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Functions of the APC tumor suppressor protein dependent and independent of canonical WNT signaling: Implications for therapeutic targeting

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Abstract

The acquisition of biallelic mutations in the *APC* gene is a rate-limiting step in the development of most colorectal cancers and occurs in the earliest lesions. *APC* encodes a 312-kDa protein that localizes to multiple subcellular compartments and performs diverse functions. APC participates in a cytoplasmic complex that promotes the destruction of the transcriptional licensing factor β-catenin; *APC* mutations that abolish this function trigger constitutive activation of the canonical WNT signaling pathway, a characteristic found in almost all colorectal cancers. By negatively regulating canonical WNT signaling, APC counteracts proliferation, promotes differentiation, facilitates apoptosis and suppresses invasion and tumor progression. APC further antagonizes canonical WNT signaling by interacting with and counteracting β-catenin in the nucleus.

APC also suppresses tumor initiation and progression in the colorectal epithelium through functions that are independent of canonical WNT signaling. APC regulates the mitotic spindle to facilitate proper chromosome segregation, localizes to the cell periphery and cell protrusions to establish cell polarity and appropriate directional migration, and inhibits DNA replication by interacting directly with DNA. Mutations in *APC* are often frameshifts, insertions or deletions that introduce premature stop codons and lead to the production of truncated APC proteins that lack its normal functions and possess tumorigenic properties. Therapeutic approaches in development for the treatment of APC-deficient tumors are focused on the inhibition of canonical WNT signaling, especially through targets downstream of APC in the pathway, or on the restoration of wild-type APC expression.

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Keywords

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Driving questions in colorectal cancer research

Colorectal cancer has emerged as a family of diseases that can develop along a number of different histological and molecular trajectories (Fig. 1). As the understanding of colorectal cancer at the molecular level becomes increasingly detailed, intense research interest surrounds the ability of these histological or molecular characteristics to predict prognosis. 65% of colorectal cancer patients survive 5 years or more after diagnosis, although individual outcomes are heavily dependent upon stage. Localized colorectal cancers are associated with 5-year survival of approximately 70–90% (depending on the degree of invasiveness) [1], while cancers that have spread to nearby lymph nodes are associated with only 40–50% 5-year survival. Metastatic colorectal cancers that have spread to the liver or other distant sites are associated with only 5–15% survival over a 5-year period, yet represent more than 20% of diagnoses [1]. These statistics indicate the extent to which surgical resection remains the best tool for curing colorectal cancer, while therapeutic options against cancers that have disseminated to other sites quickly become exhausted.

The discovery and characterization of the genetic changes acquired along the malignant pathway have informed the search for novel therapeutic options. However, significant questions remain unanswered, such as whether or how colorectal tumors tend to acquire these key mutations in a particular order [2]. The field has begun to identify the patterns of mutations or aberrations in gene expression that distinguish short-term survivors from long-term survivors of advanced disease [3], or that correlate with responsiveness to certain interventions, including emerging antibody-based therapies [4]. These clinically-oriented questions may eventually be answered through a more complete understanding of how particular genetic changes translate into phenotypic changes.

The *APC* gene

Biallelic mutation of the *APC* gene occurs in 45%–80% of colorectal cancers [5–7] and is observed in the earliest detectable lesions [2]. The *APC* locus was originally identified based on its link to familial adenomatous polyposis coli (FAP), an inherited syndrome of cancer predisposition [8–11]. Inherited mutations in the *APC* gene cause affected individuals to develop hundreds to thousands of adenomatous polyps, resulting in the onset of CRC typically before the age of 40 [12]. Individuals with FAP inherit a loss-of-function mutation in a single allele of *APC*, followed by an additional acquired mutation in the second allele of *APC* in the adenomas and adenocarcinomas that develop [13–15]. Thus, the acquisition of biallelic *APC* mutations represents an early and rate-limiting step in all FAP-associated and most sporadic colorectal tumors.

In studies of colorectal cancer as a whole, *APC* mutational status does not strongly correlate with outcome [16, 17]. Nevertheless, *APC* mutations exhibit an interesting pattern of differential distribution in the recognized subtypes of colorectal cancer (Fig. 1). *APC*

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mutations correlate strongly with a large subset of colorectal cancers associated with intermediate prognosis [18]. On the other hand, *APC* mutations occur infrequently within a smaller subset derived from sessile serrated adenomas and associated with microsatellite instability and good prognosis [18]. This latter subset exhibits a relatively high proportion of activating mutations in the gene encoding β-catenin (*CTNNB1*) [19] that are mutually exclusive of *APC* mutations [20, 21]. Interestingly, *CTNNB1* mutations are significantly more prevalent in small adenomas than in large adenomas or adenocarcinomas, [18], whereas *APC* mutations are well-represented across all stages of tumorigenesis. It has recently emerged that *APC* mutational status has value as a predictive marker of poor prognosis in Stage III colorectal cancers [17], raising the possibility that *APC* mutations not only initiate colorectal cancer development, but drive clinical phenotypes relevant to progression and metastasis as well.

Functions of the APC tumor suppressor protein

The *APC* gene encodes a 312-kDa protein (Fig. 2) that performs diverse cellular functions and localizes to multiple subcellular compartments. Mutations in *APC* are often frameshifts, insertions or deletions that introduce premature stop codons and lead to the production of a truncated APC protein. Amino acids 1000 to 1600 of APC have been identified as a mutation cluster region that represents roughly 20% of the total (2,843-amino acid) protein yet contains about 60% of all identified mutation sites [7]. The following sections detail functions of the wild-type APC protein, all of which are weakened or lost through the acquisition of pathogenic mutations. Also discussed are dominant functions of truncated APC proteins that contribute to tumorigenesis.

