The activity of TCFs is tightly controlled. In the absence of Wnt signaling, TCF target gene transcription is actively repressed by negative regulators that bind to TCF. These can be corepressors of the Groucho family (Cavallo et al., 1998; Roose et al., 1998), or the *Xenopus* C-Terminal Binding Protein (XCtBP) (Brannon et al., 1999). Another negative regulator, the histone acetyltransferase CBP/p300, acetylates TCF and interferes with its binding to Armadillo, and the phenotype of dCBP mutant embryos suggests that this activity of dCBP primarily antagonizes Wnt signaling in cells that are weakly stimulated (Waltzer and Bienz, 1998). In contrast, in mammalian cells that contain high levels of activated β-catenin, CBP behaves as a transcriptional coactivator of TCF and β-catenin (Hecht et al., 2000; Takemaru and Moon, 2000).

Clearly, the stability of free cytoplasmic β-catenin/Armadillo is at the heart of the canonical Wnt pathway. Two lines of genetic evidence from *Drosophila* demonstrate that most if not all effects of Wingless signaling are mediated by nuclear Armadillo and TCF. First, signaling-deficient alleles of *armadillo* suppress all known defects of Wingless overactivation in the embryo (Noordermeer et al., 1994; Peifer et al., 1994; Siegfried et al., 1994; Yu et al., 1996). Second, *Drosophila dTCF* mutant embryos

closely resemble *armadillo* and *wingless* mutant embryos (Brunner et al., 1997; van de Wetering et al., 1997). However, it is worth noting that some Wnt ligands and Frizzled receptors effect responses, including the control of planar polarity, that are not transduced by β-catenin/Armadillo and TCF (e.g., Boutros et al., 2000). These pathways are thus distinct from the above-described canonical pathway. Furthermore, as already mentioned, β-catenin/Armadillo has an additional function which is genetically and physically separable from its function as a Wnt transducer (Figure 1): it binds to the homotypic adhesion molecule E-cadherin and links the cadherin junctions to the actin cytoskeleton to mediate cellular adhesion (Ben-Ze'ev and Geiger, 1998). The two functions of β-catenin/Armadillo are executed by separate protein pools, the free cytoplasmic pool and the membrane-bound junctional pool which are in equilibrium with each other (Fagotto et al., 1996; Orsulic and Peifer, 1996; Sanson et al., 1996).

As mentioned above, the canonical Wnt pathway is highly conserved between *Drosophila* and vertebrates. Indeed, some of the Wnt-transducing components function almost interchangeably between *Drosophila* and mammalian cells (e.g., Siegfried et al., 1992; Hayashi et al., 1997; Hamada et al., 1999). However, the pathway is less conserved in the nematode *C. elegans*. For example, the nematode genome does not appear to encode an Axin homolog, nor a true functional homolog of APC since its only APC-related protein APR-1 (Rocheleau et al., 1997) fails to bind to β-catenin/BAR-1 (Korswagen et al., 2000). Also, *C. elegans* has three β-catenin-like genes. One of these, HMP-2, is dedicated to cellular adhesion, while another homolog, BAR-1, functions exclusively in the canonical Wnt pathway to mediate TCF coactivation (Korswagen et al., 2000). The best-studied nematode β-catenin, WRM-1, signals during the polarization of early embryonic cells (Thorpe et al., 1997). To date, no functional homolog of WRM-1 has been described in other species.

Early Events during Colorectal Tumorigenesis

Some 85% of all sporadic and hereditary colorectal tumors show loss of APC function (Kinzler and Vogelstein, 1996). Colon cancer cells with mutant *APC* contain high levels of free β-catenin which can be downregulated by exogenous wild-type APC (Munemitsu et al., 1995). Stabilized β-catenin effects a transcriptional response that is thought to be critical in tumorigenesis (Korinek et al., 1997). It is now commonly accepted that the key tumor suppressor function of APC lies in its ability to destabilize free β-catenin (Smits et al., 1999; von Kries et al., 2000). Likewise, the main function of APC during normal development of flies and frogs is to antagonize Armadillo and β-catenin (Ahmed et al., 1998; Yu et al., 1999; McCartney et al., 1999; Farr et al., 2000).