Cytoplasmic APC negatively regulates canonical WNT signaling

The best-known function of APC is its ability to interact with β-catenin in the cytoplasm and promote β-catenin phosphorylation, ubiquitination and subsequent proteolytic degradation (Fig. 3) [22–25]. This function occurs within the context of a cytoplasmic complex [23, 26] that includes glycogen synthase kinase 3β (GSK-3β) [27], AXIN1 [24, 28] or AXIN2 [23, 29], and other kinases and phosphatases [30, 31]. The interaction of APC with this complex maps to a region in the center of the protein that contains three serine-alanine-methionine-proline (SAMP) repeats that mediate the binding of APC to AXIN1/AXIN2 [23], as well as three repeats of 15 amino acids each and seven repeats of 20 amino acids each. β-catenin interacts with the 15-amino acid repeats constitutively [32], and with the 20-amino acid repeats inducibly [33] following their phosphorylation by GSK-3β [34] and casein kinase I [31, 35]. Both the 15-amino acid and 20-amino acid repeats are necessary to enable APC to promote β-catenin degradation [33]; truncating mutations to APC generally disrupt these repeats either partially or completely.

The role of APC in this cytoplasmic complex negatively regulates the canonical WNT signaling pathway [26], whose ability to mediate transcriptional changes requires the licensing factor β-catenin to bind to transcription factors of the TCF/LEF family [36]. The canonical WNT signaling pathway alters the transcription of key target genes, including activation of the genes encoding c-MYC [37], Cyclin D1 [38, 39] and LGR5 [40, 41]. These

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and other changes in gene expression collectively drive proliferation, survival and maintenance of an undifferentiated state in progenitor cells of the colorectal epithelium [38, 42–45]. The pathway is critical to normal tissue homeostasis [46], as mature cells lining the colon and rectum must be frequently replaced as they become damaged, undergo apoptosis and are sloughed into the intestinal lumen [47]. Progenitor cells of the colorectal epithelium are characterized by activated canonical WNT signaling, while maturing cells are characterized by inactivation of the pathway and increasing expression of APC [48, 49]. Disruption of this balance initiates colorectal cancer, as the progeny of a colorectal progenitor cell lacking functional APC are unable to stop proliferating, differentiate or undergo apoptosis [48]. APC loss stabilizes β-catenin and constitutively activates the pathway even in the absence of a WNT signal [33, 50]. The importance of this particular APC function in the process of tumorigenesis is underscored by the observation that a significant percentage of the colorectal cancers with wild-type APC exhibit a point mutation in *CTNNB1*, the gene encoding β-catenin [20] that makes the protein resistant to degradation [21]. Together, APC and β-catenin mutations form part of a larger group of molecular changes by which approximately 93% of colorectal cancers exhibit activation of the canonical WNT signaling pathway [5].

Nuclear APC negatively regulates canonical WNT signaling

The APC protein further counteracts canonical WNT signaling by participating in at least two other mechanisms that take place in the nucleus. Nuclear import of APC is dependent upon two nuclear localization sequences (NLS) as well as a separate Armadillo domain that also promotes nuclear import [51, 52]. Once inside, nuclear APC interacts with β-catenin and facilitates its export to the cytoplasm [53–56]. This activity is dependent upon two recognized nuclear export sequences (NES) within APC [54]. The ratio of nuclear APC to cytoplasmic APC decreases as proliferation slows down and cells enter a quiescent state [57–59], and is controlled in part by phosphorylation of key residues near each APC NLS [51, 57]. The nuclear interaction between APC and β-catenin occurs in part within the chromatin fraction, in which APC promotes the removal of β-catenin from specific genomic loci (Fig. 4) [60]. Nuclear APC interacts not only with β-catenin but also with C-terminal binding protein (CtBP), a transcriptional co-repressor [61]. A detailed mechanistic study has demonstrated that APC and CtBP transiently interact with β-catenin at the WNT-activated *MYC* promoter and promote the removal of β-catenin from this locus, coinciding with the appearance of the more stable co-repressors TLE-1 and HDAC1 [60]. This function of chromatin-associated APC negatively regulates WNT activation of the *MYC* gene [60], as well as WNT activation of the *AXIN2*, *DKK1* and *SP5* genes [62]. Truncated APC proteins observed in colorectal cancer generally lack both NLS, which are located in the missing C-terminal half of the protein. However, truncated APC retains the ability to move between the nucleus and cytoplasm because of the preserved Armadillo domain [63]. It is difficult to compare the relative importance of the cytoplasmic and nuclear mechanisms by which APC negatively regulates canonical WNT signaling, as both are disrupted by pathogenic *APC* mutations [64]. Evidence for the importance of nuclear APC includes the observations that biallelic point mutations abolishing both NLS within APC increase proliferation and

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expression of canonical WNT signaling targets in the mouse intestine, and that a single mutant allele increases polyp number and size in *Apc*$^{Min/+}$ mice [65].

APC in mitotic spindle dynamics and genomic stability

Colorectal cancers segregate into two mutually exclusive categories based on exhibiting genomic instability at either the microsatellite level (due defects in mismatch repair) or the chromosomal level [66, 67]. *APC* mutations are particularly well-represented within the subset of colorectal cancers that feature widespread chromosomal damage [68], and also play an important role in this phenotype [69]. The ability of APC to interact with the microtubule network facilitates its localization to the kinetochore [69, 70], a protein structure that mediates the attachment of microtubules to sister chromatids during mitosis. Murine cells with *Apc* mutations exhibit chromosomal abnormalities as well as mitotic spindles characterized by numerous microtubules lacking proper connections to the kinetochore [69]. *APC* silencing in human colorectal cancer cells similarly decreases inter-kinetochore tension during metaphase, and results in defective progression through mitosis [71]. The APC-interacting protein EB1 collaborates with APC to regulate the mitotic spindle, and the loss of this APC-dependent mechanism facilitates errors in chromosome alignment without halting cell division [72].