The tenet that stabilized β-catenin can initiate malignant development was considerably strengthened by discoveries of mutations in other Wnt pathway genes which, like loss of *APC*, cause free β-catenin to accumulate. Among the 15% colon carcinomas that retain wild-type *APC*, point mutations were found in β-catenin which change any of the four serine/threonine residues in its N terminus, the putative targets of GSK3 (Morin et al., 1997). These mutations render β-catenin refractory to destruction by the Axin complex (see above). Similar β-catenin mutations were detected in a wide variety of other tumor types, including melanomas, gastric cancer, hepatocellular carcinomas, and hair follicle tumors (Rubinfeld et al., 1997; Polakis, 2000). Furthermore, a fraction of hepatocellular carcinomas with wild-type β-catenin show Axin mutations (Satoh et al., 2000). Finally, Conductin mutations have recently been found among DNA mismatch repair–deficient colorectal cancers with intact APC (Liu et al., 2000). These Axin and Conductin mutations apparently only affect one allele; therefore, their oncogenic effect may be due to a dominant-negative activity of the mutant Axin/Conductin truncations which causes stabilization of free β-catenin (see Behrens et al., 1998; Hart et al., 1998).

The evidence is now compelling that stabilized free β-catenin is an early event during, and perhaps even initiates, tumorigenesis in the mammalian intestinal tract. First, the smallest detectable intestinal adenomas in heterozygous $Apc^{\Delta 716}$ knockout mice lack the remaining wild-type copy of the *Apc* gene (Oshima et al., 1995). In humans too, inactivation of both *APC* alleles has been found in a majority of small colorectal adenomas (Nagase and Nakamura, 1993; Kinzler and Vogelstein, 1996). Second, conditional knockin mutation in mice of one of the β-catenin alleles to an oncogenic form (bearing an N-terminal deletion which removes the GSK3 target sites) produces adenomatous polyps in the intestine and microadenomas in the colon (Harada et al., 1999). Also, in the mouse skin, transgenic expression in the epidermis of β-catenin with a similar N-terminal deletion causes hair follicle tumors (Gat et al., 1998).

What are the initial effects of APC loss or stabilized β-catenin in the mammalian intestine? To address this question, we briefly introduce the structure and cell biology of the mammalian gut epithelium. Although there are morphological differences between the different sections along the intestinal axis (e.g., between the small intestine and the colorectal section), the basic design of the intestinal epithelium remains essentially the same. The epithelium of the small intestine comprises alternating crypts and villi (Potten and Loeffler, 1990) (Figure 2A). The crypts contain the stem cell compartment at their bases from which proliferating cells slowly migrate up toward the villus while differentiating into various distinct cell types. Thus, cell differentiation and migration are intimately linked in this process. On reaching the villar apex, the differentiated cells eventually die and are shed into the gut lumen. This cycle of proliferation and subsequent differentiation is remarkably active and fast: in the small intestine of the mouse, 60% of crypt cells pass through the cell cycle every 12 hr, and the enterocytes, the principal epithelial cells, pass from the base to the top of a villus in 2–3 days. More than 10^8 cells are shed per day in the mouse, $\sim 10^{11}$ cells in the human, small intestine (Potten and Loeffler, 1990). Interestingly, mouse Tcf4, a TCF family member specifically expressed in the epithelia of the mammary gland and the intestinal tract, is required for the maintenance of the undifferentiated cycling crypt cell compartment (Korinek et al., 1998). These observations suggest that Tcf4 may be activated in the adult crypt by a localized Wnt signal emanating from the surrounding mesenchymal cells.

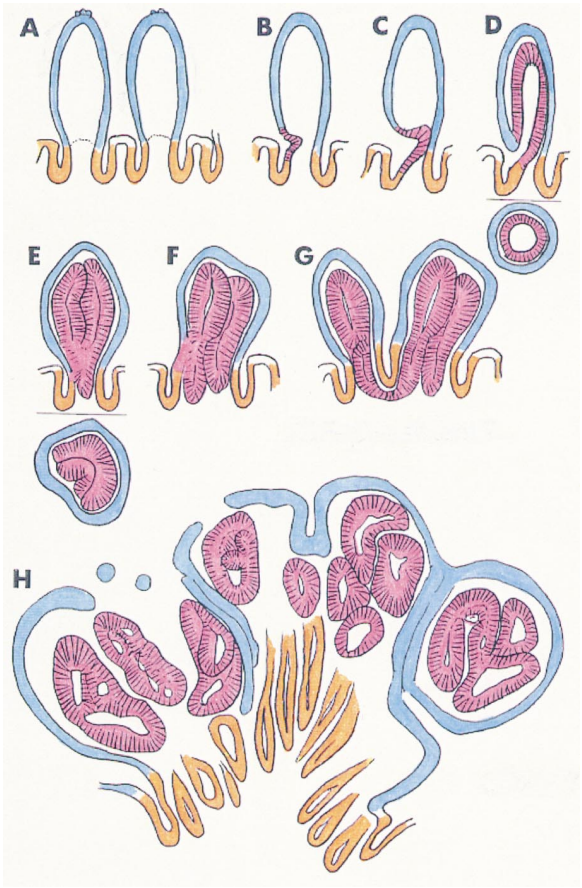

Figure 2. Adenomatous Polyp Formation in *Apc* Mutant Mice

(A) Normal intestinal epithelium (yellow, crypt cells; blue, differentiated cells in the villi). (B and C) Outpocketing pouches form at the proliferative zone of the crypt and protrude into the lacteal side of neighboring villi. (D–F) Microadenoma formation within single villi. (G) Expansion of a microadenoma into a neighboring villus. (H) Multivillus polyp formed by fusion of multiple adenomas, in the process of rupturing through the villus epithelium to protrude into the gut lumen. Adenomatous tissue in pink. This figure is reproduced from Oshima et al. (1997).

However, it is not known at present whether any of the Wnts are expressed in this tissue at this stage.

Taketo and colleagues have studied the early effects of APC loss in their heterozygous $Apc^{\Delta 716}$ knockout mice which develop intestinal adenomas at high frequency (Oshima et al., 1995, 1997). Polyp formation is initiated by abnormal outpocketing of the intestinal epithelium at the crypt–villus boundary (Figure 2B). The adenomal pocket protrudes into the inside of the adjacent villus, and subsequently grows to fill this space (Figure 2C-G) while remaining covered by the villar epithelium. Even after filling several adjacent villi and subsequent fusion, much of the villar epithelium remains intact, and the polyp is barely exposed to the gut lumen (Figure 2H). It is interesting that polyps are polyclonal, and that this polyclonality appears to be obligatory for tumor progression (Merritt et al., 1997). Note that all these events precede the transition to malignancy. Importantly, there is no increase in the rate of proliferation during the early stage of polyp formation. A similar sequence of events is observed in mice overexpressing N-terminally truncated β-catenin (Harada et al., 1999).

Thus, the direct consequence of APC loss is an increase in size of the proliferating crypt compartment. This results in an abnormal tissue architecture which presents as a polyp. Given that Tcf4 is normally required for crypt maintenance (see above), it is conceivable that inappropriate activation of Tcf4 by stabilized β-catenin accounts for this apparent expansion of the crypt compartment. In other words, the polyp may be built from cells that would normally migrate out of the crypt compartment to become differentiated, but that have been misspecified by the activated Tcf4 to remain crypt cells. Note that suppressive effects of activated β-catenin on cellular differentiation have been observed (Harada et al., 1999). All this is consistent with the notion that the initial consequences of APC loss may reflect cell fate changes due to the transcriptional activity of β-catenin/Tcf4. Alternatively, these consequences could be due to a defect in cell migration or adhesion of APC mutant cells that are independent of nuclear β-catenin (see below).