APC is modified during mitosis by phosphorylation [73], including by the spindle checkpoint kinases Bub1 and BubR1, suggesting that its contributions to mitotic spindle integrity and chromosome segregation are accompanied by a role in the spindle checkpoint [70]. Interestingly, overexpression of a truncated APC protein exerts a dominant negative effect that compromises the spindle checkpoint [74], in which defects are often linked to chromosomal instability. These functional data collectively suggest that truncating APC mutations drive genomic instability at the chromosomal level through both loss-of-function and gain-of-function mechanisms that occur independently of its role in canonical WNT signaling.

APC controls DNA replication and cell cycle progression

In addition to its role in the spindle checkpoint and mitotic progression, APC controls the cell cycle by regulating the G1/S transition. Various APC-deficient colorectal cancer cell lines stably transfected to express wild-type APC exogenously exhibit dramatic changes in doubling time and inhibition of G1/S phase progression [75]. Overexpression of RB pathway components such as Cyclin D1/CDK4, Cyclin E/CDK2, E1A, and E2F overrides cell cycle inhibition by APC, suggesting that APC re-expression restores the G1/S checkpoint [76, 77]. Negative regulation of canonical WNT signaling by APC contributes to this function by downregulating targets including Cyclin D1 itself [38, 39]. The physical interaction of APC with other proteins such as DLG may also be involved, as the C-terminal DLG-binding residues within APC are required for complete inhibition of S-phase entry in a mouse fibroblast cell model, while DLG overexpression in itself is sufficient to arrest cells at the G1/S transition [78]. The interaction of the APC C-terminus with A/T-rich DNA also blocks entry into or progression through S-phase by blocking DNA replication [79, 80]. These findings are consistent with evidence that defective G1/S-phase progression due to

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APC overexpression is only partially alleviated by co-transfection of a constitutively active mutant β-catenin [77]. APC thus contributes to the regulation of cell cycle progression through a combination of WNT-dependent and WNT-independent mechanisms.

Pro-apoptotic functions of APC

APC exhibits a gradient of increasing expression in the luminal direction along the colorectal crypt-villus axis [49], coinciding with mature, non-proliferative areas of the crypt where apoptotic cell death and cell shedding occur. Functional evidence linking APC to apoptosis began to accumulate with the observation that exogenously restoring APC expression in an APC-deficient colon cancer cell line triggered a 10-fold increase in the proportion of apoptotic cells [43]. In addition, overexpression of WNT-inhibitory PDZ domain-containing peptides from the Dishevelled protein induce apoptosis in an APC-dependent manner [81]. The link between APC and apoptosis is mediated at least in part by its role in canonical WNT signaling, whose targets include the *BIRC5* gene that encodes the anti-apoptotic Survivin protein [82].

The ability of APC to sensitize cells to apoptosis is also partially WNT-independent [83]. Caspase family members proteolytically cleave APC in apoptotic cells [84], producing an N-terminal fragment that localizes to mitochondria and interacts with hTID-1 to promote caspase activity and cell sensitivity to apoptosis [85]. While truncated APC proteins in colorectal cancer also exhibit mitochondrial localization, they appear to exert anti-apoptotic effects instead, as their knockdown promotes apoptosis and mitochondrial membrane permeability [86]. Truncated APC proteins interact with and promote mitochondrial localization of the anti-apoptotic BCL2 protein [86]. The role of APC loss in shifting the balance between apoptosis and survival therefore is mediated by multiple mechanisms, both direct and indirect.

The role of APC in differentiation

Just as the pattern of APC expression along the colorectal crypt-villus axis indicates its role in apoptosis, the coincidence of APC expression with areas of mature, functional epithelium provided the first clue that APC affects differentiation as well. This luminal upper half of the colorectal crypt is populated with a mixture of terminally-differentiated columnar epithelial cells, goblet cells that produce mucus and neuroendocrine cells, all of which are derived from multipotent stem cells at the base of the crypt. Apc loss in the mouse small intestine disrupts commitment to all three cell fates while driving commitment to the basally-located Paneth cell lineage through a WNT-dependent mechanism [87]. Apc likely drives differentiation through suppression of canonical WNT signaling as well, as mice deficient in the canonical WNT signaling transcription factor Tcf7L2 exhibit dramatic and lethal defects in the proliferation of intestinal stem cells [88]. Consistent with these findings, canonical WNT signaling activates the expression of Lgr5, a critical marker of intestinal stem cells [89].

Interestingly, loss of Apc in the zebrafish gut produces a differentiation defect that occurs prior to nuclear β-catenin accumulation or changes in proliferation [90]. This may result

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from the loss of an Apc function in promoting proteasome-dependent degradation of the transcriptional co-repressor Ctbp1 [91]. Upon Apc loss, Ctbp1 suppresses the transcription of genes involved in retinoic acid biosynthesis [91], a process required for differentiation in the zebrafish gut [90, 92]. Differentiation in this model is accomplished through the ability of retinoic acid to antagonize the expression of demethylase genes, resulting in the methylation of gene promoters critical to maintaining progenitor-like phenotypes [93]. APC similarly regulates CtBP1 expression in human colon cancer cell lines [91], indicating that the zebrafish mechanism by which it drives differentiation through retinoic acid biosynthesis may be present in the human colorectal epithelium as well. Studies from the zebrafish model also indicate that impaired intestinal differentiation following Apc loss is mediated in part by a decrease in the expression of mitochondrial pyruvate carrier 1 (*Mpc1*), resulting in defective pyruvate metabolism that is independent of the WNT pathway and potentially linked to the Warburg effect [94]. Since differentiation and cell migration along the crypt-villus axis are coupled processes [95], APC may help link cell fate and cell migration in the colorectal epithelium.