Why is the intestinal epithelium uniquely prone to cancer as a consequence of APC loss? This is essentially not understood. Conceivably, the intestinal epithelium may be unique in that misspecification by inappropriately activated TCF in this tissue results in cells remaining in the stem cell compartment. It is also possible that APC loss may confer growth arrest or even apoptosis in other tissues rather than net cell survival and growth. Further explanations may include the possible role of APC in migration and cellular adhesion, as outlined below. Finally, APC loss may be compensated for by the second APC gene (Nakagawa et al., 1998; van Es et al., 1999) in other tissues. Whatever the case, recall that FAP patients who start life with one APC allele already inactivated develop tumors in tissues other than the colorectum. Interestingly, a recently developed sensitive detection method based on yeast has revealed inactivating APC mutations in a significant fraction of primary breast cancers (Furuuchi et al., 2000). However, this finding awaits confirmation since it contrasts earlier attempts to find such mutations. Note also that breast cancer is neither prevalent in FAP patients, nor have activating β-catenin mutants been found in this type of cancer.

Progression to Malignancy

For the colorectal adenomas to progress to malignancy and invasiveness, further genetic changes need to occur, as previously reviewed (Kinzler and Vogelstein, 1996). One of the best documented ones is the apparent loss of the response to TGF-β. TGF-β signaling can inhibit the growth rate of epithelial cells, and the response to TGF-β is often lost in cancers (Polyak, 1996). In the mammalian intestinal epithelium, TGF-β ligands and receptors are normally expressed in the villi (e.g., Oshima et al., 1997), but it is not known whether these signaling molecules function during the process of normal cell shedding in the intestine. TGF-β receptor type II mutations are relatively common in replication error–prone colorectal tumor cell lines (Markowitz et al., 1995). Furthermore, a small but significant fraction of colorectal

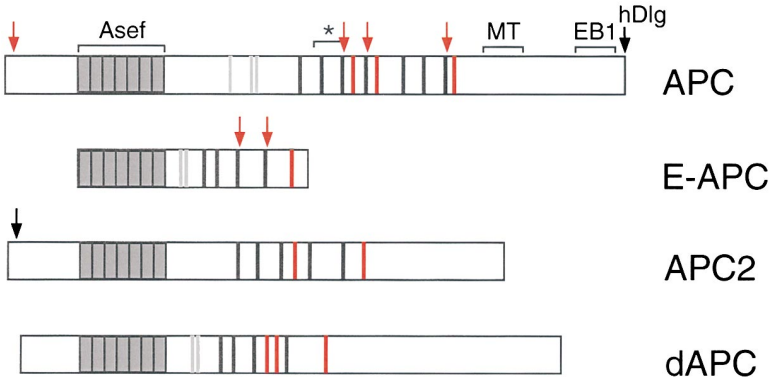

Figure 3. APC Proteins in Human and *Drosophila*

Top, ubiquitous proteins (human APC, *Drosophila* E-APC); bottom, proteins predominantly expressed in the nervous system (human APC2, *Drosophila* dAPC). Gray, Armadillo repeat domain; gray and black bars, 15 and 20 amino acid repeats, respectively, the domains binding to β-catenin; red bars, Axin binding motifs. Arrows point to functional NESs in APC and E-APC (red; an N-terminal NES conserved in APC2 is indicated by black arrow). The MCR is marked by asterisk; regions in APC that bind to Asef, microtubules (MT) and EB1 are bracketed; the C-terminal peptide T/SXV binds to human Dlg.