Cytoskeletal functions link APC to adhesion, migration and cell polarity

In addition to its roles in the cytoplasm and nucleus, APC localizes to the cell border [63] and participates in at least three mechanisms regulating epithelial organization and cell migration. APC localization to the cell periphery is dependent upon the actin cytoskeleton [96] as well as the Armadillo domain within APC [97], although truncated APC proteins retaining this region still localize there with reduced efficiency [96]. The scaffolding protein IQGAP1 mediates an indirect interaction of APC with actin and also links APC with Rac1 and Cdc42, two Ras-family GTPases that regulate actin structures [98]. APC also regulates the activity of these GTPases to influence actin organization [99]. APC also interacts with actin filaments and stress fibers and has the ability to bundle actin filaments *in vitro* [100], raising the possibility that it connects and coordinates crosstalk between the actin and microtubule cytoskeletons. APC collaborates with the formin mDia1 to nucleate actin filament formation [101], and this function is required for directed cell migration and focal adhesion turnover in cultured cells [102].

APC interacts with both β-catenin and plakoglobin (γ-catenin) at cell-cell junctions [103], which function to connect adjacent epithelial cells, organize them into layers and promote cell polarity. β-catenin and plakoglobin physically connect cytoskeletal components including actin and intermediate filaments with transmembrane adhesion molecules such as cadherins. APC loss promotes a decrease in cell-cell and cell-matrix adhesion by altering the subcellular distribution of E-cadherin [104, 105]. This consequence of APC loss occurs at the protein level, independently of canonical WNT signaling [104]. Truncated APC proteins contribute in a dominant negative manner to this loss of migration directionality [106]. APC is furthermore linked to cell polarity through interactions with DLG [107] and hScrib [108], the respective human homologues of the *Drosophila* proteins Discs Large and Scribble. DLG interacts with PDZ domain-binding residues at the C-terminus of APC, and the two proteins form a complex with hScrib at lateral regions of cell-cell contact in canine kidney epithelial cells [108].

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Finally, APC localizes to leading edges of migrating cells in a microtubule-dependent manner [109]. This function of APC maps not to its N-terminal Armadillo domain, but to C-terminal regions of APC that directly contact the microtubule network [110]. Specifically, amino acids 2200–2400 are enriched for basic residues that facilitate microtubule binding and that promote polymerization [110] and bundling [111, 112] of microtubules *in vitro*. The APC C-terminal region also binds the microtubule-interacting protein EB1, which specifically interacts with and localizes APC to microtubule plus ends [113]. These microtubule-related functions of APC are proposed to drive the formation of membrane protrusions and influence the balance between adhesion and motility [114]. The well-characterized decrease in cell migration that follows APC loss *in vivo* [45] may be explained at least in part by the decreased formation of membrane protrusions and alterations in microtubule stability that follow APC loss in cultured cells [115]. APC also functions in mouse fibroblasts as an RNA-binding protein that localizes key RNA molecules to cell protrusions with consequences for cell migration [116]. In dying cells, APC may be required to target microtubules to the appropriate side of the cell, promoting proper extrusion from the apical rather than the basal side of the epithelium [117]. Collectively, these studies of APC at the cell periphery argue for roles in linking the actin cytoskeleton with the microtubule network and in the establishment of cell polarity and suppression of invasive behaviors [118].

APC counteracts invasion, cancer progression and metastasis through targets of canonical WNT signaling

The previous section discussed non-transcriptional roles of APC at the cell periphery, which explain at least in part the phenotype of defective intestinal epithelial cell migration observed along crypt-villus axes in both Apc-deficient [119] and Apc-overexpressing [120] mice. Additional evidence has linked APC loss to cell motility and invasive phenotypes through its role in the regulation of canonical WNT signaling. At first glance, this appears to conflict with the common view that *APC* mutations initiate colorectal tumorigenesis and drive adenoma formation, while other subsequent hits drive invasion and metastasis. This point of view is supported by the findings that *APC* mutations occur early in colorectal tumorigenesis [6] and are typically concordant both between paired primary and metastatic tumors [121] and between metastatic and non-metastatic intratumoral lineages [122]. On the other hand, histological analysis of β-catenin staining in primary colorectal tumors indicates that most tumors exhibit a predominantly peripheral rather than nuclear distribution of β-catenin, coinciding with a more differentiated epithelial growth phenotype, while nuclear β-catenin staining tends to characterize a smaller population of poorly-differentiated cells at the invasive front that lack membrane-associated E-cadherin staining [123]. Two representative cases (Figs. 5A and 5B) depict a higher proportion of tumor cells at the invasive edge exhibiting nuclear β-catenin staining relative to their counterparts in the tumor center. This evidence has led to the hypothesis that the tumor microenvironment at these sites drives loss of differentiation and epithelial-to-mesenchymal transition to promote invasion in a manner dependent on nuclear β-catenin [123].

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This finding raises the question of which targets of canonical WNT signaling might mediate cell migration, invasion and metastatic dissemination. Activation of canonical WNT signaling downregulates expression of E-cadherin, a key driver of epithelial cell identity that counteracts both invasion and metastasis [124]. The extent to which this regulation occurs directly through WNT transcriptional activity [125] or indirectly through WNT targeting of the transcription factor Slug (SNAI2) [126] is not clear. Other targets of canonical WNT signaling that facilitate invasive and/or metastatic phenotypes include extracellular matrix components such as fibronectin (FN1) [127] and LAMC2 [128], matrix-remodeling enzymes such as matrilysin (MMP7) [129, 130], MMP14 [131] and ADAM10 [132], and cell adhesion receptors such as uPAR [133], CD44 [134], Nr-CAM [135] and L1-CAM [136]. Relevant targets also include ligands and receptor tyrosine kinases of the EphB/EphrinB pathway [137] and fascin (FSCN1), which induces the formation of filopodia [138]. Some of these targets are more highly expressed at the invasive front relative to the well-differentiated central region of colorectal tumors [139], consistent with the proposed role of elevated nuclear β-catenin at these sites driving invasion. CD44 is a particularly interesting target, as the variant isoform CD44v6 marks cancer stem cells with metastatic potential within primary colorectal tumors, exhibits higher expression in mesenchymal-like cells at the invasive front, and enhances both cell migration and metastatic capability [140]. CD44v6 expression remains elevated in metastases [140] despite the fact that these lesions are generally characterized by a predominantly differentiated epithelial histology and cell peripheral β-catenin staining pattern similar to their primary tumor counterparts [123]. Overall, APC is linked to the suppression of colorectal cancer progression and metastasis not only through its role in maintaining chromosomal stability, but also through both WNT-dependent and WNT-independent contributions to cell migration and invasion.

Targeted treatment of APC-deficient colorectal cancers

Re-establishing Apc expression can restore normal development and differentiation of the intestinal crypt epithelium in mice with Apc-deficient intestinal adenomas and adenocarcinomas [46]. This is consistent with studies in human colon cancer cell lines in which re-expression of APC modifies cancer-related characteristics such as proliferation [75], adhesion [104] and sensitivity to apoptosis [43]. These reports on re-establishing APC function as a therapeutic strategy, and have led to the exploration of several different methodologies for restoring APC expression. Tumors with nonsense mutations in *APC* respond to aminoglycoside and macrolide agents that promote readthrough of premature stop codons [141]. These treatments partially restore APC expression in an APC-deficient human colon cancer cell line sufficiently to reduce tumor size in a mouse xenograft model [141]. Treatment with the macrolide compound tylosin reduces the size and number of intestinal tumors and extends the lifespan of $Apc^{Min/+}$ mice possessing a nonsense mutation at codon 850 of the *Apc* gene [141]. In breast and lung cancer cell lines with hypermethylation of the *APC* promoter, APC expression has been successfully restored by treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine [142]. Finally, herpesvirus particles tested as vehicles for delivery of wild-type *APC* to APC-deficient colon cancer cell lines cause a reduction in both their proliferation and migration in culture [143]. In spite of the demonstrated benefits of reversing the effects of APC loss in multiple model

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systems, clinical application of this strategy is not considered technically feasible and has not been explored outside of preliminary macrolide studies in patients with FAP.

Research strategies to improve the treatment of APC-deficient colorectal cancers have also focused on the development of agents targeting the canonical WNT signaling pathway, as several lines of evidence point to the pathway's activation as a key tumorigenic consequence of APC loss [20, 21, 46, 144–146] and a driver of key cancer stem cell lineages within colorectal tumors [147]. In spite of significant efforts to develop inhibitors of canonical WNT signaling for cancer treatment, clinically relevant inhibitors do not yet exist as of 2017. One speculated reason has been the lack of convenient enzymatic targets in canonical WNT signaling relative to other developmental signaling pathways [148], as well as the challenge of targeting components of the pathway downstream of APC in colorectal cancers harboring *APC* mutations [149]. Another proposed obstacle has been the possibility that the synthetic TOPFLASH reporter system used for candidate discovery fails to recapitulate the endogenous complexity of WNT activation and inactivation sufficiently to facilitate identification of clinically relevant targets [150]. Finally, even an effective WNT inhibitor may be accompanied by significant safety concerns due to the critical role of canonical WNT signaling in normal processes such as somatic stem cell maintenance [148].

Many of the more promising inhibitors of canonical WNT signaling target early steps of the pathway that lie upstream of APC, and therefore are less effective in the absence of functional APC [148]. Nevertheless, evidence suggests that APC-deficient cells rely on endogenous WNT ligands to fine-tune the signaling pathway to optimal levels [151], and inhibitors of Porcupine O-Acyltransferase (required for WNT ligand palmitoylation and secretion) [152] effectively reduce the ability of colon cancer cell lines to proliferate and form tumors in xenograft mouse models [153]. The Porcupine inhibitors LGK974 [154] and ETC-159 [155] are currently in clinical trials for treatment of a subset of colorectal cancers harboring *BRAF* mutations as well as for other solid tumors. Other antagonists of WNT signal transduction currently in clinical trials include inhibitors of the WNT receptor Frizzled, such as the antibody vantictumab [156] and the fusion protein ipafricept [157].

Potential therapeutic targets in the canonical WNT signaling pathway include other components of the cytoplasmic complex through which APC negatively regulates β-catenin stability. β-catenin degradation can be promoted therapeutically by activators of casein kinase that can enhance β-catenin phosphorylation [158], and by inhibitors of tankyrase enzymes that modulate the stability of the key scaffolding component AXIN [159]. The potential efficacy of these strategies in the treatment of APC-deficient colorectal cancers remains a matter of some debate [160], and tankyrase inhibition in particular has been shown to partially reverse the effects of APC loss on β-catenin levels in cell culture models [161]. Other mechanisms negatively regulate β-catenin levels independently of canonical WNT signaling, including activation of the Vitamin D signaling pathway [162–164].