tumors show loss of the tumor suppressor *DPC4*, the gene encoding human Smad4 which cotransduces all TGF-β-like signals, and some harbor mutations in the TGF-β transducing Smad2 (Moskaluk and Kern, 1996; Riggins et al., 1996). Finally, mice double-heterozygous for *Apc* and *Smad4* mutations develop large intestinal tumors that exhibit both hyperproliferation and invasive behavior (Takaku et al., 1998). These tumors all show loss of the second *Apc* and *Smad4* alleles. This suggests that, in the mouse intestinal tract, the loss of the response to TGF-β may suffice for the adenoma to progress to malignancy and invasiveness. Consistent with this, in humans, the earliest mutations in the TGF-β receptor are found at the late adenoma stage, apparently correlating with the transition from benign adenoma to malignant carcinoma (Grady et al., 1998). However, there is no experimental proof at present that the loss of TGF-β responsiveness brings about this transition.

Oncogenic activation of K-ras and mutations in *p53* are also frequently observed in colorectal tumors, but neither of these events alone appears to be sufficient to initiate tumorigenesis (Kinzler and Vogelstein, 1996). In humans, oncogenic K-ras apparently contributes to tumor progression relatively early, namely during the transition from moderate to late adenomas (Lamlum et al., 2000). Surprisingly, activating *ras* mutations do not seem to play a significant role in tumor progression in the mouse. For example, no oncogenic *K-ras* mutations have been detected in adenomas and adenocarcinomas of *Apc* mutant mice (Oshima et al., 1997; Smits et al., 1997; Takaku et al., 1998). On the other hand, deficiency of *p53* mildly enhances the multiplicity and invasiveness of intestinal adenomas in *Apc* mutant mice (Halberg et al., 2000). Thus, there appear to be some differences between mice and humans in the factors that are important for tumor progression. An explanation for this could be that APC loss and β-catenin activation lead to tumors predominantly in the small intestine of the mouse, but in the colorectum of humans. Furthermore, some of the apparent differences may reflect the different life and survival spans—on the order of months in mice, but decades in humans.

The Molecular Function of APC

Humans and *Drosophila* both have two related APC genes (Figure 3). Each of these is capable, like the tumor suppressor, of reducing the β-catenin levels in APC mutant colon cancer cells (Hayashi et al., 1997; Hamada et al., 1999; van Es et al., 1999). They differ mainly by their expression patterns: like APC, *Drosophila* E-APC/dAPC2 is expressed ubiquitously (McCartney et al., 1999; Yu and Bienz, 1999), while dAPC and APC2/APCL are primarily expressed in the nervous system (Hayashi et al., 1997; Nakagawa et al., 1998; van Es et al., 1999). The four genes share several conserved sequence blocks (Figure 3). The most conserved of these is the Armadillo repeat domain for which there is no well-documented function as yet, although there is a recent report that a G protein exchange factor, termed Asef, interacts with this domain (Kawasaki et al., 2000; see below). Each APC protein also contains multiple β-catenin binding motifs (called 15 and 20 amino acid repeats; note that the 15 amino acid repeats are missing in APC2/APCL) and Axin/Conductin binding sites ("SAMP" repeats). Finally, each except E-APC has a long C terminus, with scattered homology blocks throughout, which includes a region that in the human protein mediates binding to microtubules, to proteins of the EB/RP family and to the human homolog of the *Drosophila* tumor suppressor Discs large (Dlg). This C terminus is also present in the known mouse, rat, and *Xenopus* APC proteins each of which is closely related to the human tumor suppressor. These and other proteins binding to APC have been discussed elsewhere (e.g., Polakis, 2000).

Almost all APC mutations in colorectal tumors result in protein truncations, and the great majority of the mutations in sporadic tumors are clustered in the central domain of APC, the mutation cluster region (MCR) (Nagase and Nakamura, 1993) (Figure 3). Typically, these truncations remove all binding sites for Axin/Conductin, and a strong case can be made that APC's ability to bind to Axin is critical for its tumor suppressor function (Smits et al., 1999; von Kries et al., 2000). However, additional functions may be encoded by sequences 3′ to the MCR that contribute critically to this function.