Other promising preclinical studies have characterized small molecules designed to disrupt the nuclear interaction between β-catenin and TCF/LEF family transcription factors [165] or between β-catenin and the transcriptional coactivator CBP [166]. Disruptors of the β-catenin and CBP interaction include PRI-724 (also known as ICG-001), currently in clinical trials

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for newly-diagnosed metastatic colorectal cancer [150]. Small molecule antagonists of the β-catenin / TCF complex include PKF115-584 and CGP049090, which appear to function by binding directly β-catenin [165] but are not currently in clinical trials.

The Traf2 and Nck-interacting kinase (TNIK) is a required collaborator of β-catenin and TCF7L2 in nuclear transcription factor complexes [167], and is under investigation as a potential therapeutic target for APC-deficient colorectal cancers [149, 168, 169]. A recently-discovered TNIK inhibitor, NCB-0846, has not yet been studied in a clinical context [169]. Other more targeted strategies have been designed to manipulate a single gene activated by canonical WNT signaling, particularly the *MYC* proto-oncogene [170]. The specificity of these strategies may help mitigate the anticipated shortcoming of more general WNT antagonists in that the canonical WNT signaling pathway functions to maintain the somatic stem cells responsible for normal homeostasis of the colorectal epithelium and other tissues [88, 148]. Toxicity to normal cells is therefore a significant concern as these therapeutic candidates for APC-deficient colorectal cancer continue to progress into clinical studies.

Summary

In conclusion, the APC tumor suppressor protein performs multiple functions that contribute to its role in preventing colorectal tumorigenesis. It is difficult to assign relative importance to individual functions, other than to point out that APC contributions to relevant phenotypes such as cytoskeletal regulation, cell cycle progression, chromosomal stability, sensitivity to apoptosis, differentiation and invasion/metastasis are mediated by multiple mechanisms, both WNT-mediated and WNT-independent. Dominant negative effects of truncated APC proteins further counteract wild-type APC functions by promoting migration, chromosomal instability and evasion of apoptosis. Therapeutic strategies to restore functions of the APC tumor suppressor protein are not yet fully developed but are progressing along several different lines of investigation, particularly in the form of inhibitors of the canonical WNT signaling pathway.

Materials and Methods

Immunohistochemical analysis

Stage II colorectal cancer tumor samples were previously formalin-fixed, embedded in paraffin and prepared as 4 μm sections by the Department of Pathology at The Ohio State University College of Medicine. Antigen retrieval was performed with a high pH EDTA solution (Agilent Technologies, Santa Clara, CA) before immunohistochemical staining with an anti-human β-catenin antibody (Agilent Technologies, clone b-catenin-1; 1:400 dilution). Detection was performed with the Novolink polymer detection system (Leica Biosystems, Buffalo Grove, IL) and an Autostainer Link 48 (Agilent Technologies). Image capture was performed at 10x, 20x and 40x magnification.

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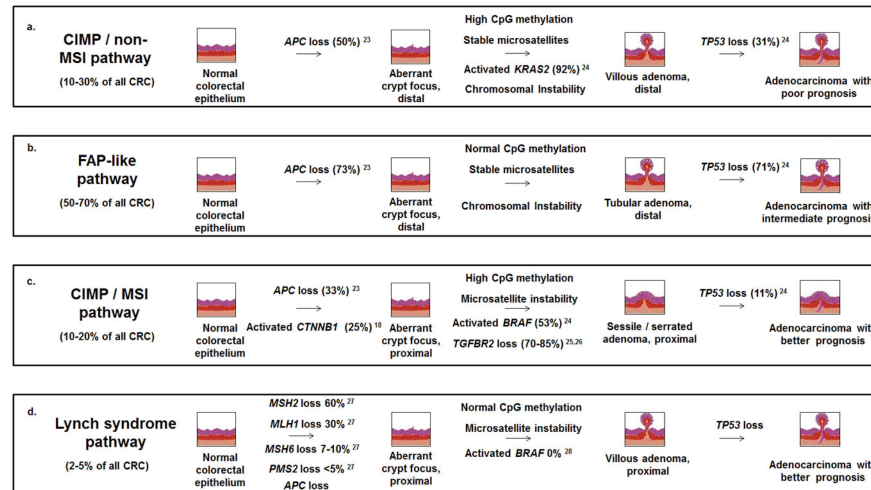

Figure 1. *APC* mutations are unequally distributed across different colorectal cancer pathways

Molecular analysis of colorectal cancers has identified at least four subsets associated with different prognoses [66, 171]. Subsets are defined by the presence or absence of the CpG island methylator phenotype (CIMP) and the microsatellite instability phenotype (MSI), two often-linked characteristics which together with the chromosomal instability phenotype (CIN) generate mutations that drive disease progression. Most colorectal cancers are characterized by neither CIMP nor MSI (B), but follow a trajectory similar to that observed in familial adenomatous polyposis (FAP), characterized by mutations in *APC* and *TP53* [68, 172]. CIMP in the absence of MSI is associated with poor prognosis (A), while CIMP resulting in MSI [19, 173, 174] is associated with good prognosis (C), similar to the MSI-driven cancers observed in Lynch syndrome [175, 176] (D).

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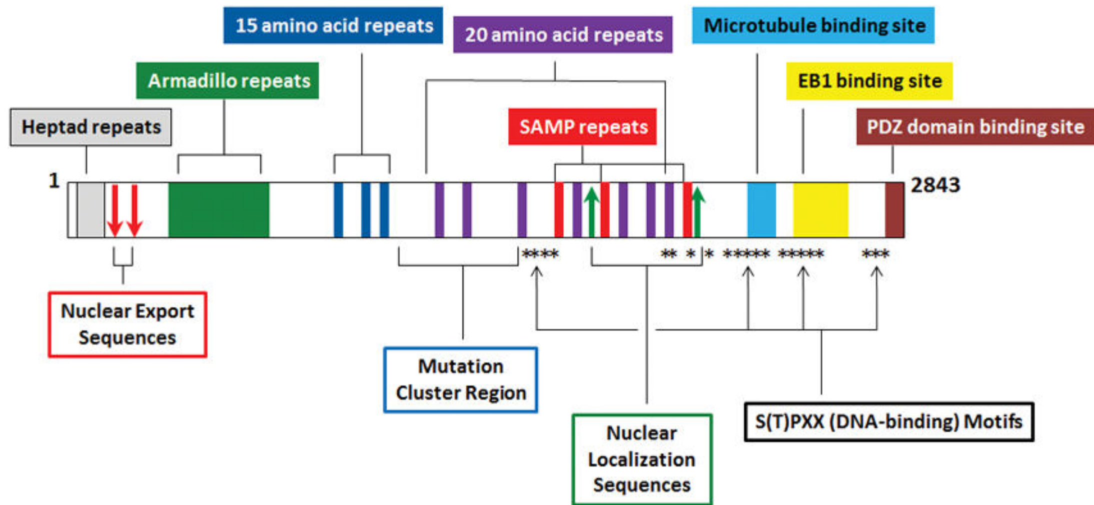

Figure 2. Truncating mutations disrupt key structural features in the central and C-terminal regions of the human APC protein

Key structural features of the human APC protein that are disrupted by most truncating mutations include its 20-amino acid repeats (purple), SAMP repeats (red), nuclear localization sequences (green arrows), microtubule binding region (light blue), EB1 binding region (yellow), DNA-interacting S(T)PXX motifs (asterisks), and C-terminal PDZ domain-binding region (brown). Truncated N-terminal APC proteins retain some ability to interact with β-catenin due to the preservation of the 15-amino acid repeats and some of the 20-amino acid repeats, while retention of the Armadillo domain preserves the ability localize to the nucleus.

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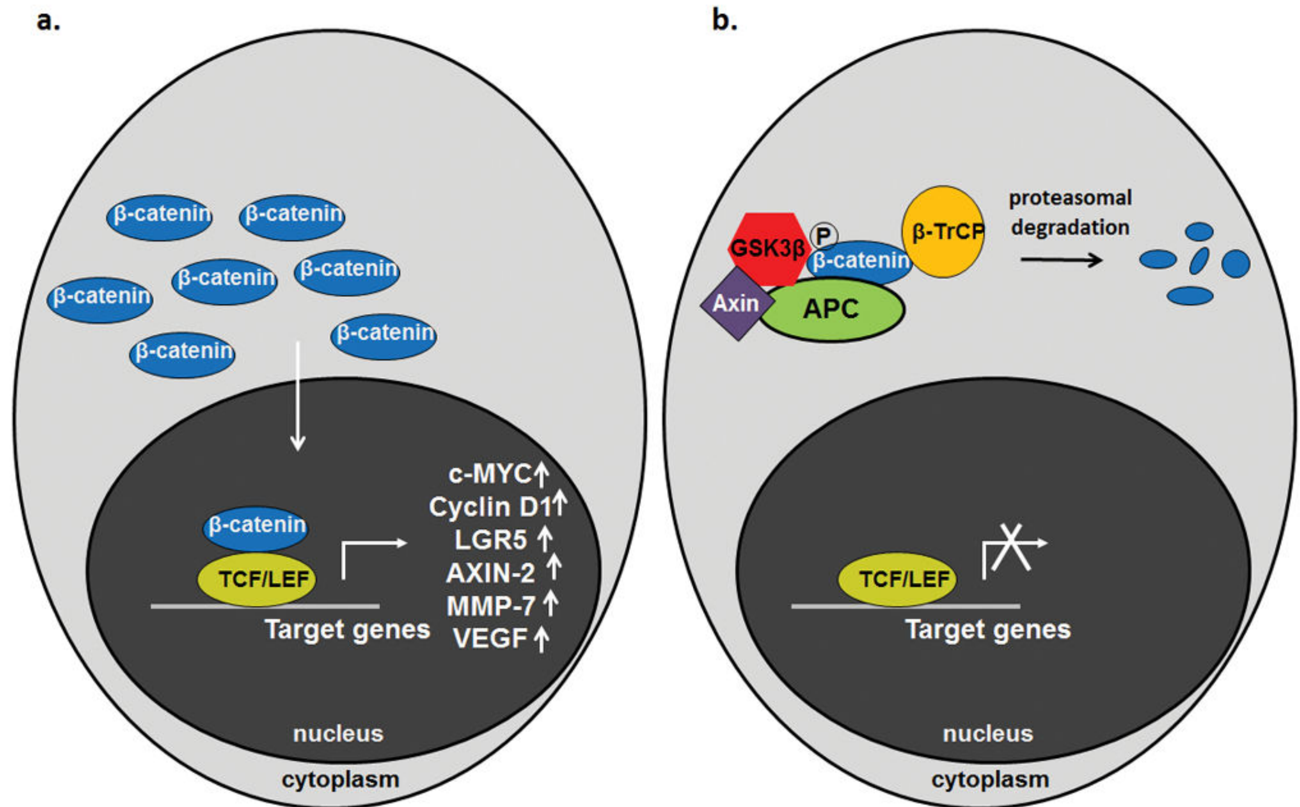

Figure 3. Cytoplasmic APC protein negatively regulates the canonical WNT signaling pathway

The critical difference between the active (A) and inactive (B) states of the canonical WNT signaling pathway is the accumulation of the licensing factor β-catenin, especially in the nucleus, where it binds to TCF/LEF family transcription factors to promote changes in gene transcription. The active state of the pathway (A) is characteristic of colorectal cancers and colorectal progenitor cells, as it favors proliferation and survival at the expense of differentiation and sensitivity to apoptosis. The inactive state (B) is characteristic of mature, differentiated cells of the colorectal epithelium that express APC. APC (green) is a required component of the cytoplasmic complex that limits β-catenin availability by promoting its proteolytic degradation.