Nuclear β-catenin/Armadillo and TCF together bring about changes in gene transcription. This nuclear response is thought to be an early event in tumorigenesis (Korinek et al., 1997). Indeed, mutational inactivation of *Tcf1*, a target gene of β-catenin/Tcf4 in mouse epithelial cells, leads to adenomas in multiple tissues including the gut (Roose et al., 1999). *Tcf1* loss synergizes strongly with *Apc* heterozygosity in dramatically enhancing the number and size of intestinal tumors. This apparent tumor suppressor function of mouse Tcf1 was unex-

pected, given that TCFs typically promote Wnt pathway activity (Figure 1). However, a plausible explanation for this apparent paradox is that the most abundantly expressed epithelial isoform of Tcf1 is a dominant-negative TCF which lacks its β-catenin binding domain (Roose et al., 1999).

Another TCF target gene that may contribute to tumor progression in mice and humans encodes the metalloproteinase matrilysin (Brabletz et al., 1999; Crawford et al., 1999). The functional significance of this finding is supported by genetic evidence: the incidence and size of intestinal tumors is markedly suppressed in *Apc* mutant mice which also lack *matrilysin* function (Wilson et al., 1997). Furthermore, the cell cycle promoting genes c-*myc* and *cyclin D1* have been identified as TCF target genes (He et al., 1998; Tetsu and McCormick, 1999), as well as PPARδ, a putative target protein in colorectal cancer for the beneficial effects of certain nonsteroidal anti-inflammatory drugs (He et al., 1999). However, there is no genetic evidence as yet to indicate the functional relevance of these TCF target genes during normal development or malignant transformation of the intestinal epithelium.

How does APC destabilize β-catenin? This is poorly understood at the molecular level. As mentioned above, the Axin complex to which APC can bind earmarks β-catenin for degradation by the proteasome pathway. However, overexpression of Axin in *APC* mutant colon cancer cells is sufficient to reduce the high β-catenin levels in these cells, thus bypassing the requirement for APC in this process (Behrens et al., 1998; Hart et al., 1998). This indicates a regulatory role of APC. A specific suggestion has been that APC may somehow derepress Axin to facilitate its activity in the β-catenin-destabilizing complex (Hart et al., 1998). Alternatively, it has been proposed that APC may have a spatial regulatory role, shuttling β-catenin around the cell (Bienz, 1999) (Figure 4). This model was inspired by the striking subcellular distribution of *Drosophila* E-APC which is associated with apical adhesive junctions, but is also found in the cytoplasm and in the nucleus (McCartney et al., 1999; Yu et al., 1999). According to the shuttling model, APC binds to β-catenin/Armadillo in the cytoplasm and in the nucleus and shuttles it to the junctional zones where it delivers it to the Axin complex for subsequent degradation, or to E-cadherin for incorporation into adhesive junctions. The model implies that APC connects the two subcellular pools (free and junctional) of β-catenin/Armadillo and maintains an equilibrium between them.

Support for this model has recently come from the discovery that APC contains nuclear export signals (NES) which allow it to exit from the nucleus (Rosin-Arbesfeld et al., 2000; Henderson, 2000). Of particular interest is the pair of NESs immediately downstream of the MCR, conserved in all known APC proteins (Figure 3), since these are deleted in almost all APC truncations found in sporadic colorectal tumors (Rosin-Arbesfeld et al., 2000). Consequently, truncated APC (only retaining the N-terminal NES) is nuclear in cells derived from APC mutant tumors. These cells also show high levels of nuclear β-catenin, and these can only be reduced efficiently by exogenous APC which retain a minimum of two NESs. It thus appears that the truncated APC typically observed in colon cancers may be unable to export

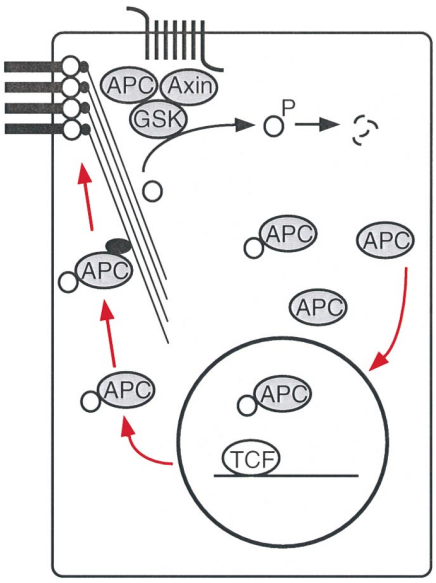

Figure 4. Shuttling Model of APC Function

According to this model, APC shuttles β-catenin/Armadillo from the nucleus and the cytoplasm to the junctional compartment of epithelial cells. Here, β-catenin/Armadillo is delivered either to the Axin complex to be earmarked for degradation, or to E-cadherin for incoporation into adherens junctions. Actin filaments could serve as tracks to which APC may attach via a motor protein (black oval). Thus, APC may connect the two functional pools, junctional and the free cytoplasmic, of β-catenin/Armadillo (see also Bienz, 1999).

β-catenin efficiently from the nucleus, or may even trap β-catenin in the nuclei of these cells. Whatever the case, the nuclear export function of APC appears to be critical for its tumor suppressor function (Rosin-Arbesfeld et al., 2000). Of note, β-catenin has been observed to be nuclear in colorectal tumors (Brabletz et al., 1998; Kolligs et al., 2000).

Functions of APC in Cell Migration and Adhesion?

A corollary of the shuttling model is that APC loss may not only affect transcription via nuclear β-catenin/Armadillo, but also cellular adhesion via the junctional pool of β-catenin/Armadillo. Indeed, it is beginning to emerge that APC may have functions at the cell periphery that are separate from its function effected by nuclear β-catenin. Below, we shall discuss recent evidence from mammalian and *Drosophila* cells that suggest a role of APC in cellular migration and adhesion.

An indication that APC may affect cell migration came from experiments in migratory mammalian tissue culture cells. In these cells, APC was found to cluster in membrane extensions at the plus ends of microtubules (Näthke et al., 1996). Indeed, tagging of APC with green fluorescent protein allowed direct visualization of its ability to track along microtubules to peripheral cellular sites (Mimori-Kiyosue et al., 2000), illustrating the mobility of APC within cells (see Figure 4). Intriguingly, overexpression of N-terminal truncations of β-catenin that bind more stably to APC than wild-type β-catenin impairs the migratory properties of these cells (Barth et al., 1997). Nelson and colleagues have argued that this is a conse-

quence of the dynamics of APC–β-catenin interaction; it is conceivable that these APC "superbinders" may exert dominant-negative effects on the shuttling of normal β-catenin by APC. Finally, it was discovered recently that APC binds to Asef, an exchange factor that apparently activates the small G protein Rac which in turn controls the actin cytoskeleton (Kawasaki et al., 2000). Evidence based on overexpression of exogenous proteins suggested that APC activates Asef at the membrane periphery and, as a consequence, induces cell flattening and membrane ruffling, both signs of migratory activity. This is perhaps the most persuasive evidence yet that APC may regulate cell migration. It seems to do so by remodeling the actin cytoskeleton via Asef and Rac (Kawasaki et al., 2000).