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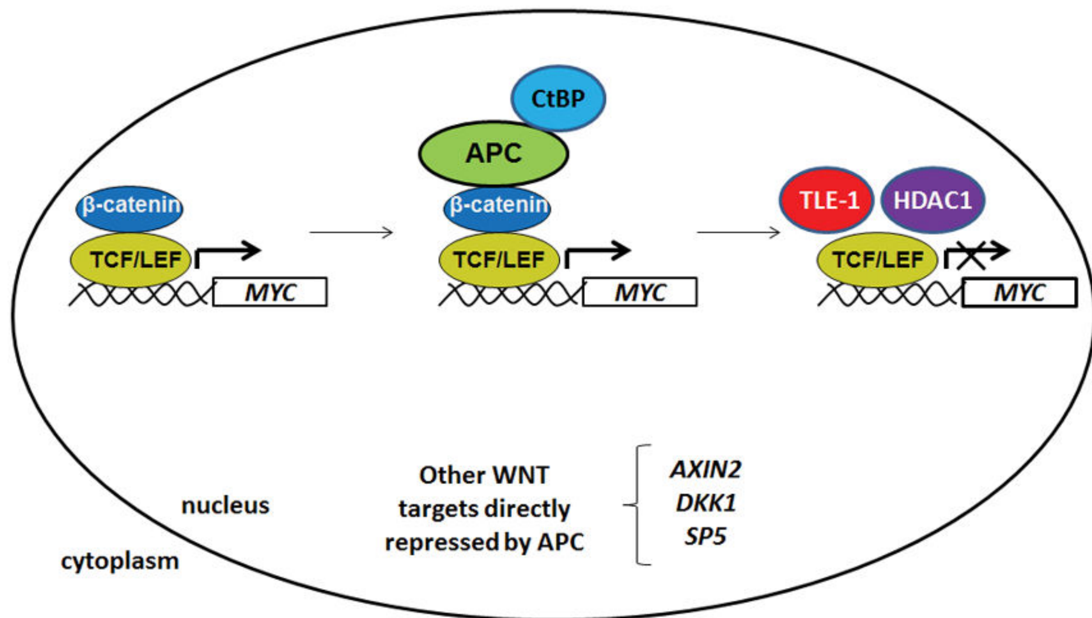

Figure 4. Chromatin-associated APC negatively regulates canonical WNT activation of specific target genes by removing the licensing factor β-catenin

The nuclear fraction of APC further antagonizes canonical WNT signaling in colorectal cancer cells by interacting with chromatin-associated β-catenin at WNT-activated target genes such as *MYC*. This transient interaction leads to the removal of β-catenin, the appearance of co-repressors such as TLE-1 and HDAC1, and transcriptional repression [60, 62].

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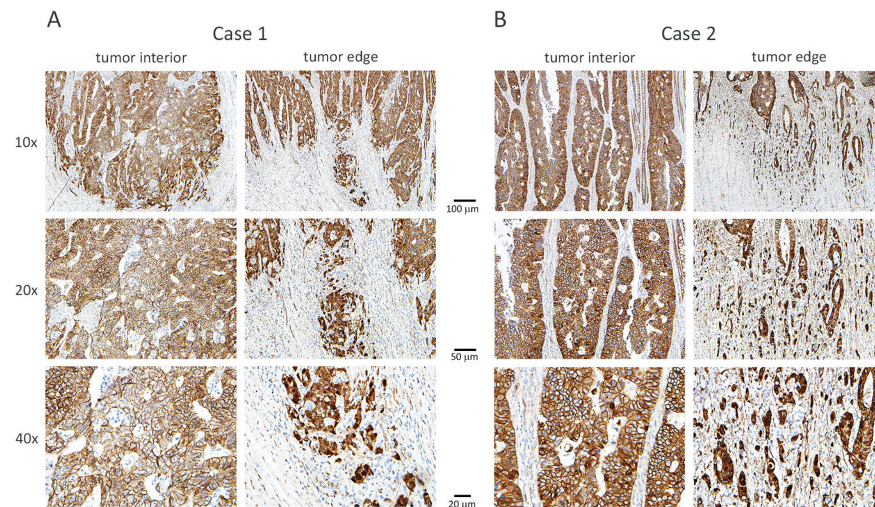

Figure 5. Colorectal tumor cells at the invasive front exhibit higher levels of nuclear β-catenin than cells at the tumor center

Stage II colorectal cancer cases were selected from The Ohio State University College of Medicine, Department of Pathology archives and stained immunohistochemically to characterize β-catenin localization. Two representative cases are shown in Figures 5A and 5B and include cells in the tumor center and at the invasive tumor edge at 10x, 20x and 40x magnification. Cases were selected representatives of a common pattern in which a higher proportion of tumor cells at the invasive edge exhibit nuclear β-catenin staining relative to their counterparts in the tumor center. These observations are consistent with published reports of variations in β-catenin staining across tumor sub-compartments as well as with evidence that some canonical WNT targets are differentially activated at the invasive tumor edge.