Further suggestions that APC may affect directly cell shape and adhesion came from *Drosophila*. First, in the developing eye, loss of the neuronal APC causes cell shape changes that are mediated by Armadillo but not by TCF (Ahmed et al., 1998). Second, depletion of E-APC in the embryo produces subtle defects in the junctional association of Armadillo (Yu et al., 1999). Finally, a weak mutation in *E-APC* that abolishes the association of the mutant protein with junctional membranes causes partial detachment of Armadillo from cadherin junctions as well as a mild mutant phenotype in the ovary reminiscent of the phenotype due to impaired cell adhesion in this tissue (Townsley and Bienz, 2000). Of note, the cellular consequences of complete loss-of-function of APC in *Drosophila* are not known at present. Nevertheless, these observations implicate *Drosophila* E-APC in the maintenance of junctional integrity. It may be of interest in this context that human APC binds to the homolog of *Drosophila* Dlg (Matsumine et al., 1995), a septate junction protein in *Drosophila* which is required for cell adhesion, and whose loss-of-function causes cell overgrowth in this organism (Woods et al., 1996).

Further experimental support is needed to consolidate this putative role of APC in cellular migration and adhesion. Such a role could be relevant for APC's function as a tumor suppressor both at the early adenoma stage as well as at late stages of tumor progression. As mentioned above, one of the earliest manifestations of APC loss in the intestinal epithelium is an abnormal tissue architecture which may reflect a defect in cell migration or adhesion. Furthermore, it is well established that cadherin-mediated adhesion is typically lost at the invasive front of tumors where cells become migratory, and that this loss accompanies the progression from benign to malignant tumors (Birchmeier and Behrens, 1994). Intriguingly, in human colorectal tumors, the cells at the invasive front rather than those in the tumor center tend to show high levels of nuclear β-catenin (Brabletz et al., 1998). Thus, a self-reinforcing loop might operate in APC mutant cells: a scenario could be envisaged according to which impaired nuclear export of β-catenin due to APC loss may cause subtle depletion of junctional β-catenin, thus initiating the gradual dismantling of cadherin junctions. This in turn would tend not only to facilitate migration and, ultimately, invasive behavior of cells, it would also further increase the levels of nuclear β-catenin as the junctional β-catenin is progressively released from the membrane-bound pool. New TCF target genes may be switched on as a consequence of the high nuclear β-catenin levels that result from this putative shift in equilibrium between the two β-catenin pools which may be due to APC loss.

In summary, as initially suggested (e.g., Hülsken et al., 1994), APC could operate to suppress tumorigenesis at the level of cell migration and adhesion. Both of these processes might be affected by mutant forms of β-catenin that interfere with the interaction between APC and normal β-catenin (see above), but perhaps not by β-catenins with point mutations such as those found in cancers. Thus, although the latter appear to be as potent as APC loss regarding transcriptional activation (Morin et al., 1997), APC loss may have wider functional consequences than oncogenic activation of β-catenin. Consistent with this, oncogenic β-catenin mutations are relatively common among small adenomas, but are very infrequent among invasive carcinomas, and oncogenic β-catenin is associated with less aggressive tumors than APC loss (Samowitz et al., 1999). Note also that γ-catenin has recently been found to be regulated, like β-catenin, by APC, and was shown to have oncogenic properties (Kolligs et al., 2000). All of this indicates that APC loss is more powerful than oncogenic activation of β-catenin in promoting malignant development.

Epilog

The convergence of developmental biology and molecular oncology has outlined a crucial role for mutations activating the Wnt pathway in causing colorectal tumors. The pivotal event appears to be the formation of a constitutively active β-catenin/Tcf4 complex in the intestinal epithelial cell. Additional functions of the APC tumor suppressor in cell migration and adhesion may critically contribute to the progression of tumors.

It will be of great interest to determine the complete spectrum of genes that are activated in response to stabilized β-catenin during normal and malignant development of the intestine. The advent of DNA array technology will provide such insights. The β-catenin/Tcf4 complex presents a well-defined target for therapeutic intervention in cancer. However, it remains to be demonstrated whether the disruption of this complex by small molecular compounds in an adenoma, let alone in a carcinoma with multiple additional mutations, will have beneficial effects by reversing the course of carcinogenesis.

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